JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

EDITOR

MICHAEL LEDERER (Rome)

EDITORIAL BOARD

P. Boulanger (Lille), C. B. Coulson (Legon, Ghana), G. Duyckaerts (Liège), J. E. Falk (Canberra), I. M. Hais (Hradec Králové), A. T. James (Sharnbrook), J. Janák (Brno), A. I. M. Keulemans (Eindhoven), K. A. Kraus (Oak Ridge, Tenn.), E. Lederer (Gif-sur-Yvette, S. et O.), A. Liberti (Naples), J. F. W. McOmie (Bristol), G. B. Marini-Bettòlo (Rome), R. Neher (Basel), F. H. Pollard (Bristol), J. Schubert (Pittsburgh, Pa.), G. M. Schwab (Munich), J. Shankar (Bombay), A. Tiselius (Uppsala), H. Tuppy (Vienna), O. Westphal (Freiburg-Zähringen).

EDITORS, BIBLIOGRAPHY SECTION

K. Macek (Prague)
J. Janák (Brno)

VOL. 16 1964

ELSEVIER PUBLISHING COMPANY

AMSTERDAM

All rights reserved

ELSEVIER PUBLISHING COMPANY

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

ZUR BESTIMMUNG VON ADSORPTIONSENTHALPIEN MIT HILFE DER GASCHROMATOGRAPHIE

HELMUT KNÖZINGER UND HANS SPANNHEIMER
Physikalisch-Chemisches Institut der Universität München (Deutschland)

(Eingegangen den 10. Februar 1964)

Im Rahmen heterogen katalytischer Untersuchungen ist es immer von Interesse. Aussagen auch über den mit der Adsorption verbundenen Energieumsatz zu gewinnen, Bekanntlich können die interessierenden Grössen auch mittels der Gaschromatographie gewonnen werden. CREMER und Mitarbeiter haben sich zuerst eingehend mit dieser Aufgabe befasst[1-4]. Da kommerzielle Geräte im allgemeinen wegen der zu geringen Genauigkeit besonders der darin verwendeten Manometer und Strömungsmesser und eventuell auftretender Temperaturgefälle längs der Säule für die Bestimmung physikalischer Grössen nicht geeignet sind[5], haben wir ein diese Voraussetzungen berücksichtigendes Gerät gebaut. Die Auswertung der Chromatogramme erfolgt nach einem gegenüber den aus der Literatur bekannten Methoden etwas abgewandelten und vereinfachten Verfahren.

DER GASCHROMATOGRAPH

Der Gesamtanordnung wurde das allgemein bekannte Bauprinzip eines Gaschromatographen zu Grunde gelegt. Als Druckmesser konnte bei den kleinen benötigten Säulenvordrucken ein Quecksilbermanometer verwendet werden, an dem der Druck auf $\pm$ 0.1 Torr abgelesen werden konnte. Die Strömungsmesser am Ende von Vergleichs- und Messgasweg waren zwei Seifenblasenströmungsmesser, die eine Messgenauigkeit von $\pm$ 0.1 ml/min zuliessen.

Es wurde ein Betrieb auch bei Temperaturen bis 800°C angestrebt. Einmal hat man dann die Möglichkeit, das Adsorbens in der Säule selbst bei erhöhter Temperatur zu reinigen und zu sintern, zum anderen kann man den gesamten katalytisch interessanten Temperaturbereich überstreichen. Zu diesem Zweck wurden spiralförmige Säulen aus Quarzglas mit einem Innendurchmesser von 7 mm, einem Spiraldurchmesser von 80 mm und einer Länge bis zu 750 mm hergestellt. Diese Säulen wurden in einem ringförmigen Hohlraum in einem aus zwei Teilen bestehenden, zylinderförmigen Metallblock aus hitzbeständigem Spezialstahl* beheizt. (Fig. 1). Die Säulentemperatur wurde mit einem im Zentrum des Metallblocks angebrachten Thermoelement bestimmt, nachdem festgestellt war, dass nach Einstellung des Temperaturgleichgewichts kein radialer Temperaturgradient auftrat. Längs der Säule wurde eine Temperaturkonstanz besser als 1°C erreicht. Der Säulenofen wurde mittels eines Regeltransformators

* AZ 18 der Stahlwerke Südwestfalen AG, Geisweid, Kreis Siegen.

mit Spannung versorgt. Eine Zweipunktregelung mit Grundlast (Fallbügelregler) ergab bei 250°C eine Temperaturkonstanz von ± 1°C.

Als Detektor diente eine Wärmeleitfähigkeitsbrücke*. Die Beheizung des Zellenblocks erfolgte elektrisch. Die Wicklung wurde auf einen an den Stirnflächen verschlossenen Messingzylinder gebracht. Die Wände des Zylinders hatten vom Zellenblock einen Abstand von 50 mm. Der so entstandene Luftraum dient zur Dämpfung

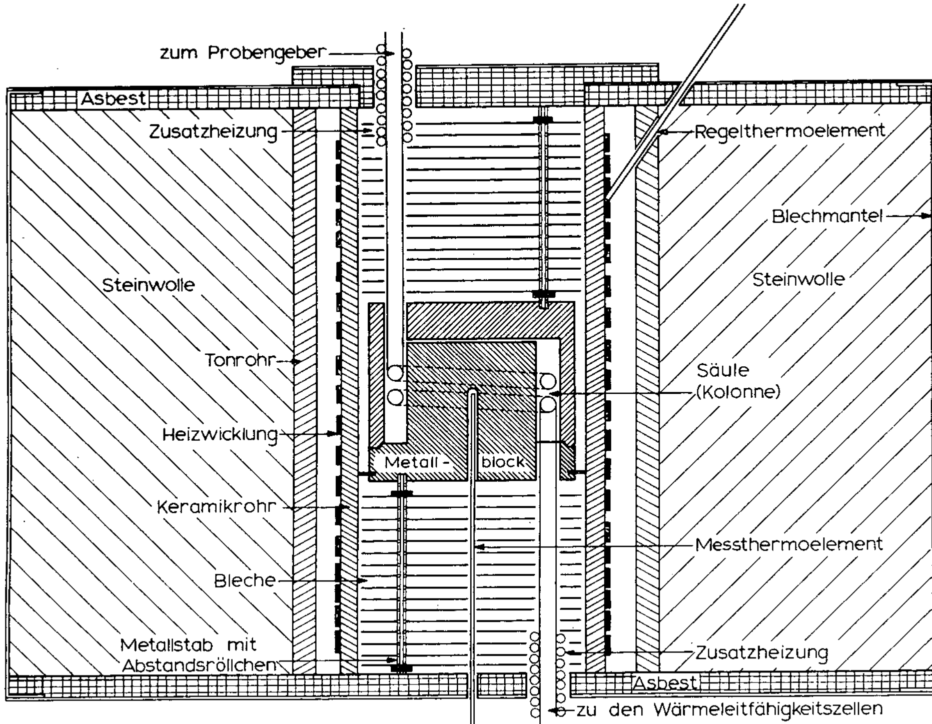


Fig. 1. Der Kolonnenofen.

kurzzeitiger, asymmetrischer Temperaturschwankungen, gegen die die Nullinienkonstanz sehr empfindlich ist. Diese Anordnung wurde noch in ein gut verschlossenes Gehäuse aus Wärmedämmplatten eingesetzt. Die Heizspannung wurde durch einen Magnetstabilisator konstant gehalten. Der Messgasanschluss wurde unmittelbar mit dem Säulenausgang verbunden und zur Kondensationsverhütung mit einer Zusatzheizung versehen. Um eine lineare Anzeige des Detektors zu erhalten, sollte die Wärmeleitfähigkeitsbrücke mit konstantem Strom gespeist werden. Eine verhältnismässig einfache Rechnung zeigt jedoch, dass im Bereich der auftretenden Konzentrationen auch Spannungskonstanz (Akkumulator) noch Linearität liefert, was auch experimentell bestätigt wird.

Die Aufgabe der flüssigen Proben erfolgte in der üblichen Weise mit einer 1 μl bzw. 10 μl Hamiltonspritze.

---

* Fa. Beckman Instruments, Inc.

AUSWERTUNG DER CHROMATOGRAMME

Die Verwendung der bekannten Beziehung[1,6,7]:

$$\ln V_g{}^m = \Delta H/RT_s + \text{const.}^* \tag{1}$$

zur Bestimmung der Adsorptionsenthalpie ΔH birgt wesentlich drei Nachteile. Erstens: das spezifische Retentionsvolumen des Bandenmaximums hängt in unbekannter Weise von der Probenmenge ab. Zweitens: bei breiten Banden lässt sich das Maximum oft nur mit grosser Unsicherheit feststellen. Drittens: die Belegungsdichte des Adsorbens bleibt unbekannt, wenn auch in der Literatur berichtet wird, die so gewonnenen Werte entsprächen einer gegen Null gehenden Bedeckung[8]. Es ist deshalb eine von CREMER UND HUBER[4] eingeführte und auch von HUBER UND KEULEMANS[9] diskutierte Methode vorzuziehen, die zunächst aus dem Eluierungsdiagramm mit Hilfe der Gleichung:

$$V_g = f'(c) \tag{2}$$

die Adsorptionsisothermen und daraus über die Adsorptionsisosteren die Adsorptionsenthalpien bei einer bestimmten Belegung ergibt. Die Rechnung liefert die beiden Gleichungen:

$$f'(c) = \frac{\lambda_r \cdot f_k \cdot F_m \cdot T_s}{\dot{\lambda} \cdot W \cdot T_m} \tag{3}$$

und

$$c = \frac{T_m \cdot n \cdot \dot{\lambda}}{f_k \cdot F_m \cdot T_s \cdot A} \cdot h \tag{4}$$

bzw. in analoger Weise für den Druck:

$$p = \frac{T_m \cdot n \cdot R \cdot \dot{\lambda}}{f_k \cdot F_m \cdot A} \cdot h \tag{4a}$$

die im wesentlichen auch CREMER UND HUBER[4] und HUBER UND KEULEMANS[9] zu Grunde legen. Die letzteren allerdings beziehen im Laufe der Rechnung die Empfindlichkeit auf Detektortemperatur, was nur richtig ist, wenn diese gleich der Säulentemperatur ist. Die beiden Gleichungen (3) und (4a) sind eine Parameterdarstellung der Funktion $f'(p)$ mit den Parametern λ_r und h, die selbst einander durch den Rücken der Durchbruchzacke zugeordnet sind. Man kann $f'(p)$ als Funktion von p auftragen, die graphische Integration von $f'(p)$ liefert die Adsorptionsisotherme. Nach dieser Methode verfuhren CREMER UND HUBER[4]. Die Auswertung lässt sich vereinfachen, wenn man den Rücken der Durchbruchzacke als eine Funktion $f'(p)$ auffasst. Denn es besteht ein linearer Zusammenhang zwischen p und h einerseits und λ_r und $f'(p)$ andererseits. Die p-Achse ist eine Senkrechte auf die Nullinie, der Nullpunkt ist derjenige Punkt, an dem eine Inertgasbande erscheint, die Nullinie bildet die f'-Achse. Die Integration der Rückfront liefert direkt $h(p)$. Der Masstab für die p-Achse ist durch die Gleichung (4a) gegeben. Es gilt:

$$f(p) = \int_0^p (V_g \cdot dp')/RT_s \tag{5}$$

* Die verwendeten Symbole sind auf S. 5 zusammengestellt.

Mit:

$$V_g = \frac{t_r \cdot f_k \cdot F_m \cdot T_s}{W \cdot T_m} \tag{6}$$

und Gleichung (4a) ergibt sich:

$$f(p) = \frac{n}{W \cdot A} \int_0^p \lambda_r \cdot dh = \frac{n}{W} \frac{\int_0^p \lambda_r \cdot dh}{\int_{-\infty}^{+\infty} h \cdot d\lambda_r} \tag{7}$$

Die Gleichungen (4a) und (7) liefern die Adsorptionsisotherme. Dabei werden die beiden auftretenden Integrale als Flächen mit Hilfe eines Planimeters bestimmt. Dieser Weg der Auswertung der Chromatogramme bringt gegenüber der Methode von CREMER UND HUBER[4] den Vorteil geringeren Zeitaufwands und der Verringerung der Fehlerquellen, da nur vier direkt gemessene Grössen an Stelle der sonst notwendigen acht eingehen.

Da die Ausgangsgleichung (2) nur für ideale Chromatographie gilt[9], sind die auf diesem Wege gewonnenen Ergebnisse im allgemeinen noch von vielfach diskutierten[5,10,11] sekundären zur Bandenverbreiterung führenden Effekten (insbesondere Diffusionseffekten) verfälscht. BACHMANN, BECHTOLD UND CREMER[12] geben zur Eliminierung der Diffusionseinflüsse zwei mögliche Korrektionen an. Die dabei notwendigen Voraussetzungen (z.B. Gleichheit der Verbreiterungsgeschwindigkeit an Front und Rücken) gelten jedoch nur für wenige Sonderfälle. Überdies wird bei der Korrektur die Bedingung konstanter Bandenfläche offensichtlich nicht immer eingehalten. Es erscheint deshalb vorteilhafter, den von HUBER UND KEULEMANS[9] vorgeschlagenen Weg zu verfolgen. Demnach hat man alle chromatographischen Bedingungen so zu wählen, dass sich in der Kolonne flache Konzentrationsprofile mit nur kleinen Konzentrationsgradienten ausbilden. Unter Verwendung kleiner Proben-

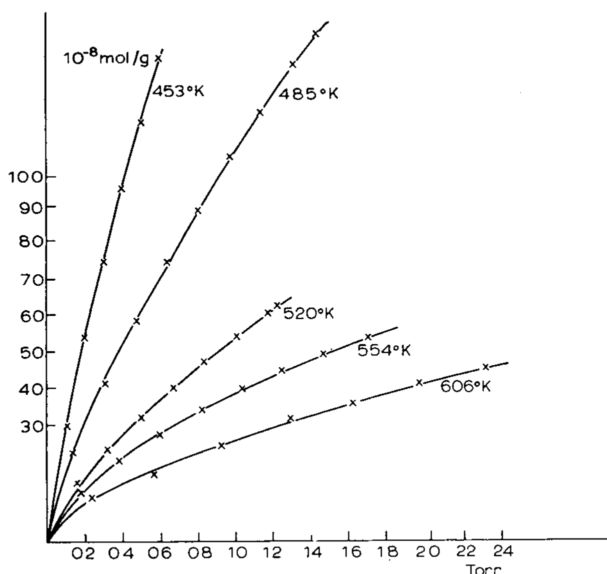


Fig. 2. Adsorptionsisothermen von Benzol an Silikagel.

mengen und langer Kolonnen (grosser wirksamer Oberfläche) lassen sich mit statischen Experimenten gut übereinstimmende Ergebnisse erhalten[9].

BEISPIEL

Es sei ein nach dem geschilderten Verfahren gewonnenes Ergebnis angeführt. In eine 750 mm lange Kolonne wurden 16.3 g Silikagel (Beckman Instruments Inc.) eingefüllt. Die Korngrösse lag zwischen 0.3 und 0.5 mm, die spezifische Oberfläche (BET) betrug 620 $m^2/g \pm 10\%$. Die Reinigung des Adsorbens erfolgte im Heliumstrom (5 ml/min) über 20 Stunden bei 550°C. Als Adsorbend wurden 1–2 μl Benzol aufgegeben. Fig. 2 zeigt die Adsorptionsisothermen bei verschiedenen Temperaturen. Die Adsorptionsenthalpien lagen bei Bedeckungen von $5 \cdot 10^{-7}$ Mol/g im Temperaturbereich von 200° bis 320°C zwischen 8 und 11 kcal/Mol. Die Schwankungen der Einzelmessungen lagen bei etwa 5 %, hervorgerufen insbesondere durch die schlechte Reproduzierbarkeit der Einspritzmenge. Die Fehler aller übrigen Messwerte sind kleiner.

ZUSAMMENSTELLUNG DER VERWENDETEN SYMBOLE

V_g	Spezifisches Retentionsvolumen in ml
V_g^m	Spezifisches Retentionsvolumen des Bandenmaximums in ml
ΔH	Adsorptionsenthalpie
$t_r = \frac{\lambda_r}{\lambda}$	Reine Retentionszeit (Aufenthaltszeit) in min
R	Allgemeine Gaskonstante
T_s	Säulentemperatur in °K
T_m	Temperatur des Strömungsmessers in °K
$f'(c)$	1. Ableitung der Adsorptionsisothermen nach der Konzentration
λ_r	Retentionszeit in cm Papiervorschub des Schreibers
λ	Papiervorschubgeschwindigkeit des Schreibers
f_k	Martinfaktor zur Korrektur für die Kompressibilität des Trägergases
F_m	Volumengeschwindigkeit des Trägergases bei der Temperatur des Strömungsmessers in ml/min
W	Menge des Adsorbens in g
c	Konzentration des Adsorbenden im Trägergas in Mol/ml
p	Partialdruck des Adsorbenden im Trägergas in Torr
n	Menge des Adsorbenden in Mol
A	Bandenfläche in cm^2
h	Höhe eines Punktes über der Nullinie in cm

DANK

Der Deutschen Forschungsgemeinschaft und dem Fond Chemie sei für Sachbeihilfen zu dieser Arbeit gedankt, Herrn Prof. Dr. G.-M. SCHWAB für sein Interesse und seine Unterstützung.

ZUSAMMENFASSUNG

Es wird der Aufbau eines Gaschromatographen zur Bestimmung von Adsorptionsenthalpien, sowie die Auswertung der gewonnenen Chromatogramme geschildert.

SUMMARY

The construction of a gas chromatograph with which heats of adsorption can be determined, is described, as well as the evaluation of the chromatograms obtained.

LITERATUR

[1] E. CREMER UND F. PRIOR, *Z. Elektrochem.*, 55 (1951) 66.
[2] E. CREMER, *Angew. Chem.*, 71 (1959) 512.
[3] E. CREMER, *Monatsh. Chem.*, 92 (1961) 112.
[4] E. CREMER UND H. HUBER, *Angew. Chem.*, 73 (1961) 461.
[5] R. KAISER, *Chromatographie in der Gasphase*, Bd. 1 und 3, Bibliographisches Institut AG, Mannheim, 1960.
[6] A. B. LITTLEWOOD, C. S. G. PHILLIPS UND D. T. PRICE, *J. Chem. Soc.*, (1955) 1480.
[7] D. AMBROSE, A. I. M. KEULEMANS UND J. H. PURNELL, *Anal. Chem.*, 30 (1958) 1582.
[8] S. ROSS, J. K. SAELENS UND P. OLIVIER, *J. Phys. Chem.*, 66 (1962) 696.
[9] J. F. K. HUBER UND A. I. M. KEULEMANS, *4. Intern. Gas Chromatography Symposium, Hamburg, 1962*, Butterworths, London, 1962, S. 255.
[10] A. I. M. KEULEMANS, *Gas Chromatography*, Reinhold, New York, 1957.
[11] P. C. VAN BERGE, P. HARHOFF UND V. PRETORIUS, *Trans. Faraday Soc.*, 58 (1962) 2272.
[12] L. BACHMANN, E. BECHTOLD UND E. CREMER, *J. Catalysis*, 1 (1962) 113.

DOSAGE CHROMATOGRAPHIQUE DE FAIBLES TENEURS EN VAPEUR D'EAU DANS UNE ATMOSPHÈRE GAZEUSE

R. AUBEAU, L. CHAMPEIX ET MME J. REISS

Commissariat à l'Énergie Atomique, Centre d'Études Nucléaires de Saclay, Département de Métallurgie, Service de Chimie des Solides, Section d'Étude de la Corrosion par Gaz et Métaux Liquides, Gif-sur-Yvette, S. et O. (France)

(Reçu le 12 février 1964)

INTRODUCTION

L'eau est pratiquement toujours présente à l'état de traces dans les atmosphères gazeuses et dans les liquides supposés très secs; elle s'adsorbe également sur la plupart des solides. On sait par ailleurs que la réactivité de deux ou plusieurs phases entre elles peut dépendre notablement de la quantité d'eau contenue dans chacune d'elles. Ainsi, dans le domaine de la corrosion des métaux ou alliages par les gaz à température élevée, le degré de dessiccation de l'atmosphère en présence se montre bien souvent déterminant; cette circonstance caractérise en particulier le problème de la compatibilité du béryllium avec le gaz carbonique sous pression dès 600°C[1], problème des plus importants en vue des applications nucléaires de ce métal. Il est alors nécessaire de disposer d'une méthode très sensible permettant de doser la vapeur d'eau à l'état de traces dans la phase gazeuse, pratiquement jusqu'au volume par million (v.p.m.).

Les techniques utilisées pour analyser la vapeur d'eau contenue dans un gaz dépendent essentiellement de sa plus ou moins grande concentration dans ce gaz et du volume gazeux disponible. Pour les quantités relativement fortes (quelques mg), on peut utiliser la pesée d'un tube absorbeur, ou la détermination du point de rosée si l'eau est le seul gaz condensable. La détermination de traces (de quelques v.p.m. à 1000 v.p.m.) peut s'effectuer commodément à l'aide d'appareils tels que les hygromètres électrolytiques, si l'on dispose d'un débit non négligeable (> 5 l/h) et constant[2,3]. La méthode de Karl Fischer est aussi utilisée, même pour des concentrations de quelques v.p.m.

Cependant, durant ces dernières années, quelques auteurs ont essayé de doser l'eau par chromatographie en phase gazeuse. CARLSTROM *et al.*[4] l'ont analysée dans du butane au moyen d'une colonne de brique réfractaire imprégnée de polyéthylèneglycol 200; pour les teneurs inférieures à 200 v.p.m., une colonne de concentration de même nature permettrait d'atteindre une sensibilité inférieure au v.p.m.; la reproductibilité est de 20 % environ. Dans d'autres cas, on a préféré transformer l'eau en acétylène dans un tube contenant du carbure de calcium; l'acétylène ainsi formé est analysé sur une colonne de gel de silice[5,6] ou au moyen du mélange diméthylsulfolane et squalane déposé sur brique réfractaire[7]. Malgré les techniques très diverses utilisées[5-7] pour rendre l'analyse quantitative acceptable, une imprécision notable persiste et la reproductibilité est très aléatoire.

Lorsque l'on dispose d'un volume limité de mélange gazeux, ce qui est souvent le

cas dans notre laboratoire (dégazage d'échantillons solides, atmosphère d'essais de corrosion), seules les méthodes de Karl Fischer et chromatographiques peuvent alors convenir. Cependant, la mise en oeuvre délicate de la méthode de Karl Fischer, en particulier le maintien des réactifs à l'état anhydre, constitute un obstacle important pour un laboratoire qui n'utilise que rarement les techniques de dosage par voie chimique. Aussi nous sommes-nous orientés vers la mise au point d'une technique chromatographique, technique que nous utilisons couramment pour l'analyse des gaz permanents.

Dans le présent mémoire, nous examinerons donc les possibilités des différentes méthodes chromatographiques, en ayant pour principal objectif la détermination aisée de faibles teneurs en humidité (quelques v.p.m.) dans des volumes limités de mélanges gazeux.

APPAREILLAGE ET MODE OPERATOIRE

L'analyse chromatographique de la vapeur d'eau dans un gaz peut être envisagée de deux manières: soit directement en chromatographiant l'eau à l'état gazeux, soit indirectement en dosant un gaz issu de la réaction de l'eau avec un composé solide.

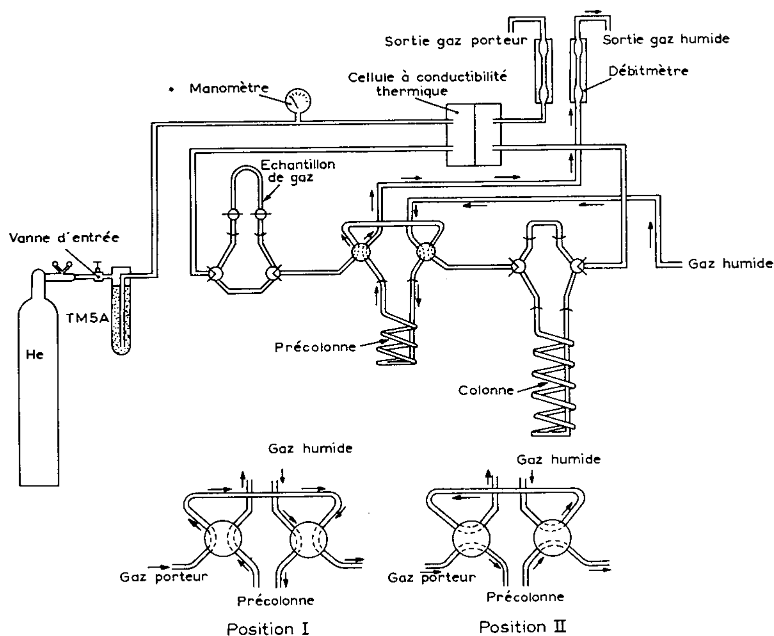


Fig. 1. Schéma du chromatographe.

(a) *Dosage de la vapeur d'eau par voie indirecte*

L'eau est transformée, dans un tube de réaction, en un gaz qui est séparé des autres constituants de l'échantillon gazeux sur une colonne de chromatographie.

L'appareil utilisé est représenté par la Fig. 1*. Les canalisations et les robinets sont en pyrex; les volumes morts sont aussi faibles que possible.

* Le tube de réaction remplace la précolonne et les robinets sont en position II.

Deux ensembles "tube de réaction–colonne" ont été utilisés:

Premier ensemble. Le tube de réaction est rempli de carbure de calcium et la colonne de gel de silice.

Le tube de réaction, en forme d'U, de 8 mm de diamètre et de 20 cm de longueur, contient environ 10 g de carbure de calcium concassé et tamisé; lors des manipulations, on évite le plus possible une exposition prolongée à l'humidité de l'air. La colonne, en forme de spirale, a 50 cm de longueur et 3 mm de diamètre; elle contient environ 8 g de gel de silice déshydraté à 150°. Avec ce montage, l'eau contenue dans l'échantillon de gaz réagit avec le carbure de calcium pour donner de l'acétylène que l'on sépare des autres gaz sur la colonne. Comme le montre la Fig. 2, cette méthode permet de doser, au moins qualitativement, en une seule opération, l'eau et le gaz carbonique contenus dans l'échantillon, les gaz permanents usuels sortant en un seul pic.

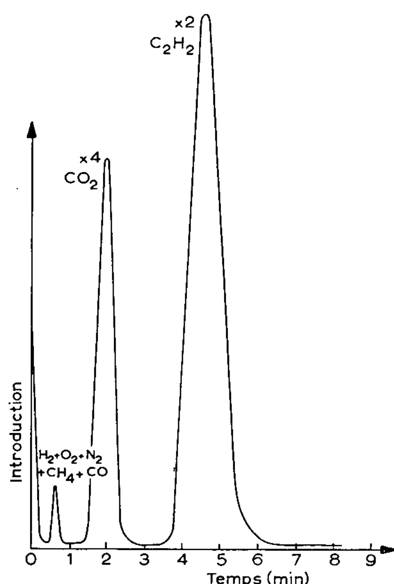


Fig. 2. Séparation de l'anhydride carbonique et de l'acétylène sur gel de silice. Colonne: gel de silice, 0.50 m. Température: 50°. Gaz porteur: hélium, 50 cm^3/min. Échantillon: CO_2, 40 mm^3 N.T.P.; C_2H_2, 60 mm^3 N.T.P.

Deuxième ensemble. Le tube de réaction, de mêmes dimensions que le précédent, contient de l'hydrure de calcium (10 g). La colonne est remplie de tamis moléculaire 5 A déshydraté à 250° sous vide[8–10]; elle a 2 m de longueur et 3 mm de diamètre intérieur. Cette méthode est particulièrement intéressante sous l'angle de la grande sensibilité propre à la détection de l'hydrogène. Précisons aussi qu'elle peut éventuellement permettre de doser en même temps, sur une même prise d'essai, les différents gaz indiqués sur la Fig. 3.

(*b*) *Dosage de la vapeur d'eau par voie directe*

Pour séparer les corps polaires par chromatographie, on utilise généralement des polyéthylèneglycols. La plupart de nos essais ont donc été réalisés avec du poly-

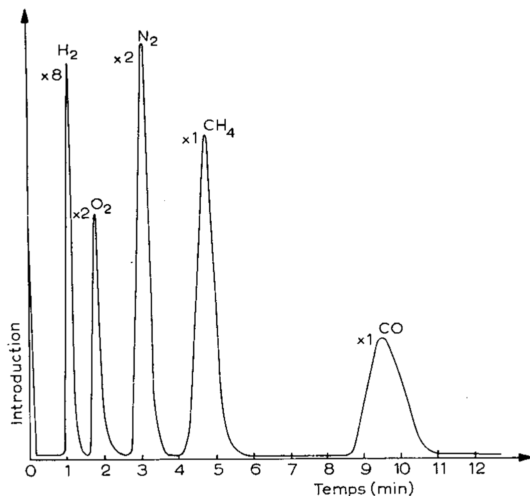


Fig. 3. Séparation des gaz permanents sur T.M.5A. Colonne: T.M.5A, 2 m. Température: 50°. Gaz porteur: argon, 40 cm³/min. Échantillon: H_2, 5.4 mm³ N.T.P.; O_2, 13.4 mm³ N.T.P.; N_2, 50.0 mm³ N.T.P.; CH_4, 10.0 mm³ N.T.P.; CO, 20.5 mm³ N.T.P.

éthylèneglycol 1500 comme phase stationnaire, en raison de son poids moléculaire important, donc de sa faible tension de vapeur aux températures relativement élevées (100°). La polyéthylèneglycol 200 n'a été utilisé qu'à titre comparatif. Les traînées des pics des corps polaires étant généralement attribuées à l'effet des sites d'adsorption du support, nous avons expérimenté le téflon en poudre, outre les supports les plus couramment utilisées (Chromosorb P, Chromosorb W, Chromosorb HMDS*) (Figs. 4 et 5). Comme le montrent ces figures, et surtout pour de faibles quantités d'eau, la traînée du pic est très importante, sauf avec le support en téflon; celui-ci a généralement été utilisé récemment par LANDAULT ET GUIOCHON[11], et, dans l'ensemble, les conclusions de ces auteurs sont en bon accord avec les nôtres. Il semble toutefois que nous ayons eu beaucoup moins de difficultés pour réaliser le remplissage des colonnes: à condition de battre la colonne tout en introduisant doucement le téflon imprégné de phase stationnaire, une colonne d'un mètre peut être remplie en une heure environ, sans avoir recours à l'emploi du vide ou à une surpression.

Actuellement, la poudre de téflon pour chromatographie est vendue sous deux variétés appelées Fluoropak et Haloport. Bien que nous ne possédions pas d'informations sur la texture de ces poudres, nous avons constaté qu'elles présentent des efficacités tout à fait similaires (Fig. 5); toutefois le remplissage des colonnes est plus facile avec le Fluoropak, le Haloport ayant tendance à s'agglomérer en formant des bouchons. Les colonnes que nous utilisons pour les analyses courantes ont donc été fabriquées avec du Fluoropak comme support; elles ont généralement un mètre de longueur et 3 mm de diamètre, contiennent environ 12 g de Fluoropak imprégné de polyéthylèneglycol 1500 et sont thermostatées à 90°. Le gaz porteur est l'hélium, sous un débit de 100 cm³/min. Le taux d'imprégnation adopté est de 20 % en poids; un

* Le traitement HMDS [hexaméthyldisilazane: $Si(CH_3)_3$–NH–$Si(CH_3)_3$] doit permettre de neutraliser les centres d'adsorption les plus actifs.

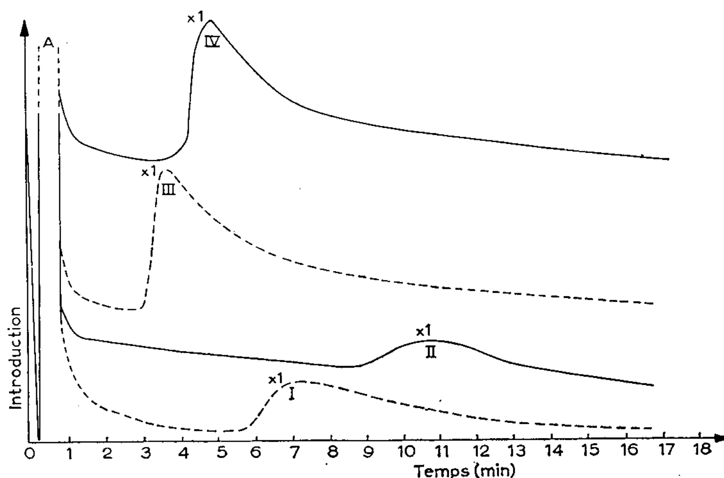


Fig. 4. Influence du support sur l'analyse de la vapeur d'eau. Phase stationnaire: polyéthylène-glycol 1500. Taux d'imprégnation: 20%. Longueur de la colonne: 1 m. Température: 90°. Gaz porteur: hélium, 100 cm³/min. Échantillon: 32 μg H_2O. (I) Support: Chromosorb P, 8.8 g; phase stationnaire, 1.8 g. (II) Support: Chromosorb P traité HMDS, 9.7 g; phase stationnaire, 1.9 g. (III) Support: Chromosorb W, 4.3. g; phase stationnaire, 0.9 g. (IV) Support: Chromosorb W traité HMDS, 7.1 g; phase stationnaire, 1.4 g.

taux de 10 % est trop faible, car bien que l'on obtienne une bonne séparation (Fig. 5), le temps de rétention de l'eau est trop court, de sorte que ce pic peut être perturbé par la trainée du pic des gaz permanents qui constituent l'élément principal de l'échantillon, ou confondu avec le pic de l'alcool éthylique, s'il existe. L'emploi d'une température de colonne inférieure à 90° diminue la sensibilité, en augmentant le temps de rétention ainsi que la dissymétrie du pic. Pour une température plus élevée, la phase stationnaire possède une tension de vapeur non négligeable. On remarque enfin que la trainée du pic est notablement plus importante quand la quantité d'eau analysée est plus faible (Fig. 6).

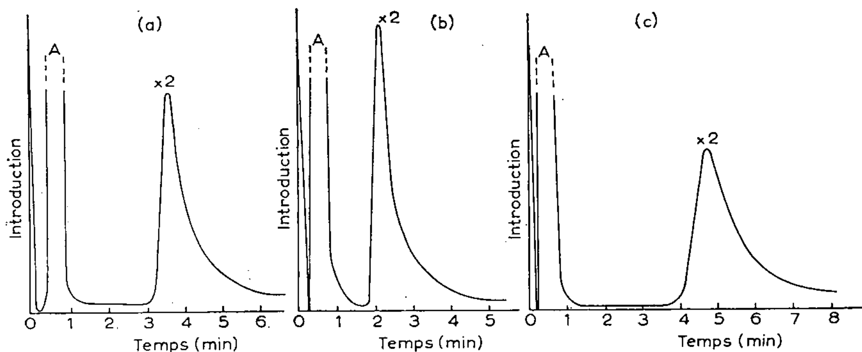


Fig. 5. Influence du support sur l'analyse de la vapeur d'eau. Phase stationnaire: polyéthylène-glycol 1500. Longueur de la colonne: 1 m. Température: 90°. Gaz porteur: hélium, 100 cm³/min. Échantillon: 32 μg H_2O. (a) Support: téflon (Haloport), 9.7 g. Taux d'imprégnation: 20%. (b) Support: téflon (Fluoropak), 11.7 g. Taux d'imprégnation: 10%. (c) Support: téflon (Fluoropak), 13.2 g. Taux d'imprégnation: 20%.

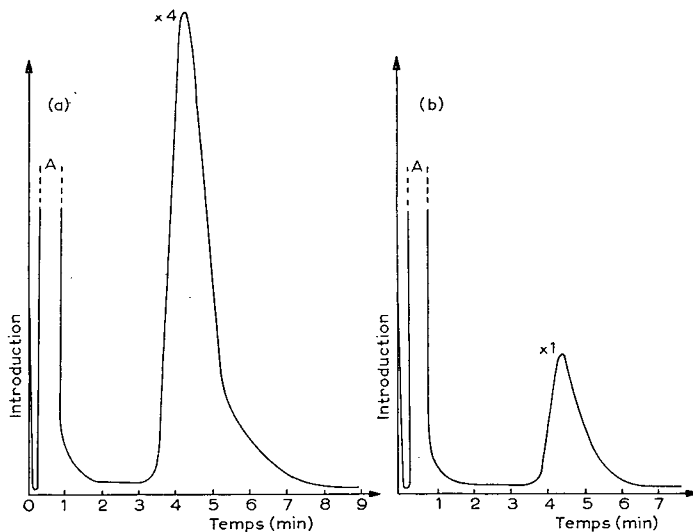


Fig. 6. Dosage de l'eau par la méthode directe. Colonne: longueur, 1 m; support, Fluoropak, 13.2 g; phase stationnaire: polyéthylèneglycol 1500, 2.6 g; taux d'imprégnation, 20%. Température: 90°. Gaz porteur: hélium, 100 cm³/min. (a) Volume argon introduit: 15 cm³ N.T.P. contenant 212 µg H_2O. (b) Volume argon introduit: 2 cm³ N.T.P. contenant 17 µg H_2O.

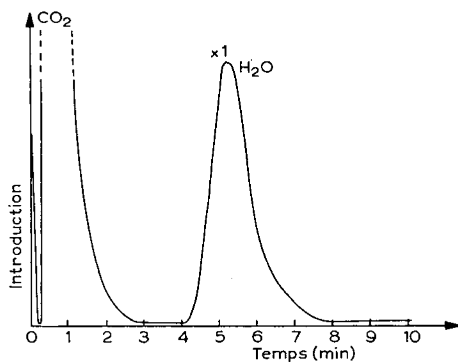


Fig. 7. Dosage de l'eau par la méthode de concentration. Précolonne: longueur, 0.50 m; support: Fluoropak, 5.8 g; phase stationnaire: polyéthylèneglycol 1500, 1.2 g; taux d'imprégnation, 20%; température de concentration, 0°; température de réchauffement, 90–100°. Colonne: longueur, 1 m; support: Fluoropak, 13.2 g; phase stationnaire: polyéthylèneglycol 1500, 2.6 g; taux d'imprégnation, 20%; température: 90°. Gaz porteur: hélium, 100 cm³/min. Gaz analysé: anhydride carbonique; débit: 28 cm³/min; temps de concentration: 20 min; volume prélevé: 560 cm³ N.T.P; teneur en H_2O: 95 v.p.m.

Pour les faibles teneurs en eau d'un gaz (moins de 100 v.p.m.), nous avons eu recours à une méthode de concentration. Le gaz humide circule* dans une précolonne de concentration, de même nature que la colonne, mais thermostatée à 0°, tandis que le gaz porteur circule uniquement dans la colonne (Fig. 1 – position I). Au bout d'un temps connu, une quantité déterminée de gaz humide ayant traversé la précolonne, on réchauffe cette dernière à 100° (robinets en position II), et l'eau qui se désorbe est chromatographiée sur la colonne (Fig. 7).

ANALYSE QUANTITATIVE

La reproductibilité des différentes techniques expérimentées a été examinée en utilisant divers gaz permanents usuels (argon, gaz carbonique, azote, etc.) dans lesquels a été introduite une quantité connue et souvent faible de vapeur d'eau. Les gaz ainsi humidifiés ont également permis d'étalonner les méthodes retenues pour les analyses courantes.

Introduction d'une teneur connue de vapeur d'eau dans un gaz

L'humidification d'un gaz a été réalisée selon deux procédés: électrolyse ou barbotage. Pour les faibles teneurs ($\tau < 100$ v.p.m.) en particulier, nous avons utilisé une cellule d'électrolyse contenant de l'eau acidulée.

La Fig. 8 représente l'appareillage dans son ensemble. Un courant de gaz (argon par exemple), à débit constant (20 cm^3/min), entraîne l'hydrogène et l'oxygène

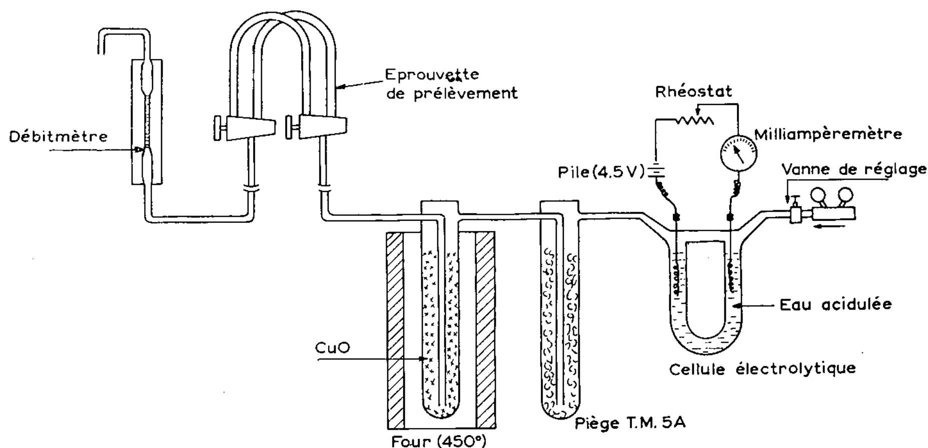


Fig. 8. Humidification d'un gaz par électrolyse.

électrolysés. Ce mélange est desséché dans un piège rempli de tamis moléculaire, à la température ambiante**. L'hydrogène est ensuite transformé en eau dans un tube

* Le sens du courant de gaz humide indiqué sur la Fig. 1 doit être respecté si l'on veut obtenir une désorption rapide de l'eau au moment de l'analyse et éviter ainsi une trainée importante du pic relatif à l'eau.

** La méthode d'analyse par concentration exposée ci-dessus nous a montré que, dans nos conditions opératoires, la teneur en eau du gaz (argon), en aval du piège à tamis moléculaire, est inférieure à 2 v.p.m.

contenant de l'oxyde de cuivre porté à 400–450°*. L'éprouvette de prélèvement permet finalement d'introduire une quantité connue de ce gaz humide dans le chromatographe et d'avoir ainsi un pic représentatif de la vapeur d'eau. Les gaz à forte teneur en eau (0.1 à 1 %) ont en général été obtenus par barbotage à saturation, au moyen de l'appareillage représenté par la Fig. 9.

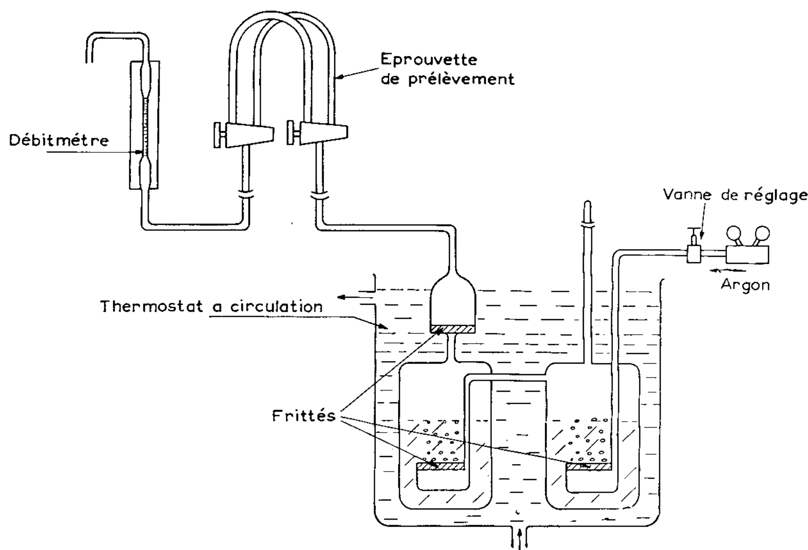


Fig. 9. Humidification d'un gaz par barbotage.

Avec cette technique, l'entraînement de gouttelettes d'eau par le gaz conduisait, lors des premiers essais, à une sursaturation de ce dernier à la température du thermostat. Nous y avons remédié en plaçant, sur le trajet du gaz, un fritté à la sortie du barboteur; ce fritté est bien évidemment dans l'enceinte thermostatée (Fig. 9). Comme précédemment, une quantité connue de ce gaz est ensuite introduite dans le courant de gaz porteur du chromatographe, au moyen de l'éprouvette à gaz.

Etude de la reproductibilité

Le gaz humidifié par l'une ou l'autre des deux techniques précédentes, nous a permis d'étudier la reproductibilité de chacune des deux méthodes de dosage de l'eau dont le principe a été décrit plus haut.

(*1*) *Méthode indirecte.* Si, avec cette méthode, l'analyse qualitative est séduisante, comme nous venons de le montrer, la reproductibilité, facteur essentiel de l'analyse quantitative, est très mauvaise. Sans entrer dans le détail des nombreux essais que nous avons réalisés, nous sommes en droit de penser que la dispersion obtenue est principalement due à l'adsorption chimique ou physique d'une partie de l'eau de l'échantillon à analyser sur la chaux [CaO et $Ca(OH)_2$] présente dans le tube de

* En analysant par chromatographie (avec l'argon comme gaz porteur[8]) la teneur en hydrogène du gaz en aval du four à CuO, nous avons observé qu'il fallait au moins 400 g de CuO broyé pour que la conversion $H_2 \rightarrow H_2O$ soit totale ($H_2 < 2$ v.p.m.). Pour des quantités plus faibles de CuO, il reste une quantité notable d'hydrogène.

TABLEAU I

REPRODUCTIBILITÉ DE LA MÉTHODE D'ANALYSE DE L'EAU PAR VOIE DIRECTE

Quantité d'eau introduite (µg)	*Hauteur du pic (cm)*	*Surface du pic (cm²)*
1.7	0.35*	0.30*
	0.35*	0.35*
	0.40*	0.35*
3.1	0.65*	0.55*
	0.70*	0.55*
	0.65*	0.55*
9.5	1.85*	1.80*
	1.90*	1.90*
	1.95*	2.10*
11.4	2.30*	2.60*
	2.35*	3.00*
	2.25**	2.80**
	2.20*	2.60*
18.9	3.70*	3.80*
	4.00**	3.70**
	3.75**	4.20**
	4.15**	4.55**
	3.60**	3.70**
	3.70*	3.70*
	3.70*	4.10*
	3.80**	4.10**
35.1	7.90**	9.90**
	8.50**	9.20**
	8.20**	9.50**
	8.30**	9.00**

* Gaz humidifié par électrolyse
** Gaz humidifié par barbotage

transformation. La chaux peut en effet se former dès le remplissage et elle apparaît en tout cas lors de l'analyse. Il en résulte que des introductions successives d'échantillons gazeux contenant de l'eau augmentent la quantité de chaux dans le tube de transformation, et conduisent donc à des pics de plus en plus faibles dans les meilleurs cas (Fig. 10). Il n'est cependant pas rare d'obtenir un nuage de points distribués au hasard. Nous avons tenté en vain d'améliorer cette technique en faisant varier le temps de séjour de l'eau dans le tube de transformation ou en augmentant la température. On observe en particulier qu'au-dessus de 50° l'hydrure de calcium se décompose (Fig. 11).

(2) *Méthode directe.* La reproductibilité de cette technique, avec ou sans colonne de concentration, est généralement acceptable (Tableau I). Accidentellement, nous avons obtenu des dispersions de l'ordre de 20 à 30 %. Cependant, il à été montré que ces écarts notables provenaient essentiellement des échantillons de gaz humides analysés, ou plutôt du degré de dessiccation de l'éprouvette de prélèvement. A condition d'employer des éprouvettes nettoyées à l'alcool et séchées à l'étuve sous vide à 100°, on peut obtenir une reproductibilité de 8 % environ, même pour de faibles quantités de vapeur d'eau.

ห้องสมุด กรมวิทยาศาสตร์

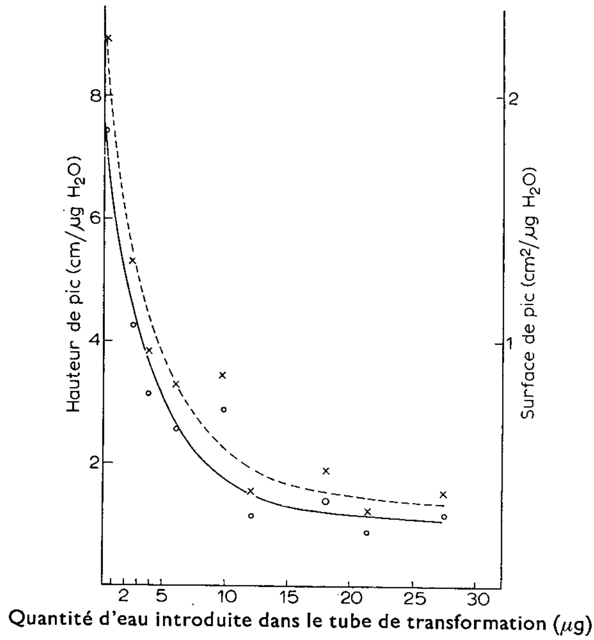


Fig. 10. Évolution de la sensibilité du dosage en fonction de la quantité d'eau introduite dans le tube de transformation. O—O = hauteur de pic. ×--× = surface de pic.

