JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

VOL. 22 1966

ELSEVIER PUBLISHING COMPANY

AMSTERDAM

PRINTED IN THE NETHERLANDS

CENTRIFUGAL CHROMATOGRAPHY ON PLASTER OF PARIS

A. AFFONSO
Goa College of Pharmacy, Goa (India)
(Received September 21st, 1965)

The use of thick and thin strips of set plaster of Paris as supporting media in ascending chromatography has been described in an earlier paper[1]. Since ascending chromatography is a slow process limited by the capillary forces causing the ascension, it was decided to investigate the possibility of accelerating the separation, using either a forced positive pressure flow or causing an increased flow rate by the use of centrifugal force. The results of the centrifugal method are reported here.

Centrifugal chromatography was used by McDONALD *et al.*[2] on filter paper discs, sandwiched between plastic discs shaped as saucers. The discs and the paper were rotated at 300 to 1,000 r.p.m., solvent being fed to a point near the center of the filter paper disc. The sample mixture to be separated, was applied at a point a little off-center. The drawbacks of this method are sputtering of the separated bands and also diffuse bands. However, the method is fast and it is possible to make a rapid analysis of comparatively simple mixtures of water soluble compounds[3]. The limitation is that the use of filter paper does not permit the application of sizable amounts of sample mixture and, when volatile solvents are used, evaporation of these interferes with the separation. In contrast to filter paper, a set layer of plaster of Paris does not suffer from these limitations. Apparatuses using the centrifugal force for chromatographic separation have also been adapted for industrial purposes. The chromatofuge described by HOPF *et al.*[4,5] is a centrifuge packed with adsorbent. This paper describes the use of discs of set plaster of Paris in centrifugal chromatography. The sample mixture is applied in a circle away from the center of the disc. The disc is rotated at speeds ranging from 500 to 3,000 r.p.m. and solvent is added at a controlled rate at a point slightly off-center. The circular bands of the separated constituents move towards the periphery, leave the disc due to centrifugal force, and are collected in a special fraction collector. To illustrate the efficiency of the method, separation of two dyes and two alkaloids has been tried and achieved in a matter of minutes.

EXPERIMENTAL

Apparatus and methods

The experimental set up is shown in Fig. 1. The flame proof motor (A) has a speed regulator so that speeds ranging from 500 to 3,000 r.p.m. can be obtained. The fraction collector (H) is a cylinder made of translucent, hard polyethylene. On the inner side of the cylinder 12 closely fitting rings, G_1, G_2, . . . G_{12}, of hard polyethylene having a deep groove on one side were fixed at a slant of 25° to the horizontal. In this

way 12 circular, inclined collection troughs were obtained. At the lowest point of the rings, a hole was made so that pieces of hard polyethylene tubing, E_1, E_2, ... E_{12}, could be fitted thus making delivery tubes for the liquid that would be collected in the troughs. The slant of the rings makes complete drainage of the liquid collected into 12 small test tubes possible.

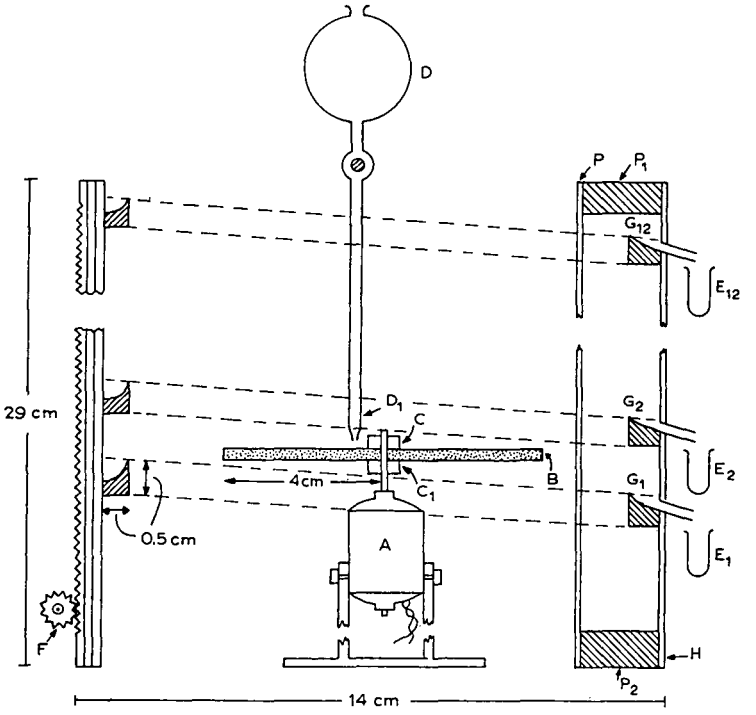


Fig. 1. Set-up for centrifugal chromatography.

The fraction collector was mounted in a wheel and ratchet arrangement (F) so that each collection trough could be brought in line with the set plaster of Paris disc B. The plaster disc was fixed directly to the motor spindle by sandwiching it between two pieces CC_1 of tightly fitting thick pressure rubber or polyethylene tubing. The hole in the disc should fit snugly the motor spindle.

On one side of the cylinder H, two rests P_1 and P_2 were fixed so that a 2 cm broad strip of set plaster of Paris could be fixed with tape. The purpose of the strip was to serve as a pilot for the separation. Since it moves with cylinder H, as the separated fractions leave the disc and are collected in the troughs, a part of each of them impinges on the strip leaving thus a print. When the strip is removed and developed with iodine or Dragendorff reagent, in case of alkaloids, it is possible to fix exactly in which test tube the different constituents have been collected.

The solvent is fed from a separating funnel D having at its end a glass tube D_1 drawn to a point. The solvent could therefore be added in a regulated rate at a place slightly off the center.

Preparation of set plaster of Paris discs and strips

50 g of plaster of Paris manufactured by A.D.T.D. Ltd. England (Calspar Brand) is made into a paste by the addition of 40 c.c. of distilled water. Air bubbles are removed by means of a vibrator and a 3 mm thick layer of set plaster of Paris was obtained by allowing the plaster to set between two 25 cm × 25 cm glass plates separated from each other by 3 mm thick glass strips placed on opposite sides[1]. A circle of 4 cm radius was etched on the dried plate by rotating a sharp pointed divider several times. The depth of the groove should be about ½ mm. A square containing the etched circle was broken off from the original plate in the same way as described previously[1]. A perfectly round disc is obtained by breaking carefully, piece by piece, the plaster along the circular groove. More discs can be obtained in this way from the remaining original plate. Any irregularities were filed off. With the help of a drill bit ½ cm diameter, which was equal to the diameter of the motor spindle, a circular hole was made exactly in the centre of the disc. The disc was now ready to be mounted on the motor spindle as described above. The strip of plaster P (Fig. 1) was cut in the same way as described previously[1]. It was 2 cm broad and 20 cm long.

Preparation of sample mixtures

In order to test the effectiveness of the method, the mixtures used were: (a) Mixture of eosin and malachite green 2 % in acetic acid. (b) Veratrine and atropine hydrochlorides 400 mg/c.c. in 5 % dilute HCl.

Application of sample mixtures

The sample mixtures, 0.05 c.c., were applied to each disc with the aid of the applicator, described previously[1], by rotating the disc at 120 r.p.m. and touching the applicator at a point 1.7 cm away from the centre. In this way a perfectly uniform circular deposition line was obtained. The discs were dried.

Solvent

For the separation of eosin and malachite green, 30 % acetic acid was used. For the separation of atropine and veratrine, the solvent mixture was: benzene–isopropyl alcohol–conc. HCl (15 c.c.:2 c.c.:0.05 c.c.).

Depending on the hydrochlorides of the alkaloids to be separated, the amount of isopropyl alcohol has to be adjusted.

Adjustment of solvent rate

The success of the separation depends on the correct rate of addition of the solvent. In order to fix the solvent addition rate, a blank experiment is carried out in the following way. An identical dry disc of set plaster of Paris is fixed in the centre to a vertical support and the solvent is added at such a rate so that it spreads evenly by capillarity over the whole disc. The surface where the solvent drops should not show excess liquid. Since the centrifugal force helps the distribution of the solvent throughout the disc and since the movement of rotation will evaporate the volatile constituents of the solvent, the rate at which the solvent has to be added in the actual working, can be 1.5 times faster. In the experiment described here, the rates were:

(a) For separation of eosin and malachite green 10 c.c. per min.

(b) For separation of atropine and veratine 15 c.c. per min.

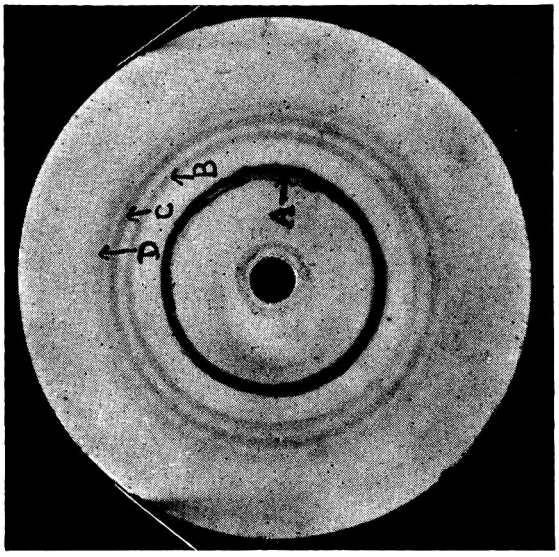


Fig. 3. A = Start; B = atropine; C = veratrine; D = front.

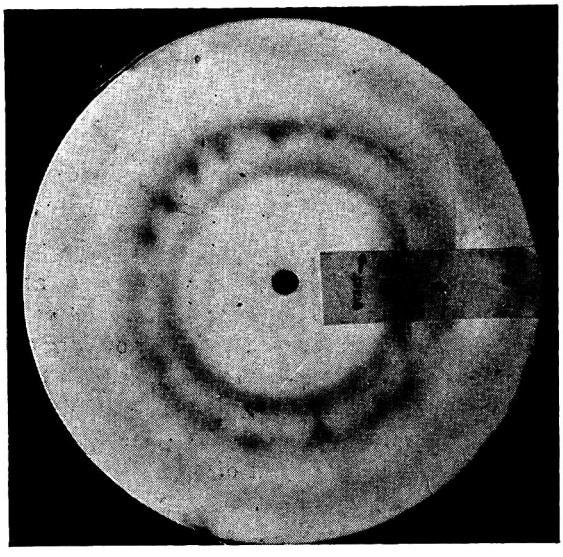


Fig. 2. A = Malachite green; B = eosin; C = eosin impurity.

Collection of separated fractions

The separated constituents in the case of alkaloids are collected in the 12 troughs. Each trough is placed in position for 1 min. In order to find out where the constituents have been collected, the strip P (Fig. 1) is developed with iodine or Dragendorff reagent. The alkaloids appear as sharp bands. By placing the developed strip in its original position, the troughs which contain each alkaloid can be easily located.

RESULTS

Fig. 2 shows a photograph of the progress of separation of eosin and malachite green. The bands are marked A, B, C. The dyes were not very pure and this explains the additional band in case of eosin. Time for separation = 1 min. Time for collection of eosin was 3 min after the start, and for malachite green 4 to 6 min after the start.

Fig. 3 shows a photograph of the progress of the separation of atropine and veratrine. Time for separation = 1½ min. Time for collection was 3 to 5 min after the start for veratrine and 7 to 8 min for atropine.

The purity of each alkaloid was established by re-chromatography on plaster strips as described previously[1].

SUMMARY

A method of centrifugal chromatography using set plaster of Paris discs is described. Solvents suitable for separating a mixture of two dyes and two alkaloids are also described. A specially constructed fraction collector is described and results show that both separation and recovery are possible within a few minutes. The method offers a rapid way to chromatographic separation and studies are in progress on its use for other types of substances and on the use of gradient elution techniques. Studies on industrial applications are also being made.

REFERENCES

1 A. AFFONSO, *J. Chromatog.*, 21 (1966) 332.
2 H. J. MCDONALD, E. W. BERMES, JR. AND H. G. SHEPHERD, JR., *Chromatog. Methods*, 2 (1957) 1.
3 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press, New York, 1958, p. 49.
4 P. P. HOPF, *Ind. Eng. Chem.*, 39 (1947) 983.
5 P. P. HOPF, C. G. LYNAM AND H. WEIL, *Brit. Pat.*, 585,224 (1947); *C.A.*, 14 (1950) 3750c.

EINE METHODE ZUR CHARAKTERISIERUNG VON GASCHROMATOGRAPHISCHEN TRENNFLÜSSIGKEITEN

L. ROHRSCHNEIDER
Untersuchungsabteilung der Chemischen Werke Hüls A.G., Marl (Deutschland)
(Eingegangen den 27. September 1965)

Die Klassifizierung von Trennflüssigkeiten ist eng mit dem Problem der Vorausberechnung von Retentionsdaten verbunden. Nur eine Beschreibung von stationären Flüssigkeiten, die eine Vorausberechnung der Retention gestattet, hat praktische Bedeutung. Voraussagen über Retentionen sind nur möglich, wenn die stationären Flüssigkeiten und die gelösten Stoffe durch eine oder mehrere Grössen so beschrieben werden, dass aus der Kombination dieser Daten Retentionszeiten errechnet werden können.

Zur Beschreibung von stationären Flüssigkeiten wurden von mehreren Autoren für homologe Reihen charakteristische Grössen angegeben. PIEROTTI und Mitarbeiter[1] geben eine Formel zur Berechnung der Aktivitätskoeffizienten für homologe Reihen an, deren empirische Konstanten experimentellen Daten entstammen. TENNEY[2] beschreibt die Selektivität von stationären Flüssigkeiten durch das Retentionsverhältnis gleichsiedender Stoffe zweier homologer Reihen. WERHLI UND KOVATS[3] schlagen zur Beschreibung von Trennflüssigkeiten die Angabe von 13 für homologe Reihen charakteristischen Grössen als "Retentionsdispersion" vor. EVANS UND SMITH[4] definieren einen für homologe Reihen charakteristischen Δ Me-Wert.

BROWN[5] klassifiziert stationäre Flüssigkeiten durch drei Grössen, die von der Elektronen-Donator- und Akzeptoreigenschaft der Trennsäule abhängen. ROHRSCHNEIDER[6,7] gibt Zahlenangaben für die "Polarität" bzw. Polaritätsfaktoren. LITTLEWOOD[8] schlägt das Lösevermögen für *n*-Alkane als umfassendes Charakteristikum für Trennflüssigkeiten vor. Eine ähnliche Grösse wird von CHOVIN[9] als "Polarität" bezeichnet.

Sieht man von der Extrapolation homologer Reihen ab, so führten Voraussagen von Retentionszeiten bisher MARTIRE[10] und ROHRSCHNEIDER[6] aus. Beide Methoden zur Vorausberechnung gelten nur für ein beschränktes Material und erfordern nur schwierig zu gewinnende Daten zur Beschreibung der stationären Flüssigkeiten.

Im folgenden soll eine einfache Methode zur Charakterisierung von stationären Flüssigkeiten beschrieben werden, die eine Voraussage der Retention erlaubt. Ihre Grundlage ist die Additivität zwischenmolekularer Kräfte, wesentlicher Bestandteil die Angabe der Retention in Indexeinheiten nach KOVATS und die Beziehung auf eine unpolare Standardsäule.

DER RETENTIONSINDEX

Der Retentionsindex von KOVATS[3,11], eine experimentell leicht zugängliche, genau bestimmbare und relativ temperaturunabhängige, logarithmische Retentionsangabe erweist sich als geeignet zur Untersuchung der Wechselwirkungen zwischen Gelöstem und stationärer Flüssigkeit. Der Retentionsindex gibt an, mit welchem *n*-Paraffin (gebrochener C-Zahl) gleichzeitig ein Stoff aus einer Trennsäule austritt.

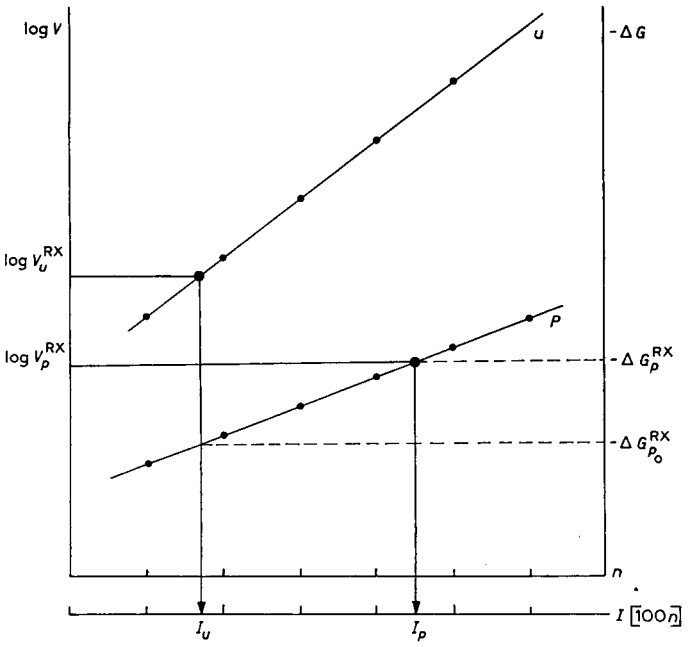


Fig. 1. Schema des Indexsystems.

In der schematischen Fig. 1 sind die Logarithmen der Retentionsvolumina des Stoffes RX sowie der *n*-Paraffine gegen die C-Zahl für eine unpolare (*u*) und eine polare (*p*) Trennsäule aufgetragen. RX sei ein Stoff mit dem Alkylrest R- und der funktionellen Gruppe -X. Die homologen *n*-Alkane mit der C-Zahl *n* bilden auf beiden Säulen Gerade oder leicht gekrümmte Kurven, die sich in guter Näherung, wenn auch nicht exakt durch die Gleichung:

$$\log V = A + Bn$$

beschreiben lassen. Der Retentionsindex I eines Stoffes ist das 100-fache der (gebrochenen) C-Zahl des Alkans mit der gleichen Retentionszeit.

Setzen wir nach JAMES, GIDDINGS UND KELLER[12]:

$$\Delta G = -RT \ln K,$$

wobei ΔG die freie Energieänderung beim Lösen eines Stoffes in der stationären Flüssigkeit unter den Analysenbedingungen ist. ΔG ist ein Mass für die Kräfte, die

auf das gelöste Molekül einwirken. Der Verteilungskoeffizient K ist mit dem spezifischen Retentionsvolumen V_g durch die Gleichung:

$$K = \frac{V_g T \rho}{273}$$ verknüpft.

(T = Säulentemperatur, ρ = Dichte der stationären Flüssigkeit).
Damit ist:

$$\log V = -\Delta G^{RX} + k_s$$

wobei k_s eine von der stationären Flüssigkeit und der Temperatur abhängige Konstante ist. Die Steigung der Geraden für die Alkane in Fig. 1 ist:

$$B = \frac{\log V^{\text{Alkan}_{n+1}} - \log V^{\text{Alkan}_n}}{100} = \frac{-(\Delta G^{n+1} - \Delta G^n)}{100}$$

Der Unterschied zwischen den Retentionsvolumen der n-Paraffine mit den C-Zahlen $n + 1$ und n wird durch die Methylengruppen $-CH_2-$ verursacht.

$$B = \frac{-\Delta G^{-CH_2-}}{100}$$

"POLARITÄT" DER TRENNFLÜSSIGKEIT

Unterscheiden wir zwischen polaren und unpolaren zwischenmolekularen Wechselwirkungen, wobei Dispersions- oder London-Kräfte als unpolar, Induktions-, Orientierungs-, Ladungsüberführungskräfte und Wasserstoffbrückenbindungen als polar gelten sollen, so lässt sich ein grosser Teil der unpolaren Kräfte durch Vergleich der gemessenen Retentionsindices mit denen auf einer "unpolaren" Standardsäule eliminieren. Eine solche unpolare stationäre Flüssigkeit ist Squalan, ein verzweigtes C_{30}-Paraffin. Auf dieser Trennflüssigkeit sollen auf das Gelöste keine polaren, sondern allein Dispersionskräfte einwirken.

Der Stoff erscheint auf Squalan mit dem Retentionsindex I_u^{RX} gleichzeitig mit dem n-Paraffin der C-Zahl i. Auf einer polaren Trennsäule würde der Stoff RX wiederum gleichzeitig mit dem n-Paraffin der C-Zahl i und deshalb mit dem Retentionsindex I_u^{RX} austreten, wenn auf den Stoff RX nur die gleiche Art und Stärke von zwischenmolekularen Kräften einwirkte, wie auf das n-Paraffin mit der C-Zahl i. Wirken jedoch ausser den unpolaren Wechselwirkungskräften, die die Retention der n-Alkane bestimmen, noch zusätzliche zwischenmolekulare Kräfte auf den Stoff RX ein, so erhöht sich der Retentionsindex auf der polaren Säule um einen Betrag ΔI^{RX} der diesen zusätzlichen Wechselwirkungsenergien entspricht:

$$I_p^{RX} = I_u^{RX} + \Delta I^{RX}$$

Die Indexdifferenz ist, wie sich aus Fig. 1 entnehmen lässt, proportional der Differenz zwischen der gesamten freien Löseenergie des Stoffes RX in der polaren

stationären Flüssigkeit ΔG_p^{RX} und dem Anteil $\Delta G_{p_0}^{RX}$ an der gesamten freien Löseenergie, der den unpolaren Wechselwirkungskräften in Squalan entspricht.

$$\Delta I^{RX} = I_p^{RX} - I_u^{RX} = \frac{100\,(\Delta G_p^{RX} - \Delta G_{p_0}^{RX})}{\Delta G_p^{-CH_2-}}$$

ADDITIVITÄT ZWISCHENMOLEKULARER KRÄFTE

Die Indexdifferenz ΔI wird durch verschiedenartige zwischenmolekulare Wechselwirkungsenergien verursacht. Nimmt man an, dass die einzelnen Wechselwirkungskräfte unabhängig voneinander sind, so ist ΔG_p^{RX} und damit ΔI additiv aus Anteilen der verschiedenen Wechselwirkungsarten zusammengesetzt:

$$\Delta G_p^{RX} = \Delta G_{p_0}^{RX} + \Delta G_{p_1}^{RX} + \Delta G_{p_2}^{RX} + \cdots + \Delta G_{p_n}^{RX}$$

$$\Delta I = \frac{100\,\Delta G_{p_1}^{RX}}{\Delta G_p^{-CH_2-}} + \frac{100\,\Delta G_{p_2}^{RX}}{\Delta G_p^{-CH_2-}} + \cdots + \frac{100\,\Delta G_{p_n}^{RX}}{\Delta G_p^{-CH_2-}}$$

Nimmt man weiter an, dass ähnlich wie bei den Orientierungskräften und wie von PULLIN UND WERNER[13] bei der Wasserstoffbrückenbindung beobachtet, für jeden Typ polarer intermolekularer Wechselwirkung die Stärke der Wechselwirkungsenergien proportional ist einer stoffcharakteristischen Grösse A_i und einer Grösse X_i, die für die Trennflüssigkeit charakteristisch ist,

$$\Delta G_{p_i}^{RX} = A_i \cdot X_i$$

so muss auch ΔI aus mehreren Produkten $(a_i \cdot x_i)$ zusammengesetzt sein:

$$\Delta I^{RX} = \Sigma\,(a_i \cdot x_i)$$

Bei dem Versuch, Indexdifferenzen von 13 Stoffen auf 20 Trennflüssigkeiten durch eine derartige Formel zu beschreiben[6], benutzten wir drei Produkte von Polaritätsfaktoren:

$$\Delta I = ax + by + cz$$

Aus dieser Untersuchung ergab sich, dass ausser den zwischenmolekularen Kräften, die z.B. Benzol stärker festhalten als die Alkane, ein gesonderter Faktor für die Alkohole (z.B. Äthanol) und die Karbonylverbindungen (z.B. Methyläthylketon) berücksichtigt werden muss. In Fortführung dieser Arbeiten[14] zeigte sich, dass das Retentionsverhalten von Nitromethan, Chloroform und Phenylacetylen sich nicht vollständig durch die Kräfte beschreiben lässt, die die Retention von Benzol, Äthanol und Methyläthylketon verursachen und eine vierte Grösse, die durch die Retention von Nitromethan charakterisiert wird, eingeführt werden muss. Im Laufe der hier vorliegenden Untersuchung stellte sich heraus, dass für Pyridin und Dioxan noch ein fünfter Wechselwirkungsanteil berücksichtigt werden muss.

Jeder Stoff und jede Trennflüssigkeit sind danach durch mindestens fünf Grössen charakterisiert, aus der Summe deren Produkte sich Indexdifferenzen errechnen lassen:

$$\Delta I^{RX} = (a^{RX} \cdot x_p) + (b^{RX} \cdot y_p) + (c^{RX} \cdot z_p) + (d^{RX} \cdot u_p) + (e^{RX} \cdot s_p) \qquad (1)$$

Die Indexdifferenz des Stoffes RX auf der Trennflüssigkeit p setzt sich aus fünf Produkten mit je einem stoff- und säulenspezifischen Polaritätsfaktor zusammen.

BESTIMMUNG DER RETENTIONSWERTE

Zur Prüfung der Beziehung (1) war es notwendig, die Retentionsindices von möglichst vielen verschiedenen Stoffen auf möglichst vielen verschiedenen Trennflüssigkeiten zu messen und mit berechneten Werten zu vergleichen. Wir wählten 30 Substanzen, die aus 25 verschiedenen homologen Reihen stammen, sich aus den Atomen C, H, O, S, N, F, Cl, Br und J zusammensetzten und 12 verschiedene funktionelle Gruppen enthalten. Als stationäre Flüssigkeiten wurden 22 Stoffe verwendet, die sich z.T. in unserem Laboratorium bewährt haben, z.T. häufig in der Literatur erwähnt werden und sich gleichmässig über die Polaritätsskala verteilen. Auf extrem polare Trennflüssigkeiten (Glycerin, Diglycerin, Triäthanolamin) musste verzichtet werden, da die Retentionszeiten für die Alkane nicht mehr mit der genügenden konzentrationsunabhängigen Reproduzierbarkeit zu bestimmen waren.

Alle Trennsäulen enthielten 20 Gew. % stationäre Flüssigkeit auf Kieselgur (Merck) oder Embacel (May & Baker). Zur Verminderung der Schwanzbildung (tailing) wurde einigen stationären Flüssigkeiten 0.1 % Alkaterge T nach einem Vorschlag von AVERILL[15] zugesetzt. Länge, Alkatergzusatz und die Bezeichnung der verwendeten Trennflüssigkeiten sind aus Tabelle I zu ersehen.

Sämtliche Retentionszeiten wurden bei 100° mit Wasserstoff als Trägergas auf einem Fraktometer F6/2H der Firma Perkin-Elmer gemessen. Die Trägergasgeschwindigkeit wurde so eingestellt, dass die Stoffe mit den längsten Retentionszeiten nach 30 bis 45 Minuten austraten. In manchen Fällen wurde zur genaueren Messung der Retention schnell durchlaufender Substanzen die Strömungsgeschwindigkeit vermindert. Die Probenmenge wurde so gering wie möglich gehalten (0.1–0.3 μl für Mehrkomponentengemische). Aus den vom Luftpeak aus gemessenen Retentionszeiten wurde der Retentionsindex für jeden Stoff nach der üblichen Formel berechnet und aus dem Retentionsindex jedes Stoffes auf jeder Trennflüssigkeit insgesamt 660 Indexdifferenzen gebildet:

$$\Delta I^{RX} = I_p{}^{RX} - I_{Squalan}{}^{RX}$$

Die gemessenen Retentionsindices sind in Tabelle II zusammengestellt. Die Trennleistungen der einzelnen Säulen waren recht unterschiedlich. Um eine Vergleichsmöglichkeit mit dem Messfehler und dem Fehler der Vorausberechnung zu bekommen, geben wir die Trennleistung gleichfalls in Indexeinheiten an. Die Trennschärfe der verwendeten Säulen soll dabei durch die Halbwertsbreite (Peakbreite in halber Höhe) von Benzol in Indexeinheiten gekennzeichnet sein. Diese Grösse ist in Tabelle I angegeben.

TABELLE I

SÄULENPOLARITÄTEN

Länge	*Trennflüssigkeit*	*Alkaterg-zusatz*	*Trennleistung: Halbwertsbreite Benzol*	*ΔI/100*					F_m
				Benzol	*Äthanol*	*Methyl-äthylketon*	*Nitro-methan*	*Pyridin*	
				x	*y*	*z*	*u*	*s*	
4 m	Silicon-Öl DC 200	+	9	0.15	0.56	0.47	0.78	0.44	6.2
5 m	Apiezon L	+	9	0.32	0.39	0.25	0.48	0.55	5.5
2 m	Diäthylhexylsebacat	+	10	0.73	1.65	1.15	2.20	1.24	4.9
2 m	Celanese-Ester Nr. 9	+	9	0.83	1.76	1.28	2.36	1.46	5.1
2 m	Diisodecylphthalat	+	9	0.83	1.65	1.43	2.53	1.54	5.0
2 m	Silicon-Öl DC 710	+	14	1.05	1.50	1.61	2.51	1.90	6.0
5 m	Fluoro-Silicon QF1	+	11	1.09	1.86	3.00	3.94	2.41	6.9
2 m	Polypropylenglykol (UCON 550x)		10	1.14	2.76	1.68	3.12	2.08	5.7
2 m	Acetyltributylcitrat		10	1.36	2.66	2.11	3.70	2.33	6.6
2 m	Trikresylphosphat	+	13	1.74	3.22	2.58	4.14	2.95	7.9
5 m	Polyphenyläther (5-ring)	+	8	1.75	2.27	2.34	3.26	2.84	8.8
2 m	Marlophen 87		13	1.78	3.72	2.53	4.49	3.34	5.7
2 m	Polypropylensebacat		16	1.93	3.38	2.58	4.36	3.27	4.0
2 m	Marlophen 814		14	2.22	4.23	2.92	5.35	3.78	3.7
5 m	Neopentylglykolsuccinat	+	21	2.68	4.88	3.87	6.13	5.21	6.3
5 m	Silicon-Fluid-Nitril XF 1150	+	17	2.86	4.80	4.49	6.82	4.89	8.8
2 m	Carbowax 20 M		16	3.18	5.33	3.81	7.02	5.04	3.9
2 m	Carbowax 4000		13	3.22	5.46	3.86	7.15	5.17	3.2
2 m	Reoplex 400		20	3.56	5.75	4.44	7.43	5.95	4.2
5 m	Diäthylenglykolsuccinat		23	4.93	7.58	6.14	9.50	8.37	5.4
2 m	Äthylenglykol-bis-cyanäthyläther		17	5.19	7.60	7.00	10.41	8.12	5.7
5 m	1,2,3-Tris-2-cyanäthoxypropan		11	6.00	8.71	7.94	11.53	9.40	4.7
5 m	Squalan	+	6	0	0	0	0	0	
	Retentionsindex I_u (Squalan)			649	384	531	457	695	

TABELLE II

RETENTIONSINDEX UND EINZELDIFFERENZEN

	Squalan	*Silicon-Öl DC 200*	*Apiezon L*	*Diäthylhexylsebacat*	*Celanese-Ester Nr. 9*	*Diisodecylphthalat*	*Silicon-Öl DC 710*	*Fluoro-Silicon QF 1*	*Polypropylenglykol*
2,4-DM-Pentan	631	633	627	632	631	630	625	647	625
		0	+ 1	— 3	— 2	+ 1	+ 4	— 3	+ 3
2-Äthylhexen-1	780	788	783	796	799	799	806	807	803
		— 4	+ 4	0	0	0	— 2	+ 2	+ 1
Cyclohexan	675	675	689	680	682	684	708	692	692
		— 2	0	+ 2	+ 5	+ 6	— 1	— 1	— 4
Toluol	757	767	787	827	837	837	858	865	866
		+ 4	+ 1	0	0	— 1	0	— 3	+ 2
Styrol	874	885	914	968	981	981	999	1010	1015
		+ 9	— 1	+ 2	0	0	+ 5	— 1	+ 5
Phenylacetylen	833	868	875	964	978	975	995	987	1044
		— 5	+ 9	+ 7	+ 5	+ 6	— 1	— 8	— 5
Aceton	422	472	450	546	558	572	593	740	599
		0	— 1	— 2	0	+ 2	0	— 3	0
Propionaldehyd	437	475	460	540	551	568	590	696	589
		+ 3	+ 3	+ 3	+ 4	0	— 6	0	+ 4
Crotonaldehyd	579	631	619	720	734	758	774	918	786
		+ 5	— 5	+ 4	+ 5	+ 1	+ 3	+ 6	— 1
n-Butylacetat	743	794	756	835	849	855	891	991	894
		—12	+13	+ 4	+ 3	+11	— 7	— 2	—11
Acetonitril	393	460	444	581	595	619	635	787	668
		+ 4	— 9	+ 7	+ 9	+ 5	— 4	0	— 4
Nitroäthan	554	623	598	748	764	782	793	945	825
		+ 3	— 4	— 1	— 2	+ 1	— 4	+ 9	— 1
Dioxan	651	697	687	759	778	779	823	876	826
		—10	+ 6	0	— 1	+ 6	— 6	+ 8	— 3
n-Dibutyläther	857	875	859	888	890	896	916	914	909
		— 7	+ 7	+ 1	+ 3	— 2	—15	+13	0

Acetyltributylcitrat	*Trikresylphosphat*	*Polyphenyläther*	*Marlophen 87*	*Polypropylensebacat*	*Marlophen 814*	*Neopentylglykolsuccinat*	*Silicon-Fluid-Nitril XF 1150*	*Carbowax 20 M*	*Carbowax 4000*	*Reoplex 400*	*Diäthylenglykolsuccinat*	*Äthylenglykol-bis-cyanäthyläther*	*1,2,3-Tris-2-cyanäthoxy-propan*
631	627	620	621	625	623	626	617	609	612	611	606	613	604
— 3	0	+ 3	+ 4	— 2	— 2	— 2	+10	+ 3	0	+ 2	+ 1	— 2	+ 3
814	820	821	817	823	826	841	844	847	848	858	890	898	916
— 4	— 1	— 2	+ 1	— 1	+ 1	— 2	+ 1	0	0	— 1	— 2	— 2	— 3
688	715	732	711	715	726	740	745	752	757	779	814	790	830
+ 9	— 8	— 5	0	+ 5	— 4	+ 7	—10	+ 1	— 2	— 8	+ 3	+13	— 4
887	925	930	930	948	972	1022	1038	1066	1069	1110	1238	1273	1353
+ 2	+ 4	+ 1	— 1	— 2	+ 1	— 5	+ 1	+ 1	+ 2	— 4	+ 5	+ 2	+ 4
1045	1098	1089	1097	1121	1152	1211	1231	1275	1279	1324	1468	1518	1600
+ 3	— 6	— 2	+ 1	— 5	+ 4	— 9	— 1	+ 2	+ 3	— 8	+ 9	— 3	+11
1070	1115	1074	1138	1148	1212	1256	1255	1370	1378	1404	1561	1593	1676
— 2	— 8	— 1	0	— 2	+ 3	— 6	+10	— 1	0	— 9	+ 4	— 7	+ 9
647	691	661	692	689	733	827	887	824	833	885	1071	1149	1247
— 2	+ 1	+ 5	— 2	+ 3	— 1	+ 4	+ 5	+ 7	— 1	+ 5	— 5	+ 3	+ 1
630	676	651	677	674	716	792	844	798	804	849	1009	1078	1166
+ 1	— 6	+ 1	— 5	+ 2	— 5	+ 2	+ 1	+ 1	0	+ 5	+ 2	+ 5	+ 4
835	902	866	894	892	944	1037	1103	1059	1067	1121	1314	1395	1496
+ 4	—18	—16	— 2	0	+ 1	+ 9	+ 4	+ 2	+ 1	+ 2	— 3	0	— 1
929	955	955	965	961	997	1076	1103	1078	1085	1131	1277	1338	1411
— 8	+ 3	—13	— 4	+ 2	— 2	+ 3	+15	— 2	— 3	— 2	— 3	— 8	— 4
724	775	709	800	782	871	949	1039	1025	1030	1061	1267	1365	1478
+ 4	— 4	— 3	— 9	+ 5	— 3	+ 4	— 8	— 5	0	+ 7	0	+ 5	— 2
887	940	871	947	949	1018	1115	1200	1168	1179	1223	1423	1532	1635
— 1	— 9	— 6	+ 2	— 5	+ 5	— 3	— 9	+ 1	0	— 4	— 3	— 7	— 3
860	902	919	925	931	971	1080	1091	1081	1091	1159	1363	1402	1517
— 3	+11	—10	— 1	+ 1	— 1	— 1	+ 3	+ 4	+ 2	+ 1	+ 5	— 5	— 5
925	941	942	934	931	943	977	974	970	972	993	1055	1088	1115
— 8	— 6	—11	0	+ 5	+ 3	— 2	+14	+ 6	+ 5	+ 5	+ 2	—11	— 3

(*Fortsetzung S. 14*)

TABELLE II (*Fortsetzung*)

	Squalan	*Silicon-Öl DC 200*	*Apiezon L*	*Diäthylhexylsebacat*	*Celanese-Ester Nr. 9*	*Diisodecylphthalat*	*Silicon-Öl DC 710*	*Fluoro-Silicon QF 1*	*Polypropylenglykol*
Thiophen	652	671 0	690 + 2	742 + 2	755 + 1	751 + 5	779 — 1	773 — 2	799 — 5
Chloroform	587	610 + 8	622 + 2	717 — 6	722 — 3	715 — 2	710 — 2	686 + 7	776 + 1
Tetrachlorkohlenstoff	656	669 — 6	682 + 4	705 + 1	709 + 5	709 + 6	733 + 1	706 0	736 —4
Methyljodid	516	524 + 9	554 — 5	590 + 1	597 + 4	598 + 5	631 —11	605 +10	634 — 6
Äthylbromid	504	519 + 5	529 0	576 — 1	580 + 4	589 — 1	610 — 8	627 + 3	610 — 1
Difluortetrachloräthan	681	709 —14	691 +12	727 + 3	729 + 8	733 + 9	747 + 5	771 —14	768 —13
n-Propanol	485	547 — 2	523 — 1	658 — 1	669 — 2	658 — 2	645 — 9	672 + 7	768 + 2
Isopropanol	431	486 — 1	463 + 2	589 — 7	600 — 7	589 — 6	573 — 4	613 + 3	674 +11
Allylalkohol	467	539 — 7	512 — 1	666 + 1	679 — 1	667 — 3	645 — 8	664 + 6	794 0
tert.-Butanol	471	514 + 4	493 + 7	618 —18	626 —16	616 —12	593 + 3	649 + 3	673 +13
Cyclopentanol	719	782 — 4	767 — 2	894 + 1	909 0	900 + 2	902 — 9	916 +17	1001 + 5

Acetyltributylcitrat	*Trikresylphosphat*	*Polyphenyläther*	*Marlophen 87*	*Polypropylensebacat*	*Marlophen 814*	*Neopentylglykolsuccinat*	*Silicon-Fluid-Nitril XF 1150*	*Carbowax 20 M*	*Carbowax 4000*	*Reoplex 400*	*Diäthylenglykolsuccinat*	*Äthylenglykol-bis-cyanäthyläther*	*1,2,3-Tris-2-cyanäthoxy-propan*
815	861	861	873	886	927	975	988	1046	1050	1084	1234	1246	1340
+ 4	— 4	—10	— 1	— 1	0	— 1	— 3	— 3	— 1	— 4	— 1	0	— 3
785	824	769	843	845	906	931	937	1026	1030	1036	1157	1159	1231
+ 2	— 9	— 9	+ 9	— 1	+ 6	— 5	— 7	+ 1	+ 6	0	— 2	+ 2	+ 2
747	784	787	784	792	819	836	829	895	896	916	1015	987	1045
+ 1	—17	—14	+ 1	+ 2	0	+14	+ 7	0	+ 4	+ 1	— 7	+ 4	— 5
635	685	687	698	700	740	768	793	833	832	857	972	970	1050
+16	— 8	—15	— 4	— 3	— 2	+ 8	—14	— 1	+ 6	+ 1	+2	+ 9	— 7
629	666	654	668	674	707	740	772	783	778	806	922	935	1004
+ 3	— 7	— 4	— 1	— 1	— 5	+ 5	—10	— 4	+ 5	+ 2	— 7	+ 5	— 4
778	796	778	793	799	821	846	830	888	885	903	985	960	997
— 8	—13	0	+ 8	+ 2	+ 4	+13	+20	— 7	+ 1	— 3	—12	+ 4	+ 4
756	812	714	861	828	911	977	971	1021	1037	1062	1240	1249	1358
+ 4	+ 2	— 6	+ 4	0	+ 6	— 1	+ 2	+ 5	+ 2	+ 1	— 2	— 3	— 5
682	724	634	774	741	810	880	867	903	917	951	1129	1127	1231
— 7	+ 4	+ 4	— 1	— 2	+ 5	+ 2	+ 9	+ 6	+ 4	+ 1	— 8	— 2	— 4
779	839	727	898	858	963	1015	1015	1098	1114	1135	1327	1344	1461
+ 5	+ 2	— 8	+ 2	+ 3	+ 4	0	+ 4	+ 3	+ 2	— 4	— 4	— 7	— 5
700	726	649	741	753	798	879	862	875	886	929	1093	1101	1193
—17	+ 6	+14	+23	—14	+ 2	—12	+ 9	+ 7	+ 6	— 3	—11	— 3	— 1
998	1068	989	1113	1089	1169	1255	1247	1298	1314	1351	1540	1555	1669
+ 9	— 5	—16	+ 4	— 3	+ 7	— 4	— 6	+ 6	+ 5	— 1	+ 8	— 7	— 5

Der mittlere Fehler einer Doppelbestimmung betrug für 16 Stoffe, die auf 16 zweimal im grossen Zeitabstand mit der gleichen stationären Flüssigkeit belegten Trennsäulen gemessen wurden, 3.5 Indexeinheiten. Der Messfehler ist stark säulenabhängig (1.2 Einheiten für Dioktylsebacat; 5.7 Einheiten für Carbowax 4000) und wenig stoffabhängig (2.9 Einheiten für 2-Äthylhexen-1; 5.2 Einheiten für Cyclopentanol).

BERECHNUNG DER ERGEBNISSE

Zur Berechnung der für die Stoffe und Trennflüssigkeiten charakteristischen Daten, die im folgenden Stoff- bzw. Säulenpolaritäten genannt werden sollen, stehen 660 Indexdifferenzen von 30 Stoffen auf 22 stationären Flüssigkeiten zur Verfügung. Gesucht sind $5 \times 30 = 150$ Stoffpolaritäten und $5 \times 22 = 110$ Säulenpolaritäten. Für 5 Stoffe auf einer Trennsäule ergeben sich fünf Gleichungen mit 25 unbekannten Stoff- und 5 unbekannten Säulenpolaritäten, die alle der Form:

$$\Delta I = ax + by + cz + du + es$$

gehorchen.

Diese fünf Gleichungen sind 25-fach unterbestimmt, wir müssen für 25 Unbekannte (willkürliche) Zahlen setzen, um die Gleichung lösen zu können. Da physikalisch sinnvolle Grössen für die Wechselwirkung der einzelnen Stoffe mit den Trennflüssigkeiten nicht bekannt sind, setzen wir willkürliche Zahlen, die für eine Berechnung von Indexdifferenzen ausreichen, für eine quantitative Betrachtung der intermolekularen Wechselwirkungen aber unzureichend sind. Für die fünf Standardsubstanzen Benzol, Äthanol, Methyläthylketon, Nitromethan und Pyridin werden als Stoffpolaritäten die in der Tabelle III angegebenen Werte eingesetzt.

TABELLE III

	a	*b*	*c*	*d*	*e*
Benzol	100	0	0	0	0
Äthanol	0	100	0	0	0
Methyläthylketon	0	0	100	0	0
Nitromethan	0	0	0	100	0
Pyridin	0	0	0	0	100

Für jede stationäre Flüssigkeiten ist dann:

$x = \Delta I_{\text{Benzol}} : 100$

$y = \Delta I_{\text{Äthanol}} : 100$

$z = \Delta I_{\text{Methyläthylketon}} : 100$

$u = \Delta I_{\text{Nitromethan}} : 100$

$s = \Delta I_{\text{Pyridin}} : 100.$

In Tabelle I sind die für jede Trennflüssigkeit charakteristischen Säulenpolaritäten für die in dieser Arbeit verwendeten 23 stationären Flüssigkeiten angegeben.

Die Polaritäten eines Stoffes können aus den Indexdifferenzen auf fünf Trennflüssigkeiten, deren Säulenpolaritäten bekannt sind, errechnet werden. Da jede Indexdifferenz mit einem Messfehler behaftet ist, würden die so ermittelten Stoffpolaritäten von der Wahl der zur Berechnung verwendeten fünf Trennflüssigkeiten abhängen.

Es wurde deshalb eine über alle Säulen gemittelte Stoffpolarität für jeden Stoff bestimmt. Die damit berechneten Indexdifferenzen haben dann einen Fehler, der sich gleichmässig über alle Trennflüssigkeiten verteilt und einen geringeren mittleren Fehler zwischen berechneten und gemessenen Indexdifferenzen. Zu Bestimmung der gemittelten Stoffpolaritäten wird das Verfahren der kleinsten Fehlerquadrate herangezogen. Für einen Stoff auf allen Trennflüssigkeiten gilt:

$$\Sigma\,(\Delta I_i - ax_i - by_i - cz_i - du_i - es_i)^2 \equiv \text{Minimum} \qquad (2)$$

Damit die Summe der positiven Abweichung der Messwerte von den berechneten Indexdifferenzen den negativen gleich werden, ist weiter:

$$\Sigma\,(\Delta I_i - ax_i - by_i - cz_i - du_i - es_i) = 0 \qquad (3)$$

Zur Lösung von Gl. (2) werden die partiellen Differentialquotienten der Fehlerquadratsumme nach a, b, c, d und e gleich Null gesetzt. Nach Ausmultiplizieren und Division durch -2 werden die Gleichungen über alle Säulen summiert und aus den entstandenen Gleichungen die Stoffpolaritäten isoliert. Auch aus Gleichung (3) lassen sich die Stoffpolaritäten nach Summierung über alle Säulen isolieren. Setzt man jeweils die beiden Ausdrücke für a, b, c, d und e gleich, bringt beide Seiten der Gleichungen auf einen gemeinsamen Nenner, teilt dann die Gleichungen schliesslich durch diesen, so erhält man folgendes System von 5 Gleichungen mit 5 unbekannten Stoffpolaritäten:

$$\begin{aligned}
&b(\Sigma xy\cdot\Sigma x - \Sigma y\cdot\Sigma x^2) + c(\Sigma xz\cdot\Sigma x - \Sigma z\cdot\Sigma x^2) + d(\Sigma xu\cdot\Sigma x - \Sigma u\cdot\Sigma x^2) + \\
&\quad + e(\Sigma xs\cdot\Sigma x - \Sigma s\cdot\Sigma x^2) = \Sigma Mx\cdot\Sigma x - \Sigma M\cdot\Sigma x^2 \\
&a(\Sigma xy\cdot\Sigma y - \Sigma x\cdot\Sigma y^2) + c(\Sigma yz\cdot\Sigma y - \Sigma z\cdot\Sigma y^2) + d(\Sigma yu.\Sigma y - \Sigma u\cdot\Sigma y^2) + \\
&\quad + e(\Sigma ys\cdot\Sigma y - \Sigma s\cdot\Sigma y^2) = \Sigma My\cdot\Sigma y - \Sigma M\cdot\Sigma y^2 \\
&a(\Sigma zx\cdot\Sigma z - \Sigma x\cdot\Sigma z^2) + b(\Sigma yz\cdot\Sigma z - \Sigma y\cdot\Sigma z^2) + d(\Sigma uz\cdot\Sigma z - \Sigma u\cdot\Sigma z^2) + \\
&\quad + e(\Sigma sz\cdot\Sigma z - \Sigma s\cdot\Sigma z^2) = \Sigma Mz\cdot\Sigma z - \Sigma M\cdot\Sigma z^2 \\
&a(\Sigma ux\cdot\Sigma u - \Sigma x\cdot\Sigma u^2) + b(\Sigma yu\cdot\Sigma u - \Sigma y.\Sigma u^2) + c(\Sigma zu\cdot\Sigma u - \Sigma z\cdot\Sigma u^2) + \\
&\quad + e(\Sigma su\cdot\Sigma u - \Sigma s\cdot\Sigma u^2) = \Sigma Mu\cdot\Sigma u\cdot\Sigma M\cdot\Sigma u^2 \\
&a(\Sigma xs\cdot\Sigma s - \Sigma x\cdot\Sigma s^2) + b(\Sigma ys\cdot\Sigma s - \Sigma y\cdot\Sigma s^2) + c(\Sigma zs\cdot\Sigma s - \Sigma z\cdot\Sigma s^2) + \\
&\quad + d(\Sigma us\cdot\Sigma s - \Sigma u\cdot\Sigma s^2) = \Sigma Ms\cdot\Sigma s - \Sigma M\cdot\Sigma s^2
\end{aligned}$$

Hierbei sind x, y, z, u, s die oben angegebenen Werte für die Indexdifferenzen der fünf Standardsubstanzen auf der jeweiligen Trennflüssigkeit und M die gemessene Indexdifferenz des Stoffes mit den Polaritätsfaktoren a, b, c, d und e. Dieses Glei-

chungssystem lässt sich für jeden Stoff lösen und ergibt die in Tabelle IV zusammengestellten Stoffpolaritäten.

Mit diesen Daten können die Indexdifferenzen aller untersuchten Stoffe auf allen Säulen mit Hilfe der fünf Standard-Indexdifferenzen berechnet werden. So beträgt z.B. die Indexdifferenz von Acetonitril mit den Stoffpolaritäten aus Tabelle IV auf allen Säulen:

$$\Delta I = 2.86\frac{\Delta I_{\text{Benzol}}}{100} - 16.38\frac{\Delta I_{\text{Äth.}}}{100} + 30.09\frac{\Delta I_{\text{MÄ K}}}{100} + 84.10\frac{\Delta I_{\text{Nim.}}}{100} + 0.03\frac{\Delta I_{\text{Pyr.}}}{100}$$

und auf Carbowax 20 M:

$$\Delta I = 2.86 \cdot 3.18 - 16.38 \cdot 5.33 + 30.09 \cdot 3.81 + 84.10 \cdot 7.02 + 0.03 \cdot 5.04 = 627$$

Der experimentell gefundene Wert beträgt 632, die Differenz 5 Indexeinheiten. Auf diesem Weg wurden sämtliche Indexdifferenzen berechnet und mit den gemessenen verglichen. In Tabelle IV sind die mittleren (F_m) und die maximalen Differenzen (F_{max}) zwischen berechneter und gemessener Indexdifferenz für jeden Stoff angegeben. Der mittlere Fehler für sämtliche Indexdifferenzen beträgt 6 Index-

TABELLE IV

STOFFPOLARITÄTEN

		a	*b*	*c*	*d*	*e*	F_m	F_{max}
1	Benzol	100.00	0.00	0.00	0.00	0.00	—	—
2	Äthanol	0.00	100.00	0.00	0.00	0.00	—	—
3	Methyläthylketon	0.00	0.00	100.00	0.00	0.00	—	—
4	Nitromethan	0.00	0.00	0.00	100.00	0.00	—	—
5	Pyridin	0.00	0.00	0.00	0.00	100.00	—	—
6	2,4-DM-Pentan	19.63	0.74	12.97	— 1.94	0.68	3.2	10
7	2-Äthylhexen-1	19.14	— 0.69	4.86	— 1.29	0.10	2.0	4
8	Cyclohexan	32.06	—22.47	—21.64	4.07	29.72	5.7	13
9	Toluol	108.33	3.77	8.75	— 7.01	— 7.61	2.6	5
10	Styrol	127.00	0.02	— 8.08	10.67	— 8.94	5.3	11
11	Phenylacetylen	125.20	2.53	—74.82	57.97	0.90	5.9	10
12	Aceton	— 5.30	— 4.61	94.94	7.90	5.64	3.1	7
13	Propionaldehyd	13.27	— 1.01	74.86	4.79	1.33	3.4	6
14	Crotonaldehyd	— 7.86	—17.59	65.75	37.49	17.21	6.2	18
15	*n*-Butylacetat	— 3.77	—13.31	57.29	13.88	19.98	7.5	15
16	Acetonitril	2.86	—16.38	30.09	84.10	0.03	5.3	9
17	Nitroäthan	— 5.41	—11.07	43.66	75.66	— 1.28	4.6	9
18	Dioxan	45.86	— 2.89	40.20	— 7.49	40.24	5.3	11
19	*n*-Dibutyläther	17.34	9.77	29.73	—12.48	— 2.79	7.3	15
20	Thiophen	105.69	— 4.19	—31.53	20.10	11.22	3.4	10
21	Chloroform	69.71	28.91	—72.62	53.05	— 6.29	5.1	9
22	Tetrachlorkohlenstoff	63.28	—20.94	—57.47	28.28	33.75	6.6	17
23	Methyljodid	71.06	—14.95	—42.57	31.77	21.58	8.0	16
24	Äthylbromid	46.05	— 7.74	— 1.28	18.34	9.11	4.7	10
25	$C_2F_2Cl_4$	14.89	—16.12	—35.03	29.09	33.35	9.6	20
26	*n*-Propanol	— 9.42	105.26	0.25	6.63	— 7.49	3.9	9
27	Isopropanol	—18.15	95.89	15.76	— 6.53	2.09	5.3	11
28	Allylalkohol	18.11	116.45	—21.27	22.06	—23.35	4.3	8
29	*tert.*-Butanol	—11.42	76.51	33.75	—12.77	0.21	11.0	23
30	Cyclopentanol	2.08	77.84	—20.48	21.69	17.75	7.1	17

einheiten. Der maximale Fehler ist von gleicher Grössenordnung wie die halbe Peakbreite des Benzols. Die Differenzen zwischen den experimentell bestimmten und den berechneten Indexdifferenzen sind in Tabelle II jeweils unter dem gemessenen Retentionsindex eingetragen.

VORAUSBERECHNUNGEN DER RETENTIONSDATEN

Zur Prüfung, mit welcher Genauigkeit sich Indexdifferenzen voraussagen lassen, wenn von einer Trennsäule nur die Indexdifferenzen der fünf Standardsubstanzen bekannt sind, wurden die 25 Indexdifferenzen aller Stoffe auf Carbowax-Dioleat, einer stationären Flüssigkeit, die nicht in die statistische Berechnung der Stoffpolaritäten einbezogen worden ist, vorausberechnet.

In Tabelle V sind die gemessenen und die vorausberechneten Indexdifferenzen zusammengestellt. Die mittlere Differenz zwischen den vorausberechneten und gemessenen Werten beträgt 4.1 Indexeinheiten.

TABELLE V

INDEXDIFFERENZEN AUF CARBOWAX-DIOLEAT

	Berechnet	*Gefunden*
Benzol		285
Äthanol		504
Methyläthylketon		360
Nitromethan		649
Pyridin		476
2,4-Dimethylpentan	— 15	— 10
2-Äthylhexen-1	61	62
Cyclohexan	68	69
Toluol	278	276
Styrol	360	356
Phenylacetylen	481	476
Aceton	382	385
Propionaldehyd	340	342
Crotonaldehyd	451	449
n-Butylacetat	314	312
Acetonitril	580	585
Nitroäthan	571	569
Dioxan	404	398
n-Dibutyläther	111	104
Thiophen	350	348
Chloroform	397	393
Tetrachlorkohlenstoff	212	209
Methyljodid	283	280
Äthylbromid	250	252
Difluortetrachloräthan	183	180
n-Propanol	512	506
Isopropanol	456	453
Allylalkohol	594	591
tert.-Butanol	393	392
Cyclopentanol	550	542

DISKUSSION DER ERGEBNISSE

Mit den erhaltenen Stoffpolaritäten lassen sich zwar mit guter Genauigkeit Retentionszeiten berechnen, Aussagen über die Stärke der zwischenmolekularen Wechselwirkungsenergien sind aber aus den stoffspezifischen Grössen nur annähernd zu erhalten. So sind alle negativen Polaritäten unwahrscheinlich, da die Summe der zwischenmolekularen Wechselwirkungsenergien sich nur aus positiven Anteilen zusammensetzt, wenn wir von Abstossungskräften absehen wollen. Die Ursache der negativen Stoffpolaritäten sind die willkürlich eingesetzten, zu kleinen Polaritätsfaktoren von 0 anstelle einer positiven, endlichen Zahl für die Standardsubstanzen. Chloroform, Tetrachlorkohlenstoff und Phenylacetylen haben ein relativ hohes *d* von 53, 28 und 58 Einheiten. Diese drei Stoffe weisen aber auch ein hohes negatives *c* von —73, —57 und —75 Einheiten auf. Wenn Nitromethan neben seinen 100 *d*-Einheiten nicht 0, sondern *ca.* 170 *c*-Einheiten erhielte, würden die *c*-Glieder der drei Stoffe positiv sein oder bei 0 liegen. Um aus den gefundenen Stoffpolaritäten sinnvolle physikalische Grössen zu machen, müssten sinnvolle Werte für die Stoffpolaritäten der fünf Standardsubstanzen eingesetzt werden. Hierzu ist es notwendig, die physikalische Bedeutung der 5 Polaritätsfaktoren zu ermitteln.

Die Grösse *a* ist charakteristisch für die besonderen zwischenmolekularen Kräfte, die Benzol einzugehen vermag. Aus der Tabelle IV der Stoffpolaritäten ist zu ersehen, dass alle Aromaten, aber auch Halogenalkyle und Cyclohexan einen hohen *a*-Wert aufweisen. Aus Untersuchungen von Elektronen-Donator-Akzeptor-Komplexen ist bekannt, dass die eben aufgezählten Stoffe σ- oder π-Donatoreigenschaften aufweisen. (Siehe hierzu BRIEGLEB[16].) Tetrachlorkohlenstoff allerdings gilt wegen seiner von WATANABE[17] bestimmten hohen Ionisierungsenergie von 11.5 eV nicht als Elektronendonator. Möglicherweise spielen auch Induktionskräfte für die Grösse *a* eine Rolle oder Grössenunterschiede gegenüber den Paraffinen.

Die Grösse *b* ist charakteristisch für Alkohole und deren Fähigkeit H-Atome zur Wasserstoffbrückenbindung abzugeben. Nur Alkohole und, wie auch LITTLEWOOD[18] feststellte, Chloroform besitzen diese Eigenschaft in stärkerem Masse und weisen entsprechend hohe *b*-Werte auf.

Die Grösse *c* ist durch den willkürlich eingesetzten *c*-Wert von 0 für Nitromethan stark verschoben. Werden die *c*-Werte der Stoffe mit hohem *d* nach $c_{\text{korr.}} = c + 1.70\,d$ korrigiert, so ergeben sich die folgenden Werte für $c_{\text{korr.}}$ (Tabelle VI). Zum Vergleich sind die Dipolmomente (μ) der Stoffe in Debye und eine dem Quadrat der Dipolmomente proportionale Grösse mitangegeben.

TABELLE VI

	c_{korr}	μ	$10\,\mu^2$
Cyclohexan	— 14	0	0
Tetrachlorkohlenstoff	— 10	0	0
Dioxan	27	0	0
Chloroform	18	1.02	10
Dibutyläther	8	1.18	14
Methyläthylketon	100	2.79	78
Crotonaldehyd	130	3.69	136
Acetonitril	173	3.96	156

Die korrigierten *c*-Werte sind exponentiell abhängig vom Dipolmoment der einzelnen Stoffe. Die Grösse *c* sollte dann ein Mass für die Orientierungskräfte sein. Bemerkenswert ist der hohe *c*-Wert von Dioxan, obwohl Dioxan ein Dipolmoment von o hat. Dieses gemessene Dipolmoment setzt sich aber aus zwei entgegengesetzt gerichteten Einzelmomenten zusammen, die bei der zwischenmolekularen Wechselwirkung in Erscheinung treten können.

Die Grösse *d* wird am stärksten durch die Nitro- und Nitrilgruppe verursacht. Nitroverbindungen und Nitrile gelten als Elektronenakzeptoren, wenn diese funktionellen Gruppen gehäuft in einem Molekül auftreten. Akzeptoreigenschaften von Nitromethan und Acetonitril wurden bisher noch nicht beobachtet. LOVELOCK[19] beobachtete eine bemerkenswert hohe Elektronenaffinität von Tetrachlorkohlenstoff und Chloroform, die sich beide durch einen hohen *d*-Wert auszeichnen. Elektronenaffinitäten dienen als Mass für die Akzeptoreigenschaften organischer Moleküle (BRIEGLEB[16]).

Die Grösse *c* mit ihrem typischen Vertreter Pyridin und Dioxan kann charakteristisch für *n*-Donatoreigenschaften sein oder für die Fähigkeit, H-Atome zur Wasserstoffbrückenbindung aufzunehmen.

Die fünf Stoffpolaritäten können damit als Mass für Orientierungskräfte (Faktor *c*), Ladungsüberführungskräfte (Donator *a*, Akzeptor *d*) und die Wasserstoffbrückenbindung (H-Donator *b*, H-Akzeptor *e*) gedeutet werden. Da quantitative Beziehungen zwischen molekularen physikalischen Grössen und den gefundenen Polaritätsfaktoren nicht aufgefunden werden konnten, ist diese Deutung bisher nur eine Spekulation.

Der relativ hohe Fehler von *tert.*-Butanol kann auf den Einfluss sterischer Effekte zurückgeführt werden, die eine Behinderung der H-Brückenbindung durch die benachbarten Methylgruppen verursachen. Sterische Einflüsse können damit die Vorausberechnung von Retentionszeiten nach der angegebenen Methode erschweren, wenn die sterische Hinderung einer zwischenmolekularen Bindung von der stationären Flüssigkeit abhängig ist.

Bemerkenswert ist die gute Berechenbarkeit auch für die als unpolar geltenden Stoffe Cyclohexan und Dimethylpentan.

Die Indexdifferenz ist damit nicht nur additiv aus Inkrementen für einzelne Haftzonen zusammengesetzt, wie KOVATS[20] feststellte, sondern die Indexdifferenzen der Haftzonen wiederum aus Anteilen für verschiedene intermolekulare Wechselwirkungsenergien.

DANK

Der Autor dankt H. LORKOWSKI für seine Hilfe bei der Durchführung der umfangreichen Rechnungen.

ZUSAMMENFASSUNG

Es wird eine Methode zur Charakterisierung von gaschromatographischen Trennflüssigkeiten durch die Retentionsindex-Differenzen von 5 ausgewählten Stoffen (Benzol, Äthanol, Methyläthylketon, Nitromethan und Pyridin) angegeben, die auf der Additivität zwischenmolekularer Wechselwirkungsenergien beruht.

22 Trennflüssigkeiten und 30 Stoffe werden durch je 5 Grössen charakterisiert, so dass 660 Indexdifferenzen mit einem mittleren Fehler von 6 Indexeinheiten berechnet werden können. Der Retentionsindex von 30 Stoffen auf 22 Trennflüssigkeiten wird angegeben. 25 Indexdifferenzen auf einer polaren Trennflüssigkeit werden mit einem mittleren Fehler von 4 Indexeinheiten vorausberechnet.

SUMMARY

Stationary liquids can be characterized by the retention index differences of 5 selected compounds (benzene, ethanol, ethyl methyl ketone, nitromethane and pyridine). This method is based on the additivity of intermolecular forces. Twenty-two stationary liquids and 30 substances have been characterized from the data for the 5 compounds. Thus 660 index differences can be calculated with a mean error of 6 index units. Twenty-five index differences on a polar stationary liquid are predicted with a mean error of 4 units.

LITERATUR

1 G. J. PIEROTTI, C. H. DEAL, E. L. DERR UND P. E. PORTER, *J. Am. Chem. Soc.*, 78 (1956) 2989.
2 H. M. TENNEY, *Anal. Chem.*, 30 (1958) 2.
3 A. WEHRLI UND E. KOVATS, *Helv. Chim. Acta*, 42 (1959) 2709.
4 M. B. EVANS UND J. F. SMITH, *Nature*, 190 (1961) 905.
5 J. BROWN, *J. Chromatog.*, 10 (1963) 284.
6 L. ROHRSCHNEIDER, *J. Chromatog.*, 17 (1965) 1.
7 L. ROHRSCHNEIDER, *Z. Anal. Chem.*, 170 (1959) 256.
8 A. B. LITTLEWOOD, *J. Gas Chromatog.*, 1, No. 11 (1963) 16.
9 P. CHOVIN, *J. Gas Chromatog.*, 2, No. 3 (1964) 83.
10 D. E. MARTIRE, *Anal. Chem.*, 33 (1962) 1143.
11 E. KOVATS, *Z. Anal. Chem.*, 181 (1961) 359.
12 M. R. JAMES, J. C. GIDDINGS UND R. A. KELLER, *J. Gas Chromatog.*, 3, No. 2 (1956) 57.
13 I. A. PULLIN UND R. L. WERNER, *Nature*, 206 (1965) 393.
14 L. ROHRSCHNEIDER, *Z. Anal. Chem.*, 211 (1965) 18.
15 W. AVERILL, in N. BRENNER, J. E. CALLEN AND D. WEISSNAUGATUCK (Herausgeber), *Gas-Chromatography*, Academic Press, New York, 1962, S. 1.
16 G. BRIEGLEB, *Elektronen-Donator-Akzeptor-Komplexe*, Springer-Verlag, Berlin, 1961.
17 K. WATANABE, *J. Chem. Phys.*, 26 (1958) 542.
18 A. B. LITTLEWOOD, *Anal. Chem.*, 36 (1964) 1441.
19 J. E. LOVELOCK, *Nature*, 189 (1961) 729.
20 E. KOVATS, *Helv. Chim. Acta*, 41 (1958) 1915.

A NEW GAS–LIQUID CHROMATOGRAPHIC PHASE

E. W. ABEL, F. H. POLLARD*, P. C. UDEN AND G. NICKLESS**
Department of Chemistry, The University, Bristol (Great Britain)
(Received September 27th, 1965)

This paper is a preliminary communication which describes the preparation of a new type of gas–liquid chromatographic phase where the stationary phase is chemically bonded and attached to the support phase.

The existence of silanol groups on various silica gels has been shown by thermal decomposition, lithium aluminium hydride reduction, and infra-red spectroscopy. WARTMANN AND DEUEL[1] have formed a number of organic derivatives of silica gels with Si–O–C bonds:

$$\text{e.g.}\quad \text{Si—OH} + \text{SOCl}_2 \rightarrow \text{SiCl} + \text{SO}_2 + \text{HCl}$$

$$\text{Si—Cl} + \text{ROH} \rightarrow \text{Si—O—R} + \text{HCl}$$

Although "R" groups containing up to 18 carbon atoms were attached to such silica gels, all were relatively easily hydrolysed. Derivatives of "silica chloride" having Si–C bonds were also prepared:

$$\text{(a)}\quad \text{Si—Cl} + \text{ClMgR} \rightarrow \text{Si—R} + \text{MgCl}_2$$

$$\text{(b)}\quad \text{Si—Cl} + \text{LiR} \rightarrow \text{Si—R} + \text{LiCl}$$

Similar reactions coupling, organic groups to colloidal silicic acid, silica, and even quartz have been described by WEISS and co-workers[2–4].

EXPERIMENTAL

Preparation of the phase

Celite 545 (acid and alkali–methanol washed) was dried by heating at 600° for 10 h.

To this dry Celite 545 (39.70 g) was added pure redistilled *n*-hexadecyltrichlorosilane (11.40 g) and about 500 ml of sodium dried light petroleum spirit (b.p. 60–80°). The resultant mixture was then shaken for 5 h in a closed vessel, and the light petroleum was then removed under reduced pressure (0.1 mm). The powder

* Deceased October 16th, 1965.
** To whom enquiries should be addressed.

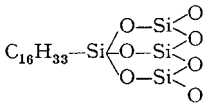
so formed was then shaken vigorously in a flask fitted with a Dreschel head, through which was passed a continuous stream of warm air saturated with water vapour. Copious fumes of hydrogen chloride were evolved for some hours, and the air stream was stopped when no further hydrogen chloride could be detected in the exit stream – after about 12 h.

The resultant damp material was dried for about 12 h at 60°, 0.1 mm pressure. The dried material was then used for gas chromatography (Phase I).

Subsequently a batch of Phase I was treated with hexamethyldisilazane after the manner of PERRETT AND PURNELL[5], in order to remove any "active" centres.

Thermal analysis of the phase

On the assumption that the organic grouping n-$C_{16}H_{33}$ is attached to the Celite surface in the form of:



then from the example of the preparation given above, the total organic content ($C_{16}H_{33}$) would be expected to be 14.5 % w/w of the whole material.

Thermal analysis of the final product gave an ash of 86.1 %, by weight, giving an indirectly analysed organic content of 13.9 %. This was thought to be in excellent agreement with the value predicted from the reactant quantities.

Chromatographic characteristics of hexadecyltrichlorosilane/Celite combined phase

Gas chromatography was carried out on a Griffin and George D. 6 Gas Density Balance chromatograph, using a 2 m, 0.6 cm O.D. stainless steel U column containing about 12 g of the combined phase/support. The column was operated at successively increasing temperatures up to 250°. At each stage, after a little initial bleed-off of volatile material, consistent retention and chromatographic characteristics were obtained over substantial operating periods. There was no evidence of any decomposition of the phase in continual operation at 250°.

The phase was compared under similar operating conditions with a freshly prepared phase of 15 % E. 301 Silicone Oil on 36–60 mesh acid and methanol washed Celite which gave closely similar retention times.

Examples of various classes of compound, and various mixtures were successfully chromatographed: benzene, acetone, neohexane, octene-1, heptyne-1, nonadiyne-1,8, n-pentyl acetate, tetrachloroethylene, n-pentanol, trimethyl-n-hexylsilane, trimethylethoxysilane, tri-n-propylsilane, trimethylstannane (thermal breakdown above 75°), petroleum ethers in ranges from 40–60° and 300–325°, methyl-n-butyldichlorosilane, tetraethylplumbane, essential oil extracts, and phosphonitrilic fluorides and chlorides.

No effective chromatography was possible for methanol, formic acid, n-butylamine, thiophene and silylamines, these either showing breakdown or greatly distorted peak shape. However, similar characteristics were observed on Silicone Oil for these compounds.

In general, peak symmetry and column efficiency was greater for the combined phase than for the silicone oil phase. The separation of a complex tretaalkylsilane mixture is shown in Fig. 1, using both phases, the boiling range being 26° to 280°. The much improved selectivity for close-boiling isomers is seen. A comparison of column efficiency estimated for the same component (*n*-propyltri-*n*-butylsilane) in each chromatogram, gave values for *n* (the number of theoretical plates) as $\simeq$ 1750 for the silicone oil column as against $\simeq$ 2300 for the combined phase.

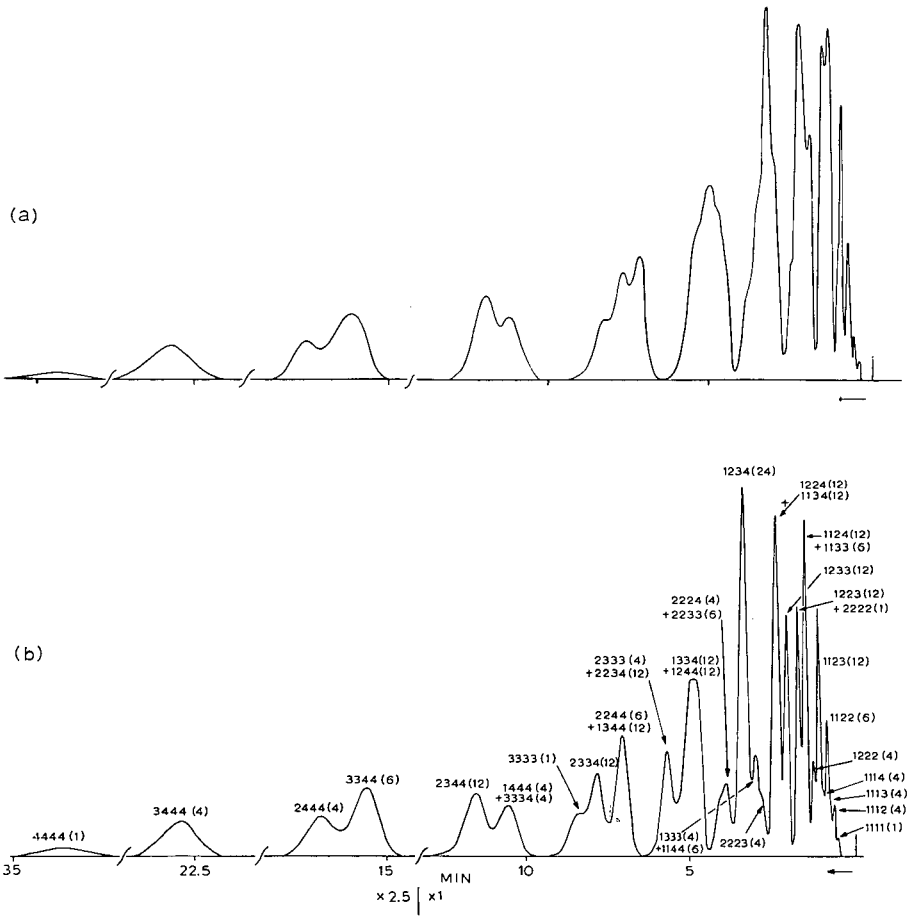


Fig. 1 (a) Chromatogram of redistribution products from methylethyl-*n*-propyl-*n*-butylsilane on 15% (w/w) E.301 Silicone Oil on Celite at 150°. (b) Chromatogram of redistribution products from methylethyl-*n*-propyl-*n*-butylsilane on the "combined hexadecylsilane/Celite phase." 1 = methyl; 2 = ethyl; 3 = *n*-propyl; 4 = *n*-butyl. Theoretical proportions for random redistribution are shown in brackets.

It was noted that less tailing of peaks occurred on the combined phase for all the classes of compounds studied.

Although relative selectivity between different types of compound has not been extensively studied, appreciable selectivity is given in Table I.

Only in the case of chlorosilanes containing the highly active Si–Cl groups was any interaction with the phase found (presumably due to reaction with unblocked Si–OH groups). Small percentages of these materials present in mixtures were absorbed, even after treatment of the phase with hexamethyldisilazane, although only a small proportion of a complete chlorosilane sample was lost under similar conditions.

TABLE I

Compound	t_R' *(combined phase)** / t_R' *E.301*
$(CH_3)_3SiC_6H_{13}{}^n$	1.15
$(C_3H_7{}^n)_3SiH$	1.26
$CH_3 \cdot C_4H_9Si^nCl_2$	1.24

* t_R' = adjusted retention time.

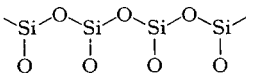
This effect has, in fact, proved useful in distinguishing small quantities of chlorosilane by-products produced in catalysed redistribution reactions of alkylsilanes.

DISCUSSION

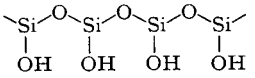
Structure of the phase

If it is assumed that the Celite support material is a complex matrix, then two possibilities are open for the bonding of the hexadecylsilyl group to it; they are (1) by direct reaction, and (2) by adsorption and hydrolysis.

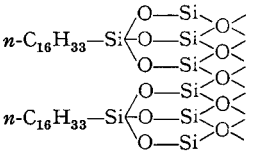
(1) *Direct reaction.* Assume initially that after initial drying the silicate structure is of the form:



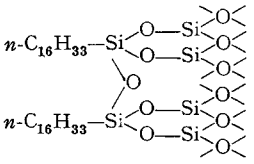
When this is perfectly dry, hexadecyltrichlorosilane will have no reaction with the chain, but by the passage of moist air, we can achieve structures as follows:



This may then react with the chloro-groups on the long chain silicon compound forming structures of the type:



However, if some hydrolysis between chloro-groups also takes place, then the following type of structure is also feasible:



In this case the Celite surface would be covered by a "skin of combined polymer".

(2) *Adsorption and hydrolysis.* The alternative to (1) is to postulate that after the initial drying of the Celite, it adsorbs the *n*-hexadecyltrichlorosilane in the usual manner. When damp air is passed through the mixture, the chloro-groups hydrolyse and combine amongst themselves forming networks of polysiloxanes in three dimensions throughout the three-dimensional silicate network of the original Celite.

This type of reaction is naturally very difficult to differentiate from (1) but it is felt that if (2) does take place it is to a minor extent only. The reasons are as follows:

(a) With (2) we would expect to notice that different batches of the same material would show irreproducible retention data, due to inhomogeneity of the phase through inhomogeneous condensation reactions. This is not the case.

(b) When subjected to thermal pyrolysis there was no gradual loss in weight, as might be expected if low molecular weight polymers were present. The phase broke down sharply at around 320°, the region expected for C–Si bonds under the oxidising conditions of the thermobalance. Also treatment of the phase with a number of solvents in attempts to solvent extract low molecular weight polymers failed completely.

Thus it is thought probable that this type of phase is of the form described in (1), although the possibility of (2) is not discounted.

Advantages of the combined phase

The active solvent group is chemically bonded to the –Si–O matrix with no apparent diminution of solute–solvent interaction, and thus there is no danger of deterioration of resolving efficiency due to breakdown of uniformity of phase coating on the support particles. Such decrease in column efficiency is commonly observed in the use of Apiezon-type greases and high molecular weight Silicone Oils at column temperatures up to 250°.

The greatly enhanced temperature stability enables the resolution which might be obtained from a n-$C_{16}H_{34}$ hydrocarbon solvent to be employed up to at least 250°. The preparative methods could perhaps be extended to include other active solvent groups in such combination, giving specific selectivity for various classes of compound.

Resolution is notably superior to that obtained on a silicone oil column operated under similar conditions, both improved peak symmetry and better resolution being noted for a wide range of compounds.

There is an added advantage that it should be possible to clean the phase, should it become contaminated, by the use of suitable solvents, since there is little possibility of Si–O–C bond breakage. Once any low-boiling material is removed, there should be no bleed-off under any normal operating conditions.

The phase could well prove useful for employing with ionisation detectors, where high stability and minimum bleed-off is essential.

SUMMARY

The preparation of a new type of gas–liquid chromatographic phase (where the stationary phase is most probably chemically bonded to the support phase) is described. A brief comparison between this phase and a conventional silicone oil phase for gas chromatography is made.

REFERENCES

1 J. WARTMANN AND H. DEUEL, *Chimia*, 12 (1958) 455.
2 A. WEISS AND A. WEISS, *Proc. I.U.P.A.C. Colloquium, Munster, September, 1954*, p. 41.
3 A. WEISS, M. EUGEN AND A. WEISS, *Hydrogen Bonding*, Pergamon Press, London, 1957, p. 495.
4 A. WEISS, G. REIFF AND A. WEISS, *Z. Anorg. Allgem. Chem.*, 311 (1961) 151.
5 R. H. PERRETT AND J. H. PURNELL, *J. Chromatog.*, 7 (1962) 455.

THIN-LAYER CHROMATOGRAPHY OF STEREOISOMERIC 2,4-DINITROPHENYLHYDRAZONES OF ALIPHATIC ALDEHYDES

HARDY M. EDWARDS, Jr.*
Department of Physiological Chemistry, University of Lund, Lund (Sweden)
(Received September 27th, 1965)

A number of articles have been published recently on chromatographic methods of separating aliphatic aldehyde 2,4-dinitrophenylhydrazones (2,4-DNP)[1–4], with no mention of encountering stereoisomeric forms. Theoretically, two stereoisomeric forms of each aldehyde 2,4-DNP and unsymmetrical ketone 2,4-DNP are possible. There are reports in the literature of the occurrence of two forms of 2,4-dinitrophenylhydrazones[5–8]. BRYANT[5] reported two distinct crystalline forms of acetaldehyde 2,4-DNP designated as stable and metastable, distinguished mainly by optical crystallographic constants. GORDON and coworkers[6] noted that the 2,4-DNP of normal chain aldehydes and methyl ethyl ketone gave two bands when column chromatography was used to separate them. RAMIREZ AND KIRBY[7] utilized crystallization to obtain the *syn* and *anti* form of 2-bromo-acetophenone 2,4-DNP and proved their difference by melting point studies and spectrographic studies. VAN DUIN[8] separated two forms, designated the α-isomer and the β-isomer, of the 2,4-DNP of α-keto-acid esters by reverse-phase column chromatography, using a nitromethane–silica gel stationary phase and light petroleum ether as a mobile phase.

While developing a method for localization of double bonds in fatty acids by identification of 2,4-DNP derivatives of reduced ozonides by thin-layer chromatography[9], it was noted that two bands always appeared for each aldehyde 2,4-DNP derivative. This communication presents observations on the occurrence of these two bands and evidence that they are stereoisomeric forms of these derivatives.

EXPERIMENTAL

Materials

The aliphatic aldehydes (propanol through nonanal) were obtained from Fluka AG, Switzerland, and were of extremely high purity (> 95 %) as determined by gas–liquid chromatography on a silicone column. The ketones used were older products that had been carefully distilled and stored and were of high purity (> 90 %) by chromatography. The 2,4-DNP were prepared from these products by mixing 0.5 g of the aldehyde or ketone with 0.8 g 2,4-dinitrophenylhydrazine, 4 ml of concentrated sulfuric acid, 50 ml of ethanol and 3 ml of distilled water. The 2,4-DNP were then twice recrystallized from ethanol. Uncorrected melting points were within 2° of values reported in the literature.

* On leave from the University of Georgia, Athens, Ga. (U.S.A.).

Chromatographic systems

All of the systems used were reverse-phase thin-layer chromatography.

1. Stationary phase: *n*-undecane (applied to plate as 10% *n*-undecane in petroleum ether); moving phase: 75% methanol–25% distilled water; plate coating: Kieselgel G according to STAHL.

2. Stationary phase: silicone oil (petroleum ether); moving phase: 75% methanol–25% distilled water; plate coating Kieselgel G according to STAHL.

3. Stationary phase: 2-phenoxyethanol (absolute ethanol); moving phase: *n*-heptane; plate coating: Kieselgur G. This is the system developed by URBACH[4].

4. Stationary phase: dimethylformamide (absolute ethanol); moving phase: *n*-heptane; plate coating: (a) for separation of homologous series and isomers of aliphatic aldehydes, Kieselgur G, (b) for maximum separation of isomers and much less separation of homologous series, Aluminum Oxide according to STAHL.

Smooth glass plates either 6.66 × 6.66 cm, or 20 × 20 cm were used with the plate coating 0.25 mm or 0.50 mm thick respectively. The plates were allowed to air dry for at least 24 h after pouring. They were impregnated with the stationary phase immediately before use either by dipping into the solution or by allowing the plates to develop in a tank containing 2–3 cm of the stationary phase solution. They were removed, allowed to air dry and all samples applied with a microsyringe. The plates were then developed by the ascending method in regular tanks.

When the developing solution was methanol–water the plates were developed only one time. However, when heptane was the mobile phase, they were developed two or three times.

For routine laboratory study, the spots were observed under ultraviolet light, no detecting agent being required. Spraying with a small amount of 0.2% of 4,5-dichlorofluorescein in 96% ethanol often sharpened the spots and made for better detection of small spots. The plates were sprayed with aqueous 5 *N* sodium hydroxide solution before photographs were taken.

Removal from chromatographic plates

A. When the non-polar stationary phases were used, the spots were scraped from the plates and placed in elution tubes. The material was extracted twice with 2–5 ml of petroleum ether (the diameter of the elution tube and amount of petroleum ether used depended on the amount of material removed from the plate) to remove most of the stationary phase material. The 2,4-DNP was then removed by two extractions with 5 ml of acetone.

B. When the polar solvents were the stationary phase the spots were removed to the elution tube and the 2,4-DNP eluted with two extractions of 5 ml of acetone. Since this also contained all the stationary phase further extraction was necessary to obtain the pure compound. This involved taking the acetone solution to dryness, dissolving the 2,4-DNP in a large volume of petroleum ether, extraction of this with water several times, drying with anhydrous sodium sulfate, filtration and reduction of volume.

RESULTS AND DISCUSSION

When the purified crystalline 2,4-DNP of aliphatic aldehydes were dissolved in

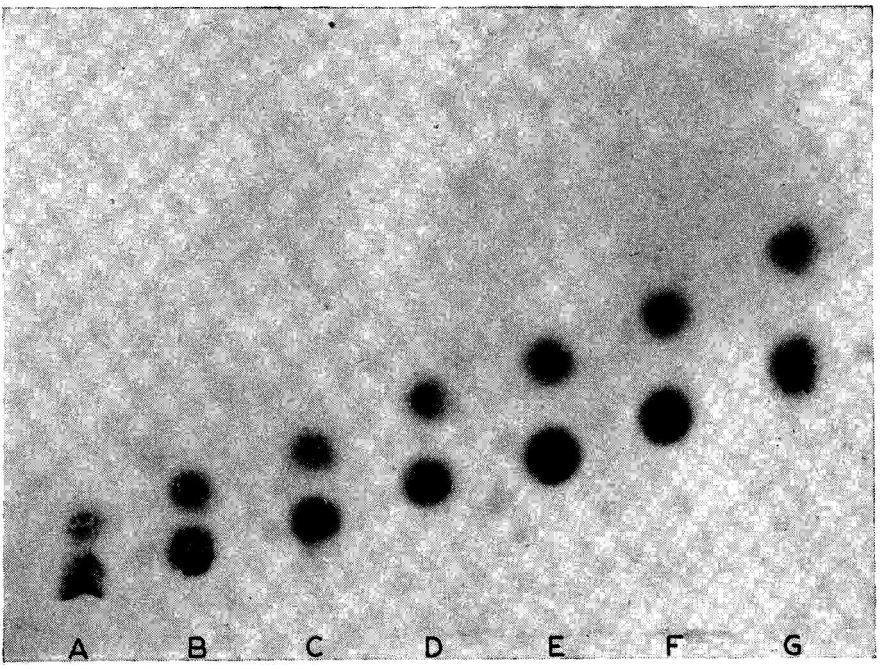


Fig. 1. TLC separation of stereoisomers of aliphatic aldehyde 2,4-DNP using phenoxyethanol on Kieselgur and developing two times with *n*-heptane. The 2,4-DNP were dissolved in chloroform for placing on the plate. A = propanal; B = butanal; C = pentanal; D = hexanal; E = heptanal; F = octanal; G = nonanal. Note the separation of both isomers and homologous series. The *syn* form is the lower form.

chloroform and then separated by thin-layer chromatography in the phenoxyethanol system, each phenylhydrazone showed two spots (Fig. 1). This separation had been observed in three other thin-layer reverse-phase chromatographic systems, consisting of either non-polar stationary phases such as undecane and silicone oil or the polar phase dimethylformamide. For reasons to be discussed later, the dark lower spot (Fig. 1) will be called the *syn* form and the lighter upper spot the *anti* form. When these phenylhydrazones were separated on thin-layer systems with non-polar stationary phases, the short chain aldehyde 2,4-DNP traveled farthest from the origin and the *syn* form traveled further than the *anti*. These two forms were apparently normal products of the reaction to produce the hydrazones from the hydrazine. Direct chromatography of reaction mixtures of chloroform, acetone or petroleum ether extracts of the reaction mixtures showed two forms.

When 2,4-DNP recrystallized several times from ethanol were placed in solution in petroleum ether, heptane, acetone, methanol, or ethanol, only the *syn* form was apparent from chromatography. When the same crystalline material was placed in solution in chloroform, two forms were apparent from chromatography (Fig. 2, D and E). When a small amount of hydrochloric acid was added to an acetone or methanol solution of crystals that were only the *syn* form, racemization took place and the two forms appeared. It is believed that the racemization that took place in the chloroform solution (laboratory distilled chloroform with 1.5% methanol added as a stabilizer) was due to small amounts of hydrochloric acid formed by

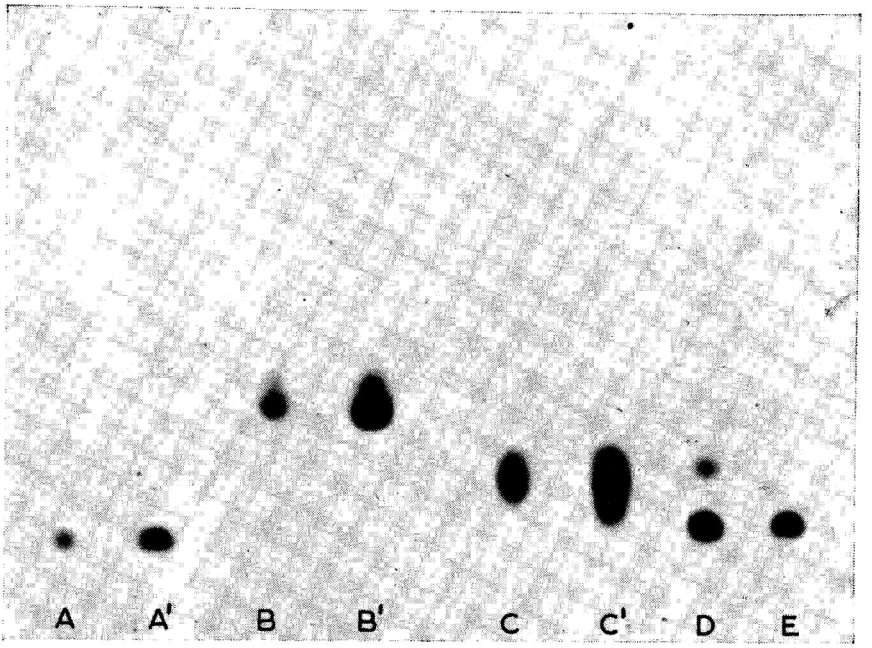


Fig. 2. TLC separations of 2,4-dinitrophenylhydrazones using phenoxyethanol on Kieselgur and developing two times with *n*-heptane. A and A′ = acetone; B and B′ = methyl *n*-amyl ketone; C and C′ = methyl *n*-propyl ketone; D = crystalline 2,4-DNP of pentanal dissolved in chloroform for placing on the plate; E = same as D, but dissolved in acetone for placing on the plate.

chloroform breakdown. No success was obtained in attempts to force the equilibrium so that only the *syn* or *anti* form was produced in a solution that originally contained the two forms. While hydrochloric acid addition caused racemization, this proceeded only to an equilibrium of 75 % *syn* form and 25 % *anti*. Larger amounts of acid or refluxing for several hours with additional acid had no further effect. The addition of sodium hydroxide caused an acetone solution containing both the *syn* and *anti* forms or the *syn* form alone to turn dark purple or black. There was no effect on the equilibrium between the two forms and no conversion of the *syn* form to the *anti* form when it was the only form present. Since VAN DUIN[8] reported that ultraviolet light stimulated the conversion of the *anti* to *syn* (he reported α-isomers to β-isomers), this procedure was subjected to preliminary investigation. These observations were at first interpreted to indicate that a small amount of irradiation decreased the *anti* form. With greater irradiation, it was apparent that destruction was taking place and that both forms were being destroyed. While the ratio remained constant, the decrease in the intensity of the faint spot (*anti* form) was first observed. This gave the false impression that it was being converted to the *syn* form.

The equilibrium of all of the 2,4-DNP of aldehydes tested was constant in chloroform solution (75 % *syn* and 25 % *anti* form). This was not the ratio observed in reaction mixtures where the solvents were primarily ethanol with small amounts of sulfuric acid and water. When these mixes were analyzed directly or when the 2,4-DNP were extracted into petroleum ether and then analyzed, the ratio was approximately 90 % *syn* and 10 % *anti* form.

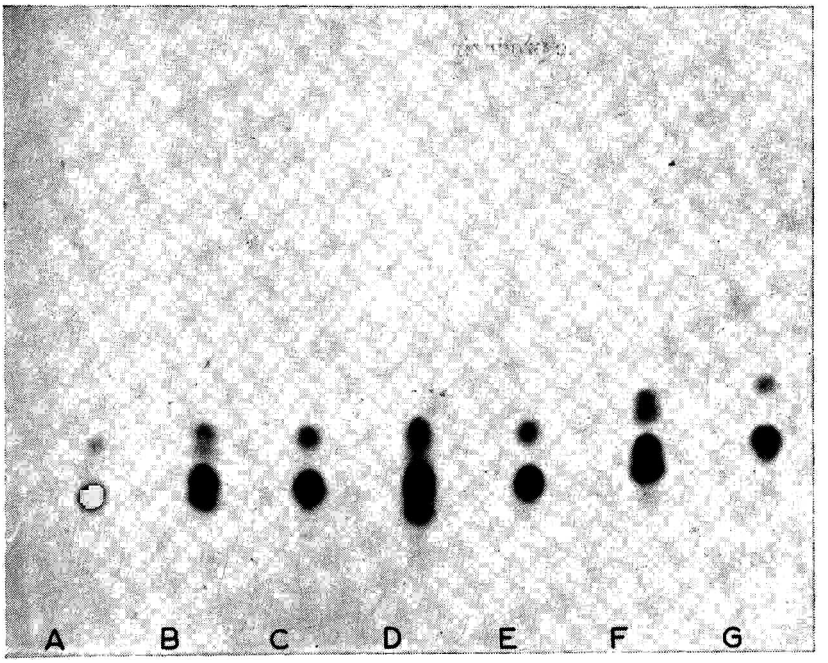


Fig. 3. TLC separation of stereoisomers of some aliphatic aldehyde 2,4-DNP using dimethylformamide as a stationary phase on Aluminum Oxide and developing two times with *n*-heptane. A = pentanal; B = pentanal and heptanal; C = heptanal; D = pentanal, heptanal and nonanal; E = heptanal; F = heptanal and nonanal; G = nonanal. Note the very good separation of isomers, but limited separation of homologous series.

Pure *anti* form 2,4-DNP of a particular aldehyde was easily obtained by elution of the band scraped from the thin-layer plate. The material isolated was a light yellow liquid or liquid-crystal, as contrasted to the *syn* form which was an orange crystal. The material was chromatographically pure and had a slight difference in adsorption maximum from the *syn* form ((352 mμ for the *anti* form of the 2,4-DNP of pentanal as compared to 358 mμ for the *syn* form).

The 2,4-DNPs produced from methyl *n*-amyl ketone also gave two spots when chromatographed, but they did not separate as well as the aliphatic aldehyde derivatives. Since a symmetrical ketone such as dimethyl ketone (acetone) should produce a 2,4-DNP of only one form, this was tested and the results of such a comparison are presented in Fig. 2. It is apparent from this chromatoplate that the 2,4-DNP of acetone gives only one spot, while that of methyl *n*-amyl ketone or methyl *n*-propyl ketone gives two spots.

A chromatographic system was found that gives good separation of the two stereoisomeric forms of aliphatic aldehyde 2,4-DNP while giving very poor separation of the homologous series. This system utilizing dimethylformamide as a stationary phase and heptane as the mobile phase was unique in that if Kieselgur G was used instead of Aluminum Oxide, all other things equal, the system separated both isomers and homologous series equally well. Fig. 3 shows a chromatoplate using this system. This system used in conjunction with the phenoxyethanol system was of value in resolving mixtures of *syn* and *anti* forms of mixtures of aliphatic aldehyde 2,4-DNP.

The undecane and silicone systems were very effective in separating the homologous series while at the same time producing a minimum separation of the isomers. With this system, there was always overlap between the *anti* form of a 2,4-DNP of an aliphatic aldehyde and the *syn* form of the 2,4-DNP of the next member in the homologous series. There was no other overlap among members of the homologous series. The spots were not as distinct in this system and when equilibrium conditions were not carefully set up there was a tendency to get excessive streaking. The phenoxyethanol system was described and discussed in detail by URBACH[4]. An excellent system in many respects, it gave good separation of both homologous series and *syn* and *anti* forms. The result was that the *anti* form of the heptanal derivative was always partly in front of and partly mixed with the *syn* form of the nonanal derivative.

Probably the best evidence for accurate assignment of configurations to stereoisomeric forms of 2,4-DNP was that of RAMIREZ AND KIRBY[7] (see discussion in WHELAND[10]). Their work showed that the form that was proven to be *syn* was the darker color crystalline form (red or orange *versus* yellow), had the highest melting point, was easily crystallized from the reaction mixture and had an absorption maximum in the ultraviolet spectra at somewhat longer wave lengths. Comparing these characteristics with the forms studied in this report, the *syn* and *anti* terms were assigned to the respective materials.

ACKNOWLEDGEMENTS

Thanks are due to Prof. B. BORGSTROM for providing laboratory facilities used to conduct this research. Mrs. ULLA-BRITT CARLSON gave technical assistance and Miss INGRID EKVALL conducted the photographic work. The investigation was supported in part by U.S. Public Health Service Research Grants No. 6338 and 05302 (Met.) and a Research Career Program Award No. 18,411 from the Arthritis and Metabolic Diseases Institute.

SUMMARY

The stereoisomeric forms of 2,4-dinitrophenylhydrazones (2,4-DNP) of some aliphatic aldehydes and unsymmetrical ketones were separated by several reverse-phase thin-layer chromatographic procedures. Evidence indicated that the reaction to produce the 2,4-DNP produces both the *syn* and *anti* form, but that repeated recrystallization in ethanol yielded the pure *syn* crystalline form. Racemization of the crystalline *syn* form could be stimulated by dissolving it in chloroform or by the addition of a small amount of hydrochloric acid to an acetone or methanol solution. Certain thin-layer chromatographic systems separated the homologous series to the greatest extent and the isomers the least (undecane/75 % methanol–25 % water). Another system separated the isomers to a considerable extent but was not too effective in separating the homologous series (dimethylformamide/heptane). Combinations of these systems should be of value in resolving mixtures of *syn* and *anti* forms of mixtures of aliphatic aldehyde 2,4-DNP.

REFERENCES

1 A. I. SCHEPARTZ, *J. Chromatog.*, 6 (1961) 185.
2 J. ROMUS AND Z. DEYL, *J. Chromatog.*, 6 (1961) 187.
3 A. M. ASATOOR, *J. Chromatog.*, 7 (1962) 417.
4 G. URBACH, *J. Chromatog.*, 12 (1963) 196.
5 W. M. BRYANT, *J. Am. Chem. Soc.*, 58 (1936) 2335.
6 B. E. GORDON, F. WOPAT, Jr., H. D. BURNHAM AND L. C. JONES, Jr., *Anal. Chem.*, 23 (1951) 1754.
7 F. RAMIREZ AND A. F. KIRBY, *J. Am. Chem. Soc.*, 75 (1953) 6026.
8 H. VAN DUIN, *Rec. Trav. Chim.*, 73 (1954) 78.
9 H. M. EDWARDS, Jr., *Lipids*, 1 (1966) 1.
10 G. W. WHELAND, *Advanced Organic Chemistry*, John Wiley and Sons, New York, 1960, p. 453.

THIN-LAYER CHROMATOGRAPHY OF SUBSTITUTED BENZALDEHYDE 2,4-DINITROPHENYLHYDRAZONES

K. THOMAS FINLEY AND ROBERT E. GILMAN
Department of Chemistry, Rochester Institute of Technology, Rochester, N.Y. (U.S.A.)
(Received September 27th, 1965)

INTRODUCTION

Since its introduction 35 years ago, 2,4-dinitrophenylhydrazone (2,4-DNPH) has proven to be generally useful as a derivative for carbonyl compounds[1,2]. Numerous studies of the chromatographic separation of these compounds have been reported in the literature. These include: column[3], paper[4], and gas[5] applications as well as the more recent thin-layer technique[6,7].

Recent interest in the thin-layer chromatography of aromatic aldehyde 2,4-DNPH's[8] prompts us to report our experiments with an extentive series of mono-substituted benzaldehyde 2,4-DNPH's. A further purpose of this work was to examine and evaluate the Eastman Chromagram precoated sheet and sandwich-type developing chamber.

EXPERIMENTAL

Materials

The 2,4-DNPH derivatives of the benzaldehydes included in this study were all prepared by the procedure of SHRINER, FUSON AND CURTIN[9] and were recrystallized until the melting points agreed with the literature values. In most cases methanol was the solvent used, but with the nitro- and amino-substituted compounds tetrahydrofuran was preferable.

Solvents used for spotting, developing, and recrystallizing were Eastman "White Label" and were used without further purification.

The adsorbent employed was silica gel in all cases. The major portion was supplied by Eastman on Chromagram sheet at a thickness of 100 μ. For comparison, a 250 μ layer of Merck Silica Gel G spread on glass plates using STAHL's method was adopted.

All chromatograms were developed with benzene.

Procedure

Since an evaluation of apparatus and conditions was desired in addition to the separations themselves, careful standardization of procedure was rigorously observed[10]. This was especially important in view of the fact that a large number of inexperienced workers were obtaining the data. The following standard procedures were used:

(1) Spots of 3–5 λ (6–10 λ for mixtures) were applied from 0.1 mg per ml solutions in chloroform or tetrahydrofuran so that their diameter did not exceed 3 mm.

(2) Spots were air dried for 15 min.

(3) Desaga tanks were lined with Whatman No. 1 filter paper and the ground glass covers were sealed with silicone grease.

(4) Sandwiches were carefully dried before each development.

(5) Chromagram sheets and glass plates were activated for 30 and 60 min, respectively. Chromagram sheets were stored in a desiccated chamber and glass plates were used immediately.

(6) All development was over a distance of 100 mm.

(7) Spots were located 20 mm from the bottom of the sheet or glass plate. The distance from the edge and between adjacent spots was also 20 mm.

(8) Three spots of a given binary mixture (benzaldehyde and a substituted benzaldehyde 2,4-DNPH) and three spots of each component of the mixture were chromatographed on a 20 × 20 cm sheet or glass plate as illustrated in Fig. 1.

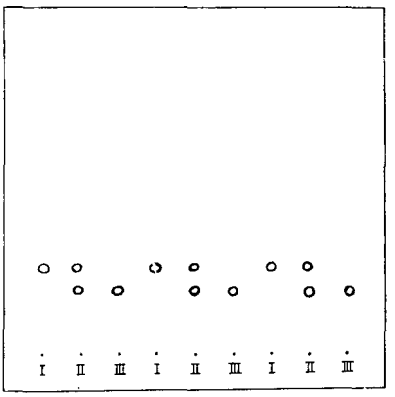


Fig. 1. Separation of 2,4-DNPH mixtures. Adsorbent: silica gel. Solvent: benzene. I = Benzaldehyde 2,4-DNPH; II = mixture of benzaldehyde and 3-methoxybenzaldehyde 2,4-DNPH's; III = 3-methoxybenzaldehyde 2,4-DNPH.

RESULTS

It is generally agreed that a much more useful comparison of chromatographic values is found when they are reported relative to a standard compound[7,8]. For our purposes, the unsubstituted benzaldehyde is most convenient; thus, we define:

$$R_{\text{benzaldehyde}} = \frac{R_F \text{ substituted benzaldehyde 2,4-DNPH}}{R_F \text{ benzaldehyde 2,4-DNPH}}$$

The relative values obtained are presented in Table I. A comparison of the precision of the various apparatus and conditions studied is presented in Table II.

DISCUSSION

Separation of binary mixtures

Ten of the nineteen substituted benzaldehyde 2,4-DNPH's that were chromato-

TABLE I

$R_{benzaldehyde}$ VALUES OF SUBSTITUTED BENZALDEHYDE 2,4-DNPH's

Substituent	*1**	*2*	*3*
4-OH	0.07	0.07	0.09
3-OH	0.09	0.10	0.11
4-CN	0.36	0.37	0.30
4-NO_2	0.51	0.53	0.54
3-NO_2	0.64	0.63	0.63
3-OCH_3	0.73	0.82	0.76
4-OCH_3	0.73	0.78	0.72
4-$N(CH_3)_2$	0.73	0.75	0.72
2-OH	0.78	0.82	0.70
4-$N(C_2H_5)_2$	0.93	0.91	0.87
4-F	0.96	0.98	0.93
4-Cl	0.98	0.97	0.96
4-Br	0.98	1.04	1.11
4-$CH(CH_3)_2$	0.98	1.04	1.06
3-Cl	1.00	1.01	1.00
3-Br	1.00	0.99	1.06
3-F	1.02	1.01	0.96
3-CH_3	1.07	1.00	1.04
4-CH_3	1.07	1.03	1.00

* Apparatus and conditions: (1) tank, benzene, Chromagram sheet, silica gel; (2) Chromagram sandwich, benzene, Chromagram sheet, silica gel; (3) tank, benzene, glass plate, silica gel G.

graphed as components of binary mixtures with benzaldehyde 2,4-DNPH were found to be separable. This may in all cases be attributed to the presence of a polar substituent. It was further found that the methoxy-, hydroxy-, nitro-, cyano-, and amino-compounds all migrate more slowly than benzaldehyde 2,4-DNPH.

The extremely small R_F values for 3- and 4-hydroxybenzaldehyde 2,4-DNPH's are due to the strong hydrogen bonding between the polar group and the silica gel. This effect, as well as the large R_F value for 2-hydroxybenzaldehyde 2,4-DNPH, has been reported in an earlier publication[8]. The influence of the 2-hydroxy group can be easily understood as a consequence of intramolecular hydrogen bonding.

TABLE II

PRECISION OF BENZALDEHYDE 2,4-DNPH R_F VALUES

*Apparatus and conditions**	R_F	±	σ
1	0.45	±	0.022
2	0.74	±	0.033
3	0.46	±	0.028

* For footnote, see Table I.

The conditions used fail to separate all isomeric compounds with the exception of the 2-hydroxy just discussed and the strongly polar nitrosubstituent.

An interesting case is found in the observed results with 4-dimethylamino- and 4-diethylaminobenzaldehyde 2,4-DNPH's. Under the conditions studied, the former is clearly separable while the latter shows an $R_{\text{benzaldehyde}}$ value much closer to 1.00 and in several cases was only partially separated. This may be a result of the greater hydrocarbon character of the diethyl molecule.

The remaining nine compounds, those containing either a halogen or alkyl substituent, all showed R_F values close to that of benzaldehyde 2,4-DNPH and when chromatographed as mixtures were totally inseparable. In early attempts to improve the separations, a substantial number of additional solvent systems were tried. Ethyl acetate, chloroform and tetrahydrofuran, as well as the mixtures benzene–chloroform, benzene–ligroine, and benzene–ethyl acetate were investigated. All of these attempts failed to produce the desired results and in most cases were less satisfactory.

Since at the time of this study Eastman Chromagram sheet was available only with silica gel coating, it was not possible to vary the nature of the adsorbent.

Comparison of apparatus and conditions

The precision of R_F values was determined as the standard deviation of 100 values submitted by 44 different workers. The maximum value of $\pm$ 0.033 obtained in this manner is well within the limits of $\pm$ 0.05 that is generally considered satisfactory. This is true of tanks and sandwiches, Chromagram sheet, and glass plates. Typical results are shown in Table II.

Agreement among a smaller number of determinations (12 to 24) for the various substituted compounds was generally better than $\pm$ 0.02. A given individual was able to reproduce R_F values with still greater precision. In neither instance did more than an occasional average deviation exceed $\pm$ 0.02.

A striking observation is the large difference in R_F values for a given compound in tanks and sandwiches. The large R_F values in the sandwiches result from the inherent difference in the two techniques. The tanks are presaturated with solvent whereas the atmosphere inside the sandwich is saturated by the moving solvent front as it advances. Since more solvent passes through a given spot of compound in the sandwich, the R_F value is larger[11].

The use of the relative value, $R_{\text{benzaldehyde}}$, produced the agreement shown in Table I and discussed above. It should be noted that this is true not only for the comparison of Chromagram sheets in tanks and sandwiches, but includes the values obtained on glass plates as well. This clearly illustrates the greater utility of R_{standard} values as compared to simple R_F values.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to the 44 high school students and teachers who performed the experiments described in this communication. The Eastman Chromagram sheet and apparatus were obtained from Distillation Products Industries. This research was supported in part by the National Science Foundation under CCSS/PES grant No. GE-7660.

SUMMARY

Benzaldehyde 2,4-DNPH and 19 mono-substituted benzaldehyde 2,4-DNPH's have been chromatographed by thin-layer techniques. Conditions are reported for the separation of ten of the substituted compounds from binary mixtures with benzaldehyde 2,4-DNPH.

Comparisons are reported of Eastman Chromagram precoated sheets with glass plates and of Desaga tanks with Chromagram sandwiches.

REFERENCES

1 C. F. H. ALLEN, *J. Am. Chem. Soc.*, 52 (1930) 2955.
2 C. F. H. ALLEN AND J. H. RICHMOND, *J. Org. Chem.*, 2 (1937) 222.
3 A. A. ROSEN, K. V. Y. SUNDSTROM AND W. F. VOGEL, *Anal. Chem.*, 24 (1952) 412.
4 A. M. GADDIS AND R. ELLIS, *Anal. Chem.*, 31 (1959) 870.
5 R. J. SOUKUP, R. J. SCARPELLINO AND E. DANIELCZIK, *Anal. Chem.*, 36 (1964) 2255.
6 G. URBACH, *J. Chromatog.*, 12 (1963) 196.
7 E. DENTI AND M. P. LUBOZ, *J. Chromatog.*, 18 (1965) 325.
8 G. RUFFINI, *J. Chromatog.*, 17 (1965) 483.
9 R. L. SHRINER, R. C. FUSON AND D. Y. CURTIN, *The Systematic Identification of Organic Compounds*, 5th Ed., Wiley, New York, 1965, p. 253.
10 E. STAHL, *Thin-Layer Chromatography*, Academic Press, New York, 1965, p. 27.
11 E. STAHL, *Thin-Layer Chromatography*, Academic Press, New York, 1965, p. 14.

QUANTITATIVE SEPARATION OF STEROIDS IN OILY SOLUTIONS BY MEANS OF THIN-LAYER CHROMATOGRAPHY WITH CONTINUOUS ELUTION

G. CAVINA AND G. MORETTI
Istituto Superiore di Sanità, Rome (Italy)
(Received September 10th, 1965)

In previous work conducted in this laboratory on microtechniques applicable to the isolation and determination of steroids possessing hormonal activity in the presence of lipid organ extracts, a description of a method of separation and determination of corticosteroid and lipid constituents present in the total lipid extract of rat adrenal glands using thin-layer chromatography was given[1].

Further studies dealing with the extension of this technique to the general problem regarding the separation of steroids less polar than corticosteroids, such as progesterone, testosterone and 19-nortestosterone esters and estrogens, from mixtures of lipids such as incubates, organ extracts and oily solutions, resulted in a method for the isolation and determination of some steroid hormones in oily solutions used for pharmaceutical purposes.

The methods described in the literature for the separation of steroids in such oily pharmaceutical preparations are based on physico-chemical separations, such as countercurrent extraction with ethanol and hydrocarbons[2,3], adsorption chromatography on Florisil as described by UMBERGER[4] and TAPPI *et al.*[5], or partition chromatography using either ethanol on ground silica and iso-octane[2], or polyethylene glycol 600 on ground silica and hexane[6] or nitromethane on ground silica and heptane[7]. These methods have generally been described for individual problems.

Some applications of paper chromatography to the separation of steroids in oily solutions have also been reported, although they are less numerous than those described for other pharmaceutical forms or for purity controls[8–11]. ROBERTS AND FLOREY[12] have described the determination of long chain esters of testosterone and 17α-hydroxyprogesterone in concentrated oily solutions (125–250 mg/ml) using paper chromatography followed by quantitative determination with isonicotinic hydrazide reagent in acid methanol as described by UMBERGER[4] using the same reagent as the means of elution.

A similar technique[13] to that for the determination of the 3-cyclopentyl enol ether of progesterone (Quingesterone) in oily solutions in the presence of its transformation products[14], has recently been described for the separation and determination of 19-nortestosterone decanoate (nandrolone decanoate) from oily solutions (concentration 50 mg/ml).

Thin-layer chromatography has been applied to the quantitative analysis of steroids of various degrees of purity[15], to corticosteroids and natural cortical extracts[16],

to the determination of 17α-ethynyl-estradiol 3-methyl ether in the presence of synthetic progesterones and compared with gas chromatographic analysis[17], to the determination of 17β-hydroxy-Δ^4-estrene-17α derivatives in tablets[18] and to the analysis of 6-chloro-17α-hydroxypregnane-4,6-diene-3,20-dione acetate, also in mixtures with 17α-ethynylestradiol 3-methyl ether[19].

Applications of this technique to the analysis of steroids in oily solutions are only qualitative. KORZUN AND BRODY[20] have described the identification of some steroids of pharmaceutical interest using various colorimetric reactions after silica gel thin-layer chromatography using various solvents which permitted separation from a greater part of the oily solvent.

Our studies have been directed towards the development of a continuous elution technique for ascending thin-layer chromatography that is at once simple and effective, and permits the complete separation of the steroids not only from triglycerides, but also from the minor components of the oils such as diglycerides and free fatty acids that cannot be separated from the steroids under the normal conditions of chromatography, and also allows the spectrophotometric determination in the U.V. of the steroids with a satisfactory recovery.

The eluates were obtained in high purity conditions and identification was possible throughout the entire U.V. spectrum. It is also possible to apply other reactions for the determination such as that with isonicotinic hydrazide.

EXPERIMENTAL*

Preparation of the standard solution and samples

Steroids purified for pharmaceutical use were employed in the preparation of solutions in spectrograde ethanol at a concentration of 5 mg/ml (250 mg/50 ml).

The compounds used for quantitative analysis included testosterone propionate, progesterone, 19-nortestosterone propionate, and estradiol cyclopentylpropionate. Solutions of testosterone cyclopentylpropionate and estradiol benzoate at the same concentration were also used for the qualitative analysis.

The absorbance index in ethanol (absorptivity as described by *Chemical Abstracts*, $E^{1\%}_{1\,cm}$ according to previous denomination) was determined for each steroid by diluting the 5 mg/ml ethanol solution. Samples were withdrawn with a Hamilton microsyringe, tared to 50 μl and diluted to 25 ml with ethanol. The values obtained were in good agreement with the values reported in the literature[21,22]. To obtain greater exactness of the chromatography recovery values, the values of the absorbance index as determined above were used. They were 491 for testosterone propionate and 535 for progesterone.

The steroids so analysed were used for the preparation of solutions in pure olive oil for pharmaceutical use with the addition of 1 % benzyl alcohol. The solutions prepared for the quantitative tests were the following:

(1) testosterone propionate (25 mg/ml);
(2) progesterone (10 mg/ml);
(3) 19-nortestosterone propionate (10 mg/ml);
(4) estradiol cyclopentylpropionate (5 mg/ml).

* The experimental work was carried out in collaboration with A. MOLLICA.

Thin-layer chromatography

20 × 20 cm plates coated with Merck Silica Gel G washed in a Soxhlet for 24 h with chloroform and mixed with 0.3 % Dupont Luminescent Chemical 609 were used. The thickness was 0.5 mm; activation was carried out for 30 min at 105°.

The following mixtures were used as solvents:

(1) petroleum ether b.p. 65°–peroxide-free ethyl ether–acetic acid (70:30:1);

(2) petroleum ether b.p. 65°–peroxide-free ethyl ether–acetic acid (50:50:1).

Glass tanks 18 × 25 × 22 cm fitted with smaller internal glass vessels having capacities of 100 ml for the solvents were used for the continuous chromatography. The inner vessels were placed on glass supports 5 cm from the bottom in such a manner that the upper edge of the plate, tilted at about 50° with respect to the base, was a few mm under the cover of the tank. The samples were applied as single spots or as a wide band on a line 3 cm from the lower edge of the plate. Continuous elution was carried out by placing 4 strips of Munktell 20 paper 3.5 × 20 cm* and another one of 15 × 20 cm on the upper edge of the plate. The strips were held firmly in place by means of a 20 × 5 cm glass slide and two clamps. The strips were so pressed on to the layer over an area of 3.5 × 20 cm at the upper edge of the plate. In this way, absorption of most of the triglycerides into the four 3.5 × 20 cm sheets was possible. The 15 × 20 cm sheet acted as an extension of the plate with the purpose of syphoning the solvent out of the container so as to allow continuous elution.

The prepared plate, described above, was so arranged in the tank as to allow most of the filter paper (20 × 15 cm) to pass through a narrow slit left between the cover and edge of the tank.

Solvent was put in the inner vessel which contained the lower part of the plate and the chromatogram was allowed to develop for the necessary time. In these experiments solvent 1 was run for 8 h and solvent 2 for 3 h. Under these conditions the bands of the various steroids ran mean distances of about 3.5–7.5 cm. They were clearly separated from the bands of the two diglyceride isomers (1,2 and 1,3), which ran for 10.2 and 11.3 cm (the distance is calculated from the starting line to the central points of the bands).

Quantitative separation of the steroids from oily solutions

The steroids were prepared in oily solutions as described above. They were diluted with heptane for the purpose of making them more fluid and to reduce the concentration of the steroids to 5 or 2.5 mg/ml. A Hamilton micro-syringe set at 50 μl or 100 μl was used for the deposition. The sample was distributed in small drops of equal size along a continuous line of 7 cm.

250 μg of steroids were placed on each line. On every 20 × 20 cm plate it was possible to chromatograph in parallel either two samples or one sample and a control sample of oil or the adsorbent. The heptane dilutions and the amount of oil to be separated by chromatography are shown in Table I.

Plates were developed with solvent 1 for 8 h (1st system) or with solvent 2 for 3 h (2nd system). They were then examined under U.V. light at a wavelength of 254 mμ and the zones corresponding to the steroids were marked together with one at the same height for the control.

* Other types of chromatography paper that are thick and porous can also be used.

TABLE I

HEPTANE DILUTIONS OF OIL SOLUTIONS OF STEROIDS

Sample	*Concentration (mg/ml)*	*Dilution with heptane, v/v*	*Quantity of steroids in 50 µl (µg)*	*Quantity of oil in 50 µl (µl)*
Testosterone propionate	25	1:5	250	10
Progesterone	10	1:2	250	25
19-Nortestosterone propionate	10	1:2	250	25
Estradiol cyclopentylpropionate	5	1:2	125	25

Quantitative separation of the steroids from alcoholic solutions

Solutions of pure steroids, concentration 5 mg/ml, were used for the purpose of determining the absorbance values of the solutions obtained by elution of the steroid band. This was done so as to serve as a reference for the determination of the recovery from the oily solutions.

As described above, 50 µl of steroid solution, concentration 5 mg/ml, were placed on each plate using a Hamilton microsyringe. An empty zone was left at the side for the control test of the adsorbent.

The plates were developed and examined with U.V. light as described previously. Five or six tests were done for each steroid and each variation in the chromatographic conditions so that values for the mean absorbance, standard deviation and average error could be obtained.

Elution and spectrophotometric determination in the U.V.

The marked areas which corresponded to the zones of migration of the steroids and the controls were carefully scraped off and the silica gel was quantitatively transferred to 50 ml separating funnels equipped with teflon stopcocks. 10 ml of water were added and the suspension was extracted four times using 5 ml of chloroform each time. These extracts were then filtered through anhydrous sodium sulfate and brought to a volume of 25 ml. 10 ml were removed and dried with nitrogen in graduated test tubes and then brought again to a volume of 10 ml with spectrograde ethanol. The spectrophotometric measurements were carried out over the range 225–250 mµ, particular attention being given to the maximum at 240–242 mµ. A Beckman DU spectrophotometer was used with 1 cm thick cuvettes. The quantity of steroid recovered was calculated using the following formula:

$$\frac{A_m - A_b}{a_{\text{det.}}} \times 10{,}000 \times 25 = \mu\text{g of steroid eluted}$$

where A_m was the steroid absorbance, A_b the blank control absorbance and $a_{\text{det.}}$ ($E^{1\%}_{1\,\text{cm}}$) was the value for the absorptivity obtained in the recovery tests using the steroid in alcoholic solution.

Determination by means of the reaction with isonicotinic hydrazide

The reagent consisted of 0.1 g of isonicotinic hydrazide and 0.125 ml of concentrated HCl in 100 ml of methanol as described by UMBERGER and has been widely used in this laboratory[23,24].

Five ml aliquots of the chloroform solution obtained in the extraction of the silica gel as described above were brought to dryness in glass stoppered test tubes; then 2 ml of methanol were added and, after shaking, 2 ml of the reagent. The solutions were protected from light and left for 30 min at room temperature. Absorbance at 380 mμ was determined against the reagent controls.

The formula used for the calculations is analogous to that used for the spectrophotometric determinations:

$$\frac{A_m - A_b}{a_{\text{det.}}} \times 10{,}000 \times 4 \times \frac{25}{5} = \mu\text{g of steroids eluted}$$

where A_m is the steroid absorbance, A_b is the blank control absorbance, $a_{\text{det.}}$ ($E^{1\%}_{1\,\text{cm}}$) is the value for the absorptivity obtained in the recovery test using the steroids in alcoholic solution and referred to the reaction mixture volume (4 ml) while 5 ml is the aliquot taken and 25 ml the total volume of the eluate.

RESULTS AND DISCUSSION

The continuous elution technique

A solution of testosterone propionate in olive oil at a concentration level of 25 mg/ml, diluted 1:5 with heptane, can be submitted to thin-layer chromatography with a petroleum ether (b.p. 65°)–ethyl ether–acetic acid (70:30:1) mixture; the steroid ranging in quantity from 100–250 μg and the oil varying between 2.5–10 mg.

If normal operating conditions as described by STAHL are followed, *i.e.* the solvent is allowed to rise to about 15 cm from the starting line, and the development time being about 45 min, it is possible to separate most of the triglycerides from the steroids, which, however, move together with the less mobile band of the diglycerides (Fig. 1a). This type of separation may be useful for the identification, but quantitative determination will not be precise because of the high and variable values of

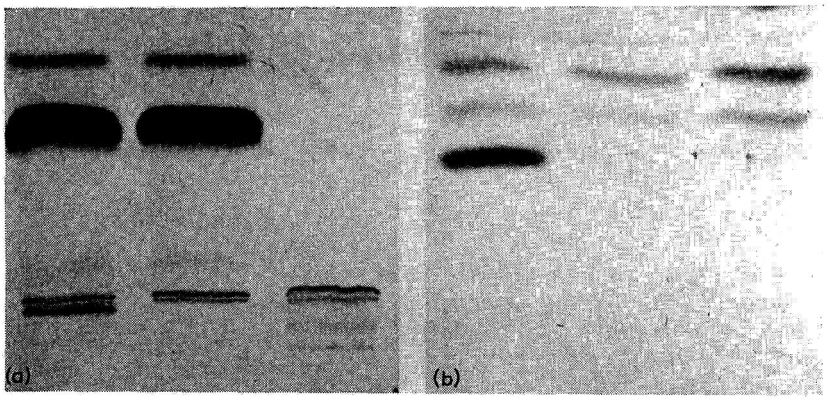


Fig. 1. (a) Chromatogram with solvent 1, development for 45 min. From left to right: testosterone propionate, oily solution; reference oil; diglyceride mixture. From bottom to top: monoglycerides (traces), two bands of diglycerides, free fatty acids, triglycerides, sterol esters. Detection: sulfuric acid. (b) Chromatogram with solvent 1, continuous development for 8 h. From left to right: same sample disposition as in (a). From bottom to top on the left: testosterone propionate clearly separated from the two bands of diglycerides. Detection: sulfuric acid.

the blank controls. It also is not suitable for general application as it would be necessary to have available for comparative testing the oil used in the steroid solution.

If our method described here is used instead, the elution time can be extended up to eight hours, along with a proportional increase in the elution volume. It also permits the separation of substances which have small differences in R_F value. This is demonstrated in Fig. 1b where the substances to be separated are identical to those in Fig. 1a; and where the two bands corresponding to the diglycerides are easily identifiable and are clearly separated from each other and from the steroid.

This type of separation is also applicable to oily solutions of steroids other than testosterone propionate as is shown by Figs. 2a and 2b. These figures clearly show the similar behaviour of 19-nortestosterone propionate, testosterone cyclopentylpropionate, progesterone and estradiol benzoate.

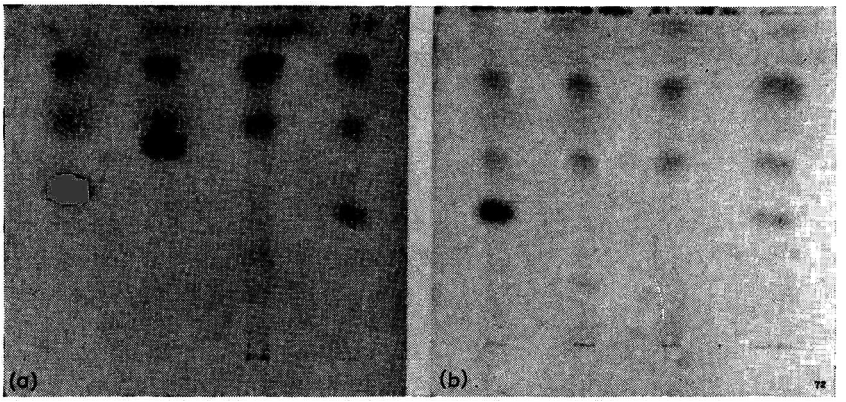


Fig. 2. (a) Chromatogram with solvent 1, continuous development for 8 h. From left to right: testosterone propionate, oily solution (steroid 50 μg, oil 2 mg); testosterone cyclopentylpropionate, oily solution (steroid 50 μg, oil 2 mg); reference oil (2 mg); 19-nortestosterone propionate, oily solution (steroid 20 μg, oil 2 mg). From bottom to top: steroid and the two bands of diglycerides. Detection: sulfuric acid. (b) Chromatogram with solvent 1, continuous development for 8 h. From left to right: testosterone propionate, oily solution (steroid reference); progesterone, oily solution (steroid 20 μg, oil 2 mg); reference oil (2 mg); estradiol benzoate (steroid 10 μg, oil 2 mg). From bottom to top: steroid and the two bands of diglycerides. Detection: sulfuric acid.

This technique of continuous development can be applied also with more polar solvents, reducing the development time proportionally. It was possible, using a petroleum ether b.p. 65°–ethyl ether–acetic acid (50:50:1) mixture for three hours, to obtain excellent separations of both progesterone and testosterone propionate.

Quantitative analysis

The application of this continuous development method to the quantitative analysis of steroids is shown in Tables II and III.

Table II reports the results obtained with pure steroids in alcoholic solution for the purpose of studying their behaviour in the presence of the adsorbent, their recovery, and the determination of their absorptivity along with the standard deviation and the standard error of the mean.

The absorptivity calculated in this manner can then be used to determine the amounts of the various steroids isolated from the oily solutions as shown in

TABLE II

ABSORPTIVITY OF PURE STEROIDS IN ETHANOL SOLUTION

Sample	*Solvent system*	*Quantity of steroid spotted on plate, μg*	*Absorptivity at 240 mμ obtained after elution, a**	*Absorptivity at 380 mμ obtained after elution and reaction with isonicotinic hydrazide, a**
Testosterone propionate, ethanol solution, 5 mg/cm³	1	250	448	328
	1	250	437	338
	1	250	450	327
	1	250	—	326
	1	250	450	357
	1	250	440	326
			445 ± 6.1**	334 ± 12.3**
			± 2.7***	± 4.0***
Testosterone propionate, ethanol solution, 5 mg/cm³	2	250	465	—
	2	250	476	—
	2	250	452	—
	2	250	446	—
	2	250	443	—
	2	250	465	—
	2	250	462	—
			458 ± 11.9**	
			± 4.5***	
Progesterone, ethanol solution, 5 mg/cm³	2	250	526	411
	2	250	526	396
	2	250	527	395
	2	250	524	415
	2	250	536	405
			527 ± 4.8**	404 ± 8.9**
			± 2.1***	± 4.0***

* a = absorbance index, absorptivity, $(E^{1\%}_{1\,cm})$.
** Standard deviation.
*** Standard error of the mean.

Table III, as other authors previously mentioned[12–15] have done in the quantitative applications of thin-layer and paper chromatography. The recovery values in these cases were always referred, although with some variations in the different methods, to a standard chromatographed under the same conditions as the sample.

These results allow us to draw the following conclusions. The absorptivity values used as references in the tests with testosterone propionate and progesterone were respectively 491 and 535, in good agreement with those reported in the literature[21] (490 and 540). Absolute recovery ran from 98.5 % for progesterone to 93.3 % for testosterone propionate after a chromatographic development lasting 3 h, and to 90.6 % for the latter steroid after a development lasting 8 h. These results confirm the observation that the behaviour of several steroids may vary with regards to the adsorbent and the chromatographic conditions.

Higher absolute recovery values for progesterone with respect to testosterone

TABLE III

RECOVERY OF STEROIDS IN OILY SOLUTIONS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Sample	*Solvent system*	*Quantity of steroid spotted on plate*	*Quantity recovered*				*Absorptivity value used in spectrophotometric analysis, a**	*Absorptivity value used in colorimetric analysis, a**
			Spectrophotometric		*Colorimetric*			
		μg	*μg*	%	*μg*	%		
Testosterone propionate, oily solution, 25 mg/cm³	1	250	237.5	95.0	238.3	95.3	445	334
	1	250	239.3	95.7	240.8	96.3		
	1	250	241.0	96.4	242.5	97.0		
	1	250	237.0	94.8	239.0	95.6		
	1	250	242.6	96.1	255.3	102.1		
	1	250	241.0	96.4	—	—		
			239.7 ±2.2**	95.9	243.1 ±7.0**	97.2		
Testosterone propionate, oily solution, 25 mg/cm³	2	250	248.3	99.3	—	—	458	—
	2	250	243.8	97.5	—	—		
	2	250	251.0	100.4	—	—		
	2	250	243.8	97.5	—	—		
	2	250	243.3	97.3	—	—		
			246.0 ±3.4**	98.4				
Progesterone, oily solution, 10 mg/cm³	2	250	242.8	97.1	250.5	100.2	527	404
	2	250	242.8	97.1	244.8	97.9		
	2	250	241.3	96.5	244.0	96.7		
	2	250	243.3	97.3	254.6	101.8		
	2	250	247.0	98.8	248.8	99.5		
			243.4 ±2.1**	97.4	248.5 ±4.9**	99.4		
Estradiol cyclopentylpropionate, oily solution, 5 mg/cm³	2	250	259.2	103.7	—	—	53.6	—
	2	250	254.0	101.6	—	—		
19-Nortestosterone propionate, oily solution, 10 mg/cm³	2	250	239.1	95.6	—	—	487	—
	2	250	236.0	94.4	—	—		

* a = absorbance index, absorptivity, ($E^{1\%}_{1cm}$).
** Standard deviation.

were observed by MATTHEWS and others[15] under different chromatographic conditions. This confirms additionally the necessity for accurate verification of quantitative thin-layer chromatography under standard working conditions, a criterion which is usual for gas–liquid chromatography.

The results reported in Table III for the experiments done with progesterone and testosterone propionate, and compared with the absorptivity values determined using steroids in ethanol solutions, show excellent recoveries of the same order for the different conditions of chromatography and for the various steroids, *viz.* 97.4, 95.9 and 98.4, all values higher than 95 % in agreement with the findings of ROBERTS AND FLOREY[12].

This technique can be considered of general use for steroids in oily solutions having a Δ^4-3-keto configuration, as is demonstrated by the experiments with 19-nortestosterone propionate. Regarding the estrogens, we limited our tests to estradiol cyclopentylpropionate where the spectrophotometric determinations at the wavelength 282 mμ were possible. In any case the low concentrations of estrogens in oily solutions generally used in pharmaceutical chemistry, and the low absorptivity values in the U.V. of the estrogens cause the measurements to be less precise than for those of the Δ^4-3-keto steroids.

In subsequent tests using oily solutions of estrogens the sulfuric acid–hydroquinone colorimetric method described by BROWN[25] was used to full advantage. This will be referred to in another paper.

It must be pointed out that the satisfactory precision obtainable by this method depends not only on the efficiency of the separation as indicated in Fig. 3, but also on the elution techniques which furnish consistent and low values for the controls.

Absorbance values for the controls averaged 0.024 $\pm$ 0.006 (standard deviation calculated from 17 tests) in comparison to sample absorbance values of 0.445–0.527,

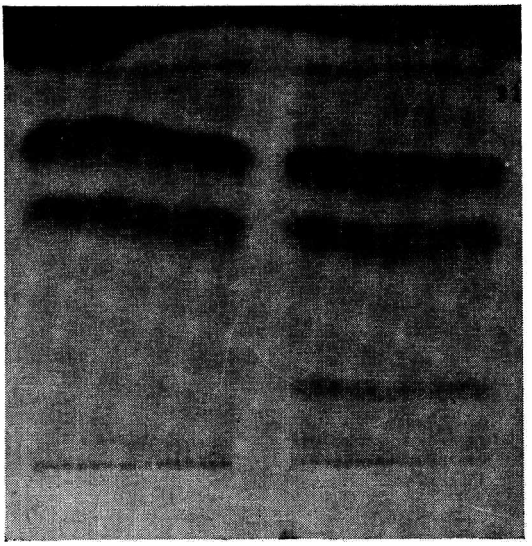


Fig. 3. Chromatogram with solvent 2, continuous development for 3 h. Left: oil 25 mg; right: progesterone 250 μg in 25 mg oil. From bottom to top: progesterone clearly separated from the two diglyceride bands. Detection: iodine vapor.

for equal areas eluted at the same dilution. Performing the elution with ethanol resulted in average extinction values of 0.135 with appreciable differences between various samples. The efficiency of the method with respect to the separation of steroids from fats is shown in Fig. 4, where the absorption spectra of progesterone, the oil control and the adsorbent layer only are reported. The absorbance/wavelength plots for the two types of control are practically coincident, thereby showing the clear separation of the steroid from interfering substances contained in the oil and the possibility of eliminating the control-oil test.

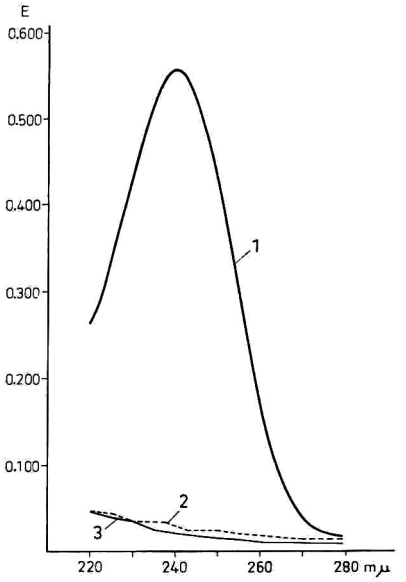


Fig. 4. U.V. absorption spectra of (1) progesterone eluate, (2) reference oil eluate, and (3) adsorbent eluate, from equivalent areas.

In some cases, attempts were made to perform, simultaneously along with the spectrophotometric determinations of the Δ^4-3-keto steroids, determinations with the isonicotinic hydrazide according to the method of UMBERGER[4] which has been widely applied[12–14] in the determination of steroids eluted by paper chromatography. The results obtained were in good agreement with those by the U.V. measurements. Again in this case, the control values were extremely low (oil blank 0.003, adsorbent blank 0.002).

It can be said in conclusion that the simple continuous elution technique developed in our laboratory would possibly give excellent results in the separation of many classes of steroids from lipids. The petroleum ether (b.p. 65°)–ethyl ether–acetic acid mixtures as proposed by MANGOLD[26] for the separation of lipids in classes have wider application to this kind of important separation.

Furthermore this technique gives satisfactory results even in the quantitative analysis of steroids in lipid–steroid mixtures.

SUMMARY

The present paper describes a thin-layer chromatographic method for the determination of some less polar hormonal steroids in oily solution.

By using an ascending technique with continuous elution it is possible to considerably increase the time of the chromatographic run. In this way a ready separation of the steroids is possible, not only from the principal components of the oils (triglycerides) but also from those components (diglycerides and free fatty acids) which under normal conditions of chromatography interfere because of their R_F values.

It is possible to determine testosterone, 19-nortestosterone and estradiol esters and also progesterone spectrophotometrically in the U.V. Other colorimetric determinations may be performed on the same chromatographic eluate.

REFERENCES

1 R. ANGELICO, G. CAVINA, A. D'ANTONA AND G. GIOCOLI, *J. Chromatog.*, 18 (1965) 57.
2 J. CAROL, *J. Assoc. Offic. Agr. Chemists*, 36 (1953) 1000.
3 *Pharmacopoeia of United States*, XV Ed., 1955, p. 717.
4 E. J. UMBERGER, *Anal. Chem.*, 27 (1955) 768.
5 G. TAPPI, E. M. ANDREOLI AND E. FREA, *Pharm. Weekblad*, 93 (1958) 231.
6 D. W. SNAIR AND L. A. SCHWINGHAMER, *J. Pharm. Pharmacol.*, 13 (1961) 148.
7 J. WOLFF, *J. Pharm. Sci.*, 52 (1963) 93.
8 H. J. PAZDERA, W. H. MCMULLEN, L. L. CIACCIO, S. R. MISSAN AND T. C. GREENFELL, *Anal. Chem.*, 29 (1957) 1649.
9 L. M. REINECKE, *Anal. Chem.*, 8 (1956) 1853.
10 G. CAVINA, E. CINGOLANI AND A. GIRALDEZ, *Farmaco (Pavia), Ed. Prat.*, 17 (1962) 149.
11 F. M. KUNZE, *J. Pharm. Sci.*, 53 (1964) 1170.
12 H. R. ROBERTS AND K. FLOREY, *J. Pharm. Sci.*, 51 (1962) 794.
13 Drug Standard Laboratory, *J. Pharm. Sci.*, 53 (1964) 98.
14 J. M. TALMAGE, M. H. PENNER AND M. GELLER, *J. Pharm. Sci.*, 53 (1964) 76.
15 J. S. MATTHEWS, A. L. PEREDA AND A. AGUILERA, *J. Chromatog.*, 9 (1962) 331.
16 G. CAVINA AND C. VICARI, in G. B. MARINI-BETTÒLO (Editor), *Thin-layer Chromatography*, Elsevier, Amsterdam, 1064; *Farmaco (Pavia), Ed. Prat.*, 19 (1964) 348.
17 E. P. SCHULZ, *J. Pharm. Sci.*, 54 (1965) 146.
18 H. G. GANSHIRT AND J. POLDERMAN, *J. Chromatog.*, 16 (1964) 510.
19 H. L. BIRD, JR., H. F. BRICKLEY, J. P. COMER, E. P. HARTSAW AND M. L. JOHNSON, *Anal. Chem.*, 35 (1963) 346.
20 B. P. KORZUN AND S. BRÓDY, *J. Pharm. Sci.*, 52 (1963) 206.
21 *British Pharmacopoeia*, 1963, pp. 16 and 666.
22 *National Formulary*, XI Ed., Am. Pharm. Assoc., 1960, p. 131.
23 E. CINGOLANI, G. CAVINA AND V. AMORMINO, *Farmaco (Pavia), Ed. Prat.* 15, (1960) 301.
24 G. CAVINA, E. CINGOLANI, V. AMORMINO AND A. GIRALDEZ, *Farmaco! (Pavia), Ed. Prat.*, 17 (1962) 393.
25 J. B. BROWN, *Biochem. J.*, 60 (1955) 185.
26 H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.

QUANTITATIVE DETERMINATION OF CHOLESTEROL IN AUTO-OXIDATION MIXTURES BY THIN-LAYER CHROMATOGRAPHY

CSABA HORVATH*

Physics Research Laboratory, Massachusetts General Hospital, Boston, Mass. (U.S.A.)

(Received September 13th, 1965)

An analytical method was needed to follow the degradation of crystalline cholesterol in a study of its thermal and radiation induced decomposition, the results of which will be reported at a later date.

Cholesterol readily undergoes auto-oxidation and decomposition by heat or by irradation[1]. Some of the products have been identified as 7-ketocholesterol, the α- and β-7-hydroxycholesterols, cholestan-3β,5α,6β-triol, $\Delta^{3,5}$-cholestadien-7-one and 25-hydroxycholesterol.

As the thin layer chromatogram of our samples demonstrates (Fig. 1) a great number of products arose from the decomposition of crystalline cholesterol. Presumably, most of the products are closely related to cholesterol. No simple and reliable method has been reported for the quantitative determination of cholesterol in such mixtures.

Although NORCIA[2] has suggested the SCHOENHEIMER-SPERRY method, modified by SPERRY AND WEBB[3] for quantitative determination of cholesterol in auto-oxidation mixtures the specificity of this technique was doubtful in the present case. Values of cholesterol were expected to be higher than the actual ones, since large amounts of the epimeric 7-hydroxycholesterols can interfere[4,5] and some of the unknown degradation products could also precipitate with digitonin and react with the Liebermann-Burchard reagent. Although gas chromatography is being successfully used for the determination of cholesterol, this method might result in unreliable low cholesterol values for our application[6]. Some of the degradation products, particularly peroxides and hydroperoxides may react with cholesterol at high temperatures in the gas chromatograph decreasing the cholesterol values.

As a conclusion, it was necessary to develop a specific analytical method in order to obtain reliable values for cholesterol in mixtures which contain both its oxidation and degradation products. Thin-layer chromatography (TLC) has been found to be an expeditious and simple method for separation of cholesterol from these products. The color reaction with the perchloric acid–phosphoric acid–ferric chloride (PPF) reagent introduced by MOMOSE *et al.*[7] is relatively insensitive to the impurities of the reagents and gives a simple method for the quantitative determination of the separated cholesterol.

The method described here utilizes a combination of this color reaction with TLC.

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* Present address: Yale University School of Medicine, Department of Internal Medicine, 333 Cedar Street, New Haven, Conn.

EXPERIMENTAL

Materials

Cholesterol, reagent grade, was recrystallized from glacial acetic acid and then from ethyl acetate. Silica Gel H and aluminium oxide H were supplied by E. Merck AG (Germany). Reagent grade solvents and $SbCl_3$ were used throughout the study.

Preparation of the samples

Five different samples prepared under the conditions given in Table I were analyzed. Crystalline cholesterol was placed in glass vessels of 20 mm inner diameter and air, saturated with water at 20°, was passed through the vessel at a slow flow rate.

TABLE I

DECOMPOSITION OF CHOLESTEROL

Sample	*Treatment*	*Temperature* (°C)	*Approx. dose* (*mrads*)	*Duration of treatment* (*h*)	*Remaining cholesterol*[a] (%)
A	Thermal	105	—	171.5	52.0
B	Thermal	98	—	143.0	45.7
C	γ-irradiation	50	51	89.4	15.0
D	γ-irradiation	50	79	142.3[b]	15.4
E	γ-irradiation	50	24.2	43.0[c]	74.2

[a] Determined by the present method.
[b] In air saturated with water and hydrogen peroxide.
[c] In dry air.

Each batch containing 3 g cholesterol was either heated in an oil bath or irradiated in a ^{60}Co-irradiator[8]. The samples were then dried *in vacuo* and powdered. Solutions were made up in chloroform which contained about 15 mg of the solid material per ml.

Separation of the samples

In order to obtain information about their composition, the samples were separated by the thin-layer chromatography (TLC) technique successfully used by VAN DAM *et al.*[9] to separate some oxidation products of cholesterol. For our purpose, a mixture of chloroform and acetone, 5:2 by volume, was found to be a suitable solvent system for plates coated with silica gel H. Fig. 1 shows the separation of the five different samples A–E.

The plate was divided in 20 mm strips and for optimal separation of the products the strips were narrowed to a width of 5 mm below the starting line by removing excess silica gel.

The plate was activated at 120° for 2 h and allowed to cool in air. The separation was obtained at approximately 27° and 40% relative humidity. 25 μl samples of the chloroform solutions containing 360–380 μg of auto-oxidized cholesterol were applied 30 mm from the bottom edge. The plate was irrigated with the above mentioned solvent system in a lined chamber filled to a height of 1 cm. The solvent was allowed to migrate 13 cm from the starting line (55 min) and the plate was dried at 100° for 3 min. The irrigation and drying were repeated in order to obtain a better separation.

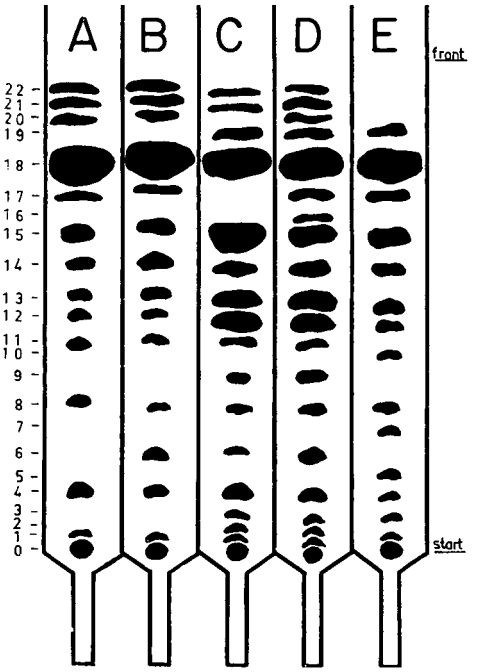


Fig. 1. Separation of auto-oxidized cholesterol samples A–E.

The components were detected by spraying with a saturated solution of $SbCl_3$ in chloroform, and after recording the color the plate was heated at 100° for 3 min. A 300 μg sample of the original cholesterol proved to be pure by this procedure.

The R_{Chol}-values, *i.e.* the R_F-values related to cholesterol, and the colors of the spots are given in Table II. Although the goal of the present work was not the identification of the products, some information has been obtained by using reference substances and detection methods for peroxides. Spot No. 18 was identified as cholesterol and spot No. 4 as cholestane-3β,5α,6β,-triol. Most probably, spots No. 12–13 represent the epimeric 7-hydroxy-cholesterols and No. 15 the cholesterol-5-hydroperoxide. The last one might arise from rearrangement of cholesterol-5-hydroperoxide in chloroform[10]. 7-Ketocholesterol and $\Delta^{3,5}$-cholestadien-7-one have been found in samples A and B using H_2SO_4 charring for detection. The position of these compounds corresponds to that of the spots No. 21–22 in Fig. 1. The presence of peroxidic compounds was confirmed by spraying with potassium iodide and starch solutions[11] or with a mixture of ferro-ammonium-sulfate and ammonium-thiocyanate-solutions[12].

PROCEDURE FOR QUANTITATIVE ANALYSIS

Reagents and solutions

Mixture of chloroform and acetone, 4:1 by volume. Glacial acetic acid containing 0.1 % $FeCl_3 \cdot 6H_2O$. Color reagent: 4 g of $FeCl_3 \cdot 6H_2O$ dissolved in a mixture of 100 ml of 70 % perchloric acid and 300 ml of 85 % phosphoric acid. Standard cholesterol stock solution prepared by dissolving 500 mg of cholesterol in 50 ml chloroform.

TABLE II

R_{Chol}-VALUES AND COLORS OF THE SPOTS IN FIG. 1

Colors were observed immediately after spraying with $SbCl_3$ solution and after heating the plate at 100° for 3 min. The results of the second observation are given in brackets. The peroxide reactions were obtained on other plates. The U.V. fluorescence was observed in long-wave ultraviolet light.

No.	R_{Chol}[a]	*Colors of the spots from samples*		
		A and B	*C and D*	*E*
0	0.00	blue (gray)[c]	orange (brown)[b, c]	pink (violet)[b, c]
1	0.04	— —	pink (brown)[b]	pink (violet)
2	0.07	— —	pink (brown)[b]	— —
3	0.09	— —	pink (brown)[b]	pink (yellow)
4	0.15	blue (yellow)[d]	blue (yellow)[d]	blue (yellow)[d]
5	0.20	— —	— —	— —
6	0.24	— —	pink (violet)	— —
7	0.31	— —	— —	— (brown)
8	0.37	blue (brown)	blue (brown)	— (brown)
9	0.46	— —	blue (brown)	— —
10	0.52	— —	— —	— (brown)
11	0.55	blue (brown)	— (violet)	— —
12	0.60	blue (blue)	blue (blue)	blue (blue)
13	0.65	blue (blue)	blue (blue)	blue (blue)
14	0.75	pink (brown)	pink (violet)	— (violet)
15	0.82	blue (blue)[b, c]	blue (blue)[b, c]	violet (violet)[b, c]
16	0.86	— —	— (brown)	— —
17	0.93	— (violet)	blue (brown)[c]	pink (violet)
18	1.00	orange (maroon)	orange (maroon)	orange (maroon)
19	1.08	— —	[d]	— (crimson)
20	1.12	blue (blue)	blue (blue)[d]	— —
21	1.16	violet (maroon)	— (maroon)[d]	— —
22	1.19	blue (violet)	violet (violet)[d]	— —

[a] R_F value related to cholesterol.
[b] Peroxidic reaction with KI–starch[11].
[c] Peroxidic reaction with Fe^{2+}–SCN^-.[12]
[d] Fluorescence in U.V.

Thin-layer chromatography

Glass plates 200 × 200 mm were coated uniformly with silica gel H to a thickness of 0.4 mm according to the procedure suggested by the supplier. After coating, the plates were dried in the atmosphere. Beginning at 10 mm from one edge, 1 mm channels spaced 20 mm apart were engraved with a jeweler's screwdriver. Nine strips of 20 mm were obtained, allowing nine separate samples to be run simultaneously. The plates were washed overnight in the usual manner in a chamber with ethanol and activated at 100° for 2 h. Immediately after cooling, the samples containing 20–200 μg cholesterol in a solution of chloroform were applied at a point in the center of each strip, 30 mm from the lower edge. For sampling, 50 μl Hamilton syringes with Chaney-adaptors were used and heat was applied to the bottom of the plates for samples larger than 20 μl in order to facilitate the evaporation of the solvent.

The plates were placed in a chromatography jar lined on three sides by filter paper and immersed to a depth of 10 mm from the lower edge in a mixture of chloroform–acetone, 4:1 by volume. The solvent was allowed to rise to a height of 160 mm above the starting points. This development required about 35 min. The plates were

removed and dried at 100° for 5 min and upon cooling were sprayed with distilled water. The spraying was started at a safe distance in order to avoid the peeling of dry silica gel by the air stream. Upon spraying, the cholesterol spots ($R_F \sim 0.6$) became visible by viewing the plate on black paper. From the center of the spots, distances of 8 mm in the starting direction and 12 mm in the flow direction were marked off on the strips. The marked parts of the wet silica gel strips were then transferred into 15 ml centrifuge tubes by using a micro spatula which had been ground flat at the end and bent 5 mm from this edge. The silica gel remaining on the spatula was removed with a stirring rod which was put in each centrifuge tube. Using two spatulas, one bent and one straight, the operation could be carried out more quickly. The centrifuge tubes were dried for 10 min at 100° and the silica gel scraped from the walls to the bottom by the stirring rods. To avoid the oxidation of the finely dispersed cholesterol, the samples must be worked up immediately.

Color development

2 ml of glacial acetic acid containing 0.1 % ferric chloride were added to each tube and the mixture was stirred vigorously with the stirring rod. The tubes were then heated in a water bath at 80° for 6 min.

After cooling, 1 ml color reagent was added to each tube and the contents were mixed thoroughly. The tubes were heated in an oil bath at 100° for 20 min while the liquid level was kept above the surface of the contents of the tubes. The tubes were allowed to cool in cold water and after removing the glass rods they were centrifuged at 3000 r.p.m. for 15 min. The supernatant solution was poured carefully into glass cuvettes of 10 mm path length and the absorbance was read using a Beckmann DK-2 spectrophotometer against a reagent blank at 450 mμ. The absorbance remained constant for about 2 h. The reagent blank was made from 2 ml of glacial acetic acid containing 0.1 % ferric chloride and 1 ml color reagent in the previously described manner omitting the centrifugation. From each batch of thin-layer plates, blanks were made with silica gel, which was scraped from a 4 cm^2 area at the edge of the plate. The absorbance of these blanks was found to be equal to that of the reagent blanks at 450 mμ.

Calibration curve and calculation

Solutions were prepared by diluting 2,4,6 and 8 ml of standard cholesterol stock solution to 10 ml with chloroform, and 20 μl samples containing 20, 40, 80, 120, 160 and 200 μg cholesterol were applied. The absorbances were measured after the described procedure above. The calibration curve obtained by plotting absorbance *versus* micrograms of cholesterol is a straight line passing through the origin. The cholesterol values of the samples were calculated from the calibration curve or conveniently from the ratio of the absorbance of the unknown sample to that of a standard cholesterol sample.

RESULTS AND DISCUSSION

Fig. 1 shows the pattern of the products in samples oxidized under different conditions such as elevated temperature or gamma irradiation. It is seen that the number of the degradation products is much greater than has been assumed on the

basis of previously reported results. However, there may be additional products which have not been separated or detected with the present technique. Although the qualitative and quantitative make-up of the samples varies with the conditions, a large number of identical products are present in each sample. This indicates that the present method also could be used for cholesterol determination in samples arising from other autoxidation processes. The separation of the samples shown in Fig. 1 has been achieved on specially prepared plates and with double irrigation in a tedious procedure. The quantitative determination of cholesterol does not require the separation of the products from each other but that of cholesterol from the products. The procedure used for this purpose is simple and gives reliable results as shown by the following tests.

Marking the spot area

For an equal amount of cholesterol the visible spot area is smaller after spraying with water than with $SbCl_3$-solution. Spots representing 80–300 μg of cholesterol were examined in order to mark that part of the silica gel strips which had to be transferred. After spraying with water, the spot lengths in the flow direction varied from 6 to 11 mm when the plates were examined on underlying black paper.

Spot lengths of 15–20 mm were measured after drying and spraying with $SbCl_3$-solution in transparent light on the same plate. The centers of the colored spots were generally 2 mm closer to the front of the spots. In agreement with this observation up to 250 μg of cholesterol can be transferred quantitatively after spraying with water if 20 mm of the strip beginning 8 mm below the center of the spots are scraped off. This order of marking has been verified by the following tests. 360–380 μg of samples A–E were separated. Silica gel areas containing cholesterol from triplicate samples were combined and extracted with 1.5 ml of chloroform. The solutions were concentrated to about 60 μl in a water bath and 25 μl samples were applied on two TLC-plates. One of them was coated with silica gel H, the other with aluminium oxide H. The plates were prepared and irrigated as described above. After treatment with $SbCl_3$, no spots were found close to that of cholesterol. However, spots were found with R_{Chol}-values of 0.63, 0.82 and 1.17 which correspond to those of spots No. 12–13, No. 15 and No. 21 in Fig. 1. These compounds should have been separated from cholesterol in the normal procedure in consideration of their R_{Chol}-values. The areas of these spots were significantly larger when the silica gel containing cholesterol was stored in air at room temperature for several days. Compounds showing the same spots arose from pure cholesterol which was adsorbed on silica gel, exposed to air at room temperature for a week and chromatographed as above. All these indicate that they are the primary autoxidation products of cholesterol. Because of the rapid autoxidation of cholesterol adsorbed on silica gel, the separated cholesterol must be processed immediately.

The existence of cholesterol outside of the marked area has been investigated. The contiguous 10 mm parts of the silica gel strips on both sides of the removed cholesterol spot areas were scraped off, extracted and chromatographed as above. No cholesterol was detected on spraying with $SbCl_3$ solution. Absorption spectra of the developed color between 300–1000 mμ were checked in the quantitative analysis of the five samples. In all cases, the shape of the spectrum was the same as that of the standard cholesterol samples. As expected, absorption spectra of the five samples

obtained by the color reaction without TLC separation were significantly different from that of cholesterol.

Effect of the sample volume

Chloroform solutions of sample A (see Table I) containing 0.8 μg, 3.2 μg and 40.0 μg solid material per μl were prepared. Sample volumes of 3000 μl, 75 μl and 6 μl, respectively were applied on TLC plates and analyzed in the described manner. The starting spot areas were less than 7 mm in diameter. Each sample contained 240 μg of sample A, *i.e.* 124.8 μg of cholesterol. The relative standard deviation of the measurement was 1.9 % from 10 determinations.

Reproducibiltiy of the measurement

Chloroform solutions of samples B and C (see Table I) containing 231 μg and 91.8 μg cholesterol in 30 μl were analyzed. The fractional standard deviations of each cholesterol value at 7 and 6 degrees of freedom were 1.81 and 1.75 %.

However, this loss and the decrease of color by silica gel are proportional to the amount of cholesterol in the range of 20–250 μg as indicated by good linearity *i.e.*, 0.34 % relative standard deviation of the calibration curve.

Comparison of different methods

The five samples examined have been analysed for cholesterol by three other methods which are:

(a) The SCHOENHEIMER-SPERRY method modified by SPERRY AND WEBB[3] which involves a digitonin separation.

(b) The BARR method[13] which is essentially the Liebermann-Buchard color reaction.

(c) The PPF-color reaction[7].

Microliter volumes of the chloroform solutions of samples A–E and of the standard cholesterol solutions used in the foregoing study were evaporated. The residues were dissolved in the particular solvent of each method and processed according to the descriptions.

Table III shows the results of these analyses compared with those obtained by the method described in this paper. As expected, the color reaction of cholesterol

TABLE III

CHOLESTEROL VALUES DETERMINED BY DIFFERENT METHODS

The figures in brackets represent the obtained values normalized to those measured by the present method.

Method	*Cholesterol (wt. %)*				
	A	*B*	*C*	*D*	*E*
SCHOENHEIMER-SPERRY method modified by SPERRY AND WEBB	57.6 (1.11)	53.3 (1.16)	17.7 (1.18)	21.4 (1.39)	82.6 (1.11)
BARR method	76.2 (1.46)	67.2 (1.26)	39.8 (2.65)	34.1 (2.21)	91.2 (1.22)
Direct color reaction with the PPF-reagent	63.6 (1.20)	61.9 (1.35)	34.4 (2.29)	19.4 (1.26)	80.6 (1.09)
Present method	52.0	45.7	15.0	15.4	74.2

separated with thin-layer chromatography results in lower values than the other methods where cholesterol was not or only partly separated. The extent of the differences depends on the qualitative and quantitative composition of the samples and varies with the methods. Generally, the variation is smaller in case of samples of high cholesterol content as the relative amount of interfering products is smaller. The best agreement with the results of the present method has been obtained by the SCHOENHEIMER-SPERRY method probably due to the separation by the digitonin precipitation. However, none of the compared previously known methods can be considered as reliable for analyzing such mixtures.

ACKNOWLEDGEMENTS

The author gratefully acknowledges and appreciates the assistance of G. L. BROWNELL under whose direction this work was performed.

This work was supported by the American Cancer Society, Grant No. T-82F and the U.S. Public Health Service, Grant No. 5T1 GM889-08.

SUMMARY

A method is presented for the quantitative determination of cholesterol in the presence of its oxidation and degradation products arising from thermal and radiation induced decomposition. The samples are chromatographed on silica gel coated thin layer plates with a chloroform–acetone mixture. Cholesterol becomes visible upon spraying with water. The spot is scraped off and transferred with the wet silica gel into a centrifuge tube. After drying, glacial acetic acid containing ferric chloride is added, and color is developed with perchloric acid–phosphoric acid–ferric chloride reagent in the slurry. After centrifugation the absorption of the liquid is measured at 450 mμ. The method can be used for the determination of 20–200 μg cholesterol with less than 2 % error.

REFERENCES

1 S. BERGSTRÖM AND B. SAMUELSON, in W. O. LUNDBERG (Editor), *Autoxidation and Antioxidants*, Vol. 1, Interscience, New York, 1961, p. 233.
2 L. N. NORCIA, *J. Am. Oil Chemists' Soc.*, 38 (1961) 238.
3 W. M. SPERRY AND M. J. WEBB, *J. Biol. Chem.*, 187 (1950) 97.
4 F. RADT (Editor), *Elsevier's Encyclopaedia of Organic Chemistry*, Vol. 14, Suppl., A. GEORG (Editor), *Steroids*, Elsevier, Amsterdam, 1956, pp. 2066s, 2068s.
5 A. E. SOBEL, M. GOLDBERG AND S. R. SLATER, *Anal. Chem.*, 25 (1953) 629.
6 L. S. ETTRE, personal communication.
7 T. MOMOSE, Y. UEDA, K. YAMAMOTO, T. MASUMURA AND K. OHTA, *Anal. Chem.*, 35 (1963) 1751.
8 G. L. BROWNELL, K. STRATTON AND J. L. PINTER, *Phys. Med. Biol.*, 8 (1963) 265.
9 M. J. D. VAN DAM, G. J. DE KLEUVER AND J. G. DE HEUS, *J. Chromatog.*, 4 (1960) 26.
10 G. O. SCHENCK, O. A. NEUMÜLLER AND W. EISFELD, *Ann.*, 618 (1958) 185.
11 K. RANDERATH, *Thin Layer Chromatography*, Academic Press, New York, 1963, p. 210.
12 M. H. ABRAHAM, A. G. DAVIES, D. R. LLEWELLYN AND E. M. THAIN, *Anal. Chim. Acta*, 17 (1957) 499.
13 W. R. BLOOR, *J. Biol. Chem.*, 24 (1916) 227.

QUANTITATIVE SCANNING OF FLUORESCENT OR LIGHT-ABSORBING SPOTS ON THIN-LAYER CHROMATOGRAMS

H. T. GORDON
Division of Entomology and Acarology, University of California, Berkeley, Calif. (U.S.A.)
(Received September 23rd, 1965)

Many different instruments have been used for the direct measurement of light absorbance, fluorescence, or radioactivity on chromatograms, although this is generally considered to be less accurate than elution of spots for quantitative analysis[1–3]. During current research on thin-layer chromatography (TLC) of fluorescent urethanes of sterols and other higher alcohols it became clear that a fluorescence scanning apparatus was essential to provide permanent and at least roughly quantitative records of the chromatograms. Few scanners are available commercially, and their performance is not usually evaluated thoroughly. Our laboratory uses the relatively new Aminco Fluoro-Microphotometer (American Instrument Company, Silver Spring, Md.) for fluorimetry of solutions. This instrument is sensitive and stable but does not yet have an accessory for scanning thin-layer plates. Such an accessory has therefore been designed and constructed, and its performance is being reported in this paper.

DESCRIPTIONS OF SCANNING ACCESSORY

The instrument was designed to fit the Aminco Fluoro-Microphotometer with a minimum of modification and to be quickly insertable and removable to allow normal use as a solution fluorimeter or colorimeter. The sample chamber of the Fluoro-Microphotometer is a metal well 25 mm², with a depth easily extendable to 25 cm. Light (from interchangeable ultraviolet or visible lamps) enters through a 32 × 18 mm first aperture into a primary filter compartment that allows use of interchangeable light filters 50 mm² and then passes through a 32 × 18 mm second aperture into the sample chamber. Fluoresced, scattered, or reflected light passes through a 32 × 18 mm exit aperture at a right angle to the entry aperture, through a secondary filter compartment to a photomultiplier tube.

The mechanical and electrical systems of the scanner were designed and constructed by J. DIMICK (Dimick Enterprises, El Cerrito, Calif.) to perform according to the specifications of the author. The metal frame slides in vertically to the full depth of the Aminco sample chamber and is attached by a set screw. The frame carries a vertical metal movable plate, 250 × 28 mm, which is positioned diagonally in the sample chamber and can support glass plates having maximum thickness of about 3.2 mm, width of 26 mm, and length of about 200 mm. Such plates are routinely used in our laboratory for one-dimensional TLC. To the back of the metal slide is affixed a helical rack, which is driven by a helical pinion attached to a 1 r.p.m.

reversible synchronous motor, swivel-mounted so that the pinion can be quickly disengaged from the rack. The vertical motion is at a rate of 18.7 mm/min. There are upper and lower limit switches that restrict the travel to a maximum of 183 mm, starting about 3 mm from one end. A full scan takes 9.8 min. A reversing switch allows immediate shifting from downwards to upward motion. A tubular metal cover protects the light chamber from interference by room light.

The optical systems were designed by the author. For fluorescence scanning it is desirable to project on the thin-layer surface a horizontal band of light, as intense and uniform as possible. This is approximated by using a cylindrical lens (5.5 mm wide, focal length 5.6 mm) mounted on an interchangeable metal slide within the sample chamber and directly in front of the second entry aperture. The lens projects a widened image of a 1 × 3 mm rectangular slit in an interchangeable plastic slide mounted in front of the first entry aperture near the fluorescent lamp. Since the image is projected on a surface set at a 45° angle to the lens and the slit is illuminated by the very diffuse light from a GE F4T4/BL fluorescent lamp the band of light on the thin-layer surface is about 16 mm wide and not perfectly uniform in thickness or intensity.

The nature of the light band produced by this optical system was analysed by applying to a thin-layer plate a series of 1 μl spots of a fluorescent solution, positioned vertically 5 or 10 mm apart and horizontally at distances from 0 to 5 mm to the right or left of the center line. The spots had a radius of about 2 mm. When this test plate was scanned, the peak fluoresced light intensity proved to be the same for spots positioned 0 to 3 mm on either side of the center, but it decreased to 90 % of the maximum value at 4 mm and 80 % at 5 mm. Resolving power was measured by the intensity of fluoresced light in the center of the 1-mm wide dark region between spots separated by a vertical distance of 5 mm. This intensity was about 7 % of the maximum in the region from 0 to 5 mm to the left of center where the distance from lens to surface varies from 3 to 8 mm. To the right of center resolving power decreased, minimum light intensity being 25 % of maximum at 5 mm to the right. While resolving power is somewhat increased by using a 0.5 × 3 mm light slit, the major factor lowering resolution seems to be broadening of the effective light band by internal reflections within the thin layer.

The light system for fluorescence scanning can be used for reflectance scanning of light-absorbing spots, but the reflected blank light is so intense that a 0.1 × 3 mm slit is preferable; it may even be necessary to introduce a light-reducing aperture in the secondary filter compartment. The extremely wide band has the advantage that the full width of the absorbing spot is scanned, *i.e.*, all the material in the spot contributes to absorption of light. However, sensitivity (as measured by peak reduction in light intensity) is relatively low since a large fraction of the band of light never encounters any absorbing material. If a pinpoint light source is used instead of a slit the cylindrical lens still spreads it out into a relatively wide but non-uniform band of light. Therefore a second lens system, a double-convex lens (5 mm in diameter, focal length 7 mm) mounted on an interchangeable metal slide, is used to replace the cylindrical lens. The circular lens, used with a pinpoint source, can project a very small spot of light on the center of the thin-layer plate. Such a system, however, is very sensitive to small changes in shape and centering of the spot; even if the substance being chromatographed is applied as a wide band, perfect uniformity is diffi-

cult to achieve. Use of a pinpoint source has therefore been discontinued. For high-sensitivity scanning, the combination of the circular lens with a 0.1 × 3 mm slit was adopted; this projects a thin uniform band of light about 4 mm wide on the plate. This is wide enough to cover most spots or at least to have an averaging effect over the greater part of the spot width, and yet not so wide that a large fraction of the light fails to encounter the spot. It is affected by serious off-centering of the spot; however, this can be minimized by spotting a fairly wide band of material initially. Even if the initial band is not homogeneous, the averaging effect of scanning with a band of light tends to correct for this.

OPERATION, CONTROLS AND RECORDING

The recorder output of the Aminco photometer is 50 mV and any potentiometric recorder of 100 mV or higher sensitivity, 1 sec or faster response, and chart speed of the order of 20 mm/min will give satisfactory scans. We have used a Bausch and Lomb VOM-6 recorder at 25 mV sensitivity and chart speed of 25 mm/min; this amplifies the photometer output by 2, and its zero-setting control is sometimes useful to keep scans on-scale.

Except for occassional use of the zero-setting control of the recorder (and changes of lens and slit systems) all operating adjustments are made with the Fluoro-Microphotometer controls. The zero-adjust control allows setting the recorder pen to zero or 100 in the blank region of a chromatogram at the start of a scan. The meter-multiplier control allows switching of photometer sensitivity over a 1000-fold range in steps of 1.0, 0.3, 0.1, etc. The light systems of the scanner are designed so that the maximal sensitivity required is usually not greater than 0.01, where zero stability of the photometer is very high. Even the most intense spots can then be scanned at a sensitivity of 1.0.

The first scan is normally in the downward direction at maximum sensitivity. If an intense spot gives an off-scale scan the chromatogram can be re-scanned in the upward direction at a lower sensitivity by using the scanner reversing switch. Time can often be saved by reversing the scanner as soon as an off-scale spot has been scanned, simultaneously decreasing sensitivity 10-fold and readjusting the zero setting. If the reverse-scan is still off-scale or now gives too small a peak, the scanner is again reversed and sensitivity readjusted. This allows satisfactory scanning of the intense spot in a few minutes. While the area of a scanned spot can be measured with a planimeter, it is more convenient to approximate it to an isosceles triangle and measure peak height and base width in mm. The product of height × base × photometer sensitivity is the "relative area", which measures the light intensity change caused by the spot. If a spot is so spread out that it does not scan as a triangle, it is probably best to spot a more dilute solution and chromatograph it.

Scanner performance with fluorescent spots using adsorption chromatography on silica gel G

In all tests the scanning system has always given identical repetitive scans of any one thin-layer plate. The performance tests indicate the sensitivity and range normally attainable, but the variability found is entirely in the chromatographic operation.

The fluorescent test substance used was a crystalline preparation of the fluoranthenyl urethane of cholesterol, m.p. 184–186°, prepared from recrystallized cholesterol (m.p. 144–145°) and fluoranthenyl-3-isocyanate synthesized from commercial 3-aminofluoranthene and phosgene. A stock solution in toluene at 0.163 μg/μl (equivalent to 0.1 μg/μl of cholesterol), and a 1:8 dilution in toluene (equivalent to 0.0125 μg/μl of cholesterol) were prepared. Test spots were applied to the silica gel G plates (thickness of layer about 850 μ wet, 625 μ dry) in the center 10 mm from one end. A Hamilton PB-600-1 repeating dispenser with a Hamilton 705 50-μl syringe (Hamilton Company, Inc., Whittier, Calif.) was used to deliver 1-μl droplets. Quantities of 1, 2, 4, 8 and 16 μl were applied, each droplet being allowed to dry before applying the next. Chromatograms were developed in a 1:1 mixture of cyclohexane and benzene, completed in less than an hour, then dried for 10–15 min before scanning. When a plate was re-scanned several hours later the scan was identical, but after 24 h there was slight loss of fluorescence intensity.

The use of unusually thick layers made the plates even more fragile than the usual 200–300 μ layers. The intention was to increase the capacity, but the greater fragility is an inconvenience during chromatography and scanning of thinner layers would normally be preferable.

Table I lists the data for triplicate runs. Scanning used the 1 × 3 mm slit and cylindrical lens, with a primary filter of peak transmittance at 365 mμ (Corning 7-60) and a secondary filter cutting off wavelengths below 485 mμ (Wratten 8). The relative areas of triplicates show standard deviations of the order of 20%, caused by large deviations in peak heights or base widths. The mean relative area per μg of cholesterol, however, is remarkably constant over a wide range, although it declines above 0.4 μg of cholesterol. Spotting of a large volume of a dilute solution or a small volume of more concentrated solution does not seem to affect the scans. Base width of peaks is increased less than 2-fold by a 16-fold increase in volume spotted and a 100-fold increase in quantity spotted. R_F values vary; but there is no striking change with increasing load, nor any clear correlation between R_F and relative area in replicates. It seems probable that most of the fluoresced light is emitted from the upper 100 μ of the thin layer. The variability may be caused by variations in the quantity of fluorescent substance migrating upward into this surface layer as the chromatogram dries. It would probably be less if a 200 or 300 μ thick layer were used, and might also be reduced by making drying conditions uniform.

Scanner performance with fluorescent spots using adsorption and partition chromatography on Avicel–silica

Avicel–silica forms extremely tough layers on glass, and is used routinely in our laboratory for partition chromatography, in which the layer must be pre-coated with a stationary phase liquid.

The microcrystalline cellulose, Avicel (American Viscose Division of FMC Corporation, Newark, Del., U.S.A.) was introduced for TLC by WOLFROM *et al.*[4]; in our experience, layers of pure Avicel have been less satisfactory than 1:1 mixtures of Avicel and silica (without binder). The 1:1 Avicel is prepared by mixing 20 g of Avicel and 20 g of silica with 200 ml of distilled water in a Waring blendor at high speed for 15 min. The warm, creamy mixture is briefly subjected to a water-pump vacuum while gently shaking, to remove air bubbles. It is then spread on the glass

TABLE I

SPOT SCAN DATA FOR ABSORPTION CHROMATOGRAMS OF FLUORANTHENYL URETHANE OF CHOLESTEROL ON SILICA GEL G

μl spotted		*Solution**	*μg cholesterol*	*Photometer sensitivity*	*Height × base (mm × mm)*	*Relative area ***	*Mean rel. area*	*Std. dev. (%)*	*Rel. area per μg cholesterol*	$R_F \times 100$
	a				44 × 14	1.848				44
1	b	A	0.0125	0.003	47 × 14	1.974	2.002	0.175	160	45
	c				52 × 14	2.184		(8.7%)		41
	a				33 × 12	3.96				47
2	b	A	0.025	0.01	62 × 10	6.2	4.59	1.41	184	43
	c				30 × 12	3.6		(31%)		39
	a				62 × 18	11.16				44
4	b	A	0.05	0.01	58 × 18	10.44	9.35	2.53	187	42
	c				37 × 18	6.46		(27%)		42
	a				39 × 14	15.38				49
8	b	A	0.10	0.03	50 × 11	16.5	18.62	4.76	186	50
	c				47 × 17	23.97		(25.6%)		46
	a				36 × 21	26.68				47
16	b	A	0.20	0.03	76 × 19	43.32	31.32	9.21	157	42
	c				56 × 20	33.6		(29.6%)		48
	a				37 × 17	18.87				50
1	b	B	0.10	0.03	40 × 17	15.3	17.69	2.07	177	51
	c				35 × 18	18.9		(11.7%)		46
	a				59 × 17	30.09				43
2	b	B	0.20	0.03	109 × 10	32.7	35.33	6.81	177	45
	c				90 × 16	43.2		(19.3%)		42
	a			0.03	109 × 21	68.67				41
4	b	B	0.40	0.1	63 × 15	94.5	73.39	22.0	183	53
	c			0.1	30 × 17	51.0		(30%)		42
	a				32 × 21	67.2				
8	b	B	0.80	0.1	47 × 22	103.4	82.1	19.0	103	47
	c				36 × 21	75.6		(23%)		
	a			0.1	67 × 24	160.8				51
16	b	B	1.60	0.1	95 × 24	228	185.5	23.5	116	
	c			0.3	43 × 13	167.7		(12.7%)		57

* Solution A contains cholesterol (as the fluoranthenyl urethane) at 0.0125 μg/μl and solution B at 0.10 μg/μl.

** Relative area is peak height × base × photometer sensitivity.

TABLE II

SPOT SCAN DATA FOR 3 TYPES OF CHROMATOGRAMS OF FLUORANTHENYL URETHANE OF CHOLESTEROL ON AVICEL–SILICA

μl spotted		*Type of chromatogram**	*Solution***	*μg cholesterol*	*Photometer sensitivity*	*Height × base (mm × mm)*	*Relative area****	*Mean rel. area*	*Std. dev. (%)*	*Rel. area per μg cholesterol*	R_F
	a					15 × 13	1.95				0.35
1	b	A	A	0.0125	0.01	21 × 13	2.73	2.44	0.43	195	0.39
	c					24 × 11	2.64		(17.6%)		0.45
	a					43 × 12	5.16				0.23
1	b	P-1	A	0.0125	0.01	32 × 12	3.84	4.60	0.69	368	0.17
	c					40 × 12	4.80		(15.0%)		0.27
	a					18 × 18	3.24				0.47
1	b	P-2	A	0.0125	0.01	27 × 12	3.24	3.12	0.21	249	0.57
	c					24 × 12	2.88		(6.7%)		0.49
	a					34 × 12	12.24				0.44
1	b	A	B	0.10	0.03	28 × 15	12.6	12.36	0.21	123.6	0.45
	c					34 × 12	12.24		(1.85%)		0.50
	a					52 × 15	23.4				0.23
1	b	P-1	B	0.10	0.03	47 × 17	23.97	24.61	1.64	246.1	0.38
	c					63 × 14	26.46		(6.66%)		0.15
	a					36 × 18	19.44				0.59
1	b	P-2	B	0.10	0.03	37 × 21	23.31	20.71	2.27	207.1	0.58
	c					38 × 17	19.38		(11.0%)		0.58
	a					26 × 21	163.8				0.57
16	b	A	B	1.60	0.3	26 × 19	148.2	148.8	14.8	93	0.54
	c					28 × 16	134.4		(9.94%)		0.66
	a					56 × 16	268.8				0.36
16	b	P-1	B	1.60	0.3	38 × 28	319.2	287.2	28.0	180	0.24
	c					38 × 24	273.6		(9.75%)		0.24
	a					53 × 23	365.7				0.82
16	b	P-2	B	1.60	0.3	43 × 22	283.8	314.5	45.1	197	0.55
	c					49 × 20	294.0		(13.9%)		0.68

* A signifies adsorption chromatograms developed in cyclohexane–benzene (1:1); P-1 signifies partition chromatograms using 7.5% phenylacetonitrile–12.5% β-methoxypropionitrile in acetone to form the stationary phase, with development in isooctane; P-2 signifies partition chromatograms using 25% β-methoxypropionitrile in acetone to form the stationary phase, with development in isooctane.

** Solution A contains cholesterol (as the fluoranthenyl urethane) at 0.0125 μg/μl and solution B at 0.10 μg/μl.

*** Relative area is peak height × base × photometer sensitivity.

TABLE III

BAND SCAN DATA FOR PARTITION CHROMATOGRAMS OF *n*-OCTANOYLAMIDE OF 1-PHENYLAZO-2-AMINONAPHTHALENE ON AVICEL–SILICA

Partition chromatograms using 25% β-methoxypropionitrile in acetone to form the stationary phase, followed by development in isooctane. Most of the chromatograms showed some tailing, especially at higher loads, but only the main roughly triangular peak area was measured. Tailing may be caused by irreversible adsorption of part of the load, and contributes to the variance in relative area of replicates. The 12 chromatograms (3a to 24c) were scanned 3 times. The first set of scans (run at times from 24 to 62 min after chromatography) and the second set (run at 72 ± 3 h after chromatography) used the 0.1 × 3 mm slit and cylindrical lens system. The third set (run at 74 ± 2 h after chromatography) used the 0.1 × 3 mm slit and circular lens system. The higher variance of the scanning, especially with 3a and 6c, is caused by off-centering of the band.

μl spotted	*Time interval**	*μg*	*Height × base (mm × mm)*	*Relative area* **	*Mean rel. area*	*Std. dev. (%)*	*Rel. area per μg*	*K****	*R_F*
a	50 min		12 × 14	5.04					0.72
3 b	44 min	3	13 × 15	5.85	4.93	1.2	1.64	44	0.67
c	58 min		10 × 13	3.90		(24.2%)			0.70
a	40 min		34 × 15	15.3					0.70
6 b	46 min	6	19 × 14	7.98	10.32	3.49	1.69	52	0.67
c	43 min		16 × 16	7.68		(33.8%)			0.67
a	50 min		30 × 18	16.2					0.67
12 b	56 min	12	41 × 15	18.4	17.3	0.9	1.44	52	0.76
c	62 min		44 × 13	17.2		(5.2%)			0.57
a	40 min		42 × 18	22.7					0.68
24 b	24 min	24	46 × 16	22.1	25.5	4.44	1.06	51	0.77
c	30 min		59 × 18	31.8		(17.4%)			0.64
a			19 × 16	9.10					
3 b	72 h	3	18 × 16	8.63	7.71	2.01	2.57	69	
c			12 × 15	5.40		(26.1%)			

6	a b c	72 h	6	38 × 16 23 × 16 20 × 16	18.2 11.0 9.6	12.9	4.62 (35.8%)	2.15	65
12	a b c	72 h	12	37 × 19 54 × 15 51 × 13	21.1 24.3 19.9	21.8	2.27 (10.4%)	1.82	65
24	a b c	72 h	24	47 × 18 52 × 18 63 × 17	25.4 28.1 32.2	28.6	3.42 (12.0%)	1.19	57
3	a b c	74 h	3	20 × 14 47 × 17 33 × 17	8.40 23.97 16.83	16.40	7.79 (47.5%)	5.47	147.5
6	a b c	74 h	6	66 × 16 70 × 19 16 × 18	31.68 39.90 8.62	26.73	16.2 (61.2%)	4.45	134
12	a b c	74 h	12	53 × 20 92 × 15 94 × 14	31.2 41.4 39.4	37.3	5.4 (14.5%)	3.11	112
24	a b c	74 h	24	101 × 21 82 × 16 117 × 19	63.8 39.3 66.8	56.6	15.1 (26.8%)	2.36	113

* The time interval in min or h between removal of a plate from the developing solvent and beginning of the scan. No notable changes were noted during the first hour or two. Intervals marked 72 h in fact varied from 68 to 74 h.

** All scans were run at a photometer sensitivity of 0.03. Relative area is peak height × base × photometer sensitivity.

*** The hyperbolic constant, K, is (relative area) × (24 + load in μg)/(load in μg).

plates, at wet thicknesses from 400 to 800 μ (adequate to cover one or two 20 $\times$ 20 cm plates), which dry to layers of about half this thickness after standing overnight at room temperature. The layers (like those of pure Avicel) are so firm that plates can be stacked together without damaging the layer, and also can be written on with pencil. They give excellent results both in adsorption and partition chromatography, even with solvents containing 20 % water. For partition chromatography, spots are applied and allowed to dry and then the plates are coated with a solution of a stationary phase liquid in acetone, using a 1-ml pipet. The solution is first applied to the region below the spot, then directly above the spot, moving away from the spot to the upper end. Since the solution flows into the spotted area, the spots are compressed into fairly narrow bands. After air-drying for a few minutes to allow evaporation of the acetone, the plates are dipped (to a depth of 4 or 5 mm) in the mobile phase solvent for development, in a chamber saturated with stationary phase solvent vapor.

Only 3 levels of the fluoranthenyl urethane of cholesterol were tried, equivalent to 0.0125, 0.1 and 1.6 μg cholesterol. However, 3 different chromatographic systems were used: (1) adsorption chromatography using cyclohexane–benzene (1:1), as with silica gel G, (2) partition chromatography using a pre-coat of 7.5 % phenylacetonitrile and 12.5 % β-methoxypropionitrile in acetone, followed by development in isooctane, and (3) partition chromatography using a precoat of 25 % β-methoxypropionitrile in acetone, followed by development in isooctane. In partition chromatograms some shifting of the moving spot to the right or left of the center frequently occurs, since perfectly uniform pre-coating is technically difficult. Fortunately, the width of the scanning band of light is usually sufficient to encompass such deviations from the true center.

The data are listed in Table II. Scanning used the same conditions as in the work with silica gel G. There is less variation among triplicates than in the tests with silica gel G, possibly because the layers were thinner. The partition chromatograms show less variation than the adsorption chromatograms. As with silica gel G there is relatively little change in base width with increasing load. The relative area per μg of cholesterol, however, shows a consistent tendency to decrease as load increases, *i.e.*, calibration curves would be less linear than those for silica gel G. Highest sensitivity at low loads is shown by the phenylacetonitrile–β-methoxypropionitrile chromatograms. The phenylacetonitrile is of low volatility and has high solvent power; during drying it would tend to rise to the surface of the thin layer, tending to retain small loads of the fluorescent compound in solution. With large loads, however, a smaller fraction of the load can thus be carried into the upper surface. Chromatograms coated with β-methoxypropionitrile, which is a moderately volatile liquid, seem to carry a more constant fraction to the surface during drying at all loads, giving relatively better linearity. Adsorption chromatograms using the volatile benzene–cyclohexane solvent show a less efficient and constant surface-transfer effect, with lower sensitivity and less linearity. Avicel–silica layers are less translucent than silica gel G layers of equal thickness, so that less efficient light penetration is to be expected; this may enhance the effect of variation in concentration into the surface layer during drying. Nevertheless, sensitivity is of the same order of magnitude for all types of chromatography; spots barely detectable by the human eye can be quantitatively scanned. Sensitivity is also of the same order of magnitude

attained by fluorimetry in 2 ml volumes of chloroform. The major limiting factor is the apparent background fluorescence; as pointed out by UDENFRIEND[5] this includes Raman scatter, and adsorption and quenching effects may also lower sensitivity.

Scanner performance with light-absorbing spots

Only Avicel–silica layers, about 350 μ thick, were used. The test substance was a crystalline sample of the *n*-octanoylamide of 1-phenylazo-2-aminonaphthalene (m.p. 91–92°), prepared by the author, as a 1 μg/μl solution in toluene. This yellow azo dye absorbs 360 mμ light strongly, and chromatograms were scanned with the long-wave U.V. source and primary filter used for the fluorescence work but without a secondary filter. A light-reducing slit 2 mm wide was used in the secondary filter compartment to lower the light intensity on the photomultiplier tube. The optical system consisted of the 0.1 × 3 mm slit and cylindrical lens.

Three 1-μl spots (total 3 μg) centered 3 mm apart were applied to the center region of a starting line, 10 mm from one end of the plate. These generate a band 6 to 12 mm wide when chromatographed. Loads were increased by overspotting the initial spots to give 6, 12 and 24 μg. If protected from light (which decomposes azo dyes) spots do not fade with time; in fact, there is an increase in intensity after 1 or 2 days.

The scanning data are presented in Table III. Relative areas (over the limited 8-fold range tested) are neither a linear nor a logarithmic function of the load; the relation seems to be hyperbolic, the factor K [(mean rel. area) × (24 + load in μg)/(load in μg)] being nearly constant. The reason seems to be that at loads of the order of 10 μg the TLC system becomes saturated so that peak density no longer increases much with load; differences in scans are then largely caused by increases in spot size. The width of the 12 and 24 μg spots extends beyond the linear range of the light band formed by the cylindrical lens. Table III also presents data from the re-scanning of the same plates after storage in the dark for 2 days. All peak heights are increased, suggesting continued migration of dye into the upper surface of the thin layer. Variability among replicates remains high. The approximately hyperbolic relation between relative area and load still holds.

Absorbance scanning therefore seems useful over a no more than 10-fold range in load, unlike fluorescence scanning which has a 100-fold range (and 100-fold greater sensitivity). The optimal conditions for absorbance measurement are probably spotting of an initial band at least 10 mm wide (*e.g.*, four 1-μl spots 3 mm apart) and scanning with the 4-mm wide light band formed by the circular lens. Although only a fraction of the absorbing band will be scanned, it will be a relatively constant fraction and relatively unaffected by off-center migration. This technique should be satisfactory for a 2 to 20 μg range (depending on the molecular extinction coefficient, this may be higher or lower). Quantities greater than 20 μg usually exceed the capacity of TLC, so that compactness of bands and resolving power are diminished.

One can conclude that the limiting factor in quantitative scanning is the reproducibility of replicate chromatograms. Presumably this can be improved by extreme care in controlling the nature of the thin layer and the chromatography and drying of the plate. Even so, quantitative data should be based on triplicate runs to allow estimation of the variance, and on calibration curves of standards run under identical conditions.

ACKNOWLEDGEMENTS

The author is indebted to Dr. D. JENSEN and Dr. R. L. USINGER for their support of this work, and to JOHN DIMICK for his major contribution to the design and construction of the scanning accessory. Most of the chromatography and scanning was done by Miss JANICE JOHNSON, and Miss GLORIA BRO assisted in the preparation of the manuscript.

SUMMARY

The construction and operation of a scanning accessory for 25 × 200 mm TLC plates adapted to the Aminco Fluoro-Microphotometer are described. Fluorescent compounds can be quantitatively scanned in the range from 0.005 to 5 μg, but calibration curves are not linear. Repeated scans of any one plate are identical, but the standard deviation of triplicates is high (10 to 20 %) because of variations in dispersion of the fluorescent compound in the thin layer during chromatography and drying. Light-absorbing compounds of high molecular extinction coefficient can be quantitatively scanned by reflectance in the range from 1 to 20 μg; calibration curves are not linear and triplicates also show high variance caused by chromatography and drying.

REFERENCES

1 I. E. BUSH, *Methods Biochem. Anal.*, 11 (1963) 149.
2 J. ROSENBERG AND M. BOLGAR, *Anal. Chem.*, 35 (1963) 1559.
3 R. L. SQUIBB, *Nature*, 198 (1963) 317.
4 M. L. WOLFROM, D. L. PATIN AND R. M. DE LEDERKREMER, *Chem. Ind. (London)*, (1964) 1065.
5 S. UDENFRIEND, *Fluorescence Assay in Biology and Medicine*, Academic Press, New York, 1962, 505 pp.

CHROMATOGRAPHIE DER ZWISCHENPRODUKTE DER SYNTHESE VON ISOPHYTOL UND VITAMIN A

K. MACEK UND S. VANĚČEK
Forschungsinstitut für Pharmazie und Biochemie, Prag (Tschechoslowakei)
(Eingegangen den 21. September 1965)

Pseudojonon gehört zu den wichtigen Parfümerie-Produkten, und ist nebenbei auch ein Zwischenprodukt der Synthese von Vitamin A. Wenngleich auch für die Darstellung des Pseudojonons verschiedene Reaktionsbedingungen ausgenutzt werden, haben die Mehrzahl der Verfahren die Zwischenprodukte 2-Methyl-2-hepten-6-on, Dehydrolinalool und Citral gemeinsam. Pseudojonon kann auch weiterhin als Ausgangsprodukt für die Synthese des Isophytols dienen, und daher auch des Vitamins E und K. Die Synthese des Isophytols ist allgemein durch eine Verlängerung der Kette um 1 bis 3 Kohlenstoffatome charakterisiert. Einige der bedeutendsten Vorgänge der Synthese des Vitamins A sind mit dem Aufbau seines Zwanzigkohlenstoff-moleküls begründet, und zwar aus den Bestandteilen C_6 (3-Methyl-2-penten-4-in-1-ol) und C_{14} (β-C_{14}-Aldehyd, das aus Pseudojonon über β-Jonon gewonnen wurde). Die Mehrzahl dieser Verfahren kann in einer Reihe von Übersichtsreferaten (z.B. FRAGNER[1], ISLER u. Mitarb.[2,3], BELOV UND SHKVORTSOVA[4] u.a.) gefunden werden.

In Anbetracht der komplizierten Synthese der angeführten Substanzen, wobei in vielen Stufen ein reiches Gemisch von Stoffen flüssigen Charakters, oft Stereoisomeren, entsteht, versuchten wir ein einheitliches Analysenverfahren einiger möglicher Zwischenprodukte unter Anwendung der chromatographischen Methoden auszuarbeiten. Mit Hinsicht auf den Charakter der Substanzen und da die Notwendigkeit besteht, schnelle und quantitative Ergebnisse zu erzielen, wählten wir als Grundmethode die Gaschromatographie. Nur in wenigen Fällen war es notwendig, die Dünnschicht- oder Papierchromatographie anzuwenden.

Die Anwendung der chromatographischen Verfahren für die Verfolgung der gesamten Synthese des Isophytols und des Vitamins A ist bisher noch nicht beschrieben worden. Abgesehen von den schon mehrmals chromatographierten Substanzen mit niedrigem Siedepunkt in der ersten Phase der Synthese von Pseudojonon, sind von Substanzen dieser Gruppe bisher meistens die Parfümerie-Produkte, das Pseudojonon und das Citral, chromatographiert worden. NAVES[5] chromatographierte beispielsweise Isomere des Citrals auf Siliconöl D.C. 200 bei einer Temperatur von 160°, Isomere des Pseudojonons bei 220°, KINGSTON[6] Citral auf Carbowax bei 131° und YANOTOVSKIĬ[7] die Isomere des Citrals und des Pseudojonons auf Polyäthylenglycol 4000 bei 180°. Diese Substanzen wurden ebenfalls auf Dünnschichten chromatographiert: STAHL[8] trennte Methyl-2-hepten-6-on und Citral auf Kieselgel G durch Entwicklung mit Benzol, wobei er die Flecke mit 2,4-Dinitrophenylhydrazin sichtbar machte, und DHONT UND DIJKMAN[9] analysierten Pseudojonon in derselben Anordnung, jedoch unter Anwendung von sechsfacher Entwicklung.

Chromatographische Methoden wurden auch bei der Synthese des Isophytols mehrmals angewendet. Die Gaschromatographie verwendete z.B. RUDENKO u. Mitarb.[10] für die Bewertung von Pseudojonon und des hydrogenierten Produktes. Sie arbeiteten auf Siliconelastomer bei einer Temperatur von 170°, TEISSERE UND CORBIER[11] dagegen trennten ähnliche Produkte auf Propylenglycolpolysebacinat bei einer Temperatur von 150°. Für die Chromatographie des Phytadiens wurde von MAYER u. Mitarb.[12] Polyäthylenglycoladipat als stationäre Phase verwendet. DEMOLE UND LEDERER[13] trennten Phytol von Isophytol mittels der Chromatographie auf Kieselgel-G-Schichten und entwickelten mit einem Gemisch von *n*-Hexan–Äthylacetat (85:15), STAHL[8] dagegen verankerte auf Kieselgel G das Paraffin und entwickelte mit 70 % Methanol.

Die meisten Arbeiten waren der Bewertung der Synthese von Vitamin A aus Pseudojonon gewidmet, und dies einerseits zur Trennung des Pseudojonons von β-Jonon, jedoch hauptsächlich für die Bewertung des Endproduktes, des Vitamins A und seiner verschiedenen Ester. Die Gaschromatographie wurde von mehreren Autoren angewendet. YANOTOVSKIĬ[7] trennte Pseudojonon, β-Jonon und β-C_{14}-Aldehyd auf Polyäthylenglycol 4000 bei 180° und auf Siliconelastomer bei 120°, RUDENKO u. Mitarb.[10] trennten Pseudojonon und β-Jonon gleichfalls auf Siliconelastomer bei 170°, GRISLER[14] trennte Pseudojonon von α- und β-Jonon auf Apiezon L bei 180° und ARATANI u. Mitarb.[15] dieselbe Stoffe auf Asphalt bei 180° bzw. 200°. Mit Ausnahme der Trennung von Pseudojonon und β-Jonon (DHONT UND DIJKMAN[9]) wurde die Dünnschichtchromatographie in dieser Phase hauptsächlich für die Bewertung des Vitamins A und seiner verschiedenen Ester angewendet (siehe Übersicht von BOLLIGER[16]). Für dengleichen Zweck fand auch die Papierchromatographie häufige Anwendung (siehe Übersicht beispielsweise von HAIS[17]).

EXPERIMENTELLER TEIL

Gaschromatographie

Geräte. Beckman GC-2 mit Flammenionisationsdetektor (FID).

Beckman GC-2 mit programmierter Temperatur (Thermotrac); Wärmeleitfähigkeitszelle.

Griffin VPC-II Apparatus mit Wärmeleitfähigkeitszelle.

Kolonnen und Füllungsmaterial. Siliconelastomer E 301 (30%) verankert auf Celit 545 (60/80 mesh); Glaskolonne in U-Form, 6 Fuss lang (für Griffin-Gerät).

Siliconelastomer SE-30 (20 %) auf Firebrick C-22 (42/60 mesh); ein Paar Aluminiumkolonnen, spiralförmig, 6 Fuss lang (für Beckman-Thermotrac).

Reoplex 400 Merck (20 %) auf Celit 545 (60/80 mesh); Glaskolonne in U-Form, 6 Fuss lang (für Griffin-Gerät).

Diäthylenglycolsuccinat Polymer (DEGS) (15 %) auf Chromosorb W (40/60 mesh); Kolonne aus rostfreiem Stahl, 6 Fuss lang (für Beckman FID).

Apiezon L (20 %) auf Celit 545 (60/80 mesh); Glaskolonne in U-Form, 6 Fuss lang (für Griffin-Gerät).

Substanzmenge für die Analyse. Für die Chromatographie mit dem Beckman-Geräten verwendeten wir für die Analyse 1–2 μl, mit dem Griffin-Gerät 10–20 μl Substanz.

Quantitative Analyse. Quantitative Analyse erfolgte durch die Integration der Bandenfläche mittels Disc-Integrator.

Dünnschichtchromatographie

Technik. Die Chromatographie führten wir auf Glasplatten 10 × 20 cm (Schichtdicke 250 μ) durch. Wir entwickelten aufsteigend bei einer Temperatur von 20°. Von der Analysensubstanz trugen wir 20–200 μg auf.

Sorbente. Als Sorbente verwendeten wir Kieselgel G Merck und weiterhin Kieselgel G mit Zugabe von $AgNO_3$: für die Zubereitung von letztgenannten Schichten verwendeten wir statt des Wassers eine $AgNO_3$-Lösung, damit die $AgNO_3$-Konzentration im Kieselgel 10 % beträgt.

Mobile Phase. S_1: Benzol–Äthylacetat (9:1);

S_2: Benzol-Äthylacetat (8:2).

Nachweis. Die Schichten haben wir einerseits in U.V. Licht einer Niederdruck-Quecksilberlampe (Chromatolite) bewertet, andererseits chemisch durch Besprühung mit 50 %iger Schwefelsäurelösung und Erwärmung auf 150°. Oxoverbindungen wurden noch ausserdem mit 0.2 %iger 2,4-Dinitrophenylhydrazinlösung in 0.1 *N* methanolischer HCl nachgewiesen und verschiedene Formen des Vitamins A und des Pseudojonons mit einer gesättigten Antimon(III)-chlorid-Lösung in Chloroform.

Papierchromatographie

Technik. Für die Chromatographie verwendeten wir das Papier Whatman 4 oder Schleicher-Schüll 2040a unter Anwendung der absteigenden Entwicklung bei einer Temperatur von 20°. Bei der Chromatographie der 3,5-Dinitrobenzoesäureester trugen wir 50 μg Substanz auf das Papier auf, bei C_{20}-Diolen 500 μg und bei Vitamin A bzw. seiner Ester 100–200 μg.

Lösungsmittelsysteme. S_3: 10 %iges Paraffinöl/Methanol–Dimethylformamid–Wasser (10:10:1);

S_4: 50 %iges Dimethylformamid/Cyclohexan;

S_5: 20 %iges Petroleum/Eisessig.

Nachweis. Die Chromatogramme bewerteten wir zunächst im U.V. Licht (Chromatolite). 3,5-Dinitrobenzoesäureester machten wir nach der Reduktion mit Zinn(IV)-chlorid durch Besprühen mit *p*-Dimethylaminobenzaldehyd sichtbar[18], die Glycole mit einer Kaliumpermanganatlösung[18] und Vitamin A bzw. verschiedene Ester mit gesättigter Antimon(III)-chlorid-Lösung in Chloroform.

Darstellung der 3,5-Dinitrobenzoesäureester. In einem Gemisch von 3 ml Benzol, 1 ml Pyridin und 0.5 g 3,5-Dinitrobenzoylchlorid lösten wir 50 mg eines Alcohols auf und erhitzten 30 Minuten am siedenden Wasserbad. In einem Scheidetrichter schüttelten wir hierauf das Gemisch mit 5 ml 50 %igem KOH und nach Verdünnung mit 50 ml Wasser liessen wir die Wasserschicht ab. Diesen ganzen Vorgang wiederholten wir. Die Benzolschicht schüttelten wir dann jeweils zweimal mit 5 ml Wasser, zweimal mit 5 ml 15–20 %iger HCl, dreimal mit 5 ml Wasser, liessen sie in einen Messzylinder ab, füllten mit Benzol auf 5 ml auf und trockneten mit wasserfreiem Natriumsulfat. Einen Teil der Benzolschicht trugen wir dann auf das chromatographische Papier auf.

ERGEBNISSE UND DISKUSSION

Eines der Hauptzwischenprodukte der Synthese von Pseudojonon ist das 2-Methyl-2-hepten-6-on. Bei seiner Darstellung kommt die Analyse einiger Zwischenstufen in Frage, bei der Stoffe mit verschiedenem Charakter auftreten. Es handelt

TABELLE I

GASCHROMATOGRAPHIE EINIGER ZWISCHENPRODUKTE DER SYNTHESE VON 2-METHYL-2-HEPTEN-6-ON

	Gerät:	*FID*	*Griffin*	*Griffin*	*Thermotrac*
	Kolonne:	*DEGS*	*Apiezon L*	*E 301*	*SE-30*
	Trägergas:	N_2	*He*	H_2	H_2
	Durchfluss (Druck):	*1.5 atm.*	*2.6 l/h*	*1.8 l/h*	*3 l/h*
	Temperatur:	*70°*	*90°*	*90°*	*Programm 140–200°/15 min*
		Relative Retentionswerte			
Aceton		0.46			
Dimethyläthinylcarbinol		2.55			
Dimethylvinylcarbinol		1.00			
Dimethyläthylcarbinol		0.84			
Isopren			0.21	0.21	
3-Methyl-1-chlor-2-buten			1.0	1.0	
3-Methyl-1,3-dichlorbutan			2.5	2.1	
3-Methyl-3-chlor-1-buten			0.42	0.45	
Äthyl-(3-methyl-2-butenyl)-acetacetat					1.63
Äthyl-bis(3-methyl-2-butenyl)-acetacetat					4.0
2-Methyl-2-hepten-6-on		6.6	4.1		0.62
Undecan		1.0			1.0

sich ausschliesslich um Stoffe mit nicht zu hohem Siedepunkt, für deren Analyse sich die Gaschromatographie am besten eignet. Carbinole und Oxoverbindungen liessen sich am besten auf polaren Phasen trennen, wie Diäthylenglycolsuccinat oder Reoplex (Fig. 1), chlorierte Kohlenwasserstoffe auf Apiezon L oder auf Siliconelastomer, und Ester gemeinsam mit dem Endprodukt auf Siliconelastomer, bzw. bei Ausnützung der programmierten Temperatur. Die Ergebnisse der Trennung sind aus der Tabelle I ersichtlich.

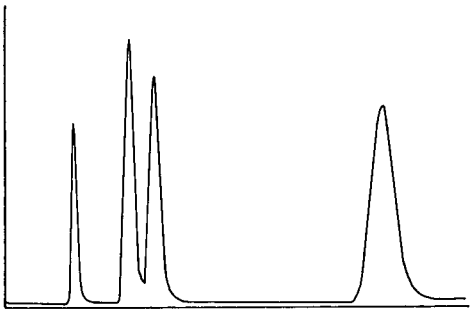


Fig. 1. Trennung eines Gemisches von Aceton, Dimethyläthylcarbinol, Dimethylvinylcarbinol und Dimethyläthinylcarbinol (von links nach rechts) mittels Gaschromatographie. Stationäre Phase: DEGS. Temperatur: 70°. Trägergas: N_2.

Bei den gewählten Bedingungen kommt es zu sehr guter Trennung der meisten Substanzen. Dimethylvinylcarbinol kann von Dimethyläthylcarbinol noch vollkommener auf Siliconelastomer bei 70° getrennt werden. Die Bedingungen für die Analyse des 2-Methyl-2-hepten-6-ons sind aus den Tabellen I und II ersichtlich: Die Auswahl richteten wir je nach den Stoffen, die wir neben dem 2-Methyl-2-hepten-6-on bestimmen wollten. Für die Analyse des 3-Methyl-1-chlor-2-butens neben dem 2-Methyl-2-hepten-6-on eignete sich als stationäre Phase Apiezon L oder das Siliconelastomer bei einer Temperatur von 90°, oder besser die programmierte Temperatur, da bei Temperaturen die höher als 90° sind eine Zersetzung des 3-Methyl-1-chlor-2-butens auftreten kann. Für die Bestimmung des Äthyl-(3-methyl-2-butenyl)-acetacetats neben Äthyl-bis(3-methyl-2-butenyl)-acetacetat und 2-Methyl-2-hepten-6-on bewährte sich am besten Siliconelastomer und die programmierte Temperatur von 140–200°. Die angeführten Ester könnten sich bei einer höheren Probeneinlass-Temperatur zersetzen. Für die Bewertung des Äthyl-(3-methyl-2-butenyl)-acetacetats wählten wir daher eine Probeneinlass-Temperatur von 150°, bei welcher die Zersetzung minimal war, für Äthyl-bis(3-methyl-2-butenyl)-acetacetat 220°.

