JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

VOL. 29 1967

ELSEVIER PUBLISHING COMPANY

AMSTERDAM

PRINTED IN THE NETHERLANDS

GAS–LIQUID CHROMATOGRAPHIC STUDIES OF ELECTRON-DONOR-ACCEPTOR SYSTEMS

IV. DI-*n*-NONYL TETRACHLOROPHTHALATE AS AN ELECTRON-ACCEPTOR STATIONARY PHASE

A. R. COOPER AND C. W. P. CROWNE
Department of Chemistry, Sir John Cass College, London, E.C.3 (Great Britain)
AND
P. G. FARRELL
Department of Chemistry, Queen Elizabeth College, London, W.8 (Great Britain)
(Received December 27th, 1966)

INTRODUCTION

LANGER *et al.*[1] have described the use of tetrachlorophthalate esters as stationary phases in gas–liquid chromatography (GLC), for the separation of aromatic compounds. They attribute the selectivity of these esters in the retention of such compounds in part to the formation of charge transfer (CT) complexes, following earlier work in which complexes of hexamethylbenzene with these esters were isolated and coloured solutions formed on adding electron donors[2], among them N,N-dimethylaniline[3]. The chromatographic experiments[4] showed the emergence of *p*-xylene after *m*-xylene, in the reverse order of volatility but also in the reverse order of donor strength.

Investigations of other CT acceptors as stationary phases have been reported[5,6] correlating retention data with CT association constants determined spectrophotometrically. This correlation was examined for substituted anilines, aromatic hydrocarbons and heterocyclic compounds which are stronger donors than those employed by LANGER *et al.*[1] in the chromatographic studies of the tetrahalophthalate esters.

Retention data have now been obtained for these stronger donors chromatographed on di-*n*-nonyl tetrachlorophthalate (NTCP). Di-*n*-nonyl phthalate has been used extensively as a stationary phase in GLC[7] and it was found that the tetrachloro-compound also has the low volatility required for use at these temperatures. The esters used by LANGER *et al.*[1] are too volatile to be used as stationary phases at temperatures in the region of $\sim 200°$, these temperatures being desirable for the study of methylated naphthalenes and quinolines. The results are compared with the corresponding values for elution from 2,4,7-trinitrofluorenone (TNF)[5], a stronger acceptor than the ester, and with retention data[8,9] for non-complexing polar stationary phases. Such a comparison supports the contention that CT association contributes to the retention of the donors chromatographed on NTCP columns, but implies that NTCP is a very weak electron acceptor.

TABLE I

RETENTION DATA AND EXCESS THERMODYNAMIC FUNCTIONS OF SOLUTION FOR SUBSTITUTED ANILINES

No.	Compound	$V_{g(SO)}$ (ml) 180°	$V_{g(NTCP)}$ (ml) 180°	γ_{NTCP} 180°	R_{NTCP} 180°	$V_{g(NTCP)}$ (ml) 195°	$\Delta\bar{G}_e^\circ$ (cal) 180°	$\Delta\bar{H}_e^\circ$ (cal)	$\Delta\bar{S}_e^\circ$ (cal deg.$^{-1}$)	R_{TNF}/R_{NTCP}
1	Aniline	24.6	71.5	1.11	2.91	51.6	99.5	182	3.81	2.34
2	*o*-Toluidine	37.7	117.7	1.02	3.12	86.1	21.6	252	5.51	2.32
3	*m*-Toluidine	38.7	121.6	1.08	3.14	88.9	71.7	281	6.05	2.45
4	*p*-Toluidine	35.3	117.9	1.04	3.34	86.8	37.9	283	6.16	2.48
5	*p*-Ethylaniline	57.1	182.1	0.75	3.20	127.5	—256.7	117	3.14	1.78
6	2,4-Xylidine	57.1	193.8	0.90	3.39	131.7	93.9	176	4.08	2.46
7	N-Methylaniline	36.6	106.5	1.02	2.91	77.0	19.7	188	4.11	1.88
8	N,N-Dimethylaniline	41.3	106.8	0.95	2.59	77.6	—46.2	234	5.28	1.32
9	N,N-Diethylaniline	72.3	170.1	1.10	2.32	119.4	85.2	235	5.00	0.64
10	N,N-Dimethyl-*o*-toluidine	37.9	74.8	1.08	1.97	57.5	69.2	343	7.43	0.39
11	N,N-Dimethyl-*p*-toluidine	62.0	164.1	0.96	2.65	118.1	— 32.1	234	5.24	1.47
12	N,N-Dimethyl-*p*-*tert.*-butyl-aniline	155.9	416.3	1.17	2.67	281.1	141.4	199	4.09	0.57
13	N,N-Dimethyl-2,4-xylidine	57.4	115.3	1.05	2.01	84.1	40.6	245	5.31	0.36
14	N,N-Dimethyl-2,6-xylidine	50.7	96.0	1.10	1.89	72.6	85.2	324	7.00	0.30
15	2,6-Xylidine	57.4	186.1		3.24					2.25
16	2-Ethylaniline	54.5	168.8		3.10					1.51
17	2,6-Diethylaniline	173.2	350.5		2.02					0.94

EXPERIMENTAL

The apparatus and technique for measuring specific retention data have been described elsewhere[5]. Two-metre glass columns of 4 mm I.D. were packed with hexamethyldisilazane-treated Celite (44–52 mesh) coated with 10–15 % w/w di-*n*-nonyl tetrachlorophthalate.

Retention measurements were made relative to 1,2,3,5-tetramethylbenzene or naphthalene. The fall in retention time for these reference compounds was used to monitor the loss of stationary phase and a column rejected if this loss exceeded 10 % of the weight of stationary phases.

Materials

Di-n-nonyl tetrachlorophthalate. This compound was prepared by the method used by NORLANDER AND CASS[10] for the corresponding octyl and decyl compounds. After distilling excess alcohol from the reaction mixture the product was fractionally distilled at reduced pressure. Di-*n*-nonyl tetrachlorophthalate had a b.p. of 260–270° at 1–2 mm; $n_D^{20} = 1.5155$. (Found: C, 55.2; H, 6.6. Calc. for $C_{26}H_{38}O_4Cl_4$: C, 56.1; H, 6.9 %.)

The presence of anhydride in the stationary phase markedly increases the retention of these donors. Extreme care was taken to exclude it from the ester in these experiments.

In addition to the diester a decarboxylation product, *n-nonyl tetrachlorobenzoate*, was produced. This had a b.p. of 210–215° at 2 mm; m.p. 33–35°. (Found: C, 50.8; H, 5.5. Calc. for $C_{16}H_{20}O_2Cl_4$: C, 49.8; H, 5.2 %.)

Anilines. All samples used were purified in accordance with the literature, redistilled and collected at their recorded boiling points.

Hydrocarbons and heterocycles. Chromatographically pure commercial samples were used.

RESULTS AND DISCUSSION

Anilines

In contrast to the elution of aromatic amines from TNF, where there is a major change in the elution order from that found with silicone oil, the selectivity of NTCP is far weaker with only one instance of a change in elution order, N,N-dimethylaniline emerging after N,N-dimethyl-2,6-xylidine. In spite of the absence of large changes in retention order, the influence of CT interactions can be seen in the data of Table I.

In Table I are shown values for the specific retention volumes V_g at 180 and 195° and the calculated[5] activity coefficients γ, at infinite dilution and 180°, in NTCP for a series of aromatic amines. Also included are the specific retention volumes in silicone oil $V_{g(SO)}$ and $R_{NTCP} = V_{g(NTCP)}/V_{g(SO)}$, at 180°. We have calculated the excess partial molar free energy of solution $\Delta\bar{G}_e°$ at 180° together with the corresponding excess enthalpy $\Delta\bar{H}_e°$ and entropy $\Delta\bar{S}_e°$ as in previous work[5]. The solution process is endothermic and the excess entropy of solution positive; therefore any contribution from CT complexing must be very weak. The values of $\Delta\bar{H}_e°$ are similar to those found for the weaker complexes between TNF and N,N-dimethyl-*o*-toluidine or N,N-dimethyl-2,6-xylidine.

TABLE II

RETENTION DATA FOR HYDROCARBONS AND HETEROCYCLES

Compound	$V_{g(NTCP)}$ (*ml*) 180°	R_{NTCP} 180°	R_{TNF}[a] 180°	*Compound*	$V_{g(NTCP)}$ (*ml*) 180°	R_{NTCP} 180°	R_{TNF}[a] 180°
Decalin (*cis*)	91.3	1.78	(0.21)[b]	2-Methylnaphthalene	380.3	3.32	6.83
Decalin (*trans*)	72.8	1.72	(0.25)[b]	1,6-Dimethylnaphthalene	709.8	4.19	10.21
Tetralin	152.9	2.45	1.39	2,6-Dimethylnaphthalene	634.6	3.91	9.58
Indan	75.5	2.10	1.19	2,3-Dimethylnaphthalene	709.8	4.23	11.63
Indene	91.3	2.54	2.84	1-Allylnaphthalene	837.5	4.12	5.08
1,2,3,4-Tetramethylbenzene	146.4	2.57	2.15	1-Bromonaphthalene	1018.3	4.41	8.48
1,2,4,5-Tetramethylbenzene	1111.1	2.29	1.65	2-Bromonaphthalene	1035.4	4.54	8.08
1,2,3,5-Tetramethylbenzene	114.8	2.28	1.56	Quinoline	310.2	3.74	7.71
Pentamethylbenzene	273.5	2.79	2.66	2-Methylquinoline	405.4	3.72	5.86
Hexamethylbenzene	676.8	3.34	3.73	4-Methylquinoline	634.6	4.33	10.13
Diphenyl	504.3	3.54	4.00	6-Methylquinoline	546.5	4.21	9.41
Benzo[*b*]thiophene	225.5	3.24	5.70	7-Methylquinoline	555.3	4.21	9.50
Indole	482.6	5.10	24.49	8-Methylquinoline	413.7	3.62	5.41
Benzofuran	72.4	2.44	3.36	Isoquinoline	370.3	4.04	8.73
Dibenzofuran	1172.8	4.77	13.92	3-Methylisoquinoline	471.8	4.00	7.24
Naphthalene	222.9	3.30	5.84	2,4-Dimethylquinoline	771.1	4.50	—
1-Methylnaphthalene	417.5	3.45	6.92	2,6-Dimethylquinoline	695.1	4.40	—

[a] Values taken from ref. 6.

[b] Values less accurate than other R values because of the low retention volumes on TNF.

The weaker selectivity of NTCP compared with TNF can be seen in the smaller range of activity coefficients for these solutes in NTCP. Stronger interaction between solute and TNF is reflected by $\gamma < 1$ for compounds 1, 2, 3, 4, 5, 6, 7, 8 and 11 whereas in NTCP $\gamma < 1$ is found only for compounds 5, 6, 8 and 11. The tertiary amines 8 and 11 are the strongest donors of the series as also are 5 and 6 in the primary amine series but in addition the latter are likely to form a hydrogen bond with the acceptor. In view of the paucity of vapour pressure data for many organic donors solute–solvent interaction is discussed in the light of retention parameters alone.

The last column of Table I illustrates the relative selectivity of these stationary phases. Although both solvents are capable of acting as acceptors in hydrogen bonding, the donor hydrogen bonding solutes are held more strongly on TNF. The ratio R_{TNF}/R_{NTCP} for compounds 1, 2, 3, 4 and 6 is roughly constant suggesting that this type of association is a constant factor in each solvent. *p*-Ethylaniline with a larger substituent and N-methylaniline with only one free N-H fall a little way out of this class as they did in the plot of R_{TNF} *vs.* K_x (ref. 5), where K_x is the CT association constant. Compounds 8 and 11, which are the most strongly complexed on TNF, are also the most strongly selected by NTCP relative to other N,N-dimethylated compounds, though the interaction is weaker than on TNF. The more heavily substituted compounds 9, 10, 12, 13 and 14 are favoured by NTCP. It may be that the single aromatic ring and aliphatic side chain are sterically more favourable than TNF.

R_{NTCP} follows the pattern of R_{TNF}, *i.e.* increasing alkyl substitution increases R, the behaviour associated with CT complexing. This is contrary to the effect obtained using the non-complexing stationary phases HCEM and diglycerol[6]. The pattern is repeated exactly for the compounds 1 to 6, the values for the three toluidines being greater than that for *p*-ethylaniline, which is greater than R_{NTCP} for aniline. Ethyl substitution adjacent to the amino group, as in 2-ethylaniline, increases R_{NTCP} whereas the opposite behaviour is observed with TNF. Possibly the steric hindrance is greater in this latter case. This might also explain why 2,6-diethylaniline shows only a small reduction in R_{NTCP} relative to aniline whereas in TNF the effect is considerable.

Aromatic hydrocarbons and heterocyles

The specific retention volumes at 180° and R_{NTCP} values for these compounds are shown, together with previously determined R_{TNF} values[6], in Table II. The range of R_{NTCP} values (1.72–4.77 excluding the H-bonding indole) is much less than that for R_{TNF} (0.21–13.92), showing TNF to be a much more selective solvent. The low values for R_{TNF} for compounds in Table II up to pentamethylbenzene relative to R_{NTCP}, excluding indene, show poor solvent qualities for TNF towards molecules with some aliphatic character. This is most marked with the decalins. The stronger donor hexamethylbenzene would appear to be a clear example of CT interaction exerting considerable influence in spite of aliphatic TNF repulsions. In the series of substituted benzenes the order of R_{NTCP} values is the same as the order of R_{TNF} values. These same substitution effects suggest that CT interactions do contribute to retention on NTCP but to a smaller extent than on TNF.

The compounds following pentamethylbenzene in Table II are all more strongly retained by TNF, the stronger CT acceptor. Dibenzofuran, the most soluble substance

in TNF, is still the most soluble in NTCP, though its "escaping tendency" is much higher than for TNF. The R_{NTCP} and R_{TNF} values both suggest an order of decreasing CT complexing given by indole > benzo[*b*]thiophene > benzofuran, which was the order found spectroscopically with tetracyanoethylene as acceptor[11].

The R_{NTCP} and R_{TNF} figures for the methyl-substituted naphthalenes show the same order of solubility in both solvents, with the stronger complexing dimethyl derivatives showing the greater solubility.

The R_{NTCP} values for the quinoline series parallel the R_{TNF} values with one exception, the R values for quinoline and 3-methylisoquinoline, which lie close together in both solvents, are reversed. 2,4-Dimethylquinoline and 2,6-dimethylquinoline, which we were unable to elute satisfactorily from TNF, have R_{NTCP} values that are slightly larger than the monomethylquinolines (complexing effect). These disubstituted quinolines are both substituted in an unfavourable position (2-) for strong complexing and because of this the R_{NTCP} values are not greatly increased over their corresponding monosubstituted compounds, 4-methylquinoline and 6-methylquinoline, respectively. As expected the effect of the position of methyl groups on R_{NTCP} is in accord with 4-methyl > 6-methyl.

ACKNOWLEDGEMENT

We are grateful to Sir John Cass College for financial support (to A. R. C.).

SUMMARY

Di-*n*-nonyl tetrachlorophthalate has been found to be a good selective stationary phase for the separation of aromatic compounds. It acts as a weak electron acceptor, but comparison of elution data with those obtained for other stationary phases implies that charge-transfer associations do contribute to the retention of the aromatic donors.

REFERENCES

1 S. H. LANGER, C. ZAHN AND G. PANTAZOPLOS, *J. Chromatog.*, 3 (1960) 154.
2 S. H. LANGER, C. ZAHN AND M. H. VIAL, *J. Org. Chem.*, 24 (1959) 423.
3 P. PFEIFFER AND E. FLATER, *Chem. Ber.*, 55 (1922) 413.
4 S. H. LANGER, C. ZAHN AND G. PANTAZOPLOS, *Chem. Ind. (London)*, (1958) 1145.
5 A. R. COOPER, C. W. P. CROWNE AND P. G. FARRELL, *Trans. Faraday Soc.*, 62 (1966) 2725.
6 A. R. COOPER, C. W. P. CROWNE AND P. G. FARRELL, *Trans. Faraday Soc.*, in press.
7 e.g. S. EVERED AND F. H. POLLARD, *J. Chromatog.*, 4 (1960) 451.
8 J. S. FITZGERALD, *Australian J. Appl. Sci.*, 12 (1961) 51.
9 J. JANÁK AND M. HŘIVNÁČ, *J. Chromatog.*, 3 (1960) 297.
10 B. W. NORLANDER AND W. E. CASS, *J. Am. Chem. Soc.*, 69 (1947) 2675.
11 A. R. COOPER, C. W. P. CROWNE AND P. G. FARRELL, *Trans. Faraday Soc.*, 62 (1966) 18.

RETENTION TIMES AND MOLECULAR SHAPES: THE USE OF DESOXYCHOLIC ACID IN COLUMN LIQUIDS FOR GAS CHROMATOGRAPHY

A. O. S. MACZEK* AND C. S. G. PHILLIPS
Inorganic Chemistry Laboratory, Oxford (Great Britain)
(Received December 27th, 1966)

INTRODUCTION

In an earlier investigation[1], it has been shown that saturated solutions of tri-*o*-thymotide (TT) used as column liquids for gas chromatography are able to bring about selective separations based on molecular shape. The saturated solution of TT is such that narrow, straight-chain molecules are able to enter holes or channels in its structure, whereas broad, branched-chain molecules are excluded from such holes or channels and are consequently less retarded. Desoxycholic acid (DCA), a bile acid, is known to form crystalline inclusion compounds[2] with a variety of molecules of restricted width (*viz.* acids[3], esters[4], and straight chain hydrocarbons[5]) but not with broad molecules (branched-chain hydrocarbons[5]). An investigation of column substrates consisting of saturated solutions of DCA should serve to demonstrate if the effects found with TT are more generally widespread.

EXPERIMENTAL

Pure DCA (B.D.H. Ltd., Poole, Dorset) was recrystallised from *tert.*-butyl acetate and then heated *in vacuo* at 100° for several hours.

Found: C = 73.0 % H = 10.5 % M.p. = 175–6°

Required for DCA: C = 73.43 % H = 10.27 % M.p. = 176°

DCA was found to dissolve in 1:1 proportions in benzyldiphenyl (BDP) and tritolyl phosphate (TTP) at 150°; the resultant solutions start to precipitate DCA at about 80° and 100°, respectively. Solutions in these two solvents were used (at 78.5° and 100°) in subsequent investigations. Column packings were prepared by mixing solutions of DCA and BDP or TTP in *tert.*-butyl acetate with celite support material (15 % w/w stationary liquid). A flame ionisation detector was used, and retention times were determined with a stop watch. All retention times were measured relative to a value of 100 for 2,2,5-trimethylhexane (a molecule which is probably too broad to form an inclusion compound with DCA), or relative to other secondary standards calibrated directly against 2,2,5-trimethylhexane. Samples ranging from 2–5 μl of 1 % solutions in ether were used.

---

* Present address: Department of Physical Chemistry, The University, Leeds 2 (Great Britain).

Solution of n-alkanes and n-alkenes in DCA/TTP

The relative retentions of various classes of compounds are shown in Fig. 1 in which the logarithms of relative retentions in the mixed DCA/TTP phase are plotted against those in pure TTP. In such a comparison of most pairs of stationary phases[6,7] there results a family of almost parallel straight lines, one for each homologous series of compounds. The gradient of a line gives an indication of the relative affinity of the hydrocarbon part of the compound in question for either of the phases, and the relative separation of the lines indicates a greater or lesser affinity of the functional group of the homologous series for either of the phases. The frequently occurring family of nearly parallel lines, indicates that the affinity of the hydrocarbon chain for most phases is virtually independent of the functional group attached to the chain.

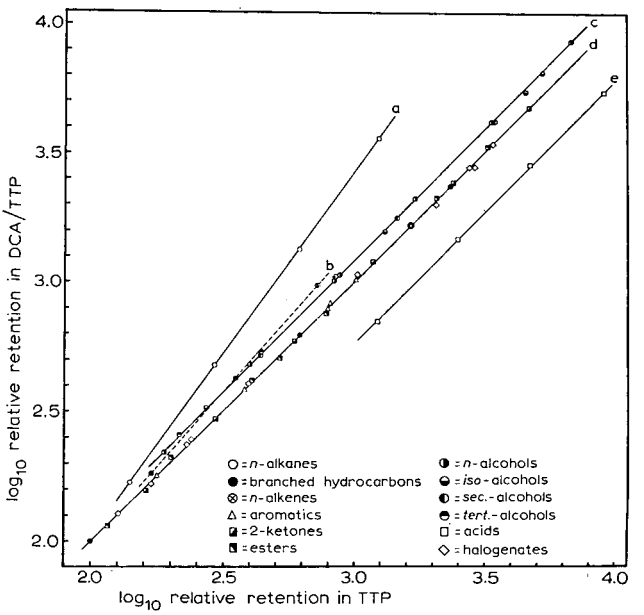


Fig. 1. The selectivity of desoxycholic acid dissolved in tritolyl phosphate at 100°. Compounds (in order of increasing retention): (a) Hydrocarbons: *n*-octane, *n*-nonane, *n*-decane, *n*-undecane. (b) Olefines: *n*-octene, *n*-nonene, *n*-decene. (c) Alcohols: *n*-propyl, *tert.*-amyl, isobutyl, *n*-butyl, *sec.*-amyl, *tert.*-hexyl, *n*-amyl, *sec.*-hexyl, isohexyl, *sec.*-heptyl, *n*-hexyl, *n*-heptyl, *sec.*-octyl, isooctyl, isononyl, *n*-octyl. (d) Miscellaneous: isononane, ethyl acetate, carbon tetrachloride, 2-butanone, chloroform, benzene, ethyl propionate, dichloroethane, butyl bromide, 2-pentanone, toluene, butyl iodide, ethyl butyrate, butyl acetate, 2-hexanone, pinene, propyl butyrate, *p*-xylene, *m*-xylene, *o*-xylene, hexyl bromide, 2-heptanone, mesitylene, phellandrene, bromoform, amyl butyrate, *trans*-decalin, 2-octanone, tetrachloroethane, pentachloroethane, hexyl butyrate, 2-nonanone. (e) Acids: formic, acetic, propionic, butyric. The *n*-alkene line has been dotted-in to avoid confusion on the diagram.

From Fig. 1 it is clear that the majority of compounds behave in a regular and predictable way. Thus 32 compounds in 5 widely different families (branched hydrocarbons, aromatics, esters, 2-ketones, and halogenated hydrocarbons) fall on the same straight line (line d) of gradient = 1, which passes through the origin. For these compounds, and the families to which they belong, there is no discernable qualitative

difference between pure tritolyl phosphate and the mixed phase (though of course it is to be expected that absolute retention volumes would show a quantitative difference between the two phases). Both the hydrocarbon chains and the functional groups behave in the same way in both liquid phases.

Equally it is clear that all classes of alcohols (line c) show a preference for the mixed DCA/TTP phase and that acids (line e) show a reverse effect and a greater affinity for the pure TTP phase. Yet both acids and alcohols lie on lines parallel to the line on which most other compounds fall. Thus, though these functional groups discriminate between the two phases, the hydrocarbon chains quite clearly do not.

Of these two anomalies, the preference of alcohols for the mixed phase is not surprising, for the opportunities for strong hydrogen bonding by alcohols are considerably enhanced in the carboxylic mixed phase compared with the pure TTP phase, and preferential retention of alcohols naturally results. The preference of carboxylic acids for pure TTP is only a little less expected. The solubility of an acid in an acid saturated phase might well be expected to be less than in a non-acidic phase. The hydrogen bonding ability of the mixed phase towards acids is already fully satisfied by the acid group of desoxycholic acid. This observation is in accord with LITTLEWOOD[8] who has concluded that polyglycol phases dissolve alcohols less than do phases with a lower hydroxyl-group concentration.

The most unusual selectivity of the mixed phase is shown towards *n*-alkanes (line a) and, to a lesser extent, towards *n*-alkenes (line b). These straight-chain hydrocarbons, in marked contrast to all other straight-chain compounds and also to branched-chain hydrocarbons, lie on a line inclined towards the mixed-phase axis. Thus while in pure TTP the addition of a methylene group to an *n*-alkane or an *n*-alkene (or indeed to any compound) adds 0.31 log units to the $\log_{10}$ retention, in the mixed-phase addition of a methylene group to an *n*-hydrocarbon adds 0.45 log units to the $\log_{10}$ retention for *n*-alkanes, and 0.36 log units for *n*-alkenes. This may be differently expressed by saying that in TTP all compounds approximately double their retentions for each addition of a methylene group, whereas in the mixed phase, while most compounds double their retentions per methylene group, *n*-alkenes increase theirs by a factor of 2.3, and *n*-alkanes by an unusual factor of 2.8.

Accompanying this large selective retardation of *n*-alkanes (and to a lesser extent of *n*-alkenes) there is a very considerable loss of column efficiency. The *n*-hydrocarbon peaks are markedly asymmetrical and very broad. Efficiency decreases with increasing retention to the extent that the emergence of an *n*-dodecane peak has never been observed. Other peaks are of more normal width. This is illustrated in Fig. 2, which shows a plot of the ratio of retention distance to peak width measured at the base line between tangents to the points of inflection (which is a measure of column efficiency) against relative retention. For most stationary phases such a plot has the shape of curve (d) in Fig. 2; column efficiency is low for compounds eluted rapidly but reaches a steady higher value for later components. This also holds true for most of the compounds which behave normally on DCA/TTP. The curve for branched hydrocarbons (d') is a little lower than that for many other species, but not unduly so. The curve for *n*-alcohols (c) is different from the norm (d) and flattens off at a lower column efficiency. In contrast, the curve for carboxylic acids (e) is a very shallow, though it seems to approach the same maximum as the alcohols. The acid peaks are slightly asymmetrical, but such behaviour is not uncommon with acids on most phases. Normal

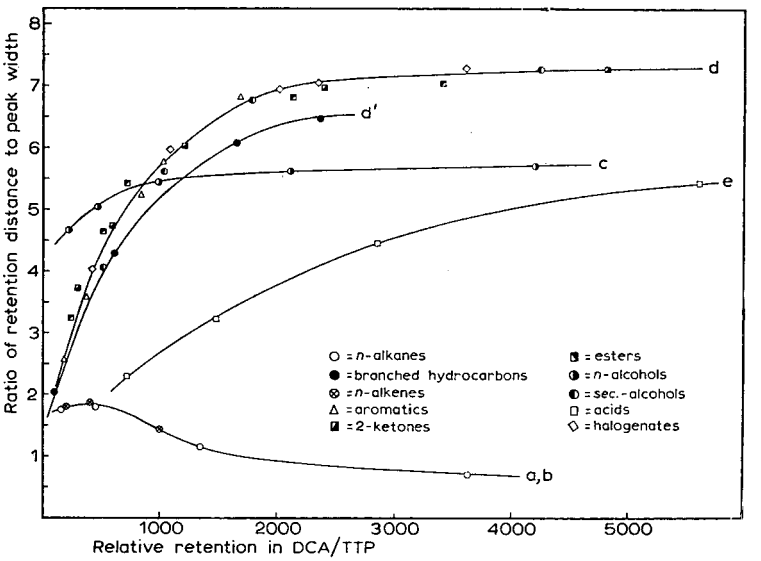


Fig. 2. Peak width variations on desoxycholic acid/tritolyl phosphate (DCA/TTP). Number of theoretical plates = 16 × (ratio of retention distance to peak width)^2.

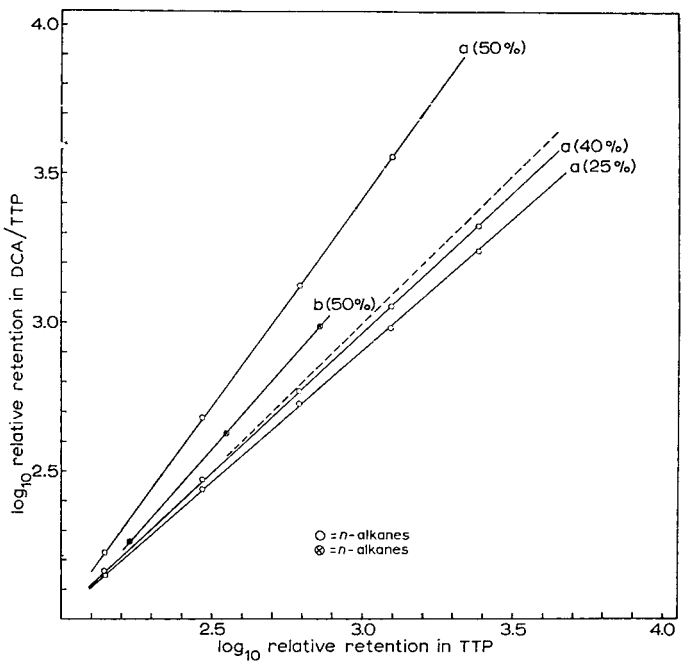


Fig. 3. The effect of desoxycholic acid concentration on the retention of hydrocarbons. The 40% and 25% values for *n*-alkenes are not shown, in order to avoid congestion on the diagram. (a) *n*-Alkanes: octane to dodecane. (b) *n*-Alkenes: octene to decene. Figures refer to percentage of DCA in TTP. The broken line is of unit slope and represents a situation in which there is no relative selectivity between the phases.

alkanes and alkenes (a, b) show unexpectedly high peak-widths—between 2 and 10 times those of other compounds. Moreover, there is an anomalous and marked decrease in efficiency with increasing retention. It seems that accompanying the considerable retention of these compounds, there is a correspondingly slow equilibrium on the column, with a consequent decrease in efficiency.

Solution of alkanes in more dilute DCA/TTP mixtures

A 40 % solution of DCA in TTP does not saturate till well below 100°. A 25 % solution is unsaturated at room temperature. The behaviour of *n*-hydrocarbons in these solvents at 100° was investigated; the results are plotted in Fig. 3. It can be seen that the selectivity shown by the saturated DCA/TTP solution is not shown by these unsaturated solutions. What is more, the addition of less DCA than is required to form a saturated solution decreases the hydrocarbon selectivity of the mixed phase to a value below that of pure TTP. This is quite marked in the case of the 25 % solution.

Solution of alkanes in DCA/BDP

Measurements have been made with a column at 78.5° containing a saturated solution of DCA in BDP. As with tri-*o*-thymotide in BDP[1] a settling down effect was noticed; with the exception of aromatic hydrocarbons, the initial retentions in DCA/BDP are in general lower than the equilibrium retentions measured after the column had been run at 78.5° for several days. This is shown in Fig. 4 in which equilibrium $\log_{10}$ relative retention of a series of compounds are plotted against log values obtained initially.

The preference which is shown for the DCA/BDP phase at equilibrium may be interpreted as the results of slow dissolution of DCA in BDP, which leads to an increased relative retention for polar compounds. The increase in the relative retention of *n*-alkanes is not however open to this interpretation, and will be discussed later.

DCA/BDP does not show the marked selectivity for *n*-alkanes or *n*-alkenes which was observed in DCA/TTP. Indeed, the equilibrium retention values of these hydrocarbons in DCA/BDP are little different from the retention values found in BDP alone; the intial relative retentions in Fig. 4 for *n*-alkanes are in fact lower than those found for pure BDP. Peak widths too are quite regular.

Addition of hydrocarbons to saturated DCA/TTP and DCA/BDP solutions under static conditions

Small drops (~ 0.05 ml) of *n*-nonane and 2,2,5-trimethyl hexane were added to clear saturated TTP and BDP solutions above some residual undissolved desoxycholic acid in test tubes at 100°. Both the branched and straight-chain isomers produced a marked precipitate when added to the DCA/TTP solution; the precipitate in the case of DCA/BDP was much less marked and required several drops of hydrocarbon to produce effects similar to those produced by one drop in DCA/TTP. Gas chromatographic analysis of extracts from these precipitates showed some traces of *n*-nonane; no 2,2,5-trimethylhexane was found.

DISCUSSION

A saturated solution of DCA in TTP strongly retards *n*-alkanes and to a lesser extent *n*-alkenes; this retention is accompanied by a loss of column efficiency and

asymmetrical peaks. At the same time, the hydrocarbon chains of other compounds behave in the mixed phase in much the way they do in pure TTP; their retentions follow the same pattern as the retentions of branched hydrocarbons. A saturated solution of DCA in BDP retards *n*-alkanes and *n*-alkenes in much the same way as does BDP alone. Unsaturated solutions of DCA/TTP are less selective towards *n*-alkanes and *n*-alkenes than is pure TTP; the same is true of DCA/BDP. Saturated solutions of DCA in TTP precipitate *n*-alkane complexes when *n*-alkanes are added; this effect with saturated solutions of DCA in BDP is less marked. Branched alkanes produce precipitates which do not contain trapped hydrocarbon molecules.

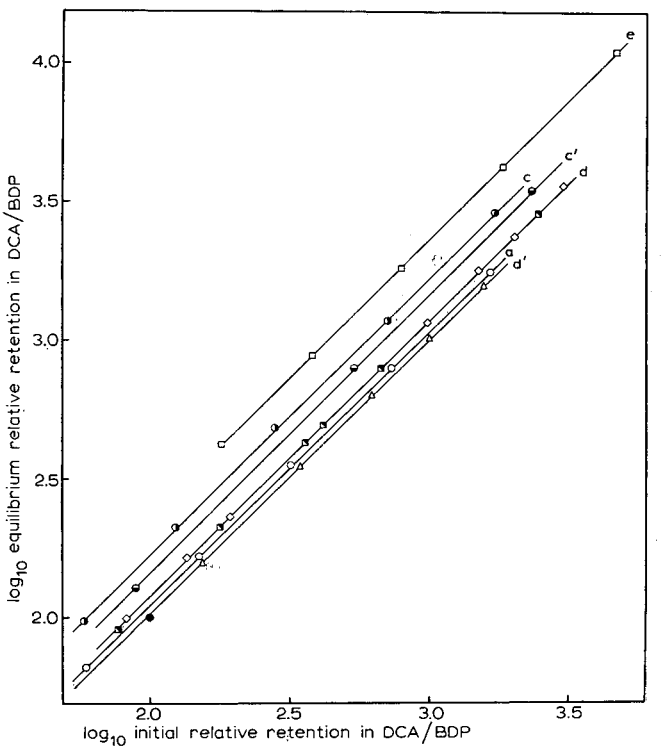


Fig. 4. Equilibrium effects in desoxycholic acid/benzyldiphenyl (DCA/BDP). For key, see Fig. 1. (a) *n*-Alkanes: octane to undecane. (c) *n*-Alcohols: propyl to heptyl. (c') *iso*-Alcohols: butyl, hexyl, octyl. (d) Esters and halogenates: ethyl acetate, chloroform, dichloroethane, ethyl propionate, butyl bromide, ethyl butyrate, butyl acetate, propyl butyrate, hexyl bromide, bromoform, tetrachloroethane, pentachloroethane, amyl butyrate, hexachloroethane. (d') Branched alkane and aromatics: isononane, benzene to mesitylene.

These observations show that there is a quite marked difference between desoxycholic acid dissolved in tritolyl phosphate and comparable tri-*o*-thymotide solutions[1]. Tri-*o*-thymotide differentiates quite distinctly between branched and straight chains, but does so uniformly, whatever the functional group attached to the hydrocarbon chain. Desoxycholic acid, on the other hand, differentiates between

branched and straight chains only in hydrocarbons; the presence of polar functional groups removes any shape specific selectivity.

We suggest that the shape selectivity of saturated DCA/TTP solutions is the result of the ability of straight chain hydrocarbons to form inclusions in the open DCA crystal structure, inclusions for which branched chains are too broad. A saturated solution of DCA in TTP may be considered to have a fairly open structure into which straight chains can readily fit. Furthermore, the presence of hydrocarbons in the solution, which causes precipitation on a macro-scale, may well cause straight chains to become partially trapped in the solution the GLC column. Such a trapping process, followed by a slow dissolution of the inclusion complex, would cause an increase in the resistance to mass transfer across the gas–liquid interface, a consequent loss of column efficiency, and asymmetrical peaks.

It is commonly found that polar solvents (tritolyl phosphate, benzyldiphenyl, 7:8-benzoquinoline etc.) discriminate quite extensively between hydrocarbon shapes, whereas the emergence order of hydrocarbons on columns containing non-polar solvents is in strict order of boiling points. It may well be that intermolecular interaction forces in polar solvents give the liquids a measure of open structure similar to DCA/TTP. The effect of introducing a bulky hydrocarbon molecule into such solvents will tend to disrupt solvent intermolecular forces to a greater extent than would the introduction of a narrow, straight-chain hydrocarbon molecule. The effect of size differences on the average solvent forces will be most noticeable if the solvent interaction forces are large (polar solvent–intermolecular forces), and it is therefore reasonable that such effects should be most obvious in the case of the dissolution of hydrocarbons, for these inert molecules have no means of compensating for any disruption which they cause; polar solutes can replace solvent intermolecular polar forces by solvent–solute intermolecular polar forces. This explanation accounts for the loss of selectivity in unsaturated solutions of DCA in TTP or BDP. In both BDP and TTP, the pure solvent possesses a shape selective structure in its own right. In the case of TTP, saturation with DCA produces a solution with a better shape selective structure than the parent solvent; with BDP the result is a solution whose properties are little different from the parent solvent. Less than adequate amounts of DCA succeed only in destroying the shape selectivity of the pure solvent by disrupting whatever solvent structure is responsible for shape selectivity, without replacing it by a new and (as with DCA/TTP) more effective structure.

The failure of DCA/BDP solutions to act in the same way as DCA/TTP is probably due to the greater affinity of hydrocarbons for the hydrocarbon BDP than for polar TTP. The fact that the introduction of hydrocarbons into saturated solutions has less tendency to cause DCA to precipitate from DCA/BDP than from DCA/TTP, supports this view. It may be that the presence of polar TTP molecules forces hydrocarbon molecules into the vicinity of DCA molecules and consequently makes precipitation and inclusion an easier process.

CONCLUSIONS

The use of a saturated solution of DCA in TTP produces an enhanced *n*-/*iso*-hydrocarbon separation, but does so only at the cost of an increasingly diminished column efficiency for the anomalously retarded component.

ACKNOWLEDGEMENTS

The authors are indebted to A. B. LITTLEWOOD and H. M. POWELL for helpful discussions, D. H. DESTY for a gift of branched hydrocarbons, Imperial Chemical Industries for the loan of a recorder and the Scientific Research Council for a maintenance grant (A.O.S.M.).

SUMMARY

Desoxycholic acid is known to form inclusion compounds with molecules of restricted width. Retention times have been measured using column liquids containing desoxycholic acid dissolved in either tritolyl phosphate or benzyldiphenyl, and these have been compared with retention times for pure tritolyl phosphate and benzyldiphenyl. The mixed phase containing benzyldiphenyl does not show any appreciable selectivity for *n*-hydrocarbons relative to their branched homologues, but that containing tritolyl phosphate strongly retards *n*-hydrocarbons with an unexpectedly large log retention increment per methylene group. Accompanying this large retention, the anomalously retarded peaks are asymmetrical and unusually broad, to the extent that it is impossible to detect the emergence of *n*-dodecane from this column. The peaks of all other compounds are of more normal width, and their retentions conform to established patterns.

REFERENCES

1 A. O. S. MACZEK AND C. S. G. PHILLIPS, in R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, London, 1961, p. 284.
2 F. CRAMER, *Einschlussverbindungen*, Springer, Berlin, 1954, p. 30.
3 H. WIELAND AND H. SORGE, *Z. Physiol. Chem.*, 97 (1916) 1.
4 O. KRATKY AND G. GIACOMELLI, *Makromol. Chem.*, 69 (1936) 4.
5 L. FIESER AND M. NEWMAN, *J. Am. Chem. Soc.*, 57 (1935) 1602.
6 A. B. LITTLEWOOD, *Gas Chromatography*, Academic Press, New York and London, 1962, p. 104–106.
7 H. PURNELL, *Gas Chromatography*, Wiley, New York, 1962, pp. 227–228.
8 A. B. LITTLEWOOD, *J. Gas Chromatog.*, 1 (1963) 16.

AROMATIC INTERACTIONS IN GAS CHROMATOGRAPHY: THE USE OF 2,4,6-TRINITROPHENETOLE AS A COLUMN LIQUID

A. O. S. MACZEK* AND C. S. G. PHILLIPS
Inorganic Chemistry Laboratory, Oxford (Great Britain)
(Received December 27th, 1966)

INTRODUCTION

Several workers have tried to take advantage of π-interactions to separate aromatic hydrocarbon isomers—most especially *m*- and *p*-xylene. The most commonly used substrate has been a 1:1 mixture of fluorene and picric acid[1–6] though FABRIZIO *et al.*[7] have used dibutylphthalate and picric acid, and SMITH[8] has used *p*-nitroaniline picrate. PETRANEK AND SLOSAR[9] have recommended the 3,5-dinitrobenzoate of ethylene glycol, which they claim has better temperature stability than fluorene–picric acid, and is moreover more selective for ethyl benzene and higher alkylbenzenes. NORMAN[10] has advocated 2,4,7-trinitrofluorenone as a selective aromatic phase, and this has been used to separate *o*-, *m* and *p*-nitrotoluenes by ACHE *et al.*[11]. Other suggested aromatic selective substrates include phenanthrene and 7,8-benzoquinoline[12], α-napthylamine[13], nitronaphthalene and chloronaphthalene[14], and *m*-phenylenediamine[15]. JANAK AND HRIVNAC[16] have reviewed several cases of aromatic selectivity in terms of π-interactions. The investigations of LANGER *et al.*[17,18] and PINES AND CHEN[19] have shown that tetrahalophthalate esters provide a good means of separating *m*- and *p*-isomers. These esters appear to act differently from other aromatic selective phases in that they reverse the usual *p*-, *m*-xylene emergence order. More recently LITTLEWOOD[20] has questioned the existence of specific aromatic π-interactions in many cases where such interactions are invoked to explain experimental data.

The work described below attempts to determine the existence and extent of some specifically aromatic interactions in gas chromatography. The substrate used, 2,4,6-trinitrophenetole, has a conveniently low melting point (78.5°) and is closely related to picric acid, whose ability to form aromatic π-complexes is well established.

PREPARATION OF THE COLUMN

2,4,6-Trinitrophenetole was produced *in situ* in the column by coating picryl chloride (B.D.H.Ltd.) onto prepared celite from ethanol solution, and then passing carrier gas, saturated at room temperature with ethanol, through the column at 100° for several hours.

The column was originally packed with 30 % of picryl chloride by weight. Micro-analysis of the column material after passing ethanol showed less than 0.1 % residual chlorine.

* Present address: Department of Physical Chemistry, The University, Leeds 2 (Great Britain).

RESULTS

Several compounds were run on 2,4,6-trinitrophenetole (TNP) at 83.5°. The results are plotted in Fig. 1 with tritolyl phosphate (TTP) as a reference phase. Retentions in both these substrates are measured relative to benzene taken as an arbitrary 100, and the logarithms of relative retentions are compared.

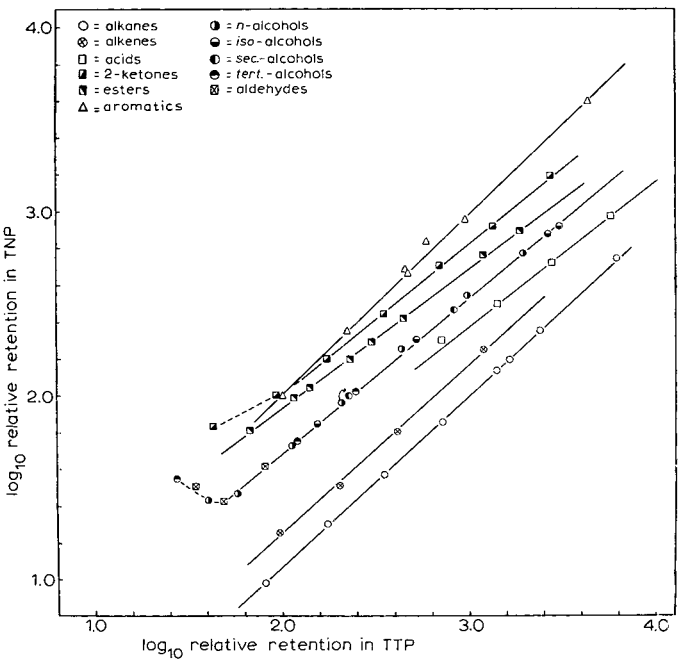


Fig. 1. The selectivity of 2,4,6-trinitrophenetole relative to tritolyl phosphate. Compounds (in order of increasing retention): alkanes: *n*-octane, *n*-nonane, *n*-decane, *n*-undecane, *n*-dodecane, *trans*-dekalin, *cis*-dekalin, *n*-tetradecane; alkenes: *n*-octene, *n*-nonene, *n*-decene, *n*-dodecene; acids: formic, acetic, propionic, butyric; alcohols: *tert.*-butyl, methyl, ethyl, *n*-propyl, *tert.*-amyl, isobutyl, *n*-butyl, *sec.*-amyl, *tert.*-hexyl, *n*-amyl, isohexyl, *sec.*-hexyl, *n*-hexyl, *n*-heptyl, isooctyl, isononyl; aldehydes: formaldehyde, acetaldehyde, propionaldehyde; esters: ethyl acetate, propyl acetate, butyl acetate, ethyl propionate, ethyl butyrate, propyl butyrate, amyl butyrate, ethyl caproate; methyl ketones: methyl, ethyl, propyl, amyl, hexyl, heptyl; aromatics: benzene, toluene, *m*-xylene, *p*-xylene, *o*-xylene, mesitylene, tetralin.

Several trends are clear from Fig. 1. Relative to benzene, all classes of compounds other than aromatic hydrocarbons show a preference for the TTP phase. Of these, the most marked preference for TTP is shown by aliphatic hydrocarbons. These are so strongly rejected by the TNP column that on this latter, benzene, boiling at 80.1°, emerges mid-way between *n*-undecane (b.p. = 195.8°) and *n*-dodecane (b.p. = 214.5°). The most strongly alkane/aromatic discriminating columns in the literature are β,β′-oxydipropionitrile (KELKER[21]) and 1,2,3-tris(2-cyanoethoxy)propane (MCNAIR[22]), on both of which benzene emerges between *n*-decane and *n*-undecane *i.e.*, one carbon number lower than on 2,4,6-trinitrophenetole. This rejection of alkanes makes the measurement of alkane retentions subject to quite appreciable error—

especially since those *n*-alkanes with appreciable retentions do not give very good peaks due to difficulties of volatilisation. Olefines have a predictably greater preference for the TNP medium than alkanes.

The straight line plots for different classes of compounds (hydrocarbons, acids, alcohols, ketones and esters) are not strictly parallel to each other. The lines for alkanes, alkenes and aromatic hydrocarbons all have a gradient = 1; the remainder have lesser slopes (~ 0.80–0.83) and the first member of several homologous series (*e.g.* formaldehyde, methanol, dimethyl ketone, formic acid) shows a preference towards the TNP phase. These trends may be the result of a progressive rejection of polar compounds as their hydrocarbon chains increase in length, a rejection analogous to the overall rejection of aliphatic hydrocarbons by TNP.

o- and *p*-xylene show an appreciable specific retardation on the TNP substrate. The order *p*-, *m*-, *o*- on TTP becomes *m*-, *p*-, *o*- on 2,4,6-trinitrophenetole. Table I shows relative retentions of *o*-, *m*- and *p*-xylene taking *o*-xylene as an arbitrary 100. The bulk of the data are taken from LITTLEWOOD[23] and 2,4,6-trinitrophenetole is shown for comparison.

It may be seen that TNP gives a very good *o*-xylene/*m*-xylene separation, though its *m*-/*p*-separation (= 0.966) is not so good as that of dipropyl tetrachlorophthalate (= 0.959). It seems probable, in view of the reverse *m*-/*p*-elution order, that 2,4,6-trinitrophenetole exerts on both *o*- and *p*-xylene a specific effect involving aromatic π-bonding. This point will be discussed further in the next section.

TABLE I

RELATIVE RETENTIONS OF XYLENES

	Squalane	*Benzyl-diphenyl*	*Tritolyl phosphate*	*7,8-Benzo-quinoline*	*Dipropyl tetrachloro-phthalate*	*Methyl propyl tetrachloro-phthalate*	*2,4,6-Trinitro-phenetole*
	78.5°	*78.5°*	*78.5°*	*78.5°*	*110°*	*110°*	*83.5°*
o-Xylene	100	100	100	100	100	100	100
m-Xylene	83.9	80.0	78.2	78.0	75.7	75.0	68.8
p-Xylene	82.8	76.2	76.1	72.2	79.0	78.0	71.1

AROMATIC INTERACTIONS

Aromatic interactions of the kind considered here can conveniently be presented in the form of a special plot developed by ROHRSCHNEIDER[24], an example of which is shown in Fig. 2. In such a plot, log *r*, the logarithm of the retention of any substance relative to a standard (*n*-octane in this case), is measured along the vertical axis. The standard itself is represented by a horizontal line log $r = 0$. The vertical axis then represents a paraffinic stationary phase (*e.g.* squalane). A line of unit slope is now drawn from a point on the vertical axis at a value of log *r* for a reference compound (*n*-octene here). This line now represents this reference compound. Any line drawn parallel to the vertical axis intersects both the reference and the standard lines; the distance between the two intersections gives a particular value for log *r* for these two compounds. Therefore, if such lines are fitted along the standard line such that the

distance between the intersections is an experimental value of log r for a given stationary phase, stationary phases will be arranged in order along an axis calibrated in terms of the relative retentions of the two (reference and standard) compounds.

LITTLEWOOD[20] has surveyed the selectivity of stationary phases in GC using such plots. As a part of his survey he has extended the data of ROHRSCHNEIDER[24] to include an examination of the assumed π-interactions of aromatic hydrocarbons.

Table II shows the retentions of *n*-octene (reference) and benzene, relative to

TABLE II

RETENTIONS RELATIVE TO *n*-OCTANE = 1.00

Stationary phase	*n-Octene*		*Benzene*		*Source*
	Relative retention	*log r*	*Relative retention*	*log r*	
BDP (benzyldiphenyl)	1.135	0.055	0.892	—0.05	
TTP (tritolyl phosphate)	1.218	0.086	1.235	0.092	
DNP (dinonyl phthalate)	1.021	0.009	0.616	—0.210	MCNAIR AND DEVRIES[22]
TCEP [1,2,3-tris(2-cyano-ethoxy)propane]	1.890	0.276	8.34	0.921	MCNAIR AND DEVRIES[22]
SQN (squalane)	0.886	—0.053	0.332	—0.480	MCNAIR AND DEVRIES[22]
DBTCP (di-*n*-butyl tetra-chlorophthalate)	1.071	0.030	0.871	—0.060	LANGER *et al.*[18]
TNP (2,4,6-trinitrophenetole)	1.845	0.266	10.41	1.017	

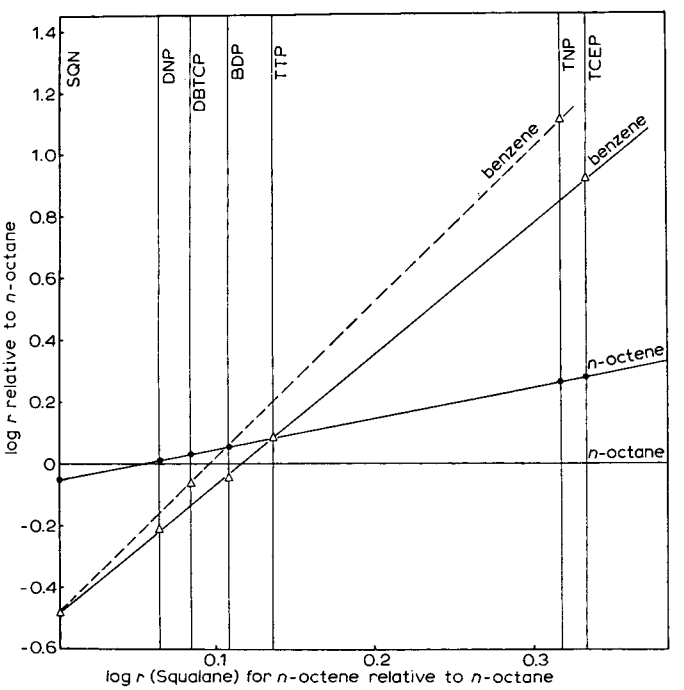


Fig. 2. ROHRSCHNEIDER plot: benzene relative to *n*-octane and *n*-octene. SQN = Squalane; DNP = dinonyl phthalate; DBTCP = dibutyl tetrachlorophthalate; BDP = benzyldiphenyl; TTP = tritolyl phosphate; TNP = 2,4,6-trinitrophenetole; TCEP = 1,2,3-tris(2-cyanoethoxy)-propane.

n-octane (standard). These include our results on TNP, and values taken from the references indicated. The Rohrschneider plot of these data is shown in Fig. 2; it has an extended horizontal scale for added clarity.

It may be seen that on five phases (squalane, dinonyl phthalate, benzyldiphenyl, tritolyl phosphate and 1,2,3-tris(2-cyanoethoxy)propane) the order of elution of benzene is the same as for octene. This has already been pointed out by LITTLEWOOD[20] who deduced from his results that there was no specific aromatic π-bonding in such solvents. 1,2,3-tris(2-cyanoethoxy)propane in particular is surprisingly normal, despite its apparent preferential strong retention of benzene mentioned earlier. Two substrates in Fig. 2, however, retain benzene appreciably more than the others. On dibutyl tetrachlorophthalate (DBTCP) and on 2,4,6-trinitrophenetole (TNP) (for which the benzene points lie on a straight line which passes through the point for benzene on the squalane reference phase) there is probably a real π-aromatic interaction effect. It is significant that on both of these substrates the *m*-xylene–*p*-xylene elution order is different from that on all the other phases (see Table I).

It is unfortunately not possible to include any other supposedly π-interacting substrates on Fig. 2 owing to a lack of suitable data (*n*-octane: *n*-octene: benzene) in the literature. Fig. 3 however, shows a ROHRSCHNEIDER plot of benzene relative to cyclohexane and cyclohexene; retention values are taken from RAUPP[5] and BROOKS[25]. The linearity of the benzene plot is once again remarkably good, and this time covers six substrates: nujol, diethylhexyl sebacate, dinonyl phthalate, polypropylene glycol,

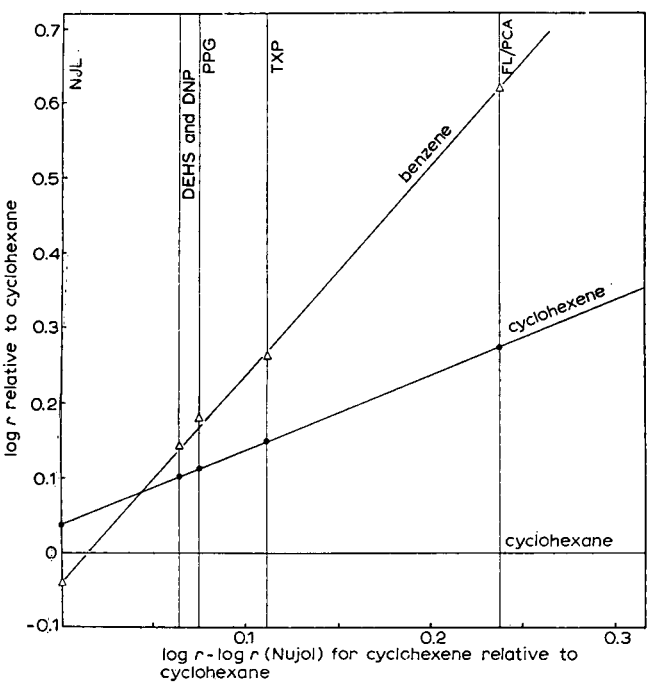


Fig. 3. ROHRSCHNEIDER plot: benzene relative to cyclohexane and cyclohexene. NJL = Nujol; DEHS = diethylhexyl sebacate; DPN = dinonyl phthalate; PPG = polypropylene glycol; TXP = trixylenyl phosphate; FL/PCA = fluorene–picric acid.

trixylenyl phosphate and 1:1 fluorene–picric acid (*i.e.* the fluorene picrate π-complex). This latter liquid behaves no differently from the others so far as aromatic selectivity is concerned, which could perhaps have been foretold from its quite normal elution order (*p*-, *m*-, *o*-xylene). Moreover it is perhaps too optimistic to expect the strong fluorene picrate complex to dissociate in favour of a weaker benzene complex, which would have to happen for there to be a specific selective effect on benzene. A substrate with more than 1:1 picric acid in fluorene may well belong to the DBTCP, TNP π-complexing class of compounds.

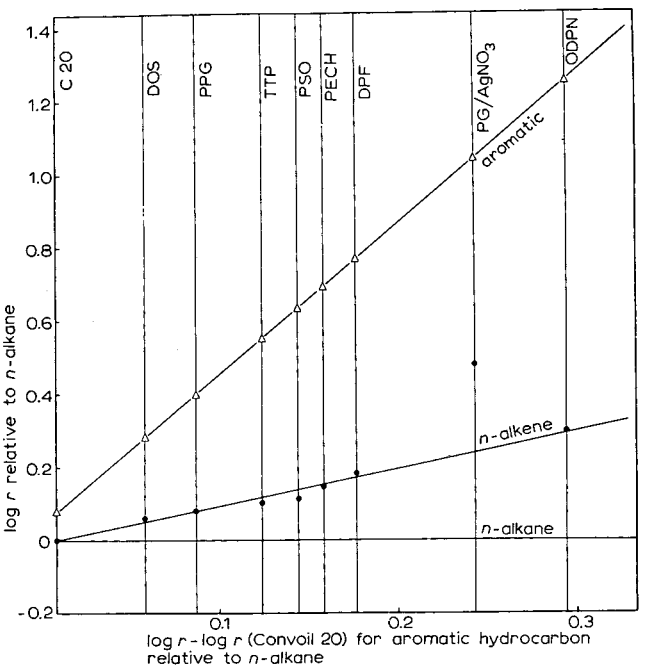


Fig. 4. ROHRSCHNEIDER plot: aromatic hydrocarbon relative to *n*-alkane and *n*-alkene all at a common boiling point of 100°. C20 = Convoil 20; DOS = dioctyl sebacate; PPG = polypropylene glycol; TTP = tritolyl phosphate; PSO = polystyrene oxide; PECH = poly-epichlorohydrin; DPF = diphenyl formamide; $PG/AgNO_3$ = propylene glycol–$AgNO_3$; ODPN = β,β'-oxydipropionitrile.

Finally, Fig. 4 illustrates specific olefine selectivity. This plot is for three hypothetical compounds, an *n*-alkane, an *n*-alkene, and an aromatic hydrocarbon at a common boiling level of 100°, and shows the effect of silver nitrate on olefine retentions. Retention values are from the data of TENNEY[26] in which the hypothetical retentions of compounds at a common boiling level of 100° are obtained from plots of log retention against boiling point. In this case, however, substrates are arranged in the order of their retention of the aromatic relative to the alkane. The gradient of the aromatic line is arbitrarily fixed at a value of 4 for convenience.

It is clear that there is still a general overall agreement between the retentions of alkanes, aromatics and alkenes (though it is a less accurate agreement than in the previous figures—polystyrene oxide, for example, seems either to reject alkenes or to

retard aromatics specifically) except in the case of propylene glycol–silver nitrate. In this phase, the *n*-alkene is retained appreciably beyond the retention predicted from the trend in the other substrates. It can be seen that the presence of silver nitrate causes the *n*-alkene to be retained selectively by a factor of 1.74 ($\log r = 0.24$) on propylene glycol–silver nitrate, and that the well established use of silver nitrate for olefine separations is based on a real specific interaction.

FURTHER AROMATIC INTERACTIONS ON 2,4,6-TRINITROPHENETOLE

The results obtained with aromatics in the two previous sections and particularly the enhanced *o*-xylene–*m*-xylene separation, have prompted an investigation of a wider range of alkyl benzenes on 2,4,6-trinitrophenetole. Table III shows some relative retentions on TNP and tritolyl phosphate (TTP). In Fig. 5, relative retentions on TNP are plotted against those on TTP.

TABLE III

RETENTIONS OF SOME AROMATIC HYDROCARBONS RELATIVE TO BENZENE (= 100)

	2,4,6-Trinitrophenetole (TNP) (78.5°)	*Tritolyl phosphate (TTP)* (100°)
1. Benzene	100	100
2. Toluene	224	224
3. Ethylbenzene	308	448
4. *p*-Xylene	480	440
5. *m*-Xylene	464	468
6. *o*-Xylene	675	585
7. Isopropylbenzene	320	640
8. *n*-Propylbenzene	428	790
9. 1,3,5-Trimethylbenzene	900	920
10. 1,2,3-Trimethylbenzene	746	680
11. 1,2,4-Trimethylbenzene	1,290	1,090
12. *tert.*-Butylbenzene	437	985
13. *sec.*-Butylbenzene	390	1,090
14. *n*-Butylbenzene	730	1,590
15. *tert.*-Amylbenzene	633	1,690
16. 1,2,4,5-Tetramethylbenzene	3,800	2,730
17. Pentamethylbenzene	14,600	8,860
18. Styrene	905	731
19. Tetralin	3,870	4,300

In addition to the already mentioned *p*- and *o*-xylene selectivity which 2,4,6-trinitrophenetole exhibits, Fig. 5 shows that this substrate is specifically selective towards several inter-related categories. The first and largest division is between the set of compounds containing benzene, its methylated homologues and styrene, and the set consisting of other alkyl benzenes, each with more than one carbon residue in the alkyl chain. Within the two sets formed by these compounds there is further discrimination.

Thus, benzene, toluene, *m*-xylene and mesitylene all fall on the same line, of gradient 1, whereas *p*-xylene, *o*-xylene, 1,2,3-trimethylbenzene and 1,2,4-trimethylbenzene show an increasing retardation greater than the first set. The second set con-

sists of methyl compounds which contain one or more *o*- or *p*-grouping, whereas those without substituents of the latter kind are all in the first set. Styrene is selectively retarded even more than any of these *o*- and *p*-substituted methylbenzenes, probably as a result of its additional double-bond. The alkylbenzenes with more complex side chains fall into three interrelated sets which derive from toluene. Thus the *n*-alkylbenzenes fall on one straight line, *tert.*-alkylbenzenes on another and *sec.*-alkylbenzenes on a third.

Since benzene shows a specific interaction with TNP (Fig. 2) it is probable that benzene, toluene, and the other similarly retarded homologues all interact specifically with TNP to a similar extent. It seems that an *o*- or *p*-disposal of methyl groups enhances this specific interaction, and that it is further enhanced by the presence of an olefinic grouping in the alkyl chain.

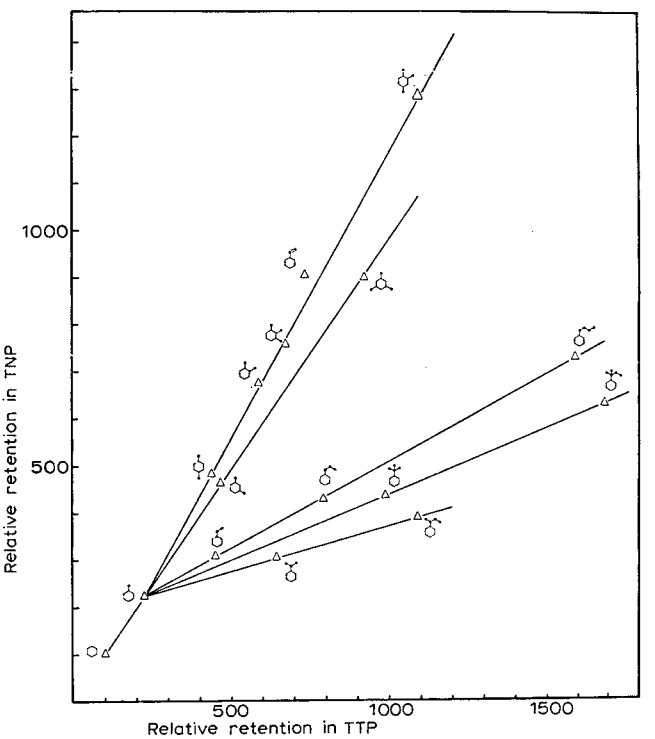


Fig. 5. Aromatic selectivity in 2,4,6-trinitrophenetole.

An increase in the length of the side chain beyond one carbon atom markedly decreases the affinity of the aromatics for TNP. This is almost certainly a size consideration; the bulk of an interacting molecule must play a large part in the resultant strength of π-interactions. The effect, though regular, is not altogether simple, for although these alkyl benzenes are less retarded on TNP than they are on TTP, tertiary isomers are retarded more on the TNP phase than are secondary ones. Again this is probably the result of steric considerations, albeit somewhat unusual ones.

ACKNOWLEDGEMENTS

The authors are indebted to A. B. LITTLEWOOD and H. M. POWELL for helpful discussions, to Imperial Chemical Industries for the loan of a recorder, and to the Scientific Research Council for a maintenance grant (A.O.S.M.).

SUMMARY

Retention times measured on a column containing 2,4,6-trinitrophenetole show that this substrate possesses considerable aromatic selectivity. On 2,4,6-trinitrophenetole, benzene emerges between *n*-undecane and *n*-dodecane, and *m*-xylene precedes *p*-xylene, which is the reverse of the usual order. The major part of the difference in the retention of aliphatic and aromatic compounds appears to act by a mechanism which rejects aliphatics rather than one which preferentially retains aromatics. However, an analysis of the results using a ROHRSCHNEIDER plot shows that this substrate is nevertheless specifically selective towards aromatic compounds. Moreover, compared with tritolyl phosphate, 2,4,6-trinitrophenetole strongly retards *o*- and *p*-xylene and retards methyl substituted aromatic hydrocarbons significantly more than it retards those substituted with more bulky groups.

REFERENCES

1 A. I. M. KEULEMANS, A. KWANTES AND P. ZAAL, *Anal. Chim. Acta*, 13 (1955) 357.
2 B. T. WHITMAN, in D. H. DESTY (Editor), *Vapour Phase Chromatography*, Butterworths, London, 1957, p. 194.
3 B. T. WHITMAN, in D. H. DESTY (Editor), *Vapour Phase Chromatography*, Butterworths, London, 1957, p. 395.
4 R. E. RIPPERE, in V. J. COATES, H. J. NOEBELS AND A. S. FAGERSON (Editors), *Gas Chromatography*, Academic Press, New York, 1958, p. 223.
5 G. Z. RAUPP, *Anal. Chem.*, 164 (1958) 135.
6 T. MAEDA AND M. FUJII, *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 62 (1959) 649.
7 F. A. FABRIZIO, R. W. KING, C. C. CERATO AND J. W. LOVELAND, *Anal. Chem.*, 31 (1959) 2060.
8 B. SMITH, *Acta Chem. Scand.*, 13 (1959) 887.
9 J. PETRANEK AND J. SLOSAR, *Collection Czech. Chem. Commun.*, 26 (1961) 2667.
10 R. O. C. NORMAN, *Proc. Chem. Soc.*, (1958) 151.
11 H. J. ACHE, W. HERR AND A. THIEMANN, *Int. At. Energy Agency Symp., Vienna, 1961*, preprints paper TTS/10.
12 A. GOLDUP AND W. T. SWANTON, *Brit. Pat.*, 864611 (1961).
13 T. ARAKI AND R. GORO, *Bull. Chem. Soc. Japan*, 33 (1960) 115.
14 A. ZLATKIS, S. LING AND H. R. KAUFMAN, *Anal. Chem.*, 31 (1959) 945.
15 L. C. CASE, *J. Chromatog.*, 6 (1961) 381.
16 J. JANAK AND M. HRIVNAC, *J. Chromatog.*, 3 (1960) 297.
17 S. H. LANGER, C. ZAHN AND M. H. VIAL, *J. Org. Chem.*, 24 (1959) 423.
18 S. H. LANGER, C. ZAHN AND G. PANTAZOPLOS, *J. Chromatog.*, 3 (1960) 154.
19 H. PINES AND C. T. CHEN, *J. Am. Chem. Soc.*, 82 (1960) 3562.
20 A. B. LITTLEWOOD, *J. Gas Chromatog.*, 1 (1963) 16.
21 H. KELKER, *Angew. Chem.*, 71 (1959) 128.
22 H. MCNAIR AND T. DEVRIES, *Anal. Chem.*, 33 (1961) 806.
23 A. B. LITTLEWOOD, *Gas Chromatography*, Academic Press, New York, London, 1962, p. 405.
24 L. ROHRSCHNEIDER, *Z. Anal. Chem.*, 170 (1959) 256.
25 V. T. BROOKES AND G. A. COLLINS, *Chem. Ind. (London)*, (1959) 921.
26 H. M. TENNEY, *Anal. Chem.*, 30 (1958) 2.

ÜBER DIE PROGRAMMIERTE GASCHROMATOGRAPHIE

III. DIE DOPPELT PROGRAMMIERTE GASCHROMATOGRAPHIE UND IHRE ANALYTISCHE ANWENDUNG

L. MÁZOR UND J. TAKÁCS
Institut für Allgemeine und Analytische Chemie, Technische Universität, Budapest (Ungarn)
(Eingegangen den 21. April 1966)

Bei der Gaschromatographie kommt es oft vor, besonders wenn man bei der oberen Temperaturgrenze der Kolonnen-Verteilungsflüssigkeit arbeitet, dass gewisse Komponenten sehr verzerrt und mit hoher Retentionszeit erscheinen, da die übrigen Komponenten der Probe bei dieser Temperatur keine höhere Strömungsgeschwindigkeit gestatten. Dies war u.a. unsere Erfahrung bei der gaschromatographischen Analyse von Lavendelölen, nachdem das optimale Temperaturprogramm erreicht wurde (Fig. 1). In solchen Fällen bestehen nur zwei Möglichkeiten zur Verminderung der Analysenzeit bzw. zur Verbesserung des verzerrten Teiles des Chromatogramms: Trägergasprogrammierung[1] und Rückströmung[2].

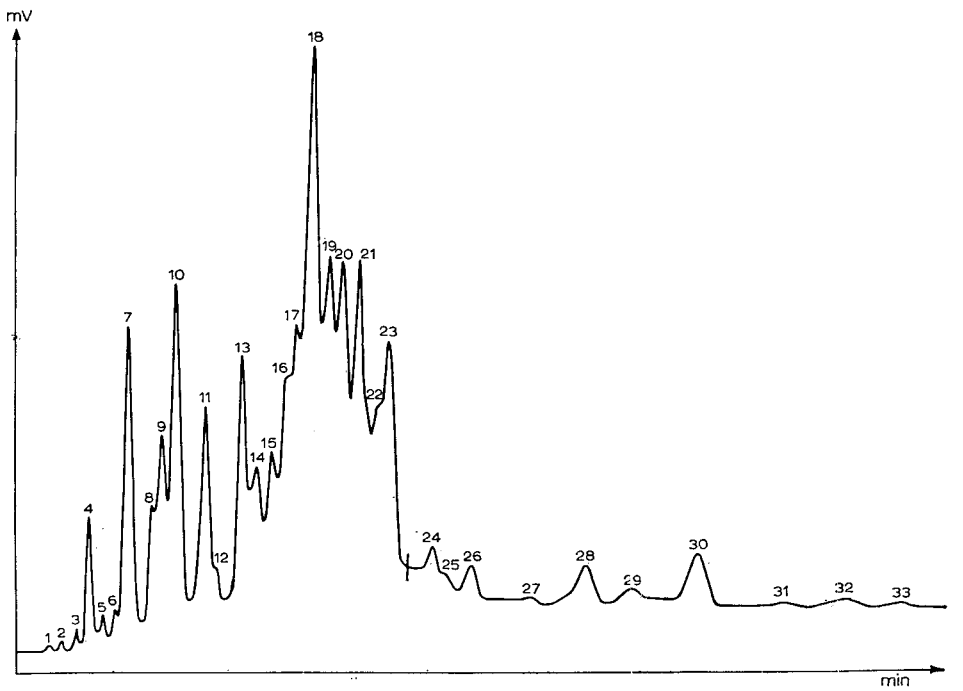


Fig. 1. Chromatogramm von Lavendelöl bei optimalem Temperaturprogramm.

Auf Grund unserer früheren Erfahrungen mit Trägergasprogrammierung wählten wir die erste Möglichkeit. Das mit Trägergasprogrammierung erhaltene Chromatogramm der Lavendelöle (Fig. 2) zeigt einerseits die in dem kritischen Gebiet der Fig. 1 erscheinenden Komponenten mit deutlichen Zacken und geringeren Retentionszeiten, andererseits auch Zacken von solchen Komponenten, die in dem gewöhnlichen Chromatogramm infolge Diffusionserscheinungen nicht zu sehen sind.

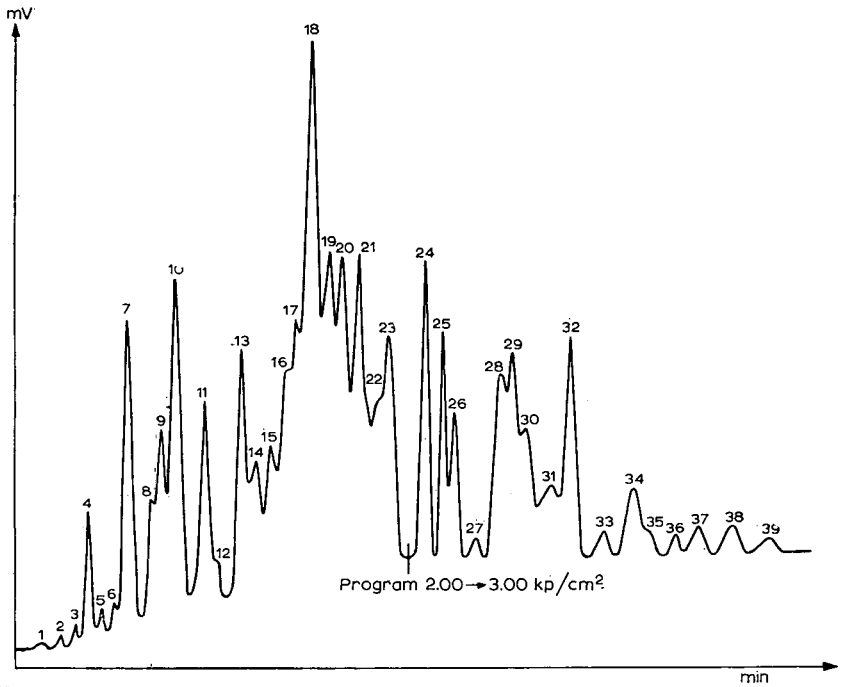


Fig. 2. Doppelt programmiertes Chromatogramm von Lavendelöl.

Nach diesen gelungenen Versuchen begannen wir, theoretisch und praktisch die Methode der doppelten Programmierung ("double programmed gas chromatography" = DPGC) zu studieren. Doppelte Programmierung nennen wir die Methode, bei der innerhalb derselben Analyse Temperatur- und Trägergasprogrammierung angewandt werden. Hierzu bestehen prinzipiell fünferlei Möglichkeiten:

1. Temperaturprogramm–Trägergasprogramm nacheinander.
2. Trägergasprogramm–Temperaturprogramm nacheinander.
3. Trägergasprogramm und Temperaturprogramm gleichzeitig.
4. Temperaturprogramm, teilweise überdeckt mit Trägergasprogramm.
5. Trägergasprogramm, teilweise überdeckt mit Temperaturprogramm.

In dieser Arbeit wollen wir nur Möglichkeit (1) behandeln.

Zum Studium der Grundkombination von Temperaturprogrammierung–Trägergasprogrammierung nacheinander benötigen wir ein Grundchromatogramm: Ein bei dem optimalen Temperaturprogramm und mit einer der Mehrzahl der Komponenten entsprechenden konstanten Strömungsgeschwindigkeit aufgenommenes Chromatogramm, das die Temperaturwerte nicht übertreffen können. Dem weiteren

Temperaturanstieg kann die obere Temperaturgrenze der verwendeten Verteilungsflüssigkeit oder der Umstand, dass bei höherer Temperatur einzelne Zacken ineinanderfliessen, Einhalt gebieten. Dieses Grundchromatogramm dient zum Aufbau des Trägergasprogrammes, das seinem Charakter nach auch von zweierlei Art sein kann, weshalb beide Möglichkeiten separat behandelt werden sollen. Zur einfacheren Übersicht dient ein doppelt programmiertes Chromatogramm (Fig. 3), indem dem

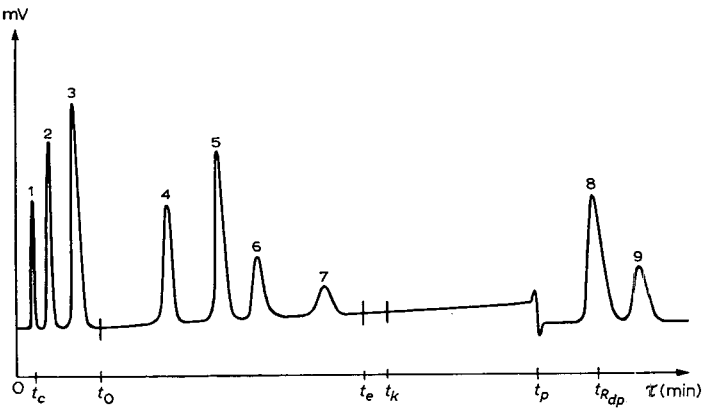


Fig. 3. Doppelt programmiertes Chromatogramm des ersten Modellgemisches.

Temperaturprogramm ein kombiniertes Trägergasprogramm folgt. Die Gleichungen beziehen sich auf die achte Komponente des Chromatogramms. Das Chromatogramm lässt sich in verschiedene Strecken einteilen:

1. Zwischen o und t_0 besteht es aus einer isotherm-isorheischen–isobaren Strecke,

2. zwischen t_0 und t_e aus einer isorheischen Strecke mit programmierter Temperatur,

3. zwischen t_e und t_k aus einer isotherm-isorheischen–isobaren Strecke,

4. zwischen t_k und t_p aus einer isothermen Strecke mit programmierter Strömung und

5. zwischen t_p und t_{Rdp} aus einer isotherm-isorheischen–isobaren Strecke.

(Es ist anzunehmen, dass das Anfahren des etappenmässigen Trägergasprogramms und die Einstellung des neuen Gleichgewichts augenblicklich erfolgt.)

Diese Einteilung in Strecken erleichtert das Aufstellen der Gleichungen. Als Beispiel diene die zur Berechnung des netto Retentionsvolumens der achten Komponente dienende Beziehung:

$$V_N = jF_c(t_0 - t_c) + F_c \int_{t_0}^{t_e} j[p_i(t)]\mathrm{d}t + j_t F_c(t_k - t_e) + \int_{t_k}^{t_p} jF_c[p_i(t)]\mathrm{d}t + \\ + j_c F_{cp}(t_{Rdp} - t_p). \qquad (1)$$

Das Einschalten eines etappenmässigen Trägergasprogrammes nach dem Temperaturprogramm modifiziert Gleichung (1) folgendermassen:

$$V_N = jF_c(t_0 - t_c) + F_c \int_{t_0}^{t_e} j[p_i(t)]\mathrm{d}t + j_t F_c(t_p - t_e) + j_c F_{cp}(t^R_{dp} - t_p). \qquad (2)$$

Folgt dem Temperaturprogramm ein kontinuierliches Trägergasprogramm, so hat Gleichung (1) folgende Form:

$$V_N = jF_c(t_0 - t_c) + F_c \int_{t_0}^{t_e} j[p_i(t)]\mathrm{d}t + j_t F_c(t_k - t_e) + \int_{t_k}^{t_{Rdp}} jF_c[p_i(t)]\mathrm{d}t. \quad (3)$$

Prinzipiell kann das Temperaturprogramm gleichzeitig mit dem Beginn des Trägergasprogrammes ausgeschaltet werden, d.h. $(t_k - t_e)$ bzw. $(t_p - t_e)$ sind gleich Null. Da in der Praxis jedoch dies nur auf Kosten von Reproduzierbarkeit und Genauigkeit erreicht werden könnte, ist es ratsam, den Wert der obigen beiden Differenzen auf ungefähr 1 Minute zu bemessen. Ebenso könnte prinzipiell das Temperaturprogramm gleichzeitig mit der Einwaage, dh. bei $t_0 = 0$, beginnen. In der Praxis wird auch dies zweckmässig vermieden. Wenn jedoch Einwaage und Temperaturprogramm von zwei verschiedenen Personen durchgeführt werden, so kann man die bei Temperaturprogrammen übliche Reproduzierbarkeit und Genauigkeit erzielen. Der erste Teil der Gleichung (1) ändert sich in diesen Fällen natürlich grundsätzlich. Für den Fall $t_0 = 0$ hat die Gleichung folgende Form:

$$V_N = F_c\left(\int_0^{t_e} j[p_i(t)]\mathrm{d}t - \int_0^{t_{cp}} j[p_i(t)]\mathrm{d}t\right) + j_t F_c(t_k - t_e) + \int_{t_k}^{t_p} jF_c[p_i(t)]\mathrm{d}t +$$

$$+ j_c F_{cp}(t_{Rdp} - t_p). \quad (4)$$

Vergleicht man Gleichungen (1), (2) und (3), so sieht man, dass ihre ersten beiden Glieder identisch sind. Sie sind für das Grundchromatogramm kennzeichnend und sind an der Trägergasprogrammierung effektiv unbeteiligt. Bei der Berechnung der Trägergasprogrammierung braucht man sie deshalb nicht zu berücksichtigen. Es ist die Strecke $(t_{Rp} - t_e)$ des Grundchromatogramms, die durch kombiniertes oder kontinuierliches Trägergasprogramm modifiziert werden kann. Da auf dieser Strecke schon isotherme Verhältnisse bestehen, kann man die folgenden Gleichungen aufstellen:

(a) bei etappenmässiger Trägergasprogrammierung:

$$jF_c(t_{Rp} - t_p) = j_c F_{cp}(t_{Rdp} - t_p). \quad (5)$$

(b) Bei linearer Trägergasprogrammierung:

$$jF_c(t_{Rp} - t_k) = \left(\frac{2j_t F_c + (t_{Rdp} - t_k - 1)L}{2}\right)(t_{Rdp} - t_k). \quad (6)$$

Gleichungen (5) und (6) ermöglichen nach Substitution der entsprechenden Daten des Grundchromatogramms die Berechnung der doppelt programmierten Retentionszeit und danach, auf Grund ihrer Kenntnis, die Kritik bzw. Korrektur der Programmierung. Für die niedrigste Analysenzeitdauer wäre es wünschenswert, den Koeffizienten $j_c F_{cp}$ in Gleichung (5) und den Faktor L in Gleichung (6) möglichst hoch zu wählen. Diese Werte können jedoch nicht beliebig gewählt werden, da nach dem Überschreiten eines Maximalwertes die Aufeinanderprogrammierung der Komponenten erfolgen würde. Wenn es sich nur um die Programmierung einer einzelnen Komponente handelt, setzt der maximalen Höhe des Trägergasprogramms natürlich nur die Konstruktion des Geräts Grenzen. In der Praxis sind aber im allgemeinen mehrere

Komponenten zu programmieren, weshalb der Maximalwert des Trägergasprogramms berechnet werden muss. Dies bleibt jedoch nur auf die Berechnung des maximalen Wertes von $j_c F_{cp}$ und L beschränkt.

Den Höchstwert von $j_c F_{cp}$ erhält man folgendermassen:

$$(j_c F_{cp})_{max} = \frac{\Delta V_{min}}{2\left[\left(\frac{1}{\lambda z}\right) + \left(\frac{1}{\lambda z - 1}\right)\right]}. \tag{7}$$

Die Berechnung des maximalen Werts von L ist jedoch komplizierter, da bei linearer Trägergasprogrammierung für jede zu programmierende Komponente paarweise zu bestimmen ist, ob eine Aufeinanderprogrammierung vorliegt. Im allgemeinen kann man annehmen, dass das Programm dann maximal ist, wenn für alle Komponenten die Gleichung

$$t_{Rdp(z)} - t_{Rdp(z-1)} = 2\left[\left(\frac{1}{\lambda z}\right) + \left(\frac{1}{\lambda z - 1}\right)\right] \tag{8}$$

erfüllt ist.

Das maximale Programm kann für alle z Werte infolge der Zusammensetzung der Proben meistens nicht verwirklicht werden. Deshalb muss man so arbeiten, dass der Wert L_{max} soweit wie möglich erreicht wird.

Mit dem aus Gleichung (7) errechneten Wert $j_c F_{cp}$ lässt sich die doppelt programmierte Retentionszeit der Komponente im voraus berechnen:

$$t_{Rdp} = \frac{(t_{Rp} - t_p) j F_c}{(j_c F_{cp})_{max}} + t_p. \tag{9}$$

Unsere Versuche wurden mit einem Apparat Carlo Erba Fractovap Modell C bzw. D an künstlichen Modellen durchgeführt. Das in Tabelle I angegebene Gemisch ergab Fig. 3, das in Tabelle II angegebene Fig. 4 und 5. Die Nummerierungen der

TABELLE I

Komponente	*Sdp.* (C°)
1 Luft	
2 Aceton	56.5
3 Benzol	80.08
4 Toluol	110.80
5 *m*- und *p*-Xylol	138.8–138.0
6 *o*-Xylol	144.0
7 Nitrobenzol	210.9
8 *o*-Nitroäthylbenzol	223.5
9 *p*-Nitroäthylbenzol	241.50

TABELLE II

Komponente	*Sdp.* (C°)
1 Aceton	56.5
2 Benzol	80.08
3 Toluol	110.80
4 Äthylbenzol	136.15
5 Methylsalicylat	223.3

Komponenten stimmen in Tabellen und Abbildungen überein. Es wurde Flammenionisationsnachweis angewandt. Zum Registrieren diente ein Gerät Speedomax G mit 2.5 mV Vollausschlag und 0.5 inch/min Papiergeschwindigkeit. Die übrigen Versuchsparameter, u.a. Kolonnen, Analysetemperatur usw. wurden je nach den Versuchsserien geändert.

In Fig. 4 ist das Grundchromatogramm, in Fig. 5 das doppelt programmierte Chromatogramm des Modellgemisches von Tabelle II zu sehen. Diese zwei Aufnahmen

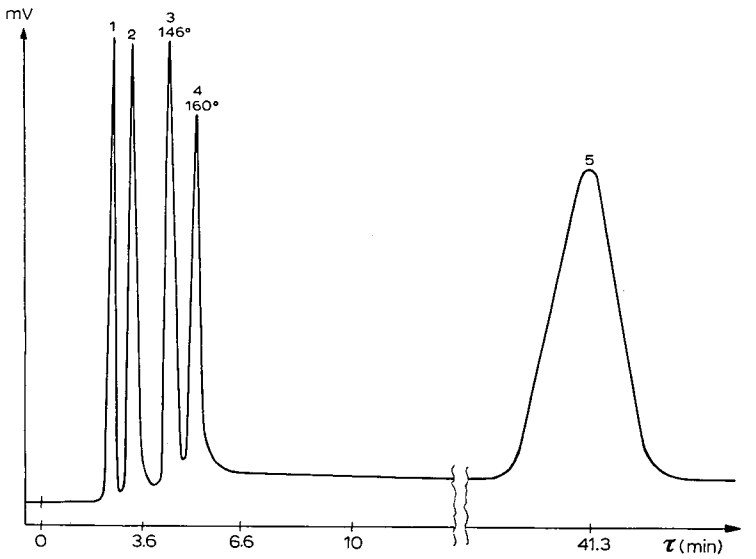


Fig. 4. Grundchromatogramm des zweiten Modellgemisches.

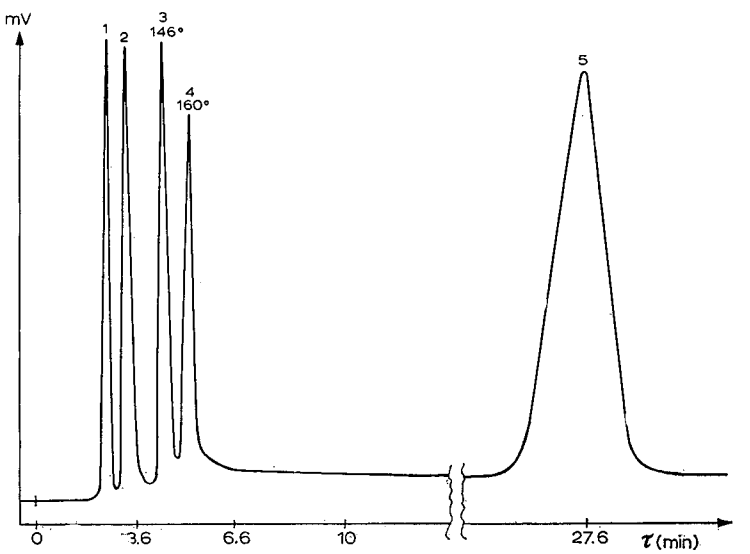


Fig. 5. Doppelt programmiertes Chromatogramm des zweiten Modellgemisches.

dienten zum numerischen Rechnen auf Grund von Gleichung (5). Die doppelt programmierte Retentionszeit der Komponente 5 wurde nämlich nach Aufnahme des Grundchromatogramms zu 27.4 Minuten bemessen. Das kombinierte Trägergasprogramm hatte in der 7. Minute einzusetzen, da das Temperaturprogramm (16°/min) nach 6.6 Minuten ausgeschalten wurde (bei der oberen Temperaturgrenze von Carbowax 1500). Der jF_c Wert des Grundchromatogramms betrug 15.0 ml/min, während $t_R = 41.3$ min war.

Geordnet und nach Substitution erhält man aus Gleichung (5)

$$j_c F_{cp} = \frac{15(41.3 - 7)}{(27.4 - 7)} = 25.2 \text{ ml/min.}$$

Mit Hilfe einer Funktion $jF_c - p_i$ wurde der zu 25.2 ml/min gehörende p_i-Wert bestimmt und die doppelte Programmierung durchgeführt (Chromatogramm in Fig. 5). Da eine Komponente programmiert wurde, war es unnötig, das Maximum des Programms mit Hilfe von Gleichung (7) zu berechnen. Das angewandte Programm erreichte übrigens nicht die maximale Leistungsfähigkeit des Apparats und diente ausschliesslich zur Lösung der erwähnten Aufgabe.

Mit doppelt programmierter Gaschromatographie gelang es uns, verschiedene analytische Probleme zu lösen. Ausser der schon erwähnten Lavendelölanalyse haben wir z.B. sehr gute Ergebnisse bei der Analyse von Phenyl-äthoxy-silanen erzielt. Die Erfahrung zeigte, dass man gut reproduzierbare Ergebnisse nur durch Anschluss von etappenmässiger oder linearer Trägergasprogrammierung erhält, während die qualitative und quantitative Analyse nur unter Verwendung von reinen Standardsubstanzen entsprechend genaue Resultate liefert.

BEZEICHNUNGEN

t_0 = die zum Anfahren des Temperaturprogramms gehörende Zeitkoordinate (min)
t_e = die zum Ausschalten des Temperaturprogramms gehörende Zeitkoordinate (min)
t_k = die dem Beginn des kontinuierlichen (linearen) Trägergasprogramms entsprechende Zeitkoordinate (min)
t_p = die dem Beginn der etappenmässigen Trägergasprogrammierung entsprechende Zeitkomponente (min)
t_{Rdp} = die doppelt programmierte Retentionszeit der Komponente (min)
V_N = netto Retentionsvolumen der Komponente (ml Trägergas)
j = Korrekturfaktor des Druckgefälles
F_c = Trägergasvolumgeschwindigkeit (ml/min) bei Druck und Temperatur des Kolonnenauslaufs
t_c = Durchströmzeit von Luft (Argon, Helium) (min)
p_i = Eintrittsdruck des Trägergases (kp/cm^2)
t = Zeit (min)
j_t = Korrekturfaktor des Druckgefälles nach Ausschalten des Temperaturprogramms
j_c = Korrekturfaktor des Druckgefälles während der etappenmässigen Trägergasprogrammierung für $n = 1$

n = Zahl der etappenmässigen Programmbeginne, die der Erscheinung der Höchstkonzentration der Komponente vorangehen
F_{cp} = Volumströmungsgeschwindigkeit des Trägergases bei etappenmässiger Trägergasprogrammierung für $n = 1$ (ml/min)
t_{Rp} = (Temperatur oder Trägergas) programmierte Retentionszeit der Komponente (min)
L = Kennzahl des linearen Trägergasprogramms (ml/min^2)
ΔV_{min} = Minimum der aus dem netto Retentionsvolumen der Komponenten errechneten ΔV-Werten (ml Trägergas)
ΔV = $V_{N(z)} - V_{N(z-1)}$
z = Zahl der Komponenten
λ = Verteilungsparameter (min^{-1})
t_{cp} = programmierte Durchströmzeit von Luft (Argon, Helium) (min)

ZUSAMMENFASSUNG

Einige theoretische und praktische Probleme der doppelt programmierten Gaschromatographie wurden untersucht. Es wurde gefunden, dass die Kombination Temperaturprogramm–Trägergasprogramm zur Lösung von analytischen Aufgaben am geeignetsten ist, besonders wenn sich ein etappenmässiges oder lineares Trägergasprogramm dem Temperaturprogramm anschliesst. Die Methode ist besonders erfolgreich, wenn bei der oberen Temperaturgrenze der Verteilungsflüssigkeit der Kolonne gearbeitet wird.

SUMMARY

Several theoretical and practical problems of double programmed gas chromatography are investigated. It was found that the combination temperature programme–carrier gas programme is most suitable for the solution of analytical problems, particularly in those cases where a temperature programme is followed by a stepwise or linear carrier gas programme. The technique is particularly successful when performed at the upper temperature limit of the partition liquid of the column.

LITERATUR

1 L. MÁZOR UND J. TAKÁCS, *J. Chromatog.*, 23 (1966) 42.
2 J. M. VERGNAUD, *J. Chromatog.*, 19 (1965) 495.

SEPARATION OF HYDROGEN ISOTOPES BY GAS–SOLID CHROMATOGRAPHY

M. L. CONTI AND M. LESIMPLE
Département Radioéléments, CEN Saclay, CEA, B.P.2, Gif Sur Yvette, Essone (France)
(Received December 22nd, 1966)

INTRODUCTION

Several methods of separating hydrogen isotopes and isomers by gas–solid chromatography have been published in the last few years[1–13].

In all these methods, excepting that studied by GANT AND YANG[8], the separation is carried out at liquid nitrogen temperature.

At this temperature hydrogen is retained permanently with helium as carrier gas by several absorbant materials including Molecular Sieve 4A[7]. In a study of the static absorption of hydrogen isotopes by PANCHENKOV *et al.*[14] this absorbant phase shows the best selection capacity with respect to the other Zeolites A and X, the Mordenites, the aluminosilicate catalyst, silica and aluminium oxide.

We studied the use of this stationary phase in the separation of hydrogen isotopes at temperatures above 77°K, using helium, argon and neon as carrier gases. We found that in the range —110 to —160°C, with helium as carrier, we obtain a total resolution for the six isotopic species in the region of —140°C. Argon gives a separation of hydrogen, hydrogen tritide and tritium, but with poorer resolution than that obtained with helium, while neon behaves in the same way as helium.

We were also able to calculate the heats of absorption for hydrogen deuteride, hydrogen tritide, deuterium, deuterium tritide, tritium.

EXPERIMENTAL

The diagram of the apparatus used is given in Fig. 1. It consists of a Perkin-Elmer chromatograph F6–3T with carrier gas flow-rate regulator and thermal conductivity detector employing thermistors, and a cryostat containing the column. This cryostat, the 2 ml ionisation chamber and the glass injector connecting the chromatograph to the vacuum apparatus, were built in the laboratory. The cryostat allows the temperature to be regulated to $\pm$ 1°C. The columns were prepared with Molecular Sieve 4A from the Union Carbide Corp. Two methods of activation were used: helium or nitrogen sweeping of the filled column for 4 to 8 h at 250°–300°C, and degassing of the sieve under vacuum at 450°–500°C before filling.

The columns are made of copper, with an internal diameter of 2 mm and length varying between 30 and 200 cm. The samples of isotopic mixtures were prepared with tritium produced by the Radioelements Department (Saclay), hydrogen from the "Air Liquide" Company, and deuterium from the Stable Isotope Section (Saclay), equilibrated by electric discharge. All the carrier gases were purified by passage over Molecular Sieve 5A at liquid nitrogen temperature.

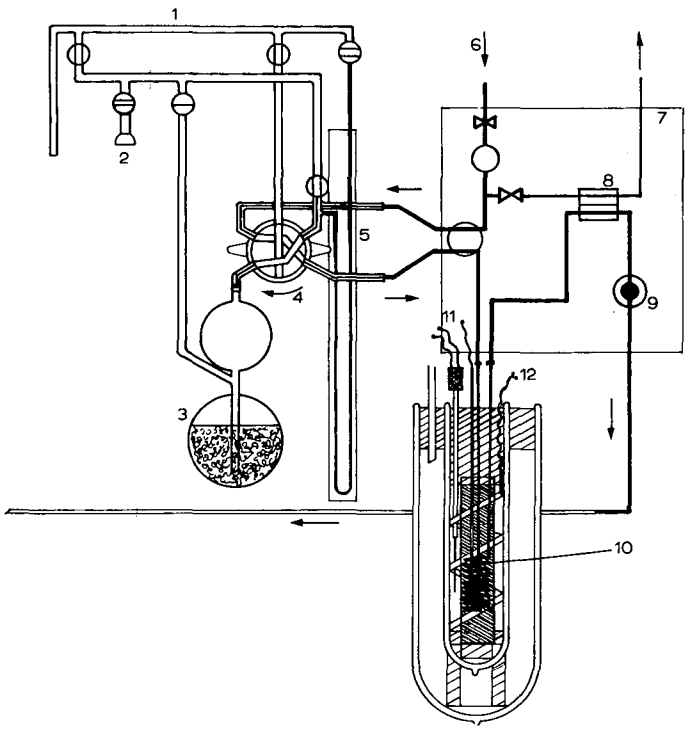


Fig. 1. Diagram of apparatus: 1 = vacuum apparatus; 2 = sample glass bulbs; 3 = Toepler pump; 4 = injector; 5 = mercury manometer; 6 = carrier gas; 7 = Perkin-Elmer chromatograph; 8 = thermal conductivity detector; 9 = ionization chamber; 10 = column; 11 = probe and thermocouple; 12 = heating element.

RESULTS AND DISCUSSION

Helium carrier

Ortho–para hydrogen separation. The ortho–para hydrogen separation can be achieved in the temperature range from —135° to —160°C. Fig. 2 shows the chromatograms obtained on a column of Molecular Sieve 4A, 2 m long and 2 mm in diameter, activated at 250°C under a gas flow.

It can be seen that in spite of the distance between the peak maxima (the separation factor reaches $\alpha = 1.21$), we fail to obtain total resolution because of the presence of an intermediary plateau.

The presence and size of this plateau depends on the activation of the column. In highly activated columns (500°C under vacuum), no separation is obtained and the hydrogen appears as a single peak (Fig. 3). The rate of passage of the hydrogen through the column also has some influence on this separation. An incipient separation can be observed on a highly activated column with high flow rates, whereas with low flow rates a single peak is obtained (Fig. 4a and b). As a general rule a better separation is obtained at very low temperature with a fast flow. It seems that highly activated Molecular Sieve 4A (checked for absence of iron) can have a catalytic effect on the

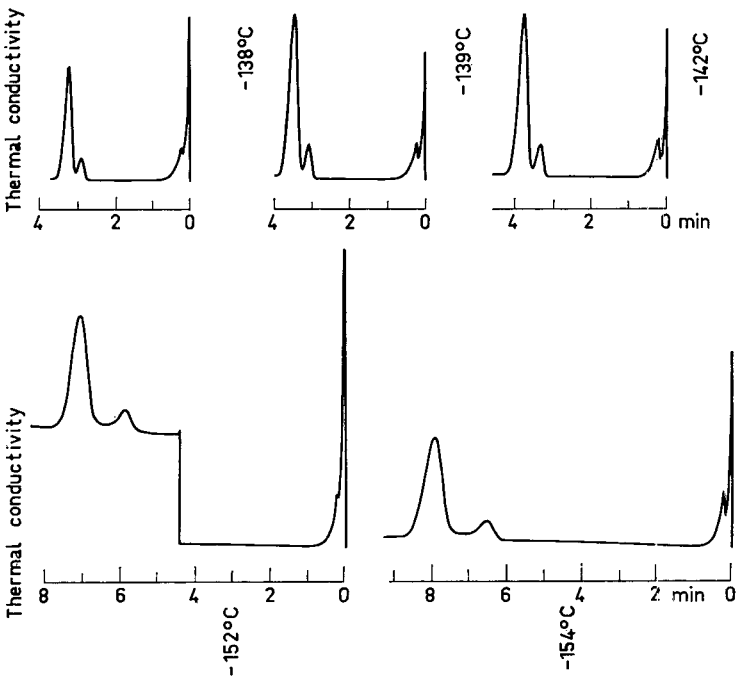


Fig. 2. Separation of ortho and para-hydrogen on a Molecular Sieve 4A column at different temperatures. Experimental conditions: column 2 m × 2 mm, particle size 0.31–0.4 mm, activation at 250° under helium flow, carrier gas flow rate 85 ml/min.

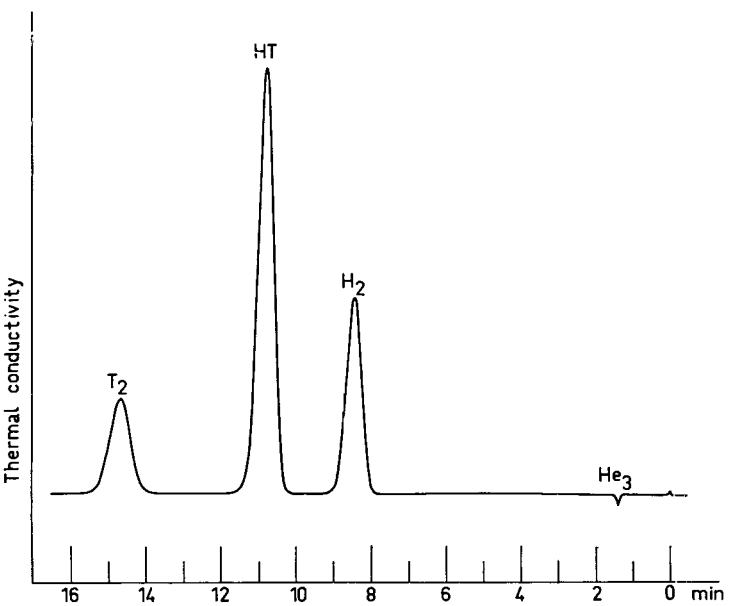


Fig. 3. Separation of H_2, HT, T_2 on a Molecular Sieve 4A column. Experimental conditions: column, 2 m × 2 mm; particle size, 0.16–0.25 mm; activation at 500°C under vacuum; carrier gas flow rate, 25 ml/min; temperature, −133°C.

ortho–para hydrogen conversion. This effect has been observed by MOORE AND WARD and by SMITH AND CARTER[3–5] with highly activated alumina.

Separation of isotopic species. Hydrogen, hydrogen tritide and tritium are completely separated between —125°C and —160°C with carrier gas flow rates between 10 and 90 ml/min on 2 m columns; even on moderately activated columns (250°C under gas flow) (Fig. 3).

It should be mentioned that between —115° and —125°C a separation is obtained, but the resolution is only complete with highly activated columns and very low flow rates. Below —160°C the separation cannot be used for an analysis because of the broadening of the peaks and the excessively long analysis time.

The six isotopic species: hydrogen, hydrogen deuteride, hydrogen tritide, deuterium, deuterium tritide, tritium are separated between —140°C and —160°C with flow rates between 15 and 50 ml/min; the resolution is complete.

Fig. 4 shows chromatograms of the separations obtained on two columns: one highly and the other moderately activated.

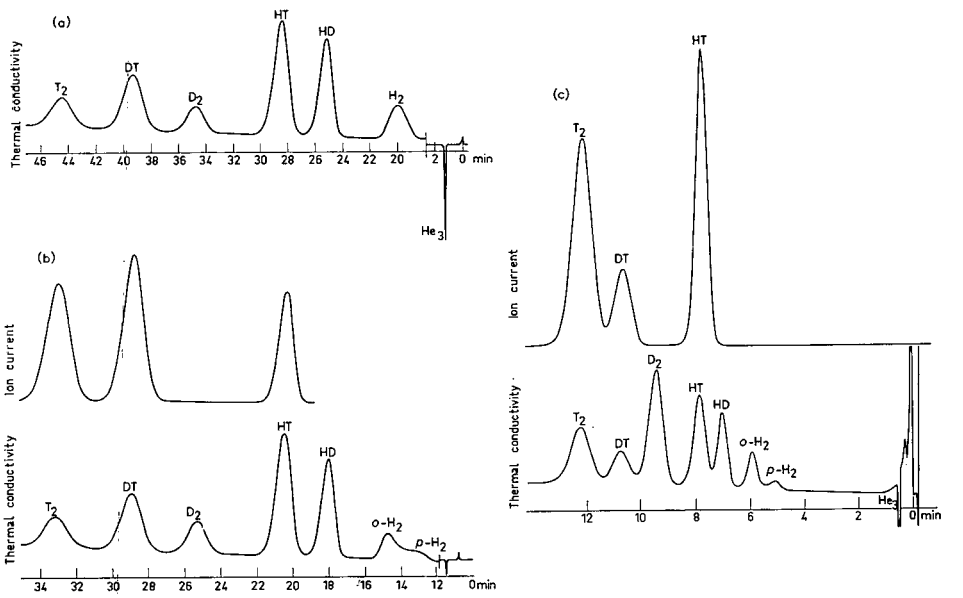


Fig. 4. Separation of o-H_2, p-H_2,HD,HT,D_2,DT,T_2 on different columns of Molecular Sieve 4A. Experimental conditions: (a) column, 2 m × 2 mm; particle size, 0.16–0.25 mm; activation at 500°C under vacuum; carrier gas flow rate, 28 ml/min; temperature, —149°C; (b) column, 2.20 m × 2 mm; particle size, 0.25–0.31 mm; activation at 500°C under vacuum; carrier gas flow rate, 55 ml/min; temperature —151°C; (c) column, 2 m × 2 mm; particle size, 0.25–0.31 mm; activation at 250°C under helium flow; carrier gas flow rate, 80 ml/min; temperature —148°C.

We noted that the HD peak is not superimposed on the o-H_2 peak as is the case with separation on alumina. On the other hand the HT–HD couple is only separated completely on highly activated columns. Table I shows the separation factors obtained for columns of different particle size, length and activation as a function of temperature. For purposes of comparison, the published values of separation factors obtained with other absorbants at 77°K are also given.

TABLE I

SEPARATION FACTORS

Columns: (a) 2 m column, particle size 0.25–0.31 mm (40–60 mesh), activation under gas flow at 250°C.
(b) 2 m column, particle size 0.31–0.4 mm (35–40 mesh), activation under gas flow at 250°C.
(c) 2 m column, particle size 0.16–0.25 mm (60–80 mesh), activation under vacuum at 500°C.
(d) 2.20 m column, particle size 0.25–0.31 mm (40–60 mesh), activation under vacuum at 500°C.
(e) 2 m column, particle size 0.31–0.4 mm (35–40 mesh), activation under vacuum at 500°C.

Temperature		oH_2/pH_2	HD/H_2	HT/H_2	D_2/H_2	DT/H_2	T_2/H_2	HT/HD	D_2/HD
	(a)			1.21*			1.58*		
	(b)								
−119°C	(c)								
154°K	(d)								
	(e)			1.24			1.57		
	(a)			1.25*			1.76*		
	(b)								
−130°C	(c)								
143°K	(d)								
	(e)			1.27			1.74		
	(a)								
	(b)			1.24*			1.70*		
−133°C	(c)			1.33			1.89		
140°K	(d)								
	(e)								
	(a)								
	(b)	1.12		1.29*	1.47*		1.84*		
−135°C	(c)								
138°K	(d)								
	(e)								
	(a)			1.35*			2.15*		
	(b)	1.16		1.35*			2.11*		
−146°C	(c)		1.27	1.43	1.76	1.99	2.27	1.13	1.38
127°K	(d)								
	(e)		1.22	1.38	1.69	1.91	2.18	1.12	1.38
	(a)		1.19*	1.35*	1.65*	1.88*	2.16*	1.13	1.37
	(b)	1.18	1.19*	1.34*	1.62*	1.83*	2.15*	1.12	1.35
−148°C	(c)		1.28	1.45	1.81	2.08	2.39	1.13	1.41
125°K	(d)								
	(e)								
	(a)			1.37*	1.66*		2.19*		
	(b)	1.21	1.20*	1.36*	1.66*	1.90*	2.16*	1.13	1.37
−151°C	(c)								
122°K	(d)		1.23	1.40	1.75	2.00	2.30	1.13	1.42
	(e)		1.26	1.44	1.82	2.10	2.45	1.14	1.44
	(a)			1.39*			2.23*		
	(b)								
−152°C	(c)								
121°K	(d)								
	(e)		1.27	1.46	1.85	2.16	2.53	1.15	1.46
	(a)		1.22*	1.41*	1.77*	2.08*	2.46*	1.15	1.44
	(b)								
−153°C	(c)								
120°K	(d)		1.24	1.42	1.78	2.07	2.40	1.14	1.44
	(e)								

DT/HD	T_2/HD	D_2/HT	DT/HT	T_2/HT	DT/D_2	T_2/D_2	T_2/DT
				1.30			
				1.27			
				1.33			
				1.33			
				1.36			
				1.31**			
				1.35			
				1.41			
		1.14		1.42		1.25	
				1.58			
				1.57			
1.57	1.79	1.23	1.39	1.58	1.13	1.29	1.14
1.56	1.78	1.22	1.38	1.58	1.13	1.29	1.14
				1.60**			
1.57	1.80	1.22	1.39	1.59	1.14	1.31	1.14
1.53	1.72	1.21	1.37	1.58	1.13	1.27	1.12
1.63	1.87	1.24	1.43	1.64	1.15	1.32	1.15
		1.22		1.60		1.32	
1.57	1.79	1.22	1.39	1.58	1.14	1.30	1.14
1.63	1.87	1.24	1.42	1.64	1.14	1.31	1.15
1.67	1.94	1.26	1.45	1.69	1.15	1.34	1.16
				1.61			
				1.54**			
1.70	1.99	1.26	1.47	1.73	1.16	1.36	1.17
				1.70			
1.70	2.00	1.25	1.48	1.74	1.17	1.38	1.17
1.66	1.93	1.25	1.45	1.68	1.15	1.34	1.16

(continued on p. 38)

TABLE I (*continued*)

Temperature		oH_2/pH_2	HD/H_2	HT/H_2	D_2/H_2	DT/H_2	T_2/H_2	HT/HD	D_2/HD
	(a)			1.42*			2.42*		
	(b)								
−156°C	(c)								
117°K	(d)		1.24	1.43	1.79	2.08	2.41	1.15	1.44
	(e)								
*Published values***									
	(13)		1.09	1.20	1.26	1.45	1.66	1.10	1.15
−195.8°C	(6)		1.13	1.23	1.38		1.68	1.09	1.22
77°K	(5)		1.22	1.42	1.49	1.85	2.08	1.22	1.17
	(7)	1.37							1.53
	(7)								1.39
	(11)		1.14	1.20					1.39
	(11)	1.19							1.23
	(11)								1.47

* These values refer to the separation with respect to ortho-hydrogen.
** Line (a) these values correspond to a column 80 cm long; line (b) the column was 30 cm.
*** The separation factors calculated here and those given in references 7 and 11 are obtained from the ratios of the retention times corrected for dead time, whilst those given in references 13, 6 and 5 are calculated from the retention times taken following injection.

The introduction of different quantities of the various components between $2 \cdot 10^{-4}$ and 0.2 ml NTP gave no measurable variations in the retention times for a given temperature and degree of activation.

In addition the absolute retention volumes at constant temperature are independent of the carrier gas flow rate. We are thus in the linear region of absorption isotherm.

Fig. 5 shows the $HETP_{eff}$ curve for HT and T_2 as a function of flow rate. The optimum flow rate is 25 ml/min. The efficiency (number of effective theoretical plates

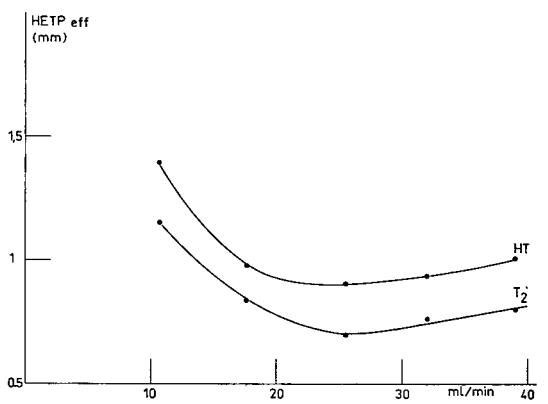


Fig. 5. Plots of $HETP_{eff}$ against carrier gas flow rate for the peaks of HT and T_2.

DT/HD	T_2/HD	D_2/HT	*DT/HT*	T_2/HT	DT/D_2	T_2/D_2	T_2/DT	
				1.70				
				1.69**				
1.67	1.93	1.25	1.45	1.68	1.15	1.34	1.16	
								Exptl. cond.
1.33	1.52	1.05	1.21	1.38	1.15	1.32	1.14	$Al_2O_3 + Fe_2O_3$ – He carrier
	1.48	1.12		1.30		1.22		– d° –
1.52	1.71	1.05	1.30	1.47	1.24	1.40	1.13	– d° – M.S.13X–He carrier
				1.62		1.34		M.S. 5A – H_2 carrier
				1.73	1.25		1.05	Al_2O_3–cap. col. He carrier
								M.S.13X–cap. col. He carrier
								M.S.5A–cap. col. He carrier

per metre) is a function of particle size and especially of activation. It increases slightly with decrease in temperature and reaches a maximum between −145° and −155°C. The values, which vary according to the isotopic species considered, are 500 (700–800 at the maximum point) for columns activated under a gas flow and 1000 (1300–1500 maximum) for those activated under vacuum.

Table II lists the resolutions obtained on a highly activated column of particle size 0.16–0.25 mm, with the corresponding capacity ratios and number of theoretical plates. This chromatographic separation method was used in the quantitative analysis of H_2, HT, and T_2 mixtures.

The calibration curves are linear up to 100 mm³ NTP for hydrogen, and over the whole range studied, *i.e.* up to 200 mm³ NTP, for HT and T_2. The minima detectable (we take as the detection limit an elongation of 10 mm equal to 0.08 mV) are 0.57 mm³ NTP of T_2, 0.5 mm³ NTP of HT and 3 mm³ NTP of H_2 (these figures were obtained with a 2 m column activated under vacuum at 500°C, carrier gas flow rate 37 ml/min, temperature −130°C, Perkin-Elmer thermistor detector).

If an ionization chamber is used as detector it is possible to determine down to 0.005 mm³ NTP of tritium in the form of HT or T_2.

Determination of heats of absorption. From the general equation

$$\frac{\mathrm{d} \ln V_g}{\mathrm{d}(1/T)} = \frac{\Delta H}{R}$$

the graph of $\ln V_g$ as a function of $1/T$ gives straight lines (Fig. 6) and the heats of

TABLE II

RESOLUTION FACTORS

Temperature	oH_2/pH_2	HD/H_2	HT/H_2	HT/HD	D_2/HT	DT/D_2	T_2/DT	D_2/HD	T_2/HT	*Flow rate*	K_{HT}*	H_{T_2}*	$N_{eff}T_2$**
−133			2.93						4.36	25.5 ml/min	6.71	9.51	2840
−146		2.23	3.38	1.38	2.50	1.58	1.65	3.98	5.56	24 ml/min	15.59	24.70	2700
−150		2.77	3.52	1.56	2.76	1.77	1.78	4.49	6.02	28 ml/min	20.82	33.55	3092

* Capacity ratio, $-K = t_{dr}/t_m$.

** Number of effective theoretical plates for T_2 peak.

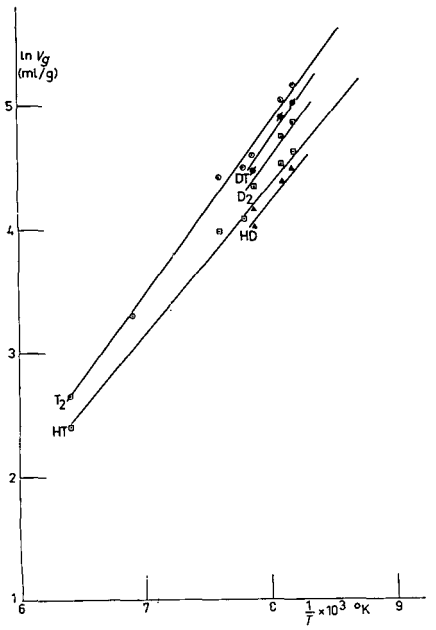


Fig. 6. Plot of ln Vg against inverse temperature.

absorption H can be calculated from their slopes [15,16]. The heats of absorption thus obtained are as follows:

$\Delta H_{HD} = 2.30 \pm 0.1$ kcal
$\Delta H_{HT} = 2.40 \pm 0.05$ kcal
$\Delta H_{D_2} = 2.64 \pm 0.1$ kcal
$\Delta H_{DT} = 2.74 \pm 0.1$ kcal
$\Delta H_{T_2} = 2.80 \pm 0.05$ kcal

The precision of the ΔH_{HT} and ΔH_{T_2} values is greater because of the larger number of experimental points obtained.

Argon carrier

The use of argon as carrier gas was studied because of the greater sensitivity obtainable with a thermal conductivity detector in the measurement of hydrogen and with an ionization chamber in the determination of HT and T_2.

This gas can be used down to —160°C and separates He,H_2,HT,T_2; because of its high absorption on Molecular Sieve 4A, the retention times for the same column and under the same flow rate and temperature conditions are shorter than with helium and the separation factors and resolutions are lower. Under the best temperature conditions, —143–150°C, with a flow rate of 40–50 ml/min, we obtain: $\alpha(T_2/HT) = 1.61$–1.69; $\alpha(HT/H_2) = 1.34$; $R(T_2/HT) = 2.09$–2.20 $R(HT/H_2) = 1.42$, (the columns used were moderately activated).

Neon carrier

With neon as carrier total separation of H_2, HT, D_2, DT, T_2 can be obtained.

The separation factors are slightly better than those obtained with helium for the same column and under the same temperature and flow-rate conditions.

At −155°C we obtain: $\alpha(HD/H_2) = 1.33$; $\alpha(HT/HD) = 1.17$; $\alpha(D_2/HT) = 1.30$; $\alpha(DT/D_2) = 1.18$; $\alpha(T_2/DT) = 1.15$; $\alpha(T_2/HT) = 1.77$.

Unfortunately because of the high cost of the gas this separation cannot be used for routine analysis.

CONCLUSION

Molecular Sieve 4A can be used as the stationary phase in gas–solid chromatographic separation of hydrogen isotopes. In the temperature range −140–150°C, with helium as carrier gas and flow-rates between 15 and 50 ml/min, total resolution of the six isotopic species is obtained.

SYMBOLS AND FORMULAE

t_r = retention time (injection measurement)

t_m = retention time of a non-absorbed component. In our system this dead time was determined from the retention time of He_3, which is not absorbed in the temperature range studied.

t_{dr} = corrected retention time, $t_{dr} = t_r - t_m$

F = carrier gas flow rate in ml/min measured at room temperature and at the column output pressure P_s (atmospheric pressure under our experimental conditions).

V_c = retention volume, $V_c = t_r \times F$

V_m = dead volume, $V_m = t_m \times F$

V_n = absolute retention volume calculated thus:

$$V_n = (V_c - V_m) \times \frac{T_c}{T_s} \times j$$

where

T_c = temperature of column in °K,

T_s = absolute temperature at which the flow rate is measured,

j = pressure gradient correction factor of JAMES AND MARTIN.

$$j = \frac{3(P_e/P_s)^2 - 1}{2(P_e/P_s)^3 - 1}$$

V_g = specific retention volume (brought to 273°C and 1 g of the stationary phase),

$$V_g = \frac{V_n \times 273}{m \times T_c}$$

α = separation factor,

$$\alpha = \frac{t_{dr_1}}{t_{dr_2}}$$

R = resolution,

$$R = 2\,\frac{(t_{dr_2} - t_{dr_1})}{W_2 + W_1},$$

with t_{dr_1} and t_{dr_2} corrected retention times of components 1 and 2, and W_1 and W_2 widths at the base of peaks 1 and 2.

n = number of effective theoretical plates,

$$n = 16 \left(\frac{t_{dr}}{W}\right)^2$$

k = capacity ratio,

$$k = \frac{t_{dr}}{t_m} = \frac{t_r}{t_m} - 1$$

SUMMARY

Molecular Sieve 4A can be used as the stationary phase in the gas–solid chromatographic separation of hydrogen isotopes and isomers: the separation is studied as a function of the different factors involved (temperature, flow rate, activation of the stationary phase).

From the determination of the specific retention volumes as a function of temperature it was possible to calculate the heats of absorption of hydrogen deuteride, hydrogen tritide deuterium, deuterium tritide and tritium.

REFERENCES

1 H. A. SMITH AND P. P. HUNT, *J. Phys. Chem.*, 64 (1960) 383.
2 P. P. HUNT AND H. A. SMITH, *J. Phys. Chem.*, 65 (1961) 87.
3 W. R. MOORE AND H. R. WARD, *J. Phys. Chem.*, 64 (1960) 832.
4 W. R. MOORE AND H. R. WARD, *J. Am. Chem. Soc.*, 80 (1958) 2909.
5 E. M. CARTER JR. AND H. A. SMITH, *J. Phys. Chem.*, 67 (1963) 1512.
6 J. KING JR., *J. Phys. Chem.*, 67 (1963) 1397.
7 F. BOTTER, G. DE LA PERRIÈRE AND S. TISTCHENKO, *CEA Rept.* No. 1962 (1961).
8 P. L. GANT AND K. YANG, *Science*, 129 (1959) 1548.
9 S. OHKOSHI, S. TENMA, Y. FUJITA AND T. KWAN, *Bull. Chem. Soc. Japan*, 31 (1958) 773.
10 C. CERCY, F. BOTTER AND S. TISTCHENKO, *Bull. Soc. Chim. France*, (1962) 2315.
11 C. CERCY AND F. BOTTER, *Bull. Soc. Chim. France*, (1965) 3383.
12 S. AKHTAR AND H. A. SMITH, *Chem. Rev.*, 64 (1964) 261.
13 D. L. WEST AND A. L. MARSTON, *J. Am. Chem. Soc.*, 86 (1964) 4731.
14 G. M. PANCHENKOV, A. M. TOLMACHEV AND T. W. ZOTOVA, *Zh. Fiz. Khim.*, 38 (1964) 1361; transl. *CEA Rept.* No. *R* 1879, (1965).
15 S. A. GREENE AND H. PUST, *J. Phys. Chem.*, 62 (1958) 55.
16 A. B. LITTLEWOOD, *Gas Chromatography*, Academic Press, New York, 1962, pp. 73, 76, 112.

THE ANALYSIS OF OILS AND FATS BY GAS CHROMATOGRAPHY

IV. THE ALKALINE ISOMERISATION OF LINOLEYL ACETATE AND OCTADECA-9,12-DIENE

GEORGE R. JAMIESON AND ELIZABETH H. REID
Chemistry Department, The Paisley College of Technology, Paisley, Renfrewshire (Great Britain)
(Received December 23rd, 1966)

When linoleic acid is heated with alkali, rearrangement to conjugated isomers takes place. It has been found[1] that the amount of rearrangement to these isomers is negligible at reaction temperatures up to 120° but increasing amounts of isomerisation products are formed as the reaction temperature is increased. At a reaction temperature of 234°, about 2 % of the linoleic acid has not been isomerised. When four vegetable oils were heated at 234° it was found that about 5 % of the linoleic acid originally present had not been isomerised. Other investigators[2–5] have found that varying amounts (up to 6 %) of linoleic acid remain unisomerised after reaction with alkali at 180°.

It has also been shown[6] that, when carboxylic acids are converted to the corresponding acetates and hydrocarbons, gas chromatographic retention data for the corresponding methyl esters, alcohols and acetates are very similar when they are chromatographed under the same conditions.

The purpose of the present work is to investigate the action of alkali on linoleyl acetate and on octadeca-9,12-diene and to separate the conjugated isomers by means of gas chromatography. A comparison can then be made with the amounts of conjugated isomers obtained from the corresponding acid, acetate and hydrocarbon.

EXPERIMENTAL

Preparation of derivatives

Octadeca-9,12-dienyl acetate (linoleyl acetate). Linoleic acid (puriss. Koch-Light Laboratories Ltd.) was reduced to octadeca-9,12-dienol with aluminium lithium hydride and this alcohol was converted to its acetate by reaction with acetyl chloride[6].

Octadeca-9,12-diene. Octadeca-9,12-dienol was converted to its *p*-toluenesulphonate and this derivative was reduced to the diunsaturated hydrocarbon using the method of DYEN, HAMMAN AND SWERN[7].

Alkali isomerisation

The derivative (200 mg) was heated with potassium hydroxide–diethylene glycol for 1 h as described previously[1]. In the case of the acetate, reaction with the alkali brought about hydrolysis and therefore, after isolation of the isomerisation products from the reaction mixture, they were converted to the acetates by reaction with acetyl chloride.

Gas–liquid chromatography

Gas chromatographic separations were carried out, as described previously[8], using a Perkin Elmer 800 chromatograph with butanediol succinate (BDS) as the stationary liquid. The acetate derivatives were separated at a column temperature of 210° and the hydrocarbons at 150°.

RESULTS AND DISCUSSION

When octadeca-9,12-dienyl acetate and octadeca-9,12-diene are heated with potassium hydroxide–diethylene glycol it is found that isomerisation to conjugated derivatives takes place and also that the amounts of the isomerisation products increase with an increase in reaction temperature (Table I). The results obtained for the acetate and hydrocarbon are similar to those obtained previously[1] for linoleic acid under similar reaction conditions except that the amount of hydrocarbon unisomerised at 180° is greater than that found for both the acid and the acetate. This is probably due to the fact that the hydrocarbon was not very soluble in the reaction mixture and a homogeneous solution could not be obtained.

TABLE I

PERCENTAGE AMOUNTS OF PRODUCTS FROM ISOMERISATION REACTION

	Original	*Temperature of reaction*			
	(%)	*120°*	*150°*	*180°*	*234°*
Acetate					
$18:1^{9}$	0.9	0.9	0.9	0.7	0.8
$18:2^{9,12}$	99.1	98.0	86.7	5.0	4.5
18:2conj. *cis,trans*	—	1.1	11.0	85.5	54.2
18:2conj. *cis,cis*	—	—	1.4	3.9	13.8
18:2conj. *trans,trans*	—	—	—	4.9	26.6
Hydrocarbon					
$18:1^{9}$	0.8	0.8	0.8	0.9	0.7
$18:2^{9,12}$	99.2	99.2	96.4	46.9	5.1
18:2conj. *cis,trans*	—	—	1.6	45.7	39.2
18:2conj. *cis,cis*	—	—	0.5	3.0	13.8
18:2conj. *trans,trans*	—	—	0.7	3.5	41.2

Retention data and ECL values of the various C_{18} derivatives are shown in Table II. It was found that the peak assigned to the conjugated *cis,trans* acetate was broad and misshapen and at a column temperature of 190° this was resolved into two incompletely separated peaks. The corresponding hydrocarbon gave a broad misshapen peak which could not be resolved by lowering the column temperature to 120°. We have found[6] that methyl esters and the corresponding alcohols and acetates give similar retention times and ECL values when chromatographed under the same conditions. Since there is a very close agreement between the relative retention times and ECL values found in the present investigation for the various acetates and those for the corresponding methyl esters found previously, we have tentatively identified the peaks due to the various isomerisation products of linoleyl acetate. The retention data for the hydrocarbon isomerisation products followed a similar pattern to those

TABLE II

RETENTION DATA AND EQUIVALENT CHAIN LENGTHS

	Column temperature				
	190°	*200°*	*210°*	*140°*	*150°*
	Relative retention times				
	Acetates			*Hydrocarbons*	
18:0	1.00	1.00	1.00	1.00	1.00
18:1[9]	1.11	1.10	1.10	1.12	1.12
18:2[9,12]	1.32	1.31	1.30	1.38	1.37
18:2conj. *cis,trans*	1.79 1.85	1.78	1.77	2.06	1.96
18:2conj. *cis,cis*	1.98	1.95	1.92	2.19	2.16
18:2conj. *trans,trans*	2.15	2.12	2.09	2.50	2.41
	Equivalent chain lengths				
18:0	18.00	18.00	18.00	18.00	18.00
18:1[9]	18.30	18.31	18.31	18.27	18.27
18:2[9,12]	18.85	18.86	18.88	18.73	18.75
18:2conj. *cis,trans*	19.80 19.91	19.88	19.95	19.65	19.62
18:2conj. *cis.cis*	20.13	20.18	20.22	19.79	19.85
18:2conj. *trans,trans*	20.38	20.44	20.50	20.09	20.16

given by the methyl esters and the acetates. Octadeca-9(*trans*),11(*trans*)-dienyl acetate and octadeca-9(*trans*),11(*trans*)-diene were prepared from the corresponding methyl ester[9] and the retention times found for these compounds agreed with those obtained for the peaks assigned to the conjugated *trans,trans* isomerisation product.

The relative amounts of the isomerisation products formed are shown in Table III and they are similar to the amounts obtained from vegetable oils[1]. At reaction temperatures of 150° and 180° the main products are the *cis,trans* isomers. At a reaction temperature of 234° there are decreases in the relative amounts of *cis,trans* isomers and these decreases are accompanied by increases in the relative amounts of the *cis,cis* isomers and larger increases in the relative amounts of the *trans,trans* isomers. These results obtained by gas–liquid chromatography are supported by the

TABLE III

RELATIVE PROPORTIONS (PER CENT) OF ISOMERISATION PRODUCTS

	Temperature of reaction		
	150°	*180°*	*234°*
Acetate			
18:2conj. *cis,trans*	89	91	57
18:2conj. *cis,cis*	11	4	15
18:2conj. *trans,trans*	—	5	28
Hydrocarbon			
18:2conj. *cis,trans*	95	87	42
18:2conj. *cis,cis*	2	6	15
18:2conj. *trans,trans*	3	7	43

results obtained by infra-red spectroscopy when the absorptions at 985 cm^{-1} and 946 cm^{-1} are compared for the samples obtained from reaction at different temperatures.

We find that, with both the acetate and hydrocarbon, there is about 5 % of unisomerised linoleic derivative at a reaction temperature of 234°. We have previously found that about 2 % of linoleic acid was unisomerised at this temperature. DANIELS AND RICHMOND[2] found that, when oil extracted from flour was heated at 180° with potassium hydroxide–ethylene glycol, 2.9 % of the linoleic acid was unisomerised.

SREENIVASAN *et al.*[3] have shown that not all the C_{18} dienes in corn oil were the 9,12-isomers and also that 3.9 % of the linoleic acid was not conjugated after heating

TABLE IV

AMOUNTS OF OXIDATION PRODUCTS

Methyl ester[a]	*Weight (%)*	
	Calculated[b]	*Found*
C_5 mc	—	0.7
C_6 mc	37.1	34.2
C_9 mc	0.4	0.6
C_7 dc	—	0.3
C_8 dc	—	2.1
C_9 dc	62.5	58.1
C_{10} dc	—	0.3
Unidentified	—	3.7

[a] mc = Monocarboxylic ester; dc = dicarboxylic ester.
[b] From GLC analysis of original ester, assuming normal positions of the double bonds.

for 25 min at 180° with potassium hydroxide–ethylene glycol. BEADLE, JUST, MORGAN AND REINERS[4] found that for a number of corn oils the linoleic acid content determined by gas chromatography was higher by 2.4–6.0 % than that determined by the alkali isomerisation method.

CARTONI, LIBERTI AND RUGGIERI[5], using capillary columns, have shown that a commercial sample of linoleic acid contained 89.4 % 9,12-*cis,cis* isomer, 5.4 % 9,12-*cis-trans* isomer and 5.2 % 9,12-*trans,cis* + *trans,trans* isomers. Since it would not be expected to be able to separate these isomers on a conventional packed column, this sample of linoleic acid would be taken to be 100 % 9,12-*cis,cis*-linoleic acid when analysed on these packed columns. When this commercial sample was isomerised by heating with alkali at 180° it was found that 99.5 % of the *cis,cis* isomer had been converted to conjugated derivatives but only 61 % of the *cis,trans* isomer and 75 % of the remaining isomers had been isomerised. If this isomerised sample had been chromatographed on a conventional packed column the results would have indicated that 2.9 % of the linoleic acid had not been isomerised.

A sample of the linoleic acid used in the present investigation was oxidised with periodate–permanganate[10]. The main oxidation products found were hexanoic acid and azelaic acid in the relative amounts expected from the oxidation of octadeca-9,12-dienoic acid. However a number of minor products were found (Table IV). Some of these minor products may have been produced by secondary oxidation although a pure sample of azelaic acid on being treated with periodate–permanganate under the same conditions as those used for the oxidation of linoleic acid did not give any

detectable oxidation products. The difficulties of differentiating secondary oxidation products from the primary oxidation products of isomers present in amounts of about 1 % have been discussed by KUEMMEL[10].

SUMMARY

Isomerisation occurs when octadeca-9,12-dienyl acetate and octadeca-9,12-diene are heated with potassium hydroxide–diethylene glycol at temperatures above 120°. The amounts and nature of the isomerisation products parallel those obtained from linoleic acid. It is found that about 5 % of both the acetate and diene are not isomerised even at 234°. It is possible that, in the original linoleic acid, some geometrical and positional isomers are present which either do not isomerise to conjugated compounds or do not isomerise at the same rate as the 9,12-*cis,cis* isomer.

REFERENCES

1 G. R. JAMIESON AND E. H. REID, *J. Chromatog.*, 20 (1965) 232.
2 N. W. R. DANIELS AND J. W. RICHMOND, *Nature*, 187 (1960) 55.
3 B. SREENIVASAN, J. B. BROWN, E. J. JONES, V. L. DAVISON AND J. NOWAKOWSKA, *J. Am. Oil Chemists' Soc.*, 39 (1962) 255.
4 J. B. BEADLE, D. E. JUST, R. E. MORGAN AND R. A. REINERS, *J. Am. Oil Chemists' Soc.*, 42 (1965) 90.
5 G. CARTONI, A. LIBERTI AND G. RUGGIERI, *Riv. Ital. Sostanze Grasse*, 40 (1966) 431.
6 G. R. JAMIESON AND E. H. REID, *J. Chromatog.*, 26 (1967) 8.
7 M. E. DYEN, H. C. HAMANN AND D. SWERN, *J. Am. Oil Chemists' Soc.*, 43 (1966) 431.
8 G. R. JAMIESON AND E. H. REID, *J. Chromatog.*, 17 (1965) 230.
9 J. GRIGOR, D. M. MACINNES AND J. MCLEAN, *J. Chem. Soc.*, (1955) 1069.
10 D. F. KUEMMEL, *Anal. Chem.*, 36 (1964) 426.

GAS CHROMATOGRAPHY OF VOLATILE AMINO ACID DERIVATIVES

IV. MIXED STATIONARY PHASES FOR THE SEPARATION OF N-TRIFLUOROACETYLATED AMINO ACID *n*-AMYL ESTERS

A. DARBRE AND K. BLAU*
Department of Biochemistry, King's College, London (Great Britain)
(Received December 21st, 1966)

INTRODUCTION

In previous attempts to find stationary phases for the complete isothermal resolution of mixtures of protein amino acids as their N-trifluoroacetylated *n*-amyl esters we showed that in spite of the wide range of phases tried[1] no single one gave a complete resolution of the most volatile of these amino acid derivatives (those of alanine, valine, glycine, isoleucine, leucine, threonine and serine). The most important factors operating against achieving the desired resolution were the lack of sufficient selectivity on the part of the phases tried and also the use of packed columns giving only 3,000 total theoretical plates with some stationary phases and much less with other phases.

With the second group of amino acid derivatives (those of cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid and glutamic acid) a phase was found which gave the necessary resolution[2], but on further study tyrosine, which had not been included in this investigation, was found to be insufficiently separated from aspartic acid[3].

These difficulties were largely overcome by the use of mixtures of packings prepared from different stationary phases and by using columns with a higher total number of theoretical plates. The choice of stationary phases was deliberately restricted to those with high thermal stability and low bleed-rates.

MATERIALS AND METHODS

Apparatus

A D6 gas density balance gas chromatograph (Griffin & George Ltd., Alperton, Middlesex) was used. Nitrogen (99.9 % "White spot" from British Oxygen Co. Ltd., and "high-purity oxygen-free" from Air Products Ltd.) was used as carrier gas. Some work was also done with the D1 single column modification of this instrument, which permitted the use of higher operating pressures, and had a flash heater on the inlet. The final results were checked on a MicroTek MT 220 dual-column gas chromatograph with flame-ionisation detectors to prove their general application.

* Present address: Laboratory for the Study of Neurometabolic Disorders, Biochemistry Department, School of Medicine, University of North Carolina, Chapel Hill, U.S.A.

Columns

In order to increase the total number of theoretical plates, glass columns were made from Pyrex tubing of 0.4 or 0.25 cm internal diameter, with a "paper-clip" configuration and in this way columns of any required length could be used. The sector plates that normally hold the columns in the oven of the D6 chromatograph were fitted with column-top fittings which had been modified by brazing on at the lower end, $^1/_8$ in. "Swagelok" male couplings drilled out to accept the ends of the glass column. The ends of the column inserted into the couplings were made gas tight with two $^1/_4$-in. "Viton" O-rings and locked in position with nuts. A similar arrangement was used to fit the same glass columns into the D1 modification of the instrument.

Column packings

Since the preparation of Silocel C22 as used previously was time-consuming, "10-mesh cuts" of Anakrom acid and base washed and vacuum siliconized (ABS) were used. These are commercially available and were found to give high efficiency columns. Anakrom has a lower packing density and surface area per g than Silocel C22, but for equivalent weights of stationary phase in the column the separations achieved were found to be comparable.

The stationary phases were dissolved in a suitable solvent (usually ethyl methyl ketone) in a 1 l round-bottomed flask with four dimples in the side to promote mixing. The correct weight of Anakrom ABS (90–100 or 100–110 mesh) (Analabs, through Gas Chromatography Ltd., Maidenhead, Berks.) was added, and the solvent was evaporated off under reduced pressure using first a water pump and then an oil pump on a rotary evaporator. The flask was turned slowly by hand to prevent the production of "fines". The packings were prepared from single stationary phases only, and mixed phases were made up subsequently by blending together the calculated proportions of the dried packings by weight. These blended packings were filled into the column through a funnel attached at one end, while suction was applied from an oil-pump at the other end. The packing was compacted by vibration and gentle tapping. The column and the glass yarn used to plug the ends of the column were cleaned with a chromic acid–sulphuric acid mixture, washed free of acid with distilled water, dried, deactivated with 5 % dimethyldichlorosilane in toluene for 2 h, rinsed twice with toluene, washed with methanol and dried before using.

Preparation of derivatives

Modifications were introduced in methods previously described[1]. Owing to difficulty in esterifying lysine hydrochloride with amyl alcohol[3] the amino acids (0.5–2.0 mg of each) were first dissolved in trifluoroacetic acid (0.2 ml) in a B 14 test tube. Amyl alcohol (2 ml) was then added and dry HCl gas was bubbled continuously through the reaction mixture at 108° ± 2° for 25 min. The alcohol was then removed with the rotary evaporator using first a water pump then an oil pump. As the amyl ester of arginine gives no volatile trifluoroacetylated derivative on standing in trifluoroacetic anhydride at room temperature it was necessary to use higher temperatures[4]. The amino acid esters were taken up in a minimum of methyl alcohol and an aliquot transferred by means of a microlitre syringe to a small Pyrex tube. The alcohol was removed with the rotary evaporator or alternatively by standing the tube in an oven at 70°. Trifluoroacetic anhydride (0.1 ml) was added and the tube sealed and

placed in the oven at 140° for 5 min. The tube was then cracked open, the TFA anhydride removed on the rotary evaporator and the residue taken up in a known volume of dry ethyl methyl ketone for injection onto the column.

RESULTS AND DISCUSSION

In isothermal gas chromatography of the most volatile amino acid derivatives it was found necessary to restrict the amino acids to alanine, valine, glycine, threonine, isoleucine, leucine and serine. The next most volatile derivatives, those of cysteine and proline, have much longer retention times than those of leucine and serine.

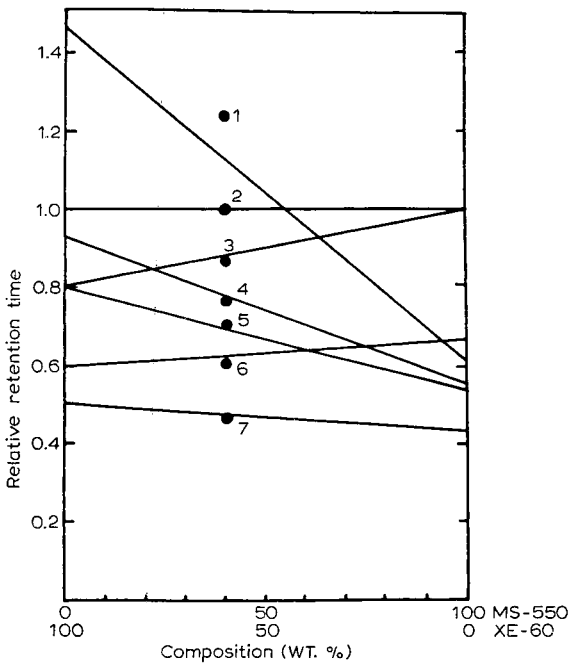


Fig. 1. The relationship between the relative retention time (leucine = 1.0) of the trifluoroacetylated *n*-amyl esters of seven amino acids and the composition (wt. %) of the mixed stationary phase XE-60 and MS-550 at 140°. 1 = Serine; 2 = leucine; 3 = isoleucine; 4 = glycine; 5 = threonine; 6 = valine; 7 = alanine.

The selection of mixed stationary phases was made by plotting the retention times of the derivatives relative to the leucine derivative on one stationary phase against the relative retention times of the same derivatives on a second phase using data obtained at the same temperature. This procedure eliminates combinations that will not improve resolution and also indicates the best proportions to be used in combinations that are likely to be effective. Three such combinations were found to give complete resolution of the derivatives of this first group of seven amino acid derivatives:

61 % XE-60 and 39 % Duo-seal pump oil;
27 % XE-60 and 73 % XF-1105;
60 % XE-60 and 40 % MS-550.

The individual packings were prepared to contain 5 % of the stationary phase.

Fig. 1 shows the result of applying such a graphical method for this third mixed phase. Serine is the only amino acid to give a relative retention time deviating considerably from the expected value. Packings containing Duo-seal pump oil and XF-1105 were not developed further, since these stationary phases were found to bleed considerably at temperatures above 150°. Quite appreciable bleed-rates which are often not very noticeable with the gas density balance detector are unsatisfactory with the flame-ionisation detector due to the base-line drift which becomes apparent at higher sensitivities.

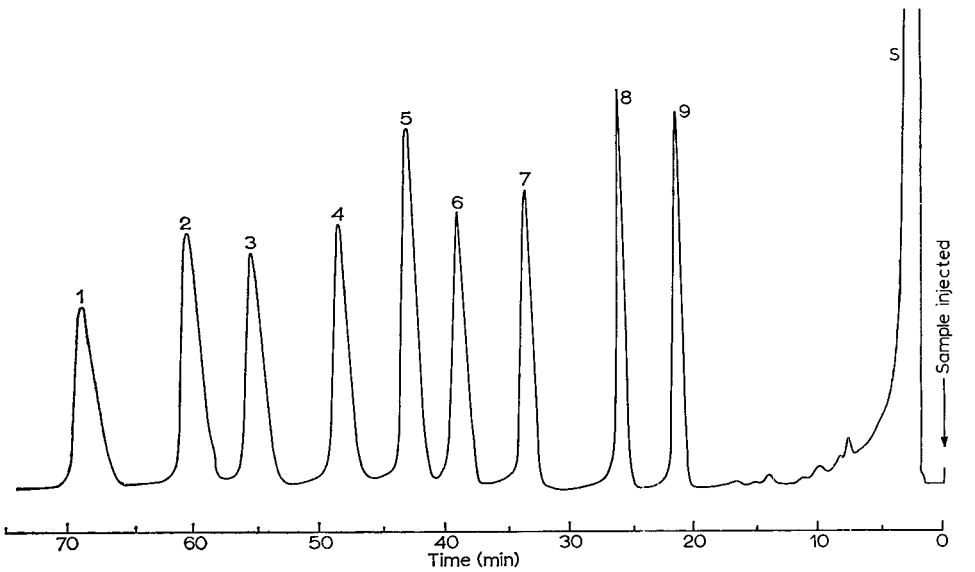


Fig. 2. Separation of a mixture of 9 trifluoroacetylated amino acid *n*-amyl esters on Anakrom ABS 90–100 mesh with the mixed phase XE-60 and MS-550. The packings were prepared separately to contain 5% w/w of the stationary phase and the column was filled with a blend of 60% XE-60 and 40% MS-550. The glass column was 5 m long with internal diameter 0.25 cm. Column temperature: 135°; gas flow: 24 ml N_2/min; inlet pressure: 25 p.s.i. D6 chromatograph with gas density balance detector. Sample size: 2.0 μl. Attenuation: × 1. Total number of theoretical plates for the leucine derivative (45 μg): 7300. 1 = Serine; 2 = β-amino-*n*-butyric acid; 3 = leucine; 4 = isoleucine; 5 = glycine; 6 = threonine; 7 = valine; 8 = alanine; 9 = α-amino-isobutyric acid. S = Solvent.

We found very little difference in performance between the columns filled with the blended packings and those produced by filling consecutive sections of the columns with different packings (segmented packings: see HAAHTI, VANDENHEUVEL AND HORNING[5]). However, it was found that the blended packings were more convenient, because it was easier to achieve the desired proportions and to alter the proportions if they required adjustment. It was also found that columns prepared from blended packings gave slightly higher efficiencies. When "mixed-film" packings were prepared by dissolving both stationary phases in the same solvent in the desired proportions prior to coating onto the support they were not always found to give the relative retention times that were expected. They gave slightly lower efficiencies and also the proportions of the stationary phases could not be altered.

Fig. 2 shows the separation of the first group of amino acids on a column packed with a mixture of the stationary phases XE-60 and MS-550. Two non-protein amino

acids, α-amino-isobutyric acid and β-amino-*n*-butyric acid are included, as these may be conveniently used as internal standards. It is interesting to note the difference in retention times for this pair of isomers. For certain quantitative work it may be preferable to add a known volume of a solution containing an external standard to the final reaction mixture. Naphthalene or ethyl benzoate are suitable external standards and emerge before the α-amino-isobutyric acid peak. Biphenyl and bibenzyl[1] do not have suitable retention times for this mixed column.

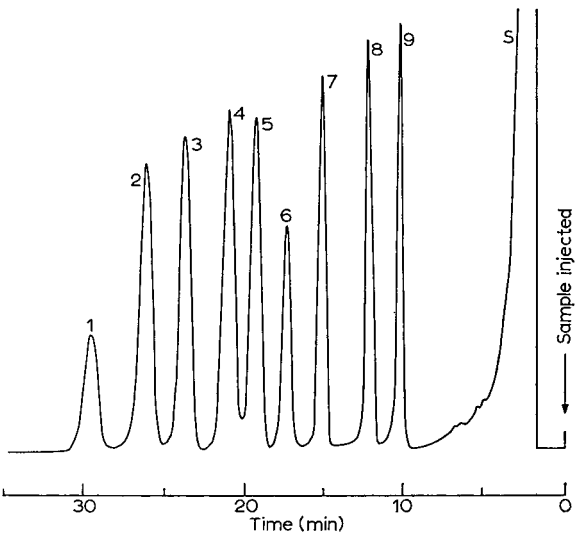


Fig. 3. Separation of same mixture as in Fig. 2 diluted eight-fold with methyl ethyl ketone. Column packing as for Fig. 2. Glass column 320 cm long with internal diameter 0.25 cm. Column temperature: 135°; gas flow: 24 ml/min. MicroTek MT 220 chromatograph with flame ionization detector. Sample size: 0.2 μl. Attenuation: 0.1 × 32. Total number of theoretical plates for the leucine derivative (~ 0.5 μg): 5100.

The relative retention times of these derivatives (relative to leucine taken as 1.0) vary with temperature. If the relative retention times are plotted against temperature, straight lines are obtained, whose slopes vary considerably. In practice, these temperature effects may be exploited to optimize the separation of these seven protein amino acids. With the mixed phases studied the separation of glycine, threonine and valine always presented the greatest difficulties and it was found that by raising or lowering the temperature by only a few degrees it was possible to adjust the separation to give the best resolution. Changing the gas flow-rate was not found to improve separations. The highest efficiencies were obtained with nitrogen gas flow rates of 38 ml/min and 25 ml/min for 0.4 and 0.25 cm internal diameter columns respectively.

The gas density balance detector was used to obtain the separation in Fig. 2. In Fig. 3 the same sample of mixed amino acids was separated with the MicroTek MT 220 gas chromatograph. In this separation the absolute amount of sample applied to the column was reduced to take advantage of the high sensitivity of the flame ionization detector and the total time taken has been reduced from 71 min to 31 min. The relative peak heights in Figs. 2 and 3 are not the same. This is due to the fundamental differences between the two types of detector used.

For the second group of derivatives (those of cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid, glutamic acid, tyrosine, lysine, arginine, tryptophan and cystine) the major problem was to get adequate resolution of cysteine, proline and hydroxyproline without extending the time of analysis too much for the less volatile derivatives such as lysine, arginine and tryptophan. To separate the amino acid derivatives in this group a mixed phase was finally developed after carrying out the procedure of plotting relative retention times as shown in Fig. 1. For convenience aspartic acid was taken as 1.0. Only one mixed phase was found to give adequate resolution: 53 % QF-1 and 47 % MS-710. Both these stationary phases have high maximum operating temperatures and low rates of bleeding, so that they are suitable for use with highly sensitive detectors. Fig. 4 shows the separation of 11 amino acids on this mixed phase at 170°. The two non-protein amino acids, ethionine and ornithine are included and may be used as internal standards. All the amino acids shown in Figs. 2 and 3 have very short retention times under these conditions and

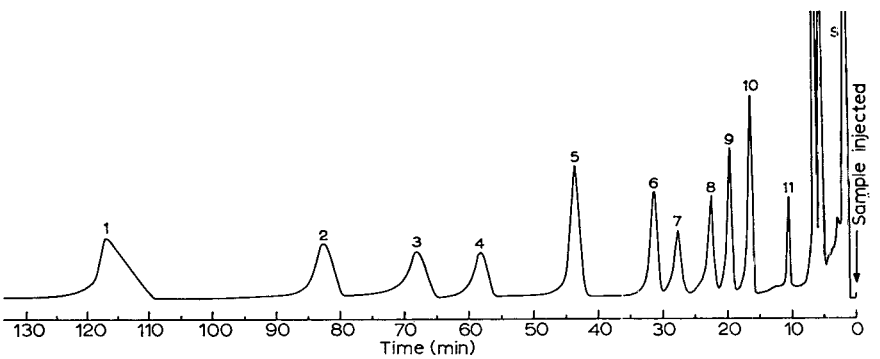


Fig. 4. Separation of a mixture of 11 trifluoroacetylated amino acid *n*-amyl esters on Anakrom ABS 100–110 mesh with the mixed phase QF-1 and MS-710. The packings were prepared separately to contain 5 % w/w of the stationary phase and the column was filled with a blend of 53 % QF-1 and 47 % MS-710. The glass column was 320 cm long with internal diameter 0.4 cm. Column temperature: 170°; gas flow: 38 ml N_2/min; inlet pressure: 16 p.s.i. D6 chromatograph with gas density balance detector. Sample size: 2.0 μl. Attenuation: × 1. Total number of theoretical plates for the aspartic acid derivative (40 μg): 3500. 1 = Lysine; 2 = glutamic acid; 3 = ornithine; 4 = tyrosine; 5 = aspartic acid; 6 = phenylalanine; 7 = ethionine; 8 = methionine; 9 = hydroxyproline; 10 = proline; 11 = cysteine. S = Solvent.

emerge as an unresolved set of peaks after the solvent but before cysteine. On this column the peaks of arginine and tryptophan are not completely resolved. They have a relative retention time of 4.5 (aspartic acid taken as 1.0). No identifiable volatile derivative has been obtained from histidine as the trifluoroacetylated *n*-amyl ester derivative. However, other workers have shown peaks for the trifluoroacetylated histidine methyl[6–8] and butyl[9] esters. The retention time of the cystine derivative is estimated to be about 6 h under these conditions and it may be advantageous to reduce cystine to cysteine[6] prior to making the derivative. Attempts to make a volatile derivative with cysteic acid using the methods described here have failed. The isothermal separation of the protein amino acids as their trifluoroacetylated methyl ester derivatives was carried out by MAKISUMI AND SAROFF[7] and these authors found it necessary to separate the amino acids in three groups, each at a different temperature.

Although no survey of stationary phases comparable to that carried out for the

trifluoroacetylated amino acid *n*-amyl esters has been made for the corresponding methyl esters, our results on a limited number of different columns indicate that at any one temperature the retention times of many of the methyl derivatives are about one third of those of the corresponding amyl derivatives. Similar results were also

TABLE I

RETENTION TIMES OF TRIFLUOROACETYLATED ESTERS OF ASPARTIC AND GLUTAMIC ACIDS

5 % w/w QF-1 and 5 % w/w MS-710 both on Silocel C22, 90–100 mesh acid washed and deactivated[1], blended in the proportion 60:40 and packed into two stainless steel tubes[1] with total length 182 cm. Gas flow: 38 ml N_2/min; column temperature: 185°.

N-TFA ester	R_T *(min)*
Aspartic acid	
dimethyl	3.3
methyl-amyl	8.5
diamyl	22.5
Glutamic acid	
dimethyl	5.5
methyl-amyl	15.2
diamyl	41.0

found by MAKISUMI, NICHOLLS AND SAROFF[10]. They quote a ratio of 1:3.4 for the relative retention times of the N-TFA leucine methyl and amyl esters. However, where two ester groups on the same molecule are involved the retention times of the methyl derivatives are about $^1/_7$ of the corresponding amyl derivatives. Table I shows

TABLE II

RETENTION TIMES OF TRIFLUOROACETYLATED CYSTINE ESTERS

5 % w/w SE-30 on Anakrom ABS 90–100 mesh packed into a glass column 152 cm long with 0.4 cm internal diameter. Gas flow: 38 ml N_2/min; column temperature: 210°.

N,N'-Di-TFA cystine ester	R_T *(min)*
Dimethyl	6.8
Diethyl	10.0
Dipropyl	14.5
Dibutyl	28.0
Diamyl	48.0

the increase in retention time for the dimethyl, the monomethyl-monoamyl and the diamyl esters of both aspartic and glutamic acids. Table II shows the increase in retention time for the homologous series of esters of cystine. In practice it is found that the methyl ester derivatives may be conveniently chromatographed with the column maintained at a temperature 30° lower than that required for the amyl esters. Using various stationary phases for isothermal separations the trifluoroacetylated methyl ester derivatives show a different order of emergence from the column to that of the trifluoroacetylated amyl esters, (*cf.* MAKISUMI AND SAROFF[7] and IKEKAWA[11] with refs. 1, 2 and 3). Where we have carried out isothermal and temperature-pro-

grammed separations on the same column, no difference was found in the order of emergence of our amyl ester derivatives.

It may be added that the trifluoroacetylated amino acid esters of pentan-2-ol could be resolved into two peaks on packed columns. These derivatives are more volatile than the corresponding pentan-1-ol esters. Fig. 5 shows the double peak

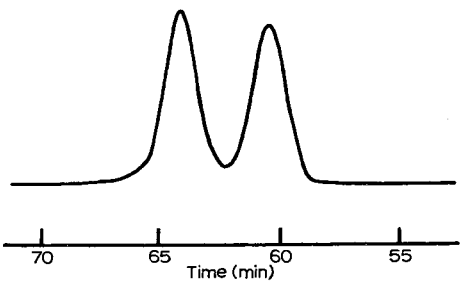


Fig. 5. Resolution into two peaks of the trifluoroacetylated D,L-valine D,L-pentan-2-ol ester. Same column in MicroTek MT 220 as used for Fig. 3. Column temperature: 100°; gas flow: 27 ml N_2/min. Total number of theoretical plates: 8500.

obtained with valine. No allocation of stereoisomers to the peaks has been made. Using capillary columns, GIL-AV, CHARLES AND FISCHER[12] and POLLOCK AND OYAMA[13] showed a similar resolution with the trifluoroacetylated octan-2-ol and butan-2-ol amino acid esters, respectively.

ACKNOWLEDGEMENTS

The D6 gas chromatograph and the D1 modification used in this investigation were purchased with grants from the Central Research Fund of the University of London. The MicroTek MT 220 gas chromatograph was purchased with a grant from the Medical Research Council.

We wish to thank Miss U. LAWRENCE for technical assistance.

We are grateful to Prof. HARRY HARRIS, F. R. S., and Prof. H. R. V. ARNSTEIN for their interest and support.