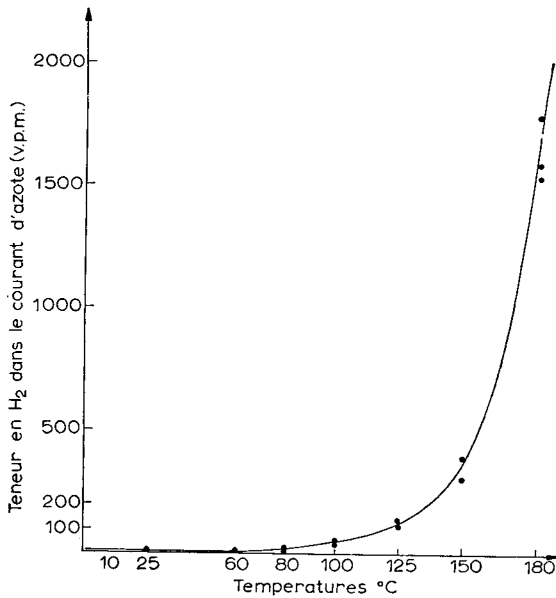


Fig. 11. Décomposition de CaH_2 en fonction de la température. Poids de CaH_2: 9 g. Débit d'azote sec: 20 cm^3/min.

Étalonnage

La méthode directe, qui conduit à des résultats reproductibles, a été seule étalonnée. Comme pour les gaz permanents[8–10], l'étalonnage s'effectue en introduisant des quantités d'eau variables, mais connues, dans le chromatographe. On y parvient en utilisant des éprouvettes de volumes différents, contenant un gaz à teneur en eau constante, ou en introduisant un volume constant d'un gaz de degré hygrométrique variable, ces gaz étant humidifiés selon l'un ou l'autre des deux procédés exposés. On aboutit ainsi,

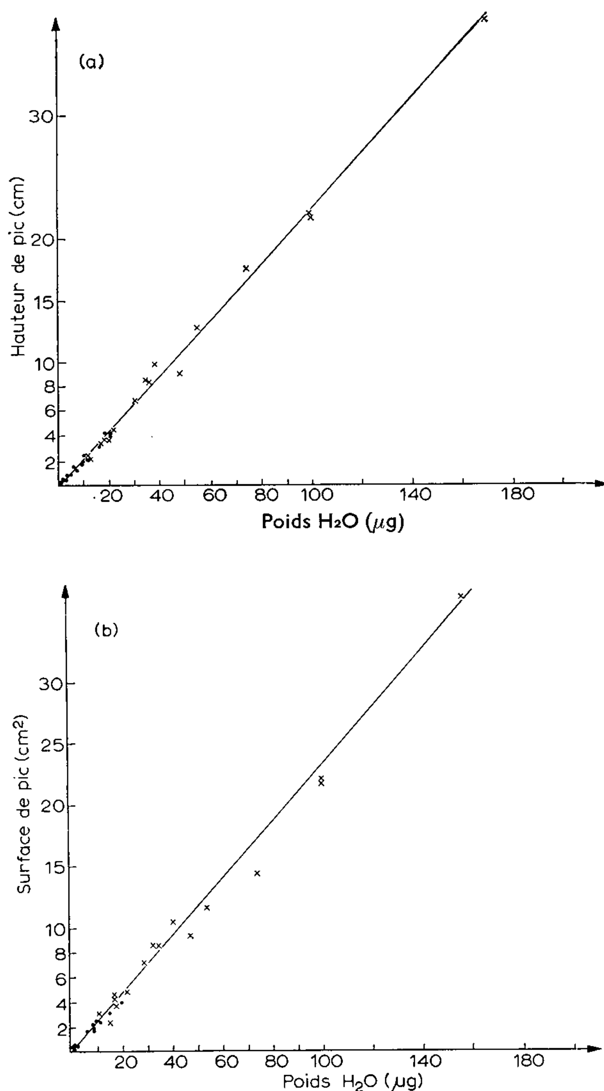


Fig. 12. Étalonnage (méthode directe). Colonne: longueur, 1 m; support: Fluoropak, 13.2 g; phase stationnaire: polyéthylèneglycol 1500, 2.6 g; taux d'imprégnation, 20%. Température: 90°. Gaz porteur: hélium, 100 cm³/min. ● = humidification par électrolyse. × = humidification par barbotage. (a) Étalonnage avec concentration. (b) Étalonnage sans concentration.

pour des quantités d'eau relativement faibles, à une courbe d'étalonnage linéaire en utilisant la hauteur (Fig. 12a) ou la surface du pic* (Fig. 12b). La dispersion, inférieure à 7 % en moyenne, montre entre autres que l'adsorption de l'eau sur les parois de l'éprouvette est faible et qu'en tout cas la quantité d'eau adsorbée est négligeable devant celle qui se trouve en phase gazeuse, du moins pour les concentrations utilisées.

Lorsque l'on utilise la technique de concentration, l'étalonnage est effectué en remplaçant, dans le circuit d'humidification (Fig. 8), l'éprouvette de prélèvement par la colonne de concentration (Figs. 13a et 13b).

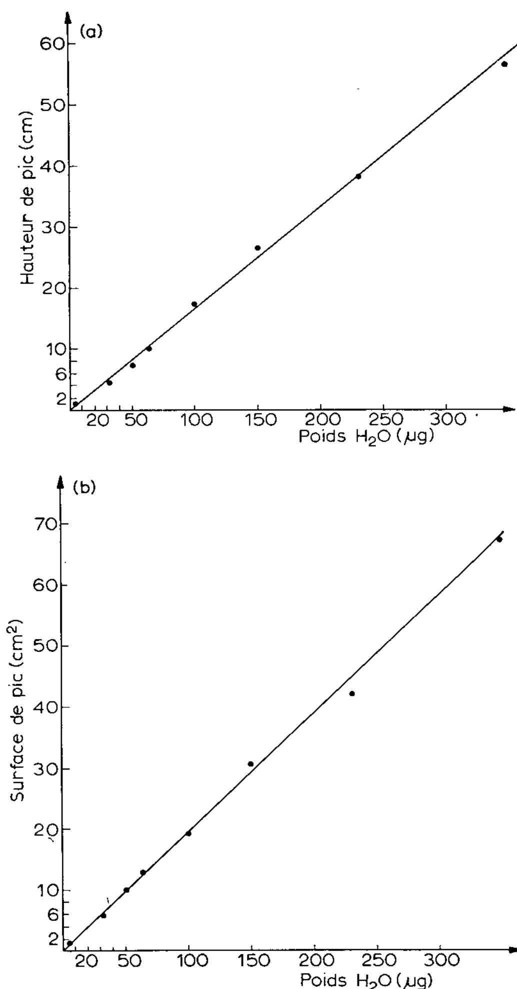


Fig. 13. Étalonnage (technique de concentration). Précolonne: longueur, 0.50 m; support: Fluoropak, 5.8 g; phase stationnaire: polyéthylèneglycol 1500, 1.2 g; taux d'imprégnation, 20 %; température de concentration, 0°; température de réchauffement, 90–100°. Colonne: longueur, 1 m; support: Fluoropak, 13.2 g; phase stationnaire: polyéthylèneglycol 1500, 2.6 g; taux d'imprégnation, 20 %; température, 90°. Gaz porteur: hélium, 100 cm^3/min. Gaz humidifié par électrolyse: argon, 20 cm^3/min. (a) Étalonnage avec concentration. (b) Étalonnage sans concentration.

* La surface du pic est mesurée au planimètre.

On remarque que les pentes des droites d'étalonnage avec et sans concentration sont légèrement différentes, comme on le constate en comparant les Figs. 12a et 13a d'une part et les Figs. 12b et 13b d'autre part.

DISCUSSION

Les techniques opératoires assez inhabituelles utilisées pour analyser l'eau après transformation en acétylène[5–7] appellent certains commentaires. Certains auteurs ont observé, comme nous, que la dispersion importante des résultats était généralement due à l'adsorption de l'eau sur la chaux contenue dans le tube de réaction. Pour atténuer ce phénomène, DUSWALT ET BRANDT[5] limitent l'utilisation d'un tube de transformation à trois analyses, afin que la quantité de chaux formée soit faible. Ces auteurs précisent d'ailleurs que la sensibilité varie d'un tube à un autre et qu'il est nécessaire de procéder à un étalonnage avant chaque analyse. SUNDBERG ET MARESH[6] ne précisent pas l'ordre de grandeur de la dispersion de leurs mesures, la quantité d'eau analysée étant relativement grande (1 mg). KNIGHT ET WEISS[7] abaissent la dispersion en fabricant de la chaux dans le tube de réaction avant son emploi, par passage d'air ambiant. Ces différentes techniques conduisent DUSWALT ET BRANDT[5] et SUNDBERG ET MARESH[6] à une précision de 0.2 %. KNIGHT ET WEISS[7] obtiennent une reproductibilité de 7 à 20 % pour des teneurs en eau de quelques v.p.m.

Il est bien clair que de telles méthodes analytiques, par leur mode opératoire à la fois complexe et peu sûr, ne peuvent être utilisées couramment dans un laboratoire de contrôle. Toutefois, le chercheur peu préoccupé par la durée d'une opération pourrait certainement utiliser ces modes opératoires, à condition de vérifier scrupuleusement l'étalonnage et la reproductibilité avant chaque analyse. Par ailleurs la précision annoncée par DUSWALT ET BRANDT[5] et SUNDBERG ET MARESH[6] paraît optimiste, vu les résultats que nous avons obtenus dans des conditions similaires.

La méthode d'analyse par voie directe paraît plus séduisante. Cependant, dans les mêmes conditions opératoires, nous n'avons pas pu obtenir de pics symétriques, contrairement à ce qu'avancent les auteurs précédents; la trainée du pic de l'eau est même très importante. L'étalonnage de la méthode de concentration, effectuée avec de l'air (1 à 3 cm^3) saturé d'eau à température ambiante, peut être une source d'erreur, ainsi que nous l'avons déjà remarqué (Figs. 12 a et 13a).

Ainsi, la comparaison des différentes techniques chromatographiques d'analyse de l'eau montre que la méthode par voie directe est mieux adaptée aux analyses de contrôle que la méthode avec transformation. Il est à noter, cependant, que l'utilisation, en analyse directe, du téflon comme support de la phase stationnaire, augmente à la fois la sensibilité et la reproductibilité, en diminuant considérablement l'asymétrie du pic. Quoi qu'il en soit, cette méthode permet d'analyser l'eau aussi commodément que n'importe quel gaz permanent, avec une précision seulement très légèrement inférieure.

CONCLUSION

La teneur en vapeur d'eau d'un gaz peut être déterminée par chromatographie de partage. Cette méthodes s'accommode d'échantillons gazeux de quelques cm^3, renfermant 100 v.p.m. d'eau ou davantage. Grâce à une technique de concentration, une teneur d'un v.p.m., ou même inférieure, peut être analysée si l'on dispose d'un

volume de gaz assez important (1 litre au moins), et ce avec une précision de l'ordre de 5 à 8 %.

RÉSUMÉ

L'analyse par chromatographie gazeuse de la vapeur d'eau contenue dans un gaz a été envisagée de deux façons: soit avec tube de transformation et colonne d'adsorption (méthode indirecte), soit avec colonne unique de partage (méthode directe).

Dans la méthode indirecte, l'eau est tout d'abord transformée en acétylène ou en hydrogène; ces deux gaz sont séparés respectivement sur une colonne de gel de silice et sur une colonne de tamis moléculaire. Cependant la reproductibilité médiocre procurée par cette méthode rend l'analyse quantitative imprécise, voire inexacte.

Dans la méthode directe, la vapeur d'eau est séparée des gaz présents sur du polyéthylèneglycol 1500. La traînée du pic, due à l'adsorption sur la plupart des supports usuels, a été fortement atténuée en utilisant du téflon en poudre comme support de la phase stationnaire. Ainsi, avec une prise d'essai de quelques cm^3, on peut doser 100 v.p.m. ou davantage de vapeur d'eau dans un gaz. Une technique de concentration avec une précolonne de même nature que la colonne de partage permet d'atteindre unse sensibilité de 1 v.p.m. et même moins.

La méthode directe, avec ou sans système de concentration, conduit à une précision de l'ordre de 5 à 8 % environ, ce qui est largement suffisant dans la plupart des applications.

SUMMARY

The determination of the content of water vapour in a gas by gas chromatography was examined using two different methods, *viz.* by means of a reactor tube and adsorption column (indirect method), or with a single separation column (direct method).

In the indirect method the water is first converted into acetylene or into hydrogen, which gases are then separated from the other gases present, a silica gel column being used for acetylene and a molecular sieve column for hydrogen. The poor reproducibility obtained in this method makes quantitative analysis unprecise and even inexact.

In the direct method the water vapour is separated from the gases by means of polyethylene glycol 1500. The tailing of the peak due to adsorption on most of the usual supports is considerably reduced when powdered teflon is used as support for the stationary phase. In this way, taking a sample of a few cm^3, amounts of 100 v.p.m. (volume per million) or more of water vapour in a gas can be determined. By preliminary concentration on a pre-column of the same kind as the column used for separation, a sensitivity of 1 v.p.m. or even less, can be attained.

In the direct method, with or without a system of preliminary concentration, the precision is of the order of approximately 5–8 %, which is sufficient for most purposes.

REFERENCES

1 R. DARRAS, *Bull. Inform. Sci. Tech. (Paris)*, 62 (1962) 43; *Energie Nucl.*, 5 (1963) 588.
2 A. A. CRAWSHAW ET F. G. DAVIDSON, *J. Sci. Instr.*, 36 (1959) 121.
3 E. S. TAYLOR, *Refrig. Eng.*, 64 (1956) 41.
4 A. A. CARLSTROM, C. F. SPENCER ET J. F. JOHNSON, *Anal. Chem.*, 32 (1960) 8.
5 A. A. DUSWALT ET W. W. BRANDT, *Anal. Chem.*, 32 (1960) 272.
6 O. E. SUNDBERG ET C. MARESH, *Anal. Chem.*, 32 (1960) 274.

[7] H. S. KNIGHT ET F. T. WEISS, *Anal. Chem.*, 34 (1962) 749.
[8] R. AUBEAU ET L. CHAMPEIX, *Ind. Atom.* (*Suisse*), 11–12 (1960) 78.
[9] R. AUBEAU, L. CHAMPEIX ET J. REISS, *J. Chromatog.*, 6 (1961) 209.
[10] R. AUBEAU, J. REISS, L. CHAMPEIX ET V. RAVNIK, *J. Nucl. Mater.*, 6, No. 3 (1962) 271.
[11] C. LANDAULT ET G. GUIOCHON, *J. Chromatog.*, 9 (1962) 133.

J. Chromatog., 16 (1964) 7–21

GAS CHROMATOGRAPHIC DETERMINATION OF SMALL VAPOUR PRESSURES

DETERMINATION OF THE VAPOUR PRESSURES OF SOME TRIAZINE HERBICIDES

K. FRIEDRICH AND K. STAMMBACH
Analytical Laboratories, J. R. Geigy A.G., Basle (Switzerland)

(Received February 6th, 1964)

INTRODUCTION

Various trisubstituted symmetrical triazines are used nowadays as herbicides[1-3]. GAST[4] has examined triazines of this kind with respect to their properties as herbicides, and found that even the vapour of these substances had an effect upon plants. In order to obtain information on the amount of herbicide present in the vapour phase, we determined the vapour pressure of 10 different herbicides.

The vapour pressure of substances can be determined in various ways[5,6], a simple procedure being the gas saturation method[5-7]. A stream of dry inert gas is passed over the substance under examination in such a way that a saturated vapour results, the latter being in equilibrium with the solid phase. The saturation pressure of the substance is represented by its partial vapour pressure. Subsequently, the vapour is condensed, the amount of solid substance is determined by suitable means and the vapour pressure is finally obtained by calculation. For pressures in the order of magnitude of 10^{-4} to 10^{-5} mm Hg, the amount of substance transferred, assuming an average molecular weight of 250, is in the order of 10^{-6} g/l carrier gas. The amount of substance to be transferred depends on the sensitivity of the analytical procedure used. The measurement of vapour pressures of this order requires either a considerable amount of inert gas being passed through the system or, alternatively, a very sensitive method for determining minute quantities of condensed substance. High sensitivities are obtainable with methods like spectrophotometry, mass spectroscopy, etc. For our purpose, we thought gas chromatography with a flame ionisation detector the most suitable method. Gas chromatography is also highly selective and is therefore more advantageous than gravimetric, photometric or other non-specific methods. The substances to be examined is not required in an absolutely pure form. Any small impurities with a relatively higher vapour pressure do not cause an incorrect partial vapour pressure since the desired compound is obtained selectively in its pure form by gas chromatographic separation.

It will be shown later in this report that a simultaneous determination of the vapour pressures of several substances is possible without detrimental mutual interference. A further advantage consists in the fact that by using a gas chromatographic technique, the time-consuming degassing of the substance, in order to remove interfering substances which have relatively high vapour pressure, is unnecessary.

PRINCIPLE

The stream of gas leaving the saturation column 6 (Fig. 1) is fed to a gas chromatographic column 7, the latter being packed with a stationary phase suitable for the substance to be examined. Column 7 is cooled by solid carbon dioxide; the triazine therefore condenses. The total amount of gas passed through the system is obtained by measurement of the volume of water being forced from the gasometer. The substance condensed in column 7 is subsequently eluted in the gas chromatograph at a given temperature.

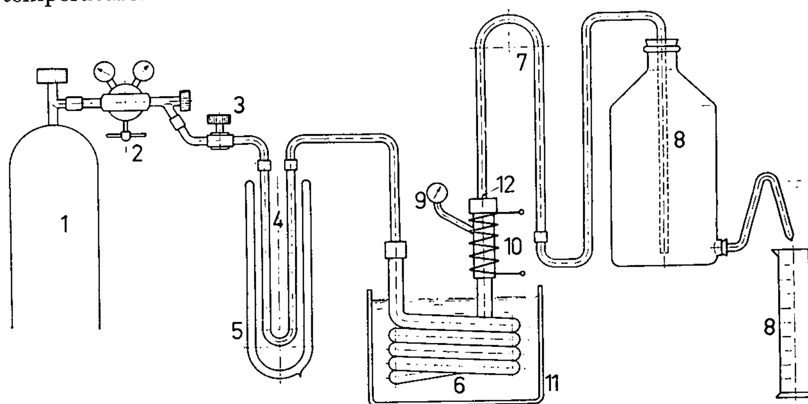


Fig. 1. Scheme of the apparatus. 1 = Nitrogen cylinder; 2 = pressure valve; 3 = fine adjustment of pressure; 4 = drying column packed with 5 Å molecular sieve; 5 = Dewar flask; 6 = saturation column; 7 = absorption column; 8 = measurement of the volume of inert gas; 9 = pressure at the outlet of the saturation column; 10 = heating between the saturation column and the gas chromatographic column; 11 = constant temperature bath; 12 = condensation of the transferred substance by means of dry ice.

If the total amount of gas used (V), the condensed amount of vapour (m), the molecular weight (M) and the experimental temperature (T) are known, the saturation pressure (p_s) is calculated by equation (1):

$$p_s = \frac{m}{M} \cdot \frac{22.415}{V_c} \cdot \frac{T}{273} \cdot \frac{p_B}{p_C} \cdot p_B \qquad (1)$$

where p_s = saturation pressure (mm Hg),
m = weight of transported substance (g),
M = molecular weight of substance,
V_c = volume of inert gas, as measured at the outlet of the gas chromatographic column (ml),
T = absolute temperature of the saturation column,
p_B = pressure of the inert gas at the outlet of the saturation column (mm Hg),
p_C = pressure of the inert gas at the outlet of the absorption column (mm Hg).

The ratio p_B/p_C corrects the gas volume at the outlet of the absorption column to that at the outlet of the saturation column. The *conditio sine qua non* for the application of the saturation method is the total saturation of the vapour leaving the column. This can easily be checked by carrying out a test series with the same compound at

varying gas flow rates. It can be shown that in a packed column having a pressure drop, a continuous saturation of the carrier gas from the inlet to the outlet takes place (Fig. 2). Due to the pressure drop between point A and B of the saturation column and between point B and C of the absorption column, the inert gas continually expands ($V_C > V_B > V_A$ if $p_C < p_B < p_A$). In order to establish the equilibrium, continuous

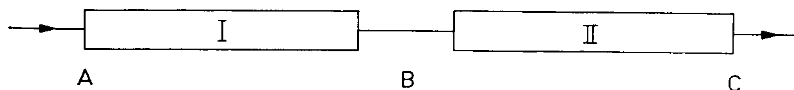


Fig. 2. I = Saturation column; II = absorption column (gas chromatographic column); A = inlet of inert gas; B = passage between I and II; C = outlet of the absorption column (point of integral measurement of the volume of the carrier gas at atmospheric pressure).

vaporisation throughout the saturation column is necessary. There is a further pressure gradient between point B and C in the absorption column (7), but no more solid is available here. The volume of carrier gas measured at point C (V_C) is compressed to V_B at point B:

$$V_B = V_C \cdot \frac{p_C}{p_B}$$

The volume of gas measured at point C has to be corrected for the conditions existing at point B because no further equilibrium between vapour and solid occurs during the absorption in the gas chromatographic column.

EXPERIMENTAL

Apparatus (see Fig. 1)

Pure nitrogen (99.99 %) is used as the transport gas. Traces of moisture are removed by passing the gas through a 5 Å molecular sieve column (4), which is cooled by solid carbon dioxide (5). The dried gas is now led into the saturation column (6), the latter consisting of a stainless steel tube of 2 m length and having an internal diameter of 8 mm. The column is packed with a mechanical mixture of 1 part of triazine and 6 parts of celite (20/30 mesh). The addition of celite increases the surface and improves the permeability of the column. Experiments showed that the same values for the vapour pressure were obtained whether the column had been packed with a mixture of triazines and celite or whether the solid phase had been impregnated with the triazines dissolved in a suitable solvent. The saturation column is plugged at each end by means of glass wool. A Haake-Ultra-Thermostat (11) maintains the desired temperature of the column to within $\pm$ 0.2°. The outlet (10) of the saturation column above the heating bath is electrically heated in order to avoid condensation of the volatile material in this part of the column. This heating equipment consists of a 100 W-cartridge heater, its output being controlled by a Variac (Fig. 3). The chromatographic column (7) extends a few mm into this heated zone from above. The column is joined to the saturation column by means of Ermeto tube-fittings so as to make the junction air tight. The condensation of the triazines in the gas chromatographic column is effected 5 cm above the level of the tube connection by cooling (9) with solid carbon dioxide.

A gas chromatographic column of 1 m in length and 4 mm I.D. packed with

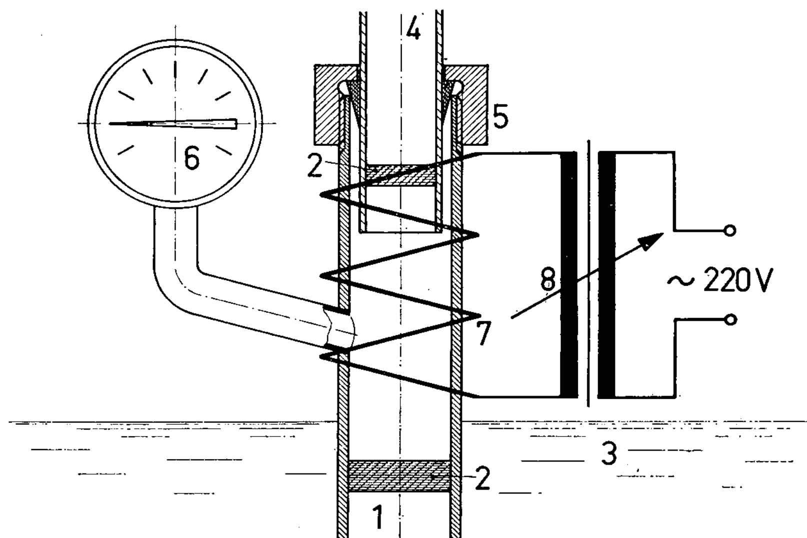


Fig. 3. Detail of tube connections. 1 = Saturation column; 2 = glass wool plugs; 3 = constant temperature bath; 4 = absorption column (gas chromatographic column); 5 = Ermeto tube fittings; 6 = pressure gauge 0–0.15 atm; 7 = heating 100 V; 8 = Variac.

1 % Reoplex 400 on kieselguhr (40/60 mesh) is very suitable for the absorption and the subsequent separation of the triazines. The determination is finally completed by disconnecting the chromatographic column from the saturation column and putting the former into the gas chromatograph. The type used in our laboratory was Perkin-Elmer, Fraktometer 116 E, equipped with flame ionization detector.

Working conditions

Pre-conditioning of the saturation column. This may be achieved before the substance is transferred to the absorption column, in order to remove the more volatile impurities. The necessity for pre-conditioning the substance under examination depends on the purity, volatility and possibility of interference in gas chromatographic determination. As an example, although this is not generally necessary, a column charged with Prometryne [2-methylmercapto-4,6-bis-(isopropylamino)-*s*-triazine] was pre-conditioned for 16 h at 130° and at a flow rate of 50 ml/min. The other triazines, even as mixtures, may be conditioned in a similar way.

Selection of flow rate. By altering the gas flow rate, one can ascertain whether the gas stream is completely saturated with the vapour of the substance under investigation. It is known that high flow rates result in incomplete saturation of the gas as the amount of substance transported is too small. Our experiments on the triazine herbicides showed saturation over a wide range of flow rates. Although the experiments discussed in this paper were carried out at a flow rate of 25 ml/min, doubling this value still did not lead to a decrease in transported substance.

Procedure. The cooled gas chromatographic column is connected to the saturation column for a given time, the total amount of carrier gas depending on the vapour pressure. Normally it is sufficient to transport 0.2–50 μg of triazine. Thereupon the gas chromatographic column is disconnected from the saturation apparatus and 10 μl

of a suitable standard are injected into the former by means of a Hamilton syringe. The triazine peaks and the standard peak on the chart should be of the same order of magnitude, this being achieved by selecting the proper concentration of the standard solution.

Other related triazines which differed completely from the peaks of the triazines in the mixture under investigation were chosen as standards. The correction factors were determined in separate runs. The absorption column is now placed in the gas chromatograph, the latter being already heated to the separation temperature. The flow rate of the carrier gas is then adjusted and the chromatogram is recorded. The chromatographic separation of the various triazines was made under the following conditions:

Column: 1 m, 4 mm I.D., 1 % Reoplex 400 on kieselguhr (acid washed 40/60 mesh),

Separating temperature: 180°,

Carrier gas: Nitrogen,

Flow rate: 30 ml/min,

Duration: 10 to 40 min, depending on the triazine examined.

The peak areas of the standard and sample are determined by planimetry and the total amount of transferred substance is calculated from the figures of the standard added, correction factor and peak areas.

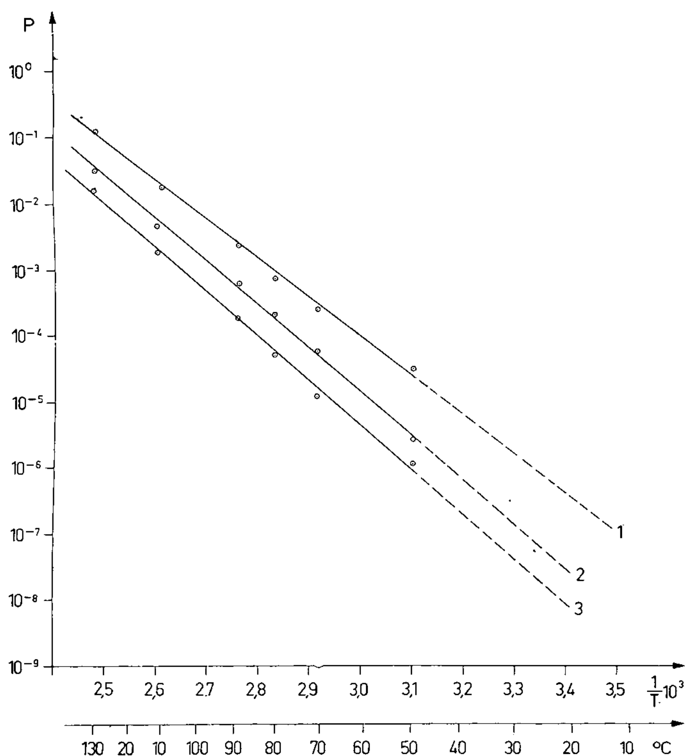


Fig. 4. Vapour pressure diagrams of 3 triazine herbicides. 1 = Atrazine; 2 = Propazine; 3 = Simazine.

RESULTS AND DISCUSSION

The vapour pressures of 10 triazines were determined in the range of 50° to 130° and the data are expressed by means of the vapour pressure constants A and B of the equation (2)

$$\log p = A - \frac{B}{T} \tag{2}$$

which is derived[7] from the Clausius-Clapeyron equation (3)

$$\frac{d \ln p}{d (1/T)} = \frac{-\Delta H}{R} \tag{3}$$

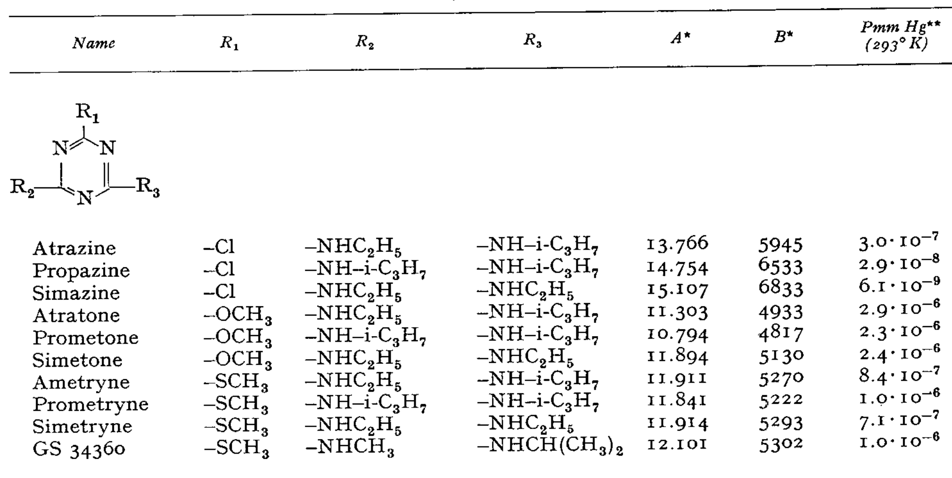
where A and B = vapour pressure constants,
T = absolute temperature,
ΔH = heat of vaporization per mole,
R = gas constant.

In Fig. 4, the vapour pressures obtained experimentally are plotted against the reciprocal absolute temperature for Azatrine, Propazine and Simazine. The straight lines indicate that eqn. (2) is quite accurate. The values for the vapour pressure constants of ten different triazines are given in Table I. The last column contains the vapour pressure extrapolated to 20°.

A considerable saving of time was achieved by the simultaneous determination of the vapour pressures of several triazines. The purity of the triazines under investigation ranged between 95–100 %. None of the impurities present had a detrimental effect on the results. By using a flame ionisation detector, only minute quanti-

TABLE I

VAPOUR PRESSURES AND VAPOUR PRESSURE CONSTANTS OF TEN SUBSTITUTED SYMMETRICAL TRIAZINES



Name	R_1	R_2	R_3	A*	B*	*Pmm Hg*** (293° K)
Atrazine	$-Cl$	$-NHC_2H_5$	$-NH-i-C_3H_7$	13.766	5945	$3.0 \cdot 10^{-7}$
Propazine	$-Cl$	$-NH-i-C_3H_7$	$-NH-i-C_3H_7$	14.754	6533	$2.9 \cdot 10^{-8}$
Simazine	$-Cl$	$-NHC_2H_5$	$-NHC_2H_5$	15.107	6833	$6.1 \cdot 10^{-9}$
Atratone	$-OCH_3$	$-NHC_2H_5$	$-NH-i-C_3H_7$	11.303	4933	$2.9 \cdot 10^{-6}$
Prometone	$-OCH_3$	$-NH-i-C_3H_7$	$-NH-i-C_3H_7$	10.794	4817	$2.3 \cdot 10^{-6}$
Simetone	$-OCH_3$	$-NHC_2H_5$	$-NHC_2H_5$	11.894	5130	$2.4 \cdot 10^{-6}$
Ametryne	$-SCH_3$	$-NHC_2H_5$	$-NH-i-C_3H_7$	11.911	5270	$8.4 \cdot 10^{-7}$
Prometryne	$-SCH_3$	$-NH-i-C_3H_7$	$-NH-i-C_3H_7$	11.841	5222	$1.0 \cdot 10^{-6}$
Simetryne	$-SCH_3$	$-NHC_2H_5$	$-NHC_2H_5$	11.914	5293	$7.1 \cdot 10^{-7}$
GS 34360	$-SCH_3$	$-NHCH_3$	$-NHCH(CH_3)_2$	12.101	5302	$1.0 \cdot 10^{-6}$

* A and B = vapour pressure constants (see ref. 6).
** Pmm Hg = vapour pressures at 293° K, calculated by eqn. (2).

ties of transferred substance are required for one determination and the transition time is low. On the other hand the determination of very low vapour pressures is achieved by using a minimum amount of transfer gas.

SUMMARY

A new method for the determination of vapour pressures by means of gas chromatography is described. A stream of inert gas is passed through a saturation column and the transferred substance is condensed in a cooled gas chromatographic column. A solution of a suitable standard is injected into the column and the condensed substance is subsequently eluted in a gas chromatograph at a preselected temperature. Vapour pressures in the order of 10^{-5} to 10^{-6} mm Hg can easily be determined. Ten 1,3,5-triazines substituted in the 2,4,6-positions were investigated in the temperature range of 50° to 130°, the vapour pressure constants were calculated and the extrapolated figures of the vapour pressures at 20° are given.

REFERENCES

[1] H. GYSIN AND E. KNÜSLI, *Proc. 3rd Brit. Weed Control Conf., Blackpool, 1956*, pp. 615–622.
[2] H. GYSIN AND E. KNÜSLI, *Proc. 4th Brit. Weed Control Conf., Brighton, 1960*, pp. 225–233.
[3] E. KNÜSLI, *Phytiat.-Phytopharm.*, 7 (1958) 81.
[4] A. GAST, *Mededel. Landbouwhogeschool Opzoekings-stat. Staat Gent*, 27 (1962) 3.
[5] H. KIENITZ, *Bestimmung des Dampfdruckes*, in HOUBEN-WEYL (Editors), *Methoden der organischen Chemie*. Band III. *Physikalische Forschungsmethoden*. Teil 1, Georg Thieme-Verlag, Stuttgart, 1953, p. 255ff.
[6] A. WEISSBERGER, *Physical Methods of Organic Chemistry*, Vol. 1, Part 1, Interscience, New York, 1959, pp. 401ff.
[7] W. KUHN AND P. MASSINI, *Helv. Chim. Acta*, 32 (1949) 1530.

COMPOSITION OF GUM TURPENTINES OF *PINUS HALEPENSIS* AND *PINUS BRUTIA* GROWN IN GREECE

N. ICONOMOU, G. VALKANAS AND J. BÜCHI
Abteilung für Pharmazeutische Chemie des Pharmazeutischen Institutes und Institut für Organisch-Chemische Technologie der Eidgenössischen Technischen Hochschule, Zürich (Switzerland)

(Received January 27th, 1964)

INTRODUCTION

Variations in the composition of gum turpentine oil (*i.e.* the steam volatile fraction of the oleoresin obtained by wounding a tree of the genus *Pinus*) have been reported for more than 90 well described and characterised species[1]. The detailed analysis of turpentine, which is a mixture of mainly terpenic hydrocarbons, has been hampered by the special difficulties involved in the analysis of this category of compounds. Gas chromatographic analysis is particularly attractive for the identification and characterisation of terpene mixtures. The high separation efficiencies, the inertness of the atmosphere during analysis, and the rapidity of the method, make it a most useful tool for analytical work[2–7]. The analysis of turpentine oil, however, has not been studied to the same extent as the analysis of other essential oils[8–11]. STANLEY AND MIROV[12] were the first to employ gas chromatographic methods in the analysis of American turpentine. The composition of gum turpentine from 22 species of *Pinus* grown in New Zealand has been similarly examined by WILLIAMS AND BANNISTER[10]. The characterisation of a number of turpentine oils of different origin has also been carried out in connection with the requirements of the Pharmacopœa Helvetica V[13].

Little is known about the composition of Greek turpentine. Some early studies are connected only with the determination of physical constants and the qualitative analysis of the mixture, only major components having been detected and characterised[14]. In Greece the production of turpentine oil had already been commercialised in olden times and many centuries of cultivation have resulted in a selection of the genus. The *Pinus halepensis* Mill., which is the main variety abundant on Greek soil, is known to have produced yearly 3–4 kg of oleoresin per tree for over 60 years, the highest reported production in the world[15]. Another variety, *Pinus brutia*, grows in only a few distinct districts of the country and is of minor commercial importance. *Pinus halepensis* grows in regions adjacent to the Mediterranean sea. The composition of this turpentine is reported in early analytical work as 95 % α-*d*-pinene for the low boiling destillate; the higher boiling fraction (tailing), accounting for less than 5 % of the product, being attributed to bornyl acetate (1.4 %) and to higher sesquiterpenes[16]. MIROV[17] has reported a composition of α-*d*-pinene 87 %, myrcene 2 %, sesquiterpenes 4 %, for a product of specific rotation $+41.25°$. *Pinus brutia* grows in the

Italian province of Calabria (ancient Brutium), Syria, Turkey, Greece and Cyprus. It is reported to give a laevorotatory turpentine, a first analysis of which gave a composition widely different from that of turpentine from *Pinus halepensis* (*l*- and *dl*-α-pinene 62 %, β-pinene 17 %, Δ^3-carene 13 %, terpinolene 2 %, sesquiterpenes 4.6 %), thus supporting the suggestion that the two pines are in fact different species[1]. Some botanists consider *Pinus brutia* to be a variety of *Pinus halepensis*, others believe that the two names are synonyms. *Pinus brutia* crosses naturally with *Pinus halepensis*[18].

EXPERIMENTAL AND RESULTS

To gather information on the change in turpentine oil composition with change in habitat, sampling was carried out in geographically different parts of the country. Samples of oleoresin of *Pinus halepensis* were collected in the districts of Corinth, Attica and Chalkidiki, whereas those of *Pinus brutia* come from Eubœa, where this species is very abundant. The samples were collected from a limited number of trees growing in representative areas of high growth density. Time of sampling and period of collecting were identical in all cases. The turpentine oil was separated by steam distillation on a laboratory scale and stored. Analyses performed at once and after storage indicated no changes in composition.

The standard Perkin-Elmer vapour fractometer model 116 used was equipped with a thermal conductivity detector and a 2.5 mV Siemens recorder. From a variety

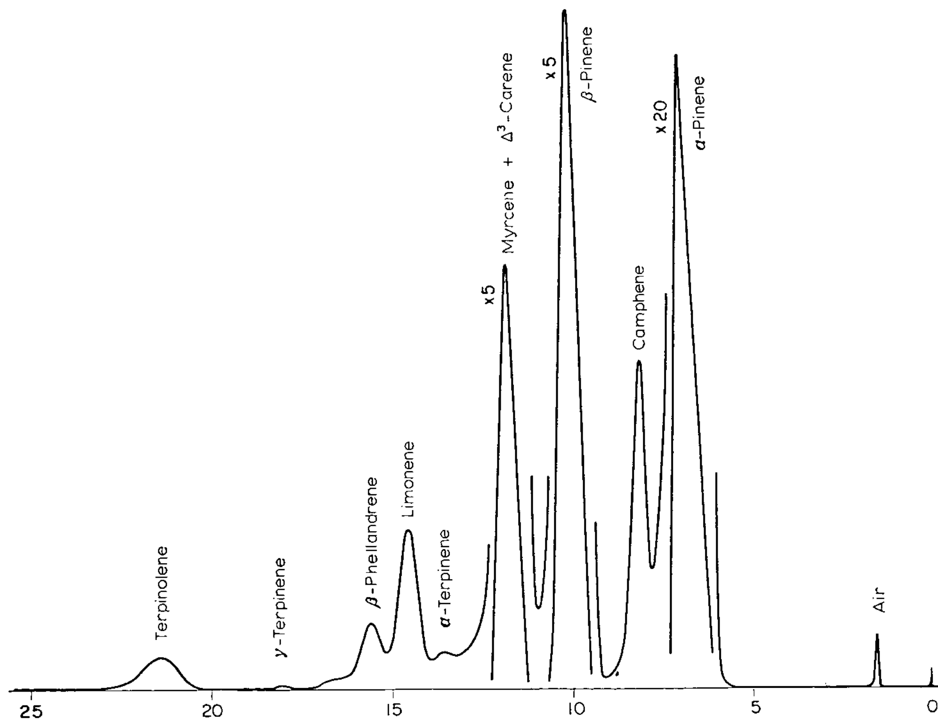


Fig. 1. Chromatogram of turpentine from *Pinus brutia* (Eubœa) on 4 m Carbowax 1500 column at 100° and helium flow rate 84 ml/min.

of columns tested the most satisfactory overall separation of the constituents of the turpentines was obtained with 4 m of Carbowax 1500, 16 % on Chromosorb W, and 4 m diisodecyl phthalate, 20 % on Celite 545, at temperatures of 100° and 162°, and helium flow rates of 84 ml/min and 86 ml/min respectively (Figs. 1 and 2).

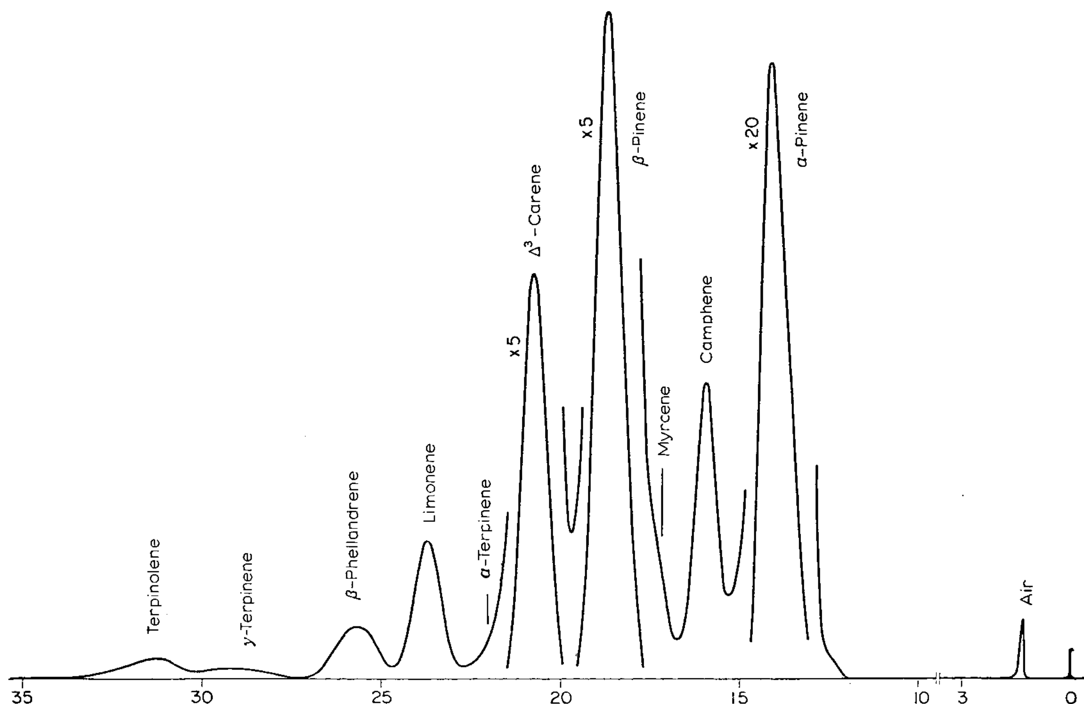


Fig. 2. Chromatogram of turpentine from *Pinus brutia* (Eubœa) on a 4 m diisodecyl phthalate column at 102° and helium flow rate 86 ml/min.

To identify the peaks obtained in the chromatograms, retention data were compared with those of authentic samples taken under the same conditions. Further proofs of identity were obtained by correlating our data with literature results, as described in another paper[19]. A Carbowax 1500 column gave better shaped peaks, more suitable for quantitative analysis, while a diisodecyl phthalate column gave a better separation. Myrcene and Δ^3-carene, which appear together on the first column, were well resolved on the second column, where myrcene precedes β-pinene. The results of the quantitative analyses, calculated by the methods already described[9], are identical for both columns within the limits of experimental error (Table I). The different temperatures employed show that there is no isomerisation under these analytical conditions; this has also been established in other studies[7].

There are practically no differences in the composition of the *Pinus halepensis* samples, which indicates that this variety on Greek soil does not show variation of turpentine oil composition with change in habitat. Since turpentine oil composition is connected with the tree physiology[20] this would infer that the *Pinus halepensis* variety in Greece is of unique genus. The composition of *Pinus brutia* turpentine differs

TABLE I

GAS CHROMATOGRAPHIC ANALYSIS OF TURPENTINES OF *Pinus halepensis* AND *Pinus brutia*

Compound	*Pinus halepensis*						*Pinus brutia Eubœa*	
	Attica		*Corinth*		*Chalkidiki*			
	Carbowax 1500	*Diisodecyl phthalate*	*Carbowax 1500*	*Diisodecyl phthalate*	*Carbowax 1500*	*Diisodecyl phthalate*	*Carbowax 1500*	*Diisodecyl phthalate*
α-Pinene, %	96.2	96.3	96.1	96.2	96.0	96.1	68.1	67.1
Camphene, %	0.7	0.65	0.7	0.7	0.8	0.7	0.7	0.9
β-Pinene, %	0.9	0.9	0.8	0.8	0.7	0.6	16.6	16.9
Unidentified	traces		traces		traces		traces	
Myrcene, %		0.75		0.8		0.5		0.9
	1.05		1.1		0.8		12.5	
Δ^3-Carene, %		0.25		0.3		0.2		11.6
α-Terpinene, %	—	—	—	—	—	—	0.1	0.1
Limonene, %	1.0	1.0	1.1	1.0	1.45	1.5	0.8	0.7
β-Phellandrene, %	0.05	0.05	0.1	0.05	0.05	0.1	0.5	0.5
p-Cymene, %	traces		traces		traces		traces	
γ-Terpinene, %	—	—	—	—	traces		traces	
Terpinolene, %	0.1	0.1	0.1	0.15	0.2	0.25	0.7	0.7

greatly from that of *Pinus halepensis* in having a high content of β-pinene and Δ^3-carene. This supports the theory that the two varieties are different species[1].

The relative retention times of components of turpentines from *Pinus halepensis* and *Pinus brutia* are shown in Table II.

TABLE II

RELATIVE RETENTION TIMES OF TERPENE HYDROCARBONS OF TURPENTINES FROM *Pinus halepensis* AND *Pinus brutia* COMPUTED FROM CHROMATOGRAMS 1 AND 2

(α-Pinene = 1.00)

Compound	*Stationary phase*	
	Carbowax 1500	*Diisodecyl phthalate*
α-Pinene	1.00	1.00
Camphene	1.32	1.15
β-Pinene	1.67	1.36
Myrcene	2.04	1.28
Δ^3-Carene	2.05	1.53
α-Terpinene	2.42	1.67
Limonene	2.67	1.79
β-Phellandrene	2.86	1.95
γ-Terpinene	3.4	2.08
Terpinolene	4.16	2.46

SUMMARY

Separate samples of gum turpentine *Pinus halepensis* and *Pinus brutia* grown in Greece were analysed by gas–liquid partition chromatography using two stationary phases of different polarity. It was found that the turpentine of *Pinus halepensis* consisted mainly of *d*-α-pinene (about 96 %), while that of *Pinus brutia* contained less

d-α-pinene (about 68 %) and substantially amounts of β-pinene (about 16 %) and Δ^3-carene (about 12 %). Other identified compounds were: camphene, myrcene, α-terpinene, limonene, β-phellandrene, *p*-cymene, γ-terpinene and terpinolene.