Zwischenprodukte, die sich bei einigen der möglichen Synthesen des Pseudojonons aus 2-Methyl-2-hepten-6-on ergeben und deren gaschromatographisches Verhalten sind am besten aus der Tabelle II ersichtlich. Es handelt sich hier vorwiegend um Substanzen mit verhältnismässig hohem Siedepunkt, die einige Doppelbindungen oder dreifache Bindungen und Sauerstoffunktion aufweisen. Bei zwei Stoffen, dem Citral und dem Pseudojonon, kommt die *cis–trans*-Isomerie in Frage. Nach RUDENKO u. Mitarb.[10] und auch YANOTOVSKIĬ[7] haben *cis*-Isomere auf Siliconelastomer, wie auch auf Polyäthylenglycol ein geringeres Elutionsvolumen als die entsprechenden *trans*-Isomere. Für die Analyse von Substanzen dieser Gruppe bewährte sich am besten als

TABELLE II

CHROMATOGRAPHIE EINIGER ZWISCHENPRODUKTE DER SYNTHESE VON PSEUDOJONON

	Gaschromatographie			*Dünnschichtchromatographie*	
Gerät:	*Griffin*	*FID*	*Thermotrac*		
Kolonne:	*E 301*	*DEGS*	*SE-30*		
Trägergas:	*He*	N_2	H_2		
Durchfluss (Druck):	*4.2 l/h*	*1.5 atm.*	*3 l/h*		
Temperatur:	*140°*	*160°*	*Programm 120–200°/15 min*		
Sorbent:				*Kieselgel G*	*Kieselgel G + $AgNO_3$*
Mobile Phase:				S_1	S_1
	Relative Retentionswerte			R_F	R_F
2-Methyl-2-hepten-6-on	1.0	1.0	1.0	0.57	0.62
Dehydrolinalool	1.68	2.4	1.51	0.47	0.07*
Dehydrolinalylacetat	4.08	2.45	2.47	0.68	0.06*
cis-Citral		3.15	2.44	0.57	0.60
trans-Citral		3.65	2.62	0.57	0.60
cis-Pseudojonon		10.0	4.53	0.56	0.57
trans-Pseudojonon		13.9	4.97	0.56	0.57
α-Jonon			3.75		
β-Jonon			4.25	0.57	0.68
Undecan			1.65		

* Langgezogener Fleck vom Start.

stationäre Phase Diäthylenglycolsuccinat Polymer bei dem eine Trennung der Substanzen schon bei einer Temperatur von 160° möglich war. Bei der Arbeit mit Siliconelastomer war es notwendig höhere Temperatur (bis 180°) anzuwenden. Bei höheren Temperaturen ergab sich jedoch die Gefahr der Zersetzung einiger Substanzen während des Entwickelns, die z.B. bei Dehydrolinalylacetat, Citral und Pseudojonon aufgetreten sind. Daher war es vorteilhafter, für die Analyse dieser Substanzen eine niedrigere Konzentration der stationären Phase und einen höheren Durchfluss des Trägergases bei niedrigerer Arbeitstemperatur anzuwenden. Auf Diäthylenglycolsuccinat Polymer wurde jedoch Dehydrolinalool und Dehydrolinalylacetat nicht getrennt, und wir verwendeten für ihre Trennung Siliconelastomer und die Temperatur von 140°. Unter diesen Bedingungen kam es aber wiederum nicht zur Trennung des Dehydrolinalylacetats und des *cis*-Citrals. Für die Trennung einer grösseren Anzahl von Substanzen dieser Gruppe nebeneinander war die Anwendung der programmierten Temperatur von 120–200° vorteilhafter (Fig. 2).

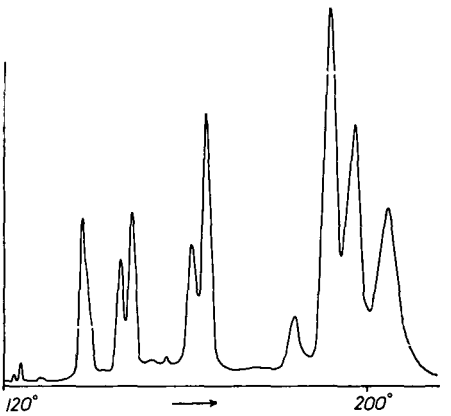


Fig. 2. Trennung eines Gemisches von 2-Methyl-2-hepten-6-on, Dehydrolinalool, Undecan, *cis*-Citral, *trans*-Citral, α-Jonon, β-Jonon, *cis*-Pseudojonon und *trans*-Pseudojonon (von links nach rechts) mittels Gaschromatographie. Stationäre Phase: Siliconelastomer SE-30. Programmierte Temperatur: 120–200°/15 min. Trägergas: H_2.

Mit Hinsicht auf die geringe Flüchtigkeit dieser Substanzen bei Laboratoriumstemperatur kann für ihre Analyse auch die Chromatographie auf Kieselgelschichten dienen, wobei sich z.B. 2-Methyl-2-hepten-6-on, Dehydrolinalool und Dehydrolinalylacetat trennen lassen, und ausserdem noch eine Reihe von Verunreinigungen bestimmt werden kann. Für eine vollkommenere Unterscheidung der Substanzen mit dreifacher Bindung war die Anwendung von Kieselgel mit einem Zusatz von Silbernitrat von Vorteil, da Substanzen mit dreifacher Bindung nahe dem Start aufgehalten werden. Die Dünnschichtchromatographie hat sich für die Trennung von Pseudojonon und Citral nicht bewährt. Auch die Unterscheidung von *cis-trans*-Isomeren brachte unter den gewählten Bedingungen keinen Erfolg.

Eine weitere Phase stellte die Analyse einiger Zwischenprodukte der Synthese von Isophytol vor (Tabelle III). Als stationäre Phase bewährte sich für die Trennung dieser Substanzen Diäthylenglycosuccinat. Wenn auch in vielen Fällen Siliconelastomer Verwendung finden konnte, gaben wir dem Diäthylenglycolsuccinat den Vorzug, da die

TABELLE III

CHROMATOGRAPHIE EINIGER ZWISCHENPRODUKTE DER SYNTHESE VON ISOPHYTOL

	Gas-chromatographie	*Dünnschichtchromatographie*		*Papier-chromatographie*
Gerät:	*FID*			
Kolonne:	*DEGS*			
Trägergas:	N_2			
Druck:	*1.5 atm.*			
Temperatur:	*190°*			
Sorbent:		*Kieselgel G*	*Kieselgel G + $AgNO_3$*	
System:		S_2	S_2	S_3
Derivat:				*3,5-DNB*
	Relative Retentionswerte	R_F	R_F	R_F
2-Methyl-2-hepten-6-on	1.0			
cis-Pseudojonon	5.8	0.65	0.52	
trans-Pseudojonon	7.6	0.65	0.52	
Hexahydropseudojonon	1.75	0.65	0.50	
3,7,11-Trimethyl-3-hydroxy-1-dodecin	3.35	0.65	0.07	
3,7,11-Trimethyl-3-hydroxy-1-dodecen	2.35	0.62	0.40	
6,10,14-Trimethyl-5-pentadecen-2-on	5.6	0.73	0.65	
6,10,14-Trimethyl-5-pentadecen-2-on, Isomer	6.2	0.73	0.65	
Phyton	5.0	0.72	0.65	
3,7,11,15-Tetramethyl-3-hydroxy-1-hexadecin	10.5	0.65	0.09	0.76
Isophytol	6.95	0.63	0.42	0.46
Dihydroisophytol	6.95	0.59	0.56	0.33
Phytadien	2.45	0.82	0.75	
3,5-Dinitrobenzoesäure				0.85
Undecan	0.6			

Arbeit bei einer niedrigeren Temperatur durchgeführt werden konnte. Für die ganze Gruppe wählten wir eine Arbeitstemperatur von 190° und für den Probeneinlass 270°. Es ergab sich die Möglichkeit, Hexahydropseudojonon, 3,7,11-Trimethyl-3-hydroxy-1-dodecin und 3,7,11-Trimethyl-3-hydroxy-1-dodecen auch bei 150° zu chromatographieren, wobei auch die Temperatur des Probeneinlasses wesentlich niedriger gehalten werden konnte. Die angeführten Bedingungen ermöglichten die Lösung praktisch aller Aufgaben, die bei dieser Gruppe in Betracht kommen. Nur Pseudojonon konnte neben 6,10,14-Trimethyl-5-pentadecen-2-on oder Phyton nicht bestimmt werden, doch dies kommt in der Praxis nicht in Frage. Für solche Trennungen wäre die Anwendung von Siliconelastomer und die Temperatur von 210° unter erhöhtem Durchfluss des Trägergases notwendig. Auf diese Weise kam es nicht zur Trennung der beiden Isomere, des 6,10,14-Trimethyl-5-pentadecen-2-ons von Phyton. In der weiteren Phase war die Bestimmung der Isophytols neben dem Dihydroisophytol schwierig, da beide Stoffe ähnliche Elutionsvolumina besitzen*. Für die Analyse von Phytol neben den angeführten Substanzen war es notwendig, die Temperatur 200–210° und erhöhten Trägergasdurchfluss anzuwenden.

Die angeführten Schwierigkeiten können durch Anwendung der Dünnschicht- oder Papierchromatographie ausgeschaltet werden. Die dünnschichtchromatographische Trennung der Stoffe ist unter Benützung der gebräuchlichen Sorbenten nicht gelungen, da sich diese zwischen den R_F-Werten 0.60–0.80 bewegten. Mit Hinsicht darauf, dass es sich um Substanzen handelt, die sich durch Anwesenheit und Anzahl der Doppelbindungen und dreifachen Bindungen unterscheiden, versuchten wir dieses Problem durch Zugabe von Silbernitrat zu dem Sorbenten zu lösen. In diesem Falle wurden die Substanzen mit dreifachen Bindungen besonders stark aufgehalten (3,7,11-Trimethyl-3-hydroxy-1-dodecin und 3,7,11,15-Tetramethyl-3-hydroxy-1-hexadecin), sodass es gelang, auch die Gruppe 3,7,11,15-Tetramethyl-3-hydroxy-1-hexadecin, Isophytol, Dihydroisophytol und Phytadien zu trennen (Fig. 3). Dieses Problem

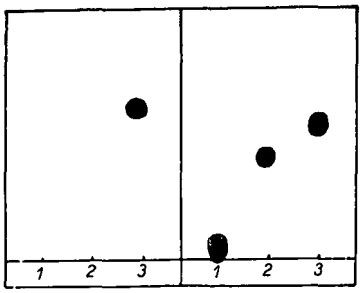


Fig. 3. Trennung eines Gemisches von 3,7,11,15-Tetramethyl-3-hydroxy-1-hexadecin (1), Isophytol (2) und Dihydroisophytol (3) mittels Dünnschichtchromatographie. Links: Kieselgel G. Rechts: Kieselgel G mit $AgNO_3$.

kann auch mit Hilfe der Papierchromatographie gelöst werden, nach Überführung der entsprechenden Alkohole in 3,5-Dinitrobenzoesäureester. Infolge der langwierigen Darstellung der Derivate gaben wir jedoch der Dünnschichtchromatographie den Vorzug.

* Nach Privatmitteilung von H. BOBER (München) können beide Substanzen an Kolonnen von 1/8″ unter Einspritzen von 0.1 μl Proben getrennt werden.

TABELLE IV

GASCHROMATOGRAPHIE EINIGER ZWISCHENPRODUKTE DER SYNTHESE VON 3-METHYL-2-PENTEN-4-IN-1-OL

	Gerät:	*Griffin*	*Griffin*	*Thermotrac*
	Kolonne:	*Reoplex*	*Reoplex*	*SE-30*
	Trägergas:	H_2	H_2	H_2
	Durchfluss:	*1.8 l/h*	*1.8 l/h*	*3 l/h*
	Temperatur:	*70°*	*120°*	*70°*
		Relative Retentionswerte		
3-Chlor-2-buten-1-ol			11.0	
3-Chlor-2-buten-1-ol, Isomer			16.1	
1,3-Dichlor-2-buten		3.15	2.45	
1,3-Dichlor-2-buten, Isomer		4.57	3.2	
Methylvinylketon		1.0	1.0	
Methylvinyläthinylcarbinol				1.0
Dimethyläthinylcarbinol				0.6
3-Methyl-2-penten-4-in-1-ol				3.6
3-Methyl-2-penten-4-in-1-ol, Isomer				4.5

TABELLE V

CHROMATOGRAPHIE EINIGER ZWISCHENPRODUKTE DER SYNTHESE VON VITAMIN A

	Gaschromatographie		*Dünnschicht-chromatographie*		*Papier-chromatographie*	
Gerät:	*Thermotrac*	*Thermotrac*				
Kolonne:	*SE-30*	*SE-30*				
Trägergas:	H_2	H_2				
Durchfluss:	*3 l/h*	*3 l/h*				
Temperatur:	*Programm 160–190°*	*170°*				
Sorbent:			*Kieselgel G*	*Kieselgel G + $AgNO_3$*		
System:			S_1	S_1	S_4	S_5
	Relative Retentionswerte		R_F	R_F	R_F	R_F
cis-Pseudojonon	1.22		0.47	0.46		
trans-Pseudojonon	1.32		0.47	0.46		
α-Jonon	1.0	1.0				
β-Jonon	1.13	1.21	0.53	0.59		
β-C_{14}-Aldehyd		2.0				
Undecan	0.45					
3,7-Dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)-nona-2,7-dien-4-in-1,6-diol			0.06	0.75	0.26	
3,7-Dimethyl-9-(2,6,6-methylcyclohexen-1-yl)-nona-2,4,7-trien-1,6-diol			0.06	0.12	0.64	
3,7-Dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl)-nona-2,4,7-trien-1,6-diol, Acetat			0.32	0.40	0.96	
Vitamin A, Alcohol			0.27	0.25		0.80
Vitamin A, Acetat			0.69	0.72		0.58
Vitamin A, Palmitat			0.87	0.89		0.08

Bei der letzten Phase der Synthese von Vitamin A analysierten wir mittels Gaschromatographie die Zwischenprodukte des 3-Methyl-2-penten-4-in-1-ols, die sich auf Reoplex (bis zu Methylvinylketon) und auf Siliconelastomer (Carbinole) chromatographieren lassen. Die Bedingungen und Resultate sind aus der Tabelle IV ersichtlich.

Weiterhin kam die Bewertung von Pseudojonon neben α- und β-Jonon und β-C_{14}-Aldehyd in Betracht, für welche sich als stationäre Phase Siliconelastomer und die programmierte Temperatur von 160–190° bewährte. Weitere Stufen stellen dann schon feste Substanzen mit zu hohem Siedepunkt vor. Für deren Analyse benutzten wir die Papier- oder Dünnschichtchromatographie. Die Analyse der C_{20}-Diole führten wir auf Kieselgel-G-Schichten oder auf Papier im System Dimethylformamid/Cyclohexan durch. Bei ähnlichem Zeitanspruch der beiden Methoden besteht der Vorzug der Dünnschichtchromatographie in dem empfindlicheren Nachweis. Ähnlich kann das Vitamin A und seine Ester durch beide Methoden analysiert werden. In diesem Falle ist auch bei der Papierchromatographie der Nachweis sehr empfindlich, wenn die Stoffe auf Grund ihrer Fluoreszenz im U.V. licht oder chemisch durch empfindliche Reaktion mit Antimontrichlorid bewertet werden. Die Ergebnisse der Trennung sind aus der Tabelle V ersichtlich.

DANK

Wir danken Frau J. STANISLAVOVÁ für die technische Mitarbeit bei der Dünnschichtchromatographie, Herrn Dr. J. WEICHET für die Durchlesung des Manuskripts und Herrn Dr. J. WEICHET, Ing. L. BLÁHA und Ing. K. KALINA für die Überlassung einer Reihe von Substanzen.

ZUSAMMENFASSUNG

Zur Verfolgung der möglichen Zwischenprodukte der Synthese von Pseudojonon, Isophytol und Vitamin A wurden Gas-, Dünnschicht- und Papierchromatographie angewendet. Die Gaschromatographie wurde für die Verfolgung aller Zwischenprodukte, mit Ausnahme der Endphasen der Synthese von Vitamin A, benutzt. Die Dünnschichtchromatographie hat sich besonders für einige Zwischenprodukte der Synthese des Isophytols bewährt, die sich durch Anwesenheit und Anzahl von Doppel- oder dreifachen Bindungen unterscheiden. Ähnliche Substanzen, sowie die Endphasen der Synthese des Vitamins A, wurden gleichfalls mit Hilfe der Papierchromatographie bewertet.

SUMMARY

In order to follow the production of the possible intermediates in the synthesis of pseudo-ionone, isophytol and vitamin A, gas, thin-layer and paper chromatography were employed. Gas chromatography was used to follow and determine all the intermediates, with the exception of those formed during the last stages in the synthesis of vitamin A. Thin-layer chromatography was found to be particularly useful for determining some of the intermediates in the synthesis of isophytol, which differ by the presence and number of double or triple bonds. Similar substances as well as those

formed during the last stages of the synthesis of vitamin A, were also determined by paper chromatography.

LITERATUR

1 J. FRAGNER (Herausgeber), *Vitamine*, Fischer, Jena, 1964.
2 O. ISLER UND P. ZELLER, in R. S. HARRIS UND D. J. INGLE (Herausgeber), *Vitamins and Hormones*, Vol. 15, Academic Press, New York, 1957, S. 31.
3 O. ISLER, R. RÜEGG, U. SCHWIETER UND J. WÜRSCH, in R. S. HARRIS UND D. J. INGLE (Herausgeber), *Vitamins and Hormones*, Vol. 18, Academic Press, New York, 1960, S. 295.
4 V. N. BELOV UND N. I. SHKVORTSOVA, *Usp. Khim.*, 32 (1963) 265.
5 Y. R. NAVES, *Bull. Soc. Chim. France*, (1958) 377.
6 B. H. KINGSTON, *Mfg. Chemist*, 33 (1962) 510.
7 M. C. YANOTOVSKIĬ, *Zh. Analit. Khim.*, 19 (1964) 262.
8 E. STAHL, *Dünnschichtchromatographie*, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1962, S. 200.
9 J. H. DHONT UND G. J. C. DIJKMAN, *Analyst*, 89 (1964) 682.
10 B. A. RUDENKO, V. F. KUCHEROV, V. A. SMIT UND A. V. SEMENOVSKIĬ, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1962) 236.
11 P. TEISSERE UND B. CORBIER, *Riv. Ital. Essenze-Profumi-Piante Offic. Aromi-Saponi-Cosmet.*, 45 (1963) 392; *C.A.*, 60 (1964) 4186h.
12 H. MAYER, P. SCHUDEL, R. RÜEGG UND O. ISLER, *Helv. Chim. Acta*, 46 (1963) 650.
13 E. DEMOLE UND E. LEDERER, *Bull. Soc. Chim. France*, (1958) 1128.
14 O. GRISLER, *Anal. Chem.*, 31 (1959) 1516.
15 T. ARATANI, H. KOMAE UND T. MATSUURA, *Kogyo Kagaku Zasshi*, 65 (1962) 1565; *C.A.*, 59 (1963) 3959.
16 H. R. BOLLIGER, in E. STAHL (Herausgeber), *Dünnschichtchromatographie*, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1962, S. 217.
17 I. M. HAIS, in I. M. HAIS UND K. MACEK (Herausgeber), *Handbuch der Papierchromatographie*, Band I, 2. Ausgabe, Fischer, Jena, 1963, S. 755.
18 I. M. HAIS UND K. MACEK, *Handbuch der Papierchromatographie*, Band I, 2. Ausgabe, Fischer, Jena, 1963, S. 903*ff*.

DETECTION OF REARRANGEMENT REACTION OF NATURAL GLYCERIDES BY CHROMATOGRAPHY

M. M. CHAKRABARTY, D. BHATTACHARYYA AND A. GUPTA
Department of Applied Chemistry, Calcutta University, Calcutta (India)
(Received September 27th, 1965)

Rearrangement reactions of glycerides[1-4] have acquired importance in recent years in the preparation of new types of fats by inter- and intramolecular exchange of acyl groups under the influence of catalysts. However, dependable and rapid methods for detecting the change induced have not been developed so far, although detailed[4] analysis may ultimately give the information. It was considered worthwhile therefore to investigate methods for detecting new glycerides formed by this rearrangement reaction.

In recent years chromatographic techniques such as paper and thin layer chromatography are finding enormous applications in the analysis of natural and modified triglycerides. The possibility of applying these in the study of ester rearrangement reactions was therefore investigated. The literature reveals some of the recent studies in this field. Paper chromatography of the more common natural triglycerides has been studied exhaustively by KAUFMANN and co-workers. Improved procedures have been described by KAUFMANN, WESSELS AND VISWANATHAN[5], who separated linseed and sunflower oil glycerides on paper coated with a nonpolar stationary phase like tetradecane and using acetic acid–acetonitrile (8:2) or acetone–methanol (9:1) as mobile phases. STEINER AND BONAR[6] separated cocoa butter glycerides on paper impregnated with 5% liquid paraffin and using acetone–methanol (9:1) as mobile phase.

INOUYE AND NODA[7] separated mixtures of triglycerides by first converting them to mercuric acetate adducts and then fractionating them on paper on the basis of the number of double bonds per glyceride molecule.

Separations and identification of synthetic and natural triglycerides on thin layers of suitable adsorbent have also been studied mainly by KAUFMANN and co-workers[8], who resolved many synthetic triglycerides and the component triglycerides of corn, sunflower, sesame, olive and linseed oils, lard and beef tallow on Kieselguhr plates impregnated with a 5% solution of tetradecane in petroleum ether and using acetone–acetonitrile (8:2) as developing solvent. Using an improved technique, KAUFMANN AND KHOE[9] chromatographed cocoa butter and olive oil on thin layers of calcium sulphate coated with tetradecane. KAUFMANN *et al.*[10] also described the hydrogenation and bromination of the glycerides on plates as steps towards complete separation of the superimposing components. Similar separations of the glycerides of seed oils have been reported by MICHALEC *et al.*[11] on thin layers of silicic acid impregnated with paraffin oil and with acetic acid as developing solvent.

Since the time DE VRIES[12] initially showed that the methyl esters of palmitic,

oleic, and linoleic acids can be separated on the basis of unsaturation on a silicic acid column impregnated with silver nitrate, many investigators have extended this method for elucidation of synthetic and natural, as well as modified, triglycerides on thin layers of silica gel containing silver nitrate. BARRET *et al.*[13] separated and estimated quantitatively triglycerides of lard, interesterified lard, cocoa butter, palm oil, peanut oil, soybean oil, and cotton seed oil on silica gel G plates impregnated with a 12.5 % solution of silver nitrate. Solvents used were 99.5 % chloroform and 0.5 % acetic acid, and a more selective solvent consisting of carbon tetrachloride (60 vol.), chloroform (40 vol.), and acetic acid (0.5 vol), to which variable small amounts of ethanol are added for separation of unsaturated glycerides. After development the plates are charred by spraying with 50 % phosphoric acid and heating to 340°. DE VRIES[14] also separated synthetic glyceride mixtures on silica gel columns impregnated with $AgNO_3$ on the basis of unsaturation and isomeric configurations. REISER *et al.*[15] separated the triglycerides of *Cuphea ilavia var.* miniata seed fat according to the number of double bonds per molecule using preparative thin layer chromatography on silicic acid impregnated with silver ions. The recovered fractions were determined quantitatively by the chromotropic acid technique. The multiple chromatography procedure resolved *Cuphea ilavia* triglycerides into seventeen different components. Chloroform containing 1 % ethanol was used as developing solvent. JURRIENS and co-workers[16] analysed the triglycerides of cocoa butter, Sumatra palm oil, lard, groundnut oil, soybean and cotton seed oils according to their degree of unsaturation by means of thin layer chromatography on silica gel G impregnated with $AgNO_3$. The glycerides are extracted from the adsorbent and the amount in each fraction is determined by glycerol determination with periodic acid after saponification. PRIVETT *et al.*[17] separated a randomised mixture of synthetic triglycerides containing palmitic, oleic and linoleic as well as palmitic, oleic and linolenic on silicic acid impregnated with silver nitrate as described by BARRETT *et al*[13]. The less unsaturated triglycerides (3 or less double bonds) are chromatographed with 0.8 % methanol in chloroform. Triglycerides containing more than three double bonds are chromatographed with 2–3 % methanol in chloroform.

KAUFMANN AND WESSELS[18] separated the glycerides of sunflower oil by first fractionating on silver nitrate impregnated silica gel plate and effecting further separation in a reversed phase system.

In the present study, thin layer and paper chromatography techniques have been used to separate the glycerides of some seed oils, before and after rearrangement reactions, by the reversed phase multiple development principle. The object of such separations has been to note their pattern and the nature of the new component glycerides produced as a result of random rearrangement.

EXPERIMENTAL

Paper chromatography

Whatman No. 1 chromatography paper was used for the separation of the glycerides. The paper strips (14 in. × 3 in.) were impregnated with a 5 % solution of liquid paraffin (B.P., B.D.H.) in petroleum ether (b.p. 40–60°). 100 μg of the original and randomised samples were then spotted on the impregnated papers and three developments were carried out in the ascending manner using acetone–methanol

TABLE I

PAPER CHROMATOGRAPHY OF NATURAL AND RANDOMISED GLYCERIDES

Glyceride sample	*Number of spots*	*R_F value of spot* (× 100)*											
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Natural groundnut	6	10.4	16.8	24.1	31.8	39.1	90.0						
Randomised groundnut	9	7.7	14.1	20.0	26.3	33.2	40.4	87.7	92.7	97.2			
Natural sesame	6	16.8	23.6	30.0	33.2	45.4	92.7						
Randomised sesame	8	16.0	23.6	30.4	33.2	45.0	84.0	91.3	97.3				
Natural cottonseed	5	34.0	41.6	50.4	60.0	90.0							
Randomised cottonseed	6	34.0	42.4	51.2	60.0	88.0	92.0						
Natural safflower	6	37.6	45.0	53.6	61.6	77.3	90.8						
Randomised safflower	7	37.0	46.0	54.4	62.4	69.2	88.8	93.2					
Natural linseed	9	38.0	45.6	53.2	61.2	68.8	76.0	80.8	89.2	93.6			
Randomised linseed	10	38.4	45.2	54.4	62.0	69.2	76.0	82.0	86.8	91.6	96.8		
Natural mustard	6	14.4	20.4	26.8	32.0	38.0	46.0						
Randomised mustard	12	10.0	15.6	20.4	26.0	31.6	38.4	46.0	52.8	74.8	78.4	83.2	88.4

* Spots serially numbered from the base line.

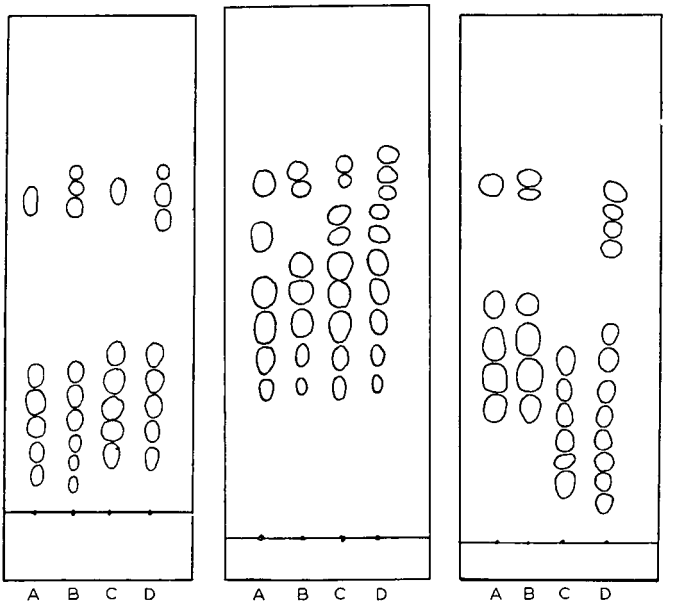


Fig. 1. Chromatographic separation of: (A) Natural groundnut glycerides; (B) Randomised groundnut glycerides; (C) Natural sesame glycerides; (D) Randomised sesame glycerides.

Fig. 2. Chromatographic separation of: (A) Natural safflower glycerides; (B) Randomised safflower glycerides; (C) Natural linseed glycerides; (D) Randomised linseed glycerides.

Fig. 3. Chromatographic separation of: (A) Natural cotton seed glycerides; (B) Randomised cotton seed glycerides; (C) Natural mustard glycerides; (D) Randomised mustard glycerides.

(8:2) as eluting solvent. After development, the papers were freed from solvent and exposed to iodine vapour for 30 min, and the back was sprayed with 1 % starch solution[6] when white spots on a blue background were observed.

The number of spots and their R_F values are given in Table I.

The separations are shown in Figs. 1, 2 and 3.

Thin layer chromatography

The separation of triglycerides was carried out on Kieselguhr plates impregnated with liquid paraffin. The plates were prepared by pouring a slurry of 6 g Kieselguhr (E. Merck), 0.6 g plaster of Paris (E. Merck) and 13.2 ml of distilled water in an applicator designed in the laboratory and adjusted to a clearance of about 0.3 mm and drawing the applicator along the glass plates (20 × 10 cm). The plates were allowed to set for 15 min, then baked at 110° for 1 h and finally stored over fused calcium chloride in a desiccator.

The plates were impregnated with 5 % solution of liquid paraffin (B.P., B.D.H.) in petroleum ether (b.p. 40–60°) and left in the air for 10 min to remove petroleum ether. After spotting exactly similar amounts of the original and randomised samples (as a 1 % solution in benzene; E. Merck) on the impregnated plates, the plates were developed in closed chambers with 8:2 and 7:4 acetone–methanol, previously saturated with liquid paraffin. The glycerides of groundnut, sesame and mustard oils were separated by acetone–methanol (8:2) and acetone–methanol (7:4) was employed for safflower, cotton seed and linseed oil glycerides. The plates were developed twice except in the case of the mustard oil plate which was developed four times. The plates, after development, were freed from solvent and exposed to iodine vapour for about 5 min. When brown spots appeared the plates were taken out and sprayed with 1 % α-cyclodextrin in 30 % ethanol when blue spots appeared against a white background.

The number of spots and their R_F values are given in Table II. Separations are shown in Figs. 4, 5, 6 and 7.

DISCUSSION

In the present investigation seed oils containing high percentages of unsaturated acids have been randomly rearranged. The component glycerides of these oils usually have a high mobility. After the rearrangement some of the component glycerides were found to separate with even higher mobility. These higher mobility components are obviously more unsaturated glycerides and are composed, wholly or predominantly, of C_{18} unsaturated acids. There were, however, two extra spots at the bottom of the TLC plate and one on the paper in the case of mustard seed oils which are likely to contain erucic acid or other higher saturated acids (C_{20} and above), and the mobility of the glycerides of these acid radicals is quite low. The number of spots separated may represent either individual glycerides or critical partners. There was a difference between the original and rearranged seed oils in the number of spots and the rearranged oils showed an increase in the number of component glycerides. In fact, rearrangement in general increases the number of component glycerides. Although the number of spots obtained by thin layer and paper chromatography differ, the separations achieved demonstrate that these two techniques can be readily used for rapid detection of rearrangement reactions. It is also obvious that a rearrangement

TABLE II

THIN LAYER CHROMATOGRAPHY OF NATURAL AND RANDOMISED GLYCERIDES

Glyceride sample	*No. of spots*	*R_F value of spot* (× 100)*									
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Natural groundnut	8	29.7	36.2	42.4	50.7	59.4	68.1	75.3	81.1	—	—
Randomised groundnut	10	29.0	34.7	41.3	48.5	58.0	66.6	73.1	79.7	85.5	93.4
Natural sesame	5	39.1	47.1	55.8	63.7	71.7					
Randomised sesame	7	40.6	48.5	57.2	65.2	73.9	86.9	94.2			
Natural cotton seed	4	52.9	61.6	70.3	78.2						
Randomised cottonseed	5	52.2	61.6	70.3	77.5	96.4					
Natural safflower	4	55.8	63.0	71.1	78.2						
Randomised safflower	6	55.8	63.0	71.1	78.2	84.0	96.4				
Natural linseed	7	50.7	55.7	62.8	69.2	74.2	80.0	82.9			
Randomised linseed	8	51.4	57.7	65.5	71.1	76.0	80.0	84.5	97.1		
Natural mustard	6	57.2	61.7	69.1	76.6	84.0	89.5				
Randomised mustard	9	40.9	47.6	55.0	62.1	69.1	76.6	84.0	89.2	94.4	

* Spots serially numbered from the baseline.

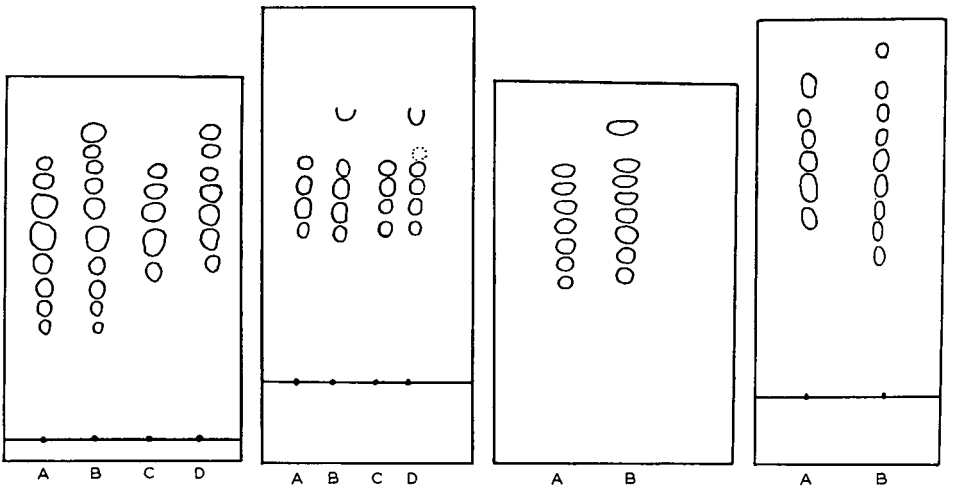


Fig. 4. Chromatographic separation of: (A) Natural groundnut glycerides; (B) Randomised groundnut glycerides; (C) Natural sesame glycerides; (D) Randomised sesame glycerides.

Fig. 5. Chromatographic separation of: (A) Natural cotton seed glycerides; (B) Randomised cotton seed glycerides; (C) Natural safflower glycerides; (D) Randomised safflower glycerides.

Fig. 6. Chromatographic separation of: (A) Natural linseed glycerides; (B) Randomised linseed glycerides.

Fig. 7. Chromatographic separation of: (A) Natural mustard glycerides; (B) Randomised mustard glycerides.

reaction which alters the glyceride composition and results in an increase of the number of spots compared with the original oil can also be used for the detection of adulteration of fats, as adulteration with any other oil would lead to a change in the number and intensity of the spots.

Further work for identification of new component glycerides formed by the rearrangement reaction is in progress.

SUMMARY

The techniques of reverse phase paper and thin layer chromatography have been applied for the rapid detection of the rearrangement reaction involving randomisation of some seed oils containing high percentages of unsaturated acids. It has been found that the two techniques separate the component glycerides readily permitting visualization and detection of the effect of such rearrangements on glyceride composition.

REFERENCES

1 F. A. NORRIS AND K. F. MATTIL, *Oil and Soap*, 23 (1946) 289.
2 E. W. ECKEY, *Ind. Eng. Chem.*, 40 (1948) 1183.
3 A. E. BAILEY, *Industrial Oil and Fat Products*, 2nd ed., Interscience, New York, 1951, 828–829.
4 L. H. WIEDERMANN, J. T. WEISS, A. G. JACOBSON AND K. F. MATTIL, *J. Am. Oil Chemists' Soc.*, 38 (1961) 389.
5 H. P. KAUFMANN, H. WESSELS AND C. V. VISWANATHAN, *Fette, Seifen, Anstrichmittel*, 64 (1962) 509.
6 E. H. STEINER AND A. R. BONAR, *J. Sci. Food Agr.*, 3 (1961) 247.
7 Y. INOUYE AND M. NODA, *J. Agr. Chem. Soc. Japan*, 33 (1959) 452.
8 H. P. KAUFMANN, Z. MAKUS AND B. DAS, *Fette, Seifen, Anstrichmittel*, 63 (1961) 807.
9 H. P. KAUFMANN AND T. H. KHOE, *Fette, Seifen, Anstrichmittel*, 64 (1962) 81.
10 H. P. KAUFMANN, Z. MAKUS AND T. H. KHOE, *Fette, Seifen, Anstrichmittel*, 64 (1962) 1.
11 C. MICHALEC, M. SULC AND J. MESTAN, *Nature*, 193 (1962) 63.
12 B. DE VRIES, *Chem. Ind. (London)*, (1962) 1049.
13 C. B. BARRETT, M. S. J. DALLAS AND F. B. PADLEY, *J. Am. Oil Chemists' Soc.*, 40 (1963) 580.
14 B. DE VRIES, *J. Am. Oil Chemists' Soc.*, 41 (1964) 403.
15 R. REISER, M. FARQUHAR AND C. LITCHFIELD, *J. Am. Oil Chemists' Soc.*, 41 (1964) 588.
16 G. JURRIENS AND A. C. J. KROESEN, *J. Am. Oil Chemists' Soc.*, 42 (1965) 9.
17 O. S. PRIVETT, B. VERDINO AND M. L. BLANK, *J. Am. Oil Chemists' Soc.*, 42 (1965) 87.
18 H. P. KAUFMANN AND H. WESSELS, *Fette, Seifen, Anstrichmittel*, 66 (1964) 81.

THIN-LAYER GEL FILTRATION OF PROTEINS AND MUCOPOLYSACCHARIDES

G. P. ROBERTS
National Institute for Research in Dairying, Shinfield, Reading, Berks. (Great Britain)
(Received August 27th, 1965)

Since its introduction a few years ago the chromatographic technique of gel filtration has become an important fractionation procedure and has been the subject of many recent papers. GRANATH AND FLODIN[1] showed that fractionation of dextrans occurred according to molecular weight and this was extended by ANDREWS[2] to provide a method of molecular weight determination by correlating the elution volumes with the logarithms of the molecular weights of the proteins examined. A similar approach was used in this laboratory[3] for the investigation of the relative molecular sizes of fragments obtained by degradation of epithelial mucopolysaccharides, the agar columns used being first calibrated with dextrans of known molecular weight ranges. Although this procedure has proved extremely valuable in degradative studies on proteins and mucopolysaccharides, the necessity for prior calibration of the columns has tended to make the method time consuming. It appeared feasible that the technique of thin-layer chromatography might be used to advantage in this problem, the standards and material under investigation being run at the same time, hence eliminating any errors caused by differences in column performances during different runs.

The application of Sephadex G-25 and G-75 to thin-layer chromatography has already been reported from two laboratories[4,5] and whilst this paper was in preparation other papers[6–8] appeared describing the thin-layer gel filtration of proteins on Sephadex G-100 and G-200. The present paper describes an improved detection procedure which can be used to detect proteins, mucopolysaccharides, peptides and amino sugars.

EXPERIMENTAL

Materials

The gel filtration media used were the commercially available Sephadex G-25 fine and G-50 fine. Samples of Sephadex G-100 and G-200 < 400 mesh size and blue dextran 2,000 were generously supplies by Pharmacia, Uppsala, Sweden. The proteins used were samples kindly supplied by Dr. P. ANDREWS and their source has been described by him[2]. The mucopolysaccharides and mucoproteins were as used by GIBBONS AND ROBERTS[3].

Preparation and development of thin-layer chromatograms

The Sephadexes used were allowed to soak in an excess of 0.05 *M* sodium

phosphate buffer (pH 7.0) containing 0.15 *M* potassium chloride and saturated with chloroform for at least 72 h before use. Thin-layer chromatograms were prepared by pouring off the excess buffer from the gels and spreading the resulting free flowing suspension on degreased plates (20 × 10 × 0.4 cm) using a spreader manufactured by C. Desaga G.m.b.H., Heidelberg, with a slit width of 0.25 mm. The plates were allowed to dry in air for a few minutes until the excess moisture had drained off leaving a moist gel surface. As a development tank a plastic bowl covered with a sheet of polythene was found quite adequate. A glass histological dish (11 × 11 × 4 cm) served to hold the buffer and the thin-layer plate was inclined against this at an angle of approximately 10°, contact with the buffer being made by means of a filter paper strip. The plate was allowed to equilibrate overnight with 0.05 *M* sodium phosphate, pH 7, containing 0.15 *M* potassium chloride before use. As a spotting guide a strip of paper marked with an origin line was placed underneath the glass plate at a distance of about 2 cm from the upper end of the plate. Along this origin line 1 % (w/v) protein solution (1 or 2 μl) together with a guide spot of india ink were spotted using calibrated capillary tubes and the plate developed until the india ink spot had reached within a few cm of the bottom of the plate. The time required for development varied with the height of buffer in the reservoir and with the Sephadex used. Under the conditions used it was approximately 2–3 h for G-25 and G-50, 4 h for G-100 and about 12 h for G-200. India ink was not entirely satisfactory as a marker because irreversible adsorption occurred occasionally and in later work it was replaced by blue dextran 2,000 which did not suffer from this disadvantage.

Detection and recording procedure

The procedure used to stain proteins and mucopolysaccharides was a modification of the chlorination technique first described by RYDON AND SMITH[9]. After excess moisture had been removed from the developed plates by allowing them to stand at 37° for 5–10 min they were placed in an atmosphere of chlorine for 10–15 min then allowed to stand in a stream of air for 15 min and sprayed with a solution containing 20 % (w/v) ammonium sulphate and 5 % (w/v) sodium bicarbonate. This treatment, which was the main modification to the original method of RYDON AND SMITH[9], served to destroy all the excess chlorine leaving the N-chloro derivatives intact. After allowing the plate to stand for a further 15 min, the N-chloro derivatives were located as blue spots on a white background by spraying with a solution containing 1 % (w/v) starch and 1 % (w/v) potassium iodide.

A permanent record of the plate was obtained by placing it on a sheet of photographic contact paper and illuminating it with a photocopying lamp held at a distance of 4 cm above the plate for about 5 sec. The correct exposure time depended on the intensities of the spots and was found by trial and error. The photographic paper was then developed and fixed in the usual manner and in this way the proteins were shown up as a series of white spots against a black background. The distance of the centres of these spots from the origin could then be measured with some accuracy.

RESULTS AND DISCUSSION

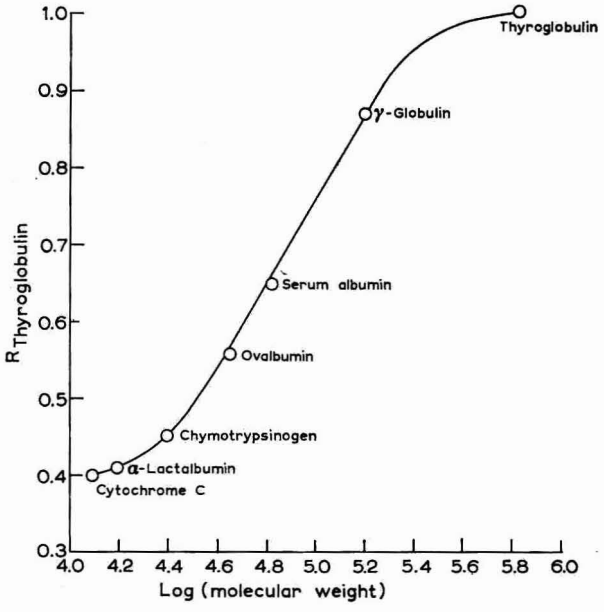
In the previous papers published on thin-layer gel filtration the detection of peptides and proteins was accomplished by staining with ninhydrin or amido black.

These techniques were found inconvenient for the detection of large proteins on the more open Sephadexes G-100 and G-200 because of the large amount of shrinking of the gel which occurred on drying. They are also very insensitive for carbohydrate-rich glycoproteins. As an alternative method for detecting proteins, staining in an atmosphere of iodine was first used in this laboratory[10] but was found to be too insensitive. The chlorination staining technique was found to be much superior and by using this method less than 1 μg of protein could be detected and a permanent record of the plate produced. This staining technique has also been used satisfactorily with mucopolysaccharides although the sensitivity is slightly less here because of their lower nitrogen content. It has also proved extremely useful for detecting oligosaccharides containing hexosamines and other nitrogenous compounds on paper chromatograms but in this case it was necessary to wash the chromatographic paper with 2 *N* acetic acid and water before use. This modified staining procedure possesses the advantages of taking less time and being more reproducible than RYDON AND SMITH'S[9] original chlorination method but still retains the wide specificity of the original method so that it can be applied for the detection of small molecules as well as macromolecules. This wide specificity is a great advantage when carrying out degradative studies since the usual protein stains would not detect any smaller fragments which might be released.

Other workers[2,11] have shown that with gel filtration columns a linear relationship exists between elution volume of proteins and the logarithm of their molecular weights within certain limits of molecular weights determined by the gel filtration media used. When using thin-layer chromatograms, $R_{thyroglobulin}$ defined as:

$$\frac{\text{distance travelled by protein}}{\text{distance travelled by thyroglobulin}}$$

was found to be a convenient index of the rate of travel of a protein. Fig. 1 shows a plot of $R_{thyroglobulin}$ against log (molecular weight) for a number of proteins separated on a Sephadex G-200 thin-layer chromatogram. This is seen to be linear within certain limits of molecular weight but as might be expected it deviates from linearity at the lower and higher ends of the molecular weight range. This linear relation can only be expected to exist for molecules which are of similar shape; since most of the proteins examined are compact folded structures this is true but would not hold if the more asymmetric polysaccharides and mucopolysaccharide molecules were examined on the same scale. A comparison of the behaviour of proteins on the different Sephadexes is shown in the form of $R_{thyroglobulin}$ values in Table I. These cannot be taken as absolute values since they show slight variation from plate to plate, this possibly being due to small changes in the equilibrium of the gel media with the aqueous phase. Approximate molecular weights of unknown proteins can be obtained using thin-layer gel filtration provided the standards and unknown proteins are run on the same plate. For this purpose when working with G-200 it was found convenient to have a standard mixture of thyroglobulin, human γ-globulin, bovine serum albumin, ovalbumin, and α-lactalbumin. A 1.0 % (w/v) mixture of these proteins was completely resolved into discrete spots on Sephadex G-200 and a typical thin-layer chromatogram is shown in Fig. 2. Occasionally diglycylglycine was added to this mixture in order to show the complete inclusion limit but as shown in Fig. 2, this material although separated



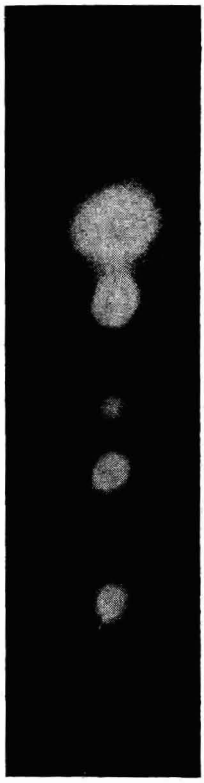


Fig. 1. Plot of $R_{thyroglobulin}$ against logarithm (molecular weight) for thin-layer gel filtration of proteins on Sephadex G-200.

Fig. 2. Thin-layer chromatogram on Sephadex G-200. Mixture containing from top to bottom respectively, diglycylglycine, α-lactalbumin, ovalbumin, bovine serum albumin, human γ-globulin and thyroglobulin.

TABLE I

COMPARISON OF $R_{thyroglobulin}$ VALUES OF PROTEINS ON THIN-LAYER CHROMATOGRAMS USING DIFFERENT SEPHADEXES

Protein	*Molecular weight*	$R_{thyroglobulin}$		
		G-50	*G-100*	*G-200*
Diglycylglycine	189	0.53	0.45	0.30
Cytochrome c	12,400	0.82	0.52	0.40
α-Lactalbumin	15,500	0.85	0.55	0.41
Chymotrypsinogen	25,000	0.89	0.63	0.45
Ovalbumin	45,000	1	0.74	0.56
Bovine serum albumin	67,000	1	0.82	0.65
Human γ-globulin	160,000	1	0.97	0.87
Thyroglobulin	670,000	1	1	1

from α-lactalbumin tended to give a larger spot due to the greater amount of diffusion with the smaller molecule. Although more precise values for molecular weights may be obtained by gel filtration on columns, 10–20 % accuracy is obtainable by thin-layer gel filtration and for many purposes this is sufficient. This is especially true of degradative studies when approximate molecular sizes of the degraded molecule are required for comparison with the intact molecule which can be run on the same plate. As with the column method there is always the possibility of interaction of the protein with the gel media by adsorption or ionic interaction thus producing an erroneous result. In the case of such an anomalous result occurring thin-layer gel filtration possesses an advantage over the column method since it is possible to see exactly what is happening on the thin-layer plate and in this case the wastage of material is negligible if the gel has to be discarded on account of protein adsorption.

ACKNOWLEDGEMENTS

The author is indebted to Pharmacia, Uppsala, Sweden for gifts of Sephadex and blue dextran and to Dr. P. ANDREWS for samples of proteins.

This work was supported by a grant from the Population Council, Rockefeller Institute, New York.

SUMMARY

A method of detecting proteins and mucopolysaccharides on thin-layer gel filtration chromatograms which is sensitive to less than 1 μg of protein has been developed and a procedure for making a permanent record of the chromatogram is described. A correlation between the rate of travel of proteins on Sephadex G-200 and the logarithm of their molecular weights has been obtained.

REFERENCES

1 K. A. GRANATH AND P. FLODIN, *Makromol. Chem.*, 48 (1961) 160.
2 P. ANDREWS, *Nature*, 196 (1962) 35.
3 R. A. GIBBONS AND G. P. ROBERTS, *Ann. N.Y. Acad. Sci.*, 106 (1963) 218.
4 B. G. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 16 (1962) 2067.
5 H. DETERMANN, *Experientia*, 18 (1962) 430.
6 B. G. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 18 (1964) 217.
7 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
8 C. J. O. R. MORRIS, *J. Chromatog.*, 16 (1964) 167.
9 H. M. RYDON AND P. W. G. SMITH, *Nature*, 169 (1952) 922.
10 G. P. ROBERTS, unpublished work.
11 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.

A RAPID QUANTITATIVE METHOD FOR ROUTINE DETERMINATION OF MONOSACCHARIDES AND OLIGOSACCHARIDES FROM PLANTS BY PAPER CHROMATOGRAPHY*

BOCK G. CHAN AND JOHN C. CAIN
New York Agricultural Experiment Station, Cornell University, Geneva, N.Y. (U.S.A.)
(Received September 21st, 1965)

INTRODUCTION

Methods of quantitative determination of sugars by paper chromatography can be divided into two groups. The first group quantitatively determines the colored compound that is formed on a chromatogram upon oxidation of a sugar by an oxidizing agent. The colored compound is either extracted and determined spectrophotometrically or determined by a transmittance densitometer. Such a compound is triphenyltetrazolium chloride which is reduced to a formazon compound which is extracted and determined by a spectrophotometer (WALLENFELS[1], MATTSON AND JENSEN[2] and TRAVELYAN *et al.*[3]). Another such compound is ammoniacal silver nitrate which upon reduction by a reducing sugar to silver black is measured with a transmittance densitometer (MCFARREN *et al.*[4]) or a radiometer (BEER[5]).

The second group of methods extracts or elutes the sugar from a cut-out spot on a paper chromatogram in which the sugar was first located by a guide strip technique. Then the sugar is determined by a standard micro-colorimetric method (WHISTLER[6] and HOUGH AND JONES[7]). The latter method is preferred because sugars are easily and quantitatively extracted or eluted off the chromatogram with water and the color development which is formed by the reaction of a sugar and a reagent is more easily controlled in a test tube than that on the paper chromatogram. Extraction is done either by an extractor (DUFF AND EASTWOOD[8]) or simply by immersing the spot on the paper in water (WHISTLER AND HICKSON[9] and DUBOIS *et al.*[10]). SHALLENBERGER AND MOORES[11] simultaneously extracted and determined sugars separated on Whatman No. 1 paper. After paper blank correction, standard deviation was found to be less than 5 μg in the range of 10–200 μg of sugar. DIMLER *et al.*[12] modified the elution method of DENT[13] for sugar analysis and recommended that elution should be carried out at room temperature and the rate of elution should be carefully controlled to avoid channeling which causes incomplete elution.

This paper describes a rapid quantitative procedure with high reproducibility suitable for the large number of determinations involved in routine analysis for sugars in plants. The solvent which was developed gave good separation of sucrose, glucose and fructose, all of which are commonly found in plants. The sugars from

* Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1454, Sept. 14, 1965.

the paper chromatogram were eluted by means of a micro-elution tube directly into the test tube used in a standard micro-colorimeter method.

MATERIAL AND METHODS

Extraction and purification of samples

Samples of 0.25 to 1 g of freeze-dried peach (*Prunus persica* var. Halehaven) endosperm were extracted with 50 ml of 70 % ethanol or by the water–methanol–chloroform extracting method of BIELINSKI AND YOUNG[14]. The 70 % alcoholic extract was partially purified by dilution with water and extraction with chloroform in the following tertiary solvent system: alcoholic extract, chloroform and water (6:3:2, v/v). The chloroform phase containing the fat-soluble materials was discarded. The aqueous phase was further purified by passing through a column of Dowex 50 resin in H^+ form and then a column of Amberlite IR 45 resin in OH^- form. Dowex 50 resin removed the cations, and Amberlite IR 45 resin removed the anions. The neutral filtrate and washings were combined and dried over concentrated sulfuric acid *in vacuo* in a desiccator. This residue was dissolved in a volume of 10 % ethanol to make about 10 μg per λ, preparatory to colorimetric determinations of sugars and chromatography.

Determination of sugar concentration

The Somogyi copper reagent (SOMOGYI[15]) was prepared and stored in 2 parts (BELL[16]). A: 28 g anhydrous disodium phosphate and 40 g Rochelle salt were dissolved in 700 ml distilled water. 4 g sodium hydroxide were dissolved in 100 ml distilled water and added with mixing. 180 g anhydrous sodium sulfate were added and the whole was made to 900 ml. B: 8 g cupric sulfate (crystalline) were dissolved in 100 ml distilled water with a drop of sulfuric acid. Nine parts of reagent A and one part of reagent B were mixed just prior to use.

Arsenomolybdate reagent (NELSON[17]) was prepared as follows: 25 g ammonium molybdate were dissolved in 450 ml distilled water and 21 ml concentrated sulfuric acid were added slowly. Then 3 g dibasic sodium orthoarsenate pentahydrate in 25 ml distilled water were added with mixing. The solution was aged at 37° for 48 h before use and stored in a dark brown bottle.

One percent (v/v) invertase solution was prepared from concentrated invertase solution (Nutritional Biochemical Company). Two drops of the 1 % invertase solution were added to each test tube containing sucrose and oligosaccharides with sucrose type of linkage (melizitose, raffinose, gentianose and stachyose). One to two hours at 20–25° should elapse before determination of sugar concentration.

The concentration of total reducing sugar and total sugar by hydrolysis with invertase was determined on the partially purified extracts. A specific volume of the partially purified sugar extract giving about 100 μg of sugar in 1 ml distilled water was mixed with 1 ml of the mixed Somogyi copper reagent. The mixed solution was heated in a water bath at 100° for 20 min, then cooled immediately in a water bath at room temperature. The 1 ml of arsenomolybdate reagent was added and mixed vigorously. After 5 min the solution was diluted to 25 ml final volume with distilled water and mixed, then measured at 630 mμ with a Bausch and Lomb Spectronic 20. The concentration of sugar was calculated from a standard glucose

curve established for each batch of reagents. 2 ml of the reagents instead of 1 ml was used in the sugar determination on eluates from paper chromatograms because the volumes of eluates were approximately 2 ml.

Chromatography

Four spots of each sample of sugar solution were placed 4 cm apart on Whatman No. 3MM paper with a Misco spotting pipette. The amount of sugar per spot should give a concentration of 10–150 μg for each sugar determined. When multiple spotting was found necessary to give this concentration, the spot was dried in an air stream of 60° before respotting. A standard sugar mixture consisting of raffinose, sucrose, glucose, fructose, arabinose and xylose was spotted on the edge of each chromatogram as references. A solvent which gave good separation of sucrose, glucose and fructose was water-saturated *n*-butanol, 95 % ethanol and trichloroethylene (6:2:2, v/v). Chromatograms were developed descendingly for 32 h at 32° without equilibration. Longer developing time was needed to separate the higher oligosaccharides.

Development temperature of 32° was chosen because it improved definition, separated in less time (particularly with the slowly moving oligosaccharides), permitted handling of a larger quantity of sugars, and it was easy to maintain (CHAN[18]).

The chromatograms were dried at room temperature. The chromatogram was then cut into strips. One strip of the sample (the guide strip) and the reference strip

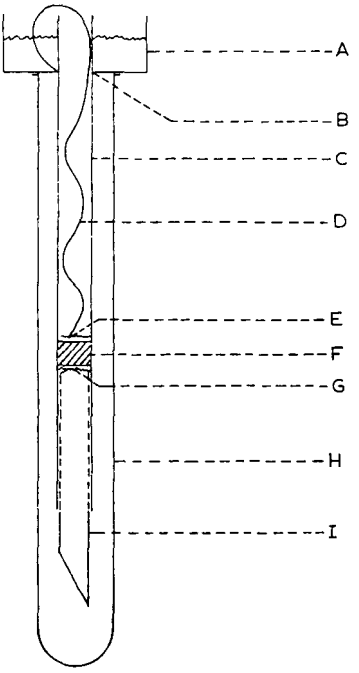


Fig. 1. Specifications for the assembly of the micro-elution tube. (A) a 1 cm in height × 3 cm in diameter plastic cap holding 3 ml water; (B) seal with paraffin; (C) 10 cm of a glass tube with 0.5 cm bore; (D) a strand from a glass wool wick 10 cm long; (E) loose cellulose powder; (F) 0.4 cm filter paper plug; (G) small plug of glass wool; (H) 15–150 mm test tube; (I) a rolled-up "cut-out square" of a paper chromatogram.

of the standard sugars were sprayed with the following improved benzidine reagent (CHAN AND CAIN[19]): A mixture of 10 ml of 5 % benzidine in glacial acetic acid plus 10 ml of 40 % aqueous trichloroacetic acid was diluted to 100 ml with 95 % ethanol. Aldoses were revealed as brown spots after heating at 70° for 10–15 min, then ketoses were revealed as yellow spots after additional spraying with 1.0 % HCl in 95 % ethanol and heating again at 70° for 10–15 min. These colored spots were marked with aid of a black light lamp. The chromatogram was reassembled. The sites of sugars on the three unsprayed strips were located by reference to the guide strip with a centimeter allowance on either end of the sugar spots.

The squares containing the sugars were cut out from the unsprayed strips of paper chromatogram and eluted by distilled water with a micro-elution tube. The specifications for the micro-elution tube are shown in Fig. 1.

The "cut-out square" of paper was rolled up diagonally with forceps. It was inserted half way into the micro-elution tube so that it touched the filter paper plug, which was adjustable. The pointed tip of the paper was squeezed, and the micro-elution tube was placed in a graduated test tube in a slightly tilted rack so that the tip of the paper was in contact with the test tube wall. This facilitated the run-off of the eluate. 3 ml distilled water were added to the plastic cup. The rate of capillary siphoning by the glass wool wick should be approximately equal to that of the paper so that little or no water accumulates above the filter paper plug. This held back excess water if siphoning was too rapid. The whole set-up was enclosed in a humidified box while elution proceeded overnight. To those test tubes collecting eluates containing sucrose, raffinose, or stachyose, two drops of 1 % invertase were added before elution. This allowed hydrolysis of these sugars as they were eluted into the test tube.

RESULTS

A solvent for paper chromatography was developed to give a good separation of sucrose, glucose and fructose. After a series of studies on various proportions of water-saturated *n*-butanol, 95 % ethanol and trichloroethylene, 6:2:2, v/v was found to be a good combination. Table I shows the comparison of the R_G values (reference to glucose) of some sugars by this solvent with several solvents studied by PARTRIDGE AND WESTALL[20]. Its R_G values compared favorably with the slowly developing *n*-butanol–NH_3 (1 % w/v) and better than the *n*-butanol–acetic acid–water (4:1:5, v/v).

Complete elution in the range of 47–142 μg glucose from 15 cm^2 Whatman No. 3MM paper was accomplished with 2.4 ml distilled water (Table II). Percentage of recovery varied from 101 to 102 %. The standard deviation of the mean was less than 1.1 μg glucose in the 47–142 μg range tested. 100 μg raffinose was eluted completely as shown by testing the paper with improved benzidine reagent afterward.

Sucrose, glucose, fructose and ribose were used to test the percentage of recovery and the reproducibility of the procedure. Table III shows a 91 to 99 % recovery with a standard deviation of less than 2 μg and with less than 3 % error.

The endosperm of peach, *Prunus persica* var. Halehaven, was extracted with 70 % ethanol and prepared for paper chromatography as described. Total reducing sugar and total sugar were determined on the partially purified extract. The total sugar was determined after hydrolysis by 1 % invertase. The difference between

TABLE I

A COMPARISON OF THE R_G VALUES OF SOME SUGARS IN WATER-SATURATED *n*-BUTANOL–TRICHLOROETHYLENE–95% ETHANOL (6:2:2, v/v) WITH SEVERAL SOLVENTS[20]

Sugar	R_G *value*	R_G *value calculated from Partridge and Westall*[20]		
	Water-saturated n-butanol–TCE–95% ethanol (6:2:2, v/v) at 22° for 36 h	*Phenol–HCN–NH_3 (1% w/v)*	*n-Butanol–HAc–water (4:1:5, v/v)*	*n-Butanol–NH_3 (1% w/v)*
Raffinose	0.07	0.69	0.28	—
Melibiose	0.16	—	—	—
Cellobiose	0.19	—	—	—
Sucrose	0.41	1.00	0.78	—
Galactose	0.76	1.13	0.89	0.86
Glucose	1.00	1.00	1.00	1.00
Fructose	1.42	1.31	1.28	1.43
Mannose	1.28	1.15	1.11	1.43
Arabinose	1.39	1.39	1.17	1.43
Xylose	1.83	1.13	1.56	1.79
Ribose	2.09	1.51	1.72	2.57

TABLE II

COMPARISON OF RECOVERY OF GLUCOSE BY ELUTION WITH DIRECT DETERMINATION BY A STANDARD COLORIMETRIC METHOD

Glucose (μg)		*Recovery (%)*	*Standard deviation (μg)*
Spotted	*Found**		
46.3	47.1	101.5	0.163
92.5	94.0	101.6	0.349
139.0	142.0	102.1	1.020
92.5**	—	—	1.455

* Mean of nine determinations.
** Mean of twelve determinations on 1 ml sugar solution.

TABLE III

RECOVERY OF SOME SUGARS FROM PAPER CHROMATOGRAMS

Sugar	*Quantity (μg)**		*Recovery (%)*	*Standard deviations (μg)*	*Errors (%)*
	Spotted	*Found*			
Sucrose	78.0	70.8	90.8	1.88	2.66
Glucose	71.0	70.5	99.3	2.01	2.85
Fructose	62.0	60.1	96.9	1.17	1.96
Ribose	92.1	90.5	98.3	1.49	1.62

* Mean of nine determinations.

TABLE IV

COMPARISON OF SUGAR DETERMINATIONS ON PEACH ENDOSPERM BEFORE AND AFTER CHROMATOGRAPHY

(mg glucose per g dried endosperm)

	Unidentified sugar	*Sucrose*	*Glucose*	*Fructose*	*Total reducing sugar*	*Total sugar*
Before chromatography	—	(29.2)*	—	—	100.8	130.0
After chromatography	4.20	26.6	46.3	38.1	(88.6)*	(115.1)*
Standard deviations (μg)**	0.114	0.174	0.028	0.199	0.220	0.078

* Values in parentheses are calculated.
** Standard deviation of nine determinations.

total sugar and total reducing sugar represented non-reducing sugar, the sucrose fraction. 91 % of this sucrose fraction was recovered as sucrose after chromatography (Table IV). Sucrose was further identified by invertase hydrolysis on the chromatogram as follows. The chromatogram was sprayed with 1 % invertase on the area containing sucrose and kept in a humidified chamber for several hours. It was developed at right angles to the first development with the solvent. When dry it was sprayed with benzidine reagent. The monomers of sucrose were identified as glucose and fructose by comparison with standards. Other sugars found in appreciable quantities were fructose, glucose and an unidentified reducing sugar; the percentage of errors was from less than 1 % for glucose to 2.7 % for the unidentified sugar.

DISCUSSION

The described method provided a simple procedure for quantitative sugar analysis of plants by paper chromatography with good reproducibility. The chromatographic solvent which was developed gave a good separation of sugars which was comparable to that by the *n*-butanol–NH_3 (1 % w/v) solvent of PARTRIDGE AND WESTALL[20]. The percentage of recovery was 90.8 for sucrose and 99.3 for glucose. SHALLENBERGER AND MOORES[11] found 93.7 % recovery of sugars in the 10–200 μg range. The high percentage of recovery of sugars from paper chromatograms is because only 1 % sugar was lost during chromatography due to lag or absorption between the original spot and the final spot (KOWKABANY AND HORDIS[21]). Percent error of the present method varied from 1.6 for ribose to 2.9 for glucose. This is in the order of the 2 % error of the Somogyi-Nelson copper colorimetric method assessed by SHU[22].

Ion exchange resins are commonly used for partial purification of sugar extracts. WILLIAMS *et al.*[23,24] studied the use of resins for this purpose and found the resins were superior and more convenient than the old lead precipitation procedure. However, HULME[25] warned against the use of strongly basic anion exchange resins which may decompose the sugar to lactic acid. Amberlite IR 45 (OH^-) adsorbed the organic acids which could be eluted with a volatile acid and were suitable for organic acid analysis. Dowex 50 (H^+) adsorbed the amino acids which could be eluted with 10% NH_4OH and were suitable for amino acid analysis. Thus, this method of sugar analysis fitted into a general analysis of plant material.

The method described in this paper could handle a large number of determinations such as are often required in plant analysis. This facility was due in large part to the efficiency of the micro-elution tube. It allowed a small volume of eluent to be used for complete elution which made possible the micro-colorimetric determination directly in the test tube. It eliminated certain problems of other elution procedures (DIMLER *et al.*[12]), the problem of extraction (DUFF AND EASTWOOD[8], WHISTLER AND HICKSON[9] and DUBOIS *et al.*[10]), and the blank correction (SHALLENBERGER AND MOORES[11]). The micro-elution tube unit could be used for the elution of other chromatographed substances on paper, providing suitable eluent is used. It was easily constructed from ordinary laboratory materials.

SUMMARY

A simple quantitative procedure for sugar analysis of plant materials by paper chromatography with good reproducibility is described. The alcoholic extract was partially purified by a tertiary solvent system and ion exchange resins and then paper chromatography with the following solvent: water-saturated *n*-butanol, 95% ethanol and trichloroethylene (6:2:2, v/v). The sugars were selected by a guide strip technique, and eluted from the "cut-out squares" of paper with 3 ml distilled water by a micro-elution tube, here described. They were determined by a micro-colorimetric method. The method permitted 72 determinations to be done at one time.

REFERENCES

1 K. WALLENFELS, *Naturwiss.*, 37 (1950) 491.
2 A. M. MATTSON AND C. O. JENSEN, *Anal. Chem.*, 22 (1950) 182.
3 W. E. TRAVELYAN, D. P. PROCTOR AND J. S. HARRISON, *Nature*, 166 (1950) 444.
4 E. F. MCFARREN, K. BRAND AND H. R. RUTKOWSKI, *Anal. Chem.*, 23 (1951) 1146.
5 J. Z. BEER, *J. Chromatog.*, 11 (1963) 247.
6 R. L. WHISTLER, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, pp. 395–399.
7 L. HOUGH AND J. K. N. JONES, in R. L. WHISTLER AND M. L. WOLFROM (Editors), *Methods in Carbohydrate Chemistry*, Vol. 1, Academic Press, New York, 1962, pp. 299–309.
8 R. B. DUFF AND D. J. EASTWOOD, *Nature*, 165 (1950) 848.
9 R. L. WHISTLER AND J. L. HICKSON, *Anal. Chem.*, 27 (1955) 1514.
10 M. DUBOIS, K. GILLES, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Nature*, 168 (1951) 167.
11 R. S. SHALLENBERGER AND R. G. MOORES, *Anal. Chem.*, 29 (1957) 27.
12 R. J. DIMLER, W. C. SCHAEFER, C. S. WISE AND C. E. RIST, *Anal. Chem.*, 24 (1952) 1411.
13 C. E. DENT, *Biochem. J.*, 41 (1947) 240.
14 R. L. BIELINSKI AND R. E. YOUNG, *Anal. Biochem.*, 6 (1963) 54.
15 M. SOMOGYI, *J. Biol. Chem.*, 160 (1945) 61.
16 D. J. BELL, in K. PAECH AND N. M. TRACEY (Editors), *Modern Methods of Plant Analysis*, Vol. 2, Springer, Berlin, 1955, pp. 1–52.
17 N. NELSON, *J. Biol. Chem.*, 153 (1944) 375.
18 B. G. CHAN, *M. S. Thesis*, Cornell University, 1960, p. 49–59.
19 B. G. CHAN AND J. C. CAIN, *Nature*, 192 (1961) 69.
20 S. M. PARTRIDGE AND R. G. WESTALL, *Biochem. J.*, 42 (1948) 238.
21 G. N. KOWKABANY AND C. K. HORDIS, *Abstracts, 128th Meeting, Am. Chem. Soc.*, 1955, p. 7D.
22 P. SHU, *Can. J. Res.*, 28B (1950) 527.
23 K. T. WILLIAMS, E. F. POTTER, A. BEVENUE AND W. R. SCURZI, *J. Assoc. Offic. Agr. Chemists*, 32 (1949) 698.
24 K. T. WILLIAMS, A. BEVENUE AND B. WASHAUER, *J. Assoc. Offic. Agr. Chemists*, 33 (1950) 986.
25 A. C. HULME, *Nature*, 171 (1953) 610.

ANION EXCHANGE SEPARATION OF ORGANIC ACIDS IN ACETATE MEDIUM: INFLUENCE OF TEMPERATURE

ULLA-BRITT LARSSON, INGER NORSTEDT AND OLOF SAMUELSON
Department of Engineering Chemistry, Chalmers Tekniska Högskola, Göteborg (Sweden)
(Received September 27th, 1965)

A decreased selectivity at elevated temperatures has been demonstrated by KRAUS, RARIDON AND HOLCOMB[1] in a study of the temperature coefficient of the Br^-–Cl^- reaction with anion exchange resins. Similar results have been reported by many authors (*cf.* refs. 2 and 3) for monovalent cation exchanges.

Improved separations of various ionic species have, however, often been observed when chromatographic separations are carried out at an elevated temperature. The chief explanation for this is that the rate of diffusion inside the resin particles increases with increasing temperature and therefore less broadening of the elution curves occurs (*cf.* ref. 2).

In connection with our work on carbohydrates and on organic acids formed during the degradation of carbohydrates it has been found that in sugar separations by partition chromatography on ion exchange resins a great improvement is obtained when working at an elevated temperature[4], however, in separations of hydroxy acids by anion exchange chromatography in borate medium an increased temperature can result in serious complications[5]. Another technique used in our work is the separation of organic acids by chromatographic elution with sodium acetate solution[6]. The aim of this work is to investigate the influence of the temperature upon this separation.

EXPERIMENTAL

The experimental technique was the same as that used previously[5] with the exception that the eluant was preheated to the desired temperature in a separate column before entering the ion exchange column. This refinement of the technique is of importance only in runs at high flow rate. The anion exchanger was Dowex 1 X-8. Unless otherwise stated, the particle size was 40–60 μ. Different batches were used, but all results reported in each figure were obtained in runs on the same column. The eluate was analyzed in Technicon's Auto Analyzer using the dichromate method described in an earlier paper[7]. The aldobionic acids were prepared in earlier work[8] and all other chemicals were obtained from commercial sources.

The volume distribution coefficients (D_v) were calculated from the peak elution volumes by the conventional method[2].

RESULTS AND DISCUSSION

General

Anion exchange separations of carboxylic acids in sodium acetate medium are based upon simple ion exchange processes in which no consideration has to be given to the formation of covalent bonds or complexes. The volume distribution coefficients (D_v) of a monoprotic acid (HB) can be calculated from the following equation (*cf.* ref. 2):

$$\log D_v = -\log [A] + \log (\gamma_A/\gamma_B)_r - \log (\gamma_A/\gamma_B) + \log [A]_r + \log \rho_r$$

where [A] is the acetate concentration in the external solution (no subscript) and in the resin phase (subscript r), γ the activity coefficients, and ρ_r the mass of dry resin per cm^3 of the column.

Since acetate ions are present in large excess, $[A]_r$ can be considered independent of the external concentration and of the temperature over a large interval. Similarly, the changes in swelling are so small that ρ_r can be considered constant. In most systems the ratio between the activity coefficients in the external solution, independent of the temperature, can be assumed to approach unity, provided that the eluant concentration is low. At constant temperature, the ratio $(\gamma_A/\gamma_B)_r$ is determined by $[A]_r$ which is constant when [A] is varied within such limits that electrolyte invasion can be neglected. Since the resin phase behaves like a concentrated electrolyte solution of a complicated nature, this ratio can differ widely from unity[9] and can be dependent upon temperature. Hence, it would be expected that at constant temperature there would exist a linear relationship between log D_v and —log [A], the slope of which is equal to one.

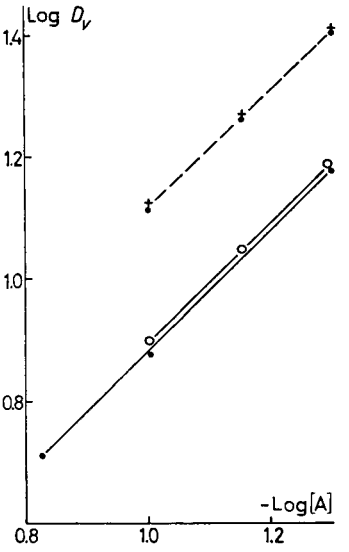


Fig. 1. Influence of the sodium acetate concentration (A moles/l) upon the volume distribution coefficient at various temperatures. – – – glyceric acid at 29° (●), and 80° (+). ——— gluconic acid at 29° (●), and 60° (○).

This has been demonstrated earlier in experiments with various hydroxy acids in sodium acetate solution carried out at room temperature[6,8]. As can be seen from Fig. 1 the same relationship holds true at elevated temperatures. With glycerate ions, which exhibit an ion exchange affinity close to that of acetate ions, no influence of temperature is detected. With gluconate ions, which are held less firmly than acetate ions $[(\gamma_A/\gamma_B)_r < 1]$, an increase in temperature results in a larger distribution coefficient.

A number of other anions, both those which are held less firmly than acetate ions and those which are held more firmly, were studied. From the D_v values determined at 28° and 80° (or in some experiments 60° to avoid serious decomposition) the temperature coefficient was calculated. The results of experiments carried out at two different acetate concentrations are given in Table I. It can be seen that ions

TABLE I

DISTRIBUTION COEFFICIENTS AND TEMPERATURE COEFFICIENTS (dD_v/dt) OF VARIOUS ORGANIC ACIDS

Acid	*Eluant (M)*	D_v *at 28°*	dD_v/dt
Lactobionic	0.05	7.35	0.01
Maltobionic	0.05	8.17	0.01
Cellobionic	0.05	8.26	0.01
Gluconic	0.05	15.1	0.01
Glyceric	0.05	25.5	0
Glyceric	0.1	12.6	0
Lactic	0.1	14.3	0.02
Glycolic	0.1	15.7	—0.01
Formic	0.1	26.2	—0.07
Pyruvic	0.1	40.4	—0.14

with low distribution coefficients exhibit an increased distribution coefficient at high temperature whereas those which are firmly held by the resin show a lowered ion exchange affinity. Hence, in most systems, the selectivity is lower at high temperature. Most interesting is the behavior of lactate ions which in the affinity series take an intermediate position. From the distribution coefficient it might be expected that lactate ions would have a negative temperature coefficient, but determinations carried out with two batches of resin showed that the temperature coefficient is positive. This means that the separation of lactic and glyceric acids is improved at elevated temperature whereas that of lactic and glycolic acids is jeopardized. Both conclusions have been verified in chromatographic runs with these acids.

From the observation that with most ions the selectivity decreases with an increased temperature it can be concluded that, with these anions, heat is evolved on the uptake of a preferred anion. No simple correlation exists, however, between the temperature coefficient and the distribution coefficient which means that there is no generally valid correlation between the selectivity coefficient and the heat of ion exchange.

The influence of temperature upon the shape of the elution curves also deserves some comments. The result of two typical runs with glyceric, formic and pyruvic acids are reproduced in Fig. 2. It is seen that a clear-cut separation was obtained

both at 28° and at 80°. In these runs very fine and carefully fractionated resin particles were used. It is seen that at the flow rate used in these experiments a sharpening of the elution curves occurred at the elevated temperature. This can be explained not only by the lower peak elution volumes, but also by a decrease in the height of a theoretical plate due to more rapid diffusion (*cf.* ref. 2). With less regular resin particles no sharpening of the curves was observed. This is explained by the fact that the non-uniform flow over the cross section of the column has a predominant influence upon the broadening of the elution curves.

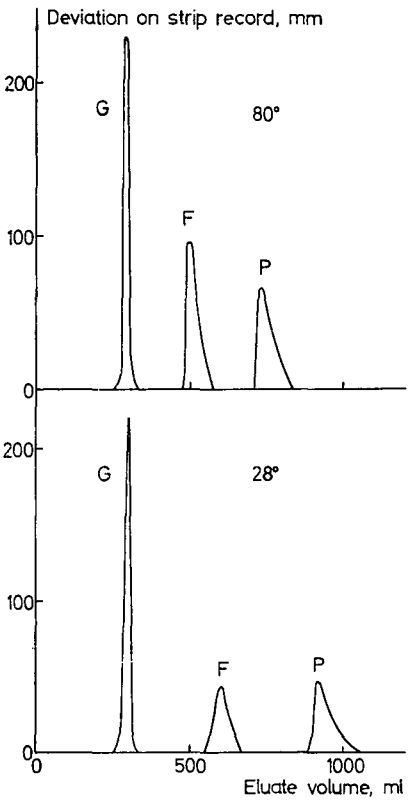


Fig. 2. Separation of 11 mg glyceric acid (G), 21 mg formic acid (F) and 30 mg pyruvic acid (P). Eluant: 0.1 *M* sodium acetate. Column: 6 × 805 mm Dowex 1 X-8, 18–23 μ. Flow rate: 3.3 ml cm^{-2} min^{-1}.

The experiments referred to in Fig. 2 were carried out with a batch of resin other than those used in Fig. 1 and Table I. It is seen that with both resins the position of the curve corresponding to glycerate ions is only slightly affected by the temperature, whereas those corresponding to formate and pyruvate appear earlier in the chromatogram at the elevated temperature. When only these acids are involved, the decreased selectivity at high temperature has no detrimental effect upon the separation.

Another observation worth mentioning is that with the resins used in this

work the separations of lactic and glycolic acids were not as good as those obtained under comparable conditions with another batch of the same resin used in previous work (*cf.* ref. 6). A comparison between the chromatograms showed that this is explained by less favorable distribution coefficients with the new resin. On the other hand glyceric acid could not be separated quantitatively from lactic acid with the resin used previously, whereas with the resin used in the present work an effective separation could be obtained independent of the temperature. The different results obtained with the two batches of resin can be fully explained by differing selectivity coefficients, *i.e.*, by the influence of the resin structure upon the equilibrium uptake of various ionic species.

Epimerization of aldonic acids

In experiments with aldonic acids at high temperatures serious complications occurred. In runs at high flow-rate these complications were less important and hardly detectable in the chromatogram. A comparison between the chromatogram obtained with gluconic acid at 28° (Fig. 3A) and that from a run at high speed at 65° (Fig. 3B)

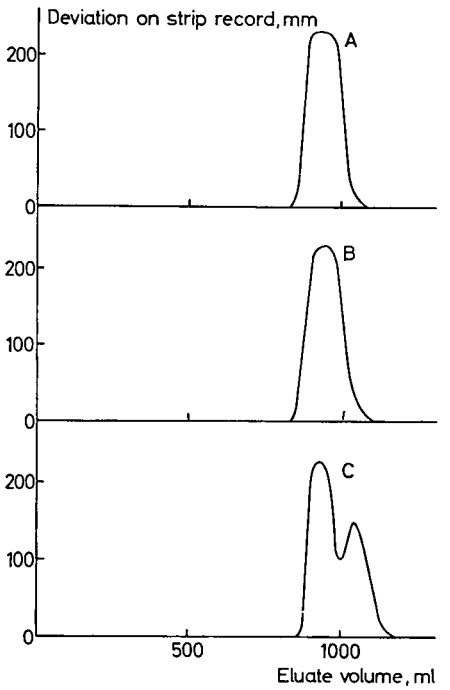


Fig. 3. Elution of gluconic acid (50 mg) with 0.1 *M* sodium acetate solution. Column: 15 × 900 mm. (A) 28°; 0.6 ml cm^{-2} min^{-1}. (B) 65°; 3.9 ml cm^{-2} min^{-1}. (C) 65°; 0.6 ml cm^{-2} min^{-1}.

shows that very small changes occurred. It can be concluded that the peak elution volume of gluconic acid is only slightly affected by changes in temperture. Experiments with mannonic and arabinonic acids showed that these acids behaved in a similar manner.

In experiments at elevated temperature and a low flow rate chromatograms with two overlapping elution bands were recorded. A typical example is given in Fig. 3C. The peak elution volume of the first band corresponded to that of gluconic acid whereas the second band, which was more or less well developed, depending upon working conditions (temperature and flow rate), had a position between that of gluconic and mannonic acids. Separate runs with mannonic acid on the same column showed that its peak elution volume was equal to 1250 ml. The eluate fractions corresponding to the second band in the chromatogram were rechromatographed at low temperature and gave a peak elution volume corresponding to that of mannonic acid. After reduction with sodium borohydride, mannose was identified by paper chromatography[10]. These experiments show that during runs at elevated temperature in acetate medium (pH about 8) gluconic acid is partially converted to mannonic acid. The position of the mannonic acid is displaced to the left because mannonic acid is successively formed during the passage of gluconic acid down the column.

The partial conversion (epimerization) of gluconic acid to mannonic acid in strongly alkaline medium after heating for several days has been established earlier[11], but as can be seen from the results presented above the epimerization is also of importance under comparatively mild conditions. In connection with this, it can be mentioned that an epimerization of gluconic acid was observed after heating an aqueous solution at pH 7 or slightly below 7 for 96 h on a steam bath. The ion exchange method was used to separate the acids.

The reverse reaction, *i.e.* the conversion of mannonic acid to gluconic acid, has also been established in experiments carried out in strongly alkaline solution[11]. This reaction seems to be slower, since no significant formation of gluconic acid was observable during the elution of mannonic acid with 0.1 *M* sodium acetate at elevated temperatures. Heating a solution of mannonic acid at pH 8 for 100 h on a steam bath, however, gave a chromatogram which exhibited bands corresponding to both gluconic and mannonic acids.

On the column which was used in the experiments reproduced in Fig. 1, arabinonic acid was found to give a peak elution volume of 1150 ml at 28°. The value obtained at 80° (1200 ml) differed only slightly from that obtained at the lower temperature. Both elution bands were sharp. By analogy with the behavior of gluconic acid, a conversion of arabinonic acid into ribonic acid was suspected. In separate experiments the elution behavior of ribonic acid was, however, shown to differ so slightly from that of arabinonic acid that these acids could not be distinguished from each other.

On the other hand an elution of these acids with 0.1 *M* sodium tetraborate solution resulted in a clear-cut separation (Fig. 4). This separation method was therefore employed to study the possible conversion of arabinonic acid into ribonic acid upon heating the solution in weakly alkaline medium. The results given in Fig. 5 show that this reaction occurs to a remarkable extent at pH 8.

An analogous experiment carried out with ribonic acid showed that the reverse reaction also occurred. The chromatogram showed two distinct elution bands, a major band corresponding to that of ribonic acid and a minor band corresponding to arabinonic acid. The presence of arabinonic acid in the eluate was further established by reduction with sodium borohydride. The sugar formed from the reduction was identified by paper chromatography.

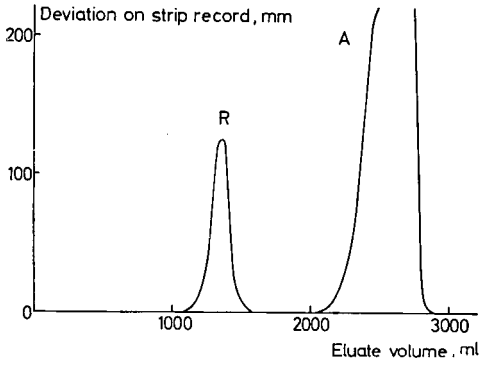


Fig. 4. Elution of ribonic (R; 26 mg) and arabinonic (A; 124 mg) acids with 0.1 *M* sodium tetraborate solution (28°). Column: 10 × 910 mm. Flow rate: 2.5 ml cm^{-2} min^{-1}.

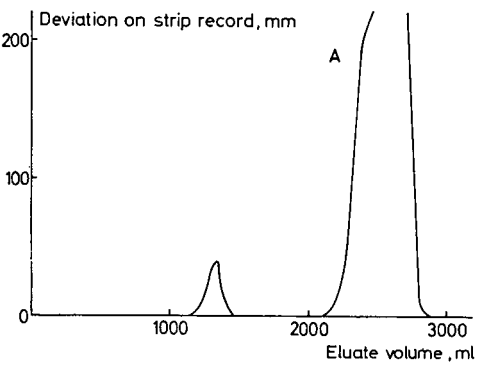


Fig. 5. Elution of arabinonic acid (A; 154 mg) after heating at pH 8 for 96 h on a steam bath. Eluant: 0.1 *M* sodium tetraborate solution (28°). Column: 10 × 910 mm. Flow rate: 2.5 ml cm^{-2} min^{-1}.

The same technique was used to study the epimerization of galactonic acid. On the borate column galactonic acid gave a peak elution volume of 4300 ml whereas the elution curve of talonic acid showed a maximum at 1770 ml. After heating at pH 8, under the same conditions as given in Fig. 5, the galactonic acid solution gave two distinct elution bands on the borate column. The position of the first band corresponded to that of talonic acid whereas the position of the main band was the same as that of galactonic acid. The acid present in the first band was reduced with sodium borohydride and gave a paper chromatogram which showed excellent agreement with that obtained after reduction of an authentic sample of talonic acid.

These results show that the aldonic acids epimerize very easily in alkaline medium. Heating of the solutions and addition of excess alkali should therefore be avoided when the lactones are saponified before separation of the acids.

Destruction of uronic acids

Elution with sodium acetate solution at room temperature is a useful technique in separations of various uronic acids[12]. Experiments carried out at elevated temperature showed that serious destruction occurred. In an experiment at 60°, on a column with the dimensions 15 × 950 mm, run at a flow rate of 1.7 ml·cm^{-2}·min^{-1}, only about 50 % of the added glucuronic acid was recovered in the eluate. At 80° the corresponding value was about 10 %. Some destruction products appeared ahead of the uronic acid band while some were held so strongly that they were not recorded on the chromatogram. Galacturonic acid exhibited similar behavior. With both acids the temperature had only a slight influence upon the peak elution volume of the remaining uronic acid. In a run carried out at a 28° the recovery of the uronic acids was complete.

ACKNOWLEDGEMENT

The financial support of the Swedish Technical Research Council is gratefully acknowledged.

SUMMARY

The elution of organic acids from anion exchange columns with sodium acetate solution at elevated temperatures was studied. In most systems very little can be gained by working at elevated temperatures when using a resin of low particle size. In some separations the decreased selectivity seriously affects the result.

With aldonic acids an interfering epimerization can be detected even at 60°, and with uronic acids serious destruction occurs at this temperature.

REFERENCES

1 K. A. KRAUS, R. J. RARIDON AND D. L. HOLCOMB, *J. Chromatog.*, 3 (1960) 178.
2 O. SAMUELSON, *Ion Exchange Separations in Analytical Chemistry*, Almqvist and Wiksell, Stockholm, and Wiley, New York, 1963.
3 G. E. BOYD, F. VASLOW AND S. LINDENBAUM, *J. Phys. Chem.*, 68 (1964) 590.
4 B. ARWIDI AND O. SAMUELSON, *Svensk Kem. Tidskr.*, 77 (1965) 84.
5 U.-B. LARSSON AND O. SAMUELSON, *J. Chromatog.*, 19 (1965) 404.
6 B. ALFREDSSON, S. BERGDAHL AND O. SAMUELSON, *Anal. Chim. Acta*, 28 (1963) 371.
7 O. SAMUELSON AND R. SIMONSON, *Svensk Papperstid.*, 65 (1962) 363.
8 O. SAMUELSON AND L. O. WALLENIUS, *J. Chromatog.*, 12 (1963) 236.
9 O. SAMUELSON, *Studier Rörande Jonbytande Fasta Ämnen*, Esselte, Stockholm, 1944.
10 O. SAMUELSON, K. J. LJUNGQUIST AND C. PARCK, *Svensk Papperstid.*, 61 (1958) 1043.
11 H. T. BONNETT AND F. W. UPSON, *J. Am. Chem. Soc.*, 55 (1933) 1245.
12 O. SAMUELSON AND L. WICTORIN, *Svensk Papperstid.*, 67 (1964) 555.

ION-EXCHANGE THIN-LAYER CHROMATOGRAPHY

XV. PREPARATION, PROPERTIES AND APPLICATIONS OF PAPER-LIKE PEI-CELLULOSE SHEETS*

K. RANDERATH AND E. RANDERATH

Biochemical Research Laboratory and John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Mass. (U.S.A.)

(Received September 27th, 1965)

Until recently thin layers have been prepared in the laboratory almost exclusively on glass plates[1]. A few authors reported the preparation of thin layers on different materials, *e.g.* aluminum plates[2], aluminum foil[3], and plastic sheets[4,5].

In our experience with ion-exchange and cellulose thin layers, plastic sheets have been found to offer many advantages when compared with glass plates. The present communication describes the preparation of PEI-cellulose** anion-exchange thin layers on plastic sheets and their application in qualitative and quantitative nucleotide analysis.

EXPERIMENTAL

Materials

Bakelite® Rigid Vinyl Sheets (21 × 50 in.), type VSA 3310 Clear 31 Matte 06, 0.010 in., manufactured by Union Carbide Corporation, Cincinnati, Ohio, U.S.A., were obtained from Commercial Plastics and Supply Corporation, 630 Broadway, New York 12, N.Y., U.S.A. MN 300 cellulose powder, manufactured by Macherey und Nagel, Düren, West-Germany, and a STAHL type applicator[1] with adjustable slit width were supplied by Brinkmann Instruments, Westbury, N.Y., U.S.A. Poly-(ethyleneimine) (Polymin P "BASF") was obtained from Chemirad Corporation, East Brunswick, N.J., U.S.A. Nucleotides were supplied by Sigma Chemical Company, St. Louis, Missouri, U.S.A., and by Calbiochem, Los Angeles, California, U.S.A.

Preparation of sheets

The conventional procedure is modified in the following way: Instead of a series

* This work has been supported by grants-in-aid from the U.S. Atomic Energy Commission (AT(30-1)–2643), the U.S. Public Health Service (CA 5018–08), the National Science Foundation (22138), and the Wellcome Trust. This is publication No. 1240 of the Cancer Commission of Harvard University.

** A cellulose anion-exchange material obtained by impregnating chromatography cellulose with poly(ethyleneimine)[6]. Abbreviations for nucleotides: dAMP = deoxyadenosine-5′-monophosphate; GMP = guanosine-5′-monophosphate; GDP = guanosine diphosphate; TDP = thymidine diphosphate; ATP = adenosine triphosphate; GDP-glucose = guanosine diphosphate glucose; GDP-mannose = guanosine diphosphate mannose; UDP-glucose = uridine diphosphate glucose.

of glass plates one long sheet of the plastic material (21.0 cm × full length*) is coated at a time, and a glass plate (127 × 21.5 × 0.6 cm) is used as a mounting board instead of a plastic template**.

The glass template is laid on the bench with one long side protruding a few mm over the edge. The bench top should be horizontal, otherwise the template must be leveled. This may be done by attaching thick adhesive tape to the bottom of the template.

Prior to coating the plastic sheet is degreased on one side using a commercial cleanser and is rinsed on both sides under tap water. After excess water has been allowed to drain, the wet sheet is placed on the glass template in such a way that the front edge protrudes about 1 mm over the corresponding edge of the template***. The sheet is being attached firmly to the template due to adhesion forces. Subsequently, the surface of the sheet is dried by wiping with a towel.

After the slit width has been adjusted to 0.5 mm, the applicator is placed on the right hand side of the sheet so that its guide bar is in contact with the template edge protruding over the bench. To ensure an even surface the empty spreader is moved twice over the sheet. It is then filled with a PEI-cellulose suspension which is prepared as follows:

20 g commercial 50% poly(ethyleneimine) solution is diluted with distilled water (700 ml), adjusted to pH 6 with concentrated HCl, and made up to 1 l with water (final concentration: 1%).

A suspension of 22 g cellulose MN 300 in 145 ml of the 1% PEI hydrochloride solution is homogenized for 15–20 sec in an electric mixer. In order to remove air bubbles the suspension is transferred to a beaker and is stirred about 1 min with a glass rod. Subsequently, it is poured into the applicator and is stirred a few seconds in the applicator chamber. The sheet is then coated immediately. (A narrow band along one side of the sheet will be left uncoated due to the fact that the sheet is 1 cm wider than the slit of the spreader.)

The coated sheets are allowed to dry overnight at room temperature. This will result in a layer with optimal ion-exchange properties. Under no circumstances should drying of the layer be accelerated by heating.

After drying, the sheet is cut into shorter pieces; 7–10 cm wide strips from both ends of the long sheet are discarded. For cutting the coated sheets, a cutting board is recommended.

After cutting, the layers are washed to remove impurities which would interfere with separations and assays. The following procedures may be used:

1. Sheets are washed by ascending irrigation with distilled water in the same way as PEI-cellulose layers on glass plates[7,§]. The water is allowed to rise in a direction perpendicular to the coating direction. Impurities are removed most effectively if a wick of thick absorbent paper (*e.g.*, Whatman No. 3 MM) is attached to the top region by stapling.

* The commercial sheets may be cut with scissors. Alternatively, they can be obtained cut-to-size from local dealers.

** A glass template is not required if a hard and completely level bench is available; in this case the sheet may be laid directly on the bench top for coating.

*** This will prevent the suspension from entering the space between glass template and sheet which would alter the layer composition close to this edge of the sheet.

§ It was found unnecessary to "scratch" the bottom part of the layer as described for glass plates[7,8], because PEI-cellulose layers are bound more firmly to plastic material than to glass.

2. Sheets are washed using the ascending NaCl/water procedure described for PEI-cellulose on glass plates[8,9]. Attaching a wick of absorbent paper to the top part of the layer (see under 1) is again recommended.

3. Each sheet is soaked for 1 min in 10 % NaCl solution (800–1000 ml)*. Immersion is started from one end of the sheet and continued in slow and steady motion. After excess solution has been allowed to drain, the sheet is dried in the air for several hours. (It may be suspended using a clip attached to a cord.) It is then soaked for 5 min in 800–1000 ml distilled water and is again dried. Subsequently, the layer is washed by ascending irrigation with distilled water. A paper wick may be attached to the top region, see under 1.

Dialysis of the poly(ethyleneimine) solution[7,8] is not required if impurities are transferred to the paper wick (procedures 1 and 2) or if the more thorough procedure 3 is used. Procedure 1 is sufficient for one-dimensional separations; for two-dimensional separations and for quantitative techniques (see below) procedures 2 and 3 are preferable.

After washing and drying at room temperature, five to ten sheets are placed on top of each other, wrapped in foil and are stored in a freezer. They are still suited for qualitative and quantitative work after several months at —10 to —20°.

Qualitative procedures

Ascending chromatography is carried out essentially as described for PEI-cellulose on glass plates[7,8]. Nucleotides are applied 2 cm from the lower edge.

For *descending chromatography*, compounds are applied about 4.5 cm from the upper end and the sheet is folded along a line 3.5 cm from this end so that the layer forms an angle of about 45 degrees. The antisiphon rod of commercial paper chromatography equipment is attached with adhesive tape to the uncoated side of the sheet between 3.0 and 3.4 cm from the upper end. This arrangement allows the sheet to hang vertically without touching the wall of the solvent trough. The solvent assembly may be supported by two glass rods (7 cm long) attached to the walls of a rectangular tank (about 28 × 25 × 7.5 cm) by rubber tubing.

For *continuous-flow chromatography*, a paper wick is attached to the distant part of the sheet (see above). In ascending chromatography, it may be folded back behind the uncoated side of the sheet. In descending continuous-flow chromatography for longer periods of time, a long paper wick (> 20 cm) is attached, and the separation is carried out using equipment for descending paper chromatography. To facilitate draining of the solvent, the distant edge of the paper may be serrated.

Two-dimensional chromatography is carried out essentially as described for glass plates[9,10], and the same procedures are used to remove electrolytes prior to development in the second direction. The front area of the first dimension is cut off and discarded. If desired the chromatogram may be cut perpendicularly to the first dimension into several strips containing different groups of compounds which can then be chromatographed in the second dimension with different solvents.

Rechromatography of fractions separated on a PEI-cellulose sheet may be carried out in the following way: the area of the compound(s) to be rechromatographed is cut out using a cutting board. The cut-out is desalted by soaking in anhydrous

* The NaCl solution may be used repeatedly, *e.g.*, for all pieces obtained from one long sheet.

methanol (see refs. 7 and 9) and is dried. Its layer is then brought into contact with the starting area of a fresh sheet and is held in place by attaching two flat magnetic bars or a magnetic bar and a metal sheet to the uncoated sides. Subsequently, chromatography is carried out in the usual way. The compound(s) is (are) transferred quantitatively to the fresh sheet during development.

*Quantitative procedures**

Transfer procedure. This technique, which comprises direct transfer of the nucleotide from the layer to a paper wick, subsequent elution from the paper and spectrophotometry[8], may be used on plastic sheets. A triangle-shaped area containing the substance spot is cut out with scissors rather than isolated *in situ*[8]. To hold the triangle its uncoated side is attached to an uncoated sheet of the same plastic material using a small drop of acetone. About 3 mm substance-free layer between the triangle base and the spot is scraped off with a razor blade, and the paper wick is brought into contact with the straight-cut edge thus obtained. Eluant is applied to the opposite corner with a micropipet as described[8].

Direct procedure. This technique is made possible by the paper-like properties of the ion-exchange sheets. The area of the compound to be assayed is located under short-wave ultraviolet light. A rectangle around the spot is marked with a pencil and is cut out using a cutting board or sharp scissors. Care must be taken that the uncoated side is clean. The rectangle is transferred, layer side up, to the bottom of a tube. Eluant (1.0 ml) is added slowly from a capillary pipet, *e.g.*, a 1000-μl Carlsberg pipet. We found 0.7 *M* $MgCl_2$–2.0 *M* Tris hydrochloride, pH 7.4 (100:1, v/v) to be capable of quantitatively eluting all common mononucleotides, *e.g.*, nucleoside diphosphate sugars, nucleoside mono-, di-, and triphosphates. After an extraction time of 1 h, which is sufficient for these compounds, the eluate is transferred to a centrifuge tube with a capillary pipet and is centrifuged at 2000 r.p.m. for 5 min. Subsequently, extinction at 260 mμ is measured against a blank value from an adjacent area of the sheet processed in the same manner as the compound area. A Zeiss spectrophotometer (model PMQ II) or a Cary spectrophotometer (model 14) equipped with a sensitive slide wire may be used. It is possible to scale down the quantitative procedure to the 1 mμmole range by reducing the eluant volume and using microcuvettes.

As special examples, quantitative determinations of dAMP, UDP-glucose, TDP and ATP were carried out on sheets prewashed according to procedure 3 (see above). For the elution experiments, 5 μl of solutions containing 1.4–2.0 mμmoles nucleotide/μl each were spotted in replicate. Compounds were chromatographed using the following solvents: 0.5 *M* LiCl (dAMP), 0.3 *M* LiCl (UDP-glucose), and 1.0 *M* LiCl (TDP). Development distance was 10 cm. Stepwise elution[7] was used for ATP: 0.5 *M* $(NH_4)_2SO_4$ solution was allowed to ascend to 3 cm from the origin, followed, without intermediate drying, by 0.7 *M* $(NH_4)_2SO_4$ up to 10 cm. After drying, direct extraction was carried out as described above.

Radioactive nucleotides (^{32}P) may be assayed prior to elution: rectangles are cut out as described, transferred to planchets for counting and are subsequently eluted for spectrophotometry. For semiquantitative assays of labeled compounds, sheets are scanned in standard equipment for scanning paper chromatograms.

* Since assays of very small quantities (a few mμmoles) are intended, precautions have to be taken to ensure a low and even background, see ref. 8.

RESULTS

Qualitative results

Properties of the ion-exchange sheets. The ion-exchange cellulose adheres very well to the VSA 3310 plastic material, and layers obtained are homogeneous. Mechanically, they are considerably more stable than plain cellulose layers on the same material[5,11] due to a binding effect of poly(ethyleneimine). Bending or folding of the sheets (see above) does not disrupt the continuity of the ion-exchange coating. Sheets are sufficiently rigid to stand upright when leaned against the wall of a chromatography chamber. The plastic material is stable against all aqueous solvents used in ion-exchange thin-layer chromatography[7,8,10] and against alcohols, aliphatic hydrocarbons, and ethers.

Chromatographic results. Nucleotide mobilities were found not to depend on the support of the PEI-cellulose layer. R_F values given previously for PEI-cellulose on glass[7] are therefore correct for sheets. Spot patterns on two-dimensional chromatograms[9,10] are the same in both cases.

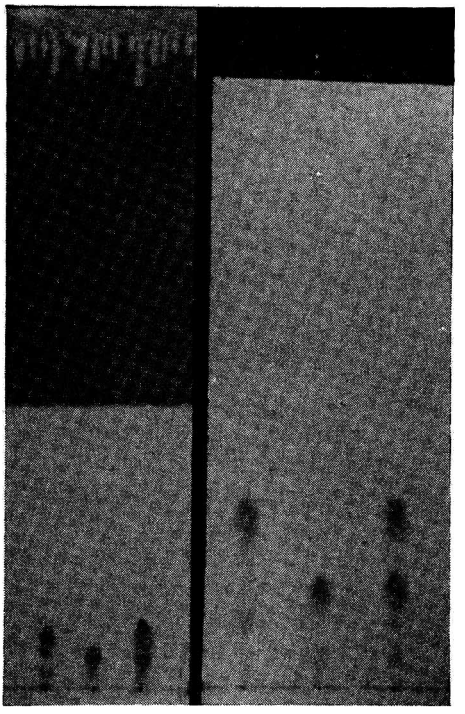


Fig. 1. Comparison between ascending and continuous-flow descending chromatography of closely related nucleotides. 0.5 mm (wet thickness) PEI-cellulose layers on VSA 3310 plastic sheets. About 20 mμmoles of each compound. From left to right: GDP-glucose; GDP-mannose; GDP-glucose plus GDP-mannose. Solvent: a solution of 6 g $Na_2B_4O_7 \cdot 10\ H_2O$, 3 g H_3BO_3, and 25 ml ethylene glycol in 70 ml water. Left: Ascending development for about 3 h (13 cm development distance; R_F values: GDP-mannose 0.05 and GDP-glucose 0.08). Right: Descending continuous-flow development for 20 h; a 25 cm long sheet of Whatman No. 3 MM paper was attached to the ion-exchange sheet at a distance of 12 cm from the origin. Impurities (close to the origin) are GMP and GDP. Photographed by short-wave ultraviolet light. Background fluoresces behind a secondary front.

The sheets may be cut to any size desired. Microscopic slide size sheets were found to be useful for preliminary experiments and for development of new solvent systems. The borate system for separation of nucleotide sugars (see Fig. 1) was developed using a series of such sheets. On the other hand, descending separations of complex mixtures may be carried out on 30–40 cm long sheets.

For many separations, ascending chromatography is sufficient, and resolution of complex mixtures[9,10] can be achieved by two-dimensional ascending chromatography on ion-exchange layers. Some closely related mononucleotides, however, are difficult to separate by simple ascending development, *e.g.*, the GDP-glucose/GDP-mannose pair. These nucleotides can be partially resolved by ascending development with a borate solvent (Fig. 1); descending continuous-flow development, using the same solvent, results, however, in a much more distinct resolution (see Fig. 1)*. Separation of these compounds on paper requires development times of 70 hours and 7 days, respectively, depending on the solvent[12]. As a rule, solvents of low elution power (R_F values of the compounds to be separated < 0.2, preferably 0.05–0.15) are best suited for continuous-flow chromatography on ion-exchange layers.

In the case of two-dimensional separations, cutting the sheet after chromatography in the first dimension (see above) and individually developing each section obtained makes possible a resolution of practically all common mononucleotides on one plate**.

The novel rechromatography procedure described under Experimental facilitates further analysis and purification without time-consuming desalting steps.

Although chromatography paper impregnated with poly(ethyleneimine)[14,15] resembles PEI-cellulose layers in many respects, a comparison[13] clearly shows the latter to be superior with regard to sensitivity and resolution power. For a comparison between PEI-paper and other anion-exchange papers, see ref. 15.

Quantitative results

In Table I data are listed which illustrate the reliability and effectiveness of the direct elution procedure described. The results closely resemble those obtained with the transfer procedure previously described for glass plates[8]: accurate and precise spectrophotometric determinations of small quantities of individual nucleotides can be performed. The direct technique, however, is less time-consuming: quantitative analysis of a compound can be completed within 75–90 min after separation. U.V. spectra may also be recorded.

DISCUSSION

The results presented show PEI-cellulose thin-layer sheets to combine advantages of both paper and thin-layer chromatography. Due to the thinness of the plastic material (0.25 mm) used as a support for the anion-exchanger and to the stability of the layer the sheets can be cut with scissors or a cutting board. They may be folded without disrupting the layer continuity, and a paper wick may be attached by stapling. These three operations make possible the following analytical procedures:

* The solvent used is also capable of separating other nucleoside diphosphate sugars according to the hexose moiety[13].

** Unpublished experiments.

TABLE I

QUANTITATIVE ELUTION OF MONONUCLEOTIDES (7–10 mμMOLES EACH) FROM PEI-CELLULOSE SHEETS (DIRECT PROCEDURE)

Eluant: 1.0 ml 0.7 *M* $MgCl_2$–2.0 *M* Tris hydrochloride, pH 7.4 (100:1, v/v).

Nucleotides	N^*	*Applied* ($E_{260\ m\mu,\ 1\ cm}$)	*Recovered in replicate (mean)*	σ^{**}	σ_{rel}^{***} (%)	*Recovery* (%)
dAMP	10	0.107	0.1077	0.0025	2.3	100.5
dAMP	10	0.109	0.1113	0.0029	2.6	102.1
ATP	8	0.149	0.1468	0.0033	2.3	98.5
UDP-glucose	8	0.087	0.0867	0.0017	2.0	99.7
TDP	8	0.067	0.0650	0.0015	2.3	97.1

* Number of determinations
** Standard deviation = (variance)$^{1}/_{2}$.
*** Relative standard deviation = $\sigma \cdot 100$/mean %.

1. Direct quantitative evaluation (spectrophotometry or counting) of a cut-out.
2. Direct rechromatography.
3. Chromatography with different solvents in the second dimension.
4. Descending chromatography.
5. Continuous-flow development.

Descending and continuous-flow chromatography would be more difficult to perform on glass plates; direct quantitative assays and rechromatography could not possibly be carried out on glass plates.

As an additional advantage, homogeneous layers are more readily obtained on the plastic material than on conventional small glass plates, because one long sheet is coated in one operation. As a consequence, layer preparation and analytical techniques can more easily be standardized than on glass plates.

The plastic sheets should also be useful in micropreparative work: 1–10 mg of a nucleotide may be chromatographed as a band on a 10 cm wide sheet. After cutting out the band, the compound may be eluted by a transfer procedure[8] or directly with electrolyte solutions in the way described for quantitative assays. If the nucleotide is sufficiently stable against acid or alkali, dilute hydrochloric acid or dilute ammonia may be used for elution; otherwise, salt or buffer solutions (preferably volatile) are suitable. In our experience with the direct procedure, 15 min are sufficient for 90% extraction if the eluant is capable of quantitative extraction in 1 h. Since temperature has little effect on the rate of the ion-exchange process, elution may be carried out at low temperature if required. For removal of non-volatile salts, the procedure reported by DANECK *et al.*[16] should be considered.

Due to their properties the thin-layer sheets are well suited for qualitative and quantitative routine analyses, *e.g.*, of biological extracts and of reaction mixtures. They are useful tools in both biological and preparative chemistry of nucleotides.

ACKNOWLEDGEMENT

The authors are indebted to Prof. H. M. KALCKAR and Prof. P. C. ZAMECNIK for generous support of this work.

SUMMARY

The present communication describes preparation and properties of anion-exchange (PEI-cellulose) thin layers on plastic sheets. The ion-exchange sheets are shown to combine advantages of paper and ion-exchange thin-layer chromatography. Procedures are reported which are difficult to perform or cannot be performed on the conventional glass plates. These include techniques for quantitative assays, rechromatography, descending and continuous-flow chromatography.

REFERENCES

1 E. STAHL, *Chemiker-Z.*, 82 (1958) 323.
2 F. SNYDER, *Anal. Chem.*, 35 (1963) 599.
3 F. W. KOSS AND D. JERCHEL, *Naturwiss.*, 51 (1964) 382.
4 R. L. SQUIBB, *Nature*, 198 (1963) 317.
5 E. SAWICKI, T. W. STANLEY AND H. JOHNSON, *Microchem. J.*, 8 (1964) 257.
6 K. RANDERATH, *Angew. Chem.*, 74 (1962) 780; *Angew. Chem., Intern. Ed. (Engl.)*, 1 (1962) 553.
7 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 16 (1964) 111.
8 E. RANDERATH AND K. RANDERATH, *Anal. Biochem.*, 12 (1965) 83.
9 E. RANDERATH AND K. RANDERATH, *J. Chromatog.*, 16 (1964) 126.
10 J. NEUHARD, E. RANDERATH AND K. RANDERATH, *Anal. Biochem.*, 13 (1965) 211.
11 K. RANDERATH AND E. RANDERATH, in L. GROSSMAN AND K. MOLDAVE (Editors), *Nucleic Acids*, a volume of S. P. COLOWICK AND N. O. KAPLAN (Editors-in-chief), *Methods in Enzymology*, Academic Press, New York, in preparation.
12 H. CARMINATTI, S. PASSERON, M. DANKERT AND E. RECONDO, *J. Chromatog.*, 18 (1965) 342.
13 K. RANDERATH AND E. RANDERATH, *Anal. Biochem.*, in press.
14 K. RANDERATH, *J. Chromatog.*, 10 (1963) 235.
15 H. VERACHTERT, S. T. BASS, J. WILDER AND R. G. HANSEN, *Anal. Biochem.*, 11 (1965) 497.
16 K. DANECK, G. WEIMANN AND F. CRAMER, *Experientia*, 21 (1965) 417.

PAPER CHROMATOGRAPHIC DATA FOR PURINES, PYRIMIDINES AND DERIVATIVES IN A VARIETY OF SOLVENTS

KAY FINK AND WILLIAM S. ADAMS

Department of Medicine and the Department of Biophysics and Nuclear Medicine, School of Medicine, University of California at Los Angeles, Los Angeles, Calif. (U.S.A.)

(Received September 13th, 1965)

Filter paper chromatographic data in ten solvent systems were reported by FINK, CLINE, AND FINK[1] for several classes of compounds, including amino acids, purines, pyrimidines, sugars, organic acids and compounds reacting with acidic *p*-dimethylaminobenzaldehyde. Such data are useful as an aid in identification of metabolites, particularly radioactive ones which may be detected on chromatograms at concentrations far less than that required for classification by color reactions[2]. In addition, the data provide information both for selection of solvent systems for separation of two or more substances, and for recognition of compounds which might overlap and interfere.

In the course of continuing investigation of purine and pyrimidine metabolism[3,4], chromatographic properties for an additional 112 ultraviolet absorbing or fluorescing compounds have been obtained. The data for these have been combined here with the 103 purines and pyrimidines reported earlier[1] to provide a single chromatographic table for these two classes of substances detected by ultraviolet light.

EXPERIMENTAL

The basic procedure is essentially the same as reported previously[1]. Small aliquots of solutions of the various compounds (usually about 3 μl of 0.01 *M* solutions) were applied along a base line on sheets of filter paper (Whatman No. 1, 20 × 21 cm). The sheets were fastened into cylinders by insertion of two plastic loops through 2 mm holes made with a paper punch near the sides and about 5 cm from the top and bottom of the paper. The loops were made from ½ in. I.D. polyethylene (5/8 in. O.D.) or teflon (0.56 O.D.) tubing, by cutting a spiral about 1½ cm wide with a sharp razor blade, and the plastic spiral was then cut to give about 1.1/4 turns per loop. Each paper cylinder was developed by ascending chromatography at 28° in a wide-mouth half-gallon fruit jar (Ball special) which contained 50 ml of solvent and was sealed by a lid lined with "Parafilm". With a two-phase solvent system, a 20 ml beaker containing about 15 ml of the aqueous phase was centered at the bottom of the jar, and the contents allowed to equilibrate at 28° before use.

The composition of the various solvents is tabulated below (Table I). Instead of measuring each component separately before mixing, we now use stock solutions of water and acid, water and ammonium hydroxide, and *tert.*-butyl alcohol and methyl ethyl ketone combined in the proper ratios, to reduce the number of individual measurements for routine preparation of the one-phase solvent systems.

TABLE 1

COMPOSITION OF THE SOLVENT SYSTEMS

Nomenclature	*Mixture*
FORM	*tert.*-Butyl alcohol–methyl ethyl ketone–formic acid–water (40:30:15:15)
t-Bu	
NH_3	*tert.*-Butyl alcohol–methyl ethyl ketone–water–ammonium hydroxide
t-Bu	(40:30:20:10)
HAc	*n*-Butyl alcohol–glacial acetic acid–water (50:25:25)
n-Bu	
s&t	Upper phase from a mixture of water–*sec.*-butyl alcohol–*tert.*-butyl alcohol
Bu	(48.4:43:8.6)
HCl	Isopropyl alcohol–water–concentrated HCl (65:18.4:16.6)
i-Pr	
s-Bu	Upper phase from a mixture of *sec.*-butyl alcohol and water
FORM	Ethyl acetate–formic acid–water (70:20:10)
EtAc	
EtAc	Upper phase from a mixture of ethyl acetate–water–formic acid (60:35:5)
form	
t-Bu	*tert.*-Butyl alcohol–methyl ethyl ketone–water–formic acid (44:44:11:0.26)
form	

The compounds were detected by examination of the paper over a short wave ultraviolet lamp (253.7 mμ).

RESULTS AND DISCUSSION

The chromatographic data are tabulated in Table II according to R_F values of the compounds in the first solvent, and a letter was appended to permit individual designations for compounds with the same R_F value in that solvent. To determine whether a specific compound has been listed and where it is located in Table II, the compounds have been arranged alphabetically in Table III with the R_F value and a letter designation for the first solvent. A discussion of the collection of the data and its use has been made in the previous publication[1]. The data for the 115 additional compounds presented here were obtained using a constant temperature cabinet, rather than a room, and under these slightly different environmental conditions, we find a somewhat wider variation in repeated R_F determinations with two-phase solvent systems than previously. Chromatographic data with the phenol solvent included in the earlier paper have been omitted here, since phenol gives a pronounced background under ultraviolet light.

Many of the compounds listed were generously contributed over the years by the individuals who initially synthesized or isolated them, and we wish to again express our appreciation to them.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the competent assistance of ILDONG PARK. The research was supported in part by the American Cancer Society Grant P-213A, United States Public Health Service Grant CA 02433, and Cancer Research Funds of the University of California.

TABLE II

CHROMATOGRAPHIC DATA FOR PURINES, PYRIMIDINES AND DERIVATIVES ARRANGED ACCORDING TO INCREASING R_F VALUE IN THE FIRST SOLVENT SYSTEM

Compound	*R_F values (× 100) in various solvent systems**								
	FORM t-Bu	*NH_3 t-Bu*	*HAc n-Bu*	*s&t-Bu*	*HCl i-Pr*	*s-Bu*	*FORM EtAc*	*EtAc form*	*t-Bu form*
Uridine-5′-diphosphate	0a	0	7	12	66	0	0	0	0
Adenosine-5′-triphosphate	0b	4	6	13	39	0	0	0	0
Uridine diphosphate glucose	0c	6	8	16	69	2	0	0	0
Adenosine-5′-diphosphate	2a	4	6	12	40	0	0	0	0
Uridine-5′-triphosphate	3a	3	5	15	63	0	0	0	0
2,5-Diamino-4,6-dioxypyrimidine	3b	3	8	14	—	2	0	0	0
Guanosine-5′-phosphate	5a	4	17	22	39	2	0	0	0
S-Adenosylmethionine	5b	35	17	14	7	8	1	0	0
Cytidine-5′-phosphate	7a	6	22	20	50	4	2	0	0
Orotidine monophosphate	8a	2	12	12	74	1	1	0	0
Adenosine-5′-phosphate	8b	8	27	25	42	5	3	0	0
Cytosine-5-carboxylic acid	9a	23	—	22	26	8	19	0	3
Uridine-5′-phosphate	12a	4	23	24	70	6	5	0	0
2,6-Diamino-5-formylamino-4-hydroxypyrimidine	12b	10	30	20	19	11	9	0	1
Uric acid	14a	18	30	27	22	5	7	4	12
5-Aminouridine	14b	23	35	42	24	25	7	0	14
Deoxycytidine-5′-phosphate	15a	7	29	22	64	4	6	0	0
6-Amino-2,8-dihydroxypurine	15b	9	31	23	33	—	4,17	0	5
6-Amino-5-formylaminouracil	15c	16	31	26	22	16	12	0	6
Orotidine	17a	17	30	20	66	7	12	0	0
Xanthosine	18a	32	34	28	26	13	12	0	10
5-Methyldeoxycytidine-5′-phosphate	19a	7	36	27	65	8	10	0	0
2-Amino-8-hydroxypurine	19b	24	28	37	21	24	8	0	6
Guanosine	20a	27	36	44	29	32	11	0	11
Deoxyuridine-5′-phosphate	21a	4	38	17	83	6	12	0	0
6-Amino-5-formylamino-4-hydroxypyrimidine	21b	22	40	30	19	18	21	0	7
5-Ribosyluracil	21c	25	35	43	53	34	12	2	0
8-Hydroxy-7-methylguanine	22a	15	46	43	43	35	23	4	11
6-Amino-5-formylamino-1-methyluracil	22b	27	42	35	27	19	22	1	7
3-Methyluric acid	22c	28	41	38	36	24	19	3	18
6,8-Dihydroxypurine	23a	18	38	42	41	36	20	2	19
Guanine	23b	19	45	42	22	36	13	0	11
Thymidine-5′-phosphate	24a	5	36	27	88	6	13	0	0
Xanthine	24b	27	45	46	25	41	21	0	22
5-Hydroxymethyluridine	24c	31	38	48	59	32	13	0	19
Inosine	24d	36	39	48	30	35	14	0	14
1-Methyluric acid	25a	28	42 ±	42	—	27	20	3	30

5-Aminouracil	25b	31	41	40	21	35	12	5	22
7-Methylinosine	26a	unstable	38	29	31	15	13	0	4
5-Hydroxymethylorotic acid	26b	22	32	30	53	15	19	0	5
7-Methyluric acid	26c	25	49	37	54	22	20	3	22
5-Hydroxymethyldeoxycytidine	26d	56	53	44	54	34	—	—	—
2,6-Diamino-7-methylpurine	28a	30	42,52	33	14	16	15	4,24	0
7-Methylguanosine	28b	unstable	45	28	40	16	15	0	5
5-Amino-6-carboxyuracil	29a	16	43	25	24	10	27	11	10
6,8-Dihydroxy-2-methylpurine	29b	23	54	50	43	36	29	5	18
1,7-Dimethylguanine	29c	60	49	39	25	23	17	0	7
6-Carboxypurine	30a	16	43	22	35	10	27	4	4
6-Amino-2-hydroxypurine	30b	22	52	34	32	24	19	0	6
1-Methylguanine sulfate	30c	36	54	48	20	35	18	2	14
5-Formyluridine	30d	43	38	45	56	34	20	4	31
1-Methylguanosine	30e	49	50	47	38	32	16	1	13
6-Methylaminopurine deoxyribonucleoside	30f	52	53	26	38	12	18	0	6
6-Amino-8-hydroxypurine	31a	27	51	55	44	52	10	0	19
5-Hydroxydeoxyuridine	32a	18	47	57	68	46	25	6	40
5-Amino-6-methyluracil	32b	31	54	46	25	40	16	1	28
2-Aminopurine	32c	37	57	60	17	48	23	1	23
Cytidine	32d	47	47	45	45	29	19	0	12
1-Methyladenosine	32e	51	53	28	42	15	22	0	5
4-Amino-5-imidazolecarboxamide ribonucleoside	32f	57	50	48	48	36	21	0	19
Adenosine	32g	64	56	63	33	51	20	3	25
Deoxyguanosine	33a	36	50	55	24	39	18	1	20
Isobarbituric acid	34a	4,14	44	53	57	42	31	9	36
6-Aminouracil	34b	22	47	46	pink str.	38	32	6	22
2,6-Diaminopurine hemisulfate	34c	31	56	39	16	28	29	0	11
5-Methylaminouracil	34d	40	61	61	35	53	10,21	11	45
2-Amino-4,6-dioxypyrimidine	35a	21	51	48	pink str.	39	33	6	22
7-Methylguanine	35b	24	55	46	26	35	19	2	11
Uridine	35c	33	46	55	67	42	25	6	35
3-Methylguanine	36a	22	53	34	33	25	21	2	7
2-Hydroxy-6-methylpurine	36b	30	55	45	40	33	27	2	11
5-Hydroxymethyldeoxyuridine	36c	39	49	59	73	47	25	4	36
6-Azauridine	36d	42	49	53	63	42	27	9	39
5-Methylcytidine	36e	47	53	41	49	30	20	0	12
5-Hydroxymethyluracil	37a	36	48	56	65	46	29	8	33
4,5,6-Triaminopyrimidine sulfate	37b	48	57	40	22	32	29	0	2,14
1-Methylinosine	37c	56	54	45	49	37	30	3	19
2-Amino-4,6-dioxy-5-methylpyrimidine	38a	17	56	45	68	33	38	5	14
Orotic acid	38b	23	39	31	57	15	34	9	6
6-Amino-5-formylamino-3-methyluracil	38c	26	54	50	50	38	31	5	25

(*continued on p. 122*)

TABLE II (*continued*)

Compound	*R_F values (× 100) in various solvent systems*								
	FORM t-Bu	*NH_3 t-Bu*	*HAc n-Bu*	*s&t-Bu*	*HCl i-Pr*	*s-Bu*	*FORM EtAc*	*EtAc form*	*t-Bu form*
2,4-Diamino-6-oxypyrimidine	38d	28	54	36	51 pink	23	29	3	9
6-Methylamino-5-(N-formylmethylamino)uracil	38e	29	57	42	60	31	45	7	19
3-Methylhypoxanthine	38f	30	52	40	30	31	27	4	11
Hypoxanthine	38g	35	53	57	28	49	25	6	26
5-Hydroxymethylcytosine	38h	42	49	40	44	30	23	0	10
Deoxyinosine	38i	42	49	58	31	45	26	4	24
3-Methylxanthine	38j	43	55	60	35	51	38	16	31
6-Amino-5-formylamino-3-methyl-4-oxypyrimidine	38k	45	53	35	30	25	40	6	13
4,6-Diamino-5-formylamino-2-methylpyrimidine	38l	48	57	37	40	20	31	0	0
6-Amino-5-formylamino-1-methyl-4-oxypyrimidine	39a	30	55	42	39	33	41	6	17
3,7-Dimethyluric acid	39b	38	60	50	57	40 ±	41	9	34
1-Methylguanine	40a	25	50	45	32	30	23	0	7
7-Methylxanthine	40b	32	56	57	32	47	42	15	28
2-Methyladenine	41a	46	65	71	41	64	33	0	12
8-Hydroxypurine	42a	40	63	76	31	66	26	13	51
2-Amino-6-methylpurine	42b	43	62	64	27	59	32	2	26
5-Formylcytosine	42c	48	55	52	35	40	33	7	26
5-Formyldeoxyuridine	42d	63	55	64	73	53	26	5	44
6-Methylaminopurine ribonucleoside	42e	77	68	70	51	62	25	7	43
Uracil-6-acetic acid	43a	17	51	26	65	15	34	14	33
5-Methyl-5-hydroxybarbituric acid	43b	25	53	51	63	45	43	25	52
4,6-Diamino-2-oxypyrimidine	43c	29	42	19	52	13	49	35	10
Adenine-N′-oxide	43d	29	61	41	32	32	37	5	8
Barbituric acid	43e	37	45	28	51	12	52	31	21
5-Methoxymethyluridine	43f	37	55	58	74	47	35	7	39
Deoxyadenosine	43g	74	66	70	35	62	31	5	35
Uracil-5-carboxylic acid	44a	9	46	23	54	10	38	26	44
5-Acetylamino-6-amino-3-methyluracil	44b	26	59	48	78	39	40	0	25
5-Hydroxymethyl-6-methyluracil	44c	39	55	55	75	49	40	9	34
5-Formyluracil	44d	56	50	56	58	47	39	24	54
2-Methylhypoxanthine	45a	32	62	60	39	50	35	8	28
5-Hydroxymethylorotic acid lactone	45b	37	49	46	49	38	47	37	49
5-Methyluridine	45c	46	57	64	74	55	35	8	47
Cytosine	45d	49	53	48	45	37	32	0	15
2-Methyl-4,5,6-triaminopyrimidine sulfate	45e	55	64	43	35	32	36	0	15 ±
7-Methyladenine	45f	57	60	58	30	42	35	0	15
4-Amino-5-imidazolecarboxamide	45g	58	58	56	50	48	36	1	25
5-Bromouridine	46a	49	58	60	78	65	39	17	65

1-Methyladenine	46b	47	59	35	31	18	41	1	8
6-Succinoaminopurine	47a	7	61	17	55	6	27	4	45
2-Thio-orotic acid	47b	25	44	—	69	—	44	21	13
8-Methylhypoxanthine	47c	33	63	62	46	57	30	5	33
1,7-Dimethyluric acid	47d	35 ±	65	63	82	48	40	17	43
6-Hydroxy-2-methylaminopurine	47e	34	58	60 ±	49	50 ±	30	0	25
1,3-Dimethyluric acid	47f	43	64	60	67	49	42	16	42
5-Formylamino-3-methyl-6-methylaminouracil	48a	30	63	52	54	39	49	7	22
Adenine	48b	40	65	71	33	63	37	6	33
5′-Thiomethylinosine	48c	53	61	62	45	53	38	11	44
Deoxycytidine	48d	61	57	55	58	40	29	0	18
7-Methylhypoxanthine	49a	35	62	64	36	50	42	12	28
6-Amino-1-methyluracil	49b	57	58	53	pink str.	44	51	11	27
2-Amino-4-hydroxypyrimidine	50a	35	66	66	55	57	40	4	33
9-Methylhypoxanthine	50b	37	59	57	36	47	42	11	25
6-Amino-2-thiouracil	50c	38	57	str	65	57	45	18	53
Uracil	50d	41	56	65	69	55	46	21	47
Deoxyuridine	50e	45	58	67	80	59	41	13	52
1-Methyl-6-methylamino-5-(N-formylmethylamino)-uracil	50f	46	64	52	75	44	49	12	32
2,6-Diamino-5-formylamino-4-methoxypyrimidine	50g	52	61	47	51	26	40	0	5
6-Amino-1,3-dimethyl-5-formylaminouracil	50h	58	61	51	51	43	48	8	27
2-Dimethylamino-6-hydroxypurine	51a	36	68	61	37	52	42	4	28
1-Methylhypoxanthine	51b	37	63	62	36	51	42	13	30
1-Methylxanthine	52a	35	67	69	47	65	49	25	51
5-Methylcytosine	53a	55	60	55	52	44	38	0	15
3-Methyladenine	53b	55	61	48	46	43	45	4	12
4,6-Diaminopyrimidine	53c	60	63	56	47	46	49	3	15
6-Dimethylaminopurine ribonucleoside	53d	83	74	80	58	74	36	13	61
5-Fluorouracil	54a	42	62	70	75	63	55	38	64
2-Hydroxy-4-methylpyrimidine	54b	48	66	60	49	55	41	7	38
2-Thiobarbituric acid	54c	50	46	41	72	21	58	39	15
Spongothymidine	55a	63	61	79	81	66	42	12	59
3,8-Dimethylxanthine	56a	38	70	69	48	64	56	24	45
5-Ethoxymethyluridine	56b	51	66	70	85	63	—	—	—
5-Methyldeoxycytidine	56c	67	65	59	63	46	35	1	21
5-Methylorotic acid	57a	27	44	—	78	18	51	8	16
2-Methyladenine sulfate	57b	51	69	76	44	68	46	3	30
5-Acetyluracil	57c	54	66	70	71	64	52	36	63
1-Methylcytosine	57d	61	63	55	55	45	44	2	18
9-Methyladenine	57e	67	71	66	40	59	47	12	30
5′-Thiomethyladenosine	57f	87	75	82	54	77	45	14	54
2-Thiocytosine	58a	34,93	59	89	55	87	45	5	33

(*continued on p. 124*)

TABLE II (*continued*)

Compound	*R_F values (× 100) in various solvent systems*								
	FORM t-Bu	*NH_3 t-Bu*	*HAc n-Bu*	*s&t-Bu*	*HCl i-Pr*	*s-Bu*	*FORM EtAc*	*EtAc form*	*t-Bu form*
2-Amino-4-hydroxy-6-methylpyrimidine	58b	38	72	70	63	63	44	6	36
4-Hydroxypyrimidine	58c	46	68	70	45	65	54	25	51
3,7-Dimethylxanthine	58d	46	70	64	52	56	62	30	42
6-Methylpurine	58e	52	76	81	46	77	46	22	57
Purine	58f	53	73	73	35	69	45	22	52
2,4-Dihydroxypyrimidine-6-methylsulfone	58g	60	61	39	66	23	62	50	61
6-Amino-3-methyl-5-(N-formylmethylamino)uracil	59a	35	64	64	83	58	52	3	52
5-Fluorodeoxyuridine	59b	42	65	72	86	66	50	29	73
6-Methyluracil	59c	50	68	71	77	66	59	29	55
4,6-Diamino-2-thiopyrimidine	59d	80	62	68	56	52	44	2	25
5-Methylbarbituric acid	60a	33	54	24 ±	63	10	65	55	46 ±
2-Amino-5-methyl-4-oxypyrimidine	61a	47	72	71	67	64	48	7	38
5-Methoxymethyluracil	61b	50	68	72	81	65	57	29	58
6-Methylaminopurine	61c	61	77	85	49	80	47	8	53
Thymine	62a	56	68	77	80	70	61	34	61
Thymidine	62b	59	68	77	87	71	48	19	61
2-Aminopyrimidine	62c	80	78	77	52	72	58	35	60
6-Methoxypurine ribonucleoside	62d	81	78	77	59	70	60	29	58
3-Methyluridine	62e	83	68	70	92	65	50	18	59
4,5-Diamino-6-methylpyrimidine	63a	69	64	60	49	55	52	0	13
6-Dimethylaminopurine	63b	71	77	88	52	82	55	12	65
5-Bromodeoxyuridine	64a	53	68	81	85	75	56	32	77
1,7-Dimethylxanthine	66a	42	76	77	58	68	70	42	56
4-Hydroxy-6-methylpyrimidine	66b	47	76	75	54	72	58	33	59
8-Mercapto-6-methylpurine	66c	49	73	77	49	77	58	45	74
1-Methyluracil	66d	50	69	70	81	60	64	38	57
1,7-Dimethylhypoxanthine	66e	70	73	64	52	57	60	29	40
3-Methyl-6-methylamino-5-(N-formylmethylamino)-uracil	67a	41	76	64	89	56	70	27	51
5,6-Dimethyluracil	69a	70	75	80	85	77	69	40	66
2-Amino-4-methylpyrimidine	69b	88	82	87	63	83	65	39	72
1,3-Dimethylxanthine	70a	55	78	80	61	75	67	50	65
6-Azathymine	71a	46	73	82	78	83	70	66	77
6-Methoxypurine	72a	54	82	84	58	84	68	52	69
6-Methyl-2-thiouracil	72b	56	76	79	78	73	77	64	75
6-Amino-1,3-dimethyluracil	72c	83	75	76	pink str	75	74	38	61
5-Ethoxymethyluracil	73a	59	78	86	89	79	70	49	75
6-Amino-2,4-dimethylpyrimidine	73b	87	72	69	75	70 ±	69	4	16,30

2-Amino-4,6-dimethylpyrimidine	74a	92	82	89	78	86	70	31	67
1-Methylthymine	75a	67	78	72	85	69	75	56	68
3-Methyl-6-methylaminouracil	76a	43	78	84	pink str.	77	73	38	67
Dithiouracil	76b	73	77	88	88	86	83	90	89
Theophylline-7-acetic acid	77a	45	74	36	88	17	72	54	54
3-Methyluracil	77b	61	78	81	91	77	75	58	70
2-Thiothymine	77c	63	77	92	87	83	75	70	84
5-Ethoxymethy-6-methyluracil	77d	69	81	88	81	84	78	53	76
6-Furfurylaminopurine	78a	85	87	94	79	89	77	58	85
6-Methylmercaptopurine	79a	68	82	90	55	82	78	71	77
1,3,7,9-Tetramethyluric acid	79b	87	80	80	93	71	79	58	65
1,3,8-Trimethylxanthine	80a	61	84	86	75	83	80	63	76
1,3,7-Trimethylxanthine	82a	88	84	81	79	79	85	74	73
4-Hydroxy-2-methylmercaptopyrimidine	83a	59	82	88	76	85	86	78	81
4,6-Dimethylpyrimidine	84a	—	89	—	74	—	81	80	77
1,3-Dimethyluracil	86a	91	85	87	97	84	88	82	83
Propyl-2-thiouracil	88a	76	88	93	95	95	93	92	92
5-Butoxymethyluracil	88b	85	86	96	95	93	89	87	87
2,4-Dimethoxy-5-methylpyrimidine	90a	95	93	93	89	92	91	97	93

* ± following a figure indicates an abnormally high variability in replicate determinations of the R_F values.

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TABLE III

PURINES, PYRIMIDINES, AND DERIVATIVES DETECTED WITH SHORT WAVE ULTRAVIOLET LIGHT (ALPHABETICAL ARRANGEMENT OF COMPOUNDS WITH R_F NOTATION FOR THE FIRST SOLVENT IN TABLE II)

5-Acetylamino-6-amino-3-methyluracil	44b	5-Aminouridine	14b
5-Acetyluracil	57c	6-Azathymine	71a
Adenine	48b	6-Azauridine	36d
Adenine-N′-oxide	43d	Barbituric acid	43e
Adenosine	32g	5-Bromodeoxyuridine	64a
Adenosine-5′-diphosphate	2a	5-Bromouridine	46a
Adenosine-5′-phosphate	8b	5-Butoxymethyluracil	88b
Adenosine-5′-triphosphate	0b	6-Carboxypurine	30a
S-Adenosylmethionine	5b	Cytidine	32d
5-Amino-6-carboxyuracil	29a	Cytidine-5′-phosphate	7a
6-Amino-2,8-dihydroxypurine	15b	Cytosine	45d
6-Amino-1,3-dimethyl-5-formylaminouracil	50h	Cytosine-5-carboxylic acid	9a
2-Amino-4,6-dimethylpyrimidine	74a	Deoxyadenosine	43g
6-Amino-2,4-dimethylpyrimidine	73b	Deoxycytidine	48d
6-Amino-1,3-dimethyluracil	72c	Deoxycytidine-5′-phosphate	15a
2-Amino-4,6-dioxy-5-methylpyrimidine	38a	Deoxyguanosine	33a
2-Amino-4,6-dioxypyrimidine	35a	Deoxyinosine	38i
6-Amino-5-formylamino-4-hydroxypyrimidine	21b	Deoxyuridine	50e
6-Amino-5-formylamino-1-methyl-4-oxypyrimidine	39a	Deoxyuridine-5′-phosphate	21a
6-Amino-5-formylamino-3-methyl-4-oxypyrimidine	38k	2,5-Diamino-4,6-dioxypyrimidine	3b
6-Amino-5-formylamino-1-methyluracil	22b	2,6-Diamino-5-formylamino-4-hydroxypyrimidine	12b
6-Amino-5-formylamino-3-methyluracil	38c	2,6-Diamino-5-formylamino-4-methoxypyrimidine	50g
6-Amino-5-formylaminouracil	15c	4,6-Diamino-5-formylamino-2-methylpyrimidine	38l
2-Amino-4-hydroxy-6-methylpyrimidine	58b	2,6-Diamino-7-methylpurine	28a
2-Amino-8-hydroxypurine	19b	4,5-Diamino-6-methylpyrimidine	63a
6-Amino-2-hydroxypurine	30b	2,4-Diamino-6-oxypyrimidine	38d
6-Amino-8-hydroxypurine	31a	4,6-Diamino-2-oxypyrimidine	43c
2-Amino-4-hydroxypyrimidine	50a	2,6-Diaminopurine hemisulfate	34c
4-Amino-5-imidazolecarboxamide	45g	4,6-Diaminopyrimidine	53c
4-Amino-5-imidazolecarboxamide ribonucleoside	32f	4,6-Diamino-2-thiopyrimidine	59d
6-Amino-3-methyl-5(N-formylmethylamino) uracil	59a	6,8-Dihydroxy-2-methylpurine	29b
2-Amino-5-methyl-4-oxypyrimidine	61a	6,8-Dihydroxypurine	23a
2-Amino-6-methylpurine	42b	2,4-Dihydroxypyrimidine-6-methylsulfone	58g
2-Amino-4-methylpyrimidine	69b	2,4-Dimethoxy-5-methylpyrimidine	90a
5-Amino-6-methyluracil	32b	2-Dimethylamino-6-hydroxypurine	51a
6-Amino-1-methyluracil	49b	6-Dimethylaminopurine	63b
2-Aminopurine	32c	6-Dimethylaminopurine ribonucleoside	53d
2-Aminopyrimidine	62c	1,7-Dimethylguanine	29c
6-Amino-2-thiouracil	50c	1,7-Dimethylhypoxanthine	66e
5-Aminouracil	25b	4,6-Dimethylpyrimidine	84a
6-Aminouracil	34b	1,3-Dimethyluracil	86a

5,6-Dimethyluracil	69a
1,3-Dimethyluric acid	47f
1,7-Dimethyluric acid	47d
3,7-Dimethyluric acid	39b
1,3-Dimethylxanthine	70a
1,7-Dimethylxanthine	66e
3,7-Dimethylxanthine	58d
3,8-Dimethylxanthine	56a
Dithiouracil	76b
5-Ethoxymethyl-6-methyluracil	77d
5-Ethoxymethyluracil	73a
5-Ethoxymethyluridine	56b
5-Fluorodeoxyuridine	59b
5-Fluorouracil	54a
5-Formylamino-3-methyl-6-methylaminouracil	48a
5-Formylcytosine	42c
5-Formyldeoxyuridine	42d
5-Formyluracil	44d
5-Formyluridine	30d
6-Furfurylaminopurine	78a
Guanine	23b
Guanosine	20a
Guanosine-5′-phosphate	5a
5-Hydroxydeoxyuridine	32a
6-Hydroxy-2-methylaminopurine	47e
5-Hydroxymethylcytosine	38h
5-Hydroxymethyldeoxycytidine	26d
5-Hydroxymethyldeoxyuridine	36c
8-Hydroxy-7-methylguanine	22a
4-Hydroxy-2-methylmercaptopyrimidine	83a
5-Hydroxymethyl-6-methyluracil	44c
5-Hydroxymethylorotic acid	26b
5-Hydroxymethylorotic acid lactone	45b
2-Hydroxy-6-methylpurine	36b
2-Hydroxy-4-methylpyrimidine	54b
4-Hydroxy-6-methylpyrimidine	66b
5-Hydroxymethyluracil	37a
5-Hydroxymethyluridine	24c
8-Hydroxypurine	42a
4-Hydroxypyrimidine	58c
Hypoxanthine	38g
Inosine	24d
Isobarbituric acid	34a
8-Mercapto-6-methylpurine	66c
5-Methoxymethyluracil	61b
5-Methoxymethyluridine	43f
6-Methoxypurine	72a
6-Methoxypurine ribonucleoside	62d
1-Methyladenine	46b
2-Methyladenine	41a
3-Methyladenine	53b
7-Methyladenine	45f
9-Methyladenine	57e
2-Methyladenine sulfate	57b
1-Methyladenosine	32e
6-Methylamino-5-(N-formylmethylamino)uracil	38e
6-Methylaminopurine	61c
6-Methylaminopurine deoxyribonucleoside	30f
6-Methylaminopurine ribonucleoside	42e
5-Methylaminouracil	34d
5-Methylbarbituric acid	60a
5-Methylcytidine	36e
1-Methylcytosine	57d
5-Methylcytosine	53a
5-Methyldeoxycytidine	56c
5-Methyldeoxycytidine-5′-phosphate	19a
1-Methylguanine	40a
1-Methylguanine sulfate	30c
3-Methylguanine	36a
7-Methylguanine	35b
1-Methylguanosine	30e
7-Methylguanosine	28b
5-Methyl-5-hydroxybarbituric acid	43b
1-Methylhypoxanthine	51b
2-Methylhypoxanthine	45a
3-Methylhypoxanthine	38f
7-Methylhypoxanthine	49a
8-Methylhypoxanthine	47c
9-Methylhypoxanthine	50b
1-Methylinosine	37c
7-Methylinosine	26a
6-Methylmercaptopurine	79a
1-Methyl-6-methylamino-5-(N-formylmethylamino)uracil	50f
3-Methyl-6-methylamino-5-(N-formylmethylamino)uracil	67a
3-Methyl-6-methylaminouracil	76a
5-Methylorotic acid	57a

(*continued on p.* 128)

TABLE III (*continued*)

6-Methylpurine	58e	Theophylline-7-acetic acid	77a
6-Methyl-2-thiouracil	72b	2-Thiobarbituric acid	54c
1-Methylthymine	75a	2-Thiocytosine	58a
2-Methyl-4,5,6-triaminopyrimidine sulfate	45e	5′-Thiomethyladenosine	57f
1-Methyluracil	66d	5′-Thiomethylinosine	48c
3-Methyluracil	77b	2-Thio-orotic acid	47b
6-Methyluracil	59c	2-Thiothymine	77c
1-Methyluric acid	25a	Thymidine	62b
3-Methyluric acid	22c	Thymidine-5′-phosphate	24a
7-Methyluric acid	26c	Thymine	62b
3-Methyluridine	62e	4,5,6-Triaminopyrimidine sulfate	37b
5-Methyluridine	45c	1,3,7-Trimethylxanthine	82a
1-Methylxanthine	52a	Uracil	50d
3-Methylxanthine	38j	1,3,8-Trimethylxanthine	80a
7-Methylxanthine	40b	Uracil-6-acetic acid	43a
Orotic acid	38b	Uracil-5-carboxylic acid	44a
Orotidine	17a	Uric acid	14a
Orotidine monophosphate	8a	Uridine	35c
Propyl-2-thiouracil	88a	Uridine-5′-diphosphate	0a
Purine	58f	Uridine diphosphate glucose	0c
5-Ribosyluracil	21c	Uridine-5′-phosphate	12a
Spongothymidine	55a	Uridine-5′-triphosphate	3a
6-Succinoaminopurine	47a	Xanthine	24b
1,3,7,9-Tetramethyluric acid	79b	Xanthosine	18a

SUMMARY

Paper chromatographic data for 215 purines, pyrimidines and derivatives in nine solvent systems have been reported. The data are useful as an aid in identification of compounds, and for selection of solvents to perform various separations.

REFERENCES

1 K. FINK, R. E. CLINE AND R. M. FINK, *Anal. Chem.*, 35 (1963) 389.
2 R. M. FINK, C. E. DENT AND K. FINK, *Nature*, 160 (1947) 801.
3 K. FINK, W. S. ADAMS, F. W. DAVIS AND M. NAKATANI, *Cancer Res.*, 23 (1963) 1824.
4 K. FINK, W. S. ADAMS AND W. PFLEIDERER, *J. Biol. Chem.*, 239 (1964) 4250.

HISTAMINE CHROMATOGRAPHY AND ELECTROPHORESIS

THE *o*-PHTHALALDEHYDE FLUOROGRAM*

WALTER B. SHELLEY AND LENNART JUHLIN

Department of Dermatology, University of Pennsylvania, School of Medicine, Philadelphia, Pa. (U.S.A.)

(Received September 21st, 1965)

Although histamine has long been biologically assayed by its effect on smooth muscle, the best modern quantitation technics are based on spectrofluorometry[1–3]. Nevertheless, both approaches remain time consuming and require meticulous attention to detail. With a view to simplifying and accelerating the quantitative estimation of histamine we have turned to the new worlds of chromatography and electrophoresis.

Youthful and vigorous, the field of chromatography has attracted many investigators as five comprehensive review volumes will attest[4–8]. Of the several branches available for study, we chose paper and thin-layer partition chromatography. The other popular branch, gas chromatography, is rapid and gives remarkable separation of compounds in an ultra micro sample. Indeed, it has been used in the determination of histamine[9] but it requires specimens with far higher concentrations of histamine than exist biologically, except in cell depots.

Paper and thin-layer chromatography allow separation and identification of compounds by virtue of their partition coefficients in carefully selected solvents moving over paper or a thin layer of a supportive solid. In practical use for over twenty years, chromatographic observations have been made on an unbelievable number of substances. These have included histamine, but the majority of this literature relates to histamine as an incidental member of a series of compounds, *e.g.* the amino acids. Table I summarizes the salient composite data from the previous reports[10–20]. Nearly all of these values relate to paper chromatography. The newer thin-layer techniques have been recently tried[7,8,19], and appear to offer the advantages of speed and sensitivity.

Reviewing Table I, it can be concluded that alkalinization greatly accelerates the migration of histamine. Thus almost any degree of movement can be achieved by using the proper solvent. Colorimetric evocation of the histamine spot has been achieved either by employing the relatively insensitive non-specific ninhydrin or by the more sensitive Pauly reagent, an azo dye (diazotized sulfanilic acid or *p*-nitroaniline). A surprising degree of sensitivity is achieved in certain laboratories, some detecting as little as 0.05 γ on silica gel thin-layer plates. The general average appears to be of the order of magnitude of 1 γ. All of the paper chromatographic methods are time consuming requiring hours, whereas the thin-layer techniques are much more rapid.

* This work was supported by a grant from the John A. Hartford Foundation, Inc.

The application of an electrical field to paper thin-layer strips[4] and more particularly to gels[21] has been another way of separating compounds. Here, the ion mobilities are the critical determinant rather than partition coefficients in carefully selected solvents. As a small highly charged molecule, histamine would appear particularly suited to such iontophoresis or electrophoresis. Studies on histamine have not appeared in the gel electrophoresis literature, but there is one report concerned with thin-layer electrophoretic systems[22].

TABLE I

REPRESENTATIVE DATA FROM PREVIOUS CHROMATOGRAPHIC METHODS OF DETECTING HISTAMINE
Whatman No. 1 paper, ascending.

Approx. R_F*	*Solvent set (ratios)*	*Visualization spray*	*Sensitivity* (μg)
0.0	Isobutyric acid–water (8:2)		
0.1	*n*-Butanol–acetic acid–water (4:1:5)		
0.2	*n*-Butanol–acetic acid–water (8:2:2)	Ninhydrin 0.2% in water saturated *n*-butanol	1
0.3	2,6-Lutidine–water (65:35)	or	
0.4	2,6-Lutidine–collidine–water (1:1:1)	Diazotized sulfanilic acid (Pauly reagent)	
0.5	2,6-Lutidine (water satd.)–collidine (3:1)		
0.6	Propanol–0.2 *N* ammonia (3:1)		
0.7	Pyridine–water (65:35)		
0.8	*n*-Butanol–95% ethanol–conc. ammonium hydroxide (8:1:3)	*p*-nitroaniline	0.3
0.9	Phenol–water (ammonia atmosphere) (10:2)		

* $R_F = \frac{\text{distance histamine carried}}{\text{distance solvent front moved}}$.

The above techniques may be intermingled with the result that some workers have employed two dimensional chromatography (two solvent sets), others, paper chromatography followed by electrophoresis, and a few, chromatography coupled with elution and subsequent biologic or spectrophotometric assay.

Our survey of the literature indicated that histamine has been but a stepchild in chromatographic studies. It has received scant primary attention. Accordingly, the present investigation was undertaken to determine directly the most sensitive, specific, rapid method of separating and detecting histamine using the latest chromatographic techniques. This study of histamine has thus included comparative observations on both paper and thin-layer chromatography as well as paper, thin-layer and gel electrophoresis. Particular emphasis has been laid on developing a more sensitive and specific means of staining for histamine.

OBSERVATIONS AND RESULTS

Initial studies centered on spray reagents for detecting the histamine spot in as sensitive and specific a manner as possible. None of the previously employed visualization techniques seemed to offer as much as might be achieved by employing a new compound, *o*-phthalaldehyde (OPT). This compound readily forms a fluorescent condensate with histamine in alkaline solution and this reaction serves as the basis for the most popular and sensitive fluorometric test for histamine today[2,3]. Although

OPT had previously been employed by PATTON AND FOREMAN[23] as a simple colorimetric agent for the detection of glycine, histidine and tryptophan in paper chromatograms, it has never been used in detecting histamine chromatographically.

Preliminary tests showed that unless the histamine were in aqueous solution, *i.e.* ionized, it did not form a fluorochrome with OPT. It also became apparent that for ultimate sensitivity the OPT would have to be applied in a solvent in which neither histamine nor the histamine-OPT condensate would dissolve. Indeed the following requisites proved desirable for the OPT spray solvent:

(1) Dissolves OPT, and provides stable non-reactive milieu.
(2) Immiscible with water.
(3) Does not dissolve histamine.
(4) Releases OPT to aqueous phase.
(5) Does not interfere with histamine-OPT condensate formation.
(6) Does not dissolve condensate.
(7) Neither fluoresces nor quenches fluorescence.

Over 125 solvents were screened by these seven criteria, and it was found that xylene, *p*-xylene and diethyl ether were the best. They met all of the requirements. α-Chloronaphthalene and dibutyl carbitol were two other satisfactory solvents, but xylene and *p*-xylene proved to be best in general usage. Spraying solutions of OPT in any of these solvents on an alkaline spot of dilute histamine on Whatman No. 1 paper was regularly followed by the appearance of a blue color when viewed under ultraviolet light (3600 Å). This color was faint and evanescent at the greatest dilutions, apparently due to the photosensitive nature of the condensate formed. It should be noted that unlike histamine the condensate is thermolabile.

Next, it was shown that OPT in a 0.2–1% (w/v) concentration gave the optimal sensitivity. Samples from different companies showed variations, but the best and most consistent results were obtained by using OPT from California Biochemical Corporation. Immediately preceding the application of the OPT spray, the chromatogram was rendered alkaline to promote OPT-histamine condensate formation. For this, the best spray proved to be 0.2 *N* sodium hydroxide (fluorescent grade, Hartman-Leddon Company, Inc.), although a pH 11 buffer was also satisfactory. Under exceptional instances, *e.g.* when using the magnesium silicate plates, this alkaline spray step could be eliminated.

Using the 0.2 *N* NaOH–1% OPT *p*-xylene sprays, sixty papers and thin-layer plates were screened to see which afforded the greatest degree of spot test sensitivity (Table II). Magnesium silicate thin layer proved to be the most satisfactory of all the supportive media studied. Although similar sensitivity could be achieved with other paper and plates, magnesium silicate gave the most vivid and lasting spot reaction. Viewing was best done under an intense Wood light.

Having established the technique of *o*-phthalaldehyde fluorescent spot testing for histamine, we turned to examine its specificity (Table III). Previous authors had reported that OPT formed fluorescent condensates with certain biologic compounds. Under the conditions of testing in our laboratory, only histidine and glutathione (reduced) were found to interfere significantly. It was found that these two compounds could readily be separated from histamine by either chromatographic or electrophoretic means. With this background we turned to the application of the OPT technique to chromatography and electrophoresis.

TABLE II

SPOT TEST FOR HISTAMINE HYDROCHLORIDE. COMPARISON OF PAPERS AND THIN-LAYER PLATES

Factors: sample, 0.01 *N* NaOH, 1 % OPT in *p*-xylene (0.01 ml each); U.V. light viewing: blue spot.

Paper	*Limit of detection* (*μg*)
Whatman No. 1	0.02
Whatman No. 2	0.02
Sepraphore III (Gelman)	0.02
Schleicher & Schüll 576	0.05
Schleicher & Schüll 2045	0.05

Thin-layer plates	*Limit of detection* (*μg*)
Magnesium silicate*	0.01
Alumina Basic*	0.02
Absorbasil	0.02
Cellulose MN 300	0.03
Silica H	0.05

* No buffer added. Read at once.

The following papers were less satisfactory or not suitable: Whatman Nos. 3 MM, 4, 5, 7, 11, 20, 31, 40, 42, 44, 50, 52, 54, 541. Schleicher & Schüll 507, 589 (red, white, blue, orange, green, black), 2041, 602, 2040A, 2043A, 2045B, 2043B, 598-4D, 598, 470A, 470. Cellogel (Colab), Millipore UF, RA, HAB, OH, HA, Duralon. Oxoid Electrophoresis Strips.

The following plates were less satisfactory or not suitable: Neutral Alumina, Acidic Alumina, Alumina G, Alumina GF, Cellulose ECTEOLA, Cellulose DEAE, Talc, Kieselguhr, Silica G, Polyamide, Eastman Silica Paper.

TABLE III

BIOLOGIC SPECIFICITY OF *o*-PHTHALALDEHYDE SPOT TEST REACTION

Whatman No. 1 paper, 0.01 *N* NaOH, 1 % OPT in *p*-xylene, immediate reading of blue color under U.V. light.

Reactive biologic compounds	*Minimal amount detectable* (*μg*)	*Color*
Histamine	0.02	Blue
Histidine	0.1	Blue
Glutathione (reduced)	1.0	Blue
Citrulline	10.0	Blue

See Fig. 1 for thin-gel data.

Non-reactive compounds (100 *μg* or less) include: acetophenetidin, *l*-alanine, ammonia, amphetamine, arginine, asparagine, *dl*-aspartic acid, creatine, creatinine, cysteine, cystine, dopa, dopamine, *dl*-ethionine, galactosamine, glucosamine, glutamine, glycine, glycogen (rabbit liver), glycogen (shellfish), glucose 6-phosphate, guanidine, heparin, homocitrullin, *dl*-homocystine, hyaluronic acid, 5-hydroxy-3-indoleacetic acid, hydroxyproline, imidazole, indole, isoleucine, *l*-leucine, *dl*-lysine, methionine, *d*-*β*-naphthol, nor-epinephrine, norleucine, *dl*-norvaline, *dl*-ornithine·HCl, *l*-proline, procaine, pyribenzamine, sarcosine, serotonin-creatinine sulfate, *dl*-serine, spermidine, spermine, streptomycin, sulfanilamide, taurine, thiamine, thiourea, threonine, tryptophan, tryptamine, tyramine·HCl, *l*-tyrosine, urea, uric acid, urocanic acid, *l*-valine.

1. Paper chromatography

Despite its technical simplicity, paper chromatography is ordinarily a slow process requiring many hours. Hence only limited studies were done in this area since both thin-layer chromatography and electrophoresis gave answers within minutes.

Ascending chromatograms could be satisfactorily prepared using Whatman No. 1 paper and a solvent system of propanol–0.2 *N* ammonia (3:1). The ammonia had to be removed by heating (150°, 10 min) before OPT was added to prevent non-specific staining of ammonia by the OPT. It should be noted that, although histamine is heat stable, it is not stable in alkaline solutions so that prolonged periods in such a solvent may lower the yield and makes this approach unsatisfactory for detecting trace amounts. Thus, amounts less than 1 μg could not be visualized well.

2. Thin-layer chromatography

In this procedure, 0.01 ml test solution is applied to carrier plates coated with a uniformly thin layer (250 μ) of powdered adsorbent. The commercially prepared plates (Mann Research Laboratories, Inc.) given in Table IV were used.

Ascending chromatograms were made using propanol–0.2 *N* ammonia (3:1). Varying degrees of separation and sensitivity can be achieved but for general work, the Cellulose 300 MN plates proved to be the best.

TABLE IV

PLATES FOR THIN-LAYER CHROMATOGRAPHY

No.	*Commercial name*	*Absorbent*	*Binder*	*Reaction*
1	Absorbasil	Silica	Calcium sulfate	Neutral
2	Alumina-Acidic	Aluminum oxide	None	Acidic
3	Alumina-Basic	Aluminum oxide	None	Basic
4	Alumina-Neutral	Aluminum oxide	None	Neutral
5	Alumina G	Aluminum oxide	Calcium sulfate	Basic
6	Alumina GF	Same as 5 with zinc fluorescent agent added		
7	Cellulose MN 300	Cellulose powder	None	Neutral
8	Cellulose MN 300 ECTEOLA	Epichlorohydrin-triethanolamine cellulose (anion exchange resin)	None	Neutral
9	Cellulose MN 300 DEAE	Diethylaminoethyl cellulose (ion exchange resin)	None	Basic
10	Hy-Flo Talc	Magnesium silicate	None	Neutral
11	Kieselguhr	Diatomaceous earth	Calcium sulfate	Neutral
12	Magnesium silicate	Magnesium silicate	Calcium sulfate	Basic
13	Polyamide	Polyamide	None	Neutral
14	Silica G	Silica gel	Calcium sulfate	Acidic
15	Silica GF	Same as 14 with zinc fluorescent agent added		
16	Silica H	Silica gel	Hydrated silica	Acidic

3. Electrophoresis

The addition of an electrical potential to the basic chromatographic techniques greatly accelerated and simplified separation techniques. Using horizontal strip electrophoresis apparatus (Arthur H. Thomas Model or Turner Model 310), it was possible to increase sensitivity and shorten separation time to as little as 10 min. Although these rapid clear separations were achieved with paper, thin-layer and gel techniques, the thin-gel procedure proved to be the best.

(*a*) *Paper electrophoresis*. Using either Whatman No. 1 paper or Gelman Sepraphore III, excellent separation of histamine, histidine and glutathione was attained. The following general technique was employed:

Add 0.01 ml sample to 2 × 8 in. strip and dry.

Spray lightly with pH 4.6 buffer (potassium acid phthalate–sodium phosphate).

Place face up in apparatus rack.

Run 10–30 min (Thomas unit, 300 V, 5 mA/8 in. strip) with pH 4.6 buffer in side wells.

Dry paper in oven at 150° for 5 min.

Spray lightly with 0.2 *N* NaOH.

Spray lightly with 1 % OPT in *p*-xylene.

Read blue spots under Wood light immediately.

Within 30 min, 0.050 μg of histamine in aqueous solution may be separated and detected.

(*b*) *Thin-layer electrochromatography*. Thin-layer plates as described above were all tested and Cellulose 300 MN proved to be the most satisfactory. Thus, using this plate with the same technique outlined for paper electrophoresis, with the exception that the plates are not dried in the oven, histamine moves 8 cm toward the cathode in 30 min. Histidine moves about half as far and glutathione is stationary, with a resultant excellent separation. It is possible to detect a 1/600,000 dilution of histamine hydrochloride. Histamine 1/100,000 added to plasma can be separated and identified by this means. Alumina G and Absorbasil plates were also useful, but gave a lower order of sensitivity. The other plates were distinctly less satisfactory or completely unusable.

(*c*) *Gel electrophoresis*. This proved to be the most sensitive and generally satisfactory technique for detecting, identifying, separating and quantitating trace amounts of histamine in fluids, blood and tissue extracts. It is possible to separate and detect as little as 2 ng (0.002 μg) of the free base of histamine under optimal conditions, using the following OPT–thin-layer method:

Alcohol cleansed glass plates, 4 × 3 ¼ in., are covered with 5 ml of hot 1 % agar (Ionagar® No. 2) in pH 4.6 potassium acid phthalate, sodium phosphate buffer. Plate is kept at absolute level during cooling.

Seven 1 mm wells are punched out in a line.

Add 0.004 ml samples to each well.

Plate placed face down in Turner Model 310 with pH 4.6 buffer in side wells.

Run for 5–10 min., 200 V.

Remove and spray lightly with 0.2 *N* NaOH.

Spray lightly with 1% OPT in *p*-xylene.

Read instantly in intense transmitted U.V. light. Direct Wood light is much less satisfactory. All data recorded is based on reading with the transmitted light of a microscope mercury vapor lamp (Osram HBO 200 W, Filter UG 1).

Thick gels are employed in disc electrophoresis (Canalco®) and vertical gel electrophoresis (E. C. Apparatus®) were extensively tried but proved of no value for histamine detection. Both polyacrylamide and agar gels were tried in varying concentration. It should be noted that the tris and riboflavin commonly added to these

media had to be eliminated due to fluorescence. Polyacrylamide could, however, be used as a gel in the thin-gel procedure described above (5 % in a pH 4.6 buffer). The high voltage (10,000 V) electrophoretic apparatus was not tried since separation of histamine from plasma could be made in 5 to 10 min with the present method.

Unfortunately at this stage the fluorescence cannot be recorded quantitatively since routine densitometers and reflectance fluorometers have not proved adequate.

Two dimensional thin-gel electrophoresis may be undertaken by making a second run employing a 9.6 buffer, thus eliminating all OPT reactive substances with an isoelectric point between 4.6 and 9.6.

Histamine, histidine and glutathione can be sharply separated electrophoretically by virtue of their electrical charge in solutions of varying pH. The distance separating histamine and histidine remains approximately the same throughout the range pH 2.6 to 11.0. However, both move to the cathode if the pH is less than 8. At its isoelectric point (pH 8) histidine remains stationary whereas histamine moves still to the cathode. Between pH 8 and 10 histidine moves toward the anode whereas histamine is still moving toward the cathode. Finally, at pH 11 histamine is stationary, whereas histidine migrates even more toward the anode. In view of the fact that separation distance is essentially the same, we elected to work at an acid pH 4.6 since here histamine has maximal stability, and glutathione never interferes inasmuch as it is moving toward the opposite pole.

Extraction tests with this thin-gel–OPT method revealed histamine in the cases given in Table V.

TABLE V

DETECTION OF HISTAMINE BY THIN-GEL ELECTROPHORESIS AND OPT

Source	*Extraction technique*
Rat mast cells	Freeze-thaw
Rabbit platelets	Freeze-thaw
Rat tongue	Butanol–benzene
Rat skin	Butanol–benzene
Human stratum corneum	Trichloracetic acid
Human stratum normal	Trichloracetic acid
Human stratum psoriatic	Trichloracetic acid
Human stomach	Trichloracetic acid
Guinea pig lung	Trichloracetic acid
Rat skin	Trichloracetic acid
Rat tongue	Trichloracetic acid

DISCUSSION

We have found the *o*-phthalaldehyde fluorogram a simple, rapid and specific means of analyzing for trace amounts of histamine. The procedure is based on the application of the OPT reagent in an organic solvent which dissolves neither the histamine nor the OPT-histamine fluorescent condensate. This permits precise chromatographic localization of very small amounts of histamine.

The OPT reaction is not rigidly specific for histamine. Hence the spot test is of limited value, although if an extraction method has been employed which excludes

interfering biologic compounds (Table III), it is possible to detect rapidly traces of histamine in an aqueous medium. With the employment of chromatography of varying types, it has been possible to isolate and identify histamine with precision. In our experience, neither paper chromatography nor thin-layer chromatography was ideal, since sensitivity was relatively low. With the addition of an electrical field, these techniques became more satisfactory. However, in all of our studies the singular and best approach was thin-gel electrophoresis. This had all of the advantages of reproducibility, speed and sensitivity (Table VI and Fig. 1). The electrical field re-

TABLE VI

SUMMARY OF RECOMMENDED TECHNIQUES FOR FLUOROGRAM DETECTION OF AQUEOUS HISTAMINE HYDROCHLORIDE

Alkaline spray followed by 1% OPT in *p*-xylene. Read in U.V. light.

Technique	*Absorbent system*	*Solvent*	*Time*	*Detection limit (μg)*
Spot test: thin-layer plate	Magnesium silicate	None	Instantaneous	0.01
Paper chromatography	Whatman No. 1	Propanol–0.2 *N* ammonia (3:1)	3 h	1.0
Thin-layer chromatography	Cellulose MN 300	Propanol–0.2 *N* ammonia (3:1)	30 min	0.1
Paper electrophoresis	Whatman No. 1	Buffer pH 4.6	30 min	0.05
Thin-layer electrophoresis	Cellulose MN 300	Buffer pH 4.6	30 min	0.02
Thin-gel electrophoresis	Ionagar 1%	Buffer pH 4.6	10 min	0.005

sulted in marked separation of all of the interfering substances which give fluorescent condensates with OPT. Thus in our laboratories this technique has been employed regularly with success.

Quantitation of the fluorogram has proved to be a difficult problem. None of the equipment tested by us proved satisfactory, but this is still under study. It is possible, however, to elute the histamine spot in the chromatogram and reap the elution photo-fluorometrically.

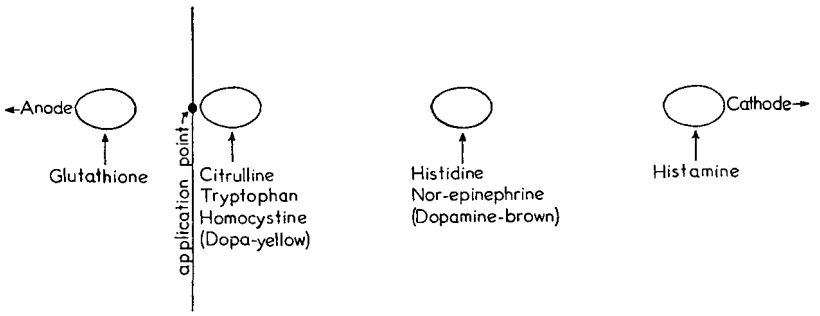


Fig. 1. Specific separation of histamine on *o*-phthalaldehyde fluorogram. Thin-gel (agar) electrophoresis (10 min, 200 V, 0.004 ml sample, pH 4.6 buffer). Localization and size of spot drawn to scale. Dilution limits for identification in 0.004 ml sample: 10^{-6} histamine, histidine; 10^{-5} nor-epinephrine, citrulline, glutathione; 10^{-4} tryptophan, dopamine, dopa; homocystine (saturated solution). Over sixty other biologic compounds gave no fluorescence at dilutions of 10^{-3} or greater.

SUMMARY

Rapid new sensitive chromatographic techniques are described for the isolation and detection of trace amounts of histamine. These are all based on the fact that in an alkaline medium, *o*-phthalaldehyde (OPT) and histamine immediately form a relatively specific and highly fluorescent condensate. Such OPT fluorograms may be made employing spot tests, paper and thin-layer chromatography, as well as paper, thin-layer and gel electrophoresis. With spot tests, thin-layer magnesium silicate plates proved to be the most sensitive, allowing the detection of less than 0.01 μg of histamine. For separation and more definitive identification of histamine, agar thin-gel electrophoresis was found to be the best method. Employing this, it was possible within 10 minutes to isolate and identify free histamine base in quantities as small as 0.002 μg.

REFERENCES

1 C. F. CODE AND F. C. MCINTIRE, *Methods Biochem. Anal.*, 3 (1956) 49.
2 P. A. SHORE, A. BURKHALTER AND V. H. COHN, Jr., *J. Pharmacol. Exptl. Therap.*, 127 (1959) 182.
3 L. T. KREMZNER AND I. B. WILSON, *Biochim. Biophys. Acta*, 50 (1961) 364.
4 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New York, 1955, 484 pp.
5 I. M. HAIS AND K. MACEK (Editors), *Paper Chromatography*, Academic Press, New York, 1963, 955 pp.
6 J. M. BOBBITT, *Thin Layer Chromatography*, Reinhold Publishing Corp., New York, 1963, 208 pp.
7 K. RANDERATH, *Thin-Layer Chromatography*, Academic Press, New York, 1963, 250 pp.
8 E. STAHL (Editor), *Thin-Layer Chromatography. A Laboratory Handbook*, Academic Press, New York, 1965, 553 pp.
9 H. M. FALES AND J. J. PISANO, *Anal. Biochem.*, 3 (1962) 337.
10 K. F. URBACH AND L. GISCAFRE, *Proc. Soc. Exptl. Biol. Med.*, 68 (1948) 430.
11 C. E. DENT, *Biochem. J.*, 43 (1948) 169.
12 H. K. BERRY, H. E. SUTTON, L. CAIN AND J. S. BERRY, *Development of Paper Chromatography for Use in the Study of Metabolic Patterns*, Univ. of Texas Publ., No. 5109, May 1, 1951, pp. 22–55.
13 B. N. AMES AND H. K. MITCHELL, *J. Am. Chem. Soc.*, 74 (1952) 252.
14 G. V. R. BORN AND J. R. VANE, *Brit. J. Pharmacol.*, 7 (1952) 298.
15 G. B. WEST AND J. F. RILEY, *Nature*, 174 (1954) 882.
16 L. I. GARDNER AND A. A. TICE, *Pediatrics*, 21 (1958) 805.
17 G. B. WEST, *J. Pharm. Pharmacol.*, 11 (1959) 595.
18 M. SHIFRINE, E. OUSTERHOUT, C. R. GRAU AND R. H. VAUGHN, *Appl. Microbiol.*, 7 (1959) 45.
19 A. H. BECKETT AND N. H. CHOULIS, *J. Pharm. Pharmacol.*, 15 (1963) 236T.
20 M. SHIFRINE AND G. ZWEIG, *J. Chromatog.*, 14 (1964) 297.
21 J. F. FREDERICK (Editor), *Gel Electrophoresis, Ann. N.Y. Acad. Sci.*, 121 (1964) 305.
22 C. G. HONEGGER, in E. STAHL (Editor), *Thin-Layer Chromatography. A Laboratory Handbook*, Academic Press, New York, 1965, pp. 433–435.
23 A. R. PATTON AND E. M. FOREMAN, *Science*, 109 (1949) 339.

CHROMATOGRAPHIC STUDIES OF PHOSPHORUS COMPOUNDS

PART XI. THE REACTION OF SODIUM HYDROXIDE WITH PHOSPHORYL CHLORIDE

F. H. POLLARD†, G. NICKLESS* AND J. D. MURRAY
Department of Chemistry, The University, Bristol (Great Britain)
(Received September 27th, 1965)

The hydrolysis of orthophosphoryl chloride with water has been studied by several early authors[1–3], and more recently by GRUNZE *et al.*[4,5] and HUDSON AND MOSS[6]. Grunze The latter concluded that the primary product of hydrolysis is dichlorophosphoric acid. Furthermore, he claimed that by self-condensation of this acid and co-condensation with monophosphoric acid, chloro-derivatives of polyphosphoric acids can be formed. VAN WAZER[7] *et al.* suggest that condensed acids are produced but with little or no chlorine in their structure.

We have applied paper and anion-exchange chromatographic techniques to investigate the reaction occurring when $POCl_3$ and sodium hydroxide solution are mixed in various mole ratios.

EXPERIMENTAL

Method

The correct amount of 1 *N* NaOH solution was placed in a small conical flask surrounded by an ice–water mixture. The solution was stirred using a magnetic stirrer. After 15 min the $POCl_3$ (purified by distillation) was added by means of a syringe pipette. There was some agitation between the immiscible liquids and then a sudden evolution of hydrogen chloride vapour occurred. Liberation of HCl continued slowly but upon heating the rate of evolution increased.

When the $POCl_3$:NaOH mole ratio was 50:1, a white precipitate was evident after the sudden HCl evolution. However, if the mixture was refluxed above 100° for one hour or longer, the precipitate disappeared, and now there was seen a clear viscous phase beneath a transparent labile liquid.

A white precipitate was also produced when the mole ratio was 25:1 but after one hour's refluxing, a viscous mixture was produced with no separation of phases. When the ratio was 100:1, no white precipitate was formed initially, but the viscous and labile layers were evident after refluxing. If excess NaOH was present, an homogeneous solution resulted after HCl evolution which was not noticeably affected by heat.

† Deceased October 16th, 1965.
* To whom enquiries should be addressed.

Analysis

(1) *Paper chromatography.* The white precipitates and viscous phases were dissolved in distilled water and neutralised to restrict degradation. The labile phases were found to react with water and so were applied directly to the chromatogram.

Descending methods of elution were used with Whatman No. 541 chromatographic paper. Many solvents with different ratios of components and different pH's were tried. The most successful was BIBERACHER's basic solvent[8]. After drying, the chromatograms were lightly sprayed with KARL-KROUPA's reagent[9] and then they were redried. The developed spots were identified as far as possible by comparison with a standard mixture of condensed phosphates which was developed on the same chromatogram.

(2) *Anion-exchange chromatography.* The neutral solutions obtained from the white precipitates or viscous phases were diluted sufficiently so that 1 ml containing 0.01 g of phosphorus could be introduced on to a column of Dowex 1X8 resin of mesh 100–200. The resin was contained in a column of length 50 cm, diameter 1.0 cm. This was eluted with potassium chloride buffered to pH 11.4 with ammonia. A linear gradient was employed by placing the reservoir and mixing vessel (which were both of 1 l volume and equal diameter) on the same horizontal level with the strong KCl solution (55 g/l) siphoning from the reservoir over into the weak KCl (5 g/l) in the mixing vessel.

The collected 10 ml fractions were warmed with 10 ml of concentrated nitric acid for one hour to convert all phosphate species to monophosphate. Then, after cooling, the solution was made up to 100 ml with distilled water after 5 ml of 5 g/l ammonium metavanadate solution (containing 2 % conc. HNO_3) and 10 ml of 10 % ammonium molybdate solution had been added. The optical density of the resulting phosphovanadomolybdate complex was measured by comparison with a blank using an Unicam spectrophotometer S.P. 500. Species were identified as far as possible by comparison of retention volumes with those of standard condensed phosphates and also by addition of a known quantity of a standard to the neutral solution under investigation.

RESULTS AND DISCUSSION

While the paper chromatographic findings were purely qualitative, the results derived from the anion-exchange investigation may be described as semi-quantitative. As the $POCl_3$ and NaOH were immiscible, the relative amount of each product depends to some extent on the surface area common to both components. The area of interface is not reproducible, therefore the results cannot be completely quantitative.

The transparent and labile phases resulting before and after refluxing gave rise to spots corresponding to mono-, di- and triphosphates and trimetaphosphate (Table I). It was also found that a spot of pure $POCl_3$ produced spots corresponding to the same species (reaction must occur in moist air or solvent vapour; streaking would have resulted if the liquid solvent had reacted with the $POCl_3$). Indeed the transparent liquid present with the white precipitate had the appearance and characteristic odour of $POCl_3$ while the labile phase produced after refluxing although having a similar smell was a less dense liquid—this was more likely dichlorophosphoric acid.

TABLE I

CONDENSED PHOSPHATES DETECTED BY PAPER CHROMATOGRAPHY

$POCl_3$:NaOH	*Phase*	*Species identified*
1:1	Homogeneous solution, unaffected by heat	Monophosphate and trace of diphosphate
25:1	Initial white precipitate. Viscous mixture (after refluxing)	Mono- and diphosphates
50:1	Initial white precipitate	Monophosphate and traces of diphosphate and higher polyphosphates
	Viscous phase (after 1 h refluxing	Mono-, di-, triphosphates, higher polyphosphates and trimetaphosphate
100:1	No white precipitate. Viscous layer (after 1 h refluxing)	Mono-, di-, triphosphates, higher polyphosphates and trimetaphosphate

Figs. 1, 2 and 3 show the anion-exchange separation of a mixture of polyphosphates, the elution patterns for the white precipitate, and for the viscous phases respectively.

The final large peak in the viscous phase is presumably due to higher polyphosphates which have not separated from one another. Such a separation could most likely be achieved, using a longer column, and a "shallower" gradient elution method.

When the mixture was refluxed for six hours instead of one hour, there was no significant change in the relative quantities of products.

It is interesting that GRUNZE found polyphosphoric acid derivatives were produced after refluxing a $POCl_3$–H_2O (1:1) mixture for a few hours[5]. The water content of this mixture is approximately the same as in our $POCl_3$–NaOH (50:1) mixture. Therefore the hydrolyses depend on water content and not OH^- ion concentration. There is no white precipitate formed in the primary step of the water

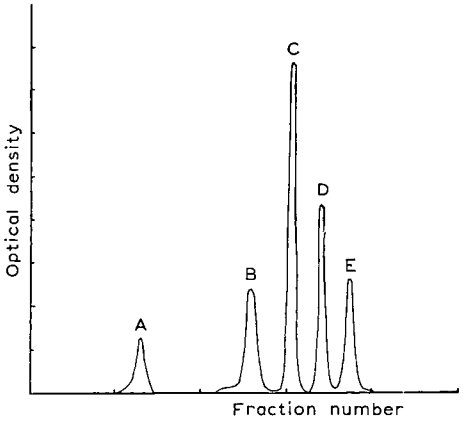


Fig. 1. Chromatographic separation of a synthetic standard mixture of polyphosphates. A = monophosphate; B = diphosphate (pyrophosphate); C = tripolyphosphate; D = tetrametaphosphate; E = trimetaphosphate.

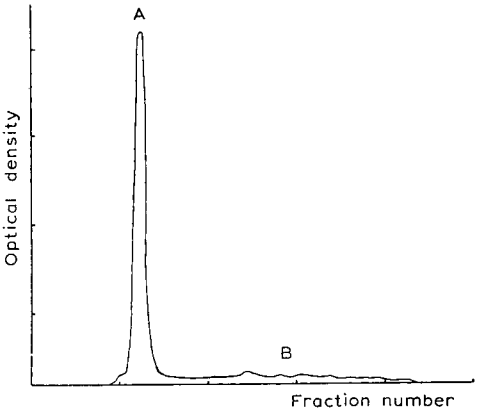


Fig. 2. Chromatogram obtained upon analysis of the white precipitate. A = monophosphate; B = traces of diphosphate and higher polyphosphates.

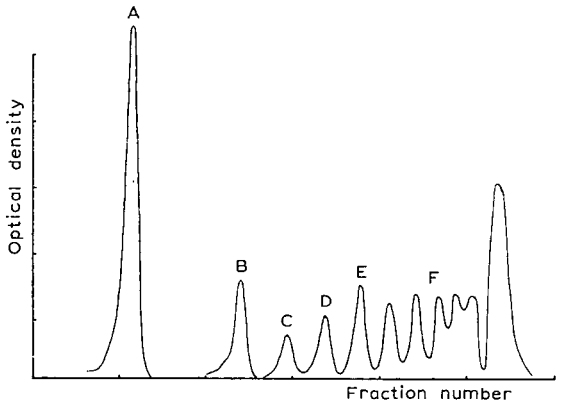


Fig. 3. Chromatogram obtained upon analysis of the viscous phase. A = monophosphate; B = diphosphate; C = tripolyphosphate; D = tetrametaphosphate; E = trimetaphosphate; F = higher polyphosphates (4 or more phosphorus atoms in the chain length).

hydrolysis because acids are produced and not sodium salts of the lower condensed acids which are insoluble in $POCl_3$.

GRUNZE also claimed[5] that polyphosphoric acids were produced when $POCl_3$:$H_2O < 2$. We checked this water hydrolysis and could identify only mono- and diphosphoric acids for $POCl_3$:$H_2O = 2$, just as only mono- and diphosphates were produced in the $POCl_3$–NaOH (25:1) mixture. As the relative amount of water is increased, one would expect the replacement of –Cl by –OH to take priority over the condensation occurring by elimination of HCl molecules.

It must be pointed out that any branched chain products would undergo degradation upon dissolution in water and also if chloro-derivatives were present, they would probably be converted to chloride-free condensed phosphates when the neutralisation was performed with strong base.

SUMMARY

The hydrolysis of phosphoryl chloride with aqueous base has been investigated. Separation and identification of the products by means of paper and anion-exchange chromatography has shown that salts are formed which have a similar composition to the acids formed by hydrolysis with water in neutral solution.

REFERENCES

1 C. A. WURTZ, *Ann. Chim. Phys.*, 20 (1847) 472.
2 A. BESSON, *Compt. Rend.*, 124 (1897) 1099.
3 H. MEERWEIN AND K. BODENDORF, *Ber. Deut. Chem. Ges.*, 62 (1929) 1952.
4 H. ROUX, E. THILO, H. GRUNZE AND M. VISCONTINI, *Helv. Chim. Acta*, 38 (1955) 25.
5 H. GRUNZE, *Z. Anorg. Allgem. Chem.*, 313 (1961–1962) 316.
6 R. F. HUDSON AND G. MOSS, *J. Chem. Soc.*, (1962) 3599.
7 J. R. VAN WAZER, L. C. D. GROENWEGHE AND J. H. PAYNE, *J. Am. Chem. Soc.*, 82 (1960) 5305.
8 G. BIBERACHER, *Z. Anorg. Allgem. Chem.*, 285 (1956) 86.
9 E. KARL-KROUPA, *Anal. Chem.*, 28 (1956) 1091.

THE SEPARATION OF SCANDIUM BY CATION EXCHANGE IN ACID AMMONIUM SULFATE MEDIA

ROKURO KURODA, YOSHIHITO NAKAGOMI AND KOJI ISHIDA
Laboratory for Analytical Chemistry, Faculty of Engineering, University of Chiba, Yayoi-cho, Chiba (Japan)
(Received September 27th, 1965)

The considerable data regarding the ion exchange behavior of Sc(III) in different media already available and recent results have been reviewed in several papers[1-4], so that they will not be discussed here. A systematic survey of the cation exchange behavior of Sc(III) and many other metal ions in acid $(NH_4)_2SO_4$ media indicates that the distribution coefficient of Sc(III) differed sufficiently from that of many other metal ions to ensure good separation. Sc(III) exhibits weak adsorption, while many other ions adsorb strongly from the sulfate media on a strong acid cation exchanger, such as Dowex 50W-X8.

Cation exchange distribution coefficients with BIO-RAD AG50W-X8 have currently been presented for 45 cations in H_2SO_4 media by STRELOW, RETHEMEYER AND BOTHMA[5]. However, no information is available on the behavior of Sc(III) in sulfate media. STRELOW AND BOTHMA[3] have also effected the cation exchange separation of Sc(III) from Y(III) and the rare earths by elution with 2 *N* H_2SO_4 using a column of AG Dowex 50W-X8 resin. The Sc(III), which is eluted first, shows a marked tailing effect. Flow rates are also critical because of the slow exchange rate of Sc(III) in H_2SO_4 media. These difficulties have been overcome completely in this work. It will be demonstrated that the Sc(III) can quickly be separated as a narrow band from Y(III) and the rare earths, as well as many other cations, by elution with 0.3 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4 solution, which is a considerably lower sulfate concentration than that employed by STRELOW AND BOTHMA.

EXPERIMENTAL

Apparatus and reagents

Ion-exchange resin. A strong acid type cation exchanger, Dowex 50W-X8, hydrogen form, 100–200 mesh was used. The resin was pretreated in a large column by washing with 2 *M* NH_4SCN–0.5 *M* HCl solution, followed by 3 *M* HCl solution, and finally with deionized water. The resin was removed from the column and stored in a desiccator over a saturated KBr solution.

Ion-exchange column. Five grams of dried resin were slurried with water and poured into a conventional ion exchange column, 0.9 cm I.D., pulled to a tip, and plugged with glass wool at the outlet of the column. The resin bed was approximately 10 cm long. Unless otherwise mentioned, this size of column was employed throughout the work. For difficult separations a somewhat longer column, 1.0 cm I.D. and 14 cm

long (bed height), was used. The eluent was fed from a 200 ml separatory funnel whose stem was attached to the top of the column with rubber tubing.

Stock solutions. Stock solutions of metal ions were mostly prepared by dissolving their oxide, chloride or sulfate in HCl to give 10 to 20 mg of each metal ion per ml of 0.5 *M* HCl solution. Exceptions are the stock solutions of Mo(VI), Ti(IV) and Bi(III). Appropriate amounts of $(NH_4)_6Mo_7O_{24}\cdot 4\ H_2O$ were dissolved in 0.025 *M* H_2SO_4 to give about 25 mg Mo(VI) per ml. The solution of Ti(IV) was prepared by dissolving titanium metal in HF–HNO_3 mixture, fuming with H_2SO_4 to expel HF and finally dissolving the residue in 1 *M* H_2SO_4 to give 0.6 mg Ti(IV) per ml. A solution approximately 20 mg of Bi(III) per ml of 1 *M* HNO_3 was prepared. The strength of each stock solution was determined by conventional analytical methods (see Table I).

TABLE I

ANALYTICAL METHODS USED

Ions	*Method*
Sc(III), Y(III), La(III), Sm(III), Lu(III), Bi(III)	Titration with EDTA using xylenol orange as indicator
Mg(II), Mn(II), Zn(II), Cd(II)	Titration with EDTA using eriochrome black T as indicator
Fe(III)	Titration with EDTA using variamine blue B as indicator
Al(III), V(IV), Co(II), Ni(II), Ga(III), In(III)	Titration with EDTA using a mixture of 1-(2-pyridylazo)-2-naphthol and Cu EDTA as indicator
Ca(II)	Titration with EDTA using 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthioic acid as indicator
Cu(II)	Titration with EDTA using 1-(2-pyridylazo)-2-naphthol as indicator
Cr(III)	Back-titration with Mn(II) ion in excess of EDTA using eriochrome black T as indicator
Hg(II)	Substitution-titration with EDTA in presence of MgEDTA using eriochrome black T as indicator
Ti(IV)	Colorimetrically with H_2O_2 as reagent
Zr(IV)	Gravimetrically with NH_4OH as precipitant
Mo(VI)	Gravimetrically with α-benzoinoxime as precipitant

Distribution coefficient measurement

The distribution coefficient of Sc(III) and the rare earths was determined by a column elution method and a batch method, respectively. About 1 mg of Sc(III) was loaded on to the top of the column and eluted with $(NH_4)_2SO_4$ solutions of various concentrations, keeping the concentration of free sulfuric acid constant at 0.025 *M*. From the elution profile curve thus obtained the distribution coefficient, K_d, was calculated as described previously[6]. The distribution coefficients for the rare earths were obtained in a similar way by the batch method[6].

Procedure

The sample mixture is loaded on to the column in dilute mineral acid solution so that a sharp adsorption band develops near the top of the column. Excess free acid in the sample solution should be avoided to ensure good separation. Heavy loading should also be avoided, the loading usually being kept less than about 10 % of the total exchange capacity of the column. If necessary, the column is washed down with small quantities of water. Sc(III) is first removed from the column by elution

with 0.3 M $(NH_4)_2SO_4$–0.025 M H_2SO_4, and is quantitatively recovered in the fraction of effluent ranging between 20 and 35 ml. The flow rate is not critical and is usually adjusted to 0.5 to 1.5 ml per min. The other metal ions listed in Table II remain adsorbed on the column. These ions can easily be stripped from the column with 3 to 6 M HCl. Useful eluents for In(III), Cr(III), and Fe(III) are given in the footnote to Table II.

TABLE II

SEPARATION OF Sc(III) FROM FOREIGN METALS IN $(NH_4)_2SO_4$ MEDIA

Sc (mg)		*Foreign ions (mg)*		
Added	*Found*		*Added*	*Found*
0.863	0.863	Al(III)	11.9	12.1
0.863	0.869	Ca(II)	37.6	38.4
0.863	0.865	Cd(II)	17.2	17.1
0.863	0.863	Co(II)	11.8	11.7
0.863	0.881	Cr(III)**	10.2	10.2
0.863	0.859	Cu(II)	25.9	26.1
0.863	0.869	Fe(III)***	7.1	7.2
0.863	0.885	Ga(III)	18.6	18.9
0.863	0.885	In(III)*,***	11.4	11.7
0.863	0.858	La(III)	98.4	101.4
		La(III)	52.8	Total of
0.863	0.857	Sm(III)	30.0	100.2 %
		Y(III)	22.6	recovered
0.863	0.865	Lu(III)	1.27	1.29
0.863	0.865	Mg(II)*	7.15	7.29
0.863	0.859	Mn(II)	32.4	32.3
0.863	0.861	Ni(II)*	30.5	30.9
0.863	0.864	Sm(III)	97.7	97.6
0.863	0.861	Sm(III)	135.2	138.3
0.863	0.863	Y(III)	45.2	45.3
0.883	0.863	Zn(II)	14.8	14.9

* Longer column used.
** Eluted by 10 ml of 10 % H_2SO_4 after removal of Sc(III).
*** Eluted by 40 ml of 1 M $(NH_4)_2SO_4$–0.025 M H_2SO_4 after removal of Sc(III).

When Sc(III) is accompanied by Fe(III), Sc(III) is preferentially eluted with about 110 ml of 0.15 M $(NH_4)_2$ SO_4–0.025 M H_2SO_4 solution. Sc(III) is recovered first in the effluent fraction, 70 to 110 ml, and can thus be separated from Fe(III). To ensure the effective separation of Sc(III) from Hg(II), V(IV), Mo(VI), Ti(IV) and Bi(III), the sample solution is treated with a suitable complexing agent for the interfering ions so that they show only slight or no adsorption on the resin. Sc(III), which remains on the column, is then stripped with 0.3 M $(NH_4)_2$ SO_4–0.025 M H_2SO_4 as before. The necessary information is outlined in Table III. The methods used for analysis of the effluents are listed in Table I.

RESULTS AND DISCUSSION

Fig. 1 gives the values for the distribution coefficients of Sc(III) as a function of $(NH_4)_2SO_4$ concentration. For comparison the values obtained by STRELOW *et al.*[5] for Sc(III) in H_2SO_4 media are also shown. It can be seen that the values for

TABLE III

QUANTITATIVE SEPARATION OF Sc(III) FROM FOREIGN METAL IONS IN DIFFERENT MEDIA

Sc (mg)		*Foreign ions (mg)*			*Eluent used for metal ions*
Added	*Found*		*Added*	*Found*	
0.863	0.877	Bi(III)	37.6	37.7	Bi eluted in the effluent from 2 *M* HNO_3 sample solution
0.863	0.854	Hg(II)	180.0	193.4	Hg eluted in the effluent from 0.5 *M* HCl sample solution
0.863	0.861	Mo(VI)	25.8	25.4	Mo eluted in the effluent from 0.3 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4 solution
0.863	0.864	Ti(IV)	5.8	5.8	Ti eluted with 40 ml of 2% H_2O_2–1 *M* H_2SO_4
0.863	0.881	V(IV)	18.3	18.3	V eluted with 15 ml of 1% H_2O_2
0.863	0.871	Zr(IV)*	2.30	2.36	Zr eluted with 8 ml of 1 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4

* Longer column used.

the distribution coefficient of Sc(III) are significantly lower in sulfate media than in H_2SO_4 media. Therefore, the sulfate media would provide more efficient removal of Sc(III) from the column, ensuring good separation. Difference in slope of the distribution curves suggests that the dominant complex species formed should differ between sulfate and H_2SO_4 media.

In Fig. 2 single elution curves for Sc(III) are shown; these were obtained by elution with both $(NH_4)_2SO_4$ and H_2SO_4 solutions. Contrasted to the curves obtained with H_2SO_4, those with $(NH_4)_2SO_4$ show a fairly sharp elution band. In order to elute Sc(III) within a 20 ml fraction of effluent, the concentration of H_2SO_4 would have to be raised to more than 3 *N*, which would cause serious difficulties in the subsequent effluent analysis of Sc(III). Flow rate is not critical in the $(NH_4)_2SO_4$ elution, the shape of the elution band being unaffected by increasing the rate up to at least 1.5 ml per min.

The values for the distribution coefficients of "average" rare earths are also shown in Fig. 1. The values for the rare earths ranging from La(III), through Sm(III), to Lu(III) do not differ markedly from each other, so that they may be approximated as single values for the "average" rare earths. The distribution coefficient of average rare earths diminishes more rapidly with increasing concentration of $(NH_4)_2SO_4$ than that of Sc(III). The separation factor, $K_{d_{av.R.E.}}/K_{d_{Sc}}$, works out to be 2.2 and 15 at 1 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4 and 0.3 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4 respectively. The separation factor appears to favor the separation of Sc(III) and the rare earths over the $(NH_4)_2SO_4$ concentration range tested with a shorter column. The results for a separation of Sc(III), with 0.3 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4 elution, from Y(III) and the rare earths are quoted in Table II. Small quantities of Sc(III) can quantitatively be recovered in as little as a 15 ml fraction of effluent, while 100 times more Y(III) and rare earths as Sc(III) remain adsorbed on the column. They can be removed from the column by elution with 6 *M* HCl. Many other cations including Al(III), Ca(II), Cd(II), Co(II), Cr(III), Cu(II), Ga(III), In(III), Mg(II), Mn(II), Ni(II) and Zn(II) are easily separated in this way. The results of these separations are also listed in Table II. Th(IV) and Be(II) behave like Sc(III) so that their separation is difficult in this cation exchange–sulfate system.

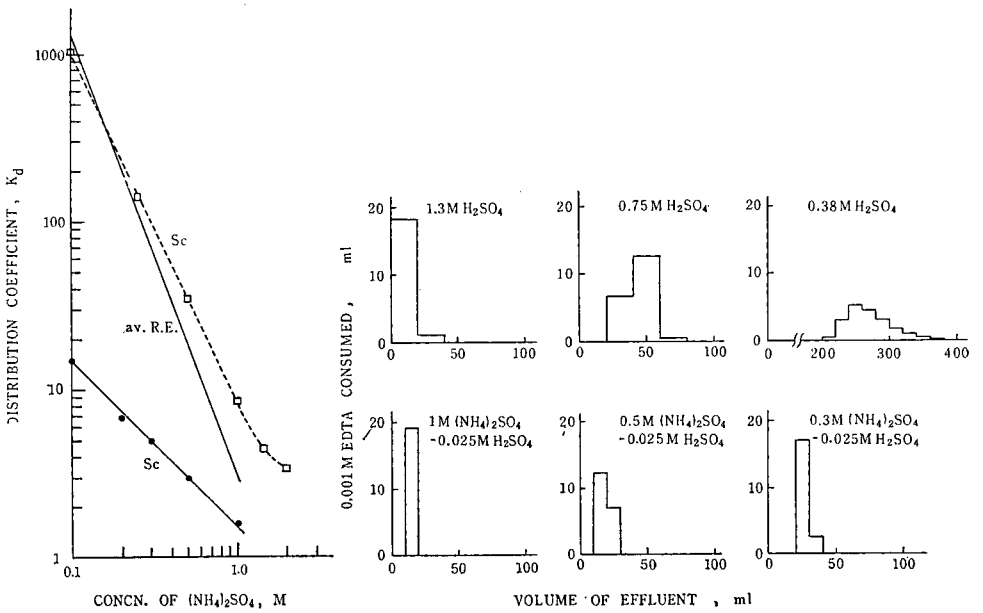


Fig. 1. Distribution coefficients of Sc(III) and "average" rare earths as a function of $(NH_4)_2SO_4$ concentration. Free acid concentration is kept constant at 0.025 *M* H_2SO_4. Resin, Dowex 50W-X8 (●——●) = Sc(III); (———) = "Average" rare earths; (□- - -□) = Values for Sc(III) in H_2SO_4 media as given by STRELOW, RETHEMEYER AND BOTHMA[5]. Numerical values of abscissa stand for H_2SO_4 concentration.

Fig. 2. Single elution curves for Sc(III). Upper: Eluted with H_2SO_4 of various concentrations. Lower: Eluted with $(NH_4)_2SO_4$ of various concentrations. Free acid concentration is kept constant at 0.025 *M* H_2SO_4.

Anion exchange chromatography in $(NH_4)_2SO_4$ media[4] permits the separation of Sc(III) from them, although it takes a considerable time to achieve the chromatographic run.

Mo(VI) does not adsorb from 0.3 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4 solution to any great extent on the cation exchange resin. By feeding the sample, adjusted to 0.3 *M* sulfate in 0.025 *M* H_2SO_4, on to the column and subsequent washing with the same solution, Mo(VI) can be eluted from the sample solution prior to the breakthrough of Sc(III). Zr(IV) exhibits a slight adsorption from the acid sulfate media. It can be separated from Sc(III) by elution with 1 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4, thus being eluted as a very sharp band, immediately followed by Sc(III). V(IV) and Ti(IV) are eluted rapidly prior to Sc(III) with 1 % H_2O_2 and 2 % H_2O_2–1 *M* H_2SO_4, respectively. Sc(III) is then removed by passing 20 ml of 0.3 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4 down the column. The separation of Hg(II) and Bi(III) from Sc(III) is simply conducted by feeding the sample solution, adjusted to 0.5 *M* in HCl for Hg(II) and to 2 *M* in HNO_3 for Bi(III), respectively, on to the column and then washing with the same solution. Both elements are eluted in the effluent from the sample solution, while Sc(III) remains on the top of the column. The results of the separations are given in Table III. The present ion exchange method for Sc(III) offers advantages in speed, selectivity and ease of effluent analysis for Sc(III).

SUMMARY

A study of the adsorption of metal ions, in acid sulfate media, on a strong-acid type cation exchange resin, Dowex 50W-X8, indicates that the difference between the distribution coefficients of Sc(III) and many other ions is sufficiently large for good separation. This fact makes it possible to develop a cation exchange chromatographic procedure for the separation of Sc(III) from other ions. Sc(III) can be eluted with 0.3 *M* $(NH_4)_2SO_4$–0.025 *M* H_2SO_4, while Al(III), Ca(II), Cd(II), Co(II), Cr(III), Cu(II), Ga(III), In(III), Mg(II), Mn(II), Ni(II), Y(III) and the rare earths, and Zn(II) remain adsorbed on the column. Ions forming stable sulfatocomplexes or other types of complex are eluted earlier in the effluent from the sample solution. Suitable elution systems for these ions are given. Separation of Sc(III) from 23 metal ions is reported.

REFERENCES

1 H. HAMAGUCHI, R. KURODA, K. AOKI, R. SUGISITA AND N. ONUMA, *Talanta*, 10 (1963) 153.
2 H. HAMAGUCHI, N. ONUMA, M. KISHI AND R. KURODA, *Talanta*, 11 (1964) 495.
3 F. W. E. STRELOW AND C. J. C. BOTHMA, *Anal. Chem.*, 36 (1964) 1217.
4 H. HAMAGUCHI, A. OHUCHI, T. SHIMIZU, N. ONUMA AND R. KURODA, *Anal. Chem.*, 36 (1964) 2034.
5 F. W. E. STRELOW, R. RETHEMEYER AND C. J. C. BOTHMA, *Anal. Chem.*, 37 (1965) 106.
6 H. HAMAGUCHI, K. KAWABUCHI AND R. KURODA, *Anal. Chem.*, 36 (1964) 1654.

SUR LE DOSAGE SIMULTANÉ DU CADMIUM, DU COBALT, DU ZINC, DU NICKEL ET DU CUIVRE PAR CHROMATOGRAPHIE DE DÉPLACEMENT SUR RÉSINE ÉCHANGEUSE D'IONS: ÉTUDE DES PERTURBATIONS CAUSÉES PAR CERTAINS IONS

J. P. QUINCHE ET S. SAX
Station Fédérale d'Essais Agricoles, Lausanne (Suisse)
(Reçu le 20 septembre 1965)

INTRODUCTION

Dans une étude antérieure[1], nous avons décrit une méthode permettant le dosage simultané des cations Co^{2+}, Zn^{2+}, Ni^{2+} et Cu^{2+} à l'aide d'une technique de chromatographie de déplacement sur échangeur d'ions. Depuis lors, nous avons apporté divers perfectionnements à ce procédé. Il est ainsi possible de doser également le cadmium en même temps que les 4 cations cités plus haut. Dans cet article nous décrivons des exemples d'analyses chromatographiques de mélanges comprenant les 5 cations (= M^{2+}) étudiés, ainsi que quelques exemples d'analyses de solutions contenant un ou deux cations incolores (p.ex. mélanges $Cd^{2+} + Zn^{2+}$) montrant l'utilisation d'éléments séparateurs (p.ex. Co^{2+} et Ni^{2+}). D'autre part, dans le but de préciser les conditions d'application de cette technique chromatographique, nous avons étudié expérimentalement l'influence de divers ions sur la marche de l'analyse et sur la précision des dosages. De plus, un exemple d'application de la méthode est décrit en détail.

PRINCIPE DE LA MÉTHODE

Le mélange des ions M^{2+} à analyser est fixé sur un échangeur cationique fortement acide, sous forme H^+, puis élué au moyen d'une solution tampon à base de glycocolle et d'ammoniaque. Dès le début de la chromatographie, des ions complexes $[MG_3]^-$ se forment au sommet de la colonne:

(G^- = anion glycocollate $H_2N{-}CH_2{-}COO^-$)

$$\overline{M^{2+}} + 3\ G^- + 2\ NH_4^+ \longrightarrow [MG_3]^- + 2\ \overline{NH_4^+}$$

(symboles surlignés = ions fixés sur l'échangeur d'ions).

On a en plus, pour 2 cations M_a^{2+} et M_b^{2+}, un équilibre:

$$[M_b\ G_3]^- + \overline{M_a^{2+}} \rightleftarrows [M_a\ G_3]^- + \overline{M_b^{2+}}$$

lequel est en faveur de $[M_aG_3]^-$ si $K_a > K_b$, où $K = \dfrac{([MG_3]^-)}{(M^{2+}) \cdot (G^-)^3}$.

Les ions $[M_aG_3]^-$ formés se décomposent lorsqu'ils arrivent au contact de la résine H^+ constituant la "barrière":

$$[M_a\,G_3]^- + \overline{2\,H^+} \longrightarrow \overline{M_a^{2+}} + 2\,GH + G^-$$

Il en résulte que les éléments M se séparent et s'ordonnent du haut en bas de la colonne en zones adjacentes et d'après la stabilité croissante de leurs complexes[2]. Les zones de Co^{2+}, Ni^{2+} et Cu^{2+} sont colorées respectivement en rouge carmin, vert et bleu, ces colorations étant celles des ions complexes $[MG_3]^-$ superposées à celles des cations M^{2+} fixés sur la résine. Par contre, les ions Zn^{2+} et Cd^{2+} étant incolores, les zones correspondantes sont aussi incolores. Cependant la mesure de la longueur de la zone du zinc ne présente pas de difficultés car cette dernière est intercallée en général entre deux zones colorées (Co et Ni ou Co et Cu, voir réf. 1). Par contre, la zone du cadmium se situe au-dessus de celle du cobalt, du fait que sa constante d'équilibre est la plus faible de celles de la série des cations M^{2+}. Pour déceler la frontière supérieure de la bande de Cd^{2+}, il existe deux possibilités:

(A) Si l'on utilise une résine Dowex 50 W X2, 200–400 mesh, on peut introduire dans la colonne une faible quantité de Fe^{2+} qui, une fois le chromatogramme développé, donnera une zone vert clair située au-dessus de celle de Cd^{2+}. A l'aide d'une lampe U.V. (filtre 366 mμ), on observe dans l'obscurité que la zone de Cd^{2+} est bleu clair et bien délimitée à sa partie supérieure par la zone de Fe^{2+}, laquelle apparaît colorée en brun foncé (Fig. 1).

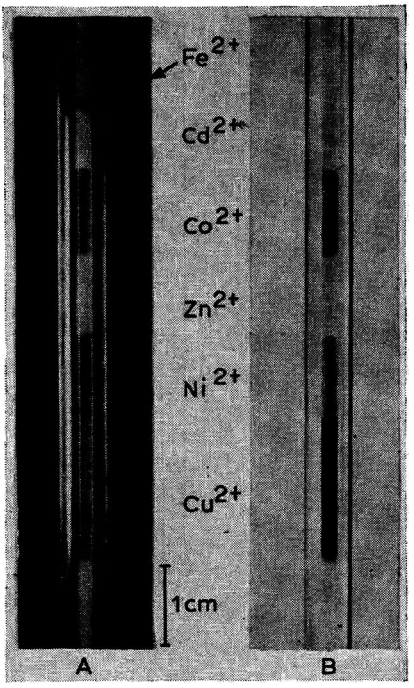


Fig. 1. Séparation Cd^{2+}–Co^{2+}–Zn^{2+}–Ni^{2+}–Cu^{2+} (5 × 0.100 méquiv.-g) + traces Fe^{2+}. (A) Aspect en lumière U.V. (366 mμ). (B) Aspect en lumière du jour.

(B) Si l'on utilise une résine Dowex 50 W X2, 100–200 mesh, la présence de Fe^{2+} dans la colonne est inutile. En effet, la limite supérieure de la zone de Cd^{2+} est nettement visible si l'on opère à la lumière U.V. filtrée et dans l'obscurité complète.

Notons qu'il n'est pas possible de distinguer, même avec la lampe U.V., la frontière entre une zone de Cd^{2+} et une zone de Zn^{2+} lorsque ces dernières sont adjacentes: l'emploi d'un "élément séparateur" — le cobalt — est indispensable. Il faut également introduire un peu de Ni^{2+} ou de Cu^{2+} pour délimiter la zone de Zn^{2+} à sa partie inférieure.