SUMMARY

A gas chromatographic separation of the trifluoroacetylated *n*-amyl esters of α-amino-isobutyric acid, alanine, valine, threonine, glycine, isoleucine, leucine, β-amino-*n*-butyric acid and serine in that order has been achieved isothermally using mixed stationary phases. With a different mixed phase at a higher temperature the following amino acids were separated: cysteine, proline, hydroxyproline, methionine, ethionine, phenylalanine, aspartic acid, tyrosine, ornithine, glutamic acid and lysine. The difficulties with other protein amino acids are discussed.

REFERENCES

1 A. DARBRE AND K. BLAU, *J. Chromatog.*, 17 (1965) 31.
2 K. BLAU AND A. DARBRE, *J. Chromatog.*, 17 (1965) 445.
3 K. BLAU AND A. DARBRE, *J. Chromatog.*, 26 (1967) 35.
4 D. L. STALLING AND C. W. GEHRKE, *Biochem. Biophys. Res. Commun.*, 22 (1966) 329.
5 E. O. A. HAAHTI, W. J. A. VANDENHEUVEL AND E. C. HORNING, *Anal. Biochem.*, 2 (1961) 344.
6 P. A. CRUICKSHANK AND J. C. SHEEHAN, *Anal. Chem.*, 36 (1964) 1191.
7 S. MAKISUMI AND H. A. SAROFF, *J. Gas Chromatog.*, 3 (1965) 21.
8 P. B. HAGEN AND W. BLACK, *Can. J. Biochem.* 43 (1965) 309.
9 C. W. GEHRKE AND F. SHAHROKHI, *Anal. Biochem.*, 15 (1966) 97.
10 S. MAKISUMI, C. H. NICHOLLS AND H. A. SAROFF, *J. Chromatog.*, 12 (1963) 106.
11 N. IKEKAWA, *J. Biochem.*, 54 (1963) 279.
12 E. GIL-AV, R. CHARLES AND G. FISCHER, *J. Chromatog.*, 17 (1965) 408.
13 G. E. POLLOCK AND V. I. OYAMA, *J. Gas Chromatog.*, 4 (1966) 126.

GAS CHROMATOGRAPHY AND STRUCTURAL CORRELATION OF SUBSTITUTED AZIRIDINES*

WALTER L. ZIELINSKI, Jr., LAWRENCE FISHBEIN, RICHARD O. THOMAS AND THOMAS E. WELSKO

Bionetics Research Laboratories, Inc., Falls Church, Va. (U.S.A.)

(Received January 10th, 1967)

Ethylenimine and its derivatives, the substituted aziridines, are an important class of compounds in industrial and biomedical areas. N-Substituted[1,2], N-carbamoyl[3], sulfur[4,5], and phosphorus[6–10] containing aziridines, have been utilized as insect chemosterilants. Aziridines have also been studied in neoplasms[11–14], as monoamine oxidase inhibitors[15], ion-exchange copolymers[16]; and wear and water proofing[17,18], textile[19–22] and paper improving agents[23,24].

Analysis of aziridines has been effected by colorimetry with γ-(4-nitrobenzoyl)-pyridine[25] and 1,2-naphthoquinone-4-sulfonate[26] and by direct[27] and potentiometric titration[28]. Gas chromatographic analysis of aziridines, however, has been limited to the separation of a *cis* and *trans* alkyl aziridine[29].

This study describes the elution behavior of N-aryl- and N-alkyl-carbamoyl-aziridines on five liquid phases and relates the chromatographic data obtained for the aryl derivatives to the separation of solute moiety–solvent interaction *via* consideration of linear aryl moiety values as interaction products of a solute moiety value and a partitioning phase value.

EXPERIMENTAL

The N-carbamoylaziridine derivatives were synthesized by reaction of ethylenimine with a selection of aryl and alkyl isocyanates in benzene. Ethylenimine was obtained from Chemirad, Inc., East Brunswick, N.J. (U.S.A.). The isocyanates were obtained from various commercial sources.

Gas chromatographic analysis was carried out on 6 ft. by 0.25 in. O.D. glass coiled columns containing alternatively, 3 % Carbowax 20M (polyglycol), 4 % DC QF-1 (trifluoropropylmethyl silicone fluid), 15 % GE Versilube F-50 (chlorophenylmethyl silicone fluid), and 15 % GE XE-60 (cyanoethyl methyl/dimethyl silicone gum), housed in a modified (kit from Applied Science Labs., State College, Pa., U.S.A.) F & M Model 1609 flame ionization instrument such that the samples were injected on-column with the effluent passing directly to the detector. Column temperature was set by an F & M Model 240 power-proportioning controller and the carrier flow was maintained through a Brooks ELF Model 8943 constant downstream flow controller. Analysis on 12 % GE SE-30 (methyl silicone gum) was performed on a 5.5 ft. by 0.25

* Presented to the Division of Analytical Chemistry, 152nd Meeting, Am. Chem. Soc., New York, N.Y., September 1966.

in. O.D. copper coiled column in an F & M Model 720 oven containing a hot-wire detector. The specific conditions of analysis for all chromatographic columns are summarized in Table I.

Physical constants of the synthesized aziridines and the respective isocyanates together with literature values where available are given in Table II. With the exception of several of the alkyl derivatives and N-β-phenylethyl-carbamoylaziridine, the synthesized aziridines were all solids. The melting points were obtained on a Fisher-Johns Melting Point Apparatus. Injected samples were of 0.1 to 0.2 μl, generally in acetone solution.

TABLE I

EXPERIMENTAL CONDITIONS

	Columns				
	(1) *QF-1*	(2) *Versilube F-50*	(3) *Carbowax 20M*	(4) *XE-60*	(5) *SE-30*
Column					
% Coating	4 %	15 %	3 %	15 %	12 %
Support	Chromosorb W 80–100 mesh (HMDS)	Chromosorb W 60–80 mesh (HMDS)	Chromosorb G 60–80 mesh (AW-DMCS)	Chromosorb W 60–80 mesh (HMDS)	Chromosorb W 60–80 mesh (HMDS)
Dimensions	6 ft. × 1/4 in. (glass)	6 ft. × 1/4 in. (glass)	6 ft. × 1/4 in. (glass)	6 ft. × 1/4 in. (glass)	5.5 ft. × 1/4 in. (copper)
Conditions					
Column temperature	110° 150°	170° 210°	140° 200°	165°	180°
Injection port	255°	255°	255°	255°	245°
Detector temperature	200°	200°	200°	200°	250°
N_2, ml/min	33 37	45 46	69 119	61	33[a]
Detector	F.I.	F.I.	F.I.	F.I.	T.C.

[a] He, ml/min.

RESULTS AND DISCUSSION

Retention data for the aziridine derivatives, relative to N-*n*-propyl-carbamoylaziridine, on the QF-1 and Versilube F-50 liquid phases are given in Table III. Overall, retention and resolution were generally greater on the latter than on the former phase. Elution distinctions for isomeric aryl families may be discerned, as may the categoric retention order of $NO_2 > CN > CH_3O$, $Br > Cl > CH_3 > F$.

The chromatographic results of the aziridines measured on four of the partitioning phases employed (chromatography on XE-60 to be discussed below), expressed relative to N-phenyl-carbamoylaziridine and as KOVÁTS hydrocarbon retention indices, are shown in Table IV. The isocyanate precursors to the respective aziridine derivatives were included in the analyses performed on QF-1. It was observed with interest and concern that the retention of the aryl derivatives was fairly close to that of their respective isocyanates in a number of instances. The slight differences obtained, however, were consistently reproducible—the isocyanate (with the exception of the naphthyl and *p*-cyanophenyl derivatives) always eluting slightly ahead of the corresponding aziridine. On the polar Carbowax 20M liquid phase, N-phenyl-carbamoylaziridine had an elution time of 1.80 min, while phenyl isocyanate eluted in

TABLE II

PHYSICAL CONSTANTS (M.P. AND B.P.) OF ISOCYANATES AND N-CARBAMOYLAZIRIDINES

	$R-N=C=O$	$R-N(H)-C(=O)-N\langle CH_2-CH_2 \rangle$	
R	*Isocyanate (°C) (mm)*	*Aziridine (°C) (mm)*	
		Found	*Literature*
Methyl	37–39[a]	35–37 (0.4)[a]	—
Ethyl	59–61[a]	51–53 (0.4)[a]	—
Propyl	87–88[a]	69–71 (0.4)[a]	—
Isopropyl	73–75[a]	50–52	—
Butyl	110–114[a]	84–86 (0.4)[a]	85–87 (0.4)[a,b]
Phenyl	54–55 (13)[a]	79–81	82–83[d]
Cyclohexyl	166–168[a]	78–80	81–82[c]
o-Tolyl	184–186[a]	73.5–75	75–76[b]
m-Tolyl	75–76 (12)[a]	69–70.5	70.5–71.5[b]
p-Tolyl	70–72 (10)[a]	97–99	98.5–99.5[b]
p-Fluorophenyl	71–73 (29)[a]	73–75	—
o-Methoxyphenyl	93–94 (7)[a]	63–64.5	64–65[b]
p-Methoxyphenyl	(−11)–(−9)[a]	114–116	114–115.5[b]
o-Chlorophenyl	(−5)–(−3)[a]	51–53	52–53.5[b]
m-Chlorophenyl	76–78 (10)[a]	92–94	92.5–94[b]
p-Chlorophenyl	29.5–30.5	133–135	132–133.5[b]
m-Trifluormethylphenyl	54 (11)[a]	73–74	—
p-Bromophenyl	41–42.5	141–143	138.8–139.5[d]
β-Phenethyl	98–100 (10)[a]	127–129	—
α-Naphthyl	3–5	107–108	108–109[b]
p-Cyanophenyl	102–104	131–133	—
o-Nitrophenyl	38–39	227–230	—
m-Nitrophenyl	52–53.5	135–137	—
p-Nitrophenyl	57–59	171–172	164 dec[b]

[a] Boiling points.
[b] A. B. BOŘKOVEC AND C. W. WOODS, *J. Med. Pharm. Chem.*, 8 (1965) 545.
[c] E. BESTIAN, J. HEYNA, A. BAUER, G. EHLERS, B. HIRSEKORN, T. JACOBS, W. NOLL, W. WEIBEZAHN AND F. ROMER, *Ann.*, 566 (1950) 210.
[d] B. C. FISCHBACK AND G. H. HARRIS, *U.S. Pat.*, 2,775,587, Dec. 25, 1956; *C.A.*, 51 (1957) 9700.

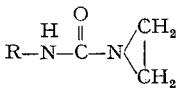
0.7 min (column temperature, 140°). A considerable retention difference was observed for the N-alkyl-carbamoylaziridines *vs.* their respective isocyanates, illustrating the contribution to retention introduced by condensation of the ethylenimine ring with the low boiling alkyl isocyanates. The retention relative to N-phenyl-carbamoyl-aziridine was less for hydrocarbon structures (*e.g.* the tolyl derivatives) on Carbowax 20M than on the silicone phases. For the same reason (*i.e.* diminished extent of interaction of Carbowax 20M with non-polar hydrocarbon solutes*), the highest index values were obtained on the polar Carbowax 20M phase.

Anomalous results were obtained on the XE-60 stationary phase, where it was

* Index values were determined using a C-10 to C-18 *n*-hydrocarbon mixture obtained from Applied Science Laboratories, State College, Pa. (U.S.A.).

TABLE III

N-CARBAMOYLAZIRIDINES RELATIVE TO N-*n*-PROPYLCARBAMOYLAZIRIDINE



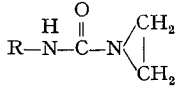
Compound No.	*R*	*QF-1*[a]	*Versilube F-50*[b]
1	ϕ	0.24	0.50
2	*p*-Fϕ	0.30	0.55
3	Cyclohexyl	0.36	—
4	*m*-$CF_3\phi$	0.38	0.59
5	*o*-Tolyl	0.41	0.73
6	*m*-Tolyl	0.43	0.81
7	*p*-Tolyl	0.44	0.92
8	Methyl	0.47	0.61
9	Ethyl	0.62	0.69
10	*o*-Clϕ	0.66	0.94
11	*m*-Clϕ	0.64	1.14
12	*p*-Clϕ	0.69	1.28
13	β-ϕC_2H_4	0.75	–
14	Isopropyl	0.90	0.74
15	*n*-Propyl[c]	1.00	1.00
16	*p*-Brϕ	1.14	1.74
17	*o*-Methoxyϕ	1.19	1.53
18	*p*-Methoxyϕ	1.32	1.62
19	*n*-Butyl	1.43	1.71
20	α-Naphthyl	3.05	4.53
21	*p*-CNϕ	3.33	3.31
22	*o*-$NO_2\phi$	4.23	—
23	*m*-$NO_2\phi$	4.66	—
24	*p*-$NO_2\phi$	4.72	—

[a] Column temperature 110° for compounds 1–19; 150° for compounds 20–24.
[b] Column temperature 210° for compounds 20 and 21; 170° for all other compounds.
[c] Representative adjusted retention time (min) for the N-*n*-propyl carbamoylaziridine standard: 6.82 on QF-1 (110°), 2.00 on QF-1 (150°), 3.30 on Versilube F-50 (170°), 1.25 on Versilube F-50 (210°).

observed that chromatography resulted in two peaks on the recorder chart for each sample analyzed, including the N-phenyl-carbamoylaziridine standard. Neither of the peaks were commonly present in all samples analyzed, belying lysis to a common degradation product (*e.g.* a fragment structure containing the ethylenimine ring). The first peak was found to coincide precisely with the respective parent isocyanate while the second peak matched the respective aniline derivative. The formation of aniline derivatives was further substantiated by the positive Hinsberg test obtained by bubbling the effluent gas from the chromatograph directly into a small amount of test reagent in a small tube. This latter step was carried out on an F & M Model 500 chromatograph equipped with a Model 720 dual column oven and a hot-wire detector. A copper column (8 ft. by 0.125 in. O.D.) was used with the same liquid phase load

TABLE IV

N-CARBAMOYLAZIRIDINES RELATIVE TO N-PHENYLCARBAMOYLAZIRIDINE AND KOVÁTS INDICES



Compound No.	*R*	*SE-30*		*Versilube F-50*[a]		*QF-1*[b]			*Carbowax 20M*[c]	
		Aziridine/ N-Phenyl	*Index*	*Aziridine/ N-Phenyl*	*Index*	*Aziridine/ N-Phenyl*	*Isocyanate/ N-Phenyl*	*Aziridine Index*	*Aziridine/ N-Phenyl*	*Index*
1	ϕ[d]	1.00	1007	1.00	976	1.00	1.00	1187	1.00	1638
2	*p*-Fϕ	1.06	1020	1.10	997	1.24	1.14	1228	1.78	1759
3	Cyclohexyl	—	—	—	—	1.48	1.48	1261	0.21	568
4	*m*-$CF_3\phi$	1.13	1035	1.18	1010	1.58	1.29	1273	2.74	1850
5	*o*-Tolyl	1.46	1097	1.46	1054	1.70	1.69	1287	0.56	1516
6	*m*-Tolyl	1.50	1103	1.61	1075	1.77	1.72	1294	1.00	1638
7	*p*-Tolyl	1.60	1118	1.84	1103	1.83	1.79	1301	1.03	1645
8	Methyl	—	—	1.21	1016	1.96	—0.03	1314	—	—
9	Ethyl	—	—	1.38	1043	2.58	0.03	1365	—	—
10	*o*-Clϕ	1.82	1148	1.87	1106	2.74	2.62	1377	3.53	1902
11	*m*-Clϕ	2.32	1204	2.28	1148	2.65	2.45	1371	[e]	[e]
12	*p*-Clϕ	2.37	1208	2.56	1172	2.86	2.72	1385	[e]	[e]
13	β-øC_2H_4	—	—	—	—	3.09	3.03	1399	—	—
14	Isopropyl	—	—	1.47	1056	3.72	0.03	1436	—	—
15	*n*-Propyl	—	—	2.00	1121	4.13	0.11	1456	—	—
16	*p*-Brϕ	4.52	1348	3.48	1236	4.71	4.71	1482	13.4	2182
17	*o*-Methoxyϕ	2.53	1223	3.06	1208	4.93	4.76	1491	2.59	1838
18	*p*-Methoxyϕ	2.76	1242	3.24	1221	5.45	5.24	1511	[f]	[f]
19	*n*-Butyl	—	—	3.41	1232	5.90	0.29	1526	[g]	[g]
20	α-Naphthyl	—	—	9.06	1523	12.6	12.7	1807	—	—
21	*p*-CNϕ	6.22	1420	6.62	1439	12.1	13.7	1830	[f]	[f]
22	*o*-$NO_2\phi$	—	—	—	—	17.5	15.9	1887	—	—
23	*m*-$NO_2\phi$	—	—	—	—	19.3	16.6	1912	—	—
24	*p*-$NO_2\phi$	—	—	—	—	19.5	18.1	1915	—	—

[a] Column temperature 210° for compounds 20 and 21; 170° for all other compounds.

[b] Column temperature 110° for compounds 1–19; 150° for compounds 20–24.

[c] Column temperature 200° for compounds 16 and 18; 140° for all other compounds.

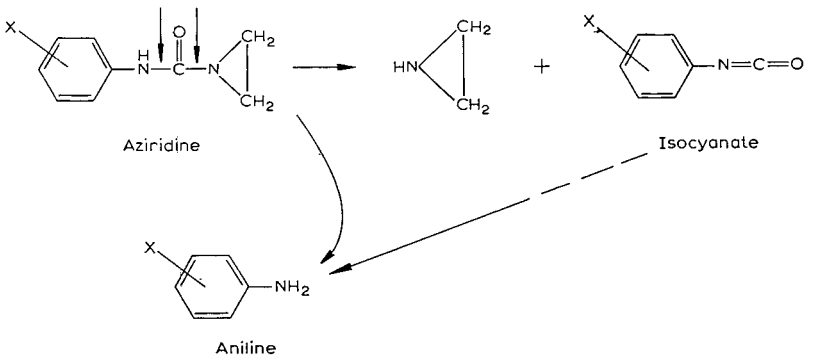
[d] Representative adjusted retention time (min) for N-phenyl-carbamoylaziridine standard: 1.30 on SE-30, 1.70 on Versilube F-50 (170°), 0.65 on Versilube F-50 (210°), 1.65 on QF-1 (110°), 0.50 on QF-1 (150°), 1.80 on Carbowax 20M (140°), 0.28 on Carbowax 20M (200°).

[e] Decomposed.

[f] Not detected.

[g] Eluted in solvent peak.

(15% XE-60 on 60–80 mesh HMDS-Chromosorb W). The chromatographic observations on XE-60 may be illustrated as follows:

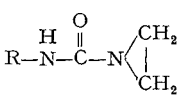


Owing to the difficulty involved in accounting for the conversion of isocyanate to aniline, it is felt that the aniline products may result directly during initial cleavage of the aziridines.

As may be seen in Table V, the N-alkyl derivatives did not degrade. Degradation of the isomeric tolyl derivatives interestingly gave varying aniline/isocyanate peak area ratios (Table VI). The *ortho* isomer produced considerably less aniline product. The

TABLE V

CHROMATOGRAPHY ON 15% XE-60



Compound No.	*R*	*Isocyanate index*[b]	*Aniline index*[b]
1	ϕ	1340	1506
2	*p*-Fϕ	1334	1563
5	*o*-$CH_3\phi$	1400	1573
6	*m*-$CH_3\phi$	1407	1605
7	*p*-$CH_3\phi$	1407	1569
11	*o*-Clϕ	—	1666
17	*o*-$CH_3O\phi$	1643	1698
18	*p*-$CH_3O\phi$	1659	1808
10	*m*-Clϕ	—	1823
12	*p*-Clϕ	—	1829
16	*p*-Brϕ	—	1947
21	*p*-CNϕ[a]	—	—
		Index	
N-Butyl-carbamoylaziridine		1796	
Butyl isocyanate		965	
Butylamine		in solvent front	

[a] Not detected.

[b] Representative adjusted retention time (min) for the standard hydrocarbon mixture: C-10 (0.25), C-12 (0.81), C-14 (1.85), C-16 (4.20), C-18 (9.45).

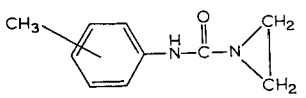
reasons for solute lysis on XE-60 and not on other substrates is not known although there is apparently an early catalytic interaction of the XE-60 solvent phase with the injected solute molecules.

The relative influence of various *para* substituents on the elution of N-aryl-carbamoylaziridines is presented in Table VII for each of the stationary phases employed. The phases are roughly arranged in order of increasing index values (see also Table IV), thereby suggesting their relative order of increasing polarity. With the exception of XE-60, on which aziridine degradation effects were noted, a general increase in the relative influence values of Table VII was observed (relative to the non-polar *p*-methyl substituent) with increasing stationary phase polarity. This should be greater with the more polar solutes as a result of the enhanced dipole–dipole interactions with the stationary solvent.

In interpretation of the analytical chromatographic data, one may go further and ascribe values to the individual aryl moieties based upon the numerical loga-

TABLE VI

DEGRADATION OF ISOMERIC N-TOLYL-CARBAMOYLAZIRIDINES ON XE-60

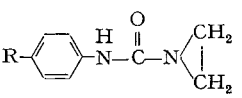


Position	*Area (%)*[a]		*Aniline/isocyanate*
	Isocyanate	*Aniline*	
Ortho	65.3	34.7	0.5
Meta	25.9	74.1	2.9
Para	22.8	77.2	3.6

[a] Based on relative chromatogram peak areas.

TABLE VII

RELATIVE INFLUENCE OF *para* SUBSTITUENTS OF N-ARYL-CARBAMOYLAZIRIDINES ON RETENTION[a]



R	*Mol. wt. of R*	*Relative influence*[a]				
		SE-30	*F-50*	*QF-1*	*XE-60*[b]	*C20-M*
Methyl	15.0	1.00	1.00	1.00	1.00/1.12	1.00
Fluoro	19.0	0.91	0.90	0.94	0.95/1.11	1.07
Cyano	26.0	1.27	1.31	1.41	—	—
Methoxy	31.0	1.11	1.11	1.16	1.18/1.28	—
Chloro	35.4	1.08	1.06	1.06	— /1.30	—
Nitro	50.0	—	—	1.47	—	—
Bromo	79.9	1.21	1.12	1.14	— /1.38	1.33

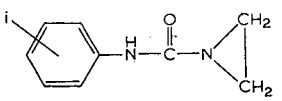
[a] Obtained by ratioing the hydrocarbon index values of the aziridines to the index value obtained for *p*-tolyl-carbamoylaziridine on each liquid phase.

[b] Values given as isocyanate/aniline, relative to *p*-tolyl isocyanate.

rithmic differences of the elution data between the various substituted N-aryl-carbamoylaziridines and N-phenyl-carbamoylaziridine, for each liquid phase. This method had been used earlier for evaluating the relative moiety contributions to the gas chromatographic retention of 3,4-methylenedioxyphenyl derivatives in which the log differences were determined relative to methylenedioxybenzene[30]. The rationale for this type of calculation obviously lies with the well known observation that isothermal gas chromatographic elution is a logarithmic function. The contribution of structural moieties to chromatographic behavior was early recognized by MARTIN[31] prior to the gas chromatographic era. Moiety values resulting from logarithmic calculations with retention data were shown to be additive by KOVÁTS[32,33], KNIGHTS[34,35] and in earlier work by the authors with N-substituted and simple carbamates[36]. Moiety values for the aryl substituents in the N-aryl-carbamoylaziridines are given Table VIII. They infer the relative linear contributions of the aryl substituents toward the chromatographic behavior reported in Tables III and IV, and suggest the relative overall degree of interaction of the solute moiety with the liquid phase.

TABLE VIII

ARYL MOIETY VALUES (M)[a]



i	(*s*) *SE-30*	*F-50*	*QF-1*	*Carbowax 20M*
H	0.000	0.000	0.000	0.000
p-F	0.025	0.041	0.093	0.250
m-CF_3	0.053	0.072	0.199	—
o-CH_3	0.164	0.164	0.230	−0.252
m-CH_3	0.176	0.207	0.248	0.000
p-CH_3	0.204	0.265	0.263	0.013
o-Cl	0.260	0.272	0.438	0.548
m-Cl	0.366	0.358	0.423	—
p-Cl	0.375	0.408	0.456	—
o-CH_3O	0.403	0.486	0.693	0.413
p-CH_3O	0.441	0.510	0.736	—
p-Br	0.665	0.543	0.673	1.127
p-CN	0.794	0.821	1.083	—
o-NO_2	—	—	1.243	—
m-NO_2	—	—	1.290	—

[a] Obtained from: $\log R.E._{x+i} - \log R.E._x$.

It is possible to delineate these interactions somewhat by a consideration of the moiety values of Table VIII as interaction products of the solute moiety with the non-mobile solvent phase in a manner analogous to the Hammett calculations for substituent values from equilibrium or rate constants in organic reactions[37]. If such dissection were quantitatively descriptive, moiety values could be obtained which, in principle, would be independent of the liquid phase, and a liquid phase selectivity scale might be attainable. Such a simplified approach obviously contains several inherent errors (*e.g.* the aggregation of induction, dispersion and hydrogen bonding

forces into a net interaction value, and the assumption that the differences in the electronic induction effect of the various aryl substituents on the "polarity" of the residual portion of the aziridine molecule and relative to N-phenyl-carbamoyl-aziridine, are negligible). The results of this semi-quantitative/qualitative approach towards a selectivity scale for the liquid phases employed in this study is, nonetheless, somewhat interesting and may be described as follows:

The relatively low polar (methyl silicone polymer) SE-30 phase was arbitrarily designated a relative selectivity of 1.00. Consideration of the Hammett equation proposes a similar equation for gas chromatographic interaction as

$$\log R.E._{x+i} - \log R.E._x = M_i = ps$$

where $R.E.$ = relative elution; M_i = moiety value (additive); p = relative selectivity of liquid phase; s = substituent value; and the M_i values for SE-30 in Table VIII become the s substituent values.

In the expression $M_i = ps$, one has a linear equation possessing a slope of p and an intercept of zero. The greatest point scatter was obtained for Carbowax 20M. The calculated slopes together with their standard deviations are given in Table IX. The observation that this approach suggests a selectivity scale for the liquid phases employed in this study towards the aziridine solutes analyzed is clear when the difference $M_i - M_j = p_A(s_i - s_j)$ for two substituents on column A is algebraically compared to $p_B(s_i - s_j)$ for the same two substituents on column B. If $p_B(s_i - s_j) > p_A(s_i - s_j)$ and $(s_i - s_j)$ approximates a constant, then $p_B > p_A$, which proposes that column B is more selective than column A for the separation of the two aziridines containing substituents i and j, respectively.

TABLE IX

SELECTIVITY SCALE FOR LIQUID PHASES

Phase	*P*	*Standard deviation*
SE-30	1.00	0.00
Versilube F-50	1.18	0.23
QF-1	1.42	0.24
Carbowax 20M	1.60	0.45

The paucity of ample data in the approach discussed above for delineation of solute–solvent interactions is readily conceded. Doubtlessly more reliable and, perhaps, more general selectivity values could be determined if a model block-type experiment could be designed such that varying degrees of polarity in solutes and stationary phases were represented. Delineation of solute–solvent interactions would likewise be improved if one could quantitatively isolate the various attractive forces comprising such interactions.

ACKNOWLEDGEMENT

This study was supported by Research Contract PH 43-64-57, National Cancer Institute, National Institutes of Health, Public Health Service, and represents Paper No. 29 of this Contract.

SUMMARY

A diverse selection of alkyl and substituted aryl N-carbamoylaziridines were prepared *via* reaction of ethylenimine and various isocyanates. The gas chromatographic behavior of these derivatives was reported on five liquid phases and the data obtained reported relative to N-phenyl-carbamoylaziridine and as KOVÁTS' indices. Degradation to the isocyanate and the aniline derivatives was observed for aryl aziridines on XE-60. The N-alkyl-aziridines were found to chromatograph without thermal rupture. To correlate structural features with the analytical results, structural and positional moiety values were obtained from the log differences of the N-aryl-carbamoylaziridines with N-phenyl-carbamoylaziridine in a manner similar to which the sigma–rho product is obtained in the Hammett equation from equilibrium or rate constants in organic reactions. Separations of *o*, *m* and *p* substituted N-chlorophenyl derivatives were successful.

The influence of functionality in the *para* position of N-aryl derivatives revealed an elution order of $F < CH_3 < Cl < Br$, $CH_3O < CN$.

REFERENCES

1 A. B. BOŘKOVEC, *Residue Rev.*, 6 (1964) 87.
2 C. W. WOODS, A. B. BOŘKOVEC AND P. M. HART, *J. Med. Pharm. Chem.*, 7 (1964) 371.
3 A. B. BOŘKOVEC AND C. W. WOODS, *J. Med. Pharm. Chem.*, 8 (1965) 545.
4 A. B. BOŘKOVEC AND C. W. WOODS, *Advan. Chem. Ser.*, 41 (1963) 47.
5 J. C. PARISH AND B. W. ARTHUR, *J. Econ. Entomol.*, 58 (1965) 699.
6 A. B. BOŘKOVEC, *Science*, 137 (1962) 1034.
7 S. C. CHANG AND A. B. BOŘKOVEC, *J. Econ. Entomol.*, 57 (1964) 488.
8 W. J. HAYER, Jr., *Bull. World Health Organ.*, 31 (1964) 721.
9 T. J. HENNEBERRY AND A. N. KISHABA, *J. Econ. Entomol.*, 59 (1966) 156.
10 G. C. LABREQUE, *J. Econ. Entomol.*, 54 (1961) 684.
11 S. S. BROWN, *Advances in Pharmacology*, Vol. 2, Academic Press, New York, 1963, p. 243.
12 L. F. LARIONOV, *Acta Unio Intern. Contra Cancrum*, 13 (1957) 393; *C.A.*, 52 (1958) 4853.
13 M. UCHIDA AND H. TAKAGI, *Gann*, 48 (1957) 205.
14 O. V. ZUBOVA, *Akad. Med. Nauk SSSR*, 2 (1960) 75; *C.A.*, 60 (1964) 1010.
15 J. N. WELLS, A. V. SHIRODKAR AND A. M. KNEVEL, *J. Med. Pharm. Chem.*, 9 (1966) 195.
16 G. MANECKE AND K. H. HELLER, *German Pat.*, 1, 160, 183 (Dec. 27th, 1963); *C.A.*, 60 (1964) 9440.
17 H. CHOU AND W. L. YANG, *Fang Chih Chi Shu.*, No. 2 (1964) 37; *C.A.*, 62 (1965) 13308.
18 Y. OHARA, *Japan Pat.*, 12,232 (Aug. 30, 1960); *C.A.*, 55 (1961) 10914.
19 E. FRIESER, *Z. Ges. Textil-Ind.*, 60 (1958) 977; *C.A.*, 53 (1959) 8636.
20 F. B. JONES, H. G. HAMMON, R. I. LEININGER AND R. G. HEILIGMANN, *Textile Res. J.*, 31 (1961) 57.
21 K. KAMIYA AND Y. JO, *Sen-i Kôgyô Shikensho Ihô*, 45 (1958) 79; *C.A.*, 55 (1961) 4968.
22 P. S. UGRYUMOV, *Tekstil'n. Prom.*, 20 (1960) 45.
23 R. E. REIZIN AND A. TUPURAINE, *Akad. Nauk Latv. SSR*, 25 (1963) 107; *C.A.*, 60 (1964) 8225.
24 H. S. STANGER AND W. SANNE, *Brit. Pat.*, 985,716 (March 10th, 1965); *C.A.*, 62 (1965) 16507.
25 J. EPSTEIN, R. W. ROSENTHAL AND R. J. ESS, *Anal. Chem.*, 27 (1955) 1435.
26 D. H. ROSENBLATT, P. HLINKA AND J. EPSTEIN, *Anal. Chem.*, 27 (1955) 1290.
27 R. R. JAY, *Anal. Chem.*, 36 (1964) 667.
28 M. LIDAKS, J. LICIS AND A. VEISS, *Latvijas PSR Zinatnu Akad. Vestis*, 2 (1960) 101; *C.A.*, 55 (1961) 25602.
29 R. L. VAN ETTEN AND A. T. BOTTINI, *J. Chromatog.*, 21 (1966) 408.
30 W. L. ZIELINSKI, Jr. AND L. FISHBEIN, *Anal. Chem.*, 38 (1966) 41.
31 A. J. P. MARTIN, *Biochem. Soc. Symp. (Cambridge, England)*, 3 (1949) 4.
32 E. KOVÁTS, *Helv. Chim. Acta*, 46 (1963) 2705.
33 E. KOVÁTS AND P. B. WEISZ, *Z. Physik. Chem.*, 65 (1965) 812.
34 B. A. KNIGHTS, *J. Gas Chromatog.*, 4 (1966) 329.
35 B. A. KNIGHTS AND G. H. THOMAS, *Anal. Chem.*, 34 (1962) 1046.
36 W. L. ZIELINSKI, Jr. AND L. FISHBEIN, *J. Gas Chromatog.*, 3 (1965) 260.
37 L. P. HAMMETT, *Physical Organic Chemistry*, McGraw-Hill, New York, 1940, p. 184.

GAS–LIQUID CHROMATOGRAPHY OF SUBMICROGRAM AMOUNTS OF DRUGS

III. ANALYSIS OF ALKALOIDS IN BIOLOGICAL MEDIA

HAROLD V. STREET
Department of Forensic Medicine, University of Edinburgh (Great Britain)
(Received December 2nd, 1966)

In this paper, the term "alkaloid" is used to denote any basic nitrogenous compound which is extractable from aqueous alkaline solution by an organic solvent. Not only are there a large number of drugs included under "alkaloids" by this classification but also these drugs themselves display a wide variety of functional groups and cover an extensive range of molecular weight. This is precisely where gas–liquid chromatography (GLC) is of inestimable value owing to its capability of dealing with the separation of compounds possessing almost any thermostable non-ionic functional group encountered in drugs. Furthermore, GLC can handle drugs varying in molecular weight from less than 100, *e.g.* ethanol, to about 500, *e.g.* emetine, and it may be that future improvements in the thermal stability of liquid phases and in the sensitivity of detectors will permit alkaloids of molecular weight greater than 500 to be successfully separated by GLC. In this respect it is interesting to note that SUBBARAM[1] claims to have separated a mixture of cholesteryl palmitate and cholesteryl stearate (mol. wt. 624 and 652, respectively) on a column of Anakrome ABS coated with 2 % SE 30 programmed from 200° to 340° at 3° per min. Even metallo-porphyrins have been separated in a high pressure (1830 p.s.i.) column by KLESPER, CORWIN AND TURNER[2] using dichlorodifluoromethane as carrier gas although, in this case, the compounds were not eluted but were located within the column after the "run". These examples serve to illustrate that ultimately the limiting factor may not be the molecular weights but may be the thermal stability in nitrogen (or other carrier gas) of the compounds being separated. However, it is perhaps fortunate for the analytical toxicologist that the majority of alkaloids in current therapeutic use have a molecular weight below 500.

In 1960, LLOYD *et al.*[3] described the separation of alkaloids by gas chromatography using 2–3/100 SE 30 at 160°–222° but no mention was made of acid-washing or of silanising the Chromosorb W. A chromatogram was shown displaying the separation of six alkaloids of the papaveraceae group but the exact weight of alkaloid injected was not stated. The peaks also showed marked tailing. Working on the quantitative determination of morphine in opium by gas–liquid chromatography, BROCHMANN-HANSSEN AND SVENDSEN[4] in 1963 found that "phenolic alkaloids, such as morphine, are difficult to gas chromatograph even on a treated support". They overcame their difficulty by reacting the phenolic-OH group with hexamethyldisilazane (HMDS). The trimethylsilyl ethers so formed showed no tailing at 183° on

their column of acid- and base-washed Gas Chrom P, HMDS-treated, and coated first with 0.1 % polyethylene glycol 9000 and then with 4 % SE 30. Even so, the weight of morphine injected corresponded to between 20 and 30 μg.

Many other papers have appeared in the literature dealing with gas–liquid chromatography of alkaloids but in may cases. *e.g.* ANDERS AND MANNERING[5]; SHAW[6]; BROCHMANN-HANSSEN AND SVENDSEN[7]; LINDSTEDT[8], it has been found necessary to form a derivative in order to obtain a satisfactory "run" of the compound. ANDERS AND MANNERING[9] in another article, described the separation of a number of phenothiazine derivatives. These were "run" as free bases but the amounts of drugs used were from 5 to 10 μg. BROCHMANN-HANSSEN AND FONTAN[10,11] have also published two articles dealing with the gas chromatography of alkaloids with polar stationary liquids. In neither of these articles is there any mention of the amount of alkaloid injected. It seems quite probable that the tailing which they would encounter with their polar phases would inevitably lead to dependence of retention time on amount of drug injected. Quite recently, MASSINGILL AND HODGKINS[12] have tabulated their results of GLC of alkaloids using four packed columns. The liquid phases used were Epon 1001 Resin, XE 60, SE-52 and JXR. Again, amounts of drugs injected did not go below 1 μg; furthermore, no peak shapes were illustrated.

MCMARTIN AND STREET[13] have recently described their work on the preparation of columns displaying reduced amounts of adsorption and have discussed the scope and limitations of such columns. In a further paper, MCMARTIN AND STREET[14] showed that these columns could be applied successfully, in the sub-microgram region, to the analysis of barbiturates and related compounds in biological material.

The present paper describes the analysis of sub-microgram amounts of alkaloids by the application of columns prepared in a similar manner, with slight but important modifications, to that given by MCMARTIN AND STREET[13].

EXPERIMENTAL

Gas chromatography details

Gas–liquid chromatography was carried out with a Perkin-Elmer Model 800 or with an F. & M. Model 810. In each case, an all-metal system fitted with a flame ionisation detector was used. Injector temperature was generally about 50° above column temperature; detector temperature was usually about the same as column temperature. The flow rate of the carrier gas (oxygen-free nitrogen) was adjusted to give the shortest retention time consistent with good peak shape and resolution of various mixtures, but was generally between 50 and 60 ml per min. The flow rates of air and hydrogen were optimised. Detector signals were recorded on a Honeywell recorder (—0.25 to +2.5 mV). Chart speed was 1 in. in 4 min. unless otherwise stated. The procedures of MCMARTIN AND STREET[13,14] for preparation of steel columns and for treatment and coating of diatomaceous earth have been modified in several ways. To avoid confusion, the complete details incorporating the modifications are given below.

Preparation of metal column. A 6 ft. length of stainless steel tube $^1/_8$ in. O.D. and 0.085 in. I.D. was coiled into a helix about 3 in. in diameter and 18 in. long. A steel sintered plug was pressed into one end of the tube. The column was heated in air in a furnace at 800° for 18 h, removed from the furnace and allowed to cool in air. Swagelock ferrules and compression nuts were then fitted on the steel tubing and air was

drawn through the column for several minutes. The inside surface was then washed by drawing about 50 ml of toluene through the column. The first 10 ml or so of toluene washings were distinctly yellow as they emerged from the column. Traces of toluene were removed by drawing air through the column and then by heating the column in a stream (60 ml per min) of oxygen-free nitrogen at 360° for 1 h.

Preparation of SE 30 solution. Dissolve 50 g of silicone gum rubber (SE 30) in 500 ml of hot toluene, shaking the mixture frequently over a period of several hours. Allow the liquid to cool to room temperature, and make up the volume to 500 ml. Shake 110 ml of the clear, viscous solution with 5 ml of water. (The shaking should be quite vigorous for about 5 min.) Allow the water-saturated SE 30 solution to stand overnight in a separating funnel. Remove and discard the excess water which has separated out. The "cloudy" SE 30 solution is then ready for coating the diatomaceous earth.

Preparation of column packing. About 250 ml of Chromosorb W (100–120 mesh) were washed several times with concentrated hydrochloric acid and the powder was then boiled in the acid in a conical flask for 10 min. The powder was rinsed several times with concentrated hydrochloric acid and then with water until the supernatant liquid was neutral to a pH paper. The suspension of the powder in water was then boiled for 10 min, rinsed several times with water, the "fines" decanted after each rinsing and excess water removed by vacuum filtration. The powder was placed in flat glass dishes and dried, with frequent stirring, on a boiling water bath.

60 ml of this washed Chromosorb W were put into a 400 ml beaker. 200 ml of toluene were added and the suspension was stirred thoroughly with a glass rod. The powder was allowed to settle and the "fines" were decanted. The washing with toluene was then repeated and as much toluene removed as was possible by decantation. 50 ml of toluene were then added, followed by 100 ml of 10% water-saturated SE 30 solution (prepared as described above) and the mixture was thoroughly stirred. Excess toluene was removed by vacuum filtration, and the coated powder was dried in four separate portions with stirring on a hot-plate. The four portions were placed in a Pyrex glass tube measuring 2.5 cm in diameter and 40 cm long and fitted at one end with a sintered glass disc. Oxygen-free nitrogen was passed (30 ml per min) through the powder in the glass tube first at room temperature for 5 min, and then whilst the tube was heated in a furnace at 370° for 1.5 h. At the end of this period, the tube was removed from the furnace and allowed to cool down to room temperature with the nitrogen flowing. The powder was removed by suction in three separate fractions. These will be referred to as F1, F2 and F3 where fraction F1 is nearest to the nitrogen input. Fraction F1 was packed into the stainless steel column (prepared as described above) using the packing procedure described by McMARTIN AND STREET[13], and the column heated at 340° in a stream of oxygen-free nitrogen (50 ml per min) for 18 h. The column was then emptied, packed with fraction F3 and heated at 370° in oxygen-free nitrogen (30 ml per min) for 1 h. This packed column was then ready for use.

Compounds studied

The drugs listed in Table 1 were subjected to GLC as pure solutions in ethanol or chloroform. Several of these drugs were added to blood and/or urine and then taken through one of the extraction procedures described below. Reagent blanks were carried through each procedure by starting with water in place of biological sample. Where possible, the 6th edition of Merck Index was used for drug nomenclature.

TABLE I

RETENTION TIME (IN MINUTES) OF ALKALOIDS SUBJECTED TO GAS–LIQUID CHROMATOGRAPHY AT VARIOUS COLUMN TEMPERATURES

With the exceptions noted below, the amount of alkaloid injected was 0.1 μg in 1 μl of solution at attenuation × 20. This is not the minimum detectable amount (MDA) but represents the amount necessary to produce a peak height of at least 1 in. at *attenuation × 20*, which is a practical attenuation limit for the extraction procedures described (see text). Exceptions—MDA at attenuation × 20 are: a = 0.2 μg; b = 0.3 μg; c = 0.5 μg.

Alkaloid	*Mol. wt.*	*Column temperature*									
		130°	*140°*	*160°*	*180°*	*205°*	*215°*	*235°*	*270°*	*290°*	*310°*
Amphetamine	135	1.9	1.5								
Phentermine	149		1.7								
Methylamphetamine	149	2.45	1.85								
Mephentermine	163		2.65	1.5							
Chlorphentermine	184		4.1	2.25	1.25						
Ephedrine[b]	165	6.4	4.4	2.3	1.3						
Phenmetrazine	177		6.0	2.65	1.7						
Hordenine[b]	165			3.2							
Meclophenoxate	242			4.4	2.3	1.2					
Metaraminol[c]	167					1.6					
Pheniramine	240					2.85					
Metyrapone	250					3.5	2.65				
Orphenadrine	269				9.1		2.85				
Aribine	182				10.0	4.25	3.2				
Chlorpheniramine	275						3.9	2.1			
Adiphenine	311						6.85	3.5			
Nortriptyline[a]	263							3.7			
Chlorcyclizine	301						7.6	3.9			
Diazepam	285							6.6	2.4		
Chlorpromazine	319							7.9	2.75		
Pentaquine[a]	301							9.3	2.95		
Acetylpromazine	326							13.5	4.3		
Quinine	324							16.4	5.05		
Papaverine	339							18.4	5.15		
Proclorperazine[b]	374								7.6	4.0	
Octaverine	397							33.2	8.3	4.05	
Prolixin[c]	421								8.8	4.5	
Strychnine[a]	334										3.1
Brucine[a]	394										5.7

Purification of reagents

Ether. Anaesthetic ether (diethyl ether) was shaken with 10 % sodium hydroxide solution to remove anti-oxidant additives from the ether. This ether was then used to wash a fresh solution of 10 % sodium hydroxide to remove impurities from the alkali. The purified alkali was then used to wash a fresh batch of untreated anaesthetic ether. This washed ether was then shaken with several portions of water until the washings were neutral.

Anhydrous sodium sulphate. 1 kg of anhydrous "Analar" Na_2SO_4 was washed with three separate 2-litre portions of absolute ethanol. After the ethanol washings had been decanted, the ethanol-wet Na_2SO_4 was blotted with filter paper and then heated in a furnace at 600° for 20 h. When cold, the purified Na_2SO_4 was stored in a suitable dry container. This purification of Na_2SO_4 is absolutely essential if "peak-free" blanks are to be obtained.

Sodium hydrogen carbonate. Solid $NaHCO_3$ is purified by washing with liberal amounts of purified ether.

TREATMENT OF URINE SAMPLES

Basic alkaloids

5 ml of urine are acidified by addition of 0.1 ml of 2 *N* sulphuric acid solution. The mixture is extracted with 30 ml of washed ether. The layers are allowed to separate and the organic solvent is discarded. 0.2 ml of 2 *N* sodium hydroxide solution is added and the mixture is tested with an indicator paper to make sure the pH is not less than 10 units and then extracted with two separate 30 ml portions of washed ether. The aqueous phase is kept for extraction of amphoteric alkaloids (see below).

The combined ether extracts are shaken with about 1 g of purified anhydrous sodium sulphate. The dried ether extract is carefully evaporated to dryness in a 15 ml conical centrifuge tube at about 20° using a stream of dry nitrogen. If the analysis is not to include the more volatile alkaloids, the ether may then be carefully removed by holding the tube in the steam from a boiling-water bath.

Amphoteric alkaloids

Morphine and N-allylnormorphine are the chief drugs in this group. Other substances which may be present include those basic drugs containing an aromatic ring which may have become hydroxylated by microsomal enzymes thus giving rise to an (acidic) phenolic group.

"Free" (*i.e.* unconjugated) morphine is extracted from the aqueous phase remaining after extraction of basic alkaloids (see above). 0.1 ml of 2 *N* sulphuric acid is added first and then 1 ml of a saturated solution of purified sodium hydrogen carbonate. The mixture is extracted with two separate 30 ml portions of washed ether. The ether is dried by shaking with about 1 g of purified anhydrous sodium sulphate and evaporated carefully to dryness in a 15 ml conical centrifuge tube held in the steam from a boiling-water bath.

TREATMENT OF BLOOD AND LIVER SAMPLES

In the following description of the analysis of blood and liver, the procedure given is for *basic alkaloids only*. If other fractions are required a procedure similar to that described for urine (*q.v.*) may be applied after preliminary treatment.

The first part of the extraction procedure for blood and for liver is based on CURRY's[15] modification of the method described by DUBOST AND PASCAL[16] for analysis of phenothiazine derivatives. As CURRY[15] has pointed out, the treatment with hot hydrochloric acid gives increased yields of alkaloids other than the phenothiazines. This is, presumably, due to liberation of more protein-bound drug. It is suggested, therefore, that, *provided the alkaloid is stable under such conditions*, the hot HCl procedure may be used for analysis of all alkaloids in protein-containing fluids and tissues.

Procedure for blood

1 ml of water and 4 ml of concentrated hydrochloric acid are added to 5 ml of

blood. The mixture is placed in a boiling water bath for 5 min, then cooled in ice-water. 6 ml of ether-washed 60 % potassium hydroxide solution is added making certain that the mixture is at least pH 10. The mixture is then shaken with two 30 ml portions of washed ether, the combined ether extracts are washed successively with 5 ml of 2.5 % ether-washed sodium hydroxide solution and two 5 ml portions of water. The washed ether extract is shaken with 5 ml of 0.1 *N* sulphuric acid; the two phases are separated and the aqueous phase is made alkaline (again to about pH 10) by dropwise addition of 60 % ether-washed potassium hydroxide solution and extracted with 20 ml of washed ether. The ether phase is dried with purified anhydrous sodium sulphate and evaporated to dryness in a conical centrifuge tube held in the steam from a boiling water bath.

Procedure for liver

20 g of liver is macerated with 20 ml of water and 27 ml of concentrated hydrochloric acid and the mixture is heated in a boiling water bath for 5 min and then cooled in ice-water. 24 ml of ether-washed 60 % potassium hydroxide solution is added slowly with stirring, making sure that the mixture is at least pH 10, cooled in ice-water and then shaken with two 100 ml portions of washed ether. The combined ether extracts are washed first with 20 ml of 2.5 % sodium hydroxide solution, then with 20 ml of water and finally with 10 ml of water. The washed ether extract is shaken with 10 ml of 0.1 *N* sulphuric acid and the aqueous phase is made alkaline (again, pH not less than 10 units) with ether-washed 60 % potassium hydroxide solution and extracted with 20 ml of washed ether. The ether is shaken with about 1 g of purified anhydrous sodium sulphate and evaporated to dryness in a conical centrifuge tube over a boiling water bath.

GLC ANALYSIS OF THE SEPARATED FRACTIONS

The residue from each of the above fractions is dissolved in 100 μl of ethanol and 5 μl of this solution is injected into the GLC apparatus. For quantitative results, an internal standard may be included in the ethanol. If there is a relatively large amount of residue in the conical tube, it may be necessary to use more than 100 μl of ethanol and then to *inject* a larger volume of the solution.

RESULTS AND DISCUSSION

Amount of drug introduced into gas chromatograph

Many published articles dealing with gas–liquid chromatography do not give the actual weight of compound introduced into the chromatograph. In some cases, it is stated, for example, that 1–3 μl of a 0.5 to 1.0 % solution of a drug was injected. A tracing of the chromatogram is shown but the reader does not know whether the peak he sees was obtained with 1 μl of a 0.5 % solution or 3 μl of a 1.0 % solution (or any other combination), *i.e.* it is not clear whether 5 μg or 30 μg of drug were responsible for the peak shown. Certain manufacturers too have a habit of showing very tall thin peaks displaying very little tailing and of stating that these peaks were obtained by injection of "*x*" μl of solution but without stating the *concentration* of drug in the solution. The reporting of results in this form is most unsatisfactory; in the sub-

microgram region it may be completely misleading. For instance, there is no difficulty at all in obtaining reasonably good peak shapes when 30 μg of morphine are used on some *conventional* columns but 1 μg of morphine usually fails to emerge at all from such columns.

When the weight of compound injected is not stated it becomes very difficult, and in some cases impossible, to assess the value of other workers' results. This is especially so when, in many cases, the exact details of column preparation are not given. Where amounts *are stated* for alkaloids, these are usually greater than 1 μg, and are often of the order of 20 μg. Also, because peak shapes are not shown in many cases, and where they *are* shown tailing may be quite marked, it is unwise to extrapolate to the sub-microgram region. It is my experience that, with *conventionally* "silanised" supports in metal columns, very few alkaloids can be "run" satisfactorily in amounts less than 2 μg.

Column preparation

In the preparation of packed stainless steel columns there are two very important factors to be considered. One is the treatment of the diatomaceous earth, the other is the treatment given to the metal column itself. McMARTIN AND STREET[13,14] incorporated tristearin in their procedure for coating the diatomaceous earth because the ester was found to increase peak heights without, apparently, affecting the tailing. Such columns are stable at temperatures up to 245°. However, after our papers had been published, I observed that when "tristearin-columns" are run for several hours at temperatures around 310°, such as may be required to run brucine in a "reasonable" time, they subsequently show very bad tailing not only at 245° with some of the alkaloids but also when the temperature is dropped to about 160° to "run" the barbituric acid derivatives. This difficulty has been overcome by omitting tristearin and modifying the "silanising" procedure in the manner described below.

We noted in our original article (McMARTIN AND STREET[13]) that we obtained effective "silanisation" using *wet* Chromosorb W and dichlorodimethylsilane (DDS). It is interesting to note that it has now been found that heating the diatomaceous earth (in the absence of air) with a *water-saturated* SE 30 solution produces powders which show remarkably little adsorption and which are stable at temperatures up to 320° with sub-microgram amounts of a wide range of drugs. The preparations are also reproducible. Similar results can be produced by heating *wet* diatomaceous earth with "dry" SE 30 solution in toluene. However, the final preparation in this case is not always reproducible, possibly because it is difficult to obtain an even distribution of water throughout the powder. It is possible that water may be required for hydrolysis of the SE 30 and that the breakdown products so produced, effectively "silanise" the diatomaceous earth *at 370°*.

The second important factor is the treatment of the steel column. For the production of low adsorption columns, it is *essential* that a new steel surface be heated with a silicone gum rubber in the absence of air. It is not sufficient to pour a solution of SE 30 down the tube and then to heat this, probably because the coating of the metal is thereby uneven. The simplest way is to pack the tube with one of the SE 30 coated fractions and to heat this in a stream of nitrogen. A possible explanation of the effect of this treatment is that a thermal breakdown product of SE 30 may react with the metal oxide on the inner surface of the metal column to produce a layer which is less

polar than the oxide itself. (N.B. that the thermal breakdown product *must* be produced in the *absence* of free oxygen.) This could then lead to less adsorption and/or less destruction of the compounds being "run". Such a reaction might also account for the fact that less "tailing" is observed when steel columns are heated *in air* prior to packing, thus forming the oxide or partial oxide necessary for reaction with the SE 30 product. Furthermore, it has been found that steel injector blocks can be treated in a similar manner to give improved over-all performance of the GLC apparatus. In fact, with this type of treatment, it has been found possible to run testosterone (Δ^4-androsten-17β-ol-3-one) in an "all-metal" system, using a liquid phase of SE 30, in amounts down to 5×10^{-9} g. However, it is intended in a future article to deal with the analysis of sub-microgram amounts of steroids using steel columns.

Alkaloids in pure solution

The results obtained with pure solutions of alkaloids are presented in Table I in terms of retention time at different column temperatures. With the exceptions noted in Table I, where the figures in brackets indicate minimum detectable amounts, all the alkaloids were injected into the GLC apparatus in 0.1 μg amounts in a volume of 1 μl of solution. However, 0.1 μg is not the smallest amount of alkaloid detectable by this procedure. Indeed, in may cases, the lower limit for pure solutions of drugs is around the 0.01 μg region.

Extraction procedures

In body fluids and tissues, it is usually analytically simpler to look for the *unchanged* drug rather than the metabolic transformation product(s) of the drug. This is because the body's detoxication mechanisms operate mainly to convert the (toxic) drug into more polar and more water-soluble compounds which can then be more readily excreted by the kidneys. Hence, in many cases, *e.g.* in conjugates with glucuronic acid, the metabolite cannot be extracted from aqueous solution by the usual organic solvents. For this reason, the procedures described in this paper have been deliberately aimed at identifying the unchanged drug. This is not an entirely satisfactory approach because, ideally, one should search for both unchanged drug *and* its metabolites. Although it is true that, in many cases, the metabolites are no longer toxic, there are cases where the metabolite is the compound actually responsible for the pharmacological action ascribed to the parent drug, *e.g.* imipramine and its metabolite mono-desmethylimipramine; phenacetin and *p*-acetamidophenol. A more complicated example is seen in the case of phenylbutazone. BURNS *et al.*[17], state that phenylbutazone is converted in man into two metabolic products. According to YÜ *et al.*[18], one of these metabolites, oxyphenbutazone, is responsible for the anti-rheumatic action attributed to phenylbutazone, whilst the second metabolite, in which the butyl side-chain of phenylbutazone is hydroxylated, is stated by BRODIE *et al.*[19] to account for the uricosuric action of the parent drug. However, until considerable improvements are made in analytical procedures, either in extraction or analysis *without* extraction, toxicologists will, in general, have to be content with a search for unchanged drugs.

With the extraction procedures described, quantitative experiments, based on peak height comparison, indicate that recovery varies with the particular drug being studied. In general, recoveries of alkaloids are about 80 % when the drugs are present

in the sample at a concentration of 0.5 μg per ml of sample. In some cases, *e.g.* trifluoperazine, recovery may be up to 90 % whilst in other cases *e.g.* morphine, recovery may be as low as 50 %. Morphine and other alkaloids possessing an acidic group in addition to their basic amino group represent a special case because of their amphoteric nature. Under the conditions described, using diethyl ether as the extracting solvent, the recovery of morphine is never greater than 60 %. Although mixtures of *n*-butanol and benzene, or isopropanol and chloroform are more efficient extracting solvents, ether has been deliberately chosen because ether is more easily purified than the other solvents referred to, it can also be kept in a purified state, and it is more convenient to remove, after extraction, because of its high volatility. Again, in the case of strychnine and brucine, ether is not the ideal extracting solvent but, in my opinion, solvent purity is especially important when working with microgram and sub-microgram amounts of drugs, relatively large volumes of solvent, and low attenuation settings on the gas chromatograph. It must also be remembered that we are dealing here with the transfer from glass vessel to glass vessel of *microgram* amounts of polar compounds and that adsorption of the drugs could be expected to occur on the glass surfaces. The adsorption might be even greater on *ground* glass surfaces. Indeed the surprising thing is that the recoveries are so *high*.

In the extraction procedures described above for blood and liver, it was suggested that the brief pre-treatment with hot concentrated hydrochloric acid could be used for those alkaloids which have been shown to be stable under such conditions. An extension of this procedure is to use it in cases where the alkaloid is *unstable* under such conditions but which is converted (preferably quantitatively) into a product or products which can be readily identified. In this connexion, we have observed that *d*-propoxyphene is converted by the hot HCl treatment into two compounds which are separable by GLC on one of our SE 30 columns. This fact was recently used by us in one of our routine cases as evidence to suggest that *d*-propoxyphene had been ingested. Neither direct extraction nor tungstic acid treatment revealed the presence of *d*-propoxyphene. We have not yet identified the compounds responsible for the two GLC peaks, but there is no evidence for the presence of unchanged *d*-propoxyphene following the acid treatment.

Alkaloids in biological media

In the section on alkaloids in pure solution, it was noted that the detection limit for many drugs is of the order of 0.01 μg. When these drugs are extracted from biological material, using one of the procedures described above, the "background" collected during the process usually prevents the use of the GLC apparatus at maximum sensitivity. Thus an attenuation of sensitivity of not less than 20 has been used and it is at this attenuation level (1/20 of maximum sensitivity) that 0.1 μg of the drugs is readily detected, *i.e.* give a recorder peak height of *at least* 1/10 of full scale deflection. The tracings shown in this paper are given merely as examples of what can be achieved with these columns, and anyone intending to use the procedure should establish his own conditions to suit his particular purpose.

For a given alkaloid, the retention times, in many cases, were identical for 1 μg and 0.1 μg. In *all* cases, the increase in retention time, as the injected amount was changed from 1 μg to 0.1 μg, was not greater than 5 %.

Although it is of great importance to display the *shapes* of the peaks obtained

and to illustrate that, for a given alkaloid, the retention time is independent of the amount of alkaloid injected, it is not practicable to show here the chromatograms obtained for *each* drug studied at *each* concentration because of lack of space. However, a few representative tracings are shown of chromatograms obtained from extracts of urine containing alkaloids.

Fig. 1 shows the results obtained from an ether extract of 5 ml of urine containing 1.0 μg each of brucine and strychnine per ml of urine. One twentieth (5 μl) of the final extract was injected into the GLC apparatus at a column temperature of 300°. Even at this temperature there is very little background interference. The "shoulder" on each of the peaks is probably due to impurity present in the original solid sample of alkaloid. Complete resolution is achieved within 10 min.

The results shown in Fig. 2 were obtained from an ether extract of 5 ml of urine containing 0.5 μg each of imipramine, promazine, chlorpromazine and mepazine per ml of urine. Again, one twentieth (5 μl) of the final extract was injected into the gas chromatograph but in this case the column temperature was 240°. Resolution of the four drugs is obtained within 9 min.

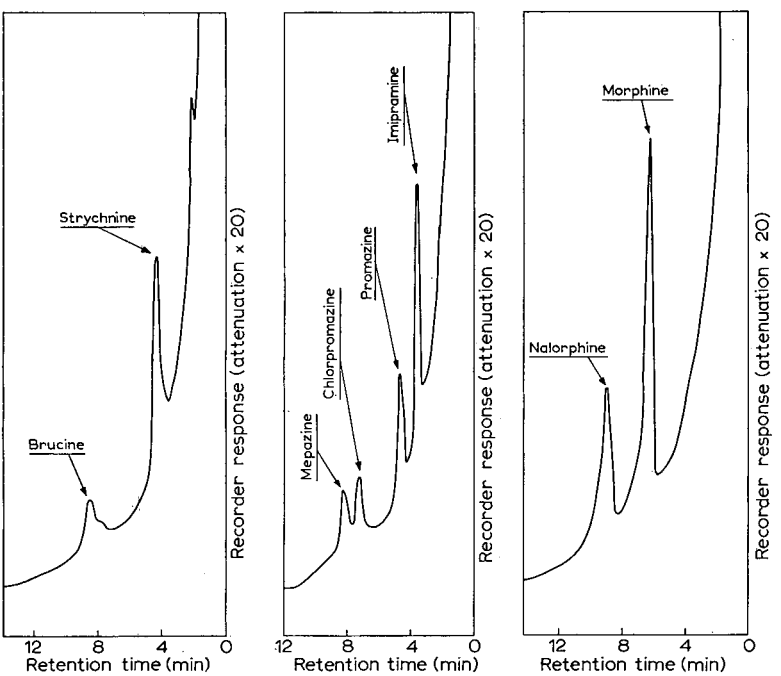


Fig. 1. A chromatogram of an ether extract (see text) of 5 ml of urine containing 1 μg each of strychnine and brucine per ml of urine. Residue was dissolved in 100 μl of ethanol; 5 μl of this solution was injected into the gas chromatograph. Column temperature: 300°; attenuation × 20.

Fig. 2. A chromatogram of an ether extract (see text) of 5 ml of urine containing 0.5 μg each of imipramine, promazine, chlorpromazine and mepazine per ml of urine. Residue was dissolved in 100 μl of ethanol; 5 μl of this solution was injected into the gas chromatograph. Column temperature: 240°; attenuation × 20.

Fig. 3. A chromatogram of an ether extract (see text) of 2.5 ml of urine containing 7.5 μg each of morphine and nalorphine per ml of urine. Residue was dissolved in 100 μl of ethanol; 5 μl of this solution was injected into the gas chromatograph. Column temperature: 240°; attenuation × 20.

Resolution of a mixture of morphine and nalorphine is seen in the chromatogram presented in Fig. 3. In this case, each drug was present in the urine at a concentration of 7.5 μg per ml of urine. 2.5 ml of urine was extracted with ether and one twentieth (5 μl) of the final solution was injected into the GLC apparatus, at a column temperature of 240°,

FELDSTEIN AND KLENDSHOJ[20] analysed the urine of hospital patients who had received from 1/4 to 1 grain of morphine during their stay in hospital and found, on average, about 1 mg of "free" (unconjugated) morphine per 100 ml of urine, *i.e.* 10 μg per ml. They also found the ratio of "bound" morphine to "free" morphine to vary from 1.6 to 2.2. Hence, the results shown in Fig. 3 illustrate that our GLC procedure could be used to detect morphine in the urine of patients receiving the drug in therapeutic amounts.

The procedure for alkaloids is intended mainly as a rapid screening test for the *presence* of drugs excreted in urine. However, it is probable that a measurement of the height of the GLC peak and reference to a calibration curve, prepared from peak heights obtained by injection of different concentrations of the appropriate standard drug solution, will provide a quantitative assessment which will be of acceptable accuracy in most cases. The recoveries referred to in the previous section should, of course, be taken into account. But it must be remembered that, in the interpretation of quantitative results of urine analysis, it is often difficult to decide whether a particular concentration of a drug in the urine represents the excretion of a recently administered therapeutic dose or the final stages of excretion of a large overdose taken some time previously.

It is suggested, however, that such a procedure could be adopted for the quantitative analysis of alkaloids *in plasma* although, here again, the *interpretation* of plasma alkaloid levels may be difficult in some cases. One such difficulty is illustrated in a paper by CURRY[21] where he showed that in a fatal case involving Tofranil, the level of imipramine in the liver was over sixty times greater than the level in the blood. This order of difference between blood and liver imipramine levels is in keeping with my own findings as described below.

Recently, a case involving ingestion of an overdose of Tofranil was encountered in our Department. Analysis by CURRY'S[15] modification of the method described by DUBOST AND PASCAL[16] showed that the blood contained 0.5 mg of imipramine per 100 ml and that the liver contained 36.1 mg of imipramine per 100 g. Imipramine was identified by the blue colour formed on addition of dilute nitric acid solution, by the elevated temperature reversed-phase paper chromatography procedure described by STREET[22–25], and also by its retention time in one of the columns prepared as described in this paper. It is pertinent to note here that, using a liquid phase of nitrile silicone gum (XE 60) in place of SE 30 it has been found possible to resolve a mixture containing sub-microgram amounts of mono-desmethylimipramine and di-desmethylimipramine.

As I have already pointed out (see STREET[26]), the fundamental problem of the analytical chemical toxicologist is the *qualitative* identification of the poisons. Once the drugs have been identified, their quantitative estimation does not usually present great difficulties. Although resolution by GLC of *submicrogram quantities* of drugs extracted from gram amounts of biological material will assist the toxicologist in his identification of toxic agents in cases of poisoning, the question of *absolute* identifi-

cation still remains. It is suggested that a combination of GLC and mass spectrometry will provide the final answer to this question.

ACKNOWLEDGEMENT

This research programme has been supported by a grant from the Scottish Hospital Endowments Research Trust.

SUMMARY

The surface properties of diatomaceous earth and of oxidised stainless steel can be modified to produce GLC columns which show very little adsorption and which can by used for GLC *analysis of submicrogram amounts of alkaloids*. The modification is effected by heating with a water-saturated silicone polymer at about 370° in a stream of nitrogen. GLC stainless steel columns containing such a preparation have been used to analyse 2.5 ml urine samples containing 7.5 μg morphine and nalorphine per ml; and 5 ml urine samples containing 1 μg brucine and strychnine per ml, and 0.5 μg of many other basic alkaloids, *e.g.* imipramine, promazine, chlorpromazine and mepazine per ml of urine. Details of extraction procedures for alkaloids in blood, urine and liver are described. Retention times are given of 29 alkaloids at various column temperatures.

REFERENCES

1 M. R. SUBBARAM, *J. Chromatog.*, 15 (1964) 79.
2 E. KLESPER, A. H. CORWIN AND D. A. TURNER, *J. Org. Chem.*, 27 (1962) 700.
3 H. A. LLOYD, H. M. FALES, P. F. HIGHET, W. J. A. VANDENHEUVEL AND W. D. WILDMAN, *J. Am. Chem. Soc.*, 82 (1960) 3791.
4 E. BROCHMANN-HANSSEN AND A. B. SVENDSEN, *J. Pharm. Sci.*, 52 (1963) 1134.
5 M. W. ANDERS AND G. J. MANNERING, *Anal. Chem.*, 34 (1962) 730.
6 P. D. SHAW, *Anal. Chem.*, 35 (1963) 1581.
7 E. BROCHMANN-HANSSEN AND A. B. SVENDSEN, *J. Pharm. Sci.*, 51 (1962) 938.
8 S. LINDSTEDT, *Clin. Chim. Acta*, 9 (1964) 309.
9 M. W. ANDERS AND G. J. MANNERING, *J. Chromatog.*, 7 (1962) 258.
10 E. BROCHMANN-HANSSEN AND C. R. FONTAN, *J. Chromatog.*, 19 (1965) 296.
11 E. BROCHMANN-HANSSEN AND C. R. FONTAN, *J. Chromatog.*, 20 (1965) 394.
12 J. L. MASSINGILL AND J. E. HODGKINS, *Anal. Chem.*, 37 (1965) 952.
13 C. MCMARTIN AND H. V. STREET, *J. Chromatog.*, 22 (1966) 274.
14 C. MCMARTIN AND H. V. STREET, *J. Chromatog.*, 23 (1966) 232.
15 A. S. CURRY, *Poison Detection in Human Organs*, Thomas, Springfield, Ill., 1963.
16 P. DUBOST AND S. PASCAL, *Ann. Pharm. Franc.*, 11 (1953) 615.
17 J. J. BURNS, R. K. ROSE, S. GOODWIN, J. REICHENTHAL, E. C. HORNING AND B. B. BRODIE, *J. Pharmacol. Exptl. Therap.*, 113 (1955) 481.
18 T. F. YÜ, J. J. BURNS, B. C. PATON, A. B. GUTMAN AND B. B. BRODIE, *J. Pharmacol. Exptl. Therap.*, 123 (1958) 63.
19 B. B. BRODIE, T. F. YÜ, J. J. BURNS, T. CHENKIN, B. C. PATON, M. J. STEELE AND A. B. GUTMAN, *Proc. Soc. Exptl. Biol. (Med.)*, 86 (1954) 884.
20 M. FELDSTEIN AND N. C. KLENDSHOJ, *J. Forensic Sci.*, 1 (1956) 47.
21 A. S. CURRY, *J. Pharm. Pharmacol.*, 16 (1964) 265.
22 H. V. STREET, *J. Forensic Sci. Soc.*, 2 (1962) 118.
23 H. V. STREET, *J. Forensic Sci.*, 7 (1962) 222.
24 H. V. STREET, *Acta Pharmacol. Toxicol.*, 19 (1962) 312.
25 H. V. STREET, *Acta Pharmacol. Toxicol.*, 19 (1962) 325.
26 H. V. STREET, in A. STOLMAN (Editor), *Progress in Chemical Toxicology*, Vol. 2, Academic Press, London, New York, 1965.

REACTION CHROMATOGRAPHY

I. GAS–LIQUID/THIN-LAYER CHROMATOGRAPHIC DERIVATIZATION TECHNIQUE FOR THE IDENTIFICATION OF CARBONYL COMPOUNDS*

J. H. TUMLINSON, J. P. MINYARD, P. A. HEDIN AND A. C. THOMPSON

Boll Weevil Research Laboratory, Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, State College, Miss. (U.S.A.)

(Received December 16th, 1966)

Identification of oxygenated flavor- or aroma-producing compounds from food and plant essential oils is sometimes complicated by the small quantities present. In lieu of positive spectral proof, tentative gas chromatographic identifications necessarily require confirmative evidence by derivative behavior. A simple and inexpensive technique readily available in most laboratories has been developed for obtaining such evidence for carbonyl compounds. In an extension of the gas–liquid/thin layer chromatographic (GLC/TLC) derivatization technique reported by CASU AND CAVALOTTI[1], 2,4-dinitrophenylhydrazones (DNPHs), *p*-nitrophenylhydrazones (PNPHs), and 2,4-dinitrophenylsemicarbazones (DNPSCs) were found to be suitable carbonyl compound derivatives which form readily on TLC plates at the exhaust of a gas chromatograph. TLC behavior of these derivatives in several systems and subsequent possible manipulations are described.

The technique involves isolation of the carbonyl compounds from the essential oil by the Girard T technique[2], GLC of the regenerated compounds, and derivative formation on TLC plates of each eluting maximum at the chromatograph exhaust port. After development of the TLC plates in a suitable system, the purified derivative spot is scraped from the plate, eluted from the adsorbent, and subsequently examined in other TLC or GLC systems. Also, U.V.-visible spectra may be determined, or melting point taken. The Girard T isolation is a desirable but not essential feature in the process. The utility of these techniques has been demonstrated in the isolation and identification of carbonyl compounds from the essential oil of the cotton bud[3].

EXPERIMENTAL

Reagents and apparatus

Gas chromatograph. Barber-Colman Model 5000, modified with exhaust splitter and Luer-Lok exhaust line discharging vertically downward, equipped with a 10 ft. × ¼ in. column packed with Apiezon L (20 %, w/w) on Gas Chrom P.

2,4-Dinitrophenylhydrazine and *p*-nitrophenylhydrazine (Matheson, Coleman,

* Part of a thesis submitted by J. P. MINYARD in partial fulfillment of requirements for the Ph.D. degree from Mississippi State University, State College, Miss.

and Bell) and 2,4-dinitrophenylsemicarbazide (K & K Laboratories) were used without further purification.

Reagents for gas chromatographic derivatization on thin layer plates were prepared as follows:

DNPH. Reagent was dissolved in H_3PO_4 and ethanol according to JOHNSON[4].

PNPH. p-Nitrophenylhydrazine (0.5 g) was added to 30 ml ethanol, 6 ml of methylal, and 5 drops of concentrated H_3PO_4 catalyst.

DNPSC. 2,4-Dinitrophenylsemicarbazide (0.15 g) was dissolved in 20 ml of boiling ethanol (95 %), and 6 drops of concentrated HCl were added. On cooling, some of the reagent precipitated out of solution, but this was ignored as it caused no ill effects.

TLC plates (250 μ adsorbent depth) were prepared with Brinkman apparatus on 20 × 20 cm × 3 mm glass. Silica Gel G (SGG) and polyamide powder (Merck) were used as adsorbents. In addition, SGG plates were coated with Carbowax 600 by immersing in 20 % (v/v) Carbowax 600–acetone solution, removing, and allowing the acetone to evaporate at room temperature. Polyamide and Carbowax coated SGG plates were used without further treatment; the SGG plates were activated at 115° for 1 h before use.

All aldehydes and ketones examined were commercial samples available in our laboratory or obtained from previous work with the cotton bud essential oil[3].

Formation of standard derivatives

DNPHs and PNPHs were prepared according to SHRINER AND FUSON[5]. Purification was by recrystallization[5], except for some of the PNPHs which were oils. These were purified by preparative TLC utilizing system A (see later).

DNPSCs were prepared by the method of MCVEIGH AND ROSE[6]. Saturated aliphatics were recrystallized from 95 % ethanol and the unsaturated aliphatic and aromatic derivatives from a mixture of N,N-dimethylformamide (DMF) and 95 % ethanol. In the latter case, a minimum of DMF was added to the boiling ethanol to dissolve the derivative. Melting points and micro-Kjeldahl nitrogen analyses were obtained to establish purity and identity.

Formation of derivatives from gas chromatograph

A thin layer plate was placed on a lab jack so the starting line of the plate was under the exhaust port of the gas chromatograph. With the jack, the distance of the plate from the exhaust port could be adjusted as desired. For best results, the plate was kept as close as possible to the port during derivatization without allowing it to touch. A drop of reagent (*ca.* 10 μl) was spotted on the starting line of the plate just before elution of each GLC maximum, and the eluant was allowed to flow into the center of the reagent spot. Extra reagent was added to the spot during elution as needed to keep it moist. Ordinarily eight GLC maxima were reacted on each plate on 2 cm centers, and a drop of unreacted reagent was added at a vacant site for comparison. If the eluted sample was larger than *ca.* 2 μl, it was usually necessary to derivatize on more than one spot to prevent reagent caking and overloading of the adsorbent. The temperature of the exhaust port was maintained at 185° for maximum yield of derivative.

With all three reagents, 50 μg or less of a carbonyl compound injected into the

gas chromatograph could be derivatized by this method in sufficient amounts to allow development, elution, and rechromatography on other systems.

Thin-layer chromatography

Samples were spotted 1.5 cm from the bottom edge of the plate (starting line) and 2 cm apart in every case. All plates were allowed to develop to a height of 10 cm from the starting line. Tanks containing solvents were equilibrated before introduction of plates.

Systems used to separate the derivatives were:

(A) Benzene–petroleum ether (38–50°) (4:1); solid adsorbent—SGG.

(B) Methanol–water (95:5); solid adsorbent—polyamide powder.

(C) Heptane–benzene (4:1); solid adsorbent—SGG coated with Carbowax 600.

(D) Benzene–ethyl acetate (4:1); solid adsorbent–SGG.

All DNPHs were readily visualized on the plates by their bright yellow to deep orange-red color. The PNPHs and DNPSCs of saturated aliphatic aldehydes were not visible under ordinary light but were readily distinguishable under U.V., since they are normally strong absorbers. The aromatic and α,β-unsaturated aldehyde PNPHs fluoresce yellow to yellow-green under U.V. light.

Test mixture (Desaga, Heidelberg) was spotted on each plate and R_Y values, based on the Butter Yellow spot in the mixture, were determined for each sample spot. Standard derivatives and those prepared from the gas chromatograph were spotted side by side for comparison.

All derivatives prepared from the GLC were made on SGG plates. The DNPHs and PNPHs were developed in system A and the DNPSCs in system D. A reagent blank was run on each plate with the derivatives. The major derivative spot from each sample, normally 80+ % of the total quantity of derivatives formed, was scraped from the plate, and the derivative was eluted from the silica gel with solvent. GLC unresolved carbonyl impurities in the commercial standard, reagent impurities, and pyrolytic or other artifacts from the reaction gave rise to the several small spots usually observed to accompany the major derivative spot. There was no difficulty in establishing which spot was the derivative since it was the darkest and largest with the exception of the unused reagent. The DNPHs and PNPHs were eluted with methylal; the less soluble DNPSCs were eluted with methylal–DMF mixtures. These derivatives were respotted and developed in other systems and R_Y values calculated.

RESULTS AND DISCUSSION

Several general features are desirable or necessary for a suitable GLC/TLC derivatization system of a compound class. The derivatives must form rapidly in good yield, since the exposure to the heating effect of the exhaust gas is brief. They also must be separable from unexpended reagent or post-reaction modified reagent by some TLC system, and must either be visible or capable of being visualized by non-destructive techniques so they may be transferred to other systems or otherwise examined. At least two distinctly different derivatives would be desirable for more positive identification of compounds of a given functionality type.

Compounds which are labile under the reaction conditions may or may not be suitable for characterization by this method, depending on the relative rates of

decomposition and derivatization. This is true, of course, for the same reaction in a test tube, but on the TLC plate, catalytic action of the adsorbent may favor one reaction path over the other. Terpenoids of known acid lability thus may be unsuited to this method, since all the reagents used here are acidic. However, parallel GLC/TLC derivatization of a standard and unknown gives reproducible and closely comparable results. In summary, if a derivative can be formed of a compound on a macro scale, and if additional requirements outlined above are met, GLC/TLC derivatization appears to be feasible.

Adsorbents for the reaction preferably should be physically strong and adherent to the glass plate support to withstand repeated spotting, handling, strong reagents, and elevated localized temperatures. Alumina and silica gel both serve well, but silica was preferred for its handling properties in elution of the spots. Both DPNHs and DNPSCs appear to be stable on silica gel; the PNPHs undergo changes with time, as explained later.

The three derivatives reported here satisfy most of the requirements mentioned. The sensitivities were found to be roughly equal, and they were equally sensitive for aromatic and aliphatic compounds. The carbonyl compound detection threshold is below 50 μg for all three. Aromatic and conjugated unsaturated aliphatic derivatives were noticeably deeper yellow or yellow-red than the saturated aliphatics.

All three derivatives ran at higher R_Y values on the reaction plate systems than the reagent, with the exception of aromatic DNPSCs. These latter offered no problem, however, for several reasons explained more fully later.

The reproducibility of R_Y values was not very good since they varied as much

TABLE I

TLC R_Y[a] VALUES OF 2,4-DINITROPHENYLHYDRAZONES (DNPH)

Solvent systems: (A) Silica Gel G; benzene–petroleum ether (38–50°) (4:1). (B) Polyamide; methanol–water (95:5). (C) Carbowax 600/Silica Gel G; heptane–benzene (4:1).

DNPH compound	R_Y		
	A	*B*	*C*
Hexanal	1.04	1.12	0.70
Heptanal	1.07	1.02	0.89
Nonanal	1.16	0.85	1.10
Isovaleraldehyde	0.95	1.35	0.64
2-Hexenal	0.93	0.86	0.54
2-Heptenal	1.00	0.80	0.65
2-Octenal	1.05	0.74	0.75
2-Nonenal	1.08	0.65	0.84
trans-2, *cis*-6-Nonadienal	1.02	0.81	0.53
Benzaldehyde	0.92	0.54	0.13
p-Tolualdehyde	1.22	0.33	0.25
Acetone	0.33	1.24	0.35
2-Butanone	0.87	1.33	0.70
2-Nonanone	1.08	1.04	1.16
6-Methyl-5-hepten-2-one	1.03	1.21	1.03
Acetophenone	0.98	1.41	0.42
l-Carvone	1.19	0.64	1.03
Menthone	1.16	0.84	1.17

[a] R_Y is travel ratio of unknown to Butter Yellow dye.

TABLE II

TLC R_Y[a] VALUES OF *p*-NITROPHENYLHYDRAZONES (PNPH)

Solvent systems: (A) Silica Gel G; benzene–petroleum ether (38–50°) (4:1). (B) Polyamide; methanol–water (95:5).

PNPH compound	R_Y	
	A	*B*
Propionaldehyde	0.22	1.13
Butyraldehyde	0.27	1.10
Valeraldehyde	0.29	1.02
Hexanal	0.32	0.94
Heptanal	0.36	0.86
Nonanal	0.41	0.73
Decanal	0.43	0.63
Undecanal	0.46	0.61.
Dodecanal	0.48	0.57
Isobutyraldehyde	0.33	1.15
Isovaleraldehyde	0.31	1.13
Crotonaldehyde	0.23	0.80[b]
2-Hexenal	0.35	0.76[b]
2-Nonenal	0.43	0.56[b]
Benzaldehyde	0.31	0.52[b]
p-Isopropylbenzaldehyde	0.41	0.51[b]
Salicylaldehyde	0.15	0.35[b]
Cinnamaldehyde	0.26	0.28[b]
2-Butanone	0.22	1.15
2-Nonanone	0.35	0.87
6-Methyl-5-hepten-2-one	0.27	1.08
Acetophenone	0.31	0.58[b]
l-Carvone	0.55	0.55[b]
Menthone	0.50	0.80

[a] R_Y is travel ratio of unknown to butter Yellow dye.
[b] Fluorescent under U.V. radiation.

as ± 15 % (relative) from one plate to another for the same compound. However, standard derivatives and those prepared from the gas chromatograph gave values which agreed within 3 % (relative) when run side by side. R_Y values in Tables I–III indicate the relative behavior of various compounds in different systems. The compounds reported for each derivative type were run on a single plate in each system several times, so the values given are internally consistent.

DNPHs of some representative compounds and their R_Y values in three TLC systems are listed in Table I. R_Y values for system A were not calculated from the reaction plates but from plates on which the derivative had been respotted and developed. In all cases the derivatives ran out ahead of the reagent on the reaction plate, but excess reagent on this plate tended to retard the derivatives somewhat, giving false R_Y values.

If the exhaust port is held too close to one spot on the plate for too long, the derivative appears to pyrolyze or darken considerably. This is particularly true of highly unsaturated compounds. The reagent also may cake, in which case the solvent does not move through it easily and the derivative may not move off the starting line.

Elution of the DNPHs from the reaction plate with a suitable solvent provides

them in purified form for examination by several methods. Other TLC systems[7,8], and several paper chromatographic systems[9,10] have been reported to give good separations of these derivatives. In addition, they may be subjected to gas chromatography[11] and visible spectrophotometry[12] or melting points may be taken after solvent evaporation.

Table II lists R_Y values for PNPHs of several representative compounds. Values for system A, in which the reaction plates were developed, were calculated from a second plate after elution from the reaction plate, respotting, and development.

The PNPHs appear to be rather unstable and darken on the SGG plates on standing in air. Additionally, several spots were observed for each sample in system A. For this reason, the major spot from each sample was scraped from the reaction plate and eluted immediately after development. To avoid possible degradation, even in solution, the PNPHs should be examined as soon as possible after elution.

So far as can be found, this work is the first report of TLC of PNPHs, although

TABLE III

TLC R_Y[a] VALUES AND MELTING POINTS OF 2,4-DINITROPHENYLSEMICARBAZONES (DNPSC)
Solvent systems: (B) Polyamide; methanol–water (95:5). (D) Silica Gel G; heptane–benzene (4:1).

DNPSC compound	R_Y		*M.p.*[b] (°C)
	B	*D*	
Valeraldehyde	1.00	0.54	185–187
Hexanal	0.88	0.59	155–157
Heptanal	0.77	0.61	150–151
Nonanal	0.68	0.65	138–139
Decanal	0.54	0.71	133–135
Undecanal	0.51	0.67	130–132
Dodecanal	0.42	0.68	134–136
Isobutyraldehyde[c]	1.09	0.53	207–208
Isovaleraldehyde	1.11	0.56	198–200
2-Methylvaleraldehyde	1.01	0.64	171–173
Crotonaldehyde[c]	0.82	0.51	228–230
2-Hexenal	0.69	0.62	204–206
2-Nonenal	0.54	0.68	177–179
Citral[d]	0.61	0.80	171–174
	0.52	0.64	
Citronellal	0.87	0.70	140–142
Benzaldehyde[c]	0.27	0.52	225–254 (dec.)[e]
p-Isopropylbenzaldehyde	0.21	0.57	267–269
Salicylaldehyde	0.22	0.31	258–261
Cinnamaldehyde[c]	0.19	0.52	228–230 (dec.)
2-Butanone	0.84	0.54	248–249 (dec.)
2-Nonanone	0.54	0.67	191–192
6-Methyl-5-hepten-2-one	0.59	0.63	221–222
Acetophenone[c]	0.27	0.58	264–266 (dec.)[f]
l-Carvone	0.32	0.90	222–225
Menthone	0.59	0.80	212–214

[a] R_Y is travel ratio of unknown to Butter Yellow dye.
[b] Fisher John's block, uncorrected.
[c] Reported by McVEIGH AND ROSE[6].
[d] Commercial; contains geranial and neral by GLC
[e] McVEIGH AND ROSE[6] report 232°.
[f] McVEIGH AND ROSE[6] report 245° (dec.).

paper chromatographic systems are available in which alkaline sprays are reported to be of some value in identification, especially under U.V. light[13]. Gas chromatography[14] and visible spectrophotometry[15] also may yield useful data for compound identification. Melting points may be taken in many cases, but some PNPHs are oils or are very difficult to crystallize. This may create problems in preparation and purification of standards, but preparative TLC purification of these derivatives appears to be satisfactory.

Of the DNPSCs listed in Table III, only five have been reported previously[6], and no reports could be found of TLC, paper chromatography, or GLC of these derivatives. The melting point and R_Y values in two systems for each standard derivative are listed in the table. The DNPSC reagent reacts rapidly with carbonyl compounds and provides an excellent derivative which may be recrystallized from 95 % ethanol or DMF–ethanol mixtures. On the SGG plates the reaction is almost quantitative, consuming most of the reagent. Neither pyrolysis nor caking was observed with any of the DNSPCs investigated.

In system D, the R_Y values of the DNPSCs of aliphatic carbonyl compounds were greater than the R_Y of the reagent, except for crotonaldehyde which had a value about equal to that of the reagent. The R_Y values of the aromatics were usually less than that of the reagent in this system. No solvent combination was found which would move these latter out of the reagent, which tended to streak badly from the starting line up to R_Y 0.50. However, the aromatic compounds investigated reacted so thoroughly that little excess reagent was left to contaminate the derivative spot. In system B, the R_Y of the reagent was greater than any of the derivative values, and very little streaking of the reagent was observed. This was of value, since any remaining reagent in the aromatics from reaction system D was removed in this second system.

It has been reported that the visible spectra of DNPSCs are very characteristic of the various carbonyl compound classes[16].

ACKNOWLEDGEMENTS

We are indebted to Mr. R. D. HENSON for nitrogen analyses and to Mrs. MODERIA PETTY for technical assistance.

Mention of a proprietary product or company does not necessarily imply endorsement of the company or proprietary product by the U.S. Department of Agriculture.

SUMMARY

A technique for identifying small quantities of carbonyl compounds present in essential oils is described. These compounds are derivatized on thin-layer plates as they are eluted from the exhaust port of a gas chromatograph. Subsequently, they may be examined by thin-layer chromatography, gas–liquid chromatography, paper chromatography, or several other methods for confirmation of identity. 2,4-Dinitrophenylhydrazones, *p*-nitrophenylhydrazones, and 2,4-dinitrophenylsemicarbazides were utilized in this study, and thin-layer chromatographic data are given for each. In addition, melting points are listed for some previously unreported 2,4-dinitrophenylsemicarbazides.

REFERENCES

1 B. CASU AND L. CAVALOTTI, *Anal. Chem.*, 34 (1962) 1514.
2 C. L. TEITELBAUM, *J. Org. Chem.*, 23 (1958) 646.
3 J. P. MINYARD, J. H. TUMLINSON, A. C. THOMPSON AND P. A. HEDIN, *J. Agr. Food Chem.*, 15 (1967) 517.
4 G. D. JOHNSON, *J. Am. Chem. Soc.*, 73 (1951) 5888.
5 R. L. SHRINER AND R. C. FUSON, *The Systematic Identification of Organic Compounds*, 3rd Ed., Wiley, New York, 1948.
6 J. L. MCVEIGH AND J. D. ROSE, *J. Chem. Soc.*, (1945) 713.
7 H. T. BADINGS AND J. G. WASSINK, *Neth. Milk Dairy J.*, 17 (1963) 132.
8 G. A. BYRNES, *J. Chromatog.*, 20 (1965) 528.
9 E. BREWER, H. LEADER AND S. SAREL. *Bull. Res. Council Israel, Sect. A.*, 9 (1960) 43.
10 D. A. FORSS, E. A. DUNSTONE, J. F. HORWOOD AND W. STARK, *Australian J. Chem.*, 15 (1962) 163.
11 R. J. SOUKUP, J. SCARPELLINO AND E. DANIELSZIK, *Anal. Chem.*, 36 (1964) 2255.
12 J. P. PHILLIPS, *J. Org. Chem.*, 27 (1962) 1443.
13 W. J. SCHMITT, E. J. MORICONI AND W. F. O'CONNOR, *Anal. Chem.*, 28 (1956) 249.
14 E. FEDELI AND M. CIRIMELE, *J. Chromatog.*, 15 (1964) 435.
15 H. H. HODGSON AND K. E. COOPER, *J. Chem. Soc.*, (1929) 231.
16 A. E. GILLAM AND D. G. MOSS, *J. Chem. Soc.*, (1947) 1387.

REACTION CHROMATOGRAPHY

II. GAS–LIQUID/THIN-LAYER CHROMATOGRAPHIC DERIVATIZATION TECHNIQUE FOR THE IDENTIFICATION OF ALCOHOLS*

J. P. MINYARD, J. H. TUMLINSON, A. C. THOMPSON AND P. A. HEDIN

Boll Weevil Research Laboratory, Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, State College, Miss. (U.S.A.)

(Received December 16th, 1966)

The preceding paper[1] outlined requirements for successful application of the gas–liquid/thin-layer chromatographic (GLC/TLC) derivatization technique. The procedure was developed for use with compounds containing reactive group functions and successfully applied to carbonyl compounds. Components eluted from the GLC exhaust into appropriate reagents spotted on thin-layer plates form derivatives which then may be chromatographed and removed for subsequent examination.

This work extends the concept to alcohols with the use of 3,5-dinitrobenzoate (DNB) and *o*-nitrophenylurethan (ONPU) derivatives. Thin-layer chromatographic migration behavior of these derivatives on several new systems is tabulated.

EXPERIMENTAL

Reagents and apparatus

The apparatus and methods employed are essentially those reported previously except that a thermometer was wrapped in contact with the GLC exhaust with heating tape to allow exhaust temperature measurement. GLC stationary phases employed to separate the alcohols were Carbowax 4000 and Apiezon L.

3,5-Dinitrobenzoyl chloride and *o*-nitrophenylisocyanate (Eastman Organic Chemicals) were used without purification to prepare reagents as follows:

ONPU. *o*-Nitrophenylisocyanate (10.0 g) was dissolved by heating in 100 ml of benzene redistilled over calcium oxide.

DNB. 3,5-Dinitrobenzoyl chloride (1.0 g) was dissolved in 7.5 ml *p*-xylene and 1.0 ml tetrahydrofuran.

TLC plates were prepared and activated as described previously[1]. Additionally, Silica Gel G (SGG) plates were coated with vaseline by immersing in a 5 % (w/v) vaseline–heptane solution, removing, and allowing the heptane to evaporate at room temperature for 20 min. Fluorescent SGG plates for DNBs were prepared by adding 200 μg 2′,7′-dichlorofluorescein (Eastman Organic Chemicals) per 20 × 20 cm plate during adsorbent slurry preparation.

All alcohols used were commercial samples available in our laboratory.

* Part of a thesis submitted by J. P. MINYARD in partial fulfillment of requirements for the Ph.D. degree from Mississippi State University, State College, Mississippi.

Formation of standard derivatives

DNBs and ONPUs were prepared according to SHRINER AND FUSON[2], except for the terpene ONPUs which were prepared as described by ATTAWAY *et al.*[4]. The DNBs were purified by recrystallization from aqueous alcohol or preparative TLC on system A (see later). The ONPUs were purified by preparative TLC on system B (see later), since many were very slow in crystallizing from the reaction mixture; their authenticity was established by comparing paper chromatographic R_F values with those reported by ATTAWAY *et al.*[3,4].

Formation of derivatives from gas chromatograph

The reagent spotting and GLC derivatization were accomplished as before[1]. In some instances, a 20-gauge hypodermic needle 1 cm long was attached to the Luer-Lok GLC exhaust port to cool the emerging compounds for better derivatization.

Control of the exhaust port temperature for optimum derivatization was necessary with the DNBs. Primary and all terpene alcohols investigated reacted better at an exhaust temperature of 185° with the needle off; the secondary alcohols gave much better yields at 155° with the needle on. ONPUs were prepared with the needle off and the outlet at 185°.

After formation of the DNBs, excess reagent was hydrolyzed and fixed at the TLC plate starting line by adding one or two drops of 10 % aqueous sodium hydroxide to each spot. The ONPUs ran well ahead of the unexpended reagent on system B (see later) so no post-reaction treatment before TLC was necessary.

Thin layer chromatography

Samples spotted 1.5 cm from the bottom edge of the plate and 2 cm apart were developed to a height of 10 cm in solvent vapor equilibrated tanks. Systems used to separate the derivatives were:

(A) Benzene–petroleum ether (50–68°) (1:1); solid adsorbent—SGG with 200 μg/plate of 2′,7′-dichlorofluorescein incorporated.

(B) Heptane–acetone (4:1); solid adsorbent—SGG.

(C) Methanol–water (9:1); solid adsorbent—polyamide powder.

(D) Methanol–water (95:5); solid adsorbent—SGG coated with 5 % vaseline.

The DNBs appeared as light pink spots on the fluorescent plates (system A) by daylight and as dark purple absorbing spots on a yellow green background under U.V. light. On polyamide plates, they were initially colorless by daylight but dark purple U.V. absorbers. If the polyamide plates were exposed to U.V. light for more than about 2 min, the DNBs changed appearance and began to fluoresce deep fuchsia. Afterwards, they were clearly visible by daylight as light violet spots, the color persisting for several hours but gradually fading. The ONPUs were visible as yellow spots on all systems and even more distinct as strong absorbers under U.V. radiation.

Test mixture (Desaga, Heidelberg) was spotted on each plate and R_F values were calculated relative to Butter Yellow for adjacent standard and GLC derivatives.

All reactions were performed on SGG plates. The DNBs were developed in system A and the ONPUs in system B, with reagent blanks run on each derivative plate. The derivative spot from each sample was scraped from the plate and eluted with methylal. The DNBs then were spotted and run on systems A and C, the ONPUs on systems B, C, and D, and R_Y values obtained.

RESULTS AND DISCUSSION

The hydrolysis of unexpended DNB reagent with aqueous alkali illustrates the requirement mentioned in the previous paper[1], *i.e.*, the derivative should be separable from unexpended reagent by some TLC system. This principle should be applicable generally when large quantities of unused reagent overload the TLC adsorbent and mask the derivative spot upon development. This was the case with the DNBs as well as with the α-naphthylurethans which were examined initially. In the latter case, derivatization was successful, but since all the derivatives had nearly the same R_Y values in all TLC systems tried, the work was terminated. The use of mildly acidic or basic aqueous solutions was preferred for the hydrolysis since the salts formed were unmoved by the organic developing solvents.

Certain secondary and tertiary alcohols, particularly some of the terpenoids, are quite sensitive to thermal or acid catalyzed rearrangements and dehydrations. The reaction conditions for DNB formation (localized HCl production plus elevated temperature) may be too severe for such sensitive compounds, especially since non-deactivated silica gel has been shown to be an effective catalyst for terpenoid rearrangements[5]. However, the ONPUs seem to form without any major attendant rearrangements or degradation. Whenever the identity of the GLC prepared derivative is uncertain due to possible rearrangements, standard and unknown derivatives should be prepared in an identical manner by GLC/TLC for comparison.

The ONPUs and DNBs prepared by GLC/TLC agreed in R_Y values in all systems with the standards ($\pm$ 3 % relative).

tert.-Butyl and *tert.*-amyl alcohols, the only two nonterpenoid tertiary compounds tested, did not form DNB or ONPU derivatives from the gas chromatograph at the 10 μl level, either because of lowered reactivity or degradation occurring in the GLC separation. The yields of derivatives from linalool and α-terpineol by GLC/TLC also were considerably smaller than those from secondary and primary alcohols. Thus this technique may have limited use in identification of tertiary alcohols.

DNBs of primary and secondary alcohols were formed in detectable amounts from 0.5 mg or slightly less of compound. Primary alcohols consistently gave better yields than secondary alcohols. Tertiary alcohols which formed DNBs did so only at the 5 mg or higher level. Primary and secondary alcohols formed ONPUs from 0.05 mg of sample, but the tertiary terpene alcohols required a minimum of 0.5 mg to give detectable spots. Primary alcohols formed ONPUs in better yields than secondary alcohols. The ONPUs thus seem to be excellent derivatives for primary, secondary, and some tertiary alcohols.

Tables I and II give relative TLC behavior of representative alcohols in several systems. All values reported for a given system and derivative type were obtained from runs of all samples on single plates and thus are internally consistent.

DNB R_Y values in two systems are listed in Table I. Values for the derivatization system (A) were calculated from plates on which the derivative had been respotted and developed. Because unhydrolyzed 3,5-dinitrobenzoyl chloride (or the acid) streaked from the starting line up to about R_Y 2.0, much above all the derivatives, the alkaline hydrolysis postreaction step was necessary. All the DNBs ran well ahead of the hydrolyzed reagent, which stayed at the starting line.

The DNBs can be visualized with a 1 % ethanolic α-naphthylamine spray[6], but

TABLE I

TLC R_Y[a] VALUES OF 3,5-DINITROBENZOATES (DNB)
Solvent systems: (A) Silica Gel G with 2′,7′-dichlorofluorescein; benzene–petroleum ether (38–50°) (1:1). (C) Polyamide; methanol–water (9:1).

DNB	R_Y	
Compound	*A*	*C*
1-Propanol	0.68	2.01
1-Butanol	0.82	1.90
1-Pentanol	0.96	1.71
1-Hexanol	1.04	1.57
1-Heptanol	1.08	1.42
1-Octanol	1.15	1.28
1-Nonanol	1.19	1.17
1-Decanol	1.24	1.03
1-Undecanol	1.28	0.91
1-Dodecanol	1.35	0.78
2-Propanol	0.79	2.08
2-Butanol	0.92	2.02
2-Pentanol	1.05	1.89
2-Heptanol	1.15	1.77
2-Octanol	1.23	1.54
3-Pentanol	1.05	1.91
4-Heptanol	1.42	1.72
2-Methyl-1-butanol	1.01	1.76
3-Methyl-1-butanol	0.96	1.84
3-Hexen-1-ol	0.94	1.73
Phenylethyl alcohol	0.61	1.54
Cinnamyl alcohol	0.66	1.31
α-Terpineol	1.15	1.17
Geraniol	1.04	1.44
Citronellol	1.08	1.40
Linaloöl	1.07	1.46
Menthol	1.46	1.44

[a] R_Y is travel ratio of unknown to Butter Yellow dye.

sprayed derivatives run at slightly low R_Y values when rechromatographed in the same or other systems. The 2′,7′-dichlorofluorescein dye from system A plates did not affect the TLC behavior of recovered DNBs on rechromatography.

Other TLC systems[6,7] and paper chromatographic systems[8–11] have been recommended for DNB separations. GALETTO, KEPNER AND WEBB recently reported successful GLC of DNBs on an SE-30 column[12].

Table II gives TLC R_Y values for ONPUs of representative alcohols on systems B, C, and D. TLC of ONPUs has not been reported previously, to our knowledge. Again, system B (reaction system) values were obtained by rechromatography of the derivatives removed from the reaction plate. Because the derivatives run with nearly the same R_Y values and because of its handling properties during derivatization and elution, system B is desirable for the initial formation of ONPU's but is of little use for distinguishing between alcohols.

Urethan derivatives appear to form readily by GLC/TLC, especially if activating *ortho* or *para* electron withdrawing groups are present in the aryl isocyanate. The structure of the residue attached to the isocyanate function appears to have a large influence on the TLC separability of the urethans formed, judging by our experience

TABLE II

TLC R_Y[a] VALUES OF *o*-NITROPHENYLURETHANS (ONPU)
Solvent systems: (B) Silica Gel G; heptane–acetone (4:1). (C) Polyamide; methanol–water (9:1). (D) Silica Gel G coated with 5% vaseline; methanol–water (95:5).

ONPU	R_Y		
Compound	*B*	*C*	*D*
1-Propanol	0.98	2.05	1.19
1-Butanol	1.08	1.94	1.11
1-Pentanol	1.21	1.80	1.04
1-Hexanol	1.21	1.61	1.00
1-Heptanol	1.23	1.45	0.91
1-Octanol	1.28	1.30	0.84
1-Nonanol	1.30	1.12	0.75
1-Decanol	1.30	1.00	0.66
1-Undecanol	1.30	0.87	0.52
1-Dodecanol	1.30	0.75	0.41
2-Propanol	1.06	2.30	1.26
2-Butanol	1.09	2.22	1.19
2-Pentanol	1.15	2.16	1.07
2-Hexanol	1.23	1.86	1.05
2-Heptanol	1.25	1.70	0.98
2-Octanol	1.31	1.54	0.86
4-Heptanol	1.27	1.89	0.93
2-Methyl-1-butanol	1.14	1.96	1.10
3-Methyl-1-butanol	1.16	1.82	1.13
3-Hexen-1-ol	1.28	1.76	1.02
Phenylethyl alcohol	0.89	1.70	1.19
α-Terpineol	1.23	1.35	0.84
Geraniol	1.20	1.38	0.73
Citronellol	1.27	1.34	0.63
Linaloöl	1.17	1.55	0.88
Menthol	1.28	1.35	0.72
Fenchyl alcohol	1.45	1.35	0.88

[a] R_Y is travel ratio of unknown to Butter Yellow dye.

with the α-naphthylurethans and the work of ATTAWAY *et al.*[3,4]. *p*-Phenylazophenyl-, *m*-nitrophenyl-, and *p*-nitrophenylurethans also might provide effective GLC/TLC derivatives. Traces of water present a major difficulty in the formation of urethans in test tube reactions, but this can be avoided nicely by the GLC/TLC technique if the SGG plates are used immediately after activation and if the reagent solvent is carefully dried. Urethans also avoid the possibly detrimental effect of acidic reagents in terpenoid derivatizations.

ACKNOWLEDGEMENTS

We thank Mrs. MODERIA PETTY and Mrs. LAVENIA MILES for technical assistance.

Mention of a proprietary product or company does not necessarily imply endorsement of the company or proprietary product by the U.S. Department of Agriculture.

SUMMARY

The technique for the identification of reactive compounds by derivative formation on thin-layer plates at the exhaust of a gas chromatograph (gas–liquid/thin-layer chromatography) was applied to alcohols. Advantages, limitations, and reaction conditions for 3,5-dinitrobenzoates (DNBs) and *o*-nitrophenylurethans (ONPUs) as derivatives of representative alcohols used in this study are discussed and thin-layer chromatographic behavior in several systems is tabulated. DNBs are readily formed from 0.5 mg and ONPUs from 0.05 mg of the same primary and secondary alcohols. Some tertiary alcohols failed to give either derivative from 10 mg of reactant, while 0.5–5 mg of others reacted discernibly.

REFERENCES

1 J. H. TUMLINSON, J. P. MINYARD, P. A. HEDIN AND A. C. THOMPSON, *J. Chromatog.*, 29 (1967) 80.
2 R. L. SHRINER AND R. C. FUSON, *The Systematic Identification of Organic Compounds*, 3rd Ed., Wiley, New York, 1948, pp. 163–166.
3 J. A. ATTAWAY, R. W. WOLFORD, G. E. ALBERDING AND G. J. EDWARDS, *Anal. Chem.*, 34 (1962) 671.
4 J. A. ATTAWAY, R. W. WOLFORD, G. E. ALBERDING AND G. J. EDWARDS, *Anal. Chem.*, 35 (1963) 234.
5 R. E. WROLSTAD AND W. G. JENNINGS, *J. Chromatog.*, 18 (1965) 318.
6 J. H. DHONT AND C. DE ROOY, *Analyst*, 86 (1961) 527.
7 A. MEHLITZ, K. GIERSCHNER AND T. MINAS, *Chemiker-Ztg.*, 89(6) (1964) 175.
8 D. F. MEIGH, *Nature*, 169 (1952) 706.
9 A. MEHLITZ AND B. MATZIK, *Ind. Obst- Gemüseverwert.*, 42 (1957) 359.
10 R. C. RICE, JR., G. KELLER, JR. AND G. KIRCHNER, *Anal. Chem.*, 23 (1951) 194.
11 J. W. WHITE, *Food Res.*, 15 (1950) 68.
12 W. G. GALETTO, R. E. KEPNER AND A. D. WEBB, *Anal. Chem.*, 38 (1966) 34.

CHROMATOGRAPHIC SEPARATION OF DIASTEREOISOMERIC ESTERS

I. LACTATE AND MANDELATE OF BUTANOL-2*

HARVEY D. SPITZ**, HERBERT L. ROTHBART*** AND WILLIAM RIEMAN III
Ralph G. Wright Chemical Laboratory, Rutgers—The State University, New Brunswick, N. J. (U.S.A.)
(Received December 27th, 1966)

The resolution of racemic alcohols is generally a more difficult task than the resolution of acids or amines. The classical method for alcohols, which is still widely used, consists in converting the alcohol to the acid ester of a diprotic acid and then applying fractional crystallization to the salt of this derivative with an optically active amine. Several workers have studied the separation of diastereoisomeric esters by gas–liquid[1–4], liquid–liquid[5], and solid–liquid[6,7] chromatography; but none of them has developed a method that is conspicuously better than the classical.

The purpose of this paper is to describe the resolution of racemic alcohols by chromatography of their diastereoisomers with an ordinary, optically inactive, ion-exchange resin as the stationary phase and aqueous solutions of salts or nonelectrolytes as the mobile phase.

EXPERIMENTAL

Synthesis of the diastereoisomeric esters

The mandelic acids were obtained from Aldrich Chemical Company. The D(—)-mandelic acid had $[\alpha]_D^{23.5} = -154.1$ ($C = 2.077$ in H_2O) and $[\alpha]_D^{21.0} = -153.9$ ($C = 2.917$ in H_2O). The menthol, lactic acid, and butanol-2 were obtained from Eastman, Mallinckrodt, and Matheson, Coleman and Bell respectively.

Equal weights of the alcohol and acid were mixed with about 0.6 ml of concentrated sulfuric acid (hydrochloric acid in the case of menthyl mandelate) and 75 ml of benzene. The mixture was refluxed on a water bath until no more water was obtained in a Dean-Stark tube. It was then dissolved in ether and washed with water, aqueous sodium carbonate, and again with water until it was neutral. It was dried with anhydrous sodium sulfate and filtered. After distillation of the ether and benzene, the residual ester was purified by distillation at low pressure. Yields were between 50 and 78 %. The DL-*sec.*-butyl D(—)-mandelate melted at 26–27°; $[\alpha]_D^{21.0} = -96.6$ ($C = 0.969$ in EtOH). The DL-*sec.*-butyl DL-lactate boiled between 57.0 and 58.5° at 8 mm.

Chromatography

The dimensions of the several columns will be given with the results. The 11.3-m column was made from five lengths of glass tubing. Neoprene gaskets were inserted between the flanged ends and clamped together. This column was located in a shaft

* Supported in part by U.S.P.H.S. Grant GM 09687.
** Present address: Johnson and Johnson Company, New Brunswick, N.J., U.S.A.
*** Present address: U. S. Department of Agriculture, Philadelphia, Pa., U.S.A.

that extended from the basement to the third floor of Wright Laboratory. Although automatic temperature control was not available, the internal brick walls of the shaft provided a much more nearly constant temperature than prevails in an ordinary laboratory. All columns were vertical, and the mobile phases moved downward at flow rates controlled by a Minipump*. Fractions of effluent were generally collected automatically. Glass tubing was used as much as possible to conduct the liquid from the reservoir through the pump and column to the fraction collector. Where necessary, glass-to-glass joints were connected with Tygon tubing. These precautions decreased the dissolution of ultraviolet-absorbing compounds from plastic or rubber tubing[8]. In the case of butyl lactate, the effluent passed into a flow-through cell contained in a Beckman DB spectrophotometer; and the absorbance was recorded automatically. The Dowex resins used in the columns had been treated by standard procedures to remove the excessively fine particles and—as much as practicable—the soluble organic constituents.

Solubility measurements

The solubility of the esters was determined by shaking the appropriate solvent (water or aqueous solutions of ethanol or sodium chloride) with excess of the ester for 24 h, separating the two phases, and measuring the absorbance of the aqueous phase at 257.5 mμ. Although the accuracy of these measurements is not very great, it is sufficient for the present purpose.

A Beckman DU spectrophotometer and a Rudolph Model 80 polarimeter were used in the examination of the fractions.

RESULTS AND DISCUSSION

D(−)-*Menthyl* DL-*mandelate*

The solubility of this compound in water is so small that aqueous ethanol (35 to 50 vol. %) was used as the eluent in elution chromatography. Then the peak of the graph appeared shortly after the interstitial volume, and no separation could be observed. The addition of lithium or sodium chloride to the eluent did not help. The substitution of dioxane for ethanol was also fruitless.

The unsuccessful attempt to separate these diastereoisomers may be attributed to the following factors:

(1) Concentrations of organic solvents necessary to give sufficient solubility to the ester cause very small separation factors.

(2) Solubilization chromatography has been found to be useful only for compounds of about 12 or fewer carbon atoms.[9]

(3) Entrance of the ester into the resin is inhibited because of its small solubility and large molecular volume. The steric effects caused by the ester are due to the bulky groups on the menthol and its nonplanar nature. (Menthol is predominantly in the chair form.)

DL-*sec.*-*Butyl* DL-*mandelate*

The solubility of this ester in water and some aqueous solutions is given in Table I.

* Milton Roy Company, 1300 E. Mermaid Lane, Philadelphia, Pa., U.S.A.

Four ml of nearly saturated aqueous solution was eluted with pure water through a column 150.5 cm × 0.785 cm^2 of Dowex 50W-X2, 200–400 mesh, sodium form, at 0.32 cm per min. Fractions of 3.60 ml were analyzed spectrophotometrically at 257.5 mμ. The graph showed two very distinct but badly overlapping peaks. It is clear that the original mixture of four diastereoisomers had been partly separated into a mixture of the D–L and the L–D esters and a mixture of the L–L and the D–D esters. It could not be ascertained from the graph which mixture constituted each peak. The distribution ratios of the two pairs of diastereoisomers were calculated[10] from the retention volumes to be $C_1 = 11.2$, $C_2 = 12.1$.

TABLE I

SOLUBILITY OF DL-*sec.*-BUTYL ESTERS AT ROOM TEMPERATURE

Solvent	*Solubility (moles per l)*	
	DL-*Mandelate*	DL-*Lactate*
Water	0.015	0.505
10% aqueous EtOH (v/v)	0.023	
20% aqueous EtOH (v/v)	0.035	
30% aqueous EtOH (v/v)	0.068	
0.10*M* NaCl	0.015	
0.50*M* NaCl	0.013	0.400
1.0*M* NaCl	0.012	
1.5*M* NaCl		0.250
2.0*M* NaCl		0.222
3.0*M* NaCl		0.130

In the hope of getting a better separation, the elution was repeated with 0.50*M* and 1.0*M* aqueous sodium chloride as eluent[11]. In the former case, the values of C_1 and C_2 were 17.6 and 19.4. The small increase in the ratio C_2/C_1 did not compensate for the additional time required for the elution because of the increase in C values. Therefore salting-out chromatography of this ester was abandoned. An elution was done under similar conditions except that the eluent was a 15 vol.% solution of ethanol in water to take advantage of the greater solubility of the ester in this solvent. The separation was less complete than with pure water as eluent; hence work with solubilization chromatography[12] was discontinued.

DL-*sec.-Butyl* D(—)-*mandelate*

Five ml of a saturated solution of this ester in pure water (containing 0.074 mmole) was eluted through a column 151.6 cm × 0.785 cm^2 of sodium-form Dowex 50W-X2, 200–400 mesh, at 0.65 cm per min with pure water as eluent. Repetition of the foregoing experiment at a flow rate of 0.32 cm per min significantly improved the separation. The elution graph of the latter experiment, Fig. 1, is very similar to that of the elution of DL-*sec.*-butyl DL-mandelate with water. By the application of previously published equations[10] to this graph, the following values were calculated: $C_1 = 10.8$, $C_2 = 11.6$, P_1 (calculated from the rising slope of the first peak) = 9.7, P_2 (calculated from the falling slope of the second peak) = 7.8, H = the height of column required for a chromatographic separation 99.9% complete = 1000 cm.

The elution was repeated under identical conditions except that the resin was

in the barium form, but no advantage was found. The hydrogen form would catalyze the hydrolysis of the ester. All subsequent work was done with the sodium form.

Elutions of DL-*sec.-butyl* D(—)-*mandelate through the long column.* Fifty ml of a saturated aqueous solution of this ester was added to a column 10.1 m × 1.29 cm^2 of sodium-form Dowex 50W-X2, 200–400 mesh. This was eluted with water at a flow rate of 0.26 cm per min. Fractions of 7.73 ml were collected and examined as before. The elution graph, Fig. 2, shows a nearly quantitative separation of the two diastereoisomers.

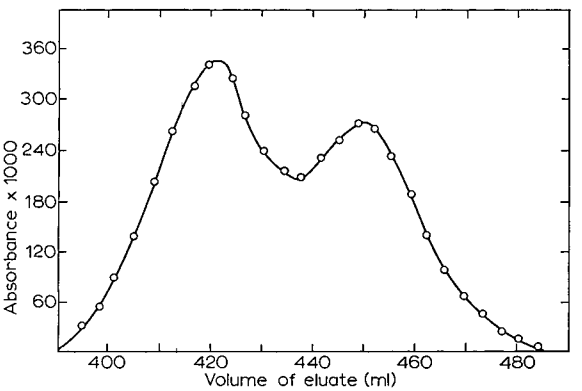


Fig. 1. Elution graph of DL-*sec.*-butyl D(—)-mandelate with a 152-cm column.

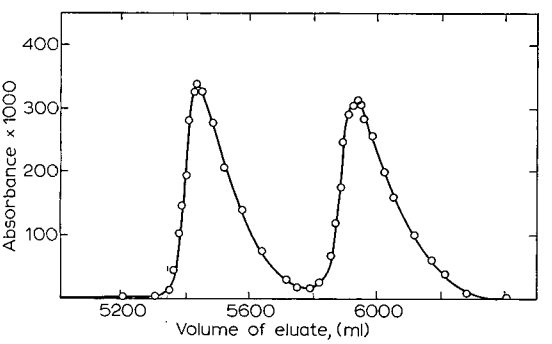


Fig. 2. Elution graph of DL-*sec.*-butyl D(—)-mandelate with a 10-m column.

In spite of elaborate precautions to wash soluble organic impurities from the resin before use, troublesome absorbances were observed in the fractions of eluate before the breakthrough of the first diastereoisomer and after the complete elution of the second. These absorbances were greater after than before the elution. Fig. 2 was drawn after correcting for the blanks by assuming that the blank increased linearly from a point just before the breakthrough to a point just after the complete elution.

Both elution curves of Fig. 2 show distinct tailing. This is probably due in part to the variation in the blank correction, which skews the graphs so as to aggravate tailing. Failure to attain equilibrium within the column would also contribute to the tailing.

For equal concentrations of esters, the fractions under the first curve had more

positive rotations than those under the second. This indicates that L(+)-*sec.*-butyl D(—)-mandelate was eluted before the D(—)-*sec.*-butyl ester. The areas under the two curves had a ratio of 1.01, indicating that the sample contained the two diastereoisomers in equal amounts within the experimental error.

Ultraviolet scans of fractions from each peak coincided with those of the pure diastereoisomers when the effluent (before the solute emerges) was used as the blank.

The values of C_1 and C_2 calculated from Fig. 2 are 12.75 and 14.02. A repetition of this elution gave 12.71 and 13.98. These results are in very good agreement with each other. The failure to agree well with the results from the shorter columns is due to the fact that a different batch of Dowex 50W-X2 was used to fill the long column.

Frontal chromatography of DL-*sec.-butyl* D(—)-*mandelate on the long column.* In spite of the nearly quantitative separation of the diastereoisomers achieved by elution chromatography on the long column, this procedure is not a practicable method of resolving racemic butanol-2 because of the very severe limitation on the size of the sample, 0.037 mmole of each diastereoisomeric ester with the column used. Larger samples can be accommodated by frontal chromatography.

A nearly saturated aqueous solution of DL-*sec.*-butyl D(—)-mandelate (0.0074 *M* with respect to each diastereoisomer) was fed to the 10.1-m column at 0.24 cm per min until 5,710 ml of effluent was collected. Then the ester solution in the tube above the resin was removed and water was fed into the column at the same rate until both diastereoisomers were removed from the column. Fractions of 10.0 ml of effluent were collected.

In order to eliminate the large blank readings of absorbance at 257.5 mμ, the concentrations of ester in the fractions were determined by a slight modification of the spectrophotometric dichromate method[13]. Instead of the recommended volumes, 4 ml of sample solution and 14 ml of water were added to 18 ml of the 0.1 *N* dichromate in concentrated sulphuric acid. Since water dissolves from the resin only very small concentrations of solutes having extremely large absorptivities in the ultraviolet, the use of the dichromate method eliminated the blank correction almost entirely.

Fig. 3 shows the chromatographic curve for the entire cycle, *i.e.*, the frontal and the subsequent elution of the esters from the resin. The first isomer, L(+)-*sec.*-butyl D(—)-mandelate, appeared in the effluent at U (volume of effluent) = 5,260. Its concentration rose rapidly to 0.0081 *M*. Then it remained essentially constant. The second diastereoisomer appeared in the effluent at U = 5,710. The total concentration

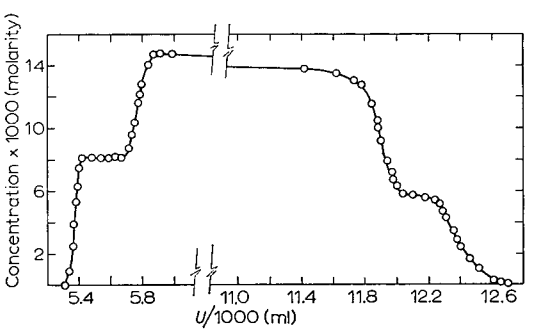


Fig. 3. Frontal graph of DL-*sec.*-butyl D(—)-mandelate with a 10-m column.

of ester rose rapidly and reached a plateau at 0.00147 M at $U = 5{,}870$. The concentration of the first diastereoisomer started to decrease at $U = 11{,}420$ and became zero at $U = 12{,}100$. At this point the second ester was emerging from the column with a concentration of about 0.0057 M. Its concentration started to decrease at $U = 12{,}180$ and reached zero at $U = 12{,}710$.

The theory of frontal partition chromatography with ion-exchange resins as the stationary phase has been discussed, and data were presented that support the theory very well[14]. Application of the equations of this paper to the data of Fig. 3 permits the calculation of the C values of each ester from the midpoints of their breakthroughs. The values are 12.55 and 13.50 respectively. The distribution ratios can also be calculated by the equation

$$U_d^* - U_c = CV + V \qquad (1)$$

where U_c is the volume of effluent collected when the input into the column was changed to water and U_d^* is the volume of effluent at the midpoint of the descending part of the graph for any one solute. This equation gave values of 12.35 and 13.67, respectively, in fair agreement with those obtained from the first part of this experiment and from the elutions performed with the same batch of resin.

Fig. 3 has some unexpected features: (1) The first plateau, corresponding to the emergence of pure L(+)-*sec.*-butyl D(—)-mandelate from the column, occurred at a concentration slightly greater than the concentration of D(—)-*sec.*-butyl D(—)-mandelate when it finally reached the second plateau. (2) The second plateau had a small negative slope; and the third plateau, corresponding to the removal of pure D(—)-*sec.*-butyl D(—)-mandelate from the column, occurred at an apparent concentration lower than that calculated from the difference between the second and first plateaus. (3) The portion of the graph corresponding to the emergence of pure L(+)-*sec.*-butyl ester ($U = 5{,}300$ to $5{,}700$) is more nearly rectangular than the portion corresponding to the emergence of pure D(—)-*sec.*-butyl ester ($U = 12{,}000$ to $12{,}700$).

The area under the graph from $U = 5{,}300$ to $U = 5{,}700$ represents 2.6 mmole of very nearly pure D(—)-*sec.*-butyl D(—)-mandelate. The area from $U = 12{,}000$ to $U = 12{,}700$ represents approximately the same quantity (2.3 mmole) of very nearly pure L(+)-*sec.*-butyl mandelate.

The fractions of effluent from $U = 5{,}300$ to $U = 5{,}700$ that had not been destroyed in the determinations of concentration were pooled. The ester was extracted by ether and hydrolyzed by aqueous sodium hydroxide. The L(+)-butanol-2 was extracted by ether. The rotation of this solution was +0.207°. An ethereal solution of butanol-2 recovered analogously from the effluent from $U = 12{,}000$ to U 12,700 was —0.156°. These data indicate that a resolution of the butanol had been accomplished. Unfortunately, these rotations cannot be used to check the yields of diastereoisomers previously mentioned (2.6 and 2.3 mmole) because the authors were unable to find in the literature a value of $[\alpha]_D$ of butanol-2 in ether and because extensive loss by evaporation occurred during the saponification.

Repetitive frontal chromatography of DL-*sec.-butyl* D(—)-*mandelate on the long column.* A chromatographic experiment was performed like the last except that the sequence of feed to the column was 720 ml of aqueous ester solution, 2095 ml of water, 700 ml of ester solution, 2100 ml of water, 680 ml of ester solution, and finally sufficient

water to remove all the esters from the column. It is suggested that this type of separation be called *repetitive frontal chromatography*.

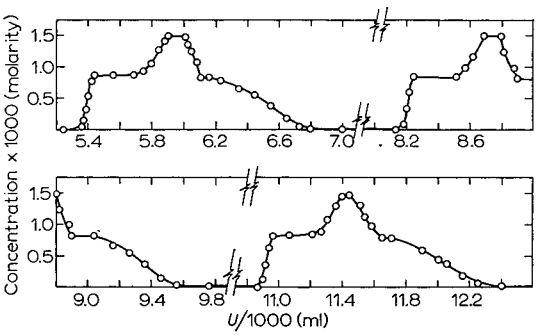


Fig. 4. Graph of repetitive chromatography of DL-*sec.*-butyl D(—)-mandelate with a 10-m column.

A comparison of Figs. 3 and 4 reveals the advantage of this procedure. Fig. 4 has three regions representing pure L(+)-*sec.*-butyl D(—)-mandelate, each approximately equal in area to the single analogous region of Fig. 3. The same statement is applicable to the D(—)-*sec.*-butyl ester. Six independent values of C of the L(+)-*sec.*-butyl ester and six of the D(—)-*sec.*-butyl ester can be calculated from the data of Fig. 4. The mean results are 12.41 and 13.53 with standard deviations of 0.14 and 0.10, respectively, in satisfactory agreement with the values obtained in the previously discussed frontal and elution experiments. On the other hand, the calculated[12] values of P, the number of plates per cm of column, are in poor agreement among themselves and with the values obtained in elution chromatography. This may be due to the fall in concentration of the L(+)-isomer emerging from the column when the D(—)-isomer starts to emerge. This would make the second plateau lower than the theoretical value, cause small errors in the C values estimated from the midpoint between the first and second plateaus, also from the midpoint between the second and third plateaus, and make any calculations of P evaluated in these regions highly dubious.

From the foregoing experiment, results show that 360 ml of frontal solution was required to reach the end of the first plateau. Therefore, the addition of the frontal-feed solution beyond this volume is actually unnecessary as the second plateau represents a mixture of the two diastereoisomers. One could have also reduced the volume of eluent between the end of the "elution" and the next frontal breakthrough by at least one liter. If these modifications were made, then one could add seven frontal samples instead of three within a total volume of 11,800 ml. This would have more than doubled the amounts of the optically active alcohols that could have been recovered. In comparison to the initial frontal-elution experiment, the total amounts of optically active isomer would have been seven times as great. This technique could be applied to other two-component systems.

There are two features that militate against the convenience of this method of resolving racemic alcohols: (1) The small value of C_2/C_1 together with the large values of these constants requires the use of an inconveniently long column. (2) The small solubility of the mandelate esters requires the use of dilute feed solutions and hence

involves the recovery of the active alcohols from dilute solutions of their esters. The substitution of lactate esters for mandelate esters should permit the use of more concentrated feed solutions.

DL-*sec.-Butyl* DL-*lactate*

The much greater solubility of this ester in comparison with the mandelate (Table I) is an important advantage because it permits the use of more concentrated solutions.

Samples of this ester were eluted through columns, about 150 cm × 0.785 cm^2, of sodium-form Dowex 50W-X2, 200–400 mesh, with water and several different concentrations of sodium chloride as eluents at flow rates of 0.30 cm per min. Water gave a single broad peak. 0.5 *M* sodium chloride gave two barely distinguishable peaks. More concentrated aqueous solutions of sodium chloride gave two distinct but badly overlapping peaks. This indicates a partial separation of the four diastereoisomers into one mixture containing principally D-*sec.*-butyl D-lactate and L-*sec.*-butyl L-lactate and another mixture containing mostly D-*sec.*-butyl L-lactate and L-*sec.*-butyl D-lactate. It also indicates that racemic butanol-2 can be resolved by the chromatographic separation of its esters with active lactic acid.

The best eluent for the separation seemed to be 1.5 *M* aqueous sodium chloride. From the elution graph, Fig. 5, it was calculated[10] that a column of 11 m will give a quantitative separation.

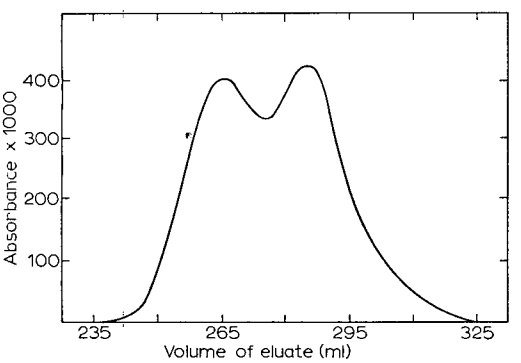


Fig. 5. Elution graph of 1.50 mmole of DL-*sec.*-butyl DL-lactate with a 150-cm column.

Additional details on the work described in this paper can be found elsewhere[15].

Work on the resolution of racemic alcohols by the chromatographic separation of their D-lactates is continuing.

ACKNOWLEDGEMENT

The authors express their gratitude to the Dow Chemical Company for the generous gift of the resins used in this work.

SUMMARY

A chromatographic procedure is described in which aqueous solutions of DL-*sec.*-butyl D(—)-mandelate and pure water were fed alternately to a column, 10 m × 1.29 cm², of Dowex 50W-X2. The time required for the chromatography was 680 h. About 7 mmole of D-*sec.*-butyl D-mandelate (contained in 1.1 l of water) and an equal amount of L-*sec.*-butyl D-mandelate (contained in 2.4 l of water) were isolated. Evidence is presented that much larger yields of the active *sec.*-butyl D-lactates can be obtained under approximately the same conditions because of the much greater solubility of the lactates.

REFERENCES

1 E. GIL-AV, R. CHARLES-SIGLER, G. FISCHER AND D. NUROK, *J. Gas Chromatog.*, 4 (1966) 51.
2 H. C. ROSE, R. L. STERN AND B. L. KARGER, *Anal. Chem.*, 38 (1966) 51.
3 J. CASANOVA AND E. J. COREY, *Chem. Ind. (London)*, (1961) 1664.
4 G. E. POLLOCK, V. OYAMA AND R. D. JOHNSTON, *J. Gas Chromatog.*, 3 (1965) 174.
5 S. BLACKBURN AND P. TETLY, *Biochim. Biophys. Acta*, 20 (1956) 423.
6 R. CURTI AND V. COLOMBO, *J. Am. Chem. Soc.*, 74 (1952) 3961.
7 M. M. JAMISON AND E. E. TURNER, *J. Chem. Soc.*, (1942) 611.
8 J. S. FRITZ AND G. E. HEDRICK, *Anal. Chem.*, 37 (1965) 1015.
9 W. RIEMAN, *J. Chem. Educ.*, 38 (1961) 338.
10 J. BEUKENKAMP, W. RIEMAN AND S. LINDENBAUM, *Anal. Chem.*, 26 (1954) 505.
11 R. SARGENT AND W. RIEMAN, *J. Phys. Chem.*, 61 (1957) 354.
12 J. SHERMA AND W. RIEMAN, *Anal. Chim. Acta*, 18 (1958) 214.
13 R. SARGENT AND W. RIEMAN, *Anal. Chim. Acta*, 14 (1956) 381.
14 H. D. SPITZ, H. L. ROTHBART AND W. RIEMAN, *Talanta*, 12 (1965) 395.
15 H. D. SPITZ, *Thesis*, Rutgers, The State University, New Brunswick, N.J., 1966.

COUNTERCURRENT DISTRIBUTION OF LABELED ANOMERIC METHYL ARABINOSIDES AND METHYL α-D-MANNOSIDE*

LAWRENCE M. MARSHALL, W. PAUL WALKER, JOYCELYNN A. GUNN AND LUCY PANTON

Department of Biochemistry, Howard University College of Medicine, Washington, D.C. 20001 (U.S.A.)

(Received December 20th, 1966)

It was previously reported that lyxose-1-^{14}C [1] and arabinose 1-^{14}C showed an isotope effect during countercurrent distribution in cyclohexane–ethanol (2:1). For arabinose, at least, the effect was a positional one; for example arabinose-5-^{14}C showed the same mobility as its unlabeled counterpart. With the possibility that the isotope effect could be related to the anomerization of the sugar or to the distribution of the anomers at equilibrium, it was thought that the examination of the mobility of the glycosides, where the anomerization under the conditions could not prevail, would aid the understanding of the earlier observations. If the isotope effect were the same for the glycosides and the sugars from which they were synthesized, a role of the isotope effect in the anomerization would be excluded. On the other hand, a difference between the isotope effect for the sugar and this derivative would allow the possibility, if not prove, that the isotope could either influence the equilibrium mixture of the anomers or the rate at which mutarotation occurs.

METHODS

Preparation of methyl β-D-arabinoside-1-^{14}C

Seven grams of D-arabinose-1-^{14}C, specific activity, 1.3 μC per mmole, 2 g Dowex 50 (H^+) and 20 ml methanol were placed in a 50 ml double necked round bottom flask and heated under reflux with stirring. After 24 h, the Dowex was removed from the reaction mixture by filtration and washed with several 15 ml portions of methanol. The filtrate and washings were concentrated under vacuum. The residue was dissolved by suspending it in 20 ml of ethanol and boiling under reflux. On cooling the product from crystallization was methyl β-D-arabinoside-1-^{14}C, m.p. 167–170°, $[\alpha]_D^{22}$ + 248.

When the product was mixed with commercial methyl β-D-arabinoside there was no depression of melting point. When the product was chromatographed on paper with ethyl acetate–*n*-propanol–water (5:3:2), the mobility of the radioactive material was identical with that of the spot obtained after first spraying with 0.5 % NaIO and then after 5 min with benzidine dihydrochloride. The triacetate of the methyl D-arabinoside was prepared from 125 mg of the product, 15 mg of anhydrous sodium acetate and 0.3 ml acetic anhydride. The mixture was refluxed at 50° for 4 h.

* Supported in part by a grant from the National Science Foundation (GB-2251) and by U.S. Public Health Training Grants, GM-481 and T5-GM-1645.

After adding 3.5 ml water, the mixture was stirred for 2 h and filtered. The filtrate contained methyl β-D-arabinoside triacetate, m.p. 69°.

Preparation of methyl α-D-arabinoside-1-^{14}C

Eight grams of D-arabinose-1-^{14}C, specific activity, 1.13 μC per mmole, and 4 g Dowex 50 (H^+) were added to 40 ml of anhydrous methanol. The mixture was refluxed at 40°—instead of at boiling as in the above preparation—for 30 h, after which the Dowex was filtered off and discarded. Methyl β-D-arabinoside-1-^{14}C was precipitated from the filtrate on standing for less than 5 min. The filtrate contained methyl α-D-arabinoside-1-^{14}C, m.p. 128–131°.

Preparation of methyl α-D-mannopyranoside-1-^{14}C

The procedure closely followed that of MOWERY[2]. The chromatography of the products in the filtrate from the reaction mixture with Dowex appears in Fig. 1. The zones designated by Roman numerals represent the chromatographic positions

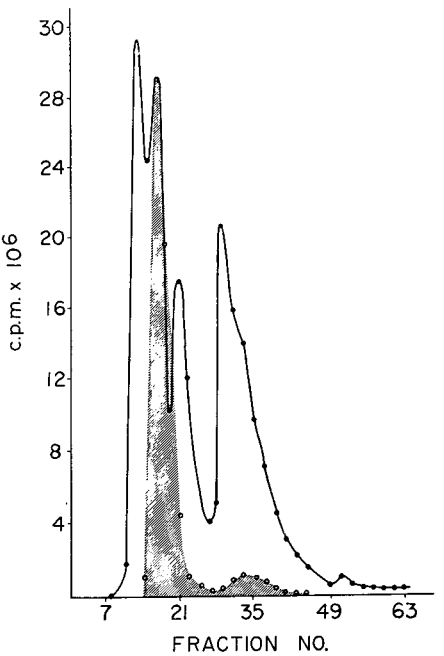


Fig. 1. Graphical representation of the chromatography of the filtrate of the reaction mixture with mannose, methanol and Dowex 50. Collected fractions were 2.5 ml. Columns were 1 × 17.5 cm. The shaded area represents the chromatography of α-D-mannopyranoside after a single crystallization.

for the methyl α-D-furanoside, α-D-pyranoside, β-D-furanoside and β-D-pyranoside of mannose. In this study no effort was made to identify solutes other than the solute from zone II. The shaded area of the plot, Fig. 1, represents the distribution on the cellulose columns of the product of a single crystallization from the reaction mixture. The twice crystallized product, methyl α-D-mannopyranoside-1-^{14}C, m.p. 185–187°, was a solute for the countercurrent distribution studies below.

Countercurrent distribution

The details for the distributions, employing the all glass machine of CRAIG AND POST, have been previously described[3]. All distributions were carried out in cyclohexane–ethanol (2:1). The assays for radioactivity were carried out on aliquots using a dioxane–naphthalene solvent, 2,5-diphenyloxazole (PPO) and 2,2′-*p*-phenylenebis(5-phenyloxazole) (POPOP) in the Nuclear Liquid Scintillation Counter, Model No. 8264. Glycosides after hydrolysis, as well as the pure sugars, were measured by the *o*-aminobiphenyl reaction through the absorption of the glycosylamine at 370 mμ[4].

RESULTS AND DISCUSSION

The data in Fig. 2 indicate that the isotope effect during the countercurrent distribution of methyl α-D-arabinoside-1-^{14}C is less than that of the arabinose resulting after the hydrolysis of the glycoside. Similar data were obtained for the methyl β-derivative (Fig. 2B). Plots of the type shown in Fig. 2 would ideally graphically represent an absence of an isotope effect as a straight line parallel to the abscissa;

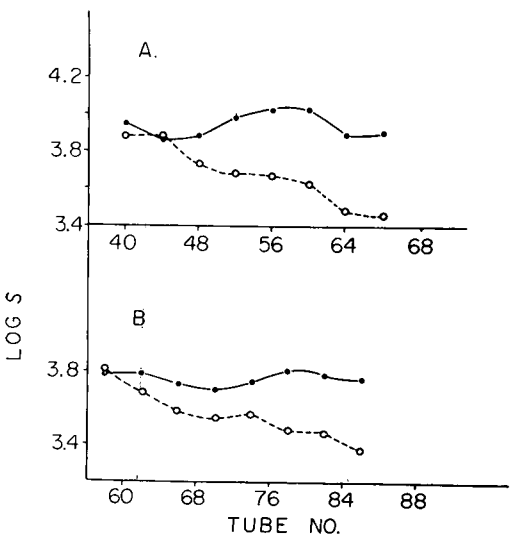
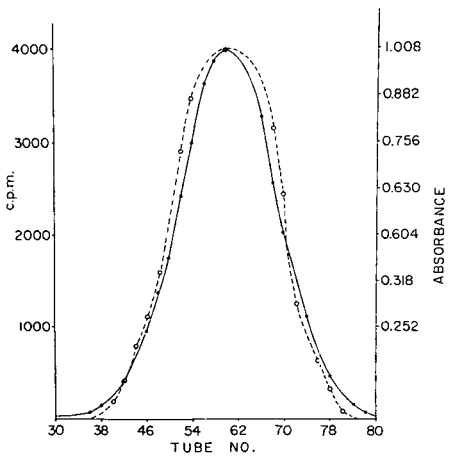


Fig. 2. Plots of log specific activity, log S, against tube number, X, in accordance with $\ln S = [(M_1 - M_2)X/\sigma^2] + [(M_2^2 - M_1^2)/2\sigma^2]$ derived from the ratio of the curves (absorbance, ^{14}C-activity) assuming the normal distribution and that they have the same standard deviation 6, but M_1, the mean of the absorbance curve, differs from M_2, the mean of the ^{14}C activity curve; thus the slope of the line is the index of resolution for the distribution. (A) Solid line: methyl α-D-arabinoside-1-^{14}C after 500 transfers along with the pentose from it on hydrolysis; dotted line, after 590 transfers. (B) Solid line: methyl β-D-arabinoside-1-^{14}C after 600 transfers along with the pentose from it after 800 transfers.

the presence of an isotope effect that is characterized by a decrease in specific activity across the countercurrent zone will appear as a straight line with a negative slope. While the scattering of the points in Fig. 2 does not permit straight lines, lines drawn through the most points in A and B would show a slope for the glycoside that is compatible with the view that the slopes for the two are unlike. Thereby, the

influence of the labeled carbon on the mobility of the pentose during the distribution differs from its effect on the glycoside. With respect to arabinose-1-^{14}C, the observations for the pentose—not the derivative—are similar to those previously reported[3] and add support, since the initial glycoside gave evidence of reasonable purity, to the earlier interpretations of the data, namely, that impurities could not have accounted for the isotope effect during the distribution of this pentose.

Methyl α-mannoside showed no isotope effect during countercurrent distribution (Fig. 3). When mannose from the hydrolyzed glycoside was distributed in the cyclohexane–ethanol system, the data (Fig. 4) from the dissymmetry of the curves suggest a greater heterogeneity than was observed with the glycoside. Indeed, this



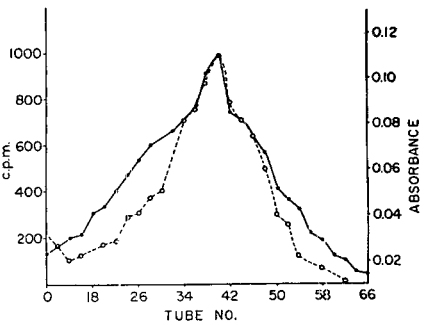


Fig. 3. Five hundred transfer countercurrent distribution of methyl α-D-mannopyranoside-1-^{14}C. The solvent system was an equilibrated mixture of 2 parts of cyclohexane with 1 part 95% ethanol at 22°. The upper phase composition, by volume in percent of water, ethanol and cyclohexane, was 0.8, 14.5 and 84.5 as determined by matching spectra of synthetic mixtures of the components using the Perkin-Elmer infrared spectrophotometer Model 21. Solid line, counts/min; dotted line, absorbance at 370 mμ of the mannosylamine.

Fig. 4. Five hundred transfer countercurrent distribution of mannose-1-^{14}C from methyl α-D-mannopyranoside-1-^{14}C. Solid line, counts/min; dotted line, absorbance. Except that the aliquot for the absorbance was one half that used for Fig. 3, all other conditions were the same.

heterogeneity is reminiscent of that earlier found for arabinose. While the data for mannose-1-^{14}C suggest from the left side of the curve of Fig. 4 that the labeled molecules have a slightly lower K than the unlabeled pentose population, the plot of the right side of the band lends no support to an isotope effect. Commercial mannose-1-^{14}C that had not undergone glycosidation and hydrolysis showed similar apparent heterogeneity when the dissymmetry of the plots of the countercurrent distribution zone was the index.

The data allow the possibility that the anomerization that contributes to molecular heterogeneity can be influenced by the observed isotope effect previously reported for arabinose-1-^{14}C. The most reasonable explanation for this overlooked relationship would be that the isotope contributes to the formation of one anomer more than the other during mutarotation. Such an influence is compatible with the

earlier suggestion[5] that a difference in electronegativity between carbon-12 and carbon-14 could account for such isotope effects. Through this suggested mechanism an inductive effect would be expected to influence the non-bonded interaction between groups of the pentose with the consequence that the stability of one anomer more than the other would be augmented.

SUMMARY

The isotope effect observed during the countercurrent distribution of arabinose-1-^{14}C did not appear with the methyl glycoside of this labeled pentose. This observation along with the difference in the symmetry of plots for methyl α-D-mannoside and D-mannose were considered in the discussion of such isotope effects.

REFERENCES

1 L. M. MARSHALL AND D. MAGEE, *J. Chromatog.*, 15 (1964) 97.
2 D. F. MOWERY, Jr, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. II, Academic Press, New York, 1963, pp. 328–331.
3 L. M. MARSHALL AND R. E. COOK, *J. Am. Chem. Soc.*, 84 (1962) 2647.
4 T. E. TIMELL, C. P. J. GLAUDEMANS AND A. L. CURRIE, *Anal. Chem.*, 28 (1956) 1916.
5 K. A. PIEZ AND H. EAGLE, *Science*, 122 (1955) 969.

THIN-LAYER CHROMATOGRAPHY OF POLYCYCLIC AROMATIC HYDROCARBONS

RICHARD H. WHITE AND JOHN W. HOWARD
Division of Food Chemistry, Bureau of Science, Food and Drug Administration, Washington, D.C. 20204 (U.S.A.)
(Received January 13th, 1967)

INTRODUCTION

The applications of thin-layer chromatography (TLC) to the separation of polycyclic aromatic hydrocarbons have been reviewed by several investigators[1,2]. Various absorbents including silica gel, alumina, cellulose, and cellulose acetate with different types of developing solvents have been evaluated for this purpose. In general, it can be stated that there is no universal or one method which is superior to another. The choice of a particular system or systems will depend upon the hydrocarbons to be separated and the chemical characteristics of the background material from which they are to be isolated. It is often found that where one system fails for a specific group of hydrocarbons, another may prove satisfactory. Consequently, because of the wide variety of polycyclic aromatic hydrocarbons it may be necessary to resort to several different systems to achieve the desired separations.

During the past few years we have been concerned with the development of sensitive, practical analytical methods for the determination of polycyclic aromatic hydrocarbons in foods[3,4]. In general, these procedures consisted of an initial extraction of the hydrocarbons, followed by a partition step between dimethyl sulfoxide and an aliphatic solvent. Column chromatography followed by paper and thin-layer separation is then used to reduce interfering background material and isolate the polycyclic compounds. Depending on the type of product under analysis and the specific hydrocarbons to be isolated, it may be necessary to employ all of these techniques to obtain satisfactory ultraviolet and spectrophotofluorometric spectra for characterization and estimation of the compounds present. If only benzo[*a*]pyrene is to be determined, the procedure may be shortened considerably by using thin-layer chromatography on cellulose acetate directly without the lengthy paper chromatographic method. In our work on the specific polycyclic hydrocarbons in smoked foods[3], we attempted to shorten the method by substituting thin-layer chromatography for paper chromatography. An examination of various thin-layer techniques indicated that good results were obtainable on cellulose plates of 500 μ thickness when 35 % N,N-dimethylformamide (DMF) in ethyl ether was used as the immobile solvent and iso-octane as the mobile solvent. For example, excellent separations and recoveries were attained when mixtures of standard solutions of pyrene, 4-methylpyrene, benz[*a*]anthracene, benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene, and benzo[*g*,*h*,*i*]perylene were applied to the plate. In the actual isolation of the extracted hydrocarbons from food, however, the

bands were close together or overlapping, and problems were encountered in quantitatively recovering the compounds from the plate. It was also noted that the higher condensed ring compounds (4-, 5- and 6-ring types) were localized near the starting line in the presence of small amounts of extraneous background material. In subsequent studies with refined vegetable oils[5], these difficulties were overcome by altering the concentration of DMF to 20 %, thereby achieving good overall separation. This report describes the separation of polycyclic hydrocarbons by this technique of reverse phase thin-layer chromatography.

MATERIALS

Apparatus

Thin-layer chromatography apparatus. (1) Glass plates, 20 × 20 cm (25–10–11); (2) standard adjustable applicator, model S-II (25–09–00); (3) Plexiglass mounting board, standard size for plates up to 20 cm wide (04-10-00); (4) drying rack, with 10 shelves for 20 × 20 plates (25-09-15); (5) standard, rectangular developing tank, 22 cm deep × 8.5 cm wide × 20.5 cm long (25–10–22); (6) stainless steel desiccating storage cabinet, 30 cm wide × 25 cm deep (25-09-40) (Desaga/Brinkmann Instruments, Inc., Westbury, N.Y., or equivalent).

Dipping tank. Type 303, capacity 370 ml, inside dimensions 8 3/8 × 3/16 × 8 3/8″ equipped with cover (3106-H50) (A. H. Thomas Co., Phila., Pa., or equivalent).

Ultraviolet equipment. (1) Lamps: longwave, 3660 Å; shortwave, 2537 Å; and (2) Chromato-Vue Cabinet (Ultraviolet Products, Inc., San Gabriel, Calif., or equivalent).

Evaporation flasks. 250 ml capacity all-glass flasks (K-61725), with 24/40 S/T stopper (K-33175) having inlet and outlet tubes to permit passage of nitrogen across the surface of contained liquid to be evaporated. The inlet tube of the stopper used to convey the nitrogen is cut off 2 cm below the joint, and the outlet tube is constricted at the end and bent downward at a 45° angle to prevent flow-back of the condensate into the flask (Kontes Glass Co., Vineland, N.J., or equivalent).

Pressure filter. 30 ml capacity, fine porosity filter (K-95500) modified to include a 24/40 S/T outer joint and an adapter equipped with a 24/40 S/T inner joint (K-18300) for connection to a tank of nitrogen (Kontes Glass Co., or equivalent).

Recording spectrophotometer and accessories. (1) Cary 11 (Applied Physics Corp., Monrovia, Calif., or equivalent). (2) Cells: (a) fused rectangular quartz cells, optical path length 10 ± 0.005 mm, 1.5 ml capacity (5-503 QS); (b) fused quartz cells, optical path length 50 ± 0.05 mm (2-228 Q), tolerance A (Optical Cell Co., Inc., Brentwood, Md., or equivalent).

Blender and accessories. Waring Blender, Model LB-1 (Waring Products Co., Winsted, Conn., or equivalent).

Reagents

All the reagents purified by distillation were distilled with an air-cooled reflux condenser (about 300 mm long) between the reservoir and the water-cooled condenser. The solvents were distilled in 2 l lots; the first 200 ml distillate was discarded and the next 1600 ml collected for use.

Iso-octane and benzene were purified to meet the specifications of the following test:

To the specified quantity of solvent in a 250 ml evaporation flask, 1 ml of purified *n*-hexadecane was added and the container was placed on the steam bath. The tube assembly was inserted; the inlet tube was connected to the nitrogen supply and the outlet tube to the solvent trap and vacuum line. Evaporation was discontinued when 1 ml of residue remained. (To the benzene residue 10 ml of purified iso-octane was added and the solution was re-evaporated. This procedure was repeated to insure the complete removal of benzene.) The 1 ml of hexadecane residue was dissolved in iso-octane and the volume was adjusted to 25 ml. The absorbance was determined in the 5 cm path length cells compared to iso-octane as reference. The absorbance of the solution of the solvent residue should not exceed 0.01 per cm path length between 280 and 400 mμ.

Iso-octane (2,2,4-trimethylpentane). Purified by distillation or by passage through a column of activated silica gel (Grade 12, Davison Chemical Co., Baltimore, Md., or equivalent) about 90 cm long and 5–8 cm diameter. 180 ml was used for the test described in the previous paragraph.

Benzene. ACS reagent grade. Purified by distillation and 160 ml was used for the test.

Methanol. ACS reagent grade. Purified as follows: 2 l of alcohol was refluxed with 10 g of KOH and 25 g of zinc dust for 3 h. Distillation was carried out with the air-cooled reflux condenser connected to a water-cooled condenser and the collection flask was provided with a drying tube to protect the distilled solvent from moisture.

50 ml of distilled methanol was placed in a 125 ml evaporation flask, 1 ml of *n*-hexadecane added, the tube assembly inserted, and evaporation to a 1 ml residue was carried out as previously described for benzene. 10 ml of purified iso-octane was added, the mixture re-evaporated, and then once again. The 1.0 ml of hexadecane was transferred into the 1 cm path length (total capacity 1.5 ml) and the ultraviolet spectrum recorded with iso-octane in the reference cell. The absorbance values should not exceed 0.03 per cm path length between 250 and 275 mμ, 0.015 between 275 and 300 mμ, 0.010 between 300 and 350 mμ, and 0.00 between 350 and 400 mμ.

n-Hexadecane. 99 % olefin-free. This is purified by percolation through a column of activated silica gel (Grade 12, Davison Chemical Co., or equivalent). 1 ml of *n*-hexadecane is transferred into the 1 cm path length cell (total capacity 1.5 ml) and the ultraviolet spectrum is recorded with iso-octane in the reference cell. The absorbance values should not exceed 0.02 per cm path length between 225 and 250 mμ, 0.010 between 250 and 275 mμ, and 0.00 between 275 and 400 mμ.

N,N-Dimethylformamide (DMF). Redistilled before use. (Matheson Co., Inc., East Rutherford, N. J., or equivalent.)

Ethyl ether. Analytical reagent.

Cellulose. Ultra Pure (MN300-HR, Brinkman Instruments, Inc.).

Polycyclic aromatic hydrocarbons. The compounds were obtained from various sources (see Acknowledgments). Purity of the compounds was checked by thin-layer chromatography before use.

Ethanol. USP grade. Redistilled before use.

Acetylated linters powder. 21 % acetylated (No. 124/21 ac, Schleicher and Schuell Co., Keene, N. H., or equivalent).

Toluene. Redistilled before use.

Developing solvents. (1) Cellulose plates: mobile phase, iso-octane; immobile

phase, 20% DMF in ethyl ether (v/v). (2) Cellulose acetate plates: mobile phase, ethanol–toluene–water (17:4:4, v/v/v).

EXPERIMENTAL

Preparation of plates

The adsorbent is prepared by placing 20 g of cellulose and 100 ml of water in a Waring blender and homogenizing at high speed for 3 min. With a thin-layer applicator, the slurry is applied to the plates (20 × 20 cm) with a thickness of 0.5 mm or 500 μ. (Five plates can be prepared with this amount of slurry.) After coating they are allowed to completely air-dry. Before use, each of the prepared plates is washed in a chromatographic tank by allowing the mobile solvent, iso-octane, to migrate to the top of the plate. The plate is removed from the tank and the excess iso-octane allowed to evaporate. The plates are then stored in a desiccator until needed.

General development procedure

50 ml of the mobile phase, iso-octane, is poured into the development chamber and allowed to equilibrate for at least 30 min. The polycyclic hydrocarbons are prepared in benzene and spotted at levels of 0.2–0.5 μg along the starting line drawn 1.5 cm from the bottom of the pre-washed plates. In addition, 0.5 μg of benzo[*a*]pyrene is also spotted on the plate. Using a glass funnel the dipping tank is then filled to 1/8 in. of the rim with the immobile phase (20% v/v DMF in ethyl ether). The plate is inverted and carefully immersed in the dipping tank to within 0.5 cm of the starting line of the spots. The plate is then removed from the tank and the excess immobile phase allowed to drain for about 15 sec. The plate is inverted and placed (with the starting line facing downward) in the developing chamber containing the mobile phase, iso-octane. The chromatogram is allowed to develop in the dark until the solvent front has reached the top of the plate (about 1.25 h). Then the plate is removed from the chamber and immediately examined under both long and short wave ultraviolet light in the Chromato-Vue Cabinet. With the aid of a labeling template, the position of each fluorescent spot is determined and the R_F value calculated.

Quantitative estimation of hydrocarbons

The fluorescent spots on the adsorbent are outlined in the Chromato-Vue Cabinet. The plate is removed from the cabinet and the adsorbent around the spots is scraped off with a spatula and discarded. Each outlined spot of the adsorbent is then collected in a 125 ml beaker and the polycyclic hydrocarbon is eluted by extracting with 5 to 10 ml portions of hot methanol until fluorescence under ultraviolet light can no longer be seen in the last portion of solvent. (Three or four extractions are ordinarily enough to remove the polycyclic aromatic compounds from the adsorbent.) The flask is swirled repeatedly during the extraction operation and the individual extracts successively filtered through the 30 ml pressure filter under nitrogen pressure into a 50 ml glass-stoppered Erlenmeyer flask. The combined methanol eluate is concentrated to 0.5 ml or less on a steam bath under nitrogen. (Do *not* evaporate to dryness!) The solution is then transferred to a 1 cm cell, the volume adjusted to 1 ml with methanol and the ultraviolet spectra recorded using methanol in the reference cell. Any maxima observed are compared with those in the spectra of known polycyclic aromatic hydro-

carbons. Estimation of the quantities of the identified hydrocarbons is made by the baseline technique in conjunction with spectra of the hydrocarbon standard solutions recorded under the same instrumental conditions. Identification of the compound is also confirmed by applying the technique of spectrophotofluorometry. *Note*: If the spectra indicated the presence of hydrocarbon mixtures or extraneous background absorbance material, the solution is transferred quantitatively with small portions of benzene to a 50 ml glass-stoppered Erlenmeyer flask. The solvent is evaporated on the steam bath under nitrogen to 0.5 ml or less. 5 ml of benzene is added and the solution again concentrated to 0.5 ml or less. The concentrate is reserved for thin-layer chromatography on cellulose acetate plates[4].

RESULTS AND DISCUSSION

The R_F values for 29 polycyclic aromatic hydrocarbons on cellulose (immobile phase, 20% DMF in ethyl ether; mobile phase, iso-octane) and cellulose acetate (ethanol–toluene–water) are reported in Table I. These data indicate that each of the

TABLE I

R_F VALUES[a] OF POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic hydrocarbon	*Cellulose*[b]		*Acetylated cellulose*[c]	
	Range	*Av.*	*Range*	*Av.*
7,12-Dimethylbenz[*a*]anthracene	0.71–0.77	0.74	0.49–0.51	0.50
4-Methylpyrene	0.64–0.72	0.70	0.67–0.72	0.70
5,6-Dimethylchrysene	0.68–0.71	0.69	0.41–0.43	0.42
3-Methylcholanthrene	0.67–0.71	0.69	0.53–0.61	0.57
Anthracene	0.63–0.67	0.65	0.53–0.60	0.57
12-Methylbenz[*a*]anthracene	0.62–0.64	0.63	0.34–0.35	0.34
7,8-Dimethylbenz[*a*]anthracene	0.62–0.64	0.63	0.42–0.42	0.42
Benzo[*c*]phenanthrene	0.59–0.63	0.62	0.52–0.56	0.54
1,2-Dihydrobenz[*e*]aceanthrylene	0.59–0.63	0.62	0.38–0.39	0.39
Cholanthrene	0.59–0.60	0.60	0.49–0.50	0.50
5-Methylchrysene	0.58–0.59	0.59	0.39–0.39	0.39
Pyrene	0.57–0.59	0.58	0.59–0.64	0.62
Fluoranthene	0.56–0.57	0.57	0.58–0.62	0.60
7-Methylbenz[*a*]anthracene	0.56–0.56	0.56	0.44–0.45	0.45
4-Methylbenzo[*a*]pyrene	0.50–0.52	0.51	0.37–0.37	0.37
Benz[*a*]anthracene	0.44–0.47	0.45	0.42–0.42	0.42
Chrysene	0.43–0.44	0.44	0.35–0.36	0.35
Triphenylene	0.43–0.44	0.44	0.48–0.50	0.49
Benzo[*a*]pyrene	0.39–0.41	0.40	0.23–0.23	0.23
Benzo[*k*]fluoranthene	0.39–0.41	0.40	0.39–0.41	0.40
Benzo[*e*]pyrene	0.37–0.40	0.39	0.55–0.59	0.56
Anthanthrene	0.35–0.37	0.36	0.32–0.33	0.32
Perylene	0.32–0.33	0.33	0.45–0.49	0.47
Benzo[*g,h,i*]perylene	0.32–0.33	0.33	0.50–0.52	0.51
Dibenz[*a,h*]anthracene	0.30–0.30	0.30	0.47–0.49	0.48
Dibenzo[*a,i*]pyrene	0.28–0.28	0.28	0.24–0.24	0.24
Dibenzo[*a,i*]phenanthrene	0.27–0.28	0.27	0.55–0.59	0.57
Dibenzo[*a,e*]pyrene	0.25–0.27	0.26	0.38–0.38	0.38
Dibenz[*b,i*]anthracene	0.18–0.19	0.19	0.51–0.56	0.54

[a] Average of five determinations.
[b] Solvent system: immobile phase, 20% DMF in ethyl ether; mobile phase, iso-octane.
[c] Solvent system: ethanol–toluene–water, 17:4:4 (v/v/v).

systems has inherent advantages in the separation of various polycyclic hydrocarbons.