REFERENCES

1. N. T. Mirov, *U.S. Dept. Agr. Forest Service, Bull.*, No. 1239 (1961).
2. A. Liberti and G. P. Cartoni, in D. H. Desty (Editor), *Gas Chromatography 1958*, Butterworths, London, 1958.
3. E. von Rudloff, *Can. J. Chem.*, 38 (1960) 631.
4. H. Westaway and J. F. Williams, *J. Appl. Chem. (London)*, 9 (1959) 440.
5. G. Eglinton, *Chem. Ind. (London)*, (1959) 955.
6. E. Stahl and L. Trennheuser, *Arch. Pharm.*, 293 (1960) 826.
7. M. H. Klouwen and R. Ter Heide, *J. Chromatog.*, 7 (1962) 297.
8. M. H. Bannister, A. L. Williams, I. R. C. McDonald and M. B. Forde, *New Zealand J. Sci.*, 5 (1962) 486.
9. W. J. Zubyk and A. Z. Conner, *Anal. Chem.*, 32 (1960) 912.
10. A. L. Williams and M. H. Bannister, *J. Pharm. Sci.*, 51 (1962) 970.
11. J. Haslam and A. R. Jeffs, *Analyst.*, 87 (1962) 659.
12. R. G. Stanley and N. T. Mirov, *133rd National Meeting, Am. Chem. Soc., San Francisco*, April 1958, Abstr., p. 7A, No. 16.
13. N. Iconomou, G. Valkanas and J. Buchi, *Pharm. Acta Helv.*, 38 (1963) 875.
14. D. E. Tsakalotos, *J. Pharm. Chim. (Athens)*, 11 (1915) 70.
15. B. Pejoski, *Fette, Seifen, Anstrichmittel*, 62 (1960) 626.
16. G. Dupont, *Chim. Ind. (Paris)*, 8 (1922) 320.
17. N. T. Mirov, *J. Am. Pharm. Assoc., Sci. Ed.*, 43 (1954) 378.
18. I. Papajoannou, *Forstwiss. Zbl.*, 58 (1936) 194.
19. G. Valkanas and N. Iconomou, *J. Chromatog.*, 12 (1963) 536.
20. R. G. Stanley, *Proc. Intern. Congr. Biochem. 4th, Vienna*, 2 (1958) 48; *C.A.*, 54 (1960) 15536.

APPLICATION DE LA CHROMATOGRAPHIE EN PHASE GAZEUSE À L'ÉTUDE DES PRODUITS DE PYROLYSE D'ALCALOÏDES INDOLIQUES

I. OBTENTION DES PYROGRAMMES

G. VAN BINST, L. DENOLIN-DEWAERSEGGER ET R. H. MARTIN

Service de Chimie Organique, Université Libre de Bruxelles, Bruxelles (Belgique)*

(Reçu le 19 février 1964)

La détermination de structure de substances complexes peu volatiles et de poid-moléculaire élevé, et principalement de polymères, peut être effectuée par chromatos graphie gazeuse de leurs pyrolysats dont les fragments sont identifiés aisément par comparaison à des référents connus (le plus souvent des monomères entrant dans la composition des polymères). Les techniques de pyrolyse utilisées sont variées: le four à induction[1-2], le four à résistance chauffante[3-5], le filament[6-8]. Pour la détermination de structure d'alcaloïdes, la pyrolyse est un moyen classique utilisé depuis longtemps. Cette réaction n'a cependant été associée à la chromatographie gazeuse que récemment: plusieurs études ont été entreprises par JANÁK[9-11] sur l'atropine et la cocaïne, par NELSON ET KIRK[12] et par FRANC ET BLAHA[13]. Ces pyrolyses ont été effectuées sur filament.

Pour notre travail, nous avons choisi la technique de pyrolyse en tube capillaire scellé sous vide, plongé dans un bain métallique, telle qu'elle était décrite auparavant[14]. Ce moyen de pyrolyse nous a permis de mettre au point les conditions de séparation indépendamment des conditions de pyrolyse tout en présentant l'avantage de permettre le piégeage des fragments en quantité suffisante pour l'identification par spectroscopie I.R. ou U.V.

Les alcaloïdes étudiés sont les suivants (Fig. 1):

Groupe A – Cycle C hexatomique

β-Yohimbine	I
Réserpiline	II
Réscinamine	III
Corynanthine	IV

Groupe B – Cycle C hexatomique ponté

Voachalotine	V
Tombozine	VI
Ajmaline	VII
Déshydroxyméthyl-voachalotinol	VIII

* Directeur: Prof. R. H. MARTIN.

Groupe B' – Cycle C hexatomique ponté avec ouverture en 3,4

Vobasine	IX
N-Cyanoapodéshydroxy-méthylvoachalotine	X

Groupe C – Cycle C heptatomique

Ibogaïne	XI
Voacangine	XII
N-Méthylibogaïne	XIII

La quantité minimum requise pour l'obtention d'un pyrogramme est de 0.5 mg. L'allure du pyrogramme est peu sensible à la durée de pyrolyse: celle-ci a été fixée à 1 min.

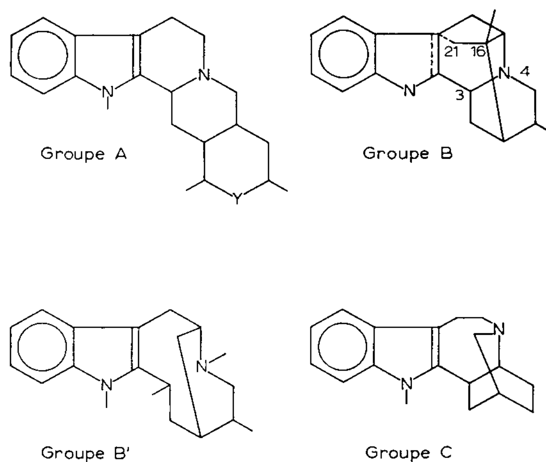


Fig. 1. Structure des alcaloïdes étudiés.

RECHERCHE DES CONDITIONS OPTIMUMS DE PYROLYSE

Dans le domaine de température où l'on observe un pyrogramme, seule l'intensité relative des pics varie, et non leur nature et nous pouvons en déduire que l'interprétation des pyrogrammes ne dépend que très faiblement des conditions de pyrolyse. Nous considérons que la température idéale de pyrolyse est celle pour laquelle le pic de l'alcaloïde inchangé n'apparaît plus et où on remarque des pics intenses aussi bien parmi les substances de temps de rétention courts que parmi celles de temps de rétention longs. Les pyrolyses ont été effectuées de 50 en 50° entre 250 et 450°. Une fois un intervalle fixé de cette façon, celui-ci a été exploré de 10 en 10°.

Les températures optimales trouvées sont les suivantes:

Alcaloïdes du groupe A: 350°.
Alcaloïdes du groupe B: 375°.
Alcaloïdes du groupe C: 425°.

RECHERCHE DES CONDITIONS IDÉALES DE SÉPARATION*

Il apparaît des résultats dans le Tableau I que les deux meilleures phases liquides sont le silicone gum rubber (SE 30) et le diéthylèneglycoladipate (DGA). Malheureusement cette dernière phase ne permet pas de dépasser 225° et ne peut donc servir qu'à identifier les premiers pics des pyrogrammes. Les pyrogrammes complets discutés dans ce qui suit ont donc été obtenus par chromatographie sur une colonne de 1 m de SE 30 à 20 % utilisée en programme de température de 75 à 300° à 4.2° par min, et ensuite à température constante à 300°.

TABLEAU I

CONDITIONS DE SÉPARATION

Phase liquide	*Phase solide*	*n*	*Température °C*	*Longueur m*	*HETP mm*
Carbowax 3 % + silicone gum rubber SE 30 7 %	Chromosorb P	200	260	2	10
		colonne instable			
Apiézon L 20 %	Chromosorb W	1450	300	2	1.4
Dow Corning QF_1 10 %	Gas Chrom P présiliconé	660	200	1	1.5
Silicone gum rubber SE 30 20 %	Gas Chrom P	950	250	1	1
Silicone gum nitrile 10 %	Diatoport S	520	250	1	1.9
Diéthylèneglycoladipate 10 % (DGA)	Chromosorb W	1200	175	1	0.8

DISCUSSION DES PYROGRAMMES

Alcaloïdes à cycle C à 6 atomes pontés (groupe B et B')

Ces alcaloïdes se distinguent entre eux par la présence ou l'absence du groupement N^{α}-CH_3, par la nature des substituants en C_{16}, par la stéréochimie de ceux-ci au niveau du C_{16}, et, pour l'ajmaline, par la présence d'un noyau dihydroindolique, du groupement OH en C_{21} et de la chaîne saturée C_2H_5 au lieu de la chaîne éthylidène. Remarquons cependant que la voachalotine se transforme préalablement en déshydroxyméthylvoachalotine[15] et qu'en fait la pyrolyse s'effectue sur ce dernier composé.

La comparaison des pyrogrammes (Fig. 2) dont l'allure caractérise très bien ce groupe d'alcaloïdes permet de mettre en évidence deux pics intenses communs aux quatre substances: 14.5 et 15 min. D'autre part, dans le cas des trois alcaloïdes N^{α}-méthylés, il apparaît un pic à 22 min et un pic à 35 min. Ceux-ci ne se retrouvent pas dans la tombozine, mais, dans le pyrogramme de la tombozine, on observe un pic caractéristique à 30.6 min.

Il est remarquable de constater que l'ajmaline, alcaloïde dihydroindolique, fournit le même pyrogramme que les alcaloïdes indoliques pontés. Il faut donc supposer que l'un des premiers stades de la pyrolyse est la rupture du pont suivie de la formation de la double liaison indolique. A partir de ce moment, le schéma de pyrolyse devient analogue à celui des autres alcaloïdes indoliques pontés. Nous n'avons pu mettre en évidence des différences dues à la présence d'une chaîne éthylidène ou éthyle dans ces alcaloïdes.

* Les pyrogrammes ont été relevés sur des appareils F et M modèles 720 et 1609.

Les pyrogrammes de la N-cyanoapodéshydroxyméthylvoachalotine (X), produit d'ouverture du cycle C, dérivé de la voachalotine et de la vobasine (IX) de structures semblables, ont été comparés à celui de la voachalotine. Les trois pyrogrammes présentent de nombreuses similitudes ce qui permet de supposer, soit que la liaison 3,4 de la déshydroxyméthylvoachalotine se rompt au début de la fragmentation, soit

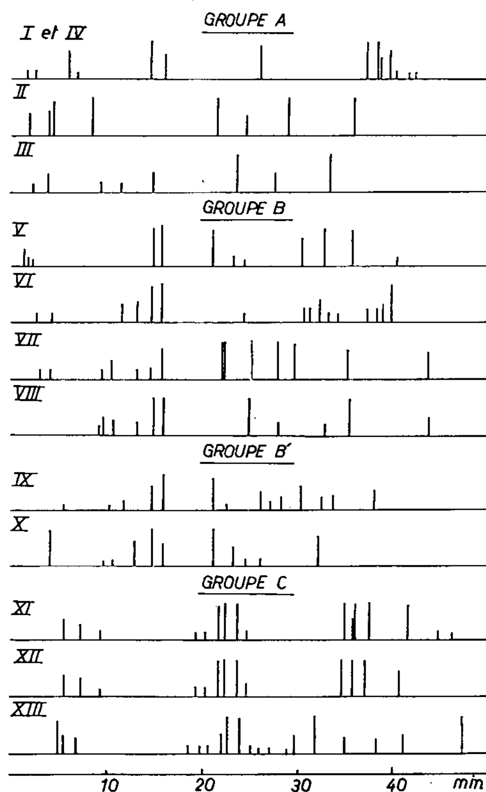


Fig. 2. Schéma des pyrogrammes.

que dans les dérivés de la vobasine une recyclisation a lieu. Dans la vobasine il y a cependant un centre de rupture supplémentaire engendré par la présence de la fonction C=O. Aussi, dans le pyrogramme de cet alcaloïde, n'y a-t'il pas de fragments prépondérants: tous les pics sont d'intensité à peu près équivalente.

En conclusion, tous les alcaloïdes apparentés au groupe à cycle C hexatomique ponté, ou leurs dérivés, sont reconnaissables à leur pyrogramme grâce aux pics de temps de rétention: 14.5; 15.5; 22; 24.5; 26 et 34 min.

Alcaloïdes à cycle C à 6 atomes du type yohimbine et hétéroyohimbine

Si l'on compare les pyrogrammes (Fig. 2) de ce groupe-ci à ceux du groupe précédent, on remarque immédiatement que les fragments sont moins nombreux. Ceci peut s'expliquer par une diminution de centres de rupture due à l'absence du pont. La β-yohimbine possède en commun avec le groupe précédent les pics à 14.5, 33 et 35.7

min. Par contre, il n'existe aucune similitude entre la β-yohimbine, la réserpiline et la réscinamine. Remarquons toutefois que la réscinamine est monométhoxylée et que la réserpiline est diméthoxylée ce qui justifierait un déplacement des pics vers des temps de rétention plus élevés.

Alcaloïdes à cycle C à 7 atomes

Les trois alcaloïdes que nous avons étudiés dans ce groupe sont monométhoxylés. Leurs pyrogrammes (Fig. 2) sont pratiquement identiques et sont très caractéristiques de ce type de structure. Dans le cas de la N-méthylibogaïne, on remarque cependant un retard d'une minute environ pour les fragments de temps de rétention "moyen" (21, 23 et 24 min) par rapport à ceux de l'ibogaïne et de la voacangine.

CONCLUSIONS

L'étude des pyrogrammes de trois types d'alcaloïdes permet de caractériser avec certitude les alcaloïdes à cycle à 7 atomes et à 6 atomes pontés. Les échantillons du troisième type, les analogues de la yohimbine dont nous disposons actuellement présentent une diversité structurale trop variée pour tirer des règles générales.

Nous poursuivons nos travaux sur un plus grand nombre d'échantillons et l'identification des fragments par spectroscopie I.R. et U.V. est en cours actuellement.

REMERCIEMENTS

Nous remercions Monsieur J. PECHER, chargé de cours associé, pour les discussions fructueuses que nous avons eues avec lui et pour plusieurs échantillons de dérivés de la voachalotine.

Que le Docteur X. MONSEUR (Laboratoire des Recherches Chimiques du Ministère de l'Agriculture à Tervuren), qui nous a remis un échantillon de tombozine, trouve ici l'expression de notre gratitude.

Nous remercions l'Institut pour l'Encouragement à la Recherche Scientifique dans l'Industrie et l'Agriculture pour la bourse de spécialisation accordée à l'un de nous (L.D.) et le Fonds de la Recherche Fondamentale Collective qui a octroyé un subside au laboratoire.

RÉSUMÉ

Les pyrogrammes permettent de distinguer et de caractériser neuf alcaloïdes indoliques contenant un cycle C à 7 atomes ou un cycle ponté à 6 atomes.

Les structures des quatre alcaloïdes du type yohimbine étudiés jusqu'à présent sont trop différentes pour permettre une comparaison de l'allure des pyrogrammes. L'identification des fragments par spectroscopie IR, UV et spectrométrie de masse est en cours ainsi que la pyrolyse de nouveaux alcaloïdes.

SUMMARY

Nine indole alkaloids containing either a seven-membered or a bridged six-membered C ring can be distinguished and characterized by their pyrograms. The structures of the four yohimbine-type alkaloids studied so far differed too much to allow a reason-

able comparison. Identification of the fragments by I.R., U.V., and mass spectrometry is being studied while work on more alkaloids is in progress.

BIBLIOGRAPHIE

1 F. A. LEHMANN ET G. M. BRAUER, *Anal. Chem.*, 33 (1961) 673.
2 K. ETTRE ET P. F. VÁRADI, *Anal. Chem.*, 34 (1962) 752.
3 C. G. HERVITT ET B. T. WHITHAM, *Analyst*, 86 (1961) 643.
4 C. G. SMITH, *Analyst.*, 86 (1961) 480.
5 C. G. HERVITT ET B. T. WHITHAM, *Analyst.*, 86 (1961) 645.
6 R. S. LEHRLE ET J. C. ROBB, *Nature*, 183 (1959) 1671.
7 C. R. JONES, dans R. P. W. SCOTT (Éditeur), *Gas Chromatography, 1960*, Butterworths, London, 1960.
8 J. STRASSBURGER, G. M. BRAUER, M. TRYON ET A. F. FORZIATI, *Anal. Chem.*, 32 (1960) 454.
9 J. JANÁK, dans R. P. W. SCOTT (Éditeur), *Gas Chromatography, 1960*, Butterworths, London, 1960.
10 J. JANÁK, *Nature*, 185 (1960) 684.
11 J. JANÁK, *Collection Czech. Chem. Commun.*, 151 (1960) 780.
12 D. F. NELSON ET P. L. KIRK, *Anal. Chem.*, 34 (1962) 899.
13 J. FRANC ET J. BLAHA, *J. Chromatog.*, 6 (1962) 396.
14 R. GOUTAREL, F. PERCHERON ET M. M. JANOT, *Compt. Rend.*, 243 (1956) 1670.
15 N. DEFAY, M. KAISIN, J. PECHER ET R. H. MARTIN, *Bull. Soc. Chim. Belg.*, 70 (1961) 475.

CHANGES IN THE IMMOBILE AQUEOUS PHASE OF CELLULOSE DURING CHROMATOGRAMS FORMED WITH ABSOLUTE ETHANOL

BETTY B. WEINBERG AND ROY A. KELLER
Department of Chemistry, University of Arizona, Tucson, Ariz. (U.S.A.)

(First received August 27th, 1962)
(Modified December 31st, 1963)

For a chromatographic system operated at near-equilibrium conditions, the fraction of solute molecules in the mobile phase at any time in any infinitesimal segment, dx, of the migration path is given by[1,2]

$$R_i = \frac{1}{1 + \alpha_i(A_L/A_M) + \beta_i(A_S/A_M)} \qquad (1)$$

where α_i is the ratio of the concentration of the ith solute in the stationary liquid to that in the mobile fluid, β_i is the ratio of the solute concentration adsorbed on the solid support to that in the mobile phase, A_L is the cross-sectional area of the immobile liquid, A_S is the "cross-sectional area" of the solid support, *i.e.*, it is some measure of the extent of the solid surface, and A_M is the cross-section of the mobile fluid. The term $\alpha_i(A_L/A_M)$ measures solute retention by the partitioning liquid and $\beta_i(A_S/A_M)$ measures solute retention by the partitioner support. If β_i is very small, *i.e.*, the support is essentially inactive, or if $A_L \gg A_S$ (*cf.* ref. 3) then $\beta_i(A_S/A_M)$ is negligible and

$$R_i = \frac{1}{1 + \alpha_i(A_L/A_M)} \qquad (2)$$

If α_i, A_L, A_M, and the velocity of the mobile fluid are constant in the direction of solvent flow (x-direction), then for liquid-liquid chromatograms, $R_i = R_{Fi}$ where R_{Fi} is the ratio of the distance moved by the center of the zone of the ith solute to the distance moved by the mobile fluid front. Insufficient consideration has been given to the dependency of A_L and A_M on the coordinate x. GIDDINGS, STEWART AND RUOFF[4] have shown that A_M is not constant along the migration path in paper chromatography. This leads to a considerable dependency of R_F upon x for certain R_F-values. In gas–liquid chromatography, A_L may be reduced at the column inlet by evaporation of the liquid partitioner[5–7]. Similarly for liquid–liquid systems, if the mobile phase is not saturated with the liquid acting as the immobile phase, it will extract the latter from the support until saturated. Not only is A_L a function of the x-coordinate but also, in the region of partitioner loss, A_L may be so reduced that the solid adsorption term of eqn. (1) cannot be ignored while further along the path, eqn. (2) is appropriate. It may also be that α_i has one value in the region for saturated mobile phase and a different value in the region of unsaturated carrier. Generally this problem can be

avoided when the mobile and immobile liquids used are immiscible in the bulk by saturating each with the other before forming the chromatogram. Nonequilibrium between these two phases has led to anomalous results, *e.g.*, the appearance of two zones for a single solute[8].

A problem arises when the mobile phase is a liquid which is completely miscible with the liquid of the immobile phase[9-12]. The equilibrium concentration of partitioner in the mobile phase is not known and if there is nonequilibrium, A_L will not be constant.

The system selected for study here was paper, where the immobile phase is water, and a mobile phase of absolute ethanol. Because of the difficulty of analyzing paper for its water content, we elected to measure it in the eluant fractions much as one seeks partitioner in the effluent gas in gas-liquid chromatography.

EXPERIMENTAL

Gas chromatographic analysis

The problem was the analysis of 0.2 ml eluant fractions from paper and cellulose pulp column chromatograms where the amount of water in the ethanol was 10 % or less. Gas chromatography seemed the best method for fast, simple, and duplicate analysis.

The equipment was a Cenco No. 70130 Vapor Phase Analyzer (Central Scientific Co., Chicago, Ill., U.S.A.) with a thermal conductivity cell. The machine had nine sensitivity settings by which the response, and hence the height of the concentration profile, could be adjusted. Column, detector, and sample injection unit were all at the same temperature. Concentration profiles were recorded on a Leeds and Northrup Speedomax Model S, variable range, variable sensitivity recorder of one second response time and 30 in./h chart speed. Driving pressure was measured by a mercury manometer at the column inlet and flowrate by a soap-film flowmeter at the outlet. Sample introduction was by a 100 μl Hamilton syringe.

A difficulty with any analysis of a binary mixture where the proportion of one component to the other is very small (trace analysis) is that the peak of the principal constituent is very much larger than that of the other. There is considerable error in measuring the small peak areas[13-15]. The equipment lacked an automatic attenuation device to reduce the response of the detector in proportion to the signal. Attenuation was accomplished by manual adjustment. The alcohol peak was kept on scale by using a large millivolt range on the recorder and a low sensitivity setting on the gas chromatograph. After the alcohol peak had passed, the millivolt range was reduced on the recorder, and the sensitivity on the gas chromatograph was increased which magnified the following water peak. This procedure required a sufficient difference in retention times of the ethanol and water so that the adjustment could be made and a reliable base line established. The retention times themselves could not be too large since this broadened and flattened the water peak so that it was indistinguishable from the background circuit noise.

The column used was 3 ft. of coiled 0.25 in. O.D. copper tubing packed with 12.1 g of 30-50 mesh of Neutraport-T, a fluorocarbon (Micro-Tek Instruments Inc., Baton Rouge, La., U.S.A.), bearing THEED (tetrahydroxy-ethylenediamine)[16,17] (Applied Science Laboratory, State College, Pa., U.S.A.) prepared by evaporation of an acetone solution of the partitioner while in contact with the support. Repeated

extraction of the packing indicated a 10.5 % liquid load. The column was conditioned by passing helium through it at 23.3 ml/min for 7 h at 100°. THEED is very hygroscopic. A water peak always appeared on the record whenever the machine was started. Before every series of analyses, the machine was allowed to run at the operating conditions until this water peak was eluted and a constant base line established. A driving pressure of 10 cm Hg gave a consistent carrier flow of 30 ml/min at 84°. The retention times of ethanol and water were 5.0 min and 24 min respectively and permitted the adjustment of the machine between peaks without introducing appreciable tailing or peak broadening. Tailing was never completely eliminated. The retention times varied slightly with sample size. After nine months of use, the retention time of ethanol was reduced to about 3.5 min while that of water was about 18 min, which is the type of change that one would expect with loss and redistribution of partitioner[5-7].

Samples of known composition were prepared from absolute ethanol (U.S. Industrial Chemicals Co., New York, U.S.A.) and water. Peak areas were measured with a planimeter and the average of three measurements used to compute the per cent area of the water and ethanol peaks. No adjustment was made for the attenuation. A least squares line was fitted to the data points for the variation of area per cent *vs.* water content for the prepared samples of known composition from 1.16 to 10.66 % weight by water (16 data points, 20 μl samples) and 0.59 to 2.28 % (12 data points, 70 μl samples) using both weight per cent and mole per cent and a linear regression coefficient[18] computed in each case. Both plots were slightly nonlinear with virtually identical regression coefficients (0.9869 and 0.9861 respectively). We used weight per cent since it reduced the computations. The deviations of the area per cents from the mean were computed for each sample and their squares pooled to calculate a standard deviation. An average estimate of the error based on twice the standard deviation is 0.45 % water for the range of 0.59 to 10.66 % water. Samples containing 0.5 % water gave small but measurable peaks. This was the lower limit of this method of analysis. For samples of 0.35 % water or less, peaks were not detected. Between these extremes, the water content of samples was estimated by visual comparison of the tracing with those of standards. Samples of the same composition and differing in size by less than 20 % gave per cent areas which varied within experimental error. Outside of this limit, peak area per cents were detectable as dependent upon the sample size.

Because of the change in the properties of the column with use as indicated by the change in retention volume, calibrations were interspersed with analyses. There was insufficient change in the standard curves to warrant the construction of new calibration curves during the collection of pertinent data.

Sample storage

Sample collection tubes were fastened firmly to the outlet of the chromatographic system by means of a tight fitting cork with a small vent on its side and collections were made at room temperature. Tubes, with their solvent fractions, were tightly stoppered with corks and stored in a glass bottle with a tightly fitting screw cap. This bottle was placed in a desiccator over calcium chloride until analyzed. Even with this, the water content of the stored samples increased as will be pointed out later.

Cellulose pulp columns

Ethanol was added to a 22 × 2.2 O.D. cm chromatographic tube from a separatory funnel protected from atmospheric moisture by a drying tube containing calcium chloride. Collection was made in carefully dried test tubes, calibrated for 0.2 ml. Fig. 1 shows the percentage water as a function of total eluant volume for a column

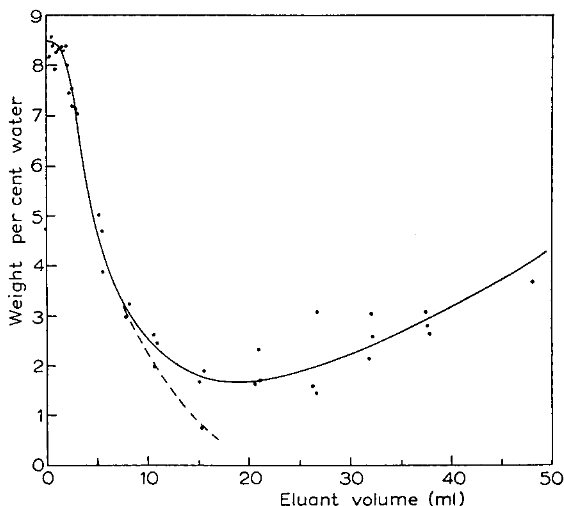


Fig. 1. Weight per cent water in the eluant as a function of the total eluant volume for the 17 cm column of cellulose pulp.

of cellulose pulp (Whatman Cellulose Powder, Standard Grade) 17 cm in length and weighing 18.21 g. Fig. 2 shows the data for a column, 9 cm long, weighing 10.72 g. Analysis of the alcohol in the reservoir on completion of the chromatograms did not detect water.

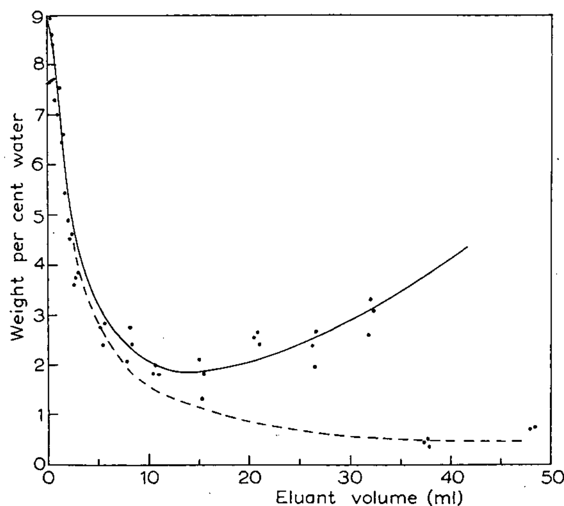


Fig. 2. Weight per cent in the eluant as a function of the total eluant volume for the 9 cm column of cellulose pulp.

Both Figs. 1 and 2 show about 9 % water in the first eluant fractions. It was not thought that the increasing amount of water present in later eluant fractions had meaning in terms of the chromatographic process but was due to contamination of the samples in storage. Fig. 1 represents 92 individual analyses and Fig. 2 represents 103 analyses. As rapid as gas chromatography was as an analytical method, a period of over a week elapsed before the last samples were analyzed and there was ample time for contamination. Fig. 1 shows a sample of 15 ml total eluant volume where the water content was much lower than the others. This particular sample was analyzed much earlier than the others in this region. Fig. 2 shows fractions at 37 and 48 ml total eluant volume which were also among the first analyses. Their water content was about 0.5 % while those at 20 to 30 ml total eluant volume showed 2.5 to 3 % water. For these reasons, the dotted lines in Figs. 1 and 2 are probably better estimates of the water content of successive eluant samples than are the solid lines.

Each point of Figs. 1 and 2 represents the mean of from two to four analyses of an eluant fraction. A deviation from the mean was computed for the analysis of each 0.2 ml fraction and the squares of these combined to calculate a pooled standard deviation corresponding to a precision of 0.60 % at the 95 % confidence limit.

Paper strip chromatography

The chamber for the paper chromatography consisted of a glass pipe 19.4 cm in length and 7.0 cm I.D. stoppered at both ends. An aluminum cradle hung from the top stopper and held a glass solvent boat. Solvent was added to the boat through a hole in the stopper which was closed during the experiments. The lower stopper held a thistle tube which collected the drippings from the paper and delivered them to calibrated test tubes.

Whatman Filter Paper No. 1 was cut into strips measuring 22.6 × 4.1 cm. The solvent passed through about 19.1 cm of paper in its journey from the solvent surface in the boat to the end of the strip. A typical strip weighed 0.803 g, which meant that the solvent passed through 0.678 g of cellulose. Before use the chamber was rinsed twice with absolute ethanol and 25 ml of ethanol was placed in the bottom of the chamber so that an alcohol saturated atmosphere was established in the chamber.

All experiments were performed at room temperature (26–28°).The papers were treated in several ways. (1) The paper strip was suspended in the chamber overnight in contact with ethanol vapors. The solvent of absolute ethanol reached the bottom of the strip in about two hours and 1.5 to 1.8 h were required to collect 0.2 ml of eluant. Analysis showed no detectable water. (2) The strip was placed in the chamber and the solvent flow was started immediately. Analysis showed 0.35 % or less water in one experiment and 0.50 % water in another. A detectable amount of water was present in the second case. (3) The paper strip was allowed to stand overnight in an atmosphere saturated with water, dried at room temperature for 5 h, hung overnight in ethanol vapors in the chromatographic chamber, and the experiment performed. Analysis gave no detectable water. (4) The paper strip was hung overnight in a water saturated atmosphere, dried at room temperature for 5 h, placed in the chromatographic chamber and the experiment begun immediately. Analysis gave 0.42 and 0.43 % water.

Discussion

For the gas chromatographic analysis reported here, water can be analyzed in solution in ethanol in the range of 0.5 to 10 % water with an average error of 0.6 % water. Water in a concentration as low as 0.35 % can be detected but between 0.35 and 0.50 %, the analysis is only approximate.

Absolute ethanol is capable of removing water from cellulose pulp to reduce the amount of the immobile phase and perhaps alter the chromatographic properties of the system, *i.e.*, introduce retention by the support. This water appears in the first eluant from the column to give a sharp change in the composition of the solvent flowing from the column. The concentration of water (about 9 %) is independent of the length of the column and the quantity of packing. The total amount of water in the eluant depends upon the amount of cellulose pulp. The area under the dotted curve above 1 % water content for Fig. 1 is 1.6 times the same area for Fig. 2. These areas are measures of the total amount of water in the eluant. The ratio of the weight of cellulose pulp in the two columns was 1.7 which shows a good correlation between the water content of the eluant and the weight of the packing materials. If these same areas are used to calculate the total amount of water removed per gram of cellulose, the result for the 17 cm column is 0.024 g and for the 9 cm column is 0.026 g to give an average of 0.025 g. If cellulose pulp is assumed to have normally 10 % by weight water[19] the ethanol removed 25 % of this bound solvent.

Using the data for the cellulose powder, the ethanol, in passing through the 0.678 g of cellulose of the paper strip, should have removed 0.017 g of water. If this appeared in the first 0.2 ml fraction, the percentage water would have been 8.5 %. This was not the case. Water could not be detected in the eluant from papers allowed to stand in vapors of ethanol overnight and was just detectable in eluants from papers used immediately after placing them in the ethanol atmosphere. This was true whether the paper was presaturated with water or not. The absence of water in the eluant does not mean that the paper was not dehydrated. Such a hypothesis would rest on the assumption that the cellulose of the column was different from the cellulose of the paper. The explanation must lie in the presence of ethanol vapors in the paper chromatographic chamber but which were not present in the column. As shown by the columns, the ethanol extracts water from the paper as it migrates down the paper. This solution, because of its water content, exerts a higher vapor pressure than ethanol (95 % ethanol boils at 78.15° while ethanol boils at 78.3°) and will evaporate into the atmosphere to remove water and reduce the concentration of water in the mobile phase. This can be seen by considering the fractional distillation of a solution of water content less than 5 %. The composition of the vapor approaches that of the azeotrope while the composition of the residue approaches pure ethanol. There is sufficient time for this to occur since very nearly 2 h were required for the ethanol to reach the end of the paper and nearly 1.5 h was required for a drop to form.

Apparently water is also lost from the immobile phase by a similar process if the paper is allowed to stand in the ethanol atmosphere because no water was found in the eluant under these circumstances whereas a trace of water was found where there was no equilibration.

ACKNOWLEDGEMENTS

We have appreciated the support of the National Institutes of Health, RG 7046 Bio

(Cl), for this research. The assistance of Miss MARY HAWKE proved essential to the project and we gratefully acknowledge her participation.

SUMMARY

Developers which are miscible with water are capable of removing some of the immobile aqueous phase from cellulose if they are initially anhydrous. Eluant fractions from cellulose columns developed with absolute ethanol showed a water content of about 9 % (w/w) and a total content equivalent to removal of about 25 % of the immobile liquid phase. Eluants from paper chromatograms showed no water probably due to evaporation of the solution from the paper in the chamber. Analysis was by gas chromatography using THEED supported by a fluorocarbon. The average error was 0.6 % water in the range 0.5 to 10 % water. Water could not be detected below 0.35 %.

REFERENCES

1 J. C. GIDDINGS AND R. A. KELLER, in E. HEFTMANN (Editor), *Chromatography*, Reinhold Publishing Co., New York, 1961, p. 92.
2 A. J. P. MARTIN, *Endeavour*, 6 (1947) 21.
3 R. A. KELLER AND G. H. STEWART, *Anal. Chem.*, 34 (1962) 1834.
4 J. C. GIDDINGS, G. H. STEWART AND A. L. RUOFF, *J. Chromatog.*, 3 (1960) 239.
5 R. A. KELLER, R. BATE, B. COSTA AND P. FORMAN, *J. Chromatog.*, 8 (1962) 157.
6 R. A. KELLER AND G. H. STEWART, *J. Chromatog.*, 9 (1962) 1.
7 G. H. STEWART AND R. A. KELLER, *J. Chromatog.* 12 (1963) 150.
8 R. A. KELLER AND J. C. GIDDINGS, *J. Chromatog.*, 3 (1960) 205, in M. LEDERER (Editor), *Chromatographic Reviews*, Vol. 3, Elsevier, Amsterdam, 1961, p. 1.
9 E. LEDERER, *Bull. Soc. Chim. France*, [5] 19 (1952) 815.
10 A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 35 (1941) 1358.
11 A. J. P. MARTIN, *Ann. Rev. Biochem.*, 19 (1950) 517.
12 S. MOORE AND W. H. STEIN, *Ann. Rev. Biochem.* 21 (1952) 521.
13 J. JANAK, R. KOMERS AND J. SIMA, *Chem. Listy.*, 52 (1958) 2296; *Collection Czech. Chem. Commun.*, 24 (1959) 1492.
14 J. JANAK, *J. Chromatog.*, 3 (1960) 308.
15 S. CHINO, K. KASAMATSU AND K. SUZUKI, *J. Japan Petrol. Inst.*, 4 (1961) 288.
16 *Gas Chromatography Bulletin No. 3*, Fisher Scientific Co., Chicago, Ill., 1960.
17 H. G. NADEAU AND D. M. OAKS, *Anal. Chem.*, 32 (1960) 1760.
18 P. G. HOEL, *Introduction to Mathematical Statistics*, John Wiley & Sons, Inc., New York, 1947.
19 P. H. HERMANS, *Physics and Chemistry of Cellulose Fibres*, Elsevier, Amsterdam, 1949 p. 186.

AN INVESTIGATION OF LIQUID–LIQUID CHROMATOGRAPHY WITH A RECORDING DETECTOR

KARL J. BOMBAUGH AND JAMES N. LITTLE*

Spencer Chemical Company, Research and Development Division, Merriam, Kan. (U.S.A.)

(Received January 27th, 1964)

INTRODUCTION

The object of this work was to attempt to fit liquid–liquid chromatography to the gas chromatography model. The need for this seems evident. Even though liquid partition chromatography preceded gas chromatography by many years[1,2], the techniques of liquid chromatography, by and large, have remained what they were a decade ago. The equipment, method of elution and band detection have in no way paralleled the advance of gas chromatography. A relatively small number of workers have been using continuously recording U.V. or I.R. spectrometers, differential refractometers and interferometers. Some instruments were obtained commercially, others were designed and built in the respective laboratories using commercially obtained components. In spite of this advanced work by a few, it is reasonable to state that when a chromatographer shifted from gas chromatography to liquid chromatography, the changes in technique and equipment have been phenomenal: recording instruments have been replaced by fraction collectors, analysis time has increased 5 to 10 fold and separation efficiencies have decreased. The intent here is not to criticize liquid chromatography as a technique, but to look at both gas and liquid chromatography objectively in order to establish a correspondence between the two; then to attempt to fit liquid chromatography to a gas chromatography model to gain some of the benefits of gas chromatography in simplicity and speed of operation; perhaps in diversity of application.

The importance of liquid–liquid chromatography to analytical chemistry should not be under-rated. Even though high temperature gas chromatography, lightly loaded columns and glass bead supports have pushed the temperature limitations of gas chromatography to levels beyond expectation, the analytical chemist is still confronted with a multitude of materials which can not be volatilized, in mixtures which must be separated. For many of these problems, liquid–liquid chromatography affords the solution.

A comparison of the fundamental requirements of liquid–liquid chromatography and gas–liquid chromatography is contained in Table I. Discussion of this comparison is provided in this report along with the reported developmental results.

---

* Present address: Massachusetts Institute of Technology.

TABLE I

COMPARISON OF REQUIREMENTS FOR GAS AND LIQUID SYSTEMS

	Gas chromatography	*Liquid–liquid chromatography*
Carrier	Gas	Liquid
Motion	Cylinder pressure	Gravity Pump Gas pressure
Sample inlet	Septum injection	Open column loading Septum injection
Column	Size and shape may be the same for either	
Solid support	Chromosorb	Chromosorb
Liquid phases	Low vapor pressure Suitable solvent characteristics	Immiscible with carrier Suitable solvent characteristics
Detector	Thermal detector } universal Ionization detector } universal	Visual, Chemical, U.V., I.R. } not universal Thermal, Refractometer, Interferometer, Mass } universal

EXPERIMENTAL

Detector

A universal detector was needed to carry out this investigation. A Waters Associates differential refractometer[3] was selected because:

1. It was reasonably universal.
2. It provided high sensitivity.
3. It provided small detector volume which is imperative to fast response.
4. It was moderately priced when compared to such devices as recording spectrometers or interferometers.

The schematic diagram in Fig. 1 shows its operating principle. The chief limitation of this detector as found in this work was that its full dynamic range is not as readily available as is customary with gas chromatography detectors.

The detector was built into a system as shown in the block diagram in Fig. 2.

Sample injection

The sample injection assemblies were prepared from Swagelok fittings. These were prepared either from Swagelok Tee's or from bulkhead fittings. The body of the bulkhead fitting was drilled with a No. 52 drill and a piece of $^1/_8$ in. O.D. tubing silver soldered into it. The sharp leading edge of the Swagelok fitting was ground flat to provide a seal for the septum. Injectors were used with either metal or glass columns ranging in size from $^1/_8$ in. to $^1/_2$ in. tubing.

Carrier

The carrier or moving phase in liquid–liquid chromatography should be a comparatively poorer solvent than the stationary phase for the sample components. With the refractometer as a detector, the carrier liquid should have a refractive index different from the sample. The moving phase should also be less strongly retained by the solid

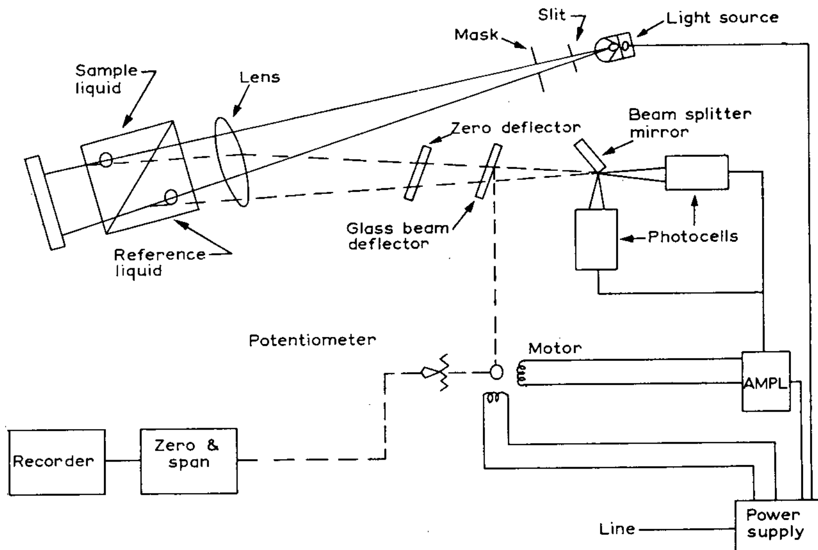


Fig. 1. Schematic diagram of Waters Associates liquid chromatography detector.

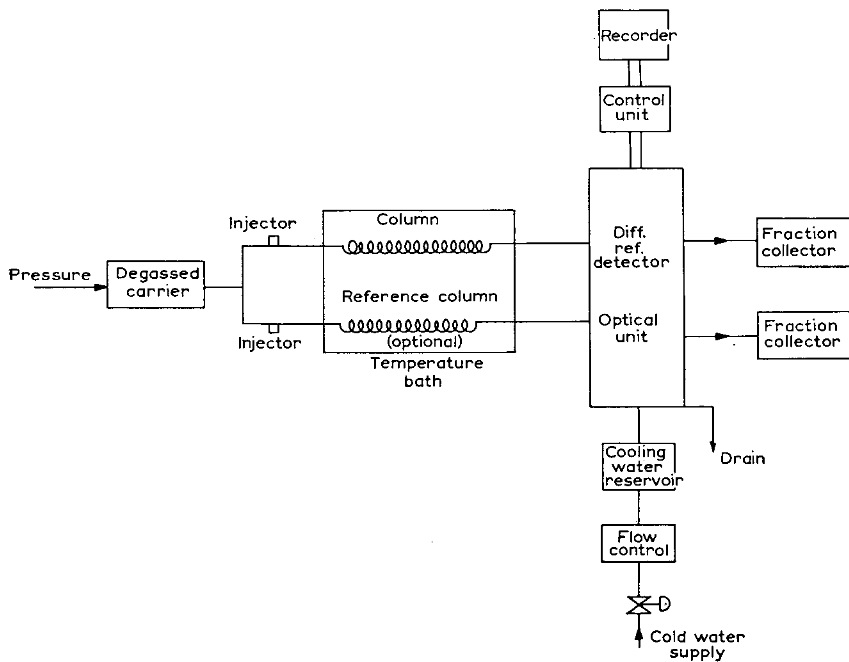


Fig. 2. Block diagram of liquid chromatography system.

support than the stationary phase. Immiscibility between the stationary and moving phases is desirable but not essential. Immiscibility implies a difference in polarity between the two phases. Materials ranging in polarity from water to isooctane may be used. Carrier liquids used in this investigation were *n*-heptane, ligroin, iso-octane, methanol, ethanol, and water.

Carrier motion

Moving the carrier is a problem of choice in liquid–liquid chromatography. Many types of pumps and constant head devices have been reported. In this work, three methods of carrier motion were used:

1. Gravity plus a hydrostatic head using a dip leg.
2. A microbellows pump supplied by Research Appliances Co., Allison Park, Pa.
3. Helium head pressure from a cylinder, controlled by a 2-stage regulator.

Much of this work was done with the microbellows pump arranged into an assembly with an auxiliary bellows used to provide the analog of a resistance-capacitance filter to smooth flow[4]. The method was only partially successful because smoothing was not complete and an occasionally encountered high pressure drop column caused rupturing of bellows. The preferred operation was obtained with method 3.

Degassing the carrier liquid

Degassing carrier liquids prior to use was found to be imperative for smooth operation of pressurized systems. Fig. 3 shows the difference between chromatograms produced with air-saturated carrier solvent and degassed carrier solvent. In the investigation a carrier was degassed by refluxing and allowing it to cool in a closed vessel. Under gravity flow with low head pressure, degassing the carrier was not necessary. However, when pressure was applied to the carrier liquid, the pressure drop across the column permitted outgassing. The gas bubbles in the line produced spiking shown in Fig. 3.

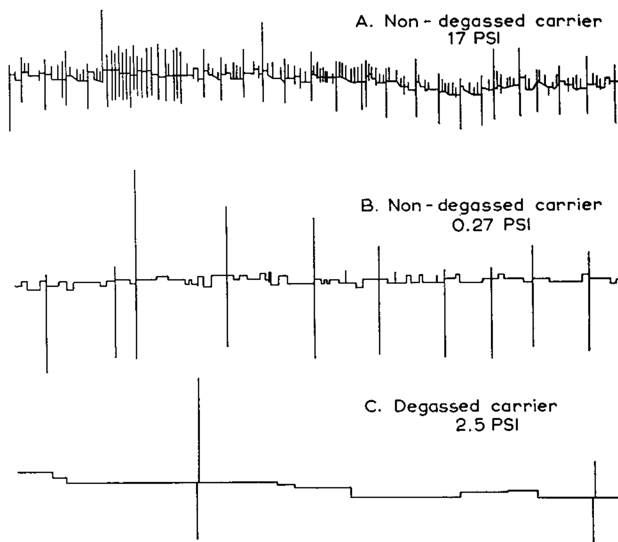


Fig. 3. Effect of gas in moving phase.

The stationary phase

Ideally the stationary phase should be completely insoluble in the carrier liquid. In practice, this is virtually unattainable. It has been the practice, therefore, to saturate the moving phase with the stationary phase to prevent depletion of the stationary phase during use. This technique has found limited application in gas chromatography also[5]. Although the saturated carrier solves the problem of stationary phase "permanence" it introduces a new problem in that the stationary phase contaminates the sample with materials that may be difficult to remove. The stationary phase usually must be similar in functionality to the sample component in order to provide the desired solubility and therefore desired retention of the sample components.

As a result the stationary phase may be as difficult to separate from the components of interest as the original contaminants in the sample. To avoid this problem, the stationary phase must be chosen with discretion. This in turn limits the choice of stationary phases available to a need. An approach to this problem is to use high polymers as stationary phases. A copolymer stationary phase for example can provide multiple functionality and be very sparsely soluble in a moving phase. Of necessity, the solubility of the sample components in the copolymer substrate will be very small and will require the use of very small sample loads monitored by a high sensitivity detector. We tried this approach with moderate success with a separation of nonane and tetradecane on a column of ethylene–methyl acrylate copolymer using methanol as a carrier. Many problems remain to be solved, but the results are sufficiently promising to warrant continued investigation.

Columns

A number of column systems were investigated. These are shown in Table II. The ethylene glycol–heptane system is a well known system. It was used in this work to evaluate the equipment and to develop a working knowledge of the technique. The polyglycols, copolymer and Sephadex systems were extensions into the realm of high polymers.