APPAREILLAGE

Nous avons employé l'appareillage de la Fig. 2, excepté lors de l'étude préliminaire du comportement chromatographique du cadmium (Tableau I) pendant laquelle nous avons utilisé l'appareil simple décrit antérieurement[1]; il se compose d'une colonne de chromatographie A (tube capillaire en verre Pyrex, d'environ 2 mm de diamètre interne, de 500 mm de longueur et de section connue exactement) à laquelle est connectée par l'intermédiaire d'un rodage (NS 14.5/23 DIN 12242) une pièce intermédiaire E ou F munie à sa partie inférieure d'une fritte de porosité élevée (P 1). La pièce E, dont le volume utile est de 40 ml de résine, est utilisée pour la chromatographie des cations M^{2+} lorsque les teneurs en ions étrangers Me^{n+} des solutions à analyser sont élevées. Pour une résine Dowex 50 W X 2 (100–200 ou 200–400 mesh) dont la capacité est de 0.75 méquiv.-g/ml de résine humide, sous forme H^+, la capacité totale d'une pièce du type E est de 30 méquiv.-g de cations.

Nous n'introduisons pas plus de 15 méquiv.-g de cations dans cette dernière,

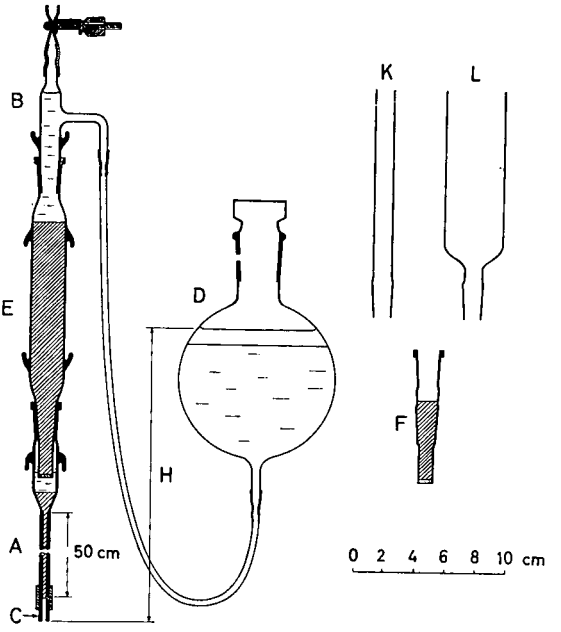


Fig. 2. Appareillage pour la chromatographie de déplacement sur résine échangeuse d'ions.

afin d'avoir une certaine marge de sécurité. Pour les chromatographies d'étalonnement (0.5 à 0.75 méquiv.-g M^{2+}), ainsi que pour des chromatographies en présence de petites quantités de cations étrangers (jusqu'à 1.5 méquiv.-g Me^{n+}), on se sert d'une pièce intermédiaire F dont le contenu en résine est de 6 à 7 ml au maximum (capacité: 4.5 à 5.3 méquiv.-g, au maximum).

Les rallonges K et L se fixent sur les pièces F et E respectivement et facilitent la charge des solutions à analyser sur la résine ainsi que les lavages. La résine est maintenue en place dans la colonne A grâce à une rondelle de tissu de nylon (diamètre 7 mm) intercalée entre la colonne de chromatographie et un petit tube C fixé à cette dernière par un tuyau de plastic.

Les pièces E ou F sont reliées au réservoir d'éluant D par une pièce B à rodage (NS 14.5/23) et par l'intermédiaire d'un tuyau de caoutchouc "para". La partie supérieure de la pièce B est munie d'un petit tuyau de PCV qui peut être ouvert ou fermé à l'aide d'une pince de serrage. D'autre part, une couche de kérosène d'environ un cm d'épaisseur placée sur l'éluant permet d'éviter une variation du pH de ce dernier par perte d'ammoniac. On utilise aussi une lampe U.V. d'analyse (p. ex. lampe Hanau, filtre 366 mμ).

PARTIE EXPÉRIMENTALE

Eluants et résines échangeuses d'ions

On utilise comme éluant une solution aqueuse de glycocolle à 40.0 g/litre (soit 0.533 *M*) dont le pH est amené à 8.80 (mesuré à 24° à l'aide d'un pH-mètre à électrode de verre) avec NH_4OH, NaOH, ou parfois LiOH.

La résine cationique Dowex 50 W X 2, sous forme H^+, donne satisfaction; pour les premiers essais, nous avons utilisé une résine de 200 à 400 mesh, puis nous avons constaté qu'une résine de 100 à 200 mesh permettait de travailler plus rapidement sans pour autant que la précision des dosages soit diminuée. Avant l'emploi, la résine doit être traitée au citrate biammonique 0.5 *M* afin d'éliminer les traces de métaux lourds, lavée à l'eau déminéralisée, mise sous forme H^+ en la traitant avec une solution d'HCl puriss. (1 vol. HCl 25 % + 3 vol. d'eau), puis finalement lavée à fond avec de l'eau. On utilise pour ces opérations une colonne de chromatographie (500 × 40 mm) permettant de traiter 500 g de résine à la fois. Pour le remplissage des colonnes capillaires, on agite la résine dans un flacon de verre à large col avec de l'eau afin d'obtenir un mélange relativement fluide, puis on laisse décanter pendant quelques minutes; on munit la colonne de son petit filtre de nylon et du petit tube C de verre; puis on plonge la tête de la colonne dans la résine et on aspire à l'aide d'une trompe à eau. On maintient l'aspiration jusqu'à ce que tous les grains de résine soient immobiles dans le capillaire. Finalement, on laisse une couche de résine d'environ 1 cm de hauteur dans la tête de la colonne (voir Fig. 2).

Solutions à analyser et solutions standards

On utilise comme solutions standards des solutions de chlorures ou de sulfates à une teneur de 1,000 méquiv.-g M^{2+}/10 ml. La concentration des solutions est contrôlée par titrages complexométriques au moyen d'une solution 0.05 *M* de Na_2H_2-édta. $2H_2O$ (édta = éthylène-diaminetétraacétate). Les indicateurs employés sont la murexide pour le titrage de Co^{2+}, Ni^{2+} et Cu^{2+}, en milieu ammoniacal, et le noir

ériochrome T pour le titrage de Cd^{2+} et Zn^{2+}, en milieu $NH_4OH + NH_4Cl$ (tampon de Schwarzenbach). Pour l'étude de l'influence de divers cations étrangers sur la précision des analyses chromatographiques, nous avons préparé une solution standard renfermant les 5 cations M^{2+} en qualités équivalentes (1,000 méquiv.-g de chaque cation M^{2+}/100 ml) à partir des 5 solutions à 1,000 méquiv.-g M^{2+}/10 ml.

Mise en marche des chromatographies

On place la pièce intermédiaire contenant la résine chargée du mélange des cations à étudier au-dessus de la colonne de chromatographie préparée à l'avance, après avoir graissé les parties rodées à l'aide d'une graisse de silicone de viscosité élevée. On s'arrange pour que la surface inférieure de la fritte des pièces E ou F soit située 3 à 4 mm au-dessous du niveau de l'eau contenue dans la tête de la colonne. Ce mode de faire permet d'éviter une entrée d'air dans la couche de résine, à travers la fritte, lorsqu'on diminue la pression hydrostatique de travail.

Développement des chromatogrammes et mesures des zones

(A) Lorsque la quantité des cations à chromatographier ne dépasse pas 2.5 méquiv.-g, on peut utiliser le montage de la Fig. 2, mais en utilisant une pièce frittée du type F.

On commence la chromatographie en plaçant le réservoir D contenant l'éluant à une hauteur telle que la différence de niveau H soit d'environ 100 à 120 cm. Lorsque les cations M^{2+} commencent à pénétrer dans le capillaire de la colonne A, on amène H à environ 60 cm et on maintient cette différence de niveau constante jusqu'à ce que tous les cations M^{2+} aient pénétrés dans le capillaire. Puis, on abaisse H jusqu'à 3 ou 4 cm. Lorsque les frontières des zones sont devenues nettes et pratiquement horizontales, on mesure leurs longueurs, en évitant les erreurs dues à la parallaxe. Dans ce but, on peut se servir d'une réglette de 10 cm de longueur, graduée en mm, que l'on applique derrière la colonne de chromatographie. On répète 2 à 3 fois les mesures après avoir fait progresser chaque fois les zones de 5 à 7 cm dans le capillaire. Les mesures se font dans l'obscurité, à la lumière U.V. filtrée (filtre 366 mμ).

(B) Si la quantité totale des cations à chromatographier est supérieure à 2.5 méquiv.-g, on doit utiliser une pièce à fritte du type E, d'un diamètre intérieur d'environ 20 mm dont la longueur est fonction de la quantité de cations mise en jeu. Après avoir chargé ces derniers sur la résine, on lave à l'eau puis on élue. On ne relie la pièce E à une colonne capillaire A que lorsque les zones des cations qui nous intéressent sont arrivées à environ 2 à 3 cm au-dessus de la fritte. On poursuit alors la chromatographie comme sous (A).

Essais de chromatographie du cadmium sur échangeur d'ions

Dans le but d'établir si la mesure des longueurs des zones de cadmium permet d'envisager un dosage de cet élément, nous avons procédé à deux séries de chromatographies. Dans la première, nous avons chromatographié des mélanges de quantités connues des cations Cd^{2+} et Co^{2+}, tandis que dans la deuxième série, nous avons chromatographié des mélanges étalons de Cd^{2+} et de Cu^{2+}. Les conditions de travail et les résultats sont consignés dans le Tableau I; les résultats sont, de plus, représentés graphiquement dans la Fig. 3, où les volumes des zones (en mm^3) sont portés en abscisse, tandis que les teneurs des solutions à analyser sont portées en ordonnée

TABLEAU I

CHROMATOGRAPHIES D'ÉTALONNEMENT (Cd^{2+} + Co^{2+} ET Cd^{2+} + Cu^{2+})

Résine: Dowex 50 W X2, 200–400 mesh, forme H^+, lot no. 4. Eluant: glycocolle (40.00 g/l) + NH_4OH (pH 8.80 à 24°). Elément séparateur: 0.05 méquiv.-g Fe^{2+} par chromatographie.

Cd^{2+}			Co^{2+}		
Méquiv.-g	*Zone* (mm^3)	*Moyenne* (mm^3)	*Méquiv.-g*	*Zone* (mm^3)	*Moyenne* (mm^3)
0.050	26.4	27.00	0.050	33.6	33.45
0.050	27.6		0.050	33.3	
0.100	52.9	52.75	0.100	67.1	67.05
0.100	52.6		0.100	67.0	
0.150	80.2	80.05	0.150	102.8	102.50
0.150	79.9		0.150	102.2	
0.200	103.0		0.200	130.4	
0.200	114.0	109.30	0.200	137.0	134.90
0.200	110.9		0.200	137.3	

Cd^{2+}			Cu^{2+}		
Méquiv.-g	*Zone* (mm^3)	*Moyenne* (mm^3)	*Méquiv.-g*	*Zone* (mm^3)	*Moyenne* (mm^3)
0.050	26.7	26.90	0.050	51.6	50.60
0.050	27.1		0.050	49.6	
0.100	54.5	54.20	0.100	101.2	100.20
0.100	53.9		0.100	99.2	
0.150	83.8	81.60	0.150	152.7	151.90
0.150	79.4		0.150	151.1	
0.200	105.3		0.200	204.9	
0.200	111.3	108.67	0.200	194.0	199.87
0.200	109.4		0.200	200.7	

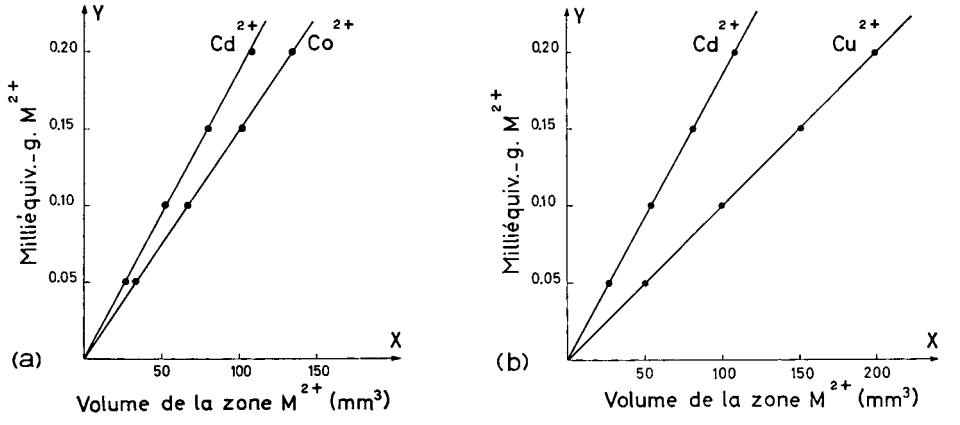


Fig. 3. Droites d'étalonnement (voir Tableau I).

(en méquiv.-g). On obtient 4 droites passant par l'origine, dont les pentes, déterminées graphiquement sont les suivantes:

(a) pour Cd^{2+}: $1.85 \cdot 10^{-3}$; pour Co^{2+}: $1.48 \cdot 10^{-3}$,

(b) pour Cd^{2+}: $1.85 \cdot 10^{-3}$; pour Cu^{2+}: $1.00 \cdot 10^{-3}$.

Il faut relever la bonne reproductibilité des résultats puisque les deux droites d'étalonnement de Cd^{2+} ont la même pente. L'expérience montre donc que, pour un lot de résine et un éluant déterminés, les volumes des zones de Cd^{2+} sont directement proportionnels aux teneurs de la solution analysée en cet élément.

Notons également que, à équivalent égaux, les zones de Cd^{2+} sont près de deux fois moins longues que celles de Cu^{2+}.

Exemples d'analyses chromatographiques de mélanges synthétiques des cinq cations M^{2+}

Le calcul des résultats se fait comme d'habitude à l'aide de la relation:

$$\text{méquiv.-g } M_x^{2+} = \frac{V_x \cdot M_e}{V_e}$$

où V_x = volume de la zone de M_x^{2+}, V_e = volume de la zone correspondante de la chromatographie-étalon, M_e = quantité de cation M_x^{2+} dans la chromatographie-étalon.

TABLEAU II

ANALYSES CHROMATOGRAPHIQUES DE MÉLANGES SYNTHÉTIQUES DES 5 CATIONS M^{2+}

Résine: Dowex 50 W X2, 200–400 mesh; forme H^+, lot no. 4. Eluant: glycocolle (40.00 g/l) + NH_4OH (pH 8.80 à 24°). Elément séparateur: 0.05 méquiv.-g Fe^{2+} par chromatographie. Chaque chromatographie a été faite en double: seuls les volumes moyens des zones correspondantes sont indiqués.

Chromatographie étalon E 1–2

M^{2+}	*Donné (méquiv.-g)*	*Zone (mm^3)*
Cd^{2+}	0.1500	81.3
Co^{2+}	0.1500	105.2
Zn^{2+}	0.1500	101.3
Ni^{2+}	0.1500	99.8
Cu^{2+}	0.1500	168.1

Analyses A 1 et A 2

	M^{2+}	*Donné (méquiv.-g)*	*Zone (mm^3)*	*Trouvé (méquiv.-g)*	*Erreur relative (%)*
A_1	Cd^{2+}	0.1000	55.4	0.1022	+2.2
	Co^{2+}	0.1000	70.2	0.1001	+0.1
	Zn^{2+}	0.1000	66.6	0.0986	−1.4
	Ni^{2+}	0.1000	67.3	0.1012	+1.2
	Cu^{2+}	0.1000	112.6	0.1005	+0.5
A_2	Cd^{2+}	0.2000	111.7	0.2061	+3.1
	Co^{2+}	0.2000	138.8	0.1979	−1.1
	Zn^{2+}	0.2000	135.5	0.2006	+0.3
	Ni^{2+}	0.2000	133.1	0.2000	0.0
	Cu^{2+}	0.2000	223.1	0.1991	−0.5

TABLEAU III

ANALYSES CHROMATOGRAPHIQUES DE MÉLANGES SYNTHÉTIQUES DES 5 CATIONS M^{2+}

Résine: Dowex 50 W X 2, 100–200 mesh, forme H^+, lot no. 1. Eluant: glycocolle (40.00 g/l) + NH_4OH (pH 8.80 à 24°). Elément séparateur: aucun. Chaque chromatographie a été faite en double: seuls les volumes moyens des zones correspondantes sont indiqués.

Chromatographie étalon E 3–4

M^{2+}	*Résultats calculés après 6 h (une seule mesure)*		*Résultats calculés après 47 à 55 h (5 à 6 mesures)*
	Méquiv.-g	*Zone (mm^3)*	*Zone (mm^3)*
Cd^{2+}	0.1000	79.5	76.3
Co^{2+}	0.1000	90.2	90.2
Zn^{2+}	0.1000	85.7	87.6
Ni^{2+}	0.1000	86.6	86.5
Cu^{2+}	0.1000	190.8	181.6

Analyses A 3 et A 4

	M^{2+}	*Résultats calculés après 6 h (une seule mesure)*				*Résultats calculés après 47 à 55 h (5 à 6 mesures)*		
		Donné (méquiv.-g)	*Zone (mm^3)*	*Trouvé (méquiv.-g)*	*Erreur relative (%)*	*Zone (mm^3)*	*Trouvé (méquiv.-g)*	*Erreur relative (%)*
A_3	Cd^{2+}	0.0500	38.8	0.0488	−2.4	38.9	0.0510	+2.0
	Co^{2+}	0.1500	133.9	0.1484	−1.1	135.0	0.1497	−0.2
	Zn^{2+}	0.0500	43.9	0.0512	+2.4	43.7	0.0499	−0.2
	Ni^{2+}	0.1500	128.4	0.1481	−1.3	130.3	0.1503	+0.2
	Cu^{2+}	0.0500	95.2	0.0491	−1.8	92.9	0.0512	+2.4
A_4	Cd^{2+}	0.1500	115.4	0.1453	−3.1	114.4	0.1500	0.0
	Co^{2+}	0.0500	45.8	0.0508	+1.6	44.8	0.0497	−0.6
	Zn^{2+}	0.1500	129.4	0.1509	+0.6	131.7	0.1502	+0.1
	Ni^{2+}	0.0500	42.4	0.0483	−3.4	43.0	0.0497	−0.6
	Cu^{2+}	0.1500	279.7	0.1465	−2.3	273.6	0.1507	+0.5

Si nous admettons une erreur absolue de $\pm$ 0.4 mm sur la mesure de la longueur d'une zone, une erreur relative de $\pm$ 0.5% sur la détermination des sections internes des colonnes, et une erreur relative de $\pm$ 0.5% sur M_e, il est simple de calculer l'erreur relative à craindre sur M_x^{2+}.

Dans le Tableau II sont consignés deux exemples d'analyses de mélanges synthétiques des cinq cations M^{2+}. Les erreurs relatives observées sont toutes comprises dans les limites admissibles, qui s'étendent de $\pm$ 2.8% dans le cas de Cu^{2+} (0.2000 méquiv.-g; zone de 223.1 mm^3) à $\pm$ 5.3% dans le cas de Cd^{2+} (0.1000 méquiv.-g; zone de 55.4 mm^3). Cependant, la durée des chromatographies est trop longue (6 à 7 jours pour 7 à 8 mesures). Cela provient du fait que le lot de résine utilisé était constitué d'une proportion particulièrement élevée de particules très fines. L'emploi d'une résine plus grossière nous a permis d'obtenir des résultats acceptables déjà 6 heures après la mise en route des analyses (Tableau III). Les résultats obtenus sont tous situés dans les limites d'erreurs admissibles. Toutefois, la prolongation de la durée des analyses et des mesures répétées des longueurs des zones conduisent à une amélioration sensible de la précision des résultats.

Exemples d'analyses chromatographiques de mélanges synthétiques de cations M^{2+} nécessitant l'emploi d'éléments séparateurs

L'introduction d'une petite quantité d'éléments séparateurs est indispensable dans la chromatographie de certains mélanges de cations tels que $Cd^{2+} + Zn^{2+}$ ou $Zn^{2+} + Cu^{2+}$. Dans le premier cas, on utilise un peu de Co^{2+}, qui se place entre les zones de cadmium et de zinc, et de Ni^{2+} pour délimiter la zone de zinc. Dans le second cas, l'incorporation d'une faible quantité de Co^{2+} à la solution à analyser est nécessaire pour pouvoir repérer la frontière supérieure de la bande de zinc. Les résultats obtenus sont exposés dans les Tableaux IV et V.

TABLEAU IV

ANALYSES CHROMATOGRAPHIQUES DE MÉLANGES DES CATIONS Cd^{2+} ET Zn^{2+}

Résine: Dowex 50 W X2, 100–200 mesh, forme H^+, lot no. 1. Eluant: glycocolle (40.00 g/l) + NH_4OH (pH 8.80 à 24°). Eléments séparateurs: 0.05 méquiv.-g Co^{2+} + 0.05 méquiv.-g Ni^{2+} par chromatographie.

Chromatographie-étalon E 5–9
(Chiffres moyens de 2 chromatographies faites en parallèle)

M^{2+}	*Résultats calculés après 6 h (une seule mesure)*		*Résultats calculés après 25 h (4 mesures)*
	Méquiv.-g	*Zone (mm^3)*	*Zone (mm^3)*
Cd^{2+}	0.3000	224.7	221.6
Zn^{2+}	0.2500	209.7	210.9

Analyses A 5 à A 9

	M^{2+}	*Résultats calculés après 6 h (une seule mesure)*				*Résultats calculés après 25 h (4 mesures)*		
		Donné (méquiv.-g)	*Zone (mm^3)*	*Trouvé (méquiv.-g)*	*Erreur relative (%)*	*Zone (mm^3)*	*Trouvé (méquiv.-g)*	*Erreur relative (%)*
A_5	Cd^{2+}	0.0250	18.5	0.0247	—1.2	18.5	0.0250	0.0
	Zn^{2+}	0.5000	393.3	0.4688*	—6.2	419.6	0.4978	—0.4
A_6	Cd^{2+}	0.5000	364.2	0.4862*	—2.8	362.5	0.4907	—1.9
	Zn^{2+}	0.0500	42.1	0.0502	+0.4	41.7	0.0495	—1.0
A_7	Cd^{2+}	0.0500	36.5	0.0487	—2.6	35.8	0.0485	—3.0
	Zn^{2+}	0.5000	412.8	0.4920	—1.6	419.9	0.4981	—0.4
A_8	Cd^{2+}	0.5000	362.9	0.4845*	—3.1	360.9	0.4886	—2.3
	Zn^{2+}	0.0250	20.2	0.0241	—3.6	20.5	0.0243	—2.8
A_9	Cd^{2+}	0.5000	370.2	0.4943	—1.1	367.2	0.4969	—0.6
	Zn^{2+}	0.0100	7.0	0.0083	—17.0	8.0	0.0095	—5.0

* Résultats situés hors des limites d'erreurs admissibles.

On constate que dans la série d'analyses de mélanges $Cd^{2+} + Zn^{2+}$ (Tableau IV) trois résultats sont situés hors des limites admissibles d'erreurs (qui sont de ± 2.4% dans les trois cas) si les mesures sont exécutées 6 heures après le début des chromatographies. Par contre, si l'on procède à 4 mesures des longueurs des zones (durée totale des analyses: 25 heures), tous les résultats sont compris dans les limites d'erreurs admissibles. Dans la série d'analyses de mélanges $Zn^{2+} + Cu^{2+}$, on observe qu'un seul

TABLEAU V

ANALYSES CHROMATOGRAPHIQUES DE MÉLANGES DES CATIONS Zn^{2+} ET Cu^{2+}

Résine: Dowex 50 W X2, 100–200 mesh, forme H^+, lot no. 1. Eluant: glycocolle (40.00 g/l) + NH_4OH (pH 8.80 à 24°). Elément séparateur: 0.05 méquiv.-g Co^{2+} par chromatographie.

Chromatographie-étalon E 10–14
(Chiffres moyens de 2 chromatographies faites en parallèle)

M^{2+}	*Résultats calculés après 6 h (une seule mesure)*		*Résultats calculés après 48 h (5 à 7 mesures)*
	Méquiv.-g	*Zone (mm^3)*	*Zone (mm^3)*
Zn^{2+}	0.1000	84.9	85.6
Cu^{2+}	0.1000	172.7	170.0

Analyses A 10 à A 14

	M^{2+}	*Résultats calculés après 6 h (une seule mesure)*				*Résultats calculés après 48 h (5 à 7 mesures)*		
		Donné (méquiv.-g)	*Zone (mm^3)*	*Trouvé (méquiv.-g)*	*Erreur relative (%)*	*Zone (mm^3)*	*Trouvé (méquiv.-g)*	*Erreur relative (%)*
A_{10}	Zn^{2+}	0.0400	34.4	0.0405	+1.3	34.4	0.0401	+0.3
	Cu^{2+}	0.2000	327.8	0.1907*	−4.7	328.8	0.1934*	−3.3
A_{11}	Zn^{2+}	0.2000	168.6	0.1986	−0.7	171.7	0.2006	+0.3
	Cu^{2+}	0.0400	69.1	0.0400	0.0	69.8	0.0411	+2.7
A_{12}	Zn^{2+}	0.0100	9.1	0.0107	+7.0	8.8	0.0103	+3.0
	Cu^{2+}	0.2000	344.2	0.1993	−0.4	340.5	0.2003	+0.2
A_{13}	Zn^{2+}	0.2000	164.4	0.1937	−3.2	166.1	0.1940	−3.0
	Cu^{2+}	0.0100	17.8	0.0103	+3.0	18.5	0.0109	+9.0
A_{14}	Zn^{2+}	0.0200	16.9	0.0199	−0.5	17.3	0.0202	+1.0
	Cu^{2+}	0.2000	348.6	0.2018	+0.9	347.6	0.2045	+2.3

* Résultats situés hors des limites d'erreurs admissibles.

des résultats est situé hors des limites d'erreurs (qui sont de ± 2.6% pour le dosage de Cu^{2+} dans l'analyse A 10); des mesures répétées durant 48 heures ne permettent pas dans ce cas de ramener l'erreur à une valeur acceptable.

Recherche des ions perturbateurs

Pour la recherche des cations perturbateurs, nous avons additionné à la solution des 5 cations M^{2+} (5 × 0.100 méquiv.-g) des quantités déterminées de cations étrangers. Ces derniers ont été introduits sous forme de chlorures, de nitrates ou de sulfates (qualité pour analyses). Dans certains cas, ils ont été fixés sur la résine après le passage de la solution des 5 ions M^{2+} et après lavage à l'eau, afin d'éviter la formation de composés insolubles (p.ex. tels que $PbSO_4$, AgCl). Les chromatographies d'étalonnement ont été effectuées en parallèle, dans les mêmes conditions, mais sans l'élément étranger étudié.

Parmi les cations monovalents, on constate que la présence de Li^+ ou de Na^+ conduit à des résultats trop élevés pour le dosage de Cd^{2+}, ceci par suite d'une pénétration des ions Li^+ ou Na^+ dans la zone de Cd^{2+}. Cependant, l'utilisation d'un

éluant à base de glycocolle + LiOH ou de glycocolle + NaOH, nous a permis d'obtenir des résultats satisfaisants pour le dosage de Cd^{2+} en présence de Li^+ ou de Na^+ respectivement (voir Tableau VI). Notons encore que, en l'absence de Cd^{2+} dans le mélange à analyser, les cations Li^+ ou Na^+ ne perturbent pas les dosages des ions Co^{2+}, Zn^{2+}, Ni^{2+} et Cu^{2+}. D'autre part, nous n'avons pas observé de perturbations en présence des ions K^+ (au moins jusqu'à 10 méquiv.-g), Rb^+ (au moins jusqu'à 1 méquiv.-g) et NH_4^+ (au moins jusqu'à 1 méquiv.-g). Par contre, la présence des cations Ag^+ dans les solutions à analyser doit être exclue. En effet, à la suite de la réduction des ions Ag^+, on observe la formation de dépôts d'argent métallique sur les grains de résine et sur la paroi intérieure de la colonne, si bien que la limite supérieure de la zone de Cd^{2+} est masquée. De plus, la plus grande partie du cobalt quitte la colonne sous forme d'une solution rose: il y a oxydation de Co(II) en Co(III) par Ag^+ fixé sur la résine, et formation du complexe neutre et stable de formule CoG_3. Signalons que le spectre d'absorption de ce composé présente 2 maximums situés dans la partie visible du spectre (375 mμ et 520 mμ). Les dosages de Cd^{2+} et de Co^{2+} ne sont donc pas possibles en présence des ions Ag^+, tandis que les résultats obtenus pour Zn^{2+}, Ni^{2+} et Cu^{2+} sont souvent trop élevés.

Parmi les cations bivalents, nous avons constaté que Ca^{2+}, Mg^{2+} et Mn^{2+} ne gênent pas, même s'ils sont présents dans les solutions à analyser à des teneurs de 10 méquiv.-g. Précisons que Ca^{2+} est pratiquement bloqué en tête de la colonne, que Mg^{2+} s'étale dans une certaine mesure pendant l'élution et que Mn^{2+} se détache du haut de la colonne et forme une bande diffuse qui s'étend jusqu'à la limite supérieure de la zone de Cd^{2+}. Les cations Ba^{2+} et Sr^{2+} ne se déplacent pas durant la chromatographie et ne perturbent pas (au moins jusqu'à une teneur de 1 méquiv.-g). Par contre, le cation UO_2^{2+} n'est pas complètement retenu au sommet des colonnes durant les chromatographies, si bien que l'on observe parfois des valeurs trop élevées pour Cd^{2+} et Cu^{2+} (voir Tableau VI). De même, les ions VO^{2+} conduisent à des résultats trop élevés pour Ni^{2+} et Cu^{2+} par suite d'une certaine incorporation du vanadyle dans les zones correspondantes.

D'autre part, la présence des cations Sn^{2+} est à éviter dans les solutions à analyser, car on observe de nettes perturbations dans les dosages de Co^{2+}, de Zn^{2+}, de Ni^{2+} et surtout de Cu^{2+}, et ceci déjà à une teneur de 0.5 méquiv.-g Sn^{2+}. Les pertes en cuivre sont dues au fait que la fixation des ions Cu^{2+} pendant la charge n'est pas complète par suite du faible pH de la solution à analyser, une certaine acidité étant nécessaire pour éviter l'hydrolyse de l'étain. Si de très petites quantités de Fe^{2+} (jusqu'à 0.05 méquiv.-g) ne gênent pas, on observe par contre des valeurs trop faibles pour le dosage de Cd^{2+}, et ceci déjà pour des teneurs en Fe^{2+} de l'ordre de 0.1 méquiv.-g. En effet, en présence de Fe^{2+}, une partie du cadmium traîne un peu au sommet de la colonne; ce fait a été établi par l'utilisation de ^{115}Cd comme traceur. En présence de Pb^{2+}, on observe souvent des valeurs trop élevées pour le cuivre, ce qui est curieux car le plomb ne pénètre pratiquement pas dans les zones des 5 ions M^{2+} durant la chromatographie, mais forme une bande diffuse qui se détache du sommet de la colonne et s'étend jusqu'à la limite supérieure de la zone de cadmium.

De grandes quantités de Fe^{3+} ou d'Al^{3+} ne perturbent pas (jusqu'à au moins 10 méquiv.-g): Al^{3+} reste totalement bloqué en tête de colonne; Fe^{3+} est également retenu au sommet, mais durant l'élution une faible proportion de ces ions se réduit en Fe^{2+} qui forme alors une bande étroite (épaisseur de l'ordre de 1 mm) au-dessus

TABLEAU VI

EFFETS DE DIVERS CATIONS ÉTRANGERS SUR LA PRÉCISION DES DOSAGES DES CATIONS M^{2+}

Résine: Dowex 50 W X2, 100–200 mesh, sous forme H^+.

Eluant pH 8.80 à 24° (40.00 g glycocolle/l)	*Cation étranger (méquiv.-g)*	*Erreur relative (%) sur le dosage des cations M^{2+}*				
		Cd^{2+}	Co^{2+}	Zn^{2+}	Ni^{2+}	Cu^{2+}
Glycocolle + NH_4OH	0.1 Li^+	+24.3*	+ 1.6	— 0.3	— 1.6	— 1.0
	0.5 Li^+	+27.2*	+ 3.1	+ 1.7	+ 0.4	+ 0.6
	1.0 Li^+	+26.8*	+ 2.6	+ 1.2	+ 1.5	+ 3.2
Glycocolle + LiOH	0.1 Li^+	— 0.8	+ 0.5	— 1.7	— 1.5	— 0.2
	0.5 Li^+	+ 1.6	+ 2.2	+ 0.9	— 0.5	— 2.8
	1.0 Li^+	— 0.7	— 0.2	— 0.6	— 1.3	— 1.3
Glycocolle + NH_4OH	0.1 Li^+	—	+ 1.8	+ 0.6	— 0.9	— 2.1
	0.5 Li^+	—	+ 3.1	+ 2.2	+ 1.6	+ 0.9
	1.0 Li^+	—	— 0.6	— 0.7	— 1.5	— 3.5
Glycocolle + NH_4OH	0.1 Na^+	+ 8.6*	+ 1.6	— 0.6	— 0.8	— 4.2*
	0.5 Na^+	+12.6*	+ 0.6	— 2.2	+ 0.1	— 3.5
	1.0 Na^+	+12.5*	+ 3.5	+ 0.5	— 0.1	+ 0.4
	10.0 Na^+	+16.4*	— 1.4	— 2.4	— 2.5	— 2.0
Glycocolle + NaOH	0.1 Na^+	+ 5.0	+ 1.7	+ 2.0	+ 2.2	— 1.8
	0.5 Na^+	— 2.3	— 3.3	— 2.1	— 2.1	— 2.4
	1.0 Na^+	+ 2.7	— 1.6	+ 0.3	+ 0.2	+ 2.6
	10.0 Na^+	+ 0.5	— 8.0*	+ 0.6	+ 0.1	+ 2.1
	10.0 Na^+	— 1.2	— 1.9	— 1.6	— 1.6	— 3.3
Glycocolle + NH_4OH	0.1 Na^+	—	— 3.0	— 0.5	— 3.0	— 3.4
	0.5 Na^+	—	— 1.0	— 0.9	— 1.2	— 1.6
	1.0 Na^+	—	— 2.5	— 2.9	— 0.7	— 2.2
Glycocolle + NH_4OH	0.05 Ag^+	indosable	indosable	+ 6.5*	+ 2.6	+10.1*
	0.1 Ag^+	indosable	indosable	+23.0*	+15.5*	+ 8.4*
	0.2 Ag^+	indosable	indosable	+ 0.6	+ 5.2*	+33.4*
	0.3 Ag^+	indosable	indosable	+ 2.3	+ 2.0	+12.3*
Glycocolle + NH_4OH	0.1 UO_2^{2+}	+ 8.5*	+ 0.7	+ 2.1	+ 0.3	+ 6.5*
	1.0 UO_2^{2+}	+ 3.2	+ 1.5	+ 2.1	+ 1.4	+ 9.2*
Glycocolle + NH_4OH	0.1 VO^{2+}	— 1.7	+ 1.5	+ 2.3	+12.7*	+ 3.7*
	0.5 VO^{2+}	— 2.2	+ 0.1	+ 1.6	+12.1*	+ 5.3*
Glycocolle + NH_4OH	0.1 Sn^{2+}	+ 2.6	+ 3.5	+ 2.5	+ 2.8	— 4.6*
	0.5 Sn^{2+}	+ 5.4	+ 6.0*	+ 4.6	+ 5.1*	— 9.3*
	1.0 Sn^{2+}	+ 4.2	+ 5.0*	+ 2.6	+ 2.5	—51.3*
Glycocolle + NH_4OH	0.05 Fe^{2+}	— 1.6	+ 3.5	+ 1.6	+ 0.7	0.0
	0.1 Fe^{2+}	— 6.7*	+ 1.8	+ 0.6	0.0	+ 1.6
	1.0 Fe^{2+}	— 7.5*	+ 0.8	— 3.5	— 1.2	+ 3.0
Glycocolle + NH_4OH	0.1 Pb^{2+}	— 0.1	+ 0.7	+ 1.1	— 0.4	+ 8.3*
	0.2 Pb^{2+}	— 0.2	+ 1.0	+ 0.1	— 1.1	+ 1.6
	0.3 Pb^{2+}	— 2.7	+ 0.2	— 1.7	— 1.2	+ 8.3*
	0.5 Pb^{2+}	+ 3.4	+ 1.6	+ 1.8	+ 0.2	+18.1*
	1.0 Pb^{2+}	0.0	+ 0.6	+ 1.4	+ 0.9	+ 2.4
	1.0 Pb^{2+}	— 1.4	+ 2.0	+ 1.7	+ 1.5	+ 5.1*
	1.0 Pb^{2+}	— 1.0	+ 2.1	0.0	— 0.3	+ 0.8
Glycocolle + NH_4OH	0.1 Cr^{3+}	+ 0.6	+ 2.3	— 0.9	+ 2.8	— 0.5
	0.2 Cr^{3+}	— 4.6	— 7.3*	—12.1*	+ 2.8	+ 6.4*
	0.3 Cr^{3+}	— 3.0	—17.2*	— 3.0	— 6.3*	+ 6.9*
Glycocolle + NH_4OH	0.1 Sn^{4+}	indosable	— 0.4	— 0.7	— 0.8	— 2.0
	0.5 Sn^{4+}	indosable	+ 1.6	— 3.1	— 0.3	— 0.3
	1.0 Sn^{4+}	indosable	0.0	— 3.3	+ 0.2	+ 1.1

* Résultats situés hors des limites d'erreurs admissibles.

de la zone de Cd^{2+}, et qui est visible à la lumière U.V. (366 mμ). Les cations Bi^{3+}, Y^{3+}, La^{3+} et Lu^{3+} ne gênent pas, au moins pour des teneurs jusqu'à 1 méquiv.-g; ces 4 derniers ions ne se déplacent pas durant l'élution et restent fixés en tête de colonne. Pour l'étude de l'influence de Cr^{3+}, nous avons utilisé le sel $[CrCl_2 \cdot (H_2O)_4]Cl.2H_2O$. On constate que, si de petites quantités de Cr^{3+} ne dérangent pas, des teneurs supérieures à 0.1 méquiv.-g causent de sérieuses perturbations; en effet, on observe que des complexes du chrome fortement colorés en violet se forment et se répandent dans les zones des cations M^{2+} de telle sorte que la mesure des longueurs de ces dernières devient difficile, voire même impossible pour des teneurs en Cr^{3+} supérieures à 0.4 méquiv.-g. Dans le Tableau VI sont consignés les résultats de 3 chromatographies effectuées en présence de Cr^{3+}. On remarque que les valeurs obtenues pour le Co^{2+} sont en général trop faibles; à l'aide de ^{60}Co nous avons pu montrer qu'il y avait, dans ces conditions, une certaine rétention de Co^{2+} au sommet de la colonne. Il se produit un phénomène analogue avec le Zn^{2+} dont la longueur de la zone augmente régulièrement au cours de l'élution, sans arriver parfois à une valeur stable.

Th^{4+} qui, lui aussi, reste totalement bloqué en tête de la colonne pendant l'analyse ne perturbe pas. Par contre, en présence de Sn^{4+} dans les solutions à analyser, le dosage de Cd^{2+} n'est guère possible, car de faibles quantités d'hydroxyde d'étain se forment dans la colonne et masquent le front supérieur de la zone de cadmium. D'ailleurs, dans ces conditions, une partie importance du cadmium n'est pas retenue par la résine lors de la charge (ce fait a été établi à l'aide de ^{115}Cd).

Les anions F^-, PO_4^{3-}, $C_2O_4^{2-}$, CH_3COO^-, introduits dans les solutions sous forme de sels de potassium, n'ont pas causé de perturbations, au moins pour des teneurs inférieures ou égales à 1 méquiv.-g.

Pour conclure, relevons que les dosages les plus sujets à des perturbations sont ceux de Cd^{2+} et de Cu^{2+}, tandis que ceux de Co^{2+} et de Ni^{2+} le sont beaucoup moins. L'analyse de Zn^{2+} n'est perturbée que par 2 cations (Ag^+ et Cr^{3+}) sur les 24 qui ont été étudiés. Il est d'ailleurs possible d'éviter un certain nombre des perturbations signalées, ceci à l'aide de divers moyens: par utilisation d'un éluant composé de glycocolle + LiOH ou de glycocolle + NaOH dans le cas des cations étrangers Li^+ ou Na^+, respectivement; par élimination de Ag^+ des solutions (précipitation sous forme AgCl); par oxydation préalable de Sn^{2+} ou de Fe^{2+} en Sn^{4+} ou Fe^{3+}, respectivement.

Signalons aussi que diverses possibilités de séparations découlent de notre étude: en effet, les cations restant bloqués quantitativement en tête de la colonne durant la chromatographie (Ca^{2+}, Sr^{2+}, Ba^{2+}, Bi^{3+}, Al^{3+}, Y^{3+}, La^{3+}, Lu^{3+} et Th^{4+}) peuvent être séparés des cations M^{2+}. La séparation Mg^{2+} – M^{2+} est également réalisable en tenant compte du fait que la zone de Mg^{2+} s'allonge durant l'élution, mais qu'elle ne pénètre cependant pas dans les bandes des cations M^{2+}.

EXEMPLES D'APPLICATIONS

(1) *Dosage simultané du zinc et du cuivre dans un aliment composé pour porcs*

Afin d'éliminer l'effet d'une distribution éventuellement hétérogène des oligo-éléments dans l'aliment, nous avons préparé à partir de ce dernier une solution stock (= solution A) qui nous a servi pour toute la série d'analyses chromatographiques, ainsi que pour les analyses comparatives par polarographie et par spectrophotométrie.

Cette solution a été préparée de la manière suivante: nous avons calciné 10 prises de 20 g d'aliment (placées dans 10 capsules de platine) d'abord sur une rampe de chauffage électrique, puis finalement au four électrique à 450°. Les cendres ainsi obtenues ont été mises ensuite en solution dans HCl puriss en léger excès et à chaud. Après filtration, les 10 filtrats sont réunis dans un ballon jaugé de 1000 ml. On amène au trait avec de l'eau déminéralisée et on homogénéise (= solution A). A partir de cette solution, nous avons procédé aux 10 analyses chromatographiques dont les résultats figurent dans le Tableau VII. Signalons que l'aliment étudié renfermait environ 3 mg de cobalt par kg; il en résultait dans chaque chromatogramme une zone de Co^{2+} d'environ 0.2 mm d'épaisseur qui permettait de délimiter la zone du zinc.

TABLEAU VII

DOSAGE SIMULTANÉ DU ZINC ET DU CUIVRE DANS UN ALIMENT MÉLANGÉ POUR PORCS

Résine: Dowex 50 W X2, 100–200 mesh, forme H^+. Eluant 1: glycocolle 40.0 g/l + NH_4OH (pH 8.80 à 24°). Eluant 2: glycocolle 40.0 g/l + NaOH (pH 8.80 à 24°). Prises: 50 ml de solution A (soit 10 g d'aliment). Montage: selon Fig. 2 (fritte type E).

	Série d'analyses avec l'éluant 1		*Série d'analyses avec l'éluant 2*	
	Zn (p.p.m.)	*Cu (p.p.m.)*	*Zn (p.p.m.)*	*Cu (p.p.m.)*
	134.4	148.8	135.0	155.6
	129.4	145.6	139.6	149.0
	130.9	152.4	139.3	143.9
	141.2	151.2	138.6	147.1
	137.7	146.7	135.0	156.6
Moyenne	134.7	148.9	137.5	150.4
Erreur moyenne	3.8	2.3	2.0	4.5
Erreur type	4.8	2.9	2.3	5.5

Dosage colorimétrique du cuivre: 154.5 p.p.m.
Dosage polarographique du zinc: 133.7 p.p.m.

De plus, on pouvait observer à l'aide de la lampe U.V. une zone brune de Fe^{2+} d'environ 5 mm de hauteur, située au-dessus de l'étroite zone de cobalt. Cette bande de fer ne perturbe pas et peut même servir pour délimiter le front supérieur de la zone de zinc lorsqu'un aliment ne renferme pas de cobalt.

Aux fins de comparaison, nous avons dosé colorimétriquement le cuivre dans la solution A (méthode au diéthyldithiocarbamate[3], spectrophotomètre Beckman DB) et le zinc par polarographie[4] (après élimination des ions PO_4^{3-} par échangeur cationique; polarographe Metrohm E 261 R; milieu $NH_4OH + NH_4Cl$).

On constate que les résultats des analyses chromatographiques de la solution A concordent à peu de chose près avec les valeurs obtenues par des méthodes classiques.

(2) *Analyse de mélanges de lanthanides et de cations M^{2+}*

Les cations trivalents des lanthanides (= R^{3+}) restant bloqués au sommet des colonnes lors de l'élution avec une solution de glycocolle (40.00 g/litre + NH_4OH, pH:8.80 à 24°), on pourrait doser d'abord les ions M^{2+} par chromatographie de dé-

placement sur un échangeur d'ions sous forme H^+ (Dowex 50 W X2, 100–200 mesh) en utilisant l'éluant ci-dessus; puis, après interruption de cette première chromatographie, il suffirait de laver à l'eau la résine chargée de cations R^{3+} (ainsi que d'ions NH_4^+), de placer la fritte contenant celle-ci au-dessus d'une colonne capillaire remplie d'une résine sous forme Cu^{2+}/H^+ (Dowex 50 W X2, 200–400 mesh), puis d'éluer à l'aide d'une solution de $(NH_4)_3$H-édta tamponnée avec CH_3COONH_4 ou avec $HCOONH_4$[5–7]. Après dévelopement du chromatogramme, la mesure de la longueur des diverses zones de terres rares (à la lumière U.V. 366 mμ) permettrait le calcul des teneurs relatives des éléments R dans le mélange analysé. Une chromatographie-étalon exécutée en parallèle avec des quantités connues de lanthanides permettrait la détermination des teneurs absolues des divers ions R^{3+}.

RÉSUMÉ

Nous décrivons une technique de chromatographie de déplacement sur résine échangeuse de cations permettant le dosage simultané de Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} et Cu^{2+}. Nous avons étudié l'effet de 24 cations étrangers et de 4 anions sur la précision des analyses. Nous donnons un exemple d'application de la méthode (dosage simultané du zinc et du cuivre dans un aliment pour porcs).

SUMMARY

A technique of displacement chromatography on a cation exchange resin is described permitting the simultaneous quantitative analysis of Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Cu^{2+}. We have studied the influence of 24 other cations and 4 anions on the accuracy of the analysis. An example of the application of this method is given (simultaneous determination of zinc and copper in pig feed).

BIBLIOGRAPHIE

1 J. P. QUINCHE, *J. Chromatog.*, 13 (1964) 203.
2 L. G. SILLÉN ET A. E. MARTELL, *Stability Constants of Metal-ion Complexes*, Chem. Society, London, 1964.
3 G. CHARLOT, *Les Méthodes de la Chimie Analytique*, Masson, Paris, 1961.
4 G. W. C. MILNER, *The Principles and Applications of Polarography*, Longmans, London, New York, Toronto, 1957.
5 G. BRUNISHOLZ ET J. P. QUINCHE, *Chimia (Aarau)*, 13 (1959) 331.
6 G. BRUNISHOLZ ET J. P. QUINCHE, *Chimia (Aarau)*, 14 (1960) 343.
7 R. MORET ET G. BRUNISHOLZ, *Chimia (Aarau)*, 15 (1961) 313.

SÉPARATION DES LANTHANIDES ET DES ACTINIDES PAR L'ACIDE HYDROXYÉTHYLÈNEDIAMINOTRIACÉTIQUE

I. ÉTUDE DE L'EFFET DE SEL SUR L'EFFICACITÉ DE LA COLONNE

E. MERCINY ET G. DUYCKAERTS
Laboratoires de Chimie Analytique, Université de Liège (Belgique)
(Reçu le 21 septembre 1965)

INTRODUCTION

La qualité d'une séparation chromatographique sur résine échangeuse d'ions dépend essentiellement de deux grandeurs:

(*a*) *Le facteur de séparation*

Il est fonction des affinités relatives des ions pour la résine et surtout, dans le cas d'une élution avec un complexant, des constantes de stabilité des complexes formés.

(*b*) *L'efficacité de la colonne se traduisant dans la largeur des pics d'élution*

L'efficacité d'une colonne est régie par les phénomènes cinétiques suivants:

1. La réaction d'échange proprement dite, que les auteurs considèrent en général comme rapide[1,2].

2. La diffusion des ions au travers de la couche de liquide stationnaire entourant les particules de résine: diffusion laminaire.

3. La diffusion des ions dans l'échangeur: diffusion particulaire.

GLUECKAUF[3] a établi une formule permettant de chiffrer l'importance de ces différents facteurs:

$$\Delta = 1.64\, r + \frac{K_D}{(K_D + \alpha)^2} \frac{0.124\, r^2 F}{D_S} + \frac{K_D^2}{(K_D + \alpha)^2} \frac{0.266\, r^2 F}{D_L\,(1 + 70\, rF)} \qquad (1)$$

Δ = hauteur équivalente à un plateau théorique.
r = rayon des particules (cm).
K_D = coefficient de distribution calculable par la formule:

$$K_D = \left(\frac{V_M}{V_L} - 1\right)\alpha$$

α = rapport du volume libre (V_L) au volume total d'une colonne de résine.
V_M = volume d'élution correspondant au maximum d'un pic.
D_S = coefficient de diffusion particulaire ($cm^2 \cdot sec^{-1}$).
D_L = coefficient de diffusion laminaire ($cm^2 \cdot sec^{-1}$).
F = débit de la colonne ($ml \cdot sec^{-1} \cdot cm^{-2}$).

Signalons encore que dans la discussion et l'interprétation de ses résultats, FUGER[4] a été conduit à admettre un quatrième facteur qui dépend de la vitesse de décomplexation et qui peut prendre une importance dominante pour certains complexes très stables.

Il nous a paru intéressant d'essayer d'améliorer l'efficacité de la colonne et nous avons choisi, pour cette étude, comme agent éluant, l'acide hydroxyéthylènediaminotriacétique (HEDTA) et comme séparation celle d'un mélange de terres rares, ou d'actinides.

Cette note fournit les premiers résultats expérimentaux que nous avons obtenus.

RAPPEL THÉORIQUE

Dans le cas de l'emploi d'un agent complexant H_nY, le coéfficient de distribution entre la résine et la solution est donné par la formule suivante:

$$K_d = K_d^\circ \frac{1}{1 + \frac{[H_nY]_t}{\Theta K_c}} \tag{2}$$

K_d = rapport des concentrations [Me]résine/Σ[Me]sol.
K_d° = le coefficient de distribution en l'absence de complexant.
$(H_nY)_t$ = la concentration totale en complexant dans la phase liquide.
Θ = la fonction de distribution du complexant qui dépend du pH et des différents pK

$$\Theta = 1 + \frac{[H^+]^n}{K_1K_2K_n} + \frac{[H^+]^{n-1}}{K_2 \cdots K_n} + \cdots \frac{[H^+]^2}{K_{n-1}K_n} + \frac{[H^+]}{K_n} \tag{3}$$

K_c = constante de dissociation du complexe.

Le nombre de plateaux d'une colonne, calculé à partir de la largeur du pic d'élution, est donné par la formule suivante:

$$N = 8\left(\frac{V_M}{\beta}\right)^2 \tag{4}$$

V_M = volume d'élution correspondant au maximum du pic d'élution.
β = largeur du pic à une hauteur correspondant à 1/e du maximum.
N = nombre de plateaux théoriques.

CONDITIONS EXPÉRIMENTALES

L'acide hydroxyéthylènediaminotriacétique (HEDTA) est tribasique (P.M. = 278, T_F = 159°); les constantes d'acidité ont été déterminées par CHABEREG ET MARTELL[5]:

$pK_1 = 2.64$ $pK_2 = 5.33$ $pK_3 = 9.73$.

Dans la Fig. 1, nous avons représenté log Θ, calculé d'après ces valeurs, en fonction du pH.

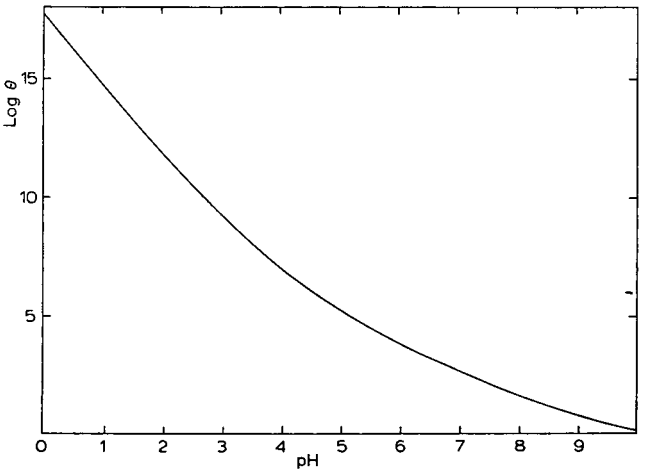


Fig. 1. Log Θ en fonction du pH pour l'acide hydroxyéthylènediaminotriacétique. $pK_1 = 2.64$; $pK_2 = 5.33$; $pK_3 = 9.73$.

La Fig. 2 reproduit les constantes de stabilité des complexes des terres rares en fonction du rayon ionique: ces valeurs ont été déterminées par SPEDDING *et al.*[6] par potentiométrie, à la température de 25°, dans un milieu de force ionique égale à 0.1 (KCl).

La solubilité dans l'eau à 22° est de 4.4 % en poids (solution 0.16 molaire); la solubilité est très élevée à plus haute température.

La résine Dowex 50 X 8 (200–400 mesh) de pureté pour analyse que nous avons utilisée pour la majorité des essais, a subi le conditionnement suivant:

(a) Élimination des grains de diamètre inférieur à 36 μ par sédimentation.

(b) Élimination des grains de diamètre supérieur à 74 μ par tamisage.

(c) Purification par lavages successifs avec 10 volumes libres des solutions suivantes: HCl N, HCl 6 N, NaOH 2 N, EDTA $2.5 \cdot 10^{-2}$ M (pH = 4.2), eau désionisée.

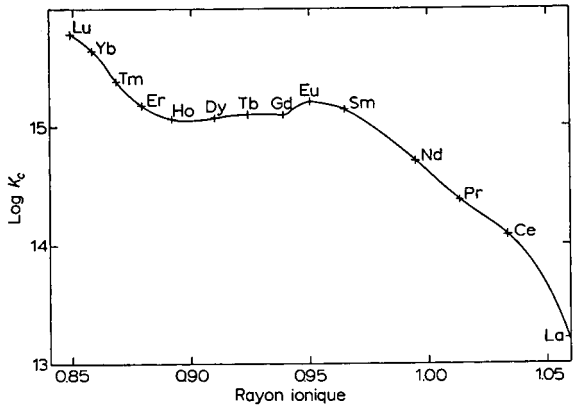


Fig. 2. Log K_C en fonction du rayon ionique pour l'acide HEDTA. La = 13.22; Ce = 14.08; Pr = 14.39; Nd = 14.71; Sm = 15.15; Eu = 15.21; Gd = 15.10; Tb = 15.10; Dy = 15.08; Ho = 15.06; Er = 15.17; Tm = 15.38; Yb = 15.64; Lu = 15.79.

(d) Séchage à poids constant (105–110°) et conservation dans un exsiccateur sous vide.

La colonne de chromatographie est thermostatisée à 80° par circulation de benzène à l'ébullition. Sa section est de 1.09 cm^2 et sa hauteur de 33 cm.

Les expériences ont été effectuées au moyen de terres rares marquées; celles-ci ont été obtenues par irradiation de leur oxyde au réacteur B.R.1. (Mol) dans des ampoules en quartz. Les ampoules ont été ouvertes sous liquide afin d'éviter tout risque de contamination; les oxydes sont mis en solution dans HCl 0.1 N.

RÉSULTATS EXPÉRIMENTAUX ET DISCUSSIONS

Il est à prévoir, d'après la formule (2) qu'en modifiant simultanément la concentration totale en complexant $[H_nY]_t$ et le pH de l'éluant c'est à dire Θ, il soit possible de maintenir inchangée la position d'un pic d'élution: il suffit en effet de maintenir constant le rapport $[H_nY]_t/\Theta$.

Mais, on peut se demander si cette modification a une influence sur le nombre de plateaux théoriques de la colonne.

Deux expériences de ce genre ont été réalisées avec le thulium: l'une à pH = 3.3 et à une concentration en HEDTA de 10^{-2} M, l'autre à pH = 2.9 et une concentration en complexant de 10^{-1} M; de cette façon, le rapport $[H_3Y]_t/\Theta$ vaut dans les deux cas $10^{-10.5}$.

Les autres conditions expérimentales de ces deux expériences sont consignées dans la légende de la Fig. 3. Il est à remarquer que pour avoir dans les deux expériences, une même concentration en ions sodium, nous avons dû, dans le cas de la solution 10^{-2} M en HEDTA, ajouter une certaine quantité de NaCl (25.5 ml de NaCl N par litre de solution éluante).

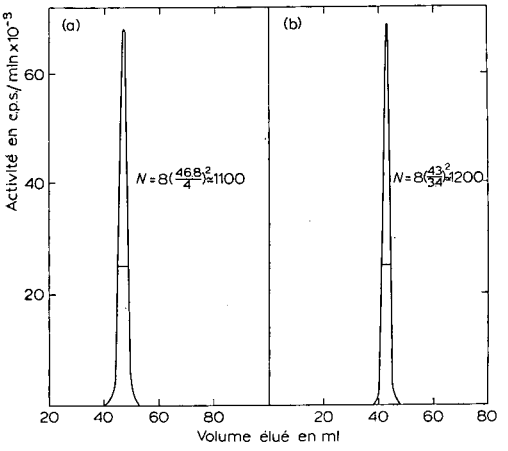


Fig. 3. Élution de Tm sur résine Dowex 50 X8 (200–400 mesh). Colonne: 33.6 cm de hauteur; température, 80°. Vitesse d'élution: 0.2 ml/min. V_L: 14 ml. Éluant: (a) HEDTA, 0.01 M; pH 3.3; concentration en Na^+, 0.06 M; (b) HEDTA, 0.1 M; pH 2.9; concentration en Na^+, 0.06 M.

Ainsi que le montrent les deux chromatogrammes de la Fig. 3, les positions des deux pics sont sensiblement les mêmes et le nombre de plateaux théoriques ne semble

pas affecté par ces modifications de pH et de concentration en HEDTA, pour autant toutefois, comme nous allons le voir, que la concentration en ions sodium soit maintenue constante. En effet, si nous comparons ces résultats à ceux de la Fig. 4 obtenus avec une solution éluante à pH = 3.58 et $[HEDTA]_t = 2.5 \cdot 10^{-2}$ M pour laquelle la

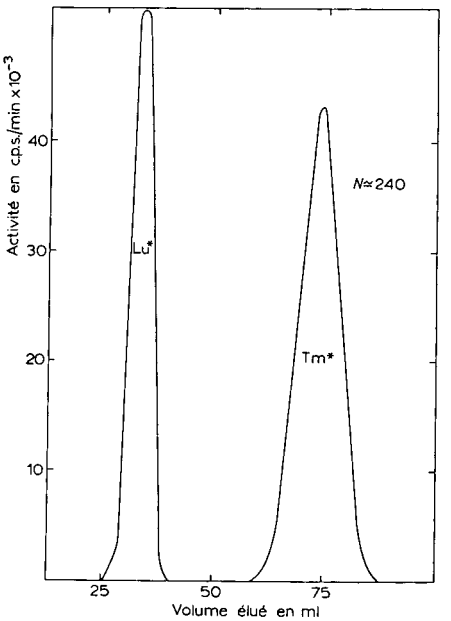


Fig. 4. Séparation Lu–Tm sur résine Dowex 50 X 8, 200–400 mesh. Colonne: 33 cm de hauteur de résine; 14.95 g; température, 80°. Vitesse d'élution: 0.2 ml/min. V_L: 13 ml. Éluant: HEDTA, $2.5 \cdot 10^{-2}$ M; pH 3.58.

concentration en Na^+ est beaucoup plus faible (les ions Na^+ proviennent de la quantité de NaOH nécessaire pour amener la solution de HEDTA au pH voulu), il apparait clairement que le nombre de plateaux dans cette dernière expérience est considérablement plus faible. Toutes conditions égales, il semble donc que la concentration en ions sodium joue un rôle important dans l'efficacité de la colonne.

Etude de l'influence de la concentration en Na^+ sur l'efficacité de la colonne

Ces résultats nous ont amenés tout naturellement à étudier systématiquement l'influence de la concentration en Na^+ en maintenant constants tous les autres paramètres expérimentaux: ces conditions expérimentales sont renseignées dans la légende de la Fig. 5. Notons cependant, au préalable, que pour ne pas avoir un volume d'élution trop important avec la dernière solution, nous avons augmenté, pour celle-ci, le pH de 3 à 3.5.

Il est à remarquer qu'une augmentation de la concentration en Na^+ se traduit non seulement par une diminution du volume éluant (V_M) mais encore par une augmentation considérable du nombre de plateaux théoriques qui passe de 320 à 2,300. Si la diminution de V_M, lorsque $[Na^+]$ augmente, s'explique tout naturellement par la loi d'action de masses, l'augmentation considérable de l'efficacité de la colonne est assez inattendue; en effet, d'après la formule de GLUECKAUF (1), on doit s'attendre à

une influence du K_D sur la hauteur du plateau théorique; ainsi, si l'on se trouve dans la région du phénomène contrôlé en ordre principal par la diffusion particulaire, on doit trouver, en première approximation, une augmentation du nombre de plateaux proportionnelle au K_D.

Il en résulte que l'accroissement du nombre de plateaux avec la concentration en ions Na^+ est encore plus important que celui qui ressort des valeurs calculées et indiquées sur la Fig. 5 puisque celles-ci devraient encore être corrigées pour tenir compte des variations du K_D.

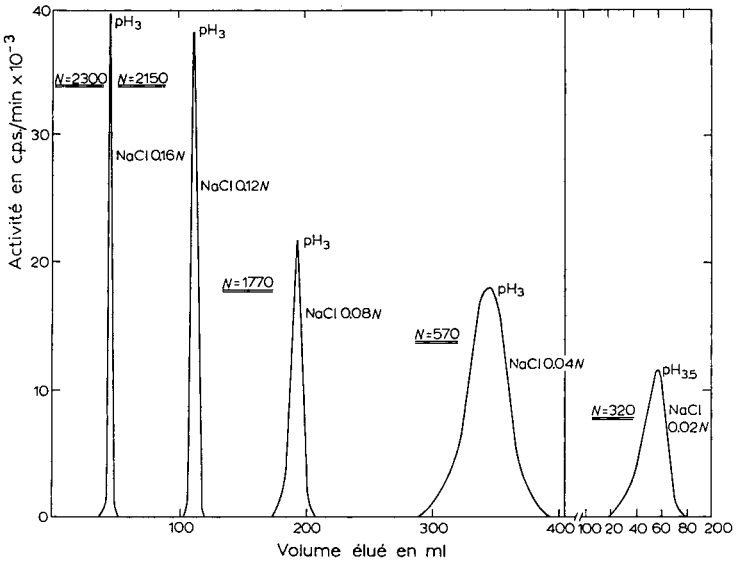


Fig. 5. Influence de la concentration en Na^+ sur la cinétique d'échange. Colonne: 33 cm de résine Dowex 50 X 8, 400 mesh; section, 1.09 cm^2. Vitesse d'élution: 0.2 ml/min. V_L: 13 ml. Éluant: HEDTA, 10^{-2} M. Isotope: Tm.

Influence de la nature du cation

Pour arriver à comprendre ce phénomène, il était indiqué d'examiner l'influence de la nature de l'ion et, à cet effet, nous avons fait une série d'expériences comparatives avec les ions monovalents suivants: Li^+, Na^+, K^+, NH_4^+, Ag^+.

Ces expériences ont été effectuées dans les conditions suivantes:

Résine Dowex 50 X 8, 200–400 mesh préparée sous forme lithique, sodique etc., suivant que l'éluant contient le chlorure de lithium ou de sodium etc. La résine a été conditionnée avec un volume d'éluant égal à 10 fois le volume libre puis lavée à l'eau désionisée jusqu'à réaction négative de l'éluant au nitrate d'argent.

La température de travail est de 80°, le volume de résine de 30 ml et la vitesse d'élution de 0.2 ml/min. L'isotope utilisé est le Tm.

La solution éluante est 0.01 M en HEDTA et 0.1 M en chlorure du cation expérimenté.

Le pH de la solution éluante était de 3.

Dans ces conditions, nous avons trouvé les nombres de plateaux suivants: LiCl: N = 900 plateaux; NaCl: N = 1,200 plateaux; KCl: N = 1,600 plateaux.

Il n'a pas été possible d'obtenir des valeurs constantes dans le cas de NH_4Cl;

le mélange éluant maintenu à 80° accuse une perte continuelle en NH_3 entraînant une diminution progressive du pH.

Dans le cas du nitrate d'argent, on a observé une réaction de réduction du $AgNO_3$ par le groupement alcoolique du HEDTA.

En conclusion, on peut dire que l'effet de sel qui se marque sur l'efficacité de la colonne augmente lorsque le rayon ionique hydraté du cation diminue.

Influence de la nature de l'anion du sel

Par contre, la nature de l'anion ne semble jouer aucun rôle: des expériences effectuées successivement avec KCl, KNO_3, $KClO_4$, KBr fournissent des résultats identiques du point de vue du nombre de plateaux.

Influence du degré de réticulation de la résine

Il était intéressant de voir si l'effet de sel était influencé par le degré de réticulation de la résine utilisée: à cet effet, des expériences similaires ont été faites successivement avec des résines Dowex 50 calibrées (200–400 mesh) X2, X4, X8, X12.

Pour chaque résine, nous avons effectué deux expériences dans des conditions identiques de concentration en HEDTA, de température, de vitesse d'élution, l'une sans addition de KCl et l'autre avec du KCl 0.1 *M*; les pH dans les deux expériences étaient différents de façon à obtenir le pic à la même place et éliminer ainsi l'influence du K_D sur le nombre de plateaux. Les résultats trouvés sont rassemblés dans le Tableau I.

TABLEAU I

NOMBRE DE PLATEAUX THÉORIQUES

Dowex 50	*X 2*	*X 4*	*X 8*	*X 12*
Sans KCl supplémentaire	220	100	70	30
Avec KCl 0.1 *M*	3,400	1,500	1,100	460
Rapport	15.5	15	15.7	15.3

Remarquons d'abord que dans les expériences avec ou sans KCl, le nombre de plateaux diminue lorsque le degré de réticulation de la résine augmente; comme l'augmentation du degré de réticulation entraîne une diminution du coefficient de diffusion particulaire D_S, il semble que dans les conditions expérimentales que nous avons adoptées, la cinétique globale soit régie essentiellement par la diffusion particulaire (second terme de la formule de GLUECKAUF).

Par ailleurs, il est intéressant de constater que l'effet de sel est quantitativement le même quel que soit le degré de réticulation de la résine utilisée.

Influence de la granulométrie de la résine

Enfin, l'on sait par la formule de GLUECKAUF, que la hauteur équivalente à un plateau théorique augmente fortement avec le diamètre des grains de résine et il était dès lors indiqué de se rendre compte de l'effet de sel avec des résines de granulométries différentes.

Les expériences ont été effectuées avec des résines calibrées de 50–100 mesh, 100–200 mesh, 200–400 mesh, > 400 mesh.

Comme dans le paragraphe précédent, les essais ont été faits dans des conditions identiques, avec ou sans KCl et à des pH légèrement différents pour obtenir des V_M identiques. Les résultats obtenus sont consignés dans le Tableau II.

TABLEAU II

NOMBRE DE PLATEAUX THÉORIQUES

Dowex 50 X 8	*50–100 mesh*	*100–200 mesh*	*200–400 mesh*	*> 400 mesh*
Sans KCl supplémentaire	30	55	65	80
Avec KCl 0.1 *M*	150	540	970	1,300
Rapport	5	10	15	16

Ici encore on observe dans les expériences sans KCl comme dans celles avec KCl que le nombre de plateaux augmente lorsque la granulométrie diminue (en accord avec la théorie de GLUECKAUF) mais, contrairement aux résultats trouvés pour le degré de réticulation, on peut voir que la granulométrie joue un rôle beaucoup plus important dans le cas des expériences avec addition de KCl.

CONCLUSIONS

Les conclusions que nous pouvons tirer dès à présent de cette étude qui se poursuit, sont essentiellement d'ordres qualitatifs et pratiques; en effet, la justification quantitative du phénomène observé nous paraît à l'heure actuelle encore difficile: tout au plus, pouvons-nous constater que dans nos conditions expérimentales, le phénomène cinétique lent qui paraît dominer et fixer le nombre de plateaux est la diffusion particulaire.

Dans ces conditions, on peut imaginer que la vitesse d'établissement de l'équilibre de distribution qui fixe en partie le nombre de plateaux, doit augmenter:

(1) avec la diminution du degré de réticulation;

(2) avec la diminution de la granulométrie;

(3) avec la diminution du rayon ionique hydraté des ions (Li^+, Na^+, K^+);

(4) avec l'augmentation de la concentration en ions (Li^+, Na^+, K^+) dans la solution. Cette dernière augmenterait la vitesse de diffusion.

Du point de vue pratique, on peut dire que, tout au moins dans le cas des séparations en doses traceurs, il est avantageux de substituer une partie importante du complexant par un sel comme KCl sans pour autant diminuer l'efficacité de la colonne.

Nous voulons illustrer cette conclusion au moyen de deux séparations représentées dans les Figs. 6 et 7.

La Fig. 6 concerne la séparation de douze terres rares au cours d'une seule élution au moyen d'une solution de HEDTA 0.01 *M* contenant du KCl $1.5 \cdot 10^{-1}$ *M*.

Signalons encore pour terminer la différence entre les facteurs de séparation que

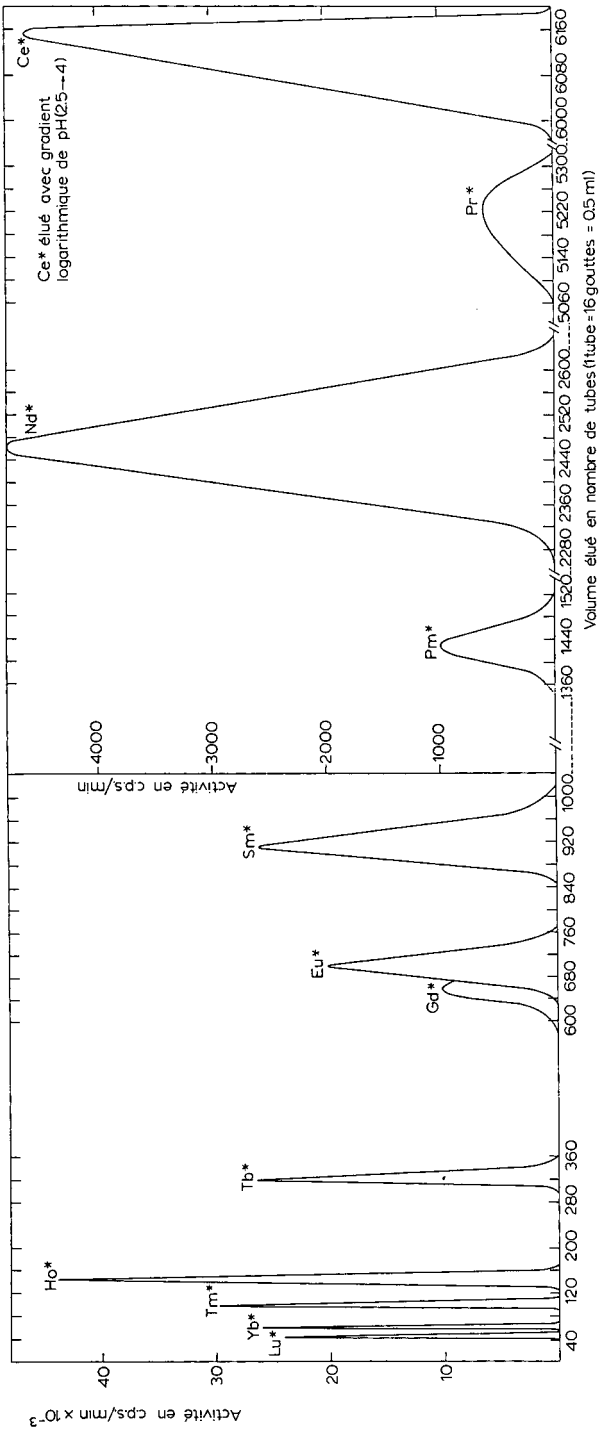


Fig. 6. Séparation de douze terres rares. Colonne: 30 cm de hauteur de résine Dowex 50 X 8, 200–400 mesh; section, 1.09 cm². Vitesse d'élution: 0.30 ml/min. V_L: 12 ml. Éluant: HEDTA 10^{-2} *M*; pH 2.5; température, 80°; concentration en KCl, $1.5 \cdot 10^{-1}$ *M*. Identification des lanthanides au spectromètre RIDL 400 Canaux. Comptage Gd–Eu au spectromètre RIDL 400 Canaux.

nous obtenons expérimentalement et ceux que l'on peut prévoir à partir des valeurs de K_c données par SPEDDING *et al.*[6] (Fig. 2).

Les valeurs des facteurs de séparation des lanthanides étudiés, par rapport au praseodyme pris comme référence, sont comparées sur la Fig. 8.

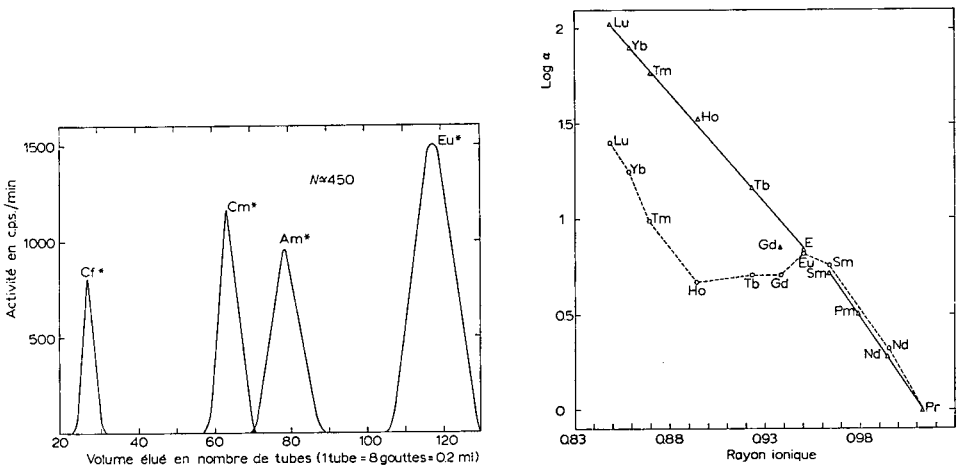


Fig. 7. Séparation de trois actinides et d'un lanthanide. Colonne: 14 cm de hauteur de résine Dowex 50 X 8, 200–400 mesh; section, 0.12 cm². Vitesse d'élution: 0.5 ml/min. V_L.: 0.7 ml. Éluant: HEDTA 10^{-2} M; pH 2.4; température, 80°, concentration en KCl, $1.5 \cdot 10^{-1}$ M.

Fig. 8. Comparaison des valeurs des facteurs de séparation des lanthanides. △ = Valeurs expérimentales; ○ = valeurs calculées après les K_C donnés par SPEDDING *et al.*[6].

La Fig. 7 a trait à la séparation de trois actinides et d'un lanthanide sur une colonne de 14 cm de hauteur et de 4 mm de diamètre; les conditions expérimentales sont renseignées dans la légende.

On voit à nouveau les résultats excellents que l'on peut obtenir même avec une solution diluée de HEDTA (0.01 M) à condition d'ajouter une concentration suffisante en sel (KCl $1.5 \cdot 10^{-1}$ M).

REMERCIEMENTS

Il nous est agréable de remercier ici l'I.I.S.N. pour les subsides accordés qui nous ont permis de mener à bien ces recherches.

RÉSUMÉ

Dans le but d'améliorer la qualité d'une séparation chromatographique sur résine d'échangeurs d'ions, nous avons étudié l'influence sur la cinétique d'échange de différents sels (LiCl, NaCl, KCl) et de résines de granulométrie et de cross-linking différents.

Les expériences d'élution ont été réalisées avec l'acide hydroxyéthylènediaminotriacétique (HEDTA) et un mélange de terres rares marquées.

SUMMARY

With the aim of improving chromatographic separation on ion-exchange resins, we have studied the influence on the kinetics of exchange of different salts (LiCl, NaCl, KCl) and of resins of different grain size and cross-linking.

The experiments were carried out with hydroxyethylenediaminetriacetic acid (HEDTA) and a mixture of labelled rare earths.

BIBLIOGRAPHIE

1 G. E. BOYD, A. W. ADAMSON ET L. S. MYERS, *J. Am. Chem. Soc.*, 69 (1947) 2837.
2 H. C. THOMAS, *J. Am. Chem. Soc.*, 66 (1944) 1664.
3 E. GLUECKAUF, *A.E.R.E. C/R* 1356 (1957).
4 J. FUGER, *Thèse de doctorat*, Université de Liège, 1959.
5 S. CHABEREK, Jr., ET A. E. MARTELL, *J. Am. Chem. Soc.*, 77 (1955) 1477.
6 F. H. SPEDDING, J. E. POWELL, ET E. J. WHEELWRIGHT, *J. Am. Chem. Soc.*, 78 (1956) 34.
7 E. GLUECKAUF ET J. J. COATES, *J. Chem. Soc.*, (1947) 1315.

Notes

Production of pure helium carrier gas by diffusion

The successful use of a helium ionization detector for permanent gases in gas chromatography requires the production of a very pure carrier gas containing no more than 1 p.p.m. (by volume) of impurities in total. The impurities in commercial helium amount to 10 p.p.m. (by volume) approximately, excluding water, and are principally neon, nitrogen, hydrogen, oxygen, argon and carbon dioxide.

The methods of purifying helium proposed so far have been the use of traps cooled in liquid helium, traps containing molecular sieve cooled in liquid nitrogen[1], or a chemical purification train[2]. This last method is the most commonly used. The train is composed of absorbers containing molecular sieve at both room and liquid nitrogen temperatures and furnaces containing titanium at 600° and Hopcalite (mixed copper and manganese oxides) at 350°. We have used such a purification train and find it difficult and expensive to set up as it requires meticulous attention to cleanliness and a long period of preliminary operation before stability is reached. The life of absorbers is limited and the train requires periodic replacement. Although BOURKE *et al.*[3] have proposed a simpler chemical system the same criticisms may be applied.

The principle of stress enhanced diffusion of helium through quartz, investigated by MCAFEE[4], has not so far been proposed as a means of preparing a pure carrier gas for helium ionization chromatography. We have found that a commercially available diffusion cell* will deliver helium of acceptable purity within hours of starting the flow (the impurities are stated by the manufacturers to total less than 0.5 p.p.m. (by volume)). The construction of this cell is similar to that of MELNYK AND HABGOOD[5]. It consists of a steel tube containing a bundle of quartz capillary tubes, which are individually sealed at one end. The sealed ends are exposed to the impure helium at high pressure. The tubes pass through a resin seal, with their open ends terminating on the low pressure side, into which they release the diffused helium. This cell was connected directly to the helium cylinder by means of the hose and pressure regulator supplied by the manufacturers. The diffusion area of the cell was wrapped with heating tapes dissipating a maximum of 500 W and supplied by a variable transformer. The seal area of the cell was water cooled. A pressure release valve** set at 35 p.s.i. was inserted downstream of the cell to protect the chromatograph.

Maximum temperature obtainable in the diffusion area was 600° and at 1,500 p.s.i. inlet pressure 450 ml/min of pure helium could be obtained at atmospheric pressure. Normal operation at 375° and 500 p.s.i. at the inlet provided 70 ml/min.

When the output from this diffusion cell was substituted for the output from a chemical purification train supplying a chromatograph, the standing current in the ionization detector of the chromatograph fell markedly.

* Electron Technology Inc., 625 Schuyler Ave., Kearney, N.J.
** Seatru Ltd., 43 Corn St., Bristol 1, Great Britain.

Some comparative values of detector current are given in Table I, the last figure of which obtained by BOURKE *et al.*[6], probably represents the best obtainable by chemical means.

TABLE I

COMPARATIVE VALUES OF DETECTOR CURRENT

Instrument No.	*Carrier gas purifier*	*Radiation source (mC)*		*Detector*	
		^{3}H	^{90}Sr	*Volts*	*Amps*
1	chemical	100		500	$1.1 \cdot 10^{-8}$
1	diffusion	100		500	$4.2 \cdot 10^{-9}$
2[2]	chemical	100		750	$1 \cdot 10^{-8}$
			10	—	$1 \cdot 10^{-8}$
3[6]	chemical		10	1,000	$1.8 \cdot 10^{-9}$

Several cylinders of helium have now been processed by this method without difficulty. Rapid venting of the high pressure gas in the diffuser when changing the supply appears to be sufficient to prevent excessive accumulation of impurities and there has been no indication that the life of the system will be limited in any way.

United Kingdom Atomic Energy Authority (Production Group), Capenhurst Works, Chester (Great Britain) ERIC G. JOHNSON

1 J. E. LOVELOCK, *Res. Develop.*, 2 (1962) 36.
2 R. BERRY, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962, p. 231.
3 P. J. BOURKE, M. D. GRAY AND W. H. DENTON, *J. Chromatog.*, 19 (1965) 189.
4 K. B. MCAFEE, *J. Chem. Phys.*, 28 (1958) 218 and 226.
5 N. MELNYK AND H. W. HABGOOD, *Can. Mining Met. Bull.*, (1961) 768.
6 P. J. BOURKE, R. W. DAWSON AND W. H. DENTON, *J. Chromatog.*, 14 (1964) 387.

Received October 4th, 1965

R_M values in simplified four-component systems of the type: ternary mixed solvent/pure solvent

Multi-component solvents, which are more versatile for the choice of optimal conditions of separation, are often used in paper and thin-layer chromatography. It is easier to analyse the effect of phase composition on the partition chromatographic behaviour of solutes when the two liquid phases are essentially immiscible, that is, when the stationary phase held by the supporting material is insoluble in the developing solvent (*e.g.*, the system carbon tetrachloride–benzene–chloroform/formamide[1]). Nevertheless, even in these cases, the R_F–composition relationships for four-

component solvent systems have to be represented by space models, or by employing the method of iso-lines[2]. However, the relationships can be simplified if certain rules are applied when choosing the composition of the system; thus, as shown in an earlier work[3], quaternary systems of the type binary phase/binary phase (2/2) give simple relationships of the type binary phase/pure solvent (2/1) or *vice versa* (1/2) when the composition of one of the phases is fixed. Similar simple relationships are obtained in systems of the type binary solvent/aqueous buffer solution (2/pH) when the pH of the water phase is kept constant[4]. In the present work the choice of composition of the mixed ternary phase is considered, permitting the simplification of the R_M–composition relationships in systems of the type 3/1 or 1/3 (ternary mixed phase/pure solvent, or *vice versa*); the experimental data are, at the same time, a test of the thesis that (in an idealized case) the R_M value of a solute is additive with respect to the composition of the mixed phase[2]:

$$R_M = u_1 R_{M1} + u_2 R_{M2} + u_3 R_{M3} \qquad (1)$$

In a strictly thermodynamic approach, both the composition (u) of the mixed phase and the concentrations determining the partition ratios K and R_M values should be expressed in mole fractions (*cf.* BUCHOWSKI[5] for partition coefficients). If, however, the molar volumes of the component solvents do not differ to a greater extent, then the conventional R_M values may be used (in which the concentrations in the extraction coefficient are expressed in the mol/l scale) and the composition of the mixed phase expressed in volume fractions, which is advantageous for practical purposes. Moreover, the use of volume compositions and conventional extraction coefficients may in certain cases compensate for deviations from ideal behaviour of the mixed phase; thus, equations like (1) (u-volume fraction) are useful approximate semi-empirical relationships.

It follows from eqn. (1) that when the composition of the ternary phase is represented on a Gibbs' diagram and the R_M values are plotted above the triangle, then the R_M–composition relationship is given by a plane surface passing through the three points corresponding to R_M values for the three pure component solvents[2]. Therefore, a straight line lying in this plane surface, projected horizontally on to the side walls of the prism, will also be represented by straight lines which are simple plots of an R_M–composition relationship of the type binary solvent/pure solvent. This allows one to choose series of ternary mixtures resulting in simplified R_M–composition plots.

Experimental

(a) A constant proportion of components 1 and 2 is employed, the content of component 3 being varied (Fig. 1a). The mixtures are prepared simply by mixing a standard mixture of 1 and 2 with various proportions of 3. In this case the R_M value should be linearly dependent on the percentage of component 3 in the mixed phase.

(b) A constant percentage of one of the component solvents is maintained (Fig. 1b), while the content of the remaining solvents is varied. In the case illustrated in Fig. 1b, the R_M value should be linearly dependent on the percentage of component 3 (or 1). *Cf.*, for instance, WALDI[6].

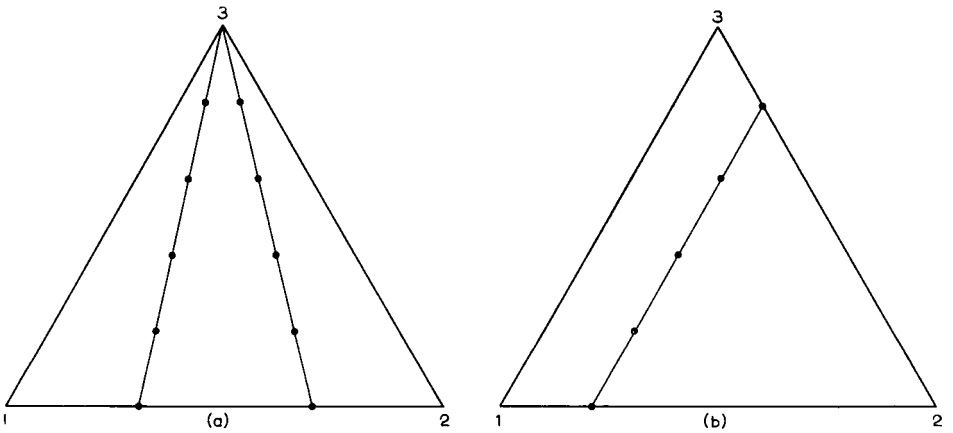


Fig. 1. Linear series of compositions in ternary mixtures. a = constant proportions of components 1 and 2; b = constant content of component 2.

In order to test this hypothesis, R_F and R_M values were determined for a few quinoline bases in solvent systems of the type cyclohexane (1)–carbon tetrachloride (2)–trichloroethylene (3)/aqueous buffer solution. The solvent system was chosen merely as an example in which three component solvents of the mobile phase are practically immiscible with the other phase. The pH of the water phase (3.0) was chosen so that the R_M values of each solute were in the range of optimal accuracy (R_F 0.1–0.7) for any composition of the mixed phase. The "moist buffered paper" technique, described in earlier papers (*cf.*, ref. 4) was employed. The experiments were carried out at 20.0 ± 0.5°.

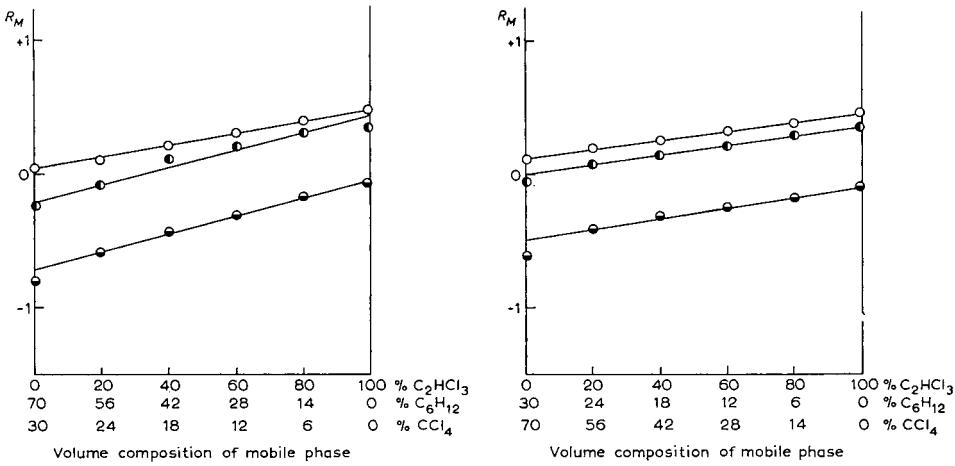


Fig. 2. Simplified R_M *vs.* volume composition of mobile phase relationship. Choice of compositions of the ternary phase: *cf.* Fig. 1a. ◐ = quinoline; ◒ = iso-quinoline; ○ = 8-hydroxyquinoline. C_6H_{12}:CCl_4 = 7:3.

Fig. 3. Simplified R_M *vs.* volume composition of mobile phase relationship. Data as in Fig. 2. C_6H_{12}:CCl_4 = 3:7.

Results

The experimental results are represented in Figs. 2 and 3 (case (a), constant proportion of cyclohexane and carbon tetrachloride, *cf.* Fig. 1a) and Fig. 4 (case (b), constant content of carbon tetrachloride, *cf.* Fig. 1b). The compositions of the mobile phase are given under the abscissa axes. R_M is defined here according to REICHL:

$$R_M = \log \frac{R_F}{1 - R_F}$$

It can be seen that, in accordance with theoretical anticipations, the R_M values vary linearly with the concentration of trichloroethylene in the mixed phase. Only the extreme points tend to lie below the straight lines. Thus, in spite of a number of

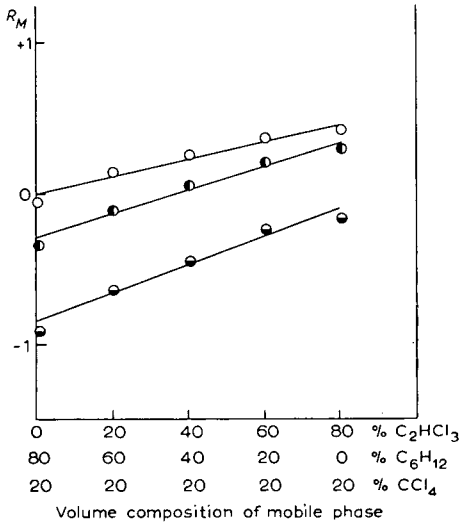


Fig. 4. Simplified R_M *vs.* volume composition of mobile phase relationship. Choice of composition of the ternary phase: *cf.* Fig. 1b. $u_{CCl_4} = 0.20$.

simplifications assumed in the theoretical treatment, the experimental results confirm both the additivity of R_M values with respect to the composition of the mixed phase, and the possibility of choosing series of mixed phases which result in simplified relationships, analogous to simple systems of the type: binary phase/pure solvent.

Department of Inorganic Chemistry,
Medical Academy, Lublin (Poland)

EDWARD SOCZEWIŃSKI
WIESŁAWA MACIEJEWICZ

1 M. PÖHM AND L. FUCHS, *Naturwiss.*, 41 (1954) 63.
2 E. SOCZEWIŃSKI, *J. Chromatog.*, 8 (1962) 119; *Chem. Anal. (Warsaw)*, 8 (1963) 337.
3 E. SOCZEWIŃSKI, *J. Chromatog.*, 11 (1963) 275.
4 E. SOCZEWIŃSKI AND W. MACIEJEWICZ, *J. Chromatog.*, 17 (1965) 333.
5 H. BUCHOWSKI, *Bull. Acad. Polon. Sci., Ser. Chim.*, 10 (1962) 195; *The Effect of the Properties of Solvents on Partition Coefficients of Nonelectrolytes between Water and Organic Solvents* (in Polish), Dissertation Univ. Varsoviensis (Poland) No. 4, PWN, Warsaw, 1963.
6 D. WALDI, in *Some General Problems of Paper Chromatography, Liblice Symposium 1961, 1st*, CSAV, Prague, 1962, p. 139.

Received October 22nd, 1965

Quantitative separation of chloro-xylenols by thin layer chromatography

Methods for the qualitative separation of chloro-derivatives of cresols and xylenols by paper and thin layer chromatographic techniques have been described by us recently[1,2]. This paper presents a thin layer chromatographic (TLC) method for quantitative estimation of some xylenols and their chloro-derivatives using, essentially, the techniques described by BLOCK[3], ROCKLAND AND DUNN[4] and PRIVETT AND BLANK[5] for the estimation of amino acids and glycerides, respectively. An automatic Joyce Chromoscan[6] was used to measure the optical densities of the spots; these are converted by the unit into absolute areas from which the relative percentages of the components are readily calculated.

Materials and methods

The xylenols and their chloro-derivatives were obtained from commercial sources[7] and repeatedly recrystallized till chromatographically pure. The plates (20 × 2.1 cm) were coated with silica gel G and developed as described earlier[2]. Microslides (7.2 × 2.4 cm) were prepared by dipping in a well-stirred suspension of silica gel G (30 g) in 80 ml of chloroform and 20 ml of methanol[8]. Both plates and microslides were scanned by the densitometer to determine the degree and uniformity of the background density.

Known volumes of various dilutions of compounds in acetic acid were applied with an Agla micrometer syringe[9] on the TLC plates and the optimum dilution range determined. The sample was then spotted (*ca.* 50 μg) with a glass capillary or microsyringe. The chromatogram was developed in xylene saturated with formamide, sprayed with a sufficient quantity of phosphotungstomolybdic acid (Folin-Denis reagent) and the plate was then exposed to ammonia vapour. The compounds appeared as blue spots on a white background.

For scanning, the developed plate (Fig. 1) was held in the densitometer and the

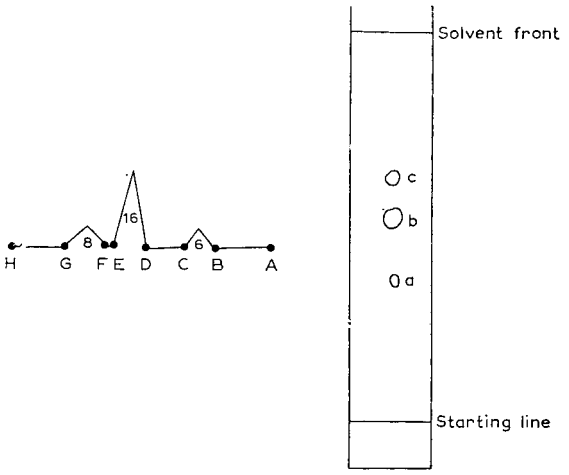


Fig. 1. Thin-layer chromatography diagram and corresponding peaks obtained on Chromoscan. B–C and a = 4-chloro-3,5-xylenol (20.0%); D–E and b = 4,6-dichloro-3,5-xylenol (53.33%); F–G and c = 2,4,6-trichloro-3,5-xylenol (26.67%).

base line adjusted for stability and maximum peak recording. The plate was then scanned with a slit opening of 1 × 10 mm when peaks were obtained for each coloured spot (Fig. 1). After this initial scanning, the process was repeated at the corners of the bases of the peaks (points B to C, D to E and F to G in Fig. 1) to obtain integrated numbers for the peak areas. The relative percentage of each component was calculated from these numbers.

Results and discussion

The optimum quantity for spotting was determined by resolving and scanning various dilutions of mixed 4-chloro-3,5-xylenol and 2,4,6-trichloro-3,5-xylenol in acetic acid on TLC plates (Table I). Though reasonable accuracy was obtained on

TABLE I

SENSITIVITY OF THE METHOD

Mixture used: (a) 4-chloro-3,5-xylenol 27.97%; (b) 2,4,6-trichloro-3,5-xylenol 72.03%

Mixture (*μl*)	*Quantity spotted* (*μg*)	*Composition found* (%)	*Difference* (%)
25.32	253.2	(a) 44.19 (b) 55.81	16.22
12.66	126.6	(a) 36.11 (b) 63.89	8.14
8.44	84.4	(a) 29.63 (b) 70.37	1.66
5.06	50.6	(a) 28.57 (b) 71.43	0.6
3.61	36.1	(a) 29.02 (b) 70.98	1.05

84.4 μg of the mixture, *ca.* 50 μg was selected for further studies. With higher quantities spots were obtained of diameter greater than 1 cm which were not fully scanned with a slit opening of this diameter.

Xylenols and chloro-xylenols taken in varying proportions were estimated by this method using 20 × 2.1 cm plates with the results shown in Table II. It is evident that this technique can be employed for the quantitative estimation of mixtures of xylenols and their chloro-derivative with a degree of accuracy of *ca.* 3%.

Overlapping or partly resolved spots, given for example by xylenols and their 4-chloro-derivatives were recorded during scanning by the densitometer as single or as incompletely resolved peaks. In such cases, the integrated peak areas of the two components together were taken. Results obtained on microslides were as good as those with larger plates (Table III). Since the scanning of a microslide takes about 2 min and entire procedure about 10 min, the technique can well be used as a control method while chlorinating unknown mixtures of phenols.

TABLE II

ACTUAL AND DETERMINED PERCENTAGE COMPOSITION OF CHLORO-XYLENOL MIXTURES

Components	*Composition (%)*		
	Taken	*Found*	*Difference*
A. 2,3-Xylenol and its chloro-derivatives			
(1) 4-Chloro-2,3-xylenol	73.89	76.40	2.51
4,6-Dichloro-2,3-xylenol	26.11	23.60	
(2) 4-Chloro-2,3-xylenol	22.98	25.37	2.39
4,6-Dichloro-2,3-xylenol	77.02	74.63	
(3) 2,3-Xylenol	24.13	60.22	1.35
4-Chloro-2,3-xylenol	34.74		
4,6-Dichloro-2,3-xylenol	41.13	39.78	
(4) 2,3-Xylenol	28.45	59.83	1.08
4-Chloro-2,3-xylenol	30.30		
4,6-Dichloro-2,3-xylenol	41.25	40.17	
(5) 2,3-Xylenol	9.3	34.61	2.01
4-Chloro-2,3-xylenol	23.30		
4,6-Dichloro-2,3-xylenol	67.40	65.39	
(6) 2,3-Xylenol	10.56	21.21	0.24
4-Chloro-2,3-xylenol	10.41		
4,6-Dichloro-2,3-xylenol	79.03	78.79	
(7) 2,3-Xylenol	11.88	29.80	1.72
4-Chloro-2,3-xylenol	16.20		
4,6-Dichloro-2,3-xylenol	71.92	70.20	
B. 2,5-Xylenol and its chloro-derivatives			
(8) 4-Chloro-2,5-xylenol	23.95	23.26	0.69
4,6-Dichloro-2,5-xylenol	76.05	76.74	
(9) 4-Chloro-2,5-xylenol	59.55	57.58	1.97
4,6-Dichloro-2,5-xylenol	40.45	42.42	
(10) 4-Chloro-2,5-xylenol	5.74	8.00	2.26
4,6-Dichloro-2,5-xylenol	94.26	92.00	
(11) 2,5-Xylenol	28.81	50.43	1.27
4-Chloro-2,5-xylenol	22.89		
4,6-Dichloro-2,5-xylenol	48.30	49.57	
(12) 2,5-Xylenol	7.97	15.00	1.36
4-Chloro-2,5-xylenol	8.39		
4,6-Dichloro-2,5-xylenol	83.64	85.00	
C. 3,5-Xylenol and its chloro-derivatives			
(13) 2,4-Dichloro-3,5-xylenol	31.16	34.38	3.22
2,4,6-Trichloro-3,5-xylenol	68.84	65.62	
(14) 2,4-Dichloro-3,5-xylenol	23.87	25.00	1.13
2,4,6-Trichloro-3,5-xylenol	76.13	75.00	
(15) 2,4-Dichloro-3,5-xylenol	77.00	76.00	1.00
2,4,6-Trichloro-3,5-xylenol	23.00	24.00	
(16) 4-Chloro-3,5-xylenol	22.06	20.00	2.06
2,4-Dichloro-3,5-xylenol	53.19	53.33	0.14
2,4,6-Trichloro-3,5-xylenol	24.75	26.67	1.92
(17) 4-Chloro-3,5-xylenol	20.92	22.22	1.30
2,4-Dichloro-3,5-xylenol	20.08	22.22	2.14
2,4,6-Trichloro-3,5-xylenol	59.00	55.56	3.44
(18) 4-Chloro-3,5-xylenol	20.83	23.37	2.54
2,4-Dichloro-3,5-xylenol	42.62	42.85	0.23
2,4,6-Trichloro-3,5-xylenol	36.55	33.78	2.77

(*continued on p. 183*)

TABLE II (*continued*)

Components	*Composition* (%)		
	Taken	*Found*	*Difference*
(19) 3,5-Xylenol	24.47	48.87	0.42
4-Chloro-3,5-xylenol	24.82		
2,4-Dichloro-3,5-xylenol	32.07	34.09	2.02
2,4,6-Trichloro-3,5-xylenol	18.64	17.04	1.60
(20) 3,5-Xylenol	22.17	38.30	2.59
4-Chloro-3,5-xylenol	13.54		
2,4-Dichloro-3,5-xylenol	32.43	34.04	1.61
2,4,6-Trichloro-3,5-xylenol	31.86	27.66	4.2
(21) 3,5-Xylenol	15.85	16.66	0.81
4-Chloro-3,5-xylenol	12.62	12.50	0.12
2,4-Dichloro-3,5-xylenol	37.36	41.67	4.31
2,4,6-Trichloro-3,5-xylenol	34.17	29.17	5.00
D. 3,4-Xylenol and its chloro-derivatives			
(22) 6-Chloro-3,4-xylenol	43.16	41.93	1.23
2,6-Dichloro-3,4-xylenol	56.84	58.07	
(23) 6.-Chloro-3,4-xylenol	13.12	16.66	3.54
2,6-Dichloro-3,4-xylenol	86.88	83.34	
(24) 6-Chloro-3,4-xylenol	60.37	57.15	3.22
2,6-Dichloro-3,4-xylenol	39.63	42.85	
(25) 3,4-Xylenol	21.76	22.21	0.45
6-Chloro-3,4-xylenol	31.86	33.34	1.48
2,6-Dichloro-3,4-xylenol	46.38	44.45	1.93
(26) 3,4-Xylenol	15.80	18.37	2.57
6-Chloro-3,4-xylenol	10.28	12.24	1.96
2,6-Dichloro-3,4-xylenol	73.92	69.39	4.53
(27) 3,4-Xylenol	26.33	24.23	2.10
6-Chloro-3,4-xylenol	13.39	13.76	0.37
2,6-Dichloro-3,4-xylenol	60.28	62.01	1.73

TABLE III

COMPARATIVE QUANTITATIVE STUDY ON 20 × 2.1 CM AND 7.2 × 2.4 CM MICROSLIDE PLATES

Components	*Taken* (%)	*Found*		*Difference* (%)
		On 20 × 2.1 cm plates (%)	*On 7.2 × 2.4 cm plates* (%)	
4-Chloro-3,5-xylenol	27.97	29.63	29.41	— 0.22
2,4,6-Trichloro-3,5-xylenol	72.03	70.37	70.59	+ 0.22
4-Chloro-3,5-xylenol	68.82	68.42	68.75	+ 0.33
2,4,6-Trichloro-3,5-xylenol	31.18	31.58	31.25	— 0.33
4-Chloro-2,3-xylenol	Unknown mixture obtained during chlorination of 2,3-xylenol with one mole of SO_2Cl_2	61.53	61.30	— 0.23
4,6-Dichloro-2,3-xylenol		38.47	38.70	+ 0.23

Acknowledgement

Our thanks are due to Dr. R. VAIDYESWARAN, SHRI D. P. AGRAWAL and SHRI B. GOPINATH for the helpful discussions and also to Dr. G. S. SIDHU, Director for his keen interest.

Regional Research Laboratory, Hyderabad (India) SAJID HUSAIN
P. ANANDA SWAROOP

1 S. HUSAIN, *J. Chromatog.*, 18 (1965) 197.
2 S. HUSAIN, *J. Chromatog.*, 18 (1965) 419.
3 R. J. BLOCK, *Proc. Soc. Exptl. Biol. Med.*, 72 (1949) 337.
4 L. B. ROCKLAND AND M. S. DUNN, *J. Am. Chem. Soc.*, 71 (1949) 4121.
5 O. S. PRIVETT AND M. L. BLANK, *J. Lipid Res.*, 2 (1961) 37.
6 *Mannual M 64/7/CSC. Joyce, Loebl & Co. Ltd.*, Princesway Team Valley, Gateshed-on-Tyne, England.
7 S. HUSAIN AND R. VAIDYESWARAN, *Indian J. Chem.*, 2 (1964) 417.
8 J. J. PEIFER, *Mikrochim. Acta*, (1962) 529.
9 J. W. TREVAN, *Biochem. J.*, 19 (1925) 1111.

Received October 4th, 1965

J. Chromatog., 22 (1966) 180–184

Ultraviolet-induced isomerization of β-D-glucosyl o-hydroxycinnamic acid on filter paper*

Coumarinic acid glucoside (β-D-glucosyl *cis-o*-hydroxycinnamic acid) and *o*-coumaric acid glucoside (β-D-glucosyl *trans-o*-hydroxycinnamic acid) are readily detected as absorbing areas on filter paper chromatograms exposed to ultraviolet light at wavelengths near 260 mμ. Long wavelength ultraviolet radiation is frequently used to detect fluorescent compounds closely related to these two glucosides. The foregoing facts prompted this investigation concerning the influence of both long and short wavelength ultraviolet light on small amounts of coumarinic acid glucoside and *o*-coumaric acid glucoside, air-dried on filter paper strips. Ultraviolet-induced interconversion of these two isomers in aqueous solutions is well known[1].

Procedure

The two glucosides were isolated from hot water extracts of sweetclover leaves by paper chromatography. The solvent consisted of 2% acetic acid[2]. In this system R_F values for coumarinic acid glucoside and *o*-coumaric acid glucoside are 0.90 and 0.66, respectively. The glucosides were detected on test strips cut from chromatographic sheets; this prevented exposure of the entire chromatograms to ultraviolet light. Bands representing the two glucosides were cut out and eluted with water; eluates were assayed[3] and then diluted with water to a final concentration of 1 μmole/ml.

* Cooperative investigations of the Crops Research Division, Agricultural Research Service, Department of Agriculture, and the Nebraska Agricultural Experiment Station. Supported in part by the National Science Foundation (Grant GB1148). Published with the approval of the Director as Paper 1805, Journal Series, Nebraska Agricultural Experiment Station.

A 0.1-ml aliquot of the coumarinic acid glucoside solution was applied to each of thirty-six 1 × 11-in. strips of Whatman No. 1 filter paper, along a 9-in. line marked in the center of the strip. Similarly, *o*-coumaric acid glucoside was applied to 36 filter paper strips. All strips were air-dried, after which they were irradiated at a distance of approximately 12 in., with either a Mineralight model R53* lamp (peak intensity near 254 mμ) or a Gates MR4* lamp equipped with the TF8 tube (peak intensity near 360 mμ). Strips were irradiated in duplicate for the times indicated in Fig. 1.

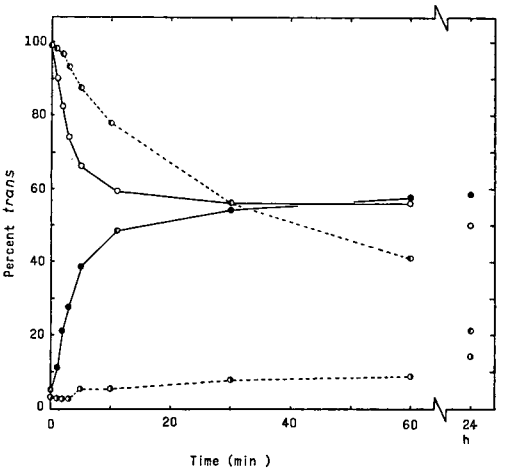


Fig. 1. Influence of short (peak near 254 mμ) and long (peak near 360 mμ) wavelength ultraviolet light upon coumarinic acid glucoside (*cis*) and *o*-coumaric acid glucoside (*trans*) dried on filter paper strips. See text for description of conditions used. Isomer at start: *cis*, ●———● (254 mμ), ◑ – – – ◑ (360 mμ); *trans*, ○———○ (254 mμ), ◐ – – – ◐ (360 mμ).

After treatment, strips were placed immediately in a freezer where they were stored for subsequent elution and assay. For parts of the study requiring more than 0.1 μmole of compound per strip, successive 0.1-ml applications of the glucoside solutions were made, with drying between applications, until the desired quantities had been applied.

All treated strips were eluted with water in a descending chromatography apparatus until 0.5 ml of eluate was collected from each. Eluates were diluted to 2.0 ml with water and were then assayed for the glucosides of coumarinic and *o*-coumaric acids as previously indicated. All work was done either in a dark room or in the laboratory under subdued light.

Results and discussion

As shown in Fig. 1, both sources of ultraviolet light were effective in interconverting the *cis* and *trans* isomers of β-D-glucosyl *o*-hydroxycinnamic acid on filter paper. Under the conditions used, the Mineralight source effected a much more rapid interconversion of the two isomers than did the longer wavelength Gates lamp. With the Mineralight, extensive isomerization occurred with exposures of 2 min or less.

* Mention of specific instruments is for identification only and does not imply endorsement by the U. S. Department of Agriculture.

Thus, unless precautions are taken, appreciable isomerization is likely to occur during routine examination of chromatograms with short wavelength ultraviolet light.

The equilibrium point of the isomerization differed with the wavelengths of the ultraviolet light used. Thus, with the Mineralight lamp, at equilibrium approximately 55% of the glucoside was present as the *trans* isomer. Although equilibrium apparently was not reached during the 24 h of treatment with the Gates lamp, available evidence indicates that at equilibrium, between 15% and 20% of the compound would be in the *trans* form.

As might be expected, extended irradiation with the Mineralight lamp was highly destructive to β-D-glucosyl *o*-hydroxycinnamic acid. Recovery measurements indicated that only about 40% of the compound remained intact at the end of the 24-h treatment. No significant destruction was observed as a result of irradiation with the Mineralight source for 30 min or less, or from any of the treatments with the Gates lamp. The glucosides did not appear to be hydrolyzed by irradiation.

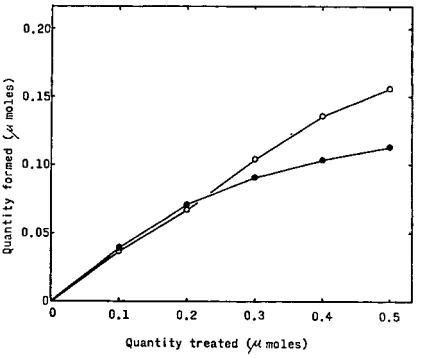


Fig. 2. Influence of concentration on extent of isomerization of β-D-glucosyl *o*-hydroxycinnamic acid dried on filter paper. Light source: Mineralight lamp (peak near 254 mμ). Treatment duration: 5 min. O———O *trans* to *cis* conversion; ●———● *cis* to *trans* conversion.

The extent of isomerization was influenced by the concentration of glucoside present on the paper when a 5-min duration of Mineralight irradiation was used (Fig. 2). The concentration effect was more pronounced in the case of coumarinic acid glucoside, indicating a difference in the spectral properties of the two glucosides in the dried condition. In aqueous solution, coumarinic acid glucoside is known to absorb maximally at 254 mμ, and *o*-coumaric acid glucoside at 270 mμ[2].

Department of Agronomy, University of Nebraska, Lincoln, Nebr. (U.S.A.)

ANDRIS KLEINHOFS
F. A. HASKINS
H. J. GORZ

1 F. A. HASKINS, L. G. WILLIAMS AND H. J. GORZ, *Plant Physiol.*, 39 (1964) 777.
2 T. KOSUGE, *Arch. Biochem. Biophys.*, 95 (1961) 211.
3 F. A. HASKINS AND H. J. GORZ, *Biochem. Biophys. Res. Commun.*, 6 (1961) 298.

Received October 8th, 1965

Papierchromatographische Trennung von ε-Aminocapronsäure und α-Aminosäuren sowie homologer Oligo-ε-aminocapronsäuren

ε-Aminocapronsäure spielt in jüngster Zeit als Proteasen-Hemmstoff und insbesondere als Antifibrinolytikum eine Rolle in Medizin und Biochemie[1–8]. Ihre Anwendung eröffnet auch im Hinblick auf das Krebsproblem interessante Aspekte (siehe dazu Lit. 9).

Bei *in vivo*-Versuchen mit ε-Aminocapronsäure benötigten wir zum chromatographischen Nachweis dieser unphysiologischen Aminosäure in Gewebeextrakten ein Lösungsmittelsystem, das ihre Erkennung neben den im Gewebe vorkommenden freien α-Aminosäuren gestattet. Nach unseren Erfahrungen mit dem Gemisch 2,4,6-Collidin/Boratpuffer pH 9 konnten wir mit diesem System eine gute Trennung erwarten. Ein ähnliches Gemisch (Collidin–Lutidin/Boratpuffer pH 9) wurde erst-

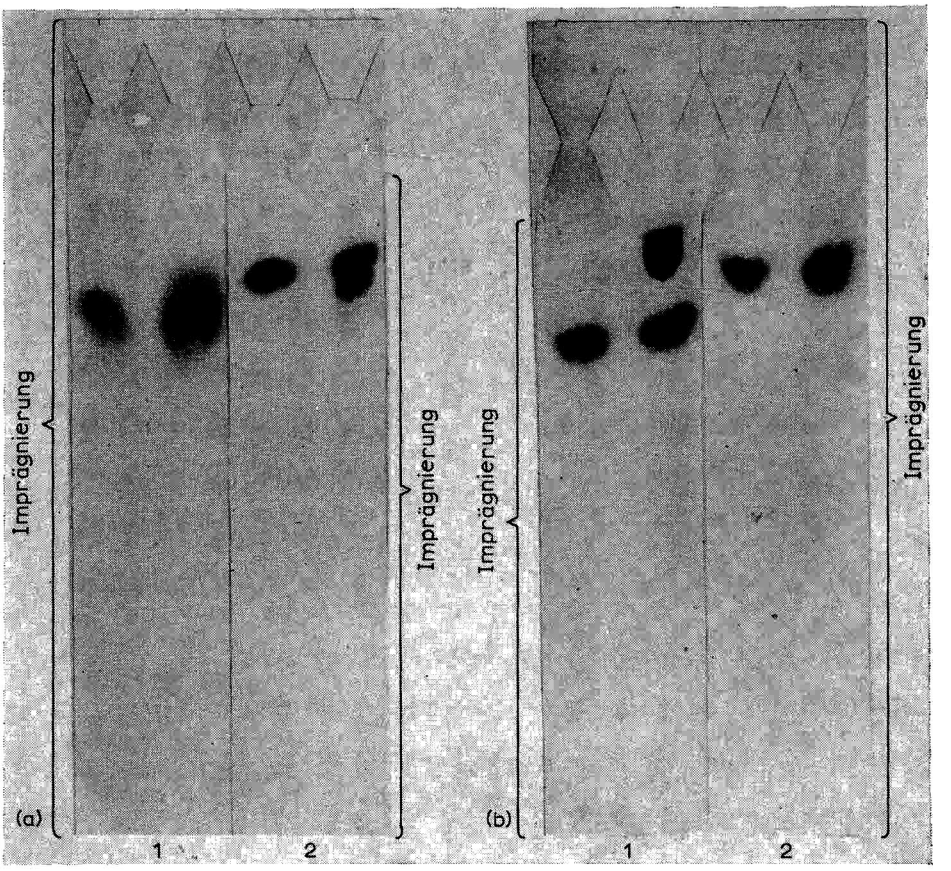


Fig. 1. (a) Trennung von ε-Aminocapronsäure und Threonin. Temperatur: 20 ± 2°. (1) Auf vollständig imprägniertem Papier. Links: ε-NH_2-Cap; rechts: ε-NH_2-Cap/Thre. Durchlauf: 72 St. (2) Auf teilweise imprägniertem Papier. Links: ε-NH_2-Cap; rechts: ε-NH_2-Cap/Thre. Durchlauf: 48 St. (b) Trennung von ε-Aminocapronsäure und Arginin. (1) Auf teilweise imprägniertem Papier. Durchlauf: 48 St. (2) Auf vollständig imprägniertem Papier. Durchlauf: 48 St.

malig von McFARREN[10] zur Trennung von α-Aminosäuren auf mit Puffer imprägniertem Papier benutzt (siehe dazu auch Lit. 11).

Experimenteller Teil

Zur Verwendung kam 2,4,6-Collidin, das durch Schütteln mit Brom, Natriumthiosulfat, stehen über NaOH und Destillation[12] gereinigt wurde. Boratpuffer vom pH 9 wurde nach HAIS[13] bereitet und bei Versuchstemperatur (20°) mit Collidin gesättigt. Die obere Phase wurde als Laufmittel benutzt. Zur Imprägnierung wurde das Papier mit dem Puffer getränkt und danach an der Luft bei Raumtemperatur getrocknet. Als Chromatographiepapier verwendeten wir FN 4 (VEB Spezialpapierfabrik Niederschlag, Erzgeb.) und 2043b mgl (Schleicher/Schüll). Die Chromatographie erfolgte nach einer modifizierten Keilstreifenmethode (siehe Fig. 1 und 2) absteigend. Freie Aminosäuren und Peptide wurden mit Ninhydrin, N-acylierte Derivate mit Chlor/*o*-Tolidin–KJ nach ZAHN UND REXROTH[14] sichtbar gemacht.

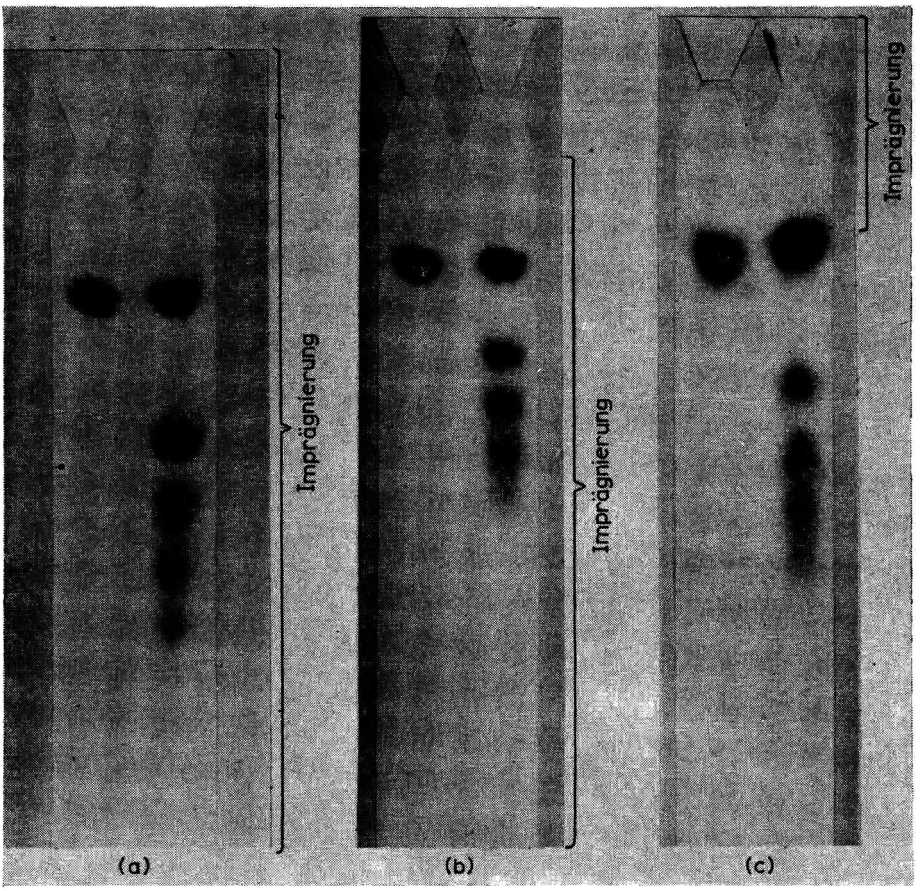


Fig. 2. Oligomerhomologen-Trennung auf verschieden imprägniertem Papier. Links jeweils ε-Aminocapronsäure; rechts von oben nach unten Mono-, Di-, Tri-, Tetra- und Penta-aminocapronsäure. Temperatur: 20 ± 2°. (a) Vollständige Imprägnierung. Durchlauf: 96 St. (b) Imprägnierung 3 cm vom Start. Durchlauf: 48 St. (c) Imprägnierung der ersten Laufstrecke bis 6 cm unterhalb vom Start. Durchlauf: 48 St.

Ergebnisse

Trennung von ε-Aminocapronsäure und α-Aminosäuren. Nach vollständiger Imprägnierung des Papiers liessen sich die meisten α-Aminosäuren neben ε-Aminocapronsäure eindeutig erkennen. Eine Trennung von ε-Aminocapronsäure, Threonin, Histidin, Arginin und Alanin war nicht möglich. In Tabelle I sind die entsprechenden R_F-Werte aufgeführt.

TABELLE I

R_F-WERTE VON ε-AMINOCAPRONSÄURE (ε-NH_2-CAP) UND α-AMINOSÄUREN
Temperatur: 20 ± 1°, 44 St.

Aminosäure	*FN4*	*2043b mgl*	*Aminosäure*	*FN4*	*2043b mgl*
Cys	0.03	0.03	Thre	0.14	0.13
Glu	0.04	0.04	Pro	0.16	0.14
Asp	0.04	0.04	Val	0.24	0.21
Lys	0.09	0.08	Met	0.28	0.25
Ser	0.10	0.09	Ileu	0.31	0.27
Gly	0.10	0.09	Leu	0.34	0.31
Arg	0.13	0.12	Phe	0.39	0.35
Ala	0.13	0.12	Tyr	0.48	0.43
ε-NH_2-Cap	0.13	0.11	Try	0.54	0.50
His	0.14	0.13			

Zur Verbesserung des Trenneffektes haben wir die Imprägnierung des Papiers derartig vorgenommen, dass die Streifen erst 3 cm vom Startfleck entfernt mit dem Puffer getränkt wurden. (Ein ähnliches Verfahren der teilweisen Imprägnierung wurde von CEREPKO[15] für die Chromatographie der cyclischen Oligoamide der ε-Aminocapronsäure in wässrg. Thymol auf mit methanol. Thymol getränktem Papier angewandt.) Die Aminosäuren durchlaufen so erst eine unimprägnierte Strecke mit grösserer Geschwindigkeit. In der darauffolgenden imprägnierten Zone wird ihr Lauf verlangsamt. Fig. 1a zeigt die so erfolgte Trennung von ε-Aminocapronsäure (ε-NH_2-Cap) und Threonin neben dem ungetrennten Gemisch ε-NH_2-Cap/Thre auf vollständig imprägniertem Papier, Fig. 1b die entsprechende Trennung von ε-NH_2-Cap/Arg. ε-Amino-capronsäure und Arginin lassen sich auch auf unimprägniertem Papier im gleichen System ausgezeichnet trennen. Eine Trennung von ε-NH_2-Cap/Ala und ε-NH_2-Cap/His war auch mit dieser Methode nicht möglich; ebensowenig führten andere Variationen der Imprägnierung (s.u.) zum Erfolg. Eine Unterscheidung dieser Aminosäuren kann aber in Butanol–Eisessig–Wasser Systemen erfolgen (siehe dazu HAIS[16]).

Trennung von Oligo-ε-aminocapronsäuren. Das 2,4,6-Collidin/Boratpuffer-System erwies sich als sehr wirksam zur Trennung oligomerer Peptide der ε-Aminocapronsäure. Die Trennung der Oligomeren erfolgte bisher entweder nach ZAHN UND HILDEBRAND[17] in den Systemen 2-Butanol–Ameisensäure–Wasser und 2-Butanol–wässrg. Ammoniak oder nach ROTHE[18] im System *n*-Butanol–Eisessig–Wasser (BEW). Die genannten

Systeme ergeben aber keine ausreichende Trennung der homologen Aminocapronsäuren. Wegen der relativ grossen R_F-Werte im BEW-System (Di-Aminocapronsäure 0.60, Tri-, Tetra- und Pentameres > 0.60) ist auch eine chromatographische Trennung im Durchlauf nicht möglich. Das 2,4,6-Collidin/Boratpuffer-Gemisch ergibt nach vollständiger Imprägnierung des Papiers für die oligomeren ε-Aminocapronsäuren folgende R_F-Werte (siehe Tabelle II).

TABELLE II

R_F-WERTE FÜR OLIGO-ε-AMINOCAPRONSÄUREN UND EINIGE DERIVATE

Z = $C_6H_5CH_2OCO$–, Cap = –$NH(CH_2)_5CO$– nach Lit. 17, Bz = $C_6H_5CH_2$–
Temp.: 20 ± 1°C, 40 St.

Substanz	*FN4*	*2043b mgl*	*Substanz*	*FN4*	*2043b mgl*
H(Cap)OH	0.13	0.11	Z(Cap)OH	0.72	0.70
$H(Cap)_2OH$	0.25	0.21	$Z(Cap)_2OH$	0.76	0.72
$H(Cap)_3OH$	0.30	0.27	$Z(Cap)_3OH$	0.78	0.74
$H(Cap)_4OH$	0.34	0.31	$Z(Cap)_4OH$	0.79	0.75
$H(Cap)_5OH$	0.38	0.35	$Z(Cap)_5OH$	0.80	0.78
			$Z(Cap)_6OH$	0.81	0.79
			$Z(Cap)_2OBz$	0.96	0.93
			$Z(Cap)_3OBz$	0.96	0.93

Die Substanzen laufen als gut abgegrenzte Flecke geringer Ausdehnung, was ihre Trennung sehr erleichtert. Nach 90-stündiger Chromatographie im Durchlauf liegen sämtliche Homologe bis zum Pentameren gut getrennt neben einander vor (Fig. 2a). Eine Auftrennung noch höherer Oligomere ist im angewandten System nicht mehr möglich. Hexa-aminocapronsäure verbleibt z.T. am Start, ein Teil läuft mit dem Pentameren.

Änderung der Imprägnierung führte auch hier zu einer Verbesserung der Trennung. Fig. 2b und c zeigen Mono- bis Penta-aminocapronsäure nach verschiedener Imprägnierung des Papiers. Eine gute Trennung ist hier bereits ohne Durchlauf des Lösungsmittelgemisches nach 48 Stunden erfolgt. Teilweise Imprägnierung erst 3 cm vom Start entfernt (Fig. 2b) ergibt kleine, gut abgesetzte Flecke. Lässt man die Substanzen aus einer imprägnierten Zone in eine unimprägnierte Zone laufen, dann vergrössern sich die Flecke mit der Laufgeschwindigkeit. Dieses Verfahren (Fig. 2c) liefert ebenfalls eine befriedigende Trennung. Als recht günstig erwies sich ferner eine streifenweise Imprägnierung (1 cm breite Streifen in jeweils 2 cm Abstand) quer zur Laufrichtung. Auf unimprägniertem Papier wurden im gleichen System nur die niederen Oligomeren scharf getrennt. Diese modifizierten Verfahren sind also dann zu empfehlen, wenn man auf die zeitraubende Durchlaufchromatographie verzichten will. Sie erlauben allerdings nicht in jedem Fall eine scharfe Trennung von Tetra- und Pentameren.

Trennung von Derivaten der ε-Aminocapronsäure. Das Collidin/Boratpuffer-System ermöglicht weiterhin die Erkennung von Carbobenzoxy(Z)-oligo-aminocapronsäuren neben Z-oligo-aminocapronsäurebenzylestern (R_F-Werte siehe Tabelle II).

Das ist wichtig zur Reinheitsprüfung der Z-Oligoamid-benzylestern nach der Kupplung von Z-Oligoamiden mit Oligoamid-benzylester mit Hilfe peptidchemischer Methoden[20]. In den bisher angewandten Systemen BEW, TCW (Tetrahydrofuran–Cyclohexan–Wasser)[19] und TE (Tetrachlorkohlenstoff–Eisessig)[21] konnte Z-Aminocapronsäure nicht von Z-Diaminocapronsäurebenzylester getrennt werden[20].

Für gewissenhafte technische Mitarbeit habe ich Frau G. HÄNOLD zu danken.

*Forschungstelle für Experimentelle Onkologie** KLAUS DIETER SCHWENKE
der Deutschen Akademie der Wissenschaften zu Berlin
in Potsdam-Rehbrücke (D.D.R.)

1 F. B. ABLONDI, J. J. HAGAN, M. PHILIPS UND E. C. DERENZO, *Arch. Biochem.*, 82 (1959) 153.
2 N. ALKJAERSIK, A. P. FLETCHER UND S. SHERRY, *J. Biol. Chem.*, 234 (1959) 832.
3 W. DOLESCHEL, W. AUERSWALD UND A. V. LÜTZOW, *Thromb. Diath. Haemorrhag.*, 8 (1962) 101.
4 W. AUERSWALD UND W. DOLESCHEL, *Wien. Med. Wschr.*, 112 (1962) 619.
5 J. D. GERATZ, *Biochim. Biophys. Acta*, 56 (1962) 599.
6 J. D. HUREAU, H. AUDHOUI, P. VAYRE UND E. VAIREL, *Compt. Rend. Soc. Biol.*, 158 (1964) 17.
7 H. W. LECHTENBERG, *Münch. Med. Wochschr.*, 106 (1964) 615.
8 A. DETTER, *Deut. Apotheker-Ztg.*, 104 (1964) 1185.
9 W. F. BALE, I. L. SPAR, R. L. GOODLAND UND M. J. IZZO, *Federation Proc.*, 20 (1961) 58.
10 E. F. MCFARREN, *Anal. Chem.*, 23 (1951) 168.
11 I. M. HAIS, in I. M. HAIS UND K. MACEK (Herausgeber), *Handbuch der Papierchromatographie*, Band I, 2. Aufl., Fischer, Jena, 1963, S. 527.
12 S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238;
ref. bei F. CRAMER, *Papierchromatographie*, 4. Aufl., Verlag Chemie, Weinheim, 1958, S. 44.
13 I. M. HAIS, in I. M. HAIS UND K. MACEK (Herausgeber), *Handbuch der Papierchromatographie*, Band I, 2. Aufl., Fischer, Jena, 1963, S. 959.
14 H. ZAHN UND E. REXROTH, *Z. Anal. Chem.*, 148 (1955) 181.
15 K. CEREPKO, *J. Chromatog.*, 9 (1962) 199.
16 I. M. HAIS, in I. M. HAIS UND K. MACEK (Herausgeber), *Handbuch der Papierchromatographie*, Band I, 2. Aufl., Fischer, Jena, 1963, S. 536.
17 H. ZAHN UND E. HILDEBRAND, *Chem. Ber.*, 90 (1957) 320; *ibid.*, 92 (1959) 1963.
18 M. ROTHE, *Habilitationsschrift*, Universität Halle, 1960.
19 M. ROTHE, *J. Polymer Sci.*, 30 (1958) 227.
20 K. D. SCHWENKE, *Faserforsch. Textiltech.*, 16 (1965) 61.
21 K. MACEK UND Z. PROCHAZKA, in I. M. HAIS UND K. MACEK (Herausgeber), *Handbuch der Papierchromatographie*, Band I, 2. Aufl., Fischer, Jena, 1963, S. 132;
G. REINISCH UND W. JÄGER, *Faserforsch. Textiltech.*, 16 (1965) 583.

Eingegangen den 27. September 1965

* Direktor: Dr. med. habil. F. SCHMIDT.

Resolution of steroid 2,4-dinitrophenylhydrazone isomers by thin-layer chromatography*

Geometrical isomerism of 2,4-dinitrophenylhydrazones of various ketones and aldehydes has been known for many years. Such isomerism of steroid derivatives has apparently not been encountered, although the possibility has been suggested[1]. Recently, in the separation of dinitrophenylhydrazones of steroids of biological origin multiple spots were detected on the thin-layer chromatograms of supposedly pure steroid derivatives. The finding prompted an investigation of these reaction products of steroid ketones with dinitrophenylhydrazine. Progesterone was selected initially as a "model" compound.

Experimental

The progesterone used was re-crystallized several times from *n*-hexane and methanol–water, and had a constant melting point of 128–130° (uncorr.). It was shown to be chromatographically pure in the paper partition systems cyclohexane–propylene glycol, cyclohexane–90% acetic acid and cyclohexane–85% methanol, and in the thin-layer systems methanol–methylene chloride (1:99) and cyclohexane–ethyl acetate (1:3).

2,4-Dinitrophenylhydrazone derivatives were prepared according to the general method of REICH[2] using well established conditions. The dinitrophenylhydrazine was freshly recrystallized as the hydrochloride prepared from Eastman reagent grade material by recrystallization from ethanol–conc. HCl (5:1) and ethanol–conc. HCl (19:1).

The products, 4-pregnene-3,20-dione-3-dinitrophenylhydrazone (progesterone-mono-DNPH) and 4-pregnene-3,20-dione-3,20-bis-dinitrophenylhydrazone (progesterone-bis-DNPH) were freed from contaminants by chromatography on neutral alumina columns[2] and silica gel thin-layer plates, and recrystallized from absolute ethanol and benzene–methanol. Progesterone-mono-DNPH showed a λ_{max} in chloroform at 388 mμ (E 20,100) while progesterone-bis-DNPH showed a λ_{max} at 380 mμ (E 49,050). These compounds had melting points of 215–216.5° and 284–286° (uncorr.), respectively, agreeing with literature values[3].

These steroid derivatives were chromatographed in a wide variety of thin-layer solvent systems on Silica Gel G and alumina plates to demonstrate geometrical isomerism (Table I).

Whereas most thin-layer systems showed only one symmetrical spot for each compound, certain systems resolved these areas into two closely running zones, of which the less polar predominated in all cases. Notable among these systems were benzene–chloroform and benzene–methylene chloride. Where separation of two isomers occurred, the less polar was orange and the more polar yellow. Upon elution, the difference in colour vanished. Spraying the plates with 0.01 N NaOH in 80% aqueous methanol showed no difference in colour between the two zones, in contrast to findings with keto-acid DNPH derivatives[4]. Cleavage of residues of chloroform extracts of these areas with hydrochloric acid–acetone[5] gave good yields of progeste-

* Supported by a grant, HD-01117-01, from the Public Health Service, U.S. Department of Health, Education, and Welfare.

TABLE I

R_F VALUES OF STEROID DERIVATIVES IN VARIOUS TLC SYSTEMS (SILICA GEL)

System	*Progesterone-mono-DNPH*	*Progesterone-bis-DNPH*	*Pregnenolone-20-DNPH*
Acetone–benzene (1:4)	0.72	0.75	0.48
Acetone–benzene (1:19)	0.56	0.70	0.18
Chloroform–benzene (3:1)	0.15, 0.25	0.29, 0.40	0.07
Acetone–chloroform–benzene (1:5:5)	0.58	0.68	0.26
Ether–chloroform (3:1)	0.72	0.80	0.50
Chloroform	0.36	0.53	0.09
Methylene chloride	0.42, 0.45	0.67	0.13
Methanol–chloroform (1:199)	0.55	0.69	0.22
Benzene–acetic acid (9:1)	0.52	0.67	0.37
Benzene–acetic acid (19:1)	0.42	0.57, 0.61	0.22
Chloroform–acetic acid (98:2)*	0.49	0.67	0.17
Chloroform–pyridine (98:2)*	0.40, 0.43	0.64	0.20
Benzene–ethyl acetate (1:2)	0.70	0.82	0.40

* These systems showed two solvent fronts due to breakdown of the mobile phase.

rone as the only detectable ketone in all cases. While the different forms of the dinitrophenylhydrazones were fairly stable in the dry state, standing for several days in chloroform solution resulted in rearrangement of the polar forms to the less polar forms, demonstrated by thin-layer chromatography in benzene–chloroform (1:3). No such rearrangement occurred with the less polar isomers, indicating ultimate equilibration to the less polar forms. This is in contrast with findings with keto-acid derivatives, where each isomer may be resolved into the two forms[4,6].

Ultraviolet spectroscopy in chloroform showed identical spectra for the progesterone-mono-DNPH isomers (λ_{max} 388–390 mμ) and for the progesterone-bis-DNPH isomers (λ_{max} 380 mμ). Infrared spectra, while essentially similar, did show differences for the isomeric forms, noticeably in the 1500–1535 cm^{-1}, 1300–1310 cm^{-1}, and fingerprint regions for progesterone-bis-DNPH and the 1600–1630 cm^{-1}, 1500–1540 cm^{-1}, 1420–1450 cm^{-1}, and fingerprint regions for progesterone-mono-DNPH.

The rapid rearrangement of the more polar isomer during attempts to recrystallize it for melting point determinations did not permit the use of standard techniques. Samples of each isomer of progesterone-bis-DNPH were prepared by allowing drops of concentrated chloroform eluates of freshly chromatographed material to evaporate spontaneously on cover slips.

Material which appeared crystalline under the microscope was carefully heated on a hot stage and the melting points observed. The more polar isomer had a melting point of 274.5–276° (uncorr.) while the less polar form had a m.p. of 282–283.5 (uncorr.). The latter melting point agrees with that of material crystallized from absolute ethanol, and shows that the crystals were of material completely rearranged to the less polar form.

Discussion

The lack of further resolution of the progesterone-bis-DNPH, as well as the failure to resolve 5-pregnen-3β-ol-20-one-20-DNPH (Table I) would indicate that for progesterone the 3-keto derivative only is capable of isomerism. The data of

GÖNDÖS *et al.*[7], showing isomerism of 17-ketosteroid-17-oximes, indicate that the apparent non-existence of C-20 isomerism may be a peculiarity of the 20-ketone, possibly due to the steric hindrance exerted by the steroid nucleus. This is supported by the resolution of 17β-acetoxy-4-androsten-17β-ol-3-one-3-DNPH, prepared by acetylating the testosterone-DNPH, into two isomers with R_F values of 0.28 and 0.33 in the system benzene–chloroform (1:1). Further work is being done in this area to elucidate this, among other questions.

This demonstration of isomerism in the C_{21} steroid-DNPH derivatives indicates the need for caution in interpreting chromatograms of such derivatives to avoid mistaking the isomeric forms of the derivative of one compound from biological samples for two dissimilar derivatives. As the application of this reaction for steroidal identification is widespread, involving steroids from such diverse sources as human pregnancy blood[8] and botanical extracts[9], such a need to distinguish between isomeric forms of the desired steroid derivative and products of unknown ketones is apparent.

Acknowledgements

We wish to acknowledge the able assistance of Mr. GEORGE F. SCRIMSHAW in the preparation and interpretation of the infrared spectra, the continued interest of Dr. SUMNER H. BURSTEIN, and the suggestions made by Dr. IAN BUSH and Dr. HOWARD RINGOLD, all of this Foundation.

Worcester Foundation for Experimental Biology, Shrewsbury, Mass. (U.S.A.)

DAVID J. WATSON
ELIJAH B. ROMANOFF

1 I. E. BUSH, *Chromatography of Steroids*, Pergamon Press, Oxford, 1961, p. 361.
2 H. REICH, S. J. SANFILIPPO AND K. F. CRANE, *J. Biol. Chem.*, 198 (1952) 713.
3 H. REICH, K. F. CRANE AND S. J. SANFILIPPO, *J. Org. Chem.*, 18 (1953) 822.
4 I. E. BUSH AND T. D. R. HOCKADAY, *J. Chromatog.*, 8 (1962) 433.
5 J. DEMAECKER AND R. H. MARTIN, *Nature*, 173 (1954) 266.
6 F. A. ISHERWOOD AND D. H. CRUICKSHANK, *Nature*, 173 (1954) 121.
7 G. Y. GÖNDÖS, B. MATKOVICS AND O. KOVACS, *Microchem. J.*, 8 (1964) 415.
8 D. OTT AND H. PELZER, *Klin. Wochschr.*, 36 (1958) 487.
9 R. D. BENNETT AND E. HEFTMANN, *Science*, 149 (1965) 652.

Received August 23rd, 1965

Isolation of 1-kestose and nystose by chromatography on a cation exchange resin

In studies of the transformation of sugars in sugar beets during storage it was desirable to determine 1-kestose [O-α-D-glucopyranosyl-(1 → 2)-O-β-D-fructofuranosyl-β-D-fructofuranoside] quantitatively by measuring the density of spots on chromatographic paper sheets developed by alkaline silver nitrate. A supply of 1-kestose was required as a standard because different sugars give different densities upon reaction with alkaline silver nitrate. Kestose is not commercially available, but preparations of several kestoses by paper chromatography and 1-kestose by carbon column chromatography have been described[1]. Recently BINKLEY isolated both 1-kestose and 6-kestose from cane final molasses by column chromatography with several different stationary phases[2]. BINKLEY AND ALTENBURG also isolated a tetrasaccharide fructosyl-1-kestose [O-α-D-glucopyranosyl-(1 → 2)-O-β-D-fructofuranosyl-(1 → 2)-O-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranoside] which was named nystose[3]. They obtained this sugar from a mixture of oligosaccharides by column chromatography with granular carbon as the adsorbent.

Procedures described for separating sugar mixtures[1] include paper chromatography[4], gas–liquid partition chromatography[5], thin-layer chromatography[6], gel filtration[7], and chromatography on ion exchange resins[8]. Chromatography on ion exchange resins appears to be one of the simplest methods available and it seemed worthwhile to describe the preparation of crystalline 1-kestose and nystose by this procedure.

Experimental

Preparation of oligosaccharide mixture. After the method of GROSS[2], 20 g of sucrose in 80 ml of water and 0.2 *M* phosphate buffer (2 ml) of pH 7 was incubated 48 h with a dialyzed solution of Taka-diastase* (10 g) in water (50 ml) for 24 h at 20°, boiling for 3 min stopped the reaction. The solution was freed from coagulated protein by filtration.

Paper chromatography. Paper chromatography was run in the organic layer of a mixture of 1-butanol, glacial acetic acid, and deionized water (4:1:5, v/v). A sample of 1 μl (about 10 % solids) was placed on Schleicher & Schüll No. 2043-B paper sheets and allowed to develop descending for 20 h. Air-dried papers were dipped in an indicator containing 1 ml of saturated silver nitrate in 200 ml of acetone, dried, and dipped in 0.5 % sodium hydroxide in ethanol. The sheets were air dried for about 1 h, then dipped first into saturated sodium thiosulfate in 60 % alcohol and then into 60% ethanol. The alcohol-washed chromatograms were dried in air.

Separation of oligosaccharides. A column 4.5 cm across × 167 cm high with a coarse fritted disc as a support was prepared. The resin bed (4.5 cm × 122 cm) was formed from slurry of 200–400 mesh Dowex 50W X4 (4% cross-linkage with divinylbenzene in the K^+ form). The resin was conditioned and eluted with 0.2 % potassium benzoate to prevent microbial growth and 2.5 mmoles each of glucose, sucrose, and

* Diastase, Pharmaceutical Grade (*Aspergillus oryzae*), Mann Research Laboratory, Inc., New York 6, N. Y. Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

raffinose (5.57 g) in 25 ml was separated at a flow rate of 0.5 ml/min to test the efficiency of the column. Two-ml fractions were collected and assayed by paper chromatography. The recovery of raffinose crystallized from ethanol–water was 99 %. Glucose and sucrose were separated, but no attempts were made to crystallize them.

An amount of 25 ml of oligosaccharide mixture (10 % solids) was added to the top of the column and allowed to drain to the top of the resin. The developing solvent, 0.2 % potassium benzoate at pH 7.3, was then added carefully, and elution commenced at a flow rate of 0.5 ml/min.

Two-ml fractions from 200 tubes were assayed by paper chromatography as described and were grouped as 1-kestose, tetrasaccharide(s), and higher oligosaccharides. Fig. 1 shows a typical fractionation for the preparation of 1-kestose and nystose. The 1-kestose and nystose fractions were combined separately and evaporated to 100 ml each.

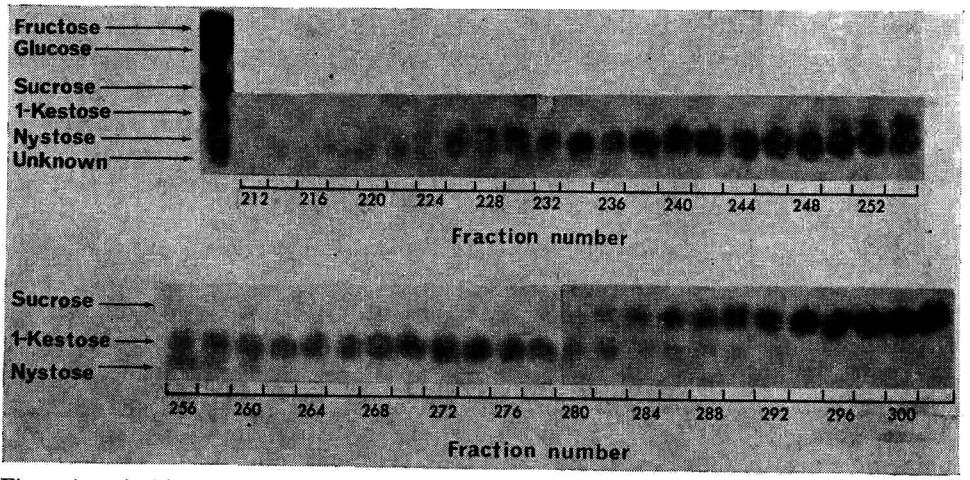


Fig. 1. A typical fractionation of the oligosaccharide mixture on an ion exchange resin and portions analyzed by paper chromatography.

Crystallization of 1-kestose and nystose. Potassium benzoate was removed from the sugar fractions by batch ion exchange in about 30 min at 5°. A magnetic stirrer agitated 1 g of 50–100 mesh Dowex 50W X8 (H^+) with 2 g of 60–80 mesh Permutit A (OH^-). The resin was filtered off and the filtrates containing the samples were adjusted to pH 7.5–8.0 with dilute ammonium hydroxide and evaporated to dryness at 40°. Three runs combined yielded 1.2 g of 1-kestose syrup and 1.3 g of tetrasaccharide syrup. The first syrup taken up in 2 ml of anhydrous methanol and seeded with crystals of authentic 1-kestose crystallized overnight. Upon recrystallization from water and anhydrous methanol, fine white crystals of 1-kestose were obtained. From the second syrup the tetrasaccharide, nystose, crystallized as elongated plates from anhydrous methanol[3]. The compounds gave single spots by paper chromatography. Recrystallized 1-kestose melted at 198–200°, $[\alpha]_D^{25}$ +28.4° (*c* water 2 %), $R_{glucose}$= 0.187. Recrystallized nystose melted at 130–133°, $[\alpha]_D^{25}$ +9.7° (*c* water 2 %), $R_{glucose}$= 0.104. Melting points are uncorrected and were measured on a Kofler hot stage. Both 1-kestose and nystose are non-reducing and gave negative RAYBIN tests[9].

Acknowledgement

We thank Dr. W. W. BINKLEY for supplying the 1-kestose seed crystals.

*Western Regional Research Laboratory**, R. M. MCCREADY
Albany, Calif. (U.S.A.) J. C. GOODWIN

1 R. L. WHISTLER AND M. L. WOLFROM, *Methods in Carbohydrate Chemistry*. Vol. 1. *Analysis and Preparation of Sugars*, Academic Press, New York, 1962.
2 W. W. BINKLEY, *Intern. Sugar J.*, 66 (1964) 46.
3 W. W. BINKLEY AND F. W. ALTENBURG, *Intern. Sugar J.*, 67 (1965) 110.
4 R. I. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press; New York, 1958.
5 C. T. BISHOP, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 10, Interscience Publishers, New York, 1962, p. 1.
6 E. STAHL, *Thin Layer Chromatography, A Laboratory Handbook*, Academic Press, New York, 1965.
7 P. FLODIN, *J. Chromatog.* 5 (1961) 103;
P. FLODIN AND K. ASPBERG, in T. W. GOODWIN (Editor), *Biological Structure and Function*, Vol. 1, Academic Press, New York, 1961, p. 345;
A. LUNDBLAD AND I. BERGGÅRD, *Biochim. Biophys. Acta*, 57 (1962) 129.
8 J. K. N. JONES, R. A. WALL AND A. O. PITTET, *Chem. Ind. (London)*, (1959) 1196; *Can. J. Chem.*, 38 (1960) 2285, 2290.
9 H. W. RAYBIN, *J. Am. Chem. Soc.*, 55 (1933) 2603; 59 (1937) 1402.

Received October 15th, 1965

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

Chromatography of some metal ions on paper impregnated with stannic phosphate

Papers impregnated with zirconium precipitates have been found useful for the separation of metal ions[1–3]. Preliminary studies[4] in these laboratories indicated that stannic phosphate (SP) papers would also offer similar possibilities. The present work was therefore undertaken to study the preparation and properties of SP papers for the separation of metal ions.

Experimental

15 × 3 cm strips were developed in 20 × 5 cm glass jars by the ascending technique and the solvent was allowed to migrate 11 cm from the point of application.

Test solutions

0.1 *M* solutions of metal ions were used. Bismuth and antimony chlorides were dissolved in 3 *N* and 2.7 *N* hydrochloric acid, respectively. Cerium, Ti, Pd and Y chlorides were dissolved in 1 *N* HCl. Gold chloride, platinum chloride, sodium tungstate and ammonium molybdate were dissolved in water. The selenium oxide was dissolved in water and made just alkaline with 1 *N* KOH. Tellurium metal was dissolved in conc. HNO_3 and the oxide thus obtained was dissolved in the least quantity of 1 *N* KOH. All other cations were taken as nitrates and dissolved in 0.1 *N* HNO_3. Usual methods for the detection of spots were used.

Preparation of ion-exchange papers

A 17.6% solution of stannic chloride pentahydrate and a 10% solution of phosphoric acid (89% w/w; sp. gr. 1.75) were prepared in water. Whatman No. 1 strips were dipped in the hot stannic chloride solution. The excess solution was removed by placing the strips on a filter sheet. The strips were then passed through hot phosphoric acid solution for a few seconds. The excess phosphoric acid was drained off. The strips were dried at room temperature and then washed with water till the wash water had a pH of 4. The strips were again dried and used as such.

The behaviour of group IB elements was studied on SP papers and on a SP column. The column was prepared in a 25 ml burette with an outer diameter of 1 cm. The burette was filled to a height of 2 cm with SP (50–100 mesh) prepared as described earlier[5]. It was found that Au(III) as $AuCl_4'$ was not retained on the SP column. Tests with a solution of Au(III) and Cu(II) (as $CuCl_2$) showed that only Cu(II) was retained.

Ag(I) and Cu(II) as sulphates were retained on the column and could be readily separated by eluting first with 0.1 *N* HCl to remove the Cu(II) and then with NH_4OH–NH_4Cl (4 *N*) to remove the silver.

Au(III) had the same R_F value on SP paper as on ordinary Whatman No. 1 paper (R_F 0.47) with 0.1 *N* or 1 *N* HCl.

When a mixture of Ag(I) and Cu(II) sulphates was developed on SP paper with 0.1 *N* HCl, Ag(I) stayed at a point of application and Cu(II) had an R_F value of 0.61. Thus the SP papers and SP column showed analogous behaviour, differing only with respect to adsorption on cellulose in the case of the paper.

TABLE I

R_F VALUES OF SOME METAL IONS ON PLAIN AND SP PAPERS

Metal ions	R_F *values*	
	Whatman No. 1 (0.1 N HCl)	*Stannic phosphate paper (0.1 N HCl)*
Pb(II)	0.83	0.09
Cu(II)	0.95	0.61
Fe(III)	0.96	0.05
Fe(II)	0.94	0.00
Ni(II)	0.94	0.73
Co(II)	0.95	0.50
UO_2(II)	0.95	0.06

To study the effect of impregnation a few metal ions were chromatographed on plain and SP papers. The results are summarized in Table I.

In order to investigate the utility of SP papers in qualitative analysis, numerous metal ions were chromatographed using HCl of different concentrations. The results are summarized in Table II. Hg(I), Al(III), Th(IV), Zr(IV) and Ti(IV) had an R_F of 0.00, in all the three HCl concentrations studied.

A few metal ions were also developed at higher acid concentrations. The results are summarized in Table III.

TABLE II

R_F VALUES OF SOME METAL IONS IN A FEW HCl SYSTEMS

Metal ions	R_F *values**		
	0.01 N HCl	*0.1 N HCl*	*1 N HCl*
Ag(I)	0.07	0.00	0.00
Tl(I)	0.16	0.24T	0.21T
Pb(II)	0.04	0.09	0.47T
Cu(II)	0.18	0.61	0.87
Cd(II)	0.09	0.71	0.87
Bi(III)	0.03	0.16T	0.87
Sb(III)	0.00	0.00	0.09
Fe(III)	0.03	0.05	0.06
Ni(II)	0.25T	0.73	0.92
Co(II)	0.07	0.50	0.89
UO_2(II)	0.05	0.06	0.35E
Pd(II)	0.87	0.90	0.94
Pt(IV)	0.90	0.94	0.94
Mo(VI)	0.04	0.03	0.07
W(VI)	0.00	0.00	0.13
Au(III)	0.50	0.47	0.47
Ce(III)	0.14T	0.15T	0.65T
La(III)	0.17T	0.32	0.65
Selenite	0.30T	0.30T	0.45E
Tellurite	0.00	0.03	0.12

* T = tails; E = elongated.

TABLE III

A FEW R_F VALUES IN SOME HCl AND $HClO_4$ SYSTEMS

Metal ions	R_F *values**					
	HCl			$HClO_4$		
	1 N	*3 N*	*5 N*	*1 N*	*3 N*	*5 N*
Sb(III)	0.09	0.82	0.87	0.04	0.17	0.20
Fe(III)	0.06	0.46E	0.71	0.04	0.13	0.15
Zr(IV)	0.00	0.00	0.00	—	—	—
Th(IV)	0.00	0.09	0.15	0.03	0.03	0.13
Ti(IV)	0.00	0.00	0.00	—	—	—
UO_2(II)	0.35E	0.54E	0.74E	0.37E	0.32E	0.32E

* E = elongated.

The R_F values of some metal ions, *e.g.* Pb(II), Cu(II), Cd(II), Bi(III), Ni(II), Co(II), Pd(II), U(VI), Ce(III), La(III) and Y(III) increase with increase in hydrochloric acid concentration (Table II). However, an increase in the perchloric acid concentration has no effect upon them (Table III). This shows that the change in R_F values is probably due to complex formation.

Acknowledgements

The authors are grateful to Prof. A. R. KIDWAI, Head of the Department of Chemistry for his interest and encouragement. One of us (S.Z.Q.) thanks the Government of India (MSRCA), for financial assistance.

The Chemical Laboratories,
Aligarh Muslim University,
Aligarh, U.P. (India)

MOHSIN QURESHI
SAIDUL ZAFAR QURESHI

1 G. ALBERTI AND G. GRASSINI, *J. Chromatog.*, 4 (1960) 83.
2 J. M. P. CARBAL, *J. Chromatog.*, 4 (1960) 86.
3 M. J. NUNES DA COSTA AND M. A. S. JERÓNIMO, *J. Chromatog.*, 5 (1961) 546.
4 K. HUSAIN, *M. Sc. Thesis*, A.M.U., Aligarh, 1964.
5 Y. INOUE, *Bull. Chem. Soc. (Japan)*, 36 (1963) 1316.

First received June 29th, 1965
Modified October 26th, 1965

J. Chromatog., 22 (1966) 198–200

The adsorption of metal ions on exchangers with phosphoric groups from perchloric acid solutions

The curious increase of adsorption of metal ions on Dowex-50 with an increase in the concentration of $HClO_4$ was first described by NELSON *et al.*[1], and we have since shown[2,3] that it occurs also on cellulose sulphonic exchangers and from solutions of

sodium perchlorate. In the present communication we have extended the study of the adsorption of metal ions from $HClO_4$ to two further exchangers, zirconium phosphate and cellulose phosphate. The two differ in their behaviour with $HClO_4$; zirconium phosphate seems to break down at higher $HClO_4$ concentrations resulting in a desorption of the metal ions, while on cellulose phosphate there is the same increase of adsorption as on sulphonic exchangers. We have investigated a large number of metal ions on cellulose phosphate as there are several analytical possibilities. However, it should be kept in mind that cellulose phosphate also decomposes in higher acidities and is only stable if the development time is rather short (less than one hour or so).

Experimental

(*a*) *Zirconium phosphate.* Macherey, Nagel & Co. paper holding 15 % zirconium phosphate was used in this work, 11.7 *N* $HClO_4$ (Carlo Erba) was employed and all other concentrations were prepared by diluting it with water. All chromatograms were developed by the ascending method and the spots detected with the usual spray reagents. The results with a number of metal ions are shown in Fig. 1.

The only ions which do not adsorb on cellulose (Pb and Bi do adsorb) and which are relatively strongly adsorbed on zirconium phosphate are Fe(III) and UO_2^{2+}. After an initial increase to 1.7 *N*, the R_F values remain almost constant up to 5.8 *N* and then both ions desorb rapidly. Most other metal ions do not adsorb above about 1 *N* $HClO_4$[4] and hence little further data can be obtained about them. However, comparing the results for Fe(III) and UO_2^{2+} with those on cellulose phosphate (below), it seems likely that the desorption is due to the properties of the adsorbant (*i.e.* its destruction) and not to the alteration of the ionic state of the metal ions.

(*b*) *Cellulose phosphate.* Whatman cellulose phosphate paper P 20 was converted to the hydrogen form by washing with 1 *N* HCl and water and then drying. Fig. 2 shows the R_F values of numerous metal ions in up to 7 *N* $HClO_4$.

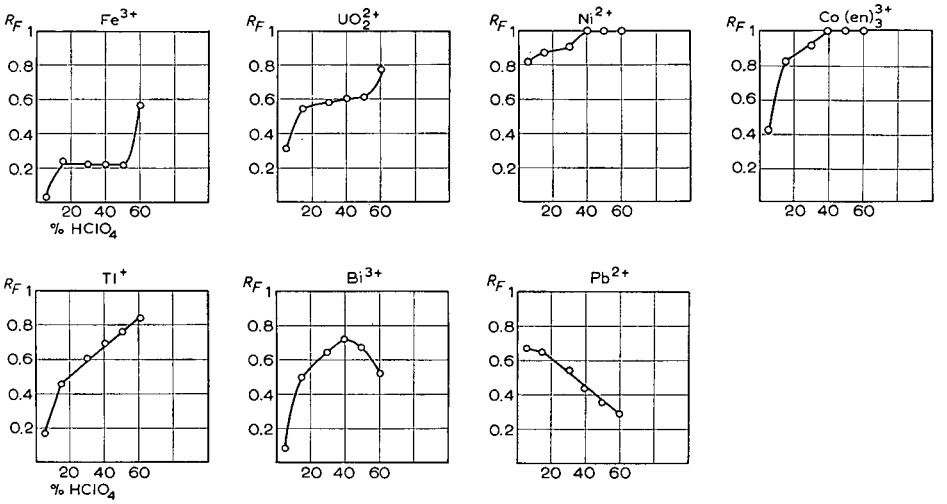


Fig. 1. R_F values of some metal ions plotted against the $HClO_4$ concentration on Macherey, Nagel & Co. zirconium phosphate paper (15 % ZP). The $HClO_4$ concentration is expressed in % (100 % = 11.7 *N*).

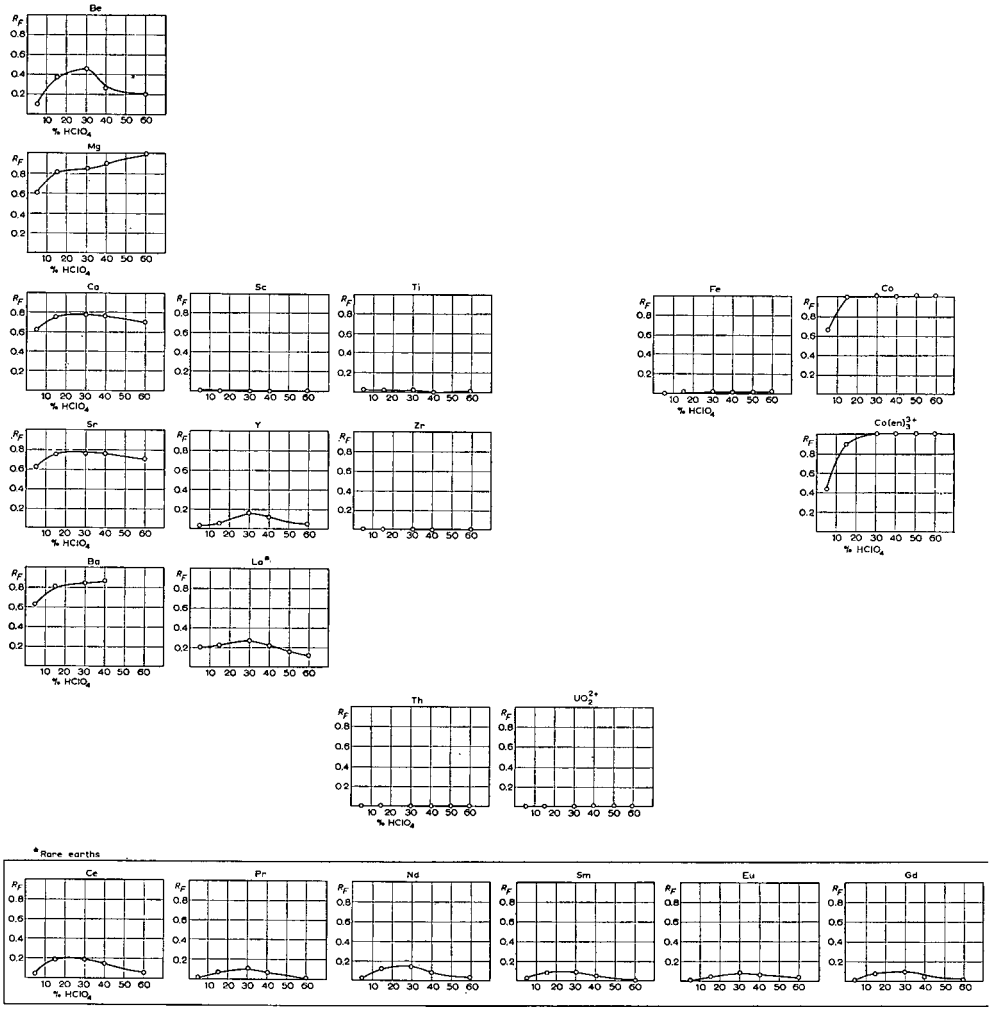
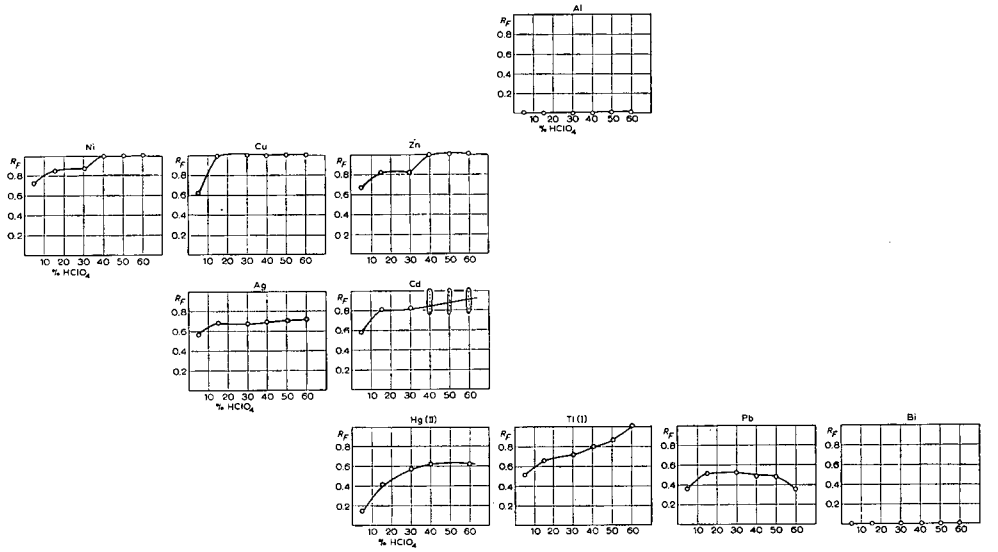


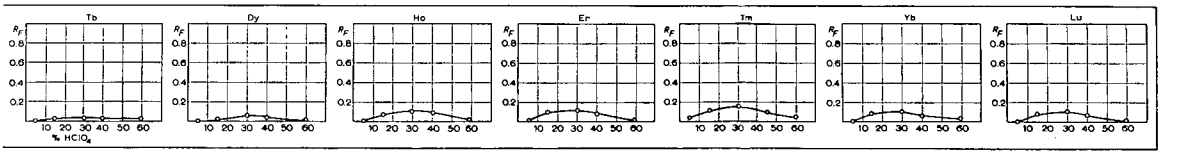
Fig. 2. R_F values of some metal ions on Whatman cellulose phosphate paper P 20. The $HClO_4$ concentration is expressed in % (100% = 11.7 N).

Most transition metals are only little adsorbed while the tetravalent ions such as Zr, Th and Ti remain near R_F 0. The rare earths, yttrium and beryllium show the bell shaped R_F curve which is typical for adsorptions from $HClO_4$ on sulphonic exchangers with a maximum around 3.5 N.

There seem to be well defined R_F differences between the rare earths, the light ones being less adsorbed than the intermediate ones, however, as stated above this cannot be exploited readily with a lengthy development owing to the unstable nature of cellulose phosphate.

The very strong adsorption of Fe(III) and Al(III) and the weak adsorption of $Co(en)_3^{3+}$ emphasises again that the adsorption does not bear much relation to the charge of the metal ion as to complexation with the phosphoric groups.





This work was carried out under a Contract (No. 265/RB) with the International Atomic Energy Agency (Vienna).

Laboratorio di Cromatografia del C.N.R., M. LEDERER
Istituto di Chimica Generale ed Inorganica, Rome (Italy) L. OSSICINI

1 F. NELSON, T. MURASE AND K. A. KRAUS, *J. Chromatog.*, 13 (1964) 503.
2 M. LEDERER AND F. SARACINO, *J. Chromatog.*, 15 (1964) 80.
3 F. SARACINO, *J. Chromatog.*, 17 (1965) 425.
4 G. ALBERTI, F. DOBICI AND G. GRASSINI, *J. Chromatog.*, 8 (1962) 103.

Received September 21st, 1965

Bibliography Section

Paper Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

KOWALCZYK, J. AND NOWOSZ-ARKUSZEWSKA, I.: (The relation between chromatographic behavior and chemical structures of organic compounds). *Wiadomosci Chem.*, 18 (1964) 291–311; *C.A.*, 62 (1965) 13887e.

VACEK, Z., ŠŤOTA, Z. AND STANĚK, J.: pH-Chromatography of chlorinated phenols. *J. Chromatog.*, 19 (1965) 572–579 — phenol and 19 chlorophenols were studied.

3. TECHNIQUES I

SAINI, A. S., SINGH, I. D. AND POONIA, N. S.: Method to reduce solvent flow in paper chromatography. *Indian J. Physiol. Pharmacol.*, 8 (1964) 93–99; *C.A.*, 62 (1965) 13817h.

6. ALCOHOLS

BROWNELL, H. H.: The detection of compounds containing hydroxyl groups on paper chromatograms. *J. Chromatog.*, 19 (1965) 556–563 — 77 compounds were tested.

TIKHOMIROVA, G. P. AND KALITVYANSKAYA, V. I.: (Paper chromatographic determination of phytol). *Zh. Analit. Khim.*, 20 (1965) 276–277; *C.A.*, 62 (1965) 15433f.

7. PHENOLS

GLABISZ, U.: (Separation and identification of products of the chlorine dioxide–phenol reaction by adsorption paper chromatography). *Chem. Anal. (Warsaw)*, 10 (1965) 873–879.

HALMEKOSKI, J. AND HANNIKAINEN, H.: (R_M values of hydroxy-substituted benzoic acids and partition coefficients of the acids between water and aliphatic alcohols). *Suomen Kemistilehti*, Pt. B, 37 (1964) 221–226; *C.A.*, 62 (1965) 15404a.

NIMZ, H.: Isolierung des Dimethyl-pyrogallylglycerins. *Chem. Ber.*, 98 (1965) 3153–3159 — R_F values of 40 phenolic aldehydes, alcohols and acids.

POSTAWKA, A. AND PRAJER-JANCZEWSKA, L.: (Paper chromatography of 2-hydroxy-(bromo)-naphthalenes. XIII. Bromonaphthyl esters). *Chem. Anal. (Warsaw)*, 10 (1965) 977–982.

THIEME, H.: Die Phenylglykoside der Salicaceen. 5. Untersuchungen über die Glykosidspektren und den Glykosidgehalt der mitteldeutschen Salixarten. *Pharmazie*, 20 (1965) 570–574.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

BIRKOFER, L., KAISER, C. AND BECKER, F.: Bignonosid, ein acyliertes Flavon aus *Catalpa bignonioides*. *Z. Naturforsch.*, 20b (1965) 923.

LEWAK, S.: Leucocyanidin of the leaves of hawthorn (*Crataegus oxyacantha*). *Roczniki Chem.*, 38 (1964) 1773–1780; *C.A.*, 62 (1965) 14615c.

TIKOTSKAYA, K. M. AND POPOVA, L. N.: (Determination of rutin in some biological materials). *Vopr. Pitaniya*, 22, No. 4 (1963) 71–76; *Anal. Abstr.*, 12 (1965) 1299.

9. OXO COMPOUNDS

BENTLEY, R. AND GATENBECK, S.: Naphthoquinone biosynthesis in molds. The mechanism for formation of mollisin. *Biochemistry*, 4 (1965) 1150–1156.

DABROWSKI, B. AND MALEK, B.: (Isolation and chromatographic separation of crude glucofrangulin from *Rhamnus frangula* bark). *Acta Polon. Pharm.*, 21 (1964) 281–286; *C.A.*, 62 (1965) 15990f.

PEN, R. Z.: (Modification of the micromethod for alkaline nitrobenzene oxidation of lignified materials). *Zh. Analit. Khim.*, 20 (1965) 277–278; *C.A.*, 62 (1965) 15434c.

10. CARBOHYDRATES

AKASHI, S., GOTO, H., SASAKI, M., TSUYAMA, A., SAI, Y. AND KUNO, T.: Chemical, immunochemical and electromicroscopical studies on the polysaccharide produced by a certain mucous strain of *E. coli*. *Bull. Soc. Chim. Biol.*, 47 (1965) 1011–1031.

ASPINALL, G. O. AND FRASER, R. N.: Plant gums of the genus *Sterculia*. II. *Sterculia caudata* gum. *J. Chem. Soc.*, (1965) 4318–4325.

ASPINALL, G. O., FRASER, R. N. AND SANDERSON, G. R.: Plant gums of the genus *Sterculia*. III. *Sterculia setigera* and *Cochlospermum gossypium* gums. *J. Chem. Soc.*, (1965) 4325–4329.

BARRY, G. T.: Un nouveau mucopolyoside des enterobactéries. *Bull. Soc. Chim. Biol.*, 47 (1965) 529–544.

GROLLMAN, A. P., HALL, C. W. AND GINSBURG, V.: Biosynthesis of fucosyllactose and other oligosaccharides found in milk. *J. Biol. Chem.*, 240 (1965) 975–981.

HU, C. L., RENDIG, V. V. AND MCCOMB, E. A.: Evidence for the ninhydrin-positive reaction of some ketoses. *J. Chromatog.*, 19 (1965) 622–623.

TOMASHEVICH, G. N. AND KOPYLOVA, V. D.: (Paper chromatography in the analysis of compounds containing carbohydrates and encountered in the restoration of works of art and monuments of material culture). *Molekul. Khromatog., Akad. Nauk SSSR, Inst. Fiz. Khim.*, (1964) 152–157; *C.A.*, 62 (1965) 13809f.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

FALKOWSKI, L., ZIMINSKI, T., MECHLINSKI, W. AND BOROWSKI, E.: (Synthesis of 1-methylpentadecanedicarboxylic acid). *Roczniki Chem.*, 39 (1965) 225–229; *C.A.*, 62 (1965) 16043c — PC and TLC.

GERCHUK, M. P., VALOVA, V. D. AND PORTNYKH, N. V.: (Reversed-phase, partition and thin-layer chromatography for the identification and characterization of nordihydroguaiaretic acid). *Molekul. Khromatog., Akad. Nauk SSSR, Inst. Fiz. Khim.*, (1964) 106–111; *C.A.*, 62 (1965) 13848f — PC and TLC.

REEVES, H. C. AND AJL, S. J.: Enzymatic synthesis and metabolism of hydroxypyruvic aldehyde. *J. Biol. Chem.*, 240 (1965) 569–573.

SAINI, A. S.: New solvents for paper chromatography of phenolic acids. *J. Chromatog.*, 19 (1965) 619–622 — R_F values for 26 acids in 5 solvent systems at various temperatures.

SOKOL'SKAYA, E. V., VISNEVSKAYA, G. L. AND EGOROV, A. S.: (Identification of esters and aldehydes by paper chromatography). *Tr. Ukr. Nauchn.-Issled. Inst. Spirt. i Likero-Vodochn. Prom.*, No. 9 (1964) 38–44; *C.A.*, 62 (1965) 15431c.

STECK, W. AND WENDER, S. H.: Paper chromatography in benzene–acetic acid–water as an aid in estimating free hydroxyl groups in phenolic compounds. *J. Chromatog.*, 19 (1965) 564–571 — R_F values for 86 compounds.

STUDNITZ, W. VON: Vergleichende Studien über die 3-Hydroxy-4-methoxy- und 3-Methoxy-4-hydroxymandelsäure. *Clin. Chim. Acta*, 12 (1965) 330–334.

THOMAS, H. AND DIRSCHERL, W.: 4-Hydroxy-3-methoxy-phenylglyoxylsäure als Metabolit von Adrenalin und Noradrenalin. *Z. Physiol. Chem.*, 339 (1964) 115–121 — Whatman 542.

ZANE, A. AND WENDER, S. H.: A synthesis of 1-O-, 4-O- and 5-O-feruloylquinic acids. *Chem. Ind. (London)*, (1965) 1034–1035.

11b. Lipids and their constituents

GRAY, G. M.: The isolation and partial characterization of the glycolipids of BP8/C3H ascites-sarcoma cells. *Biochem. J.*, 94 (1965) 91–98 — PC and TLC.

See also TLC section.

13. STEROIDS

EXLEY, D.: Urinary excretion of C-20-reduction products of corticosterone. *Biochem. J.*, 94 (1965) 271–278.

FEHÉR, T.: (Über die verbesserte Trennung bei der Papierchromatographie der Steroide). *Magyar Kem. Folyoirat*, 70 (1964) 318–325; from *Z. Anal. Chem.*, 213 (1965) 316.

FEHÉR, T.: Structural analysis of C_{19}-steroids by means of chromatography. *J. Chromatog.*, 19 (1965) 551–555 — ΔR_{Mr} for 5 reactions on paper and thin layer; 38 compounds were studied.

HAIS, I. M. AND MYANT, N. B.: Photolysis of cholesterol during biological experiments. *Biochem. J.*, 94 (1965) 85–90.

HUSMANN, F. AND KLEINFELDER, H.: Eine neue Methode zur Bestimmung der hexahydrierten Cortisol-Metaboliten. *Z. Physiol. Chem.*, 339 (1964) 75–84.

PASQUALINI, J. R.: Métabolisme des ester-sulfates de corticostéroides. *Bull. Soc. Chim. Biol.*, 47 (1965) 471–480.

SHARMA, D. C., RAHEJA, M. C., DORFMAN, R. I. AND GABRILOVE, J. L.: Studies on a feminizing human adrenal tumor. I. Biosynthesis *in vitro* of steroid hormones from pregnenolone-4-^{14}C and progesterone-4-^{14}C. *J. Biol. Chem.*, 240 (1965) 1045–1053 — PC and TLC.

14. STEROID GLYCOSIDES AND SAPONINS

BLEIER, W., KUBELKA, W., WEISS, EK. AND WICHTL, M.: Lokunjosid, ein Hauptglykosid aus Blättern, Blüten und Samen von *Convallaria majalis* L. *Pharm. Acta Helv.*, 40 (1965) 554–563.

15. TERPENE DERIVATIVES

DALIDOWICZ, J. E. AND MCDONALD, H. J.: Site of the *in vitro* inhibition of cholesterol biosynthesis by tolbutamide and phenethylbiguanide. *Biochemistry*, 4 (1965) 1138–1143.

GIANNOTTI, C.: (Separation by paper partition chromatography and ion-exchange chromatography of the azulenes obtained by gas phase dehydrogenation of azulenogenic and sesquiterpenes). *Compt. Rend.*, 259 (1964) 823–826; *C.A.*, 62 (1965) 15400c.

JENSEN, A.: (Paper chromatography of carotenes and carotenoids). *Wiss. Veroeffentl. Deut. Ges. Ernaehrung*, 9 (1963) 119–127, Discussion 128; *C.A.*, 62 (1965) 15990c.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

FLOCH, H.: (R_F values of some catechol amines and their derivatives). *Biol. Med. (Paris)*, 53 (1964) 36; from *J. Chromatog., Chromatog. Data*, 19 (1965) D 4 — R_F values of 8 acids and 9 amines.

KLÖCKING, H. P., MARKWARDT, F. AND RICHTER, M.: Zur Analytik von *p*-Aminomethylbenzoesäure (PAMBA), einem neuen Antifibrinolyticum. *Pharmazie*, 20 (1965) 554–560 — PC and TLC.

18. AMINO ACIDS

BOYLAND, E. AND NERY, R.: The metabolism of urethane and related compounds. *Biochem. J.*, 94 (1965) 198–208 — PC and TLC; urethane and its potential and actual metabolites; 15 compounds.

CALAM, D. H.: Some new compounds in the lens. *Exptl. Eye Res.*, 1 (1962) 436–442.

FUJIWARA, Y.: Effect of copper in filter-paper on chromatographic studies of amino acids. *Oita Daigaku Gakugei Gakubu Kenkyu Kiyo, Shizen Kogaku*, No. 2 (1963) 9–14; *Anal. Abstr.*, 12 (1965) 1888.

KAGAN, H. K., MANNING, L. R. AND MEISTER, A.: Stereospecific synthesis of α-methyl-L-glutamine by glutamine synthetase. *Biochemistry*, 4 (1965) 1063–1068.

OERIU, S. AND TANASE, I.: Séparation et détermination de la proline, de l'hydroproline et de 3-hydroxyproline des produits biologiques par chromatographie sur papier. *Bull. Soc. Chim. Biol.*, 47 (1965) 979–985.

ROBINSON, N. AND WILLIAMS, C. B.: Amino acids in human brain. *Clin. Chim. Acta*, 12 (1965) 311–317.

SATWEKAR, K. AND RADHAKRISHNAN, A. N.: Free and bound amino acids in human urine from normal subjects and patients with tropical sprue. *Clin. Chim. Acta*, 12 (1965) 394–402.

VIGOUROUX, E. AND JOST, A.: Séparation sur résine échangeuse d'ions ou par chromatographie sur papier des composés iodés plasmatiques, marqués par ^{131}I, chez le lapin. *Bull. Soc. Chim. Biol.*, 47 (1965) 1053–1071.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

BISERTE, G. AND HAN, K.: Peptides résiduels de l'hydrolyse enzymatique de la gliadine du blé. *Bull. Soc. Chim. Biol.*, 47 (1965) 597–610.

CHEFTEL, R. I., CLOAREC, L., MORETTI, J. AND JAYLE, M. F.: Structure des glycopeptides obtenus par protéolyse de l'haptoglobine humaine. *Bull. Soc. Chim. Biol.*, 47 (1965) 385–393.

STEGEMANN, H., BERNHARD, G. AND O'NEIL, J. A.: Endgruppen von Anaphylatoxin. Einfache Sequenzanalyse von Carboxyl-endständigen Aminosäuren. *Z. Physiol. Chem.*, 339 (1964) 9–13 — R_F values of 38 DNP-amino acids.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BOLLACK, C., KEITH, G. AND EBEL, J. P.: Alcoylation des acides nucléiques. I. Étude comparée de la méthylation des acides ribonucléiques en solution aqueuse et organique. *Bull. Soc. Chim. Biol.*, 47 (1965) 765–785.

DUSPIVA, F. AND HANSEN-DELKERSKAMP, E.: Die Thymidin-Phosphorylierung bei verschiedenen Entwicklungsstadien von *Bufo bufo* L. *Z. Naturforsch.*, 20b (1965) 582–587 — Whatman DE 20 paper.

DUVAL, J. AND EBEL, J. P.: Halogénation des acides nucléiques. II. Bromuration des acides ribonucléiques de levure en milieu diméthylformamide. *Bull. Soc. Chim. Biol.*, 47 (1965) 787–806.

FURLONG, N. B.: A simple and rapid method for determining the chain length of oligonucleotides randomly labeled with P^{32}. *Anal. Biochem.*, 12 (1965) 349–356.
IMAI, K. AND HONJO, M.: Synthesis of 5-substituted pyrimidine nucleosides. *Chem. Pharm. Bull. (Tokyo)*, 13 (1965) 7–16; *C.A.*, 62 (1965) 16359g — PC and TLC.
KAUSS, H.: Uridine diphosphate rhamnose from the golden brown alga *Ochromonas*. *Biochem. Biophys. Res. Commun.*, 18 (1965) 170–173.
LINN, S. AND LEHMAN, I. R.: An endonuclease from *Neurospora crassa* specific for polynucleotides lacking an ordered structure. II. Studies of enzyme specificity. *J. Biol. Chem.*, 240 (1965) 1294–1304.
MERLEVEDE, M.: (Chromatography of barbiturates). *Pharm. Tijdschr. Belg.*, 42 (1965) 1–8; *C.A.*, 62 (1965) 15989f.
NIKAIDO, H.: Biosynthesis of cell wall polysaccharide in mutant of *Salmonella*. III. Transfer of L-rhamnose and D-galactose. *Biochemistry*, 4 (1965) 1550–1561 — PC and TLC.
SCHWARZ, E.: Zur Genese von Imidazol-Derivaten der wasserlöslichen Substanzen epidermaler Hornschicht. *In-vitro*-Untersuchungen mit (2-^{14}C) adenin. *Z. Physiol. Chem.*, 339 (1964) 110–114.
VENNER, H.: Untersuchungen an Nucleinsäuren. IX. Stabilität der N-glykosidischen Bindung von Nucleosiden. *Z. Physiol. Chem.*, 339 (1964) 14–27.

22. ALKALOIDS

BOISSIER, J. R. AND COMBES, G.: (Tarennine, a pharmacologically active alkaloid of the *Rubiaceae*). *Fr. Pat.*, 1,391,764 (Cl. A 61*k*, C 07*c*), March 12 (1965), Appl. Nov. 22 (1963) 11 pp.; *C.A.*, 62 (1965) 15996g.
KALBERER, P.: Breakdown of caffeine in the leaves of *Coffea arabica* L. *Nature*, 205 (1965) 597–598.
KHOI, D.-H.: (Analytical chemistry of the alkaloid rotundine of *Stephania rotunda*). *Pharm. Zentralhalle*, 103 (1964) 99–108; *Anal. Abstr.*, 12 (1965) 2446 — PC and TLC.
MASAMUNE, T., TAKASUGI, M. AND MORI, Y.: C-Nor-D-homosteroids and related alkaloids. III. C-9 configuration of jervine and related alkaloids. *Tetrahedron Letters*, (1965) 489–495; *C.A.*, 62 (1965) 14774c.
MORIMOTO, H. AND OSHIO, H.: (Alkaloids. V. Components of *Lespedeza bicolor* var. *japonica*. 1. Lespedamine, a new alkaloid). *Ann. Chem.*, 682 (1965) 212–218; *C.A.*, 62 (1965) 14740a.
ROPER, E. C., BLOMSTER, R. N., FARNSWORTH, N. R. AND DRAUS, F. J.: Studies on alkaloid detecting reagents. II. Stability and sensitivity of modified Dragendorff's reagents. *Planta Med.*, 13 (1965) 98–103; *C.A.*, 62 (1965) 15990g.
STEINEGGER, E. AND WICKY, K.: Die Alkaloide von *Genista lusitanica* L. = *Echinospartum lusitanicum* (L.) Rothm. *Pharm. Acta Helv.*, 40 (1965) 610–619.
VOLKOVA, A. AND KOLUSHEVA, A.: (Determination of galanthamine in drugs). *Farmatsiya (Sofia)*, 14, No. 6 (1964) 32–36; *C.A.*, 62 (1965) 15991g.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

BARTHOLINI, G., DA PRADA, M. AND PLETSCHER, A.: Inhibition of 5-hydroxytryptamine liberation from blood platelets by reserpine. *Nature*, 205 (1965) 400–401.
CHAYKIN, S., DAGANI, M., JOHNSON, L. AND SAMLI, M.: The fate of nicotinamide in the mouse. Urinary metabolites. *J. Biol. Chem.*, 240 (1965) 932–938.
CHIERICI, L. AND ARTUSI, G. C.: (Chromatographic study of pyrrole acids. Paper chromatography of C-pyrrolecarboxylic acids). *Ric. Sci., Rend., Sez. A*, 6 (1964) 170–172; *C.A.*, 62 (1965) 13849d.
DUBOVSKÝ, J. AND DUBOVSKÁ, E.: Urinary imidazolelactic acid in normal subjects and in liver cirrhosis. *Clin. Chim. Acta*, 12 (1965) 360–362.
LULY, A. M. AND SAKODYNSKY, K.: A paper chromatographic study of aza-heterocyclic hydrocarbons using aqueous solvents. *J. Chromatog.*, 19 (1965) 624–629 — R_F values in 4 solvent systems for 21 compounds.
MOSEBACH, K. O., HUEP, W. W. AND RIECK, G.: Über den Abbau von Imidazolylessigsäure im Säugetierorganismus. *Z. Physiol. Chem.*, 339 (1964) 150–156.

24. ORGANIC SULPHUR COMPOUNDS

GALSWORTHY, S. B. AND METZENBERG, R. L.: Sulphur-containing metabolites secreted by an ethionine-resistant mutant of *Neurospora*. *Biochemistry*, 4 (1965) 1183–1188.
WAGNER, G. AND SCHMIDT, R.: Über die Synthese von Glucosiden von Mercaptochinolinen. *Pharmazie*, 20 (1965) 549–553.

25. ORGANIC PHOSPHORUS COMPOUNDS

HARTMAN, F. C. AND BARKER, R.: An exploration of the active site of aldolase using structural analogs of fructose diphosphate. *Biochemistry*, 4 (1965) 1068–1075.

27. VITAMINS

DESCIMON, H.: Identification de la sépiaptérine dans les ailes des *Pieridae* (*Lepidoptera rhopalocera*). *Bull. Soc. Chim. Biol.*, 47 (1965) 1095–1100 — PC and TLC.

FUKUI, S., OHISHI, N., KISHIMOTO, S., TAKAMIZAWA, A. AND HAMAZIMA, Y.: Formation of "thiaminosuccinic acid" as an intermediate in the transformation of oxythiamine to thiamine by a thiamineless mutant of *Escherichia coli*. *J. Biol. Chem.*, 240 (1965) 1315–1321.

LINGENS, F. AND VOLLPRECHT, P.: Zur Biosynthese der Nicotinsäure in Streptomyceten, Algen, Phycomyceten und Hefe. *Z. Physiol. Chem.*, 339 (1964) 64–74.

MISITI, D., MOORE, H. W. AND FOLKERS, K.: Synthesis of 2-solanesyl-1,4-naphthoquinone. New member of a vitamin K_2 group. *Biochemistry*, 4 (1965) 1156–1160 — PC and TLC.

28. ANTIBIOTICS

COX, W. H. AND GREENWELL, B. E.: Paper chromatographic method for detecting phenethicillin contamination in drugs. *J. Pharm. Sci.*, 54 (1965) 1076–1077.

SZILÁGYI, I., VÁLYI-NAGY, T. AND KERESZTES, T.: Formation of two spots caused by "secondary adsorption" in paper chromatography of primycin. *Nature*, 205 (1965) 1225–1227.

30. SYNTHETIC AND NATURAL DYES

CALIN, C. AND SANDULESCU, V.: (Some methods for identifying dyes of unknown structure). *Ind. Textila* (*Bucharest*), 15 (1964) 531–534; *C.A.*, 62 (1965) 14859b.

GRUENE, A.: (Paper chromatography of synthetic dyes and intermediate products). *Lab. Sci.* (*Milan*), 12, No. 4 (1964) 97–118; *C.A.*, 62 (1965) 16413f.

KIEL, E. G. AND KUYPERS, G. H. A.: (Paper chromatography of dyes. VIII. Direct azo dyes). *Tex*, 22 (1963) 779–781; *C.A.*, 62 (1965) 15401g.

KIEL, E. G. AND KUYPERS, G. H. A.: (Paper chromatography of dyes. IX. Acid dyes). *Tex*, 22 (1963) 873–875; *C.A.*, 62 (1965) 15401g.

RUIZ, I. S. L.: (Zur Papierchromatographie von blauen Triphenylmethanfarbstoffen). *Ann. Fals. Expert. Chim.*, 57 (1964) 115–118; from *Z. Anal. Chem.*, 214 (1965) 230.

See also TLC section.

33. INORGANIC SUBSTANCES

ACKERMANN, G. AND SCHLOSSER, G.: (Rapid qualitative analysis of aluminium alloys by paper chromatography). *Z. Chem.*, 5, No. 2 (1965) 66; *C.A.*, 62 (1965) 15401h.

CVJETICANIN, N.: Chromatographic separation of rare earths on paper treated with di-*n*-butyl phosphate. *Bull. Boris Kidric Inst. Nucl. Sci.*, 15 (1964) 201–203; *C.A.*, 62 (1965) 15400g.

SASTRI, M. N., RAO, A. P. AND SARMA, A. R. K.: Chromatographic behaviour of metal ions on papers treated with primary, secondary, tertiary and quaternary amines. *J. Chromatog.*, 19 (1965) 630–633.

WINAND, L.: (Effect of cations on the paper chromatography of polyphosphates). *Bull. Soc. Chim. Belges*, 74 (1965) 8–11; *C.A.*, 62 (1965) 13817g.

34. RADIOACTIVE AND OTHER ISOTOPIC COMPOUNDS

SHKOLNIK, R. YA., DOMAN, N. G., SPEKTOROV, K. S. AND LINKOVA, E. A.: (Intermediate photosynthesis products of a synchronous culture of *Chlorella pyrenoidosa* at different development stages). *Dokl. Akad. Nauk SSSR*, 161 (1965) 1231.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

HÁZNAGY, A., SZENDREI, K. AND TÓTH, L.: Die Dünnschichtchromatographie und ihre Bedeutung in der Pharmakognosie. 1. Literaturübersicht. *Pharmazie*, 20 (1965) 541–548 — a review with 228 references.

PATAKI, G.: *Dünnschichtchromatographie in der Aminosäure- und Peptid-Chemie*, Walter de Gruyter, Berlin, 1966, 250 pp.

YAMAGUCHI, M.: Thin-layer chromatography. *Hakko Kyokaishi*, 21 (1963) 361–365; *C.A.*, 62 (1965) 13811c.

2. FUNDAMENTALS, THEORY AND GENERAL

HESSE, G., ENGELHARDT, H. AND KOWALLIK, W.: Dünnschichtchromatographie an standardisierten Aluminiumoxiden. *Z. Anal. Chem.*, 214 (1965) 81–88.

GEISS, F., SCHLICHT, H. AND KLOSE, A.: Vorbeladung von Dünnschicht-Sorbentien und chromatographische Eigenschaften. *Z. Anal. Chem.*, 213 (1965) 321–330.

GEISS, F., SCHLITT, H. AND KLOSE, A.: Zur Reproduzierbarkeit in der Dünnschichtchromatographie. Einfluss von Luftfeuchtigkeit, Kammerform und -atmosphäre auf das chromatographische Resultat. *Z. Anal. Chem.*, 213 (1965) 331–346.

3. TECHNIQUES I

BUCHTELA, K. AND LESIGANG, M.: Dünnschichtchromatographie an Salzen von Heteropolysäuren. *Mikrochim. Acta*, (1965) 67–74.

DITULLIO, V.: A thin layer chromatography applicator. *Chem. Ind. (London)*, (1965) 1026.

SCHERZ, H., BANCHER, E. AND KAINDL, K.: Über eine Kombination der Dünnschichtchromatographie mit der Ringofenmethode nach WEISZ. *Mikrochim. Acta*, (1965) 255–257.

SIRÉN, M. J.: New method for thin-layer chromatography. *Scand. J. Clin. Lab. Invest.*, 16 (1964) 123; *Anal. Abstr.*, 12 (1965) 2064 — preparation of the plates by sedimentation.

ŚLIWIOK, J.: (Regeneration of the Kieselgur G carrier for thin-layer chromatography of higher fatty acids). *Chem. Anal. (Warsaw)*, 10 (1965) 1037–1038.

4. TECHNIQUES II

Systematic analysis, automation and preparative scale thin-layer chromatography

BELL, C. E.: A modified spreader for the application of adsorbent layers for preparative thin-layer chromatography. *Chem. Ind. (London)*, (1965) 1025–1026.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

PETROWITZ, H. J.: Zur Dünnschichtchromatographie und Gaschromatographie von Verbindungen der Cyclododecan-Reihe. *Z. Anal. Chem.*, 213 (1965) 194–197.

6. ALCOHOLS

KATES, M., PALAMETA, B. AND YENGOYAN, L. S.: Aliphatic diether analogs of glyceride-derived lipids. II. Synthesis of naturally occurring L-2,3-di-O-3′,7′,11′,15′-tetramethylhexadecyl glycerol and its D-isomer. *Biochemistry*, 4 (1965) 1595–1599.

7. PHENOLS

MATHEW, T. V. AND MITRA, S. N.: Separation and identification of antioxidants in oils and fats by thin-layer chromatography. *Indian J. Technol.*, 3, No. 3 (1965) 102; *C.A.*, 62 (1965) 16524b.

ZOWALL, H. AND LEWANDOWSKA, T.: (Chromatography of Dian (2,2-bis(4-hydroxyphenylpropane)) and its impurities on thin layers). *Chem. Anal. (Warsaw)*, 10 (1965) 947–952.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

MIZELLE, J. W., DUNCAP, W. J., HAGEN, R. E., WENDER, S. H., LIME, B. J., ALBACH, R. F. AND GRIFFITHS, F. P.: Isolation and identification of some flavanone rutinosides of the grapefruit. *Anal. Biochem.*, 12 (1965) 316–324.

O'HARA, M. J., KINNARD, W. J. AND BUCKLEY, J. P.: Preliminary characterization of aqueous extracts of *Piper methysticum* (kava, Kawa Kawa). *J. Pharm. Sci.*, 54 (1965) 1021–1025.

9. OXO COMPOUNDS

DANILOVIĆ, M. AND NAUMOVIĆ-STEVANOVIĆ, O.: The separation of some anthraquinone derivatives by thin-layer chromatography. *J. Chromatog.*, 19 (1965) 613–614.

GUDRINIECE, E. AND KREICBERGA, D.: (Chromatographic separation of diketones. II. Thin-layer chromatography of 1,3-indandione). *Latvijas PSR Zinatnu Akad. Vestis, Kim. Ser.*, (1964) 419–423; *C.A.*, 62 (1965) 15402e.

KREICBERGA, D. AND GUDRINIECE, E.: (Chromatographic separation of diketones. III. Chromatography of derivatives of 2-phenyl-1,3-indandione). *Latvijas PSR Zinatnu Akad. Vestis, Kim. Ser.*, (1964) 424–428; *C.A.*, 62 (1965) 15402f.

OBRUBA, K., NAVRÁTIL, F., SPĚVÁK, A. AND NETUŠIL, Z.: (Chromatographic identification and estimation of a mixture of nitroanthraquinones). *Chem. Prumysl*, 15, No. 2 (1965) 95–99; *C.A.*, 62 (1965) 13847g.

SAWICKI, E., STANLEY, T. W., ELBERT, W. C. AND MORGAN, M.: Column and thin-layer chromatographic separation of polynuclear ring-carbonyl compounds. *Talanta*, 12 (1965) 605–616; *C.A.*, 62 (1965) 15399d.

VETTIG, K.: (Die Dünnschichtchromatographie von Chinonen und einigen polycyklischen Kohlenwasserstoffen). *Gigiena i Sanit.*, 29, No. 12 (1964) 56–57; from *Z. Anal. Chem.*, 214 (1965) 121.

10. CARBOHYDRATES

MODI, B. D., PATIL, J. R. AND BOSE, J. L.: Separation of isopropylidene derivatives of monosaccharides by thin-layer chromatography. *Indian J. Chem.*, 2 (1964) 32–34; *Anal. Abstr.*, 12 (1965) 2253.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

ÄNGGÅRD, E. AND SAMUELSSON, B.: Prostaglandins and related factors. XLII. Metabolism of prostaglandin E_3 in guinea pig lung. *Biochemistry*, 4 (1965) 1864–1871.

PALLOTTA, U. AND MATARESE, L.: (Detection of elaidic acid in fats by thin-layer chromatography). *Riv. Ital. Sostanze Grasse*, 40 (1963) 579–583; *Anal. Abstr.*, 12 (1965) 1442.

STICHER, O.: Über die antibakterielle Wirksamkeit von *Lichen islandicus* mit besonderer Berücksichtigung der Inhaltsstoffe. *Pharm. Acta Helv.*, 40 (1965) 483–495.

TING, I. P. AND DUGGER, Jr., W. M.: Separation and detection of organic acids on silica gel. *Anal. Biochem.*, 12 (1965) 571–578 — R_F values of 23 acids.

See also PC section.

11b. Lipids and their constituents

BOISSY, M. C. AND PERLÉS, R.: Étude d'un phospholipide à inositol des graines de sorgho. *Bull. Soc. Chim. Biol.*, 47 (1965) 859–866.

CHAKRABARTY, M. M. AND BHATTACHARYYA, D.: Resolution of natural and rearranged glycerides by multiple-development chromatography on thin layer and paper. *J. Oil Technologists' Assoc. India*, 18 (1963) 317–318; *C.A.*, 62 (1965) 14940b — TLC and PC.

DUNPHY, P. J., WHITTLE, K. J. AND PENNOCK, J. F.: On the use of fluorescein as non-destructive stains for lipids. *Chem. Ind. (London)*, (1965) 1217–1218.

ETIENNE, J., MASSÉ, M. AND POLONOVSKI, J.: Les phospholipides de *Serratia marcescens*. *Bull. Soc. Chim. Biol.*, 47 (1965) 851–858 — TLC and PC.

KANFER, J.: Observations on cerebroside metabolism *in vivo*. *J. Biol. Chem.*, 240 (1965) 609–612.

KUHN, N. J. AND LYNEN, F.: Phosphatidic acid synthesis in yeast. *Biochem. J.*, 94 (1965) 240–246.

MEHL, E. AND JATZKEWITZ, H.: Eine Cerebrosidsulfatase aus Schweineniere. *Z. Physiol. Chem.*, 339 (1964) 260–276.

MUSTAKALLIO, K. K., KIISTALA, U., NIEMINEN, E. AND LEIKOLA, E.: Les lipides de l'épiderme humain. Séparation de l'épiderme *in vivo* par la technique de succion pour l'étude des lipides par chromatographie en couche mince. *Arch. Biochim. Cosmetol.*, No. 72 (1964) 11–16.

PAYSANT, M., WALD, R. AND POLONOVSKI, J.: Métabolisme des lysolécithines et des lécithines dans la rate de rat. *Bull. Soc. Chim. Biol.*, 47 (1965) 621–630.

POLONOVSKI, J., WALD, R. AND PETEK, F.: Isolement des glucolipides de *Staphylococcus aureus* et identification du diglucosyldiglycéride. *Bull. Soc. Chim. Biol.*, 47 (1965) 409–416 — TLC of lipids, PC of sugars.

POMERANZ, Y. AND CHUNG, O.: The lipid composition of a single wheat kernel and its structural parts. *J. Chromatog.*, 19 (1965) 540–550.

PRIVETT, O. S., BLANK, M. L., CODDING, D. W. AND NICKELL, E. C.: Lipid analysis by quantitative thin-layer chromatography. *J. Am. Oil Chemists' Soc.*, 42 (1965) 381–393 — a review with 104 references.

ROOTS, B. I. AND JOHNSTON, P. V.: Lipids of isolated neurons. *Biochem. J.*, 94 (1965) 61–64.

ŚLIWIOK, J.: Nachweis fremder Pflanzenöle in Olivenöl durch Dünnschichtchromatographie. *Mikrochim. Acta*, (1965) 294–296.

YOUNG, O. M. AND KANFER, J. N.: An improved separation of sphingolipids by thin-layer chromatography. *J. Chromatog.*, 19 (1965) 611–613.

See also PC section.

13. STEROIDS

BEGUE, J. A.: Dosage des sulfates de déhydroépiandrostérone et d'androstérone dans le sang. *Bull. Soc. Chim. Biol.*, 47 (1965) 885–889.

CRÉPY, O. AND JAYLE, M. F.: Incubation du sulfate d'oestrone en présence de coupes de foie de lapin. *Bull. Soc. Chim. Biol.*, 47 (1965) 427–441.

FEHÉR, T.: Application of thin-layer chromatography on purification, separation and quantitative determination of steroid metabolites. *Mikrochim. Acta*, (1965) 105–116.

LUISI, M.: (Determination of urinary oestrogens by thin-layer chromatography). *Ric. Sci.*, B3 (1963) 369–377; *Anal. Abstr.*, 12 (1965) 1925.

RIESS, J.: Identification et séparation chromatographique des acides organo-phosphoriques par leurs esters méthyliques. *J. Chromatog.*, 19 (1965) 527–530.

See also PC section.

14. STEROID GLYCOSIDES AND SAPONINS

SUN, N.-C. AND LANG, H.-Y.: Application of thin-layer chromatography in the study of natural products. III. Identification of cardiac glycosides. *Yao Hsueh Hsueh Pao*, 11 (1964) 101–107; *Anal. Abstr.*, 12 (1965) 2441.

TSCHESCHE, R., SCHMIDT, W. AND WULFF, G.: Über Triterpene. XIX. Reindarstellung und Strukturermittlung der Saponine des Efeus (*Hedera helix* L.). *Z. Naturforsch.*, 20b (1965) 708–709.

15. TERPENE DERIVATIVES

MESSERSCHMIDT, W.: (Gas and thin-layer chromatography of the essential oils of several *Thymus* species. II. The influence of various origins on the composition of essential oils of thyme herb and of *Thymus serpyllum* herb and proposals for their chromatographic evaluation). *Planta Med.*, 13 (1965) 56–72; *C.A.*, 62 (1965) 15988g.

STAHL, E., BOLLIGER, H. R. AND LEHNERT, L.: (Thin-layer chromatography of carotene and carotenoid mixtures). *Wiss. Veroeffentl. Deut. Ges. Ernaehrung*, 9 (1963) 129–134; *C.A.*, 62 (1965) 15988b.

STAHL, E. AND VOLLMANN, H.: (Thin-layer chromatography. XV. Separation of terpenes and sesquiterpene alcohols on silver nitrate–silica gel layers). *Talanta*, 12 (1965) 525–528; *C.A.*, 62 (1965) 13818c.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

MANTHEY, J. A. AND EMUNDSON, M. E.: Improved thin-layer chromatographic separations using alcoholic solutions of inorganic salts as solvents. *J. Chromatog.*, 19 (1965) 522–526.

WOLF, V., BLOCK, W. AND PIATER, H.: (Alkyne amines. IX. Conversion of 1-bromo-3-methyl-1-pentyn-3-ol and its methyl ether with secondary amines). *Ann. Chem.*, 682 (1965) 112–123; *C.A.*, 62 (1965) 14476c.

See also PC section.

18. AMINO ACIDS

FAIRCLOTH, M. A., WILLIAMS, A. D. AND FLORSHEIM, W. H.: A thin-layer chromatographic method for the analysis of thyroidal iodoamino acids. *Anal. Biochem.*, 12 (1965) 437–443.

GOEDDE, H. W., BRUNSCHEDE, H. AND HOFFBAUER, R.: Quantitative dünnschichtchromatographische Bestimmung von β-Aminoisobuttersäure. *Angew. Chem.*, 77 (1965) 683.

See also PC section and TLC section 1.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BERGQUIST, P. L.: A thin-layer method for the mapping of enzymatic digests of ribonucleic acids. *J. Chromatog.*, 19 (1965) 615–618.

BOVÉ, J. M. AND YOT, P.: Contrôle des hydrolyses d'acides nucléiques par chromatographie en couche mince. *Bull. Soc. Chim. Biol.*, 47 (1965) 1286–1290.

OFENGAND, J.: A chemical method for the selective modification of pseudouridine in the presence of other nucleosides. *Biochem. Biophys. Res. Commun.*, 18 (1965) 192–201.

RANDERATH, K.: Two-dimensional separation of nucleic acid bases on cellulose layers. *Nature*, 205 (1965) 908 — cellulose layers.

WEICHSEL, H.: Nachweis und Bestimmung von Barbitursäurederivaten. *Mikrochim. Acta*, (1965) 325–330.

See also PC section

22. ALKALOIDS

FARNSWORTH, N. R., BLOMSTER, R. N., DAMRATOSKI, D., MEER, W. A. AND CAMMARATO, L. V.: *Catharanthus* alkaloids. VI. Evaluation by means of thin-layer chromatography and ceric ammonium sulfate spray reagent. *Lloydia*, 27 (1964) 302–314; *C.A.*, 62 (1965) 16316e.