The cellulose reverse phase system is more effective in separating the compounds into groups according to their ring structure. The cellulose acetate multiphase technique is superior in the separation of the individual 4-, 5- and 6-ring compounds. For example, the hydrocarbon pairs, benzo[*a*]pyrene and benzo[*e*]pyrene and benz[*a*]-anthracene and chrysene, difficult to separate on cellulose, were readily separated with the other system. When the two systems are used in conjunction with one another, only four of the 29 compounds studied could not be adequately separated for subsequent quantitative analysis, *viz.*, pyrene and fluoranthene and perylene and benzo-[*g,h,i*]perylene. In our experience, other available methods also fail to give sufficient separation of the first hydrocarbon pair for quantitative estimations. However, both pairs of compounds can be identified and estimated from the ultraviolet absorbance spectrum. As discussed in a previous report[3], either the baseline or the variable reference technique can be utilized advantageously. For the quantitative determination of the polycyclic aromatic hydrocarbons it is estimated that a difference in R_F units of 0.03 is required for the reverse phase system and 0.05 units for the multiphase system.

SAWICKI *et al.*[1], in a study of the application of thin-layer to the analysis of atmospheric pollutants, compared the following adsorbents and developers: alumina with pentane–ether (19:1, v/v); cellulose with DMF–water (1:1, v/v); and cellulose acetate with ethanol–toluene–water (17:4:4, v/v/v).

The procedure using alumina was found to yield poor separations of individual polycyclic hydrocarbons but was more effective than the other systems in isolating the compounds from organic fractions of airborne and air pollution source particulates. Since this adsorbent effectively separates classes of aromatic compounds, it has been recommended as a preliminary separative technique. The best overall separations of polycyclic hydrocarbons were obtained on cellulose plates with aqueous DMF as the mobile phase but this system worked poorly for the so-called "benzpyrene fraction," *e.g.* benzo[*a*]pyrene, benzo[*e*]pyrene, benzo[*k*]fluoranthene, and perylene[1]. Other compound pairs which may not be completely resolved, as evidenced by the closeness of the reported R_B values, include dibenz[*a,h*]anthracene and benzo[*g,h,i*]perylene, chrysene and pyrene, benz[*a*]anthracene and triphenylene, and phenanthrene and anthracene. The aforementioned system and other types where high percentages of water were utilized in the developing solvent have been studied in our laboratory. One of the disadvantages noted was the long developing time, 2–4 h. With the reverse phase system, the chromatogram is developed in about 1.25–1.5 h when the solvent front is allowed to migrate a distance of 18.5 cm. The cellulose acetate system was found by SAWICKI *et al.* to be the most effective in the separation of the "benzpyrene" fraction, whereas most other paper and thin-layer chromatographic procedures fail in these respects. This procedure has also been used after paper chromatography in our studies on the determination of polycyclic hydrocarbons in smoked foods[3] with excellent results.

GUNTHER AND BUZZETTI[2], in their review of thin-layer procedures, report that better resolution of polycyclic compounds is obtained with Silica Gel G than alumina. According to these authors, the efficiency of extraction of the hydrocarbons from both of these adsorbents for quantitative work is often poor. It is also pointed out that the possibilities of accelerated oxidation (air, ultraviolet light) must be considered when sensitive polycyclic aromatic hydrocarbons are adsorbed on active surfaces, such as

alumina. For example, SAWICKI *et al.*[1] reported recoveries of 50–80 % for benzo[*a*]-pyrene after thin-layer chromatography on alumina. With the cellulose and cellulose acetate systems described here, benzo[*a*]pyrene recoveries ranged from 93 to 98 %, thus demonstrating that quantitative recoveries can be obtained in the application and transferral of the compound from one plate to the other. Similar recovery values have been obtained for benz[*a*]anthracene, benzo[*g,h,i*]perylene, and dibenz[*a,h*]-anthracene. In the actual isolation of trace quantities of polycyclic aromatic hydrocarbons from food products, such as refined vegetable oils[5], the extracts are best resolved when applied to the cellulose adsorbent in a narrow streak rather than a spot. With this technique, more definitive separations are afforded and the background material extracted from the oils tends to move more rapidly and completely with the solvent front.

ACKNOWLEDGEMENTS

We should like to express our appreciation to E. O. HAENNI of the Division of Food Chemistry for his interest and helpful suggestions.

The authors are also indebted to the Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, and to the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institute of Health, Bethesda, Md., U.S.A., for supplying some of the polycyclic aromatic hydrocarbons used.

SUMMARY

R_F values have been obtained for thin-layer chromatography of 29 polycyclic aromatic hydrocarbons with the following systems: cellulose (immobile phase, dimethylformamide in ethyl ether; mobile phase, iso-octane) and cellulose acetate (ethanol–toluene–water, 17:4:4, v/v/v). The cellulose reverse phase system more effectively separated the compounds into groups according to their ring structure. The cellulose acetate multi-phase technique was superior in separating the individual 4-, 5- and 6-ring compounds. This technique is rapid and has been successfully used in the preliminary separation of these hydrocarbons from vegetable oils.

REFERENCES

1 E. SAWICKI, T. W. STANLEY, W. C. ELBERT AND J. D. PFAFF, *Anal. Chem.*, 36 (1964) 497.
2 F. A. GUNTHER AND F. BUZZETTI, *Residue Rev.*, 9 (1964) 90.
3 J. W. HOWARD, R. T. TEAGUE, Jr., R. H. WHITE AND B. E. FRY, Jr., *J. Assoc. Offic. Agr. Chemists*, 49 (1966) 595.
4 J. W. HOWARD, R. H. WHITE, B. E. FRY, Jr. AND E. W. TURICCHI, *J. Assoc. Offic. Agr. Chemists*, 49 (1966) 611.
5 J. W. HOWARD, E. W. TURICCHI, R. H. WHITE AND T. FAZIO, *J. Assoc. Offic. Agr. Chemists*, 49 (1966) 1236.

SEPARATION OF CHITIN OLIGOSACCHARIDES BY THIN-LAYER CHROMATOGRAPHY

R. F. POWNING AND H. IRZYKIEWICZ
Division of Entomology, CSIRO, Canberra, A.C.T. (Australia)
(Received December 27th, 1966)

INTRODUCTION

Chromatography of chitin oligosaccharides on paper has already been described[1,2]. Thin-layer chromatography is generally regarded as giving better results than paper chromatography and the following investigations were carried out to determine the best conditions for the thin-layer technique. The separation of acetylglucosamine and oligosaccharides of glucosamine on thin layers was described by TAKEDA *et al.*[3] but the N-acetylated oligosaccharides were not examined.

MATERIALS AND METHODS

Chitin oligosaccharides were prepared substantially according to BARKER *et al.*[1] and further purified by passage through Sephadex G-25.

Layer materials

Kieselgel G, Kieselgel HR, Kieselguhr G were all Merck products. Plates, 20 × 5 cm, were spread at 0.25 mm thickness according to standard procedures with "Shandon" apparatus. After air-drying overnight, about 2 mm of the layer was removed from the edges and then the plates were stored in a sealed cabinet without further activation. Samples were applied in 0.5 μl spots along a line 3 cm from the lower end of the plate. A line was scraped in the layer material 10 cm from the origin and the plates were removed from the developing solvent immediately the solvent reached this line.

Chromatography

The solvents used in this work after preliminary trial runs with microslides were as shown in Table I. Development was carried out at room temperature (24–25°) in glass cylinders 22 cm high by 6.5 cm diameter, fitted with gas-tight glass lids. Solvent (40 ml) was added to the jar and the plate positioned so that the layer was on the inner side of the plate. No attempt was made to saturate the atmosphere in the jars by paper wetted with solvent. The solvent was removed from the plates by heating in an oven at 70° for ½–1 h.

Spray reagents

The chlorination starch–iodide technique as described for paper chromatograms[2] was modified slightly. The plates were humidified over water at 40° for 15 min and then

TABLE I

CHROMATOGRAPHY SOLVENTS

Solvent No.	*Solvent composition**								*Time (h) on*	
	n-Pro	*Isopro*	*tert.-Bu*	*n-Bu*	*Isoam*	*Eth*	*Water*	*Amn*	*Silica Gel HR*	*Silica Gel G*
1	70	—	—	—	—	—	30	—	1.6	2.8
1A	70	—	—	—	—	—	30	1	1.5	2.8
2	—	72	—	—	—	—	27	—	2.7	3.5
2A	—	72	—	—	—	—	27	1	2.8	3.5
3	—	—	70	—	—	—	35	—	3.6	5.8
3A	—	—	70	—	—	—	35	1	3.7	6.7
4	—	—	—	50	—	70	40	—	1.7	2.5
4A	—	—	—	50	—	70	40	1	1.2	2.5
5	—	—	—	—	50	60	30	—	1.6	2.8
5A	—	—	—	—	50	60	30	1	1.8	2.8
6	—	—	50	—	—	70	40	—	2.3	3.4
6A	—	—	50	—	—	70	40	1	2.3	3.3

* The above components were *n*-propanol, isopropanol and *tert.*-butanol (BDH laboratory grade) and *n*-butanol, isoamyl alcohol, ethanol and ammonia (sp.gr. 0.91) (BDH analytical reagent).

chlorinated over a solution of chlorine in carbon tetrachloride for 30 min. These procedures were carried out in covered glass troughs in which the plates were supported face up above the water or the chlorine solution. After aeration for 1 h the plates were sprayed with starch–iodide reagent. With this technique 0.5 μg of oligosaccharide could easily be detected. A similar sensitivity could be obtained by spraying the plate with a solution of 0.2 *N* potassium permanganate in 4 *N* sulphuric acid and warming slightly for about 15 min. The originally pink plate changes to a brown background with white spots. Although the chromatograms can be examined at this stage, a much greater contrast can be obtained by neutralising the acid on the plate over ammonia and then spraying with saturated benzidine hydrochloride (or *o*-tolidine) in 2 % acetic acid. The spots then appear white on a dark blue background.

RESULTS

Preliminary experiments showed that results with thin-layers of microcrystalline cellulose were similar to those found for paper chromatography[2], and that alumina thin layers were inferior to those made with silica gel. On Kieselguhr G with the solvents listed in Table I, no separation was observed between the oligosaccharides, and all migrated close to the solvent front. Modification of solvents by decreasing water content had the effect of leaving an increasing amount of oligosaccharide at the origin with the remainder going to the solvent front. Silica Gel G and HR were selected for further study and the R_F values obtained are listed in Table II. All the results show a linear relationship between R_M value, log $(1/R_F-1)$, and degree of polymerisation of the oligosaccharide. A selection of these relationships is given in Fig. 1. Addition of ammonia to solvents for silica gel plates invariably reduced the R_F values and it also appeared to increase resolution slightly and to reduce tailing. The type of chromatogram obtained can be seen in Fig. 2. When two developments on Silica Gel G were used, excellent resolution of oligosaccharides was obtained (Fig. 2). Due to the re-

tarding effect of ammonia addition, solvent 1 A is thought to be most satisfactory for multiple runs. R_{F_2} values for chitohexaose were quite high, and the data closely approximated the equation, $R_{F_2} = 2R_{F_1} - R_{F_1}^2$, of STARKA AND HAMPL[4].

TABLE II

R_F VALUES FOR CHITIN OLIGOSACCHARIDES ON SILICA GEL

Silica	*Saccharide**	*Solvent*											
		1	*1A*	*2*	*2A*	*3*	*3A*	*4*	*4A*	*5*	*5A*	*6*	*6A*
HR	Nag	0.72	0.48	**	0.54	0.77	0.50	0.76	0.59	0.63	0.46	**	0.67
	biose	0.63	0.37		0.41	0.70	0.38	0.70	0.47	0.51	0.31		0.56
	triose	0.53	0.27		0.31	0.62	0.27	0.63	0.37	0.40	0.20		0.45
	tetraose	0.45	0.19		0.22	0.55	0.19	0.56	0.27	0.31	0.12		0.36
	pentaose	0.37	0.13		0.16	0.47	0.13	0.48	0.21	0.23	0.07		0.27
	hexaose	0.30	0.09		0.11	0.38	0.09	0.41	0.14	0.15	0.05		0.19
G	Nag	0.63	0.45	0.74	0.54	0.68	0.51	0.66	0.53	0.54	0.40	0.73	0.61
	biose	0.52	0.34	0.66	0.42	0.58	0.39	0.57	0.41	0.42	0.27	0.68	0.49
	triose	0.42	0.24	0.58	0.31	0.48	0.29	0.48	0.32	0.30	0.16	0.61	0.38
	tetraose	0.33	0.17	0.50	0.22	0.39	0.21	0.40	0.23	0.22	0.10	0.53	0.28
	pentaose	0.25	0.12	0.42	0.16	0.31	0.15	0.32	0.17	0.14	0.06	0.45	0.21
	hexaose	0.19	0.08	0.33	0.10	0.23	0.11	0.25	0.11	0.09	0.03	0.37	0.14

* Nag→hexaose represents the oligosaccharide series from acetylglucosamine.
** Little differentiation and bad tailing.

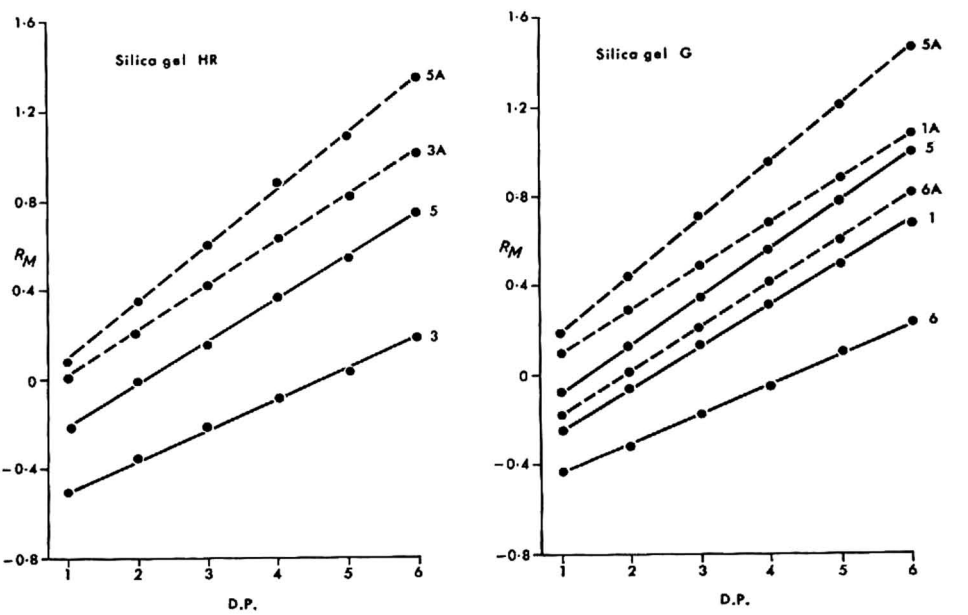


Fig. 1. Relationship between R_M value and degree of polymerisation (D.P.) of chitin oligosaccharides on silica gel thin-layer plates. Numbers on graphs refer to solvents listed in Table I. Broken lines represent solvents with added ammonia.

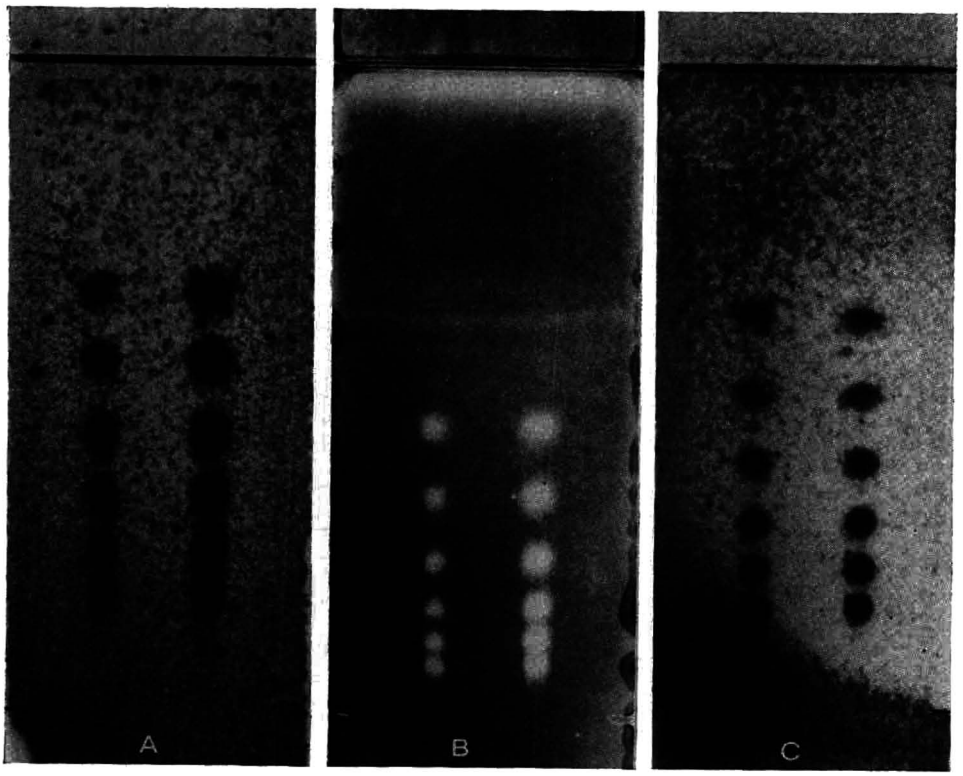


Fig. 2. Chromatograms of chitin oligosaccharides (1 and 2 μg) on Silica Gel G. (A) Single development in solvent 1; chlorine, starch–iodide treatment. (B) Single development in solvent 1A; permanganate, sulphuric acid, benzidine treatment. (C) Double development in solvent 1A; chlorine, starch–iodide treatment.

DISCUSSION

All solvents (Table I) used with Silica Gel G plates permit satisfactory separations of chitin oligosaccharides up to the hexaose. Despite slower development rates, Silica Gel G is the preferred layer material as results with plates made with Silica Gel HR were slightly inferior due to tailing. Solvents containing a lower alcohol such as *n*-propanol travelled faster than those containing higher alcohols, in particular *tert.*-butanol, however, the spots on plates developed in the latter appeared to be more compact. It is probable that the higher viscosity of *tert.*-butanol was responsible for both the longer development time and the lower diffusion of solute.

Since none of the solvents gave any resolution on Kieselguhr G plates, it appears that in this material the chromatographic process is very different from that in silica gel. Kieselguhr is a less active adsorbent than silica gel and is generally recommended for partition chromatography[5]. If the mechanism with chitin oligosaccharides on Kieselguhr G is mainly partition, the failure on this layer is probably due to the rapid decrease of solubility with increased size of the oligosaccharides. Despite the fact that the solvents used with silica gel have high percentages of water, the mechanism in this

layer is at least partly adsorption and this is supported by the effects of ammonia.

Oligosaccharides higher than chitohexaose were not available to test their behaviour in thin-layer chromatography. However, the results of double development on Silica Gel G plates show that the resolution obtained and the movement of the hexasaccharide are satisfactory for resolution at least up to the octasaccharide.

In view of the linear relationship established between size of oligosaccharide and R_M value, and the improved resolution of spots, it may be concluded that thin-layer chromatography is preferable to paper chromatography for many purposes in oligosaccharide analysis.

SUMMARY

A variety of solvents for use in thin-layer chromatography have been examined, and from these can be selected solvents appropriate for single run separation of chitin oligosaccharides at least up to the hexasaccharide, or for multiple run chromatograms with higher oligosaccharides. Best results were obtained on Silica Gel G plates. A linear relationship has been established between R_M value and degree of polymerisation of oligosaccharide for all solvents used. Ammonia addition to solvents decreased R_F values and increased resolution.

REFERENCES

1 S. A. BARKER, A. B. FOSTER, M. STACEY AND J. M. WEBBER, *J. Chem. Soc.*, (1958) 2218.
2 R. F. POWNING AND H. IRZYKIEWICZ, *J. Chromatog.*, 17 (1965) 621.
3 M. TAKEDA, T. TOMIDA, T. OMURA AND H. KATSUURA, *J. Shimonoseki Coll. Fisheries*, 14 (1965) 1.
4 L. STARKA AND R. HAMPL, *J. Chromatog.*, 12 (1963) 347.
5 D. WALDI, in E. STAHL (Editor), *Thin-layer Chromatography*, Academic Press, New York, 1965, p. 33.

CHARGE-TRANSFER COMPLEXES OF 2,4,6-TRINITROTOLUENE AND *m*-DINITROBENZENE WITH SOME AMINES

A. K. DWIVEDY, D. B. PARIHAR, S. P. SHARMA AND K. K. VERMA
Defence Science Laboratory, Metcalfe House, Delhi-6 (India)
(Received November 1st, 1966)

The field of charge-transfer complexes has been investigated recently by several chromatographic techniques. Unsaturated lipids have been resolved on columns of silica gel impregnated with silver nitrate[1]. Gas chromatography with stationary phases containing silver nitrate has been widely applied in the separation of olefins and other unsaturated compounds[2–6]. Benzene and some alkylbenzenes have been separated by GC on E-301 containing 2,4-dinitro-chlorobenzene[7]. In addition, the study of the complexes between olefins and 1,3,5-trinitrobenzene by GC is also reported[8]. Unsaturated fatty acid methyl esters and triglycerides have been resolved as their silver ion π-complexes by reversed phase paper partition chromatography[9]. Thin-layer chromatography has been employed in the investigations of the complexes of silver with terpenes[10–13], glycerides[14] and allylic-propenylic isomers[15]. The separation of the π-complexes of *m*-dinitrobenzene; 2,4-dinitrotoluene; 2,4,6-trinitrotoluene; 2,4,6-trinitroanisole and tetryl with some hydrocarbons on TLC has been carried out[16].

Aromatic amines, being π-donors, form complexes with hybrid structures involving a dative and no bond[17]. The formation of charge-transfer complexes by nitro compounds with aromatic donors is attributed to the polarization of the nitro group. On the basis of the study of the complexes of *o*-, *m*,- *p*-aminobenzoic acids and their sodium salts with *m*-dinitrobenzene (*m*-DNB) it has been proposed that only one nitro group participates in the formation of a 1:1 complex[18].

$$O_2N \cdot C_6H_4 \cdot N^+(O)(O^-) \; — \; NH_2 \cdot C_6H_4 \cdot COOH$$

The very weak physical bonding between amines and nitro aromatic compounds is readily ruptured by solvents, adsorption forces, temperature, etc. Probably in view of these difficulties it has not been possible to study such complexes by TLC in the past.

The present paper describes a study of the π-complexes of a number of aromatic amines with 2,4,6-trinitrotoluene (*s*-TNT) and *m*-dinitrobenzene (*m*-DNB) employing a thin-layer chromatographic procedure. It has been possible to clearly resolve the charge transfer complexes formed from 1–2 μg of the individual amines.

EXPERIMENTAL

The different solvents employed were dried and freshly distilled. Kodak photographic plates (22 × 22 cm) were used as supports and the ascending irrigation

technique adopted for resolution of the compounds. The distances travelled by solvent fronts in the cases of *s*-TNT and *m*-DNB complexes were 18.5 and 17.0 cm, respectively. The temperature of irrigation was 18 ± 1°. The highly colored complexes could be easily located.

Adsorbents

(1) Kieselgel G (E. Merck).

(2) Cellulose powder, ashless (Whatman, 100 mesh, B.S.S.) containing 1% calcium sulphate.

(3) Cellulose acetate (acetyl content 28.6%, 100 mesh, B.S.S.) containing 1% calcium sulphate.

(4) Kieselguhr G (E. Merck).

Compounds

The complexes were prepared by taking one mole each of *s*-TNT or *m*-DNB and amine in a suitable solvent (10 mole), warming the mixture till the solution was clear and allowing it to cool in a refrigerator. The complexes were further crystallized from the same solvent till there was no rise in melting point. For *s*-TNT complexes ethanol was used and for *m*-DNB complexes benzene.

Preparation, spotting and irrigation of plates

The plates were coated by taking a freshly prepared slurry of the adsorbent in an appropriate solvent, pouring it on the plates and tilting them from side to side. The plates were left overnight at room temperature and activated at 110° for one hour (except cellulose acetate plates, which were activated at 60° in a vacuum oven for 3 h). These were impregnated with *s*-TNT/*m*-DNB by irrigating with a 3% solution of these compounds in acetone. The amounts of the adsorbents, solvents etc., are given in Table I.

TABLE I

DETAILS OF THIN-LAYER COATINGS OF VARIOUS ADSORBENTS

No.	*Coating*	*Wt. of the adsorbent (g)*	*Solvent*	*Average coating of adsorbent (mg/cm²)*
1	Kieselgel G	26	Chloroform (50 cc) –methanol (20 cc)	8.2
2	Cellulose–$CaSO_4$	26.25	Water (90 cc)	7.1
3	Cellulose acetate–$CaSO_4$	26.25	Water (90 cc)	7.3
4	Kieselguhr G	32	Ethanol (65 cc)	6.8

Ethanolic solutions of different amines (10 μl) containing 1–2 μg were spotted with a standard microcapillary on the plates. This resulted in deep-colored spots of the complexes. Spots of crystalline complexes as well as those formed in solution were also placed adjacent to the above spots. The plates were left in a desiccator for one hour, after which they were irrigated with different solvent systems. Table II gives the melting points of the complexes, solvent systems, and R_F values.

TABLE II

R_P VALUES OF VARIOUS CHARGE-TRANSFER COMPLEXES

S. No.	Complexes of s-TNT/ m-DNB with the following amines	s-TNT complexes		Adsorbents: Kieselgel G impregnated with 3% s-TNT		
		M.p. observed	M.p. reported	Solvents:	Toluene–ethylene dichloride* (95:5)	Toluene–monochloro-benzene* (3:2)	Benzene–petroleum ether* (1:1)
1	N-Methylaniline	—	—		0.82	0.67	0.68
2	N,N-Dimethylaniline	45–46	—		0.86	0.76	0.90
3	Aniline	84–85	83–84[19]		0.57	0.50	0.31
4	α-Naphthylamine	144	141.5[19]		0.63	0.52	0.26
5	β-Naphthylamine	144	113.5[19]		0.58	0.42	0.21
6	o-Anisidine	82	—		0.54	0.43	0.34
7	p-Anisidine	50–51	—		0.32	0.22	0.14
8	m-Chloroaniline	71–72	—		0.68	0.61	0.58
9	p-Chloroaniline	59	—		0.60	0.51	0.44
10	Diphenylamine	34–35	31[19]		0.88	0.78	0.91
11	o-Toluidine	61–62	53–55[19]		0.61	0.56	0.49
12	m-Toluidine	67–68	62–63[19]		0.55	0.45	0.41
13	p-Toluidine	70–71	63–64[20]		0.49	0.41	0.35
14	m-Phenylenediamine	78–79	—		0.09	0.05	0.00

* Solvent contained 3% s-TNT/m-DNB.
** Solvent saturated with m-DNB.

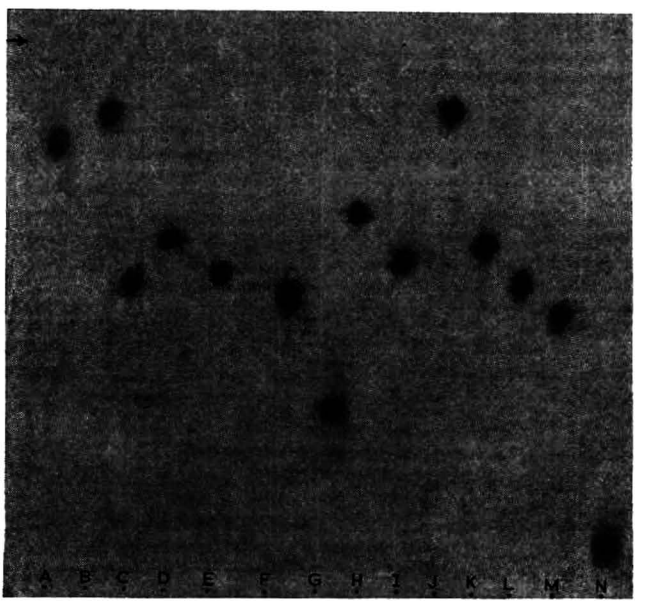


Fig. 1. Chromatoplate showing the separation of charge-transfer complexes of 2,4,6-trinitrotoluene with the following amines: A = N-Methylaniline; B = N,N-dimethylaniline; C = aniline; D = α-naphthylamine; E = β-naphthylamine; F = o-anisidine; G = p-anisidine; H = m-chloroaniline; I = p-chloroaniline; J = diphenylamine; K = o-toluidine; L = m-toluidine; M = p-toluidine; N = m-phenylenediamine. Adsorbent: Kieselgel G impregnated with 3% s-TNT; solvent: toluene–ethylene dichloride (95:5) containing 3% s-TNT; system: ascending.

	m-DNB complexes						
	M.p. observed	*M.p. reported*	*Adsorbents: Kieselgel G impregnated with 3% m-DNB*				
*Xylene–ethylene dichloride** (4:1)			*Solvents:*	*Toluene–monochloro-benzene** (3:2)	*Petroleum ether–ethyl acetate*** (9:1)	*Petroleum ether–ethylene dichloride** (1:1)	*Petroleum ether–ethyl acetate*** (95:5)
0.61	—	—		0.72	0.90	0.83	0.60
0.70	—	—		0.82	1.00	0.91	0.97
0.41	40–41	41.5[21], 44.2[22]		0.48	0.80	0.59	0.27
0.46	67	—		0.50	0.73	0.64	0.21
0.43	58	63.8[21]		0.43	0.62	0.57	0.17
0.44	—	—		0.40	0.82	0.59	0.30
0.26	—	—		0.22	0.43	0.31	0.08
0.56	—	—		0.61	0.87	0.70	0.38
0.43	—	—		0.46	0.72	0.62	0.23
0.79	—	—		0.84	1.00	0.93	0.83
0.45	—	—		0.47	0.87	0.65	0.41
0.36	—	—		0.40	0.81	0.61	0.33
0.28	—	—		0.37	0.75	0.55	0.28
0.06	—	—		0.07	0.07	0.06	0.00

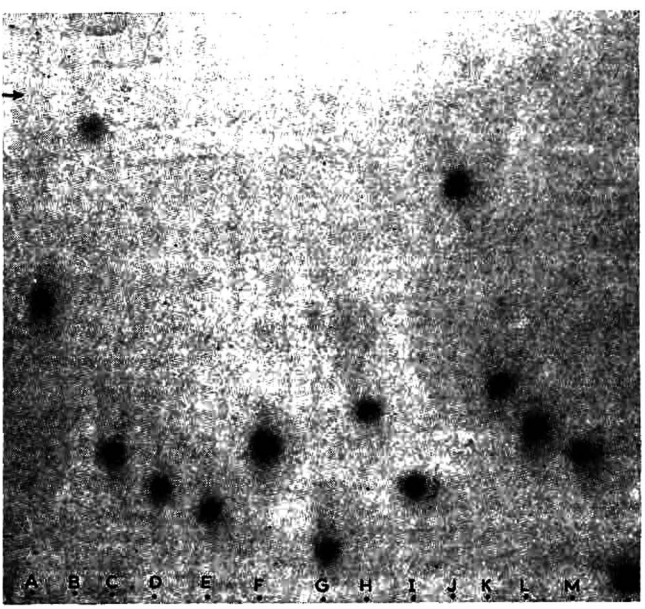


Fig. 2. Chromatoplate depicting the resolution of charge-transfer complexes of *m*-dinitrobenzene with amines (A—N). Adsorbent: Kieselgel G with 3% *m*-DNB; solvent: petroleum ether–ethyl acetate (95:5) saturated with *m*-DNB; system: ascending.

Specimen chromatoplates depicting the resolutions of charge-transfer complexes of 2,4,6-trinitrotoluene and *m*-dinitrobenzene with amines are represented in Figs. 1 and 2, respectively.

DISCUSSION AND RESULTS

The crystalline complexes of *s*-TNT and *m*-DNB with amines when run on Kieselgel G, cellulose–$CaSO_4$, and cellulose acetate–$CaSO_4$ plates (untreated with *s*-TNT/*m*-DNB) broke down completely into nitroaromatic compounds and amines in all possible polar and non-polar solvents tried. The adsorption forces proved to be so strong that irrigation even at —10° was a failure. Only in the case of Kieselguhr G did the complexes not break and travel up to the solvent front when irrigated with solvents like dioxan, benzene, toluene, xylene, chloroform–xylene (80:20).

Kieselgel G impregnated with 3 % *s*-TNT or *m*-DNB proved to be the best adsorbent for the sharp separation of the complexes. In order to further check the breakdown of the complexes a 3 % addition of *s*-TNT/*m*-DNB to the irrigating solvent was found necessary. It was observed that the general patterns of migrations of both *s*-TNT and *m*-DNB complexes were the same. The mobilities of the complexes were almost in the order of the basicities of their amines. Aniline being a stronger base compared to N-methylaniline and N,N-dimethylaniline, its complexes were strongly adsorbed on the plates resulting in lower R_F values. The complexes of *p*-anisidine migrate less than those of *p*-chloroaniline. *p*-Toluidine being more basic than aniline, its complexes have lower R_F values than that of the latter. The low R_F values of *m*-phenylenediamine complexes are due to the strong basicity of the π-donor. Further, the R_F values of the complexes of *p*-substituted amines were lower than those of *meta* and *ortho* substituted amines, *e.g.* *p*-anisidine complex < *o*-anisidine complex; *p*-toluidine complex < *m*-toluidine complex < *o*-toluidine complex; *p*-chloroaniline complex < *m*-chloroaniline complex.

Attempts to prepare and run the complexes of 2,4-dinitrodiphenylamine with *s*-TNT and *m*-DNB failed. This could be due to the presence of electron attracting groups in the donor molecule, which renders the charge-transfer linkage highly unstable.

It was noted that the colors of *s*-TNT and *m*-DNB complexes with N-methyl- and N,N-dimethylaniline disappeared within half an hour on drying the plates after irrigation. This shows that their complexes are weaker by comparison with aniline which could be due to partial neutralization of the basic charge on the amino group by methyl groups.

ACKNOWLEDGEMENTS

Thanks are due to Dr. H. K. ACHARYA, Assistant Director (Chemistry) and Dr. KARTAR SINGH, Director, Defence Science Laboratory, Delhi, for encouragement.

SUMMARY

Charge-transfer complexes of 2,4,6-trinitrotoluene and *m*-dinitrobenzene with aromatic amines have been studied by employing a thin-layer chromatographic

technique. Excellent resolution of the complexes was achieved on Kieselgel G plates impregnated with the acceptor molecule and employing nonpolar solvents. It was possible to characterize distinctly the complexes formed from 1–2 μg of individual amines.

REFERENCES

1 B. DE VRIES, *Chem. Ind. (London)*, (1962) 1049.
2 J. SHABTAI, *J. Chromatog.*, 18 (1965) 302.
3 J. SHABTAI, *Israel J. Chem.*, 1 (1963) 300.
4 J. SHABTAI, J. HERLING AND E. GIL-AV, *J. Chromatog*, 11 (1963) 32.
5 R. J. CVETANOVIC, F. J. DUNCAN AND W. E. FALCONER, *Can. J. Chem.*, 41 (1963) 2095.
6 E. K. C. LEE AND F. S. ROWLAND, *Anal. Chem.*, 36 (1964) 2181.
7 K. MALINOWSKA, *Chem. Anal. (Warsaw)*, 9 (1964) 353.
8 R. J. CVETANOVIC, F. J. DUNCAN AND W. E. FALCONER, *Can. J. Chem.*, 42 (1964) 2410.
9 A. G. VERESHCHAGIN, *J. Chromatog.*, 17 (1965) 382.
10 R. IKAN, *J. Chromatog*, 17 (1965) 591.
11 M. V. SCHANTZ, S. JUVONEN AND R. HEMMING, *J. Chromatog.*, 20 (1965) 618.
12 V. A. PASESHNICHENKO AND A. R. GUSEVA, *Prikl. Biokhim. Mikrobiol.*, 1 (1965) 559.
13 E. STAHL AND H. VOLLMANN, *Talanta*, 12 (1965) 525.
14 C. B. BARRETT, M. S. J. DALLAS AND F. B. PADLEY, *Chem. Ind. (London)*, (1962) 1050.
15 G. M. NANO AND A. MARTELLI, *J. Chromatog.*, 21 (1966) 349.
16 M. FRANĆK-NEUMANN AND P. JÖSSANG, *J. Chromatog.*, 14 (1964) 280.
17 R. E. MILLER AND W. F. K. WYNNE-JONES, *J. Chem. Soc.*, (1959) 2375.
18 B. V. TRONOV AND N. V. SYRNEVA, *Izvest. Vysshikh Ucheb. Zavedenii, Khim. i Khim. Tecknol.*, 3 No. 4 (1960) 752.
19 T. URBANSKI, *Chem. Technol. Explosives, (Warszawa)*, 1 (1964) 311.
20 I. ISENBERG AND S. L. BAIRD, *J. Am. Chem. Soc.*, 84 (1962) 3803.
21 T. URBANSKI, *Chem. Technol. Explosives (Warszawa)*, 1 (1964) 239.
22 P. V. ROMBURGH, *Chem. Zentr.*, II (1911) 444.

THIN-LAYER CHROMATOGRAPHY OF NUCLEIC ACID BASES, NUCLEOSIDES, NUCLEOTIDES AND RELATED COMPOUNDS

III. SEPARATION OF COMPLEX MIXTURES ON CELLULOSE LAYERS*

G. PATAKI
Analytical Department, Laboratory for Chromatography, Robapharm Ltd., Basle (Switzerland)
(Received December 19th, 1966)

Thin-layer chromatography (TLC) has been initially developed for the analysis of lipophilic substances[1,2]. This technique is now also, however, a standard method in the separation of hydrophilic substances, *e.g.* amino acids and derivatives[3] or constituents of nucleic acids[4]. The separation of nucleotides has been investigated by RANDERATH *et al.*[5–18] in great detail. These authors have used TLC on ion-exchange cellulose for the separation of complex mixtures of nucleotides. Other investigators have also published a number of papers on TLC of nucleo-derivatives (for review *cf.* ref. 4). In connection with our work concerning the isolation, detection and structure elucidation of substances in organ extracts we wanted a chromatographic technique which allows the separation and characterization of a very great number of nucleo-derivatives. Earlier experiments in our laboratory have shown that two-dimensional paper chromatography is very useful for the separation of nucleo-derivatives in organ extracts. It was felt that this technique may also be applicable, when cellulose thin layers are used instead of paper as carrier. The TLC method, reported in this communication, allows the characterization of more than sixty nucleo-derivatives and related compounds on cellulose layers, and is especially suitable for the detection of nucleo-derivatives in biological material. Moreover this technique allows the detection of nucleic acid bases, nucleosides and nucleotides in the same chromatogram**.

EXPERIMENTAL

Purification of the cellulose powder

60 g MN-300 cellulose powder (Macherey, Nagel & Co., Düren, Germany) are suspended in 500 ml *n*-propanol–25 % ammonia–water (6:3:1, v/v) and mixed by vigorous shaking (30 min). After filtration, the powder is suspended in 400 ml *n*-propanol, shaken vigorously for 15–30 min and filtered again. The solid residue is purified by washing with *n*-propanol and is again filtered. The powder is homogenized, vacuum-dried at 60° until all the ammonia is removed, and dried again 2 × 12 h (60°, high vacuum).

---

* For Part II of this series, see ref. 19.
** For preliminary report, *cf.* ref. 19.

Preparation of the plates

15 g purified cellulose powder are suspended in 90 ml water and homogenized 30 sec with an electric mixer. The slurry is spread, after removing air bubbles (about 5 min), over 5 glass plates (20 × 20 cm) with 0.5 mm slot width, using STAHL's apparatus (Desaga, Heidelberg, Germany), followed by drying overnight at ambient temperature.

Application of the solutes

Standard solutions as well as organ extracts are applied in portions of 5 μl 3 cm from the edges of the plate and each portion is dried in a stream of cold air. For studying the chromatographic behaviour, the chromatograms are loaded with 1–5 μg of pure compound dissolved in 5 μl solvent (we use generally 0.04 % NaOH for nucleobases and nucleosides or 10 % isopropanol for nucleotides).

Separation procedure and location of spots

For bidimensional chromatography* (descending technique) the following solvent systems were used:

1st direction: *n*-propanol–25 % ammonia–water (6:3:1, v/v)

2nd direction: isopropanol–saturated ammonium sulphate–water (2:79:19, v/v).

Shandon multiplate-chromatotanks (Shandon, London) were filled with 500 ml of the first solvent system and 6 plates (layer always against layer) were immediately introduced. The solvent system for the first dimension migrated about 18 cm after 3 h. The chromatograms are then dried 20 min in a stream of cold air, turned through 90° and put into the second Shandon multiplate-chromatotank, which is at the same time filled with 500 ml of the second solvent system.

In this solvent the front again migrates about 18 cm within 3 h. The chromatograms are taken out, dried 20 min in a stream of cold air and put under U.V. light* at 254 and 360 nm (U.V. lamp supplied by Camag, Muttenz, Switzerland). The absorption spots are outlined and hatched with a pencil, the fluorescent spots are surrounded by an interrupted line. Much better for documentation is, of course, direct photography under U.V. light. The layer is illuminated with two U.V.-lamps (Camag, Muttenz, Switzerland) at 254 nm. Distance between sheet and lamps: 7 cm. The film (Agfa-Isopan IFF 13 DIN) is exposed for 4 min. Distance between camera (Leica M3, aperture 5.6 with filter No. 302/5—Omag/Switzerland) and back of the glass plate: 50 cm (using Leitz diapositiv repro DIN A4 for M cameras with Elmar-objectiv 50 mm). *All photographs were taken from the back.* Development of photographs was carried out with Neofin-blau (Tetenal, Hamburg/Germany) for 8 min at 20° using Agfa BW-1. The fluorescence spots remain, of course, invisible or almost invisible.

RESULTS AND DISCUSSION

Various techniques have been described for TLC of nucleo-derivatives[4]. The separation of complex mixtures, *e.g.* nucleotides, can be carried out on ion-exchange cellulose layers[9,13–18]. However, as far as we know, there are no methods in the literature on the separation of complex mixtures containing nucleic acid bases, nucleosides, nucleotides and related compounds. It was felt that a technique, which allows the

* The chromatograms should be viewed not only on the surface, but also from the back.

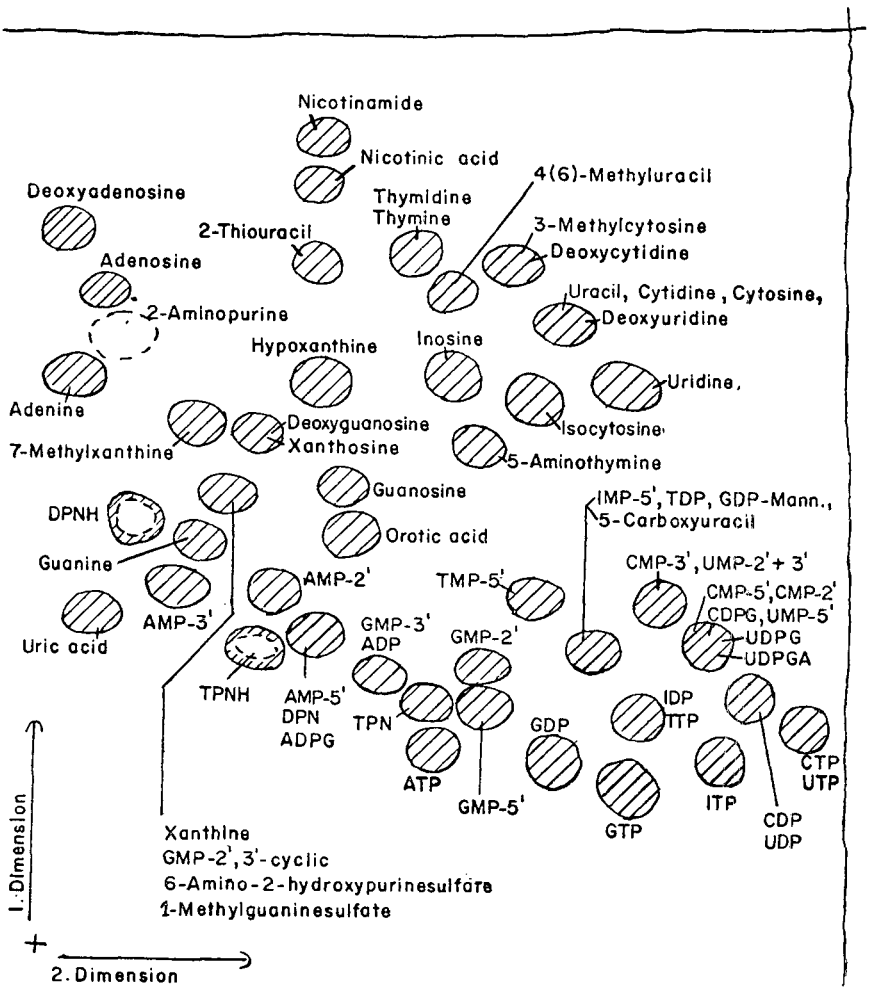


Fig. 1. Separation of nucleo-derivatives on purified cellulose layers. 1st dimension: *n*-propanol–25% ammonia–water (6:3:1, v/v); 2nd dimension: isopropanol–saturated ammonium sulphate–water (2:79:19, v/v). Absorption spots are hatched, fluorescence spots are surrounded by an interrupted line. For remaining conditions *cf.* experimental part. Note: DPNH and TPNH show fluorescence, which changes after several hours drying to absorption. R_F value of CTP in the second dimension: 0.83. (schematical)

separation of the compounds considered above, will be very useful for characterization of nucleo-derivatives in biological material as well as for the detection of nucleo-derivatives isolated from organ extracts by column chromatography. Since in our laboratory paper chromatography is a well established technique for the separation of nucleo-derivatives, we began to use our solvent systems for TLC on cellulose layers. Nucleic acid bases, nucleosides, nucleotides and related compounds can be separated on cellulose layers using the solvent systems given in the experimental part. It is a requisite that chromatography is carried out *without* chamber saturation (*cf.* Experimental). The ammonium sulphate solution, which is used for development in

the second direction, must be *completely saturated*, otherwise the separation in the second dimension will be altered. *These two conditions must be strictly fulfilled.* When MN-300-cellulose is used for the chromatography of nucleoside mono-, di- and triphosphates good separations are obtained. However, the resolution of nucleic acid bases and nucleosides on such layers is less effective and the spots tail. In order to

TABLE I

SEPARATION OF DIFFERENT GROUPS IN CHROMATOGRAMS ACCORDING TO FIG. I

Group	*Spots*
Adenine	Adenine, adenosine, deoxyadenosine, AMP-3′, AMP-2′, AMP-5′+ADPG, ADP, ATP
Guanine	Guanine, guanosine, deoxyguanosine, GMP-3′, GMP-2′, GMP-5′, GDP, GTP, GDP-Mann
Hypoxanthine	Hypoxanthine, inosine, IMP-5′, IDP, ITP
Cytosine	Cytidine + cytosine, deoxycytidine, CMP-3′, CMP-2′ + CMP-5′, CDP, CTP, CDPG
Uracil	Uracil + deoxyuridine, uridine, UMP-2′ + 3′, UMP-5′, UDP, UTP, UDPG + UDPGA
Thymine	Thymine + thymidine, TMP-5′, TDP, TTP

overcome the difficulties caused by the cellulose, a purification procedure has been developed which rectifies this shortcoming*. The chromatographic behaviour of more than sixty nucleo-derivatives is shown in Fig. 1**.

The resolving power of our chromatographic system can be judged *e.g.* by the quality of the separation of substances which belong to the same base (see Table I). *Nucleic acid bases* and the *corresponding ribosides* are, apart from cytidine and cytosine, well separated. It is remarkable that the *ribosides* and *deoxyribosides* under investigation show different chromatographic behaviour. On the other hand, uracil and deoxyuridine as well as thymine and thymidine, that means the *base* and its corresponding *deoxyriboside*, travel together. Nucleoside mono-, di- and triphosphates are separated in every group. It should be mentioned that di- and triphosphates show tailing in the first dimension. Finally, the pyridine nucleotides are also resolved in this chromatographic system. As we can see in Fig. 1, some spots contain more than one substance. For the differentiation of compounds which are not resolved in chromato-

* For quantitative estimation of nucleo-derivatives by direct fluorometry, the use of purified cellulose is indispensable[19–21].

** Abbreviations: adenosine-5′-monophosphate=AMP-5′; adenosine-2′-monophosphate=AMP-2′; adenosine-3′-monophosphate=AMP-3′; adenosine diphosphate=ADP; adenosine triphosphate=ATP; adenosine diphosphate glucose=ADPG; guanosine-5′-monophosphate=GMP-5′; guanosine-2′-monophosphate=GMP-2′; guanosine-3′-monophosphate=GMP-3′; guanosine-2′,3′-cyclic-phosphate=GMP-2′,3′-cyclic; guanosine diphosphate=GDP; guanosine triphosphate=GTP; guanosine diphosphate mannose=GDP-Mann; inosine-5′-monophosphate=IMP-5′; inosine diphosphate=IDP; inosine triphosphate=ITP; cytidine-5′-monophosphate=CMP-5′; cytidine-2′-monophosphate=CMP-2′; cytidine-3′-monophosphate=CMP-3′; cytidine diphosphate=CDP; cytidine triphosphate=CTP; cytidine diphosphate glucose=CDPG; uridine-5′-monophosphate=UMP-5′; uridine-2′+3′-monophosphate=UMP-2′+3′; uridine diphosphate=UDP; uridine triphosphate=UTP; uridine diphosphate glucose=UDPG; uridine diphosphate glucuronic acid=UDPGA; thymidine-5′-monophosphate=TMP-5′; thymidine diphosphate=TDP; thymidine triphosphate=TTP; diphosphopyridine nucleotide=DPN; diphosphopyridine nucleotide red.=DPNH; triphosphopyridine nucleotide=TPN; triphosphopyridine nucleotide red.=TPNH.

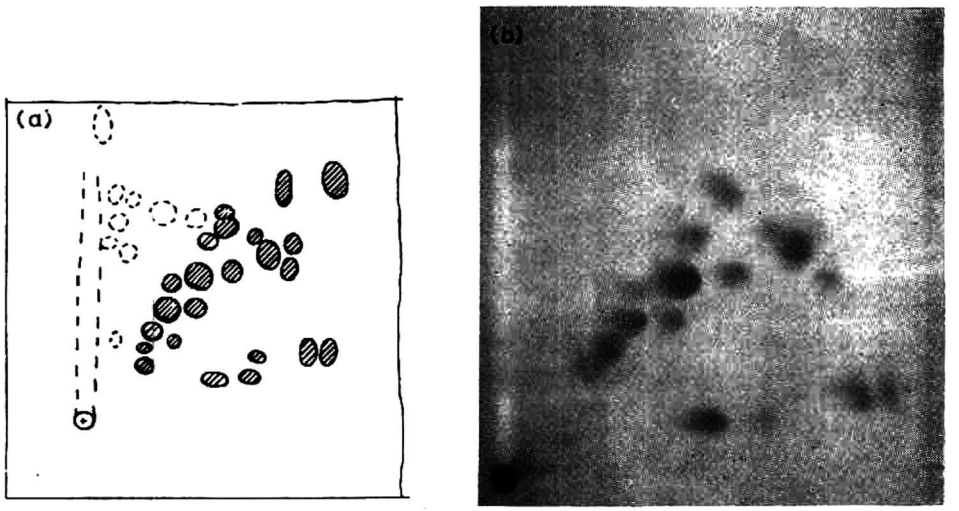


Fig. 2. (a) Detection of nucleo-derivatives in Robuden UD. Load: 5 μl 10:1 concentrated solution. For remaining conditions *cf.* Fig. 1 and the experimental part. (b) U.V. photography of chromatogram corresponding to Fig. 2a (*cf.* experimental part). Note: Some of the minor spots are not visible on the reproduction.

grams according to Fig. 1, additional chromatographic systems are necessary. The combined use of the technique described in this paper together with chromatography on poly(ethyleneimine) layers[9,13–18,21] is the method of choice for the identification of nucleotides and will now be investigated in our laboratory. Chromatographic systems for separation of the other groups not resolved on chromatograms according to Fig. 1 are mostly known from the literature (*cf.* ref. 4). Finally, it should be pointed out that the direct rechromatography of spots as suggested by RANDERATH[18] allows rapid and easy identification; however, chromatography must be performed on sheets

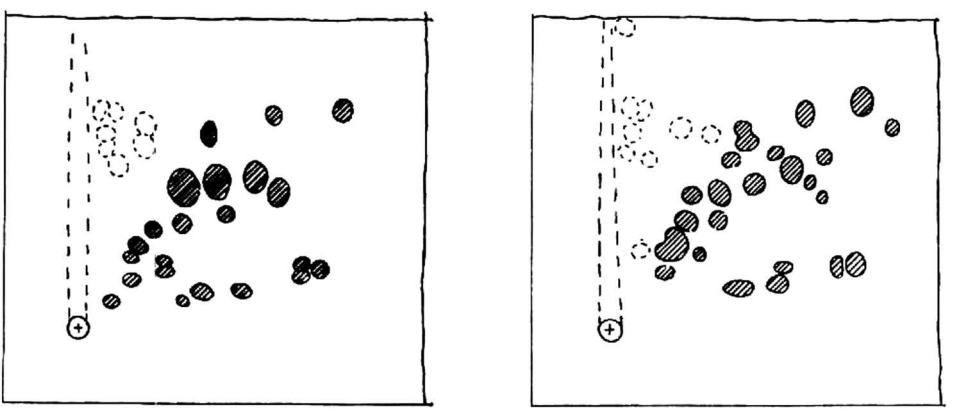


Fig. 3. Detection of nucleo-derivatives in Robuden UV. Load: 5 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

Fig. 4. Detection of nucleo-derivatives in Raveron. Load: 5 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

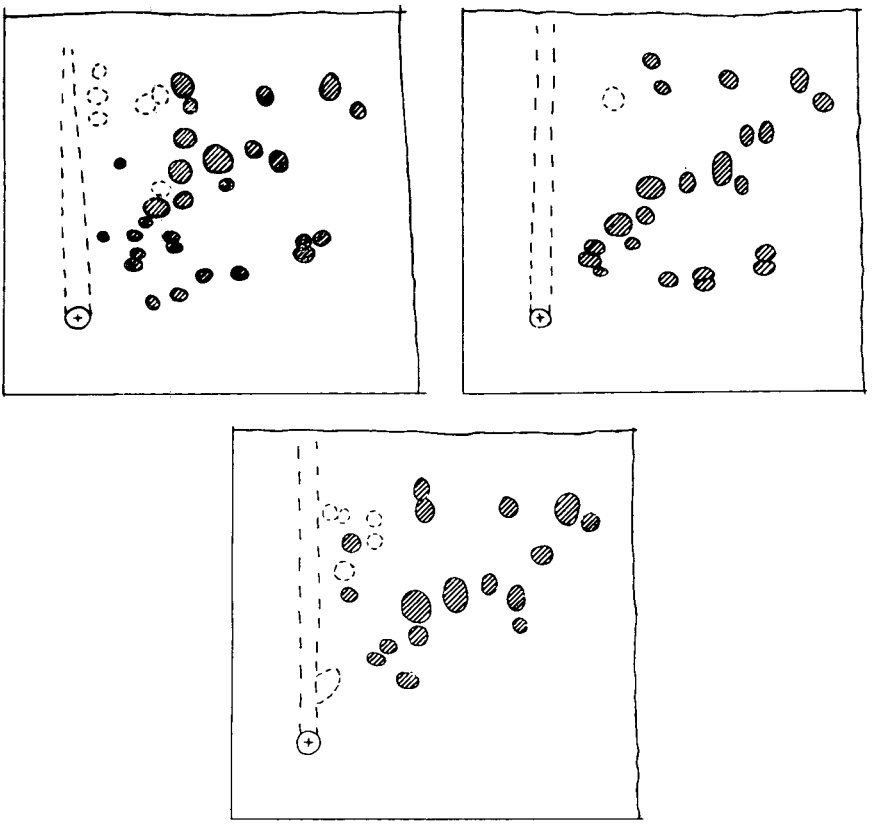


Fig. 5. Detection of nucleo-derivatives in Ripason. Load: 5 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

Fig. 6. Detection of nucleo-derivatives in Rumalon. Load: 15 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

Fig. 7. Detection of nucleo-derivatives in Recosen. Load: 5 μl 10:1 concentrated solution. For remaining conditions, *cf.* Fig. 1 and the experimental part.

instead of glass plates. Preliminary investigations have shown that cellulose thin layer sheets* can be used for the separation of nucleo-derivatives. Such sheets give chromatograms in which the separation is the same as on unpurified cellulose layers[19]. The same is true for precoated cellulose plates, which are now available**.

As we have already mentioned in the introduction to this paper, experiments have been made to apply our technique to the detection of nucleo-derivatives in biological material. Figs. 2–7 are schematic chromatograms and show the detection of nucleo-derivatives in different organ extracts***.

Fig. 2b is an original photograph (*cf.* Methods) corresponding to Fig. 2a.

* Macherey, Nagel & Co., Düren, Germany.
** Camag, Muttenz, Switzerland.
*** Recosen, Raveron, Robuden UD, Robuden UV, Ripason and Rumalon. Products of Robapharm Ltd., Basle, Switzerland.

Chromatograms from organ extracts, as shown in Figs. 2–7, were made *without* any pretreatment. The extracts were applied to the layer in portions of 5 μl (intermediate drying with a stream of cold air between each application). The quality of separation depends on the diameter of the starting spot. When the starting spot is too large the separation is less effective. On the other hand, if the spot applied to the chromatogram is too small, we again obtain poor resolution. In our experience the best chromatograms result when we use the procedure given above for spotting the organ extracts. However, the technique of application may depend on the nature of the biological material. Identification can be made by co-chromatography with known substances and by rechromatography in other chromatographic systems as discussed above. In this connection infrared spectroscopy on a microgram scale should be mentioned (*cf. e.g.* ref. 22).

ACKNOWLEDGEMENTS

The author wishes to thank Mr. A. KUNZ and Mr. M. KUNZ for technical assistance.

SUMMARY

A method is described for the characterization of more than sixty nucleo-derivatives on purified cellulose layers using *n*-propanol–25 % ammonia–water (6:3:1, v/v) in the first and isopropanol–saturated ammonium sulphate–water (2:79:19, v/v) in the second dimension. The technique is suitable for detection of nucleo-derivatives in biological material without any pretreatment.

REFERENCES

1 E. STAHL (Editor), *Thin-Layer Chromatography*, Springer, Berlin and Academic Press, New York, 1965.
2 K. RANDERATH, *Thin-Layer Chromatography*, Verlag Chemie, Weinheim and Academic Press, New York, 1966.
3 G. PATAKI, *Dünnschichtchromatographie in der Aminosäure- und Peptidchemie*, Walter de Gruyter, Berlin, 1966.
4 G. PATAKI in C. GIDDINGS AND R. A. KELLER (Editors), *Advances in Chromatography*, Dekker, New York, in preparation.
5 K. RANDERATH, *Angew. Chem.*, 73 (1961) 674.
6 K. RANDERATH AND H. J. STRUCK, *J. Chromatog.*, 6 (1961) 365.
7 K. RANDERATH, *Angew. Chem.*, 74 (1962) 484.
8 K. RANDERATH, *Biochem. Biophys. Res. Commun.*, 6 (1961/62) 452.
9 K. RANDERATH, *Biochim. Biophys. Acta*, 61 (1962) 852.
10 K. RANDERATH, *Biochim. Biophys. Acta*, 76 (1963) 622.
11 G. WEIMANN AND K. RANDERATH, *Experientia*, 19 (1963) 49.
12 K. RANDERATH, *Experientia*, 20 (1964) 406.
13 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 16 (1964) 111.
14 E. RANDERATH AND K. RANDERATH, *J. Chromatog.*, 16 (1964) 126.
15 E. RANDERATH AND K. RANDERATH, *Anal. Biochem.*, 12 (1965) 83.
16 J. NEUHARD, E. RANDERATH AND K. RANDERATH, *Anal. Biochem.*, 13 (1965) 211.
17 K. RANDERATH AND E. RANDERATH, *Anal. Biochem.*, 13 (1965) 575.
18 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 22 (1966) 110.
19 G. PATAKI AND ED. STRASKY, *4th Symp. Chromatog., Brussels, 1966*, in press.
20 G. PATAKI AND A. KUNZ, *J. Chromatog.*, 23 (1966) 465.
21 G. PATAKI AND A. NIEDERWIESER, *J. Chromatog.*, 29 (1967) 133.
22 P. BAUDET, CL. OETTEN AND E. CHERBULIEZ, *Helv. Chim. Acta*, 47 (1964) 2430; *Arch. Sci. (Geneva)*, 18 (1965) 287.

THIN-LAYER CHROMATOGRAPHY OF NUCLEIC ACID BASES, NUCLEOSIDES, NUCLEOTIDES AND RELATED COMPOUNDS

IV. SEPARATION ON PEI-CELLULOSE LAYERS USING GRADIENT ELUTION AND DIRECT FLUOROMETRY OF SPOTS*,**

G. PATAKI
Analytical Department, Laboratory for Chromatography, Robapharm Ltd., Basle (Switzerland)
AND
A. NIEDERWIESER
Forschungslaboratorium für Medizinische Akustik, Biochemische Abteilung, Universität Düsseldorf, Düsseldorf (Germany)
(Received December 27th, 1966)

In a previous communication[1] a method was described for the characterization of more than sixty nucleo-derivatives and related compounds on cellulose thin layers. This technique is especially suitable for the detection of nucleo-derivatives in biological material. However, the separation of some groups of nucleotides is poor; nucleoside tri- and diphosphates show some tailing. Fortunately nucleotides can be separated very sharply on PEI-cellulose layers according to RANDERATH[2–4]. For the chromatography of nucleotides on PEI-cellulose layers stepwise elution with different solvents has been used[3–6] (for a review, see ref. 7). The quantitative estimation of these compounds, using a micro-technique, has also been described[6].

It was felt that gradient elution[8] may be useful for the chromatography of nucleo-derivatives on PEI-cellulose layers, when separation and detection of nucleic acid bases, nucleosides and nucleotides on the same plate is required. Since direct fluorometry of spots was found to be suitable for the quantitative analysis of compounds, *e.g.* nucleo-derivatives[9,10] and amino acids[11,12], resolved on silica gel or on cellulose layers, there is no doubt that this method can also be used for direct scanning of spots on PEI-cellulose chromatograms.

In this communication we would like to describe some results concerning gradient elution chromatography of nucleo-derivatives on PEI-cellulose layers. Additionally the direct fluorometry of these compounds will also be reported.

EXPERIMENTAL

Preparation of sheets

RANDERATH's method[13] was used with slight modifications. Plastic sheets (Type DC, GA 1190, Galenopharm, Geneva, Switzerland) 20 × 130 cm are cleaned

* For Part III of this series, see ref. 1.
** 6th communication on "Quantitative Thin-Layer Chromatography". 5th communication, see ref. 12.

and fixed on the plastic holder of the applicator (Desaga, Heidelberg, Germany) using scotch tape. A suspension of 22 g MN-300-cellulose (*Procedure a*) or 22 g purified MN-300-cellulose[1,10] (*Procedure b*) in 145 ml of a 1 % PEI-solution (cellulose powder supplied by Macherey, Nagel & Co., Düren, Germany, preparation of PEI-solution according to RANDERATH[13]) is homogenized for 1 min in an electric mixer. After stirring for about 1 min in a beaker to remove air bubbles, the suspension is placed in the applicator, the sheet coated immediately (slot width 0.5 mm) and allowed to dry overnight at room temperature.

Pretreatment of sheets

Ascending chromatography on the sheets (20 × 20 cm sheets are fixed with a rubber band on glass plates) is carried out in Shandon multiplate-chromatotanks (Shandon, London). In order to avoid loss of layer and to remove the impurities, filter paper strips (*e.g.* prefolded paper strips for BN-chamber, Desaga, Heidelberg Germany) are attached to the top and to the bottom. The sheets are developed with 10 % NaCl for 5 cm, and then, without intermediate drying, with distilled water up to 20 cm. After drying for about 3 h the sheets are redeveloped with water for 20 cm. After drying overnight at room temperature they are ready for use. For quantitative estimations it is necessary to remove impurities as far as possible. It is therefore advisable to examine the sheets in U.V. light before use, and if there are still visible impurities, another development with water should be carried out. *All runs are made perpendicular to the coating direction.*

Preparation of plates

RANDERATH's method[2–4] has been used with slight modifications. 100 g Polymin P (Badische Anilin- und Sodafabrik, Ludwigshafen, Germany) and 250 ml distilled water are stirred, neutralized with conc. HCl to pH 6, and made up to 500 ml with distilled water. 25 ml of this solution is dialyzed against 4 l distilled water (using a magnetic stirrer). After 24 h, the dialyzed solution is made up to 250 ml. 40 g purified MN-300-cellulose[1,10] (*Procedure c*) and the dialyzed PEI-solution are homogenized in an electric mixer for 1 min. The slurry is spread over 10 glass plates, 20 × 20 cm (slot width 0.5 mm) using *e.g.* STAHL's applicator (Desaga, Heidelberg, Germany), followed by drying overnight at room temperature.

Pretreatment of plates

In order to obtain an even solvent front it is advisable to remove a 0.5 cm strip from both edges of the plate. To avoid cracking it is also advisable to scratch lines into the layer as described by RANDERATH[14]. In addition, the attachment of filter paper strips to the top and the bottom is also necessary (*cf.* pretreatment of sheets).

Ascending chromatography, drying, etc. is made as described under "Pretreatment of sheets", however, *all runs should be in the coating direction.* Before spotting and chromatography all parts of the layer into which lines were scratched are scraped off.

Chromatography

For chromatography, a sandwich type chamber (BN-chamber, Desaga, Heidelberg, Germany) cooled with tap water and combined with a device for gradient elution previously described[8] is used. A similar apparatus for gradient elution thin-layer

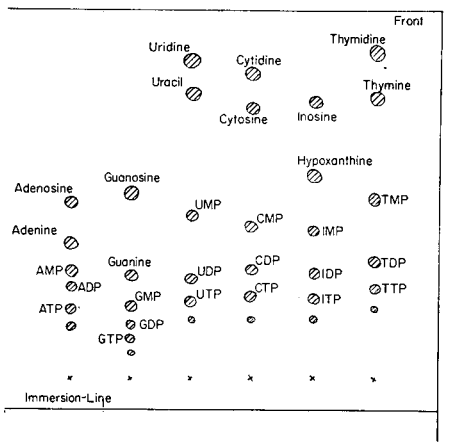
chromatography is now available (Desaga, Heidelberg, Germany). Total volume of the capillary, 4 ml; length, 276 cm, inner diameter, 1.5 mm. In this study we have used mainly the following solvent systems (designed as standard gradient) in which the capillary is filled (speed, 0.05 ml/sec), successively, with:

(1) 0.6 ml 0.1 *M* LiCl
(2) 0.6 ml 0.2 *M* LiCl
(3) 0.6 ml 0.5 *M* LiCl
(4) 0.6 ml 1 *M* LiCl
(5) 1.2 ml 2 *M* LiCl.

combined with the feed pipe in such a way that chromatography starts with the solvent of lowest salt concentration (here 0.1 *M* LiCl). Chromatography is perpendicular to the coating direction on the sheets. Development on plates is in the coating direction. Distance between immersion line and starting points was 1.5 cm.

Scanning of spots

For direct fluorometry[9–12] a Turner-Fluorometer 111 fitted with a door for thin-layer chromatograms (Camag, Muttenz, Switzerland) is used. Spots on the plates or sheets can be scanned by illuminating the chromatograms with a short wave-length



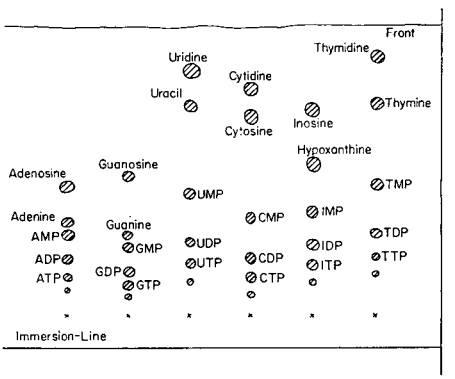


Fig. 1. Separation of the adenine, guanine, uracil, cytosine, hypoxanthine and thymine group on PEI-cellulose sheet (*cf.* procedure *b* in Experimental). Standard gradient (capillary is filled with: 0.6 ml 0.1 *M* LiCl; 0.6 ml 0.2 *M* LiCl; 0.6 ml 0.5 *M* LiCl; 0.6 ml 1 *M* LiCl; and 1.2 ml 2 *M* LiCl). Load: base, nucleoside and monophosphate 1 μg each; diphosphate and triphosphate 2 μg each. Distance between starting point and immersion line: 1.5 cm. Running time for 3.6 ml solvent: 160 min; relative air humidity: 65%.

Fig. 2. Separation of the adenine, guanine, uracil, cytosine, hypoxanthine and thymine group on PEI-cellulose plate (*cf.* procedure *c* in Methods). Experimental conditions as in Fig. 1. Running time: 95 min; relative air humidity: 37%.

fluorescent lamp (110–851) at 254 nm and by using a Corning 7–54 filter as primary filter (transmitting between 230–400 nm) and a Kodak-Wratten 2A (transmitting only above 405 nm) as secondary filter. The conditions are*: at door full open;

* Recently, some improvements were introduced by the manufacturer. The new apparatus probably requires the same variations in the experimental conditions as given in this paper and published previously[9–12].

sensitivity 10 ×; recording of fluorometer units by a Hi-Speed-Recorder 201, supplied by Kontron/Zürich (speed, 8 cm/min; voltage, 10 mV). Before scanning the chromatograms are dried 30 min in a stream of cold air (designed as "$t = 0$").

RESULTS AND DISCUSSION

The chromatographic behaviour of nucleo-derivatives on PEI-cellulose *sheets* prepared according to procedure *b* and on PEI-cellulose *plates* prepared according to procedure *c* (*cf.* Methods) with our standard gradient is shown in Figs. 1 and 2. As can be seen, there is a good separation in every group between nucleoside-mono-, di- and triphosphates as well as between nucleoside and the corresponding base. It is especially remarkable that in each group phosphates, nucleosides and the corresponding bases can be detected on the *same plate*, a fact which makes possible the analysis of substances from three different polarity classes.

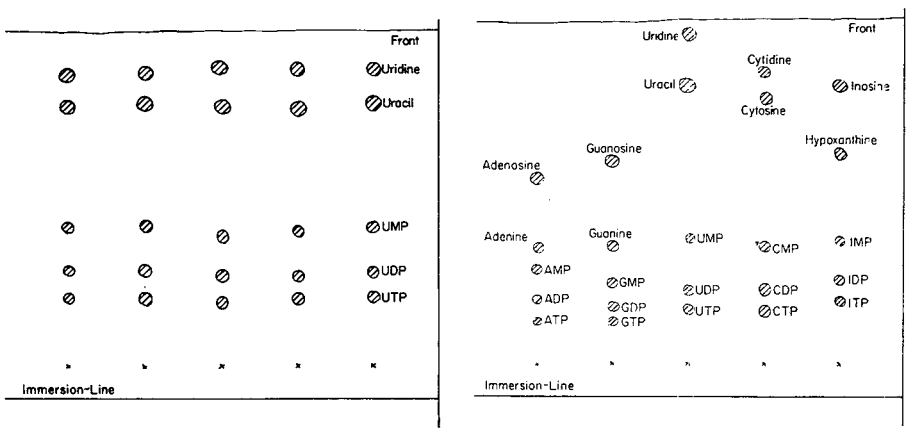


Fig. 3. Reproducibility of the standard gradient (*cf.* Methods) on the same PEI-cellulose sheet (*cf.* procedure *b* in Methods). Experimental conditions as in Fig. 1. Running time: 165 min; relative air humidity: 45%.

Fig. 4. Separation of different groups of nucleo-derivatives on PEI-cellulose plate (procedure *c* in Methods). Gradient: (1) 0.4 ml water; (2) 0.6 ml 0.1 *M* LiCl; (3) 0.6 ml 0.2 *M* LiCl; (4) 0.6 ml 0.5 *M* LiCl; (5) 0.6 ml 1 *M* LiCl; (6) 1.2 ml 2 *M* LiCl. For the remaining conditions *cf.* Fig. 1. Running time: 120 min for 4 ml solvent; relative air humidity: 50%.

The separation will not be altered if we use PEI-cellulose sheets prepared according to procedure *a* (impregnation of unpurified cellulose with poly(ethyleneimine); *cf.* Methods). The reproducibility of the standard gradient (*cf.* Methods) on the same sheet is shown in Fig. 3. R_F values of compounds observed on *different sheets* under *different conditions* suggest that the variance of R_F values in gradient elution thin-layer chromatography is nearly of the same order of magnitude as that generally encountered in thin-layer chromatography[15].

The separation of nucleotides from their corresponding bases and nucleosides in each group is given in Figs. 1 and 2. However, adenine and guanine move approximately as fast as IMP and CMP and slower than UMP (*e.g.* Figs. 1 and 2). When we use gradient elution for the separation of complex mixtures containing nucleotides nucleic acid bases and nucleosides a distinct differentiation is required between *all*

nucleotides on the one hand, and *all* nucleosides and nucleic acid bases on the other hand. Since the R_F values of nucleic acid bases and nucleosides do not depend on the salt concentration of the solvent, these compounds can be separated from nucleotides and other acidic substances by a development with distilled water[3]. We have therefore modified our standard gradient slightly to obtain a better separation between the compounds discussed above. Starting with 0.4 ml distilled water instead of 0.1 *M* LiCl causes only a minor change (compare the positions of adenine, guanine, UMP, CMP and IMP in Figs. 2 and 4). Gradients containing 1 and 1.8 ml water, resp., as starting solvent are capable of separating all the nucleic acid bases and nucleosides

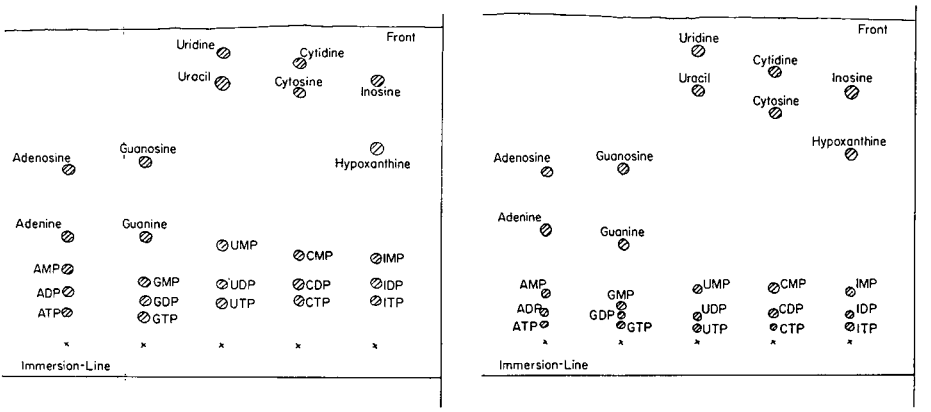


Fig. 5. Separation of different groups of nucleo-derivatives on PEI-cellulose plate (procedure *c* in Methods). Gradient: (1) 1 ml water; (2) 0.5 ml 0.1 *M* LiCl; (3) 0.5 ml 0.2 *M* LiCl ;(4) 0.5 ml 0.5 *M* LiCl; (5) 0.5 ml 1 *M* LiCl; (6) 1 ml 2 *M* LiCl. For the remaining conditions *cf.* Fig. 1. Running time: 95 min for 4 ml solvent; relative air humidity: 33%.

Fig. 6. Separation of different groups of nucleo-derivatives on PEI-cellulose plate (procedure *c* in Methods). Gradient: (1) 1.8 ml water; (2) 0.4 ml 0.1 *M* LiCl; (3) 0.4 ml 0.2 *M* LiCl; (4) 0.4 ml 0.5 *M* LiCl; (5) 0.4 ml 1 *M* LiCl; (6) 0.6 ml 2 *M* LiCl. For the remaining conditions *cf.* Fig. 1. Running time: 105 min for 4 ml solvent; relative air humidity: 33%.

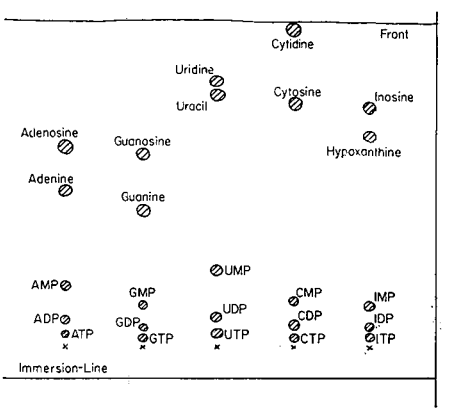
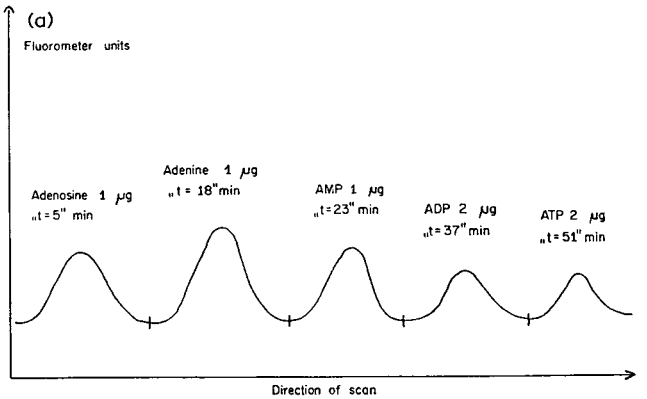
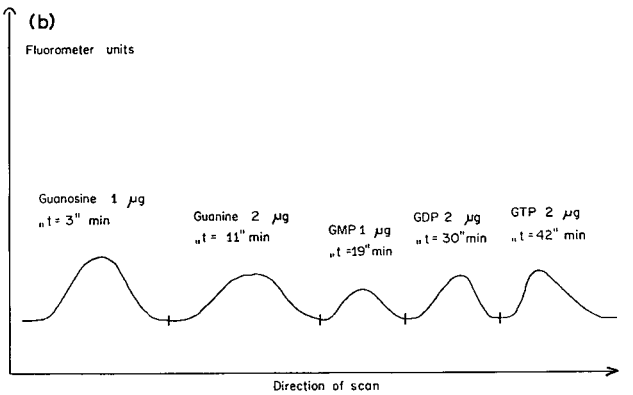
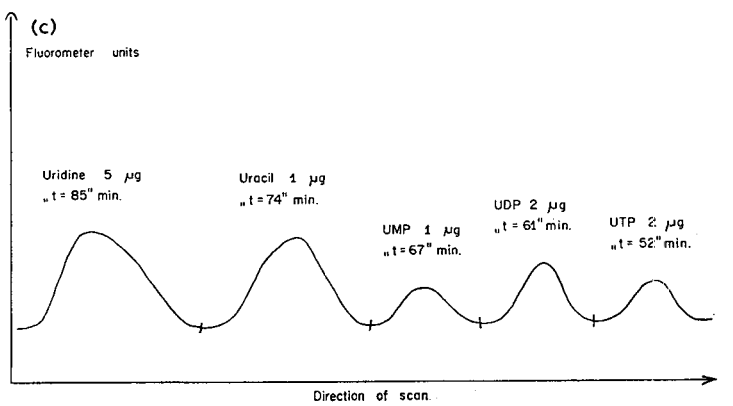
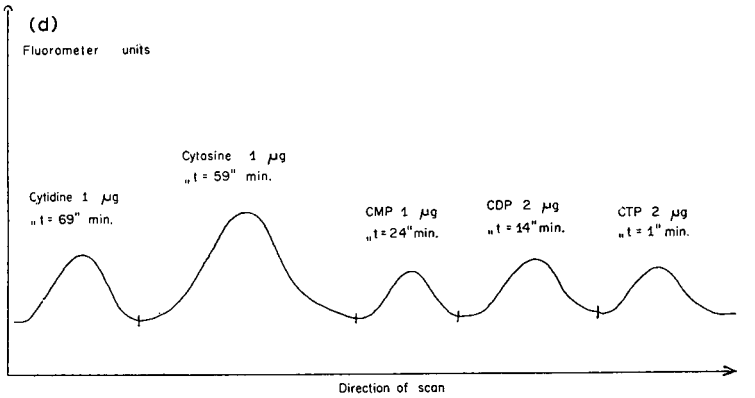


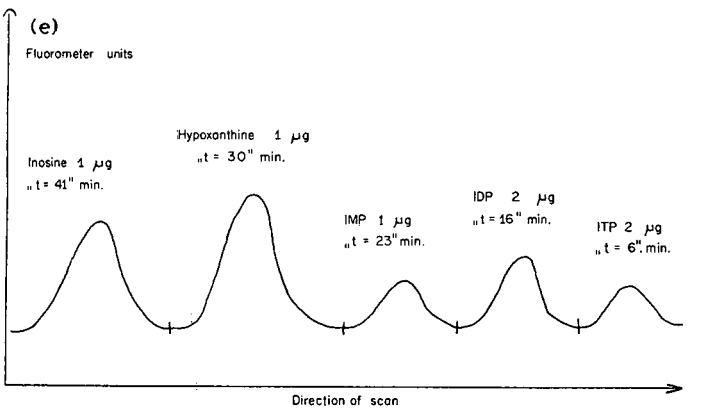
Fig. 7. Separation of different groups of nucleo-derivatives on PEI-cellulose plate (procedure *c* in Methods). Gradient: (1) 1 ml methanol–water (1:1); (2) 0.6 ml 0.1 *M* LiCl; (3) 0.6 ml 0.2 *M* LiCl; (4) 0.6 ml 0.5 *M* LiCl; (5) 0.6 ml 1 *M* LiCl; (6) 0.6 ml 2 *M* LiCl. For the remaining conditions *cf.* Fig. 1. Running time: 180 min for 4 ml solvent; relative air humidity: 36%.


(a)
Fluorometer units
Adenosine 1 µg
„t = 5" min
Adenine 1 µg
„t = 18" min
AMP 1 µg
„t = 23" min
ADP 2 µg
„t = 37" min
ATP 2 µg
„t = 51" min
Direction of scan


(b)
Fluorometer units
Guanosine 1 µg
„t = 3" min
Guanine 2 µg
„t = 11" min
GMP 1 µg
„t = 19" min
GDP 2 µg
„t = 30" min
GTP 2 µg
„t = 42" min
Direction of scan


(c)
Fluorometer units
Uridine 5 µg
„t = 85" min.
Uracil 1 µg
„t = 74" min.
UMP 1 µg
„t = 67" min.
UDP 2 µg
„t = 61" min.
UTP 2 µg
„t = 52" min.
Direction of scan.





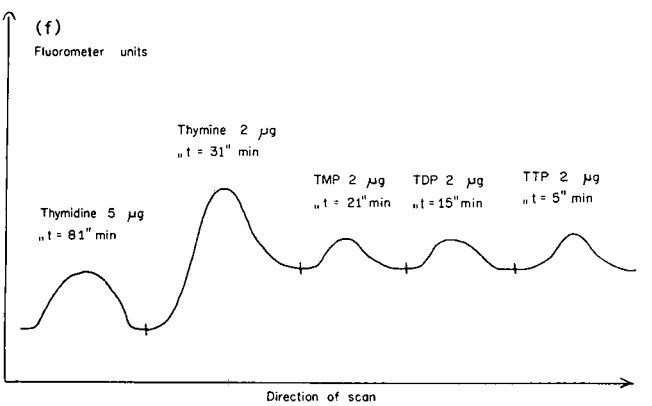


Fig. 8. Scanning of spots (perpendicular to the direction of run) on PEI-cellulose sheets (*cf.* procedure *b* in Methods). The sheet with the substances was covered, except a small strip containing the spot under investigation, with another plain PEI-cellulose sheet. Before scanning ("t = 0" min) the chromatogram was dried 30 min in a stream of cold air. For the remaining conditions *cf.* Methods. Impurities in nucleotide di- and triphosphates are not given here. Loads are given in micrograms (µg). a = Adenine group; b = guanine group; c = uridine group; d = cytosine group; e = hypoxanthine group; f = thymine group.

under investigation from the nucleotides. However, the resolution of the nucleotides in these cases is somewhat less effective (Figs. 5 and 6). Finally Fig. 7 shows a chromatogram with a gradient containing 1 ml methanol–water (1:1) as starting solvent. Higher amounts of methanol give chromatograms in which the nucleic acid bases and nucleosides tail.

As already mentioned, attempts have been made to apply the direct fluorometric procedure previously reported[9–12] for scanning of spots on PEI-cellulose chromatograms. Earlier experiments* showed that direct fluorometry cannot be performed on unpurified cellulose layers. In order to overcome the difficulties caused by the inconstancy of the zero line, we have developed a purification procedure[1,10] (*cf.* Methods), which partly rectifies this shortcoming. Experiments not reported here, have shown that scanning of the spots is impossible on a PEI-cellulose layer prepared according to procedure *a* (*cf.* Methods) and that purification of the cellulose powder is indispensable. Fortunately the separation of the nucleo-derivatives is not altered by use of purified cellulose for the preparation of PEI-cellulose layers (procedures *b* and *c* in Methods). We have therefore carried out all measurements on PEI-cellulose layers prepared from purified cellulose powder (procedures *b* and *c*). The spots are scanned by illuminating the chromatogram with a fluorescent lamp (254 nm) and by using a primary filter transmitting between 230 and 400 nm (*cf.* refs. 9, 10, 11 and 12). Typical peaks** produced by the scanner are shown in Fig. 8. The fluorescence quenching, that means the peak area, as reported earlier[12] for amino acid derivatives, will be altered by the time of drying***. The drying time therefore has to be standardized. Thus the peak areas can only be compared with reservations. Nevertheless, they give an idea of the sensitivity of the method. In our opinion about 1 μg nucleoside phosphate and about 0.5 μg nucleoside or nucleic acid base can be estimated by this method. In some cases, however, it may be useful to increase the peaks by scale expansion.

It has to be pointed out that impurities in the layer, which, in some cases, can be seen in the region of the front, may disturb the scanning of substances with high mobilities.

All the precautions given under Pretreatment of sheets and Pretreatment of plates must therefore be followed. The scanning should, of course, be made with care as described previously[10] (*cf.* also ref. 7).

The application of gradient elution thin-layer chromatography on PEI-cellulose

---

* Unpublished results.

** The amounts of nucleoside phosphates, given in Fig. 8, correspond to the compounds listed here: Adenosine-5′-monophosphoric acid monohydrate; adenosine diphosphate sodium salt; adenosine triphosphate disodium salt; cytidine-5′-monophosphate disodium salt; cytidine diphosphate sodium salt·$4H_2O$; cytidine triphosphate sodium salt·$4H_2O$; thymidylic acid ammonium salt; thymidine diphosphate trisodium salt; thymidine triphosphate trisodium salt; uridine monophosphate disodium salt; uridine diphosphate disodium salt; uridine triphosphate trisodium salt; guanosine monophosphate sodium salt·H_2O; guanosine diphosphate sodium salt; guanosine triphosphate sodium salt; inosine-monophosphoric acid; inosine diphosphate disodium salt·$5\frac{1}{2}H_2O$; inosine-triphosphoric acid.

Nucleoside di- and triphosphates contain the corresponding mono- and diphosphates, resp. The amounts of these impurities, however, vary from one lot to another. Some samples of nucleoside triphosphates also contain a substance with a lower mobility on PEI-cellulose layers. These spots are probably nucleoside tetraphosphates and their amounts again differ from one lot to another.

*** The standard deviation between "t = 150" min and "t = 600" min (31 measurements in intervals of each 15 min) is 6.9%.

and the direct fluorometry of spots to the separation and quantitative estimation of complex mixtures is to be investigated in our laboratories.

The combined use of the technique described in this paper and our earlier published method[1] for the analysis of nucleo-derivatives in biological material will be published in a subsequent communication.

ACKNOWLEDGEMENT

We wish to thank Miss H. ZÜRCHER, Miss OTGERT BULL and Mr. A. KUNZ for technical assistance.

SUMMARY

The separation of nucleic acid bases, nucleosides and nucleotides on PEI-cellulose layers using gradient elution technique has been investigated. The standard gradient (0.1 *M* LiCl → 2 *M* LiCl) described in this paper allows the detection of nucleic acid base, nucleoside and nucleotide on the same plate or sheet.

Special reference is made to the direct fluorometry of compounds resolved on PEI-cellulose layers.

REFERENCES

1 G. PATAKI, *J. Chromatog.*, 29 (1967) 126.
2 K. RANDERATH, *Biochim. Biophys. Acta*, 61 (1962) 852.
3 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 16 (1964) 111.
4 E. RANDERATH AND K. RANDERATH, *J. Chromatog.*, 16 (1964) 126.
5 G. WEIMANN AND K. RANDERATH, *Experientia*, 19 (1963) 49.
6 E. RANDERATH AND K. RANDERATH, *Anal. Biochem.*, 12 (1965) 83.
7 G. PATAKI, in C. GIDDINGS AND R. A. KELLER (Editors), *Advances in Chromatography*, Dekker, New York, in preparation.
8 A. NIEDERWIESER AND C. G. HONEGGER, in C. GIDDINGS AND R. A. KELLER (Editors), *Advances in Chromatography*, Vol. II, Dekker, New York, 1966, p. 123.
9 G. PATAKI AND A. KUNZ, *J. Chromatog.*, 23 (1966) 465.
10 G. PATAKI AND ED. STRASKY, *4th Symp. Chromatog., Brussels, 1966*, in press.
11 G. PATAKI, *4th Symp. Chromatog., Brussels, 1966*, in press.
12 G. PATAKI AND ED. STRASKY, *Chimia*, 20 (1966) 361.
13 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 22 (1966) 110.
14 K. RANDERATH, *Dünnschichtchromatographie*, Verlag Chemie, Weinheim, 1965, p. 41.
15 G. PATAKI, *Dünnschichtchromatographie in der Aminosäure- und Peptidchemie*, Walter de Gruyter, Berlin, 1966, p. 47 *ff*.

DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG ORGANISCHER SULFOXIDE UND DINITROTHIOÄTHER. EINIGE BEMERKUNGEN ZUR REPRODUZIERBARKEIT UND ZUM STRUKTUREINFLUSS

I. MITT. EINFLUSS DER RELATIVEN FEUCHTE

H. W. PRINZLER UND H. TAUCHMANN

Technische Hochschule für Chemie "Carl Schorlemmer", Institut für Petrolchemie, Leuna-Merseburg, 42 Merseburg (D.D.R.)

(Eingegangen den 5. September 1966)

Thioäther lassen sich aus nativen Kohlenwasserstoffgemischen durch selektive Oxydation zu ihren Sulfoxiden und anschliessende Chromatographie leicht abtrennen, wobei die hohen Polaritätsunterschiede zwischen Kohlenwasserstoffen und Sulfoxiden die Trennung begünstigen[1–3]. Zur weiteren Untersuchung der resultierenden Sulfoxidgemische fanden wir die Dünnschichtchromatographie (DC) als gut brauchbar. Gleichzeitig erschien uns diese Stoffklasse zum Studium der Einflussfaktoren in der DC als besonders geeignet, umfasst doch die homologe Reihe der Alkylsulfoxide sowohl hydrophile als auch mehr oder weniger hydrophobe Verbindungstypen.

ZUR REPRODUZIERBARKEIT IN DER DC

In der DC gut reproduzierbare Ergebnisse zu erhalten, ist problematisch. Das aus der grossen Anwendungsbreite der DC resultierende Interesse an grundsätzlichen Studien findet sich in zahlreichen Arbeiten bestätigt[5–18,25,35].

Die Einflussfaktoren lassen sich in zwei Gruppen einteilen:

(a) Faktoren, die nur eine anteilige Verschiebung des R_F-Wertes bedingen, das Trennbild also nicht verändern.

(b) Faktoren, die das Trennverhalten der Substanzen beeinflussen.

Zur ersten Gruppe gehören die den Fliessmitteldurchsatz verändernden Parameter wie Schichtdicke, Temperatur (bei gleicher relativer Feuchte)[13,14] und auch die Chromatographie-Technik, sofern hierbei das reduzierte Fliessmittelprofil[4] auf der Platte gleichbleibt und auch die Aktivität der Schicht nicht verändert wird.

Der Einfluss dieser Faktoren lässt sich dadurch eliminieren, indem man die R_F-Werte auf eine Standard-Substanz bezieht (R_{St}-Wert). Hierbei wird vorausgesetzt, dass sich, beispielsweise bei unterschiedlicher Chromatographie-Technik, die mittleren Wanderungsgeschwindigkeiten der Substanzen um den gleichen Faktor ändern.

Zur zweiten Gruppe gehören alle die Struktur des Adsorbens verändernden Faktoren (Art der Herstellung des Gels, thermische Behandlung, Deaktivierung mit mehr oder minder polaren Stoffen u.a.) sowie Unstetigkeiten im reduzierten Fliessmittelprofil, die z.B. durch unterschiedliche Sättigung hervorgerufen werden können.

Weichen die relativen Änderungen der R_F-Werte nicht stark von der Norm ab,

so kann man oft dadurch reproduzierbare Ergebnisse erreichen, indem man die Retentionswerte auf zwei Substanzen bezieht[27] (Tabelle II).

Für die meisten Trennaufgaben — insbesondere bei Verwendung mehrkomponentiger Fliessmittelgemische — wird es indes unumgänglich sein, die DC unter "kontrollierten" Bedingungen durchzuführen[36]. GEISS UND SCHLITT schlugen kürzlich eine Klimakammer unter Verwendung mehrerer Salztröge vor[14], Variationen der S-Kammer beschreiben DAVIES[21], JÄNCHEN[22], WASICKY[23] und SACHS UND SZEREDAY[24].

OBERFLÄCHENBESCHAFFENHEIT VON Al_2O_3 IN ABHÄNGIGKEIT VOM RELATIVEN WASSERDAMPFDRUCK

Obwohl Oberfläche und Porengrösse und damit auch die Menge des adsorbierten Wassers stark vom Herstellungsprozess des Al_2O_3 abhängig sind, kann man doch — wie vor allem GLEMSER UND RIECK[29], CORNELIUS *et al.*[30], DE BOER *et al.*[31,32], JUHASZ[33] und PERI[34] zeigen konnten — in Abhängigkeit von der relativen Feuchte (r.F.) typische Oberflächenstrukturen antreffen.

So sollen bis zu einer r.F. von etwa 15 % die aktivsten Zentren abgesättigt, d.h., die Monoschicht ausgebildet sein, während > 60 % r.F. Kapillarkondensation eintritt.

Für die Desorptionsenergien des adsorbierten Wassers[30,33] lassen sich ähnliche Bereiche erkennen. Im Bereich mittlerer relativer Dampfdrücke verlaufen die Adsorptions-Potentialkurven waagerecht.

Wir haben an dem von uns verwendeten Al_2O_3-D (Greiz-Dölau, D.D.R.) Wasserdampf-Isothermen aufgenommen (Fig. 1), wobei der im Al_2O_3-D enthaltene Gips (15 %) durch einstündiges Erhitzen auf 480° in die nichthygroskopische Form ($CaSO_4$ II) umgewandelt wurde[37].

Die vorklimatisierten Proben wurden bis zur Einstellung des Gleichgewichts

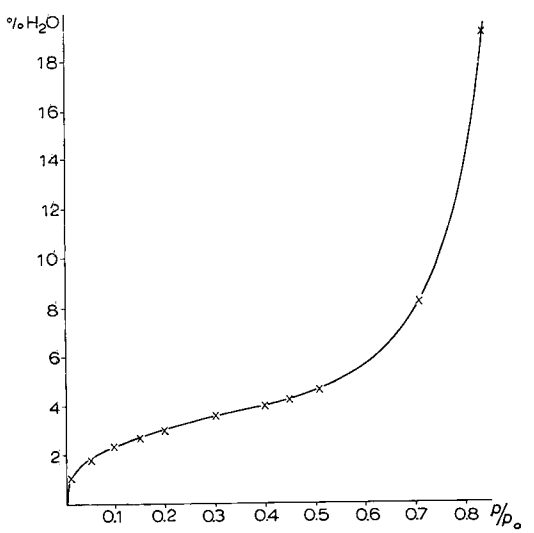


Fig. 1. Wasserdampf-Adsorptionsisotherme (20°) auf Al_2O_3-D.

entsprechenden H_2O-Dampfdrücken (erzeugt über Schwefelsäure bestimmter Konzentration bzw. für $p/p_0 > 0.5$ über Salzlösungen) ausgesetzt und die jeweilige Gewichtszunahme pro Gramm Trockengewicht (einstündiges Erhitzen der Proben auf 120°) bestimmt.

Die Wasserdesorption über 120° war sehr gering, wie thermogravimetrisch aufgenommene Isobaren zeigten.

Im Bereich zwischen 10 und 50 % r.F. ist der lineare Verlauf der Isotherme, ebenso die geringe Zunahme der Menge des adsorbierten Wassers bemerkenswert. Hier würde nur ein geringer Einfluss auf das chromatographische Verhalten zu erwarten sein.

Wir haben mittels der BET-Gleichung aus der Isotherme bei 20°, die dem Typ II nach der Klassifikation von BRUNAUER, DEMING u.a.[38] entspricht, die Oberflächendaten bestimmt. Die graphische Lösung (Fig. 2) ergab folgende Werte:

$c = 59.5 \qquad x_m = 26.5$ mg H_2O/g Adsorbens
$= 95.7$ m²/g (1 Molekül $H_2O \triangleq 10.8$ Å²)

Demnach wäre also bei etwa 14 % r.F. die Monoschicht vollständig aufgebaut.

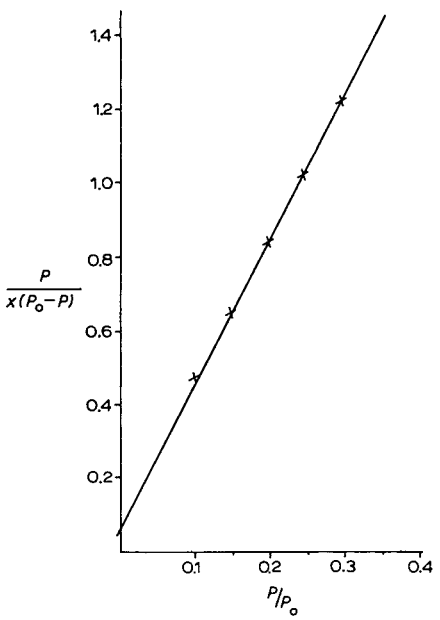


Fig. 2. Ermittlung von c und x_m nach der BET-Gleichung.

CHROMATOGRAPHISCHES VERHALTEN DER SULFOXIDE IN ABHÄNGIGKEIT VON DER RELATIVEN FEUCHTE

Noch bedeutungsvoller als in der Papierchromatographie[26,28] ist der Einfluss der Feuchtigkeit in der DC für adsorptionschromatographische Trennungen, wie neben PITRA *et al.*[17], MATSUSHIKA[19] und KURTSCHENINOWA[20] vor allem GEISS UND SCHLITT[13,14] sowie DALLAS[18] feststellen konnten.

Wir untersuchten den Einfluss der relativen Luftfeuchte auf die dünnschichtchromatographische Trennung der Sulfoxide bei Verwendung eines Fliessmittelgemisches und Al_2O_3 als stationäre Phase. Ferner ermittelten wir die Abhängigkeit der Fliessmittelentmischung vom Wassergehalt des Al_2O_3.

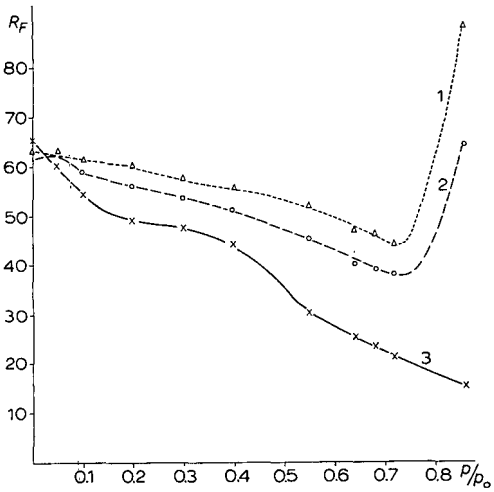


Fig. 3. Abhängigkeit der Retentionswerte der Sulfoxide von der r.F. Stationäre Phase: Al_2O_3-D; mobile Phase: Benzol–Methanol (9:1). 1 = Dioktylsulfoxid; 2 = Dibutylsulfoxid; 3 = Dimethylsulfoxid.

Die niederen Glieder der homologen Reihe der Sulfoxide lassen sich auf "aktivem" Al_2O_3-D mit Benzol–Methanol (Vol. Verh. 9:1) trennen. Aus Fig. 3 geht hervor, dass die R_F-Werte* mit steigender r.F. sinken, das Adsorbens also scheinbar aktiver wird und erst bei relativen Dampfdrücken >0.7 wieder ansteigen, allerdings nur für die hydrophoberen Sulfoxide. Zwischen 1 und 5 % r.F. tritt keine Trennung ein, während von 20 bis 60 % r.F. die R_F-Wert-Änderungen im Verhältnis zu anderen Bereichen gering sind; die R_{St}-Werte (bezogen auf Dioktylsulfoxid) sind nur annähernd konstant (Tabelle I).

Errechnet man unter Annahme der empirischen Beziehung[27]

$$R_F{}^0 = aR_F + b$$

die Standard-R_F-Werte ($R_F{}^0$) für $p/p_0 = 0.4$, so ergibt sich sogar zwischen 20 und 68 % r.F. eine hinreichende Konstanz (Tabelle II); a und b sind Konstanten, jedoch abhängig von der relativen Feuchte und anderen Chromatographie-Bedingungen.)

DISKUSSION

Es hat nicht an Versuchen gefehlt, einen für die Dünnschichtchromatographie günstigen Bereich der relativen Feuchte zu finden. GEISS und Mitarbeiter[13] geben für Polyphenylgemische als solchen 28–50 % r.F. an (reproduzierbares Plateau). An Hand

* Die R_F-Werte (üblicher R_F-Wert × 100) wurden bewusst auf die α-Front bezogen, obzwar eine Entmischung des Elutionsmittels (nicht sichtbar!) eintritt.

TABELLE I

RETENTIONSWERTE VON SULFOXIDEN AUF Al_2O_3
(Mobile Phase: Benzol–Methanol (9:1), S-Ka)

Relative Feuchte	*20*	*30*	*40*	*55*	*68*	*20*	*30*	*40*	*55*	*68*
Sulfoxid	*R_F-Werte** (± 1.5)					*R_{St}-Werte*				
Dimethyl-	49	47	44	30	25	81,5	81	81.5	55.5	53
Dibutyl-	56	53.5	51	45	40	93	92.5	94.5	86.5	85
Dioktyl-	60	58	54	52	47	100	100	100	100	100
Thiacyclopentyl-	51	49	46.5	35	29	85	84.5	86	67.5	61.5

* Üblicher R_F-Wert × 100.

TABELLE II

STANDARD-RETENTIONSWERTE (R_F^0) VON SULFOXIDEN
(Bezogen auf 40% r.F.)

Relative Feuchte	*20*	*30*	*40*	*55*	*68*
Sulfoxid					
Dibutyl-	50.4	49.8	51*	50.8	50.8
Thiacyclopentyl-	45.8	45.8	46.5*	46.3	45.8
a	0.9091	0.9091	1.0	0.4545	0.4545
b	−0.546	1.272	0	30.365	32.638

* Bei 40% r.F. gemessene Werte × 100.

$$a = \frac{(R_F^0)_A - (R_F^0)_B}{(R_F)_A - (R_F)_B}$$

$$b = (R_F^0)_A - a(R_F)_A$$

A = Dioktylsulfoxid; B = Dimethylsulfoxid.
R_F bei der entspr. r.F. gemessenen Werte × 100.

dünnschichtchromatographischer Trennungen eines Farbstoffgemisches[14] weisen sie jedoch später nach, dass ein Niveau, innerhalb dessen adsorptionschromatographisch erhaltene R_F-Werte konstant sind, nicht existiert, sondern dass die R_F-Werte eindeutig von der Oberflächenbeschaffenheit des Adsorbens abhängen. In der LEAC kommt dies in der Abhängigkeit der Aktivitätsparameter vom Hydratationsgrad ebenfalls zum Ausdruck[39].

Dennoch existieren Bereiche der r.F. — vor allem bei Aluminiumoxiden mit kleiner Oberfläche — in deren Grenzen sich die R_F-Werte nur wenig verändern; zumeist erfolgt eine annähernde Parallelverschiebung (z.B. für die Sulfoxide zwischen 20–40 bzw. 68 oder für das von GEISS u. Mitarb. untersuchte Farbstoffgemisch zwischen 38 und 63 % r.F.). PITRA *et al.*[17] gibt für Silicagel als günstigen Arbeitsbereich einen 10–20 %igen Wassergehalt des Adsorbens an.

Man kann nun für diesen Bereich, wie es Tabellen I und II zeigt, durch Verwendung von ein oder zwei Substanzen des jeweiligen Stoffgemisches als Bezugssubstanzen

die Einflussfaktoren rechnerisch weitestgehend eliminieren. Dieser für die Reproduzierbarkeit günstige, nicht notwendigerweise für die Trennung optimale Bereich dürfte immer innerhalb der geringsten Neigung der Wasser-Adsorptionsisotherme liegen.

Trotzdem erklärt auch der Verlauf dieser Isotherme das chromatographische Verhalten der Sulfoxide nicht vollständig. So müsste ausserhalb des linearen Teils der Isotherme ($0.55 < p/p_0 < 0.14$) eine extreme Veränderung der R_F-Werte erfolgen (Aufbau der monomolekularen Schicht sowie Einsetzen der Kapillarkondensation). Diese Veränderungen erfolgen nur bei dem zwischen 0–5 und > 70 % r.F. vorklimatisierten Adsorbens.

Bedenkt man, dass am Aufbau der stationären Phase auch das Fliessmittel beteiligt ist, so wird verständlich, dass die Grenzen dieser fast linearen Änderung des Adsorptionsvermögens (bzw. des Phasenverhältnisses der Benzol–Methanol-Mischung) durchaus verschoben sein können.

So gibt das wasserreiche Al_2O_3 sowohl an die mobile Phase Wasser ab (wie aus der Literatur[40,41] bekannt und wir an der Säule nachweisen konnten) als auch durch Benzoladsorption an den Gasraum, wie GEISS[14] in einem anderen Beispiel feststellen konnte.

Die von uns an klimatisierter Al_2O_3-Säule beobachtete Entmischung des Benzol–Methanol-Gemisches (Fig. 4) erklärt recht eindeutig das Absinken der R_F-Werte mit steigendem Wassergehalt des Adsorbens. Die Fliessmittelprofile der Säule und der Platte sind sicher nicht identisch, dennoch dürften sich beide bei Änderung eines Parameters analog verändern. Die Desorption des Sulfoxide durch Benzol ist minimal, so dass diese praktisch erst mit dem Durchbruch des Methanols wandern (Fig. 5). Je schneller die Methanolkonzentration zunimmt (Anstieg der Durchbruchskurven vergrössert sich), desto kleiner wird die Differenz der R_F-Werte zwischen den Sulfoxiden mit unterschiedlichem Adsorptionsvermögen.

Zwischen 0 und 5 % r.F. bricht die dünnschichtchromatographische Trennung restlos zusammen (Inversion!), obwohl auch bei aktivem Adsorbens eine Entmischung (Gradient zwar sehr steil) auf der Säule stattfindet.

Hier dürfte in der Sandwichkammer (S-Ka) über die Gasphase eine Deaktivie-

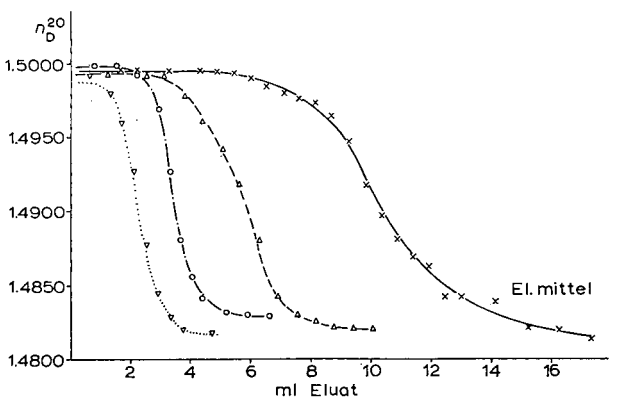


Fig. 4. Entmischungsverlauf eines Benzol–Methanol-Gemisches (9:1) an Al_2O_3-D, das bei verschiedenen Partialdampfdrücken hydratisiert wurde. p/p_0 = 0.79 (—); 0.40 (– – –); 0.10 (–·–·); 0.0 (····).

rung des Adsorbens mit Methanoldämpfen eingetreten sein. Aus gaschromatographischen Untersuchungen des Kammervolumens (S-Ka) ging hervor, dass die Methanolkonzentration über der aktiven freien Schicht des Adsorbens (Al_2O_3-D) während der Elution mit einem Benzol–Methanol-Gemisch (9:1) praktisch gleich 0 ist. (Hiermit ist bewiesen, dass die übliche S-Kammer ungesättigt ist.) Das bedeutet, dass in S-Kammern die Diffusionsgeschwindigkeit der Elutionsmittelmoleküle in den Gasraum für die auf der noch freien Schicht des Adsorbens adsorbierten Menge entscheidend ist.

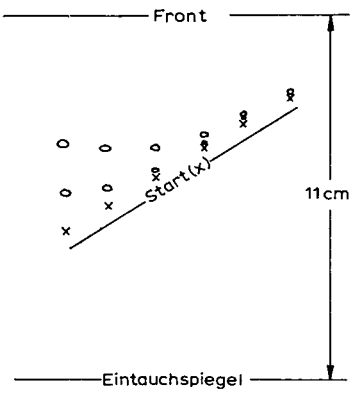


Fig. 5. Wanderungstendenzen der Sulfoxide entsprechend dem Fliessmittelprofil auf der DC-Platte. Obere Reihe: Dioktylsulfoxid; untere Reihe: Dimethylsulfoxid. Stationäre Phase: Al_2O_3-D, 40% r.F.; mobile Phase: Benzol–Methanol (9:1).

Die Diffusionsgeschwindigkeit ist bei niedrigen Methanolkonzentrationen unabhängig von derselben im Gasraum. Die Konzentrationsänderung betrug in unserem Fall (Fig. 6) durchschnittlich $2 \cdot 10^{-2}$ mg/ml·min. Daraus errechnete sich unter Berücksichtigung des Kammervolumens und der mittleren Laufzeit eines Chromatogramms die vom trockenen Adsorbens adsorbierte Methanolmenge zu mindestens 0.5 %. Diese Menge macht einen beträchtlichen Teil der Kapazität der Monoschicht aus und scheint auszureichen, einer Entmischung des Benzol–Methanolgemisches so-

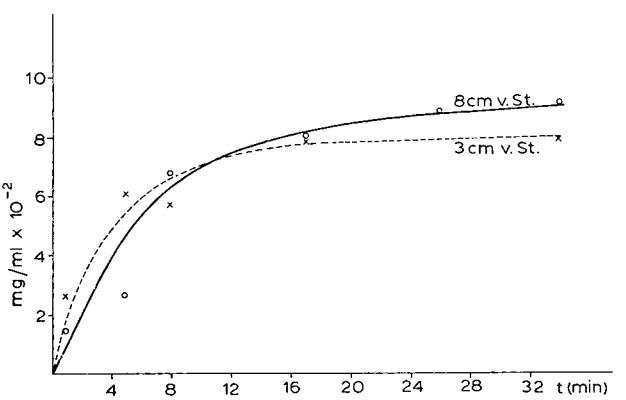


Fig. 6. Methanolkonzentration der S-Ka (ohne Adsorbens) in Abhängigkeit von der Zeit und dem Abstand vom übl. Start.

weit entgegenzuwirken, dass sich die unterschiedlichen Polarisierbarkeiten der Sulfoxide nicht mehr bemerkbar machen können.

Demzufolge erscheint uns fraglich, inwieweit es sinnvoll ist, aktivierte Adsorbentien bei Elution mit einem Fliessmittelgemisch, das eine leicht flüchtige polare Komponente enthält, einzusetzen.

ZUSAMMENFASSUNG

Die dünnschichtchromatographisch erhaltenen R_F-Werte der adsorptiv an Al_2O_3 getrennten Sulfoxide hängen ebenso wie die Entmischung des Elutionsmittels beträchtlich vom Hydratisierungsgrad des Adsorbens ab. Dennoch existieren Bereiche, innerhalb derselben man auf recht einfache Weise Retentionswerte von annähernder Konstanz (bezogen auf eine bestimmte r.F.) errechnen kann. Wie die Adsorptionsisotherme und die hieraus mittels der BET-Beziehung errechneten Oberflächen zeigen, liegt der für die Reproduzierbarkeit günstige Bereich im Gebiet der geringsten Neigung der Adsorptionsisotherme. Der Verlauf der Isotherme erklärt das chromatographische Verhalten der Sulfoxide in Abhängigkeit von der relativen Feuchte nicht vollständig; Fliessmittelentmischung und Veränderung der stationären Phase durch Fliessmitteldämpfe müssen berücksichtigt werden.

SUMMARY

The R_F values of sulphoxides, obtained by thin-layer adsorption chromatography on Al_2O_3, as well as the composition of the eluting solvent depend to a great extent on the degree of hydration of the adsorbent. There are, however, ranges within which it is possible to calculate in a very simple manner retention values that are approximately constant (with reference to a certain relative humidity). It is clear from the adsorption isotherms and the areas calculated therefrom by means of the BET equation, that the range favourable for reproducibility lies in the region where the adsorption isotherm has the least slope. The shape of the isotherm does not completely explain the chromatography behaviour of the sulphoxides as regards dependence on the relative humidity; alterations in the composition of the eluting solvent and variation of the stationary phase due to vapours of the solvent should also be considered.

LITERATUR

1 E. N. KARAULOWA UND D. G. GALPERN, *Metody analisa organitscheskich sojedinenii nefti, ich smesjej i proiswodnych, Akad. Nauk SSSR, Moskau*, 1960, S. 101–106.
2 E. N. KARAULOWA, G. D. HALPERN UND W. A. SMIRNOW, *Tetrahedron*, 18 (1962) 1115.
3 INDER JIT, *Dissertation*, Technische Hochschule für Chemie "Carl Schorlemmer", Leuna-Merseburg, Merseburg, 1965.
4 E. STAHL, *Dünnschicht-Chromatographie*, Springer, Berlin, 1962, S. 109.
5 H. G. BUNGENBERG DE JONG UND J. T. HOOGEVEEN, *Proc., Koninkl. Ned. Akad. Wetenschap., Ser. B*, 64 (1961) 18, 167, 183.
6 L. R. SNYDER, *J. Chromatog.*, 16 (1964) 55.
7 L. S. BARK, R. J. T. GRAHAM UND D. MCCORMICK, *Talanta*, 12 (1965) 122.
8 M. BRENNER, A. NIEDERWIESER, G. PATAKI UND A. FAHMY, *Experientia*, 18 (1962) 101.
9 G. PATAKI UND M. KELLER, *Helv. Chim. Acta*, 46 (1963) 1054.
10 G. PATAKI UND J. KELEMEN, *J. Chromatog.*, 11 (1963) 50.
11 J. KELEMEN UND G. PATAKI, *Z. Anal. Chem.*, 195 (1963) 81.

12 G. PATAKI, *Helv. Chim. Acta*, 47 (1964) 784.
13 F. GEISS, H. SCHLITT, F. J. RITTER UND W. M. WEIMAR, *J. Chromatog.*, 12 (1963) 469.
14 F. GEISS, H. SCHLITT UND F. KLOSE, *Z. Anal. Chem.*, 213 (1965) 321, 331.
15 G. C. HONEGGER, *Helv. Chim. Acta*, 46 (1963) 1730, 1772.
16 J. P. GAREL, *Bull. Soc. Chim. France*, (1964) 653.
17 J. PITRA, J. REICHELT UND Z. CEKAN, *Collection Czech. Chem. Commun.*, 28 (1963) 3072.
18 M. S. J. DALLAS, *J. Chromatog.*, 17 (1965) 267.
19 H. MATSUSHITA, Y. SUZUKI UND H. SAKABE, *Bull. Chem. Soc. Japan*, 36 (1963) 1371.
20 N. K. KURTSCHENINOWA, L. CH. WINOGRADOW UND R. A. SSALOWA, *Zavodsk. Lab.*, 30 (1964) 1076.
21 B. H. DAVIES, *J. Chromatog.*, 10 (1963) 518.
22 D. JÄNCHEN, *J. Chromatog.*, 14 (1964) 261.
23 R. WASICKY, *Naturwiss.*, 50 (1963) 569.
24 L. SACHS UND Z. SZEREDAY, *J. Chromatog.*, 18 (1965) 170.
25 E. PORGES UND A. PORGESOVA, *Chem. Zvesti*, 19 (1965) 497.
26 A. J. TOMISEK UND P. W. ALLAN, *J. Chromatog.*, 14 (1964) 232.
27 D. S. GALANOS UND V. M. KAPOULAS, *J. Chromatog.*, 13 (1964) 128.
28 G. ACKERMANN UND J. MICHAL, *Talanta*, 11 (1964) 451.
29 O. GLEMSER UND G. RIECK, *Angew. Chem.*, 68 (1956) 182.
30 E. B. CORNELIUS, T. H. MILLIKEN, G. A. MILLIS UND G. A. OBLAD, *J. Phys. Chem.*, 59 (1955) 809.
31 J. H. DE BOER, *Angew. Chem.*, 70 (1958) 383.
32 J. H. DE BOER, J. M. H. FORTUIN, B. C. LIPPENS UND W. J. MEIJS, *J. Catalysis*, 2 (1963) 1.
33 Z. JUHASZ, *Földt. Közl.* 93, *Sonderheft Tonmineralien* (1963) 132.
34 J. B. PERI, *J. Phys. Chem.*, 69 (1965) 211, 220.
35 A. NIEDERWIESER UND M. BRENNER, *Experientia*, 21 (1965) 50, 105.
36 H. T. BADINGS, *J. Chromatog.*, 14 (1964) 265.
37 *Gmelins Handbuch der anorganischen Chemie*, 8 Aufl., 28 (Teil B, Lief. 3), 1961, S. 684.
38 S. BRUNAUER, L. S. DEMING, W. S. DEMING UND E. TELLER, *J. Am. Chem. Soc.*, 62 (1940) 1723.
39 L. R. SNYDER, *J. Chromatog.*, 20 (1965) 463.
40 G. HESSE UND G. ROSCHER, *Z. Anal. Chem.*, 200 (1964) 3.
41 L. R. SNYDER, *J. Chromatog.*, 6 (1961) 22.

DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG ORGANISCHER SULFOXIDE UND DINITROTHIOÄTHER. EINIGE BEMERKUNGEN ZUR REPRODUZIERBARKEIT UND ZUM STRUKTUREINFLUSS

II. MITT. TRENNUNG VON SULFOXIDGEMISCHEN MITTELS EIN- UND ZWEIDIMENSIONALER DÜNNSCHICHTCHROMATOGRAPHIE

H. W. PRINZLER, H. TAUCHMANN UND C. TZSCHARNKE

Technische Hochschule für Chemie "Carl Schorlemmer", Institut für Petrolchemie, Leuna-Merseburg, 42 Merseburg (D.D.R.)

(Eingegangen den 5. September 1966)

Sulfoxide sind von ihren korrespondierenden Sulfiden und Sulfonen von ERTEL UND HORNER[1] auf Kieselgel mit Chloroform als Laufmittel, TEPPKE[2] in einem zweidimensionalen Verfahren und neuerdings von FISHBEIN UND FAWKES[3] dünnschichtchromatographisch mit Erfolg getrennt worden. Letztere Autoren geben Toluol–Äthylacetat sowie Benzol–Aceton als Elutionsmittel an.

Wir untersuchten das chromatographische Verhalten einiger repräsentativer Vertreter homologer Reihen der *n*-Alkyl- sowie *n*-Alkylarylsulfoxide an Aluminiumoxid.

ADSORPTIONSCHROMATOGRAPHIE SYMMETRISCHER UND ASYMMETRISCHER *n*-ALKYL- SOWIE DER PHENYL-*n*-ALKYLSULFOXIDE

Wie aus unserem 1. Beitrag[4] hervorging, erwies sich für die Reproduzierbarkeit adsorptionschromatographisch erhaltener Retentionswerte von Sulfoxiden (Elutionsmittel Benzol–Methanol) eine Vorklimatisierung des Adsorbens bei 20–40 % relativer Feuchte (r.F.) als günstig. Wir führten daher alle unten beschriebenen adsorptionschromatographischen Trennungen mit bei *ca.* 40 % r. F. klimatisierten DC-Platten durch. — Die Schichtdicke betrug in allen Fällen ungefähr 0.4 mm, die Laufstrecke 10 cm. Entwickelt wurde aufsteigend bei einem Neigungswinkel von 65°; die Detektion erfolgte mit 0.5 %iger wässriger Permanganatlösung (helle Flecken durch Reduktion).

Obzwar eine Mehrfachentwicklung mit Benzol–Methanol als Laufmittel eine Verbesserung bringt[5], erweisen sich Benzol–Pyridin (Vol.verh. 20:1) und Dioxan[6] für die dünnschichtchromatographische Trennung niedermolekularer Sulfoxide homologer Reihen als geeigneter.

Bei dem Fliessmittelgemisch trat während der Elution eine sichtbare Entmischung ein. Da sämtliche Substanzen unterhalb der 2. Front wanderten, wurden die R_F-Werte auf diese β-Front bezogen. Die Substanzflecke sind ebenso wie mit Benzol–Methanol als Fliessmittel durch die Gradientenwirkung des Gemisches recht klein und scharf begrenzt.

Tabelle I gibt die R_F-Werte mit dem jeweiligen Schwankungsbereich einiger

TABELLE I

R_F-WERTE DER DIALKYLSULFOXIDE UND PHENYL-*n*-ALKYLSULFOXIDE
Stationäre Phase: Al_2O_3-D; mobile Phase: Benzol–Pyridin 20:1.

Sulfoxid	$\overline{R_F(\beta)}$	S_{R_F}
Dimethyl-	0.06	0.023
Di-*n*-butyl-	0.36	0.018
Di-*n*-hexyl-	0.48	0.015
Di-*n*-oktyl-	0.62	0.014
Di-*n*-dodecyl-	0.77	0.019
Phenylmethyl-	0.41	0.013
Phenyläthyl-	0.56	0.016
Phenyl-*n*-butyl-	0.79	0.014
Phenyl-*n*-hexyl-	0.88	0.019
Phenyl-*n*-decyl-	0.94	0.018

$$S_{R_F} = \sqrt{\frac{\Sigma(R_{F(k)} - \overline{R_{F(k)}})^2}{n - 1}}$$

n = Anzahl der Messungen; $R_{F(k)}$ = R_F-Wert der Einzelmessung.

TABELLE II

R_F-WERTE ASYMMETRISCHER ALKYLSULFOXIDE AUF Al_2O_3-D

Mobile Phase	*Dioxan*		*Essigester*		*Benzol–Pyridin (20:1)*
Sulfoxid	$\overline{R_F}$	S_{R_F}	R_F	S_{R_F}	R_F
Dimethyl-	0.29	0.0	0.17	0.008	0.06
Methyl-äthyl-	0.39	0.018	0.22	0.022	
Methyl-*n*-propyl-	0.51	0.023	0.29	0.002	
Methyl-*n*-butyl-	0.60	0.005	0.35	0.008	0.23
Methyl-*n*-hexyl-	0.69	0.015	0.40	0.010	0.25*
Methyl-*n*-dodecyl-	0.72	0.014	0.44	0.009	0.27
Diäthyl-	0.57	0.008	0.35	0.002	—
Äthyl-*n*-butyl-	0.75	0.016	0.50	0.027	—
Äthyl-*n*-hexyl-	0.82	0.013	0.59	0.025	0.54**

* Methyl-*n*-oktylsulfoxid.
** Äthyl-*n*-oktylsulfoxid.

symmetrischer Dialkylsulfoxide und Phenyl-*n*-alkylsulfoxide mit Benzol–Pyridin als Laufmittel an. In Tabelle II sind die R_F-Werte von unsymmetrischen *n*-Alkylsulfoxiden mit Benzol–Pyridin (20:1), Essigester und Dioxan als mobiler Phase angeführt.

Aus den Tabellen I und II folgt, dass die Trennung der höhermolekularen Homologen adsorptiv nur unvollkommen gelingt. Hier führte die Verteilungschromatographie zum Erfolg.

VERTEILUNGS-DC (UMGEKEHRTE PHASE) IN HOMOLOGEN REIHEN DER *n*-ALKYL- SOWIE ARYL-*n*-ALKYLSULFOXIDE

Als Hydrophobiermittel wurden Cetan, Methylsilikonöl, Methylphenylsilikonöl, Trikresylphosphat, Phthalsäure-di-3,5,5-trimethylester, β,β'-Oxypropionitril und Dinonylphthalat (DNP) verwendet, wobei die Imprägnierung der Schicht mit letzterem in Form einer 5 %igen acetonischen Lösung die besten Ergebnisse lieferte. Als mobile Phase erwies sich ein Methanol–Wasser–Pyridingemisch (Vol.Verh. 5:1:1) als geeignet.

In den Tabellen III und IV sind die erhaltenen R_F-Werte angeführt. Die Reproduzierbarkeit erwies sich als gut.

TABELLE III

R_F-WERTE SYMMETRISCHER *n*-ALKYLSULFOXIDE SOWIE DER PHENYL-*n*-ALKYLSULFOXIDE
Stationäre Phase: Al_2O_3-D/DNP; mobile Phase: Methanol–Wasser–Pyridin (5:1:1).

Sulfoxid	$\overline{R_F}$	S_{R_F}
Dimethyl-	0.93	0.024
Di-*n*-butyl-	0.91	0.020
Di-*n*-hexyl-	0.82	0.015
Di-*n*-oktyl-	0.64	0.015
Di-*n*-dodecyl	0.24	0.017
Phenyl-methyl-	0.93	0.015
Phenyl-äthyl-	0.91	0.017
Phenyl-*n*-butyl-	0.88	0.011
Phenyl-*n*-hexyl-	0.82	0.020
Phenyl-*n*-decyl-	0.62	0.010

TABELLE IV

R_F-WERTE EINIGER ASYMMETRISCHER *n*-ALKYLSULFOXIDE
Stationäre Phase: Al_2O_3-D/DNP; mobile Phase: Methanol–Wasser–Pyridin (5:1:1).

Sulfoxid	R_F	S_{R_F}
Methyl-*n*-butyl-	0.90	0.023
Methyl-*n*-oktyl-	0.86	0.017
Methyl-*n*-dodecyl-	0.80	0.025
Oktyl-*n*-äthyl-	0.84	0.026
Oktyl-*n*-hexyl-	0.76	0.013
Oktyl-*n*-dodecyl-	0.38	0.018

ZWEIDIMENSIONALE DC

Wie oben beschrieben kann ein komplexes Sulfoxidgemisch weder adsorptions- noch verteilungschromatographisch restlos getrennt werden; kombiniert man jedoch beide Arbeitsweisen, so ist dies weitgehendst möglich.

Fig. 1 und 2 veranschaulichen dies an Hand der Trennung der Oktyl-*n*-alkyl- und Methyl-*n*-alkylsulfoxide sowie der Phenyl-*n*-alkyl-sulfoxide. In Fig. 2 kommt gleichzeitig die gute Trennmöglichkeit von den korrespondierenden Sulfiden zum Ausdruck.

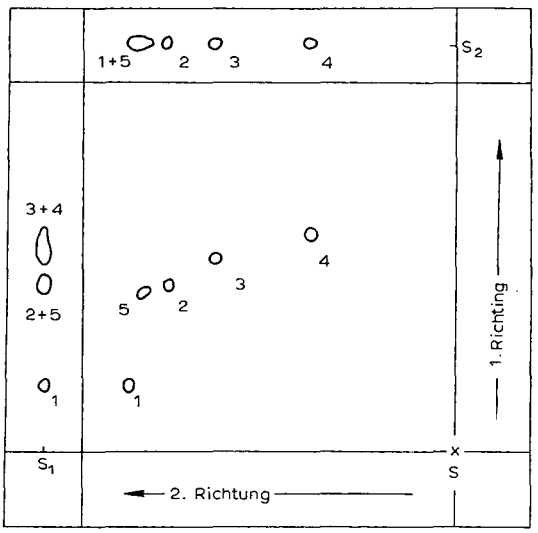


Fig. 1. Zweidimensionale Trennung in der Reihe der *n*-Oktyl-alkylsulfoxide. Stationäre Phase: Al_2O_3-D/DNP; mobile Phase: 1. Richtung: Benzol–Pyridin (20:1); 2. Richtung: Methanol–Wasser–Pyridin (5:1:1). (1) Oktylmethylsulfoxid; (2) Oktyl-*n*-hexylsulfoxid; (3) Oktyl-*n*-oktylsulfoxid; (4) Oktyl-*n*-dodecylsulfoxid; (5) Oktyl-*n*-äthylsulfoxid.

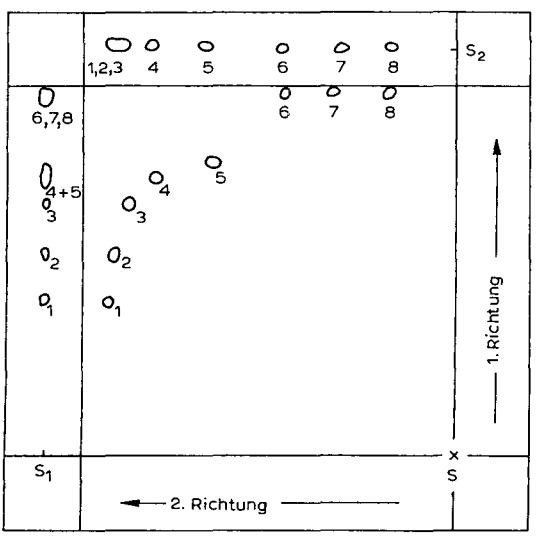


Fig. 2. Zweidimensionale Trennung der Phenyl-*n*-alkylsulfoxide und ihrer Sulfide. Bedingungen wie Fig. 1. (1) Phenylmethylsulfoxid;(2) Phenyläthylsulfoxid; (3) Phenylbutylsulfoxid; (4) Phenylhexylsulfoxid; (5) Phenyldecylsulfoxid; (6) Phenylbutylsulfid; (7) Phenylhexylsulfid; (8) Phenyldecylsulfid.

Das Gemisch wurde im Punkt S aufgetragen; S_1 und S_2 sind die Vergleichs-Starts für die jeweilige Richtung. Zunächst wurde adsorptiv mit Benzol–Pyridin (20:1) eluiert. Nach einer kurzen Zwischentrocknung wurde die Schicht mit DNP imprägniert und im Winkel von 90° zur ersten Richtung mit Methanol–Wasser–Pyridin erneut eluiert.

DISKUSSION

Obzwar sich die permanenten Dipolmomente der symmetrischen Dialkylsulfoxide nur unwesentlich unterscheiden (alternierend in der "homologen" Reihe!)[7], ist adsorptiv — natürlich abhängig von der jeweiligen mobilen Phase — eine dünnschichtchromatographische Trennung der niederen Homologen möglich. So gelingt mit Benzol–Methanol als Laufmittel eine Trennung der symmetrischen Dialkylsulfoxide nur bis zum Di-*n*-pentylsulfoxid[4], während mit Benzol–Pyridin dieselbe noch bis zum Di-*n*-nonylsulfoxid möglich ist. Die Phenyl-*n*-alkylsulfoxide lassen sich mit Benzol–Methanol nicht ($R_F > 0.60$), wohl aber mit einem Benzol–Pyridingemisch bis zum Phenylhexylsulfid trennen (Fig. 2). Dioxan erweist sich vor allem für die asymmetrischen *n*-Alkylsulfoxide als recht brauchbares Elutionsmittel, jedoch ist ab Hexylmethyl(äthyl)sulfoxid eine Trennung nicht mehr möglich.

Auch in anderen homologen Reihen (z.B. der Dinitrophenylthioäther) gelingt adsorptiv nur eine Trennung der kurzkettigen Verbindungen. Diese Feststellung traf schon MANGOLD[8] für die Dinitrophenylderivate primärer Amine.

Hier wird die polarisierende Wirkung der stationären Phase deutlich, denn mit wachsender Kettenlänge dürften die Unterschiede in der Polarisierbarkeit zwischen den aufeinanderfolgenden Homologen abnehmen und folglich nur die ersten Glieder der homologen Reihen adsorptiv mit Erfolg getrennt werden können.

Bemerkenswert ist in diesem Zusammenhang, dass die Sulfone, die gegenüber den Sulfoxiden ein weit höheres Dipolmoment aufweisen, schwächer als ihre korrespondierenden Sulfoxide adsorbiert werden[5]. Auch hier möchten wir die unterschiedliche Polarisierbarkeit für den offensichtlich verschiedenen Mechanismus der Adsorption verantwortlich machen.

Verteilungschromatographisch gelingt an DNP eine Trennung der Sulfoxide mit einer Gesamt-Kohlenstoffzahl > 12 innerhalb der homologen Reihen mit Methanol–Wasser–Pyridin als Laufmittel, ausgenommen sind hiervon die Methyl- und die Äthyl-*n*-alkylsulfoxide. Ferner dürften die R_F-Werte der langkettigen *n*-Alkylsulfoxide im wesentlichen nur von der C-Zahl und nicht oder nur wenig von der unterschiedlichen Grösse der Alkylreste abhängen. (So haben z.B. Oktyl-*n*-hexyl- (0.76) und Di-n-heptylsulfoxid (interpol. 0.74) fast gleiche R_F-Werte.)

Überall dort, wo eine destillative oder extraktive grobe Fraktionierung schwierig ist (Arbeiten mit geringen Substanzmengen), gewinnt die dünnschichtchromatographische Trennmöglichkeit von Gemischen, die kurz- und langkettige Verbindungstypen enthalten, an Bedeutung. — Die Kombination der Adsorptions- mit der Verteilungschromatographie im zweidimensionalen Trennverfahren, deren Vorteile schon KAUFMANN und Mitarbeiter[9] erkannten, ermöglicht die Analyse solcher Gemische.

Wie aus Fig. 1 hervorgeht, gelingt adsorptiv in der Reihe der Oktyl-*n*-alkylsulfoxide mit Benzol–Pyridin die Trennung der Methyl- und Äthylverbindung (verteilungschromatographisch nicht möglich), während in der 2. Richtung durch

Verteilung die Verbindungen ab Pentyloktylsulfoxid getrennt werden können. Die Phenylalkylsulfoxide lassen sich adsorptiv bis zum Phenylhexylsulfoxid (1. Richtung) und von hier ab verteilungschromatographisch (2. Richtung) trennen (Fig. 2).

Oft genügt jedoch ein Laufmittel zur Charakterisierung einer Verbindung nicht. Ebenso sind Kombinationen verschiedener Laufmittel im zweidimensionalen Verfahren angebracht.

Liegen z.B. Dialkylsulfoxide, Methyl-*n*-alkyl- und Äthyl-*n*-alkylsulfoxide sowie Phenyl-*n*-alkylsulfoxide nebeneinander vor, so könnte man mit Benzol–Methanol zunächst letztere abtrennen. In der zweiten Dimension kann man adsorptiv die kurzkettigen und zum anderen verteilungschromatographisch die langkettigen Verbindungen trennen.

ZUSAMMENFASSUNG

Die R_F-Werte repräsentativer Vertreter der Dialkyl-, Methylalkyl-, Äthylalkyl- sowie der Phenylalkylsulfoxide auf Aluminiumoxid wurden für verschiedene Laufmittel bestimmt. Benzol–Pyridin (20:1) und Dioxan erwiesen sich für adsorptive Trennungen, Methanol–Wasser–Pyridin (5:1:1) zur Trennung langkettiger Verbindungen mittels Verteilungschromatographie (stationäre Phase Dinonylphthalat) am geeignetsten. Durch Anwendung zweidimensionaler Verfahren ist eine Identifizierung der Verbindungen eines komplexen Sulfoxidgemisches weitgehendst möglich.

SUMMARY

The R_F values of representative dialkyl, methyl alkyl, ethyl alkyl, and phenyl alkyl sulphoxides were determined on aluminium oxide for various eluants. It was found that benzene–pyridine (20:1) and dioxane were most suitable for adsorption chromatography, while for the separation of long-chain compounds by partition chromatography (stationary phase dinonyl phthalate) a mixture of methanol–water–pyridine (5:1:1) could best be used. By using a two-dimensional procedure, the identification of the components of a complex mixture of sulphoxides is largely possible.

LITERATUR

1 H. ERTEL UND L. HORNER, *J. Chromatog.*, 7 (1962) 268.
2 M. TEPPKE, *Diplomarbeit*, Technische Hochschule für Chemie "Carl Schorlemmer", Leuna-Merseburg, Merseburg, 1964.
3 L. FISHBEIN UND J. FAWKES, *J. Chromatog.*, 22 (1966) 323.
4 H. W. PRINZLER UND H. TAUCHMANN, *J. Chromatog.*, 29 (1967) 142.
5 H. W. PRINZLER, D. PAPE, H. TAUCHMANN, M. TEPPKE UND C. TZSCHARNKE, *Ropa a Uhlie*, 8 (16) (1966) 13.
6 H. BURAU, *Diplomarbeit*, Technische Hochschule für Chemie "Carl Schorlemmer", Leuna-Merseburg, Merseburg, 1966.
7 C. W. N. CUMPER, J. F. READ UND A. I. VOGEL, *J. Chem. Soc.*, (1965) 5323.
8 K. MANGOLD in E. STAHL *Dünnschichtchromatographie*, Springer-Verlag, 1962, S. 108.
9 H. P. KAUFMANN UND Z. MAKUS, *Fette, Seifen, Anstrichmittel*, 62 (1960) 1014.

THE QUANTITATIVE ESTIMATION OF SUBSTANCES ON PAPER CHROMATOGRAMS

II. AN APPARATUS FOR THE RAPID QUANTITATIVE PHOTOMETRY OF PAPER CHROMATOGRAMS

I. E. BUSH

The Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, Mass. 01545 (U.S.A.)

(Received November 28th, 1966)

INTRODUCTION

The general rationale of direct quantitative estimation of substances on paper chromatograms has been discussed in the first paper of this series[1] and in earlier articles[2,3]. It is well-known that this method, commonly known as the "scanning" of paper chromatograms, fell into disrepute around 1954 because of the apparently insuperable difficulties encountered in attempts to reduce its errors to acceptable limits (see *e.g.*, refs. 2 and 4). In 1954, the author discovered numerous elementary mechanical and optical errors in a commercially available manual scanner for paper electropherograms and decided to reinvestigate the method[5]. This investigation led to a useful method for determining reducing steroids on paper chromatograms[6] and to the design and construction of a mechanically driven scanner which has been described previously but incompletely[2,3,7]. Although this apparatus is now over seven years old, it seems to possess numerous advantages over any commercially produced instrument available at present, and, in some aspects at least, to surpass other published instruments of this type (see Discussion). Although designed to be operated in conjunction with the chromatogram-processing machine described in the first paper of this series[1], it can be used quite independently whenever necessary or convenient. This paper will be devoted to a description of the apparatus, some variations that have been tried out, and to the general principles governing its design and method of operation. Special details of chemical techniques and solid-state versions of the electronics will be reserved for later papers in this series.

CONSTRUCTION OF THE APPARATUS

The apparatus consists of three main sections, the light-source and monochromator; the paper tracker and photomultiplier (the scanner proper); and the electronic recording apparatus.

(1) *Light source and monochromator*

The monochromator is based on the optical components and light path of the

Unicam (Cambridge, England) S.P. 900 Flame Photometer. A variety of light sources have been used, including tungsten lamps (6 or 12 V d.c.) for absorptiometry in the visual range of wavelengths, mercury arc for ultraviolet-excited fluorimetry, and a.c. and d.c. xenon arcs (Osram-Neco, XBO-150) for both absorptiometry and fluorimetry. The most reliable arrangement and the one used for the greater part of the working life of this scanner is described here.

The components of the S.P. 900 Flame Photometer's monochromator were secured by their usual mountings to an aluminum alloy base plate (0.635 × 47 × 36 cm). The sides and a top were fitted to the base to form a closed light-proof box (Fig. 1). For reasons of economy simple wedges and cams were used to control the width of home-made slits (Fig. 2), and the angle of the Littrow mirror required for wavelength selection (Fig. 3). A double quartz lens condenser (diameter 5.1 cm, focal length 5.1 cm) and plane diagonal mirror (7.5 × 5.1 cm) were mounted so as to form an image of the exit slit (S_2, Fig. 1) on the paper strip, in the horizontal plane 3.3 cm above the top surface of the lid which was pierced with a 5.1 cm diameter hole (Fig. 1c, H_2) on the axis of the diagonal mirror.

The light source used most frequently was a xenon arc (a.c., 375 W, Westinghouse type now obsolete, and later a d.c. arc, type XBO-150, Osram-Neco). This was mounted vertically using a large Tufnol (bonded plastic) base (0.635 × 15.2 cm diameter) to insulate the anode terminal from a subsidiary aluminum alloy base plate. The lamp was surrounded by a stout cylindrical metal housing perforated with ventilation louvres and supporting a crude focussing mirror made by aluminizing a 10.2 cm diameter watchglass. The outlet hole was directed toward the inlet slit (S_1, Fig. 1) and the light path between the two was covered by a removable metal cover. The igniter-circuit and power-supply to the xenon arc were mounted on the side of of the lamp away from the monochromator.

(2) *Paper-tracker and photomultiplier housing*

This was constructed as a separate unit which could be lifted on and off the lid of the monochromator (Fig. 3). Its optical axis was aligned with that of the diagonal mirror of the monochromator by the adjustment screw AS (Fig. 1b).

The photomultiplier (PMT) was housed in a bushing holding the base-connector, which slid into the side of the brass cylinder C (Fig. 3). The dynode resistances were soldered directly to the pins of the base-connector and covered with a light aluminum cover made out of a commercially available can. The main brass cylinder (C) was fitted to the top of the paper-tracking device by pressing it into a snugly-fitting hole in the well (W) of the latter.

The main frame of the paper-tracker consists of two 0.635 cm thick aluminum side plates (P, Fig. 3) fixed to aluminum spacing rods (8.1 cm × 2.5 cm diameter,

Fig. 1. Monochromator. (a) Plan view showing layout of main optical components and the position of the paper-tracking unit (1) and its photomultiplier housing (2). (b) Elevation of monochromator and paper-tracking unit. (c) Vertical section through axes of slits S_1 and S_2 to show arrangement of cross-beam (B) holding them, the condenser L_1 and the diagonal plane mirror, M_1. S_1 = entry slit; S_2 = exit slit; D_1 = first diagonal; D_2 = second diagonal; CM_1 = first collimating spherical mirror; CM_2 = second collimating spherical mirror; P = dispersing prism; LM = Littrow mirror (plane); L_1 = condenser; WS = wavelength adjustment knob; AS = longitudinal adjustment screw; H_1 = hole and cover for collimation of source S_1, S_2 and L_1; H_2 = hole in lid of monochromator below paper-tracking unit.

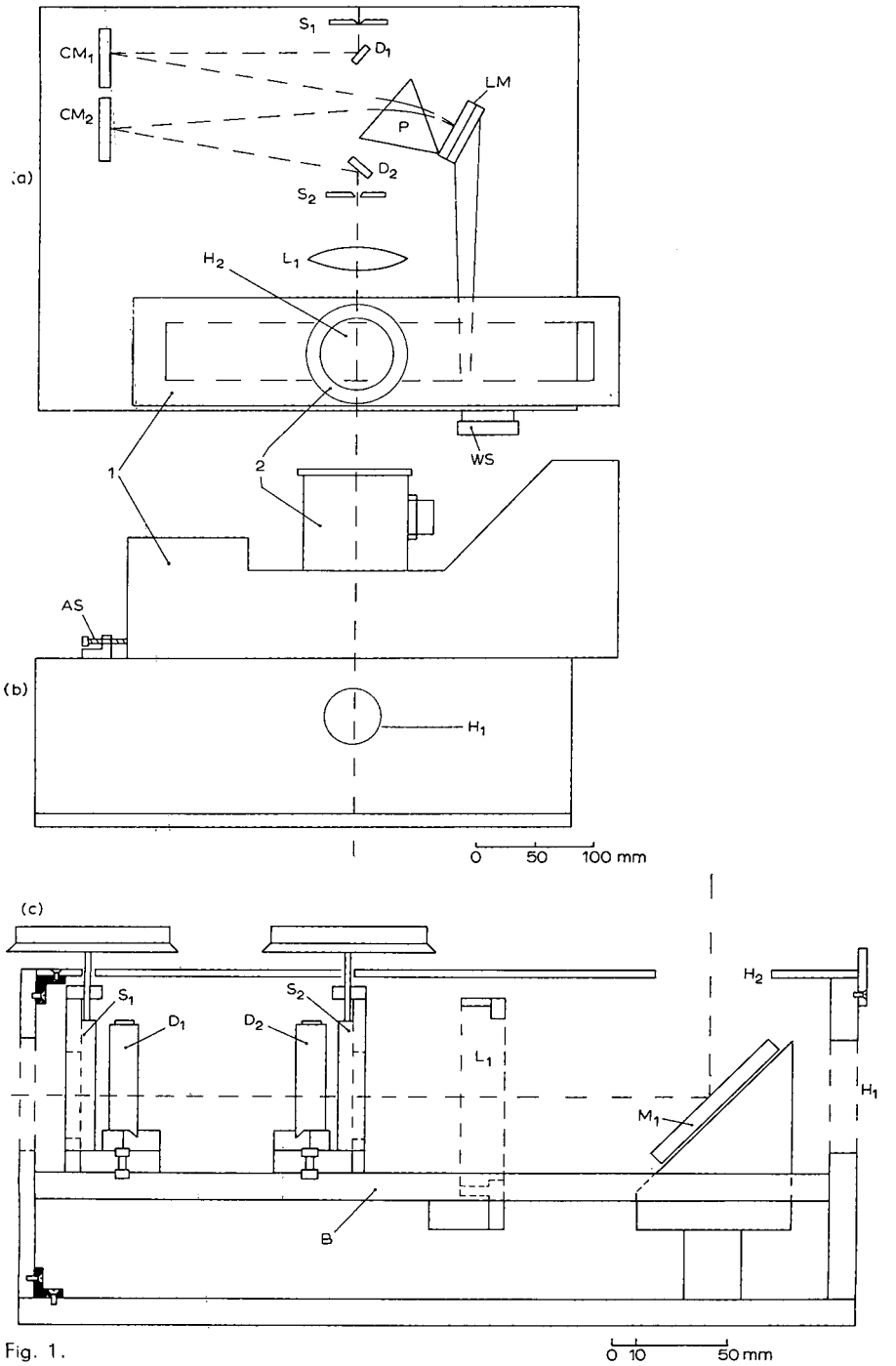

(a)
S1
D1
CM1
CM2
LM
P
D2
S2
H2
L1
WS
1
2
AS
(b)
H1
0 50 100 mm
(c)
S1
D1
S2
D2
L1
H2
H1
M1
B
0 10 50 mm

Fig. 1.

SP, Fig. 3; and 8.1 × 0.95 cm diameter, sp, Fig. 3). Knurled rollers (R_1–R_3, Fig. 3) drive paper strips 5.0–5.2 cm wide by means of spring-loaded idling rollers (r_1–r_3, Fig. 3) the paper sliding in the guide-rails (G) milled from aluminum alloy. The paper enters at R_1—r_1 *via* a metal guide chute (Fig. 4) and is ejected by rollers R_3—r_3. The correct direction and identical speeds of rotation of the drive-rollers are obtained by a train of fiber spur gears. These are fixed to the outside face of the tracker by spongy-bronze bearings on stub shafts screwed to the (back) main side-plate P.

The track of the paper is straight and horizontal in the region around the optical axis of the scanner and the guides G are supplemented by cross-plates and diaphragms (– – –, Fig. 3) to minimize the entrance of extraneous light. The end-plate of the tracker (EP, Fig. 3) is extended upwards to form the main body of the entrance paper-guide (Fig. 5). A flap of black paper closes the exit guide of the device and is lifted by each strip of paper as it emerges.

The front main side-plate (P_1) is pierced by two rectangular holes (3.8 × 0.6 cm) centered on the optical axis of the scanner which hold the slits and auxiliary filter holders S_3 and S_4 (Fig. 3). The well W whose upper ridged circumference receives the PMT housing C has a ridge machined at its lower end on which can be mounted the collimating lens L_2 and auxiliary optical components, including secondary filters for fluorimetry, and the field-stop FS.

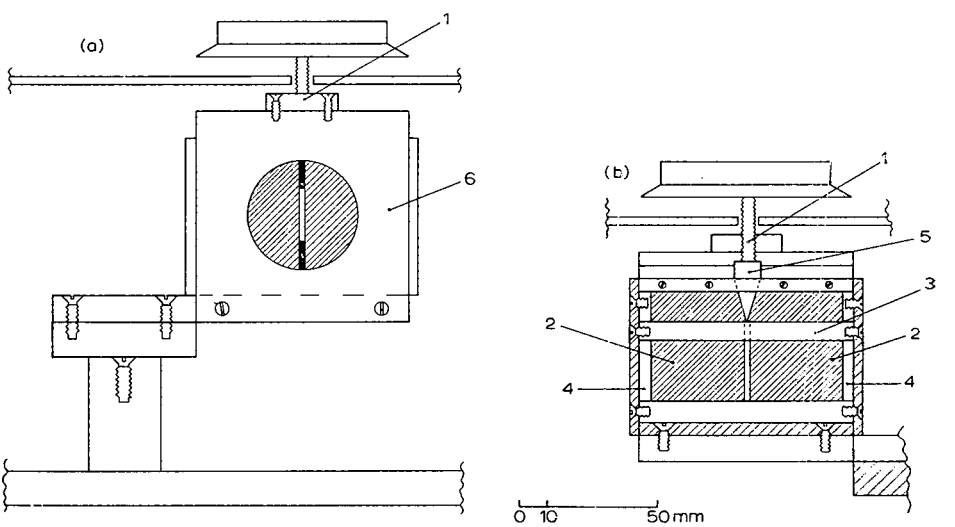


Fig. 2. Slit mechanism. (a) Incident and (b) emergent view of home-made slits for the monochromator. See Fig. 1c for sectional view and mounting beam. 1 = Screw adjustment with knob; 2 = jaws of slit; 3 = retaining cross-bar; 4 = flat springs; 5 = wedge driving slits apart; 6 = main plate supporting mechanism. In order to obtain a linear final image on the paper, the exit slit (S_2, Fig. 1a) was made linear and the entry slit (S_1, Fig. 1a) machined to a curve obtaining full correction of spherical aberration.

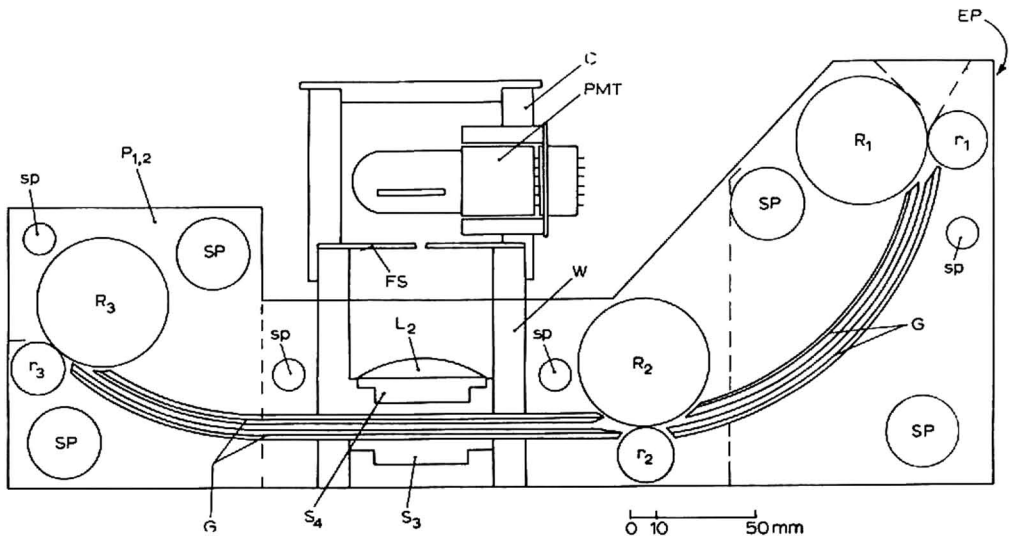


Fig. 3. Paper tracking-unit and photomultiplier housing. Diagrammatic elevation and section. See text for details. The entry guide for the paper strips is omitted from this view but is seen in Fig. 5.

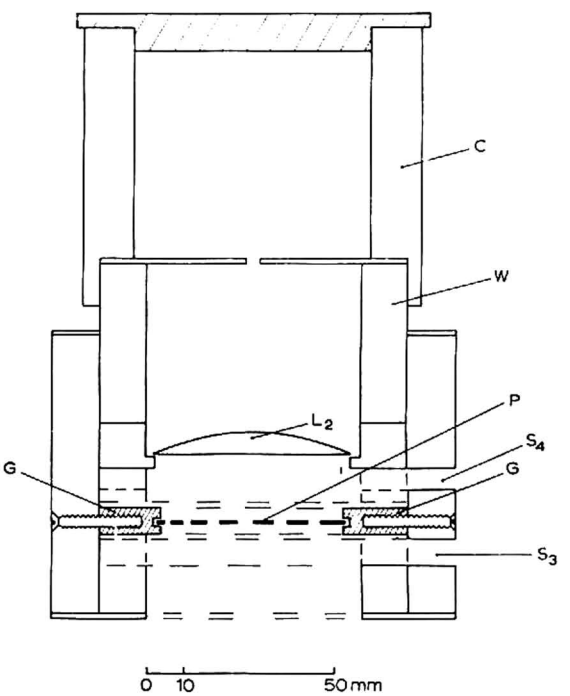


Fig. 4. Cross-section of photomultiplier section. C = Photomultiplier housing; W = well for lens, etc.; L_2 = collimating lens; G,G = guide rails for paper strip; P = paper chromatogram strip; S_3,S_4 = slits for second slits, filters, etc.

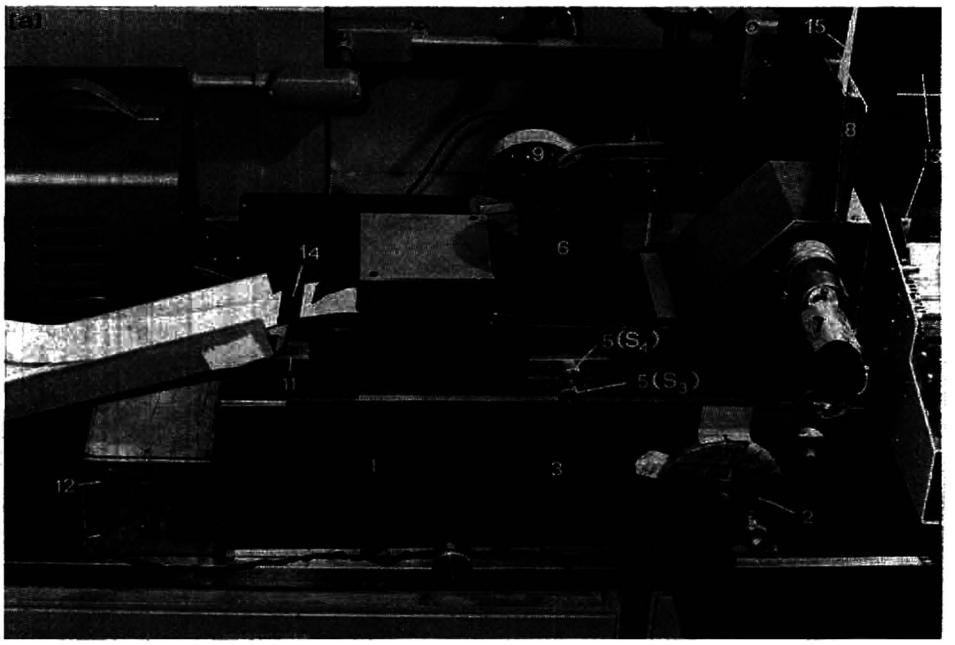


Fig. 5a. General view of the scanner. 1 = Monochromator; 2 = wavelength control; 3 = collimating aperture (covered) (H_1); 4 = paper-tracking unit; 5 = secondary slits (and filter) holder (S_3,S_4); 6 = photomultiplier housing (C); 7 = synchronous motor driving R_1; 8 = inlet guide attached to EP; 9 = tungsten lamp; 10 = stabilized rectifier and power supply for lamp; 11 = adjustment screw; 12 = magnetic clutch power supply for motor drive; 13 = recorder and electronic apparatus (see Fig. 5b); 14 = train of chromatograms emerging from scanner; 15 = last of the train entering scanner; 16 = one of fiber gears in train linking drive from R_1 to R_2 and R_3. Letters in brackets refer to codes of Fig. 3.