TABLE II

COLUMN SYSTEMS USED IN THIS INVESTIGATION

No.	*Moving phase*	*Stationary phase*	*Solid support*	*Length in meters*
1	*n*-Heptane*	Ethylene glycol	Chromosorb P	1.3
2	*n*-Heptane*	PEG 400	Celite	1.3
3	*n*-Heptane*	PEG 400	Celite	5.0
4	Methanol	Polyethylene	Chromosorb P	4.0
5	Methanol	Ethylene–methyl acrylate copolymer	Chromosorb P	5.0
6	*n*-Heptane	Ethylene–methyl acrylate copolymer	Chromosorb P	5.0
7	0.5 % and 0.2 % butanol in *n*-heptane	Butanol	Silica Gel	5.0
8	*n*-Heptane*	Ethylene glycol	Chromosorb	1.7
9	Ligroin*	Ethylene glycol	Chromosorb	1.7
10	Ligroin*	Carbitol	Chromosorb	1.5
11	*n*-Heptane*	Water	Chromosorb	5.0
12	*n*-Heptane*	Water	Silica Gel	4.5
13	0.4 % and 10 % butanol in *n*-heptane*	Water	Silica Gel	4.5

* These materials were saturated with stationary phase for use.

Sephadex G-25–water–ethanol

A relatively ideal column for use with polar solvents was prepared from the polydextran Sephadex G-25 (Pharmacia, Uppsala, Sweden[6]). Normally, this material is used in a fully swelled state for gel filtration by molecular exclusion. Its use is usually limited to large molecules with molecular weights above 2000. Its range was extended to smaller molecules by using it in a partially swelled state. Fig. 4 shows a separation

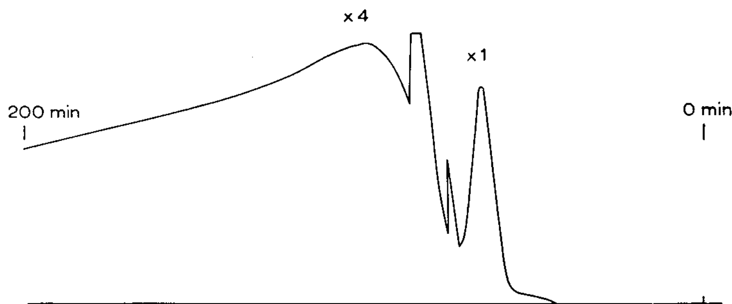


Fig. 4. Chromatogram showing separation of methyl naphthalene sodium sulfonate from ammonium nitrate. Column: 120 cm × 1.3 cm, Sephadex G-25. Carrier: 95% ethanol–5% water. Flow rate: 1.22 ml/min.

of methylnaphthalene sodium sulfonate from ammonium nitrate on a 120 cm × 13 cm O.D. column of partially swelled Sephadex using water as the stationary phase and ethanol–water (95:5) as the moving phase.

This separation was made in 2 h with an efficiency of 312 plates[7]. More complete

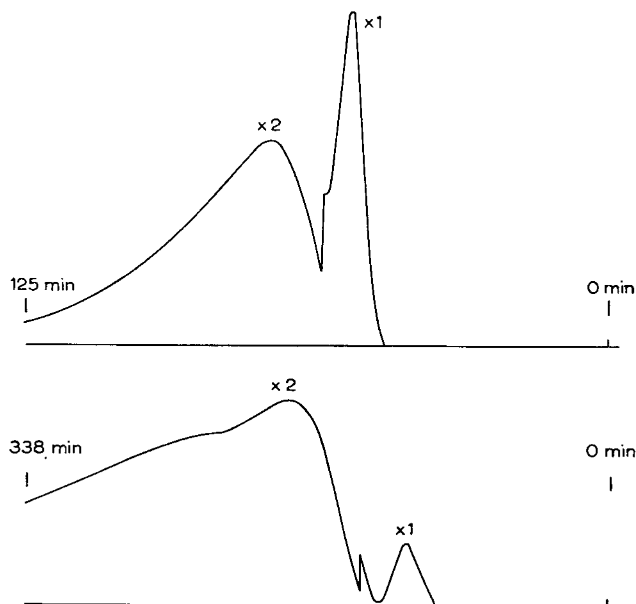


Fig. 5. Effect of flow rate on separation. Column and carrier same as Fig. 4. Flow rate: top 1.7 ml/min; bottom, 0.5 ml/min.

resolution was obtained at a slower flow rate as shown in Fig. 5, but analysis time was much longer. Carrier flow rate was maintained by a head pressure of helium using a 2-stage regulator. The top chromatogram in Fig. 5 was prepared at a head pressure of 5 p.s.i. which produced a flow rate of 1.7 ml/min. The bottom was prepared at 0.27 p.s.i. with a flow rate of 0.5 ml/min. Separation times were 125 and 338 min, resp.

Chromosorb–water

Columns containing water on red chromosorb were shown to be useful in liquid–liquid chromatography in the separation of surfactants. Many of these high molecular weight polyfunctional molecules are not sufficiently volatile to permit their separation by gas chromatography. They are not readily separated by chemical methods or by simple extraction techniques. They may be separated by liquid–liquid chromatography using water as the stationary phase and *n*-heptane as the moving phase. Table III shows the relative retention of a number of surfactants with a number of different chemical compositions. They include anionic, nonionic and cationic materials.

TABLE III

RETENTION OF SURFACTANTS (RELATIVE TO BUTANOL) ON COLUMN II

Surfactant	*No. of ethylene oxide units*	*HLB index*	*Relative retention*
Triton X-15	1	—	0.00
Span 85	—	1.8	0.03
Span 80	—	4.3	0.05
Ethofat 60/15	5	—	0.35
Span 60	—	4.7	0.41
Triton X-35	3	—	0.46
Triton X-45	5	—	0.46
Duponol C	—	—	0.67
Span 40	20	6.7	0.93
Ethofat 60/25	15	—	1.04
Tween 65	20	10.5	2.37
Myrj 45	—	11.1	2.37
Siponate DS-11	—	—	2.37
Morpholinium Oleate	—	—	2.37
Triton X-102	12	—	2.76
Tween 20	—	16.7	> 3.00

They are separated by their solubilities in heptane relative to water. Elution is in order of their HLB (hydrophylic–lipophylic balance) index[8]. More hydrophylic materials which exhibit larger HLB indices are retained longer than the lipophylic materials. Within any class of polyoxyethylene type material, retention is a direct function of the number of ethylene oxide units.

With a reversed phase system, elution order should be reversed.

Celite–PEG 400

Alcohols and acids are eluted from a PEG column in order of increasing ionization constant.

Although this separation was poor by gas chromatography standards, the information is included to illustrate the reverse order separation. Carboxylic acids and their salts may be eluted in order of carbon number or ionization constant in liquid–

liquid chromatography by choosing the proper moving and stationary phases. In gas chromatography elution order is predominantly a function of boiling point or carbon number in any series.

Gradient elution in liquid–liquid chromatography

Gradient elution is generally used in liquid absorption chromatography where progressively more polar solvents are used to elute solutes from the column in order of increasing polarity. Gradient elution may be used to great advantage in liquid–liquid chromatography, but it has no parallel in conventional gas–liquid chromatography.

A separation of butyne-1,4-diol from 4-hydroxy-2-butynyl N-(3-chlorophenyl)-carbamate was required. Using dichloroethane as the moving phase and water as the stationary phase, the diol was permanently retained on the column. It could be eluted from the column readily with water. By a two-solvent gradient elution technique the separation was complete on a very short column.

The separation could have been made with a single solvent system. For example, by using an appropriate mixture of benzene, ethanol and water the familiar differential chromatogram could have been obtained. However, a longer column and greater attention to conditions would have been required to effect a complete separation.

SUMMARY

A liquid–liquid chromatography system patterned after the gas chromatography model was built and investigated. Liquid–liquid chromatography and gas–liquid chromatography were found to have many principles in common. Much of the gas chromatography instrument technology was applicable to liquid–liquid chromatography. The system built for this investigation afforded simplified operation and provided rapid separations which were not readily attainable by other methods. Although problems remain to be solved, the results warrant continued investigation. The subtle differences which separate the techniques were found to result from basic differences between gas–liquid equilibrium and liquid–liquid equilibrium. When properly treated, however, these differences may be used to advantage.

In the unitized system, water was shown to be a desirable stationary phase, particularly when working with surfactants. Both chromosorb and the polydextran Sephadex G-25 were found to be desirable solid supports. Multifunctional copolymers showed promise as stationary phases and warrant further investigation.

REFERENCES

1 A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, 35 (1941) 1358.
2 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
3 L. E. Maley, *A New Laboratory Recording Refractometer*, Bulletin 2-1660 (1962), Waters Associates, Framingham, Mass.
4 S. M. Lambert, Shell Development Co., personal communication.
5 A. Kwantes and G. W. A. Rijnders, in D. H. Desty (Editor), *Gas Chromatography*, Academic Press, New York, 1958, pp. 125–136.
6 J. Porath, *Clin. Chim. Acta.*, 4 (1959) 776.
7 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 681.
8 W. C. Griffin, *J. Soc. Cosmetic Chemists*, 1 (1949) 311.

LINEAR ELUTION ADSORPTION CHROMATOGRAPHY

IX. STRONG ELUENTS AND ALUMINA. THE BASIS OF ELUENT STRENGTH

LLOYD R. SNYDER

Union Oil Company of California, Union Research Center, Brea, Calif. (U.S.A.)

(Received January 24th, 1964)

INTRODUCTION

The role of the eluent in adsorption chromatographic separation has been discussed in previous papers[1-5]. For linear isotherm separation (linear elution adsorption chromatography, LEAC), solute equivalent retention volume $\underline{R}^\circ$ (ml/g) varies with eluent strength (adsorption energy per unit area) ε° according to eqn. (1):

$$\log \underline{R}^\circ = \log \underline{R}_p - \alpha\varepsilon^\circ A_s \quad (1)$$

Here, $\underline{R}_p$ is the value of $\underline{R}^\circ$ for elution by the standard weak eluent pentane (ε° equal 0.00), α is an adsorbent activity function, and A_s is the "effective surface volume" of the solute. A_s is normally proportional to the surface area required by the solute upon adsorption (almost all solutes for adsorption on alumina[1,2], weakly adsorbed solutes on silica[3] and Florisil[4]). For certain strongly adsorbing solutes on silica[3] and Florisil[4], A_s is larger than predicted because of the localization of strongly adsorbed solutes on strong adsorbent sites[5].

With few exceptions, previous studies of the eluent role in LEAC separation have been restricted to weak eluents or eluents of intermediate strength. The behavior of very strong eluents might be expected to differ in some fundamental respects. First, the validity of eqn. (1) requires that the solution forces between non-adsorbed solute and eluent molecules be weak, so that the net loss in solution energy upon adsorption for these molecules will be small[1,5]. Strong eluents are for the most part highly polar solvents, for which this assumption would seem less applicable than in the case of previously studied weaker eluents. Similarly, the accuracy of eqn. (1) in previously studied chromatographic systems is believed to reflect relatively weak, non-specific (non-bonding) interactions between adsorbed eluent, solute, and adsorbent. As the strength of the eluent is increased, however, these interactions must eventually take on the character of strong chemical bonds, again with a breakdown in the reliability of eqn. (1).

A second complication in very strong eluent systems concerns solute configuration. Most chromatographic separations so far studied by us[1,2,5,6] find the solute adsorbing parallel to the adsorbent surface (flat) rather than in a perpendicular (vertical) or intermediate configuration. For elution by very strong eluents, however, certain solute types might be excepted to adopt a vertical configuration because of

the competition between eluent and less strongly adsorbing parts of the solute for a place on the adsorbent surface. This has already been observed in the case of *n*-alkyl substituted solutes, where the alkyl group is largely confined to the adsorbed phase for elution by weak eluents[1], but is largely desorbed for elution by strong eluents[2,7]. Similarly, for the elution of the diphenyl disulfides by strong eluents[2,8], only one phenyl ring and one sulfur atom are adsorbed, the remainder of the molecule dangling out in solution; in weaker eluents, progressively more of the solute adsorbs. In very strong eluent systems, this variablity in configuration of the adsorbed solute should be much more common.

A final, experimental difficulty in elution by very strong eluents is possible in the case of water-deactivated adsorbents. The strong eluent may successfully compete with the adsorbed water for adsorption on the strong adsorbent sites, and continued elution can then lead to displacement of water from the adsorbent with a concommitant increase in adsorbent activity (as in the activation of adsorbents by solvent washing[9]). Eqn. (1) of course assumes that adsorbent activity is constant throughout separation.

The present paper is concerned with the general nature of LEAC separation in elution by strong eluents from alumina. In this connection, we propose to examine the significance of each of the above discussed effects, to acquire eluent strength data (ε° values) for a variety of strong eluents, and to determine the chromatographic properties of a number of strongly adsorbing solutes for the first time. Previous papers[2,5] have presented a preliminary analysis of the dependence of eluent strength on molecular structure, one of the more challenging theoretical problems of adsorption chromatography[10]. In the present study, with its determination of a number of new eluent strength values to add to the considerable number already tabulated for alumina[2], we re-examine our original theory in an attempt to make it quantitatively compatible with experiment.

EXPERIMENTAL

Retention volume data described in the present communication were obtained essentially as in previous studies[2]; 2 to $5 \cdot 10^{-5}$ g/g of solute were charged to prewet columns of water-deactivated Alcoa F-20 alumina (chromatographically standardized), and each column discarded after a single $\underline{R}^\circ$ determination. Column linear capacity was determined for a number of representative solutes, and it appears that the above column loadings insure the linearity of presently reported $\underline{R}^\circ$ values. It was necessary to add up to 0.5 % water to eluents containing methyl or isopropyl alcohol, in order to prevent elution of adsorbed water. This is described in a following section. The various eluents used were reagent grade solvents, prepurified over calcined alumina. Commercial dioxane was observed to contain a strongly adsorbing impurity which was not readily removed by percolation over alumina (as evidenced by decline in retention volume for repeated elution of a given solute from the same alumina column by dioxane). Retention volume data for this eluent and its solutions are probably less reliable than for the other eluents studied.

The standardization of the adsorbents studied in the present series of investigations has been discussed in detail[1,3,6]. It has been shown that an adsorbent activity function α and the surface area of the adsorbent (or surface volume V_a) adequately characterize adsorptive properties. For certain commercial adsorbents, whose

properties remain relatively constant from batch to batch, it has been possible to average any variation in V_a and α into a single adsorbent parameter: effective water content (% H_2O) or chromatographic activity, as inferred from the $\underline{R}°$ value for a standard solute–eluent system (naphthalene eluted by pentane). In this respect, it appears that the last tabulation[1] of values of V_a, α, and $\underline{R}°$ (naphthalene–pentane) *versus* % H_2O–Al_2O_3 (Alcoa F-20 alumina) is now somewhat in error, as a result of two effects. First, the initial calculation[1] of values of V_a gave slightly low values relative to a later procedure[3], and second, the properties of Alcoa F-20 alumina acquired during the last several years seem slightly different from those of the sample originally reported on[1], and acquired in 1957. The error in the original table of V_a, α, and $\underline{R}°$ *versus* % H_2O–Al_2O_3, as applied to presently available adsorbent samples, has had a negligible effect on the accuracy of predicting and correlating retention volume data in the various systems subsequently studied by us, particularly since most of these data have been measured for 3.6–4.0 % H_2O–Al_2O_3, where the old and new adsorbent parameter values are identical. The main limitation on these originally tabulated adsorbent parameters for Alcoa F-20 alumina occurs in the preparation of adsorbent of a certain activity by water deactivation of calcined adsorbent. Thus, addition of 2 % water to recent samples of calcined Alcoa F-20 generally gives adsorbent with a chromatographic activity equal on the old scale[1] to 1.3 % H_2O–Al_2O_3. A similar discrepancy occurs for most other adsorbent activities, and occasionally this has been experimentally inconvenient. Fig. 1 shows the relationship between

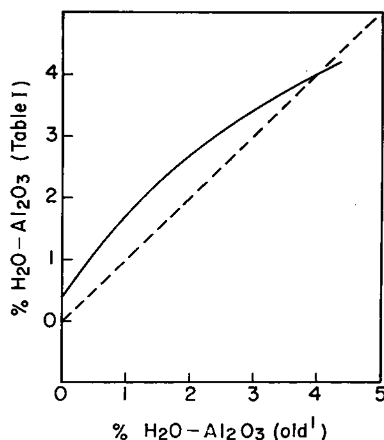


Fig. 1. Comparison of chromatographic activity as determined from old and new standard $\underline{R}°$ values, expressed in terms of water content for equal chromatographic activity (solid curve).

chromatographic activities by the old and revised scales, and Table I summarizes the relationship between the adsorbent parameters and adsorbent water content according to the *revised* scale. Subsequent references to the chromatographically standardized water contents of Alcoa F-20 alumina samples will be based on the data given in Table I, as well as report standard $\underline{R}°$ values (naphthalene–pentane) in order to minimize confusion in the change-over from the old standardization relationship.

TABLE I

REVISED (NEW) ADSORBENT PARAMETERS FOR ALCOA F-20 ALUMINA

% H_2O–Al_2O_3	V_a	log V_a	α	$\underline{R}^{\circ a}$
0.0	0.054	−1.27	1.00	68
0.5	0.049	−1.31	0.90	30
1.0	0.044	−1.36	0.84	17
2.0	0.034	−1.47	0.75	7.1
3.0	0.024	−1.62	0.69	3.3
4.0	0.014	−1.85	0.63	1.3

[a] Elution of naphthalene by pentane.

DISCUSSION

Relative to the weaker eluents, elution from alumina by certain strong eluents exhibits a number of new and interesting effects. These are covered in detail in the following sections. The primary purpose of the present section is to summarize the practical applications of these later sections. Additionally, previous papers in this series have developed the correlational eqn. (2) for predicting retention volume in LEAC separation over alumina:

$$\log \underline{R}_p = \log V_a + \alpha \left[\sum^{i} Q^\circ_i + \sum^{j} q^\circ_j - f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i\right] \qquad (2)$$

V_a is the adsorbent surface volume, α is the adsorbent activity function, Q°_i is the adsorption energy per solute group i, q°_j is a solute energy term associated with certain solute geometries, and $f(Q^\circ_k)$ is a solute localization function. Examples of the application of eqns. (1) and (2) to the calculation of $\underline{R}^\circ$ in strong eluent systems will be offered at the end of the present section, for a number of cases which reflect peculiarities in elution by strong eluents.

The use of the alcohols (or solutions of the alcohols) as eluents in separation over water-deactivated alumina results in the rapid stripping of water from the adsorbent and a resulting increase in adsorbent activity. Other eluents summarized in Table II do not appear to activate the adsorbent in this manner. Adsorbent activation during elution is an inherently undesirable phenomenon. In normal elution chromatography there is always the tendency for later eluted solute bands to broaden and $\underline{R}^\circ$ values to increase exponentially, thereby increasing separation time and decreasing detection sensitivity. Eluent activation increases the severity of this effect. If sufficient water is added to the eluent to effect equilibrium with adsorbent water, adsorbent activation will not occur. For the alcohols and their solutions, this usually requires addition of 0.1–0.5 % water. The determination of eluent equilibrium water content (water required in the eluent to avoid adsorbent activation) is simple and straightforward, as detailed in the immediately following section.

The eluent strengths of 9 strong eluents ($0.38 \leqslant \varepsilon^\circ \leqslant 0.95$) were determined for the first time and are reported in Table II, along with some other properties of these solvents pertinent to their use as eluents. Data for some previously studied eluents are also included in Table II. Additional eluent strength values are predicted in Table III for another 26 eluents, most of which are strong. These latter data were

TABLE II

PROPERTIES OF THE ELUENT: ELUTION FROM ALUMINA

Eluent	ε°			n_b	Cut off wavelength ($m\mu$)	Comments
	Exptl.	Calc.[a]	Calc.[b]			
n-Pentane	0.00	0.00	0.00	5.3	210	
Isooctane	0.01	—0.01	—0.01	7.4	210	
n-Hexane	0.01	0.00	0.00	6.0	210	
n-Octane	0.01	0.01	0.01	7.4	210	
Cyclohexane	0.04	0.03	0.03	6.0	210	
n-Decane	0.04	0.01	0.01	8.8	210	
Cyclopentane	0.05	0.03	0.03	5.0	210	
Carbon tetrachloride	0.18	—	—	4.4	260	
Carbon disulfide	0.26	—	—	3.0	380	
Isopropyl chloride	0.29	0.44	0.33	3.4	255	
Toluene	0.29	0.28	0.28	6.9	285	
n-Propyl chloride	0.32	0.44	0.33	3.4	225	
Benzene	0.32	0.31	0.31	6.0	280	
Isopropyl ether	0.28	0.43	0.32	4.7	235	c
Ethyl sulfide	0.38	0.42	0.31	4.4	290	
Phenetole	0.40	0.41	0.41	8.2	300	
Chloroform	0.40	—	—	4.4	245	
Methylene chloride	0.42	—	—	3.6	235	
Ethyl ether	0.38	0.49	0.38	4.1	210	c
Ethyl benzoate	0.48	0.41	0.50	9.5	300	
Acetone	0.56	0.55	0.55	4.0	330	
Ethyl acetate	0.58	0.41	0.50	5.5	250	
Methyl acetate	0.60	0.44	0.53	4.9	255	
Dioxane	0.63	0.73	0.62	5.5	250	
Diethylamine	0.63	—	—	—	275	d
Nitromethane	0.64	0.68	0.77	3.4	380	
Pyridine	0.71	0.73	0.73	5.6	300	d
Isopropanol	0.82	0.85	0.74	8.0[f]	210	e
Methanol	0.95	1.05	0.94	8.0[f]	210	e

[a] Using eqns. (6a) or (6b).
[b] Corrected for further localization or delocalization of large groups or small aliphatic groups.
[c] Δ_{es} values tend to be negative for solutes with strongly adsorbing groups (see Table IV).
[d] Δ_{es} values tend to be positive for solutes with potentially acidic groups (see Table XI).
[e] Δ_{es} values tend to be variable, amino groups showing generally large positive values.
[f] Value is larger than predicted from area of eluent and used in calculating ε° values.

calculated as described in a following section. As seen from Table II, by comparison of experimental and calculated (b) ε° values, it is possible to predict ε° in most cases with a precision ($\pm$ 0.03 units, average) approaching the experimental accuracy of the measured value. Together, the data of Tables II and III summarize the elution properties of more than 50 pure solvents, for ready application to experimental separation problems involving alumina as adsorbent. Since the eluent properties of any binary[2] or ternary[11] eluent mixture can in turn be calculated from the data of Tables II and III for the pure constituent solvents, the elution characteristics of a vast array of widely differing eluents can be easily obtained.

TABLE III

PREDICTED PROPERTIES FOR SEVERAL NEW ELUENTS[a]: ELUTION FROM ALUMINA

Eluent	n_b	ε°	*Comments*
Diisobutylene	7.1	0.06	
1-Pentene	5.0	0.08	
m,p-Xylene	7.8	0.25	
Amyl chloride	4.0	0.26	
o-Xylene	7.7	0.27	
Chlorobenzene	6.8	0.30	
Bromobenzene	7.1	0.31	
Ethyl bromide	3.4	0.37	
1,2-Diethoxyethane	7.4	0.43	c
Methyl isobutyl ketone	5.1	0.43	
Benzonitrile	7.2	0.44	
Triethylamine	5.8	0.48	d
1,2-Dichloroethane	5.0	0.49	
Acetophenone	7.9	0.50	
Nitrobenzene	7.3	0.52	
Nitropropane	4.1	0.53[b]	
Benzaldehyde	7.2	0.54	
Dimethyl phthalate	12.0	0.59	
Amyl alcohol	~ 8[f]	0.61	e
α-Picoline	6.5	0.62	d
Aniline	6.9	0.62	d
β,γ-Picoline	6.5	0.68	d
Pyrrole	5.0	0.72	e
Butyl cellosolve (C_4H_9–O–C_2H_4OH)	~ 8[f]	0.74	
Acetonitrile	2.8[f]	0.79	
Ethylene glycol	~ 8	1.11	e
Formamide, N,N-dimethylformamide		> 1.0	
Carboxylic acids		> 1.00	

[a] Estimated from eqn. (6a) or (6b), plus correction factors.
[b] Estimated from ε° for nitromethane.
[c] Δ_{es} values tend to be negative for solutes with strongly adsorbing groups (see Table IV).
[d] Δ_{es} values tend to be positive for solutes with potentially acidic groups (see Table XI).
[e] Δ_{es} values tend to be variable, amino groups showing generally large positive values.
[f] See Table II, note f.

A preceding paper[7] has discussed the advantages of different solvent pairs in gradient elution chromatography. In general, it is desirable that both solvents be relatively non-viscous or low boiling. Additionally, the stronger of the two solvents should have a reasonably large ε° value and as small a value of n_b as possible. Examination of the data of Tables II and III shows the use of the aliphatic amines and alcohols to be undesirable in this regard, while such solvents as acetone, nitromethane, and acetonitrile look especially promising. For the weaker solvent, pentane, propyl chloride, or carbon disulfide appear suitable. At first glance, the solvent pair pentane–ethyl ether might appear ideal on several counts: low viscosity, transparency in the far ultraviolet, widely differing eluent strengths, and a small n_b value for the ethyl ether. However, the failure of eqn. (1) for this system (which we will examine) results in a practical compression of the eluent strength range, and in some cases poorer separation.

Eqn. (1) breaks down in some cases involving certain strongly adsorbing eluents and solutes. This appears to be the result generally of strong interactions between eluent or solute and the adsorbent surface. Eqn. (1) predicts a constant separation order for solutes of similar size (A_s value), independent of eluent, so that two solutes of the same size which are inseparable by one eluent are predicted to be inseparable by all eluents. Consequently, the failure of eqn. (1) raises the possibility of separating such theoretically inseparable solute pairs in some cases. The ability to classify such instances and predict when they will occur is therefore of great practical interest. The breakdown of eqn. (1) in the case of specific solute–eluent combinations can be remedied by defining a solute–eluent interaction term Δ_{es} such that eqn. (3) applies exactly in all cases:

$$\log \underline{R}^{\circ} = \log \underline{R}_p - \alpha\, \varepsilon^{\circ} A_s + \alpha\, \Delta_{es} \quad (3)$$

It has been found that for given binary eluents, Δ_{es} will be essentially constant for all compositions richer than 5 % in the stronger eluent. Tables II and III summarize those cases where values of Δ_{es} are significantly different from zero (under "comments" column). One example of the failure of eqn. (1) occurs in the case of the aliphatic ethers as eluents (footnote c). Values of Δ_{es} tend to be negative for solutes with strongly adsorbing groups, so that elution by the ethers tends to speed up the elution of compounds with strongly adsorbing groups such as –COOR or –N=. Table IV provides some experimental values of Δ_{es} for ethyl ether and isopropyl ether as eluents,

TABLE IV

Δ_{es} VALUES FOR THE ALIPHATIC ETHERS AS ELUENTS

Strongest solute group	Q°_k	Δ_{es}	
		Ethyl ether	*Isopropyl ether*
–N= (3:4-benzacridine)	0.8	—0.24	—0.11
–O–R	1.8		—0.58
$-NO_2$	2.8		—0.01
$-CO_2R$	3.3	—0.57	—0.74
–N= (quinoline)	4.1	—0.64	—0.61
–N= (pyridine)	4.6	—0.89	

and various solutes containing the indicated groups. The value of Δ_{es} for a solute is determined in this case only by the single strongest adsorbing group, additional groups not affecting Δ_{es}. Thus, Δ_{es} for methyl benzoate, dimethyl phthalate, and methoxyl methyl benzoate are all predicted equal. Unfortunately, the failure of eqn. (1) in the case of the ether eluents does not appear to result in many unique separation possibilities. Separation problems in which the data of Table IV might suggest use of the ethers as eluents will frequently succumb to the use of "normal" eluents (*i.e.* for which Δ_{es} equal zero).

Another example of the failure of eqn. (1) occurs with the basic eluents such as diethylamine and pyridine (footnote d of Tables II and III). Solutes which are acidic (*e.g.* phenol, carbazole) or *potentially* acidic (*e.g.* aniline) are less readily eluted by the basic eluents than by normal eluents of comparable strength. The potentially acidic solutes presumably include all compounds having a proton bonded to an

electronegative element such as oxygen or nitrogen. Δ_{es} values for the basic eluents range from $+0.8$ for a solute such as aniline to $+2.0$ for the most acidic solutes such as phenol and carbazole. For solutes of intermediate acidity, Δ_{es} parallels acid strength. This failure of eqn. (1) for basic eluents seems potentially useful for certain otherwise difficult separations.

A final example of the failure of eqn. (1) has been noted in elution by the alcohols and their solutions. With these eluents all solutes substituted by $-NH_2$ are preferentially retained relative to elution by normal eluents of comparable strength, while the phenol and carbazole type of solutes are relatively unaffected (Δ_{es} approximately zero). Insufficient data have been obtained to generalize on the solute types for which eqn. (1) fails in elution by the alcohols, but it is likely that other compound types also exhibit non-zero Δ_{es} values. For this reason, some strongly adsorbed solutes which are inseparable by "normal" eluents may be separable upon elution by one of the alcohols or its solution with a weaker eluent.

For elution by strong eluents, it has definitely been established that the phenols and substituted phenols adsorb perpendicular to the adsorbent surface (vertical), with only the hydroxyl group contained in the adsorbed phase. A_s for the phenols is therefore smaller than predicted for the molecule in the flat configuration. This effect is somewhat counteracted by a larger than normal contribution of the hydroxyl group to A_s, as in the case of the pyrrole derivatives[6]. Similarly, substituent groups on the phenols do not contribute directly to the adsorption energy of the phenol, and $\sum^{i} Q^{\circ}_i$ for the phenols equals Q°_i for the hydroxyl group. This does not mean that all phenols are adsorbed equally strongly on alumina, and hence have equal retention volumes. Substituents on the phenol ring change the acidity of the phenol, and hence effect the adsorption energy of the phenol group (which is increased by increased acidity). This solute geometry effect q°_j has been related to the HAMMETT σ function[12] of the substituent on the phenol ring:

$$q^{\circ}_j = 2.03\ \sigma \qquad (4)$$

The phenols can therefore be separated on alumina according to their relative acidity. It is believed that other acidic and hydroxylic solutes such as the thiophenols, oximes, and alcohols behave similarly to the phenols as regards configuration and response to substituents. The adsorption energy of the phenol hydroxyl is strongly affected by bulky *ortho* substituents such as *tert.*-butyl. Two such groups decrease the adsorption energy of the hydroxyl group by a factor of over 5. Table V summarizes Q°_i and a_i values for several strongly adsorbing solute groups of the present type, as well as other solute groups investigated during the present study.

In a first example of the calculation of retention volume in these strong eluent systems, consider the elution of methyl benzoate by 15 %v ethyl ether–pentane from 3.8 % H_2O–Al_2O_3. For elution by "normal" eluents from this adsorbent, log $\underline{R}_p$ equals 1.56 (ref. 8), and A_s can be calculated[2] as 9.0. The eluent strength of 15 %v ether–pentane can be calculated from eqn. (5) (following section) and Tables I and II as 0.099. The "comments" column in Table II notes that ether is an abnormal eluent, and Table IV summarizes values of Δ_{es} for various solute groups; the strongest adsorbing group in methyl benzoate is the ester group, for which Δ_{es} equals -0.57 from Table IV. Finally, from Table I, α equals 0.64. Substituting into eqn. (3):

$$\log \underline{R}^\circ = 1.56 - 0.099\,(9.0) + 0.64\,(-0.57) = 0.31.$$

The experimental value was 0.25. If ether had been a normal eluent, the $\alpha\Delta_{es}$ term would be zero, and log $\underline{R}^\circ$ would be higher by 0.36 units.

In a second example, consider the separation of aniline and quinoline over 3.9 % H_2O–Al_2O_3. These two solutes have similar log $\underline{R}_p$ (2.29 and 2.31, respectively) and A_s (7.5 and 8.0) values[2,8], and elution by normal eluents would generally show little separation. For example, using an eluent for which $\alpha\varepsilon^\circ$ equal 0.302, we calculate from eqn. (1):

for *aniline* $$\log \underline{R}^\circ = 2.29 - 0.302\,(7.5) = 0.03$$

and for *quinoline* $$\log \underline{R}^\circ = 2.31 - 0.302\,(8.0) = -0.10.$$

The resulting separation (determined by difference in $\underline{R}^\circ$) is seen to be poor. Use of a basic eluent, such as 20 % v diethylamine–pentane ($\alpha\varepsilon^\circ = 0.302$), however, will preferentially retain the aniline because of its acidic hydrogens (–NH). The $\alpha\Delta_{es}$ value for this solute–eluent combination is observed to be +0.53. Consequently, for aniline eluted by the latter eluent, substituting into eqn. (3):

$$\log \underline{R}^\circ = 2.29 - 0.302\,(7.5) + 0.53 = 0.56.$$

An experimental value of 0.49 was observed, *i.e.* the predicted better separation of aniline ($\underline{R}^\circ = 3.7$ ml/g) from quinoline ($\underline{R}^\circ = 0.8$ ml/g) now occurs.

In a final example, consider the elution of *m*-acetophenol by 20 % v isopropanol–pentane from 3.9 % H_2O–Al_2O_3. α equals 0.64 and log V_a is −1.82 (Table I). A vertical

TABLE V

GROUP ADSORPTION ENERGIES FOR VARIOUS STRONGLY ADSORBED SOLUTES

Solute group, X	Q°_i			a_i
	R)–X	*Ar)–X*	*Ar)–X–(Ar*	
–OH	6.5	7.4		6.0[d]
		7.1[a]		7.0[d]
		1.3[b]		
–SH		8.7		6.0[d]
–CH=N–OH		7.2		6.0[d]
$-C(CH_3)=N-OH$		6.6		6.0[d]
$-CONH_2$		(6.1)[c]		2.0
$-NH-CO-CH_3$ [6]		6.2		3.0
$=CH-)CO-N(CH_3)-$ (N-methylquinolone)			6.2	4.0
$-NO_2$	5.4			2.5

[a] In 2,6-dimethylphenol.
[b] In 2,6-di-*tert.*-butyl-*p*-cresol.
[c] Approximate value, extrapolated from isopropanol solutions; $\alpha\Delta_{es}$ estimated at 1.0.
[d] For total solute ($a_i = A_s$); assumes strong eluent, vertical adsorption.

configuration of the solute is assumed, so from Table V, $Q^\circ_i = 7.4 = \sum^{i} Q^\circ_i$, and $A_s = a_i = 6.0$. From eqn. (4), $q^\circ_j = 2.03\,(0.306) = +0.62$. Since no solute groups other than the hydroxyl are adsorbed, $\sum^{i \neq k} Q^\circ_i = 0.00$. $\alpha\varepsilon^\circ$ for the eluent may be calculated from eqn. (5) and Table II as 0.452. Finally, $\alpha\Delta_{es}$ for phenol (solute) and isopropanol (eluent) is $+0.30$. Substituting into eqn. (2):

$$\log \underline{R}_p = -1.82 + 0.64\,(7.4 + 0.62 - 0) = 3.31.$$

From eqn. (3):

$$\log \underline{R} = 3.31 - 0.452\,(6.0) + 0.30^\circ = 0.90.$$

An experimental value of 0.84 was found. It should be noted that log $\underline{R}_p$ as calculated above is for a vertical configuration, whereas in the weak eluent (pentane), the molecule is undoubtedly adsorbed flat. This is of no practical consequences, however, since in any eluent system strong enough to elute this phenol at a reasonable rate, the configuration would be vertical, and the hypothetical (vertical) configuration used to calculate R_p would be applicable.

ADSORBENT ACTIVATION BY THE ALCOHOLS AND THEIR SOLUTIONS DURING ELUTION

Adsorbent activation or the removal of adsorbed water has been accomplished in some laboratories[9] by washing the adsorbent with certain polar solvents. *Elution* by polar solvents from water-deactivated adsorbents may therefore result in the progressive activation of the adsorbent during the course of separation. This would not greatly affect the retention volumes of the first eluted sample components, which would have left the column before appreciable activation of the adsorbent had occurred, but would increase the retention volumes of later eluted compounds. Such an effect would be disadvantageous in most separations, since it leads to inconveniently long separation times and excessive solute band broadening with loss in detection sensitivity. That is, adsorbent activation during elution would accentuate those problems inherent in normal fixed eluent elution, and which have led to the development of the gradient elution technique[11]. For the adsorbents studied in the present investigation (3.8 to 3.0 % H_2O–Al_2O_3), only the alcohols and their solutions were found to give appreciable adsorbent activation during elution. The remaining solvents of Table II appear to give no problem in this respect.

Adsorbent activation by the eluent is illustrated in the data of Fig. 2 for the elution of benzaldoxime from 3.9 % H_2O–Al_2O_3 by 5 %v isopropanol–ethyl ether. The circles refer to $\underline{R}^\circ$ values determined in the usual manner: prewetting the column, charging the solute, and eluting the solute from the column. The squares refer to $\underline{R}^\circ$ values determined in this manner *after* initial passage of 8 ml/g of eluent through the column (for possible adsorbent activation). These $\underline{R}^\circ$ values are plotted *versus* the amount of water added to the eluent prior to elution, and both sets of data show $\underline{R}^\circ$ decreasing with increasing eluent water content. The two curves intersect at approximately 0.21 % water in the eluent, and it is presumed that for eluent of this composition the water on the initial adsorbent and in the initial eluent are at equilibrium, since no change in adsorbent activity occurs with continued elution. For

dryer eluent compositions (< 0.21 % H_2O), retention volumes and adsorbent activity increase with continued elution, since the eluent is water-poor with respect to starting adsorbent, and hence can remove water and increase activity. This dependence of $\underline{R}°$ on eluent water content and extent of pre-elution is just that expected for adsorbent activation (and deactivation) during elution by a polar solvent.

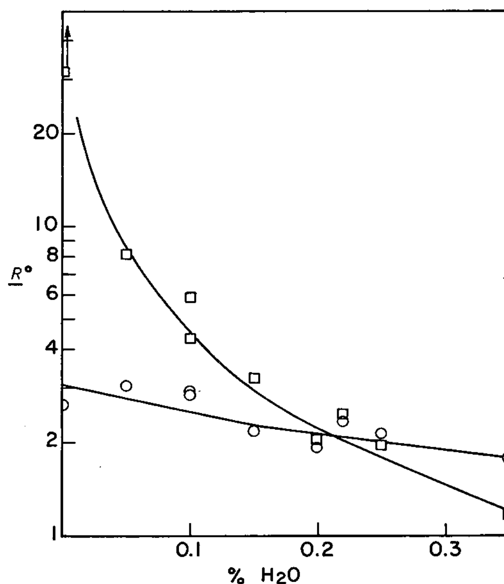


Fig. 2. Activation and deactivation of the adsorbent during elution, *versus* eluent water content. Elution of benzaldoxime by 5 %v isopropanol–ethyl ether from 3.9 % H_2O–Al_2O_3. ○ = simple column pre-wetting (1 ml/g pre-elution); □ = pre-elution by 8 ml/g of eluent.

Preservation of the adsorbent activity at its initial value requires the addition of a certain amount of water to the eluent (0.21 % in the system of Fig. 2). The amount of water needed will vary with the adsorbent activity and eluent composition, and may be determined by experiment as in Fig. 2. In normal separation, and particularly in theoretical investigations such as the present one, it will be important to use water-equilibrated eluent whenever the eluent is capable of stripping water from the column. In some separation systems, however, it might prove advantageous to use higher than equilibrium water content so as to effect the gradual deactivation of the adsorbent during separation. Practically, this would confer some of the advantages of gradient elution separation without use of the special equipment normally required. Because of the limited solubility of water in most of the weaker eluents, this technique would be limited to separations with strong eluents or eluents of intermediate strength.

The addition of water to the eluent might be expected to change its effective strength ($\varepsilon°$) as an eluent, as well as prevent adsorbent deactivation. The data of Fig. 2 suggest that this effect will be small, inasmuch as the $\underline{R}°$ data for 1 ml/g pre-elution (circles) show little dependence on water content (about what could have been expected from adsorbent activation and deactivation, from comparison with the 8 ml/g pre-elution data). The small amounts of water required in eluent equili-

bration (0–0.5 %) and the great strength of the alcohols and their solutions as eluents appear to explain the lack of dependence of eluent strength on water content in studies such as that of Fig. 2.

Previous studies of elution from alumina have not involved the alcohols as eluents, and hence have not been subject to the adsorbent activation effect. A later study will show that adsorbent activation occurs much more readily with silica as adsorbent, although prior studies with silica are not thereby in error (because of the measuring technique used, equivalent to simple prewetting data of Fig. 2, and the less polar solvents employed as eluents).

ANOMALIES IN ELUTION FROM ALUMINA BY STRONG ELUENTS

For elution from alumina by eluents of weak or intermediate strength, the role of the eluent in determining solute retention volume (eqn. 1) has been thoroughly tested[1,2,6,7]. The only separation variables that contribute to an eluent effect in these cases are eluent strength ε° (or adsorption energy per unit area) and solute effective area (or surface volume A_s). The applicability of eqn. (1) in given situations suggests two corollary conclusions: (a) solution effects are relatively unimportant, or the solubility of the solute in the eluent plays no major role in determining its retention volume; (b) strong chemical bonds between specific adsorbent sites and reactive eluent atoms on groups are absent. As the polarity and adsorption strength of the eluent increase (these two eluent properties are roughly related), however, the validity of the latter two conclusions becomes progressively more suspect, and eqn. (1) should become less accurate for elution by the strongest eluents. Previously reported data on the applicability of eqn. (1) have been largely restricted to eluents whose strengths ε° lie between pentane (0.00) and methylene chloride (0.42). Such data as have been reported for stronger eluents[2] do in fact suggest a lesser accuracy in the application of eqn. (1).

A large number of data for elution by strong eluents were obtained in the present investigation, leading to the expanded eluent strength scale of Table II. In the course of acquiring these data, it has become apparent that eqn. (1) as it stands is in many cases a poor approximation for elution in these strong eluent systems. While this complication of the simple eluent theory (eqn. 1) may appear lamentable from the standpoint of comprehending adsorption chromatographic separation, the number of separation possibilities are greatly increased in separations using strong eluents or their binary solutions. The use of strong eluents (or their solutions in weaker eluents) therefore complicates the task of the theoretical chromatographer but simplifies the work of the practical chromatographer. The value of simple eluent theory (eqn. 1) in these more complex strong eluent separations is as a tool for the recognition, quantitation, and classification of strong eluent anomalies (for eventual exploitation in new separation schemes), and as a necessary preliminary to the development of a satisfactory theory of adsorption chromatography in strong eluent systems.

The "ether anomaly"

One type of strong eluent anomaly or failure of eqn. (1) is illustrated by the data of Tables VI and VII for elution of a number of solutes from 3.8 % H_2O–Al_2O_3 by several

ether–pentane binary eluents. If values of $\alpha\varepsilon°$ are calculated from these $\underline{R}°$ values by means of eqn. (1) and plotted *versus* eluent composition as in Fig. 3, the resulting plot scatters badly. This means that $\alpha\varepsilon°$ for these binary eluents appears to be a function of both eluent composition and solute type, whereas for normal eluents $\alpha\varepsilon°$ is independent of solute type. The dark circles of Fig. 3 for the non-hydrocarbon

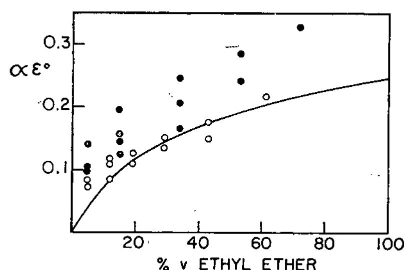


Fig. 3. Experimental eluent strength data *versus* eluent composition and solute type. Elution of solutes of Tables VI and VII by ether–pentane solutions from 3.8 % H_2O–Al_2O_3. ○ = experimental data for hydrocarbon solutes; ● = experimental data for non-hydrocarbon solutes; —— calculated from eqn. (5) and data of Table II for ethyl ether.

solutes lie consistently above the data for the hydrocarbon solutes (open circles). If we assume that the hydrocarbon $\alpha\varepsilon°$ values of Table VI represent the "true" eluent strengths of these binaries (as previously[2]), it is possible to calculate values of log $\underline{R}°$ for the non-hydrocarbons of Table IV by means of eqn. (1), and the differences $\alpha\Delta_{es}$ between experimental and calculated log $\underline{R}°$ values by means of eqn. (3). Values of Δes are shown in parentheses in Table VII. For those solutes in Table VII which have the eluent varied over a wide range of compositions (*e.g.* quinoline, pyridine), values of $\alpha\Delta_{es}$ are seen to be essentially independent of eluent compositions. This is particularly true if the $\alpha\Delta_{es}$ values for the most dilute ether–pentane eluents ($\leqslant$ 5 %) are excepted. This lack of dependence of $\alpha\Delta_{es}$ on eluent composition strongly suggests that solution effects (preferential solubility of the solute in different eluents) are *not* responsible for the anomalous behavior represented by the large $\alpha\Delta_{es}$ values of Table VII. Rather, some peculiarity in the interaction of solute and eluent with the adsorbent is suggested, since for ether concentrations in excess of 10 % the adsorbent

TABLE VI

ELUTION OF HYDROCARBON SOLUTES FROM 3.8 % H_2O–Al_2O_3 BY ETHYL ETHER–PENTANE BINARY ELUENTS

Solute	*log $\underline{R}_p$*	*log $\underline{R}°$ for indicated eluents*[a]						
		2.0	*5.0*	*12.0*	*19.0*	*29.0*	*43.0*	*61.0*
Phenanthrene	1.14	0.64	0.31	−0.04				
Chrysene	1.90	1.46	1.05	0.59				
Picene	2.58			1.42	1.05	0.72	0.51	
Benzocoronene	3.75				1.61	1.20	0.81	0.08
$\alpha\varepsilon°$ exptl.		0.043	0.077	0.103	0.118	0.141	0.160	0.216
calc.[b]		0.017	0.039	0.085	0.114	0.144	0.176	0.205

[a] % v Ethyl ether–pentane.
[b] Eqn. (5), data of Table II.

TABLE VII

ELUTION OF NON-HYDROCARBON SOLUTES FROM 3.8 % H_2O–Al_2O_3 BY ETHYL ETHER–PENTANE BINARY ELUENTS

Solute	*log $\underline{R}_p$*	*Q°_k*	*log $\underline{R}^\circ$ for indicated eluents*[a]						*av. $\alpha\Delta_{es}$*[b]
			5.0	*15.0*	*34.0*	*53.0*	*72.0*	*100.0*	
3:4-Benzacridine	2.15	0.8	1.75 (0.00)	0.67 (—0.22)	0.19 (—0.08)				—0.15
Methyl benzoate	1.56	3.3	0.68 (—0.19)	0.25 (—0.35)					—0.35
1,2,4-Tricarbomethoxy-benzene	4.31	3.3						0.24 (—0.37)	—0.37
Quinoline	2.30	4.1		1.45 (—0.38)	1.06 (—0.38)	0.67 (—0.46)	0.38 (—0.47)	—0.06 (—0.38)	(—0.41)
6-Methoxyquinoline	2.99	4.1						0.12 (—0.40)	—0.40
Pyridine	2.26	4.6	1.40 (—0.38)	1.09 (—0.53)	0.79 (—0.53)	0.56 (—0.54)	0.32 (—0.62)	0.15 (—0.63)	(—0.57)
$\alpha\varepsilon^\circ$ (best values)			0.077	0.107	0.156	0.193	0.220	0.247	

[a] % v Ethyl ether; parentheses refer to experimental log $\underline{R}^\circ$ values minus calculated values (eqn. 1), $\alpha\Delta_{es}$.
[b] Exclusive of data for 5 % ether solutions.

surface is covered primarily with ether, and hence the composition of the adsorbed phase does not vary much over the eluent composition range of 10–100 % ether. If the averaged values of $\alpha\Delta_{es}$ for each solute are plotted *versus* the adsorption energy Q°_k of the strongest solute group k, as in Fig. 4, an approximate correlation is noted, indicative of localization effects as in the adsorption of strong solute groups on specific adsorbent sites[8].