GENEST, K.: A direct densitometric method on thin-layer plates for the determination of lysergic acid amide, isolysergic acid amide and clavine alkaloids in morning glory seeds. *J. Chromatog.*, 19 (1965) 531–539.

KURONO, G., OGURA, K. AND SASAKI, K.: Determination of constituents in crude drugs by thin-layer chromatography. I. Determination of berberine in *Coptis japonica* by thin-layer chromatography. *Yakugaku Zasshi*, 85 (1965) 262–266; *C.A.*, 62 (1965) 15991d.

LI, L.-N. AND FANG, C.-C.: Application of thin-layer chromatography in the study of natural products. II. Determination of ergometrine, ergotamine and ergotoxine. *Yao Hsueh Hsueh Pao*, 11 (1964) 189–193; *Anal. Abstr.*, 12 (1965) 2441.

NONCLERCQ, M. AND NYS, C.: (Determination of codeine and ethylmorphine in certain sirups). *J. Pharm. Belg.*, 19 (1964) 421–435; *C.A.*, 62 (1965) 14421f.

RAM, M. AND HANDA, K. L.: Isolation and estimation of ergot alkaloids. *Indian J. Chem.*, 3 (1965) 42–43; *C.A.*, 62 (1965) 14421g.

RENNER, U.: (Alkaloids from *Schizozygia caffaeoides*. III. Structural relationship between schizozygine and some accessory alkaloids). *Lloydia*, 27 (1964) 406–415; *C.A.*, 62 (1965) 14746h.

See also PC section.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

BERNASCONI, R.: Untersuchungen über die Stabilität von N,N-Dimethylenoxid-bis-(pyridinium-4-aldoxim)-dichlorid in wässriger Lösung. *Pharm. Acta Helv.*, 40 (1965) 564–574.

CHIERICI, L. AND PERANI, M.: (Chromatographic study of pyrrole acids. Thin-layer chromatography of the C-pyrrolecarboxylic acids). *Ric. Sci., Rend., Sez. A*, 6 (1964) 168–169; *C.A.*, 62 (1965) 13814d.

GONNARD, P., ROUCAYROL, J. C., CAMIER, M. AND PERRIN, J.: Étude radiochromatographique sur la stabilité de la (7-^{14}C) benzoyl hydrazone de phospho-5′-pyridoxal en présence de pyridoxalo-enzymes. *Bull. Soc. Chim. Biol.*, 47 (1965) 481–491.

GRANDBERG, I. I., FAIZOVA, G. K. AND KOST, A. N.: (Chromatography of pyridine derivatives on a thin unfixed layer of aluminium oxide). *Zh. Analit. Khim.*, 20 (1965) 268–271; *C.A.*, 62 (1965) 15402b.

24. ORGANIC SULPHUR COMPOUNDS

MEYER-DULHEUER, K. H. AND RITTER, E.: (Thin-layer chromatography of sulfonamides in drugs). *Pharm. Ztg.*, 110 (1965) 260–261; *C.A.*, 62 (1965) 15994h.

SEILER, N.: Nachweis und Bestimmung von Aminen im 10^{-10} Mol-Massstab. Trennung von 1-Dimethylamino-naphthalin-5-sulfonsäureamiden auf Dünnschichtchromatogrammen. *Angew. Chem.*, 77 (1965) 684.

25. ORGANIC PHOSPHORUS COMPOUNDS

BIELESKI, R. L.: Separation of phosphate esters by thin-layer chromatography and electrophoresis. *Anal. Biochem.*, 12 (1965) 230–234.

HANKIEWICZ, J. AND STUDNIARSKI, K.: (Dünnschichtchromatographische Identifikation mancher lipophiler organischer Phosphorverbindungen). *Chem. Anal. (Warsaw)*, 10 (1965) 941–946.

PIETSCH, H. P. AND MEYER, R.: (Thin-layer chromatography of tricresyl phosphate and phthalates on alumina). *Nahrung*, 9 (1965) 151; *C.A.*, 62 (1965) 16449h.

WILEY, R. A. AND GODWIN, H. N.: Synthesis and biological activity of some diphenylphosphine derivatives. *J. Pharm. Sci.*, 54 (1965) 1063–1065.

27. VITAMINS

GOODHUE, C. T.: Specific growth of a soil microorganism on the natural isomer of α-tocopherol. *Biochemistry*, 4 (1965) 1822–1824.

See also PC section.

28. ANTIBIOTICS

LACHARME, J. AND NETIEN, G.: (Separation of chloramphenicol and certain nitro derivatives by thin-layer chromatography). *Bull. Trav. Soc. Pharm. Lyon*, 8, No. 3 (1964) 122–128; *C.A.*, 62 (1965) 13818f.

NAKAMURA, S., CHIKAIKE, T., YONEHARA, H. AND UMEZAWA, H.: Structure of bottromycin A and B. *J. Antibiotics (Tokyo), Ser. A.*, 18 (1965) 60–61; *C.A.*, 62 (1965) 16379a.

NUSSBAUMER, P. A. AND SCHORDERET, M.: Injectable de streptomycine. Elaboration d'une méthode de contrôle faisant appel à la chromatographie en couche mince et à la microbiologie. *Pharm. Acta Helv.*, 40 (1965) 477–482.

30. SYNTHETIC AND NATURAL DYES

BAJO, M. S. P.: (Toxic action of some dyes found in hair tinctures). *Anales Fac. Quim. Farm., Univ. Chile*, 15 (1963, pub. 1964) 192–197; *C.A.*, 62 (1965) 14418h — TLC and PC.

CAPELLA, P., FEDELI, E., LANZANI, A., JACINI, G. AND POLESELLO, A.: (Separation and determination of carotenoids in oils or food-stuffs). *Riv. Ital. Sostanze Grasse*, 40 (1963) 666–669; *Anal. Abstr.*, 12 (1965) 2001.

GOODWIN, T. W. AND WILLIAMS, R. J. H.: A mechanism for the cyclization of an acyclic precursor to form β-carotene. *Biochem. J.*, 94 (1965) 5C-7C–β-carotene counted in Packard Tri-Carb spectrometer after removal from the plate and decolorization with U.V. light.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

DANHIER, A.: (Analysis of dibenzazepine derivatives — trimeprimine, imipramine, demethylimipramine and opipramol). *J. Pharm. Belg.*, 19 (1964) 385–400; *C.A.*, 62 (1965) 14424c.

EL GENDI, S., KISSER, W. AND MACHATA, G.: Isolierung und Nachweis basischer Arzneimittel in der Toxikologie. *Mikrochim. Acta*, (1965) 120–132.

PINZON, R. AND KAPÉTANIDIS, I.: Séparation et identification, par chromatographie en couche mince, de six agents conservateurs. Recherche qualitative dans deux préparations galéniques. *Mikrochim. Acta*, (1965) 269–273.

33. INORGANIC SUBSTANCES

CANIČ, D. V., PETROVIČ, S. M. AND BEM, A. K.: Trennung der Kationen mit Hilfe der Dünnschichtchromatographie auf Stärke. *Z. Anal. Chem.*, 213 (1965) 251–253.

HRANISAVLJEVIĆ-JAKOVLJEVIĆ, M., PEJKOVIĆ-TADIĆ, I. AND MILJKOVIĆ-STOJANOVIĆ, J.: Thin-layer chromatography of inorganic ions. III. Separation of some noble metal dithizonates. *Mikrochim. Acta*, (1965) 141–143.

34. RADIOACTIVE AND OTHER ISOTOPIC COMPOUNDS

BLEECKEN, S., KAUFMANN, G. AND KUMMER, K.: Quantitative Bestimmung tritiummarkierter Verbindungen auf Dünnschichtchromatogrammen. *J. Chromatog.*, 19 (1965) 105–113.

JOLCHINE, G.: (Method for detecting ^{14}C- and ^{3}H-labelled substances on thin-layer chromatograms). *Physiol. Vegetale*, 2 (1964) 341–346; *C.A.*, 62 (1965) 15402h.

LÜTHI, U. AND WASER, P. G.: Low-temperature fluorography induced by tritium-labelled compounds on thin-layer chromatograms. *Nature*, 205 (1965) 1190–1191.

SHIKITA, M., KAKIZAKI, H. AND TAMAOKI, B.: Thin-layer chromatography of radioactive substances. *Kagaku No Ryoiki, Zokan*, No. 64 (1964) 45–50; *C.A.*, 62 (1965) 10799h — a review with 9 references.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

BEY, K.: Die dünnschichtchromatographische Analyse auf dem Gebiete der Tenside. *Fette, Seifen, Anstrichmittel*, 67 (1965) 217–221.

HOSOGAI, Y.: Thin-layer chromatography of organic chlorine compounds. *Kagaku No Ryoiki, Zokan*, No. 64 (1964) 185–196; *C.A.*, 62 (1965) 10800b — a review with 27 references.

KOLATTUKUDY, P. E.: Biosynthesis of wax in *Brassica oleracea*. *Biochemistry*, 4 (1965) 1844–1855.

LIU, K.: Thin-layer chromatography applied to natural products. VII. Identification of the principal constituents in essential oils. *Yao Hsueh Hsueh Pao*, 12 (1965) 108–118; *C.A.*, 26 (1965) 15987d.

Gas Chromatography

1. REVIEWS AND BOOKS

GIDDINGS, J. C.: *Dynamics of Chromatography. Part I. Priciples and Theory*, Marcel Dekker, New York, 1965, p. 323 — a book the object of which is to interpret theoretical developments in chromatography in a physical sense.

STRUPPE, H. G.: V. Symposium über Gas-Chromatographie vom 18. bis 21. Mai 1965 in Berlin. *Z. Chem.*, 5 (1965) 355–359 — a report (theory, 7 papers; detection, 7 papers; adsorption, 5 papers; application, 19 papers).

2. FUNDAMENTALS, THEORY AND GENERAL

2b. Gas–solid systems

HAWKES, S. J., RUSSELL, C. P. AND GIDDINGS, J. C.: Test of the theory of glass bead columns in gas liquid chromatography. *Anal. Chem.*, 37 (1965) 1523–1526 — with glass beads with diameters ranging from 0.004 to 0.023 cm and liquid loadings from 0.1 to 3.9%, theory and experiment agreed within 10%.

2c. Thermodynamics and theoretical relationships

CHOVIN, P. AND GUIOCHON, G.: Comparison des performances de colonnes de chromatographie en phase gazeuse de natures différentes. I. Résolution de pics et efficacité de la colonne. *Bull. Soc. Chim. France*, (1965) 3391–3395 — a nomogram relates the resolution power of a column given by number of theoretical plates, N and R, relative retention α, and dead volume of the column.

CHOVIN, P. AND GUIOCHON, G.: Comparaison des performances de colonnes de chromatographie en phase gazeuse de natures différentes. II. Méthode de comparaison et application à des colonnes fonctionnant à une même température. *Bull. Soc. Chim. France*, (1965) 3396–3403 — investigation of the influence of K (10, 100, 1000, ∞) on column performance at the same resolution ($R = 1$).

CHOVIN, P. AND GUIOCHON, G.: Comparaison des performances de colonnes de chromatographie en phase gazeuse de natures différentes. III. Application à des colonnes fonctionnant à des températures différentes. *Bull. Soc. Chim. France*, (1965) 3404–3407 — investigation of the influence of $d\alpha/dT = 0, > 0, < 0$ at the same resolution ($R = 1$).

GENKIN, A. N., BOGUSLAVSKAJA, B. I., BRESLER, L. S. AND NEMCOV, M. D.: (The thermodynamic functions of the interaction of substances with polar solvents as determined by gas–liquid chromatography). *Dokl. Akad. Nauk SSSR*, 164 (1965) 1089–1092.

KAMINSKIĬ, V. A., TIMASHEV, S. F. AND TUNITSKIĬ, N. N.: (On the shape of chromatographic peaks). *Zh. Fiz. Khim.*, 39 (1965) 2540–2546 — calculation of the first six moments of a chromatographic peak developed in terms of longitudinal diffusion and sorption coefficient only.

LAZARRE, F. AND ROUMAZEILLES, S.: Polarité des phases liquides et sélectivité des colonnes chromatographiques. *Bull. Soc. Chim. France*, (1965) 3371–3373 — $T \cdot \alpha$ = const. (where $\alpha = e^{A+B/T}$) within 0.5% for 13 investigated stationary phases in the range 300–470°K.

SAKHAROV, V. M.: (Functions of retention volume values independent of the kind of solvent in gas–liquid chromatography). *Neftekhimiya*, 5 (1965) 771–775 — on the basis of the equation $p_i = (\ln V_{i(1)} - \ln V_{i(2)})/(\ln V_{\text{standard}(1)} - \ln V_{\text{standard}(2)} = \text{inv. } (P)$.

SAKHAROV, V. M.: (Comparative calculating of retention volume values in gas–liquid chromatography). *Neftekhimiya*, 5 (1965) 762–770 — the retention volume can be calculated from the equation $\ln V_i = p \ln V_{\text{standard}} + B$ on the basis of 100 C_2–C_7 hydrocarbons (p and B are coefficients for a given substance).

2d. General

ANDREEV, L. V., USHKINA, T. A. AND VIGDERGAUZ, M. S.: (Correction coefficients for peak areas in gas chromatography). *Zh. Fiz. Khim.*, 39 (1965) 2425–2429 — increments for functional groups and for different bond types for thermal conductivity measurement.

BELENKY, B. G., ORESTOVA, I. A. AND EĬZNER, YU. E.: (Increase of the accuracy of the quantitative analysis on a gas chromatograph with an argon ionisation detector). *Zh. Analit. Khim.*, 20 (1965) 934–940 — area ratio determination when two peaks overlap and detector signal is nonlinear.

GUIOCHON, G.: Analyse qualitative par chromatographie en phase gazeuse: emploi des indices de rétention en température programmée. *Bull. Soc. Chim. France*, (1965) 3420–3423.

3. TECHNIQUES I

3a. Detectors

BALANDINA, V. A., KLESHCHEVA, M. S., KUZNETSOVA, G. S. AND TURKOVA, L. D.: (Quantitative interpretation of chromatograms made with thermal conductivity detector). *Zh. Analit. Khim.*, 18 (1963) 808–810 — on DOSebacate + sebacic acid at 105°.

FRITZ, D., GARZÓ, G., SZÉKELY, T. AND TILL, F.: Anomalous response of the flame ionization detector to organosilicon compounds. *Acta Chim. Hung.*, 45 (1965) 301–311 — Si-components give a peak inversion on FID under conditions in which hydrocarbons give normal peak shapes; inverted peaks permit C/Si ratio determinations.

JORDAN, J. AND KEBBEKUS, B. B.: Mixed carrier gases for quantitative chromatography: A new approach for linearizing thermal conductivity based on kinetic theory. *Anal. Chem.*, 37 (1965) 1572–1573 — a mixture of 10–60 mole % He and 90–40 mole % N_2 is suitable for the determination of CO_2 in a sample range of 10–1000 μmoles.

LOVELOCK, J. E.: The (W) value detector for high concentrations of gases and vapor in air. *Anal. Chem.*, 37 (1965) 583–584 — minimal detectable concentration is in the region of 0.03 vol. %; detector is of a great stability.

MCCORMACK, A. J., TONG, S. C. AND COOKE, W. D.: Sensitive selective gas chromatography detector based on emission spectrometry of organic compounds. *Anal. Chem.*, 37 (1965) 1470–1476 — 2450-Mc electrode-less discharge plasma and photoelectrical detection in the U.V.-visible region; sensititivy $2 \cdot 10^{-16}$ g of hexane/sec; relative area response factors (to *n*-hexane) at 3852 Å per unit volume are given for 14 compounds.

3b. Column performance and filling studies

BLANDENET, G.: Étude de la texture d'échantillons de graphite en vue de leur utilisation comme supports de chromatographie gaz–liquide. *Bull. Soc. Chim. France*, (1965) 3412–3415 — surface 1.3–2.5 m^2/g, pore volume 0.092–0.196 ml/g, impregnation 0.25–4 % of squalane.

BORISENKO, I. V., KISELEV, V. A., PETROVA, R. S., CHUYKINA, V. K. AND SHCHERBAKOVA, K. D.: (Chemical modification of silica gel surface for gas chromatography by methylchlorosilanes). *Zh. Fiz. Khim.*, 39 (1965) 2685–2690 — different type of modification followed in terms of retention volume per square meter, V_S.

DAL NOGARE, S.: New developments in gas chromatography. *Anal. Chem.*, 37 (1965) 1450–1453 — use of organic solids as sorbents.

GIDDINGS, J. C.: Practical criterion for minimum coil diameter in gas chromatography. *Anal. Chem.*, 37 (1965) 1580–1581 — $R_0/r_c > d_c/d_p$, where R_0/r_c is the ratio of coil to column radius and d_c/d_p the ratio of column to particle diameter.

GUIOCHON, G.: Écoulement des gaz dans les milieux poreux. Problèmes posés par le fonctionnement des colonnes de chromatographie en phase gazeuse. *Bull. Soc. Chim. France*, (1965) 3367–3371 — laminar and turbulent flow and molecular diffusion are discussed in terms of Poisseuille's and Darcy's laws.

KIRKLAND, J. J.: Porous thin-layer modified glass bead supports for gas liquid chromatography. *Anal. Chem.*, 37 (1965) 1458–1461 — 0.8 mm HETP on silica sol-modified 60–80 mesh glass beads coated with a silicone oil has been achieved.

KUDRYAVTSEVA, N. A., TARASOV, A. I., LULOVA, N. I. AND SHCHIPANOVA, A. I.: (Optimal conditions of chromatographic resolution of support material TZK). *Khim. Tekhnol. Topliv i Masel*, No. 10 (1965) 55–58.

MYERS, M. N. AND GIDDINGS, J. C.: High column efficiency in gas liquid chromatography at inlet pressures to 2500 p.s.i.: *Anal. Chem.*, 37 (1965) 1453–1457 — performance (N/t = number of theoretical plates per time) on 650 to 4000 ft. columns at an inlet pressure of 10–150 atm. was investigated.

SCHLUNEGGER, U. P.: Zur gaschromatographischen Trennung von aliphatischen, sauerstoffhaltigen Substanzen in Wasser. I. Eine neue polare, flüssige phase. *J. Chromatog.*, 20 (1965) 23–26 — 1-nonylphenoxy-2,3-propanediol up to 130° with flame ionization detector.

3c. Apparatus, accessories and materials for GC

EDWARDS, R. A. AND FAGERSON, I. S.: Collection of gas chromatographic fractions for infrared analysis. *Anal. Chem.*, 37 (1965) 1630–1631.

GRAVEN, W. M. AND HARMON, H. R.: All-Teflon sampling valve made for gas chromatography. *Anal. Chem.*, 37 (1965) 1626.

JOHNSON, Jr., H. W.: Evaluation of a computer-based technique for estimating the limit of detection of chromatographic detectors. *Anal. Chem.*, 37 (1965) 1581–1583.

WAHL, W. H. AND MEISELS, G. G.: Inexpensive, simple high vacuum injection system for gas chromatography. *Rev. Sci. Instr.*, 36 (1965) 1508–1509 — transfer of trace amounts of less than 0.1 ml (vacuum in sample loop 10^{-5} Torr).

4. TECHNIQUES II

4c. High speed GC

CHÉNEAU, M., FARRÉ-RIUS, F., GUIOCHON, G., JACQUÉ, L., LANDAULT, C. AND MERLE D'AUBIGNÉ, J.: Analyse rapide par chromatographie en phase gazeuse. *Bull. Soc. Chim. France*, (1965) 3416–3420.

4d. Special microtechniques

JUVET, Jr., R. S. AND TURNER, L. P.: Study of organic structure via mercury-sensitized photolysis and gas chromatography. Alcohols and esters. *Anal. Chem.*, 37 (1965) 1464–1470 — a pyrolysis technique: photolytic degradation–GLC is described; samples of the order of 1 mg.

WORMAN, J. C. AND GREEN, L. E.: Simulated distillation of high boiling petroleum fractions. *Anal. Chem.*, 37 (1965) 1620–1621.

4f. Measurement of physico-chemical and related values

GAVRILOVA, T. B. AND KISELEV, A. V.: (Measurement of adsorption isotherms and rapid determination of specific surface area by thermal desorption followed by gas chromatography). *Zh. Fiz. Khim.*, 39 (1965) 2582–2585 — an application of NELSEN AND EGGERTSEN's method (*Anal. Chem.*, 30 (1958) 1387) and comparison with B.E.T. (N_2 and Kr) for surface areas $Z = 180$ m²/g.

SAINT-YRIEIX, A.: Détermination par chromatographie gaz–solide des isothermes d'adsorption et de la surface spécifique des solides. *Bull. Soc. Chim. France*, (1965) 3407–3411 — an interesting review rather than a contribution to the problem.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5b. Other hydrocarbon types

BAUMANN, F., STRAUS, A. E. AND JOHNSON, J. F.: Boiling points of C_{10}–C_{15} straight chain alkylbenzene isomers from gas chromatographic retention data. *J. Chromatog.*, 20 (1965) 1–8 — retention data of 30 C_{10}–C_{14} straight-chain alkylbenzenes on SF-96 at 190° and Apiezon L at 210° and 240°.

CHAPMAN, L. R. AND KUEMMEL, D. F.: Liquid–solid and capillary gas–liquid chromatography of internal olefin isomers. *Anal. Chem.*, 37 (1965) 1598–1600 — separation of *trans-* and *cis*-olefins by Al_2O_3 impregnated with $AgNO_3$ and determination of the number of C atoms by GLC analysis.

DEMBOVSKAJA, E. A., KONYASHINA, R. A., MEZHLUMOVA, A. I. AND PAL'CHIKOV, G. F.: (Investigation of the chemical composition of gas oil from catalytic cracking). *Khim. Tekhnol. Topliv i Masel*, No. 11 (1965) 16–19 — chromatographic scheme of investigation is of interest; GC on PEGA.

EYZEN, D. G. AND LUYGA, P. O.: (Use of preparative-scale gas chromatography for analysis of hydrocarbon mixtures). In A. A. ZHUKHOVITSKII *et al.* (Editors), *Gazovaya Khromatografiya*, Izdat. Nauka, Moscow, 1964, pp. 216–222.

GERBER, M. I., TERENT'EVA, E. M., ORLOVA, V. S. AND KONDRAT'EV, V. P.: (Boiling points and retention volumes of C_9–C_{18} hydrocarbons). *Neftekhimiya*, 5 (1965) 776–785 — retention volumes of 78 hydrocarbons, 36 cyclanes, 38 aromatics, 4 of mixed type on PFMS-4 (polyphenylmethyl siloxane) and E-301 at 234° (both) and 300° (PFMS-4).

KÁNTOR, M. AND KERÉNYI, E.: (Individual hydrocarbons analysis of our inland gasolines by gas–liquid chromatography). *Res. Rept. Hung. Inst. Petrol. Gas*, No. 6 (1965) 9–18 — retention data of 47 C_5–C_{10} hydrocarbons on squalane at 60°, 70°, 80°, 100° and 120°, on β,β'-oxydipropionitrile at 70°, and on Carbowax 1000 at 100°.

SCHULTHEISZ, I., BÉLAFI, K., BÖRZSÖNYI, M., KÁNTOR, M. AND KERÉNYI, E.: (Synthesis of some branched-chain hydrocarbons). *Res. Rept. Hung. Inst. Petrol. Gas*, No. 6 (1965) 35–44 — purity investigation on dimethylsulfolane at 36° and squalane at 120°.

SHEPARD, R. C.: Determination of triphenylene in irradiated organic reactor coolant and high-boiling samples by gas chromatography. *U.S. At. Energy Comm.*, Rept. NAA-SR-Memo-10,133, (1964) 9 — on DC-11 at 305°; errors ± 2%.

5c. Halogen derivatives of hydrocarbons

JOHNSON, R. L. AND BURTON, D. J.: Gas chromatographic analysis of some polyfluorinated alicyclic olefins. *J. Chromatog.*, 20 (1965) 138–140 — on SE-30, DNP and β,β'-oxydipropionitrile at 40°.

6. ALCOHOLS

SATO, M., MIYAKE, H., MITOOKA, M. AND ASANO, K.: A simple method for the analysis of a homologous series of alcohols using gas chromatography. *Bull. Chem. Soc. Japan*, 38 (1965) 884–887.

7. PHENOLS

KRUMBHOLZ, K. AND VOLLERT, U.: Gas-chromatographische Untersuchungen tertiär-butylierter Phenole. *Chem. Tech. (Berlin)*, 17 (1965) 693–694 — retention data of 22 phenols on silicone rubber Gi 7100/6 at 200°.

9. OXO COMPOUNDS

ANDRONOV, L. M. AND NORIKOV, YU. D.: (Analysis of hydroxyaldehydes by gas–liquid chromatography). *Zh. Anal. Khim.*, 20 (1965) 1007–1009 — on Apiezon N at 165° and 200°.

BELEN'KIĬ, B. G. AND ORESTOVA, V. A.: (Gas chromatographic analysis of methyl- and ethylacrolein). *Zavodsk. Lab.*, 31 (1965) 1328–1329 — on PEG 1000 at 75°.

SOKOLOV, D. N. AND GOLOVATENKO, P. T.: (Gas chromatographic analysis of products of polyformaldehyde plant). *Zavodsk. Lab.*, 31 (1965) 1321–1324 — retention data of methylol, ethylol, benzene, 1,3-dioxolane, dimethyl ether, dioxymethylene glycol, trioxane and formaldehyde at 90°.

10. CARBOHYDRATES

DUTTON, G. G. S. AND UNRAU, A. M.: Some applications of paper and vapour-phase chromatography to polysaccharides. Model studies on a synthetic D-mannan. *J. Chromatog.*, 20 (1965) 78–88 — some retention data on O-methyl or O-trimethylsilyl derivatives on Versamid or SF-96 at 235°.

SAWARDEKER, J. S., SLONEKER, J. H. AND JEANES, A.: Quantitative determination of monosaccharides as their alditol acetates by gas–liquid chromatography. *Anal. Chem.*, 37 (1965) 1602–1604 — retention data of 11 sugars on Carbowax 20M and ECNSS-M at 190° and on XE-60 at 160°.

11. ORGANIC ACIDS AND THEIR CONSTITUENTS

11a. Organic acids

DRUSKINA, E. Z. AND SHAPOSHNIKOV, YU. K.: (Analysis of products from propionic acid plant by gas–liquid chromatography). *Khim. Pererabotka Drevesiny, Ref. Inf.*, No. 9 (1965) 9–10 — C_1–C_4 fatty acids on dioctyl sebacate + sebacic acid at 120°.

GERSHON, H. AND RENWICK, J. A. A.: Gas chromatography of fluorinated fatty acids. I. Separation and identification of lower 2-fluorofatty acids. *J. Chromatog.*, 20 (1965) 134–137 — retention data of C_2–C_6 fatty and fluorofatty acids on Tween 80 + 85% H_3PO_4 at 132°.

HOFFMAN, N. E. AND WHITE, I. R.: Gas chromatographic analysis of malonic acids. *Anal. Chem.*, 37 (1965) 1541–1543 — malonic acids are decarboxylated in the chromatographic injector at 190–220° and the resulting monocarboxylic acids are separated on Carbowax 20M–terephthalic acid at 110–155°.

JÜTTNER, B. AND BERTLING, H.: Oxydative Zerlegung von Ruhrkohlenarten. *Brennstoff-Chem.*, 46 (1965) 277–280 — benzene mono- to hexacarboxylic acid methyl ester by PTGC 150–310°, 2°/min.

POTATUEV, A. A., SHELOMOV, I. K. AND PARIMSKIĬ, A. I.: (Rapid gas chromatographic analysis of multi-component mixtures). *Zavodsk. Lab.*, 31 (1965) 1328 — C_7–C_{17} fatty acid methyl esters in 120 sec.

11b. Lipids and their constituents

HENLY, R. S.: Preparative GLC of lipids. *J. Am. Oil Chemists' Soc.*, 42 (1965) 673–681 — a critical review on methods and applications; 24 references.

POKORNÝ, J., ŽÁK, L., JANÍČEK, G., ZEMAN, I. AND HAVRÁNKOVÁ, J.: (Investigation of chemical composition of cereals. III. Properties of wheat lipid fractions). *Rostlinna Vyroba*, 11 (1965) 789–798 — on PEGA at 220°.

ZEMAN, I.: (Composition of fatty acids present in rape-seed and winter rape-seed oils). *Průmysl Potravin*, 15 (1964) 287–288 — Apiezon M at 200° and Apiezon L at 240°.

15. TERPENE DERIVATIVES

CALVARANO, M. AND CALVARANO, I.: (Composition of bergamot oil. II. Use of ultra-violet spectrophotometry and gas chromatography in the analysis). *Essenze, Deriv. Agrumari*, 34 (1964) 71–92.

GLEN, A. T., LAWRIE, W., MCLEAN, J. AND EL-GARBY YOUNES, M.: Isolation of a new triterpenoid from Rose-bay Willow-herb. *Chem. Ind. (London)*, (1965) 1908.

HARTLEY, R. D. AND NEVE, R. A.: Relationships between constituents of cones and male flowers of the hop (*Humulus lupulus* L.). *Nature*, 208 (1965) 804–805 — on Apiezon L at 175°.

16. NITRO AND NITROSO COMPOUNDS

COURTIER, J. C., ÉTIENNE, L., TRANCHANT, J. AND VERTALIER, S.: Analyse par chromatographie en phase gazeuse des dérivés nitrés du toluène. Reproducibilité et précision de l'analyse quantitative. *Bull. Soc. Chim. France*, (1965), 3181–3186 — retention data of mono- and dinitro-derivatives on silicone Rhodosil 47V300 at 200°, on Apiezon L at 188°, and on XE-60 at 175° and 200°; relative responses for flame ionization are given.

18. AMINO ACIDS

SMITH, E. D. AND SHEPPARD, Jr., H.: Quantitative gas chromatography of amino-acids as trimethylsilyl derivatives. *Nature*, 208 (1965) 878–880 — relative errors ± 3%, average yields of TMS-derivatives 70–85%.

22. ALKALOIDS

MCNIVEN, N. L., RAISINGHANI, K. H., PATASHNIK, S. AND DORFMAN, R. I.: Determination of nicotine in smoker's urine by gas chromatography. *Nature*, 208 (1965) 778–789 — on SE-30 at 200°(nicotine and cotinine have been found.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

DEMOSS, R. D. AND GAGE, V.: Identification and estimation of indole analogs by gas–liquid chromatography. *Anal. Biochem.*, 13 (1965) 116–120 — retention data of 12 derivatives on silicone oil DC-550 at 160–164° and 200°.

ZIELINSKI, Jr., W. L. AND FISHBEIN, L.: Gas chromatography of derivatives of maleic hydrazide. II. Trimethylsilyl maleic hydrazide. *J. Chromatog.*, 20 (1965) 140–142 — retention data on Carbowax 20M and SE-30.

24. ORGANIC SULPHUR COMPOUNDS

MASHKINA, A. V. AND SAVOSTIN, YU. A.: (Chromatography of sulfolenes preceded by their pyrolysis). *Neftekhimiya*, 5 (1965) 760–761 — hydrocarbons + SO_2 on DNP at 50°.

26. METALLO-ORGANIC COMPOUNDS

GEISSLER, H. AND KRIEGSMANN, H.: Trennflüssigkeiten zur gas-chromatographischen Untersuchung einiger *n*-Butylzinnverbindungen. *Z. Chem.*, 5 (1965) 423–424 — retention data of mono- to tetra-*n*-butyl derivatives on 11 stationary phases at different temperatures between 162° and 194°.

MORIE, G. P. AND SWEET, T. R.: Analysis of mixtures of aluminum, gallium, and indium by solvent extraction and gas chromatography. *Anal. Chem.*, 37 (1965) 1552–1555 — as trifluoroacetylacetonates on glass microbeads coated with DC-550, in a column of borosilicate glass; column and support were silanized; 128°C; standard deviation 2% Al, 2.5% Ga and 5.2% In.

29. INSECTICIDES AND OTHER PESTICIDES

BACHE, C. A. AND LISK, D. J.: Determination of organophosphorus insecticide residues using the emission spectrometric detector. *Anal. Chem.*, 37 (1965) 1477–1480 — measurement of the P-line at 2535.65 Å; sample 5 μl; recoveries of insecticides 0.03–0.6 p.p.m. from foods.

FISHBEIN, L. AND ZIELINSKI, Jr., W. L.: Gas chromatography of trimethylsilyl derivatives. I. Pesticidal carbamates and ureas. *J. Chromatog.*, 20 (1965) 9–14 — retention data on QF-1, SE-30 and Carbowax 20M at 130°.

HRIVŇÁK, J., ŠŤOTA, Z. AND DOLEŽAL, J.: Verhalten einiger 2,4-Dinitro-6-*sek*.-butylphenylcarbonate bei der Gaschromatographie. *J. Chromatog.*, 20 (1965) 143–146 — retention data of 17 derivatives on Apiezon L at 205°, E-301 and PEGA at 200° and PEG 1570 at 190°.

31. PLASTICS AND THEIR INTERMEDIATES

BERTON, A.: Analyse de matières plastiques et autres substances organiques par chromatographie gazeuse et détection galvanique sélective de leurs produits de pyrolyse. *Chim. Anal.*, 47 (1965) 502–511.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

BECKETT, A. H. AND ROWLAND, M.: Determination and identification of amphetamine in urine. *J. Pharm. Pharmacol.*, 17 (1965) 59–60 — retention data of some amino-derivatives of pharmacological interest on Carbowax 6000 at 140°.

HOLTZMAN, J. L.: Detection of nanogram quantities of chloroquine by gas-liquid chromatography. *Anal. Biochem.*, 13 (1965) 66–70 — on polyvinylpyrrolidone and NGS at 220°.

MOSS, M. S. AND KENYON, M. W.: The detection of trichloro sedatives in urine. *Analyst*, 89 (1964) 802–803.

SCHULTZ, O. E. AND MOHRMANN, H. L.: Beitrag zur Analyse der Inhaltsstoffe von Knoblauch — *Allium sativum* L. 2. Mitt. Gaschromatographie des Knoblauchöls. *Pharmazie*, 20 (1965) 441–447 — on 10 stationary phases (the best: Apiezon L, butoxyethyl phthalate + tricresyl phosphate) at 154°–170.

TÖLGYESSY, J., ŠARŠÚNOVÁ, M. AND HAIS, I. M.: The use of radionuclides in some chromatographic methods and their application in pharmacy and drug research. *Cesk. Farmac.*, 14 (1965) 9–21.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

ATTRILL, J. E., BOYD, C. M. AND MEYER, Jr., A. S.: Gas chromatographic detection of permanent gases in helium at reduced sample pressures. *Anal. Chem.*, 37 (1965) 1543–1546 — H_2, O_2, N_2, CH_4, CO and CO_2 monitoring in He at 100 mm Hg pressure.

RENNHAK, S., DÖRING, C. E., SCHMIDT, G., SCHNELLER, D., STÜRTZ, H. AND WERNER, E.: Gaschromatographische Spurenuntersuchungen in C_2–C_4-Olefinfraktionen. *Chem. Tech. (Berlin)*, 17 (1965) 688–692 — retention data of C_2–C_4 higher unsaturated hydrocarbons on 10 stationary phases at 15–30°.

SHERTSOV, V. E. AND ZHUKHOVITSKIĬ, A. A.: (Analysis of blast-furnace gas by chromatography without carrier gas). *Zavodsk. Lab.*, 31 (1965) 1318–1321 — a special frontal type of analysis; errors: H_2, 0.23%; N_2, 1.79%; CO, 1.3%; CO_2, 0.35%; time of analysis 14–18 min.

33b. Volatile inorganic compounds

DURAND, J. C., CHAUDRON, T. AND MONTUELLE, J.: Utilisation de la chromatographie en phase gazeuse pour le dosage des traces de carbone dans les métaux de pureté élevée. *Bull. Soc. Chim. France*, (1965) 3109–3112 — p.p.m. of carbon in 500-mg samples of metal; no references or world literature on the same topic.

OTTO, R., REINHARDT, M., HOFFMANN, G. AND KÖLLNER, H.: Gas-chromatographische Bestimmung von Chloroform- und Schwefelkohlenstoffspuren in Tetrachlorkohlenstoff mit einer Wärmeleitfähigkeitsmesszelle. *Chem. Tech. (Berlin)*, 17 (1965) 621–622 — on squalane at 85° in a concentration range of 100–5000 p.p.m.

STEINDORF, W., JUST, E. AND ARDELT, H. W.: Zur gaschromatographischen Bestimmung des Phosphorwasserstoffs im Acetylen. *Z. Chem.*, 5 (1965) 388 — on silica gel; mean deviation 0.003% at a concentration of 0.065 vol. %.

SZEPESY, L., ILLÉS, V. AND CSIKÓS, R.: (Investigations concerning the elimination of impurities in carbon dioxide gas). *Res. Rept. Hung. Inst. Petrol. Gas*, No. 6 (1965) 127–140 — investigation of adsorption on charcoal at 50–60 atm followed by GC.

34. RADIOACTIVE AND ISOTOPIC COMPOUNDS

BOTTER, F., MENES, J., TISTCHENKO, S. AND DIRIAN, G.: Chromatographies frontales et de déplacement de bande des isotopes de l'hydrogène sur colonnes de palladium. *Bull. Soc. Chim. France*, (1965) 3374–3382 — theoretical treatment of H_2–D_2 and H_2–T_2 systems.

CERCY, C. AND BOTTER, F.: Possibilités de la chromatographie capillaire dans l'analyse des isomères et des isotopes de l'hydrogène. *Bull. Soc. Chim. France*, (1965) 3383–3390 — retention data of *p*- and *o*-H_2, H_2, D_2, T_2, HD, HT and DT on alumina, molecular sieves 13X and 5A with He and Ne as carrier gas.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

ARNI, P. C., COCHRANE, G. C. AND GRAY, J. D.: The emission of corrosive vapour by wood. II. The analysis of the vapour emitted by certain freshly felled hardwoods and softwoods by gas chromatography and spectrophotometry. *J. Appl. Chem.*, 15 (1965) 463–468 — C_1–C_4 alcohols and esters were studied.

KOLESNIKOVA, L. P., SIMONYANTS, E. G. AND KRYUKOV, YU. B.: (Chromatographic determination of C_1–C_5 alcohols in mixtures with aliphatic hydrocarbons and compounds containing oxygen). *Zavodsk. Lab.*, 31 (1965) 1330 — on triethanolamine at 85°.

MARKOVETZ, A. J. AND KLUG, M. J.: Detection and recovery of biological oxidation products of hydrocarbons by gas chromatography. *Anal. Chem.*, 37 (1965) 1590 — fatty acids and alcohols from *Pseudomonas aeruginosa* on Carbowax 20M–isophthalic acid column at 175°.

PICHLER, H. AND OBENAUS, F.: Die katalytische Oxydation von Methylaromaten mit Luft in der Gasphase. *Brennstoff-Chem.*, 46 (1965) 258–264.

SCHMELTZ, I., STILLS, C. D., CHAMBERLAIN, W. J. AND STEDMAN, R. L.: Analysis of cigarette smoke fraction by combined gas chromatography–infrared spectrophotometry. *Anal. Chem.*, 37 (1965) 1614–1616.

THOMSON, G. F. AND SMITH, K.: Application of on-column acid-base reactions to the gas chromatographic separation of free fatty acids and amines. *Anal. Chem.*, 37 (1965) 1591–1592 — conversion of potassium salts of C_2–C_8 acids in solution to the free acids on an acid-packed fore-column and separation of the free acids on DEGA at 130°; liberation of amines from their salts on a base-packed fore-column and separation on Apiezon L (or Teflon) at 100°.

CHROMATOGRAPHIE EN PHASE GAZEUSE

INFLUENCE DE LA TEMPÉRATURE SUR L'ÉLUTION DES PARAFFINES ET DES CYCLOPARAFFINES

J. BRICTEUX
Institut National de l'Industrie Charbonnière (INICHAR), Liège (Belgique)
ET
G. DUYCKAERTS
Laboratoire de Chimie Analytique, Université de Liège, Liège (Belgique)
(Reçu le 12 octobre 1965)

La relation existant entre la température et l'ordre d'élution des substances en chromatographie en phase gazeuse est parfois fort complexe; ainsi HOARE ET PURNELL[1,2] et PURNELL[3] ont montré que, dans le cas de composés chimiques différents, une augmentation de la température pouvait provoquer des inversions dans l'ordre d'élution, fait qu'ils ont mis en relation avec les valeurs respectives des pressions de vapeur et le rapport entre la chaleur molaire de solution à dilution infinie et la chaleur latente de vaporisation. Récemment, BLAUSTEIN, ZAHN ET PANTAGES[4] ont observé des modifications dans l'ordre d'élution de certains hydrocarbures renfermant de 5 à 8 atomes de carbone chromatographiés en travaillant sur colonne de tricrésylphosphate, soit à 40, soit à 80°; les mêmes auteurs relèvent dans la littérature quelques exemples d'inversion provoquée par une modification de la température, la plupart de ces exemples se rapportant à des mélanges où des composés cycliques sont élués dans le voisinage de composés non cycliques. ROBERTS[5] qui a remarqué des inversions dans l'ordre d'élution des farnésène et caryophyllène chromatographiés sur Apiezon L en passant de 125 à 175° interprète ces faits sur la base d'une évolution différente des courbes de tension de vapeur en fonction de la température.

Au cours de l'examen sur phase stationnaire de graisse de silicone, de mélanges contenant des paraffines et des cycloparaffines, nous avons aussi observé qu'une augmentation de la température retarde de façon systématique les cycloparaffines par rapport aux paraffines et provoque parfois des inversions dans l'ordre d'élution. Ce comportement différent des paraffines et des cycloparaffines présente un intérêt certain en analyse qualitative car il permet, en réalisant des chromatographies à différentes températures, de distinguer les deux types d'hydrocarbures.

PARTIE EXPÉRIMENTALE

Le chromatographe utilisé est un appareil "Perkin-Elmer" modèle 116, équipé d'un détecteur à thermistances; le gaz porteur est l'hélium. Les colonnes constituées de tube de cuivre de $^1/_4$ pouce sont garnies de graisse de silicone "Dow Corning

High Vacuum Grease" déposée sur Firebrick C_{22} à raison de 20 % en poids; la longueur des colonnes est de 18 mètres.

Les hydrocarbures mis en oeuvre au cours de ce travail sont d'origine commerciale ou ont été synthétisés par des méthodes classiques de la chimie organique. La pureté des hydrocarbures a été vérifiée par chromatographie analytique en phase gazeuse et, quand cela s'est avéré nécessaire, la purification a été réalisée par chromatographie préparative en phase gazeuse. La caractérisation des produits préparés a été faite à l'aide des températures d'ébullition, des indices de réfraction et des spectres infrarouges, ces différentes propriétés étant comparées à celles données dans les tables et les catalogues de l'American Petroleum Institute[6,7].

ASPECT THÉORIQUE

La Fig. 1 montre l'influence de la température sur l'ordre d'élution des paraffines et des cycloparaffines dans le cas d'un mélange composé de *n*-heptane (H), de 2,2-diméthylhexane (2,2-DMh), de méthylcyclohexane (MCh) et de 2,5-diméthylhexane (2,5-DMh). On remarque que si à 70° l'ordre d'élution est H, 2,2-DMh, MCh, 2,5-DMh, il devient à 130°: H, 2,2-DMh, 2,5-DMh, MCh; il y a donc inversion dans l'ordre d'élution des méthylcyclohexane et 2,5-diméthylhexane.

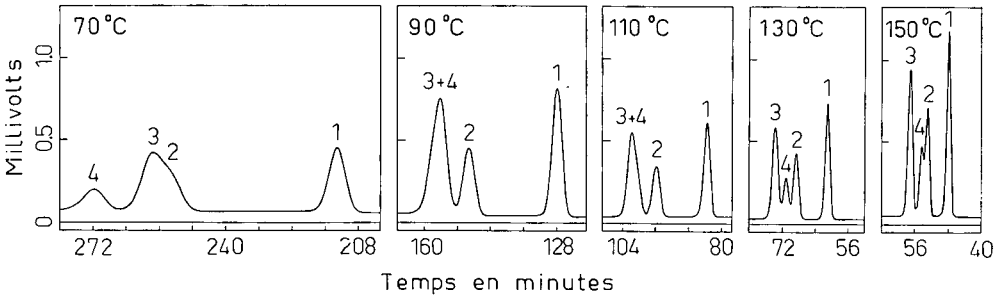


Fig. 1. Influence de la température sur l'ordre d'élution des *n*-heptane (1), 2,2-diméthylhexane (2), méthylcyclohexane (3) et 2,5-diméthylhexane (4) chromatographiés sur graisse de silicone.

La façon la plus simple d'expliquer ce phénomène est de considérer la rétention relative, α, et d'exprimer les coefficients de partage de la façon définie par PORTER et coll.[8] et PIEROTTI et coll.[9]. Dans ces conditions, pour deux solutés 1 et 2, on peut écrire, 1 étant le soluté de référence:

$$\alpha_{1,2} = \frac{(t_r - t_A)_2}{(t_r - t_A)_1} = \frac{K_2}{K_1} = \frac{\gamma_1 p^\circ_1}{\gamma_2 p^\circ_2}$$

Dans cette expression:

$t_r - t_A$ est la différence entre le temps de rétention expérimental et le temps de rétention de l'air; nous avons assimilé aux temps de rétention, les distances de rétention mesurées directement sur le chromatogramme;

K est le coefficient de partage;

p° est la tension de vapeur du soluté pur à la température opérationnelle;

γ est le coefficient d'activité du soluté dans la phase stationnaire considérée.

Nous avons, en vue de l'application de la formule donnée ci-dessus, choisi le *n*-heptane comme élément de référence. C'est donc par rapport à cet hydrocarbure que nous aurons à considérer l'évolution, en fonction de la température, des temps de rétention, tensions de vapeur et coefficients d'activité.

Les temps de rétention relatifs sont déduits directement des chromatogrammes.

Les tensions de vapeur ont été calculées aux diverses températures mises en oeuvre au moyen de la formule d'Antoine:

$$\log_{10} p^\circ = A - \frac{B}{C + t}$$

dans laquelle p°, tension de vapeur, est exprimée en mm Hg et t, température, en °C. Les valeurs des coefficients A, B et C ont été extraites des tables de l'American Petroleum Institute[6].

TABLEAU I

CALCUL DES TEMPS DE RÉTENTION RELATIFS AU *n*-HEPTANE ET DES RAPPORTS DES PRESSIONS DE VAPEUR ET DES COEFFICIENTS D'ACTIVITÉ

Temp. (°C)	$\frac{(t_r - t_A)_X}{(t_r - t_A)_H} = T_R{}^H$			$\frac{p^\circ_H}{p^\circ_X}$			$\frac{\gamma_H}{\gamma_X}$		
	2,2-DMh	*MCh*	*2,5-DMh*	*2,2-DMh*	*MCh*	*2,5-DMh*	*2,2-DMh*	*MCh*	*2,5-DMh*
70	1.20	1.22	1.29	1.30	1.05	1.41	0.92	1.16	0.91
90	1.19	1.25	1.26	1.28	1.07	1.38	0.93	1.17	0.91
110	1.17	1.26	1.26	1.27	1.08	1.35	0.92	1.17	0.93
130	1.16	1.27	1.21	1.26	1.10	1.33	0.92	1.15	0.91
150	1.15	1.28	1.19	1.25	1.11	1.31	0.92	1.15	0.91

A partir de la connaissance des rapports des temps de rétention et des tensions de vapeur, on détermine les valeurs des rapports des coefficients d'activité. Le Tableau I rend compte des valeurs des différents rapports et la Fig. 2 traduit leur évolution.

D'après la formule précédente on peut écrire que:

$$\log \alpha_{1,2} = \log \frac{p^\circ_1}{p^\circ_2} + \log \frac{\gamma_1}{\gamma_2}$$

Dans ces conditions, l'inversion qui correspond à une intersection des deux droites de $\log \alpha_{1,2}$ en fonction de la température absolue $(1/T)$ (Fig. 2B), dépend de deux facteurs:

(a) la valeur et l'évolution des tensions de vapeur avec la température;

(b) la valeur et l'évolution éventuelle des coefficients d'activité avec la température.

Or, l'expérience montre (Tableau I et Fig. 2A) que les rapports des coefficients d'activité ne dépendent pas de la température. Dans un système de solvant et solutés non polaires, les forces de cohésion se résument aux seules forces de dispersion qui sont indépendantes de la température. Ces forces de dispersion sont néanmoins présentes et le fait que les rapports des coefficients d'activité soient inférieurs ou

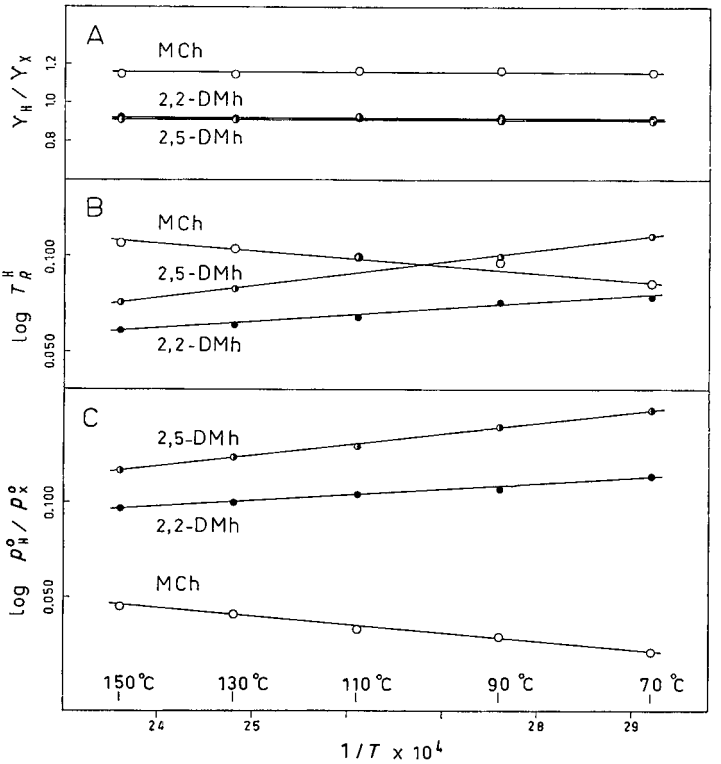


Fig. 2. Evolution en fonction de la température des rapports des coefficients d'activité (A), des temps de rétention relatifs au *n*-heptane (B) et des pressions de vapeur (C).

supérieurs à l'unité suivant que l'on a affaire aux paraffines ramifiées ou au méthylcyclohexane est probablement lié aux grandeurs respectives des molécules. En effet, dans le cas du squalane, phase stationnaire qui ne met également en jeu que des forces de dispersion, les cycloparaffines sont aussi retardées par rapport aux paraffines et ceci serait dû, d'après DESTY ET SWANTON[10], au fait que les molécules des cycloparaffines, plus petites que les molécules des paraffines à même nombre d'atomes de carbone se logeraient plus facilement dans le réseau du solvant et seraient retenues plus longtemps.

Par ailleurs, le coefficient angulaire des droites représentant les paraffines ramifiées est positif, tandis que celui de la droite du méthylcyclohexane est négatif.

Il en résulte que l'inversion est conditionnée d'une part par les courbes des tensions de vapeur (valeur et pente, Fig. 2C) et d'autre part, par le rapport des coefficients d'activité.

D'après les résultats qui vont suivre, il semble que ce phénomène soit tout à fait général pour des mélanges de paraffines et de cycloparaffines.

ASPECT PRATIQUE

Ce comportement particulier des paraffines et des cycloparaffines en fonction de la température, présente un intérêt certain en analyse qualitative.

Soit un mélange composé des hydrocarbures normaux, ramifiés et cycliques suivants: 2-méthylbutane (1), *n*-pentane (2), 2-méthylpentane (3), cyclopentane (4), 3-méthylpentane (5), *n*-hexane (6), méthylcyclopentane (7), 2-méthylhexane (8), cyclohexane (9), 3-méthylhexane (10), *n*-heptane (11), 2,2-diméthylhexane (12), 2,5-diméthylhexane (13), méthylcyclohexane (14), 2-méthylheptane (15), 1,1,2-triméthylcyclopentane (16), 3-méthylheptane (17), 1,1-diméthylcyclohexane (18), *n*-octane (19), 2,2-diméthylheptane (20), 2,6-diméthylheptane (21), propylcyclopentane (22), éthylcyclohexane (23), 1,1,4-triméthylcyclohexane (24), 2-méthyloctane (25), 3-méthyloctane (26), *n*-nonane (27), propylcyclohexane (28).

Ce mélange a été chromatographié sur une colonne de 18 mètres de graisse de silicone à 95, 110, 125 et 140°. Les valeurs des logarithmes des temps de rétention relatifs au 2-méthylheptane, choisi ici comme hydrocarbure de référence, sont groupées dans le Tableau II et portées en graphique en fonction de la température absolue ($1/T$) dans la Fig. 3; les droites ont été calculées par la méthode des moindres carrés.

On voit, d'après cette figure, que les droites correspondant aux différents hydrocarbures paraffiniques, comme celles correspondant aux différentes cycloparaffines, se présentent les unes et les autres comme des faisceaux de droites divergentes issues de deux points différents. Ce fait peut être mis à profit dans l'identification de mé-

TABLEAU II

LOGARITHMES DES TEMPS DE RÉTENTION RELATIFS AU 2-MÉTHYLHEPTANE À DIVERSES TEMPÉRATURES

Hydrocarbures	*95°*	*110°*	*125°*	*140°*
2-Méthylbutane	—0.879	—0.813	—0.750	—0.688
n-Pentane	—0.807	—0.750	—0.690	—0.635
2-Méthylpentane	—0.588	—0.548	—0.502	—0.462
Cyclopentane	—0.588	—0.536	—0.483	—0.438
3-Méthylpentane	—0.542	—0.502	—0.460	—0.421
n-Hexane	—0.499	—0.462	—0.426	—0.392
Méthylcyclopentane	—0.393	—0.357	—0.321	—0.286
2-Méthylhexane	—0.292	—0.272	—0.250	—0.229
Cyclohexane	—0.288	—0.251	—0.223	—0.198
3-Méthylhexane	—0.264	—0.251	—0.223	—0.198
n-Heptane	—0.198	—0.184	—0.170	—0.157
2,2-Diméthylhexane	—0.126	—0.114	—0.104	—0.094
2,5-Diméthylhexane	—0.101	—0.087	—0.084	—0.076
Méthylcyclohexane	—0.101	—0.087	—0.067	—0.051
2-Méthylheptane	0	0	0	0
1,1,2-Triméthylcyclopentane	0.018	0.025	0.033	0.041
3-Méthylheptane	0.022	0.025	0.023	0.022
1,1-Diméthylcyclohexane	0.086	0.092	0.097	0.100
n-Octane	0.099	0.092	0.082	0.075
2,2-Diméthylheptane	0.161	0.151	0.140	0.130
2,6-Diméthylheptane	0.188	0.176	0.162	0.149
Propylcyclopentane	0.207	0.199	0.190	0.183
Ethylcyclohexane	0.216	0.213	0.206	0.200
1,1,4-Triméthylcyclohexane	0.237	0.236	0.229	0.227
2-Méthyloctane	0.289	0.270	0.247	0.227
3-Méthyloctane	0.309	0.290	0.267	0.247
n-Nonane	0.385	0.362	0.331	0.305
Propylcyclohexane	0.487	0.462	0.437	0.411

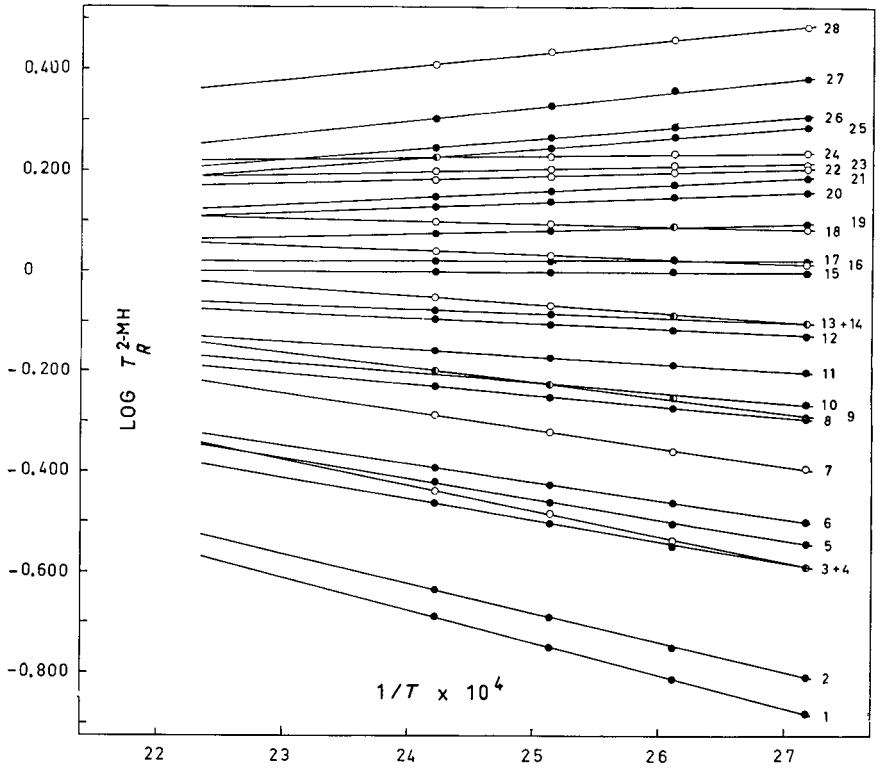


Fig. 3. Logarithmes des temps de rétention relatifs au 2-méthylheptane en fonction de la température (pour la signification des nombres: voir texte).

langes complexes en permettant de classer directement les composés dans l'une ou l'autre catégorie d'hydrocarbures.

Une autre méthode plus simple pour arriver à ce résultat, consiste à porter les logarithmes des temps de rétention à une température en fonction des logarithmes des temps de rétention à une autre température. Comme le montre la Fig. 4, le diagramme résultant rappelle ceux que l'on obtient en portant les logarithmes des temps de rétention sur une phase stationnaire en fonction des logarithmes des temps de rétention sur une autre phase stationnaire et permet directement de faire le classement en hydrocarbures paraffiniques et cycloparaffiniques.

Cette façon de distinguer les paraffines des cycloparaffines est utile aux hautes températures, domaine où les phases stationnaires les plus sélectives sont inutilisables. Mais c'est surtout dans le cas de mélanges complexes où l'un des deux types d'hydrocarbures est présent en forte concentration et l'autre à l'état de traces, que le procédé présente le plus d'intérêt. En effet, en opérant à des températures de plus en plus élevées, on obtient, tout en modifiant le profil d'élution, des pics de plus en plus effilés et on peut mettre en évidence des hydrocarbures présents en très faible concentration. Au cours d'un travail ayant pour objet la connaissance de la composition des goudrons de carbonisation des houilles à basse température, nous avons pu ainsi réaliser des analyses détaillées de fractions paraffiniques complexes renfermant

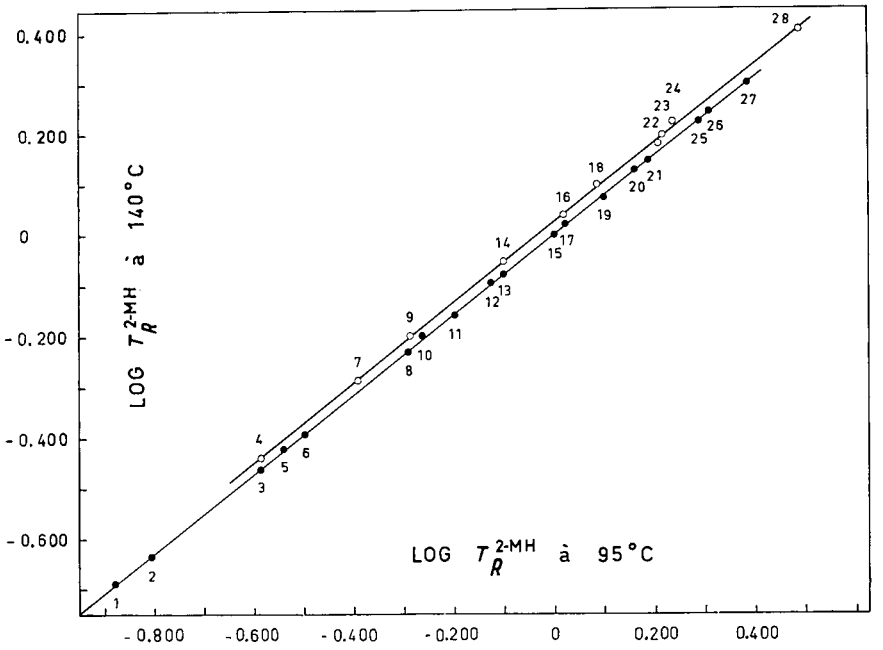


Fig. 4. Log $T_R^{2\text{-MH}}$ à 95° en fonction log $T_R^{2\text{-MH}}$ à 140° (pour la signification des nombres: voir texte).

jusqu'à onze atomes de carbone[11] et y déceler des traces de paraffine masquées, à certaines températures, dans un pic important correspondant à une cycloparaffine et inversement.

RÉSUMÉ

En chromatographie en phase gazeuse réalisée sur graisse de silicone, une augmentation de la température a pour effet de retarder les cycloparaffines par rapport aux paraffines et provoque même, dans certains cas, des inversions dans l'ordre d'élution.

En réalisant des chromatographies à deux températures, on peut donc distinguer les paraffines des cycloparaffines. Ce procédé est particulièrement utile aux hautes températures et dans le cas de mélanges complexes où l'un des deux types d'hydrocarbure est présent en forte concentration et l'autre à l'état de traces.

Le comportement particulier des paraffines et cycloparaffines est probablement lié à une évolution différente des pressions de vapeur en fonction de la température.

SUMMARY

In gas–liquid chromatography carried out on silicone grease, an increase in temperature retards the cycloparaffins in relation to the paraffins and, in some cases, even brings about inversions in the order of elution.

By chromatographing at two temperatures, it is therefore possible to distin-

guish the cycloparaffins from the paraffins. This process is particularly useful at high temperatures and in the case of complex mixtures in which there is a high concentration of one of the two types of hydrocarbon and only traces of the other.

The particular behaviour of the paraffins and cycloparaffins is probably linked with a different evolution of the vapour pressures in relation to the temperature.

BIBLIOGRAPHIE

1 M. R. HOARE ET J. H. PURNELL, *Research*, 8 (1955) S 41.
2 M. R. HOARE ET J. H. PURNELL, *Trans. Faraday Soc.*, 52 (1956) 222.
3 J. H. PURNELL, in D. H. DESTY (Editor), *Vapour Phase Chromatography*, Butterworths, London, 1957, p. 52.
4 B. D. BLAUSTEIN, C. ZAHN ET G. PANTAGES, *J. Chromatog.*, 12 (1963) 104.
5 J. B. ROBERTS, *Nature*, 193 (1962) 1071.
6 *Selected Values of Properties of Hydrocarbons*, American Petroleum Institute, Research Project 44, Carnegie Press, Pittsburgh, Pa., 1953.
7 *Catalog of Infrared Spectral Data*, American Petroleum Institute, Research Project 44, Agric. and Mech. College of Texas, College Station, Texas.
8 P. E. PORTER, C. H. DEAL ET F. H. STROSS, *J. Am. Chem. Soc.*, 78 (1956) 2999.
9 G. J. PIEROTTI, C. H. DEAL, E. L. DERR ET P. E. PORTER, *J. Am. Chem. Soc.*, 78 (1956) 2989.
10 D. H. DESTY ET W. T. SWANTON, *J. Phys. Chem.*, 65 (1961) 766.
11 J. BRICTEUX, *Thèse de doctorat*, Université de Liège, 1965.

ZUR GASCHROMATOGRAPHISCHEN TRENNUNG VON ALIPHATISCHEN, SAUERSTOFFHALTIGEN SUBSTANZEN IN WASSER

III. EIN NEUES TRENNMATERIAL ZUM NACHWEIS KLEINER METHANOLMENGEN IN WÄSSERIGEN LÖSUNGEN

U. P. SCHLUNEGGER
Gerichtlich-Medizinisches Institut, Universität Bern (Schweiz)
(Eingegangen den 19. November 1965)

EINLEITUNG

Auf der Suche nach stationären Phasen zur Trennung der niedermolekularen, sauerstoffhaltigen Substanzen in Wasser liess sich zeigen, dass Materialien des Typs Nonylphenol-glycerinäther recht gute Trenneigenschaften mit Wasser- und Temperatur-Beständigkeiten vereinigen. Allerdings wird die Trennfähigkeit dieser stationären Phasen stark durch Wasser beeinträchtigt. Wässerige Lösungen mit kleinern Substanzgehalten als *ca.* 1 % werden infolge eines verstärkten "tailings" nur mehr schlecht aufgetrennt. Nach einer systematischen Untersuchung des Einflusses von verschiedenen Substituenten am aromatischen Kern ergab sich dann, dass für den Nachweis und die Auftrennung kleinster Substanzmengen in Wasser — weniger als $1^0/_{00}$ — ein verätherter Dihydroxyalkylbenzol geeignet sein dürfte, d.h. etwa ein verätherter Alkylresorcin[1].

Wir synthetisierten deshalb 4-Isooctyl-1,3-bis-(dihydroxypropanoxy)-benzol (Octylresorcin-glycerinäther, ORGA) durch Acylierung von Resorcin nach FRIEDEL-CRAFTS[2], Reduktion des Keto-Körpers nach CLEMMENSEN[3] und anschliessender Verätherung der beiden Phenolgruppen mit Glycerin nach WILLIAMSON[4]. Die einzelnen Synthese-Zwischenprodukte wurden jeweils rein dargestellt und die Reaktionsabläufe dünnschichtchromatographisch verfolgt.

Im Folgenden sind nun einige Eigenschaften dieses Materials — im Weiteren ORGA genannt — beschrieben und besonders die gaschromatographischen Trennfähigkeiten an ausgewählten Testgemischen dargelegt.

APPARATIVES

Gaschromatograph: Beckman GC-2 mit Flammenionisations-Detektor. 10 ft. Stahlsäule: 3/16 in. Durchmesser, Säulentemperatur 70°. Trägergas: Stickstoff, Eingangsdruck 2 Atü. Trägermaterial: Chromosorb A-NAW, 60–80 mesh (Johns-Manville), Belegungsdichte 10 % flüssige Phase.

RESULTATE

1. *Einfluss des Trägermaterials*

Versuche zeigten, dass sich weitgehend inaktivierte Trägermaterialien, wie Chromosorb G-AWDMCS, nur unter Schwierigkeiten mit ORGA imprägnieren liessen. Demzufolge resultierten auch ausgesprochen schlechte Auftrennungen der Testgemische im Gaschromatographen. Auch aktivere Träger der Chromosorb-Reihe wie W-AWHMDS, W-AW und P-HMDS, wie auch Kieselgur (Merck) zeigten ein analoges Verhalten. Immerhin zeichnete sich eine Verbesserung der Trennfähigkeiten mit zunehmender Aktivität des Trägers ab. Die ersten annehmbaren Resultate lieferte Chromosorb P-MS. Mit Abstand die besten Resultate liessen sich aber unter Verwendung von Chromosorb A-NAW erzielen, das sich ohne Schwierigkeiten mit einer Lösung des — teilweise wasserlöslichen — ORGA in Aceton imprägnieren liess. Dieses Trennmaterial wurde demzufolge für die weiteren Untersuchungen beibehalten.

2. *Selektivität*

Zum Abschätzen der Selektivität von ORGA wurde die Elutionsreihenfolge einiger Substanzen mit ähnlichen Siedepunkten, aber unterschiedlichen Strukturen und Polaritäten ermittelt. Diese ist in der Tabelle I zusammengestellt:

3. *Auflösegrade*

Die Auflösegrade[5] einiger schwieriger zu trennenden Stoffgemische wurden bei 70° mit dem Flammenionisations-Detektor bestimmt. Es wurden jeweils 5 μl von wässerigen Lösungen eingespritzt, die je 1 Promille der einzelnen Komponenten des zu analysierenden Stoffpaares enthielten. Die erhaltenen Werte sind in Tabelle II

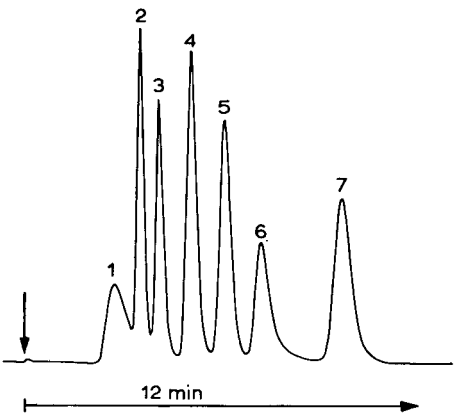


Fig. 1. Auftrennung diverser Substanzen in Wasser (je 0.1‰) bei 70° (Beckman GC2; 10 ft. Stahlsäule 3/16 in.; 10% ORGA auf Chromosorb A-NAW 60–80 mesh; N_2: 35 p.s.i. Eingangsdruck). 1 = Acetaldehyd; 2 = Äthylformiat; 3 = Methylacetat; 4 = Aceton; 5 = Äthylacetat; 6 = Methanol; 7 = Äthanol.

zusammengestellt. Ausserdem ist in Fig. 1 ein Chromatogramm eines Gemisches diverser Substanzen in Wasser (je 0.1‰) abgebildet.

TABELLE I

ELUTIONSREIHENFOLGE DIVERSER SUBSTANZEN AUF ORGA

	Kp	*Reihenfolge*	*Retentionszeit (Min.)*
Äthylacetat	77.15	3	6.6
Äthanol	78.5	6	11.5
Methyläthylketon	79.6	5	9.6
Benzol	80.1	2	5.9
Propylformiat	81.3	4	6.7
Cyclohexan	81.4	1	3.6
Isopropanol	82.3	7	12.4

TABELLE II

AUFLÖSEFAKTOREN VON EINIGEN SUBSTANZPAAREN IN WASSER (JE 1‰) BEI 70°, EINSPRITZMENGE 5 μl

Stoffgemisch		*Auflösefaktor*
Methanol und	Äthanol	0.96
	Methyläthylketon	0.70
	Äthylacetat	0.94
	Propylformiat	0.94
	Aceton	1
Aceton und	Äthylacetat	0.97
	Propylformiat	0.97
	Methylacetat	0.97
	Äthylformiat	1.0
	Acetaldehyd	1.0
Äthanol und	Isopropanol	0.06
	tert.-Butanol	0.10
	Methyläthylketon	0.90

TABELLE III

AUFLÖSEFAKTOREN NICHT VOLL AUFGETRENNTER SUBSTANZPAARE IN ABHÄNGIGKEIT VON DER KONZENTRATION IN WASSER

Konzentration (pro Substanz, ‰)	*Acetaldehyd–Äthylformiat*	*Aceton–Äthylacetat*
10	0.87	0.97
1	0.86	0.97
0.1	0.85	0.96
0.01	0.83	0.94
0.005	0.82	0.5

4. *Temperaturstabilität*

ORGA lässt sich im Vakuum (10^{-4} mm Hg) bei 200–220° überdestillieren, wobei allerdings mit einer geringgradigen Zersetzung zu rechnen ist (Wasserabspaltung?). Bei Temperaturen bis zu 180° scheint ORGA dagegen beständig zu sein.

Die mit ORGA imprägnierten Trennmaterialien wurden jeweils 12 h bei 130° unter Stickstoff ausgeheizt. Entsprechende Versuche zeigten, dass diese Behandlung keine Verschlechterung der Trennfaktoren bewirkte. Auch eine längere Ausheizzeit von 48 Std. bei 130° ergab keine Veränderung der Trennfähigkeiten.

5. *Chemische Eigenschaften*

ORGA kann in alkoholischer NaOH oder HCl — (mit oder ohne Wasser) — ohne merkbare Zersetzung gekocht werden. Sauerstoff scheint nur in stark alkalischem Milieu auf nicht voll verätherte Bestandteile (Phenolgruppen) einen Einfluss zu haben. ORGA ist gut löslich in Methanol, Äthanol, Aceton und Methylenchlorid, teilweise löslich in Wasser, wenig oder nicht löslich in apolaren, organischen Lösemitteln.

ORGA ist eine bei Zimmertemperatur sehr zähflüssige, klebrige Masse, deren Viskosität mit steigender Temperatur rasch abnimmt.

6. *Einfluss des Wassers auf die Trennfähigkeit*

Die Beeinflussung der Trennfähigkeit durch Wasser wurde anhand der Trennfaktoren bestimmter nicht ganz aufgetrennter Substanzpaare verfolgt. Als Beispiel seien die Auflösegrade der beiden Gemische Acetaldehyd–Äthylformiat und Aceton–Äthylacetat bei diversen Konzentrationen in Wasser aufgeführt (Tabelle III).

DISKUSSION

Nach der Elutions-Reihenfolge von Substanzen verschiedener Polarität (Abschnitt 2), wie auch aus Fig. 1 ist zu schliessen, dass ORGA eine ausgeprägte Selektivität für alkoholische Verbindungen aufweist. Diese Eigenschaft ist nach früheren Untersuchungen[1] vor allem auf die beiden Phenol-glycerinäther-Gruppen ($R–O–CH_2–CHO–CH_2OH$) zurückzuführen. Diese stark polaren Stellen im Molekül — insbesondere die vier freien Hydroxylgruppen — sind verantwortlich für die verhältnismässig kleine Flüchtigkeit, die teilweise Löslichkeit von ORGA in Wasser, aber auch für die auf *ca.* 180° begrenzte Temperaturstabilität (Abschnitt 4 und 5). Ebenso wird die Tatsache erhellt, dass sich nur aktivere Trägermaterialien mit ORGA gut belegen lassen und annehmbare Trennleistungen ergeben (Abschnitt 1). Die besten Resultate bezüglich Trennung und kurzer Analysenzeit wurden entsprechend mit Chromosorb A erzielt.

Wie aus Abschnitt 3 hervorgeht, lassen sich die niederen Aldehyde, Alkohole, Ketone, sowie die Ester mit 3 und 4 C-Atomen innerhalb von *ca.* 12 Min. ohne weiteres direkt in wässerigen Lösungen von Methanol abtrennen (Tabelle II). Das Methanol selbst weist auch bei kleinen Konzentrationen nur ein verhältnismässig schwaches "tailing" auf. Aber auch andere Substanzen, wie etwa das Aceton, werden praktisch vollständig von benachbarten Substanzen abgetrennt. Dagegen gelingt die Auflösung von Äthanol, Isopropanol und *tert.*-Butanol nur unvollständig oder nicht.

Bemerkenswerterweise nimmt die Trennfähigkeit der ORGA-Säule mit stei-

gender Verdünnung der zu analysierenden, wässerigen Lösungen bis zu 10 p.p.m. nur unmerklich ab, wie dies aus Abschnitt 6 ersichtlich ist. Die Auflösefaktoren werden mit zunehmendem Wassergehalt nur um wenige Prozente kleiner und auch das "tailing" des Methanols wird nicht wesentlich erhöht. Erst bei Konzentrationen unter 10 p.p.m. (0.01‰) ist mit einer störenden Verkleinerung der Trennfaktoren zu rechnen. Demzufolge eignet sich ORGA als stationäre Phase zur direkten Bestimmung z.B. von Methanol in wässerigen Lösungen bis hinunter zu Konzentrationen von mehreren p.p.m.

DANK

Meinen technischen Mitarbeitern, Fräulein CHRISTA BECKER und Herrn A. BUSSLINGER, sei für die Hilfe bei der Synthese und den gaschromatographischen Überprüfungen bestens gedankt.

ZUSAMMENFASSUNG

Isooctyl-1,3-bis-(dihydroxypropanoxy)-benzol ("Octylresorcinglycerinäther") kann als stationäre Phase zur direkten gas-chromatographischen Bestimmung von kleinen Methanolmengen in wässerigen Lösungen (p.p.m.-Bereich) verwendet werden. Diese und einige weitere Eigenschaften des neuartigen Trennmaterials wurden beschrieben.

SUMMARY

Iso-octyl-1,3-bis-(dihydroxypropanoxy)-benzene ("Octylresorcinol glycerol ether") is suitable as stationary phase for the direct gas-chromatographic determination of small amounts of methanol in water (p.p.m. range). This and some further properties of the new stationary phase are reported.

LITERATUR

1 U. P. SCHLUNEGGER, *J. Chromatog.*, 20 (1966) 452.
2 E. BERLINER, *Org. Reactions*, 5 (1949) 229.
3 J. C. BELL, W. BRIDGE UND A. ROBERTSON, *J. Chem. Soc.*, (1937) 1544.
4 *Organikum*, Technische Universität Dresden, Deutscher Verlag der Wissenschaften Berlin, 1964, S. 167.
5 U. P. SCHLUNEGGER, *J. Chromatog.*, 20 (1966) 23.

ÉTUDE D'UNE RÉACTION D'ÉCHANGE ISOTOPIQUE DE L'IODE PAR RADIOCHROMATOGRAPHIE GAZEUSE*

R. MACHIROUX
Laboratoire de Chimie Analytique, Université de Liège (Belgique)
(Reçu le 9 septembre 1965)

INTRODUCTION

L'échange isotopique d'iode entre les iodures d'alkyle, l'iode moléculaire et l'ion I^- a permis de mettre en évidence trois types principaux de mécanismes de réaction (Tableau I).

TABLEAU I

MÉCANISMES D'ÉCHANGE D'IODE

Type de réaction	*Nature du mécanisme*	*Equation cinétique*	$\Delta k/\Delta\varepsilon$	*Influence de la lumière*	*Influence de la structure*[a]	*Réf.*
$RI-I_2$	Formation du complexe RI_3:					
	(a) avec échange intramoléculaire	$R = k[RI]\,[I_2]$	> 0	—	+	(1) (2) (3)
	(b) avec échange intermoléculaire	$R = k[RI]\,[I_2]^2$	> 0	nulle	+	(3)
	Réaction par radicaux libres	$R = k[RI]\,[I_2]^{1/2}$	> 0	accélération	+	(4)
$RI-I^-$	Réaction ion-dipole $I^{-*}\cdots R—I \rightarrow I^*—R\cdots I^-$	$R = k[RI]\,[NaI]$	< 0	—	—	(5) (6) (7) (8)

[a] + = accélération par allongement ou ramification de la chaîne carbonée; — = ralentissement par allongement ou ramification de la chaîne carbonée.

Nous nous sommes posé la question de savoir si deux iodures d'alkyle, dissous dans un solvant, étaient susceptibles d'échanger leur atome d'iode et par quel type de mécanisme ce processus pouvait avoir lieu. Il est apparu que la chromatographie en phase gazeuse constituait la technique la plus adéquate pour apporter une réponse à ces questions.

* L'appareillage utilisé au cours de ce travail a fait l'objet d'une publication distincte dans l'*Ind. Chim. Belge* (sous presse).

TECHNIQUE EXPÉRIMENTALE

Réactifs et solvants

Les réactifs commerciaux (CH_3I et C_2H_5I, p.a.) ont subi le cycle de purification suivant:

(a) lavage au Na_2CO_3 dilué;
(b) lavage à l'eau distillée jusqu'à neutralité;
(c) desiccation sur Na_2SO_4 sec;
(d) fractionnement par distillation.

Les produits purifiés ont été conservés à l'abri de la lumière et de l'humidité. Juste avant la préparation des solutions, ils ont subi un transfert sous vide, en présence de charbon actif, à la température de N_2 liquide.

La pureté des iodures d'alkyle a été vérifiée par spectrophotométrie dans le visible (traces d'iode libre $< 3 \cdot 10^{-3}$ m %) et par chromatographie gazeuse (C_2H_5I dans $CH_3I < 0.01$ %).

Les solvants ont été soigneusement distillés et déshydratés par les moyens courants (CaO, Mg, P_2O_5). Leur teneur en eau a été déterminée par la méthode de Karl Fisher ($C_{H_2O} < 10^{-3}$ % en poids).

Choix de l'isotope et marquage de C_2H_5I

Parmi les isotopes radioactifs de l'iode, notre choix s'est porté sur ^{125}I, émetteur γ de 0.035 MeV d'énergie et de 57 jours de période. A côté de la faible énergie de son rayonnement, cet isotope présente l'avantage de posséder une période assez longue pour permettre de négliger la correction de décroissance.

Le marquage de l'iodure d'éthyle a été réalisé par synthèse directe (alcoolyse de PI_3*). L'iodure d'éthyle marqué a subi le même cycle de purification que les réactifs inactifs. Sa pureté a été contrôlée uniquement par chromatographie en phase gazeuse.

Conditions de séparation

Pour la séparation des iodures d'alkyle, la littérature[9–11] préconise l'emploi de phases liquides de faible polarité (esters, Octoil S, etc.). Ces liquides stationnaires donnent cependant lieu à des séparations incomplètes lorsque, à côté des iodures d'alkyle, on tente de séparer des solvants polaires (alcools, acétonitrile, acétone, etc.). Dans pareils cas, force nous a été de préparer des colonnes polaires pour retarder sélectivement les corps à moment dipolaire élevé. Ceci ne va pas sans affecter les pics des iodures d'une légère traînée, insuffisante cependant pour altérer les mesures quantitatives. La Fig. 1 montre les pics inactifs et radioactifs des iodures de méthyle et d'éthyle obtenus dans les conditions suivantes:

(a) *Conditions de séparation*
Temp.: 58°
Colonne: 2 m, 20 % en poids de Carbowax 1500, Fire-Brick 60–80 m/in.
Gaz porteur: He, 28.5 ml/min NTP
Détecteur à ionisation: HT = 300 V
Electromètre: V_{ch} = 4.5 V
Att. × 200

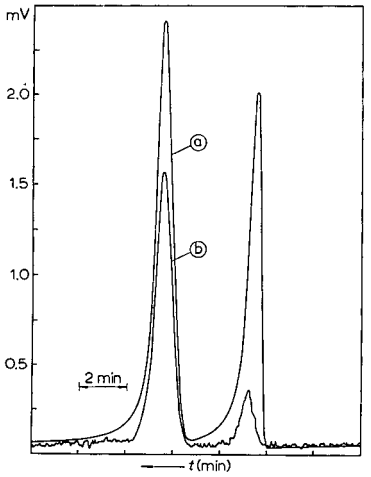


Fig. 1. Chromatogrammes inactif (a) et radioactif (b) d'une solution alcoolique de CH_3I et C_2H_5I.

(*b*) *Conditions de mesure radiométrique*
Photomultiplicateur: HT = 1160 V
Ratemeter: gamme 300 c.p.s.
c^te de temps 5 sec

Le pic du solvant (alcool éthylique), fortement retardé ne figure pas sur l'enregistrement.

Expériences d'échange

Les réactifs et solvants destinés à la préparation des solutions ont subi le cycle de purification immédiatement avant l'emploi.

Les concentrations ont été déterminées par pesée. Les solutions ont ensuite été réparties entre une douzaine d'ampoules en Pyrex par fractions de 0.2 ml environ. Les ampoules ont été scellées sous vide après un cycle de dégazage destiné à éliminer les gaz dissous. Les ampoules ont été conservées à l'abri de la lumière dans un mélange acétone–carboglace. Elles ont alors été introduites séparément, pour des laps de temps connus, dans un thermostat à l'huile, à 92.4 ± 0.05°. A leur sortie du thermostat les ampoules ont été plongées rapidement dans l'azote liquide pour arrêter la réaction. Après ouverture, les solutions ont été conservées dans le mélange réfrigérant, à l'abri de la lumière.

Chaque échantillon a été mesuré en double exemplaire, nous avons respectivement mesuré, au planimètre, les surfaces des pics inactifs et radioactifs correspondants. Pour mémoire, rappelons que l'activité spécifique a été mesurée, à une constante près, par le rapport des surfaces du pic inactif et radioactif correspondant.

Traitement des données expérimentales

La connaissance des activités spécifiques à l'origine (AS_0), au temps t (AS) et à l'équilibre (AS_∞) permet de calculer la fraction échangée F.

$$F = \frac{(AS) - (AS_0)}{(AS_\infty) - (AS_0)}$$

Les valeurs de F associées aux valeurs de t et des concentrations conduisent à la valeur de R, vitesse d'échange, pour un couple déterminé de concentrations A et B (CH_3I et C_2H_5I).

$$R = -\frac{1}{t}\frac{[A]\,[B]}{[A] + [B]} \ln (1 - F)$$

En fait, nous avons calculé R en mesurant la pente des droites $-\ln (1 - F) = f(t)$.

Les vitesses d'échange obtenues pour des concentrations variables nous fourniront la relation qui unit ces grandeurs (ordre de la réaction). De la formule:

$$R = k[A]^m[B]^n$$

il sera possible de tirer la valeur de k (constante de vitesse) dans différents milieux (variation de ε). L'ensemble de ces données nous permettra de tirer des conclusions quant à la nature du mécanisme de la réaction d'échange.

RÉSULTATS EXPÉRIMENTAUX

Échange dans le p-dioxane

Il ne nous a pas été possible d'observer d'échange isotopique dans ce milieu.

L'étude de la réaction dans le p-dioxane met en évidence les faits suivants:

(a) après 183 min de réaction, la fraction échangée est inférieure à 2 %;

(b) si on laisse évoluer le système pendant 16 h, la solution se colore fortement en brun (iode moléculaire);

(c) après ce temps de réaction, aucune activité n'est plus détectable dans les iodures et on assiste à une diminution de la surface des pics inactifs pour un volume injecté constant.

Ces expériences d'orientation mettent en évidence deux faits principaux:

(a) dans un solvant de basse constante diélectrique (ε = 2.070 à 92.4°), la réaction d'échange est extrêmement lente, pour ne pas dire nulle;

(b) les iodures subissent une décomposition chimique.

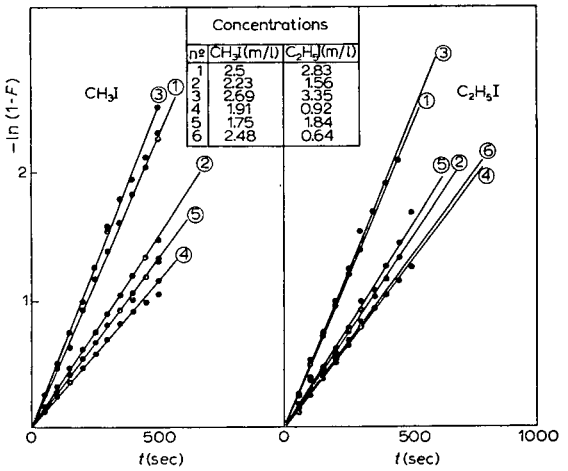


Fig. 2. Courbes d'échange — $\ln(1 - F) = f(t)$. Solvant: C_2H_5OH. Température: 92.4°.

TABLEAU II

VITESSE D'ÉCHANGE

No.	$[CH_3I]$ (m/l)	$[C_2H_5I]$ (m/l)	$R \times 10^3$ $(m \cdot l^{-1} \cdot sec^{-1})$	$R/[CH_3I] \times 10^3$ (sec^{-1})	$R/[C_2H_5I] \times 10^3$ (sec^{-1})
1	2.50	2.83	6.06–6.42	2.43–2.57	2.14–2.27
2	2.23	1.56	2.68	1.20	1.72
3	2.69	3.35	7.49	2.78	2.23
4	1.91	0.92	1.44–1.61	0.76–0.85	1.57–1.75
5	1.75	1.84	2.78	1.59	1.61
6	2.48	0.64	1.36	0.55	2.13

Échange dans C_2H_5OH

Dans ce milieu, nous avons pu observer une réaction d'échange typique. Dans la Fig. 2, nous avons rassemblé les courbes $-\ln(1 - F) = f(t)$ obtenues à concentrations variables. Dans le Tableau II, nous avons rassemblé les valeurs de R, calculées à partir de ces courbes et les valeurs de $R/[CH_3I]$ et $R/[C_2H_5I]$. Ces dernières ont été portées respectivement en fonction de C_2H_5I et de CH_3I.

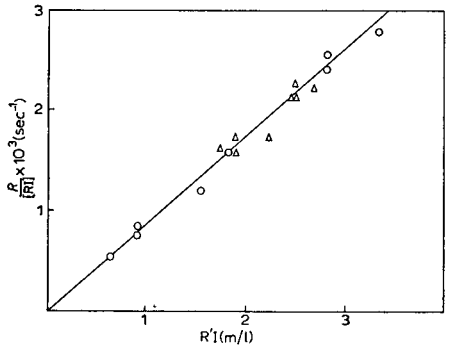


Fig. 3. Graphique $R/[RI] = f(R'I)$. (○) $RI \equiv CH_3I$; $R'I \equiv C_2H_5I$; (△) $RI \equiv C_2H_5I$; $R'I \equiv CH_3I$.

La Fig. 3 montre que les valeurs de $R/[RI]$ ($RI = CH_3I$ et C_2H_5I) se groupent autour d'une droite passant par l'origine et de pente exprimée par:

$$\Delta\,(R/[RI])/\Delta\,([R'I]) = (0.87 \pm 0.09)10^{-3}\ l \cdot m^{-1} \cdot sec^{-1}.$$

Influence de la constante diélectrique

Outre l'alcool éthylique, nous avons utilisé, comme solvants, l'acétonitrile et les alcools méthylique, *n*-propylique et isopropylique. Les valeurs des constantes diélectriques des alcools à 92.4° ont été extraites des travaux de DANNHAUSER ET BAHE[12], celle de CH_3-CN n'est connue qu'à 82°. Les valeurs de R sont réunies dans le Tableau III.

INTERPRÉTATION DES RÉSULTATS

Nature ionique du mécanisme

Les résultats obtenus dans le *p*-dioxane semblent assez nettement établis pour pouvoir éliminer, sans grand risque, deux types de mécanismes:

TABLEAU III

INFLUENCE DE LA CONSTANTE DIÉLECTRIQUE

	CH_3–CN ($\varepsilon = 25.9$)	CH_3OH ($\varepsilon = 21.7$)	n-C_3H_7OH ($\varepsilon = 12.3$)	iso-C_3H_7OH ($\varepsilon = 10.5$)
$[CH_3I]$ (m/l)	1.84	1.90	1.78	1.84
$[C_2H_5I]$ (m/l)	1.41	1.44	1.37	1.42
$R \times 10^3$ ($m \cdot l^{-1} \cdot sec^{-1}$)	0.42–0.43	0.38–0.60	1.29–1.46	1.26

(a) le mécanisme radicalaire.

(b) le mécanisme de formation de complexe.

A propos de ce dernier, il convient de faire la remarque suivante.

Les complexes RI_3 sont du type de transfert de charge. Il faudrait que, des deux iodures d'alkyle, l'un présentât le caractère de donneur et l'autre, d'accepteur de charge; ce qui nous paraît assez peu vraisemblable étant donné l'analogie de structure des deux corps.

Nous pensons donc bien pouvoir attribuer au mécanisme de la réaction, un caractère ionique. Cette affirmation repose sur deux observations:

(a) la réaction d'échange a lieu uniquement dans des solvants de constante diélectrique élevée;

(b) indépendamment de la réaction d'échange, nous avons observé une réaction secondaire qui s'interprète aisément sur la base de la dissociation ionique des iodures d'alkyle.

En effet dans tous les chromatogrammes inactifs des solutions dans les milieux hydroxylés, nous avons relevé les pics d'éthers-oxydes mixtes (R—O—R') et symétriques (R—O—R) dont on peut expliquer la formation par la séquence de réactions:

$R—OH \rightleftharpoons RO^- + H^+$ ($R = CH_3$, C_2H_5, n-C_3H_7, iso-C_3H_7)

$R'I \rightleftharpoons R'^+ + I^-$ ($R' = CH_3$, C_2H_5)

$RO^- + R'^+ \rightarrow R—O—R'$ (ou R—O—R: $CH_3—O—CH_3$,

$H^+ + I^- \rightleftharpoons HI$ $C_2H_5—O—C_2H_5$).

Cette réaction a déjà été étudiée par HECHT ET CONRAD[13] pour des solutions d'iodures de méthyle ou d'éthyle et d'éthylate de Na dans C_2H_5OH. Nous consacrerons un paragraphe particulier à ce phénomène.

Ordre de la réaction

SWART ET LE ROUX[5,6] ont montré que dans le cas de RI/NaI, la réaction d'échange est d'ordre global égal à 2; l'équation cinétique étant constituée d'un terme unique, même dans un solvant comme l'eau.

Les données du Tableau II et de la Fig. 3 sont compatibles avec une équation cinétique d'ordre global égal à 2.

$$R = k[CH_3I]\,[C_2H_5I]$$

avec $k = (0.87 \pm 0.09)10^{-3}\ l \cdot m^{-1} \cdot sec^{-1}$.

Ceci constitue un argument supplémentaire en faveur de la nature ionique du mécanisme.

Influence de la constante diélectrique

Un autre argument en ce sens peut être trouvé dans l'influence de la constante diélectrique du solvant sur la constante de vitesse k.

Sur la base de l'hypothèse d'un mécanisme d'ordre 2, nous avons calculé la constante de vitesse dans les différents solvants, à 92.4° et nous avons réuni les valeurs dans le Tableau IV.

TABLEAU IV

CONSTANTES DE VITESSE

	CH_3–CN	CH_3OH	C_2H_5OH	n-C_3H_7OH	iso-C_3H_7OH
ε à 92.4°	25.9	21.7	15.8	12.3	10.5
$k \times 10^3$ ($l \cdot m^{-1} \cdot sec^{-1}$)	0.16	0.33–0.26	0.87	0.60–0.53	0.48

Les valeurs de k ont été portées en coordonnées semilogarithmiques en fonction de $1/\varepsilon$ (Fig. 4). Dans la première partie du graphique ($25.9 \geqslant \varepsilon \geqslant 15.8$), la relation linéaire attendue d'après les travaux de SWART ET LE ROUX semble bien être respectée: l'augmentation de ε se traduit par une diminution de k. Au delà de $\varepsilon = 15.8$, où k présente, pour nous, une valeur maximum, on assiste à une diminution de k en même temps que ε.

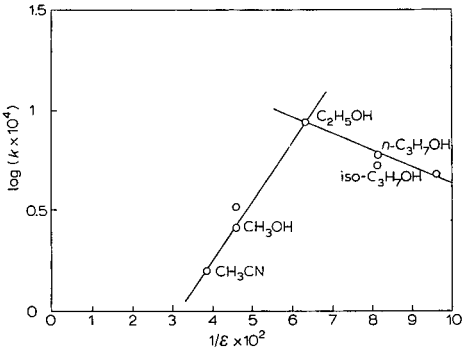


Fig. 4. Influence de ε sur k. Température: 92.4°.

Nous ne pensons pas que le simple fait de changer la nature du solvant sans modifier la structure des réactifs puisse être tenu pour responsable d'une modification profonde du mécanisme. Il nous paraît plus logique d'interpréter l'influence de la constante diélectrique par deux actions antagonistes simultanées:

La diminution de ε produit:

(a) une décroissance de l'énergie d'activation;

(b) une diminution des coefficients de dissociation ionique des deux iodures.

La variation de ε entre 25.9 (acétonitrile) et 15.8 (C_2H_5OH) nous permettrait d'assister à la prépondérance de la première action avec une augmentation de k. Pour $\varepsilon < 15.8$, cet effet serait contrebalancé par la diminution des coefficients de dissociation. L'effet global se traduirait alors par la décroissance de k.

ÉTUDE DE LA RÉACTION SECONDAIRE

La réaction secondaire présente un caractère perturbateur en ce sens qu'elle conduit à la formation d'acide iodhydrique. Cette substance a été mise en évidence, en ordre principal, par la production, dans les alcools *n*- et iso-propyliques, des dérivés iodés correspondants:

$$n(\text{iso})\text{-}C_3H_7OH + HI \rightleftharpoons n(\text{iso})\text{-}C_3H_7I + H_2O.$$

Toutefois, nous n'avons pu déceler aucun signe de la présence de HI dans les radiochromatogrammes, soit à la suite de sa décomposition, soit à la suite d'une réaction avec le liquide stationnaire.

Vitesse de formation de HI

Sur la base des travaux de HECHT ET CONRAD, il n'est pas illogique d'admettre que la réaction

$$R—OH + R'I \rightarrow R—O—R' + HI$$

est d'ordre 2. La vitesse de formation HI est décrite par l'équation:

$$\frac{d[HI]}{dt} = k\,[ROH]\,[R'I] = \frac{d[ROR']}{dt}$$

Nous disposons d'une méthode indirecte pour étudier les variations de concentration en HI, en portant, en fonction du temps, les hauteurs des pics des éthers-oxydes qui, comme on le sait, sont proportionnelles aux concentrations. Les graphi-

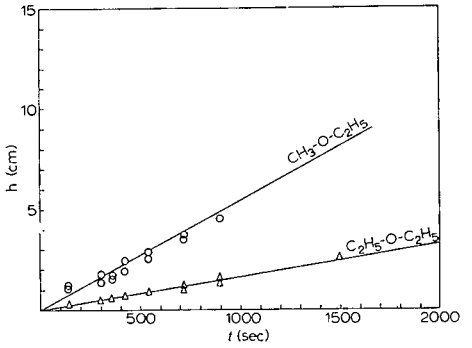


Fig. 5. Vitesses de formation de $CH_3—O—C_2H_5$ et $C_2H_5—O—C_2H_5$. Solvant: C_2H_5OH. Température: 92.4°.

ques obtenus (Fig. 5) sont des droites passant par l'origine, dans les quatre alcools.

On peut écrire:

$$[ROR'] = [HI] = \mathscr{K}t$$

ou encore

$$\frac{d[HI]}{dt} = \mathscr{K}$$

TABLEAU V

CONSTANTES DE VITESSE DE FORMATION

Solvants	CH_3OH	C_2H_5OH	$n\text{-}C_3H_7OH$	$iso\text{-}C_3H_7OH$
Réactifs				
CH_3I	$0.33 \cdot 10^{-3}$	$0.19\text{–}0.21 \cdot 10^{-3}$	$0.16 \cdot 10^{-3}$	$0.19 \cdot 10^{-3}$
C_2H_5I	$0.30 \cdot 10^{-3}$	$0.12\text{–}0.13 \cdot 10^{-3}$	$0.11 \cdot 10^{-3}$	$0.11 \cdot 10^{-3}$

La comparaison des deux expressions de d[HI]/dt montre que la réaction, dans les limites de nos observations est d'ordre apparent égal à zéro. A partir de $\mathscr{K}$ et des concentrations, nous avons calculé k (Tableau V). Les valeurs données dans ce tableau ne sont que des valeurs relatives: elles ne tiennent pas compte des réponses relatives des différents éthers-oxydes. C'est pourquoi, les valeurs des constantes reprises dans le Tableau V sont exprimées en $cm \cdot sec^{-1} \cdot (m/l)^{-2}$.

Les valeurs données dans le cas de C_2H_5OH sont les limites inférieure et supérieure obtenues pour des variations de concentration comprises entre 1.75 et 2.69 m/l pour CH_3I, 0.64 et 3.35 m/l pour C_2H_5I et entre 9.86 et 18.6 m/l pour C_2H_5OH.

Influence de cette réaction sur les concentrations des réactifs

Comme on peut s'en rendre compte aisément, cette réaction fait apparaître dans le milieu réactionnel une forme supplémentaire d'iode échangeable mais, de plus, elle altère les valeurs des concentrations des réactifs. Cependant, il semble bien que les fractions consommées par cette réaction puissent être tenues pour négligeables vis-à-vis des concentrations globales:

(a) pour des volumes d'échantillon grossièrement constants ($\pm$ 5 %) la surface des pics inactifs oscille autour d'une valeur moyenne, dans toutes les expériences;

(b) si les concentrations des iodures variaient, il ne serait pas possible d'observer un ordre apparent nul pour la réaction secondaire;

(c) si on calcule par la méthode rapide $[S_{\text{éther oxyde}}/(S_{\text{éther oxyde}} + S_{\text{iodure}})]$ la fraction transformée, on peut contrôler que celle-ci oscille autour de 0.002 pour les deux iodures.

Il ne faut toutefois pas perdre de vue que cette réaction conduit à la formation d'acide iodhydrique qui, dans les milieux de forte constante diélectrique, est probablement dissocié beaucoup plus fortement que les iodures d'alkyle de telle sorte que de faibles quantités de HI sont susceptibles de perturber sensiblement le déroulement de la réaction d'échange d'iode.

CONCLUSIONS

Il semble bien prouvé que les iodures d'alkyle échangent leur atome d'iode par un mécanisme de nature ionique. La réaction principale s'accompagne, dans les alcools de la formation d'éthers-oxydes et d'acide iodhydrique. Il semblerait bien que cette réaction soit sans effet appréciable sur l'échange proprement dit mais ce point de nos conclusions doit encore faire l'objet d'une étude détaillée avant de pouvoir être confirmé en toute certitude.

REMERCIEMENTS

Nous remercions Monsieur le Prof. G. DUYCKAERTS qui a bien voulu nous guider dans l'élaboration et la rédaction de ce travail.

RÉSUMÉ

La réaction d'échange d'iode entre CH_3I et C_2H_5I a été étudiée à 92.4° dans le *p*-dioxane, les alcools éthylique, méthylique, *n*-propylique et isopropylique et dans l'acétonitrile.

Il paraît bien que la réaction procède par un mécanisme ionique d'ordre 2 et de constante de vitesse:

$$k = (0.87 \pm 0.09) \cdot 10^{-3}\ l \cdot m^{-1} \cdot sec^{-1}$$

L'influence de la constante diélectrique du solvant confirme le caractère ionique du mécanisme.

La réaction principale s'accompagne dans les solvants hydroxylés, d'un processus secondaire:

$$RI + R'OH \rightarrow R{-}O{-}R' + HI \quad (R \equiv CH_3\text{-}, C_2H_5\text{-}) \ (R' \equiv CH_3\text{-}, C_2H_5\text{-}, n\text{- et iso-}C_3H_7\text{-})$$

Cette réaction secondaire donnant naissance à de l'acide iodhydrique échangeable, semble ne pas affecter le déroulement de la réaction d'échange principale, tout au moins pendant la période initiale.

SUMMARY

The exchange reaction of iodine between CH_3I and C_2H_5I has been studied at 92.4° in *p*-dioxane, ethyl alcohol, methyl alcohol, *n*- and isopropyl alcohol and acetonitrile.

It appears that the reaction proceeds by an ionic mechanism of the second order and that the rate constant is:

$$k = (0.87 \pm 0.09) \cdot 10^{-3}\ (l \cdot m^{-1} \cdot sec^{-1})$$

The effect of the dielectric constant of the solvent confirms the ionic character of the reaction.

The principal reaction that takes place in the hydroxylated solvents is accompanied by a secondary process:

$$RI + R'OH \longrightarrow R{-}O{-}R' + HI$$
$$(R \equiv CH_3\text{-}, C_2H_5\text{-}) \ (R' \equiv CH_3\text{-}, C_2H_5\text{-}, n\text{- and iso-}C_3H_7\text{-})$$

This secondary reaction, giving rise to exchangeable hydriodic acid, does not seem to affect the course of the principal exchange reaction, at least during the initial period.

BIBLIOGRAPHIE

1 H. SCHMIED ET R. W. FINK, *J. Chem. Phys.*, 27 (1957) 1034.
2 L. DARBEE ET G. M. HARRIS, *J. Phys. Chem.*, 61 (1957) 111.
3 J. E. BUJAKE, Jr. ET R. M. NOYES, *J. Am. Chem. Soc.*, 83 (1961) 1555.
4 J. E. BUJAKE, Jr., M. W. T. PRATT ET R. M. NOYES, *J. Am. Chem. Soc.*, 83 (1961) 1547.
5 E. R. SWART ET L. J. LE ROUX, *J. Chem. Soc.*, (1956) 2110.
6 E. R. SWART ET L. J. LE ROUX, *J. Chem. Soc.*, (1957) 406.
7 H. SEELIG ET D. E. HULL, *J. Am. Chem. Soc.*, 64 (1942) 940.
8 H. A. C. MACKAY, *J. Am. Chem. Soc.*, 65 (1943) 703.
9 A. J. JAMES, *Research*, 8 (1955) 8.
10 CL. MOUSSEBOIS, *Thèse non publiée*, Université de Liège, 1957.
11 R. KAYSER, *Gas Chromatography*, Butterworths, London, 1963.
12 W. DANNHAUSER AND L. W. BAHE, *J. Chem. Phys.*, 40 (1964) 3058.
13 W. HECHT ET M. CONRAD, *Z. Physik. Chem. (Leipzig)*, 3 (1889) 450.

GAS CHROMATOGRAPHIC ANALYSIS OF HISTAMINE METABOLITES IN URINE

EXCRETION OF LABELLED MATERIAL IN DOGS*

RICHARD THAM

Department of Pharmacology and Department of Toxicology, Swedish Medical Research Council, Karolinska Institutet, Stockholm (Sweden)

(Received October 13th, 1965)

Studies by SCHAYER of urinary metabolites following injection of ^{14}C-labelled histamine have demonstrated that this compound is metabolized in various ways[1,2]. In man, ring-N-methylation constitutes one of the main catabolic pathways[3] (Fig. 1). The ring-N-methylated histamine undergoes further oxidative deamination in the side-chain to N-methylated imidazoleacetic acid. Identification of the urinary metabolites by isotope dilution technique has shown that ring-methylation takes place only at the nitrogen remote from the side-chain. In man, about 50 % of injected ^{14}C-labelled histamine can be recovered from urine as 1-methylimidazole-4-acetic acid (1,4-MeImAA). The isomeric 1-methylimidazole-5-acetic acid (1,5-MeImAA) could not be detected with this method[3].

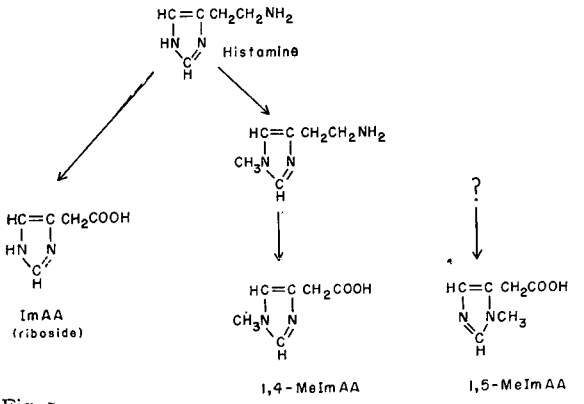


Fig. 1.

Studies in our laboratories on "endogenous" urinary metabolites gave results which are contradictory to these findings. Gas chromatographic analysis of human urine without previous administration of labelled histamine has shown that both isomers of ring-methylated imidazoleacetic acid are normally excreted[4].

A number of "biological" explanations for the difference between these results

* This research was supported by the Swedish Medical Research Council, Project No. 40X-677-01, and Reservationsanslaget, Karolinska Institutet.

and those of SCHAYER *et al.* have been put forward in a previous paper[4]. One purpose of the present investigation has been to eliminate the possibility of experimental errors, since differences in the sensitivities of the two methods could also possibly explain the diversity of the results. Another purpose has been to find out if the intake of histamine or histidine in food is reflected in the excreted amount of 1,4-MeImAA and 1,5-MeImAA.

METHODS

2-^{14}C-Histamine dihydrochloride and 2-^{14}C-*l*-histidine were obtained from The Radiochemical Centre, Amersham, England. The specific activities were 192 μC/mg of free base and 226 μC/mg, respectively. The labelled histamine, dissolved in saline, was injected subcutaneously in dogs weighing 8–18 kg. In other experiments, labelled histamine or histidine was suspended in milk and given to the dogs perorally. No food restrictions were imposed on the animals before or during the experiments. Urine was collected in metabolic cages or by catheterization during 6 or 24 h after administration of the labelled compound.

An aliquot of the collected urine, corresponding to 50 mg creatinine, was made slightly alkaline and run through an anion ion exchange column. The imidazolic acids retained by the column were eluted with acetic acid, the eluate was dried, and the acids esterified with methanol containing hydrogen chloride. The esters were extracted with chloroform (for details see ref. 4).

In order to test the recovery, 0.1–0.5 ml of the crude urine and 5–10 μl of the final extract were each added to 15 ml of scintillation liquid (toluene–ethylene glycol monoethyl ether (1:1, v/v), containing 0.4 % PPO and 0.01 % dimethyl POPOP[5]) in plastic tubes, and the radioactivity counted in a Packard Tricarb liquid scintillation counter, Model 314 Ex-2. The values were corrected for background and for quenching by addition of internal standard. 1 mμC of authentic labelled histamine or histidine gave about 1000 c.p.m. under the given conditions.

Gas chromatographic analysis of the urine extract was performed with an F & M gas chromatograph, Model 400, equipped with a flame ionization detector and a splitter system. Two columns with different stationary phases–10 % ethylene glycol adipate (EGA) and 7 % neopentyl glycol succinate (NGS)–were used (for details, see ref. 4). The splitter divides the gas stream at the outlet side of the column into two parts, one of which passes to the detector. The other part passes to a port in which teflon tubes can be inserted. The compounds emerging in the effluent from the column were allowed to condense on the walls of the teflon tube. At suitable time intervals the teflon tube was removed, and a new one inserted into the splitter port. The teflon tubes were then connected to a burette and 15 ml of scintillation liquid was allowed to drop slowly through the tubes into vials, the radioactivity of which was then counted. The values were corrected for background activity and quenching. The method for measuring the radioactivity in the effluent from the gas chromatograph was tested with authentic ^{14}C-1,4-MeImAA (methyl ester). Samples containing 1–10 μg, with a total radioactivity of 50–2000 c.p.m., were injected into the gas chromatograph. The effluent was collected when the ester peak appeared. With a splitter ratio of 1:1, about 35 % of the injected radioactivity could be recovered (50 % escaped through the detector).

RESULTS AND DISCUSSION

Previous investigators[6] have pointed to methylation of the ring-nitrogen as the major route of catabolism of injected histamine in dogs. Gas chromatographic analysis of imidazolic acids in the urine now demonstrated that dogs like humans excreted both isomers of methylimidazoleacetic acid (Fig. 2). The identity of the two isomers was established on two columns as described in a previous publication[4]. Using a high sensitivity adjustment, a peak with a retention time coinciding with that of authentic imidazoleacetic acid (ImAA, methyl ester) could also be identified (Fig. 2). The excretion pattern of imidazoleacetic acids in the dog thus seems to be very similar to that of man.

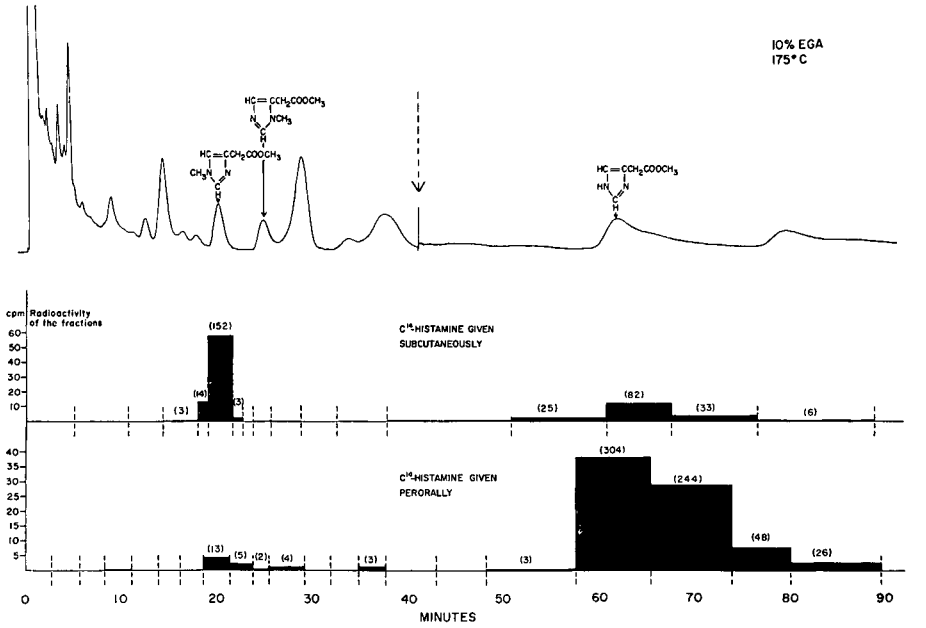


Fig. 2. Upper panel: Gas chromatographic analysis of urine extract from a dog. The dotted arrow represents an adjustment of a 4 times higher sensitivity of the recorder. Middle panel: Radioactivity in the gas chromatographic effluent from a urine extract of the same dog. Labelled histamine in μg amounts had been given to the dog subcutaneously. The dotted lines represent changes of trapping tubes. The total radioactivity in c.p.m. trapped in each tube is seen within the parentheses. The ordinate scale represents the radioactivity trapped per min. The total radioactivity of the injected sample was 880 c.p.m. Splitter ratio: 1:1. Lower panel: Radioactivity of the gas chromatographic effluent from a urine extract of the same dog. Labelled histamine in μg amounts had been given to the dog perorally. The radioactivity is expressed as above. The total radioactivity of the injected sample was 2120 c.p.m. Splitter ratio: 1:1.

After subcutaneous administration of labelled histamine (0.2 μg/kg) to a dog, about 45 % of the total radioactivity could be recovered during the next 6 h. Discounting losses in the procedure, 75 % of this radioactivity corresponds to aromatic acids and other compounds, which are retained by an anionic resin and could be extracted by chloroform after esterification (see Methods). Gas chromatographic analysis of the chloroform extract with simultaneous recording of the radioactivity in the

effluent demonstrated that the radioactivity is confined to the peaks representing 1,4-MeImAA (methyl ester) and ImAA (methyl ester) (Fig. 2). No radioactivity is recovered in the peak corresponding to 1,5-MeImAA (methyl ester). It is thus evident that in spite of the normal occurrence of 1,5-MeImAA in urine, subcutaneously administered histamine is methylated only at the nitrogen remote from the side-chain. The experiments are in agreement with previous investigations by SCHAYER *et al.*

The same amount of labelled histamine, with or without addition of non-labelled carrier (46 mg/kg), was also given to dogs perorally. Upon collection of urine during the next 6 or 24 h, 50 % and 90 % of the given total radioactivity was recovered respectively. Gas chromatography with simultaneous radioactivity analysis showed that most of the administrated histamine is excreted as non-methylated ImAA (Figs. 2 and 3). In these experiments, where comparatively large amounts of non-labelled histamine were given, the gas chromatographic peak corresponding to ImAA

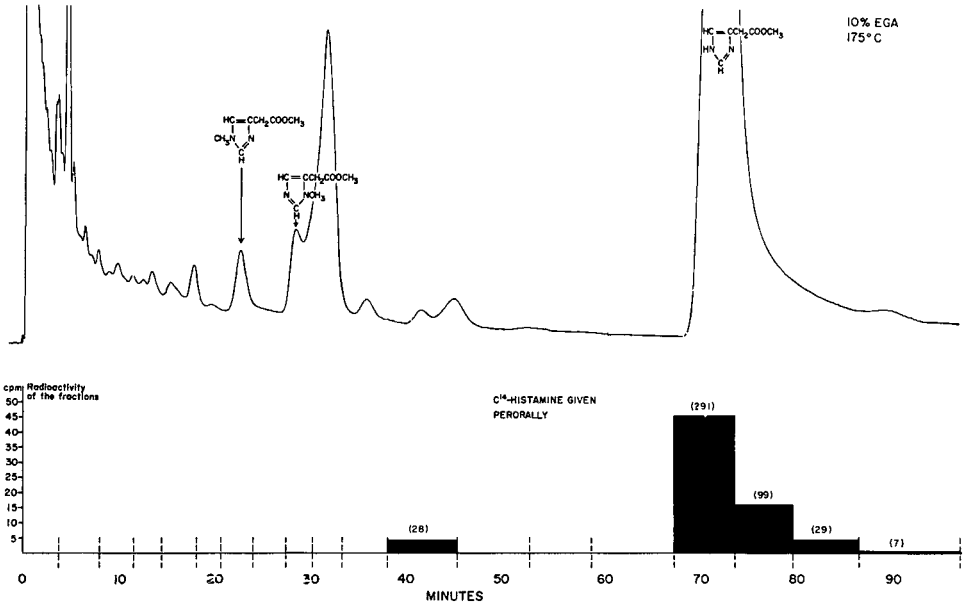


Fig. 3. Gas chromatographic analysis of urine extract from a dog given mg amounts of labelled histamine. Gas chromatogram: upper panel. Radioactivity in the effluent: lower panel. The radioactivity is expressed as in Fig. 2. The total radioactivity of the injected sample was 450 c.p.m. Splitter ratio: 1:1.

(methyl ester) was very high (Fig. 3). No radioactivity corresponding to 1,5-MeImAA (methyl ester) could be detected in the above experiments (Fig. 3). It is thus unlikely that the metabolic origin of 1,5-MeImAA is histamine absorbed from the intestine.

The fact that most of the histamine administered perorally is excreted as ImAA may be attributed to the high concentration of diamineoxidase (histaminase) in the intestinal mucosa[7,8]. Previous investigators have shown that *in vitro* histamine is degraded by intestinal mucosa of dogs mainly to ImAA[8]. It is notable that ^{14}C-labelled histamine given perorally to man is excreted mainly as 1,4-MeImAA[6].

In the above experiments, no attempts were made to demonstrate the ex-

cretion of the riboside of ImAA. According to previous investigators, however, dogs do not conjugate appreciable quantities of ImAA with ribose[6,9].

^{14}C-Labelled histidine (11 μg/kg) mixed with a large amount of the non-labelled amino acid (256 mg/kg) was also administered to dogs. About 25 % of the radioactivity could be recovered in urine during 24 h. About 1 % of this represented compounds which under the given conditions were retained by an anion exchanger column and could be extracted by chloroform after esterification. The gas chromatogram revealed a peak corresponding to ImAA (methyl ester), which was much higher than normal (Fig. 4). A part of the radioactivity was also confined to this peak (Fig. 4). No radioactivity was found corresponding to the peaks of 1,4-MeImAA (methyl ester) and 1,5-MeImAA (methyl ester). Radioactivity was also found in a peak the identity of which is at present unknown.

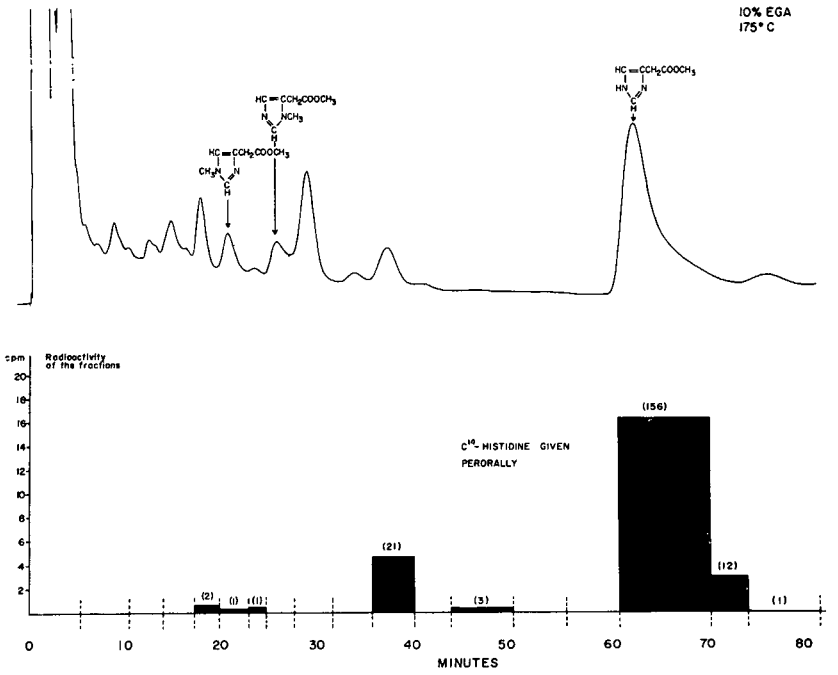


Fig. 4. Gas chromatographic analysis of a urine extract from a dog given labelled histidine perorally. Gas chromatogram: upper panel. Radioactivity in the effluent: lower panel. The radioactivity is expressed as in Fig. 2. The total radioactivity of the injected sample was 1240 c.p.m. Splitter ratio: 1:1.

Several investigators have reported the identification of ImAA as a metabolite of histidine[10–12]. It has been suggested that histidine is transformed into imidazole-pyruvic acid, part of which is then oxidized to ImAA. Ingested histidine may also be decarboxylated to histamine, which secondarily may be deaminated to ImAA. This histamine production appears to be insignificant relative to other reactions in histidine catabolism[12,13]. The excretion of ImAA thus depends on the histidine intake in food and will not specifically reflect the endogenous liberation of histamine. From the above experiments it can also be concluded that the excretion of 1,4-MeImAA is

independent of histidine intake. This result is in agreement with studies by BROWN *et al.*[12] on urinary metabolites of labelled histidine in monkeys, humans, and rats. Neither does histidine in food seem to be the metabolic origin of 1,5-MeImAA.

SUMMARY

Gas chromatographic analysis of urine has demonstrated that 1-methylimidazole-4-acetic acid as well as 1-methylimidazole-5-acetic acid is excreted by dogs. Subcutaneously administered labelled histamine in microgram quantities is excreted as 1-methylimidazole-4-acetic acid and imidazoleacetic acid, but does not contribute to the urine content of 1-methylimidazole-5-acetic acid. Histamine or histidine absorbed from the intestine do not seem to contribute to the next 24 hours' excretion of 1-methylimidazole-5-acetic or 1-methylimidazole-4-acetic acid.

REFERENCES

1 W. R. SCHAYER, *J. Biol. Chem.*, 196 (1952) 469.
2 W. R. SCHAYER AND S. A. KARJALA, *J. Biol. Chem.*, 221 (1956) 307.
3 W. R. SCHAYER AND J. A. D. COOPER, *J. Appl. Physiol.*, 9 (1956) 481.
4 R. THAM AND B. HOLMSTEDT, *J. Chromatog.*, 19 (1965) 286.
5 G. BRUNO AND J. E. CHRISTIAN, *Anal. Chem.*, 33 (1961) 1216.
6 W. R. SCHAYER, *Brit. J. Pharm.*, 11 (1956) 472.
7 C. H. BEST AND E. W. MCHENRY, *J. Physiol.*, 70 (1930) 349.
8 Y. KOBAYASHI AND A. C. IVY, *Am. J. Physiol.*, 195 (1958) 525.
9 S.-E. LINDELL AND W. R. SCHAYER, *Brit. J. Pharm.*, 13 (1958) 44.
10 G. WOLF, P. L. WU AND W. W. HECK, *J. Biol. Chem.*, 222 (1956) 159.
11 R. C. BALDRIDGE AND C. D. TOURTELLOTTE, *J. Biol. Chem.*, 233 (1958) 125.
12 D. D. BROWN, O. L. SILVA, P. B. MCDONALD, S. H. SNYDER AND M. W. KIES, *J. Biol. Chem.*, 235 (1960) 154.
13 W. R. SCHAYER, *J. Biol. Chem.*, 199 (1952) 245.

GAS–LIQUID CHROMATOGRAPHY OF VOLATILE FATTY ACIDS FROM FORMIC ACID TO VALERIC ACID

II. THE INSTABILITY OF SILICONE OIL–FATTY ACID STATIONARY PHASES

R. B. JACKSON*
Fodder Conservation Section, C.S.I.R.O., Highett, Victoria (Australia)
(Received October 26th, 1965)

JAMES AND MARTIN[1] reported that silicone DC550–stearic acid columns retained their resolving power for volatile fatty acids indefinitely when operated at temperatures up to 137°. However, several workers have reported rapid loss of efficiency under these conditions[2,3,4,5].

Both MCINNES[3] and BOER[2] noticed a loss of stearic acid from the column; if loss of stearic acid had caused deterioration of the columns the retention volumes of the acids should have decreased, but this was not the case. Behenic acid was lost at a slower rate than stearic acid but complete suppression of acid bleeding was obtained by using a fatty acid mixture prepared from Carnauba wax (BOER[2]). This permitted about 20 determinations before resolution became unsatisfactory. MCINNES[3] replaced stearic acid with varying proportions of monocarboxylic acids (C_{20} and C_{22}) and dicarboxylic acids (C_{13}, C_{18} and C_{20}). However, replacing stearic acid by less volatile acids did not increase the life of the columns to any appreciable extent.

The importance of water in the sample and carrier gas has been noticed by several workers[1,6,7,8]. GRAHAM[8] reported that some batches of Celite gave poor resolution of C_1–C_5 fatty acids when using dry nitrogen. This was improved by saturating the carrier gas with water at room temperature and he found that the columns tolerated a considerable proportion of water in the samples.

HAWKE[9], using wet carrier gas, found no deterioration of a silicone MS550–behenic acid–phosphoric acid column at 137°.

In view of the conflicting reports about the stability of silicone–fatty acid columns and the effect of water on their performance, a systematic study of these aspects has been made.

APPARATUS AND MATERIALS

Chromatograph

The glass column, 120 cm long and 4 mm inside diameter, was supported inside an electrically heated air jacket. Temperature was controlled by means of a variable transformer. The column terminated in a ground-glass joint which fitted into a titration cell. Acids emerging from the column were detected by automatic titration

* Present address: C.S.I.R.O. Division of Soils, Glen Osmond, South Australia.

with a Radiometer Titrigraph[10]. The carrier gas was commercial dry nitrogen which was either passed through water, or dried by passing through a column of activated Union Carbide Molecular Sieve, Type 5A, before entering the column.

Materials for preparing columns

Chromosorb W, acid-washed, 80–100 mesh; batch I was obtained from F & M. Scientific Corporation, batch II from Johns-Manville Products Corporation; Haloport F (F & M. Scientific Corporation); Paraffin oil (British Pharmacopoeia grade); Silicone DC 550 (Dow-Corning); Stearic acid (Eastman-Kodak); Behenic acid (Hopkin & Williams), recrystallized from acetone; Phosphoric acid (B.D.H. Analar); Acetone and chloroform (May and Baker reagent grade).

Organic acids

The carboxylic acids used were commercial samples. The mixture used for testing the columns contained approximately equi-molar amounts of formic, acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids. In some cases formic, isobutyric and isovaleric acids were omitted from the test mixture.

EXPERIMENTAL PROCEDURE AND RESULTS

Preparation of columns

The materials for the liquid phases were dissolved in acetone except for that containing paraffin oil; the packing containing paraffin oil was prepared in chloroform. The solid support was added and the solvent removed on a rotary evaporator.

Operating conditions

The temperature of the columns was maintained at 130 $\pm$ 1°. The nitrogen flow-rate was 10, 15 or 30 ml/min depending upon the retention times of the acids on the column being tested.

The performance of a column was tested by applying 1 μl of the mixture of acids to a plug of glass wool at the top of the packing.

Chromosorb W–silicone DC 550–stearic acid packing

This packing consisted of acid-washed Chromosorb W, batch I (10 parts), silicone DC 550 (4 parts) and stearic acid (0.4 parts). Curves A to D (Fig. 1) show the separation of the mixture of acids after the column had been operating with dry nitrogen at 15 ml/min for 0, 4, 10 and 24 h, respectively. The first noticeable change was loss of resolution between iso- and *n*-butyric acids after 4 h. After 10 h all the acids showed increased tailing and there was little separation of the butyric acid isomers from one another. Even greater loss of resolution was apparent after 24 h. At that stage the carrier gas was passed through water instead of the column of molecular sieve. There was no immediate change in the resolving power of the column, but 4 h after changing to wet gas some reduction of tailing was evident (curve A, Fig. 2). The performance of the column 8, 24 and 48 h after changing to wet gas is shown by curves B, C and D (Fig. 2). The separation of the butyric isomers was not quite as good as that obtained with the freshly prepared column but otherwise the resolving power was restored. Operation of the column for a further 28 h produced no further

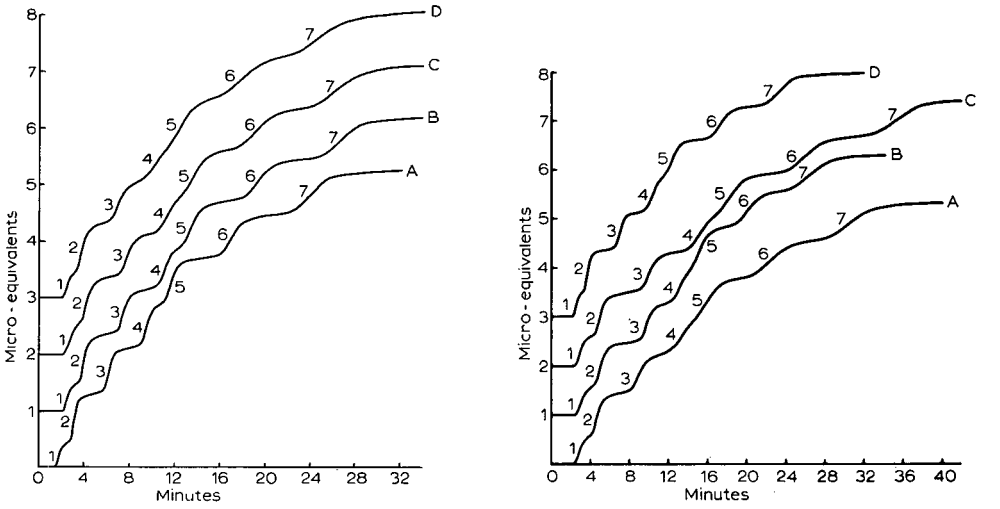


Fig. 1. Chromatograms of a mixture of C_1 to C_5 acids showing deterioration of a column of 40% (w/w) silicone DC 550 and 4% (w/w) stearic acid on acid-washed Chromosorb W (batch I) when operated with dry carrier gas for (A) 0 h, (B) 4 h, (C) 10 h and (D) 24 h. Column temperature: 130 ± 1°. Flow rate: 15 ml/min. Acids: 1 = formic; 2 = acetic; 3 = propionic; 4 = isobutyric; 5 = *n*-butyric; 6 = isovaleric; 7 = *n*-valeric.

Fig. 2. Restoration of the column described in Fig. 1 (D) when operated with wet nitrogen for (A) 4 h, (B) 8 h, (C) 24 h and (D) 48 h. Temperature and flow rate as for Fig. 1. Acids: as in Fig. 1.

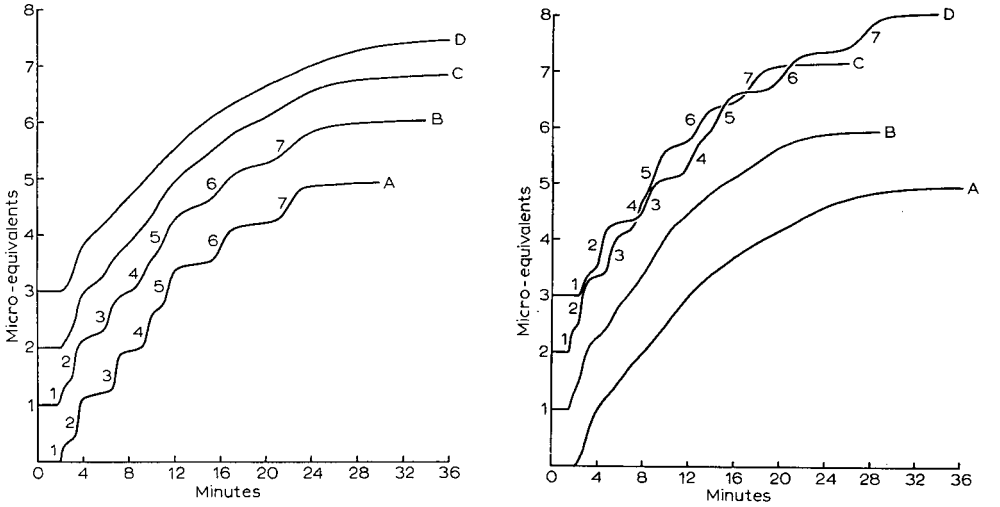


Fig. 3. Chromatograms of a mixture of C_1 to C_5 acids showing deterioration of a column of 40% (w/w) silicone DC 550 and 4% (w/w) behenic acid on acid-washed Chromosorb W (batch I) when operated with dry nitrogen for (A) 0 h, (B) 24 h, (C) 76 h and (D) 193 h. Column temperature: 130 ± 1°. Flow rate: 15 ml/min. Acids: as in Fig. 1.

Fig. 4. Restoration of the resolving power of the column described in Fig. 3 (D) when operated with wet nitrogen for (A) 0 h, (B) 2 h, (C) 24 h and (D) 48 h. Column temperature and flow rate as in Fig. 3. Acids: as in Fig. 1.

change in performance. At this stage the column had been run for a total of 100 h and had lost an appreciable amount of stearic acid. The performance was improved slightly by repacking the column with the addition of more stearic acid and then was comparable with that of a freshly prepared column run with wet carrier gas.

Chromosorb W–silicone DC 550–behenic acid packing

This packing consisted of acid-washed Chromosorb W, batch I (10 parts), silicone DC 550 (4 parts) and behenic acid (0.4 parts).

The performance of this packing with dry carrier gas is illustrated in Fig. 3. The pattern of change was very similar to that found for silicone–stearic acid columns operated with dry gas. Curve B (Fig. 3) shows the result obtained after 24 h; excessive tailing of all acids is apparent and there is practically no separation of iso- and *n*-butyric acids. After 76 h the loss of separating power was almost complete (curve C, Fig. 3). The column was run for an extended period prior to testing the reversibility of the changes. After 193 h curve D (Fig. 3) was obtained.

At this stage the carrier gas was changed to wet nitrogen; there was no immediate change in performance (curve A, Fig. 4), but after 2 h a slight separation was apparent (curve B, Fig. 4). After 24 h operation with wet gas (curve C, Fig. 4), all components of the mixture except the butyric acid isomers were identifiable although they still tailed considerably. After 48 h (curve D, Fig. 4) the resolving power of the column was only slightly inferior to that of a freshly prepared column, except that iso- and *n*-butyric acids overlapped considerably. The column was run for an additional 70 h without further change in behaviour being apparent. For comparison, a freshly prepared silicone–behenic acid column was run with wet carrier gas from the beginning. Performance after 0, 4, 10 and 84 h is shown in Fig. 5. There was an early loss of some resolving power for iso- and *n*-butyric acids and a slow increase in the tailing of formic acid, otherwise the behaviour of the column was stable.

Effect of phosphoric acid

JAMES AND MARTIN[1] found that the addition of orthophosphoric acid to a Celite–silicone–stearic acid column eliminated tailing and improved the separation of formic and acetic acids. HAWKE[9] also obtained an excellent separation of formic and acetic acids on a Celite–silicone oil–behenic acid–phosphoric acid column using wet carrier gas. As this seemed to be a useful packing, its stability under wet and dry conditions was investigated. The packing consisted of acid-washed Chromosorb W, batch I (10 parts), silicone DC 550 (4 parts), behenic acid (0.4 parts) and orthophosphoric acid (0.4 parts). The nitrogen flow rate was 30 ml/min.

Curve A (Fig. 6) was obtained with a freshly prepared column using dry carrier gas. Formic and acetic acids were not separated but the separation of the remaining acids was good. After 9 h some resolution of formic and acetic acids became apparent. Thereafter little further change took place, curves B, C, and D (Fig. 6) being obtained after 24, 48 and 336 h, respectively. Wet carrier gas was then used and the retention volumes of the acids decreased immediately (curve A, Fig. 7). There appeared to be no separation of formic and acetic acids but any separation may have been obscured because the addition of alkali for this run was too slow for the rate at which these acids were eluted. The decrease in retention volumes continued over the first 3–4 h; thereafter, the performance of the column showed little change, curves B and C

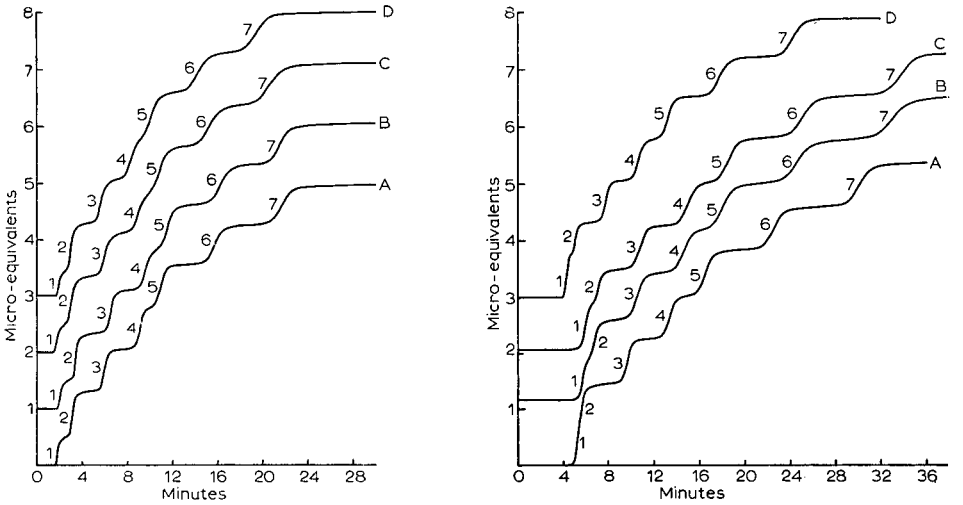


Fig. 5. Separation of a mixture of C_1 to C_5 acids on a column of 40% (w/w) silicone DC 550 and 4% (w/w) behenic acid on acid-washed Chromosorb W (batch I) after operating with wet nitrogen for (A) 0 h, (B) 4 h, (C) 10 h and (D) 84 h. Column temperature: 130 ± 1°. Flow rate: 15 ml/min. Acids: as in Fig. 1.

Fig. 6. Separation of a mixture of C_1 to C_5 acids on a column of 40% (w/w) silicone DC 550, 4% (w/w) behenic and 4% (w/w) orthophosphoric acids on acid-washed Chromosorb W (batch I) after operating with dry nitrogen for (A) 0 h, (B) 24 h, (C) 48 h and (D) 336 h. Column temperature: 130 ± 1°. Flow rate: 30 ml/min. Acids: as in Fig. 1.

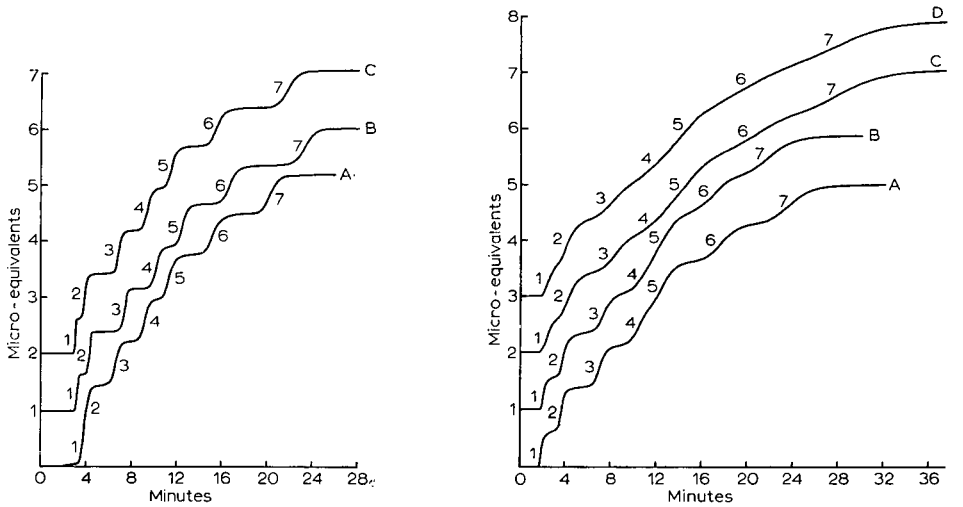


Fig. 7. Separation of a mixture of C_1 to C_5 acids on the column described in Fig. 6 (D) after operating with wet nitrogen for (A) 0 h, (B) 24 h and (C) 180 h. Column temperature and flow rate as in Fig. 6. Acids: as in Fig. 1.

Fig. 8. Chromatograms of a mixture of C_1 to C_5 acids showing deterioration of a column of 13.3% (w/w) silicone DC 550 and 1.3% (w/w) behenic acid on Haloport F after operating with dry nitrogen for (A) 0 h, (B) 24 h, (C) 72 h and (D) 144 h. Column temperature: 130 ± 1°. Flow rate: 15 ml/min during separations, otherwise 30 ml/min. Acids: as in Fig. 1.

(Fig. 7) being obtained after 24 and 180 h, respectively. At this stage of the investigation the poor performance of the silicone–fatty acid phases was attributed to tailing caused by adsorption of the volatile acids by the Chromosorb support. Addition of water to the carrier gas was thought to prevent tailing by saturating the adsorption sites. The resolution obtained under dry conditions when phosphoric acid was added to the liquid phase was likewise attributed to suppression of adsorption.

Haloport F–silicone DC 550–behenic acid packing

To obtain further evidence on this point, Chromosorb W was replaced by the perfluorocarbon, Haloport F. There was negligible adsorption on this material since

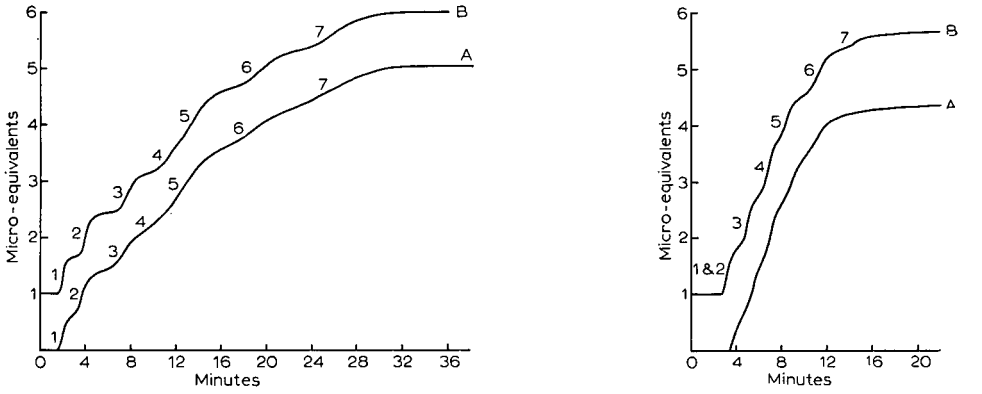


Fig. 9. Restoration of the column described in Fig. 8 (D) when operated with wet nitrogen for (A) 5 h and (B) 48 h. Temperature and flow rates as in Fig. 8. Acids: as in Fig. 1.

Fig. 10. Chromatograms of a mixture of C_1 to C_5 acids on a column of 40% (w/w) silicone DC 550 on acid-washed Chromosorb W (batch II). (A) Dry nitrogen, freshly prepared column; (B) after 144 h with wet nitrogen. Column temperature: 130 ± 1°. Flow rate 10 ml/min during separations, otherwise 30 ml/min. Acids: as in Fig. 1.

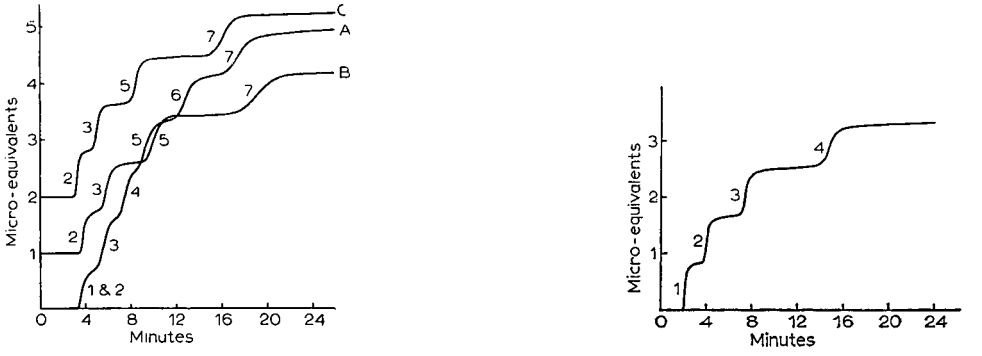


Fig. 11. Chromatograms of mixtures of C_1 to C_5 and C_2 to C_5 acids on a column of 4% (w/w) behenic acid on acid-washed Chromosorb W (batch II). (A) wet nitrogen, freshly prepared column; (B) after 72 h with wet nitrogen; (C) after 24 h with dry nitrogen. Column temperature: 130 ± 1°. Flow rate: 10 ml/min during separations, otherwise 30 ml/min. Acids: as in Fig. 1.

Fig. 12. Chromatogram of a mixture of C_2 to C_5 acids on a column of 40% (w/w) paraffin oil and 4% (w/w) behenic acid on acid-washed Chromosorb W (batch II) after 72 h operation with dry nitrogen. Column temperature: 130 ± 1°. Flow rate: 30 ml/min. Acids: as in Fig. 1.

when the acids were run on a column with no liquid phase, they were completely eluted in less than 15 ml. The column packing consisted of Haloport F (15 parts), silicone DC 550 (2 parts) and behenic acid (0.2 parts) and contained about the same weight of liquid phase as that prepared on Chromosorb W. This column was run with a flow rate of 30 ml/min except that 15 ml/min was used for testing performance. The curves obtained after 0, 24, 72 and 144 h with dry gas are shown in Fig. 8. Wet nitrogen was then passed through the same column. There was no immediate change but some improvement was evident after 5 h (curve A, Fig. 9) and after 48 h (curve B, Fig. 9) the performance was almost as good as when freshly prepared. Further evidence that the support was not involved in the deterioration of the Chromosorb W–silicone oil–behenic acid packing was provided by the observation that wetting the carrier gas had no apparent effect on the adsorption of acids by a Chromosorb W column with no liquid phase.

The results obtained with the Haloport F column and the column packed only with Chromosorb W implicated the liquid phase as the source of instability when dry carrier gas was used. To determine whether one or both components were involved, columns were prepared in which each component of the liquid phase was omitted in turn.

Chromosorb W–silicone DC 550 packing

This packing consisted of acid-washed Chromosorb W, batch II (10 parts) and silicone DC 550 (4 parts). Performance was tested at a flow rate of 10 ml/min, otherwise the flow rate was 30 ml/min. Curve A (Fig. 10) was obtained with the freshly prepared column and there was no apparent change in performance after 48 h. Wet nitrogen was then used and the performance slowly improved. After 144 h there was some resolution of six of the seven acids (curve B, Fig. 10). Thereafter no further improvement took place.

Chromosorb W–behenic acid packing

This packing consisted of acid-washed Chromosorb W, batch II (10 parts) and behenic acid (0.4 parts). The flow rate was maintained at 30 ml/min but performance was tested with a flow rate of 10 ml/min. Curve A (Fig. 11) was obtained when the freshly prepared column was run with wet nitrogen. Formic and acetic acids were not resolved and most of the acids appeared to tail badly. Later it was found that formic acid tailed so badly on columns prepared with batch II Chromosorb* that it overlapped all the acids of the test mixture except *n*-valeric acid. Consequently a mixture containing acetic, propionic, *n*-butyric and *n*-valeric acids was used for testing performance and curve B (Fig. 11) was obtained after 72 h. Dry carrier gas was then used and after 24 h operation curve C (Fig. 11) was obtained. The column was run for a total of 168 h without any apparent deterioration.

Chromosorb W–paraffin oil–behenic acid packing

Although the experiments described above clearly show that the deterioration

* This material was later found to contain appreciable quantities of acid-soluble iron which may have caused tailing of formic acid. With the exception of the behaviour of formic acid, columns prepared with silicone oil–behenic acid on batch II Chromosorb W exhibited the same loss of resolving power under dry conditions and restoration with wet carrier gas, as those prepared with batch I.

of silicone oil–fatty acid packings under dry conditions is attributable to the silicone oil, further confirmation was sought by re-placing the silicone with paraffin oil. The packing consisted of acid-washed Chromosorb W, batch II (10 parts), paraffin oil (4 parts) and behenic acid (0.4 parts). The column was operated with dry carrier gas at a flow-rate of 30 ml/min. No change in performance was apparent after 72 h and excellent separation as shown in Fig. 12 was obtained.

Effect of water in the sample

In view of the improvement effected by adding water to the carrier gas and the conflicting reports about the effects of water in the sample, a few experiments were carried out to determine the effect of water in the sample on the separation by a silicone oil–behenic acid–phosphoric acid column, using wet carrier gas. A freshly prepared column was used and curves A, B, C and D (Fig. 13) were obtained when 0, 1, 2 and 4 μl of water, respectively, were added to 1 μl samples of fatty acid mixture.

That the effects observed were not due to deterioration of the packing was demonstrated by subsequent application of an anhydrous sample, which gave a curve practically identical to curve A, Fig. 13. Amounts of water up to 1 μl did not interfere with the separation but amounts in excess of this caused poor separations, particularly of the lower acids.

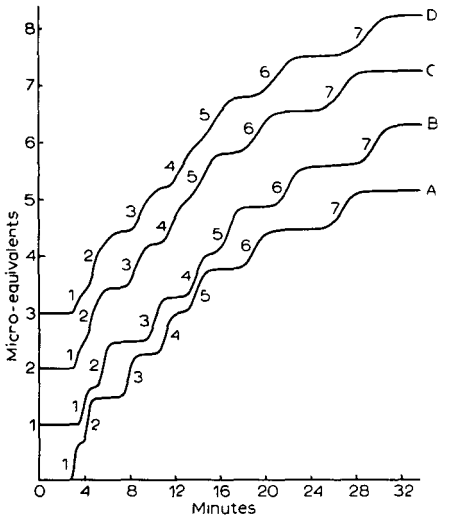


Fig. 13. Effect of water in the sample on the separation of a mixture of C_1 to C_5 acids on a column of 40% (w/w) silicone DC 550, 4% (w/w) behenic and 4% (w/w) orthophosphoric acids on acid-washed Chromosorb W (batch II). Samples: 1 μl mixture of acids without added water (A) or with the following amounts of water added: 1 μl (B), 2 μl (C) and 4 μl (D). Wet nitrogen flow rate: 15 ml/min. Column temperature: 130 ± 1°. Acids: as in Fig. 1.

DISCUSSION

The results of the present study clearly show that silicone oil–stearic acid and silicone oil–behenic acid stationary phases have very short useful lives under dry conditions at 130°. Deterioration can be prevented by continuously adding a small

amount of water to the carrier gas or by incorporating a small amount of orthophosphoric acid in the stationary phase. The latter modification, however, may cause partial decomposition of any formic acid present in the sample[5]. The satisfactory performance of freshly prepared columns is probably due to the presence of small amounts of water which, however, are eluted from the column during the first few hours of operation[8].

The experiments reported above clearly show that the silicone oil component of these packings is a source of instability under dry conditions. The possibility that the Chromosorb W support is also involved has not been eliminated completely. However, the performances of the paraffin oil–behenic acid and the lightly loaded behenic acid columns strongly suggest that the support is not involved.

KELLER, BATE, COSTA AND FORMAN[11] have reviewed the literature dealing with changes which occur in the immobile liquid phase during gas–liquid chromatography. The effect of these changes on retention volumes was discussed further by KELLER AND STEWART[12]. The stationary phase of a gas chromatographic column may undergo physical and chemical changes during use. KELLER AND STEWART[12] classified as physical changes, those which change the total amount of liquid and/or its distribution on the support. It is conceivable that physical changes as defined by KELLER AND STEWART[12] could, on the Chromosorb W support, give rise to adsorption effects which would be reversed by addition of water to the carrier gas[13]. However, the fact that the resolving power of the silicone–behenic acid phase was lost also on the non-adsorptive support, Haloport F, under dry conditions, shows that deterioration was not due to unmasking of adsorption sites by movement of liquid phase.

KELLER *et al.*[11] cited a number of reports of chemical changes in silicone liquid phases but most of these changes were observed at temperatures considerably higher than 130°. The loss of resolving power under dry conditions and its restoration by water suggest that the changes observed in the present study may involve dehydration and hydration reactions in the liquid phase. MARTIN[14,15] proposed that adsorption of the solute can occur on the surface of the liquid phase as well as on the surface of the support.

If carboxylic acids are adsorbed at the liquid–gas interface under dry conditions, then addition of water or phosphoric acid to the system presumably prevents this by saturating the adsorption sites. It might be expected that even under dry conditions the adsorption sites would be masked by the behenic acid in the stationary phase. However, the long hydrocarbon chains of the behenic acid molecules may prevent them reaching adsorption sites at the gas–liquid interface. Some evidence consistent with this is MCINNES' observation[3] that longer chain volatile acids (heptanoic to decanoic) can be separated on DC 550 silicone columns.

The necessity for using a wet carrier gas with silicone oil–behenic acid (or stearic acid) columns for separation of volatile fatty acids precludes their use with detectors that respond to, or are dampened by water (*e.g.* thermal conductivity and argon ionisation detectors*): If formic acid is absent from the samples, loss of resolving power may be prevented by including phosphoric acid in the stationary phase. Some of the difficulties associated with the separation of mixtures containing formic acid have been discussed[5,10].

* FOSTER AND MURFIN[16] recently reported that the response of the hydrogen-flame ionization detector too, is depressed by water.

Although only a limited investigation of the effect of water in the sample was carried out, the results support the contention of JAMES AND MARTIN[1] that even small amounts of water in the sample upset the separation. However, this appears to be the case only when the sample is applied directly to the column. The experiences of HAWKE[9], GRAHAM[8] and LANIGAN AND JACKSON[10] indicate that, provided the water is vaporized before entering the packing, considerable amounts of water can be tolerated.

ACKNOWLEDGEMENT

This investigation was financed by a grant from the Australian Dairy Produce Research Committee.

SUMMARY

The stability of silicone DC 550–stearic acid and silicone DC 550–behenic acid stationary phases has been investigated. Columns containing these liquid phases rapidly lost their resolving power for volatile fatty acids when operated with a dry carrier gas. Resolving power was restored by passing the carrier gas through water for about 48 h. Deterioration could be prevented by using a wet carrier gas or by incorporating orthophosphoric acid in the liquid phase; the latter, however, may cause some decomposition of formic acid when the carrier gas is dry.

The silicone oil was shown to be the source of instability in the stationary phase.

Some possible reasons for the deterioration under dry conditions have been discussed.

REFERENCES

1 A. T. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 50 (1952) 679.
2 H. BOER, *World Petrol. Congr., 4th, Rome*, 1955, Section V/A, Paper 1.
3 A. G. MCINNES, in D. H. DESTY (Editor), *Vapour Phase Chromatography*, Butterworths, London, 1956, p. 304.
4 E. BAYER, *Gas Chromatography*, Elsevier, Amsterdam, 1961, p. 107.
5 R. B. JACKSON, *J. Chromatog.*, 16 (1964) 306.
6 C. L. HANKINSON, W. J. HARPER AND E. MIKOLAJCIK, *J. Dairy Sci.*, 41 (1958) 1502.
7 C. W. GEHRKE AND W. M. LAMKIN, *J. Agr. Food Chem.*, 9 (1961) 85.
8 A. R. GRAHAM, in D. H. DESTY (Editor), *Vapour Phase Chromatography*, Butterworths, London. 1956, p. 315.
9 J. C. HAWKE, *J. Dairy Res.*, 24 (1957) 366.
10 G. W. LANIGAN AND R. B. JACKSON, *J. Chromatog.*, 17 (1965) 238.
11 R. A. KELLER, R. BATE, B. COSTA AND P. FORMAN, *J. Chromatog.*, 8 (1962) 157.
12 R. A. KELLER AND G. H. STEWART, *J. Chromatog.*, 9 (1962) 1.
13 D. M. OTTENSTEIN, *J. Gas Chromatog.*, 1, No. 4 (1963) 11.
14 R. L. MARTIN, *Anal. Chem.*, 33 (1961) 347.
15 R. L. MARTIN, *Anal. Chem.*, 35 (1963) 116.
16 J. S. FOSTER AND J. W. MURFIN, *Analyst*, 90 (1965) 118.

GAS–LIQUID CHROMATOGRAPHY OF VOLATILE FATTY ACIDS FROM FORMIC ACID TO VALERIC ACID

III. ANALYSIS OF DILUTE ETHEREAL SOLUTIONS USING A THERMAL CONDUCTIVITY DETECTOR

R. B. JACKSON*
Fodder Conservation Section, C.S.I.R.O., Highett, Victoria (Australia)
(Received November 2nd, 1965)

INTRODUCTION

The work reported in this paper arose out of difficulties that were experienced using a commercial gas chromatograph equipped with a thermal conductivity detector, for the quantitative analysis of volatile fatty acids isolated from biological materials. The volatile fatty acids present in biological materials are usually isolated by steam distillation, followed by titration to give an aqueous solution of sodium or potassium salts. For estimation by gas–liquid chromatography the free acids must be prepared, usually in an anhydrous state. JAMES AND MARTIN[1] described a method for preparing an anhydrous ethereal solution of the free acids from their sodium salts. Other workers, however, have been unable to obtain quantitative recoveries with the method and various alternative procedures for obtaining the free acids and applying them to columns[2–6] have been described. Most of these involve acidification and subsequent extraction into ether. To ensure complete recovery of the acids, comparatively large volumes of ether are required and the combined extracts are correspondingly dilute. With the sensitive hydrogen flame and argon ionization detectors the volume of such extracts required to give adequate detector response is unlikely to be excessive. By contrast, the low sensitivity of thermal conductivity detectors necessitates the injection of comparatively large volumes of dilute ethereal solutions into the column system.

SMITH *et al.*[7] used "a 10 ft. Ucon (polar) column" to determine the C_2 to C_4 acids in 0.1 ml samples of ethereal solutions. However, with most of the packings that have been described for separating volatile fatty acids, samples of ethereal solutions greater than about 50 μl give ether peaks which seriously overlap those of the lower fatty acids.

Ethereal solutions of volatile fatty acids cannot be concentrated without loss, except by elaborate and time consuming methods[5,8]. JAMES AND MARTIN[1] applied acids, in ethereal extracts, to columns by evaporating the ether and acids in a stream of air drawn through the columns, which were at room temperature. VAN DE KAMER *et al.*[2] and MCINNES[3] modified the end of the column so that a solution of the acids

* Present address: Division of Soils, C.S.I.R.O., Glen Osmond, South Australia.

in ether could be placed in a bulb and then distilled on to the column in the carrier gas stream. LANIGAN AND JACKSON[9] acidified the dried sodium salts of the acids in the carrier gas stream which then carried the free acids on to the column. Many of the workers mentioned above[1–4,9] used acid–base titration to detect the acids, so that ether and water in the sample did not interfere, provided they were insufficient to upset the separation of acids on the column.

EXPERIMENTAL AND RESULTS

Apparatus and materials

The instrument, some of the materials, the columns, and the method for preparing the column packings have been described previously[10]. Haloport F, Ucon LB-550-X (a polypropylene glycol) and diethylene glycol succinate were purchased from F & M Scientific Corporation and Tween 80 (a product of Honeywell-Atlas Ltd.) from a local distributor.

The columns were conditioned at 150° for 16 h with a carrier gas flow rate of 5 ml/min.

Sample application

In seeking a solution to the problem discussed above, 0.5 ml of an ethereal solution was adopted as the maximum sample size.

Various attempts were made to distil acids on to columns by methods similar to those used by other authors[1–4,9]. Although these experiments indicated that the acids could be separated from 0.5 ml ether on some columns using an appropriate application procedure, it became apparent that the separation of water from the acids would be a much more difficult task. Therefore, a search was undertaken for a column packing which would not only resolve a mixture of the volatile fatty acids from formic to *n*-valeric, but also separate the acids from 0.5 ml of ether and the small amount of water present in ether extracts dried with sodium sulphate.

Column packings

Liquid phases of the silicone oil–fatty acid type were unsuitable for reasons discussed earlier[10,11]. Exploratory experiments indicated that diethylene glycol succinate–phosphoric acid[12] might be suitable for separating ether and water from the acids. However, this phase did not adequately separate formic acid from acetic and propionic acids. The stationary phases described previously[10] did not give a satisfactory separation of the lower acids from water and the large volume of diethyl ether.

On Tween 80 columns (SMITH[13]) formic acid had a retention time between that of acetic and propionic acids; because of the excessive tailing of the acids, formic acid seriously overlapped both of the higher ones. Furthermore, the separation of water from the acids was not adequate for accurate work.

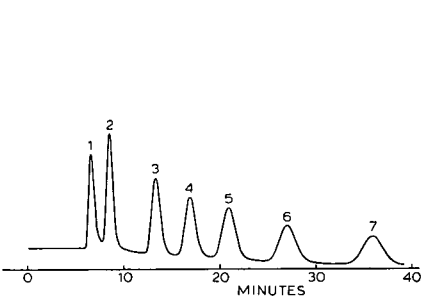
Addition of 10% orthophosphoric acid to the Tween 80 reversed the elution order of acetic and formic acids, but formic acid tailed to an even greater extent than in the absence of phosphoric acid.

The stationary phase of dioctyl sebacate and sebacic acid (RAUPP[14]) gave satisfactory separations of the C_1 to C_5 acids, but the retention times in the tempera-

ture range 125 to 145° were too short to permit adequate separation from water and a large volume of ether. Some improvement was effected by operating the column at 65° for the first ten minutes and then raising the temperature to 135°, but separation of ether and water from the acids was still unsatisfactory.

4 ft columns packed with 20% Ucon LB-550-X (SMITH *et al.*[7]) on acid-washed Chromosorb W did not separate formic and acetic acids. Furthermore, the peaks of all the acids were asymmetrical and tailed into the peaks of the following acids. A notable feature of this packing was the long retention times of the acids at lower temperatures, *e.g.* 65°. Replacement of 20% of the Ucon with behenic acid practically eliminated tailing, but formic and acetic acids were still not resolved. A mixture of equal parts of Ucon and behenic acid gave a partial separation of formic from acetic acid. When behenic acid was replaced by sebacic acid, the separation of formic from acetic acid was improved considerably (Fig. 1); a better separation of the butyric acid isomers was also obtained.

The columns finally adopted for the analytical procedure described below, were of stainless steel, 122 cm long and 4 mm internal diameter, packed with a



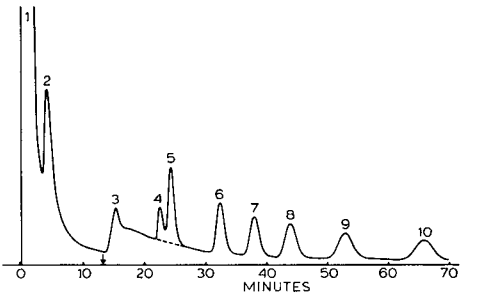


Fig. 1. Separation of a mixture of C_1 to C_5 acids on a column of 10% (w/w) Ucon LB-550-X and 10% (w/w) sebacic acid on acid-washed Chromosorb W. Sample, 2 μl mixed acids. Column temperature 145°. Helium flow-rate 30 ml/min. Attenuation × 8. Peaks: 1 = formic acid; 2 = acetic acid; 3 = propionic acid; 4 = isobutyric acid; 5 = *n*-butyric acid; 6 = isovaleric acid; 7 = *n*-valeric acid.

Fig. 2. Separation of acids in an ethereal solution prepared from a mixture of sodium salts of C_1 to C_5 acids on the column described in Fig. 1. Sample, 0.5 ml ethereal solution containing 1 mg mixed acids. Column temperature 65°, then raised to 135° where indicated ↓. Helium flow-rate 30 ml/min. Attenuation × 4. Peaks: 1 = ether, 2 and 3 = water; 4 = formic acid; 5 = acetic acid; 6 = propionic acid; 7 = isobutyric acid; 8 = *n*-butyric acid; 9 = isovaleric acid; 10 = *n*-valeric acid. Broken line represents true base-line of peaks 4 and 5.

mixture of Ucon LB-550-X (1 part), sebacic acid (1 part) and acid-washed Chromosorb W, 80–100 mesh (F & M Scientific Corporation) (10 parts). This column gave complete separation of acids from acetic to *n*-valeric; when the acids were applied as a solvent-free mixture the separation of formic from acetic acid was almost complete too (Fig. 1), but resolution was poorer when the acids were dissolved in 0.5 ml of ether. Water also had a deleterious effect, but the separations obtained with ethereal extracts dried with sodium sulphate (Fig. 2), were adequate for estimation of peak-areas by triangulation. Further drying with calcium sulphate might be worthwhile in some circumstances.

Analytical procedure

Ethereal solutions of volatile fatty acids were prepared from aqueous solutions of sodium salts by the method of McINNES[3].

The carrier-gas flow rate was 30 ml/min and the injection port was maintained at about 160°. With the column at 65°, up to 0.5 ml of an ethereal solution of volatile fatty acids was introduced into the injection port with a gas-tight syringe (Hamilton Co., Inc.).

10 to 15 min after injection of the sample the column temperature was raised rapidly (at about 80°/min) to 135°. A typical chromatogram is shown in Fig. 2; with large samples the water peak still overlapped the formic and acetic acid peaks. In such cases the broken line shown in Fig. 2 was used as a baseline for determining peak-areas for formic and acetic acids.

DISCUSSION

Although thermal conductivity detectors have been superceded to a considerable extent by more sensitive detectors, gas chromatographs employing the former are still used in many laboratories. In the field of volatile acid analysis they have an advantage over hydrogen flame detectors in that they respond to formic acid. However, when the solutions available for analysis are very dilute the low sensitivity of thermal conductivity detectors is a serious disadvantage.

The procedure described above permits the determination of the volatile fatty acids in dilute ethereal solutions using a gas chromatograph with a thermal conductivity detector. The success of the method depends, to a large extent, on the long retention times of the acids compared with ether and water, on columns containing Ucon LB-550-X at 65°. The stability of the column described was satisfactory; more than sixty separations were carried out on one column without detectable loss in efficiency.

For quantitative work without an internal standard, it is desirable to use a gas-tight syringe, since the pressure developed on injecting a large volume of ether into the injection port, may force some of the sample past the plunger of the syringe.

The method described above has been used in this laboratory for the determination of volatile fatty acids isolated from rumen contents and silages.

ACKNOWLEDGEMENT

This work was supported by a grant from the Australian Dairy Produce Research Committee.

SUMMARY

Some of the difficulties associated with gas–liquid chromatography of C_1 to C_5 fatty acids isolated from biological materials are discussed. Attention is directed particularly to the problems which arise when thermal conductivity detectors are used.

Column packings described previously for the separation of volatile fatty acids were unsatisfactory for analysing dilute ethereal solutions of the acids, especially if they contained small quantities of water.

A new stationary phase which adequately separated the acids from water and up to 0.5 ml of ether is described.

A procedure is described for the estimation of C_1 to C_5 fatty acids, isolated from biological material, using a gas chromatograph with a thermal conductivity detector.

REFERENCES

1 A. T. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 50 (1952) 679.
2 J. H. VAN DE KAMER, K. W. GERRITSMA AND E. J. WANSINK, *Biochem. J.*, 61 (1955) 174.
3 A. G. MCINNES, in D. H. DESTY (Editor), *Vapour Phase Chromatography*, Butterworths, London, 1956, p. 304.
4 C. L. HANKINSON, W. J. HARPER AND E. MIKOLAJCIK, *J. Dairy Sci.*, 41 (1958) 1502.
5 C. W. GEHRKE AND W. M. LAMKIN, *J. Agr. Food Chem.*, 9 (1961) 85.
6 RUTH N. SHELLEY, H. SALWIN AND W. HORWITZ, *J. Assoc. Offic. Agr. Chem.*, 46 (1963) 486.
7 E. E. SMITH, G. D. GEOTSCH AND H. D. JACKSON, *Arch. Biochem. Biophys.*, 95 (1961) 256.
8 V. M. RIDDLE, *Anal. Chem.*, 35 (1963) 853.
9 G. W. LANIGAN AND R. B. JACKSON, *J. Chromatog.*, 17 (1965) 238.
10 R. B. JACKSON, *J. Chromatog.*, 16 (1964) 306.
11 R. B. JACKSON, *J. Chromatog.*, 22 (1966) 251.
12 L. D. METCALFE, *Nature*, 188 (1960) 142.
13 B. SMITH, *Acta Chem. Scand.*, 13 (1959) 480.
14 G. P. RAUPP, *Angew. Chem.*, 71 (1959) 284.

CHROMATOGRAPHIE EN PHASE GAZEUSE ET LIPOCHIMIE

X. ANALYSE DE TRIGLYCÉRIDES MIXTES

D. LEFORT, R. PERRON, A. POURCHEZ, C. MADELMONT ET J. PETIT
Laboratoire de Lipochimie du C.N.R.S., Bellevue (France)

(Reçu d'abord le 27 janvier 1965)
(Reçu enfin le 18 octobre 1965)

INTRODUCTION

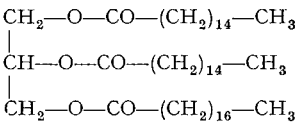
L'application de la chromatographie en phase gazeuse à l'étude des glycérides a conduit à des résultats suffisamment précis et certains pour que l'on puisse songer à aborder maintenant des problèmes plus délicats quant à la structure de ces composés.

En effet si les premiers essais dans ce domaine ont porté sur les mono- et diglycérides après acétylation des fonctions hydroxyle libres[1] ou sur les mono-glycérides sous forme d'esters allyliques[2], plusieurs auteurs ont montré que les triglycérides pouvaient être analysés correctement par chromatographie en phase gazeuse. FRYER *et al.*[3] utilisant un taux d'imprégnation de 30 % de graisse de Silicone ont publié des chromatogrammes significatifs mais dont le tracé laissait penser que l'on pouvait obtenir mieux. En diminuant le taux d'imprégnation (0.75 %) PELICK *et al.*[4] analysaient un mélange de triglycérides du trilauride au tristéaride en 40 minutes. Les uns et les autres constataient d'ailleurs que les phases stationnaires Polyesters ou Apiezon ne donnaient pas de bons résultats.

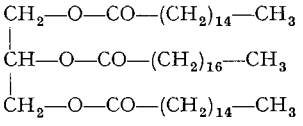
Simultanément, HUEBNER[5] et KUKSIS ET MCCARTHY[6] amélioraient les séparations par l'utilisation de la programmation de température permettant ainsi d'analyser un mélange de triglycérides simples de la triacétine au tristéaride en des temps raisonnables (20 à 40 min). Les températures pratiquées relativement élevées (300 à 350° pour le tristéaride) pouvaient faire craindre une décomposition des triglycérides sur la colonne; en fait le tracé des chromatogrammes et l'étude par Infra-Rouge des produits piégés à la sortie de la colonne autorisent les précédents auteurs à dire qu'il n'en est rien. Bien entendu après l'étude qualitative de mélanges synthétiques de triglycérides simples la méthode fut appliquée à l'analyse des huiles naturelles[5,6] et même d'un point de vue quantitatif à la détection des fraudes dans le beurre[7,8]. Dans les chromatogrammes des produits naturels on observe, certes, les pics correspondant aux triglycérides simples mais également d'autres pics auxquels les auteurs ont accordé, par interpolation, des structures brutes de triglycérides mixtes sans que ceux-ci aient pu être comparés avec des échantillons authentiques.

Dans le cadre d'une étude en analyse thermique différentielle effectuée sur les triglycérides, certains triglycérides mixtes ont été synthétisés, et pour vérifier la pureté des produits, nous en avons fait l'analyse par chromatographie en phase gazeuse et par chromatographie en couche mince.

Pour simplifier l'écriture nous désignerons, par exemple, l'α,β-dipalmityl-α'-stéaride soit:



par le sigle PPS;
l'α,α'-dipalmityl-β-stéaride soit:



par le sigle PSP et bien entendu les glycérides simples trimyristide, tripalmitide, tristéaride respectivement par les sigles M_3, P_3 et S_3, et ainsi de suite en désignant toujours par les lettres P, M et S les radicaux acyle correspondant respectivement aux acides palmitique, myristique et stéarique. Nous avons eu à étudier deux séries de triglycérides mixtes à deux termes homologues d'acides différents: acides palmitique et stéarique d'une part (soit SPP, PSP, PSS et SPS), acides palmitique et myristique d'autre part (soit de la même façon MPP, PMP, PMM et MPM).

Ces triglycérides ont été préparés de deux façons différentes:

Méthode A. Réaction d'un monoglycéride ou d'un diglycéride sur un acide gras adéquat en solution dans le chloroforme, à ébullition, en présence de quantités catalytiques d'acide *p*-toluènesulfonique.

Méthode B. Réaction d'un monoglycéride ou d'un diglycéride sur un chlorure d'acide gras convenable, en milieu chloroformique et en présence de pyridine à l'ébullition.

En outre, les triglycérides homogènes M_3, P_3 et S_3 ont été préparés par action directe des acides gras correspondants sur le glycérol également en milieu chloroformique à l'ébullition, et en présence d'acide *p*-toluènesulfonique comme catalyseur.

Les deux méthodes A et B appliquées aux triglycérides mixtes, ont conduit à des résultats très différents comme nous allons le voir.

TRIGLYCÉRIDES SYNTHÉTISÉS PAR LA MÉTHODE A

D'une façon générale les chromatogrammes des glycérides obtenus par cette méthode, présentent tous plusieurs pics.

Triglycérides d'acides stéarique et palmitique

Le chromatogramme d'un mélange de glycérides simples ($M_3 + P_3 + S_3$) donne un seul pic pour chacun des constituants, parfaitement symétrique et séparé des voisins comme le montre la Fig. 1, alors que celui d'un glycéride mixte (SPS, PSS, PPS ou PSP) est toujours constitué de trois ou quatre pics proches les uns des autres et d'importances relatives variables selon les échantillons. Par interpolation, d'après les températures d'élution comparées à celles d'un mélange témoin ($M_3 + P_3 + S_3$)

analysé dans les mêmes conditions on est en droit de supposer que le glycéride PSS, par exemple, obtenu par cette méthode de synthèse, est en fait un mélange de P_3 (308°), P_2S (318°), PS_2 (327°) et S_3 (334°) (Fig. 2).

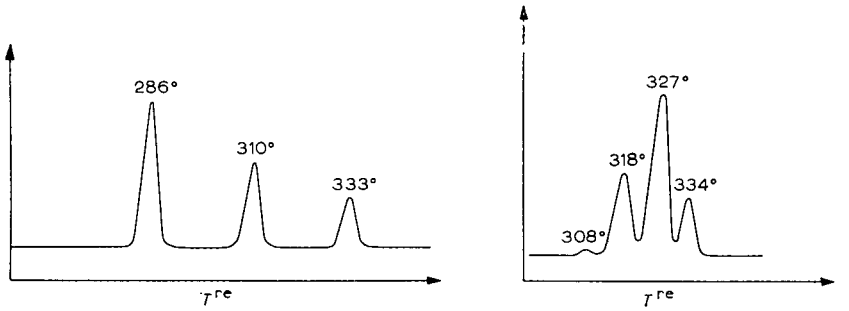


Fig. 1. Mélange de glycérides simples M_3–P_3–S_3. Colonne 50 cm; Silicone SE 52 — 4 % sur Chromosorb 60/80 traité HMDS. Température programmée: 4° par minute.

Fig. 2. Glycéride mixte PSS préparé selon méthode A. Colonne 50 cm; Silicone SE 52 — 4 % sur Chromosorb 60/80 traité HMDS. Température programmée: 4° par minute.

Toutefois, il peut être objecté également que le produit injecté était pur mais qu'il a subi au contact de la colonne et sous l'influence de la température des réarrangements intermoléculaires conduisant à divers glycérides.

Nous nous sommes efforcés de démontrer que précisément il n'y avait pas réarrangements et que les différents pics représentaient bien les glycérides correspondants, présents dans le produit initial. D'ailleurs la seconde méthode de synthèse a confirmé ce résultat puisqu'elle conduit, comme nous le verrons, à un produit pur ne donnant qu'un seul pic.

Nous avons travaillé sur un échantillon de SP_2 dont le chromatogramme comporte trois pics (Fig. 3) et les arguments prouvant qu'il s'agit d'un mélange sont

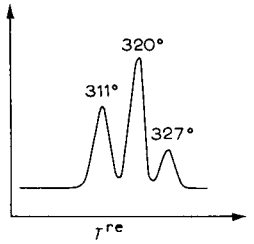


Fig. 3. Glycéride mixte PPS préparé selon méthode A. Colonne 50 cm; Silicone SE 52 — 4 % sur Chromosorb 60/80 traité HMDS. Température programmée: 4° par minute.

également valables pour les autres glycérides mixtes. Ces arguments sont les suivants:

(1) Un mélange des trois glycérides simples M_3, P_3 et S_3 donne trois pics parfaitement symétriques et de géométrie irréprochable. L'absence rigoureuse de pics parasites intermédiaires prouve qu'il n'y a pas d'échanges intermoléculaires. Dès lors nous voyons mal pourquoi ces échanges auraient lieu quand il s'agit de molécules de glycérides mixtes et non dans le cas de glycérides simples.

(2) En admettant que ces échanges existent et que la colonne en soit la cause, si nous faisons varier le temps de contact, en affichant une température initiale plus ou moins élevée et en opérant à une vitesse de programmation plus ou moins grande, nous devrions observer des modifications dans les proportions relatives des différents pics. Rien de tel ne se produit et, aux erreurs d'expériences près (de l'ordre de 5 %), les surfaces respectives sont équivalentes d'une expérience à l'autre comme le montre le Tableau I.

TABLEAU I

Gaz vecteur: Azote 47.5 ml/min. Temp. injecteur: 325°.

T° départ	*Progr.*	*Temp. élution des 3 pics*			*% Calculé par triangulation*			*Temps d'élution des 3 pics*		
200°	+4°	310	319	325	35.7	54.2	10.1	30′10″	32′32″	34′12″
200°	+3°	302	310.5	318	38	54.3	7.7	39′03″	42′22″	44′37″
225°	+4°	308	317.5	324	32	55	13	22′36″	24′05″	26′52″
225°	+3°	301	310	317	34.3	53.7	12	28′59″	32′18″	34′33″
225°	+2°	294	302	315	38	52.5	9.5	38′13″	44′08″	47′27″
250°	+4°	306	315	321.5	34.7	50.8	14.5	15′44″	18′06″	19′46″
250°	+3°	300	309	315	32.4	53.1	14.5	19′39″	22′50″	25′05″
250°	+2°	294	301	308	34.7	54	11.3	24′30″	28′45″	32′11″
275°	+4°	307.5	315	321	32.1	51.6	16.3	8′45″	10′46″	12′25″
275°	+3°	301	309	315	30.3	53.7	10	10′32″	13′22″	15′30″
275°	+2°	296	302.5	308	30.6	53.6	15.8	12′04″	15′44″	18′42″
275°	+1°	288	293.5	298	31.4	52.9	15.7	15′02″	20′57″	25′48″

(3) Les spectres I.R. du produit de départ et du produit récupéré en totalité à la sortie de la colonne, effectués en solution à même concentration, sont identiques qualitativement et quantitativement à l'exception d'une légère altération, dans le spectre du produit récupéré vers 1710 cm^{-1}. On observe d'ailleurs la même altération, insignifiante dans le spectre d'un glycéride simple, pur, récupéré après chromatographie.

(4) Le chromatogramme du glycéride SP_2 comporte trois pics (Fig. 3) dont nous avons attribué l'origine, dans l'ordre, aux glycérides P_3 (311°), SP_2 (320°), S_2P (327°); chacun de ces pics a été "piégé" à la sortie de la colonne, opération délicate étant donné la résolution relativement faible et la quantité de substance injectée réduite et qui a demandé des chromatographies répétées.

Chacun des produits ainsi récupérés a été identifié de la façon suivante:

(a) Nouvelle chromatographie en phase gazeuse: on obtient alors un seul pic élué à la température initialement observée dans le chromatogramme du produit de départ.

(b) Transformation des acides gras constitutifs des glycérides en esters méthyliques et détermination quantitative de la composition du mélange ainsi obtenu. Les résultats rassemblés dans le Tableau II, compte tenu du fait que la proximité des pics sur le chromatogramme du glycéride SP_2 ne permet pas une récupération excluant rigoureusement les produits voisins, confirment qu'il s'agit bien de la structure assignée d'après la température d'élution.

Nous pensons ainsi avoir démontré qu'un glycéride mixte préparé par la mé-

TABLEAU II

Désignation du pic	*Temp. d'élution (°C)*	*Composition % en esters méthyliques*			
		Trouvé		*Calculé*	
		Palmitique	*Stéarique*	*Palmitique*	*Stéarique*
No. 1 P_3	311	91	9	110.5	0
No. 2 SP_2	320	61	39	64.5	35.5
No. 3 S_2P	327	38	62	31.2	68.8

thode A était en fait un mélange de plusieurs glycérides, que les divers pics obtenus par chromatographie en phase gazeuse correspondaient à des glycérides existant dans le produit final et n'étaient pas la conséquence d'échanges intermoléculaires sur la colonne.

Triglycérides mixtes d'acides palmitique et myristique

D'une façon similaire la chromatographie en phase gazeuse révèle que les produits PMM, MPM, MPP et PMP sont également des mélanges de glycérides; par exemple le composé auquel le processus de synthèse pourrait à première vue attribuer la structure PMM est en fait constitué de M_3 (297°), M_2P (302°), MP_2 (308°) et P_3 (314°).

Ces résultats ont pour seul but de montrer que la méthode de synthèse utilisée conduit à un mélange et non à un corps pur et aucune autre interprétation ne peut être donnée des différences de composition selon les échantillons.

TRIGLYCERIDES SYNTHÉTISÉS PAR LA MÉTHODE B

Les glycérides mixtes d'acides stéarique et palmitique (SPP, PSP, SSP et SPS) obtenus par cette méthode de synthèse sont purs et ne donnent qu'un seul pic parfaitement symétrique en chromatographie en phase gazeuse; il n'est pas possible de différencier les isomères de position (SPP et PSP) et (PSS et SPS) dont les températures d'élution respectives (311° et 315°) s'intercalent entre celles du tripalmitide

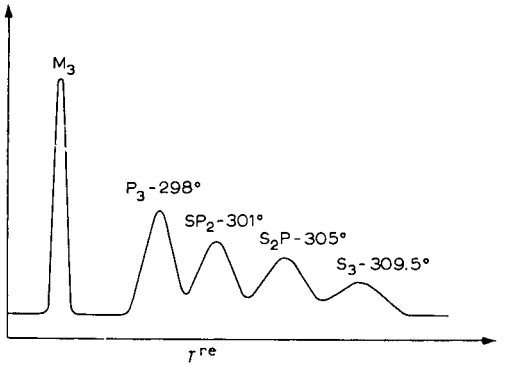


Fig. 4. Mélange de glycérides M_3–P_3–SP_2–S_2P–S_3. Colonne 50 cm; Silicone SE 52 — 4% sur Chromosorb 60/80 traité HMDS. Température programmée: 1° par minute.

(309°) et du tristéaride (319°) pour des conditions opératoires de colonne et de programmation de températures identiques bien entendu.

La séparation des glycérides P_3, SP_2, S_2P et S_3 est relativement aisée (Fig. 4) et apporte ainsi une première preuve du bien fondé des suppositions faites par divers auteurs dans l'interprétation des chromatogrammes de glycérides d'huiles naturelles.

CONFIRMATION DES RÉSULTATS PAR CHROMATOGRAPHIE EN COUCHES MINCES

Les différents composés ont été testés par la méthode de KAUFMAN *et al.*[9]. Cette méthode, d'emploi très différent n'a pu être valablement exploitée que dans le cas des triglycérides d'acides myristique et palmitique.

Les chromatogrammes obtenus sont représentés Fig. 5.

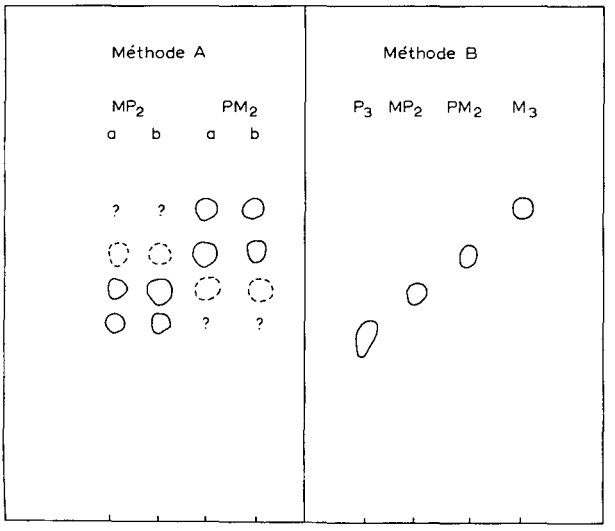


Fig. 5. Chromatographie en couche mince selon KAUFMANN *et al.*[9] des triglycérides dérivant des acides myristique et palmitique préparés par les méthodes A et B.

Les chromatogrammes de la partie gauche de cette figure se rapportent aux produits résultant de la méthode A, les désignations a et b indiquant que la synthèse a été faite respectivement à partir d'un mono ou d'un diglycéride.

Ces chromatogrammes montrent clairement que la méthode A conduit bien à un mélange de triglycérides, et confirment les résultats de la chromatographie gazeuse.

Dans la partie droite de la Fig. 5, les composés MP_2 et PM_2 préparés selon la méthode B, et les deux triglycérides homogènes M_3 et P_3, servant de témoins pour les chromatogrammes de la partie gauche, ne donnent effectivement qu'un seul spot, confirmant la pureté des produits obtenus par cette méthode.

CONCLUSIONS

La chromatographie en phase gazeuse et, conjointement, la chromatographie en couche mince, ont permis de montrer d'une façon certaine que la méthode A de

synthèse (glycéride partiel + acide gras, en présence d'acide *p*-toluènesulfonique) conduisait à des glycérides mixtes qui en fait se trouvent être des mélanges de divers glycérides mixtes et simples alors que la méthode B (glycéride partiel + chlorure d'acide en présence de pyridine) permet d'obtenir les produits purs attendus, et par déduction de confirmer le fait que l'acide *p*-toluènesulfonique est un excellent catalyseur de transestérification.

D'autre part la chromatographie en phase gazeuse permet de séparer et de caractériser des triglycérides dont le nombre total d'atomes de carbone diffère de deux unités.

PARTIE EXPÉRIMENTALE

Synthèse des glycérides

Avant d'appliquer les méthodes A ou B aux mono- et diglycérides convenables, nous avons préparé ceux-ci selon les procédés suivants:

Dans le premier cas (monoglycérides), et selon HARTMAN[10], en formant d'abord l'isopropylidène-glycérol en présence d'acide *p*-toluènesulfonique dans le chloroforme, puis en faisant réagir l'acide gras approprié sur ce composé, et en hydrolysant ensuite après formation d'un complexe de l'acide borique introduit dans le méthoxy-2 éthanol.

Les produits bruts ont été recristallisés plusieurs fois et successivement, dans l'éthanol et l'acétone.

Dans le second cas (α,α'-diglycéride), en faisant agir sur le glycérol, le chlorure d'acide correspondant, en milieu chloroformique, et en présence de pyridine et de N-diméthylformamide, selon HARTMAN[11]. Les produits bruts ont été recristallisés successivement dans l'éthanol et l'hexane.

Méthode A. Nous avons fait réagir, en transposant la méthode de HARTMAN[10] citée plus haut, les acides gras purs sur les mono- et diglycérides préparés précédemment, en milieu chloroformique, à l'ébullition, en présence d'acide *p*-toluènesulfonique, l'eau de la réaction ayant été éliminée par passage des vapeurs chloroformiques sur carbonate de potassium anhydre.

Les triglycérides homogènes ont été avantageusement synthétisés par le même procédé.

Méthode B. Les différents glycérides mixtes purs ont été préparés selon CRAIG *et al.*[12], en faisant agir le chlorure d'acide convenable sur les mono- ou diglycérides, en milieu chloroformique et en présence de pyridine, à reflux. Cristallisation des produits dans l'éthanol et l'acétone.

Les caractéristiques des divers produits préparés, tels les points de fusion ou de solidification, sont données dans une autre publication.

Chromatographie en phase gazeuse

Les chromatogrammes analytiques ont été réalisés avec un appareil Aérograph Hy-Fi A-600 à détecteur ionisation de flamme, muni d'un programmeur de température, dans les conditions suivantes:

Colonne: 50 cm × 0.2 cm; Chromosorb 60/80 traité HMDS. Silicone SE 52 — 4%; Gaz vecteur: Azote 60 cm³/min; Température injecteur: 350°; Température colonne: de 260° à 340°.

Les récupérations d'échantillon ont été faites sur un appareil Aérograph A 90 S, à détecteur catharomètre, dans les conditions suivantes:

Colonne: 50 cm × 0.45 cm; Chromosorb 60/80 traité HMDS. Silicone SE 52 — 15 %; Gaz vecteur: Hydrogène; Température injecteur: 350°; Température colonne: 320°.

Spectres I.R.

Les spectres I.R. ont été effectués en solution dans le tétrachlorure de carbone, avec un appareil Beckman IR-5.

Chromatographie en couches minces

(1) La pureté de tous les produits préparés, quant à leur identité de mono-, di- ou triglycéride, a été contrôlée selon la technique préconisée par PRIVETT *et al.*[13].

(2) Les produits MP_2, PM_2, P_3 et M_3 provenant des méthodes A et B, ont été chromatographiés selon KAUFMANN *et al.*[9], utilisant comme support du Kieselguhr G imprégné de tétradécane, et en éluant par le mélange acétonitrile–acétone (20:80) saturé à 80 % de tétradécane.

Les mêmes conditions appliquées aux glycérides dérivés des acides palmitique et stéarique, n'ont pas permis de bonnes séparations.

RÉSUMÉ

La chromatographie en phase gazeuse permet d'identifier et donc de vérifier la pureté, des divers glycérides mixtes d'acides myristique, palmitique et stéarique sans qu'il soit possible toutefois de différencier les isomères de position. Les auteurs ont démontré qu'un glycéride mixte ne subissait pas de modification au cours de l'opération.

SUMMARY

By means of gas chromatography it is possible to identify and verify the purity of various mixed glycerides of myristic, palmitic and stearic acid. Positional isomers can, however, not be differentiated. It is shown that a mixed glyceride does not undergo transformation during the procedure.

BIBLIOGRAPHIE

1 V. HUEBNER, *J. Am. Oil Chemists' Soc.*, 36 (1959) 262.
2 A. MCINNES, N. TATTRIE ET M. KATES, *J. Am. Oil Chemists' Soc.*, 37 (1960) 7.
3 F. FRYER, W. ORMAND ET G. CRUMP, *J. Am. Oil Chemists' Soc.*, 37 (1960) 589.
4 N. PELICK, W. SUPINA ET A. ROSE, *J. Am. Oil Chemists' Soc.*, 38 (1961) 506.
5 V. HUEBNER, *J. Am. Oil Chemists' Soc.*, 38 (1961) 628.
6 A. KUKSIS ET M. MCCARTHY, *Can. J. Biochem. Physiol.*, 40 (1962) 679.
7 A. KUKSIS, M. MCCARTHY ET J. BEVERIDGE, *J. Am. Oil Chemists' Soc.*, 40 (1963) 530.
8 A. KUKSIS ET M. MCCARTHY, *J. Am. Oil Chemists' Soc.*, 41 (1964) 17.
9 H. KAUFMANN, Z. MAKUS ET B. DAS, *Fette, Seifen, Anstrichmittel*, 63 (1961) 807.
10 L. HARTMAN, *Chem. Ind. (London)*, (1960) 711.
11 L. HARTMAN, *J. Chem. Soc.*, (1957) 3572.
12 B. CRAIG, W. LUNDBERG ET W. GEDDES, *J. Am. Oil Chemists' Soc.*, 29 (1952) 169.
13 C. PRIVETT, M. BLANK ET W. LUNDBERG, *J. Am. Oil Chemists' Soc.*, 38 (1961) 312.

GAS–LIQUID CHROMATOGRAPHY OF SUBMICROGRAM AMOUNTS OF DRUGS

I. PREPARATION, SCOPE AND LIMITATION OF COLUMNS

COLIN McMARTIN AND HAROLD V. STREET
Department of Forensic Medicine, University of Edinburgh (Great Britain)
(Received October 25th, 1965)

For certain aspects of toxicological work, it is essential to be able to carry out analyses *rapidly*. If gas–liquid chromatography is to be used in such analyses, the retention times of the drugs involved must, therefore, be made quite short. Retention times may be reduced by (a) using a high flow-rate of carrier gas, (b) shortening the column, (c) raising the column temperature or (d) reducing the percentage of liquid phase on the column. However, (a) and (b) are limited because if the flow-rate is too high or the column too short the resolving power of the column is lost, and there is an obvious limitation under (c) depending on the thermal stability of the stationary phase and of the sample. This means that support material coated with a small amount of a non-polar liquid phase must be used if short retention times are to be obtained for polar compounds of high molecular weight. Unfortunately, the use of column packings prepared from diatomaceous earth coated lightly with non-polar liquids for gas chromatography of drugs and other polar compounds results in an unsatisfactory peak shape, manifested by a sharp leading edge and a flat trailing edge which returns slowly to the baseline. When smaller samples are used, the time between injection and peak maximum increases and the peak shape is more distorted. Use-preconditioning may also occur. These effects can be explained in terms of adsorption of the sample by the surface of the support material. Drugs with hydrogen atoms capable of forming hydrogen bonds seem to be adsorbed strongly and this would suggest that adsorption may involve hydrogen bond formation. Several attempts to reduce this adsorption have been described.

Various support materials have been investigated by other workers. Of these, supports prepared from diatomaceous earth seem to have been used most widely and a number of ways of treating calcined diatomaceous earth have been reported. Using glass microbeads PARKER, FONTAN AND KIRK[1] found that the resolution of the column was poor and peak tailing occurred. A support material prepared from "Tide" was described by DECORA AND DINNEEN[2] and shown to give better results than an[3] acid-washed support prepared from diatomaceous earth. SMITH AND RADFORD compared a number of diatomaceous supports and showed that the adsorption by firebrick was greater than that of several flux-calcined products.

Acid-washing of support material was described by JAMES AND MARTIN[4] in an early paper on gas chromatography. Celite was washed with concentrated hydrochloric acid and then with water until neutral. The function of acid-washing

was stated to be the removal of metallic oxides. Acid-washing has often been used in conjunction with other procedures for improving support material and this makes it difficult to assess the importance of acid-washing. PARKER AND KIRK[5] described the behaviour of barbituric acid derivatives on acid-washed firebrick coated with SE-30. 1 to 10 μg of derivative were used but the method of acid-washing was not stated.

Treatment of supports with dimethyl-dichlorosilane (DDS) was described by HORNING, MOSCATELLI AND SWEELEY[6] who exposed their support material, which had been washed with concentrated hydrochloric acid, to DDS vapour. HOLMES AND STACK[7] found the conditions for adequate treatment with DDS to be critical. They recommend a detailed procedure in which acid-washed support is treated with a very dilute solution of DDS. ANDERS AND MANNERING[8,9] have reported results for a column packed with acid-washed, DDS-treated support used for phenothiazine derivatives and other drugs.

Hexamethyldisilazane (HMDS)-treatment of support materials was first described by BOHEMEN, LANGER, PERRETT AND PURNELL[10] who believed that acid-washing was unimportant and that their treatment, which involved refluxing support material with HMDS in petroleum ether, was superior to that involving DDS. BROCHMANN-HANSSEN AND SVENDSEN[11,12] used a column packed with acid-washed, HMDS-treated, support for barbiturates and sympathomimetic amines. Improvement of columns by *injection* of HMDS has been described by ATKINSON AND TUEY[13]. HMDS and DDS are believed to react with hydroxyl groups on the surface of the support material and thus remove adsorbing sites.

PARKER, FONTAN AND KIRK[14] described the application of a column containing support material coated with potassium hydroxide to the analysis of sympathomimetic amines. They found, not surprisingly, that barbiturates could not be run on this column. The use of polar additives to suppress tailing sometimes makes a column unsuitable for a particular class of drugs. Furthermore, the additives may be thermally unstable and thus limit the temperature range of the column.

The large number of variations involved make it virtually impossible to draw satisfactory comparisons of the various treatments of support material described by other workers. Because of this, it was decided to investigate some of these treatments. Using Chromosorb W, the effects of acid-washing and treatment with HMDS or DDS were tested by packing treated, but uncoated, powder into a column. The uncoated powder was used because it was thought that this would provide a more critical test of adsorption. These experiments indicated that acid-washing followed by treatment with DDS gave the best results. Further study indicated that poor results were obtained when very *dry* support material was treated with DDS. This discovery led to the treatment of *damp* support material with DDS. It was found that under these conditions remarkably good results could be obtained.

During these investigations we found that support material treated with dimethyldichlorosilane improves on heating. This prompted us to investigate the effect of heat on acid-washed Chromosorb W which had been coated with the silicone polymer SE-30. An improvement in results was obtained with temperatures above 300° in the absence of oxygen. In this connection, it is interesting to note that PARKER AND KIRK[5] describe the heating of a column, packed with acid-washed firebrick coated with SE-30, in argon (30 ml/min) at 280° for 24 h, and that GOLDBAUM,

SCHLOEGEL AND DOMINGUEZ[15] recommend that columns packed with SE-30 or QF-1 coated Chromosorb W should be heated at 300° for 8 h. These workers have not reported any development of this procedure and it is not clear from their articles whether they appreciated the effect that heating could have on column performance. The heating may have been intended to remove, from the column, material which caused a high recorder base-line.

It is relevant to note here that a procedure has been described for the production of water-repellent glass surfaces involving coating the surface with a silicone and then heating in air. JOHANNSON AND TOROK[16] stress that the surface must be degreased before coating, either by heating at 400° in air for 1 h, or by cleaning with a solvent. After coating, heating at 300° for 30 min or at lower temperatures for longer periods of time gives the best results. In an article on the thermal and oxidation stabilities of polymethylsiloxanes, ATKINS, MURPHY AND SAUNDERS[17] report that changes occur when these compounds are heated in an oxidising atmosphere at 200° and that cracking occurs in an inert atmosphere at 250°. It is likely that the treatment of surfaces by heating with silicone polymers will be more effective in an *inert* atmosphere, where the effect of increasing the temperature and duration of the treatment may be studied without fear of oxidation occurring. HUNTER *et al.*[18] have reported experiments on the treatment of glass surfaces with a number of silane derivatives, including polymers and lauryl and stearyl derivatives. Their experiments on the effect of heating were rather surprisingly carried out in air.

In a further investigation of the effect of heating support materials with SE-30, we varied the conditions of heating. Good results were obtained when the diatomaceous earth, coated with SE-30, was placed in a slow stream of nitrogen and heated in a furnace at 350° for 1 h. It was, however, not possible to obtain good results when the experiment was repeated. This led to a study of the factors which govern the effectiveness of the treatment. It was observed that a number of factors influence the behaviour of the columns prepared from support material which has been treated in the above manner with SE-30. The method of coating the support material before baking would seem to be critical. After much trial and error, a satisfactory coating procedure was developed in which the diatomaceous earth, which had not been thoroughly dried, was boiled with a solution of SE-30. After excess solution had been removed by filtration, the remaining solvent was removed from the powder by evaporation whilst stirring.

Another important factor is the position of the powder in the tube during the heating at 350°. In an experiment in which the flow rate of nitrogen during heating was much faster than usual, support material with very poor characteristics was obtained. It seemed possible that this was due to the removal of SE-30 or its products from the diatomaceous earth and it occurred to us that this might apply, to a lesser extent, to the powder into which nitrogen at the slower flow rate passed first. To test this, an experiment was performed in which an excess of powder was heated in a slow stream of nitrogen and only powder which had been well down-stream was used for packing the column. On some occasions very good results were obtained by this method.

On one occasion a remarkable improvement in a column which had formerly given poor results, occurred after a liver extract had been injected into the column. This led us to try the effect of tristearin on the performance of the column. When

tristearin was injected and the injector temperature was raised for a short time and then lowered, the results were similar to those obtained with the liver extract. Unfortunately, in some cases, this improvement was only temporary but the results were sufficiently encouraging to warrant further investigation involving tristearin. This was done by heating support material with SE-30, removing excess SE-30 by rinsing with petroleum or toluene and recoating with a mixture of tristearin and SE-30. This procedure yielded very good results. One of the features of tristearin treatment is that the peak height increases even when the tailing is not reduced very much.

Anomalous behaviour of certain metal columns led us to compare a number of stainless steel columns which were packed with portions of the same packing material. Some of the columns, including a new column which had not been used before, gave very much worse results than others. It was subsequently discovered that such columns could be rendered satisfactory by heating them in air to about 550° and then heating them packed with SE-30 coated support material with carrier gas passing.

The elucidation of many of these effects requires further investigation but we feel that it is important that some of the results we have obtained so far should be available for those interested in developing methods for the preparation of columns suitable for gas–liquid chromatography of small quantities of certain compounds.

Although the application of gas chromatography to drugs has been described by a number of workers, little attention seems to have been paid to the *minimum* amounts of drugs which could be run satisfactorily on the columns used. In general, the minimum amounts stated to have been used have been of the order of 1 or 2 μg. This paper includes a description of the gas-chromatographic behaviour of a number of different types of drugs on columns prepared by our new technique (see below). The limitations of these columns have been investigated and it has been found that, in general, the minimum amounts of drugs required for satisfactory analysis are about 25 times smaller than those described by most previous workers.

LLOYD *et al.*[19] give results for the gas chromatography of a number of high molecular weight alkaloids. The size of sample they used was between 5 and 30 μg and the column was packed with unwashed Chromosorb W coated with SE-30. The actual size of samples with which the reported results were obtained was not given.

PARKER, FONTAN AND KIRK[1,14] state their findings for 1 to 8 μg of tranquillizers on a column containing glass microbeads, and for 1 to 10 μg of a large range of drugs on columns packed with acid-washed Chromosorb W coated with SE-30. These drugs include barbiturates, tranquillizers and alkaloids such as morphine, quinine, emetine and strychnine. ANDERS AND MANNERING[8,9] have applied a column packed with dimethyldichlorosilane-treated Gas-Chrom S, coated with SE-30, to some phenothiazine derivatives and a number of other compounds. Results are given for 5 to 10 μg of phenothiazines, for 2.5 μg amphetamine and 30 μg of morphine.

KAZYAK AND KNOBLOCK[20] used 10 to 30 μg of sample in their analysis of a wide range of drugs including morphine, quinine and strychnine. These workers used a column, conditioned at 300° for 8 h, packed with Anakrom ABS coated with SE-30. They show a chromatogram obtained with 25 μg of morphine after extraction from urine. BROCHMANN-HANSSEN AND SVENDSEN[11,12] report results for 5 to 10 μg of a large number of barbituric acid derivatives and sympathomimetic amines. Barbiturates were analysed on a column containing SE-30 at 137° and amphetamine was

run on SE-30 at 82°. In both columns, the support material was Chromosorb W, acid-washed and treated with hexamethyldisilane. CIEPLINSKI[21] describes the preparation of a column for barbituric acid derivatives and shows results for 10 μg per μl of solution but he does not state what volume of this solution was injected.

JAIN, FONTAN AND KIRK[22] describe a column and procedure by which 0.08 μg of a barbituric acid derivative can be analysed after a simple extraction from blood. The column temperature they used was 230°, which would make their column unsuitable for high molecular weight compounds. VANDENHEUVEL, HAAHTI AND HORNING[23] describe the application of a column to a number of compounds (including barbiturates) but they do not state the amount of samples used in the recordings which they reproduce. They do, however, imply that they could "recognize" down to about 0.1 μg but, again, the meaning of their statement is not clear.

Our experimental investigations of some of the factors limiting the sensitivity of columns used for the gas chromatography of drugs resulted in the preparation of columns which gave satisfactory results with 0.04 μg of some barbituric acid derivatives at 160°. Some of these drugs could be detected at the 0.01 μg level. Also, a number of drugs either containing groups which we thought might cause the drug to run badly (*e.g.* with severe tailing) or else of high molecular weight were tested. The temperature of the column was adjusted to give a retention time of 2 to 8 min and the response for different quantities of sample was obtained.

EXPERIMENTAL

A Perkin-Elmer model 800 Gas Chromatograph equipped with a flame ionisation detector was used. The signal was recorded on a Honeywell —0.25 to +2.5 mV recorder. The carrier gas was nitrogen (oxygen free) 30 ml/min.

Preparation of column packing

About 200 ml of Chromosorb W 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 on a boiling water bath. 50 ml of this washed powder were then boiled for 10 min with an excess of toluene. A volume of a 10 % (w/v) solution of SE-30 in toluene, equal to the volume of toluene present was added to the mixture which was then thoroughly stirred and boiled briefly. The powder was then drained in three portions by vacuum filtration and each portion was dried with stirring on a hot-plate. The three 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. The tube and contents were then heated in a stream of nitrogen (about 30 ml/min) in a furnace at 350° for 1 h. That portion which had lain between the other two portions during the heating was placed in a sintered glass filter funnel and rinsed 4 times with toluene and 3 times with a solution containing 2 % (w/v) SE-30 and 0.1 % (w/v) tristearin in toluene. Each rinse involved stirring followed by vacuum filtration. The final rinse was followed by thorough

filtration and the powder was then dried with stirring on a hot-plate. The powder was packed into a metal column prepared as described below.