The top and ends of the tracker are closed with thin (approx. 0.2 cm thick) aluminum alloy plates screwed to the side-plates P_1 and P_2. These and other metal–metal joints were sealed internally before assembly with matt-black paper or felt, glued or taped to the metal. A cross-section of the PMT housing, paper guides and related parts is shown in Fig. 4.

(3) Electronic and recording apparatus

Various arrangements have been used successfully but all of those used for absorptiometry have been based on the circuit of SWEET[8], the output of which is approximately a linear function of the logarithm of changes of light intensity. Changes of output are thus directly proportional to changes in the optical density of media placed in the light path.

Earlier versions employed a tube circuit very similar to that of SWEET with an RCA-931A photomultiplier. This gave signals from chromatographic zones of average intensity in the range 0–100 mV which were recorded by a Sunvic (A.E.I., London, England) RSP2 potentiometric chart-recorder with a two-channel digital integrator. More recently we have used GORDY's solid-state version of SWEET's photometer[9] and used the output to drive a Bausch and Lomb VOM-6 potentiometric chart recorder with the range setting 0–2.5 mV. In this case the photomultiplier was the RCA-IP-21.

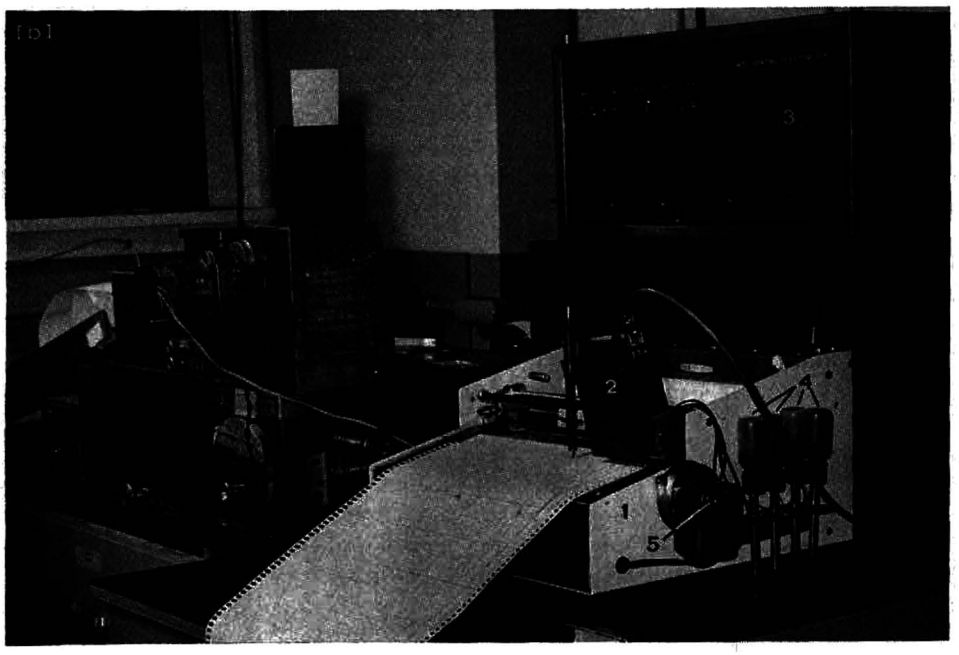


Fig. 5b. View of scanner with recorder and associated electronics. 1 = Heathkit EUW-20A chart recorder; 2 = 4-pen integral output unit; 3 = cabinet containing Sweet logarithmic output circuit, voltage-to-frequency converter, decade dividers, Schmidt triggers and pen drivers; 4 = ink wells; 5 = motor exteriorised to provide high-speed gearing to chart-drive.

Full details of the circuitry are given by GORDY *et al.*[9] In order to bring the output of GORDY's instrument on to the working range of the chart-recorder, however, an external backing-off voltage must be applied. This is conveniently provided by a standard mercury cell with a potential divider such as is commonly used for standardizing potentiometric recorders (*e.g.*, Eveready, E42N, 1.35 V). This is connected in the appropriate sense in either of the output leads to the recorder.

The most convenient arrangement is to place a good variable potentiometer of high precision (0–1000 Ω) in the output lead of a Sweet-type circuit as a potential divider. A smooth continuous variation in sensitivity is then obtainable so that an optical density change of 0.3 above the paper background which is nearly the maximum that can be measured reliably in practice, can be used to generate an output signal of up to 100 mV. A wide variety of commercially-available potentiometric chart-recorders can then be used with the apparatus.

Two aspects of the electronic apparatus are especially important. The paper is driven at 1 cm/sec through the tracker so that fast recorders are needed if accurate results are to be achieved. We have found that the A.E.I. Sunvic RSP2 specially modified to give full-scale deflection (f.s.d.) in 0.5 sec, the Bausch and Lomb VOM-6 rated at an f.s.d. of 0.5 sec, and to a lesser extent the Heathkit EU20A rated at an f.s.d. of 1.5 sec give reasonably good reproducibility with scanning speeds of 1 cm/sec and paper chromatograms 40–50 cm long. All these recorders however are extremely sensitive to stray a.c. noise so that it has usually been necessary to connect a capa-

citor of 200 μF across the input terminals of the recorder. Recently, trials with an Esterline Angus Lab Graph (Series S) recorder with an f.s.d. time of 0.2 sec have given the best results we have obtained so far.

A second important feature of the recording apparatus has been the provision of a cumulative integral record in digital form in parallel with the direct record of optical density. The A.E.I. Sunvic RSP2 provides an integral output in the form of two pens acting as "event markers" along one side of the chart. The solenoid-driven pens are fired by pulses from an inertia-less "integrating motor" which is driven by a voltage supplied from a retransmitting slidewire mounted on the same drum as the servo-driven potentiometer of the recorder itself. The rate of firing is directly proportional to the voltage provided by the retransmitting slidewire and hence to the voltage being recorded. The two pens are arranged to fire units and tens up to a certain level, above which the unit pen changes over to fire in hundreds.

The most successful arrangement has been a four-channel integral marker (units, tens, hundreds and thousands) which was driven, *via* a chain of decades, either by an external photoelectrically based integrating motor of very low inertia (Series 5300 Integrator, Electromethods Ltd., Stevenage, Herts., England), or by a solid-state voltage-to-frequency converter of the type described by HOWARD[10] driving the 4-pen unit *via* suitable dividers, decades and pen-drivers, the effective voltage being drawn from a retransmitting 1000 Ω potentiometer mounted on the servo-potentiometer of the Heathkit EU20A recorder, and a stabilized d.c. source.

All the electronic circuits that have been used with the scanner over the last eight years have been simple adaptations of standard or published apparatus. In the last year, solid-state modifications have been developed which offer considerable advantages in stability and reliability. This final version of the electronic system will be described in detail in the next paper of this series.

METHOD OF OPERATION

Collimation (*See Fig. 1*)

The light source is placed on the optical axis of the monochromator by sighting the (unlighted!) arc through the centers of the slits S_1 and S_2 *via* the hole H_1. The diagonals D_1 and D_2 are now inserted and the arc lighted. The focussing mirror of the lamp-house is then adjusted to give the best available diffuse image of the arc covering or filling the slit S_1. Vertical alignment is now checked and corrected if necessary by caliper measurement of the heights of the centers of the arc and of S_1. The components of the monochromator are now aligned in the usual way using card diaphragms marked with a convenient graticule to center images and beams on each component in the optical pathway. The lens L_1 is next aligned *via* the hole H_1. The diagonal mirror M_1 is now inserted and adjusted until a sharp image of the exit slit S_2 is obtained normal to the axis of the paper strip at the calculated position above the hole H_2. The lid of the monochromator casing is then placed in position and the diagonal mirror M_1 is now re-aligned if necessary, by centering the diffuse image of S_2 on a ruled paper diaphragm placed in the exit hole of the lid. The cover plate for H_1 is then fixed in place.

The collimation of the monochromator and light source is now checked by selecting a wavelength close to 580 mμ (yellow) and observing the image of S_2 with the eye close to the image plane. The eye is then moved to and fro along the image. At

this wavelength any serious misalignment is readily detected as vignetting at either end of the image, or as a change of apparent color over the length of the image.

The visual range of the monochromator can now be calibrated approximately on the wavelength dial by matching the color of the image with standard filters. If the standard wavelength drum control of the Unicam SP 900 is purchased, one adjustment with yellow light of 585 mμ is sufficient at this stage.

The scanner is now placed on the lid of the monochromator casing and 4.8 × 0.5 mm slits placed in the slit holders S_3 and S_4 (Fig. 3). Approximate alignment is now obtained by removing the PMT housing and looking down through S_3 and S_4. The screw (Fig. 1b) is now turned until the position of the scanner secures maximum illumination *via* S_3 and S_4, and the PMT housing is placed in position.

The final adjustment of the scanner and the PMT itself is made after checking the electronic equipment and will be described later.

Adjustment of the electronic equipment

If the SWEET circuit is based on electronic tubes it should include a delaying relay which switches on the E.H.T. supply only after the heater circuit of the power tetrode has brought the cathode to full emission[8], and preferably a fast safety cut-out relay which switches off or reduces the E.H.T. whenever the anode current from the PMT exceeds a safe threshold. This protects the photocathode from damage due to failure of the feedback controlling the dynode voltages, or to its possible inadequacy during accidental exposure to strong light. In any event it is best to minimize the light reaching the photocathode whenever the scanner is being adjusted or left to warm up by inserting a blank strip of filter paper in the scanner or by reducing the widths of S_1 and S_2 to less than 0.1 mm.

A blank strip of paper is therefore fed into the scanner by hand until the leading edge has just emerged from the exit rollers R_3—r_3 (Fig. 3). The slits S_1 and S_2 are now reduced to 0.3 mm width and a convenient visual wavelength selected on the monochromator dial. The power supplies to the SWEET circuit and to the potentiometric recorder are then switched on, and the usual period of warming-up is allowed to elapse if tube circuits are used.

The backing-off voltage is now adjusted to bring the potentiometric recorder to a reading of approximately 10 % of fullscale. (It is assumed that the recorder itself has been adjusted previously.) If the potentiometer controlling the backing-off voltage has to be turned too far towards either extreme of its range the slits S_1 and S_2 must be adjusted until the recorder can be brought to near zero without employing an extreme position of the backing-off potentiometer.

Using Whatman No. 2 or 3MM paper in the scanner, it will usually be found that a zero adjustment of the chart recorder will be obtained with slit widths (S_1 and S_2) of approximately 0.6 mm in the wavelength range 450–600 mμ.

The sensitivity is now reduced to a minimum by means of the potentiometer forming the potential divider in the output of the SWEET circuit. A small adjustment of the backing-off voltage may be required to zero the recorder. It will then be found that complete closure of the slits S_1 and S_2 produces a small or negligible increase in the reading of the chart recorder. The sensitivity control is then turned up until, after zeroing with the backing-off potentiometer, complete closure of the slits S_1 and S_2 just produces a full-scale deflection of the chart recorder. This setting of the sen-

sitivity control is then recorded or marked as the *lowest* practicable sensitivity of the instrument as a whole.

Because of the large background optical density of filter paper (see later) a check should be made before every use of the apparatus that the instrument is adjusted to be on the working range of the feedback controlling the dynode voltage of the PMT. Thus for wavelengths distant from the optimum of the photocathode's response the slits S_1 and S_2 must be widened so that the recorder is brought to zero with approximately the same backing-off voltage as obtained for the optimum wavelength. If the adjustment required to zero the recorder on changing wavelength is carried out entirely by changing the backing-off voltage, it is often found that the maximum response on closing slits S_1 and S_2 is less than 25 % of full-scale even at high sensitivity. The simplest way of ensuring that one is indeed on the working range of the instrument is to ensure that with the estimated starting slit-width and backing-off voltage needed to zero the recorder, a full-scale deflection is achieved by reducing S_1 and S_2 to less than 0.1 mm width.

Final optical adjustment

The recorder is brought to 50 % full-scale by reducing the width of S_1 or S_2. The position of the scanner is now adjusted with the screw (Fig. 1b) until a *minimum* reading is obtained on the recorder. If the original alignment was very faulty, or if the sensitivity setting is very high, readjustment of the backing-off voltage or further reduction of slit width (S_1 and S_2) may be needed to keep the recorder pen above zero.

On completion of this adjustment, the photomultiplier housing C is rotated in the well W to achieve a minimum reading, and finally the same manoeuvre is carried out rotating the PMT bushing (Fig. 3). These adjustments bring the axes of slits S_3 and S_4 (Fig. 3) on to the optical axis of the emerging beam of the monochromator, and the photomultiplier into the orientation securing optimal illumination of the photocathode. This minimizes the slit widths of S_1 and S_2 required to secure the full working-range at any wavelength, and maximizes the range of wavelengths that can be used with any given photomultiplier.

Checking the performance of the instrument

The machine is designed to give a signal proportional to the optical density of an absorbing zone on a paper strip and to produce a record on which the areas of peaks are directly proportional to the quantity of substance producing the peak. The sources of error are thus as follows:

(1) Limitations on the validity of the Beer–Lambert law as applied to absorbing materials deposited in a relatively thin medium of variable thickness and heterogeneous refractive index.

(2) Variations with time of the intensity, orientation, and wavelength of the light irradiating the chromatogram.

(3) Deviation of the output of the SWEET circuit from a strictly linear function of the logarithm of the intensity of light reaching the photocathode.

(4) Errors in the linearity and stability of the potentiometric chart recorder.

(5) Non-linearity of the integrating system (*i.e.*, either of the retransmitting slidewire or of the voltage-to-frequency converter itself).

(6) Time-lag in response of any part of the circuitry due to the large velocity at which the paper is scanned (1.0 cm/sec or 10–20 "effective zones"/sec).

(7) Variations with time of the velocity of the chromatogram through the scanner.

(8) Variations of chart-speed with time in the absence of a direct, time-controlled integrating system.

The first source of error is not an instrumental one and the features of the instrument designed to minimize the optical factors in this source of error will be discussed later.

As long as satisfactory mechanical rigidity has been assured by the usual means the second source of error can be checked by running the chart-recorder at a slow speed with the instrument switched on at high sensitivity and a stationary strip of paper in the scanner. Assuming that the stability of the SWEET circuit and the recorder have been checked and confirmed, any drift or more rapid shifts in the chart record will indicate instability of the illuminating system. The most likely cause of this is arc-jumping or variations in power supplies with xenon arcs, and filament vibration, overrated lamps, or variations in supply voltage with tungsten sources. The effects of arc-jumping and filament vibration are minimized by using the crude system described above to focus the arc on the slit S_1 which produces a diffuse image which is approximately 1.5 times as long, and perhaps as much as 10 times the width of S_1.

It is difficult to stabilize xenon arcs and much experience suggests that the more elaborate types of current-stabilizers (N.B. voltage stabilization is not adequate with these arcs) are not usually worth the moderate advantages they possess over the conventional stabilized power-supplies usually recommended by the manufacturers. Stability over periods of a few minutes is adequate since each chromatogram only takes 40–60 seconds to scan and average peaks are completely scanned in 2–5 seconds. It has usually not been difficult to secure stability to within $\pm$ 2 % full-scale deflection for periods of 30–60 minutes (equivalent to 40–80 chromatograms scanned) with conventionally stabilized xenon arcs, and to within $\pm$ 0.05 % with stabilized d.c. tungsten lamps.

The linearity of the response of the SWEET circuit has been discussed fully before[8,9]. Almost any desired degree of linearity can be achieved, although different types of photomultiplier design can affect the relative ease of doing so. The instrument described here was checked by using neutral grey filters of optical densities 1.00, 2.02, 3.01, and 4.01 which had been standardized by the National Physical Laboratory, Teddington, England. The three circuits that have been used during the seven and a half years' operation of the instrument were linear to within $\pm$ 2 % or better over the range optical density 1.0–4.0. Since the working range *with chromatographic zones* is usually only 0.5 optical density units or less (see later) and the effect of this error is reduced by a reasonable standard of uniformity of the methods of preparing and running paper chromatograms, this error is of small influence on the overall precision of the instrument.

The linearity and stability of the chart-recorders has been checked by standard electronic means. The linearity of the integrating system and its effective calibration curve was checked by feeding stable voltages to the recorder and running the chart for periods of 10–15 sec at each voltage. The integrating motor (Electro Methods Ltd.,

Series 5300) gave excellent results using a 1000 Ω retransmitting potentiometer and although rated at $\pm$ 1 % usually gave results within 0.5 % of linearity (Fig. 6).

Time-lag in the recorder is checked easily by running through the scanner a test chromatogram containing a moderately dense zone of light-absorbing substance, using a sensitivity-setting that gives 80–90 % full-scale deflection. The chromatogram is then passed through the scanner manually and positioned to give the maximum optical density of the peak. With the strip stationary in this position the chart is run for a few seconds to record this density as a short straight line. If the time-response of the system as a whole is adequate this reading will equal that obtained at the peak when the chromatogram was scanned at the normal operating speed. If the moving peak gives a result more than 1–2 % below the stationary recording the time-response of the recorder is probably at fault and should be checked electronically. If the recorder performs at its rated response-speed on a noise-free source pulse it is probable that the output of the SWEET circuit is conducting a.c. noise. If a simple filter fails to eliminate this (*e.g.*, a 20–100 μF capacitor across the output of the circuit) the nature and source of the a.c. interference should be sought with an oscilloscope in the usual fashion.

The source of a.c. interference is often eliminated by screening or improved earthing, but may sometimes be due to mains ripple or higher frequency noise in the power-supply to the xenon arc. Ordinary 50–60 cycles per sec mains ripple should not seriously affect a recorder protected by the simple filter described above. A "noisy" mains supply, however (*e.g.*, one supplying a large number of unsophisticated motors in a building), can produce serious effects, either directly *via* power supplies or stray capacitances to the circuitry, or indirectly *via* the xenon arc which is capable of "following" very high frequency oscillations of current.

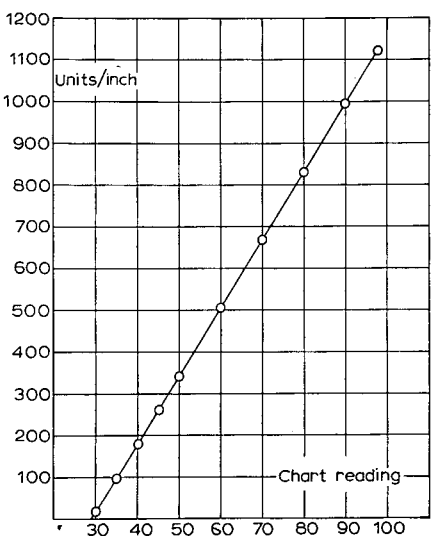


Fig. 6. Calibration curve for integrator. The input to the recorder was short-circuited and the pen manually placed at a desired chart-setting (zero at 28). The chart was then run at the usual working speed until approximately 10 cm of record had been obtained. This was repeated at various chart settings. The integrator's counting rate per length of chart was then obtained by counting over a minimum of 7.6 cm. (The results are expressed in inches (2.54 cm) which are the units used for the readings on the printed chart.)

The speed of the paper through the scanner is easily checked by ruling pencil lines at known intervals across a strip of Whatman No. 2 paper and repeatedly passing it through the scanner. If the speed of the chart of the recorder has been checked independently (*e.g.*, with a stroboscope or with an electronic pulse generator triggering pen) one can then measure the intervals between the pen deflections obtained from the pencil marks on the strip of chromatography paper. This suffices to provide estimates of the velocity of scanning and its variance. Using two different synchronous motors over the past seven and a half years, the variance has never exceeded 0.5 % for 10 cm intervals and has in fact been too small to measure precisely by this method. Any larger errors over intervals of this size would probably be the result of eccentricity or other machining faults in the main driving rollers of the scanner.

When these several characteristics and sources of error have been checked independently, the overall performance of the apparatus has been assessed by repeatedly scanning single test chromatograms containing zones of small, moderate and large optical density. It is best to test both moderately broad zones near the middle of such strips, and also the sharper zones found nearer the origin. Tests of this kind have invariably shown an unexpectedly high degree of reproducibility, suggesting that despite the numerous potential sources of errors in the whole system, the sources of systematic errors are reasonably stable and that the rest are truly random and mutually cancelling in their overall effect. Thus, even when the Heathkit EUW20A Recorder which is the slowest that has been used (f.s.d. rated at 1.5 sec), the standard deviation of the integrated value of peak area in five repeated scans of a moderately sharp peak (cortisone, estimated with alkaline blue tetrazolium) giving 75 % f.s.d. at the peak was only 0.87 % of the mean.

Absorptiometric characteristics of paper chromatograms

When off-axis scattered light is reduced to a minimum by the use of the slits S_3 and S_4, the collimating lens L_2, and the field stop (Fig. 3), the optical density of untreated filter paper (Whatman No. 2) was found to be approximately 2.5 in yellow light (585 mμ). This was measured as follows. The filter paper strip was fed into the scanner by hand and the recorder brought to 25 % f.s.d. with the backing-off potentiometer using a low sensitivity setting. The paper was then removed, and standardized neutral grey filters of optical density 2.02, 3.01 and 4.01 inserted between S_3 and S_4 (Fig. 3). The signals recorded on the chart for each filter were measured and plotted as an absolute calibration curve of optical density. The optical density of the paper strip was then read from this calibration curve. To overcome small errors due to variable positioning of the neutral filters these measurements were repeated three or four times for each filter and paper strip. The background optical density of a strip of Whatman No. 2 paper treated with alkaline blue tetrazolium[6] was found by this method to be approximately 2.64 (range $\pm$ 0.01, four measurements). Untreated Whatman 3MM paper gave an optical density of 2.79 (range $\pm$ 0.005, three measurements).

Using the same procedure the optical densities of some typical colored zones on test chromatograms were measured. A 45 μg zone of tetrahydrocortisone approximately 18 cm from the origin of a 45 cm long chromatogram, filling the width of a 5.08 cm wide strip of Whatman No. 2 paper, and approximately 2.8 cm long (head to tail) was revealed with alkaline blue tetrazolium by the method of BUSH AND WILLOUGHBY[6]. The optical density at the peak's maximum was 0.19 optical density units above back-

ground. Subsequent use of GORDY's circuit which provides a direct meter readout in optical density units has confirmed these results. Zones containing approximately 80 μg of cortisone represent the upper limit of linearity of the effective calibration curve for this method. Zones of 2 μg represent the minimum quantity that is reliably detected on scanning records made at a sensitivity giving 90% full-scale deflection with 50 μg zones.

The scanning records at various sensitivity settings of an untreated strip of Whatman No. 2 paper are shown in Fig. 7. The record is characterized by a continuous series of short period fluctuations (approx. 6 cycles per cm of paper), less regular fluctuations in which cycles of 1–3 per cm predominate, and very irregular fluctuations 2–8 cm in length. If scanning records of the same strip of paper are made at different wavelengths the records are almost exactly superimposable, except for a small and smooth increase in the amplitude of the short period fluctuations in the ultraviolet. Very similar results have been obtained with Whatman 3MM paper in several experiments of this type.

Such records provide a good objective assessment of the "texture" of different grades of filter paper although the short period of variations in optical density will not be recorded faithfully at very high sensitivity-settings due to the relatively slow responses of pen-recorders. Thus the records for Schleicher and Schuell papers Nos. 287 and 2495 were extremely uneven, and those for Whatman papers No. 1, 3 and 4, moderately uneven. Whatman Nos. 2, 3MM, 20 and 520 possessed the smallest fluctuations in optical density and are probably thus the most suitable for this type of work.

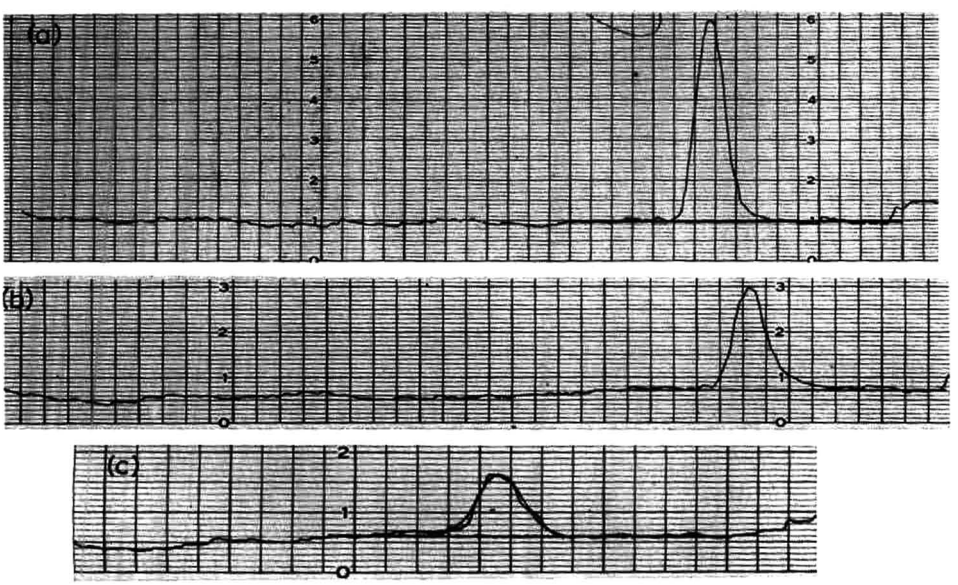


Fig. 7. Scanning records. (a, b, c) 25, 10 and 5 μg of 11-deoxycorticosterone with the alkaline BT method[6]. Low sensitivity, moderate damping, Bausch and Lomb VOM-6 recorder. Whatman No. 2 paper washed 36 h with 90% aq. methanol.

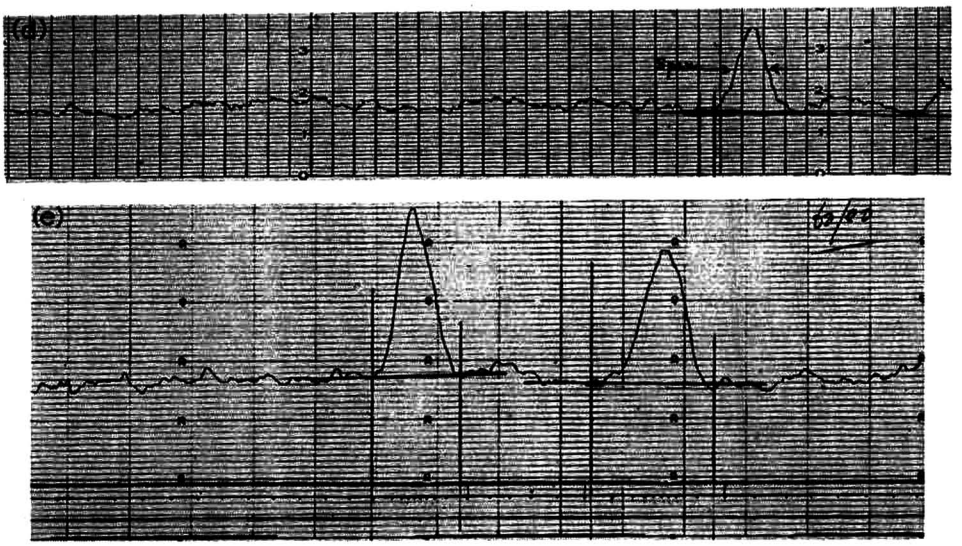


Fig. 7. Scanning records. (d) 5 μg deoxycorticosterone as in (c) but using minimal damping, moderate sensitivity, and unwashed Whatman No. 2 paper. (e) 10 μg each of androsterone (A) and dehydroepiandrosterone (DHA) with the Zimmermann reagent on washed Whatman No. 2 paper. Moderate sensitivity, minimal damping, A.E.I. Sunvic RSP-2 recorder. Chart record thickened by hand for photography. An earlier integrator was used in which the "thousands" pen is at the bottom and the "ones" at the top.

Adaptation of the instrument for fluorimetry

The instrument is not optically designed for fluorescence measurements but has been adapted successfully to the fluorimetry of N-DANSYL-amino acids [N-(1-dimethylaminonaphthalene-5-sulphonyl)-amino acids][11,12]. A wooden box with the same dimensions as the monochromator casing was constructed and fitted with a diagonal mirror in the position of M_1 of the monochromator (Fig. 1c). A mercury arc, filters, and a focussing quartz lens (as in GRAY AND HARTLEY[11]) were fitted and the scanner placed over the exit hole H_2 (Fig. 1c) and aligned in the same fashion as when used with the S.P. 900 monochromator. Secondary filters were mounted in S_4 or in the well below the PMT housing in place of the collimating lens, L_2, and field-stop, FS (Fig. 3). The PMT was now connected directly to the E.H.T. supply and amplifier unit of a Bowman-Aminco spectrophotofluorimeter and the oscilloscope output of the latter fed to the chart-recorder and integrator via a suitable potential divider.

While far from achieving optimal conditions, the apparatus was capable of detecting as little as 0.5×10^{-3} μmole of the majority of common amino acids, and the useful working range for their estimation was from $1.0\text{–}20.0 \times 10^{-3}$ μmole.

Routine operation for quantitative absorptiometry

Ideally, a batch of chromatograms is assembled which includes three or four on which suitable quantities of a standardizing substance have been run, and on which all the "unknowns" are present in quantities smaller than that of the largest standard. The chromatogram of the latter is fed into the scanner by hand after switching on the power supplies and selecting the appropriate wavelength on the monochromator dial.

The chromatogram is brought to a position at which a section of background is under the scanner's slits (S_3 and S_4, Fig. 3) and the pen of the recorder brought to a position between 2 % and 5 % of full-scale deflection by a combination of adjustment of slit width (S_1 and S_2, Fig. 1) and the backing-off potentiometer. The slits S_1 and S_2 are now closed to ensure that a full-scale deflection is obtained. If not, a wider initial slit width must be used and the backing-off voltage reduced. The chromatogram is then passed slowly through the scanner by manual rotation of one of the driving gears and the variation of the background is noted. If the pen of the recorder falls to zero at any point it is brought to just above zero (approx. 1 % of full-scale deflection) by reducing the backing-off voltage. When the zone of absorbing material passes under the scanner's slits a positive deflection of the pen is obtained. The sensitivity-setting and backing-off voltage are now adjusted if necessary until the maximum of the peak gives 90–95 % full-scale deflection, when the background has been restored to its original position. The rest of the chromatogram is now passed through the scanner by

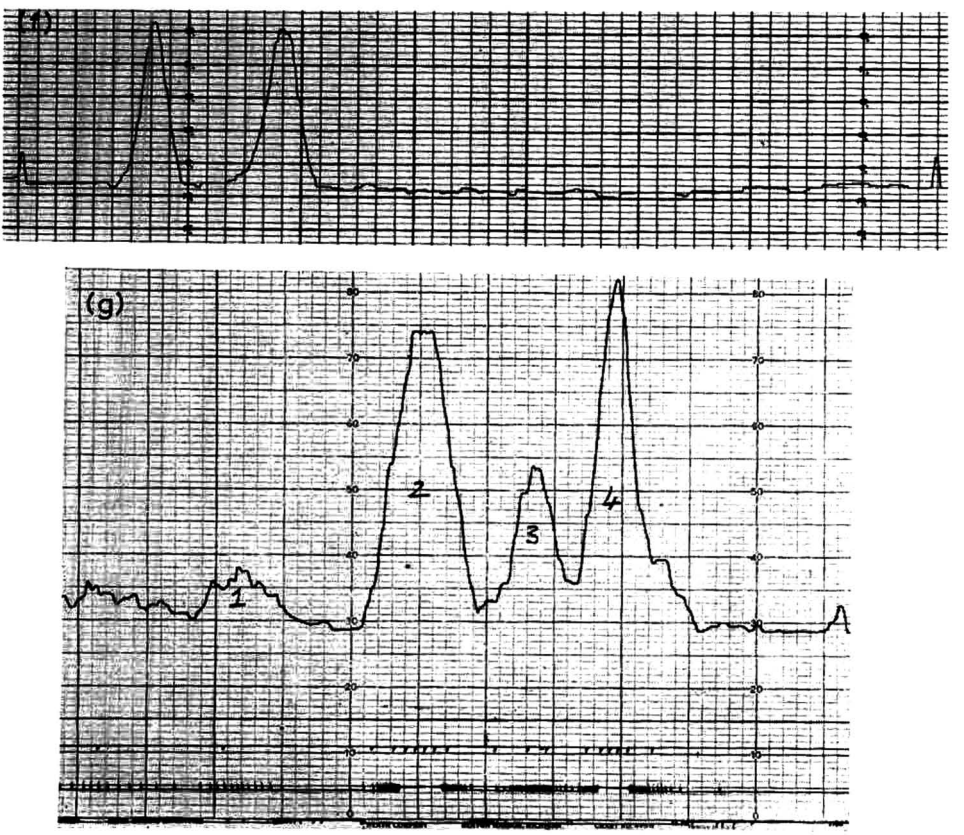


Fig. 7. Scanning records. (f) 25 μg of cortisol (f) and cortisone (E) by the alkaline BT method. Z_1, 0, pencil lines at front and origin respectively. Esterline Angus Labgraph recorder, low sensitivity, minimal damping. Whatman SG81 paper, unwashed. (g) Typical record of the main reducing steroids of human urine using alkaline BT[6]. 1 = Cortisol; 2 = tetrahydrocortisone; 3 = *allo*tetrahydrocortisol; 4 = tetrahydrocortisol. Heathkit EUW-20A recorder with four-pen integrator (see text). Moderate sensitivity, minimal damping.

hand observing the background fluctuations, making further adjustments of the backing-off voltage if necessary to keep the background always above zero on the chart-recorder.

The chromatogram of the largest standard is now run through mechanically by engaging the magnetic clutch on the synchronous motor-drive of the scanner, and the chart recorder is switched on to obtain a record. A reasonably satisfactory standard chromatogram should give a record in which all "background" zones are within ± 3 % (f.s.d. units) of a mean which lies between 3 % and 5 % of full-scale deflection, and in which the largest peak has a maximum between 90 % and 98 % of full-scale deflection.

The chart recorder is now re-started and the chromatograms are fed by hand into the scanner in sequence. The chart is marked with a pencil to indicate the reference number of each chromatogram. With a long series of "unknowns" it is useful to provide a check on stability of the apparatus by re-scanning one or more of the standards once or twice during the middle or at the end of the series. The smoothest operation is secured by linking the "unknowns" strips head-to-tail with short lengths of adhesive tape and piling them in zig-zag fashion in a shallow tray at the level of the inlet to the scanner. The first strip is introduced by hand, following which the whole series is pulled through without further manual assistance. The beginning and end of each scanning record is conveniently indicated on the chart record by brief excursions of the pen to (or below) the zero line, caused by the sudden increases of light transmission in the gaps between successive strips; or by excursions to full-scale deflection if the strips are linked by opaque adhesive tape.

The problems that may arise during this procedure are of two sorts. The first are those due to instability or failure of any part of the electronic apparatus or light-source. It is useful to have spares of any particularly vulnerable components at hand for such emergencies. The second class of problems comprise those which arise from defects or intrinsically unavoidable features of the chromatograms. The commonest problem is the need to measure both large and small peaks on the same chromatograms. Sometimes one or two unknowns may contain unexpectedly large peaks which exceed the largest standard. Unless two recorders can be run in parallel at conveniently different sensitivities the only way to overcome the first problem is to carry out two series of scans at different sensitivities, including the standards so that two calibration curves can be constructed. Peaks overshooting full-scale deflection in the high-sensitivity run are neglected, and the smaller peaks are measured only from the record taken at high sensitivity.

An "unknown" peak which exceeds in height the peak given by the largest standard can be measured by extrapolation in the following manner. The strip containing the largest unknown is fed into the scanner by hand and the background level and sensitivity setting adjusted until the peak gives 90–95 % full-scale deflection. The scanner and chart-recorder are now run mechanically and all "excessive" unknowns are scanned at the new setting in company with the two or three largest standards. If the areas and extrapolated estimates of quantities obtained for the "unknowns" are within the range known from previous studies to give linear calibration curves with the absorptiometric method in question, these extrapolated values can be used with reasonable confidence.

In the case of methods involving a color reaction carried out on the paper, the limiting factors on the linearity of calibration curves will almost certainly be chemical

in origin and not optical or instrumental[2,7]. If, however, light-absorbing derivatives have been prepared by some standardized method before chromatography the limitations are confined to those of optical or instrumental origin. In such cases good or reasonable estimates of unexpected and excessive amounts of "unknowns" can be obtained by scanning at wavelengths away from the maxima usually used. Thus, in the absence of non-specific absorption, excessive amounts of keto-acid dinitrophenylhydrazones which are normally scanned at 390 mμ (BUSH AND HOCKADAY[13]) can be scanned in the visual range at approximately 480 mμ (at which the molecular extinction coefficient is much smaller) and estimated by an extrapolation of the calibration curve of the standards carried out at the same wavelength. It is more convenient when possible to use samples which contain quantities small enough to be in the normal working range of the instrument with the color reaction in question.

Processing the chart records

The basic details of this procedure have been discussed previously[7] so that the account here is confined to an example showing how the 4-channel digital read-out is used. Fig. 8 shows an untouched section of a chart-record containing a typical well-defined peak of large size (cortisone revealed by alkaline BT, approx. 50 μg) and below

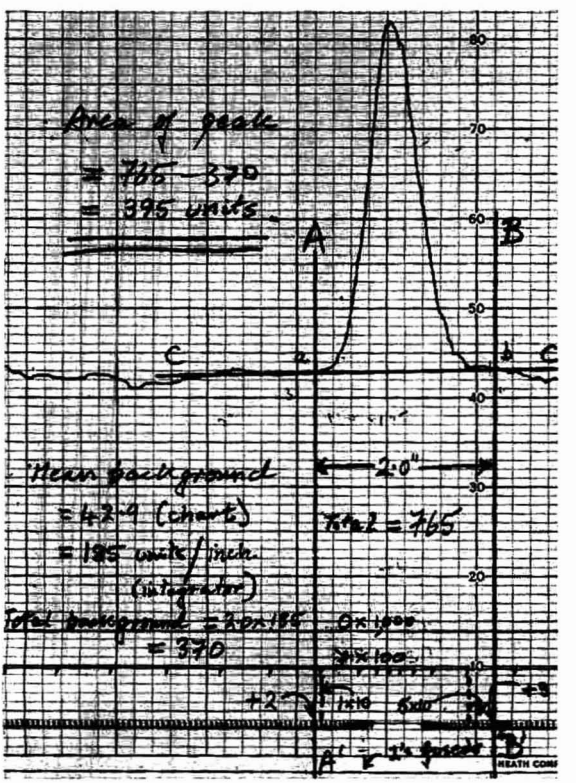


Fig. 8. Section of a scanning record of a paper chromatogram illustrating the method of calculating areas under a single peak from the 4-channel digital record. See text for full description.

it the cumulative integral record. The lines AA′ and BB′ were drawn at right-angles to the long axis of the chart so as to be close to the limits of the peak and yet to coincide with the nearest integral or simple decimal-fractional distance between them. The background level is taken as the line CC′. The first and last deflections of each pen between AA′ and BB′ were then lightly marked and the number of deflections counted and marked as shown by dotted lines so as to obtain rapidly the total number of "counts" between AA′ and BB′. The background was set higher than absolutely necessary in this run (the recorder is zeroed at 30 on the chart to make room for the 4-pen integrator unit), so that the "ones" pen was fused (rate > 25/sec). The units were estimated by eye from the "tens" pen using a magnifying glass ("+ 2" and "+ 3"). Artifacts, or difficulties of alignment between pens, are eliminated by checking sums across two adjacent pens or by counting back to areas where the record is absolutely clear.

The points *a* and *b* are now read from the chart as ordinates and their mean (42.9) taken as the level of the background under the peak. The firing rate of the integrator at this level (185 units/inch) was then read from the calibration curve of the integrator and multiplied by the length of chart between AA′ and BB′ to give the background "count". This was subtracted from the total count to give the digital value of the peak area.

Note that with isolated well-defined peaks and a good background the exact position of AA′ and BB′ is not crucial since any excess over the "true" width of the peak is compensated automatically by an increase in the total background count. Thus, to speed up computation it is best whenever possible to place AA′ and BB′ at a distance apart which gives a convenient value of the *length* by which the background firing rate must be multiplied to give the total background count. Using a slide-rule or a desk calculator such calculations can then be carried out very rapidly. Overlapping peaks are dealt with by an obvious extension of the method described earlier in detail[7].

If the background near the peak is excessively "noisy", the baseline for the peak is drawn according to an arbitrary rule as follows (see Fig. 7d). A length of background equivalent to approximately half the "span" of the peak (width at half the height of the maximum) is marked off ahead and behind the limits of the peak. A point midway along each of these segments of background is drawn at the mean of the highest and lowest values of background within each segment. The line joining these points is drawn and its intersections *a* and *b* with AA′ and BB′ are taken as the ordinates whose mean gives the best estimate of the baseline for the peak itself.

The digital values for the areas of the standards are used to construct a three or four-point calibration curve from which the quantities of unknowns are read once their areas have been calculated.

DISCUSSION

Principal features of design and construction

The present apparatus suffers from a number of limitations, but conforms better than most or all other available instruments of this type to the general principles required for an efficient absorptiometric scanner[2,5,7,14]. The most important features are as follows.

First it is essential to ensure that the incident light beam be monochromatic and

as nearly as possible in the form of parallel or only slightly convergent rays (see *e.g.*, BROWN AND MARSH[14] and EICHORN[15]). This is partly achieved in the present instrument by the combination of the lens L_1 and the slit S_3 (Fig. 3). Even more essential, the transmitted light must be well collimated and any non-perpendicular (off-axis), scattered light rejected. This is achieved adequately in planes parallel to the axis of the paper strip by the slit S_4, but the lens L_2 and field-stop FS (Fig. 3) are needed to eliminate non-perpendicular scattered light in the plane of the slit S_4. The positions of the stop and photomultiplier are designed to provide a diffuse patch of unfocussed light on the photocathode. This minimizes desensitization of the photocathode with age and avoids sensitivity to positional or directional variations in the photocathode's response to illumination.

Since the complete elimination of non-perpendicular scattered light is difficult or impossible to achieve, the position of the paper relative to the slits S_3 and S_4 (Fig. 3) must be maintained very precisely. This is achieved by the guiderails, G,G, of the scanner (Figs. 3, 4) which provide a groove 0.8 mm wide and 2.4 mm deep in which the edges of the paper strip slide. These grooves and the slits S_3 and S_4 also ensure that no light passes outside the edges of the strip.

While this arrangement is questionably not quite as satisfactory as to mount the strips in an accurately machined frame or drum (*e.g.*, LAURENCE[19]; Photovolt Corporation, Densicord 542), it appears to provide an adequately precise location of the paper strip during the scanning process, and has the very considerable advantage that unmounted strips can be passed directly and rapidly into the scanner either singly or as trains of strips linked together with adhesive tape.

Another important feature is the relatively high velocity of the strips in the scanner. The velocity of 1 cm/sec was adopted in order to match the first part of the combined apparatus described previously[1]. While the response-time of the SWEET circuit gives a large safety factor when using such velocities[8] with chromatograms of conventional size (30–60 cm long) the same is not true of some potentiometric recorders and integrators of the type described in this paper. There are however a number of suitable recorders now available which have f.s.d. times of 1 sec or less and several of these have proved entirely adequate to the task, with the exception of very sharp zones such as are found near the origins of typical paper chromatograms. It is probably best to use recorders rated at 0.5 sec f.s.d. or less, and preliminary experience with an Esterline Angus Series S Lab Graph recorder (f.s.d. $\leq$ 0.2 sec) has confirmed this view.

It would be entirely feasible at the present time to scan at even greater velocities. However, this is unlikely to be worthwhile, since the mechanical difficulties of moving the fairly fragile strips of paper at these speeds are likely to be considerable, and the speed of the present apparatus is already sufficient to produce results at a rate which can only be dealt with satisfactorily by a digital computer (see below).

The main defects of the present apparatus are as follows:

(1) The glass prism monochromator is inadequate for work in the ultraviolet below about 360 mμ as is the photomultiplier used at present (RCA-931A or IP-21).

(2) The optical pathway is poorly adapted to fluorimetry despite the very satisfactory results obtained with DANSYL-amino acids[12].

(3) The optical pathway requires a wastefully powerful light source for several reasons. First, the avoidance of trouble from the instability of xenon arcs is secured

by using a large, poorly focussed image on the entrance slit of the monochromator. Second, the elimination of incident and scattered light in directions non-perpendicular to the paper is achieved mainly by collimating slits, and the dimensions of the monochromator do not lend themselves to an inexpensive condensing system.

(4) The grooved paper guides produce an undesirable amount of friction to the entry of paper strips along the first curved part of the guides. The finely grooved section of the guides should be confined to the central straight portion of the paper's track immediately below the photomultiplier, and should preferably be highly polished.

(5) The lack of a cross-scanning device (WIEME[17]) means that good quantitative estimations can only be obtained with chromatograms prepared and run so that the resulting absorbing zones are regular bands at right-angles to the axis of the strip, and fill the whole width of the strip[2,7]. Techniques to secure such zones are available but require slightly more skill and time than those which will suffice for good zones in the form of "spots"[2].

A large number of scanners for paper and thin-layer chromatograms, and for electrophoretograms have been described (*e.g.*, refs. 19–23). The earlier generation of such instruments (up to around 1954) have been discussed previously[2,4,18]. One of the most satisfactory of these, and the only one in which a careful appraisal of the optical pathway appears to have been made, was that of BROWN AND MARSH[14]. This was designed to fit the Beckman Model DU spectrophotometer, the output of which was fed to a suitable potentiometric chart recorder. Both absorptiometric and fluorometric measurements could be made by using appropriate filters. These authors were the first to point out that the irregular background absorption of filter paper did not seriously affect the accuracy with which quantitative estimation could be carried out by this means. In another pioneering study REES, FILDES AND LAURENCE[16] defined carefully the requirements for accurate quantitative scanning of paper electrophoretograms of proteins stained with dyes such as bromphenol blue. WIEME[17] made a major contribution by devising a scanner which also scanned rapidly across the paper strip. The majority of other scanning instruments have been designed as attachments to commercially available spectrophotometers or fluorometers (*e.g.*, refs. 14, 22–24).

The latter and all the available commercially supplied scanners suffer from more or less serious defects. The three principal defects to be found in these instruments are:

(1) The optical pathway does not meet the requirements for efficient operation either as absorptiometers or fluorometers[2,7].

(2) The paper or thin-layer chromatogram must be mounted on a drum or linear frame for transport across the scanning beam of light.

(3) The movement is unnecessarily slow.

The last two defects make the procedure unnecessarily lengthy and tedious. The first makes the method almost totally dependent upon absolutely reproducible chromatography (*i.e.*, shapes of zones) for any accuracy of quantitative measurement, and it is not usually possible to obtain linear calibration curves.

The present instrument has been designed so as to avoid these defects. Unmounted strips are fed into the input orifice of the apparatus and scanned at 1 cm/sec. A continuous train of chromatograms linked by adhesive tape can be fed into the machine so that its operation is fully automatic for an hour or more, during which time up to 72 strips of 50 cm length can be processed per hour. The most important

point to be observed, however, is that the desired optical pathway is not difficult to achieve, but is *absolutely essential* if reliable performance is to be achieved.

The only commercially available paper chromatogram scanner which embodies many of the principles of the instrument described in this paper is the recently announced fluorimetric scanner of the Shandon Scientific Company attributed to BOULTON, CHARD AND GRANT[25,26]. As far as can be judged from the brief publications which are available, the Shandon instrument is a more or less direct copy of the fluorimetric adaptation described above (p. 171), apart from the addition of a digital data-output, a heating unit on the paper inlet to intensify the fluorescence of certain substances, and an altered packaging of the components[25,27]. It is not clear whether the Shandon scanner can be used for absorptiometric measurements. Until full details of this instrument are published, it is impossible to compare it with the instrument described here. However, all the specifications and details of performance that have been released so far[25-27] are, apart from the use of filters in place of a monochromator, *identical* with those of the instrument described in this paper. One must conclude that it probably resembles the latter in being of low efficiency for fluorimetric scanning because the main optical pathway was designed for *absorptiometric* purposes, a fact apparently not sufficiently understood by BOULTON *et al.*[25].

The optical characteristics of filter paper

The general subject of scattering in heterogeneous materials has been well reviewed by SHIBATA[28], and the consequences for the scanning method have been discussed previously[2]. One point of interest has emerged since the earlier article was written. Much previous work had shown that the apparent optical density of absorbing zones in heterogeneous media was much greater than in a homogeneous medium[28]. It is similarly a general rule that the apparent optical densities of colored zones on dry paper chromatograms and electropherograms are over twice those obtained when the paper is wetted with water or "cleared" with an oil of suitable refractive index[2,19]. This suggested that the high optical density of dry filter paper itself was largely due to internal scattering and absorption. The measurements with Whatman papers Nos. 2 and 3MM however (see above) suggest that reflection, or scattering in a relatively thin surface layer towards the incident light may be the main source of the high apparent optical density of filter paper. Thus, the optical density (or more strictly "attenuance"[28]) of Whatman No. 2 (0.160 mm thick) at 584 mμ was found to be 2.5: that of Whatman No. 3MM which is 0.318 mm thick was 2.79, a surprisingly small increase. This and the actual absolute values which have been obtained may well lead to a reconsideration of the relative advantages of using dried or "cleared" strips for scanning[2,16]. Thus, the two main advantages of using dried strip rather than "cleared" strips are an approximately doubled optical density of absorbing zones, and the omission of a time-consuming process which is both somewhat troublesome and a potential source of error. Since, however, the limitations on the sensitivity of the scanning method appear to be almost entirely a question of signal-to-noise ratio one could well afford a loss of optical density if a more than comparable reduction in "noise" were gained thereby.

Some operational characteristics of the method

The acceptability of the analytical accuracy of the method of direct scanning

of paper chromatograms has been argued previously in detail[2,7]. There seems little doubt that the present instrument performs with an overall instrumental and computational standard error of approximately ± 1 % as expected from the ratings of the electronic equipment that is employed. On the basis of past experience one can expect to be able to adapt any well-established absorptiometric method for quantitative scanning of paper chromatograms, as long as it does not employ reagents and conditions which destroy or caramelize filter paper. With the equipment described in this and a previous paper[1], 95 % confidence limits of from ± 2 % to ± 4 % should be achieved without too much difficulty. A considerable number of methods which employ aqueous reagents or other easily controlled conditions should be adaptable for the *manual* treatment of the chromatograms with reagent followed by the use of the scanner. This is the case for instance with the alkaline blue tetrazolium method for reducing steroids[6] and of BAROLLIER's ninhydrin–cadmium reagent for amino acids[2].

Although acceptable analytical performance compared with other chromatographic methods is an essential feature of direct scanning of paper chromatograms, and could not have been expected on the basis of published results up to 1954, the main advantage of the method is its speed and potential productivity. Although conventionally sized paper chromatograms are slow to run compared with gas–liquid chromatograms, large numbers can be run at the same time in cheap and relatively compact apparatus, and the running time involves no personnel working time[2,3]. The overall productivity of the scanning method is thus very much greater than any other existing method of quantitative estimation by chromatography for comparable capital and running costs. The present machine produces a chart record of a 50 cm chromatogram (equivalent to approx. 2000 theoretical plates for a typically overrun strip[29]) in 50 seconds, *i.e.*, at rates of 504 chromatograms in seven hours' running.

In fact, the present machine has never been used to process more than about 200 chromatograms in any one day even though it has serviced simultaneously up to four separate research projects most of its working life. The preparation of extracts suitable for chromatography, and the processing of the chart records are the rate-limiting steps in analytical procedures of this type. The present machine has processed approximately 25,000 chromatograms in its working life despite a considerable amount of "down time" spent on modification, development work, and transport between laboratories. In the last 12 months one research group carrying out a large scale survey of ten individual urinary steroids by a modification of the general fractionation scheme of BUSH AND WILLOUGHBY[6] has been able to process over 1500 urines (*i.e.*, approx. 5000 chromatograms) using this apparatus, at the same time as it was being used for two other major research projects.

Chromatograms of the usual size may contain ten or more separated substances that are detected by any one colorimetric method, but it is unusual to obtain well-separated zones with more than eight components on each strip. With many research problems one adopts a system giving five or fewer zones to be measured per strip. Direct measurements in our laboratory showed that a skilled worker takes approximately 1.8 min per zone for the computation of peak areas by triangulation (MOORE, STEIN AND SPACKMAN[30]) and 0.8 min for computation from the 4-channel integral record described here. For a batch of chromatograms 50 cm long containing an average of five zones per strip, therefore, 50 sec work scanning the strip produces 4.0 min computational work, plus approximately 10 min per batch of chromatograms for

construction of a calibration curve, checking dubious results, etc. Reading quantities from the calibration curve and tabulation of the results take an average 0.4 min per peak. Thus, a batch of 50 chromatograms would require approximately 42 min scanning time plus approximately 10 min for warming up and initial adjustments, or a total of 52 min work with the instrument. This would then need approximately 5 h of computation and tabulation. These figures agree well with the usual routine experience of our laboratory with this method.

It will be obvious that the problems of processing chart records of this type are entirely similar in principle to those of processing the record of gas–liquid or other types of column-effluent-monitored chromatography. Specialized digital computers such as the Infotronics peak integrator (Infotronics Corp., Houston, Texas, U.S.A.) have been used successfully for peak area computation in ion-exchange chromatography of amino acids and in gas–liquid chromatography[31]. The background "noise", however, is much greater from paper scanning than from liquid or gaseous effluent monitoring methods. This, and the rapid speed of the scanner described here, made it impossible to achieve reliably reproducible results with the Infotronics instrument during a recent trial. It seems likely that the best method of overcoming the bottleneck imposed by the computational part of the procedure is to convert the photometric signal to a convenient digital form on paper or magnetic tape and process this off-line with a general purpose digital computer. A variety of computer programs have been reported recently which greatly facilitate this part of the work[32–36]. Most of them have been used for the calculation of amino acid analyses using the automatic ion-exchange method of SPACKMAN, MOORE AND STEIN[30]. The calculation of quantities from peak areas on chromatogram records of whatever sort is complex unless attention is confined to "ideal" chromatograms with zero background drift and noise, and perfectly separated peaks[7,31]. There is little doubt, however, that the use of fast digital computers will enable the full productivity of chromatogram-scanners to be achieved[3]. A detailed consideration of this topic will be given in a later paper of this series.

ACKNOWLEDGEMENTS

I am extremely grateful to the Squibb Institute for Therapeutic Research and the Rockefeller Foundation for grants which defrayed the costs of the monochromator, paper-tracking unit, and Sunvic RSP2 recorder. Subsequent modifications have been partly financed by grants from the Nuffield Foundation (U.K.) and the American Cancer Society for which I am very grateful. My greatest debt is to Dr. EDGAR SCHUSTER (Oxford, U.K.) who constructed the monochromator and paper-tracking unit for cost of materials only during a financial emergency.

I am also indebted to Mr. DEREK GROVES, Mr. ALAN WHITE, Mr. NORMAN CHARD, Mr. L. GRANT and Mr. PAUL HOFFMAN for electronic servicing and modifications to the original SWEET circuit and integrator; and to Dr. ALAN BOULTON and Mr. CHARD for constructing the fluorimetric mock-up adaptation of the scanner to my design. I am grateful to Dr. W. GRAY (Cambridge, U.K.) for advance details of the light-source and filters needed for the latter design.

The initial work could not have been brought to a successful conclusion without the longstanding support and encouragement of Professor Sir GEORGE PICKERING, F.R.S., in whose department the first instrument was begun and completed.

SUMMARY

Electronic apparatus for photometric scanners of paper chromatograms is described, together with its testing and use. The apparatus includes a logarithmic densitometer based on SWEET's circuit, a stabilized supply for a tungsten light source, a voltage-to-frequency converter for providing integral records, a four-pen event marker with decade-counters and pen-drivers to give a convenient 4-digit integral record alongside the direct chart record of the scan, and a stabilized voltage source for a retransmitting slidewire capable of providing a 0 to —10 V output from the scanner (*via* the recorder) suitable for driving the voltage-to-frequency converter or other types of analogue-to-digital conversion apparatus.

REFERENCES

1 I. E. BUSH, *J. Chromatog.*, 23 (1966) 94.
2 I. E. BUSH, in D. GLICK (Editor) *Methods of Biochemical Analysis*, Vol. 11, Interscience, New York, 1964, p. 149.
3 I. E. BUSH, *Science*, 154 (1966) 77.
4 A. C. CHIBNALL, *Brit. Med. Bull.*, 10 (1954) 183.
5 I. E. BUSH, *Mem. Soc. Endocrinol.*, 8 (1960) 24.
6 I. E. BUSH AND M. L. N. WILLOUGHBY, *Biochem. J.*, 67 (1957) 689.
7 I. E. BUSH. *The Chromatography of Steroids*, Pergamon, Oxford, 1961.
8 M. H. SWEET, *Electronics*, November (1946) 105.
9 E. GORDY, P. HASENPUSCH AND G. F. SIEBER, *Electron. Eng.*, 36 (1964) 808.
10 W. G. HOWARD, *Frequency*, 2 (1964) 16.
11 W. R. GRAY AND B. S. HARTLEY, *Biochem. J.*, 89 (1963) 379.
12 A. A. BOULTON AND I. E. BUSH, *Biochem. J.*, 92 (1964) 11P.
13 I. E. BUSH AND T. D. R. HOCKADAY, *J. Chromatog.*, 8 (1962) 433.
14 J. A. BROWN AND M. M. MARSH, *Anal. Chem.*, 25 (1953) 1865.
15 R. M. EICHORN, *Ind. Eng. Chem.*, 53 (1961) 67.
16 V. H. REES, J. E. FILDES AND D. J. R. LAURENCE, *J. Clin. Pathol.*, 7 (1955) 336.
17 R. J. WIEME, *J. Chromatog.*, 1 (1958) 166.
18 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New York, 1958.
19 D. J. R. LAURENCE, *J. Sci. Instr.*, 31 (1954) 137.
20 M. S. SHIPALOV, M. A. BOKUCHAVA AND G. A. SOBOLEVA, *Biokhimiya*, 23 (1958) 390.
21 F. FRANEK AND J. MASTNER, *Chem. Listy*, 51 (1957) 1773.
22 F. M. GAVIS, F. BRAYER, W. KREMER, J. HOWLAND, T. D. BROWN AND P. D. BIUNTA, *U.S. At. Energy Comm. Rep. UR-613*, 1962.
23 A. BRODIE AND J. F. TAIT, in R. I. DORFMAN (Editor), *Methods in Hormone Research*, Academic Press, New York, Ch. 7, in press.
24 A. PALACKY, *Experientia*, 13 (1957) 377.
25 A. A. BOULTON, N. CHARD AND L. GRANT, *Biochem. J.*, 96 (1965) 69P.
26 A. A. BOULTON, N. CHARD AND L. GRANT, *Biochem. J.*, 96 (1965) 82P.
27 A. A. BOULTON, N. CHARD AND L. GRANT, *Biochem. J.*, 96 (1965) 83P.
28 K. SHIBATA, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 7, Interscience, New York, 1959, p. 77.
29 J. F. TAIT AND S. A. TAIT, *Mem. Soc. Endocrinol.*, 8 (1960) 40.
30 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
31 H. J. JONES AND D. W. SPENCE, *Application Notes No. 1*, Infotronics Corp., Houston, Texas.
32 A. YONDAR, D. L. FILMER, H. PATE, N. ALONZON AND C. H. W. HIRS, *Anal. Biochem.*, 10 (1965) 53.
33 G. N. GRAHAM AND B. SHELDRICK, *Biochem. J.*, 96 (1965) 517.
34 R. D. FRAZER, A. S. INGLIS AND A. MILLER, *Anal. Biochem.*, 7 (1964) 247.
35 A. A. BOULTON, R. L. HOLDER AND H. F. ROSS, *Biochem. J.*, 96 (1965) 70P.
36 A. J. ROBINS, R. A. EVANS, J. A. DE SIRIWARDENE AND A. J. THOMAS, *Biochem. J.*, 99 (1966) 46P.

ASSAY OF FLAVINES AND LUMAZINES IN THE STUDY OF FLAVINOGENESIS IN *ASHBYA GOSSYPII*

PAOLO CERLETTI, ROBERTO STROM, SILVIA GIOVENCO, DONATELLA BARRA AND MARIA ADELAIDE GIOVENCO

Departments of Biological Chemistry of the Universities of Camerino and Rome (Italy)

(Received January 18th, 1967)

The separation of ribolumazines from flavines has become an important problem in recent times since complex mixtures of these compounds are encountered in studies of flavine biosynthesis in flavinogenic organisms.

Ashbya gossypii and the related flavinogenic ascomycetes *Eremothecium ashbyii* produce riboflavine and appreciable amounts of the intermediate, in riboflavine synthesis, 6,7-dimethyl-8-ribityllumazine and of its oxidation product 6-methyl-7-hydroxy-8-ribityllumazine[1–4]. Large amounts of flavine coenzymes are formed in *Eremothecium ashbyii*, but neither FAD* nor FMN have so far been reported in *Ashbya gossypii*.

Analytical methods based on paper chromatography are reported in the literature[1,2,5,6]. However they do not appear adequate for quantitative work, particularly if aimed at the estimation of all the compounds in question.

Some procedures using column chromatography have been described more recently[7–9]. Two of them may be used in sequence[9] to resolve mixtures containing the lumazines, FMN and riboflavine. For routine work on broth extracts it seemed desirable to have procedures which allow a ready separation of the lumazines and all flavine coenzymes (FAD included), over a wide range of concentration of each component relative to the others. Indeed the assay of FAD and FMN in *Ashbya gossypii* is hindered by differences of several orders of magnitude in the content of these compounds, riboflavine and the ribolumazines. Conditions for a complete analysis were therefore studied.

The methods developed are reported in the present paper, as well as their application to the study of flavinogenesis in *Ashbya gossypii*.

MATERIALS

Riboflavine was kindly supplied by Hoffmann-La Roche. FMN was from Sigma Chemical Co. FAD was prepared by the procedure of CERLETTI AND SILIPRANDI[10]. 6,7-Dimethyl-8-ribityllumazine and 6-methyl-7-hydroxy-8-ribityllumazine were isolated from *Eremothecium ashbyii* as described by MASUDA[1] and by KUWADA *et al*[3].

Fluorimetric measurements were made either with a Farrand spectrofluorimeter, equipped with two grating monochromators and a photomultiplier tube 1P28, or with a Klett fluorimeter, lamp filter Corning glass 5543 (transmittance band

* Abbreviations used: FAD = flavine-adenine dinucleotide; FMN = flavine mononucleotide.

between 350 and 505 mμ), photocell filter Corning 3385 (transmittance above 475 mμ).

Fluorescent and U.V. absorbing spots were detected on paper chromatograms with the aid of Mineralight 3660 and 3537 U.V. lamps.

Amberlite IRC 50, Rohm & Haas, Philadelphia, U.S.A.; Munktell cellulose powder; and Whatman paper No. 4 were used.

Ashbya gossypii was grown in submerged cultures[11]. The mycelium was obtained by filtration of the broth and flavines and lumazines were extracted from it with 0.01 *M* KCl–(0.03 *M* KH_2PO_4–K_2HPO_4 buffer, pH 6.8) (96:4) at 80°[12].

METHODS AND RESULTS

Throughout this study, the composition of the mixtures and the purity of the isolated compounds were checked by the paper chromatography procedures described in the text.

The quantitative determination of flavine was done by spectrophotometry and/or fluorimetry[12,13]. Lumazines were determined spectrophotometrically[1,3,14]; they were also assayed fluorimetrically, as described in the text.

For quantitative fluorimetric work internal standards of the pure compounds were added to the samples. This procedure overcomes the influence of varying salt concentration on fluorescence[12]. Organic solvents, if necessary, were removed before assay.

The lower limit of sensitivity of the spectrofluorimetric determination was about 0.2 mμ moles/ml of each compound with the equipment used in this research. Paper chromatography detected $2 \cdot 10^{-3}$ μmole flavine and about $1 \cdot 10^{-2}$ μmole lumazines.

Paper chromatography

Good separations were obtained with acidic solvents which had *n*-butanol as the main constituent. To improve resolution, it was necessary to increase the amount of water dissolved in the solvents. This was accomplished by adding alcohols, which mix freely with both water and *n*-butanol, and then saturating again with water. Methanol and *tert.*-butanol were found most suitable. The influence of the amount of *tert.*-butanol in the solvent is shown in Table I. With alkaline solvents, it was observed that, in addition to the nature and amounts of the alcohols, variations in the pH affect the mobility of the various compounds in quite different ways.

As a result of this study, the following solvents were routinely used:

In the acidic range, solvent 3 (Table I).

In the alkaline range a solvent composed of: *tert.*-butanol–33 % (v/w) trimethylamine–H_2O (60:0.95:39.05); pH 10.9.

Cellulose column chromatography

As shown in Fig. 1 chromatography on a Munktell cellulose column resolves mixtures of pure ribolumazines and flavine coenzymes. Elution is performed with solvent 3 (Table I) followed by H_2O to displace FAD. Since FMN and FAD can be determined quantitatively in the presence of each other[12], the analysis is speeded up by washing out both components with water as soon as FMN appears into the alcoholic eluate. Recovery is between 95 and 100 %. The separation is obtained over a wide range of concentration of each component relative to the others. The procedure sepa-

TABLE I

PAPER CHROMATOGRAPHY OF FLAVINES AND LUMAZINES IN ACIDIC SOLVENTS

Chromatograms (on Whatman paper No. 4) were developed by the descending technique at a temperature of 18–22°. The main constituent of the solvents was the upper alcoholic phase of the mixture: *n*-butanol–1 *N* HCl–H_2O (100:2:148). Additions to it were made as shown below.

Solvent No.	*Addition to 100 ml of butanol phase*	R_F				
		6-Methyl-7-hydroxy-8-ribityllumazine	*6,7-Dimethyl-8-ribityllumazine*	*Riboflavine*	*FMN*	*FAD*
1	None	0.01	0.02	0.05	0	0
2	*tert.*-Butanol (40 ml) + 0.01 *N* HCl (2.5 ml) + H_2O (30 ml)	0.06	0.09	0.23	0.01	0
3	*tert.*-Butanol (50 ml) + 0.01 *N* HCl (2.5 ml) + H_2O (40 ml)	0.08	0.11	0.24	0.04	0
4	*tert.*-Butanol (60 ml) + 0.01 *N* HCl (2.5 ml) + H_2O (50 ml)	0.08	0.12	0.25	0.25	0.01
5	Methanol (10 ml) + H_2O (13 ml)	0.14	0.17	0.35	0.11	0.04

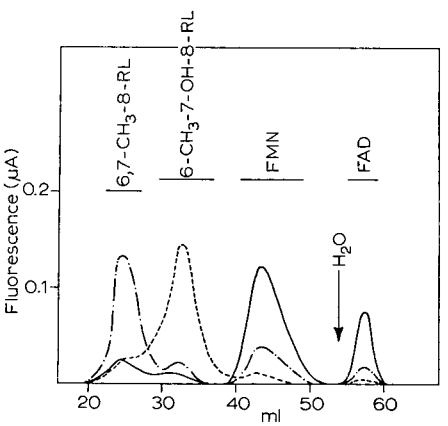


Fig. 1. Separation of flavines and lumazines on a cellulose column. A mixture containing 10 μg each of the following compounds: FMN, FAD, 6,7-dimethyl-8-ribityllumazine (6,7-CH_3-8-RL) and 6-methyl-7-hydroxy-8-ribityllumazine (6-CH_3-7-OH-8-RL), was applied on a column, 200 mm × 75 mm^3, packed with Munktell cellulose powder. Eluant was solvent 3 (Table I) followed by water, at a flow rate of 15 ml/h. 1.5-ml fractions were collected. Ordinate: fluorescence intensity of the eluate in μA: (– – – –) excitation at 340 mμ, emission at 425 mμ; (–·–·–·–) excitation at 415 mμ, emission at 475 mμ; (————) excitation at 445 mμ, emission at 520 mμ. Upper horizontal lines indicate the compounds identified by paper chromatography in the eluate.

rates larger quantities than paper chromatography and is used for the quantitative assay of mixtures and for the isolation of the compounds on a semipreparative scale.

The elution can be followed spectrophotometrically by recording the absorption at 350 mμ, 400 mμ and 450 mμ, corresponding respectively to 6-methyl-7-hydroxy-8-ribityllumazine, 6,7-dimethyl-8-ribityllumazine and the flavines. The absorption spectra of the above compounds show however considerable overlaps, and if separations are not clear cut, spectrophotometric measurements do not permit identification and quantitative estimation of the components in the eluate. Fluorescence measurements are more selective and afford a higher sensitivity. Spectral and emission properties of some of the compounds under study have been reported in the literature[13]. Those lacking were determined on the pure substances and the data are summarized in Table II. Determinations in the eluate were made at the following wavelengths, 6,7-dimethyl-8-ribityllumazine, 415 mμ excitation, 475 mμ emission; 6-methyl-7-hydroxy ribolumazine, 340 mμ excitation, 425 mμ emission; flavines, 445 mμ excitation, 520 mμ emission. The resolving power of fluorimetry is nevertheless not absolute since excitation and emission bands of some of the above compounds show a minor overlap. Emission may therefore be recorded also at wavelengths corresponding to a compound which in reality is not present. This is what happens in the peaks of Fig. 1 for fluorescence at 520 mμ excited at 445 mμ (first and second peak), fluorescence at 475 mμ excited at 415 mμ (second and fourth peak) and fluorescence at 425 mμ excited at 430 mμ (first, third and fourth peak). The same is observed in the first peak of Fig. 2A for the emission at 475 mμ excited at 415 mμ, and for the fluorescence at 520 mμ excited at 445 mμ in the second peak of the same figure. Relative intensities at specific wavelengths however permit a decision about the identity of the compound, which if necessary can be confirmed *e.g.* by paper chromatography.

TABLE II

FLUORESCENCE CHARACTERISTICS OF PURIFIED LUMAZINES AND CONTENT OF FLAVINE COENZYMES AND PRECURSORS IN FLAVINOGENIC AND NON-FLAVINOGENIC STRAINS OF *Ashbya gossypii*

Fluorescence spectra were recorded at pH 6, in 0.01 *M* phosphate buffer.

Compound	*Fluorescence*		*μmoles/g of protein*	
	Maximum of exciting light (mμ)	*Maximum of emission (mμ)*	*In flavinogenic strains*	*In non-flavinogenic strains*
6,7-Dimethyl-8-ribityllumazine	415	475	3.4	0
6-Methyl-7-hydroxy-8-ribityllumazine	287	425	30	0
	342	425		
Riboflavine	370	520[13]	148	0.103
	445	520		
FMN	370	520[13]	0.123	0.115
	445	520		
FAD	370	520[13]	0.065	0.028
	445	520		

Ion exchange chromatography

Previous work has established that flavine mixtures could be resolved readily on the weak cation exchanger Amberlite IRC 50, H form[12,15]. Experiments with the pure compounds showed that on this resin lumazines could also be satisfactorily separated from riboflavine. Flavine coenzymes and 6-methyl-7-hydroxy-8-ribityllumazine are eluted from the resin with water in a first peak, 6,7-dimethylribolumazine in a second. Riboflavine is eluted only by 1 *N* HCl. Recoveries are 95 % for flavine coenzymes and lumazines and about 85 % for riboflavine.

The removal of riboflavine and of 6,7-dimethyl-8-ribityllumazine by this procedure facilitates the subsequent analysis of other components in mixtures where the concentration of the above compounds far exceeds that of the other ones.

Application to flavinogenic and non-flavinogenic strains of Ashbya gossypii

Flavines and lumazines were determined by the methods described above in flavinogenic strains of *Ashbya gossypii*, and in strains which apparently did not accumulate riboflavine, obtained by U.V. irradiation of the mold as described elsewhere[11].

Ion exchange chromatography on Amberlite IRC 50 was used as a preliminary

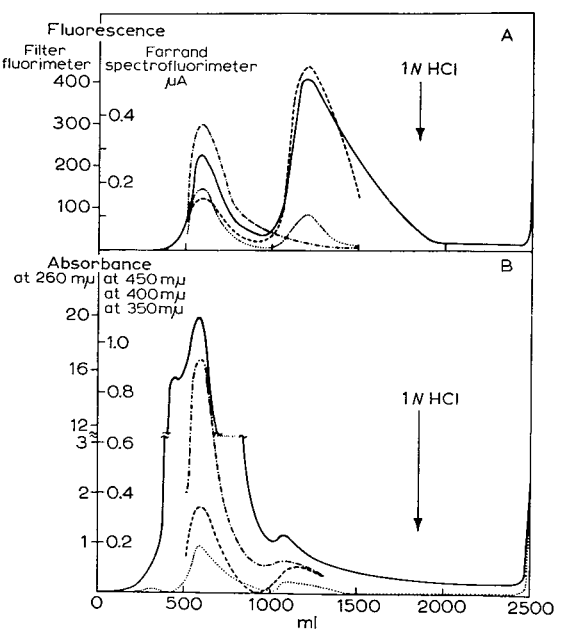


Fig. 2. Elution pattern on a carboxylic ion exchanger of the extract of a flavinogenic strain of *Ashbya gossypii*. 37.8 g of mycelium (wet weight) from a submerged culture at the fourth day of fermentation, corresponding to 1.3 g of proteins, were separated from the broth by filtration, and extracted with 0.01 *M* KCl–(0.03 *M* KH_2PO_4–K_2HPO_4 buffer, pH 6.8) (94:4) at 80°. The extract was applied to a column 100 cm × 75 cm^2, packed with Amberlite IRC 50, H form. Eluant was H_2O, followed by 1 *N* HCl, at a rate of 40 ml/h. 8-ml fractions were collected at 2°. (A) Fluorescence intensity of the eluate, in μA; measured with a spectrofluorimeter: (–·–·–·–) excitation at 340 mμ, emission at 425 mμ; (– – – –) excitation at 415 mμ, emission at 475 mμ; (· · · ·) excitation at 445 mμ, emission at 520 mμ; (————) fluorescence intensity measured with a filter fluorimeter as compared to a standard containing 0.079 μg fluorescein/ml taken as 100. (B) Absorbance of the eluate: (————) at 260 mμ; (–·–·–·–) at 350 mμ; (– – – –) at 400 mμ; (· · · ·) at 450 mμ.

step for the assay of mold extracts. The elution was followed by recording the absorption at 260 mμ. Each peak was then measured at 350 mμ, 400 mμ and 450 mμ. As a first approach fluorescence was measured throughout the elution with a filter fluorimeter. Each peak was then assayed at the excitation and emission wavelengths characteristic for each compound. The elution pattern of the mycelial extract from a flavinogenic strain is shown in Fig. 2. Fig. 3 shows the pattern obtained, under identical conditions with a non-flavinogenic strain. In this case only one peak, containing FMN and FAD, is eluted by water. Regarding the sensitivity of the methods, no 6-methyl-7-hydroxy-8-ribityllumazine was detected and the second peak due to 6,7-dimethyl-ribolumazine, did not appear. Only small amounts of riboflavine were eluted by 1 *N* HCl.

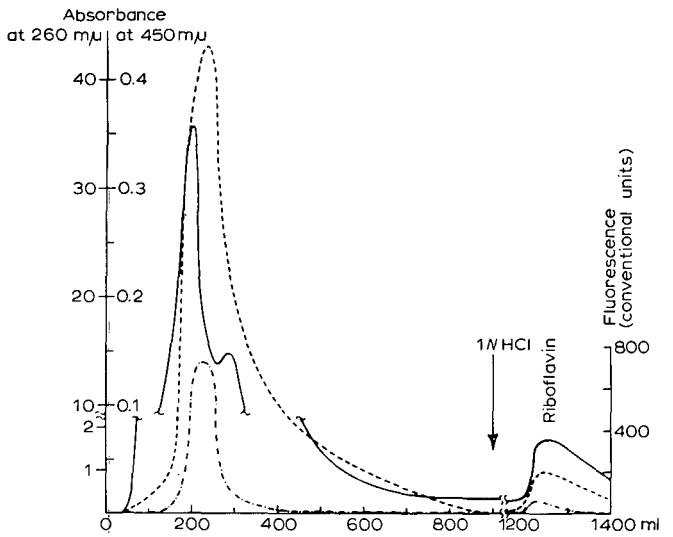


Fig. 3. Elution pattern on a carboxylic ion-exchanger of the extract of a non-flavinogenic strain of *Ashbya gossypii*. 83 g of mycelium (wet weight) from a submerged culture at the fourth day of fermentation, corresponding to 3.44 g of proteins, were separated from the broth by filtration, and extracted with 0.01 *M* KCl–(0.03 *M* KH_2PO_4–K_2HPO_4 buffer, pH 6.8) (94:4) at 80°. The extract was applied to a column, 50 cm × 3 cm^2, packed with Amberlite IRC 50, H form. Eluant was H_2O followed by 1 *N* HCl, at a rate of 30 ml/h. 5-ml fractions were collected at 2°. Ordinates: (————) absorbance at 260 mμ; (– – – –) absorbance at 450 mμ; (–·–·–·–) fluorescence intensity measured with a filter fluorimeter as compared to a standard containing 0.079 μg fluorescein/ml taken as 100.

The first peak eluted by H_2O from the Amberlite column, containing the flavine coenzymes and, in flavinogenic strains 6-methyl-7-hydroxy-8-ribityllumazine, was fractionated by chromatography on a cellulose column. A preliminary treatment was required. Indeed, other non-identified U.V. absorbing substances from the mold contaminate the eluate from the ion exchanger and on the cellulose column interfere with the separation of 6-methyl-7-hydroxy-8-ribityllumazine from FMN. The ribolumazine is therefore extracted quantitatively from the effluent from the ion-exchanger with *n*-butanol followed by ether. Traces of FMN in this extract are readily separated on the cellulose column. FAD and the bulk of FMN remain in the water phase with U.V. absorbing contaminants. Contaminants are satisfactorily separated

on the cellulose column where they move faster than FMN in the alcoholic solvent.

The analytical data are summarized in Table II. FAD and FMN were demonstrated in both types of strains and a relationship was found between accumulation of riboflavine and presence of lumazines.

DISCUSSION

The methods developed in the present work permit the separation both on an analytical and on a preparative scale, and the quantitative estimation of flavines and lumazines. The procedures described in the literature do not generally consider simultaneously both groups of compounds. Those aimed at the determination of flavines in tissues[12,16,17] do not account for a simultaneous estimation of lumazines. Chromatography on a cellulose column yields in one operation a satisfactory separation on a semi-preparative scale of mixtures of all pure lumazines and flavines. If necessary, the treatment described in the text also permits the analysis of extracts contaminated by other material of biological origin.

The combination of methods outlined permits quantitation of the compounds in a mixture containing FAD in amounts about 2,300 times less than riboflavine and 500 times less than 6-methyl-7-hydroxy-8-ribityllumazine. This proved extremely useful in the study of *Ashbya gossypii*, since it facilitated the determination of small amounts of flavine coenzymes in strains which accumulate large quantities of riboflavine.

Flavine coenzymes could be predicted as necessary cofactors for oxidative enzymes in *Ashbya gossypii* since this ascomycetes is a strictly aerobic mold, and flavinogenesis is greatly enhanced by aeration of the culture. So far however FAD and FMN had never been demonstrated, and it is of interest that strains which do or do not accumulate riboflavine synthesize, on a protein basis, similar amounts of flavine coenzymes despite the very different production of riboflavine. It has been shown previously[11] that the low content of FMN and FAD is not to be attributed to breakdown of these compounds by the mold. The ratio of vitamin to coenzymes in non-flavinogenic strains is about the same as that existing in animal tissues[12] and in many microorganisms[18]. Lumazines, if present, are less than $1 \cdot 10^{-3}$ μmoles/g of mycelial protein.

Despite their dramatic accumulation of riboflavine, flavinogenic strains have a growth rate similar to non-flavinogenic ones[11]. Previous data[11] indicate that riboflavine accumulation in flavinogenic mold is not, at least in *Ashbya gossypii*, the consequence of a shift from a cytochrome type of terminal respiration to the flavinoprotein type, as has been suggested for *Eremothecium ashbyii*[19]. Flavine accumulation is more likely a side event in the life of the mold, and is limited to the free vitamin and to its near precursors.

The lack of lumazines, at least in detectable amounts, in non-flavinogenic strains, suggests that flavinogenesis may be differently regulated in flavinogenic and non-flavinogenic strains, at a level involving the precursors of the vitamin.

ACKNOWLEDGEMENT

This research was supported by grants of the Impresa di Enzimologia of the Consiglio Nazionale delle Ricerche (Italy).

SUMMARY

The separation both on an analytical and on a preparative scale of flavines and ribolumazines is described. The quantitation of these compounds in flavinogenic and non-flavinogenic strains of *Ashbya gossypii* is reported. The content of each coenzyme in flavinogenic strains is similar to the corresponding flavine in the non-flavinogenic strain. Flavinogenic strains contain about 2,300 times more riboflavine than FAD. Lumazines were not detected in non-flavinogenic strains.

REFERENCES

1 T. MASUDA, *Pharm. Bull.* (*Tokyo*), 4 (1956) 71 and 375.
2 T. MASUDA, *Pharm. Bull.* (*Tokyo*), 5 (1957) 28 and 136.
3 S. KUWADA, T. MASUDA, T. KISHI AND M. ASAI, *Pharm. Bull.* (*Tokyo*) 6 (1958) 447.
4 G. W. E. PLAUT, *Ann. Rev. Biochem.*, 30 (1961) 409.
5 E. LEDERER, *Chromatographie en chimie organique et biologique*, Masson, Paris, 1960.
6 H. KATAGIRI, I. TAKEDA AND K. IMAI, *J. Vitaminol.* (*Kyoto*), 4 (1958) 207, 211 and 278.
7 G. W. E. PLAUT, *J. Biol. Chem.*, 238 (1963) 2225.
8 H. WACKER, R. A. HARVEY, C. H. WINESTOCK AND G. W. E. PLAUT, *J. Biol. Chem.*, 239 (1964) 3493.
9 D. J. HOWELLS, AND G. W. E. PLAUT, *Biochem. J.*, 94 (1965) 755.
10 P. CERLETTI AND N. SILIPRANDI, *Arch. Biochem. Biophys.*, 76 (1958) 214.
11 P. CERLETTI, R. STROM, M. G. GIORDANO, D. BARRA AND S. GIOVENCO, *J. Biochem.* (*Tokyo*), 57 (1965) 773.
12 P. CERLETTI AND P. L. IPATA, *Biochem. J.*, 75 (1960) 119.
13 H. BEINERT, in P. D. BOYER, H. LARDY AND K. MYRBACK (Editors), *The Enzymes*, Vol. 2A, Academic Press, New York, 1960, p. 360.
14 G. F. MALEY AND G. W. E. PLAUT, *J. Biol. Chem.*, 234 (1959) 641.
15 P. CERLETTI, G. MONTESI AND N. SILIPRANDI, *Arch. Sci. Biol.* (*Bologna*), 41 (1957) 554.
16 O. A. BESSEY, O. H. LOWRY AND R. H. LOVE, *J. Biol. Chem.*, 180 (1949) 755.
17 K. YAGI, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 10, Interscience, New York, 1962, p. 319.
18 J. L. PEEL, *Biochem. J.*, 69 (1958) 403.
19 F. KAPRALEK, *J. Gen. Microbiol.*, 29 (1962) 403.

QUELQUES APPLICATIONS DE LA CHROMATOGRAPHIE SUR COUCHES MINCES D'ÉCHANGEURS D'IONS EN ANALYSE MINÉRALE

J. A. BERGER, G. MEYNIEL ET J. PETIT

Laboratoires de Chimie analytique et de Biophysique médicale de la Faculté de Médecine et de Pharmacie, B.P. 38, 63 Clermont-Ferrand (France)

(Reçu le 28 octobre 1966)

Depuis 1960, la chromatographie sur couches minces a connu un essor considérable et son emploi s'est pratiquement généralisé à tous les laboratoires d'analyse. Les premiers essais, dûs à ISMAÏLOV ET SCHRAÏBER[1], datent seulement d'un quart de siècle et successivement CROWE[2], WILLIAMS[3], MEINHART ET HALL[4], KIRCHNER, MILLER ET KELLER[5], REITSEMA[6] contribuèrent à l'amélioration d'une technique encore imparfaite. Une codification pratiquement définitive de la méthode fût proposée par STAHL dès 1958 et suivie d'une normalisation progressive de l'appareillage[7]. Depuis 1960, l'application de la chromatographie sur couches minces à l'analyse minérale a fait l'objet de quelques travaux, en particulier de SEILER[8–12], TURINA *et al.*[13] et HRANISAVLJEVIČ[14]. Pour notre part, nous avons orienté nos recherches dès 1962 vers l'utilisation des couches minces d'échangeurs d'ions et ce sont les résultats obtenus en analyse minérale, que nous exposons plus loin.

PRÉPARATION DES COUCHES MINCES DE RÉSINES ÉCHANGEUSES D'IONS

Notre idée directrice fût essentiellement la transposition à l'échelle microanalytique de procédés de séparation, déjà codifiés sur colonne de résines échangeuses d'ions, tant en chimie minérale qu'en chimie organique. Les premiers essais furent effectués sur des couches minces, obtenues par étalement de suspensions de résine dans l'eau distillée, mais que le craquellement, provoqué par la dessication, rendait rapidement inutilisables. Nous avons donc été amenés à ajouter une certaine proportion de cellulose à la suspension de résine, pour conférer à la masse à étaler une stabilité suffisante. La composition suivante a été finalement retenue (pour une surface de 0.2 m^2):

Cellulose M.N. 300 (sans plâtre), 5 g.

Résine analytique, essorée sur verre fritté et préparée sous la forme ionique désirée (maille de 200 à 400 mesh), 30 g.

Eau distillée, 60 ml.

La cellulose est d'abord divisée au mortier avec quelques ml d'eau distillée pour obtenir des couches très régulières; on ajoute ensuite 20 à 30 ml d'eau par petites portions, en poursuivant la trituration et, dans le mélange relativement fluide obtenu, on introduit la totalité de la résine, puis progressivement le reste d'eau distillée. La masse parfaitement homogène est alors prête à être étalée selon la technique habi-

TABLEAU I

DD

PROPRIÉTÉS DES RÉSINES

D'après Biorad Laboratories, Richmond, Calif. U.S.A.

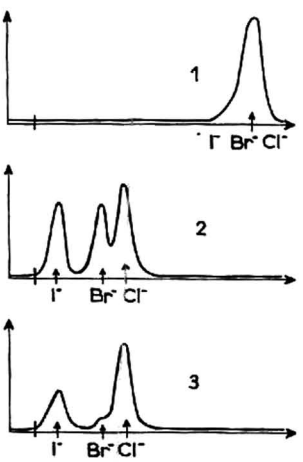
Résine: marque	*Biorad AG 50* *Dowex 50* *Amberlite CG 120*	*Biorad AG1* *Dowex 1*	*Biorad AG3* *Dowex 3*	*Biorad Chelex 100* *Dowex A1*	*Biorad Biorex 5* *Duolite A 30*
Type	Cationique Échangeur fort	Anionique Échangeur fort	Anionique Échangeur faible	Cationique Complexante	Anionique Échangeur moyen
Groupement actif	ϕ-SO_3^-	ϕ-$N(CH_3)_3^+$	Polyamine $\rightarrow NH^+$	ϕ-$N\langle^{CH_2COO^-}_{CH_2COO^-}$	R-$N^+\langle^{(CH_3)_2}_{C_2H_5\text{-}OH}$
Forme ionique	H^+, NH_4^+	Cl^-, OH^-, SO_4^-	Cl^-	Na^+, H^+, Ba^{2+}	Cl^-
Sélectivité des ions (ordre décroissant)					
Monovalents	Ag, Rb, Cs, K, NH_4, Na, H, Li	I, NO_3, Br, Cl, HCO_3, IO_3, HCOO, CH_3COO, OH, F	ϕ-SO_3, citrate, CrO_3, SO_4, tartrate, C_2O_4, PO_4, AsO_4, NO_3, I, Br, Cl, F, HCOO, CH_3COO, CO_3H	H, Li, Na, K	
Divalents	Zn, Cu, Ni, Co, Ba, Sr, Ca			Cu, Pb, Fe(III), Al, Cr(III), Ni, Zn, Ag, Co, Cd, Fe(II), Mn, Ca, Sr, Ba	

tuelle* et les plaques sont sêchées à l'air libre et stockées dans une armoire étanche. Il faut noter que les couches minces de résines échangeuses d'ions doivent de préférence être utilisées dans les huit jours qui suivent leur préparation, car le vieillissement des couches se traduit par une diminution marquée de leur pouvoir séparateur. Dans le tableau I sont mentionnées les caractéristiques essentielles des quelques résines utilisées pour les expériences décrites plus loin.

SÉPARATIONS RÉALISÉES SUR COUCHES MINCES DE RÉSINES ÉCHANGEUSES D'ANIONS

Halogènes

Nous référant à la séparation sur colonne d'un mélange d'halogénures, obtenue par de GEISO et collaborateurs[15], nous avons réalisé le fractionnement en chromatographie ascendante d'un mélange de $^{36}Cl^-$, $^{82}Br^-$ et $^{131}I^-$ radioactifs, sur couche mince de Dowex 1 X 10, sous forme OH^-. Le solvant, qui nous a donné les meilleurs résultats, est une solution molaire de $NaNO_3$[16,17]. La position des différents spots est déterminée dans un premier temps avec un scintillateur à NaI (Tl) (Fig. 1, courbe 2). La localisation des éléments est obtenue par spectrométrie gamma sur les pics photoélectriques de 780 keV pour ^{82}Br et de 364 keV pour ^{131}I. La position de $^{36}Cl^-$, émetteur béta pur, est déduite par différence.



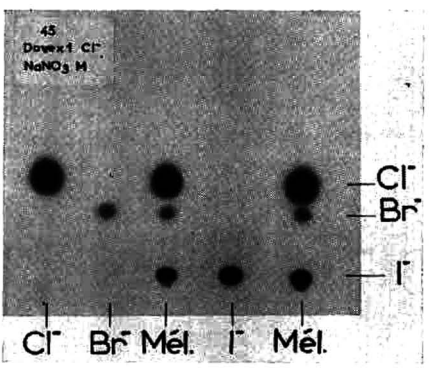


Fig. 1. Courbes d'activité des radio-chromatogrammes d'un mélange d'halogénures radioactifs ($^{36}Cl^-$, $^{82}Br^-$, $^{131}I^-$). Sur cellulose (courbe 1): aucune séparation; sur résine anionique Dowex 1 X 10 (courbe 2): séparation des trois constituants; la courbe 3, obtenue huit jours après, objective la décroissance d'activité de ^{82}Br et de ^{131}I.

Fig. 2. Radio-chromatogramme d'un mélange d'halogénures radioactifs et des témoins correspondants, obtenu sur résine anionique Dowex 1 X 10.

Les périodes très différentes du brôme (36 h), de l'iode (8 jours) et du chlore (3.1×10^5 ans) ont permis, en renouvellant les mesures à huit jours d'intervalle, de confirmer l'ordre de leur séparation (Fig. 1, courbe 3). Une chromatographie du même mélange, effectuée dans les mêmes conditions et avec le même solvant, mais sur une

* Appareillage pour chromatographie sur C. M.: Desaga (Heidelberg), importateur: Roucaire, France.

couche mince de cellulose pure, s'est avérée incapable de séparer les trois halogénures (Fig. 1, courbe 1). Ceci prouve donc le rôle fondamental de la résine échangeuse d'anions dans cette séparation chromatographique.

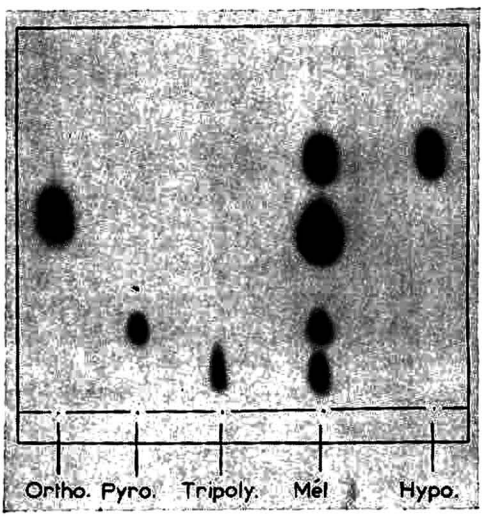
La Fig. 2 montre une autoradiographie d'un chromatogramme du même mélange et des témoins correspondants, constitués par des solutions pures des trois halogénures radioactifs.

Phosphates

Sur résine Biorex 5 (Cl^-), une chromatographie ascendante, réalisée avec une solution aqueuse molaire de $NaNO_3$, a permis de résoudre un mélange de dérivés oxygénés du phosphore: orthophosphate, pyrophosphate, tripolyphosphate et hypophosphite. La révélation des spots est effectuée, après sêchage des plaques, par pulvérisation d'une solution aqueuse à 10 % de molybdate d'ammonium en milieu sulfurique, puis d'une solution à 10 % de chlorure stanneux dans l'acide chlorhydrique normal. Les spots, correspondants aux divers composés, se colorent immédiatement en bleu sur le fond beige de la résine. La coloration dûe à l'hypophosphite est très fugace et disparait rapidement après la vaporisation du révélateur. La Fig. 3 représente le chromatogramme du mélange précédent, ainsi que des témoins correspondants, photographié immédiatement après coloration.

Arsénites et arséniates

Une chromatographie ascendante sur la même résine Biorex 5 (Cl^-), avec une solution aqueuse de NaCl 2 *M*, a permis le fractionnement des arsénites et arséniates alcalins. La révélation, à l'aide des mêmes réactifs que précédemment, doit être précédée ici d'une oxydation des arsénites, obtenue par vaporisation de la plaque avec une solution d'eau oxygénée à 10 volumes (Fig. 4).



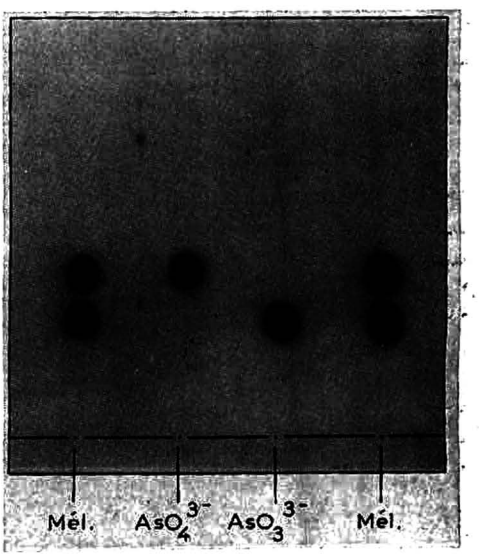


Fig. 3. Chromatogramme d'un mélange d'anions oxygénés du phosphore sur résine anionique Biorex 5.

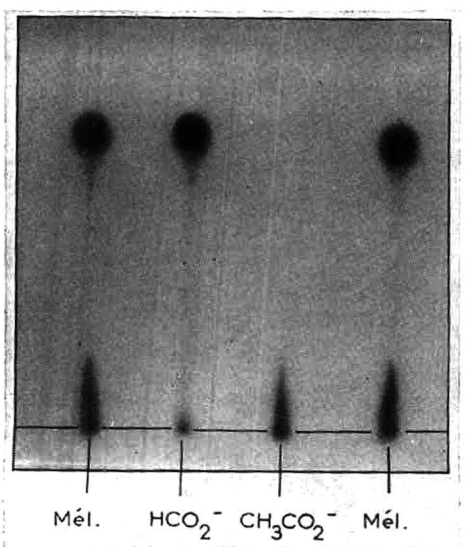
Fig. 4. Chromatogramme d'un mélange d'arsénite et d'arséniate sur résine anionique Biorex 5.

Anions distillables: formiates et acétates

Une mélange équimoléculaire de formiate et d'acétate alcalins a été résolu en chromatographie ascendante sur résine Biorex 5 (Cl^-) et à l'aide du même solvant que précédemment (NaCl 2 *M*). La révélation est obtenue, après sêchage de la plaque, par vaporisation d'une solution à 0.1 % de pourpre de bromocrésol dans l'éthanol, additionnée d'ammoniaque diluée jusqu'à virage de l'indicateur. Les deux anions apparaissent alors en bleu sur fond jaune. Sur la Fig. 5, on constate que si l'ion acétate ne donne qu'une seule tache, sous forme de trainée, très près du point de départ, le spot du formiate se dédouble en une tache régulière migrant à 12 cm environ du dépôt et en une tache de très faible intensité, de migration nulle, dont le R_F se rapproche donc de celui de l'ion acétate. Il s'agit vraisemblablement de traces très faibles d'acétate, que l'on sait d'ailleurs accompagner toujours les formiates, même les plus purs. Cette méthode chromatographique, transposable d'ailleurs sur colonne de résine, pourrait donc servir à préparer un formiate de haute pureté.

SÉPARATIONS RÉALISÉES SUR COUCHES MINCES DE CRISTAUX ÉCHANGEURS D'ANIONS

Les cristaux échangeurs sont des agrégats minéraux de synthèse, de structure microcristalline et qui possèdent une capacité d'échange d'ions semblable à celle des résines organiques conventionnelles. La préparation des couches minces et les proportions des divers constituants de la masse à étaler sont les mêmes que celles indiquées précédemment pour les couches minces de résine. Nous avons utilisé indifféremment soit des cristaux de 2 à 44 μ, préparés spécialement pour couches minces, soit des cristaux ordinaires de 100 à 200 mesh destinés plus spécialement à la chromatographie sur colonne.



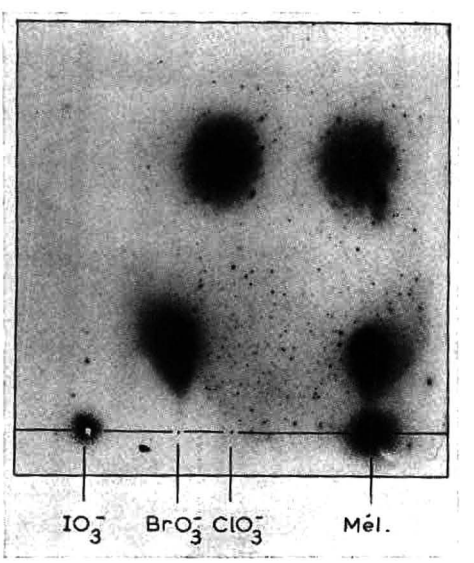


Fig. 5. Séparation chromatographique des anions formiate et acétate sur résine anionique Biorex 5.

Fig. 6. Chromatogramme d'un mélange de chlorate, bromate et iodate sur couche mince de cristaux d'oxyde de zirconium hydraté (HZO-1, Biorad).

Chlorate, bromate, iodate

Une excellente séparation de ces trois anions a été obtenue par chromatographie ascendante sur cristaux HZO-1 (Biorad), constitués par de l'oxyde de zirconium hydraté, à l'aide d'une solution aqueuse de NaCl 2*M*. La révélation des spots est obtenue par pulvérisation d'une solution à 5 % d'iodure de potassium dans HCl normal. Les spots de BrO_3^- et de IO_3^- se colorent immédiatement en brun, celui de ClO_3^- plus tardivement (Fig. 6).

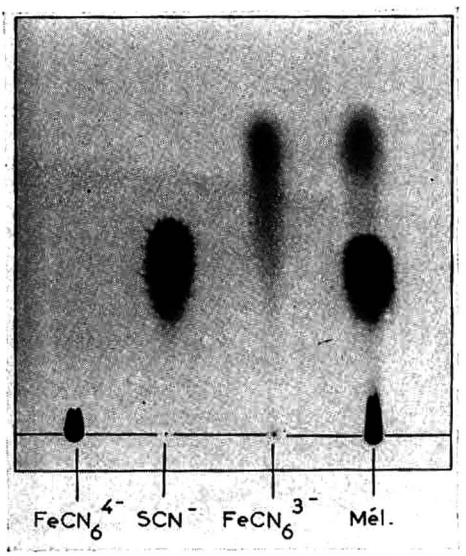


Fig. 7. Séparation chromatographique des complexes cyanés du fer sur cristaux échangeurs HZO-1 (Biorad).

Anions complexes cyanés: thiocyanate, ferro- et ferri-cyanures

Une chromatographie ascendante sur les mêmes cristaux échangeurs d'anions, mais avec NaCl 3*M* comme solvant, permet un excellent fractionnement du mélange de ces trois anions complexes: SCN^-, $Fe(CN)_6^{4-}$ et $Fe(CN)_6^{3-}$. Après séchage de la chromatoplaque, la révélation est obtenue par vaporisation d'une solution aqueuse à 10 % de chlorure ferrique (Fig. 7).

SÉPARATIONS RÉALISÉES SUR COUCHES MINCES DE RÉSINES ÉCHANGEUSES DE CATIONS

Alcalins

Les premiers essais, que nous rapportons, portent sur la transposition sur couches minces de la technique de COHN[19], concernant la séparation des cations alcalins sur colonne de résine Dowex 50. A un mélange de sodium, potassium, rubidium et césium, nous avons ajouté les traceurs radioactifs suivants: ^{24}Na (15 h), ^{42}K (12.5 h), ^{86}Rb (18.5 j) et ^{131}Cs (9.9 j). Ce dernier radio-élément, préparé par irradiation aux neutrons du ^{130}Ba, contient des quantités importantes de ^{131}Ba (12 j). Une solution aqueuse molaire de LiCl entraine, en chromatographie ascendante sur couche mince de résine Dowex 50 WX 2 (H^+), une séparation partielle des éléments précédents. Par ordre de mobilité croissante, on distingue successivement Ba^{2+}, Cs^+, bloc $Rb^+ + K^+$ et Na^+.

A la partie supérieure de la Fig. 8, ont été tracées les courbes de radioactivité, obtenues à un mois d'intervalle, à l'aide d'un compteur G.M. A la partie inférieure de la même figure, les autoradiographies du mélange et des témoins, correspondant aux courbes précédentes, permettent d'identifier les divers constituants[17].

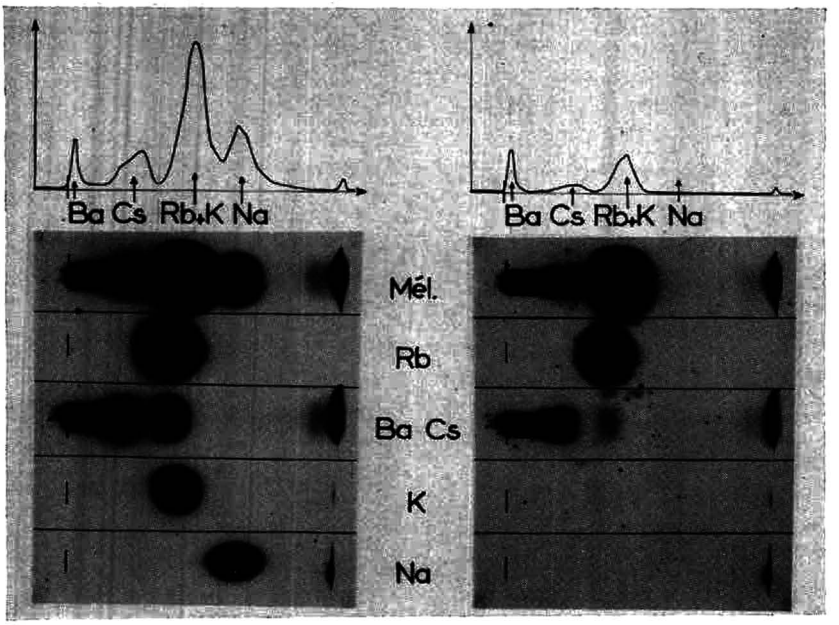


Fig. 8. Radio-chromatogramme d'un mélange de cations alcalins radioactifs, avec les courbes d'activité et les témoins correspondants, obtenu sur résine cationique Dowex 50 WX 2 (à droite: courbe et autoradiographie réalisées un mois après).

Dans un travail plus récent[20] nous avons utilisé un autre type de résine cationique, la résine complexante à groupements aminodiacétate Chelex 100 (Na^+). Deux blocs ont été séparés à partir du même mélange d'alcalins: un premier bloc contenant Na^+ et K^+, qui, non complexés, migrent avec le front du solvant (LiCl 0.25 M); un second bloc, comprenant Rb^+ et Cs^+, plus fortement retenus par la résine, avec un R_F d'environ 0.5.

Alcalino-terreux

Nous référant à la séparation de cations alcalino-terreux radioactifs sur colonne de résine cationique, réalisée par TOMPKINS et collaborateurs[21], nous avons effectué des essais sur un mélange de calcium, strontium et baryum, contenant les radioisotopes suivants: $^{45}Ca^{2+}$ (165 j), $^{89}Sr^{2+}$ (51 j) et $^{131}Ba^{2+}$ (12 j), ce dernier contenant des quantités variables de $^{131}Cs^+$, qui est son produit de transformation. Sur couche minces de résine Dowex 50 WX 2 (H^+), en chromatographie ascendante et avec une solution aqueuse de lactate d'ammonium 0.75 M, ont été séparés, par ordre de mobilité croissante, Ba^{2+}, Sr^{2+}, puis Ca^{2+}, alors que le césium monovalent, moins solidement retenu par la résine, est entrainé avec le front du solvant (Fig. 9).

L'autoradiographie comparée des chromatogrammes du mélange et des témoins, ainsi que l'étude de la décroissance de la radioactivité un mois après (partie droite de

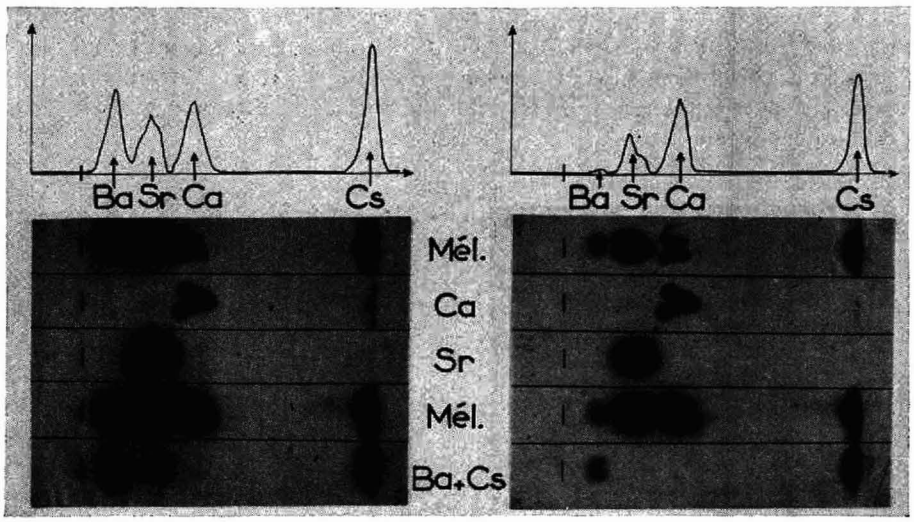


Fig. 9. Radio-chromatogramme d'un mélange de cations alcalino-terreux sur résine cationique Dowex 50 WX 2 (à droite: courbe et autoradiographie réalisées un mois après).

la Fig. 9) ont apporté une confirmation supplémentaire de l'identité des éléments séparés, grâce à leurs périodes différentes. De très bons résultats ont également été obtenus sur couches minces de résine complexante Chelex 100 (Na^+) avec une solution aqueuse de NH_4NO_3 2 *M*. On observe un ordre inverse dans la séparation des alcalino-terreux, les complexes avec la résine ayant une stabilité croissante du baryum au calcium (Fig 10).

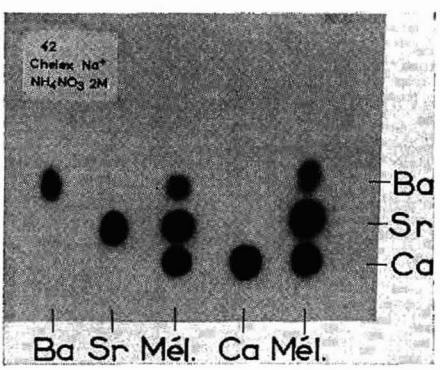


Fig. 10. Radio-chromatogramme d'un mélange de cations alcalino-terreux obtenu sur résine cationique complexante Chelex 100.

Métaux lourds

Toutes les chromatographies des mélanges de cations lourds ont été realisées sur résine Chelex 100 (H^+)[18].

Premier groupe: Ag^+, Hg^+, Pb^{2+}. La séparation de ces trois éléments a été obtenue à l'aide de la technique dite des "Développements répétés". Trois chromatographies ascendantes successives ont été réalisées: la première avec HNO_3 2 *M*

sépare Pb^{2+} des deux autres cations, qui restent pratiquement au point de départ; après séchage de la plaque à l'air, deux autres chromatographies successives avec KCN aqueux à 5 % comme solvant, entrecoupées d'un séchage à l'air, différencient Ag^+ de Hg^+, ce dernier migrant le moins loin (Fig. 11). La révélation est faite à l'aide de H_2S en milieu alcalin.

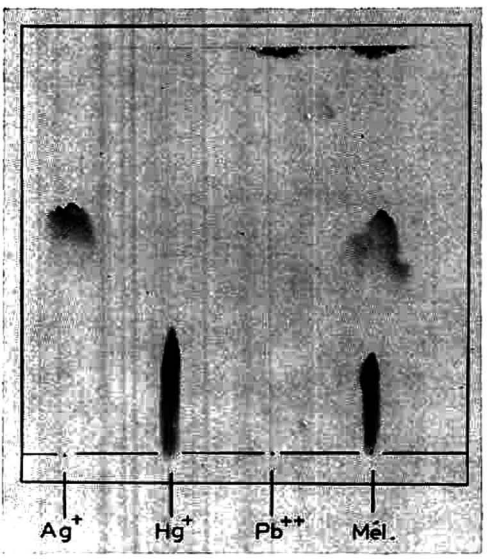


Fig. 11. Chromatogramme du mélange des cations du 1er groupe (Ag^+, Hg^+, Pb^{2+}), sur résine complexante Chelex 100.

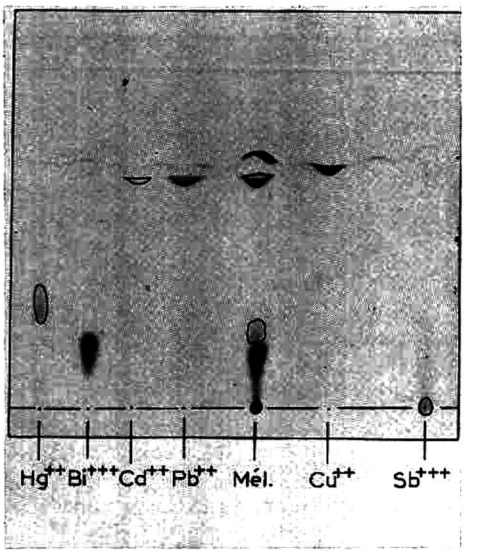


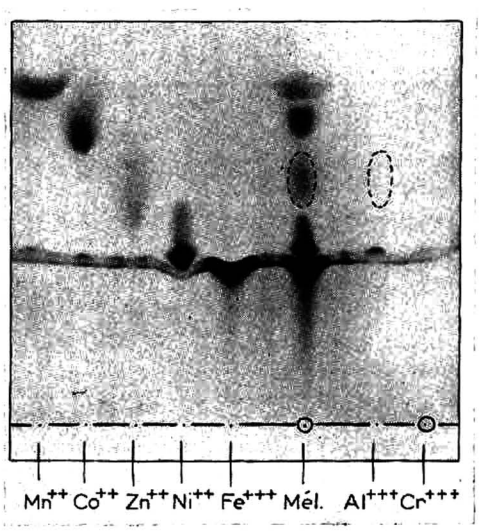
Fig. 12. Chromatogramme du mélange de cations du 2ème groupe (Hg^{2+}, Bi^{3+}, Cd^{2+}, Pb^{2+}, Cu^{2+}, Sb^{3+}) sur résine complexante Chelex 100.

Second groupe: Sb^{3+}, Bi^{3+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}. Ces cations ont été séparés en chromatographie ascendante à l'aide d'une solution aqueuse molaire de HNO_3 et leur révélation obtenue toujours par H_2S en milieu alcalin (Fig. 12). La faible intensité de coloration de certains spots, tels que ceux de Hg^{2+}, Cd^{2+}, Sb^{3+}, nous a contraints à en souligner les contours en noir, pour la reproduction photographique.

Troisième et quatrième groupes: Al^{3+}, Fe^{3+}, Cr^{3+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}. Un certain nombre d'essais préliminaires nous ont conduits à considérer finalement comme possible le fractionnement d'un mélange comprenant la totalité des cations de ces deux groupes. Le solvant utilisé est encore à base d'acide nitrique, mais en concentration plus faible: HNO_3 0.5*M*. La révélation se fait par vaporisation d'une solution méthanolique à 0.2 % de pyridyl-azo napthtol ou P.A.N., suivie d'une exposition aux vapeurs d'ammoniaque. On obtient des spots de teintes variées pour Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Fe^{3+}. La migration de Al^{3+}, dont l'emplacement est entouré d'un pointillé, est identique à celle du zinc, mais ce cation ne se révèle pas par la méthode précédente; par contre il devient parfaitement visible en lumière U.V., après vaporisation d'une solution méthanolique à 1 % de 8-hydroxy-quinoléine, suivie d'une exposition aux vapeurs d'ammoniaque (transformation en oxinate fluorescent). Dans le cas où Al^{3+} et Zn^{2+} sont présents simultanément, leur différentiation est possible en effectuant les deux révélations successivement.

COUCHES MINCES SUCCESSIVES DE NATURE DIFFÉRENTE ET JUXTAPOSÉES SUR LA MÊME PLAQUE DE VERRE

Le principe de cette nouvelle modalité technique est fondé sur la juxtaposition sur la même plaque de verre de deux ou plusieurs couches minces de nature différente, au sein desquelles le mélange à séparer migre successivement, abandonnant ainsi progressivement ses constituants aux différents milieux traversés. Il s'agit donc effectivement d'une chromatographie continue, faisant appel au cours d'une même opération aux pouvoirs séparateurs spécifiques de différents milieux et susceptible de mettre en jeu tour à tour l'adsorption, le partage, l'échange d'ions ou même des réactions chimiques variées. Cette méthode nous a permis de résoudre très rapidement certains problèmes analytiques complexes et d'effectuer, en une seule fois, des séparations chromatographiques irréalisables sur des couches minces constituées par un support unique[17,22,23]. Dans les exemples que nous donnons ici, l'utilisation de couches doubles permet de séparer les deux types de constituants d'un mélange, de propriétés chimiques différentes. On peut également envisager le cas, où la première couche jouerait un rôle essentiellement "épurateur" vis à vis de certaines substances gênant la séparation ultérieure des autres constituants sur la seconde couche juxtaposée.



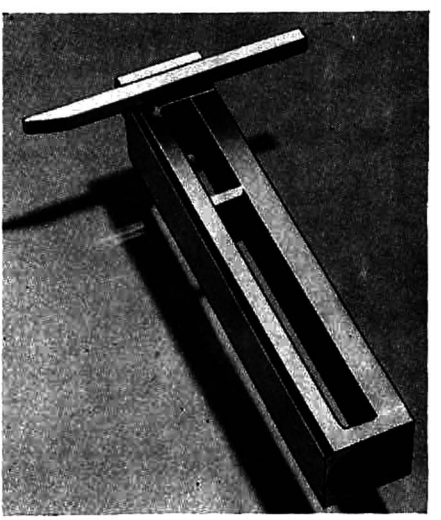


Fig. 13. Chromatogramme d'un mélange de cations des 3ème et 4ème groupes (Mn^{2+}, Co^{2+}, Zn^{2+} Ni^{2+}, Fe^{3+}, Al^{3+}, Cr^{3+}) sur résine complexante Chelex 100.

Fig. 14. Appareil d'étalement modifié pour la réalisation des doubles couches juxtaposées.

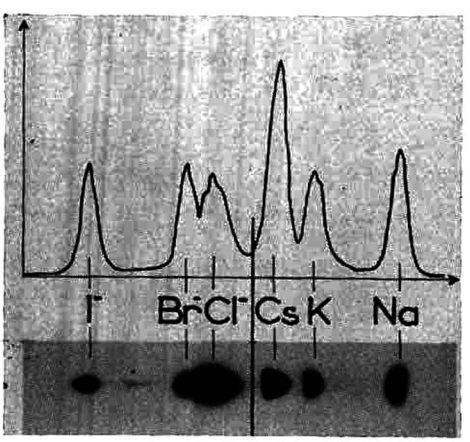
Préparation des couches multiples juxtaposées

Grâce à une modification relativement simple de l'étaleur classique de Desaga, nous avons mis au point un appareil permettant la juxtaposition très régulière de deux ou plusieurs milieux différents sur la même plaque de verre. Seul a été conservé

le chassis extérieur de l'appareil initial, qui sert de cuve réservoir; les ouvertures circulaires, situées sur les côtés, sont obturées à l'aide de deux disques en plexiglass; une cloison mobile, également en plexiglass, permet l'aménagement de deux compartiments de longueur et de capacité variables. De diamètre identique au diamètre intérieur de la cuve, cette cloison est prolongée par une languette, de même largeur que l'orifice rectangulaire de l'appareil et qui, taillée en biseau, présente une arête rectiligne très fine située au contact de la plaque de verre au cours de l'étalement (Fig. 14). Il est également possible d'utiliser une ou plusiers autres cloisons identiques à la première, de manière à réaliser des couches triples ou quadruples par exemple. Les suspensions des différents milieux à étaler sont préparées séparément et chacune d'elles est introduite dans le compartiment désiré, juste avant l'étalement.

Séparations réalisées sur doubles couches résine cationique/résine anionique

Mélange d'halogénures alcalins. Les trois sels suivants ont été utilisés: NaI, KBr et CsCl et leur solution aqueuse a été additionnée des isotopes radio-actifs correspondants, à savoir: ^{131}I, ^{82}Br et ^{36}Cl pour les anions et ^{24}Na, ^{42}K et ^{134}Cs pour les cations. Une première couche de résine anionique Dowex 1 X 10 (OH^-) différencie d'abord les trois halogènes, alors qu'une seconde couche de rèsine cationique Dowex 50 WX 2 (H^+), juxtaposée à la première et d'égale longueur, sépare les cations alcalins. En nous référant aux résultats précédents, tant pour les alcalins que pour les halogènes seuls, nous avons choisi comme éluant une solution aqueuse molaire de $LiNO_3$, éluant qui s'est d'ailleurs avéré efficace d'emblée, en chromatographie ascendante (Fig. 15).



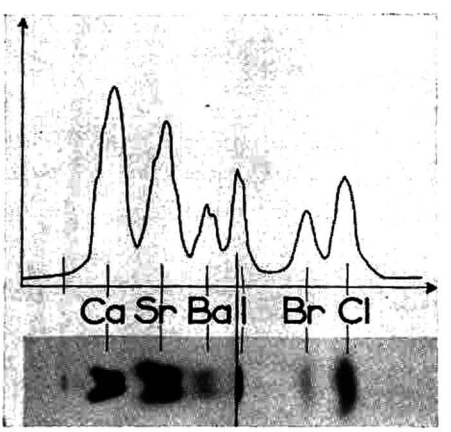


Fig. 15. Radio-chromatogramme et courbe d'activité correspondante d'un mélange d'halogénures alcalins sur double couche résine anionique Dowex 1 X 10/résine cationique Dowex 50 WX 2.

Fig. 16. Radio-chromatogramme et courbe d'activité correspondante d'un mélange d'halogénures alcalino-terreux sur double couche résine cationique complexante Chelex 100/résine anionique Dowex 1 X 10.

Mélange d'halogénures alcalino-terreux. Les solutions aqueuses des sels suivants: $CaBr_2$, SrI_2, $BaCl_2$ ont été utilisées, additionnées également des divers isotopes radio-actifs correspondants. La chromatographie ascendante des alcalino-terreux est

réalisée sur la première couche de résine aminodiacétate Chelex 100 (Na^+) et celle des halogènes sur une seconde couche de résine anionique Dowex 1 X 10 (Cl^-). Pour choisir un solvant unique, convenant à la fois aux anions et aux cations, nous avons tenu compte évidemment, comme dans l'expérience précédente, des résultats antérieurs obtenus sur des mélanges simples et nous nous sommes arrêtés à une solution aqueuse de NH_4NO_3 2*M*. La Fig. 16 montre l'excellente séparation des alcalino-terreux puis des halogènes et les activités de chaque isotope séparé, mesurées au compteur G.M., sont objectivées sur la courbe correspondante, située à la partie supérieure de la figure.

CONCLUSIONS

Depuis longtemps déjà, l'utilisation des échangeurs d'ions en chromatographie sur colonnes appartient à la routine du laboratoire industriel comme du laboratoire de recherches, dans les domaines les plus variés, allant de la chimie biologique à la chimie nucléaire.

Les possibilités techniques des échangeurs d'ions synthétiques, minéraux ou organiques, sont immenses et chaque jour de nouvelles applications sont proposées. De très nombreuses méthodes de séparation font appel aux échangeurs d'ions et les procédés analytiques, mis au point au laboratoire, sont très souvent transposés avec succès sur une plus grande échelle, dans le domaine industriel.

La chromatographie sur couches minces d'échangeurs d'ions représente une étape de plus réalisée dans cette voie à l'échelle micro-analytique. En effet, les quantités de substances, susceptibles d'être séparées et décelées par cette méthode, sont comprises couramment entre 10^{-8} et 10^{-7} atômes ou molécules-gramme et l'application des traceurs radio-actifs permet de descendre beaucoup plus bas encore dans l'échelle des valeurs.

C'est pourquoi, ce nouveau procédé semble présenter un intérêt tout particulier en analyse radiochimique, non seulement dans le domaine minéral, dont font partie les quelques applications que nous venons d'exposer, mais aussi et surtout en analyse organique et biochimique, où l'utilisation des traceurs et des molécules marquées prend chaque jour une place plus importante.

RÉSUMÉ

Des séparations chromatographiques d'ions minéraux ont été réalisées sur des couches minces de résines ou de cristaux échangeurs d'ions et ont permis de résoudre avec succès des mélanges d'anions et de cations de propriétés voisines, tels que les halogénures, les phosphates, les alcalins ou les alcalino-terreux.

A l'aide de "couches multiples", obtenues par juxtaposition de deux ou de plusieurs couches minces d'échangeurs différents, il a été possible de séparer successivement les anions et les cations, contenus dans les mélanges de sels minéraux, tels que les halogénures alcalins ou alcalino-terreux.

L'utilisation de radio-isotopes comme traceurs, au cours de ces expériences, a souvent facilité la détection de certains ions et la mise au point définitive des techniques chromatographiques correspondantes.

SUMMARY

Inorganic ions were separated by chromatography on thin layers of ion exchange resins or crystalline ion exchangers. Successful separation of mixtures of anions and cations with very similar properties, such as halides, phosphates, alkali or alkaline-earth metals, was achieved.

By means of "multiple layers", obtained by juxtaposition of two or more thin layers of different ion exchangers, it was possible to separate successively the anions and cations present in mixtures of inorganic salts, such as alkali or alkaline-earth halides.

The use of radioisotopes as tracers in these experiments has often facilitated the detection of certain ions and helped to give a definite improvement of the corresponding chromatographic techniques.

BIBLIOGRAPHIE

1 N. A. ISMAÏLOV ET M. S. SCHRAÏBER, *Farmacia*, 3 (1938) 1.
2 M. CROWE, *Anal. Chem.*, 13 (1941) 845.
3 T. I. WILLIAMS, *Introduction to Chromatography*, Blackie, Glasgow, 1947.
4 J. E. MEINHARD ET N. F. HALL, *Anal. Chem.*, 21 (1949) 185.
5 I. G. KIRCHNER, J. M. MILLER ET G. I. KELLER, *Anal. Chem.*, 23 (1951) 420.
6 R. H. REITSEMA, *Anal. Chem.*, 26 (1954) 960.
7 E. STAHL, *Dünnschicht Chromatographie*, Springer, Berlin, 1962; *Thin-Layer Chromatography*, Springer, Berlin et Academic Press, New York, 1963.
8 H. SEILER ET M. SEILER, *Helv. Chim. Acta*, 43 (1960) 1939.
9 H. SEILER ET W. ROTHWEILER, *Helv. Chim. Acta*, 44 (1961) 941.
10 H. SEILER ET T. KAFFENBERGER, *Helv. Chim. Acta*, 44 (1961) 1282.
11 H. SEILER, *Helv. Chim. Acta*, 44 (1961) 1753.
12 H. SEILER, dans E. STAHL (Rédacteur), *Dünnschicht-Chromatographie*, Springer, Berlin, 1962, p. 491.
13 S. TURINA, V. MARJANOVIC-KRAJOVAN ET V. OBRADOVIC, *Anal. Chem.*, 36 (1964) 1905.
14 M. HRANISAVLJEVIČ-JAKOVLJEVIČ, I. PEJKOVIČ-TADIČ ET K. JAKOVLJEVIČ, dans G. B. MARINI-BETTÒLO (Rédacteur), *Thin-layer Chromatography*, Elsevier, Amsterdam, 1964, p. 221.
15 R. C. DE GEISO, W. RIEMAN ET S. LINDENBAUM, *Anal. Chem.*, 26 (1954) 1840.
16 J. A. BERGER, G. MEYNIEL ET J. PETIT, *Compt. Rend.*, 255 (1962) 1116.
17 J. A. BERGER, G. MEYNIEL, J. PETIT ET P. BLANQUET, *Bull. Soc. Chim. France*, (1963) 2662.
18 J. PETIT, *Thèse doctorat Pharmacie (État)*, Clermont-Ferrand, 1965.
19 W. E. COHN ET H. W. KOHN, *J. Am. Chem. Soc.*, 70 (1948) 1986.
20 J. A. BERGER, G. MEYNIEL ET J. PETIT, *Bull. Soc. Chim. France*, (1964) 3176.
21 E. R. TOMPKINS, J. X. KHYM ET W. E. COHN, *J. Am. Chem. Soc.*, 69 (1947) 2769.
22 J. A. BERGER, G. MEYNIEL, P. BLANQUET ET J. PETIT, *Compt. Rend.*, 257 (1963) 1534.
23 J. A. BERGER, G. MEYNIEL ET J. PETIT, *Bull. Soc. Chim. France*, (1964) 3179.

A RAPID METHOD FOR THE CARRIER-FREE DETERMINATION OF INDIVIDUAL RARE EARTHS BY ION EXCHANGE AT ROOM TEMPERATURE

S. C. FOTI AND L. WISH
U. S. Naval Radiological Defense Laboratory, San Francisco, Calif. 94135 (U.S.A.)
(Received January 2nd, 1967)

INTRODUCTION

Rare earth and yttrium fission product radionuclides of comparatively short half-lives, *e.g.*, 9-h ^{156}Sm, 5.9-h ^{145}Pr, 3.9-h ^{141}La, are difficult to study because of the amount of time at present required for their chemical separation and recovery. A faster separation procedure is thus desirable. Also, a decrease in the time for separations permits more analyses in a given period of time.

In a previous report on a quantitative separation and recovery procedure[1] carrier-free yttrium and the rare-earth radionuclides were separated first from a gross fission product mixture by liquid–liquid extraction with di-(2-ethylhexyl) phosphoric acid (HDEHP) and then were absorbed on a Dowex 50W-X4 cation exchange resin. Yttrium and the individual rare earths were separated from each other at room temperature in 35 h by gradient elution with 1 *M* α-hydroxyisobutyric acid (AHIB) between pH 2.79 and 3.40 at a flow rate of 1 ml per 10 min. Ion exchange at room temperature[2,3] can be performed with simpler apparatus and more easily than by previous methods using high temperatures[4,5]. The absence of carriers[2,6] makes possible better separations because of less tailing.

Preliminary experiments indicated that a faster ion exchange separation may be accomplished by decreasing the ligand and hydrogen ion concentrations. This approach was investigated more thoroughly because of its simplicity and because of the high cost of the eluant α-hydroxyisobutyric acid. Conditions were established which permit the quantitative separation and recovery of individual rare earth radionuclides by ion exchange at room temperature in 14 h. This work is described in the following.

EXPERIMENTAL

The gradient elution technique[6–8] was used in the separation scheme developed in this study. A schematic diagram of the apparatus employed is shown in Fig. 1. Two vessels, operated under air pressure, served as the reservoirs for the eluant. The air pressure from the laboratory compressed air line was controlled through a pressure gauge. Each vessel was 7 in. long with an internal diameter of 1 in. The Tygon tubing leading from the low pH (3.83) reservoir to the resin column was 3/16 in. I.D. × 22 in. and contained 11 ml of the low pH eluant. A 3-in. piece of Tygon tubing of 3/16 in.

I.D. connected the low and high pH (8.34) reservoirs. A small magnetic stirrer was used during the elution in order to ensure a complete and continuous mixing of the solution in the low pH reservoir. Since both vessels had the same dimensions, the volume removed from the low pH reservoir was replaced with half as much volume from the higher pH reservoir which resulted in a gradual increase in the pH of the eluant.

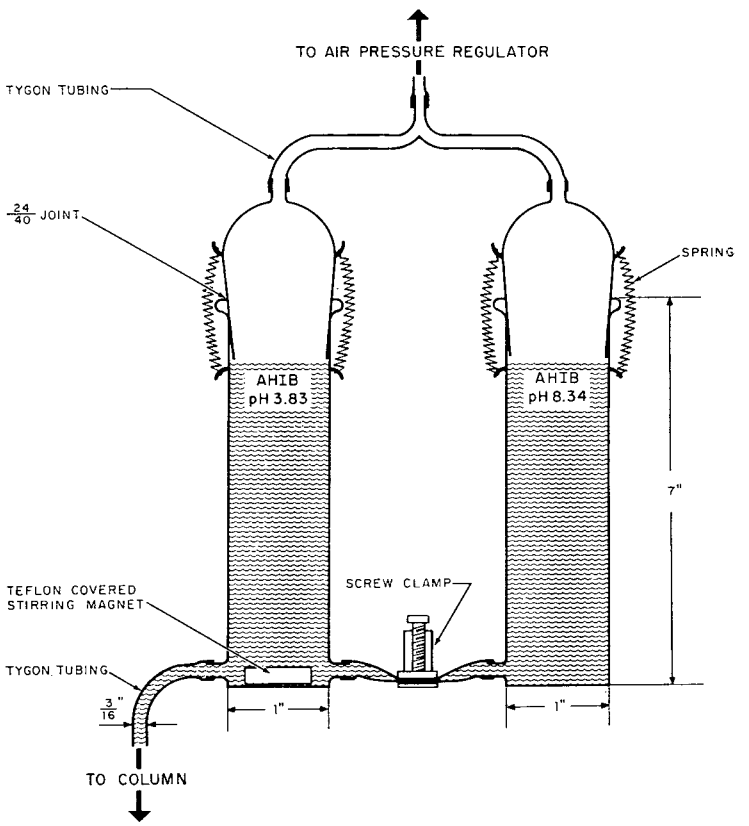


Fig. 1. Gradient elution equipment.

A 51-in. long column was made from 3-mm precision capillary glass tubing. The top of the column had a ground glass joint attachment, and the bottom of the column contained a sintered glass disc of medium porosity. The column was loaded with a slurry of the Dowex 50W-X4 resin in the ammonium form. The resin was equilibrated with the initial eluant before each run. The height of the resin was 47.25 $\pm$ 0.25 in. after equilibration when the column was operated between 5 and 10 p.s.i.g. The volume of the eluant above the resin in the column was about 1 ml. The fractions were collected in 10-ml glass tubes placed in an automatic sample changer. These tubes were transferred directly to a gamma-ray well scintillation instrument for counting.

Reagents

The AHIB was obtained from the Aldrich Chemical Company of Milwaukee, Wis. and used without further purification. Initial solutions were made 2 *M* in the reagent and were slightly turbid. They were filtered and diluted to 0.25 *M*. The pH of the 0.25 *M* AHIB stock solution was 2.4. The eluants were made from this stock solution by adjusting the pH with small amounts of concentrated ammonium hydroxide. The Dowex 50W-X4 cation exchange resin (200 to 400 mesh) was purchased from the Bio-Rad Laboratories of Berkeley, Calif.

Radionuclides

Yttrium-91, europium-155, praseodymium-143, cerium-144 and lanthanum-140 were carrier-free tracers obtained commercially or separated from fission products. Terbium-160, gadolinium-159, samarium-145, promethium-145, and neodymium-147 were of high specific activity and were made by neutron-irradiation of suitable stable isotopes. The purity of the isotopes was checked by comparing the gamma-ray spectra with previously published data. These longer-lived radionuclides were used for convenience in the experimental approach.

Procedure

The resin column was equilibrated initially with 0.25 *M* AHIB at pH 3.83. During the equilibration the flow rate was adjusted to 1 ml per 10 min by regulating the air pressure. The volume of each fraction was determined by weighing the effluent. The eluant density had been determined previously by weighing a known volume.

A mixture of the yttrium and rare earth radionuclides in nitric acid solution was evaporated to dryness. The residue was taken up with 300 μl of the pH 2.4 AHIB stock solution and transferred quantitatively to the top of the resin column using a small amount of the AHIB solution at pH 3.83 as a wash. The space above the resin in the column was filled with 0.25 *M* AHIB at pH 3.83. The reservoirs contained 50 ml of the pH 3.83 and 52 ml of 0.25 *M* AHIB eluant at pH 8.34. The additional 2 ml of pH 8.34 AHIB eluant in the high pH reservoir compensated for the space between the two vessels. A small Teflon-covered magnet was placed in the low pH vessel. The vessels were attached to the air pressure system and the delivery tube connected to the resin column. The magnetic stirrer under the low pH vessel was started. Air pressure was applied to give a flow rate of 1 ml per 10 min (1 drop every 53 to 57 sec). The first ml of effluent, which consisted mainly of the loading solution, was discarded. The automatic fraction collector, set with a time interval of 10 min, was then started. After the first 2 h the pressure was increased to give a flow rate of 1.25 ml per 10 min (1 drop every 42 to 45 sec). This increase in the pressure effected a decrease of 3 h in the total elution time without causing any measurable cross-contamination.

RESULTS AND DISCUSSION

Fig. 2 shows the elution curves for yttrium and the rare earth tracers from a synthetic mixture. The first radionuclide out, yttrium, started to elute in about 1 h. The last, cerium, was recovered in 13.5 h. Lanthanum remains on the ion exchange column after the elution of cerium and can be eluted within 0.5 h by a sharp increase

TABLE I

ELUTION* OF INDIVIDUAL RARE EARTHS FROM DOWEX 50W-X4 AT ROOM TEMPERATURE

Flow rate 1 ml per 10 min, increased to 1.25 ml per 10 min after the first 2 h.

Column	*Y*	*Tb*	*Gd*	*Eu*	*Sm*	*Pm*	*Nd*	*Pr*	*Ce*
	Run 1								
1	6– 10	11– 14	18 – 22	23 – 29	32 – 36	41 – 49	52 – 58	60 – 68	70 – 79
2	6– 10	11– 14	—	24 – 30	32 – 38	43 – 50	52 – 60	61 – 68	71 – 80
3	6– 10	11– 15	—	23 – 28	32 – 37	40 – 48	51 – 59	60 – 68	69 – 78
	Run 2								
1	6– 11	11– 15	—	23 – 28	32 – 37	41 – 48	50 – 59	60 – 68	69 – 80
2	7– 11	11– 14	—	23 – 30	31 – 37	41 – 48	51 – 58	61 – 68	70 – 78
3	6– 11	11– 15	—	23 – 29	31 – 38	41 – 49	52 – 59	—	70 – 79
Elution range	6– 10	11– 15	16 – 22	23 – 30	31 – 39	40 – 49	50 – 59	60 – 68	69 – 80
Volume (ml	6– 11	11– 17.0	17.0– 25.7	25.7– 35.7	35.7– 47.0	47.0– 59.5	59.5– 72.0	72.0– 83.2	83.2– 98.2
Time (min)	0–100	110–150	150 – 220	220 –300	300 –390	390 –490	490 –590	590 –680 (13.5 h)	680 –810

* Characterized by index number of 10 min fractions in which element appeared.

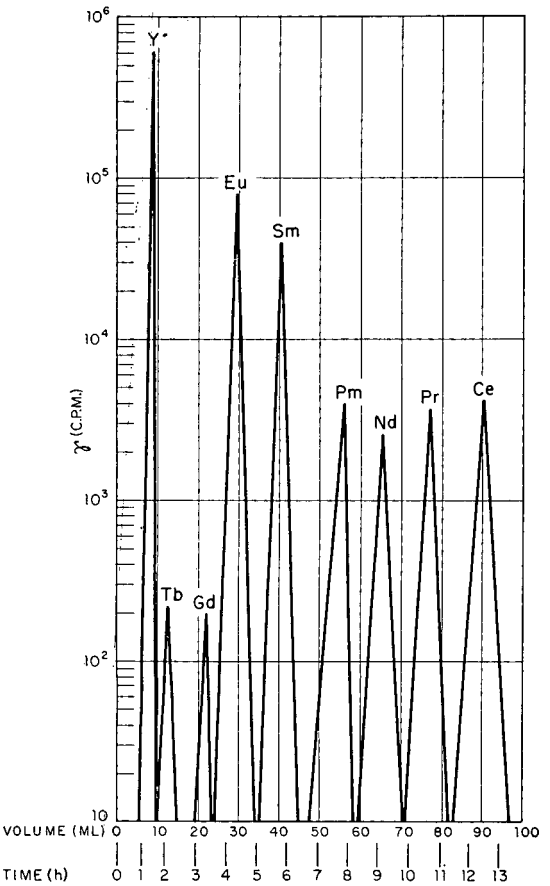


Fig. 2. Elution of yttrium and rare earth radionuclides from Dowex 50W-X4 cation exchange column at room temperature.

in the pH of the eluant. In the elution of both the gadolinium–europium and the neodymium–praseodymium pairs, the times between pair nuclide separations were small. Therefore extra care should be taken for these pairs. The gamma-ray spectra of the individual fractions showed no evidence of cross-contamination. The beta-ray emitting ^{143}Pr, which gives a bremsstrahlung spectrum, showed no gamma-ray contamination. The reproducibility of elution was determined on each of three columns with essentially the same flow rate. The results are given in Table I, which lists the 10-min fraction range in which each element was eluted. In the first run on each of the three columns all of the elements were clearly separated from each other; for these experiments the ratio of the yttrium to the terbium gamma-ray activity (gamma c.p.m.) in the rare earth synthetic mixture was 3.5×10^2. In these three cases yttrium started to elute on the sixth fraction and was recovered by the tenth fraction. Terbium was found in the next five fractions, etc. In fission product samples the ratio of yttrium to terbium gamma-ray activity greatly exceeds 10^3. Therefore, to test the yttrium–

terbium separation in such samples, the procedure was repeated on a rare earth synthetic mixture containing a yttrium-to-terbium gamma-ray activity ratio of 1.5×10^3. The ion exchange resin in each of the three columns was equilibrated back to pH 3.83 with AHIB before the second run. Yttrium was found in the eleventh fraction and contaminated the terbium fraction. All the other elements were separated cleanly. Gadolinium was available for only the first run column 1, and praseodymium for all except the second run, column 3. At the bottom of Table I are the range, volume, and times which best cover the elution of the nine tracers.

TABLE II

PERCENT RECOVERY OF YTTRIUM AND RARE EARTH TRACERS FROM A SYNTHETIC MIXTURE

Column	*Y*	*Tb*	*Gd*	*Eu*	*Sm*	*Pm*	*Nd*	*Pr*	*Ce*
	Run 1								
1	100.5	100.8	100.3	99.6	100.8	99.6	99.6	98.2	98.2
2	99.5	101.0	—	100.4	101.4	99.8	99.6	100.4	100.2
	Run 2								
1	99.9	118.0	—	98.8	101.2	99.4	99.4	101.7	102.3
2	99.9	117.4	—	99.4	99.6	100.0	99.0	98.5	100.2
Mean	99.9	—	—	99.6	100.6	99.7	99.4	99.7	100.6
S.D.	±0.4			±0.5	±0.8	±0.3	±0.3	±1.7	±0.4

For each of four runs (column 1, runs 1 and 2; column 2, runs 1 and 2) the fractions for each tracer were combined in accordance with the elution range results in Table I. The recovery of the nine tracers is given in Table II. The recovery of each radionuclide was obtained as the ratio of the eluted activity to the intitial activity. The average recovery for each radionuclide with the exception of terbium in the second run, column 1 and 2, was 99 to 101 % with a standard deviation of 0.3 to 1.7 %. The terbium in the second run was contaminated with yttrium. This contamination resulted in apparent recoveries of 117.4 and 118 %. The yttrium contamination of the terbium can be eliminated by recycling the terbium fraction through another cation column.

The data in Tables I and II show that the carrier-free elution of rare earth

TABLE III

TIME FOR RECOVERY OF FISSION PRODUCT RARE EARTH RADIONUCLIDES BY THE PRESENT AND PREVIOUS METHOD

Radionuclide	*Present time (h)*	*Previous time (h)*
Y	2.5	4.3
Tb	3.5	7.7
Gd	4.6	11.0
Eu	6.0	14.0
Sm	7.5	19.6
Pm	9.2	24.3
Nd	10.8	27.7
Pr	12.3	31.0
Ce	14.5	36.0
La	15.0	36.5

elements was sufficiently reproducible to permit the collection of these elements in a predetermined time interval. Each column requires calibration and the flow rate must be kept within narrow limits, *e.g.*, 1.0 ± 0.05 ml. per 10 min. Table III compares the time required for the quantitative separation and recovery of fission product rare earth radionuclides by our present and previous method of elution. One hour was required to separate the total rare earth group from the fission product mixture by solvent extraction in preparation for the ion exchange procedure. This time was added to the elution time for the separation and recovery of the individual rare earth radionuclides.

The half-lives of ^{141}La, ^{145}Pr and ^{156}Sm are such that 7, 22.7 and 56 % of the respective activities would remain after separation by the present method while only 0.15, 2.3 and 22.2 % remain by the longer elution method (Table III).

SUMMARY

The time for separations of rare earth radionuclides by ion exchange was reduced to 14 h by the use of one tenth of the concentration of the eluant (α-hydroxyisobutyric acid) and a higher pH. This reduction in time of separations will make it possible to study shorter-lived rare earth radionuclides, *e.g.*, ^{156}Sm, ^{145}Pr, ^{141}La. In addition, more analyses can now be performed in a given period of time.

REFERENCES

1 L. WISH AND S. C. FOTI, *J. Chromatog.*, 20 (1965) 585.
2 H. L. SMITH AND D. C. HOFFMAN, *J. Inorg. Nucl. Chem.*, 3 (1956) 243.
3 K. WOLFSBERG, *Anal. Chem.*, 34 (1962) 518.
4 G. R. CHOPPIN AND R. J. SILVA, *J. Inorg. Nucl. Chem.*, 3 (1956) 153.
5 K. SEYB AND G. HERRMANN, *Electrochem.*, 64 (1960) 1065.
6 W. E. NERVIK, *J. Phys. Chem.*, 59 (1955) 690.
7 R. M. BOCK AND N. S. LING, *Anal. Chem.*, 26 (1954) 1543.
8 L. R. BUNNEY, E. C. FREILING, L. D. MCISAAC AND E. M. SCADDEN, *Nucleonics*, 15 (1957) 81.

AUGMENTING THE SEPARATION OF GADOLINIUM AND EUROPIUM AND EUROPIUM AND SAMARIUM MIXTURES IN ION EXCHANGE ELUTIONS WITH EDTA*

J. E. POWELL AND H. R. BURKHOLDER

Institute for Atomic Research and Department of Chemistry, Iowa State University, Ames, Iowa (U.S.A.)

(Received December 27th, 1966)

INTRODUCTION

The major difficulty encountered in resolving mixtures of europium and gadolinium by ion exchange has been the small magnitude of the separation factor afforded by difference in stabilities of europium and gadolinium chelates[1,2] (see upper curve in Fig. 1). When EDTA is employed as the eluant at 25° for example, $\alpha_{\mathrm{Eu}}^{\mathrm{Gd}} = K_{\mathrm{GdCh}}/K_{\mathrm{Eu\ Ch}} \cong 1.1$ (ref. 2); and it is necessary to elute a band of sorbed Eu–Gd mixture approximately 10 times its length down an ion exchange system to attain the steady state. At this point the inherent overlap between 99.9 % pure, 99.99 % pure, etc., Gd and Eu products is $L = 6h/\log \alpha_{\mathrm{Eu}}^{\mathrm{Gd}}$, $L = 8h/\log \alpha_{\mathrm{Eu}}^{\mathrm{Gd}}$, etc., where h is the height equivalent to a theoretical plate (usually 1 cm or more)[1,2]. Obviously the length of the sorbed band must exceed L if any "pure" product is to be had. The purpose of this work is first to examine the theories regarding chelate stabilities in order to decide whether or not it is feasible to improve the Gd–Eu and Eu–Sm separation factors, and then to perform confirmatory experiments.

It has been suggested by some on the basis of stability constant data[3] that the ligand in ethylenediamine-N,N,N′,N′-tetraacetato complexes of the trivalent rare earths behaves hexadentately in combining with the lighter rare earth cations but only pentadentately when bonding to the heavier, but smaller, rare earth cations and to yttrium. Others[4] have speculated that the EDTA anion is pentadentate in its chelates with the lighter rare earth cations but only tetradentate in its association with the heavier lanthanons. These arguments were advanced to account for the non-monotonic behavior of $\Delta F°$, $\Delta H°$ and $\Delta S°$ for the reaction:

$$R(OH_2)_n^{3+} + Ch^{4-} \longrightarrow [R(OH_2)_{n-m}Ch]^- + mH_2O$$

across the rare earth series[5]. (See Fig. 2.) Both of these views have been nullified by recent precision X-ray diffraction data[6].

From lanthanum through terbium (at least) the $[Ln(OH_2)_3Ch]^-$ anion is the dominant species in crystalline salts having the stoichiometry $MLnCh \cdot 8H_2O$, where M

* Work was performed in the Ames Laboratory of the U.S. Atomic Energy Commission. Contribution No. 2014.

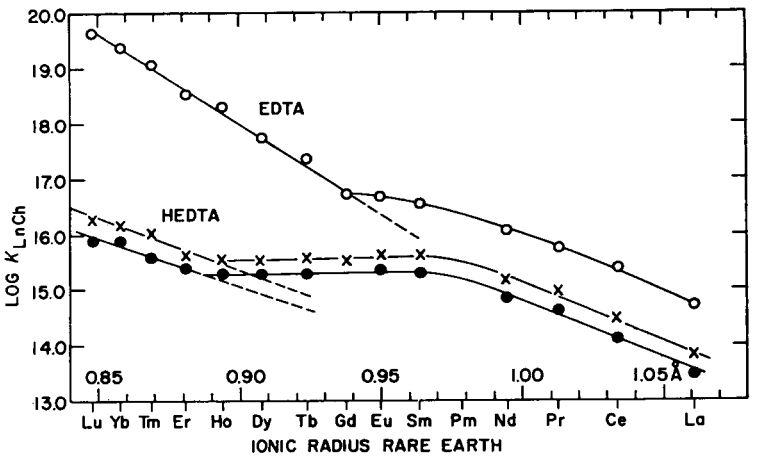


Fig. 1. Stabilities of rare earth EDTA and HEDTA chelates as a function of cationic radius: (○) WHEELWRIGHT, SPEDDING AND SCHWARZENBACH[3]; (×) MACKEY, HILLER AND POWELL[10]; (●) MOELLER AND FERRÚS[11].

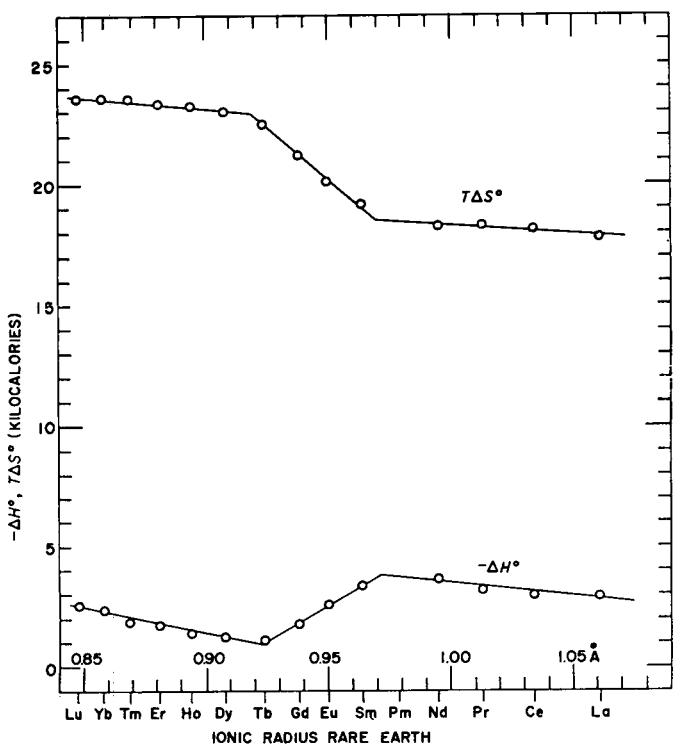


Fig. 2. Enthalpy and entropy contributions to $RT \ln K_{Ln(EDTA)}$ ($-\Delta F^\circ$) as a function of cationic radius.

is ammonium, sodium or potassium, Ln is a rare earth and Ch designates EDTA. This nine-coordinated configuration is believed to persist on through lutetium. The lanthanum and terbium compounds have been found to be completely isostructural so that the anomaly occurring between Sm and Gd can no longer be attributed to a change in the basic dentate character of the EDTA anion or to a change in the coordination number of the chelated rare earth ion.

It is possible, however, that part of the over-all bond energy of the lighter rare earth chelates is derived in a more subtle manner through a type of internal bonding in which the four uncomplexed carboxyl oxygen atoms augment the negativity of the electron donating oxygen atoms of the three adjacent coordinated water molecules. It is not implied that quasi-chelate rings form directly from carboxyl oxygen atoms to hydrogen atoms of the coordinately bound water molecules but circuitously through other water molecules located just outside the primary coordination sphere. Such an effect would impart a dentate character to the EDTA anion nominally greater than six in the case of the larger rare earth cations. Decreasing radius might produce such a strain that the quasi-chelate structure would gradually diminish and perhaps fail to exist at all with Ln^{3+} cations having radii less than about 0.93 Å. A regular decrease in stability with increased radius is consistent with an unvarying bonding mode, and the stability would be expected to continue decreasing along the dashed extension of the straight line through the Lu–Gd data in Fig. 1 if the Ln–EDTA bond strengths were not enhanced in some way from samarium through lanthanum.

An even greater anomaly, of the type observed near the middle of the rare-earth EDTA chelate stability sequence, occurs in the case of β-hydroxyethylethylenediaminetriacetato (HEDTA) chelates, but the transition occurring is more drastic, being manifested from neodymium through holmium rather than reaching completion at gadolinium (see lower curves of Fig. 1). The stability defect in the case of the heavier rare earth chelate species is much more obvious with HEDTA than with EDTA. $\log K_{Ln(EDTA)} - \log K_{Ln(HEDTA)}$ from Ln = La to Ln = Pm is remarkably constant and less than one log unit. This constant small depression of the $\log K_{Ln(HEDTA)}$ values relative to $\log K_{Ln(EDTA)}$ reflects the difference in bonding potential of the CH_2CH_2OH and CH_2COO^- groups; plus, of course, any related difference in hydrogen bonding. It would not be amiss then to suggest that HEDTA, as well as EDTA, behaves hexadentately in coupling to the lighter rare earths. From Nd through Gd, in both cases, one can envisage a progressive rupture of hydrogen bonds between carboxyl oxygen atoms and water molecules. The rapid divergence of the stability curves from Gd through Ho heralds a marked deviation in the bonding characters of HEDTA and EDTA. While increased nuclear charge draws both nitrogen atoms and four carboxyl oxygen atoms of EDTA ever closer, the effect on HEDTA is to pull the two nitrogen atoms and three carboxyl oxygen atoms closer in such a way that a strain results which tends to prevent the hydroxyl oxygen atom from freely occupying a coordination site. Probably a water molecule enters the equatorial site in an increasing percentage of cases, and the change in dentate character is gradual rather than abrupt. One would judge then that the straight line through the (Lu–Gd) EDTA data and its dashed extension represents the stability curve for an uncomplicated $[Ln(OH_2)_3(EDTA)]^-$ anion in which EDTA is hexadentate and, likewise, that the straight line through the (Lu–Ho) HEDTA data represents the stability curve for a simple $[Ln(OH_2)_4(HEDTA)]^-$ anion in which HEDTA is pentadentate.

The models suggested then for EDTA chelate formation are: (1) at least enneahydrated rare earth cations initially in all cases, since X-ray diffraction studies indicate 9 moles of water in coordination sites about the cations in lanthanum, praseodymium, erbium, and yttrium ethylsulfates[7,8]; (2) a nine-coordinated chelate species $[Ln(OH_2)_3Ch]^-$ from lanthanum through neodymium (perhaps promethium) in which a number of hydrogen bonds form between uncoordinated carboxyl oxygen atoms and coordinated water molecules, and tend to enhance the chelate stability above that expected to be due to a simple hexadentate attachment; (3) a gradual change in the number of existing hydrogen bonds, due to strains introduced as Ln decreases in radius from Sm to Eu to Gd, accompanied by progressive reduction in the nominal dentate character of the ligand anion to a minimum value of six; (4) an uncomplicated nine-coordinated chelate species $[Ln(OH_2)_3Ch]^-$ with Ln = Tb, Dy, Ho, Er, Tm, Yb, Lu, and Y, which receives no stability contribution at all from hydrogen bonding.

Now it is immediately apparent that, if something were done to cause Eu^{3+}, Sm^{3+}, etc., to form lesser hydrogen bonded chelate species, the stability constants of the EDTA chelates of these lanthanons would drop to values represented by the extension of a straight line through the Lu–Gd data in Fig. 1. The formation reaction would become less exothermic and the entropy would increase (Fig. 2). It is also apparent that a rise in temperature would accomplish this end since temperature elevation would tend to increase the entropy of the system by destroying the tenuous hydrogen bonded structure between the uncoordinated carboxyl oxygen atoms and the coordinated water molecules.

One way to test the above hypothesis is, of course, to determine painstakingly the temperature dependence of individual Ln–EDTA stability constants; but a simpler test can be made by taking advantage of the fact that the minimum number of band displacements required to resolve a mixture by displacement chromatography depends solely on the mole fraction of the component which elutes first in the original mixture and the separation factor[1,2], that is:

$$\nu = \frac{1 + (\alpha - 1)N_0}{(\alpha - 1)} \tag{1}$$

Another convenient means of ascertaining the separation factor at 92° is to prepare an excess of a known mixture of two dilute pure rare earth EDTA solutions, heat it, and pass it slowly through a section of H^+-form resin bed maintained at 92° until the composition of the effluent solution coincides with that of the feed solution. Then the rare earth mixture is stripped from the resin bed to determine the molar ratio of the components sorbed.

$$\alpha_B^A = \frac{\text{ratio of A to B in solution}}{\text{ratio of A to B in resin bed}} \tag{2}$$

Actually when the value of $\alpha - 1 = \varepsilon$ is small, it is more precise in view of analytical considerations to compute ε directly[9] instead of α. This is possible from material balance data obtained from such an experiment since

$$\varepsilon = \frac{\Delta n}{X_0 Q(1 - X_0 - \Delta n/Q)} \tag{3}$$

where Δn is the total number of moles of component B missing from a series of weighed rare earth oxide samples derived from individual fractions of the effluent solution collected prior to achieving equilibrium (*i.e.*, sorbed on the resin bed in excess of what would have resulted had no separation of components occurred), X_0 is the original mole fraction of B in the aqueous solution, and Q is the total capacity of the resin bed (total number of moles of rare earth mixture sorbed).

EXPERIMENTAL

The experimental set-up consisted of a number of identical ion exchange columns constructed of standard, 2-in. I.D., flanged Pyrex pipe, 5 ft. long, fitted with Teflon resin-bed supports and gaskets, and Plexiglas end closures (reinforced externally by steel plates). The columns were interconnected when it was so desired by means of 6-mm Pyrex tubing and Beckman Teflon fittings. Each column unit was mounted on a rack in a steam heated, thermostated, 10 × 10 × 8 ft., insulated room and filled to a height of 45 in. with 40–50 mesh spheres of Dowex 50-X8 cation-exchange resin in the H^+-cycle.

In the first experiment, the columns were backwashed with de-aerated distilled water, and two columns of the set were charged with an excess of dilute (Eu–Gd) chloride solution applied by back flow to obtain a uniform band of sorbed Eu–Gd mixture. The excess chlorides were removed by further backwashing with de-aerated distilled water, and the rare earth saturated resin beds were allowed to settle.

In the second experiment, two columns were saturated with an excess of (Sm–Eu) chloride mixture and the excess rare earths were removed as described above.