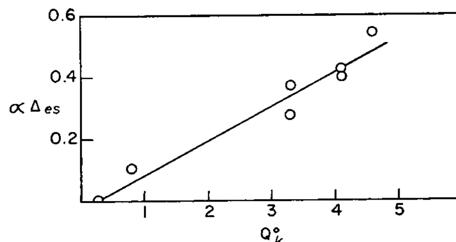


Fig. 4. Dependence of $\alpha\Delta_{es}$ on adsorption energy of strongest adsorbing solute group. Ethyl ether–pentane binary eluents, 3.8 % H_2O–Al_2O_3.

The eluent anomaly which occurs in the case of ethyl ether and its solutions cannot be recognized in a solvent without obtaining extensive experimental data of the type given in Tables VI and VII. Additionally, the experimental analysis of the "ether effect" becomes almost impossible for eluents stronger than ethyl ether (or of comparable strength and with n_b larger). A simpler procedure is therefore required for assessing the importance of this "ether anomaly" in other strong eluents. We will now show that the dependence of non-hydrocarbon $\alpha\varepsilon^\circ$ values on binary eluent composition can be used to detect the presence or absence of the "ether anomaly" in the strong eluent.

The strength of an eluent binary ε°_{AB} has previously[2] been related to the strengths of the constituent eluents A (weak) and B (strong):

$$\varepsilon^\circ_{AB} = \varepsilon^\circ_A + \frac{\log\,[X_B \cdot 10^{\alpha n_b(\varepsilon^\circ_B - \varepsilon^\circ_A)} + 1 - X_B}{\alpha n_b} \quad (5)$$

X_B is the mole fraction of B in the binary mixture, and n_b is the eluent surface volume (equal to its value of A_s for the eluent considered as solute). ε°_A and ε°_B are the strengths of the two pure eluents A and B. The values of n_b in Tables II and III differ slightly from previous values[2,5], reflecting improvements in the calculation procedure (as for analogous A_s values [5]).

Eqn. (5) accurately correlates (solid line) the $\alpha\varepsilon^\circ$ values of Fig. 3 for the hydrocarbons, and satisfactory correlations of the same type have been noted for many "normal" eluents and both hydrocarbon and non-hydrocarbon solute types[2]. Attempts to fit the non-hydrocarbon data of Fig. 3 by means of eqn. (5) do not give a good fit, even when the value of ε° for ethyl ether is permitted to vary. If the value of n_b for ethyl ether is permitted to vary too, however, rather than being restricted to the real, calculated value (4.1), the non-hydrocarbon data of Fig. 3 can be brought into good agreement with eqn. (3). The deviation of experimental (6.0, optimized) and calculated (4.1) values of n_b then provides a direct, quantitative measure of the failure of eqn. (5) for these non-hydrocarbon data. The form of eqn. (5) and of the

deviations from eqn. (1) in Table VII suggests that deviations between experimental and calculated n_b values can be used to evaluate the presence or absence of an "ether anomaly" for other eluents.

Table VIII summarizes a number of eluent strength data for elution of both hydrocarbons and non-hydrocarbons by binary eluents previously reported or described for the first time in Table VI. These data were fitted by least squares to a best value of n_b ($\pm$ 0.5 units) by comparison with calculated values of $\alpha\varepsilon°$ from eqn. (5). For the first nine binary eluent systems, the average deviation of experimental and and calculated n_b values is only $\pm$ 0.7 units, within the variability of the $\alpha\varepsilon°$ determinations. Where the difference between experimental and calculated n_b values exceeds 0.5 unit, the experimental value is invariably negative, as might have been expected from the crowding of eluent molecules on the adsorbent surface, with reduction in the area required for adsorption. In the last five binary eluent systems, the experimental n_b values *for the non-hydrocarbon data* are all greater than $+1.5$ (although normal for the hydrocarbon data), suggesting the possible existence of an "ether anomaly" effect for these three strong eluents (ethyl sulfide, ethyl ether, and diethylamine). The remaining strong eluents of Table VIII appear quite normal in this respect.

These various observations on the "ether anomaly" effect suggest a fairly detailed picture of its origin. The effect is associated with the adsorbed, rather than solution, phase; it is most prominent in the case of strongly adsorbed solute groups, implying some peculiar interaction between strong adsorbent sites, solute, and/or eluent, and it *seems* to occur for the strongly adsorbing, saturated, aliphatic eluents (ethyl sulfide, ethyl ether, diethylamine), rather than for aromatic counterparts (*e.g.* phenetole) or unsaturated aliphatics (*e.g.* acetone). The adsorption of ethyl ether on the alumina surface is visualized in Fig. 5A. It is presumed that the primary

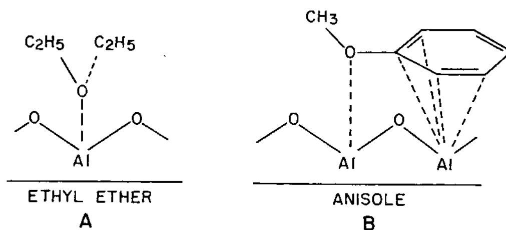


Fig. 5. Origin of the "ether anomaly". Configuration of adsorbed ethyl ether and anisole.

interaction is between the basic oxygen atom and an acidic aluminum atom, as in the adsorption of the basic nitrogen derivatives[6]. The geometry around the oxygen atom in ethyl ether favors the formation of a localized, strong bond, in contrast with the adsorption of the "normal" eluent anisole, shown in Fig. 5B. Thus, the concommitant adsorption of the phenyl ring in the latter eluent requires that the oxygen atom of anisole remain at a greater distance from the alumina surface than in the case of the ethyl ether oxygen group. The energy required to desorb the ether oxygen in these two situations will clearly be greater in the case of the ethyl ether molecule. More important, because of the proximity of oxygen and aluminum atoms which is possible in the adsorption of ethyl ether, the resulting interaction will assume the

TABLE VIII

BINARY ELUENT STRENGTH AND THE "ETHER ANOMALY"

	Binary eluent system[a]	*Adsorbent activity*[b]	*%vB*	$\alpha\varepsilon°$	n_b *Exptl.*[c]	n_b *Calc.*[d]	$(n_b)^c - (n_b)^d$	*Reference*
(1)	CCl_4–pentane (H)	2.0	5	0.016	3.5	4.4	−0.9	13
			10	0.028				
			25	0.059				
			50	0.083				
	CCl_4–pentane (NH)	3.8	10	0.028	3.5	4.4	−0.9	2
			25	0.044				
			50	0.072				
(2)	Benzene–pentane (H)	0.0	1.5	0.095	6.5	6.0	+0.5	2
			5.0	0.141				
			15	0.194				
			30	0.235				
			70	0.293				
(3)	CH_2Cl_2–pentane (NH)	3.8	5	0.052	2.5	3.6	−1.1	2
			10	0.084				
			25	0.146				
			50	0.206				
			70	0.248				
(4)	Phenetole–pentane (NH)	3.9	30	0.178	7.0	8.4	−1.4	Table IX
(5)	Acetone–pentane (NH)	3.9	10	0.170	4.0	4.0	0.0	Table IX
			25	0.259				
			50	0.316				
(6)	Dioxane–pentane (NH)	3.8	10	0.242	5.0	5.5	−0.5	2
			25	0.307				
			50	0.357				
(7)	Pyridine–pentane (NH)	3.9	0.4	0.132	4.5	5.6	−1.1	Table IX
			1.0	0.155				
			4.0	0.238				
			10	0.274				
			25	0.356				
			50	0.417				
(8)	Nitromethane–benzene (NH)	3.9	3	0.233	3.5	3.4	+0.1	Table IX
			8.3	0.266				
			25	0.328				
(9)	Ethyl sulfide–pentane (NH)	3.9	10	0.100	6.0	4.4	+1.6	Table IX
			30	0.164				
(10)	Ethyl ether–pentane (H)	2.0	10	0.139	4.0	4.1	−0.1	14
(11)	Ethyl ether–CCl_4 (H)	2.0	25	0.240	5.0	4.1	+0.9	14
(12)	Ethyl ether–pentane (H)	3.8	(2–61)		2.5[e]	4.1	−1.6	Table VI
	Ethyl ether–pentane (NH)	3.8	(5–72)		6.0	4.1	+1.9	Table VII
(13)	Diethylamine–pentane (NH)	3.9	5	0.233	7.5	4.4	+3.1	Table IX
			20	0.302				
			50	0.368				

[a] Strong eluent (B) given first; (H) refers to data for hydrocarbon solutes, (NH) to non-hydrocarbons.
[b] % H_2O–Al_2O_3 (chromatographic).
[c] Least squares fit to eqn. (5).
[d] Calculated from molecular dimensions of molecule as in ref. 5.
[e] Assumes $\varepsilon°$ value calculated from non-hydrocarbons.

TABLE IX

RETENTION VOLUME DATA FOR CALCULATING ELUENT STRENGTH IN SOME STRONG ELUENT SYSTEMS; 3.9% H_2O–Al_2O_3

Eluent	*log R°*										
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*
Ethyl sulfide			0.29					0.67			
10% v ethyl sulfide–pentane			1.51								
30% v ethyl sulfide–pentane			1.00								
Nitromethane											
3% v nitromethane–benzene								0.67			
8.3% v nitromethane–benzene								0.34			
25% v nitromethane–benzene								—0.28			
Ethyl benzoate								—0.04			
Phenetole			0.27					0.52			
30% v phenetole–pentane			0.89								
Acetone										0.54	
10% v acetone–pentane								1.30		1.04	
25% v acetone–pentane								0.41			
50% v acetone–pentane								—0.21			
Ethylamine											0.60
5% v ethylamine–pentane		0.01	0.26						0.59		
20% v ethylamine–pentane			—0.37						—0.05		1.56
50% v ethylamine–pentane											1.02
Pyridine											0.23
0.4% v pyridine–pentane		0.63	1.04	1.06							
1% v pyridine–pentane		0.40	0.79	0.83							
4% v pyridine–pentane				0.06		0.51	0.67				
10% v pyridine–pentane					0.20	0.10	0.40				
25% v pyridine–pentane											1.04
50% v pyridine–pentane											0.43

	Solute	*log R_p*		*Solute*	*log R_p*
I	acetophenone	1.76	VII	6-methoxyquinoline	2.95
II	methyl 1-naphthoate	2.05	VIII	*m*-nitroaniline	3.00
III	quinoline	2.31	IX	dimethyl phthalate	3.33
IV	1-acetonaphthone	2.47	X	2,3-diaminonaphthalene	4.46
V	phenanthridine	2.68	XI	1,10-phenanthroline	4.60
VI	isoquinoline	2.73			

character of a strong chemical bond, and the disruption of this bond upon adsorption of a solute will clearly require a good deal more energy than is required on less active sites. Consequently, the adsorption of solutes with strongly adsorbing groups k, which have a preference for adsorption on the strongest sites (localization), will suffer most when ether or similar solvent is eluent, because the ether–site bonds tend to be stronger than for normal eluents, which are incapable of forming strong bonds with strong adsorbent sites. The localization of ether molecules on strong adsorbent sites thus appears to explain the basis of the "ether anomaly".

Strongly adsorbing aliphatic eluents such as acetone do not exhibit the "ether anomaly", presumably because the p electrons of the ether oxygen atom favor the formation of a strong, directed bond to aluminum while the more diffuse π electrons of acetone do not. Other strongly adsorbing aliphatic eluents capable of the "ether anomaly" can be predicted: aliphatic halides, other ethers, alkyl sulfides and alkylamines. Additional data for the first three eluent types were obtained to evaluate this possibility, and these are summarized in Table X. Comparison of hydrocarbon and non-hydrocarbon $\alpha\varepsilon^\circ$ values in Table X shows the *absence* of the "ether anomaly" in the case of ethyl sulfide and propyl chloride, and confirms its presence in the case of isopropyl ether. Values of $\alpha\Delta_{es}$ for isopropyl ether and various solute groups are quite similar to corresponding values for ethyl ether, as seen in the averages of Table IV. The absence of an "ether anomaly" in the case of the chlorides and sulfides need not be surprising since, the "ether anomaly" must be a function of the bonding energies of the localizing eluent group and aluminum atom, the sensitivity of this energy to the distance of separation, and the steric environment around the localizing eluent group. The presence or absence of the "ether anomaly" in eluents as strong as diethylamine cannot be directly verified and is therefore not of much practical importance; the experimental ε° value for this eluent in Table II includes the effect of Δ_{es} terms.

The "basic eluent anomaly"

The data of Table IX for elution by diethylamine and pyridine include no $\underline{R}^\circ$ values for solutes possessing either N–H or O–H groups (*i e.* acidic hydrogens). Table XI summarizes a number of such experimental $\underline{R}^\circ$ values, along with values of $\alpha\Delta_{es}$ in parentheses calculated as previously, using the $\alpha\varepsilon^\circ$ values of Table VIII for the pyridine and diethylamine solutions. As in the elution of non-hydrocarbons by the ethers, we find large values of $\alpha\Delta_{es}$ for these solutes, which are essentially independent of eluent composition. This suggests, as in the case of the "ether anomaly", that some peculiar interaction in the *adsorbed* phase is occurring. We also note in Table XI that the values of $\alpha\Delta_{es}$ are invariably positive, and generally increase with the acidity of the N–H or O–H group. Thus, the acidity of the aniline derivatives increases in the order shown in Table XI (aniline weakest, nitroaniline strongest), paralleling the increase in σ[12] for the substituent group on the aniline. Similarly, acidity should increase in the order aniline, carbazole, phenol. To the extent that acidic solute groups interact with these basic eluents in *solution*, increasing acidity of the solute group should decrease adsorption and hence lead to *negative* $\alpha\Delta_{es}$ values. It is therefore clear that the effect arises in the adsorbed phase, in agreement with the constancy of the $\alpha\Delta_{es}$ terms as eluent composition is varied. It is proposed that this "basic eluent anomaly" arises from a complex interaction of adsorbed eluent

TABLE X

TEST OF THE "ETHER ANOMALY" FOR PROPYL CHLORIDE, ETHYL SULFIDE, AND ISOPROPYL ETHER; 3.9% H_2O–Al_2O_3

Solute	*log $\underline{R}_p$*	*log $\underline{R}°$*: *n-Propyl chloride–pentane binaries*[a]				*Ethyl sulfide–pentane binaries*[b]			*Isopropyl ether–pentane binaries*[c]			
		10	*25*	*50*	*100*	*10*	*30*	*100*	*10*	*25*	*50*	*100*
Chrysene	1.85	1.20	0.70	0.15		0.62			1.08	0.69	0.29	
1:2;4:5-Dibenzopyrene	3.10				0.14		0.71				1.08	0.41
3:4-Benzacridine	2.10					1.09	0.31		1.29 (—0.04)[d]	0.84 (—0.10)[d]		
Dimethyl phthalate	3.33									1.75 (—0.42)[d]	1.32 (—0.43)[d]	0.62 (—0.56)[d]
Quinoline	2.31				0.68	1.51	1.00	0.29		1.11 (—0.42)[d]	0.83 (—0.42)[d]	0.56 (0.32)[d]
m-Nitroaniline	3.00							0.67				
p-Diethoxybenzene	1.53								0.52 (—0.37)[d]			
m-Dinitrobenzene	2.28								1.57 (—0.01)[d]			
$\alpha\varepsilon°$ exptl. (hydrocarbons)		0.054	0.096	0.142	0.204	0.103	0.159	—	0.064	0.097	0.133	0.179
(non-hydrocarbons)					0.204	0.091	0.155	0.242	0.078	0.126	0.175	0.223
calc.		0.052	0.101	0.150	(0.204)	0.074	0.145	(0.242)	0.038	0.076	0.121	(0.179)

[a] % v Propyl chloride.
[b] % v Ethyl sulfide.
[c] % v Isopropyl ether.
[d] $\alpha\Delta_{es}$

TABLE XI

THE "BASIC ELUENT" ANOMALY FOR PYRIDINE AND DIETHYLAMINE AS ELUENTS; 3.9% H_2O–Al_2O_3

Solute	*log R_p*	*log $R°$*				
		Diethylamine–pentane solutions[a]				*50 % v Pyridine–pentane*
		5	*20*	*50*	*100*	
aε°		*0.233*	*0.302*	*0.368*	*0.400*	*0.417*
Carbazole	2.64	1.22 (0.78)				
Phenol	2.93				1.60 (1.07)	
Aniline	2.29	1.15 (0.60)	0.49 (0.47)	0.05 (0.52)		
p-Fluoroaniline	2.33		0.81 (0.76)			
p-Chloroaniline	2.36		0.85 (1.06)			
m-Chloroaniline	2.36		0.74 (1.05)			
m-Nitroaniline	3.00		1.14 (1.16)	0.50 (1.18)	0.24 (1.24)	0.19 (1.36)

[a] % v Diethylamine.

and solute, as shown in Fig. 6 for the adsorption of aniline from diethylamine solutions. The amino group of the aniline is first assumed to localize on a strong adsorbent site (aluminum atom) with electron transfer[6]. This greatly increases the acidity of the N–H group in the aniline molecule, and the proton on the aniline nitrogen can then interact by hydrogen bonding or proton transfer with an adjacent amino group. Since not all eluent molecules can localize on strong adsorbent sites (see discussion of following section), the amine to which hydrogen bonding occurs is presumed to be non-localized, with little loss in its normal basicity. Similarly, the amine displaced by the aniline nitrogen upon adsorption is less acidic than the corresponding aniline amino group, so that competitive interaction between two eluent groups, as in Fig. 6 between an eluent and a solute, is less favored.

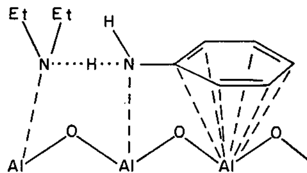


Fig. 6. Origin of the "basic eluent anomaly". Configuration of adsorbed aniline–diethylamine complex.

The magnitude of the "basic eluent" effect is similar for both diethylamine and pyridine solutions, and this may be true of other basic eluents as well. The "basic eluent anomaly" can be used to great advantage in the separation of solutes differing in the possession of N–H or O–H groups, but not in other chromatographic properties

(A_s, $\underline{R}_p$). It is important to realize that the acidity of the solute *in the adsorbed phase* determines the magnitude of the "basic eluent anomaly", rather than the more familiar solution acidity of the substance. Thus, phenol is certainly a stronger acid than *p*-chloroaniline, yet the values of $\alpha\Delta_{es}$ in each case are identical. Presumably, the strong, electron transfer interaction of the aniline nitrogen with an adsorbent aluminum atom results in an appreciably greater increase in the aniline acidity than occurs for the phenol upon adsorption.

The alcohols and their solutions as eluents

The alcohols and their solutions represent a limiting case as eluents, being among the strongest useful eluents*, and giving rise to the strongest solution interactions. Additionally, specific adsorbent–solute–eluent interactions as in Fig. 6 can be readily conceived. It might therefore seem unlikely that eqn. (1) could have more than qualitative significance in systems using the alcohols as eluents. Table XII summarizes $\underline{R}^\circ$ data for elution of various solutes by several isopropanol binaries. Varying amounts of water were added to the eluent as needed to preserve adsorbent activity at its initial value. Using 1,10-phenanthroline as standard solute, value of $\alpha\varepsilon^\circ$ were calculated for the 1 to 35 % isopropanol–pentane solutions, and these $\alpha\varepsilon$ values in turn used to calculate values of $\alpha\Delta_{es}$ for most of the remaining solutes. For the first seven solutes, these values of $\alpha\Delta_{es}$ are appreciable, but apparently randomly distributed as a function of solute structure; the average of the $\alpha\Delta_{es}$ values for the 1 to 10 % isopropanol solutions, where sufficient solute data are available for a valid comparison of experimental values with those calculated from eqn. (1), is $\pm$ 0.15 log units. This is reasonably small considering our previous remarks. The last four solutes of Table XII show much larger values of $\alpha\Delta_{es}$, and it may be significant that each of these solutes possesses an amino group. This suggests interaction of adsorbed solute and eluent, similar to that shown in Fig. 6 for the basic eluents. The relative constancy of these $\alpha\Delta_{es}$ values for each amino-substituted solute confirms the importance of interactions in the adsorbed rather than solution phase. The model of Fig. 6 fails for the other acidic solutes of Table XII (azaindole, acetylindole, phenol), which should also be strongly retained (large positive $\alpha\Delta_{es}$ values) if a "basic eluent anomaly" were operating in elution by the alcohols. While there are insufficient data in Table XII to permit any firm conclusions as to the origin of these alcohol eluent $\alpha\Delta_{es}$ values for the amino-substituted solutes, the following qualitative explanation may have some merit.

The two indole derivatives of Table XII and phenol have a relatively acidic hydrogen in each case, and it is believed (ref. 6 and following section) that the principal contribution to the adsorption energy of these solutes arises as a result of proton transfer from solute to the surface oxygen atoms of the alumina. For elution by basic eluents, such as the amines and pyridine, the basic eluent competes favorably with the surface oxygen atoms for proton transfer from the solute, and the adsorption energy for the solute is enhanced by proton transfer to the eluent, as in Fig. 6. For eluents such as the alcohols, the alcohol oxygen atom is considerably less basic, and it is postulated that interactions such as those of Fig. 6 are insufficiently strong to pre-empt the primary proton bonding between solute and surface

* The aliphatic acids are stronger, but chemically alter the alumina adsorbent and do not appear to constitute useful eluents.

TABLE XII

ISOPROPANOL–PENTANE BINARY ELUENTS; 3.9% H_2O–Al_2O_3

Solute	og $\underline{R}_p$	log $\underline{R}^o$								
		Isopropanol-pentane solutions[a]							CH_2Cl_2	Dioxane
		1	2	5	10	20	35	100		
7-Azaindole	3.36		0.35	0.02	—0.26					0.35
			(—0.35)	(—0.35)	(—0.38)					
3-Acetylpyridine	3.02	0.25	0.02							
		(—0.26)	(—0.17)							
3-Acetylindole	4.61		1.38	0.90						
			(0.13)	(0.07)						
Dimethyl phthalate	3.33	—0.44								
		—								
N-Methylquinolone	3.69		0.29	0.04					1.02	—0.46
			(0.14)	(0.33)						
1:10-Phenanthroline	4.60	1.44	1.05	0.62	0.29	—0.01	—0.24			0.59
		—	—	—	—	—	—			
Phenol	2.92			0.90	0.59	0.34	0.30			
				(0.36)	(0.25)	(0.18)	(0.27)			
Benzyl alcohol	2.33[b]		0.66	0.24	—0.11					
Benzamide	3.23[c]		1.43	1.29	0.80		0.26			
2-Aminopyridine	3.04	1.58	1.16	0.73	0.39	0.09	—0.13			0.03
		(0.79)	(0.78)	(0.68)	(0.62)	(0.51)	(0.46)			
Aniline	2.16	0.34								
		(0.53)								
m-Nitroaniline	3.00	1.18	0.70	0.64	0.31	0.02				
		(1.32)	(1.24)	(1.62)	(1.62)	(1.63)				
2,3-Diaminonaphthalene	4.46						1.19	0.77		
							(2.05)	—		
$\alpha\varepsilon^{o}$[d]		0.314	0.354	0.398	0.431	0.461	0.484	0.522[e]		

[a] % v Isopropanol.
[b] Assumes A_s equal 6.0, $\alpha\Delta_{es}$ equal 0.3.
[c] Assumes A_s equal 8.0, $\alpha\Delta_{es}$ equal 1.0.
[d] Calculated from 1,10-phenanthroline data.
[e] Calculated from 2,3-diaminonaphthalene data.

oxygen atoms. Furthermore, the alcohols themselves are believed selectively adsorbed by proton transfer to the surface oxygen atoms, which in turn selectively restricts the adsorption of acidic solutes such as the indoles and phenols. Solutes such as aniline do not adsorb principally by proton transfer, and any additional adsorption energy contributed by interactions of the type shown in Fig. 6 should increase the adsorption energy of the anilines and hence give positive values of $\alpha\Delta_{es}$. While this explanation of the data of Table XII is qualitatively satisfactory, the $\alpha\Delta_{es}$ values for the amino derivatives of Table XII tend to be higher than comparable values in Table XI, whereas the present theory requires that they be lower. Undoubtedly, the actual situation is considerably more complex than we have represented it.

If the $\alpha\varepsilon^{\circ}$ values of Table XII for the isopropanol–pentane binaries are correlated by means of eqn. (5), it is found that a very poor fit results with the calculated n_b value (3.2), but a reasonable fit is obtained for n_b equal 8. This is similar to the case of diethylamine as eluent, and may reflect the existence of an "ether anomaly" effect with the alcohols.

An ε° value for pure isopropanol may be calculated from the data of Table XII, equal to 0.82. The pure alcohols do not appear suitable as eluents since they tend to wash out the smaller adsorbent particles, which usually is experimentally inconvenient. Retention volume values for methanol–ethyl ether binaries (25 and 50 % v) and 2,3-diaminonaphthalene as solute (log $\underline{R}^{\circ}$ equal 0.33 and 0.10, respectively) suggest an ε° value for pure methanol equal to 0.95.

THE RELATIONSHIP OF ELUENT STRENGTH TO MOLECULAR STRUCTURE

The relative strength of the eluent in adsorption chromatography has been investigated by a number of workers, as reviewed by LEDERER AND LEDERER[15], and more recently by STRAIN *et al.*[16]. Previous studies have led to eluent strength series generally similar to that of Table II, although there has been occasional disagreement about the relative elution efficiency of certain solvents. Such differences of opinion are scarcely surprising, in view of effects such as the "ether anomaly" and "basic eluent anomaly", which if ignored lead to eluent strength series which depend upon the solute studied. The importance of such effects in previous studies has been magnified by the tendency of previous workers to study only a limited number of solutes, as well as by past ignorance of the theoretical relationship between binary eluent strength and eluent composition (eqn. 5).

The theoretical basis of the relationship between eluent strength and molecular structure has long been an important problem in the theory of adsorption chromatography. Early workers noted the general dependence of eluent strength on "polarity" (dipole moment, dielectric constant, etc.), but such relationships have never been found to be more than rough correlations with numerous exceptions. The eluent has been generally assumed to achieve its elution power by competitive interactions with solute in both adsorbed and non-sorbed phases[16]. The importance of net solution interactions in this respect, however, is contradicted by the general applicability of eqn. (1) for the weaker eluents, and the association of deviations from eqn. (1) for the stronger eluents (*e.g.* ethyl ether, ethylamine, isopropanol), with adsorbed phase interactions. Direct comparison[5] of the magnitudes of solution and adsorption

terms in typical chromatographic systems also challenges the importance of solution interactions in affecting adsorption chromatographic separation. Consequently, a reasonable theory of the dependence of eluent strength on molecular structure must emphasize the interactions in the adsorbed phase.

Eluent strengths of pure solvents

An earlier paper[2] in the present series has suggested that the strength of a compound used as eluent (ε°) can be related to the adsorption energy of the same compound when considered as solute. Specifically, it was predicted that ε° would be determined by the adsorption energy of the solute S° per unit area A_s:

$$\varepsilon^\circ = \frac{S^\circ}{A_s} = \frac{\sum^{i} Q^\circ_i + \sum^{j} q^\circ_j - f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i}{A_s} \tag{6}$$

Eqn. (6) completely ignores solution energy terms. Application of eqn. (6) to a number of eluents of known strengths[2] showed good agreement between calculated and experimental ε° values for a number of weak and moderately strong eluents, but calculated values for the stronger eluents were generally high by a factor of at least two. This was originally rationalized in terms of localization effects. Solutes with strongly adsorbing groups tend to localize upon adsorption[8], with the strongest adsorbing solute group fixed to a strong adsorbent site. Eqn. (6) takes solute localization into account, but localization of the adsorbed eluent would be expected to differ markedly from localization of the adsorbed solute, because of greater competition for adsorbent sites among eluent molecules. The failure of the *average* eluent molecule to adsorb on a strong adsorbent site would lead to a lower energy of adsorption of the eluent than predicted by eqn. (6), as is in fact observed.

The actual adsorption energy per eluent molecule can be estimated if we assume that localization is absent in the average adsorbed eluent molecule, an assumption which appears realistic since the average strong adsorbent sites appear widely separated[6]. The various group adsorption energies Q°_i (and solute geometry terms q°_j) of eqn. (6) must then be replaced by $[1 - f(Q^\circ_k)]Q^\circ_i$, the energy per non-localized group in the eluent as solute[8]. $f(Q^\circ_k)$, the localization function for the strongest adsorbing solute group k, has been previously defined[8]. The resulting relationship between eluent strength and structure is then given by:

$$\varepsilon^\circ = \frac{[1 - f(Q^\circ_k)]\,[\sum^{i} Q^\circ_i + \sum^{j} q^\circ_j]}{A_s} \tag{6a}$$

Before applying eqn. (6a) to eluent strength calculations, however, two additional complications must be recognized. First, previous treatments of solute adsorption energy (and tabulations of Q°_i values) ignore the delocalization of benzene rings attached directly to a localizing group k. Since the benzene ring is delocalized in the eluent considered as solute, it cannot undergo further delocalization when the solute is considered as eluent. Consequently, for aromatic eluents eqn. (6a) assumes the alternate form:

$$\varepsilon^\circ = \frac{n_a Q^\circ_c + [1 - f(Q^\circ_k)]\,[\sum^{i} Q^\circ_i + \sum^{j} q^\circ_j - n_a Q^\circ_c]}{A_s} \tag{6b}$$

Here, n_a refers to the number of aromatic carbon atoms (usually six) in the ring attached to the group k, and $Q°_c$ is the value of $Q°_i$ for an aromatic carbon atom (0.31 for alumina).

A second correction is required for eluents where k is attached to alkyl carbon. In this case, the localization function calculated from aromatic solutes does not apply, again because of the convention of ignoring the delocalization of the benzene ring attached to the localizing group k. That is, if delocalization of the aromatic ring in such solutes as anisole had been originally considered, the calculated net adsorption energy of the benzene ring would have been less, and the $Q°_i$ value of the methoxy group (k) larger. This would in turn have led to a larger calculated value of $f(Q°_k)$ in such solutes as dimethoxybenzene. Fig. 7 summarizes the complete re-interpretation

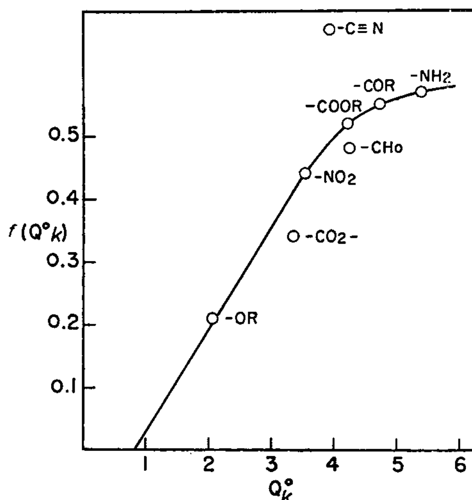


Fig. 7. Revised localization function for both aromatic and aliphatic solutes and eluents (delocalization of attached ring considered).

of Table VI of ref. 8 (where the localization function was first derived) in terms of a model assuming the delocalization of the benzene ring attached to each localizing group k, as well as delocalization of other groups. As expected, the revised relationship between $f(Q°_k)$ and $Q°_k$ differs from the previous relationship (Fig. 2, ref. 8) based on non-delocalization of one aromatic ring. The localization function of Fig. 7 could be used with the revised $Q°_k$ values (also noted in Fig. 7) to calculate the adsorption energies of aromatic solutes with a precision equal to the previous treatment[8]. Alternately, these same $Q°_k$ and $f(Q°_k)$ values of Fig. 7 could be used in eqn. (6a), rather than the original values in eqn. (6b), to calculate the eluent strengths of aromatic eluents. For purposes of convenience and consistency, however, we will retain the former convention of ignoring ring delocalization for the aromatic solutes and eluents. The revised function of Fig. 7 has the unique merit of permitting eluent strength calculations for *any* solvent according to eqn. (6a), since the new localization function (Fig. 7) is of general applicability.

Values of A_s for the various eluents of Tables II and III were calculated from the estimated configuration of adsorbed eluent and the known molecular dimensions

and Van der Waals radii, as previously in the calculation of solute group area values[5]. For alkyl groups longer than methyl, the contribution to A_s was taken from experimental data for alkyl-substituted solutes (obtained by eluent variation studies[11]). $\varepsilon°$ values for 24 of the 29 eluents of Table II could then be calculated using either eqn. (6a) for aliphatic eluents, or (6b) for aromatic eluents, and $Q°_i$ and $q°_j$ values from the present study (Table V) or a previous tabulation[8]. Five of the eluents of Table II could not be treated by eqn. (6a) or (6b), either because unknown $Q°_i$ values were involved (carbon disulfide, diethylamine), or more than one strongly adsorbing group was attached to the same carbon atom (carbon disulfide, CH_2Cl_2, $CHCl_3$, CCl_4), with resulting serious steric and electronic interaction (of unknown magnitude) among these strong eluent groups.

The agreement between experimental and calculated (no correction) $\varepsilon°$ values in Table II is semi-quantitative, the average deviation being $\pm$ 0.06 units for a total range of $0.00 \leqslant \varepsilon \leqslant 0.95$. Seven eluents deviate from the calculated value by more than 0.10 unit. Several sources of error in this calculation can be recognized, apart from possible inadequacies in the theoretical development. Several of the $Q°_i$ values for aliphatic eluents (R–CO_2R, R–Cl, R–O–R) are estimates, based on extrapolated data[8]. In other cases (R–OH), the $Q°_i$ values are relatively uncertain for other reasons. The various A_s values can hardly be more accurate than $\pm$ 10%. Finally, the calculation of the localization function in Fig. 7 is somewhat ambiguous; a previous communication[6] has observed that the localization of aromatic rings and of single groups (*e.g.* nitro, amino, etc.) has a fundamentally different basis, and that the resultant localization functions are only fortuitously equal for certain solute types. The delocalization of a ring attached to a strongly adsorbing group may in fact be somewhat greater than assumed in Fig. 7. With these considerations in mind, the uncorrected correlation of Table II is in fact seen to be quite reasonable. Since we have ignored solution interactions completely in this treatment, this is excellent additional evidence for the general unimportance of such interactions in determining the properties of an eluent.

It is worth while to consider one additional contribution to eluent strength, particularly as it permits an approximate correction of the $\varepsilon°$ values calculated above. We have already observed the special ability of ethyl ether to adopt a preferred adsorbed configuration (as in Fig. 5A), and it has been previously postulated[8] that all monatomic adsorbing groups have a greater than average localization function because of their ability to form a single strong bond with an adsorbent site. If the eluents capable of ether-like interaction with the adsorbent are considered separately (8 compounds in Table II of the general type R–X or R–X–R, where X is monatomic exclusive of hydrogens, and R is alkyl), it is found that the calculated values are high by an average of 0.11 $\pm$ 0.04 units. This would have been predicted if localization of the eluent molecule (as solute, but not as eluent) as in Fig. 5A occurs, since the true delocalization function is then effectively higher. It also suggests a correction (—0.11 units) for the calculated $\varepsilon°$ values of eluents of this type. The second column of calculated $\varepsilon°$ values in Table II (corrected) have been corrected in this fashion. Similarly, it seems possible that eluents which contain a tri-atomic k group (exclusive of hydrogen) might tend to have *smaller* than normal localization functions, because of the more diffuse interaction of the group k with a point adsorbent site, and resulting greater freedom (lesser delocalization) of the remainder of the solute to adopt an

optimum configuration on the adsorbent surface. In confirmation of this expectation, the four eluents of this type in Table II (nitromethane, ethyl benzoate, methyl acetate, ethyl acetate) show calculated ε° values which are *low* by 0.09 $\pm$ 0.08 units. It therefore seems that eluents which possess strong groups containing three or more atoms will generally show ε° values higher than calculated, and that the calculated values should be corrected by adding 0.09 units. The second column of calculated ε° values in the Table II (corrected) have been corrected in this fashion.

The 24 calculated ε° values of Table II, corrected in the above fashion, deviate from the experimental values by an average of only $\pm$ 0.03 units with only one calculated ε° value in error by more than 0.10 unit. This correlation of experimental and calculated ε° values is better shown in the plot of Fig. 8. Considering the range

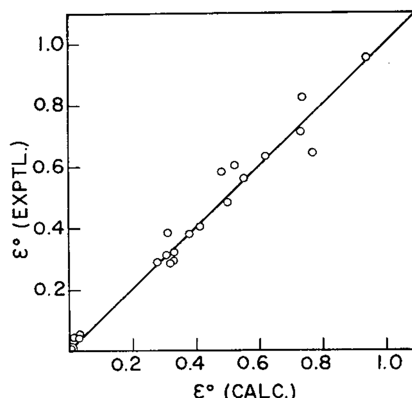


Fig. 8. Comparison of experimental *versus* calculated (corrected) ε° values for the eluents of Table II.

in eluent strengths and properties, the numerous approximations represented in the Q°_i and A_s values, and the simplicity of the theoretical derivation, this is a surprisingly good fit of experiment and theory. Fig. 8 confirms that the major aspects of the role of the eluent in adsorption chromatography now rest upon a firm fundamental basis. Only the more general problem remains of relating *solute* adsorption energies to structure in terms of fundamental interactions between adsorbate and adsorbent.

A recent approach[17] to the relationship between eluent strength and structure deserves brief comment; it has been suggested that eluent strength can be correlated with surface tension *versus* water. While this correlation appears experimentally more satisfactory than previous correlations on eluent dielectric constant or dipole moment, it seems clear that only a qualitative, empirical relationship is involved, which cannot apply to all systems.

Eqns. (6a) and (6b) can be used to predict the strengths of a number of eluents for which ε° values have not yet been measured. Table III summarizes several such values. In most cases these calculated ε° values should be accurate within $\pm$ 0.1 units. The value for nitropropane was estimated from the experimental value for nitroethane, in view of the large error in the calculated value for that eluent.

Eluent strengths of binary mixtures

For molecules which localize on the adsorbent surface, the adsorption energy differs

depending upon whether the molecule functions as an eluent or a solute. As an eluent, the entire adsorbent surface is covered by the localizing molecule, the competition for strong adsorbent sites is keen and most molecules are incompletely localized, and average adsorption energy is low. As a solute, very little of the adsorbent surface is covered by localizing molecule, there is *no* competition among solute molecules for strong adsorbent sites, and average solute adsorption energy is high. In binary eluent solutions of the localizing molecule and a weaker adsorbing solvent, the average adsorption energy of the localizing molecule (as eluent) approaches that of pure eluent at high surface coverages, and that of the molecule as solute at low surface coverages. Eqn. (5) should therefore be most accurate at high concentrations of the strong eluent.

Similarly, in binary eluents containing a strong eluent which exhibits "ether" or "basic eluent" anomalies, $\alpha\Delta_{es}$ has been observed to be constant as eluent composition varies. This can only be true at relatively high surface coverages, however, $\alpha\Delta_{es}$ eventually approaching the value applicable to the weaker binary eluent as the concentration of the strong eluent in solution and adsorbed phases decreases. Adsorbent surface coverage by the strong eluent in a binary eluent system can be calculated as previously[2], and compared with experimental $\alpha\varepsilon^\circ$ and $\alpha\Delta_{es}$ values in selected systems, in order to evaluate the importance of these effects. Such comparisons can also offer additional evidence in behalf of the present theory of eluent localization in the adsorbed phase.

In Fig. 9a, $\alpha\varepsilon^\circ$ values for dilute ethyl ether–pentane solutions are plotted *versus* eluent composition. These are taken from the hydrocarbon solute data of Table VI, plus a similarly determined value for 2% ether–pentane. The lower solid line ("eluent") of Fig. 9a is calculated from eqn. (5), using the ε° value for ether as eluent.

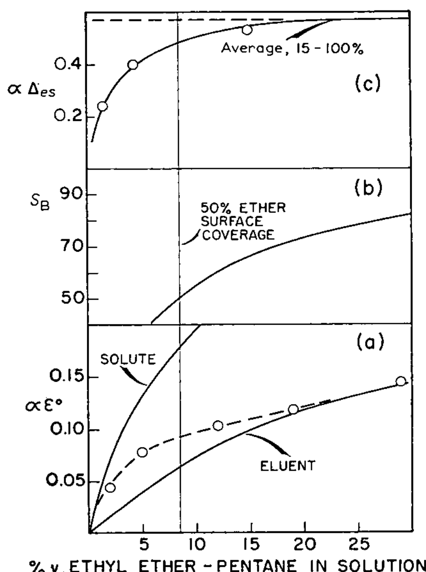


Fig. 9. Eluent strength and the "ether anomaly" in dilute solutions of ethyl ether–pentane. 3.8% H_2O–Al_2O_3. (a) $\alpha\varepsilon^\circ$ data for hydrocarbon solutes (see Table VI); (b) % surface coverage S_B by ether, calculated from eqn. 6, ref. 2; (c) $\alpha\Delta_{es}$ values for pyridine as solute (see Table VII).

The upper solid line of Fig. 9a ("solute") is calculated from eqn. (5), using a calculated ε° value (eqn. 6) for ether at low surface coverages (as solute). As seen, the experimental data follow the "eluent" curve at higher ether concentrations, and approach the "solute" curve at low ether concentrations. In Fig. 9b, calculated surface coverage, S_B, by ether (eqn. 6, ref. 2) is plotted *versus* ether solution concentration. As ether surface coverage drops to about 50 %, we might expect the ether adsorption energy to change from that characteristic of the eluent to that characteristic of the solute. Fig. 9a confirms this, the $\alpha\varepsilon^\circ$ data falling about midway between the "eluent" and "solute" curves when ether surface coverage is 40–50 %.

Similarly, for the plot of $\alpha\Delta_{es}$ *versus* ether concentration in Fig. 9c, the $\alpha\Delta_{es}$ values are essentially constant for ether surface coverages above about 70 %, and begin to drop significantly when ether surface coverage approaches 50 %. It is seen that the breakdown of eqn. (5) and the dependence of $\alpha\Delta_{es}$ on eluent composition first occurs in the system of Fig. 9 at ether concentrations of 5–10 %. Since other localizing eluents are generally as strong or stronger than ethyl ether, have bigger n_b values, and the adsorbent of Fig. 9 is relatively deactivated, it may be anticipated that *effects of the type shown in Fig. 9 (failure of eqn. 5, variation of* $\alpha\Delta_{es}$ *with % strong eluent) will in general not be important when the strong eluent concentration in a binary exceeds 5 %.*

THE PHENOLS AND RELATED COMPOUNDS AS SOLUTES

Whereas most solutes are adsorbed on alumina in a parallel (flat) configuration, the adsorption of the phenols is commonly assumed[19] to give a perpendicular (vertical) configuration. These two possible orientations of the adsorbed phenol molecule are illustrated in Fig. 10. It is of both practical and theoretical interest to confirm the true configuration of the adsorbed phenols at low surface coverages.

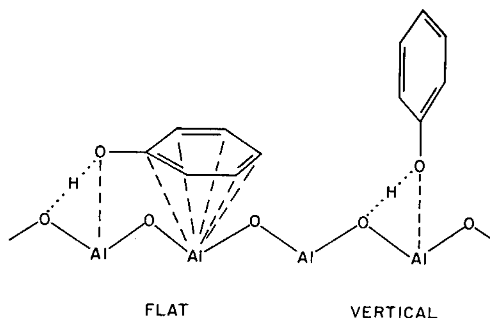


Fig. 10. Possible orientations of adsorbed phenol on adsorbent surface.

Tables XIII and XIV summarize a number of retention volume data for various substituted phenols and some related compounds. The variation of $\underline{R}^\circ$ with changing eluent can in principle provide a value of A_s and hence specify solute configuration[2]. This procedure is not particularly applicable to the data of Table XIII (phenol, dimethylphenol) because of the lesser accuracy of eqn. (1) in strong eluent systems. Additionaly, it appears that the A_s values of acidic solutes (such as carbazole) are higher than calculated[6], further confusing the interpretation of such data. An alter-

TABLE XIII

RETENTION VOLUMES FOR MISCELLANEOUS STRONGLY ADSORBING SOLUTES; 3.9% H_2O–Al_2O_3

Solute	*log $\underline{R}°$*								*log $\underline{R}_p$*
	P[a]	*50% M–P*[b]	*10% D–P*[c]	*CH_2Cl_2*	*Ethyl ether*	*25% D–P*[d]	*Dioxane*	*20% IPA–P*[e]	
Phenol[f]			1.58			0.98	0.53	0.34	2.93
2,6-Dimethylphenol[g]		1.35	0.88	0.85	0.78	0.17	−0.12		2.64
2,6-Di-*tert*.-butyl-*p*-cresol	0.20								
Thiophenol[h]								1.12	3.7
Benzaldoxime[h]								0.23	2.8
Acetophenone oxime[h]								0.20	2.4
$\alpha\varepsilon°$		0.210	0.235	0.267	0.293	0.298	0.401	0.461	

[a] Pentane.
[b] 50% v Methylene chloride–pentane.
[c] 10% v Dioxane–pentane.
[d] 25% v Dioxane–pentane.
[e] 20% v Isopropyl alcohol–pentane.
[f] Assumes A_s equal 6.0.
[g] Assumes A_s equal 7.0.
[h] Assumes A_s equal 6.0, $\alpha\Delta_{es}$ equal +0.30.

native analysis is offered by the variation of $\underline{R}°$ among the substituted phenols, using a single system, as in Table XIV. From eqns. (1) and (2), and the $\underline{R}°$ value for phenol, $\underline{R}°$ values for the substituted phenols may be calculated if we ignore the (unknown) geometry term $\sum^{j} q°_j$. The difference between experimental and calculated experimental values Δ_f is then equal to the solute geometry term. This calculation assumes flat adsorption of the phenols. Alternatively, for vertical adsorption, $\sum^{j} q°_j$ or Δ_v is simply the difference between the experimental values of $\underline{R}°$ for the substituted phenol and

TABLE XIV

RETENTION VOLUMES OF *m*- AND *p*-SUBSTITUTED PHENOLS; 3.9% H_2O–Al_2O_3, 20% V ISOPROPANOL–PENTANE ELUENT

Solute	*log $\underline{R}°$*	Δ_v	Δ_f	σ^{18}
p-Methoxyphenol	0.30	−0.04	0.26	−0.268
3,4-Dimethylphenol	0.15	−0.19	0.71	−0.239
p-Methylphenol	0.27	−0.07	0.38	−0.170
3,5-Dimethylphenol	−0.03	−0.37	0.53	−0.138
Phenol	0.34	0.00	0.00	0.000
m-Methoxyphenol	0.40	0.06	0.36	0.115
p-Chlorophenol	0.75	0.41	0.80	0.227
m-Acetophenol	0.84	0.50	0.10	0.306
m-Chlorophenol	0.70	0.36	0.75	0.373
p-Acetophenol	1.54	1.20	0.80	0.874[a]
p-Formylphenol	1.93	1.59	0.87	1.126[a]
p-Nitrophenol	> 2.00	> 1.64	> 1.87	1.27[a]

[a] σ^* Values for reactions of anilines and phenols.

phenol. In either case, these $\overset{j}{\Sigma} q^{\circ}{}_j$ values should be linearly related to the HAMMETT σ parameter[12] since phenol acidity is so related, and the adsorption of the phenols on alumina is assumed to be an acid–base type reaction[19]. The best correlation of $\overset{j}{\Sigma} q^{\circ}{}_j$ on σ should therefore indicate the actual configuration of the adsorbed phenols. Table XIV and Fig. 11 summarize these two possibilities. As clearly seen in Fig. 11, for the vertical adsorption case, this configuration gives a quite satisfactory correlation on δ, whereas the correlation for the horizontal adsorption case in Fig. 11b is very poor. It is therefore concluded that the phenols are adsorbed in the vertical configuration from the solvent of Table XIV. The correlation of Fig. 11a also permits $\overset{j}{\Sigma} q^{\circ}{}_j$ for the substituted phenols to be related to σ (eqn. 4).

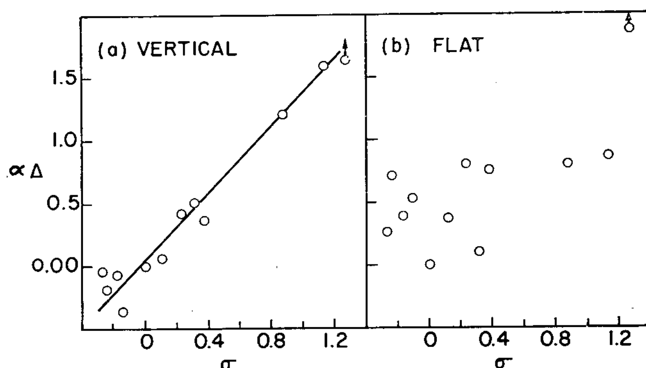


Fig. 11. Correlation of phenol Δ values for flat and vertical configurations *versus* HAMMETT σ values.