Preparation of metal column

The column consisted of a 6 ft. length of stainless steel tube $^1/_8$ in. O.D. and 0.085 in. I.D., 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 then heated in air in a furnace at 550° for 1 h. After cooling, the column was packed (as described below) with one of the end-portions of powder which had been coated with SE-30 and heated as described above. This column was then heated at 300° in the gas chromatograph with carrier gas flowing at 30 ml/min for 2 h. The column was then removed, emptied, and repacked with the middle fraction prepared as describe above. After heating in the gas chromatograph at 250° for 2 h, the column was ready for use.

Procedure for packing the column

The sintered steel disc end of the column was connected to a water pump. The open end of the column was held uppermost and powder was poured into it through a small filter funnel attached by rubber tubing. The column was tapped gently until no more powder entered. This procedure took less than 5 min. The open end of the column was then plugged with glass wool which had been silanised by wetting it with a 1 % (v/v) solution of DDS in petroleum ether, rinsing with methanol and then with petroleum ether, and drying.

Drug solutions

These were prepared in ethanolic solution and were injected into the gas-chromatograph with a 10 μl Hamilton graduated syringe. For nomenclature of the drugs, we have used that given in the Merck Index 1960.

RESULTS AND DISCUSSION

The results with Chromosorb W which was *rapidly* washed with concentrated hydrochloric acid and not allowed to stand with the acid were as good as those obtained with Chromosorb W which had *prolonged* contact with acid. Washing with aqua regia and concentrated nitric acid did not give better results than washing with concentrated hydrochloric acid.

Injection of HMDS or DDS into columns containing these acid-washed Chromosorb W preparations caused a large improvement. Silanisation of the acid-washed powder gave much better results than those of the silanised non-acid-washed powder. Treatment with HMDS similar to that described by BOHEMEN *et al.*[10] gave good results which deteriorated when the column was heated to 260°.

A commonly given explanation of the effects of silanising agents on support material is the formation of silyl ethers of reactive groups on the surface of the support. BOHEMEN *et al.*[10] suggest that HMDS reacts with $-\overset{|}{\underset{|}{Si}}-OH$ groups to produce the inert group $-\overset{|}{\underset{|}{Si}}-O-Si(CH_3)_3$. If this explanation is correct it would appear that this ether is not stable at 260°. In the same way reaction with DDS might be supposed to involve reaction of a DDS molecule with two neighbouring surface

–Si–OH groups or with one surface group and with a molecule of methanol. If methanol rinsing were omitted, this scheme suggests that free –Si–Cl groups would hydrolyse to form harmful –Si–OH groups. In fact, under these circumstances, DDS-treatment was found to be ineffective.

An adequate explanation of the effect of DDS must also take into account the importance of water and the improvement of treated support material on heating. A possible explanation of the importance of water is that adsorbing surface groups (which might be metallic oxides or derived from silica) are converted to a reactive form in the presence of water, *i.e.* metallic oxides might hydrate to give a hydroxide. In this connection, it is of interest that treatment of *wet* Chromosorb W (which had not been acid-washed) with DDS was successful. It is possible, however, that in this case the hydrochloric acid, released in the reaction of DDS with water, fulfilled the same function as acid-washing.

A rather different explanation of the results is also possible. If only a few of the adsorbing sites on the surface of the powder were reactive with DDS, DDS molecules would only be attached to a few sites along the surface. In the presence of water the formation of polymers on these sites would occur and this might lead to a situation in which adsorbing sites were screened by layers of silicone polymers on the surface.

A certain amount of information has been collected about the changes which occur when support material is heated with SE-30. When nitrogen was passed through coated support material and then through uncoated support material, both portions of support material being heated at 350°, the uncoated support was found to improve, but not to the same extent as occurred when *coated* support was heated under the same conditions. The improvement obtained by heating support material with SE-30 was still considerable even after heating in nitrogen at 500° for 1 h, by which time the stationary phase had been removed, (presumably by thermal decomposition and vaporisation). When powder which had been heated with SE-30 was rinsed thoroughly with toluene or with petroleum ether and recoated with SE-30 the results were still very good. These observations suggest a reaction in which parts of silicone polymers are chemically bound to the surface of the support material. It would appear that a reaction can occur between the support material and volatile products formed by heating SE-30.

The importance of a number of other factors must also be taken into account in an explanation of the effect of heating with SE-30. The presence of water on the support material and the coating procedure are important. Also when powder is heated in a high flow-rate of nitrogen poor results are obtained. Furthermore, that portion of the powder (in the glass heating tube) through which the nitrogen passed first yielded inferior results. Possibly the contact of SE-30 with the surface is essential for obtaining good results, and hence uniform coating of the surface is important. The role of water might then be connected with the spreading of the SE-30, or the water might be involved in the reaction. The adverse effect of a high flowrate of nitrogen might be due to traces of oxygen in the nitrogen or it might be connected with removal of SE-30 from the powder through which the nitrogen first passes. These suggestions are only tentative and further investigation of the effect of heating with SE-30 on support materials may yield useful results.

The improvement that occurs when tristearin is injected has some unexpected features. The quantity of tristearin required is very low (500 μg) and, in some cases, there is a large change in peak height with little change in tailing. The mode of action of the tristearin is not known but the results indicate that a process other than simple adsorption can influence the results obtained in gas–liquid chromatography.

Adsorption which affects the results must lie somewhere between the extremes of weak adsorption by a large number of sites and strong adsorption by a few sites. These types of adsorption will exert different effects on the results. With weak adsorption, distortion of the peak shape will occur but it should be possible to run small quantities through the column although the retention time may increase slightly. Strong adsorption will be characterised by the removal of a fixed amount of sample and by the fact that good results can be obtained for a sufficiently large sample (which saturates the adsorbing sites). A column with strongly adsorbing sites may show marked use-conditioning.

It might, therefore, be thought that tristearin occupies a few strongly adsorbing sites. Tristearin, however, does not behave as a polar phase because a 0.1 % coating of tristearin does not alter the retention times of a column. In view of this, if the removal of adsorbing sites occurs after the injection of tristearin it must be caused either by polar decomposition products or by reaction.

The difficulty with this explanation is that tristearin affects the peak heights over a wide range of sample sizes. If, for instance, 0.1 μg of a sample were strongly adsorbed the results with 0.1 μg would be poor and with 1 μg they would be good. Suppose injection of tristearin reduced the adsorption to 0.01 μg. The results with 0.1 μg would improve enormously and the peak height with 1 μg by a factor of 10/9. In practice, on some occasions, the peak heights from 0.04 to 2 μg of barbituric acid derivatives approximately doubled following injection of tristearin.

It would appear then that although adsorption may play an important part in the non-ideal behaviour of packed columns, another effect operates which prevents a certain fraction of sample from emerging in the main sample peak and this effect is controlled by tristearin.

Two suggestions of possible causes of this phenomenon may clarify the situation. The carrier gas used in this work was oxygen-free nitrogen. This gas may contain up to 5 p.p.m. of oxygen and a calculation shows that, if a sample is in the column for 4 min, enough oxygen will have passed through to oxidise about a μg of sample or, alternatively, to produce sites which would capture a similar quantity of sample. Suppose this reaction were slow and the rate depended on the concentration of sample in the gas phase, an amount of sample proportional to sample size would be eliminated. Tristearin might remove oxygen by reaction or it might react with sites as they were produced. However, an attempt to eliminate oxygen from the carrier gas did not produce better results.

An alternative explanation is that after reaction with SE-30 a layer of molecules attached to the surface forms a screen through which sample can diffuse slowly to adsorbing sites which have not reacted. The control of the rate of adsorption can be seen in terms of closely spaced polymer molecules which will occasionally adopt a configuration allowing access to the surface. The tristearin or its products might slowly diffuse through the polymer layer and thus modify this effect. The rate of sample diffusion might depend on concentration and hence similar fractions of sample

would be removed. The importance of the coating procedure before baking might, therefore, be due to the production of a dense layer of silicone polymers chemically bound to the surface.

The condition of stainless steel columns and methods for improving such columns are of great importance. The inferior results obtained with some untreated columns may be due to contamination but, if this is the case, the contamination must be such that it could not be removed by toluene, petroleum ether or methanol. Metal columns have some obvious advantages over glass columns; they are strong, can be bent without heating into almost any shape desired and are not fragile. This means that packing can be done rapidly and firmly because the column can be tapped without fear of breakage whilst it is being packed.

The following approximate quantities, calculated for the materials and apparatus used in this work may be useful in a consideration of factors which limit the column performance.

1. The surface area of the powder in one of our packed columns is about 10 square metres.

2. The surface area of the inside of one of our columns is about 0.01 square metres.

3. A 2% (w/w) loading of the powder with SE-30 should give a layer, on average, 3 molecules deep.

4. 250 μg of tristearin could cover an area of 0.2 square metres.

5. 2 μg of sample could cover an area of 0.002 square metres.

As regards the scope of the column prepared as described above, the results show that there is a wide variation in the performance of the column for different compounds. Table I shows the retention times for a given column temperature and the minimum detectable amounts of various drugs using our column. The drugs were selected because they contained a group or groupings which might be expected to prevent the compound from running satisfactorily on the column. These drugs were,

TABLE I

MINIMUM DETECTABLE AMOUNT OF DRUGS CHOSEN TO TEST THE SCOPE OF THE COLUMN

Drug	*Column temperature (°C)*	*Retention time (min)*	*Minimum amount of drug detectable (μg)*
Amphetamine	120	2.5	0.2
Phenelzine	140	3.1	1
Salicylic acid	160	1.5	2
Secobarbital	160	9.2	0.01
4-Hydroxyacetanilide	180	3.5	2
Caffeine	180	4.9	0.04
Cyclizine	215	2.7	0.01
Phenylbutazone	215	7.2	0.01
Scopolamine	225	4.6	0.2
Morphine	245	3.2	0.2
Librium*	245	3.6 and 7.3	0.04
Trifluoperazine	245	6.9	0.04

* Librium shows two peaks. The first peak to emerge may be a decomposition product of librium.

therefore, used as a test of the ability of the column to handle small amounts of compounds of a range of chemical types encountered in toxicological analysis. It is difficult to identify the features of a drug which cause it to run badly from the small selection of drugs considered here but the following observations can be made.

The highly conjugated drugs phenylbutazone, caffeine and librium can be detected in amounts less than 0.04 μg. Drugs containing the piperazine ring, substituted so as to give two tertiary amine groups, such as cyclizine and trifluoperazine can be detected in small quantities whereas the same amount of scopolamine, which contains a tertiary amine group and an alcohol group, does not give as satisfactory a result. 0.2 μg of morphine (a tertiary amine, a phenol and an alcohol) and amphetamine (a primary amine) is the minimum quantity to give satisfactory results. More than 1 μg of phenelzine (a derivative of hydrazine), salicylic acid (a carboxylic acid and a phenol) and 4-hydroxy-acetanilide are required for satisfactory results. It is interesting to note that *p*-aminophenol gives better results than its N-acetylated derivative 4-hydroxyacetanilide. The high molecular weight drugs quinine, brucine and emetine were not detected when up to 10 μg were injected at 245°. Retention times for these compounds have been reported by other workers but in some cases the reported retention time is so short that it seems likely that the observed peak was due to a decomposition product.

One of the most important facts which emerges from our results is that, with most of the drugs we have used, the retention time does not increase as the concentration of the injected drug is reduced. For example, identical retention times were obtained for 0.2 μg and 0.01 μg of secobarbital; 0.2 μg and 0.01 μg of phenylbutazone; for 0.2 μg and 0.04 μg of librium; and for 0.2 μg and 0.04 μg of trifluoperazine. In all these examples, the volume of solution injected was 1 μl. This point is discussed further in our article dealing specifically with the analysis of barbiturates and some related compounds in biological media (see McMARTIN AND STREET[24]).

Our results suggest that our column could be used to develop a procedure to screen biological samples for the presence of an unknown drug in poisoning cases, where the quantity of drug present would be sufficiently large for the technique to be used to cover rapidly a large number of drugs. Work along these lines is now being carried out.

The column we used was designed to have short retention times so that high molecular weight compounds could be analysed at reasonable temperatures and, hence, so that the column would be applicable, with temperature-programming, to a large range of drugs. For work on a single drug, it would be possible to use a column containing more phase or a more polar phase which, whilst more limited in the range of drugs to which it were applicable, would give results with smaller quantities of certain drugs than those described in this paper.

A technique capable of estimating 0.01 μg or less of a drug would be very useful in studies connected with the correlation of the "free" drug level in the plasma and therapeutic effectiveness in the human.

ACKNOWLEDGEMENT

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

SUMMARY

Experiments on Chromosorb W show that if hexamethyldisilazane (HMDS) or dimethyldichlorosilane (DDS) is to be used to reduce adsorption then acid-washing is also necessary in addition to the silanisation to obtain satisfactory results with drugs. Treatment with HMDS is not thermostable. Acid-washed Chromosorb W which has been treated with DDS improves after heating at 260°. The presence of water on the acid-washed support is necessary for satisfactory DDS treatment. A column packing which gives satisfactory results with 0.04 μg of some barbiturates at 200° has been prepared by treatment of *wet* Chromosorb W with DDS.

Some of the factors limiting the quantities of certain drugs which can be analysed by GLC are described. Support material suitable for small quantities of certain barbituric acid derivatives was prepared by heating the support material, coated with SE-30 at 350° in a stream of nitrogen. Consistent results required special procedures for coating and heating the powder. A remarkable improvement in poor columns sometimes occurred following the injection of tristearin. The detailed preparation of SE-30–tristearin columns is described. The effect of tristearin has been exploited in the preparation of good columns. The state of the metal column is important if good results are to be obtained and even new columns may be unsatisfactory. A satisfactory procedure for improving poor metal columns is described. Good results were obtained with 0.04 μg of certain barbituric acid derivatives at 160° using an SE-30–tristearin column.

The scope of this type of column was tested with drugs chosen to represent a wide range of chemical types. For each drug the temperature was adjusted to give a retention time of about 4 min and the minimum quantity of drug to give satisfactory results under these conditions was determined. This quantity varied from 5 μg for 4-hydroxyacetanilide to 0.01 μg for cyclizine and phenylbutazone. 0.04 μg of a number of drugs can be analysed successfully using an all-metal system.

REFERENCES

1 K. D. PARKER, C. R. FONTAN AND P. L. KIRK, *Anal. Chem.*, 34 (1962) 757.
2 A. W. DECORA AND G. U. DINNEEN, *Anal. Chem.*, 32 (1960) 164.
3 E. D. SMITH AND R. D. RADFORD, *Anal. Chem.*, 33 (1961) 1160.
4 A. T. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 50 (1952) 679.
5 K. D. PARKER AND P. L. KIRK, *Anal. Chem.*, 33 (1961) 1378.
6 E. C. HORNING, E. A. MOSCATELLI AND C. C. SWEELEY, *Chem. Ind. (London)*, (1959) 751.
7 W. L. HOLMES AND E. STACK, *Biochim. Biophys. Acta*, 56 (1962) 163.
8 M. W. ANDERS AND G. J. MANNERING, *J. Chromatog.*, 7 (1962) 258.
9 M. W. ANDERS AND G. J. MANNERING, *Anal. Chem.*, 34 (1962) 730.
10 J. BOHEMEN, S. H. LANGER, R. H. PERRETT AND J. H. PURNELL, *J. Chem. Soc.*, (1960) 2444.
11 A. BROCHMANN-HANSSEN AND A. B. SVENDSEN, *J. Pharm. Sci.*, 51 (1962) 318.
12 A. BROCHMANN-HANSSEN AND A. B. SVENDSEN, *J. Pharm. Sci.*, 51 (1962) 938.
13 A. P. ATKINSON AND G. A. P. TUEY, *Nature*, 199 (1963) 482.
14 K. D. PARKER, C. R. FONTAN AND P. L. KIRK, *Anal. Chem.*, 35 (1963) 356.
15 L. R. GOLDBAUM, E. L. SCHLOEGEL AND A. M. DOMINGUEZ, in A. STOLMAN (Editor), *Progress in Chemical Toxicology*, Academic Press, London, New York, 1963, p. 41.
16 O. K. JOHANNSON AND J. J. TOROK, *Proc. Inst. Radio Engrs.*, 34 (1946) 296.
17 D. C. ATKINS, C. M. MURPHY AND C. E. SAUNDERS, *Ind. Eng. Chem.*, 39 (1947) 1395.
18 M. J. HUNTER, M. S. GORDON, A. J. BARRY, J. F. HYDE AND R. D. HEIDENREICH, *Ind. Eng. Chem.*, 39 (1947) 1389.
19 H. A. LLOYD, H. M. FALES, P. F. HIGHET, W. J. A. VANDENHEUVEL AND W. C. WILDMAN, *J. Am. Chem. Soc.*, 82 (1960) 3791.

20 L. KAZYAK AND E. C. KNOBLOCK, *Anal. Chem.*, 35 (1963) 1448.
21 E. W. CIEPLINSKI, *Anal. Chem.*, 35 (1963) 256.
22 N. C. JAIN, C. R. FONTAN AND P. L. KIRK, *Microchem. J.*, 8 (1964) 28.
23 W. J. A. VANDENHEUVEL, E. O. A. HAAHTI AND E. C. HORNING, *Clin. Chem.*, 8 (1962) 351.
24 C. MCMARTIN AND H. V. STREET, *J. Chromatog.*, 1966, in press.

CHROMATOGRAPHIC STUDIES ON ORGANOSILICON COMPOUNDS

PART II. PYROLYSIS OF ARYLTRIMETHYLSILANES

F. H. POLLARD*, G. NICKLESS** AND D. B. THOMAS
Department of Chemistry, The University, Bristol (Great Britain)
(Received September 27th, 1965)

INTRODUCTION

Detailed investigations on the thermal pyrolysis of tetraalkylsilanes have been carried out[1–6] using gas chromatography combined with mass, infra-red, and nuclear magnetic resonance spectroscopic techniques to elucidate the numerous products produced. The chemical cleavage of silicon to carbon bonds, especially where at least one of the carbon atoms attached to the silicon is also part of an aromatic system has been thoroughly examined[7]. However, little work could be found concerning the thermal decomposition of such arylsilanes[8], and hence it seemed profitable to initiate studies on these systems. This paper describes qualitative aspects of studies made on these compounds using gas chromatographic methods. Briefly, samples of the silanes were injected into a reactor, and the products swept directly on to a gas chromatography column. The products were subsequently identified by comparison of their retention times with those of known compounds. Later, it is intended to publish the results of quantitative kinetic studies on alkylarylsilanes.

RESULTS

All compounds observed except trimethylsilane were identified using retention time techniques, the latter being identified by inference since no sample of trimethylsilane was available for comparison purposes. Later work carried out when trimethylsilane was available, has shown that the assumption was completely justified[9]. A graph of log retention volume against carbon number also indicated that our assignment was unambiguous. Fig. 1 shows a plot of boiling points (°C) *versus* log corrected retention time (t'_R).

PYROLYSES OF INDIVIDUAL SILANES

(*a*) *Phenyltrimethylsilane*

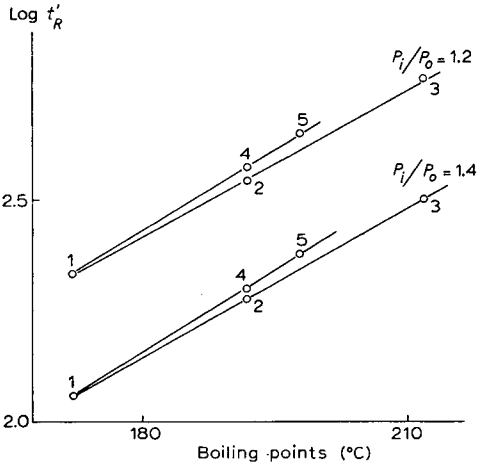
This compound gave a measurable cracking pattern commencing at a reactor temperature of 755°, when trimethylsilane and benzene were the major products. Traces of methane, and toluene were also formed. On increasing the temperature,

* Deceased October 16th, 1965.
** To whom enquiries should be addressed.

the amount of cracking increases, and all four products are clearly observable at 830°, where the sample was 37 % decomposed (see Fig. 2).

(*b*) *Benzyltrimethylsilane*

This compound commenced decomposing at about 700°, and was 32 % decomposed at 755°. The major products were methane, trimethylsilane, toluene, with small amounts *p*-xylene, *o*-xylene, phenyltrimethylsilane, and either *o*- or *p*-methylbenzyltrimethylsilane. At 830° the compound was 95 % decomposed (see Fig. 3).



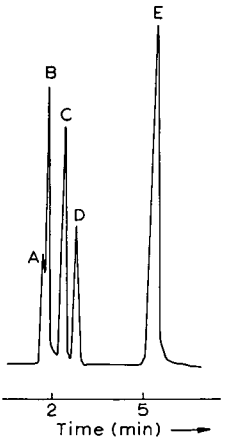


Fig. 1. Graph of $\log_{10}(t'_R)$ against boiling points for a series of aryltrimethylsilanes at various P_i/P_o ratios. 1 = Phenyltrimethylsilane; 2 = benzyltrimethylsilane; 3 = *p*-methylbenzyltrimethylsilane; 4 = *o*-tolyltrimethylsilane; 5 = *p*-tolyltrimethylsilane.

Fig. 2. Pyrolysis pattern of phenyltrimethylsilane. A = CH_4; B = $(CH_3)_3SiH$; C = C_6H_6; D = $C_6H_5CH_3$; E = $C_6H_5Si(CH_3)_3$.

(*c*) *p-Methylbenzyltrimethylsilane*

The decomposition pattern appeared at 700°, and the extent of decomposition at various temperatures was found to be similar to that of benzyltrimethylsilane.

The products formed were, methane, trimethylsilane, *p*-xylene with smaller amounts of benzene, toluene, phenyltrimethylsilane and benzyltrimethylsilane.

An unknown compound boiling at a higher temperature than the starting material was also formed (see Fig. 4).

(*d*) *o-Tolyltrimethylsilane*

This compound showed less signs of decomposition than the other silanes at 700°.

However, the decomposition pattern became clear at temperatures around 750°, where it was degraded to the extent of about 12 %. At 830° *o*-tolyltrimethylsilane is 70 % decomposed.

The major products were methane, trimethylsilane and toluene, with smaller amounts of *p*-xylene, phenyltrimethylsilane, and some *o*- or *p*-methylbenzyltrimethylsilane (see Fig. 5).

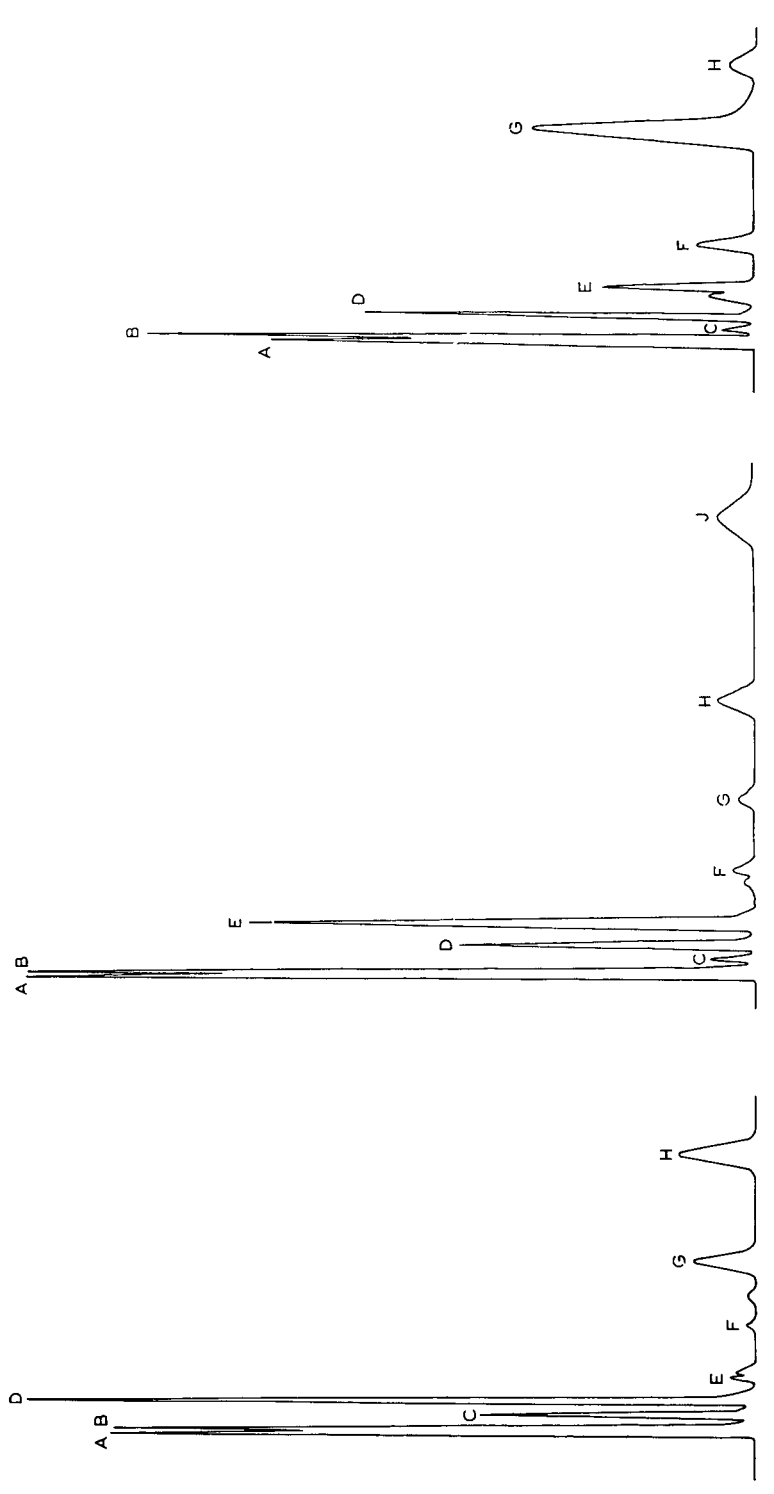


Fig. 3. Pyrolysis pattern of benzyltrimethylsilane. A = CH_4; B = $(CH_3)_3SiH$; C = C_6H_6; D = $C_6H_5CH_3$; E = xylenes; F = $C_6H_5Si(CH_3)_3$; G = $C_6H_5CH_2Si(CH_3)_3$; H = *o*- or *p*-$CH_3C_6H_4CH_2Si(CH_3)_3$.

Fig. 4. Pyrolysis pattern of *p*-methylbenzyltrimethylsilane. A = CH_4; B = $(CH_3)_3SiH$; C = C_6H_6; D = $C_6H_5CH_3$; E = *p*-xylene; F = C_6H_5-$Si(CH_3)_3$; G = $C_6H_5CH_2Si(CH_3)_3$; H = *p*-$CH_3C_6H_4CH_2Si(CH_3)_3$; J = unknown.

Fig. 5. Pyrolysis pattern of *o*-tolyltrimethylsilane. A = CH_4; B = $(CH_3)_3SiH$; C = C_6H_6; D = $C_6H_5CH_3$; E = *p*-xylene; F = $C_6H_5Si(CH_3)_3$; G = *o*-$CH_3C_6H_4Si(CH_3)_3$; H = *o*- or *p*-$CH_3C_6H_4CH_2Si(CH_3)_3$.

(e) p-Tolyltrimethylsilane

This compound began to show a decomposition pattern at 755°, and at 830° was degraded to an extent of 44 %.

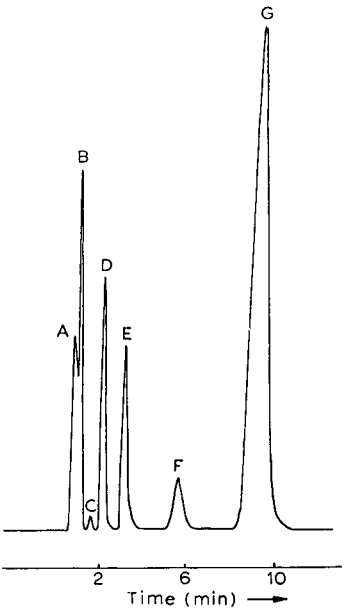
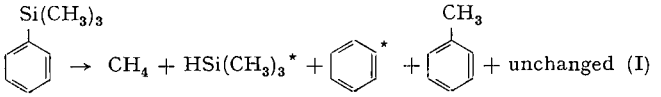


Fig. 6. Pyrolysis pattern of *p*-tolyltrimethylsilane. A = CH_4; B = $(CH_3)_3SiH$; C = C_6H_6; D = $C_6H_5CH_3$; E = p-$CH_3C_6H_4CH_3$; F = $C_6H_5Si(CH_3)_3$; G = p-$CH_3C_6H_4Si(CH_3)_3$.

The products were methane, trimethylsilane, *p*-xylene and phenyltrimethylsilane (see Fig. 6).

DISCUSSION

(a) Phenyltrimethylsilane



It is clear that the main process is the cleavage of the Si–C_6H_5 bond. Hydrogen is obtained from the carrier gas.

Methyl radicals may be present due to breakdown of the benzene rings. These probably react with phenyl radicals in competition with hydrogen to form toluene.

The mechanism can be postulated as follows:

Initiation: $C_6H_5Si(CH_3)_3 \rightarrow C_6H_5^{\bullet} + {}^{\bullet}Si(CH_3)_3$ (homolytic cleavage)

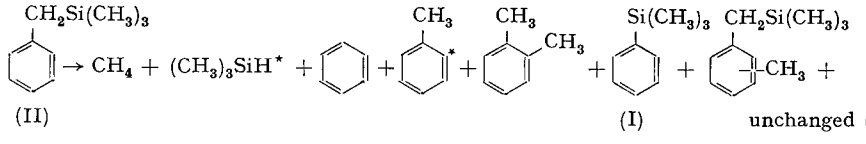
$H_2 + {}^{\bullet}Si(CH_3)_3 \rightarrow HSi(CH_3)_3 + H^{\bullet}$

$C_6H_5 + H^{\bullet} \rightarrow C_6H_6$

* Indicates major products.

It is likely that it is the trimethylsilyl radical which attacks the hydrogen molecule in preference to the phenyl radical which is stabilised by delocalisation of the odd electron into the π-orbitals of the ring.

(*b*) *Benzyltrimethylsilane*



This sequence indicates that the Si–CH_2 bond is cleaved more easily than the CH_2–C_6H_5 bond. This is due to the fact that the former has a bond energy of 76 kcal/mole, while the latter has a value of 82.7 kcal/mole.

Secondary cracking of toluene gives a small amount of benzene and methane.

It also appears that methyl radicals also attack toluene molecules to produce a very small amount of *o*-xylene.

The reaction is interesting in that a small amount of phenyltrimethylsilane is formed together with a substantial amount of *o*- and/or *p*-methylbenzyltrimethylsilane.

The mechanism may be:

Initiation: $C_6H_5CH_2Si(CH_3)_3 \rightarrow C_6H_5CH_2^{\bullet} + {}^{\bullet}Si(CH_3)_3$

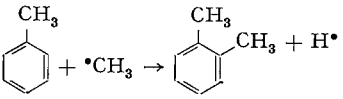
$(CH_3)_3Si^{\bullet} + H_2 \rightarrow (CH_3)_3SiH + H^{\bullet}$

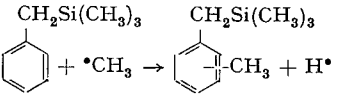
$C_6H_5CH_2^{\bullet} + H^{\bullet} \rightarrow C_6H_5CH_3$

$C_6H_5CH_3 \rightarrow C_6H_5^{\bullet} + CH_3^{\bullet}$

$C_6H_5^{\bullet} + {}^{\bullet}Si(CH_3)_3 \rightarrow C_6H_5Si(CH_3)_3$

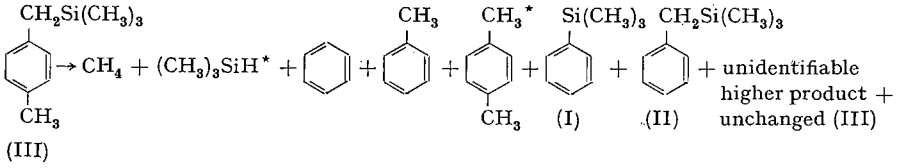
$C_6H_5^{\bullet} + H_2 \rightarrow C_6H_6 + H^{\bullet}$





The reason for the formation of a substantial amount of the latter compound is not clear since it has been shown that it has the same stability as the benzyltrimethylsilane from which it is formed.

(*c*) *p-Methylbenzyltrimethylsilane*



The main products were again formed by cleavage of the Si–CH_2 bond.

Benzene and toluene are problably formed as degradation products of *p*-xylene. A considerable amount of toluene is formed but only a small amount of benzene, probably due to the relatively short residence time in the furnace. Consequently only a small amount of phenyltrimethylsilane is formed.

The fact that xylene is the main product indicates clearly that the Si–CH_2 bond breaks first. This would also follow from bond energy considerations. Thus the mechanism for the formation of benzyltrimethylsilane must be due to reaction of toluene with trimethylsilyl radicals, rather than by the shearing off of a methyl radical with subsequent replacement by a hydrogen radical.

The boiling point of the unknown product is estimated to be in the range 230° to 250°. This estimate was inferred by extrapolating the graphs of log (retention time) against boiling points for the aromatic series and the silane series (see Fig. 7).

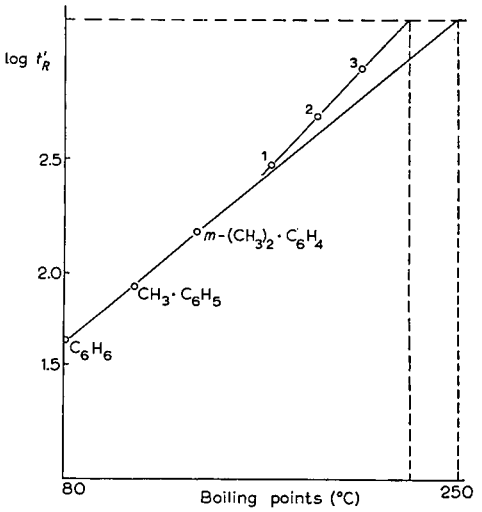
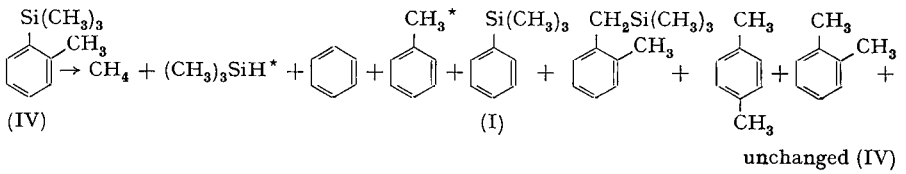


Fig. 7. Extrapolation of $\log_{10} t'_R$ against boiling points for (i) aromatic hydrocarbons and (ii) aryltrimethylsilanes. 1 = phenyltrimethylsilane; 2 = benzyltrimethylsilane; 3 = *p*-methylbenzyltrimethylsilane.

(d) o-Tolyltrimethylsilane



Again cleavage of the bond between silicon and the ring occurs. This takes place in preference to cleavage of the bond between the methyl group and the ring, since toluene is one of the main products.

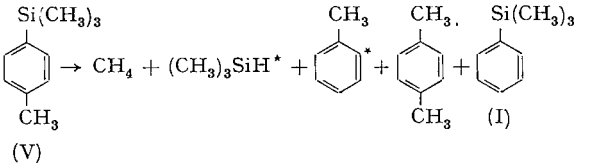
The formation of phenyltrimethylsilane is explained as before. (Very little benzene is formed.)

The other silane formed, namely the methylbenzyltrimethylsilane is likely to be the *ortho-* isomer. Evidence for this is that it has a longer retention time than the *para-* isomer, the retention time of which is known. It would be expected to have a higher boiling point than the *para-* isomer (*cf.* *o-* and *p*-tolyltrimethylsilanes).

This compound is probably formed by attack by a trimethylsilyl radical on an *o*-xylene molecule.

It is uncertain how the xylene itself is formed, but it is definitely present in a small amount, the amount increasing with increasing temperature. A suggestion is that it formed by methylation of toluene in the lower, cooler part of the furnace.

(*e*) *p-Tolyltrimethylsilane*



(V) (I)

The silane and toluene are formed as explained previously.

Phenyltrimethylsilane is also formed in small amounts. It is probably formed by combination of a trimethylsilyl radical with a phenyl radical formed from the degradation of a toluene molecule. This is offered as an explanation for the absence of benzene in the products.

The order of decreasing stability at 755° is:

$$C_6H_5Si(CH_3)_3 > p\text{-}CH_3C_6H_4Si(CH_3)_3 > o\text{-}CH_3C_6H_4Si(CH_3)_3 > \begin{cases} C_6H_5CH_2Si(CH_3)_3 \\ p\text{-}CH_3C_6H_4CH_2Si(CH_3)_3 \end{cases}$$

At 830° the order of the first two silanes is reversed (see Fig. 8).

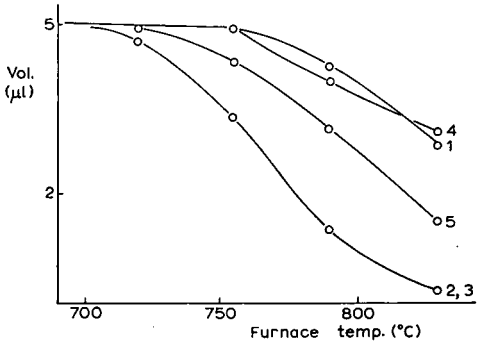


Fig. 8. Cracking rate for the aryltrimethylsilanes as a function of temperature. 1 = phenyltrimethylsilane; 2 = benzyltrimethylsilane; 3 = *p*-methyltrimethylsilane; 4 = *o*-tolyltrimethylsilane; 5 = *p*-tolyltrimethylsilane.

Rate of cracking

This was calculated using the results at 790°. Relative values were found due to the uncertainty in the value for the residence time in the furnace, due to the fact

that the furnace was not heated uniformly, and the length of furnace at the temperature quoted was uncertain.

The following first-order rate equation was used:

$$R = 1/t \ln C_0/C$$

where: R = rate constant, t = residence time, C_0 = initial concentration of sample, C = final concentration of sample.

The ratios of the rate constants were found to be:

$$C_6H_5Si(CH_3)_3: \begin{cases} C_6H_5CH_2Si(CH_3)_3 : o\text{-}CH_3C_6H_4Si(CH_3)_3 : p\text{-}CH_3C_6H_4Si(CH_3)_3 = 1 : 8.8 : 3 : 1.4 \\ p\text{-}CH_3C_6H_4CH_2Si(CH_3)_3 \end{cases}$$

Comparison of the behaviour of the silanes with the corresponding hydrocarbons

Data are available only for the simpler substituted hydrocarbons. Comparison can be made between *tert.*-butylbenzene and phenyltrimethylsilane. The former has been shown to crack at 600°[10,11], while the latter cracks only above 700°.

Thus it is apparent that the Si–C_6H_5 bond is more stable than the C–C_6H_5 bond although theoretically the C–C bond is stronger than the C–Si bond.

The stability of phenyltrimethylsilane is probably due to the back-donation of π-electrons from the benzene ring into the vacant *d*-orbitals of the silicon. Thus the C_6H_5–Si bond order is increased[12].

EXPERIMENTAL

Preparation of aryltrimethylsilanes

(*a*) *Phenyltrimethylsilane.* The phenyl Grignard reagent was prepared from magnesium turnings (15 g) and bromobenzene (60 g) in sodium-dried ether (600 ml).

Trimethylchlorosilane (41 g) was added dropwise to the Grignard reagent, and upon complete addition, the mixture was refluxed for 4 h. Fractional distillation produced the required product in 41 % yield.

$C_6H_5Si(CH_3)_3$. Analyses: C (found) = 71.96 %, H (found) = 9.39 %; required C = 71.94 %, H = 9.39 %; $n_{20°}^{D}$ = 1.4896.

(*b*) *Benzyltrimethylsilane.* The benzyl Grignard reagent was prepared from magnesium turnings (12 g) and benzyl bromide (57.5 g) in sodium-dried ether (450 ml).

Trimethylchlorosilane (36.6 g) was added dropwise over a period of 90 min, during which time precipitation occurred.

On fractional distillation the product was obtained in 79 % yield.

$C_6H_5CH_2Si(CH_3)_3$. Analyses: C (found) = 74.67 %, H (found) = 10.00 %; required C = 73.09 %, H = 9.82 % (consistent analytical figures for carbon were difficult to obtain, the value above is an average), $n_{20°}^{D}$ = 1.4954.

(*c*) *p-Methylbenzyltrimethylsilane.* The *p*-methylbenzyl Grignard reagent was prepared from magnesium turnings (12 g) and *p*-methylbenzyl chloride (46.9 g) in sodium-dried ether (500 ml).

Trimethylchlorosilane (30.2 g) was added to the Grignard reagent over a period of 45 min. The resulting mixture was refluxed and stirred for 3 h and then left to reflux overnight.

On fractional distillation the product was obtained in 29 % yield.

$CH_3C_6H_4CH_2Si(CH_3)_3$. Analyses: C (found) = 74.20 %; H (found) = 9.83 %; required C = 74.08 %, H = 10.17 %, $n_{20°}^{D}$ = 1.4950.

(*d*) *o-Tolyltrimethylsilane.* A block of sodium metal (29 g) was cut up into small pieces and melted in boiling toluene (75 ml). A mixture *o*-tolyl chloride (54.32 g) and trimethylchlorosilane (63.30 g) was placed in a dropping funnel and added dropwise to the molten sodium over a period of 45 min. The resulting mixture was refluxed for 22 h. Ethanol was added to remove unreacted sodium and the solution was washed with water three times.

The non-aqueous layer was fractionally distilled and yielded the product in 30 % yield.

$CH_3C_6H_4Si(CH_3)_3$. Analyses: C (found) = 72.51 %; H (found) = 9.43 %; required C = 73.09 %, H = 9.82 %, $n_{20°}^{D}$ = 1.5050.

(*e*) *p-Tolyltrimethylsilane.* The solid *p*-bromotoluene (25.7 g) was dissolved in dry ether (80 ml) and added dropwise to magnesium turnings (5 g) in sodium-dried ether (250 ml).

Trimethylchlorosilane (16.29 g) was added to the resulting Grignard reagent and the mixture was refluxed for 15 h. The reaction mixture was fractionally distilled and the product was obtained in 50 % yield.

$CH_3C_6H_4Si(CH_3)_3$. Analyses: C (found) = 73.00 %, H (found) = 9.58 %; required C = 73.09 %, H = 9.82 %, $n_{20°}^{D}$ = 1.4930.

The I.R. spectra of these compounds were taken on an Unicam S.P. 200 spectrophotometer employing thin films.

Apparatus

The column used was a copper U-tube 1.8 m in length and 0.5 cm I.D. It was heated by means of a vapour jacket containing either benzene or toluene. The former was used for the greatest part whilst the latter was used in order to improve the resolution of the first two peaks.

The carrier gas used was B.O.C. hydrogen. The flow rate was measured by means of a soap-bubble flow-meter at the end of the system. The pressure of the gas entering the system was measured by means of a mercury manometer.

The katharometer detector was situated directly at the end of the column and was kept at a constant temperature. It was a four-filament type and the filament current was maintained at 140 mA. The output of the detectors was measured directly by a 10 mV F.S.D. Elliot recorder.

The furnace unit was composed of a copper tube 20 cm in length and 0.4 cm I.D. This was enclosed in a silica-glass tube, around which a heating wire 80 cm in length was wound. This, in turn, was covered with a silica tape which supported the outer cement case. The unit was positioned in the flow system directly before the column.

Samples were injected by means of a 10 μl Hamilton syringe, through a self-sealing silicone seal, which was placed in the open end of the T-piece connecting the furnace to the gas stream. The injection port was surrounded by a tube, in the form of a loop, carrying cooling water to prevent deterioration of the seal at high temperatures.

The furnace temperature was obtained by means of a calibrated chromel-alumel thermocouple.

The heating current was produced by means of a transformer giving 15 A and a stepped-down mains voltage of up to 90 V. The voltage applied to it was varied by means of a rheostat and this afforded a convenient method of varying the furnace temperature.

The furnace was packed with copper turnings to increase the hot surface area.

The flow rate employed was 0.79 ml/sec. Samples of 2 μl phenyltrimethylsilane were taken and samples of 5 μl of the other silanes.

The temperature range covered was 450° to 830°.

SUMMARY

This paper describes a preliminary investigation into the pyrolysis of some aryltrimethylsilanes in the presence of hydrogen in a continuous-flow reactor. Gas–liquid chromatography was used to detect the products, which were subsequently identified by comparison of their retention times with those of pure known compounds.

The nature of the products is in accordance with bond energy considerations and mechanisms for the reactions are suggested.

It is intended to examine quantitative aspects of these reactions with a view to obtaining activation energy values.

REFERENCES

1 D. F. HELM AND E. MACK, *J. Am. Chem. Soc.*, 59 (1937) 60.
2 C. E. WARING, *Trans. Faraday Soc.*, 36 (1940) 1142.
3 G. FRITZ AND B. RAABE, *Z. Anorg. Allgem. Chem.*, 299 (1959) 232.
4 G. FRITZ AND J. GROBE, *Z. Anorg. Allgem. Chem.*, 315 (1962) 157.
5 G. FRITZ, H. BAHL, J. GROBE, D. AULINGER AND W. REERINK, *Z. Anorg. Allgem. Chem.*, 312 (1961) 301.
6 G. FRITZ, *Fortschr. Chem. Forsch.*, 44 (1963) 459.
7 C. EABORN, *Organo-Silicon Compounds*, Butterworths, London, 1960, pp. 146–159.
8 V. CHVALOVSKY, private communication.
9 D. B. THOMAS, unpublished observations.
10 A. DOBRYANSKII, E. K. KANEP AND S. V. KATZMAN, *Trans. Exptl. Research Lab. "Khemgas" (Leningrad)*, 3 (1936) 1.
11 A. DOBRYANSKII, E. K. KANEP AND S. V. KATZMAN, *Trans. Exptl. Research Lab. "Khemgas" (Leningrad)*, 2 (1935) 60.
12 J. A. BEDFORD, J. R. BOLTON, A. CARRINGTON AND R. H. PRINCE, *Trans. Faraday Soc.*, 59 (1963) 53.

LIQUID–LIQUID PARTITION CHROMATOGRAPHY WITH THE SYSTEM CHLOROFORM–CYCLOHEXANE–NITROMETHANE

WENDELL F. SMITH, Jr. AND KENNETH L. EDDY
Research Laboratories, Eastman Kodak Company, Rochester, N.Y. 14650 (U.S.A.)
(Received October 22nd, 1965)

INTRODUCTION

Liquid–liquid partition chromatography (LLC) can be divided into two major categories: (1) column partition chromatography, and (2) paper chromatography. Both techniques were introduced in the early 1940's by MARTIN and his co-workers[1,2]. Since that time paper chromatography has seen a phenomenal development, whereas column partition chromatography has progressed at a much slower rate. This is particularly true with regard to instrumentation which, when compared to the elegant tools available for gas chromatography, is still in a stage of infancy. At least two laboratories are currently working in this field[3–5]. The popularity of paper chromatography has undoubtedly been due in large part to the convenience with which small amounts of materials may be separated.

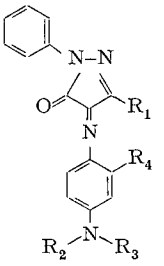
A similar situation exists in the separation of synthetic dyestuffs. The vast majority of dye separations by partition chromatography* have been carried out with paper as the support[6,7]. Furthermore, since most synthetic dyestuffs have acidic or basic groups, practically all of these separations have employed water or hydroxylated solvents as the stationary phase. DEREPENTIGNY AND JAMES[10] have reported one of the few separations of dyes by column partition chromatography. These workers separated two isomeric aminofluoresceins using 0.2 *M* sodium phosphate buffer supported on kieselguhr as the stationary phase, and *n*-butanol–cyclohexane mixtures for the mobile phase. In this paper, we describe a completely organic system which has been found highly efficient for the separation of many hydrophobic dyes.

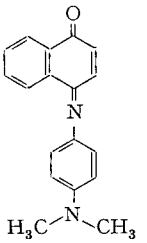
It was of interest to separate quantitatively, as well as qualitatively, mixtures of azomethine and indoaniline dyes, such as those with structures I–XIII: I–X are magenta dyes, XI is a cyan dye, and XII and XIII are yellow dyes. These dyes are, in general, sensitive towards acids and bases and consequently often deteriorate when examined by adsorption chromatography on the common adsorbents such as alumina and silica gel. Although we have been able to resolve some mixtures on polyamide columns with no loss of dye, more often we have found that alumina or silica gel is required.

To solve our problem we turned to liquid–liquid partition chromatography. We have investigated several liquid–liquid systems for separating azomethine and

* We are not here concerned with the incorporation of indicator dyes in the stationary phase to render zones of colorless acidic materials, such as fatty acids, visible on a column[1,8,9].

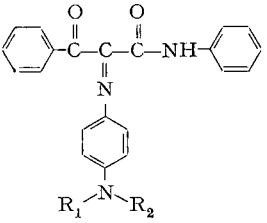
indoaniline dyes and have found the chloroform–cyclohexane–nitromethane system to be the most satisfactory. With this system, we have also effected separations within other classes of materials, including chloroplast pigments and azo dyes such as those (XIV–XIX) used by BROCKMANN AND SCHODDER[11] for determining the activity of alumina.



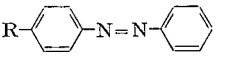


(XI)

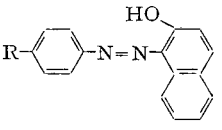
(I) $R_1 = CH_3$; $R_2 = R_3 = C_2H_5$; $R_4 = H$
(II) $R_1 = R_2 = CH_3$; $R_3 = C_2H_5$; $R_4 = H$
(III) $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$
(IV) $R_1 = CH_3$; $R_2 = C_2H_5$; $R_3 = R_4 = H$
(V) $R_1 = R_2 = CH_3$; $R_3 = R_4 = H$
(VI) $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$
(VII) $R_1 = C_6H_5$; $R_2 = R_3 = CH_3$; $R_4 = H$
(VIII) $R_1 = R_2 = R_3 = R_4 = CH_3$
(IX) $R_1 = NHCOC_6H_5$; $R_2 = R_3 = R_4 = CH_3$
(X) $R_1 = C_6H_5$; $R_2 = CH_3$; $R_3 = R_4 = H$



(XII) $R_1 = R_2 = CH_3$
(XIII) $R_1 = CH_3$; $R_2 = H$



(XIV) R = H
(XV) R = OCH_3
(XVI) R = NH_2
(XVII) R = OH



(XVIII) R = H (Sudan yellow)
(XIX) R = $C_6H_5N = N$ (Sudan red).

RESULTS AND DISCUSSION

The system chloroform–cyclohexane–nitromethane

The binoidal curve (Fig. 1) for the ternary system was determined at laboratory temperature (24°) by titration, with the same solvents which were to be used for chromatographic purposes. The solvents were Eastman Kodak Spectro Grade nitromethane, Practical Grade chloroform, and Eastman Grade cyclohexane. The addition of chloroform to cyclohexane–nitromethane mixtures is endothermic. Almost all of the

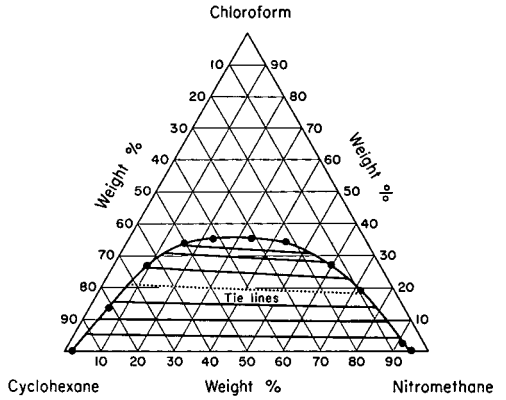


Fig. 1. Solubility curve at 24° for the system chloroform–cyclohexane–nitromethane.

chloroform necessary to give one phase was therefore added to the cyclohexane–nitromethane mixtures, and these solutions were then allowed to stand at room temperature for about two hours before the additional chloroform necessary to give one phase was added. The results are given in Table I and Fig. 1.

To establish the ends of the tie lines, refractive index measurements were used. The refractive index of the upper (non-polar) phase changes slowly with changing composition, but it changes more rapidly with changing composition of the lower

TABLE I

SOLUBILITY CURVE AT 24° FOR CHLOROFORM–CYCLOHEXANE–NITROMETHANE

Chloroform (*wt.* %)	*Cyclohexane* (*wt.* %)	*Nitromethane* (*wt.* %)
0.0	97.8*	2.2*
13.7	77.6	5.7
26.8	63.9	9.3
33.9	50.1	16.0
35.4	41.3	23.3
35.7	30.7	33.6
34.3	22.3	43.4
27.2	13.5	59.3
18.5	9.8	71.7
2.3	6.3	91.4
0.0	5.1*	94.9*

* Ref. 12 (value at 25°).

phase. A point of each tie line was determined by the composition of a two-phase mixture. A second point was established by measuring the refractive index of the lower phase. The data are presented in Table II and Fig. 1. The dotted tie line in Fig. 1 indicates the phase pair which we have found satisfactory for our separations. The composition of the phases are (by weight): lower (stationary) phase: 18.1 % $CHCl_3$, 9.7 % C_6H_{12}, 72.2 % CH_3NO_2; upper (mobile) phase: 21.1 % $CHCl_3$, 72.1 % C_6H_{12}, 6.8 % CH_3NO_2. Other phase pairs may prove better for other classes of compounds.

TABLE II

THE LINE DATA FOR CHLOROFORM–CYCLOHEXANE–NITROMETHANE

	Chloroform (*wt.* %)	*Cyclohexane* (*wt.* %)	*Nitromethane* (*wt.* %)
Upper phase	5.5	91.8	2.7
Lower phase	4.8	5.7	89.5
Upper phase	10.3	86.1	3.6
Lower phase	9.3	7.0	83.7
Upper phase	15.8	79.1	5.1
Lower phase	13.7	8.1	78.2
Upper phase	21.1	72.1	6.8
Lower phase	18.1	9.7	72.2
Upper phase	26.2	64.5	9.3
Lower phase	21.9	11.3	66.8
Upper phase	31.0	56.5	12.5
Lower phase	28.0	14.0	58.0
Upper phase	33.5	51.0	15.5
Lower phase	30.8	17.0	52.2

Preparation of phases and columns

To prepare the mobile and stationary phases, any mixture on the selected tie line, properly equilibrated, may be used. From the practical standpoint, one usually desires a larger quantity of mobile than of stationary phase (for elution), although occasionally the reverse is true (for coating the support). For the former case, we use (*cf.* dotted tie line, Fig. 1) 796 ml of $CHCl_3$, 496 ml of CH_3NO_2 and 5000 ml of C_6H_{12}, stirred for at least three hours. The mobile (upper) phase is then stored in bottles over a few milliliters of stationary phase.

As a support, powdered cellulose (Whatman CF 11 Chromedia) is excellent. Although silica gel is capable of supporting more stationary phase than cellulose and therefore gives columns of higher capacity, separations are much slower on such columns because some adsorption still occurs. Consequently, dyes are more apt to decompose on LLC columns in which silica gel is the support. Cellulose has also proved more satisfactory than several diatomaceous-earth preparations.

The column design is important. It is essential that the neck of the column (consisting of a standard-taper outer joint to accommodate a solvent reservoir) be

slightly larger in diameter than the rest of the column. This permits a machined cylindrical Teflon packing plug, approximately 1 in. long, to fit snugly in the column. The plug has a hole drilled in the center of one end. The hole is threaded to fit the threaded end of a stainless-steel rod of a length convenient for packing purposes. If the Teflon plug does not fit snugly, the column may be packed unevenly, *i.e.*, one side tighter than the other, and uneven bands and streaking may result during a separation.

A typical procedure for preparing a 1-in. diameter by 30-in. long column follows:

(1) *Coating the cellulose.* A 2-l, three-necked Morton-flask[13], equipped with a dropping funnel and an air-driven, propeller-type stirrer, is charged with 150 g of cellulose powder (Whatman CF 11 Chromedia) and enough mobile phase to cover the cellulose completely. To this is slowly added, with rapid stirring, 45 g (30 % by weight of the cellulose) of nitromethane *or* 45 g of lower phase* (Table II). The coated cellulose is kept covered with mobile phase at all times.

(2) *Packing the column.* The column is packed by the conventional wet-packing technique. Enough cellulose is added at a time to give about 2 in. of packed material. The material is compressed using the tool described above, about 65 lb./sq.in. pressure being applied. Pressure rings do not adversely affect the performance of these columns. When the cellulose is packed, its upper surface is covered with a little sand, and approximately 1 in. of mobile phase is left above the sand for column storage. It is advisable not to leave a large amount of mobile phase over a column (*e.g.*, in a solvent reservoir) since temperature fluctuations in the laboratory may cause some phase separation. If this happens droplets of (mainly) nitromethane form on top of the sand; the droplets are readily removed with a syringe, however, and this must be done before the column is put into use. Thermostatting the column and reservoir would probably circumvent this problem and also prolong the life of the column. We have found that our columns give satisfactory separations for about twenty chromatograms, the efficiency slowly decreasing with use.

Solutes are readily recovered from the eluents by rotary evaporation with a Rinco evaporator at room temperature. Traces of residual nitromethane may be removed by adding benzene or methanol and re-evaporating the solution.

Column efficiency

The height equivalent to a theoretical plate (H.E.T.P.) was measured for a freshly packed, 1-in. diameter column, by Method 2 of JAMES AND MARTIN[14]. With dye III as solute and an elution rate of 3.0 ml/min, the H.E.T.P. was 0.5 mm.

R_F values

The R_F values were calculated from the formula:

$$R_F = R\left(\frac{A_m}{A}\right)$$

* Columns prepared with cellulose coated with nitromethane give initially better separations than those prepared with cellulose coated with lower phase. However, with use the two become equivalent.

in which[1]:

R = (movement of position of maximum concentration of solute)/(simultaneous movement of surface of developing fluid in empty part of tube above chromatogram),

A = area of cross section of the column,

A_m = area of cross section of the mobile phase.

The values were determined with 1-in. diameter columns, by using an elution rate of approximately 3 ml/min. The results for the dyes are given in Table III, in which the R_F values by LLC are also compared with those by TLC (Al_2O_3 plates with benzene as eluent). The least-squares relationship (Fig. 2) is R_F (LLC) $\cong$ 0.30 + 0.81 R_F (TLC) $\pm$ 0.10. This allows at least a rough estimate to be made of the separation to be expected by LLC on the basis of a quick TLC experiment.

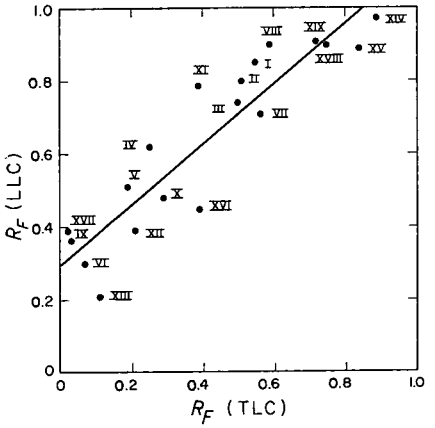


Fig. 2. R_F values by TLC (alumina plates, benzene eluent) *versus* R_F values by LLC.

The R_F values for the plant pigments of a grass-leaf extract are given in Table IV. Identification is based upon the TLC data of ANWAR[15] and ROLLINS[16]. On a 36-in.

TABLE III

R_F VALUES OF DYES I–XIX

Dye	*TLC**	*LLC*	*Dye*	*TLC**	*LLC*
I	0.55	0.85	XI	0.39	0.79
II	0.51	0.80	XII	0.21	0.39
III	0.50	0.74	XIII	0.11	0.21
IV	0.25	0.62	XIV	0.89	0.97
V	0.19	0.51	XV	0.84	0.89
VI	0.07	0.30	XVI	0.39	0.45
VII	0.56	0.71	XVII	0.02	0.39
VIII	0.59	0.90	XVIII	0.75	0.90
IX	0.03	0.36	XIX	0.72	0.91
X	0.29	0.48			

* Elution with benzene on alumina plates.

column, complete resolution of four of the xanthophylls is obtained. Chlorophylls *a* and *b* are resolved, but each is contaminated with a carotene, pheophytin, or xanthophyll.

TABLE IV

R_F VALUES OF PLANT-LEAF PIGMENTS

Band	*Identification*	R_F *value*
Yellow	Carotene	0.54
Gray	Pheophytin	0.53
Blue-green	Chlorophyll *a*	0.50
Yellow	Xanthophyll	0.47+
Green	Chlorophyll *b*	0.47—
Yellow	Xanthophyll	0.41
Yellow	Xanthophyll	0.36
Yellow	Xanthophyll	0.32
Yellow	Xanthophyll	0.21

SUMMARY

The system chloroform–cyclohexane–nitromethane has been investigated at 24°, and the application of one phase pair to liquid–liquid partition chromatography described. The use of powdered cellulose as the support for the stationary (polar) phase has given excellent separations of some plant-leaf pigments and azomethine, indoaniline, and azo dyes.

REFERENCES

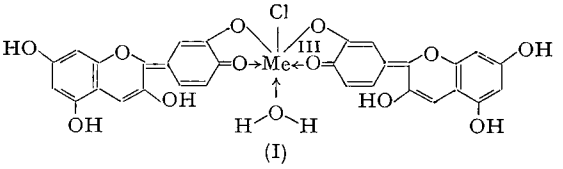
1 A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 35 (1941) 1358.
2 R. CONSDEN, A. H. GORDEN AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
3 S. M. LAMBERT AND P. E. PORTER, *Anal. Chem.*, 36 (1964) 99.
4 S. M. LAMBERT, *Anal. Chem.*, 37 (1965) 959.
5 K. J. BOMBAUGH AND J. N. LITTLE, *J. Chromatog.*, 16 (1964) 47.
6 E. LEDERER AND M. LEDERER, *Chromatography*, Elsevier, New York, 1937, Chap. 15.
7 K. VENKATARAMAN, *The Chemistry of Synthetic Dyes*, Academic Press, New York, 1952, pp. 1326–1331.
8 A. H. GORDON, A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 37 (1943) 79.
9 H. F. LIDDELL AND H. N. RYDON, *Biochem. J.*, 38 (1944) 68.
10 J. DEREPENTIGNY AND A. T. JAMES, *Nature*, 174 (1954) 927.
11 H. BROCKMANN AND H. SCHODDER, *Ber.*, 74B (1941) 73.
12 H. J. WECK AND H. HUNT, *Ind. Eng. Chem.*, 46 (1954) 2521.
13 A. A. MORTON AND D. M. KNOTT, *Ind. Eng. Chem. (Anal. Ed.)*, 13 (1941) 649.
14 A. J. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 50 (1952) 681.
15 M. H. ANWAR, *J. Chem. Educ.*, 40 (1963) 29.
16 C. ROLLINS, *J. Chem. Educ.*, 40 (1963) 32.

TRENNUNG VON ANTHOCYANGEMISCHEN DURCH KOMPLEXBILDUNG AN ALUMINIUMOXID

LEONHARD BIRKOFER, CHRISTELMARGOT KAISER UND MANFRED DONIKE
Institut für Organische Chemie der Universität Köln (Deutschland)
(Eingegangen den 21. Oktober 1965)

Im Verlauf unserer Untersuchungen[1-3] über acylierte Anthocyane ergab sich das Problem, grössere Mengen der mittels Kationenaustauscher gewonnenen Anthocyangemische[2] aufzutrennen.

Die Isolierung der Einzelpigmente durch präparative Papierchromatographie ist nicht nur zeitraubend, sondern auch recht kostspielig. Die verteilungschromatographische Trennung über Zellulose-[4] bzw. Kieselgelsäulen[5] gelingt nicht immer befriedigend und erfordert erheblichen Aufwand, speziell bei der Vorbereitung der Säulen[6]. KARRER UND STRONG[7] verwendeten Aluminiumoxid für die Gewinnung von Anthocyanen; andere Autoren[8] halten dieses Adsorbens jedoch für unwirksam. Da die chromatographischen Verfahren kein befriedigendes Ergebnis versprachen, versuchten wir, ob die Komplexbildung mit Metallionen, die als Farbreaktion zum Nachweis von *ortho*-ständigen Hydroxylgruppen im Seitenphenylring des Anthocyans schon lange bekannt ist[9], zum Ziel führt. Wie BAYER[10] bei der Untersuchung der Eisen- und Aluminiumkomplexe des Cyanidins feststellte, weisen sie bei etwa pH 5.0 nicht nur die grösste Farbintensität, sondern auch eine geringe Löslichkeit auf. Er schlägt im Falle des Cyanidins Formel I vor, bei der 2 Moleküle Cyanidin mit einem Me^{+++}–Ion[11,12] verknüpft sind.

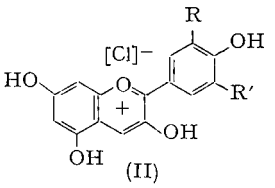


(I)

Pelargonidin (IIa) vermag nach den Untersuchungen von BAYER[11] keine schwer löslichen Komplexe zu bilden. Offen jedoch war die Frage, ob ein zur Hydroxylgruppe vicinaler Methoxylrest zur Komplexbildung befähigt ist. Wie wir fanden, bilden Päonidin (IId) und Malvidin (IIf) bei pH 5.0 auch keine stabilen Eisen- oder Aluminiumkomplexe.

Das gleiche Verhalten ist bei der Komplexbildung von Reduktonen (III) beschrieben[13], bei denen durch Methylierung des mittelständigen Hydroxyls die Fähigkeit zur Chelatbildung verloren geht.

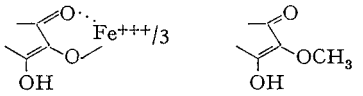
Demnach ist für die Entstehung eines stabilen Komplexes der Anthocyanidine Voraussetzung, dass sie im Seitenphenylring mindestens zwei vicinale Hydroxyl-



(II)

IIa: R=R'=H Pelargonidin; IIb: R=OH, R'=H Cyanidin;
IIc: R=R'=OH Delphinidin; IId: R=OCH_3, R'=H Päonidin;
IIe: R=OCH_3, R'=OH Petunidin; IIf: R=R'=OCH_3 Malvidin.

gruppen besitzen, also sich von Cyanidin (IIb), Delphinidin (IIc) und Petunidin (IIe) ableiten.



(III)

Auf Grund dieser Erkenntnisse bot sich die Möglichkeit, Anthocyangemische in komplex- und nicht komplexbildende Komponenten zu zerlegen. Zur Trennung stellten wir anionotropes Aluminiumoxid durch Zugabe von Natronlauge auf pH 5.0 ein und gaben auf das so vorbehandelte und in eine Säule gefüllte Adsorbens eine auf den gleichen pH-Wert eingestellte Anthocyanlösung. Durch Elution mit Wasser, Methanol oder Äthanol liessen sich die nicht komplexbildenden Anthocyane quantitativ entfernen (Fig. 1). Nachfolgend konnten die adsorbierten Pigmente durch Zusatz von 1 % Salzsäure zu dem Elutionsmittel unter Zerstörung des Komplexes abgelöst werden.

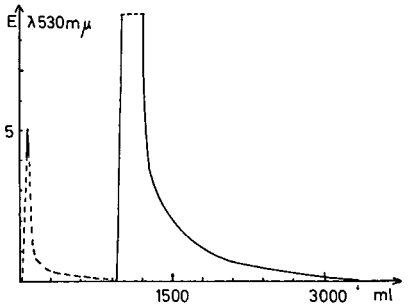


Fig. 1. Trennung von 100 mg Mischkristallen aus der Petuniensorte A 6. – – – Elutionskurve des Negreteins mit Methanol (90 %), ——— Elutionskurve des Petanins mit Methanol (90 %), das 1 % HCl enthält. Negretein = Malvidin-3-[4-(*p*-cumaroyl)-rhamnosyl (1 → 6)-glucosido]-5-glucosid; Petanin = Petunidin-3-[4-(*p*-cumaroyl)-rhamnosyl (1 → 6)-glucosido]-5-glucosid.

Die systematische Variation der Wasserstoffionenkonzentration der Aluminiumoxid-Suspension ergab, dass zur Trennung von komplex- und nicht komplexbildenden Anthocyanen der pH-Bereich von 4.5 bis 5.2 am günstigsten ist. Bei einem pH-Wert von 5.5 und grösser konnte z.B. das Malvidinderivat Negretein nicht mehr mit den oben erwähnten neutralen Lösungsmitteln eluiert werden, da es wahrscheinlich als

Anion adsorbiert wird. Bei Erniedrigung des pH-Wertes begannen ab pH 4.0 die Anthocyankomplexe in zunehmendem Masse zu wandern. Dies führte zu einer unvollständigen Trennung, wenn die Aluminiumoxidschicht in der Säule nicht entsprechend vergrössert wurde. Wesentlich für uns war, dass sich auch acylierte Anthocyane bei diesem Verfahren nicht verändert hatten, insbesondere keine Abspaltung von Säuren auftrat. Ferner spielte das prozentuale Verhältnis von komplex- zu nicht komplexbildenden Anthocyanen keine Rolle.

Tabelle I zeigt die allgemeine Anwendbarkeit der Methode für die Trennung von Anthocyanen bei verschiedenen Pflanzen.

TABELLE I

ANTHOCYANTRENNUNG AN ALUMINIUMOXID

Pflanzen	*Aglyka* (%)	
	Komplexbildend	*Nicht komplexbildend*
Petunia hybrida A 6	Petunidin (86) Delphinidin (1)	Malvidin (13)
Petunia hybrida P 14	Petunidin (10)	Malvidin (90)
Petunia hybrida V 79 a	Petunidin (3) Cyanidin (3) Delphinidin (2)	Päonidin (90) Malvidin (2)
Petunia hybrida V 78a	Petunidin (30) Delphinidin (40)	Malvidin (30)
Tulipa spec. (Tulpen)	Cyanidin (50)	Pelargonidin (50)
Fragaria hybrida (Erdbeeren)	Cyanidin (2)	Pelargonidin (98)
Raphanus sativus (Radieschen)	Cyanidin (1)	Pelargonidin (99)

In diesem Zusammenhang lag die Vermutung nahe, dass auch die gute Trennung von Anthocyanen auf einer Kieselgel-Dünnschicht[14] im Laufmittel Äthylacetat–Ameisensäure–Wasser[15] auf der Wechselwirkung der Pigmente mit den im Kieselgel vorhandenen komplexbildenden Eisen- oder Aluminiumionen beruht. Nach unseren Befunden besitzt eisen- und aluminium-ionenfreies Kieselgel tatsächlich keine Trennfähigkeit mehr für Anthocyane einer Glykosidklasse. Der gleiche Effekt zeigt sich, wenn man den pH-Wert des Laufmittels durch Zugabe von Salzsäure erniedrigt und damit die Komplexbildung verhindert.

EXPERIMENTELLES

1. Lösungsmittel

Als Elutionsmittel können Wasser, Methanol oder Äthanol dienen. Wir verwendeten meist Methanol (90 %), da nach Entfernung des Methanols im Rotationsverdampfer die Acylanthocyane aus der wässrigen Lösung ausfallen.

2. Bereitung der Aluminiumoxidsäule

100 ml Aluminiumoxid Fluka (sauer, Typ 504C, Aktivität I nach BROCKMANN) werden in 200 ml Wasser suspendiert. Das Wasser wird nach 10 Min. abdekantiert und durch mehrmaliges Aufschlämmen durch Methanol (90 %) ersetzt. Den pH-Wert

der überstehenden Lösung bringt man durch tropfenweises Zugeben von 2 *N* Natronlauge auf pH 5.0 (gemessen mit einer Glaselektrode). Erst nach mehreren Stunden tritt Konstanz des pH-Wertes ein, da das Aluminiumoxid Austauschereigenschaften besitzt.

Von dem so vorbereiteten Aluminiumoxid wird in eine Säule (20 mm Ø) auf eine Schicht Seesand so viel mit Methanol (90 %) eingeschlämmt, dass eine 10 cm hohe Schicht entsteht. Die Säule wird dann mit 1 l Methanol (90 %) gewaschen.

3. Trennung von in Lösung vorliegenden Anthocyanen

Liegen die Anthocyane in saurer Lösung vor, so wird nach Zugabe von 5 ml Aluminiumoxid (sauer) der pH-Wert unter Rühren und fortlaufender Messung durch Zutropfen von 2 *N* Natronlauge auf 5.0 eingestellt. Wenn die überstehende Lösung fast farblos ist, so reicht die Menge des Aluminiumoxids aus, um die komplexbildenden Anthocyane zu binden. Andernfalls muss noch mehr Aluminiumoxid zugefügt werden.

Diese Suspension füllt man auf eine wie unter (2) bereitete Säule und wäscht so lange mit Methanol (90 %), bis beim Eintropfen in vorgelegte Salzsäure (im allgemeinen 1 ml konz. Salzsäure) keine Rotfärbung mehr auftritt.

4. Trennung von Anthocyan-Mischkristallen

Zur Trennung von Mischkristallen in die Einzelpigmente werden über der nach (2) bereiteten Säule *ca.* 10 ml des zum Waschen benutzten Methanols (90 %) belassen. Hierzu kann man bis zu 300 mg kristallines Anthocyangemisch geben. Nach dem Lösen der Kristalle in der überstehenden Flüssigkeit wirbelt man durch Rühren mit einem Glasstab etwa 3 cm der sich darunter befindenden Aluminiumoxidschicht auf. Sogleich wird auf dem Aluminiumoxid die blaue Farbe des Komplexes sichtbar. Nach dem Einsickern des überstehenden Methanols kann mit dem Auswaschen der Nichtkomplexbildner begonnen werden.

5. Ablösen der Komplexe von der Aluminiumoxidsäule

Die auf Aluminiumoxid als Komplexe fixierten Pigmente lassen sich mit salzsaurem Methanol ablösen. Im allgemeinen ist 1 % Salzsäure ausreichend, obwohl niedrigere und höhere Konzentrationen verwendbar sind.

Im Falle der Petuniensorte A 6 verbleibt auch nach längerer Elution mit Methanol (90 %), das 1 % Salzsäure enthält, ein Pigment auf der Säule, das erst durch 2 *N* Salzsäure entfernbar ist. Durch spektroskopische und chromatographische Bestimmungen konnte das Aglykon als Delphinidin erkannt werden.

ZUSAMMENFASSUNG

Anthocyane, die Päonidin oder Malvidin als Aglyka enthalten, bilden ebensowenig wie Pelargonidin-glykoside bei pH 5.0 stabile Aluminiumkomplexe. Daher ist eine schnelle und einfache Trennung von den komplexbildenden Pigmenten (Cyanidin-, Petunidin- und Delphinidin-glykoside) an einer Aluminiumoxidsäule in präparativem Masstab möglich. Bei günstiger Zusammensetzung eines Anthocyangemisches führt die Methode zu chromatographisch reinen, kristallinen Verbindungen. Im Gegensatz zur Verteilungschromatographie können hierbei unbegrenzte Mengen getrennt werden.

SUMMARY

Anthocyanins containing peonidin or malvidin as aglycones, are like pelargonidin-glycosides not able to form stable aluminium complexes at pH 5.0. Therefore a rapid and simple separation of the complex-forming pigments (cyanidin-, petunidin- and delphinidin-glycosides) can be achieved on alumina on a preparative scale. With favourable compositions of anthocyanin mixtures the method yields chromatographically pure and crystalline compounds. In contrast to liquid–liquid partition chromatography, unlimited amounts of material can be separated.

LITERATUR

1 L. BIRKOFER UND C. KAISER, *Z. Naturforsch.*, 18b (1963) 337.
2 L. BIRKOFER, C. KAISER, W. KOCH, M. DONIKE UND D. WOLF, *Z. Naturforsch.*, 18b (1963) 631.
3 L. BIRKOFER, C. KAISER, M. DONIKE UND W. KOCH, *Z. Naturforsch.*, 20b (1965) 424.
4 B. V. CHANDLER UND K. A. HARPER, *Nature*, 181 (1958) 131.
5 KUANG C. LI UND A. C. WAGENKNECHT, *Nature*, 182 (1958) 657.
6 W. DIEMAIR, W. POSTEL UND H. SENGEWALD, *Z. Lebensm.-Untersuch. Forsch.*, 120 (1963) 173.
7 P. KARRER UND F. M. STRONG, *Helv. Chim. Acta*, 19 (1936) 25.
8 M. K. SEIKEL, in T. A. GEISSMAN, *The Chemistry of Flavonoid Compounds*, Pergamon, Oxford, London, New York, Paris, 1962, S. 37.
9 G. M. ROBINSON UND R. ROBINSON, *Biochem. J.*, 25 (1931) 1687.
10 E. BAYER, *Chem. Ber.*, 91 (1958) 1115.
11 E. BAYER, *Chem. Ber.*, 92 (1959) 1062.
12 E. BAYER, K. NETHER UND H. EGETER, *Chem. Ber.*, 93 (1960) 2871.
13 F. ARNDT, L. LOEWE UND E. AYÇA, *Chem. Ber.*, 84 (1951) 333; 85 (1952) 1150.
14 E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, Göttingen, Heidelberg, 1962.
15 D. HESS UND C. MEYER, *Z. Naturforsch.*, 17b (1962) 853.

ENHANCEMENT AND MODIFICATION OF ISOTOPE FRACTIONATION DURING THE PARTITION CHROMATOGRAPHY OF ^{3}H AND ^{14}C LABELED STEROIDS*

VACLAV CEJKA**, ELIZABETH M. VENNEMAN AND NEL BELT-VAN DEN BOSCH
*Department of Internal Medicine***, University of Amsterdam, Binnengasthuis, Amsterdam (The Netherlands)*
AND
PETER D. KLEIN
Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill. (U.S.A.)
(Received October 12th, 1965)

There is a growing awareness of the occurrence of isotope fractionation during preparative or analytical resolution of labeled steroids by chromatographic means. Such instances have been observed by JENSEN AND JACOBSEN[1] for estradiol-4-^{14}C and estradiol-1,2-^{3}H in paper chromatographic systems; by TAIT[2], CEJKA AND VENNEMAN[3] and by LARAGH, SEALEY AND KLEIN[4] for aldosterone-1,2-^{3}H diacetate-1-^{14}C on celite columns and paper chromatograms, and by KIRSCHNER AND LIPSETT[5] for testosterone-1,2-^{3}H acetate-1-^{14}C on gas–liquid chromatograms. The general occurrence of such fractionation in analytical separations has recently been reviewed by KLEIN[6].

Isotope fractionation of doubly labeled steroids is a particularly vexing consequence in the separation processes when isotope dilution measurements are being carried out, since the anticipated criterion of purity (a constant isotope ratio) is not to be found. LARAGH, SEALEY AND KLEIN[4] have described the mathematical basis for determining the isotope dilution when isotope fractionation is present, but an alternative approach is to ascertain whether the degree of fractionation itself can be altered. Such an approach, if fruitful, might also yield information on the mechanism(s) whereby one isotopically substituted steroid exhibits a different mobility from the same steroid with a different label.

One indication that the isotope fractionation of aldosterone-1,2-^{3}H diacetate from aldosterone diacetate-1-^{14}C might be modified by experimental circumstances came to light in an exchange of data between the Binnengasthuis and Argonne Laboratories. In the system used by CEJKA AND VENNEMAN, the displacement between the ^{3}H and ^{14}C forms was 1.85 % whereas LARAGH AND SEALEY (at Columbia University College of Physicians and Surgeons) had found the displacement to be less than half as much: 0.65 %. A point-by-point comparison of procedures suggested that the composition of the stationary phase influenced the degree of separation and that further modifications might be possible. The present report is a verification of this possibility.

* Work supported in part by the U.S. Atomic Energy Commission.
** Present address: Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill., U.S.A.
*** Head: Prof. Dr. J. G. G. BORST.

The data to be presented indicate that the degree of fractionation can be varied over more than a twofold range for doubly labeled aldosterone and over a tenfold range for dually labeled cortisone. These appear to be the largest changes in isotope fractionation in response to a procedural variation in the fractionation system that have been reported to date.

METHODS

Aldosterone-1,2-^{3}H (specific activity 31.3 C/m*M*), cortisone-4-^{14}C (specific activity 42 mC/m*M*), and cortisone-1,2-^{3}H (specific activity 721 mC/m*M*) were purchased from New England Nuclear Corp., Boston, U.S.A., and freed from radioactive impurities by column chromatography prior to use. Aldosterone-4-^{14}C (specific activity 46 mC/m*M*) was obtained through the courtesy of Dr. MORRIS M. GRAFF from the Endocrinology Study Section of the U.S.N.I.H.

The mobile phase of the solvent systems consisted of a mixture of four parts toluene (Analar, British Drug House) and one part ligroin (b.p. 80–100°; Brocades and Stheeman Pharmacia, Amsterdam). The stationary phase consisted of various aqueous methanol concentrations as indicated in the experiments. The solvent systems were equilibrated at room temperature for at least 24 h. Columns of 1 cm diameter were packed with acid washed celite 545 (Johns-Manville) mixed with stationary phase (2:1, w/v) using 1 g of celite per 3 cm column length.

The alcoholic solutions of radioactive steroids to be chromatographed were evaporated *in vacuo*, dissolved in 0.25 ml of stationary phase, and mixed with 0.5 g of celite. Mobile phase was added to the celite and after mixing with Sudan red the slurry was applied to the top of the column. A new column was packed for every experiment.

The collection of fractions (2 or 5 ml) was started when Sudan red emerged from the column. After transferring to glass counting vials the column eluates were mixed with 10 ml scintillation solution containing 0.5 g P.P.O. and 0.03 g dimethyl-P.O.P.O.P. per 100 ml toluene.

^{3}H and ^{14}C radioactivity was measured simultaneously in a Tricarb liquid scintillation counter model 314-EX-2 on high voltage 1205 and channel settings: 10 % gain (gate 3–10) and 100 % gain (gate 1–10). The counting efficiencies were:

Channel	^{14}C	^{3}H
10 % gain	34.8 %	0.0 %
100 % gain	20.1 %	31.2 %

Computations

The scintillation counting data expressed as dpm^{3}H or dpm^{14}C were used to compute a probit analysis for each radioactive peak[7]. From this was obtained a mean retention volume M and a peak dispersion σ, as well as the standard errors of these quantities. From the isotope ratio ø for successive fractions of the mixed peak, the displacement ΔM % and its standard error were computed, using the equations derived by KLEIN, SIMBORG AND SZCZEPANIK[8], which include correction for any

differences in dispersion between the two peaks. The overall computer programs for these calculations were prepared by BARBARA KUNZE-FALKNER of the Division of Biological and Medical Research.

RESULTS

An example of the fractionation of ^{3}H labeled aldosterone from ^{14}C labeled aldosterone is illustrated in Fig. 1. The isotope ratio exhibits a remarkable increase in the mixed peak, indicative of the difference in mobility between the two isotopically labeled aldosterones.

Table I lists the pertinent data for 13 columns in which the stationary phase was successively: water, 10 %, 30 %, 50 %, and 80 % methanol and the mobile phase was toluene–ligroin (4:1). Despite the fact that within each series the columns were

TABLE I

CHROMATOGRAPHY OF ALDOSTERONE-1,2-^{3}H AND ALDOSTERONE-4-^{14}C ON CELITE PARTITION COLUMNS WITH VARIOUS STATIONARY PHASES

Stationary phase	*Column length (cm)*	*Retention volume (ml) ± S.E.*		*Dispersion (ml) ± S.E.*	
		^{14}C	^{3}H	^{14}C	^{3}H
H_2O	15	177.67 ± 0.30	178.90 ± 0.26	4.25 ± 0.27	4.15 ± 0.23
		169.56 ± 0.28	170.75 ± 0.32	4.21 ± 0.31	4.17 ± 0.31
		183.19 ± 0.11	184.45 ± 0.12	4.67 ± 0.12	4.31 ± 0.12
10 % methanol	15	106.69 ± 0.05	108.50 ± 0.14	5.75 ± 0.05	6.06 ± 0.14
		116.80 ± 0.08	118.73 ± 0.10	7.02 ± 0.08	7.18 ± 0.10
30 % methanol	30	85.33 ± 0.13	86.62 ± 0.09	6.47 ± 0.13	6.68 ± 0.10
		84.34 ± 0.20	85.77 ± 0.23	5.64 ± 0.20	5.82 ± 0.22
50 % methanol	40	104.30 ± 0.04	105.98 ± 0.01	4.46 ± 0.04	4.54 ± 0.02
		103.15 ± 0.09	104.76 ± 0.10	4.05 ± 0.08	4.06 ± 0.10
		101.26 ± 0.07	102.75 ± 0.06	4.79 ± 0.06	4.88 ± 0.04
		98.67 ± 0.06	100.13 ± 0.04	4.43 ± 0.05	4.54 ± 0.04
80 % methanol	40	116.16 ± 0.25	117.11 ± 0.24	6.20 ± 0.25	6.40 ± 0.24
		114.20 ± 0.16	115.26 ± 0.14	5.10 ± 0.17	5.21 ± 0.15

completely repacked after every run, the retention volumes and dispersions from run to run were closely reproducible. For this reason, even the small number of columns used in these studies were sufficient to obtain meaningful comparisons between the stationary phases. Table II lists the per cent displacement of the ^{3}H-aldosterone from the ^{14}C-aldosterone for each of the five series; there is a twofold increase in displacement when the stationary phase is changed from water to 10 % methanol and this displacement is maintained over most of the range, declining somewhat when the methanol concentration reaches 80 %. A plot of these displacements *versus* the methanol concentration is shown in Fig. 2, which illustrates the close agreement between the average displacements measured by probit analysis (*i.e.*, from the differences in means, Table I) and those obtained from the isotope ratio.

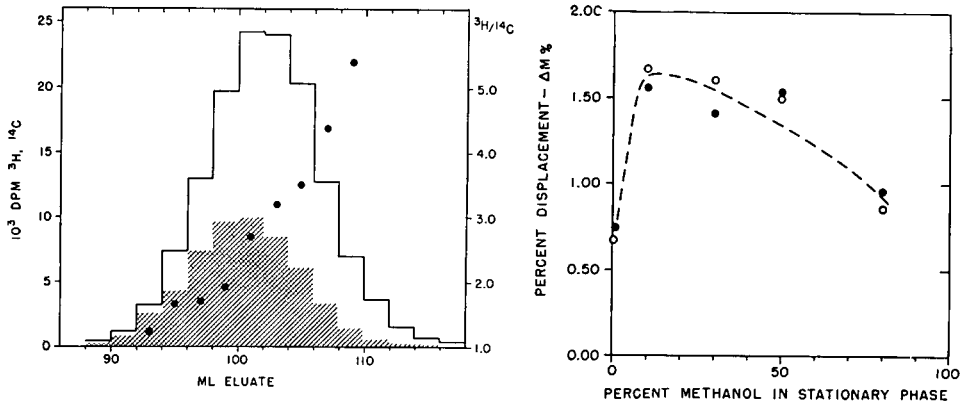


Fig. 1. The fractionation of aldosterone-1,2-^{3}H from aldosterone-4-^{14}C during partition chromatography on 40 cm celite column. Cross hatched area: ^{14}C; outline: ^{3}H; dots: ^{3}H/^{14}C. (Solvent system toluene–ligroin (4:1)/50% methanol.)

Fig. 2. The effect of methanol concentration on the isotopic fractionation of ^{3}H-aldosterone from ^{14}C-aldosterone. Open circles: as determined by probit analysis; solid circles: from isotope ratio measurement.

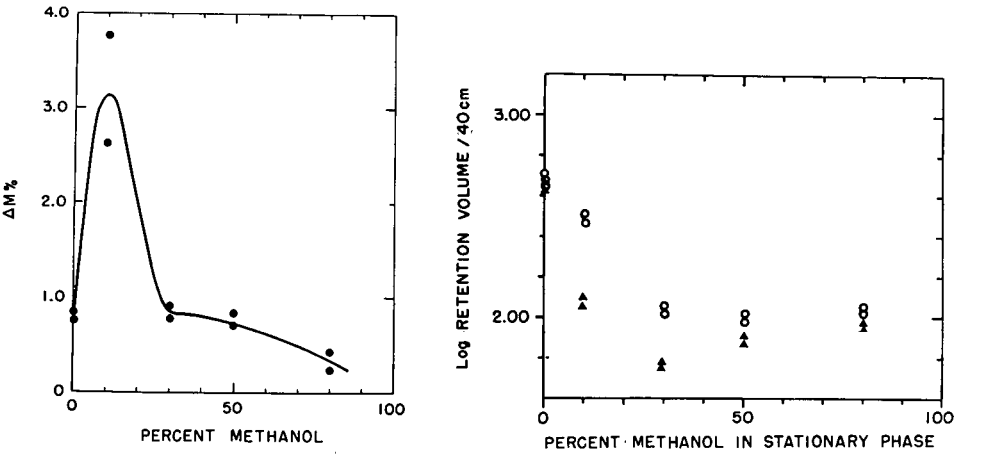


Fig. 3. The effect of methanol concentration in stationary phase on the isotopic fractionation of ^{3}H-cortisone from ^{14}C-cortisone.

Fig. 4. The effect of methanol concentration in the stationary phase on the log retention volumes of aldosterone (circles) and cortisone (triangles).

TABLE II

ISOTOPE FRACTIONATION FACTORS DETERMINED FROM ISOTOPE RATIO MEASUREMENT FOR ALDOSTERONE-4-^{14}C AND ALDOSTERONE-1,2-^{3}H ON VARIOUS STATIONARY PHASES

Run	H_2O	*10% Methanol*	*30% Methanol*	*50% Methanol*	*80% Methanol*
	ΔM% ± S.E.	*ΔM% ± S.E.*	*ΔM% ± S.E.*	*ΔM% ± S.E.*	*ΔM% ± S.E.*
1	0.73 ± 0.07	1.50 ± 0.14	1.32 ± 0.11	1.53 ± 0.07	0.88 ± 0.10
2	0.68 ± 0.11	1.63 ± 0.10	1.53 ± 0.17	1.73 ± 0.15	1.05 ± 0.10
3	0.85 ± 0.04			1.43 ± 0.06	
4				1.47 ± 0.06	
Average	0.75 ± 0.06	1.56 ± 0.06	1.41 ± 0.09	1.54 ± 0.09	0.96 ± 0.08

A similar series of columns was packed and run to determine the effect of the stationary phase composition of the separation of ^{3}H-cortisone from ^{14}C-cortisone. The column data for these runs are shown in Table III and the displacements obtained are shown in Fig. 3. An even more striking effect of the methanol concentration can be seen in the region of 10% methanol, where the fractionation effect attains a value of more than 3% and then declines to less than 0.3% at 80% methanol.

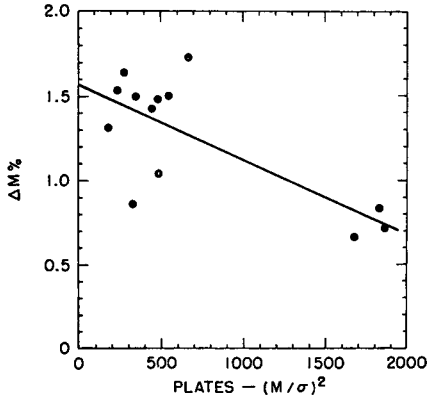
The effect of the stationary phase composition on the log retention volume of aldosterone and cortisone is shown for a standardized column length of 40 cm in Fig. 4. Instead of the anticipated straight line, there are two components to the relationship, one decreasing the retention volume, the other maintaining or increasing it. These intersect somewhere in the region of 30% methanol for both steroids. Furthermore, cortisone, which is less polar than aldosterone and should be expected to have a lower retention volume at all methanol concentrations, displays an anomalously high retention volume on those columns in which water is the stationary phase. There is, however, no anomalous behavior in either series ascribable to, or related to, the changes in isotope fractionation.

TABLE III

CHROMATOGRAPHY OF CORTISONE-1,2-^{3}H AND CORTISONE-4-^{14}C ON CELITE PARTITION COLUMNS WITH VARIOUS STATIONARY PHASES

Stationary phase	*Column length (cm)*	*Retention volume (ml) ± S.E.*		*Dispersion (ml) ± S.E.*	
		^{14}C	^{3}H	^{14}C	^{3}H
H_2O	15	241.54 ± 0.20	243.40 ± 0.17	12.70 ± 0.20	13.20 ± 0.18
		249.66 ± 0.14	251.35 ± 0.22	13.56 ± 0.16	13.53 ± 0.23
10% methanol	15	52.14 ± 0.07	53.38 ± 0.13	5.24 ± 0.08	4.93 ± 0.14
		52.56 ± 0.04	53.73 ± 0.22	4.36 ± 0.05	4.42 ± 0.28
30% methanol	40	58.35 ± 0.05	58.92 ± 0.05	3.18 ± 0.05	3.23 ± 0.05
		60.39 ± 0.02	60.86 ± 0.02	3.52 ± 0.02	3.54 ± 0.02
50% methanol	40	82.10 ± 0.12	82.47 ± 0.11	4.63 ± 0.12	4.42 ± 0.11
		78.46 ± 0.08	78.90 ± 0.04	4.02 ± 0.07	3.87 ± 0.03
80% methanol	60	166.48 ± 0.03	167.04 ± 0.05	7.32 ± 0.03	7.18 ± 0.05
		149.19 ± 0.10	149.74 ± 0.04	7.02 ± 0.10	6.67 ± 0.04

When the numbers of plates generated in the columns (computed as $(M/\sigma)^2$) were plotted against the per cent displacement obtained, a significant relationship was observed and is shown for aldosterone in Fig. 5 and for cortisone in Fig. 6. Both figures indicate that the greater the number of plates in the column, the smaller the per cent displacement which occurs between the two labeled forms of the steroid. In the case of aldosterone, a linear regression of the points yielded a slope of $-4.272 \cdot 10^{-5}$ with a standard error of $1.212 \cdot 10^{-5}$; the probability of this being a zero slope is 0.002. In the cortisone data, the curvature of the relationship precluded a linear regression analysis, but the inverse correlation of displacement with plate number is easily seen.



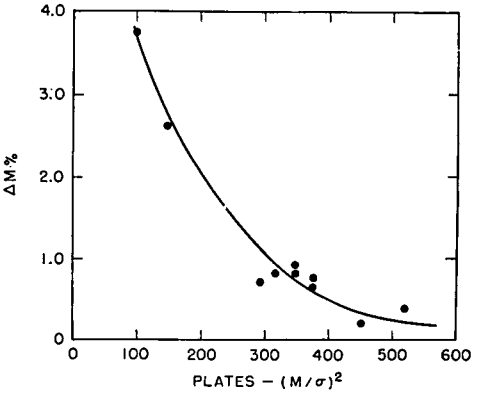


Fig. 5. The relationship between per cent displacement of ^{3}H-aldosterone from ^{14}C-aldosterone and the numbers of plates generated in the column.

Fig. 6. The relationship between per cent displacement of ^{3}H-cortisone from ^{14}C-cortisone and the numbers of plates generated in the column.

The unexpected *inverse* relationship shown in Figs. 5 and 6 suggested that at least a portion of the fractionation was due to an isotope effect exerted through some associative process present in the early stages of the chromatography. If present, such associative interaction would decay as the number of plates (and hence the number of transfer from stationary to mobile phase and back) increased; that is to say, it should be maximal with a short column and diminish as the column length increases. To investigate this possibility, two columns were packed with celite in which the stationary phase was water and the mobile phase was toluene. The column lengths were 8.5 and 28.5 cm and the fractionation of dual-labeled cortisone was determined on each column. The results are shown in Table IV. They indicate that on the short column, the per cent displacement of the two labeled forms of cortisone was more than threefold larger than on the long column, or conversely, extending the length of the column reduced the difference in migration rates by nearly three-quarters.

DISCUSSION

There have been few systematic attempts to influence the degree of isotope fractionation in heteroatomic molecules. LIBERTI, CARTONI AND BRUNER[9] have shown

TABLE IV

VARIATION OF ISOTOPE DISPLACEMENT WITH COLUMN LENGTH DURING THE PARTITION CHROMATOGRAPHY OF CORTISONE-1,2-^{3}H AND CORTISONE-4-^{14}C

Column length (cm)	*Retention volume (ml) ± S.E.*		*Dispersion (ml) ± S.E.*		*ΔM %*	*Plates generated in column*
	^{14}C	^{3}H	^{14}C	^{3}H		
8.5	65.80 ± 0.27	66.72 ± 0.32	9.51 ± 0.29	9.36 ± 0.35	2.33 ± 0.60	48
28.5	253.84 ± 0.23	255.56 ± 0.20	12.70 ± 0.24	12.93 ± 0.21	0.64 ± 0.04	400

that the stationary phase of a gas–liquid chromatographic column affects the degree to which perdeuterobenzene is resolved from benzene. They determined the separation factors on squalene and on silicone oil at various temperatures and have computed the heats of solution in each phase as well as the entropy change. DAVIDSON, MANN AND SHELINE[10] have measured the influence of resin cross-linking in the isotopic fractionation of $H^{14}COOH$ from $H^{12}COOH$ by ion exchange chromatography. They found an increase in the separation factor from 1.0028 at 2 % cross linking to 1.0044 at 8 % and a maximum effect of 1.0059 (equivalent ΔM %: 0.59) at 10 %. KLEIN, SIMBORG AND SZCZEPANIK[8] have shown that the spatial separation of ^{3}H and ^{14}C in the cholesterol acetate molecule influences the degree of fractionation found during absorption chromatography: the closer the ^{3}H to the ^{14}C, the greater the displacement which was observed.

The results which have been obtained with doubly labeled aldosterone and cortisone indicate that parameters in the chromatographic system itself can influence the degree of separation. These are perhaps the most interesting of the possibilities to date, since they are much more amenable to experimental variation and study. The ability to enhance the fractionation to the degree where the difference in mobility is more than 3 % and then to reduce this by more than tenfold by changing the stationary phase composition offers opportunities for study not heretofore available. From the standpoint of the primary objective, that of reducing the degree of fractionation, these experiments offer encouragement that solvent systems in which fractionation is minimal can be found, though whether or not fractionation can be eliminated is still problematic.

It is difficult to conceive of these responses to stationary phase composition as being those of primary isotope effects in the partition process. Although such effects do occur, and may indeed be present in these chromatograms, the existence of maxima in the effect of methanol concentrations and the fractionation response to changes in column length are much more characteristic of associative processes such as micelle formation. Since the degree of displacement (*i.e.*, the effective separation factor) *decreases* under conditions presumed to disrupt such associative forms, the quite surprising inference appears to be that the formation of such aggregates may itself be the isotope-sensitive process. A somewhat comparable interpretation has been offered for the unusually large isotope effects observed during the distillation of aqueous ^{3}H-formaldehyde by SIMON AND HEUBACH[11]. Inasmuch as the distillation of HOH from HTO resulted in far less enrichment than for the formaldehydes, the fractionation could not be due to a primary effect of ^{3}H on vapor pressures. They attributed

the fractionation to a difference in the hydration equilibrium of formaldehyde in which the formation of the hydrate was favored for the labeled formaldehyde over the unlabeled formaldehyde. In confirmation of this mechanism, they were able to show that as the pressure at which the distillation was carried out was reduced, the fractionation effect became correspondingly smaller. Further experiments in the present series to determine the proportion of fractionation effects which are derived from each source are being carried out. These will establish the irreducible minimum component due to isotope effects in the partition process itself.

SUMMARY

The isotope fractionation occurring during the partition chromatography of aldosterone-1,2-^{3}H and aldosterone-4-^{14}C and of cortisone-1,2-^{3}H and cortisone-4-^{14}C has been shown to be influenced by the composition of the stationary phase used to prepare the column. Displacements corresponding to separation factors as high as 1.030 and as low as 1.003 have been observed for the two labeled varieties of cortisone.

REFERENCES

1 E. V. JENSEN AND H. I. JACOBSEN, *Recent Progr. Hormone Res.*, 18 (1962) 387.
2 J. F. TAIT, in E. E. BAULIEU AND P. ROBEL (Editors), *Aldosterone*, Blackwell, Oxford, 1965, p. 19.
3 V. CEJKA AND E. M. VENNEMAN, *Clin. Chim. Acta*, 11 (1965) 188.
4 J. H. LARAGH, J. E. SEALEY AND P. D. KLEIN, *Trans. IAEA Symp. Radiochem. Methods Anal., Salzburg, 1964*, Vol. 2, IAEA, Vienna, 1965, p. 253.
5 M. A. KIRSCHNER AND M. B. LIPSETT, *J. Lipid Res.*, 6 (1965) 7.
6 P. D. KLEIN, in R. A. KELLER AND J. C. GIDDINGS (Editors), *Advances in Chromatography*, Vol. III, Dekker, New York, 1966, in press.
7 D. J. FINNEY, *Probit Analysis*, Cambridge University Press, London, 1952.
8 P. D. KLEIN, D. W. SIMBORG AND P. A. SZCZEPANIK, *Pure Appl. Chem.*, 8 (1964) 357.
9 A. LIBERTI, G. CARTONI AND F. BRUNER, *J. Chromatog.*, 12 (1963) 8.
10 C. N. DAVIDSON, C. K. MANN AND R. K. SHELINE, *J. Phys. Chem.*, 67 (1963) 1519.
11 H. SIMON AND G. HEUBACH, *Z. Naturforsch.*, 18 (1963) 160.

ANALYTISCHE UNTERSUCHUNGEN ÜBER O,O-DIMETHYL-S-N-METHYL-CARBAMYLMETHYL-DITHIOPHOSPHAT (DIMETHOAT)

2. MITT. DÜNNSCHICHTCHROMATOGRAPHISCHE BESTIMMUNG VON DIMETHOAT IN FORMULIERUNGEN*

F. GRIMMER, W. SPICHALE, R. KLICHE UND D. QUAAS

*Forschungslaboratorium des VEB Elektrochemisches Kombinat Bitterfeld (Deutsche Demokratische Republik)***

(Eingegangen den 16. November 1965)

Das Insektizid Dimethoat wird zumeist als Emulsionskonzentrat gehandelt. Solche Konzentrate enthalten gewöhnlich 20–50 % Dimethoat, organische Lösungsmittel und geringe Mengen hochwirksamer Emulgatoren; besonders letztere erschweren durch ihre emulgierenden Eigenschaften und ihre komplexe chemische Zusammensetzung die analytische Bestimmung des Dimethoats erheblich. Es bedarf daher zur Ermittlung des Dimethoat-Gehaltes in derartigen Formulierungen absolut spezifischer Bestimmungsreaktionen oder sehr leistungsfähiger Trennoperationen. Als Trennverfahren kommen praktisch nur chromatographische Methoden in Betracht.

In der Literatur sind säulenchromatographische Trennverfahren von DUPPÉE, GARDNER UND NEWTON[2] sowie DAUTERMAN und Mitarb.[3] beschrieben worden. Diesen Methoden haftet der Nachteil eines relativ hohen Zeitaufwandes und einer Unsicherheit bezüglich der Lage der Dimethoat-Fraktion an. Italienische Verfasser[4] gaben ein quantitativ-papierchromatographisches Analysenverfahren für ROGOR-Formulierungen an, das jedoch zeitraubend und umständlich ist.

Wir versuchten, mit Hilfe der Dünnschichtchromatographie, die bisher nur selten zur quantitativen Analyse von Thiophosphorsäureester-Formulierungen herangezogen wurde (z.B. von WALKER UND BEROZA zur quantitativen Analyse der Formulierung CO-RAL[5]), ein rasches und zuverlässiges Analysenverfahren zu erarbeiten. BÄUMLER UND RIPPSTEIN[6] trennten dünnschichtchromatographisch verschiedene Thiophosphorsäureester auf Kieselgel G mit Aceton–*n*-Hexan (1:4) als Laufmittel. Als Sprühreagens diente 0.5 %ige $PdCl_2$-Lösung[7], die Thiophosphorsäureester werden durch gelbe bis braune Flecken auf schwach bräunlichem Untergrund angezeigt.

Für eine quantitative Auswertung der Chromatogramme direkt auf der Platte müssen folgende Voraussetzungen gegeben sein:

1. Die Farbreaktion muss stöchiometrisch verlaufen.
2. Die Beschichtung der Platten muss völlig gleichmässig sein.

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* 1. Mitteilung, siehe Lit. 1.

** Leiter Dr. H. KALTWASSER.

Wir konnten die angegebenen Voraussetzungen nicht erfüllen. Die Farbreaktion von Thiophosphorsäureestern mit Palladiumchlorid verläuft nicht stöchiometrisch, bei unterschiedlichen Konzentrationen an Dimethoat erhält man Färbungen, die von gelb über gelbbraun bis braun variieren. Auch die Konstanthaltung der Schichtdicke bereitet Schwierigkeiten. Deshalb musste die Bestimmung des Dimethoats in Lösung erfolgen.

OPTIMIERUNG DES TRENNVERFAHRENS

Wir arbeiteten auf Aluminiumoxid D als Adsorptionsmittel. In Anlehnung an BÄUMLER UND RIPPSTEIN[6] erzielten wir mit Aceton–*n*-Heptan (1:2) gute Trennungen. Hinsichtlich einer möglichst grossen Menge an aufzutragender Substanz bei ausreichender Trennschärfe ermittelten wir 0.75 mm als optimale Schichtdicke. Eventuellen R_F-Wert-Verschiebungen, die durch ungenügende Kammersättigung, Veränderungen der Laufmittelzusammensetzung usf. verursacht sein können, begegneten wir durch die individuelle Bestimmung der Lage des Dimethoatfleckes auf jedem Chromatogramm durch Besprühen einer auf der gleichen Platte mitgelaufenen Vergleichsprobe. Zur Extraktion des Dimethoats aus dem Adsorbens eignen sich polare Lösungsmittel wie Aceton oder Chloroform; ungeeignet ist Methanol wegen der Bildung schwer zerstörbarer Suspensionen mit Aluminiumoxid D.

DIMETHOAT-BESTIMMUNG IM ELUAT

Die Dimethoatbestimmung erfolgte spektrophotometrisch als Molybdatovanadato-phosphat[2]. Dazu wurde das Dimethoat nach Verkochen des Elutionsmittels unter Wasserzusatz durch oxydativen Aufschluss mit Salpetersäure und Perchlorsäure in Orthophosphorsäure umgewandelt. Geringe Minderbefunde durch unvollständige Extraktion des Dimethoats aus dem Adsorbens eliminierten wir durch Aufstellen einer Eichkurve unter gleichen Bedingungen.

EXPERIMENTELLER TEIL

Apparatives

Aufstreichgerät nach STAHL (Hersteller: VEB Glaswerk Ilmenau).

Universalspektrophotometer VSU 1 (Hersteller: VEB Carl Zeiss Jena), 3 cm-Quarzküvetten, Wellenlänge 470 nm, Spaltbreite 0.03 mm.

Chromatographiersäulen (lichte Weite 12 mm, Länge 100 mm).

Glasplatten 100 × 200 mm.

Entwicklungskammern.

Sprühgerät für die Chromatographie.

Reagentien

Aluminiumoxid D zur Dünnschichtchromatographie (VEB Chemiewerk Greiz-Dölau).

Aceton, p.a., getrocknet und über eine Kolonne destilliert.

n-Heptan, p.a.

Methanol, p.a.

Salpetersäure, p.a., $d = 1{,}42$.
Perchlorsäure, p.a., 72 %ig.
Ammoniumvanadat-Lösung (2.5 g/l, schwach salpetersauer).
Ammoniummolybdat-Lösung (50 g/l).
Palladiumchlorid-Lösung (0.5 %ig, schwach salzsauer).

Arbeitsvorschrift

1. *Dünnschichtchromatographie.* Die Glasplatten werden 0.75 mm stark mit einer wässrigen Suspension von Aluminiumoxid D beschichtet und nach dem Abbinden 45 Min. im Trockenschrank bei 130° aktiviert. Etwa 2 g der zu untersuchenden Probe (30–40 %ige Formulierung) werden in einen 50-ml-Masskolben genau eingewogen und mit Methanol zur Marke aufgefüllt. 100 µl der methanolischen Lösung werden auf 5 nebeneinander liegende Punkte an der linken Plattenseite verteilt aufgebracht, auf die rechte Plattenseite bringt man 20 µl dieser Lösung als Vergleichsprobe und 20 µl einer Lösung von 0.8 g reinem Dimethoat in 50 ml Methanol auf (Fig. 1). Die Auftragsflecken sind möglichst klein zu halten.

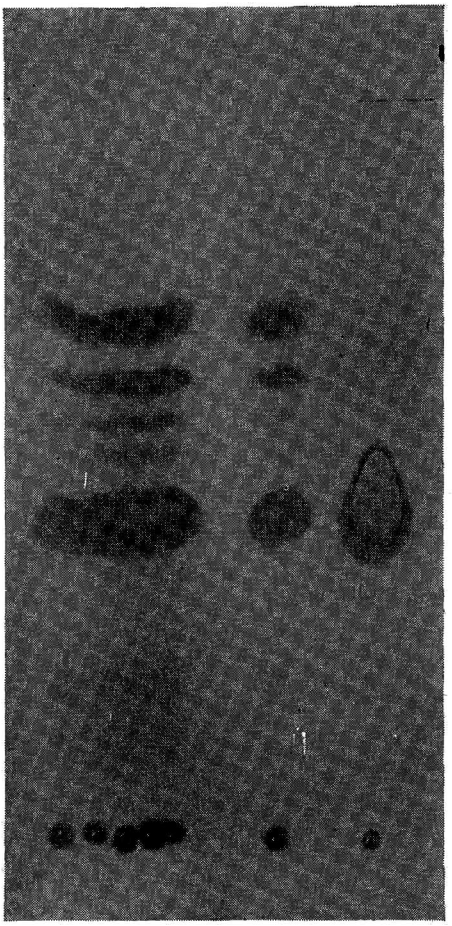
Die Platten werden zum Entwickeln in eine dicht verschliessbare Chromatographierkammer eingestellt, in die zur Kammersättigung eine Stunde zuvor das Laufmittelgemisch, trockenes Aceton–*n*-Heptan (1:2), eingefüllt wurde. Man entwickelt die Platten bis zu einer Laufstrecke von 15–17 cm (Laufzeit 20–30 Min.), trocknet sie an der Luft und besprüht die Vergleichsproben (rechte Plattenseite) mit 0.5 %iger Palladiumchlorid-Lösung, wobei man die linke Seite des Platte sorgfältig abdeckt. Der R_F-Wert des Dimethoats beträgt etwa 0.45–0.55 (abhängig von der Schichtdicke, Laufmittelzusammensetzung, Raumtemperatur usw.). Man markiert grob die Lage des Dimethoatfleckes auf der zuvor abgedeckten, unbesprühten Plattenseite und besprüht in gleicher Art die Zonen ausserhalb der Markierung, um zu ermitteln, ob das Chromatogramm gleichmässig gelaufen ist (Fig. 2). Dabei ist der Dimethoatfleck sorgfältig abzudecken!

Die dem Dimethoatfleck der Analysenprobe entsprechende unbesprühte Aluminiumoxid-Schicht wird dann quantitativ mit einem Spatel von der Platte in ein 50-ml-Becherglas geschabt und das Aluminiumoxid mit 5 ml trockenem Aceton suspendiert.

2. *Elution.* Eine Chromatographiersäule wird am Auslauf mit einem Glaswollebausch versehen und mit einer acetonischen Suspension von 2 g Aluminiumoxid D gefüllt. Man lässt absitzen und so lange Aceton durch die Schicht laufen, bis die Flüssigkeit klar abläuft. Danach wird die acetonische Aufschlämmung des beladenen Aluminiumoxids in die Säule gespült und in Portionen zu je 5 ml, die man zuvor zum Ausspülen des Becherglases benutzt, mit insgesamt 40 ml Aceton eluiert. Die Eluate sammelt man in einem 100-ml-Kjeldahlkolben.

3. *Aufschluss.* Zum Kjeldahlkolben mit der acetonischen Lösung setzt man 4 ml Wasser zu und vertreibt das Aceton. Nach dem vollständigen Verkochen des Acetons leitet man den Aufschluss mit 4 ml konz. Salpetersäure ein, fügt noch 2 ml Perchlorsäure zu und erwärmt erst mässig, später stärker, bis alle Salpetersäure vertrieben ist und die Probe nur noch konz. Perchlorsäure enthält (farblose Flüssigkeit von 1–2 ml Volumen). Man hält noch kurze Zeit am Sieden, lässt abkühlen, fügt 8 ml dest. Wasser zu, kocht die Probe etwa 5 Min. auf und kühlt ab.

4. *Photometrische Messung.* Den Inhalt des Kjeldahlkolbens giesst man in



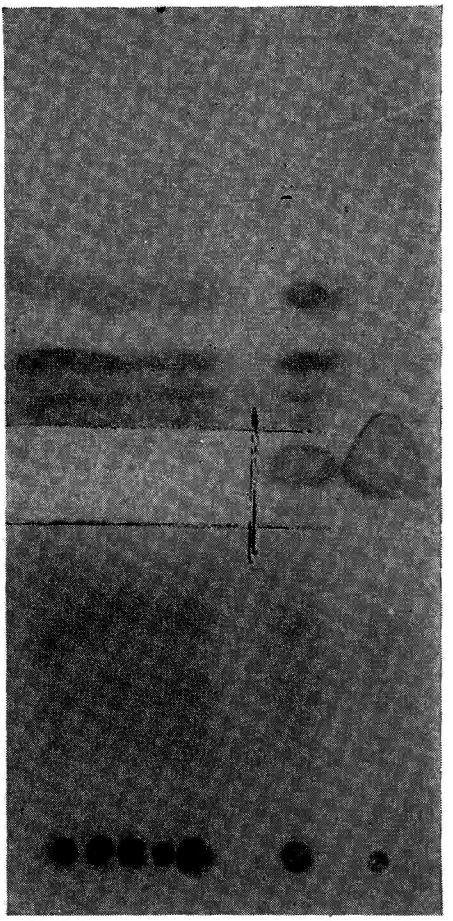


Fig. 1. Dünnschichtchromatogramm einer Testformulierung, die Lösungsmittel, Emulgatoren und stark verunreinigten technischen Wirkstoff enthält. Aufgetragene Substanzen (v.l.n.r.): Analysenprobe (5 Punkte und Vergleichsfleck), Dimethoat.

Fig. 2. Dünnschichtchromatogramm 1 zur Analyse vorbereitet.

einen 25-ml-Masskolben und benutzt folgende Reagenslösungen in der angegebenen Reihenfolge zum Nachspülen des Kjeldahlkolbens:

5 ml Ammoniumvanadat-Lösung,
5 ml Ammoniummolybdat-Lösung
und dest. Wasser bis zur Eichmarke.

Eine Blindprobe stellt man in folgender Weise her: 42 ml Aceton (die gleiche Charge, die zur Analyse verwendet wurde) werden in einem Kjeldahlkolben mit 4 ml Wasser versetzt und — wie oben beschrieben — weiterverarbeitet.

Die Proben werden nach 30–90 Min. spektrophotometrisch bei 470 nm ausgemessen.

Aufstellen der Eichkurve

0.8 g reines Dimethoat, dessen Gehalt man zuvor nach einer der bekannten

TABELLE I

EICHMESSUNGEN MIT KRISTALLINEM DIMETHOAT 16 mg/ml; 94.6 % Reinheit

Einsatzmenge		*Extinktion*	*Mittlere Extinktion*
µl	*mg*		
50	0.757	0.218; 0.214; 0.211; 0.214; 0.224	0.216
80	1.211	0.351; 0.330; 0.344; 0.358; 0.346	0.346
100	1.514	0.436; 0.435; 0.435; 0.431; 0.415	0.430

Methoden bestimmt hat[1], werden in einem 50-ml-Masskolben in Methanol gelöst. Man füllt bis zur Marke auf, trägt — wie bereits beschrieben — 50, 75, 100, 125 µl der Lösung (und 10, 15, 20, 25 µl Vergleichsprobe) auf Aluminiumoxid-Dünnschichtplatten auf und arbeitet wie oben weiter. Die Extinktion wird als Funktion der Einsatzmenge Dimethoat graphisch dargestellt.

ERGEBNISSE UND DISKUSSION

In Tabelle I sind Werte zur Aufstellung einer Eichkurve dargestellt. Die Eichlösung enthielt 0.800 g technisches Dimethoat in 50 ml methanolischer Lösung, dessen Reinheit nach Methode[1] zu 94.6 % bestimmt wurde. Die Eichkurve verläuft bis 2 mg streng linear.

TABELLE II

ANALYSEN TECHNISCHER FORMULIERUNGEN

Probe	*Dimethoatgehalt gefunden (Gew.-%)*	*Mittelwert (Gew.-%)*	*Abweichung vom Mittelwert (% relativ)*
1*	39.5		+ 1.8
	37.9	38.8	— 2.3
	38.9		+ 0.3
2*	35.5		— 3.5
	37.8	36.8	+ 2.7
	37.0		+ 0.5
3*	24.3		— 2.0
	25.6	24.8	+ 3.2
	24.4		— 1.6
4**	35.6		+ 2.0
	35.0	34.9	+ 0.3
	34.1		— 2.3

* Hersteller: VEB Elektrochemisches Kombinat Bitterfeld; Probe 3 künstlich gealtert (500 Std. bei 70°).

** Hersteller: Kleinholz & Co., Essen; 1 Jahr gelagert.

Tabelle II zeigt Analysen einiger technischer Formulierungen. Die Einzelbestimmungen stimmen recht gut überein, der relative Fehler der Bestimmung übersteigt bei Formulierungen mit 40 % Dimethoatgehalt nicht $\pm$ 3 %. Die Methode eignet sich auch zur Ermittlung des Dimethoatgehaltes gealterter Proben (z.B. Proben 3 und 4). Besonders während der Alterung bei erhöhter Temperatur treten sehr viele chemisch ähnliche Substanzen auf, die die Untersuchung nach den Analysenverfahren[2,4] sehr erschweren (vgl. Fig. 3).

Einen Vergleich der Analysenergebnisse, die wir nach dem beschriebenen dünnschichtchromatographischen und dem säulenchromatographischen Verfahren von DUPPÉE und Mitarb.[2] erhielten, zeigt Tabelle III. Die Ergebnisse nach beiden Analysenverfahren stimmen innerhalb der Fehlergrenzen überein. Die dünnschichtchromatographische Arbeitsweise besitzt den Vorteil eines erheblich geringeren Arbeitszeitaufwandes: eine Arbeitskraft kann täglich etwa 7 Bestimmungen ausführen.

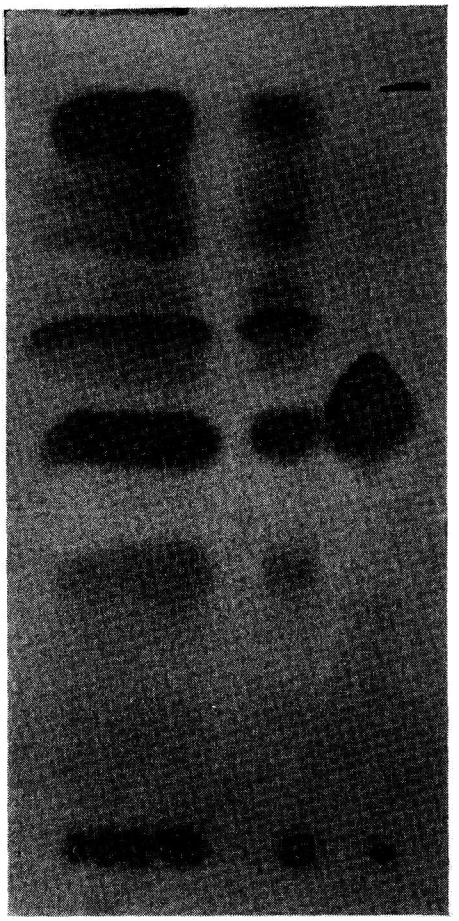


Fig. 3. Dünnschichtchromatogramm einer künstlich gealterten Dimethoat-Formulierung (500 Std. bei 70°).

TABELLE III

VERGLEICH DER ANALYSENERGEBNISSE NACH DEM SÄULENCHROMATOGRAPHISCHEN[2] UND DEM DÜNNSCHICHTCHROMATOGRAPHISCHEN VERFAHREN

Probe	*Gefundener Dimethoatgehalt (Gew.-%)**	
	säulenchromatographisch	*dünnschichtchromatographisch*
1**	30.8	32.0
2***	35.1	34.9

* Es sind Mittelwerte aus 3–4 Einzelbestimmungen angeführt.
** Testformulierung mit verunreinigtem technischem Wirkstoff (*ca.* 72% Dimethoatgehalt).
*** Hersteller: Kleinholz & Co., Essen; 1 Jahr gelagert.

ZUSAMMENFASSUNG

Es wird ein universelles Analysenverfahren für Dimethoat-Emulsionskonzentrate angegeben. Dimethoat wird dünnschichtchromatographisch abgetrennt, vom Adsorptionsmittel extrahiert und über seinen Phosphorgehalt als Molybdato-vanadato-phosphat spektrophotometrisch bei 470 nm bestimmt. Der Fehler der Bestimmung liegt unterhalb $\pm$ 3% relativ, der Zeitaufwand ist geringer als der bisher publizierter Analysenverfahren.

SUMMARY

An analytical method for Dimethoate emulsion concentrates is described. Dimethoate is separated by thin-layer chromatography, extracted from the adsorbent, and determined spectrophotometrically as molybdato-vanadato-phosphate at 470 nm. The relative error of determination is below $\pm$ 3%, and the time consumption is lower than that of analytical methods already published.

LITERATUR

1 F. GRIMMER, K. SCHULZE, D. QUAAS AND W. SPICHALE, *Z. Chem.*, 5 (1965) 234.
2 L. F. DUPPÉE, K. GARDNER AND P. NEWTON, *Analyst*, 85 (1960) 177, 924.
3 W. C. DAUTERMAN, J. E. CASIDA, J. B. KNAAK AND T. KOWALCZYK, *J. Agr. Food Chem.*, 7 (1959) 188.
4 B. BAZZI AND R. SANTI, *Montecatini-Werksmitteilung*, Mailand, 1963.
5 K. C. WALKER AND M. BEROZA, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 250.
6 J. BÄUMLER AND S. RIPPSTEIN, *Helv. Chim. Acta*, 44 (1961) 1162.
7 O. KÖNIG AND W. R. CROWELL, *Mikrochemie Ver. Mikrochim. Acta*, 33 (1948) 298.

DETECTION AND THIN-LAYER CHROMATOGRAPHY OF SULFUR COMPOUNDS

I. SULFOXIDES, SULFONES AND SULFIDES

LAWRENCE FISHBEIN AND JOHN FAWKES
Bionetics Research Laboratories, Inc., Falls Church, Va. (U.S.A.)
(Received November 3rd, 1965)

INTRODUCTION

A number of feeding and metabolic studies of pesticidal sulfones and sulfoxides in our laboratory has prompted the need to investigate various detecting reagents and thin-layer chromatography as to their possible utility for subsequent differentiation, identification and separation of the above moieties.

Recent literature has focused on the ever expanding utility of dimethyl sulfoxide, specifically, as a radioprotective agent[1], preservative for red-blood cells and tissues[2–4], vehicle for steroids[5], proteins[6], drugs[7,8], and agricultural toxicants[9]. Other sulfoxides, as well as sulfones and sulfides exhibit a broad spectrum of applications, *e.g.*, as insecticides[10–12], fungicides[13–14], herbicides[15–16], synergistic agents[17,18], and rocket fuels[19].

The metabolic oxidation–reduction interrelationships of R_2S, R_2SO and R_2SO_2 respectively, have also been well documented[20–28].

Sulfoxides have been detected on paper by Dragendorff's reagent[29], molybdophosphoric acid[29], and hydriodic acid[30], on thin-layer chromatograms by permanganate–sulfuric acid[31] and separated by column[32,33] and gas chromatographic techniques[34]. They have also been determined classically by prior reduction with hydriodic acid[35,36] and titanous chloride[37], as well as titrated directly as bases in acetic anhydride[38].

Sulfones have been separated by column[39] and gas chromatography[40,41], and detected on paper[42] and columns[43] by Ehrlich's and N-(1-naphthyl)-ethylene diamine reagents, respectively.

Sulfides have been detected on paper[44], columns[45] and thin-layer chromatograms[46] utilizing potassium iodoplatinate, a gold-iodide complex and, Rhodamine 6G, respectively. Gas chromatographic techniques[47,48] have also been employed for the separation of sulfides.

EXPERIMENTAL

Preparation of chromatoplates

The silicic acid chromatoplates were prepared according to the method of MORLEY AND CHIBA[49]. Silica gel DF-5* was applied on (8 × 8 in.) glass plates to a

* Obtained from Camag, Muttenz, Switzerland.

thickness of 280 μ. After air drying, the plates were activated in an oven at 75° for 30 min. Acetone solutions (1–2 μl containing 5–10 μg) of test substance were applied along a line 2.5 cm from the lower end of the plate and developed by the ascending method, till the solvent front was about 13 cm from the starting line. After evaporation of the solvent, the spots were located on the plate by spraying with one of the chromogenic reagents, then exposure of the plates to ammonia vapors.

The developing solvent systems utilized were:

(A) 2.5 % acetone in benzene.

(B) Toluene–ethyl acetate (1:1).

Detecting reagents were:

(1) TCNE reagent: 2 % tetracyanoethylene in benzene.

(2) DDQ reagent: 2 % 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in benzene.

(3) Chloranil: 1 % tetrachloro-*p*-benzoquinone in benzene.

(4) N,2,6-Trichloro-*p*-benzoquinoneimine: 2 % solution in ethanol.

(5) Gibbs reagent: 2 % 2,6-dibromo-N-chloro-*p*-benzoquinoneimine in benzene.

Materials

Compounds 1, 14, 21, 33, 35, 41–43, chloranil and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone were obtained from J. T. Baker Chem. Co., Phillipsburg, N.J., USA; compounds 2,3,5,7,8,11–13, 15, 16, 19, 20, 22, 23, 28, 32, 39, 40, 45, and 46 were obtained from K & K Laboratories, Inc., Plainview, N.Y., USA; compounds 4, 9, 10, 24–27, 30, 31, 34, 36–38 and 44 from Aldrich Chemical Co., Milwaukee, Wisc., USA; compound 29 from FMC Corporation, Middleport, N.Y., USA; tetracyanoethylene, N,2,6-trichloro-*p*-benzoquinoneimine, and 2,6-dibromo-N-chloro-*p*-benzoquinoneimine from Eastman-Kodak Laboratories, Rochester, N.Y., USA; compounds 6, 17 and 18 from Wateree Chemical Co., Lugoff, S.C., USA.

RESULTS AND DISCUSSION

Tables I and II depict the spot colors of various classes of sulfur compounds on paper and thin-layers utilizing detecting reagents 1–5, followed by exposure of the chromatograms to ammonia vapors.

A number of general observations as to their chromogenic behavior to the variety of detecting reagents can be made.

1. Sulfoxides are more reactive than sulfones toward the detecting reagents screened both on paper and thin layers, *e.g.* sulfoxides yield immediate colors whereas the sulfones are detected after several minutes or require development at 80°. The greater chemical stability of the sulfone group as compared to the lower oxidized state (sulfoxides) is well recognized[50–53]. Sulfoxides have been found to form strong hydrogen bonds with alcohols[54] and phenols[54–56] in addition to forming complexes with iodine[57], iodine cyanide[58] and antimony pentachloride[59]. Sulfones, conversely, are weaker bases (poorer donors) due to the net decrease in the electron density on the sulfur and oxygens[57].

2. The substituted *p*-benzoquinone type detectors, *e.g.* DDQ reagent (detector 2) and chloranil reagent (detector 3) were more sensitive than the halogenated quinoneimine detectors. The Gibbs reagent and N-2,6-trichloro-*p*-benzoquinoneimine, with the DDQ reagent being the detector of choice for the differentiation of sulfoxides,

TABLE I

SPOT COLORS OF SULFUR COMPOUNDS ON WHATMAN NO. 1 PAPER

Designation of colors: B = blue; Bn = brown; C = crimson; G = green; Gr = grey; L = lilac; O = orange; sl = slow (2–3 min); T = tan; V = violet; wk = weak; Y = yellow.

Class of compound	*Detecting reagents*									
	Before NH_3 exposure					*After NH_3 exposure*				
	*1**	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*	*5*
Sulfoxides										
Alkyl	C-T	O-C	Y	Y	Y-O	—	Y-O	B-Gr	Y (wk)	Bn
Aryl	C-T	O-C	Y	Y	Y-O	—	Y-O	B-Gr	Y (wk)	Bn
Cyclic	C-T	O-C	Y-G	Y-G	Y	—	Y-O	L	Y (wk)	Bn
Sulfones										
Alkyl	L**	V**	L**	—	—	Y	T	Gr (wk)	—	L
Aryl	L**	V**	L**	—	—	Y	T	Gr (wk)	—	L
Cyclic	L**	V**	L**	—	—	Y	T	Gr	—	L
Sulfides										
Alkyl	Y (sl)	T	Bn	T	Y (wk)	—	—	—	—	O
Aryl	Y (sl)	T	Bn	T	Y (wk)	—	—	—	—	O
Cyclic	Y	T	Bn	Bn	Y	—	—	—	—	Y-O
Sultones	—	V (sl)	—	—	—	Y	B-G	—	—	—
Thiols (alkyl)	—	—	—	—	T (wk)	—	Y-T (wk)	—	Y (sl)	Y (wk)

* For key detecting reagents 1–5, see text.
** Colors developed after heating at 80° for one minute.

TABLE II

SPOT COLORS OF SULFUR COMPOUNDS ON SILICA GEL DF-5 PLATES

Designation of colors: B = blue; Bk = black; Bn = brown; C = crimson; d = deep; G = green; L = lilac; O = orange; P = purple; Pk = pink; R = rose; sl = slow (2–3 min); T = tan; V = violet; W = white; wk = weak; Y = yellow.

Class of compound	*Detecting reagents*									
	Before NH_3 exposure					*After NH_3 exposure*				
	*1**	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*	*5*
Sulfoxides										
Alkyl	Y	O-C	Y	Y	Y-O	W	O-C	Y	Y	Y
Aryl	Y	O-C	Y	Y	Y	W	O-C	Y	Y	Y
Cyclic	Y	O-C	Y	Y	Y	W	O-C	Y	Y	Y
Sulfones										
Alkyl	Pk (sl)	L (sl)	Pk**	V (wk)	V**	Y (d)	Y-G	V	W	T
Aryl	Pk (sl)	L (sl)	Pk**	V (wk)	V**	Y (d)	Y-G	V	W	T
Cyclic	Pk (sl)	L (sl)	Pk**	V (wk)	V**	Y (d)	Y-G	V	W	T
Sulfides										
Alkyl	O-C	P-B	Y	Y-Bn	Y-Bn	—	O	P-O	Y-G	B-Bk
Aryl	O-C	P-B	Bn	Y-Bn	Y-Bn	—	O	P-O	Y-G	B-Bk
Cyclic	O-C	P-B	Y	Y-Bn	Y-Bn	—	O	P-O	T-G	B-Bk
Sultones	L (wk)	T-O	—	—	—	Y (d)	V	—	—	—
Thiols (alkyl)	R (sl)	W	—	—	Y	—	Y-T (sl)	—	Y (wk)	Y

* For key to detecting reagents 1–5 see text.
** Colors developed after heating at 80° for one minute.

TABLE III

R_F VALUES OF SULFOXIDES, SULFONES AND SULFIDES ON SILICA GEL DF-5 CHROMATOPLATES

No.	*Compound*	*2.5% acetone–benzene*	*Toluene–ethyl acetate (1:1)*
	Sulfoxide		
1	Dimethyl	0.04	0.15
2	Di-*n*-propyl	0.09	0.23
3	Di-*n*-butyl	0.12	0.27
4	Di-iso-butyl	0.10	0.23
5	Di-*n*-amyl	0.15	0.32
6	Dihexyl	0.18	0.35
7	Diphenyl	0.19	0.37
8	Dibenzyl	0.22	0.41
9	Bis-(*p*-tolyl)	0.25	0.44
10	Bis-(*p*-chlorophenyl)	0.39	0.56
11	Tetramethylene	0.10	0.25
	Sulfone		
12	Dimethyl	0.08	0.29
13	Diethyl	0.11	0.34
14	Di-*n*-propyl	0.15	0.38
15	Di-iso-propyl	0.12	0.33
16	Di-*n*-butyl	0.19	0.43
17	Di-iso-butyl	0.15	0.40
18	Di-*sec.*-butyl	0.16	0.40
19	Di-*n*-amyl	0.24	0.48
20	Di-iso-amyl	0.20	0.43
21	Divinyl	0.12	0.36
22	Diphenyl	0.28	0.54
23	Bis-(*p*-tolyl)	0.32	0.60
24	Bis-(*p*-chlorophenyl)	0.47	0.73
25	Bis-(*p*-fluorophenyl)	0.42	0.69
26	Bis-(*p*-aminophenyl)	0.05	0.30
27	Bis-(*m*-aminophenyl)	0.09	0.35
28	*p*-Chlorophenyl-methyl	0.49	0.70
29	*p*-Chlorophenyl-2,4,5-trichlorophenyl	0.47	0.77
30	*p*-Chlorophenyl-phenyl	0.41	0.69
31	*p*-Fluoro-*m*-nitrophenyl	0.45	0.73
32	Tetramethylene	0.35	0.49
	Sulfide		
33	Di-*n*-propyl	0.54	0.70
34	Di-iso-propyl	0.50	0.66
35	Di-*n*-butyl	0.59	0.75
36	Di-iso-butyl	0.54	0.71
37	Di-*sec.*-butyl	0.53	0.71
38	Di-*tert.*-butyl	0.46	0.62
39	Di-*n*-amyl	0.64	0.80
40	Di-iso-amyl	0.60	0.76
41	Di-*n*-hexyl	0.68	0.84
42	Diphenyl	0.68	0.83
43	Dibenzyl	0.72	0.86
44	Bis-(*p*-chlorophenyl)	0.82	0.91
45	Bis-(*p*-aminophenyl)	0.42	0.50
46	Tetramethylene	0.56	0.74

sulfones and sulfides both on paper and thin layers. DDQ has been shown to be a strong electron acceptor readily forming complexes with suitable electron donors[60,61]. The oxidative potential of DDQ is about 1.0 V compared with 0.71 V for chloranil[62,63] suggesting the more active role of the former reagent towards complex formation. ANDERSEN[64] has indicated the utility of DDQ for the detection of aromatic amines, hydrocarbons and phenols on paper chromatograms, with the resultant color reaction being attributed to the formation of π-complexes. The hypothesis is advanced that in the case of sulfoxides (although sulfur as well as oxygen possesses a lone pair of electrons)[57,58] complexing with DDQ may occur via oxygen, as in the previously noted complexes of sulfoxides with iodine and phenols[57].

3. The halogenated quinoneimine reagents studied in this work (detectors 4 and 5) as well as the TCNE reagent also exhibit usefulness for the differentiation of sulfoxides, sulfones and sulfides on silica gel chromatoplates, both before and following ammonia-vapor exposure. SEARLE[65] and MCALLISTER[66] have indicated the ability of quinoneimine reagents to form complexes with glyoxaline-2-thiols and 2-alkylthioglyoxalines, respectively. The TCNE reagent has been previously shown to form colored complexes with a variety of aromatic hydrocarbons[67,68].

4. Sultones and alkyl thiols also tested, were shown not to interfere in the differentiation of sulfoxides, sulfones, and sulfides, by the detection reagents utilized in this study.

5. The exposure of both paper and thin-layer chromatograms to ammonia vapors (after the application of the detector reagents) generally resulted in the accentuation of spots. GRAHAM[69] had previously noted the utility of ammonia exposure after the utilization of N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine for the detection of halogenated aromatic hydrocarbons on paper chromatograms.

Table III illustrates the R_F values of sulfoxides, sulfones and sulfides as determined in the two solvent systems on silica gel DF-5 chromatoplates. For both the 2.5 % acetone–benzene and toluene–ethyl acetate (1:1) systems the R_F relationships of the above moieties is as follows:

(a) sulfides > sulfones > sulfoxides; (b) for the substituted aryl sulfones; *p*-chlorophenyl > *p*-fluorophenyl > *p*-aminophenyl; (c) for the isomeric aminophenyl sulfones; *m*-aminophenyl > *p*-aminophenyl.

For the alkyl sulfoxides, sulfones and sulfides studied, utilizing both solvent systems, there is essentially a linear relationship when the R_F values are plotted *versus* the number of substituent carbon atoms, with the R_F values increasing with increasing chain length.

Although the separation of *n*-, iso-, and *tert.*-butyl sulfides can be affected by both solvent systems, neither system could resolve iso- and *sec.*-butyl sulfides or iso- and *sec.*-butyl sulfones.

ACKNOWLEDGEMENTS

The technical assistance of Mrs. P. H. TURNER in this study is gratefully acknowledged.

This study was supported by Research Contract PH-43-64-57, National Cancer Institute, National Institutes of Health, Public Health Service.

SUMMARY

The chromatogenic and chromogenic behavior of 11 sulfoxides, 20 sulfones and 14 sulfides is described. Useful differentiation of these compounds can best be accomplished using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone or tetracyanoethylene detecting reagents. Separation has been accomplished on thin-layer chromatograms utilizing a toluene ethyl acetate (1:1) or 2.5 % acetone–benzene solvent system.

REFERENCES

1 M. J. ASHWOOD-SMITH, *Intern. J. Radiation Biol.*, 3 (1961) 41.
2 J. S. PORTERFIELD AND M. J. ASHWOOD-SMITH, *Nature*, 193 (1962) 548.
3 M. J. ASHWOOD-SMITH, *Blood*, 23 (1964) 494.
4 J. E. LOVELOCK AND M. W. H. BISHOP, *Nature*, 183 (1959) 1394.
5 H. ROSENKRANTZ, Z. HADIDIAN, H. SEAY AND M. M. MASON, *Cancer Chemotherapy Rept.*, No. 31 (1963) 7.
6 E. D. REES AND J. J. SINGER, *Arch. Biochem. Biophys.*, 63 (1956) 144.
7 R. L. DIXON, R. H. ADAMSON, M. BEN AND D. P. RALL, *Proc. Soc. Exptl. Biol. Med.*, 118 (1965) 756.
8 S. W. JACOBS, R. J. HERSCHLER AND M. G. BISCHELL, *Belg. Pat.*, 644,613, July 1, 1964; *Chem. Abstr.*, 63 (1965) 9759.
9 H. L. KIEL, *Agr. Chem.*, 1 (1965) April.
10 J. R. GEIGY A.-G., *Swiss. Pat.*, 255,886, September 1, 1949; *Chem. Abstr.*, 43 (1949) 7633.
11 P. G. CAMPBELL, *U.S. Pat.*, 2,645,592, July 14, 1953; *Chem. Abstr.*, 47 (1953) 10171.
12 E. J. SOBOCZENSKI, *U.S. Pat.*, 3,148,111, September 8, 1964; *Chem. Abstr.*, 62 (1965) 8336.
13 H. A. STEVENSON, J. R. MARSHALL AND A. F. HAMS, *Brit. Pat.*, 938,980, February 3, 1961; *Chem. Abstr.*, 60 (1964) 2842.
14 W. A. HENSLEY, J. T. FITZPATRICK AND D. C. TORGESON, *U.S. Pat.*, 3,136,687, June 9, 1964.
15 R. J. WINEMAN AND G. J. MARCO, *U.S. Pat.*, 3,124,447, August 10, 1959; *Chem. Abstr.*, 60 (1964) 15072.
16 SHELL INTERNATIONAL RESEARCH MAATSCHAPPIJ, N.V., *Dutch Pat.*, Appl. 6, 6,408,099, February 9, 1965.
17 R. B. MARCH, R. L. METCALF AND L. L. LEWALLEN, *J. Econ. Entomol.*, 45 (1952) 851.
18 M. E. SYNERHOLM, *U.S. Pat.*, 2,486,445, November 1, 1949; *Chem. Abstr.*, 44 (1950) 2170.
19 T. F. DOUMANI AND C. S. COE, *U.S. Pat.*, 3,196,607, July 27, 1965; *Chem. Abstr.*, 63 (1965) 9737.
20 V. D. STEFANO AND H. H. BORGSTEDT, *Science*, 144 (1964) 1137.
21 M. A. BENNETT, *Biochem. J.*, 33 (1939) 1794.
22 F. L. ROSE AND A. SPINKS, *Biochem. J.*, 43 (1948) VII.
23 G. A. SNOW, *Biochem. J.*, 65 (1957) 77.
24 G. MEDES, *J. Biol. Chem.*, 109 (1935) LXIV.
25 S. TSUTSUMI, *Repura*, 29 (1960) 88; *Chem. Abstr.*, 61 (1964) 11193.
26 T. L. SOURKES AND Y. TRANO, *Arch. Biochem. Biophys.*, 42 (1953) 321.
27 S. BLACK, E. M. HARTE, B. HUDSON AND L. WARTOFSKY, *J. Biol. Chem.*, 235 (1960) 2910.
28 K. I. H. WILLIAMS, K. S. WHITTEMORE, T. N. MELLIN AND D. S. LAYNE, *Science*, 149 (1965) 203.
29 L. SUCHOMELOVA, V. HORAK AND J. ZYKA, *Microchem. J.*, 9 (1965) 196.
30 J. F. THOMPSON, W. N. ARNOLD AND C. J. MORRIS, *Nature*, 197 (1963) 380.
31 H. ERTEL AND L. HORNER, *J. Chromatog.*, 7 (1962) 268.
32 V. HORAK AND J. PECKA, *J. Chromatog.*, 14 (1964) 97.
33 J. B. HAUS, L. MANOLEV AND R. W. PRICE, *J. Assoc. Offic. Agr. Chemists*, 47 (1964) 264.
34 V. E. CATES AND C. E. MELOAN, *Anal. Chem.*, 35 (1963) 658.
35 H. HOGEVEEN AND F. MONTANARI, *Gazz. Chim. Ital.*, 94 (1964) 176.
36 F. JANCIK AND J. KOERBL, *Cesk. Farm.*, 11 (1962) 305; *Chem. Abstr.*, 61 (1964) 2491.
37 D. BARNARD AND K. R. HARGRAVE, *Anal. Chim. Acta*, 5 (1951) 536.
38 D. C. WIMER, *Anal. Chem.*, 30 (1958) 2060.
39 H. S. HUMAN, *J. Assoc. Offic. Agr. Chemists.*, 46 (1963) 195.
40 V. E. CATES AND C. E. MELOAN, *J. Chromatog.*, 11 (1963) 472.
41 J. BURKE AND L. JOHNSON, *J. Assoc. Offic. Agr. Chemists*, 45 (1962) 328.
42 W. H. LONGENECKER, *Anal. Chem.*, 21 (1949) 1402.
43 A. C. BRATTON AND E. K. MARSHALL, *J. Biol. Chem.*, 128 (1939) 537.
44 I. M. HAIS AND M. MACEK, *Paper Chromatography*, Academic Press, New York, 1963, p. 629.
45 A. L. LEROSEN, *Anal. Chem.*, 24 (1952) 1335.

46 M. T. J. MURPHY, B. NAGY, G. ROUSER AND G. KRITCHEVSKY, *J. Am. Oil. Chemist. Soc.*, 42 (1965) 475.
47 B. H. ANDREEN, D. V. KNIEBES AND P. B. TARMAN, *Inst. Gas Technol., Tech. Rept.*, No. 7 (1963) 32; *Chem. Abstr.*, 62 (1965) 2645h.
48 T. J. WALLACE AND J. J. MATLOW, *Nature*, 201 (1964) 817.
49 H. V. MORLEY AND M. CHIBA, *J. Assoc. Offic. Agr. Chemists.*, 47 (1964) 306.
50 E. D. AMSTUTZ, I. M. HUNSBERGER AND J. J. CHESSICK, *J. Am. Chem. Soc.*, 73 (1951) 1220.
51 D. BARNARD, J. H. FABIAN AND H. P. KOCH, *J. Chem. Soc.*, (1949) 2442.
52 H. SZMANT, in N. KHARASCH (Editor), *Organic Sulfur Compounds*, Vol. I, Pergamon Press, Oxford, 1961, p. 154.
53 N. KHARASCH, *Organic Sulfur Compounds*, Vol I, Pergamon Press, Oxford, 1961, pp. 146–150.
54 M. TAMRES AND S. SEARLES, Jr., *J. Am. Chem. Soc.*, 81 (1959) 2100.
55 S. GHERSETTI AND A. LUSA, *Spectrochim. Acta*, 21 (1965) 1067.
56 T. GRAMSTAD, *Acta Chem. Scand.*, 18 (1964) 27.
57 R. S. DRAGO, B. WAYLAND AND R. L. CARLSON, *J. Am. Chem. Soc.*, 85 (1963) 3125.
58 P. KLAEBOE, *Acta Chem. Scand.*, 18 (1964) 27.
59 P. KLAEBOE, *Acta Chem. Scand.*, 18 (1964) 999.
60 I. LINDQUIST AND M. ZACKRISSON, *Acta Chem. Scand.*, 14 (1960) 453.
61 A. OTTENBERG, R. L. BRANDON AND M. E. BROWNE, *Nature*, (1964) 1119.
62 H. D. BECKER, *J. Org. Chem.*, 30 (1965) 982, 989.
63 D. H. REID, M. FRASER, B. B. MOLLOY AND H. A. S. PAYNE, *Tetrahedron Letters*, 15 (1961) 530.
64 L. ANDERSEN, *Finska Kemistsamfundets Medd.*, 70 (1961) 31.
65 C. E. SEARLE, *J. Appl. Chem.*, 5 (1955) 313.
66 R. A. MCALLISTER, *J. Clin. Pathol.*, 4 (1951) 432.
67 P. V. PEURIFOY AND S. C. SLAYMAKER AND M. ONAGER, *Anal. Chem.*, 31 (1959) 1740.
68 R. E. MERRIFIELD AND W. D. PHILLIPS, *J. Am. Chem. Soc.*, 80 (1958) 2778.
69 S. O. GRAHAM, *Science*, 139 (1967) 875.

THIN LAYER CHROMATOGRAPHY OF INDOLYL-ACRYLYL-GLYCINE AND OTHER URINARY INDOLES

I. L. HANSEN AND M. A. CRAWFORD*
Department of Physiology, Makerere University College Medical School, Kampala (Uganda)
(Received September 27th, 1965)

It has been shown that apparently healthy people in both East and West Africa utilizing the plantain (*Musa sp.*) as a staple food, excrete indoles to an extent which would be considered as pathological in individuals living on a European type of diet[1-2].

The plantain contains large amounts of 5-hydroxytryptamine (5-HT) (*ca.* 50 μg/g pulp) which is excreted mainly as 5-OH-indolyl-acetic acid (5-HIAA). The presence in the urine of other 5-HT metabolites such as 5-HIAA-glycine and -glucuronide, N-acetyl-5-HT-glucuronide and 5-OH-tryptophol has been reported in the literature[3,4,5]. Further it has recently been shown that urine from banana-eaters frequently contains indolyl-acrylyl-glycine[6], a substance which so far only has been found in large amounts in the urine of Hartnup disease[7].

In order to investigate the reasons for the presence of the indolyl-acrylyl-glycine in the urine it therefore became necessary to establish a simple and rapid technique for identification of this and other indoles.

Routine determinations of urinary indoles have so far mainly been carried out by paper chromatography[8]; however, the development of thin layer chromatography offers excellent possibilities for efficient and rapid separations. The present investigation was carried out to separate indolyl-acrylyl-glycine and some of the most common urinary indoles by thin layer chromatography.

MATERIALS

Adsorbents

Kieselgel G according to Stahl;
Kieselgel GF_{254} according to Stahl;
Kieselguhr G according to Stahl;
Aluminiumoxyd G according to Stahl.

Solvents

Analytical grade reagents were used without further purification.

Standards

The non-conjugated indoles examined (5-HT as creatinine-sulphate and tryptamine as HCl) were available from commercial sources.

* Present address: Department of Biochemistry, Nuffield Institute of Comparative Medicine, Regent's Park, London, N.W. 1.

Standards for the conjugated indoles were prepared biologically from primates and lower animals: The IAA-glycine and IAA-glucuronide were obtained after oral administration of 0.5 g IAA to a guinea pig[7] and the glutamine-conjugate after interperitoneal injection of 0.75 g IAA to a Vervet monkey[9].

Indolyl-acrylyl-glycine was obtained from urine from a banana-eater after ingestion of indolyl-acrylic acid.

Location-reagents

The spots were detected on the chromatograms by the following: (a) U.V. light; (b) Ehrlich-reagent[10]; (c) ninhydrin/acetic acid[8]; and (d) cinnamaldehyde[8].

METHODS

Chromatoplates were prepared according to STAHL[11]. Non-activated (*i.e.* air-dried) plates of Kieselgel G were used in a few cases, but were not found suitable. In all experiments described below the plates were activated by heating at 105° for 30 min.

During attempts to find systems for separation of indolic amino acids and amines, the suitability of Kieselguhr G and Aluminiumoxyd G was also examined; however, with all the solvents studied both these adsorbents caused "streaking".

RESULTS AND DISCUSSION

It was not found possible to obtain efficient separation of all the urinary indoles examined with one single solvent system. Instead an attempt was made to separate the indoles within the four following groups: (A) Indolic acids; (B) Conjugates of indolic acids; (C) Indolic amines; (D) Indolic amino acids.

By dividing the indoles in these groups a reduction in the number of indoles on each chromatoplate was obtained leading to better separation and thus facilitating elution for eventual quantitative determination.

The systems which were considered suitable are given in Table I and those applied for each of the groups A–D are discussed separately below.

TABLE I

COMPOSITION OF SOLVENT SYSTEMS

All chromatograms were run over a distance of 11–12 cm except where both media II and III were employed; in this case the chromatogram was run over a distance of 15 cm.

Medium	*Constituents*	*Proportions*	*Time (min)*
I	Ether–petrol ether (60–80°)–HCOOH	75:25:2	30
Ia	Ether–petrol ether (60–80°)–CH_3COOH	75:25:2	30
II	Acetone–NH_3 (25%)	100:1	30
III	Acetone–$CHCl_3$–acetic acid–water	40:40:20:5	60
IV	Ether–acetone–acetic acid	2:2:1	45
V	Ethyl acetate–acetic acid	4:1	45
VI	Ethyl acetate–benzene–acetic acid	4:1:1	45
VII	Ether–acetic acid	100:1	30

(*A*) *Indolic acids*

Medium I: Diethyl ether–petrol ether (60–80°)–formic acid (75:25:2).

Medium Ia: Diethyl ether–petrol ether (60–80°)–acetic acid (75:25:2).

Medium VII: Diethyl ether–acetic acid (100:1).

Indolic acids were separated in acidic systems containing small amounts of either acetic or formic acids. It is known that the indole nucleus is unstable in presence of strong acid and it would be natural to prefer the weaker acetic to the formic. Since double chromatography (*i.e.* 2-dimensional chromatograms using the same solvent in both directions) did not show any decomposition of 4 of the 5 indolic acids examined, and since formic gives sharper spots than acetic acid, system I was preferred.

Two spots could be detected after chromatography of indolyl-acrylic acid with either of the systems I and Ia. Bands of the two substances located under U.V. were eluted from the Kieselgel GF_{254} layer with acetone–water (1:1). In both cases chromatography of each of the two fractions resulted in two spots identical with the original. Repetition of this procedure with either spot reproduced two spots at the original R_F values; on elution U.V. absorption peaks were identical for both spots. It was therefore assumed that the presence of the two spots on the chromatograms was due to isomerization rather than to decomposition.

The presence of two isomers could be explained by the fact that indole in solutions will normally be present in both the indole and indolenine form (see Fig. 1).

Since the double-bond in the side-chain in indolyl-acrylic acid is conjugated with the double bond in the pyrrole ring, it is possible that both the *cis* and *trans* form of the indolyl-acrylic acid could be found in a tautomeric equilibrium.

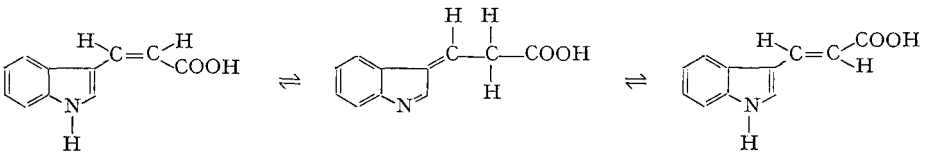


Fig. 1. Diagramatic representation of the possible tautomeric rearrangements of indolyl-acrylic acid which could give rise to the *cis–trans* isomers and double spots on the chromatogram.

It was obvious that the amount of the less polar and less stable of the two isomers increased during storage of a solution. A double chromatogram also suggested that isomerization took place during the chromatographic procedure. Attempts to further prove the assumed isomeric character of the two spots were considered to be outside the scope of the present investigation.

The solvent systems I and Ia were the only ones of the systems investigated which were able to separate both indolyl-propionic acid, IAA, indolyl-acrylic acid and 5-HIAA. It was, however, not found possible to obtain separation between indolyl-lactic acid and 5-HIAA. Separation of these two substances would be achieved using system VII.

These three systems will only allow migration of indolic acids whereas the other indolic groups B–D plus urea remain at origin.

(B) Conjugated indoles

Medium IV: Diethyl ether–acetone–acetic acid (2:2:1).

Medium V: Ethyl acetate–acetic acid (4:1).

Medium VI: Ethyl acetate–acetic acid–benzene (4:1:1).

Conjugated indolic acids, being more polar than the corresponding free acids, could be separated in more polar systems. Also in this case, sharper spots could often be obtained by substitution of acetic acid with formic acid.

As seen from Table II it was found difficult to obtain good separation between indolyl-acetyl-glutamine/urea (IV and V) and urea/indolyl-acetyl-glycine (IV and VI). Since, however, IAA-glycine and IAA-glutamine will rarely be present in large amounts in the same urine sample, one of the three systems should be suitable for any urine sample.

TABLE II

R_F VALUES × 100

The column labelled II/III represents the final R_F value reached after consecutive chromatography in these two systems (see Fig. 2).

Indole group	*Solvent system*								
	I	*Ia*	*II*	*III*	*II/III*	*IV*	*V*	*VI*	*VII*
A									
Indolyl-acetic acid	61	59	—	93	93	88	85	85	96
Indolyl-acrylic acid	50	43	—	98	98	98	85	85	84
Indolyl-lactic acid	24	4	—	75	75	74	59	57	22
Indolyl-propionic acid	68	64	—	96	96	98	86	85	84
5-OH-Indolyl-acetic acid	28	28	—	86	86	78	83	77	73
B									
Indolyl-acetyl-glucuronide	—	—	—	45	45	30	15	8	—
Indolyl-acetyl-glutamine	—	—	—	68	68	58	42	21	—
Indolyl-acetyl-glycine	—	—	—	82	82	72	66	52	—
Indolyl-acrylyl-glycine	—	—	—	83	83	72	69	55	—
C									
Tryptophan	—	—	—	38	38	16	—	—	—
5-OH-Tryptophan	—	—	—	24	24	11	—	—	—
D									
Tryptamine	—	—	55	51	64	10	—	—	—
5-OH-Tryptamine	—	—	36	38	46	7	—	—	—
E									
Indoxyl sulphate	—	—	24	33	73	54	27	17	—
Kynurenine sulphate	—	—	—	25	30	—	—	—	—
Skatole	96	97	98	100	100	100	90	91	100
Urea	—	—	23	73	73	63	56	49	10

After chromatography of the standard sample of indolyl-acrylyl-glycine with medium VI two spots giving the characteristic red colour with Ehrlich's reagent appeared on the chromatograms. It was assumed that this phenomenon again could be explained by *cis–trans* isomerism.

(C) *Indolic amines*

Medium II: Acetone–ammonia (25 %) (100:1).

Indolic amines could be separated in many different systems. One of the most simple of these media, acetone containing 1 % of a concentrated NH_3 solution, gave a rapid and efficient separation of tryptamine and 5-HT leaving the indole groups (A, B and D) at the origin. Indoles containing neither NH_2- nor COOH-groups were taken to the solvent front.

(D) *Indolic amino acids*

Medium II–III.

The greatest difficulties were met during the attempts to establish a system for separation of 5HT, tryptophan and 5-OH-tryptophan. It was possible to separate the two latter compounds in several systems, but most of these did not give sufficient separation of 5HT and tryptophan.

An interesting combination led to the required result: in many acidic systems a good separation was obtained between 5HT and tryptophan and between 5HT and tryptamine (*e.g.* in system III). If after this separation the chromatoplate was dried and then developed with system II the amino acids would remain stationary whereas the amines would migrate further. Consequently a complete separation between all the four compounds discussed was achieved (see Fig. 2). It is essential to dry the chromatogram thoroughly before development in the second system; heating at 110° for 20 min apparently did not destroy any of these four compounds. Also the free acids can be separated by this procedure, but since they are partly decomposed during the heat treatment separation with system I is still to be preferred for these substances.

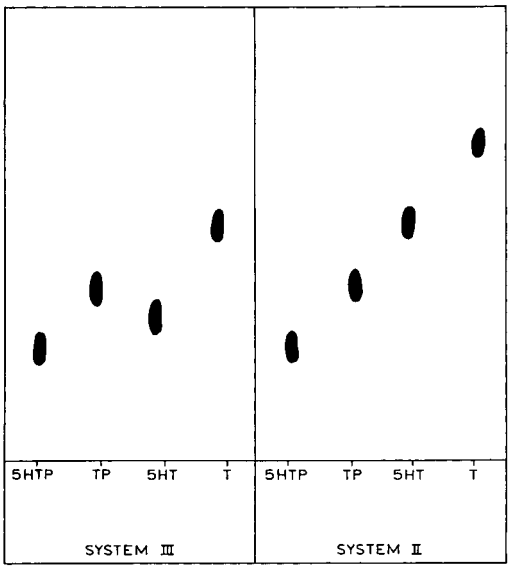


Fig. 2. Diagramatic representation of the separation of the amino acids from the amines by developing the results of solvent III in II. System III separates the amino acids and amines from other indoles but does not separate amines and amino acids. System II then causes further migration of the amines, separating them from the amino acids.

The method described here has the advantage over the procedure earlier reported by SCHMID *et al.*[12] in that it also separated the indolic amino acids from the free acids. The method of SCHMID[12] gives an excellent separation among the four compounds discussed here but all acidic indoles are concentrated in a very small area on the chromatogram.

The location reagents used were the same as those normally used for paper chromatography. Substitution of Kieselgel G with Kieselgel GF_{254} with incorporated fluorescence indicator made all the spots visible under U.V. but they all now appeared blue on a yellow-green background and fluorescent characterization of different indoles was therefore impossible.

All the systems described could be applied successfully for qualitative determination of indoles in neat urine, as even rather large concentrations of urea apparently did not disturb the separation of the indoles.

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Technical Co-operation and the Tropical Medical Research Board for financial assistance and guidance.

SUMMARY

Urinary indoles were separated on chromatoplates of Kieselgel G. The indoles were divided into four groups according to polarity and pK values, and solvent systems were established for separation of the compounds within these four groups. A weakly polar system was used for the free indolic acids, a more polar system for the corresponding conjugates and a strongly polar basic system for the amines. Separation of tryptophan, 5-HTP, 5-HT and tryptamine was achieved by application of an acidic system followed by an alkaline system in a one-way chromatogram; this procedure will separate most indoles present in an indole mixture.

The separation of indolyl-acrylic acid and its glycine conjugate from other urinary indoles was achieved; the double spot formation of indolyl-acrylic acid and its conjugates is discussed in terms of *cis–trans* isomerism.

REFERENCES

1 M. A. CRAWFORD, *Lancet*, 1 (1962) 352.
2 J. M. FOY AND J. R. PARRATT, *J. Pharm. Pharmacol.*, 12 (1960) 360.
3 W. M. MCISAAC AND I. H. PAGE, *J. Biol. Chem.*, 234 (1959) 858.
4 H. WEISSBACH, B. G. REDFIELD AND S. UDENFRIEND, *Federation Proc.*, 17 (1958) Abstr. 1653.
5 S. KVEDER, S. ISKRIC AND D. KEGLEVIC, *Biochem. J.*, 85 (1962) 447.
6 J. BANWELL AND M. A. CRAWFORD, *Biochem. J.*, 89 (1963) 69P.
6a M. A. CRAWFORD, *E. African Med. J.*, 41 (1964) 228.
7 M. D. MILNE, M. A. CRAWFORD, C. B. GIRAO AND L. LOUGHRIDGE, *Clin. Sci.*, 19 (1960) 165.
8 J. B. JEPSON, in I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*, Vol. I, Heinemann, London, 1960, pp. 183*ff*.
9 R. Z. PATEL AND M. A. CRAWFORD, *Biochem. J.*, 89 (1963) 81P.
10 A. B. DURKEE AND J. C. SIROIS, *J. Chromatog.*, 13 (1964) 173.
11 E. STAHL, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin, Göttingen, Heidelberg, 1962.
12 E. SCHMID, L. ZICHA, J. KRAUTHEIM AND J. BLUMBERG, *Med. Exptl.*, 7 (1962) 8.

QUELQUES MODALITÉS EXPÉRIMENTALES NOUVELLES DANS LE DOMAINE DE LA CHROMATOGRAPHIE ET DE LA CHROMATO-ÉLECTROPHORÈSE EN COUCHE MINCE DE POUDRE DE CELLULOSE DES SUBSTANCES HYDROSOLUBLES

ROGER L. MUNIER ET GISÈLE SARRAZIN
Institut Pasteur, Service de Biochimie Cellulaire, Paris (France)
(Reçu le 14 juillet 1965)

Lorsqu'on désire procéder à des analyses chromatographiques de routine dans le domaine de la détermination de la structure primaire des protéines (enchaînement des aminoacides), il faut être en mesure de répéter un grand nombre d'essais d'identification d'aminoacides ou de certains de leurs dérivés. Il est donc extrêmement intéressant de pouvoir réaliser ces essais à l'aide d'un matériel très simple, peu couteux et peu encombrant. Connaissant l'intérêt que présentent dans ce domaine les techniques de la chromatographie en couche mince, nous nous sommes attachés à la mise au point de conditions opératoires et d'un matériel permettant de réaliser simultanément un grand nombre de chromatogrammes sur de très faibles quantités de substance et dans des conditions expérimentales très variées. Pour réaliser ce programme, nous avons mis au point une modification de la cuve de BRENNER ET NIEDERWIESER[1]. Le modèle de cuve que nous proposons est d'un emploi très général; en particulier, il permet de réaliser les différents types possibles d'opérations chromatographiques: développement de chromatogrammes en atmosphère conditionnée ou non, développement avec ou sans écoulement continu du solvant, écoulement continu des solvants hors des limites de la couche mince avec ou *sans* évaporation du solvant mobile en extrémité de couche, développement en seconde dimension d'une portion choisie du chromatogramme déjà développé en totalité en 1-ère dimension etc....Grâce aux procédés que nous allons décrire, on peut réaliser des chromatogrammes à deux dimensions ou des chromato-électrogrammes avec ou sans écoulement continu des solvants et ainsi répartir les taches de substances sur la surface totale d'une couche mince (200 × 200 mm). Des méthodes très simples et efficaces de couplage électrophorèse-chromatographie seront également décrites.

Ce matériel et les modalités de son emploi ont donné d'excellents résultats dans le domaine de la chromatographie en couche mince de poudre de cellulose des aminoacides libres[2,3] et des dinitrophényl-aminoacides[4,5]. Le faible encombrement que représente chaque dispositif de chromatographie et la possibilité de faire des empilements font qu'il est possible de réaliser simultanément jusqu'à 80 chromatogrammes sur un plan de travail d'un mètre carré et en une journée de laboratoire.

Quoique les méthodes décrites aient été mises au point pour la chromatographie des substances hydrosolubles sur couche mince de poudre de cellulose sans liant, elles peuvent aussi être employées, sous certaines conditions, pour la chromato-

graphie dans des couches minces formées d'autres matières et donc dans des couches mécaniquement moins résistantes. Ainsi les méthodes décrites peuvent, avec quelques modifications, s'appliquer à la chromatographie des substances liposolubles.

I. TECHNIQUES EXPÉRIMENTALES ET MATÉRIEL UTILISÉ

Couches minces

Les couches minces (250 μ) utilisées sont formées, sur plaque de verre 200 × 200 × 4 mm, à partir de poudre de cellulose sans liant (Macherey et Nagel No. MN300; taille moyenne des particules: 10 μ) de la manière décrite dans les références[2,5]. Ces couches sont mécaniquement très résistantes; avec des solvants mobiles aqueux ou organiques on peut réaliser sur ces plaques des chromatographies en "couche mince plafond" ou en "couche mince plancher", c'est à dire dans des conditions telles que, dans la cuve à chromatographie, la couche mince soit portée par la plaque de verre supérieure ou par la plaque de verre inférieure.

Cuve à chromatographie

Dans sa réalisation et sa mise en oeuvre la plus simple, la cuve à chromatographie utilisée est celle qui est représentée par le schéma de la Fig. 1. Les deux grands côtés de la cuve sont formés par deux plaques de verre (200 × 200 × 4 mm) espacées par un étrier (u) en forme de u *plus long* que les plaques. Cet étrier est formé à partir d'une baguette de verre pyrex de 6 mm de diamètre. La plaque de verre supérieure

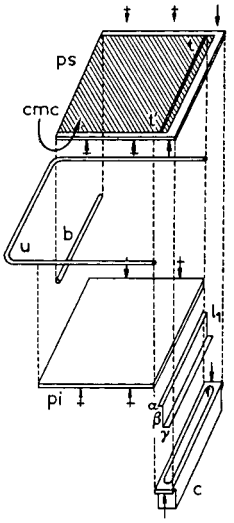


Fig. 1. Principe de montage d'une "auto-cuve", pour développement d'un chromatogramme en "couche mince plafond", mettant en oeuvre une cuvette à solvant modifiée. cmc = couche mince de poudre de cellulose sur la face inférieure de la plaque (200 × 200 × 4 mm) de verre supérieure (ps); b = baguette (diamètre: 6 mm) de fermeture de l'autocuve; c = cuvette à solvant comportant, aux deux extrémités, un rebord permettant sa fixation facile à la plaque de verre supérieure (ps) à l'aide de pinces à dessin; l_1 = languette (17 × 4.2 cm), de papier Whatman No. 2, amenant le solvant de la cuvette (c) à la couche mince; pi = plaque inférieure (200 × 200 × 4 mm) de verre; u = étrier de verre (diamètre: 6 mm) séparant les deux plaques de verre (ps, pi); l'ensemble est maintenu en place grâce à des pinces à dessin de petite (↓, largeur × ouverture: 20–40 × 6 mm) ou de grande (⏚, largeur × ouverture: 80 × 10–15 mm) taille.

(ps), ici, porte la couche mince sur sa face inférieure. L'ensemble, constituant quatre des côtés de la cuve, est maintenu en place à l'aide de pinces à dessin. Le solvant contenu dans la cuvette (c) est amené à la couche mince par une languette de papier (l_1) pliée en trois segments (α,β,γ). Une extrémité (β,γ) de cette languette (l_1) baigne dans le solvant tandis que celle (α) qui repose sur le bord de la cuvette à solvant se trouve mise au contact de la couche mince (trace tt' de la partie α de la languette l_1 sur la couche mince). A l'aide de deux petites pinces à dessin et grâce aux rebords (5 × 5 × 25 mm) ménagés dans ses deux petits côtés, la cuvette à solvant est fixée à la plaque de verre supérieure; ainsi cette cuvette assure également la fermeture du 5-ème côté de la cuve à chromatographie. Dans le cas de la Fig. 1, le 6-ème côté de la cuve est fermé par une baguette de verre (diamètre 6 mm). Nous verrons que cette fermeture du 6-ème côté de la cuve à chromatographie peut être assurée par d'autres dispositifs (voir Fig. 4) que la baguette de verre (b). Dans certains montages, l'étrier de verre (u), la baguette de verre (b) peuvent être remplacés par des pièces de polyéthylène haute pression ou de polytetrafluoroéthylène. Une cuve à chromatographie similaire peut être construite pour des plaques de verre 400 × 200 × 4 mm.

Pour la plupart des solvants employés en chromatographie de partage direct et en chromatographie de relargage une cuvette à solvant usinée dans un bloc (2.5 × 2.5 × 20 cm) de chlorure de polyvinyle (Afcodur, Leucoflex) suffit; lorsque la chromatographie est réalisée dans des solvants attaquant le chlorure de polyvinyle (exemple, mélange méthyl-éthylcétone–acétone–eau), on emploie des cuvettes en polyéthylène basse pression.

La cuve à chromatographie ainsi décrite sera désignée sous le nom d'"auto-cuve"; lorsque ses composants sont assemblés avec des pinces suffisamment puissantes, la cuve obtenue est d'une étanchéité satisfaisante qui permet son emploi dans tous les domaines de la chromatographie en couche mince.

On peut remarquer que le modèle d'"auto-cuve" décrit diffère en trois points de celui de BRENNER ET NIEDERWIESER[1]: plus grand espace ménagé entre les deux plaques de verre, plus grande accessibilité de l'ouverture de la cuve se trouvant à l'opposé de la cuvette à solvant, plus grande facilité de fixation de la cuvette à solvant sur la plaque de verre supérieure. Ces modifications rendent cette auto-cuve plus maniable et permettent de l'employer dans un plus grand nombre de cas.

Cellules d'électrophorèse

Pour obtenir des séparations électrophorétiques sur couches minces (250 μ), portées sur plaques 200 × 200 (ou 400) × 4 mm, en 1-ère ou 2-ème dimension, nous avons mis au point des dispositifs simples (voir Fig. 2) utilisant, en général, des récipients en matières plastiques d'origine commerciale. Dans la plupart des cas, avec des gradients de potentiels compris entre 15 et 20 V par cm, il est possible d'obtenir par électrorhéophorèse[6,7] en couche mince, la séparation des substances, sous forme de petites taches très nettes, en 30 à 240 min. La cellule d'électrophorèse A de la Fig. 2 permet d'utiliser des plaques 200 × 200 mm, les cellules B et C des plaques 200 × 400 mm; le champ est appliqué respectivement sur 200 mm dans le montage B et sur 400 mm dans le montage C.

Dans tous les cas, nous utilisons un même générateur de tension continue constitué par un transformateur dont le circuit secondaire est à point moyen (350 — 0 — 350 $V_{eff.}$), une valve biplaque 5Z3 (chauffage: 5 $V_{eff.}$, 3A $_{eff.}$), une cellule de filtrage

en π (16 μF, t.s. 1000 V; 10 H, 300 Ω, c.s. 120 mA; 8 μF, t.s. 1000 V); la cellule d'électrophorèse est montée en parallèle avec une résistance de l'ordre de 100,000 Ω (10 watts) montée en potentiomètre aux bornes de sortie du générateur de tension continue.

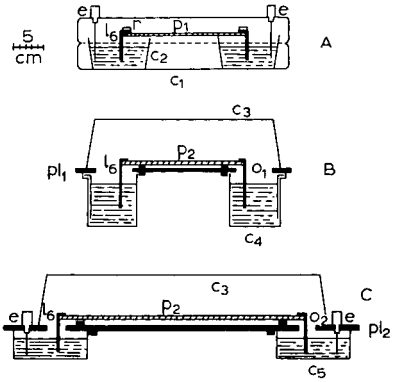


Fig. 2. Dispositifs simples pour l'électrophorèse "basse tension" en couche mince de poudre de cellulose. (A) pour plaque 200 × 200 × 4 mm. (B) pour plaque 200 × 400 × 4 mm, sens d'électrophorèse 200 mm. (C) pour plaque 200 × 400 × 4 mm, sens d'électrophorèse 400 mm. c_1 = 2 cuves en plexiglass 34 × 23 × 4.2 cm; c_2 = cuve en polypropylène 21 × 10 × 6 cm pour 800 ml de liquide, ou 21 × 14 × 6 cm pour 1 l de liquide; c_3 = cuve en polypropylène 48 × 32 × 7.5 cm; c_4 = cuve en chlorure de polyvinyle 44 × 9 × 8 cm, à électrode incorporée, pour 2 l de liquide; c_5 = cuve en plexiglass 24 × 12 × 5 cm pour 900 ml de liquide; e = électrode amovible; l_6 = languette (19 × 6 cm) de papier Whatman No. 2; o_1 = orifice (41 × 6 cm) percé dans le plateau (pl_1) à 3 cm du bord; o_2 = orifice (21 × 3 cm) percé dans le plateau (pl_2) à 7 cm du bord; p_1 = plaque de verre 200 × 200 × 4 mm; p_2 = plaque de verre 200 × 400 × 4 mm; p_1,p_2 portent la couche mince (250 μ) de poudre de cellulose; pl_1 = plateau en chlorure de polyvinyle (35 × 50 cm); pl_2 = plateau (35 × 58 cm); r = réglette de plexiglass.

RÉSULTATS ET DISCUSSION

BRENNER ET NIEDERWIESER[1] ont proposé un dispositif ingénieux pour réaliser le développement continu des chromatogrammes sur couche mince par évaporation du solvant mobile en extrémité de plaque. Du point de vue réalisation, on peut cependant reprocher au dispositif tel qu'il est décrit par ses auteurs[1] d'exiger l'emploi d'un dispositif de serrage encombrant pour la fixation de la cuvette à solvant et de ne laisser qu'un écartement trop faible entre les plaques de verre. Ainsi les gouttelettes de solvant, qui se condensent sur la plaque de verre supérieure ne portant pas de couche mince ont tendance à mouiller la couche mince portée par la plaque de verre inférieure. Enfin, et surtout, le modèle de cuve à chromatographie en couche mince proposé par BRENNER ET NIEDERWIESER[1] n'a été conçu qu'en vue de son emploi en chromatographie avec écoulement continu du solvant par évaporation de ce dernier lors de son arrivée en extrémité de couche mince.

Reprenant le principe proposé par ces auteurs[1], à savoir la possibilité de former une cuve à chromatographie en couche mince avec deux plaques de verre dont l'une porte la couche mince, nous avons mis au point un modèle de cuve, dont le montage n'exige qu'un matériel simple et courant, et qui peut être utilisé dans tous les cas possibles de chromatographie: développement avec ou sans écoulement continu du solvant, développement d'une couche mince fixée sur la plaque de verre inférieure

ou supérieure, développement simultané de deux chromatogrammes sur deux couches minces se faisant face, développement en atmosphère conditionnée, etc.

Dans sa mise en oeuvre la plus simple, ce dispositif est décrit p. 337 et dans la Fig. 1; dans cette figure, on a représenté le développement (sans écoulement continu) d'un chromatogramme en "couche mince plafond" (voir p. 337).

Chromatographie sans écoulement continu du solvant

Sur la Fig. 3, sont donnés des schémas qui montrent différents cas de mise en oeuvre de la chromatographie en couche mince de poudre de cellulose à l'aide d'"auto-cuve" sans écoulement continu du solvant. Dans tous les cas, le côté de la cuve, qui est à l'opposé de la cuvette à solvant, est fermé par une baguette de verre (b). Le schéma A montre le montage qui permet de développer un chromatogramme sur "couche mince plafond"; dans ce cas, l'alimentation en solvant est faite à travers la languette (l_1) de papier dont l'extrémité mise au contact de la couche mince, est serrée entre la plaque de verre supérieure (ps) et le bord de la cuvette à solvant (c).

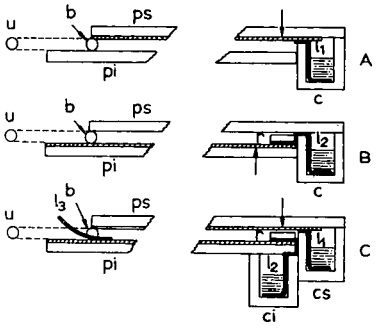


Fig. 3. Schémas montrant différents cas de mise en oeuvre de la chromatographie en couche mince de poudre de cellulose à l'aide d'"auto-cuves"; développement: (A) d'une "couche mince plafond"; (B) d'une "couche mince plancher"; (C) d'une "couche mince plafond" (ps) avec écoulement préalable ou (et) simultané, dans la couche mince inférieure (pi) d'un solvant créant l'atmosphère de la cuve. b = baguettes de verre (diamètre 6 mm) formant les "auto-cuves"; c = cuvette à solvant; ci = cuvette inférieure à solvant; cs = cuvette supérieure à solvant; l_1,l_2 = languettes (respectivement: 17 × 4.2 et 17 × 5.5 cm) de papier Whatman No. 2, amenant les solvants des cuvettes (c, ci, cs) aux bords des couches minces de poudre de cellulose (surfaces hachurées); l_3 = languette (17 × 6 cm) de papier permettant éventuellement l'écoulement continu du solvant incréant l'atmosphère de la cuve; pi, ps = respectivement, plaques de verre (200 × 200 × 4 mm) inférieure et supérieure; r = réglette (180 × 20 × 4 mm) de verre; u = trace de l'étrier de verre (u, de la Fig. 1); ↓ = positions de dépôt des substances à soumettre à la chromatographie.

Dans le schéma B, on a représenté le montage utilisé pour développer un chromatogramme en "couche mince plancher"; l'alimentation en solvant de la couche mince est obtenue par l'intermédiaire de la languette de papier (l_2) qui est appliquée sur le bord de la couche mince grâce à la réglette de verre (r) d'épaisseur plus faible que l'espace existant entre les deux plaques de verre (ps, pi). Lorsqu'on utilise des couches minces de poudre de cellulose, l'une ou l'autre des méthodes précédentes de développement (A ou B, Fig. 3) peuvent être utilisées; en général, on préfère cependant employer le montage le plus simple, c'est à dire celui représenté dans le schéma A. Lorsqu'il s'agit de couches minces fragiles (SiO_2, Al_2O_3) pour lesquelles le développement du chromatogramme ne peut donc avoir lieu qu'en "couche mince plancher"

on utilise le montage B (Fig. 3)*. Pour les couches minces de support activé (chromatographie d'adsorption) on remplace l'étrier de verre (u), la baguette de verre (b) et la réglette de verre (r) par un cadre (épaisseur 6 mm) taillé dans une plaque de polyéthylène haute pression. Dans ce cas, le cadre maintient en place la languette l_2 (comme dans le schéma B de la Fig. 3), ferme la cuve, permet de maintenir la séparation entre les deux plaques de verre (ps et pi) et, enfin, protège la couche mince de la désactivation par l'humidité atmosphérique.

Le schéma C de la Fig. 3 montre le montage utilisé pour développer un chromatogramme en couche mince, dans une atmosphère conditionnée. Ici, l'auto-cuve contient deux couches minces se faisant face. Dans un premier temps, l'atmosphère de la cuve à chromatographie est créée par l'irrigation de la couche mince inférieure par un solvant volatil approprié; pour obtenir ce résultat, le solvant introduit dans la cuvette inférieure (ci) est amené à la couche mince à l'aide de la languette de papier (l_2). Après un temps convenable, le solvant devant permettre le développement du chromatogramme dans la couche mince supérieure (ps) est introduit dans la cuvette supérieure (cs). Ainsi le développement du chromatogramme (alimentation en solvant à travers la languette de papier l_1) peut avoir lieu en atmosphère conditionnée (vapeurs de solvants organiques, ammoniac, amines aliphatiques volatiles, pyridine, acides volatils etc....). Remarquons qu'il peut être intéressant de mettre, au contact de l'extrémité de la couche mince inférieure, une languette de papier (l_3) afin de permettre l'écoulement continu éventuel du solvant devant créer l'atmosphère de la cuve.

Dans l'exemple que nous venons de décrire, les deux couches minces représentées dans le schéma C (Fig. 3) sont à base de poudre de cellulose et sont, donc, mécaniquement très résistantes. Lorsqu'il s'agit de faire une chromatographie dans une couche mince fragile, le développement du chromatogramme doit être réalisé dans la couche mince inférieure et le solvant qui va créer l'atmosphère de la cuve devra donc se déplacer dans la couche mince supérieure à base de poudre de cellulose; dans ce cas, la languette de papier (l_3) est, évidemment, mise au contact de la couche mince supérieure et son extrémité est repliée sur la face supérieure de la plaque de verre supérieure.

Le dispositif représenté dans le schéma C (Fig. 3) est extrêmement intéressant puisqu'il permet de faire des chromatographies dans une atmosphère rigoureusement contrôlée, opération qui ne pouvait être réalisée, jusqu'ici, qu'en chromatographie sur papier. Signalons que ce dispositif a donné d'excellents résultats[4,5] pour la chromatographie, en atmosphère fortement ammoniacale, des dinitro-phényl-amino-acides sur couche mince de poudre de cellulose.

Remarquons, enfin, que dans le cas du montage représenté dans le schéma C (Fig. 3) le modèle adopté pour la cuvette à solvant (c) de l'auto-cuve (voir Fig. 1) rend très aisée la fixation (à l'aide de *petites* pinces à dessin) des cuvettes inférieure et supérieure aux plaques de verre.

Chromatographie avec écoulement continu du solvant

Si dans les dispositifs A et B de la Fig. 3, on tire légèrement vers l'extérieur la baguette de verre (b), lorsque le front du solvant a atteint l'ouverture de la cuve à

* Dans ce cas, on peut remplacer la réglette de verre r par une réglette (verre ou polyéthylène haute pression) de même épaisseur que l'espace existant entre les deux plaques de verre (pi et ps).

chromatographie, on peut réaliser à l'instar de BRENNER ET NIEDERWIESER un développement continu du chromatogramme par évaporation du solvant en extrémité de plaque. En fait ceci ne peut être pratiqué que pour des temps courts de développement continu et avec des solvants dont tous les constituants sont également (et suffisamment) volatils. Pratiquement, dans beaucoup de cas, ce procédé donne des résultats très peu reproductibles; ceci est dû au fait que, d'une part, le dispositif est extrêmement sensible à l'action néfaste des courants d'air et que d'autre part, les impuretés contenues dans la poudre de cellulose de la couche mince et dans les constituants du solvant mobile s'accumulent en extrémité de plaque et bloquent plus ou moins rapidement la progression du solvant.

Pour pallier tous ces inconvénients, nous avons mis au point un procédé nouveau de développement continu des chromatogrammes en couche mince. A la place de la baguette (b) de verre (voir schéma A de la Fig. 3) qui forme le 6-ème côté de l'auto-cuve, nous avons mis un accordéon (l_4) de papier (voir schéma A de la Fig. 4) dont une des faces du premier pli est au contact de l'extrémité de la couche

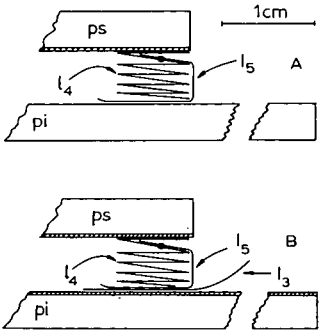


Fig. 4. Dispositif simple et maniable permettant l'écoulement continu du solvant hors des limites de la couche mince; seule la partie terminale de la couche mince, mise en oeuvre dans une "auto-cuve", est représentée dans le schema ci-dessus. En A: développement d'une "couche mince plafond" de poudre de cellulose; en B: développement d'une "couche mince plafond" (ps), avec écoulement simultané dans la "couche mince plancher" (pi) d'un solvant volatil maintenant une atmosphère conditionnée dans la cuve de développement; l_5 = languette de papier Whatman No. 2 permettant l'écoulement continu du solvant créant l'atmosphère de la cuve; l_4 = languette (185 × 220 cm) de papier Whatman No. 2 pliée (12–14 plis) en accordéon et emballée, dès la 2-ème face du 1-er pli, dans une languette (180 × 35 × 0.2 mm) de polyéthylène; pi, ps = respectivement, les plaques de verre inférieure et supérieure de l'auto-cuve.

mince. Dès que le front du solvant arrive en fin de couche mince, il continue à progresser dans le papier et ainsi on réalise un développement continu du chromatogramme. Afin de rendre le dispositif insensible à l'action des courants d'air, une languette (l_5) de polyéthylène haute pression (agrafée sur le second côté du premier pli) recouvre la partie externe de l'accordéon de papier. Ce dispositif a permis d'obtenir des séparations intéressantes dans le domaine de la chromatographie des amino-acides[2,3] et de certains dinitro-phényl-aminoacides[4,5]. En combinant les dispositifs A de la Fig. 4 et C de la Fig. 3, on obtient un montage qui permet de réaliser, en atmosphère conditionnée, le développement continu d'un chromatogramme "en couche mince plafond". On trouvera, dans le schéma B de la Fig. 4, une représentation de l'extrémité de ce montage.

Pour le développement continu de chromatogrammes en "couche mince plancher", des dispositifs similaires aux précédents peuvent être montés; dans ce cas, la languette de papier (l_3), l'accordéon de papier (l_4) partiellement entouré par la languette de polyéthylène (l_5) sont mis dans une position inverse de celle qui est indiquée dans les schémas A et B de la Fig. 4; la partie de couche mince se trouvant hors de l'auto-cuve est éliminée si l'on n'utilise pas une plaque de verre supérieure de 200 × 225 mm.

Remarquons que le procédé de développement continu des chromatogrammes que nous proposons peut aussi être appliqué lorsqu'il s'agit de chromatographies réalisées dans des bacs de verre (voir Fig. 5). Suivant les cas, l'accordéon de papier (l_4) et la réglette de verre (r) peuvent être maintenus en place à l'aide de bracelets de caoutchouc ou de petites pièces en acier inoxydable.

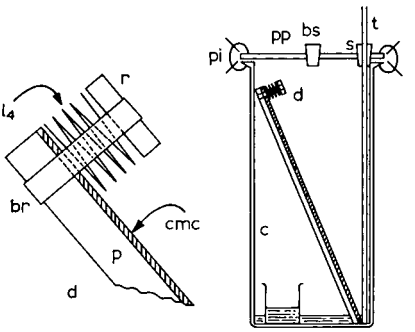


Fig. 5. Schema d'un dispositif permettant l'écoulement continu du solvant hors des limites de la couche mince (250 μ); développement sur plaques (p) 200 × 200 × 4 mm dans les cuves 21 × 21 × 9 cm (dimensions internes); couvercle en polyéthylène (pp) de la cuve (c) percé de deux trous pour recevoir un bouchon de caoutchouc de silicone (bs) et un bouchon percé (s) muni d'un tube de verre (t); pi = pince à dessin maintenant le couvercle (pp) en place; d = dispositif d'absorption du solvant; r = réglette (200 × 200 × 4 mm) de verre, l_4 = accordéon (12–14 plis) de papier (19 × 22 cm) Whatman No. 2; br = bracelet de caoutchouc; cmc = couche mince de poudre de cellulose.

Développement en seconde dimension d'une portion de chromatogramme

Lorsque dans un procédé chromatographique, le mélange des substances soumis à l'analyse se trouve séparé (en première dimension) en divers groupes, il est quelquefois intéressant de pouvoir développer (en 2-ème direction) avec des solvants différents les portions de couche mince portant ces divers groupes de substances ou même de pouvoir soumettre certaines d'entre elles à une électrophorèse de zone.

En chromatographie sur couches minces portées par des plaques de verre, ce procédé est utilisé avec sûreté lorsque la séparation des substances peut être obtenue, en première dimension, par une électrophorèse de zone; en effet, dans ce cas, les positions atteintes par les taches de substances sont identiques d'un essai à l'autre si les conditions d'électrophorèse sont les mêmes; il est ainsi possible de déterminer en toute rigueur les positions que doivent avoir les bandes étroites (vv', Fig. 6) où la couche mince sera éliminée pour être divisée en portions avant les développements en 2-ème dimension.

Il était donc intéressant de mettre au point un dispositif mettant en oeuvre une "auto-cuve" et permettant de ne développer, en seconde dimension, qu'une portion

d'un chromatogramme déjà développé, dans sa totalité, en 1-ère dimension. Un tel dispositif est représenté à la Fig. 6. Dans ce schéma, on voit qu'après développement, en 1-ère dimension, le chromatogramme a été divisé en deux portions (cmc_1, cmc_2) par élimination de la couche mince (à l'aide d'un scalpel et d'un pinceau) sur une bande étroite (vv'). L'alimentation en solvant de la partie (cmc_1) de la couche mince est assurée à travers la languette de papier (l_1) dont la largeur est égale à celle de la

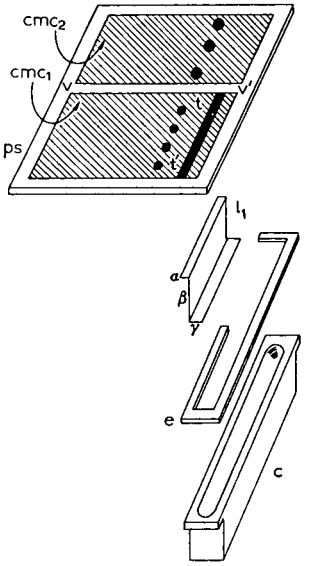


Fig. 6. Schema de montage d'une "auto-cuve" en vue du développement en 2-ème dimension d'une portion (cmc_1) de couche mince déjà développée en 1-ère dimension sur toute sa surface. Couche mince de poudre de cellulose (surfaces hachurées) divisée en deux portions (cmc_1, cmc_2) par élimination d'une bande étroite (vv') de la couche mince; c = cuvette à solvant; l_1 = languette de papier Whatman No. 2 amenant le solvant de la cuvette (c) à la portion (cmc_1) de couche mince; e = cale taillée dans une languette de polyéthylène (200 × 25 × 1 mm); seule la plaque supérieure (ps) porteuse de la couche mince a été représentée dans ce schéma, mais l'étrier de verre (u), la baguette de fermeture (b) et la plaque de verre inférieure (pi), représentés dans la Fig. 1, sont également utilisés.

zone que l'on souhaite irriguer par le solvant. Pour isoler la portion (cmc_2) de couche mince ne devant pas être irriguée par le solvant, une cale (e)* est placée entre la languette de papier (l_1) et la face supérieure de la cuvette à solvant (c); l'ensemble — cuvette à solvant (c), cale (e), languette (l_1) de papier — est attaché à la plaque de verre supérieure à l'aide d'une pince à dessin. Le montage de l'auto-cuve est, pour le reste, identique à celui qui est représenté à la Fig. 1.

Dans l'étape chromatographique suivante la seconde portion (cmc_2) de couche mince est, par exemple, développée par un autre solvant; une autre cale de taille et de forme appropriées isole la portion (cmc_1) de couche mince déjà développée.

Sous certaines conditions, ce procédé a l'avantage de pouvoir permettre la séparation d'un plus grand nombre de substances que dans le cas où le chromatogramme est développé en totalité dans les deux dimensions.

* Découpée à la taille convenable par l'expérimentateur dans une mince (1 mm) plaque de polyéthylène haute pression ou de polytetrafluoroéthylène.

Association chromatographie-électrophorèse, électrophorèse de zone à deux dimensions

Les dispositifs décrits dans le schéma de la Fig. 2 sont ceux que nous avons utilisés pour l'électrophorèse en couche mince de poudre de cellulose (250 μ) soit pour des plaques de verre 200 × 200 × 4 mm, soit pour des plaques 200 × 400 × 4 mm; suivant les cas, le champ est appliqué sur 200 ou 400 mm. Dans des couplages chromatographie-électrophorèse ces montages extrêmement simples* nous ont permis d'obtenir d'excellentes séparations dans le domaine de la chromatographie des aminoacides[2,3] et des dinitro-phényl-aminoacides hydrosolubles[4,5]. La présence d'une plaque épaisse de verre (4 mm) comme support de la couche mince permet de travailler avec un gradient de potentiel relativement élevé: une partie de l'énergie dégagée par effet Joule se trouve utilisée pour porter la plaque de la température ambiante à une valeur de régime plus élevée. L'épaisseur de la plaque de verre joue un rôle non négligeable dans les essais n'exigeant l'application du champ électrique que pour un temps relativement court. Dans tous les cas, pour rendre petites les taches de substances et pour les maintenir plus longtemps sur la couche mince** malgré l'action d'un gradient de potentiel relativement élevé***, nous réglons l'évaporation uniforme de la solution d'électrolyte par unité de surface de couche mince à une valeur convenable (pour un exemple, voir la séparation des dinitro-phényl-aminoacides hydroacidosolubles par chromato-électrophorèse[4,5]). Ce résultat est obtenu en amenant à une valeur satisfaisante la conductivité de la solution d'électrolyte imprégnant la couche mince. Dans certains cas on peut faire varier la concentration de la solution saline à pouvoir tampon utilisée; dans d'autres et lorsque cette dernière est constituée par des électrolytes faibles, il est intéressant de régler la conductivité de la solution par addition d'un sel neutre (exemple: NaCl, Na_2SO_4) jusqu'à obtention d'une concentration convenable.

RÉSUMÉ

Des méthodes et des dispositifs expérimentaux simples, peu coûteux et efficaces permettant de réaliser la chromatographie à deux dimensions et la chromato-électrophorèse sur couche mince, sont présentés. En particulier, il est possible, avec ce matériel, de faire des chromatogrammes avec écoulement continu des solvants sans évaporation de ces derniers en extrémité de plaque et (ou) de les développer dans une atmosphère conditionnée. Ces procédés permettent de réaliser simultanément, dans des conditions rigoureusement reproductibles, un grand nombre de chromatogrammes sur une faible surface de travail. Ces méthodes ont donné, en particulier, d'excellents résultats dans le domaine de la chromatographie sur couche mince de poudre de cellulose des aminoacides et de leurs 2,4-dinitro-phényl dérivés.

SUMMARY

Cheap and efficacious methods and simple apparatus are described for two-dimensional chromatography and chromato-electrophoresis on thin layers. With this material, it is in particular possible to run chromatograms with continuous solvent

* Sans les dispositifs compliqués de refroidissement qui rendent les appareils onéreux.
** Et donc sous l'action séparatrice du champ électrique appliqué.
*** C'est à dire pour bénéficier des avantages fondamentaux de l'électrorhéophorèse[6,7].

flow without evaporation of the solvent at the end of the plate and/or to develop them in a conditioned atmosphere. These procedures permit, under closely controlled conditions, the simultaneous running of a large number of chromatograms on a fragile working surface. The methods have given excellent results, particularly in the field of chromatography of amino acids and their 2,4-dinitrophenyl derivatives on thin layers of powdered cellulose.

BIBLIOGRAPHIE

1 M. BRENNER ET A. NIEDERWIESER, *Experientia*, 17 (1961) 237.
2 R. L. MUNIER ET G. SARRAZIN, *Bull. Soc. Chim. France*, (1965) 1490.
3 R. L. MUNIER ET G. SARRAZIN, *Bull. Soc. Chim. France*, (1966) sous presse.
4 R. L. MUNIER ET G. SARRAZIN, *Bull. Soc. Chim. France*, (1965) 2959.
5 R. L. MUNIER ET G. SARRAZIN, *J. Chromatog.*, 22 (1966) 347.
6 M. MACHEBOEUF, P. REBEYROTTE ET J. M. DUBERT, *Bull. Soc. Chim. Biol.*, 35 (1953) 334 et 346; *Chem. Weekblad*, 49 (1953) 237.
7 R. L. MUNIER, *Chim. Anal. (Paris)*, 36 (1954) 253, 283 et 340.

CHROMATOGRAPHIE À DEUX DIMENSIONS DES DINITROPHÉNYL-AMINOACIDES ÉTHÉROSOLUBLES ET CHROMATO-ÉLECTROPHORÈSE DES DNP-AMINOACIDES HYDRO-ACIDO-SOLUBLES, EN COUCHE MINCE DE POUDRE DE CELLULOSE*

ROGER L. MUNIER ET GISÈLE SARRAZIN
Institut Pasteur, Service de Biochimie Cellulaire, Paris (France)
(Reçu le 14 juillet 1965)

On sait qu'après transformation en 2,4-dinitrophényl-dérivés, il est extrêmement aisé de doser les aminoacides contenus dans les hydrolysats de protéines et de peptides grâce aux excellents procédés de séparation chromatographique sur papier décrits pour ces substances (BISERTE ET OSTEUX[1], LEVY[2], MUNIER ET SARRAZIN[3]). Les méthodes de la chromatographie sur papier exigent cependant un matériel encombrant, coûteux, et la mise en oeuvre d'une quantité relativement importante de produit. Lorsqu'il s'agit de procéder à un grand nombre de dosages, d'identifications des constituants des mélanges, il devient souhaitable de s'adresser aux techniques de la chromatographie en couche mince. Malheureusement ces techniques n'ont été développées pour les DNP-aminoacides que sur couche mince de gel de silice[5] et avec des combinaisons de solvants différentes de celles employées en chromatographie sur papier. Il était, à priori, intéressant de chercher à transposer les méthodes décrites pour la chromatographie sur papier à la chromatographie sur couche mince de poudre de cellulose. Ainsi pouvait-on espérer obtenir des résultats comparables par les deux méthodes.

Comme nous allons le montrer, l'application directe des conditions opératoires utilisées en chromatographie sur papier ne permet d'obtenir aucune séparation satisfaisante des DNP-aminoacides sur couche mince de poudre de cellulose. Après une étude des causes d'insuccès (trop faible conductibilité des électrolytes, trop faible teneur en NH_3 de l'atmosphère des cuves, trop lente élimination du solvant de déve-

* Abréviations utilisées: DNP = radical 2,4-dinitrophényl; Ala = DNP-alanine; β-Ala = DNP-β-alanine; α-Arg = N-α-mono-DNP-arginine; Asp = DNP-acide-aspartique; Asp-NH_2 = DNP-asparagine; CySCM = S-carboxyméthyl-cystéine; CySCM-O = produit d'oxydation à l'air de la S-carboxyméthyl-cystéine; $CySO_3H$ = acide-DNP-cystéique; di-His = di-DNP-histidine; di-Lys = di-DNP-lysine; di-Tyr = di-DNP-tyrosine; DNP-NH_2 = dinitro-aniline; DNP-OH = dinitrophénol; Etio = DNP-éthionine; "EtSO ex Etio" = artefact: fraction de la tache de DNP-éthionine transformée en DNP-éthionine sulfoxyde pendant le séchage du solvant de 1-ère dimension; Glu = acide-DNP-glutamique; Glu NH_2 = DNP-glutamine; Gly = DNP-glycocolle; Ileu = DNP-isoleucine; Leu = DNP-leucine; ε-Lys = ε-mono-DNP-lysine; Met = DNP-méthionine; "MeSO ex Met" = artefact: fraction de la tache de DNP-méthionine transformée en DNP-méthionine sulfoxyde pendant le séchage du solvant de 1-ère dimension; Me SO_2 = DNP-méthionine sulfone; N-α-mono His = N-α-mono-DNP-histidine; N-mono Tyr = N-α-mono-DNP-tyrosine; Nor leu = DNP-norleucine; Nor val = DNP-norvaline; p-F Phe = DNP-p-fluorophényl-alanine; Phe = DNP-phényl-alanine; Pro = DNP-proline; Ser = DNP-sérine; Thr = DNP-thréonine; Try = DNP-tryptophane; Val = DNP-valine.

loppement en 1-ère dimension, "dissociation capillaire" des constituants du mélange solvant au cours de son cheminement, retention excessive des constituants du solvant de 1-ère dimension dans la couche de cellulose etc.) nous avons mis au point des conditions opératoires permettant d'obtenir la séparation de l'ensemble des DNP-aminoacides éthéro-solubles, des DNP-aminoacides de faible mobilité (y compris DNPS-carboxyméthyl-cystéine, acide DNP aspartique, acide DNP-glutamique, DNP-asparagine, DNP-glutamine), des DNP-aminoacides hydro-acido-solubles (ε-mono-DNP-lysine, α-DNP-arginine, di-DNP-histidine, α-mono-DNP-histidine, acide-DNP cystéique). Les méthodes expérimentales décrites permettent de réaliser simultanément jusqu'à 20 chromatogrammes sur un plan de travail d'un quart de mètre carré avec des quantités de l'ordre de 3 à 7 mμmoles de chaque DNP-aminoacide par chromatogramme.

TECHNIQUES EXPÉRIMENTALES

Préparation des couches minces

Les couches minces (250 μ) de poudre de cellulose (sans liant) utilisées, sont formées par étalement uniforme d'une bouillie épaisse sur plaque de verre [200 × 200 (ou 400) × 4 mm] à l'aide du dispositif de STAHL[4]. La poudre de cellulose, mise en suspension dans l'eau, est homogénéisée par passage, pendant quelques secondes, dans un mélangeur type Turmix. La bouillie, versée dans un erlenmeyer, est dégazée par agitation sous vide (produit par une trompe à eau) puis est rapidement versée dans l'étaleur; les couches minces sont immédiatement formées de la manière habituelle puis placées sur une surface plane et séchées (une nuit) à la température de la pièce. Pour une proportion correcte de poudre de cellulose et d'eau (de l'ordre de 15 g pour 90 à 85 ml), à déterminer pour chaque lot de poudre, on obtient des *couches lisses* et *adhérant fortement* à la plaque de verre. Un léger excès de cellulose donnerait des couches dont la surface serait granuleuse; un léger excès d'eau entraînerait la formation de couches minces assez peu résistantes (entraînement assez facile des particules superficielles par passage d'un doigt). Avant emploi, les bords de la couche mince sont éliminés (bande de 1 cm de largeur) sur trois côtés. Le quatrième bord de la couche mince ne sera éliminé qu'avant le développement du chromatogramme en 2-ème dimension; dans toutes les figures, les bords de la plaque de verre sont indiqués par un trait plein, ceux de la couche mince par des tirés et points alternés.

Produits utilisés

Alcool octylique primaire (purifié, Prolabo), alcool octylique normal secondaire (Prolabo), alcool laurique (alcool C12 chimiquement pur, Firmenich et Cie, Genève), dodecylsulfate de sodium (pur, Serlabo), monochlorhydrine du glycol (purifiée, Prolabo), diéthylamine anhydre (Eastman Kodak), Tween 20,80, Brij 35 (Atlas Powder Co., Wilmington, U.S.A.), poudre de cellulose (taille moyenne des grains: 10 μ) sans liant No. MN300 (Macherey et Nagel Co.); aucun des solvants commerciaux utilisés (pyridine pure, toluène pur, monochlorhydrine du glycol purifiée) n'ont besoin d'être redistillés; pour les DNP-aminoacides utilisés voir la référence 3.

Préparation des phases solvantes mobiles

Le solvant "toluène"[1] est préparé, 4 h avant son emploi de la manière habituelle:

toluène–monochlorhydrine du glycol–pyridine–ammoniaque 0.8 *N* (150:90:45:90). Juste avant usage, il est passé sur deux filtres de papier plissé de taille appropriée. Dans les conditions opératoires qui nous ont donné satisfaction, la totalité du solvant (correspondant à 150 ml de toluène) obtenu après filtration est additionnée de 10 à 12 gouttes d'alcool octylique primaire (pipette compte-gouttes délivrant 1 ml d'eau en 20 gouttes à 22°)*.

Les solvants utilisés pour la chromatographie de relargage sont:

(a) Tampon Na_2PO_4H–$NaPO_4H_2$ (0.75 *M* en PO_4^{3-}), pH 6.0.
(b) Eau saturée de $(NH_4)_2SO_4$–eau saturée de NaCl–eau (1:1:6).
(c) Eau saturée de $(NH_4)_2SO_4$–eau (1:4), pH 5.5.
(d) Eau saturée de $(NH_4)_2SO_4$–eau (1:7 ou 1:9).
(e) Solution aqueuse 0.75 *M* en $NaPO_4H_2 \cdot 2\ H_2O$.
(f) Eau saturée de Na_2SO_4–eau saturée de $(NH_4)_2SO_4$–eau (10:2:18).
(g) Eau saturée de Na_2SO_4–eau (2:1).
(h) Eau saturée de Na_2SO_4–eau (4:1).
(i) Eau saturée de NaCl–eau (1:5).

Dans les conditions opératoires qui ont donné de bons résultats:

les solutions a, c, e, g, i, étaient saturées de dodécylsulfate de sodium (concentration $< 0.0025\ M$);

les solutions b, d, f, h, étaient 0.0025 *M* en dodécylsulfate de sodium.

Dans le cas de la chromatographie des DNP-aminoacides de faible mobilité (dans le solvant "toluène"), le chromatogramme est imprégné d'acétate de sodium [pulvérisation d'une solution 0.2 *M* en acétate de sodium dans un mélange alcool–eau (6:4)] puis séché (90°, quelques minutes) *rapidement* avant développement en 2-ème dimension [solvant: isobutanol–acide acétique glacial–eau (100:4:20)].

Préparation des solutions d'électrolytes et conditions d'électrophorèse

Pour l'électrophorèse des DNP-aminoacides hydrosolubles, une solution aqueuse de diéthylamine (0.033 *M*) et de chlorure de sodium (0.02 *M*) est utilisée. Après séchage du solvant de 1-ère dimension (un séchage dans un courant d'air à température ambiante suffit), la solution est pulvérisée (avec beaucoup de soin) uniformément sur la surface de la couche mince; la plaque portant la couche mince humide est disposée dans la cellule d'électrophorèse (A de la Fig. 2 de réf. 6); la connexion électrique entre la couche mince et les solutions d'électrolytes (800 ml) contenues dans les bacs à électrode est obtenue à l'aide de deux languettes (20 × 6 cm) de papier Whatman No. 2; après 15 min d'équilibre, la tension est appliquée aux bornes de la cellule d'électrophorèse. Les intensités et les tensions indiquées dans les légendes des figures correspondent aux valeurs initiales.