In both cases, the charged columns were connected in series and the sorbed band of binary rare earth mixture (two columns in length) was eluted down a series of additional resin beds at a flow rate of 65 ml/min with 0.01 M ammonium EDTA at pH 8.32 (originally made up at 25°), with the enclosure temperature maintained at 92°. The ammonium EDTA solution was heated to 90° before entering the 92° enclosure and fed through a column containing moist ammonium-form resin beads and air. The top of the resin bed in this column was maintained slightly above ambient temperature by means of an external heating tape. A venting system at the top of the first column of the experimental series allowed air bubbles issuing from the de-aerator to be discharged from the system. The eluant was forced through the system by a pump located outside the heat shield, and the eluate was discharged from the system at a point higher than the top of the last column to ensure that further degassing would not occur in the experimental columns due to a pressure drop. The Gd–Eu and Eu–Sm bands moved down the resin bed system at a rate of about 19 in. per day in both experiments and analytical profiles of the developing chromatograms were obtained by periodically analyzing small portions of solution as the bands passed between adjacent columns of the system, *i.e.*, a complete profile of each developing chromatogram was obtained approximately every half band length of travel. In this way it was possible to estimate the minimum displacement distances required to achieve steady state conditions to about a fifth of a band length (steady state is approached at a constant rate and achieved when the constant composition plateau of a developing chromatogram disappears).

The separation factors α^{Gd}_{Eu} and α^{Eu}_{Sm} were calculated by means of eqn. (1) and are given in Table I. The theoretical plate height, in each case, was calculated from a plot of the log of the ratio of the two components involved as a function of the distance from the front of the chromatogram after steady state conditions were achieved through the relationship

$$h = \Delta L \cdot \log \alpha^{A}_{B} / \Delta \log r \qquad (4)$$

where $r = [A]/[B]$.

TABLE I

SEPARATION FACTORS AND PLATE HEIGHTS CALCULATED FROM THE ELUTION OF Gd–Eu AND Eu–Sm MIXTURES WITH EDTA AT 92°

Mixture (A–B)	N_0	v	$\alpha - 1$	α^{A}_{B}	h *(cm)*
Gd–Eu	0.610	3.04 ± 0.2	0.41 ± 0.04	1.4 ± 0.05	0.7 ± 0.1
Eu–Sm	0.396	1.85 ± 0.2	0.68 ± 0.10	1.7 ± 0.1	0.7 ± 0.1

The values of α^{A}_{B} and ε (see Table II) were also evaluated by the alternate method outlined briefly above.

TABLE II

SEPARATION FACTOR DATA OBTAINED AT 92° IN THE PRESENCE OF EDTA BY AN ALTERNATE PROCEDURE

Mixture (A–B)	α^{A}_{B}	ε	$1 + \varepsilon$	α^{A}_{B} *(av.)*
Gd–Eu	1.53	0.478	1.478	1.5
Eu–Sm	1.77	0.82	1.82	1.8

It is of interest that the latter values are a bit higher than those observed in the elution experiments. It is likely that complete equilibrium was not achieved at the flow rate used in the elution experiments. If such were the case, the transport rate would be affected, a greater than the minimum number of band displacements would be required, and $\alpha - 1$ and α calculated would be low. The figures reported for α^{Gd}_{Eu} and α^{Eu}_{Sm} in Table I, therefore, probably represent minimum rather than true values.

CONCLUSIONS

The observed separation factors in these systems represent a substantial improvement over those reported previously for elution with EDTA at room temperature ($\alpha^{Gd}_{Eu} = 1.1$ and $\alpha^{Eu}_{Sm} = 1.4$)[1]. From this fact it may be inferred that the models

proposed herein are substantially correct. Although the factors were smaller than projected purely on the basis of an extension of the straight line plot through the Lu–Gd data[10] of the upper curve of Fig. 1, they are sufficiently large that elution with EDTA above 90° becomes competitive with other techniques for isolating pure europium from its concentrates. Additional benefits stemming from the application of heat are: (1) substantial improvement in theoretical plate height, allowing faster flow rates to be used; and (2) increased solubility of the H_4Ch species of EDTA, allowing H^+ retaining ion to be used in place of Cu^{2+} or Zn^{2+}, so that recycling of both eluting agent and water becomes economically attractive.

Similar enhancement of separation factors through temperature elevation should occur in the cases of Ho–Dy and Dy–Tb pairs when HEDTA rather than EDTA is the eluant, but it is by no means clear what will occur with pairs of elements from Gd through Nd in the more complex HEDTA system. That the HEDTA Ho–Dy and Dy–Tb separation factors do actually improve with elevated temperature is obvious from data published by MOELLER AND FERRÚS[11] (see Fig. 3). One can only conclude from the observed trend over a 25° temperature range that continued elevation of temperature would depress log $K_{Ln(HEDTA)}$ still further in the cases of terbium, dysprosium and holmium, yet not much affect the stabilities of the erbium, thulium and ytterbium chelates. Consequently, the Tb–Ho chelate stabilities would finally drop to levels corresponding to points lying on an extension of a straight line through the Er–Yb data.

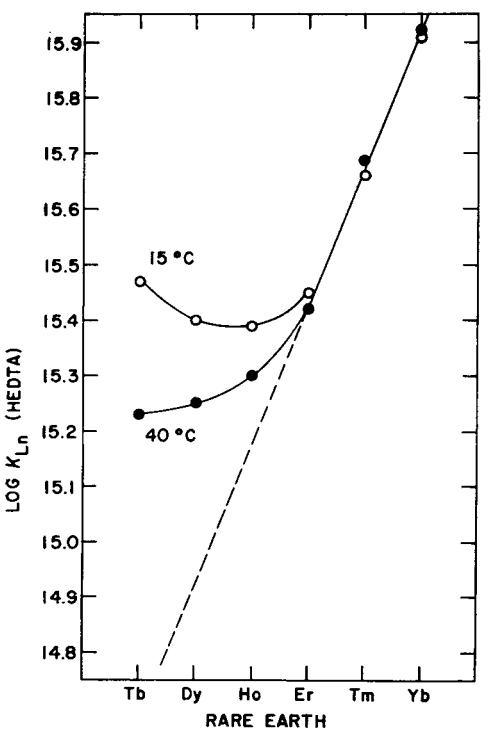


Fig. 3. The effect of increased temperature on the Tb–Yb HEDTA stability constants.

SUMMARY

Scrutiny of thermodynamic data and proposed models for the hydrated ethylenediaminetetraacetato (EDTA) chelates of the rare earths has suggested a rather obvious means of improving displacement chromatographic separations of Gd–Eu and Eu–Sm mixtures on cation exchange columns. Increasing the operating temperature from 25 to 92° was found to effect an increase in the Gd–Eu separation factor from a skimpy 1.1 to a robust 1.47 ± 0.07 and the Eu–Sm separation factor from 1.4 to 1.8, presumably by destroying a degree of internal hydrogen bonding which occurs to a varying extent in the case of the less constrained lighter rare earth EDTA chelates at room temperature. Apparently this type of internal hydrogen bonding is not possible in the case of the smaller heavier rare earth EDTA chelates (Gd through Lu). This interesting and important effect has been correlated with a similar trend noted in the stability behavior of terbium, dysprosium and holmium β-hydroxyethylethylenediaminetriacetates with increased temperature.

REFERENCES

1 J. E. POWELL AND F. H. SPEDDING, *Chem. Eng. Progr. Symp. Ser.*, No. 24, 55, (1959) 101.
2 J. E. POWELL, in L. EYRING (Editor), *Progress in the Science and Technology of the Rare Earths*, Pergamon, Oxford, 1964, p. 62.
3 E. J. WHEELWRIGHT, F. H. SPEDDING AND G. SCHWARZENBACH, *J. Am. Chem. Soc.*, 75 (1953) 4196.
4 R. H. BETTS AND O. F. DAHLINGER, *Can. J. Chem.*, 37 (1959) 91.
5 J. L. MACKEY, J. E. POWELL AND F. H. SPEDDING, *J. Am. Chem. Soc.*, 84 (1962) 2047.
6 J. L. HOARD, BYUNGKOOK LEE AND M. D. LIND, *J. Am. Chem. Soc.*, 87 (1965) 1612; BYUNGKOOK LEE, *Thesis*, Cornell University, Ithaca, N.Y. (1966).
7 E. B. HUNT, R. E. RUNDLE AND A. J. STOSICK, *Acta Cryst.*, 7 (1954) 106.
8 D. R. FITZWATER AND R. E. RUNDLE, *Z. Krist.*, 112 (1959) 362.
9 F. H. SPEDDING, J. E. POWELL AND H. J. SVEC, *J. Am. Chem. Soc.*, 77 (1955) 6125.
10 J. L. MACKEY, M. A. HILLER AND J. E. POWELL, *J. Phys. Chem.*, 66 (1962) 311.
11 T. MOELLER AND R. FERRÚS, *J. Inorg. Nucl. Chem.*, 20 (1962) 261.

Notes

Gas chromatography of isomeric pentyl halides

Research currently in progress in these laboratories is concerned with alkyl rearrangements which may occur during the interactions of the eight isomeric pentyl alcohols with halogenating agents. A number of such rearrangements have been known for a long time and were first recognised by means of refractive index measurements and tests of hydrolytic stability. Secondary alcohols and those branched at the β-carbon atom are particularly prone to rearrangement[1]. Whilst it seems unlikely that in the reaction of any one alcohol all possible isomeric halogenopentanes would be obtained, it is nevertheless important to be able to show with certainty the presence or absence of any particular isomer in a reaction product. A method has therefore been devised for the analysis of such mixtures by gas chromatography. Separations have previously been reported only for the straight chain (1-, 2- and 3-)halogenopentanes[2–4].

Experimental

Materials. 1-Halogenopentanes were obtained commercially and redistilled.

Isomerically pure 2- and 3-halogenopentanes were prepared as described previously, by the use of phosphorus trihalides under specified conditions[5].

The branched chain halogenomethylbutanes were prepared from the corresponding alcohols (Table I), as described previously for the straight chain isomers[5], and purified if necessary by preparative gas chromatography.

Neopentyl halides (1-halogeno-2,2-dimethylpropanes): The chloride was obtained by thermal decomposition of neopentyl chlorosulphite (100°/100 h), in the presence of pyridine hydrochloride (0.01 mol.)[6]. The primary product (52 % yield) contained both neo- (58 %) and *tert.*- (42 %) pentyl chlorides and was shaken vigorously with an equal volume of 2 *N* nitric acid to remove the *tert.*-isomer. Periodic examination by GLC during this process showed hydrolysis to be slow; about 12 h were necessary for equilibration of the system: *tert.*-PeCl + $H_2O \rightleftharpoons$ *tert.*-PeOH + HCl. *tert.*-Pentyl alcohol was then removed by aqueous washing and the whole process repeated eight times to achieve complete purification.

Isomerically pure neopentyl bromide and iodide were prepared by described methods, by interaction of neopentyl alcohol with triphenylphosphine dibromide[7] and triphenyl phosphite–methiodide[8,9] respectively.

The identities of all halides were confirmed by halogen analysis and n.m.r. examination. Infrared absorption frequencies were recorded in the KBr region for the purpose of assisting in the characterization of mixtures.

Apparatus. Purifications by preparative scale GLC were carried out with a 4 ft. × 3/4 in. O.D. glass column packed with squalane (15 %) on 85–100 mesh Celite. A temperature of 50° and nitrogen flow-rate of 250–300 ml/min were employed.

Analytical separations were mostly performed on a Perkin-Elmer F. 11 chromatograph with nitrogen carrier gas and flame ionization detector. Two columns were used:

TABLE I

PREPARATION OF HALOGENOMETHYLBUTANES

Alcohol (ROH)		*Reagent (mol.)*	*Reaction*		*Yield*[a] *(mol.)*	*Alkyl halide composition (%)*[b]			
R	*g. (1 mol)*		*Temp.* (°C)	*Time*		$Me_2CHCH_2CH_2X$	$EtMeCHCH_2X$	$Me_2CHCHXMe$	Me_2EtCX
$Me_2CHCH_2CH_2$	16.74	PCl_3 (0.37)	—10	15 min	0.16	100	—	—	—
	8.23	PBr_3 (0.37)	—10	15 min	0.17	100	—	—	—
	5.50	PI_3 (1.00)	20	60 h	0.32	100	—	—	—
$EtMe_2CHCH_2$	44.95	PCl_3 (0.33)	—15	15 min	0.17	—	99	—	1[c]
	32.60	PBr_3 (0.37)	—10	15 min	0.24	—	100	—	—
	11.00	PI_3 (1.00)	20	48 h	0.40	—	100	—	—
$Me_2CHCHMe$	35.54	PCl_3 (0.33)	—15	15 min	0.24	—	—	14	86[d]
	30.75	PBr_3 (1.00)	—15	15 min	0.70	—	—	20	80[d]
	11.00	PI_3 (1.00)	20	48 h	0.49	—	—	24	76[d]
Me_2EtC[e]	30.10	HCl (2.00)[f]	20	15 min	0.63	—	—	—	100
	32.40	HBr (2.00)[f]	20	15 min	0.43	—	—	—	100
	10.00	HI (2.00)[f]	20	15 min	0.25	—	—	—	100

[a] Distilled product. Crude reaction products were washed and dried before distillation.
[b] Skeletal rearrangements (*i.e.* involving methyl shifts) were not observed.
[c] *tert.*-Isomer removed by prolonged washing with 2 *N* HNO_3.
[d] Isomers separated by preparative GLC.
[e] *tert.*-Pentyl.
[f] Concentrated aqueous acid.

TABLE II

RELATIVE RETENTION TIMES AND CHARACTERIZATION DATA FOR PENTYL HALIDES

Compound	*Relative retention times*		*B.p.*	n_D^{20}	*Infrared* ν_{max} (cm^{-1})[a]			
	Squalane	*Bentone*	(°C)					
1-Chloro-2,2-dimethylpropane	1.00	1.00	83	1.4038	764(s)	722(s)	470(m)	
2-Chloro-2-methylbutane	1.00	1.13	85	1.4049	778(m)	618(m)	561(s)	
2-Chloro-3-methylbutane	1.37	1.47	93	1.4093	785(s)	673(s)	649(s)	515(s)
2-Chloropentane	1.52	1.80	96	1.4052	759(s)	747(s)	671(s)	615(s)
3-Chloropentane	1.63	1.86	97	1.4095	657(s)	634(m)	606(s)	535(m)
1-Chloro-3-methylbutane	1.68	1.70	99	1.4070	727(s)	656(s)		
1-Chloro-2-methylbutane	1.83	1.83	100	1.4120	774(s)	730(s)	683(s)	
1-Chloropentane	2.41	2.66	108	1.4120	731(s)	654(s)		
1-Bromo-2,2-dimethylpropane	1.00	1.00	105	1.4372	746(m)	656(s)	452(m)	
2-Bromo-2-methylbutane	1.00	(1.15)[b]	63/165 mm	1.4416	799(s)	772(s)	510(s)	
2-Bromo-3-methylbutane	1.33	1.52	115	1.4428	775(s)	617(s)	606(s)	456(s)
2-Bromopentane	1.42	1.86	117	1.4380	756(s)	747(s)	617(s)	551(s)
3-Bromopentane	1.55	1.93	117	1.4412	604(s)	531(s)	490(s)	
1-Bromo-3-methylbutane	1.55	1.67	120	1.4410	752(m)	647(s)	565(s)	
1-Bromo-2-methylbutane	1.68	1.82	120	1.4428	765(s)	650(s)	617(s)	
1-Bromopentane	2.15	2.69	129	1.4443	732(s)	643(s)	564(s)	
1-Iodo-2,2-dimethylpropane	1.00	1.00	40/25 mm	1.4886	742(m)	607(s)	447(m)	
2-Iodo-2-methylbutane	0.97	(1.08)[b]	40/25 mm	1.4997	794(s)	763(s)	487(s)	465(s)
2-Iodo-3-methylbutane	1.33	1.54	141	1.4993	771(s)	578(s)	423(m)	
2-Iodopentane	1.39	1.77	145	1.4915	744(s)	580(s)	491(s)	
3-Iodopentane	1.53	1.90	146	1.4982	798(s)	771(s)	574(s)	483(m) 460(m)
1-Iodo-3-methylbutane	1.53	1.58	147	1.4941	735(s)	594(s)	510(m)	
1-Iodo-2-methylbutane	1.66	1.78	148	1.4972	794(m)	763(s)	602(s)	582(s)
1-Iodopentane	2.14	2.51	155	1.4947	726(s)	592(s)	505(m)	

[a] KBr region. Weak absorptions not recorded.
[b] Determined in 1 m glass column at 20°.

(a) 4 m × 1/16 in. O.D. stainless steel column containing squalane (10 %) on 80–100 mesh Chromosorb W. An inlet pressure of 30 p.s.i. gave a nitrogen flow-rate of 13–15 ml/min. Chlorides were analyzed at 20°; bromides and iodides at 40°.

(b) 4 m × 1/8 in. O.D. stainless steel column containing bentone 34 (11.5 %) and silicone MS 555 (11.5 %) on 80–100 Chromosorb W. A nitrogen flow-rate of 25–30 ml/min (inlet pressure 25 p.s.i.) and temperature of 40° were employed for all halides.

As *tert.*-pentyl bromide and iodide were unstable under the above conditions on column (b), mixtures containing these isomers were further examined in a 1 m × 1/4 in. O.D. glass column containing the same bentone–silicone stationary phase. For this a Pye Argon chromatograph was used, with argon flow-rate of 60 ml/min and column temperature of 20°.

Infrared spectra were recorded for liquid films on a Perkin-Elmer 137 KBr spectrometer.

Results and discussion

Relative retention times for the conditions given above are shown in Table II, together with other characterization data determined for the pure isomers. The strongest infrared absorptions in the KBr region are included to assist in the identification of isomers in mixtures.

Typical chromatograms (for the chlorides) appear in Figs. 1 and 2. On squalane, the isomers eluted in order of increasing boiling point. The narrow bore squalane column afforded much greater efficiency than had been obtained in earlier work with

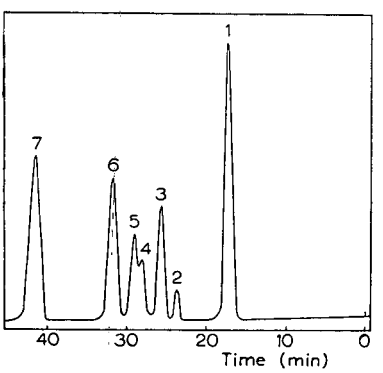


Fig. 1.

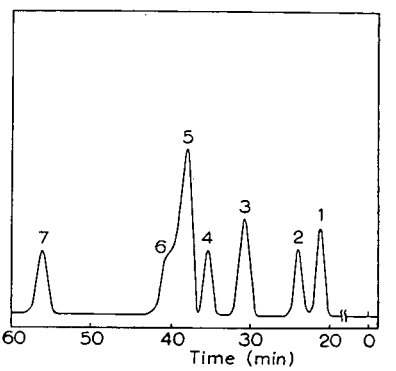


Fig. 2.

Fig. 1. Gas chromatogram of isomeric pentyl chlorides on squalane column. 1 = 1-chloro-2,2-dimethylpropane and 2-chloro-2-methylbutane; 2 = 2-chloro-3-methylbutane; 3 = 2-chloropentane; 4 = 3-chloropentane; 5 = 1-chloro-3-methylbutane; 6 = 1-chloro-2-methylbutane; 7 = 1-chloropentane.

Fig. 2. Gas chromatogram of isomeric pentyl chlorides on modified bentone column. 1 = 1-chloro-2,2-dimethylpropane; 2 = 2-chloro-2-methylbutane; 3 = 2-chloro-3-methylbutane; 4 = 1-chloro-3-methylbutane; 5 = 1-chloro-2-methylbutane and 2-chloropentane; 6 = 3-chloropentane; 7 = 1-chloropentane.

the straight chain halides[2], thus the complete resolution of 2- and 3-halogenopentanes was easily achieved. Two separations which were not possible on this column, *viz.* the 1-halogeno-2,2-dimethylpropanes (neopentyl halides) from the 2-halogeno-2-methylbutanes (*tert.*-pentyl halides), and the 3-halogenopentanes from the 1-halogeno-3-

methylbutanes, were achieved on a modified bentone column, a type which has hitherto been applied mainly to the separation of aromatic compounds and hydrocarbons[10,11]. Of a large number of other stationary phases investigated, none afforded separation of the first mentioned pair of isomers.

tert.-Pentyl bromide and iodide were unfortunately decomposed to a considerable extent in the 4 m bentone column, although not on squalane. This difficulty was overcome for the bromide by use of a short (1 m) glass column at 20°, in which good resolution from neopentyl bromide was obtained, without observable decomposition. *tert.*-Pentyl iodide was still decomposed appreciably however, even under these conditions, and is best determined by aqueous hydrolysis or infrared analysis, after preparative scale separation of all except the neo- and *tert.*-isomers on squalane. Repeated use of the bentone column for these unstable halides resulted in loss of resolving power.

The 3-halogenopentanes were the only isomers which could not be completely separated from all other pentyl structures. This represents no disadvantage unless the 2- and 3-halogenopentanes, the 1-halogeno-2-methylbutanes and the 1-halogeno-3-methylbutanes, are all present in one sample, in which case the 3-halogenopentane content may be obtained by calculation from both chromatograms. Such a combination of products is unlikely to be obtained. With boron tribromide, a reagent which favours alkyl rearrangement, the straight chain pentanols gave only straight chain halides[12]; preliminary experiments with the branched pentyl alcohols indicate that in no case would all these isomers be produced.

Acknowledgements

The authors are grateful to Mr. R. C. LESLIE (Perkin-Elmer Ltd.) for preliminary investigations with a modified bentone column, to Dr. R. G. REES for assistance with the n.m.r. analyses, and to Dr. W. GERRARD for his interest in this work.

Department of Chemistry, Northern Polytechnic, Holloway, London, N. 7. (Great Britain)

B. A. CHAUDRI
H. R. HUDSON
W. S. MURPHY

1 W. GERRARD AND H. R. HUDSON, *Chem. Rev.*, 65 (1965) 697.
2 W. GERRARD AND H. R. HUDSON, *J. Chem. Soc.*, (1963) 1059; *ibid.*, (1964) 2310.
3 H. J. WESSELMAN AND G. W. MILLS, *J. Gas Chromatog.*, (1964) 344.
4 J. CASON AND J. S. CORREIA, *J. Org. Chem.*, 26 (1961) 3645.
5 E. J. COULSON, W. GERRARD AND H. R. HUDSON, *J. Chem. Soc.*, (1965) 2364.
6 W. GERRARD AND P. TOLCHER, *J. Chem. Soc.*, (1954) 3640.
7 G. A. WILEY, R. L. HERSHKOWITZ, B. M. REIN AND B. C. CHUNG, *J. Am. Chem. Soc.*, 86 (1964) 964.
8 S. R. LANDAUER AND H. N. RYDON, *J. Chem. Soc.*, (1953) 2224.
9 N. KORNBLUM AND D. C. IFFLAND, *J. Am. Chem. Soc.*, 77 (1955) 6653.
10 J. V. MORTIMER AND P. L. GENT, *Nature*, 197 (1963) 789.
11 D. WHITE, *Nature*, 179 (1957) 1075.
12 W. GERRARD, H. R. HUDSON AND W. S. MURPHY, *J. Chem. Soc.*, (1964) 2314.

Received February 3rd, 1967

A glass–Teflon continuously variable splitter assembly for the Autoprep 705

The Autoprep 705* vapour phase chromatograph is provided with a metal flow-splittin gsystem to bleed part of the carrier gas and sample to interchangeable electron capture or ionisation cross-section detectors. Alteration of the splitting ratio requires dismantling of the system to place wires of various diameters in the splitter needle. An assembly is described below for a metal-free system for use with a glass column, which allows a wide variation in splitting ratio and adjustment of the latter without dismantling.

The splitter chamber shown in Fig. 1 was sealed on to the end of the glass column. It consisted of a section of glass tubing 2 cm long by 1 cm diameter with a 2 mm diameter bleed tube sealed into the wall. The main flow passed through a 2 mm capillary tube to a series of parallel glass traps for sample collection. A 1 mm constriction was incorporated in this outlet, near the splitter, to ensure that the greatest restriction to the gas flow occured after the splitter.

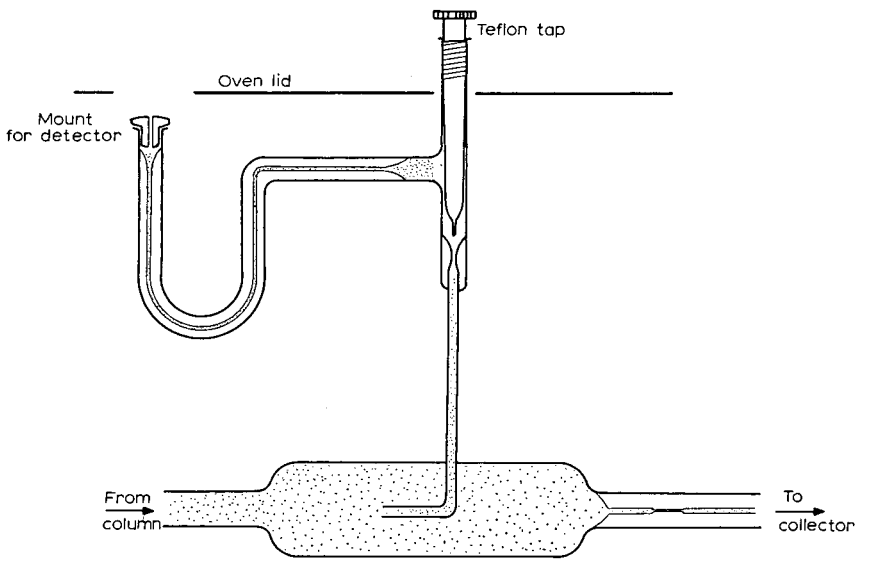


Fig. 1. Splitter chamber and bypass to detector.

The bleed tube was connected to the detector *via* a 1 ¼ mm Teflon–glass needle valve**. This allowed continuously variable control of the flow of gas by-passed to the detector. This could range from 0 to 50 ml/min with a flow through the column of 250 ml/min. The exit from the needle valve was joined by 2 mm capillary tubing to a glass socket into which plugged the Teflon mounting for the detector***. The splitter chamber and capillary leads were filled with the same inert material as was used for the support in the column.

The two detectors used were the same shape and interchangeable. Replacement

* Wilkens Instrument and Research, Aerograph, Manchester, Great Britain.
** Fischer and Porter Co., Warminster, U.S.A.
*** A gas-tight seal was obtained by first spraying the inner wall of the glass socket with a Teflon aerosol (Flucalub-H, Camlab Ltd., Cambridge).

and support of the detectors was assisted by the use of a split glass sleeve between the body and the outer casing which permitted ready alignment of the leads. This sleeve also improved the steadiness of the recorder baseline, presumably by reducing noise from static charges.

In our assembly, the splitter and detector were mounted in the column oven with the top of the Teflon tap and the detector body projecting above the (modified) oven lid. This allowed the splitting ratio to be changed during a separation. For example, a small fraction may be analysed with a large splitting ratio and then larger fractions injected for preparative separation using a smaller splitting ratio or even, by timing, with no flow to the detector at all. The system has been used up to 120° and this temperature could be raised by replacing Teflon by other materials. The use of a needle valve to control splitting ratios in an all-metal system would have similar advantages.

This system has been used in our laboratory to separate the polygermanes and their alkyl derivatives in a metal-free system.

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

K. M. MACKAY
K. J. SUTTON
R. WATT

Received January 16th, 1967

Gaschromatographie cyclischer Anhydride und Imide von Dicarbonsäuren

Cyclische Anhydride und Imide von Dicarbonsäuren stellen wichtige Zwischenprodukte für viele organische Synthesen dar. Die Analyse dieser Verbindungen in Mischung durch übliche analytische Methoden ist oft anspruchsvoll und zeitraubend.

Für die cyclischen Dicarbonsäureanhydride wurde die Bestimmung des Phthalsäureanhydrids durch Gaschromatographie in "coating resins" durch Veresterung des Anhydrids zum entsprechenden Methylester ausgearbeitet[1]. Bei der Analyse des technischen Phthalsäureanhydrids wurde Maleinsäureanhydrid und Phthalsäureanhydrid direkt bei Temperaturen von 185 und 220°[2,3] chromatographiert. Falls in der Probe Phthalsäure vorhanden ist, geht diese in das Anhydrid über.

Zwecks Unterdrückung der Dehydratation der Dicarbonsäuren haben wir Kolonnen mit einem niedrigen Gehalt an stationärer Phase ausprobiert. Gleichermassen interessierte uns die Möglichkeit der Bestimmung cyclischer Anhydride in cyclischen Imiden von Dicarbonsäuren durch Gaschromatographie.

Experimenteller Teil

Die in dieser Arbeit verwendeten cyclischen Anhydride und Imide der Dicarbonsäuren waren Chemikalien von analytischer Reinheit, die wir noch einer weiteren Reinigung unterzogen haben.

Die chromatographischen Arbeiten wurden auf dem Gerät Fractovap D (Carlo Erba, Milano) durchgeführt, das mit einem Flammenionisationsdetektor ausgerüstet war. Als Trägergas gelangte Stickstoff zur Anwendung. Sämtliche Kolonnen waren aus Glas, und der Injektionsraum wurde gleichfalls mit einem Röhrchen aus Borsilicatglas versehen.

Als stationäre Phase wurde Neopentylglykolsuccinat (NPGS), Butandiolsuccinat (BDS), Cyclohexandimethanolsuccinat (CHDMS) und Polyäthylenglykoladipat (PEGA) verwendet. Als Trägerstoff wurde Chromosorb W mit einer Körnung von 60–80 mesh benutzt, das mit 3 % der stationären Phase und 1 % Orthophosphorsäure imprägniert wurde. Es wurden Kolonnen von 80 cm Länge und mit einem Innendurchmesser von 3 mm benutzt. Die Stickstoffdurchflussmenge wurde bei einem

TABELLE I

RELATIVE ELUTIONSVOLUMINA UND KOVÁTS-INDIZES CYCLISCHER ANHYDRIDE UND IMIDE VON DICARBONSÄUREN BEI 140°

Verbindung	*3 % NPGS + 1 % H_3PO_4*		*3 % BDS + 1 % H_3PO_4*		*3 % CHDMS + 1 % H_3PO_4*		*3 % PEGA + 1 % H_3PO_4*	
	V_r	I	V_r	I	V_r	I	V_r	I
Maleinsäureanhydrid	0.06	1581	0.06	1629	0.06	1500	0.07	1645
Bernsteinsäureanhydrid	0.37	1928	0.39	1992	0.32	1844	0.38	1989
Glutarsäureanhydrid	0.74	2034	0.81	2141	0.65	1992	0.76	2137
Phthalsäureanhydrid	0.64	1998	0.68	2104	0.67	2000	0.74	2133
Δ^4-Tetrahydrophthalsäureanhydrid	1.00	2137	1.00	2186	1.00	2082	1.00	2196
Maleinsäureimid	0.28	1887	0.31	1947	0.25	1797	0.40	2002
Bernsteinsäureimid	1.12	2188	1.10	2242	0.94	2068	1.48	2279
Glutarsäureimid	0.79	2055	0.93	2165	0.69	2006	0.93	2180
Phthalsäureimid	3.65	2457	4.30	2479	4.02	2372	5.65	2565
Δ^4-Tetrahydrophthalsäureimid	4.30	2494	4.90	2408	4.28	2384	5.80	2571

Druck von 1 kp/cm² am Kolonnenanfang eingestellt. Vor der Analyse wurden die Kolonnen bei 195–205° unter Trägergasdurchfluss 8–12 Std. konditioniert. Es wurden 0.5–1.0 μl einer 1–5 %igen Lösung der Probe in wasserfreiem Aceton mittels einer Hamilton-Injektionsspritze dosiert.

Ergebnisse und Diskussion

Die relativen Elutionsvolumina der cyclischen Anhydride und Imide der Dicarbonsäuren, bezogen auf Δ^4-Tetrahydrophthalsäureanhydrid (V_r = 1.0) und deren KOVÁTS-Indexe werden in Tabelle I angeführt. Aus den Resultaten ist zu ersehen, dass die cyclischen Imide der Dicarbonsäuren stets hinter den cyclischen Anhydriden der entsprechenden Dicarbonsäuren eluiert werden. Die Unterschiede der Elutionswerte sind dazu hinreichend, dass auch Spurenkonzentrationen cyclischer Anhydride von Dicarbonsäuren in technischen cyclischen Imiden von Dicarbonsäuren durch Gaschromatographie entdeckt werden können. Die sehr verschiedenen Unterschiede zwischen den KOVÁTS-Indexen cyclischer Imide von Dicarbonsäuren und cyclischer Anhydride von Dicarbonsäuren findet seine Erklärung durch die Aktivität des Imid-Wasserstoffs, der mit der stationären Phase Wasserstoffbindungen eingeht.

Auf unpolaren stationären Phasen ergaben die dem Studium unterworfenen

Verbindungen, insbesondere cyclische Imide von Dicarbonsäuren, keine symmetrischen Elutionszacken. Deshalb haben wir den polaren stationären Phasen, denen wir Orthophosphorsäure zugesetzt haben, Aufmerksamkeit zugewandt. Da in cyclischen

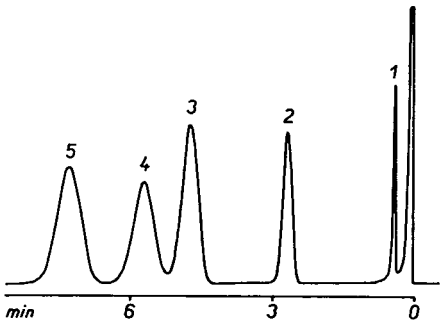


Fig. 1. Chromatogram cyclischer Anhydride: (1) Maleinsäureanhydrid; (2) Bernsteinsäureanhydrid; (3) Phthalsäureanhydrid; (4) Glutarsäureanhydrid; (5) Δ^4-Tetrahydrophtalsäureanhydrid. Kolonne: 3 % Butandiolsuccinat und 1 % H_3PO_4 auf Chromosorb W bei 125°; Stickstoff 1 kp/cm².

Imiden von Dicarbonsäuren der Imid-Wasserstoff sauren Charakter aufweist, wird durch Anwesenheit von Orthophosphorsäure die Adsorption dieser Verbindungen an der Trägerstoffoberfläche in ähnlicher Weise herabgesetzt wie dies bei Fettsäuren[4] der Fall ist. Die Anwesenheit von Orthophosphorsäure beeinflusst ebenso auch die mögliche Reaktion cyclischer Anhydride von Dicarbonsäuren mit stationären Polyester-Phasen. Auf polaren Kolonnen haben wir in sämtlichen Fällen symmetrische Elutionszacken erhalten.

Ein Chromatogramm cyclischer Anhydride von Dicarbonsäuren auf einer Kolonne mit Butandiolsuccinat (BDS) bei der Temperatur von 125° ist in Fig. 1 ersichtlich. Wie wir experimentell festgestellt haben, kommt es bei dieser Temperatur durch das Chromatographieren der einzelnen Dicarbonsäuren zu keiner Dehydratation und auf den Chromatogrammen treten keine Elutionszacken cyclischer Anhydride von Dicarbonsäuren in Erscheinung, mit Ausnahme der Malein- und Glutarsäure, bei denen bei einer Temperatur des Injektionsraumes von 125° gemäss dem Chromatogramm eine 2–5 %ige Dehydratation von der gesamten dosierten Menge eintritt.

Forschungsinstitut für agrochemische Technologie, Bratislava-Predmestie (Č.S.S.R.) JÁN HRIVŇÁK, ZDENĚK ŠŤOTA

Lehrstuhl für organische Technologie an der Slowakischen Technischen Hochschule, Bratislava (Č.S.S.R.) JÚLIUS KALAMÁR

1 G. G. ESPOSITO, *Aberdeen Proving Ground Rept.* No. CCL 132, (1962) 7; *Anal. Abstr.*, 11 (1964) zit. 1826.
2 C. E. MOORE UND S. MEINSTEIN, *Anal. Chem.*, 34 (1962) 1503.
3 H. TRACHMAN UND F. ZUCKER, *Anal. Chem.*, 36 (1964) 269.
4 L. D. METCALFE, *Nature*, 188 (1960) 142.

Eingegangen den 18. Januar 1967

The prevention of condensation of very long chain fatty acid esters in certain gas chromatographs*

In GLC analysis of samples of rapeseed fatty acid methyl esters on polyester columns in an Aerograph A-350-B dual column instrument, the erucic acid ester (22:1) peak appeared as in chromatograms (A) or (B) in Fig. 1. Although the peak area, as measured by an electromechanical integrator, could be used in quantitative analysis with reasonable accuracy, the analysis conditions were not regarded

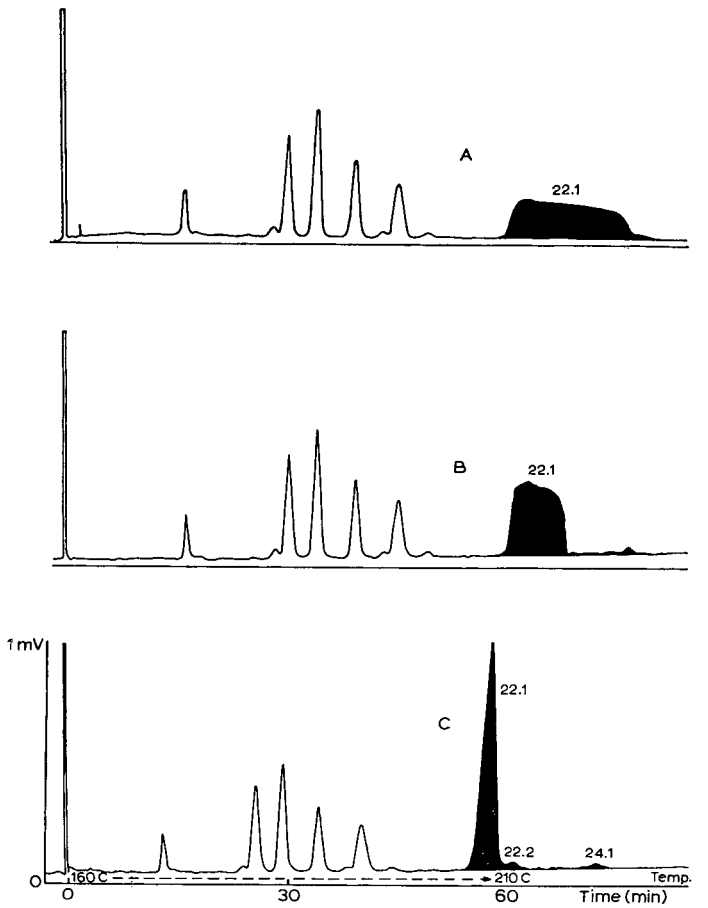


Fig. 1. Analysis of rapeseed oil fatty acid methyl esters on an Aerograph gas chromatograph model A 350-B. Analytical data: 8 ft. × ¼ in. copper columns packed with 60–80 mesh Celite containing 10% BDS. Sample size, 0.5 μl. Injector temperature, 275°; detector temperature, 240°; column temperature 160–210° at a nominal rate of 1°/min. Flow rate of helium, 100 ml/min. Attenuation × 1. The letters A, B and C are referred to within the text.

satisfactory, as the minor components, 22:2 and 24:1 were obscured. Analytical parameters such as sample size, flow rate of carrier gas and temperature programming

* Communication No. 282 from the Swedish Seed Association.

rate were changed in efforts to obtain a sharp erucic acid peak. However, such changes were ineffective in solving the problem. It was observed that if very small samples were used on columns with 10% stationary phase, a sharp erucic acid peak was obtained. With this system minor components gave peaks too small to permit calculation, although the instrument was run on the highest sensitivity. Columns with 30% stationary phase gave, on the other hand, symmetrical erucic acid peaks for larger samples but the very long retention times made this solution of the problem less attractive. Consideration of overloading the column failed to account for the abnormal erucic acid peak as much larger concentrations of methyl oleate gave symmetrical peaks. The existence of so-called "cold spots" within the chromatograph, where high boiling components could condense temporarily was then considered[1].

A few layers of asbestos cord were wound round the two tubes between the columns and the detector, the connections between which are uninsulated for a distance of 2 cm between the column oven and the detector oven. This insulation had a striking effect as the subsequent chromatograms appeared as (C) in Fig. 1. Obviously the erucic acid methyl ester had condensed in the non-insulated connecting tube, when the concentration reached a critical level. It is believed that this is the reason why columns with 30% stationary phase yielded symmetrical peaks for larger absolute amounts.

This modification worked properly until an exchange of damaged connecting tubes had to be made, whereupon chromatograms of types A and B reappeared. The phenomenon occurred if a temperature program starting at 160° was used. Isothermal runs at 210° gave symmetrical erucic acid peaks but did not resolve all components. The new connecting tubes were of stainless steel, whereas those used previously were brass, which has a higher thermal conductivity. Heating tape (Electrothermal Engineering Ltd., London), length 65 cm, was therefore wound round the tubes and connected to a 10 V a.c. supply, thereby providing about 10 W. The erucic acid peak again assumed the appearance demonstrated in chromatogram (C) in Fig. 1.

Even if this phenomenon does not appear in chromatographs equipped with more sensitive detectors, requiring smaller samples, it was thought that the solution of the problem would be of interest to all who have similar equipment in use with high boiling compounds.

The author wishes to thank Dr C. G. YOUNGS, Saskatoon, Canada, for valuable discussions; Svenska Extraktionsföreningen, Karlshamn, and Malmfonden—Swedish Foundation for Scientific Research and Industrial Development, for financial support.

Chemical Department, L.-Å. APPELQVIST
The Swedish Seed Association, Svalöf (Sweden)

1 C. G. YOUNGS, personal communication.

Received January 19th, 1967

High molecular weight alcohols of human hair lipids*

An analysis of alcoholic components of human scalp and hair lipids by gas chromatography has been advanced by GERSHBEIN AND O'NEILL[1] and good resolution of branched chain alcohols was later afforded by thin-layer chromatography (TLC) followed by gas chromatography[2]; components up to C_{30} were detected. In the present study, techniques were explored for the analysis of alcohols of even higher carbon number in such lipids.

Experimental

Alcohol mixtures. Hair cuttings were carefully collected from full-headed men who abstained from the use of any scalp or hair dressings and extracted with petroleum ether (b.p. 30–60°). The two pools of lipids (F-9 and F-10) were saponified by heating with 20 % sodium hydroxide in 95 % ethanol. Each of the unsaponifiable portions in petroleum ether was chromatographed over alumina (Alcoa F-20) and the column eluted with petroleum ether as such and containing 5 % and 10 % chloroform, then 100 % chloroform and finally absolute methanol, whereby five fractions were isolated. The last one (Fraction V) contained the alcohols and sterol. The procedures for removal and processing of the lipids as presented in greater detail in previous reports[3,4] were followed in all respects.

Fraction V was shaken mechanically with 90 % ethanol at room temperature for periods up to 1 h and the solid product after filtration from the solution rich in the lower alcohols was repeatedly extracted with fresh portions of the ethanol. The insoluble portion (Fraction V-In) made up about 5 % of the fraction or about 0.5 % or less as based on the initial hair lipid mixture.

TLC. Separation of the saturated and olefinic alcohols of Fraction V-In was achieved by TLC. For this purpose, glass plates of 20 × 5 × 0.4 cm were uniformly coated with Silica Gel G at a thickness of 0.25 mm, dried at 25° for 16 h and then heated at 110° for 30 min. The cooled plates were stored over silica gel until use. Samples of the lipid in ether were applied 2.0 cm from the edge of the plate and the latter dried at 25° then placed in a chamber containing 70 % ethanol saturated with silver nitrate and which had been equilibrated for several hours previously. Ascending development was conducted at 25° for 1 h, after which time the straight line solvent front was marked and the plates dried. The respective areas or spots were located by initial charring with sulfuric acid–dichromate mixture. With the latter as guide, chromatoplates were then prepared and the two gel portions removed and the respective pools exhaustively extracted with ethyl ether. Solvent was removed under nitrogen thereby yielding the saturated components which did not migrate from the point of application and the mobile unsaturated alcohol portion. The ratio of unsaturated to saturated components was about 1:3.

Gas chromatography. Fraction V-In and the unsaturated alcohol mixture from TLC separation were acetylated with acetic anhydride and the acetates submitted to temperature programmed chromatography to 400–410° in a Barber-Colman gas chromatograph model 5000 with hydrogen flame detector. The rate of heating was 1.5–2°/min. Resolution of components was performed in U-shaped glass columns

* Presented in part at the 5th national meeting of the Society for Applied Spectroscopy, Chicago, Ill., June 14th, 1966.

containing aged or completely stripped SE-30 (2.2 %) on 60–80 mesh Gas Chrom P. The latter was conditioned at 400° for 16 h with helium at 120 ml/min. The samples were injected in ethereal solution and the various peaks identified by comparison with

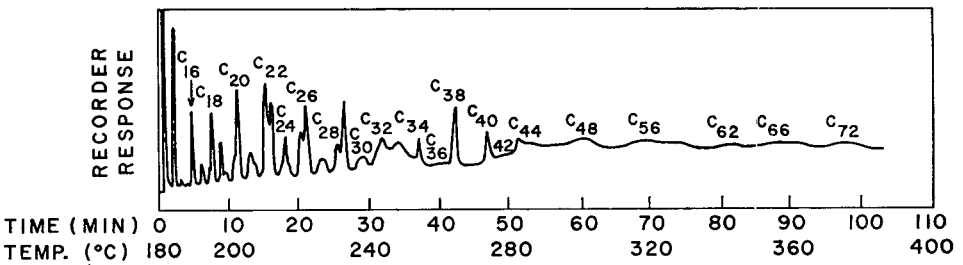


Fig. 1. Gas chromatographic resolution of alcohols of Fraction V-In from pool F-9 in a U-shaped column, 18 in. × 0.6 in. O.D. containing stripped packing, 2.2 % SE-30 on Gas Chrom P; hydrogen flame detector; He pressure: 35 lb.

the elution times. Pertinent chromatograms appear in Figs. 1 and 2. Rubber stoppers or septa became brittle and sintered in the course of the daily runs, a difficulty which was unresolved.

The effect of glass column length on the separation was also explored. A column of 6 in. gave poor resolution and greater combination of peaks, whereas long columns of about 72 in. were responsible for loss of higher molecular weight alcohols, in agreement with the findings reported by KUKSIS[5]. Resolution of components was generally effective with column lengths of 36–48 in. except for the higher components and for which, by far, the best results were achieved with 24 in. glass columns. It is imperative that vibration of the column to settle the packing be avoided as this procedure fractures particles exposing adsorptive sites; rather gentle tapping is recommended.

For Fraction V-In from one pool (F-9), the mass spectrum obtained on heating to 300° showed small but recognizable peaks up to about mass 750 (C_{52}). A minor

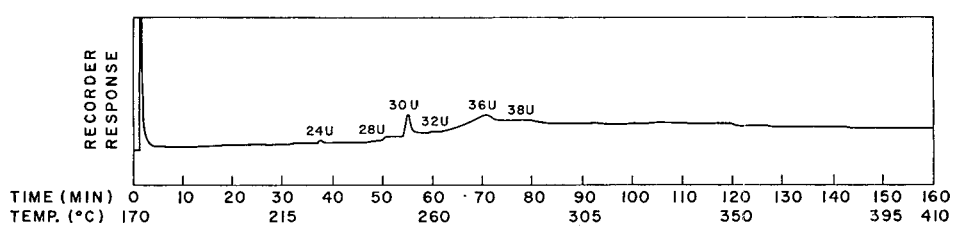


Fig. 2. Gas chromatographic analysis of unsaturated alcoholic components from TLC resolution of Fraction V-In of pool F-10. Temperature programming was carried out as per Fig. 1 except for the column size (30 in. × 0.6 in. O.D.).

portion of material was left in the sample probe. Even longer chain alcohols would have very small peaks and since the amount of components in the range of C_{72} is quite minute, the data do not preclude their presence.

Discussion

By temperature programmed chromatography, both saturated and unsaturated components occurred in the same peaks based on carbon number. It was not possible

to detect the unsaturated moieties by hydrogenation and rerunning the chromatography. Accordingly, the TLC method was instituted and the migrating unsaturated components eluted from the chromatoplates and the resulting acetates analyzed. Previous workers have employed plates impregnated with silver nitrate for such separations but a drawback is the rapid darkening of such plates. This difficulty was circumvented by employing the silver salt as complexing agent in the developing medium.

As will be noted from the chromatogram (Fig. 1), straight chain odd and even alcohols of C_{18} to C_{72} were present in Fraction V-In and possibly, branched components. Attempts at rechromatography in more "efficient" columns resulted in further shouldering and therefore, an exhaustive identification of the higher alcohols cannot be advanced presently. The olefinic members ranged up to about C_{38} or possibly even higher. Sterol was present in small amounts as observed in the gas chromatograms and by colorimetric analysis.

A combination of solvent extraction with TLC and gas chromatography techniques, shown to be of value in the elucidation of sebum higher alcoholic composition, might also be applied profitably to other natural products.

Acknowledgements

This investigation was supported by Public Health Grant, CA 06487, from the National Cancer Institute. The authors are indebted to A. H. STRUCK, Perkin-Elmer Corp. for his aid in the mass spectrometry.

Biochemical Research Laboratories, ERIC J. SINGH
Northwest Institute for Medical Research, 5656 *W. Addison St.,* LEON L. GERSHBEIN
Chicago, Ill. (U.S.A.)

1 L. L. GERSHBEIN AND H. J. O'NEILL, *J. Invest. Dermatol.*, 47 (1966) 16.
2 E. J. SINGH, L. L. GERSHBEIN AND H. J. O'NEILL, *J. Invest. Dermatol.*, 48 (1967) 96.
3 L. L. GERSHBEIN AND B. K. KROTOSZYNSKI, *J. Gas Chromatog.*, 3 (1965) 378.
4 L. L. GERSHBEIN, B. K. KROTOSZYNSKI AND E. J. SINGH, *J. Chromatog.*, 27 (1967) 431.
5 A. KUKSIS, *J. Am. Oil Chemists' Soc.*, 42 (1965) 269.

Received January 19th, 1967

Chromatographic separation of hydrogenation products of dibenz[*a*,*h*]-anthracene

Evidence shows that some partially hydrogenated derivatives of dibenz[*a*,*h*]-anthracene are effective in decreasing tumor incidence when administered with the highly carcinogenic parent hydrocarbon[1,2]. As a part of a program to prepare half-gram amounts of several of these derivatives by catalytic hydrogenation of dibenz-[*a*,*h*]anthracene, methods for separating the hydrogenation products from the parent compound and each other were required. This report describes our efforts to develop gas and column chromatographic methods for carrying out the large-scale separation.

Development column chromatographic techniques were used by LIJINSKY[3-6] for separating reduced derivatives of dibenz[*a*,*h*]anthracene. The basis of his separation was development on magnesia–Celite, after which the column material was extruded, and the segments containing the various derivatives were separated, eluted, and rechromatographed. In order to separate larger amounts of materials more rapidly, two continuous separation techniques, preparative gas chromatography and elution column chromatography, were selected for evaluation in preference to the successful but slower technique of LIJINSKY.

Several unsuccessful attempts were made to separate these hydrogenation products by preparative gas chromatography. All of the experiments were conducted on a Wilkens Model 700 Autoprep preparative gas chromatograph fitted with a 3/8 in. × 30 in. column packed with 10% SE-30 (a G.E. methyl silicone) on 60–80 mesh acid-washed Chromosorb W (Applied Science Laboratories, State College, Pa.).

Three problems combined to make this approach unsuccessful. First, the reaction products and the parent compound are solids at room temperature, and only slightly soluble in organic solvents. Thus they had to be injected as dilute solutions (0.5 ml of benzene) so that yields on each cycle were low, and much solvent in the exit stream had to be trapped and removed.

Secondly, the high melting points of the parent compound and the derivatives (*e.g.*, dibenz[*a*,*h*]anthracene, 262°; 1,2,3,4-tetrahydrodibenz[*a*,*h*]anthracene[1], 211–212.5°) required extremely high injector, column, detector, and collector temperatures and short columns in order to obtain symmetrical peaks, short retention times (to permit repetitive injections), and adequate resolution. The best separations were achieved at 345°, at which temperature the stationary phase bled too rapidly; the nonmetallic parts of the instrument, especially the gaskets, deteriorated rapidly; and evidence from repetitive injections of trapped fractions indicated that the hydrogenation products had partially decomposed.

Thirdly, the parent compound and hydrogenation products apparently existed in the exit stream as aerosols and were extremely difficult to trap in any standard collector. Many modifications of the collectors were made but much potentially carcinogenic material always penetrated the collection system so that the latter had to be installed in a special hood.

Both silica[7-11] and alumina[8,10-21] have been used for separating benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene from other polynuclear aromatic hydrocarbons by liquid adsorption column chromatography. Alumina produced the best separations with mobile phases consisting of mixtures of aromatic and alicyclic or aliphatic hydro-

carbons, or petroleum ether and acetone, benzene–cyclohexane being the most widely used combination.

Because of the previous successes with alumina, and of the expectation that the separation of these structurally similar partially hydrogenated derivatives of dibenz[*a*,*h*]anthracene from each other and the parent compound would be more difficult than the separation of polynuclear aromatic hydrocarbons, acid-washed alumina (Merck) was selected for this separation. To ensure a standard adsorbent, all of the acid-washed alumina was heated at 130° for 30 h, after which 1.7 % water was added to give a total of 13.5 %[22]. A short experiment on 7 mm × 40 mm columns showed that dibenz[*a*,*h*]anthracene could be eluted from this alumina with no apparent decomposition.

A mixture of benzene–cyclohexane was similarly selected as the eluent. Experiments on the 7 mm × 40 mm columns with a hydrogenated dibenz[*a*,*h*]anthracene mixture showed that a mixture of 15 % benzene and 85 % cyclohexane (by volume) gave adequate separation as judged by development of fluorescent bands on the column, consistent with a desirable slow flow rate.

Other experimental conditons selected for separation were: column size, 1 in. × 36 in.; column packing, 475 g of standardized alumina in a slurry of eluent (benzene–cyclohexane, 15:85); column loading, 5 g of hydrogenated dibenz[*a*,*h*]anthracene mixture in a slurry with 300 ml of the eluent (although this technique is not ideal, it was permissible because the most soluble solutes were the least strongly adsorbed, and the least soluble—dibenz[*a*,*h*]anthracene—was the most strongly adsorbed); column flow rate, not controlled but varied between 2 and 60 ml/h. In addition, the eluent was kept saturated with nitrogen to reduce oxidation on the column, and the column was covered with aluminum foil to eliminate light and consequent decomposition.

Fractions were collected with Packard and LKB automatic constant-volume fraction collectors. In some experiments, unresolved mixtures were rechromatographed on the same type of column with the same eluent. All the fractions containing single components or similar mixtures were combined and the eluent was removed with a flash evaporator. The various hydrogenation products and the parent compound were identified in the fractions by NMR spectroscopy and ultraviolet spectroscopy, using for comparison ultraviolet spectra of pure compounds furnished us by Dr. LIJINSKY.

Elution order was established by chromatography and rechromatography of several hydrogenated dibenz[*a*,*h*]anthracene mixtures, *i.e.* a mixture of highly hydrogenated unidentified materials, 5,6,12,13-tetrahydrodibenz[*a*,*h*]anthracene, 1,2,3,4,12,13-hexahydrodibenz[*a*,*h*]anthracene, 5,6-dihydrodibenz[*a*,*h*]anthracene, 1,2,3,4-tetrahydrodibenz[*a*,*h*]anthracene, and finally dibenz[*a*,*h*]anthracene. Not all of these compounds could be separated by repetitive column chromatography, and separations were terminated when the following pure compounds and mixtures were obtainable:

(a) 5,6-dihydrodibenz[*a*,*h*]anthracene, 99+ mol %;
(b) 5,6-dihydrodibenz[*a*,*h*]anthracene, 86.5 ± 2.5 mol %, and 1,2,3,4-tetrahydrodibenz[*a*,*h*]anthracene, 13.5 ± 2.5 mol %;
(c) 1,2,3,4,12,13-hexahydrodibenz[*a*,*h*]anthracene (72.5 ± 5 mol %), and 5,6,12,13-tetrahydrodibenz[*a*,*h*]anthracene (27.5 ± 5 mol %).

Estimates of the purity of these compounds were obtained by integration of NMR spectra.

Conclusion

The column chromatographic method is simpler than previously published methods for separating partially hydrogenated derivatives of dibenz[*a*,*h*]anthracene, and it gives satisfactory separations. Several of the compounds were not completely separated by the method; operating the column at optimum conditions for separation[23] or use of solvent gradients, should improve the separation. Neither of these approaches was tried in this study.

This research was conducted under Contract No. PH 43-64-567, National Cancer Institute, National Institutes of Health.

Midwest Research Institute, J. F. ENGEL
Kansas City, Mo. 64110 (U.S.A.) J. E. BARNEY II

1 W. LIJINSKY, H. GARCIA, B. TERRACINI AND U. SAFFIOTTI, *J. Natl. Cancer Inst.*, 34 (1965) 1.
2 W. LIJINSKY, W. CEFIS, H. GARCIA AND U. SAFFIOTTI, *J. Natl. Cancer Inst.*, 34 (1965) 7.
3 W. LIJINSKY AND L. ZECHMEISTER, *J. Am. Chem. Soc.*, 75 (1953) 5495.
4 W. LIJINSKY, *Anal. Chem.*, 32 (1960) 684.
5 W. LIJINSKY, *J. Org. Chem.*, 26 (1961) 3230.
6 W. LIJINSKY AND C. R. RAHA, *Toxicol. Appl. Pharmacol.*, 3 (1961) 469.
7 H. J. CAHNMANN, *Anal. Chem.*, 29 (1957) 1307.
8 S. Z. CARDON, E. T. ALVORD, H. J. RAND AND R. HITCHCOCK, *Brit. J. Cancer*, 10 (1956) 485.
9 D. HOFFMAN AND E. L. WYNDNER, *Cancer*, 13 (1960) 1062.
10 M. J. LYONS AND H. JOHNSTON, *Brit. J. Cancer*, 11 (1957) 60.
11 H. J. RAND, S. Z. CARDON, E. T. ALVORD AND A. BURHAN, *Am. J. Surg.*, 94 (1957) 438.
12 G. J. CLEARY, *J. Chromatog.*, 9 (1962) 204.
13 B. T. COMMINS, *Intern. J. Air Pollution*, 1 (1958) 14.
14 J. W. COOK AND R. SCHOENTAL, *Nature*, 167 (1951) 725.
15 J. A. S. GILBERT AND A. J. LINDSEY, *Chem. Ind. (London)*, (1956) 927.
16 D. HOFFMAN AND E. L. WYNDNER, *Cancer*, 15 (1962) 93.
17 G. E. MOORE AND M. KATZ, *Intern. J. Air Pollution*, 2 (1960) 221.
18 B. MUEL, M. HUBERT-HABART AND N. P. BUU-HOÏ, *J. Chim., Phys.*, 54 (1957) 483.
19 A. REUTER, K. O. JOHNE AND J. LIEB, *Z. Anal. Chem.*, 164 (1958) 335.
20 C. SEELKOPF, *Z. Lebensm. Untersuch. Forsch.*, 100 (1955) 218.
21 B. L. VAN DUUREN, *J. Natl. Cancer Inst.*, 21 (1958) 1.
22 Training Course Manual, *Analysis of Atmospheric Organics*, U.S. Department of Health, Education, and Welfare, Public Health Service, Bureau of State Services, Division of Air Pollution, Section III, *Use of Column Chromatography for Polynuclear Hydrocarbon Analyses*, March 1965, p. 1.
23 J. C. GIDDINGS, *Anal. Chem.*, 36 (1964) 1890.

Received January 17th, 1967

Retention behavior of steroids in gas chromatography with a series of combination columns

The present paper shows that a series of combination columns might be used as means for obtaining further information regarding the structure of steroidal compounds. Steroids predominantly ketonic showed a negative slope, while hydroxyl type compounds presented a positive slope when retention times were plotted against substrate concentration. Equal numbers of ketones and hydroxyls in a molecule resulted in little or zero slope.

Experimental

A Glowall Model A-110 with a 1 cm argon ionizing detector, containing radium, was used. Glass coil columns, 6 ft. × 4 mm I.D., were packed by the vacuum technique with Gas Chrom Q (Applied Science) coated with the stated concentration of substrate. The coating procedure involved dissolving the calculated amounts of both substrates in methylene chloride and evaporating the solvent after addition of the support. All the columns were conditioned at 240° for 18 h or more with argon flowing through at 10 p.s.i. inlet pressure. Flash temperature 260°, column and detector temperature 240°, with an inlet pressure of 30 p.s.i. are the operating conditions.

All steroids were used as obtained from the supplier. In each instance the amount of impurity was negligible for the present purpose.

Samples were made up as 0.5 $\mu g/\mu l$ solution in *tert.*-butyl alcohol and 2–4 μg were injected using a 10 μl Hamilton syringe.

Relative retention times with respect to estrone as unity were calculated by measuring distance of maximum peak height from the initial pressure or "air" peak.

The retention times were plotted against percentage concentration of substrate in the combination packing used in the columns.

The pair QF-1 (fluorosilicone polymer, Dow Corning) and L-45 (methyl silicone, General Electric) were used since these in combination have given the most satisfactory results for separation of many steroids. They represent a selective and non-selective substrate respectively. In addition as previously reported[1], this type of combination can result in greater response or recovery for certain compounds. The packings were made up so that the total substrate concentration was 5 % of support; *viz.* 3.5 % of QF-1 and 1.5 % of L-45 or either one 5 % alone.

Results

Table I gives the retention times of 16 representative steroids relative to estrone as unity. These retention times are plotted in a nomographic manner in Fig. 1. Both estrone and androsterone, each having a single ketonic and hydroxylic function, showed zero slopes in the curve presented by their retention times. Progesterone, a diketone, had a negative slope while the dihydroxyl compound estradiol-17β showed a positive slope. Compounds containing predominantly ketones had negative slopes, while those with more hydroxyls showed positive slopes. As in the case of cholesterol, molecular weight has an influence on the retention times while retaining the positive slope due to the hydroxyl function.

Preliminary attempts were made to apply regression lines to the data. If this could be done it would be possible to apply mathematical methods to predict the

J. Chromatog., 29 (1967) 235–238

TABLE I

RETENTION BEHAVIOR OF STEROIDS IN A SERIES OF COMBINATION COLUMNS RELATIVE TO ESTRONE (10 MIN) AS 100

Compound	%*QF-1*[a] %*L-45*	*Relative retention time*									
		5% 0		3.75% 1.25%		2.5% 2.5%		1.25% 3.75%		0 5%	
		1[b]	2[c]	1	2	1	2	1	2	1	2
Cholesterol		105	108	170	163	235	240	300	301	365	364
2α-Hydroxyestriol		252	253	287	—	322	322	357	—	392	370
3α,20α-Dihydroxypregnane		78	80	91	90	104	105	117	118	130	131
Estradiol-17β		65	67	75	75	86	85	95	95	105	105
Estriol		144	143	153	153	162	163	171	171	180	181
Androst-5-ene-3β,17α-diol		52	53	60	60	68	68	76	76	84	86
Pregnanolone		106	106	110	111	113	114	117	117	120	121
Androsterone		82	82	82	82	82	82	82	82	82	83
Estrone		100	100	100	100	100	100	100	100	100	100
17β-Hydroxyandrostan-3-one		106	106	103	—	100	100	97	98	94	95
Testosterone		160	159	148	148	136	136	124	124	112	114
Androstane-3,17-dione		180	179	158	161	136	136	114	111	92	92
Androst-4-ene-3,17-dione		258	259	220	226	182	185	144	143	106	109
Progesterone		332	332	292	293	252	256	212	211	172	173
17α-Hydroxyprogesterone		454	454	400	—	346	346	292	—	238	238
Androst-4-ene-3,11,17-trione		402	406	332	341	262	271	192	193	122	127

[a] The columns used are not more than three days old. Retention times will change gradually with age.
[b] Calculated data.
[c] Experimental data, each figure represents the average of three determinations.

structure of a compound. Coefficients of determination for linear curves fitted to the 16 compounds were of the order 94–97 %. This was not considered sufficient for accurate prediction of molecular structures.

It has been shown by others that equatorial configurations in the various positions of the steroid molecule influence retention times. We are now in process of enlarging the series in attempt to improve the coefficient of determination.

Since the relationship between retention times and concentration of substrate was linear, a mathematical expression was possible:

$$\frac{P_1R_1 + P_2R_2}{P_1 + P_2} = R_3$$

The percentage of a second substrate (P_2) required to give a separation (R_3) when the amount of the other substrate (P_1) is known can be calculated. The retention times of R_1 and R_2 of the individual substances with the substrates individually must be known. In the experiments so far performed substrates combinations over 15 % were not satisfactory. This type of data has been adapted for computer use by PORTER *et al.*[2].

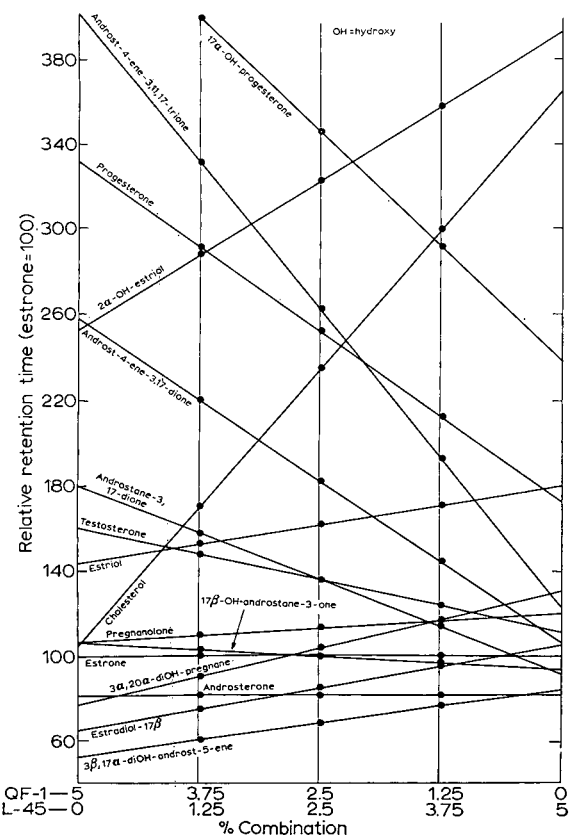


Fig. 1. Retention behavior of steroids on combination columns plotted from calculated data in Table I. OH = hydroxy.

Discussion

A number of reports have described the use of the steroid number concept[3–5]. Retention factors have been determined for a number of functions of the steroid molecule[6,7]. The KOVATS retention index system has been widely used as an aid to structural investigations[8]. KNIGHTS AND THOMAS[9] used the log of retention values which were additive for structural determination. HILDEBRAND AND REILLEY[10] showed how mathematical formulae may be used to predict the retention times of various compounds on combination columns.

There appears to be a correlation between the nature of the functional groups within the molecule and the slopes of the curves as obtained in the present work. It may be that determination of structure might be facilitated if a larger series including geometrical considerations were obtained and a mathematical expression derived.

By use of varying substrate proportions in the combination, if the relative retention times of a number of compounds are known, it is possible to calculate a combination to give the maximum resolution of a mixture. With a wide variety of substances present in biological samples this tool can be of advantage.

Acknowledgements

This work was supported in part by N.I.H. Grants HD-1199 and Research Career Development Award AM-14, 013.

Department of Obstetrics and Gynecology and Harrison Department of Surgical Research, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)

JOSEPH C. TOUCHSTONE
CHUNG-HSIU WU
ALEXANDER NIKOLSKI
TARAS MURAWEC

1 J. C. TOUCHSTONE, A. NIKOLSKI AND T. MURAWEC, *Steroids*, 5 (1965) 423.
2 R. S. PORTER, R. L. HINKINS, L. TORNHEIM AND J. F. JOHNSON, *Anal. Chem.*, 36 (1964) 260.
3 R. B. CLAYTON, *Nature*, 192 (1961) 524.
4 E. O. A. HAAHTI, W. J. A. VANDENHEUVEL AND E. C. HORNING, *Anal. Biochem.*, 2 (1961) 182.
5 S. R. LIPSKY AND R. A. LANDOWNE, *Anal. Biochem.*, 3 (1961) 818.
6 W. J. A. VANDENHEUVEL, B. G. CREECH AND E. C. HORNING, *Anal. Biochem.*, 4 (1962) 191.
7 R. B. CLAYTON, *Biochemistry*, 1 (1961) 357.
8 E. Z. KOVATS, *Z. Anal. Chem.*, 181 (1961) 357.
9 B. A. KNIGHTS AND G. H. THOMAS, *Anal. Chem.*, 34 (1962) 1046.
10 G. P. HILDEBRAND AND C. M. REILLEY, *Anal. Chem.*, 36 (1964) 47.

Received January 24th, 1967

Separation of some selenides, sulfides and ethers by gas chromatography

Many organic compounds of selenium, like those of sulfur, are known to occur in trace amounts in living organisms[1–7]. Since our attention has been focussed on these compounds, we have found that gas chromatography in view of their physico-chemical properties appears to be a suitable analytical tool for the reliable resolution of their mixtures. The method can be employed for the estimation of the compounds in living matter and in exhaled gases.

We have developed a separation procedure for these compounds and determined their behaviour in relation to analogous compounds of sulfur and oxygen.

Compounds of Se have so far received little attention in gas chromatography though EVANS AND SMITH[8] mention the determination of dibutyl selenide and there is a somewhat more extensive study by EVANS AND JOHNSON[9].

From the point of view of physiological research it was necessary first of all to obtain the purest possible preparation of dimethyl selenide, both non-radioactive and ^{14}C-labelled. In this case, gas chromatography was used as a control method for checking the purity of the compound synthesized. From an analytical point of view it was important to examine the separation of some derivatives of hydrogen selenide, both from one another and from analogous derivatives of hydrogen sulfide. So that we could compare the chromatographic data of compounds of elements of the same group of the periodic system we have also examined analogous compounds of oxygen.

Experimental

Apparatus. The measurements were carried out in a Pye Argon Chromatograph (W. G. Pye and Co. Ltd., Cambridge, England) using a ^{90}Sr ionization detector. We used a 120 cm straight glass column of internal diameter equal to 4 mm. The liquid phases used were 20% (w/w) dinonyl phthalate (May and Baker Ltd., Dagenham, England) and 20 % (w/w) squalane (B.D.H. Laboratory Chemicals Division, England). In both cases, 80–100 mesh Celite 545 (Johns-Manville, London, England) was used as carrier. The flow rate of argon was 50 ml/min. Measurements were carried out at 90° and at 110°. Dimethyl selenide was estimated at room temperature. For sample introduction we used a 10 μl syringe (Hamilton Co., Inc., Micromesure, The Hague, Holland). With radioactive dimethyl selenide we used a modified procedure wherein the gas flow was not interrupted. The sample was applied by piercing a disc made of silicone rubber which was located inside a tapered-cone stopper; the amounts applied were 0.2–0.6 μl of substance. The eluates from the column were condensed at —70°.

Substances examined. The substances investigated here can be divided into three groups: selenides, sulfides and ethers.

The first group contained: ^{14}C-dimethyl selenide, dimethyl selenide, diethyl selenide, dipropyl selenide, di-*n*-butyl selenide, phenylselenol, phenyl methyl selenide and phenyl ethyl selenide. The second group contained: dimethyl sulfide, diethyl sulfide, dipropyl sulfide, di-*n*-butyl sulfide, thiophenol, phenyl methyl sulfide and phenyl ethyl sulfide. The ether group contained: dipropyl ether, di-*n*-butyl ether, phenol, anisole and phenetole.

Synthesis of ^{14}C-dimethyl selenide (ref. 10). A mixture of 4.5 g Rongalite C and 1.25 g elementary selenium was placed in a 50 ml flask provided with a reflux condenser. While stirring with an electromagnetic stirrer and cooling with water, a

solution of 3.5 g NaOH in 15 ml water was added. After some 30 min we attached the apparatus (Fig. 1) through which 2 ml $^{14}CH_3I$ (specific activity 2.5 mC/ml) was distilled into the reaction mixture and the mixture was refluxed for 3 h. It was then distilled and the fraction boiling at 56–60° collected. This was redistilled in a collar flask and the 56–58° fraction collected. We obtained 1.4 g (81.4% yield with respect to Se) of the product with a radiochemical yield of 4.64 mC (92%). The specific activity of the dimethyl selenide was 0.36 mC/mmol.

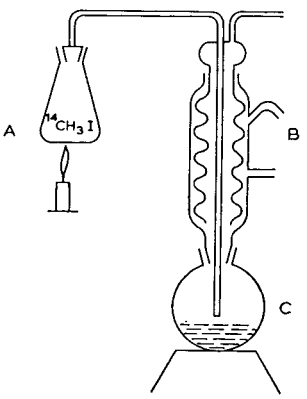


Fig. 1. Apparatus for producing ^{14}C-dimethyl selenide. A = Flask for distilling $^{14}CH_3I$ through the cooling system B into the reaction flask C, where dimethyl selenide is condensed.

Syntheses of all the other substances have been described in the papers quoted in Tables I, II and III.

Results and discussion

Characteristic chromatographic data were obtained for the series of selenides, sulfides and ethers under identical conditions in the two phases and at the two tem-

TABLE I

RELATIVE ELUTION VALUES REFERRING TO DIPROPYL SELENIDE

Selenides	*B.p.* (°C)	*20% DNPHT*		*20% Squalane*		*Number of C atoms*	*Mol. wt.*	*Ref. to method of preparation*
		90°	*110°*	*90°*	*110°*			
Dimethyl selenide	58	0.27	0.22	0.2	0.26	2	109.03	10
Diethyl selenide	108	0.28	0.25	0.23	0.26	4	137.08	10
Di-*n*-propyl selenide	159	1.00[a]	1.00[b]	1.00[c]	1.00[d]	6	165.13	10
Di-*n*-butyl selenide	81–83 (12)	4.5	3.84	4.42	3.74	8	193.18	10
Phenylselenol	183.6	1.3	1.3	0.87	0.89	6	157.07	11
Phenyl methyl selenide	79–80 (12)	6.2	5.32	3.53	3.19	7	171.09	12
Phenyl ethyl selenide	89–91 (12)	9.2	7.59	5.59	4.8	8	185.12	12

[a] V_g = 600 ml.
[b] V_g = 303 ml.
[c] V_g = 545 ml.
[d] V_g = 292 ml.

TABLE II

RELATIVE ELUTION VALUES REFERRING TO DIPROPYL SULFIDE

Sulfides	*B.p.* (°C)	*20% DNPHT*		*20% Squalane*		*Number of C atoms*	*Mol. wt.*	*Ref. to method of preparation*
		90°	*110°*	*90°*	*110°*			
Dimethyl sulfide	35	0.08	0.09	0.013	—	2	62.13	13
Diethyl sulfide	76–77	0.12	0.14	0.18	—	4	90.18	13
Di-*n*-propyl sulfide	140–146	1.00[a]	1.00[b]	1.00[c]	—	6	118.23	13
Di-*n*-butyl sulfide	182	4.59	3.88	4.55	—	8	146.28	13
Thiophenol	168–169	3.2	2.88	1.7	—	6	110.17	14
Phenyl methyl sulfide	69 (12)	7.15	5.97	4.01	—	7	124.20	12
Phenyl ethyl sulfide	96–98 (12)	10.3	8.44	6.22	—	8	138.22	12

[a] V_g = 382.5 ml.
[b] V_g = 204 ml.
[c] V_g = 320 ml.

TABLE III

ELUTION VALUES REFERRING TO DIPROPYL ETHER

Ethers	*B.p.* (°C)	*20% DNPHT*		*20% Squalane*		*Number of C atoms*	*Mol. wt.*
		90°	*110°*	*90°*	*110°*		
Dimethyl ether		—	—	—	—	2	46.07
Diethyl ether	34	—	—	—	—	4	74.12
Di-*n*-propyl ether	91	1.00[a]	1.00[b]	1.00[c]	1.00[d]	6	102.17
Di-*n*-butyl ether	142.4	4.87	6.16	5.82	4.55	8	130.22
Phenol	181.4–2	16.2	20.1	9.42	—	6	94.11
Anisole	154.5–155.5	12.1	15.0	7.00	5.44	7	108.13
Phenetole	172	19.8	23.7	12.5	9.0	8	122.16

[a] V_g = 64 ml.
[b] V_g = 24 ml.
[c] V_g = 48 ml.
[d] V_g = 36 ml.

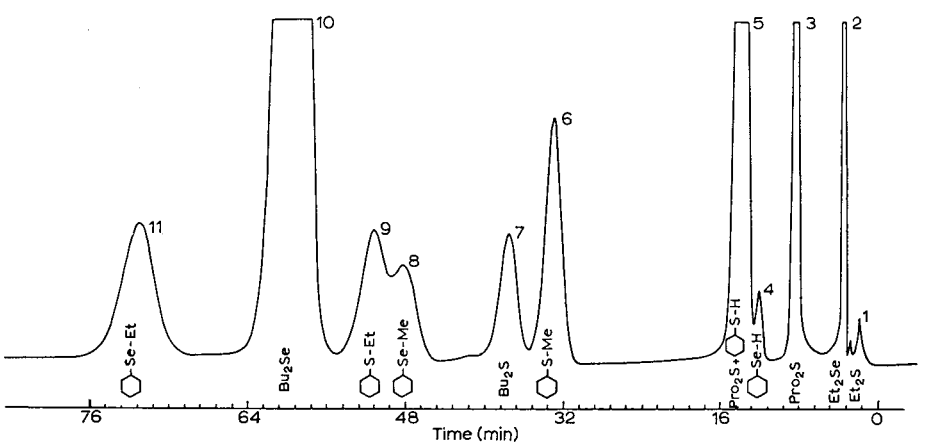


Fig. 2. Separation of the mixture of selenides and sulfides. Stationary phase, 20% squalane; column temperature, 90°; flow rate of argon, 60 ml/min; argon ionization detector.

peratures and the results are summarised in Tables I, II and III. We found 20% squalane suitable at 90° and 20% dinonyl phthalate at both 90° and 110°. At temperatures above 90°, separation on squalane is less favorable. Under these conditions, the individual representatives of the various groups are readily separated. The only exception is the separation of dimethyl selenide (sulfide) from diethyl selenide (sulfide) in view of the relatively high column temperature. For the same reason in the ether series, separation only above di-*n*-propyl ether was observed.

The effect of the character of the liquid phase on the separation of the individual compounds may be seen in the various elution times. Very close elution data are displayed by the pairs $(C_3H_7)_2X$, C_6H_5XH and $(C_4H_9)_2X$, $C_6H_5XCH_3$ (where X =

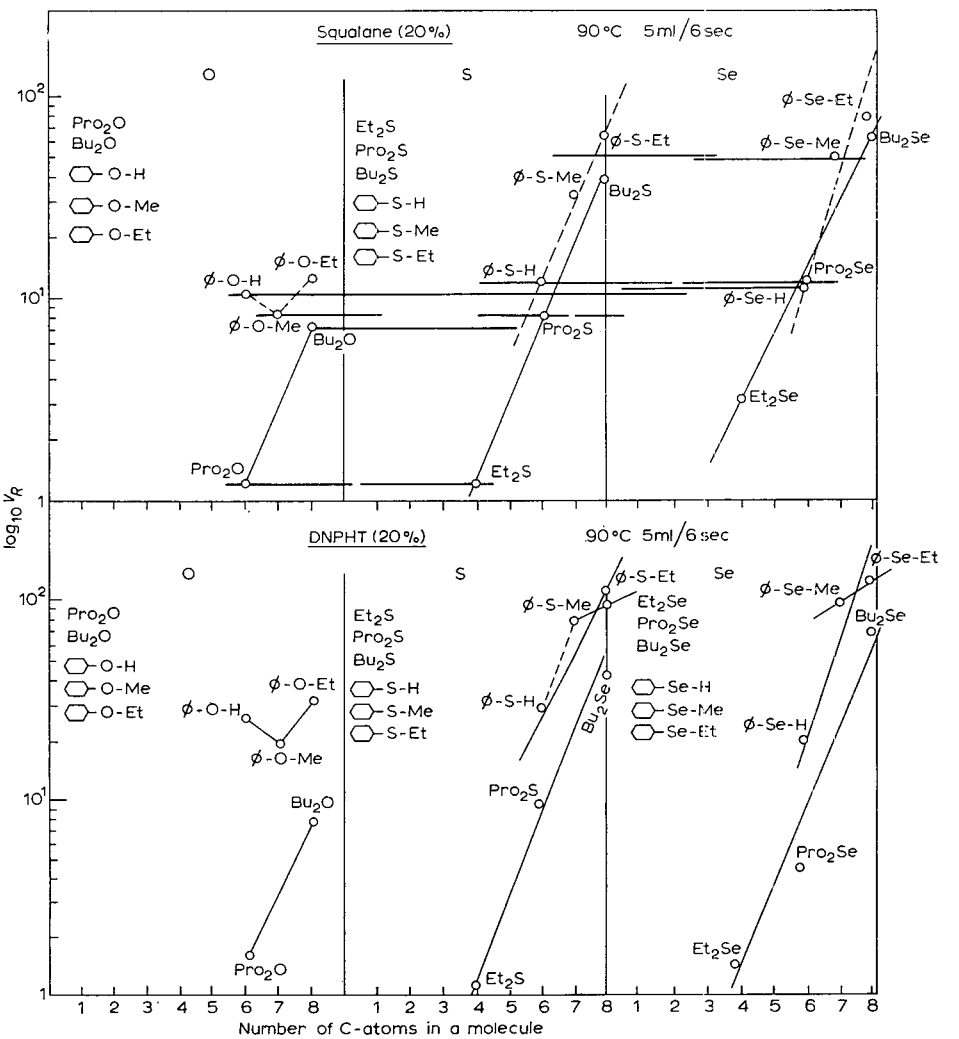


Fig. 3. The relationship between $\log_{10}V_R$ and number of carbon atoms in a molecule. Temperature, 90°; flow rate, 5 ml/6 sec. Stationary phase: (a) 20% squalane, (b) 20% dinonyl phthalate.

Se,S). The similarity of elution times is affected by the boiling points and by the molecular weights.

An example of different separation of substances on two different phases is provided by di-*n*-butyl sulfide which, on squalane at 90°, has a higher elution time than phenyl methyl sulfide, whereas on dinonyl phthalate under the same conditions the reverse is true. Similarly, phenylselenol has a shorter elution time than dipropyl selenide, and dibutyl selenide greater than phenyl methyl selenide, whereas on dinonyl phthalate it is reversed.

Optimum separation of a mixture of sulfides and selenides is achieved at 90° on 20 % squalane at a flow rate of 60 ml/min. Thiophenol is not separated from dipropyl selenide (Fig. 2).

Of the various graphical relationships, we plotted the relationship between $\log V_R$ and the number of carbon atoms in the individual derivatives on both liquid phases. Fig. 3 shows its linear course in dialkyl and alkyl aryl derivatives.

When the elution data for the various ethers, sulfides and selenides are compared it may be seen that the elution values rise from oxygen to selenium, *i.e.* with the increasing atomic weight of the central element.

Results have been summarized in tables and graphs.

Isotope Laboratory of the Biological Institutes, J. BENEŠ
Czechoslovak Academy of Sciences, Prague (Czechoslovakia) V. PROCHÁZKOVÁ

1 Nutrition Symposium: Interrelationships among vitamin E, coenzyme Q and selenium, *Federation Proc.*, 24 (1965) 55.
2 K. SCHWARZ, *Vitamins Hormones*, 20 (1962) 463.
3 I. ROSENFELD AND O. A. BEATH, *Selenium. Geobotany, Biochemistry, Toxicity and Nutrition*, Academic Press, New York, 1964.
4 F. HOFMEISTER, *Arch. Exptl. Pathol. Pharmakol.*, 33 (1893–1894) 198.
5 K. P. MCCONNELL, *J. Biol. Chem.*, 145 (1942) 55.
6 K. P. MCCONNELL AND O. W. PORTMAN, *J. Biol. Chem.*, 195 (1952) 277.
7 F. CHALLENGER, D. B. LISLE AND P. B. DRANSFIELD, *J. Chem. Soc.*, (1954) 1760.
8 M. B. EVANS AND J. F. SMITH, *J. Chromatog.*, 8 (1962) 303.
9 C. S. EVANS AND C. M. JOHNSON, *J. Chromatog.*, 21 (1966) 202.
10 M. L. BIRD AND F. CHALLENGER, *J. Chem. Soc.*, (1942) 570.
11 F. TABOURY, *Bull. Soc. Chim.*, 29 (1903) 761.
12 D. G. FOSTER AND S. F. BROWN, *J. Am. Chem. Soc.*, 50 (1928) 1182.
13 D. T. MCALLAN, T. V. CULLUM, R. A. DEAN AND F. A. FIDLER, *J. Am. Chem. Soc.*, 73 (1951) 3627.
14 E. BOURGEOIS, *Rec. Trav. Chim.*, 18 (1899) 426.

Received January 21st, 1967

Dispositif d'équilibrage vertical pour colonne à chromatographie

Pour obtenir de bonnes séparations à l'aide de chromatographie par filtration sur gel, il est nécessaire d'employer des colonnes rigoureusement verticales. Ceci est utile aussi bien lors du remplissage de la colonne avec le gel qu'ensuite, tant au moment de l'application de l'échantillon à chromatographier, que pendant sa séparation. Sans cette précaution, on obtient des traînées plus ou moins importantes qui limitent les possibilités de la technique. Afin d'effectuer correctement cette mise en position verticale, même dans des enceintes réfrigérées de petites dimensions, nous avons réalisé un dispositif formé de trois parties (Fig. 1 et 2).

(1) Une embase vissante, adaptée à la tête de la colonne et comportant à sa face inférieure deux sièges servant au positionnement des pointes d'équilibrage.

(2) Une pièce intermédiaire portant quatre pointes d'équilibrage placées deux à deux sur chacune des faces et disposées suivant deux axes perpendiculaires l'un par

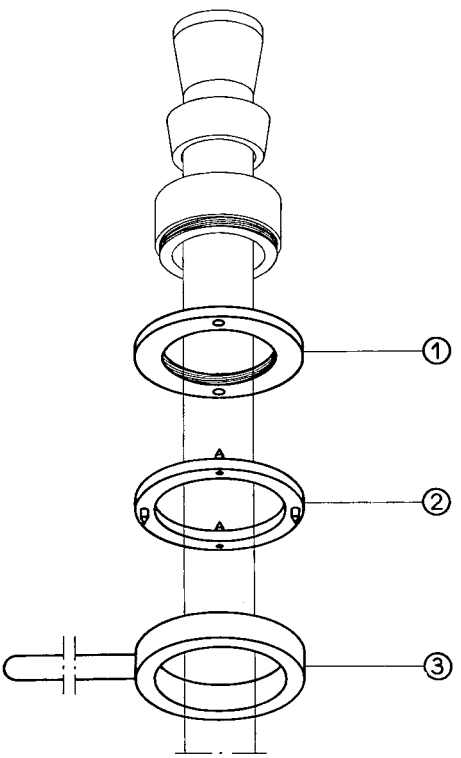


Fig. 1. Plan du dispositif.

rapport à l'autre.

(3) Une embase comportant deux sièges de positionnement à sa face supérieure ainsi qu'une queue vissée et brasée sur son champ.

L'alésage des pièces 2 et 3 est réalisé de manière à laisser un passage pour l'embout inférieur de la colonne, ce qui permet le montage et le démontage pratique de l'ensemble.

Nous employons des colonnes possédant des pièces terminales en polyxyméthylène (Pharmacia, Uppsala). Nous exécutons sur leur embout supérieur, un épaulement et un filetage qui permettent de le solidariser à la pièce 1.

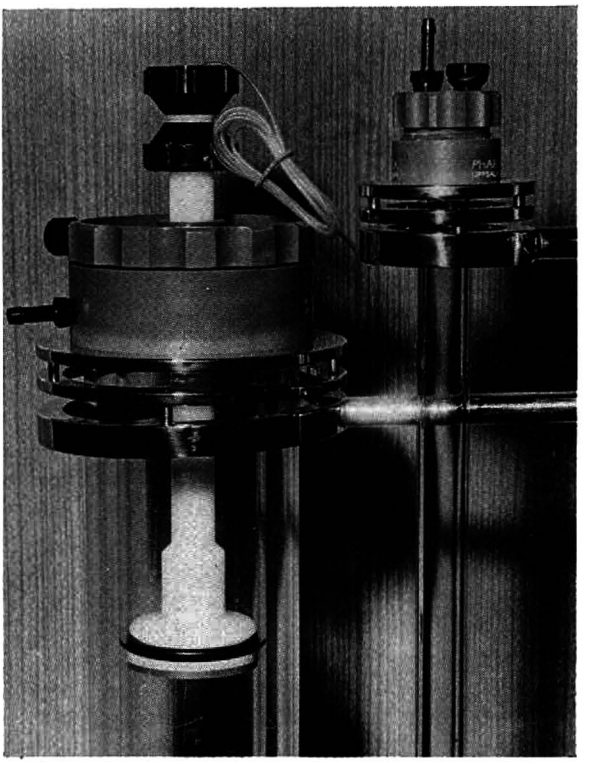


Fig. 2. Dispositifs montés sur deux colonnes Pharmacia. A gauche, colonne de 5 cm de diamètre, munie d'un système de réfrigération. A droite, colonne de 1.5 cm de diamètre.

Le positionnement correct des colonnes se fait automatiquement par gravité. Il faut toutefois faire attention aux efforts exercés par les tuyaux de connexion qui doivent être souples et légers (Rhodorsil, Prolabo).

Lorsque la colonne a pris sa position d'équilibre, il est possible de l'immobiliser à l'aide d'une pince de laboratoire qui saisie son extrémité inférieure.

Laboratoire Pasteur de l'Institut du Radium et
Laboratoire de Physique de la Fondation Curie,
26 rue d'Ulm, Paris V^e^ (France)

H. BAZIN
J. J. BARON

Reçu le 11 janvier 1967

Chromatographic separation of 17-hydroxycorticosteroids on Sephadex LH-20

It has been reported that sugars[1] and some organic acids[2] can be separated by partition chromatography on ion exchange resins, and the use of a moving phase with a lower polarity has permitted the separation of 17-hydroxycorticosteroids on cation exchange resins, Dowex 50W-X4[3] and partially esterified Amberlite IRC-50[4].

It has also been shown by NYSTROM AND SJÖVALL[5] that methylated Sephadex can be used as the stationary phase for the separation of steroids. It was suggested by these authors that this separation was based partly on liquid–liquid partition between a mobile phase and a stationary gel–solvent phase. A hydrophobic gel resembling methylated Sephadex is now available commercially under the name of Sephadex LH-20, and its use for the separation of steroid sulfate has been reported[6].

In the present paper, the successful separation of some 17-hydroxycorticosteroids is described utilizing Sephadex LH-20 as the stationary phase and a less polar solvent mixture (ethanol–benzene–*n*-hexane–water, 50:350:80:3.3, by volume) as the moving phase.

Sephadex LH-20 was classified by the sedimentation method[7] in 30 % aqueous ethanol and the particles of 40–90 μ were collected. They were first washed with 10 volumes of 99% ethanol and then with 20 volumes of the moving phase. The washed Sephadex was suspended in two volumes of the moving phase and poured into a chromatographic tube and allowed to settle. After about 200 ml of the eluent has been passed through, the column was ready for use.

The sample was dissolved in 0.5 ml of a mixture of ethanol, benzene and water (30:210:1, by volume) and then 0.2 ml of a mixture of *n*-hexane and carbon tetrachloride (5:1, by volume) was added with mixing. This solution was applied to the column and overlayered carefully with the eluent and elution was performed with the same eluent. The effluent was collected in fractions of 20 drops in test tubes using a drop count type automatic fraction collector. 17-Hydroxycorticosteroids were analyzed by the Porter-Silber reaction or by the ultraviolet absorption at 240 mμ. Each

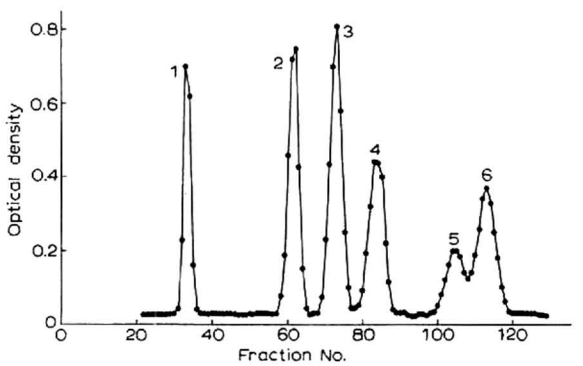


Fig. 1. Elution of standard samples of various 17-hydroxycorticosteroids. The compounds in the order of their elution from the column are: 17α,21-dihydroxypregn-4-ene-3,11,20-trione (1), 11β,17α,21-trihydroxypregn-4-ene-3,20-dione (2), 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione (3), 6β,17α,21-trihydroxypregn-4-ene-3,20-dione (4), 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one (5) and 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one (6). Column size: 0.5 × 60 cm. Fraction size: 20 drops. Flow rate: 3 fractions per hour.

fraction was allowed to evaporate at room temperature and the residue was dissolved in 2 ml of ethanol for measurement of ultraviolet absorption. For estimation of these steroids with Porter-Silber reagent, the residue was dissolved in 0.5 ml of 60% aqueous ethanol and then 1.0 ml of Porter-Silber reagent was added and mixed. The mixture was allowed to stand at room temperature for 15 h and the optical density was measured at 410 mμ. The recovery of steroids was 75 to 80%. The elution pattern is shown in Fig. 1. The column could be used repeatedly.

Since the solutes distributed themselves between the less polar outer liquid phase and the more polar swollen stationary phase which is in equilibrium with the liquid phase, the elution sequence of 17-hydroxycorticosteroids resembled that in straight phase partition chromatography. This type of partition chromatography, which utilizes synthetic cross-linked polymer of medium polarity as the stationary phase and a less polar solvent mixture consisting of smaller amounts of polar components (*e.g.*, ethanol and water) and larger amounts of less polar components (*e.g.*, benzene and *n*-hexane) as the moving phase, seems to be useful for the separation of neutral organic compounds of moderate and higher polarity.

Department of Genetics, Osaka University Medical School, Osaka (Japan) TOKUICHIRO SEKI

1 O. SAMUELSON AND B. SWENSON, *Anal. Chim. Acta*, 28 (1963) 426.
2 T. SEKI, *J. Chromatog.*, 22 (1966) 498.
3 T. SEKI AND K. MATSUMOTO, *Endocrinol. Japon.*, 13 (1966) 75.
4 T. SEKI AND K. MATSUMOTO, *J. Chromatog.*, 27 (1967) 423.
5 E. NYSTROM AND J. SJÖVALL, *Anal. Biochem.*, 12 (1965) 235.
6 R. VIHKO, *Acta Endocrinol., Suppl.*, 109 (1966) 15.
7 P. DECKER AND H. HØLLER, *J. Chromatog.*, 7 (1962) 392.

Received January 4th, 1967

The prediction of R_F values

Several theoretical studies have appeared in the chromatography literature. A large number of specific solutions to particular problems are also available. Very few studies have been made using both approaches simultaneously. A plea is made here for more studies applied to the practical problems of the selection of solvents, the determination of the conditions of chromatography, and the presentation of data.

Chromatography is essentially a stochastic process and the statistical knowledge of estimation and prediction could be applied with success here. The aim of this investigation is to show the possibilities of predicting the R_F value of a compound, given a set of conditions.

The physical factors influencing R_F values have been investigated quite extensively on filter paper[1–4]. CONSDEN, GORDON AND MARTIN[4] called attention to the factors paper, temperature, quantity of material, extraneous substances, degree of

saturation with water, supply of solvent, and distance between starting point and source of solvent. CASSIDY[1] reviewed these factors and others at the bulk and molecular levels. Contradictory data are reported in the literature, probably due to the fact that the factors investigated are very limited.

No similar study has been made on thin-layer chromatoplates. The possibility of studying the support by using different adsorbents or binary mixtures of adsorbents has created a new dimension in chromatography[5]. The article analyzes some of the factors affecting and explaining the behavior of compounds on thin-layer chromatoplates.

Material

Thin-layer plates were prepared by mixing MN Cellulose 300 and Silica Gel G in known proportions in water and applying to a thickness of 0.25 mm with the Desaga-Brinkman* applicator. The plates were dried overnight at room temperature to insure reproducibility. The compounds studied were iodine-containing compounds of medical interest: 3-monoiodotyrosine (MIT), 3,5-diiodotyrosine (DIT), 3,5,3′-triiodothyronine (T_3), 3,5,3′,5′-tetraiodothyronine (T_4) and iodide (I^-). The solvent system used was *tert.*-amyl alcohol–1 *N* ammonium hydroxide–dioxane (2:1:2). Following a 10 cm run in a saturated tank, iodide was visualized by spraying with 0.5 % palladium (II) chloride in 1 *N* hydrochloric acid and the tyrosines and thyronines by spraying with 0.5 % diazosulfanilic acid in 5 % sodium carbonate. The temperature was kept constant ($\pm$ 1°) by placing the tank in a constant temperature bath**.

Method

The mathematical technique will be presented in some detail hoping that others will follow this method of analysis.

We are presenting an analysis of the relations among a single criterion measure and two or more predictor measures. The technique of multiple regression is applied to predict the hR_F ($R_F \times 100$) value of a compound from *a priori* knowledge of the conditions of chromatography.

In the general case of an m-dimensional space, the standard regression equation has the form:

$$\hat{Z}_{mi} = \beta_1 Z_{1i} + \beta_2 Z_{2i} + \beta_3 Z_{3i} + \cdots + \beta_{m-1} Z_{(m-1)i}$$

The beta weights indicate the relative contribution of the corresponding variables to the prediction of the criterion. The Z's are defined as $Z_{1i} = (X_{1i} - \bar{X}_1/\hat{\sigma}_1)$. It is often desirable to compute the regressed deviation score $\hat{x}_{mi}$ instead of the regressed standard score $\hat{z}_{mi}$; in this case, the b weights are derived from the beta weights by scaling each beta weight by the ratio of the standard deviation of the criterion to the standard deviation of that predictor ($b_j = (\hat{\sigma}_m/\hat{\sigma}_j)\beta_j$). It is possible to compute a multiple correlation coefficient $R_{m\cdot 1,2,\ldots,\, m-1}$ in the range $0 \leq R \leq +1$. The coefficient R^2 provides an estimate of the proportion of the total variance in the criterion that can be predicted from the known variance in the predictors and is a measure of the overall

* Brinkman Instruments, Westbury, New York.
** Forma Scientific Inc., Marietta, Ohio.

effectiveness of the multiple regression. The significance of R^2 is tested by the analysis of variance as follows:

$$F^{m-1}_{N-m-2} = \frac{R^2(N-m-2)}{(1-R^2)(m-1)}$$

where N is the number of subjects in the sample. The standard error of estimate for $\hat{Z}_m$ is given by $\sqrt{1-R^2}$.

The criterion is the hR_F value of a compound. The set of predictors are listed in Table I. They touch upon the support (adsorbent), the solvent (developing time), the environment (temperature) and the compound (molecular weight and number of iodine atoms in the compound).

TABLE I

SET OF PREDICTORS

Z	*Description*
1	Adsorbent (% Silica Gel G)
2	Temperature (°C)
3	Molecular weight of compound
4	Number of iodine atoms in the molecule
5	Developing time (time for solvent to reach 10 cm)

A stepwise procedure which adds one variable to the prediction equation at a time and thus provides a number of intermediate regression equations is possible. Variables are added or dropped according to the statistical significance of their contribution to the prediction of the criterion. In this case any effort to generalize from sample to population is open to serious danger of capitalization on chance.

All computations were performed at the Harvard Computation Center on the IBM 7094 Digital Computer. The routine for multiple regression was as published by COOLEY AND LOHNES[6]. The stepwise multiple regression program was taken from the share library (SDA 3143).

Results

The results of the correlation analysis on 225 sets of data are summarized in Table II. Apart from the trivial correlation between the number of iodine atoms and

TABLE II

CORRELATION MATRIX

Variables	*1*	*2*	*3*	*4*	*5*	*6*
1	—					
2	0.02	—				
3	0.00	0.00	—			
4	0.00	0.00	0.96*	—		
5	0.06	−0.95*	0.00	0.00	—	
6	−0.06	−0.26*	0.38*	0.50*	0.25*	—

* $p < 0.001$.

the molecular weight of a compound, we have a high correlation between the temperature and the developing time. Significant correlation coefficients are observed between hR_F value and all other variables except the percent silica gel in the adsorbent, the most important factors in predicting the R_F value of a compound being the number of iodine atoms in the molecule and its molecular weight. This does not mean that there is no relation between the hR_F values of iodine-containing compounds and the modifications of the adsorbent. Very high correlations are observed for a specific compound especially with T_4, T_3 and iodide. The lumping of all the data together is the reason for the vanishing of the high correlation between hR_F and percent silica gel.

The results of the multiple regression analysis are presented in Table III. The importance of each factor (the betas of the regression equation) in the prediction of the hR_F values is given. The coefficient R^2 is 0.4632 (R = 0.6806). This coefficient is a measure of the effectiveness of our equation in predicting R_F values from a knowledge of the conditions of chromatography.

Following a stepwise multiple regression analysis, variables 2, 3 and 4 were retained as significant and are quoted in Table IV with their respective b weights and standard error.

TABLE III

SUMMARY OF MULTIPLE REGRESSION ANALYSIS

Variables	$\bar{X}$	*S.D.*	*β weights*	*b weights*
1	53.33	36.11	−0.07	−0.03
2	22.73	11.17	−0.13	−0.22
3	458.99	233.75	−1.43	−0.11
4	2.20	1.17	1.88	29.77
5	103.44	37.06	0.13	0.07
6	36.66	18.50	—	—

In spite of a high coefficient of determination, the solution to the multiple regression equation is not a unique one[7]. As can be seen from the stepwise multiple regression analysis, when variables 2 and 5 are eliminated from the set of contributing factors some of the weights change (compare Tables III and IV). The squared multiple correlation coefficients of these two equations are quite different: it was 0.46 for the complete equation and 0.68 when only the significant variables were included. The efficiency of prediction is different for both equations.

TABLE IV

SUMMARY OF STEPWISE REGRESSION ANALYSIS

Variables	*Coefficient(b)*	*Standard error of the coefficient*
2	−0.43	0.08
3	−0.11	0.01
4	29.77	2.97

Discussion

Very few studies have dealt with the problem of predicting R_F values. CONNORS[8] was able to predict the R_F values of a set of compounds in one solvent given this knowledge in a different solvent. To our knowledge, this is the only investigation oriented toward the prediction of R_F values.

Let us not be distracted by the mathematics from the more important chemical observations and let us summarize with this idea in mind.

When we say 68% of the variance in hR_F is predicted from a knowledge of temperature, molecular weight and number of iodine atoms in the compounds, we mean that this is a measure of the overall effectiveness of our predictive equation. The b weights indicate that the number of iodine atoms in the compounds is the most useful predictor. Since in only 68% of the cases can we predict the R_F value accurately, other factors are involved in determining the R_F values of a given compound; and temperature, molecular weight and number of iodine atoms in the compound are the significant predictors. All other variables did not contribute significantly to the prediction of R_F values. These other factors need to be uncovered before a very accurate prediction can be achieved. Some of these factors might be solubility of the compounds in the solvent, elution strength of the solvent, viscosity, specific gravity and surface tension of the solvent. The most important factors affecting the behavior of chromatographed compounds, such as partition, adsorption and ion exchange, are difficult to measure and express numerically. Only when we can quantify these concepts will it become possible to predict R_F values.

A most interesting aspect of this procedure is the possibility of predicting the R_F value of any member of a chemical series, given the numerical values for the set of conditions of chromatography. Unfortunately the material on which we have accumulated a large body of data comprises a very small series and forbids any attempt at prediction. Amino acids or sugar series would be ideally suited for such a study, and would allow an estimate of the accuracy of prediction. A large body of data accumulated under standardized specific conditions is needed before the usefulness of the technique described can be fully evaluated.

Physics Research Laboratory,
Massachusetts General Hospital,
Boston, Mass. 02114 (U.S.A.)

R. P. OUELETTE*
J. F. BALCIUS

1 H. C. CASSIDY, *Fundamentals of Chromatography*, Interscience, New York, 1957.
2 A. SAIFER AND I. ORESKES, *Anal. Chem.*, 25 (1963) 1539.
3 G. N. KOWKABANY AND H. G. CASSIDY, *Anal. Chem.*, 24 (1952) 643.
4 R. CONSDEN, A. H. GORDON AND A. J. P. MARTIN, *Biochem. J.*, 41 (1947) 590.
5 R. P. OUELETTE AND J. F. BALCIUS, *J. Chromatog.*, 24 (1966) 465.
6 W. W. COOLEY AND P. R. LOHNES, *Multivariate Procedures for the Behavioral Sciences*, Wiley, New York, 1962.
7 M. G. KENDALL, *A Course in Multivariate Analysis*, Hafner, New York, 1961.
8 K. A. CONNORS, *Anal. Chem.*, 37 (1965) 261.

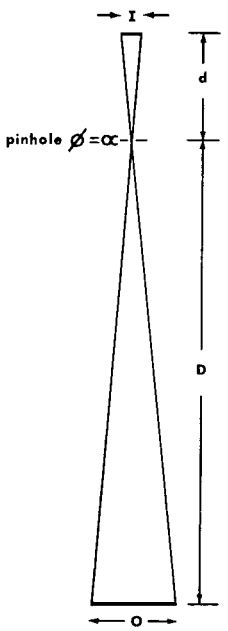
Received December 23rd, 1966

* Present address: Hazleton Laboratories, Inc., P.O. Box 40, Falls Church, Va. 22046, U.S.A.

Recording short-wave ultraviolet absorption on thin-layer chromatograms by pinhole photography*

It is often necessary to record the locations of compounds on TLC plates under short-wave ultraviolet radiation. When using longer wavelengths, *i.e.* 366 mμ, no problem exists as standard camera optics may be employed[1]. Because of the opacity of glass to short wavelengths, quartz optics are indicated but are of prohibitive cost. As will be shown in the present application this difficulty can be overcome by using the classic pinhole diaphragm.

The principle of the pinhole was first described in the sixteenth century[2] and its value lies in the fact that it will produce an image of any illuminated object placed on either side of it. (The system does not require focussing.) Landscapes, or distant objects, produce acceptably sharp images through a pinhole; however, complications



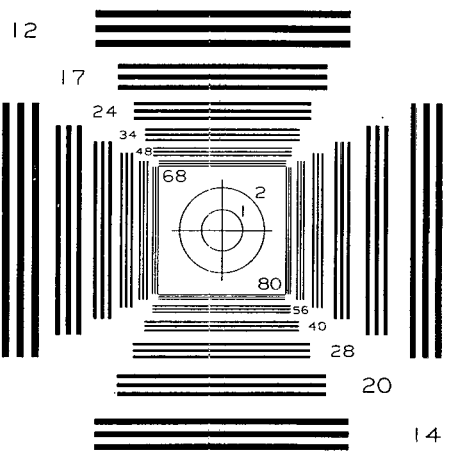


Fig. 1. Geometry of the pinhole diaphragm.
Fig. 2. N.B.S. Resolution Test Chart.

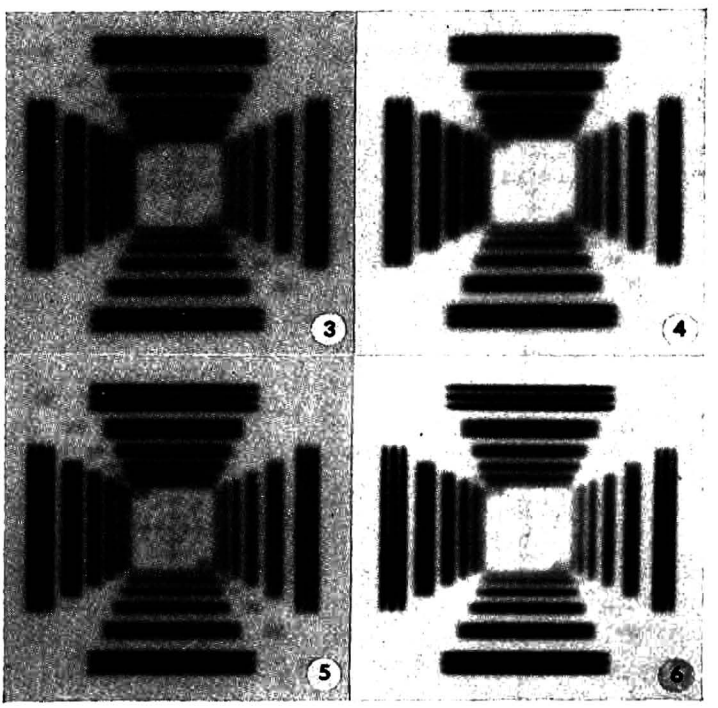
arise when this technique is used for specialized applications. As seen from the geometrical relationship indicated by the diagram in Fig. 1 the size of the image, I, is given by

$$I = Od/D \qquad (1)$$

where O is the size of the object and d and D, the distances of pinhole to image and object, respectively. Thus alteration of the d/D ratio simply changes the proportion of I to O.

* Contribution No. 34. Scientific Information Section, Research Branch, Canada Department of Agriculture, Ottawa (Canada).

The quality of the image depends on the size of the pinhole and the smoothness of its edge, down to a diameter at which diffraction effects introduce image deterioration. The definition increases as the diameter, α, decreases; or, as D increases. Aside from the necessity to avoid diffraction effects, limitations in the reduction of pinhole diameter are imposed by constructional difficulties[3] and by the necessity to avoid impractically long exposure times. Because of the low level of energy radiated by



Figs. 3 to 6. Comparisons in white light of Kodak Plus-X Pan film (Figs. 3 and 5) and Kodak High Contrast Copy film (Figs. 4 and 6) against pinholes of 0.016 in. diameter (Figs. 3 and 4) and 0.012 in. diameter (Figs. 5 and 6).

U.V. lamps, this factor was of importance in the present application. It is to be noted that the f value of the system is defined by

$$f = d/\alpha \qquad (2)$$

Testing the system resolution

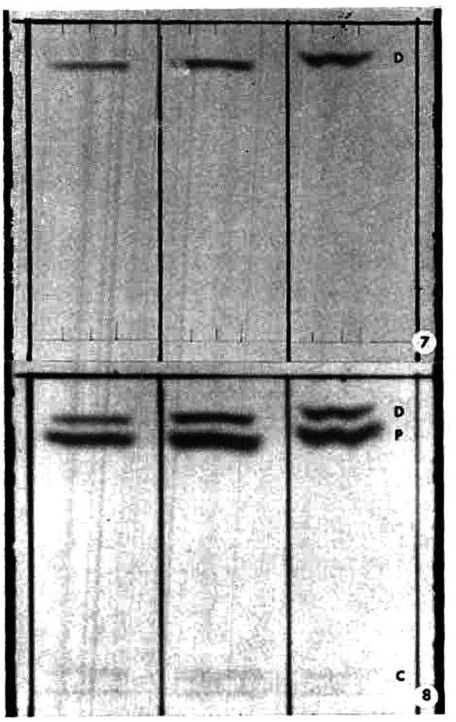
Standard 200 × 200 mm TLC plates were used in the present tests. In order to utilize the full width of 35 mm film a distance d = 100 mm of pinhole to film and a distance D = 950 mm of pinhole to TLC plate were used. Expression (1) gives I = 21 mm in this case; the I/O ratio was 1/9.5.

The degree of resolution obtainable with two pinhole sizes, *i.e.* 0.012 and 0.016 in., was determined with each of two emulsion types. The tests were conducted in white light using the NBS high resolution Test Chart[4] (Fig. 2) and the I/O ratio 1/9.5. The results, Figs. 3 to 6, were examined visually, no attempt being made to assign

numerical values for the resolution obtained. It was estimated that the definition and contrast achieved with the 0.012 in. pinhole in combination with High Contrast Copy Film (Fig. 6) were adequate to the present application.

Testing the system with short-wave U.V. light

A TLC plate was used in this test. Illumination was provided by two Mineralight lamps (Ultraviolet Products Inc., San Gabriel, Calif.), directed at 45° on each side and



Figs. 7 and 8. TLC plate in white light, Fig. 7, showing only dye band D. The same plate under shortwave U.V., Fig. 8, showing absorption in bands D, P and C.

at 10 in. distance from the center of the plate. The f value with the 0.012 in. hole and $d = 100$ mm was 330. It was found that about an 8-min exposure produced negatives of sufficient density for subsequent reproduction; direct visual evaluation from negatives was possible with 3-min exposures.

The plate, prepared and developed by applying known techniques[5], had been spotted[6] with mixtures of steroids. By ordinary light (Fig. 7) only the dye band D, used as a reference for the determination of R_b values, was visible (R_b value = 1.00). In the same plate, under U.V. illumination, absorption by this band and that corresponding to progesterone, P, produced clear zones on the negative translated as dark bands on the print (Fig. 8). Weaker absorption by a third compound produced band C.

Fluorescence induced by U.V. radiation will appear as light bands on the print, provided the energy thus emitted is higher than that reflected by the background.

Bio-graphic Unit, Scientific Information Section, ROSS JACKSON
Research Branch, Canada Agriculture, Ottawa (Canada)

1 H. R. JACKSON, *J. Chromatog.*, 20 (1965) 410.
2 P. POLLOCK, *Picture History of Photography*, Abrams, New York, 1958.
3 *Focal Encyclopedia of Photography*, Focal Press, London, 1956.
4 F. E. WASHER AND I. C. GARDNER, *National Bureau of Standards Circular 533*, Washington, D.C., 1953.
5 F. A. VANDENHEUVEL, G. J. HINDERKS AND J. C. NIXON, *J. Am. Oil. Chemists' Soc.*, 42 (1965) 283.
6 F. A. VANDENHEUVEL, *J. Chromatog.*, 25 (1966) 102.

Received January 10th, 1967

Thin-layer chromatographic separation of primary and secondary amines as 4-(phenylazo)benzenesulfonamides

The direct chromatography on thin layers of primary and secondary amines in most cases causes difficulties due to the strong adsorption of the NH-group to the adsorbents generally used. Several methods have, nevertheless, been developed for work with the free amines, but the use of derivatives is often to be preferred. Thus derivatization has been performed with reagents such as 2,4-dinitrochlorobenzene[1,2], 3,5-dinitrobenzoyl chloride[3,4], 1-dimethylaminonaphthalene-5-sulfonyl chloride[5], 4-toluenesulfonyl chloride[4,6] or benzoyl chloride[4].

In the present investigation, 4-(phenylazo)benzenesulfonyl chloride has been used for preparing derivatives of the amines. These derivatives have previously been described, and fair separations were achieved by column chromatography[7]. The 4-(phenylazo)benzenesulfonamides formed have several advantages over the free amines as well as over derivatives used before. They are thus well suited for thin-layer chromatography, *e.g.* on alumina plates, the compounds are easy to detect on the plates, as they are intensely colored, and the preparation of the derivatives can be carried out very simply even from aqueous solutions of the amines or their salts. Due to these facts the method is also very suitable for separation and characterization of volatile amines arising from the cleavage of more complicated molecules, *e.g.* of biological origin. The procedure described here was originally developed for the lower aliphatic amines, as only very few methods are available for the identification of these compounds, but results are also given for some amines of pharmacological interest. The method discussed in this paper has been included in the course in organic identification at the Technical University of Denmark.

Preparation of the derivatives

The reagent, 4-(phenylazo)benzenesulfonyl chloride, was prepared from azobenzene (Fluka, puriss.) and chlorosulfonic acid[8]. The derivatives were prepared according to the following procedure:

3 mmoles of an amine (or of an amine salt, such as the hydrochloride, sulfate or tartrate) and 2 ml of a sodium hydroxide solution (2 *N*) were poured into a centrifuge tube and 1 ml of a 1 % solution of the reagent in chloroform was added. The mixture was shaken vigorously for 3 min, centrifuged, and the upper phase (the water phase) was sucked off and rejected. The colored, organic phase was then shaken vigorously with 2 ml of a hydrochloric acid solution (4 *N*); after separation from the aqueous phase, the chloroform phase was finally shaken with 2 ml of water and used directly for thin-layer chromatography.

Chromatographic procedure

The chromatography was performed on microchromatoplates (40 × 76 mm) coated with alumina (Fluka, D5) as previously described[9]. The plates were developed in a 250 ml beaker covered with a cork. The mobile phase was a mixture of 25 ml ethyl acetate and 100 ml petroleum ether (62–82° from Shell) saturated with water. The amount of the above-mentioned chloroform solution used was about 0.1 μl. The time of the development was 10–15 min.

As the derivatives are intensely colored, they can, in most cases, be observed directly on the plates; however, the detection can be considerably improved by exposure of the plates to iodine vapour.

Results and discussion

With the procedure used, it is not possible to characterize the movement of the individual compounds by ordinary R_F values, as the chromatography is continued after the solvent front has reached the upper edge of the plate. Therefore, the data reported in this paper represent relative values, with the movement of the derivative of butylamine taken as the standard; for a few fast-moving compounds, the derivative of dipropylamine was taken as the standard. In this way, good reproducibility of the results was obtained, as deviations from the normal, due to variations in temperature, composition of the mobile phase (*cf.* however below), layer thickness etc. are eliminated to a certain degree. The reproducibility is illustrated by the results from 18 plates, chromatographed one after the other, with 2 samples of a reference mixture of the 4-(phenylazo)benzenesulfonamides (4-PABSA's) of methylamine, ethylamine, propylamine, and butylamine, one on each edge of the plate. The relative distances and the standard deviations for the 36 runs are given in Table I.

The use of relative values for the distances travelled eliminates, as mentioned, to a certain degree many of the factors of uncertainty. However, due to slight differ-

TABLE I

REPRODUCIBILITY IN CHROMATOGRAPHING 4-(PHENYLAZO)BENZENESULFONAMIDES

4-PABSA	*Average value* $\left(\pm\sqrt{\frac{\Sigma\lambda^2}{n(n-1)}}\right)$	*Standard deviation* $\left(\pm\sqrt{\frac{\Sigma\lambda^2}{n-1}}\right)$
Methylamine	0.466 ± 0.004	± 0.024
Ethylamine	0.706 ± 0.003	± 0.017
Propylamine	0.868 ± 0.002	± 0.012
Butylamine	1.00 ± 0	—

ences in the adsorption power of the stationary phase it is in most cases necessary to adjust the ethyl acetate content of the mobile phase, so as to yield an R_X value of 1.50 for dipropylamine. Both butylamine and dipropylamine have been used in this way to standardize the mobile phase employed for determining the values presented in Tables II and III.

In Table II, relative values are given for all the 4-PABSA's dealt with. Table III contains the derivatives which have R_X values greater than 1.50 relative to butylamine. As such large values are only poorly reproducible relative to butylamine, it is preferable to use a faster running compound as the standard. Dipropylamine has been chosen as standard for these compounds, and the R_X values determined on this basis are given in Table III. All the compounds were chromatographed on 3–6 different plates with a standard sample of the four 4-PABSA's, dealt with in Table I, on each edge.

TABLE II

R_X VALUES (RELATIVE TO THE DERIVATIVE OF BUTYLAMINE) ON ALUMINA FOR 4-(PHENYLAZO)-BENZENESULFONAMIDES

Primary amines			*Secondary amines*		
No.	*4-PABSA*	R_X	*No.*	*4-PABSA*	R_X
1	Methylamine	0.47	22	Dimethylamine	1.16
2	Ethylamine	0.71	23	Diethylamine	1.43
3	Propylamine	0.87	24	Dipropylamine	1.50
4	Isopropylamine	0.92	25	Dibutylamine	>1.50
5	Butylamine	1.00	26	Diisobutylamine	>1.50
6	*sec.*-Butylamine	1.04	27	Diamylamine	>1.50
7	Isobutylamine	1.06	28	Diallylamine	1.47
8	*tert.*-Butylamine	1.05	29	Dipropargylamine	1.28
9	Amylamine	1.08	30	Dibenzylamine	1.41
10	Isoamylamine	1.13	31	β-Phenylisopropylmethylamine	1.26
11	Hexylamine	1.15	32	Ephedrine	0.57
12	Octylamine	1.20	33	Pyrazole	1.38
13	Decylamine	1.27	34	Imidazole	0.94
14	Allylamine	0.81	35	Morpholine	0.99
15	Cyclopentylamine	1.00			
16	Cyclohexylamine	1.05			
17	Benzylamine	0.87			
18	α-Phenethylamine	0.86			
19	β-Phenethylamine	0.83			
20	Amphetamine	0.94			
21	Mescaline	0.15			

TABLE III

$R_X{}^+$ VALUES (RELATIVE TO THE DERIVATIVE OF DIPROPYLAMINE) ON ALUMINA FOR 4-(PHENYLAZO)-BENZENESULFONAMIDES

No.	*4-PABSA*	$R_X{}^+$
24^+	Dipropylamine	1.00
25^+	Dibutylamine	1.05
26^+	Diisobutylamine	1.06
27^+	Diamylamine	1.10

With the method described, a good separation of the first six straight-chain, saturated primary amines as well as of octylamine and decylamine was achieved, the distance travelled increasing with increasing length of the carbon chain. A branched chain causes the compounds to move faster, *e.g.* isopropylamine runs faster than propylamine. The effect caused by increased branching of the carbon chain tends to be more pronounced the closer the branch is situated to the amino group; the great difference between the distances travelled by primary and secondary amines with the same number of carbon atoms is most striking (*cf.* diethylamine and butylamine). Double bonds retard movement, and so also do alicyclic groups; phenyl groups have a very strong retarding effect.

The preparation of derivatives of compounds containing amino groups attached directly to a benzene ring is not possible. Further, it has not been possible to prepare derivatives from diamines or amines containing acidic groups (phenols etc.). Finally it has proved impossible to obtain derivatives from di-*sec.*-butylamine and phentermine, possibly due to some sort of steric hindrance.

It should be noted that alcohols, hydrazine compounds, tertiary amines, and ammonia do not interfere in the procedure outlined above.

Department of Organic Chemistry,
The Technical University, Bygning 201,
Lyngby (Denmark)

AAGE JART
ALLAN J. BIGLER

1 D. P. SCHWARTZ, R. BREWINGTON AND O. W. PARKS, *Microchem. J.*, 8 (1964) 402.
2 S. HOLDER AND H. J. BREMER, *J. Chromatog.*, 25 (1966) 48.
3 D. B. PARIHAR, S. P. SHARMA AND K. C. TEWARI, *J. Chromatog.*, 24 (1966) 443.
4 H. GERLACH AND H.-J. SENF, *Pharm. Zentralhalle*, 105 (1966) 93.
5 N. SEILER AND M. WIECHMANN, *Experientia*, 21 (1965) 203.
6 K. TEICHERT, E. MUTSCHLER AND H. ROCHELMEYER, *Deut. Apotheker Ztg.*, 100 (1960) 283.
7 E. O. WOOLFOLK, W. E. REYNOLDS AND J. L. MASON, *J. Org. Chem.*, 24 (1959) 1445.
8 R. D. DESAI AND C. V. MEHTA, *Indian J. Pharm.*, 13 (1951) 211; *C.A.*, 46 (1952) 8627.
9 A. JART AND A. J. BIGLER, *J. Chromatog.*, 23 (1966) 261.

Received February 14th, 1967

J. Chromatog., 29 (1967) 255–258

Charge-transfer complexes of 2,4,6-trinitrophenyl-N-methylnitramine (tetryl) and 2,4-dinitrochlorobenzene with some amines

Thin-layer chromatography has been efficiently employed for the investigation of various charge-transfer complexes. For example studies on complexes of terpenes[1–4], glycerides[5], hydrocarbons[6] with silver and polynitro aromatic compounds with hydrocarbons[7] have been reported. Recently the π-complexes of a number of aromatic amines with 2,4,6-trinitrotoluene (*s*-TNT) and *m*-dinitrobenzene (*m*-DNB) have been investigated[8] by this technique.

In the present paper, studies of the π-complexes of 2,4,6-trinitrophenyl-N-methylnitramine (tetryl) and 2,4-dinitrochlorobenzene (DNCB) with some amines are

reported. By employing a TLC technique it was possible to separate and characterise π-complexes obtained from 2–3 μg of different amino compounds.

Experimental

All the solvents employed for irrigation of chromatoplates were dried and freshly distilled. Kodak photographic glass plates (22 × 22 cm) were used as supports and the ascending irrigation technique was used. The distance travelled by the solvent front in each case was kept constant, *i.e.* 18.0 cm. The temperature of irrigation was 20° ± 1°. The colors of the complexes varied from yellow to deep red and could be easily located.

Adsorbents. (1) Kieselgel G (E. Merck)

(2) Kieselgel G + Kieselguhr G (9:1, E. Merck)

(3) Kieselguhr G (E. Merck)

(4) Cellulose benzoate (benzoyl content 54.8 %; 100 mesh, B.S.S.) containing 1 % $CaSO_4$.

(5) Cellulose acetate (acetyl content 38.6 %; 100 mesh, B.S.S.) containing 1 % $CaSO_4$.

(6) Cellulose powder, ashless (Whatman, 100 mesh, B.S.S.) containing 1 % $CaSO_4$.

Preparation, spotting of complexes and irrigation of the plates. The chromatoplates were prepared by taking a homogenous slurry of the adsorbent in a suitable solvent, pouring it on the glass plates and tilting them from side to side; a uniform coating of the adsorbent was thus achieved. The plates were kept at room temperature overnight and activated at 110° for 1 h (cellulose benzoate and acetate plates were activated at 60° for 3 h in a vacuum oven). The plates were impregnated with tetryl/DNCB by irrigating them with a 3 % solution in acetone before spotting. The amounts of various adsorbents, solvents and average coatings are given in Table I.

TABLE I

Serial No.	*Coating*	*Weight of the adsorbent (g)*	*Solvent*	*Average coating of adsorbent (mg/cm²)*
1	Kieselgel G	30	Chloroform (60 cc) –methanol (30 cc)	7.9
2	Kieselgel G + Kieselguhr G	30	Methanol (90 cc)	7.2
3	Kieselguhr G	32	Ethanol (70 cc)	6.2
4	Cellulose benzoate–$CaSO_4$	26.25	Water– (40 cc) ethanol (20 cc)	8.4
5	Cellulose acetate–$CaSO_4$	26.25	Water–(40 cc) ethanol (20 cc)	8.1
6	Cellulose–$CaSO_4$	27	Water (80 cc)	6.9

The tetryl/DNCB–amine complexes were prepared directly on the above plates by spotting ethanolic solutions of various amines (15 μl containing 2–4 μg) with a standard microcapillary. This gave instantaneous spots of the colored complexes. The plates were left in a desiccator for half an hour after which they were irrigated with

various solvents. Tables II and III give the R_F values of various π-complexes along with their colors. Two typical chromatoplates showing the resolutions of π-complexes of tetryl and DNCB with amines are given in Figs. 1 and 2, respectively.

TABLE II

R_F VALUES AND COLORS OF π-COMPLEXES OF TETRYL WITH AMINES

Sample No.	*Complex-forming amines*	*Color of the complex*	*Kieselgel G impregnated with 3% tetryl*			
			Monochlorobenzene–ethyl acetate (9:1)	*Toluene*	*Xylene–carbon tetrachloride (3:1)*	*Ethylene dichloride–petroleum ether (4:1)*
1	N-Methylaniline	light violet	0.70	0.49	0.39	0.89
2	N,N-Dimethylaniline	light violet	0.80	0.65	0.51	0.93
3	Aniline	yellowish brown	0.48	0.33	0.22	0.73
4	*o*-Anisidine	brownish violet	0.54	0.27	0.17	0.74
5	*p*-Anisidine	orange	0.31	0.12	0.09	0.33
6	Diphenylamine	violet	0.87	0.83	0.71	0.97
7	α-Naphthylamine	violet	0.55	0.32	0.21	0.82
8	β-Naphthylamine	brownish red	0.50	0.27	0.18	0.74
9	*o*-Toluidine	brownish red	0.51	0.23	0.23	0.77
10	*m*-Toluidine	yellowish brown	0.48	0.27	0.20	0.72
11	*p*-Toluidine	yellowish brown	0.42	0.22	0.15	0.60
12	Benzidine	brown	0.23	0.04	0.03	0.21
13	*m*-Chloroaniline	reddish brown	0.59	0.33	0.24	0.86
14	*p*-Chloroaniline	reddish brown	0.53	0.26	0.18	0.80

TABLE III

R_F VALUES AND COLORS OF π-COMPLEXES OF DNCB WITH AMINES

Sample No.	*Complex-forming amines*	*Color of the complex*	*Kieselgel G impregnated with 3% DNCB*		*Kieselgel G + Kieselguhr G (9:1) impregnated with 3% DNCB*	
			Monochlorobenzene–ethyl acetate (9:1)	*Toluene–ethylene dichloride (3:1)*	*Xylene–ethyl acetate (4:1)*	*Carbon tetrachloride–petroleum ether (3:1)*
1	N-Methylaniline	light yellow	0.66	0.58	0.76	0.84
2	N,N-Dimethylaniline	reddish brown	0.81	0.68	0.85	0.92
3	Aniline	light orange	0.49	0.36	0.56	0.62
4	*o*-Anisidine	reddish brown	0.55	0.43	0.57	0.69
5	*p*-Anisidine	orange	0.28	0.12	0.35	0.26
6	Diphenylamine	reddish brown	0.85	0.87	0.79	1.00
7	α-Naphthylamine	reddish brown	0.57	0.42	0.56	0.59
8	β-Naphthylamine	yellowish brown	0.51	0.35	0.50	0.52
9	*o*-Toluidine	pale yellow	0.56	0.47	0.55	0.68
10	*m*-Toluidine	bright yellow	0.52	0.38	0.51	0.66
11	*p*-Toluidine	bright yellow	0.44	0.27	0.47	0.42
12	Benzidine	dark orange	0.21	0.07	0.29	0.01
13	*m*-Chloroaniline	light yellow	0.63	0.58	0.62	0.72
14	*p*-Chloroaniline	light yellow	0.56	0.48	0.55	0.58

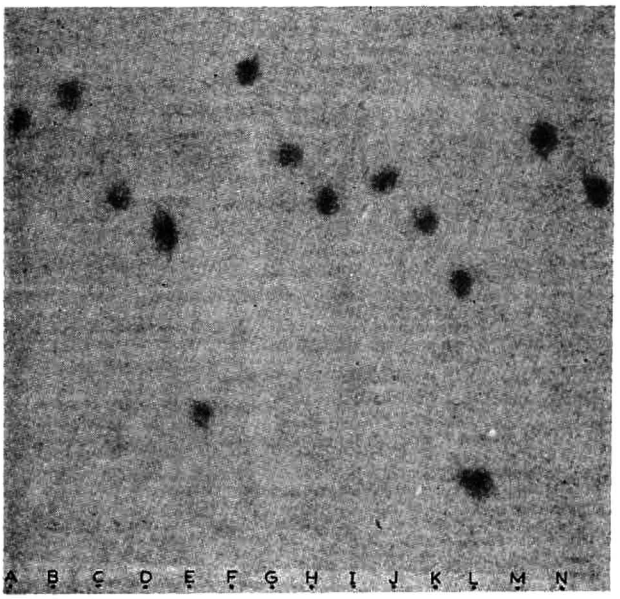


Fig. 1. Chromatoplate showing the resolutions of the charge-transfer complexes of tetryl with the following amines: A = N-Methylaniline; B = N,N-dimethylaniline; C = aniline; D = *o*-anisidine; E = *p*-anisidine; F = diphenylamine; G = α-naphthylamine; H = β-naphthylamine; I = *o*-toluidine; J = *m*-toluidine; K = *p*-toluidine; L = benzidine; M = *m*-chloroaniline and N = *p*-chloroaniline. Absorbent: Kieselgel G impregnated with 3% tetryl; solvent: ethylene dichloride–petroleum ether (4:1, with 3% tetryl); system: ascending.

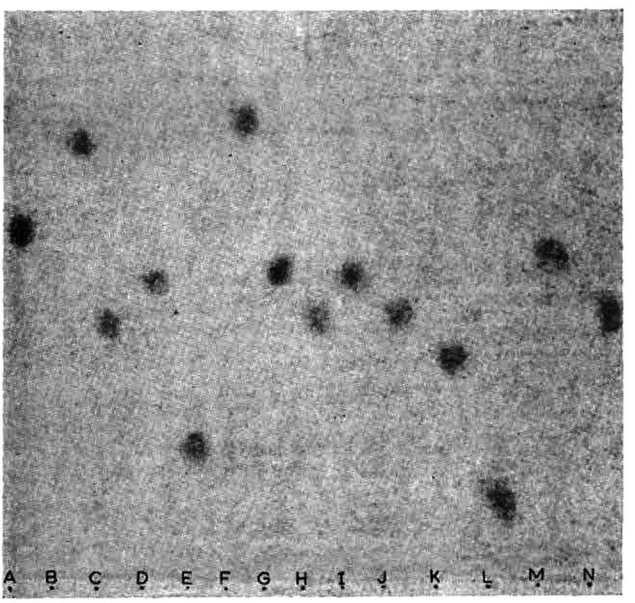


Fig. 2. Chromatoplate showing the separations of the complexes of 2,4-dinitrochlorobenzene with amines (A–N). Adsorbent: Kieselgel G containing 3% DNCB; solvent: ethyl acetate–monochlorobenzene (1:9, with 3% DNCB); system: ascending.

Discussion and results

The charge-transfer complexes of aromatic amines with tetryl and 2,4-dinitrochlorobenzene were found to be highly unstable, particularly when polar solvents were used for crystallization or irrigation on reversed phase systems. All the complexes broke down into tetryl or DNCB and the individual amines when spotted and run on different adsorbents (untreated with the nitro aromatic compounds) employing all possible irrigating solvent systems. Thus the adsorption forces were found to be even stronger than solvent forces. Since cellulose powder, due to its hydrophilic nature, had a strong rupturing effect on the weak physical linkages in the π-complexes, it was esterified. Even when highly acetylated and benzoylated cellulose samples were used as adsorbents no useful results were obtained.

In the case of Kieselguhr G the compounds travelled with the solvent front. Kieselgel G containing 3 % tetryl/DNCB proved to be the best adsorption medium for the study of these complexes. In order to prevent further break-down of the complexes, 3 % of the acceptor molecule was added to the irrigating non-polar solvent.

The distance of migration of the tetryl/DNCB–amine complexes was found to be parallel to the relative basicities of the individual amines. Complexes of more basic amines had lower R_F values than those which were less basic, *e.g.* aniline < N-methylaniline < N,N-dimethylaniline. It was found that the complexes of *para*-substituted anilines had the least mobilities compared with those which were *meta*- and *ortho*-substituted. Complexes of *p*-anisidine had lower R_F values than those of *o*-anisidine; those of *p*-chloroaniline had lower R_F values than those of *m*-chloroaniline; and of *p*-toluidine < *m*-toluidine < *o*-toluidine. Diphenylamine being a weak base, its complexes had high R_F values.

Acknowledgements

Thanks are due to Dr. H. K. ACHARYA, Assistant Director (Chemistry), and Dr. KARTAR SINGH, Director, Defence Science Laboratory, Delhi for their encouragement.

Defence Science Laboratory,
Metcalfe House, Delhi-6 (India)

D. B. PARIHAR
S. P. SHARMA
K. K. VERMA

1 R. IKAN, *J. Chromatog.*, 17 (1965) 591.
2 M. VON SCHANTZ, S. JUVONEN AND R. HEMMING, *J. Chromatog.*, 20 (1965) 618.
3 V. A. PASESHNICHENKO AND A. R. GUSEVA, *Prikl. Biokhim. i Mikrobiol.*, 1 (1965) 559.
4 E. STAHL AND H. VOLLMANN, *Talanta*, 12 (1965) 525.
5 C. B. BARRETT, M. S. J. DALLAS AND F. B. PADLEY, *Chem. Ind. (London)*, (1962) 1050.
6 G. M. NANO AND A. MARTELLI, *J. Chromatog.*, 21 (1966) 349.
7 M. FRANCK-NEUMANN AND P. JÖSSANG, *J. Chromatog.*, 14 (1964) 280.
8 A. K. DWIVEDY, D. B. PARIHAR, S. P. SHARMA AND K. K. VERMA, *J. Chromatog.*, 29 (1967) 120.

Received February 9th, 1967

Separation of *cis* and *trans* isomers of α,β-unsaturated acids by thin-layer chromatography

Chemical constituents of plants often occur as esters of different α,β-unsaturated acids like angelic acid, tiglic acid, β,β-dimethylacrylic acid etc. In such compounds, the acid part is usually identified by isolation of the acid in a pure state after saponification of the corresponding esters. In many instances the process of isolation is further complicated by the conversion of the α,β-unsaturated acid from a labile *cis* form to a more stable *trans* configuration during saponification.

In the course of chemical investigations on some plants we encountered a number of angelic acid and β,β-dimethylacrylic acid esters of some triterpenes and it was found necessary to identify extremely small amounts of these α,β-unsaturated acids, and in particular to differentiate the *cis* and *trans* isomers (*i.e.* angelic acid and tiglic acid) by TLC. Lack of any convenient method for the resolution of these compounds by TLC prompted us to develop a suitable method.

At the outset, attempts were made to separate angelic acid, tiglic acid and β,β-dimethylacrylic acid by conventional TLC on Silica Gel G using a number of different solvent systems, but without any success. All the three acids migrated to an equal extent on the thin-layer chromatogram.

Certain triglyceride mixtures have been resolved into classes according to their degree of unsaturation and within these classes, certain isomeric unsaturated triglycerides have been resolved by TLC over Silica Gel G impregnated with silver nitrate[1]. SUKH DEV *et al.*[2] have also separated certain isomeric olefinic sesquiterpenoids by TLC over Silica Gel G impregnated with silver nitrate. Following their methods, we tried to separate the aforesaid α,β-unsaturated acids. However, all the three acids again gave rise to long streaking spots which could not be employed for identification purposes. The following solvent systems were tested:

(1) Chloroform–methanol (95:5, v/v)
(2) Chloroform–methanol (9:1, v/v)
(3) Benzene–methanol–acetic acid (95:3:2, v/v).

It has been reported recently[3] that silica gel plates impregnated with ammoniacal silver nitrate, give much better resolution than silica gel plates impregnated with silver nitrate itself. Following this method, we have been able to separate the above mentioned acids. Three different solvent systems were employed:

(A) Chloroform–methanol (95:5, v/v)
(B) Chloroform–methanol (9:1, v/v)
(C) Chloroform–methanol (2:1, v/v).

The R_F values of angelic acid, tiglic acid and β,β-dimethylacrylic acid in the three solvent systems mentioned above are tabulated below:

Experimental

β,β-Dimethylacrylic acid and tiglic acid were obtained commercially and angelic acid was prepared from tiglic acid according to the method of MOCK *et al.*[4] A slurry of Silica Gel G (E. Merck, 3.0 g) in ammoniacal silver nitrate solution (3.60 ml, made by adding liquor ammonia to a 5% aq. silver nitrate solution drop by drop till the precipitate first formed completely dissolved) was applied to the glass plates (10 × 20 cm) with an improvised spreader (layer thickness 0.35 mm). The plates were air-

TABLE I

Solvent system	R_F *values*		
	Angelic acid	*Tiglic acid*	*β,β-Dimethylacrylic acid*
A	0.50	0.14	0.03
B	0.63	0.38	0.21
C	0.78	0.41	0.30

dried for 15 min and then activated by heating in an air oven at 110° for 30–40 min. The acids in chloroform solution (100–200 μg) were spotted to the plates in the usual way.

The solvent was allowed to run up to a height of 14–15 cm from the base line. The plates were then air-dried and sprayed with an ethanolic solution (0.1 %) of 2,7-dichlorofluorescein and then exposed to U.V. light. Greenish-yellow fluorescent spots were observed in case of all the three acids. All the operations were carried out in an air conditioned room at a temperature of 22–24°.

Discussion

The strong co-ordination complex formed by the electrophilic complex amino ion $Ag(NH_3)_2^+$, present in the ammoniacal silver nitrate solution, with the nucleophilic π-bonds of the olefinic compounds probably explains the better separation of the α,β-unsaturated acids by this method. Of the three solvent systems employed, best results were achieved with solvent system C, as will be seen from Table I.

Acknowledgements

The authors wish to thank Dr. D. M. Bose, Director and Dr. A. Sen, Head of the Dept. of Chemistry, Bose Institute, Calcutta, for their kind interest in the work.

Department of Chemistry, Bose Institute, S. P. Dutta
Calcutta 9 (India) A. K. Barua

1 C. B. Barett, M. S. J. Dallas and F. B. Padley, *J. Am. Oil Chemists' Soc.*, 40 (1963) 580.
2 Sukh Dev and A. S. Gupta, *J. Chromatog.*, 12 (1963) 189.
3 R. Wood and F. Snyder, *J. Am. Oil Chemists' Soc.*, 43 (1966) 53.
4 R. E. Buckles and G. V. Mock. *J. Org. Chem.*, 15 (1950) 680.

Received February 1st, 1967

The separation of some poly(ethylene terephthalate) oligomers

During the trans-esterification of dimethyl terephthalate by ethylene glycol and the subsequent polymerization of the product to form poly(ethylene terephthalate) a variety of oligomeric materials are formed. In order to study the reaction kinetics of the interchange reaction, it is desirable to be able to detect the products formed and to measure the amounts of each formed. Also, for continuous production, it is desirable to have a method of detecting any unreacted methyl groups prior to polymerization.

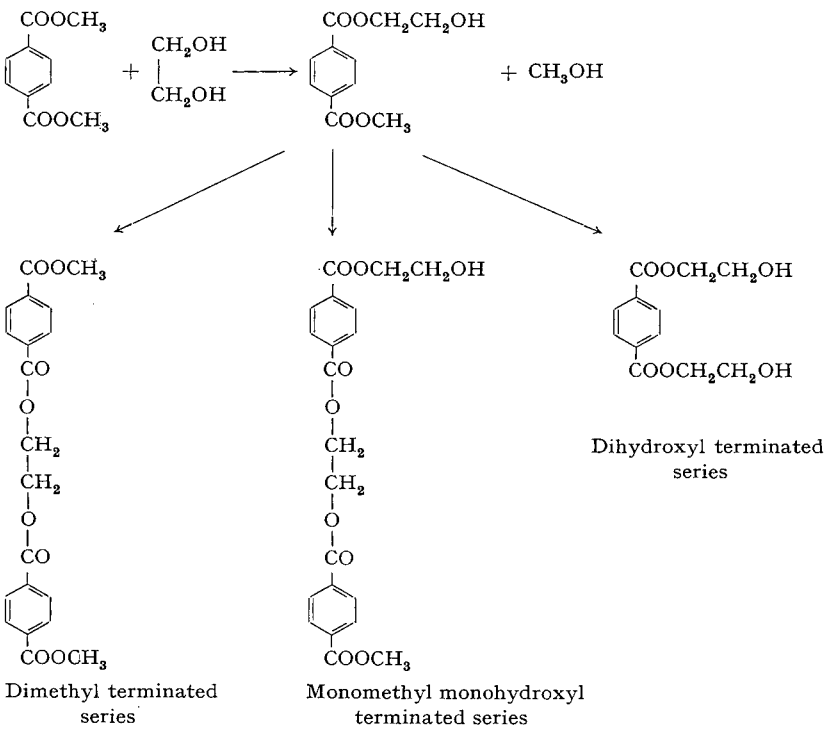
The oligomers formed can be listed as three series of compounds:

(1) Those terminated with methyl groups at both ends;

(2) those terminated with hydroxyl groups at both ends, and

(3) those terminated with one methyl and one hydroxyl group.

These series can be seen in the following scheme:



The dihydroxyl terminated series is the most important since these compounds can be polymerized by the removal of ethylene glycol whereas methyl terminated compounds will not polymerize without prior esterification.

Experimental

For calibration and identification purposes, our polymer research group synthesized monohydroxyethylenemonomethyl terephthalate, bishydroxyethylene terephthalate, dimethyl dimer and dimethyl trimer.

Thin-layer chromatographic layers were made up 200 μ thick of Silica Gel G containing 13 % calcium sulfate. The layers were activated at 120° for 30 min, cooled in a desiccator, spotted and developed for 15 cm in a closed, saturated chamber using one of three developers: (a) an 85:15 benzene–ethyl acetate mixture[1] which gives good separation of the dimethyl species; (b) a 55:45 benzene–ethyl acetate mixture which gives reasonable separation of all species with some overlap of the dimethyl species, or (c) ethyl acetate which does not separate the dimethyl species but which will separate the others.

Standards and unknowns were dissolved (about 1 % concentration) in dioxane and 5 μl spotted on the TLC layer. After development and subsequent evaporation of the solvent, the plates were sprayed with a mixture of 0.2 g methyl red, 0.2 g bromothymol blue, 100 ml 35 % formaldehyde and 400 ml 95 % ethanol[2]. About 10 min after spraying, the background had faded sufficiently for the spots to be identified, usually a pale rose color.

Table I shows the R_F values of the species in the different solvents.

TABLE I

Compound	*Solvent mixture*		
	Benzene–ethyl acetate (85:15)	*Benzene–ethyl acetate (55:45)*	*Ethyl acetate*
Dimethyl terephthalate	0.70	0.88	1.0
Dimethyl dimer	0.62	0.87	1.0
Dimethyl trimer	0.50	0.85	1.0
Monohydroxyethylene monomethyl terephthalate	0.12	0.44	0.72
Bishydroxyethylene terephthalate	0.00	0.12	0.36
Secondary front	0.72	0.88	—

If a quantitative estimation of the compounds present is required, this can be obtained by lifting the spots and surrounding support from the plate, dissolving the organic matter in methanol, and, after filtration, measuring the absorption of the solution at 240 mμ in the U.V. Previous calibration of absorption *vs.* concentration for each specie at 240 mμ then provides a measure of the amount of material present. Studies indicate that absorption is proportional to concentration up to at least 1.2 mg/100 ml.

Conclusion

The method described is fast and can be used either quantitatively or qualitatively. With practice the method is reproducible to $\pm$ 5 %.

Fibers Division Technical Center,
Allied Chemical Corporation, P.O. Box 31,
Petersburg, Va. 23804 (U.S.A.)

VICTOR A. DORMAN-SMITH

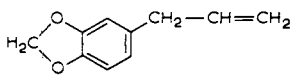
1 J. W. C. PEEREBOOM, *J. Chromatog.*, 4 (1960) 323.
2 E. STAHL, *Thin Layer Chromatography*, Academic Press, New York, 1965, p. 495.

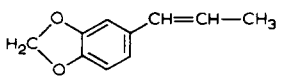
Received January 26th, 1967

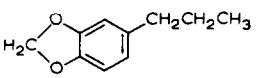
Thin-layer chromatography of rat bile and urine following intravenous administration of safrole, isosafrole, and dihydrosafrole

In a previous paper[1] we reported on the elimination of pesticidal synergists (piperonyl butoxide and tropital) and their metabolites in rat bile and urine, resulting from single intravenous administrations of the above compounds.

The purpose of this investigation was to continue the thin-layer chromatographic elaboration of rat bile and urine following the single intravenous administration of three basic methylenedioxyphenyl derivatives, *viz.*, safrole (I), isosafrole (II), and dihydrosafrole (III)*.


I


II


III

Safrole, the principal component of oil of sassafras[2], has until recently, been widely used in foods chiefly as a flavoring agent in root beer[3]. Both safrole and isosafrole are employed in the manufacture of heliotropin (piperonal) and are active synergists for pyrethrum and Sevin[4] (1-naphthyl methyl carbamate) as well as being inhibitors of the hydroxylation of naphthalene in houseflies[5].

Safrole has been shown to be a relatively weak hepatic carcinogen when fed to rats for chronic periods[6,7] while dihydrosafrole produced benign and malignant esophageal tumors during similar feeding studies with rats[8] and fatty degeneration of the liver when fed to mice[9]. Although liver changes in rats resulting from chronic feeding of isosafrole, safrole and dihydrosafrole were of the same general type (including hepatic cell enlargement, cystic necrosis and bile duct proliferation), the magnitude of the liver changes was much greater with safrole than with the other two compounds[8].

Experimental

Preparation of the plates

Silica Gel Df-5 chromatoplates were prepared as previously described[1].

Solvent systems

(A) Toluene–acetic acid–water (10:10:1)
(B) Ethyl acetate–acetic acid–methanol (70:10:20)
(C) *n*-Butanol–acetic acid–water (10:1:1)
(D) Acetone–benzene (1:39).

Detecting reagents

(1) Chromogenic agents: (a) Conc. sulfuric acid–*n*-butanol (15:85)[10]; (b) Chromotropic acid reagent[11].

(2) Radiation sources: (a) U.V. 3660Å—Mineralight, Blak-Ray Model UVL-22**; (b) U.V. 2537Å—Mineralight, Model UVS-11**.

* 3,4-Methylenedioxyallylbenzene, 3,4-methylenedioxypropenylbenzene and 3,4-methylenedioxypropylbenzene, respectively. All obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. (U.S.A.).

** Obtained from Allied Impex Corp., New York, N.Y.

TABLE I

SUMMARY OF RAT BILE R_F (× 100) DIFFERENCES ON SILICA GEL DF-5 RESULTING FROM SINGLE INTRAVENOUS ADMINISTRATION OF SAFROLE, ISOSAFROLE AND DIHYDROSAFROLE

Detectors: (1) 2537Å U.V.; (2) sulfuric acid–butanol, 3660Å; (3) sulfuric acid–butanol, visible light; (4) chromotropic acid, 3660Å; (5) chromotropic acid, visible light.
Colors: B = blue; Bl = black; Bn = brown; G = green; Gr = grey; O = orange; Ob = obscured by other 2537Å absorbing spots; Q = quench; R = red; V = violet; W = white; Y = yellow; (—) = not detected.

Developer	*Safrole*						*Isosafrole*						*Dihydrosafrole*					
	R_F	*Detector and color*					R_F	*Detector and color*					R_F	*Detector and color*				
		1	*2*	*3*	*4*	*5*		*1*	*2*	*3*	*4*	*5*		*1*	*2*	*3*	*4*	*5*
Toluene–acetic acid–water (10:10:1)	0	Ob	Q	G	Q	Bn-G	0	Ob	Q	G	Q	G	0	Ob	Q	G	Q	G
	8	—	V	V	Q	V												
	20	Q	Q	V	Q	V	20	Q	—	Bn	—	Bn						
	23	Q	R-V	Y-Bn	R-V	Y-Bn	23	Q	R-V	Y-Bn	R-V	Y-Bn						
	28	Ob	—	—	—	V	28	Ob	—	—	—	V	28	Ob	—	—	—	V
	36	B	—	—	—	—	36	B	—	—	—	—						
	38	—	—	—	—	V	38	—	—	—	—	V						
	48*	—	W	Y-O	W	Y-O	45*	—	W	Y-O	W	Y-O	45*	—	W	Y-O	W	Y-O
	55	Q	Q	Gr	Q	Gr	55	Q	—	Gr	—	Gr	55	—	—	Gr	—	Gr
	58	—	W	—	W	—												
	72	Ob	—	Gr	—	Bl	72	Ob	—	Gr	—	Bl	72	Ob	—	Gr	—	Bl
Ethyl acetate–acetic acid–methanol (70:10:20)	0	Ob	Q	Bn	Q	Bn	0	Ob	Q	Bl	Q	Bl	0	Ob	Q	Bl	Q	Bl
	25	—	V	G-Bn	V	Gr-Bn	25	—	V	Bn	V	Gr	25	—	V	Bn	V	Gr
	38	Q	R-V	Gr	R-V	Gr	38	Q	R-V	Gr	R-V	Gr						
	60	Ob	—	Gr	—	Gr	60	Ob	—	Gr	—	Gr	60	Ob	—	Gr	—	Gr
	70	Ob	—	Gr	—	Gr	70	Ob	—	Gr	—	Gr	70	—	—	Gr	—	Gr
	75*	—	W	Y-O	W	Y-O	75*	—	W	Y-O	W	Y-O	75*	—	W	Y-O	W	Y-O
	81	B	—	—	—	V	81	B	—	—	—	V						
n-Butanol–acetic acid–water (10:1:1)	0	Ob	Q	Bl	Q	Bl	0	Ob	—	G	—	G	0	Ob	—	G	—	Gr
	5	Q	Q	Gr	—	Gr	5	Q	Q	Gr	—	Gr	5	Q	Q	Gr	—	Gr
	13	—	Q	Bl	Q	Bl												
	18	—	Q	G-Bl	Q	Bn												
	22	Ob	—	V	—	V												
	28	Ob	V	V	V	V	28	Ob	—	Bn	—	Gr						
	47	Ob	—	Gr	—	Gr												
	56	—	—	V	—	V							56	Ob	—	Bn	—	Bn
	68	B	—	—	—	—	68	B	—	—	—	—						
	75*	—	W	Y-O	W	Y-O	75*	—	W	Y-O	W	Y-O	75*	—	W	Y-O	W	Y-O
	78	Ob	—	Gr	—	Gr	78	Ob	—	Gr	—	Gr	78	Ob	—	Gr	—	Gr

* Cholic acid.

Bile and urine sampling

Single intravenous injections of 0.04 ml each of safrole, isosafrole, and dihydrosafrole were given to adult male rats of the Sprague-Dawley strain, averaging 350 g in weight. Bile samples were collected by fistula and urine samples by cannulation from each rat. Details on the handling of the animals, anesthesia, surgery and sample collection and timing have previously been described[12].

At least three urine samples were collected, one before intravenous injection, a second at an appropriate interval after injection and a final sample at the termination of the bile collection. All samples were kept frozen until ready for analysis.

Photography of chromatoplates

The thin-layers plates were photographed in color and black and white using equipment and procedures previously described[1].

Procedure

Time study of metabolite formation. Twenty microliters of all bile and urine samples were applied as half-inch streaks* on Silica Gel Df-5 plates. One microliter of standard RBY dye** was applied to each plate as a reference marker. The plates were developed in ethyl acetate–acetic acid–water (10:10:1), the solvent removed with a hot air dryer and each plate photographed under U.V. at 2537Å. The plates were then treated with the chromotropic acid reagent. After spraying, all plates were heated in a circulating air oven at 120° until color development was maximum (10–20 min). The plates were then photographed under U.V. at 3660Å and visible light. R_F values and spot colors were taken directly from the photographs because many of the colors were unstable on the silica gel plates.

Metabolite characterization. Bile and urine samples exhibiting optimum concentration of metabolites were pooled and applied as a streak (60 μl/1.25 in.) on Silica Gel Df-5 plates and were compared with controls taken before injection. After solvent development, they were first photographed under U.V. at 2537Å. One half of each streak*** was sprayed with sulfuric acid–butanol reagent and the other half with the chromotropic acid reagent, then the spots developed at 120° as described above.

Results and discussion

Chromatographic differences in bile and urine samples resulting from intravenous administration of safrole, isosafrole and dihydrosafrole are summarized in Tables I and II, respectively. Tables I and II list the R_F values of each component and data regarding its characterization (means of detection, color, etc.).

It is apparent from the data shown in Table I that the presence of an unsaturated side chain, as in safrole and isosafrole, results in a larger number of metabolites in the bile than in the case of dihydrosafrole where the side chain is saturated. For safrole and isosafrole there exists the possibility of degradation at the double bond of the aliphatic side chain and/or at the methylenedioxy ring leading to the type of products that are diagrammed in Fig. 1.

---

* Bile and urine samples were applied with a RADIN-PELIDS thin-layer sample streaker obtained from Applied Science Laboratories, State College, Pa., U.S.A.

** Obtained from Camag, Muttenz, Switzerland.

*** Two thin-layer glass plates were used to cover the areas that were not to receive the spray.

J. Chromatog., 29 (1967) 267–273

TABLE II

SUMMARY OF RAT URINE R_F (× 100) DIFFERENCES ON SILICA GEL DF-5 RESULTING FROM SINGLE INTRAVENOUS ADMINISTRATION OF SAFROLE, ISOSAFROLE AND DIHYDROSAFROLE

Detectors: (1) = 2537Å; (2) = sulfuric acid–butanol, 3660Å; (3) = sulfuric acid–butanol, visible light; (4) = chromotropic acid, 3660Å; (5) = chromotropic acid, visible light.
Colors: B = blue; Bl = black; Bn = brown; G = green; Gr = grey; O = orange; Ob = obscured by other 2537Å absorbing spots; Q = quench; V = violet; W = white; Y = yellow; (—) = not detected.