A vertical configuration for phenol can be used to calculate a value of A_s equal 3.5, *versus* 7.0 for the horizontal configuration. Similarly, 2,6-dimethylphenol should have an A_s value of 4.3 in the vertical configuration. The data of Table XIII actually suggest A_s values for phenol and dimethylphenol of about 6 and 7, respectively. The higher values can presumably be ascribed to an effect similar to that encountered in the pyrrole derivatives[6], where the acidic nitrogen group requires an apparent area of adsorption (A_s value) some 2.5 units higher than calculated. Assuming a similar increase in A_s for all the solutes of Table XIII, the indicated log $\underline{R}_p$ values can be calculated, and are the basis of the values of Table V. The data taken from the 20 % isopropanol–phenol solution were arbitrarily reduced by 0.3, the value of $\alpha\Delta_{es}$ for phenol in this solvent.

Without a doubt, as eluent strength is decreased from that of 20 % isopropanol–pentane toward that of pentane, at some point the phenol molecule changes from a vertical to a flat configuration. The condition for this configuration transformation has been considered previously[2]: it should occur at that eluent strength where the adsorption energies for each configuration are equal. Conversely, configuration in a particular case may be decided from adsorption energy calculations on both flat and vertical configurations. The configuration giving the greatest energy or $\underline{R}^{\circ}$ value is the preferred one. As an example, the A_s values for flat and vertical phenol are calculated as 9.5 and 6.0 units. The adsorption energy of the benzene ring is given by

$f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i$ for the flat configuration (1.02), taking the normally neglected delocalization of the ring attached to k into account, while that for the vertically adsorbed phenol ring is 0.00. At an eluent strength ε° equal to 1.02/3.5, or 0.29, phenol should pass from a vertical configuration to a horizontal one. Similarly, we can estimate the configuration of *p*-acetophenol in the system of Table XIV. A_s for the flat and vertical configurations is calculated at 11.5 and 6.0, respectively, and the net adsorption energy $\alpha f(Q^\circ_k) \sum^{i \neq k} Q^\circ_i$ to be gained in the flat configuration is 1.94. $\alpha\varepsilon^\circ$ is 0.461 and it is calculated that the vertical configuration is favored by 0.60 units, *i.e.* by a substantial margin. Since *p*-acetophenol is the most likely of the phenols in Table XIV to adsorb in the flat configuration (the aceto group has the largest adsorption energy), this calculation supports our prior conclusion: all of these phenols adsorb vertically.

GLOSSARY OF TERMS

A_s	Solute surface volume, approximately proportional to area required by solute upon adsorption
a_i	Contribution of solute group i to A_s; $A_s = \sum^i a_i$
$f(Q^\circ_k)$	Solute localization function[8]
n_a	Number of aromatic carbon atoms in eluent molecule
n_b	Value of A_s for the strong eluent in a binary eluent solution
q°_j	Solute geometry parameter
Q°_i	Solute group adsorption energy parameter
$\underline{R}^\circ$	Linear equivalent retention volume (ml/g)
$\underline{R}_p$	Value of $\underline{R}^\circ$ for pentane eluent
S°	Solute dimensionless adsorption energy from pentane onto calcined adsorbent (defined by relationship $\log \underline{R}_p = \log V_a + S^\circ$
S_B	Fraction of adsorbent surface covered by eluent B where binary eluent (A–B) is used
V_a	Adsorbent surface volume (ml/g)
X_B	Mole fraction of strong eluent B in eluent binary (A–B)
α	Adsorbent activity function
Δ_{es}	Eluent–solute interaction parameter, for application to "anomalous" eluents; defined by eqn. (3)
ε°	Eluent strength parameter
ε°_{AB}	Eluent strength of binary solution (A–B)
ε°_A, ε°_B	Eluent strengths of solvents A and B
δ, δ^*	HAMMETT substituent constants[12,18]

ACKNOWLEDGEMENTS

The author is grateful to his associate, Dr. J. K. FOGO, for editing the original manuscript, and to Mr. F. O. WOOD for assistance in the experimental work.

SUMMARY

Experimental data are reported for elution of a number of solutes from alumina by several very strong eluents. A number of these strong eluents (the ethers, amines,

alcohols, pyridine) exhibit unique elution characteristics, leading to a number of new separation possibilities. A quantitative theory has been developed for the relationship between elution strength and eluent constitution. For elution from alumina, the eluent properties of almost any common solvent or solvent mixture can now be accurately predicted. Whereas most solutes adsorb parallel to the adsorbent surface (flat), it is shown that the phenols adsorb in a perpendicular configuration (vertical) from strong eluents.

REFERENCES

[1] L. R. SNYDER, *J. Chromatog.*, 6 (1961) 22.
[2] L. R. SNYDER, *J. Chromatog.*, 8 (1962) 178.
[3] L. R. SNYDER, *J. Chromatog.*, 11 (1963) 195.
[4] L. R. SNYDER, *J. Chromatog.*, 12 (1963) 488.
[5] L. R. SNYDER, *Advan. Anal. Chem. Instr.*, 3 (1964) 251.
[6] L. R. SNYDER, *J. Phys. Chem.*, 67 (1963) 2344.
[7] L. R. SNYDER, *J. Chromatog.*, 15 (1964) 344
[8] L. R. SNYDER, *J. Chromatog.*, 8 (1962) 319.
[9] K. N. TRUEBLOOD AND E. W. MALMBURG, *J. Am. Chem. Soc.*, 72 (1950) 4112.
[10] M. J. SABACKY, L. B. JONES, H. D. FRAME AND H. H. STRAIN, *Anal. Chem.*, 34 (1962) 306.
[11] L. R. SNYDER, *J. Chromatog.*, 13 (1964) 415.
[12] L. P. HAMMETT, *Physical organic chemistry*, McGraw-Hill Book Co., New York, 1940, p. 186.
[13] L. R. SNYDER, unreported studies.
[14] L. R. SNYDER, *J. Phys. Chem.*, 67 (1963) 234.
[15] E. LEDERER AND M. LEDERER, *Chromatography*, Elsevier, Amsterdam, 1957, p. 38.
[16] H. H. STRAIN, *Anal. Chem.*, 33 (1961) 1733.
[17] F. MUNTER, *Chem. Tech.*, 15 (1963) 244.
[18] H. H. JAFFEE, *Chem. Rev.*, 53 (1953) 191.
[19] C. H. GILES, T. H. MACEWAN, S. N. NAKHAWA AND D. SMITH, *J. Chem. Soc.*, (1960) 3973.

AN ELECTRICAL ANALOGY TO THE GEL FILTRATION PROCESS

TORVARD C. LAURENT AND ESTAN P. LAURENT
Department of Medical Chemistry, University of Uppsala, Uppsala, and
Department of Telegraphy and Telephony, Royal Institute of Technology, Stockholm (Sweden)

(Received February 26th, 1964)

INTRODUCTION

Gel filtration is a type of partition chromatography in which substances are separated largely on the basis of molecular size[1,2]. It has been suggested that this is due to a decreased available volume for other molecules in the gel network, and LAURENT AND KILLANDER[3] have shown that most experimental data are compatible with such a mechanism. Their theoretical treatment of gel filtration makes it possible to predict the elution position of a substance for the ideal case, but it does not, in general, permit calculation of the shapes of the elution curves. Therefore an analog computer has been constructed which permits analysis of the effect on the elution pattern of available volume, equilibration time, and flow rate. The computer is built according to the principle that a chromatographic column can be hypothetically subdivided into a number of plates, and that the solute moves in the liquid phase from plate to plate. The solute is assumed to attain some degree of equilibrium, partial or complete, between the liquid phase and the gel phase of each plate. Each plate is simulated by an electrical circuit containing two condensors and a resistor. A somewhat similar picture of a chromatographic process has been proposed by GOLAY[4].

The picture of dividing a chromatographic column into a number of plates is common in the theoretical treatment of chromatography[5–9]. The plates defined in this paper are equivalent to conventional "theoretical plates" if the resistance in the circuit is zero. When a finite resistance is used, the analog will give a kinetic description of the chromatographic process with local non-equilibria on the column similar to that described in the "rate theories"[8–11].

ELECTRICAL ANALOGY

It is assumed that the chromatographic bed can be represented by ninety-six (or n times ninety-six) plates of equal height. The liquid column in the bed is furthermore assumed to move stepwise along the bed at intervals of Δt seconds, each step corresponds to the height of one plate. A solute, present within a plate, is distributed between the liquid and gel phases during the time interval.

The electrical analogy of each plate is a circuit of the type shown in Fig. 1. The condensors C_G and C_L correspond to the gel and liquid phases, respectively. Their capacitances are proportional to the volumes of the phases which are available to the solute. When charged, their charges represent, respectively, the amounts of solute

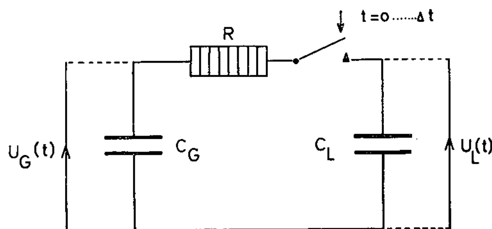


Fig. 1. Electrical analogy to the distribution process in a plate of the chromatographic column.

present in each phase. The voltages, U_G and U_L, correspond to the concentrations of solute.

The condensors will discharge over the resistance, R, when the circuit is closed. The voltages, U_G and U_L, will change during the time Δt according to the relation:

$$U_L\ (\Delta t) - U_G\ (\Delta t) = (U_L\ (0) - U_G\ (0)) \cdot e^{-\frac{\Delta t}{T}} \tag{1}$$

where T is the time constant:

$$T = R \frac{C_G \cdot C_L}{C_G + C_L} \tag{2}$$

Since charge is conserved:

$$C_G U_G\ (0) + C_L U_L\ (0) = C_G U_G\ (\Delta t) + C_L U_L\ (\Delta t) \tag{3}$$

These three relations determine $U_G\ (\Delta t)$ and $U_L\ (\Delta t)$.

The gel phase in the column is represented by 96 equal condensors, C_G', C_G'' . . ., and the liquid phase by one condensor, C_L, which may be connected successively to the gel condensors by a sliding contact. Representation of the liquid phase by a single condensor instead of a row of condensors sliding parallel to the gel condensors simplifies the construction. Each movement of the contact arm along the gel condensors corresponds to the transport along the gel bed of a volume of liquid equal to that in one plate. Any amount of liquid can be transported along the bed by repeating the procedure an appropiate number of times. The voltage retained by the condensor C_L after the sliding contact has passed all gel condensors is proportional to the concentration of solute in the liquid when it emerges from the column. The voltage is recorded by a recording voltmeter.

A particular solute introduced into a gel bed is distributed between the liquid and gel phases in a characteristic way. The partition at equilibrium between the gel phase and liquid phase is equal to the ratio C_G/C_L. It can be varied by varying C_L.

A diffusion equilibrium will not be reached in each plate if the diffusional flux between the gel phase and liquid is slow or if Δt is very short. This is analogous to a high value of the resistance, R (Fig. 1). By varying R, one can obtain various concentration distributions, which can be expressed as fractions of the initial concentration differences. Thus, a variation of R represents either a change in flow rate or a change in the time constant for diffusional exchange between gel and liquid.

CONSTRUCTION OF COMPUTER

An illustration of the instrument is shown in Fig. 2. The main parts are: (a) a con-

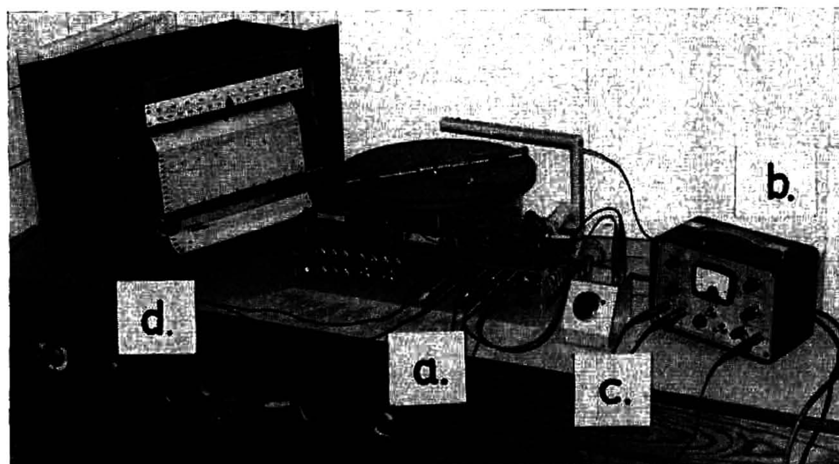


Fig. 2. View of the analog computer.

nection block containing all the condensors with a distributor on top; (b) a power supply; (c) a variable resistor; and (d) a recording voltmeter.

A general circuit diagram is shown in Fig. 3. The distributor, D, effects the connections between the condensors. It consists of a wiper arm mounted on a driving shaft which passes through the centre of a circular plate. The wiper arm is rotated at 2.2 revolutions per second by a synchronous motor. The connections to the 96 condensors (0.96 μF $\pm$ 0.5 % each) are arranged in a semicircle at the edge of the distributor. The condensors, designated C_G', C_G'' . . . etc., are connected with each end of the wiper shaft through sliding contacts.

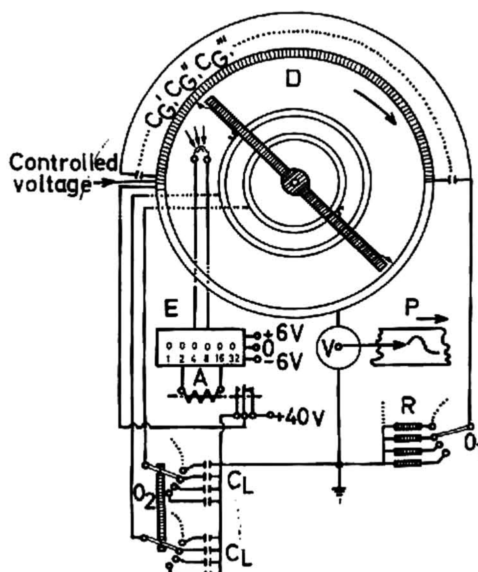


Fig. 3. General circuit diagram of the analog computer.

There are two sets of condensors corresponding to C_L. These two sets are so designed (see appendix I) that their capacitances can be varied between 0.27 and 9.12 μF. Each set of C_L is connected by a sliding contact to one side of the wiper arm and thereby to C_G. The value of C_L is varied by adjusting a bank of switches on the front panel.

Before the arm passes C_G', it will obtain an electrical impulse from a power supply (see appendix II) which is connected to the first contact in the semicircle. The number of times that the wiper shaft carries a new charge to C_G' is regulated by a relay, A, and an electronic counter, E (see appendix III). The electronic counter can be preset on the front panel for a certain number of impulses (1 to 63). More than 63 impulses can be introduced if the counter is short circuited. The voltage of the charging impulse can be varied if desired, by the use of a special arrangement (see appendix IV).

After the wiper shaft has passed the last C_G condensor, it connects C_L with the recording volt meter, P, which records the voltages on a moving paper strip. C_L is then automatically discharged before the shaft starts a new revolution.

R is a variable resistance (0–100 kΩ) on the line between C_G and C_L. It corresponds to R in Fig. 1.

The computer is operated in the following way: the capacitance of C_L, the resistance of R and the number of pulses to be introduced are selected. The recording volt meter and the motor that drives the wiper shaft are started. The first pulse is obtained by pushing the "start" button, which connects the power supply with the first contact in the semicircle. The starting time is recorded manually on the recording strip. The voltmeter then records the elution curve. When the operation is over, the instrument is discharged and the counter is returned to the zero position by pressing the reset button.

COMPARISON OF GEL FILTRATION AND ITS ELECTRICAL ANALOGY

The following symbols are used[3]:

V_0 = void volume or total volume of liquid phase in the gel bed.

V_x = total volume of gel phase.

$V_t = V_0 + V_x$ = total volume of gel bed.

K_{av} = the fraction of the gel phase available to the solute.

$K_{av} \cdot V_x$ = total volume available to the solute in the gel phase.

ε = equilibration factor for the distribution of solute between the gel phase and the liquid phase. It is equal to the change in the difference in solute concentration between the gel and liquid phases of each plate, divided by the total initial concentration difference.

Application of material

The volume of material applied to the column corresponds to the number of times that C_L is recharged before passing C_G'. The electronic impulse counter can be set at any value between 1 and 63, representing a range of sample sizes from 1–66 % of the void volume or approximately 0.3–20 % of the total volume of the column. If the counter is short circuited, a larger volume can be introduced.

Elution curve

The elution curve recorded graphically begins at the void volume of the column. The

movement of the paper strip corresponds to the flow of liquid from the column and the recorded voltage is proportional to the concentration of solute. Thus, the area under each peak is proportional to the total amount of substance in the effluent.

Void volume, V_0

Although the void volume is not recorded in the elution diagram, it can be calculated easily. Each half turn of the wiper shaft moves 1/96 of the liquid phase through the column. The liquid phase or void volume will therefore have emerged completely after 48 turns; the void volume corresponds to the distance the recording strip has moved during this time.

Total volume of gel bed, V_t

The total volume cannot be determined directly. Experiments on Sephadex G-200 gels[3] showed that the void volume in this case was in the order of 29–30 % of the total volume, *i.e.* the total volume is approximately 3.3 times that of the void volume.

Fraction of the gel phase available to the solute, K_{av}

The fraction of the gel phase available to solute molecules is determined by their sizes. However, the analog computer is not programmed with K_{av}, but with the ratio of the total available volume of the gel phase to the void volume:

$$\frac{C_G}{C_L} = \frac{K_{av} \cdot V_x}{V_0} = \frac{K_{av}\,(V_t - V_0)}{V_0} \sim \frac{K_{av} \cdot 0.7 V_t}{0.3\, V_t} \sim 2.3\, K_{av} \tag{4}$$

The analog computer cannot be programmed with K_{av} unless the exact relationship between V_t and V_0 is known.

Equilibration factor, ε

The equilibration factor is represented in the computer by the variable resistance R. It can be calculated from eqns. (1) and (2):

$$\varepsilon = 1 - \frac{U_L(\Delta t) - U_G(\Delta t)}{U_L(0) - U_G(0)} = 1 - e^{-\frac{\Delta t}{T}} \tag{5}$$

Δt is the time of contact between the wiper shaft and each C_G condensor. The latter is 1.4 msec in the instrument and the equilibration factor becomes:

$$\varepsilon = 1 - e^{-\frac{0.0014\,(C_G + C_L)}{R \cdot C_G \cdot C_L}} \tag{6}$$

In the gel filtration process, the equilibration factor is a function of the flow rate, the free diffusion rate of the solute, the size and shape of the gel grains and the sieve action of the polymer in the gel grain.

DISCUSSION

The theoretical treatment of various types of chromatography is extremely complex and the process is far from completely understood (see *e.g.* refs. 5–11). The great

success achieved in making analogies between electrical and hydrodynamical processes in general[12] induced us to determine whether an electrical analog computer for gel filtration could be constructed. Two assumptions were made; namely, that a chromatographic column could be divided hypothetically into plates and that the distribution of solute between the gel and liquid phases could be represented by charge transfer in the circuit shown in Fig. 1. The first assumption is often used in the theoretical treatment of chromatography. The second amounts to the assumption that the solute encounters resistance to diffusion only in the boundary between the gel and liquid phases, rather than in the gel phase itself. It seems to be a reasonable approximation.

The number of plates was restricted to be ninety-six for practical reasons only. An increase in the number of plates will in general increase the resolution between two components[5] and experiments with gel filtration have sometimes given resolutions comparable to 5000 plates or more[2]. A 4 × 40 cm column of Sephadex G-200, 200–270 mesh, operated at a flow rate of 25 ml/h, has a resolution for albumin corresponding to 500 plates. The selection of ninety-six plates for the analog does not, however, limit its applicability. An elution curve can be fed back into the computer by a special device (appendix IV), and the effective length of the column can thereby be increased many times by simulating the recycling operation employed experimentally[13]. Obviously, the recycling behaviour of parts of elution curves can also be studied with the analog.

The analog gives a simplified picture of the gel filtration process. It does not account for certain factors, such as longitudinal diffusion and turbulent flow. However, the former is generally considered to be negligibly small and the latter is very difficult to estimate.

When the equilibration factor is 1, *i.e.* when complete equilibrium is obtained in each plate during the process, the analog describes a counter current distribution experiment. In principle, one should be able to obtain nearly statistical distribution curves on a column with ninety-six plates[5]. Such curves are demonstrated in Fig. 4.

The ideal case of complete equilibrium is seldom obtained in practice and the analog makes possible a systematic analysis of the effects of dis-equilibrium. Non-ideal gel filtration behavior is shown most often when very large molecules (low values

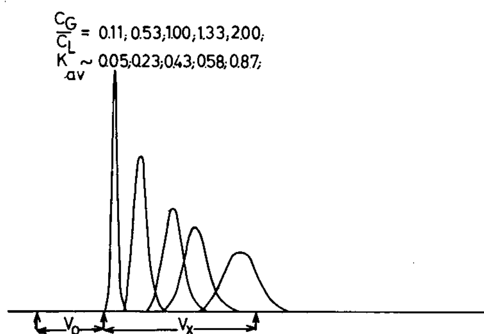


Fig. 4. Elution diagrams of substances with various partitions (C_G/C_L) between the gel and liquid phases. K_{av} values have been calculated on the assumption that $V_0 = 0.3\ V_t$. $\varepsilon = 1$. Eight electrical impulses were used, corresponding to approximately 2.5% of the total volume of the column.

of C_G/C_L) are studied at high flow rates. An example of how a decreasing equilibration factor influences the elution pattern is shown in Fig. 5. A more detailed comparison of experimental results with those obtained in the analog will be given in another paper.

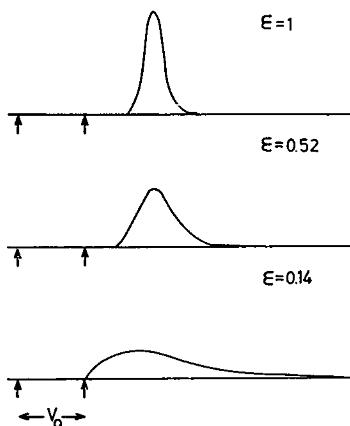


Fig. 5. Elution diagram of substances with partitions (C_G/C_L) = 1 but with varying equilibration factors. Eight impulses were used.

The analog computer described was built in order to extend the theoretical treatment of gel filtration presented in a preceding paper[3]. However, it may also be used to reproduce other kinds of chromatography or counter current distribution. The analog may be useful for the design of experimental conditions in various types of separations.

After this paper had been completed, the authors became aware that, simultaneously, VINK[14] had described chromatography in a similar fashion. He did not make an electrical analog, but, starting from equations equivalent to eqns. (1), (2) and (3), calculated two elution patterns for a column divided into thirty plates. His numerical approach is suitable for digital computers.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the valuable suggestions made by Professor TORBERN LAURENT during the progress of the work and the helpful discussions with Dr. BERTEL LAURENT and Mr. KÅRE MOSSBERG. The work has been supported by the Swedish Medical Research Council and the Swedish Cancer Society.

SUMMARY

An electrical analog computer has been constructed to study the process of gel filtration. The gel phase is represented by a series of condensors and the liquid phase by one condensor connected to a wiper shaft which moves along the row of condensors. The time for diffusion equilibrium between the phases is regulated by a resistor. The elution curve for a solute can be simulated if the partition coefficient and the time factor for the equilibration of the substance between the gel and liquid phases are known.

APPENDIX I

The condenser, C_L, is composed of a number of condensors which have different capacitances and can be connected in series or parallel in different combinations. This is shown schematically in Fig. 6. The object was to obtain a satisfactory number of

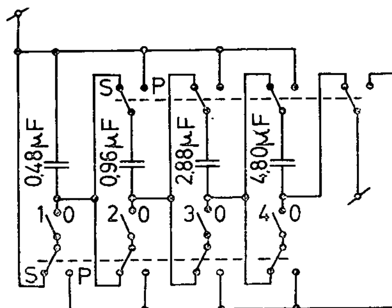


Fig. 6. Condensor (C_L) change-over switch.

possible variations from a minimum number of condensors and switches. The switches are set with the aid of a table, which shows C_G/C_L and $C_G \cdot C_L/(C_G + C_L)$ for various combinations.

APPENDIX II

The D.C. voltages needed are obtained from a 220 V A.C. source through a power supply unit, which is shown schematically in Fig. 7. It is essential that the D.C.

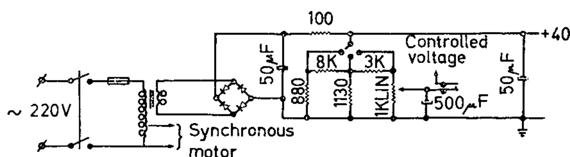


Fig. 7. Power supply circuit.

voltages are kept constant and that the charge impulses do not interfere with the electronic counter. The protection has been obtained with 50 μF electrolytic condensors.

APPENDIX III

Fig. 8 shows a block diagram of the counter and Figs. 9, 10 and 11 are the circuit diagrams of the various units.

The relay is activated in its original position, which is obtained by setting the switches 1–6 (Fig. 10) in zero position and pressing the hand-operated reset button. The relay becomes locked by the clamping circuit (Fig. 11). The number of impulses wanted (1–63) is set by the switches 1–6 in Fig. 10. The relay is released when the "start" button is pressed.

The recharge voltage is connected through relay contact 1 when the relay is non-excited (Fig. 11). The counter is reset through relay contact 2.

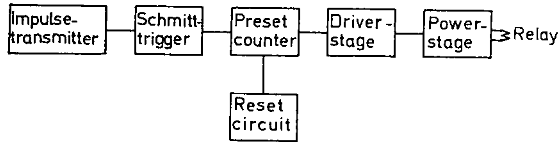


Fig. 8. Block diagram of the counter.

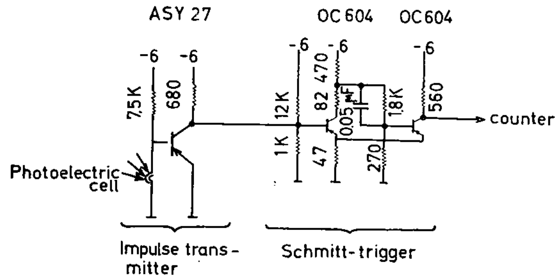


Fig. 9.

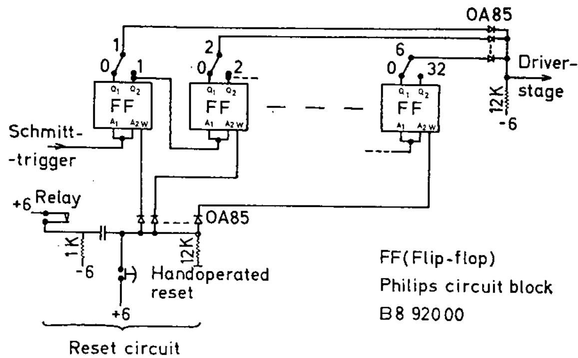


Fig. 10.

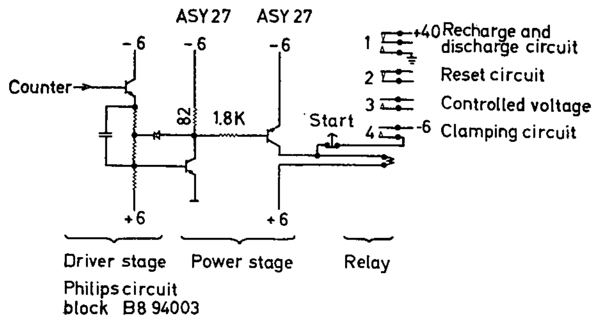


Fig. 11.

Figs. 9–11. Circuit diagrams of parts of the electronic counter.

The Schmitt-trigger, which gives the square waves to the preset counter is controlled by impulses generated by a photoelectric cell circuit. When the preset number of impulses has been transmitted, the relay becomes excited and locks.

APPENDIX IV

C_L can be charged with a voltmeter-controlled voltage instead of a constant voltage before it is discharged to the condensors C_G', C_G'' . . . etc. The voltage can be varied manually in such a way that the charge voltage has the same curve form as the recently obtained elution curve. In this way the recording voltmeter will record a new elution curve that corresponds to 192 plates instead of 96. This procedure can be repeated as many times as desired. The preceding elution curve is followed manually on the recording paper strip with a needle which is mechanically linked to a potentiometer.

REFERENCES

1 J. Porath, *Advan. Protein Chem.*, 17 (1962) 209.
2 P. Flodin, *Dextran Gels and Their Applications in Gel Filtration*, Dissertation, Uppsala, 1962.
3 T. C. Laurent and J. Killander, *J. Chromatog.*, 14 (1964) 317.
4 M. J. E. Golay, in V. J. Coates, H. J. Noebels and I. S. Fagerson (Editors), *Gas Chromatography*, Academic Press, New York, 1958.
5 A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, 35 (1941) 1358.
6 B. Glueckauf, in *Ionic Exchange and Its Applications*, Society of Chemical Industry, London, 1955.
7 E. Helfferich, *Ionenaustauscher*, Band 1, Verlag Chemie GmbH, Weinheim/Bergstr., 1959.
8 R. A. Keller, G. H. Stewart and J. C. Giddings, *Ann. Rev. Phys. Chem.*, 11 (1960) 347.
9 H. W. Habgood, *Ann. Rev. Phys. Chem.*, 13 (1962) 259.
10 J. C. Giddings, *J. Chromatog.*, 2 (1959) 44.
11 J. C. Giddings, *J. Chromatog.*, 5 (1961) 46.
12 T. Laurent, *Vierpoltheorie und Frequenztransformation*, Springer Verlag, Berlin, 1956.
13 J. Porath and H. Bennich, *Arch. Biochem. Biophys.*, Suppl. 1 (1962) 152.
14 H. Vink, *J. Chromatog.*, 15 (1964) 488.

ZUR TRENNUNG DER GIBBERELLINE IN PFLANZENEXTRAKTEN MIT HILFE DER DÜNNSCHICHTCHROMATOGRAPHIE UND DER HORIZONTALEN SÄULENCHROMATOGRAPHIE*

E. REINHARD, W. KONOPKA UND R. SACHER

Botanisches Institut der Universität Würzburg (Deutschland)

(Eingegangen den 25. Februar 1964)

Die Methode der Dünnschichtchromatographie hat sich im Laufe der letzten Jahre immer weitere Gebiete erobert und Möglichkeiten der Auftrennung von Stoffgemischen eröffnet, die mit anderen chromatographischen Methoden nicht zu erreichen waren.

Für die Trennung der neun bisher bekannten Gibberelline liegen mehrere Arbeiten vor, die die Trennung der Reinsubstanzen auf Kieselgel- und Kieselgur-Platten mit Hilfe verschiedener Laufmittel beschreiben. SEMBDNER *et al.*[1] benutzten als Laufmittel verschiedene Mischungen von Chloroform mit Essigester unter Zusatz von Eisessig. Das System Chloroform–Eisessig wurde bereits von WEST UND PHINNEY[2] zur Trennung von Gibberellinen an Kieselgel-Säulen verwendet. MCMILLAN UND SUTER[3] benutzten als Entwicklungsgemisch Di-isopropyl-äther–Eisessig (95:5), des weiteren Benzol–Eisessig–Wasser (8:3:5) und Benzol–Propionsäure–Wasser (8:3:5). KAGAWA *et al.*[4] gebrauchten Gemische von Benzol-*n*-Butanol–Eisessig (80:15:5) resp. 70:25:5), sowie eine Mischung von Tetrachlorkohlenstoff–Eisessig–Wasser (8:3:5, untere Phase), zu deren Unterphase noch 10 % resp. 20 % Äthylacetat zugefügt wurden.

Mit keinem dieser Laufmittel ist es möglich, sämtliche Gibberelline in einem einzigen Trennungsgang voneinander zu isolieren. Sie werden vielmehr in Gruppen von Gibberellinen mit nahe beieinanderliegenden R_F-Werten getrennt. Generell wandern die Gibberelline A_1,A_8,A_3 und A_2 sehr langsam, die Gibberelline A_4,A_5,A_6,A_7 bleiben in den mittleren R_F-Bereichen und A_9 wandert am nächsten zur Lösungsmittelfront, wenn die Gibberelline als freie Säuren chromatographiert werden. Die Gibberelline innerhalb dieser Gruppen können dann in einem weiteren Trennungsgang unter Verwendung eines anderen Laufmittels weiter getrennt werden. So entwickelten KUTACEK *et al.*[5] ein Verfahren zur Trennung der Gibberelline A_1 und A_3 auf Al_2O_3-Platten mit dem Laufmittel Benzol–Eisessig (10:3).

Alle diese Trennungen wurden mit Reinsubstanzen ausgeführt, die nach erfolgter Trennung auf der Dünnschichtplatte mit chemischen Methoden sichtbar gemacht werden können, wie durch Besprühen mit Kaliumpermanganat-Lösung[1], Schwefelsäure[1,3,4] oder Antimontrichlorid[4]. (Chemische Nachweismethoden siehe Zit.[6]). Selbst

* Mit Unterstützung der Deutschen Forschungsgemeinschaft. Für die freundliche Überlassung der Gibberelline sei an dieser Stelle Dr. M. RADLEY von den Imperial Chemical Industries, Akers, auf das Herzlichste gedankt.

Gibberelline, die mit den zuvor genannten Laufmitteln nur geringe R_F-Unterschiede zeigen, können bei chemischem Nachweis auf der Dünnschichtplatte noch als getrennt wahrgenommen werden.

Isoliert man jedoch Gibberelline aus Pflanzenmaterial und versucht die Gibberelline in den Extrakten — selbstverständlich nach entsprechender Vorreinigung — auf Dünnschichtplatten aufzutrennen, so ergeben sich erhebliche Komplikationen. Nun können die Gibberelline nicht mehr durch Verwendung der erwähnten Sprühreagenzien auf den Trennplatten nachgewiesen werden, da diese Reagenzien unspezifisch sind und gleiche Reaktionen auch mit anderen, in den Extrakten enthaltenen Verbindungen geben. Der Nachweis muss in diesem Falle durch biologische Teste erfolgen, d.h. die entsprechenden Zonen müssen von der Platte entfernt und rückextrahiert werden. Durch die hierbei notwendigen Manipulationen bedingt, können zwei Gibberelline nur dann sicher als getrennt wahrgenommen werden, wenn sie auf der Trennschicht wenigstens 2 cm auseinanderliegen. Ausserdem muss man berücksichtigen, dass die Platten unterschiedlich beschichtet sind und so die R_F-Werte nur in gewissen Grössenordnungen, nicht aber genau reproduziert werden können. Eine einmal aufgestellte Norm mit Reingibberellinen gilt also nicht immer. Hinzu kommen noch die verschiedenen Verunreinigungen der Pflanzenextrakte, die die Trennung auf der Dünnschichtplatte erheblich beeinflussen. Auch Reinsubstanzen am Rande der Dünnschichtplatte mit zu chromatographieren, um so die Lage eines bestimmten Gibberellins in einem aufgetrennten Pflanzenextrakt zu markieren, kann aus diesen Gründen zu Irrtümern führen. All dies lässt die Reproduzierbarkeit der Trennungen und die Charakterisierung eines Stoffes mit Hilfe der Dünnschichtchromatographie auf Grund der R_F-Werte sehr unsicher werden.

Diese Fehlerquellen lassen sich weitgehend ausschalten, wenn man den Extrakten, aus denen Gibberelline getrennt werden sollen, Farbstoffe beimischt, die in einem gegebenen Entwicklungssystem gleiche oder ähnliche R_F-Werte besitzen wie die betreffenden Gibberelline. Diese Farbstoffe unterliegen bei der Trennung auf der Dünnschichtplatte den gleichen R_F-Verschiebungen und kennzeichnen so sicher die einzelnen R_F-Zonen der Gibberelline. Das nachfolgend beschriebene Verfahren ist auf der Verwendung solcher Markierungsfarbstoffe aufgebaut. Hierbei werden besonders die Gibberelline berücksichtigt, die bisher in höheren Pflanzen gefunden wurden[7]. Als Farbstoffe dienen Frangulin*, Fluoreszein**, Eosin*** sowie Methylrot§. Als Laufmittel wird eine Mischung aus Chloroform–Äthylacetat–Eisessig (90:10:5) verwendet[1]. Die Dünnschichtplatten (20 × 20 cm) werden in der üblichen Weise mit Kieselgel G nach STAHL ausgestrichen[8]. Die Trennstrecke von 20 cm reicht allerdings nicht für eine befriedigende Trennung der Gibberelline aus. Es muss daher im Durchlaufverfahren gearbeitet werden. Hierfür hat sich die Trennkammer nach BRENNER UND NIEDERWIESER[9] besonders bewährt§§. Die Trennkammer muss während der Entwicklung der Chromatogramme unter Raumtemperatur gekühlt werden, weil sonst die Trennung durch Abtropfen von Kondensaten von der Deckplatte, gestört werden kann. Durch diese Kühlung wird gleichzeitig eine Temperatur-

---

* Frangulin, Karl Roth, Karlsruhe.

** Fluoreszeinum Erg.B.6, E. Merck, Darmstadt.

*** Eosin bläulich, Mercks Präparate für Mikroskopie und Bakteriologie.

§ Methylrot, Desaga, Heidelberg.

§§ BN-Kammer, Desaga, Heidelberg.

konstanz erzielt, die der Reproduzierbarkeit der Chromatogramme sehr förderlich ist. Bei der Entwicklung werden die Gibberelline zunächst in 3 Gruppen getrennt, und zwar in die Gruppe A_8,A_1,A_3, in die Gruppe A_4,A_5,A_6,A_7 und in Gibberellin A_9. Gibt man in den Startpunkt der aufgetragenen Gibberelline die oben genannten Farbstoffe, so markiert Frangulin die Gibberelline A_8,A_1 und A_3, Fluoreszein A_4,A_5,A_6 und A_7 und schliesslich Eosin A_9. Eosin dient gleichzeitig als Front. Die Chromatogramme werden so lange entwickelt, bis das Eosin 10 cm weit vom Startpunkt entfernt ist. Die Entwicklungszeit hierfür beträgt etwa 2 Stunden. Damit ist zunächst eine Gruppentrennung erreicht, welche die bisher in höheren Pflanzen gefundenen Gibberelline in zwei Gruppen teilt. Zu einer Gruppe gehören die Gibberelline A_8 und A_1, zur anderen die Gibberelline A_5 und A_6 (Fig. 1).

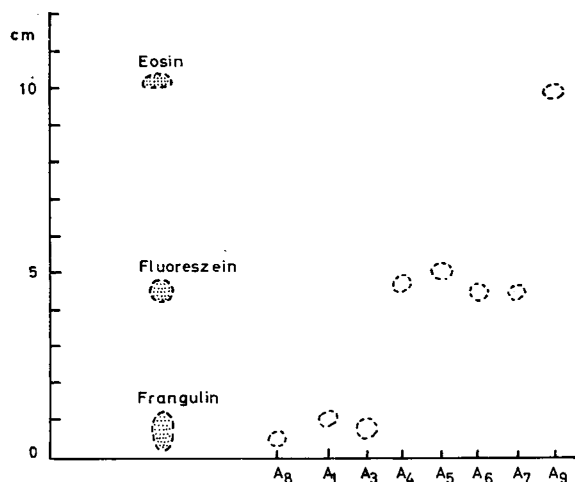


Fig. 1. Farbstoffmarkierung der Gruppentrennung der Gibberelline mit dem Laufmittel Chloroform–Äthylacetat–Eisessig (90:10:5) auf Kieselgel-G Platten. Der Farbstoff Eosin markiert das Gibberellin A_9, Fluoreszein die Gibberelline A_4,A_5,A_6 und A_7, Frangulin die Gibberelline A_8,A_1 und A_3.

In vielen Fällen ist es nicht möglich, diese erste Gruppentrennung bereits auf einer Dünnschichtplatte auszuführen, da die Extrakte noch zu viele Verunreinigungen enthalten, die eine Auftrennung nicht möglich machen. Dies gilt vor allem dann, wenn Blatt- und Stengelmaterial aufgearbeitet werden muss. In solchen Fällen kann die eben beschriebene Gruppentrennung mit Hilfe eines säulenchromatischen Trennverfahrens ausgeführt werden, das den Verhältnissen auf der Dünnschichtplatte entspricht[10].

Als Säule dient ein nahtlos gezogener Dialysierschlauch aus Zellophan im Durchmesser von 2.8 cm, der mit den Adsorbentien Kieselgel G und Kieselgur nach STAHL im Wechsel gefüllt wird. Zur Markierung der Gibberellin-Zonen werden die oben beschriebenen Farbstoffe dem aufzutrennenden Pflanzenextrakt beigefügt. Nach der Auftrennung wird die Säule in die entsprechenden Zonen zerschnitten. Zur Bereitung der Zellophansäule nimmt man ein Schlauchstück in der Länge von 20–25 cm und taucht es für einige Minuten in Wasser, um die Falten zu beseitigen. Dann wird das eine Ende auf ein Kernschliffstück aufgezogen, das andere mit einem Glasstopfen

verschlossen. Der Schlauch wird nun senkrecht gespannt, aufgeblasen und luftgetrocknet. Nach dem Trocknen erhält man eine völlig faltenlose Hülle, die leicht mit den Adsorbentien gefüllt werden kann. Zur eigentlichen Trennung der Gibberelline dient eine Schicht von Kieselgel G - Merck. Dieser wird eine 5 cm hohe Kieselgurschicht nachgeschaltet, die zur Aufnahme der schnell wandernden Begleitstoffe, Chlorophyll u.dgl. dient und die nach der Entwicklung der Säule verworfen wird. Vorgeschaltet wird der Kieselgelzone eine 1 cm hohe Kieselgurschicht, auf die der Extrakt aufgebracht wird. Diese vorgeschaltete Kieselgurschicht dient dazu, die langsam wandernden Gibberelline A_8,A_1 und A_3 in kurzer Zeit genügend weit von der Startzone zu entfernen. Die Gibberelline werden nur schwach an Kieselgur adsorbiert und durch das Laufmittel schnell an die Kieselgelzone herangetragen, an der die eigentliche Auftrennung erfolgt. In der Startzone verbleiben die meisten der noch im Extrakt vorhandenen Verunreinigungen, die die biologischen Teste stören.

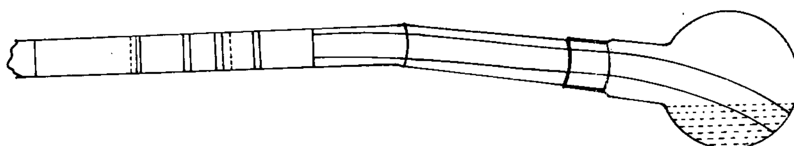


Fig. 2. Anordnung zur säulenchromatographischen Trennung der Gibberelline. Das Laufmittel befindet sich in einem Schliffkolben. Über ein Schliffzwischenstück, auf das der Dialysierschlauch aufgespannt ist, wird die Verbindung mit dem Vorratsgefäss mittels eines Dochtes hergestellt. Die Säule liegt während der Entwicklung horizontal.

Der Extrakt, der auf die Säule aufgebracht werden soll, wird nach entsprechender Vorreinigung stark eingeengt, die Markierungsfarbstoffe, Frangulin, Fluoreszein und Eosin, sowie 0.8–1 g Kieselgel G beigefügt und weiter zur Trockne eingeengt. Dieses Gemisch wird zu einem gleichmässigen Pulver verrieben, das dann in die Säule eingebracht wird. Zum Schutze der Oberfläche der Startzone wird diese noch mit weiteren 2–3 cm Kieselgur überdeckt. Die so präparierte Säule wird nun mit einem Schliffkolben verbunden, in dem sich 100 ml des Entwicklungsgemisches Chloroform–Äthylacetat–Eisessig (90:10:5) befinden. Dieses wird mit Hilfe eines Dochtes (Fig. 2) an die Kieselgurschicht herangeführt. Der Verlauf der Trennung kann jederzeit leicht mit Hilfe der beigefügten Farbstoffe im Tages- und U.V.-Licht verfolgt und kontrolliert werden (Fig. 3). Entwickelt wird so lange, bis das Eosin das Ende der Kieselgelschicht erreicht hat. Hierzu werden etwa 2–2$^1/_2$ Stunden

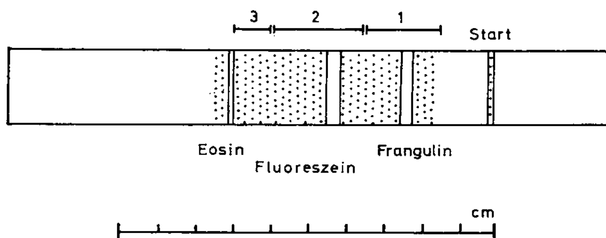


Fig. 3. Schema der Auftrennung der Gibberelline an der Säule und der Markierung durch die Farbstoffe Eosin, Fluoreszein, Frangulin. Die Trennung und Markierung entspricht völlig den Verhältnissen auf der Dünnschichtplatte.

benötigt. Nach der Auftrennung wird die Kieselgelschicht in drei Teile zerschnitten. Zone 1, markiert durch Frangulin, enthält die Gibberelline A_8,A_1 und A_3, Zone 2, markiert durch Fluoreszein, enthält die Gibberelline A_4,A_5,A_6 und A_7, während sich in der Zone 3, die durch Eosin markiert wird, nur Gibberellin A_9 findet. Aus diesen einzelnen Zonen werden die Gibberelline mit Äthylacetat wieder eluiert. Diese Eluate können in biologischen Testen auf Gibberellin-Aktivitäten geprüft werden.

Die weitere Auftrennung der Gibberelline innerhalb der Gruppen kann nun auf Dünnschichtplatten erfolgen. Dies sei im Folgenden für die Gibberelline A_1 und A_8 sowie A_5 und A_6 beschrieben, für die Gibberelline also, die bisher aus höheren Pflanzen isoliert wurden.

Von diesen finden sich die Gibberelline A_1 und A_8 in dem Eluat der Zone 1. Diese beiden Gibberelline können auf Kieselgel G-Platten mit Chloroform–Äthylacetat–Eisessig (60:40:5)[1] aufgetrennt werden. Die Trennung erfolgt im Durchlaufverfahren in der oben erwähnten Kammer von BRENNER UND NIEDERWIESER. Als Bezugsfront dient wiederum Eosin. Um gute Trennungen zu erzielen, sind allerdings lange Entwicklungszeiten von 3–4 Stunden erforderlich. Die Lage von Gibberellin A_1 auf dem Chromatogramm wird durch Frangulin markiert. Bezogen auf Frangulin hat Gibberellin A_8 einen R_{St}-Wert von 0.41 (Fig. 4). Gibberellin A_3, das

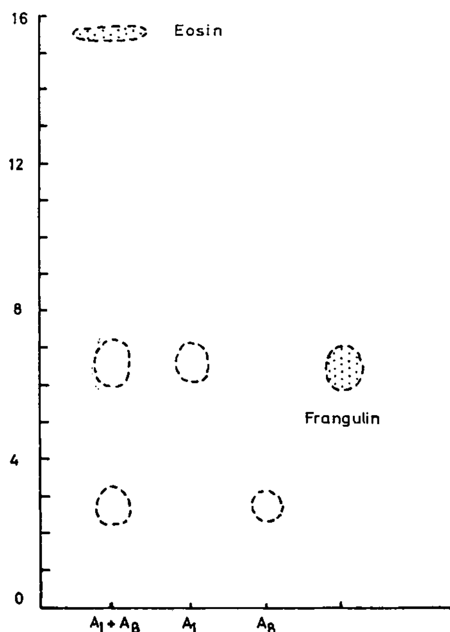


Fig. 4. Trennung der Gibberelline A_1 und A_8 auf Kieselgel-G Platten. Als Bezugs-Front dient Eosin. Gibberellin A_1 wird durch den Farbstoff Frangulin markiert.

sich noch in der gleichen Gruppe finden könnte, würde mit Gibberellin A_1 wandern und wäre hiervon nicht zu unterscheiden. Die übrigen Gibberelline, die hier allerdings bereits abgetrennt sein sollten, würden sich in der Front (Eosin) finden.