Matériel employé

Les plaques de verre portant les couches minces ont les dimensions 200 × 200 (ou 400) × 4 mm; dans tous les exemples de séparation électrophorétique présentés, l'épaisseur des plaques de verre sera toujours indiquée; en effet, ce facteur est important dans le cas où les plaques de verre ne sont pas refroidies et spécialement lorsque le temps d'électrophorèse est relativement court. En effet, en électrophorèse de zone

* 10 gouttes = 0.197 g d'alcool.

lorsque l'on ne dissipe pas rapidement l'énergie produite par effet Joule, on sait que les résultats obtenus (taille et position des taches de substance) dépendent d'un grand nombre de facteurs liés à l'importance de l'évaporation de la solution d'électrolyte contenue dans la couche mince[9,10]. La plaque de verre relativement épaisse joue ici le rôle de capacité calorifique et le résultat obtenu, toutes les autres conditions restant les mêmes, peut différer légèrement selon l'épaisseur de la plaque de verre.

L'étaleur utilisé est celui mis au point par STAHL[4] et manufacturé par Desaga (Roucaire, Paris).

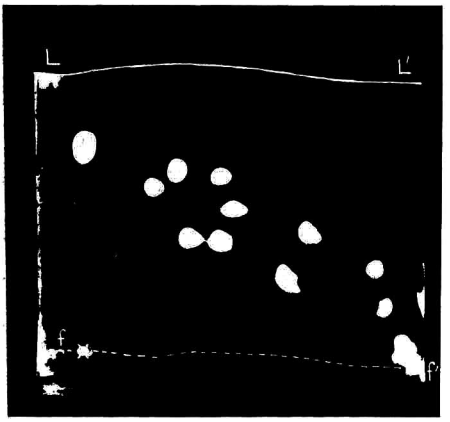
Cuves à chromatographie. Les couches minces de poudre de cellulose sans liant utilisées sont suffisamment adhérentes à la plaque de verre pour que tous les chromatogrammes puissent être développés en "couche mince plafond"[6] dans une cuve dérivant du principe de celle de BRENNER ET NIEDERWIESER[7] et selon le modèle que nous avons décrit[6].

Suivant les cas, les chromatogrammes sont développés soit en simple irrigation (voir schéma A de la Fig. 3 de réf. 6), soit en atmosphère conditionnée selon le procédé dit de double irrigation (voir le schéma C de la Fig. 3 de réf. 6). Dans ce dernier cas, le mélange à analyser étant déposé près de l'origine sur la "couche mince-plafond", la cuve est montée[6] puis on commence l'irrigation, par le solvant devant créer l'atmosphère de la cuve; lorsque le front du 1-er solvant a atteint l'extrémité de la plaque (languette l_3 de la Fig. 3C de réf. 6) le développement du chromatogramme proprement dit (couche mince déposée sur la face inférieure de la plaque supérieure de la cuve) est commencé. Dans les conditions opératoires donnant de bons résultats, le développement par le solvant "toluène" modifié (voir page 352) était réalisé en double irrigation, la couche mince portée par la face supérieure de la plaque de verre inférieure étant préalablement irriguée par de l'ammoniaque 0.8 *N*. Les développements par le solvant isobutanol–acide acétique glacial–eau (100:4:20) étaient réalisés en simple irrigation avec écoulement continu du solvant (remplacement de la baguette de fermeture de la cuve (b de la Fig. 3A de réf. 6) par un accordéon de papier (dispositif A de la Fig. 4 de réf. 6)).

Cellule d'électrophorèse. Les électrophorèses sur couche mince sont réalisées à l'aide d'une cellule (A de la Fig. 2 de réf. 6, pour plaques 200 × 200 × 4 mm) déjà décrite. Les conditions opératoires choisies permettent d'utiliser les avantages de l'électrorhéophorèse[9,10]: taches petites et bien délimitées (voir Fig. 7) restant dans le champ d'expérience malgré l'emploi d'un gradient de potentiel non négligeable.

Enregistrement photographique des chromatogrammes

Les taches de DNP aminoacides sont mises en évidence sur les chromatogrammes grâce à leur capacité d'absorber un rayonnement filtré à 360–366 mμ (lampe Mazda MAV, 75 W à filtre de Wood) par une modification du procédé utilisé en chromatographie sur papier[1]: enregistrement photographique de l'absorption d'un rayonnement filtré (pour les conditions opératoires voir réfs. 3 et 8). Ici, un papier sensible ("Lumière" R 6/5 pour reproduction de documents, mince, lisse, simple face, ultra contraste) était glissé sous la plaque de verre portant la couche mince. Cette dernière étant en contact direct avec la surface sensible, l'ensemble était éclairé à travers la lame de verre. Après développement photographique de la surface sensible, on obtenait les enregistrements présentés dans les Figs. 1, 3, 5 et 7.



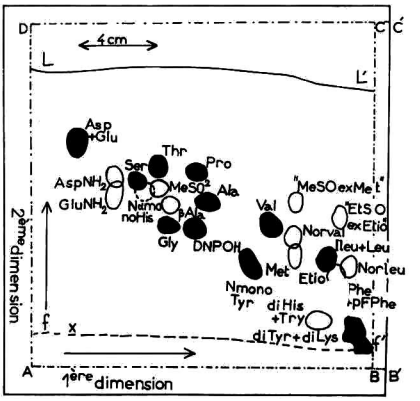


Fig. 1. Enregistrement photographique d'un chromatogramme à deux dimensions sur couche mince (250 μ) de poudre de cellulose des DNP-aminoacides naturels éthéro-solubles les plus fréquemment rencontrés; plaques 200 × 200 × 4 mm. X = position de dépôt du mélange des DNP-aminoacides; 3 à 7 mμmoles de chaque DNP-aminoacide; développement, en 1-ère dimension (1 h 15): phase organique décantée du mélange toluène–monochlorhydrine du glycol–pyridine–ammoniaque 0.8 N (150:90:45:90) et 10 à 12 gouttes (voir texte) d'alcool octylique primaire, après équilibration (1 h 15) de l'atmosphère de la cuve (voir texte) avec l'ammoniaque 0.8 N; en 2-ème dimension (1 h): eau saturée de $(NH_4)_2SO_4$–eau–dodécylsulfate de sodium (100 ml: 700 ml: 0.576 g); précautions à prendre pour obtenir cette parfaite définition des taches: voir le texte; "auto-cuve" montée en "double irrigation" pour le développement en 1-ère dimension (comme indiqué dans la Fig. 3 C de réf. 6), en "simple irrigation" pour le développement en 2-ème dimension (comme indiqué dans la Fig. 3 A de réf. 6); pour la désignation des substances voir la Fig. 2.

Fig. 2. Schéma montrant les positions des DNP-aminoacides éthéro-solubles après chromatographie à deux dimensions sur couche mince (250 μ) de poudre de cellulose; plaques 200 × 200 × 4 mm. Front du solvant, après développement en 1-ère dimension, confondu avec le bord droit de la plaque; après développement en 2-ème dimension: front du solvant en LL'———; les taches noires, correspondant aux DNP-aminoacides naturels les plus fréquemment rencontrés, sont celles des DNP-aminoacides présents sur le chromatogramme dont l'enregistrement photographique est donné dans la Fig. 1; les taches marquées par un simple trait indiquent les positions d'autres DNP-aminoacides; ——— = limites (20 × 20 cm) de la plaque de verre; —·—·—· = limites de la couche mince de poudre de cellulose; la partie BB'C'C de la couche mince est éliminée avant développement en 2-ème dimension; les autres conditions de chromatographie sont indiquées dans la légende de la Fig. 1.

Précautions à prendre lors des chromatographies et électrophorèses

Pendant tout le temps des chromatographies et électrophorèses les taches des DNP-aminoacides sont protégées de l'action destructrice de la lumière. Ceci est facilement réalisé en emballant les cuves et les chromatogrammes dans une mince feuille d'aluminium.

RÉSULTATS ET DISCUSSIONS

Cas des DNP-aminoacides éthéro-solubles

Lorsque nous avons tenté d'employer le système solvant [1-ère dimension: chromatographie de partage avec le solvant "toluène"[1]; 2-ème dimension: chromatographie de relargage avec une solution aqueuse de phosphate[2]] et les conditions opératoires qui donnent d'excellents résultats en chromatographie sur papier[1,2,12], pour la chromatographie en couche mince de poudre de cellulose, nous n'avons obtenu qu'un médiocre résultat: les 15 DNP-aminoacides éthéro-solubles appa-

raissent sous forme de 7 taches très diffuses et très allongées. C'est alors que nous avons entrepris une étude systématique de l'influence des conditions de chromatographie sur la netteté et la taille des taches de DNP-aminoacide et sur le degré de séparation obtenu.

En chromatographie sur papier des DNP-aminoacides, nous avions observé[3] que les taches de substances étaient rondes et extrêmement petites, avec des bords très nets, si, avant développement par le solvant "toluène", la feuille de papier portant la tache du mélange des DNP-aminoacides et l'atmosphère de la cuve à chromatographie, étaient équilibrées avec une grande quantité d'ammoniaque 0.8 *N*. Les taches étaient notablement diffuses si l'équilibre avait été obtenu avec la phase aqueuse décantée de la préparation du solvant "toluène" (ou avec le solvant lui-même) ou si l'ammoniac avait été éliminé de l'atmosphère de la cuve juste au moment du démarrage du développement du chromatogramme avec le solvant "toluène". En un mot, le contact des taches de DNP-aminoacides, avec une atmosphère très riche en ammoniac, *avant* et *pendant le développement* du chromatogramme par le solvant "toluène" est essentiel*. Nous avons retrouvé les mêmes résultats en chromatographie en couche mince de poudre de cellulose. Dans ce cas, pour pouvoir réaliser ce pré-équilibre de la tache du mélange des DNP-aminoacides et de l'atmosphère de la cuve à chromatographie avec l'ammoniac, nous avons imaginé une modification[6] de la cuve de BRENNER ET NIEDERWIESER[7]. Cette modification[6] permet, en particulier, de réaliser l'irrigation séparée de deux couches minces se faisant face dans une même cuve. La plaque de verre portant la couche mince supérieure [ayant reçu, près de l'origine, la tache du mélange à analyser] et la plaque de verre portant l'autre couche mince sont assemblées (voir cliché C de la Fig. 3 de réf. 6), puis on procède à l'irrigation de cette dernière par de l'ammoniaque 0.8 *N*. Après 1 à 2 h (plaques 200 × 200 mm) d'équilibre**, le développement de la couche mince supérieure par le solvant "toluène" est commencé.

Comme en chromatographie sur papier, si l'on veut maintenir la parfaite définition des taches de substance après chromatographie dans le solvant "toluène" en atmosphère ammoniacale, il faut réaliser un séchage *rapide* du chromatogramme (violent courant d'air au voisinage de l'ouverture d'une hotte presque totalement fermée). Dans le cas d'une couche mince de poudre de cellulose, au cours d'un séchage à l'étuve (80°) ou d'un séchage à température ambiante en l'absence de courant d'air violent, les taches de DNP-aminoacides deviennent rapidement très diffuses et le chromatogramme est inutilisable pour une chromatographie en deuxième dimension.

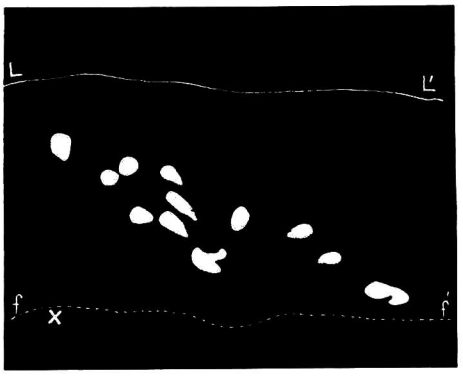
Ayant mis au point de bonnes conditions opératoires pour la chromatographie en couche mince de poudre de cellulose dans le solvant "toluène", nous avons essayé de développer les chromatogrammes en deuxième dimension avec le tampon phosphate (1.5 *M*, pH 6.0) habituel; la progression du solvant était en général curieuse-

* Le degré hygrométrique de l'atmosphère de la cuve joue aussi un grand rôle sur la définition des taches; comme exemple, indiquons que les taches de DNP-aminoacides sont très diffuses lorsque l'atmosphère de la cuve a été créée par une solution d'ammoniac dans le glycol.

** *Le temps d'irrigation* de la couche mince inférieure par de l'ammoniaque 0.8 *N*, avant le développement de la couche supérieure par le solvant "toluène", a une *grosse influence sur les séparations*; des résultats satisfaisants sont obtenus pour un temps d'irrigation de 45 min à 2 h; les meilleurs résultats (taches très nettes, absence de déformation dans certaines zones du chromatogramme) sont obtenus pour 1 h d'irrigation; au delà de 2 h (temps exigé pour un déplacement de 18 cm du front de l'ammoniaque dans la couche mince) d'irrigation, les taches comprises entre la N-mono-DNP-tyrosine et le front du solvant "toluène" sont très déformées.

ment irrégulière; on pouvait voir sur le chromatogramme 11 taches diffuses plus ou moins allongées; les séparations obtenues étaient, d'un essai à l'autre, totalement irreproductibles; nous avons alors supposé que ces irrégularités de progression du tampon étaient dues à une retention excessive des constituants du solvant de 1-ère dimension et que ceux-ci avaient partiellement lipophilisé les fibres de cellulose de la couche mince. Nous avons alors imaginé d'ajouter au tampon phosphate (0.75 *M*, pH 6.0) une petite quantité (1 %) d'agent tensio-actif (Tween 20: polyoxyéthylène sorbitane monolaurate); ceci nous a permis d'obtenir des taches rondes et petites pour les DNP-aminoacides de faible mobilité dans le solvant "toluène" (c'est à dire jusqu'à la tache du mélange DNP-alanine + DNP-OH + DNP-proline); les taches des DNP-aminoacides plus mobiles (dans le solvant "toluène") étaient déformées, comme entraînées par un second front liquide lors de la chromatographie en 2-ème dimension; l'augmentation (jusqu'à 4 %) ou la diminution (jusqu'à 0.5 %) de la concentration en Tween 20 dans le tampon phosphate n'améliorait pas les résultats obtenus d'une manière intéressante; il en était de même lorsqu'on substituait le Tween 80 (polyoxyéthylène sorbitane monooléate) au Tween 20.

Nous avons alors remarqué, sur le chromatogramme développé en deux dimensions, que la limite séparant le domaine où les taches étaient rondes et nettes et le domaine où elles étaient mêlées et déformées correspondait assez étroitement avec l'une (limite $\beta\beta'$, voir sa position par exemple, dans le schéma de la Fig. 4) des limites



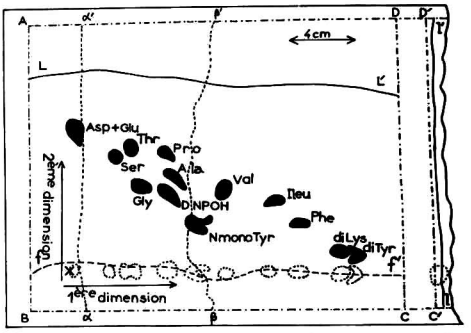


Fig. 3. Enregistrement photographique d'un chromatogramme à deux dimensions sur couche mince (250 μ) de poudre de cellulose des DNP-aminoacides naturels éthéro-solubles les plus fréquemment rencontrés; plaque 200 × 400 × 4 mm. X = position de dépôt du mélange des DNP-aminoacides; 3 à 7 mμmoles de chaque DNP-aminoacide; développement en 1-ère et 2-ème direction, respectivement, selon la plus grande et la plus petite dimension de la plaque; les conditions de chromatographie sont les mêmes que celles données dans les Figs. 1 et 2, excepté que le front du solvant de 1-ère dimension s'est déplacé de 25 cm, que le temps d'équilibre du chromatogramme avec l'ammoniaque 0.8 *N*, et le temps de développement en 1-ère dimension, sont, ici, respectivement de 1 h 30 et 1 h 30; LL' = front du solvant après développement en 2-ème dimension (1 h); pour la désignation des substances voir Fig. 4.

Fig. 4. Schéma montrant les positions des DNP-aminoacides éthéro-solubles après chromatographie à deux dimensions sur couche mince (250 μ) de poudre de cellulose; plaques 200 × 400 × 4 mm. Conditions de chromatographie données dans la légende de la Fig. 3; ll' = position atteinte par le front du solvant de développement en 1-ère dimension; DD'C'C = portion de la couche mince éliminée avant le développement en 2-ème dimension; LL' = position atteinte par le front du solvant de développement en 2-ème direction; les taches délimitées par un pointillé et les taches noires représentent les positions des DNP-aminoacides après développement du chromatogramme respectivement, en 1-ère et 2-ème dimension.

des trois zones d'opalescence différente présentes en première dimension sur le chromatogramme développé par le solvant "toluène"; ces trois zones étaient respectivement comprises entre l'origine du chromatogramme et la limite $\alpha\alpha'$, la limite $\alpha\alpha'$ et la limite $\beta\beta'$, la limite $\beta\beta'$ et le front ll' du solvant; ces limites sont données à titre indicatif dans le schéma de la Fig. 4. Ceci nous a fait penser qu'au moins deux de ces trois zones étaient dues à la "dissociation capillaire" des constituants du solvant "toluène" au cours de son cheminement chromatographique et qu'en particulier la zone la plus rapide avait considérablement "lipophilisé" les fibres de cellulose et que même en présence d'agents tensio-actifs (Tween 20, Tween 80), il n'était plus possible de réaliser une chromatographie de relargage dans cette partie du chromatogramme. Notons qu'indépendamment, nous avons été amené à cette même conclusion au cours d'études sur la cinétique de déformation des taches des DNP-aminoacides de grande mobilité dans le solvant "toluène" sur longues feuilles (75 cm) de papier. Pour essayer de réduire cette "dissociation capillaire" des constituants du solvant "toluène" nous avons ajouté à ce dernier un agent tensio-actif: l'alcool octylique primaire* (10 à 12 gouttes — voir techniques expérimentales — pour le solvant correspondant à 150 ml de toluène). Dans ces conditions opératoires, il a été possible de réaliser en 2-ème dimension, une chromatographie de relargage (tampon phosphate 0.75 *M*, pH 6.0 à 0.5 % de Tween 20) donnant des taches nettes pour l'ensemble des DNP-aminoacides; cependant la reproductibilité, d'un chromatogramme à l'autre, de la netteté des taches n'était pas encore parfaite. Le remplacement du Tween 20 par le dodécyl sulfate de sodium** (concentration finale 0.0025 *M*) dans le tampon phosphate (0.75 *M*, pH 6.0) utilisé pour le développement en 2-ème dimension allait permettre d'obtenir des chromatogrammes extrêmement reproductibles, dont toutes les taches de DNP-aminoacides étaient rondes, petites et très nettes (voir Figs. 1 et 2)***. La substitution au mélange des phosphates (Na_2PO_4H, $NaPO_4H_2$; 0.75 *M* en PO_4^{3-}, pH 6.0) d'autres sels (Na_2SO_4, $(NH_4)_2SO_4$, $NaPO_4H_2$, NaCl) de concentration convenable, soit seuls soit en mélanges, permettent également d'obtenir de bons résultats (les compositions de ces solutions (a) à (i) sont données dans la partie expérimentale) en présence de dodecylsulfate de sodium.

Conditions de chromatographie adoptées. Les conditions opératoires adoptées pour obtenir la séparation chromatographique en couche mince (250 μ) de poudre de cellulose sur plaque 200 × 200 mm, de l'ensemble des DNP aminoacides éthéro-solubles sous forme de taches nettes et rondes sont données dans la partie expérimentale (voir page 349) et dans les légendes des Figs. 1 et 2. La Fig. 1 correspond à l'enregistrement photographique du chromatogramme obtenu pour les DNP-aminoacides les plus couramment rencontrés. On remarquera la grande définition des taches obtenues. Le schéma de la Fig. 2 montre les positions de l'ensemble des DNP aminoacides éthéro-solubles; les taches noires, sur ce même schéma, correspondent aux DNP-aminoacides du chromatogramme de la Fig. 1. Comme en chromatographie sur papier, les acides DNP-glutamique et DNP-aspartique restent mêlés;

* Ou l'alcool octylique normal secondaire, l'alcool laurique.

** Ou le Brij 35 (polyoxyéthylène lauryl ether): 0.75 g/l.

*** On doit noter qu'une prolongation excessive (voir note** pag e352) du temps d'irrigation de la couche mince inférieure par l'ammoniaque 0.8 *N*, avant le développement (par le solvant "toluène") du chromatogramme proprement dit, provoque une intensification des fronts intermédiaires (et spécialement du front $\beta\beta'$ (voir Fig. 4) qui peut occasionner une déformation des taches se trouvant à son niveau; voir un exemple dans la Fig. 4).

il en est de même pour la DNP-glutamine et la DNP-asparagine, pour la di-DNP-histidine et le DNP-tryptophane. Contrairement aux résultats obtenus en chromatographie sur papier, en chromatographie sur couche mince, les taches de DNP-thréonine, DNP-sérine, DNP-méthionine sulfone sont très bien séparées les unes des autres; ceci est très important, puisqu'on sait que ce dérivé de la méthionine est la forme stable de cet aminoacide au cours de l'hydrolyse chlorhydrique des protéines[11]. Dans le schéma de la Fig. 2, la position d'un DNP-aminoacide hydro-acido-soluble l'α-mono-DNP-histidine est indiquée; en effet, dans certaines conditions d'extraction des DNP-aminoacides éthéro-solubles, un peu de di-DNP-histidine peut être entraînée dans ce groupe; ce DNP-aminoacide peu stable a tendance à donner de l'α-mono-DNP-histidine; il était donc intéressant d'indiquer la position de ce dernier dans les chromatogrammes à deux dimensions, d'autant plus qu'elle est très voisine de celle de la DNP-sérine.

On remarquera sur les Figs. 1 et 2 que la tache de di-DNP-tyrosine et celle de la di-DNP-lysine sont, quelquefois, confondues lorsque le chromatogramme est réalisé sur couche mince de 200 × 200 mm. Ceci tient au fait que ces deux DNP-aminoacides se trouvent près du front du solvant c'est à dire en un point où les impuretés les plus liposolubles contenues dans la couche mince s'accumulent et réduisent le pouvoir séparateur de la chromatographie. Ceci nous engage à conseiller de réaliser la chromatographie sur plaque 200 × 400 mm. Dans ce cas les taches des deux di-DNP-aminoacides précédents ne sont plus confondues avec le front du solvant "toluène" [qui se déplace d'environ 25 cm (ll' Fig. 4)]; les conditions opératoires sont indiquées dans la partie expérimentale page 349, et dans les légendes des Figs. 3 et 4. Le solvant "toluène" additionné d'alcool octylique, permet de développer le chromatogramme en 1-ère direction en atmosphère ammoniacale, dans la plus grande dimension (400 mm) de la plaque tandis que la chromatographie de relargage dans une solution aqueuse de sulfate d'ammonium contenant du dodécyl sulfate de sodium est réalisée parallèlement à la plus petite dimension de la plaque. Dans ces conditions, on obtient, aussi, une bonne séparation des taches de la di-DNP-tyrosine et de la di-DNP-lysine*. La séparation entre ces deux taches, après chromatographie en 1-ère dimension est déjà excellente mais elle est due à une chromatographie de partage avec déplacement (la tache de di-DNP-lysine "chasse" la tache de di-DNP-tyrosine). On peut conseiller de récupérer la partie de la couche mince portant ces deux taches avant de développer le chromatogramme par chromatographie de relargage en 2-ème dimension.

Dans les conditions de chromatographie définitivement adoptées (Figs. 1, 2, 3 et 4) on voit que l'on a préféré une solution de sulfate d'ammonium au tampon phosphate habituel pour développer les chromatogrammes en 2-ème direction. En effet, les taches des DNP-aminoacides de grande mobilité (dans le solvant "toluène") sont plus parfaites après chromatographie de relargage dans la première solution saline que dans la seconde.

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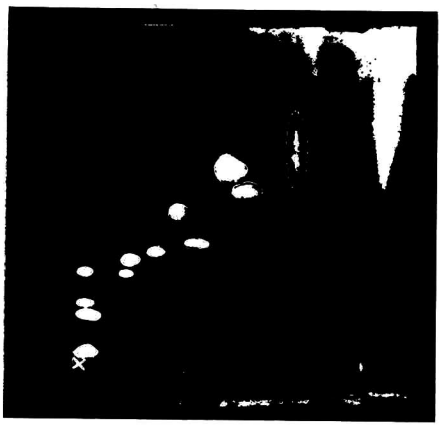
* Au cours de la chromatographie dans la solution de sulfate d'ammonium contenant du dodécylsulfate de sodium (0.0025 *M*), on voit un front lent (marqué ff' dans la Fig. 4) qui a tendance à mêler les taches de di-DNP-Tyr et de di-DNP-Lys, lorsqu'il atteint leur niveau; ce phénomène perturbateur est évité en utilisant une concentration plus faible (0.001 *M*) de dodécylsulfate ou en utilisant un détergent neutre (Brij 35).

Cas des DNP-aminoacides éthéro-solubles de faible mobilité dans le solvant "toluène"

Pour obtenir la séparation sur couche mince des DNP-aminoacides des mélanges non résolus (acide DNP aspartique + acide DNP glutamique + DNP-S-carboxyméthyl-cystéine, DNP-glutamine + DNP-asparagine) après chromatographie dans le système "toluène"–"sulfate d'ammonium", nous avons essayé de transposer le procédé que nous avons décrit[3] pour la chromatographie sur papier. Après développement en 1-ère dimension, en atmosphère ammoniacale, dans le solvant "toluène" additionné d'alcool octylique primaire (mêmes conditions opératoires que celles décrites dans le paragraphe précédent), la couche mince est imprégnée d'acétate de sodium (voir partie expérimentale et les légendes des Figs. 5 et 6) puis le développement, en 2-ème dimension, est réalisé avec un mélange d'alcool et d'acide juste saturé d'eau [isobutanol–acide acétique–eau (100:4:20)].

Sur les clichés des Figs. 5 et 6 on peut voir que la séparation sur couche mince des taches de la S-carboxyméthyl-cystéine, de son produit d'oxydation à l'air, des acides DNP aspartique et DNP glutamique, de la DNP-glutamine et de la DNP-asparagine, de la DNP-sérine, de la DNP-thréonine, du DNP-glycocolle, est aussi parfaite qu'en chromatographie sur papier[3]; les séparations sont extrêmement nettes et les taches de DNP-aminoacides sont très petites. Pour obtenir ces résultats en



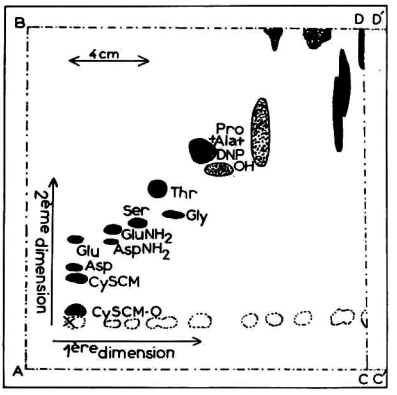


Fig. 5. Enregistrement photographique d'un chromatogramme à deux dimensions sur couche mince (250 μ) de poudre de cellulose des DNP-aminoacides éthéro-solubles de faible mobilité dans le solvant "toluène"; plaque 200 × 200 × 4 mm. X = position de dépôt du mélange des DNP-aminoacides; 3 à 7 mμmoles de chaque DNP-aminoacide; développement en 1-ère dimension (2 h 30) avec le solvant "toluène" dans les conditions de chromatographie identiques à celles utilisées pour les chromatogrammes des Figs. 1 et 2; développement (6 h) en 2-ème dimension par le solvant isobutanol–acide acétique glacial–eau (100:6:20) avec écoulement continu (dispositif A de la Fig. 4 de réf. 6), après imprégnation de la couche mince avec de l'acétate de sodium (pulvérisation, puis séchage rapide à 90°, d'une solution 0.2 *M* dans le mélange alcool–eau (6:4)); pour la désignation des substances voir Fig. 6.

Fig. 6. Schéma montrant les positions des DNP-aminoacides éthéro-solubles de faible mobilité dans le solvant "toluène" (DNP-S-carboxyméthyl-cystéine, acide DNP aspartique, acide DNP glutamique, DNP-asparagine, DNP-glutamine, DNP-sérine, DNP-glycocolle, DNP-thréonine) après chromatographie à deux dimensions. Pour les solvants utilisés et les conditions de chromatographie: voir légende de la Fig. 5; les taches délimitées par un pointillé et les taches noires représentent les positions des DNP-aminoacides après développement du chromatogramme, respectivement, en 1-ère et 2-ème dimension; la partie DD'C'C de la couche mince est éliminée avant le développement en 2-ème dimension.

chromatographie en couche mince de poudre de cellulose, deux précautions doivent être prises: imprégnation de la couche mince par une solution *hydro-alcoolique* d'acétate de sodium afin d'éviter le décollement de la couche mince, séchage *rapide* (dans une étuve à 90°) de la couche humide pour que les taches des DNP-aminoacides restent petites. L'utilisateur du procédé ne s'étonnera pas d'observer que, quelquefois, certaines taches de DNP-aminoacides deviennent allongées dans les premiers moments du développement chromatographique en 2-ème dimension; après un certain temps, la substance contenue dans la queue de la tache est ramenée par le flux de solvant dans la masse de la tache et ainsi cette dernière devient parfaitement nette.

Cas des DNP-aminoacides hydro-acido-solubles

Il était essentiel de mettre au point un procédé pour séparer les DNP-aminoacides restant dans la phase aqueuse acide (HCl) après extraction des DNP aminoacides éthéro-solubles.

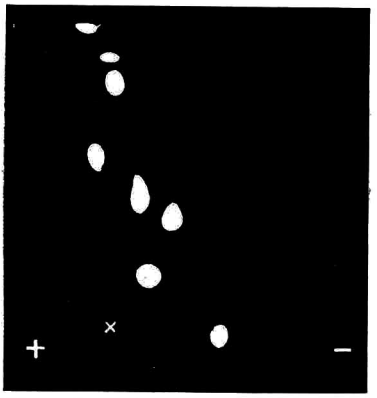
On sait qu'après chromatographie dans les solvants alcalins[1,12,15*] les taches d'α-mono-DNP-arginine et d'ε-mono-DNP-lysine sont confondues et que seule une électrophorèse en milieu fortement alcalin (ammoniaque[12], diéthylamine[3], triéthylamine[3]) permet d'obtenir la séparation de ces deux DNP-aminoacides. Le couplage d'une chromatographie dans le solvant "toluène" et d'une électrophorèse dans la diéthylamine 0.025 *M* nous a permis d'obtenir un excellent procédé de séparation sur feuille de papier de l'ensemble des DNP-aminoacides hydro-acido-solubles (acide DNP cystéique, di-DNP-histidine, α-mono-DNP-histidine, ε-mono-DNP-lysine, α-mono-DNP-arginine)[3].

Ayant pu mettre au point des conditions opératoires qui permettent d'obtenir la séparation des DNP-aminoacides éthéro-solubles sur couche mince de poudre de cellulose et qui peuvent également s'appliquer à la séparation des DNP-aminoacides hydro-acido-solubles dans le solvant "toluène" additionné d'alcool octylique primaire, nous avons essayé d'obtenir la séparation complète par chromato-électrophorèse de ces derniers DNP-aminoacides.

Les clichés de la Figs. 7 et 8 montrent que les résultats obtenus sont aussi nets en chromatographie en couche mince de poudre de cellulose qu'en chromatographie sur papier. Pour obtenir des taches petites sur couche mince (250 μ) de poudre de cellulose déposée sur plaque 200 × 200 × 4 mm, il faut ajouter à la diéthylamine (ici 0.033 *M*) une concentration convenable d'un sel neutre (0.02 *M* NaCl par exemple) afin de rendre l'électrolyte suffisamment conducteur; ainsi, les taches sont plus nettes qu'en électrophorèse sans évaporation. Les séparations ont été réalisées dans l'appareil extrêmement simple (A de la Fig. 2 de réf. 6), déjà décrit, pour l'électrophorèse en couche mince de poudre de cellulose. Les conditions opératoires permettant d'ob - tenir ce résultat, sont indiquées dans les légendes des Figs. 7 et 8.

Si la di-DNP-histidine est présente dans le mélange à analyser, il faut prendre des précautions pour éliminer le solvant "toluène" ayant servi au développement en 1-ère dimension. En effet ce DNP-aminoacide est assez sensible à l'action de la lumière et des milieux alcalins. Si le chromatogramme était séché à 80°, une tache d'α-mono-DNP-histidine, dont le pourtour est indiqué par un trait interrompu (h) sur le schéma de la Fig. 8, apparaîtrait après l'électrophorèse en 2-ème dimension. La position (p)

* Solvant "toluène"[1,12], *n*-propanol–ammoniaque à 34 % (7:3)[15], *n*-butanol–ammoniaque à 34 % (4:1)[15].



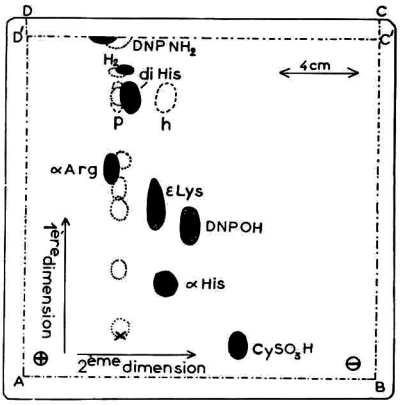


Fig. 7. Enregistrement photographique d'un chromato-électrogramme à deux dimensions sur couche mince (250 μ) de poudre de cellulose des DNP-aminoacides naturels hydro-acido-solubles: plaques 200 × 200 × 4 mm. X = position de dépôt des substances à 6 cm du futur côté — pôle négatif; 3 à 7 mμmoles de chaque DNP-aminoacide; développement chromatographique en première dimension (1 h 45 à 2 h 15) avec le solvant "toluène" en atmosphère ammoniacale (autocuve montée en "double irrigation" comme indiqué dans la Fig. 3 C de réf. 6) dans les conditions opératoires utilisées pour le développement en 1-ère dimension du chromatogramme de la Fig. 1; électrophorèse, en 2-ème dimension (9.5 mA, 385 V, 30 min, cuve d'électrophorèse A de la Fig. 2 de réf. 6; générateur de tension continue décrit dans la réf. 6) après pulvérisation d'une solution aqueuse de diéthylamine 0.033 *M* et de NaCl 0.02 *M*; précautions à prendre pour le séchage, après le développement en 1-ère dimension: voir le texte; pour la désignation des substances: voir Fig. 8.

Fig. 8. Schéma montrant les positions des DNP-aminoacides hydroacido-solubles (acide DNP cystéique, α-mono-DNP-histidine, ε-mono-DNP-lysine, α-mono-DNP-arginine, di-DNP-histidine) après chromato-électrophorèse sur couche mince (250 μ) de poudre de cellulose; plaque 200 × 200 × 4 mm; 1-ère dimension: solvant "toluène" en atmosphère ammoniacale; 2-ème dimension: électrophorèse dans la diéthylamine; pour le solvant, la solution d'électrolyte et les conditions de chromatographie et d'électrophorèse utilisés, voir la légende de la Fig. 7; front du solvant, en fin de développement du chromatogramme en première dimension, confondu avec le bord (DC) de la couche mince; X = position de dépôt des substances; DD'C'C = portion de la couche mince éliminée avant l'électrophorèse en 2-ème dimension; les taches délimitées par un pointillé et les taches noires représentent les positions des DNP-aminoacides après développement du chromatogramme, respectivement, en 1-ère et 2-ème dimension; les taches délimitées par un trait interrompu (– – –) correspondent aux positions occupées par divers artefacts possibles (voir le texte) après développement du chromatogramme en 2-ème dimension: p = tache du produit de décomposition photochimique de la di-DNP-histidine (voir le texte), h = tache d'α-mono-DNP-histidine produite au cours d'un chauffage éventuel de la tache de di-DNP-histidine lors de l'élimination du solvant de chromatographie utilisé en 1-ère dimension.

du produit de décomposition photochimique de la di-DNP-histidine est également indiquée sur ce schéma (voir légende de la Fig. 8). La décomposition de la di-DNP-histidine en α-mono-DNP-histidine au cours de l'élimination du solvant "toluène" est fortement réduite si cette élimination a lieu à la température ambiante à l'aide d'un violent courant d'air (même méthode que pour l'élimination du solvant "toluène" dans le cas des DNP-aminoacides éthéro-solubles).

DISCUSSION GÉNÉRALE ET CONCLUSION

Ainsi, nous venons de mettre au point des conditions opératoires qui permettent de séparer par chromatographie à deux dimensions et par chromato-électrophorèse en couche mince de poudre de cellulose tous les DNP-dérivés correspondants aux

aminoacides présents dans les protéines natives ou modifiées par voie chimique. Les diagrammes des positions des taches de DNP-aminoacides que nous avons obtenus (Figs. 1 à 8) sont identiques à ceux que l'on a en chromatographie sur papier[2,3,12]. Il devient donc, maintenant, extrêmement aisé de comparer les résultats obtenus par la chromatographie sur papier et par la chromatographie en couche mince. Le procédé décrit bénéficie de tous les avantages de la chromatographie en couche mince: exigence d'une faible quantité de produit (3 à 7 mμmoles, au lieu de 10 à 200 de chaque DNP-aminoacide en chromatographie sur papier), rapidité d'obtention des résultats, possibilité de réaliser simultanément un grand nombre de chromatogrammes dans un faible espace de travail. Ainsi, par exemple, on peut rapidement déterminer, grâce à plusieurs chromatographies en couche mince réalisées simultanément, la nature d'une tache de DNP-aminoacide repérée sur un chromatogramme sur papier, en ne mettant en oeuvre qu'une petite portion de l'éluat de la tache. Associé à l'emploi de témoins internes notre procédé de chromatographie en couche mince peut être très utile pour l'identification des aminoacides présents dans un hydrolysat chimique ou enzymatique d'oligopeptides naturels ou modifiés par voie chimique.

La seule méthode de chromatographie en couche mince mise au point pour les DNP-aminoacides, avant celle que nous présentons aujourd'hui, se limitait à l'analyse des DNP-aminoacides éthéro-solubles. Cette méthode employait des couches minces de gel de silice partiellement désactivé[5,15,16] et ses auteurs, après avoir utilisé le solvant "toluène" de BISERTE ET OSTEUX[1] pour développer la 1-ère dimension de leur chromatogramme* remarquaient[15] qu'il leur était impossible d'obtenir une séparation satisfaisante des DNP-aminoacides par chromatographie de relargage (tampon phosphate) en seconde dimension.

Comme nous venons de le montrer, nous avons été en mesure de réaliser la chromatographie de relargage après une chromatographie dans le solvant "toluène". Ce résultat a été obtenu car nous avons pu mettre en évidence les causes des incidents propres à la chromatographie en couche mince; incidents qui tiennent surtout, d'une part, à la rétention excessive des constituants du mélange solvant utilisé dans le développement des chromatogrammes, d'autre part, à un phénomène que nous avons désigné sous le nom de "dissociation capillaire" des constituants du mélange solvant. Ces incidents sont principalement dûs à une lipophilisation des particules formant la couche mince par certains constituants du solvant mobile utilisé en 1-ère dimension. Dans une association chromatographie de partage direct, en 1-ère dimension, chromatographie de relargage en 2-ème dimension, nous avons pu montrer que tous les incidents de la chromatographie sont considérablement réduits si l'on utilise des concentrations convenables** d'agents tensio-actifs appropriés dans les solvants employés en 1-ère et 2-ème dimension.

Pour réaliser des chromatographies à deux dimensions des DNP-aminoacides éthéro-solubles sur couche mince de gel de silice, après chromatographie dans le solvant "toluène" selon leur mode opératoire, BRENNER, NIEDERWIESER ET PATAKI ont dû s'adresser à la chromatographie d'adsorption[5] pour pouvoir développer le

* Sans employer un développement en atmosphère fortement ammoniacale mais tout au plus en prééquilibrant la plaque de gel de silice, pour la désactiver, avec les vapeurs de la phase aqueuse décantée du solvant "toluène"[5].

** Un excès d'agent tensio-actif empêche, dans certains cas, toute séparation chromatographique.

chromatogramme en seconde dimension. Ces auteurs obtiennent des séparations assez satisfaisantes des DNP-aminoacides éthéro-solubles quoiqu'il semble que la définition des taches soit moins bonne* que celle obtenue par le procédé que nous venons de mettre au point. Peut-être serait-il intéressant de reprendre l'étude de la chromatographie sur couche mince de gel de silice désactivée, en tenant compte des observations que nous avons faites en chromatographie sur couche mince de poudre de cellulose. Cependant, nous pensons que l'emploi de couches minces de poudre de cellulose est préférable à celui de couches de gel de silice car les premières n'exigent pas de liant et sont mécaniquement plus résistantes que les secondes et donc plus faciles à manipuler.

Quoiqu'il en soit, les diagrammes donnant les positions des taches des DNP-aminoacides après chromatographie selon le procedé de BRENNER, NIEDERWIESER ET PATAKI[5] ne peuvent pas être comparés avec le diagramme obtenu en chromatographie sur papier avec le système "toluène"–"phosphate"[2,12].

Dans nos conditions opératoires, quoique nous ayons utilisé un système "toluène"–"sulfate d'ammonium" sur couche mince de poudre de cellulose, le diagramme des positions des taches des DNP-aminoacides éthéro-solubles est identique à celui obtenu en chromatographie sur papier avec le système "toluène"–"phosphate".

Enfin, on doit aussi faire remarquer, que dans notre procédé, l'extrême définition des taches de DNP-aminoacides après chromatographie tient au fait que nous avons pu montrer que les DNP-aminoacides doivent être maintenus sous forme de sels d'ammonium au cours de la chromatographie dans le solvant "toluène"; ceci exige une très forte concentration en ammoniac dans l'atmosphère des cuves. Nous avions déjà pressenti cette exigence[3] en chromatographie sur papier mais, dans ce cas, elle n'était pas absolue. Il n'en pas de même en chromatographie en couche mince de poudre de cellulose où une trop faible concentration en ammoniac dans l'atmosphère des cuves donne naissance à des taches diffuses de DNP-aminoacides.

RÉSUMÉ

Des méthodes efficaces de séparation chromatographique des DNP-aminoacides sur couche mince (250 μ) de poudre de cellulose sans liant sont décrites. Dans les conditions opératoires utilisées, il est possible de séparer, sous forme de taches extrêmement bien définies tous les DNP-aminoacides du groupe des éthéro-solubles et des hydro-acido-solubles. Sur plaque 20 $\times$ 20 (ou 30) cm, la quantité optimum de chaque DNP-aminoacide pouvant être mise en oeuvre est de 3 à 7 mμmoles.

SUMMARY

Some efficient methods of chromatographic separation of DNP-amino acids on thin layers (250 μ) of powdered cellulose without binder are described. Under the operating conditions used, it is possible to separate all the DNP-amino acids of the ether-soluble and hydracid-soluble groups in the form of well defined spots. The optimum quantity of each DNP-amino acid which can be applied to a plate 20 $\times$ 20 (or 30) cm is 3–7 mμmoles.

* Voir les photographies des chromatogrammes de BRENNER et coll.[16]. (Note by editor: This comparison is not quite just, as the latter are urine samples and not synthetic mixtures.)

BIBLIOGRAPHIE

1 G. BISERTE ET R. OSTEUX, *Bull. Soc. Chim. Biol.*, 33 (1951) 50.
2 A. L. LEVY, *Nature*, 174 (1954) 126.
3 R. L. MUNIER ET G. SARRAZIN, *Bull. Soc. Chim. France*, (1963) 2939.
4 E. STAHL, *Thin layer chromatography*, Academic Press, New York, 1965, p. 6.
5 M. BRENNER, A. NIEDERWIESER ET G. PATAKI, *Experientia*, 17 (1961) 145.
6 R. L. MUNIER ET G. SARRAZIN, *J. Chromatog.*, 22 (1966) 336.
7 M. BRENNER ET A. NIEDERWIESER, *Experientia*, 17 (1961) 237.
8 R. MUNIER, dans L. LOISELEUR (Rédacteur), *Techniques de Laboratoire: Chimie Biologique, Chimie Physique*, Tome I, Fasc. 1, Masson, Paris, 1963, p. 636.
9 M. MACHEBOEUF, P. REBEYROTTE ET J. M. DUBERT, *Bull. Soc. Chim. Biol.*, 35 (1953) 334 et 346; *Chem. Weekblad*, 49 (1953) 237.
10 R. L. MUNIER, *Chim. Anal. (Paris)*, 36 (1954) 253, 283 et 340.
11 N. P. NEUMANN, S. MOORE ET W. H. STEIN, *Biochemistry*, 1 (1962) 68.
12 G. BISERTE, J. W. HOLLEMAN, J. HOLLEMAN-DEHOVE ET P. SAUTIÈRE, dans M. LEDERER (Rédacteur), *Chromatographic Reviews*, Vol. 2, Elsevier, Amsterdam, 1960, p. 59.
13 R. L. MUNIER, *Journées Internationales de Séparation Immédiate et de Chromatographie, Paris, 13, 14, 15 juin, 1961*, Gams, Paris, 1961, p. 189–192.
14 R. L. MUNIER ET G. SARRAZIN, *Compt. Rend.*, 256 (1963) 3532.
15 M. BRENNER, A. NIEDERWIESER ET G. PATAKI, dans E. STAHL (Rédacteur), *Thin Layer Chromatography*, Academic Press, New York, 1965, p. 418–419.
16 M. BRENNER, A. NIEDERWIESER ET G. PATAKI, dans A. T. JAMES ET J. L. MORRIS (Rédacteurs), *New Biochemical Separations*, Van Nostrand Co., New York, 1964, pp. 123–156.

POLYAMIDE LAYER CHROMATOGRAPHY OF DNP-AMINO ACIDS*

KUNG-TSUNG WANG, JOYCE M. K. HUANG AND IRIS S. Y. WANG
Department of Chemistry, National Taiwan University, Taipei, Taiwan (China)
(Received October 29th, 1965)

INTRODUCTION

Since SANGER's publication[2], fluorodinitrobenzene has been widely used as a reagent to determine N-terminal groups in peptides and proteins. Numerous techniques for the identification and estimation of dinitrophenylamino acids (DNP-amino acids) have been reviewed[3,4], including chromatography on paper, on columns of silica gel, kieselguhr, silicic acid–celite and polyamide. Recent advances have been made in partition chromatography on Hyflo Super-Cel columns[5,6], adsorption chromatography on a silica gel–celite column[7], chromatography on glass paper impregnated with silica gel[8], paper chromatography[9,10], paper electrophoresis[11] and gas chromatography of DNP-amino acid esters[12]. Thin-layer chromatographic separations of DNP-amino acids on silica gel G thin layers have been carried out by BRENNER[13–17] *et. al.* under strict conditions. In addition, their method needs a complicated B-N chamber for continuous development. The purpose of this paper is to describe a rapid chromatographic method for the analysis of DNP-amino acids on polyamide layers.

Polyamide is suitable for the chromatography of many organic compounds[18]; ENDRES[18], HILLE[19,20] and SCHWERDTFEGER[21] have had varied success in isolating DNP-amino acids on polyamide columns. Although the unique character of polyamide as a chromatographic adsorbent in columns is widely recognized, chromatography on polyamide thin layers is rare. In 1961 DAVIDEK AND DAVIDKOVA[22] prepared polyamide thin layers by spreading polyamide powder without a binder on glass plates and used it to separate antioxidants and flavonoids (see ref. 18 for review). Recent developments include the separation of quinones[23], plant tanning extractives[24] and antioxidants[25]. The chief difficulty seems to be in the preparation of durable layers.

However, WANG allowed a polyamide solution in formic acid to evaporate on a glass plate and obtained a fairly durable layer which was used to separate phenols[26]. Recent developments in the preparation of polyamide layers in our laboratory enable it to be used to separate sulfonamides[27], chloramphenicol[28] and estrogens[29]. Its application to the separation of DNP-amino acids showed many advantages over previous methods, *e.g.*, more rapid analysis, less broadening of the spots and easier handling.

We found ten solvents systems which were excellent for the separation of DNP-amino acids on polyamide layers. They can be classified into three groups

* Some of the preliminary experiments were submitted to *Nature* (see ref. 1).

according to the difference of R_F values. It is possible to combine these systems to get several combinations of solvent systems for two-dimensional chromatography.

Only seventeen DNP-amino acids are available to us at present. Further data will be reported when other DNP-amino acids have been obtained.

EXPERIMENTAL

Preparation of polyamide layer

Twenty grams of polyamide (CM 1007 s, Toyo Rayon Co.) was dissolved in 100 ml of 75 % formic acid to give a homogeneous viscous solution. Fifteen ml of this solution was spread evenly on each of four glass plates (15 × 15 cm) kept horizontal in the bottom of a stainless steel chromatographic cabinet (50 × 50 × 45 cm) which was saturated with water vapor. The plates were kept in the cabinet for two days to allow slow evaporation of formic acid at 26° or at 29°. The layers were then dried in an oven at 100° for 15 min to eliminate the last traces of formic acid. The dried plates can be stored for a long period without any noticeable change. The solvent travels faster on plates prepared thus rather than by the former method[26].

Both Toyo Rayon Co.'s ε-polycaprolactam resin CM 1011 and CM 1007 s have been tested. Resin CM 1011 gives a denser layer which gives nearly circular spots but requires a longer time for development which makes the spots more diffuse.

The evaporation temperature during the preparation of the polyamide layer has a profound effect on the properties of the resulting layer. A higher temperature makes the layer coarser and shortens the time for development, but some solvent systems give elongated spots on layers prepared at high temperature.

For one-dimensional chromatography, two kinds of layers were used; a CM 1007 s layer made at 26° and CM 1007 s layer made at 29°. All of the two-dimensional chromatograms were run on layers of CM 1007 s prepared at 26°.

Reagents

All of the 19 DNP derivatives available to us were kindly supplied by Prof. T.B. Lo.

Solvents

The solvents were purified by general methods to meet the chromatographic requirements.

Chromatography

The ascending method was used. For one-dimensional chromatography, DNP-amino acids (1 μg in 1 μl of methanol) were spotted 1.5 cm from the bottom edge and the solvent was allowed to ascend a distance of 10 cm from the origin. For two-dimensional chromatography, a mixture of the 19 samples (0.5 μg each in 10 μl of methanol) was spotted on one corner 2 cm from the edges. In all development processes, there was no need to saturate the polyamide layer in advance but the chamber should be lined with filter paper saturated in solvent. Because DNP-amino acids are photosensitive, the development was run in the dark. For two-dimensional chromatography, it was necessary to carry out intermediate drying under a current of hot air for 15 min.

Detection

DNP-amino acids are visible in transmitted daylight and give dark spots in transmitted ultraviolet light. Ultraviolet contact photography has a much higher sensitivity and is the most convenient way of locating the spots and filing the results. The sensitive side of photographic paper was laid on the polyamide layer and long-wave ultraviolet light was allowed to pass through the layer on to the photographic paper for 30 sec (one-dimensional chromatogram) or 18 sec (two-dimensional chromatogram). The print was developed in the usual manner to make a chromatogram of white spots on black background. This print was used as the negative to make a chromatogram of dark spots on white background.

RESULTS AND DISCUSSION

One-dimensional chromatography

A number of solvent systems suitable for the separation of DNP-amino acids on polyamide layer are summarized in Table I together with the time required for development. They are classified into three groups according to the differences of R_F values. Table II summarizes the R_F values of DNP-amino acids in the above solvent systems.

TABLE I

SOLVENT SYSTEMS FOR DNP-AMINO ACIDS ON POLYAMIDE LAYERS

Symbol	*Group*	*Components*	*v/v*	*Time required for development (min)*	
				CM 1007 s (29°)	*CM 1007 s (26°)*
I	A	Benzene–glacial acetic acid	80:20	30	60
II	A	Carbon tetrachloride–glacial acetic acid	80:20	40	90
III	A	*n*-Butyl acetate–glacial acetic acid	90:10	30	60
IV	A	Diethyl ether–glacial acetic acid	90:10	15*	60
V	A	Methyl ethyl ketone–chloroform–glacial acetic acid	10:80:10	30*	60
VI	B	Glacial acetic acid–water	50:50	60	200
VII	B	90% Formic acid–water	50:50	30	60
VIII	C	*n*-Butanol–glacial acetic acid	90:10	80	200
IX	C	Dimethylformamide–glacial acetic acid–95% ethanol–water	5:10:20:20	80	180
X	C	Dimethylformamide–sulfamic acid–95% ethanol–water	5:0.5:20:20 (ml:g:ml:ml)	80	150

* Elongated spots.

Solvent IX is similar to that used in polyamide column chromatography of DNP-amino acids[18,30,31]. We found the latter equally satisfactory for polyamide layer chromatography. The only drawback was the slow rate of ascent, so we changed the solvent ratio to shorten the development time.

Solvents I–IX contain 10–20% acetic acid or formic acid, otherwise excessive

TABLE II

R_F VALUES OF DNP-AMINO ACIDS ON POLYAMIDE LAYERS

No.	*DNP-derivatives*	*Abbreviation*	*Solvent system*									
			I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*
1	α-DNP-L-arginine	α-Arg	0.03	0.01	0.04	0.01	0.07	0.93	0.90	0.47	0.95	0.87
2	DNP-L-aspartic acid	Asp	0.07	0.03	0.11	0.10	0.05	0.50	0.55	0.12	0.36	0.48
3	DNP-DL-serine	Ser	0.09	0.03	0.21	0.17	0.10	0.56	0.61	0.20	0.44	0.54
4	ε-DNP-lysine hydrochloride	ε-Lys	0.11	0.03	0.13	0.02	0.22	0.94	0.90	0.62	0.96	0.89
5	DNP-L-glutamic acid	Glu	0.14	0.06	0.18	0.17	0.11	0.52	0.56	0.18	0.37	0.48
6	DNP-threonine	Thr	0.14	0.06	0.32	0.29	0.16	0.60	0.60	0.27	0.51	0.60
7	Bis-DNP-lysine	Bis-Lys	0.32	0.07	0.53	0.16	0.50	0.23	0.14	0.15	0.16	0.11
8	DNP-tryptophan	Try	0.28	0.09	0.47	0.39	0.31	0.24	0.12	0.23	0.30	0.26
9	DNP-glycine	Gly	0.27	0.10	0.39	0.34	0.27	0.47	0.48	0.21	0.35	0.36
10	Bis-DNP-DL-tyrosine	Bis-Tyr	0.43	0.11	0.58	0.28	0.50	0.24	0.12	0.16	0.20	0.13
11	DNP-alanine	Ala	0.44	0.22	0.55	0.63	0.46	0.52	0.49	0.33	0.42	0.48
12	2,4-Dinitroaniline	NH_2	0.48	0.23	0.75	0.91	0.74	0.59	0.49	0.64	0.65	0.49
13	DNP-L-proline	Pro	0.52	0.27	0.61	0.74	0.57	0.59	0.50	0.46	0.59	0.60
14	DNP-phenylalanine	Phe	0.52	0.31	0.56	0.70	0.54	0.37	0.23	0.33	0.38	0.39
15	DNP-DL-methionine	Met	0.52	0.31	0.55	0.64	0.54	0.47	0.41	0.32	0.39	0.46
16	DNP-DL-valine	Val	0.58	0.40	0.68	0.86	0.63	0.50	0.38	0.53	0.51	0.50
17	DNP-DL-leucine	Leu	0.64	0.49	0.71	0.87	0.66	0.47	0.32	0.53	0.48	0.48
18	DNP-isoleucine	Ileu	0.65	0.49	0.71	0.87	0.67	0.47	0.31	0.56	0.51	0.48
19	2,4-Dinitrophenol	OH	0.79	0.65	0.69	0.73	0.71	0.63	0.60	0.25	0.47	0.44

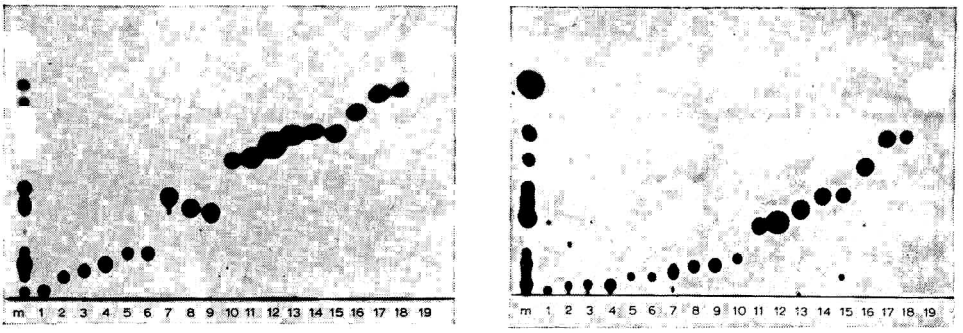


Fig. 1. One-dimensional chromatogram. Solvent: I, 60 min, 10 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 1 μg in 1 μl methanol; numbers: *cf.* Table II.

Fig. 2. One-dimensional chromatogram. Solvent: II, 90 min, 10 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 1 μg in 1 μl methanol; numbers: *cf.* Table II.

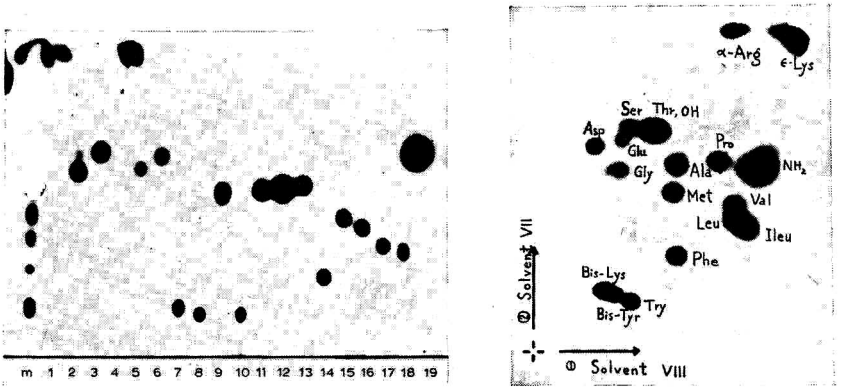
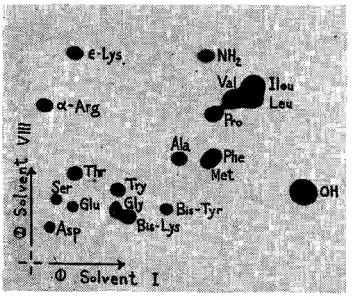


Fig. 3. One-dimensional chromatogram. Solvent: VII, 75 min, 10.5 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 1 μg in 1 μl methanol; numbers: *cf.* Table II.

Fig. 4. Two-dimensional chromatogram. Solvent: 1st dimension: VIII, 200 min, 10 cm; 2nd dimension: VII, 60 min, 11 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 0.5 μg each DNP derivative in a total of 10 μl methanol; symbols: *cf.* Table II.



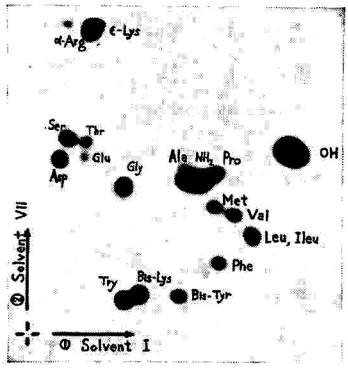


Fig. 5. Two-dimensional chromatogram. Solvent: 1st dimension: I, 60 min, 10.5 cm; 2nd dimension: VIII, 200 min, 9.5 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 0.5 μg each DNP derivative in a total of 10 μl methanol; symbols: *cf.* Table II.

Fig. 6. Two-dimensional chromatogram. Solvent: 1st dimension: I, 60 min, 10 cm; 2nd dimension: VII, 60 min, 10 cm; layer: ε-polycaprolactam resin CM 1007s (26°); loading: 0.5 μg each DNP derivative in a total of 10 μl methanol; symbols: *cf.* Table II.

tailing results. Higher concentration of acid gives circular spots and better distribution of R_F values but at the same time reduces the speed of ascent. In solvent X we used sulfamic acid instead of the acetic acid as in solvent IX and found that the order of R_F values was the same as in solvent IX.

We ran one-dimensional chromatograms on CM 1007s (26°) layers and CM 1007s (29°) layers. Both give similar R_F values except in the solvent systems IV and V, which give elongated spots on the CM 1007s (29°) layer but give circular spots on the CM 1007s (26°) layer.

We have tried using a basic solvent system, for example pyridine–benzene, but it did not give a good chromatogram on either CM 1007s (29°) or CM 1007s (26°) layers.

The notorious difficulty of separating leucine from isoleucine was overcome with a butanol–acetic acid (90:10) system. All of the other nine solvent systems failed to differentiate these two structurally similar compounds.

Figs. 1–3 show typical chromatograms of 19 DNP derivatives in solvent systems I, II and VII. Some samples give more than one spot after development due to the impurities present in the original sample.

In order to show the difference between the solvent systems, we numbered the DNP derivatives according to their R_F values in solvent system II.

Two-dimensional chromatography

Fig. 4 shows the separation of a mixture containing 0.5 μg of each DNP derivative using a combination of solvent systems VIII and VII. Of all the possible combinations of the above ten solvent systems, this combination gives the best distribution of spots on the chromatogram. DNP-valine, DNP-leucine and DNP-isoleucine can be separated satisfactorily. Bis-DNP-lysine and bis-DNP-tyrosine overlap to an appreciable extent. DNP-threonine and 2,4-dinitrophenol merge into one spot. The overall time required is about 2 h when the chromatogram is run on a CM 1007s (29°) layer or about 4 h when it is run on a CM 1007s (26°) layer.

Fig. 5 shows the two-dimensional chromatogram in solvent systems I and VIII. The separations of the bis-DNP-lysine–bis-DNP-tyrosine pair and the DNP-threonine–2,4-dinitrophenol pair have been achieved. But DNP-phenylalanine and DNP-methionine overlap to form an elliptical spot. This problem can be solved by running simultaneously another chromatogram in solvent systems I and VII (see Fig. 6). The overall time required is about 2 h for a CM 1007s (29°) layer or 4 h for a CM 1007s (26°) layer for the combination I–VIII. For the combination I–VII, the overall time required is about 1 h and 2 h for CM 1007s (29°) and CM 1007s (26°) layers, respectively.

ACKNOWLEDGEMENTS

We wish to thank Dr.'s Y. T. LIN and C. H. YANG for their generous encouragement and discussion. We are also indebted to Dr. T. B. LO for his kindness in supplying the DNP-amino acids and helpful discussion. We should like to express our sincere gratitude to Dr. I. M. CHENG who provided us with the photographic equipment. Thanks are also due to Miss C. M. CHEN for technical assistance.

SUMMARY

The separation of seventeen DNP derivatives of amino acids, 2,4-dinitroaniline and 2,4-dinitrophenol by polyamide layer chromatography is investigated. Convenient procedures for the preparation of durable polyamide layers are described. A table of R_F values in ten solvent systems and ultraviolet contact photographs of three one-dimensional chromatograms and three two-dimensional chromatograms are given. The present method is better than paper or thin-layer chromatography in speed and efficiency.

REFERENCES

1 K.-T. WANG AND J. M. K. HUANG, *Nature*, 208 (1965) 281.
2 F. SANGER, *Biochem. J.*, 39 (1945) 507.
3 J. LEGGETT BAILEY, *Techniques in Protein Chemistry*, Elsevier, Amsterdam, 1962, pp. 143–169.
4 E. HEFTMANN, *Anal. Chem.*, 36 (1964) 14R.
5 A. V. PORTUGAL, R. GREEN AND T. M. SUTHERLAND, *J. Chromatog.*, 12 (1963) 183.
6 N. A. MATHESON, *J. Biochem.*, 94 (1964) 513.
7 G. MATSUDA, T. MAITA, H. TAKEI, T. MAEKAWA, T. SHIKAYA, K. MAEDA AND M. FUJIWARA, *Acta Med. Nagasakiensia*, 8 (1963) 28.
8 H. C. DESSAUER, J. G. HAMILTON AND J. K. CALVET, *J. Chromatog.*, 18 (1965) 192.
9 R. L. MUNIER AND G. SARRAZIN, *Bull. Soc. Chim. France*, (1963) 2939.
10 G. BISERTE AND Y. MOSCHETTO, *Bull. Soc. Chim. France*, (1963) 2932.
11 R. L. MUNIER AND G. SARRAZIN, *J. Chromatog.*, 12 (1963) 542.
12 J. J. PISANO, W. J. A. VANDENHEUVEL AND E. C. HORNING, *Biochem. Biophys. Res. Commun.*, 7 (1962) 82.
13 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 17 (1961) 145.
14 D. WALZ, A. R. FAHMY, G. PATAKI, A. NIEDERWIESER AND M. BRENNER, *Experientia*, 19 (1963) 213.
15 G. PATAKI AND M. KELLER, *Helv. Chim. Acta*, 47 (1964) 787.
16 M. BRENNER, A. NIEDERWIESER AND G. PATAKI, in A. T. JAMES AND L. J. MORRIS (Editors), *New Biochemical Separations*, Van Nostrand, London, 1964, pp. 141–156.
17 F. DRAWERT, O. BACHMANN AND K. H. REUTHER, *J. Chromatog.*, 9 (1962) 376.
18 H. ENDRES AND H. HORMANN, *Angew. Chem.*, 2 (1963) 254.
19 H. STEULE AND E. HILLE, *Biochem. Z.*, 331 (1959) 220.
20 E. HILLE, *Biochem. Z.*, 333 (1960) 269.
21 E. SCHWERDTFEGER, *Z. Tierphysiol., Tierernaehr. Futtermittelk.*, 16 (1961) 262.
22 J. DAVIDEK AND E. DAVIDKOVA, *Pharmazie*, 16 (1961) 352.
23 W. GRAU AND H. ENDRES, *J. Chromatog.*, 17 (1965) 587.
24 P. SPADLER AND H. ENDRES, *J. Chromatog.*, 17 (1965) 591.
25 J. W. COPIUS-PEEREBOOM, *Nature*, 204 (1964) 748.
26 K.-T. WANG, *J. Chinese Chem. Soc.*, 8 (1961) 241;
Y.-T. LIN, K.-T. WANG AND Y.-S. LIN, *ibid.*, 9 (1962) 68;
Y.-T. LIN AND K.-T. WANG, *ibid.*, 10 (1963) 146.
27 Y.-T. LIN, K.-T. WANG AND T.-I. YANG, *J. Chromatog.*, 20 (1965) 610.
28 Y.-T. LIN, K.-T. WANG AND T.-I. YANG, *J. Chromatog.*, 21 (1966) 158.
29 Y.-T. TUNG AND K.-T. WANG, *Nature*, 208 (1965) 581.
30 H. HORMANN AND H. V. PORTATIUS, *Z. Physiol. Chem.*, 315 (1959) 141.
31 W. GRASSMANN, H. HORMANN AND H. V. PORTATIUS, *Z. Physiol. Chem.*, 321 (1960) 120.

ANALYSE DE TANINS SYNTHÉTIQUES PAR CHROMATOGRAPHIE EN COUCHE MINCE

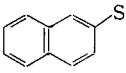
J. AURENGE, G. BARBE-RICHAUD ET L. GELPI
Progil-LCR, Avenue J. Jaurès, Décines, Isère (France)
(Reçu le 11 octobre 1965)

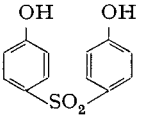
INTRODUCTION

Les tanins synthétiques sont des produits de polycondensation formaldéhydique de composés à fonctions phénoliques et sulfoniques.

L'activité tannante est liée à la présence des composés phénoliques condensés, au degré de cette condensation ainsi qu'au degré de sulfonation; le mélange et l'interaction des produits phénoliques avec les composés sulfoniques constituent le tanin définitif dit de "remplacement".

Nous avons plus spécialement étudié les tanins de remplacement à base de naphtalène et de dihydroxy-4,4'-diphényl sulfone. Les matières premières de synthèse de ces tanins sont les suivantes:

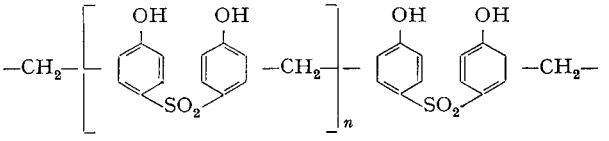
l'acide naphtalène sulfonique:  (R_1)

la dihydroxy-4,4'-diphényl sulfone:  (R_2)

le formaldehyde: HCHO

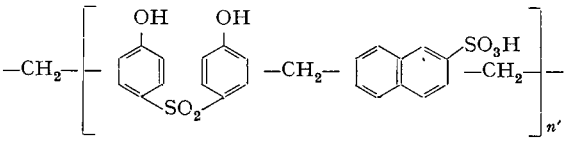
Le schéma simplifié de la synthèse des tanins de ce type est le suivant:

(1) Polycondensation de la dihydro-4,4'-diphényl sulfone en présence de formol. On obtient un résol de sulfone dont les motifs sont du type suivant:



(*n* a plusieurs valeurs)

(2) Condensation de l'acide naphtalène sulfonique sur le résol en présence de formaldéhyde: on obtient ainsi un tanin définitif dont les motifs sont du type suivant:



REICH[1] admet une structure analogue: $R_1—(CH_2—R_1)_n$; $R_1—(CH_2—R_2)_n$; $R_2(CH_2—R_2)_n$. Il existerait en outre des radicaux méthylol (—CH_2OH) fixés sur le noyau aromatique de certaines chaines.

Les études analytiques de ces mélanges complexes sont relativement rares.

Des études par chromatographie sur papier sur des tanins naturels ont déjà été réalisées; citons entre autres ROUX ET EVELYN[2] qui séparèrent des composés flavoniques et des tanins naturels au moyen d'un ternaire *n*-butanol–acide acétique–eau. ROUX ET MAIHS[3] adaptèrent différents révélateurs au cas de ces tanins naturels. Par ailleurs FREEMAN[4] a utilisé un éluant alcalin à base de *n*-butanol et ammoniaque pour la séparation de phénols alcools simples.

Pour étudier certains points particuliers de la fabrication des tanins synthétiques REICH a utilisé un couple similaire (*n*-butanol–ammoniaque, 3:1) pour séparer les dérivés méthylol du dihydroxy-4,4'-diphényl sulfone[1,5] ainsi que le ternaire *n*-butanol–acide acétique–eau (4:1:5) pour l'étude de la condensation formaldéhydique du β-naphtol et des acides β-naphtol sulfoniques.

Les séparations précitées correspondent à des stades particuliers de la synthèse des tanins. Elles ne sont pas effectuées sur le produit final complexe: la chromatographie en couche mince nous a permis de résoudre ce problème, au moins en partie.

MISE EN OEUVRE DES ANALYSES PAR CHROMATOGRAPHIE EN COUCHE MINCE

Étude de la révélation

Dans le domaine des composés flavoniques et des tanins naturels ROUX ET MAIHS[3] préconisaient les révélateurs suivants: alun ferrique, nitrate d'argent ammoniacal, benzidine diazotée, acide toluène sulfonique; REICH[1] de son côté utilisait un réactif ferrique. Sur chromatoplaque ces réactifs nous ont apporté des résultats médiocres. Par contre, l'absorption U.V. à 2540 Å sur support fluorescent (silice Merck GF 254) visualise très finement les composés.

Étude du support

Les supports déjà essayés étaient la cellulose dans le cas de la chromatographie sur papier et la poudre de polyamide dans le cas de la chromatographie sur colonne[5]. Nous avons essayé un adsorbant commun et bien adapté à la technique de CCM: la silice (silice Merck GF 254); il nous a donné toute satisfaction. L'activation est menée à 110° pendant 30 min.

Étude de l'élution

Les essais réalisés avec des éluants pour chromato d'adsorption (chloroforme, acétate d'éthyle, éther...) n'ont donné aucune séparation. Par contre, la chromatographie de partage a pu résoudre le problème.

Élution monodimensionnelle alcaline. L'éluant de REICH (*n*-butanol–ammoniaque, 3:1) ne convenait pas pour la chromatographie en couche mince sur silice, les produits

n'étant pas assez élués. Nous avons augmenté le pouvoir éluant de cette formule par adjonction d'un pourcentage d'eau. Le mélange obtenu étant hétérogène une addition d'éthanol a été nécessaire. Le solvant définitif adopté a la composition suivante: *n*-butanol–éthanol–ammoniaque RP–eau (75:10:15:10). Durée de la chromatographie 1 h 30; élution sur 15 cm.

Élution monodimensionnelle acide. L'éluant de ROUX, MAIHS ET EVELYN[2,3] donne des résultats intéressants en chromatographie sur couche mince de silice, nous l'avons adopté. Il se compose de la phase supérieure du mélange: *n*-butanol–acide acétique RP–eau (4:1:5). Durée de la chromatographie: 2 h; élution sur 15 cm.

Élution bidimensionnelle. La chromatographie avec solvant acide est entreprise d'abord, suivie dans une direction perpendiculaire d'une chromatographie avec l'éluant alcalin.

APPLICATION

Nous avons soumis simultanément à la séparation chromatographique:

–Deux tanins A et B de condensation différente. Lors de la synthèse de ceux-ci le pourcentage initial en formaldéhyde était plus élevé pour le tanin A que pour le tanin B. On pouvait donc s'attendre à un degré de réticulation (ou de condensation) plus élevé pour le tanin A, ce que, nous le verrons plus bas, la chromatographie a confirmé.

–Les matières premières de synthèse (dihydroxy-4,4'-diphényl sulfone, acide naphtalène sulfonique).

–Les produits intermédiaires que l'on peut obtenir par condensation formaldéhydique: d'une part du dihydroxy-4,4'-diphényl sulfone sur lui-même (résol de sulfone), d'autre part de l'acide naphtalène sulfonique sur lui-même.

La chromatographie sépare différents constituants qui ne sont pas obligatoirement des produits simples; nous verrons d'ailleurs que les constituants ou groupes de constituants séparés sont différents en élution acide et alcaline; c'est la technique bidimensionnelle qui donnera la meilleure résolution.

Élution alcaline

Sur la Fig. 1 qui rend compte de cette séparation, on peut souligner les points suivants:

(a) dihydroxy-4,4'-diphényl sulfone: 2 constituants dont 1 principal ($R_F = 0.5$),

(b) acide naphtalène sulfonique: 2 constituants dont 1 principal ($R_F = 0.5$),

Les produits de départ de la synthèse ne sont donc pas purs.

(c) acide naphtalène sulfonique condensé: 10 constituants ($0 \leqslant R_F \leqslant 0.5$) dont celui de l'acide de départ ($R_F = 0.5$),

(d) résol de sulfone: 9 constituants ($0 \leqslant R_F \leqslant 0.5$) dont la sulfone de départ ($R_F = 0.5$).

Ces deux derniers produits condensés peuvent être considérés comme des produits intermédiaires dans la synthèse des tanins, leur complexité illustre celle que peut avoir un tanin terminé.

(e), (f) tanins A et B: séparation de 9 et 11 constituants ($0 \leqslant R_F \leqslant 0.5$) dont la sulfone et l'acide sulfonique de départ.

Remarquons que la comparaison de ces deux tanins est malaisée à partir des résultats de cette élution, ceci a justifié la recherche d'un éluant acide.

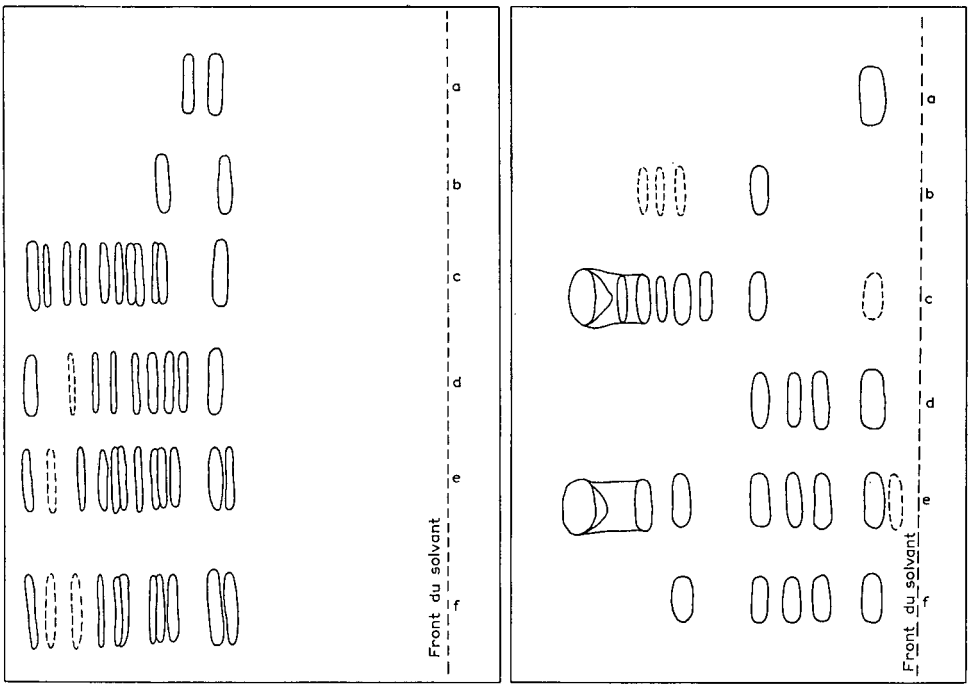


Fig. 1. Séparation de tanins synthétiques et matières premières brutes et semi-condensées. a = Dihydroxy-4,4′-diphényl sulfone; b = acide naphtalène sulfonique; c = acide naphtalène sulfonique condensé; d = résol; e = tanin A; f = tanin B. Support: Silice GF 254 (Merck). Solvant: *n*-butanol–éthanol–ammoniaque RP–eau (75:10:15:10, v/v/v/v).

Fig. 2. Séparation de tanins synthétiques et matières premières brutes et semi condensées. a–f, voir la Fig. 1. Support: Silice GF 254 (Merck). Solvant: phase supérieure du mélange *n*-butanol–acide acétique RP–eau (4:1:5, v/v/v).

Élution acide

La Fig. 2 montre les séparations obtenues:

(a) dihydroxy-4,4′-diphényl sulfone: un seul constituant (R_F = 0.87), la chromato acide ne sépare donc pas l'impureté vue en élution alcaline.

(b) acide naphtalène sulfonique: 4 constituants séparés dont un principal (R_F = 0.50),

(c) acide naphtalène sulfonique condensé: 7 constituants séparés avec traînée des produits très condensés ($0 \leqslant R_F \leqslant 0.50$) et acide sulfonique de départ,

(d) résol de sulfone: 4 constituants séparés ($0.55 \leqslant R_F \leqslant 0.87$) dont le dihydroxy-4,4′-diphényl sulfone de départ,

(e) Tanin A: 7 constituants séparés avec traînée vers les basses valeurs de R_F ($0 \leqslant R_F \leqslant 0.90$).

(f) Tanin B: 5 constituants séparés: $0.35 \leqslant R_F \leqslant 0.87$.

Élution bidimensionnelle acide–basique

Les Figs. 3 et 4 montrent les chromatogrammes à deux dimensions des tanins A et B avec les références unidimensionnelles de chaque côté. On voit que le Tanin

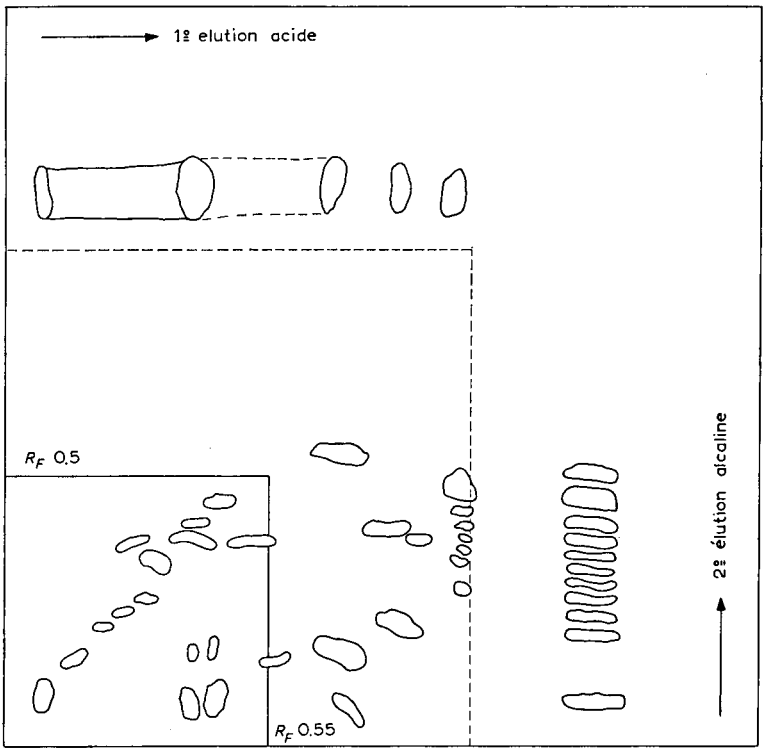


Fig. 3. Séparation en élution bidimensionnelle du tanin A. 1r solvant: phase supérieure *n*-butanol–ac.acétique–eau (4:1:5). 2e solvant: *n*-butanol–éthanol–ammoniaque RP–eau (75:10:15:10).

A est beaucoup plus complexe que le Tanin B. Le premier présente 28 constituants séparés tandis que le second en montre 13.

DISCUSSION

Nous étudierons d'abord les résultats obtenus sur les produits de condensation en présence de formaldéhyde, de la dihydroxy-4,4'-diphényl sulfone, et de l'acide naphtalène sulfonique. Nous examinerons ensuite les résultats obtenus sur les tanins proprement dits.

Sulfone condensée et acide naphtalène sulfonique condensé

L'élution alcaline (Fig. 1) fournit d'excellentes séparations. Cependant, on observe que dans cette élution, les produits de condensation formaldéhydique issus de composés de nature différente comme la dihydroxy-4,4'-diphényl sulfone et l'acide naphtalène sulfonique se placent à des R_F identiques. On peut donc penser que les séparations obtenues pour chaque produit condensé correspondent à des degrés différents de condensation ou de méthylation et non pas à la nature des produits de base.

L'élution acide (Fig. 2) par contre, répartit dans deux zones différentes du chromatogramme les produits condensés dérivant de chacun des composés de base:

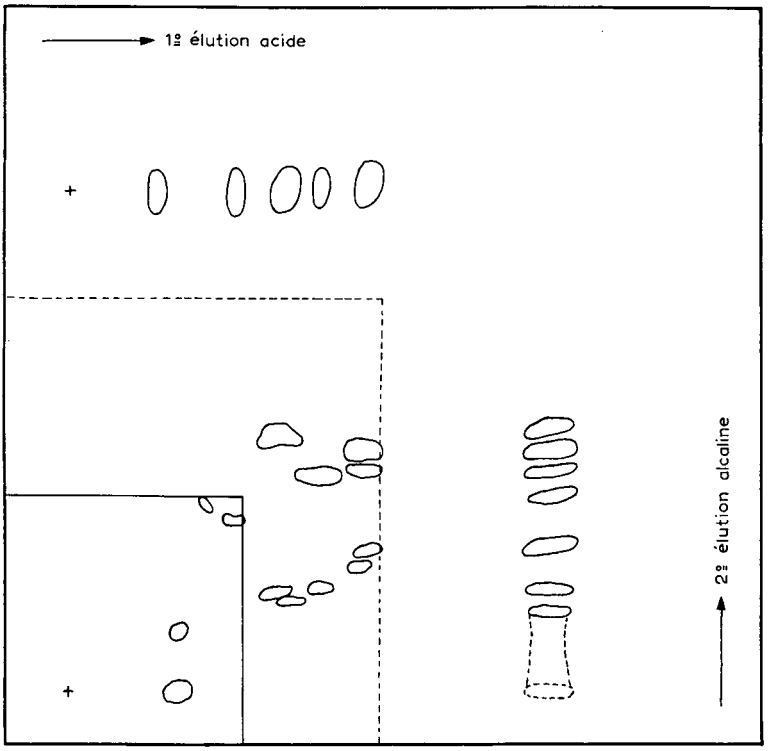


Fig. 4. Séparation en élution bidimensionnelle du tanin B. 1r solvant: phase supérieur *n*-butanol–ac.acétique–eau (4:1:5). 2e solvant: *n*-butanol–éthanol–ammoniaque RP–eau (75:10:15:10).

on observe en effet que la partie supérieure du chromatogramme ($R_F = 0.5$ à 0.9) est occupée par les produits de condensation dérivés de cette dihydroxy-4,4'-diphényl sulfone, tandis que les produits de condensation de l'acide naphtalène sulfonique s'échelonnent de $R_F = 0.0$ à $R_F = 0.5$.

En conséquence, l'élution bidimensionnelle qui associe les effets des deux élutions précédentes, doit séparer le mélange de ces composés de base condensés, à la fois selon leur nature et selon leur degré de condensation ou de méthylation.

Tanins A et B

Du fait de l'action spécifique de chaque éluant, la comparaison des tanins A et B en élution simple (Figs. 1 et 2) ne peut fournir qu'un renseignement très partiel sur la différence de constitution qui peut exister entre eux.

On note qu'en élution alcaline (Fig. 1) les deux tanins sont très semblables entre eux, de la même façon que l'acide naphtalène sulfonique condensé et la dihydroxy-4,4'-diphényl sulfone condensée présentent peu de différence.

En élution acide (Fig. 2) on remarque que le tanin A contient plus de constituants de bas R_F que le tanin B, ce qui pourrait s'interpréter comme une condensation plus poussée en chaînes naphtalènes sulfoniques.

En élution bidimensionnelle enfin (Figs. 3 et 4) ont note une augmentation très sensible du nombre de constituants dans le tanin A (environ 28) par rapport au tanin

B (environ 13); les constituants supplémentaires du tanin A sont répartis dans les deux zones de séparation déterminées par l'élution acide.

Comme nous l'avions supposé au début du paragraphe "APPLICATION", nous voyons en définitive que dans la synthèse de ces tanins, l'augmentation du taux de formaldéhyde a pour effet d'augmenter le nombre de constituants ce qui est en liaison avec une condensation plus élevée.

CONCLUSION

On peut dire en conclusion que la technique de chromatographie en couche mince a mis en évidence la complexité des tanins synthétiques. La comparaison de comportements chromatographiques des deux tanins pris comme exemples est en accord avec les différences des condensations obtenues lors des deux synthèses par variation des quantités de formaldéhyde mises en jeu.

La méthode analytique étant maintenant au point, un travail peut être actuellement envisagé pour étudier systématiquement l'influence des différents paramètres dans la synthèse de ces composés.

REMERCIEMENTS

Cette étude a été effectuée au L. C. R. PROGIL, Directeur Mr DEGEORGES, Chef du Département Mr NORMAND, que nous tenons à remercier ici.

RÉSUMÉ

La méthode décrite a permis la séparation de différents éléments composant les tanins synthétiques examinés.

Notre étude a porté essentiellement sur les tanins issus de la condensation de l'acide naphtalène sulfonique avec la dihydroxy-4,4'-diphényl sulfone en présence de formaldéhyde.

La technique de chromatographie en couche mince s'est révélée bien adaptée à la résolution de ce problème.

SUMMARY

The separation of different elements of some synthetic tannins is possible with the method described.

This study is primarily concerned with formaldehyde condensation products of 4,4'-dihydroxydiphenyl sulfone and naphthalenesulfonic acid. The application of thin-layer chromatography as an analytical tool in following the extent of condensation is indicated.

BIBLIOGRAPHIE

1 G. REICH ET G. HEBESTREIT, *J. Prakt. Chem.*, 19 (1963) 303.
2 D. G. ROUX ET S. R. EVELYN, *J. Chromatog.*, 1 (1958) 537.
3 D. G. ROUX ET A. E. MAIHS, *J. Chromatog.*, 4 (1960) 65.
4 J. H. FREEMAN, *Anal. Chem.*, 24 (1952) 955.
5 G. REIGH ET G. HEBESTREIT, *J. Prakt. Chem.*, 19 (1963) 309.

QUALITY TESTING OF CHROMATOGRAPHIC DATA WITH THE AID OF A STATISTICAL CRITERION*

GEORGE LEDIN, JR., WALDEMAR R. GUSTAVSON AND ARTHUR FURST
Institute of Chemical Biology, University of San Francisco, San Francisco, Calif. (U.S.A.)
(Received October 22nd, 1965)

INTRODUCTION

An exhaustive mathematical treatment of the chromatographic process, even if of high theoretical interest, is usually of no practical value for the practicing chemist. The interpretative mathematics of biology and chemistry may provide a reasonably close explanation of the phenomena involved, but are generally inaccessible tools for the biologist or chemist who need to analyze the experimental data on hand and obtain an answer of validity. Many good theories[1] have been written and chromatography has been presented as a convolution process, or a Poisson process[2], but again almost no literature is available on the practical aspects of evaluating the data upon completion of the experiment[3].

The criterion here derived is the result of the authors' work on the statistics of the chromatography of vitamin B_6 in which it was desirable to establish a simple test for reproducibility. This criterion is a fast test for dispersion (% error) that provides a narrow confidence band and in many cases will prove to be easier to use, quicker, and better than the very well known and often misused *t*-Student's and Chi-Square tests.

THEORY

Although the R_F of a specific compound at a fixed pH should be a constant value, in actual practice these figures vary from experiment to experiment. This variation is bounded:

$$0 \leqq x_k \leqq 1 \tag{1}$$

where x_k is the k-th R_F value.

The expression x_k, of course, can be applied to all R_F values, which naturally will have bounds within the closed interval [0,1].

For statistical purposes it is convenient to normalize the x_k by defining a new variable, the variability ratio:

$$Y_k = \frac{x_k}{\bar{X}} \tag{2}$$

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where X is the mean R_F value. One of the common forms of the coefficient of dispersion is:

$$d = \frac{s}{X} \tag{3}$$

$$d = \sqrt{\frac{\sum_{k=1}^{N} (Y_k - 1)^2}{N - 1}} \tag{4}$$

where s is the standard deviation of the sample, and N is the number of readings.

Optimum reproducibility (replicability) conditions call for a maximum coefficient of dispersion, the magnitude of which will depend upon the difficulty of the separations. The researcher who needs to check the reproducibility of the experimental data may obviously use formulas (3) or (4); but, generally speaking, this is a tedious and time-consuming process. A much quicker way of checking if the coefficient of dispersion is within the established interval is the "Y-test".

The Y-test

In order to have $d \leqslant d_0$, it is necessary to have*

$$Y_{max} = \frac{x_{max}}{X} \leq 1 + d_0 \quad \text{and} \quad Y_{min} = \frac{x_{min}}{X} \geq 1 - d_0$$

Proof. Formula (4) may be rewritten in the following approximate form:

$$d = \sqrt{\frac{N}{N - 1}} (Y_{max} - 1) \tag{5a}$$

and, solving for Y_{max}:

$$Y_{max} = 1 + \sqrt{1 - \frac{1}{N}} \cdot d \tag{5b}$$

where Y_{max} is the maximum variability ratio (or, what is the same, the ratio of the highest allowable reading to the mean) permitted for a given dispersion d. Using the binomial expansion, (5b) may be rewritten as follows:

$$Y_{max} = 1 + \left(1 - \frac{1}{2N} - \frac{1}{8N^2} - \cdots\right) d \tag{6}$$

Depending on how large N is, one of the two following approximate expressions can be used:

$$Y_{max} = 1 + \left(1 - \frac{1}{2N}\right) d \tag{7a}$$

$$Y_{max} = 1 + d \tag{7b}$$

* Necessary, but not sufficient. The speed of this test is obtained at the expense of some accuracy.

The same line of reasoning may be applied for Y_{min}. In this case, formulas (7a) and (7b) would be replaced by:

$$Y_{min} = 1 - \left(1 - \frac{1}{2N}\right)d \qquad (7a')$$

$$Y_{min} = 1 - d \qquad (7b')$$

Naturally, formulas (7) can be used to determine the Y-test for any given dispersion d. Particularly, if $d = d_0$, then, using (7b) and (7b'), $Y_{max} = 1 + d_0$, and $Y_{min} = 1 - d_0$.

EXAMPLES

To illustrate the theoretical results obtained above, two examples of vitamin B_6-amine-$5PO_4$ (synthetic compound and from mouse brain) will be analyzed. This technique of analysis is of course also applicable to pyridoxols and pyridoxals, and, in general, to any R_F data.

TABLE I

ANALYSIS OF THE R_F VALUES OF THE SYNTHETIC COMPOUND VITAMIN B_6-AMINE-5PO. Solvent at pH 6.5.

k	x_k	$\lvert X - x_k \rvert$	$\lvert X - x_k \rvert^2 \cdot 10^{-6}$
1	0.09	0.03	900
2	0.11	0.01	100
3	0.11	0.01	100
4	0.11	0.01	100
5	0.11	0.01	100
6	0.11	0.01	100
7	0.11	0.01	100
8	0.11	0.01	100
9	0.12	0.00	0
10	0.12	0.00	0
11	0.12	0.00	0
12	0.12	0.00	0
13	0.12	0.00	0
14	0.12	0.00	0
15	0.13	0.01	100
16	0.13	0.01	100
17	0.13	0.01	100
18	0.13	0.01	100
19	0.13	0.01	100
20	0.15	0.03	900

Here $N = 20$, $X = 0.12$. Let us set $d = 0.10 = 10\%$ (expecting 90% of our data to be within one standard deviation from the mean).

If we now apply the Y-test, we will obtain: $Y_{max} = 0.15/0.12 = 1.25$ and $Y_{min} = 0.09/0.12 = 0.75$, which gives $d = Y_{max} - 1 = 1 - Y_{min} = 25\%$. Hence some of our data are outside the desired confidence band. Let us assume that x_1 and x_{20} are outside.

Then, applying the Y-test again, we obtain: $Y_{max} = 0.13/0.12 = 1.08$ and $Y_{min} = 0.11/0.12 = 0.92$, which gives $d = Y_{max} - 1 = 1 - Y_{min} = 8\%$

If we were to carry out the usual computations of standard deviation, etc. we would find: $N = 20$, $X = 0.12$, $s = 0.01$, $d = 8\%$ and for the narrower band: $N = 18$, $X = 0.12$, $d = 7\%$.

A systematic procedure for a statistical analysis of this kind is the following:

1. Sort the R_F values in ascending or descending order of magnitude*.
2. Compute the mean $\dot{R}_F$ value.
3. Compute all $| X—x_k |$ and, correspondingly, all $| X—x_k |^2$.
4. From these calculations determine s and d.

The above procedure is followed in Tables I and II, and then comparisons are drawn by applying the criterion developed in Theory. The two examples show that, if the Y-test is applied, the above procedure is reduced to steps 1 and 2 only:

1. Sort the R_F values in ascending or descending order of magnitude*.
2. Compute the mean R_F value.
3. Obtain an estimate of the coefficient of dispersion by applying the Y-test (for example, $d =$ (greatest R_F)/(mean R_F) — 1), or see if the data falls within established confidence limits (for a desired d).

TABLE II

ANALYSIS OF THE R_F VALUES OF VITAMIN B_6-AMINE-5PO_4 FROM MOUSE BRAIN

k	x_k	$\lvert X - x_k \rvert$	$\lvert X - x_k \rvert^2 \cdot 10^{-6}$
1	0.10	0.04	1600
2	0.11	0.03	900
3	0.12	0.02	400
4	0.13	0.01	100
5	0.13	0.01	100
6	0.13	0.01	100
7	0.13	0.01	100
8	0.13	0.01	100
9	0.13	0.01	100
10	0.13	0.01	100
11	0.13	0.01	100
12	0.13	0.01	100
13	0.13	0.01	100
14	0.14	0.00	0
15	0.14	0.00	0
16	0.14	0.00	0
17	0.14	0.00	0
18	0.15	0.01	100
19	0.15	0.01	100
20	0.15	0.01	100
21	0.16	0.02	400
22	0.18	0.04	1600

Here $N = 22$, $X = 0.14$. Let us again set $d = 0.10 = 10\%$.

If we now apply the Y-test, we will obtain: $Y_{max} = 0.18/0.14 = 1.29$ and $Y_{min} = 0.10/0.14 = 0.71$, which gives $d = Y_{max} - 1 = 1 - Y_{min} = 29\%$. Hence some of our data are outside the desired confidence band. Let us assume that x_1, x_2, x_3, x_{21}, and x_{22} are outside. Then, applying the Y-test again, we obtain: $Y_{max} = 0.15/0.14 = 1.07$ and $Y_{min} = 0.13/0.14 = 0.93$ which gives $d = Y_{max} - 1 = 1 - Y_{min} = 7\%$.

If we were to carry out the usual computations of standard deviation, etc. we would find: $N = 22$, $X = 0.14$, $s = 0.02$, $d = 14\%$ and for the narrower band, $N = 17$, $X = 0.14$, $d = 6\%$.

* This step is not necessary. Its convenience lies in the facts that the distribution of the R_F values around the mean will be appreciated by a glance, and x_{max} and x_{min} will be easiest to pick out.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to the United States Air Force (Wright-Patterson Air Force Base, Ohio) for the financial support under which this work was carried out.

SUMMARY

Testing the reproducibility of chromatographic data with the usual statistical tests (*t*-Student's, Chi-Square, etc.) is in most cases a time-consuming error-inviting procedure. The criterion developed in this paper allows the experimenter to obtain a good estimate of the confidence bounds by applying an extremely simple test. Two examples involving the R_F values of pyridoxamine-5PO_4 illustrate the method.

REFERENCES

1 H. VINK, *J. Chromatog.*, 15 (1964) 488 and 18 (1965) 25.
2 D. A. MCQUARRIE, *J. Chem. Phys.*, 38 (1963) 437.
3 W. EDWARDS DEMING, *Statistical Adjustment of Data*, Dover Publications, New York, 1964.

PAPER CHROMATOGRAPHY OF SOME ISOMERIC MONOSUBSTITUTED PEROXYBENZOIC ACIDS

R. KAVČIČ, B. PLESNIČAR AND B. HRISTOVSKI

Organic Chemistry Laboratory, Department of Chemistry, University of Ljubljana, Ljubljana (Yugoslavia)

(Received October 7th, 1965)

INTRODUCTION

As part of our program on peroxy acids we have prepared and investigated the paper chromatographic separation of some isomeric monosubstituted peroxybenzoic acids in order to obtain information about the *ortho* effect and other structural factors influencing the properties of these compounds. The problem is a complex one, because in addition to factors such as inductive and steric effects, field and resonance, several implications of hydrogen bonding must be considered, including solvation and intramolecular (chelation) effects, as well as intermolecular associations (dimerization)[1,2,3]. Consequently, it is to be expected that the so-called *ortho* effect may vary markedly under different conditions.

Reviewing the literature on the paper chromatography one notes that aromatic peroxy acids have been ignored in the many recent applications of this technique. In general, the reports have concerned themselves with the paper chromatography of organic peroxides. Thus, RIECHE AND SCHULZ[4] were first to report on the separation of various organic peroxides and used ethyl acetate–dioxane–water (2.0:4.5:4.6) as the mobile solvent on partially acetylated paper (20 %) and a solution of *p*-aminodimethylaniline hydrochloride in methanol as spray reagent for locating peroxides. ABRAHAM and coworkers[5] used paper treated with silicone oil, the mixture ethanol–chloroform–water as the mobile phase and a solution of ferrothiocyanate as a spraying agent. MILAS AND BELIČ[6] have obtained good results for identification and separation of the organic peroxides on unimpregnated paper using dimethylformamide–decalin, N-methylformamide–decalin–*n*-butanol (45 %), and ethanol–water as the mobile solvents. CARTLIDGE AND TIPPER[7] were successful in avoiding the losses due to the volatility of the organic peroxides during the chromatographic separation by covering the chromatographic paper with two glass plates. They used a mixture of water–diethyl ether–*n*-butanol (1:10:10) for chromatography of organic peroxides on unimpregnated paper. A mixture of water–ethanol–chloroform (20:17:2) was recommended as the mobile phase on paper impregnated with silicone oil and *n*-butanol–petroleum ether (b.p. 80–100°) as the mobile solvent on paper impregnated with ethylene glycol. DOBSON AND HUGHES[8] used paper impregnated with a 5 % solution of silicone oil in petroleum ether (b.p. 80–100°) for paper chromatography of dialkyl peroxides and water–methanol as the mobile solvent. In the paper by CARTLIDGE AND TIPPER[7] the data on R_F values for lower aliphatic acids from C_1 to C_4 are also given.

This paper describes a simple procedure for the paper chromatography of isomeric monosubstituted peroxybenzoic acids and presents an attempt to investigate the effect of the orientation of substituent groups on the R_F values. Data are presented for some known aromatic peroxybenzoic acids and for a number of monosubstituted peroxybenzoic acids which have not been previously prepared.

EXPERIMENTAL

A series of isomeric monosubstituted peroxybenzoic acids was prepared for this purpose, by thus extending SWERN's procedure[9]. Crude peracids were recrystallised or purified by sublimation *in vacuo*. Thoroughly dried peracids were used then without further purification. Gas phase chromatography of peroxy acids thus prepared indicated that they contained no isomers. The chromatographic papers used were: partially acetylated paper for chromatography from Binzer (25 % acetylation), Schleicher & Schüll paper No. 2043b, and a paper impregnated in this laboratory. The latter was impregnated by standing overnight in a 20 % solution of N-methylformamide in acetone and dried in air. All chromatograms were run at 25° using the ascending technique. The papers were allowed to equilibrate 30 min in the tank before immersion. The solvents were allowed to travel 17 cm requiring 30 to 60 min. The paper sheets were sprayed with the spray reagent as soon as they were removed from the tanks and then air dried.

RESULTS AND DISCUSSION

Hexane–dioxane–dimethylformamide (25:12:12) were found to be a satisfactory mobile solvent for use in the paper chromatography of isomeric monosubstituted peroxybenzoic acids on acetylated paper using ascending development. The mixture of these solvents was vigorously shaken and allowed to stand in a separatory funnel for 3 to 4 h. A solution of hexane saturated with dioxane and dimethylformamide was used as mobile phase. Benzene was found to be satisfactory as mobile phase in the paper chromatography of the above mentioned peroxy acids on paper impregnated with N-methylformamide.

A solution of *p*-aminodimethylaniline hydrochloride in methanol (10 ml water:10 ml ethanol:1 ml glacial acetic acid:1 g reagent) was found to be a good spot-locating reagent. The solution is quite stable and gives easily discernible red-coloured spots. These spots are not permanent and disappear after a certain period of time. A solution of potassium iodide and a starch solution (5 ml of glacial acetic acid, 5 ml of a saturated solution of potassium iodide and 5 ml of a 5 % starch solution) has also been found to be a very good spray reagent. It gives black spots which are permanent, so that the paper chromatograms may be retained as a record.

Chromatographic data for various isomeric monosubstituted peroxybenzoic acids are summarized in Table I. Migration rates are given in R_b values, where R_b is the migration distance of the peroxy acid divided by the migration distance of the unsubstituted peroxybenzoic acid. The R_F values of peroxybenzoic acid averaged about 0.35 on acetylated paper and 0.36 on impregnated paper.

A comparison of the effect of isomeric groups on the separation in the first solvent shows that, in general, the lowest R_b values are obtained with *ortho* isomers. The

TABLE I

R_b VALUES* OF ISOMERIC MONOSUBSTITUTED PEROXYBENZOIC ACIDS

Peroxy acid	R_b	
	*I***	*II****
o-Chloroperoxybenzoic	0.51	1.17
m-Chloroperoxybenzoic	0.91	1.30
p-Chloroperoxybenzoic	0.80	1.42
o-Bromoperoxybenzoic	0.66	1.06
m-Bromoperoxybenzoic	1.23	1.20
p-Bromoperoxybenzoic	1.14	1.42
o-Nitroperoxybenzoic	0.11	0.33
o-Fluoroperoxybenzoic	0.57	0.89
p-Fluoroperoxybenzoic	0.10	1.06
m-methylperoxybenzoic	1.57	1.53
p-methylperoxybenzoic	1.31	1.36

* $R_b = \frac{\text{Migration distance of substituted peroxybenzoic acid}}{\text{Migration distance of peroxybenzoic acid}}$.

** 25% acetylated paper; solvent: hexane saturated with dioxane and dimethylformamide.

*** Paper impregnated with N-methylformamide; solvent: benzene.

meta isomers have the greatest R_b values. The difference in R_b values between *meta* and *para* isomers are relatively small. The so-called *ortho* effect is quite obvious among the isomeric groups. The *ortho* isomer is separate from the *meta* and *para* isomers. The R_b values of the *meta* and *para* isomers are close together, with the *meta* isomer having a slightly greater R_b value than the *para* isomer. The only exception is *m*-methyl peroxybenzoic acid which has a higher R_b value. The differences in R_b values of various isomeric groups on different chromatographic papers and/or in different solvents show many variations depending on the nature of the substituting groups and the solvents. For example, in both mobile solvents, the chloro-peroxybenzoic acids show large R_b differences between the isomers. The methyl peroxybenzoic acids, however, show little difference in R_b values in both solvents. The fluoro-peroxybenzoic acids, on the other hand, show the largest differences in the first system of mobile solvents.

Observation of the data in Table I, column II, shows that when benzene was used as the mobile phase on impregnated paper, the order is altered, for *meta* and *para* substituted peroxybenzoic acids, the *para* isomer having the higher R_b value. With the three isomers, there is a large difference between the *ortho* and *para* isomers while the *ortho* and *meta* isomers run close together.

If methanolic solutions of a monosubstituted peroxybenzoic acid were used in the paper chromatography 3 to 5 spots were detected after the development of the chromatograms. In order to elucidate this, some qualitative experiments using thin-layer chromatography were carried out and a considerably sharper separation of these spots of substances with similar R_b values was obtained. The origin of these spots is presumably due to reaction products formed between the peroxy acid and the alcohol. The explanation of this phenomenon will be the subject of a forthcoming publication.

ACKNOWLEDGEMENT

The authors wish to thank the Boris Kidrič Fund for the financial support of this work.

SUMMARY

The R_b values of a number of isomeric monosubstituted peroxybenzoic acids have been determined for two different solvent systems. The solvent systems are hexane saturated with dioxane and dimethyl formamide for use on acetylated paper and benzene for use on paper impregnated with N-methylformamide. The R_b values of isomeric peracids vary considerably, depending on the substituent group and its orientation. An attempt is made to correlate the chromatographic data with the structure of the compounds. The influence of the strong inductive and steric effects of the substituents is clearly seen.

REFERENCES

1 D. SWERN, L. P. WITNAUER, C. R. EDDY AND W. E. PARKER, *J. Am. Chem. Soc.*, 77 (1955) 5537.
2 D. BELITSKUS AND G. A. JEFFREY, *Acta Cryst.*, 18 (1965) 458.
3 M. SAX, P. BEURSKENS AND S. CHU, *Acta Cryst.*, 18 (1965) 252.
4 A. RIECHE AND M. SCHULZ, *Angew. Chem.*, 70 (1958) 694.
5 M. H. ABRAHAM, A. G. DAVIES, D. R. LLEWELLYN AND E. M. THAIN, *Anal. Chim. Acta*, 17 (1957) 499.
6 N. A. MILAS AND I. BELIČ, *J. Am. Chem. Soc.*, 81 (1959) 3358.
7 J. CARTLIDGE AND C. F. H. TIPPER, *Anal. Chim. Acta*, 22 (1960) 106.
8 G. DOBSON AND G. HUGHES, *J. Chromatog.*, 16 (1964) 416.
9 L. S. SILBERT, E. SIEGEL AND D. SWERN, *J. Org. Chem.*, 27 (1962) 1336.

BIOCHEMISTRY OF SPHINGOLIPIDS

IV. SOME NEW PAPER CHROMATOGRAPHIC SYSTEMS FOR THE CHARACTERIZATION OF SPHINGOLIPIDS

Č. MICHALEC AND Z. KOLMAN

Laboratory of Protein Metabolism and Protein Synthesis, Faculty of General Medicine, Charles University, Prague (Czechoslovakia)

(Received October 4th, 1965)

INTRODUCTION

In the last few years many papers have been published concerning the application of various chromatographic techniques to studies in sphingolipid biochemistry. Chromatography on thin layers of adsorbents, especially, has been widely used for the fractionation of cerebrosides, sulfatides, gangliosides and sphingomyelins. However, only a few publications on the paper chromatography of these substances have appeared[1–6].

For the past few years in our laboratory, we have been investigating the possibility of using paper chromatography for the topographic analysis of sphingolipids in human organs under normal and pathological conditions. This article presents our results on this subject.

EXPERIMENTAL

Preparation of purified sphingolipids

For the preparation of sphingolipids the organs were extracted by the method of FOLCH[7] *et al.* After mild alkaline hydrolysis the isolated mixture of sphingolipids was fractionated on Florisil and DEAE-cellulose. Authentic samples of cerebrosides, gangliosides and sphingomyelins (from bovine brain) were obtained from Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England).

Impregnation of paper with silica gel

Two types of paper impregnated with silica gel were used:

(A) Commercial silica gel paper, Schleicher and Schüll No. 289 containing 35–40 % of silica gel.

(B) Whatman No. 3 paper impregnated with silica gel[8].

Solvent systems

The following solvent systems were used:

System 1: chloroform–methanol–water (12:1.6:0.1);

System 2: tetrahydrofuran–diisobutyl ketone–water (45:9:4);

System 3: tetrahydrofuran–diisobutyl ketone–water (45:5:6);

System 4: chloroform–acetone–glacial acetic acid (9.5:5.7:0.4);
System 5: chloroform–acetone–propionic acid (6.2:7.2:1.0);
System 6: chloroform–methanol–12.5 % ammonium hydroxide (12:7:1.6);
System 7: chloroform–acetone–glacial acetic acid–water (12:1.6:0.4:0.05).

Detection

(*a*) *Rhodamine B.* The dry chromatogram is immersed in a 0.001 % solution of Rhodamine B in 0.25 *M* K_2HPO_4 for approximately 60 min. The excess dye is removed by washing with tap water. Sphingolipids show up as red spots. In ultraviolet light they fluoresce bright yellow or orange.

(*b*) *Chlorine–benzidine–KI.* The dry chromatogram is wetted with an ethanol–acetone mixture (1:1) and exposed to chlorine (0.1 % $KMnO_4$–10 % HCl, 1:1) for 5–10 min. Then it is immediately washed 10 min in running water. When nearly dry the chromatogram is drawn through a 0.5 % benzidine solution in ethanol–water (1:1) with the addition of few crystals of KI and allowed to dry in air. The blue spots are stable for several hours.

(*c*) *Cresyl violet.* The chromatogram is immersed in 0.02 % cresyl violet in 1 % acetic acid for 10 min at 60° or 30 min at room temperature and then washed well in 2 % acetic acid for several hours.

(*d*) *Pinacryptol yellow.* The chromatogram is drawn through an 0.05 % solution of pinacryptol yellow in water and subsequently observed in ultraviolet light. Counterstaining with cresyl violet permits precise identification of sulfatides.

(*e*) *Acriflavine–p-dimethylaminobenzaldehyde.* The dry chromatogram is dipped in 0.002 % acriflavine in 0.1 *M* citrate–HCl buffer solution, pH 2.5, for 10 min. It then is immersed in a 2 % *p*-dimethylaminobenzaldehyde solution in 20 % HCl diluted with *n*-propanol or isopropanol in the proportion (30:70, v/v) before use. As soon as the chromatogram is completely orange in colour (approximately 1–2 min) it is washed several times with water until the background is yellow. The sulfatides appear as bright orange spots. The color is unstable and disappeared after an hour.

(*f*) *Acid fuchsin–uranyl nitrate.* The dry chromatogram is dipped in an 0.02 % solution of acid fuchsin in 0.01 *N* HCl containing 0.2 % of uranyl nitrate for 30 min. Then it is washed three times with an 0.2 % solution of uranyl nitrate in 0.01 *N* HCl. After drying at laboratory temperature, sphingomyelins appeared as red spots on a white background.

The color reactions of individual sphingolipids are summarized in Table I.

TABLE I

COLORATION OF INDIVIDUAL SPHINGOLIPIDS IN SOME DETECTION REACTIONS

Substance	*a*	*b*	*c*	*d*	*e*	*f*
CMH	red-orange	blue	pale blue	—	—	—
CDH	red-orange	blue	pale blue	—	—	—
CTH	red-orange	blue	pale blue	—	—	—
AmGl	red-orange	blue	red-blue	—	—	—
GG	red-orange	weak blue	red-blue	—	—	—
CMHS	red-orange	blue	red-brown	bright yellow	brick-red	—
CDHS	red-orange	blue	red-brown	bright yellow	brick-red	—
SPH	red-orange	blue	blue	—	—	red-violet

CHROMATOGRAPHIC RESULTS

System 1

This solvent system is very suitable for the separation of CMH, CDH, CTH and AmGl*. Each fraction of these substances is further subfractionated into cerebrosides with nonhydroxy and hydroxy fatty acids, the latter having a slower mobility. CMH and CDH, especially, are very well separated. Commercial Schleicher and Schüll No. 289 silica gel paper proved to be superior to Whatman No. 3 silica gel paper, showing minimal streaking and well defined spots. Chromatograms were run on short sheets of paper (solvent front 15 cm) in most experiments. In several cases long chromatograms (solvent front 25 cm) developed by a horizontal technique resulted in a better separation of the spots (Fig. 1).

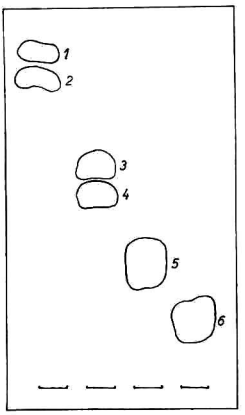


Fig. 1. Separation of human kidney cerebrosides on Schleicher and Schüll No. 289 paper impregnated with silica gel. System 1. Detection: Rhodamine B. 1 = CMH-N; 2 = CMH-OH; 3 = CDH-N; 4 = CDH-OH; 5 = CTH; 6 = AmGl.

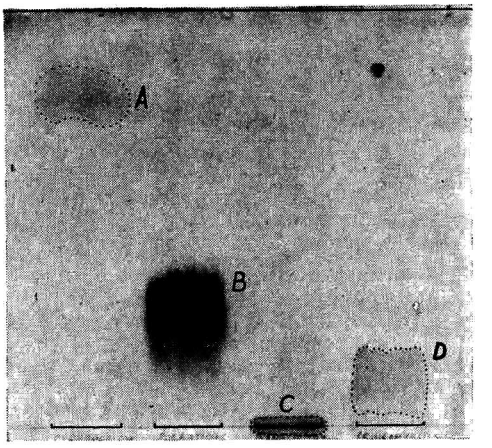


Fig. 2. Separation of human brain sphingolipids on Whatman No. 3 paper impregnated with silica gel in chloroform–methanol–water (12:2.0:0.1). Detection: cresyl violet. A = cerebrosides; B = sulfatides; C = gangliosides; D = sphingomyelins.

If the proportions of methanol or water are increased (*e.g.* chloroform–methanol–water, 12:2.0:0.1 or 12:2.5:0.2) it is possible to differentiate CTH and AmGl (high mobility) on Schleicher and Schüll No. 289 paper. In these systems CMH and CDH run close to the solvent front.

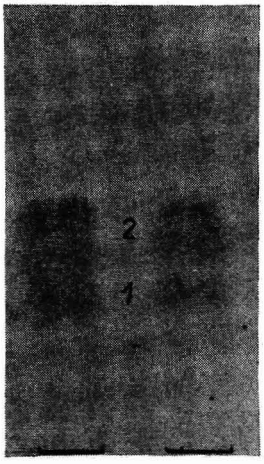
On the other hand an ascending technique using Whatman No. 3 silica gel paper developed for long periods (24 h) led to excellent separation of SPH_{18} and SPH_{24} (Fig. 3).

Sulfatides, gangliosides and sphingomyelins form compact spots without need for further subfractionation on chromatograms developed on short sheets (Fig. 2).

* Abbreviations used: CMH = ceramide-monohexosides; CDH = ceramide-dihexosides; CTH = ceramide-trihexosides; AmGl = aminoglycolipids; GG = gangliosides (G_0–G_6 = symbols for individual ganglioside fractions as used by SUZUKI[8]; CMHS = ceramide-monohexoside-sulfatide; CDHS = ceramide-dihexoside-sulfatide; SPH_{18} = more-polar sphingomyelins containing fatty acids below C_{20}; SPH_{24} = less-polar sphingomyelins containing fatty acids above C_{20}; N = fraction with nonhydroxy fatty acids; OH = fraction with hydroxy fatty acids.

System 2

This system gives a very clear separation of CMHS and CDHS and is very suitable in cases where the sulfatide fraction was purified from other sphingolipids by column chromatography.



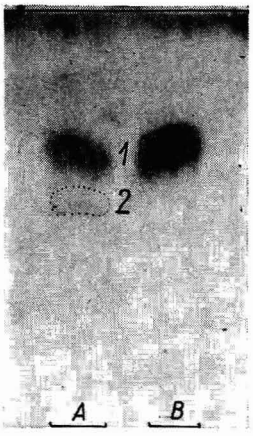


Fig. 3. Separation of human blood serum sphingomyelins by continuous paper chromatography on Whatman No. 3 paper impregnated with silica gel in System 1 visualized with acid fuchsin–uranyl nitrate. 1 = SPH_{18}; 2 = SPH_{24}.

Fig. 4. Separation of sulfatides on Schleicher and Schüll No. 289 paper impregnated with silica gel in System 3. Detection: pinacryptol yellow and cresyl violet. A = sulfatide fraction from human kidney; B = sulfatide fraction from human brain. 1 = CMHS; 2 = CDHS.

System 3

This offers the same resolution as System 2 for CMHS and CDHS but the mobilities are higher. CMH, CDH and CTH are also separated but without further subfractionation. If this system was used two-dimensionally with System 1 an excellent separation of cerebroside fractions was obtained. The spots on silica gel impregnated paper are more compact than on untreated paper as described by SVENNERHOLM[5].

System 4

This is advantageous for the differentiation nonhydroxy and hydroxy fatty acid fractions in the CMH fraction. In this system the mobilities of these two fractions are very different. Other sphingolipids remained near or on the start line.

System 5

The system which consists of chloroform and acetone with the addition of propionic acid permits satisfactory separation of CMHS fractions with nonhydroxy and hydroxy acids. A two-dimensional technique using System 3 for the first dimension and System 5 for the second makes a complete separation of all sulfatide fractions possible (Fig. 5).

System 6

The characterization of individual ganglioside fractions is possible with this

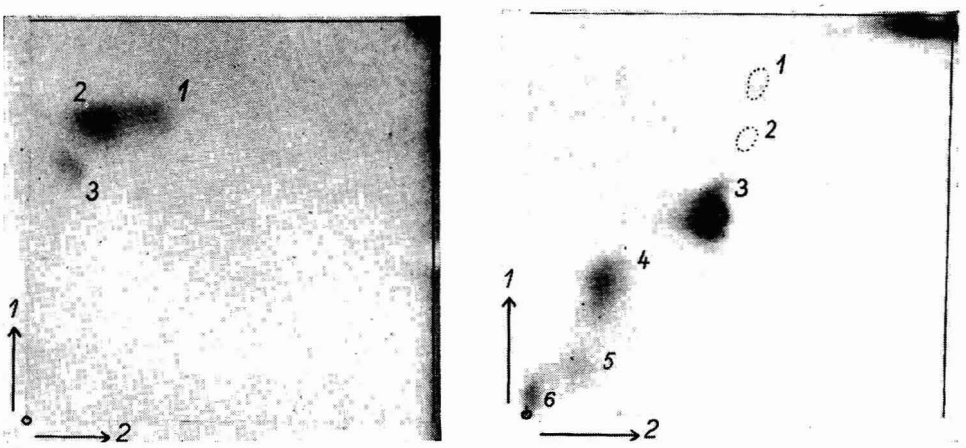


Fig. 5. Two-dimensional chromatography of human kidney sulfatides on Schleicher and Schüll No. 289 paper impregnated with silica gel. First dimension: system 3; second dimension: system 5. Detection: pinacryptol yellow and cresyl violet. 1 = CMHS-N; 2 = CMHS-OH; 3 = CDHS.

Fig. 6. Two-dimensional chromatography of human brain gangliosides on Schleicher and Schüll No. 289 paper impregnated with silica gel. First dimension: system 3; second dimension: system 6. Detection: cresyl violet. 1 = G_6?; 2 = G_5; 3 = G_4; 4 = G_3; 5 = G_2; 6 = G_1.

system. All fractions below G_4 are well separated but have high mobilities. Disialo- and trisialogangliosides eventually aminoglycolipids are only partially resolved. An increase of methanol and ammonium hydroxide concentration (*e.g.* chloroform–methanol–12.5 % ammonium hydroxide, 12:8:2.0) leads to the separation of the trisialogangliosides into 3 fractions and the disialogangliosides into 2 fractions. Ganglioside fractions below G_4 remained unseparated and run near to or with the solvent front as the other sphingolipids. The solvent mixture chloroform–methanol–12.5 % NH_4OH (10:3:0.4) is especially useful for monosialogangliosides, which it separates into 3 fractions. Other gangliosides remain at the start.

The two-dimensional combination (first dimension: system 3; second dimension: chloroform–methanol–12.5 % NH_4OH, 12:7:1.6) gives a good resolution of the whole spectrum of gangliosides (Fig. 6).

System 7

This system is useful for the identification of CMH-N and CMH-OH which are well separated near the starting line. It also appears possible to use this mixture for the separation of ceramides.

DISCUSSION

The chromatographic systems described in this paper show good potentiality for the identification of various types of sphingolipids. Excellent results were obtained especially with the purified fractions obtained after column chromatography on Florisil, DEAE cellulose, silica gel etc. By the combination with certain color reactions it is possible to characterize with good precision the whole range of sphingolipid fractions. The methods described here have a wide application in the analysis of these substances in biological material.

SUMMARY

Seven new chromatographic systems for the separation of sphingolipids on paper impregnated with silica gel are described. When used together with certain color reactions it is possible to differentiate most of these substances in biological material.

REFERENCES

1 C. B. SCRIGNAR, *J. Chromatog.*, 14 (1964) 189.
2 L. SVENNERHOLM, *J. Neurochem.*, 10 (1963) 613.
3 G. ROUSER, A. J. BAUMAN, N. NICOLAIDES AND D. HELLER, *J. Am. Oil Chemists' Soc.*, 38 (1961) 565.
4 P. S. SASTRY AND M. KATES, *Biochemistry*, 3 (1964) 1280.
5 L. SVENNERHOLM, *Acta Chem. Scand.*, 17 (1963) 1170.
6 R. SOPER, *Comp. Biochem. Physiol.*, 10 (1963) 325.
7 J. FOLCH, M. LEES AND G. H. SLOANE STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
8 Č. MICHALEC, Z. KOLMAN, M. ŠULC AND J. MĚŠŤAN, *J. Chromatog.*, 9 (1962) 237.
9 K. SUZUKI, *J. Neurochem.*, 12 (1965) 629.

A QUANTITATIVE AUTOMATED TECHNIQUE FOR THE DETERMINATION OF DIAZOTIZABLE IMIDAZOLE COMPOUNDS IN BIOLOGICAL MATERIAL

WALTER D. BLOCK, RICHARD W. HUBBARD* AND MARY H. WESTHOFF
Department of Dermatology, Biochemical Research Laboratories, University of Michigan, Medical Center, Ann Arbor, Mich. (U.S.A.)
(Received November 15th, 1965)

INTRODUCTION

Methods used to determine the metabolism of imidazole compounds in biological materials range from the use of enzymes to ion-exchange chromatography[1–5].

To facilitate studies in which a number of biological samples may be analyzed in a relatively short time for a series of imidazole compounds in which the 1, 2, and 3 positions on the ring are "non-substituted", a quantitative, automated method has been developed combining ion-exchange chromatography and the modified PAULY reaction[6]. An extract of the biological material is applied to a column containing resin, and a buffer gradient of increasing pH and ionic strength is applied to separate the imidazole compounds. The resulting eluate automatically is made alkaline, diazotized, and the color produced is determined by continuous photometry. The results are plotted automatically on a recording microammeter.

MATERIALS AND METHODS

Apparatus

Chromatographic tubes and resin. A heavy walled, jacketed chromatograph tube, 0.9 cm in diameter and 106 cm in length from the Teflon plate to the top of the tube is used. An 18/9 socket joint is attached to the top of the tube, and a 12/5 glass ball joint is affixed at the bottom. The column jacket is maintained at 50° throughout the 18 to 20 h of the chromatographic period by the circulating fluid system used by SPACKMAN *et al.*[7].

The column is filled to a height of 85–90 cm with 24–32 micron beads of unground 8% cross-linked, sulfonated polystyrene resin, sodium form (Chromo-bead resin, Type A, Technicon Instruments Corp., Chauncey, N.Y.). The resin is prepared and poured in the manner described for the preparation of columns for amino acid analysis by MOORE *et al.*[8], except that a citrate buffer, pH 3.90, is used (Table I, buffer I). Before use, the resin column is regenerated by pumping through it CO_2-free NaOH, 0.2 *M* (Na^+), followed by buffer I (Table I).

* Present address: Research Department, Beckman Instruments, Inc., Spinco Division, 1117 California Avenue, Palo Alto, California.

TABLE I

BUFFER GRADIENT

Buffer I, pH 3.90 ± 0.02, 0.20 *M* (Na^+) containing: 189.81 g citric acid·H_2O; 74.25 g NaOH (97%); 58.50 ml HCl; 0.90 ml caprylic acid; diluted to 9 l. Before use 2 ml/l of Brij-35 (50 g per 100 ml of H_2O) is added. Buffer II, pH 6.25 ± 0.02, 0.33 *M* (Na^+) containing: 291.15 g sodium citrate·2 H_2O; 0.45 g ethylenediaminetetraacetic acid; 10.80 ml HCl; 0.90 ml caprylic acid; diluted to 9 l. Before use 2 ml per liter of Brij-35 (50 g per 100 ml of H_2O) is added.

Chamber number	*Amount of buffer (ml)*	
	I	*II*
1	67	0
2	55	12
3	43	24
4	31	36
5	20	47
6	8	59
7	0	67
8	0	67
9	0	67

Buffer gradient. A 9-chambered "Block Autograd" (Technicon Instruments Corp.) supplies a continuous flow of variable buffer gradient. The quantities and composition of the buffers used in each chamber are shown in Table I. The outlet of the variable gradient device is connected by Tygon tubing, 3/16 in. I.D., to a 12/5 ball-socket joint. The socket section is joined by glass tubing, 1/16 in. I.D., to a series of 2 Teflon, 3-way stopcocks (size no. 2) united by Tygon tubing. One outlet of the first 3-way stopcock permits the buffer gradient to flow to the second stopcock; the other outlet is attached by glass tubing 1/16 in. I.D., to a reservoir containing buffer I, (Table I). The second 3-way stopcock has one inlet attached by Tygon tubing, 1/16 in. I.D., to a reservoir containing 0.2 *M* (Na^+), CO_2-free NaOH; the outlet connects with Tygon tubing, 1/16 in. I.D., to a T-bore Teflon stopcock (size no. 2), one end of which is left free to permit the operator to release air bubbles trapped in the system. The other end is connected with Tygon tubing, 1/16 in. I.D., to a Milton Roy CHMML-B-X chromatographic minipump with column valve liquid ends (Beckman Instruments, Inc., Palo Alto, Calif.) which pumps the buffer through a FP 1/16-10-G-5/81, Fischer and Porter, Warminster, Penn., flow-meter; and thence through Tygon tubing, 1/16 in. I.D., fitted with a U.S. 3823 by-pass pressure gauge (U.S. Gauge Co., Sellersville, Penn.), to an 18 mm ball with a swivel fitting (Beckman Instruments, Inc.) which is accommodated by the socket on top of the chromatographic column.

Analysis of column effluent. The column effluent is fed through a Beckman Instruments, Inc., 5/12 ball acceptor with a swivel fitting (attached with spring clips to the glass ball at the bottom of the column) and Teflon tubing, 1/32 in. I.D., into one capillary arm of a G-2 standard cactus*. Air is pumped through the second capillary arm of the cactus (Double Red Tygon tubing*) and Na_2CO_3 through the standard arm (Double Green Tygon tubing*) by a proportioning pump*, and are mixed with the effluent in a standard length mixing coil* (Fig. 1). Sulfanilic acid is

* Technicon Instruments Corp.

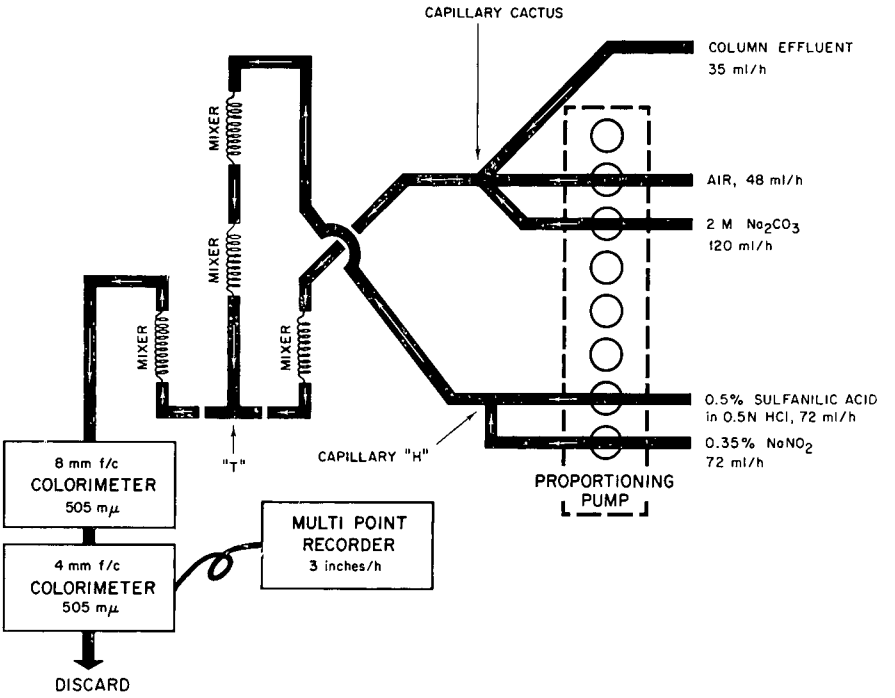


Fig. 1. Schema for automated diazotization of column effluent and recording of optical density produced. The abbreviation "f/c" denotes flow-cell.

pumped through the standard arm and $NaNO_2$ through the capillary arm of a D-1 "H"-tube* (Double Yellow Tygon tubing*) by the proportioning pump and are mixed together as they flow through 2 standard length mixing coils*. The alkaline effluent and the diazotizing mixture enter a standard length mixing coil* at room temperature (Fig. 1). The diazotized effluent proceeds directly into a pair of flow colorimeters* equipped with 8 mm and 4 mm cuvettes with wavelength filters of 505 mμ. The colorimeter signals are recorded into a multiple-point (three) Bristol recorder* with a chart speed of 3 in./h (Fig. 1). One of the 3 channels is "damped-out" to give a baseline.

Addition of sample to column. A solution of the sample to be analyzed is pipetted onto the top of the column and driven into the resin with air, 15 p.s.i. The sample is washed in with three 0.5–0.7 ml aliquots of buffer I (Table I). The ball joint from the buffer gradient system is attached, the pump started, and the buffer gradient allowed to flow through the chromatographic column for 18 to 20 h.

Regeneration of the column. The ion-exchange chromatographic column is regenerated whenever the resin is replaced, or a sample has been chromatographed. Regeneration is begun by disconnecting the "Block Autograd" at the 12/5 joint, and using a syringe to rinse the glass section between the joint and the first Teflon 3-way stopcock with buffer I (Table I). The second Teflon 3-way stopcock referred to under *Buffer gradient* is turned so the CO_2-free 0.2 *M* (Na^+) NaOH from the reservoir is

* Technicon Instruments Corp.

pumped through the system at a rate of 30 ml/h for 20 to 30 min. The stopcock is then turned to a position to receive solution through the outlet of the first stopcock, which is positioned so that buffer I (Table I) is pumped from the reservoir through the column until the eluate attains a pH of 3.90. The procedure usually requires about 3.5 h. The Autograd is filled with the proper buffers, reconnected, and the system is ready for another analysis.

Standard mixture of imidazole compounds. The apparatus is standardized by the use of a mixture of synthetic imidazole compounds, each of which was found in individual studies to be recoverable in a single peak from the ion-exchange column.

A stock solution is made by weighing 125 μmoles each of the following compounds: imidazolepyruvic acid*, imidazolelactic acid**; L-tyrosine, L-histidine and L-carnosine obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; imidazoleacetic acid·HCl, dihydrourocanic acid (imidazolepropionic acid), urocanic acid·H_2O, and 4-amino-5-imidazolecarboxamide·HCl from California Corp. for Biochem. Research, Los Angeles, Calif.; and L-homocarnosine obtained from Regis Chem. Co., Chicago, Ill. The compounds are placed into an 100 ml volumetric flask and 5 ml of 6 *M* HCl added. As some of them dissolve with difficulty, the mixture is heated slightly by placing the flask in hot water until solution is achieved. The solution is cooled, brought to volume, and stored in 5 ml amounts in ampules under N_2 at $-20°$.

Various concentrations of the stock solution which are applied to the column are obtained by diluting the solution with appropriate amounts of citrate buffer, pH 2.2 $\pm$ 0.03, prepared from the following: 21.0 g citric acid·H_2O, 8.3 g NaOH (97 %), 16.0 ml HCl, 0.1 ml caprylic acid, 20.0 ml thiodiglycol, and diluted to 1 liter with water[11].

TABLE II

CONSTANTS FOR VARIOUS IMIDAZOLE COMPOUNDS AND TYROSINE

Compound	*Concentration (μmoles)*				*Average constant*	*Maximum deviation from mean (%)*
	0.125	*0.250*	*0.500*	*1.000*		
Imidazolepyruvic acid	2.20		2.20		2.20	0.0
Imidazolelactic acid	15.76	15.96	16.20		15.97	1.4
Tyrosine	5.68	5.00	4.54		5.07	12.0
Imidazoleacetic acid	19.92	20.08	20.24	20.41	20.16	1.2
Urocanic acid	14.80	14.64	14.98	14.97	14.85	0.9
Dihydrourocanic acid (imidazolepropionic acid)	19.04	18.80	18.56	19.02	18.86	1.5
4-Amino-5-imidazole carboxamide		1.76	1.82	1.88	1.82	3.3
Histidine	19.12	18.72	18.96	19.58	19.10	2.5
Homocarnosine	12.00	12.16	11.86	12.14	12.04	1.4
Carnosine	13.12	13.80	13.50	13.66	13.67	4.0

The constants for each imidazole compound are computed in the same manner as the constants for amino acid analysis as given by SPACKMAN *et al.*[7], and are given in Table II for the apparatus used in this laboratory. Each laboratory will, of course,

* Prepared by the methods of SPOLTER AND BALDRIDGE[9] and BALDRIDGE AND AUERBACH[10].

** Obtained through the courtesy of Dr. H. BAUER, National Institutes of Health, Bethesda, Maryland.

need to do its own standardization although the constants should not vary appreciably from the ones given in Table II. The constants are used to calculate the amounts of the imidazoles in biological material[7].

Preparation of biological material

Tissue. Rat muscle, liver, and brain is frozen and ground in a Wiley mill (40 gauge screen) at the temperature of liquid nitrogen. The powdered material obtained is lyophylized, defatted by extraction with diethyl ether–absolute ethanol (3:1, v/v) for 24 h in a Soxhlet extractor. A picric acid extract of the dried material is prepared by the procedure of TALLAN *et al.*[12].

Urine. Urine is refrigerated during the collection period. Phenol should not be added as a preservative[13]. The aliquot of the 24 h specimen to be analyzed is filtered and 6 *M* HCl added until the pH is between 2.0 and 2.5.

Plasma. Plasma is deproteinized and filtered by the method of STEIN AND MOORE[14].

RESULTS AND DISCUSSION

Standard solution

Fig. 2 shows a typical chromatogram obtained with the stock solution diluted to a concentration of 0.250 μmoles. Imidazolepyruvic acid appears first, approximately 3.5 h after the buffer gradient flow is started, and is eluted in a broad band. The peak is sharper, of course, as the concentration of the imidazole is increased.

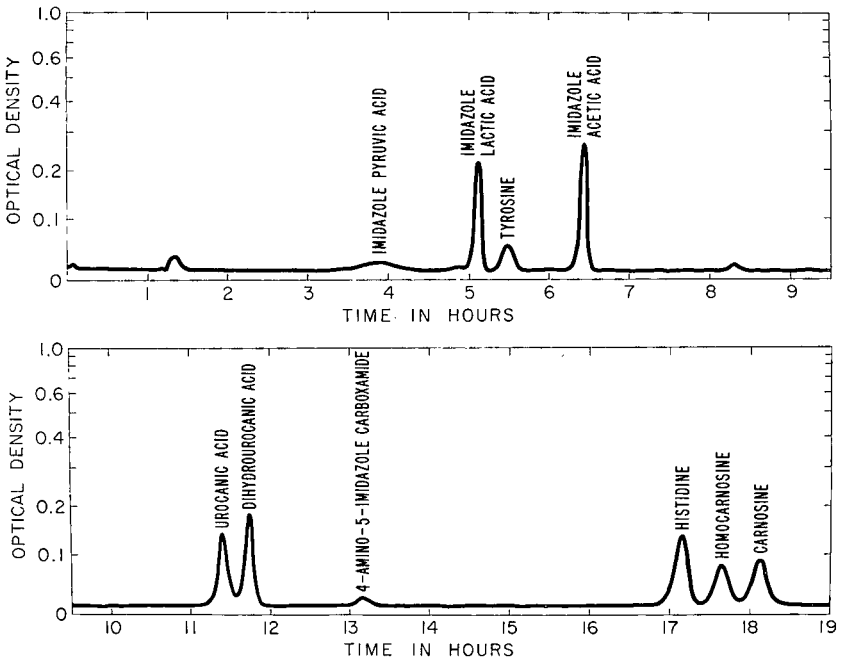


Fig. 2. Chromatogram of the standard solution containing 9 imidazole compounds plus tyrosine at a concentration of 0.250 μmoles.

The other 8 imidazole compounds and tyrosine are eluted in the order shown in Fig. 2, and give sharp and definite peaks at a concentration of 0.250 μmoles. No overlap of compounds occurs. The precision of the chromatography and the constants obtained for the various imidazole compounds did not vary more than 4% over a range of concentrations (Table II).

The two small peaks on the chromatogram eluted at approximately 1.5 and 8.5 h after initiation of the buffer gradient flow are unidentified, diazotizable material, and possibly are due to small amounts of impurities contained in one or more of the substances comprising the standard solution.

Biological material

Fig. 3 shows a chromatogram obtained with urine collected from a control male human subject ingesting an *ad libitum* diet. Imidazolelactic acid, the first diazotizable substance identified in the urine, was eluted approximately 5 h after initiation of the buffer gradient flow; carnosine, the last to be eluted, appeared approximately 13 h later. The imidazole compounds in the urine were eluted in the same order and position, relative to time of buffer gradient flow, as the synthetic imidazole compounds comprising the standard solution (Figs. 2 and 3). Each of the synthetic imidazole compounds added separately to replicate samples of the urine was eluted in a single peak. No unidentified, diazotizable compounds could be detected in the eluate from the chromatographed urine in the time interval between the appearances of imidazolelactic acid and carnosine (Fig. 3).

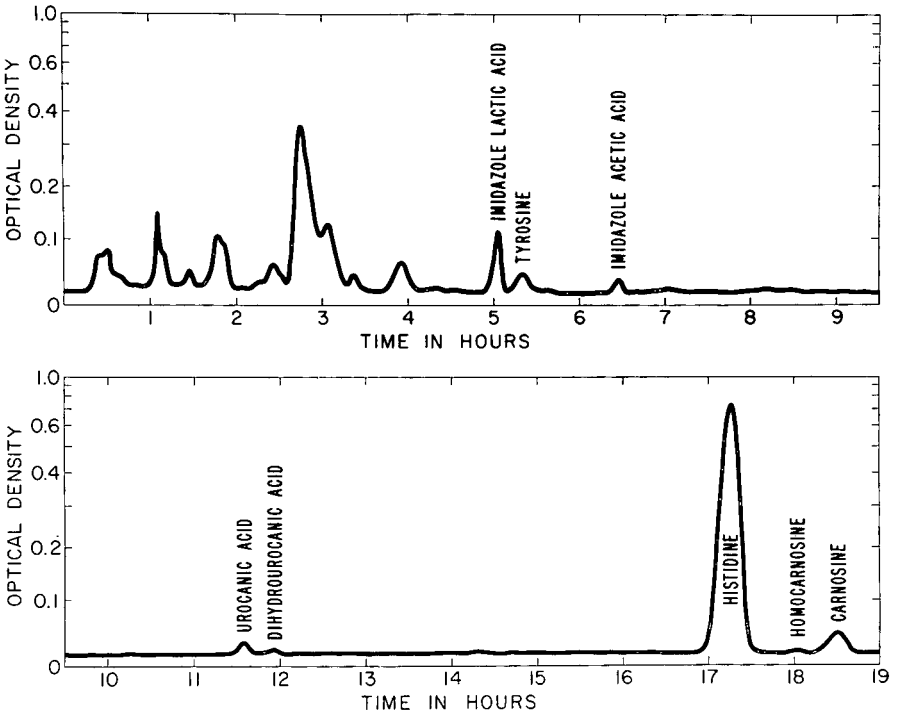


Fig. 3. Chromatogram of urine collected from a control male human subject ingesting an *ad libitum* diet.

TABLE III

IMIDAZOLE COMPOUNDS IN BIOLOGICAL MATERIAL

Compound	*Human urine*[a] *(μmoles/24 h)*		*Human plasma (μmoles/1000 ml)*	*Rat tissues*[b]		
	Av.	*Range*		*Muscle*	*Brain (μmoles/100 g)*	*Liver*
Imidazolelactic acid	55.13	41.15– 66.03 (93)[c]				
Imidazoleacetic acid	15.20	10.61– 18.67 (97)				
Urocanic acid	12.76	8.91– 15.45 (100)				
Dihydrourocanic acid (imidazolepropionic acid)	6.02	5.15– 6.75 (94)				
Histidine	650.98	565.43–921.41 (80)	4.83 (89)	91.30 (90)	70.00 (98)	239.13 (101)
Homocarnosine	<0.01	(102)			87.14 (111)	
Carnosine	101.57	25.76–183.43 (98)		1950.72 (110)	<0.01 (103)	

[a] Values for five different urine samples.
[b] Dried, defatted tissue.
[c] Percent recovery of the compound from a duplicate sample of the biological material.

Table III shows the amounts of specific diazotizable imidazole compounds present in a 24 h collection of human urine, in plasma from a fasting human subject, and in 3 tissues from the rat. The percentage recovery of the various synthetic imidazole compounds added at a concentration of 0.250 μmoles to a replicate aliquot of the biological material, also is given.

Seven diazotizable imidazole compounds were identified in urine from control human subjects. They ranged in amounts from 921.41 μmoles of histidine to less than 0.01 μmoles of homocarnosine/24 h (Table III). Imidazolepyruvic acid was not detected in the control urines; however, it was present in large amounts in urine from a patient with histidinemia*, and was separated completely from the other diazotizable substances in the abnormal urine by the automated ion-exchange chromatographic technique.

Recoveries of known amounts of synthetic imidazole compounds added to human urine ranged from 93 to 102 % (Table III), and are well within the accepted scope for biological fluids. Histidine is an exception; recovery of this compound from urine is low (80 %). The cause is not known.

Human plasma contained 4.83 μmoles of histidine/100 ml; a small amount of diazotizable material eluted at approximately 5.5 h of buffer gradient flow was identified as tyrosine.

Rat muscle contained histidine and carnosine in calculable amounts. Rat brain contained histidine, homocarnosine and carnosine; the 3 imidazoles are separated completely in a single extract of brain tissue by the ion-exchange chromatographic technique used. Rat liver contained histidine in calculable amounts. Within the working range (3.5 to 19 h of buffer gradient flow) of the method, no other diazotizable substances could be detected in amounts which could be quantitated.

Recovery of added imidazole compounds to plasma and to the different rat tissues ranged from 89 to 111 % (Table III).

ACKNOWLEDGEMENT

This study was supported in part by U.S. Public Health Service Grant AM-06825-03.

The authors wish to thank Miss MARA E. MARKOVS for able technical assistance in the initial phase of this study.

SUMMARY

A quantitative, automated apparatus is described for the simultaneous determination of a number of diazotizable imidazole compounds in the same sample of biological material. Its use in the analysis of urine, plasma and extracts of body tissues is illustrated. In practice, the biological fluid or tissue extract is applied to an ion-exchange column and a buffer gradient of increasing pH and ionic strength is used to separate the various imidazole compounds. The resulting eluate automatically is made alkaline, diazotized, and the color produced is determined by continuous photometry. The results are plotted automatically on a recording microammeter.

* Unpublished data.

Imidazolepyruvic acid, imidazolelactic acid, imidazoleacetic acid, urocanic acid, dihydrourocanic acid (imidazolepropionic acid), 4-amino-5-imidazolecarboxamide, histidine, homocarnosine and carnosine can be separated from each other with no overlapping. The values for each compound, using a solution composed of the pure compounds, can be integrated with a precision of 100 $\pm$ 4% over a range of concentrations. A complete analysis requires between 18 to 20 h.

REFERENCES

1 H. TABOR, *Methods Enzymol.*, 3 (1957) 623.
2 H. TABOR, *Methods Enzymol.*, 6 (1963) 581.
3 G. WOLF, P. WU AND W. HECK, *J. Biol. Chem.*, 222 (1956) 159.
4 J. J. PISANO, J. D. WILSON, L. COHEN, D. ABRAHAM AND S. UDENFRIEND, *J. Biol. Chem.*, 236 (1961) 499.
5 D. ABRAHAM, J. J. PISANO AND S. UDENFRIEND, *Arch. Biochem. Biophys.*, 99 (1962) 210.
6 H. PAULY, *Z. Physiol. Chem.*, 42 (1904) 508.
7 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
8 S. MOORE, D. H. SPACKMAN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1185.
9 P. D. SPOLTER AND R. C. BALDRIDGE, *J. Biol. Chem.*, 238 (1963) 2071.
10 R. C. BALDRIDGE AND V. H. AUERBACH, *J. Biol. Chem.*, 239 (1964) 1557.
11 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 893.
12 H. H. TALLAN, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 211 (1954) 927.
13 G. HUNTER, *Biochem. J.*, 16 (1922) 640.
14 W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 211 (1954) 915.

TRENNUNG DER SELTENEN ERDEN DURCH FLÜSSIGEN IONENAUSTAUSCH

IV. SELTENERDTRENNUNG DURCH PAPIERCHROMATOGRAPHIE MIT UMGEKEHRTEN PHASEN UND KOMPLEXELUTION*

GERHARD WERNER
Institut für Anorganische Chemie der Karl-Marx-Universität, Leipzig (D.D.R.)
(Eingegangen den 18. Oktober 1965)

Mit Dinonylnaphthalinsulfonsäure (DNNSS,HD) in Heptan imprägniertes Chromatographierpapier kann als stationäre Phase für eine Trennung von Metallionen verwendet werden[1]. Auch die Ionen der Seltenen Erden werden von so behandeltem Papier zurückgehalten[2]. Es finden Austauschreaktionen nach folgender Gleichung (1) statt:

$$SE^{3+} + (HD)_n \rightleftharpoons SE(H_{n-3}D_n) + 3\ H^+ \qquad (1)$$

Das Verhalten der Seltenen Erden gegenüber dem Austauscherpapier ist als echter Ionenaustausch zu beschreiben und zeigt weitgehende Analogie zum Ionenaustauschharzsystem[3]. Bei der Elution mit Salzsäure sind die R_F-Werte benachbarter Seltener Erden nahezu gleich, so dass eine Trennung praktisch nicht möglich ist. Dagegen kann durch Anwendung komplexbildender Elutionsmittel eine beträchtliche Verbesserung der Trennung erreicht werden. Hierüber wird im folgenden berichtet.

PRAKTISCHER TEIL

Dinonylnaphthalinsulfonsäure wurde durch Alkylierung von Naphthalin mit Nonen und anschliessende Sulfonierung hergestellt[4]. Mit einer Heptanlösung von DNNSS wurde Chromatographierpapier Schleicher & Schüll 2043b 30 Sec. imprägniert und anschliessend an der Luft getrocknet. 5–10 μg der Seltenen Erden (VEB Stickstoffwerk Piesteritz) wurden am Startpunkt 5 cm vom Elutionsmittel entfernt aufgegeben. Die Chromatographie wurde absteigend durchgeführt. Als Elutionsmittel dienten Milchsäure bzw. α-Hydroxyisobuttersäure, die mit Ammoniak auf den gewünschten pH-Wert und damit die gewünschte Ligandkonzentration gebracht wurden. Die Chromatogramme wurden durch Besprühen mit einer 0.1 %igen alkoholischen, ammoniakhaltigen Lösung von 1-(2-Pyridyl-azo)-2-naphthol entwickelt. Die Flecke müssen sofort markiert werden, da sie schnell verblassen.

* Auszugsweise zur Chemiedozententagung der DDR in Potsdam am 8. Juli 1965 vorgetragen.

ERGEBNISSE UND DISKUSSION

Bei der papierchromatographischen Trennung der Seltenen Erden an mit DNNSS imprägniertem Chromatographierpapier besitzen die leichten Erden kleinere R_F-Werte als die schweren[2]. Durch Komplexierung der wässrigen Phase können demnach zwei einander unterstützende Effekte zur Trennung ausgenutzt werden, nämlich (1) die unterschiedliche Affinität der Erden zum DNNSS-Papier (La > Lu) und (2) die unterschiedliche Stabilität der Komplexe in der wässrigen Phase (Lu > La).

Die Austauschreaktion lässt sich wie folgt formulieren:

$$(HD)_n + SEL_m^{3-m} \rightleftharpoons SE(H_{n-x}D_n)L_y^{3-(x+y)} + x\,H^+ + (m-y)L^- \qquad (2)$$

Hierin bedeuten: n den Assoziationsgrad der Dinonylnaphthalinsulfonsäure, m die Ligandenzahl in der wässrigen Phase, y die Ligandenzahl in der organischen Phase, x die pro Seltenerdion ausgetauschte Zahl Wasserstoffionen, SE ein beliebiges Ion der Seltenen Erden, L den negativ einwertigen Rest des Komplexbildners.

In den Gleichungen (1) und (2) wurde zur Vereinfachung die teilweise Anwesenheit von DNNSS in einer Alkaliform nicht berücksichtigt. Solange bei niedrigem pH-Wert gearbeitet wird, nehmen Alkaliionen nicht am Austausch teil. Bei der Verwendung von DNNSS-Papier in der H^+- und NH_4^+-Form treten bei gleichen Elutionsbedingungen praktisch die gleichen R_F-Werte auf (Tabelle I).

TABELLE I

R_F-WERTE VON La, Ce, Pr, Nd BEI DER ELUTION MIT 0.2 M MILCHSÄURE (pH = 3.0) AN DNNSS-PAPIER

	La	*Ce*	*Pr*	*Nd*
DNNSS H^+-Form	0.27	0.46	0.56	0.61
DNNSS NH_4^+-Form*	0.27	0.43	0.58	0.62

* DNNSS-Papier in der H^+-Form wurde 30 Sec. mit halbkonz. Ammoniak imprägniert.

Die Fig. 1 und 2 zeigen die Abhängigkeit der log R_M-Werte von der Ligandkonzentration im Elutionsmittel für α-Hydroxyisobuttersäure bzw. Milchsäure ($R_M = 1/R_F - 1$). Mit steigender Ligandkonzentration nehmen die log R_M-Werte ab. Bei diesen Versuchen wurde ohne Zusatz eines indifferenten Elektrolyten gearbeitet. Wegen der laufenden Änderung der Ionenstärke sind aus den Neigungen der Geraden der log R_M/pL-Darstellung keine Schlüsse über die Art der vorhandenen Teilchen zu ziehen. Bei Anwesenheit von $NaClO_4$ im Elutionsmittel steigen die R_F-Werte stark an, d.h. Gleichgewicht (2) wird nach der linken Seite verschoben (Tabelle II). DEELSTRA UND VERBEEK[5] erhielten mit Ionenaustauscherharzen ähnliche Ergebnisse.

Fig. 3 zeigt die Abhängigkeit der log R_M-Werte von La, Sm und Ho von der Ligandenkonzentration bei Anwesenheit von 0.203 M $NaClO_4$ im Elutionsmittel.

Der R_M-Wert ist dem Verteilungskoeffizienten VK proportional und dieser lautet:

$$VK = \frac{\Sigma\,[SE]_{Papier}}{\Sigma\,[SE]_{Lösung}} = \frac{\{[SE^{3+}] + [SEL^{2+}] + [SEL_2^+]\}_{Papier}}{\{[SE^{3+}] + [SEL^{2+}] + [SEL_2^+] + [SEL_3] + [SEL_4^-]\}_{Lösung}} \qquad (3)$$

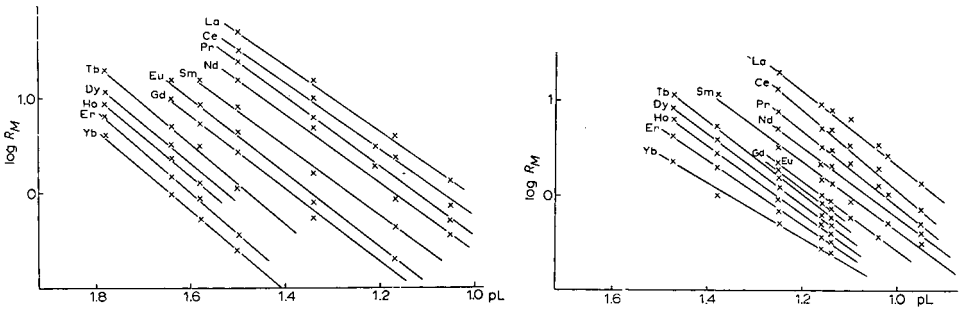


Fig. 1. Abhängigkeit der log R_M-Werte verschiedener Seltener Erden von pL der α-Hydroxyisobuttersäure. Papier: Schleicher & Schüll 2043b mit 0.1 F DNNSS in Heptan getränkt.

Fig. 2. Abhängigkeit der log R_M-Werte verschiedener Seltener Erden von pL der Milchsäure. Papier: siehe Fig. 1.

Addiert man die Konzentrationen der einzelnen Teilchen in einer Phase:

$$\{[SE^{3+}] + [SEL^{2+}] + [SEL_2^{+}]\}_{Papier} = [SEL_y^{3-y}] \text{ und}$$

$$\{[SE^{3+}] + [SEL^{2+}] + [SEL_2^{+}] + [SEL_3] + [SEL_4^{-}]\}_{Lösung} = [SEL_m^{3-m}]$$

so wird aus Gl. (3):

$$VK = \frac{[SEL_y^{3-y}]_{Papier}}{[SEL_m^{3-m}]_{Lösung}} \tag{4}$$

Hierbei seien y und m als durchschnittliche Ligandenzahlen definiert, die für y zwischen 0 und 2 und für m zwischen 0 und 4 liegen können. Mit Hilfe der Konstanten:

$$K_y = \frac{[SEL_y^{3-y}]}{[SE^{3+}]\,[L^-]^y} \text{ und } K_m = \frac{[SEL_m^{3-m}]}{[SE^{3+}]\,[L^-]^m}$$

erhält man:

$$VK = \frac{K_y\,\{[SE^{3+}]\,[L^-]^y\}_{Papier}}{K_m\,\{[SE^{3+}]\,[L^-]^m\}_{Lösung}} \tag{5}$$

Die interessierende Neigung der Geraden in Fig. 3 ergibt sich dann zu:

$$\frac{\mathrm{d}\log VK}{\mathrm{d}\,pL} = \frac{\mathrm{d}\log R_M}{\mathrm{d}\,pL} = m - y \tag{6}$$

TABELLE II

ELUTION DES LANTHANS AN DNNSS-PAPIER (0.112 F DNNSS IN HEPTAN) MIT 0.206 M MILCHSÄURE BEI GEGENWART VON $NaClO_4$ IM ELUTIONSMITTEL

$NaClO_4$-Konz. (Mol/l)	*0*	*0.05*	*0.1*	*0.2*	*0.5*	*0.8*	*1.0*
pH-Wert	3.50	3.44	3.42	3.36	3.30	3.25	3.23
R_F-Wert	0.08	0.23	0.36	0.50	0.68	0.72	0.90

Die Neigungen der Geraden in den Fig. 1 und 2 liegen bei den verschiedenen Elementen zwischen 3.2 und 4.3 und können wegen der nicht konstanten Ionenstärke nicht zur Berechnung von y verwendet werden. Bei etwa konstanter Ionenstärke in Fig. 3 ergeben sich die in Tabelle III angeführten Neigungen m-y, die mit den nach Gl. (7) berechneten Werten für m verglichen werden.

$$m = \frac{K_1[L] + 2\,K_1 \cdot K_2[L]^2 + 3\,K_1 \cdot K_2 \cdot K_3[L]^3 + 4\,K_1 \cdot K_2 \cdot K_3 \cdot K_4[L]^4}{1 + K_1[L] + K_1 \cdot K_2[L]^2 + K_1 \cdot K_2 \cdot K_3[L]^3 + K_1 \cdot K_2 \cdot K_3 \cdot K_4[L]^4} \qquad (7)$$

Die Werte für m wurden mit Hilfe der von DEELSTRA UND VERBEEK angegebenen Stabilitätskonstanten berechnet[6]. Obwohl die Bestimmung von log R_M und damit auch die von m-y mit gewissen Fehlern behaftet ist, zeigen die aus mehreren Versuchen erhaltenen Werte, dass die durchschnittliche Ligandenzahl in der organischen Phase zwischen 0.2 und 0.5 liegt. Auch an Sulfonsäureaustauschharzen werden partiell komplexierte Seltenerdionen zurückgehalten[7].

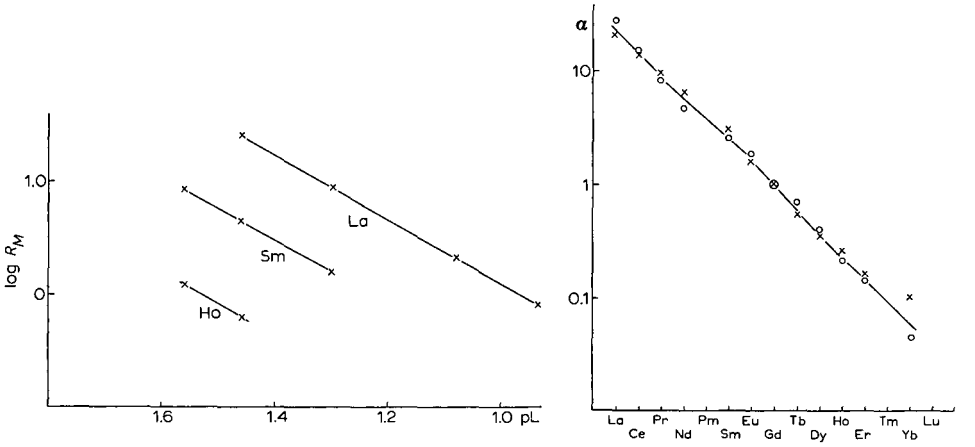


Fig. 3. Abhängigkeit der log R_M-Werte für La, Sm und Ho von pL der α-Hydroxyisobuttersäure bei Gegenwart von 0.2 M $NaClO_4$.

Fig. 4. Vergleich der papierchromatographisch und rechnerisch ermittelten Trennfaktoren für α-Hydroxyisobuttersäure. × = papierchromatographisch erhaltene Werte; O = berechnete Werte.

Die log R_M-Werte verschiedener Seltener Erden unterscheiden sich bei Abwesenheit eines Komplexbildners im Elutionsmittel nur wenig[2]. Daher sind die grossen Unterschiede bei der Komplexelution auf die Unterschiede der Komplexstabilitätskonstanten zurückzuführen. Die Fig. 4 und 5 zeigen die papierchromatographisch und rechnerisch[6] erhaltenen Trennfaktoren in Bezug auf das Element Gadolinium. Für den Trennfaktoren α der Elemente A und B gilt:

$$\alpha_A{}^B = \frac{R_M{}'^B}{R_M{}'^A} = \frac{R_M{}^B}{R_M{}^A} \times \frac{1 + \beta_1{}^A[L] + \beta_2{}^A[L]^2 + \beta_3{}^A[L]^3 + \beta_4{}^A[L]^4}{1 + \beta_1{}^B[L] + \beta_2{}^B[L]^2 + \beta_3{}^B[L]^3 + \beta_4{}^B[L]^4}$$

mit $R_M{}'$ = R_M-Wert bei Anwesenheit des Komplexbildners, R_M = R_M-Wert bei

TABELLE III

DURCHSCHNITTLICHE LIGANDENZAHLEN

pL	*La*			*Sm*			*Ho*		
	log R_M	*m*	*m-y*	*log* R_M	*m*	*m-y*	*log* R_M	*m*	*m-y*
0.93	—0.07	2.70	2.80	—	—	—	—	—	—
1.08	0.35	2.53		—	—	—	—	—	—
1.30	0.95	2.26	2.72	0.19	2.85	2.94	—	—	—
1.46	zu ungenau			0.66	2.52		—0.23	3.07	3.20
1.56	—	—	—	0.95	2.37	2.90	0.09	2.94	

Abwesenheit des Komplexbildners, β_i = Produkt der Komplexstabilitätskonstanten $K_1 \cdot K_2 \ldots \cdot K_i$.

Fig. 4 und 5 zeigen eine gute Übereinstimmung der berechneten und experimentell gefundenen Trennfaktoren. Die Abweichungen bei den letzten Elementen der Lanthanidenreihe liegen an der Schwierigkeit, sehr kleine R_M-Werte genau zu bestimmen bzw. an eventuellen Fehlern der Komplexkonstanten. Diese Betrachtungen setzen voraus, dass die Ligandenkonzentration gegenüber der Seltenerdkonzentration gross ist. Diese Bedingung ist durch die gewählte papierchromatographische Arbeitsweise erfüllt. In Fig. 6 sind die log R_M-Werte für drei verschiedene Elutionsmittel in Bezug auf das Element Gadolinium aufgeführt. Die durchschnitt-

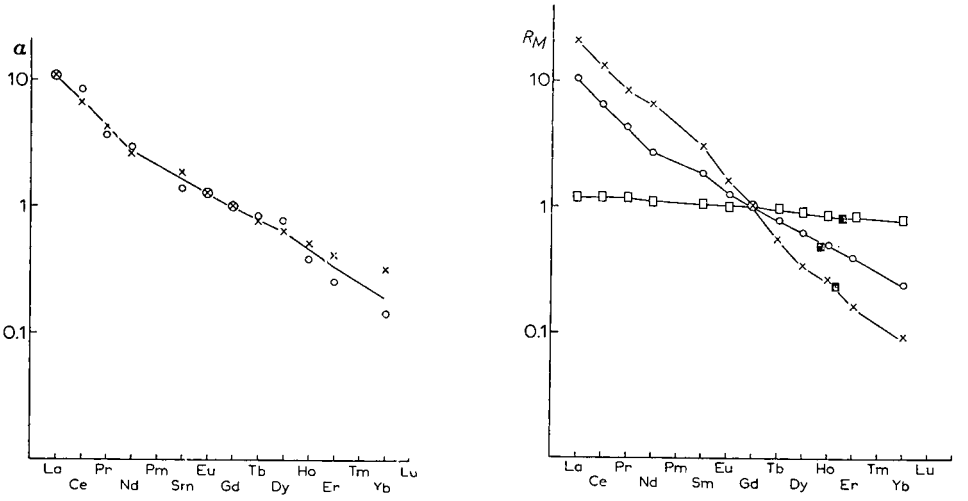


Fig. 5. Vergleich der papierchromatographisch und rechnerisch ermittelten Trennfaktoren für Milchsäure. × = papierchromatographisch erhaltene Werte; ○ = berechnete Werte.

Fig. 6. R_M-Werte der Seltenen Erden bei der Verwendung verschiedener Elutionsmittel bezogen auf Gadolinium (R_M 1). × = α-Hydroxyisobuttersäure; ○ = Milchsäure; □ = Salzsäure; ■ = Werte für Yttrium.

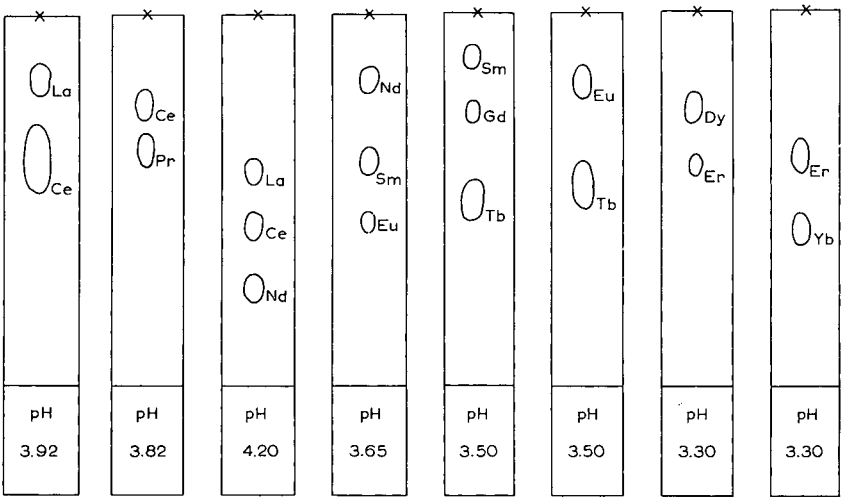


Fig. 7. Papierchromatographische Trennung einiger Seltener Erden bei Elution mit 0.12 *M* α-Hydroxyisobuttersäure.

lichen Trennfaktoren für benachbarte Elemente betragen 1.03 bei Salzsäure, 1.35 bei Milchsäure und 1.51 bei α-Hydroxyisobuttersäure. Wegen der grösseren Unterschiede der Komplexstabilitäten ist α-Hydroxyisobuttersäure am besten zur Trennung geeignet. Das Element Yttrium ordnet sich entsprechend seinem Ionenradius beim Holmium ein.

Die Fig. 7 und 8 zeigen einige massstabgetreue Chromatogramme, auf denen 2–4 Nachbarelemente getrennt wurden. Es ist möglich, unter den gewählten Elutionsbedingungen beliebige Kombinationen der Elemente Lanthan, Cer, Praseodym, Neodym, Samarium, Europium, Gadolinium, Terbium, Dysprosium, Holmium,

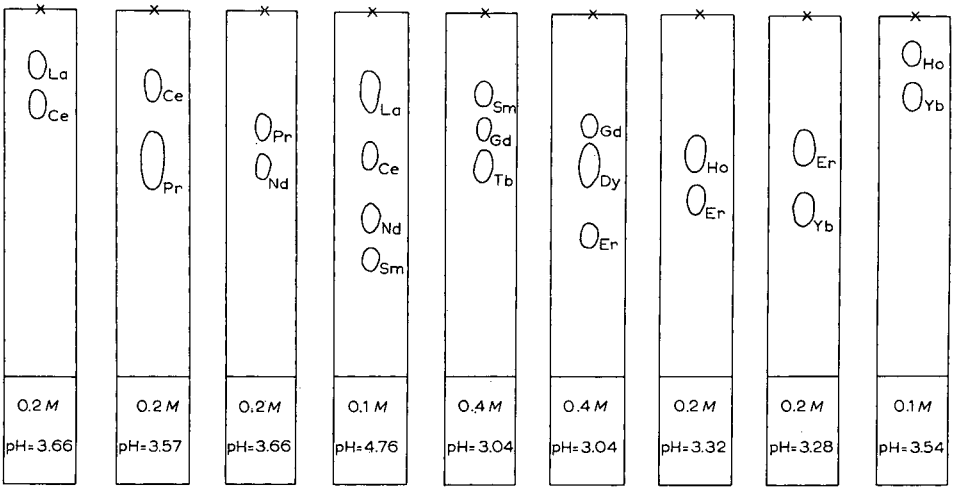


Fig. 8. Papierchromatographische Trennung einiger Seltener Erden bei Elution mit Milchsäure.

Erbium und Ytterbium mit Ausnahme der Paare Eu–Gd, Tb–Dy und Dy–Ho zu trennen.

Eine weitere Verbesserung der Trennung ist zweifellos durch Verwendung solcher Elutionsmittel zu erreichen, bei denen die Unterschiede der Komplexstabilitäten noch grösser sind. Dabei ist allerdings zu beachten, dass die absoluten Werte der Stabilitätskonstanten nicht sehr hoch sein dürfen. Bei sehr stabilen Komplexen besteht keine Möglichkeit der chemischen Indikation der Flecke.

DANK

Dem Direktor des Instituts für Anorganische Chemie der Karl-Marx-Universität, Herrn Prof. Dr. H. HOLZAPFEL, danke ich für die Möglichkeit zur Durchführung dieser Arbeit und seine stets gewährte Unterstützung.

ZUSAMMENFASSUNG

Mit Dinonylnaphthalinsulfonsäure imprägniertes Chromatographierpapier kann zur Trennung der Seltenen Erden verwendet werden. Bei der Verwendung von komplexbildenden Elutionsmitteln (Milchsäure, α-Hydroxyisobuttersäure) werden die Trennfaktoren durch die Stabilitäten der Seltenerdkomplexe bestimmt. Jeweils 2–4 benachbarte Elemente aus der Reihe Lanthan, Cer, Praseodym, Neodym, Samarium, Europium, Gadolinium, Terbium, Dysprosium, Holmium, Erbium und Ytterbium können mit Ausnahme der Paare Eu–Gd, Tb–Dy und Dy–Ho auf einem Chromatogramm getrennt werden.

SUMMARY

Paper impregnated with dinonylnaphthalenesulphonic acid can be used for the chromatographic separation of the rare earths. When using complexing eluents (lactic acid, α-hydroxybutyric acid) the separation factors are determined by the stability of the rare earth complexes. With the exception of the Eu–Gd, Tb–Dy and Dy–Ho pairs, any 2–4 adjacent elements in the series lanthanum, cerium, praseodymium, neodymium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, and ytterbium can be separated on one paper chromatogram.

LITERATUR

1 M. N. SASTRI UND A. P. RAO, *Z. Anal. Chem.*, 196 (1963) 166.
2 G. WERNER, *Z. Chem.*, 5 (1965) 147.
3 G. WERNER, *Z. Chem.*, 5 (1965) 311.
4 *Brit. Pat.*, 812,131 (1956).
5 H. DEELSTRA UND F. VERBEEK, *J. Chromatog.*, 17 (1965) 558.
6 H. DEELSTRA UND F. VERBEEK, *Anal. Chim. Acta*, 31 (1965) 251.
7 L. W. HOLM, G. R. CHOPPIN UND D. MOY, *J. Inorg. Nucl. Chem.*, 19 (1961) 251.

CHROMATOGRAPHIC TECHNIQUES USING LIQUID ANION-EXCHANGERS*

I. HCl SYSTEMS

U. A. TH. BRINKMAN**, G. DE VRIES AND E. VAN DALEN
Chemical Laboratory, Free University, Amsterdam (The Netherlands)
(Received October 1st, 1965)

INTRODUCTION

Liquid ion-exchangers have received considerable attention in analytical chemistry since their introduction in 1948[1]. These high-molecular-weight acids and bases have been used mainly as extractants and they have met with remarkable success, as is clearly shown in the reviews by COLEMAN[2], COLEMAN *et al.*[3], GREEN[4] and MOORE[5].

During recent years, however, an interesting situation has arisen, in which these liquid ion-exchangers are used in chromatographic techniques, similar procedures having been developed at the same time for high-molecular-weight neutral organophosphorus compounds (*cf.* refs. 6 and 7). The early successful work in this branch of chemistry has been reviewed by CERRAI[6], who mainly describes the results of his own group. After the initial use of TnOA***, this group has shifted its attention mainly towards HDEHP, that has been extensively used for *e.g.* rare earth separations. Other groups of workers (*cf.* literature in ref. 6 and *e.g.* refs. 8–11) have also paid attention to the use of HDEHP in chromatography and a detailed study has been made of the theoretical aspects of the column procedures[12,13].

Amines and substituted ammonium salts, however, have been used only infrequently, although far more compounds of this class are commercially available than are the high-molecular-weight acids—of which up to date only HDEHP and DNS[14,15] appear to have been used to any extent. The summary of the uses of basic liquid ion-exchangers in chromatography given in Table I shows that these compounds have hardly been investigated, though the initial results were very successful. We have therefore turned our attention to a more systematic study in order to investigate both the possibility of qualitative and quantitative separations and the correspondence between data on extraction, ion-exchange and chromatography (*cf. e.g.* ref. 6).

In order to simplify the time-consuming preliminary investigations, a rapid thin-layer technique has been developed[28].

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* A preliminary communication of our work has been published[28].
** This paper forms part of the work done by U.A.Th.B. for his Ph. D. Thesis.
*** Abbreviations: TnOA = tri-*n*-octylamine; TiOA = triisooctylamine; HDEHP = hydrogen di-(2-ethylhexyl) phosphate; DNS = dinonylnaphthalenesulphonic acid.

TABLE I

LITERATURE SURVEY OF LIQUID ION-EXCHANGE CHROMATOGRAPHY USING HIGH-MOLECULAR-WEIGHT AMINES AND SUBSTITUTED AMMONIUM SALTS*

*Solution***	*Methods*	*Eluant*	*Ions separated****	*References*
TnOA				
0.2 *M* in kerosene (b.p. 30–50°)	Paper: Whatman No. 1; radial (7 cm in 15 min) and descending	HCl (1–10 *N*) (+ HNO_3); H_2SO_4 (0.4–2.0 *N*)	HCl: U–Zr–Th; Fe–Cu–Al; Fe/Zn–Co–Mn–Ni; Zr–Hf; U–V–Ti; H_2SO_4: U/Mo–Fe/Cu–Ni; etc.	16
0.01–0.6 *M* in benzene eq. with HNO_3 or acidified nitrate (1:3, v/v) for 5 min; dried	Paper: Whatman No. 1; radial (7 cm in 15 min) and descending; 20°	HNO_3 (1–10 *N*); acidified nitrates of Li, Ca, Al (0–6 *M*), Na (0–8 *M*) and NH_4 (0–10 *M*)	R_F spectra for Th, U and La (Ce)	17
0.1 *M* in benzene eq. with HCl or NH_4NO_3 (1:2, v/v) for 10 min; dried	Column (approx. 20 × 1 cm^2; 0.2–0.3 ml/min): Whatman No. 1 cellulose powder, dried 2 h at 85° before and after equilibration; mostly 1–15 mg per element	HCl, HNO_3 and NH_4NO_3	Ni–Co–Fe; Th–Zr–U; Zr–La; Hf–Zr; etc.	18
0.2–0.25 (paper) and 0.5 (column) *M* in benzene eq. with 2 *N* HNO_3 (1:2, v/v); dried	Paper: Whatman No. 1 (CRL/1); ascending; column (140 × 13 mm^2; 0.5 ml/min): Whatman No. 1 cellulose powder, mixed with TnOA in benzene for 12 h; dried 2 h at 80°; approx. 20 mg per element	$LiNO_3$ (1–10 *M*) acidified with $2 \cdot 10^{-3}$ *N* HNO_3	Rare earth elements and Sc, Y, Th and Ba; *e.g.*: Th–Ce–Nd–Gd and Pr–Sm–Dy on paper (with 3 *M* $LiNO_3$) and Ba–Yb–Nd–La on column (gradient elution)	19
0.1 *M* in cyclohexane	Paper: Whatman No. 1; ascending	80% (v/v) methanol–HNO_3 mixtures	Rare earths; no positive results	20
0.2 *M* in benzene eq. with 2 *N* HNO_3 (1:2, v/v)	Paper: Whatman No. 1; ascending (2 h); 15°; 0.3 ml TnOA solution for 1.5 × 21 cm^2 paper	NH_4NO_3 (2–10 *M*) + HNO_3 ($2 \cdot 10^{-3}$–1 *N*)	R_F spectra for U, La, Y, Sm, Eu and Gd	21, 22
Undiluted TnOA	Column (130 × 7 mm^2; 1–2 ml/min): 1.5 g Kel-F (per column) + 1.5 ml TnOA + a few ml 4 *N* H_2SO_4 slurried into column	H_2SO_4 (0.2 *N*) and HNO_3 (1.5 *N*)	3 μg Pu (HNO_3) from 5 mg U (H_2SO_4)	23

Undiluted TnOA	Column (80 × 2.8 mm²; 0.03 ml/min): powdered Teflon (150–250 mesh) + 375 mg TnOA; washed with H_2O and 1 *N* HCl; 20°; 2 ng–60 μg per element	HCl (0.01–6 *N*) and ammoniacal NH_4 acetate (0.2 *M*)	Mn/Co/Ni/Cu or Ca/Sr/Y from Fe and Zn or Cd; Th/Pa–U	24
0.1 *M* in benzene	Paper: Whatman No. 1 (CRL/1); ascending (approx. 15 cm). One end of paper dipped in TnOA solution; after drying rest of paper treated with 0.1 *M* HDEHP in cyclohexane	HCl (1–4 *N*) and HNO_3 (6 *N*)	Fe–Zr–Gd–Pr; U–Sc–Yb–Y–La; Zn–Th–Al; etc.	25
Aliquat (and TnOA)				
0.25 *M* in benzene eq. with 0.1 *N* HCl (1:1, v/v) to remove Fe(III); after dilution to 0.1 *M*, eq. with 6 *N* HCl (1:2, v/v)	Paper: Whatman No. 1 (CRL/1)	HCl (0–11.5 *N*)	R_F spectra for Fe, Co, Ni, U, Zr and Hf, compared with those found using TnOA	26
TiOA				
0.185 *M* in ether eq. with acid (1:2, v/v) for 2 min	Thin layer (2–7 cm in 5–30 min): kieselgel G (16.8 g) + TiOA in ether 2 h at 100°; then plus $CaSO_4 \cdot 2\,H_2O$ (2.4 g) + 36 ml H_2O; dried 2 h in the air and 2 h at 100°	HCl (3 and 8 *N*); HNO_3 (5 *N*) and H_2SO_4 (2 *N*)	2- and 3-component separations from the series Mo, V, Cu, Fe, Co, Ni, Zn, U, Ti, Th, Zr, Mn, Cr(VI)	27
Tri-n-alkylamines				
0.1 *M* $(n\text{-}C_4H_9)_3N$ to $(n\text{-}C_9H_{19})_3N$ in benzene; saturated with 1 *N* HNO_3	Paper: Whatman No. 1; ascending (2 h); 15°	NH_4NO_3 (4 *M*) + HNO_3 ($2 \cdot 10^{-3}$ *N*)	U–La–Sm/Eu/Gd/Y	21
Amberlite LA-1				
0.1 *M* in $CHCl_3$ or benzene eq. with 2 *N* HCl (1:3, v/v) for 10 min; dried over Na_2SO_4	Paper: Whatman No. 1 (or S and S 2043a); ascending (15 cm in 3 h); room temp. Thin layer (approx. 3 cm in 10 min): silica gel (Fluka DO); room temp.	HCl (0.5–11.5 *N*)	R_F spectra for Ni, Co, Zn, Mn, Al, Fe, Sb(III), Cu, Cd, Hg(II), Pb and Bi	28

* A review of early chromatographic work with TnOA is included in ref. 6.

** Under this heading is given the organic solution equilibrated (eq.) with the inorganic solution under the specified conditions.

*** From articles giving merely a large number of two- to four-component separations, only a few representative ones are tabulated; this is indicated by "etc.". A/B–C means that either A or B can be separated from C.

Unless otherwise indicated, the ions mentioned were in their normal oxidation state, *e.g.*, Fe(III), U(VI), Cu(II), Ce(III), V(V) and Mo(VI).

MATERIALS AND METHODS

Chemicals

Amberlite LA-1, a fairly viscous yellow liquid (viscosity at 25° 72 c.p. (ref. 4)) is a N-dodecenyltrialkylmethyl-amine. The unsaturated amine contains 24–27 carbon atoms and has an average mol. wt. of 372.

Amberlite LA-2, also yellow coloured, but with a much lower viscosity (18 c.p. at 25° (ref. 4)) is a saturated secondary amine, N-lauryltrialkylmethyl-amine, with an average mol. wt. of 374.

Hyamine 2389, a slightly yellow coloured, very viscous "wetting agent", is a mixture of methyldodecylbenzyltrimethyl-ammonium chloride and methyldodecylxylylene-bis-(trimethylammonium chloride). The average mol. wt. was calculated to be 420, based on the assumption that the mole ratio of the chlorides was 1 : 1.

Hyamine 10X, a white crystalline powder used as a germicide or as a deodorant, is diisobutylcresoxyethoxyethyldimethylbenzyl-ammonium chloride (Lot No. 2762, with 1.2 % inert ingredient); the mol. wt. of the pure monohydrate is 480.

Hyamine 1622, also a white crystalline substituted ammonium chloride used as a germicide or deodorant, is diisobutylphenoxyethoxyethyldimethylbenzyl-ammonium chloride and the pure monohydrate has a mol. wt. of 466.

Primene JM-T is a yellow coloured primary trialkylmethylamine with 18–24 carbon atoms and an average mol. wt. of 311.

The six chemicals mentioned above are all manufactured by Rohm and Haas Co., Philadelphia, Pa. (U.S.A.).

Tetrahexylammonium iodide, a white crystalline product from Eastman Kodak, Rochester, N.Y. (U.S.A.) has a mol. wt. of 481. It is converted into the chloride by shaking the solution of the iodide in $CHCl_3$ for some minutes with a 5-fold excess of freshly precipitated Ag_2O, decanting off the supernatant solution and treating the hydroxide with HCl.

Triisooctylamine, a nearly colourless technical grade product (Lot No. 55055X) from K and K Laboratories, Plainview, N.Y. (U.S.A.) has a mol. wt. of 353. TiOA contains a mixture of dimethylhexyl, methylheptyl, etc. chains, principally 3,5-, 4,5- and 3,4-dimethylhexyl.

Tri-n-octylamine is a colourless practical quality product (mol. wt. 353) purchased both from K and K Laboratories (Lot No. 48485) and from Fluka, Buchs, S.G. (Switzerland).

Tribenzylamine, a white crystalline solid (mol. wt. 287) of purissimum (> 99 %) quality was also purchased from Fluka.

Aliquat 336, obtainable as the chloride, is a methyltri-*n*-alkyl-ammonium salt ("methyltricaprylyl-ammonium chloride") with 27–33 carbon atoms on an average. The alkyl groups mainly consist of octyl and decyl chains, and the average mol. wt. of this yellow viscous liquid was assumed to be 475.

Alamine 336, a pale yellow liquid (Lot No. 4L108), also called "tricaprylylamine", is a tertiary amine with three straight-chain alkyls, mainly octyls and decyls. The number of carbon atoms totals 26–32 and the average mol. wt. of the tertiary amine content (min. 90 %; typical 95 %) is 392. Both Alamine and Aliquat were gifts from General Mills Inc., Kankakee, Ill. (U.S.A.).

Adogen 364, a tri-*n*-alkylamine with 27 carbon atoms on an average, principally consists of octyl (60 %) and decyl (33 %) chains. It contains minimally 95 % tertiary amine. In the yellow turbid liquid a waxy deposit is formed on standing. For our work, 380 was used as average mol. wt.

Adogen 368, a pale yellow coloured tri-*n*-alkylamine, of which the alkyl groups principally consist of octyl (40 %), decyl (25 %) and dodecyl (30 %) chains, has a minimal percentage tertiary amine of 95 %. We used 430 as average mol. wt.

Adogen 464, a methyltri-*n*-alkyl-ammonium chloride with a yellow-brown colour, has a minimal percentage of quaternary ammonium salt of 92 %; its average mol. wt. is 431.

All Adogens mentioned above were received as gifts from Archer-Daniels-Midland Co., Minneapolis, Minn. (U.S.A.); the Lot numbers were 296-442-910, 281-22-306 and SR No. 27822, respectively.

All amines and substituted ammonium salts were used without further purification. They must be handled with care, as, *e.g.*, in the case of aliphatic amines the manufacturers point out that although the volatility is low, it is very important, particularly before dilution, to provide adequate ventilation to prevent inhalation of the vapours. Every precaution should be taken to avoid contact with the eyes or prolonged contact with the skin[4]. With some of the quaternary Hyamines the same rules are prescribed.

The ions tested included: Ag(I), Ni(II), Mn(II), Co(II), Zn(II), Cu(II), Pb(II), Fe(III), Sb(III) and Bi(III). The solutions of their chlorides—lead and silver were present as the nitrates—contained 2–8 μg cation per μl and were acidified as far as necessary in order to prevent hydrolysis. Hydrochloric acid solutions, made from HCl, analytical purity, were standardized to within $\pm$ 0.03 *N* of the desired normality.

Preparation of thin-layer plates and papers

The amine is converted into its HCl salt by equilibrating a 0.10 (thin layer) or 0.15 (paper) molar solution in chloroform or benzene for 10 min in a separatory funnel with 3 vol. of 2 *N* HCl (*cf.* RESULTS). The organic solution is separated, filtered and dried for some hours over sodium sulphate. Quaternary ammonium chlorides are equilibrated with HCl in the same way.

Thin layer. The amine·HCl or quaternary ammonium chloride solution, freed from sodium sulphate by filtration, is mixed thoroughly with silica gel (silica gel–$CHCl_3$ (1:2, v/v)); the resulting suspension is stored overnight and agitated again before use.

Thin-layer plates are prepared by dipping ordinary microscope slides (2.5 × 7.5 cm^2) into the silica gel–$CHCl_3$ mixture for approx. 5 sec. On leaving the glass plates for some minutes in the air, in order to evaporate off the chloroform, a thin film of amine-impregnated silica gel adheres to the slides. Using cotton wool a margin of 4 mm is made along the edges of the slides in order to avoid undesirable contact with the eluting agent.

The thin-layer plates so prepared are spotted (7 mm from the end; 2 spots per plate) with the solution to be investigated using a 1-mm wide strip of filter paper; the resulting spot ought to have a diameter of 1–2 mm. When the spots have been air-dried, the thin-layer plates are put into small vessels (Hellendahl staining jars) containing the eluting agent (approx. 3.5 ml; 6 slides per vessel) and the chromato-

gram is developed for approx. 10 min using the ascending technique. Subsequently, the plates are dried in air and the spots are visualized using conventional techniques.

Sometimes rather thin silica gel layers are obtained, when using the technique described above. This causes an uneven and slow flow of the eluants. In these cases a thicker layer may be obtained by dipping once more after the $CHCl_3$ has evaporated. This technique was used with *e.g.* Hyamine 1622 and Aliquat 336.

Paper. Air-dried Whatman paper No. 1, used without further purification, is treated with the amine·HCl or quaternary ammonium chloride solution for some seconds. This is conveniently done by continuously pulling the paper at a rate of 2–3 cm/sec through a Hellendahl staining jar, partly filled with the solution of exchanger. It is then dried in an air stream or by hanging it in the air for some minutes and cut into strips of the desired length; both 2- and 3-cm wide papers were used.

The papers are spotted (2.5 cm from the end) by using a micropipette or analogous device, so that spots with a diameter of 3–5 mm are formed. When the spots are air-dry the papers are hung in a suitable rack in glass jars (30 × 9 cm^2) containing the eluting agent (50 ml; 6 strips per jar). The chromatograms are developed for 2–3 h using an ascending technique; after air-drying, the spots are visualized using conventional techniques.

Detection

The ions were identified using conventional reagents. In some instances serious difficulties were encountered, *e.g.* with Mn and Pb. The same phenomena were occasionally found with ions not mentioned in this paper and in experiments with HDEHP-treated supports. Apparently, the liquid ion-exchangers sometimes decrease the sensitivity of normally well established spot tests. In these cases, Mn was detected by spraying with the KIO_4–tetrabase reagent[29] and Pb was identified by dipping into a sodium rhodizonate solution[29], after carefully driving off the amine by heating over a small bunsen flame.

General remarks

All experiments were done at ambient temperature (20°) and were carried out at least in duplicate; with regard to the composition of the eluting agents, acclimatisation was not deemed necessary.

In thin-layer work some suspensions altered their behaviour somewhat on standing, older suspensions giving thicker silica gel layers and sometimes also altered R_F values. Therefore care was taken to obtain roughly comparable conditions by leaving all suspensions standing for one night. Even then the first 5–10 thin layers prepared were sometimes quite unsatisfactory, but no difficulties were ever encountered after the suspension had been duly agitated again. This point needs further attention when investigating *e.g.* quantitative correlations between chromatography and extraction. In this respect paper chromatography may be advantageous, because a more even layer is obtained here.

In paper chromatography, the travelling distance of the solvent front was always taken as approx. 15 cm; development took about 3 h, with notable exceptions only in the presence of sirupy substances such as decanol (see *Paper chromatography*). In thin-layer work, 30 mm was taken as the average travelling distance; travelling time was approx. 10 min for 0.5–7 *N* HCl and 12.5–15 min for higher concentrations.

RESULTS

Development of the procedure

Preliminary investigations were carried out in order to find methods suitable for rapid and reliable thin-layer and paper chromatography.

Equilibration. Using Amberlite LA-1, equilibration was initially carried out with HCl of the same normality as that used as eluting agent. However, it was soon found that equilibration with 2 *N* HCl in all cases—irrespective of the normality of the eluting agent—gave insignificant differences only. Therefore, this technique was used in all further work.

Supports. Thin-layer plates were made using either silica gel, cellulose powder, kieselguhr or polyethylene and polyglycol powder. Layers made with silica gel were far better than all others and therefore all further research was done using this support.

The silica gel was used without further purification or drying. It contained some iron, which could be detected as a zone when Fe(III) had an R_F value of 0.6–1.0.

Paper chromatograms made using Whatman paper No. 1 and S and S paper 2043a give quite analogous results; S and S 2040a and 2045a proved somewhat less satisfactory, though still useful. Whatman paper No. 1 was used for all systematic work.

The necessity of rigorously drying both the organic solution and the paper strips was investigated using the following modifications: (a) air-dried paper is impregnated with the organic solution obtained after equilibration with 2 *N* HCl; (b) air-dried paper is impregnated with the same solution, dried for some hours over sodium sulphate; (c) paper dried for 2 h at 80° is impregnated with the sodium sulphate-dried solution.

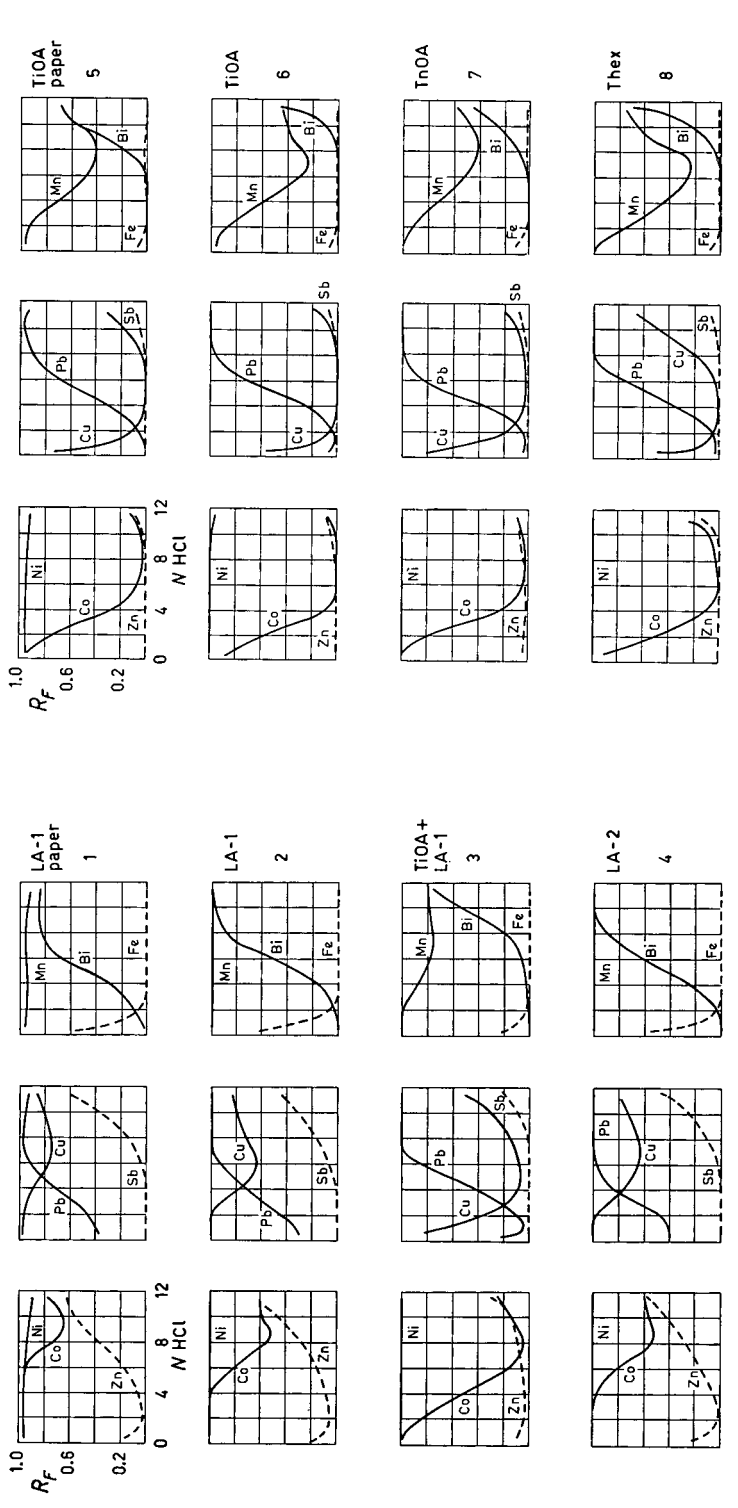
Experiments showed only relatively small differences, especially at the 0.10–0.15 *M* concentration range mostly used by us. Therefore, both techniques (b) and (c) may be recommended, but it would be better to reject (a) because the variable water content of the organic solution obtained after equilibration is an unknown factor in the subsequent processing and may adversely influence the results.

Paper strips—either air-dried or after having been heated for 2 h at 80°—were impregnated with liquid ion-exchanger both by dipping them in or pulling them through the organic solution for 2–5 sec. The organic solvent was driven off afterwards either using a fan or by hanging the strips in the air for some minutes. It was found that these and similar slight modifications did not alter the results to any significant extent.

Concentration dependence. Using the thin-layer technique, preliminary experiments were carried out using from 0.05 to 0.25 *M* Amberlite LA-1·HCl equilibration solutions. Some results are summarized in Table II, where it may be seen that the R_F values vary considerably over the 0.05–0.25 *M* concentration range, decreasing with increasing concentration, as does the migration rate of the eluant (also see DISCUSSION).

It was found that the best results were obtained with 0.10 *M* solutions, which were used for all systematic investigations. For paper chromatography, on the other hand, 0.15 *M* solutions appeared to be more suitable.

Reproducibily. The results of duplicate experiments in thin-layer chromatography generally showed good agreement ($\pm$ 0.02–0.03 in R_F value). However, when ex-



Figs. 1–4. R_F spectra for 9 ions using various amine·HCl systems. (1) 0.15 *M* Amberlite LA-1; paper. Bi forms elongated spots from 0.5 to 6 *N* HCl. (2) 0.10 *M* Amberlite LA-1; thin layer. (3) 0.10 *M* Amberlite LA-1–0.10 *M* TiOA (1:1, v/v); thin layer. (4) 0.10 *M* Amberlite LA-2; thin layer.

Figs. 5–8. R_F spectra for 9 ions using various amine·HCl and quaternary ammonium salt systems. (5) 0.15 *M* TiOA; paper. Fe forms comets at all HCl concentrations. (6) 0.10 *M* TiOA; thin layer. (7) 0.10 *M* TnOA; thin layer. Small Fe-comets at 10 *N* and conc. HCl. (8) 0.10 *M* tetrahexylammonium chloride; thin layer. Elongated spots found for Mn over the whole range, and for Co and Cu at low HCl concentrations.

periments were repeated after the silica gel suspension had been left standing for some time, somewhat altered R_F values were occasionally obtained (also see above). In view of the qualitative character of the work this aspect of our analyses has not been deeply investigated until now. In paper chromatography the results showed good reproducibility.

*Amines**

Amberlite LA-1. This secondary unsaturated amine is very suitable for chromatographic work. Figs. 1 and 2 summarize results of both thin-layer and paper chromatography, which show close correspondence, with some divergence only in the case of Co. These results indicate that many interesting qualitative separations may be realised in approx. 10 min, using the thin-layer technique developed by us, which has already been used[28] for a few cases.

TABLE II

DEPENDENCY OF R_F VALUES AND MIGRATION RATE OF ELUANT ON MOLARITY OF AMINE · HCl SOLUTION
Amberlite LA-1 was used as liquid ion-exchanger and approx. 7 *N* HCl as the eluting agent.

Ion	*R_F value at amine molarity*			
	0.05	*0.10*	*0.15*	*0.25*
Cu	0.79	0.64	0.45	0.30
Co	0.76	0.62	0.46	0.23
Bi	0.89	0.72	0.58	0.38
Zn	0.32	0.17	0.11	0.02
Length of run (mm)	35	32	26	20
Time (min)	10	10	10	20

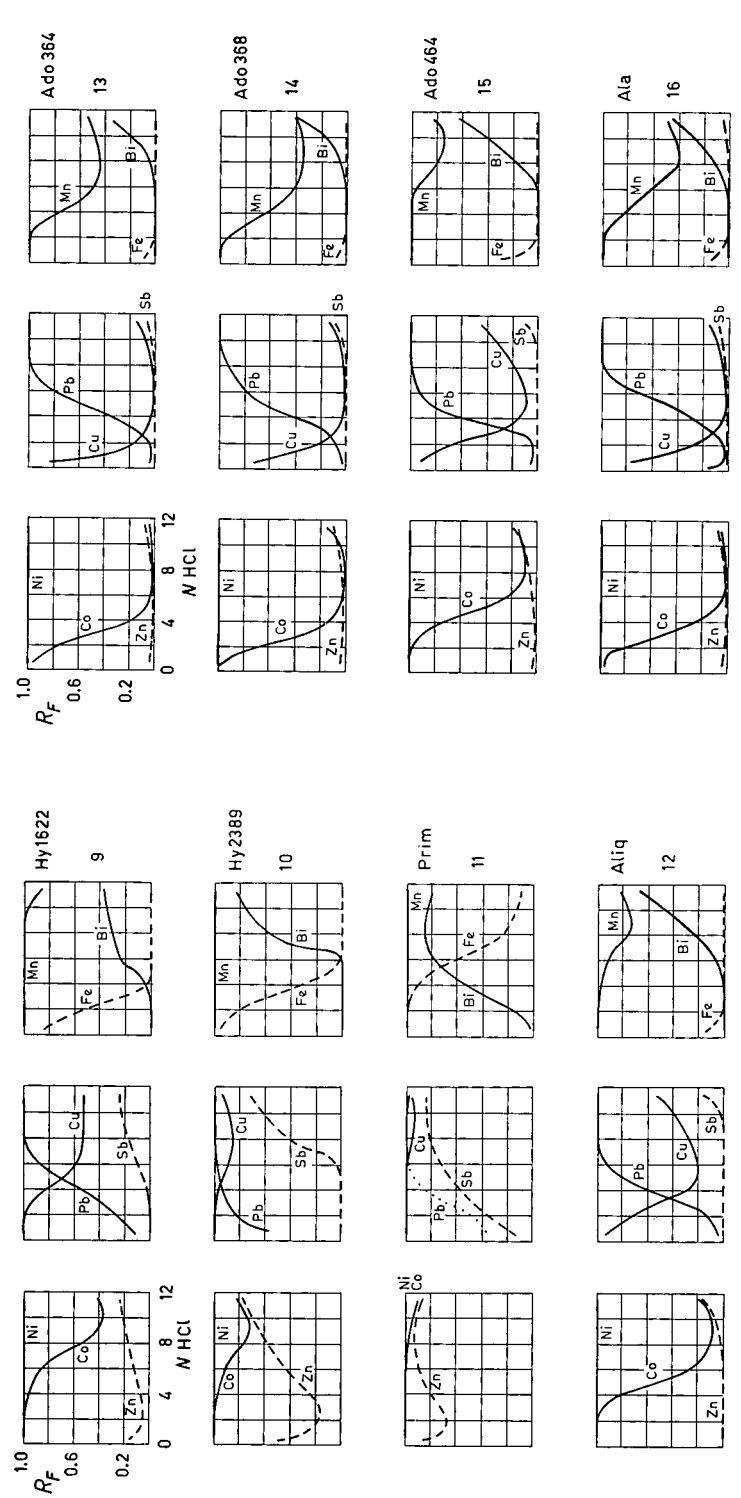
For data on Al, Hg(II) and Cd, one is referred to ref. 28; with some of the ion-exchangers discussed in this paper, these 3 ions were again investigated. It was shown, however, that Al always had $R_F = 1.0$, with a tendency to tail with conc. HCl as eluant, while Hg(II) and Cd behaved analogously to Sb(III). Therefore, they were not included in most of our experiments and are not recorded in the Figs.

Amberlite LA-2. Apart from its saturated character, Amberlite LA-2 has a structure fairly analogous to that of Amberlite LA-1. Therefore, it is not surprising that the results found with this amine agree closely with those found using Amberlite LA-1 (see Fig. 4).

Triisooctylamine. TiOA, like Amberlites LA-1 and LA-2, yields excellent thin layers, but the eluting agent travels more slowly and approx. 15 min are necessary for development. Because TiOA is strongly adsorbing** (see Fig. 6), combination of TiOA with another less powerful amine such as an Amberlite could lead to an ex-

* The term amines has sometimes been used to indicate both amines and substituted ammonium salts.

** High (low) adsorption strength means that the anion-exchanger in question strongly (weakly) retards the movements of anions, thereby leading to low (high) R_F values for those ions. Use of this term does not imply an explanation of the mechanism of the present chromatographic processes.



Figs. 9–12. R_F spectra in thin-layer chromatography for 9 ions using various amine·HCl and quaternary ammonium salt systems. (9) 0.10 *M* Hyamine 1622; Pb forms elongated spots for 0.5–4 *N* HCl. (10) 0.10 *M* Hyamine 2389. Pb and Zn form elongated spots below 4 *N* HCl; R_F spectra for Sb and Bi are irreproducible (± 0.10 R_F value) above 6 *N* HCl. (11) 0.10 *M* Primene JM-T. For 2–6 *N* HCl part of Pb is found back at the origin. (12) 0.10 *M* Aliquat 336.

Figs. 13–16. R_F spectra in thin-layer chromatography for 9 ions using various amine·HCl and quaternary ammonium salt systems. (13) 0.10 *M* Adogen 364. Fe forms comets at all HCl concentrations. (14) 0.10 *M* Adogen 368. Fe-comets more pronounced than with Adogen 364. (15) 0.10 *M* Adogen 464. Small comets for Fe at high normalities of HCl, and for Pb at 0.5–4 *N* HCl. (16) 0.10 *M* Alamine 336.

cellent mixture. Therefore, thin layers were prepared using 0.10 *M* TiOA–0.10 *M* Amberlite LA-1 (1:1, v/v) as amine mixture. Comparison of the data so obtained (Fig. 3) with those summarized in Figs. 2 and 6 shows a close agreement between that predicted and found. Elution time is also intermediate between those found with the separate components.

With TiOA, correspondence between paper (Fig. 5) and thin-layer data is as good as in the case of Amberlite LA-1.

Tri-n-octylamine. TnOA, the only amine used until now to a significant extent (*cf.* ref. 6 and Table I), yields thin layers of reasonable quality and its adsorption power is fairly high (Fig. 7).

Tetrahexylammonium chloride. This ammonium salt has approximately the same adsorbing power as TiOA (Fig. 8), but it forms many badly tailing spots, especially with Cu, Mn and Bi. Moreover, the detection of some ions, *e.g.* Sb, is difficult. Here, as with some other amines, especially Zn and Cd form thinnish spots when 10 *M* or conc. HCl are used as eluants; there is hardly any reaction with reagents such as 8-hydroxyquinoline, but the thinnish spot—looking very much like a finger-print—makes detection easy.

Hyamines 1622 and 10X. From these two very analogous quaternary ammonium salts Hyamine 1622 was chosen for thin-layer experiments. Some difficulties were encountered during equilibration, separation of the organic and aqueous layers being a time-consuming process, that took at least some hours. When preparing thin layers, the "twice dipping" technique (see under METHODS) was often used.