Developer	*Safrole*						*Isosafrole*						*Dihydrosafrole*					
	R_F	*Detector and color*					R_F	*Detector and color*					R_F	*Detector and color*				
		1	*2*	*3*	*4*	*5*		*1*	*2*	*3*	*4*	*5*		*1*	*2*	*3*	*4*	*5*
Toluene–acetic acid–water (10:10:1)	8	B	Q	Bn	Q	G	7	Ob	Q	Gr	Q	Gr	7	Ob	Q	Gr	Q	Gr
	20	Q	Q	V	Q	V	20	Q	Q	V	Q	V	20	Q	Q	Gr	Q	Gr
	23	Ob	—	Gr	—	Gr												
							40	Ob	Q	V	Q	V	40	Ob	—	Gr	—	Bl
							42	Ob	—	—	—	Bl						
							45	Ob	—	Gr	—	Bn						
	59	Q	Q	Y-G	Q	Y-G							60	Q	Q	Y-G	Q	Y-G
	72	Q	B-W	Gr	B-W	Gr												
							75	B	—	—	—	—						
													79	—	B-W	Gr	B-W	Gr
Ethyl acetate–acetic acid–methanol (70:10:20)	5–32	—	Q	Bn	Q	Bn												
							10	—	—	Bn	—	Bn	10	—	—	Bn	—	Bn
							28	Q	—	—	—	—						
													30	Ob	—	Gr	—	Gr
							57	Ob	Q	V	Q	V						
	59	Q	Q	V	Q	V												
							63	Ob	—	—	—	Gr						
							72	—	—	—	—	Bl-V						
	79	Ob	—	—	—	Bl	79	Q	—	—	—	V	79	Q	—	—	—	Bl
n-Butanol–acetic acid–water (10:10:1)	5–30	—	—	—	Q	Bn												
							10	Q	—	—	—	—						
							38	B	—	—	—	V						
	58	Q	—	—	Q	V	58	Q	—	—	Q	V	58	Q	—	—	Q	V
							68	Q	—	—	—	Gr						
	72	Q	—	—	Q	Y-G	72	B	—	—	—	Bn						
	80	Ob	—	—	B	Gr	80	Q	—	—	—	Gr						

Although the products depicted in Fig. 1 have not been unequivocally identified in this study, previous bile experiments with piperonal and piperonylic acid have indicated steps 2 → 3 of Fig. 1 and will be reported separately.

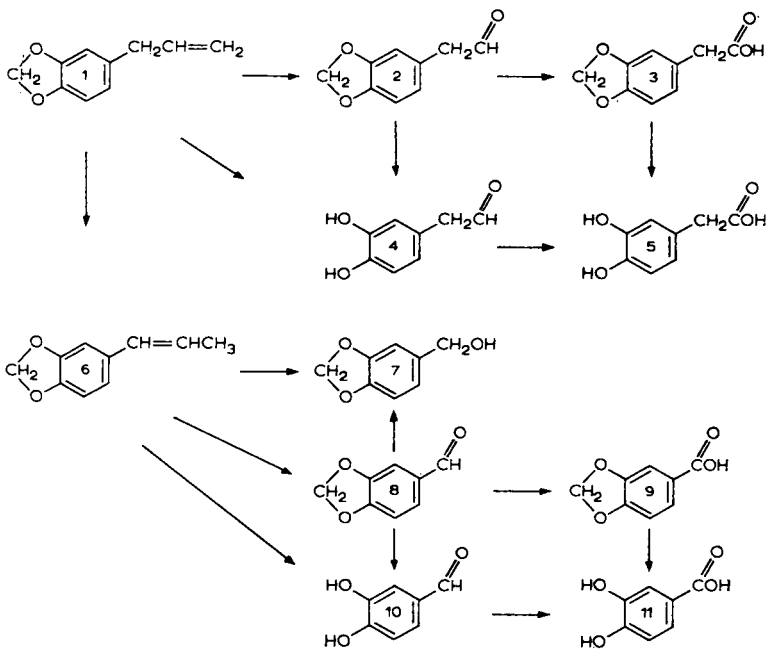
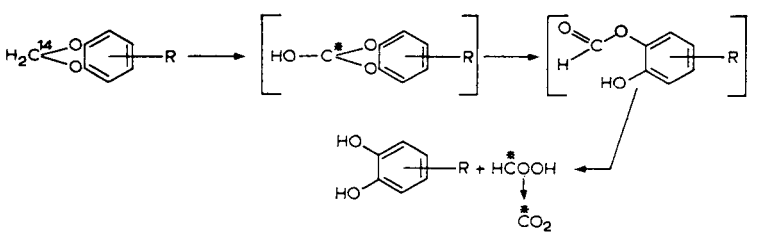


Fig. 1. Possible routes of metabolism for safrole and isosafrole. 1 = Safrole; 2 = 3,4-methylenedioxyphenylacetaldehyde; 3 = 3,4-methylenedioxyphenylacetic acid; 4 = 3,4-dihydroxyphenylacetaldehyde; 5 = 3,4-dihydroxyphenylacetic acid; 6 = isosafrole; 7 = piperonyl alcohol; 8 = piperonal; 9 = piperonylic acid; 10 = 3,4-dihydroxybenzaldehyde; 11 = 3,4-dihydroxybenzoic acid.

It is of interest to note the similarity (R_F and color) for the bile metabolites of safrole and isosafrole utilizing toluene–acetic acid–water and ethyl acetate–acetic acid–methanol developers (Table I).

CASIDA and co-workers[13] utilized methylene-^{14}C-safrole and dihydrosafrole to elaborate the metabolism in relation to synergistic action. It was found that the methylene-^{14}C group is hydroxylated as shown below:



When urine chromatograms from safrole and isosafrole were compared with those from piperonal and piperonylic acid injected rats, a similarity of grey bands occurring at R_F 0.68 for the isosafrole and piperonylic acid urines was noted.

WILLIAMS[14] has reported the isolation of piperonylic acid conjugates (in the urine) such as the ester glucuronide and a glycine conjugate after the administration of safrole and isosafrole to dogs.

Fig. 2 depicts the relationship of bile components with time after intravenous administration of safrole (the $R_F \times 100$ values shown correspond to those in Table I found utilizing *n*-butanol–acetic acid–water (10:10:1) developer). Graph (A) indicates the metabolites that occur within a few minutes after injection. Graphs (B) and (C) indicate those that begin to appear 70–90 min and 130 min, respectively, after injec-

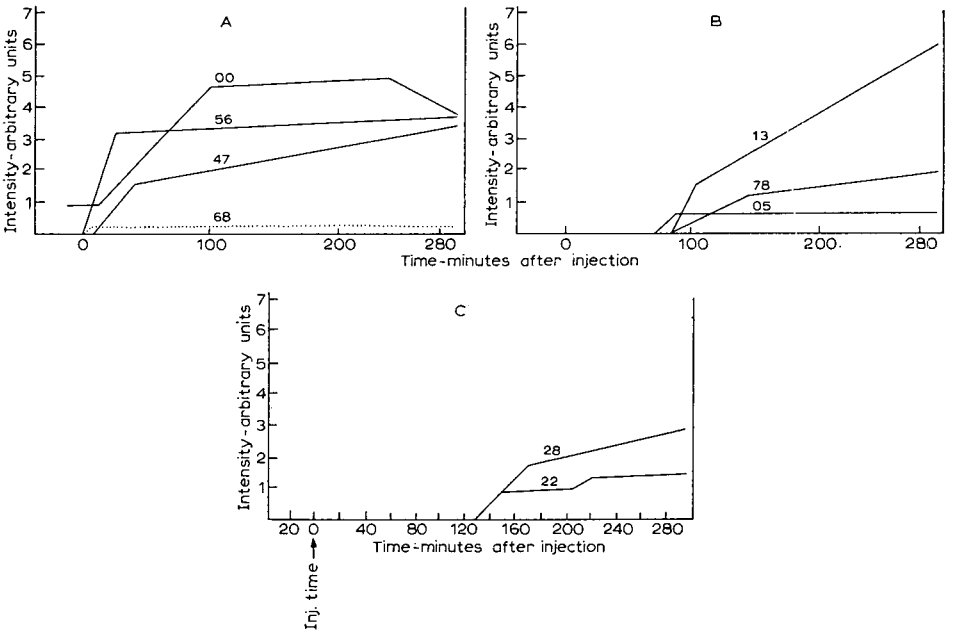


Fig. 2. Relationship of spot intensity with time of various metabolites occurring in rat bile after intravenous administration of safrole. The data shown were taken from thin-layer plates developed with *n*-butanol–acetic acid–water (10:1:1). Concentrations were estimated visually as degrees of intensity of absorbance or fluorescence. Portion (A) of figure indicates metabolites that occur within a few minutes after injection. Portions (B) and (C) indicate those that appear 70–90 min and 130 min after injection, respectively. Dotted curve (R_F 68; portion A) was viewed under U.V. at 2537 Å; all other components were plotted after spraying the plate with chromotropic acid.

tion. The bile acid, cholic acid (R_F 75) (Table I) showed no discernable change in concentration before or after safrole injection and hence was not included in Fig. 2. It is of interest to note that in analogous studies with tropital and piperonyl butoxide[1] the cholic acid concentration was shown to rise steadily throughout the length of the experiment in tropital injected animals whereas the cholic acid level remained constant in the case of piperonyl butoxide treated animals.

Conclusions

The methylenedioxyphenyl derivatives studied in these experiments were found to be altered chemically with the following apparent order of metabolism: safrole > isosafrole > dihydrosafrole. The elimination of products occurred largely in the bile after intravenous injection.

The rate of elimination of metabolites resulting from the administration of safrole does not reach a rapid peak with rapid decline thereafter, but similarly to the earlier experiments with piperonyl butoxide and tropital, suggests slow prolonged elimination of the metabolites in the bile. As suggested earlier[15], inhibition of certain detoxification mechanisms and delayed elimination from the body of pesticides and other chemicals could constitute a hazard to the health of man exposed to these compounds.

Acknowledgement

This study was supported by Research Contract PH 43-67-57, National Cancer Institute, National Institutes of Health, Public Health Service and represents Paper No. 37 of this contract.

Bionetics Research Laboratories, Inc.,
Falls Church, Va. (U.S.A.)
L. FISHBEIN
J. FAWKES

National Cancer Institute
Bethesda, Md. (U.S.A.)
H. L. FALK
S. THOMPSON

1 L. FISHBEIN, J. FAWKES, H. L. FALK AND S. THOMPSON, *J. Chromatog.*, 27 (1967) 153.
2 F. B. POWER AND C. KLEBER, *Pharm. Rev.*, 14 (1896) 101.
3 M. B. JACOBS, *Am. Perfumer. Aromat.*, 71 (1958) 57.
4 S. KUWATSUKA AND J. E. CASIDA, *J. Agr. Food Chem.*, 13 (1965) 528.
5 W. W. PHILLEO, R. D. SCHONBROD AND L. C. TERRIERE, *J. Agr. Food Chem.*, 13 (1965) 113.
6 E. L. LONG AND A. A. NELSON, *Federation Proc.*, 20 (1961) 287.
7 E. L. LONG, A. A. NELSON, O. G. FITZHUGH AND W. H. HANSON, *Arch. Pathol.*, 75 (1963) 595.
8 E. C. HAGAN, P. M. JENNER, W. I. JONES, O. G. FITZHUGH, E. L. LONG, J. W. BROUWER AND W. K. WEBB, *Toxicol. Appl. Pharmacol.*, 7 (1965) 18.
9 F. VON GRAEVENITZ, *Arch. Exptl. Pathol. Pharmakol.*, 104 (1924) 298.
10 W. L. ANTHONY AND W. T. BEHER, *J. Chromatog.*, 13 (1964) 567.
11 M. BEROZA, *J. Agr. Food Chem.*, 11 (1963) 51.
12 P. KOTIN, H. L. FALK AND R. BUSSER, *J. Natl. Cancer Inst.*, 23 (1959) 541.
13 J. E. CASIDA, J. L. ENGEL, E. G. ESSAC, F. X. KAMIENSKI AND J. KUWATSUKA, *Science*, 153 (1966) 1130.
14 R. T. WILLIAMS, *Detoxification Mechanisms*, 2nd Ed., Wiley, New York, 1959, p. 371.
15 H. L. FALK, S. THOMPSON AND P. KOTIN, *Arch. Environ. Health*, 10 (1965) 847.

Received December 9th, 1966

Chromatographie de quelques composés apparentés à la ninhydrine

L'élaboration d'une nouvelle méthode de synthèse de la ninhydrine ainsi que l'étude du mécanisme des réactions fournies par la ninhydrine avec différents produits azotés nous ont amenés à caractériser par chromatographie, sur papier ou sur couche mince, différentes substances apparentées à la ninhydrine.

Chromatographie de divers composés carbonylés indaniques

La ninhydrine, synthétisée pour la première fois par RUHEMANN[1] à partir de l'indanone-1, se prépare le plus souvent, depuis le travail de TEETERS ET SHRINER[2], par oxydation sélénieuse de l'indanedione-1,3; EGASHIRA[3] a amélioré le rendement de cette oxydation en substituant à l'indanedione-1,3 son produit de nitrosation, l'hydroximino-2-indanedione-1,3. Nous avons montré[4] que l'oxydation sélénieuse de l'indanone-1 conduit également à la ninhydrine avec un excellent rendement, avec la formation intermédiaire d'indanedione-1,2; la ninhydrine s'obtient aussi au cours de l'oxydation sélénieuse de l'oxime de l'indanone-2, de l'indanedione-1,2 et de l'hydroximino-2-indanone-1. L'identification chromatographique de ces divers composés, réalisée sur une couche mince de gel de silice G (E. Merck) est indiquée sur le Tableau I.

Les produits se colorent en jaune ou en orangé par pulvérisation d'une solution de dinitro-2,4-phénylhydrazine à 0.5 % dans HCl 2*N*. La phase solvante C nous a donné les meilleures séparations (Fig. 1).

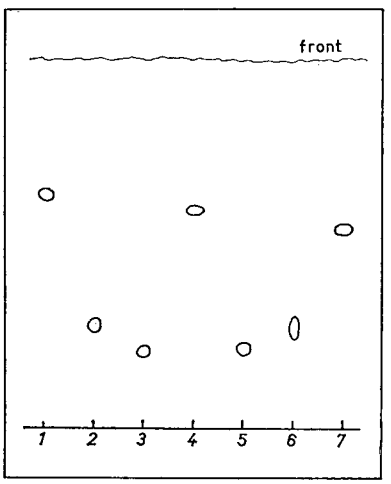


Fig. 1. Chromatographie sur couche mince de composés carbonylés indaniques. Benzène–acétate d'éthyle–acide formique (85:10:5). 1 = Indanone-2 (oxime); 2 = hydroximino-2-indanone-1; 3 = hydroximino-2-indanedione-1,3; 4 = indanone-1; 5 = ninhydrine; 6 = indanedione-1,2; 7 = indanedione-1,3.

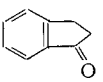
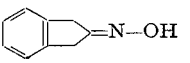
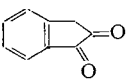
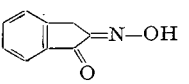
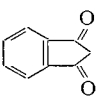
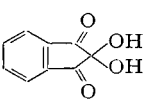
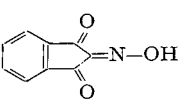
Chromatographie de produits d'oxydation de la ninhydrine

La formation inattendue du pourpre de RUHEMANN au cours de la réaction de la ninhydrine avec l'hydroximino-2-indanedione-1,3[5,6] ou avec le thiocyanate de potassium[7] implique une réaction d'oxydo-réduction au cours de laquelle la tricétone

TABLEAU I

VALEURS DES R_F DE DIFFÉRENTS COMPOSÉS CARBONYLÉS INDANIQUES SUR GEL DE SILICE

Phases solvantes: A = Benzène–acétate d'éthyle (3:1); B = Toluène–acétate d'éthyle (3:1); C = Benzène–acétate d'éthyle–acide formique (85:10:5).

Substances examinées	*Formules*	*Phase solvante*			*Couleur de la tache après révélation*
		A	*B*	*C*	
Indanone-1		0.77	0.66	0.59	orange
Indanone-2 (oxime)		0.50	0.37	0.63	jaune
Indanedione-1,2		0.13	0.00	0.27	jaune pâle
Hydroximino-2-indanone-1		0.37	0.25	0.28	jaune-orangé
Indanedione-1,3		0.50	0.60	0.54	jaune
Indanetrione (hydrate)		0.33	0.20	0.22	rouge-orangé
Hydroximino-2-indanedione-1,3		0.00	0.18	0.21	jaune

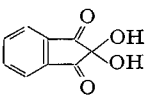
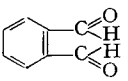
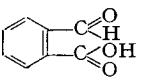
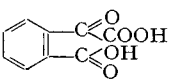
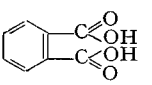
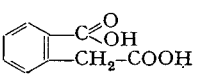
indanique s'oxyde partiellement en acide phtalonique; l'oxydo-réduction de la ninhydrine en hydrindantine et en acide phtalonique est par ailleurs catalysée par la présence d'ions cyanure[8]. La caractérisation chromatographique de différents produits résultant de la rupture oxydative du cycle indanique s'effectue dans des conditions satisfaisantes avec la phase solvante isopropanol–pyridine–eau (10:1:1), aussi bien sur papier que sur gel de silice; l'utilisation comme phase solvante d'une solution aqueuse de chlorure de sodium à 3 % permet également de résoudre quelques problèmes particuliers de séparation (Tableau II).

Les composés carbonylés sont révélés par pulvérisation d'une solution chlorhydrique de dinitro-2,4-phénylhydrazine qui fournit une coloration jaune-orangé, sauf avec l'acide phtalonique qui se colore en jaune citron (sensibilité: 20 μg); les composés acides sont mis en évidence par pulvérisation d'un aérosol de vert de bromocrésol (Sprühreagenz E. Merck).

TABLEAU II

VALEURS DES R_F DE DIFFÉRENTS PRODUITS D'OXYDATION DU CYCLE INDANIQUE

Solvants: (A) Isopropanol–pyridine–eau (10:1:1); (B) NaCl–eau (3:100, g/vol.).

Substances examinées	*Formules*	*Papier Whatman No. 3MM*		*Gel de silice*	
		A	*B*	*A*	*B*
Ninhydrine		0.77	0.76	0.93	0.79
Aldéhyde *o*-phtalique		0.88	0.90	0.88	0.70
Acide *o*-phtalaldéhydique		0.59	0.86	0.76	0.65
Acide phtalonique		0.25	0.95	0.17	0.84
Acide *o*-phtalique		0.42	0.72	0.44	0.13
Acide homophtalique		0.50	0.75	0.65	0.74

Nous avons pu ainsi confirmer la formation d'acide phtalonique au cours de l'oxydation periodique de la ninhydrine[9] et au cours de l'ébullition à l'air d'une solution alcaline de cette tricétone (pH 10). L'action de l'eau oxygénée sur la ninhydrine (1 ml de perhydrol à 110 vol. dans 5 ml d'une solution aqueuse d'hydrate d'indanetrione) conduit à la formation d'acide phtalonique et d'acide *o*-phtalique.

Laboratoire de Biochimie Médicale "B", J. HERVIEU
Faculté de Médecine et de Pharmacie, H. JENSEN
33 Bordeaux (France) E. NEUZIL

1 S. RUHEMANN, *J. Chem. Soc.*, 97 (1910) 1438.
2 W. O. TEETERS ET R. L. SHRINER, *J. Am. Chem. Soc.*, 55 (1953) 3026.
3 S. EGASHIRA, *Nippon Kagaku Zasshi*, 75 (1954) 308.
4 E. NEUZIL, A. LIERMAIN, H. JENSEN ET J. HERVIEU, *Bull. Soc. Pharm. Bordeaux*, sous presse.
5 J. SUNJIO, *Thèse Doct. Pharm. (Etat)*, Bordeaux 1965.
6 E. NEUZIL, J. SUNJIO, A. LIERMAIN, H. JENSEN ET J. HERVIEU, *Compt. Rend.*, sous presse.
7 E. NEUZIL, J. JOSSELIN, H. JENSEN ET J. HERVIEU, *Bull. Soc. Chim. France*, (1966) 1173.
8 T. C. BRUICE ET F. M. RICHARDS, *J. Org. Chem.*, 23 (1958) 145.
9 D. B. SPRINSON ET E. CHARGAFF, *J. Biol. Chem.*, 164 (1946) 433.

Reçu le 13 février 1967

Thin-layer chromatography of drugs in medicated feeds

Of the more than 40 million tons of feed manufactured annually in the United States, a very large percentage contains one or more drugs. Accurate, specific, and rapid methods of determining these drugs in feeds are needed for regulatory work. The Feed Additive Compendium[1], an authoritative manual for the feed industry, contains a compilation of various qualitative tests and assay procedures from the Manual of Microscopic Analysis of Feeding Stuffs[2], Official Methods of Analysis[3], and other sources. However, the majority of the tests described are generally nonspecific and not applicable to multicomponent preparations. In addition, many of the quantitative assay procedures for medicated feeds, *e.g.*, from Official Methods of Analysis[4], are themselves quite nonspecific.

R_F values obtained by thin-layer chromatography (TLC) have been reported for some of these drugs, namely, reserpine[5], propionic acid[6,7], griseofulvin[8,9], tetracyclines[10], penicillins[10,11], erythromycin[12], ronnel[13], and diethylstilbestrol[14].

The purpose of the work reported here was to extend the versatility, rapidity, and relative specificity of TLC to some of the other drugs commonly used in medicated feeds. Many of these drugs are commonly used in combinations such as ethopabate with amprolium. A number of these common multicomponent drug mixtures were separated and identified by TLC. To facilitate their identification the drugs were placed in categories according to similarity of structure and physical properties.

A procedure is described for the identification of 18 ingredients and 7 mixtures commonly used in medicated feeds. Various indicators have been used and lead–manganese–activated calcium silicate was incorporated in the preparation of the adsorbent to increase visibility of the spots and sensitivity of the procedure.

It should be noted that the data in this paper were obtained by working with standards of acceptable purity. Extraction and purification procedures for the drugs incorporated in the feeds are being developed and will be reported later.

These procedures will yield extracts of the feeds suitable for chromatography.

Experimental

The phenylarsonic acids were dissolved in 2 ml of 0.5 *N* sodium hydroxide and 98 ml of water. All the other reference standards were dissolved in dimethyl sulfoxide. N,N-Dimethylformamide can also be utilized as a solvent for the nitrophenyl compounds. All standards were stored in "Low-Actinic" glass-stoppered flasks to inhibit decomposition.

Preparation of plates

The adsorbent suspensions were applied to the plates with a Desaga applicator to a uniform thickness of 250μ. The plates were kept at room temperature for at least 30 min, then were activated at 100–110° for 30 min.

Alumina G. The suspension for five 20 × 20 cm plates or fourteen 10 × 15 cm plates was prepared by mixing 30 g of Alumina G (Camag DS 5) and 55 ml of water for 45 sec.

Alumina GF. A fluorescent indicator* (lead–manganese–activated calcium

* Distillation Products, Inc., Rochester, N.Y.

silicate) was added to Alumina G in a concentration of 300 mg/30 g (1 %) and a suspension was prepared as above.

Silica Gel G. A suspension of 30 g of Silica Gel G (E. Merck, Darmstadt) and 60 ml of water was prepared as for Alumina G.

Silica Gel GF. The fluorescent indicator (see Alumina GF) was added at a concentration of 1 % and the suspension was prepared as for Silica Gel G.

Polyamide G. A suspension was prepared by mixing 4.5 g of polyamide (Woelm TLC) and 500 mg of 100-mesh precipitated calcium sulfate ($CaSO_4 \cdot 2H_2O$) with 45 ml of methanol for 1 min.

Polyamide GF. 50 mg of the fluorescent indicator was added and a suspension was prepared as for Polyamide G.

Silica Gel GF on flexible plastic sheets. Eastman Chromagram Sheets, Type K 301 R, obtained from Distillation Products, Inc., Rochester, N.Y., were activated by heating for 30 min in a forced-air oven at 100° and were stored in a desiccator over silica gel.

General conditions

Table I shows the adsorbent and the developing solvent systems used in this study.

For reproducibility of the R_F values, the amount of drug applied to the plate should be in the range of 0.5–1.5 μg. The origin should be at least 2 cm from the bottom edge of the plate. It was also necessary to have the chromatographic chamber completely saturated with the solvent. To accomplish this the walls were lined with filter paper which dipped into the solvent. After 15–30 min the chamber became saturated with solvent vapor.

For solvent systems in which a volatile acid or base was used, the plate was

TABLE I

SOLVENT AND ADSORBENT SYSTEMS FOR THIN-LAYER CHROMATOGRAPHY OF DRUGS USED IN MEDICATED FEEDS

System No.	*Mobile solvent*	*Proportions*	*Min/ 10 cm*	*Adsorbent*
1	*n*-Butanol–water–acetic acid*	80:20:2.5	90	Polyamide G or GF
2	Acetone–acetic acid	100:2	15	Alumina G or GF
3	Ethanol–ammonium hydroxide	80:20	80	Alumina G or GF
4	Diethyl ether–dimethyl sulfoxide–*n*-butanol–acetic acid	98:1:1:0.2	15	Silica Gel G or GF
5a	Hexane–acetone–*n*-butanol	21:2:2	25	Silica Gel GF (flexible plastic sheet)
5b	Hexane–acetone–*n*-butanol	21:2:2	25	Silica Gel GF
6	Diethyl ether–ethanol–acetic acid	96:3:1	25	Polyamide GF
7	Acetone–*n*-butanol–acetic acid	78:20:2	20	Polyamide G or GF
8	Diethyl ether–*n*-butanol–acetic acid	73:25:2	20	Polyamide G or GF
9	*n*-Butanol–water–acetic acid	80:20:5	90	Silica Gel G or GF
10	Ethyl acetate–acetic acid	75:25	60	Silica Gel G or GF
11	Pyridine–benzene	50:50	45	Silica Gel G or GF
12	*n*-Butanol–acetic acid	75:25	60	Silica Gel G or GF

* The mixture was shaken in a separatory funnel and the lower layer was discarded.

heated in a forced-air oven for 30 min to remove all traces of the acid or base before using the detection system.

*Detection systems**

D1. A 5 % w/v solution of potassium hydroxide in methanol was prepared 2 h before use. If the spots were not visible after spraying with this reagent, the plate was viewed under U.V. light.

D2. A saturated (*ca.* 0.2 %) solution of barium diphenylamine-4-sulfonate** in methanol was prepared by first dissolving the salt in a few ml of N,N-dimethylformamide and then diluting to volume with the methanol[15]. The plates were heated at 110° for 10 min after spraying, and then were viewed under U.V. light.

D3. The plates were subjected to U.V. light.

D4. A solution was prepared by dissolving 20 mg of 4-methylumbelliferone[15] in 35 ml of ethanol, and then diluting to 100 ml with water. After the plates were sprayed, spots were seen under U.V. light. Exposure of the plates to ammonia vapor may aid in visualization of the spots.

D5. A solution of 0.2 % β-naphthoquinone-4-sulfonic acid (sodium salt)[15] in 5 % sodium carbonate was prepared 10–18 min before use. The plates were viewed under U.V. light after spraying.

D6. A 1.0 % solution of *p*-dimethylaminobenzaldehyde was prepared in ethanol which contained 1 % hydrochloric acid.

D7. A saturated (*ca.* 22 %) solution of antimony trichloride[15] in dried chloroform was prepared. This spray must be freshly prepared. The plates were sprayed, then heated at 110° for 15 min. If the spots were not visible, the plates were viewed under U.V. light.

D8. A 2 % solution of vanillin[15] in isopropanol was prepared. The plates were heated at 110° for 10 min after spraying.

Results and discussion

Table II lists the R_F values for the drugs in their respective adsorbent and solvent systems. The detection systems for the various classes of compounds are shown in Table III. When the phenylarsonic acids were chromatographed on polyamide (Table II), it was necessary to prepare the adsorbent with 10 % gypsum binder to increase the separation between carbarsone and 4-nitrophenylarsonic acid. The methanolic potassium hydroxide (Table III) was found to be a suitable secondary chromogenic agent, but did not have the sensitivity ($\leqq$ 0.25 μg) of 4-methylumbelliferone (D4). *p*-Dimethylaminobenzaldehyde (D6) was also a suitable detection agent for arsanilic acid. 4-Nitrophenylarsonic acid and 3-nitro-4-hydroxyphenylarsonic acid can be detected by barium diphenylamine-4-sulfonate (D2). 3-Nitro-4-hydroxyphenylarsonic acid can also be detected by U.V. light.

On the thin-layer chromatograms of nitrofuraldehydes (Tables II and III), furazolidone fluoresced and nitrofurazone quenched the fluorescence of U.V. light before any detection system was applied. These compounds could also be detected by potassium hydroxide (D1). Furazolidone appeared as an orange-brown spot with this reagent. The chromatograms of the heterocyclic compounds (Table II and III) were

* U.V. light at 254 mμ in all cases.

** G. Frederick Smith Chemical Co., Columbus, Ohio.

TABLE II

R_F VALUES OF DRUGS COMMONLY USED IN MEDICATED FEEDS AT 1 μg SENSITIVITY

Compound	*Trivial name*	$R_F \times 100$	*Solvent system No.*
Phenylarsonic acids			
p-Aminobenzenearsonic acid	Arsanilic acid	69	1
		25	7
		35	8
		51	9
p-Ureidobenzenearsonic acid	Carbarsone	54	1
4-Nitrophenylarsonic acid		46	1
3-Nitro-4-hydroxyphenylarsonic acid		36	1
		20	10
		45	12
Nitrofuraldehydes			
3-(5-Nitrofurfurylideneamino)-2-oxazolidone	Furazolidone	84	2
		72	8
		33	9
		28	11
5-Nitro-2-furaldehyde acetylhydrazone	Nihydrazone	64	2
1-Ethyl-3-(5-nitro-2-thiazolyl)-urea	Nithiazide	50	2
5-Nitro-2-furaldehyde semicarbarzone	Nitrofurazone	43	2
		38	11
Heterocyclic compounds			
Thiodiphenylamine	Phenothiazine	81	3
2-Sulfanilamidoquinoxaline	Sulfaquinoxaline	68	3
Hexahydropyrazine	Piperazine	58	3
1-(4-Amino-2-*n*-propyl-5-pyrimidinylmethyl)-2-picolinium chloride hydrochloride	Amprolium	16	3
		35	6
		87	7
Nitrophenyl compounds			
m,m'-Dinitrophenyl disulfide	Nitrophenide	95	4
3,5-Dinitrobenzamide		73	4
		80	10
3,5-Dinitro-*o*-toluamide	Zoalene	73	4
4,4'-Dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine	Nicarbazin	25*	4
		50*	
Aryl esters			
Diacetate of 3,4-bis-(*p*-hydroxyphenyl)-2,4-hexadiene	Dienestrol diacetate	60	5a**
		57	5b**
		77	11
		73	12
Methyl 4-acetamido-2-ethoxybenzoate	Ethopabate	25	5a
		15	5b
		80	6
Miscellaneous			
Acetyl-(*p*-nitrophenyl)-sulfanilamide		70	10

* R_F 25 is of a freshly prepared nicarbazin standard; R_F 50 is of a nicarbazin standard approximately 2 weeks old.

** 5a is Silica Gel GF on flexible plastic sheet; 5b is Silica Gel GF.

subjected to short wave U.V. light (D3) for at least 10 minutes. Phenothiazine then appeared as a reddish-blue spot and sulfaquinoxaline as a dark spot which quenched fluorescence. Piperazine appeared as a yellow spot after copious spraying with vanillin (D8). When the plates were resprayed with potassium hydroxide (D1), amprolium appeared as a red spot. Sulfaquinoxaline could be detected by *p*-dimethylaminobenzaldehyde (D6) or by barium diphenylamine sulfonate (D2). Piperazine could be detected with β-naphthoquinone-4-sulfonic acid (D5).

TABLE III

DETECTION SYSTEMS FOR TLC OF DRUGS USED IN MEDICATED FEEDS

Class of compounds	*Detection system*
Phenylarsonic acids	D4 or D1
Nitrofuraldehydes	D2 followed by D1
Heterocyclics	D3 followed by D8 followed by D1
Nitrophenyl compounds	D2
Aryl esters	D3

Nicarbazin is a molecular addition compound which decomposes upon aging. The R_F values reported (Table II) represent a standard prepared daily and a standard about 2 weeks old. Potassium hydroxide (D1) was used to distinguish zoalene from 3,5-dinitrobenzamide; zoalene appeared as a yellow spot and the 3,5-dinitrobenzamide appeared as a pink spot. These compounds could not be separated by TLC because of their structural similarity. Nicarbazin could also be detected with this detection system (D1).

Table IV lists the systems which can be used for the detection of ingredients of the common mixtures. The 3-nitro-4-hydroxyphenylarsonic acid of mixture D appeared as a bright yellow spot after it was heated at 100° for 20 min. Dienestrol diacetate appeared as a dark spot after it was copiously sprayed with the antimony trichloride reagent (D7).

Mixture E is a multicomponent drug known commercially as "Unistat". 3-Nitro-4-hydroxyphenylarsonic acid was sometimes difficult to see after it was sprayed with detection agent D2. When the plate was resprayed with potassium hydroxide (D1), the arsonic acid was visible as a yellow spot.

TABLE IV

DETECTION SYSTEMS FOR TLC OF DRUG MIXTURES USED IN MEDICATED FEEDS

Common mixtures	*Detection system*
A Ethopabate; amprolium	D3*
B Amprolium; arsanilic acid	D1
C Furazolidone; arsanilic acid	D1
D Dienestrol diacetate; 3-nitro-4-hydroxyphenylarsonic acid	D3 then D7
E 3,5-Dinitrobenzamide; acetyl-(*p*-nitrophenyl)-sulfanilamide; 3-nitro-4-hydroxyphenylarsonic acid	D2
F Dienestrol diacetate; nitrofurazone; furazolidone	D3 then D7

* Adsorbent contains phosphor.

Studies of procedures for separating these drugs from the other ingredients of feeds are now in progress.

Food and Drug Administration,
599 Delaware Ave., Buffalo, N.Y. 14202 (U.S.A.)

JOSEPH J. ANTKOWIAK
ANTHONY L. SPATORICO*

1 *Feed Additive Compendium, Feedstuffs*, Miller Pub. Co., Minneapolis, Minn., published yearly with supplements.
2 *Manual of Microscopic Analysis of Feeding Stuffs*, American Association of Feed Microscopists, obtainable from G. M. BARNHART, Missouri Dept. of Agriculture, Jefferson City, Mo., U.S.A.
3 *Official Methods of Analysis*, 9th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1960.
4 *Official Methods of Analysis*, 10th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1965.
5 M. IKRAM, G. A. MIANA AND M. ISLAM, *J. Chromatog.*, 11 (1963) 263.
6 R. D. HARTLEY AND G. J. LAWSON, *J. Chromatog.*, 7 (1962) 69.
7 V. PREY, H. BERBALK AND M. KAUSZ, *Mikrochim. Acta*, (1962) 449.
8 A. BROSSI, M. BAUMANN, M. GERECKE AND E. KYBURZ, *Helv. Chim. Acta*, 43 (1960) 1444.
9 A. BROSSI, M. BAUMANN, M. GERECKE AND E. KYBURZ, *Helv. Chim. Acta*, 43 (1960) 2071.
10 B. J. R. NICOLAUS, C. CORNELLI AND A. BINAGHI, *Farmaco (Pavia), Ed. Prat.*, 16 (1961) 349.
11 R. FISCHER AND H. LAUTNER, *Arch. Pharm.*, 294 (1) (1961) 66.
12 T. T. ANDERSON, *J. Chromatog.*, 14 (1964) 127.
13 M. F. KOVACS, *J. Assoc. Offic. Agr. Chemists*, 47 (1964) 1097.
14 D. WALDI, in E. STAHL (Editor), *Thin Layer Chromatography*, Academic Press, New York, 1965, p. 262
15 D. WALDI, in E. STAHL (Editor), *Thin Layer Chromatography*, Academic Press, New York, 1965, pp. 485–502.

Received January 23rd, 1967

* Present address: Barber-Colman Co., Rockford, Illinois.

Thin-layer chromatographic identification of thirteen medicinally important carbamates

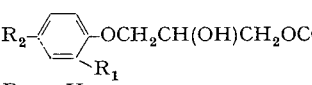
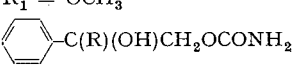
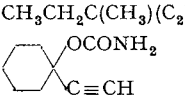
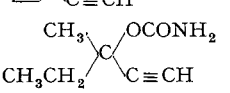
Certain carbamates are used in medicine as minor tranquillizers, skeletal muscle relaxants, anti-hypertensive agents and anti-neoplastic drugs. Most have some hypnotic effect. Since many are subject to misuse, specific tests for their identification are of value to enforcement agencies.

Identification by derivative formation is difficult if only a small amount of drug is available. Melting point determination is of little value since many of the drugs have low melting points and therefore require extensive purification in order to obtain decisive results. Although the use of paper chromatography has been reported for the separation of certain N-unsubstituted carbamates[1,2], the advantages of thin-layer chromatography have not been fully utilized. Thus carisoprodol has been separated from meprobamate and mebutamate[3] by thin-layer chromatography but the latter two carbamates were not resolved satisfactorily. Using two solvent systems, LINDFORS[4] separated ethinamate, hexapropymate and meprobamate, while HEYNDRICKX *et al.*[5] resolved hexapropymate, urethane, and meprobamate in one system. MOSS AND JACKSON[1] showed that a furfural–hydrochloric acid spray was a sensitive detecting agent for the N-unsubstituted carbamates on paper chromatograms. The use of a modified furfural spray reagent has been reported[5].

This report will show that thirteen carbamates (Table I) may be distinguished from each other by the use of three chromatographic systems and three spray reagents.

TABLE I

NOMENCLATURE AND STRUCTURAL FORMULAE OF CARBAMATES EXAMINED

Compound	*Structural formulae*
	
Chlorphenesin carbamate	$R_1 = H$ $R_2 = Cl$
Mephenesin carbamate	$R_1 = CH_3$ $R_2 = H$
Methocarbamol	$R_1 = OCH_3$ $R_2 = H$
	
Hydroxyphenamate	$R = C_2H_5$
Styramate	$R = H$
	$R_1CH_2C(R_2)(CH_3)CH_2OCONH_2$
Carisoprodol	$R_1 = OCONHC_3H_7$(iso) $R_2 = C_3H_7(n)$
Mebutamate	$R_1 = OCONH_2$ $R_2 = C_4H_9(sec)$
Meprobamate	$R_1 = OCONH_2$ $R_2 = C_3H_7(n)$
Tybamate	$R_1 = OCONHC_4H_9(n)$ $R_2 = C_3H_7(n)$
Emylcamate	$CH_3CH_2C(CH_3)(C_2H_5)OCONH_2$
Ethinamate	
Methylpentynol carbamate	
Urethane	$CH_3CH_2OCONH_2$

Materials and methods

The carbamates were obtained from pharmaceutical manufacturers or commercial suppliers. In some instances they were isolated from solid dosage forms and recrystallized. Alcohol or acetone solutions were used for chromatography. For routine analysis, tablets were finely ground in a mortar and the powder shaken with 95 % ethanol. The volume of alcohol used was that which would give an estimated concentration of 10 mg of drug per ml of alcohol. Capsule powders and liquids (*e.g.* tybamate) were treated similarly.

Glass plates (200 × 200 mm) were coated with 0.25 mm layers of absorbent, by means of the Shandon Unoplan apparatus. Two types of plates were prepared, one from Silica Gel G (Merck, Darmstadt: 30 g in 60 ml of water) and the other type from Kieselguhr G (Merck, Darmstadt: 30 g in 55 ml of water). For visualization by ultraviolet absorbance, Fluorescent Indicator Green (Woelm, Eschwege) was added to give 2 % by weight based on the dry adsorbent.

The plates were air dried for 8 h and stored in air. If urgently required the plates may be air dried for one half-hour, then heated for one half-hour at 110° without affecting the results.

The chromatograms were developed in glass tanks (21 × 20.5 × 8 cm), lined with filter paper. Fifty ml of the solvent was used to wet the filter paper, the excess poured off, and 100 ml of solvent added.

Chromatography systems

System A

Plates: Silica Gel G.

Solvent: The lower layer obtained by shaking together acetic acid–carbon tetrachloride–chloroform–water (100:60:90:50).

System B

Plates: Kieselguhr G, impregnated with formamide. The plates were dipped in a solution of 5 % formamide in methanol, and allowed to dry in air for 12 min before application of the samples.

Solvent: benzene–chloroform (30:120) saturated with formamide.

System C

Plates: Kieselguhr G impregnated as in System B.

Solvent: carbon tetrachloride saturated with formamide.

The spots on formamide impregnated plates diffuse on standing. Such plates must be developed immediately after the sample is applied, and sprayed or observed under ultraviolet light immediately after development.

To obtain accurate R_F values a small mark was made beside the spot, opposite its centre as soon as the spot was made visible by spraying or ultraviolet absorption. A short wavelength (254 mμ) ultraviolet source was used to observe absorption.

Spray reagents

(1) *Furfural–hydrochloric acid.* Furfural, if discoloured, was distilled at atmospheric pressure in a stream of nitrogen This reagent kept well if stored in a dark bottle in a refrigerator, and flooded with nitrogen whenever a portion was removed.

The plate was sprayed well, but not soaked with furfural, then immediately sprayed with concentrated hydrochloric acid.

(2) *Furfural–sulphuric acid.* This was applied as for (1) except that concentrated sulfuric acid was used, instead of hydrochloric acid.

(3) *Vanillin–sulphuric acid*[6]. Five grams of vanillin were dissolved in 100 ml of concentrated sulphuric acid. This reagent could be stored for several weeks. The plate was sprayed well, but not soaked with the reagent. Yellow spots appeared and the plate was heated in an oven at 110° until a standard spot of meprobamate, applied at the same time as the other spots, turned blue. The plate was removed from the oven, the colour of the spots noted immediately, and again 15 min after removal from the oven.

Results and discussion

The R_F values observed are shown in Table II, and the colour reactions with the various sprays in Table III. Except for the pairs carisoprodol–tybamate and methylpentynol carbamate–ethinamate, the compounds are satisfactorily resolved by means of systems A or B, with R_F differences greater than 0.05. However carisoprodol–tybamate and methylpentynol carbamate–ethinamate are well resolved by means of system C.

The characteristic colours of many of the carbamates with the vanillin–sulphuric acid spray permit distinctions between many compounds with similar R_F values. Methocarbamol and chlorphenesin carbamate are just resolved with system A and the distinctive colour reaction of methocarbamol with the vanillin reagent is ample confirmation of its difference from chlorphenesin carbamate. Where a mixture is present the upper and lower halves of the spot are usually sufficiently different in colour to show that it is not homogeneous. This is also useful for confirmation of identity, since it is only necessary to apply the unknown substance and the known substance to the same place on the starting line, and check the homogeneity of the colour developed with the vanillin reagent.

The furfural–hydrochloric acid spray is the most sensitive detection reagent for all the carbamates examined producing blue-black spots with as little as 2 to 5 μg. Emylcamate is an exception and requires approximately 12.5 μg for visualization with this spray. However, when treated with the furfural spray followed by sulphuric acid, emylcamate gives a magenta spot with as little as 1 μg of material. Recently a modified furfural spray reagent has been proposed[5]. The reagent takes more time to prepare than the simple furfural–hydrochloric acid spray, and the spots develop more slowly, but the background is better with the modified spray reagent, with a resulting increase of about four times the sensitivity of the furfural–hydrochloric acid spray.

MOSS AND JACKSON[1] reporting on the specificity of the furfural–hydrochloric acid reagent, observed that of a large number of drugs examined, only phenazone and urea gave blue-black colourations. The reaction with phenazone was reported to be very slow, but urea is well distinguished by its low R_F value in our systems. In the present investigation salicylamide in addition to phenazone was also found to give a blue-black colour with the furfural–hydrochloric acid and the HEYNDRICKX modification. It is included in Tables II and III, and is distinguished from the carbamates by its R_F value in system A and the blue-white fluorescence appearing in the centre of an absorbing spot when the plate is irradiated with ultraviolet light of 254 mμ wavelength. Of the carbamates listed in Table I only those containing a phenyl group absorb at 254 mμ.

TABLE II

R_F VALUES OF MEDICINAL CARBAMATES, UREA AND SALICYLAMIDE

Compound	*Chromatographic system*				
	A	*A*	*B*	*B*	*C*
Urea	0.13	0.12	0.00	0.00	0.03
Methocarbamol	0.22	0.22	0.31	0.33	0.00
Chlorphenesin carbamate	0.27	0.26	0.31	0.29	0.00
Styramate	0.29	0.28	0.19	0.21	0.00
Mephenesin carbamate	0.34	0.32	0.44	0.43	0.00
Meprobamate	0.37	0.37	0.35	0.34	0.00
Mebutamate	0.39	0.38	0.41	0.40	0.00
Hydroxyphenamate	0.42	0.41	0.50	0.50	0.04
Salicylamide	0.51	0.54	0.40	0.41	0.10
Urethane	0.60	0.61	0.57	0.61	0.11
Carisoprodol	0.64	0.65	0.79	0.81	0.25
Tybamate	0.65	0.70	0.78	0.84	0.37
Methylpentynol carbamate	0.70	0.72	0.70	0.71	0.25
Ethinamate	0.72	0.75	0.74	0.75	0.37
Emylcamate	0.80	0.82	0.77	0.81	0.66
Solvent front	10 cm	15 cm	10 cm	15 cm	10 cm
Running time	26 min	78 min	12 min	21 min	16 min
Drying time	—	—	12 min	15 min	12 min

TABLE III

VISUALIZATION OF CHROMATOGRAPHIC SPOTS OF MEDICINAL CARBAMATES, SALICYLAMIDE AND UREA

Compound	*Ultraviolet light,*	*Spray reagents*[a]				
	254 mμ[b]	*1*[c]	*2*[d]	*3*[e]	*3*[f]	*3*[g]
Urea	—	+	+	R	NC	NC
Methocarbamol	+	+	+	Y	Pi	R-P
Chlorphenesin carbamate	+	+	+	Y	NC	NC
Styramate	+	+	+	Y	Gr-Br	Gr-Br
Mephenesin carbamate	+	+	O	Y	P	Pi-Br
Meprobamate	—	+	+	Y	B	B
Mebutamate	—	+	+	Y	B	B-Gr
Hydroxyphenamate	+	+	+	Y	Gr-P	P-Gr
Salicylamide	+ Fl	+	—	—	F-Br	F-Br
Urethane	—	+	+	Y	F-Y	F-Y
Carisoprodol	—	+	+	Y	P	B
Tybamate	—	+	+	Y	B	D-B
Methylpentynol carbamate	—	+	O	P	P	B-Gr
Ethinamate	—	+	O	Y	B	Br-Gr
Emylcamate	—	+	P-R	P	P	P

[a] Designation of colours: B = blue; Br = brown; Gr = grey; O = orange; P = purple; Pi = pink; R = red; Y = yellow; D = dark; F = faint; NC = no colour.

[b] + Indicates absorption, + Fl, absorption, blue fluorescence at centre of spot.

[c] + Indicates blue-black spots.

[d] + Indicates blue-black spots, except as noted. The background darkens rapidly and blue-black spots merge into it.

[e] Colour immediately after spraying.

[f] Colour on heating at 110°. (See text.)

[g] Colour on standing in air for 15 min, after heating.

Bethanecol and carbachol, carbamates which lack N-substituents, react with the furfural–hydrochloric acid spray, but they have zero R_F values in all the systems included in this study. The compounds may be separated in a system reported by TAYLOR[7].

Acknowledgement

The author wishes to thank Mr. JEAN-DENIS LANTHIER for technical assistance.

Research Laboratories, Food and Drug Directorate, Tunney's Pasture, Ottawa, Ontario (Canada) T. W. MCCONNELL DAVIS

1 M. S. MOSS AND J. V. JACKSON, *J. Pharm. Pharmacol.*, 13 (1961) 361.
2 J. F. DOUGLAS AND A. SCHLOSSER, *J. Chromatog.*, 6 (1961) 540.
3 I. SUNSHINE, *Am. J. Clin. Pathol.*, 40 (1963) 576.
4 R. LINDFORS, *Ann. Med. Exptl. Biol. Fenniae (Helsinki)*, 41 (1963) 355.
5 A. HEYNDRICKX, M. SCHAUVLIEGE AND A. BLOMME, *J. Pharm. Belg.*, 20 (1965) 117.
6 M. C. NIGAM, I. C. NIGAM AND L. LEVI, *J. Soc. Cosmetic Chemists*, 16 (1965) 155.
7 E. H. TAYLOR, *Lloydia*, 27 (1964) 96.

Received December 15th, 1966

J. Chromatog., 29 (1967) 283–287

Zur Standardisierung der Dünnschichtchromatographie

II. Die R_F-Werte eines spezifischen Nachweises für Phenacetin und chemisch verwandte Verbindungen

In einer vorausgegangenen Untersuchung[1] wurden für die Dünnschichtchromatographie (DC) in tubes folgende Vorteile gegenüber den üblichen Methoden festgestellt: (1) durch die exakte Einstellbarkeit des Wassergehaltes der Kieselgelschicht sind die R_F-Werte besser als mit bisher üblichen Methoden reproduzierbar; (2) die beschichteten inaktiven tubes ermöglichen, bei verschiedenen Temperaturen aktiviert, die Auswahl der jeweils günstigen Trennungsbedingungen; (3) die standardisierten Bedingungen erlauben den direkten Vergleich der Ergebnisse mit denen anderer Untersuchungen.

Diese Untersuchungen wurden fortgesetzt. Dabei wurde eine bisher anscheinend unbekannte Reaktion von Phenacetin gefunden. Die bisher übliche Nachweismethode mit Salpetersäure[2] ist ziemlich unspezifisch, da ganze Stoffgruppen gelbe Nitrierungsprodukte ergeben können. Auch der Nachweis mittels des Eisen(III)chlorid–Kaliumhexacyanoferrat(III)-Reagenzes[3] kann nicht als spezifisch angesehen werden. Die Spezifität der Phenacetin-Nachweismethode wurde an 18 Substanzen geprüft, gleichzeitig wurde die Reproduzierbarkeit der R_F-Werte der Tube-DC mit der Plattenmethode bei Anwendung von zwei Kammern (KS und S) verglichen.

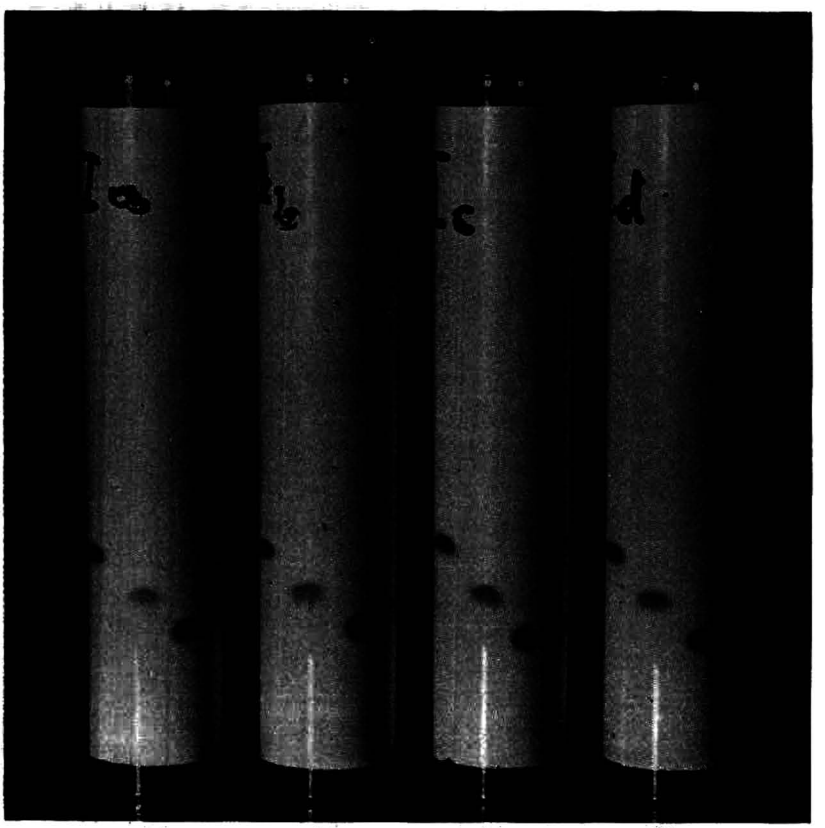
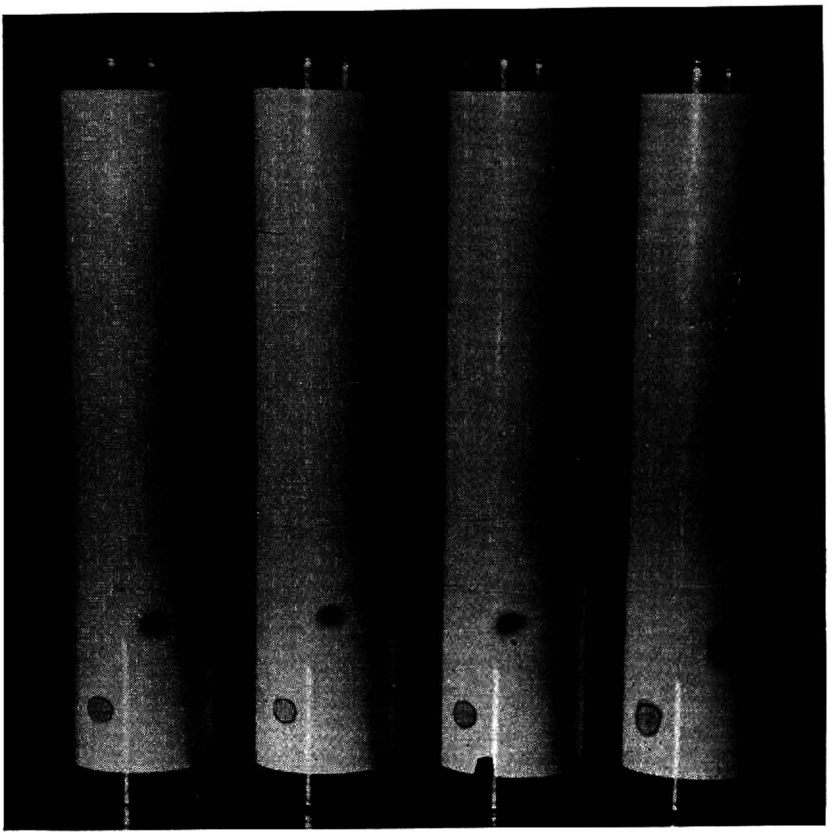


Fig. 1. Zur Demonstration der tube-DC. Von links nach rechts: *o,m,p*-Anisidin. Bedingungen: Kieselgel G bei 30° aktiviert. Laufmittel: gereinigtes Chloroform Merck p.A., Nachweis mit Bromdämpfen, nach Verdampfung 2 Min. auf 120° erhitzt.

Methode

Die Tube-DC[1] (DESAGA) beruht auf der Verwendung von innen mit Silicagel beschichteten Glasröhrchen (tubes), die gleichzeitig als Trennkammer dienen und an beiden Enden mit Gummikappen verschlossen werden können. Dadurch ist eine Standardisierung sowohl der Schichtaktivität als auch der Kammersättigung gewährleistet. Die tubes wurden auf Vorrat gehalten und vor Gebrauch in Aluminium-Wärmespeichern* im Trockenschrank 4–5 Std. auf 30° belassen. Sofort nach der Herausnahme wurden sie verschlossen, dann auf je vier die in Tabelle I angegebenen Substanzen in zwei Gruppen mit einer 2 mm^3 fassenden Mikrokapillare* aufgetragen. Unmittelbar danach wurde das tube wieder verschlossen. Als Laufmittel wurde Chloroform Merck p.A. verwendet. Dieses wurde vorher im Verhältnis 2:1 mit Aqua dest. ausgeschüttelt und über Silicagel H Merck 5 % getrocknet. Die tubes wurden, im Kaltluftstrom getrocknet, 2 Min. in einem speziellen Bedampfungsgerät* Bromdämpfen ausgesetzt. Der überschüssige Bromdampf wurde ausgetrieben, die tubes anschliessend 2 Min. auf einem Warmluftgerät auf 110° erwärmt.

* DC-Tubes sowie Zubehör sind erhältlich bei DESAGA, Heidelberg.



(a) (b) (c) (d)

Fig. 2. Von links nach rechts: *p*-Aminophenol, *p*-Phenetidin, Phenacetin. Bedingungen wie Fig. 1.

Die mit dieser Methode zu erreichenden Ergebnisse sind aus Tabelle I sowie Fig. 1 und 2 zu ersehen. Zur Platten-DC wurden die mit Silicagel G beschichteten Platten bei 30° im Trockenschrank aktiviert und je fünf in einer Kammer (KS und S) entwickelt. Die Lösungen enthielten jeweils 2 mg Substanz pro cm^3. Die Ergebnisse sind aus Tabelle II und Fig. 3 und 4 zu ersehen.

Der Vergleich der Ergebnisse zwischen der Tubemethode und den unter gleichartigen Bedingungen ausgeführten Plattenmethode ergibt eine bedeutend breitere Differenzweite der R_F-Werte in den tubes. Die bessere Trenn- und Unterscheidbarkeit der untersuchten Substanzen in den tubes ist eindeutig. Dabei sind Schwankungen der entsprechenden R_F-Werte festzustellen. Während bei der Platten-Methode sechs Substanzen nicht oder kaum wandern, sind es in den tubes nur zwei. Die R_F-Werte von *p*-Anisidin und *o*-Acetanisidin sind auf der Platte nicht zu unterscheiden. Auch die Form der peaks ist auf den Platten ungünstiger als in den tubes. Acetanilid, Acettoluidid, Anisol und Phenetol sind auf den Platten, im Gegensatz zu den tubes, überhaupt nicht sichtbar. Wie aus Tabelle I ersichtlich, gibt Phenacetin nach der oben angeführten Methode eine violette Farbreaktion in der Hitze. Ein Farbumschlag von violett nach grau-braun erfolgt erst nach einigen Stunden. Die Reaktion tritt nicht auf

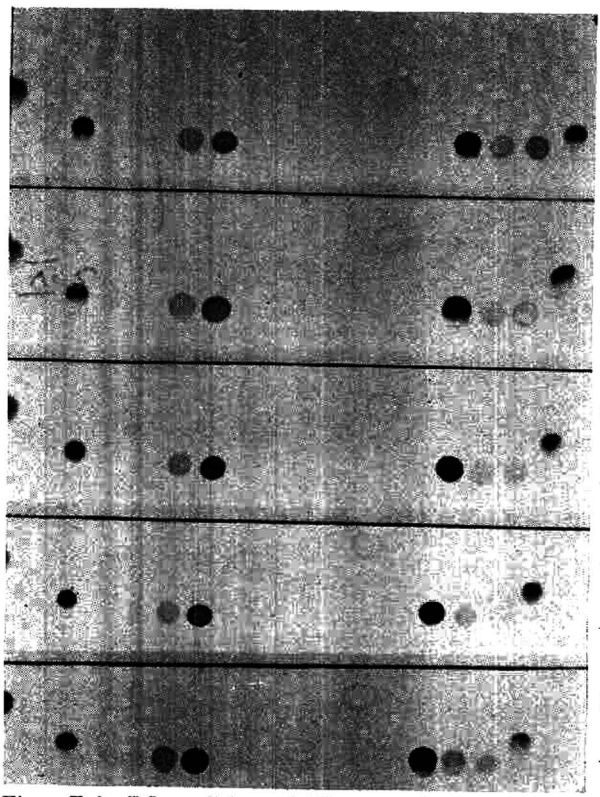


Fig. 3. Tube-DC verglichen mit Plattenmethode unter Kammersättigung. Bedingungen wie Fig. 1

TABELLE I

DÜNNSCHICHTCHROMATOGRAPHIE IN TUBES: PHENACETIN-NACHWEIS

Erläuterungen zu den Farben: br = braun; dbr = dunkelbraun; hbr = hellbraun; rbr = rotbraun; vbr = violett-braun; g = grau; bl = blau; bls = blau-schwarz; blv = blau-violett; or = orange; v = violett.

			Bromiert		R_F-*Werte*			
			Kalt	*Heiss*	*a*	*b*	*c*	*d*
I	1	*o*-Anisidin	rbr	dbr	28	28	29	28
	2	*m*-Anisidin	vbr	br	20	21	21	20
	3	*p*-Anisidin	bl	blv	15	15	14	14
	4	*o*-Acetanisidin	g	hbr	18	18	17	17
	5	*p*-Acetanisidin	g	v	3	4	4	4
	6	*o*-Acetaminophenol	g	br	4	4	4	4
	7	*p*-Essigsäureester	g	v	0	0	0	0
	8	Acetanilid	(g)	(g)	9	9	8	8
	9	Acettoluidid	(g)	(g)	11	11	12	12
II	10	Anisol	or	or	78	79	—	—
	11	Phenetol	or	or	80	82	—	—
	12	Anilin	dbr	rbr	28	29	27	28
	13	Phenol	g	g	20	21	21	21
	14	*o*-Aminophenol	g	dbr	3	4	3	4
	15	*m*-Aminophenol	g	g	2	3	3	2
	16	*p*-Aminophenol	g	v	0	0	0	0
	17	*p*-Phenetidin	bl	bls	19	18	15	17
	18	Phenacetin	g	v	5	5	3	4

TABELLE II

DC MIT ÜBLICHER KAMMER

Bedingungen wie in Tabelle I.

	R_F-Werte				
	a	*b*	*c*	*d*	*e*
(*A*) *Kammersättigung: Eckige Trennkammer*					
1	25	24	21	23	20
2	14	13	10	11	10
3	9	8	6	7	6
4	8	8	6	5	4
5	2	2	0	0	1
6	3	3	1	2	2
7	2	2	0	0	1
8	—	—	—	—	—
9	—	—	—	—	—
10	—	—	—	—	—
11	—	—	—	—	—
12	27	26	26	24	23
13	23	21	22	20	18
14	2	3	2	3	3
15	1	2	1	2	2
16	1	2	1	2	1
17	8	13	12	10	7
18	4	12	13	9	2
(*B*) *Sandwich-Trennkammer*					
1	19	18	19	17	16
2	10	10	9	8	8
3	6	5	6	4	4
4	6	5	5	5	4
5	0	0	1	0	0
6	1	1	2	1	1
7	1	0	1	0	1
8	—	—	—	—	—
9	—	—	—	—	—
10	—	—	—	—	—
11	—	—	—	—	—
12	22	23	21	23	17
13	19	18	17	17	16
14	2	2	2	2	2
15	1	1	1	1	1
16	1	1	1	1	1
17	9	10	12	13	6
18	1	1	2	2	1

Papier oder im Reagenzglas auf. Wieweit die Reaktion durch Silicagel katalysiert zu werden scheint, müsste geprüft werden. *p*-Anisidin und *p*-Phenetidin ergeben eine Blaufärbung schon in der Kälte. Nur in der Hitze gibt ausser dem Phenacetin noch *p*-Acetanisidin, *p*-Aminophenol und *p*-Acetamino-phenolessigester die violette

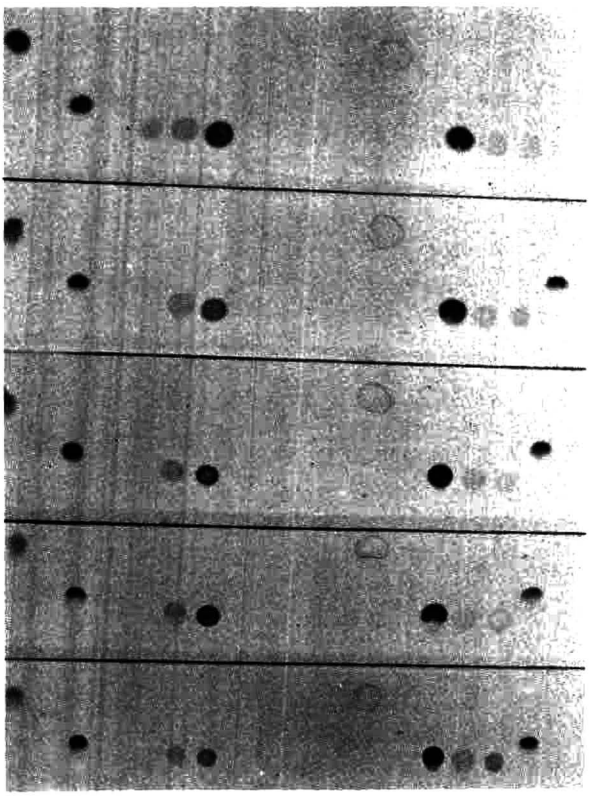


Fig. 4. Tube-DC verglichen mit Plattenmethode: Sandwich-Kammer. Bedingungen wie Fig. 1.

Farbreaktion. Die beiden letztgenannten Substanzen bleiben jedoch am Startpunkt und lassen sich dadurch von den beiden anderen Verbindungen unterscheiden. Keine Reaktion ergeben Acetanilid und Acettoluidid. Nach den bisherigen Beobachtungen scheint die Reaktion mit der Konfiguration des *p*-Aminophenol zusammenzuhängen.

*Abteilung Verkehrsmedizin,**
Institut für gerichtliche Medizin
der Universität Heidelberg (Deutschland)

P. POGACAR
B. KIENLE
P. KRAPP
KL. LÜHRSEN

1 P. POGACAR UND H. KLEIN, *Deut. Z. Ges. Gerichtl. Med.*, 60 (1967) im Druck.
2 H. LIEBICH, *Deut. Apotheker Ztg.*, 99 (1959) 1246.
3 R. BRAVO O. UND F. HERNÁNDEZ A., *J. Chromatog.*, 7 (1962) 60.

Eingegangen den 25. Januar 1967

* Leiter: Prof. Dr. H. KLEIN.

Thin-layer systems giving maximum separation of α- and β-tocopherols

No thin-layer adsorption technique has been described which will resolve α- and β-tocopherol as effectively as zinc carbonate impregnated paper[1]. SEHER[2] has reported useful separations of tocopherols on thin layers of alumina with benzene as developing solvent and on silica gel with chloroform as solvent. DILLEY AND CRANE[3] separated α- and β-tocopherol on Silica Gel G using benzene and BOLLIGER[4] reported better separation on Silica Gel G with benzene–methanol (98:2) than with cyclohexane–diethyl ether (80:20). Secondary magnesium phosphate, which gives a useful separation of α- and β-tocopherols by column chromatography[5], proved slightly inferior to silica gel as a thin layer[4].

SCHMANDKE[6], however, reported an excellent separation of α- from γ-tocopherol (which should migrate close to β-tocopherol) on a mixed layer of alumina–zinc carbonate (3:1) with chloroform as developing solvent. A mixed layer of silica gel–zinc carbonate (1:1) with benzene–chloroform (1:1) as developing solvent gave poor resolution.

A search for thin-layer chromatographic systems giving maximum separations of α- and β-tocopherol has been conducted in this laboratory and the results are shown in Table I.

Aluminium oxide–zinc carbonate layers were prepared by mixing 30 g Aluminium Oxide G (Merck) and 10 g zinc carbonate (Basic, British Drug Houses Ltd.) with 60 ml water (or 60 ml 0.001 % dichlorofluorescein in water) in a blender for 15–30 sec and allowing the bubbles to break before spreading. Kieselgur–zinc carbonate layers were prepared by blending 16 g Kieselgur G (Merck) and 8 g zinc carbonate (2:1) or 12 g Kieselgur G and 12 g zinc carbonate (1:1) with 50 ml water (or 0.001 % dichlorofluorescein) for 15–30 sec and spreading as before. Silica Gel H (Merck) and zinc carbonate were mixed in the ratio of 10:1, slurried and layers prepared. Zinc sulphate was incorporated into Silica Gel G and H layers by slurrying the absorbent with 5 % zinc sulphate solution in place of water. In one instance zinc carbonate was incorporated into a Kieselgur G layer by slurrying 10 g Kieselgur G with 10 ml water + 10 ml zinc ammonium carbonate solution[1] which was also used to impregnate papers. Florisil layers were prepared by slurrying 15 g Florisil (Floridin Co,, W. Virginia thin-layer grade as supplied by Fisher Scientific Co., New Jersey) with 45 ml water (or 0.001 % dichlorofluorescein).

All layers were 250 μ thick and were activated at 120° for 30 min immediately before use. Spots were detected by viewing under U.V. light or by staining with iodine vapour.

Best separations were achieved using mixed layers of Aluminium Oxide G–zinc carbonate (3:1) and Kieselgur G–zinc carbonate (2:1) using the solvents chloroform and benzene–cyclohexane (30:70), respectively (Table I). The efficiency of these systems in resolving α- and β-tocopherol came close to that of zinc carbonate treated paper. Occasionally when using the alumina–zinc carbonate layer with chloroform as developing solvent, the resolution was greater than that ever obtained with freshly prepared zinc carbonate papers. Incorporation of zinc carbonate or zinc sulphate into layers of Silica Gel G and H did not improve the resolution over that obtained with the respective layers alone. Increasing the ratio of zinc carbonate to either aluminium oxide or Kieselgur to 1:1 (w/w) did not increase the resolution but did allow greater

TABLE I

APPROXIMATE $R_F \times 100$ VALUES OF α- AND β-TOCOPHEROLS ON VARIOUS ADSORBENTS

Absorbent	*Solvent*	$R_F \times 100$		*Difference*	*Reference*
		α-Tocopherol	*β-Tocopherol*		
Silica Gel G	Chloroform	58	35	23[b]	2
	Benzene	50	32	18[b]	3, 7
	Benzene–methanol (98:2)	65	61	14[b]	4
	Cyclohexane–ether (80:20)	32	30	2[b]	4
Silica Gel G–zinc carbonate (1:1)	Chloroform–Benzene (50:50)	83	80 (as γ)	3[b]	5
Aluminium oxide	Benzene	56	34	22[b]	2
Aluminium Oxide G–zinc carbonate (3:1)	Chloroform	72	42 (as γ)	30[b]	5
	Chloroform	92	54	38	
	Benzene	48	18	30	
Sec. magnesium phosphate	Petroleum ether–ether (85:15)	86	76	10[b]	4
Florisil	Benzene	56	41	15	
Silica Gel G–zinc sulphate[a]	Benzene	50	32	18	
Silica Gel H	Benzene	46	30	16	
Silica Gel–zinc sulphate[a]	Benzene	46	31	15	
Silica Gel–zinc carbonate (10:1)	Benzene	44	29	15	
Kieselgur G	Cyclohexane	54	37	17	
Kieselgur–zinc ammonium carbonate[a]	Cyclohexane	55	34	21	
Kieselgur–zinc carbonate (1:1)	Cyclohexane–benzene (70:30)	47	17	30	
	Cyclohexane–benzene (50:50)	60	27	33	
	Benzene	81	49	32	
Kieselgur–zinc carbonate (2:1)	Cyclohexane–benzene (70:30)	70	33	37	
Zinc carbonate impregnated paper	Cyclohexane	73	31	42	

[a] See text.
[b] From published data.

loading of the Kieselgur G–zinc carbonate layer. Florisil and Kieselgur G layers produced elongated spots and therefore poor resolution. Both the Aluminium Oxide G–zinc carbonate and Kieselgur G–zinc carbonate layers have been used in this laboratory to assess the purity of the α-tocopherol separated from a leaf extract by TLC. The α-tocopherol isolated after a single run on Silica Gel G with benzene as the developing solvent[3,7] was rechromatographed on one of the above layers. The homogeneity of the preparation was judged by the number and size of the spots observed in this second run. Possible interference with the assay could also be checked by comparing the values obtained before and after the run on the mixed absorbent.

The Aluminium Oxide G–zinc carbonate (with dichlorofluorescein) layer has been used in two dimensions in a manner analogous to that usually employed with paper chromatography in the estimation of tocopherols in animal and plant tissues[8,9]. Acetone–petroleum ether extracts of 0.3 g of white clover and Xanthium leaf tissue[7] were applied as a 4 cm band near one corner of a 20 cm^2 layer and development in one direction carried out with benzene. After allowing the layer to dry for 2–3 min the plate was dipped into solution of 2.5 % of liquid paraffin (B.P. grade) in petroleum ether so that the level of the paraffin solution almost reached the material separated in the first run. Following evaporation of the petroleum ether, the plate was developed in the second direction using methanol as developing solvent. When viewed under U.V. light the most prominent spot, R_F 0.50 in the first direction and 0.30 (measured from limit of paraffin impregnation) in the reverse phase direction, was α-tocopherol. This was readily recoverable for analysis.

α-Tocopherol was purified by preparative TLC from DL-α-tocopherol (C-Grade, Calbiochem, Los Angeles) and β-tocopherol was a generous gift from Dr. J. GREEN, Vitamins Ltd., Tadworth, England.

Plant Physiology Division, D.S.I.R., P. G. ROUGHAN
Palmerston North (New Zealand)

1 J. GREEN, S. MARCINKIEWICZ AND P. R. WATT, *J. Sci. Food Agr.*, 6 (1955) 274.
2 A. SEHER, *Mikrochim. Acta*, (1961) 308.
3 R. A. DILLEY AND F. L. CRANE, *Anal. Biochem.*, 5 (1963) 531.
4 H. R. BOLLIGER, in E. STAHL (Editor), *Thin-Layer Chromatography*, Academic Press, New York, 1965, pp. 229–231.
5 F. BRO-RASMUSSEN AND W. HJARDE, *Acta Chem. Scand.*, 11 (1957) 34.
6 H. SCHMANDKE, *J. Chromatog.*, 14 (1964) 123.
7 P. G. ROUGHAN, *Anal. Biochem.*, 19, April issue (1967).
8 E. E. EDWIN, A. T. DIPLOCK, J. BUNYAN AND J. GREEN, *Biochem. J.*, 75 (1960) 450.
9 V. H. BOOTH, *Analyst*, 88 (1963) 627.

Received February 20th, 1967

An efficient and convenient drier for thin-layer chromatograms

The increasing use of thin-layer chromatography has created the need for a means of rapidly drying large numbers of thin-layer plates. Several stages occur when drying is necessary, *e.g.* during layer preparation, following chromatography, following spraying of chromogenic reagents etc. Ideally apparatus used for such drying should fulfil the following conditions:

(1) It should be possible to control the drying temperature, as many compounds of interest are unstable.

(2) Drying should be as rapid as possible even when carried out at room temperatures.

(3) The drying process must not disturb fragile layers.

(4) Drying must be such that migration of compounds does not occur. Elaborate procedures, such as freeze drying of chromatograms, have been recommended[1] to prevent such movement.

The following apparatus which can be readily and cheaply constructed in many laboratory workshops has been found to satisfy all these requirements.

Description of apparatus

The complete apparatus with drying rack in position is shown in Fig. 1 and a cutaway diagram and working drawing are given in Fig. 2. The case is fabricated from folded, spot welded, stainless steel. Runners and stops for the drying racks are bolted in position. All steel is 20 gauge (0.035 in.) except for the door, which is 18 gauge (0.05 in.). The door aperture (9 × 11 in.) is reinforced with a spot welded strip.

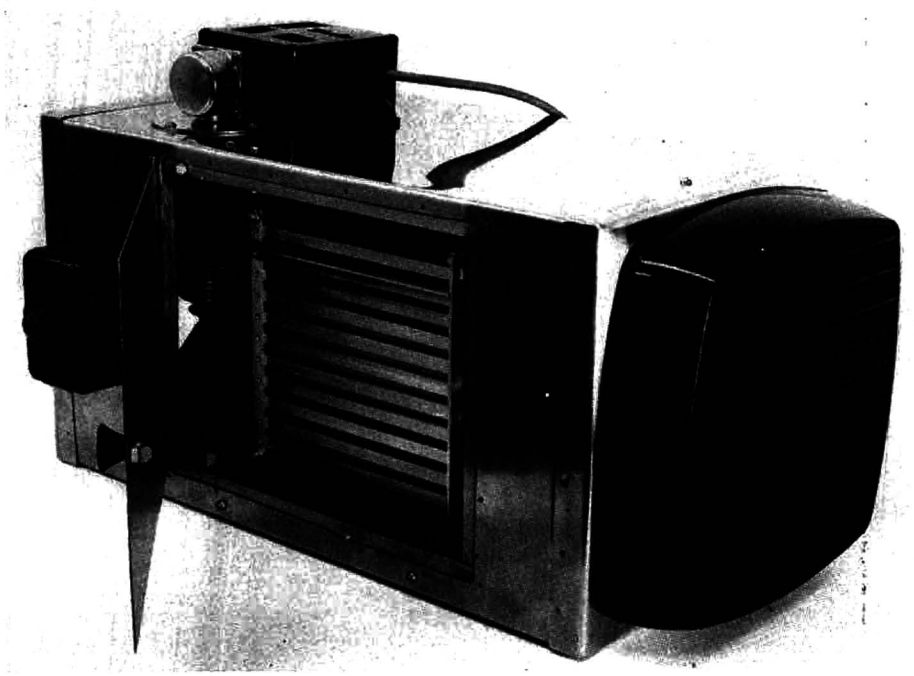


Fig. 1. Complete chromatogram drier with rack in position.

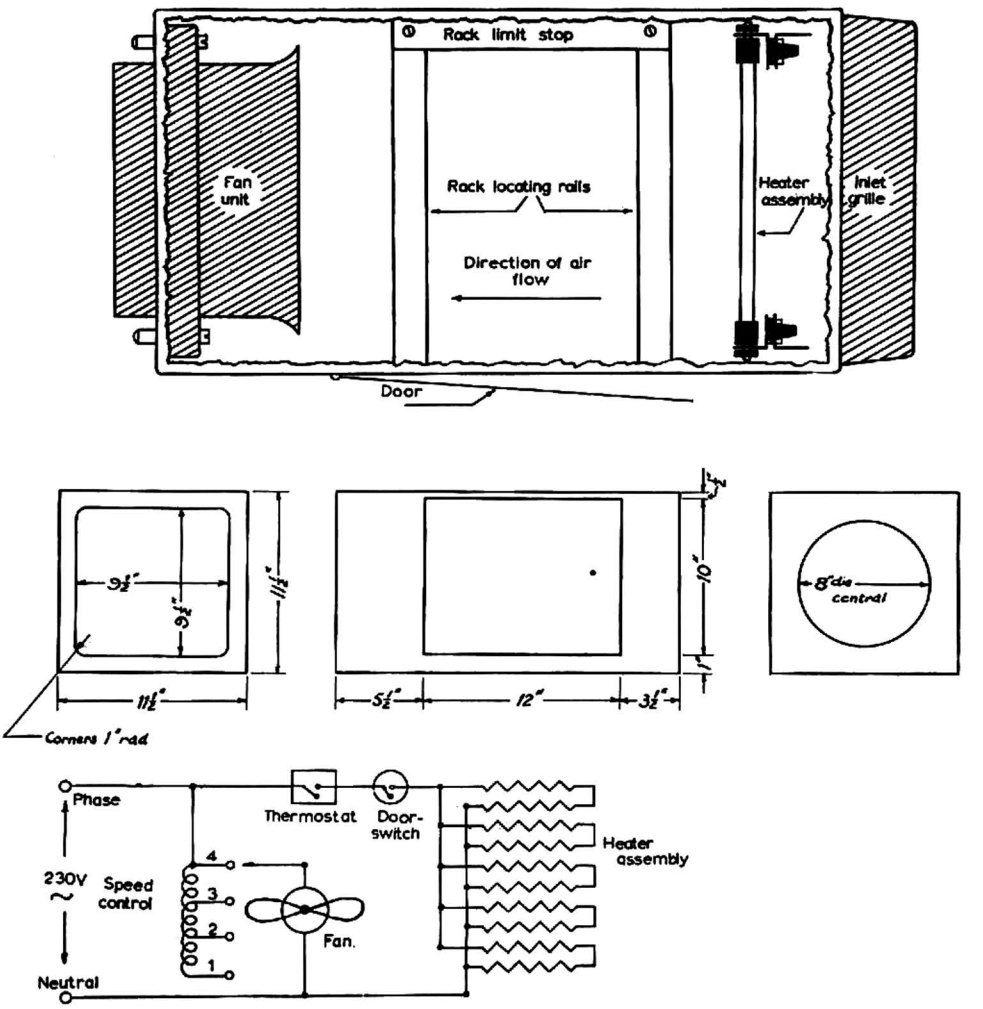


Fig. 2. Construction details of chromatogram drier.

The fan unit used is a domestic 7½ in. window fan with brushless motor and speed control. It is also desirable for the fan to be corrosion-resistant. We used a General Electric Corporation "Xpelair" fan with a capacity of 250 cu.ft./min. The normal louvre outlet of the fan is mounted at the inlet end of the cabinet to shield the heaters and assist in producing laminar air flow in the body of the drier. The fan itself is mounted at the other end so that it draws air through the cabinet.

The heating unit is built from ten silica-enclosed elements (Fig. 3), each 9 in. long and of 150 W rating. These are wired with five pairs in parallel so that for a 230 V supply each element has 115 V (Fig. 2) (for a 115 V supply all 10 elements should be in parallel). A micro-switch is mounted on the door so that power is disconnected from the heating elements whenever the door is opened. The thermostat used was an RT 126 Danfoss (15–45°).

Advantages of the apparatus

(1) *Convenience and cheapness.* The described apparatus can be made in many laboratory workshops from readily available components at a cost which is only a fraction of that of conventional drying ovens. It is portable and occupies considerably less space than other drying units. It accepts most standard thin-layer plate storage racks. Use of silica-encased heating elements obviates the risk of oxidised fragments, which might become detached from bare wire elements, contaminating the thin layers. The inclusion of a microswitch in the circuit prevents any possibility of short circuiting when loading the apparatus and also prevents overheating if the door is left open.

(2) *Speed of drying.* The times required for drying standard 20 cm × 20 cm thin-layer plates following development in different solvents are given in Table I.

These times are significantly shorter than those obtained in most conventional drying ovens operating at higher temperatures. Because of these short drying times the apparatus, though small, will handle a considerable number of plates. It has been noted that the drying times depend somewhat on the relative humidity of the room air. The figures given in Table I were obtained when the room air was at 24° and about 65% relative humidity.

All these layers were spread at a nominal thickness of 250μ. Thicker layers would be expected to take longer to dry.

The results given in Table I were obtained using a mixed layer[2] of cellulose and silica gel. Times required for drying other types of layer are not significantly different. Fragile layers (such as produced by silica gel without binder) remain completely undisturbed even when dried with fan running at full speed.

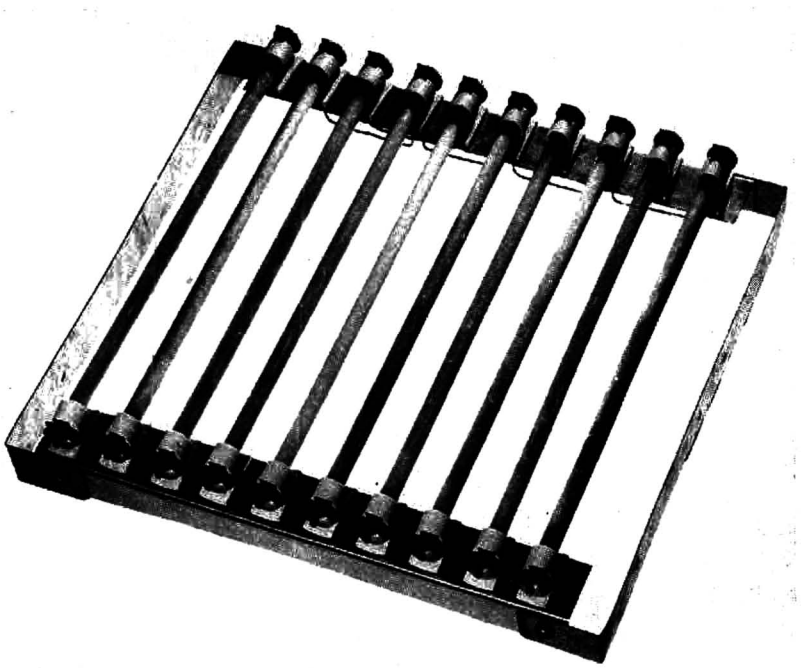


Fig. 3. Heating unit.

TABLE I

TIMES (IN MIN) REQUIRED FOR DRYING, AT 30°, THIN-LAYER PLATES RUN IN THREE CHROMATOGRAPHIC SOLVENTS

No. of plates per rack	*Solvents*		
	Water	*Butanol–acetic acid–water (5:1:4, v/v/v top phase)*	*Phenol–water (80:20, w/v)*
1	15	20	90
5	25	30	120
10	30	40	150

(3) *Evenness of drying.* Our usual procedure is to align the plates in the drier so that the solvent front is nearest to and parallel with the heating elements. Under such conditions drying starts from this front; and we find that these are the conditions for minimal migration of compounds. It is also important that the plates be kept approximately horizontal during drying.

In some recent two-dimensional procedures[3] separation in the first dimension is carried out with the origin as a short (2–5 cm long) band. Prior to separation in the second dimension it is necessary to elute the resulting series of bands back to spot size with water or other solvent. Following such elution rapid and even drying is particularly essential and this can be satisfactorily achieved in the described apparatus.

An essential requirement in the apparatus is that the fan draw (rather than push) a laminar flow of air over the plates. Air pushed over the plates by the same fan apparently travels helically and with much greater turbulence and does not produce the desired pattern of even drying from the front. The various patterns of drying obtained can be followed directly by observing the colour changes that occur during the drying of a layer which has been wet with a dilute solution of cobalt chloride.

Acknowledgements

The authors wish to thank Mr. D. ROGERS for preparing the working drawings and Drs. E. G. BOLLARD and R. L. BIELESKI for their interest in this work.

Plant Diseases Division and Fruit Research Division, Department of Scientific and Industrial Research, Auckland (New Zealand)

W. E. BARNARD
N. A. TURNER

1 W. J. CRIDDLE, G. J. MOODY AND J. D. R. THOMAS, *J. Chromatog.*, 18 (1965) 530.
2 N. A. TURNER AND R. J. REDGWELL, *J. Chromatog.*, 21 (1966) 129.
3 R. L. BIELESKI AND N. A. TURNER, *Anal. Biochem.*, 17 (1966) 278.

Received February 16th, 1967

Identification of guanidine derivatives by coupled electrophoresis–paper chromatography

For some time now, we have adopted a technique for the identification of the nitrogen components in biological material, fundamentally based on the ideas of BISERTE *et al.*[1], into which we have introduced some modifications[2].

Although bibliographical references to electrophoretic and chromatographic methods of investigation are very numerous when dealing with amine compounds, they are quite small in the field of guanidine derivatives, and even then the number of compounds studied is very small[3–5]. Recently new natural guanidine derivatives have been isolated, especially in different invertebrates[6–9], about which there are very few details which can lead to their identification in other biological materials by their electrophoretic and chromatographic characteristics. Furthermore, the study of the intermediary metabolism of guanidine derivatives raises difficult problems in connection with the identification of compounds, which are sometimes formed in minimum quantities.

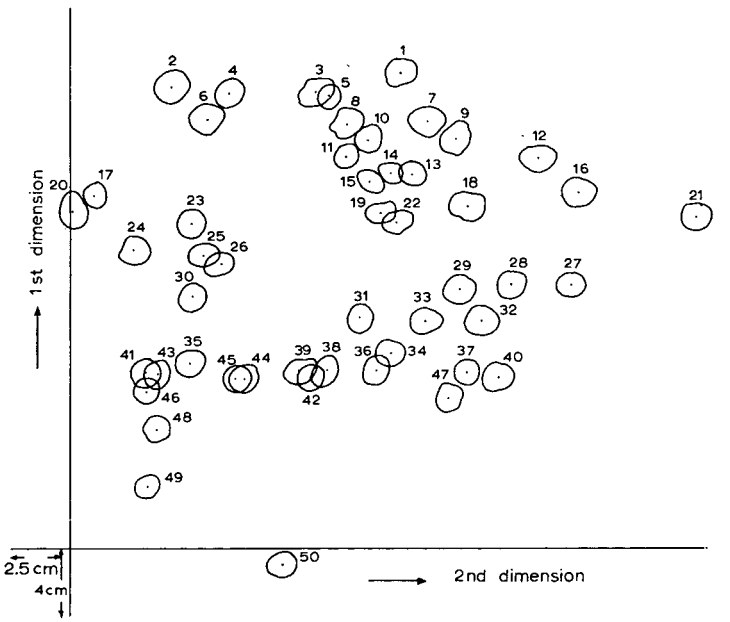


Fig. 1. Two-dimensional electrophoresis–chromatography. Paper: Schleicher & Schüll 2043b (30 × 29 cm). First dimension: electrophoresis; buffer, pyridine–acetic acid–water (3:10:487), pH 3.9; electrical potential: 350 V; time: 1.5 h. Second dimension: ascending chromatography; solvent system, *n*-butanol–acetic acid–water (12:3:5); time: 25 h.

We have considered it interesting to collect the data we obtained during several years of work on guanidine bases, during which we have frequently been confronted with problems of identification. In addition, we think that it would be useful to complete these data by the inclusion of the guanidine derivatives recently isolated, which we have synthesized because they are not commercially available.

Schematically, the technique of fractionation consists of two-dimensional

coupled electrophoresis–chromatography, carried out on sheets of Schleicher & Schüll 2043b paper 30 cm × 29 cm. Electrophoretic development is carried out in a tank in an inverted "V" with a pyridine–acetic acid–water buffer (3:10:487) of pH 3.9, at 350 V for 1 h 30 min, with subsequent chromatographic development in a *n*-butanol–acetic acid–water system (12:3:5), by the ascending method, for 25 h.

The reagent of Sakaguchi as modified by JEPSON *et al.*[10] was employed as the

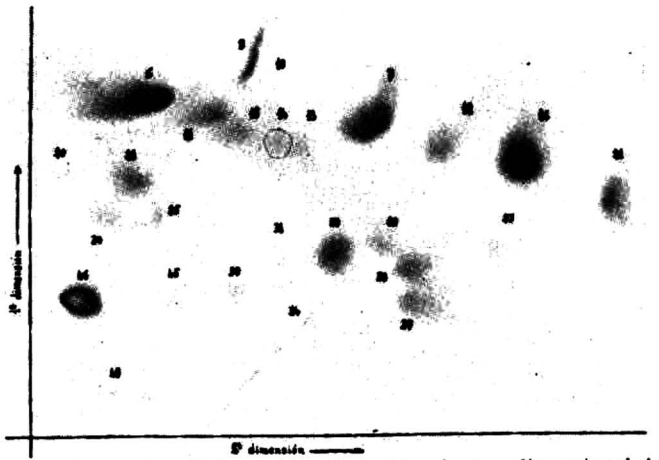


Fig. 2. Separation of 26 guanidine derivatives by two-dimensional electrophoresis–chromatography.

basic developer. Other visualisation methods used were the ninhydrin reaction[11], the diacetyl–α-naphthol system[12], the Jaffé reaction modified by AMES AND RISLEY[13], the reaction of carbonyl derivatives with 2,4-dinitrophenylhydrazine[14], and the Ehrlich reaction for urea and derivatives[15].

TABLE I

ABSOLUTE AND RELATIVE MOBILITIES OF GUANIDINE COMPOUNDS

R_G = mobility relative to that of glycine. Paper: Schleicher & Schüll 2043b (30 × 29 cm). Electrophoresis: pyridine–acetic acid–water (3:10:487) buffer, pH 3.9; 350 V for 1 h 30 min. Chromatography: *n*-butanol–acetic acid–water (12:3:5), ascending technique for 25 h. Colour of guanidine compounds with various specific reagents. D = Diacetyl–α-naphthol; N = ninhydrin; J = Jaffé; P = 2,4-dinitrophenylhydrazine; E = Ehrlich. c = reaction + only with previous heating at 100° for 2 h.

Substance	*Electrophoresis*		*Chromatography*		*Sakaguchi*	*D*	*N*	*J*	*P*	*E*
	cm	R_G	*cm*	R_G						
1 Guanidine	19.5	2.8	13.3	1.9		+				
2 N-Aminopropylagmatine	18.9	2.7	4.1	0.6	+ red	+	+			
3 Agmatine	18.8	2.7	9.9	1.4	+ red	+	+			
4 Histaguanidine	18.6	2.7	6.4	0.9	+ purple	+				
5 Homoagmatine	18.5	2.6	10.4	1.5	+ red	+	+			
6 Hirudonine	17.6	2.5	5.5	0.8	+ red	+				
7 Methylguanidine	17.6	2.5	14.4	2.1	+ orange	+				
8 Arcain	17.4	2.5	11.2	1.6	+ red	+				
9 N,N-Dimethylguanidine	16.9	2.4	15.7	2.2		+				
10 Audouine	16.7	2.4	12.0	1.7	+ red	+				

(continued on p. 302)

TABLE I *(continued)*

Substance	*Electrophoresis*		*Chromatography*		*Sakaguchi*	*D*	*N*	*J*	*P*	*E*
	cm	R_G	*cm*	R_G						
11 Mercaptoethylguanidine	16.1	2.3	11.1	1.6	+ red	+				
12 Ethylguanidine	16.0	2.3	19.0	2.7	+ orange	+				
13 Guanidinoethanol	15.5	2.2	13.7	2.0	+ pink	+				
14 Hydroxyguanidine	15.3	2.2	13.0	1.9	+ yellow					
15 γ-Guanidinobutanol	15.0	2.1	12.0	1.7	+ orange	+				
16 Propylguanidine	14.8	2.1	20.6	2.9	+ orange	+				
17 Streptidine	14.4	2.1	0.9	0.1	+ pink	+				
18 γ-Guanidinobutyraldehyde	14.0	2.0	16.1	2.3	+ orange	+			+	
19 γ-Guanidinobutyramide	13.7	2.0	12.6	1.8	+ orange	+				
20 Streptomycin	13.7	2.0	0.0	0.0	+ pink	+				
21 Isoleucaguanidine	13.6	1.9	25.5	3.6	+ red	+				
22 Creatinine	13.4	1.9	13.2	1.9				+		
23 α-Amino-β-guanidinopropionic acid	13.2	1.9	4.9	0.7	+ red	+	+			
24 Canavanine	12.2	1.7	2.6	0.4	+ brown	+	+			
25 Arginine	12.0	1.7	5.5	0.8	+ orange	+	+			
26 Homoarginine	11.6	1.7	6.0	0.9	+ orange	+	+			
27 ε-Guanidinocaproic acid	10.8	1.5	20.4	2.9	+ orange	+				
28 δ-Guanidinovaleric acid	10.8	1.5	17.9	2.6	+ orange	+				
29 γ-Guanidinobutyric acid	10.7	1.5	15.8	2.3	+ orange	+				
30 Hydroxyhomoarginine	10.3	1.5	4.9	0.7	+ pink	+	+			
31 β-Hydroxy-γ-guanidinobutyric acid	9.4	1.3	11.8	1.7	+ pink	+				
32 β-Guanidinoisobutyric acid	9.3	1.3	16.8	2.4	+ orange	+				
33 β-Guanidinopropionic acid	9.3	1.3	14.5	2.1	+ orange	+				
34 Argininic acid	7.9	1.1	13.0	1.9	+ orange	+				
35 α-Keto-γ-guanidoxybutyric acid	7.6	1.1	4.9	0.7	+ brown	+			+	
36 α-Guanidinopropionic acid	7.3	1.0	12.5	1.8	+ red	+				
37 α-Guanidinobutyric acid	7.2	1.0	16.2	2.3	+ orange	+				
38 Creatine	7.2	1.0	10.4	1.5		+		+ c		
39 Guanidinoacetic acid	7.2	1.0	9.4	1.3	+ orange	+		+ c		
40 α-Chloro-δ-guanidinovaleric acid	7.1	1.0	17.4	2.5	+ orange	+				
41 Lombricine	7.0	1.0	3.1	0.4	+ orange	+	+			
42 α-Keto-δ-guanidinovaleric acid	7.0	1.0	9.7	1.4	+ orange	+			+	
43 Octopine	7.0	1.0	3.4	0.5	+ red	+				
Glycine	7.0	1.0	7.0	1.0						
44 Citrulline	6.9	1.0	6.9	1.0			+			+
45 Taurocyamine	6.9	1.0	6.8	1.0	+ orange	+				
46 Desmethyloctopine	6.4	0.9	3.1	0.4	+ red	+				
47 Urea	6.3	0.9	15.4	2.2						+
48 Arginosuccinic acid	4.9	0.7	3.6	0.5		+	+			
49 Arginine-N$^{\alpha}$,N$^{\alpha}$-diacetic acid	2.5	0.4	3.1	0.4	+ orange	+				
50 Creatine phosphate	−0.7	−0.1	8.7	1.2		+		+ c		

The origin of the different compounds studied was as follows: creatinine, canavanine, arginine, creatine, citrulline and guanidinoacetic acid, were obtained from The Nutritional Biochemical Corporation, creatine phosphate, α-amino-β-guanidinopropionic acid, α-guanidinopropionic acid and arginosuccinic acid from Calbiochem, guanidine from Doesder, urea from Merck, and streptomycin from Antibioticos S.A.

Agmatine, methylguanidine, arcain, ethylguanidine, guanidinoethanol, N,N-

dimethylguanidine, N-aminopropylagmatine, hirudonine, propylguanidine, mercaptoethylguanidine, isoleucaguanidine, histaguanidine, hydroxyguanidine, hydroxyhomoarginine, audouine, homoagmatine, taurocyamine, homoarginine, and α-guanidinobutyric acid, ε-guanidinocaproic acid, β-hydroxy-γ-guanidinobutyric acid, δ-guanidinovaleric acid, γ-guanidinobutyric acid, β-guanidinoisobutyric acid and β-guanidinopropionic acid, were synthetized by us starting from the corresponding amino derivative and S-ethylthiourea, according to the method described for the synthesis of guanidinoacetic acid by BRAND AND BRAND[16].

Argininic acid and α-chloro-δ-guanidinovaleric acid were synthetized in our laboratory according to HAMILTON AND ORTIZ[17]. Octopine was prepared according to the technique of HERBST AND SWART[18] with α-bromopropionic acid; desmethyloctopine and arginine-Nα,Nα-diacetic acid were synthetized in the same way with monochloroacetic acid.

α-Keto-δ-guanidinovaleric acid and α-keto-γ-guanidoxybutyric acid were obtained enzymatically, according to LACOMBE *et al.*[19], starting from arginine and canavanine, respectively, and L-amino acid oxidase of viper poison. Streptidine was prepared from streptomycin, according to HUNTER *et al.*[20].

We synthetized γ-guanidinobutyraldehyde by adaptation of the method described by WITT AND HOLZER[21] for the synthesis of succinic semialdehyde. γ-Guanidinobutyramide was obtained from γ-guanidinobutyric acid with thionyl chloride, and γ-guanidinobutanol by the sulphydric reduction of γ-guanidinobutyraldehyde.

Finally, lombricine was kindly supplied to us by Prof. A. H. ENNOR (Department of Biochemistry, John Curtin School of Medical Research, Canberra, Australia).

In Table I a summary is made of all the compounds tested, with their mobilities after electrophoretic and chromatographic development expressed in cm (absolute), and relative to those of glycine (R_G). An indication is also given of their behaviour with the detecting reagents tested, with special reference to the reagent of Sakaguchi.

Fig. 1 shows the spacial disposition of all the substances tested in the two-dimensional development, while Fig. 2 represents a real separation of 26 of the guanidine derivatives which reacted positively with the Sakaguchi reagent.

Section of Physioparasitology,
"López-Neyra" Institute of Parasitology,
Granada (Spain)

JULIO LOPEZ-GORGE
MIGUEL MONTEOLIVA

1 G. BISERTE, P. BOULANGER AND P. PAYSANT, *Bull. Soc. Chim. Biol.*, 40 (1958) 2067.
2 J. LOPEZ-GORGE, M. MONTEOLIVA AND F. MAYOR, *Anal. Fis. Quim.*, in press.
3 J. P. BLASS, *Biochem. J.*, 77 (1960) 484.
4 R. PANT, *Biochem. J.*, 73 (1959) 30.
5 S. LISSITZKY, I. GARCIA AND J. ROCHE, *Experientia*, 10 (1954) 379.
6 J. ROCHE, N.-V. THOAI, Y. ROBIN AND L. A. PRADEL, *Compt. Rend. Soc. Biol.*, 150 (1956) 1684.
7 N.-V. THOAI, J. ROCHE, Y. ROBIN AND N.-V. THIEM, *Compt. Rend. Soc. Biol.*, 147 (1953) 1670.
8 J. ROCHE, C. AUDIT AND Y. ROBIN, *Compt. Rend. Soc. Biol.*, 260 (1965) 7023.
9 E. A. BELL, *Biochem. J.*, 83 (1961) 225.
10 J. B. JEPSON AND I. SMITH, *Nature*, 172 (1953) 1100.
11 G. TOENNIS AND J. J. KOLB, *Anal. Chem.*, 23 (1951) 823.
12 J. ROCHE, N.-V. THOAI AND J. L. HATT, *Biochim. Biophys. Acta*, 14 (1954) 71.
13 S. R. AMES AND H. A. RISLEY, *Proc. Soc. Exptl. Biol. Med.*, 69 (1948) 267.
14 D. E. BLAND, *Nature*, 164 (1949) 1093.

15 C. E. DALGLIESH, *Biochem. J.*, 52 (1952) 3.
16 E. BRAND AND F. C. BRAND, *Org. Syn.*, 22 (1942) 59.
17 P. B. HAMILTON AND P. J. ORTIZ, *Biochem. Prep.*, 4 (1955) 76.
18 R. M. HERBST AND E. A. SWART, *J. Org. Chem.*, 11 (1946) 368.
19 G. LACOMBE, N.-V. THIEM, N.-V. THOAI AND J. ROCHE, *Compt. Rend. Soc. Biol.*, 158 (1964) 43.
20 G. D. HUNTER, M. HERBERT AND J. D. HOCKENHULL, *Biochem. J.*, 58 (1954) 249.
21 I. WITT AND H. HOLZER, *Acta Biol. Med. Ger.*, 11 (1963) 712.

Received November 22nd, 1966

J. Chromatog., 29 (1967) 300–304

The effect of solvent purity on the chromatography of indole-3-acetic acid

In the course of an investigation on the growth substance relationships of diploid and tetraploid races of *Ranunculus ficaria* L. certain anomalies were found with regard to the R_F values quoted for indole-3-acetic acid in various chromatographic solvents.

NITSCH[1] stated that the R_F of indole-3-acetic acid (IAA) using a solvent system of isobutanol, methanol and water (16:1:3, by volume) was 0.24, whilst we have consistently obtained values of 0.80–0.90 using BDH Analar materials. Certain other observations on the effects of solvents on bio-assays prompted us to investigate the possibility of there being present an impurity in one or more of the solvent components causing the differences in R_F quoted. Purification of the isobutanol was achieved by refluxing the alcohol over potassium hydroxide for 2 h, and then fractionating the liquid. The fraction distilling over between 106° and 108° was collected. Methanol was purified by the method of GORDON AND PALEG[2], whilst the water used was doubly glass distilled and passed through a deionizer.

The R_F values obtained using combinations of repurified and un-repurified components of the solvent mixture are shown in Table I. In all cases purified water was used. Chromatography was carried out using strips of Whatman No. 3 paper, spotted with 2 μl of a methanolic solution of IAA (10^3 p.p.m.). The strips were spotted

TABLE I

THE EFFECT OF DISTILLATION OF ALCOHOL COMPONENTS OF THE SOLVENT SYSTEM ON THE R_F VALUE FOR PURE IAA

Distilled, deionized water used.

Solvent composition	*R_F value of IAA*
Distilled isobutanol / Distilled methanol	0.20–0.30
Distilled isobutanol / Undistilled methanol	0.20–0.30
Undistilled isobutanol / Distilled methanol	0.80–0.90
Both undistilled	0.80–0.90

in red light from a photographic Safe-Light, equilibrated over the solvent for 4 h, and run by ascending flow at 25°. The spots were located by their fluorescence under ultraviolet light (Wood's light) and also by Salkowski's reagent.

It is reasonably clear that the R_F value of the IAA depends on the purity of the isobutanol component, and not on that of the methanol. In this connection it is of interest to note that using a solvent of *tert.*-butanol and water (4:1 by volume), GUERN[3] quotes an R_F for IAA of 0.27, whilst SEN AND LEOPOLD[4] using the same solvent system give an R_F value of 0.80. Our own cursory examination of this latter solvent has yielded an R_F of 0.80 for IAA.

It is essential, at least in the conditions obtaining here, to use the purified isobutanol at once after distillation. Table II shows the effect of storage under various conditions of the isobutanol component on the R_F value for pure IAA.

TABLE II

THE EFFECTS OF STORAGE CONDITIONS OF THE ISOBUTANOL COMPONENT ON THE R_F VALUES OF PURE IAA

Conditions of storage of the redistilled isobutanol	*Time elapsed after distillation (h)*		
	0	*24*	*48*
In light, room temperature	0.20–0.30	Streak	0.80–0.90
In dark, room temperature	0.20–0.30	Streak	0.80–0.90
In dark, —10°	0.20–0.30	Streak	0.80–0.90

At present we have no clear indication of the nature of the causes of these effects, but from the results reported here, it seems clear that considerable attention must be paid to the purity of the solvents used in the chromatography of indoles.

Botany Department, Chelsea College of Science and Technology, London, S.W. 3 (Great Britain)

E. R. TURNER
B. YOUNG*

1 J. P. NITSCH, in R. L. WAIN AND F. WIGHTMAN (Editors), *The Chemistry and Mode of Action of Plant Growth Substances*, Butterworth, London, 1956, pp. 3–31.
2 S. A. GORDON AND L. G. PALEG, *Physiol. Plantarum*, 10 (1957) 39.
3 J. GUERN, *Trav. Dipl.*, Fac. Sci., Paris, 1958.
4 S. P. SEN AND A. C. LEOPOLD, *Physiol. Plantarum*, 7 (1954) 98.

* Present address: Department of Botany, University of Alberta, Edmonton, Alberta (Canada).

Received January 30th, 1967

Chromatography on ion exchange papers

The behaviour of some metal ions on carboxylic exchangers

Ion exchange data for metal ions are available for many aqueous systems, both for strong cation and anion exchangers, however, relatively little work has been done with carboxylic resins as their adsorption from strongly acid solutions is poor. Ion exchange papers with carboxylic exchangers have been used to our knowledge only in one separation, that of Ag–Pb–Bi–Hg by WIELAND AND BERG[1], who worked with a laboratory-made carboxylic paper and developed with ammonium acetate (0.1 *M* at pH 3.).

We thus thought it interesting to extend our survey of the chromatographic behaviour of metal ions on ion exchange papers to two carboxylic ion exchange papers, the carboxymethyl-cellulose paper (Whatman CM-50) and the Amberlite WA-2 paper (containing about 45 % of Amberlite IRC-50). As eluant we used buffers with equal molarities of sodium acetate and acetic acid and hoped to avoid pH gradients by working on the "plateau" of the buffer. This proved adequate for the Whatman CM-50 paper which could be employed unwashed as supplied by the manufacturers.

TABLE I

R_F VALUES OF METAL IONS ON WHATMAN CM-50 PAPER

Eluents: 1 = 1 *N* sodium acetate–1 *N* acetic acid;
2 = 0.5 *N* sodium acetate–0.5 *N* acetic acid;
3 = 0.1 *N* sodium acetate–0.1 *N* acetic acid.

Ion	*Eluent*		
	1	*2*	*3*
Ag^+	0.5	0.36	0.17
Te^+	0.48	0.46	0.25
Bi^{+++}	0.88	0.6	0.05
Cd^{++}	0.72	0.44	0.08
Pb^{++}	0.53	0.27	0.04
Hg^{++}	0.78	0.56	0.09
Cu^{++}	0.63	0.34	0.04
Fe^{+++}	elongated comet		0.03
Co^{++}	0.70	0.52	0.10
Ni^{++}	0.68	0.52	0.10
Mn^{++}	0.75	0.52	0.13
Zn^{++}	0.72	0.45	0.09
Al^{+++}	0.78	0.36	0.04
Sc^{+++}	0.75	0.44	0.04
Y^{+++}	0.79	0.50	0.04
La^{+++}	0.66	0.32	0.02
Ce^{+++}	0.69	0.29	0.03
Zr^{++++}	comet to LF	comet → 0.39	comet → 0.08
Be^{++}	0.85	0.71	0.12
Mg^{++}	0.84	0.62	0.17
Ca^{++}	0.65	0.45	0.08
Sr^{++}	0.62	0.48	0.11
Ba^{++}	0.52	0.35	0.05
$Co(NH_3)_6^{+++}$	0.55	0.27	0.02
$Co(en)_3^{+++}$	0.66	0.29	0.03
ReO_4^-	0.72	0.76	0.94

The Amberlite WA-2 paper required washing with HCl and distilled water as well as placing the spots 3 cm behind the liquid front to avoid the formation of double spots. The ions to be chromatographed were dissolved in the acetate buffer as their nitrates. Metal ions which are usually stable as anionic complexes (*e.g.* $AuCl_4^-$) were not studied. Uranyl ions precipitated in the buffer. The R_F values obtained with various molarities of sodium acetate–acetic acid are shown in Tables I and II.

TABLE II

R_F VALUES OF METAL IONS ON AMBERLITE WA-2 PAPER

Eluents: 1 = 3 *N* sodium acetate–3 *N* acetic acid;
2 = 2 *N* sodium acetate–2 *N* acetic acid;
3 = 1 *N* sodium acetate–1 *N* acetic acid.

Ion	*Eluent*		
	1	*2*	*3*
Ag^+	0.17	0.13	0.06
Tl^+	0.33	0.24	0.20
Bi^{+++}	0.02	0.00	0.00
Cd^{++}	0.21	0.14	0.06
Pb^{++}	0.15	0.06	0.02
Hg^{++}	0.05	0.03	0.00
Cu^{++}	0.21	0.15	0.04
Fe^{+++}	0.00	0.00	0.00
Co^{++}	0.58	0.48	0.25
Ni^{++}	0.57	0.46	0.27
Mn^{++}	0.64	0.50	0.31
Zn^{++}	0.35	0.23	0.13
Al^{+++}	0.00, 0.52	0.00, 0.41	0.00, 0.41
Sc^{+++}	0.04	0.02	0.00
Y^{+++}	0.23	0.11	0.03
La^{+++}	0.14	0.07	0.03
Ce^{+++}	0.16	0.08	0.02
Zr^{++++}	0.03	0.03	0.03
Be^{++}	0.18, 0.46	0.10, 0.33	0.05, 0.18
Mg^{++}	0.79	0.64	0.48
Ca^{++}	0.49	0.38	0.23
Sr^{++}	0.48	0.37	0.27
Ba^{++}	0.32	0.26	0.15
$Co(NH_3)_6^{+++}$	0.04	0.02	0.02
$Co(en)_3^{+++}$	0.01	0.01	0.00
ReO_4^-	0.75	0.68	0.55

Fe(III) and Zr(IV) yield diffuse comets on Whatman CM-50 paper. Beryllium and aluminium give double spots on the Amberlite WA-2 paper. Most other ions give elongated spots on the Whatman CM-50 paper and very compact sharp spots on the Amberlite WA-2 paper. Perrhenate was chromatographed as it is a monovalent anion which is strongly adsorbed on neutral surfaces and gives thus an indication of the adsorption effect. $Co(NH_3)_6^{+++}$ and $Co(en)_3^{+++}$ were examined to see whether there are great differences between trivalent ions which could complex with the carboxylic groups and those (*i.e.* the complexes) which could not.

As shown in the tables the cobalt complexes have R_F values of the same order as the trivalent metal ions and hence there seems to be little indication that complex formation is predominant.

TABLE III

R_F VALUES OF METAL IONS ON AMBERLITE WA-2 PAPER

Eluents: 1 = 1 *N* sodium acetate–1 *N* acetic acid in 20% aqueous methanol;
2 = 1 *N* sodium acetate–1 *N* acetic acid in 40% aqueous methanol;
3 = 1 *N* sodium acetate–1 *N* acetic acid in 60% aqueous methanol;
4 = 1 *N* sodium acetate–1 *N* acetic acid in 80% aqueous methanol.

Ion	*Eluent*			
	1	*2*	*3*	*4*
Fe^{+++}	0.00	0.00	0.00*	0.00*
Co^{++}	0.29	0.26	0.16*	0.13*
Ni^{++}	0.27	0.24	0.14	0.12
Zn^{++}	0.10	0.08	0.07	0.03
Mg^{++}	0.46	0.45	0.44	0.39
Ca^{++}	0.24	0.20	0.16	0.10
Sr^{++}	0.24	0.18	0.12	0.05
ReO_4^-	0.54	0.60	0.54	0.51

* Placed 4 cm behind the liquid front to avoid double spotting.

In all there are few interesting separations, (except perhaps with Ag^+–Tl^+–Pb^{++}) most ions adsorbing with similar R_F values and there is good agreement with what can be expected from the law of mass action. The only interesting results concern the alkaline earths where there is the usual difference between Ba and Sr but not between Sr and Ca. Sr and Ca are inseparable on both papers with if anything slightly higher R_F values for Sr than for Ca. Similarly in group 3, yttrium has higher R_F values than Sc inverting the order to La–Sc–Y.

We would like to suggest that the lack of separation of Ca–Sr is due to a number of equilibria involving hydration, complex formation with acetate as well as with the carboxylic groups of the papers. Table III shows some data with methanol–water mixtures. In 60% methanol the difference between Sr and Ca is already sufficient to permit a separation into adjacent spots.

This work was carried out as part of the contract No. 265/RB with the International Atomic Energy Agency (Vienna) with the technical collaboration of M. MAZZEI and R. MARINI-BETTÒLO.

Laboratorio di Cromatografia del C.N.R., Istituto di Chimica Generale ed Inorganica, Rome (Italy) M. LEDERER

1 TH. WIELAND AND A. BERG, *Angew. Chem.*, 64 (1952) 418.

Received December 17th, 1966

Separation of americium from rare earths by reversed-phase partition chromatography

Solvent extraction with quaternary ammonium salts of high molecular weight has been proposed recently by MOORE[1] and, independently, by the present authors[2], as an alternative method of separating trivalent actinides from lanthanides in thiocyanate media.

The same chemical system, basically, has been used by us in a series of reversed-phase partition chromatography experiments to effect laboratory-scale separations of americium (III) from the rare earths. Some separations between single rare earth elements have also been obtained.

Experimental

Celite (80–120 mesh) obtained from the BDH Ltd. England, made hydrophobic by immersion in a (5 %) ether solution of dichlorodimethylsilane was used as the supporting solid. Column beds were prepared by mixing the treated Celite with 0.3 *M*

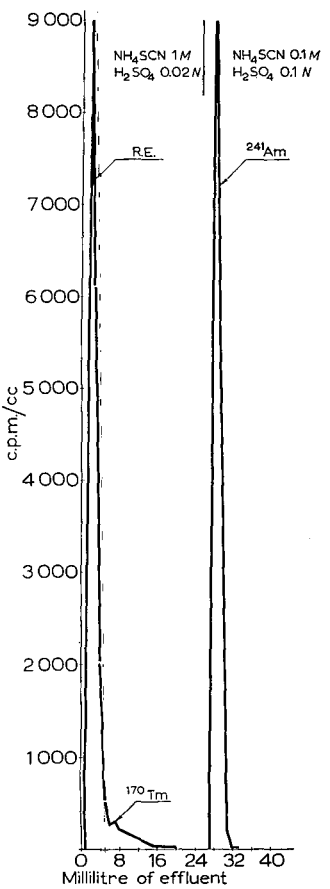


Fig. 1. Separation of americium from rare earths.

solution of Aliquat-336 (methyl-tri(*n*-alkyl)ammonium, principally octyl and decyl, obtained from the General Mills Inc., U.S.A.) in the thiocyanate form and transferring the resulting slurry to a 5.4 mm internal diameter glass column plugged with glass wool. The excess of extractant, not adsorbed on the solid support, was displaced by washing the column with the thiocyanate solution used for the first elution step. The height of the bed was 10 cm and the eluent flux 0.78 ml $cm^{-2}min^{-1}$. A small aliquot (50 λ) of a thiocyanate solution containing the tracers to be separated was transferred to the top of the resin bed and elution was carried out by the usual chromatographic techniques.

^{241}Am, ^{144}Ce, ^{147}Pm, and $^{152+154}Eu$ were obtained from the Radiochemical Centre, Amersham, England. ^{140}La and ^{170}Tm were prepared by neutron activation of high purity lanthanum and thulium in the nuclear reactor G.Galilei of CAMEN. The samples taken from the elution steps were gamma-counted using a well-type NaI

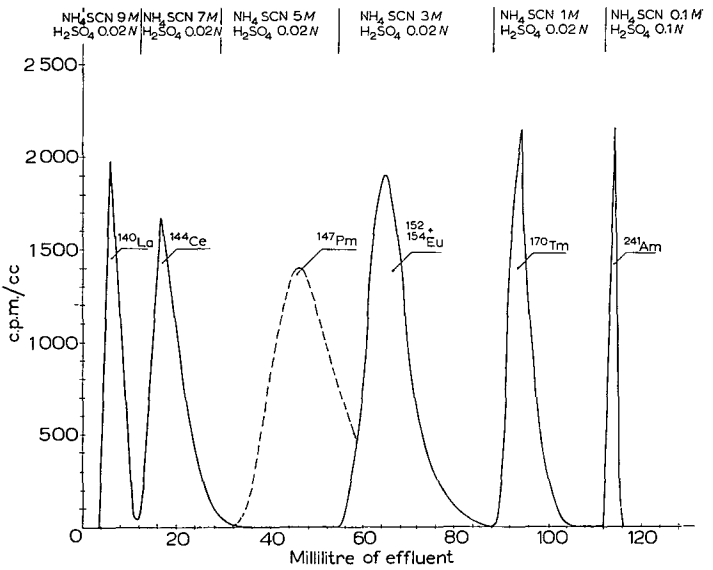


Fig. 2. Separation of ^{140}La, ^{144}Ce, ^{147}Pm, $^{152+154}Eu$, ^{170}Tm and ^{241}Am.

(Tl) scintillation counter except for samples of ^{147}Pm which were deposited on glass discs and beta-counted with a G.M. counter.

Results

A relatively rapid separation of americium from the rare earth group was obtained using the eluent composition specified in Fig. 1. A small quantity of sulfuric acid was added to the thiocyanate eluent to enhance the separation as suggested by MOORE[1]. In Fig. 2 a selective elution of lanthanum, cerium, promethium, europium, thulium and americium is reported, obtained by gradient elution with thiocyanate solutions of decreasing concentration.

A group separation of americium from the rare earth elements was also obtained using an ascending chromatography technique with paper (Whatman No. 1) strips

treated with Aliquat-336 in the thiocyanate form and a developing solution consisting of 0.2*M* NH_4SCN + 0.05*N* H_2SO_4 (Am, R_F 0.35; Tm, R_F 0.77; Eu, R_F 0.81).

Acknowledgement

The authors are grateful to Dr. P. GERONTOPULOS for his helpful cooperation in this work.

CAMEN, San Piero a Grado, Pisa (Italy) P. G. BARBANO
L. RIGALI

1 F. L. MOORE, *Anal. Chem.*, 36 (1964) 2158.
2 P. TH. GERONTOPULOS, L. RIGALI AND P. G. BARBANO, *Radiochimica Acta*, 4 (1965) 75.

Received January 2nd, 1967

Application of reversed-phase column chromatography to the determination of tungsten in stainless steels by activation analysis

As is well known, activation analysis is a very useful tool for the determination of trace elements in various materials, and tungsten is a normal minor component in stainless steels. However, when steel is irradiated, several other radio-isotopes besides tungsten are produced, for example, when samples are irradiated for one hour and then "cooled" for one day, the gamma-activity due to the long-lived nuclides ^{51}Cr, ^{59}Fe, ^{58}Co, ^{76}As, ^{187}W remains. As shown in Fig. 1, the resolution of gamma-spectrometry is not sufficient to resolve the complex spectrum obtained under these conditions, and therefore it is impossible to determine tungsten without previous chemical separation. On the other hand, determination of tungsten by the classical method of tungstic acid precipitation does not give a good quantitative separation because of the co-precipitation of other elements such as As, Mo, and so on[1]. Better results have been obtained by isolating tungsten, as WO_4^-, on chromatographic columns of an inert support impregnated with 8-hydroxyquinoline[2] or on anion-exchange resins[3]. In this work, we have used reversed-phase partition chromatography[4–6] to isolate tungsten from the unwanted major components of the steel.

HAMLIN *et al.*[7] used polytrifluorochloroethylene (Kel-F) as an inert support for tri-*n*-octylamine (TNOA); this compound behaves just like a "liquid anion exchanger": the anionic metal complexes are retained on the top of the chromatographic column, while the elements which do not form anionic complexes are quickly eluted. Generally the separative properties of the column depend on the values of the complex formation constants and on the distribution coefficients between the organic and the aqueous phase. The amine extraction technique is particularly convenient because it also works efficiently in strong acid solutions.

Experimental

Equipment and reagents. The samples were irradiated in the TRIGA reactor of C.S.N. Casaccia (Rome) and examined by gamma-ray spectrometry.

The gamma-ray spectrometer consisted of an Intertechnique 400-channel pulse height analyzer with a 3 × 3 in. NaI (Tl) crystal mounted on a photomultiplier, Dumont 6393.

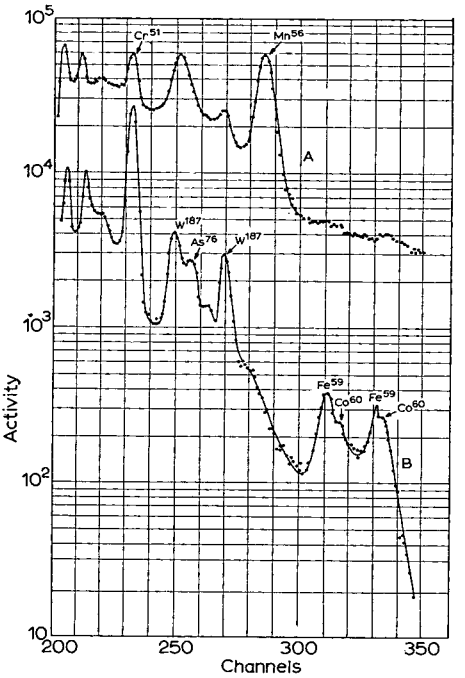


Fig. 1. Gamma-spectra of a EN 58 B sample irradiated one hour before chemical separation. (A) After 18 hours, the contribution of some nuclides at 0.5 MeV and of ^{56}Mn at 0.82 MeV overlaps the tungsten photopeak; (B) after 10 days, only ^{76}As, ^{187}W and ^{58}Co, together with a high chromium background, remain.

The columns were prepared by mixing (1:1) Kel-F moulding powder (140–200 mesh) and TNOA.

The standard solution contained 1 mg/ml of W as spectrographically pure Na_2WO_4 (Johnson & Matthey Co., London).

Preparation of the chromatographic columns. A mixture was made by adding 6 g of Kel-F to 6 ml of TNOA 50 % in xylene. 20 ml of 10*M* HCl were then added and the suspension poured into the column. The columns, which had an internal diameter of 1 cm, and an effective length of 10 cm, were fitted with a glass disc at the bottom. Flow rate was regulated by gentle pressure from a mercury device.

Irradiation. Samples and comparative standard were irradiated in the pneumatic tube of the reactor. The flux was $1.6 \cdot 10^{12}$ n cm^{-2} sec^{-1}, and the irradiation time was one hour. The samples were rectangular pieces of stainless steel (25–30 mg). The standard consisted of 0.2–0.3 ml of the Na_2WO_4 solution contained in a polyethylene

tube sealed by heating. Samples and comparative standards were irradiated simultaneously and placed side by side in a polyethylene irradiation capsule.

Counting. Gamma-ray spectrometry was used to determine the amount of radioactivity and to ascertain the radiochemical purity of the isolated nuclides.

The eluted fractions were standardized to an equal volume in a polyethylene flask, which was accurately adjusted to the supporting ring in order to obtain identical counting geometry. Calculations were made by considering the 0.48 MeV photopeak area of ^{187}W.

Chromatographic procedure. Before irradiation each sample was cleaned by treating with concentrated hydrochloric acid and distilled water to eliminate the probable impurities. As the activity of ^{60}Co produced during the one hour of irradiation was not sufficient, this radionuclide was added as a tracer to the irradiated sample conveniently dissolved in concentrated hydrochloric acid. After evaporation to dryness the residue was dissolved with the minimum quantity of 10 *M* HCl (2–3 ml) and the solution placed on the chromatographic column: 30 ml of 10 *M* HCl were then added to elute Cr(III) which does not give anionic hydrochloric complexes. Cobalt moved very slowly and Fe(III) was firmly held on the top of the column. Tungsten remained on the top of the column together with Co(II) and Fe(III). After the elution of chromium, a 7 *M* HCl–1 *M* HF system[3] was utilized to elute tungsten, which appeared immediately and was totally recovered. Cobalt was rapidly eluted with 3 *M* HCl, and iron with 1 *M* NHO_3. Fig. 2 shows the elution curves for the four metal ions,

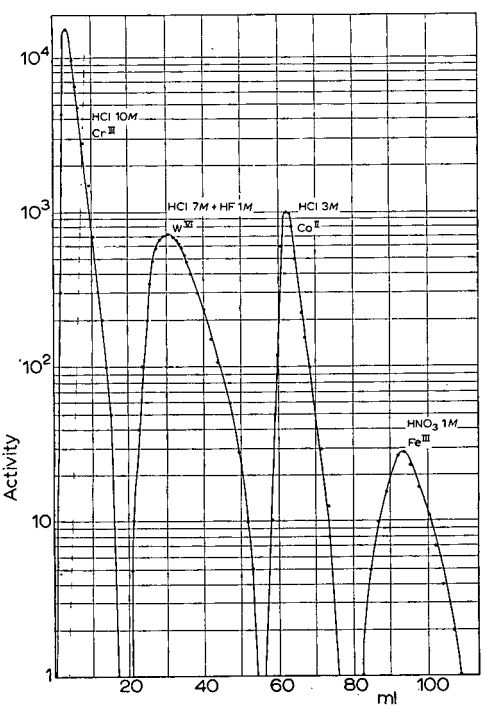


Fig. 2. Elution curves of Cr(III), W(VI), Co(II) and Fe(III) with a Kel-F–TNOA column. Length of the bed: 10 cm; volume: 9 ml; flow rate: 0.5 ml/min.

it shows that it would be also possible to use this method for the isolation of Co(II) from Fe(III) and hence for cobalt determination in the stainless steels.

Results and discussion

Some EN58B, AISI 321 and X15 UNI 1808 stainless steel samples were analyzed by means of the described technique: the results are reported in Table I,

TABLE I

TUNGSTEN CONTENTS IN 18-8 TYPE STEELS

Type	*Sample No.*	*Weight (mg)*	*Tungsten (%)*	*Mean value (p.p.m.)*	*Mean error*	
					(p.p.m.)	*(%)*
EN 58 B	1	31.0	$1.190 \cdot 10^{-1}$	1,158	± 49	4
	2	39.7	$1.087 \cdot 10^{-1}$			
	3	25.3	$1.160 \cdot 10^{-1}$			
	4	21.1	$1.195 \cdot 10^{-1}$			
AISI 321	1	26.5	$1.05 \cdot 10^{-2}$	123	± 6	5
	2	25.6	$1.20 \cdot 10^{-2}$			
	3	25.5	$1.35 \cdot 10^{-2}$			
	4	25.6	$1.30 \cdot 10^{-2}$			
UNI 1808	1	28.7	$6.4 \cdot 10^{-3}$	67.2	± 3.2	6
	2	26.2	$6.5 \cdot 10^{-3}$			
	3	22.3	$6.9 \cdot 10^{-3}$			
	4	25.1	$7.1 \cdot 10^{-3}$			

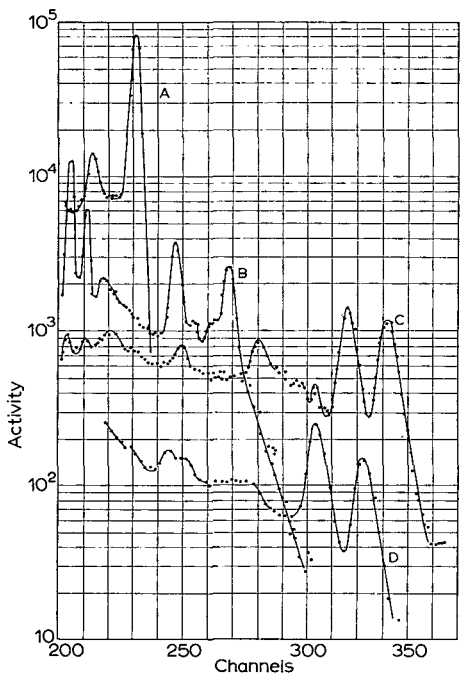


Fig. 3. Gamma-spectra of the eluted fractions: (A) chromium; (B) tungsten; (C) cobalt; (D) iron (the absence of cobalt was tested on the sum peak at 2.5 MeV).

which shows that this method is suitable both for low and medium tungsten concentrations. The relative mean error (5 % for four determinations) is a normal one for activation analysis techniques.

The tungsten fraction and the other three fractions were radiochemically pure, as shown in Fig. 3.

Kel-F–TNOA columns could be used for 10 consecutive cycles. On the basis of these results the Kel-F–TNOA columns were found suitable for the isolation and determination of tungsten, and subsequently cobalt in stainless steels. They have a great capacity and selectivity, very well defined elution curves, and give a radiochemically pure tungsten fraction. The time required for this method is about three hours including the irradiation time.

Acknowledgements

The authors gratefully thank Doctors C. TESTA, C. CESARANO and A. MOAURO for the helpful discussions throughout the course of this work. Thanks are also due to the authority of C.S.N. Casaccia of CNEN (Rome).

Laboratorio de Radioisótopos,
Facultad de Ingeniería,
Universidad de Buenos Aires (Argentina)

CARLOS E. ESPAÑOL*
ANA MARÍA MARAFUSCHI

1 G. LELIAERT, H. HOSTE AND Z. EECKHAUT, *Talanta*, 2 (1959) 115.
2 G. ROBINSON, *Metallurgia*, 37 (1947) 45.
3 K. A. KRAUS AND F. NELSON, *Symposium on Ion Exchange and Chromatography*, ASTN Special Technical Publication No. 195, 1958.
4 B. T. KENNA, *AEC (U.S.A.) Rept., TID-16296* (1963).
5 E. CERRAI AND C. TESTA, *J. Chromatog.*, 6 (1961) 443.
6 E. CERRAI AND C. TESTA, *J. Chromatog.*, 9 (1962) 216.
7 A. G. HAMLIN, B. J. ROBERTS, W. LOUGHLIN AND S. G. WALKER, *Anal. Chem.*, 33 (1961) 1547.

Received December 19th, 1966

* Fellowship of the Consejo Nacional de Investigaciones Científicas de la República Argentina

Erratum

J. Chromatog., 26 (1967) 276–279

Page 278, the formula:

$$V\ (\mathrm{ml}) = \frac{(1 - E_a \times V_a)}{E_b \times V_b} \times V_p$$

should be replaced by:

$$V\ (\mathrm{ml}) = \left(1 - \frac{E_a \times V_a}{E_b \times V_b}\right) \times V_p.$$

Bibliography Section

Paper Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

SNÍŽKOVÁ, A. AND VACÍK, J.: Effect of the specific surface on the chromatographic distribution function. *Collection Czech. Chem. Commun.*, 21 (1966) 1872–1875.

SOCZEWINSKI, E. AND PRZYBOROWSKA, M.: R_F–pH relationships for lipophilic organic electrolytes in the reversed phase chromatography with buffered aqueous phase. *Chem. Anal. (Warsaw)*, 11 (1966) 849–852.

3. TECHNIQUES I

GASPARIČ, J.: Identifizierung organischer Verbindungen. LXII. Die Zinkstaubdestillation in Verbindung mit der Papierchromatographie — ein Mittel zur Konstitutionsaufklärung polycyklischer organischer Stoffe. *Mikrochim. Acta*, (1966) 288–294 — identification of the skeleton of polycyclic compounds.

SZCZEPANIAK, S. AND KRZECZKOWSKA, I.: (A modification of the one-dimensional paper chromatographic technique). *Chem. Anal. (Warsaw)*, 11 (1966) 1197–1202.

See also TLC section.

7. PHENOLS

POSPÍŠIL, J. AND TAIMR, L.: Antioxidation agents and stabilizers. XI. The synthesis of 1,1,3,3,5,5-hexamethylhexylphenols. *Collection Czech. Chem. Commun.*, 31 (1966) 1839–1847 — R_F values of 12 phenols.

SIMONART, P. AND VERACHTERT, H.: Composés phénoliques produits par *Aspergillus fumigatus* Fresenius et par *Penicillium spinulosum* Thom. *Bull. Soc. Chim. Biol.*, 48 (1966) 943–950 — R_F values of 13 compounds.

WOJDYLO, S. AND ZIELENSKI, K.: (Studies on the chromatographic separation of monohydroxylic phenols). *Chem. Anal. (Warsaw)*, 11 (1966) 1127–1134 — R_F values of 10 phenols; influence of pH.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

OASHI, S.: Paper chromatographic determination of naringin in the naringinase-treated Natsumikan orange. *Nippon Shokuhin Kogyo Gakkaishi*, 11 (1964) 376–380; *C.A.*, 64 (1966) 14875b.

PARIS, R.-R., PARIS, M. AND FRIES, J.: Sur les polyphénols (anthocyanes, flavonoïdes) des fleurs de Digitale officinale (*Digitalis purpurea*) L. *Ann. Pharm. Franc.*, 24 (1966) 245–249.

PECKET, R. C.: Development of anthocyanin pigmentation in flowers of *Lathyrus odoratus*. *J. Exptl. Botany*, 17 (1966) 177–184; *C.A.*, 64 (1966) 14593g.

9. OXO COMPOUNDS

JUNEK, H. AND HAMBÖCK, H.: Eine Farbreaktion zum Nachweis von *o*-Chinonen der Naphthalin- und Phenanthrenreihe. *Mikrochim. Acta*, (1966) 522–525.

10. CARBOHYDRATES

FARKAŠ, J.: Nucleic acid components and their analogues. LXXIX. Synthesis of methyl-1-deoxy-D-psicofuranosides substituted at $C_{(1)}$ with halo atoms or a mercapto group. *Collection Czech. Chem. Commun.*, 31 (1966) 1535–1543 — PC and TLC of deoxypsicose derivatives.

GHALAMBOR, M. A., LEVINE, E. M. AND HEATH, E. C.: The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*. III. The isolation and characterization of 3-deoxyoctulosonic acid. *J. Biol. Chem.*, 241 (1966) 3207–3215.

JARÝ, J. AND KEFURT, K.: Über Lactone. X. δ-Lacton der 4,6-Didesoxy-L-xylohexonsäure. *Collection Czech. Chem. Commun.*, 31 (1966) 2059–2067.

KAHL, W., ROSZKOWSKI, A. AND ZUROWSKA, A.: (Studies on the application of Polish active charcoals in chromatography of sugars). *Chem. Anal. (Warsaw)*, 11 (1966) 1229–1237.

LEE, Y. C.: Isolation and characterization of lipopolysaccharides containing 6-O-methyl-D-glucose from *Mycobacterium* species. *J. Biol. Chem.*, 241 (1966) 1899–1908.

MAKINO, M. AND YAMASHINA, I.: Periodate oxidation of glycopeptides from ovalbumin. *J. Biochem. (Tokyo)*, 60 (1966) 262–267.

OCARANZA, E. R., DE COBOS, F. M., DE IGLESIAS, D. A. AND DE GEPNER, A. CH.: (New reagents for chromatographic separation of aldoses and ketoses). *Arch. Bioquim., Quim. Farm., Tucuman*, 11, No. 1–2 (1963–64) 101–108; *C.A.*, 64 (1966) 14576g — aminonaphtholsulphonic acid; phenylsemicarbazide.

ROSÍK, J., ZITKO, V., BAUER, Š. AND KUBALA, J.: The structural features of cherry-tree gum (*Prunus arium* L. var. *juliana* L.). *Collection Czech. Chem. Commun.*, 31 (1966) 1072–1078.

ROSÍK, J., ZITKO, V. AND KUBALA, J.: Structure of sour cherry-tree gum (*Prunus cerasus* L.). *Collection Czech. Chem. Commun.*, 31 (1966) 1569–1577.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

CHURÁČEK, J.: Substituierte Benzyl- und Phenazyl-halogenide als Reagenzien zur papierchromatographischen Trennung und Identifizierung von Fettsäuren. *Mikrochim. Acta*, (1966) 196–201.

HARRIS, T. M., HARRIS, C. M. AND LIGHT, R. J.: The isolation of triacetic acid lactone from cultures of *Penicillium patulum*. *Biochim. Biophys. Acta*, 121 (1966) 420–423 — PC and TLC.

HOLOWCZAK, J. A., KOFFLER, H., GARNER, H. R. AND ELBEIN, A. D.: Characterization of new glycosides from the fermentation broths of *Streptomyces hygroscopicus*. *J. Biol. Chem.*, 241 (1966) 3270–3275.

STRÁNSKÝ, Z., STUŽKA, V. AND RUŽIČKA, E.: Beitrag zur Identifizierung aliphatischer Säuren als 2-Alkylbenzimidazole. *Mikrochim. Acta*, (1966) 77–82 — PC and TLC; R_F values of 14 derivatives.

11b. Lipids and their constituents

GUINAND, M. AND MICHEL, G.: Structure d'un peptidolipide isolé de *Nocardia asteroides*, la peptidolipine NA. *Biochim. Biophys. Acta*, 125 (1966) 75–91 — PC of peptidolipid, TLC of components.

WUTHIER, R. E.: Two-dimensional chromatography on silica gel-loaded paper for the microanalysis of polar lipids. *J. Lipid Res.*, 7 (1966) 544–550.

See also TLC section.

13. STEROIDS

MONARO, J. M., MATTOS DE SPREMOLLA, I., MAGGIOLO, J., MANE, F. AND CERVINO, J. M.: (Assay of pregnanetriolone in urine by paper chromatography: its use in several endocrine diseases). *Anales Fac. Med., Montevideo*, 49 (1964) 509–514; *C.A.*, 64 (1966) 14517a.

14. STEROID GLYCOSIDES AND SAPONINS

EPPENBERGER, U., KAUFMANN, H., STÖCKLIN, W. AND REICHSTEIN, T.: Die Glykoside der Samen von *Stapelia gigantea* N.E.Br. *Helv. Chim. Acta*, 49 (1966) 1492–1504 — PC and TLC.

SAUER, H. H., WEISS, EK. AND REICHSTEIN, T.: Die Glykoside der Samen von *Dregea volubilis* (L.) Benth. ex Hook. 2. Mitt. Isolierung weiterer Drevogenine. *Helv. Chim. Acta*, 49 (1966) 1625–1632 — PC and TLC.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BORECKÝ, J.: Identifizierung organischer Verbindungen. LXI. Über die Anwendung der Papierchromatographie zur Identifizierung technisch wichtiger Amide. *Mikrochim. Acta*, (1966) 279–287 — R_F values of 24 amines.

CEE, A. AND GASPARIČ, J.: Identifizierung organischer Verbindungen. LXIII. Papierchromatographische Trennung und Identifizierung primärer aromatischer Amine in wässrigen Lösungsmittelsystemen. *Mikrochim. Acta*, (1966) 295–309 — R_F values of 169 various amine derivatives.

GASPARIČ, J. AND KLOUČEK, B.: Identifizierung organischer Verbindungen. LVIII. Beitrag zur Konstitutionsermittlung von Aryliden aromatischer Hydroxycarbonsäuren. *Collection Czech. Chem. Commun.*, 31 (1966) 106–112 — R_F values of 20 amines and 7 phenols.

GEMZOVÁ, I. UND GASPARIČ, J.: Identifizierung organischer Verbindungen. LXIV. Papierchromatographie wasserunlöslicher primärer aromatischer Amine in Systemen mit polarer stationärer Phase. *Mikrochim. Acta*, (1966) 310–321 — R_F values of 102 aromatic amines.

18. AMINO ACIDS

JOLLÈS-BERGERET, B.: Synthèse et propriétés des acides L- et DL-homocystéinesulfiniques et de l'acide L-homocystéique. *Bull. Soc. Chim. Biol.*, 48 (1966) 1265–1278 — PC and TLC.

RADHAKRISHNAMURTHY, B. AND BERENSON, G. S.: Glycopeptides from bovine aorta glycoprotein. *J. Biol. Chem.*, 241 (1966) 2106–2112 — PC of amino acids and sugars; 2,4-DNP-amino acids on glass paper.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

MCDONALD, J. K., ELLIS, S. AND REILLY, T. J.: Properties of dipeptidyl arylamidase I of the pituitary. Chloride and sulfhydryl activation of seryltyrosyl-β-naphthylamide hydrolysis. *J. Biol. Chem.*, 241 (1966) 1494–1501.

MOMOSE, K. AND KAJI, A.: Soluble amino acid-incorporating system. *J. Biol. Chem.*, 241 (1966) 3294–3307.

MORÁVEK, L., KLUH, I., JUNGE, J. M., MELOUN, B. AND ŠORM, F.: On proteins. CIII. Structure of peptides isolated from chymotryptic digest of bovine S-carboxymethyl-chymotrypsinogen A. *Collection Czech. Chem. Commun.*, 31 (1966) 1604–1627.

NAGASAWA, S., MIZUSHIMA, Y., SATO, T., IWANAGA, S. AND SUZUKI, T.: Studies on the chemical nature of bovine bradykininogen: determination of amino acid, carbohydrate, amino and carboxyl terminal residues. *J. Biochem. (Tokyo)*, 60 (1966) 643–652.

TOKUYAMA, H., KUBO, S. AND TONOMURA, Y.: On the active site of myosin A-adenosine triphosphatase. VI. Existence of asparaginyl-prolyl-prolyl-lysine near the active site. *J. Biochem. (Tokyo)*, 60 (1966) 701–706.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BERNFIELD, M. R.: Ribonuclease and oligoribonucleotide synthesis. II. Synthesis of oligonucleotides of specific sequence. *J. Biol. Chem.*, 241 (1966) 2014–2023 — DEAE cellulose paper.

CHLÁDEK, S., ŽEMLIČKA, J. AND ŠORM, F.: Oligonucleotidic compounds. XII. Synthesis of cytidylyl-(3′→5′)-cytidylyl-(3′→5′)-adenosine (CpCpA), an acceptor sequence of soluble ribonucleic acid. *Collection Czech. Chem. Commun.*, 31 (1966) 1785–1802 — R_F values of 19 compounds.

COLE, C. V. AND ROSS, C.: Extraction, separation, and quantitative estimation of soluble nucleotides and sugar phosphates in plant tissues. *Anal. Biochem.*, 17 (1966) 526–539 — PC and TLC.

ELEY, J. AND ROTH, J. S.: The specificity of a nuclease from chicken pancreas. *J. Biol. Chem.*, 241 (1966) 3070–3077.

HOLÝ, A. AND ŠORM, F.: Nucleic acids components and their analogues. LXXX. Preparation of nucleoside phosphites by reaction of nucleosides with triphenyl phosphite. *Collection Czech. Chem. Commun.*, 31 (1966) 1544–1561 — R_F values of 35 derivatives.

HOLÝ, A. AND ŠORM, F.: Nucleic acids components and their analogues. LXXXI. A selective synthesis of ribonucleoside-2′(3′)-phosphites. *Collection Czech. Chem. Commun.*, 31 (1966) 1564–1568 — R_F values of 16 derivatives.

KOWOLLIK, G. AND LANGEN, P.: Nucleotidanaloge Thymidinsulfate. *Chem. Ber.*, 99 (1966) 2725–2735 — PC and TLC of 8 compounds.

PRYSTAŠ, M. AND ŠORM, F.: Nucleic acid components and their analogues. LXXV. Synthesis of 6-furfurylthionebularine. *Collection Czech. Chem. Commun.*, 31 (1966) 1028–1034 — PC and TLC.

WAGNER, O. W., LEE, Jr., H. A., FREY, P. A. AND ABELES, R. H.: Studies on the mechanism of action of cobamide coenzymes. Chemical properties of the enzyme–coenzyme complex. *J. Biol. Chem.*, 241 (1966) 1751–1762 — PC and TLC.

See also TLC section.

22. ALKALOIDS

PAWELCZYK, E. AND WACHOWIAK, R.: (Analysis of decomposition products of drugs. XVI. Chromatographic evaluation of ageing solutions of quinidine sulphate). *Chem. Anal. (Warsaw)*, 11 (1966) 885–890 — R_F values of 19 decomposition products; quinidine sulphate was determined colorimetrically after elution.

SLAVÍKOVÁ, L. AND SLAVÍK, J.: Alkaloide der Mohngewächse (Papaveraceae). XXXII. Über die Alkaloide aus *Hunnemannia fumariaefolia* Sweet und über die Konstitution des Alkaloids HF 1. *Collection Czech. Chem. Commun.*, 31 (1966) 1355–1362.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

RATAJEWICZ, D.: (Influence of the structure of certain acridine derivatives on their distribution and adsorption during chromatography on wet buffered paper). *Chem. Anal. (Warsaw)*, 11 (1966) 929–936.

WAY, J. L., FEITKNECHT, U. F., CORDER, C. N. AND MIRANDA, P. M.: Isolation and characterization of a metabolite of 1,1′-trimethylene bis(4-aldoximinopyridinium) dibromide. *Biochim. Biophys. Acta*, 121 (1966) 432–434.

See also TLC section.

24. ORGANIC SULPHUR COMPOUNDS

LATINÁK, J.: Chromatographie von Farbstoffzwischenprodukten. XV. Chromatographische Trennung von Naphthalinsulfonsäuren mit Elektrolytlösungen. *Mikrochim. Acta*, (1966) 349–356 — R_F values of 11 derivatives.

27. VITAMINS

ATLAS, S. J. AND PINTER, K. G.: Glass-fiber paper chromatography of vitamin E. *Anal. Biochem.*, 17 (1966) 258–262.

SUGIURA, K. AND GOTO, M.: Isolation and identification of 6-hydroxymethyllumazine from spinach. *J. Biochem. (Tokyo)*, 60 (1966) 335–337.

WAITE, M. AND WAKIL, S. J.: Studies on the mechanism of action of acetyl coenzyme A carboxylase. III. Enzyme-bound 1-N-carboxybiotin as the carboxylation intermediate. *J. Biol. Chem.*, 241 (1966) 1909–1914 — acetylated paper.

28. ANTIBIOTICS

HUANG, H. T. AND ENGLISH, A. R.: Production of 6-aminopenicillanic acid. *U.S. Pat.*, 3,239,427 (Cl. 195–36) March 8, 1966, Appl. Oct. 12, 1959; *C.A.*, 64 (1966) 14925f.

SCHNEIDER, C. H. AND DE WECK, A. L.: Chemische Aspekte der Penicillin-Allergie: Die direkte Penicilloylierung von ε-Aminogruppen durch Penicilline bei pH 7.4. *Helv. Chim. Acta*, 49 (1966) 1695–1707.

SCHNEIDER, C. H. AND DE WECK, A. L.: Chemische Aspekte der Penicillin-Allergie: die direkte Bildung der Penicilloyl-Determinanten aus Penicillin. *Helv. Chim. Acta*, 49 (1966) 1707–1714.

YUKIOKA, M., SAITO, Y. AND OTANI, S.: Enzymatic hydrolysis of gramicidin S. *J. Biochem. (Tokyo)*, 60 (1966) 295–302.

30. SYNTHETIC AND NATURAL DYES

CEE, A. AND GASPARIČ, J.: Identifizierung organischer Verbindungen. LX. Identifizierung der Pyrazolonkomponenten in Azofarbstoffen. *Mikrochim. Acta*, (1966) 268–278 — R_F values of 23 4-aminopyrazolone derivatives.

GASPARIČ, J., GEMZOVÁ, I. AND ŠNOBL, D.: Identifizierung organischer Verbindungen. LIX. Untersuchung über die Beziehung zwischen der Struktur und dem chromatographischen Verhalten wasserunlöslicher Azofarbstoffe. *Collection Czech. Chem. Commun.*, 31 (1966) 1712–1741 — R_F and R_M values of 88 azo dyes.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

DEBELMAS, J. AND HÉRISSET, A.: Etude de la conservation des fleurs de Chamomille romaine par lyophilisation. I. Essais préliminaires. *Ann. Pharm. Franc.*, 24 (1966) 93–99 — PC and TLC.

33. INORGANIC SUBSTANCES

HEJTMÁNEK, M. AND HOZMANOVÁ, E.: Zur spektrophotometrischen Bestimmung von Cäsium und Rubidium als Pikrate nach papierchromatographischer Trennung. *Mikrochim. Acta*, (1966) 97–104.

PRZESZLAKOWSKI, S. AND SOCZEWINSKI, E.: (Chromatography of anionic bromide and iodide complexes of metals on paper impregnated with tri-*n*-octylamine). *Chem. Anal. (Warsaw)*, 11 (1966) 895–903.

ROMANOWSKI, H.: (Attempted paper-chromatographic and complexometric estimation of zinc impurities in air). *Med. Pracy*, 16 (1965) 461–464; *C.A.*, 64 (1966) 14852f.

34. RADIOACTIVE AND ISOTOPIC COMPOUNDS

SORANTIN, H.: Herstellung radioaktiver Standards für papierchromatographische Trennungen. 2. Mitt.: Trennung von ^{90}Sr und ^{90}Y mit Hilfe des Ringofens. *Mikrochim. Acta*, (1966) 957–964.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

FRANC, J., KOVÁŘ, V. AND MIKEŠ, F.: Identification of stilbene derivatives by paper and gas chromatography and paper electrophoresis. *Mikrochim. Acta*, (1966) 133–146 — R_F values of 35 substances.

POSPÍŠIL, J., PETRÁNEK, J. AND TAIMR, L.: Antioxydantien und Stabilisatoren. X. Bemerkung zur Darstellung einiger Hydrochinon-Alkylderivate. *Collection Czech. Chem. Commun.*, 31 (1966) 98–105 — R_F values of 15 quinones and phenols.

Thin-layer Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

HEŘMÁNEK, S. AND PLEŠEK, J.: Chemistry of boranes. III. Relationship between structure and chromatographic behaviour of boranes and their addition compounds with Lewis bases. *Collection Czech. Chem. Commun.*, 31 (1966) 1975–1984.

3. TECHNIQUES I

ACHÁVAL, A. AND ELLEFSON, R. D.: Device for applying samples as a narrow band to thin-layer chromatograms. *J. Lipid Res.*, 7 (1966) 329–330.

BANCROFT, K. C. C.: Cheap "Double-S-tank" for thin layer chromatography. *Chem. Ind. (London)*, (1966) 621.

COLLINGS, A. J.: A simple thin layer chromatography spreading device. *Chem. Ind. (London)*, (1966) 576.

CURTIS, P. J.: Apparatus for applying samples to thin layer chromatograms. *Chem. Ind. (London)*, (1966) 247–248.

DITTRICH, S.: Feststellung der Anzahl Hydroxylgruppen in organischen Substanzen durch Dünnschichtchromatographie. *Mikrochim. Acta*, (1966) 447–450 — acetylation.

JORK, H.: Direkte spektralphotometrische Auswertung von Dünnschicht-Chromatogrammen im UV-Bereich. *Z. Anal. Chem.*, 221 (1966) 17–33.

LORENTZ, K. AND HOFFMEISTER, H.: Untersuchungen über den Einfluss der Wasserstoffionenkonzentration auf die Spezifität der TTC-Reaktion. *Mikrochim. Acta*, (1966) 1062–1067 — TLC and PC.

PIORR, W. AND TÓTH, L.: Ein allgemein anwendbares Bindemittel (Henkel-Metylan-Spezial) zur Herstellung von wässrigen Streichmittel-Suspensionen für die Dünnschichtchromatographie. *Z. Anal. Chem.*, 223 (1966) 33–34.

PITRA, J.: Separation methods for natural products. XI. Detection with morin in chromatography on silicagel. *Collection Czech. Chem. Commun.*, 31 (1966) 1402–1404.

SCOTT, T. W. AND BEESTON, J. W. U.: Apparatus for applying samples to thin-layer chromatograms. *J. Lipid Res.*, 7 (1966) 456–457.

STAHL, E.: Gradient- und Transfer-Techniken in der Chromatographie. *Z. Anal. Chem.*, 221 (1966) 3–17.

WAKSMUNDZKI, A. AND RÓZYLO, J.: (An apparatus for direct, rapid and precise evaluation of R_F values in thin-layer chromatography). *Chem. Anal. (Warsaw)*, 11 (1966) 1255–1256.

4. TECHNIQUES II

GRINDLAY, J. W. AND WRIGHT, J. C.: Simple preparative scale TLC apparatus. *Chem. Ind. (London)*, (1966) 1377.

7. PHENOLS

BREITBURD, P., PUISIEUX, F. AND LE HIR, A.: Identification des polyoxyéthylèneglycols par chromatographie sur couches minces. *Ann. Pharm. Franc.*, 24 (1966) 191–199.

9. OXO COMPOUNDS

HASHMI, H. M. AND SHAHID, M. A.: Circular thin-layer chromatography of aromatic and α,β-unsaturated aldehydes. *Mikrochim. Acta*, (1966) 518–521 — oxo compounds as isonicotinoylhydrazones.

10. CARBOHYDRATES

LAMKIN, W. M., WARD, D. N. AND WALBORG, Jr., E. F.: Quantitative determination of neutral monosaccharide residues in glycoproteins and glycopeptides by thin-layer densitometry. *Anal. Biochem.*, 17 (1966) 485–494 — cellulose layers.

See also PC section.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

JONES, J. A. AND BLECHER, M.: Synthesis and characterization of 3-ketohexadecanoic acid-1-^{14}C, DL-3-hydroxyhexadecanoic acid-1-^{14}C, and *trans*-2-hexadecenoic acid-1-^{14}C. *J. Lipid Res.*, 7 (1966) 422–426.

MORRIS, J. L. AND MARSHALL, M. O.: Occurrence of stearolic acid in Santalaceae seed oils. *Chem. Ind. (London)*, (1966) 460–461.

MYERS, W. F. AND HUANG, K.-Y.: Separation of intermediates of the citric acid cycle and related compounds by thin-layer chromatography. *Anal. Biochem.*, 17 (1966) 210–213 — two-dimensional TLC of 13 acids on cellulose layers.

See also PC section.

11b. Lipids and their constituents

BOOTH, D. A., GOODWIN, H. AND CUMINGS, J. N.: Abnormal gangliosides in Tay-Sachs disease, Niemann-Pick's disease, and gargoylism. *J. Lipid Res.*, 7 (1966) 337–340.

CARLIER, A., PUISIEUX, F., MIET, C. AND LE HIR, A.: Analyse des pommades. II. Application de la chromatographie sur couches minces à l'identification des constituants des huilles et des cires. *Ann. Pharm. Franc.*, 24 (1966) 349–356.

CLARK, A. H., FLINT, C. F. AND MCGEE, D. L.: A thin layer chromatographic study of sulphurised fatty acids and their esters. *Chem. Ind. (London)*, (1966) 729–730.

DODGE, J. T. AND PHILLIPS, G. B.: Autoxidation as a cause of altered lipid distribution in extracts from human red cells. *J. Lipid Res.*, 7 (1966) 387–395.

FREEMAN, C. P. AND WEST, D.: Complete separation of lipid classes on a single thin-layer plate. *J. Lipid Res.*, 7 (1966) 324–327.

GLOMSET, J. A., JANSSEN, E. T., KENNEDY, R. AND DOBBINS, J.: Role of plasma lecithin:cholesterol acyltransferase in the metabolism of high density lipoproteins. *J. Lipid Res.*, 7 (1966) 638–648.

GLUCK, L., KULOVICH, M. V. AND BRODY, S. J.: Rapid quantitative measurement of lung tissue phospholipids. *J. Lipid Res.*, 7 (1966) 570–574.

HAJRA, A. K., BOWEN, D. M., KISHIMOTO, Y. AND RADIN, N. S.: Cerebroside galactosidase of brain. *J. Lipid Res.*, 7 (1966) 379–386.

ITASAKA, O.: Biochemistry of Shellfish lipid. VI. Occurrence of 3-O-methylfucose in *Corbicula* glycolipid. *J. Biochem. (Tokyo)*, 60 (1966) 435–438 — TLC and PC.

KEAN, E. L.: Separation of gluco- and galactocerebrosides by means of borate thin-layer chromatography. *J. Lipid Res.*, 7 (1966) 449–452.

KLOPFENSTEIN, W. E. AND SHIGLEY, J. W.: Method for recovering sulfolipid from plant lipid extracts. *J. Lipid Res.*, 7 (1966) 564–565 — combination of column and TL chromatography.

LUBOCHINSKY, B., MEURY, J. AND STOLKOWSKI, J.: Transport du potassium et synthèse des phospholipides chez *l'Escherichia coli*. II. Cinétiques comparées de l'incorporation de (^{32}P) phosphate dans les phospholipides et du transport simultané de K dans les souches B163 et B525. *Bull. Soc. Chim. Biol.*, 48 (1966) 873–885.

MAKITA, A., SUZUKI, CH. AND YOSIZAWA, Z.: Chemical and immunological characterization of the Forssman hapten isolated from equine organs. *J. Biochem. (Tokyo)*, 60 (1966) 502–513.

PANGBORN, M. C. AND MCKINNEY, J. A.: Purification of serologically active phosphoinositides of *Mycobacterium tuberculosis*. *J. Lipid Res.*, 7 (1966) 627–633.

POPOVIC, M.: Sphingosine and its lower homologues from bovine heart sphingomyelin fraction. *Biochim. Biophys. Acta*, 125 (1966) 178–182.

RAO, P. B. AND SREENIVASAN, B.: Separation of mixtures of fatty acids, alcohols and esters or glycerides by thin-layer chromatography. *Chem. Ind. (London)*, (1966) 1376–1377.

ROOTS, B. I. AND JOHNSTON, P. V.: Lipids and pigments of the chloragosomes of the earthworm *Lumbricus terrestris*. *Comp. Biochem. Physiol.*, 17 (1966) 285–288; *C.A.*, 64 (1966) 14481g.

SCHMID, H. H. O. AND MANGOLD, H. K.: Alkoxylipids. II. "Neutral plasmalogens" in the liver oil of the ratfish (*Hydrolagus colliei*). *Biochim. Biophys. Acta*, 125 (1966) 182–184.