Die Auftrennung der Gibberelline A_5 und A_6 wird auf Kieselgur-Platten (Kiesel-

gur G nach STAHL, Merck) durchgeführt. Als Laufmittel dient Cyclohexan–Eisessig (80:5). Die Farbmarkierung erfolgt durch Methylrot. Auch bei dieser Trennung wird im Durchlaufverfahren gearbeitet. Die Entwicklungszeit beträgt 4–5 Stunden. Methylrot wandert zwischen den Gibberellinen A_5 and A_6 (Fig. 5). Die Gibberelline

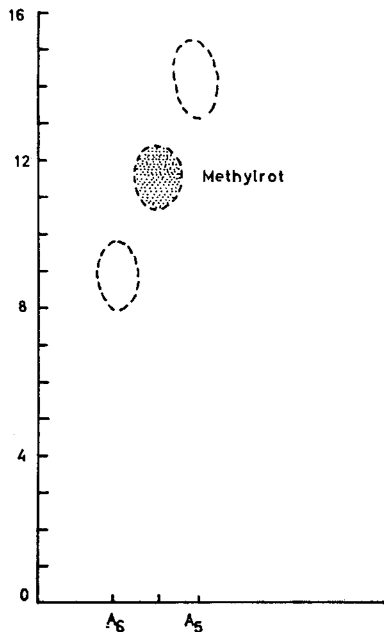


Fig. 5. Trennung der Gibberelline A_5 und A_6 auf Kieselgur-G Platten mit dem Laufmittel Cyclohexan–Eisessig (80:5). Zur Markierung dient Methylrot, das zwischen den beiden Gibberellinen wandert.

A_4 und A_7, die sich noch in der Zone 2 der Säule finden können, würden hier in der Front (Eosin) wandern. Ebenso Gibberellin A_9. Die Gibberelline A_8,A_1 und A_3, die hier allerdings nicht mehr auftreten sollten, würden am Startpunkt zurückbleiben.

Durch Beimengung von Farbstoffen zu den Pflanzenextrakten wird das Auffinden der einzelnen Gibberelline auf den Trennschichten wesentlich erleichtert. Auch lassen sich die eingangs geschilderten Fehlerquellen weitgehend ausschalten. Die in geringen Mengen beigefügten Farbstoffe, die zusammen mit den Gibberellinen aus den Trennschichten eluiert werden, beeinträchtigen die anschliessenden biologischen Teste zum Nachweis der Gibberelline nicht.

ZUSAMMENFASSUNG

Es wird ein dünnschichtchromatographisches und säulenchromatographisches Verfahren zur Trennung von Gibberellinen aus Pflanzenextrakten beschrieben. Den Pflanzenextrakten werden vor der Trennung Farbstoffe beigefügt. Diese Farbstoffe geben dann eine sichtbare Kontrolle des Trennverlaufes und erleichtern das Auffinden der Gibberelline auf den Dünnschichtplatten und Säulen. In den beschriebenen Lauf-

mitteln dient Frangulin zur Markierung der Gibberelline A_1,A_3,A_8, Fluoreszein zur Markierung der Gibberelline A_4,A_5,A_6,A_7 und Eosin markiert Gibberellin A_9. Diese erste Gruppentrennung kann auch an Kieselgel-Säulen ausgeführt werden. Die Trennung der Gibberelline A_1 von A_8 resp. A_5 von A_6 erfolgt in weiteren Trennungsgängen, wobei Frangulin zur Markierung von Gibberellin A_1 dient, während Methylrot zwischen den Gibberellinen A_5 und A_6 wandert.

SUMMARY

A method based on thin-layer and column chromatography is described for the separation of gibberellins in plant extracts. Colored compounds are added to the plant extracts which have the same R_F values as certain gibberellins. These colored compounds provide a visible control of the separation and mark the positions of the gibberellins on the thin-layer plates and the columns. Using the solvent mixtures described, frangulin marks gibberellins A_1,A_3,A_8, fluorescein marks gibberellins A_4,A_5,A_6,A_7 and eosin marks gibberellin A_9. This initial group separation can be carried out on thin-layer plates as well as on silica gel columns. The separation of gibberellins A_1 from A_8 and A_5 from A_6 is achieved by further separation steps on thin layers where frangulin serves as marker for gibberellin A_1, while methyl red migrates between A_5 and A_6.

LITERATUR

1 G. Sembdner, R. Gross und K. Schreiber, *Experientia*, 18 (1962) 584.
2 C. A. West and B. O. Phinney, *J. Am. Chem. Soc.*, 81 (1959) 2424.
3 I. MacMillan and P. I. Suter, *Nature*, 197 (1963) 790.
4 T. Kagawa, T. Fukinbara and Y. Sumiki, *Agr. Biol. Chem. (Tokyo)*, 27 (1963) 598.
5 M. Kutacek, J. Rosmus and Z. Deyl, *Biol. Plant. Acad. Sci. Bohemoslov.*, 4 (1962) 226.
6 G. Kallistratos, D. Padval und A. Pfau, in R. Knapp (Herausgeber), *Eigenschaften und Wirkungen der Gibberelline*, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1962, S. 20–24.
7 I. MacMillan, I. C. Seaton and P. I. Suter, *Advan. Chem. Ser.*, 28 (1961) 18.
8 E. Stahl, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1962.
9 M. Brenner und A. Niederwieser, *Experientia*, 17 (1961) 237.
10 H. Dahn und H. Fuchs, *Helv. Chim. Acta*, 45 (1962) 261.

SEPARATION OF FATTY ACETYLENIC, ETHYLENIC AND SATURATED COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY

M. W. ROOMI, M. R. SUBBARAM AND K. T. ACHAYA
Regional Research Laboratory, Hyderabad (India)

(Received February 10th, 1964)

Acetylenic compounds are being increasingly used as intermediates in fatty acid synthesis. Methods for their identification in the presence of corresponding ethylenic and saturated materials are scarce. BALLANCE AND CROMBIE[1] used reversed-phase paper chromatography, which takes about 20 hours, for their separation. In this paper, resolutions by thin-layer chromatography (TLC) of a series of purified fatty acids, esters and alcohols, comprising acetylenic, ethylenic and saturated compounds have been studied both by direct and reversed-phase TLC. Useful systems for specific requirements can be deduced from the results.

MATERIALS AND METHODS

All compounds were of high purity. The ethylenic acids, erucic, oleic, petroselinic and undecenoic, were prepared from mustard, olive, parsley seed and castor oils respectively. Bromination and dehydrobromination[2] gave the corresponding acetylenic acids, and hydrogenation in ethanol with a Pd/C catalyst gave the corresponding saturated acids; these derived products were thereafter purified by repeated crystallisation. The acids were converted using methanol–sulphuric acid to methyl esters, and reduced with lithium aluminium hydride in tetrahydrofuran[3] to the alcohols, unconverted acids being washed away with alkali.

The direct TLC procedure in use in this laboratory, employing Desaga equipment, plates of silica gel G, and ether–light petroleum for development, has been described previously[4]. For reversed-phase TLC, the dried, coated plate was uniformly impregnated with silicone oil (Dow Corning silicone fluid, 200) by allowing a 5 % solution in ether to ascend the plate in a developing chamber (S. VENKOB RAO, unpublished). This procedure has been found superior to the immersion technique[5], which requires a large volume of solution and often results in loosening of silica gel from the plate. With acids and esters, only one solvent system was used for reversed-phase TLC, *viz.*, 70 % acetonitrile, 10 % acetic acid and 20 % water (v/v). With alcohols, in addition to this solvent system, three others were also used, *viz.*, 70, 80 and 90 % aqueous acetic acids. These acetic acid systems were also tried with acids and esters, but since only the acetylenic and ethylenic products were resolved, results are reported for the acetonitrile system only.

TABLE I

SEPARATION OF ACIDS, METHYL ESTERS AND ALCOHOLS BY DIRECT AND REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

(All values as $R_F \times 100$)

Compound			*Acids*		*Methyl esters*		*Alcohols*				
								Reversed-phase			
Chain length	*Place of unsaturation*	*Common name of acid*	*Direct, 5% ether–pet. ether*	*Reversed-phase, acetonitrile–acetic acid–water (70:10:20)*	*Direct, 2% ether–pet. ether*	*Reversed-phase, acetonitrile–acetic acid–water (70:10:20)*	*Direct, 20% ether–pet. ether*	*Acetonitrile–acetic acid–water (70:10:20)*	*Acetic acid–water (70:30)*	*Acetic acid–water (80:20)*	*Acetic acid–water (90:10)*
					Acetylenic						
22	13,14	Behenolic	43	40	60	14	64	21	7	15	28
18	9,10	Stearolic	34	64	58	39	61	38	18	26	43
18	6,7	Tariric	34	62	58	40	61	38	18	26	43
11	10,11	Undecynoic	19	87	39	81	39	71	44	55	61
					Ethylenic						
22	13,14	Erucic	52	20	74	7	58	15	3	9	18
18	9,10	Oleic	41	50	73	29	54	28	12	18	34
18	6,7	Petroselinic	41	52	73	30	54	28	12	18	34
11	10,11	Undecenoic	26	75	57	70	34	55	34	44	51
18	9,10; 12,13	Linoleic	—	62	—	40	54	38	18	26	43
18	9,10; 12,13; 15,16	Linolenic	—	71	—	50	54	48	23	35	53
					Saturated						
22	nil	Behenic	52	3	74	0	58	0	0	0	4
18	nil	Stearic	45	40	71	14	54	21	7	15	28
11	nil	Undecanoic	28	66	57	60	34	38	26	32	38
20	nil	Arachidic	52	20	71	7	—	—	—	—	—
16	nil	Palmitic	44	50	73	29	—	28	12	18	34
14	nil	Myristic	44	64	73	39	—	38	18	26	43
9	nil	Pelargonic	28	75	59	70	—	—	—	—	—
12	nil	Lauric	—	71	—	50	—	48	23	35	53

TABLE II

DEGREES OF SEPARATION OBTAINED BY THIN-LAYER CHROMATOGRAPHY AND METHODS OF CHOICE

Separation of compounds		*Item No.*	*Acids*		*Esters*		*Alcohols*		*Recommended method*
			Direct	*Reserved-phase*	*Direct*	*Reversed-phase*	*Direct*	*Reversed-phase*[a]	*Compound and system*[b]
Same chain length									
Acetylenic from ethylenic		1	Poor	Good	Excellent	Fair	Poor	Fair	Esters, D, 2% ether
Ethylenic from saturated		2	Impossible	Good	Impossible	Good	Impossible	Good	Esters, RP, 70% acetonitrile
Acetylenic from saturated		3	Poor	Excellent	Fair	Excellent	Poor	Good	Esters, RP, 70% acetonitrile
Similar material of various chain lengths									
Acetylenics	22 from 18	4	Fair	Good	Poor	Good	Poor	Fair	Esters, RP, 70% acetonitrile
	18 from 11	5	Good	Good	Poor	Excellent	Very Good	Excellent	Esters, RP, 70% acetonitrile
Ethylenics	22 from 18	6	Poor	Excellent	Poor	Good	Poor	Fair	Acids, RP, 70%, acetonitrile
	18 from 11	7	Fair	Excellent	Fair	Excellent	Very Good	Excellent	Esters, RP, 70% acetonitrile
Saturated	22 from 18	8	Fair	Excellent	Poor	Fair	Poor	Good	Acids, RP, 70% acetonitrile
	18 from 11	9	Fair	Excellent	Fair	Excellent	Very Good	Fair	Esters, RP, 70% acetonitrile
Certain difficult pairs									
Acetylenic from saturated of 4C less		10	Impossible	Impossible	Good	Impossible	Poor	Impossible	Esters, D, 2% ether
Ethylenic from saturated of 2C less		11	Impossible	Impossible	Impossible	Impossible	—	Impossible	None
Saturated from next homologue		12	Impossible	Fair	Impossible	Fair	Impossible	Fair	Esters or Acids, RP, 70% acetonitrile
One acetylenic from two ethylenics		13	—	Impossible	—	Impossible	Fair	Impossible	Alcohols D, 20% ether
Two ethylenics from three ethylenics		14	—	Fair		Fair	Impossible	Fair	Acids, esters or alcohols, RP, 70% acetonitrile
Common C_{18} natural mixture									
Stearic, oleic, linoleic, linolenic		15	Impossible	Fair	Impossible	Good	Impossible	Fair	Esters, RP, 70% acetonitrile
Unresolved pairs[c], item numbers			2,10,11,12,15	10,11,13	2,11,12,15	10,11,13	2,12,14,15	10,11,13	

[a] Best of several solvent systems tried.
[b] D = Direct, RP = reversed-phase; composition of systems only identified briefly, for details see Table I.
[c] Positional isomers, acetylenic or ethylenic, are also not resolved by any of the systems tried.

RESULTS AND DISCUSSION

In Table I the results of separation of the various acetylenic, ethylenic and saturated acids, methyl esters and alcohols examined are shown, in terms of $R_F \times 100$ values. Table II evaluates these separations under various classified categories, using an arbitrary descriptive code based on $R_F \times 100$ values as follows: 0–2 impossible; 2–5 poor; 5–10 fair; 10–20 good; and over 20 excellent. Methods of choice for these compounds are shown, while those for specific separations can be deduced.

In direct systems, separations as acids and as alcohols are comparable, but ester separations stand slightly apart. Thus in three instances, direct TLC is the preferred separation method: (a) for resolution of acetylenic from ethylenic esters of the same chain length, *e.g.* stearolate from oleate; here a good reversed-phase system using acids is also available; (b) for separation of an acetylenic from a saturated acid shorter in chain length by four carbon atoms; and (c) for separation of an acetylenic from a diethylenic alcohol, *e.g.* stearoyl from linoleoyl. In the latter two instances, all other direct and reversed-phase systems are inadequate.

Generally reversed-phase systems are preferable to direct systems for resolution of these compounds. Thus in Table II a larger number of pairs are unresolvable by direct TLC in each category. By reversed-phase TLC, acids, esters and alcohols all show similar separation patterns. In several instances, *e.g.* for separation of oleic from stearic or undecenoic, or of behenolic from stearolic and from undecynoic, or of various homologous saturated products, esters are more advantageous than acids or alcohols for direct TLC separation. Sometimes the difference between the use of esters and acids or alcohols is only marginal, and either can be employed.

In separating fatty alcohols by reversed-phase TLC, a simple 90 % acetic acid system gives separations similar to those with a 70 % acetonitrile, 10 % acetic acid and 20 % water system.

Separation of a mixture of common C_{18} fatty acids, stearic, oleic, linoleic and linolenic, can best be effected by reversed-phase TLC of the esters using 70 % acetonitrile, 10 % acetic acid and 20 % water. Palmitic is critical with oleic and myristic with linoleic. Mixtures which include all these acids are best determined as esters by resolution (a) as such, (b) after complete hydrogenation, and (c) after conversion of unsaturated acids into bromo- or hydroxy derivatives, as recently suggested[6].

The following example illustrates the usefulness of these TLC procedures. Quantitative hydrogenation of 9,10-stearolic and 13,14-behenolic acids with LINDLAR catalyst[7] till two atoms of hydrogen had been taken up gave the corresponding ethylenic acids. Reversed-phase TLC and column chromatography of the reduction products showed the presence of only ethylenic acids. In addition, the specificity of LINDLAR catalyst was shown by the *cis* configuration of the ethylenic acid, inferred from the absence of a peak at 10.36 μ in the infrared spectra of the corresponding esters.

SUMMARY

Direct or reversed-phase thin-layer chromatography was used to separate acetylenic, ethylenic and saturated acids, methyl esters and alcohols of 22, 18 and 11 carbon chain lengths. Products corresponding to pelargonic, myristic, palmitic, arachidic,

linoleic and linolenic acids were also included. Systems are described which will resolve: (a) compounds of the same chain length but of different types of unsaturation; (b) compounds of different chain length but carrying the same type of unsaturation; and (c) certain difficult pairs, such as various saturated homologues. Palmitic and oleic acids were critical under all conditions, as were positional isomers. Thin-layer chromatography, column chromatography and infrared spectroscopy were used to confirm the specificity of LINDLAR catalyst for acetylenic to *cis*-ethylenic reduction.

REFERENCES

[1] P. E. BALLANCE AND W. M. CROMBIE, *Biochem. J.*, 69 (1958) 632.
[2] E. VON GERICHTEN AND A. KÖHLER, *Ber.*, 42 (1909) 1638.
[3] A. I. VOGEL, *A Text Book of Practical Organic Chemistry*, Longmans, Green & Co., London, 1959, p. 878.
[4] R. SUBBARAO, M. W. ROOMI, M. R. SUBBARAM AND K. T. ACHAYA, *J. Chromatog.*, 9 (1962) 295.
[5] D. C. MALINS AND H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 37 (1960) 576.
[6] D. SGOUTAS AND F. A. KUMMEROW, *J. Am. Oil Chemists' Soc.*, 40 (1963) 138.
[7] H. LINDLAR, *Helv. Chim. Acta*, 35 (1952) 446.

ION-EXCHANGE CHROMATOGRAPHY OF NUCLEOTIDES ON POLY(ETHYLENEIMINE)-CELLULOSE THIN LAYERS*,**

K. RANDERATH AND E. RANDERATH

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

(Received January 2nd, 1964)

Although it has clearly been shown by COHN and his collaborators[1,2] (for reviews see refs. 3 and 4) over ten years ago that for preparative separations of nucleic acid derivatives ion-exchange column chromatography on polystyrene resins is the best method, no effective analytical ion-exchange technique was known until recently by which traces of nucleotides (0.05–1 μg) can be separated and identified. Partition paper chromatography (for reviews see refs. 5 and 6) and paper electrophoresis (for reviews see refs. 6 and 7) are also not applicable in case of such small amounts. Because these methods give less sharp resolutions than ion-exchange column chromatography, they cannot be used to analyze very complex nucleotide mixtures in extracts from bacterial, plant and animal cells.

As we could demonstrate[8] (for a review see ref. 9), excellent and rapid separations of very small amounts of nucleic acid derivatives are obtained on ion-exchange thin layers. Chromatography on commercial cellulose ion-exchange papers gives more diffuse substance spots and hence poorer resolutions[10].

During the past three years special finely powdered cellulose cation- and anion-exchange materials have been developed which can be used in thin-layer chromatography. The following cellulose ion-exchangers for thin-layer chromatography

* Supported by grants-in-aid from the U.S. Atomic Energy Commission, the U.S. Public Health Service, the National Science Foundation, and the Burroughs Wellcome Fund. This is Publication No. 1158 of the Cancer Commission of Harvard University.

** The following abbreviations will be used: PEI-cellulose = a cellulose anion-exchange material obtained by impregnating chromatography cellulose with poly(ethyleneimine); ECTEOLA-cellulose = a cellulose anion-exchange material obtained by treating sodium cellulose with epichlorohydrin and triethanolamine; DEAE-cellulose = diethylaminoethyl-cellulose; QA-cellulose = a cellulose anion-exchange material containing quaternary ammonium groups; P-cellulose = cellulose phosphate; SE-cellulose = sulfoethyl-cellulose; CM-cellulose = carboxymethyl-cellulose; PP-cellulose = cellulose cation-exchange material containing polyphosphate. AMP, GMP, IMP, CMP, UMP = adenosine-, guanosine-, inosine-, cytidine-, and uridine-5′-monophosphates; ADP, GDP, IDP, CDP, UDP = the corresponding diphosphates; ATP, GTP, ITP, CTP, UTP = the corresponding triphosphates. The prefix d- indicates that the compound is a deoxyribonucleotide. TMP = thymidine monophosphate; TTP = thymidine triphosphate. ADPG = adenosine diphosphate-glucose; GDPM = guanosine diphosphate-mannose; CDPG = cytidine diphosphate-glucose; UDPG = uridine diphosphate-glucose; UDPGA = uridine diphosphate-glucuronic acid; UDPAG = uridine diphosphate-N-acetyl-glucosamine.

are at present commercially available: ECTEOLA-cellulose[*,**,***], DEAE-cellulose[*,**,***,§], PEI-cellulose[*,**], and QA-cellulose[§] (anion-exchangers); P-cellulose[*,**,***], SE-cellulose[*,§], and CM-cellulose[*,**,***,§] (cation-exchangers). PP-cellulose[**], a strongly acid cation-exchanger which is obtained by treating cellulose anion-exchange materials of low capacity with a polyphosphate solution[11], is suitable for separating nucleic acid bases, nucleosides, and other cationic compounds.

Ion-exchange thin-layer chromatography is more sensitive than partition paper chromatography, ion-exchange paper chromatography, paper electrophoresis, and partition thin-layer chromatography on unmodified cellulose layers. Amounts of 0.1 μg or less of nucleic acid bases, nucleosides, and nucleotides can be detected by examining the ion-exchange thin-layer chromatograms under a suitable short-wave ultraviolet lamp[§§] in a dark room.

Thin-layer chromatography of nucleotide coenzymes and of constituent nucleotides of nucleic acids can be carried out on layers of silica gel[8,12], unmodified cellulose[13–15], ECTEOLA-cellulose[8,9], DEAE-cellulose[9,16–18], and PEI-cellulose[9,10,19,20]. Also the separation on DEAE-Sephadex thin layers of a mixture of 5'-AMP, ADP and ATP has been reported[21]. For reasons which have been mentioned elsewhere[9], partition thin-layer chromatography of nucleotides on inorganic layers (silica gel, aluminum oxide) cannot be recommended. Layers of unmodified cellulose give sharper separations than chromatography paper[14]. They can be used for the resolution of not too complex mixtures. Many solvents developed for paper chromatography of nucleic acid derivatives are suitable in cellulose thin-layer chromatography[9,13–15].

As we showed earlier[11,22], cellulose ion-exchange materials can be prepared by treating unmodified or modified celluloses with high-molecular-weight basic or acid compounds. Anion-exchange materials which give especially sharp separations in thin-layer chromatography[9,10,19,20] are obtained[22] by impregnation with poly(ethyleneimine) (molecular weight 30,000–40,000) which is fixed substantively on cellulose fibers[23].

The separation of a number of mono-[9,20] and oligonucleotides[10,19] on PEI-cellulose thin-layers has been described previously. The present paper gives a detailed description of the behavior on PEI-cellulose layers of ribo- and deoxyribomononucleotides. The layers are prepared by suspending unmodified cellulose powder for thin-layer chromatography in a poly(ethyleneimine) hydrochloride solution and coating the suspension on glass plates in the usual manner[§§§].

MATERIALS AND METHODS

Materials

ADPG was a kind gift of Prof. L. F. LELOIR, Buenos Aires, to Prof. H. M. KALCKAR, Boston. D-Arabinosyl CMP, D-arabinosyl CDP, and D-arabinosyl CTP were kindly

---

* Serva-Entwicklungslabor, Heidelberg (Germany). Agent in the U.S.A.: Gallard-Schlesinger Company, Garden City, L.I., N.Y.

** Macherey und Nagel, Düren (Germany). Agent in the U.S.A.: C.A. Brinkmann, Great Neck, L.I., N.Y.

*** Brown Company, Berlin, N.H.; Schleicher and Schuell, Keene, N.H.

§ Whatman Thin-layer Chromedia (Reeve Angel, London, Great Britain. Agent in the U.S.A.: Scientifica, Clifton, N.J.).

§§ Mineralight Models R 51 or UVS 12 (Ultraviolet Products, San Gabriel, Calif.).

§§§ Layers prepared with commercial PEI-cellulose powders give separations that are different from those described in this paper.

provided by Prof. S. S. COHEN, Philadelphia. The other nucleotides were commercial products obtained from Sigma Chemical Company, St. Louis, and from California Corporation for Biochemical Research, Los Angeles.

A 50 % (w/v) solution of poly(ethyleneimine) (free base) in water, manufactured by Badische Anilin- und Soda-Fabrik, Ludwigshafen, Germany, was obtained from the Chemirad Corporation, East Brunswick, N.J. All solvents were prepared from analytical reagent grade materials.

Preparation of plates

A dialyzed 1 % poly(ethyleneimine) hydrochloride solution is prepared from a 10 % poly(ethyleneimine) hydrochloride solution (pH about 6.0) as described earlier[9]. A suspension of unmodified cellulose powder for thin-layer chromatography[9] (30 g) in the dialyzed solution (200 ml) is homogenized in an electric mixer for about 30 sec. Approximately 0.5 mm thick layers are then prepared on degreased glass plates (10 × 20 or 20 × 20 cm) by means of the Desaga-Brinkmann applicator model S II or by means of the Camag-Thomas apparatus, see ref. 9. In case of narrow plates (5 × 20 or 10 × 20 cm) the Serva applicator[9] can be used.

In order to avoid edge effects the plates should be separated from each other immediately after coating. They are allowed to dry overnight on a horizontal support at room temperature. The resulting layers are mechanically very stable so that one can write on them with pencil or ball-point. They have a capacity of approximately 1.5 mequiv. N per g cellulose.

Layers of lower capacity ($<$ 1 mequiv. N per g cellulose) can be prepared without previous dialysis of the poly(ethyleneimine) solution in the following manner. 50 % poly(ethyleneimine) solution (10 g) is diluted with distilled water (700 ml), brought to pH 6 with concentrated hydrochloric acid, and finally made up with distilled water to 1 l. Cellulose powder MN 300 (30 g) is suspended in the poly(ethyleneimine) solution (200 ml). Subsequently the layers are prepared according to the procedure described above. These layers have a capacity of 0.7–0.8 mequiv. N per g cellulose.

In order to obtain a straight solvent front dividing lines are scratched through the layer at a distance of 4–5 mm from the edges, and parallel lines are scratched into the bottom parts of the plates as described earlier[9]. Each plate is given a preliminary ascending development with distilled water[9,20]. The plates are then allowed to dry at room temperature for at least 12 h. Drying at elevated temperature is not advisable.

If the plates are not used within a few days they should be stored in darkness in the cold (0–4°) where they can be kept for several months. Whereas poly(ethyleneimine) solutions are stable, suspensions of cellulose in poly(ethyleneimine) solutions and PEI-cellulose layers can be kept for only a limited period at room temperature.

Chromatography

The samples are applied on a starting line drawn with a soft pencil 3.0 cm from the lower edge of the plate. 0.002 M solutions of sodium or lithium salts of nucleotides in distilled water were used throughout this investigation. After spotting all plates are exposed to a current of cold air for about 3 min.

Ascending chromatography is carried out in closed tanks filled with solvent to a height of 0.7–1.0 cm. Chromatography in open vessels gives results which are only slightly different from those described in this paper.

For the determination of R_F values 1 μl of the stock solutions (2 mμmoles) was spotted on layers impregnated with a dialyzed 1 % poly(ethyleneimine) solution. All chromatograms were developed up to a dividing line previously scratched through the layer 10.0 cm above the starting line. The elution was carried out perpendicular to the coating direction. The development time was 40–65 min depending on the composition of the solvent. Subsequently the plates were dried in a stream of hot air. The compounds were located by examining the plates in incident short-wave ultraviolet light and marked on the plate with a pencil.

Recording the chromatograms by photography in incident ultraviolet light

In order to obtain permanent records the chromatograms can be photographed in incident ultraviolet light, *cf.* Fig. 3. This photograph was obtained in the following way. A Polaroid MP-3 Land Camera (Polaroid Corporation, Cambridge 38, Mass.) was erected vertically over the chromatogram at a distance of 40–50 cm. An ultraviolet filter (Kodak daylight filter for type F color films No. 85 C) was placed in front of the lens (Rodenstock-Ysaron 1:4.7, f = 127 mm). Two short-wave ultraviolet lamps (Mineralight Model No. R 51), mounted at a distance of about 20 cm from the plate, were used to give even illumination in an otherwise completely darkened room. The exposure was 3 sec (lens opening f: 8; PolaPan 200 type 52 film). The conditions will of course depend on the properties of the ultraviolet lamps used.

Removal of interfering salts

Although generally speaking ion-exchange chromatography is less sensitive than partition chromatography to the presence of salts in the samples to be analyzed, a large excess of undesirable anions does interfere seriously with the separations on PEI-cellulose layers (see Figs. 5 and 6). Interfering salts are best removed by adsorption and subsequent elution of the nucleotides from activated charcoal (see *e.g.* refs. 24 and 25). A drawback of this technique is that recovery of nucleotides, especially in case of compounds containing guanine[25], is not quantitative. A further possibility is as follows. The samples are spotted in the usual manner, and after drying in a stream of air the plates are laid in a flat dish for about 10 min with anhydrous reagent grade methanol (300–600 ml). Subsequently the layers are dried, and chromatography is carried out as described above. This treatment removes excess salts, whereas in general more than 90 % of the nucleotides remain on the points of origin (see Figs. 5 and 6).

RESULTS AND DISCUSSION

General behavior of mononucleotides on PEI-cellulose layers

In Figs. 1 and 2 the R_F values of various derivatives of adenine and uracil are plotted against the lithium chloride concentration of the solvent used for elution. R_F values higher than 0.8 are not given because compounds migrating close to the solvent front have a tendency to form elongated spots so that exact R_F values cannot be measured. As can be seen from Figs. 1 and 2, the migration rate of nucleotides depends on the electrolyte concentration of the solvent in a way characteristic for each compound.

In general, the slope of the curves of pyrophosphoric acid diesters (I) (*e.g.*, ADPG, TPN) is steeper than that of monoesters of phosphoric, pyrophosphoric, or

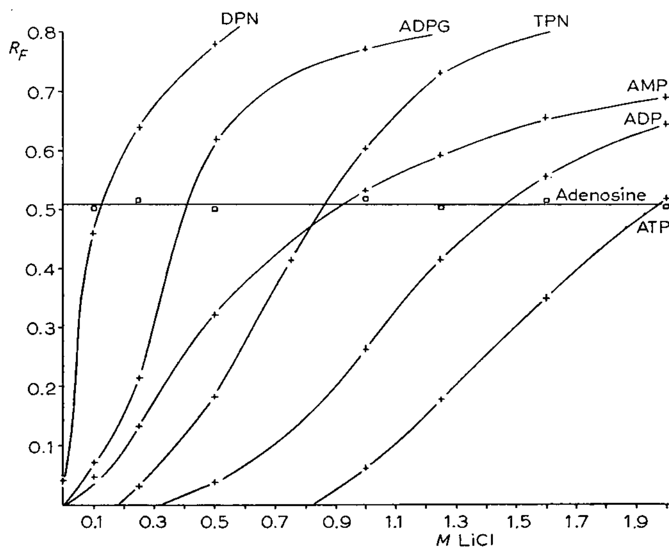


Fig. 1. Relationship between R_F values of adenine compounds and LiCl concentration of the solvent. Chromatography on 0.5 mm thick PEI-cellulose layers as described in the text.

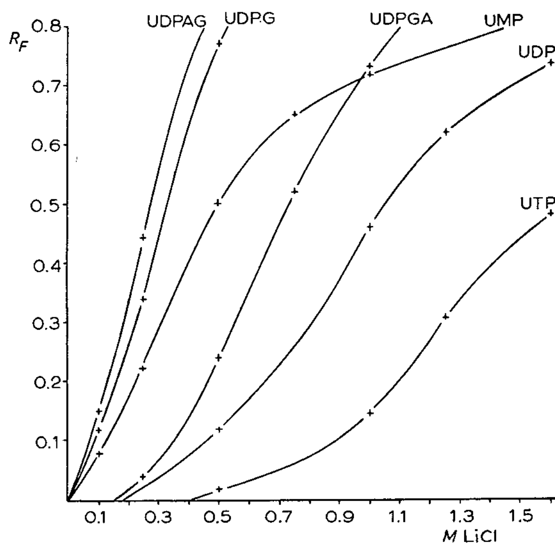
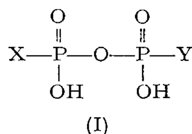


Fig. 2. Relationship between R_F values of uracil compounds and LiCl concentration of the solvent. 0.5 mm thick PEI-cellulose layers.

triphosphoric acid (*e.g.*, AMP, ADP, ATP). In some cases (see Figs. 1 and 2) a crossing of monoester and diester curves can be noticed.



(I)

The reproducibility of the R_F values is good if they are measured on plates obtained with a single poly(ethyleneimine) preparation. Slight variations (up to about $\pm$ 0.05) are observed if different poly(ethyleneimine) preparations are compared. The spot pattern, however, remains the same in each case.

In contrast to the nucleotides, R_F values of nucleic acid bases and nucleosides do not depend on the salt concentration of the solvent. This is correct for salt concentrations up to about 2 *M* NaCl or LiCl, see the data given for adenosine in Fig. 1. Uridine (not shown in Fig. 2) migrates with a R_F value of about 0.85 throughout the concentration range given in Fig. 2. At very high salt concentrations a decrease of the R_F values of some bases and nucleosides is observed. This is presumably a salting out phenomenon.

Figs. 1 and 2 show that it is possible to separate basic (or neutral) substances from nucleotides (or other acid substances) by a preliminary development with distilled water. Under these conditions all nucleotides, with the exception of DPN (Fig. 1), remain at the origin, whereas all water-soluble basic or neutral compounds migrate. The second development with electrolyte solutions for separating the nucleotides can be carried out in the direction of the first development or, preferably, perpendicular to it.

In Tables I–IV are listed the R_F data of a number of naturally occurring ribo- and deoxyribomononucleotides. It is apparent from these data that, under a given set of eluting conditions, the behavior of each nucleotide is influenced by the base, the sugar, and the phosphate residue of the molecule.

Elution under neutral conditions

At neutral pH (Table I and Figs. 1 and 2) the rate of migration decreases in the order: nucleotide sugars* (+ DPN) > monophosphates (+ TPN) > diphosphates > triphosphates containing the same base. Differences in net charge form the principal basis for this separation.

The order of elution predicted by net charge considerations is, however, subject to modifications. For example, the great rate of migration of the sugar nucleotide group cannot be explained by net charge considerations alone, since both sugar nucleotides (*e.g.*, UDPG) and nucleoside monophosphates (*e.g.*, UMP) carry a net charge of —2 at neutral pH. Furthermore, although the bases carry no net positive charge at neutral pH[3], the rate of migration in each group decreases in the order: uracil > cytosine > hypoxanthine > adenine > guanine derivatives.

Size, physical properties, and arrangement of nonionic substituents seem to in-

* Oxidation of a nucleotide sugar (*e.g.*, UDPG) to the corresponding uronic acid (UDPGA) results in a decreased migration rate, see also Fig. 3.

fluence the strength of the bonds established between compound and adsorbent. For example, one has to assume that purine compounds are adsorbed to the impregnated cellulose ion-exchanger more strongly than pyrimidine compounds of the same structural type, see also Fig. 3. Moreover, the net surface charge density on the molecule which is determined by the spatial arrangement of ionizable groups can be expected to be an important factor.

Elution under acid conditions

At pH 3.4 (Table II) the rate of migration decreases in the order: monophosphates (+ DPN) > nucleotide sugars (+ TPN) > diphosphates > triphosphates, and

TABLE I

R_F VALUES OF NUCLEOTIDES AT NEUTRAL pH

Compound	*Solvent: LiCl in water*		
	0.25 M	*1.0 M*	*1.6 M*
5'-AMP	0.11	0.52	0.65
5'-IMP	0.13	0.59	0.74
5'-GMP	0.06	0.40	0.51
5'-CMP	0.15	0.64	0.75
5'-UMP	0.20	0.74	0.80
ADP	0.00	0.26	0.54
IDP	0.00	0.30	0.63
GDP	0.00	0.17	0.45
CDP	0.00	0.33	0.64
UDP	0.00	0.41	0.71
ATP	0.00	0.06	0.34
ITP	0.00	0.09	0.39
GTP	0.00	0.05	0.25
CTP	0.00	0.11	0.41
UTP	0.00	0.14	0.49
d-AMP	0.11	0.52	—
d-GMP	0.06	0.41	—
d-CMP	0.18	0.65	—
TMP	0.24	0.74	—
d-ATP	0.00	—	0.35
d-GTP	0.00	—	0.26
d-CTP	0.00	—	0.43
TTP	0.00	—	0.52
DPN	0.64	0.80	> 0.80
DPNH	0.20	0.71	—
TPN	0.03	0.60	—
TPNH	0.00	0.34	—
ADPG	0.22	0.77	> 0.80
GDPM	0.12	0.72	> 0.80
CDPG	0.27	> 0.80	> 0.80
UDPG	0.34	> 0.80	> 0.80
UDPAG	0.44	> 0.80	> 0.80
UDPGA	0.04	0.73	—

— = not investigated.

TABLE II

R_F VALUES OF NUCLEOTIDES AT pH 3.4

Compound	Solvent: formic acid–sodium formate buffer pH 3.4			
	0.5 M	*1.0 M*	*2.0 M*	*4.0 M*
5'-AMP	0.68[a]	> 0.80	> 0.80	> 0.80
5'-IMP	0.40	0.60	0.73	> 0.80
5'-GMP	0.28	0.45	0.57	0.65[b]
5'-CMP	0.70[a]	> 0.80	—	—
5'-UMP	0.51	0.72	> 0.80	—
ADP	0.03	0.10	0.32	0.75[b]
IDP	0.00	0.04	0.14	0.49
GDP	0.00	0.02	0.09	0.34
CDP	0.08	0.20	0.45	> 0.80
UDP	0.02	0.07	0.24	0.60
ATP	0.00	0.00	0.04	0.24
ITP	0.00	0.00	0.02	0.11
GTP	0.00	0.00	0.00	0.07
CTP	0.00	0.02	0.05	0.29
UTP	0.00	0.00	0.02	0.17
DPN	0.68[a]	> 0.80	> 0.80	—
TPN	0.14	0.43	> 0.80	—
ADPG	0.30	0.57	> 0.80	—
GDPM	0.07	0.18	0.51	—
CDPG	0.45	0.67	> 0.80	—
UDPG	0.17	0.39[b]	0.77	—
UDPAG	0.32	0.55[b]	> 0.80	—
UDPGA	—	0.03	—	—

— = not investigated.
[a] Spot in second front.
[b] Elongated spot.

cytosine > adenine > uracil > hypoxanthine > guanine derivatives. At this pH the primary phosphoric acid groups are fully dissociated, and the secondary groups are totally undissociated. That monophosphates (*e.g.*, UMP) precede nucleotide sugars (*e.g.*, UDPG) under these conditions can be predicted from net charge considerations: because uracil carries no positive charge at pH 3.4, the net negative charges of UMP and UDPG are —1 and —2, respectively. However, the rather great differences between nucleotide sugars and nucleoside diphosphates (Table II) are not due to charge differences. The net charge of both UDPG and UDP is —2 at pH 3.4.

Since the pK_a values of hypoxanthine, uracil, guanine, adenine, and cytosine are different[3], the net charge on the individual nucleotides within each group is different. The order cytosine > adenine > guanine is predicted by net charge considerations. The relatively slow migration rates of hypoxanthine and guanine derivatives must, however, be explained by the greater attraction of the poly(ethyleneimine)-cellulose for these purines over the pyrimidines.

Good separations are obtained at pH 4.4 (R_F values not given). Acetic acid–sodium acetate buffers, with or without addition of lithium chloride, can be used as solvents. In this pH region uracil derivatives precede adenine derivatives of the same type.

At pH 2 (Table III) the rate of migration with regard to the bases is cytosine > adenine > guanine > uracil > hypoxanthine derivatives of the same type.

In general, smaller and sharper spots are obtained at acid pH values than under neutral or alkaline conditions. 1–3 *N* formic acid gives particularly sharp group separations. The addition of LiCl (Table III) increases the eluting power considerably.

TABLE III

R_F VALUES OF NUCLEOTIDES AT pH 2

Solvent 1 = 1.0 *N* HCOOH.
Solvent 2 = 2.0 *N* HCOOH–0.5 *M* LiCl (1:1).
Solvent 3 = 2.0 *N* HCOOH–2.0 *M* LiCl (1:1).

Compound	*Solvent*		
	1	*2*	*3*
5'-AMP	> 0.80	> 0.80	> 0.80
5'-IMP	0.19	0.53	0.78
5'-GMP	0.41	0.50[a]	0.72[a]
5'-CMP	> 0.80	> 0.80	> 0.80
5'-UMP	0.20	0.64	> 0.80
ADP	0.03	0.29	0.70
IDP	0.00	0.08	0.55
GDP	0.00	0.13	0.61
CDP	0.04	0.35	0.73
UDP	0.00	0.11	0.60
ATP	0.00	0.04	0.33
ITP	0.00	0.02	0.17
GTP	0.00	0.02	0.24
CTP	0.00	0.04	0.37
UTP	0.00	0.02	0.20
DPN	> 0.80	> 0.80	> 0.80
TPN	0.10	0.44[a]	> 0.80
ADPG	0.09	0.51[a]	> 0.80
GDPM	0.01	0.34	0.73
CDPG	0.13	0.60	> 0.80
UDPG	0.01	0.27[b]	> 0.80
UDPAG	0.02	0.38[b]	—
UDPGA	0.00	0.09	0.66

— = not investigated.
[a] Spot in second front.
[b] Elongated spot.

The formation of so-called second fronts is sometimes observed in the chromatog raphy with acid solvents. These second fronts correspond to sudden pH changes within the layer, as can be detected by spraying the plates with suitable indicator solutions.

Because they do not interfere seriously with the chromatography, equilibration of the layers with solvent prior to chromatography is seldom necessary. It must be mentioned, however, that anomalies of R_F values can be observed sometimes if nucleotides migrate with, or close to, a second front. An example is the R_F value of GMP determined after chromatography with HCOOH–LiCl mixtures, see Table III.

Separation of deoxyribo- and D-arabinosylnucleotides from ribonucleotides

As shown in Table IV, deoxyribonucleotides can be separated from their ribonucleotide-analogues on PEI-cellulose layers by chromatography with solutions of LiCl in aqueous boric acid[26]. Under the conditions indicated in Table IV some ribonucleotide spots appear slightly elongated. This might be due to a partial dissociation of the borate complexes formed by these compounds.

TABLE IV

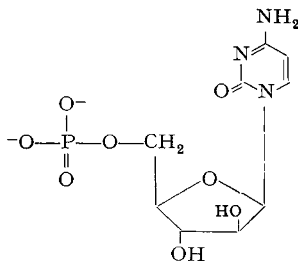
R_F VALUES OF NUCLEOTIDES IN THE PRESENCE OF BORIC ACID

Solvent 1 = 2% H_3BO_3–2 M LiCl (2:1).
Solvent 2 = 4% H_3BO_3–4 M LiCl (4:3).

Compound	Solvent	
	1	*2*
AMP	0.24	—
d-AMP	0.42	—
GMP	0.08	—
d-GMP	0.30	—
CMP	0.28	—
d-CMP	0.55	—
UMP	0.37	—
TMP	0.63	—
ADP	0.04	0.56
ATP	—	0.33
d-ATP	—	0.46
GTP	—	0.17
d-GTP	—	0.37
CTP	—	0.36
d-CTP	—	0.61
UTP	—	0.48
TTP	—	0.70

— = not investigated.

Also D-arabinosyl CMP (II), D-arabinosyl CDP, and D-arabinosyl CTP are separated from their ribo- and deoxyribo-analogues with solvents containing boric



(II)

acid. The rate of migration decreases in the order deoxyribo- > D-arabinosyl- > ribonucleotides of the same structural type.

Stepwise elution

As can be seen from Fig. 1, a mixture of DPN, TPN, ADPG, AMP, ADP, and ATP cannot be resolved with one and the same solvent, since the adsorption affinities of these compounds for PEI-cellulose are too different. In such a case the chromatogram is developed with a series of solvents of increasing electrolyte concentrations. In Fig. 3 examples are given showing separations of adenine and uracil nucleotide mixtures. The discontinuous, stepwise procedure used for elution comprised three transfers of the plate without intermediate drying from one tank to another tank containing a higher lithium chloride concentration.

When compared with constant concentration elution, development with increasing concentrations gives sharper and more circular substance spots.

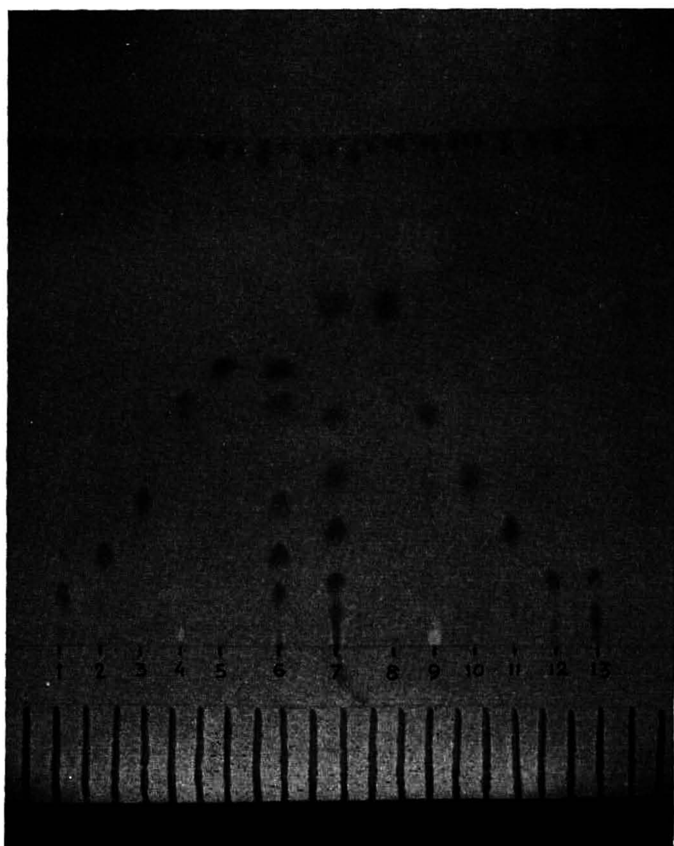


Fig. 3. Separations of adenine and uracil nucleotides on a 0.5 mm thick PEI-cellulose layer. The plate was developed (stepwise elution, see text) for 1 min with 0.1 *M* LiCl, for 5 min with 0.3 *M* LiCl, for 15 min with 0.7 *M* LiCl, and for 25 min with 1.5 *M* LiCl. Development distance: 9.3 cm in 46 min. Amounts applied: 5–10 mμ moles of each compound. 1 = UTP; 2 = UDP; 3 = UDPGA; 4 = 5'-UMP; 5 = UDPG; 6 = 1–5 together; 7 = 8–13 together; 8 = DPN; 9 = ADPG; 10 = 5'-AMP; 11 = TPN; 12 = ADP; 13 = ATP. Some impurities are visible, for example, ADPG contained a small amount of AMP, and ATP contained ADP and an unidentified compound (close to the start). Photographed by short-wave U.V. light. To increase the contrast, the plate was soaked in anhydrous methanolic 0.002 % fluorescein solution for 1$\frac{1}{2}$ min and dried before photography.

The choice of the solvent depends on the composition of the mixture to be analyzed: for example, if the mixture contains only nucleotide sugars and nucleoside monophosphates, LiCl molarities between 0.1 and 1.0 are suitable. On the other hand, if a mixture of nucleoside di- and triphosphates is to be analyzed, the concentration range would be between 0.6–0.8 M for the first solvent and 1.7–2.0 M for the last solvent. The number of "steps" sufficient in most cases is 2–5.

Influence of the poly(ethyleneimine) concentration

Fig. 4 shows that the chromatographic results depend on the poly(ethyleneimine) concentration of the solution used for preparing the plates. In general, lowering of the concentration results in larger, more elongated spots and in higher R_F values. Whereas a 0.5 % (w/v) poly(ethyleneimine) solution gives good and for many purposes sufficient separations, the resolution becomes very poor if a 0.1 % solution is used (Fig. 4).

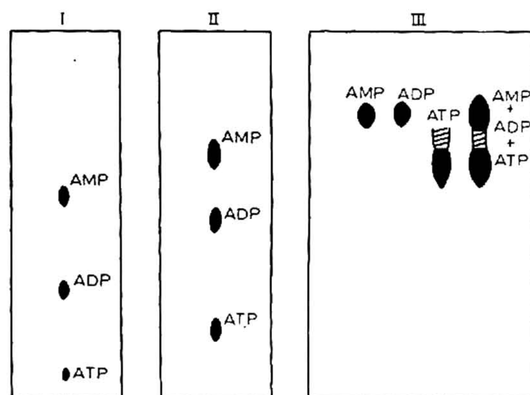


Fig. 4. Influence of poly(ethyleneimine) content (capacity). Chromatography with 1.0 M LiCl. The layers (0.5 mm thick) were prepared with dialyzed 1 % (I), 0.5 % (II) and 0.1 % (III) poly(ethyleneimine) hydrochloride solutions.