Fig. 9 shows that Hyamine 1622 adsorbs more strongly than the Amberlites, but cannot bear comparison with most of the other compounds tested. Results found with low normalities of HCl (0.5–2.0 *N*) looked somewhat less reliable, though no real difficulties were encountered.

Hyamine 2389. This mixture of quaternary salts yields very thin silica gel layers sometimes, but the average elution time is still fairly short, being 7.5 min only. Therefore, Hyamine 2389 may have advantages in this respect, but the experimental results are not too satisfactory, irreproducible R_F spectra being obtained for *e.g.* Sb and Bi (*cf.* legend to Fig. 10). Moreover, the adsorbing power is less than with the Amberlites, even though the 0.1 *M* solution has a higher effective strength as one of the components bears two functional groups.

As regards the problems met when using the Hyamines in paper chromatography, one is referred to the section on paper chromatography below.

Primene JM-T. This primary amine with which the migration rate of the eluant varies surprisingly little with HCl normality, has fairly weak adsorbing qualities. It contains quite a few impurities, that form a broad fluorescing zone at the solvent front with 8-hydroxyquinoline, which gives difficulties when detecting Mn. The identification of Pb also proved difficult, probably partly due to the long tailing spots obtained at intermediate HCl strengths.

Primene yields deviating results with Bi (*cf.* Fig. 11 with *e.g.* Figs. 2, 6 and 12); similar deviations were found with the Hyamines (Figs. 9 and 10).

Aliquat 336. The Aliquat–silica gel suspension is remarkably unstable and the silica gel settles faster than with any of the other amines tested: within a few hours a hard cake forms on the bottom of the container, which can, however, be suspended again by thoroughly mixing for some minutes, so that this phenomenon causes no

real trouble. The silica gel layers are more or less scaly in character, unlike those found with *e.g.* the Amberlites and Adogen 368, which are more even with some small clots superimposed. With Aliquat, the "twice-dipping" technique was repeatedly used. Fe, present as an impurity, may be partly removed by washing with 3 vol. of 0.1 *N* HCl (ref. 26).

Fig. 12 shows that Aliquat has good adsorbing qualities and it may therefore be recommended for chromatographic work (*cf.* ref. 26).

Adogen 364. Adogen 364, like Adogen 368, gives excellent thin layers, that resemble those obtained with the Amberlites. The data summarized in Fig. 13 show that Adogen 364 has high adsorption strength and may be compared with TiOA in this respect.

Adogen 368. Fig. 14 shows that the adsorption behaviour of Adogen 368 is almost identical with that of Adogen 364; on the other hand, the migration rate of the eluant *vs.* HCl normality diagrams are quite unlike, Adogen 368 giving a surprisingly low movement for 0.5–2.0 *N* HCl. The colour of the Adogen 368·HCl solution turns to pink-yellow within a few hours.

Adogen 464. This fairly dark coloured quaternary ammonium salt forms scaly layers, and the suspensions in $CHCl_3$ settle fairly rapidly. Adogen 464 combines the advantages of fairly high adsorption power (Fig. 15), comparable with that of Aliquat, and short elution time (7.5–10 min).

All the Adogens form rapidly drying thin layers and detection rarely causes difficulties; the Fe(III)-8-hydroxyquinoline reaction is, however, not too distinct. Moreover, the Fe-spots form comets at higher HCl normalities, a phenomenon not found with other amines of comparable strength.

Alamine 336. Results obtained with Alamine (Fig. 16) are satisfactory: the adsorption strength is high and is comparable with that of Adogens 364 and 368, while the elution time is reasonably short.

TABLE III

R_F VALUES OF SOME CATIONS ON PAPER IMPREGNATED WITH LIQUID ANION-EXCHANGER·HCl SYSTEMS

All experiments were carried out at room temperature using 6.5 *N* HCl as eluting agent.

Ion-exchanger	*R_F values found for*							
	Al	*Pb*	*Ag*	*Mn*	*Cu*	*Co*	*Zn*	*Cd*
Amberlite LA-1	0.97	0.95	0.67	0.95	0.69	0.90	0.20	0.09
Amberlite LA-2	0.98	0.95	0.70	—	0.66	0.88	0.20	0.10
TiOA	0.98	0.75	0.53	0.45	0.04	0.07	0.00	0.00
TnOA	0.95	0.69	0.55	0.60	0.07	0.11	0.00	0.00
Tetrahexylammonium chloride	0.97	0.77	0.61	0.57	0.18	0.08	0.00	0.00
0.15 *M* Hyamine 1622–decanol (4:1, v/v)	0.93	0.83	0.70	0.91	0.65	—	0.20	—
0.15 *M* Hyamine 10X–decanol (1:1, v/v)	0.96	0.70	0.63	0.89	0.69	—	0.24	0.23
Primene JM-T	0.95	0.88	—	0.97	0.83	0.92	0.78	—
Aliquat 336	0.96	0.83	0.70	0.87	0.33	—	0.03	0.00
Adogen 364	0.97	0.75	0.54	0.67	0.07	0.08	0.00	0.00
Adogen 368	0.95	0.75	0.55	0.71	0.06	0.08	0.00	0.00
Adogen 464	0.97	0.84	0.67	0.88	0.35	0.51	0.02	0.00
Alamine 336	—	0.73	0.56	0.60	0.06	0.10	0.00	0.00

Tribenzylamine. This aromatic amine forms thin layers that can hardly be used: the solvent fronts are very irregular and the eluting agents move only slowly. To improve the character of the thin layers, viscous substances (see below) were added to the suspension. This gave slightly better results, but the general picture was still unsatisfactory. We therefore only mention that Fe had R_F values of approx. 0.8 and 0.0 with 2 and 4 *N* HCl, respectively. Zn and Cd gave elongated spots with R_F 0.0–0.4 at both normalities. These results and those mentioned in the section on paper chromatography clearly indicate that tribenzylamine has only low adsorption power and is not suitable for our purposes.

Paper chromatography

As mentioned above in the paragraphs on Amberlite LA-1 and TiOA, R_F spectra obtained in paper chromatography are very analogous to those found using thin-layer techniques. We therefore omitted further systematic work on paper and limited ourselves to a short test on the usefulness of the amines by eluting 5–6 cations at one normality of HCl using papers, impregnated with each of these ion-exchangers.

Apart from a fairly good correspondence between the data so obtained (Table III) and those found in thin-layer work, our research showed as the more important aspect an overall usefulness of the basic liquid ion-exchangers in paper chromatography, excepting the three Hyamines and tribenzylamine, which yielded highly irregular solvent fronts and very elongated badly formed spots.

It is highly probable that these difficulties are brought about by the crystalline character of the ion-exchangers in question. Therefore, certain substances, *viz.* decanol and the condensation product of glycerol and benzhydryl chloride (synthesized according to ref. 30), were selected in order to retard the crystallisation of these compounds on the paper and were added to their solutions in $CHCl_3$. The experiments showed that the results were improved to a considerable extent in this way, especially in the presence of the benzhydryl ether. However, the enormously increased elution time (7 h against 3 h normally) completely offsets this advantage. Therefore, these ion-exchangers are not suitable for use in the paper (and/or thin-layer) techniques described by us.

DISCUSSION

In the last decade liquid ion-exchangers[6] have been introduced in paper and column chromatography, and it has been shown that all the advantages of selectivity and versatility of these compounds can be transferred into the field of thin-layer chromatography, where these processes can be understood by assuming that the supports treated with ion-exchangers behave like a thin film of ion resin.

Therefore, the great similarity between the processes in extraction using liquid ion-exchangers, in resinous ion-exchange and in the chromatographic techniques considered here may be emphasized: they can all be expressed either as ion-exchange, implying transport of ions, or as adduct formation, implying transport of neutral species, alternatives which are thermodynamically equivalent[2]. On the other hand, this comparison should not be carried too far, since the physical aspects of these systems are obviously substantially different[6].

We have now studied more systematically high-molecular-weight amines and

substituted ammonium salts, starting with HCl systems, because more data are available here for comparison. From the experiments carried out with a number of cations selected with regard to their different behaviour in HCl media, we may conclude (see Figs. 1–16 and RESULTS) that nearly all the exchangers tested gave satisfactory results. Difficulties were encountered only with some crystalline compounds—which are therefore not true liquid ion-exchangers. In these cases addition of viscous substances only moderately improved the results (see *Paper chromatography*).

Figs. 1–16 and Table III reveal a very satisfactory correspondence between paper and thin-layer experiments, especially when taking into account the difference in support and in concentration of the ion-exchangers in the equilibration solution (0.15 *vs.* 0.10 *M*). The discrepancies sometimes occurring do not severely detract from this general picture, as may be established by comparing *e.g.* Co in Figs. 1 and 2 and Mn and Bi in Figs. 5 and 6.

Our data moreover indicate that each of the ions investigated shows more or less the same behaviour with all exchangers. For most ions the analogy is even very pronounced (*cf.* the analogous results of CERRAI AND GHERSINI[26] when using TnOA and Aliquat 336) and it follows clearly from the figures that the exchangers may be classified according to their adsorption strength in HCl medium as follows:

TiOA > Adogen 364 ~ Adogen 368 ~ Alamine 336 ~ tetrahexylammonium ~ TnOA > Aliquat 336 ~ Adogen 464 > Hyamine 1622 > Amberlite LA-1 ~ Amberlite LA-2 > Hyamine 2389 > Primene JM-T.

Despite the apparent presence of a certain sequence, it is not yet possible to obtain far-reaching conclusions as regards *e.g.* the correspondence between structure and adsorption strength, owing to the complicated composition of some of the products used and possibly the moderate reproducibility of some of the experiments.

However, it may be tentatively assumed that tertiary and quaternary compounds are generally superior in HCl media; on the other hand their adsorption strength does not show much variability in the range of 24 to 30 carbon atoms. Reference may be made here to a paper by PANG AND LIANG[21], who did not find a distinct relationship between the number of carbon atoms in the amines and the R_F values of 6 elements studied, when using tri-*n*-alkylamines ranging from 12 to 27 carbon atoms.

The correspondence of our data and those on extraction using liquid ion-exchangers (see review by COLEMAN[2]) seems to indicate that the sequence proposed above also holds in liquid–liquid extraction—at least in HCl media—Primene JM-T being a weaker extractant than Amberlite LA-1, which in its turn is clearly surpassed by Aliquat 336, TiOA and Alamine 336. A less satisfactory result is obtained for Hyamine 1622, but here the diluent, dichloroethane, possibly plays an important role, because the other exchangers were used dissolved in xylene, kerosene or diethylbenzene, which are probably less suitable in extracting ionic species.

The fundamental aspects of liquid–liquid extraction and chromatography on treated papers may be correlated through the quantitative relationship[6,31]:

$$\log E_a^\circ = k \cdot R_M$$

where E_a° is the extraction coefficient, k a constant and $R_M = \log (1/R_F - 1)$, where the R_F values have been obtained using the same extractant and aqueous phase. As an illustration, Fig. 17 gives a comparison of data on Amberlite LA-1, which indeed

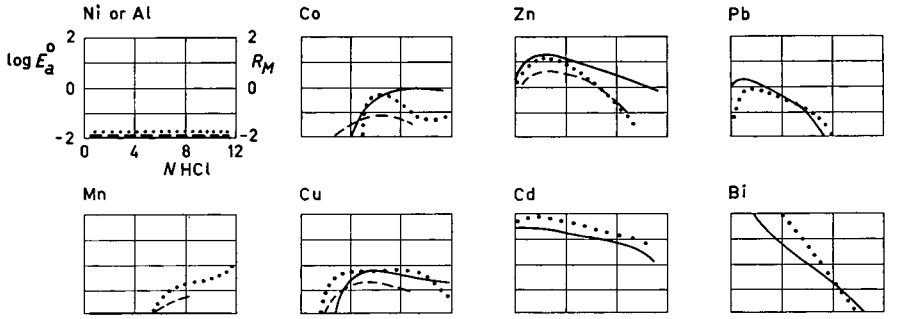


Fig. 17. Comparison of log E_a^o and R_M for Amberlite LA-1, when plotted against normality of HCl. E_a^o data have been extracted from ref. 2: (– – –) 0.3 M Amberlite in xylene; (· · ·) 0.1 M Amberlite in diethylbenzene. R_M values (———) have been calculated using the data in Fig. 2 and in ref. 28. The 0.3 M Amberlite curve for Co has a curvature more pronounced than that normally found. With Mn, we found appreciable adsorption only when using amines stronger adsorbing than Amberlite LA-1; in these cases, the R_M curves resembled the log E_a^o data.

show a satisfactory resemblance of the log E_a^o and the R_M curve. Similar results were obtained for the other liquid ion-exchangers mentioned above. In view of the many theoretical difficulties inherent in these problems, a quantitative discussion of the data seems premature at this stage of our investigation.

As regards comparison with ion-exchange data, one is mostly referred to the papers by KRAUS, NELSON and coworkers[32], but it is also worthwhile to call attention to the publications by LEDERER AND OSSICINI[33]. Our data (also see ref. 28) show an agreeable correspondence with the results of LEDERER who used the strong base resin paper Amberlite SB-2 (containing Amberlite IRA-400, a polystyrene resin). Supports impregnated with Aliquat 336 and all stronger adsorbing amines generally give the same or lower R_F values as SB-2 paper, while the Amberlites LA-1 and LA-2 are still more strongly adsorbing than both DE-20 and AE-30 cellulose anion-exchange paper[33]. It may be remarked that the curvature in LEDERER's diagrams is mostly less pronounced than in ours.

Reference may also be made to work[7,34] on the use of tri-*n*-butyl phosphate, tri-*n*-octylphosphine oxide and bis-(di-*n*-hexylphosphinyl)methane, which are generally more powerful extractants in the order given. Two conclusions reached there are worth mentioning as they correspond with our experiences: (a) the large amounts of the weak extractant tri-*n*-butyl phosphate required on the paper alter its physical characteristics and thereby cause an uneven and slow flow of the mobile phase; (b) tri-*n*-octylphosphine oxide is not too suitable in concentrated (0.2 M) solution[7], because it is a solid which tends to crystallize on the paper, but a more dilute, *e.g.* 0.025 M, equilibration solution is very satisfactory[34]. The most interesting observation is, however, that the curves found for these three organophosphorus compounds in HCl media closely resemble those obtained by us when using basic liquid ion-exchangers. Therefore, it seems that these neutral compounds—which are assumed to react via a coordination through the phosphoryl oxygen directly to the metal ion displacing water of hydration[7]—may also be described as anion-exchangers (*cf.* ref. 7, p. 71).

The form of the curves for the various ions (Figs. 1–16) may be tentatively interpreted (*cf.* ref. 32) by assuming the existence of a neutral (and/or positive) species

at low ligand concentration (R_F approx. 1.0); increased adsorption once a reasonable amount of the first negative ion has been formed (decreasing R_F); maximum adsorption of the same or a higher negative ion in or close to the minimum of the R_F curve, and lastly desorption owing to competition of excess ligand, or possibly other causes such as formation of less adsorbed higher charged negative complexes or decreasing dissociation of complex acids $H_n[MCl_p]$. Naturally, this simple picture can be considerably complicated by phenomena such as adsorption in the network of the support (*e.g.* with $AuCl_4^-$; *cf.* ref. 33) or precipitation. Parenthetically, experiments carried out with silica gel layers not treated with liquid ion-exchangers did not show any adsorption ($R_F \geqslant 0.9$) for the ions used, except for Sb(III) below 4 *N* HCl, probably due to hydrolysis.

CONCLUSIONS

The experimental results show that the application of the thin-layer chromatographic technique opens the route to quickly obtain a considerable amount of information, though the results are at best semi-quantitative as regards their value in theoretical calculations.

It is advantageous that the need for a diluent is eliminated; which is important, as it has been repeatedly shown that this so-called "inert" solvent plays a significant role in liquid–liquid extraction; moreover, prior acclimatisation can be omitted.

Our data indicate that the Adogens 364, 368 and 464, Alamine 336, Aliquat 336, TnOA and TiOA (and tetrahexylammonium chloride) all show an analogous behaviour, which also holds for the Amberlites LA-1 and LA-2 and lastly for Primene JM-T, though they adsorb more weakly. Inexpensive technical-grade products may therefore be chosen to replace more expensive ones, such as tetrahexylammonium salts and TnOA. By selecting a few amines with divergent adsorption strength the R_F values for a cation in a certain medium may be varied either by varying the molarity of the equilibration solution (*cf.* Table II; however, higher molarities will lengthen the elution time) or by mixing two or more of the selected amines (*cf.* Figs. 2, 3 and 6).

Moreover, our methods, in some respects, compare favourably with those involving the use of solid resin-impregnated papers. The latter are far more expensive and not so handy either, the use of HCl being limited to normalities up to 8 *N* HCl. On the other hand, the use of partly organic solvent mixtures (ref. 20 and own unpublished experiments) is possible only using the resin paper. LEDERER's investigations[35] using anionic, cationic and neutral papers may also be transferred into our field by using paper impregnated with *e.g.* HDEHP and DNS as cation exchange paper.

Our results show the necessity to test further some of the selected anion-exchangers with respect to their behaviour with other strong monobasic acids. This work, that is currently being done, may shed more light on the reliability of the adsorption sequence tentatively proposed above. Moreover, it may show which systems are most promising for further qualitative and quantitative separations. Lastly, despite the fair correspondence between chromatography, extraction and ion-exchange found until now, it will be necessary to support this evidence by collecting data on more ions.

ACKNOWLEDGEMENTS

Our sincere thanks are due to Mr. H. VELTKAMP for his skilful assistance in carrying out the experimental work.

We are indebted also to General Mills Inc., Kankakee, Ill. (U.S.A.) and Archer-Daniels-Midland Co., Minneapolis, Minn. (U.S.A.) for receiving free samples of some of the basic liquid ion-exchangers.

SUMMARY

Paper and thin-layer chromatography have been carried out in HCl media, using supports impregnated with basic liquid ion-exchangers. Approximately ten high-molecular-weight amines and substituted ammonium salts have been successfully investigated in combination with 9 cations. From the R_F spectra so obtained a sequence for the adsorption strength of these liquid ion-exchangers has been tentatively derived.

The R_F spectra are favourably compared with LEDERER's data on papers impregnated with solid anion resin; a remarkable correspondence with results obtained when using neutral organophosphorus compounds is also pointed out. The curves are discussed assuming among other things the formation of chloro anions and desorption owing to competition by the ligand ion.

Advantages of the chromatographic techniques with respect to speed, low cost, elimination of diluent and overall versatility are outlined. Some possible applications for practical and theoretical purposes are considered.

REFERENCES

1 E. L. SMITH AND J. E. PAGE, *J. Soc. Chem. Ind.*, 67 (1948) 48.
2 C. F. COLEMAN, *Nucl. Sci. Eng.*, 17 (1963) 274.
3 C. F. COLEMAN, C. L. BLAKE, JR. AND K. B. BROWN, *Talanta*, 9 (1962) 297.
4 H. GREEN, *Talanta*, 11 (1964) 1561.
5 F. L. MOORE, *U.S. At. Energy Comm. Rept., NAS-NS 3101*, 1960.
6 E. CERRAI, in M. LEDERER (Editor), *Chromatographic Reviews*, Vol. 6, Elsevier, Amsterdam, 1964, p. 129.
7 J. W. O'LAUGHLIN AND C. V. BANKS, *U.S. At. Energy Comm. Rept., IS-737*, 1963.
8 T. B. PIERCE AND R. F. FLINT, *Anal. Chim. Acta*, 31 (1964) 595.
9 T. B. PIERCE AND P. F. PECK, *Analyst*, 89 (1964) 662.
10 S.-W. PANG AND S.-C. LIANG, *Hua Hsueh Hsueh Pao*, 29 (1963) 319; and 30 (1964) 237.
11 H.-W. CHENG, C.-H. KE, C.-Y. LIN AND J. W. WINCHESTER, *J. Chinese Chem. Soc. (Taiwan)*, 10 (1963) 80.
12 H. GROSSE-RUYKEN AND J. BOSHOLM, *J. Prakt. Chem.*, 25 (1964) 79; and 26 (1964) 83.
13 S. SIEKIERSKI AND R. J. SOCHACKA, *J. Chromatog.*, 16 (1964) 376 and 385.
14 M. N. SASTRI AND A. P. RAO, *Z. Anal. Chem.*, 196 (1963) 166; and 207 (1965) 409.
15 G. WERNER, *Z. Chem.*, 5 (1965) 147.
16 C. TESTA, *J. Chromatog.*, 5 (1961) 236.
17 E. CERRAI AND C. TESTA, *J. Chromatog.*, 5 (1961) 442.
18 E. CERRAI AND C. TESTA, *J. Chromatog.*, 6 (1961) 443.
19 C. TESTA, *Anal. Chem.*, 34 (1962) 1556.
20 E. CERRAI AND C. TRIULZI, *J. Chromatog.*, 16 (1964) 365.
21 S.-W. PANG AND S.-C. LIANG, *Hua Hsueh Hsueh Pao*, 30 (1964) 401.
22 S.-W. PANG AND S.-C. LIANG, *K'o Hsueh T'ung Pao*, (1964) 156; *C.A.*, 61 (1964) 15324f.
23 A. G. HAMLIN, B. J. ROBERTS, W. LOUGHLIN AND S. G. WALKER, *Anal. Chem.*, 33 (1961) 1547.
24 J. MIKULSKI AND I. STROŃSKI, *J. Chromatog.*, 17 (1965) 197.
25 E. CERRAI AND C. TESTA, *J. Chromatog.*, 8 (1962) 232.
26 E. CERRAI AND G. GHERSINI, *Energia Nucl. Milan*, 11 (1964) 441.

27 P. MARKL AND F. HECHT, *Mikrochim. Acta*, (1963) 970.
28 U. A. TH. BRINKMAN AND G. DE VRIES, *J. Chromatog.*, 18 (1965) 142.
29 F. FEIGL, *Spot Tests in Inorganic Analysis*, 5th Ed., Elsevier, Amsterdam, 1958.
30 P. E. VERKADE, J. VAN DER LEE AND W. MEERBURG, *Rec. Trav. Chim.*, 54 (1935) 716.
31 A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 35 (1941) 1358.
32 K. A. KRAUS AND F. NELSON, *Proc. Intern. Conf. Peaceful Uses At. Energy, Geneva, 1955*, 7 (1956) 113.
33 M. LEDERER AND L. OSSICINI, *J. Chromatog.*, 13 (1964) 188.
34 E. CERRAI AND C. TESTA, *J. Chromatog.*, 7 (1962) 112.
35 M. LEDERER AND F. RALLO, *J. Chromatog.*, 7 (1962) 552.

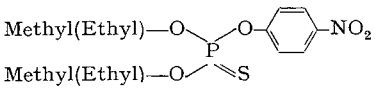
REVERSED-PHASE PAPER CHROMATOGRAPHY OF SOME CATIONS WITH TWO NITROPHENYLTHIOPHOSPHATE DERIVATIVES AS STATIONARY PHASES

E. CERRAI AND G. GHERSINI
Laboratori CISE, Segrate, Milano (Italy)
(Received November 16th, 1965)

Many organophosphorus compounds, such as the well-known tri-butyl phosphate, tri-butyl phosphine oxide, and di(2-ethylhexyl)orthophosphoric acid, have been shown to play an outstanding role in solvent extraction of inorganic ions, owing to the solvation properties of the phosphoryl group P=O.

HANDLEY AND DEAN[1] reported that, when the P=O group was substituted in some compounds by a P=S group, special selectivity characteristics could be achieved, as in the case of the octyl thiophosphates or butyl thiophosphates, with silver and mercury in a nitrate medium.

In our laboratory the properties of some commercial thiophosphate derivatives were investigated. As already stated elsewhere, a considerable amount of information on the behaviour of a liquid–liquid system can be obtained through a reversed-phase chromatographic technique[2]. Therefore, this method was adopted for a rapid study of two products, namely methylparathion and ethylparathion. Methylparathion (O,O-dimethyl O-*p*-nitrophenyl thiophosphate, mol. wt. 263, sp. gr. 1.36) and ethylparathion (O,O-diethyl O-*p*-nitrophenyl thiophosphate, mol. wt. 291, sp. gr. 1.26), are liquid insecticides, nearly insoluble in water, with the structural formula:



The R_F values obtained by eluting a spot of a given cation with a given aqueous phase on a paper previously treated with a given extractant, can be related to the extraction coefficient E_a^o for the same cation in the analogous liquid–liquid system, by the equation

$$\log E_a^o = \log \left(\frac{1}{R_F} - 1 \right) + \text{constant}$$

where the constant depends on the experimental conditions. The main advantage of the chromatographic technique is the practicability of obtaining basic information on the extraction behaviour in a very short time.

EXPERIMENTAL

The insecticides used in the present investigation were technical grade products obtained from different sources which are listed in Table I. During this work no difference among them was noticed with respect to their extraction behaviour.

The paper used was Whatman No. 1, CRL/1 type, which enabled us to carry out a great number of simultaneous elutions running a front of about 9 cm. The reagents and the solvents used were C. Erba R.P. products, supplied by C. Erba (Milan).

TABLE I

COMPOUNDS USED AND THEIR SOURCES

Compound	*Source*
Methylparathion, 80%	Bombrini Parodi-Delfino (Rome)
Ethylparathion, 94%	Bombrini Parodi-Delfino (Rome)
Methylparathion, techn. grade	Montecatini (Milan)
Ethylparathion, techn. grade	Montecatini (Milan)
Methylparathion, 80%	Cheminova (Denmark), supplied by Nymco (Milan)
Ethylparathion, 98%	Cheminova (Denmark), supplied by Nymco (Milan)

Solutions for the paper treatment were obtained by diluting parathion with toluene; the already mentioned molecular weights and specific gravities for the pure products were used for calculations.

The diluted technical product was usually purified by treating it with twice its volume of a 5% Na_2CO_3 solution: this treatment was repeated until the aqueous layer remained colourless after shaking for 5 min.

After purification, the extractant solution was washed with water, and then equilibrated with twice its volume of a 1 *M* nitric acid solution. The paper was treated as described previously[3]. The apparatus for the ascending development has also been described already[4].

Drops containing from $1 \cdot 10^{-7}$ to $5 \cdot 10^{-6}$ equivalents of a metal ion, depending on the sensitivity attainable by the detection method, were individually applied on the paper; they were taken from solutions to which, when necessary, enough nitric acid had been added to prevent hydrolysis.

At the beginning, an investigation was carried out to obtain some preliminary information on the system. The ions chosen to be eluted were among those reported by HANDLEY AND DEAN[1] and by HANDLEY[5] as being extractable by thio-organophosphorus compounds. A few additional representative ions were investigated by us. Thus, the behaviour of titanium(IV), chromium(III), iron(III), copper(II), gallium(III), palladium(II), silver(I), cadmium(II), tin(II), gold(III), and mercury(II) were investigated, using nitric acid at various concentrations as the eluent. After the elution, the spots were detected by spraying the paper with a 0.1% solution of quercetin in 95% ethanol for Ti, Ga and Sn; with 0.1% diphenylcarbazide for Cr; with 0.1% 8-hydroxyquinoline for Fe and Cu; and with a 0.1% solution of dithizone in chloroform for the other cations.

RESULTS AND DISCUSSION

At the early stage of the work, unpurified ethylparathion was used. When the paper was treated with concentrated solutions of the product as supplied it was nearly hydrorepellent, and for this reason very dilute solutions of parathion were used, namely 0.04 *M* and 0.02 *M* in toluene. Because of the low capacity of the stationary phase, due to the small amount of extractant present on the paper, the spots after the elution were often very large, and thus the R_F values obtained had an appreciable uncertainty.

Results of this preliminary investigation are reported in Table II as R_F values for the individual cations at the corresponding nitric acid concentration in the eluent. For paper treated with 0.04 *M* ethylparathion, two R_F values are reported for palladium; under these conditions this cation showed a double spot. When the paper was treated with the more dilute solution, only one spot was visible, the R_F of which is reported in the table.

TABLE II

R_F VALUES FOR SOME CATIONS AS FUNCTIONS OF THE MOLARITY OF HNO_3 AS ELUENT

Paper treated with 0.02 *M* and 0.04 *M* ethylparathion in toluene. Operating temperature 24 ± 1°.

Ethylparathion molarity	*Cation*	R_F							
		Molarity of HNO_3							
		0.0001	*0.001*	*0.01*	*0.1*	*1.0*	*5.0*	*10.0*	*14.7*
0.02	Ti^{4+}	0.94	0.94	0.95	0.96	0.82	0.82	0.75	0.68
	Cr^{3+}	0.92	0.92	0.95	0.92	0.95	0.92	0.92	0.92
	Fe^{3+}	0.44	0.72	0.68	0.95	0.95	0.90	0.88	0.82
	Cu^{2+}	0.92	0.92	0.92	0.95	0.94	0.92	0.94	0.95
	Ga^{3+}	0.91	0.91	0.91	0.97	0.88	0.90	0.95	0.71
	Pd^{2+}	0.80	0.79	0.44	0.82	0.85	0.90	0.14	0.37
	Ag^{1+}	0.00	0.26	0.57	0.90	0.92	0.95	0.95	0.92
	Cd^{2+}	0.87	0.87	0.87	0.87	0.87	0.87	0.88	0.87
	Sn^{2+}	—	0.84	0.88	0.82	0.80	0.74	0.73	0.76
	Au^{3+}	0.62	0.67	—	0.70	0.22	0.42	0.67	0.63
	Hg^{2+}	0.74	0.70	0.70	0.71	0.71	0.75	0.77	0.81
0.04	Ti^{4+}	0.95	0.97	0.95	0.95	0.97	0.95	0.90	0.70
	Cr^{3+}	0.92	0.95	0.92	0.95	0.95	0.85	0.82	0.76
	Cu^{2+}	0.92	0.95	0.92	0.92	0.95	0.97	0.95	0.92
	Ga^{3+}	0.92	0.92	0.92	0.93	0.92	0.94	0.94	0.70
	Pd^{2+}	0.50	0.57	0.60	0.62	0.61	0.70	0.73	0.82
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.31
	Ag^{1+}	0.00	0.00	0.00	0.45	0.79	0.95	0.92	0.95
	Cd^{2+}	0.34	0.50	0.80	0.95	0.92	0.95	0.70	0.65
	Sn^{2+}	0.92	0.96	0.96	0.97	0.82	0.77	0.75	0.62
	Au^{3+}	0.00	0.00	0.00	0.00	0.00	0.06	0.77	0.82
	Hg^{2+}	0.50	0.63	0.58	0.62	0.62	0.63	0.77	0.70

The low R_F values for iron in the lowest range of the nitric acid concentration are attributable to partial hydrolysis; on the other hand, the lowering of R_F values of nearly all cations at the higher nitric acid concentrations may be ascribed to the dehydration phenomena of the ions which enhance retention, as already found in

other chromatographic systems. However, the presence of parathion on the paper was undoubtedly responsible for the retention of palladium, silver, cadmium, gold, and mercury. The investigation was therefore continued by considering only the five above mentioned cations.

The paper treated with purified methylparathion and ethylparathion was found to be far more wettable than the paper treated with the unpurified products. It was then possible to treat the paper with more concentrated solutions, namely 1 *M* in toluene for both compounds. Because of the higher capacity of stationary phase thus achieved, the spots obtained after the elutions were sufficiently small and very reproducible.

Representative R_F values obtained by eluting palladium, silver, cadmium, gold, and mercury with nitric acid at various concentrations on paper treated with 1 *M* methylparathion and ethylparathion are collected in Table III and are plotted in Fig. 1 against the logarithm of the nitric acid concentration.

TABLE III

R_F VALUES FOR PALLADIUM, SILVER, CADMIUM, GOLD AND MERCURY AS FUNCTIONS OF THE MOLARITY OF HNO_3 AS ELUENT

Paper treated with 1.0 *M* methylparathion or ethylparathion in toluene. Operating temperature 24 ± 1°.

Parathion used	*Cation*	R_F												
		Molarity of HNO_3												
		0.0001	*0.0005*	*0.001*	*0.005*	*0.01*	*0.05*	*0.1*	*0.5*	*1.0*	*5.0*	*8.0*	*10.0*	*14.7*
Methyl	Pd^{2+}	0.00	0.05	0.05	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	—
	Ag^{1+}	0.00	0.19	0.25	0.20	0.17	0.15	0.18	0.16	0.18	0.25	0.28	0.30	—
	Cd^{2+}	0.10	0.20	0.25	0.55	0.64	0.77	0.79	0.85	0.82	0.95	0.97	0.95	—
	Au^{3+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.15	0.25	—
	Hg^{2+}	0.60	0.62	0.61	0.61	0.62	0.67	0.68	0.66	0.67	0.82	0.88	0.67	—
		Molarity of HNO_3												
		0.0001	*0.001*	*0.002*	*0.005*	*0.02*	*0.1*	*0.2*	*1.0*	*5.0*	*8.0*	*10.0*	*12.0*	*14.7*
Ethyl	Pd^{2+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.08
	Ag^{1+}	0.00	0.00	0.00	0.00	0.00	—	—	0.00	0.32	—	0.60	0.84	0.82
	Cd^{2+}	0.00	0.25	0.55	0.76	—	0.90	0.83	0.85	0.95	0.95	0.97	0.92	0.87
	Au^{3+}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Hg^{2+}	0.47	0.45	0.46	0.45	0.47	0.45	0.46	0.48	0.50	0.25	0.13	0.10	0.16

In Fig. 1, the behaviour at low nitric acid concentrations was omitted when the R_F in this range had the same value as for 1 *M* acid.

Data for paper treated with methylparathion and eluted with nitric acid at concentrations higher than 10 *M* are not reported, since at these concentrations of eluent the methylparathion present on the paper was washed away by the aqueous phase.

No attempt was made to explain the mechanism of retention, since for such purposes more than the results obtained with this system would be necessary. The

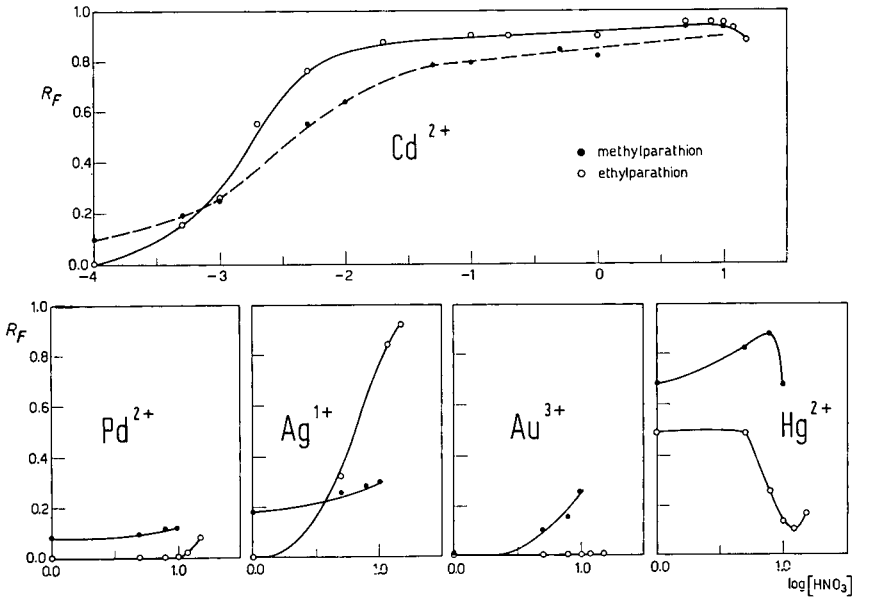


Fig. 1. R_F values for cadmium, palladium, silver, gold, and mercury plotted *vs.* log molarity HNO_3. Paper treated with 1 *M* methylparathion and 1 *M* ethylparathion in toluene. Operating temperature 24 ± 1°.

experimental evidence, however, showed that both methylparathion and ethylparathion possess a retention power when on the paper, which is undoubtedly related to an analogous extraction power in the liquid–liquid systems.

With the aim of checking the chromatographic results, some liquid–liquid extraction experiments were planned. Unfortunately, after this chromatographic investigation was accomplished, our parathion specimens were almost completely used up. Nevertheless, some preliminary experiments on silver were possible, and the extraction properties of both compounds were confirmed. Data collected in Table IV show that a certain dependence exists between percentage extraction and the concentration of silver in the aqueous phase; but this feature was not investigated further.

TABLE IV

EXTRACTION OF SILVER BY METHYLPARATHION AND ETHYLPARATHION IN TOLUENE FROM AQUEOUS NITRIC ACID SOLUTIONS

Organic phase: 5 ml of extractant 1 *M* in toluene. Aqueous phase: 5 ml of nitric acid at various concentrations containing variable amounts of silver. Shaking time: 30 min. Temperature: 24 ± 1°.

Extractant	*Molarity of* HNO_3	*μg Ag in the aqueous phase*		*% Ag extracted*
		Before extraction	*After extraction*	
Methylparathion	0.0001	62.0	38.0	44
Ethylparathion	0.0001	12.2	9.8	31
Ethylparathion	0.0001	30.4	15.7	43
Ethylparathion	0.0001	152	60.0	60
Ethylparathion	0.4	152	78.0	48

During the present work, Emmatos (O,O-dimethyldithiophosphate of diethylmercaptosuccinate, produced by BPD, Rome) was also tested in liquid–liquid extractions. An 8 % solution of this compound in ethyl ether was found to extract completely 1 mg/ml of silver from a 3 *M* nitric acid solution, with a 1:1 volume phase ratio.

From the results of the purely preliminary research reported here, it was felt that further investigations would be worthwhile on the use of thiophosphorus industrial insecticides as extractants in inorganic chemistry. Although the high toxicity of these compounds is undoubtedly a disadvantage for their use in the laboratory, on the other hand they are currently produced by industrial processes and thus are relatively cheap.

ACKNOWLEDGEMENTS

The kindness of the firms of Bombrini Parodi-Delfino, Montecatini and Nymco in supplying the specimens of the insecticides used throughout this work, is gratefully acknowledged.

SUMMARY

Two industrial thiophosphorus insecticides, namely methylparathion and ethylparathion, were used as impregnants for paper for reversed-phase chromatography of several cations with nitric acid as the eluent.

Palladium, silver, cadmium, gold, and mercury were shown to be more or less retained by the stationary phase. The behaviour of silver was checked also by liquid–liquid extraction.

The use of the above mentioned insecticides, and of Emmatos (malathion) as liquid–liquid extractants was suggested.

REFERENCES

1 T. H. HANDLEY AND J. A. DEAN, *Anal. Chem.*, 32 (1960) 1878.
2 E. CERRAI, in M. LEDERER (Editor), *Chromatographic Reviews*, Vol. 6, Elsevier, Amsterdam, 1964, 129.
3 E. CERRAI AND C. TESTA, *J. Chromatog.*, 8 (1962) 232.
4 E. CERRAI AND G. GHERSINI, *J. Chromatog.*, 13 (1964) 211.
5 T. H. HANDLEY, *Nucl. Sci. Eng.*, 16 (1963) 440.

ELEKTROPHORESE VON RADIONUKLIDEN AUF CELLOGEL

K. AITZETMÜLLER, K. BUCHTELA UND F. GRASS
Atominstitut der Österreichischen Hochschulen, Wien (Österreich)
(Eingegangen den 25. Oktober 1965)

In früher erschienenen Arbeiten[1,2] wurden bereits radiochemische Trennungen mittels Elektrophorese auf Cellogel erwähnt. Es wurde über die Ergebnisse von Trennungen der Seltenen Erden bzw. der Glieder natürlicher radioaktiver Zerfallsreihen berichtet. In der vorliegenden Mitteilung soll die Arbeitstechnik für die elektrophoretische Trennung anorganischer Ionen auf Cellogelstreifen beschrieben werden.

DAS TRÄGERMATERIAL

Cellogel ist nach Angabe der Herstellerfirma* "pure gelatinized cellulose acetate" und hat eine weiche, gelartige Konsistenz. Bisher wurde das Material hauptsächlich für die Auftrennung von Eiweissstoffen verwendet.

Der Hauptvorteil besteht in der grösseren Homogenität und geringeren Adsorptionstendenz des Mediums, das schärfere Trennungen ermöglicht und dabei gleichmässiger Fleckenformen bzw. Zonen liefert als andere Trägermaterialien. Die Streifen sind mit 250–300 μ etwas dicker als andere handelsübliche Acetylcellulosefolien (z.B. Su.S. "Membranfolien zur Elektrophorese" 150–200 μ). Dies bedeutet, dass bei der Elektrophorese Kühlungsprobleme stärker ins Gewicht fallen und keine sehr hohen Spannungen verwendet werden können. Die Streifen werden in Glasgefässen** in 50 %igem Methanol geliefert und sollen stets unter Methanol aufbewahrt werden***. Die Arbeitsanleitung der Herstellerfirma empfiehlt, dass die im Vorratsgefäss zusammengerollten Streifen mit der konkaven Seite oben verwendet werden sollen.

Cellogel-Streifen sind in einer Reihe von Lösungsmitteln löslich und können nach erfolgter Elektrophorese in verschiedener Weise aufgearbeitet werden. Die mit Pufferlösung getränkten Streifen können durch einfaches Erwärmen transparent gemacht werden. Löslich sind die Streifen z.B. in Aceton, Dioxan, Eisessig, Chloroform, Methylenchlorid und in Mischungen wie z.B. Isopropanol–Chloroform im Volumenverhältnis 2 : 1 oder in einer Mischung aus 85 ml Aceton, 5 g Phenol und 10 ml Eisessig.

Wie schon in einer früheren Arbeit erwähnt wurde[1], sind die Substanzflecken nach einer Elektrophorese auf Cellogel weit weniger verzerrt als bei der Anwendung von Chromatographierpapier als Trägermittel. Besonders bei geringen Substanzmengen (trägerfreien Radionukliden) und bei niedriger Zahl von zu trennenden

* Chemetron Chimica, Mailand.
** Neuerdings auch in Plastikbeuteln.
*** Die Streifen scheinen aus 2 Schichten zu bestehen. Bei einzelnen der uns gelieferten Chargen zeigte sich eine sehr dünne Oberflächenschichte, die sich schon im Vorratsbehälter stellenweise von der dicken Unterlage abzulösen begann.

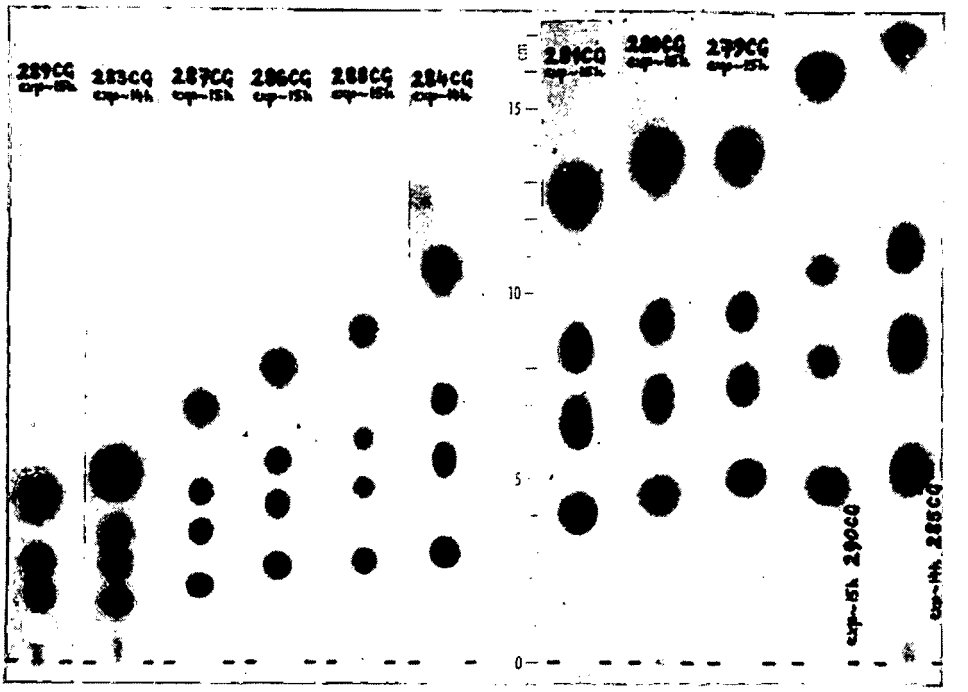


Fig. 1. Elektrophoresen auf Cellogel. Trennung einer aus vier Komponenten bestehenden Mischung Seltener Erden. Bei Verwendung kleiner Mengen erhält man meist gleichmässig runde Flecken. Die Reihenfolge der Wanderungsweiten ist Ce > Eu > Tb > Tm. Die Markierungen am unteren Rand der Autoradiogramme bezeichnen den Startpunkt. 0.4 *M* α-Hydroxyisobuttersäure; *ca.* 46 V/cm; 4.5–6.5 mA; Trennzeiten von 8.5 bis 34 Min. Radionuklide: ^{144}Ce; $^{152+154}Eu$; ^{160}Tb; ^{170}Tm.

Komponenten werden sehr gleichmässige, runde Substanzflecken erhalten. Dieses Ergebnis kann, wie Fig. 1 zeigt, auch bei verschiedenen Wanderungsweiten bzw. verschiedener Zeitdauer der Elektrophorese reproduziert werden. In der erwähnten Figur wird die Auftrennung von Cer, Europium, Terbium und Thulium als Beispiel angeführt. Bei diesem Beispiel wandert das Ion mit der niedrigsten Ordnungszahl am raschesten. Fig. 1 ist die Reproduktion einer Serie von Autoradiogrammen.

Meist verwendeten wir α-Hydroxyisobuttersäure als Elektrolyt. Ähnliche Ergebnisse können aber auch mit anderen α-Hydroxy-Carbonsäuren, z.B. Milchsäure, erhalten werden. Die Reihenfolge der gewanderten Substanzen bleibt bei Milchsäure und α-Hydroxyisobuttersäure gewöhnlich gleich, doch zeigen die Wanderungsweiten oft beträchtliche Unterschiede. Fig. 2 zeigt zum Vergleich die Ergebnisse einer Trennung in Milchsäure und α-Hydroxyisobuttersäure.

ARBEITSTECHNIK

AUFBEWAHRUNG BZW. VORBEREITUNG DER CELLOGELSTREIFEN

Bei allen Manipulationen ist stets darauf zu achten, dass die empfindliche Oberfläche bzw. der Streifen selbst nicht mechanisch verletzt werden. Die Cellogelstreifen werden aus der methanolischen Lösung herausgenommen, mehrmals mit destilliertem

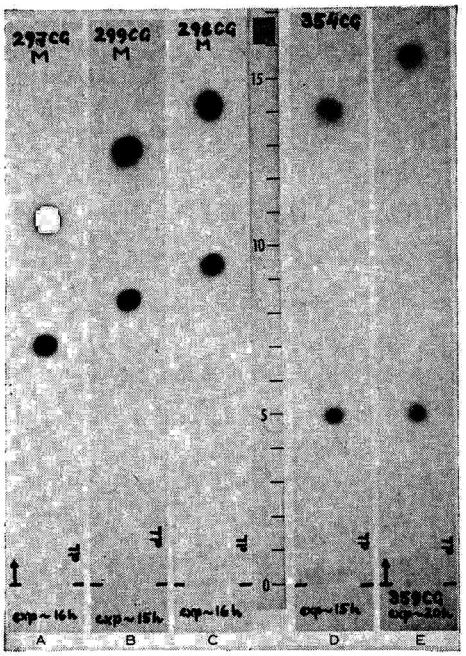


Fig. 2. Auch in Milchsäurelösung ergeben Cellogel-Elektrophoresen gleichmässig geformte Flecken. Cer wandert weiter als Thulium. Je *ca.* 46 V/cm. (A, B, C) 0.5 *M* Milchsäure; 8.8–10.2 mA; (A) 15 Min, (B) 19 Min. (C) 21 Min. (D) 0.4 *M* α-Hydroxyisobuttersäure; 5.7–6.8 mA; 23.5 Min. (E) 0.5 *M* α-Hydroxyisobuttersäure; 5.7–6.7 mA; 26.75 Min. Radionuklide: ^{144}Ce; ^{170}Tm. Der Vergleich der Autoradiogramme A, B und C (0.5 *M* Milchsäure) mit E (0.5 *M* α-Hydroxyisobuttersäure) demonstriert auch anschaulich die bessere Trennung der Erden in α-Hydroxyisobuttersäure[5].

Wasser abgespült und zusammengerollt in ein Becherglas gelegt, das mit der jeweils verwendeten Grundelektrolytlösung gefüllt ist. Bei einer Serie wurde das 50%ige Methanol gleich im Originalbehälter zur Gänze durch 0.8 *M* α-Hydroxyisobuttersäure ersetzt und die Streifen in dieser Lösung ein bis zwei Wochen lang aufbewahrt; dies war ohne Einfluss auf die Güte der Trennungen. Im allgemeinen wurden die Cellogelstreifen etwa eine Stunde vor der Elektrophorese in die hiezu verwendete Säure- bzw. Pufferlösung gegeben.

DIE ELEKTROPHORESEAPPARATUR

Die Elektrophoresen selbst wurden in Kammern durchgeführt, die aus Polyvinylchlorid und Glas bestanden. Die Cellogelstreifen lagen dabei auf einer dünnen Glasplatte, die von unten durch fliessendes Leitungswasser gekühlt wurde, während Abdeckungen seitlich und oben dafür sorgten, dass eine Konvektion der Luft unterbunden wurde und der Streifen sich praktisch in einer feuchtigkeitsgesättigten Kammer befand.

Bei den Ausführungen für längere Streifen (30–50 cm) wurde in etwa 1 cm Höhe über dem Cellogelstreifen ein mit destilliertem Wasser getränktes Stück Filterpapier darübergespannt und mit einer Deckplatte aus Glas oder Blei beschwert

(Siehe Fig. 3). Bei der Ausarbeitung von Trennmethoden für Mischungen radioaktiver Substanzen von zunächst unbekannter Wanderungsgeschwindigkeit ist es von Vorteil, in der obersten Deckplatte ein oder zwei Löcher nahe dem Ende des Cellogelstreifens anzubringen. Über diesen Öffnungen befinden sich Geiger–Müller-Zählrohre. Sobald sich die am raschesten wandernde radioaktive Substanz dem Ende des Cellogelstreifens nähert, wird z.B. ein akustisches Signal gegeben. Auf diese Weise kann man die Elektrophorese abbrechen, wenn eine bestmögliche Ausnützung der vorhandenen Streifenlänge gegeben ist.

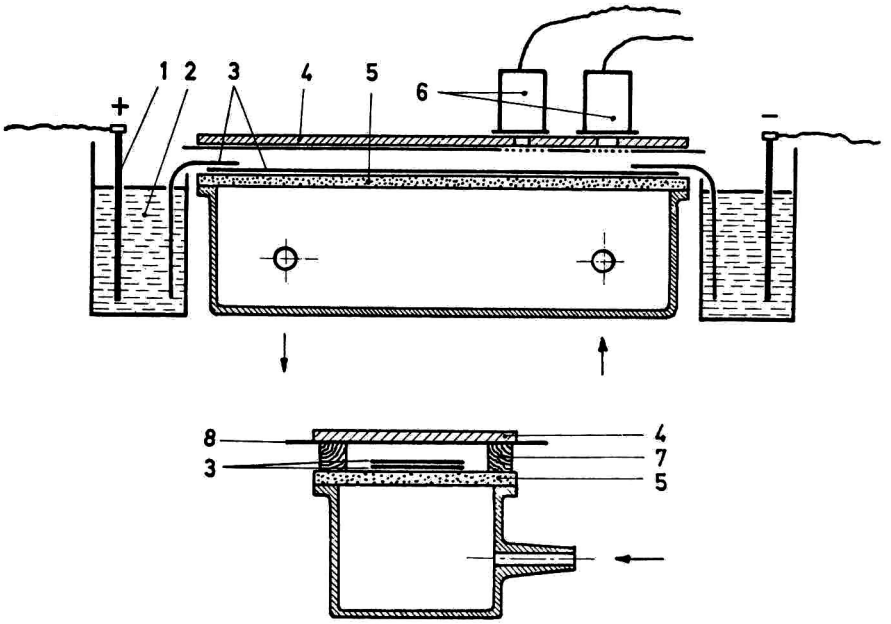


Fig. 3. Skizze der für Cellogel verwendeten Elektrophoreseapparatur. 1 = Platinelektrode; 2 = Elektrolyt; 3 = Cellogelstreifen und Zuführung aus Filterpapier; 4 = Bleiplatte mit Löchern oder Schlitzen; 5 = Glasplatte des Elektrophoresetisches (von unten durch fliessendes Wasser gekühlt); 6 = GM-Zählrohre; 7 = seitliche Begrenzung aus nichtleitendem Material (z.B. PVC); 8 = mit destilliertem Wasser getränkter Filterpapierstreifen.

DURCHFÜHRUNG DER ELEKTROPHORESE

Der Cellogelstreifen wird aus der Säure- bzw. Pufferlösung herausgenommen, flach auf die Glasplatte gelegt und der überschüssige Elektrolyt mit Filterpapierstreifen vorsichtig abgetupft. Der Einschluss von Luftblasen zwischen Streifen und Unterlage soll möglichst vermieden werden. Durch einen kleinen Einschnitt am Rande des Streifens oder besser durch zwei kurze Striche mit einem Kugelschreiber* wird der Startpunkt markiert. Die Zuführungen zu den Elektrodenräumen werden aus je zwei *ca.* 3 cm breiten und 5 cm langen Filterpapierstreifen hergestellt. Sie sollen *ca.* 5 mm mit den Enden des Cellogelstreifens überlappen. Der Cellogelstreifen soll zwischen oder unter den Enden der beiden Papierstreifen liegen. Die Enden der

* Häufig beginnt der Kugelschreiberfarbstoff im elektrischen Feld zu wandern. Trotzdem ist aber der Startpunkt auch nach dem Trocknen des Streifens noch gut erkennbar.

Papierstreifen neigen dazu, sich aufzuwölben, wodurch der leitende Kontakt verlorengehen kann. Dies lässt sich durch Beschweren mit einem kleinen Gewicht, etwa in Form eines 3 cm langen Glasstäbchens von rechteckigem Querschnitt, vermeiden.

Beim Auftragen der Substanzmischung darf die Oberfläche des Cellogelstreifens nicht verletzt werden. Ein dünner Polyäthylenschlauch (innerer Durchmesser 0.7 mm), der an einer Injektionsspritze befestigt ist und ein Aufsaugen und Aufbringen von sehr geringen Flüssigkeitsmengen (0.5 μl) möglich macht, hat sich bei unseren Versuchen gut bewährt. Beim Arbeiten mit radioaktivem Material kann das Schlauchstück leicht ausgewechselt werden. So lässt sich eine Kontamination der Injektionsspritze vermeiden, da bei den verwendeten geringen Flüssigkeitsmengen die aktive Lösung mit dem Glaskolben selbst gar nicht in Berührung kommt. Vorteilhalft ist auch ein feiner Pinsel* zum Aufbringen des Substanzgemisches. Man macht mit der Pinselspitze einen feinen Strich am Start oder man setzt die Pinselspitze auf den Startpunkt an und überlässt es der eigenen Saugfähigkeit des Cellogels, eine gewisse Flüssigkeitsmenge aus dem Pinsel herauszusaugen[3]. Wenn man nicht eine bestimmte Substanzmenge quantitativ auf den Streifen übertragen muss, sondern es nur auf eine Bestimmung der relativen Mengenverhältnisse der Komponenten der Mischung ankommt (wie z.B. der Seltenen Erden bei der Bestimmung der Herkunft von Opium[4]), so kann man die Pinselmethode anwenden. Bei geringen Unterschieden in der Wanderungsgeschwindigkeit ist dieses Verfahren vorteilhaft.

BEHANDLUNG DES STREIFENS NACH DER ELEKTROPHORESE

Nach dem Abschalten der Spannung werden die Filterpapierzuführungen entfernt, die beiden Enden des Cellogelstreifens kurz abgetupft, der Streifen von der Glasunterlage abgelöst und auf eine andere Glasplatte geeigneter Länge so aufgelegt, dass zwischen dem Streifen und der Glasoberfläche möglichst keine Luftblasen eingeschlossen werden. Die Platte mit dem Streifen wird nun für 5–10 Min in einen auf 80–100° aufgeheizten Trockenschrank gelegt, wobei der Streifen transparent wird. Bei Anwesenheit grösserer Mengen mancher anorganischer Kationen erkennt man den gewanderten Substanzfleck auf dem Cellogelstreifen auch daran, dass die sonst praktisch glasklare Folie an dieser Stelle trüb bis undurchsichtig erscheint. Besonders deutlich war dies bei unseren Arbeiten mit Strontium-90, Barium-140 und Scandium-46. Strontium-90 und Yttrium-90, sowie Barium-140 und Lanthan-140 lagen praktisch trägerfrei vor, während Scandium durch Neutronen im Reaktor aktiviert worden war und beträchtliche Mengen inaktives Scandium enthielt. Bei diesen Streifen war nach dem Erwärmen im Trockenschrank der Scandiumfleck stets mit freiem Auge erkennbar, während die trägerfreien Nuklide nur durch ihre Strahlung lokalisiert werden konnten. Die auf dem Cellogelstreifen vorliegenden radioaktiven Substanzen sind nach dem Erhitzen im Trockenschrank ganz im nunmehr transparenten Cellogel eingebettet. Mit den so behandelten Streifen kann verhältnismässig bequem hantiert werden, da diese auch beim Wischtest keine nennenswerten Aktivitäten abgeben. In einigen Fällen gelang es nicht, den Streifen transparent zu erhalten. Bei den mit Glykolsäure, Milchsäure oder α-Hydroxyisobuttersäure getränkten Streifen war dies z.B. dann der Fall, wenn die Konzentration der Säure unter 0.2 molar war.

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* Marderhaar, Grösse O (für Aquarelle).

AUTORADIOGRAPHIE

Sobald der Streifen transparent ist, wird die Glasplatte mit der Folie aus dem Trockenschrank genommen und nach dem Abkühlen mit Startmarkierungen versehen. Wir verwenden gewöhnlich Klebefolien, die auf eine Glasfläche geklebt, mit punktförmigen Aktivitäten von Promethium-147 versehen und mit einer Rasierklinge in schmale Streifen zerschnitten werden. Diese Streifen werden neben die Cellogelfolie so auf die Glasplatte geklebt, dass sich die radioaktive Markierung auf gleicher Höhe mit dem Start des Elektropherogramms am Autoradiogramm abbildet. So ist der Abstand der gewanderten Substanzen vom Start leicht erkennbar.

Es ist zweckmässig, für die Startmarkierung einen weichen Betastrahler zu verwenden, da dieser eine Seite des beidseitig beschichteten Röntgenfilms stärker schwärzt. Beim Betrachten des Autoradiogramms im reflektierten Licht erkennt man dann sofort, welche Seite des Films dem aktiven Streifen zugekehrt war, was besonders für das seitenrichtige Herausschneiden eventuell schräg liegender Zonen des Cellogelstreifens von Bedeutung ist.

Nach Anbringen der Startmarkierung wird der Cellogelstreifen mit einer Polyesterabdeckfolie bedeckt, um eine Chemographie und radioaktive Kontamination des darauf gelegten Röntgenfilms zu vermeiden. Bei Autoradiographie von Alphastrahlern verwendeten wir Polyesterfolien von 0.65 mg/cm². Der Röntgenfilm wird mit einer zweiten Glasplatte angedrückt. Nach geeigneter Expositionsdauer wird der Film entwickelt. Das fertige Autoradiogramm wird dann seitenrichtig unter die Glasplatte gelegt und mit den Startmarkierungen am Film und am Cellogelstreifen zur Deckung gebracht. Auf der Deckfolie werden die aktiven Zonen angezeichnet. Die aktiven Flecken werden nun mit einer Rasierklinge direkt aus dem Cellogelstreifen ausgeschnitten und können dann weiterverarbeitet werden.

WEITERVERARBEITUNG DER CELLOGELSTREIFEN

Dieses Problem soll in der vorliegenden Arbeit nur kurz gestreift werden.

Bei absoluten oder auch bei vergleichenden quantitativen Messungen der Radioaktivität der gewanderten Substanzflecken ist eine Messung unter definierten geometrischen Bedingungen notwendig. Messungen am Elektropherogramm direkt liefern oft nicht die gewünschte Genauigkeit. Es wurde daher versucht, dünne Folien mit gleichmässiger Verteilung der Radioaktivität zu erhalten. Dazu wurden die aus dem Cellogelstreifen herausgeschnittenen aktiven Zonen in organischen Lösungsmitteln gelöst und die Lösung auf einer geeigneten Unterlage eingedunstet. Brauchbare Ergebnisse erzielten wir mit Aceton als Lösungsmittel und Polyäthylen als Unterlage. Um die Fläche, auf der die Lösung zur Verdunstung aufgegossen wurde, zu begrenzen, wurden Ringe aus Polyäthylen auf die Unterlage gelegt. Nach dem Eindunsten konnten die Folien mit einiger Sorgfalt sauber von der Unterlage abgelöst werden und hafteten am Polyäthylenring. Zur Messung wurde ein kreisförmiges Mittelstück herausgestanzt und z.B. an einem Trägerrahmen für die 4 π-Zählung montiert. Folien der Dicke 0.2–0.5 mg/cm² liessen sich unschwer herstellen. Für eine chemische Weiterverarbeitung kann man das Cellogelmaterial in Methylenchlorid lösen und das Radionuklid extrahieren. Da bei der Extraktion die Acetylcellulose meist flockig ausfällt, muss unter Umständen ein inaktives Trägerisotop zugesetzt werden, um durch Adsorption auftretende Fehler zu vermindern.

EXPERIMENTELLE BEISPIELE

Eine Reihe interessanter radiochemischer Trennungen wurde mittels Elektrophorese auf Cellogelstreifen in Lösungen von α-Hydroxyisobuttersäure oder Milchsäure untersucht. Fig. 4 soll ein Beispiel zeigen. Um Zirkon-95 und Niob-95 zu trennen, wurde die ursprünglich vorliegende Lösung der Oxalatkomplexe dieser Spaltprodukte mehrmals mit konzentrierter Salpetersäure und Perhydrol eingedampft, in einer Lösung von α-Hydroxyisobuttersäure aufgenommen und am Start des Elektropherogramms aufgetragen. Beide Ionen wanderten als anionische Komplexe, jedoch blieb, wie aus Fig. 4a ersehen werden kann, ein grosser Teil am Start zurück, vermutlich in hydrolysierter bzw. unlöslicher Form. Die γ-Spektren der beiden Substanzflecke sind deutlich voneinander verschieden, wie man aus den übereinander projizierten Spektren in Fig. 4b erkennen kan (Zirkon-95; 0.75 + 0.72 MeV; Niob-95, 0.76 MeV). Durch

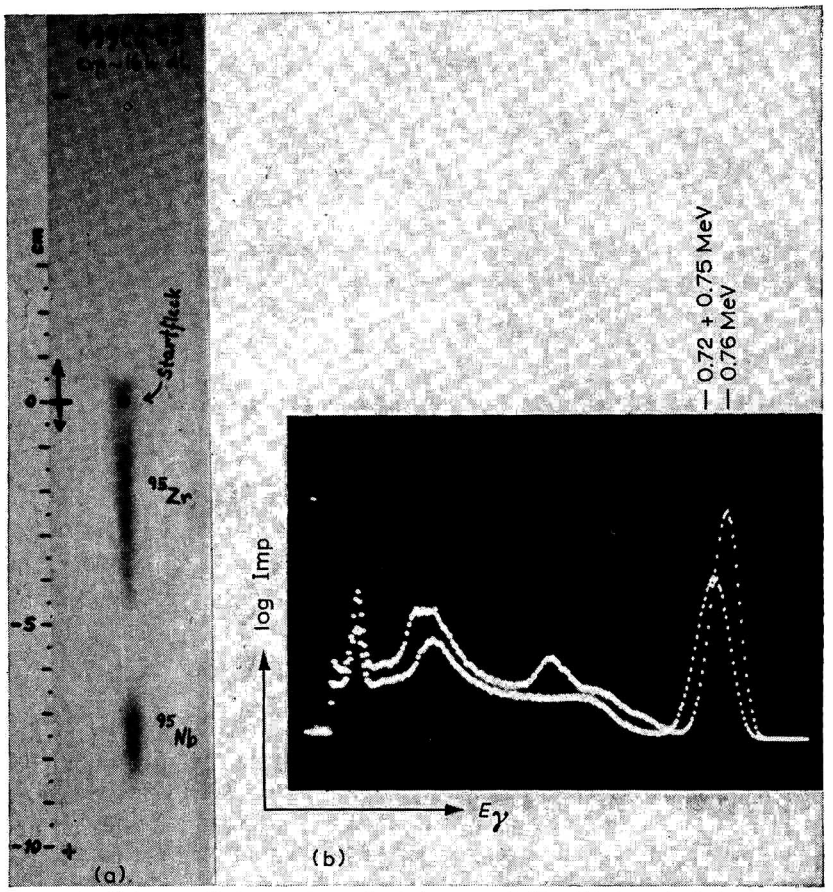


Fig. 4. (a) ^{95}Zr und ^{95}Nb wandern als Anionen. 0.8 *M* α-Hydroxyisobuttersäure; *ca.* 50 V/cm; 6.0–6.3 mA; 34.3 Min. Autoradiogramm 16 h exponiert. (b) Vergleich der γ-Spektren der beiden als Anionen gewanderten Aktivitäten der Fig. 4a. Der weiter gewanderte Fleck (^{95}Nb) liefert einen charakteristischen γ-Peak bei 0.76 MeV; der andere Fleck zeigt einen Peak bei deutlich niedrigerer Energie (^{95}Zr, E_γ = 0.72 und 0.75 MeV). Es handelt sich also nicht um verschiedene Komplexe eines Elements (wie bei Ru, Fig. 5), sondern um eine Trennung von Zr und Nb.

diese gammaspektrometrischen Messungen sollte gezeigt werden, dass es sich nicht um eine Auftrennung verschiedener Komplexe eines der beiden eingesetzten Spaltprodukte handelt, sondern eine Trennung von Niob und Zirkon erreicht werden konnte.

Eine Trennung verschiedener Komplexe eines Ions zeigt die autoradiographische Auswertung eines Elektropherogramms in Fig. 5a. Am Startpunkt wurde ein Tropfen einer schwach salzsauren Ruthenium-106-Lösung aufgetragen. Im Diagramm in Fig. 5b erkennt man die Aktivitätsverteilung entlang des Elektropherogramms.

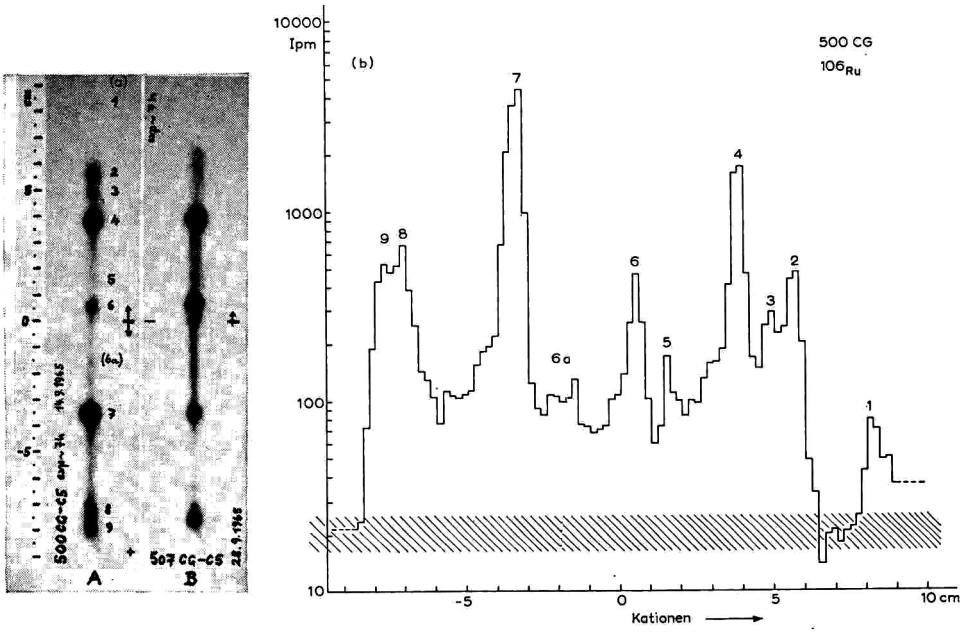


Fig. 5. (a) Elektropherogramme des Spaltproduktes ^{106}Ru zeigen mehrere Aktivitätsmaxima, von denen jedes einer definierten ionischen Spezies entsprechen muss. Bei einigen der Flecken dürfte es sich um Chlorokomplexe handeln, da das verwendete ^{106}Ru aus einer HCl-sauren Lösung stammte. 0.8 *M* α-Hydroxyisobuttersäure. (A) *ca.* 55 V/cm; 6–7 mA; 7.5 Minuten. (B) *ca.* 65 V/cm; 7–7.9 mA; 6.4 Minuten. Die gleiche Ru-Probe wie in A, aber 14 Tage lufttrocken aufbewahrt. (b) Die durch Zerschneiden des Cellogelstreifens in 2 mm breite Abschnitte erhaltene Aktivitätsverteilung. Die Zahlen bezeichnen die einzelnen Maxima entsprechend Fig. 5a. Der schraffierte Bereich zeigt den durchschnittlichen Leerwert ($19 \pm \sqrt{19}$ Ipm).

Gammaspektrometrische Vergleichsaufnahmen zeigten, dass alle Aktivitätsmaxima auf Ruthenium-106/Rhodium-106 zurückzuführen sind. Ein Gleichgewicht zwischen den einzelnen Komplexen scheint sich während der Dauer der Elektrophorese nicht oder nur sehr langsam einzustellen, weil die einzelnen Schwärzungen kreisrund und gut abgegrenzt am Autoradiogramm erscheinen. Eine genauere Bestimmung der Zusammensetzung der Komplexe wurde von uns bisher nicht durchgeführt.

Auch für die Analyse der in einer natürlichen radioaktiven Zerfallsreihe vorliegenden Elemente hat sich das Verfahren bewährt. Fig. 6 zeigt die Auftrennung von Gliedern der Actinium-Zerfallsreihe. Darüber wurde bereits ausführlich berichtet[2].

In an anderer Stelle erschienenen Arbeiten berichteten wir über Trennungen

der Elemente mit den Ordnungszahlen 57–71[1,5]. Fig. 7 zeigt ein Beispiel für die Elektrophorese eines Gemisches aus einigen Seltenen Erden.

Eine Mischung von Holmium, Erbium, Thulium, Ytterbium und Lutetium wurde im Reaktor aktiviert und anschliessend aufgetrennt. Bei diesen Trennungen erweist sich das Auftragen der Substanzmischung am Start mittels eines Pinsels als besonders vorteilhaft. Es können dann auf einer relativ kurzen Wanderungsstrecke eine grössere Anzahl verschiedener Ionen aufgetrennt und autoradiographisch nachgewiesen werden. Fig. 8 zeigt zum Vergleich das Ergebnis einer Trennung, bei der das Substanzgemisch am Start mittels eines Pinselstriches aufgetragen wurde.

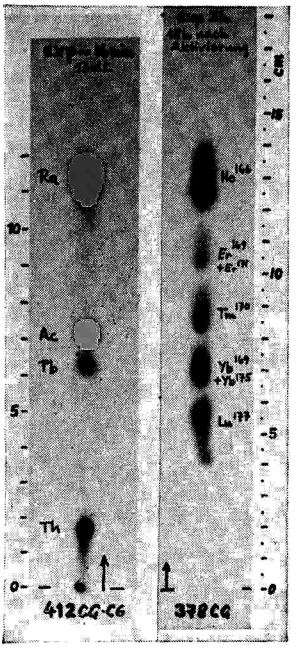


Fig. 6. Trennung von Gliedern der ^{227}Actinium-Zerfallsreihe auf Cellogel. 0.8 *M* α-Hydroxyisobuttersäure; 52 V/cm; 5.7–5.8 mA; 9.7 Min.

Fig. 7. Trennung eines Gemisches Seltener Erden auf Cellogel. 0.8 *M* α-Hydroxyisobuttersäure; *ca.* 45 V/cm; 6–7.5 mA; 90 Min.

Cellogelstreifen eignen sich auch zur elektrophoretischen Auftrennung von radioaktiven Spaltprodukten. Fig. 9 zeigt die autoradiographische Auswertung eines Elektropherogramms, auf dem Cäsium-137, Strontium- und Yttrium-90, sowie Cer-144 aufgetrennt sind. Die Identifizierung kann durch Gammaspektrometrie (Cäsium-137, Cer-144) bzw. durch die zeitliche Änderung der Aktivität (Strontium-90, Yttrium-90) erfolgen. Zur genauen Untersuchung wurde das Elektropherogramm in 2 mm breite Streifen geschnitten und diese unter dem GM-Zählrohr gemessen. Das Diagramm in Fig. 10 lässt die gute Trennung deutlich erkennen. Allerdings trat bei Strontium-90 eine leichte Schwanzbildung auf. Dies konnte häufig bei der Auftrennung von Erdalkalikationen auf Cellogel bemerkt werden. Auch bei Radium in Fig. 6 und bei Calcium, Strontium und Barium in Fig. 11 zeigt sich dieser Effekt.

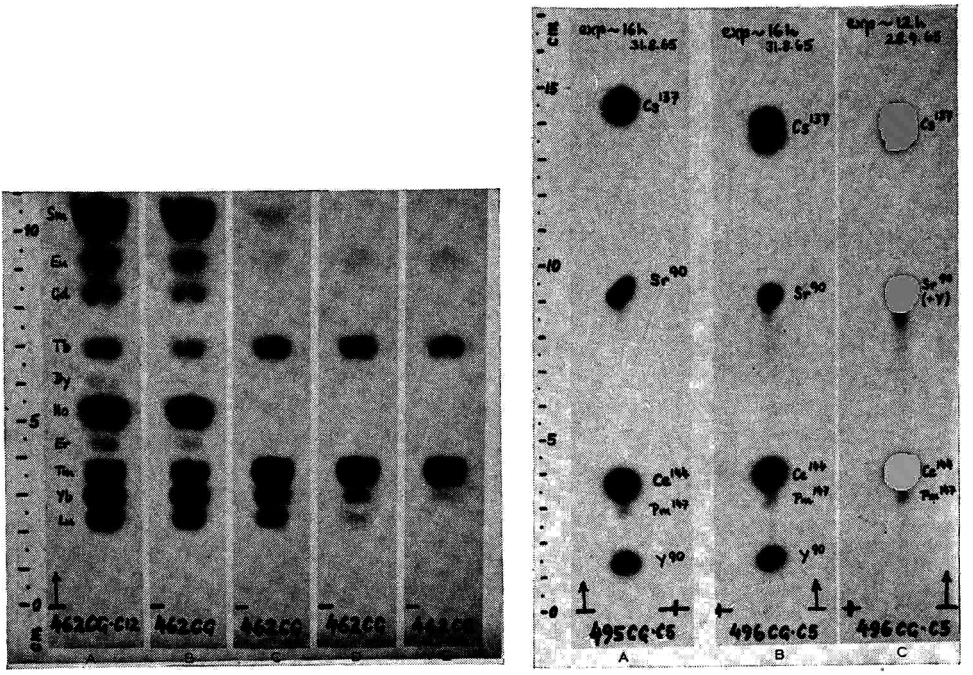


Fig. 8. Auftragen der zu trennenden Mischung mit einem Pinsel. Bei kurzen Laufstrecken bleibt die Form des Striches im wesentlichen erhalten. 0.8 *M* α-Hydroxyisobuttersäure; 47 V/cm; 9–9.5 mA; 33 Min. (A, B) 2 h exponiert; (C. D, E) je 3 h exponiert. (A) 1 h nach der Trennung, (B) 5 h, (C) 11 Tage, (D) 27 Tage, (E) 61 Tage nach der Trennung. Die Figur zeigt das Abklingen der verschiedenen Aktivitäten. Radionuklide: ^{153}Sm, $^{152m+152+154}$Eu, ^{159}Gd, ^{160}Tb, ^{165}Dy, ^{166}Ho, $^{169+171}$Er, ^{170}Tm, $^{169+175}$Yb und $^{176m+177}$Lu.

Fig. 9. Trennung einer Spaltproduktmischung. 0.8 *M* α-Hydroxyisobuttersäure; 60 V/cm; 8.3 Min. (A) 7.0–7.9 mA, (B) 5.2–5.7 mA. (A und B) am Tag der Trennung 16 h exponiert. (C) der gleiche Streifen wie (B), 28 Tage später 12 h exponiert. ^{90}Y (HWZ 64.5 h) ist hier abgeklungen, während der ^{90}Sr Fleck durch nachgewachsenes ^{90}Y stärker aktiv erscheint. ^{147}Pm war in dem von uns verwendeten ^{144}Ce als Verunreinigung enthalten.

Fig. 11a bringt ein Beispiel für die Trennung der Erdalkaliionen Calcium, Strontium und Barium und der dreiwertigen Kationen Lanthan, Yttrium und Scandium. Diese Mischung liegt vor, wenn die erwähnten Erdalkalikationen in radioaktiver Form mit ebenfalls aktiven Tochternukliden vorhanden sind. Die Spaltungsprodukte Strontium-90 und Barium-140 bilden radioaktive Folgeprodukte mit kürzeren Halbwertszeiten (Yttrium-90: 64,5 h; Lanthan-140: 40.2 h), daher ist nach einiger Zeit keine Aktivität an der Stelle der Yttrium- und Lanthanflecken nachweisbar. Auch Barium-140 selbst klingt mit einer Halbwertszeit von 12.8 Tagen ab. Die unterschiedlichen Halbwertszeiten können zur Identifizierung der Flecken auf dem Elektropherogramm verwendet werden. An dem einige Monate nach der Trennung nochmals exponierten Streifen fehlen also die kurzlebigen Radionuklide (Fig. 11a). Für Calcium und Scandium wurde nicht das Mutter–Tochter System eingesetzt, sondern Calcium-45 und Scandium-46 verwendet. Die Identität des Scandium-46 konnte gammaspektrometrisch sichergestellt werden (Fig. 11b).

Eine besondere Identifizierung des Scandiumflecks erwies sich als wünschens-

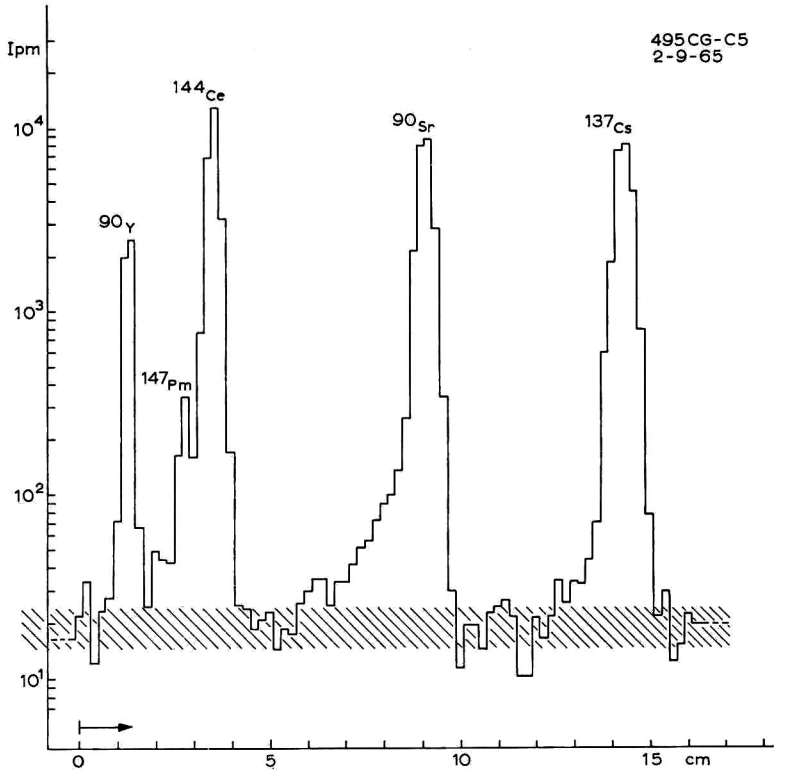


Fig. 10. Durch Zerschneiden des Streifens der Fig. 9A in 2 mm breite Abschnitte erhaltene Aktivitätsverteilung. Der schraffierte Bereich bezeichnet den Leerwert ($19 \pm \sqrt{19}$ Ipm). Zwischen den Maxima sinkt die Aktivität praktisch auf den Leerwert. ^{90}Sr zeigt eine Schwanzbildung.

wert, da andere Autoren[6] über eine Reihenfolge von Yttrium, Lanthan und Scandium bei der Papierelektrophorese in Milchsäure berichten, die nicht mit der von uns in α-Hydroxyisobuttersäure gefundenen übereinstimmt. Die Reihenfolge der anderen auf dem Elektropherogramm erkennbaren Flecken ist aus früheren eigenen Arbeiten[5] bekannt.

AKTIVIERUNGSANALYTISCHER NACHWEIS AM CELLOGELSTREIFEN

Für den Nachweis inaktiver anorganischer Kationen nach der elektrophoretischen Auftrennung schien die Aktivierungsanalyse im Trägermedium direkt ein brauchbares Verfahren zu sein. Jedoch erweisen sich bei einem Neutronenfluss von etwa 10^{12}n/cm²/sec Bestrahlungszeiten von über 15 Min. als ungünstig, da die Aktivität der Verunreinigungen im Cellogel selbst (Natrium, Phosphor, Chlor) zu hoch wird. Dies wird durch die Autoradiographie in Fig. 12 anschaulich dargestellt. Bei längerer Aktivierung im Reaktor wird nicht nur eine zu hohe Untergrundschwärzung bei Autoradiogrammen erhalten, sondern es bilden sich auch störende Abbildungen von Unregelmässigkeiten der Cellogelfolien. Der Gehalt der aktivierten Cellogelfolien an Natrium-24, Chlor-38 und Phosphor-32 konnte gammaspektrometrisch bzw. durch

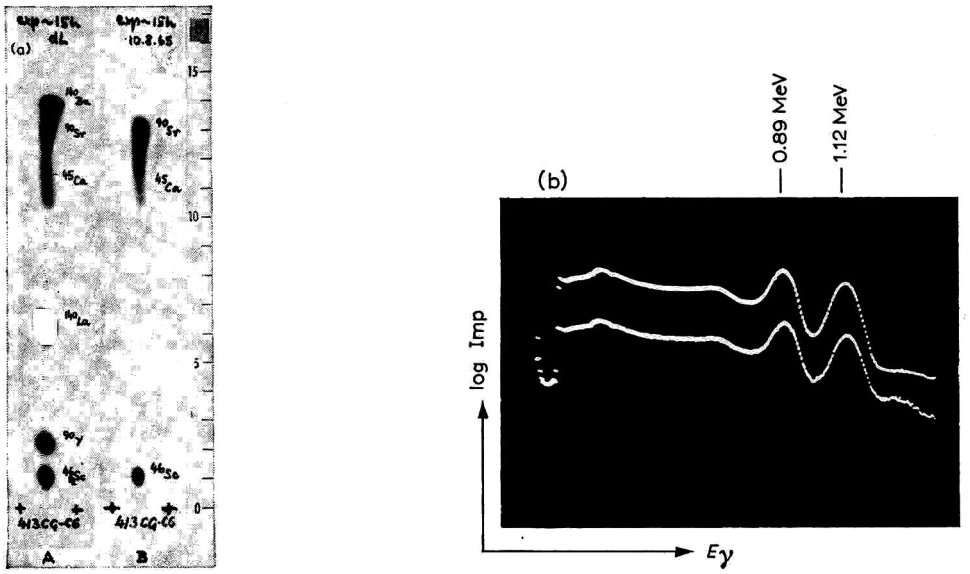


Fig. 11. (a) Elektropherogramm von Ba, Sr, Ca, La, Y und Sc auf Cellogel. 0.8 *M* α-Hydroxyisobuttersäure; *ca.* 50 V/cm; 5.1 mA; Trennzeit: 15 Min. (A) am Tag der Trennung 15 h exponiert. (B) 175 Tage später, 15 h exponiert. ^{45}Ca (HWZ 160 d) und ^{46}Sc (HWZ 85 d) haben merklich abgenommen. ^{140}Ba (HWZ 12.8 d), ^{140}La (HWZ 40.2 h) und ^{90}Y (HWZ 64.5 h) sind verschwunden. (b) Obere Kurve: γ-Spektrum eines ^{46}Sc-Standards. Untere Kurve: γ-Spektrum des als Sc bezeichneten Flecks der Fig. 11a.

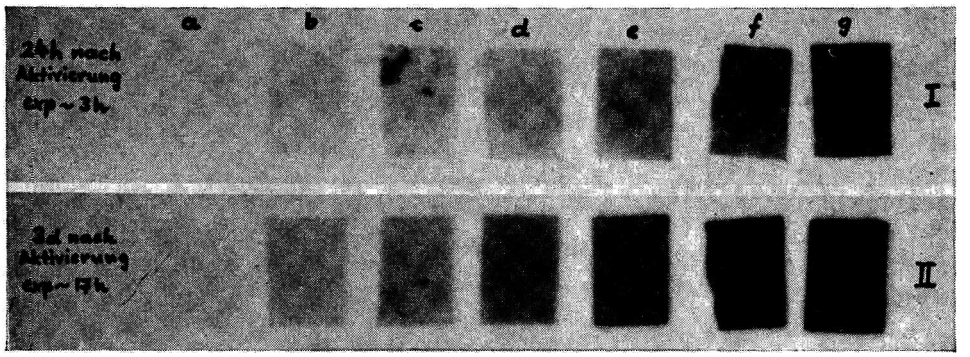


Fig. 12. Neutronenaktivierung von in 0.8 *M* α-Hydroxyisobuttersäure getränkten Cellogelstreifen. (I) 24 h nach Aktivierung; (II) 3 Tage nach Aktivierung. (a) 5, (b) 15, (c) 30 Min., (d) 1, (e) 2, (f) 3, (g) 4 h bei einem Fluss von $1.75 \cdot 10^{12}$ *n* cm^{-2} sec^{-1} aktiviert.

Aufnahme von β-Absorptionskurven bewiesen werden. Nach einer Elektrophorese können daher durch Neutronenbestrahlung nur Substanzen mit relativ grossen Einfangquerschnitten für Neutronen und geeigneten Halbwertszeiten der radioaktiven Bestrahlungsprodukte nachgewiesen werden. Dieser Nachweis ist für eine Reihe von Lanthaniden gut geeignet. Versuche in dieser Richtung werden in einer folgenden Arbeit erwähnt werden. An dieser Stelle sei nur ein aktivierungsanalytischer Nachweis von Samarium angeführt. Fig. 13 zeigt eine Trennung von Europium und Samarium. Nach einigen Tagen war das Samarium abgeklungen, konnte aber durch nachträgliche Aktivierung wieder autoradiographisch nachgewiesen werden. Fig. 13 zeigt die Autoradiogramme des Elektropherogramms vor und nach der Neutronenbestrahlung.

Nach Trennversuchen im Bereich der Lanthaniden lag es nahe, Cellogel auch auf seine Verwendbarkeit im Bereich der dreiwertigen Transurane zu prüfen. Über eine Trennung von Cer, Americium und Thulium auf Cellogel ist berichtet worden. Von den höheren Transuranen stand uns leider nur Americium-241 zur Verfügung.

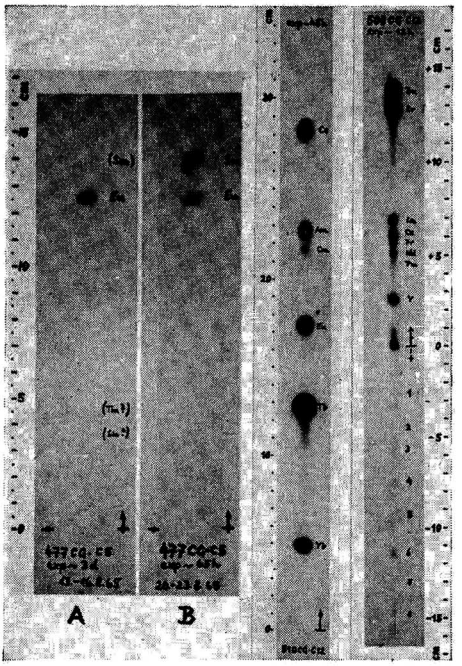


Fig. 13. Nachaktivierung eines Cellogelstreifens mit einer Sm–Eu-Trennung. 0.8 *M* α-Hydroxyisobuttersäure; *ca.* 60 V/cm; 7–7.5 mA; 34 Min. Radionuklide: ^{153}Sm, ^{155}Eu. (A) Abtrennung des ^{155}Eu von bereits abgeklungenem Sm. Das Autoradiogramm zeigt zunächst nur den Ort des ^{155}Eu. Exposition *ca.* 70 h. (B) Nach einer Minute Aktivierung des ganzen Streifens bei einem Neutronenfluss von $1.75 \cdot 10^{12}$ n cm^{-2} sec^{-1} tritt auch der Sm-Spot hervor (^{153}Sm, 47 h). Europium wird nicht mitaktiviert, da das ^{155}Eu trägerfrei vorlag. Exposition *ca.* 65 h.

Fig. 14. Trennung von Ce, Am, Cm, Eu, Tb und Yb. 0.8 *M* α-Hydroxyisobuttersäure; *ca.* 48 V/cm; 8–9 mA; 72.6 Min. Radionuklide: ^{144}Ce, ^{241}Am, ^{242}Cm, $^{152+154}$Eu, ^{160}Tb, ^{169}Yb. Die Mischung enthielt eine übergrosse Menge Tb.

Fig. 15. Trennung eines bei der Spaltung von Plutonium-239 entstehenden Gemisches. 0.8 *M* α-Hydroxyisobuttersäure; *ca.* 48 V/cm; 7.3–8.2 mA; 14.5 Min. 3 h nach der Trennung 12 h lang exponiert. Die Ziffern 1 bis 8 bezeichnen nicht näher identifizierte Anionen.

Durch Neutronen-Aktivierung im Reaktor konnte jedoch Americium-242 erhalten werden, welches durch β-Zerfall in Curium-242 übergeht. Dieses konnte nun elektrophoretisch abgetrennt werden. Fig. 14 zeigt autoradiographische Aufnahmen der auf Cellogel getrennten Radionuklide. In einer früheren Arbeit[1] wurde eine Vorhersage über die Wanderungsweiten der Transurane bei der Elektrophorese getroffen, die sich verifizieren liess. Demnach müsste bei höheren Transuranen (Bk, Cf) die Trennung noch besser sein.

Fig. 15 zeigt eine elektrophoretische Trennung eines bei der Spaltung von Plutonium-239 entstehenden Gemisches. Eine salpetersaure $Pu(NO_3)_4$-Lösung wurde unter der Infrarotlampe eingedunstet, mit α-Hydroxyisobuttersäure versetzt und wieder eingedunstet. Diese Probe wurde erst 5 h und 3 Tage später noch einmal 4 h lang einem thermischen Neutronenfluss von $1.75 \cdot 10^{12}$ n cm^{-2} sec^{-1} ausgesetzt und dann 24 h abklingen gelassen. Die Probe wurde mit 2 Tropfen destilliertem Wasser versetzt, wobei das Plutonium anscheinend fast quantitativ als voluminöser grüner Rückstand zurückblieb, während Spaltproduktaktivitäten in Lösung gingen. Die Spots von Ba, La und Ce konnten γ-spektrometrisch identifiziert werden. Die Identität der als Sr, Pr, Nd, Pm und Y bezeichneten Spots ergibt sich aus der Sequenz. Die restlichen Schwärzungen (besonders die zahlreichen Anionen) konnten wegen ihrer zu geringen Aktivität nicht identifiziert werden.

VERHALTEN DES CELLOGELS BEI DER AUFTRENNUNG GRÖSSERER SUBSTANZMENGEN

Werden zu grosse Substanzmengen aufgetragen, so zeigen sich am Elektropherogramm grosse und verformte Substanzflecken. Auch werden die Wanderungsweiten der im Elektropherogramm nachfolgenden Ionen beeinflusst. Diese wandern hinter dem in grösserer Menge vorliegenden Ion etwas rascher.

Wie aus den berichteten Ergebnissen hervorgeht, eignet sich Cellogel sehr gut für die elektrophoretische Trennung anorganischer Kationen. Besonders bei der Anwendung auf sehr geringe Substanzmengen (trägerfreie Radionuklide) werden gute Ergebnisse erhalten. Die Resultate sind einwandfrei reproduzierbar und die Arbeitstechnik einfach.

ZUSAMMENFASSUNG

Es wird die Arbeitstechnik für die Elektrophorese anorganischer radioaktiver Kationen auf einem Acetylcelluloseträgermaterial (Cellogel) beschrieben. Die Vorteile dieser Technik werden an Hand von zahlreichen Beispielen dargelegt. Besonders erwähnt werden Trennungen von Seltenen Erden, Erdalkalien und Spaltprodukten.

SUMMARY

A method for the electrophoresis of inorganic radioactive cations on cellulose acetate (Cellogel) is described. The advantages of this technique are illustrated by numerous examples. Separations of the rare earth metals, the alkaline earths and fission products are mentioned particularly.

LITERATUR

1 K. BUCHTELA, K. AITZETMÜLLER UND F. GRASS, *Atomkernenergie*, 10 (1965) 264.
2 K. AITZETMÜLLER, K. BUCHTELA UND F. GRASS, *Atomkernenergie*, 10 (1965) 269.
3 R. CLOTTEN UND A. CLOTTEN, *Hochspannungselektrophorese*, Thieme, Stuttgart, 1962, S. 116.
4 A. PAPPAS, J. ALSTAD UND G. LUNDE, *Radiochim. Acta*, 1 (1963) 109.
5 K. AITZETMÜLLER, K. BUCHTELA, F. GRASS UND F. HECHT, *Mikrochim. Ichnoanal. Acta*, (1964/6) 1089.
6 T. R. SATO, W. P. NORRIS UND H. H. STRAIN, *Anal. Chem.*, 27 (1955) 521;
T. R. SATO, H. DIAMOND, W. P. NORRIS UND H. H. STRAIN, *J. Am. Chem. Soc.*, 74 (1952) 6154.

Notes

Ein Beitrag zur Gas-Chromatographie von Phenoxyalkancarbonsäuren und anderer herbizid-wirksamer Säuren

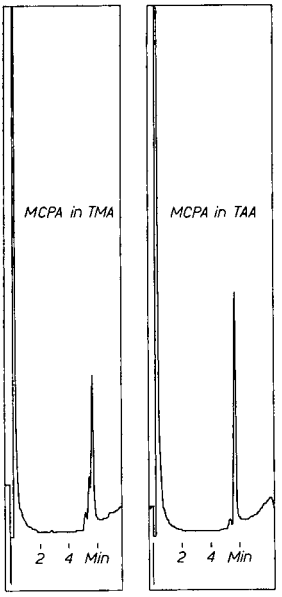
Vor einiger Zeit wurde von ROBB UND WESTBROOK [1] eine Methode zur direkten Herstellung von Carbonsäuremethylestern im Einspritzblock eines Gas-Chromatographen beschrieben, die auf dem 1936 von PRELOG UND PIZENTANIDA[2] mitgeteilten Darstellungsverfahren der Methylester durch thermische Zersetzung von Carbonsäuretetramethylammoniumsalzen beruht. Injiziert man Lösungen solcher Salze direkt in einen hochgeheizten Einspritzblock, so bilden sich die Ester, die im Gegensatz zu den freien Säuren sehr gut chromatographiert werden können.

Wir befanden uns in unserem Laboratorium seit längerer Zeit auf der Suche nach einer einfachen und schnellen Methode zur gas-chromatographischen Bestimmung von freien Phenoxyalkancarbonsäuren. Diese Säuren besitzen auf Grund ihrer Wuchsstoffwirkung grosse Bedeutung als Herbizide. Um diese Methode zur Rückstandsbestimmung in Bodenextrakten anwenden zu können, musste die vorherige Isolierung der Tetramethylammoniumsalze, wie sie von ROBB UND WESTBROOK vorgenommen wurde, umgangen werden. Wir haben daher die Säuren direkt in einer 10 %-igen wässr. Tetramethylammoniumhydroxid(TMA)-Lösung gelöst und diese Lösung in einen auf 380–400° aufgeheizten Einspritzblock injiziert. Die Phenoxyalkancarbonsäuren geben unter diesen Bedingungen jedoch nicht nur die gewünschten Methylester; es entstehen ausserdem noch einige Nebenprodukte. Wie wir schon bei früheren Versuchen festgestellt haben, ist das hier im grossen Überschuss vorhandene TMA unter den angewendeten Bedingungen sehr reaktionsfähig und vermag, eventuell über gebildete Methylionen oder -radikale, eine Reihe von Nebenreaktionen auszulösen[3]. Diese Eigenschaft zeigt das stabilere Tetraäthylammoniumhydroxid (TAA) in sehr viel geringerem Masse (Fig. 1). Löst man Phenoxyalkancarbonsäuren in einer 20 %-igen wässr. TAA-Lösung, so erhält man praktisch nur den Äthylester. Die Ausbeuten an Methylester lagen bei den von ROBB UND WESTBROOK untersuchten Alkancarbonsäuren zwischen 80 und 100 %. Die Ausbeuten an Phenoxyalkancarbon-

TABELLE I

AUSBEUTE AN ÄTHYLESTER

Phenoxyalkancarbonsäure	*Ausbeute an Äthylester* (%)
2-Methyl-4-chlorphenoxyessigsäure (MCPA)	85
2,4-Dichlorphenoxyessigsäure (2,4-DA)	33
2,4,5-Trichlorphenoxyessigsäure (2,4,5-TA)	35
2-(2-Methyl-4-chlorphenoxy)-propionsäure (α-MCPP)	20
2-(2,4-Dichlorphenoxy)-propionsäure (2-(2,4-DP))	55
2-(2,4,5-Trichlorphenoxy)-propionsäure (2-(2,4,5-TP))	24



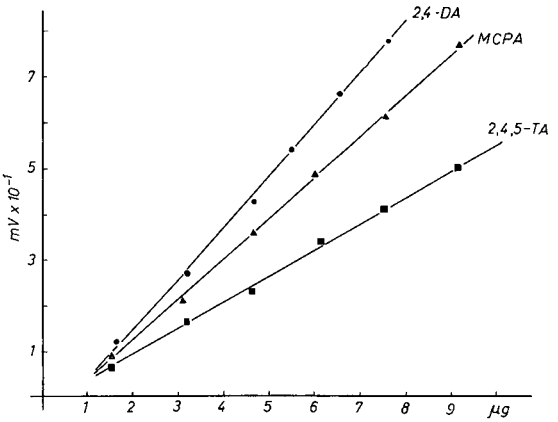
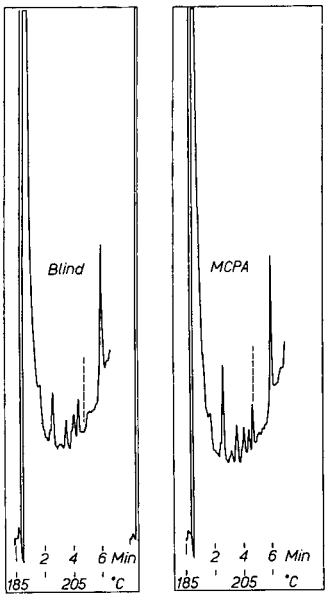


Fig. 1. 2-Methyl-4-chlorphenoxyessigsäure in 10%-iger wässr. TMA-Lösung (links) und in 20%-iger wässr. TAA-Lösung (rechts).

Fig. 2. Äthylester der Phenoxyalkancarbonsäuren: µg Säure pro µl gegen mV × 10^{-1} (Recorderanzeige).



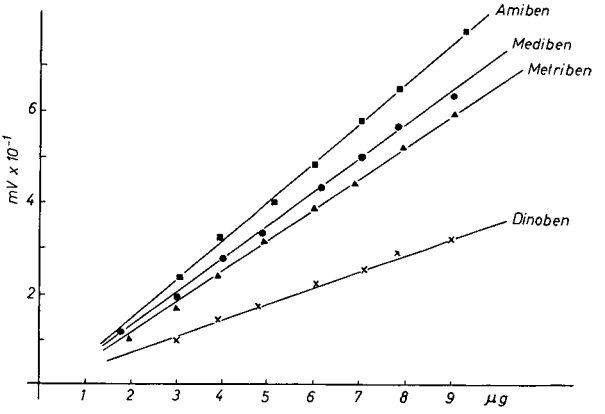


Fig. 3. Bodenextrakt in 20%-iger wässr. TAA-Lösung mit 1 p.p.m. MCPA neben einem Blindextrakt.

Fig. 4. Äthylester der Benzoesäuren: µg Säure pro µl gegen mV × 10^{-1} (Recorderanzeige).

säureäthylester liegen dagegen zum Teil wesentlich niedriger (Tabelle I). Die sehr gute Reproduzierbarkeit, sowie die lineare Abhängigkeit der Esterbildung von der Konzentration an freier Säure bis zu 1 %-igen Lösungen (Fig. 2) erlauben es jedoch, diese Methode zur quantitativen Bestimmung anzuwenden.

Auch die Anwendung zur Rückstands-Bestimmung in ungereinigten Bodenextrakten ist möglich, obwohl durch das TAA offensichtlich einige höhermolekulare Extraktbestandteile in flüchtigere Verbindungen umgesetzt werden, was zu einer Zunahme der Signalzahl in dem Bereich führt, in dem auch die Äthylester erscheinen (Fig. 3). Quantitative Bestimmungen und Identifizierungen lassen sich jedoch ohne Schwierigkeiten noch bis in den 0.5 p.p.m.-Bereich hinein durchführen.

Das Verfahren wurde ausserdem an einigen herbizid-wirksamen, substituierten Benzoesäuren erprobt. Für die Versuche wurden verwendet:

Mediben = 2-Methoxy-3,6-dichlorbenzoesäure,
Metriben = 2-Methoxy-3,5,6-trichlorbenzoesäure,
Amiben = 3-Amino-2,5-dichlorbenzoesäure,
Dinoben = 3-Nitro-2,5-dichlorbenzoesäure.

Wie die Fig. 4 zeigt, ist die Konzentrationsabhängigkeit bis zu 1 %-igen Lösungen ebenfalls hinreichend linear. Während die Reproduzierbarkeit beim Mediben und Metriben gut ist, streuen die Werte bei der amino- und der nitrogruppen-haltigen Benzoesäure etwas stärker.

Experimente

Die Untersuchungen wurden am Modell 810 der Firma F & M, Avondale, U.S.A., mit Flammenionisationsdetektor durchgeführt.

Kolonne. 1.25 % Versamid 900 auf Chromosorb G-AW-DMCS, 70–80 US-mesh, in 1.70 m Cu-Rohr, Innen-ϕ 4.5 mm, Aussen-ϕ $^1/_4$-Inch. Die günstigsten Temperaturen für die einzelnen Äthylester sind in Tabelle II zusammengestellt.

TABELLE II

KOLONNEN-TEMPERATUR (ISOTHERM) FÜR ÄTHYLESTER

Äthylester von	*Temp.* (°C)	*Äthylester von*	*Temp.* (°C)
MCPA	195	Mediben	150
2,4-DA	210	Metriben	185
2,4,5-TA	230	Amiben	265
α-MCPP	185	Dinoben	230
2-(2,4-DP)	202		
2-(2,4,5-TP)	225		

Zur Bestimmung in Bodenextrakten wurde im allgemeinen 10° unterhalb dieser Temperatur begonnen und mit einer Rate von 10°/Min. bis 275° programmiert. Temperaturen: Einspritzblock 390°; Detektor 310°. Gase: Trägergas 110 ml He/Min., 40 p.s.i.g. Vordruck; Wasserstoff 50 ml/Min., 16 p.s.i.g. Vordruck; Luft 220 ml/Min., 15 p.s.i.g. Vordruck. Schreiber: 1 mV Recorder mit 0.25 Inch/Min. Vorschub.

Als *Testlösungen* wurden die Säuren in 20 %-iger wässr. TAA-Lösung (Th. Schuchardt, München) gelöst.

Die *Bodenextrakte* wurden aus 100 g getrocknetem Dahlemer Sandboden

(Humusgehalt 1.10 %) durch 30 Min. Schütteln mit 150 ml 0.5 %-iger Sodalösung erhalten. Danach wird das Gemisch 20 Min. bei 6000 U/Min. zentrifugiert und die wässr. Phase mit 25 ml Äther extrahiert. Der Äther wird verworfen. Die wässr. Phase wird mit verdünnter H_2SO_4 auf pH 2 gebracht, zweimal mit 50 ml Äther extrahiert und die Ätherlösung über Na_2SO_4 getrocknet. Der Äther wird dann am Vakuum bei 25–30° abgesaugt und der Rückstand mit 1 ml 20 %-iger wässr. TAA-Lösung aufgenommen. Von dieser Lösung werden *ca.* 5–10 μl in den Gas-Chromatographen möglichst langsam injiziert.

Institut für Pflanzenschutzmittelforschung in der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem (Deutschland)

HANNS G. HENKEL*

1 E. W. ROBB UND J. J. WESTBROOK, *Anal. Chem.*, 35 (1963) 1644.
2 V. PRELOG UND M. Z. PIZENTANIDA, *Physiol. Chem.*, 244 (1936) 56.
3 H. G. HENKEL, *J. Chromatog.*, 21 (1966) 346.

Eingegangen den 1. November 1965

* Neue Anschrift: Société Internationale de Recherche BP, Épernon (Eure-et-Loire), B.P. 12, Frankreich.

Chromatographie en phase gazeuse des premiers membres de la série homologue Si_nCl_{2n+2}

Au cours de nos recherches concernant les chlorures volatils de certains métaux, nous avons rencontré le cas intéressant de la formation du $(SiCl_2)_x$. Ce dichlorure de silicium, que SCHMEISSER ET VOSS[1] ainsi que SCHENK ET BLOCHING[2] furent les premiers à obtenir, a l'aspect d'un liquide visqueux; il se forme par l'action du tétrachlorure de silicium sur le silicium métallique à une température voisine de 1100° sous vide.

Par pyrolyse ou encore par action du chlore sous pression normale sur le dichlorure de silicium il est possible de produire un mélange des premiers membres de la série homologue Si_nCl_{2n+2}.

Le Tableau I donne ces substances, ainsi que leur point d'ébullition et la pression correspondante.

Nos recherches personnelles visaient à étudier par chromatographie en phase gazeuse les premiers membres de la série dont nous avions obtenu un mélange par chlorolyse de dichlorure de silicium à une température voisine de 60° et sous pression normale.

L'emploi de la chromatographie en phase gazeuse pour la séparation de ces substances nous a paru avantageux étant donné que leur séparation par distillation entraîne de grandes pertes et quelquefois, en raison de la température élevée cause la destruction partielle de certains membres.

TABLEAU I

POINTS D'ÉBULLITION, ET PRESSION CORRESPONDANTE DES PREMIERS MEMBRES DE LA SÉRIE HOMOLOGUE Si_nCl_{2n+2}

Chlorures de silicium	*Point d'ébullition (°C)*	*Pression (mm Hg)*
$SiCl_4$	57	760
Si_2Cl_6	146	760
Si_3Cl_8	215	760
Si_4Cl_{10}	95	10^{-3}
Si_5Cl_{12}	145	10^{-3}
Si_6Cl_{14}	185 (subl.)	10^{-3}

Dans la littérature, nous n'avons trouvé aucune indication spécifique sur les possibilités de séparation et d'identification de ces substances à l'aide de la chromatographie en phase gazeuse.

Étant donné le caractère fortement corrosif des substances à étudier et en même temps leur tendance prononcée à s'hydrolyser, nous nous sommes vus forcés de construire en partie un chromatographe de laboratoire car le travail avec un chromatographe de commerce nous a paru trop risqué.

L'injection des substances examinées s'est effectuée au moyen d'une seringue médicale. Cependant nous avons eu de grandes difficultés surtout au moment du transport et de l'injection dans l'appareil chromatographique, difficultés provenant de la tendance prononcée de ces substances à s'hydrolyser et du caractère visqueux de quelques mélanges contenant des homologues supérieurs.

Nous avons résolu le problème de la viscosité en utilisant le benzol comme diluant, et pour surmonter les difficultés provenant de la tendance de ces substances à subir l'hydrolyse, nous avons toujours travaillé dans une atmosphère exempte d'humidité, en utilisant la technique suivante:

(a) La partie arrière du piston de la seringue médicale était toujours enduite de vaseline afin d'éviter le contact de la substance avec l'air.

(b) L'extrémité de l'aiguille était toujours couverte d'un capuchon protecteur de polyéthylène.

Cette même technique a été utilisée également par WILKE, LOSSE ET SACKMANN[3].

Le Silicon Oil 550 sur Celite 30–60 mesh 20% s'est montré très approprié pour le remplissage de la colonne dont la longueur était 1 m et le diamètre intérieur de 6 mm.

Comme détecteur nous avons employé une cellule à conductibilité thermique et comme gaz vecteur l'azote (débit 28 ml/min à 150°).

Les essais expérimentaux pour les substances $SiCl_4$, Si_2Cl_6, Si_3Cl_8, ont été effectués à la température de 150° isotherm. Pour l'étude chromatographique des membres $SiCl_4$, Si_2Cl_6, Si_3Cl_8, Si_4Cl_{10} et Si_5Cl_{12} de la série homologue, la température a été progressivement élevée de 150° jusqu'à 290° (14°/min).

La température du détecteur était de 150° pour les essais isothermes et de 200° pour les essais à température progressivement élevée.

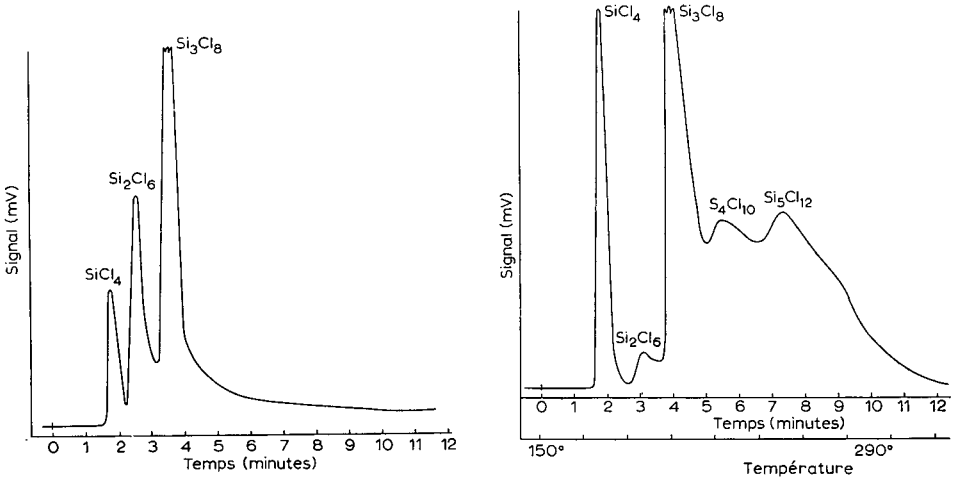


Fig. 1. Chromatogramme d'un mélange contenant les trois premiers membres de la série homologue Si_nCl_{2n+2}. Chromatographe: construit partiellement par les auteurs. Colonne: 1 m verre; diamètre intérieur 6 mm; diamètre extérieur 7.8 mm. Support solide: Celite 30–60 mesh. Phase stationnaire: Silicon Oil 550, 20%. Température de la colonne: 150°. Gaz vecteur: azote débit 28 cm^3/min (150°) pression d'entrée 1.53 kg/cm^2, pression de sortie 1 kg/cm^2. Détecteur: cellule à conductibilité thermique; polarisation 11 V; température 150°. Enregistreur: VARIAN G 40, 1 mV.

Fig. 2. Chromatogramme d'un mélange contenant les cinq membres de la série Si_nCl_{2n+2}. Chromatographe: construit partiellement par les auteurs. Colonne: 1 m verre; diamètre intérieur 6 mm; diamètre extérieur 7.8 mm. Support solide: Celite 30–60 mesh. Phase stationnaire: Silicon Oil 550, 20%. Température de la colonne: progressivement élevée de 150° à 290° (14°/min). Gaz vecteur: azote débit 28 cm^3/min (150°); pression d'entrée 1.53 kg/cm^2; pression de sortie 1 kg/cm^2. Détecteur: cellule à conductibilité thermique; polarisation 11 V; température 200°. Enregistreur: VARIAN G 40, 1 mV.

Résultats

Les Figs. 1 et 2 représentent une partie des résultats obtenus.

L'identification des pics a été effectuée en utilisant des substances pures et en comparant le temps de rétention.

Pour les trois premiers membres de la série homologue, la fonction logarithme du temps de rétention envers le nombre d'atomes de silicium est linéaire.

Remerciements

Les auteurs remercient le Comité Scientifique de l'OTAN, pour la bourse accordée à l'un d'eux (D.V.-P.) pour cette étude.

Laboratoire de Chimie Analytique, D. VRANDI-PISKOU
Université Nationale Technique d'Athènes (Grèce) G. PARISSAKIS

1 M. SCHMEISSER ET P. VOSS, *Z. Anorg. Allgem. Chem.*, 334 (1964) 50.
2 P. W. SCHENK ET H. BLOCHING, *Z. Anorg. Allgem. Chem.*, 334 (1964) 57.
3 J. WILKE, A. LOSSE ET H. SACKMANN, *J. Chromatog.*, 18 (1965) 482.

Reçu le 22 novembre 1965

Chromatography of amino acids on plaster of Paris

The use of thin and thick strips of set plaster of Paris in the chromatographic separation of alkaloids has been described in an earlier paper[1]. It was decided to investigate the use of such strips in the chromatographic separation of amino acids and this paper deals with the method evolved and the results obtained.

Thin layer chromatography of amino acids has already been studied[2] and the method proved to be faster than the classical separation on paper. The thin layer plates require delicate handling throughout the chromatography operations while strips of set plaster of Paris are mechanically stronger and offer better conditions especially when developing coloured spots. The chromatographic separation of amino acids in set plaster of Paris is very fast and the medium can be used for ascending, descending, circular and two-dimensional chromatography. In the present paper, ascending and circular chromatography methods are described and it has been found that there was sharp and clear separation in a matter of minutes of amino acids from protein hydrolysate, mixtures of known amino acids and amino acids in urine. Since the size of the sample which can be applied to set plaster of Paris strips is much larger than that possible on paper, colour development with ninhydrin is fast and the tone and depth of the colours obtained is better. The results obtained with thick and thin plates of set plaster of Paris are essentially the same, but in this paper 1 mm thick plates have been used because the size of the sample was not of importance as no preparative purpose was envisaged.

Preparation of thin plates of set plaster of Paris

30 g of plaster of Paris were mixed with 27 cc. of distilled water. Plates of 1 mm thickness were prepared by the technique described previously[1]. The plate, when fully set, was allowed to dry overnight at room temperature, and was then cut as described previously[1] in strips 2 cm broad, 10 cm long, and also in squares of 5 cm. The strips and squares were dried at 100° for 3 h.

Application of sample

The sample of mixture of amino acids, protein hydrolysate and 24 h collection of urine, were applied to the strips as spots with a graduated capillary pipette, the end of which was wrapped in cotton wool to form a swab. For the squares, the sample was applied to one corner by touching the latter with the pipette. In all cases the size of sample was 5 μl. In order to obtain quickly uniform and concentrated spots, the strips and squares were heated for 3 min on either side by the radiant heat of a 700 watt open-coil heater at a distance of 5 cm above the heater. The sample was applied to the hot strip or corner of the squares and further drying was carried out over the heater for 2 min. The strips and squares were allowed to stand at room temperature for 10 min before use.

Apparatus for chromatography

For ascending chromatography of the strips, a conventional chamber was used. For circular chromatography of the squares, a chamber consisting of Petri dishes arranged as shown in Fig. 1 was used. The corner of the square of set plaster of Paris G where the sample has been applied touches the filter paper wick E which is fixed

to a polyethylene hollow bottle stopper D having a hole in the centre. The polyethylene stopper has holes on its sides to allow the solvent H in Petri dish cover C free access to the wick. Petri dish A with cover B has a small amount of solvent

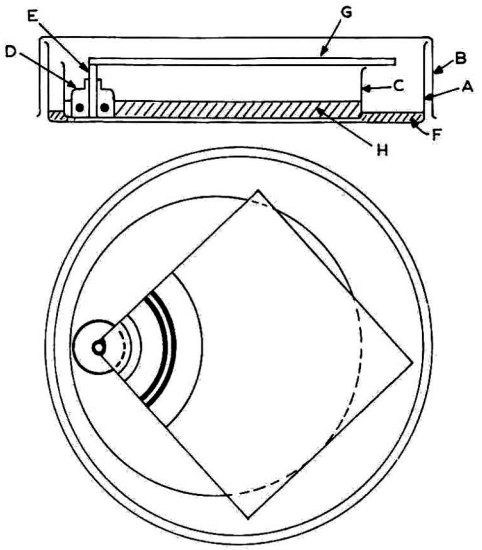


Fig. 1. Chamber for circular chromatography.

F which saturates the atmosphere easily. The square of set plaster of Paris rests on its three corners on Petri dish cover C and the fourth corner touches the wick.

Solvent

It is possible to work out different compositions of solvents suitable for separating different amino acids such as has been done on paper[3], but for the purpose of the material investigated here, the following solvent is suitable: acetone–chloroform–99–100 % acetic acid–acetate buffer[4], pH 4 (4 cc.:1 cc.:0.5 cc.:1.5 cc).

Instead of subsequently spraying or painting of the strips and squares with an 0.5 % acetone solution of ninhydrin, it has been found more convenient to add 35 mg of ninhydrin to the above solvent. The presence of ninhydrin does not affect the R_F values of the amino acids.

Chromatographic separations

Chromatography was carried out for 45 min in all cases. Longer times increase the distance between separated bands but decrease the colour intensity of the spots making photographic recording difficult.

Development of colour of spots and bands

As pointed out earlier, since ninhydrin is already included in the solvent, there is no need to spray or paint the dried strips and squares. If spraying or painting is needed for any purpose, it can also be carried out without smearing. The strips and squares are dried in an oven at 100° for 20 min for full colour development.

Results

Fig. 2 shows photographs of the following separations:

(A) Ascending chromatographic separation of a protein hydrolysate injection USP made by Bengal Immunity Co. Ltd. Calcutta.

(B) Circular chromatography of a mixture of pure amino acids showing (1) aspartic acid; (2) histidine; (3) tryptophan; and (4) tyrosine. The mixture applied contained 0.25 % aspartic acid; 0.5 % histidine; 0.5 % tryptophan; and 0.35 % tyrosine.

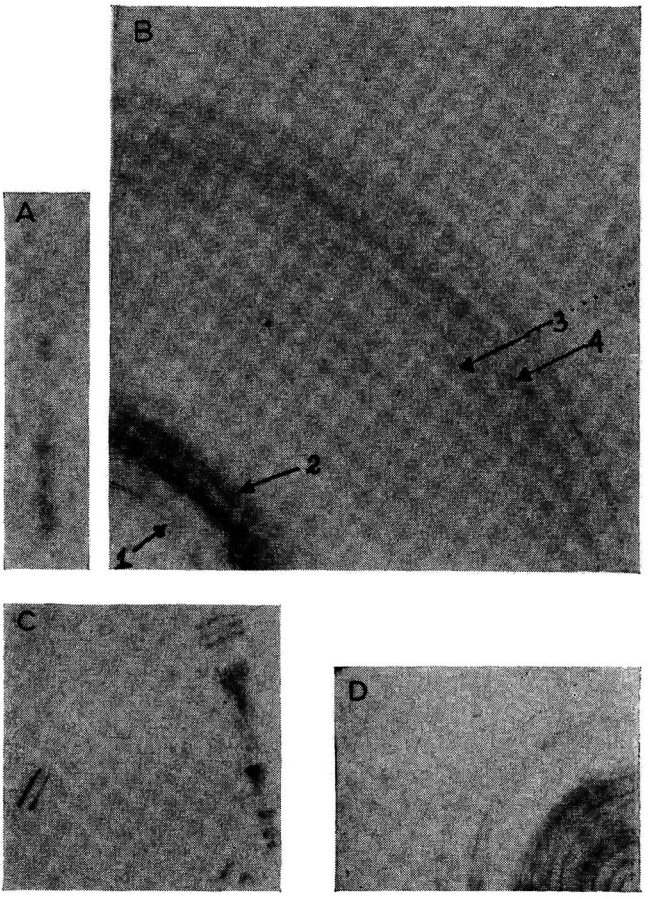


Fig. 2. (A) Chromatographic separation of amino acids in protein hydrolysate. (B) Circular chromatogram of a mixture of pure amino acids. (C) Simultaneous circular chromatogram of protein hydrolysate and a mixture of pure amino acids. (D) Circular chromatogram of protein hydrolysate.

(C) Circular chromatogram of simultaneous spots of (A) and (B). The four amino acids of (B) also exist in the protein hydrolysate (A). Only in this case the size of each sample was reduced from 5 μl to 3 μl.

(D) Circular chromatogram of protein hydrolysate as in A. Twelve amino acids could be distinguished clearly in the original square.

Fig. 3 shows the separation of amino acids present in a 24 h collection of urine from a normal healthy male on a non-vegetarian diet.

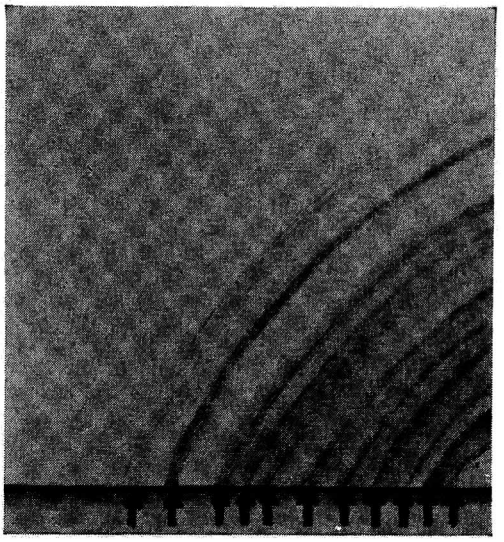


Fig. 3. Chromatogram of a sample of urine.

Discussion

Results show that the use of set plaster of Paris offers a convenient method of separating groups of amino acids very easily. It has been possible to separate clearly with the same medium individual amino acids by the uni-dimensional method, or with a longer time for chromatography by the bi-dimensional method; but the aim in the present work was to develop a quick method for screening most of the amino acids in urine or body fluids in an attempt to study the clinical aspects of amino acid excretion. Results of this investigation will be communicated later. Studies are also being made of the use of set plaster of Paris discs in centrifugal chromatography of amino acids for preparative purposes in much the same way as has been communicated in an earlier paper[5] for plant alkaloids.

Goa College of Pharmacy, Goa (India) A. AFFONSO

1 A. AFFONSO, *J. Chromatog.*, 21 (1966) 332.
2 H. W. GOEDDE AND H. BRUNSCHEDE, *Clin. Chim. Acta*, 11 (1965) 485.
3 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press, New York, 1958, p. 151.
4 G. S. WALPOLE, *J. Chem. Soc.*, 105 (1914) 2501.
5 A. AFFONSO, *J. Chromatog.*, 22 (1966) 1.

Received November 9th, 1965

Separation with uni-dimensional TLC of all neutral lipid classes

A method was devised to separate all classes of neutral lipid (cholesterol ester, triglyceride, fatty acid, cholesterol, 1,3-diglyceride, 1,2-diglyceride and monoglyceride) by TLC in a single dimension. This procedure permitted multiple samples to be applied to each plate.

No solvent system has been reported which will resolve all neutral lipid classes[1]. Solvent systems which separate the more rapidly migrating components leave the diglyceride isomers and cholesterol unresolved. Solvent systems designed to resolve the latter leave the cholesterol esters and triglycerides together at the solvent front. Bi-dimensional TLC has the distinct disadvantage of permitting application of only single samples per plate. The procedure described below utilizes sequential development in two different solvent systems to resolve all neutral lipid components.

Eight-inch square plates of Silica Gel G of 250 μ thickness were prepared on a Shandon spreader from a 2:1 (w/w) water-powder slurry and dried at 110°. Ten to forty μl of lipid sample in chloroform–methanol were applied at 3 cm from the bottom of the plate. The plates were then developed, first in a solvent of petroleum ether–ether–acetic acid (70:30:1, v/v) to a height of 16 cm. The plates were air-dried and then developed in the same dimension to a height of 11 cm (a position just below the triglyceride spot) in a solvent of ether–petroleum ether–acetic acid (70:30:1, v/v).

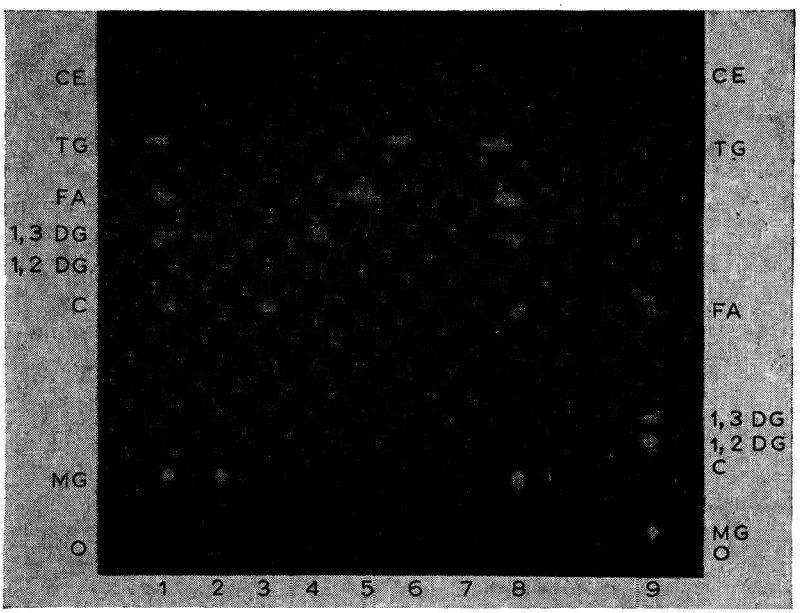


Fig. 1. CE = cholesterol ester; TG = triglyceride; FA = fatty acid; 1,3-DG = 1,3-diglyceride; 1,2-DG = 1,2-diglyceride; C = cholesterol; MG = monoglyceride; 1 and 8 = standard mixture; 2 = monopalmitin; 3 = palmitic acid; 4 = dipalmitin; 5 = cholesterol; 6 = tripalmitin; 7 = cholesterol palmitate; (1) through (8) are developed in both solvent systems; 9 = standard mixture after development in first solvent only. 10 μl of 1 mg/ml solution were applied. Standards were purchased from the Hormel Foundation, Austin, Minnesota, USA. The plate was visualized with 2% dichlorofluorescein in methanol and photographed under U.V. light.

The first solvent served to isolate the cholesterol esters and triglycerides in a position near the top of the plate. The second solvent served to separate cholesterol and the two diglyceride isomers. Phospholipids remain at the origin.

A typical separation is shown in Fig. 1.

This technique was used to chromatograph lipid extracts of serum enzyme digests of ^{14}C-labeled lipid preparations. It was thus possible to observe the localization and appearance of reaction products with considerable accuracy.

Acknowledgements

Work supported in part by USPHS Research Grant No. HE-07149 from the National Heart Institute, and by General Research Support Grant No. FR-05525 from the National Institutes of Health, U.S. Public Health Service.

The author thanks Miss DONNA J. NUTTER for valuable technical assistance.

Bio-Research Institute, Cambridge, Mass. (U.S.A.) THOMAS F. KELLEY

1 E. LEVIN AND C. HEAD, *Anal. Biochem.*, 10 (1965) 23.

Received November 22nd, 1965

J. Chromatog., 22 (1966) 456–457

Separation of *p*-nitrophenol and *p*-nitro-*m*-cresol from urine pigments by thin layer chromatography

Metabolism products of organophosphorus insecticides O,O-diethyl-O-(4-nitrophenyl) thiophosphate (Parathion) and O,O-dimethyl-O-(3-methyl-4-nitrophenyl) thiophosphate, known under the name of Metathion[1], Sumithion[2] or Folithion[3], are *p*-nitrophenol (PNP)[4] and *p*-nitro-*m*-cresol (PNMC)[5].

The determination of PNP in urine of persons exposed to Parathion serves as a toxicological test. The original method[4] of determination was modified by various authors[6–12], especially with regard to the separation of PNP from urine pigments which interfere. None of the above mentioned methods completely separate the interfering urine coextracts from PNP.

In the present work coextracts from urine were separated from PNP and PNMC chromatographically on thin layers of silica gel. In comparison with the methods mentioned complete separation of PNP and PNMC from urinary pigments is achieved by this method.

Work was carried out with artificial mixtures of extracts from human urine and PNP or PNMC. Both compounds were of chromatographic purity.

Silica gel precipitated from water glass with a grain size of 0.07–0.05 mm with a 15 % addition of plaster was used. A coating mechanism from ČSAV—Prague was used to prepare the thin layers. A layer 0.4 mm thick was prepared on glass plates 20 × 20 cm and activated for 60 min at a temperature of 120°.

PNP and PNMC are excreted in the urine in the form of esters[13] from which

they are set free by hydrolysis and then isolated by extraction from the hydrolysate. The samples to be spotted on the plates were prepared in the following way: Urine acidified with HCl (5:1) is hydrolysed for 1 h on a boiling water bath. After filtration an aliquot of the hydrolysate is extracted 3 times by means of a mixture consisting of 4 parts of petrol ether and 1 part of ethyl ether. A minimum of urine pigments pass into the ether layer. A residue containing a small quantity of urine pigments is obtained by evaporation of the solvents, from the bulked extracts on a water bath. To further extracts from 20 ml of urine is added 0.02 ml of an acetone solution containing either 5 μg PNP or 10 μg PNMC. A mixture of 20% acetone and 80% *n*-hexane proved to be the best solvent system for chromatography. Complete separation of PNP and PNMC from urine coextracts was reached at a distance of 15–16 cm.

Detection of the spots is by either ultraviolet light of by the action of ammonia vapour. In the first case PNP and PNMC quench the fluorescence (giving black spots), and in the latter case give lemon-yellow spots. Coextracts from urine give a yellow-brown colour in an ammonical medium and intensely fluorescent spots in ultraviolet light.

Fig. 1 shows a chromatogram in which the spots were detected by ammonia vapour. On the starting line the spots are spotted in the following order: 1 = urine extract with PNP (5 μg); 2 = standard PNP (5 μg); 3 = urine extract; 4 = standard PNMC (10 μg); 5 = urine extract with PNMC (10 μg). It can be seen from Fig. 1 that the spots of PNP and PNMC are well separated from the almost continuous line of coextracts.

In Table I the R_F values of components of the chromatogram detected by ultraviolet light are given. It is obvious from the table that although urine coextracts are a variable mixture of compounds they do not interfere with PNP and PNMC.

However, as can be seen from Fig. 2, PNP and PNMC are difficult to separate from each other in the system used, their R_F values being too close; this has already

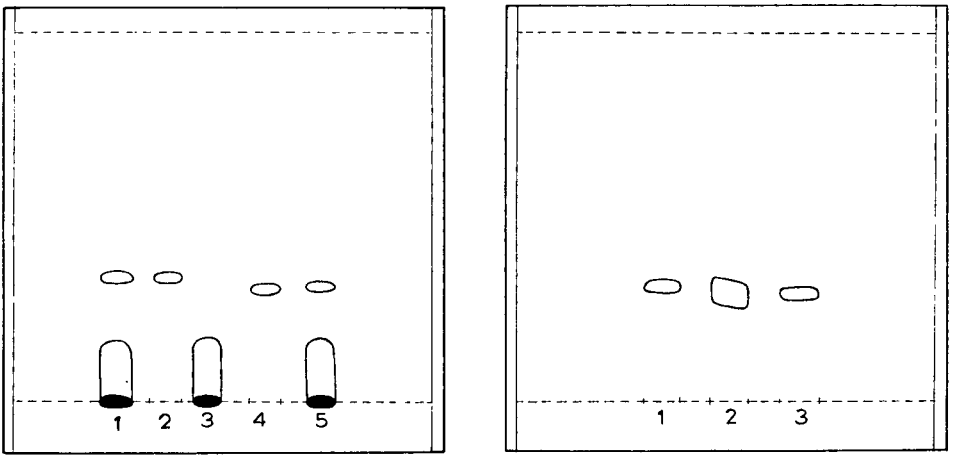


Fig. 1. Separation of PNMC and PNP from urine pigments on a thin layer of silica gel. Solvent: 20% acetone and 80% *n*-hexane. 1 = urine extract and PNP (5 μg); 2 = standard solution PNP (5 μg); 3 = urine extract; 4 = standard PNMC (10 μg); 5 = urine extract and PNMC (10 μg).

Fig. 2. Separation of PNMC and PNP on a thin layer of silica gel. Solvent: 20% acetone and 80% *n*-hexane. 1 = PNP; 2 = mixture of PNP and PNMC; 3 = PNMC.

TABLE I

R_F VALUES OF COMPONENTS DETECTED IN ULTRAVIOLET LIGHT

R_F values of spots					
Extract from urine with PNP	*PNP*	*Extract from urine*	*PNMC*	*Extract from urine with PNMC*	*Colour of fluorescing spot*
origin		origin		origin	yellow
0.03		0.03		0.03	blue
0.05		0.05		0.05	pink
0.08		0.08		0.08	blue
0.12		0.12		0.12	blue
0.33	0.33		0.31	0.31	black
0.84		0.84		0.84	white
front		front		front	bluish

been mentioned by GASPARIČ[14]. The presence of PNP may still be discerned at a level of 0.15 μg and PNMC at 0.25 μg. Similar results are obtained in the analysis of urines of experimental animals given Parathion or Metathion.

The developing systems described by other authors for the separation of phenolic compounds[15–18] did not prove to be successful in our case, *i.e.* for the separation of PNMC and PNP from urine extracts.

Institute of Industrial Hygiene and Occupational Medicine, and Department of Analytical Chemistry, Slovakian Technical University, Bratislava (Czechoslovakia)

A. HLADKÁ
Z. HLADKÝ

1 J. DRÁBEK AND Š. TRUCHLÍK, *ČSSR Pat.*, 89,124 (1957).
2 Y. NISHIZAWA, K. FUJII, T. KADOTA, J. MIYAMOTO AND H. SAKAMOTO, *Agr. Biol. Chem. (Tokyo)*, 25 (1961) 605.
3 G. SCHRADER, *Angew. Chem.*, 73 (1961) 331.
4 J. T. MOUNTAIN, H. ZLOTOLOW AND G. T. O'CONOR, *Ind. Health Monthly*, 11 (1951) 88.
5 A. HLADKÁ, *Pracovni Lekar.*, 18 (1966) 6.
6 S. EICKEN, *Angew. Chem.*, 66 (1954) 551.
7 J. W. ELLIOTT, K. C. WALKER, A. E. PENICK AND W. F. DURHAM, *J. Agr. Food Chem.*, 8 (1960) 111.
8 J. F. GARDOCKI AND L. V. HAZLETON, *J. Am. Pharm. Assoc.*, 40 (1951) 491.
9 D. J. LAWFORD AND D. G. HARVEY, *Analyst*, 78 (1953) 63.
10 D. ROBINSON, J. N. SMITH AND R. T. WILLIAMS, *Biochem. J.*, 50 (1951) 221.
11 D. VLACHOVÁ, *Pracovni Lekar.*, 8 (1956) 283.
12 R. K. WALDMAN AND L. A. KRAUSE, *Occupational Health*, 12 (1952) 37.
13 R. T. WILLIAMS, *Detoxication Mechanisms*, Chapman-Hall, London, 1958, p. 296.
14 J. GASPARIČ, *J. Chromatog.*, 13 (1964) 459.
15 K. RANDERATH, *Dünnschicht-Chromatographie*, Verlag Chemie, Weinheim, 1963, pp. 173–177.
16 E. V. TRUTER, *Thin Film Chromatography*, Cleaver-Hume, London, 1963, pp. 160–163.
17 L. LÁBLER, V. SCHWARZ *et al.*, *Chromatografie na tenké vrstvě*, Nakl. ČSAV, Prague, 1965, p. 275.
18 H. SEEBOTH, *Chem. Tech. Berlin*, 15 (1963) 34.

Received November 11th, 1965

Dünnschichtchromatographische Trennung von Diastereomeren an Kieselgel-D

Zur einfachen Identifizierung des Produktverhältnisses diastereomerer Verbindungen wird deren dünnschichtchromatographische Trennung für folgende Substanzen untersucht:

(a) *trans*- und *cis*-1-Amino-2-hydroxy-indan,
(b) *erythro*- und *threo*-1,3-Diphenyl-3-amino-propanol-(1),
(c) *erythro*- und *threo*-1,2-Diphenyl-3-amino-propanol-(1),
(d) *erythro*- und *threo*-1,2-Diphenyl-2-amino-äthanol-(1),
(e) *trans*- und *cis*-1-Amino-2-hydroxy-tetralin,
(f) *trans*- und *cis*-2-Phenyl-2-hydroxy-1-amino-cyclohexan,
(g) *meso*- und DL-1,2-Diphenyl-1,2-dibrom-äthan (Stilbendibromid),
(h) *meso*- und DL-1,2-Diphenyl-1,2-dichlor-äthan (Stilbendichlorid),
(i) *meso*- und DL-1,2-Diphenyl-1,2-dinitro-äthan.

Die Wanderungsgeschwindigkeit und der daraus resultierende R_F-Wert einer Substanz ist das Ergebnis ihrer Wechselwirkungen mit dem Lösungsmittel und dem Adsorbens. Die Art der Wechselwirkungen reicht von den v. d. Waals'schen Kräften über Wasserstoffbrückenbindungen bis zu Komplexbildungen in Lösung[1].

Unsere Untersuchungen erstrecken sich auf Diastereomere mit und ohne intramolekularer Wasserstoffbrückenbindung.

Aus den IR-Spektren der genannten Aminoalkohole[2] ergeben sich aus sterischen Gründen unterschiedliche Wasserstoffbrückenbindungsstärken innerhalb eines Diastereomerenpaares. Wenn das Laufmittelsystem selbst eine zur Wasserstoffbrückenbindung befähigte Komponente aufweist, so sollte deren Wechselwirkung mit dem Diastereomeren ohne oder geringerer intramolekularer Wasserstoffbrücke grösser sein als mit der isomeren Verbindung. Demzufolge würde die Substanz mit der stärkeren intramolekularen Wasserstoffbrücke langsamer laufen als die diastereomere Verbindung.

Im Falle des *trans*- und *cis*-1-Amino-2-hydroxy-indan, einem weitgehend starren System, zeigt das IR-Spektrum der *cis*-Verbindung keine freie OH-Bande mehr, während die *trans*-Verbindung ausschliesslich freie OH-Bande aufweist. Dadurch wird es auch leicht verständlich, dass die *cis*-Verbindung mit der wasserstoffbrückenbildenden Laufmittelkomponente weniger in Wechselwirkung treten kann und im Chromatogramm langsamer wandert als der *trans*-1,2-Aminoalkohol des Indans. Diese Anschauungen werden experimentell bestätigt. Bei den anderen untersuchten Aminoalkoholen ist die Rotation um die C–C-Bindung bzw. das Umklappen der einen cyclischen Form in die andere auf Grund der sterischen Beeinflussung durch die grossen Substituenten nur teilweise gehindert.

Die Folge hiervon ist ein gleichzeitiges Auftreten von gebundener und freier OH-Bande bei jedem Aminoalkohol. Relativ grosse Unterschiede in der Wasserstoffbrückenbindungsstärke ergeben sich noch beim *trans*- und *cis*-1-Amino-2-hydroxy-tetralin, wo ebenfalls gute Trennungen erzielt werden. Bei allen anderen untersuchten Aminoalkoholen werden unter den Versuchsbedingungen keine oder nur sehr geringe Trenneffekte erreicht.

In der Literatur[3–7] finden sich Hinweise, dass Borsäure einen positiven Einfluss

auf die Trennung von Diastereomeren ausübt. Wir haben dieses Verfahren auf die genannten Aminoalkohole angewendet und mit Hilfe der Borsäureimprägnierung des Kieselgel-D in allen Fällen eine Trennung erreicht. Durch Variation der Borsäurekonzentration kann deren Einfluss auf die Trennwirkung untersucht werden.

trans- und *cis*-1-Amino-2-hydroxy-indan und *trans*- und *cis*-1-Amino-2-hydroxy-tetralin geben auch ohne Zusatz von Borsäure zum Adsorbens eine Trennung. Das Maximum der Trennung liegt jedoch bei beiden diastereomeren Aminoalkoholpaaren bei Verwendung von 0.1 *N* Borsäurelösung. Insgesamt werden mit dem Laufmittelgemisch Dioxan–Methanol verschiedener Zusammensetzungen die besten Trenneffekte erhalten.

Die übrigen untersuchten Aminoalkoholpaare *erythro*- und *threo*-1,3-Diphenyl-3-amino-propanol-(1), *erythro*- und *threo*-1,2-Diphenyl-3-amino-propanol-(1), *erythro*- und *threo*-1,2-Diphenyl-2-amino-äthanol-(1) und *trans*- und *cis*-2-Phenyl-2-hydroxy-1-amino-cyclohexan können nur durch Borsäurezusatz befriedigend getrennt werden. Das Maximum der Trennung liegt bei Verwendung von 0.1–0.2 *N* Borsäurelösung. Die *threo*- bzw. die *cis*-Formen der genannten Aminoalkohole treten mit dem Komplexbildner stärker in Wechselwirkung, woraus eine geringere Wanderungsgeschwindigkeit im Chromatogramm folgt. Eine Ausnahme bildet nur 1,3-Diphenyl-3-amino-propanol-(1). Hier sind die Verhältnisse der Wasserstoffbrückenbindungsstärke ungekehrt, so dass bei diesem Aminoalkoholpaar die *erythro*-Form vom Komplexbildner stärker festgehalten wird. Die Tabellen I–VI geben eine Übersicht über die erzielten Trennungen.

Die R_F-Werte sind Mittelwerte einer Vielzahl von Versuchen. In den Tabellen ist neben den R_F-Werten ihre Differenz angeführt, da der R_F-Wert von vielen Faktoren beeinflusst wird, die seine genaue Reproduktion erschweren. Die Angabe der R_F-Wertdifferenz behebt diese Schwierigkeit insofern, als bei der Standardlaufstrecke von 10 cm das Verhältnis der Wanderungsgeschwindigkeit für das Diastereomerenpaar gleichbleibt.

Aus den Trennungsergebnissen geht hervor, dass die unterschiedliche Wasserstoffbrückenbildungstendenz nur teilweise zur dünnschichtchromatographischen Trennung ausgenutzt werden kann.

Die Komplexbildung mit Borsäure führt jedoch bei allen untersuchten Aminoalkoholen zu einer Trennung. Die Wechselwirkungen zwischen Substanz und Adsorbens sind sehr gross, denn zum Entwickeln des Chromatogramms werden stark eluierende Laufmittelgemische verwendet.

Bei der Untersuchung von *meso*- und DL-1,2-Diphenyl-1,2-dibrom-äthan, *meso*- und DL-1,2-Diphenyl-1,2-dichlor-äthan und *meso*- und DL-1,2-Diphenyl-1,2-dinitro-äthan können ohne Komplexbildner keine oder nur sehr geringe Trennungen erzielt werden. Diese Substanzen lassen sich nur mit den am schwächsten eluierenden Laufmitteln wie Hexan, Heptan und Cyclohexan entwickeln, da sie sonst zu nahe an der Lösungsmittelfront liegen.

Als Komplexbildner werden bei diesen Verbindungen 0.1 *N* Borsäure und 0.1 *M* Harnstofflösung verwendet.

Die diastereomeren 1,2-Diphenyl-1,2-dibrom-äthane und die 1,2-Diphenyl-1,2-dinitro-äthane geben gute Trennungen, wie aus den Tabellen VII und VIII hervorgeht. Lediglich beim DL-1,2-Diphenyl-1,2-dinitro-äthan tritt eine Isomerisierung zum *meso*-Produkt auf, wenn Harnstoff der Komplexbildner ist. Die diastereomeren 1,2-

TABELLE I

trans- UND *cis*-1-AMINO-2-HYDROXY-INDAN

Dioxan–Methanol	*Borsäure-konzentration*	R_F-*Wert*		
		trans	*cis*	*Differenz*
17:3	0	0.56	0.36	0.20
17:3	0.1–0.3	0.30	0.07	0.23
10:10	0	0.60	0.40	0.20
10:10	0.1–0.3	0.40	0.15	0.25
8:12	0.1–0.3	0.25	0.10	0.15

TABELLE II

erythro- UND *threo*-1,3-DIPHENYL-3-AMINO-PROPANOL-(1)

Dioxan–Methanol	*Borsäure-konzentration*	R_F-*Wert*		
		erythro	*threo*	*Differenz*
17:3	0.1	0.15	0.40	0.25
10:10	0.1	0.34	0.55	0.21
8:12	0.1	0.30	0.45	0.15

TABELLE III

erythro- UND *threo*-1,2-DIPHENYL-3-AMINO-PROPANOL-(1)

Dioxan–Methanol	*Borsäure-konzentration*	R_F-*Wert*		
		erythro	*threo*	*Differenz*
17:3	0.1–0.3	0.25	0.10	0.15
10:10	0.1–0.3	0.35	0.15	0.20
8:12	0.1–0.3	0.35	0.20	0.15
6:14	0.1–0.3	0.20	0.10	0.10

TABELLE IV

erythro- UND *threo*-1,2-DIPHENYL-2-AMINO-ÄTHANOL-(1)

Dioxan–Methanol	*Borsäure-konzentration*	R_F-*Wert*		
		erythro	*threo*	*Differenz*
17:3	0.1	0.60	0.45	0.15
10:10	0.1	0.50	0.40	0.10
8:12	0.1	0.50	0.40	0.10

TABELLE V

trans- UND *cis*-1-AMINO-2-HYDROXY-TETRALIN

Dioxan–Methanol	*Borsäure-konzentration*	R_F-*Wert*		
		trans	*cis*	*Differenz*
17:3	0	0.70	0.60	0.10
17:3	0.1–0.3	0.25	0.07	0.18
10:10	0	0.90	0.75	0.15
10:10	0.1–0.3	0.30	0.15	0.15
8:12	0.1–0.4	0.35	0.20	0.15
6:14	0.1–0.4	0.30	0.20	0.10

TABELLE VI

trans- UND *cis*-2-PHENYL-2-HYDROXY-1-AMINO-CYCLOHEXAN

Dioxan–Methanol	*Borsäure-konzentration*	R_F-*Wert*		
		trans	*cis*	*Differenz*
17:3	0.1–0.2	0.60	0.45	0.15
10:10	0.1	0.75	0.65	0.10
8:12	0.1	0.80	0.70	0.10
6:14	0.1	0.80	0.65	0.15

TABELLE VII

meso- UND DL-1,2-DIPHENYL-1,2-DIBROM-ÄTHAN

Laufmittel	*Komplexbildner*	R_F-*Wert*		
		meso	DL	*Differenz*
Heptan	0.1 *N* Borsäure	0.00	0.60	0.60
Heptan	0.1 *N* Borsäure	0.00	0.60	0.60
Heptan	0.1 *M* Harnstoff	0.00	0.64	0.64

TABELLE VIII

meso- UND DL-1,2-DIPHENYL-1,2-DINITRO-ÄTHAN

Laufmittel	*Komplexbildner*	R_F-*Wert*		
		meso	DL	*Differenz*
Heptan	0.1 *N* Borsäure	0.00	0.70	0.70
Heptan	0.1 *M* Harnstoff	0.00	0.70	0.70*

* Bei der DL-Form tritt Isomerisierung zum *meso*-Produkt auf.

Diphenyl-1,2-dichlor-äthane lassen sich unter diesen Versuchsbedingungen nicht trennen.

Experimenteller Teil

Als Adsorbens wird Kieselgel-D (VEB Chemiewerk Greiz-Dölau) verwendet. Die Glasplatten haben eine Abmessung von 10 × 20 cm. 7.5 g Kieselgel-D werden mit 15 ml Wasser verrührt und anschliessend mit einem Plaststreicher auf der Platte ausgezogen. Die Platte bleibt bis zum Verschwinden des Glanzes (*ca.* 15 Min.) an der Luft liegen und wird dann im Trockenschrank 50 Min. bei 110° aktiviert. Bei Einsatz von Borsäure oder Harnstoff als Komplexbildner wird an Stelle von Wasser Borsäure- oder Harnstofflösung der jeweiligen Konzentration verwendet. Nach dem Aktivieren werden die Platten in einem Exsikkator über Blaugel aufbewahrt.

Aufzutragende Mengen und Sichtbarmachung. Bei den Aminoalkoholen darf nur sehr wenig Substanz aufgebracht werden (~ 5 μg), da für die Sichtbarmachung mit Ninhydrin schon geringste Mengen genügen und sonst eine lästige Schwanzbildung auftritt. Bei Verwendung des Morgan-Elson-Reagenzes werden ~ 30 μg auf die Platte aufgetragen.

Dichlorstilben, Dibromstilben und Dinitrostilben werden mit Chromschwefelsäure sichtbar gemacht. Dazu müssen ungefähr 50 μg aufgetragen werden.

Ninhydrin. 0.3 g/100 ml abs. Äthanol. Das Laufmittel wird nach der Entwicklung abgedampft, die Platte anschliessend mit Ninhydrin besprüht und für *trans*- und *cis*-1-Amino-2-hydroxy-indan, *trans*- und *cis*-1-Amino-2-hydroxy-tetralin und *trans*- und *cis*-2-Phenyl-2-hydroxy-1-amino-cyclohexan *ca.* 10 Min. auf 120° erhitzt. Bei den restlichen Aminoalkoholen wird für 1 Std. auf 120° erhitzt.

Morgan-Elson-Reagenz[8]. Sprühlösung (1) 0.5 ml einer Mischung aus 5 ml 50% wässriger KOH und 20 ml Äthanol werden unmittelbar vor Gebrauch mit 10 ml einer Mischung aus 0.5 ml Acetylaceton und 50 ml *n*-Butanol zusammengegeben.

Sprühlösung (2): 1 g *p*-Dimethylaminobenzaldehyd wird in 30 ml Äthanol gelöst. Die Lösung wird mit 30 ml konz. HCl versetzt. Bei Bedarf wird mit 180 ml *n*-Butanol verdünnt.

Vorgang: Nach Besprühen mit (1) 5 Min. auf 105° erwärmen, mit (2) nachsprühen und 5 Min. bei 90° trocknen.

Institut für Organische Chemie und Biochemie,
Friedrich-Schiller-Universität, Jena (D.D.R.)

GÜNTHER DREFAHL
GÜNTHER HEUBLEIN
KARL SILBERMANN

1 S. HERMANEK, V. SCHWARZ UND Z. CEKAN, *Collection Czech. Chem. Commun.*, 28 (1963) 2031.
2 G. DREFAHL UND G. HEUBLEIN, *Chem. Ber.*, 94 (1961) 915, 922.
3 E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, 1962, S. 185, 476.
4 G. PASTUSKA, *Z. Anal. Chem.*, 179 (1961) 427.
5 L. J. MORRIS, *Chem. Ind. (London)*, (1962) 1238.
6 L. J. MORRIS, *Lab. Pract.*, 13 (1964) 284.
7 H. U. MANGOLD, H. SCHMID UND E. STAHL, *Methods Biochem. Anal.*, 12 (1964) 379.
8 E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, 1962, S. 502.

Eingegangen den 17. November 1965

Quantitative separation and estimation of steroids by thin-layer chromatography

I. Determination of progesterone and testosterone propionate in oil solutions

Introduction

Within the last few years there has been a great increase in the use of thin-layer chromatography for the separation of steroids including progesterone and testosterone propionate. However, all these reports have been concerned mainly with the procedure as a qualitative rather than a quantitative technique.

The present communication describes a simple and accurate method for the estimation, on a micro scale, of progesterone and testosterone propionate in mixtures of the two compounds. We feel that the proposed method will contribute to simplicity and accuracy obtainable for quantitative thin-layer chromatography in assaying mixtures of steroids.

The elaborated method has been adapted to the determination of steroid mixtures in commercially available vegetable oil solutions.

Experimental

Materials. Reagents: The INH reagent was prepared by dissolving 0.4 g of isonicotinic hydrazide in methanol; 0.5 ml hydrochloric acid (37 %) was added and filled up to 100 ml with methanol. The reagent is set aside for 24 h before use. The solvents used were of p.a. purity grade.

Adsorbent: Fluorescent Kieselgel HF_{254}, E. Merck, Darmstadt.

Reference standards: Progesterone, Testosterone propionate, Organon, Oss (The Netherlands).

Apparatus. Thin-layer chromatography outfit with regulation thickness spreader, Desaga, Heidelberg.

Agla micrometer syringe, Burroughs Welcome & Co, London.

Ultraviolet lamp (254 mμ), Hanau.

Preparation of plates

Chromatoplates were prepared following the technique described by STAHL[1]. Plates 20 × 20 cm were coated (layer 0.5 mm thick) with a slurry prepared by mixing 35 g Kieselgel HF_{254} with 85 ml of water in a mortar with the aid of a pestle. A batch suffices for six plates. The plates were air dried for 10 min at room temperature and thereafter activated by heating at 130° for 4 h and stored in a desiccator until use.

Determinations were performed in duplicate against standards run on the same plate.

Procedure

3.0 ml of the oil solution (30 mg progesterone and 45 mg testosterone propionate) were diluted with 6.0 ml chloroform. Twice 20 μl of this solution and 20 μl of each corresponding standard solution were applied with the aid of a microsyringe on to a Kieselgel HF_{254} coated plate along the starting line as 2 cm horizontal lines. The chromatogram was run by the ascending technique with 100 ml of the solvent cyclohexane–ether (8:2, v/v), which had been poured into the chromatographic chamber

previously. When the solvent had reached a point within 1 cm from the upper edge of the plate (about 50 min) the plate was removed from the chamber, air dried for about 5 min and the separated steroids were located by means of low-wavelength

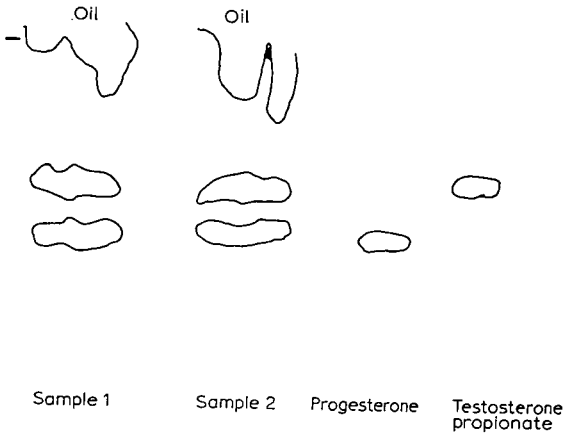


Fig. 1. Separation of steroids in oil solution.

ultraviolet light (Fig. 1). The oil migrated with the front and was quantitatively separated from the steroids. The U.V. absorbing zones were marked with an ample margin around the zone and quantitatively scraped off the plate each into a 50 ml glass stoppered flask. After the addition of 5 ml of methanol the flasks were well shaken for about 30 min to ensure complete extraction of the steroids and then centrifuged.

Progesterone

3.0 ml aliquots of each supernatant centrifuged progesterone solution of the samples and standard were pipetted into a 50 ml flask. After the addition of 4 ml INH reagent to each flask the solutions were allowed to stand for 1 h and the absorbancies determined at 380 mμ against a reagent blank.

$$\text{Per cent of progesterone in the sample} = \frac{E_{\text{sample solution}}}{E_{\text{standard solution}}}$$

where $E_{\text{sample solution}}$ = mean of extinction of sample 1 and sample 2.

Testosterone propionate

3.0 ml aliquots of each supernatant centrifuged testosterone solution of the samples and standard were pipetted into 50 ml glass stoppered flasks and the determination of testosterone propionate was carried out as described for progesterone.

Standard solution of progesterone

Dissolve 33.3 mg progesterone in chloroform and fill up to 10 ml with chloroform.

Standard solution of testosterone propionate

Dissolve 50.0 mg of testosterone propionate in chloroform and fill up to 10 ml with chloroform.

Results and discussion

The work reported here is the result of many trials with different adsorbents, solvent systems, and extraction, detection and estimation procedures.

For the sample application on the chromatoplate chloroform proved to be the most convenient solvent. The more polar solvents, such as methanol or ethanol, tended to leave the steroid as a ring rather than a zone. Warming up the chromatoplate while applying the solution in order to increase the rate of evaporation and thus speed up the solution application was found to be unnecessary.

The sensitivity of the method used for the quantification of steroids extracted from the adsorbent permits the determination of ketosteroids in quantities as small as 50 μg. This is important since steroids, because of their high physiological potency, are generally used in low concentrations (0.2–2 mg/ml). For this reason the very sensitive method suggested by UMBERGER[2] was the method of choice. This method is based on the rapid formation in acidified ethanol solution of isonicotinylhydrazones of Δ^4-3-ketosteroids having a double bond conjugated with a carbonyl group (ε = about 11.000).

To study the precision of the procedure six analyses were carried out with standard steroid vegetable oil solutions prepared in our laboratory and containing exactly known quantities of ketosteroids. As can be seen from Table I good reproducibility with a satisfactory standard deviation was obtained.

TABLE I

ANALYSIS OF STANDARD STEROID OIL SOLUTIONS

Analysis No.	*Progesterone 39.6 µg added*	*Testosterone propionate 60.0 µg added*
	µg found	*µg found*
1	41.65	60.0
2	38.72	58.08
3	38.21	60.47
4	39.48	59.77
5	37.39	57.51
6	39.05	58.93
Mean	39.08	59.12
Standard deviation P = 0.05	1.65	1.85

In Table II results of determinations with commercial samples are given. As can be seen the obtained results are in good agreement with the labelled amount of the respective steroid.

TABLE II

ANALYSIS OF COMMERCIAL STEROID OIL SOLUTIONS

Preparation No.	*Progesterone 10 mg labelled*	*Testosterone propionate 15 mg labelled*
	mg found	*mg found*
1	9.45	14.23
2	9.47	14.90
3	9.53	14.75

Work on the quantification of other steroid mixtures after thin-layer chromatographic separation is being continued in this laboratory.

Acknowledgement

The author is grateful to Mrs. A. HARAPIN for her valuable technical assistance.

*Institute for the Control of Drugs**, *Zagreb (Yugoslavia)*

T. BIĆAN-FIŠTER

1 E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, 1962.
2 E. J. UMBERGER, *Anal. Chem.*, 27 (1955) 768.

Received November 11th, 1965

* Director: V. KUŠEVIĆ, Ph. D.

A sensitive non-specific localization test for thin-layer chromatography

During the chromatography of unknown mixtures considerable time and effort can be saved if the total number of components and their R_F values can be determined immediately.

This requires a spotting or localization agent capable of reacting with a large variety of compounds of different chemical structure.

Once the number and R_F values of all the constituents are determined, more specific localization and identification tests can be applied.

Among the spotting agents of wide applicability, the sulfuric acid test, the chlorine-*p*-toluidine test (ref. 1, Test No. 32) and the iodine vapor test (ref. 1, Test No. 72 and No. 73) are the most widely used.

The sulfuric acid test requires a highly corrosive spray and if used as a 50% aqueous solution as usually recommended, will often give a very non-uniform droplet size thus capable of damaging the thin layer of adsorbent.

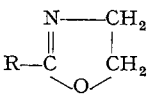
We find, furthermore, that with certain classes of compounds both tests exhibit poor sensitivity. The iodine test is sensitive but the spots fade very rapidly.

Convenient and sensitive detection is accomplished by exposing the dried and developed chromatogram to an atmosphere of ozone at periods up to 30 min, driving off excess ozone with a gentle current of air and then detecting the oxidation products (hydroperoxides, aldehydes, ketones, etc.) by well-known spotting sprays. Among

TABLE I

SENSITIVITY OF VARIOUS ORGANIC COMPOUNDS TO OZONE TEST

Spotting agent: aqueous 2,4-dinitrophenylhydrazine

Compound	2.5 γ*	5 γ*	10 γ*	*Notes*
Aromatics				
n-Decylbenzene	+	++	++	
Chlorobenzene	−	−	−	
Anthracene	+	++	+++	
Naphthalene	+	++	+++	
2,4-Dichlorobenzene	−	−	+−	
Alcohols				
n-Decyl alcohol	+−	+−	+	
2-Methylcyclohexanol	+−	+	+	
Pentaerythritol	+	+	+	
Glycerol	+−	+	++	
Amines				
Aniline	+	++	+++	
Cyclohexylamine	+	++	+++	
Pyridine	+−	++	+++	
Dodecylamine	+	++	+++	
Olefins				
Styrene	+	+	++	
Oleic acid	+	+	++	
Saturated hydrocarbons				
Tetradecane	−	−	+−	
Pentadecane	−	−	+−	Ozone treated at 55°: + at 20γ
Decalin	−	−	+−	
Stearic acid	−	−	+−	
Ether, polyether				
Dipropylene glycol	−	−	+−	+ at 20γ
1,2-Diethoxyethane	−	−	+−	
Ethoxylated alfol (59.5% ethylene oxide)	−	−	+−	
Amides				
$RCONH-CH_2CH_2OH$	+−	+	+	++ at 20γ
Oxazolines				
	+	++	++	+++ at 20γ

* +− = perceptible; + = clear spot; ++ = strong spot; +++ = very strong spot.

these an acid aqueous solution of 2,4-dinitrophenylhydrazine or an ethanolic solution of phosphomolybdic acid (ref. 1, Test No. 120) gives very satisfactory results.

Very inert compounds such as paraffinic hydrocarbons or aliphatic amides are exposed to the ozone atmosphere at 55–90° instead of room temperature and will be easily detected.

A large variety of chemical classes were tested by this procedure and approximate sensitivities determined. The data are presented in Table I.

Of particular interest is the high sensitivity to aromatic compounds and this should find many applications in the field of air and water pollution.

The only prior reference to the use of ozone for spot localization is that of KAUFMANN AND NITSCH[2] where it was used to oxidize primary alcohols to acids followed by identification of the latter by the copper salt–potassium ferricyanide test.

Limitations of the method

Since ozone will oxidize almost any organic compound, it is extremely important to drive off the developing solvent before the ozone exposure, so as to avoid a colored background after the hydrazine or phosphomolybdic acid spray.

Since some ozone remains adsorbed, a better background is obtained if the carbonyl detection agent (such as the hydrazine) is used in aqueous solution instead of an ethanolic solution. Since ethanol will be oxidized by the adsorbed ozone to acetaldehyde, a yellow background will obscure the spots of the compounds to be detected. This is less important with the phosphomolybdic acid spray since the blue spots are easily detected on a white or yellow background.

The ozone test can be combined with other more specific tests, such as the ninhydrin test for amines or amino acids, by first carrying out the specific test and marking R_F and spot size of the detected compounds, followed by the usual procedure for the ozone exposure. The areas known to contain the less reactive compounds have to be protected from the ninhydrin by covering it with a sheet of aluminum foil or plastic.

Sensitivities

Sensitivities vary with the structure of the compound to be detected.

Within a class of compounds itself sensitivity can also vary, though generally on a somewhat lower scale. Thus aromatic compounds are easily detected with polycyclics being more sensitive than alkylbenzenes and these in turn are more sensitive than halogenated aromatics. This is most probably due to the fact that the color is produced by ozonide formation and a larger concentration of ozonide could be achieved with the former compounds.

The best sensitivities are obtained with aromatics and amines (0.5–5 μg) followed by olefins (2.5 μg), alcohols (2.5–5 μg), polyether alcohols (10–20 μg) and saturated hydrocarbons (10–20 μg).

These sensitivities refer to the 2,4-dinitrophenylhydrazine test. From the limited amount of data on the phosphomolybdic acid test it appears that the latter is much more sensitive: alcohols and ethoxylated alcohols can be detected to within 1 μg.

Experimental

A 0.25 mm Silica Gel G (Desaga) layer applied with the Desaga template and

spreader* is used in all experiments. 10 mm^3 solution of the appropriate concentration is applied to provide spots containing from 0.5 to 50 μg of material to the plate. (The plates were previously activated for 1 h at 110° and stored in a desiccator over P_2O_5.)

The plates are then exposed to an atmosphere of ozone (2–4 % ozone in air) for 30 min at the required temperature. A rectangular developing tank (30 × 26 × 6 cm) with a ground glass top is provided with an aluminum cover-plate. This metal plate is provided with an ozone inlet tube extending to the tank bottom and an exit tube leading to a water bubbler. Ozone is generated from a commercial instrument (Welshbach, Model T-23). All lines are made of Tygon. The unreacted ozone is led away from the bubbler into the hood exhaust chimney. Another convenient container is available commercially*.

When a higher reaction temperature is desired the tank is immersed in a thermostated oil bath.

After the ozone exposure the plate is placed under a gentle air stream, then sprayed with a 0.4 % of 2,4-dinitrophenylhydrazine (0.4 % aqueous solution in 2 *N* HCl) or a 5 % ethanolic solution of phosphomolybdic acid. The first reagent gives yellow or orange spots on a white or light yellow background while the second reagent gives blue-black spots on a light yellow background.

The sensitivities thus determined are somewhat better than those obtained after actual development but will serve as a very good guide in the choice of concentrations required for practical separations.

Allied Chemical Corporation, Central Research Laboratory, R. FUHRMANN
Morristown, N.J. (U.S.A.) D. JERALOMON

1 E. STAHL, *Dünnschicht-Chromatographie*, Springer Verlag, Berlin, Göttingen, Heidelberg, 1962, p. 501, 506, 511.
2 H. P. KAUFMANN AND W. H. NITSCH, *Fette, Seifen, Anstrichmittel*, 56 (1954) 154; H. P. KAUFMANN AND H. G. KOHLMEYER, *Fette, Seifen, Anstrichmittel*, 57 (1955) 231.

Received October 22nd, 1965

* Desaga standard developing tank with controlled atmosphere cover. Brinkmann Instruments Catalogues Nos. 25–10–20 and 25–10–29, respectively. Brinkmann Instruments, Inc., 115 Cutter Mill Road, Great Neck, Long Island, N.Y.

An apparatus for quantitative application of samples as streaks in thin-layer chromatography

The use of thin-layer chromatography for quantitative analysis of chemical mixtures has been described recently and the merits of various techniques in sample application discussed[1]. One problem has been the transfer of enough material to the chromatoplate to allow analysis of the separated components. This has been overcome

by applying the material as a series of closely spaced spots from a row of capillaries[2] or from a mechanically operated micrometer syringe[3]. The main disadvantage of these methods is the volume of liquid required to fill the various devices, as the amount available for analysis is often small.

The device described below overcomes this problem; it is simple to construct and small volumes can be quantitatively transferred to a silica-gel plate as a narrow uniform line without damaging the silica-gel surface. It is illustrated in Figs. 1 and 2, while Fig. 3 shows the separation of an insecticide mixture applied by this method.

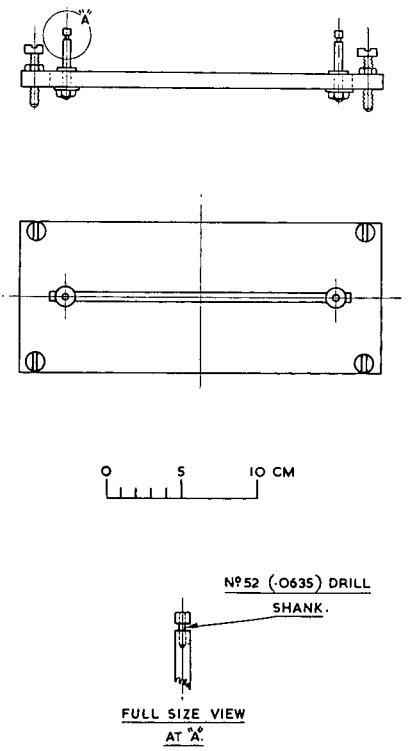
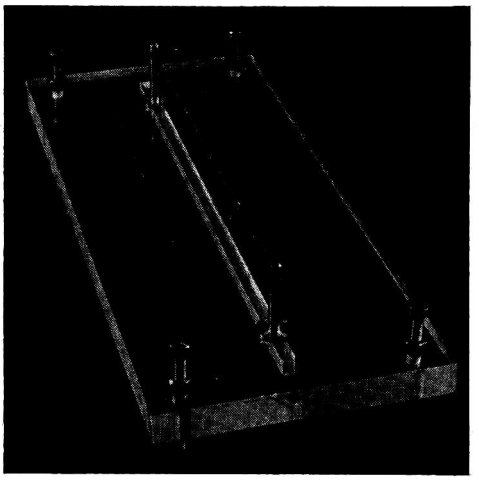


Fig. 1. General arrangement of the applicator.

The parallel horizontal strands of terylene thread are held between two vertical movable rods which can be adjusted to ensure the lines are taut and the length of thread chosen to suit the chromatoplate in use. The sample is applied from a 0.1 ml pipette by laying the pipette tip across the line and moving along the length. The liquid is held by surface tension as an even film between the two threads; a distance of 70 mm and a gap of 1.5 mm will easily support 100 μl of most common solvents. The surface of the chromatoplate is brought in contact with the film and a narrow even streak is produced. The line is washed by repeating the procedure with pure solvent and quantitative transfer can be achieved. The line will support the film more easily if it has been lightly rubbed between fine emery paper. This produces small fibres which increase the contact points between the liquid and the line.



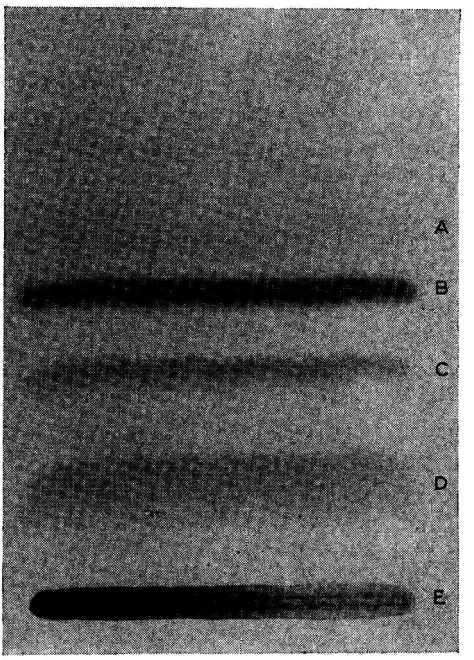


Fig. 2. The applicator with 100 μl of solution applied to the threads.

Fig. 3. Application and separation of an insecticide mixture from a soil extract on a 300 μ layer silica gel plate. Solvent: redistilled chloroform. A = soil material; B = 3,4-dimethyl-6-chlorophenyl N-acetyl N-methylcarbamate; C = 3,4-dimethyl-6-chlorophenyl N-methylcarbamate; D = soil material; E = origin and soil material.

The method has been used in this laboratory for the application of solutions of insecticide residues to thin-layer plates. As an example, 3,4-dimethyl-6-chlorophenyl-N-methylcarbamate and 3,4-dimethyl-6-chlorophenyl N-acetyl N-methylcarbamate were analysed in soil extracts. Control experiments with known amounts of the two compounds applied at a concentration of 800 μg in 200 μl could be recovered almost quantitatively. The recoveries were 99% with a range of ± 3% for 3,4-dimethyl-6-chlorophenyl N-methylcarbamate and 99 % with a range of ± 1 % for 3,4-dimethyl-6-chlorophenyl N-acetyl N-methylcarbamate on six and four determinations respectively.

Tropical Pesticides Research Unit, C. R. TURNER
Porton Down, nr. Salisbury (Great Britain)

1 M. A. MILLET, W. E. MOORE AND J. F. SAEMAN, *Anal. Chem.*, 36 (1964) 491.
2 M. E. MORGAN, *J. Chromatog.*, 9 (1962) 379.
3 M. F. BACON, *J. Chromatog.*, 16 (1964) 552.

Received October 25th, 1965

Quantitative thin-layer chromatography of sugars on microcrystalline cellulose

The successful use in this laboratory of microcrystalline cellulose as an adsorbent for the qualitative thin-layer chromatographic separation of sugars[1] prompted us to study its application for the quantitative analysis of sugars.

Only a few quantitative estimations of unsubstituted sugars by thin-layer chromatography, on silica gel or kieselguhr, have been reported[2–5] and these were mainly devised to solve specific problems. As microcrystalline cellulose has many advantages[1] over silica gel for the thin-layer chromatography of water-soluble compounds, a quantitative method for the estimation of sugars separated on cellulose layers was desirable. The aniline hydrogen phthalate method[6] is widely used for the determination of reducing sugars on papergrams. This reagent gives a colored background when used for spraying "Avicel" plates. We have found that the microcrystalline cellulose can be reduced with sodium borohydride to give a modified cellulose that still maintains all of its former chromatographic properties but gives an almost white background with the aniline phthalate reagent[6], thus making it suitable for quantitative analysis.

Experimental

The microcrystalline cellulose (50 g, Avicel—Technical Grade, Avicel Sales Division of American Viscose Division, FMC Corp., Marcus Hook, Pa.) was stirred mechanically for 10 h at 25° with 1000 ml of an aqueous solution of 0.1 *N* sodium borohydride. After the solid had settled, the supernatant was decanted and the process was repeated with fresh borohydride. The cellulose was then filtered, washed to neutrality, dried over phosphorus pentaoxide in a vacuum desiccator and ground to a powder in a mortar. The chromatoplates were prepared by blending the reduced "Avicel" (100 g) with 430 ml of water for 30 sec at high speed. Smoother plates were obtained when the slurry was deaerated in a filter flask by applying vacuum for about 1 min. The homogeneous slurry was spread on glass plates (20 × 20 × 0.4 cm) with a Desaga applicator at 0.5 mm thickness. The plates were air dried overnight. The sugar solutions were applied in 1.25 μl quantities by means of a syringe microburet; if a larger volume was required, the plate was dried between applications until a total of 10 to 100 μg of sugar was spotted on the plate. The solvent systems reported for paper chromatography were satisfactory. The developed plate was dried with the aid of a commercial hair drier and was sprayed evenly with aniline phthalate reagent (prepared by dissolving 1.66 g of *o*-phthalic acid and 0.91 ml of pure aniline in 48 ml of 1-butanol, 48 ml of ethyl ether, and 4 ml of water)[6]. The plate was heated in an oven at 105–110° until the spots appeared (5–7 min). Rectangular areas around the spots were excised with a razor blade and transferred quantitatively from the plate to a test tube. The areas cut were the same for all the spots of each sugar. A blank was cut out from the plate at the height of the sugar spots. To the test tubes, 0.5 ml of the aniline phthalate reagent was added and the tubes were heated in an oven at 105–110° for 1 h. After cooling, the solid material was broken up with a thin glass rod and to this was added 4 ml of eluting agent, prepared by adding 4 ml of concentrated hydrochloric acid to 100 ml of acetone. The tubes were closed with Teflon stoppers and allowed to stand for 1 h with oc-

casional shaking. The tubes were centrifuged for 3 min and the supernatant was transferred to 1-cm quartz cells with a syringe. The absorbances were measured against the blank, in a Beckman DU spectrophotometer, at 390 mμ for the hexoses and rhamnose and at 360 mμ for the pentoses.

Results

D-Glucose, D-galactose, D-mannose, L-rhamnose, D-arabinose, and D-xylose gave a linear relationship between the light absorption and the concentration. It was established for D-xylose and D-glucose that this linearity holds within the range 0–150 μg. The amount of sugar in the unknown was determined by reference to a standard curve obtained from known sugars that were run on the same plate as the unknowns. Ethyl acetate–pyridine–water (2:1:2, v/v, upper phase)[7] was used in the major part of our study as it gave a good resolution in the shortest time. A mixture of D-glucose and D-xylose was separated in 75 min with this solvent. The coefficient of variation obtained for mixtures containing 20 to 100 μg, respectively, of each sugar was ± 3 %.

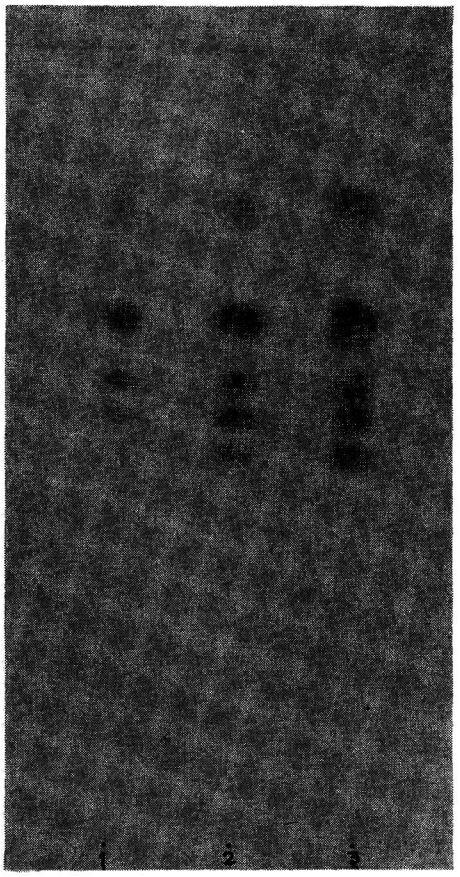


Fig. 1. Thin-layer chromatogram of a mixture of D-galactose, D-glucose, D-mannose, D-xylose, and L-rhamnose (ascending order) after four developments with ethyl acetate–pyridine–water (2:1:2, v/v, upper phase); from left to right: 10 μg, 30 μg, and 50 μg of each sugar, respectively.

Synthetic mixtures of equal amounts of D-galactose, D-glucose, D-mannose, D-xylose, and L-rhamnose in 10, 30, and 50 μg amounts of each were separated readily by four developments, with intermittent drying (hair drier, about 10 min), with ethyl acetate–pyridine–water (2:1:2, v/v); R_G 0.90, 1.00, 1.10, 1.30, 1.55, respectively (Fig. 1). A qualitative analysis of this complex mixture could be accomplished by a double development; for a quantitative determination, four developments afforded a satisfactory separation in 5 h; a similar separation by paper chromatography requires at least 24 h. A mixture of equal parts of D-galactose and D-glucose was determined with a 3 % error for D-galactose and 6 % for D-glucose when the concentration of each sugar per spot was maintained within the range 10 to 60 μg.

Acknowledgement

Acknowledgement is made to support from the Corn Industries Research Foundation.

Department of Chemistry, The Ohio State University, Columbus, Ohio (U.S.A.)

M. L. WOLFROM
ROSA M. DE LEDERKREMER
GERHART SCHWAB

1 M. L. WOLFROM, D. L. PATIN AND R. M. DE LEDERKREMER, *Chem. Ind. (London)*, (1964) 1065; M. L. WOLFROM, D. L. PATIN AND R. M. DE LEDERKREMER, *J. Chromatog.*, 17 (1965) 488.
2 G. PASTUSKA, *Z. Anal. Chem.*, 179 (1961) 427.
3 M. GEE, *J. Chromatog.*, 9 (1962) 278.
4 E. BAUCHER, H. SCHERZ AND K. KAINDE, *Mikrochim. Ichnoanal. Acta*, (1964) 652.
5 G. THOLEY AND B. WURTZ, *Bull. Soc. Chim. Biol.*, 46 (1964) 769.
6 C. M. WILSON, *Anal. Chem.*, 31 (1959) 1199.
7 M. A. JERMYN AND F. A. ISHERWOOD, *Biochem. J.*, 44 (1949) 402.

Received October 29th, 1965

J. Chromatog., 22 (1966) 474–476

Separation of polyphenyls by thin-layer chromatography

Recent interest in polyphenyls as reactor coolants has necessitated both qualitative and quantitative techniques for the separation of the isomers. DENTI *et al.*[1] have shown that sulfonation products of polyphenyls can be separated by paper chromatography. GEISS *et al.*[2] obtained a separation of the polyphenyl isomers by thin-layer chromatography (TLC) but their technique was complicated and reproducibility was difficult to achieve.

The purpose of this paper is to report the separation of the polyphenyl isomers by TLC using a single developing solvent system.

Experimental

Materials. Desaga TLC apparatus and tanks were used. Aluminum oxide G according to STAHL was used to coat glass plates 20 × 20 × 0.4 cm. Solvents were reagent grade. Polyphenyl isomers and azobenzene were obtained from Eastman

Kodak Co., Rochester, N.Y. HB-40, a partially hydrogenated terphenyl mixture, was supplied by Monsanto Chemical Co., St. Louis, Missouri.

Preparation of plates. 50 g of aluminum oxide G were suspended in 100 ml of distilled water and applied as a layer 250 μ thick to the glass plates with a Desaga spreader. The plates were air dried for at least 2 h, then activated 0.5 h at 120° and stored in a desiccator over silica gel and phosphorus pentoxide until use. Just before use each plate was activated again for 0.5 h at 120° and cooled in the desiccator for 20 min.

Preparation of the solvent. The developing solvent was prepared by mixing cyclohexane and benzene in the volume ratio of 98:2. The solvent was stored in the refrigerator until use. The developing tanks were lined with filter paper. 400 ml of solvent and a beaker containing about 100 g of phosphorus pentoxide were placed in the tank and the system was allowed to equilibrate overnight at room temperature.

Chromatographic technique. The separations were carried out by the ascending technique. Solutions of azobenzene, biphenyl, *o*-, *m*- and *p*-terphenyl in carbon tetrachloride were prepared and spotted 1.5 cm from the lower edge of the plate. 10 μl containing 5 μg of compound was the amount spotted. A synthetic mixture of biphenyl and the terphenyl isomers in carbon tetrachloride was prepared. 10 μl containing 5 μg of each component was spotted. HB-40 in carbon tetrachloride was applied at 20 μg per 10 μl spot. Azobenzene was used as a reference dye. After spotting the plate was introduced into the developing tank on to a rack above the level of the solvent and allowed to equilibrate for 0.5 h, after which it was lowered into the solvent and run to a distance of 10 cm. The developing time was about 28 min.

After development the plates were air dried for 5 min at room temperature. Higher temperatures or longer drying times caused the sublimation of the biphenyl off the plate. Detection of the spots was by visible light (for azobenzene), ultraviolet light, iodine vapours and for some plates by oxidation with potassium permanganate in sulphuric acid. The oxidation method gave the best results and consisted of a spray of 0.5 g $KMnO_4$ in 15 ml conc. H_2SO_4. The limit of detection using this method was 0.1 μg/spot for all the isomers. With time the biphenyl spot would fade due to sublimation.

Results

The mean R_F values (centre of spot) were determined from six independent runs and are given in Table I. The absolute R_F values were somewhat variable be-

TABLE I

R_F VALUES OF THE POLYPHENYLS

Compound	*Mean* $R_F \pm \sigma$	*Adjusted* $R_F \pm \sigma$
Biphenyl	0.53 ± 0.02	0.53 ± 0.02
o-Terphenyl	0.43 ± 0.03	0.43 ± 0.01
m-Terphenyl	0.33 ± 0.03	0.33 ± 0.01
p-Terphenyl	0.26 ± 0.02	0.26 ± 0.01
HB-40 spot A	0.86 ± 0.04	0.86 ± 0.02
HB-40 spot B	0.76 ± 0.04	0.77 ± 0.01
HB-40 spot C	0.56 ± 0.02	0.56 ± 0.02
HB-40 spot D	0.46 ± 0.03	0.47 ± 0.01

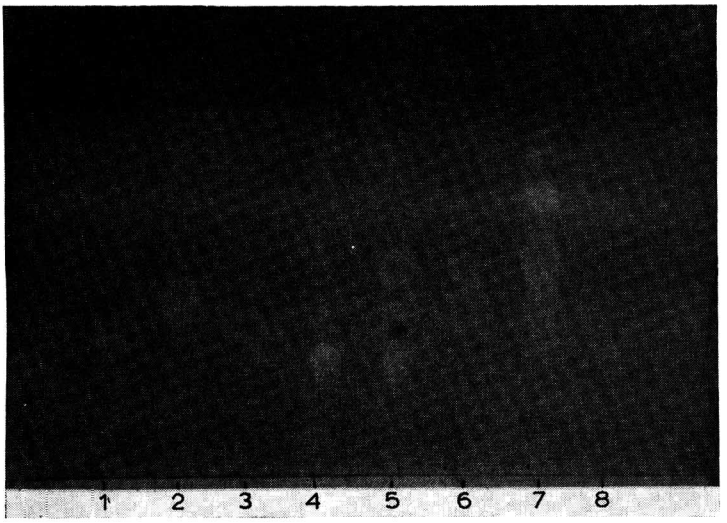


Fig. 1. Polyphenyl isomers on aluminum oxide G layer, 250 μ thick, activated for 30 min at 120°. Solvent: cyclohexane–benzene (98:2). Distance travelled: 10 cm (28 min). Spray: $KMnO_4$ in H_2SO_4. 1 = Azobenzene; 2 = *o*-terphenyl; 3 = *m*-terphenyl; 4 = *p*-terphenyl; 5 = mixture; 6 = biphenyl; 7 = HB-40; 8 = azobenzene.

tween plates but, if activation differences were accounted for using a predetermined mean R_F value (0.31) for azobenzene, the variations were reduced as indicated by the adjusted R_F values of Table I. Partial hydrogenation of the terphenyl isomers increases their mobility on TLC plates. Fig. 1 is a photograph of a typical TLC separation.

Medical Research Section, W. S. CHELACK
Whiteshell Nuclear Research Establishment, H. L. RAYNER
Atomic Energy of Canada Limited, Pinawa, Manitoba (Canada)

1 E. DENTI, M. P. LUBOZ AND A. MASSAGLIA, *J. Chromatog.*, 11 (1963) 339.
2 F. GEISS, H. SCHLITT, F. J. RITTER AND W. M. WEIMAR, *J. Chromatog.*, 12 (1963) 469.

Received October 25th, 1965

J. Chromatog., 22 (1966) 476–478

Plastic sheet thin-layer chromatography in a round tank

The recently developed thin-layer chromatographic sheets with flexible plastic backs can be used in a regular rectangular chromatographic tank or in a special developing apparatus such as the "Eastman Chromatogram" sheet and developing apparatus*. However, it is possible to combine both methods by the use of a spiral

* Supplied by Distillation Products Industries, Rochester, New York.

sheet holder. This holder can be made of plastic, glass or stainless steel wire, see Fig. 1. The advantages of this system are that:

The usual 8 × 8 in. sheets can be used in a small 9 × 2.25 in. round tank* which many laboratories already possess.

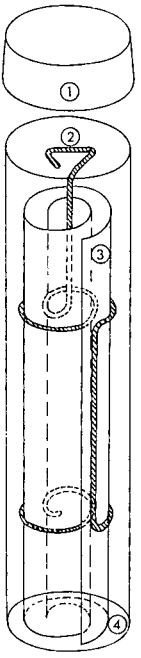


Fig. 1. TLC in a round tank using a flexible chromatography sheet. 1 = Flexible top; 2 = stainless wire; 3 = plastic TLC sheet; 4 = chromatography tank.

Since the tank is covered, volatile and flammable developing liquids may be run on the laboratory bench and there is less chance of contaminating the developing solvent or of having its composition change during a run by selective vaporization.

The thin layer coating material touches nothing but solvent.

If equilibration is desired the fit can be such that the sheet and holder will stay above the solvent until pressed down through the flexible tank top for development.

It is easily constructed from one piece of 3/32 in. stainless steel wire in a few minutes.

Veterans Administration Center, Los Angeles, Calif. (U.S.A.) GRANT G. SLATER

Received November 12th, 1965

* Research Specialties Company, Richmond, California.

The utility of pi-electron acceptors for the detection of 3,4-methylenedioxyphenyl derivatives

A number of substituted methylenedioxybenzenes have been reported to function as synergists for classes of pesticides such as pyrethrins[1–4] and carbamates[5–8], as well as inhibitors of the conversion of aldrin to dieldrin[9].

The analysis of MDO*-phenyl derivatives has been generally achieved by colorimetric techniques, *e.g.* sulfuric acid and chromotropic acids[10–12], and sulfuric and gallic acids[13–16], that are based on the liberation of formaldehyde from the methylenedioxy group and, hence, do not differentiate between related compounds containing this configuration.

A previous report[17] from our laboratory described the utility of a number of reagents (including tetracyanoethylene) for the detection of a variety of MDO-phenyl, and MDO-benzyl derivatives on thin-layer chromatograms.

It was of interest to further elaborate the general detection utility of reagents belonging to other pi-electron acceptor classes, *e.g.* halogenated benzoquinones, naphthoquinones, benzoquinoneimines and nitrofluorenones; and concomitantly relate various functional groupings on the basic 3,4-MDO-phenyl moiety with chromogenic behavior.

Experimental

Detecting reagents were:

(1) DDQ reagent: 2 % 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone in benzene.
(2) Chloranil: 1 % Tetrachloro-*p*-benzoquinone in benzene.
(3) N-2,6-Trichloro-*p*-benzoquinoneimine: 2 % solution in benzene.
(4) Gibbs reagent: 2 % 2,6-Dibromo-N-chloro-*p*-benzoquinoneimine in benzene.
(5) TCNE reagent: 2 % Tetracyanoethylene in benzene.
(6) TNF reagent: 2 % 2,4,7-Trinitrofluorenone in benzene.
(7) 2,4,5,7-Tetranitrofluorenone: 2 % solution in benzene.
(8) 9-(Dicyanomethylene)-2,4,7-trinitrofluorenone: 2 % in benzene.
(9) 2,3-Dichloronaphthoquinone: 2 % solution in benzene.

Materials

Compounds 1, 11, 15, 16 and 2,4,7-trinitrofluorenone were obtained from K&K Laboratories, Inc., Plainview, N.Y., U.S.A.; compounds 2–6, 12 and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone from J. T. Baker Corp., Phillipsburg, N.J., U.S.A.; compounds 7, 8, 10 and tetranitrofluorenone from Frinton Laboratories, Inc., Vineland, N.J., U.S.A.; compounds 9, 13, 14, 17 and 20 from Aldrich Chemical Co., New York, N.Y., U.S.A.; compound 19 (sesamex) was provided by Dr. M. BEROZA, U.S. Dept. of Agriculture, Beltsville, Md., U.S.A.; tetracyanoethylene; N,2,6-trichloro-*p*-benzoquinone; 2,6-dibromo-N-chloro-*p*-quinoneimine and 9-(dicyanomethylene)-2,4,7-trinitrofluorenone were from Distillation Industries, Rochester, N.Y., U.S.A.

Color development

The 3,4-MDO-phenyl derivatives (from 2–5 μl containing 20–50 μg) were applied in acetone solution to both Whatman No. 1 paper and silica gel DF-5 chromato-

* MDO-phenyl = 3,4-methylenedioxyphenyl.

plates (prepared as previously described[17]). After air-drying, the paper and plates were sprayed with the specific detecting reagents described above and the initial color development as well as subsequent color changes noted. The sprayed papers and plates were then exposed briefly to ammonia vapors with the results described in Tables I and II.

TABLE I

SPOT COLORS OF 3,4-METHYLENEDIOXYPHENYL DERIVATIVES AND RELATED COMPOUNDS ON SILICA GEL DF-5 PLATES FOLLOWING APPLICATION OF DETECTOR REAGENTS AND EXPOSURE TO AMMONIA

Designation of colors developed at room temperature: B = blue; Bg = beige; Br = brown; C = crimson; dk = dark; G = green; Go = gold; Gr = grey; L = lilac; O = orange; Ol = olive; P = purple; Pk = pink; R = rose; T = tan; V = violet; W = white; wk = weak; Y = yellow.

No.	*MDO derivative*	*Detecting reagents*								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1	Benzene	—	P→T	B-Gr	O-Bn	O-Y	—	Bg	T-G	Bg
2	Propylbenzene	Y	B-Gr	R-C	P→R	O-Y	Bg	T	T-G	O-C
3	Allylbenzene	Y	V→B-Gr	Y-O	P→Y-Bn	O-Y	Bg	T	T-G	O-C
4	Propenylbenzene	Y	B→Bn	Bn-C	Bn-O	Y	R	Gr	T-G	V
5	Benzaldehyde	V	O-C→Bg	Bg	Bg	O-Y	Ol	Y	Bg	Y
6	Phenol	G (dk)	Bn	B (dk)	B→Ol-G	Bn	V	Bn	T-G	V-Bn
7	Bromobenzene	O-C	R-P→Bn	O-C	O→R	Gr	Bn	O-C	Bg	O
8	Nitrobenzene	T	Y-G	Y	T	Y-O	Y	Y	G (dk)	Y
9	Benzylamine	G-T	Y-G→T	Bn (dk)	T	B*	Bn	Bn	G-Bn	Bn
10	Acetanilide	O-C	B	V	T→R-V	Y-O	R	V	T-G	R
11	Acetophenone	Bn-G	O-C→T	Y-T	Y→T	T	Y	T	T-G	Ol
12	Benzoic acid	O-Bn	R→V	Y-O	R→V	Bg	Y-T	—	Bg	T
13	Phenylmethanol	Y	L-V→V	R	R→V	Bg	Y-T	T	Bg	O-T
14	Cinnamic acid	—	L (wk)	R	Gr-Bn →V	—	T	T (wk)	T-O	R
15	Phenylacetic acid	—	L (wk)	R	Gr-Bn →V	—	T	T (wk)	T-O	R
16	Nitrostyrene	Y-Bn	Y-Gr	Go	Y-G→ Ol-G	Y-O	Y	Y	Y	Y-Bn
	Miscellaneous									
17	Piperonyl sulfoxide	T	T	R-O	Y-G	Y-G	T	Gr	V	T-O
18	Piperonyl butoxide	Y	T	V	Bg	T	C	T	Bg	R
19	Sesamex	Y (wk)	V	V-Bn	G	Gr-Bn	Y	V	T	V
20	Piperine	Gr	B (pale)	V	Bg	R-V*	Bn-V	Gr	Y-G*	P-Bn

* Fluorescence after spraying.

Results and discussion

Tables III and IV depict the spot colors of MDO-phenyl derivatives obtained with nine detecting reagents on silica gel plates and Whatman No. 1 paper, respectively.

A number of salient observations are possible in regard to both the utility of the various classes of detecting reagents and the relationship of structure to chromogenic activity.

MDO-phenyl derivatives bearing electron donating (*o,p*-directing) substituents enhance color (complex) formation. For example, substituents such as $—CH_2NH_2$,

TABLE II

SPOT COLORS OF 3,4-METHYLENEDIOXYPHENYL DERIVATIVES AND RELATED COMPOUNDS ON WHATMAN NO. 1 PAPER FOLLOWING APPLICATION OF DETECTOR REAGENTS AND EXPOSURE TO AMMONIA

Designation of colors developed at room temperature: B = Blue; Bg = Beige; Br = Brown; C = Crimson; dk = dark; G = Green; Gr = Grey; L = Lilac; O = Orange; Ol = Olive; P = Purple; Pk = Pink; R = Rose; T = Tan; V = Violet; W = White; wk = weak; Y = Yellow.

No.	*MDO derivative*	*Detector reagents*								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1	Benzene	W	—	—	—	W	—	—	—	—
2	Propylbenzene	W	—	O-C	L-Gr	W	R	L	B-Gr	—
3	Allylbenzene	W	V	O-C	L-Gr	W	O	Pk	B-Gr	—
4	Propenylbenzene	W	—	V	V	W	V	Gr	B-Gr	—
5	Benzaldehyde	Y	—	Y	T	R	Y	Y	O	—
6	Phenol	Y	O-Bn	Bn-V (dk)	O	Bn	V	Gr	T	V
7	Bromobenzene	Y	V	V	L	L	O	O-C	T	O (wk)
8	Nitrobenzene	Y	V (wk)	O-C	Y	—	Y	Y	Y	V (wk)
9	Benzylamine	—	—	Ol-Bn	Ol-G	G*	Bn-Gr	Bn (dk)	Bn-Gr	Bn (dk)
10	Acetanilide	—	—	Pk	L	R	R	Gr	T	—
11	Acetophenone	—	—	—	Gr	Y	Y	T	T	—
12	Benzoic acid	—	—	—	—	—	—	—	—	—
13	Phenylmethanol	Y	—	O-C	Gr	O	O	O	T	—
14	Cinnamic acid	—	—	—	—	R	—	—	—	—
15	Phenylacetic acid	—	—	—	—	R	—	—	—	—
16	Nitrostyrene	Y	Y	Y	Y	Y-O	Y	Y	Y	Y
	Miscellaneous									
17	Piperonyl sulfoxide	—	T	Bn	Ol	Ol	T	T	V	O-C
18	Piperonyl butoxide	Y	V	O-C	O-T	Y-O	Y-O	T	V	—
19	Sesamex	T	V	Bn	B	O	Y-O	T	T	T
20	Piperine	R	L	O-C	V	Y*	O-C	Gr	Bg*	Y

* Fluorescence after spraying.

—OH, —$NHCOCH_3$, —$CH_2CH_2CH_3$, —CH_2—CH=CH_2, —CH=CH—CH_3, as well as unsubstituted benzene, and —Br (compounds 9, 6, 10, 2, 3, 4 and 7 respectively) are most reactive towards all of the pi-acceptor detectors screened both on silica gel and paper. The above substituents yielded colored spots symbolic of displacement toward longer wavelengths, *e.g.* visible blue, blue-green and green.

Conversely, electron withdrawing groups (*meta* directing) such as —NO_2, —CHO, —$COCH_3$, —COOH, —CH=CH—COOH, and —CH_2COOH (compounds 8, 5, 11, 12, 14, 15) that induce a positive charge on the methylenedioxy moiety, as well as very weak electron donors (—CH_2OH, compound 13) are generally unreactive toward the detecting reagents yielding pale yellow to tan colors except with the strongest electron acceptors DDQ, chloranil and TCNE reagents.

The observed reactivity amongst the reagents treated category-wise is as follows:

(a) halogenated benzoquinones: DDQ > chloranil;

(b) halogenated benzoquinoneimines: N,2,6-trichloro > 2,6-dibromo-N-chloro;

(c) nitrofluorenones: 9-(dicyanomethylene)-2,4,7-trinitro > 2,4,7-trinitro > 2,4,5,7-tetranitro.

TABLE III

SPOT COLORS OF 3,4-METHYLENEDIOXYPHENYL DERIVATIVES AND RELATED COMPOUNDS ON SILICA GEL DF-5 PLATES

Designation of colors developed at room temperature: B = blue; Bg = beige; Bn = brown; C = crimson; dk = dark; G = green; Gr = grey; L = lilac; O = orange; P = purple; Pk = pink; R = rose; T = tan; V = violet; W = white; wk = weak; Y = yellow.

No.	*MDO derivative*	*Detecting reagents*									
		U.V.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1	Benzene	—	B	V	O	L	B→Bn	Y→Y-O	T-O	Gr-O	O
2	Propylbenzene	—	B-Gr	P	O-C	Pk-P	B→Gr	O	T	O-C	O-C
3	Allylbenzene	—	Gr→T-6	O-C	P-C	Bn	B→G	O	T	O-C	O-C
4	Propenylbenzene	—	B→G	B	O-Bn	V	G→Y (pale)	O-C	V→T-Gr	B-Gr	V
5	Benzaldehyde	—	R	O-C	Y-O	O (pale)	Bn-O (pale)	Y	Y	Y-O	Y
6	Phenol	B[e]	P (pale)	P	B	B	P	O-C	Bn→T-Gr	P-Gr	V
7	Bromobenzene	—	B-G	R-P	O-C	O-C	B→B-G	O-Y	O-C	R	R
8	Nitrobenzene	—	Y	Y	Y	Y	Y	Y	Y	Y	Y
9	Benzylamine	—	Y-Bn	Y-G	Bn	Bn (wk)	B[e]	Bn-V	B-Bn	B-G[e]	O→O-Bn
10	Acetanilide	—	B-G	B-Gr	P	V	B-G	O-C	Gr	Gr	V→R
11	Acetophenone	—	B-G→V	O-C	Y	O-T	T→V	Y	Y	Y	Y
12	Benzoic acid	B[e]	Pk	R	Y-O	O-Y	G (wk)	Y	Y	Y-T	Y-T
13	Phenylmethanol	—	L-P	L-V	R	O-T	Gr-B→B	O-Y	T	R	O-T
14	Cinnamic acid	B[e]	Gr-G→Gr	L	R	Pk	Gr	T (wk)	T (wk)	L→T	O-T
15	Phenylacetic acid	B[e]	Gr-G→Gr	L	R	Pk	Gr	T (wk)	T (wk)	L→T	T
16	Nitrostyrene	—	Y-G	T	Y-O	Y-O	Ol-G	Y-O	T	Y-O	Y-Bn
	Miscellaneous										
17	Piperonyl sulfoxide[a]	B[e]	Gr	V-T	T→R	O-T	P-Gr	T	T	T	T-O
18	Piperonyl butoxide[b]	B[e]	B[e]	V	Y→O-C	V	B	O-C	T	V	R
19	Sesamex[c]	—	L→Y	V	V→B	V	B-Gr	O→T	T-V	V	O-C
20	Piperine[d]	B[e]	G	B-Gr→G[e]	V	V-B	B-G[e]	Bn	T→Gr	V-B	P-B

[a] 1-Methyl-2-(3,4-methylenedioxyphenyl) ethyl octyl sulfoxide.
[b] α-[2-(2-Butoxyethoxy)-ethoxy]-4,5-(methylenedioxy)-2-propyl-toluene.
[c] [2-(2-Ethoxyethoxy)-ethyl]-3,4-methylenedioxyphenyl acetal of acetaldehyde.
[d] 1-Piperoylpiperidine.
[e] Fluorescence after spraying.

Overall, in order of decreasing utility: DDQ, TCNE > chloranil > N,2,6-trichlorobenzoquinoneimine > 9-(dicyanomethylene)-2,4,7-trinitrofluorenone > Gibbs > TNF > 2,3-dichloronaphthoquinone > 2,4,5,7-tetranitrofluorenone.

It has been possible to distinguish the isomeric pair safrole and isosafrole (compounds 3 and 4) as well as the related dihydrosafrole (compound 2) from one another by utilizing any of the reagents 1–5 on silica gel, and by reagents 1, 2 and 5 on Whatman paper.

TABLE IV

SPOT COLORS OF 3,4-METHYLENEDIOXYPHENYL DERIVATIVES AND RELATED COMPOUNDS ON WHATMAN NO. 1 PAPER

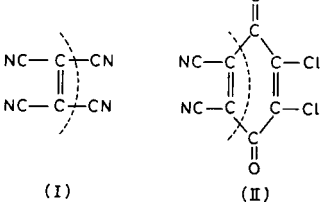
Designation of colors developed at room temperature: B = blue; Bg = beige; Br = brown; C = crimson; dk = dark; G = green; Gr = grey; L = lilac; O = orange; Ol = olive; P = purple; Pk = pink; R = rose; T = tan; V = violet; W = white; wk = weak; Y = yellow.

No.	*MDO derivatives*	*Detecting reagents*								
		U.V.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1	Benzene	—	B-G	O-C	O-L	—	V (fades)	—	—	—	—
2	Propylbenzene	—	B-G	L-Bn	O-C	O-C	B	O	O-C	V	O
3	Allylbenzene	—	B (pale)	V (dk)	O-C	O-C	B-G	O	O-C	V	O
4	Propenylbenzene	—	G	B	B	V	G	R→V	Gr-Bn	B (pale)	C
5	Benzaldehyde	—	V	O-R	Y	Y	O-Bn	Y	Y	O	Y
6	Phenol	—	V	B	P	V	V	Bn	T	Gr-T	P
7	Bromobenzene	—	B-R	Pk	Pk	O	B (pale)	Y	O	O-C	Y-O
8	Nitrobenzene	—	Y	Y	Y	Y	Y (wk)	Y	Y	Y	Y (wk)
9	Benzylamine	—	Ol-G	Ol-G	Bn-V	Ol-G	Ol-G	Bn	P-Bn	Gr-Bn	B
10	Acetanilide	—	L	L	V	V	L	Bg	L (wk)	Gr	Bg
11	Acetophenone	—	R	—	Y	Y-T	R	Y	T (wk)	O	—
12	Benzoic acid	—	—	—	—	W	—	W	—	Y (wk)	—
13	Phenyl methanol	—	B-V	L	O-C	O-C	B-V	O	O-C	O-C	O-C (wk)
14	Cinnamic acid	B*	L	—	—	L (wk)	L	Bg (wk)	T (wk)	T (wk)	—
15	Phenylacetic acid	B*	—	—	—	—	—	W	—	—	—
16	Nitrostyrene	—	Y-G	Y	Y	Y	Y	Y	Y	Y	Y
	Miscellaneous										
17	Piperonyl sulfoxide	B*	L	T	O	Ol-G	Ol-G	T	T	O-Bn	T
18	Piperonyl butoxide	B*	B	T	O-C	O-Bn	B	O	T	V (wk)	O-C
19	Sesamex	—	B→OC	O-Bn	R	V-Bn	G	Y-O	O-Bn	V	R
20	Piperine	B*	L→T	L	O-C	V	G-Bn	O-C	T-Gr	O-Bn	O-C

* Fluorescence after spraying.