SERDAREVICH, B. AND CARROLL, K. K.: Synthesis and characterization of 1- and 2-monoglycerides of anteiso fatty acids. *J. Lipid Res.*, 7 (1966) 277–284 — silica gel impregnated with boric acid.

SVENNERHOLM, E., STÄLLBERG-STENHAGEN, S. AND SVENNERHOLM, L.: Fatty acid composition of sphingomyelins in blood, spleen, placenta, liver, lung and kidney. *Biochim. Biophys. Acta*, 125 (1966) 60–69 — TLC and PC.

WHITTLE, K. J., DUNPHY, P. J. AND PENNOCK, J. F.: Detection of lipids on reversed-phase chromatograms with fluorescein. *Chem. Ind. (London)*, (1966) 1303 — TLC and PC; only kieselguhr plates permit the localization of both U.V.-absorbing and U.V.-transparent substances.

YESAIR, D. W., ROGERS, W. I., FUNKHOUSER, J. T. AND KENSLER, C. J.: Purification and characterization of phthalanilide–lipid complexes from tissues. *J. Lipid Res.*, 7 (1966) 492–500 — quantitative analysis of glycerol after elution.

YURKOWSKI, M. AND BROCKERHOFF, H.: Fatty acid distribution of triglycerides determined by deacylation with methyl magnesium bromide. *Biochim. Biophys. Acta*, 125 (1966) 55–59.

See also PC section.

13. STEROIDS

BETSUKI, S.: Stero-bile acids and bile alcohols. LXXXIV. The metabolism of trihydroxycoprostane in Bull Frog. *J. Biochem. (Tokyo)*, 60 (1966) 411–416.

DANIELSSON, H. AND EINARSSON, K.: On the conversion of cholesterol to 7α,12α-dihydroxycholest-4-en-3-one. *J. Biol. Chem.*, 241 (1966) 1449–1454.

ENEROTH, P., GORDON, B., RYHAGE, R. AND SJÖVALL, J.: Identification of mono- and dihydroxy bile acids in human feces by gas–liquid chromatography and mass spectrometry. *J. Lipid Res.*, 7 (1966) 511–523 — purification of Silica Gel G Merck.

GREGG, J. A.: New solvent systems for thin-layer chromatography of bile acids. *J. Lipid Res.*, 7 (1966) 579–581 — R_F values of 12 bile acids.

SHEPHERD, I. S., ROSS, L. F. AND MORTON, I. D.: The detection of sterols on silica gel layers. *Chem. Ind. (London)*, (1966) 1706–1707 — potassium dichromate–sulphuric acid.

14. STEROID GLYCOSIDES AND SAPONINS

EUW, J. VON AND REICHSTEIN, T.: Die Biosynthese der Cardenolid- und Spirostan-Glykoside in *Digitalis lanata* Ehrh. Vergleich der Bildungsraten. *Helv. Chim. Acta*, 49 (1966) 1468–1474.

See also PC section.

15. TERPENE DERIVATIVES

PATTON, S. AND BENSON, A. A.: Phytol metabolism in the bovine. *Biochim. Biophys. Acta*, 125 (1966) 22–32.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

OBRUBA, K., ŠLOSAR, J. AND ARIENT, J.: Identifizierung und Bestimmung der Amino-anthrachinone durch Chromatographie auf Aluminiumoxid. *Mikrochim. Acta*, (1966) 257–267 — R_F values of 64 aminoanthraquinones.

18. AMINO ACIDS

BIELESKI, R. L. AND TURNER, N. A.: Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. *Anal. Biochem.*, 17 (1966) 278–293 — two-dimensional combination of electrophoresis and chromatography.

KÄSER, H. AND GUGLER, E.: (Die dünnschichtchromatographische Bestimmung der ε-Aminocapronsäure in Serum und Urin). *Z. Klin. Chem.*, 3 (1965) 33–35; from *Z. Anal. Chem.*, 222 (1966) 460 — cellulose layers.

WILLEMOT, J. AND PARRY, G.: Recherche rapide de traces d'arginine dans l'ornithine et ses sels et vice versa par chromatographie sur couche mince. *Ann. Pharm. Franc.*, 24 (1966) 209–215.

See also PC section.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

CHRISTIANSON, D. D., SINCLAIR, H. B. AND PAULIS, J. W.: Chromatography of nucleotides in extracts containing salts on layers of anionic, microcrystalline cellulose. *Biochim. Biophys. Acta*, 121 (1966) 412–413.

GERZON, K., JOHNSON, I. S., BODER, G. B., CLINE, J. C., SIMPSON, P. J. AND SPETH, C.: Biological activities of 3-isoadenosine. *Biochim. Biophys. Acta*, 119 (1966) 445–461 — TLC and PC.

PRYSTAŠ, M. AND ŠORM, F.: Nucleic acids components and their analogues. LXXVI. The Hilbert-Johnson reaction of 2,4-dialkoxypyrimidines with 2,3,5-tri-O-benzoyl-D-ribofuranosyl chloride. *Collection Czech. Chem. Commun.*, 31 (1966) 1035–1052.

PRYSTAŠ, M. AND ŠORM, F.: Nucleic acids components and their analogues. LXXVII. Synthesis of anomeric 1-(2-deoxy-D-ribofuranosyl) and 1-(D-ribofuranosyl) derivatives of 5-hydroxymethyluracil. *Collection Czech. Chem. Commun.*, 31 (1966) 1053–1063.

See also PC section.

22. ALKALOIDS

ADAM, G. AND SCHREIBER, K.: Solanum Alkaloide. LXXIV. Intramolekulare Katalyse und 1,4-N →O-Acylwanderung bei (22*S*:25*R*)-22.26-Imino-5α-cholestandiol-(3β.16β)-Derivaten. *Chem. Ber.*, 99 (1966) 3173–3182.

DEBRAY, M., PLAT, M. AND LE MEN, J.: Alcaloïdes des Ménispermacées africaines *Epinetrum cordifolium* et *Epinetrum mangenotii*: isolement de la cycléanin, de la nor-cycléanin et de isochondrodendrine. *Ann. Pharm. Franc.*, 24 (1966) 551–558.

KNABE, J. AND DETERING, K.: Zum Mechanismus der prototropen Umlagerung von 2-Methyl-1,2-dihydropapaverin. *Chem. Ber.*, 99 (1966) 2873–2879.

VIGNOLI, L., GUILLOT, J., GOUEZO, F. AND CATALIN, J.: Séparation chromatographique sur couches minces des alcaloïdes de l'opium et de certains de leurs dérivés acétylés. I. Mise au point des protocoles expérimentaux. *Ann. Pharm. Franc.*, 24 (1966) 461–468.

VIGNOLI, L., GUILLOT, J., GOUEZO, F. AND CATALIN, J.: Séparation chromatographique sur couches minces des alcaloïdes de l'opium et de certains de leurs dérivés acétylés. II. Application à quelques échantillons de morphine et d'héroïne d'origine illicite. *Ann. Pharm. Franc.*, 24 (1966) 529–532.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

MCKENNIS, JR., H., TURNBULL, L. B., BOWMAN, E. R. AND LUKHARD, C. N.: Urinary excretion of conjugate forms of 1-(3-pyridyl)ethanol after administration of 3-acetylpyridine. *J. Biol. Chem.*, 241 (1966) 1878–1881 — TLC on chromagram sheets and PC.

24. ORGANIC SULPHUR COMPOUNDS

BUTKIEWICZ, K. AND KOWALCZYK, E.: (Determination of sulphanilamide, sulphaguanidine and sulphamethatine in technical products by thin-layer chromatography). *Chem. Anal. (Warsaw)*, 11 (1966) 1209–1213.

KASUYA, M. AND TAKASHINA, H.: Tryptic digestion of myosin A labeled with 1-dimethylamino-naphthalene-5-sulfonyl chloride. *J. Biochem. (Tokyo)*, 60 (1966) 459–461.

26. ORGANOMETALLIC COMPOUNDS

HEŘMÁNEK, S., PLEŠEK, J. AND GREGOR, V.: Chemistry of boranes. II. Thin layer chromatography of non-ionic boron compounds. *Collection Czech. Chem. Commun.*, 31 (1966) 1281–1287 — R_F values of 20 compounds.

27. VITAMINS

TIRIMANNA, A. S. L. AND WICKREMASINGHE, R. L.: Occurrence of vitamin E in black tea. *Chem. Ind. (London)*, (1966) 1719.

29. INSECTICIDES AND OTHER PESTICIDES

BROOKS, G. T. AND HARRISON, A.: Thin-layer chromatography of trimethylsilyl esters of hydroxylated derivatives of cyclodiene insecticides. *Chem. Ind. (London)*, (1966) 1414–1415.

DELFEL, N. E.: Thin-layer chromatography of rotenone and related compounds. *J. Agr. Food Chem.*, 14 (1966) 130–132; *C.A.*, 64 (1966) 14890h.

30. SYNTHETIC AND NATURAL DYES

BRUSTIER, V., BOURBON, P., AMSELEM, A., ACHOR, G., CASABONNE, M. AND FERRAND, J. C.: Contribution à l'étude de l'identification des colorants de synthèse dans les préparations pharmaceutiques. *Ann. Pharm. Franc.*, 24 (1966) 51–56.

31. PLASTICS AND THEIR INTERMEDIATES

KNAPPE, E. AND ROHDEWALD, I.: Identifizierung von Stickstoffderivaten der Kohlensäure und Untersuchung ihrer Hydrolysate mittels Dünnschichtchromatographie. *Z. Anal. Chem.*, 223 (1966) 174–181.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

BIEDER, A., BRUNEL, P. AND MAZEAU, L.: Identification de trois nouveaux métabolites de l'éthionamide: chromatographie, spectrophotométrie, polarographie. *Ann. Pharm. Franc.*, 24 (1966) 493–500.

POETHKE, W. AND BEHRENDT, H.: (Dünnschichtchromatographische Untersuchungen von Extrakten aus verschiedenen Sprossteilen von *Oreoherzogia fallax*). *Pharm. Zentralhalle*, 104 (1965) 549–553; from *Z. Anal. Chem.*, 223 (1966) 214.

See also PC section.

33. INORGANIC SUBSTANCES

GAGLIARDI, E. AND POKORNY, G.: Beiträge zur quantitativen Dünnschichtchromatographie. II. Trennung der Ammoniumsulfidgruppe; MDCM; als Reagens Bestimmung des Nickels. *Mikrochim. Acta*, (1966) 577–586.

LESIGANG-BUCHTELA, M.: Dünnschichtchromatographie anorganischer Ionen. *Mikrochim. Acta*, (1966) 408–414.

34. RADIOACTIVE AND OTHER ISOTOPIC COMPOUNDS

SCHILDKNECHT, H. *et al.*: Elektronenbrenzen, eine neue Methode zur Analyse kleinster Mengen organischer Substanzen. *Angew. Chem.*, 78 (1966) 841–850 — splitting of organic compounds by means of tritiated water and identification of the fragments by TLC.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

DICKES, G. J.: (Über den dünnschichtchromatographischen Nachweis von Süsstoffen, Konservierungsmitteln und Antioxydantien in Nahrungsmitteln). *J. Assoc. Public Analysts*, 3 (1965) 118–123; from *Z. Anal. Chem.*, 223 (1966) 153.

FREIMUTH, U., FOERSTER, K. AND LUDWIG, E.: (Ein dünnschichtchromatographisches Verfahren zum Nachweis von phenolischen Antioxydantien in Schmalz, Margarine, Fleisch- und Wurstkonserven und Gebäck). *Nahrung*, 9 (1965) 155; from *Z. Anal. Chem.*, 223 (1966) 155.

Gas Chromatography

1. REVIEWS AND BOOKS

BEREZKIN, V. G.: (*Analytical Reaction Gas Chromatography*). Nauka, Moscow, 1966, 184 pp. — a book of 8 chapters giving selected literature on various topics.

GAVRILINA, L. YA. AND VYAKHIREV, D. A.: (Gas chromatography in catalytic studies). *Usp. Khim.*, 36 (1967) 362–375 — 202 references.

PALFRAMAN, J. F. AND WALKER, E. A.: Techniques in gas chromatography. Part I. Choice of solid supports. A review. *Analyst*, 92 (1967) 71–82 — 66 references.

POMMIER, C.: Application de la chromatographie en phase gazeuse à la séparation des composés minéraux et organométalliques. *Rev. Chim. Minerale*, 3 (1966) 401–437 — a review with 206 references (halogenides, chelates, hydrides, and organometallic compounds).

SCOTT, R. P. W.: Gas–liquid chromatography: Recent developments in apparatus and technique. *Brit. Med. Bull.*, 22 (1966) 131–136.

2. FUNDAMENTALS, THEORY AND GENERAL

2c. Thermodynamics and theoretical relationships

MYERS, M. N.: High inlet pressures in gas chromatography. *Dissertation Abstr.*, 26 (1966) 4271.

PECSAR, R. E.: Solution thermodynamics from gas–liquid chromatography. *Dissertation Abstr.*, 26 (1965) 3190.

PECSAR, R. E. AND MARTIN, J. J.: Solution thermodynamics from gas–liquid chromatography. *Anal. Chem.*, 38 (1966) 1661–1669 — data were successfully correlated with a linear relation between the partial molar excess free energy and hydrocarbon chain length of the solute for a homologous series in a given solvent.

VINOGRADOVA, L. M., VYAKHIREV, D. A. AND ZHUKHOVITSKIĬ, A. A.: (Relative role of external and internal diffusion in broadening the chromatographic peaks). *Zh. Fiz. Khim.*, 61 (1967) 233–236 — the partition process was found to be controlled by external diffusion in GSC and by internal diffusion in GLC (low partition coefficients); computer technique used for calculation.

2d. General

FRASER, R. D. B. AND SUZUKI, E.: Resolution of overlapping absorption bands by least squares procedures. *Anal. Chem.*, 38 (1966) 1770–1773 — also applicable for GC purposes.

SAKHAROV, V. M., LEONT'EVA, S. A. AND LULOVA, N. I.: (Method of identification of complex mixtures by means of gas chromatography). *Khim. i Tekhnol. Topliv i Masel*, No. 1 (1967) 59–62 — the functions $\varphi = (\ln V_{g(i)} - \ln V_{g(1)})/(\ln V_{g(2)} - \ln V_{g(1)})$ and $\varphi_T = (T_i - T_1)/(T_2 - T_1)$ are recommended; T = boiling point temperature.

ZHOU, L.-M. AND LU, P.-CH.: Contribution to the dynamic theory of gas chromatography. Application of the lumped film model. *Sci. Sinica (Peking)*, 14 (1965) 859–868.

3. TECHNIQUES I

3a. Detectors

ARNIKAR, H. J., RAO, T. S. AND KARMARKAR, K. H.: The use of an electrodeless discharge as a detector in gas chromatography. *J. Chromatog.*, 26 (1967) 30–34 — a Siemens type all-glass discharge tube at 50 c/s; scheme given; 10^{-4} A for 1 μmole of H_2, O_2 and Ar.

BOURKE, P. J. AND DAWSON, R. W.: Sensitivity to oxygen of an ionization detector with helium neon as carrier gases. *Nature*, 211 (1966) 409–410.

CRAMER, W. A., HOUTMAN, J. P. W., KOCH, R. O. AND PIET, G. J.: Measurement of radioactivity in effluents of a gas chromatograph. The flame ionization detector used as a combustion chamber in combination with specially designed adsorbers. *Intern. J. Appl. Radiation Isotopes*, 17 (1966) 97–101.

HAMPTON, W. C.: Radiofrequency cell for gas chromatography. *U.S. Pat.*, 3,223,873 (Dec. 14, 1965).

HENZE, J. C. AND MCLEOD, P. C.: Square cavity whistle detector. *U.S. Pat.*, 3,229,501 (Jan. 18, 1966).

HOFFMANN, R. L. AND EVANS, C. D.: Thermal conductivity effect of carrier gases on flame ionization detector sensitivity. *Science*, 153 (1966) 172–173.

LEVY, E. J. AND MIKKELSON, L.: Detection method and apparatus for gas chromatography. *U.S. Pat.*, 3,257,847 (June 28, 1966).

RICHARDSON, B. J. AND NARAIN, C.: Some notes on the direct current discharge detector. *J. Chromatog.*, 26 (1967) 248–251.

3b. Column performance and filling studies

ASSMANN, K., SERFAS, O. AND GEPPERT, G.: Über eine temperaturbeständige flüssige Phase zur Trennung von Polyolen. *J. Chromatog.*, 26 (1967) 495–501 — polyvinylformalpropionitrile as liquid phase, separation by PTGC 150–290°, 10°/min.

BORISENKO, I. V., BRYZGALOVA, N. I., GAVRILOVA, T. B. AND KISELEV, A. V.: (Silica gel as a support in gas–liquid chromatography). *Neftekhimiya*, 6 (1966) 776–783 — properties and synthesis of silica gels with specific surface area 5–6 m^2/g given; GC behaviour is equivallent to Celite 545.

GOLOVNIYA, R. V. AND URALETS, V. P.: (Use of Kováts indices in gas chromatographic analysis on polar stationary phases). *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1967) 185–186 — the effect of the low solubility in β,β'-oxydipropionitrile of *n*-paraffin standards is discussed.

3c. Apparatus, accessories and materials for GC

BIRRELL, G.: Building an inexpensive vapor phase chromatograph. *Chemistry*, 38 (1965) 26–29.

BORTH, R., CANOSSA, A. AND NORYMBERSKI, J. K.: A simple device for the automatic introduction of samples in gas–liquid chromatography. *J. Chromatog.*, 26 (1967) 258–261 — details of the design given.

BUROW, F. H.: Sample preparation and collection means for gas chromatography columns. *U.S. Pat.*, 3,225,520 (Dec. 28, 1965).

BUROW, F. H.: Serially connected thermochromatographic columns. *U.S. Pat.*, 3,225,521 (Dec. 28, 1965).

BUROW, F. H., CLARK, R. O., LICHTENFELS, D. H. AND PETROCELLI, J. A.: Gas chromatography apparatus. *U.S. Pat.*, 3,232,093 (Feb. 1, 1966).

GUILLEMIN, C. L. AND WETROFF, G.: Process for filling columns for separation by gaseous chromatography. *U.S. Pat.*, 3,248,856 (May 3, 1966).

HOFFMANN, R. L. AND EVANS, C. D.: A stream splitter and functional group tester for use with a flame ionization detector. *J. Chromatog.*, 26 (1967) 491–494 — construction details given.

PRISCOTT, B. H.: A simple low cost gas chromatograph for the determination of fatty acids. *Analyst*, 92 (1967) 57–60 — construction details given.

TESAŘÍK, K., HÁNA, K. AND KEPRT, L.: (Working properties of a process gas chromatograph CH-O3). *Chem. Prumysl*, 17 (1967) 88–90.

WILCOX, J. W.: How we developed a mass spectrometer/gas chromatograph interface. *Lab. Management*, 4 (1966) 34–35.

YOUNG, E. T.: Multifunction valve for chromatography. *U.S. Pat.*, 3,223,123 (Dec. 14, 1965).

4. TECHNIQUES II

4a. Preparative-scale GC

BORKA, L. AND PRIVETT, O. S.: Collection of methyl esters in preparative gas–liquid chromatography via electrostatic precipitation techniques. *J. Am. Oil Chemists' Soc.*, 42 (Aug. 1965) 459A (Abr. No. 86).

HARGROVE, G. L. AND SAWYER, D. T.: Experimental studies of mass transfer contributions in preparative gas chromatography. *Anal. Chem.*, 38 (1966) 1634–1638 — the gas-phase mass transfer term is approximately 10 times larger for a 1-in. preparative column than it is for a ¼-in. analytical column.

VERZELE, M. AND VERSTAPPE, M.: Preparative scale gas chromatography. VIII. Further experiments. *J. Chromatog.*, 26 (1967) 485–490.

4d. Special microtechniques and functional analysis

BURKE, M. F.: Pyrolysis–gas chromatography by direct injection of solutions. *Dissertation Abstr.*, 26 (1966) 6337–6338.

FILIPSKA, M., HIPPE, Z., KRZYŻANOWSKA, T. AND STARECZEK, T.: (Gas chromatography in studies of paints and lacquers. V. Determination of alkoxy groups in etherified polycondensation resins). *Chem. Anal. (Warsaw)*, 12 (1967) 39–47.

FRANC, J. AND MIKEŠ, F.: Systematic analysis of nitrogen compounds using cleavage reactions and gas chromatography. *J. Chromatog.*, 26 (1967) 378–386 — scheme of microreactor given; developed for organic analysis of 14 various N-functional groups.

4e. Automation

CLEVETT, K. J.: On-line application of vapour-phase chromatography to process plants. *Instr. Rev.*, 12 (1965) 367–370.

FOSTER, F.: Gas analysis: the key to natural quality control. *Gas*, 41, No. 11 (1965) 72–74.

MCWILLIAMS, I. G.: Gas chromatography and its use for process control. *Australian Chem. Process. Eng.*, 19, No. 4 (1966) 16–19.

OGLE, F. T.: On-line instrumentation and control systems. *Chem. Eng. Progr.*, 61 (1965) 87–92.

OGLESBY, M. W. AND HOBBS, J. W.: Chromatograph analyzers for distillation control. *Oil Gas J.*, 64 (1966) 80–86.
PARKS, Jr., J. C.: Vaporizer for a process gas analyzer. *U.S. Pat.*, 3,245,250 (Apr. 12, 1966).
STALLINGS, J. W.: Considering a stream analyzer. *Oil Gas J.*, 64, No. 22 (1966) 89–94.
STERNBERG, J. C., KRULL, I. H. AND FRIEDEL, G. D.: Fragmentation gas chromatography. *Anal. Chem.*, 38 (1966) 1639–1648 — the breakdown of compounds is performed by electrical discharge on the inlet into the GC system, at atmospheric pressure; scheme and construction details given.

4f. Measurement of physico-chemical and related values

KURKCHI, G. A. AND IOGANSEN, A. V.: (Gas-chromatographic investigation of solutions of acetylene hydrocarbons). *Zh. Fiz. Khim.*, 41 (1967) 158–163 — V_g, α, ΔH and ΔS for C_2–C_5 acetylenes have been evaluated on N,N-dimethylformamide and N-methylpyrrolidone at 25°.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

LI-ZHI HU AND SHAO-YI PENG: A rapid method for the analysis of gaseous hydrocarbon mixtures by gas solid chromatography. *Sci. Sinica (Peking)*, 14 (1965) 1238–1240.

5b. Other hydrocarbons

BAGRIĬ, E. I., AMOSOVA, E. I. AND SANIN, P. I.: (Separation of adamantane from some crude oils of Balakhansk and Surakhansk deposits). *Neftekhimiya*, 6 (1966) 665–670 — on Apiezon N at 195°.
BAUMANN, F. AND CSICSERY, S. M.: Capillary column gas chromatography of C_6–C_{11} aromatic compounds. Synthesis of selected isomers for identification purposes. *J. Chromatog.*, 26 (1967) 262–267 — retention data of 44 aromatics on UCON LB 550X by PTGC 60–130°, 1°/min.
BRUNNOCK, J. V.: Separation and distribution of normal paraffins from petroleum heavy distillates by molecular sieve adsorption and gas chromatography. *Anal. Chem.*, 38 (1966) 1648–1652.
BRYANSKAYA, E. K., ZAKHARENKO, V. A. AND PETROV, A. A.: (Calibration mixtures of C_6–C_8 alkanes for identification of chromatograms of petroleum cuts). *Neftekhimiya*, 6 (1966) 784–787 — retention data of 31 hydrocarbons on squalane at 30°, 50° and 80°.
KUNUGI, T., FUJIMOTO, K., IKEDA, N. AND TAMURA, T.: Analysis of hydrocarbon mixture by capillary gas-chromatography. *J. Japan Petrol. Inst.*, (1964) 168.
LAPIDUS, A. L., DIACHENKO, A. I., BULANOVA, T. F. AND EIDUS, YA. T.: (Analysis of hexane–hexene fractions by the method of capillary chromatography). *Zh. Analit. Khim.*, 22 (1967) 295–298.
LIJINSKY, W. AND DOMSKY, I.: Detection of submicrogram quantities of carcinogenic polynuclear hydrocarbons using electron capture. *Develop. Appl. Spectry*, 4 (1965) 255–263.

5c. Halogen derivatives of hydrocarbons

BIELORAI, R. AND ALUMOT, E.: Determination of ethylene dibromide in fumigated feeds and foods by gas liquid chromatography. *J. Sci. Food Agr.*, 16 (1965) 594–596.

6. ALCOHOLS

PHIFER, L. H. AND PLUMMER, Jr., H. K.: Gas chromatographic separation of polar compounds using water vapor in the carrier gas and water as the liquid phase. *Anal. Chem.*, 38 (1966) 1652–1656 — C_1–C_5 alcohols, C_3–C_4 diols at 60–90°; retention behaviour dependent on solubility; modified FID as detector.
SCHLUNEGGER, U. P.: Zur gaschromatographischen Trennung von aliphatischen, sauerstoffhaltigen Substanzen in Wasser. IV. Eine neue Trennsäule zur direkten, quantitativen Bestimmung von Äthanol in wässeriger Lösung für forensische Zwecke. *J. Chromatog.*, 26 (1967) 1–7 — a mixture of nonylphenol glycerol ether + isooctyl-resorcinol glycerol ether + Alkaterge T at 85°.
WALLACE, J. E. AND DAHL, E. V.: Rapid vapor phase method for determining ethanol in blood and urine by gas chromatography. *Am. J. Clin. Pathol.*, 46 (1966) 152–154.
WEATHERALL, I. L.: Gas chromatography of diols. *J. Chromatog.*, 26 (1967) 251–253 — retention data of C_2–C_7 diols on LAC at 175°.

7. PHENOLS

GERMAN, I. A. AND CIOT, N.: A method for analyzing some chlorophenols by gas chromatography. *Rev. Chim. (Bucharest)*, 17 (1966) 177–178.

LINDGREN, B. O.: Homologous aliphatic C_{30}–C_{45} terphenols in birch wood. *Acta Chem. Scand.*, 19 (1965) 1317–1326.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

GIANTURCO, M. A. AND FRIEDEL, P.: Chemistry of tetrahydrofuranone derivatives. II. A convenient procedure for the synthesis of 3-alkyl-substituted furans. *Can. J. Chem.*, 44 (1966) 1083–1086.

SWEET, F. AND BROWN, R. K.: 2-Alkoxy-3-hydroxytetrahydropyrans and 2-alkoxy-3-hydroxytetrahydrofurans. I. Preparation, structure, and some derivatives. *Can. J. Chem.*, 44 (1966) 1571–1576.

9. OXO COMPOUNDS

BRAND, J. M.: Studies on grass lignins. II. The estimation of lignin oxidation products by gas–liquid chromatography. *J. Chromatog.*, 26 (1967) 373–377 — aldehydes like vanillin on SE-30 by PTGC 100–260°.

LOVE, S. AND BRATZLER, L. J.: Tentative identification of carbonyl compounds in wood smoke by gas chromatography. *J. Food Sci.*, 31 (1966) 218–222.

10. CARBOHYDRATES

LOTT, Jr., C. E. AND BROBST, K. M.: Gas chromatographic investigation of hydroxyethyl amylose hydrolyzates. *Anal. Chem.*, 38 (1966) 1767–1770 — as TMS derivatives on SE-52 by PTGC.

LUDLOW, C. J., HARRIS, T. M. AND WOLF, F. T.: An analysis of the free sugars of ferns by gas liquid chromatography. *Phytochemistry*, 5 (1966) 251–257.

PENICK, R. J. AND MCCLUER, R. H.: Quantitative determination of glucose and galactose in gangliosides by gas chromatography. *Biochim. Biophys. Acta*, 116 (1966) 288–295.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

HAKEN, J. K.: Retention time relationships in the gas chromatography of the methyl esters of branched chain fatty acids. *J. Chromatog.*, 26 (1967) 17–21 — retention data of C_{12}–C_{28} acids on DEGS, EGA, and Apiezon L at 200°, 197°, and 207° respectively.

SCHOLFIELD, C. R., BUTTERFIELD, R. O. AND DUTTON, H. J.: Fractionation of geometric isomers of methyl linolenate by argentation countercurrent distribution. *Anal. Chem.*, 38 (1966) 1694–1697 — 5 isomers were resolved on a capillary column with XE-GO (200 ft. × 0.01 in.).

11b. Lipids and their constituents

GORMICAN, A. T.: Use of thin-layer and gas–liquid chromatography in a study of human and fecal lipids of two healthy men during prolonged gluten feeding. *Dissertation Abstr.*, 26 (1966) 5694.

JAMIESON, G. R. AND REID, E. H.: The analysis of oils and fats by gas chromatography. III. Separation factors of acetates, alcohols and hydrocarbons. *J. Chromatog.*, 26 (1967) 8–16 — numerous retention data.

KADERAVEK, G. AND VOLONTERIO, G.: Some observations on gas chromatographic analysis of short chain free acids. *Riv. Ital. Sostanze Grasse*, 43 (1966) 9–10.

13. STEROIDS

CHALTORAJ, S. CH.: Gas chromatography in the quantitative measurement of the classical estrogens and some newer metabolites. *Dissertation Abstr.*, 26 (1965) 3013.

FUTTERWEIT, W., FREEMAN, R., SIEGEL, G. L., GRIBOFF, S. I., DORFMAN, R. I. AND SOFFER, L. J.: Clinical applications of a gas chromatographic method for the combined determination of testosterone and epitestosterone glucuronide in urine. *J. Clin. Endocrinol. Metab.*, 25 (1965) 1451–1456.

GARDINER, W. L. AND HORNING, E. C.: Gas–liquid chromatographic separation of C_{19} and C_{21} human urinary steroids by a new procedure. *Biochim. Biophys. Acta*, 115 (1966) 524–526.

HEAP, R. B. AND HOLZBAUER, M.: Gas chromatography of androgens, and progesterone derivatives in adrenal venous blood of pigs and dogs. *J. Physiol. (London)*, 183 (1966) 11P.

HEITZMAN, R. J. AND THOMAS, G. H.: Evaluation by gas chromatography of the urinary steroids of the pregnant dairy cow. *J. Endocrinol.*, 33 (1965) 455–467.

MURPHY, D. AND WEST, H. F.: Urinary 17-hydroxycorticosteroids measured by gas chromatography. *Acta Endocrinol., Suppl.*, 100 (1965) 66.

NAKAGAWA, K., MCNIVEN, N. L., FORCHIELLI, E., VERMEULEN, A. AND DORFMAN, R. I.: Determination of testosterone by gas–liquid chromatography using an electron capture detector. I. Response of halo-alkyl derivatives. *Steroids*, 7 (1966) 329–340.

PANICUCCI, F. AND TAPONECO, G.: Gas chromatographic determination of 17-ketosteroids in plasma. *Boll. Soc. Ital. Biol. Sper.*, 41 (1965) 874–877.

ROS, A., MISURALE, F., PARADIA, S. AND CAGNAZZO, G.: Gas chromatographic analysis of purified urine extracts for quantitative determination of 5β-pregnane-3α,20α-diol, 5α-pregnane-3β,20β-diol and 5β-pregnane-3α,17α,20α-triol. *Monit. Ostet.-Ginecol.*, 36 (1965) 253–262.

SPARAGANA, M.: Determination of urinary tetrahydrocortisone, tetrahydrocortisol, and 3α-allotetrahydrocortisol by gas chromatography. *Steroids*, 6 (1965) 583–596.

THIJSSEN, J. H. H.: A gas chromatographic method for the measurement of small amounts of oestrogens in urine. *Acta Endocrinol., Suppl.*, 100 (1965) 85, Abstr. No. 53.

VANDENHEUVEL, W. J. A.: The gas–liquid chromatographic behavior of sterol sulfonates. Effect of structure upon the nature of their elimination reaction. *J. Chromatog.*, 26 (1967) 396–403 — retention data of 32 sterols and sulphonate esters on SE-30 and EGSS-Z at 205°.

VANDENHEUVEL, W. J. A., GARDINER, W. L. AND HORNING, E. C.: A comparison of the behavior of the hydrocarbons used as reference standards in the determination of "steroid numbers" and "methylene units"; selective retention of steroid hydrocarbons by polar stationary phases. *J. Chromatog.*, 26 (1967) 387–395 — retention values on 14 stationary phases at 190° and on QF-1 at 170°, 180° and 190°; differences in retention have been found for polar and nonpolar stationary phases.

15. TERPENE DERIVATIVES

BUTTERY, R. G., BLACK, D. R. AND LING,L.: Methyl geranate in hop oil. *J. Inst. Brewing*, 72 (1966) 202–204.

DAGLIESH, C. E.: Gas liquid chromatography of humulones, lupulones and their rearrangement products. *Chem. Ind. (London)*, (1966) 2187–2188 — on polysiloxane oil by PTGC 140–250°, 2°/min.

18. AMINO ACIDS

BLAU, K. AND DARBRE, A.: Gas chromatography of volatile amino acid derivatives. III. Aspartic acid, lysine, ornithine, tryptophan and tyrosine. *J. Chromatog.*, 26 (1967) 35–40 — retention data of derivatives on 26 stationary phases at 190°.

EASTOE, J. E.: Separation of amino acids. *Brit. Med. Bull.*, 22 (1966) 174–179.

JOHNSON, D. E. AND GOODSON, T.: Research on analysis of amino acids by gas chromatography. *U.S. Govt. Res. Rept.*, 41 (1966) 35 pp.

MUSSINI, E. AND MARCUCCI, F.: The preparation of diazopropane and diazobutane for the esterification of amino acids. *J. Chromatog.*, 26 (1967) 481 — esterification is excellent.

USOVA, E. P.: (Separation of ω-amino alkanoic acids by gas–liquid chromatography.) *Zh. Analit. Khim.*, 22 (1967) 304–305 — C_3, C_5, C_7, C_9, and C_{11} amino acids as N-trifluoroacetyl methyl esters on PEGA at 200°.

22. ALKALOIDS

MARTIN, G. E. AND SWINEHART, J. S.: Use of silyl derivatives in the quantitative gas chromatographic determination of morphine and codeine. *Anal. Chem.*, 38 (1966) 1789–1790 — on ES-30 at 225°.

SCHMERZLER, E., YU, W., HEWITT, M. I. AND GREENBLATT, I. J.: Gas chromatographic determination of codeine in serum and urine. *J. Pharm. Sci.*, 55 (1966) 155–157.

24. ORGANIC SULPHUR COMPOUNDS

JAMES, S. P., WARING, R. H. AND WHITE, D. A.: Separation of mercapturic acids and related compounds by gas chromatography and a method for the determination of hippuric acid. *J. Chromatog.*, 26 (1967) 255–258 — retention data of 19 compounds on QF-1 at 190°, 205°, and 220°.

26. ORGANOMETALLIC COMPOUNDS

BIRKOFER, L. AND DONIKE, M.: Siliciumorganische Verbindungen. 30. Mitt. Zur Darstellung von Trimethylsilylverbindungen für gaschromatographische Untersuchungen. *J. Chromatog.*, 26 (1967) 270–273.

FUJINAGA, T., KUMAMOTO, T. AND ONO, Y.: Gas chromatographic determination of scandium as acetylacetonate and trifluoroacetylacetonate. *J. Chem. Soc. Japan, Pure Chem. Sect.*, 86 (1965) 1294–1299.

HRADIL, J. AND CHVALKOVSKÝ, V.: Organosiliciumverbindungen. IL. Radikalische Halogenierung silylsubstituierter Toluole. *Collection Czech. Chem. Commun*, 32 (1967) 171–182 — on E-301 at 170°C.

REYNOLDS, W. D.: Gas chromatographic investigation of transition metal hexafluoroacetylacetonates. *Dissertation Abstr.*, 26 (1965) 3010–3011.

27. VITAMINS AND HORMONES

RICHARDS, A. H. AND MASON, W. B.: Gas chromatographic separation of some iodine compounds of serum. *Anal. Chem.*, 38 (1966) 1751–1752 — iodinated tyrosine and thyronine on SE-30 at 250°.

VECCHI, M. AND KAISER, K.: The gas chromatographic determination of ascorbic acid in the form of its trimethylsilyl ether derivative. *J. Chromatog.*, 26 (1967) 22–29 — quantitative silylation by N-trimethyl silylacetamide (180 min); on XE-60 or SE-30 at 135° or 170°.

29. INSECTICIDES AND OTHER PESTICIDES

CHENG, K. W. AND KILGORE, W. W.: Determination of 2,6-dichloro-4-nitroaniline residues in fruits by electron-capture gas chromatography. *J. Food Sci.*, 31 (1966) 259–261.

CHENG, K. W. AND KILGORE, W. W.: Gas chromatographic determination of pentachlorophenol and sodium pentachlorophenate residues in fruits. *J. Food Sci.*, 31 (1966) 742–745.

CROSBY, D. G. AND ARCHER, T. E.: A rapid analytical method for persistent pesticides in proteinaceous samples. *Bull. Environmental Contamination Toxicol*, 1 (1966) 16–20.

DALE, W. E., COPELAND, M. F. AND HAYES, JR., W. J.: Chlorinated insecticides in the body fat of people in India. *Bull. World Health Organ.*, 33 (1965) 471–477.

DALE, W. E., CURLEY, A. AND CUETO, JR., C.: Hexane-extractable chlorinated insecticides in human blood. *Life Sci.*, 5 (1966) 47–54.

GUTENMANN, W. H. AND LISK, D. J.: Gas chromatographic determination of phenolic pesticides and residues. *J. Assoc. Offic. Agr. Chemists*, 48 (1965) 1173–1176.

JENSEN, D. J. AND SCHALL, E. D.: Determination of vapor pressures of some phenoxyacetic herbicides by gas–liquid chromatography. *J. Agr. Food Chem.*, 14 (1966) 123–125; for some corrections, see *ibid.*, p. 440.

KADIS, V. W. AND JONASSON, O. J.: The detection and persistence of chlorinated insecticides in human and animal blood. *Can. J. Public Health*, 56 (1965) 433–436.

KAWAHARA, T. AND KANAZAWA, J.: Determination of heptachlor by gas liquid chromatography. *Japan Analyst*, 14 (1965) 723–725.

MARKARIAN, H. E., HENRY, F. AND PRATT, JR., J. J.: Colorimetric and gas chromatographic measurements of Dieldrin residues in wool. A comparison of analytical methods. *Am. Dyestuff Reptr.*, 55 (1966) 31–32.

NOVAK, A. F. AND RAO, M. R. R.: Food safety program: Endrin monitoring in the Mississippi river. *Science*, 150 (1965) 1732–1733.

RUDOMSKI, J. L. AND FISEROVA-BERGEROVA, V.: The determination of pesticides in tissues with the electron capture detector without prior cleanup. *Ind. Med. Surg.*, 34 (1965) 934–939.

STEVENS, R. D. AND VAN MIDDELEM, C. H.: Analysis of Bidrin as iodoform by electron capture gas–liquid chromatography. *J. Agr. Food Chem.*, 14 (1966) 149–152.

THORNTON, J. S. AND ANDERSON, C. A.: Electron capture gas chromatographic determination of residues of Chemagro 2635 in crops and soils. *J. Agr. Food Chem.*, 13 (1965) 509–511.

31. PLASTICS AND THEIR INTERMEDIATES

ALISHOEV, V. R., BEREZKIN, V. G., KOROLEV, A. A. AND TUTORSKIĬ, I. A.: (Gas-chromatographic study of the products of rubber pyrolysis.) *Zh. Analit. Khim.*, 22 (1967) 151–156.

ČELEFÍN, P., DOSKOČILOVÁ, D. AND ŠEBENDA, J.: (Basic polymerisation of 6-caprolactam. XXVIII. Determination of monomers in polymerisation and copolymerisation products of caprolactam and C-methylcaprolactams). *Chem. Prumysl*, 17 (42) (1967) 73–78 — α, ε, $\beta + \delta$ and γ-isomers on PEGA at 160°.

DAVIS, A. AND GOLDEN, J. H.: Quantitative analysis of bisphenol A by direct gas chromatography. *J. Chromatog.*, 26 (1967) 254–255 — on Apiezon K at 250°.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

JOHNSON, D. E., RODRIGUEZ, CH. F. AND BURCHFIELD, H. P.: Determination by microcoulometric gas chromatography of chlorpromazine metabolites in human urine. *Biochem. Pharmacol.*, 14 (1965) 1453–1469.

KORZUN, B. P., BRODY, S. M., KEEGARE, P. G., LUDERS, R. C. AND REHM, C. R.: Rapid chromatographic method for the identification and estimation of glutethimide (Doriden) in blood. *J. Lab. Clin. Med.*, 68 (1966) 333–339.

KOSHY, K. T., WICKERSHAM, H. C. AND DUVALL, R. N.: Gas chromatographic analysis of phenacetin and probable contaminants—acetanilide, *p*-chloro-acetanilide, and *p*-phenetidine. *J. Pharm. Sci.*, 54 (1965) 1547–1549.

SHYLUK, J. P., YOUNGS, C. G. AND GAMBORG, O. L.: Gas chromatography of the trimethylsilyl derivatives of shikimic acid and biochemically related compounds. *J. Chromatog.*, 26 (1967) 268–269 — retention data on QF-1 at 150°, on SE-30 at 160°, and on XE-60 at 170°.

WOLFSON, B., CICARELLI, H. E. AND SIKER, E. S.: Gas chromatography using an internal standard for the estimation of methoxyflurane levels in blood. *Brit. J. Anaesthesia*, 38 (1966) 29–34.

YAMAMURA, H., WAKASUGI, B., SATO, S. AND TAKEBE, Y.: Gas chromatographic analysis of inhalation anesthetics in whole blood by an equilibrium method. *Anesthesiology*, 27 (1966) 311–317.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

BENNETT, D.: Analysis of gas mixtures by gas chromatography. *J. Chromatog.*, 26 (1967) 482–484 — combination of Porapak Q and molecular sieve 5A; column arrangement given.

DANZER, L. A., KEARNS, K. L. AND COHN, J. E.: Gas chromatography and pulmonary diffusing capacity measurements. *J. Appl. Physiol.*, 20 (1965) 1359–1361.

SMITH, T. C.: Carbon monoxide, neon, acetylene analysis in the presence of anesthetic gases. *J. Appl. Physiol.*, 21 (1966) 745–746.

33b. Volatile inorganic compounds

FOGL, J. AND FORTELNÝ, M.: (Determination of cyanogen chloride by gas chromatography). *Chem. Prumysl*, 17 (1967) 42–43 — on silicone oil at 50°.

GALLA, S. J., OTTENSTEIN, D. M. AND SANCETTA, S. M.: Analysis of nitrous oxide by gas chromatography in the measurement of cerebral and coronary blood flow. *J. Lab. Clin. Med.*, 67 (1966) 678–688.

GOLDUP, A. AND WESTAWAY, M. T.: Determination of trace quantities of water in hydrocarbons. Application of the calcium carbide–gas chromatographic method to streams containing methanol. *Anal. Chem.*, 38 (1966) 1657–1661 — the temperature of H_2O/C_2H_2 conversion has been found to be critical ($< 40°$ necessary); H_2O in propane at $-45°$, and in *n*-butane at $-6.5°$; errors 10% at 2 v.p.m. and 1% at 250 v.p.m.

OLDS, F. M. W., PATRICK, J. W. AND SHAW, F. H.: Determination of sulphur in carbons and cokes by gas chromatography. *Analyst*, 92 (1967) 54–56 — a combustion–chromatographic method.

34. RADIOACTIVE AND ISOTOPIC COMPOUNDS

KING, JR., J. AND BENSON, S. W.: Theory of low-temperature chromatographic separation of hydrogen isotopes. *J. Chem. Phys.*, 44 (1966) 1007–1014.

TODD, J. F. J.: A column-switching technique for the analysis of mixtures of fluorocarbons labelled with a short lived isotope. *J. Chromatog.*, 26 (1967) 273–275 — $CF_3{}^{18}F$, $CH_3{}^{18}F$, $C_2H_3{}^{18}F$, and $CF_2{}^{18}FI$ on silicone oil at 0°.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

ALEKSEEVA, K. V. AND NIKONOVA, V. YA.: (Investigation of composition of impurities in water layer of catalytic products of oxidative dehydrogenation of butenes). *Neftekhimiya*, 6 (1966) 792–796 — retention data of 16 oxygenated compounds on PEG 1000 and DNP at 85°.

BICHO, J. O., ZARVARIN, E. AND BRINK, D. L.: Oxidative degradation of wood. II. Products of alkaline nitrobenzene oxidation by a methylation gas chromatographic technique. *Tappi*, 49 (1966) 218–226.

JONES, K. AND WILLS, R.: Separation and composition of Scotch whiskey fusel oil. *J. Inst. Brewing*, 72 (1966) 196–201.

MERRIMANN, M. M., CHOULETT, H. AND BRINK, D. L.: Oxidative degradation of wood. I. Analysis of products of oxygen oxidation by gas chromatography. *Tappi*, 49 (1966) 34–39.

TERPENOIDS

CXVI. EVALUATION OF POLYESTERS AS STATIONARY PHASES IN GAS–LIQUID CHROMATOGRAPHY OF TERPENOIDS*

B. V. BAPAT, T. K. SANKPAL**, B. B. GHATGE AND S. C. BHATTACHARYYA***
National Chemical Laboratory, Poona-8 (India)
(Received January 19th, 1967)

In our previous communications[1–3] we have described the use of various polyesters as stationary phases in gas–liquid chromatography of terpenoids. Initially, polyesters from 1,ω-dicarboxylic acids with diethylene glycol were studied as GLC substrates[1]. It was found that the number of methylene groups in the acid fragment have a profound influence, giving an arithmetical relationship in the case of retention times of hydrocarbons. When the series of polyesters from various 1,ω-glycols and some fixed acids like adipic acid were used as substrates[3], a similar effect due to the spacing of the methylene groups in the glycolic fragment was also observed. The effects of molecular weights and temperature gradient have also been reported[2].

From these observations it was felt that polyesters having properties of both series may find wide applications. Since the long chain acids or glycols are not always easily accessible, it was thought that some of the hydroxy acids, commercially available from indigenous sources, might be useful for the synthesis of polyesters *via* self-condensation.

Previous workers[4] observed that esterified oleic acid, possibly because of its unsaturation, is useful in the separation of monoterpenic hydrocarbons and oxygenated compounds. This suggested that introduction of an "unsaturated centre" in a polyester may be advantageous in the separation of various terpenoids.

The present communication deals with some of the results obtained on polyester substrates prepared by self-polymerisation of hydroxy acids, and from saturated and unsaturated glycols with saturated dicarboxylic acids.

EXPERIMENTAL

Two varieties of polyesters have been prepared and their solvent capability on GLC columns, have been studied.

Polyesters from self-condensation of hydroxy acids

Polyesters crosslinked with diglycerol or pentaerythritol are fairly stable substrates and are known to give good separations[5]. With a view to preparing a polyester from a hydroxy acid permitting self-crosslinking, we selected aleuritic acid $HOCH_2$-$(CH_2)_5$-CHOH-CHOH-$(CH_2)_7$-COOH. It is available in abundance in India from an indigenous raw material shellac. It possesses interesting structural features. Besides

* Communication No. 1040 from the National Chemical Laboratory, Poona-8, India.
** Present address: Indian Institute of Technology, Powai, Bombay-76, India.
*** Present address: Senior Professor, Indian Institute of Technology, Powai, Bombay-76, India.

the terminal primary hydroxyl group, it contains two vicinal secondary hydroxyl groups at C-9 and C-10. Because of the presence of the vicinal hydroxyl groups in the central part of the molecule, it is capable of giving a cross-linked polymer.

Along with aleuritic acid, another straight chain monohydroxy acid, ω-hydroxy-stearic acid, $HOCH_2$-$(CH_2)_{15}$-CH_2-COOH has also been used for self-polymerisation. Unlike aleuritic acid, it is only capable of forming straight-chain polymers on self-condensation.

Preparation of polymers

Aleuritic acid. This acid when polymerised according to our previous procedure[1], gave a high-melting, rubber-like solid mass, which is almost insoluble in chloroform or ethyl acetate at their boiling points and nearly so in β-pinene at 100°.

Due to the insolubility of the polymer in most of the common solvents, it was not found practicable to use it to impregnate a solid support. Hence, a novel method of preparation of the polymer on the solid support was employed.

A known weight of aleuritic acid as such, was used to impregnate a fire-brick support by using ethyl alcohol as a solvent. The solvent was evaporated off on a water bath and the whole mass dried in an oven at 80° for 2 h. It was then placed in a 100 ml flask and heated at about 140–145° for 2 h, and then under vacuum (2 mm) for a further 2 h. This material was then used in the column.

A few initial runs on this column revealed some strange retention time data. When retention times of monoterpenic hydrocarbons were being recorded, it was found that, as time elapsed, the retention times of hydrocarbons went on increasing and at the same time, the peaks of the alcoholic and ketonic compounds showed considerable tailing. The base line stability was also poor. These observations indicated that the polymerisation of aleuritic acid was incomplete under the experimental conditions described above.

A number of experiments were therefore designed to trace this phenomenon. Each time a known weight of aleuritic acid was impregnated on the fire-brick support, dried and used.

Initially the column was stabilised at 100° or 120° and the retention time data for monoterpenic hydrocarbons and a few medium boiling nonterpenic compounds were recorded. The same column was then stabilised at 163–164° and the retention time data were recorded for monoterpenic oxygenated compounds and sesquiterpenic hydrocarbons. After this, the same column was conditioned at 200° for nearly 3 h and again the retention time data were recorded at 100° and 120° as before.

Based on the observations made during the last experiments described above, a further batch of polymer was prepared as follows: 2.425 g of aleuritic acid (on the whole column filling) were heated at 200° for 6 h with constant stirring; loss during heating was 0.381 g; the retention time data were then recorded.

Polymerisation of ω-hydroxy-stearic acid. 2.435 g of the acid (on the whole column filling) were heated at 200° for 6 h with constant stirring; loss during heating was 0.219 g.

RESULTS AND DISCUSSION

In the case of earlier polyesters, derived from 1,ω-dicarboxylic acids and diols,

the polyesters are essentially linear with only a little cross-linking. But in the case of aleuritic acid polymer, it could be expected that because of the presence of two vicinal —OH groups in the central part of the molecule, the resulting polymer would possess cross-linking, representing a reticular structure. This explains the very high melting point of the final polymer. Obviously, the solubilities of hydrocarbons or comparatively non-polar compounds are expected to be high and the results tabulated in Table I are in agreement with it.

TABLE I

RETENTION TIME (IN MINUTES) ON ALEURITIC ACID POLYMER

Temperature 120°				*Temperature 163–164°*		
Compound	*Set No. I*	*Set No. III*	*Set No. IV*	*Compound*	*Set No. II*	*Set No. V*
α-Pinene	5.91	8.83	9.18	α-Terpineol	5.25	8.00
β-Pinene	9.95	13.96	14.21	Camphor	11.67	17.1
p-Cymene	16.75	25.73	26.83	Borneol	19.0	30.8
1,8-Cineole	17.5	25.46	25.83	Longifolene	21.75	35.0
Cyclohexanone	14.00	18.25	17.00	Humulene	30.75	Broad peak

The retention time data were recorded as follows:

Set No. I — Monoterpenic hydrocarbons and other low boiling compounds at 120°.

Set No. II — Sesquiterpenic hydrocarbons and monoterpenic oxygenated compounds at 163–165°.

Set No. III — Again for monoterpenic hydrocarbons and other low boiling compounds at 120°.

Then the column was conditioned at 200° for several hours and further readings taken.

Set No. IV — Monoterpenic hydrocarbons at 120°.

Set No. V — Sesquiterpenic hydrocarbons and monoterpenic oxygenated compounds at 163–165°.

Comparison of the retention times of Sets No. III and IV, and II and V, shows that there is not much difference in the retention times of the former set, but a large difference is observed in the latter set. This can be attributed to the fact that the polymeric mass is becoming soft at the higher temperature (163–165°), thus allowing the sample vapours to dissolve better.

In the case of ω-hydroxy-stearic acid polymer the same steps were followed for the determination of retention time data.

In the case of both the columns, mentioned above, some decomposition of tertiary alcohols was observed. In order to retard this acid-catalysed decomposition the following procedure was adopted.

Aleuritic acid polymer column filling

About 3 g of KOH were dissolved in 300 ml of alcohol and this solution was added to the above mentioned filling. The mixture was stirred from time to time and finally allowed to stand for 15 min. The supernatant liquid was decanted off. The

remaining column filling was again washed with aqueous alcohol till the pH of the washings was nearly 8. The mass was dried in an oven and again used as column filling to record the retention time data. It was noted that the retention times were very low when compared to the previous values. This indicated that the alcoholic alkali must have leached out the stationary phase; however, at the same time the decomposition of monoterpenic oxygenated compounds had nearly vanished.

ω-Hydroxy-stearic acid polymer

This column filling was suspended in 100 ml of alcohol, and with constant stirring, titrated with 0.4 *N* aqueous KOH solution till it was slightly alkaline. The whole mass was slowly heated on a water bath to dryness and the resulting column filling heated in an air oven for several h. This column filling was again placed in the column and the retention time data were recorded. At this stage chromatograms of sesquiterpenic hydrocarbons as well as monoterpenic oxygenated compounds did not show any tailing or decomposition (Figs. 1a and b), but the retention time values were less by nearly 20%. This may be due to the fact that some of the stationary phase

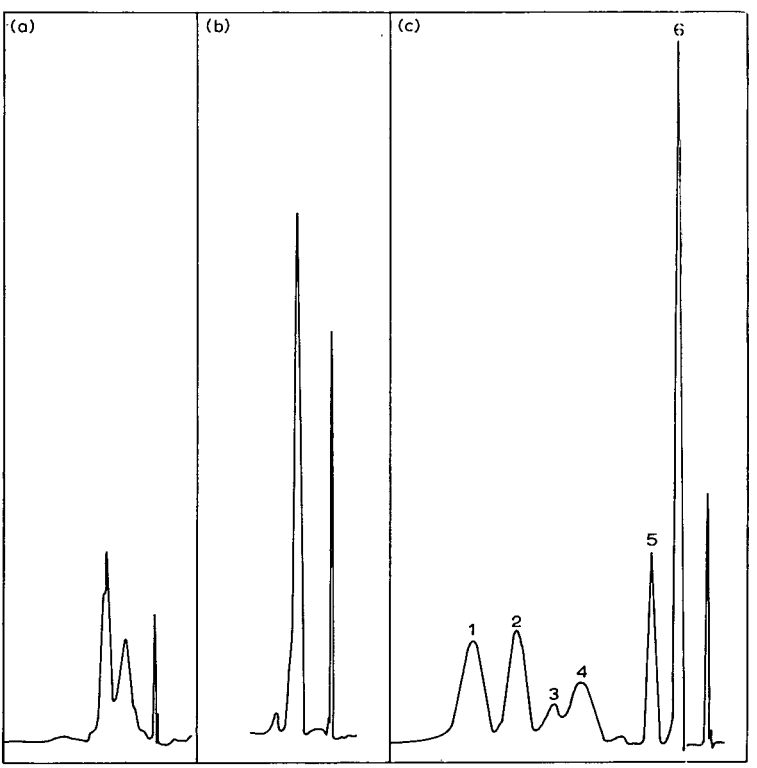


Fig. 1. Chromatogram of (a) and (b) linalool, and (c) a mixture of (1) humulene, (2) longifolene, (3) longicyclene, (4) citral, (5) linalool, and (6) methyl heptenone. Conditions: (a) temperature: 163–164°; stationary phase: ω-hydroxy-stearic acid polymer; sample: linalool; flow rate: 4 l/h H_2; (b) temperature: 163–164°; stationary phase: ω-hydroxy-stearic acid polymer (neutralized); sample: linalool; flow rate: 4 l/h H_2; (c) temperature: 180°; stationary phase: ω-hydroxy-stearic acid polymer (neutralized); flow rate: 13.5 sec/10 ml H_2.

might have been lost during the above mentioned process; or neutralisation of the free carboxylic groups might have changed the general nature of the stationary phase to some extent. Analogous observations have been recorded by previous workers[4,6].

The decomposition of monoterpenic oxygenated compounds seems to be retarded, in the present case at least, by neutralisation of the column[7] filling. It should be pointed out at this stage that some of the previous workers have incorporated 1 % sodium bicarbonate[7] in the column fillings and have also used potassium or lithium salts of fatty acids as part of the stationary phase[8]. It is interesting to note that some of the popular support materials have been found to be slightly alkaline in nature[9].

The present ω-hydroxy-stearic acid polymer showed very high solubilities for C_{15} hydrocarbons. A mixture of methyl heptenone, linalool, citral, longifolene, longicyclene and humulene was easily resolved as shown in Fig. 1, chromatogram (c). However, the geometrical isomers of citral are not resolved, though citral can easily be resolved into two geometrical isomers by using succinic acid–diethylene glycol polymer.

Effect of unsaturation

In order to study the effect of unsaturation a polyester was prepared from 1,4-butenediol ($HOCH_2$-CH=CH-CH_2OH) and adipic acid. For comparative evaluation, another polyester from 1,4-butanediol and adipic acid, was also synthesised. Both the columns were run under identical conditions. Relative retention time data recorded on these two columns are presented in Table II at 120° and Table III at 163°. Retention time data obtained on the two types of hydroxy acid polymers are also given. For comparison, retention time data compiled from our previous series of polyesters are also given in the same table for immediate reference.

Examination of the relative retention time data reveals some striking differences in the case of the stationary phases discussed above. Aromatic compounds have

TABLE II

RELATIVE RETENTION TIMES (WITH RESPECT TO LIMONENE) AT 120°

Compound	*1,4-Butanediol–adipic acid polyester*	*1,4-Butenediol–adipic acid polyester*	*ω-Hydroxy-stearic acid polymer*	*Aleuritic acid polymer*	*Pentadecane-1,15-dicarboxylic acid–diethylene glycol polyester*	*Apiezon*
α-Pinene	0.4009	0.4140	0.4574	0.4355	0.4184	0.5052
β-Pinene	0.6790	0.6719	0.6915	0.6755	0.640	0.5825
Δ^3-Carene	0.8091	0.7812	0.7939	0.8654	0.7703	0.8404
Ocimene	1.161	1.219	—	—	—	—
p-Cymene	1.416	1.50	1.151	1.196	1.144	0.9330
Limonene	9.22 ≡ 1.0	6.4 ≡ 1.0	9.4 ≡ 1.0	12.6 ≡ 1.0	12.5 ≡ 1.0	19.4 ≡ 1.0
Tricyclene	0.4122	0.4031	0.4468	0.3761	0.3960	0.4890
Camphene	0.5424	0.5671	0.5958	0.5859	0.5208	0.6081
1,8-Cineole	1.237	1.479	1.191	1.1630	1.1280	1.021
Cyclohexanone	1.728	2.369	0.9318	0.9506	0.8508	0.3299
Benzene	0.2332	0.2547	0.1329	0.1109	0.1312	0.0773
Toluene	0.4122	0.3751	0.2666	—	—	—
Methyl alcohol	0.1356	0.1719	0.046	—	—	—
Ethyl alcohol	0.1660	0.2266	0.065	—	—	—

TABLE III

RELATIVE RETENTION TIMES (WITH RESPECT TO CAMPHOR) AT 163–164°

Compound	*Succinic acid–diethylene glycol polyester*	*Azelaic acid–diethylene glycol polyester*	*Brassylic acid–diethylene glycol polyester*	*Adipic acid–1,4-butenediol polyester*	*Adipic acid–1,4-butanediol polyester*	*ω-Hydroxy-stearic acid polymer**	*Aleuritic acid polymer**
α-Terpineol	1.435	1.644	1.608	1.577	1.729	1.547	1.696
Linalool	0.6296	0.7568	0.7476	0.6893	0.7762	0.6373	0.7365
Borneol	—	—	—	1.496	1.646	1.418	1.798
Isoborneol	—	—	—	1.234	1.399	1.2019	1.560
Menthol	—	—	—	1.190	1.454	1.235	—
Camphor	5.40 = 1.0	9.25 = 1.0	10.3 = 1.0	11.1 = 1.0		5.75 = 1.0	3.95 = 1.0
Carvone	—	—	—	2.072	2.173	—	
Dihydrocarvone	—	—	—	1.365	1.473	—	
Longifolene	0.6685	1.329	1.971	1.0	1.338	2.28	1.63
Caryophyllene	—	—	—	—	1.427	2.423	
Humulene	1.019	1.909	2.617	1.432	1.904	2.869	2.15
Longicyclene	—	—	—	0.7523	1.050	1.826	—
Cyclopentadecane	—	—	—	1.712	2.301	5.217	—

* Relative retention time data were recorded on neutralised column filling.

higher relative retention values on unsaturated polyester, compared to those obtained on saturated polyester (*p*-cymene). Similarly, oxygenated compounds also show higher relative retention values on unsaturated polyester. The relative retention time of cyclohexanone is 2.37 on the unsaturated polyester, while on the saturated polyester, its relative retention value is only 1.73. In the case of the remaining terpenic hydrocarbons, there is no significant change in the relative retention values. 1,8-Cineole presents an interesting example. It has an oxygen atom in the form of "ether linkage". Its relative retention time (limonene = 1) on saturated polyester happens to be only 1.24, but rises to 1.48, when recorded on unsaturated polyester.

When we take into consideration the relative retention time data recorded at 163–165°, we find the same differences in the two cases. For example a mixture of camphor and longifolene can be easily resolved on the corresponding saturated polyester. But the same pair cannot be separated easily on the unsaturated polyester. The ω-hydroxy-stearic acid polymer column seems to be the best for the separation of camphor and longifolene. Here the relative retention value for this pair happens to be 2.28. At the same time it is interesting to note the relative retention time value for this pair on succinic acid–diethylene glycol polymer. This value happens to be 0.669, which indicates that the order of emergence of these two compounds is reversed due to the change in the nature of the polyester. It must be noted that, on the succinic acid–diethylene glycol column, camphor and humulene would overlap. But this pair can be easily separated on ω-hydroxy-stearic acid polymer. Therefore it seems that for overall separation of monoterpenic oxygenated compounds and C_{15} hydrocarbons, the ω-hydroxy-stearic acid polymer column would give a better performance.

It is interesting to observe the separation of the individual components of the monoterpenic oxygenated compounds. Separation of borneol and camphor may serve as a typical example. The relative retention time of borneol with respect to camphor, on aleuritic acid polymer, happens to be 1.80. This is the maximum value amongst all those recorded in Table III. This in turn may indicate that in the case of aleuritic acid polymer some of the –OH groups remain "free". These free groups in turn may have some strong interactions with alcohols.

Further studies are contemplated.

SUMMARY

Polyesters from ω-hydroxy acids have been prepared by self polymerisation and evaluated as substrates for gas–liquid chromatography. Two model compounds, (1) ω-hydroxy-stearic acid and (2) aleuritic acid, which are easily available from indigenous sources, were employed for polyesterification. In the case of aleuritic acid, where cross-linking is freely possible, the usual process of ester formation was not found useful. It gave a rubber-like mass, insoluble in common organic solvents. Therefore a novel method of polyester formation on the supporting material *in situ* is described for both the hydroxy acids. Such polymers, when applied to the analyses of the monoterpenic oxygenated compounds, showed acid catalysed isomerisation of the sample vapours. A successful attempt has been made to retard this process of isomerisation. These polyesters showed very high solubilities for the nonpolar sesquiterpenic hydrocarbons and can be used for the separation of monoterpenic oxygenated compounds and sesquiterpenic hydrocarbons.

Simultaneously, the effect of "unsaturation in the polyester" has also been studied by synthesising two polyesters, one from adipic acid and 1,4-butanediol and the other from adipic acid and 1,4-butenediol. The effect of "unsaturation" is found to be opposite to that which is observed in the case of long chain dicarboxylic acid diethylene glycol polyesters. The unsaturated polyester shows better solubilising powers for oxygenated compounds than for hydrocarbons.

REFERENCES

1 B. V. BAPAT, B. B. GHATGE AND S. C. BHATTACHARYYA, *J. Chromatog.*, 18 (1965) 308.
2 B. V. BAPAT, B. B. GHATGE AND S. C. BHATTACHARYYA, *J. Chromatog.*, 23 (1966) 363.
3 B. V. BAPAT, B. B. GHATGE AND S. C. BHATTACHARYYA, *J. Chromatog.*, 23 (1966) 227.
4 E. VON RUDLOFF, *Can. J. Chem.*, 39 (1961) 1190.
5 E. VON RUDLOFF, *Can. J. Chem.*, 38 (1960) 631.
6 S. R. LIPSKY, R. A. LANDOWNE AND M. R. GODET, *Biochim. Biophys. Acta*, 31 (1959) 336.
7 H. P. BURCHFIELD AND E. E. STROSS (Editors), *Biochemical Applications of Gas Chromatography*, Academic Press, New York, 1962, p. 455.
8 E. BAYER, G. KUPFER AND K. H. REUTHER, *Z. Anal. Chem.*, 164 (1958) 1.
9 S. DAL NOGARE AND R. S. JUVET (Editors), *Gas Liquid Chromatography*, Interscience, New York, 1962, p. 142.

GAS–LIQUID CHROMATOGRAPHY OF VALERIAN SESQUITERPENOIDS

TSUTOMU FURUYA AND HISASHI KOJIMA
College of Pharmaceutical Sciences, Kitasato University, Shiba, Minato-ku, Tokyo (Japan)
(Received December 19th, 1966)

We have already studied the gas–liquid chromatography of opium alkaloids[1], plant glycosides[2], flavonoids[3], lichen substances[4], anthraquinones[5], and coumarins[6].

NIGAM AND LEVI[7] and BAPAT *et al.*[8] have recently reported on the gas chromatography of sesquiterpene hydrocarbons, but not that of valerian sesquiterpenoids. We have now attempted to make an effective separation of valerian sesquiterpenoids, which will be useful from the chemotaxonomical standpoint.

The present paper describes the results of gas–liquid chromatography, using two kinds of packed columns, of fifteen standard pure samples of valerian sesquiterpenoids and five kinds of Japanese valerian oils.

The relationships between the retention volumes and the chemical structures, and the properties and the contents of sesquiterpenoids in valerian oils are discussed.

EXPERIMENTAL

Materials

All standard samples of valerian sesquiterpenoids used were donated by Prof. T. TAKEMOTO.

Five kinds of valerian plants from different sources were collected in winter, and dried. Two to three grams of dried roots were extracted with methylene chloride–ether solution (1:1, v/v) at room temperature for 7 days. The extracts, after evaporation,

TABLE I

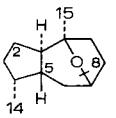
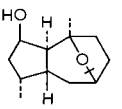
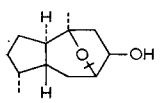
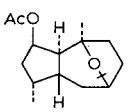
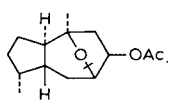
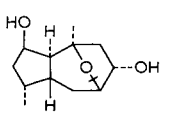
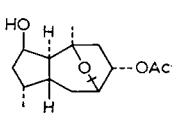
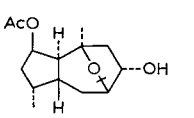
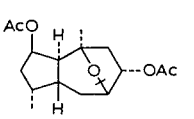
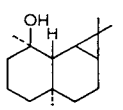
COLLECTION DATES AND OIL CONTENTS OF VALERIAN PLANTS

A = *Valeriana officinalis* var. *latifolia* (Japanese name, Ka-no-ko-so), grown wild in Kumamoto-ken; B = the same, cultivated at Toyama University; C = the same, cultivated at the Experimental Farm for the Cultivation of Medicinal Plants, the National Hygienic Laboratory; D = the same, cultivated in Tokyo Metropolitan Botanical Gardens; E = *Valeriana flaccidissima*, grown wild in Kanagawa-ken.

Sample	*Collection date*	*Valerian oil content* (%)
A	Jan. 16, 1965	2.32
B	Jan. 16, 1965	2.25
C	Jan. 16, 1965	4.27
D	Jan. 18, 1965	2.50
E	Jan. 14, 1965	1.07

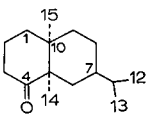
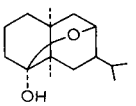
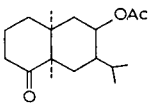
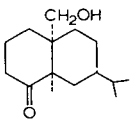
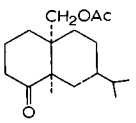
TABLE II

RETENTION TIMES AND RETENTION VOLUMES OF VALERIAN SESQUITERPENOIDS

No.	*Compound*	*Structure*	*1.5% SE-30 on Chromosorb W*		*12% DEGS on Gas Chrom P*	
			t_R *(min)*	V_R *(ml)*	t_R *(min)*	V_R *(ml)*
1	Kessane		5.8	351.5	1.5	102.0
2	α-Kessyl alcohol		10.7	648.4	7.2	489.6
3	Kessanol		12.5	757.5	12.4	843.2
4	Kessyl acetate		17.0	1030.2	7.4	503.2
5	Kessanyl acetate		17.8	1078.7	7.0	476.0
6	Kessoglycol		27.0	1636.2	74.1	5038.8
7	8-Acetoxykessan-2-ol*		35.5	2151.3	40.3	2740.4
8	2-Acetoxykessan-8-ol		40.0	2424.0	73.3	4984.4
9	Kessoglycol diacetate		53.7	3254.2	38.2	2597.6
10	Maaliol		6.6	400.0	3.2	217.6

(continued on p. 343)

TABLE II *(continued)*

No.	Compound	Structure	1.5% SE-30 on Chromosorb W		12% DEGS on Gas Chrom. P	
			t_R (min)	V_R (ml)	t_R (min)	V_R (ml)
11	Valeranone		9.8	593.9	4.8	326.4
12	Cryptofauronol		10.3	624.2	9.4	639.2
13	Fauronyl acetate		27.8	1684.7	23.8	1618.4
14	Kanokonol		28.3	1715.0	65.5	4454.0
15	Kanokonyl acetate		42.3	2563.4	36.7	2495.6

Conditions:			
	N_2 flow rate	60.6 ml/min	68.0 ml/min
	Column temp.	150°	180°
	Flash heater temp.	250°	250°
	Detector temp.	200°	200°

* Chemical structure was presumed from the result of on-column acetylation of kessoglycol.

were steam distilled and the distillates were extracted with benzene. The benzene solutions which contain valerian oil, were dried, evaporated to dryness and then dissolved in a small amount of acetone and run on the gas chromatograph.

Collection dates and oil contents of the valerian plants are shown in Table I.

Gas chromatography

Two kinds of packed columns, one with 1.5 % SE-30 on Chromosorb W (60–80 mesh) (A) and the other with 12 % DEGS on Gas Chrom P (80–100 mesh) (B), were used in this work. For the former packing a stainless steel column (2.25 m × 4 mm) was installed in a Shimadzu Model GC-1B and for the latter a glass one (1.87 m × 4 mm) in a Shimadzu Model GC-1C. Both types of column were equipped with a hydrogen flame ionization detector.

One to two μl of the acetone solution of the standard samples and valerian oils were used for injection into the gas chromatograph.

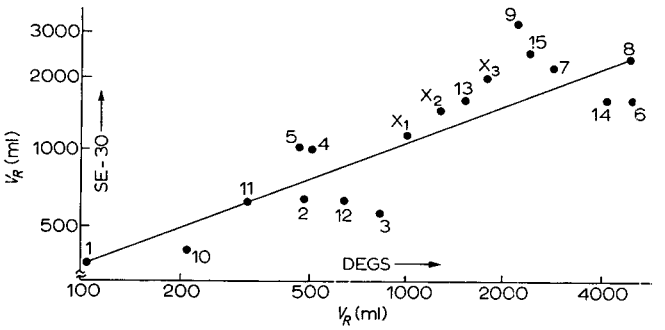


Fig. 1. Relationship between retention volumes and structures. For designations, see Table II and for X_1–X_3, see p. 346.

On-column acetylation[9]

One μl of 1 % solution of kessoglycol in acetone was first injected into the gas chromatograph, and followed as soon as possible by 3 μl of acetic anhydride. Thus acetylation instantly proceeded on the column.

RESULTS AND DISCUSSION

There are two groups of kessane and valeranane skeletons in the valerian sesquiterpenoids. Most valerian sesquiterpenoids gave single sharp peaks which do not appear to decompose. Retention times and retention volumes of valerian sesquiterpenoids are shown in Table II.

The kessane group generally has hydroxyl and acetoxyl substituents in the 2 and 8 positions, or both. The increase in retention times with increasing number of hydroxyl groups is especially notable. Kessane itself, which has no hydroxyl group, has the lowest retention time, while α-kessyl alcohol and kessanol having 2- and 8-hydroxyl groups, respectively, have a medium retention time, and kessoglycol having two hydroxyl groups is retained the longest. The retention time of α-kessyl alcohol is shorter than that of kessanol, because α-kessyl alcohol has intermolecular

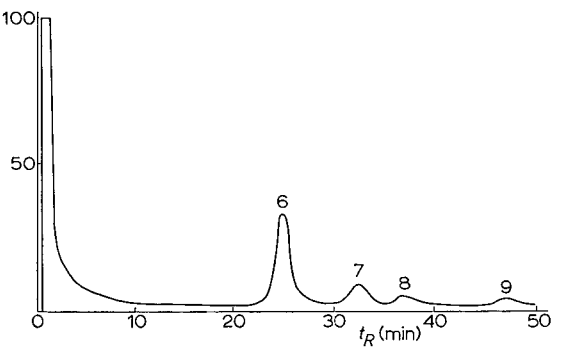


Fig. 2. Gas chromatogram resulting from on-column acetylation of kessoglycol. 6 = Kessoglycol; 7 = 8-acetoxykessan-2-ol; 8 = 2-acetoxykessan-8-ol; 9 = kessoglycol diacetate. Conditions: Shimadzu Model GC-1C, U-shaped glass column (1.87 m × 4 mm) packed with 1.5 % SE-30 on Chromosorb W (60–80 mesh). N_2 flow rate: 69.8 ml/min. Column temp. 158°; detector temp. 200°; flash heater temp. 250°.

hydrogen bonding[10] in its chemical structure, whereas there is no hydrogen bonding in kessanol.

Acetyl derivatives of the valerian sesquiterpenoids showed longer retention times than the corresponding hydroxyl compounds on the SE-30 column, especially in the case of acetylated compounds with a 2-hydroxyl group, where it was much longer. This fact is probably due to the disappearance of hydrogen bonding. However, the opposite was observed on using a DEGS column as polyester type stationary liquid phase; for example the correlations of retention times between kessyl acetate and kessanyl acetate, 8-acetoxykessan-2-ol and 2-acetoxykessan-8-ol; kanokonol and kanokonyl acetate are also worthy of note.

Employing two kinds of stationary liquid phases such as SE-30 and DEGS, logarithms of retention volumes of valerian sesquiterpenoids were plotted in the graph illustrated in Fig. 1. By joining the three points due to kessane, valeranone and 2-acetoxykessan-8-ol, a straight line was obtained. Acetyl derivatives were plotted in the upper part of the line, while hydroxyl derivatives were plotted in the lower part. Some sesquiterpenoids having no hydroxyl and no acetoxyl groups or an equal number of non-bonding hydroxyl and acetoxyl groups were recorded on the line. Thus

TABLE III

THE RELATIVE AMOUNTS OF SESQUITERPENOIDS IN SOME JAPANESE VALERIAN ROOTS

Compounds	t_R	*Samples*				
		A	*B*	*C*	*D*	*E*
1	5.8	++++	+	+	+	
	6.2	+				
10	6.6		+	+	+	+
	6.9	++	+	+	+	+
	7.4	+	+	+	+	
	8.6		+	+	+	
	9.2	+	+	+	+	+
11	9.8	+	+	+	+	+
2	10.7	++	+	+	+	
	11.8				+	+
3	12.5	+	+	+	+	
	13.1		+	+		
	14.3	++	+	+	+	+
	16.8					+
4	17.0	++++	+++	++	++	
5	17.8	?	?	?	+	
X_1	19.9	++	+	+	+	+
	21.7				+	
	22.9		+	+		+
X_2	25.3	+++	++	++	++	++
14	28.3	+	+	+	+	
X_3	32.4	++++	+++	+++	+++	+++
7	35.5	+				
15	42.3		++	++	++	
	47.6					+
9	53.7	++++	++++	++	++++	
	60.5					+

A packed column with 1.5% SE-30 on Chromosorb W was used for this analysis. Valeranone = 1; + ≦ 1; 1 < ++ ≦ 5; 5 < +++ ≦ 10; 10 < ++++.

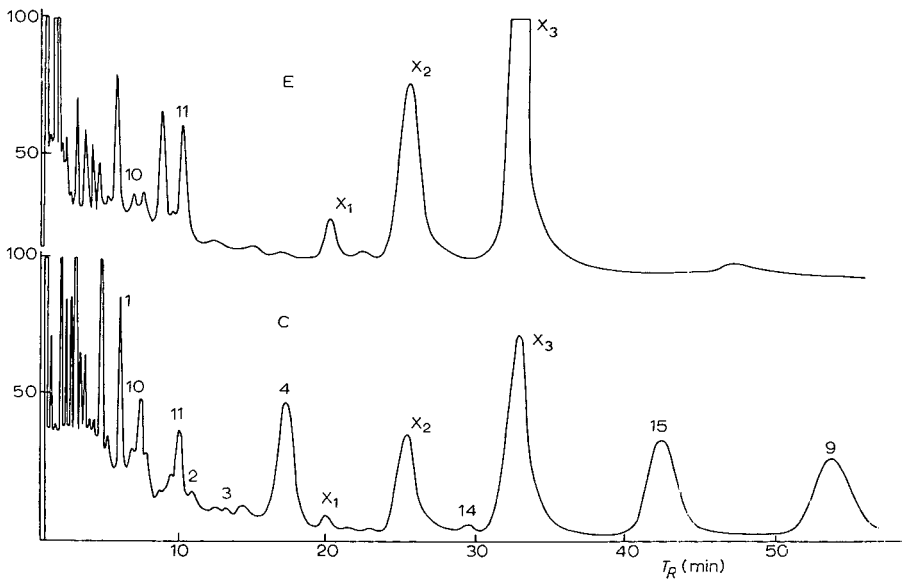


Fig. 3. Gas chromatograms of samples C and E with 1.5% SE-30. Conditions the same as in Table II. For designations, see Table II and for X_1–X_3, see p. 346.

two or three unknown substances, X_1, X_2 and X_3, which we first found gas-chromatographically, are possibly acetyl derivatives.

From this point of view gas–liquid chromatography should provide a powerful tool for the identification and structural study of valerian sesquiterpenoids.

On-column acetylation of kessoglycol resulted in acetylation of hydroxyl groups to give sharp peaks with little tailing, as shown in Fig. 2.

The peaks obtained were identical with those of authentic samples except for 8-acetoxykessan-2-ol, and are very effective for the analysis of valerian oil gas chromatograms.

The results of the gas chromatographic analysis of valerian oils are shown in Table III and some gas chromatograms in Figs. 3 and 4.

Gas chromatograms of samples A, B, C and D were very similar, except for the absence of maaliol and kanokonyl acetate in A. On the other hand, the gas chromatogram of E was distinctly different from that of the other oils. It appeared that sesquiterpenoids having a kessane nucleus are absent or only present in small amounts in E. Large amounts of kessoglycol diacetate and kessyl acetate were found in A, B, C and D but not in E. Considerable amounts of X_1, X_2, and X_3 and small amounts of valeranone, a new hypotensive and tranquilizing agent, were observed in all the valerian oils.

TAKEMOTO and his co-workers have already reported on the constituents of some Japanese[11–13] and European[14] valerian roots. We have also estimated the amounts of valerian sesquiterpenoids in five kinds of Japanese valerian oils using gas–liquid chromatography and got almost the same results in B and D as those in TAKEMOTO's paper[12], except for kessanol which they found in D only, but we in both B and D.

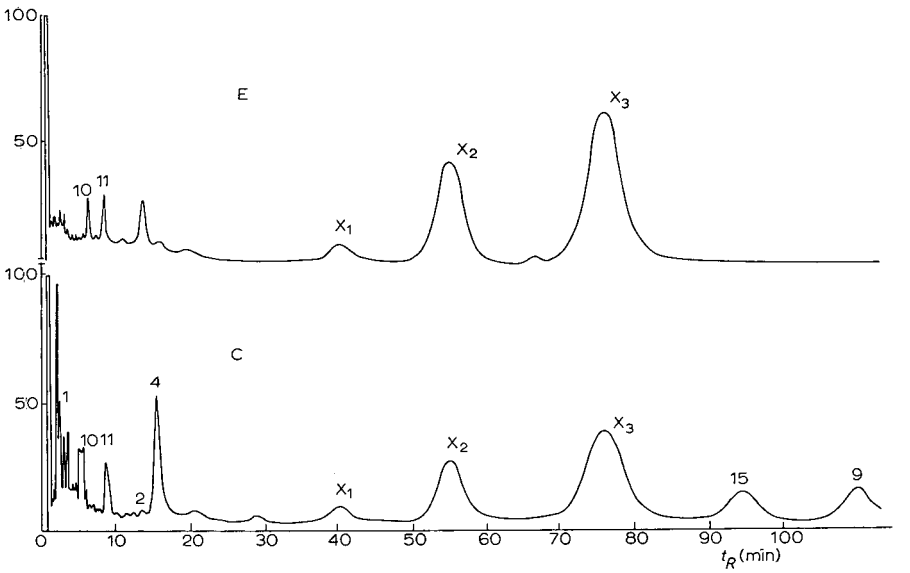


Fig. 4. Gas chromatograms of samples C and E with 12% DEGS. Conditions: A Shimadzu Model GC-1B, stainless steel column (2.25 m × 4 mm) packed with 12% DEGS on Gas Chrom P (80–100 mesh). N_2 flow rate: 75.5 ml/min. Column temperature: 152°; detector temperature: 210°; flash heater temperature: 240°. For designations, see Table II and for X_1–X_3, see p. 346.

From these results, morphological character and chromosome numbers[15], it seems likely that the original valerian plants are closely related species and fairly distant from that of E.

ACKNOWLEDGEMENTS

We are grateful to Prof. S. SHIBATA of the University of Tokyo for his kind encouragement. We are indebted to Dr. T. KURODA, Ibaraki Medical Plant Research Laboratory for the collection and oil extraction of valerian plants. The supply of standard samples of valerian sesquiterpenoids by Prof. T. TAKEMOTO of Tohoku University is gratefully acknowledged.

SUMMARY

The gas chromatographic behavior of fifteen kinds of valerian sesquiterpenoids was investigated using SE-30 and DEGS as stationary liquid phases.

The relationship between the presence of hydrogen bonding in the chemical structure and its effect on the retention time is discussed.

The present method will distinguish between various valerian oils and be useful for chemotaxonomical research into valerianaceous plants.

REFERENCES

1 E. BROCHMANN-HANSSEN AND T. FURUYA, *J. Pharm. Sci.*, 53 (1964) 1549.
2 T. FURUYA, *J. Chromatog.*, 18 (1965) 152.
3 T. FURUYA, *J. Chromatog.*, 19 (1965) 607.
4 S. SHIBATA, T. FURUYA AND H. IIZUKA, *Chem. Pharm. Bull. (Tokyo)*, 13 (1965) 1254.
5 T. FURUYA, S. SHIBATA AND H. IIZUKA, *J. Chromatog.*, 21 (1966) 116.
6 T. FURUYA AND H. KOJIMA, *J. Chromatog.*, 29 (1967) 382.
7 I. C. NIGAM AND L. LEVI, *J. Chromatog.*, 23 (1966) 217.
8 B. V. BAPAT, B. B. GHATGE AND S. C. BHATTACHARYYA, *J. Chromatog.*, 23 (1966) 363.
9 E. BROCHMANN-HANSSEN AND T. FURUYA, *Planta Med.*, 12 (1964) 328.
10 S. ITO, M. KODAMA, T. NOZOE, H. HIKINO, Y. HIKINO, Y. TAKESHITA AND T. TAKEMOTO, *Tetrahedron Letters*, (1963) 1787.
11 H. HIKINO, Y. HIKINO, H. KATO, Y. TAKESHITA AND T. TAKEMOTO, *Yakugaku Zasshi*, 83 (1963) 219.
12 H. HIKINO, Y. HIKINO, Y. TAKESHITA, Y. ISURUGI AND T. TAKEMOTO, *Yakugaku Zasshi*, 83 (1963) 555.
13 H. HIKINO, Y. HIKINO, Y. TAKESHITA, H. KATO AND T. TAKEMOTO, *Yakugaku Zasshi*, 85 (1965) 179.
14 H. HIKINO, Y. HIKINO, Y. TAKESHITA AND T. TAKEMOTO, presented at the *21st Annual Meeting Pharm. Soc. Japan, Tokushima, October 28, 1965.*
15 T. KURODA, personal communication.

SÉPARATION ET DOSAGE D'ACIDES AMINÉS EN CHROMATOGRAPHIE SUR COUCHES MINCES

R. BONDIVENNE ET N. BUSCH
Département Chimie Organique, Centre Européen de Recherches Mauvernay, Riom (France)
(Reçu le 13 décembre 1966)

Pendant de longues années, depuis la mise au point par CONSDEN, GORDON ET MARTIN[1] de la chromatographie sur papier, cette technique resta la méthode par excellence pour la séparation et la détermination des acides aminés dans un mélange quel qu'il soit.

Malgré les nouvelles possibilités qu'offrait cette méthode, les inconvénients présentés, en particulier sa lenteur d'application, devaient pousser les chercheurs à tenter de l'améliorer.

Bien que le principe de la chromatographie sur couches minces (C.C.M.) ait été décrit depuis plus d'un quart de siècle par ISMAILOV ET SHRAIBER[2] il ne fut utilisé que plus tard et presque exclusivement pour la séparation de terpènes[3,4]. Il a fallu attendre que STAHL standardise à la fois les procédés d'application et un appareillage pratique et ingénieux pour que l'on s'en serve couramment[5–9].

Le but de notre travail a été d'appliquer la chromatographie en couches minces à la séparation d'acides aminés, puis au dosage de certains d'entre eux en employant, d'une part, des solutions synthétiques, et, d'autre part, un extrait de muscle cardiaque animal.

SÉPARATION QUALITATIVE

Depuis 1959 différentes recherches furent accomplies pour séparer les acides aminés par la chromatographie sur couches minces. Les techniques employées utilisent quantités de solvants et de substrats: Le gel de silice, accompagné ou non de liant fut souvent employé pour l'étude de ces séparations et pour la mise au point de différentes méthodes, en particulier celle dite "à front perdu"[1,10–16]. L'utilisation de l'oxide d'aluminium amena, dans des applications particulières, des résultats satisfaisants[17–19]. Signalons aussi les tentatives faites sur Kieselguhr, Celite, Sephadex, sulfate de baryum[20–23]. Concurrement à ces recherches de nombreuses équipes essayèrent la cellulose: nouveaux systèmes de solvants et nouvelles techniques mono- ou bi-dimensionelles virent le jour[24–31]. Enfin BUJARD en 1964 décrit un nouveau couplage de solvant[20,24,29].

Il nous a paru hors de propos de signaler tous les travaux relatifs à la séparation des dérivés des acides aminés. La méthode ici exposée n'est pas d'une grande originalité et n'est que l'amélioration de celles ci-dessus citées, mais nous pensons que telle, elle pourra rendre des services.

Préparation des plaques

Nous avons d'abord employé la Cellulose 300 MN de Macherey et Nagel avec les quantités préconisées par le fabricant, mais du fait des difficultés de prise de

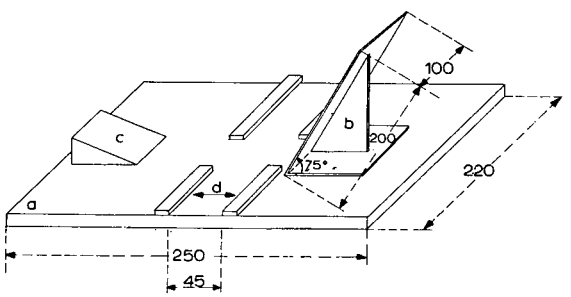
cette cellulose sur le verre, nous avons été amenés à préciser un mode opératoire.

Les plaques sont mises à tremper pendant 24 h dans la potasse alcoolique, puis après un rinçage d'environ 2 h à l'eau courante au moyen d'un système de siphon, elles sont soigneusement essuyées au papier filtre en évitant d'y laisser toute trace de doigts. D'un autre côté, on prépare un empois avec 200 mg d'amidon de maïs dans 90 ml d'eau distillée. On laisse refroidir environ 5 min et on incorpore 15 g de Cellulose 300 MN. Cette suspension devant être énergiquement agitée, on a constaté que les résultats les meilleurs étaient obtenus en malaxant avec un mixer pendant 3 fois 30 sec, avec 30 sec d'arrêt entre chaque agitation. Cette bouillie est ensuite étalée sur les plaques de verre au moyen de l'appareil mis au point par STAHL. Cet étaleur est réglé à 0.5 mm d'épaisseur de telle manière que, après séchage, la couche soit de l'ordre de 0.3 à 0.4 mm. Ces plaques sont préparées le soir et laissées à sécher toute la nuit à la température du laboratoire.

Problème de la saturation des cuves

Nos chromatographies devant être faites dans les cuves parallelépipédiques commercialisées par Desaga, il a paru bon de faire des essais de migration avec: (a) un degré de saturation normal; (b) un degré de saturation augmenté par la présence d'une feuille de papier Whatman No. 3, imbibée de solvant et appliquée sur trois des quatre parois de la cuve, d'après les conclusions de HONEGGER[32].

Ces expériences ont montré la nécessité à la fois d'une saturation préliminaire de la cuve et d'une durée de temps d'équilibrage de la plaque avant migration. Nous avons donc conçu le dispositif donné par la Fig. 1. Grâce à cet appareillage, nous opérons ainsi: 120 à 130 ml du solvant employé sont placés au fond de la cuve. On applique une feuille de papier Whatman No. 3 sur trois de ses faces (une grande et deux petites) et on laisse le solvant imbiber seul cette feuille. Pendant cette opération, la cuve est inclinée, soutenue par b et calée par c et d (Fig. 1).



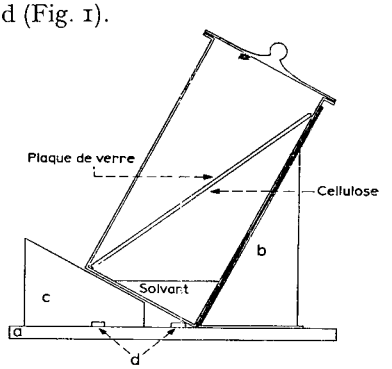


Fig. 1. Supports pour la saturation des cuves. a = Socle en bois; b = appui en polyvinyle; c = cale de bois mobile; d = bande caoutchouc mousse.

Fig. 2. Vue de profil de la cuve en position de saturation sur son support. Annotations, voir la Fig. 1.

Puis, la plaque étant prête, on l'introduit dans la cuve de telle manière que le bord inférieur ne soit pas atteint par le solvant et que la face recouverte de cellulose soit en dessous, donc dirigée vers la grande face de la cuve recouverte de papier

Whatman No. 3 (Fig. 2). On laisse la plaque s'équilibrer pendant une demi-heure en position inclinée et on démarre la migration en ôtant la cale c et en mettant la cuve en position verticale. La Fig. 3 montre deux cuves dans les deux positions ainsi décrites.

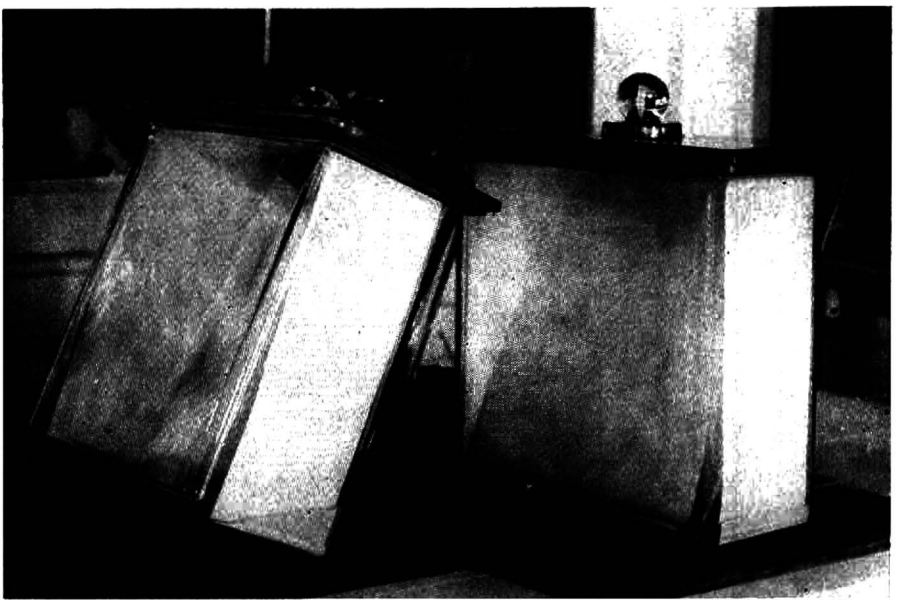


Fig. 3. Photo des cuves en position de saturation et en position de migration.

Solutions à analyser et migration des spots

Nous avons employé des acides aminés provenant de la firme suisse Fluka. Ces produits sont certifiés chromatographiquement purs par le fournisseur. Ce sont les acides aminés suivants:

(1) DL-Alanine
(2) β-Alanine
(3) DL-Phénylalanine
(4) L-Arginine
(5) DL-Asparagine
(6) Acide DL-aspartique
(7) Acide γ-*n*-aminobutyrique
(8) L-Cystéine, ClH
(9) Glycocolle
(10) L-Glutamine
(11) Acide DL-glutamique
(12) DL-Leucine
(13) DL-Lysine, ClH
(14) DL-Méthionine
(15) DL-Ornithine, 2 ClH
(16) DL-Histidine, 2 ClH
(17) DL-Proline
(18) DL-Sérine
(19) DL-Tryptophane
(20) DL-Thréonine
(21) DL-Tyrosine
(22) DL-Valine

D'autre part, le lysat de coeur étudié nous a été fourni par Riom-Laboratoires, Riom, Puy-de-Dôme, France.

Les solutions étalons de chaque acide aminé sont faites à raison de 100 mg dans 10 ml d'eau contenant 10% d'alcool isopropylique. Les spots de départ, dans ces conditions sont de 3 μl, sauf pour: DL-alanine, 10 μl; DL-proline, 8 μl; DL-Tyrosine, 20 μl.

La solution de lysat de coeur est réalisée dans le même solvant à raison de 50 mg pour 1 ml. Dans ces conditions, le spot de départ est de 15 μl.

Sur la plaque, le spot de départ est placé dans un coin, à 2 cm de chaque bord de-celle-ci. On a, auparavant, enlevé soigneusement une bande de 3 mm de large de cellulose tout autour de la plaque ainsi que sur son épaisseur, de façon qu'il ne puisse y avoir aucun contact entre la cellulose et le papier de saturation. La plaque ainsi préparée est mise à équilibrer et ensuite démarrée comme il a été expliqué précédemment.

La migration totale s'étend sur 16 cm pour les deux dimensions. La première dimension se fait perpendiculairement au sens d'étalement de la cellulose et évidemment la deuxième est parallèle à ce sens.

Systèmes de solvants

Nous avons commencé nos essais en reprenant le système de solvant préconisé par BISERTE[33], système que nous utilisions sur papier depuis de nombreuses années:

1ère dimension phase supérieure du mélange de PARTRIDGE[34];

2ème dimension phase inférieure du phénol tamponné de LEVY ET CHUNG[35].

Mais la médiocrité des résultats obtenus a incité à employer, d'abord en essais monodimensionnels, les solvants cités ou essayés par STAHL[36], FAHMY *et al.*[20] et WOLLENWEBER[24].

Finalement les meilleures séparations ont été obtenues avec le système signalé par BUJARD[29] et modifié comme suit:

1ère dimension: méthanol–chloroforme–ammoniaque à 25 % (2:2:1);

2ème dimension: méthanol–pyridine–eau (20:1:5).

Révélateur

Longtemps fidèles au révélateur ninhydrine à 1 % dans l'acétone tamponnée[35], nous avons ensuite préféré le réactif de STARON[37], qui est plus sélectif et plus sensible:

Solution I: méthylcyclohexanol–acétone (3:7, en volume)

Solution II: collidine–lutidine (1:3, en volume)

Révélateur lui-même: ninhydrine, 2 g; solution II, 2 ml; solution I, q.s.p. 100 ml.

La réaction peut se faire, soit à température ambiante, mais elle demande 12 à 15 h, soit à 65–70° et ne requiert alors que 15 à 20 min. Dans les deux cas, les couleurs peuvent être rapidement avivées par pulvérisation légère d'eau distillée.

Résultats

La séparation de tous les acides aminés envisagés est bonne comme le prouvent les Fig. 4 et 5. La Fig. 4 montre la séparation de tous les acides aminés que nous possédions, la Fig. 5 la séparation des acides aminés contenus dans le lysat de coeur.

Il faut quand même remarquer que cette méthode ne sépare pas la leucine de l'isoleucine et donne des migrations semblables pour la tyrosine et l'alanine. Le premier de ces acides aminés est plus gris que le second tout au début de la révélation, ce qui permet de reconnaître sa présence. Cette méthode, une fois mise au point, nous a permis d'aborder la deuxième partie de ce travail, c'est-à-dire le dosage de certains acides aminés dans un mélange synthétique complexe et dans le lysat de muscle cardiaque que nous possédions.

DÉTERMINATION QUANTITATIVE

Depuis l'excellente revue de JUTISZ ET DE LA LLOSA[38] et malgré nos recherches bibliographiques nous n'avons trouvés, à part la méthode originale de FRODYMA ET

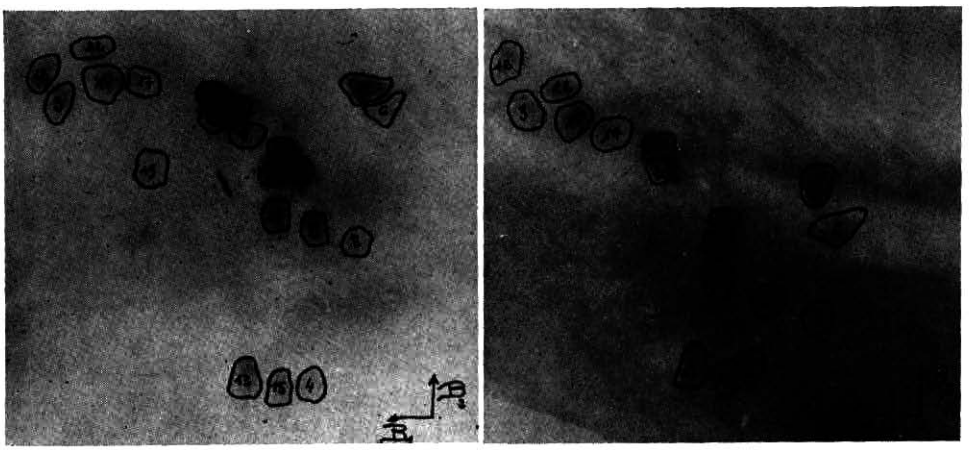


Fig. 4. Séparation des 22 acides aminés à notre disposition.

Fig. 5. Séparation des 18 acides aminés à partir du lysat de coeur.

FREI[39,40] aucun article relatif au dosage des acides aminés par colorimétrie après élution des taches séparées par chromatographie sur couches minces.

En dépit de quelques essais de révélation différentes[41–45] c'est l'emploi de la ninhydrine qui a prévalu, soit en solution seule[45–48], soit avec addition de corps donnant plus de sensibilité et de stabilité (Réf. 21, 24, 27, 29, 37, 44, 49–58). Des solutions tamponnées ont permis encore d'améliorer la sensibilité de détection[35,59–65]. De plus les problèmes de l'éluant et de la totalité de l'élution se sont posés à de nombreux auteurs (Réf. 35, 47, 50, 52, 53, 61, 66, 67).

Alors l'étude de ces diverses méthodes, et aussi certaines nécéssités experimentales nous ont conduit à mettre au point la technique suivante.

Séparation des acides aminés

Cette séparation se fait, à partir de mélange synthétique, ou à partir de solution de lysat de coeur, de la manière exposée précédemment.

Révélation

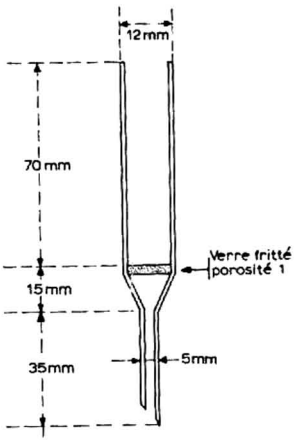
Nous employons le mélange suivant comme révélateur: ninhydrine, 1,0 g; eau bi-distillée, 100 ml; tampon collidine–lutidine, 10 gouttes.

Le tampon utilisé ici est le même que celui décrit dans la première partie de ce travail. Il permet, d'abord une dissolution complète de la ninhydrine et ensuite l'obtention d'un pH optimum de 7.1–7.2.

Les deux migrations étant faites, la plaque est séchée soigneusement sous courant d'air chaud (un sèche-cheveux du commerce), puis on pulvérise le réactif. On amorce ensuite la réaction par passage d'une demi-heure sous le même courant d'air chaud donc à une température comprise entre 50 et 60°. A la fin de cette période, les plaques sont gardées durant 45 h à une température moyenne de 27°, avec une bonne aération. Ces temps et surtout ces températures ont été déterminés de telle sorte que la révélation soit optimum, que le complexe coloré ninhydrine–acide aminé ne soit pas partiellement détruit, et que le fond du chromatogramme ne rosisse pas excessivement.

Élution des taches

Après migration et révélation, on récupère sur la plaque la totalité de la surface colorée relative à l'acide aminé envisagé, par un grattage méticuleux au moyen d'une spatule souple. On opère de même sur une surface équivalente ne contenant pas d'acides aminés et qui, ainsi, servira de "blanc". Toute la cellulose recueillie est reportée quantitativement dans un petit entonnoir à verre fritté, de porosité 1, tel qu'il est décrit par la Fig. 6.



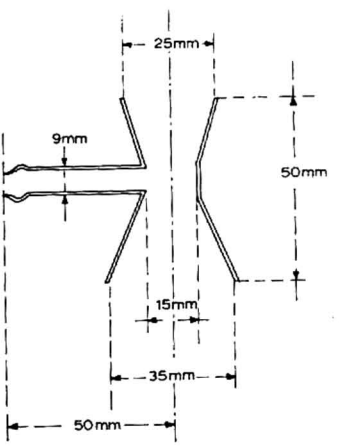


Fig. 6. Croquis du filtre construit spécialement pour l'élution.

Fig. 7. Croquis du dispositif spécial permettant la prise de vide.

Puis l'élution est faite en employant deux fois 2 ml du mélange acétone–eau (3:1). On laisse l'éluant et la cellulose en contact 5 min en agitant au moyen d'une petite spatule en inox. On aspire cet éluant, sous vide léger, dans un tube de Pyrex 18 × 180 en intercalant le petit dispositif en verre donné par la Fig. 7.

L'assemblage complet est indiqué par la Fig. 8.

Dosage lui-même

La solution colorée ainsi obtenue est passée, dans la demi-heure qui suit l'élution, au spectrophotomètre Maroc de Jobin et Yvon, à la longueur d'onde de 570 mμ et dans les conditions suivantes: cuve de verre de 1 cm d'épaisseur; sensibilité, position 5; résistance de charge, position 4.

La lecture est faite par rapport au "blanc" obtenu dans les mêmes conditions.

On a pu ainsi établir des courbes d'étalonnage de quatre acides aminés, les acides aspartique et glutamique, la lysine et la valine, et ensuite faire le dosage de ces corps dans le mélange synthétique réalisé par nous, et aussi dans le lysat de coeur mis à notre disposition.

Courbes d'étalonnage

Solutions employées

A partir des solutions mères employées précédemment soit à 100 mg pour 10 ml du mélange isopropanol–H_2O, on réalise des dilutions de manière à avoir une concentration de 10 μg/μl de solvant.

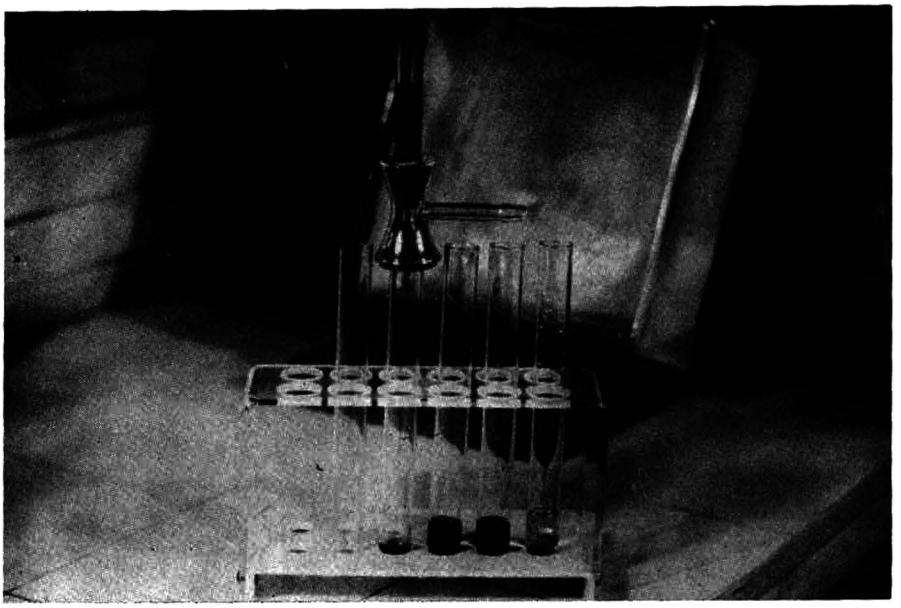


Fig. 8. Filtre et prise de vide en position d'élution sur tube à essai.

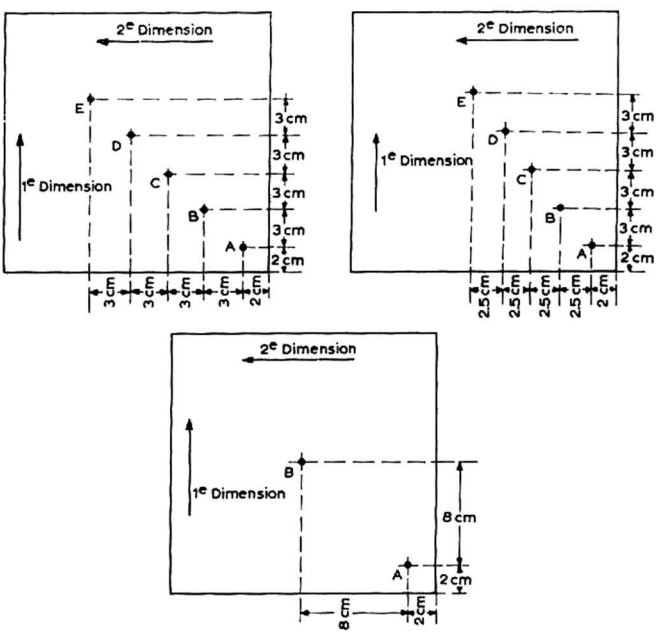


Fig. 9. Disposition des spots de départ pour les acides aspartique et glutamique.

Fig. 10. Disposition des spots de départ pour la lysine.

Fig. 11. Disposition des spots de départ pour la valine.

Préparation des plaques

Pour avoir des étalonnages comparables, nous avons opéré de telle manière que chaque tache d'acide aminé fasse une migration bi-dimensionnelle dans les mêmes conditions que la solution totale.

Nous avons donc été amenés à adopter la disposition suivante pour les spots étalons:

Acides aspartique et glutamique. Nous repérons au crayon les points où nous déposerons les quantités d'acide aminé à doser (Fig. 9). Ces quantités sont les suivantes: en a, 10 μg; en b, 15 μg; en c, 20 μg; en d, 25 μg; en e, 30 μg. Puis, nous faisons migrer les deux solvants dans les conditions précédemment indiquées.

Lysine. La même disposition que précédemment est utilisée, mais les distances entre les spots de départ sont légèrement changées. La migration des solvants se fait dans le sens indiqué sur la Fig. 10.

Valine. Ici, il existe une petite différence par rapport aux autres cas. En effet, la valine migrant loin, aussi bien dans un solvant que dans l'autre, il a fallu opérer sur deux plaques pour avoir quatre points d'étalonnage.

Nous avons donc employé deux plaques de la façon indiquée sur la Fig. 11 et les quantités suivantes ont été déposées:

1ère plaque: point a, 10 μg; point b, 15 μg;

2ème plaque: point a, 20 μg; point b, 25 μg.

Ces deux plaques ont migré simultanément. Elles ont été disposées dos-à-dos dans la même cuve.

Résultats de ces étalonnages

On trouvera sur le Tableau I, pour chaque acide aminé envisagé, les densités optiques moyennes obtenues pour chaque quantité déposée et finalement l'écart moyen sur les densités optiques ainsi obtenues après élution et dosage des taches telles qu'elles se présentent sur les Fig. 12–15.

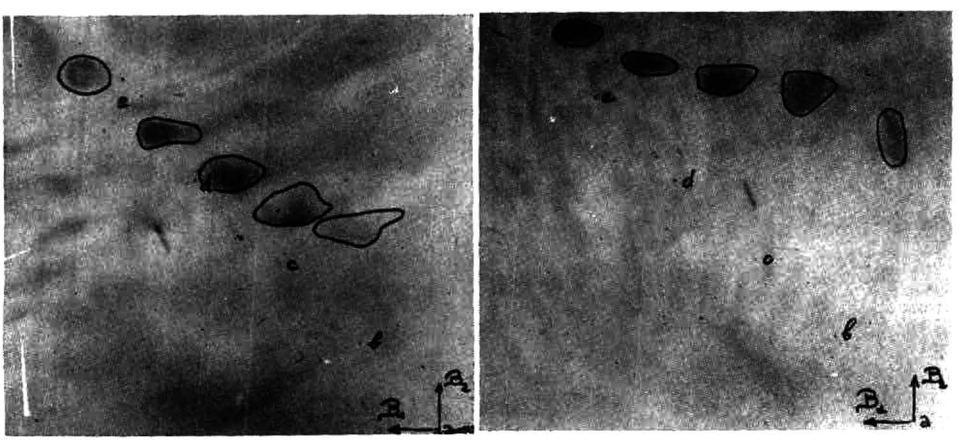


Fig. 12. Chromatographie de l'étalonnage en acide aspartique.

Fig. 13. Chromatographie de l'étalonnage en acide glutamique.

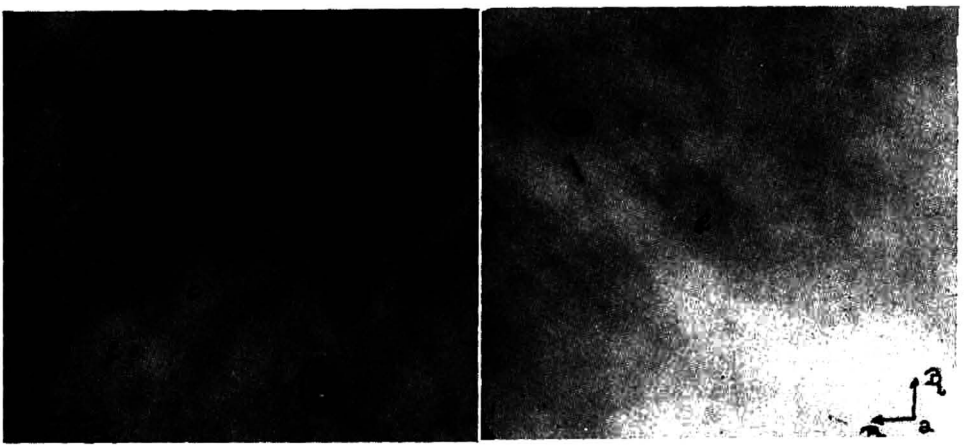


Fig. 14. Chromatographie de l'étalonnage en lysine.

Fig. 15. Chromatographie de l'étalonnage en valine.

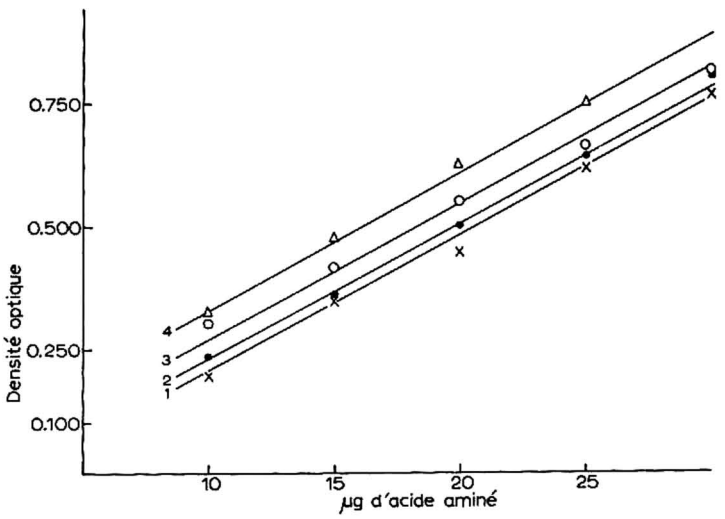


Fig. 16. Courbes d'étalonnage pour les quatre acides aminés dosés. 1 = Acide glutamique; 2 = acide aspartique; 3 = lysine; 4 = valine.

Courbes d'étalonnage. A l'aide de ces résultats, nous avons pu tracer les courbes d'étalonnage (Fig. 16), courbes auxquelles nous comparons les résultats obtenus pour les dosages des acides aminés envisagés, à la fois en solution synthétique et dans le lysat de coeur etudié.

Résultats expérimentaux

Résultats sur acides aminés en solution complexe

On a fait une solution des quatre acides aminés à doser et on a déposé au point de départ un spot contenant 20 µg de chacun de ces corps.

TABLEAU I

ÉTALONNAGE DES QUATRE ACIDES AMINÉS ENVISAGÉS

Acide aspartique				*Acide glutamique*				*Lysine*				*Valine*			
Quantité (µg)	*Densité optique moyenne*	*Écart moyen*	*% Erreur*	*Quantité (µg)*	*Densité optique moyenne*	*Écart moyen*	*% Erreur*	*Quantité (µg)*	*Densité optique moyenne*	*Écart moyen*	*% Erreur*	*Quantité (µg)*	*Densité optique moyenne*	*Écart moyen*	*% Erreur*
10	0.234	0.015	6.5	10	0.193	0.010	5.2	10	0.326	0.012	3.7	10	0.306	0.026	8.5
15	0.360	0.015	4.1	15	0.349	0.026	7.4	15	0.416	0.017	4.1	15	0.479	0.043	8.9
20	0.501	0.018	3.6	20	0.444	0.044	10.0	20	0.546	0.019	3.5	20	0.626	0.045	7.2
25	0.640	0.029	4.5	25	0.616	0.031	5.0	25	0.663	0.034	5.1	25	0.750	0.031	4.1
30	0.804	0.037	4.6	30	0.764	0.018	2.4	30	0.913	0.018	2.2				

TABLEAU II

DENSITÉS OPTIQUES OBTENUES POUR LES QUATRE ACIDES AMINÉS ENVISAGÉS DANS LE CAS D'UNE SOLUTION COMPLEXE

	Essai					*Total*	*Moyenne*	*Écart moyen*	*Quantité trouvée (µg)*	*Écart récupéré*	*% Écart*
	1	*2*	*3*	*4*	*5*						
Acide aspartique	0.438	0.498	0.478	0.458	0.578	2.450	0.490	0.038	19.6 ± 1.2	−0.4	−2.0
Acide glutamique	0.473	0.469	0.560	0.388	0.469	2.379	0.476	0.038	19.7 ± 1.2	−0.3	−1.5
Lysine	0.508	0.454	0.634	0.558	0.586	2.740	0.548	0.074	19.8 ± 2.6	−0.2	−1.0
Valine	0.540	0.550	0.730	0.720	0.640	3.180	0.636	0.075	20.9 ± 2.7	+0.9	+4.5

TABLEAU III

DENSITÉS OPTIQUES OBTENUES POUR LES QUATRE ACIDES AMINÉS DANS LE CAS DE LYSAT DE COEUR

	Acide aspartique		*Acide glutamique*		*Lysine*		*Valine*	
	Densité optique	*Écart densité optique*	*Densité optique*	*Écart densité optique*	*Densité optique*	*Écart densité optique*	*Densité optique*	*Écart densité optique*
	0.570	0.037	0.715	0.002	0.735	0.008	0.875	0.018
	0.630	0.023	0.695	0.022	0.755	0.012	0.825	0.032
	0.630	0.023	0.705	0.012	0.675	0.068	0.815	0.042
	0.625	0.018	0.680	0.037	0.795	0.052	0.875	0.018
	0.570	0.037	0.745	0.028	0.750	0.007	0.845	0.014
	0.580	0.027	0.725	0.008	0.680	0.063	0.900	0.040
	0.575	0.032	0.720	0.003	0.740	0.003	0.840	0.018
	0.600	0.007	0.735	0.018	0.755	0.012	0.895	0.035
	0.645	0.038	0.740	0.023	0.785	0.042	0.875	0.018
	0.645	0.038	0.710	0.007	0.780	0.037	0.835	0.022
Total	6.070	0.280	7.170	0.163	7.430	0.294	8.575	0.257
Moyenne	0.607	0.028	0.717	0.016	0.743	0.029	0.857	0.026
Quantité trouvée (μg)	23.6	0.095	28.4	0.60	27.1	1.10	28.9	0.90
Quantité pour 100 mg	3.146	0.063	3.796	0.40	3.612	0.70	3.852	0.6

Le Tableau II donne les différentes densités optiques trouvées, ainsi que l'écart moyen, la quantité récupérée, l'écart par rapport à la quantité déposée et le pourcentage de cet écart.

Résultats obtenus sur le lysat de coeur

Ce lysat est mis en solution aqueuse à raison de 50 mg/ml, et l'on dépose 15 μl pour former le spot de départ.

Le Tableau III donne, pour les quatre acides aminés, les densités optiques obtenues sur dix mesures, la densité optique moyenne, la quantité d'acide aminé dosé et cette même quantité ramenée à 100 mg de lysat de coeur.

Conclusions

La méthode que nous venons d'exposer offre des avantages certains:

Sa vitesse d'exécution est remarquable, car, contrairement à ce qui se passe avec de nombreuses autres méthodes, il ne faut pas une journée complète pour avoir une séparation détaillée, bi-dimensionnelle, des acides aminés. Si la partie quantitative de la méthode nécessite 48 h, c'est un temps nettement inférieur à tous ceux indiqués pour une méthode semblable faisant intervenir élution des taches et dosage colorimètrique. Evidemment, si d'autres techniques peuvent être plus rapides, elles nécessitent aussi des appareillages plus complexes et plus coûteux[39,40].

La reproductibilité qualitative, en prenant les précautions indiquées dans cet exposé, est excellente et les séparations des acides aminés sont bonnes. Le cas de l'alanine et de la tyrosine, non séparables par notre système, est évidemment à relever, mais d'autres couplages de solvants, actuellement à l'étude, permettront de

résoudre cette difficulté qui n'était que minime ici, à côté de la bonne résolution de tous les autres acides aminés envisagés.

On doit aussi souligner, du fait de la technique de préparation des plaques employées, *la robustesse des couches* de cellulose plus amidon.

Du point de vue quantitatif, on doit remarquer la bonne sensibilité de la méthode, la facilité de récupération des acides aminés ainsi que leur excellente élution se traduisant par un pourcentage d'écart de récupération inférieur ou au plus égal à 5 %, inférieur à ceux donnés par la chromatographie sur papier.

Ainsi donc, cette technique qui ne se prétend pas parfaite pourra aider les nombreux chercheurs qui se consacrent au domaine si complexe des acides aminés, protéines et peptides.

Remerciements

Nous tenons à remercier Monsieur le Professeur DUCHENE-MARULLAZ, Directeur Scientifique du C.E.R.M., pour la bienveillante attention qu'il a apportée à ce travail, ainsi que Madame CHRISTIANE FREJAT dont l'aide technique nous a été précieuse.

RÉSUMÉ

Le présent travail donne tout d'abord une nouvelle séparation chromatographique sur plaque de cellulose d'acides aminés et dans une deuxième partie, développe une méthode de dosage par colorimétrie de certains de ces acides aminés après séparation et élution des taches par un solvant approprié.

SUMMARY

A new method for the separation of α-amino acids on cellulose thin layers is described in the first part of this work. In the second part a colorimetric method for the determination of some of these α-amino acids after elution is developed.

BIBLIOGRAPHIE

1 R. CONSDEN, A. H. CORDON ET A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
2 N. A. ISMAILOV ET M. S. SHRAIBER, *Farmatsiya (Sofia)*, 3 (1938) 1.
3 J. E. MEINHARD ET N. F. HALL, *Anal. Chem.*, 21 (1949) 185.
4 J. G. KIRCHNER, J. M. MILLER ET G. E. KELLER, *Anal. Chem.*, 23 (1951) 420.
5 E. STAHL, *Pharmazie*, 11 (1956) 633.
6 E. STAHL, *Chemiker-Ztg.*, 82 (1958) 323.
7 E. STAHL, *Parfüm. Kosmetik*, 39 (1958) 564.
6 E. STAHL, *Pharm. Rundschau*, 2 (1959) 1.
9 E. STAHL, *Angew. Chem.*, 73 (1961) 646.
10 M. KANDATSU ET H. NAITO, *Nippon Nogei Kagaku Kaishi*, 33 (1959) 170.
11 M. BRENNER ET A. NIEDERWIESER, *Experientia*, 16 (1960) 378.
12 M. BRENNER ET A. NIEDERWIESER, *Experientia*, 17 (1961) 237.
13 G. PATAKI, *Z. Klin. Chem.*, 1, No. 5 (1963) 157.
14 G. PATAKI, *J. Chromatog.*, 17 (1965) 580.
15 L. CODERN, J. GADEA, E. GAL ET M. MONTAGUT, *Afinidad*, 20, No. 225 (1963) 159.
16 E. MUTSCHLER ET H. ROCHELMEYER, *Arch. Pharm.*, 292 (1959) 449.
17 M. MOTTIER, *Mitteilungsgeb. Lebensm. Hyg.*, 49 (1958) 654.
18 L. RASTEIKIENE ET T. PRANSKIENE, *Lietuvos TSR Mokslu Akad. Darbai, Ser. B*, 3 (1963) 59.
19 S. MUSHA ET H. OCHI, *Bunseki Kagaku*, 14, No. 3 (1965) 202.
20 A. R. FAHMY, A. NIEDERWIESER, G. PATAKI ET M. BRENNER, *Helv. Chim. Acta*, 44 (1961) 2022.

21 B. SHASHA ET R. L. WHISTLER, *J. Chromatog.*, 14 (1964) 532.
22 H. DÉTERMANN, *Experientia*, 18 (1962) 430.
23 D. CORBI, *Giorn. Med. Mil.*, 114, No. 2 (1964) 168.
24 P. WOLLENWEBER, *J. Chromatog.*, 9 (1962) 369.
25 T. DITTMANN, *Z. Klin. Chem.*, 1, No. 6 (1963) 190.
26 M. L. WOLFROM, D. L. PATIN ET R. M. DE LEDERKREMER, *Chem. Ind. (London)*, 25 (1964) 1065.
27 P. BLANC, P. BERTRAND, G. DE SAQUI-SANNES ET R. LESCURE, *Chim. Anal.*, 47, No. 6 (1965), 285.
28 R. L. MUNIER ET G. SARRAZIN, *Bull. Soc. Chim. France*, 5 (1965) 1490.
29 EL. BUJARD, *3e Symp. Chromatog., Bruxelles, 14 et 15 Sep. 1964*; *J. Pharm. Belg.*, 20 (1965) 413; *J. Chromatog.*, 21 (1966) 19.
30 P. DE LA LLOSA, C. TERTRIN ET M. JUTISZ, *J. Chromatog.*, 14 (1964) 136.
31 N. A. TURNER ET R. J. REDGWELL, *J. Chromatog.*, 21 (1966) 129.
32 C. G. HONEGGER, *Helv. Chim. Acta*, 46 (1963) 1730, 1772.
33 G. BISERTE, P. BOULANGER ET P. PAYSANT, *Bull. Soc. Chim. Biol.*, 40 (1958) 2067.
34 S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 241.
35 A. L. LEVY ET D. CHUNG, *Anal. Chem.*, 25 (1953) 396.
36 E. STAHL, *Thin-Layer Chromatography*, Academic Press, New-York, 1965, p. 399.
37 T. STARON, communication personelle.
38 M. JUTISZ ET P. DE LA LLOSA, *Bull. Soc. Chim. France*, 12 (1963) 2913.
39 M. M. FRODYMA ET R. W. FREI, *J. Chromatog.*, 15 (1964) 501.
40 M. M. FRODYMA ET R. W. FREI, *J. Chromatog.*, 17 (1965) 131.
41 D. MUTING, *Naturwiss.*, 39 (1952) 548.
42 J. BARROLIER, J. HEILMAN ET E. WATZKE, *Z. Physiol. Chem.*, 304 (1956) 21.
43 A. SAID ET D. H. FLEITA, *Chemist-Analyst*, 52 (1963) 79.
44 T. KNAUT, *J. Chromatog.*, 13 (1964) 560.
45 N. N. NAZIROV, *Metody Issled. Fiziol. i Biokhim. Khlopchat., Akad. Nauk Uzbek. SSR, Rezultaty Rabot Sovet.*, (1957) 128.
46 K. S. AMBE ET A. L. TAPPEL, *J. Chromatog.*, 5 (1961) 546.
47 P. BOHLEV, *Naturwiss.*, 49 (1962) 326.
48 N. A. TURNER ET R. J. REDGWELL, *J. Chromatog.*, 21 (1966) 129.
49 T. TSUKAMOTO, T. KOMORI ET N. KINOSHITA, *Chem. Pharm. Bull (Tokyo)*, 5 (1957) 363.
50 G. N. ZAITSEVA ET N. P. TYVLENEVA, *Lab. Delo*, 4, No. 3–4 (1958) 24.
51 T. TSUKAMOTO ET T. KOMORI, *Chem. Pharm. Bull. (Tokyo)*, 8 (1960) 913.
52 E. R. COOK ET M. LUSCOMBE, *J. Chromatog.*, 3 (1960) 75.
53 J. BARROLIER, *Naturwiss.*, 42 (1955) 416.
54 J. HEILMANN, J. BARROLIER ET E. WATZKE, *Z. Physiol. Chem.*, 309 (1958) 219.
55 T. BORKOWSKI ET I. MADECKA-BORKOWSKA, *Chem. Anal. (Warsaw)*, 4 (1959) 119.
56 E. MOFFAT ET R. LYTLE, *Anal. Chem.*, 31 (1956) 926.
57 E. MACEVOY-BOWE ET J. W. H. LUGG, *Biochem. J.*, 80 (1961) 616.
58 S. M. PETROVIC ET S. E. PETROVIC, *J. Chromatog.*, 21 (1966) 313.
59 S. MOORE ET W. STEIN, *J. Biochem.*, 176 (1948) 267.
60 P. H. BELL, K. S. HOWARD, R. G. SHEPHERD, B. M. FINN ET J. H. MEISENHELDER, *J. Am. Chem. Soc.*, 78 (1956) 5051, 5067.
61 Y. WATANABE, K. WATANABE, F. KOIDE, T. SAITO ET K. SHIMURA, *Nippon Nogei Kagaku Kaishi*, 34 (1960) 620.
62 B. ORCHARD, *Nature*, 198 (1963) 688.
63 R. BONDIVENNE, M. COULET, G. BETAIL, N. BUSCH ET R. Y. MAUVERNAY, *Ann. Pharm. Franc.*, 19, No. 7 (1961) 523.
64 E. A. ERMAKOVA, *Biokhimiya*, 22 (1957) 917.
65 H. WIEWIOROWSKI, J. PRZYBYLSKA ET Z. KOCIALKOWSKI, *Rocznicki Nauk Rolniczych, Ser. A*, 79 (1958) 453.
66 C. A. PORTER, D. MARGOLIS ET P. SHARP, *Contrib. Boyce Thompson Inst.*, 18 (1957) 465.
67 T. STARON, C. ALLARD ET M. M. CHAMBRE, *Compt. Rend.*, 253 (1961) 1630.

THIN LAYER CHROMATOGRAPHY OF CHLOROGENIC ACID ISOMERS

Z. GRODZIŃSKA-ZACHWIEJA, W. KAHL AND A. WARCHOŁ
Department of Organic Chemistry, School of Medicine, Cracow (Poland)
(Received January 20th, 1967)

In studies on the chemical composition of plants, chlorogenic acid is encountered with increasing frequency in nearly all plants. Although known for over 60 years[1], it is only recently that the structure of its three isomers—cryptochlorogenic, neochlorogenic and isochlorogenic acid—has been determined.

Chlorogenic acid is 3-caffeoylquinic acid[2]; cryptochlorogenic acid (known also as Band 510) is 4-caffeoylquinic acid[3]; and neochlorogenic acid is 5-caffeoylquinic acid[3]. The acid known until recently as isochlorogenic acid has proved to be a mixture of three dicaffeoylquinic acids[4].

These compounds take part in various biochemical processes, influence the growth of plants, their enzymes and defence mechanisms. In this study, trials were

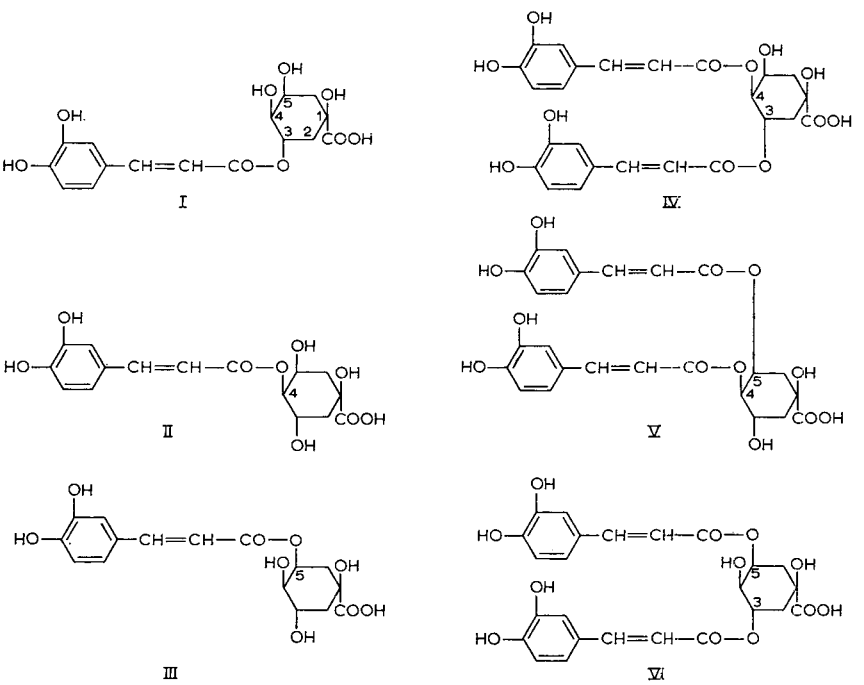


Fig. 1. Isomers of chlorogenic and isochlorogenic acid. I = Chlorogenic acid (3-caffeoylquinic acid); II = cryptochlorogenic acid (4-caffeoylquinic acid); III = neochlorogenic acid (5-caffeoylquinic acid); IV = 3,4-dicaffeoylquinic acid; V = 4,5-dicaffeoylquinic acid; VI = 3,5-dicaffeoylquinic acid.

undertaken to develop a rapid and convenient method of identifying these compounds. SCARPATI[5], in the course of studies on the structure of these compounds, employed thin layer chromatography and separated the isochlorogenic acids, but separation of chlorogenic and cryptochlorogenic acid gave unsatisfactory results. The main difficulty in the chromatographic separation of these isomers lies in the fact that, in various systems, neochlorogenic acid has an R_F value different from that of chlorogenic and cryptochlorogenic acid, while the two latter have very similar R_F values.

Attempts to separate chlorogenic acid from cryptochlorogenic and from neochlorogenic acid by means of thin layer chromatography using various solvent systems and a search for a specific colour reaction are reported here.

MATERIALS AND METHODS

Standard substances

Chlorogenic, cryptochlorogenic, neochlorogenic and isochlorogenic acids, received from Dr. E. SONDHEIMER, U.S.A., were used as standard substances in the experiments.

Adsorbent

Silica Gel G (Merck Co.) was used as adsorbent.

Preparation of plates

Three types of separation were carried out:

(1) Separation on ordinary gel using solvents containing CH_3COOH.

(2) Separation on gel impregnated with $KHSO_4$.

(3) Separation on gel impregnated with $KHSO_4$ and acidified with HCl vapour according to the method described by SCARPATI[5].

Thus, three types of plates were prepared, *viz.*: (1) plates 7 × 17 cm covered with 2 g of gel suspended in 4 ml H_2O; (2) plates 7 × 17 cm covered with 2 g of gel suspended in 5.6 ml of 2.5 % $KHSO_4$ solution; (3) plates 7 × 17 cm covered with 2 g of gel suspended in 5.6 ml of 2.5 % $KHSO_4$ solution and exposed to HCl vapour for 5 min.

Solvent systems

Of the numerous solvents that were investigated, forty, which proved of greatest interest, are listed in Tables I–III.

Colour reactions

(I) Chlorogenic acids

These are yellow at pH values above 7 and in air the colour turns brown[6]. The plates were therefore either exposed to NH_3 vapour or sprayed with 2 *N* KOH dissolved in CH_3OH.

(II) Reaction for phenols

Because of the phenolic character of the caffeic acid residue, chlorogenic acids give a colour reaction with reagents used to detect phenols, *viz.*:

(1) *$FeCl_3$*. Grey-green spots appear on plates sprayed with 2 % aqueous $FeCl_3$ solution.

(*2*) *Turnbull's blue reaction*[7]. After spraying the plates with 3 % $FeCl_3$, followed by 3 % $K_3[Fe(CN)_6]$, blue spots appear on a pale-blue background.

(*3*) *$KMnO_4$*[8]. After spraying the plates with 1 % $KMnO_4$ in 0.1 *N* H_2SO_4, yellow spots appear on a violet background.

(*4*) *Diazo-reaction.* Diazo-reaction is carried out with:

(a) Diazotized *p*-nitroaniline[8]. 5 ml of *p*-nitroaniline (0.5 % in 2 *N* HCl) is mixed with 0.5 ml of $NaNO_2$ (5 %), and 15 ml of sodium acetate (20 %) is then added. Spraying the plates with this mixture produces brown spots.

(b) Tetrazotized benzidine[7]. 1 g of benzidine is dissolved in 3 ml conc. HCl and diluted with H_2O to 200 ml. A 10 % aqueous solution of $NaNO_2$ is prepared separately. Before use, equal volumes of the two solutions are mixed. Spraying give rise to brown spots.

(c) Stabilized diazo salts[9]. An 0.05 % aqueous solution of the stabilized salts is used and coloured spots appear on sprayed plates.

(*III*) *Reactions for o-dihydroxyphenols*

(*1*) *Sodium molybdate*[10]. *o*-Dihydroxyphenols form complex red compounds with Mo. Orange-brown spots appear on plates sprayed with an 0.1 *M* aqueous solution of sodium molybdate.

(*2*) *Arnow's reaction*[11]. Arnow's reagent is prepared by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of water. After spraying the plates with this reagent orange-brown spots appear.

(*3*) *Phloroglucinol*[12]. On plates sprayed with 0.1 % solution of phloroglucinol in 1 *N* NaOH, *o*-dihydroxyphenols of the caffeic acid type give yellow spots which turn brown after heating at 80°.

RESULTS

The results of the experiments on the behaviour of chlorogenic acid, cryptochlorogenic acid and neochlorogenic acid under various condition of thin layer chromatography are summarized in Tables I, II and III.

The solvent systems used to develop the plates with ordinary gel are shown in Table I. The starting point of these experiments was the classic system of PARTRIDGE (*n*-butanol–glacial acetic acid–water, 4:1:5), in which these acids were only slightly separated. By increasing the amount of *n*-butanol in relation to water, and by increasing the acid in the organic phase, the following system was obtained: *n*-butanol–acetic acid–water (10:1.75:8). The organic phase was used, 4 ml of acetic acid per 100 ml of *n*-butanol being added. With this solvent system, the different isomers of chlorogenic acid gave markedly different R_F-values. However, in view of the long development time (140 min) and diffusion and superimposition of the spots, it was not considered entirely satisfactory.

The results of the separation of chlorogenic acid isomers on plates impregnated with $KHSO_4$ but not acidified with HCl are summarized in Table II. The best solvent system in this group was ethyl ether–acetic acid–water (50:12:50) (organic phase). Time of development was one hour. Differences between the R_F values were satisfactory, but the spots were somewhat diffuse and superimposed.

Table III lists the R_F values of chlorogenic acid isomers obtained on plates impregnated with $KHSO_4$ and acidified with HCl vapour. A system composed of aceto-

TABLE I

R_F VALUES OF CHLOROGENIC ACID ISOMERS IN VARIOUS SOLVENTS

(For plates with ordinary gel)

No.	*Solvent*	*Time* needed to reach 13 cm*	*R_F values of acid*			*Remarks*
			Neo.	*Chlor.*	*Crypt.*	
1	Water	30	—	—	—	Trails from the start to solvent front
2	*n*-Butanol	180	—	—	—	Spots at the start
3	Acetic acid, glacial	80	0.77	0.54	0.53	Similar R_F values of chlor. and crypt.
4	Acetic acid, 80%	100	0.72	0.67	0.61	Spots partly superimposed
5	Butanol–acetic acid–water (4:1:5)	150	0.49	0.44	0.39	Spots partly superimposed
6	Butanol–acetic acid–water (5:1.75:8)	130	0.55	0.55	0.55	Compact spots, but identical R_F
7	Butanol–acetic acid–water (6:1.75:8)	140	0.50	0.47	0.46	Poor separation
8	Butanol–acetic acid–water (8:1.75:8)	150	0.55	0.53	0.42	Better separation, spots superimposed
9	Butanol–acetic acid–water (10:1.75:8)	160	0.43	0.39	0.33	Better separation, spots diffuse
10	Butanol–acetic acid–water (15:1.75:8)	190	0.38	0.31	0.28	Worse separation
11	Butanol–acetic acid–water (20:1.75:8)	210	0.36	0.30	0.27	Worse separation
12	Butanol–acetic acid–water (10:1.75:8) + 1 ml**	140	0.45	0.38	0.32	Spots partly superimposed
13	Butanol–acetic acid–water (10:1.75:8) + 2 ml**	140	0.41	0.32	0.29	Better separation
14	Butanol–acetic acid–water (10:1.75:8) + 4 ml**	140	0.49	0.40	0.32	Best separation
15	Butanol–acetic acid–water (10:1.75:8) + 10 ml**	130	0.50	0.46	0.42	Worse separation

* Time in minutes.

** Systems 12, 13, 14 and 15 were prepared by shaking 100 ml of *n*-butanol with 1.75 ml of glacial acetic acid and 80 ml of water. After 24 h the layers were separated and 1, 2, 4 or 10 ml of acetic acid was added to the butanolic layers.

TABLE II

R_F VALUES OF CHLOROGENIC ACID ISOMERS IN VARIOUS SOLVENTS

(For plates with gel impregnated with $KHSO_4$, not acidified with HCl)

No.	*Solvent*	*Time* needed to reach 13 cm*	*R_F values of acid*			*Remarks*
			Neo.	*Chlor.*	*Crypt.*	
1	Ethyl ether–glacial acetic acid (50:5)	30	0.09	0.15	0.17	Compact spots, but poor separation
2	Ethyl ether–glacial acetic acid (50:10)	50	0.49	0.58	0.69	Spots diffuse
3	Ethyl ether–acetic acid–water (50:4:50) (organic layer)	60	0.03	0.07	0.06	Compact spots, chlor. and crypt. not separated
4	Ethyl ether–acetic acid–water (50:12:50)	60	0.36	0.51	0.57	Good separation, but spots superimposed
5	Ethyl ether–acetic acid–water (50:15:50)	60	0.51	0.58	0.61	Spots superimposed partly

* Time in minutes.

J. Chromatog., 29 (1967) 362–368

TABLE III

R_F VALUES OF CHLOROGENIC ACID ISOMERS IN VARIOUS SOLVENTS

(For plates with gel impregnated with $KHSO_4$, acidified with HCl)

No.	*Solvent*	*Time** (*to reach 13 cm*)	*R_F values of acid*			*Remarks*
			Neo.	*Chlor.*	*Crypt.*	
1	Non-polar solvents: *e.g.* cyclohexane, benzene	different	—	—	—	At the start
2	Ethyl ether	15	0.00	0.03	0.02	Compact spots, but poor separation
3	Polar solvents: *e.g.* methanol, ethanol, acetone	60	—	—	—	Spots diffuse, in solvent front
4	Water	30	—	—	—	Trails from start to solvent front
5	*n*-Propanol	80	0.41	0.55	0.53	Compact spots, but poor separation
6	Ethyl acetate	30	0.20	0.32	0.34	Chlor. and crypto. not separated
7	Ethyl formate	40	0.27	0.46	0.49	Chlor. and crypto. not separated
8	Dioxan	40	0.44	0.54	0.55	Chlor. and crypto. not separated
9	Methyl ethyl ketone	60	0.84	0.87	0.90	Compact spots, but poor separation
10	Acetophenone	60	0.39	0.47	0.54	Very good separation but spots diffuse
11	Acetylacetone	100	0.32	0.48	0.48	Chlor. and crypto. not separated
12	Acetic acid 80%	70	0.80	0.80	0.80	
13	Ethyl ether–dioxan (1:1)	25	0.29	0.34	0.34	As in dioxan
14	Ethyl ether–ethyl acetate (1:9)	20	0.18	0.28	0.30	As in ethyl acetate
15	Ethyl acetate–acetone (8:2)	20	0.19	0.28	0.29	Chlor. and crypto. are superimposed
16	Chloroform–dioxan (7:3)	20	0.02	0.06	0.04	Compact spots, but poor separation
17	Dioxan–ethyl acetate (1:1)	20	0.24	0.35	0.38	Better separation than in ethyl acetate
18	Acetophenone–ethyl ether (1:1)	60	0.16	0.26	0.34	Good separation, but spots are superimposed
19	Acetophenone–ethyl acetate (2:1)	60	0.27	0.37	0.45	Good separation, but spots are superimposed
20	Acetophenone–methyl ethyl ketone (1:1)	60	0.41	0.50	0.58	Excellent separation

* Time in minutes.

phenone–methyl ethyl ketone (1:1) proved excellent for the separation of the chlorogenic acid isomers. The time of development was 1 h, the R_F values of the different acids differed distinctly, and the spots were compact, giving a clear picture of the separation of the studied compounds.

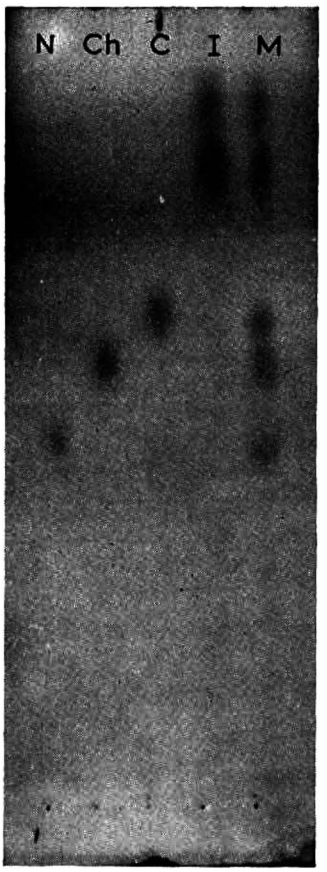


Fig. 2. Thin layer chromatography of chlorogenic acid isomers. Adsorbent: Silica Gel G impregnated with $KHSO_4$. System solvent: acetophenone–methyl ethyl ketone (1:1). Detection: NH_3 vapour. Substances separated: N = neochlorogenic acid; Ch = chlorogenic acid; C = cryptochlorogenic acid; I = isochlorogenic acid; M = mixture of the acids.

Table IV shows the reactions which served to detect the chlorogenic acids on silica gel plates. All the chlorogenic acids gave similar colour reactions with the reagents described above. Arnow's reaction proved to be the best test, giving very distinct orange-brown spots on a white background. In addition, this reaction is highly specific, giving positive reactions only with *o*-dihydroxyphenols. The reactions with ammonia vapour and diazo salts were also useful. The very sensitive but unspecific reactions with $KMnO_4$ and Turnbull's reagent can be used only for detecting plates developed in solvent systems with nonreducing properties.

TABLE IV

COLOUR REACTIONS FOR CHLOROGENIC ACIDS

No.	*Reagent*	*Colour reaction*
1	NH_3 vapour	Brown spots
2	KOH	Brown spots
3	$FeCl_3$	Grey-green spots
4	Turnbull's reagent	Blue spots on a pale-blue background
5	$KMnO_4$	Yellow spots on a violet background
6	Diazotized *p*-nitroaniline	Brown spots
7	Tetrazotized benzidine	Brown spots
8	Fast black salt K	Brown-red spots
9	Sodium molybdate	Orange-brown spots on a white background
10	Arnow's reagent	Orange-brown spots on a white background
11	Phloroglucinol	Yellow spots

ACKNOWLEDGEMENT

The authors wish to thank Dr. E. SONDHEIMER for the gift of the samples of neochlorogenic, cryptochlorogenic, and isochlorogenic acids.

SUMMARY

Chlorogenic, neochlorogenic and cryptochlorogenic acids have been separated by the use of thin layer chromatography. Silica Gel G (Merck) plates impregnated with $KHSO_4$ were used. Forty chromatographic solvent systems used to develop the acids and eleven colour reactions are listed. A solvent system composed of methyl ethyl ketone–acetophenone (1:1) and the colour test with Arnow's reagent proved best.

REFERENCES

1 K. GORTER, *Ann.*, 358 (1907) 327.
2 H. O. L. FISCHER AND G. DANGSCHAT, *Chem. Ber.*, 65 (1932) 1037.
3 M. L. SCARPATI AND P. ESPOSITO, *Ann. Chim. (Rome)*, 54 (1964) 35.
4 M. L. SCARPATI AND M. GUISO, *Tetrahedron Letters*, 39 (1964) 2851.
5 M. L. SCARPATI AND M. GUISO, *Ann. Chim. (Rome)*, 53 (1963) 1315.
6 E. SONDHEIMER, *Botan. Rev.*, 30 (1964) 667.
7 K. RANDERATH, *Thin Layer Chromatography*, Academic Press, New York, 1963, p. 175.
8 C. F. SUMERE AND G. WOLF, *J. Chromatog.*, 20 (1965) 48.
9 I. A. PEARL AND P. F. MCCOY, *Anal. Chem.*, 32 (1960) 1407.
10 J. B. PRIDHAM, *J. Chromatog.*, 2 (1959) 605.
11 E. NICHIFORESCO AND V. COUCOU, *Ann. Pharm. Franc.*, 23 (1965) 419.
12 E. EGRIVE, *Z. Anal. Chem.*, 125 (1943) 241.

COMBINED EXTRACTION AND PAPER CHROMATOGRAPHY OF FOOD PRESERVATIVES

J. RAJAMA AND P. MÄKELÄ

Laboratory for Food Research and Technology, The State Institute for Technical Research, Otaniemi (Finland)

(Received January 17th, 1967)

If a foodstuff is applied direct on paper for the chromatographic separation of food preservatives, food components interfere with the migration, and by masking the spots on the chromatogram, make this rather difficult to interpret. For this reason, a preliminary extraction step usually precedes paper chromatography of ether-soluble preservatives. A large number of extraction funnels and other items of glassware are needed for the extraction, if several analyses are to be made simultaneously. Further trouble is occasioned by emulsions; these are formed easily and can make the funnel extraction of fruit juices and jellies a rather difficult matter.

The purpose of this study is to find a remedy for these difficulties. Use is made of the well-known effect—normally detrimental in chromatographic separation—of compounds migrating with the solvent front if the solvent is too powerful. This phenomenon can be regarded as a kind of extraction on paper, as it permits the direct

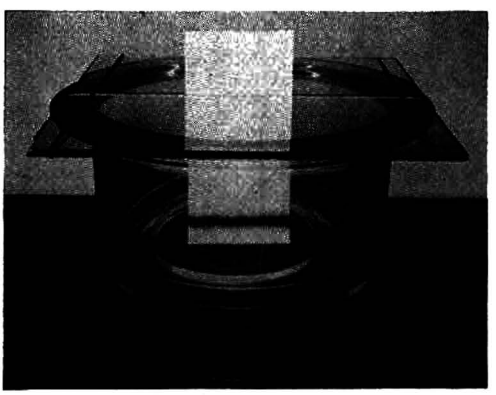


Fig. 1. Extraction vessel.

separation of ether-soluble compounds from interfering substances on chromatographic paper. The procedure is simple and rapid and does not call for any complicated apparatus. The extracting solvent concentrates the preservatives in a narrow line, well suited as a starting line for chromatography on the same paper. Moreover, some preservatives can be separated quickly and efficiently during the course of the extraction, with the help of arresting reagents. A modification of the method has been developed for fat-containing foods.

EXPERIMENTAL

Apparatus and materials

Extraction vessel (see Fig. 1). The lower part of a desiccator is used for this purpose. The arrangement allows easy regulation and change of the extraction distance. The rim of the extraction vessel should be smooth so that a saturated atmosphere is maintained within the vessel, otherwise ether evaporates from the paper before it reaches the slit between the glass plates.

Bromination chamber (see Fig. 2). This is an arrangement which resembles the extraction vessel, but a shallow dish (height about 4 cm) replaces the desiccator. Only the lowest part of the paper (starting line) comes inside the chamber through a slit between the glass plates.

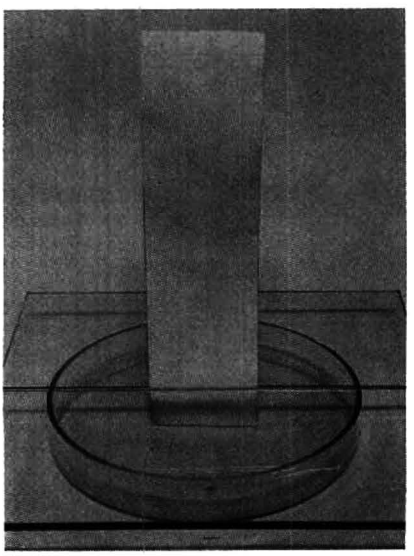


Fig. 2. Bromination chamber.

Chlorination chamber. A 2-l decanter glass used for this purpose is covered with a saran-membrane attached by means of a rubber band. Chlorine is led from a chlorine generation bottle into the chamber through a plastic tube.

U.V.-lamp. The maximum wavelength was 254 nm.

Chromatographic paper. Whatman 3 MM, or a corresponding thick paper, is cut into strips 5 cm in width; the length is varied from 15 to 30 cm according to the needs of the analysis.

Reagents

Extraction. Ethyl ether.

Impregnating solution: 1 part of 0.5 *N* sodium hydroxide, aqueous solution, and 2 parts of acetone. The acetone is added immediately before use.

Alkaline "arresting reagent": a saturated sodium bicarbonate solution.

Chromatography. Solvent system: isoamyl alcohol, ethanol and (1/10 diluted) ammonium hydroxide (6:3:2).

Identification reagents. Potassium permanganate, 0.05 % solution, 1*N* with respect to sulphuric acid.

Manganese dioxide, for the development of chlorine gas.

Hydrochloric acid, concentrated and 1 *N*.

Sodium hypochlorite: an 11 % commercial solution, diluted to 1/15 with water.

Extraction procedure

Low fat foods. A juice of low sugar concentration (10 to 20 %) can be applied as such on to the paper by pipetting 50 to 100 μl across a strip of chromatographic paper as a broad band at a distance of about 1 cm from the end of the paper. The material does not need concentration in a narrow area as in ordinary chromatographic separation; it is spread across the paper quickly, without any intermittent drying. Juices with a higher sugar content, as well as jellies and jams, should be diluted with the same or double the amount of water.

In other cases, a sample of homogenized and diluted material, corresponding to 50 to 100 mg of the original product, is transferred to the paper as described above. Application has also been effected by weighing about 50 to 100 mg of the food in a small decanter glass, diluting with double the amount of water, and transferring the total weighed and diluted material to the paper with the aid of a glass capillary.

After the sample has been applied to the paper, it is left to equilibrate in the air for a short period (3 to 7 min), so that the moisture becomes equalized at the starting line; however, it must not be allowed to dry. The presence of moisture is essential if the extraction is to succeed. With new material for analysis, some experimentation may be needed to determine the correct equilibration time. The extraction is also dependent on a certain degree of acidity, but most food materials are sufficiently acidic to make the addition of acid seldom necessary.

The strip is now pressed between the edges of two glass plates so that the lower end of the paper (about 5 to 8 cm) reaches through the slit between the plates into a dish, filled with ethyl ether, in the extraction vessel (Fig. 1). The ether is adsorbed through the paper, and evaporates on the slit, concentrating the ether-soluble material as a narrow line in the solvent front (Fig. 3). The extraction is complete within 15 min, and the paper is removed from the extraction vessel.

The evaporation line may serve as a starting line for the chromatographic separation. The paper strip is cut off one to two centimetres below the evaporation line, and the paper is transferred into a chromatographic solvent. *p*-Hydroxybenzoic acid, and its esters, are separated from sorbic and benzoic acid within an hour; 30 min may be adequate if the sample is small enough. The solvent system was described by Joux[1].

Fat-containing foods. About 50 mg of the homogenized food is weighed into a small decanter glass. A material with a medium fat content, such as fish, is mixed with double the amount of water; a fatty substance, such as margarine, is mixed with double the quantity of butanol. If the acidity of the material is insufficient, acidifi-

cation with hydrochloric acid may be necessary. The weighed and mixed material is transferred to the paper strip as described above.

The paper is placed into the extraction vessel as above, but instead of the preservatives being extracted to the solvent evaporation front, they are now arrested by alkaline arresting reagents and thus separated from the fat, which is extracted by the ether over the alkaline area up to the evaporation front. Two types of arresting reagents are used: sodium bicarbonate for free acids, and sodium hydroxide for esters.

Fig. 3. The line of sorbic and benzoic acids photographed in U.V. light on the ether evaporation front.

Sorbic, benzoic and *p*-hydroxybenzoic acids are arrested in a line of saturated sodium bicarbonate which had previously been streaked across the paper with a glass capillary at a distance of 2 to 3 cm from the start line where the food material is applied. Fat, and such neutral substances as the esters of *p*-hydroxybenzoic acid, are extracted through the bicarbonate line to the solvent front. The distance between the evaporation line and the starting line should be at least 10 cm, to leave space for paper chromatography. The long migration distance, and the need to extract an appreciably large amount of fat to the evaporation line, make a longer extraction time necessary —at least half an hour, or even more. When the extraction is completed, the paper is cut off between the foodstuff and the bicarbonate lines, and transferred to the chromatographic solvent, which directly dissolves out benzoic and sorbic acids from the bicarbonate line; *p*-hydroxybenzoic acid is retained by the bicarbonate, however.

Chromatography of PHB esters. The efficient separation of the *p*-hydroxybenzoates from each other and from free acids is possible by a single direct ether extraction. After the food has been applied to the paper as described above, the strip is dipped into a freshly prepared mixture of aqueous sodium hydroxide and acetone,

which has been added to ensure uniform impregnation. The lower border of the alkaline region is one to two cm from the foodstuff line, so that ether may collect the preservatives into a narrow line before the alkaline region where the separation take place, is reached. With foods of moderate fat content, the paper is transferred into the ether extraction vessel 5 to 7 minutes after dipping into the impregnation solution; with fats, the equilibrating time is even shorter (1 to 3 min). The migration of the esters is slower on wet paper; this facilitates the separation from the fats. An acidic milieu on the extraction line is required also in this case.

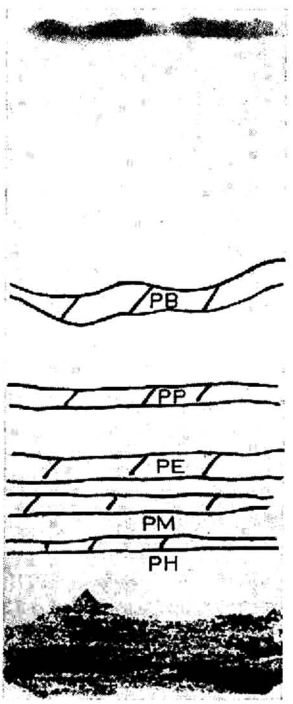


Fig. 4. 50 mg of fish homogenate (the broad dark line at the start) have been extracted with ether for 30 min on NaOH impregnated paper. Concentrations of 12 % NaCl and 12 % sugar, pH 6.2. To the material has been added *p*-hydroxybenzoic acid (PH) and methyl, ethyl, propyl and butyl *p*-hydroxybenzoates, PM, PE, PP and PB respectively, 0.02 % of each compound. The extracted fat shows as a dark line on the upper part of the chromatogram marking the evaporation front. The preservatives have been outlined with pencil in U.V. light.

While the PHB-esters are separated from each other as distinct lines in the alkaline region, the fats are extracted to the evaporation line; acid substances, such as benzoic, sorbic and *p*-hydroxybenzoic acid, are arrested at the lower border of the alkaline region (see Fig. 4).

Detection and identification

All the acids and esters mentioned above are detectable as dark blue bands in short wave, ultra-violet light. This is the simplest method of detection, and makes quantitative determination possible after elution.

Most of these compounds are distinguishable by differences in the migration rate, either in the chromatographic solvent, or simply after the ether extraction. However, two pairs of compounds could not be resolved: the butyl and benzyl *p*-hydroxybenzoates migrated at the same speed in the extraction–separation procedure for esters; and benzoic and sorbic acids had R_F values which were too close, so that separation within a short period of time was impracticable in the chromatography of these acids. However, these acids can be distinguished by their differences in reactivity with the visualization reagents.

Permanganate reaction. Sorbic acid is detectable as a white band against a pink background after the paper has been sprayed with 0.05 % potassium permanganate in 1 *N* sulphuric acid. Benzoic acid does not decolorize potassium permanganate under the same conditions. PHB and PHB esters are also discernible as white bands on the chromatogram, although they are distinguished by their different migration rates. The pink colour of the background disappears within a few minutes, but if desired, the spraying may be repeated a number of times.

Chlorination. For detection of the possible presence of benzoic acid in addition to sorbic acid, the chromatogram is cut lengthwise into two parts, and one half is put into a chamber containing chlorine gas for five minutes. Sorbic acid is no longer visible in U.V. light after the chlorination. Benzoic acid does not undergo any change, and is also visible after the chlorine treatment as a blue band in U.V. light. The chlorine is generated in a reaction bottle by the addition of concentrated hydrochloric acid to manganese dioxide, and the application of gentle warmth. The gas is led to the chlorination chamber through a plastic tube.

The chlorine treatment should be carried out while the paper is still wet after chromatography. If the paper has dried, it should be wetted by a fine water spray.

Bromination. Bromination has the same effect as chlorination, but this treatment must be effected before the chromatography. Immediately after applying the foodstuff to the paper, it is pressed between the edges of the two glass plates, so that the lowest part of the paper strip, with the still moist line of food, reaches into a shallow glass dish filled with bromine vapour—generated by a small drop of bromine at the bottom of the dish (Fig. 2). Five minutes of this bromine treatment is adequate to ensure disappearance of the U.V. absorption of sorbic acid.

Hypochlorite treatment. Disappearance of the dark blue band of sorbic acid in U.V. light is instantly achieved by spraying with 0.1 *N* sodium hypochlorite solution, to which has been added immediately before spraying two drops of concentrated hydrochloric acid per 10 ml. A similar effect is obtained by spraying first with 0.1 *N* sodium hypochlorite solution, and then with 1 *N* hydrochloric acid. Benzoic acid remains stable during this treatment.

DISCUSSION

Experiments have been made with fruit and berry material, such as juices, jams and jellies, and with foods of medium fat content, such as herring preserves. Margarine was studied as an example of fat.

To date, the preservatives studied have been: benzoic and sorbic acids, *p*-hydroxybenzoic acid and methyl, ethyl, butyl, propyl and benzyl *p*-hydroxybenzoates. After 15 min extraction on paper a chromatographic run lasting half an hour was capable of separating these compounds into three groups: (I) *p*-hydroxybenzoic acid (PH); (II) benzoic and sorbic acids (BH + SH); and (III) *p*-hydroxybenzoates (PM).

This short running time is feasible by virtue of the favourable form of the new starting line caused by evaporation of the ether front, and the absence of interfering agents.

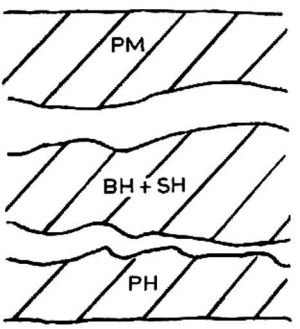


Fig. 5. Separation of extracted preservatives after a ½ h chromatographic run. The compounds have been outlined with pencil in U.V. light.

Half an hour was sufficient for the simultaneous extraction and chromatographic separation of *p*-hydroxybenzoates from non-fatty foods and foods of medium fat content on sodium hydroxide impregnated paper. One hour, or occassionally 1½ h, was required for separation from fats, such as margarine.

Stress is laid upon the critical nature of the degree of moisture of the starting line and the impregnated paper—if the paper is dried too extensively after impregnation, the esters migrate too fast, and the lines become so diffuse that the substances are not detectable, or may even be pushed into the evaporation line. Conversely, if the paper is too wet, the separation of the esters from each other may not be achieved within a reasonable length of time. An increasing amount of fat calls for less drying time, and consequently a longer extraction time.

In Fig. 6, the PHB esters were isolated from margarine, and separated by three successive 30 min runs with ether. Drying time in air after impregnation was 1 minute.

The chromatography of acid substances, and separation of PHB esters, has been effected on a single paper as follows: food material was applied to a strip of paper 25 cm in length as described above. A line of bicarbonate was drawn across the paper, 2 cm from the starting line. Fats and *p*-hydroxybenzoates were extracted by ether over a distance of 14 cm to the evaporation front. The paper was cut 2 cm below the evaporation front. The lower part of the strip was transferred to the isoamyl alcohol solvent mixture for chromatography of the acids, and the upper part was impregnated with sodium hydroxide solution and subjected to ether extraction for separation of the PHB esters.

Observations on the identification treatments

The method of generating bromine vapour is very simple, but the treatment is practicable only when conducted before the chromatographic stage; the background of the paper is dark coloured, due to adsorbed bromine, in U.V. light, and some hours are required before bromine has totally evaporated. In the arrangement employed here, the effect is limited to the starting line. This treatment is well suited to the quantitative determination of benzoic acid in the presence of sorbic acid. The examination of U.V. spectra after chromatography and elution has shown that under the conditions

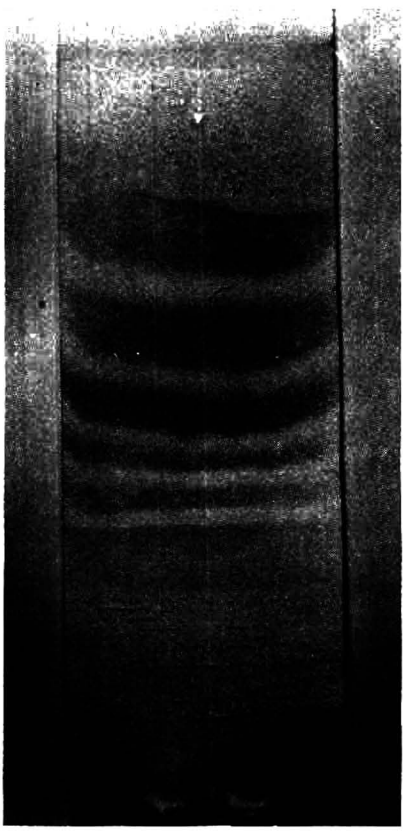


Fig. 6. The separated compounds are in a similar sequence to those in Fig. 4. The extracted fat shows as a broad light band in the upper part of the chromatogram. The compounds have been made visible by permanganate spraying.

stated sorbic acid undergoes complete reaction in 5 min, whereas benzoic acid is practically unaffected[2].

Chlorine generation is slightly more complicated than that of bromine, although the advantage here is that chlorination can be effected after chromatography. Chlorine does not impart a colour to the paper such as would hamper U.V. observations. Chlorination may be introduced before or after chromatography—and the same thing applies to hypochlorite treatment.

Treatment with hypochlorite has the advantage that ordinary spraying techniques can be applied. The spraying must not be too generous, as benzoic acid is easily eluted from the chromatogram, and may thus become undetectable. Pre-chromatographic treatment of sorbic acid has been effected not only by spraying, but also by streaking hypochlorite solution on both sides of the line containing sorbic acid, so that the reactant is brought to the action site by adsorption. The reaction has also been effected by directly mixing hypochlorite solution with the food to be analysed. The small amount of hydrochloric acid added to hypochlorite is insufficient to make the solution acidic, but activates the reagent so that it is capable of reacting with sorbic acid; in fact, if too much acid is added, the hypochlorite loses effectiveness in a very short time.

Some foodstuffs may yield interfering reaction products with the sorbic acid decomposition reagents, and thus it might be advisable to carry out the pre-chromatographic treatments not on the extraction line, but on the ether evaporation front, or on the alkaline arresting line.

CONCLUSIONS

It seems to be a reasonable assumption that modifications of this technique of extraction combined with chromatography on paper should also be suitable for other analytical applications, in cases where preliminary extraction before chromatography is necessary.

ACKNOWLEDGEMENT

This study was supported by a research grant from the Food Research Foundation in Finland.

SUMMARY

Food preservatives—sorbic, benzoic, *p*-hydroxybenzoic acid and *p*-hydroxybenzoates—have been extracted from foods directly on to chromatographic paper with ethyl ether. Paper chromatographic separation of the extracted compounds was effected on the same paper.

Methyl, ethyl and propyl *p*-hydroxybenzoates have been separated from each other during ether extraction and from the critical pair, the butyl and benzyl *p*-hydroxybenzoates, on a paper impregnated with sodium hydroxide. An examination was made of the reactions which distinguish sorbic and benzoic acids when both are present.

REFERENCES

1 J. L. JOUX, *Ann. Fals. Fraudes*, No. 581–582 (1957) 205.
2 M. PADMOYO AND E. BAUMGARTNER, *Mitt. Gebiete Lebensm. Hyg.*, 54, No. 5 (1963) 432.

THE SEPARATION AND IDENTIFICATION OF VANILMANDELIC ACID AND RELATED COMPOUNDS BY ELECTROPHORESIS ON CELLULOSE ACETATE

MARGARET M. KASER, WALDEMAR A. ROSENTHAL, KAREN NIQUETTE AND PATRICIA CULLEN

Clinical Chemistry Section, Laboratory Service, Veterans Administration Center, Wood, Wisc. (U.S.A.)

(Received January 17th, 1967)

INTRODUCTION

For a number of years the excretion of an increased amount of vanilmandelic acid (VMA) in the urine has been an indication of the presence of a pheochromocytoma. The presence of VMA and a method for its detection in urine by paper chromatography have been described by ARMSTRONG and his associates[1–3]. Paper chromatography was also used by ELLMAN[4] and GITLOW and co-workers[5]. Paper electrophoresis as a means of separation of VMA was employed by WOLF *et al.*[6], EPSTEIN, SCHIEVER AND GAMBINO[7], and KLEIN AND CHERNAIK[8]. A series of articles by VON STUDNITZ and others have described attempts to isolate VMA and related compounds by paper chromatography and paper electrophoresis with both low and high voltages[9–13].

In recent years cellulose acetate as a supporting medium has been found to be superior to paper for electrophoresis for a variety of purposes because of its lack of interaction with substances such as protein, and the rapid migration and clear resolution obtained. Also, various methods of quantitation are possible or can be developed. This paper reports the results of an investigation of cellulose acetate electrophoresis and an evaluation of the optimum conditions for the separation of VMA and related compounds.

EXPERIMENTAL

Reagents

Formate solution, 0.2 N, pH 3.0. To 575 ml of 0.2 *N* formic acid are added 100 ml of 0.2 *N* ammonium hydroxide. The pH is adjusted to pH 3.0 by the addition of formic acid or ammonium hydroxide.

Acetate buffer, 0.2 N, pH 3.6. 15 ml of 0.2 *N* sodium acetate are added to 185 ml of 0.2 *N* acetic acid. The pH is adjusted to pH 3.6 with acetic acid or sodium hydroxide.

Diazo reagent. A mixture of 10 ml of 2.5 % sodium nitrite and 7.5 ml of 0.5 % *p*-nitroaniline is freshly prepared from stock solutions kept at 5°. It is essential that the mixture be colorless.

Potassium carbonate, 5 %. 5 g potassium carbonate are dissolved in 100 ml of distilled water.

Phenolic acids

The vanilmandelic acid and related compounds used were obtained from Calbiochem and the Aldrich Chemical Company. They were dissolved in the formate solvent in concentrations of 1.0 μg/μl.

Equipment

Electrophoresis was performed on Sepraphore cellulose acetate strips with the micro electrophoresis unit of the Gelman Instrument Company.

Procedure

The cellulose acetate strips were soaked in acetate buffer for a minimum of 45 min before being placed in the electrophoresis apparatus. An aliquot of 1.0 μg of VMA in 1.0 μl of formate solvent was applied in a band at the origin near the cathode end of a strip. With other compounds amounts varying from 1.0 to 3.0 μg were similarly used. The samples were allowed to dry, and the unit was covered. Then, a current of 1.25 mA per strip or 7.5 mA for a series of 6 strips was applied for one hour at room temperature. When electrophoresis was completed, the excess buffer solution was removed by pressing the strips between sheets of filter paper. Fresh diazo reagent was poured into a long, narrow glass tray, and the cellulose acetate strips were floated one at a time on the surface for 30 sec. With a pipet 10 ml of 5 % potassium carbonate were then allowed to flow over the surface of the strip. A strip with 1.0 μg of VMA was included in each run, and the color development was continued until the VMA was clearly visible. The strips could be further dried by pressing them between two sheets of Whatman No. 1 chromatography paper at room temperature overnight or at 37° for one hour.

RESULTS

Because of our primary interest in VMA, the first experiments were designed to determine the optimum pH of a formate solution to be used as a solvent and also the pH of acetate buffer to provide the maximum rate of migration in electrophoresis on cellulose acetate. In each case the sample was 1.0 μg of VMA in 1.0 μl of formate solution. The results are given in Table I. The width of the band obtained after electrophoresis was 5 to 6 mm. The distances given are those to the center of the stained band. In a pH range of 3.0 to 4.0 the pH of the formate solvent had little influence on the

TABLE I

THE EFFECT OF VARIATIONS IN THE pH OF FORMATE SOLVENT AND ACETATE BUFFER ON THE MIGRATION OF VMA WITH CELLULOSE ACETATE ELECTROPHORESIS

pH of acetate buffer	*Distance of migration (mm) in formate solvent*		
	pH 4.0	*pH 3.6*	*pH 3.0*
4.0	32	42	38
3.6	52	51	55
3.0	14	15	15

TABLE II

ELECTROPHORETIC MIGRATION AND COLORS OBTAINED WITH DIAZOTIZED *p*-NITROANILINE WITH VMA AND RELATED COMPOUNDS

No.	*Compound*	*Migration (mm)*	*Band width (mm)*	*Color*
1	Tyrosine	0	—	Pink
2	Phenylalanine	0	—	Purple
3	Ferulic acid	0–3	3	Blue-green
4	3-Methoxy-4-hydroxyphenethylene glycol	0–5	5	Purple
5	5-Hydroxyindole-acetic acid	1–5	4	Pink
6	3,4-Dihydroxyphenylalanine	1–6	5	Yellow
7	Protocatechuic acid	3–7	4	Purple
8	Vanillic acid	3–8	5	Purple
9	Homovanillic acid	6–12	6	Brown
10	3,4-Dihydroxyphenylacetic acid	15–18	3	Olive
11	*p*-Hydroxyphenylpyruvic acid	15–18	3	Purple
12	Homogentisic acid	19–23	4	Yellow-green
13	Gentisic acid	48–51	3	Yellow-green
14	3-Methoxy-4-hydroxymandelic acid (VMA)	56–61	5	Purple
15	3,4-Dihydroxymandelic acid	60–68	8	Yellow
16	DL-*p*-Hydroxymandelic acid	64–72	8	Pink

electrophoretic migration, but the pH of the acetate buffer in the same range had a marked effect on the distance of migration. It is apparent that solution of the compounds in 0.2 *N* formate, pH 3.0, and the use of an acetate buffer, pH 3.6, for electrophoresis produced the maximum migration. Moderate variation in the concentration of these two solutions was without effect. Therefore, the indicated combination was used in all succeeding experiments.

VMA and a series of related compounds were investigated under the conditions established above. The results are shown in Table II. Samples of 1.0 to 3.0 μg were used. Good separation of these compounds was obtained, and they could be readily distinguished from VMA and each other by their rates of migration and their staining characteristics.

SUMMARY

A method has been developed for the resolution and identification of mixtures of vanilmandelic acid (VMA) and a series of related compounds by means of electrophoresis on cellulose acetate followed by staining with diazotized *p*-nitroaniline. It was established that the maximum distance of migration was obtained if the compounds were dissolved in 0.2 *N* formate solution, pH 3.0, and a 0.2 *N* acetate buffer, pH 3.6, was used for electrophoresis. The various compounds could be identified by their rates of migration and their staining characteristics. The method allows the detection of as little as 1.0 μg of each of the compounds studied.

REFERENCES

1 M. D. ARMSTRONG AND A. MCMILLAN, *Federation Proc.*, 16 (1957) 146.
2 M. D. ARMSTRONG, K. N. F. SHAW AND P. E. WALL, *J. Biol. Chem.*, 218 (1956) 293.
3 M. D. ARMSTRONG, A. MCMILLAN AND K. N. F. SHAW, *Biochim. Biophys. Acta*, 25 (1957) 422.
4 G. L. ELLMAN, *Nature*, 181 (1958) 768.
5 S. E. GITLOW, M. MENDLOWITZ, S. KHASSIS, G. COHEN AND J. SHA, *J. Clin. Invest.*, 39 (1960) 221.
6 R. L. WOLF, M. MENDLOWITZ, J. ROBOZ AND S. E. GITLOW, *J. Am. Med. Assoc.*, 188 (1964) 859.
7 S. EPSTEIN, H. SCHIEVER AND R. GAMBINO, *Clin. Chem.*, 11 (1965) 811.
8 D. KLEIN AND J. M. CHERNAIK, *Clin. Chem.*, 7 (1961) 257.
9 W. VON STUDNITZ AND A. HANSON, *Scand. J. Clin. Lab. Invest.*, 11 (1959) 101.
10 W. VON STUDNITZ, *Scand. J. Clin. Lab. Invest.*, 11 (1959) 224.
11 W. VON STUDNITZ, *Scand. J. Clin. Lab. Invest.*, 11 (1959) 309.
12 W. VON STUDNITZ, *Scand. J. Clin. Lab. Invest., Suppl.* 12, 48 (1960).
13 A. HANSON AND W. VON STUDNITZ, *Clin. Chim. Acta*, 11 (1965) 384.

Notes

Gas–liquid chromatography of coumarins

Gas–liquid chromatography is a powerful means of demonstrating the homogeneity of known and unknown coumarins and frequently serves for the purpose of identification of plant origins containing coumarins.

The applications[1] of gas–liquid chromatography to coumarins have not yet been widely explored, until we studied the gas–liquid separation of seventeen standard samples, either in the free form or as their trimethylsilyl ethers, and a few plant extracts using two kinds of column with SE-30 and HI-EFF-1BP (diethylene glycol succinate) as stationary phases.

Experimental

Materials. All coumarins used were available in this laboratory. Hexamethyldisilazane and trimethylchlorosilane were purchased from Kokusan Chemical Works, Ltd. Solvents of reagent grade were used.

Gas chromatography. A Shimadzu Model GC-1C gas chromatograph equipped with a hydrogen flame ionization detector was used in this work.

The columns, containing 1.5 % SE-30 on Chromosorb W (60–80 mesh) and 12 % HI-EFF-1BP on Gas Chrom P (80–100 mesh), respectively, were connected to the gas chromatograph, and acetone solutions of the sample in the free form or as its trimethylsilyl ether[2] were injected into the gas chromatograph with a Hamilton microsyringe. The detailed gas chromatographic conditions are shown in Table I.

Extraction and quantitative methods for furanocoumarins. Thirty grams of powdered root, 80 g of powdered leaves of *Heracleum lanatum* Michaux var. *nipponicum* Hara (Umbelliferae) and 15 g of Pimpinellae Radix were extracted according to SVENDSEN's method[3]. These neutral fractions were injected into the gas chromatograph as their acetone solutions.

The relative quantitative analysis for furanocoumarins as shown in Table II was carried out by the use of the half-width method.

Results and discussion

The results of the gas chromatographic separation of coumarins, using 1.5 % SE-30 on Chromosorb W and 12 % HI-EFF-1BP on Gas Chrom P, are shown in Table I. Three per cent XE-60 (nitrile silicone rubber) on Chromosorb W (60–80 mesh) and 3 % SE-30 on Chromosorb W (60–80 mesh) were also tried as stationary phases, but sharp peaks were not obtained.

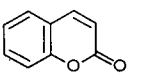
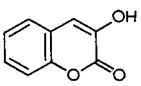
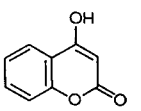
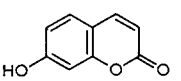
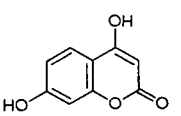
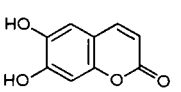
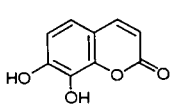
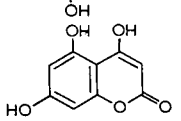
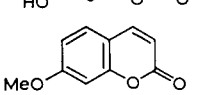
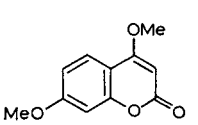
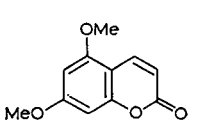
Coumarin itself, having no hydroxyl group, gave a single sharp peak, which moved very fast.

Free hydroxycoumarins did not give good results, but trimethylsilylated hydroxycoumarins gave sharp peaks which separated nicely from each other.

Amongst the monohydroxycoumarins, 3-hydroxycoumarin (t_R = 1.2 min)

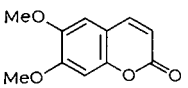
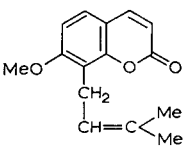
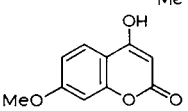
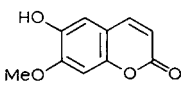
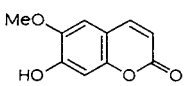
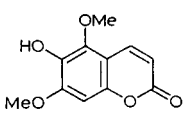
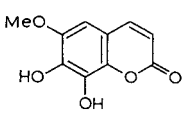
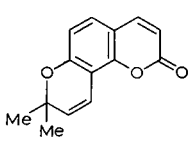
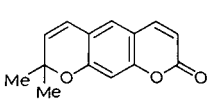
TABLE I

RETENTION TIMES OF COUMARINS

Group	*Compound*	*Structure*	t_R *(min)*		
			1.5% SE-30 on Chromosorb W (60–80 mesh)		*12% HI-EFF-1BP on Gas Chrom P (80–100 mesh)*
			Free	*TMSi**	*Free*
Coumarins	Coumarin		2.6		6.0
	3-Hydroxycoumarin			5.5	
	4-Hydroxycoumarin			9.0	
	7-Hydroxycoumarin (Umbelliferone)			8.9	
	4,7-Dihydroxycoumarin			34.5	
	6,7-Dihydroxycoumarin (Aesculetin)			23.4	
	7,8-Dihydroxycoumarin (Daphnetin)			15.5	
	4,5,7-Trihydroxycoumarin			49.5	
	7-Methoxycoumarin (Herniarin)		6.2		21.6
	4,7-Dimethoxycoumarin		17.1		74.4
	5,7-Dimethoxycoumarin (Citropten)		14.3		50.3

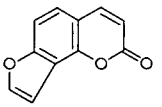
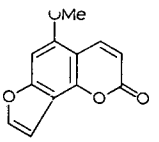
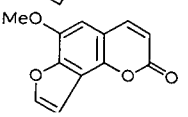
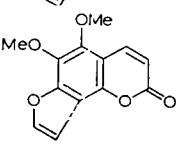
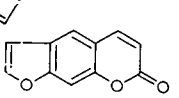
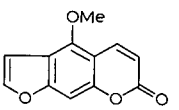
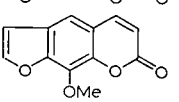
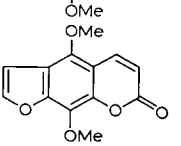
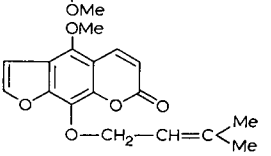
(continued on p. 384)

TABLE I (*continued*)

Group	*Compound*	*Structure*	t_R (*min*)		
			1.5% SE-30 on Chromosorb W (60–80 mesh)		*12% HI-EFF-1BP on Gas Chrom P (80–100 mesh)*
			Free	*TMSi**	*Free*
	6,7-Dimethoxycoumarin (Dimethylaesculetin)		13.1		55.1
	Osthol		8.8		37.6
	4-Hydroxy-7-methoxy-coumarin			23.3	
	6-Hydroxy-7-methoxy-coumarin (7-Methylaesculetin)			17.7	
	6-Methoxy-7-hydroxy-coumarin (Scopoletin)			17.9	
	5,7-Dimethoxy-6-hydroxycoumarin (Fraxinol)			25.5	
	6-Methoxy-7,8-dihydroxy-coumarin (Fraxetin)			29.0	
Pyrano-coumarins	Seselin		15.1		28.0
	Xanthyletin		19.4		47.2

(*continued on p. 385*)

TABLE I (*continued*)

Group	*Compound*	*Structure*	t_R (*min*)		
			1.5% SE-30 on Chromosorb W (60–80 mesh)		*12% HI-EFF-1BP on Gas Chrom P (80–100 mesh)*
			Free	*TMSi**	*Free*
Furanocoumarins	Angelicin		7.0		24.0
	Isobergapten		15.6		54.4
	Sphondin		17.0		86.9
	Pimpinellin		22.3		69.0
	Psoralen		8.4		35.4
	Bergapten		17.4		74.8
	Xanthotoxin		15.8		80.1
	Isopimpinellin		32.2		151.1
	Phellopterin		12.8**		
Conditions:	Column temp.		180°		210°
	Detector temp.		230°		230°
	Flash heater temp.		250°		250°
	N_2 flow rate		70.4 ml/min		69.1 ml/min

* TMSi = Trimethylsilyl ether.
** Broad peak.

showed the lowest retention time, 4-hydroxycoumarin (t_R = 2.3 min) a medium retention time, and umbelliferone (t_R = 3.9 min) the highest retention time using 3 % SE-30. Comparing these results with those obtained with trimethylsilylated monohydroxycoumarins, 3-hydroxycoumarin (t_R = 5.5 min) also moved the fastest, but umbelliferone (t_R = 8.9 min) showed a slightly shorter retention time than 4-hydroxycoumarin (t_R = 9.0 min) using 1.5 % SE-30. This seems likely to be due to the disappearance of intermolecular hydrogen bonding.

The correlation among carbon numbers, hydroxyl numbers and retention times is illustrated in Fig. 1.

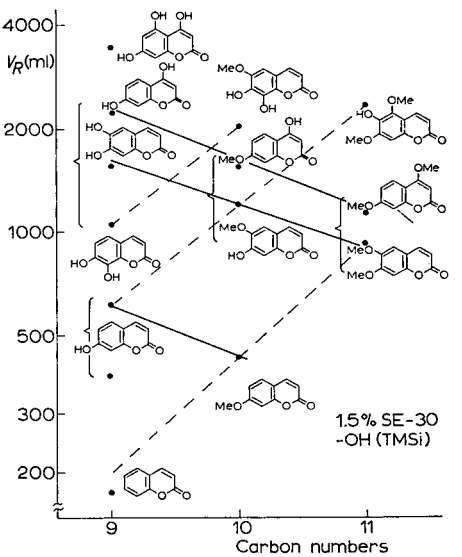


Fig. 1. Relationship between chemical structures and retention volumes.

Especially aesculetin and its methyl ethers showed a good relationship between the hydroxyl numbers and retention times, the order being as follows: t_R = 23.4 min for aesculetin bis-trimethylsilyl ether, 17.9 min for scopoletin trimethylsilyl ether, 17.7 min for 7-methylaesculetin trimethylsilyl ether, and 13.1 min for dimethylaesculetin, using 1.5 % SE-30.

Daphnetin and aesculetin, each having two hydroxyl groups in the *ortho* position, gave lower retention times than 4,7-dihydroxycoumarin.

Trihydroxycoumarin, such as 4,5,7-trihydroxycoumarin, after trimethylsilylation showed the longest retention time. Pyranocoumarins gave sharp peaks; angular types such as seselin (t_R = 15.1 min) had a lower retention time than linear ones such as xanthyletin (t_R = 19.4 min).

Furanocoumarins also gave good gas chromatograms.

Angular furanocoumarins had shorter retention times than linear ones, and the increase in the retention times with increasing number of methoxyl groups was noticeable. In the methoxyfuranocoumarins, different positions of the methoxyl group gave different retention times, as illustrated by isobergapten and sphondin (angular type) and bergapten and xanthotoxin (linear type).

Pimpinellin ran faster than sphondin using 1.5 % SE-30, but was slower when 12 % HI-EFF-1BP was used. It would appear that the presence of the two vicinal methoxyl groups in pimpinellin makes the polarization of the molecule low, and consequently the absorption to the polar liquid phase weak.

The successful separation of some furanocoumarins is illustrated in Fig. 2, and the relative contents of furanocoumarins in the root of *Heracleum lanatum* Michaux var. *nipponicum* Hara and Pimpinellae Radix, which gave almost the same results, are shown in Table II.

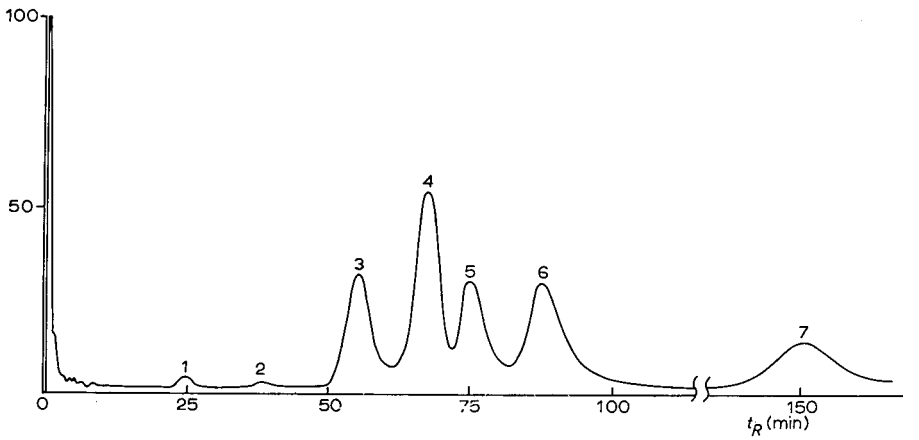


Fig. 2. Gas–liquid chromatogram of furanocoumarins from the root of *Heracleum lantum* Michaux var. *nipponicum* Hara. 1 = Angelicin; 2 = psoralen; 3 = isobergapten; 4 = pimpinellin; 5 = bergapten; 6 = sphondin; 7 = isopimpinellin.

TABLE II

CONTENT OF FURANOCOUMARINS IN CRUDE DRUGS

Compound	*Root of H. lanatum Michaux var. nipponicum Hara*		*Pimpinellae Radix*	
	Peak area (cm^2)	*Relative amount*	*Peak area (cm^2)*	*Relative amount*
Angelicin	1.4	1	0.2	1
Psoralen	trace	trace	—	—
Isobergapten	33.8	24	7.7	39
Bergapten	27.7	20	1.9	10
Sphondin	30.1	22	2.2	11
Pimpinellin	78.3	56	29.0	145
Isopimpinellin	34.4	24	10.0	50

As a result of the gas chromatographic analysis the presence of small amounts of angelicin and traces of psoralen were identified in the root of *Heracleum lanatum* Michaux var. *nipponicum* Hara, but furanocoumarins were not confirmed in the leaves as previously reported[4].

The gas–liquid chromatographic technique described above can be applied extensively to both the identification of small quantities of coumarins and to the quantitative analysis of mixtures from plant extracts.

Acknowledgements

The authors are grateful to Prof. G. SHIMADA, Meijo University, for supplying aesculetin, Prof. N. MORITA, Toyama University, for xanthyletin, and Dr. K. HATA, Kyoto University, for seselin.

College of Pharmaceutical Sciences, Kitasato University, Shiba, Minato-ku, Tokyo (Japan)

TSUTOMU FURUYA
HISASHI KOJIMA

1 S. A. BROWN AND J. P. SHYLUK, *Anal. Chem.*, 34 (1962) 1058.
2 T. FURUYA, S. SHIBATA AND H. IIZUKA, *J. Chromatog.*, 21 (1966) 116.
3 A. B. SVENDSEN AND E. OTTESTAD, *Pharm. Acta Helv.*, 32 (1957) 457.
4 M. FUJITA AND T. FURUYA, *Yakugaku Zasshi*, 76 (1956) 535.

Received November 21st, 1966

J. Chromatog., 29 (1967) 382–388

Separation of the major alkaloids of *Peganum harmala* by high voltage ionophoresis

In the opinion of CROMWELL[1] none of the existing methods for the determination of the alkaloids of *Peganum harmala* are entirely satisfactory and for this reason he considers a new analytical study of these alkaloids to be necessary. CROMWELL'S assertion continues to hold true ten years after it was first enunciated. The key point in all the determinations is the separation of harmine and harmaline. The technique is substantially the same as the fractional precipitation process followed when extracting these alkaloids from the plant. Here we shall deal with—among the different separation methods we have tried—the possibilities offered by ionophoresis on paper as the starting point for a micromethod which permits the evaluation of these tryptophan metabolism compounds in vegetables.

Several authors[2–8] have shown that a considerable number of alkaloids may be separated by ionophoresis on paper as long as they are sufficiently soluble in the buffer and do not remain adsorbed in the carrier. Most of such separations have been carried out at gradients not exceeding 10 V/cm. The advantage of using, in similar cases, fields of an intensity ten or twenty times higher is obvious. The speed of the separation will limit the broadening, distortion and overlapping of the bands due to diffusion.

But this economy of time and, above all, the desired increase of the resolving power will only be attained by either using a device capable of absorbing the heat produced by the Joule effect or adopting the necessary precautions so that the heat released is negligible. Both methods have been used in this work, with different results. We have used a heat exchanging device, cooled by brine circulation, capable of reaching temperatures of —18° for all the ionograms run in aqueous systems. When formamide or dimethylformamide have been used as the solvents the production of

heat has been so low that it has been possible to work at ordinary temperatures without the need to operate the cooling system.

1 % solutions of harmine and harmaline, in the buffer system corresponding to that with which the paper (Whatman 3MM) was impregnated, were used. In all the experiments the degree of impregnation of the paper was maintained uniform by passing the paper through a roller press, with the rollers set at the same pressure all the time so as to remove excess liquid.

As reference substance for measuring the electro-osmotic flow a solution (0.1 %) of methyl-umbelliferone was used.

The buffer system, formic acid–ammonium formate, which had yielded good results in our laboratory for the separation of the two alkaloids on an ion exchange column, was the first one tested for the ionophoresis. Since the ionization of the bases had to show the maximum difference at a pH of the buffer solution equal to half the sum of the pK's of the respective bases, the buffer pH was first set at 5.15, taking as a reference the values of the dissociation constants given by ORLOW[9]. Due to the fact that the pK of substances with ionizable groups capable of mutual interaction may be markedly affected by the ionic strength of the medium[10], experiments were carried out with the buffer values ranging from pH 5.9 to pH 3.6 within the concentration limits 0.02 M to 0.1 M. In no case was a total separation of the alkaloids achieved, tailing due to adsorption of the bases on the carrier occurred. Tests were then made with conventional buffer systems such as BRITTON's, diluted to ¼, and those of LORENZ-MÜLLER[11] ranging from pH 7 to pH 5, with ionic strengths ranging from 0.002 to 0.2 and potentials from 33 V/cm upwards.

In almost all these systems, it was possible to see, at the beginning, a difference in the migration velocity of the alkaloids, but as the operation goes on tails form and distortion occurs at the fronts. As a result the resolution is incomplete. Nevertheless with the 0.1 M LORENZ-MÜLLER buffer 4 mm separations were achieved at a pH of 6.5 and a potential of 50 V/cm after 30 min. The dissipated heat was 240 W. The addition of ethyl and isopropyl alcohol and propylene glycol to the electrolyte, with a view to attenuating the adsorption effect did not substantially improve the result. On the other hand the addition of 25 % of formamide to the LORENZ-MÜLLER acetic acid–acetate buffer prevents the formation of tails and gives rise to larger displacements. The use of pure formamide as a solvent permits the rapid displacement of the alkaloids since there is very little heat evolution and higher potentials (200 V/cm) may be used. However, the migration velocity of both alkaloids is the same in this medium and there is no separation.

Harmine and harmaline bases are soluble in the dimethylformamide–acetic acid system at ordinary temperatures. As it was known that the pH of this system did not coincide with the value it has for aqueous solutions, the pH of the different dimethylformamide–acetic acid systems was taken as that given by the pH meter when a saturated calomel electrode and a glass electrode were inserted in the system.

Fig. 1 shows the ionophoretic behaviour of the bases in each of the systems within the pH range 8–5.5. In all cases the liquid at the electrodes was the same as that with which the paper was impregnated. Ionophoresis took 30 min, the potential was 200 V/cm, the current less than 1 mA and the process could be completed without using the cooling device. Curve 1 shows the *difference* between the distances travelled by harmine and harmaline after 30 min. Curve 2 shows the distance travelled

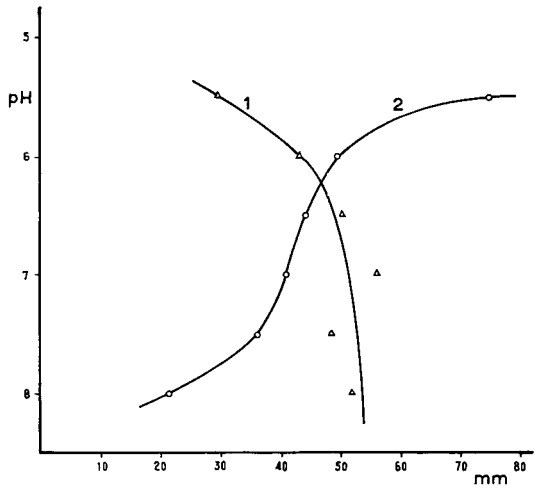


Fig. 1. Curve 1: Difference between the distances travelled by harmaline and harmine at 200 V/cm in DMF–acetic acid at different pH values. Curve 2: Distance travelled by harmaline *vs.* pH of medium.

by harmine during the same time. A close dependence between the migration velocity of the bases and acidity of the medium is observed. It can also be deduced that, at any point within the pH range investigated, migration velocities sufficient to resolve the mixture in about 10 minutes are obtained and that, in general, the optimum working range is located between pH 6.5–7.5.

Instituto "J. C. Mutis" de Farmacognosia L. CARRERAS
del C.S.I.C., Serrano 113, Madrid (Spain) M. GONZÁLEZ ALONSO

1 B. T. CROMWELL, in K. PAECH AND M. V. TRACEY (Editors), *Moderne Methoden der Pflanzenanalyse*, Band IV, Springer, Berlin, 1955, p. 470.
2 G. B. MARINI-BETTÒLO AND J. A. COCH-FRUGONI, *Gazz. Chim. Ital.*, 86 (1956) 1324.
3 G. B. MARINI-BETTÒLO AND M. LEDERER, *Nature*, 174 (1954) 133.
4 G. B. MARINI-BETTÒLO, M. LEDERER, M. A. JORIO AND A. PIMENTA, *Rend. Ist. Super. Sanità*, 18 (1955) 898.
5 H. MICHL AND F. HARBERLER, *Monatsh.*, 85 (1954) 779.
6 T. SANO AND H. KAJITA, *Klin. Wochschr.*, 33 (1955) 956.
7 J. DE WAEL, *Chem. Weekblad*, 49 (1953) 229.
8 W. DECKER AND J. SCHREIBER, *Naturwiss.*, 40 (1963) 553.
9 J. E. ORLOW, *Chem. Zentr.*, II (1932) 70.
10 A. A. GREEN, *J. Am. Chem. Soc.*, 55 (1933) 2331.
11 G. LORENZ MILLER AND R. H. GOLDER, *Arch. Biochem.*, 29 (1950) 420.

Received January 31st, 1967

Polyamide layer chromatography of opium alkaloids

Recent advances in polyamide layer chromatography techniques have resulted in the easier handling and better separation of some compounds[1]. The separation of opium alkaloids by silica gel thin layers has been reported[2] but there is no similar report for polyamide layers. In this note, the separation of seven opium alkaloids by polyamide layers will be described.

Experimental

Materials. The polyamide resin was Amilan CM 1007s (poly-ε-caprolactam) of Toyo Rayon Co., Tokyo, Japan. All alkaloids except diacetylmorphine were supplied by the Narcotic Control Administration, Ministry of Internal Affairs, Republic of China. Diacetylmorphine was synthesized[3] by acetylation of morphine. The solvents were the reagent grade of Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Preparation of polyamide layers and chromatographic techniques. The method described by WANG[1] was used.

Visualization. Two color reagents[4] were used: (1) Dragendorff's and (2) iodoplatinate reagent.

TABLE I

CHROMATOGRAPHIC DATA

No.	*Substance*	R_F *value*[a]		*Color of spot*	
		I[b]	*II*[c]	*Dragendorff*	*Iodoplatinate*
1	Morphine	0.05	0.63	orange	yellow
2	Papaverine	0.32	0.06	pink	violet
3	Codeine	0.38	0.58	orange	violet-blue
4	Ethylmorphine	0.47	0.48	orange	violet-blue
5	Noscapine	0.61	0.00	pink	violet-red
6	Dihydrocodeine	0.70	0.00	orange	yellow
7	Diacetylmorphine	0.70	0.61	orange	violet-blue

[a] The R_F values are the mean of five chromatograms.

[b] Solvent I: cyclohexane–ethyl acetate–*n*-propanol–dimethylamine (30:2.5:0.9:0.1), distance 10 cm, time required, 2 h.

[c] Solvent II: water–absolute ethanol–dimethylamine (88:12:0.1), distance 10 cm, time required, 1.5 h.

Results and discussion

Table I shows the R_F values in two solvent systems, and Fig. 1 shows the chromatogram of solvent I. It is very interesting that the R_F values in the two solvent systems are reversed. In solvent I, the result is the same as in thin-layer chromatography on silica gel; *viz.* the R_F value was increased as the hydroxyl group was substituted.

Both iodoplatinate and Dragendorff's reagent were used for visualization of the spots but iodoplatinate reagent is much preferred because of its higher sensitivity. Ten μg of alkaloids were easily detected by the iodoplatinate reagent. The violet spots changed to pale yellow after standing for a while.

The separation of morphine alkaloids by polyamide layers is most interesting

from the toxicological point of view, so that further applications to this field are in progress.

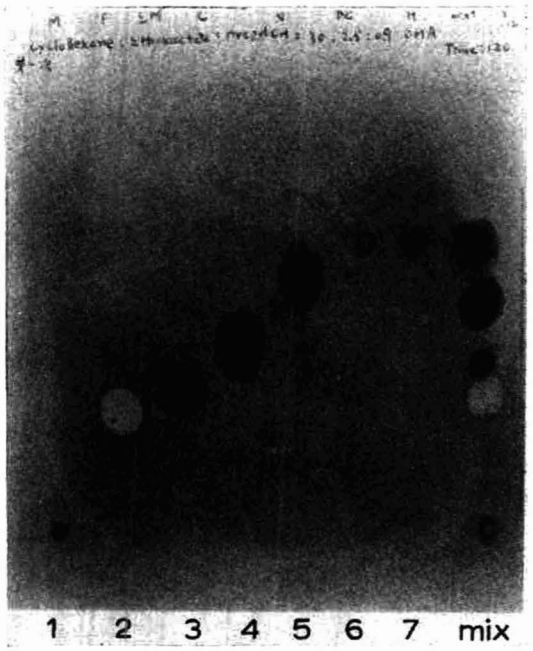


Fig. 1. One-dimensional chromatogram. Solvent: I, 2 h, 10 cm. Layer: poly-ε-caprolactam resin CM 1007s (26°). Loading: 10 μg in 0.01 ml methanol. Numbers: *cf.* Table I.

Part of this work was supported by grants from the National Council of Science Development, Republic of China, to which thanks are due.

Department of Pharmacy, Taipei Medical College JEN-TZAW HUANG
Taipei, Taiwan (Republic of China) HSING-CHIEN HSIU

Department of Chemistry, National Taiwan University KUNG-TSUNG WANG
Taipei, Taiwan (Republic of China)

1 K.-T. WANG, J. M. K. HUANG AND I. S. Y. WANG, *J. Chromatog.*, 22 (1966) 362.
2 D. WALDI, in E. STAHL (Editor), *Thin Layer Chromatography*, Academic Press, New York, 1965, pp. 284–287.
3 R. TAHARA, S. ISHIKAWA AND K. HARA, *J. Pharm. Soc. Japan*, No. 407 (1916) 94.
4 K. RANDERATH, *Thin Layer Chromatography*, Academic Press, New York, 1963, p. 74.

Received January 17th, 1967

Steroids

XXIX. Separation and characterization of various classes of steroids by thin layer chromatography*

Thin-layer chromatography is becoming increasingly popular in the separation and characterization of steroids[2-4] but so far no published paper has reported the use of a single solvent system or two solvent systems which have been found to be satisfactory in the resolution of a large variety of steroids.

In this paper we report two solvent systems which have been constantly used in our laboratory for the detection and characterization of synthetic steroids. The solvent systems consisted of mixtures of benzene, methanol and ethyl acetate or chloroform, methanol and ammonia. The steroids reported range from simple cholestane and androstane series to complex heterocyclic steroids. Each of these compounds has been synthesized in our laboratory.

At least one of these solvent systems has always been found to be satisfactory for each steroid investigated. Neither of these solvent systems has been reported in the literature in connection with the separation of steroids. The second of these systems has been used by one of us[5] previously in the separation of pyrrolizidine alkaloids and is remarkably suitable for the polar aza-steroids.

Experimental

Reagents. All reagents used were of analytical grade. The chloroform used was "Baker Analyzed Reagent Spectrophotometric, grade" and was used as such. Other solvents were further purified by passing them through basic aluminum oxide, activity I (E. Merck).

Steroids. Those used in this investigation are listed with their chemical names in Tables I and II.

Method and results

(a) All chromatograms were run at room temperature on Silica Gel G (E. Merck). The plates were activated at 110° for one hour and stored in a desiccator over calcium chloride.

(b) The spreader used for coating the plates was manufactured by Desaga-Brinkman, U.S.A. The plates, 200 × 200 × 3.7 mm, were coated to 0.25 mm thickness.

(c) Solvent systems: (1) Benzene–methanol–ethyl acetate (85:10:5)
(2) Chloroform–methanol–ammonia (85:14:1)

Solutions of the steroid samples were made in chloroform or methanol and applied at the rate of 50–100 μg of steroid as a spot on a TLC plate. The spotted plate was developed in a Desaga (Heidelberg, Germany) jar in solvent systems No. 1 or No. 2. To ensure a saturated atmosphere the jar was lined with a filter paper (Whatman No. 1) at least 45 min before the development of the plates. The steroid samples were detected from the developed chromatogram by iodine vapors.

Each experiment was conducted by running a reference standard of 5-androsten-3β-ol-17-one along with other authentic samples. Tables I and II contain the R_F,

* For Part XXVIII, see ref. 1.

R_S, and R_M values of the steroid samples studied in solvent systems 1 and 2, respectively. These values are reported for reference and to illustrate the usefulness of these solvent systems.

TABLE I

THIN-LAYER CHROMATOGRAPHY IN BENZENE–METHANOL–ETHYL ACETATE

Systematic name	R_F *value*	R_S *value*	R_M *value*
17α-Methyl-3,5-seco-4-nor-androstan-17β-ol-5-on-3-oic acid	0.48	1.09	0.033
N-Benzyl-3,5-seco-4-norcholestan-5β-ol-3-amide	0.50	1.25	0.000
4-Oxa-5α-cholestan-3-one	0.69	1.57	−0.347
4-Benzyl-4-aza-5-cholesten-3-one	0.68	1.55	−0.328
4,17α-Dimethyl-4-aza-5-androsten-17β-ol-3-one	0.27	0.61	−0.431
4-(β-Hydroxyethyl)-4-aza-5-cholesten-3-one	0.44	1.00	0.104
4-Phenyl-4-aza-5-cholesten-3-one	0.66	1.50	−0.276
4-Methyl-4-aza-5-cholesten-3-one	0.71	1.61	−0.388
4-Oxa-5α-pregnane-3,20-dione	0.57	1.29	−0.125
17α-Methyl-4-aza-5-androsten-17β-ol-3-one	0.16	0.36	0.280
4-Aza-5-cholesten-3-one	0.52	1.18	−0.022
4-Methyl-4-aza-5-pregnene-3,20-dione	0.45	1.02	0.086
4-Methyl-4-aza-5-cholestene	0.81	1.84	−0.569
4β,5β-Epoxypregnane-3,20-dione	0.68	1.49	−0.328
4α,5α-Epoxypregnane-3,20-dione	0.71	1.61	−0.388
4-Aza-5-pregnene-3,20-dione	0.42	0.95	0.170
3,5-Seco-4-norpregnan-20β-ol-5-on-3-oic acid	0.47	1.07	0.053
17α-Methyl-4-oxa-5-androsten-17β-ol-3-one acetate	0.64	1.45	−0.252
17α-Methyl-4-aza-5α-androstan-17β-ol-3-one	0.11	0.25	0.911
3-Aza-A-homo-5α-cholestan-4-one	0.29	0.66	0.389
4-Methyl-4-aza-5-pregnen-20β-ol-3-one	0.29	0.66	0.389
3,5-Seco-4-norcholestane-3,5β-diol	0.26	0.59	0.453
3-Aza-A-homo-5β-cholestan-4-one	0.37	0.84	0.230
2′-Aminothiazolo[*d*-3,2]-5α-cholest-2-ene	0.43	0.98	0.120
4-(β-Hydroxyethyl)-4-aza-5-pregnen-20β-ol-3-one	0.14	0.32	0.789
3β-Acetoxy-6-aza-B-homo-5α-cholestan-7-one	0.50	1.13	0.000
N-(β-Hydroxyethyl)-3,5-seco-4-norcholestan-5β-ol-3-amide	0.59	0.66	0.387
4-(Dimethylaminoethyl)-17α-methyl-4-aza-5-androsten-17β-ol-3-one	0.16	0.36	0.720
Thiazolo[*d*-3,2]-5α-cholest-2-ene	0.61	1.39	−0.194
4-Oxa-5α-cholestane	0.73	1.66	−0.432
6-Acetyl-6-aza-B-homo-5α-cholestan-3β-ol acetate	0.60	1.36	−0.180
Indolo[*b*-3,4]-5β-cholest-3-ene	0.70	1.59	−0.366
3,5-Cholestadien-7-one	0.33	0.75	0.307
Quinoxalino[*b*-2,3]-5α-cholestane	0.67	1.52	−0.310
2′-Aminothiazolo[*d*-3,2]-17α-methyl-5α-androst-2-en-17β-ol	0.28	0.64	0.389
1′-Nitrosoindolo[*b*-3,2]-5α-cholest-2-ene	0.67	1.52	0.310
g-Benzoquinoxalino[*b*-2,3]-5α-cholestane	0.71	1.61	−0.387
1′-Nitrosoindolo[*b*-3,4]-5β-cholest-3-ene	0.63	1.43	−0.236
1′-Aminoindolo[*b*-3,2]-5α-cholest-2-ene	0.64	1.45	−0.251
6-Methyl-6-aza-B-homo-5α-cholestan-3β-ol	0.41	0.93	0.158
6-Methyl-6-aza-B-homo-5α-cholestan-3β-ol methiodide	0.46	1.14	0.072
3,5-Seco-4-norandrostan-17β-ol-5-on-3-oic acid	0.23	0.52	0.550
1′-Methylindolo[*b*-3,2]-5α-cholest-2-ene	0.51	1.16	−0.018
1′-Methylindolo[*b*-3,4]-5β-cholest-3-ene	0.62	1.41	−0.215
7a-Aza-B-homo-5α-cholestan-3α-ol-7-one acetate	0.54	1.23	−0.076
4-Methyl-4-aza-5α-cholestan-3-one	0.57	1.30	−0.125
4-Methyl-3-phenyl-4-aza-2,5-cholestadiene	0.62	1.41	−0.208
3-Ethyl-4-methyl-4-aza-2,5-cholestadiene	0.65	1.48	−0.268
2,3-Seco-5α-cholestane-2,3-diol	0.33	0.75	0.332
B-Nor-3,5-cholestadiene	0.66	1.50	−0.276

TABLE II

THIN-LAYER CHROMATOGRAPHY IN CHLOROFORM–METHANOL–AMMONIA

Systematic name	R_F *value*	R_S *value*	R_M *value*
3α-Amino-5α-cholestane hydrochloride	0.93	1.16	—1.154
N-Benzyl-3,5-seco-4-norcholestan-5β-ol-3-amide	0.84	1.05	—0.924
4-(β-Hydroxyethyl)-4-aza-5-cholesten-3-one	0.77	0.96	—0.523
17α-Methyl-4-aza-5-androsten-17β-ol-3-one	0.71	0.89	—0.387
4α,5α-Epoxypregnane-3,20-dione	0.90	1.12	—0.959
4-Benzyl-17α-methyl-4-aza-5-androsten-17β-ol	0.79	0.99	—0.568
17β-Hydroxy-4-(β-hydroxyethyl)-17α-methyl-4-aza-5-androstene	0.51	0.64	—0.018
17α-Methyl-4-aza-5α-androstan-17β-ol-3-one	0.67	0.84	—0.310
N,N-Bis-(β-chloroethyl)-3β-amino-5α-cholestane hydrochloride	0.32	0.40	0.326
N-(β-Hydroxyethyl)-3,5-seco-4-norcholestan-5β-ol-3-amide	0.62	0.77	—0.215
6-Methyl-6-aza-B-homo-5α-cholestan-3β-ol methiodide	0.20	0.25	0.602
4-(Dimethylaminoethyl)-4-aza-5-pregnene-3,20-dione methiodide	0.20	0.25	0.602

The R_M values have been calculated according to the definition given by BATE-SMITH and WESTALL[6].

$$R_M = \log\left(\frac{1}{R_F} - 1\right)$$

The R_S value of each steroid is calculated as:

$$\frac{\text{Distance from starting point to the center of the spot}}{\text{Distance from starting point to the reference substance}}$$

As can be seen from Tables I and II, this study has the following interesting features:

(1) All of the steroids studied have been efficiently chromatographed either by solvent No. 1 or solvent No. 2.

(2) Separation of 4β,5β-epoxypregnane-3,20-dione and 4α,5α-epoxypregnane-3,20-dione has been accomplished by solvent No. 1.

(3) Strongly polar aza-steroids such as 4-benzyl-17α-methyl-4-aza-5-androsten-17β-ol, 17β-hydroxy-4-(β-hydroxyethyl)-17α-methyl-4-aza-5-androstene, and 4-aza-5-cholestene, which barely moved with solvent system No. 1, gave satisfactory R_F values with solvent system No. 2.

Acknowledgements

We thank Mr. DAVID DILLON for his competent assistance and we wish to express appreciation for the support of this program received from the National Institutes of Health (Grant No. AI-06798).

Department of Pharmaceutical Chemistry, School of Pharmacy, University of Mississippi, University, Miss. 38677 (U.S.A.)

NORMAN J. DOORENBOS
RAMESHWAR K. SHARMA

1 N. J. DOORENBOS AND S. S. VAIDYA, *Chem. Ind. (London)*, (1967) 704.
2 B. P. LISBOA, *J. Chromatog.*, 13 (1964) 391.
3 B. P. LISBOA AND E. DICZFALUSY, *Acta Endocrinol.*, 40 (1962) 60.
4 T. GOLAB AND D. S. LAYNE, *J. Chromatog.*, 9 (1962) 321.
5 R. K. SHARMA, G. S. KHAJURIA AND C. K. ATAL, *J. Chromatog.*, 19 (1965) 433.
6 E. C. BATE-SMITH AND R. G. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.

Received January 18th, 1967

Dünnschichtchromatographische Trennung homologer Reihen von *n*-Alkylhalogeniden

Die Identifizierung bei Raumtemperatur flüssiger *n*-Alkylhalogenide (AH) durch ihre Siedepunkte wie auch durch die Schmelzpunkte ihrer festen Derivate ergibt bei kleinen Mengen, insbesondere in Gemischen, experimentelle Schwierigkeiten. So zeigt sich z.B. die Problematik von Schmelzpunktsbestimmungen in den bei S-Alkylphenylthiuronium-Pikraten gefundenen Werten für folgende *n*-Alkyle:

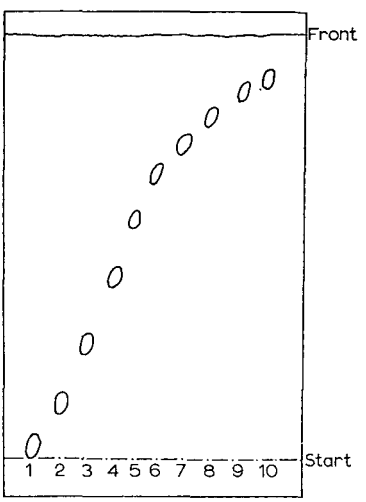
n-Heptyl = 127°	*n*-Octyl = 130°
n-Hexyl = 128°	*n*-Pentyl = 142°
n-Decyl = 128°	*n*-Butyl = 144°

Nach Überführung in N-Alkylpyridinium-Halogenide (NAPH) oder S-Alkylphenylthiuronium-Pikrate (SATP) können AH dünnschichtchromatographisch noch identifiziert werden, wenn die Auftragsmengen der festen Derivate bei etwa 20 μg liegen.

p-Alkoxyazobenzole und N-Alkylphtalimide erweisen sich hinsichtlich Darstellung und Auftrennung (Schwanzbildung) als weniger geeignet. Während die NAPH auf Kieselgur G-Schichten unzersetzt als solche wandern, erfolgt bei SATP an hydrophobiertem Kieselgel HF_{254} Auftrennung in die S-Alkylphenylthiuronium-Basen und Pikrinsäure, die mit der Front der Entwicklungsflüssigkeit durchläuft.

Experimentelles

Darstellung der NAPH. Alkylhalogenide, trockenes Pyridin und Nitromethan werden im Volumenverhältnis 1:4:10 im siedenden Wasserbad 1 Std. erhitzt. Nach Entfernung der flüchtigen Anteile am Rotationsverdampfer wird der Rückstand mit



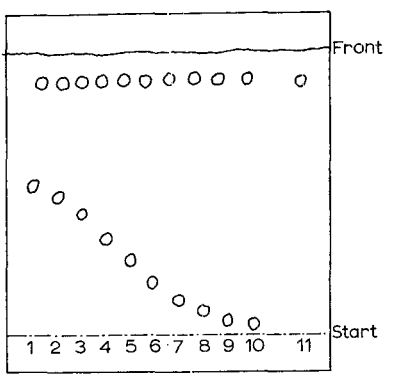


Fig. 1. Homologe Reihe der N-Alkylpyridinium-Bromide (*n*-Alkyle von C_1 bis C_{10}), aufgetragen in 1%-iger methanolischer Lösung auf Kieselgur G (Merck). Detektion mit Silbernitrat–Dichlorfluorescein.

Fig. 2. Homologe Reihe der S-Alkylphenylthiuronium-Pikrate (*n*-Alkyle von C_1 bis C_{10}), aufgetragen in 1%-iger äthanolischer Lösung auf Kieselgel HF_{254} (Merck), paraffinimprägniert (Phasenumkehr!). Punkt 11 = Pikrinsäure. Detektion im U.V.

trockenem Äther gewaschen und das Pyridiniumsalz in etwa 1 %-iger methanolischer Lösung chromatographiert.

Darstellung der SATP. 0.2 g Alkylhalogenid oder die doppelte Menge Gemisch werden mit 0.5 g Phenylthioharnstoff und 3 ml Äthanol 2 Stunden im siedenden Wasserbad erhitzt und das noch heisse Gemisch in 50 ml Pikrinsäurelösung (gesättigt in Wasser) eingerührt. Der nach 1 Std. abgesaugte Niederschlag wird 3 mal mit 5 ml Wasser gewaschen und in etwa 1 %-iger äthanolischer Lösung chromatographiert.

Chromatographie der NAPH. 30 g Kieselgur G (Merck) und 70 ml Wasser werden mittels Streichgerät auf 5 Glasplatten (20 × 20 cm) in einer Schichtdicke von 0.25 mm verteilt. Nach Lufttrocknung wird 1 Std. bei 120° aktiviert. Man entwickelt bei Kammersättigung mit Tetrachlorkohlenstoff–Essigester–Methanol (80:12:8, v/v). Nach 17 cm Laufstrecke wird mit Warmluft getrocknet und mit einem Gemisch aus 10 ml 0.1 *N* Silbernitrat und 0.5 ml Dichlorfluorescein (1 %-ig in Äthanol) besprüht, wonach die NAPH als dunkle Flecken auf rosa Untergrund erscheinen (Fig. 1 und Tabelle I).

TABELLE I

R_F-WERTE DER *n*-ALKYLE VON C_1 BIS C_{10}

	N-Alkylpyridinium-Bromide	*S-Alkylphenyl-thiuronium-Basen*
C_1	0	0.52
C_2	0.13	0.48
C_3	0.26	0.42
C_4	0.42	0.33
C_5	0.56	0.26
C_6	0.68	0.18
C_7	0.74	0.12
C_8	0.81	0.08
C_9	0.87	0.05
C_{10}	0.90	0.04

Chromatographie der SATP. Für 5 Glasplatten (20 × 20 cm) mit 0.25 mm Schichtdicke werden 30 g Kieselgel HF_{254} (Merck) und 70 ml Wasser benötigt. Nach Lufttrocknung wird mit Paraffinum subliquidum (DAB 6) in Petroläther (Kp. 40–60°) (1:10, v/v) hydrophobiert. Man eluiert mit Aceton–Wasser (50:50, paraffingesättigt) bei Kammersättigung bis 15 cm Laufstrecke. Die Flecke werden im U.V. markiert (Fig. 2 und Tablelle I).

Dem Fonds der Chemischen Industrie sind wir für die Unterstützung der Arbeit zu Dank verpflichtet.

Pharmazeutisches Institut der Universität, Bonn (Deutschland)

MELANIE RINK*
ROBERT LENHARD
HERBERT JÄGER

Eingegangen den 8. Februar 1967

* Verstorben 1965.

The separation of peptides from amino acids in urine by ligand exchange chromatography*

Normal human urine contains more than 1 g of bound amino acids in 24 h. Little is known about the origin or significance of these peptides although pathological peptiduria has been demonstrated in certain diseases. This report describes a technique, utilizing ligand exchange chromatography[1], for separating the peptides of urine from the complex mixture of amino acids. The principle on which this is based is that a transition metal is complexed with a cation exchange resin and the metallic ions can then shed their solvation shells and will form stable complexes with amino acids. The metal–resin complex will continue to extract amines from very dilute solutions until the coordinative valences of the metal ions are saturated[2]. SIEGEL AND DEGENS[1] employed this principle to concentrate the free and bound amino acids in sea water and obtained amino acid recovery rates of 100 %. The procedure described here is based on that of FAZAKERLEY AND BEST[3] who employed column chromatography with Sephadex bonded with copper, under alkaline conditions. They reported the separation of amino acids from peptides with recovery rates approaching 100 % for the amino acids. Peptide recovery rates, however, were not reported. They suggested that the use of a resin with a stronger affinity for copper than Sephadex would be more suitable for the study of peptide recoveries because of a greater retentive capacity for the metal ions.

The resin employed in this study was Chelex 100 (Biorad, Calif.) sodium form, mesh size 50–100. The active sites of this resin are iminodiacetic groups which have a strong affinity for the transition metals. The copper bonding is so strong that the complexed resin can be used in the presence of strong ionic solutions without causing the displacement of copper from the resin by other ions.

Methods

The resin was washed with ten bed volumes of deionized water, added to a saturated solution of copper sulfate and stirred at 4° for 24 h. Subsequently the supernatant copper solution was decanted and the resin washed with deionized water until the washings showed no free copper on the addition of sodium diethyldithiocarbamate. The resin was then suspended in 0.01 *M* sodium tetraborate buffer at pH 11 and 1 *N* sodium hydroxide was added to bring the suspension finally to pH 11. The prepared copper–Chelex resin was packed into a column (1.3 × 12 cm) and washed with a further 25 ml of 0.01 *M* borate buffer at pH 11 at an elution rate of 0.5 ml per min.

Samples containing 0.03 to 0.07 mmole (5–10 mg) of amino acids and a similar quantity of peptides were taken to pH 11 with 0.1 *N* sodium hydroxide and then directly applied to the top of the resin. Elution was continued by gravity at 0.5 ml per min using 0.01 *M* borate buffer at pH 11 until 50 ml of eluate had been obtained. The column eluate was maintained at 0° and collected in a container with sufficient 1 *N* hydrochloric acid at 0° to neutralize the buffer. When peptides were present in the sample, copper appeared in the eluate and removal of this could be achieved by the method recommended by FAZAKERLEY AND BEST[3]. The method requires that copper

* Supported by USPHS Grant T1 HD 108 K3 HD 22 and HD 02633-01.

be removed by a methanolic solution of sodium diethyldithiocarbamate (3.5 g in 10 ml); the mixture is shaken and then allowed to stand for some minutes and the brown derivatives and excess reagent are then removed by extraction with 500 ml of chloroform followed by 2 × 200 ml portions. The final solution is taken to dryness *in vacuo* at —60°. Finally, the dried material is redissolved in deionized water to reconstitute the original sample volume.

Amino acid analyses

All amino acid analyses were performed on a Beckman Spinco 120B Amino Acid Analyzer employing physiological resins according to the method of BENSON AND PATTERSON[4]. The use of high-sensitivity cuvettes permitted the analysis of as little as 0.01 micromoles of amino acid. Analyses were performed upon aliquots of the original sample and of the column eluant before and after hydrolysis. Hydrolysis was carried out in sealed glass containers with 6*N* hydrochloric acid at 100° for 22 h. The containers were filled with argon and subsequently evacuated before sealing. Following hydrolysis the sample was filtered to remove humin and the samples then frozen and taken to dryness *in vacuo* at —60°. The sample was then reconstituted to its original volume with 0.2 *N* pH 2.2 citrate buffer.

Results

The method was first assessed using pure mixtures of amino acids or peptides. No α-amino acids appeared in the column eluate, while dipeptides and polypeptides were not retained by the resin and were recoverable from the eluate along with large quantities of copper. Recovery rates were greater than 90 % for diglycine, triglycine, tetraglycine, glycylproline, prolylhydroxyproline and leucylglycylphenylalanine, whether these peptides were present in pure solution or admixed with solutions containing amino acids. Similar results were found for larger physiological polypeptides such as vasopressin, angiotensin II, glutathione and glucagon and also for albumin which had been predialyzed for three days. Uric acid, urea, sugars and creatinine also passed through the column under the conditions stated.

Fig. 1 shows a representative set of long column chromatograms of the acidic and neutral amino acids from an untreated normal urine (No. 1) and after passage through Chelex (No. 2), also the hydrolyzed control urine (No. 3) and the hydrolyzed column eluate (No. 4). From the diagram it willl be seen that the resin removed almost 100 % of the normal α-amino acids found in urine but did not retain many of the substances, mostly of unknown composition, which run before 4-hydroxyproline in this system. In this sample very few, if any, ninhydrin positive peptides were eluted from the column after 4-hydroxyproline or if there were any present they were not present in sufficient quantity to be detected by the system employed. By contrast, the chromatogram of the column eluant following hydrolysis, shown in Fig. 1 No. 4, shows all the physiological amino acids found in normal protein hydrolysates and the chromatogram is very similar to that of the hydrolyzed control urine (Fig. 1 No. 3).

The amino acid contents of the samples shown in Fig. 1 are presented in Table I. The poor recovery rates shown for the basic amino acids may be explained by the fact that peptides containing basic amino acids have additional free amino groups which will have a strong affinity for the copper on the resin and these may be retained by the resin. Poor recovery rates for those amino acids present in low concentrations are due

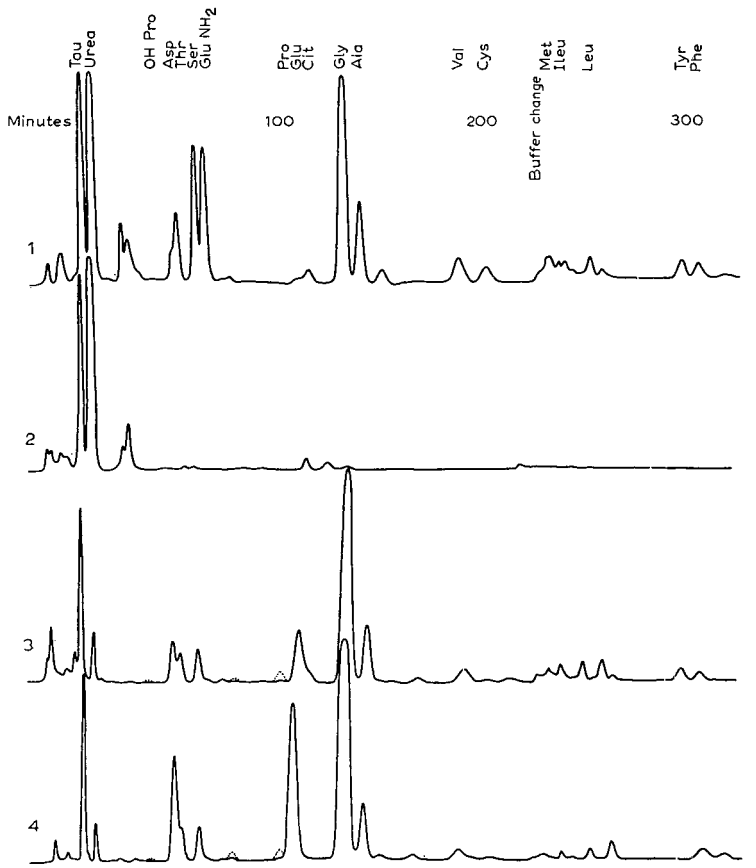


Fig. 1. The acidic and neutral amino acids of urine processed by ligand exchange chromatography before and after hydrolysis. (1) 0.1 ml control urine, unhydrolysed; (2) 0.1 ml urine after passage through resin, unhydrolysed; (3) 0.1 ml control urine, hydrolysed; (4) 0.1 ml urine after passage through resin, hydrolysed. Solid line represents reading at 570 Å. Dotted line represents reading at 440 Å.

to the inherent limits of sensitivity imposed by the analytical system employed. The apparent high recoveries of threonine are due to the progressive hydrolytic destruction of the free amino acid in the control urine[5]. An alternative explanation may be the presence of a substance in the control urine which runs in the same position as the pure amino acid during column chromatography and which is subsequently destroyed by acid hydrolysis: alteration of the conditions of hydrolysis did not materially alter the results.

The reason why amino acids are retained by the column and peptides are not is unclear, since both amino acids and peptides are possessed of free amino groups. The fact that peptides will strip copper from the resin seems to indicate that peptide bonds have an even stronger affinity for the metal than do the iminodiacetic groups and, further, that the peptide–copper complex is then configurationally unable to form a further ligand bond between its free amino groups and the copper on the resin. It will be seen in Fig. 1. that the β-amino acid taurine is not retained by the column.

TABLE I

FREE AND BOUND AMINO ACIDS (μmol/ml) IN NORMAL HUMAN URINE FOLLOWING LIGAND EXCHANGE CHROMATOGRAPHY

The figures shown in Columns 1 through 4 represent the amino acid content of the samples with the same number as shown in Fig. 1.

Amino acid or compound	*Control urine, free amino acids No. 1*	*Urine, following Chelex 100 No. 2*	*Hydrolyzed urine, total amino acid No. 3*	*Urine following Chelex, hydrolyzed peptide amino acid contents No. 4*	*Control urine, peptide amino acid content* No. 5*	*Percentage of recovery (%) No. 6*
Taurine	2.060	2.107	2.506	2.032	0.446	
Phenylacetyl-glutamine	0.204	0.170	0.279	0.019	0.075	
Unknown	0.304	0.290				
Hydroxyproline	—	—	0.230	0.080	0.230	34
Aspartic acid	+	—	1.157	0.830	0.920	90
Threonine	0.202	—	0.357	0.227	0.155	146
Serine	0.390	—	0.699	0.184	0.309	60
Glutamine	0.688	—	—			
Proline	—	—	0.442	0.345	0.442	78
Glutamic acid	0.018	—	2.925	2.074	2.219	93
Citrulline	0.050	Trace	—	—	—	
Glycine	1.664	Trace	11.115	8.897	9.451	94
Alanine	0.286	—	0.744	0.438	0.458	96
α-Aminoadipic acid	0.066	—	0.102	0.026	0.036	72
α-Amino-*n*-butyric acid	0.015	—	0.055	0.050	0.040	125
Valine	0.106	—	0.231	0.124	0.125	100
Half cystine	0.150	—	0.100	0.092	+	?
Methionine	0.052	—	0.027	0.021	—	?
Isoleucine	0.046	—	0.072	0.053	0.026	204
Leucine	0.048	—	0.167	0.103	0.119	87
Tyrosine	0.062	—	0.188	0.130	0.126	103
Phenylalanine	0.062	—	0.104	0.044	0.042	105
allo-Hydroxylysine	0	—	0.086	0.057	0.086	66
Hydroxylysine	0.092	—	0.091	0.046	—	?
γ-Aminobutyric acid	0.026	—	0.179	0.065	0.153	58
Ornithine	0.058	—	0.093	0.041	0.035	
Lysine	0.158	—	0.646	0.261	0.488	53
1-Methylhistidine	0.700	—	0.738	—	0.038	0
Histidine	1.241	—	1.301	0.024	0.060	40
3-Methylhistidine	0.492	—	0.478	0.049	—	?
Arginine	0.008	—	0.081	0.033	0.073	45
TOTALS				14.294	15.631	91

* Column 5 is derived by subtracting free from total amino acids (column 3 minus column 1).

Discussion

Techniques for the separation and identification of urinary amino acids and peptides have developed rapidly in the last two decades. A review of the subject was published in 1962 by SKARZYNSKI[6]. STEIN[7] has reported on the quantities of the bound amino acids in normal urine as released by acid hydrolysis. The figures shown in Table I are in close agreement with his results.

KING[8] has collected column eluate from a standard 22 h chromatogram of normal urine in two hourly fractions. Hydrolysis of these fractions and subsequent amino acid analysis then demonstrated the presence of bound amino acids in all portions of the chromatogram, even when it was thought that all of the ninhydrin positive peaks were definitively identified. In other words, peptides are eluted during the whole period of the standard physiological amino acid chromatogram of normal urine and they are mostly present either in quantities insufficient to be detected by the system or are not primarily ninhydrin positive. The results reported here confirm this. In 1955, WESTALL[9], starting with urine volumes of 100 l, demonstrated the presence of 22 ninhydrin positive peptides in normal urines. In this study we have analyzed only those peptides detectable in 0.25 ml of urine. It is likely that a more concentrated preparation of urine will reveal many more ninydrin positive peaks on a normal urinary amino acid chromatogram. Study of this is under progress.

The technique presented in this paper is based on the principle of ligand exchange chromatography[10]. The method can be utilized as a preparatory step in the isolation and identification of urinary peptides.

Department of Pediatric Microchemistry,
University of Colorado Medical Center,
Denver, Colo. (U.S.A.)

NEIL R. M. BUIST*
DONOUGH O'BRIEN

1 A. SIEGEL AND E. T. DEGENS, *Science*, 151 (1966) 1098.
2 F. HELFFERICH, *Ion Exchange*, McGraw-Hill, New York, 1962, pp. 222–226.
3 S. FAZAKERLEY AND D. R. BEST, *Anal. Biochem.*, 12 (1965) 290.
4 J. V. BENSON, JR. AND J. A. PATTERSON, *Anal. Biochem.*, 13 (1965) 265.
5 E. L. SMITH, M. L. MCFADDEN, A. STOCKELL AND V. BUETTNER-JANUSCH, *J. Biol. Chem.*, 214 (1955) 197.
6 B. SKARZYNSKI AND M. SARNEEKA-KELLER, *Advan. Clin. Chem.*, 5 (1962) 107.
7 W. H. STEIN, *J. Biol. Chem.*, 201 (1953) 45.
8 J. S. KING JR., *Protides of the Biological Fluids*, Elsevier, Amsterdam, 1964, pp. 292–300.
9 R. G. WESTALL, *Biochem. J.*, 60 (1955) 247.
10 P. ANDER AND A. J. SONNESSA, *Principles of Chemistry*, MacMillan Co., New York, 1965, pp. 120–222.

Received January 23rd, 1967

* Present address: Assistant Professor of Pediatrics, University of Oregon Medical School, 3181 S.W. Sam Jackson Park Road, Portland, Oreg. 97201, U.S.A.

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

CHARLETT, S. M.: Fundamentals of chromatography. *Confectionery Prod.*, 31 (1965) 648, 650–651, 653–654; *C.A.*, 65 (1966) 1422a.

TADMOR, J.: Chromatography in the preparation and purification of materials. *Ann. N.Y. Acad. Sci.*, 137 (1966) 103–126; *C.A.*, 65 (1966) 321c — PC, TLC and other methods.

2. FUNDAMENTALS, THEORY AND GENERAL

LEDERER, M., MARINI-BETTOLO, R. AND ROCH, G.: Multiple spot phenomena in the paper chromatography of sincurarine. *Ric. Sci.*, 36 (1966) 193–195; *C.A.*, 65 (1966) 2064h.

3. TECHNIQUES I

EFIMOV, B. N.: (Equipment for automatic spreading of solutions in preparative paper chromatography). *Prikl. Biokhim. i Mikrobiol.*, 2 (1966) 332–334; *C.A.*, 65 (1966) 7604e.

GLAUSER, S., WAGNER, Jr., H., ARBUCKLE, R. AND GLAUSER, E. M.: An adjunct to the interpretation of peptide fingerprints. *Biochim. Biophys. Acta*, 130 (1966) 525–527.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

KASTIEN, H. AND TOMINGAS, R.: Die Bestimmung des 3,4-Benzpyrens auf photographischem Wege. *Helv. Chim. Acta*, 49 (1966) 2348–2352.

7. PHENOLS

WAKSMUNDZKI, A. AND GROSS, J.: (R_F and R_M coefficients of some naphthols in systems of the type: nonpolar solvents + dimethyl sulphoxide + glycerol). *Roczniki Chem.*, 40 (1966) 265–270; *C.A.*, 65 (1966) 1422f.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

KOEPPEN, B. H. AND ROUX, D. G.: C-Glycosylflavonoids. The chemistry of aspalathin. *Biochem. J.*, 99 (1966) 604–609.

10. CARBOHYDRATES

ABDULLAH, M., FRENCH, D. AND ROBYT, J. F.: Multiple attack by α-amylases. *Arch. Biochem. Biophys.*, 114 (1966) 595–599.

CLARKE, A. AND STONE, B. A.: Enzymic hydrolysis of barley and other β-glucans by a β-(1→4)-glucan hydrolase. *Biochem. J.*, 99 (1966) 582–588.

CORPE, W. A. AND SALTON, M. R. J.: Properties of surface materials released from cells of *Chromobacterium violaceum*. *Biochim. Biophys. Acta*, 124 (1966) 125–135.

LINKER, A. AND JONES, R. S.: A new polysaccharide resembling alginic acid isolated from Pseudomonads. *J. Biol. Chem.*, 241 (1966) 3845–3851.

LUNDBLAD, A.: Excretion of oligosaccharides in the urine of secretors and non-secretors belonging to different blood groups. *Biochim. Biophys. Acta*, 130 (1966) 130–142.

MATSUHASHI, S., MATSUHASHI, M., BROWN, J. G. AND STROMINGER, J. L.: Enzymatic synthesis of cytidine diphosphate 3,6-dideoxyhexoses. III. Cytidine diphosphate D-glucose oxidoreductase. *J. Biol. Chem.*, 241 (1966) 4283–4287.

OSAWA, T. AND NAKAZAWA, Y.: Lysozyme substrates. Chemical synthesis of *p*-nitrophenyl O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside and its reaction with lysozyme. *Biochim. Biophys. Acta*, 130 (1966) 56–63 — PC and TLC.

PAZUR, J. H. AND OKADA, S.: A novel method for the action patterns and the differentiation of α-1,4-glucan hydrolases. *J. Biol. Chem.*, 241 (1966) 4146–4151 — two-dimensional chromatography, enzymatic hydrolysis after 1st run.

ROSÍK, J., ZITKO, V., BAUER, Š. AND KUBALA, J.: A polysaccharide from the wild cherry tree gum (*Prunus avium* L. subsp. *avium*). *Collection Czech. Chem. Commun.*, 31 (1966) 3353–3361.
ROSÍK, J., ZITKO, V., BAUER, Š. AND KUBALA, J.: Polysaccharides from *Fomes marginatus* (Fr.) Gill. *Collection Czech. Chem. Commun.*, 31 (1966) 3751–3757.
SCHNEIR, M. L. AND RAFELSON, Jr., M. E.: Isolation and characterization of two structural isomers of N-acetylneuraminyllactose from bovine colostrum. *Biochim. Biophys. Acta*, 130 (1966) 1–11.

See also TLC section.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

KUVAEV, B. E. AND IMYANITOV, N. S.: (Analysis of products of butadiene hydrocarboxylation by paper chromatography). *Zh. Analit. Khim.*, 21 (1966) 221–226; *C.A.*, 65 (1966) 36a.
RAISON, J. K., HENSON, G. AND RIENITS, K. G.: The oxidation of gentisaldehyde by nicotinamide-adenine-dinucleotide specific, aromatic aldehyde dehydrogenase from rabbit liver. *Biochim. Biophys. Acta*, 118 (1966) 285–298.
SCHÖLLNER, R. AND HERZSCHUH, R.: Zur Autoxydation ungesättigter Fettsäure-Ester in Gegenwart von Methanol und Protonen. II. Die Autoxydation von Linolsäure-methylester. *Fette, Seifen, Anstrichmittel*, 68 (1966) 616–623 — PC and TLC.
SUOMALAINEN, H. AND LINNAHALME, T.: Metabolites of α-ketomonocarboxylic acids formed by dried baker's and brewer's yeast. *Arch. Biochem. Biophys.*, 114 (1966) 502–513.

11b. Lipids and their constituents

BROWN, D. M. AND STEWART, J. C.: The structure of triphosphoinositide from beef brain. *Biochim. Biophys. Acta*, 125 (1966) 413–421.
CHIGIREV, V. S., BESINGER, E. N. AND SISAKYAN, N. M.: (A study of phosphatide-peptide compounds, connected with the biosynthesis of protein in lamellae and chloroplasts of *Phaseolus*). *Dokl. Akad. Nauk SSSR*, 169 (1966) 235–237.
HAMILTON, J. G.: Paper chromatography of lipids. *Progr. Chem. Fats Lipids*, 8 (1966) 359–372; *C.A.*, 65 (1966) 2119e.

13. STEROIDS

ISMAIL, A. A. A. AND HARKNESS, R. A.: A method for the estimation of urinary testosterone. *Biochem. J.*, 99 (1966) 717–725 — PC and TLC; an alarm clock was modified to turn the tap that filled the trough with solvent after four hours of equilibration.

See also TLC section.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

MATRKA, M., MARHOLD, J., CHMÁTAL, J. AND PÍPALOVÁ, J.: Papierchromatographie einiger möglicher Metaboliten des N,N-Dimethyl-4-aminoazobenzols. *Collection Czech. Chem. Commun.*, 31 (1966) 2605–2607.
NIKOLENKO, L. N., TOLMACHEVA, N. S., DEGTEREV, E. V. AND MAN'KO, A. A.: (Development of aromatic amines on a chromatogram). *Zh. Analit. Khim.*, 21 (1966) 382; *C.A.*, 65 (1966) 1354e — 2,3-dichloro-1,4-naphthoquinone; PC and TLC.

18. AMINO ACIDS

COVELLI, I. AND WOLFF, J.: Iodohistidine formation in ribonuclease A. *J. Biol. Chem.*, 241 (1966) 4444–4451.
GOPAL, N. G. S.: Paper chromatographic separation of thyroxine, triiodo- and diiodothyronines, and iodide. *Indian J. Pharm.*, 28, No. 2 (1966) 34–36; *C.A.*, 65 (1966) 571a.
HYÁNEK, J., CAFOURKOVÁ, Z. AND HYÁNKOVÁ, E.: (The chromatographic examination of hyperaminoacidurias). *Česk. Pediatrie*, 21 (1966) 961–969.
LUK'YANOV, V. B. AND SIMONOV, E. F.: (Accuracy of qualitative and quantitative determination of amino acids by paper chromatography). *Vestn. Mosk. Univ., Ser. II, Khim.*, 21, No. 1 (1966) 36–39; *C.A.*, 65 (1966) 1386h.
VILLANUEVA, V. R., BARBIER, M., GROS, C. AND LEDERER, E.: Sur l'"éthionine" produite par *Escherichia coli* B. *Biochim. Biophys. Acta*, 130 (1966) 329–335 — PC and TLC.
VIRUPAKSHA, T. K., SHRIFT, A. AND TARVER, H.: Metabolism of selenomethionine in selenium accumulator and non-accumulator Astragalus species. *Biochim. Biophys. Acta*, 130 (1966) 45–55.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

ANDO, K., MATSUBARA, H. AND OKUNUKI, K.: Alkylation of cytochromes *c*. I. Properties of alkylated beef cytochrome *c*. *Biochim. Biophys. Acta*, 118 (1966) 240–255.

ANDO, K., MATSUBARA, H. AND OKUNUKI, K.: Alkylation of cytochromes *c*. II. Carboxymethylation of beef and human cytochromes *c* in the oxidized and reduced forms. *Biochim. Biophys. Acta*, 118 (1966) 256–267.

CIBA LTD.: (Peptides with ACTH activity). *Neth. Pat.*, Appl. 6,510,562 (Cl. C 07*d*), Feb. 14, 1966; Swiss Appl. Aug. 13 and Dec. 2, 1964; 24 pp.; *C.A.*, 65 (1966) 2353d.

GLAZER, A. N.: Transesterification reactions catalyzed by papain. *J. Biol. Chem.*, 241 (1966) 3811–3817.

SCHWYZER, R. AND KAPPELER, H.: Synthetic heptapeptides. *U.S. Pat.*, 3,247,179 (Cl. 260–112.5), April 19, 1966; Swiss Appl. March 11, June 17 and July 15, 1960; 7 pp.; *C.A.*, 65 (1966) 803d.

TANIUCHI, H. AND ANFINSEN, C. B.: The amino acid sequence of an extracellular nuclease of *Staphylococcus aureus*. I. Linear order of the fragments produced by cleavage with cyanogen bromide. *J. Biol. Chem.*, 241 (1966) 4366–4385.

TELEHA, M. AND MÄSIAR, P.: On haemoglobin. XXI. Some differences between primary structures of haemoglobins of man and monkey (*Macacus rhesus*). *Collection Czech. Chem. Commun.*, 31 (1966) 3383–3391.

YUKIOKA, M. AND WINNICK, T.: Biosynthesis of malformin in washed cells of *Aspergillus niger*. *Biochim. Biophys. Acta*, 119 (1966) 614–623.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

ALBRECHT, G. J., BASS, S. T., SEIFERT, L. L. AND HANSEN, R. G.: Crystallization and properties of uridine diphosphate glucose pyrophosphorylase from liver. *J. Biol. Chem.*, 241 (1966) 2968–2975 — polyethyleneimine-impregnated paper.

DOSKOČIL, J.: The sequence specificity of methylation of cytosine in bacterial deoxyribonucleic acid. *Collection Czech. Chem. Commun.*, 31 (1966) 2456–2465.

HOLÝ, A. AND SMRT, J.: Oligonucleotidic compounds. XV. A general approach to the stepwise synthesis of ribooligonucleotides; synthesis of some triribonucleoside diphosphates. *Collection Czech. Chem. Commun.*, 31 (1966) 3800–3816.

MATSUHASHI, S.: Enzymatic synthesis of cytidine diphosphate 3,6-dideoxyhexoses. II. Reversible 2-epimerization of cytidine diphosphate paratose. *J. Biol. Chem.*, 241 (1966) 4275–4282.

MATSUHASHI, S., MATSUHASHI, M. AND STROMINGER, J. L.: Enzymatic synthesis of cytidine diphosphate 3,6-dideoxyhexoses. I. Over-all reactions. *J. Biol. Chem.* 241 (1966) 4267–4274.

STERN, A. I. AND AVRON, M.: An adenosine 5′-diphosphate ribose: orthophosphate adenylyltransferase from *Euglena gracilis*. *Biochim. Biophys. Acta*, 118 (1966) 577–591.

ŽEMLIČKA, J. AND CHLÁDEK, S.: Aminoacyl derivatives of nucleosides, nucleotides and polynucleotides. I. Synthesis of 2′(3′)-O-glycyl ribonucleosides and ribonucleoside 5′-phosphates via intermediates of the orthoester type. *Collection Czech. Chem. Commun.*, 31 (1966) 3775–3789 — PC and TLC.

ŽEMLIČKA, J., CHLÁDEK, S., HOLÝ, A. AND SMRT, J.: Oligonucleotidic compounds. XIV. Synthesis of some diribonucleoside phosphates using the N-dimethylaminomethylene derivatives of 2′,3′-O-ethoxymethylene ribonucleosides. *Collection Czech. Chem. Commun.*, 31 (1966) 3198–3212 — PC and TLC.

See also TLC section.

22. ALKALOIDS

BYKHOVSKII, V. YA., ZAITSEVA, N. I. AND MANTROVA, G. V.: (The effect produced by carbonyl reagents on the biosynthesis of vitamins B_{12} and porphyrins by resting cells of *Propionibacterium shermanii*). *Dokl. Akad. Nauk SSSR*, 168 (1966) 1415–1418.

PAWELCZYK, E. AND HERMANN, T.: (Chemical characterization of papaverine decomposition products. I. A method for evaluation of decomposition degree in papaverine hydrochloride). *Dissertationes Pharm.*, 17 (1966) 569–575; *C.A.*, 65 (1966) 2313h.

PREININGER, V., CROSS, A. D. AND ŠANTAVÝ, F.: Isolation and chemistry of the alkaloids from some plants of the genus *Papaver*. XXXIII. On the constitution of latericine. *Collection Czech. Chem. Commun.*, 31 (1966) 3345–3352 — PC and TLC.

SLAVÍKOVÁ, L.: Alkaloide der Mohngewächse (*Papaveraceae*). XXXIV. Alkaloide aus *Glaucium squamigerum* Kar. et Kir. *Collection Czech. Chem. Commun.*, 31 (1966) 4181–4184 — PC and TLC.

See also TLC section.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

DENKSTEIN, J. AND KADEŘÁVEK, V.: Synthesen auf dem Gebiet der Nitramine. VI. Nitrolyse von 1,3,5-Tri(cyanmethyl)hexahydro-*s*-triazin. *Collection Czech. Chem. Commun.*, 31 (1966) 2928–2937.

MARKACHEVA, T. M. AND KORNILOVA, L. I.: (Chromatography of quinoline on paper impregnated with cupric chloride). *Khim. Produkty Koksovaniya Uglei Vostoka SSSR, Poluchenie, Obrabotka, Ispol'z., Metody Analizov, Vost. Nauchn.-Issled. Uglekhim. Inst., Sb. Statei*, No. 3 (1965) 320–324; *C.A.*, 65 (1966) 10b.

MURASHIGE, K., MCDANIEL, D. AND CHAVKIN, S.: N-Methylnicotinamide oxidation in hog liver. *Biochim. Biophys. Acta*, 118 (1966) 556–565.

SMRT, J. AND CHLÁDEK, S.: Oligonucleotidic compounds. XIII. 2'-O-(1-Ethoxyethyl)-5'-O-acetyl derivatives of uridine 3'-phosphate and N-acetylcytidine 3'-phosphate. *Collection Czech. Chem. Commun.*, 31 (1966) 2978–2984.

24. ORGANIC SULPHUR COMPOUNDS

ALLAN, Z. J. AND PODSTATA, J.: Sulfurierungen. VII. Partielle Entsulfitierung und Aminierung des Adduktes von Resorcin mit Natriumhydrogensulfit. *Collection Czech. Chem. Commun.*, 31 (1966) 3573–3583.

NAVRÁTIL, O., BENDOVÁ, L. AND SVOBODA, V.: Extraktionsanalytische Anwendbarkeit der alkylierten Derivate von 4-(2-Thiazolylazo) resorcin. *Collection Czech. Chem. Commun.*, 31 (1966) 3972–3978.

PODSTATA, J., ŠNOBL, D. AND ALLAN, Z. J.: Sulfurierungen. VI. Über die Reaktion von Hydrogensulfiten mit *m*-Phenylendiamin und Resorcin. *Collection Czech. Chem. Commun.*, 31 (1966) 3563–3572.

PROCHÁZKA, M. AND PALEČEK, M.: Double bond isomerisation of cyclic sulfones. *Collection Czech. Chem. Commun.*, 31 (1966) 3744–3750.

27. VITAMINS AND HORMONES

KUTZBACH, C. AND JAENICKE, L.: (Model investigations of the biological activation of single carbon units. III. Synthesis and properties of phosphorylated models of tetrahydrofolic acid). *Ann. Chem.*, 692 (1966) 26–41; *C.A.*, 65 (1966) 701e — PC and TLC.

ROTH, J. A., CHASSY, B. M. AND MCCORMICK, D. B.: Coenzymatic activities of 2-anilino and 2-morpholino derivatives of FMN with yeast NADPH diaphorase. *Biochim. Biophys. Acta*, 118 (1966) 429–431.

See also TLC section.

28. ANTIBIOTICS

BOROWSKI, E., CHMARA, H. AND JARECZEK-MORAWSKA, E.: The antibiotic edeine. VI. Paper and thin-layer chromatography of components of the edeine complex. *Biochim. Biophys. Acta*, 130 (1966) 560–563 — PC and TLC.

DIVEKAR, P. V., VORA, V. C. AND KHAN, A. W.: Comparison of hamycin with trichomycin. *J. Antibiotics (Tokyo), Ser. A*, 19 (1966) 63–64; *C.A.*, 65 (1966) 569g.

ROSCOE, J. AND ABRAHAM, E. P.: Experiments relating to the biosynthesis of bacilysin. *Biochem. J.*, 99 (1966) 793–800 — combination of electrophoresis with chromatography.

TURKOVÁL, J., MIKEŠ, O. AND ŠORM, F.: Similarities in the composition of antibiotics albomycin and grisein. *Collection Czech. Chem. Commun.*, 31 (1966) 2444–2453.

30. SYNTHETIC AND NATURAL DYES

ALLAN, Z. J. AND PODSTATA, J.: Aromatische Diazo- und Azoverbindungen. LXVII. Austausch der Nitrogruppe gegen Chlor in *o*-Nitrobenzoldiazoniumsalzen. *Collection Czech. Chem. Commun.*, 31 (1966) 3418–3421.

ALLAN, Z. J. AND PODSTATA, J.: Aromatische Diazo- und Azoverbindungen. LXXI. Darstellung von Azofarbstoffen aus den Sulfitaddukten des Resorcins und aus Phenol-*m*-sulfonsäure. Alkalische Sulfitierung. *Collection Czech. Chem. Commun.*, 31 (1966) 4129–4144.

JARKOVSKÝ, J., ŠEJVL, J. AND ALLAN, Z. J.: Aromatische Diazo- und Azoverbindungen. LXVI. Darstellungsversuche makrocyclischer Azofarbstoffe und Darstellung der 2-Amino-7-hydroxynaphtalin-6-sulfonsäure. *Collection Czech. Chem. Commun.*, 31 (1966) 3213–3221.

PODSTATA, J. AND ALLAN, Z. J.: Aromatische Diazo- und Azoverbindungen. LXVIII. Kupplung mit *m*-Aminophenol und seinem Sulfitaddukt. *Collection Czech. Chem. Commun.*, 31 (1966) 3547–3554.

See also TLC section.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

BECKMANN, R.: Zum Verhalten von Benzilsäure-(N,N-dimethyl-2-hydroxymethyl-piperidinium)-ester-methylsulfat im Organismus. *Arzneimittel-Forsch.*, 16 (1966) 910–911 — PC and TLC.

LEWIS, D. A.: The metabolism of *cis*- and *trans*-indane-1,2-diol. *Biochem. J.*, 99 (1966) 694–702.

NĚMEČEK, O., MACEK, K., QUEISNEROVÁ, M. AND VEJDĚLEK, Z. J.: Ketophenylbutazon-Metaboliten im Harn. *Arzneimittel-Forsch.*, 16 (1966) 1339–1342 — PC and TLC.

SHTEINGART, M. V. AND NOSOVITS'KA, S. A.: (Accelerated determination of the stability of Nicoverin tablets). *Farmatsevt. Zh. (Kiev)*, 21, No. 2 (1966) 54–58; *C.A.*, 65 (1966) 2070a.

VEČERKOVÁ, J., VEČEREK, B. AND NOVOTNÁ, E.: (The use of paper chromatography in the toxicological detection of drugs. V. The rapid demonstration of amidopyrine in the urine based on the detection of its metabolits). *Česk. Farm.*, 15 (1966) 491–495.

See also TLC section.

33. INORGANIC SUBSTANCES

AGRINIER, H.: Application de la chromatographie sur papier à la détermination de certains éléments dans les minéraux et les roches. *Thesis*, Paris, 1966, 80 pp.

LANDGREBE, A. R., GILLS, T. E. AND DEVOE, J. R.: Application of radiometric techniques to quantitative paper chromatography of iron, copper, manganese, and cobalt. *Anal. Chem.*, 38 (1966) 1265–1266.

POPPER, E., FLOREAN, E., MARCU, P. AND SOSA, E.: (New organic reagent in paper chromatography. Application to inorganic substances. Determination of cadmium, bismuth, and lead). *Rev. Roumaine Chim.*, 11 (1966) 283–287; *C.A.*, 65 (1966) 1353c.

QURESHI, M., AKHTAR, I. AND VARSHNEY, K. G.: Fast quantitative separation of iron (II) and iron (III) by paper chromatography. *Anal. Chem.*, 38 (1966) 1415–1417.

VDOVENKO, M. E.: (Mechanism of separation of rare earth elements as thiocyanates by paper chromatography). *Ukr. Khim. Zh.*, 32 (1966) 284–289; *C.A.*, 65 (1966) 1355a.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

KRÖLLER, E.: Untersuchungen zum Nachweis von Emulgatoren in Lebensmitteln. 6. Mitteilung. *Fette, Seifen, Anstrichmittel*, 68 (1966) 1066–1068.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

DARDENNE, G. A.: (Thin-layer chromatography). *Ind. Chim. Belge*, 31 (1966) 323–332; *C.A.*, 65 (1966) 1354d — a review with 139 references.

PLAVSAK, M.: (Thin-layer chromatography). *Farm. Vestnik (Ljubljana)*, 16, No. 5–8 (1965) 113–116; *C.A.*, 65 (1966) 10f.

See also PC section.

3. TECHNIQUES I

HOLCZABEK, W.: (Thin-layer chromatographic investigations of tissue). *Deut. Z. Ges. Gerichtl. Med.*, 57 (1966) 211–214; *C.A.*, 65 (1966) 7605d.

KEYWORTH, D. A. AND SWENSEN, R. F.: Quantitative evaluation of thin-layer chromatograms. *Talanta*, 13 (1966) 829–836; *C.A.*, 65 (1966) 1358d.

KODAK-PATHE (BY LESTIENNE, A. AND MOYER, J. H.): (Thin-layer chromatographic products). *French Pat.*, Addn. 85,939 (Cl. B 01*d*), Nov. 5, 1965, Appl. June 15, 1964; 3 pp.; *C.A.*, 65 (1966) 1390d — ion-exchange resin and carboxymethylcellulose layers.

MCGREGOR, R. F. AND KHAN, M.: The effects of contaminated laboratory atmospheres upon thin-layer chromatographic analysis of urinary 4-hydroxy-3-methoxy-mandelic acid. *Clin. Chim. Acta*, 14 (1966) 844–846.

10. CARBOHYDRATES

HOFFMANN, ST., WEISS, EK. AND REICHSTEIN, T.: Synthese von 2-O-Methyl-6-desoxy-D-allose (D-Javose) und 4-O-Methyl-6-desoxy-D-allose. *Helv. Chim. Acta*, 49 (1966) 2209–2218 — TLC and PC.

KÄRKKÄINEN, J. E., HAAHTI, E. O. AND LEHTONEN, A. A.: Thin layer and column chromatography of carbohydrates as trimethylsilyl ethers with applications to mucopolysaccharide analysis. *Anal. Chem.*, 38 (1966) 1316–1319.

WALKER, JR., H. G., RICCI, B. A. AND GOODWIN, J. C.: Thin-layer chromatography of sugar beet carbohydrates. *J. Am. Soc. Sugar Beet Technologists*, 13 (1965) 503–508; *C.A.*, 65 (1966) 903g.

See also PC section.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

See PC section.

11b. Lipids and their constituents

AMAT, F., MARQUINEZ, E., UTRILLA, R. M. AND MARTIN, D.: (Application of thin-layer and gas-liquid chromatography to the constitutional analysis of lard). *Grasas Aceites (Seville, Spain)*, 17, No. 2 (1966) 45–50; *C.A.*, 65 (1966) 7903h.

CAVINA, G., AJELLO, M. T., CASPARRINI, G., D'ANTONA, A., MORETTA, L. AND MORETTI, G.: (Separation of lipids according to classes of compounds by means of thin-layer chromatography. Fractionation and determination of liver and serum lipids of normal rats). *Ann. Ist. Super. Sanità*, 1 (1965) 566–587; *C.A.*, 65 (1966) 7704h.

ELSBACH, P.: Phospholipid metabolism by phagocytic cells. I. A comparison of conversion of [^{32}P]-lysolecithin to lecithin and glycerylphosphorylcholine by homogenates of rabbit polymorphonuclear leucocytes and alveolar macrophages. *Biochim. Biophys. Acta*, 125 (1966) 510–524.

FIGARD, P. H. AND LEVINE, A. S.: Incorporation of labeled precursors into lipids of tumors induced by Rous sarcoma virus. *Biochim. Biophys. Acta*, 125 (1966) 428–434.

KAUFMANN, H. P., ABDEL KADER S. AHMAD AND RADWAN, S. S.: Pro- und Antioxydantien auf dem Fettgebiet. XX. Die Lipoide der Hefezellen in verschiedenen Wachstumsphasen. *Fette, Seifen, Anstrichmittel*, 68 (1966) 1010–1016.

LONG, C. AND OWENS, K.: Chromatographic behaviour of rat-liver monophosphoinositide. *Biochem. J.*, 99 (1966) 617–623 — thin layers of Kieselgel G or H are extracted before activation to remove impurities which char with H_2SO_4.

MALINS, D. C.: Recent developments in the thin-layer chromatography of lipids. *Progr. Chem. Fats Lipids*, 8 (1966) 301–358; *C.A.*, 65 (1966) 2119d.

MORRISON, M., BRIGHT, J. AND ROUSER, G.: Cytochrome c oxidase. VIII. Lipid content. *Arch. Biochem. Biophys.*, 114 (1966) 50–55.

13. STEROIDS

BERTHOLD, K. AND STAUDINGER, H.: (Quantitative determination of 6β-hydroxycortisol using thin-layer chromatography with fluorescence indicator). *Z. Klin. Chem.*, 4 (1966) 130–133; *C.A.*, 65 (1966) 7560f.

BROOKS, C. J. W. AND CARRIE, J. G.: Thin-layer chromatography of trimethylsilyl ethers. *Biochem. J.*, 99 (1966) 47P–48P.

CHAMBERLAIN, J., JAGARINEC, N. AND OFNER, P.: Catabolism of 4-^{14}C-testosterone by subcellular fractions of human prostate. *Biochem. J.*, 99 (1966) 610–616.

DÖLLEFELD, E. AND BREUER, H.: Nachweis von Oestradiol: NAD^+-17α- und 17β-Oxidoreduktasen in der Cytoplasma-Fraktion der Pferdeplacenta. *Biochim. Biophys. Acta*, 124 (1966) 187–189 — TLC and PC.

GOAD, L. J. AND GOODWIN, T. W.: The biosynthesis of sterols in higher plants. *Biochem. J.*, 99 (1966) 735–746.

KAY, H. L.: (Separation of conjugated 17-keto steroids by thin-layer chromatography on Silica Gel GF 254). *J. Pharm. Belg.*, 20 (1965) 463–466; *C.A.*, 65 (1966) 569h.

LAWRENCE, J. R. AND GRIFFITHS, K.: Oestrogen biosynthesis *in vitro* by adrenal tissue from the golden hamster. *Biochem. J.*, 99 (1966) 27C–28C — R_F values of 11 oestrogen derivatives on Silica Gel HF 254/366.

LISBOA, B. P.: (Thin-layer chromatography of steroids). *J. Pharm. Belg.*, 20 (1965) 435–449; *C.A.*, 65 (1966) 570c — R_M values of 250 steroids.

MASUI, T. AND STAPLE, E.: The formation of bile acids from cholesterol. *J. Biol. Chem.*, 241 (1966) 3889–3893.

REES, H. H., MERCER, E. I. AND GOODWIN, T. E.: The stereospecific biosynthesis of plant sterols and α- and β-amyrin. *Biochem. J.*, 99 (1966) 726–734 — Kieselgel G layers with Rhodamin 6G with or without $AgNO_3$.

SACCANI, F.: (Enzymic titration of some corticosteroid phosphates). *Boll. Chim. Farm.*, 105 (1966) 134–138; *C.A.*, 65 (1966) 571h — TLC and PC.

See also PC section.

15. TERPENE DERIVATIVES

ZIELINSKA-SOWICKA, R. AND SZEPCZYNSKA, K.: (Investigation of essential oils by thin-layer chromatography). *Ann. Acad. Med. Lodz*, 6 (1965) 35–40; *C.A.*, 65 (1966) 2060g.

16. NITRO AND NITROSO COMPOUNDS

LINGENS, F., EBERHARDT, H. UND OLTMANNS, O.: Mikrobieller Abbau des Chloramphenicols. *Biochim. Biophys. Acta*, 130 (1966) 345–354.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

GEMZOVÁ, I. AND GASPARIČ, J.: Identifizierung organischer Verbindungen. LXV. Dünnschichtchromatographie primärer aromatischer Amine an Aluminiumoxid. *Collection Czech. Chem. Commun.*, 31 (1966) 2525–2533 — R_F values of 90 amines.

See also PC section.

18. AMINO ACIDS

See PC section.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

EISLER, K., RUDINGER, J. AND ŠORM, F.: Amino acids and peptides. LXV. Analogues of oxytocin with isoleucine replaced by L-diethylalanine, L-cyclopentylglycine, and L- and D-cyclohexylglycine. *Collection Czech. Chem. Commun.*, 31 (1966) 4563–4580.

KASAFÍREK, E., RÁBEK, V., RUDINGER, J. AND ŠORM, F.: Amino acids and peptides. LXVI. Synthesis of ten extended-chain analogues of lysine vasopressin. *Collection Czech. Chem. Commun.*, 31 (1966) 4581–4591.

OPPENHEIMER, H. L., LABOUESSE, B. AND HESS, G. P.: Implication of an ionizing group in the control of conformation and activity of chymotrypsin. *J. Biol. Chem.*, 241 (1966) 2720–2730.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BOWEN, T. J., HAPPOLD, F. C. AND TAYLOR, B. F.: Studies on adenosine-5′-phosphosulphate reductase from *Thiobacillus denitrificans*. *Biochim. Biophys. Acta*, 118 (1966) 566–576 — TLC on DEAE-cellulose and PC.

CRAMER, F. AND HATA, T.: (Chemistry of high-energy phosphates. XIX. Phosphorylation with the addition product from bromocyanoacetamide and triphenylphosphine). *Ann. Chem.*, 692 (1966) 22–25; *C.A.*, 65 (1966) 636g.

See also PC section.

22. ALKALOIDS

DAL CORTIVO, L. A., BROICH, J. R., DIHRBERG, A. AND NEWMAN, B.: Identification and estimation of lysergic acid diethylamide by thin layer chromatography and fluorometry. *Anal. Chem.*, 38 (1966) 1959–1960.

GORMAN, A. A., AGWADA, V., HESSE, M., RENNER, U. AND SCHMID, H.: Zur Chemie des Benidins und Vobtusins. *Helv. Chim. Acta*, 49 (1966) 2072–2098.

GUGGISBERG, A., HESSE, M., PHILIPSBORN, W. VON, NAGARAJAN, K. AND SCHMID, H.: Die Struktur der Alkaloide Fruticosin und Fruticosamin und ihre Synthese aus Kopsin. *Helv. Chim. Acta*, 49 (1966) 2321–2337.

HORÁK, P. AND KUDRNÁČ, S.: (Determination of the individual groups of alkaloids in ergot of ergotamine type). *Česk. Farm.*, 15 (1966) 483–490 — TLC and PC.

KAESS, A. AND MATHIS, C.: Essai des préparations pharmaceutiques par la chromatographie fonctionnelle sur couche mince. II. Préparations contenant des alcaloïdes de l'opium et des Rauwolfia. *Ann. Pharm. Franc.*, 23 (1965) 739–747 — R_F values of 20 opium and 21 Rauwolfia alkaloids.

KULESHOVA, M. I.: (Separation of multicomponent mixtures of alkaloids by countercurrent extraction). *Sb. Nauchn. Tr., Tsentr. Aptechn. Nauchn.-Issled. Inst.*, 6 (1964) 114–119; *C.A.*, 65 (1966) 2063h.

PARIS, R. R. AND FAUGERAS, G.: Evaluation de la morphine dans les capsules de Pavots indigènes à l'aide de la chromatographie en couche mince. *Ann. Pharm. Franc.*, 24 (1966) 613–616.

SLAVÍKOVÁ, L. AND SLAVÍK, J.: Alkaloide der Mohngewächse (*Papaveraceae*). XXXIII. Über die Alkaloide von drei Arten der Gattung Eschscholtzia und über die Konstitution des neuen Alkaloids Escholamin. *Collection Czech. Chem. Commun.*, 31 (1966) 3362–3372 — TLC and PC.

See also PC section.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

DUDYKINA, N. V. AND ZAGOREVSKII, V. A.: (Pyran, its analogs, and related compounds. XIII. Further study of the anomalous reduction of ketoximes by lithium aluminum hydride). *Sintez Prirodn. Soedin., ikh Analogov i Fragmentov, Akad. Nauk SSSR, Otd. Obshch. i Tekhn. Khim.*, (1965) 134–138; *C.A.*, 65 (1966) 683c.

PROST, M., URBAIN, M. AND CHARLIER, R.: Préparation de propargylpipéridinols et de leurs esters. Derivés propargyliques. IV. *Helv. Chim. Acta*, 49 (1966) 2370–2394.

24. ORGANIC SULPHUR COMPOUNDS

KIGER, J. L. AND KIGER, J. G.: Procédés de diagnose différentielle rapide en série des dérivés sulfamidés usuels. *Ann. Pharm. Franc.*, 24 (1966) 593–605 —R_F values for 44 sulphonamides.

27. VITAMINS AND HORMONES

DUNAGIN, Jr., P. E., ZACHMAN, R. D. AND OLSON, J. A.: The identification of metabolites of retinal and retinoic acid in rat bile. *Biochim. Biophys. Acta*, 124 (1966) 71–85.

GOLD, P. H. AND OLSON, R. E.: Studies on coenzyme Q. The biosynthesis of coenzyme Q_9 in rat tissue slices. *J. Biol. Chem.*, 241 (1966) 3507–3516 — TLC and PC.

STURM, P. A., PARKHURST, R. M. AND SKINNER, W. A.: Quantitative determination of individual tocopherols by thin-layer chromatographic separation and spectrophotometry. *Anal. Chem.*, 38 (1966) 1244–1247.

See also PC section.

28. ANTIBIOTICS

See PC section.

29. INSECTICIDES AND OTHER PESTICIDES

BECKMAN, H. AND WINTERLIN, W.: Separation and detection of submicrogram quantities of pesticides by an improved TLC technique. *Bull. Environ. Contam. Toxicol.*, 1 (1966) 78–85; *C.A.*, 65 (1966) 7894e.

BYRDE, R. J. W., CLIFFORD, D. R. AND WOODCOCK, D.: Fungicidal activity and chemical constitution. XIII. Active components of commercial formulations of Dinocap. *Ann. Appl. Biol.*, 57 (1966) 223–230; *C.A.*, 65 (1966) 7927h.

ERNE, K.: Determination of phenoxyacetic herbicide residues in biological materials. *Acta Vet. Scand.*, 7 (1966) 77–96; *C.A.*, 65 (1966) 7893g.

HENKEL, H. G.: (Gas and thin-layer chromatography as methods for (pesticide) residue analysis). *Min. Biol. Bundesanstalt Land-, Forst-Wirtsch., Berlin-Dahlem*, 105 (1965) 103–106; *C.A.*, 65 (1966) 7895c.

HYLIN, J. W.: Thin-layer chromatography of dithiocarbamate fungicides. *Bull. Environ. Contam. Toxicol.*, 1 (1966) 76–77; *C.A.*, 65 (1966) 7928g.

30. SYNTHETIC AND NATURAL DYES

ARIENT, J., HAVLÍČKOVÁ, L. AND KNÍŽEK, J.: Imidazol-Farbstoffe. XIX. Über die Reaktionen von 1,2-Benzoylenbenzimidazol und 1,2-Naphtoylenbenzimidazol mit Grignardreagenzien. *Collection Czech. Chem. Commun.*, 31 (1966) 4682–4693.

BENK, E. AND BERGMANN, R.: (Detection of foreign dyes in orange oils). *Riechstoffe, Aromen, Koerperpflegemittel*, 16, No. 3 (1966) 79, *et seq.*; *C.A.*, 65 (1966) 567g.

DESHUSSES, J. AND DESBAUMES, P.: Identification des colorants des rouges à lèvres par chromatographie sur couche mince. *Mitt. Gebiete Lebensm. Hyg.*, 57 (1966) 373–376.

EGGER, K.: Vergleichende Untersuchung der Xanthophyllveresterung in Blüten, Früchten und Herbstlaub. *Ber. Deut. Botan. Ges.*, 77 (1964) 145–150.

EGGER, K. AND SCHWENKER, U.: Lutein-Fettsäureester im Herbstlaub. *Z. Pflanzenphysiol.*, 54 (1966) 407–416.

HAGER, A. AND MEYER-BERTENRATH, T.: (Extraction and quantitative determination of carotenoids and chlorophylls of leaves, algae, and isolated chloroplasts with the aid of TLC). *Planta*, 69 (1966) 198–217; *C.A.*, 65 (1966) 7606c.

HORI, K. AND CORMIER, M. J.: Studies on the bioluminescence of *Renilla reniformis*. VI. Some chemical properties and the tentative partial structure of luciferin. *Biochim. Biophys. Acta*, 130 (1966) 420–425 — TLC and PC.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

BLAŽEK, J.: (The study of stability of Soluglaucit). *Česk. Farm.*, 15 (1966) 478–482.

CELLARIUS, H. J. AND ZECHNER, L.: Zur Kenntnis des Conduragins. *Sci. Pharm.*, 34 (1966) 10–25 — TLC and PC.

DEBELMAS, J., BESSON, PH. AND HÉRISSET, A.: Essais de "lyophilisation industrielle" des plants médicinales. II. Camomille romaine (*Anthemis nobilis L.*). (Influence comparée de la lyophilisation et de différents modes de dessiccation sur la qualité des capitules). *Ann. Pharm. Franc.*, 24 (1966) 587–592.

FIKE, W. W.: Structure–R_F correlations in the thin layer chromatography of some basic drugs. *Anal. Chem.*, 38 (1966) 1697–1702 — R_F values for 140 basic drugs in five chromatographic systems.

MARGASINSKI, Z., DANIELAK, R. AND RAFLOWSKA, H.: (Thin-layer chromatography of benzothiadiazine derivatives). *Acta Polon. Pharm.*, 23 (1966) 35–37; *C.A.*, 65 (1966) 2066a.

See also PC section.

33. INORGANIC SUBSTANCES

HASHMI, M. H., SHAHID, M. A., AYAZ, A. A., CHUGHTAI, F. R., HASSAN, N. AND ADIL, A. S.: Identification of forty cations and nineteen anions by circular thin layer chromatography. *Anal. Chem.*, 38 (1966) 1554–1558 — systematic analysis; separation of ions into groups by solvent extraction.

PATRIARCHE, G.: (Separation of As(III), Sb(III), and Sn(II) cations by thin-layer chromatography on Silica Gel G). *J. Pharm. Belg.*, 20 (1965) 336–342; *C.A.*, 65 (1966) 1354f.

34. RADIOACTIVE AND ISOTOPIC COMPOUNDS

CECIL, R., JACK, M. AND SMITH, Jr., W. G.: Autoradiography of radioactive thin-layer chromatograms. *Contrib. Boyce Thompson Inst.*, 23 (1966) 215–217; *C.A.*, 65 (1966) 1726e.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

GASCO, M. R. AND GATTI, G.: (Application of thin-layer chromatography to cationic detergents) *Riv. Ital. Sostanze Grasse*, 43 (1966) 175–176; *C.A.*, 65 (1966) 2480f.

ISHIKAWA, S. AND KATSUI, G.: Separation and detection of antioxidants in vitamin A oil and vegetable oil by thin-layer chromatography. *J. Vitaminol.*, 12 (1966) 112–116; *C.A.*, 65 (1966) 7893c.

Gas Chromatography

1. REVIEWS AND BOOKS

KELLER, R. A.: Analytical Chemistry Summer Symposium for 1966. *Anal. Chem.*, 39, No. 1 (1967) 47A–51A — a review of 28 papers, of which 24 deal with chromatography (13 GC).
LEDERER, M. (Editor): *Chromatographic Reviews*, Vol. 8, Elsevier, Amsterdam, 1967, 298 pp. — 8 papers, 5 of which deal with GC.
LEVY, R. L.: Pyrolysis gas chromatography. A review of the technique. In M. LEDERER (Editor): *Chromatographic Reviews*, Vol. 8, Elsevier, Amsterdam, 1967, pp. 48–89 — 201 references; a most exhaustive review.
ZLATKIS, A. (Editor): *Advances in Gas Chromatography 1967*, Preston Techn. Abstr. Co., Evanston, Ill., 1967 — Proceedings of the 4th International Symposium, New York City, April 3rd–6th, 1967; 35 papers dealing with theory, column technology and detectors, applications biochemical techniques, liquid chromatography and new horizons; 213 pp.

2. FUNDAMENTALS, THEORY AND GENERAL

2d. General

GUIOCHON, G.: Flow of gases in porous media. Problems raised by the operation of gas chromatography columns. In M. LEDERER (Editor): *Chromatographic Reviews*, Vol. 8, Elsevier, Amsterdam, 1967, pp. 1–47 — 58 references; an interesting educational review.

3. TECHNIQUES I

3a. Detectors

MOCHIZUKI, M. AND KAWAKAMI, Y.: A new detector for a gas chromatograph using electron current following glow discharge. *Japan J. Physiol.*, 15 (1965) 553–559.
TSUGE, S. AND TAKEUCHI, T.: The ionization mechanism in radioactive ionization detector. *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 68 (1965) 1661–1664.

3b. Column performance and filling studies

RUSHNECK, D. R. AND DEHLER, J. L.: Cryogenically programmed gas chromatography. *Lab. Management*, 4 (1966) 49.
WILSON, A. W., BURTON, E. W. AND GOESCHL, J. D.: Chromatographic column. *U.S. Pat.*, 3,245,207 (Apr. 12, 1966).

3c. Apparatus, accessories and materials for GC

BOCHINSKI, J. H. AND PORTER, J. A.: Liquid sample reactor and evolved gas detector. *U.S. Pat.*, 3,249,403 (May 3, 1966).
BURK, M. C.: Automatic zeroing system. *U.S. Pat.*, 3,247,703 (Apr. 26, 1966).
CARLE, D. W. AND BURNELL, M. R.: Gas chromatograph. *U.S. Pat.*, 3,174,326 (March 23, 1965).
CHURCHILL, L.: A novel method for collecting samples for infrared identification. *Develop. Appl. Spectry.*, 4 (1965) 283–288.
GOESCHL, J. D.: Stacked plate chromatographic column. *U.S. Pat.*, 3,254,479 (June 7, 1966).
HAMILTON, C.: Reproducibility of injecting the gas chromatograph. *Lab. Management*, 3 (1965) 40–41.
HARRIS, R. J.: Sample injection in gas chromatography. *U.S. Pat.*, 3,205,711 (Sept. 14, 1965).
KLAVER, R. F. AND GARDINER, K. W.: Automatic integrator for chromatographic records. *U.S. Pat.*, 3,259,733 (July 5, 1966).
KOENIG, E.: Sample pick-up for gas chromatographs. *U.S. Pat.*, 3,247,704 (Apr. 26, 1966).
MCARTHUR, B. W.: Soap film type gas flow measuring device. *U.S. Pat.*, 3,248,941 (May 3, 1966).
OSTERHAUS, E.: Reinigung von Essigsäure-isoamylester zur Verwendung als Extraktionsmittel für gaschromatographische Untersuchungen. *Z. Anal. Chem.*, 215 (1965) 241–243.

4. TECHNIQUES II

4a. Preparative-scale GC

LIPPMAA, E. AND LUIGA, P.: (A study of the nonuniformity of gas flow in large-scale gas chromatographic columns). *Eesti NSV Teaduste Akad. Toimetised*, 14 (1965) 246–254.
LUIGA, P. AND LIPPMAA, E.: (The loading factor and condensation in large-scale gas chromatography). *Eesti NSV Teaduste Akad. Toimetised*, 14 (1965) 255–257.

4c. High-speed GC

LU, P. CH., ZHOU, L.-M., MA, T.-L. AND CHEN, G.-J.: High speed gas liquid chromatography for the separation of a mixture containing hydrocarbons, aldehydes, ketones and esters with a packed column. *Sci. Sinica*, 14 (1965) 1171–1175.

4d. Special microtechniques and functional analysis

CLERC, J. T. AND SIMON, W.: Limitations of a self-integrating method for the simultaneous C, H and N determination by thermal conductivity measurements. *Microchem. J.*, 7 (1963) 422–436.

HOFFMANN, R. L., LIST, G. R. AND EVANS, C. D.: Enrichment of volatile samples by syringe collection, *J. Food Sci.*, 31 (1966) 751–752.

JOHNS, T. AND MORRIS, R. A.: A new method of pyrolysis *Develop. Appl. Spectry.*, 4 (1965) 361–368.

MENDELSOHN, J. M., STEINBERG, M. A. AND MERRITT, JR., CH.: Techniques for collecting volatile components from haddock flesh for gas chromatographic analysis. *J. Food Sci.*, 31 (1966) 389–394.

4e. Automation

PEINKE, W.: Control loops with a periodic sampler and consecutive storage. *Regelungstechnik*, 9 (1961) 188–194.

4f. Measurement of physico-chemical and related values

MUEHLBAECHER, C. A., DEBON, F. L. AND FEATHERSTONE, R. M.: Further studies on the solubilities of xenon and cyclopropane in blood and protein solutions. *Mol. Pharmacol.*, 2 (1966) 86–89.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5b. Other hydrocarbons

GORDON, P. L.: Determination of the carbon structure of heptenes. *J. Inst. Petrol.*, 51 (1965) 398–400.

MOGHADAM, P. E.: New use of gas chromatography for analyzing all petrochemical products. *Iranian Petrol. Inst.*, No. 22 (1966) 754–760.

PROKSCH, E.: Gaschromatographische Untersuchungen an VÖEST-Anthracenöl. I. *Oesterr. Chemiker-Ztg.*, 67 (1966) 105–112.

RANG, S. AND EIZEN, O.: (Microanalytical determination of normal hydrocarbons in the gasoline fraction of oven pitch by molecular sieves. *Izv. Akad. Nauk Est. SSR, Ser. Fiz. Mat. Tekh. Nauk*, (1966) 245–251.

RANG, S., EIZEN, O., KUNINGAS, K. AND VYANIKVER, M.: (Chemical composition of non-aromatic hydrocarbons of oven pitch). *Izv. Akad. Nauk Est. SSR, Ser. Fiz. Mat. Tekh. Nauk*, (1966) 429–435.

RAUDE, KH., RANG, S. AND EIZEN, O.: (Composition of non-saturated hydrocarbons of the middle fraction of oven pitch). *Izv. Akad. Nauk Est. SSR, Ser. Fiz. Mat. Tekh. Nauk*, (1966) 239–244.

5c. Halogen derivatives of hydrocarbons

BURTON, D. J., JOHNSON, R. L. AND BOGAN, R. T.: The preparation and characterization of *cis*- and *trans*-2-hydro-3-chlorohexafluoro-2-butene. *Can. J. Chem.*, 44 (1966) 635–636.

KOSTYUCHENKO, V. M., GERSHENOVICH, A. I. AND TKACHEVA, L. A.: (Composition of products from chlorination of *n*-butenes). *Zh. Prikl. Khim.*, 60 (1967) 238–240.

6. ALCOHOLS

VITTE, G. AND ROQUEBERT, J.: Variations in the blood level of different aliphatic alcohols by gas chromatography. *Bull. Soc. Pharm. Bordeaux*, 103 (1964) 171–176.

WOOD, R. AND SNYDER, F.: Gas–liquid chromatographic analysis of isomeric glycerol ethers. *J. Am. Oil. Chemists' Soc.*, 42, No. 8 (1965) 456A; Abstr. No. 63.

7. PHENOLS

NELSON, F. F. AND SMITH, J. G.: The gas chromatography and infrared spectroscopy of trimethylsilyl ethers of some phenols related to lignin and ellagitannins. *Tappi*, 49 (1966) 215–218.

9. OXO COMPOUNDS

TSUGE, M. AND TANAKA, S.: (The determination of the mean degree of paraformaldehyde by gas chromatography and titration method). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 68 (1965) 477–480.

YAMAO, M.: Behavior of formaldehyde on various polyethyleneglycol columns in gas chromatography. *Japan Analyst*, 14 (1965) 926–930.

10. CARBOHYDRATES

LUDLOW, C. J., HARRIS, T. M. AND WOLF, F. T.: An analysis of the free sugars of ferns by gas liquid chromatography. *Phytochemistry*, 5 (1966) 251–257.

MARINELLI, L. AND WHITNEY, D. J.: Gas liquid chromatography of wort and beer carbohydrates. I. The characterization of wort, beer, and brewing syrup carbohydrates. *J. Inst. Brewing*, 72 (1966) 252–256.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

CHRISTIE, W. W., BRUGGER, W. E. AND HOLMAN, R. T.: Gas chromatographic characterization of cyclopropane fatty acid esters. *J. Am. Oil Chemists' Soc.*, 42 (1965) 449A.

GROMOVA, V. V., PROSKURYAKOV, V. A. AND YAKOVLEV, V. I.: (Determination of dicarboxylic acids by lithiumaluminium hydride). *Zh. Prikl. Khim.*, 60 (1967) 114–122 — retention data of acetates of C_3–C_{10} diols on PEGS at 190°.

HOFFMAN, N. E. AND CONIGLIARO, P. J.: Gas chromatographic analysis of α-hydroxycarboxylic acids. *Develop. Appl. Spectry.*, 4 (1965) 299–312.

YUNOKI, H. AND TSUTAMURA, CH.: Gas chromatography of lower unesterified fatty acids. *Hiroshima J. Med. Sci.*, 13 (1964) 323–328.

11b. Lipids and their constituents

BIGONI, G.: (Observations on the gas chromatographic analysis of fats and oils). *Riv. Ital. Sostanze Grasse*, 42 (1965) 180–183.

CATTANEO, C., DERITIS, G. C., LUCCHESI, M., ROSSI, P. AND FERRARI, S.: (Gas chromatographic analysis of fatty acids (C_{11}–C_{20}) present in mycobacteria). *Ann. Ist. "C. Forlanini"*, 25 (1965) 349–386.

CHIARLO, B.: (Investigation of the *Laurus nobilis* of Liguria. I. Gas chromatographic determination of total fatty acids in drupes). *Riv. Ital. Sostanze Grasse*, 43 (1966) 65–66.

CHUNG, A. E.: Metabolism of cyclopropane fatty acids. Oxidation of *cis*-9,10-methylene hexadecanoic and *cis*-9,10-methylene octadecanoic acids by rat liver mitochondria. *Biochim. Biophys. Acta*, 116 (1966) 205–213.

DUTTON, H. J.: Some techniques of radioactive gas chromatography for lipid research. In S. ROTHCHILD (Editor): *Advances in Tracer Methodology*, Vol. 2, Plenum Press, New York, 1965, pp. 123–134.

HEINTZ, M., GREGOIRE, J. AND LEFORT, D.: (Gas liquid chromatography and lipochemistry. XI. Contribution to the study of fatty acids composition of *Sideroxylon argania*, *Balanites aegyptiaca* and *Parinarium macrophyllum* oils). *Oleagineux*, 20 (1965) 603–608.

KISHIMOTO, Y. AND RADIN, N. S.: Determination of brain gangliosides by determination of ganglioside stearic acid. *J. Lipid Res.*, 7 (1966) 141–145.

LEE, S. AND PUTTNAM, N. A.: Gas chromatographic determination of chain-length distribution in fatty acid ethanolamides. *J. Am. Oil Chemists' Soc.*, 42 (1965) 744.

MARUYAMA, M.: (Gas chromatography of fatty acids). *J. Japan Oil Chemists Soc.*, 15 (1966) 89–93.

NODA, M. AND IKEGAMI, R.: (Lipids of leaves and seeds. II. Chromatographic separation of the lipids in rice bran lipoprotein). *Agr. Biol. Chem. (Tokyo)*, 30 (1966) 330–337.

WOOD, R. D.: Lipid metabolism and analysis. I. Cyclopropane fatty acid metabolism. II. Gas liquid chromatographic analysis of lipid alcohols. *Dissertation Abstr.*, 26 (1965) 1333–1334.

13. STEROIDS

LURIE, A. O., VILLEE, C. A. AND REID, D. E.: Progesterone in the blood. A quantitative method employing gas liquid chromatography. *J. Clin. Endocrinol. Metab.*, 26 (1966) 742–749.

MUSSER, B. O. AND CAWLEY, L. P.: Application of gas–liquid and thin layer chromatography for analysis of urinary steroids. *Am. J. Clin. Pathol.*, 44 (1965) 580.

PAPADIA, S., ROS, A., MISURALE, F. AND STORACE, A.: Gas chromatographic fractionation of total urinary 17-ketosteroids. *Monit. Ostet. Ginecol.*, 36 (1965) 333–341.

PATTI, A. A., FRAWLEY, T. F. AND STEIN, A. A.: Analysis of 17-hydroxy-corticoids in urine and blood by gas–liquid chromatography. *Am. J. Clin. Pathol.*, 45 (1966) 142–147.

15. TERPENE DERIVATIVES

BLIGHT, M. M. AND MCDONALD, I. R. C.: Sample reproducibility in pinus essential oil studies *New Zealand J. Sci.*, 7 (1964) 212–220.

BLUMER, M. AND SNYDER, W. D.: Terpenoid hydrocarbons in recent sediments: Presence of pristane and probable absence of phytane. *Science*, 150 (1965) 1588–1589.

BRIESKORN, C. H. AND DALFERTH, S.: Gas chromatography of the volatiole oils of *Salvia officinalis*, *S. lavandulaefolie* and *S. triloba*. *Deut. Apotheker-Ztg.*, 104 (1964) 1388–1392.

HEINZ, D. E., SEVENANTS, M. R. AND JENNINGS, W. G.: Preparation of fruit essences for gas chromatography. *J. Food Sci.*, 31 (1966) 63–68.

MACLEOD, Jr., W. D., MCFADDEN, W. H. AND BUIGUES, N. M.: Lemon oil analysis. II. Gas liquid chromatography on a temperature programmed, long, open tubular column. *J. Food Sci.*, 31 (1966) 591–594.

MINYARD, J. P., TUMLINSON, J. H., HEDIN, P. A. AND THOMPSON, A. C.: Constituents of the cotton bud. Terpene hydrocarbons. *J. Agr. Food Chem.*, 13 (1965) 599–602.

MINYARD, J. P., TUMLINSON, J. H., THOMAS, A. C. AND HEDIN, P. A.: Constituents of the cotton bud sesquiterpene hydrocarbons. *J. Agr. Food Chem.*, 14 (1966) 332–335.

MITZNER, B. M., MANCINI, V. J. AND LEMBERG, S.: α-Nerol and α-geraniol. *Can. J. Chem.*, 44 (1966) 2103–2105.

NIGAM, M. C., HANDA, K. L. AND GUPTA, G. N.: Gas chromatographic examination of *Ocinium kilimandscharicum*. *Perfumery Essent. Oil Record*, 56 (1965) 654–655.

RODIN, J. O., COULSON, D. M., SILVERSTEIN, R. M. AND LEEPER, R. W.: Volatile flavor and aroma components of pineapple. III. The sulfur-containing components. *J. Food Sci.*, 31 (1966) 721–725.

RUDLOFF, E. VON AND HEFENDEHL, F. W.: Gas–liquid chromatography of terpenes. XV. The volatile oil from *Mentha arvensis* var. *glabrata* Ray. *Can. J. Chem.*, 44 (1966) 2015–2022.

TAKEO, T.: Studies on the flavor of tea made from stored tea leaves at low temperature. Part. I. Examination of the essential oil of the stored tea leaves by gas liquid chromatography. *Agr. Biol. Chem. (Tokyo)*, 29 (1965) 269–274.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BULLARD, R. W.: Determination of translocated tetramine in foliage by hydrogen flame gas chromatography. *J. Agr. Food Chem.*, 14 (1966) 137–139.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

MORRIS, C. J. O. R.: Separation of peptides. *Brit. Med. Bull.*, 22 (1966) 168–173.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

FRAVOLINI, A. AND BEGLIOMINI, A. J.: Gas chromatographic determination of thiouracils in feeds. *J. Assoc. Offic. Agr. Chemists*, 48 (1965) 908–911.

26. ORGANOMETALLIC COMPOUNDS

MYAZAKI, M., IMANARI, T., KUNUGI, T. AND TAMURA, Z.: (Gas chromatography of copper(II) and nickel(II) chelates of some β-ketoimine derivatives of 2,4-pentanedione and salicylaldehyde). *Chem. Pharm. Bull. (Tokyo)*, 14 (1966) 117–120.

TACKETT, J. E.: I. Properties and infrared spectra of several metal chelates. II. Analytical gas chromatography of metal chelates. *Dissertation Abstr.*, 26 (1965) 3011–3012.

29. INSECTICIDES AND OTHER PESTICIDES

BEVENUE, A. AND BECKMAN, H.: The examination of toxaphene by gas chromatography. *Environ. Contam. Toxicol.*, 1 (1966) 1–5.

LUDWIG, G. AND KORTE, F.: Metabolism of insecticides. X. Detection of dieldrin metabolite by GLC analysis. *Life Sci.*, 4 (1965) 2027–2031.

STANLEY, CH. W.: Derivatization of pesticide-related acids and phenols for gas chromatographic determination. *J. Agr. Food Chem.*, 14 (1966) 321–323.

31. PLASTICS AND THEIR INTERMEDIATES

CARMICHAEL, J. B. AND WINGER, R.: Cyclic distribution in dimethylsiloxanes. *J. Polymer Sci., Part A*, 3 (1965) 971–984.

CIANETT, E. AND GROTTINI, A.: (Possibility of distinguishing polyethylene from polypropylene by gas chromatography of the pyrolyzates). *Rass. Chim.*, 17 (1965) 12–14.

HASSE, H. AND RAU, J.: Pyrolysis–gas chromatographic investigation of fibrous materials. *Melliand Textilber.*, 47 (1966) 434–436.
MOLDVAI, T. AND PETRESCU, G.: Methods of qualitative analysis for identifying synthetic rubbers in rubber mixtures. *Ind. Usoara (Bucharest)*, 12 (1965) 15–24.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

BRECHNER, V. L. AND BETHUNE, R. W. M.: Determination of acetone concentration in arterial blood by vapor phase chromatography of alveolar gas. *Diabetes*, 14 (1965) 663–665.
FRAM, D. H. AND GREEN, J. P.: Presence and measurement of methylhistamine in urine. *J. Biol. Chem.*, 240 (1965) 2036–2042.
LODI, F.: Limits of the use of gas chromatography in forensic toxicology. *Minerva Medicolegale*, 84 (1964) 150–153.
MATHER, A. AND ASSIMOS, A.: Evaluation of gas–liquid chromatography in assays for blood volatiles. *Clin. Chem.*, 11 (1965) 1023–1035.
MORITA, Y.: Studies on phenazines. XXIX. Mass spectrometric and chromatographic studies. *Chem. Pharm. Bull. (Tokyo)*, 14 (1966) 426–432.
NANIKAWA, R. AND KATOKU, S.: (Separation and identification of hypnotics and their detection from biological materials by gas chromatography). *Yonago Acta Med.*, 10 (1966) 49–55.
PRUCKMAYER, G. AND SANDERS, R. D.: The preparation of liquid halothane standards for gas chromatography. *Delaware Med. J.*, 38 (1966) 193–198.
STAVINOHA, W. B. AND RYAN, L. C.: Estimation of the acetylcholine content of rat brain by gas chromatography. *J. Pharmacol. Exptl. Therap.*, 150 (1965) 231–235.
SUNSHINE, I., WHITWAM, J. G., FIKE, W. W., FINKLE, B. AND LEBEAU, J.: Distribution and excretion of methohexitone in man. A study using gas chromatography and thin layer chromatography. *Brit. J. Anaesthesia*, 38 (1966) 23–28.
TAKESHITA, H.: (Basic considerations of gas chromatography for application in the anaesthesiological field). *Sapporo Med. J.*, 20 (1961) 1–11.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

CLARKE, S. A.: Chromatographic separation of gaseous mixtures. *U.S. Pat.*, 3,250,057 (1966).
EVENS, F. M. AND FASSEL, V. A.: Determination of gases in metals. *U.S. Pat.*, 3,251,217 (May 17, 1966).
TOLIVER, W. H. AND MORRIS, M. L.: Chemical analysis of permanent and organic gases in a 30-day manned experiment. *Aerospace Med.*, 37 (1966) 233–238.

33b. Volatile inorganic compounds

KONDO, T.: A gas chromatographic study of uranium oxidation mechanism in moist gases. *Dissertation Abstr.*, 26 (1966) 6633.
TURINA, S.: The possibility of the application of gaseous chromatography for the determination of sulphur. *Bull. Sci. Conseil Acad. RPF Yugoslavia*, 8 (1963) 86–87 (Abstr. of Dissertation, Fac. Technol., Univ. of Zagreb, Jan. 28th, 1960).

34. RADIOACTIVE AND ISOTOPIC COMPOUNDS

ELIAS, H.: Labeling of organic halides in gas chromatography columns. *Nucl. Sci. Abstr.*, 19 (1965) Abstr. No. 32274.
SOUTAS, D. S.: Isotope fractionation of methyl esters by gas–liquid chromatography. *Nature*, 211 (1966) 296–297.
STOECKLIN, G.: Halogen- and tritium-labeling by heterogeneous exchange reactions on gas chromatographic columns. *Nucl. Sci. Abstr.*, 19, No. 17 (1965) Abstr. No. 32275.
SWELL, L.: Gas–liquid radiochromatography of intact labeled cholesterol esters. *Proc. Soc. Exptl. Biol. Med.*, 121 (1966) 1290–1294.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

BREKKE, J. AND CONRAD, R.: Gas–liquid chromatography and vacuum oven determination of moisture in fruits and fruit products. *J. Agr. Food Chem.*, 13 (1965) 591–593.
CALZOLARI, C., CERMA, E. AND BRUNI, G.: (Application of gas chromatographic methods for the analysis of constituents in edible products). *Rass. Chim.*, 17 (1965) 18–29.
FISHER, G. E. AND NEERMAN, J. C.: Identification of brake lining constituents by pyrolysis gas chromatography. *Ind. Eng. Chem. Prod. Res. Develop.*, 5 (1966) 288–292.
FREDERICKS, A. D. AND HOOD, D. W.: A method for the determination of dissolved organic carbon in sea water by gas chromatography. *U.S. Govt. Res. Rept.*, 40 (1965) 73.

GIANTURCO, M. A., GIAMMARINO, A. S. AND FRIEDEL, P.: Volatile constituents of coffee. V. *Nature*, 210 (1966) 1358.

HENIS, Y., GOULD, J. R. AND ALEXANDER, M.: Detection and identification of bacteria by gas chromatography. *Appl. Microbiol.*, 14 (1966) 513–524.

MARTIN, G. E., SCHMIT, J. A. AND SCHOENEMAN, R. L.: Various components of bourbon whisky by thin layer and gas liquid chromatography. *J. Assoc. Offic. Agr. Chemists*, 48 (1965) 962–964.

NEUDOERFFER, T. S., SANDLER, S., ZUBECKIS, E. AND SMITH, M. D.: Detection of an undesirable anomaly in concord grape by gas chromatography. *J. Agr. Food Chem.*, 13 (1965) 584–588.

POWELL, A. D. G. AND BROWN, I. H.: The determination of beer components by gas liquid chromatography. *J. Inst. Brewing*, 72 (1966) 261–265.

ROMANI, R. J. AND KU, L.(LIM): Direct gas chromatographic analysis of volatiles produced by ripening pears. *J. Food Sci.*, 31 (1966) 558–560.

SUEMITSU, R., FUJITA, S. AND MATSUBARA, H.: Studies on cow's urine. Part. III. Examination of an oil obtained from cow's urine by gas chromatography. *Agr. Biol. Chem. (Tokyo)*, 29 (1965) 908–911.

JOURNAL OF CHROMATOGRAPHY VOLS. 1 (1958)-10 (1963)

R_F VALUE INDEX OF LIQUID–LIQUID AND LIQUID–SOLID CHROMATOGRAPHY

INTRODUCTION

The aim of this section of the *Journal of Chromatography* is to acquaint readers with those compounds for which an R_F value (absolute or relative) can be found within the numbers of the journal.

Thus for each compound listed, the volume number (in parentheses) and page will give the reference to where the R_F value may be found. The Chromatographic Data Sections of the *Journal of Chromatography* are also included in these references, and are characterized by the letter D in front of the page number, except in the case of Vol. 1 where the Data Section is paginated with Roman numerals. The references start with the initial volume of the *Journal of Chromatography* and will be brought up-to-date as quickly as possible.

On the basis of experience gained so far it has proved impossible to adopt a completely standardised nomenclature for naming of compounds because of the tremendous variation in nomenclature which different authors have given to the same compound. However, in such cases we have tried to use the most popular name as found within the *Journal of Chromatography*, and cross-references to other less popular names are given. Because of time considerations it has only proved possible to include those compounds for which an R_F value is given either in paper chromatography, thin-film chromatography, or electrophoretic patterns, and thus R_F values for ion-exchange columns are excluded.

G. NICKLESS

JOURNAL OF CHROMATOGRAPHY VOLS. 1 (1958)–10 (1963)

R_F VALUE INDEX OF LIQUID–LIQUID AND LIQUID–SOLID CHROMATOGRAPHY

JOURNAL OF CHROMATOGRAPHY VOL. 29 (1967)

AUTHOR INDEX

JOURNAL OF CHROMATOGRAPHY VOL. 29 (1967)

SUBJECT INDEX

CHROMATOGRAPHIC DATA

VOL. 29 (1967)

CHROMATOGRAPHIC DATA

SUPPLEMENT TO THE
JOURNAL OF CHROMATOGRAPHY
VOL. 29 (1967)

EDITORS:

C. G. CASINOVI (Rome)
J. GASPARIČ (Pardubice-Rybitví)
M. LEDERER (Rome)
K. MACEK (Prague)

ELSEVIER PUBLISHING COMPANY
AMSTERDAM

PRINTED IN THE NETHERLANDS

TABLE 1

TLC R_F VALUES OF SOME PHOSPHORIC ACID ESTERS

(E. BANCHER AND J. WASHÜTTL, *Mikrochim. Acta*, (1967) 225).

Thin layer: Kieselgel H (Merck) + 0.5% sodium acetate + 0.5% tylose.
Solvents: S_1 = Ethyl acetate–acetic acid–water–ammonia (6:6:2:1).
S_2 = Isopropanol–ammonia–water–acetic acid (5:3:1:1).
Detection: Ammonium molybdate–perchloric acid reagent according to C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.

Compound	R_F	
	S_1	S_2
3-Phosphoglyceric acid	0.20	0.18
Glycerol-α-phosphate	0.40	0.21
Glycerol-β-phosphate	0.36	0.26
Fructose-6-phosphate	0.33	0.10
Fructose-1-phosphate	0.26	0.19
Fructose-1,6-diphosphate	0.14	0.06
Glucose-1-phosphate	0.26	0.19
Glucose-6-phosphate	0.26	0.13
Phosphoenolpyruvic acid	0.33	0.19
Phosphoric acid	0.39	0.06

TABLE 2

TLC R_F VALUES OF SOME PESTICIDES

(H. BECKMAN AND W. WINTERLIN, *Bull. Environ. Contam. Toxicol.*, 1 (1966) 78–85; *C.A.*, 65 (1966) 7894e).

Thin layer: Silica Gel H.
Solvent: 10% Hexane in toluene.
Detection: Not stated in *C.A.*

Compound	R_F
Systox	0.44
Parathion	0.53
Guthion	0.04
Thimet	0.73
Imidan	0.09
Trithion	0.88

TABLE 3

TLC R_F VALUES OF SOME ORGANIC PEROXIDES

(E. C. J. COFFEE AND A. G. DAVIES, *J. Chem. Soc.*, C 17 (1966) 1493)

Thin layer: Kieselgel G.
Solvent: Chloroform–ether (3:1).

Compound	R_F
Hydrogen peroxide	0.13
tert-Butyl hydroperoxide	0.62
sec.-Butyl hydroperoxide	0.66
1-Methyl-1-phenylethyl hydroperoxide	0.72
1,2,3,4-Tetrahydro-1-naphthyl hydroperoxide	0.72
1-Phenylethyl hydroperoxide	0.76
1-Methyl-1-phenylpropyl hydroperoxide	0.78
Decahydro-9-naphthyl hydroperoxide	0.79
tert.-Butyl peroxyacetate	0.51
tert.-Butyl peroxybenzoate	0.89
Benzoyl peroxide	0.92
1-Methyl-1-phenylethyl xanthydryl peroxide	0.97

TABLE 4

TLC R_F VALUES OF SOME COMMON SOFTENERS

(J. H. RAU AND H. HAASE, *Melliand Textilber.*, (1965) 1318).

Thin layer: Silica Gel G activated for 30 min at 120°.
Solvent: Methylene chloride.
Detection: U.V. light at 366 nm after spraying with 0.5% solution of Rhodamine B in ethanol.

Compound	R_F
Didecyl phthalate	0.65
Diisooctyl phthalate	0.62
Dinonyl phthalate	0.60
Di-(2-ethylhexyl) phthalate	0.60
Dioctyl sebacate	0.58
Diisononyl phthalate	0.57
Dioctyl azelate	0.54
Benzyl butyl phthalate	0.53
Dibutyl phthalate	0.51
Tricresyl phosphate	0.51
Dinonyl adipate	0.50
Di-(2-ethylhexyl) adipate	0.50
Dioctyl adipate	0.49
Thiodibutyric acid di-(2-ethylhexyl) ester	0.48
Benzyl octyl adipate	0.48
Diphenyl cresyl phosphate	0.45
Triphenyl phosphate	0.44
Diethyl phthalate	0.38
Dimethyl phthalate	0.37
Benzenesulfonic acid N-butylamide	0.37
Diphenyl octyl phosphate	0.36
Trioctyl phosphate	0.24
Tributyl phosphate	0.12
Trichloroethyl phosphate	0.11

TABLE 5

TLC R_F VALUES OF SOME DEGRADATION PRODUCTS OF CHLORAMPHENICOL

(F. LINGENS, H. EBERHARDT AND O. OLTMANNS, *Biochim. Biophys. Acta*, 130 (1966) 346).

Thin layer: Silica Gel GF_{254} (Merck).
Solvents: S_1 = Benzene–methanol–acetic acid (45:8:4).
S_2 = *n*-Propanol–ammonia (7:3).
Detection: Nitro-aryl group = $SnCl_2$ and then *p*-dimethylaminobenzaldehyde.
Arylamino group = *p*-dimethylaminobenzaldehyde.
Carboxyl group = bromocresol green.
Aliphatic amino group = ninhydrin.
Oxo group = 2,4-dinitrophenylhydrazine.
Phenolic group = $FeCl_3$.

Compound	R_F	
	S_1	S_2
β-(4-Nitrophenyl)-serine	0.05	0.50
2-Amino-1-(4-nitrophenyl)-propanediol-1,3	0.09	0.68
2-Amino-1-oxo-1-(4-nitrophenyl)-propanol	0.13	0.77
4-Nitromandelic acid	0.31	
3-Hydroxy-4-acetylaminobenzoic acid	0.46	
N-Dichloroacetyl-β-(4-nitrophenyl)-serine	0.49	
Protocatechuic acid	0.50	
3-Hydroxy-4-aminobenzoic acid	0.55	
4-Hydroxylaminobenzoic acid	0.56	
Chloramphenicol	0.58	
3-Acetylprotocatechuic acid	0.61	
4-Aminobenzoic acid	0.73	
4-Nitrobenzyl alcohol	0.81	
2-Dichloroacetylamino-1-oxo-1-(4-nitrophenyl)-propanol	0.83	
4-Nitrobenzoic acid	0.85	
4-Nitrosobenzoic acid	0.85	
4-Nitromandelic acid methyl ester	0.87	
4-Nitrobenzaldehyde	0.92	

TABLE 6

TLC R_F VALUES OF SOME SULPHONAMIDES

(J. L. KIGER AND J. G. KIGER, *Ann. Pharm. Franc.*, 24 (1966) 599).

Thin-layer: Silica Gel G.
Solvents: S_1 = Chloroform–methanol (10:1).
S_2 = Chloroform–butanol–acetone–85% formic acid (4:1:1:1).
S_3 = Chloroform–methanol–butanol–2% ammonia (80:10:10:1).
Detection: Iodine atmosphere.

Sulfonamide	R_F		
	S_1	S_2	S_3
Acesulfamethoxypyridazine	0.75 (0.50)*	0.80 (0.60)	0.92
Acesulfamethoxypyrazine	0.72	0.75	0.90
Acetazolamide	**	0.45	**
Altizide	0.40	0.75	0.90
Buthiazide	0.35	0.75	0.80
Clopamide	0.55	0.42	0.80
Chlorexolone	0.75	0.87	0.90
Desaglybuzole	0.70**	0.95	0.70**
Dichlorphenamide	0.35**	0.70	**
Glybutamide	0.30	0.82	0.48
Glybuthiazole	0.40	0.70	0.45
Glyprothiazole	0.40	0.70	0.35
Glysobuzole	0.55	0.95	0.70
Maleylsulfathiazole	no migration	0.60	no migration
Methyclothiazide	0.40	0.80	0.77
Monochlorphenamide	0.25	0.58**	0.35
Phthalylsulfamethizole	no migration	0.78	no migration
Phthalylsulfathiazole	0.30 and no migration	0.55	0.50 and no migration
Salazosulfapyridine	no migration	0.80 (0.35)	no migration
Succinylsulfathiazole	no migration	0.40	no migration
Sulfacetamide	0.25	0.60 (0.10)	0.30 and no migration
Sulfachrysoidine	no migration	0.20	no migration
Sulfadiazine	0.30	0.60	0.42
Sulfadicramide	0.50	0.80	0.65
Sulfadimethoxine	0.50	0.85	0.80
Sulfadimidine	0.45	0.60	0.75
Sulfafurazole	0.40	0.70	0.40
Sulfaguanidine	0.10	0.20	0.20
Sulfamerazine	0.40	0.65	0.70
Sulfamethizole	0.40	0.60	0.25
Sulfamethoxazole	0.50	0.75	0.75
Sulfamethoxypyridazine	0.55	0.58	0.85
Sulfamethoxypyrazine	0.70	0.75	0.75
Sulfamethopyrazine	0.70	0.72	0.90
Sulfametoyl	0.40**	0.90	0.50
Sulfamidochrysoidine	no migration	no migration	no migration
Sulfanilamide	0.30	0.35	0.50
Sulfapyridine	0.40	0.50	0.70
Sulfasomizol	0.40	0.75	0.45
Sulfasuccinamide	no migration	no migration	no migration
Sulfathiazole	0.33	0.42	0.45
Sulfathiourea	0.25	0.77	0.20
Sulfisomidine	0.30	0.25	0.40
Sulforthomidine	0.75	0.80	0.78

* Values in parentheses refer to impurities.
** R_F values are inaccurate; tailing.

TABLE 7

TLC R_F VALUES OF SOME ANTIPYRETICS

(A. TSUJI AND A. WADA, *Eisei Shikensho Hokoku*, No. 82 (1964) 73–75; *C.A.*, 65 (1966) 8668g).

Thin layer: Kieselgel G.
Solvents: S_1 = Ether.
S_2 = Ethyl acetate–ether (4:1).
Detection: Not stated in *C.A.*

Compound	R_F	
	S_1	S_2
Isopropylantipyrine	0.59	0.72
Acetanilide	0.44	0.66
Ethoxybenzamide	0.32	0.57
Phenacetin	0.25	0.57
N-Acetyl-*p*-aminophenol	0.16	0.48
Aminopyrine	0.16	0.21
Caffeine	0.04	0.13
Antipyrine	0.03	0.09
Sulpyrin	0	0
Aminopropiron	0.02	0.03
Neophyllin M	0.30	0.03
Noscapine	0.49	0.71
Barbital	0.88	0.91
Allylisopropylacetylurea	0.61	0.87
Bromovalerylurea	0.61	0.85
Ethylquinine carbonate	0.09	—
Aspirin	0	0.26

TABLE 8

TLC R_F VALUES OF SOME ANALGETIC DRUG COMBINATIONS

(H. KUTLU, *Istanbul Univ. Eczacilik Fak. Mecmuasi*, 1 (1965) 128–136; *C.A.*, 65 (1966) 8676f).

Thin layer: Silica gel.
Solvent: Chloroform–acetone–water (2:9:0.5).
Detection: Spraying with 16% sulphuric acid and bromine gives yellow spots of different colour intensity.

Compound	R_F
Antipyrine	0.50
4-Dimethylaminoantipyrine	0.64
Novaminsulfone	0
Caffeine	0.05
N-Acetyl-*p*-phenetidine	0.70

TABLE 9

TLC R_F VALUES OF THIAMINE PHOSPHATES

(T. ONO AND M. HARA, *Bitamin*, 33 (1966) 512–515; *C.A.*, 65 (1966) 4239g).

Thin layer: Silica Gel G.
Solvent: Pyridine–water–ammonia–methanol–acetic acid (6:6:5:1:1).
Detection: Thiochrome fluorescence method using a mixture of ethanol–10% sodium hydroxide–2.5% potassium ferricyanide (2:1:0.05).

Compound	R_F
Thiamine	0.92
Thiamine monophosphate	0.50
Thiamine diphosphate	0.17
Thiamine triphosphate	0.08

TABLE 10

TLC R_F VALUES OF SOME *Cinchona* ALKALOIDS

(F. WARTMANN-HAFNER, *Pharm. Acta Helv.*, 41 (1966) 414).

Thin layer: Silica Gel G.
Solvent: Benzene–ether–diethylamine (20:15:5).
Detection: D_1 = U.V. light (360 nm).
D_2 = Potassium iodoplatinate.

Alkaloid	R_F	D_1	D_2
Quinamine	0.99	light violet	violet
Quinicine	0.35	violet	violet
Quinidine	0.44	blue	violet
Quinine	0.25	blue	violet
Cinchonidine	0.41	light violet	dark blue
Cinchonine	0.54	light violet	violet-blue
Hydroquinidine	0.35	blue	violet
Hydrocinchonidine	0.35	violet	grey-green
Hydrocinchonine	0.49	violet	violet

TABLE 11

TLC R_F VALUES OF SOME TROPANE ALKALOIDS

(K. C. GUVEN AND A. HINCAL, *Instanbul Univ. Eczacilik Fak. Mecmuasi*, 1 (1965) 153–156; *C.A.*, 65 (1966) 8668d).

Thin layer: Aluminium oxide.
Solvent: Ethanol–pyridine–water (10:60:40).
Detection: Potassium iodoplatinate.

Compound	R_F
Atropine	0.89
Homatropine	0.87
Methylhomatropine	0.84
Tropine	0.43
Methyltropine	0.38

TABLE 12

ELECTROPHORETIC MOBILITIES OF SOME ALKALOIDS

(A. M. EFIMENKO, *Farmatsevt. Zh. (Kiev)*, 21 (1966) 32–34; *C.A.*, 65 (1966) 2065a).

Electrolyte: Veronal buffer pH 8.6, ionic strength 0.05.
Potential: 5–7 V/cm; 0.1–0.15 mA/cm.
Time: 2–3 h.
Detection: The paper is dried at 100°, treated with a solution of 4 g potassium iodide and 2 g iodine in 100 ml water and then with a mixture of 0.1 g bromophenol blue, 50 g zinc sulphate and 50 g acetic acid and diluted with water to 1 l.

Alkaloid	*Mobility (cm)*
Atropine	14.0
Scopolamine	10.3
Platiphylline	13.0 and 11.5
Pachycarpine	15.5
Pilocarpine	8.8
Morphine	9.7
Papaverine	7.2, 4.3 and 0
Quinine	8.6 and 5.6
Strychnine	9.4
Physostigmine	11.0

CHROMATOGRAPHIC DATA VOL. 29 (1967)

AUTHOR INDEX

CHROMATOGRAPHIC DATA VOL. 29 (1967)

SUBJECT INDEX