It is interesting to note the similarity of colors elicited by DDQ and TCNE reagents towards the majority of MDO-phenyl derivatives tested both on paper and thin layer. ANDERSEN[18] has noted the chromogenic similarities of the above pair of reagents toward aromatic hydrocarbons.

The observed chromogenic similarity of DDQ and TCNE reagents noted in this study can possibly be ascribed to the basic similarity of the *functional* pi-bonding aspects of their structures. TCNE (I) is considered to be ethylene substituted by four cyano groups. In the case of DDQ (II), two cyano and two chloro groups are bonded to two ethylene molecules affixed to two common carbonyl groupings, *viz.*,



(I) (II)

MERRIFIELD AND PHILLIPS[19] have suggested (in comparing quinone with TCNE) that the highly electronegative substituents (CN) are effective in withdrawing charge from the ethylenic groups. This charge withdrawal can thus be expected to enhance the electron affinity of the ethylenic groups and thus its pi-acid strength.

Tables I and II reveal the spot colors of MDO-phenyl derivatives obtained on silica gel plates and Whatman paper respectively, after spraying with the chromogenic reagents followed by exposure to ammonia vapors.

Obliteration or conversion of many of the original colors to yellow or orange-yellow is most pronounced in cases where DDQ and TCNE were the detecting reagents (both on filter paper and thin layer). Other instances of alteration of the original spot color is observed by the nitrofluorenone type detectors. This effect has been previously reported by GORDON AND HURAUX[20] for TNF-aromatic hydrocarbon complexes.

The rapid development of colors at room temperature utilizing non-corrosive reagents, such as DDQ and TCNE, suggests obvious utility of the pi-acceptor reagents for the detection of a variety of MDO-phenyl derivatives on both paper and thin layers.

Acknowledgement

The technical assistance of Mrs. P. H. TURNER in this study is gratefully acknowledged.

This study was supported by Research Contract PH 43-64-57, National Cancer Institute, National Institutes of Health, Public Health Service.

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

LAWRENCE FISHBEIN

1 H. L. HALLER, F. B. LAFORGE AND W. N. SULLIVAN, *J. Org. Chem.*, 7 (1942) 185.
2 M. BEROZA AND W. F. BARTHEL, *J. Agr. Food Chem.*, 5 (1957) 855.
3 W. A. GERSDORFF, P. G. PIQUETT AND M. BEROZA, *J. Agr. Food Chem.*, 4 (1956) 858.
4 H. O. SCHROEDER, H. A. JONES AND A. W. LINDQUIST, *J. Econ. Entomol.*, 41 (1948) 890.
5 R. L. METCALF, T. R. FUKUTO AND M. Y. WINTON, *J. Econ. Entomol.*, 53 (1960) 828.
6 H. H. MOOREFIELD, *Contrib. Boyce Thompson Inst.*, 19 (1958) 501.
7 M. E. ELDEFRANI, R. MISCUS AND W. M. HOSKINS, *Science*, 129 (1958) 898.
8 J. E. CASIDA, *Ann. Rev. Entomol.*, 8 (1963) 39.
9 E. P. LICHTENSTEIN, K. R. SCHULZ AND G. T. COWLEY, *J. Econ. Entomol.*, 56 (1963) 485.
10 H. D. GRAHAM, *Nature*, 207 (1965) 526.
11 M. S. BLUM, *J. Agr. Food Chem.*, 3 (1955) 1344.
12 J. A. LABAT, *Bull. Soc. Chim. Biol.*, 15 (1933) 1344.
13 E. EEGRIWE, *Z. Anal. Chem.*, 110 (1937) 22.
14 M. BEROZA, *J. Agr. Food Chem.*, 11 (1963) 51.
15 M. BEROZA, *Anal. Chem.*, 34 (1962) 1029.
16 L. A. LEE, *Anal. Chem.*, 29 (1956) 1621.
17 L. FISHBEIN AND J. FAWKES, *J. Chromatog.*, 20 (1965) 521.
18 L. ANDERSEN, *Finska Kemistsamfundets Medd.*, 70 (1961) 32.
19 R. E. MERRIFIELD AND W. D. PHILLIPS, *J. Am. Chem. Soc.*, 80 (1958) 2778.
20 H. T. GORDON AND M. J. HURAUX, *Anal. Chem.*, 31 (1959) 301.

Received November 12th, 1965

Thin-layer chromatography of sugar mercaptals and sulphates

Difficulty in obtaining adequate resolution by the standard paper chromatographic techniques[1–5], of the monosaccharide diethyl dithioacetals (mercaptals) obtained during mercaptolysis of polysaccharide mucilages, has lead to the development of the thin layer procedure described. A related problem in the study of seaweed mucilages is the identification of hexose monosulphates produced during acidic fragmentation of the mucilage and this has only been partly solved by paper chromatography and paper electrophoresis. Paper chromatography in solvents giving a fair degree of separation often involves long development times and discrete spots are difficult to obtain. Furthermore, the preparation of new sugar sulphates and an interest in their chemical reactions has also emphasised the need for alternative, faster, means of chromatographic analysis. The solvent of REES[6] has been successfully applied to thin-layer chromatography (TLC) using plates coated with a mixture of silica gel and kieselgur.

Experimental

Resolution of the sugar mercaptals was achieved on 20 × 30 cm plates, coated with Kieselgel G (Merck) to an indicated thickness of 0.4 mm, and activated at 110° for one hour, with the solvent benzene–ethanol (100:15) for 110 min. The spots were detected by spraying with the mixture *p*-anisidine HCl (1 g), conc. sulphuric acid (5 ml) and butanol (100 ml), and heating at 100° for 10 min.

For the separation of the isomeric hexose sulphates, plates (20 × 30 cm), were coated with a mixture of silica gel and kieselgur (Merck, Kieselgel G and Kieselgur G in the proportion 1:2) and developed with ethyl methyl ketone saturated with water containing 1% (w/v) cetylpyridinium chloride (CPC) for 90 min. The plates were activated at 110° for one hour before use and the sugar sulphates were detected by spraying with the diphenylamine aniline reagent of BAILEY AND BOURNE[7].

Results

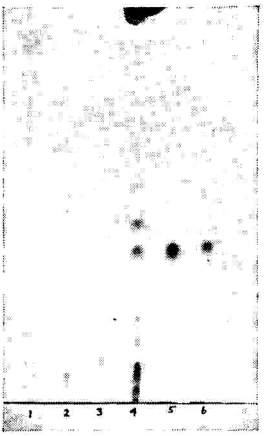
The rates of migration of the sugar mercaptals relative to 3,6-anhydrogalactose mercaptal (R_{AG}) were determined for double development in the solvent benzene–ethanol (100:15); these are listed in Table I and illustrated in Fig. 1.

The R_F (relative to the slower of the two solvent fronts) of various isomeric hexose sulphates were determined with the solvent previously described; these are listed in Table II and illustrated in Fig. 2.

TABLE I

Sugar	R_{AG}*
Xylose	0.065
Xylose mercaptal	0.29
Galactose mercaptal	0.17
6-O-Methyl galactose mercaptal	0.55
3,6-Anhydrogalactose mercaptal	1.00

* See text.



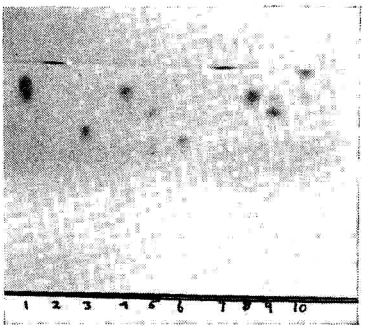


Fig. 1. Thin-layer chromatography of sugar mercaptals. 1 = 6-O-methyl-D-galactose mercaptal; 2 = D-galactose mercaptal; 3 = D-xylose and D-xylose mercaptal; 4 = mercaptolysis products from *Laurencia pinnatifida* mucilage; 5 = 3,6-anhydro-D-galactose mercaptal; 6 = mixture of 1, 2, 3, and 5.

Fig. 2. Thin-layer chromatography of hexose sulphates. 1 = Glucose 2-(barium sulphate); 2 = glucose 3-(barium sulphate); 3 = glucose 6-(barium sulphate); 4 = galactose 2-(barium sulphate); 5 = galactose 3- and 6-(barium sulphate); 6 = galactose 6-(barium sulphate); 7 = galactose 2,3-di(barium sulphate); 8 = galactose 4-(sodium sulphate); 9 = galactose 3- and 4-(sodium sulphate) and galactose; 10 = glucose 3- and 4-(sodium sulphate).

Discussion

The diethyl dithioacetals of the monosaccharides xylose, galactose, 6-O-methyl galactose and 3,6-anhydrogalactose are easily distinguished by a single development and the procedure is sufficiently rapid for the monitoring of mercaptolysis reactions.

In the procedure described for the separation of isomeric hexose sulphates the 2- and 4-sulphated mono-sulphates cannot be distinguished. However, a recent paper by PAINTER[8] describes the chromatographic resolution of these isomers. The cation associated with the sulphate grouping does not appear, in this solvent at any

TABLE II

Sugar	R_F*
Glucose 2-(barium sulphate)	0.91
Glucose 3-(barium sulphate)	1.00
Glucose 6-(barium sulphate)	0.73
Galactose 2-(barium sulphate)	0.90
Galactose 3-(barium sulphate)	0.81
Galactose 6-(barium sulphate)	0.70
Galactose 2,3-di(barium sulphate)	1.00
Galactose 4-(sodium sulphate)	0.90
Galactose 3-(sodium sulphate)	0.82
Glucose 4-(sodium sulphate)	0.90
Galactose	0.44
Xylose	0.68

* Refers to the slower of the two solvent fronts.

rate, to affect the relative R_F values significantly. R_F values should not be regarded as absolute, but merely an indication of the degree of separation that may be achieved; comparison should always be made with authentic specimens. With care the spray reagent specified may provide further help in identification. For example, the 2-isomers appear as distinct brown spots. The remainder vary from brown/green to blue.

The concentration of the sugar sulphate applied should be near the limit of detection of the reagent to avoid streaking. The proportion of CPC in the solvent is critical and may have to be varied slightly to allow for variations between different commercial samples.

Department of Chemistry, University College of North Wales, Bangor (Great Britain)

D. M. BOWKER
J. R. TURVEY

1 M. L. WOLFROM, D. HORTON AND H. G. GARG, *J. Org. Chem.*, 28 (1963) 1569.
2 K. ONODERA AND Y. MORISAWA, *Anal. Biochem.*, 2 (1961) 263.
3 T. J. PAINTER, *Can. J. Chem.*, 38 (1960) 112.
4 H. ZINNER, A. KOINE AND H. NIMZ, *Chem. Ber.*, 93 (1960) 2705.
5 M. L. WOLFROM, Z. YOSIZAWA AND B. O. JULIANO, *J. Org. Chem.*, 24 (1959) 1529.
6 D. A. REES, *Nature*, 185 (1960) 309.
7 R. W. BAILEY AND E. J. BOURNE, *J. Chromatog.*, 4 (1960) 206.
8 T. J. PAINTER, *Proc. Vth Intern. Seaweed Symp., Halifax, 1965*, Pergamon, Oxford, in the press.

Received October 28th, 1965

J. Chromatog., 22 (1966) 486–488

Detection of the polythionates on paper chromatograms

Tests have been described for the detection of the polythionates in microgram quantities on paper chromatograms[1,2]. These tests are not always suitable when detection must be followed by elution from the paper for further studies such as measurements of radioactivity. The dithionate ion can be especially difficult to detect in view of its chemical stability. In the test described by POLLARD, MCOMIE AND JONES[1] the chromatograms formed are permanent, whereas in the method of GARNIER AND DUVAL[2] the spots cannot be eluted after detection by the reagents used in a form suitable for further studies. The need for an alternative method of detection arose out of our work on the sulphur metabolism of the thiobacilli.

Although dithionates are very stable, they can be hydrolysed. The salts can only be hydrolysed slowly at elevated temperatures but the free acid can be hydrolysed at 50° quite rapidly[3] providing the basis for a test applicable on filter paper:

$$H_2S_2O_6 + H_2O \longrightarrow H_2SO_3 + H_2SO_4$$

By spraying the paper with a mixture of hydrochloric acid and hydrogen peroxide followed by gentle warmth the dithionic and other acids are converted to sulphuric

acid. Excess oxidising agent is detected by a starch–iodide spray leaving thionate spots as white areas. The sulphate formed can be eluted from the paper.

Method

Materials: Whatman No. 4 paper, 10 × 8 in. washed in turn by 0.2 *N* HCl, 0.2 *N* ammonia and distilled water followed by drying. Solvent system[4]: isopropanol 50 ml, acetone 20 ml, water 30 ml and sodium acetate 2 g. Sprays: (A) acetone 75 ml, 30 % w/v HCl 4.3 ml, 30 % H_2O_2 0.17 ml and water to 100 ml; (B) 3 % w/v potassium iodide in 1 % w/v starch solution.

The test solutions of the polythionates were run by ascending chromatography until the solvent front had travelled 8 in. The chromatograms were dried in an oven at 50° for 1 h, and, after cooling, lightly sprayed with spray A. The chromatograms were returned to the oven at 50° for 40–50 min and then sprayed with spray B. White spots appeared against a blue background.

Sulphate formed can be eluted from the paper by shaking overnight with 0.2 *N* HCl. After filtering off the paper it was washed with some more of the 0.2 *N* HCl and the sulphate precipitated in the usual way as the barium salt for radioactive assay.

Results and discussion

Dithionate had an R_F value of zero in the solvent system used. The other thionates had R_F values close to those quoted by POLLARD[1,4] and GARNIER AND DUVAL[2] in the solvent systems they used. Sulphite has an R_F value close to dithionate but does not interfere with the dithionate test since it is driven off before it is oxidised. It must be remembered that sulphite loses its SO_2 quickly after spraying with the HCl and H_2O_2 under cold conditions whereas the dithionic acid only liberates its SO_2 slowly under the higher temperature experienced after placing the chromatogram in the oven: the technique failed to detect sulphite spotted on to the paper at the same time as dithionate. A disadvantage of the method is that the chromatogram fades after 5 min but there is the advantage of being able to elute spots afterwards for radioactive assay. The sensitivity is comparable with that of the silver nitrate spray of POLLARD *et al.*[1] and the benzidine–permanganate spray of GARNIER AND DUVAL[2]. 5 μg quantities of thiosulphate, dithionate, trithionate, tetrathionate and pentathionate are detectable but not sulphite or sulphate.

Acknowledgements

One of us, W.K.T.C., wishes to thank the Science Research Council for a grant. We are grateful to Mr. D. WILSON of Sir John Cass College, London, E.C.3 for helpful correspondence.

Department of Biochemistry,
The University of Leeds (Great Britain)

T. J. BOWEN
W. K. T. COOK

1 F. H. POLLARD, J. F. W. MCOMIE AND D. J. JONES, *J. Chem. Soc.*, (1955) 4337.
2 Y. GARNIER AND C. DUVAL, *J. Chromatog.*, 2 (1959) 72.
3 R. C. BRASTED, *Comprehensive Inorganic Chemistry*. Vol. 8. *Sulfur, selenium, tellurium, polonium and oxygen*, Van Nostrand, New York, 1961, p. 166.
4 F. H. POLLARD, *Brit. Med. Bull.*, 10 (1954) 187.

Received November 1st, 1965

Über organische Rhodanverbindungen

15. Mitteilung. Die Identifizierung organischer Rhodanverbindungen nach papierchromatographischer Verteilung*

Über die papierchromatographische Verteilung von organischen Rhodanverbindungen sind bisher in der Literatur nur vereinzelte Angaben zu finden. AWE UND GROTE[1] sowie KAUFMANN UND ARENS[2] führten solche Untersuchungen an rhodanierten Fettsäuren bzw. GÖCKERITZ UND POHLOUDEK-FABINI[3] an halogen- und rhodansubstituierten Thiosemicarbaziden, Thiosemicarbazonen und Aminen durch. Erwartungsgemäss bewährten sich in diesen Fällen meistens die bei den entsprechenden Grundsubstanzen empfohlenen Verteilungsverfahren. Der Nachweis der chromatographierten Substanzen erfolgte stets über andere im Molekül vorhandene Substituenten.

Eine Identifizierung von organisch gebundenem Rhodan auf dem Papier nach papierchromatographischer Verteilung ist unseres Wissens in der Literatur noch nicht beschrieben worden. Nachstehend soll über die Ergebnisse solcher Versuche berichtet werden.

Neben Natriumrhodanid haben wir zu diesem Zweck eine Reihe von chemisch verschieden gebauten organischen Rhodanverbindungen ausgewählt, diese auf dimethylformamidimprägniertem Papier unter Verwendung von Xylol (System A)[3] oder Cyclohexan–Benzol (System B)[3] als mobile Phase verteilt und ihre R_F-Werte ermittelt (Tabelle I). Zum Vergleich wurden teils organische (Thiocarbonyl-, Sulfonamid- und Thiazolderivate) und teils anorganische rhodanfreie Schwefelverbindungen (Sulfid, Sulfit, Sulfat, Thiosulfat) herangezogen (Tabelle II). Da es uns bei diesen Untersuchungen in erster Linie um das Auffinden von Nachweisreaktionen auf dem Papier ging, haben wir diese Vergleichssubstanzen keiner Verteilung unterworfen.

Zum Sichtbarmachen der organischen Rhodanverbindungen auf dem Papier wurde eine Reihe von Reagentien auf ihre diesbezügliche Anwendbarkeit geprüft (ammoniakalische Silbernitratlösung, Millon-Reagens, Quecksilber(II)-acetatlösung, Bleiacetatlösung, Kupfer(II)-sulfatlösung, alkalische Nitroprussidnatriumlösung, Nitroprussidnatrium–Natriumcyanid-Reagens, Jod-azid-Reagens)[4]. Am besten bewährte sich eine alkalische Auflösung einer Quecksilber-Fluorescein-Additionsverbindung[5]. Dieses Reagens gibt, bei einer Mindestnachweisgrenze von 3 μg organisch gebundenem Rhodan, rote Flecke auf hellrotem Untergrund. Bei Auswertung unter U.V.-Licht kann man kräftige stahlblaue Absorptionsflecke auf gelb fluoreszierender Fläche erkennen (Tabelle I).

Ein weiteres brauchbares Reagens fanden wir im Natriumsulfid; es überführt organisch gebundenes Rhodan in anorganisches[6], wobei letzteres in bekannter Weise mit Eisen(III)-chloridlösung nachgewiesen werden kann (Fig. 1). Durch Verwendung einer äthanolischen Natriumsulfidlösung kann die Empfindlichkeit der Reaktion bedeutend erhöht werden. Die Flecke verblassen verhältnismässig rasch, so dass eine umgehende Auswertung des Chromatogrammes notwendig ist. Man erhält in den meisten Fällen nach dem Besprühen mit äthanolischer Natriumsulfidlösung und Erwärmen zunächst gelbe Flecke. Diese werden vermutlich durch die Mercapto- oder

* 14. Mitteilung: R. POHLOUDEK-FABINI, D. GÖCKERITZ UND H. BRÜCKNER, *Pharm. Zentralhalle*, 104 (1965) 315.

TABELLE I

PAPIERCHROMATOGRAPHISCHE VERTEILUNG UND NACHWEIS VON ORGANISCHEN RHODANVERBINDUNGEN MIT QUECKSILBER–FLUORESCEIN-REAGENS UND NATRIUMSULFID–EISEN(III)-CHLORID-REAGENS

Substanz	*Verteilungssystem**	*R_F-Wert*	*Quecksilber–Fluorescein-Reagens*	*Natriumsulfid–Eisen(III)-chlorid-Reagens*
4-Rhodananilin	B	0.24	+	+
4-Rhodanphenylisothiocyanat	A	0.97	+	+
4-Rhodanphenylthiosemicarbazid	A	0.78	+	+
N,N′-Bis-(4-rhodanphenyl)-thioharnstoff	A	0.80	+	+
4-Rhodanphenol	A	0.68	+	+
1-Phenyl-2,3-dimethyl-4-rhodanpyrazolon-(5)	B	0.41	+	+
5-Rhodan-2-aminopyridin	B	0.21	+	+
5-Rhodan-2-aminoanisol	B	0.25	+	+
4,6-Dirhodan-3-aminophenetol	B	0.19	+	+
ω-Rhodanacetophenon	B	0.38	+	+
Natriumrhodanid	A, B	0	—	+

* System A und B siehe Versuchsteil.

TABELLE II

DAS VERHALTEN VON RHODANFREIEN VERBINDUNGEN GEGENÜBER QUECKSILBER–FLUORESCEIN-REAGENS UND NATRIUMSULFID–EISEN(III)-CHLORID-REAGENS

Substanz	*Quecksilber–Fluorescein-Reagens*	*Natriumsulfid–Eisen(III)-chlorid-Reagens*
Anilin	—	—
4-Bromanilin	—	—
Benzonitril	—	—
Phenylisothiocyanat	+	—
4-Phenylthiosemicarbazid	+	—
N,N′-Diphenylthioharnstoff	+	—
Phenol	—	—
1-Phenyl-2,3-dimethylpyrazolon-(5)	—	—
2-Aminopyridin	—	—
2-Aminoanisol	—	—
3-Aminophenetol	—	—
ω-Bromacetophenon	—	—
Thioacetamid	+	—
Methylthiourazil	+	—
Aminothiazol	+	—
Sulfonamide	+	—
Cystein	+	—
Natriumsulfid	+	—
Natriumthiosulfat	+	—
Natriumsulfit	—	—
Natriumsulfat	—	—

Disulfidgruppe des organischen Restes hervorgerufen. Nach Besprühen mit Eisen(III)-chloridlösung schlägt die Farbe in Rot um. Die Nachweisgrenze liegt bei 5 μg organisch gebundenem Rhodan (Tabelle I).

Was nun die Spezifität anbetrifft, so reagieren beide Reagentien mit allen eingesetzten Rhodanverbindungen positiv; das Natriumsulfid–Eisen(III)-Reagens erwartungsgemäss auch mit anorganischem Rhodanid. Das stört jedoch nicht, da dieses in der Regel bei der Papierchromatographie am Startfleck bleibt (Tabelle I).

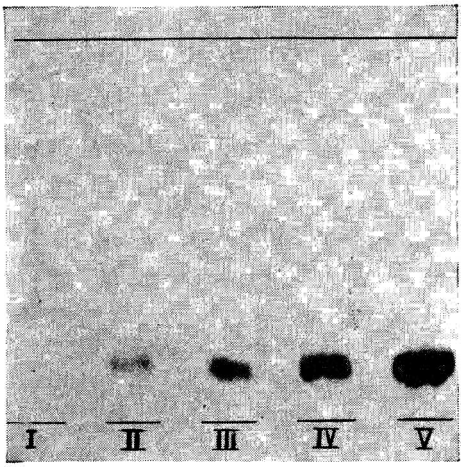


Fig. 1. Nachweis von 4-Rhodananilin (20–200 μg) mit Natriumsulfid–Eisen(III)-Reagens. (I = Blindfleck; II = 20 μg; III = 50 μg; IV = 100 μg; V = 200 μg.).

Betrachtet man die Ergebnisse bei den rhodanfreien Verbindungen, so ist das Natriumsulfid–Eisen(III)-Reagens eindeutig spezifischer auf Rhodan als das Quecksilber–Fluorescein-Reagens; dieses ist in seiner Handhabung zwar wesentlich einfacher, da nur ein Sprühvorgang notwendig ist, es reagiert jedoch auch mit anderen schwefelhaltigen Verbindungen (Tabelle II).

Speziell über das Verteilungsverhalten von organischen Rhodanverbindungen und die Möglichkeiten der quantitativen Auswertung solcher Chromatogramme sowie auch über die Trennung von rhodanhaltigen und entsprechenden rhodanfreien Substanzen wird an anderer Stelle zu berichten sein.

Versuchsteil

Papierchromatographische Verteilung

Papierbogen 29 × 30 cm (Schleicher & Schüll 2043 Bm) werden in Laufrichtung einmal langsam durch eine 50%-ige methanolische Dimethylformamidlösung gezogen und ohne vorheriges Abpressen 15 min zum Abdunsten des Methanols mit der Startlinie nach oben aufgehängt. Danach trägt man 20–50 μg der Substanz in Form einer 1%-igen acetonischen, alkoholischen oder benzolischen Lösung auf und chromatographiert aufsteigend entweder mit Xylol (System A) oder mit Cyclohexan–Benzol (3:1, V/V) (System B), nachdem vorher Kammer und Papier jeweils 2 Stunden mit der entsprechenden mobilen Phase gesättigt wurden. Die Laufzeit beträgt bei System A bei einer Steighöhe von 20 cm 2 Stunden, bei System B 3 Stunden.

Nachweis mit Quecksilber–Fluorescein-Reagens[5]

Äquimolare Mengen von Quecksilber(II)-acetat und Fluorescein werden getrennt in Eisessig unter Erwärmen gelöst. Noch warm wird die Fluoresceinlösung in die Quecksilberacetatlösung filtriert. Dabei fällt in der Regel das Präparat bereits aus. Zur vollständigen Auskristallisation des Additionsproduktes wird der Ansatz längere Zeit im Eisschrank belassen. Man saugt ab, wäscht mit Eiswasser und trocknet unter Vakuum über Blaugel. 20 mg von dieser Substanz werden in 10 ml 1 *N* Kalilauge in der Kälte gelöst. Das Sprühreagens ist etwa eine Woche haltbar.

Mit dieser Lösung wird das Chromatogramm besprüht und nach 5 min ausgewertet. Es zeigen sich rote Flecke auf hellrotem Grund. Empfindlicher gestaltet sich der Nachweis im U.V.-Licht.

Nachweis mit Natriumsulfidlösung (Lösung I) und Eisen(III)-chloridlösung (Lösung II)

Lösung I. 5.0 g Natriumsulfid (9 H_2O) werden unter leichtem Erwärmen in 20 ml Wasser gelöst und nach dem Abkühlen mit Äthanol (96 %) zu 100 ml aufgefüllt.

Lösung II. 40 ml 5 %-ige wässrige Eisen(III)-chloridlösung werden zu 100 ml mit 20 %-iger abgekochter Salpetersäure aufgefüllt.

Das Chromatogramm wird mit Lösung I besprüht, über einer heissen Kochplatte sorgfältig getrocknet (beschleunigte Bildung von Alkalirhodanid) und anschliessend mit Lösung II besprüht. Es erscheinen rote Flecke auf weissem Grund.

Pharmazeutisches Institut der Ernst-Moritz-Arndt-Universität, Greifswald (D.D.R.)

H. BRÜCKNER
D. GÖCKERITZ
R. POHLOUDEK-FABINI

1 W. AWE UND B. GROTE, *Fette, Seifen, Anstrichmittel*, 60 (1958) 806; *C.A.*, 53 (1959) 3737.
2 H. P. KAUFMANN UND M. ARENS, *Fette, Seifen, Anstrichmittel*, 60 (1958) 803; *C.A.*, 53 (1959) 3737.
3 D. GÖCKERITZ UND R. POHLOUDEK-FABINI, *Pharmazie*, 17 (1962) 679.
4 I. M. HAIS UND K. MACEK, *Handbuch der Papierchromatographie*, Bd. I, 2. Aufl., Jena, 1963, S. 903.
5 ANONYM, *Nachr. Chem. Tech.*, 11 (1963) 82.
6 C. LÖWIG, *Ann. Phys. Chemie (Poggendorf)*, [3], 7 (1846) 101.

Eingegangen den 2. November 1965

Paper chromatography of 5-substituted tryptophans*

Serotonin, one of the brain neurohumeral mediators, is formed biosynthetically from tryptophan *via* 5-hydroxytryptophan (5-HTP). On administration of either of these three compounds, only 5-HTP crosses the blood-brain barrier readily. A study was inaugurated to see if some isosteres of 5-HTP would interfere with the transport of this amino acid into the brain. If any of these 5-substituted tryptophans compete

* Supported in part by Grant No. MH06826, from the National Institute of Mental Health, U. S. Public Health Service.

with the transport of 5-HTP across the blood-brain barrier, alterations of brain serotonin levels would be noted.

Accordingly, a series of tryptophans substituted in the 5-position were synthesized and purity was determined by paper chromatography. Two independent solvent systems, butanol–acetic acid–water (B–A–W) and phenol–water (P–W) and two color developing reagents (ninhydrin and Ehrlich's reagent) were used. Surprisingly, some of the compounds with different polarities had identical R_F values. Using P–W, it was not possible to separate and hence to characterize some of the 5-substituted tryptophans by R_F values from paper chromatography alone.

Experimental

(a) *Synthesis of amino acids.* The DL-amino acids were synthesized by methods which appear in the literature. References to these procedures are given in Table I.

(b) *Paper chromatography.* Classical procedures, as described by SMITH[1], were used. The amino acids were dissolved in a solvent, spotted, dried, chromatographed in one dimension on 1.5 × 11 in. Whatman No. 1 paper strips. Experiments were repeated a minimum of five times. The apparatus used was the Gordon-Misco chromatographic tubes, as recommended by GORDON[2].

The tubes were equilibrated for a minimum of 1 h and thereafter were kept saturated. Paper strips were saturated for 20 min prior to use in all cases. The chamber temperature was constant during a run. At the end of the experiment time, usually 3 or 4 h, the papers were removed and dried overnight in a slow current of air. To develop color, one strip was sprayed with ninhydrin (0.2% w/v acetone); the other with Ehrlich reagent (1 vol. of *p*-dimethylaminobenzaldehyde 10% w/v in conc. HCl +4 vol. acetone).

The solvents were either butanol–acetic acid–water (60:15:25 v/v); or phenol–water (40 g:10 ml). All amino acids were run individually and then in pairs. DL-Tryptophan was added to the mixture of pairs to serve as a reference. For statistical evaluation the runs were repeated from six to thirty-three times. The data in Table I give the mean R_F values of the individually run amino acids and the standard deviation.

Table II gives the mean of the R_F values of compounds which were run in pairs. In cases where no separation was noted, that observation was verified by three consecutive runs.

TABLE I

R_F VALUES FOR DL-5-SUBSTITUTED TRYPTOPHANS

Substituent	*R_F value ± S.D.*				*Lab temp.* (°C)	*Exp. time* (*h*)	*Ref. to synthesis*
	B–A–W	*± S.D.*	*P–W*	*± S.D.*			
None	0.45	0.018	0.73	0.013	22	3	—
Amino	0.17	0.024	0.21	0.028	24	4	3
Nitro	0.41	0.021	0.66	0.008	24	4	3
Methyl	0.53	0.022	0.77	0.007	23	3	4
Hydroxy	0.58	0.026	0.87	0.019	22	3	Commercial
Fluoro	0.52	0.016	0.73	0.014	23	4	5
Bromo	0.58	0.007	0.73	0.028	23	4	6
Benzyloxy	0.59	0.017	0.90	0.016	24	4	Commercial

TABLE II

R_F VALUES OF MIXTURES OF TRYPTOPHANS

Substituent	*R_F value**				*Lab. temp.* (°C)	*Exp. time* (*h*)
	B–A–W		*P–W*			
	N	*E*	*N*	*E*		
Methyl	0.53	0.56	no separation		22	3
None	0.44	0.45				
Hydroxy	0.54	0.57	0.89	0.90	22	3
None	0.48	0.41	0.74	0.75		
Hydroxy	0.63	0.61	no separation		23	4
Methyl	0.51	0.51				
Nitro	0.39	—	—	—	24	4
None	0.48	0.45	—	0.74		
Amino	0.16	0.17	0.20	0.21	24	4
None	0.45	—	0.73	0.74		
Benzyloxy	0.60	0.58	0.92	0.90	24	4
None	0.44	0.43	0.74	0.73		
Methyl	0.50	0.52	0.78	0.77	23	4
Amino	0.15	0.22	0.15	0.17		
Amino	0.17	0.18	0.23	0.23	23	4
Benzyloxy	0.60	0.61	0.88	0.92		
Nitro	0.44	—	no separation		23	4
Methyl	0.53	—				

* N = ninhydrin; E = Ehrlich's reagent.

Results and conclusions

The R_F values are given in Table I, which were run on an individual basis. Table II gives the data for amino acids run in pairs. In each of these cases, DL-tryptophan was added for a reference. From Table II it can be concluded that it is not possible to separate and, hence, to characterize the following pairs when phenol–water is the solvent: 5-methyl from tryptophan, 5-hydroxy- or 5-nitro. No difficulty was encountered when butanol–acetic acid–water was used.

The pair, 5-bromo and 5-fluoro, had like R_F values in both solvent systems and thus cannot be separated.

Institute of Chemical Biology,
University of San Francisco,
San Francisco, Calif. (U.S.A.)

ARTHUR FURST
SHAKUNTLA SAXENA

1 I. SMITH (Editor), *Chromatographic and Electrophoretic Techniques*. Vol. I. *Chromatography*, Interscience, New York, 1962, p. 84, 95, 96, 193.
2 H. T. GORDON AND C. A. HEWEL, *Anal. Chem.*, 27 (1955) 1471.
3 J. DEGRAW AND L. GOODMAN, *J. Org. Chem.*, 27 (1962) 1395.
4 W. R. BOON, *J. Chem. Soc.*, (1949) 231.
5 H. RINDERKNECHT AND C. NIEMANN, *J. Am. Chem. Soc.*, 72 (1950) 2296.
6 D. G. HARVEY, *J. Chem. Soc.*, (1959) 473.

Received October 18th, 1965

The effect of urea on histones in polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) has been used for the resolution of histones and histone fractions[1,2]. Although the method of McALLISTER *et al.*[1] does appear to be one of the better methods for the separation of the apparently heterogeneous histones, the method does suffer some drawbacks and appears to lead to the formation of some artifacts. This communication reports that the incorporation of urea into the previously used buffer system eliminates the holdback at the point of application of the sample, decreases the number of bands observed in a histone sample and allows for the use of spacer gels if desired. These observed alterations may be due to the lack of aggregation of some of the histone fractions in the presence of urea[3] and thus a decrease in the number of bands in the PAGE system. The previously used PAGE systems have yielded ten or more rather diffuse bands for unfractionated calf thymus histones compared to a smaller number of more discrete bands upon urea incorporation into the buffer system.

Methods and materials

Whole calf thymus histone, as supplied by Worthington Biochemical Corporation, Freehold, New Jersey, was used for this study.

A concentrated solution of buffered Temed (N,N,N′,N′,-tetramethylethylenediamine) was prepared by adding 2.4 ml of 5 *N* KOH and 1.15 ml Temed to 53 ml of glacial acetic acid. Immediately before use 5.7 ml of this solution is added to 70 mmoles of urea and diluted to a final volume of 10 ml. The gel mixture is composed of two parts of this solution with one part of 2.8 % ammonium persulfate–7 *M* urea and one part of 60 % acrylamide–0.4 % bis (N,N′-methylenebisacrylamide). The spacer gel solution, when used, included 0.09 *M* glycinium acetate buffer (pH 4.0), 2.5 % acrylamide, 0.31 % bis, 0.5 mg % riboflavin, and 5.25 *M* urea. Sample gels were not used. The samples were dissolved in 0.09 *M* glycinium acetate buffer (pH 4.0)–1 *M* sucrose, and one ml of this solution was added to seven mmoles urea (final volume 1.3 ml, final urea concentration 5.4 *M*). This solution was placed directly on the gel and electrophoresis buffer (0.37 *M* glycinium acetate, pH 4.0, with 5.25 *M* urea) was carefully layered over it. The tubes used were 8 mm I.D. (destaining tubes as supplied by Canal Industrial Corporation, Bethesda, Maryland) with a current of 7 mA/tube for 4 h.

Staining and destaining were carried out as recommended by McALLISTER *et al.*[1]

Results and discussion

Fig. 1 compares the electrophoregrams of calf thymus histone in the presence and absence of urea. All of the bands appear more distinct in the urea containing system. It should be noted especially that in the presence of 5.4 *M* urea there is an absence of the band which appears at the point of application of the protein sample. In urea it appears that there is no hold up of the sample at this point which may indicate the lack of aggregation to form high molecular weight complexes which may form in the absence of this denaturing agent. Also, it should be pointed out that the urea containing system allows for the use of spacer gels if desired. The PAGE system run in the presence of urea appears identical with or without the spacer gel.

Another significant difference is the absence of several of the slower moving bands in the urea containing electrophoregrams. Although the molecular sieving effect is not the only factor responsible for the resolution of histones in the PAGE system, it may account for the lack of the high molecular weight species or aggregates which appear as less mobile bands in the urea free system. The difference found in the presence of urea employed here was not reported by DREIDGET *et al.*[2] in gels containing 4 *M* urea. While these authors reported no differences with 4 *M* urea, they did report

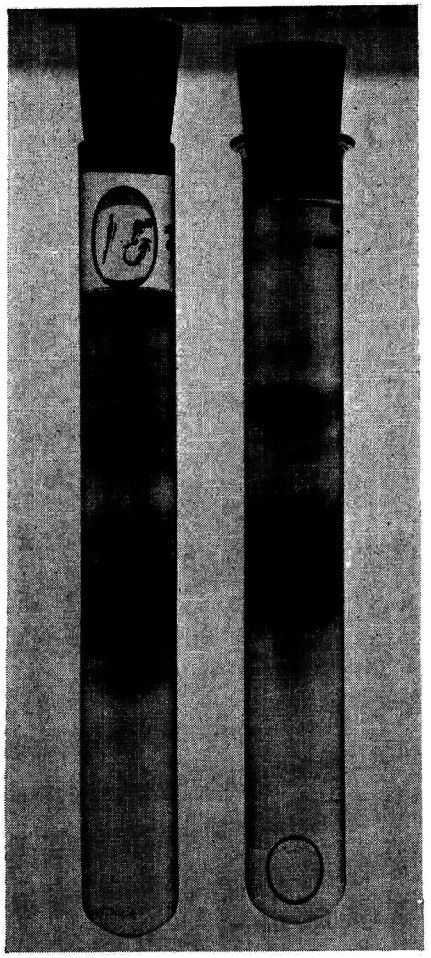


Fig. 1. The electrophoregrams of whole calf thymus histone run in the absence (left) and presence (right) of 5.4 *M* urea.

slight variation in the positions, intensities and number of bands of histones in the presence of some metal ions at a final concentration of 10^{-3} *M*. VANDE WOUDE AND DAVIS have also recently reported a considerable sharpening of histone bands when the PAGE was run in the presence of urea at concentrations up to 8 *M*[4].

These various observations point out the need for a reevaluation of the PAGE

system applied to the resolution of histones. Aggregate formation of some of the histone fractions may be one of the major factors responsible for some of the numerous bands reported in these electrophoresis systems. Employment of urea, as suggested here and in the recently reported preparative electrophoresis of proteins on polyacrylamide gels[5], may be necessary to eliminate the anomalous behavior of histones in PAGE systems.

Acknowledgement

This work was supported by Grant AM-05169 from the National Institutes of Arthritis and Metabolic Diseases, U.S. Public Health Service.

Department of Chemistry, California State College at Long Beach, Long Beach, Calif. (U.S.A.)

R. D. BAUER
W. A. THOMASSON

1 H. C. MCALLISTER, Y. C. WAN AND J. L. IRVIN, *Anal. Biochem.*, 5 (1963) 321.
2 A. DREIDGET, L. D. JOHNSON AND A. M. MARKO, *Can. J. Biochem. Physiol.*, 41 (1963) 2507.
3 K. MURRAY, *Ann. Rev. Biochem.*, 34 (1965) 209.
4 G. F. VANDE WOUDE AND F. F. DAVIS, *Anal. Biochem.*, 12 (1965) 444.
5 P. H. DUESBERG AND R. R. RUECKERT, *Anal. Biochem.*, 11 (1965) 342.

Received November 5th, 1965

J. Chromatog., 22 (1966) 496–498

Partition chromatography of some organic acids on a cation exchange resin

It is known that air dry 10% divinylbenzene cross-linked sulfonated polystyrene resin, when equilibrated with 70% aqueous acetone, absorbs water preferentially, so that the water content of the liquid absorbed is found to be higher than that of the external solution[1]. The distribution of an organic substrate which is sparingly soluble in water between these two phases will be greatly in favour of the outer solution and thus provides an explanation both for the negative adsorption and for the low catalytic activities of sulfonated polystyrene resin in the hydrolysis of various aliphatic esters in 70% acetone in water[2].

RUCKERT AND SAMUELSON[3] have also shown that strongly polar nonelectrolytes, such as sugars, can be taken up effectively from mixed solvents by means of ion exchange resins. On the basis of this, they were able to separate sugars chromatographically on anion exchange resin. Dowex 1-X8 in the sulfate form was used as the stationary phase and the eluent was 74% aqueous ethanol[4].

Separation of various organic acids using similar chromatographic systems has been attempted, in some cases successfully. In the present work cross-linked sulfonated polystyrene resin, Amberlite CG-120, in the hydrogen ion form was used. Citric acid, malic acid and tartaric acid could be separated using the mixture acetone–dichloromethane–water (160:100:9, v/v) as eluent, while an eluent containing more dichloromethane (acetone–dichloromethane–water (20:15:1, v/v), made possible the separation of fumaric acid, glutaric acid and succinic acid.

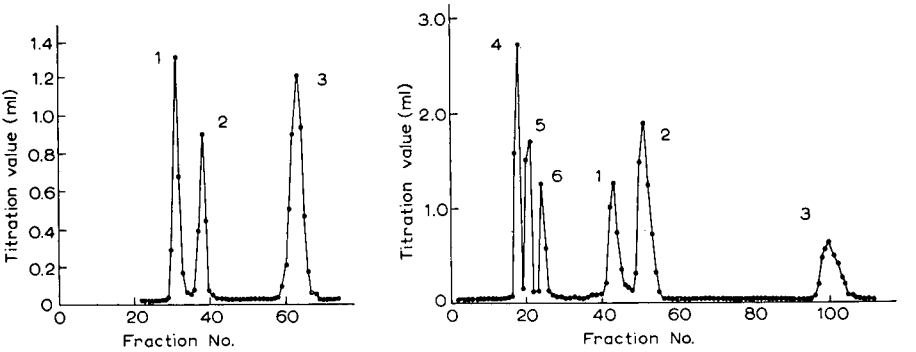


Fig. 1. Elution of organic acids. The compounds in the order of their elution from the column are: citric acid (1), malic acid (2) and tartaric acid (3). Column size: 0.75 × 75 cm. Eluent: acetone–dichloromethane–water (160:100:9, v/v). Fraction size: 40 drops.

Fig. 2. Elution of organic acids. The compounds in the order of their elution from the column are: fumaric acid (4), glutaric acid (5), succinic acid (6), citric acid (1), malic acid (2) and tartaric acid (3). Column size: 0.78 × 81 cm. Eluent: acetone–dichloromethane–water (20:15:1, v/v). Fraction size: 42 drops.

Amberlite CG-120 (200–300 mesh, screened wet in the sodium ion form) was washed successively on a glass filter with 6 *N* hydrochloric acid (10 volumes), water, 2 *N* sodium hydroxide (10 volumes), water, 2 *N* hydrochloric acid (10 volumes), water and then 95 % acetone in water (10 volumes). The washed resin was finally washed and equilibrated with the solvent to be used for chromatography, and suspended in two volumes of this solvent. The suspension was poured into the chromatographic tube and allowed to settle under gravity. When about 200 ml of the solvent had passed through the column, it was ready for use. Samples were dissolved in the solvent to be used for chromatography and 1 ml of the solution was introduced on to the column. Elution was performed at 15° to 20° and the effluent was collected in fractions of 40 or 42 drops. The flow rate was about 7 fractions per hour. Each fraction was titrated with 0.01 *N* sodium hydroxide and the titration values were plotted against the fraction number. As shown in Figs. 1 and 2, the elution sequence of the organic acids studied was similar to that observed on partition chromatography on hydrated silica gel. Recovery of these acids ranged from 90 to 100 %.

The column could be used repeatedly. The chromatographic system described above seems to be useful for the separation of acidic and neutral polar substances. Purification of sulfoxides on Dowex 50 in the hydrogen ion form, using benzene and ethanol as solvents[5], may be regarded as an example of this.

Department of Genetics, Osaka University Medical School, Osaka (Japan) TOKUICHIRO SEKI

1 C. W. DAVIES AND G. G. THOMAS, *J. Chem. Soc.*, (1952) 1607.
2 V. C. HASKEL AND L. P. HAMMETT, *J. Am. Chem. Soc.*, 71 (1949) 1284.
3 H. RUCKERT AND O. SAMUELSON, *Svensk Kem. Tidskr.*, 66 (1954) 337.
4 O. SAMUELSON AND B. SWENSON, *Anal. Chim. Acta*, 28 (1963) 426.
5 V. HORÁK AND J. PECKA, *J. Chromatog.*, 14 (1964) 97.

Received November 1st, 1965

The ion-exchange separation of a niobate–tantalate mixture

The columbite–tantalite minerals which are found in Uganda can be broken down by fusion with potassium hydroxide or potassium carbonate. Leaching the melt with water produces a solution containing niobate and tantalate ions. It was therefore decided to investigate the ion-exchange behaviour of these ions. This note presents preliminary data on the separation of niobate and tantalate ions on a column of a strongly basic ion-exchange resin, which it is hoped will provide the basis for a new and simple method of analysis for these minerals.

Procedure

A column (length 28 cm, diameter 2 cm) of Deacidite FF resin, 7–9% cross-linking, 100–200 mesh, was conditioned by passing 300 ml of a solution of 0.7 *M* potassium chloride which was also 0.035 *M* with respect to potassium hydroxide.

The adsorption was performed by passing 50 ml of a solution containing 5 mg of niobium pentoxide and 4.9 mg of tantalum pentoxide in 0.035 *M* potassium hydroxide, to which had been added 6.6 ml of 0.1 *M* potassium chloride solution. The potassium chloride was added in order to maintain the resin largely in the chloride form throughout.

The elution was carried out by passing through the column 950 ml of 0.25 *M* potassium chloride solution which was also 0.01 *M* with respect to potassium hydroxide, at a flow rate of one ml per min. An automatic fraction collector was used to obtain fractions of approximately 40 ml each. A 0.5 *M* solution of potassium chloride, which was also 0.01 *M* with respect to potassium hydroxide, was then passed down the column to elute the niobate. The fractions collected were analysed by standard colorimetric methods, and the results obtained are shown in Fig. 1.

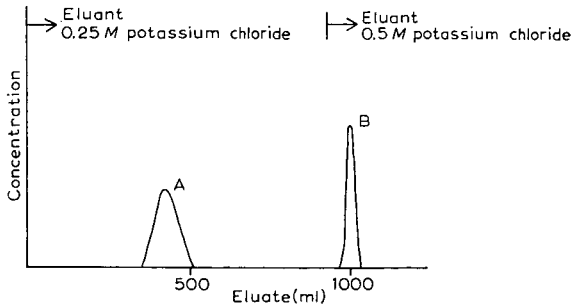


Fig. 1. Elution of niobate–tantalate. A = tantalate; B = niobate.

Acknowledgement

I wish to thank the Commissioner, Department of Geological Survey and Mines, for permission to publish this note.

Geological Survey Department, Entebbe (Uganda) F. W. SMITH

Received November 2nd, 1965

News

INTERNATIONAL SYMPOSIUM ON PHYSICAL SEPARATION METHODS IN CHEMICAL ANALYSIS

An International Symposium on Physical Separation Methods in Chemical Analysis is being organized by the Analytical Chemistry Section of the Koninklijke Nederlandse Chemische Vereniging, and will be held in the Congres-centrum, RAI, Amsterdam, from April 10th to 14th, 1967.

Scientific programme

The scientific programme will consist of invited and submitted papers. Invited papers will be presented in four morning sessions. Submitted papers will be presented and discussed in two parallel sessions on four afternoons. On April 12th there will be excursions to the research laboratories of some large industries, or to places of interest to tourists. All papers will be published in the May/June (1967) issue of *Anal. Chim. Acta*, and this issue will be sent to the participants about two weeks before the symposium. Discussions will be published later in a supplement and forwarded to the participants.

The subjects of the invited papers and the names of the lecturers are as follows:

1. Chromatography; G. W. A. RIJNDERS (Koninklijke Shell-Laboratorium, Amsterdam).
2. Gel permeation and molecular sieve effects in chromatography (lecturer to be announced).
3. Ion exchange chromatography; O. SAMUELSON (Technical University, Göteborg).
4. Zone melting; H. SCHILDKNECHT (University of Heidelberg).
5. Ultracentrifugation of synthetic polymers; H. BENOIT (Centre de recherches sur les macromolécules, Strasbourg).
6 Macromolecular interactions in gel permeation, electrophoresis and ultracentrifugation; G. A. GILBERT (University of Birmingham).
7. Electrophoresis; H. BLOEMENDAL (University of Nijmegen).

Intending authors

Those wishing to submit papers for consideration for the afternoon sessions should send an abstract of about 300 words (2 copies) to the secretary (C. L. DE LIGNY, c/o Analytisch Chemisch Laboratorium, State University, Utrecht, Netherlands) not later than August 1st 1966. In the course of August the abstracts will be screened by an international committee and the authors will be informed whether or not the papers are suitable for inclusion. In order that the proceedings may be prepared in time, the complete texts of accepted papers (about 2000 words, 3 copies) must be received before October 1st 1966. The papers should be of an original character. They can be read in French, German or (preferably) English and should have an English summary. For each paper 30 minutes will be reserved, including discussion. There will be facilities for projecting slides and films.

Social programme

An attractive social programme has been planned, including a visit to the Rijksmuseum and trips to the Keukenhof with its magnificent flowers and to the hydraulic engineering projects of the Delta works.

Registration

Intending participants can obtain further information and application forms from the Congress Bureau, St. Agnietenstraat 4, Amsterdam, Netherlands. The registration fee for delegates will be fl. 125, including proceedings and printed discussions (fl. 35 for non-participating members). The final date for registration is 31.12.1966.

Separation Techniques—Nineteenth Annual Summer Symposium

Sponsored by the Division of Analytical Chemistry and *Analytical Chemistry*, University of Alberta, June 22nd–24th, 1966.

Separation Techniques is the topic of the 19th Annual Summer Symposium on Analytical Chemistry to be held June 22nd–24th, 1966 at the University of Alberta in Edmonton, Alberta, Canada. To avoid too great a diversity of topics, the speakers are emphasizing chromatographic methods, particularly the comparison of gas and liquid techniques.

Within recent years there has been distinct evidence of feedback from developments in gas chromatography to the older technique of liquid chromatography including its more recent versions such as TLC. Exchange of ideas in both directions is obviously valuable and practitioners in all fields of chromatography will be interested in the symposium program. The 27 papers cover both theoretical and practical aspects of chromatographic separations and include such topics as comparative studies of band spreading in liquid and gas chromatography, new developments in liquid chromatography techniques, and reports on applications.

This will be the first time that the summer symposium has been held outside of the United States and will offer participants an opportunity to visit one of Canada's rapidly growing universities. Edmonton is a city of 350,000 centered in a rich farming area and also surrounded by numerous oil and gas fields.

Chairman of the symposium is Dr. Roy A. Keller of the Department of Chemistry, University of Arizona. Dr. W. E. Harris of the Department of Chemistry, University of Alberta, is in charge of local arrangements.

An International Symposium on Newer Physical Methods in Structural Chemistry

An international symposium on "Newer physical methods for the study of structural chemistry" will be held at New College, Oxford from July 18th–21st, 1966.

The symposium is being organised under the guidance of Sir Robert Robinson, Prof. C. Djerassi and Prof. W. Klyne, and several eminent workers in the U.K. and other countries have already agreed to contribute. There will be about 26 papers and the emphasis will be on mass spectroscopy, optical rotatory dispersion and circular dichroism. A detailed programme will be issued shortly. The papers will be published as a monograph as soon as possible after the symposium.

An exhibition of the latest apparatus will be held simultaneously. All enquiries and applications should be addressed to the Symposium Secretary, "Laboratory Practice", 9 Gough Square, London, E.C. 4.

Summer Symposium in Instrumental Analytical Chemistry

In keeping with Lehigh University's desire to serve the industrial community, the Department of Chemistry is pleased to announce a summer symposium in modern instrumental analytical chemistry. This symposium will follow the format of those offered in the summers of 1962, 1963 and 1964. Its purpose is to acquaint the practicing analytical chemist with the theory and techniques of modern instrumental analysis.

Scope

Each topic discussed will have at least three hours devoted to it. Initially, there will be a brief review of the principles underlying the technique of interest. Attention will then be given to the most recent applications of this technique or instrument and modifications which significantly extend its usefulness will be presented. Comparisons with competitive analytical procedures as to speed, accuracy, and cost will be made. The discussion will include both commonly available instruments and those more complex instruments and techniques which the typical analyst does not use directly but which are often available to him for specialized analyses. Consequently, it is hoped that the analyst will see how to make better use of his existing instrumentation and will also be made aware of the advantages and disadvantages of the more complex instrumental techniques.

Some of the topics to be discussed are:

Infrared spectrophotometry
X-ray fluorescence spectroscopy
Ultraviolet and visible fluorescence spectroscopy
Polarography, chronopotentiometry, and amperometric titrations
Polymer properties–light scattering and osmometry
Elemental microanalysis of organic compounds
Complexometric titrations
Liquid chromatography
Gas chromatography
Nuclear magnetic resonance
Atomic absorption and flame spectrophotometry
Mass spectrometry

In addition, a discussion of instrumental design parameters (electrical, electronic, optical, and kinematic) will be included when these areas are easily related to the topic of interest.

All theory and techniques will be thoroughly integrated with practical lecture demonstrations. Time and facilities will be made available for those who desire to repeat the demonstrations or to familiarize themselves with the manipulative techniques involved. Although this is not mandatory, it will be encouraged by the staff.

Program

The symposium will extend over one week, beginning Monday, July 11, 1966, and ending at noon Saturday, July 16, 1966. Morning sessions will be three hours in

length, beginning at 8:30 a.m. The afternoon session will begin at 1:30 p.m. and will last until 4:30 p.m. In addition, evening sessions for individuals and groups will be scheduled as the necessity or desire arises.

Each section will be limited to twenty-five or thirty participants so that individual needs can be met effectively. More than one section will be scheduled, if needed.

Staff

The symposium will be conducted by various members of the Chemistry Department faculty. Each will discuss those areas with which he is competent to speak through active research programs. Specialists will be invited to attend this symposium and to present the latest information in their subject. The staff will be available for a limited amount of time to discuss problems of an individual nature.

Eligibility

Since the greatest benefit can be gained from this symposium by persons in supervisory positions, it is strongly urged that personnel in this category be given primary consideration by the approving authority. The only prerequisite for attendance at the symposium is a bachelors degree in chemistry (or an allied field) or equivalent industrial experience. No limitation has been set on the number of participants who may wish to attend from one concern.

Costs

Texts, allied materials, and expendable supplies are included in the symposium fee of $195.00. For those who desire it, living accommodations will be made available on the Lehigh campus at $3–4 per night.

All inquiries regarding this symposium and requests for application forms should be addressed to:

Prof. ROBERT S. SPRAGUE
Department of Chemistry
Lehigh University
Bethlehem, Pa.
U.S.A.

CONFERENCE ON TRACE CHARACTERISATION, CHEMICAL AND PHYSICAL

This conference will take place in Washington, D.C., from October 3rd to 7th, 1966.

For further information contact Dr. W. W. MEINKE, Analytical Chemistry Division, National Bureau of Standards, Washington, D.C. 20234, U.S.A.

SYMPOSIUM ON GEL FILTRATION

The Istituto Superiore di Sanità is organizing a one-day Symposium on Gel Filtration, to be held on September 24th, 1966 (Viale Regina Elena 299, Roma, Italy).

The morning session will consist of three main lectures:

TH. WIELAND — Some recent developments in gel chromatography, with special reference to thin layers

H. BENNICH — Gel filtration and its application to biochemical separation problems

J. C. JANSON — Adsorption phenomena on Sephadex.

Original papers are invited for the afternoon session. Registration is free.

The lectures and papers will be published together after the Symposium.

For registration and information please write to: Prof. F. DENTICE, Istituto Superiore di Sanità, Viale Regina Elena 299, Roma, Italy.

PROJECTED AMERICAN CHEMICAL SOCIETY DIVISIONAL PROGRAMMES

Topics for ACS meetings for the Autumn 1966 (September 11th–16th, New York) and Spring 1967 (April 9th–14th, Miami Beach) include:

New York

Inorganic Ion Exchange, honouring KURT A. KRAUS, recipient of the 1965 ACS Award in Chromatography and Electrophoresis. Invited papers only. For further information contact Dr. F. NELSON, Oak Ridge National Laboratories, P.O. Box X, Oak Ridge, Tenn. 37831, U.S.A.

Miami Beach

Ion Exchange in Carbohydrate Chemistry. Invited papers only.

Erratum

J. Chromatog., 21 (1966) 366

Announcement of the Fourth International Symposium on Chromatography and Electrophoresis:

4th line from the bottom should read "The official languages at the symposium will be French, Dutch, English and German".

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

KELLER-SCHIERLEIN, W.: (Paper chromatography and the possibilities of its use in investigation of pigments and paints). *Schriftenreihe Forschgemeinsch. Schweiz. Lackfabrikanten*, (1963) 163–176; *C.A.*, 63 (1965) 4511d.

WOHLLEBEN, G.: Ausgewählte Kapitel der Chromatographie in der Drogenforschung. *Mitt. Deut. Pharm. Ges., Arch. Pharm.*, 35 (1965) 217–227 — PC and TLC.

3. TECHNIQUES I

IGLOY, M.: (Determination of the isoelectric point of amphoteric electrolytes by pH-chromatography). *Magy. Kem. Lapja*, 20 (1965) 166–167; *C.A.*, 63 (1965) 2850g.

KASATKIN, Y. N. AND FEDOROV, N. A.: (Detection of spots of compounds absorbing at 254–260 mμ on chromatograms and electropherograms (photography in ultraviolet light)). *Byul. Eksperim. Biol. i Med.*, 59, No. 6 (1965) 121–124; *C.A.*, 63 (1965) 8716h.

MAO, C. H.: Circular paper chromatography. *Hua Hsueh Tung Pao*, No. 1 (1965) 37–41; *C.A.*, 63 (1965) 3595c — a review.

MILBORROW, B. V.: A simple apparatus for holding paper chromatograms during equilibration and development. *J. Chromatog.*, 20 (1965) 180–181.

SADINI, V.: (Devices for the transfer of solutions on chromatographic paper). *Chimica (Milan)*, 37 (1961) 456–458; *C.A.*, 63 (1965) 2634d.

See also TLC section.

4. TECHNIQUES II

Systematic analysis, automation and preparative-scale paper chromatography

MACLAURIN, D. J., CROSSMAN, J. K. AND GREEN, J. W.: Peelstrip technic for monitoring preparative paper chromatography. *Chemist-Analyst*, 54 (1965) 118.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

GRIMMER, G. AND HILDEBRANDT, A.: Kohlenwasserstoffe in der Umgebung des Menschen. I. Eine Methode zur simultanen Bestimmung von dreizehn polycyclischen Kohlenwasserstoffen. *J. Chromatog.*, 20 (1965) 89–99.

6. ALCOHOLS

EDELSON, J., SCHLOSSER, A. AND DOUGLAS, J. F.: Structure of hydroxycarisoprodol, the metabolite of carisoprodol. *Biochem. Pharmacol.*, 14 (1965) 901–902; *C.A.*, 63 (1965) 4160c.

ELLIOTT, T. H., TAO, R. C. C. AND WILLIAMS, R. T.: Stereochemical aspects of the metabolism of the isomeric methylcyclohexanols and methylcyclohexanones. *Biochem. J.*, 95 (1965) 59–69.

LUK'YANOV, V. B. AND GAVRICHEV, V. S.: (The use of dispersion analysis for establishing paper chromatographic conditions for analysis of aliphatic alcohols and aldehydes). *Vestn. Mosk. Univ., Ser. II, Khim.*, 20, No. 1 (1965) 25–30; *C.A.*, 63 (1965) 1205f.

7. PHENOLS

BICKOFF, E. M., LIVINGSTON, A. L. AND GUGGOLZ, J.: Isolation of phenolic compounds from ladino clover. *J. Agr. Food Chem.*, 13 (1965) 151–155; *C.A.*, 62 (1965) 12156g.

RIMPLER, H. AND HÄNSEL, R.: Zwei neue Chalkonpigmente aus *Helichrysum bracteatum* (Vent.) Willd. *Arch. Pharm.*, 298 (1965) 838–847 — PC and TLC.

See also TLC section.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

BÉZANGER-BEAUQUESNE, L., GUILBERT, N. AND DENECK, D.: Recherches sur l'*Erythroxylon laurifolium* Lam. de la Réunion. *Ann. Pharm. Franc.*, 23 (1965) 377–384.

DREWES, S. E. AND ROUX, D. G.: Condensed tannins. Optically active diastereoisomers of (+)-mollisacacidin by epimerization. *Biochem. J.*, 94 (1965) 482–487.

GILL, S.: (Chemical composition of *Trifolium arvense*. IV. Separation and identification of kempferol 3-glycoside). *Acta Polon. Pharm.*, 21 (1964) 287–290; *C.A.*, 63 (1965) 433d.

ORLOV, YU. E. AND DZYUBA, N. P.: (A new method of estimation of khellin in raw materials by paper chromatographic resolution and polarographic estimation). *Molekul. Khromatog., Akad. Nauk SSSR, Inst. Fiz. Khim.*, (1964) 158–162; *C.A.*, 62 (1965) 12132f.

ZAPESOCHNAYA, G. G. AND BANKOVSKIĬ, A. I.: (Plant polyphenols. I. Examination of chemical constituents of the leaves of *Rhododendron aureum* Georgi). *Khim. Prirodn. Soedin., Akad. Nauk UzSSR*, 1 (1965) 289–292.

9. OXO COMPOUNDS

DIPLOCK, A. T., GREEN, J. AND BUNYAN, J.: Vitamin A and isoprenoid synthesis in the rat. *Biochem. J.*, 95 (1965) 138–143.

DOKUNIKHIN, N. S. AND KOLOKOLOV, B. N.: (Paper chromatography of anthraquinone derivatives. Chloroanthraquinones). *Zh. Analit. Khim.*, 20 (1965) 398–400; *C.A.*, 63 (1965) 1205e.

GATENBECK, S. AND BENTLEY, R.: Naphthoquinone biosynthesis in moulds: the mechanism for formation of javanicin. *Biochem. J.*, 94 (1965) 478–481.

HRDLIČKA, J. AND POKORNÝ, J.: (Formation of volatile compounds during the autoxidation of lipids. II. Volatile carbonyl compounds in autoxidized fats). *Sb. Vysoke Skoly Chem.-Technol. Praze, Potravinarske Technol.*, 7 (1963, publ. 1964) 113–124; *C.A.*, 63 (1965) 5912h.

ROTHE, M.: (Paper chromatographic identification of some bread flavor carbonyl compounds). *Wiss. Veröffentl. Deut. Ges. Ernährung*, 10 (1963) 128–135; *C.A.*, 62 (1965) 15343g.

SASAKI, K.: The volatile components in sake. I. Neutral carbonyl compounds. *Nippon Nogei Kagaku Kaishi*, 38 (1964) 309–313; *C.A.*, 63 (1965) 3585a.

10. CARBOHYDRATES

ARKHIPOVICH, N. A., ZELENINA, L. M. AND KVITA, S. N.: (Chromatographic analysis of sugar mixtures in the products of the sugar industry). *Sakharn. Prom.*, 39, No. 3 (1965) 37–43; *C.A.*, 63 (1965) 775g.

BOGS, U. AND BORMANN, G.: (Paper chromatography of calcium gluconate solutions). *Pharm. Zentralhalle*, 104 (1965) 292–294; *C.A.*, 63 (1965) 5450g.

BOUVENG, H. O.: Polysaccharides in pollen. II. The xylogalacturonan from mountain pine (*Pinus mugo* Turra) pollen. *Acta Chem. Scand.*, 19 (1965) 953–963.

BOUVENG, H. O., BREMNER, J. AND LINDBERG, B.: A mannan elaborated by a *Scarcina* species. *Acta Chem. Scand.*, 19 (1965) 967–973 — PC and TLC.

BURTON, J. S., OVEREND, W. G. AND WILLIAMS, N. R.: Branched-chain sugars. III. The introduction of branching into methyl-3,4-O-isopropylidene-3-L-arabinose and the synthesis of L-hamamelose. *J. Chem. Soc.*, (1965) 3433–3445.

CALDWELL, R. C. AND PIGMAN, W.: The carbohydrates of human submaxillary glycoproteins in secretors and non-secretors of blood group substances. *Biochim. Biophys. Acta*, 101 (1965) 157–165.

DROŻDŻ, B. AND KOWALEWSKI, Z.: Selective detection of various types of sugars present in cardiac heterosides. *J. Chromatog.*, 20 (1965) 188.

DUFF, R. B.: The occurrence of apiose in *Lemna* (duckweed) and other angiosperms. *Biochem. J.*, 94 (1965) 768–772.

DUTTON, G. G. S. AND UNRAU, A. M.: Some applications of paper and vapour-phase chromatography to polysaccharides. Model studies on a synthetic D-mannan. *J. Chromatog.*, 20 (1965) 78–88.

FEAST, A. A. J., LINDBERG, B. AND THEANDER, O.: Studies on the D-glucosaccharinic acids. *Acta Chem. Scand.*, 19 (1965) 1127–1134 — PC and TLC.

HALLER, H.: (Mellituria. A paper-chromatographic study). *Med. Welt*, (1965) 206–210 and 258–261; *C.A.*, 62 (1965) 15216e.

HIRST, E. L., REES, D. A. AND RICHARDSON, N. G.: Seed polysaccharides and their role in germination. A survey of the polysaccharides component of mustard seeds with special reference to the embryos. *Biochem. J.*, 95 (1965) 453–458.

HUTTUNEN, J. K. AND MIETTINEN, T. A.: Isolation of neuraminlactose from human male urine. *Acta Chem. Scand.*, 19 (1965) 1486–1488.

JAFFÉ, G. W. AND HANNIG, K.: Fractionation of proteins from kidney beans (*Phaseolus vulgaris*). *Arch. Biochem. Biophys.*, 109 (1965) 80–91.

KNOX, K. W.: Enzymic synthesis of O-β-D-glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose. *Biochem. J.*, 94 (1965) 534–535.

KNOX, K. W. AND HALL, E. A.: The isolation of oligosaccharides from the cell-wall polysaccharide of *Lactobacillus casei*, serological group C. *Biochem. J.*, 94 (1965) 525–533.

MICHEEL, F. AND BÖHM, R.: Neuartige Isomerisierungen von Zuckerderivaten. III. Zum Reaktionsmechanismus der Bildung von Heptaacetyl-al-DL-hexosen aus Derivaten der D-Galaktose, D-Glucose und D-Mannose. *Chem. Ber.*, 98 (1965) 1659–1667.

MURAKAMI, M. AND EYLAR, E. H.: β-(N-Acetylglucosamine)-N-glycosidase: an enzyme which catalyzes the hydrolysis of 1-β-aspartyl-2-acetamido-1,2-dideoxy-D-glucosylamine. *J. Biol. Chem.*, 240 (1965) 556–558.

PUSZTAI, A.: A study of the glucosamine-containing constituents of the seeds of kidney beans (*Phaseolus vulgaris*). *Biochem. J.*, 94 (1965) 604–610.

SCHNEIDER, F., EMMERICH, A., REICHEL, C. AND SENDOEKMEN, N.: (Determination of raffinose in beet molasses. Comparison among several methods). *Zucker*, 18 (1965) 286–292; *C.A.*, 63 (1965) 4499g.

SCHNEIDER, F. AND GEYER, H. U.: (Surface-active sugar derivatives. II. Glycosylamines and amadori compounds of long-chain aliphatic amines). *Staerke*, 16 (1964) 309–313; *C.A.*, 63 (1965) 3028a.

STANIMIROVIC, S. G., STANIMIROVIC, D. L. AND NASTIC, D. R.: (Determination of sorbitol). *Glasnik Hem. Drustva, Beograd*, 28 (1963) 377–382; *C.A.*, 63 (1965) 2854f.

STARY, Z., WARDI, A. H., TURNER, D. L. AND ALLEN, W. S.: Arabinose as a mucopolysaccharide component in human and animal brain tissue. *Arch. Biochem. Biophys.*, 110 (1965) 388–394.

STROH, H. H., ARNOLD, A. AND SCHARNOW, H. G.: Über die Reaktionsprodukte des Hydrazins mit Zuckern. *Chem. Ber.*, 98 (1965) 1404–1410 — R_F values of 11 sugar azines and 7 hydrazones.

STROH, H. H. AND SCHARNOW, H. G.: Über die Reaktion von Alkyl- und Aralkylhydrazinen mit Zuckern. *Chem. Ber.*, 98 (1965) 1588–1597 — R_F values of 53 sugar hydrazones.

SZENDEI, K.: (Carbohydrates in drugs. I. Qualitative paper-chromatographic analysis of the free mono- and oligosaccharides of some drugs). *Sci. Pharm.*, 33 (1965) 6–16; *C.A.*, 63 (1965) 2851c.

WALKER, G. J.: A transglucosylase of *Streptococcus bovis*. *Biochem. J.*, 94 (1965) 299–308.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

ADAMS, J. B.: Studies on the mucin derived from human colloid breast carcinoma. *Biochem. J.*, 94 (1965) 368–377.

BENK, E. AND TREIBER, H.: (Detection of the bitter-principle drug of artichoke leaves in preparations). *Riechstoffe Aromen*, 13 (1963) 162–163; *C.A.*, 63 (1965) 1655c.

DAWKINS, P. D. AND DICKENS, F.: The oxidation of D- and L-glycerate by rat liver. *Biochem. J.*, 94 (1965) 353–367.

EVANS, W. C., FERNLEY, H. N. AND GRIFFITHS, E.: Oxidative metabolism of phenanthrene and anthracene by soil pseudomonas. The ring-fission mechanism. *Biochem. J.*, 95 (1965) 819–831.

HASHMI, M. H., HUDA, K. Q. AND ELAHI, M.: Circular paper chromatography of critical pairs of higher fatty acids. *Pakistan J. Sci. Res.*, 16, No. 2 (1964) 78–79; *C.A.*, 63 (1965) 3178g.

HOCKADAY, T. D. R., FREDERICK, E. W., CLAYTON, J. E. AND SMITH, L. H.: Studies on primary hyperoxaluria. II. Urinary oxalate, glycolate and glyoxalate measurement by isotope dilution methods. *J. Lab. Clin. Med.*, 65 (1965) 677–687.

NICHIFORESCO, E. AND COUCOU, V.: Sur le dosage des *o*-dihydroxyphénols de type acide caféique présents dans les feuilles d'Artichaut (*Cynara scolymus* L.). *Ann. Pharm. Franc.*, 23 (1965) 419–427.

PUSKAREVS, I.: (Chromatographic method for quantitative determination of volatile fatty acids in biological samples. Volatile fatty acids in perienteric fluid of *Ascaris suum*). *Latvijas PSR Zinatnu Akad. Vestis*, No. 3 (1965) 93–99; *C.A.*, 63 (1965) 6007d.

SADYKOV, A. S., TURULOV, A. V., BURTMAN, B. G. AND ASRIYANTS, B. M.: (*Aspergillus flavus* as a producer of kojic acid). *Uzbeksk. Biol. Zh.*, 9, No. 2 (1965) 21–23; *C.A.*, 63 (1965) 3580g.

SMITH, R. M. AND OSBORNE-WHITE, W. S.: Metabolism of propionate by sheep liver. Oxidation of propionate by homogenates. *Biochem. J.*, 95 (1965) 411–422.

VIALA, A., BASTIA, B. AND STANISLAS, E.: (Organic acids of *Buxus*. I. Nonvolatile organic acids from leaves of *Buxus balearica*. II. Nonvolatile organic acids from the leaves of *Buxus sempervirens*. III. Volatile organic acids from leaves of *Buxus balearica* and *Buxus sempervirens*). *Trav. Soc. Pharm. Montpellier*, 22 (1962) 108–119; *C.A.*, 62 (1965) 15071d.

See also TLC section.

11b. Lipids and their constituents

CARTER, H. E., JOHNSON, P. AND WEBER, E. J.: Glycolipids. *Ann. Rev. Biochem.*, 34 (1965) 109–137 — many references to PC and TLC methods.

DE GRAEFF, J., DEMPSEY, E. F., LAMEYER, L. D. F. AND LEAF, A.: Phospholipids and active sodium transport in toad bladder. *Biochim. Biophys. Acta*, 106 (1965) 155–170.

GALE, E. F. AND FOLKES, J. P.: The incorporation of glycerol and lysine into the lipid fraction of *Staphylococcus aureus*. *Biochem. J.*, 94 (1965) 390–400.

SHELTAWY, A.: Dietary alterations of the mitochondrial lipid pattern. *Biochem. J.*, 95 (1965) 561–567.

TARLOV, A. R. AND KENNEDY, E. P.: The β-galactoside permease system and the metabolism of phospholipids in *Escherichia coli*. *J. Biol. Chem.*, 240 (1965) 49–53.

WAJDA, M.: Lipid composition of human bone marrow. *Biochem. J.*, 95 (1965) 252–255.

See also TLC section.

13. STEROIDS

BIRCHALL, K. AND MITCHELL, F. L.: The identification of 21-hydroxypregnenolone in the urine of newborn infants. *Steroids*, 6 (1965) 427–436 — PC and TLC.

DOMINGUEZ, O. V.: The presence of two interconvertible forms of 18-hydroxy-11-deoxycorticosterone. *Steroids*, (1965) Suppl. II, 29–49.

HOLCENBERG, J. S. AND ROSEN, W. S.: Enzymatic sulfation of steroids by bovine tissues. *Arch. Biochem. Biophys.*, 110 (1965) 551–557.

JAMES, F. AND FOTHERBY, K.: A method for the estimation of 6-oxygenated metabolites of progesterone in urine. *Biochem. J.*, 95 (1965) 459–465.

MCKENNA, J. AND RIPPON, A. E.: The extraction of plasma 3-hydroxy-17-oxosteroid sulphates and the measurement of the constituent dehydroepiandrosterone sulphate and androsterone sulphate. *Biochem. J.*, 95 (1965) 101–117.

MITROPOULOS, K. A. AND MYANT, N. B.: The metabolism of cholesterol in the presence of liver mitochondria from normal and thyroxine-treated rats. *Biochem. J.*, 94 (1965) 594–603 — PC and TLC.

PASQUALINI, J. R., UHRICH, F. AND JAYLE, M. F.: Contribution to the study of the structure of the aldosterone-conjugate isolated from human urine. *Biochim. Biophys. Acta*, 104 (1965) 515–523.

See also TLC section.

14. STEROID GLYCOSIDES AND SAPONINS

KAISER, F.: Glucodigifucosid, Neo-glucodigifucosid und Digitoxigenin-allo-methylosid aus den Blättern von *Digitalis lanata* Ehrh. *Experientia*, 21 (1965) 575.

KEMERTELUDZE, E. P.: (Cardiac glycosides of *Digitalis ciliata*. I. Paper chromatographic investigation of *Digitalis ciliata* leaves. Digitoxin from *Digitalis ciliata*). *Med. Prom. SSSR*, 19, No. 4 (1965) 22-27; *C.A.*, 63 (1965) 1653g.

MADAEVA, O. S. AND STEPANOVA, L. F.: (Mixtures of steroid sapogenins in solasodine). *Med. Prom. SSSR*, 19, No. 3 (1965) 49–51; *C.A.*, 63 (1965) 2852d.

POLÁKOVÁ, A. AND ČEKAN, Z.: (Isolation and structure of cardenolides from *Adonis vernalis*). *Cesk. Farm.*, 14 (1965) 307–314 — PC and TLC.

SEGEL, K. H.: (Digitoxigenin 3β-hemisuccinate and two of its esters). *J. Prakt. Chem.*, 27 (1965) 225–230; *C.A.*, 63 (1965) 18311f.

16. NITRO AND NITROSO COMPOUNDS

KEMULA, W. AND GEISLER, J.: (Chromato-polarographic studies. XIX. Dipole moments of isomers of aromatic nitro compounds as a criterion for the possibility of chromatographic separation in the system of two liquid phases). *Roczniki Chem.*, 38 (1964) 1691–1698; *C.A.*, 63 (1965) 4g.

MATTHEWS, R. W.: Use of diethylamine in the paper chromatographic separation of isomeric mononitrophenols. *J. Chromatog.*, 20 (1965) 190–191 — R_F values and detection reactions of 15 compounds.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BANNARD, R. A. B. AND CASSELMAN, A. A.: (Über die Papierchromatographie einiger Aminhydrochloride und -acetate in sauren Laufmittelsystemen). *Can. J. Chem.*, 43 (1965) 395–404; from *Z. Anal. Chem.*, 214 (1965) 376.

HASHIMOTO, S. AND SUNAMOTO, J.: Alkaline sulfide reductions of aromatic nitro compounds. III. Disproportionation of hydrazobenzene in the benzidine rearrangement. *Kogyo Kagaku Zasshi*, 67 (1964) 2090–2092; *C.A.*, 63 (1965) 4119h.

HRDLIČKA, J. AND JANÍČEK, G.: (Compounds affecting organoleptic properties of protein hydrolyzates. I. Amines and carbonyl derivatives in the protein hydrolyzates Vitana). *Sb. Vysoke Skoly Chem.-Technol. Praze, Potravinarske Technol.*, 7 (1963, Pub. 1964) 257–263; *C.A.*, 63 (1965) 6249c.

PINTER, I., KRAMER, M. AND KLEEBERG, J.: (Detection and determination of *p*-phenylenediamine in the presence of *p*-tolylenediamine in hair dyes). *Parfuem. Kosmetik*, 46, No. 3 (1965) 61–64; *C.A.*, 63 (1965) 1652d.

18. AMINO ACIDS

BARNABY, C. F., DAVIDSON, A. M. AND PLASKETT, L. G.: Intrathyroidal iodine metabolism in the rat. The influence of diet and the administration of thyroid-stimulating hormone. *Biochem. J.*, 95 (1965) 811–818.

BARNSLEY, E. A. AND YOUNG, L.: Biochemical studies of toxic agents. The metabolism of iodomethane. *Biochem. J.*, 95 (1965) 77–81.

BESTAGNO, M., CAMUZZINI, G. F., TANER, F. AND PERRONI, G. L.: (Evaluation of free serum thyroxine). *Arch. "E. Maragliano" Patol. Clin.*, 21 (1965) 115–127; *C.A.*, 63 (1965) 3251a.

BUESS, C. M., GIUDICI, T., KHARASCH, N., KING, W., LAWSON, D. D. AND SAHA, N. N.: The synthesis of thyromimetic substances and potential inhibitors of thyroxine. *J. Med. Chem.*, 8 (1965) 469–474; *C.A.*, 63 (1965) 4382g.

CRADDOCK, V. M.: Reaction of carcinogen dimethylnitrosamine with proteins and with thiol compounds in the intact animal. *Biochem. J.*, 94 (1965) 323–330.

DAS, J. M.: Free amino acids and carotenes in the leaves of *Moringa oleifera*. *Current Sci. (India)*, 34 (1965) 374–375; *C.A.*, 63 (1965) 8728e.

GAITONDE, M. K., DAHL, D. R. AND ELLIOTT, K. A. C.: Entry of glucose carbon into amino acids of rat brain and liver *in vivo* after injection of uniformly ^{14}C-labelled glucose. *Biochem. J.*, 94 (1965) 345–352.

GEIPEL, H., GLOEDE, J., HILGETAG, K. P. AND GROSS, H.: Über α-Aminosäuren. I. Eine einfache Synthese für β-Hydroxy-α-aminosäuren. *Chem. Ber.*, 98 (1965) 1677–1680 — R_F values for 10 unusual amino acids.

GELDMACHER-MALLINCKRODT, M. AND SOELLNER, H.: (Determination and classification of urine on the basis of free amino acids). *Arch. Kriminol.*, 135 (1965) 48–56; *C.A.*, 63 (1965) 6099f.

KRIVTSOV, V. F. AND STEPANOV, V. M.: (3-Methyl-2-thiohydantoins of amino acids. II. Synthesis and properties of 3-methyl-2-thiohydantoins of heterocyclic and N-methylated amino acids, monoamidodicarboxylic acids and their amides). *Zh. Obshch. Khim.*, 35 (1965) 556-559; *C.A.*, 63 (1965) 669e.

KUME, Y.: Improvements in quantitative paper chromatography of amino acids in foods. I. Research on procedure of development. *Nippon Nogei Kagaku Kaishi*, 37 (1963) 196–200; *C.A.*, 62 (1965) 12367h.

KUME, Y.: Improvements in quantitative paper chromatography of amino acids in foods. II. Selection of solvent systems. *Nippon Nogei Kagaku Kaishi*, 37 (1963) 201–207; *C.A.*, 62 (1965) 12367h.

LINDSTEDT, G. AND LINDSTEDT, S.: Studies on the biosynthesis of carnitine. *J. Biol. Chem.* 240 (1965) 316–321 — PC and TLC.

PERKINS, H. R.: The action of hot formamide on bacterial cell walls. *Biochem. J.*, 95 (1965) 876–882 — N-formylamino acids.

SAARIVIRTA, M. AND VIRTANEN, A. I.: Homoserine and its lactone in pea seedlings. *Acta Chem. Scand.*, 19 (1965) 1008–1009.

SANYAL, S. N.: Quantitative circular papergram of lysine in drugs. *Indian J. Pharm.*, 27, No. 5 (1965) 149–150; *C.A.*, 63 (1965) 2853e.

SODA, K., MARBLE, S. J., COLES, N. W. AND MEISTER, A.: Reduction of polylysyl soluble RNA to polylysyl lysinol by lithium borohydride. *Biochim. Biophys. Acta*, 103 (1965) 534–536.

SOLBERG, Y. J.: Improved two-dimensional paper chromatography of amino acids and some related compounds. *Acta Chem. Scand.*, 19 (1965) 1269–1270 — map of 41 amino acids.

VIRUPAKSHA, T. K. AND SHRIFT, A.: Biochemical differences between selenium accumulator and non-accumulator *Astragalus* species. *Biochim. Biophys. Acta*, 107 (1965) 69–80.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

CLAMP, J. R. AND HOUGH, L.: Studies on a glycopeptide from ovalbumin. *Biochem. J.*, 94 (1965) 502–508.

DOOLITTLE, R. F.: Characterization of lamprey fibrinopeptides. *Biochem. J.*, 94 (1965) 742–750.

DRABAREK, S. AND DU VIGNEAUD, V.: 2-D-Tyrosine-oxytocin and 2-D-tyrosine-deamino-oxytocin, diastereoisomers of oxytocin and deamino-oxytocin. *J. Am. Chem. Soc.*, 87 (1965) 3974–3978 — PC and TLC.

ELLIOTT, D. F. AND LEWIS, G. P.: Methionyl-lysyl-bradykinin, a new kinin from ox blood. *Biochem. J.*, 95 (1965) 437–447.

FOLK, J. E. AND COLE, P. W.: Chymotrypsin C. II. Enzymatic specificity toward several polypeptides. *J. Biol. Chem.*, 240 (1965) 193–197.

HEATHCOTE, J. G. AND WASHINGTON, R. J.: A dialysable peptide in normal gastric juice. *Biochem. J.*, 95 (1965) 24P.
JONES, J. G., SCOTLAND, S. M. AND DODGSON, K. S.: The biological sulphation of L-tyrosylglycine. *Biochem. J.*, 95 (1965) 17C–18C.
KHOSLA, M. C., CHATURVEDI, N. C., GARG, H. G. AND ANAN, N.: Peptides related to bacterial cell wall components. Synthesis of L-alanyl-D-(α- and γ-)-glutamyl-L-lysyl-D-alanyl-D-alanine and partially protected L-glutamyl isomer. *Indian J. Chem.*, 3, No. 3 (1965) 111–117; *C.A.*, 63 (1965) 4389b.
MANNING, M. AND DU VIGNEAUD, V.: 6-Hemi-D-cystine-oxytocin, a diastereoisomer of the posterior pituitary hormone oxytocin. *J. Am. Chem. Soc.*, 87 (1965) 3978–3982.
RICHMOND, M. H.: Wild-type variants of exopenicillinase from *Staphylococcus aureus*. *Biochem. J.*, 94 (1965) 584–593.
SATAKA, M., OKUYAMA, T., ISHIHARA, K. AND SCHMID, K.: The carbohydrate-polypeptide linkages, the amino acid sequences of the peptides adjacent to some of these bonds, and the composition and size of the carbohydrate units of α-acid glycoprotein. *Biochem. J.*, 95 (1965) 749–757.
SEN, A. B. AND YAJNIK, M. S.: Synthesis of peptides of β-amino acids. *J. Indian Chem. Soc.*, 42, No. 3 (1965) 145–148; *C.A.*, 63 (1965) 4384d.
SIMS, P. AND GROVER, P. L.: Conjugations with glutathione. The enzymic conjugation of some chlorocyclohexenes. *Biochem. J.*, 95 (1965) 156–160.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BISHOP, D. H. L. AND BRADLEY, D. E.: Determination of base ratios of six ribonucleic acid bacteriophages specific to *Escherichia coli*. *Biochem. J.*, 95 (1965) 82–93.
BROOM, A. D. AND ROBINS, R. K.: Characterization of 1,3-dimethylxanthosine (9-β-D-ribofuranosyltheophylline). *J. Heterocyclic Chem.* 2 (1965) 108–109; *C.A.*, 63 (1965) 1856b.
CORY, J. G., SUHADOLNIK, R. J., RESNICK, B. AND RICH, M. A.: Incorporation of cordycepin (3′-deoxyadenosine) into ribonucleic acid and deoxyribonucleic acid of human tumor cells. *Biochim. Biophys. Acta*, 103 (1965) 646–653.
CRAMER, F., RHAESE, H. J., RITTNER, S. AND SCHEITT, K. H.: (Oligonucleotides. IV. Synthesis of dinucleoside phosphates of the ribose series. Uridylyl-(3′ → 5′)-nucleoside and adenyl-(3′ → 5′)-nucleoside). *Ann. Chem.*, 683 (1965) 199–211; *C.A.*, 63 (1965) 5724h.
FARINA, E. AND MULE, A.: (Chemical composition of cow sternal medulla). *Boll. Soc. Ital. Biol. Sper.*, 39 (1963) 1881–1884; *C.A.*, 62 (1965) 15154a.
GERSTER, J. F. AND ROBINS, R. K.: Purine nucleosides. X. The synthesis of certain naturally occurring 2-substituted amino-9-β-D-ribofuranosylpurin-6-(1H)-ones. (N^2-Substituted guanosines). *J. Am. Chem. Soc.*, 87 (1965) 3752–3759.
GRAV, H. J. AND SMELLIE, R. M. S.: Fractionation of thymidine phosphokinase, thymidine 5′-monophosphate phosphokinase and thymidine 5-di-phosphate phosphokinase in extracts of Landschutz ascites-tumour cells. *Biochem. J.*, 94 (1965) 518–524.
HOARD, D. E. AND OTT, D. G.: Conversion of mono- and oligodeoxyribonucleotides to 5′-triphosphates. *J. Am. Chem. Soc.*, 87 (1965) 1785–1788.
HOLMES, R. E. AND ROBINS, R. K.: Purine nucleosides. IX. The synthesis of 9-β-D-ribofuranosyl uric acid and other related 8-substituted purine ribonucleosides. *J. Am. Chem. Soc.*, 87 (1965) 1772–1776.
IRRGANG, K.: Zur Pharmakologie von 5-Aethyl-5-(3-hydroxyisoamyl)-barbitursäure, einem Stoffwechselprodukt der 5-Aethyl-5-isoamyl-barbitursäure. *Arzneimittel-Forsch.*, 15 (1965) 688–691.
JACOB, T. M. AND KHORANA, H. G.: Studies on polynucleotides. XLIV. The synthesis of dodecanucleotides containing the repeating trinucleotide sequence thymidylyl-(3′→ 5′)-thymidylyl (3′ → 5′)-deoxycytidine. *J. Am. Chem. Soc.*, 87 (1965) 2971–2981.
LETHAM, D. S.: Methods for detecting and distinguishing between purines and pyrimidines on paper chromatograms. *J. Chromatog.*, 20 (1965) 184–186.
LUNT, M. R. AND NEWTON, E. A.: Glucosylated nucleotide sequences from T-even bacteriophage deoxyribonucleic acids. *Biochem. J.*, 95 (1965) 717–723.
NARANG, S. A., JACOB, T. M. AND KHORÁNA, H. G.: Studies on polynucleotides. XLVI. The synthesis of hexanucleotides containing the repeating trinucleotide sequence deoxycytidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-deoxyadenosine and deoxyguanylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-deoxyadenosine. *J. Am. Chem. Soc.*, 87 (1965) 2988–2995.
NARANG, S. A. AND KHORANA, H. G.: Studies on polynucleotides. XLV. The synthesis of dodecanucleotides containing the repeating trinucleotide sequence thymidylyl-(3′ → 5′)-thymidylyl-(3′→ 5′)-deoxyinosine. *J. Am. Chem. Soc.*, 87 (1965) 2981–2988.

OHTSUKA, E., MOON, M. W. AND KHORANA, H. G.: Studies on polynucleotides. XLIII. The synthesis of deoxyribopolynucleotides containing repeating dinucleotide sequences. *J. Am. Chem. Soc.*, 87 (1965) 2956–2970.

ONDARZA, R. N.: Characterization of a nucleotide-peptide from rat liver. *Biochim. Biophys. Acta*, 107 (1965) 112–119.

WANG, N. C., SUN, T. AND JEN, S. H.: The separation and determination of ribomononucleotides by one-dimensional filter paper chromatography. *Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao*, 5 (1965) 55–63; *C.A.*, 63 (1965) 3296b.

22. ALKALOIDS

BUKOWIECKI, H. AND FURMANOWA, M.: (Chromatographic analysis of alkaloids obtained from Polish water lilies). *Acta Polon. Pharm.*, 21 (1964) 121–126; *C.A.*, 62 (1965) 12152b.

HEYDENREICH, K. AND PFEIFER, S.: Bractavin, ein neues Papaveralkaloid. *Pharmazie*, 20 (1965) 521.

KUCHENKOVA, M. A., YULDASHEV, P. KH. AND YUNUSOV, S. YU.: (Alkaloids of *Vinca erecta*). *Dokl. Akad. Nauk Uz. SSR*, 21, No. 11 (1964) 42–45; *C.A.*, 63 (1965) 4353f.

LAVENIR, R. AND PARIS, R. R.: Sur les alcaloïdes de la Chélidonine (*Chelidonium majus* L.). Réparation dans divers organes, isolement de la stylopine à partir des fruits. *Ann. Pharm. Franc.*, 23 (1965) 307–312 — PC and TLC.

MAJLÁT, P. AND BAYER, I.: Separation of cocaine, benzoylecgonine and ecgonine by paper chromatography. *J. Chromatog.*, 20 (1965) 187.

NIJLAND, M. M.: (Magnoflorine in opium). *Pharm. Weekblad*, 99 (1964) 1165; *C.A.*, 63 (1965) 2851a.

PARIS, R. R. AND COSSON, L.: (Biogenesis of *Datura* alkaloids. Ontogenetic variations in *Datura metel* determined by a chromatographic method). *Compt. Rend.*, 260 (1965) 3148–3151; *C.A.*, 62 (1965) 15068g.

SOCZEWINSKI, E. AND SZABELSKA, B.: Chromatography and extraction of some alkaloids in systems with a buffered aqueous phase. *Dissertationes Pharm.*, 17 (1965) 53–58; *C.A.*, 63 (1965) 5450a.

WACHSMUTH, H. AND VAN KOECKHOVEN, L.: (Color reaction of benzedrine. Qualitative and quantitative applications). *J. Pharm. Belg.*, 20 (1965) 58–60; *C.A.*, 63 (1965) 5453c — PC and TLC.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

DELVIGS, P., MCISAAC, W. M. AND TABORSKY, R. G.: The metabolism of 5-methoxytryptophol. *J. Biol. Chem.*, 240 (1965) 348–350.

GOPINATHAN, K. P., SIRSI, M. AND VAIDYANATHAN, C. S.: Nicotinamide-adenine dinucleotide glycohydrolase activity in experimental tuberculosis. *Biochem. J.*, 94 (1965) 446–451.

KAEMPE, B.: Interfering substances by determination of poisons in autopsy material. IV. Tryptamine. *Acta Pharm. Toxicol.*, 22 (1965) 126–130; *C.A.*, 62 (1965) 15059h.

KHALETSKII, A. M. AND MOLDAVER, B. L.: (Pyrazoline-3,5-dione drugs). *Materialy 1-go Vseros. S'ezda Farmatsevtov, Moscow: Med. Sb.*, (1962, Pub. 1964) 330–335; *C.A.*, 63 (1965) 5625f.

KVEDER, S. AND ISKIRIĆ, S.: 5-Hydroxytryptamine metabolism by the nuclear fraction of rat-liver homogenate. *Biochem. J.*, 94 (1965) 509–512.

NYBERG, W. H., NOELL, C. W. AND CHANG, C. C.: New heterocyclic ring system: pyrimido-(4,5-*b*)-diazepine. *J. Heterocyclic Chem.*, 2 (1965) 110–111; *C.A.*, 63 (1965) 1787a.

SEN, N. P. AND MCGEER, P. L.: Preparation and properties of some compounds of biological interest. *J. Chromatog.*, 20 (1965) 147–149 — R_F values of 13 compounds.

SUBBA RAO, P. V., JEGANNATHAN, N. S. AND VAIDYANTHAN, C. S.: Enzymic conversion of 3-hydroxyanthranilic acid into cinnabarinic acid by the nuclear fraction of rat liver. *Biochem. J.*, 95 (1965) 628–632.

24. ORGANIC SULPHUR COMPOUNDS

MIRO, P. AND DOMINGUEZ, J. J. G.: (Oxidation of lanthionine). *Bull. Inst. Textile France*, No. 116 (1965) 63–67; *C.A.*, 63 (1965) 1926h.

25. ORGANIC PHOSPHORUS COMPOUNDS

VIVES, J. P., MUNOZ, A., NAVECH, J. AND MATHIS, F.: Étude de quelques esters phosphoriques cycliques. 1. Préparation et hydrolyse. *Bull. Soc. Chim. France*, (1965) 2544–2552.

27. VITAMINS

KAWASAKI, C., TOMITA, I. AND NAGAYAMA, T.: Metabolism of thiamine and related compounds. VI. Metabolism and biological activity of benzenesulfonylthiamine disulfide. *J. Vitaminol.*, 10 (1964) 14–19; *C.A.*, 62 (1965) 12325f.

KAZIRO, Y., GROSSMAN, A. AND OCHOA, S.: Metabolism of propionic acid in animal tissues. XI. Further studies on crystalline propionyl coenzyme A carboxylase. *J. Biol. Chem.*, 240 (1965) 64–67.

LAKSHMANAN, M. R., JUNGALWALA, F. B. AND CAMA, H. R.: Metabolism and biological potency of 5,6-monoepoxy-vitamin A aldehyde in the rat. *Biochem. J.*, 95 (1965) 27–34 — PC and TLC.

LAVATE, W. V., DYER, J. R., SPRINGER, C. M. AND BENTLEY, R.: Studies on coenzyme Q. The isolation, characterization and general properties of a partly reduced coenzyme Q_{10} from *Penicillium stipitatum*. *J. Biol. Chem.*, 240 (1965) 524–531.

LEHMAN, K.: Trennung, Isolierung und Identifizierung von Stoffwechselprodukten des Triamterens. *Arzneimittel-Forsch.*, 15 (1965) 812–816.

MITRA, S. K., MANDAL, R. K. AND BURMA, D. P.: A biosynthetic method for preparing ^{35}S-labelled lipoic acid. *Biochim. Biophys. Acta*, 107 (1965) 131–133.

OLSON, R. E., DIALAMEH, G. H., BENTLEY, R., SPRINGER, C. M. AND RAMSEY, V. G.: Studies on coenzyme Q. Pattern of labelling in coenzyme Q_9 after administration of isotopic acetate and aromatic amino acids to rats. *J. Biol. Chem.*, 240 (1965) 514–523.

See also TLC section.

28. ANTIBIOTICS

ABRAHAM, E. P., NEWTON, G. G. F. AND HALE, C. W.: (Derivatives of cephalosporin C). *Ger. Pat.*, 1,190,465 (Cl. C 07*d*), April 8, 1965; *Brit. Pat.*, Appl. March 17 and Aug. 29, 1958, 6 pp.; *C.A.*, 63 (1965) 1798c.

BETINA, V.: (Systematic analysis of antibiotics using paper chromatography). *Sb. Prac Chem. Fak. SVŠT (Sloven. Vysokej Školy Techn.)*, (1964) 33–40; *C.A.*, 63 (1965) 2852b.

BOGDANOVA, N. P., KOTENKO, T. V., LOVALEVA, L. A. AND ROZANOVA, A. YU.: (A comparative study of streptothricin antibiotics by using paper and ion-exchange chromatography). *Materialy 2-oi Konf. Molodykh Uchenykh Leningr. Inst. Antibiotikov, Leningrad, Sb.*, 1964, pp. 15–17; *C.A.*, 63 (1965) 5450e.

MAKSIMOVA, T. S., KOVSHAROVA, I. N. AND PROSLYAKOVA, V. V.: (Early identification of echinomycin antibiotics and systematic position of their producers). *Antibiotiki*, 10 (1965) 298–304; *C.A.*, 63 (1965) 3577f.

TAYLOR, E. C. AND HENDESS, R. W.: Synthesis of pyrrolo(2,3-*d*)pyrimidines. The aglycone of toyocamycin. *J. Am. Chem. Soc.*, 87 (1965) 1995–2003.

29. INSECTICIDES AND OTHER PESTICIDES

BALIF, G. AND GROU, E.: (Analysis of small quantities of aldrin by means of paper chromatography). *Rev. Chim. (Bucharest)*, 16 (1965) 219–220; *C.A.*, 63 (1965) 8983b.

NEUBERT, P. AND HEINISCH, E.: (Über die quantitative Papierchromatographie von DDT, Hexachlorcyclohexan und Chlordan). *J. Prakt. Chem.*, [4], 26 (1964) 67–78; from *Z. Anal. Chem.*, 214 (1965) 399.

30. SYNTHETIC AND NATURAL DYES

FRANCK, B. AND ZIMMER, I.: Mutterkorn-Farbstoffe. VIII. Konstitution und Synthese des Clavorubins. *Chem. Ber.*, 98 (1965) 1514–1521 — PC and TLC.

KIEL, E. G. AND KUYPERS, G. H. A.: (Paper chromatography of dyes. IV. Vat dyes). *Tex*, 22 (1963) 162–165; *C.A.*, 63 (1965) 4421b.

KIEL, E. G. AND KUYPERS, G. H. A.: (Paper chromatography of dyes. V. Naphthols). *Tex*, 22 (1963) 377–379; *C.A.*, 63 (1965) 4421c.

KIEL, E. G. AND KUYPERS, G. H. A.: (Paper chromatography of dyes. VI. Fast bases). *Tex*, 22 (1963) 468–469; *C.A.*, 63 (1965) 4421d.

KIEL, E. G. AND KUYPERS, G. H. A.: (Paper chromatography of dyes. VII. Basic dyes). *Tex*, 22 (1963) 533–535; *C.A.*, 63 (1965) 4421d.

KIEL, E. G. AND KUYPERS, G. H. A.: (Paper chromatography of dyes. VIII. Chrome complex dyes). *Tex*, 22 (1963) 607–611; *C.A.*, 63 (1965) 4421d.

PELLERIN, F., GAUTIER, J. A. AND CONRARD, A. M.: Identification dans les préparations pharmaceutiques des colorants organiques de synthèse autorisés. *Ann. Pharm. Franc.*, 22 (1964) 621–627.

PERKAVAC, J. AND PERPAR, M.: (Paper chromatography of developer dyes). *Kem. Ind. (Zagreb)*, 14 (1965) 9–11; *C.A.*, 63 (1965) 4924g.

SANIELEVICI, Z. AND TEODORESCU, L.: (Substantive azo dyes, derivatives of urea). *Rev. Chim. (Bucharest)*, 16 (1965) 23–27; *C.A.*, 63 (1965) 3077g.

SILVA, C. M.: (Chromatographic study of coloring materials and its application to the study of inks). *Anales Fac. Quim. Farm., Univ. Chile*, 15 (1963, Publ. 1964) 189–192; *C.A.*, 63 (1965) 4512b — PC and TLC.

SUDA, Y. AND UJIGAWA, H.: Fundamental studies on dyeing with disperse dyes. VIII. The interaction between aminoanthraquinone-type disperse dyes and esters studied by paper chromatography from the viewpoint of α-CH...X-type hydrogen bonding. *Sen-i Gakkaishi*, 19 (1963) 563–568; *C.A.*, 63 (1965) 5806b.

SUDA, Y. AND UJIGAWA, H.: Fundamental studies on dyeing with disperse dyes. X. The interaction between aminoanthraquinone-type disperse dyes and amines studied by paper chromatography from the viewpoint of RN(R')H...X-dye type hydrogen bonding. *Sen-i Gakkaishi*, 20 (1964) 176–182; *C.A.*, 63 (1965) 5806d.

SUDA, Y. AND UJIGAWA, H.: Fundamental studies on dyeing with disperse dyes. XI. The interaction between aminoanthraquinone-type disperse dyes and alcohols studied by paper chromatography. *Sen-i Gakkaishi*, 20 (1964) 324–330; *C.A.*, 63 (1965) 5806f.

SUDA, Y. AND UJIGAWA, H.: Fundamental studies on dyeing with disperse dyes. XII. The interaction between aminoanthraquinone-type disperse dyes and phenols studied by paper chromatography. *Sen-i Gakkaishi*, 20 (1964) 617–623; *C.A.*, 63 (1965) 5806g.

SUDA, Y. AND UJIGAWA, H.: Fundamental studies on dyeing with disperse dyes. XIII. One suggestion concerning X of α-CH...X-dye type hydrogen bonding in disperse dyeing. *Sen-i Gakkaishi*, 20 (1964) 731–737; *C.A.*, 63 (1965) 5806h.

SUNADA, K. V. AND STANIER, R. Y.: Observations on the pathway of carotenoid synthesis in *Rhodopseudomonas. Biochim. Biophys. Acta*, 107 (1965) 38–43.

VERMA, M. R., PURI, V. D. AND RAI, J.: Dyes used in the manufacture of writing inks. II. Chromatography of dyes for use in writing fluids and tablets. *Chem. Age India*, 15 (1964) 1179–1182; *C.A.*, 63 (1965) 1994h.

VILLANUA, L., CARBALLIDO, A., OLMEDO, R. G. AND VALDEHITA, M. T.: (Synthetic food colors. IX. Characteristics, properties, spectrophotometry and chromatography of some water-soluble artificial dyes. *Anales Bromatol. (Madrid)*, 16 (1964) 377–394; *C.A.*, 63 (1965) 4423f.

31. PLASTICS AND THEIR INTERMEDIATES

CHENE, M., MOREL, D. AND DUBOURGEAT, M.: (Identification and determination of urea–formaldehyde and melamine–formaldehyde resins by chromatography). *Papeterie*, 87 (1965) 264–269; *C.A.*, 63 (1965) 3114h — PC and TLC.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

MASIAKOWSKI, J. AND PAWELCZYK, K.: (2,4-Dinitrophenylhydrazine in pharmaceutical analysis. I. Paper chromatography of the decomposition products of amidopyrine in aqueous solution). *Dissertationes Pharm.*, 17 (1965) 87–90; *C.A.*, 63 (1965) 4094g.

PREUSS, F. R. AND MAYER, E.: Zur Struktur von Ausscheidungsprodukten nach Gabe von 1-Äthinylcyclohexylcarbamat-(1). *Arch. Pharm.*, 298 (1965) 781–787.

PREUSS, F. R. AND MAYER, E.: Zum Nachweis von O-Acetyl-Derivaten nach Gabe von 1-Äthinylcyclohexylcarbamat-(1). *Arzneimittel-Forsch.*, 15 (1965) 747–750.

PREUSS, F. R. AND VOIGT, K. M.: Über die Vorstufen und die Bildung der Methylrubazonsäure nach Einnahme von Amidopyrin. *Arzneimittel-Forsch.*, 15 (1965) 741–747 — PC and TLC.

33. INORGANIC SUBSTANCES

CHANG, C. C. AND YANG, H. H.: Separation of rare earth elements by reversed-phase paper chromatography. The TBP–HNO_3–NH_4SCN system. *Hua Hsueh Hsueh Pao*, 31 (1965) 182–185; *C.A.*, 63 (1965) 3601g.

COVELLO, M. AND CIAMPA, G.: Quantitative analysis of lithium, potassium and caesium chlorides by paper chromatography and flame spectrophotometry. *J. Chromatog.*, 20 (1965) 201–204.

DE CARVALHO, R. A. G.: (Separation of Sc, Y, lanthanides, Th, Zr and U by electrophoresis and adsorption and paper partition chromatography). *Rev. Port. Quim.*, 6, No. 2 (1964) 57–62; *C.A.*, 63 (1965) 4924c.

KNOCH, W., MUJU, B. AND LAHR, H.: The separation of some fission products and actinides on paper treated with liquid anion exchanger. *J. Chromatog.*, 20 (1965) 122–130.

LEVER, R. R.: Semiquantitative chromatographic method for estimating gold. *Australasian Inst. Mining Met. Proc.*, No. 210 (1964) 139–156; *C.A.*, 63 (1965) 3601a.

ROBINSON, H. C.: The reduction of inorganic sulphate to inorganic sulphite in the small intestine of the rat. *Biochem. J.*, 94 (1965) 687–691.

ROMMEL, M. A. L.: Paper chromatography of phosphate species. *Univ. Microfilms (Ann Arbor, Mich.)*, Order No. 65-2342, 146 pp.; *Dissertation Abstr.*, 25 (1965) 5525; *C.A.*, 63 (1965) 4922d.

SHIMODA, S.: Comparison of the recovery of inorganic iodide by paper electrophoresis or chromatography. *Endocrinology*, 77 (1965) 401–405; *C.A.*, 63 (1965) 8712c.

YAMAZOE, F., YOSHIDA, N. AND TANAKA, F.: Separation of phosphates in compound fertilizers by paper chromatography. *Nippon Dojo-Hiryogaku Zasshi*, 35 (1964) 315–318; *C.A.*, 63 (1965) 9015c.

34. RADIOACTIVE AND OTHER ISOTOPIC COMPOUNDS

ALEEM, M. I. H.: Path of carbon and assimilatory power in chemosynthetic bacteria. I. *Nitrobacter agilis*. *Biochim. Biophys. Acta*, 107 (1965) 14–28.

ELLIOTT, T. H., TAO, R. C. C. AND WILLIAMS, R. T.: The metabolism of methylcyclohexane. *Biochem. J.*, 95 (1965) 70–76.

JOHNSON, M. J.: Optimum operation of recording rate meters for radioactive paper chromatograms. *J. Chromatog.*, 20 (1965) 100–106.

JONES, J. G. AND DODGSON, K. S.: Biosynthesis of L-tyrosine O-sulphate from the methyl and ethyl esters of L-tyrosine. *Biochem. J.*, 94 (1965) 331–336 — radioactive spots are scanned and peak areas weighed.

KLEIST, H.: (Radiochromatographic investigations of fulvoacids). *Acta Biol. Med. Ger.*, 13 (1964) 949–951; *C.A.*, 62 (1965) 14987g.

LOMONOSOV, I. I.: (Measuring paper chromatograms with tritium-tagged compounds). *USSR Pat.*, 169,866 (Cl. G Oln), March 17 (1965), Appl. March 28 (1964); *C.A.*, 63 (1965) 2392f.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

LETSINGER, R. L., RAMSAY, O. B. AND MCCAIN, J. H.: Photoinduced substitution. II. Substituent effects in nucleophilic displacement on substituted nitrobenzenes. *J. Am. Chem. Soc.*, 87 (1965) 2945–2953.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

GAREL, J. P.: Chromatographie en couche mince. B. Application en chimie organique. *Bull. Soc. Chim. France*, (1965) 1563–1587 — extensive tables of R_F values.

See also PC section.

2. FUNDAMENTALS, THEORY AND GENERAL

HULTIN, E.: Thin-layer chromatography of plant extracts. I. A solvent series based on dielectric constant. *Acta Chem. Scand.*, 19 (1965) 584–587.

3. TECHNIQUES I

ANDERSEN, K. K. AND MAY, G. S.: An inexpensive TLC tank. *J. Chem. Educ.*, 42 (1965) 596.

CLITHEROE, H. J. AND GERARDE, H. W.: Use of the unopette in thin-layer chromatography. *J. Chem. Educ.*, 42 (1965) 613.

CONTRACTOR, S. F.: A simple applicator for preparative thin-layer and paper chromatography. *J. Chromatog.*, 20 (1965) 182–183 — TLC and PC.

GRUENE, A.: (Thin-layer chromatography on cellulose plate). *Riechstoffe, Aromen, Koerperpflegemittel*, 15 (1965) 127–134; *C.A.*, 63 (1965) 4922e.

MATHIS, C.: Application de la chromatographie fonctionelle sur couche mince à l'identification des substances organiques. *Ann. Pharm. Franc.*, 23 (1965) 331–334.

OTA, M.: Removing and determining of a minute amount of iron in silica gel of thin-layer chromatographic use. *Japan Analyst*, 14 (1965) 945–949.

SAMUELS, S.: Technic and enclosure for rapid chromatographic spotting. *Chemist-Analyst*, 54 (1965) 122–123 — TLC and PC.

TAKITANI, S. AND MATSUDA, K.: Automatic apparatus for the preparation of thin-layer plates. *Japan Analyst*, 14 (1965) 846–849.

VAN SUMERE, C. F., WOLF, G., TEUCHY, H. AND KINT, J.: A new thin-layer method for phenolic substances and coumarins. *J. Chromatog.*, 20 (1965) 48–60 — steamed combined silica gel–cellulose plates; R_F values for 93 compounds.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

LAM, J. AND BERG, A.: Spectrophotometric determination of polycyclic aromatic hydrocarbons separated by thin-layer chromatography, and evaluation of the light sensitivity of hydrocarbon spots. *J. Chromatog.*, 20 (1965) 168–171.

WOLOVSKY, R.: Unsaturated macrocyclic compounds. XXXVII. Cyclooctadecahexaenetriyne and cyclooctadecapentaenetetrayne. Two new dehydro[18]annulenes. *J. Am. Chem. Soc.*, 87 (1965) 3638–3644.

7. PHENOLS

BARTON, G. M.: α,α′-Dipyridyl as a phenol-detecting reagent. *J. Chromatog.*, 20 (1965) 189.

BOYLAND, E. AND SIMS, P.: Metabolism of polycyclic compounds. The metabolism of 7,12-dimethylbenz(*a*)anthracene by rat-liver homogenates. *Biochem. J.*, 95 (1965) 780–787 — TLC and PC.

BOYLAND, E. AND SIMS, P.: Metabolism of polycyclic compounds. The metabolism of 9,10-epoxy-9,10-dihydrophenanthrene in rats. *Biochem. J.*, 95 (1965) 788–792.

ERNST, W.: Umwandlung und Ausscheidung von 2-Hydroxydiphenyl bei der Ratte. *Arzneimittel-Forsch.*, 15 (1965) 632–636.

MECHOULAM, R. AND GAONI, Y.: Hashish. IV. Isolation and structure of cannabinolic, cannabidiolic and cannabigerolic acids. *Tetrahedron*, 21 (1965) 1223–1229; *C.A.*, 63 (1965) 2919c.

SIMS, P.: Metabolism of polycyclic compounds. The metabolism of 1,4-epoxy-1,4-dihydronaphthalene in rats. *Biochem. J.*, 95 (1965) 608–611 — PC and TLC.

WRIGHT, A. S., CROWNE, R. S. AND HATHWAY, D. E.: The fate of 2,4,6-tri-(3′,5′-di-*tert.*-butyl-4′-hydroxybenzyl)-mesitylene (Ionox 330) in the dog and rat. *Biochem. J.*, 95 (1965) 98–104.

See also PC section.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

BEYRICH, T.: Die dünnschichtchromatographische Verteilung von Furocumarinen. 5. Mitt. über Furocumarine. *J. Chromatog.*, 20 (1965) 173–175 — R_F values for 10 compounds.

9. OXO COMPOUNDS

AUTERHOFF, H. AND KINSKY, G.: Die Wertbestimmung von Sennesblättern. *Arch. Pharm.*, 298 (1965) 810–814.

CAMP, B. J. AND O'BRIEN, F.: Separation of the semicarbazones of some common aldehydes by thin-layer chromatography. *J. Chromatog.*, 20 (1965) 178–180 — detection with phosphomolybdic acid.

CRAIG, J. C., MARY, N. Y. AND WOLF, L.: Tertiary amine oxide rearrangements. II. N-Benzyldimethylamine oxides as models for the demethylation of nicotine. *J. Org. Chem.*, 29 (1964) 2868–2870.

THRELFALL, D. R., GRIFFITHS, W. T. AND GOODWIN, T. W.: Isolation of two analogues of plastoquinone from senescent leaves of tobacco. *Biochim. Biophys. Acta*, 102 (1965) 614–618.

10. CARBOHYDRATES

CHIBA, S. AND SHIMOMURA, T.: Thin-layer chromatography of oligosaccharides. *Agr. Biol. Chem. (Tokyo)*, 29 (1965) 486–487; *C.A.*, 63 (1965) 6006f.

DUTTON, G. G. S., GIBNEY, K. B., REID, P. E. AND SLESSOR, K. N.: Monitoring of carbohydrate reactions by thin-layer chromatography. *J. Chromatog.*, 20 (1965) 163–165.

ERDAL, K.: (Thin-layer chromatography of carbohydrates). *Brygmesteren*, 21 (1964) 281–286; *C.A.*, 62 (1965) 12408h.

HIRASAKA, Y. AND UMEMOTO, K.: Structure of D-glucurodilactone. *Chem. Pharm. Bull. (Tokyo)*, 13 (1965) 325–329; *C.A.*, 63 (1965) 3024g.

KATO, J. AND KINOSHITA, S.: Application of thin-layer chromatography for industrial analysis of sugar fatty acid esters and synthetic musks. *Kogyo Kagaku Zasshi*, 68 (1965) 192–196; *C.A.*, 63 (1965) 776h.

POZZO, A. D.: (SCHERER's reaction on thin-layer chromatography). *Boll. Chim. Farm.*, 104 (1965) 55–57; *C.A.*, 63 (1965) 430h.

WACHS, W. AND GERHARDT, K.: (Separation of sucrose monofatty acid esters by means of thin-layer chromatography, and determination of the capillary-active properties of groups of position-isomeric monoesters). *Tenside*, 2 (1965) 6–10; *C.A.*, 63 (1965) 2415c.

See also PC section.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

BENDZ, G., SANTESSON, J. AND WACHTMEISTER, C. A.: Studies on the chemistry of lichens. 22. The chemistry of the genus *Siphula*. *Acta Chem. Scand.*, 19 (1965) 1250–1252.

CALVARANO, I.: (Detection of antifermentatives in lemon and orange juices by thin-layer chromatography). *Essenze Deriv. Agrumari*, 34, No. 3 (1964) 137–143; *C.A.*, 63 (1965) 2845e.

DAGLEY, S. AND GIBSON, D. T.: The bacterial degradation of catechol. *Biochem. J.*, 95 (1965) 466–474.

HAMBERG, M. AND SAMUELSON, B.: Isolation and structure of a new prostaglandin from human seminal plasma. Prostaglandins and related factors. 43. *Biochim. Biophys. Acta*, 106 (1965) 215–217.

JOHNSTON, J. M. AND RAO, G. A.: Triglyceride biosynthesis in the intestinal mucosa. *Biochim. Biophys. Acta*, 106 (1965) 1–9.

LOURY, M., LECHARTIER, G. AND FORNEY, M.: (Identification of volatile products of the autoxidation of oleic acid, by paper and thin-layer chromatography). *Rev. Franc. Corps Gras*, 12 (1965) 253–262; *C.A.*, 63 (1965) 3177f.

MAXIMOV, O. B. AND PANTIUKHINA, L. S.: Thin-layer partition chromatography of benzenecarboxylic and hydroxybenzenecarboxylic acids. *J. Chromatog.*, 20 (1965) 160–162.

MORRIS, L. J. AND WHARRY, D. M.: Chromatographic behaviour of isomeric long-chain aliphatic compounds. I. Thin-layer chromatography of some oxygenated fatty acid derivatives. *J. Chromatog.*, 20 (1965) 27–37.

MULDER, E. AND VAN DEENEN, L. L. M.: Metabolism of red-cell lipids. I. Incorporation *in vitro* of fatty acids into phospholipids from mature erythrocytes. *Biochem. Biophys. Acta*, 106 (1965) 106–117 — TLC and PC.

11b. Lipids and their constituents

BELFRAGE, P., ELOVSON, J. AND OLIVECRONA, T.: Radioactivity in blood and liver partial glycerides, and liver phospholipids after intravenous administration to carbohydrate-fed rats of chyle containing double-labeled triglycerides. *Biochim. Biophys. Acta*, 106 (1965) 45–55.

BONSEN, P. P. M., DE HAAS, G. H. AND VAN DEENEN, L. L. M.: Synthesis and enzymic hydrolysis of an O-alanyl ester of phosphatidyl glycerol. *Biochim. Biophys. Acta*, 106 (1965) 93–105 — TLC and PC.

BRADLOW, B. A., RUBENSTEIN, R. AND LEE, J.: Quantitative thin-layer chromatography of phospholipids in normal human erythrocytes. *S. African J. Med. Sci.*, 29 (1964) 41–52; *C.A.*, 62 (1965) 15054a.

DOUSTE-BLAZY, L., SOULA, G., VALDIGUIE, P. AND DIDIER, A.: (Separation of corpuscular lipoproteins on dextran gels). *Ann. Biol. Clin. (Paris)*, 23 (1965) 265–274; *C.A.*, 62 (1965) 12241e.

HAVERKATE, F. AND VAN DEENEN, L. L. M.: Isolation and chemical characterization of phosphatidyl glycerol from spinach leaves. *Biochim. Biophys. Acta*, 106 (1965) 78–92.

HENDLER, R. W.: Uptake of nucleic acid, protein and carbohydrate precursors by hen-oviduct lipids. *Biochim. Biophys. Acta*, 106 (1965) 184–196.

JURRIENS, G.: (Quantitative analysis of oils and fats by means of thin-layer and gas chromatography). *Chem. Weekblad*, 61 (1965) 257–264; *C.A.*, 63 (1965) 4521c.

MARTINI, P. F., PIANCINO, G. AND MOLINO, G.: (Thin-layer chromatographic separation of serum phospholipids. Modification introduced into the recovery procedure). *Minerva Med.*, 56 (1965) 929–933; *C.A.*, 63 (1965) 6007g.

MICHALEC, Č.: Biochemistry of sphingolipids. I. Thin-layer chromatography of sphingosines. *Biochim. Biophys. Acta*, 106 (1965) 197–199.

MULDER, E., VAN DEN BERG, J. W. O. AND VAN DEENEN, L. L. M.: Metabolism of red-cell lipids. II. Conversions of lysophosphoglycerides. *Biochim. Biophys. Acta*, 106 (1965) 118–127 — TLC and PC.

NODA, M., KATSURA, I. AND TANAKA, K.: Lipids of leaves and seeds. I. Chromatographic separation of lipids in chlorophyll lipoprotein. *Agr. Biol. Chem. (Tokyo)*, 29 (1965) 70–76; *C.A.*, 62 (1965) 12152f — TLC and PC.

PINELLI, A. AND VINCENZI, L.: (Solvents and mixtures of solvents able to separate by thin-layer (chromatography) different lipid fractions from liver extracts). *Atti Accad. Med. Lombarda*, 19 (1964) 469–472; *C.A.*, 63 (1965) 8711e.

WHEATLEY, V. R.: Possible clinical applications of the study of cutaneous lipids. With special reference to a simple method for rapid qualitative examination of surface-liquid samples. *Bull. N.Y. Acad. Med.*, 41 (1965) 353–369; *C.A.*, 63 (1965) 8711h.

See also PC section.

13. STEROIDS

COMER, J. P. AND HARTSAW, P. E.: Thin-layer chromatographic stability assay for C^{14}-labeled steroid in a cream. *J. Pharm. Sci.*, 54 (1965) 524–527.

FARNSWORTH, W. E.: 10-Demethylation of testosterone by human prostate *in vitro*. *Steroids*, 6 (1965) 519–530.

FLANAGAN, P. J. AND THOMSON, J. B.: Steroids. V. Rotational isomerism in bisergostatrienol. *Tetrahedron Letters*, (1965) 1671–1673; *C.A.*, 63 (1965) 5707f.

FÜRST, W.: Oxydationsprodukte des Ergosterins. 1. Chromatographische Trennung der Oxydationsprodukte, Isolierung neuer Bestrahlungsprodukte des Ergosterins bei Einfluss von Sauerstoff. *Arch. Pharm.*, 298 (1965) 795–804.

GERALI, G., LUGARO, G. AND FERRARI, L.: (Steroids. IV. Chromatography of some androstene derivatives). *Farmaco (Pavia), Ed. Sci.*, 20 (1965) 148–157; *C.A.*, 63 (1965) 1825f.

KARLESKIND, A., AUDIAU, F. AND WOLFF, J. P.: (Study of sterols by vapor phase chromatography). *Rev. Franc. Corps Gras*, 12 (1965) 399–403; *C.A.*, 63 (1965) 5913g.

KREKHOVA, M. A.: (Estimation of 17-keto steroids by means of thin-layer chromatography). *Vopr. Med. Khim.*, 11, No. 2 (1965) 60–67; *C.A.*, 63 (1965) 5971h.

LIM, N. Y. AND BROOKS, R. V.: A modification of the ^{35}S-thiosemicarbazide method for the estimation of plasma testosterone. *Steroids*, 6 (1965) 561–570 — TLC and PC.

LISBOA, B. P.: Application of thin-layer chromatography to the separation of saturated 21-deoxypregnane steroids. *Steroids*, 6 (1965) 605–625 — R_F and R_{rel} values for 54 compounds.

MONDER, C. AND WHITE, A.: The 21-hydroxysteroid dehydrogenases of liver. A nicotinamide adenine dinucleotide phosphate dehydrogenase and two nicotinamide adenine dinucleotide dehydrogenases. *J. Biol. Chem.*, 240 (1965) 71–77.

MOUSSERON-CANET, M. AND LEBEEUW, B.: (Steric orientation of epoxidation in the sterols). *Compt. Rend.*, 260 (1965) 4225–4227; *C.A.*, 63 (1965) 652e.

NAKAYAMA, F. AND MIYAKE, H.: Cholesterol complexing by macromolecular fractions in human gall bladder bile. *J. Lab. Clin. Med.*, 65 (1965) 638–648.

NOMURA, T. AND TSUCHIYA, Y.: Demonstration by thin-layer chromatography of estrogenic steroids in the salted ovary of *Theragra chalcogramma pallas*. *Tohoku J. Agr. Res.*, 14 (1964) 307–313; *C.A.*, 62 (1965) 12203f.

PENG, T. C., VENA, R., OFNER, P. AND MUNSON, P. L.: A simple method for the determination of urinary dehydroepiandrosterone. *Steroids*, 6 (1965) 571–582.

SNATZKE, G. AND NISAR, A.: (Preparation of abeo- and diabeo steroids and triterpenoids). *Ann. Chem.*, 683 (1965) 159–174; *C.A.*, 63 (1965) 4346b.

SPARAGANA, M.: Determination of urinary tetrahydrocortisone, tetrahydrocortisol and 3α-allotetrahydrocortisol by gas chromatography. *Steroids*, 6 (1965) 583–596.

STEINETZ, B. G., BEACH, V. L. AND MELI, A.: Plasma and urinary metabolites of progesterone-4-C^{14} administered orally or subcutaneously to rabbits. *Steroids*, 6 (1965) 627–644.

See also PC section.

14 STEROID GLYCOSIDES AND SAPONINS

KOMATSU, M., KAMANO, Y. AND SUZUKI, M.: Thin-layer chromatography of bufo-steroids on silica gel. *Japan Analyst*, 14 (1965) 1049–1054 — R_F values of 26 bufogenin derivatives.

RUBIO LIGHTBOURN, J.: (Thin-layer chromatography of sapogen steroids). *Mexico City: U.N.A.M. Escuela Nacional de Ciencias Quimicas, 1963*; *C.A.*, 63 (1965) 3624f.

See also PC section.

15. TERPENE DERIVATIVES

BHATNAGAR, V. M.: Thin-layer chromatography of essential oils, concrete essential oils and their clathrate compounds. *Perfumery Essent. Oil Record*, 56 (1965) 374; *C.A.*, 63 (1965) 5442h.

ENZELL, C. R. AND THOMAS, B. R.: The chemistry of the order *Araucariales*. 2. The wood of *Agathus australis*. *Acta Chem. Scand.*, 19 (1965) 913–919.

NIGAM, M. C., NIGAM, I. C. AND LEVI, L.: Essential oils and constituents. XXV. Thin-layer chromatography. Some chemical and chemotaxonomic applications. *J. Soc. Cosmetic Chemists*, 16 (1965) 155–168; *C.A.*, 63 (1965) 427f.

PESNELLE, B.: (Azulenogens from galbanum. Galbanol, a mixture of sesquiterpene alcohols). *Planta Med.*, 12 (1964) 403–405; *C.A.*, 62 (1965) 12070g.

VERDERIO, E. AND VENTURINI, D.: (Thin-layer chromatography of mandarin essential oil). *Boll. Chim. Farm.*, 104 (1965) 170–176; *C.A.*, 63 (1965) 4091d.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

CHOULIS, N. H.: (Thin-layer chromatography of adrenaline, noradrenaline and dopamine). *Chim. Chronika (Athens, Greece)*, 30, No. 3 (1965) 37–39; *C.A.*, 63 (1965) 5972c.

DANNENBERG, H., SONNENBICHLER, J. AND GROSS, H. J.: (3-Amino- and 9-acetamido-1,2-cyclopentenophenanthrene). *Ann. Chem.*, 684 (1965) 200–209; *C.A.*, 63 (1965) 4222h.
DUVALL, R. N., KOSHY, K. T. AND PYLES, J. W.: Comparison of reactivity of amphetamine, methamphetamine and dimethylamphetamine with lactose and related compounds. *J. Pharm. Sci.*, 54 (1965) 607–611.
GORDON, J. E.: Adsorption chromatography of low-melting quaternary ammonium salts. *J. Chromatog.*, 20 (1965) 38–42.
GOTO, S. AND ITO, F.: Colorimetric determination of small amount of pentachloronitrobenzene. *Japan Analyst*, 14 (1965) 921–926.
GRASSHOF, H.: Dünnschicht-Chromatographie von Aminen. *J. Chromatog.*, 20 (1965) 165–167.
SCHNEIDER, F. H. AND GILLIS, C. N.: Catechol amine biosynthesis *in vitro*. Application of the thin-layer chromatography. *Biochem. Pharmacol.*, 14 (1965) 623–626; *C.A.*, 63 (1965) 3256g.
SCHWARTZ, D. P., BREWINGTON, R. AND PARKS, O. W.: Separation of homologous series of dinitrophenyl amines by thin-layer chromatography. *Microchem. J.*, 8 (1964) 402–406; *C.A.*, 62 (1965) 12136h.

18. AMINO ACIDS

BURGI, W.: (Thin-layer detection of amino acids and sugar in the urine). *Schweiz. Apotheker-Ztg.*, 103 (1964) 351–354; *C.A.*, 63 (1965) 3295h.
DERMINOT, J. AND TASDHOMME, M.: Possible direct identification by chromatography of lanthionine and cysteic acid in the presence of cystine in wool hydrolyzates). *Bull. Inst. Textile France*, No. 117 (1965) 247–254; *C.A.*, 63 (1965) 5800e.
KAESER, H. AND GUGLER, G.: (Determination of ε-aminocaproic acid (EACS) in serum and urine by thin-layer chromatography). *Z. Klin. Chem.*, 3, No. 2 (1965) 33–35; *C.A.*, 63 (1965) 6008f.
MEYNIEL, G., BERGER, J. A., ARNAUD, R. N. AND BLANQUET, P.: (Behaviour of certain thyroidal iodinate compounds on flexible chromatographic films). *Compt. Rend.*, 260 (1965) 3065–3067 *C.A.*, 63 (1965) 433g.

See also PC section.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

DAVIES, A.: Some properties of calf interferon. *Biochem. J.*, 95 (1965) 20P–21P.
DELLACHA, J. M. AND FONTANIVE, A. V.: Quantitative N-terminal amino acid analysis by thin-layer chromatography. *Experientia*, 21 (1965) 351–352.
FERGUSON, D. R.: Separation of mammalian neurohypophysial hormones by thin-layer chromatography. *J. Endocrinol.*, 32 (1965) 119–120; *C.A.*, 62 (1965) 15018c.
WALTER, R. AND DU VIGNEAUD, V.: 6-Hemi-L-selenocystine-oxytocin and 1-deamino-6-hemi-L-selenocystine-oxytocin, highly potent isologs of oxytocin and 1-deamino-oxytocin. *J. Am. Chem. Soc.*, 87 (1965) 4192–4193.

See also PC section.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

ONO, T. AND SAKAGUCHI, T.: Thin-layer chromatography of adenosine triphosphate in pharmaceutical preparations. *Japan Analyst*, 14 (1965) 891–895 — DEAE-cellulose layers.

22. ALKALOIDS

ACHENBACH, H. AND BIERMANN, K.: Isotuboflavine and norisotuboflavine. Two new alkaloids isolated from *Pleiocarpa uncitica* Benth. *J. Am. Chem. Soc.*, 87 (1965) 4177–4181.
BATTERSBY, A. R., EVANS, G. W., MARTIN, R. O., WARREN, Jr., M. E. AND RAPOPORT, H.: Configuration of reticuline in the opium poppy. *Tetrahedron Letters*, (1965) 1275–1278; *C.A.*, 63 (1965) 640h.
BHATNAGAR, A. K. AND BHATTACHARJI, S.: Thin-layer chromatography of bisbenzylisoquinoline alkaloids. *Indian J. Chem.*, 3 (1965) 43–44; *C.A.*, 63 (1965) 1214c.
BROCHMANN-HANSSEN, E. AND NIELSEN, B.: (+)-Reticuline, a new opium alkaloid. *Tetrahedron Letters*, (1965) 1271–1274; *C.A.*, 63 (1965) 639h.
CHICHIRO, V. E.: (Identification and determination of alkaloids in a dry extract of *Thermopsis lanceolata*). *Sb. Nauchn. Tr. Tsentr. Nauchn.-Issled. Aptechn. Inst.*, 5 (1964) 167–173; *C.A.*, 63 (1965) 6008e.
DUGAS, H., ELLISON, R. A., VALENTA, Z., WIESNER, K. AND WONG, C. M.: Synthesis of lycopodium alkaloids. II. Development of stereospecific synthetic methods. *Tetrahedron Letters*, (1965) 1279–1286; *C.A.*, 63 (1965) 641d.

FISCHER, R. AND WEIXLBAUMER, H.: (Determination of aconitine by thin-layer chromatography). *Pharm. Zentralhalle*, 104 (1965) 298–301; *C.A.*, 63 (1965) 5451h.
GERTIG, H.: (Alkaloids of *Eschscholtzia californica*. I. Isolation and thin-layer chromatography of alkaloid fractions from roots). *Acta Polon. Pharm.*, 21 (1964) 59–64; *C.A.*, 62 (1965) 12069e.
HODGSON, E., SMITH, E. AND GUTHRIE, F. E.: Two-dimensional thin-layer chromatography of tobacco alkaloids and related compounds. *J. Chromatog.*, 20 (1965) 176–177.
KAWANISHI, M. AND SUGASAWA, S.: The structure of so-called rotundine. *Chem. Pharm. Bull. (Tokyo)* 13 (1965) 522–524; *C.A.*, 63 (1965) 4352f.
KORNHAUSER, A. AND PERPAR, M.: Über Mutterkornfarbstoffe. 4. Alkaloid-Farbstoff-Komplexe aus Mutterkorn. *Arch. Pharm.*, 298 (1965) 890–894.
PAPP, E. AND SZABO, Z.: (Studies of alkaloid-containing plants by thin-layer chromatography. III. *Nicotiana tabacum and Sedum acre*). *Herba Hung.*, 2 (1963) 383–390; *C.A.*, 63 (1965) 8712g.
SCHUNACK, W., MUTSCHLER, E. AND ROCHELMEYER, H.: Über die dünnschichtchromatographische Identifizierung und quantitative Bestimmung von Xanthinderivaten. *Deut. Apotheker-Ztg.*, 105 (1965) 1551–1552.
SCHWENKER, G.: Zur Kenntnis der Vitalischen Farbreaktion. 1. Über β-Eliminierungen mit Carbanion-Zwischenstufen. *Arch. Pharm.*, 298 (1965) 826–838.
VAGUJFALVI, D.: (A new, sensitive, thin-layer chromatographic method for detecting alkaloids). *Planta Med.*, 13 (1965) 79–83; *C.A.*, 63 (1965) 431b.
WINEFERDNER, J. D. AND MOYER, H. A.: The application of thin-layer chromatography and phosphorimetry for the rapid determination of nicotine, nornicotine and anabasine in tobacco. *Anal. Chim. Acta*, 32 (1965) 278.

See also PC section.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

GRIMMETT, M. R. AND RICHARDS, E. L.: Separation of imidazoles by cellulose thin-layer chromatography. *J. Chromatog.*, 20 (1965) 171–173 — R_F values for 18 compounds.
GRIMMETT, M. R. AND RICHARDS, E. L.: Imidazolic compounds from the reaction of pyruvaldehyde with ammonia. *J. Chem. Soc.*, (1965) 3751–3754.

24. ORGANIC SULPHUR COMPOUNDS

KASHIWA, T. AND ITO, F.: Dry thin-layer chromatographic determination of 4-chlorophenyl-2,4,5-trichlorophenylsulfone in its formulations. *Japan Analyst*, 14 (1965) 1019–1023.
SVETLAEVA, V. M. AND ZHURAVLEV, S. V.: (Chromatography of triphtazine (10-[γ-(4-methyl-1-piperazinyl)-propyl]-2-(trifluoromethyl)-phenothiazine) and intermediate substances on a non-fixed layer of Al_2O_3). *Med. Prom. SSSR*, 18, No. 12 (1964) 38–40; *C.A.*, 63 (1965) 5f.

26. METALLO-ORGANIC COMPOUNDS

ZAIDI, S. A. H., HUSAIN, S. A. AND MAHDIHASSAN, S.: Thin-layer chromatography of a few iron-saccharate preparations. *Arzneimittel-Forsch.*, 15 (1965) 636–638.

27. VITAMINS

EGGER, K.: (The distribution of vitamin K_1 and plastoquinone in plants). *Planta*, 64 (1965) 41–61; *C.A.*, 62 (1965) 12158e.
GÜNTHER, W. H. H. AND MAUTNER, H. G.: The synthesis of selenocoenzyme A. *J. Am. Chem. Soc.*, 87 (1965) 2708–2716.
ISLER, O. AND MONTAVON, M.: La chimie des vitamines E et K et des quinones apparentées à chaine isoprénoïde. *Bull. Soc. Chim. France*, (1965) 2403–2413.
MAUREL, A., REY, S. AND REY, M.: (Separation of ascorbic acid from glucose in wine by thin-layer chromatography). *Compt. Rend. Acad. Agr. Franc.*, 50 (1964) 1081–1084; *C.A.*, 62 (1965) 12408c.
SONG, P. S., SMITH, E. C. AND METZLER, D. E.: Photochemical degradation of flavins. II. The mechanism of alkaline hydrolysis of 6,7-dimethyl-9-formylmethylisoalloxazine. *J. Am. Chem. Soc.*, 87 (1965) 4181–4184 — TLC and PC.

See also PC section.

28. ANTIBIOTICS

BREINLICH, J.: (Stability of sterilized tetracaine hydrochloride solutions). *Pharm. Ztg.*, 110 (1965) 579–582; *C.A.*, 63 (1965) 5454h.

BRODASKY, T. F. AND LUMMIS, W. L.: Quantitative thin-layer chromatographic assay for lincomycin and related antibiotics. *Antimicrobial Agents Chemotherapy*, No. 10 (1964, pub. 1965) 18–23; *C.A.*, 63 (1965) 3303e.

SARGES, R. AND WITKOP, B.: Gramicidin A. V. The structure of valine- and isoleucine-gramicidin A. *J. Am. Chem. Soc.*, 87 (1965) 2011–2020.

SARGES, R. AND WITKOP, B.: Gramicidin A. VI. The synthesis of valine- and isoleucine-gramicidin A. *J. Am. Chem. Soc.*, 87 (1965) 2020–2027.

29. INSECTICIDES AND OTHER PESTICIDES

ENGST, R. AND SPRANGER, D.: (Thin-layer chromatography of α-naphthyl-N-methylcarbamate (Servin, carbaryl) and some other pesticides). *Nahrung*, 8 (1964) 653–664; *C.A.*, 62 (1965) 12389h.

HENKEL, H. G.: (Thin-layer chromatographic behavior of compounds effective as herbicides: carbamates). *Chimia (Aarau)*, 19 (1965) 426–428; *C.A.*, 63 (1965) 8973b.

KASHIWA, T. AND ITO, F.: Dry thin-layer chromatographic determination of O,O-dimethyl-O-(3-methyl-4-(methylthio)phenyl) phosphorothioate in its formulations. *Japan Analyst*, 14 (1965) 779–784.

MELCHIORRI, P., MAFFEI, F. AND SIESTO, A. J.: (Unidimensional, multiple-unidimensional and bidimensional thin-layer chromatographic separation of organophosphorus insecticides and their residues in vegetable oils). *Farmaco (Pavia), Ed. Prat.*, 19 (1964) 610–627; *C.A.*, 62 (1965) 12369b.

30. SYNTHETIC AND NATURAL DYES

COPIUS-PEEREBOOM, J. W. AND BEEKES, H. W.: Thin-layer chromatography of dyestuffs on polyamide and "silver nitrate" layers. *J. Chromatog.*, 20 (1965) 43–47.

MATHEW, T. V., MITRA, S. N. AND ROY, A. K.: Isolation and identification of coal-tar colors in sweet-meat (Halwa) by thin-layer chromatography. *J. Proc. Inst. Chemists (India)*, 36 (1964) 301–304; *C.A.*, 62 (1965) 12368f.

See also PC section.

31. PLASTICS AND THEIR INTERMEDIATES

MILES, B. H. AND SPELL, H. L.: Applications of thin-layer and gel permeation chromatography to the characterization of epoxy resins. *Proc. Anniv. Tech. Conf., SPI (Soc. Plastics Ind.) Reinforced Plastics Div., 20th, Chicago, 1965*, 7-A, 10 pp.; *C.A.*, 63 (1965) 3114f.

See also PC section.

32. PHARMACEUTICAL AND FORENSIC APPLICATIONS; METABOLISM OF DRUGS

AMUNDSON, M. E., JOHNSON, M. L. AND MANTHEY, J. A.: Urinary excretion of *d*-propoxyphene hydrochloride in man. *J. Pharm. Sci.*, 54 (1965) 684–686.

EBERHARDT, H. AND DEBACKERE, M.: Nachweis von zentral stimulierenden Substanzen in reiner Form und nach Körperpassage mit der Dünnschichtchromatographie. *Arzneimittel-Forsch.*, 15 (1965) 929–932.

ENNET, D.: (Analysis of 1-phenyl-1-cyclohexyl-3-piperidino-1-propanol hydrochloride. I. Qualitative determination of trihexiphenidyl in medicinal preparations). *Arzneimittelstandardisier., Inform.-Dienst*, 4, No. 2 (1963) 73–80; *C.A.*, 63 (1965) 2851c.

HULTIN, E.: Thin-layer chromatography of plant extracts. II. Sequential extraction of plant material. *Acta Chem. Scand.*, 19 (1965) 588–590.

HULTIN, E.: Thin-layer chromatography of plant extracts. III. Countercurrent separation of plant extracts. *Acta Chem. Scand.*, 19 (1965) 591–594.

NOIRFALISE, A.: Mise en évidence et différenciation de quelques stimulants et dépresseurs du système nerveux central par chromatographie sur couche mince. *J. Chromatog.*, 20 (1965) 61–77 — R_F values for 36 compounds.

THOMAS, J. J. AND DRYON, L.: (Identification of psychotropic pharmaceuticals by thin-layer chromatography). *J. Pharm. Belg.*, 19 (1964) 481–504; *C.A.*, 63 (1965) 1660c.

ZICHA, L., FREYTAG, F. AND WEIST, F.: Dünnschichtchromatographische Untersuchungen über die orale und rectale Resorption von 3-Methyl-3,4-dihydroxy-4-phenyl-butin-1 und seine Ausscheidung in Galle, Harn und Liquor. *Arzneimittel-Forsch.*, 15 (1965) 777–782.

See also PC section.

33. INORGANIC SUBSTANCES

HASHMI, M. H., SHAHID, M. A. AND AYAZ, A. A.: Application of circular thin-layer chromatography to inorganic qualitative analysis. *Talanta*, 12 (1965) 713–719; *C.A.*, 63 (1965) 4924b.

HU, C. T. AND LIU, C. L.: Application of thin-layer chromatography in inorganic analysis. II. Separation and determination of Se and Te. *K'o Hsueh T'ung Pao*, (1964) 1103–1104; *C.A.*, 63 (1965) 7f.

34. RADIOACTIVE AND OTHER ISOTOPIC COMPOUNDS

JANNES, L., AMINOFF, J., HJERP, L., HUTTUNEN, R. AND RELANDER, A.: (Glutamate metabolism of *Bordetella pertussis*). *Suomen Kemistilehti*, 38B (1965) 18–20; *C.A.*, 62 (1965) 15102g.

KOSS, F. W. AND JERCHEL, D.: (Thin-layer chromatography of radioactive materials). *Radiochim. Acta*, 3 (1964) 220–224; *C.A.*, 63 (1965) 3595e.

TAYLOR, E. H.: Test for radiochemical purity of sodium-iodide (^{131}I) solution U.S.P. by thin-layer chromatography. *J. Pharm. Sci.*, 54 (1965) 639–640.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

MUTSCHLER, E.: Zur Chemie und Pharmakologie der Alkaloide Damascenin und Arecolin. *Arch. Pharm.*, 298 (1965) 861–868.

TOYOSHIMA, S. AND NISHIMOTO, Y.: Thin-layer chromatography of chelating agents. I. Reaction between the adsorbents and the chelating agents. *Yakugaku Zasshi*, 85 (1965) 235–239; *C.A.*, 63 (1965) 7c.

VERNIN, G.: (Thin-layer chromatography of some aromatic substances). *France Parfums*, 8 (1965) 145–154; *C.A.*, 63 (1965) 2845c.

YOUNG, A. L.: Chromatographic techniques in the differentiation of sagebrush taxa. *J. Range Management*, 18 (1965) 35; *C.A.*, 62 (1965) 12157a.

Gas Chromatography

1. REVIEWS AND BOOKS

BERGÉS, L. S.: (Determination of carbon, hydrogen and nitrogen (in organic compounds)). *Inform. Quim. Anal. (Madrid)*, 18 (1964) 121–127 — a review with 16 references on elemental analysis by GC.

CHOVIN, P.: Quelques faits saillants dans l'histoire de la chromatographie en phase gazeuse de ces dernières années. *Chim. Anal. (Paris)*, 47 (1965) 563–570 — 38 references.

FUTSINAGA, T.: (Theory of the height equivalent to the theoretical plate in gas chromatography). *Chemistry (Kyoto)*, 20 (1965) 427–432 — a review of papers published in 1964.

GIDDINGS, J. C.: Concept and column parameters in gas chromatography. In C. N. REILLY (Editor), *Advances in Analytical Chemistry and Instrumentation*, Vol. 3, Wiley, New York, 1964, pp. 315–367.

MOSHIER, R. W. AND SIEVERS, R. E.: *Gas chromatography of metal chelates*, Pergamon, Oxford, 1965, 8 + 163 pp.

2. FUNDAMENTALS, THEORY AND GENERAL

2a. Gas–liquid systems

VINK, H.: Theory of sorption chromatography. *J. Chromatog.*, 20 (1965) 496–499 — sorption term for solute interaction with the stationary phase of a chromatographic column; procedure for numerical solution of the problem is indicated.

2b. Gas–solid systems

DOLLIMORE, D. AND HEAL, G. R.: Errors in the calculation of surface area and average pore radius. *Nature*, 208 (1965) 1092 — in general pore radius never appears less than twice the adsorbate diameter from calculation.

KUBÍN, M.: Beitrag zur Theorie der Chromatographie. II. Einfluss der Diffusion ausserhalb und der Adsorption innerhalb des Sorbens-Korns. *Collection Czech. Chem. Commun.*, 30 (1965) 2900–2907 — not only radial and longitudinal diffusion are characterised by statistical moments of peaks, but also mass transfer through the surface of a grain and rate of sorption; no reference to E. KUČERA (*J. Chromatog.*, 19 (1965) 237).

LOCKE, D. C.: Studies in gas chromatography. *Dissertation Abstr.*, 26 (1965) 56.

LOCKE, D. C.: Gas–solid partition chromatography with real carrier gases. *J. Phys. Chem.*, 69 (1965) 3768–3773 — the effects of gas phase imperfection and mean column pressure on retention have been studied.

2d. General

COX, B. C. AND ELLIS, B.: Measurement of the areas of gas chromatographic peaks. *J. Chromatog.*, 20 (1965) 598–600 — area $A = \sum_{1}^{j} l_i + l_r$, where l_i is the length of rectangle i, and l_r is the "equivalent length" of the top part of the peak.

HAKEN, J. K. AND SOUTER, P.: Evaluation of retention data of compounds classifiable into homologous series. Part II. *n*-Alkyl ketones. *J. Gas Chromatog.*, 3 (1965) 348–349 — correlation system is an extension of the simple one-parameter treatment of JAMES AND MARTIN; nomograph for calculation with average errors of 0.61%.

VINK, H.: Theory of partition chromatography. III. *J. Chromatog.*, 20 (1965) 305–309 — steady state solutions of the differential equations for a partition column are derived for the first three moments of concentration distribution.

3. TECHNIQUES I

3a. Detectors

KARMEN, A.: Specific detection of subnanogram quantities of organophosphorus by flame ionization. *J. Gas Chromatog.*, 3 (1965) 336–339 — sensitivity of screen treatment with Li, Na, Rb and Cs chlorides to P/Cl response has been studied in the case of a flamethermionic detector.

SINGLETON, J. A., AURAND, L. W. AND BELL, T. A.: A comparison of carrier gases upon chromatograms when using a flame ionization detector with sub-ambient temperature programming. *J. Gas Chromatog.*, 3 (1965) 357–358.

SULLY, B. D. AND WILLIAMS, P. L.: Quantitative gas–liquid chromatographic analysis with detectors having a nonlinear response. *Analyst*, 90 (1965) 643–648 — the ratio of the heights or areas of the two peaks is found to be constant at different sample sizes even when detector responses are not linear (the exponential values are the same for both compounds); a discussion for an argon detector is given.

3b. Column performance and filling studies

CARTONI, C. P., LIBERTI, A. AND PALOMBARI, R.: Metal complexes of β-diketones as liquid phases in gas–liquid chromatography. *J. Chromatog.*, 20 (1965) 278–282 — retardation factors for 6 groups of compounds on Cu, Ni, Al, Be and Zn complexes of *n*-nonyl β-diketone at 120°.

DUCROS, M.: Chromatographie en phaze gazeuse. Étude du comportement d'un série homologue de phases fixes en cas particulier des esters tartrique. *Publ. Sci. Tech. Min. Air (France), Notes Tech.*, No. 126 (1963) 85 pp. — 30 references.

EWALD, G. AND ZECH, H.: Über gaschromatographische Trägermaterialien mit phosphorsäurebehandelter Oberfläche. I. Die Vorgänge bei der Einwirkung von Phosphorsäure und die Eigenschaften der entstehenden Träger. *Z. Anal. Chem.*, 215 (1965) 8–17 — GC properties of support materials are better when the support is treated thermally with H_3PO_4 than when it is simply coated with H_3PO_4.

GROB, K.: Polare Imprägnierung von Glaskapillaren für die Gas-Chromatographie. *Helv. Chim. Acta*, 48 (1965) 1362–1370.

JOHNSON, D. E., RODRIGUEZ, C. F. AND SCHLAMEUS, W.: A new method for coating glass beads for use in gas chromatography of chloropromazine and its metabolites. *J. Gas Chromatog.*, 3 (1965) 345–347 — a method for reproducible coating of glass beads with a mixture of liquid phases and particles of diatomaceous earth; HETP 0.9 mm.

MCKINNEY, R. W.: An evaluation of vermiculite as a support material for gas chromatography. *J. Gas Chromatog.*, 3 (1965) 388–389 — a hydrated magnesium-aluminium iron silicate.

SCHLUNEGGER, U. P.: Zur gaschromatographischen Trennung von aliphatischen, sauerstoffhaltigen Substanzen in Wasser. II. Über den Einfluss der Substituenten auf die Trennfähigkeit von Phenoläthern. *J. Chromatog.*, 20 (1965) 452–456 — phenyl-hydroxyalkyl-ethers with different numbers of –OH groups are investigated; retention data of water given.

SMITH, E. D. AND OATHOUT, J. M.: A statistical evaluation of some important parameters in the preparation of gas chromatographic packings. *J. Gas Chromatog.*, 3 (1965) 368–372.
WEBB, J. L.: The use of sponge spicules as a column support in gas chromatography. *J. Gas Chromatog.*, 3 (1965) 384–387 — sterrasters of *Geodia gibberose* applied for the separation of polar compounds.

3c. Apparatus, accessories and materials for GC

DECK, R. E., THOMPSON, J. A. AND CHANG, S. S.: A multiple trap carousel micro fraction collector for gas chromatography. *J. Gas Chromatog.*, 3 (1965) 392–393 — construction is shown.
EREMEEVA, K. T. AND KUZNETSOV, G. T.: (Improvement of chromatograph CHT-2M). *Koks i Khim.*, No. 12 (1965) 59–60.
HAMPTON, W. C.: Pyrolyzer assembly. *U.S. Pat.*, 3,186,801 (June 1, 1965).
HELLER, H. AND THEN, H. J.: Fluid analysis control. *U.S. Pat.*, 3,199,338 (August 10, 1965).
HILL, D. W. AND NEWELL, H. A.: The use of a continuous slow injector for the production of calibration vapour mixtures in the parts per million by volume range. *J. Sci. Instr.*, 42 (1965) 783–786 — scheme and detailed design of mixing nozzle given.
HONAKER, C. B. AND HORTON, A. D.: A simple pyrolyzer for use with gas chromatography. *J. Gas Chromatog.*, 3 (1965) 396–398 — detailed design given.
HRIVŇÁK, J.: (Injection block for gas chromatography). *Chem. Zvesti*, 19 (1965) 942–943 — details of design given.
KÖVÁRI, I. AND FÉNYI, SC.: Some experiences in the construction of gas chromatographs. *Acta Phys. Chem. Univ. Szeged*, 10 (1964) 109–113 — katharometer as detector and thermostat up to 250°.
KREJČÍ, M. AND DEML, M.: Methode und Vorrichtung für die Untersuchung der pyrolytischen Zersetzung von Substanzen bei konstanter Temperatur. *Collection Czech. Chem. Commun.*, 30 (1965) 3071–3079 — electrical circuits given; activation energy of polypropylene degradation determined.
KUČEROVSKÝ, Z. AND DOUBEK, M.: (Stabilized source and chromatographic bridge for gas chromatographs with thermal conductivity cells). *Chem. Listy*, 59 (1965) 1441–1444 — stability 12 V ± 0.5–0.75% for feed a.c. of 220 ± 20 V.
NOREM, S. D. AND HILL, H. I.: Chromatography apparatus. *U.S. Pat.*, 3,199,274 (August 10, 1965).
PEARSON, Jr., R., FINK, W. C. AND GORDUS, A. A.: Radiogaschromatography peak analyser. *J. Gas Chromatog.*, 3 (1965) 381–383 — circuit designs given.
RADIN, N. S.: The use of Kovar seals in high temperature gas chromatography with glass columns. *J. Chromatog.*, 20 (1965) 392–394.
STRICKLER, A.: Integrator. *U.S. Pat.*, 3,187,168 (June 1, 1965).
YANOTOVSKIĬ, M. I., GAZIEV, G. A., NIKIFOROV, V. P., MAKARENKO, V. G., ZIMIN, R. A., MARININ, P. I. AND FRANK, YU. A.: (Gas chromatograph with automated sampling from the stream). *Zavodsk. Lab.*, 31 (1965) 1526–1528 — scheme and complete design of the detector given.

4. TECHNIQUES II

4a. Preparative-scale GC

TARAMASSO, M., SALLUSTO, F. AND GUERRA, A.: Preparative scale gas chromatography by a laboratory rotating unit. IV. Separation of close boiling mixtures and crude reaction products. *J. Chromatog.*, 20 (1965) 226–231 — at a feed rate of 52 ml/h, *n*-pentane of 99.90% purity and isopentane of 99.8% purity were obtained.

4b. Programmed temperature and programmed pressure GC

MÁZOR, L., BALLA, J. AND TAKÁCS, J.: Über die Programmierung des Eintrittsdruckes vom Trägergas in der Gaschromatographie. *J. Chromatog.*, 20 (1965) 221–225 — the net retention volume can be calculated from the equation $V_N = \sum_{i=1}^{n+1} j_i \cdot t_{R_i} \cdot F_{C_i} - t_C \cdot j_1 \cdot F_{C_1}$.

4d. Special microtechniques

MUSHA, S. AND NISHIMURA, T.: Some applications of hydrogenation in rational and elemental analysis of organic compounds by means of a gas chromatographic technique. *Japan Analyst*, 14 (1965) 732–735.
WICHTERLOVÁ, B. AND JÍRU, P.: (Pulse method for the investigation of the rate of heterogenous reactions solid–gas phase in connection with chromatographic analysis of products). *Chem. Listy*, 59 (1965) 1451–1455.

4e. Automation

BOMBAUCH, K. J., HAYES, B. J. AND SHAW, W. R.: A preliminary study of a dual zone process chromatograph. *J. Gas Chromatog.*, 3 (1965) 373–377 — detailed flow diagrams given.

4f. Measurement of physico-chemical and related values

CLARK, R. K. AND SMITH, H. H.: Gas–liquid partition chromatography on benzene–polyphenyl systems and polymer statistical thermodynamics. *J. Phys. Chem.*, 69 (1965) 3682–3689 — activity coefficient (at 90°) and heats of solution of benzene in biphenyl, *m*- and *o*-terphenyl.

GUBBINS, K. E., CARDEN, S. N. AND WALKER, Jr., R. D.: Determination of gas solubilities in liquids. *J. Gas Chromatog.*, 3 (1965) 330 — reproducibility within ± 2%.

KONONOV, N. F., MAKAROVSKIĬ, YA. I., ROZENGART, M. I. AND KOGAN, V. B.: (Chromatographic estimation of selectivity of solutes). *Zh. Prikl. Khim.*, 38 (1965) 2522–2528 — selectivity $S = \alpha_p/\alpha$, where α_p gives the GC data and α the vapour pressure data.

SIVTSOVA, E. V., KOGAN, V. B. AND OGORODNIKOV, S. K.: (Use of gas–liquid partition chromatography for the selection of phase for extractive distillation of methylchlorosilane mixtures). *Zh. Prikl. Khim.*, 38 (1965) 2609–2611 — coefficients α of all pairs given for 30 liquids.

STASZEWSKI, R. AND POMOPOWSKI, T.: Oberflächenbestimmungen mittels einer Wärmedesorptionsmethode. *Chem. Anal. (Warsaw)*, 10 (1965) 1123–1126 — an application of NELSON AND EGGERTSEN's method (*Anal. Chem.*, 30 (1958) 1387).

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

LIAKUMOVICH, A. G., MICHUROV, YU. I., AND FEKLYAEVA, S. D.: (Determination of hydrocarbons soluted in *tert.-butyl* sulphate). *Zavodsk. Lab.*, 31 (1965) 1445–1447 — dissolved gases are stripped by CO_2 stream and collected in a nitrometer over KOH solution; no reference to the paper by J. JANÁK AND I. PÁRALOVÁ on the same principle published earlier in *Collection Czech. Chem. Commun.*, 20 (1955) 336, in Russian.

NEVMEPZHITSKAYA, E. A., BELYAEVA, A. N., POPROTSKAYA, V. A. AND KUDRYAVTSEVA, N. A.: (Investigation of composition of gas from the electrocracking of methane). *Khim. Prom.*, 41 (1965) 895–896 — on β,β'-oxydipropionitrile at 66°.

RAZUMOVSKAYA, L. V. AND KURENKOV, V. P.: (Rapid method of analysis of contact gas from butene dehydrogenation). *Khim. Prom.*, 41 (1965) 928–929 — diethylene glycol ester of *n*-butyric acid.

5b. Other hydrocarbons

CHORTYK, O. T., SCHLOTZHAUER, W. S. AND STEDMAN, R. L.: Lithium chloride as a gas chromatographic substrate for polynuclear aromatic hydrocarbons. *J. Gas Chromatog.*, 3 (1965) 394–395 — no reference to the paper by H. D. SAUERLAND (*Brennstoff-Chem.*, 45 (1964) 55), on the same problem.

GERSHBEIN, L. L. AND KROTOSZYNSKI, B. K.: Fractionation and analysis of components from human sebum. *J. Gas Chromatog.*, 3 (1965) 378–380 — C_{30} hydrocarbons.

GUVERNATOR, III, G. C., GAGER, Jr., F. L., ROBB, E. W. AND BAVLEY, A.: Electron capture detection of gas-chromatographed polycyclic hydrocarbons. *J. Gas Chromatog.*, 3 (1965) 363–367 — retention data of 19 aromatics from naphthalene to coronene at 6 temperatures from 180° to 280°; detection limits 0.01 μg.

MIYAKE, H., MITOOKA, M. AND MATSUMOTO, T.: Studies of gas chromatography with mixed stationary liquids. IV. The analysis of aromatic hydrocarbons from C_6 to C_{10} with a capillary column. *Bull. Chem. Soc. Japan*, 38 (1965) 1062–1071.

PHILIPPE, R. J., HONEYCUTT, R. G. AND RUTH, J. M.: Some C_6 hydrocarbons of the gas phase of cigarette smoke. *J. Chromatog.*, 20 (1965) 250–259 — 15 hydrocarbons have been isolated.

5c. Halogen derivatives of hydrocarbons

WANG YUNG-LUH, KUO SIN-TSENG, WANG SHOU-SUNG AND LIN YUNG-DA: (A gas chromatographic analysis of high temperature bromination products of difluorochloromethane). *Acta Chim. Sinica*, 31 (1965) 531–535 — retention data of 20 fluorocarbons on Carbowax 400 and DNP.

7. PHENOLS

HENKEL, H. G.: Phenolätherbildung im Einspritzblock eines Gaschromatographen. *J. Chromatog.*, 20 (1965) 596–597 — recoveries are given for 16 phenols.

SIDOROV, R. I. AND KABAEVA, I. I.: (Principles of gas–liquid chromatographic separation of a complex phenol mixture based on the relative polarity of stationary phases). *Izv. Sibirsk. Otd. Akad. Nauk, Ser. Khim. Nauk*, No. 2 (1965) 101–108.

10. CARBOHYDRATES

KÄRKKÄINEN, J., LEHTONEN, A. AND NIKKARI, T.: Determination of glucosamine and galactosamine by gas chromatography. *J. Chromatog.*, 20 (1965) 457–462 — retention data on QF-1 at 144° and on SE-30 at 170° and 205°.

SAWARDEKER, J. S., SLONEKER, J. H. AND DIMLER, R. J.: Detection and quantitative determination of anhydroglycoses by gas chromatography. *J. Chromatog.*, 20 (1965) 260–265 — retention data of XE-60 and SE-52 at 145°, EGS at 168° and Carbowax 20M (terminated with terephthalic acid) at 160°.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids

CALNIV, D. T.: Analysis of some organic acids by gas–liquid chromatography. *Can. J. Biochem.*, 43 (1965) 1281–1287.

FUNASAKA, W., KOJIMA, T. AND TOYOTA, T.: (Gas–liquid chromatographic analysis of napthalenesulphonic acids). *Japan Analyst.*, 14 (1965) 815–819.

JAMIESON, G. R. AND REID, E. H.: The analysis of oils and fats by gas chromatography. II The alkaline isomerisation of linoleic acid. *J. Chromatog.*, 20 (1965) 232–239 — saponification of oils at temperatures above 120° causes isomerisation, which tends to produce conjugated *cis,trans* isomers at temperatures up to 197° and *trans,trans* isomers above this temperature.

JONES, E. P., SCHOLFIELD, C. R., DAVISON, V. L. AND DUTTON, H. J.: Analysis of fatty acid isomers in two commercially hydrogenated soybean oils. *J. Am. Oil Chemists' Soc.*, 42 (1965) 727–730.

MAZLIAK, P. AND SALSAC, L.: (Gas-chromatographic analysis of plant organic acids). *Phytochemistry*, 4 (1965) 693–703.

PONS, JR., W. A. AND FRAMPTON, V. L.: Precision and accuracy in gas–liquid chromatography of C_{14}–C_{18} fatty acid methyl esters. *J. Am. Oil Chemists' Soc.*, 42 (1965) 786–789.

SANZ-BURATA, N., JULIÁ, S. AND IRURRE, J.: (Preparative separation of diastereoisomers by gas chromatography). *Afinidad*, 22 (1965) 259.

SMITS, P.: Quantitative analysis of lactone–oxo acid mixtures by gas chromatography. *J. Chromatog.*, 20 (1965) 397–398.

11b. Lipids and their constituents

HOLLA, K. S.: Estimation of glycerol in neutral glycerides, phospholipids, and cardiolipids by gas–liquid chromatography. *Dissertation Abstr.*, 25 (1965) 6930–6931.

LEEGWATER, D. C. AND VAN GEND, H. W.: Über den Nachweis von mit anderen Fetten umgeestertem Kokosfett oder Palmkernfett. *Fette, Seifen, Anstrichmittel*, 67 (1965) 1–3 — C_{32}–C_{38} triglycerides.

13. STEROIDS

ADLERCREUTZ, H. AND LUUKKAINEN, T.: Gas-chromatographic identification of pregnanediol and some of its isomers in bile of pregnant women. *Ann. Med. Exptl. Biol. Fenniae (Helsinki)*, 42 (1964) 161–167 — on QF-1, SE-30, XE-60 and 2,2-dimethylpropane-1,3-diol succinate at 175–226°.

COPIUS-PEEREBOOM, J. W.: Gas chromatography of phytosterol mixtures. Part I. Identification of phytosterols. *J. Gas Chromatog.*, 3 (1964) 325–329 — retention data of 14 acetates on SE-30 with Versamid at 220°.

HAMMOND, K. B. AND LEACH, H.: Gas chromatography of human steroids as the trimethylsilyl esters. *Clin. Chim. Acta*, 12 (1965) 363–364.

OAKS, D. M., BONELLI, E. J. AND DIMICK, K. P.: Quantitative gas chromatography of urinary steroids using a new liquid phase. *J. Gas Chromatog.*, 3 (1965) 353–357 — on STAP, a new polyester modified Carbowax liquid phase.

PANIUCCI, F. AND TAPONECO, G.: (Gas-chromatographic separation of testosterone from epitestosterone). *Boll. Soc. Ital. Biol. Sper.*, 41 (1965) 708–710.

RICCI, G., BERTOLACCINI, M. A., CRESPI, M. AND SOCCORSI, F.: Gas chromatography of bile acids in bile. *Epatologia*, 10 (1964) 215–231 — on SE-52.

15. TERPENE DERIVATIVES

RASQUINHO, L. M. A.: A practical approach of the qualitative characterization of essential oils by programmed temperature gas chromatography. *J. Gas Chromatog.*, 3 (1965) 340–344 — "retention masters" are advocated for identification; a comparison method; 50 essential oil chromatograms given.

RUDLOFF, E. VON: Gas chromatographic analysis of the volatile oil from a single conifer needle. *J. Gas Chromatog.*, 3 (1965) 390–391.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BIGHI, C., LUCCI, G. AND SAGLIETTO, G.: (Influence of alkali treatments of solid support on the separation of amines by gas–liquid chromatography). *Ann. Univ. Ferrara, Sez.* 5, 2, No. 9 (1964) 107–116.

FEDELI, E., LANZANI, A. AND VALENTINI, A. F.: (Gas-chromatographic analysis of phenylhydrazones and 2,4-dinitrophenylhydrazones). *Chim. Ind. (Milan)*, 47 (1965) 989–991.

18. AMINO ACIDS

LANDOWNE, R. A.: Quelques utilisations intéressantes de l'affinité électronique dans l'application de la chromatographie en phase gazeuse à l'analyse des acides aminés et des peptides. *Chim. Anal.*, 47 (1965) 589–591.

MUSSINI, E. AND MARCUCCI, F.: Separation of prolines and hydroxyprolines by gas chromatography. *J. Chromatog.*, 20 (1965) 266–269 — retention data of 6 prolines on Carbowax 20M and QF-1 given.

22. ALKALOIDS

BROCHMANN-HANSSEN, E. AND FONTAN, C. R.: Gas chromatography of alkaloids with polar stationary liquids. II. The effect of polyvinylpyrrolidone treatment of the support. *J. Chromatog.*, 20 (1965) 394–396 — retention data of 22 alkaloids on NGS on Gas-Chrom-P support silanized and pretreated with polyvinyl pyrrolidone.

CHALMERS, A. H., CULVENOR, C. C. J. AND SMITH, L. W.: Characterisation of pyrrolizidine alkaloids by gas, thin-layer and paper chromatography. *J. Chromatog.*, 20 (1965) 270–277 — retention data of 19 alkaloids on SE-30 at 140° and of 38 derivatives at 205°.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

TAKEUCHI, T. AND KATO, N.: (Rapid determination of impurities in pyridine by gas–liquid chromatography). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 68 (1965) 1568–1572.

24. ORGANIC SULPHUR COMPOUNDS

COLEMAN, H. J., THOMPSON, C. J., HOPKINS, R. L. AND RALL, H. T.: Identification of some naturally occurring alkylthiophenes in Wilmington, Calif., crude oil by use of a series of gas–liquid chromatography stationary phases. *J. Chromatog.*, 20 (1965) 240–249 — separation scheme given; 8 alkylthiophenes identified in a 111–150° boiling cut.

26. METALLO-ORGANIC COMPOUNDS

TANIKAWA, K. AND ARAKAWA, K.: Organometallic compounds. Part. 4. Analytical determination of ferrocene derivatives by gas chromatography. *Chem. Pharm. Bull. (Tokyo)*, 13 (1965) 926–931.

29. INSECTICIDES AND OTHER PESTICIDES

ABBOTT, D. C., HARRISON, R. B., TATTON, J. O'G. AND THOMPSON, J.: Organochlorine pesticides in the atmospheric environment. *Nature*, 208 (1965) 1317–1318 — on Apiezon and silicone grease with electron capture detector.

EBING, W.: (Gas-chromatographic analysis of insecticidal carbamates). *Chimia (Aarau)*, 19 (1965) 501–503.

GUTENMANN, W. H., BACHE, C. A. AND LISK, D. J.: Determination of imidan and imidoxon in crops by preparative thin-layer and by gas chromatography. *J. Gas Chromatog.*, 3 (1965) 350–352.

KANAZAWA, J.: (Determination of the herbicide 2,6-dichlorobenzonitrile by gas–liquid chromatography). *Japan Analyst*, 14 (1965) 720–723.

PURSLEY, P. L. AND SCHALL, E. D.: Gas chromatographic determination of 2,4-D and 2,4,5-T and their derivatives in commercial formulations. *J. Assoc. Offic. Agr. Chemists*, 48 (1965) 327–333 — retention data of 14 esters of 2,4-dichloro- and 2,4,5-trichlorophenoxyformic acid on SEK-30 at 165–175° and 200°.

STAHL, E. AND PFEIFLE, J.: Dünnschicht- und Gas Chromatographie der insektiziden Pyrethrum-Inhaltsstoffe. *Naturwiss.*, 52 (1965) 620 — on SE-52 at 225°.

TAYLOR, A., REA, R. A. AND KIRBY, D. R.: Improved procedure for the extraction of organochlorine pesticides from animal tissue. *Analyst*, 89 (1964) 497–498.

ZIELINSKI, Jr., W. L. AND FISHBEIN, L.: Gas chromatography of carbamate derivatives. II. N-Substituted carbamates. *J. Gas Chromatog.*, 3 (1965) 331–335 — relation between structure and retention data for 33 derivatives on Carbowax 20M, Versamid 900 and SE-30 is discussed.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

CASTELLO, G., BIAGINI, E. AND MUNARI, S.: The quantitative determination of hydrogen in gases, by gas chromatography with helium as the carrier gas. *J. Chromatog.*, 20 (1965) 447–451 — peak inversion is thoroughly discussed.

HANDY, J. F. C.: Gas chromatography in metallurgical analysis. *Chem. Progress (England)*, 10, No. 11 (1964) 54–59 — H_2,N_2 and CO on molecular sieve 10X after fission of metal at 1600°.

IKELS, K. G.: Gas chromatographic determination of small volumes of nitrogen dissolved in blood. *J. Gas Chromatog.*, 3 (1965) 359–362 — complete design given; accuracy is within 0.3 μl of nitrogen.

KIM YOU-CHU: (Methods of chromatographic gas analysis). *Punsoh Khvakhak (Korea)*, 4, No. 1 (1965) 35–44.

MIĘSOWICZ, H.: (Determination of argon in circulating gas from the synthesis of ammonia by gas chromatography). *Chem. Anal. (Warsaw)*, 10 (1965) 1171–1174 — on molecular sieve 5A; no reference to the paper by M. KREJČÍ *et al.* (*Collection Czech. Chem. Commun.*, 24 (1959) 3887) and further papers dealing with the same subject.

SHTOF, M. D.: (Construction of calibration graphs for the chromatographic determination of argon in gases). *Tr. Kuibyshevsk. Gos. Nauchn. Issled. Inst. Neft. Prom.*, No. 23 (1964) 202–204.

SOMOV, A. P. AND ZHUKHOVITSKIĬ, A. A.: (Determination of microvolumes of gases by a vacancy-ionization method and its use for investigation of heterogeneous processes). *Zavodsk. Lab.*, 31 (1965) 1442–1445 — sensitivity 10^{-7} ml; used for the determination of thermal dissociation of *e.g.* PbO_2.

WALKER, J. A. J.: Chromatographic separation of argon and oxygen using molecular sieve. *Nature*, 209 (1966) 197 — molecular sieve 5A must be activated at 400° for 24 h in He; separation ratio Ar = 1.57, O_2 = 1.89 when H_2 = 1.00 at 75°; slight deterioration in resolution occurred over a 5-day period.

33b. Volatile inorganic compounds

BEREZKIN, V. G. AND GORSHUNOV, O. L.: (Application of frontal-chemical concentration method for chromatographic determination of microimpurities in carbon dioxide). *Izv. Akad. Nauk SSSR, Ser. Khim.*, No. 11 (1965) 2069–2070 — 10^{-5}–10^{-6} vol. % of C_1–C_3 paraffins in 200 ml sample of CO_2; principle has often been described.

MUSHA, S. AND NISHIMURA, T.: Gas-chromatographic analysis of water by using lead tetraacetate. *Japan Analyst*, 14 (1965) 803–809.

34. RADIOACTIVE AND OTHER ISOTOPIC COMPOUNDS

BOCOLA, W., BRUNER, F. AND CARTONI, G. P.: Separation of oxygen isotopes by gas chromatography. *Nature*, 209 (1966) 200–201 — on 170 m long glass capillary etched by NaOH solution; separation ratio $^{18}O_2/^{16}O_2$ is 1.0036 at 91.2°K [$t_R(^{16}O_2)$ = 55.8 min] and 1.0107 at 80.2°K [$t_R(^{16}O_2)$ = 176 min]; enthalpy and entropy differences are 7.79 cal and 0.1 e.u.

ODELL, A. L., PACKER, J. E. AND WHITE, G. R.: Labelled products from the internal radiolysis of liquid cyclohexane saturated with tritium gas. *Nature*, 209 (1966) 72–73 — on silicone oil, di-(2-cyanoethyl) ether, and benzyldiphenyl at 25°.

PARABUZIN, V. S. AND PANCHENKOV, G. M.: (Temperature-dependence of the coefficients of separation of isotopic molecular D_2-H_2, D_2-HD, and HD-H_2 on sunthetic zeolites NaA and NaX for sorption under isobaric conditions). *Dokl. Akad. Nauk SSSR*, 164 (1965) 856–859.

VOLKOV, S. A. AND SAKODYNSKIĬ, K. I.: (Separation of labelled compounds by gas chromatography). *At. Energ. (USSR)*, 17 (1964) 70–71 — deuterated ethoxymethylsilanes on DNP at 95°.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

GIBSON, N. A. AND HOSKING, J. W.: Permanganate oxidation in non-aqueous solvents. I. Oxidation by triphenylmethylarsonium permanganate. *Australian J. Chem.*, 18 (1965) 123–125 — oxidation products by GC.

JOSIF, I. AND SCHNEIDER, I. A.: (Analysis of oxidation products of methane by a gas chromatographic method). *Studii Cercetari, Chim. Acad. RPR (Bucurest)*, 13 (1964) 453–458.

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SUPPLEMENT TO THE
JOURNAL OF CHROMATOGRAPHY
VOL. 22 (1966)

EDITORS:

C. G. CASINOVI (Rome)
J. GASPARIČ (Pardubice-Rybitví)
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K. MACEK (Prague)

ELSEVIER PUBLISHING COMPANY
AMSTERDAM

PRINTED IN THE NETHERLANDS

TABLE 1

TLC R_F VALUES OF MALTOSE ACETATES

(M. L. WOLFROM AND R. M. DE LEDERKREMER, *J. Org. Chem.*, 30 (1965) 1560)

Thin layer: Silica Gel G (Merck), 0.25 mm.
Solvents: S_1 = Ethyl acetate–benzene (1:1).
S_2 = Ethyl acetate–benzene (7:3).

Compound	R_F	
	S_1	S_2
Octa-O-acetyl-β-maltose	0.61	0.81
2,3,6,2′,3′,4′,6′-Hepta-O-acetyl-β-maltose	0.30	0.55
2,3,6,2′,3′,4′,6′-Hepta-O-acetyl-α-maltose	0.08	0.22
3,6,2′,3′,4′,6′-Hexaacetyl-β-maltose	0.10	0.27

TABLE 2

PC R_F VALUES OF CHOLINE AND RELATED COMPOUNDS

(L. M. LEWIN AND N. MARCUS, *Anal. Biochem.*, 10 (1965) 99)

Paper: Whatman No. 1.
Technique: Ascending for approximately 16 h.
Solvents: S_1 = Ethanol–water (8:1).
S_2 = *n*-Butanol–acetic acid–water–ethanol (8:1:3:2).
Detection: D_1 = Bioautography after incubating with cholinesterase.
D_2 = Bioautography after incubating with phosphatase.

Compound	R_F		*Detection*
	S_1	S_2	
Choline chloride	0.61	0.31	
Acetylcholine chloride	0.64	0.41	D_1
Propionylcholine iodide	0.71	0.55	D_1
Butyrylcholine iodide	0.77	0.66	D_1
Phosphocholine chloride	0.00	0.10	D_2
Lecithin	0.82	0.73	D_2
Lysolecithin	0.79	0.73	D_2
Methionine	0.39	0.44	

TABLE 3

PC R_F VALUES OF ISOMERIC UREAS AND THIOUREAS

(L. FISHBEIN, *Rec. Trav. Chim.*, 84 (1965) 470–472)

Paper: Whatman No. 1.
Technique: Ascending.
Temperature: 22° ± 0.5°.
Solvents: S_1 = *n*-Butanol–acetic acid–water (4:1:2).
S_2 = *n*-Butanol–95% ethanol–water (4:1:1).
S_3 = Chloroform–methanol–water (7:5:1).
Detection: D_1 = Mixture of equal volumes of 10% sodium hydroxide, 10% sodium nitro-prusside, 10% potassium ferricyanide diluted with 3 volumes of water.
D_2 = Ninhydrin.
D_3 = Grote's reagent.
D_4 = Fearon's reagent.
D_5 = Ehrlich's reagent.
D_6 = 50 ml of a *N* iodine solution added to 50 ml of 95% ethanol; 1.5 g sodium azide then dissolved in this solution.
D_7 = Phenol–hypochlorite.
D_8 = Cobalt nitrate–*p*-dimethylaminobenzaldehyde.
D_9 = The same as D_8, followed by exposure of the chromatogram to HCl vapours.
D_{10} = Sequence of D_8, D_9 followed by D_1.

Compound	$R_F \times 100$		
	S_1	S_2	S_3
Urea	53	52	34
N-Methylurea	59	55	41
N-Ethylurea	62	59	43
N-*n*-Propylurea	67	63	46
N-*n*-Butylurea	71	68	50
N-*tert.*-Butylurea	80	77	58
N-*tert.*-Amylurea	85	80	63
N-β-Hydroxyethylurea	66	62	47
N-β-Hydroxypropylurea	71	67	51
N,N′-Dimethylurea	67	59	54
N,N′-Diethylurea	70	64	57
N,N′-Di-*n*-propylurea	74	69	60
N,N′-Di-*n*-amylurea	77	74	64
N,N-Dimethylurea	68	58	55
N,N-Diethylurea	71	64	59
N,N-Di-*n*-propylurea	75	67	62
Methylpseudourea	57	46	41
Ethylpseudourea	61	49	41
Thiourea	56	41	43
N-Methylthiourea	54	43	46
N-Ethylthiourea	59	48	51
N-Isopropylthiourea	65	53	55
N-Allylthiourea	61	51	52
N-Isobutylthiourea	71	58	58
N-*n*-Heptylthiourea	88	75	70
N,N′-Diethylthiourea	88	66	62
N,N′-Diisopropylthiourea	92	70	65
N,N′-Di-*n*-butylthiourea	95	76	68
2-Methyl-2-thiopseudourea	42	28	36
2-Ethyl-2-thiopseudourea	47	31	40
2-*n*-Propyl-2-thiopseudourea	54	36	45
2-*n*-Butyl-2-thiopseudourea	61	41	48
N,N′,S-trimethylthiopseudourea	60	40	50
N,N,N′-Triethyl-S-methylthiopseudourea	80	61	73

(*continued on p.* D3)

TABLE 3 (*continued*)

Compound	$R_F \times 100$		
	S_1	S_2	S_3
2-Methyl-1(or 3)-nitro-2-thiopseudourea	44	31	40
2-Ethyl-1(or 3)-nitro-2-thiopseudourea	48	35	44
2-*n*-Propyl-1(or 3)-nitro-2-thiopseudourea	54	40	48
2-*n*-Butyl-1(or 3)-nitro-2-thiopseudourea	64	45	53
2-(β-Hydroxyethyl)-2-thiopseudourea	52	36	44
2-(δ-Hydroxypropyl)-2-thiopseudourea	60	40	47
2-(ω-Hydroxybutyl)-2-thiopseudourea	65	46	53
2-(ω-Hydroxyamyl)-2-thiopseudourea	69	49	57
2-(δ-Nitroxypropyl)-1(or 3)-nitro-2-thiopseudourea	62	46	52
2-(ω-Nitroxybutyl)-1(or 3)-nitro-2-thiopseudourea	68	53	56
2-(ω-Nitroxyamyl)-1(or 3)-nitro-2-thiopseudourea	73	58	60
1,5-Pentamethylene-bis-(2-thiourea)	34	33	41
1,10-Decamethylene-bis-(2-thiourea)	55	54	67
Ethylene-bis-(2-thiopseudourea)	22	20	26
Trimethylene-bis-(2-thiopseudourea)	26	25	30
Tetramethylene-bis-(2-thiopseudourea)	30	29	35
Pentamethylene-bis-(2-thiopseudourea)	35	35	40
Decamethylene-bis-(2-thiopseudourea)	55	57	67
Ethylene-bis-(1 or 3)-nitro-2-thiopseudourea	26	25	31
Trimethylene-bis-(1 or 3)-nitro-2-thiopseudourea	31	30	36
Tetramethylene-bis-(1 or 3)-nitro-2-thiopseudourea	36	36	40
Pentamethylene-bis-(1 or 3)-nitro-2-thiopseudourea	40	41	45
Decamethylene-bis-(1 or 3)-nitro-2-thiopseudourea	63	67	72
N,N′-Ethyleneurea	60	45	48
N,N′-Trimethyleneurea	65	49	53
Ethylenepseudourea	45	42	45
Propylenepseudourea	51	47	49
Trimethylenepseudourea	51	46	48
Ethylenethiourea	65	61	63
Propylenethiourea	69	64	68
Ethylenethiopseudourea	64	60	54
Propylenethiopseudourea	67	64	58

Class of compounds	*Spot colours of isomeric ureas and thioureas* *									
	D_1	D_2	D_3	D_4	D_5	D_6	D_7	D_8	D_9	D_{10}
Monoalkylureas	C	—	Y	—	R	—	G	Y**	Y	O
N,N′-Dialkylureas	C	—	Y	—	R	—	G	L**	Y	P-O
N,N-Dialkylureas	C	—	Y	—	R	—	Y-G	L**	Y	P-O
Alkylpseudoureas	Y	—	Y	Y	Y	—	—	—	T	Y-O
Alkylthioureas	B	—	—	B	B	W	—	B	Y-G	B-G
Dialkylthioureas	B	—	—	B	B	W	—	B-G	B-G	B-G
Alkylthiopseudoureas	P		—	O	O	—	—	—	R	P
Polymethylene-bis-(2-thiopseudoureas)	P	—	—	O	O	—	—	—	R	P
Polymethylene-bis-(2-thioureas)	B	—	—	B	B	W	—	B	B-G	B-G
Cyclic ureas	R	—	Y	—	—	—	G	Y**	Y	R
Cyclic pseudoureas	O	V	Y	O	O	—	Y-G	L**	Y	R
Cyclic thioureas	B	B	Y	B	B	W	—	Y**	Y	B
Cyclic thiopseudoureas	P-B	B	Y	P	C-P	Y	—	B**	R	B-P

* B = blue; C = crimson; G = green; L = lilac; O = orange; P = purple; R = rose; T = tan; V = violet; W = white; Y = yellow.

** Colours developed after heating at 80° for 1 min.

TABLE 4

PC R_F VALUES OF SOME ORGANOPHOSPHORUS PESTICIDES

(J. A. R. BATES, *Analyst*, 90 (1965) 457)

Paper: Whatman No. 20.

Impregnation: I_1 = 30% dimethylformamide in acetone (v/v).
I_2 = 5% liquid paraffin in diethyl ether (v/v).
I_3 = 20% formamide in acetone (v/v).

Mobile phases: S_1 = Hexane.
S_2 = Dimethylformamide–water (1:1).
S_3 = Benzene–chloroform (9:1).

Detection: D_1 = Ultraviolet light.
D_2 = 2,6-Dibromo-N-chloro-*p*-quinoneimine (0.5% solution in cyclohexane); after spraying heating to 120° for 10 min.
D_3 = Blue tetrazolium (0.1% solution in water mixed with 9 parts of 2 *N* sodium hydroxide just before spraying); after spraying dry at 50° for 5 min.
D_4 = Bromine–4-methylumbelliferone (expose the chromatogram to bromine vapours and then spray with a solution of 0.15 g 4-methylumbelliferone in 100 ml ethanol, 200 ml of water and 10 ml of 0.1 *N* ammonia, then watch in ultraviolet light).
D_5 = Silver nitrate–bromophenol blue (90 ml of a 2% solution (w/v) of silver nitrate in water are mixed with 10 ml of a 0.4% solution (w/v) of bromophenol blue in acetone just before spraying).

Compound	R_F*			*Colour with* D_2
	I_1S_1	I_2S_2	I_3S_3	
Vamidothion sulphoxide	0.00	0.97	0.06	yellow
Dimethoate	0.00	0.95	0.78	orange-brown
Vamidothion	0.00	0.95	0.5	yellow
Oxydemeton-methyl	0.00	0.90	0.18	yellow
Menazon	0.00	0.78	0.08	red
Formothion	0.09	0.96	—	red-brown
Phosphamidon	0.10	0.92	—	no colour
Mevinphos	0.15	0.92	—	no colour
Azinphos-methyl	0.18	0.92	—	orange-brown
Azinphos-ethyl	0.63	0.85	—	orange-brown
Parathion	0.82	0.65	—	red-brown
Malathion	0.85	0.85	—	orange-brown
Mecarbam	0.85	0.79	—	red-brown
Fenthion	0.91	0.65	—	red
Phorate	0.96	0.36	—	orange-brown
Diazinon	0.98	0.51	—	red-brown
Disulfoton	0.98	0.29	—	orange-brown
Ethion	0.98	0.20	—	orange-brown
"Trithion"	0.98	0.18	—	orange-brown
Phenkapton	0.98	0.10	—	yellow-brown

* Calculated from the leading edge of the spots.

TABLE 5

TLC R_F VALUES OF SOME ORGANOPHOSPHORUS COMPOUNDS

(J. HANKIEWICZ AND K. STUDNIARSKI, *Chem. Anal. (Warsaw)*, 10 (1965) 943)

Thin layer: Silicagel G.
Solvents: S_1 = Benzene–ethyl acetate–methylene chloride (2:1:1).
S_2 = Benzene–ethyl acetate–chloroform (2:1:1).
S_3 = Benzene–ethyl acetate–chloroform (4:2:1).
Detection: D_1 (for compounds 1–6, 7–10) = Potassium permanganate in sulphuric acid.
D_2 (for compounds 8 and 9) = Successive spraying with 0.5% ethanolic thymol blue, 2% ethanolic potassium hydroxide and 1% ceric sulphate in 10% sulphuric acid.

No.	*Compound*	R_F		
		S_1	S_2	S_3
1	$(CH_3O)_2P(O)–S–S–P(O)(OCH_3)_2$	0.25		0.19
2	$(CH_3O)_2P(O)–S–S–P(O)(OC_2H_5)_2$	0.38		0.26
3	$(C_2H_5O)_2P(O)–S–S–P(O)(OC_2H_5)_2$	0.54	0.31	0.39
4	$(C_2H_5O)_2P(O)–S–S–P(O)(OC_3H_7)_2$	0.61		0.44
5	$(C_3H_7O)_2P(O)–S–S–P(O)(OC_3H_7)_2$	0.75		0.58
6	$(C_2H_5O)_2P(O)–S–S–C_6H_5$	0.55	0.55	
7	$(C_2H_5O)_2P(O)–S–CH_2CH_2Cl$	0.53		
8	$(C_2H_5O)_3PS$		0.32	0.81
9	$(C_2H_5O)_2P(S)Cl$		0.41	0.95
10	$(C_6H_5)–S–CH_2CH_2Cl$	0.92		

TABLE 6

TLC R_F VALUES OF SOME ORGANOPHOSPHORUS COMPOUNDS

(J. HANKIEWICZ AND K. STUDNIARSKI, *Chem. Anal. (Warsaw)*, 10 (1965) 943)

Thin layer: Silicagel G.
Solvents: S_1 = Ligroine (60–80°)–ethyl acetate–methylene chloride (10:10:1).
S_2 = Benzene–acetone–chloroform (20:10:3).
S_3 = Acetonitrile–ethyl acetate–methylene chloride (4:1:2).
Detection: D_1 (for compounds 1–5 and 9) = Successive spraying with 2% ethanolic solution of picric acid and a 1:1 mixture of 2% ethanolic thymol blue and 5% ethanolic potassium hydroxide.
D_2 (for compounds 6, 7 and 8) = Successive spraying with 5% silver nitrate, 2% ethanolic solution of thymol blue and 2% aqueous solution of ceric sulphate.

No.	*Compound*	R_F		
		S_1	S_2	S_3
1	$(C_2H_5O)_2P(O)–O–P(O)(OC_2H_5)_2$	0.26	0.48	0.41
2	$(C_2H_5O)_2P(O)–O–P(O)(OC_3H_5)_2$	0.38	0.59	
3	$(C_3H_5O)_2P(O)–O–P(O)(OC_3H_5)_2$	0.47	0.61	
4	$(C_2H_5O)_2P(O)–O–P(O)(On\text{-}C_4H_9)_2$	0.29	0.65	
5	$(n\text{-}C_4H_9O)_2P(O)–O–P(O)(On\text{-}C_4H_9)_2$	0.33	0.79	
6	$(C_2H_5)(C_2H_5O)P(O)–O–P(O)(C_2H_5O)_2$			0.26
7	$(C_2H_5)(C_2H_5O)P(O)–O–P(O)(C_2H_5)(C_2H_5O)$			0.29
8	$(C_2H_5O)_2P(O)–P(O)(OC_2H_5)_2$			0.41
9	$(C_2H_5O)_3P$		0.20	0.75

TABLE 7

PC R_F VALUES OF PYROCATECHOL DERIVATIVES

(J. POSPÍŠIL AND L. TAIMR, *Collection Czech. Chem. Commun.*, 30 (1965) 1516)

Paper: Whatman No. 1.
Impregnation: I_1 = 20% solution of formamide in methanol.
I_2 = Solution of 5 g boric acid and 20 ml formamide in 80 ml acetone.
I_3 = 40% solution of dimethylformamide in benzene.
Mobile phases: S_1 = Diisopropyl ether.
S_2 = Chloroform.
S_3 = Heptane–benzene (1:1).
S_4 = Heptane.
Detection: $FeCl_3$ + $K_3[Fe(CN)_6]$.

Pyrocatechol derivative	R_F								
	I_1S_1	I_2S_1	I_1S_2	I_2S_2	I_1S_3	I_2S_3	I_1S_4	I_2S_4	I_3S_4
Pyrocatechol	0.28	0.20	0.08	—					
4-Methylpyrocatechol	0.43	0.33	0.15	0.10					
4,5-Dibromopyrocatechol	0.75	0.56	0.15	0.07					
3-Methylpyrocatechol	0.53	0.40	0.20	0.12					
3,4,5-Tribromopyrocatechol	0.76	0.30	0.22	0.06					
3,4,5,6-Tetrachloropyrocatechol	0.75	0.12	0.25	0.04					
4-Ethylpyrocatechol	0.57	0.49	0.25	0.18					
3,4,5,6-Tetrabromopyrocatechol	0.67	0.07	0.30	0.05					
4-Valerylpyrocatechol	0.51	0.30	0.34	0.16					
4-*n*-Propylpyrocatechol	0.70	0.63	0.38	0.30					
4-*tert.*-Butylpyrocatechol	0.78	0.72	0.47	0.38					
4-Capronylpyrocatechol	0.67	0.43	0.51	0.25					
3-Isopropylpyrocatechol	0.81	0.57	0.51	0.27					
4-*n*-Butylpyrocatechol	0.82	0.75	0.52	0.45					
4-Methyl-5-*tert.*-butylpyrocatechol	0.79	0.77	0.55	0.53					
4-*tert.*-Amylpyrocatechol	0.86	0.83	0.59	0.48					
4-Enanthoylpyrocatechol	0.78	0.56	0.64	0.40					
4-Isoamylpyrocatechol			0.64	0.56	0.15	0.12			
4-*n*-Amylpyrocatechol			0.65	0.59	0.16	0.13			
3-Methyl-5-*tert.*-butylpyrocatechol			0.69	0.52	0.25	0.14			
4-*n*-Hexylpyrocatechol			0.76	0.73	0.28	0.21			
4-*tert.*-Octylpyrocatechol			0.76	0.76	0.43	0.42			
4-Methyl-5-*tert.*-octylpyrocatechol			0.77	0.83	0.44	0.53			
3-*tert.*-Butyl-5-methylpyrocatechol			0.77	0.41	0.53	0.14			
4-*n*-Heptylpyrocatechol			0.82	0.79	0.52	0.40			
3-*tert.*-Butyl-6-methylpyrocatechol					0.63	0.07	0.18		
3-Methyl-5-*tert.*-octylpyrocatechol					0.72	0.67	0.21		0.11
3,5-Di-*tert.*-butylpyrocatechol					0.82	0.49	0.39	0.10	0.20
3-*tert.*-Octyl-5-methylpyrocatechol					0.83	0.55	0.45	0.18	0.31
3-Methyl-4,6-di-*tert.*-butylpyrocatechol					0.89	0.41	0.59	0.08	0.32
3-Methyl-6-*tert.*-octylpyrocatechol					0.91	0.39	0.64	0.09	0.30
3-*tert.*-Butyl-5-*tert.*-octylpyrocatechol							0.68	0.48	0.54
3,5-Di-*tert.*-amylpyrocatechol							0.75	0.34	0.55
3-*tert.*-Octyl-5-*tert.*-butylpyrocatechol							0.77	0.61	0.58
3,5-Di-*tert.*-octylpyrocatechol									0.75

TABLE 8

PC R_F VALUES OF SOME *o*-ALKOXYPHENOL DERIVATIVES

(J. POSPÍŠIL AND L. TAIMR, *Collection Czech. Chem. Commun.*, 30 (1965) 1518)

Paper: Whatman No. 1.
Impregnation: I_1 = 20% solution of formamide in methanol.
I_2 = 40% solution of dimethylformamide in benzene.
I_3 = 10% solution of paraffin oil in hexane.
Mobile phases: S_1 = Chloroform.
S_2 = Heptane–benzene (1:1).
S_3 = Heptane.
S_4 = 80% methanol.
Detection: D_1 = $FeCl_3$ + $K_3[Fe(CN)_6]$.
D_2 = 2,4-Dinitrophenylhydrazine.

Compound	$R_F \times 100$				
	I_1S_1	I_1S_2	I_1S_3	I_2S_3	I_3S_4
3-Methoxy-4-hydroxybenzaldehyde	64	13			
3-Hydroxy-4-ethoxybenzaldehyde	75	20			
3-Ethoxy-4-hydroxybenzaldehyde	78	33			
2-Methoxyphenol		55			
2-Hydroxy-3-methoxybenzaldehyde		63	18		
2-Methoxy-4-methylphenol		70	34	09	
2-Hydroxy-3-ethoxybenzaldehyde		79	41	23	
2-Ethoxyphenol			44	13	
2-Methoxy-4-allylphenol			53	12	
2-Ethoxy-4-methylphenol			60	20	
2-Ethoxy-5-methylphenol			61	21	
2-Methoxy-4-methyl-5-*tert.*-butylphenol			77	36	83
2-Ethoxy-6-methylphenol			76	40	76
2-Ethoxy-4-methyl-5-*tert.*-butylphenol				55	76
2-Ethoxy-4-*tert.*-butyl-5-methylphenol				59	78
2-Methoxy-4-methyl-5-*tert.*-octylphenol				66	70
2-Ethoxy-4-methyl-5-*tert.*-octylphenol				81	57
2-Ethoxy-4-*tert.*-butyl-6-methylphenol					56
2-Ethoxy-4-*tert.*-octyl-5-methylphenol					54
2-Methoxy-4-methyl-6-*tert.*-butylphenol					49
2-Ethoxy-3(5?)-*tert.*-butyl-6-methylphenol					44
2-*tert.*-Butoxy-4(5?)-*tert.*-octylphenol					43
2-Ethoxy-4-methyl-6-*tert.*-butylphenol					34
2-Methoxy-4-methyl-6-*tert.*-octylphenol					27
2-Ethoxy-4-*tert.*-octyl-6-methylphenol					27
2-Ethoxy-4-methyl-6-*tert.*-octylphenol					16
3-*tert.*-Butyl-5-*tert.*-octylpyrocatechol *tert.*-butyl ether					04

TABLE 9

PC R_F VALUES OF SOME *o*-DIALKOXYBENZENE DERIVATIVES

(J. POSPÍŠIL AND L. TAIMR, *Collection Czech. Chem. Commun.*, 30 (1965) 1519)

Paper: Whatman No. 1.
Impregnation: I_1 = 40% solution of dimethylformamide in benzene.
I_2 = 10% solution of paraffin oil in hexane.
Mobile phases: S_1 = Heptane.
S_2 = 80% methanol.
Detection: $FeCl_3$ + $K_3[Fe(CN)_6]$.

Compound	R_F	
	I_1S_1	I_2S_2
3,4-Dimethoxytoluene	0.48	0.75
3,4-Diethoxytoluene	0.74	0.64
3,4-Dimethoxy-6-*tert.*-butyltoluene	0.82	0.58
2,3-Diethoxytoluene		0.57
3,4-Diethoxy-6-*tert.*-butyltoluene		0.36
3,4-Dimethoxy-6-*tert.*-octyltoluene		0.34
2,3-Diethoxy-*x*-*tert.*-butyltoluene		0.18
3,4-Diethoxy-5-*tert.*-butyltoluene		0.17
3,4-Diethoxy-6-*tert.*-octyltoluene		0.15

TABLE 10

PC R_F VALUES OF SOME AROMATIC AMINES

(J. GASPARIČ AND B. KLOUČEK, *Collection Czech. Chem. Commun.*, 31 (1966) 108)

Paper: Whatman No. 3 impregnated with formamide (20% ethanolic solution).
Mobile phase: *n*-Heptane.
Detection: D_1 = Ehrlich reagent.
D_2 = 1-Diazo-2-chloro-4-nitrobenzene.

Amine	R_F
Aniline	0.30
2-Methylaniline	0.57
2-Amino-1,4-dimethylbenzene	0.77
4-Chloroaniline	0.38
4-Chloro-2-methylaniline	0.64
5-Chloro-2-methylaniline	0.65
2-Methoxyaniline	0.44
4-Methoxyaniline	0.13
5-Chloro-2-methoxyaniline	0.61
2-Methyl-4-methoxyaniline	0.28
2,5-Dimethoxyaniline	0.26
2,5-Dimethoxy-4-chloroaniline	0.27
2,4-Dimethoxy-5-chloroaniline	0.24
2-Ethoxyaniline	0.69
4-Ethoxyaniline	0.29
1-Naphthylamine	0.48
2-Naphthylamine	0.42
o-Tolidine	0.06
o-Dianisidine	0.04
2-Methoxy-4-chloro-5-methylaniline	0.72

TABLE 11

PC R_F VALUES OF SOME AROMATIC HYDROXY COMPOUNDS

(J. GASPARIČ AND B. KLOUČEK, *Collection Czech. Chem. Commun.*, 31 (1966) 107)

Paper: Whatman No. 3 impregnated with formamide (20% alcoholic solution).
Mobile phase: *n*-Heptane–benzene (1:1).
Detection: 0.1% solution of 1-diazo-2-chloro-4-nitrobenzene 1,5-naphthalenedisulphonate and then 5% sodium hydroxide in water–ethanol (1:1).

Compound	R_F	*Colour**	
1-Naphthol	0.43	yellow	blue
2-Naphthol	0.33	orange	orange
2-Hydroxycarbazole	0.03	yellowish brown	violet
2-Hydroxyanthracene	0.46	violet-red	blue-green
3-Hydroxydiphenylene oxide	0.41	brown	grey-green
2-Hydroxy-5,6,7,8-tetrahydronaphthalene	0.61	brown	brown
2,6-Dihydroxynaphthalene	0	reddish violet	blue-green

* The first colour is that after spraying with the solution of the diazonium salt, the second one after spraying with sodium hydroxide.

TABLE 12

PC R_F VALUES OF SOME BROMINATED 2-HYDROXYNAPHTHALENES

(A. POSTAWKA AND L. PRAJER-JANCZEWSKA, *Chem. Anal. (Warsaw)*, 10 (1965) 980)

Paper: Whatman No. 1.
Technique: Descending.
Solvents: S_1 = Water–ammonia (20:1).
S_2 = Water–methanol–ammonia (20:1:1).
S_3 = Water–pyridine (20:4).
S_4 = Water–pyridine–ammonia (20:2:1).
S_5 = Water–pyridine–ammonia (20:6:1).
S_6 = Water–pyridine–ammonia (20:4:0.5).
S_7 = Water–pyridine–ammonia (20:4:0.2).
Detection: $FeCl_3 + K_3[Fe(CN)_6]$.

Naphthalene derivative	$R_F \times 100$						
	S_1	S_2	S_3	S_4	S_5	S_6	S_7
2-Hydroxy-	59	53	49	48	59	63	56
1-Bromo-2-hydroxy-	44	42	43	38	49	62	52
3-Bromo-2-hydroxy-	44	42	43	38	48	61	52
6-Bromo-2-hydroxy-	30	27	40	25	34	53	35
8-Bromo-2-hydroxy-	39	33	40	30	40	58	41
1,6-Dibromo-2-hydroxy-	16	16	39	16	28	53	44
5,8-Dibromo-2-hydroxy-	16	16	21	13	25	44	30

TABLE 13

TLC R_F VALUES OF SOME PLASTICIZERS

(D. BRAUN, *Chimia (Aarau)*, 19 (1965) 79)

Thin layer: Kieselgel G (Merck).
Solvent: Methylene chloride.
Detections: D_1 = 20% solution of antimony pentachloride in tetrachloromethane, after spraying heat to 120°.
D_2 = Iodine.

Compound	R_F
Dimethyl phthalate	0.51
Dibutyl phthalate	0.69
Dihexyl phthalate	0.80
Dioctyl phthalate	0.86
Di-2-ethylhexyl phthalate	0.85
Didecyl phthalate	0.85
Diisodecyl phthalate	0.84
Trioctyl phosphate	0.23
Diphenyl octyl phosphate	0.42
Triphenyl phosphate	0.47
Diphenyl cresyl phosphate	0.51
Tricresyl phosphate	0.53
Dioctyl adipate	0.42
Di-2-ethylhexyl adipate	0.44
Dinonyl adipate	0.44
Adipic acid polyester	0.02
Dibutyl sebacate	0.41
Dioctyl sebacate	0.61
Di-2-ethylhexyl sebacate	0.61
Sebacic acid polyester	0.02
Triethyl citrate	0.12
Tributyl citrate	0.14
Acetyl triethyl citrate	0.15
Acetyl tributyl citrate	0.24
Acetyl tri-2-ethylhexyl citrate	0.46
Glycerol triacetate	0.13

TABLE 14

PC AND TLC R_F VALUES OF VARIOUS *Genista* ALKALOIDS

(R. BERNASCONI, ST. GILL AND E. STEINEGGER, *Pharm. Acta Helv.*, 40 (1965) 253)

Paper: Schleicher & Schüll 2045 bM.
Thin layer: Kieselgel G.
Solvents: S_1 = Isopropanol–HCl–water (8:1:1), PC.
S_2 = *n*-Butanol–acetic acid–water (50:2:17), PC.
S_3 = Cyclohexane–diethylamine (7:3), TLC.
S_4 = Chloroform–methanol (8:2), TLC.
Detection: U.V.-250 and Dragendorff reagent.

Alkaloid	R_F			
	S_1	S_2	S_3	S_4
Cytisine	0.17	0.12	0.07	0.32
Methylcytisine	0.23	0.10	0.30	0.63
Anagyrine	0.31	0.24	0.35	0.60
Lupanine	0.66	0.37	0.61	0.38
Hydroxylupanine	0.50	0.22	0.19	0.24
Lupinine	0.72	0.33	0.63	0.12
Epilupinine	0.76	0.36	0.56	0.35
Sparteine	0.59	0.45	0.93	0.10
Hydroxysparteine	0.43	0.35	0.54	0.19
17-Oxosparteine	0.82	0.37	0.74	0.53
Retamine	0.45	0.14	0.80	0.30
l-α-Isosparteine	0.52	0.56	0.92	0.03
Calycotomine	0.36	0.26	0.05	0.46

TABLE 15

PC R_F VALUES OF SOME SOLANACEOUS ALKALOIDS

(M. S. KARAWYA AND S. I. BALBAA, *J. Pharm. Sci. U. Arab Rep.*, 4 (1963) 57; *C.A.*, 63 (1965) 9745d)

Paper: Whatman No. 1 impregnated with McIlvaine buffers.
Technique: Descending.
Solvents: S_1 = Butanol satd. with McIlvaine buffer, pH 3.8.
S_2 = Butanol satd. with McIlvaine buffer, pH 5.4.
S_3 = Butanol satd. with McIlvaine buffer, pH 6.8.

Alkaloid	R_F		
	S_1	S_2	S_3
Oscine	0.045	0.029	0.07
Tropine	0.08	0.03	0.028
Belladonine	0.20	0.16	0.38
Scopolamine	0.29	0.43	0.85
Meteloidine	0.39	0.34	0.66
Hyoscyamine	0.57	0.51	0.65
Norhyoscyamine	0.60	0.48	0.56
Valeroidine	0.62	0.58	0.71
Tigloidine	0.71	0.70	0.83
Apoatropine	0.81	0.79	0.82

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