As mentioned in the section *Preparation of plates*, layers of a capacity $<$ 1 mequiv. N per g cellulose can be prepared without previous dialysis of the poly(ethyleneimine) solution. Good separations are obtained on these layers; spot size and R_F values are similar to those observed with a 0.5 % dialyzed solution (Fig. 4) if an undialyzed solution of the same concentration is used, see section *Preparation of plates*. However, the poly(ethyleneimine) concentration should not exceed 0.7 % (undialyzed) and 1.5 % (dialyzed), because otherwise it is difficult to remove low-molecular-weight impurities which are incorporated in the layer.

Interference by salts

An excess of salts or buffers in the samples to be analyzed can interfere seriously with the separations (Figs. 5 and 6). By the methanol treatment described above (see section *Removal of interfering salts*) excess electrolytes are removed. A separation of AMP, ADP, and ATP can be obtained even if the sample contains a very large excess of sodium chloride (Fig. 5b). As shown in Figs. 5 and 6, slightly higher R_F values and more elongated spots are observed on plates treated with methanol.

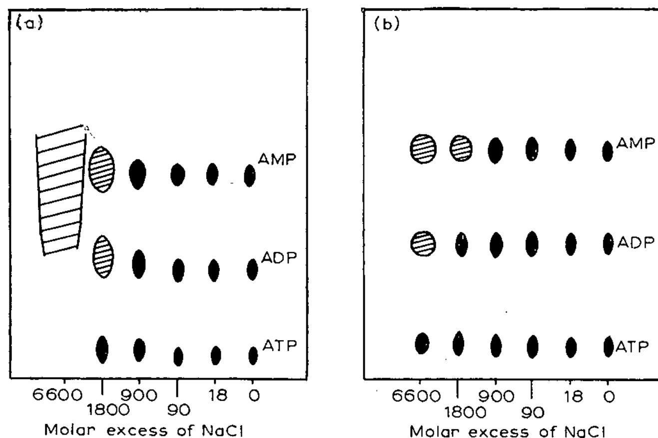


Fig. 5. Effect of interfering electrolytes. Chromatography with 1.0 M LiCl on 0.5 mm thick PEI-cellulose layers. a = without methanol treatment; b = with methanol treatment as described in the text.

CONCLUSIONS

In Table V the various methods for separating nucleotides are compared. When compared with paper chromatography[5] and paper electrophoresis[7] of nucleotides, the following advantages of ion-exchange thin-layer chromatography are evident:

1. Thin-layer chromatography is more sensitive than paper chromatography and paper electrophoresis[9]. Especially in biochemical analysis, where often only traces of the compounds being studied are available, the more than tenfold decrease in the scale of analysis is a particularly important advantage. The sensitivity is further increased if the compounds are labeled and the detection is carried out by autoradiography[27] or by scanning in a suitable instrument[9].

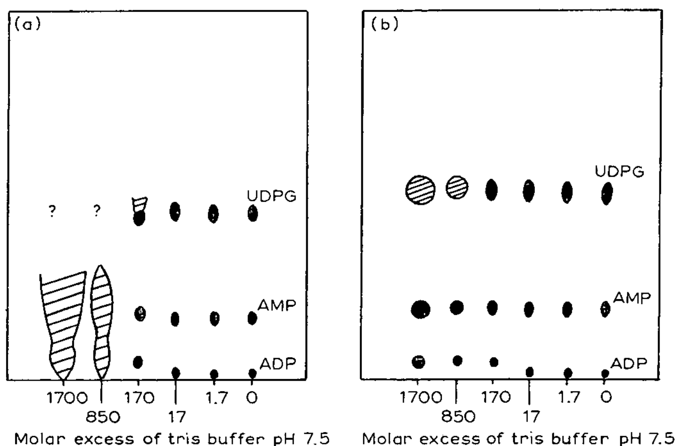


Fig. 6. Effect of interfering electrolytes. Solvent: 0.3 M LiCl. a = without methanol treatment; b = with methanol treatment as described in the text.

TABLE V

COMPARISON BETWEEN DIFFERENT METHODS FOR SEPARATION OF NUCLEOTIDES

Method	*Optimum amounts*	*Type*	*Time required*	*Resolution of complex mixtures*[a]
Paper chromatography	10–200 μg per spot	Partition (ion-exchange)	12 h–5 days	Moderate
Paper electrophoresis	100–500 μg per spot	Ionophoresis	Several h	Moderate
Column chromatography	50 μg–several hundred mg per column	Ion-exchange	Several h–10 days	Very good
Thin-layer chromatography	0.2–30 μg per spot	Ion-exchange (partition)	Several min–3 h	Very good

[a] See the following paper[28].

2. Complex mixtures of nucleotides that cannot be resolved at all by the present techniques of paper chromatography and paper electrophoresis can be separated by anion-exchange thin-layer chromatography[28].

3. The time required for separation is very short[9] (Table V).

4. Ion-exchange thin-layer chromatography can be carried out under milder conditions than paper chromatography and paper electrophoresis, sharp separations being obtained even at 0° (ref. 9). There is thus scarcely any danger of decomposition of sensitive nucleotides.

It should be kept in mind that the unequivocal identification of nucleotides is greatly facilitated by using anion-exchange thin-layer chromatography in conjunction with other methods which are based on different separation principles.

A comparison with anion-exchange column chromatography on polystyrene resins (see refs. 1–4, 25, 29) shows:

1. Both ion-exchange methods give nearly identical elution patterns at the same pH. The electrolyte concentrations required to obtain identical mobilities of individual compounds are, however, different.

2. The thin-layer chromatographic procedure results in an at least equal sharpness of resolution.

3. Thin-layer chromatography is less laborious than ion-exchange column chromatography and is therefore especially suitable for micropreparative separations (amounts of up to 2 mg per substance on a 20 × 20 cm plate). The nucleotides can be eluted with electrolyte solutions after removing the substance zones from the glass plates[9]. As in column chromatography, activated charcoal is used to reclaim the nucleotides from the eluting electrolytes.

4. In both methods a quantitative determination can be carried out by ultraviolet spectrophotometry[9]. A more detailed description of quantitative evaluation of the PEI-cellulose plates will be given in the near future.

The most important advantages of thin-layer chromatography on PEI-cellulose are excellent sharpness of separation, good reproducibility, high sensitivity, and great speed. Furthermore, the regular behavior of the nucleotides facilitates the identification of unknown compounds.

The high resolving power of the impregnated cellulose anion-exchange material is, in our opinion, to be attributed to its (a) high capacity, (b) great density of functional groups along the poly(ethyleneimine) chain, and (c) lack of cross linkage resulting in a high rate of the ion-exchange process.

SUMMARY

A great number of naturally occurring mononucleotides can be separated and identified by poly(ethyleneimine)-cellulose thin-layer chromatography. R_F data for 33 compounds are given, and the factors are discussed which influence the mobility under different elution conditions. The method is compared with other present techniques for separating nucleotides.

REFERENCES

[1] W. E. COHN, *Science*, 109 (1949) 377.
[2] W. E. COHN, *J. Am. Chem. Soc.*, 72 (1950) 1471.
[3] W. E. COHN in E. CHARGAFF AND J. N. DAVIDSON (Editors), *The Nucleic Acids*, Vol. 1, Academic Press, New York, 1955, p. 211.
[4] W. E. COHN in E. HEFTMANN (Editor), *Chromatography*, Reinhold, New York, 1961, p. 554.
[5] G. R. WYATT in E. CHARGAFF AND J. N. DAVIDSON (Editors), *The Nucleic Acids*, Vol. 1, Academic Press, New York, 1955, p. 243.
[6] D. O. JORDAN, *The Chemistry of Nucleic Acids*, Butterworths, Washington, 1960.
[7] J. D. SMITH in E. CHARGAFF AND J. N. DAVIDSON (Editors), *The Nucleic Acids*, Vol. 1, Academic Press, New York, 1955, p. 267.
[8] K. RANDERATH, *Angew. Chem.*, 73 (1961) 436.
[9] K. RANDERATH, *Thin-layer Chromatography*, Verlag Chemie, Weinheim, and Academic Press, New York, 1963.
[10] G. WEIMANN AND K. RANDERATH, *Experientia*, 19 (1963) 49.
[11] E. RANDERATH AND K. RANDERATH, *J. Chromatog.*, 10 (1963) 509.
[12] R. L. SCHEIG, R. ANNUNZIATA AND L. A. PESCH, *Anal. Biochem.*, 5 (1963) 291.
[13] K. RANDERATH, *J. Chromatog.*, 6 (1961) 365.
[14] K. RANDERATH, *Biochem. Biophys. Res. Commun.*, 6 (1961/1962) 452.
[15] L. JOSEFSSON, *Biochim. Biophys. Acta*, 72 (1963) 133.
[16] K. RANDERATH, *Angew. Chem.*, 74 (1962) 484; *Intern. Ed. Engl.*, 1 (1962) 435.
[17] R. G. COFFEY AND R. W. NEWBURGH, *J. Chromatog.*, 11 (1963) 376.
[18] T. A. DYER, *J. Chromatog.*, 11 (1963) 414.
[19] K. RANDERATH AND G. WEIMANN, *Biochim. Biophys. Acta*, 76 (1963) 129.
[20] K. RANDERATH, *Biochim. Biophys. Acta*, 61 (1962) 852.
[21] T. WIELAND, G. LÜBEN AND H. DETERMANN, *Experientia*, 18 (1962) 430.
[22] K. RANDERATH, *Angew. Chem.*, 74 (1962) 780; *Intern. Ed. Engl.*, 1 (1962) 553.
[23] H. WILFINGER, *Papier*, 2 (1948) 265.
[24] R. B. HURLBERT, in S. P. COLOWICK AND N. O. KAPLAN (Editors), *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 785.
[25] P. H. PLAISTED AND R. B. REGGIO, *Contrib. Boyce Thompson Inst.*, 22 (1963) 71.
[26] K. RANDERATH, *Biochim. Biophys. Acta*, 76 (1963) 622.
[27] K. RANDERATH AND E. RANDERATH, unpublished results.
[28] E. RANDERATH AND K. RANDERATH, *J. Chromatog.*, 16 (1964) 126.
[29] N. G. ANDERSON, J. G. GREEN, M. L. BARBER AND Sr. F. C. LADD, *Anal. Biochem.*, 6 (1963) 153.

RESOLUTION OF COMPLEX NUCLEOTIDE MIXTURES BY TWO-DIMENSIONAL ANION-EXCHANGE THIN-LAYER CHROMATOGRAPHY*,**

E. RANDERATH AND K. RANDERATH

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

(Received January 2nd, 1964)

No rapid analytical method has been described in the literature so far which permits the complete separation of complex nucleotide mixtures containing microgram and submicrogram amounts of individual compounds. Most systems described for two-dimensional paper chromatography of nucleotides require development times of 3 to 4 days. Moreover, the unequivocal detection of these, to some extent very similar, compounds in tissue extracts presents a difficult problem of analysis that cannot be completely solved by the present techniques of paper chromatography and paper electrophoresis. On the other hand, anion-exchange column chromatography is not suitable for the analysis of very small amounts of nucleotides. The complete separation of complex mixtures by this technique requires elution periods of 1 to 10 days.

We feel that a new sensitive and rapid chromatographic technique by which complex nucleotide mixtures can be resolved might be valuable in studies concerning composition and metabolism of nucleotides in bacterial, plant or animal cells. This paper describes such a technique. The separations are carried out by two-dimensional anion-exchange chromatography on PEI-cellulose thin layers. For a discussion of the general behavior of mononucleotides on PEI-cellulose layers see the preceding paper[1].

METHODS

Poly(ethyleneimine) and nucleotides, with the exception of ADPG, were obtained commercially (see the preceding paper[1]). PEI-cellulose layers (capacity about 1.5 mequiv. N per g cellulose) were prepared on 20 × 20 cm glass plates with the Desaga-Brinkmann apparatus***, as described in the preceding paper[1]. Layers prepared from commercial PEI-cellulose powders give separations that are different from those described in this paper.

* Supported by grants-in-aid from the U.S. Atomic Energy Commission, the U.S. Public Health Service, the National Science Foundation, and the Burroughs Wellcome Fund. This is Publication No. 1159 of the Cancer Commission of Harvard University.

** The abbreviations used in this paper are explained in the preceding paper[1].

*** It is also possible to prepare the layers without a spreader by pouring on the PEI-cellulose suspension and distributing it with a glass rod and by gentle tilting. Because such layers, though appearing homogeneous, give somewhat poorer and less reproducible separations, the commercial applicator was used throughout this investigation.

To remove impurities which could interfere with the chromatography in the second direction, each plate is given a preliminary development with 10% NaCl solution instead of with distilled water[1] in the following way. The plate is placed in a rectangular tank with 10% NaCl solution which is allowed to rise to a height of about 5 cm and is then developed without intermediate drying in a second tank with distilled water up to the upper edge. After drying in a current of cold air the plate is washed again with distilled water to the top edge. The plate is then allowed to dry at room temperature for 12–15 h. All plates are stored in darkness in the cold[1].

Chromatographic procedure

First dimension (perpendicular to the coating direction). After applying the nucleotide solution at the starting point S (Fig. 1) the chromatogram is developed in a closed rectangular jar for 2 min with 0.2 *M* LiCl, for 6 min with 1.0 *M* LiCl, and finally with 1.6 *M* LiCl up to a dividing line previously scratched through the layer 13.0 cm above the start (stepwise elution[1]; no intermediate drying). The plate is then dried in a current of warm air below 50°. The development time is about 75 min. It can vary slightly from batch to batch of the cellulose.

Examination of the chromatogram under a short-wave ultraviolet lamp reveals a partial resolution of the complex mixture (Fig. 1).

Second dimension. All parts of the layer which are not needed for the chromatography in the second dimension are scraped off with a sharp spatula. This includes a strip of 2.5–3 cm width below the finishing line of the first dimension.

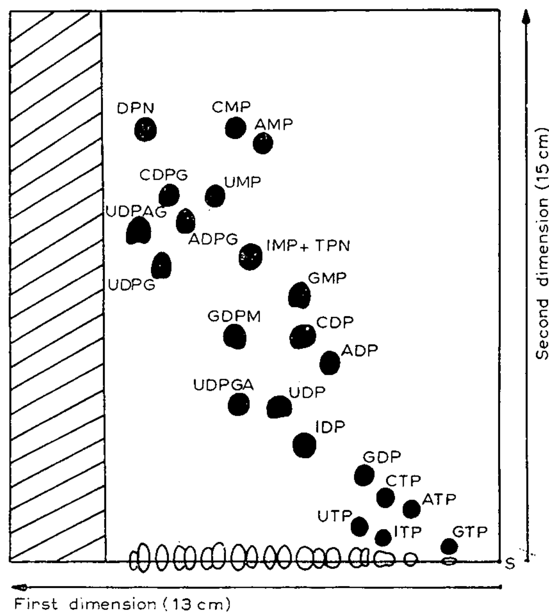


Fig. 1. Two-dimensional anion-exchange thin-layer chromatogram of mononucleotides. 0.5 mm thick PEI-cellulose layer. 0.1 ml of a solution containing 23 ribonucleotides (10–15 mμmoles each) was applied slowly in two 0.05 ml portions with intermediate drying from a micropipette to the layer at S. Elution and detection were carried out as described in the text. The hatched area was removed after the elution in the first dimension (see text).

In order to remove LiCl which would interfere with the elution in the second direction the plate is laid for about 15 min in a flat dish (25 × 25 cm) filled with 1 l anhydrous methanol. The dissolution of the salt is accelerated by occasional agitating.

After drying and scratching parallel lines into the bottom part[1] the chromatogram is developed in the second direction with formic acid–sodium formate buffers, pH 3.4[1], by a stepwise elution procedure. Solvents: 0.5 *M* buffer for 30 sec, 2.0 *M* buffer for 2 min, and 4.0 *M* buffer up to a finish line previously scratched through the layer 15.0 cm above the start of the second dimension. The development time is about 60 min. Finally the plate is dried in a current of hot air and examined under short-wave ultraviolet light.

In case of less complex mixtures the elution procedure can be simplified (see for example Fig. 2). Generally speaking, the duration of each "step" and the composition of the solvents depend on the particular compounds to be separated, see also the preceding paper[1].

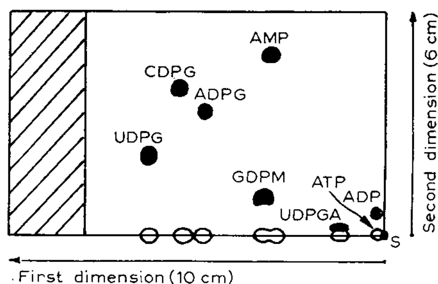


Fig. 2. Two-dimensional anion-exchange thin-layer chromatogram of nucleotide sugars, AMP, ADP, and ATP (8–15 mμmoles each). General procedure as described in the text, but no stepwise elution. The hatched area was removed after the elution in the first dimension. First dimension: 0.35 *M* LiCl, development time 55 min. Second dimension: 0.80 *M* formate buffer (pH 3.4), development time 12 min.

In order to identify a nucleotide X on the map a solution containing an excess of X was run together with the mixture. Only enough of the mixture was applied to the point of origin to make the compounds just detectable on the chromatogram. The presence of X was then apparent from the distinctly greater intensity of its spot compared with those of the other compounds.

In addition, the guanine nucleotides GDPM, GMP, GDP, and GTP were used as markers. When the plate is exposed to hydrochloric acid fumes for several minutes, nucleotides containing guanine fluoresce a brilliant light-blue in short-wave ultraviolet light, whereas derivatives of adenine, hypoxanthine, cytosine, and uracil appear as dark-blue spots.

RESULTS AND DISCUSSION

As is to be seen from Fig. 1, the 23 ribonucleotides applied at S are resolved into 22 distinct spots by the two-dimensional anion-exchange technique. IMP and TPN which are not separated under the conditions used can be resolved by one-dimensional chromatography with solvents containing formic acid, see Tables II and III of the preceding paper[1]. Fig. 2 demonstrates the resolution of a less complex model mixture containing several nucleotide sugars, AMP, ADP, and ATP.

The method is very sensitive: 0.5–2 mμmoles (0.15–1.2 μg) of each compound can be detected with a suitable ultraviolet lamp in a dark room. The sensitivity depends on the specific extinction coefficient of each nucleotide; it is, for example, lower for cytidine than for adenosine nucleotides. On the other hand, the technique can be used for micropreparative separations; mixtures containing 100 mμmoles or more of individual compounds can be resolved by two-dimensional chromatography on PEI-cellulose layers. The completeness of the resolution depends not only on the composition of the mixture being analyzed, but also on the thickness of the layer. For the separation of larger quantities 1–1.5 mm thick layers are superior to the 0.5 mm thick layers used in the present investigation.

With application of the technique described in this paper, it should be possible to detect and isolate unknown nucleotides in extracts from biological materials.

ACKNOWLEDGEMENTS

We wish to thank Prof. HERMAN M. KALCKAR and Prof. PAUL C. ZAMECNIK for their generous support during the course of this work.

SUMMARY

Very complex mononucleotide mixtures can be resolved by two-dimensional anion-exchange chromatography on poly(ethyleneimine)-cellulose thin layers. The complete resolution of a model mixture containing DPN, TPN, six nucleotide sugars, and fourteen common nucleoside-5'-mono-, di-, and triphosphates takes less than three hours. This separation cannot be achieved by the present techniques of paper chromatography and paper electrophoresis. The method is more sensitive than paper chromatography and column chromatography of nucleotides.

REFERENCE

1 K. RANDERATH AND E. RANDERATH, *J. Chromatog.*, 16 (1964) 111.

IDENTIFICATION OF FLAVONOIDS IN HOPS (*HUMULUS LUPULUS* LINNE) BY THIN-LAYER CHROMATOGRAPHY

P. R. BHANDARI

Chemisches Laboratorium der Firma M. Woelm, Eschwege (Germany)

(Received February 4th, 1964)

INTRODUCTION

A number of publications have appeared on the bitter principles and other constituents of hops, but very little has been published regarding the flavonoid constituents. The earlier workers reported the presence of quercitrin[1,2], isoquercitrin and rutin[3-5], but LEBRETON[6] approached this problem in a different way by hydrolysing the flavonoid mixture as such instead of isolating the individual compounds. Quercetin and kaempferol were identified in the hydrolysate as the aglucones and glucose and rhamnose as the sugars. The form in which the aglucones and sugars are attached to each other was not suggested.

In the present investigation use has been made of thin-layer chromatography with polyamide Woelm for isolating and identifying the various flavonoids in hops.

EXPERIMENTAL AND RESULTS

Thin-layer chromatoplates were prepared by pouring a suspension of the adsorbent in ethanol or ethanol-water mixture according to a method developed earlier[7]. A suspension of 1 g polyamide in 13.5 ml ethanol, or 3 g magnesium silicate in 13.5 ml ethanol + 1.5 ml water, or 2 g MN-Cellulosepulver 300 in 13.5 ml ethanol + 1.5 ml water, was sufficient for a plate measuring 20 × 20 cm. Except for the MN-Cellulosepulver 300, which was obtained from Macherey, Nagel and Co., the thin-layer adsorbents were made by Woelm and did not contain any binder.

1 kg of powdered hops was boiled 3 times with distilled water for 15 min each time. The filtered extract (*ca.* 30 l) was concentrated in vacuum to about 800 ml and poured into 4 l of methanol. The resulting precipitate (which was free of flavonoids) was separated by filtration. The filtrate was freed of methanol and the volume reduced to about 300 ml. This aqueous solution was shaken 50 times with 200 ml of ethyl acetate. Each extract was filtered separately and vacuum concentrated and then chromatographed on a thin-layer plate using polyamide as adsorbent and solvent mixture I for development. The first 30 ethyl acetate fractions showed four clear spots and a fifth spot of very weak intensity. The substances corresponding to the above spots have been provisionally named as substances A (R_F 0.33), B (R_F 0.24), C (R_F 0.16), D (R_F 0.07) and E (R_F 0.43). As all the thirty extracts showed identical spots they were pooled and vacuum evaporated to dryness. The residue was dissolved

in a small quantity of water, filtered and the filtrate shaken many times with ethyl acetate. This ethyl acetate fraction was concentrated and termed as Fraction I.

The remaining ethyl acetate (30–50) extracts were worked up in the same way as the first 30. Thin-layer chromatograms showed almost the same picture as for Fraction I except that the spots with R_F values 0.33 and 0.43 were almost absent. These twenty extracts were pooled, concentrated and termed Fraction II. The aqueous solution left after shaking with ethyl acetate was concentrated under vacuum, passed through a polyamide Woelm column and eluted with dilute methanol and finally with methanol. The various eluates were chromatographed as before. Thin-layer chromatograms showed three flavonoid spots with R_F values 0.08, 0.12 and 0.18. The main spot had the R_F value 0.12 (substance F); the other two being of very weak intensity. Since all the eluates gave the same chromatographic picture, they were pooled and concentrated (Fraction III).

A series of plates with polyamide were prepared and Fraction I was put on the entire length of the starting line. It was then developed with solvent mixture I. All the zones were marked under U.V. light. After scraping off the individual zones they were extracted with methanol. These methanolic extracts were concentrated separately and purified through active charcoal and allowed to crystallise. In this way the substances A, B, C, D and E from Fractions I and II and substance F from Fraction III were isolated. All the substances were crystallised a number of times from methanol.

Substance A

The melting point of substance A agreed with that of an authentic sample of astragalin[8] (176–178°) and on admixture the melting point was not depressed. The U.V. spectrum shows three maxima, *viz.* 267, 298 and 350 mμ like astragalin, for which GEISSMAN[8] gives maxima at 267, 298, 350 mμ and ÖISETH AND NORDAL[9] mention the wavelengths 269, 350 mμ and 265, 347 mμ. Colour reaction with Benedict's reagent[10] points to the fact that it is a kaempferol glucoside. Reaction with zirconium oxychloride–citric acid[11] and tests on paper with zinc–hydrochloric acid[12] indicate that it is substituted in the 3-position.

Hydrolysis. A small quantity of the substance was dissolved in methanol and boiled with 1 % H_2SO_4 for 3 h. After removal of methanol the solution was cooled and shaken with ether. After concentration the ether extract was put on a thin-layer chromatogram of cellulose acetate; a water-saturated mixture of chloroform and acetic acid (2:3) was used for development and 1 % methanolic aluminium chloride as spray reagent. A solution of authentic kaempferol was chromatographed with the ether extract and a mixture[13] of both for comparison. The thin-layer chromatogram showed that all the test substances had the same R_F value as authentic kaempferol. The aqueous extract left after ether extraction was freed of sulphuric acid by shaking with an anion exchanger (Amberlite I.R. 45) and filtered. After concentration, it was subjected to thin-layer chromatography using magnesium silicate[14] as an adsorbent and propanol–ethyl methyl ketone–water (2:1:1) as solvent. The chromatogram was dried and sprayed with 1 % $KMnO_4$ solution. The thin-layer chromatogram showed that glucose (R_F 0.49) was present in the hydrolysate of substance A.

Substance A was chromatographed in different solvent mixtures to test its purity and compared with authentic astragalin and also a mixture[13] of the two. In all the three cases the substances had the same R_F values (Table I). The above facts seem to

TABLE I

R_F VALUES OF SUBSTANCES A, B, AND F IN DIFFERENT SOLVENT MIXTURES

S	Compound	Solvent mixture				
		I	II	III	IV	V
I	Substance A	0.33	0.26	0.476	0.29	0.44
II	Astragalin (authentic)	0.33	0.26	0.476	0.29	0.44
III	Mixture of I + II	0.33	0.26	0.476	0.29	0.44
IV	Substance B	0.24	0.193	0.393	0.26	—
V	Isoquercitrin (authentic)	0.24	0.193	0.393	0.26	—
VI	Mixture of IV + V	0.24	0.193	0.393	0.26	—
VII	Substance F	0.12	0.09	0.50	0.46	—
VIII	Rutin (authentic)	0.12	0.09	0.50	0.46	—
IX	Mixture of VII + VIII	0.12	0.09	0.50	0.46	—

Solvent mixtures: I = Ethyl methyl ketone–toluene–glacial acetic acid–methanol–water (80:10:2:5:6); II = Ethyl methyl ketone–ethyl acetate–formic acid–water (3:5:1:1); III = Methanol–glacial acetic acid–water (90:5:5). IV = Water–ethanol–ethyl methyl ketone–acetylacetone (15:3:3:1); V = Ethanol–water (3:2).

indicate that substance A is identical with astragalin. The yield of substance A is very low and perhaps this is the reason for it not being reported by earlier workers.

Substance B

This substance melts at 224–226° and in admixture with an authentic sample of isoquercitrin did not depress the melting point. Hydrolytic studies show that the molecule is composed of quercetin and glucose. The zirconium oxychloride–citric acid test shows that the molecule is substituted in 3-position. A mixed and comparative thin-layer chromatogram with authentic isoquercitrin showed that substance B is identical with it. In all cases it gave the R_F value of 0.24. This was further confirmed by comparison of R_F values obtained by paper chromatography using a solvent mixture of isoamyl alcohol–glacial acetic acid–water (5:3:2) as suggested by PARIS[15]. The R_F value obtained here, *viz.* 0.54, compares well with the value of 0.56 quoted by him.

Substance C

A very high state of purity could not be obtained as the yield is very small. Hydrolytic studies show that it contains principally kaempferol and glucose together with very small traces of quercetin. The Benedict and the zirconium oxychloride–citric acid tests indicate that it is probably a 3-substituted kaempferol glucoside.

Substance D

Hydrolysis of this substance shows that the molecule is constituted of quercetin and glucose. The zirconium oxychloride–citric acid test indicates that the C-3 position of the flavonoid molecule is substituted. The quantity of the substance available was too small for further studies. This substance has a lower R_F value (0.07) than that of rutin (0.12) in solvent mixture I.

Substance E

Only very small amounts of this substance could be isolated. Benedict's colour test suggests that it is a quercetin glycoside.

Substance F

This has a m.p. of 188–189°. On admixture with authentic rutin there was no depression of the melting point. Hydrolytic studies revealed quercetin, glucose and rhamnose in the molecule. The colour tests and thin-layer chromatograms were compared with those of authentic rutin and found to be identical.

The amounts of the substances corresponding to the spots with R_F values 0.08 and 0.18 (from the aqueous extract) were too small to be isolated in a pure enough state for further investigations.

Beer

It was interesting to investigate whether the same flavonoids could be identified in a beer sample or whether they underwent any change. A concentrated solution of beer (Andreas Kloster-Brauerei, Eschwege) when worked up as above showed identical spots to those in hops on a thin-layer chromatogram.

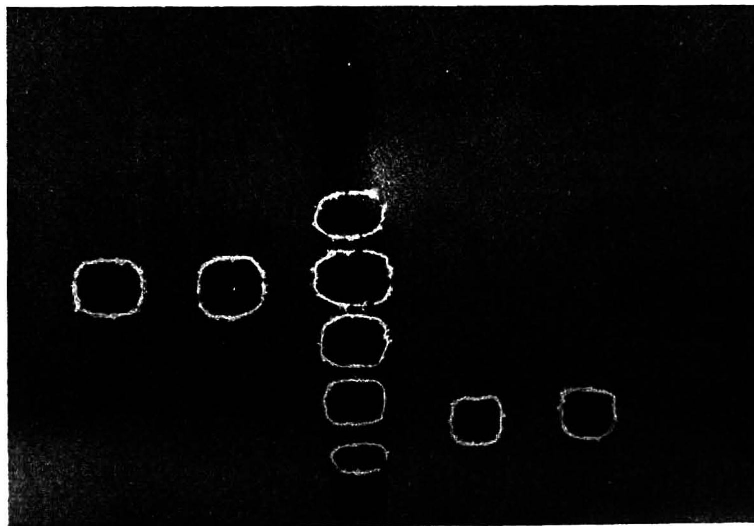


Fig. 1. Thin-layer chromatograph of hops on a polyamide plate with solvent mixture I. From left to right: (1) astragalin; (2) substance A; (3) ethyl acetate-soluble Fraction I; (4) rutin; (5) substance F.

DISCUSSION

The use of preparative thin-layer chromatography with polyamide was found to be very helpful in the isolation of various flavonoids from hops. As already indicated at least six flavonoid substances were isolated out of which three could be identified. Astragalin was found for the first time in hops. The other two flavonoids which have been identified are isoquercitrin and rutin.

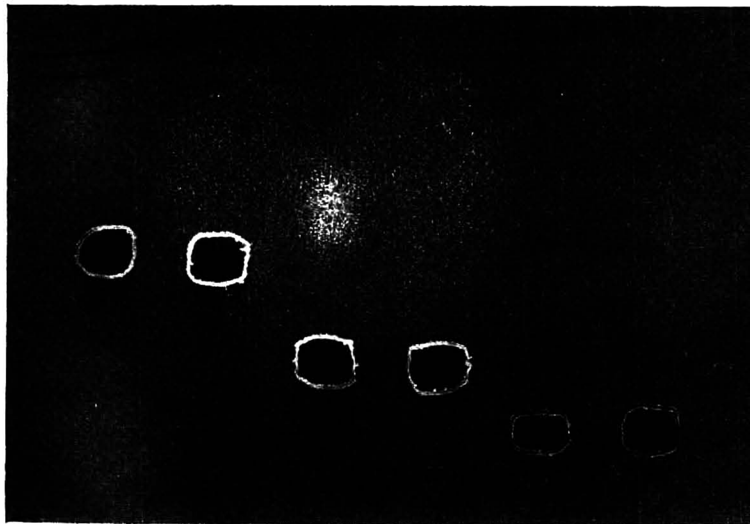


Fig. 2. Thin-layer chromatograph of substances A, B and F from hops on a polyamide plate with solvent mixture I. From left to right: (1) astragalin; (2) substance A; (3) isoquercitrin; (4) substance B; (5) rutin; (6) substance F.

The presence of quercitrin, found by earlier workers[1,2] during their investigations by paper chromatography and spectrophotometry, could not be confirmed. A paper chromatogram (Schleicher & Schüll 2043a Mgl) using Partridge mixture as solvent gave the R_F value of 0.68 for both astragalin as well as authentic quercitrin. A thin-layer chromatogram with polyamide with solvent mixture I shows however that astragalin has a slightly lower R_F value (0.33) than quercitrin with R_F 0.36. The difference is small but clear enough. The possibility of our mistaking quercitrin for astragalin is not possible because not only the colour reactions but also the hydrolytic products of the two glycosides are entirely different. It is difficult to say whether the amount of quercitrin in the particular charge of hops which we worked up was so low that it could not be isolated or whether the earlier workers[1,2] mistook astragalin for quercitrin. Some of the earlier workers[3,4,6] also failed to confirm the presence of quercitrin in hops. It should not be forgotten that the yield of flavonoids varies considerably according to the source[3].

ACKNOWLEDGEMENTS

The author wishes to thank the following for the donation of authentic flavonoid samples used in this investigation: Prof. Dr. L. HÖRHAMMER, Institut für Pharmazeutische Arzneimittellehre der Universität München, Prof. S. H. WENDER, The University of Oklahoma, Norman, Oklahoma, Dr. K. EGGER, Botanisches Institut der Universität Heidelberg, and Dr. PH. LEBRETON, Laboratoire de Chimie Biologique, 93, Rue Pasteur, Lyon.

SUMMARY

Using polyamide Woelm for thin-layer chromatography it has been possible to

identify astragalin for the first time in hops. The thin-layer chromatogram shows three more unidentified flavonoid spots besides those of rutin and isoquercitrin.

Benedict's and zirconium oxychloride–citric acid tests indicate that of these three, two seem to be quercitin glycosides and the third a kaempferol glycoside.

Beer was also investigated for its flavonoids. The thin-layer chromatogram showed spots identical to those from hops.

REFERENCES

1 J. R. Wagner, *Jahresber. Fortschr. Chem.*, (1859) 585;
T. A. Geissman, *The Chemistry of Flavonoid Compounds*, Pergamon, Oxford,1962, p. 336.
2 R. Vancraenenbroeck and R. Lontie, *Bull. Assoc. Anciens Etud. Brass. Univ. Louvain*, 51 (1955) 1.
3 Y. Umeda and M. Koshihara, *Proc. Japan Acad.*, 30 (1954) 387.
4 G. Harris, *J. Inst. Brewing*, 62 (1956) 390.
5 R. Wagner, *Chem. Zbl.*, (1859) 892;
T. A. Geissman, *The Chemistry of Flavonoid Compounds*, Pergamon, Oxford, 1962, p. 339.
6 P. Lebreton, *Brasserie*, 13 (1958) 104.
7 P. R. Bhandari, B. Lerch and G. Wohlleben, *Pharm. Ztg. Ver. Apotheker-Ztg.*, 107 (1962) 1618.
8 T. A. Geissman, *The Chemistry of Flavonoid Compounds*, Pergamon, Oxford, 1962, p. 328.
9 D. Öiseth and A. Nordal, *Pharm. Acta Helv.*, 32 (1957) 109.
10 H. Reznik and K. Egger, *Z. Anal. Chem.*, 183 (1961) 196;
K. Egger, *Z. Naturforsch.*, 176 (1962) 139.
11 L. Hörhammer and R. Hänsel, *Arch. Pharm.*, 284/56 (1951) 276; 285/57 (1952) 438;
L. Hörhammer and K. H. Müller, *Arch. Pharm.*, 287/59 (1954) 310.
12 R. Hänsel and L. Hörhammer, *Arch. Pharm.*, 287/59 (1954) 117.
13 T. B. Gage, C. D. Douglass and S. H. Wender, *Anal. Chem.*, 23 (1961) 1582.
14 H. Grasshof, *Deut. Apotheker-Ztg.*, 103 (1963) 1396.
15 R. Paris, *Journées internationales d'étude des méthodes de séparation immédiate et de chromatographie, 13–15 juin 1961*; Proceedings published in *Séparation immédiate et chromatographie*, édité par le Groupement pour l'avancement des méthodes spectrographiques (G.A.M.S.), p. 195–198.

CHARACTERIZATION OF Δ^4-3-OXO-C_{21}-STEROIDS ON THIN-LAYER CHROMATOGRAMS BY "*IN SITU*" COLOUR REACTIONS

B. P. LISBOA

Hormone Laboratory, Department of Women's Diseases, Karolinska Sjukhuset, Stockholm (Sweden)

(Received January 7th, 1964)

A previous communication described the application of thin-layer chromatography with a binder, using Silica Gel G as adsorbent, to the separation of Δ^4-3-ketosteroids of the pregnane series[31]. At that time only a small number of reactions was employed for the detection of the spots following chromatography, *e.g.* the formation of isonicotinic acid hydrazones, the reduction of phosphotungstomolybdic acid and the anisaldehyde–sulphuric acid reaction.

The application to thin-layer chromatography of reactions for α,β-unsaturated ketosteroids, reducing steroids, ketonic steroids, as well as structural reactions for the side-chain of pregnane steroids and unspecific general reactions for steroids, is reported in this paper.

EXPERIMENTAL

Materials

Reagents. In addition to the substances indicated in previous papers[31,32,34], the following reagents were used.

Phosphomolybdic acid (No. 532), triphenyltetrazolium chloride (No. 8380), potassium permanganate (No. 5083), ammonium acetate (No. 1115), 2,4-pentanedione (No. 9600), sodium nitroprusside (No. 6540), mercury(II) iodide (No. 4420), ferric ammonium sulphate (No. 3792), ammonium molybdate (No. 1182) and phenylhydrazine hydrochloride (No. 7253) were obtained from Merck A.G., Darmstadt, Germany.

Sodium arsenate, silver nitrate and potassium periodate were purchased from May & Baker, England.

Chloroform was from AB Kebo; it was purified by fractionation.

Steroids. The systematic names, trivial names, abbreviations and sources of the thirty-seven Δ^4-3-keto-C_{21}-steroids used throughout this investigation have been previously indicated[31]. One or more *hydroxy-*, *keto-* or *aldehyde*-groups are indicated in the abbreviation by *-ol*, *-one* or *-al* respectively and P denotes *pregnane*. Each systematic name can easily be derived from its abbreviation, for instance: pregna-4,6-diene-3,20-dione from $P^{4,6}$ 3,20 one (6-dehydro-progesterone). For the formation of trivial names the steroids were regarded as derivatives of the following compounds: *progesterone* (pregn-4-ene-3,20-dione), *cortexone* (21-hydroxypregn-4-ene-3,20-dione), *Reichstein's compound "S"* (17α,21-dihydroxypregn-4-ene-3,20-dione), *corticosterone* (11β,21-dihydroxypregn-4-ene-3,20-dione), *cortisol* (11β,17α,21-trihydroxypregn-4-ene-3,20-dione) and *cortisone* (17α,21-dihydroxypregn-4-ene-3,11,20-trione).

The source and systematic names of other steroids—oestrogens and androgens—cited in this work, have been indicated in previous papers[32, 33].

Ultraviolet lamp. The lamp used (λ = 254 mμ) was model PL 360 NN/15/44 from Quartzlampen GmbH, Hanau, Main, Germany.

Colours. The colours developed were recorded as previously described[34].

METHODS AND RESULTS

A. Detection of α,β-unsaturated ketosteroids

(*1*) *Absorption in ultraviolet light.* The α,β-unsaturated ketosteroids were detected by their absorption at 240 mμ after spraying with a dilute ethanolic solution of fluorescein for contrast[11]. This method of detection is not specific since many other steroids absorb in this region (see *e.g.* refs. 16, 17).

Direct observation at 240 mμ without fluorescein spraying is impossible because of the very strong absorption of Silica Gel G at this wavelength. Steroids of the pregnane series may be recovered from the plate by elution with chloroform since fluorescein does not react with these steroids.

(*2*) *Isonicotinic acid hydrazones.* The isonicotinic acid hydrazones of α,β-unsaturated ketosteroids were obtained by spraying with isonicotinic acid hydrazine as described in a previous paper[31]. This reaction permits differentiation between Δ^4-3-keto-, $\Delta^{4,6}$-3-keto-, $\Delta^{1,4}$- or Δ^1-3-keto-steroids. The presence of a $\Delta^{4,6}$-dien-3-one configuration, such as that of 6-dehydroprogesterone or 20-hydroxypregna-4,6-dien-3-one is shown by an immediate greenish yellow colour (sensitivity 1 μg), which can be differentiated from the less sensitive (4–5 μg) pale yellow colour produced by Δ^4-3-ketosteroids.

(*3*) *Bodansky-Kollonitsch's reaction*[7]. This reaction with *p*-phenylenediamine and phthalic acid, which was carried out as previously described[31], also allows differentiation between a Δ^4-3-keto- and a $\Delta^{4,6}$-3-keto-configuration.

All the Δ^4-3-ketosteroids tested revealed a yellow-olive to yellow-brown colour while 6-dehydroprogesterone and 20β-hydroxypregna-4,6-dien-3-one gave a yellow-orange to orange-brown colour. The sensitivity of the reaction is 2 to 3 μg. This reaction seems to be specific for α,β-unsaturated ketones.

Especially noteworthy is the fact that aldosterone gives an initial grey-brown colour which changes to purple as heating is continued. None of the other pregnane steroids tested gave these colours.

(*4*) *Formation of π-complexes with tetranitromethane*[63, 46]. The reaction was carried out as described by LISBOA AND DICZFALUSY[34]. The Δ^4-3-keto- and $\Delta^{4,6}$-3-ketosteroids of the pregnane series give a pale lemon colour of low sensitivity (10–15 μg). The reaction is, however, more sensitive for steroids with a benzenoid configuration, such as phenolic oestrogens, an olive-yellow colour being obtained.

(*5*) *Osmate esters.* These esters were obtained by exposure of the steroid to osmium tetroxide vapours[4, 35] in sealed tanks. A positive reaction is indicated by the formation of a black colour. Isolated double bonds such as occur in pregna-4,17-dien-3-one, 16-dehydroprogesterone, 16-dehydropregnenolone* and androst-2-ene-7,17-dione give a positive reaction after 5 to 10 min exposure to the vapours. The other Δ^4-3-keto-

---

* For a generous supply of 16-dehydropregnenolone (3β-hydroxypregna-5,16-dien-20-one) the author is indebted to Dr. O. A. DE BRUIN, N.V. PHILIPS-Duphar, Weesp, The Netherlands.

and $\Delta^{4,6}$-3-keto-steroids tested, which contained no isolated double bonds, gave a positive reaction only after 30 to 150 min exposure.

(6) *Sodium hydroxide*[10] *or tert.-butanolic sodium butoxide*[2]. It was impossible to employ these reactions on plates since no fluorescence developed.

B. Reduction reactions

(1) *Phosphomolybdic acid reagent*[29]. The plates were sprayed with a 10 % ethanolic solution of phosphomolybdic acid and heated to 90° for 15 min. The molybdenum blue colour obtained with this reagent is observed with 2 µg of steroid. Reichstein's compounds "E" and "*epi*-E" and the 20-dehydro substance "S" gave a blue-violet colour, possibly because of a side-reaction with the phosphoric acid released by the heating of phosphomolybdic acid.

(2) *Phosphotungstomolybdic acid reagent*[24]. This reagent was used as previously described by LISBOA[31]. It is less sensitive than that described above, requiring 5 µg of steroids.

(3) *Arsenomolybdic acid reagent*. This reagent, which was used for corticosteroids by SCHWARZ[54], was prepared according to NELSON[44]. The sprayed plates were heated for 5 to 10 min at 100°. The steroid is visualized as a blue spot on a yellow-green background. This is the most sensitive of the three molybdic acid reagents and as little as 0.5 µg of α,β-unsaturated steroids could be detected.

(4) *Triphenyltetrazolium reaction*. This reaction, whereby a red formazan is formed, was carried out as described by SHULL *et al.*[55]. Triphenyltetrazolium allows differentiation between α,β-unsaturated ketosteroids and α,β-ketolic steroids; the latter form a red formazan within 5 min at room teperature, with a sensitivity of 2 µg.

(5) *Alkaline potassium permanganate reagent*. The reaction was carried out as described for paper chromatography by BURTON *et al.*[9]. After spraying with a 0.2 % solution of potassium permanganate in 5 % sodium carbonate, all the Δ^4-3-ketosteroids tested immediately developed an olive-yellow colour on a violet background. When left overnight at room temperature following spraying, the steroids appeared as olive spots on a pale yellow background. This reaction is sensitive enough to detect 2 µg of steroid.

(6) *Reduction of p-amino-diethylaniline sulphur dioxide*. Plates were sprayed with a 0.5 % *p*-amino-diethylaniline sulphur dioxide in 5 % sodium bicarbonate solution and allowed to stand at room temperature overnight.

17α,21-Dihydroxy-20-ketosteroids without a hydroxyl group at C_{16}, such as compound "S", cortisol and *epi*-cortisol, give a red-orange colour with a sensitivity between 2 and 5 µg. 16α-Hydroxycortisol, 16α-hydroxy-compound "S", 16α-hydroxycortisone and the ketolic steroids, however, give an orange or yellow-orange colour. 17,20,21-Trihydroxy and 21-desoxy-Δ^4-3-keto-steroids do not reveal any colour even at 15–20 µg levels. Some oestrogenic steroids give a positive reaction: 16-keto-oestrone gives an orange colour almost immediately, while 16-ketolic oestrogens show the same colour after standing overnight.

(7) *Reduction of ferricyanide*[59]. The plates were sprayed with a solution of 0.1 % potassium ferricyanide in 0.25 % sodium carbonate and heated for 30 min at 80°. After cooling, the plates were sprayed with a 0.2 % solution of ferric ammonium sulphate, to each 100 ml of which 5 ml of concentrated (85 %) phosphoric acid had been added. The Prussian blue colour of ferric ferrocyanide appears immediately

or after a few minutes. The sensitivity of this reaction is between 2 and 5 μg.

This reaction is stronger with the 17,21-dihydroxy-20-keto- and 20,21-ketolic steroids; 17-hydroxy-C_{21}-steroids reduce more strongly than 17-deoxy-C_{21}-steroids. All other Δ^4-3-ketosteroids require at least 5 μg for detection.

(*8*) *Dragendorf reaction*[49]. The plates were sprayed with the reagent as modified by LISBOA[32]. Using this modified reagent, less than 1 μg of Δ^4-3-ketosteroid gives an orange colour on a yellow background. The latter turns to grey-lilac giving an even greater contrast. This reaction, however, is not specific.

(*9*) *Reduction of Tollens reagent.* The reaction was carried out as described for paper chromatography by ZAFFARONI *et al.*[65]. The reduction of ammoniacal silver nitrate requires more than 10 μg of an α,β-ketolic steroid. The reduction of *Nessler's reagent*[40] requires even larger quantities than the above reduction and thus is not considered suitable for use on chromatoplates.

C. Ketonic reactions

(*1*) *Reaction of Gornall and MacDonald*[21]. The plates were sprayed with a 10 % (v/v) ethanolic solution of hydrochloric acid containing 0.1 % 2,4-dinitrophenylhydrazine. The colour develops immediately upon spraying and its intensity may be enhanced by heating at 60° for a few minutes.

Steroids with only a Δ^4-3-keto group react to give an orange-red colour, *e.g.* pregn-4-en-3-one, pregna-4,17(20)-dien-3-one, 20β-hydroxypregna-4,6-dien-3-one, 20β- and 20α-hydroxypregn-4-en-3-one. If a 20-keto group is present, an orange yellow colour appears, *e.g.* progesterone, 17α-hydroxyprogesterone, 11-ketoprogesterone, 6- and 16-dehydroprogesterone. A 17α,21-dihydroxy-20-ketosteroid reacts more strongly than a 20,21-ketol; cortisol, cortisone, compound "S", 16α-hydroxy-compound "S", 16α-hydroxy-cortisone and 16α-hydroxy-cortisol give an orange colour, while corticosterone, *epi*-corticosterone, cortexone and aldosterone give a yellow colour. This reaction allows detection of 2 μg of the steroid.

The sensitivity can be increased, as suggested by STUPNICKI AND STUPNICKA[60] by using successively a solution of 0.1 % potassium permanganate in 1 % aqueous carbonate and an 0.2 % aqueous solution of ascorbic acid. If this method is applied the sensitivity of the reaction is increased, but the differentiation noted above is no longer possible.

(*2*) *The Zimmermann reaction.* This reaction for ketonic steroids with an *ortho* unsubstituted methylene group, was carried out as previously described[32]. Plates were sprayed with a solution containing equal parts of a 2 % alcoholic *m*-dinitrobenzene and a 1.25 *N* alcoholic potassium hydroxide solution (carbonate free) and dried unter a hot air stream or in a ventilated oven at 40°.

Five non-polar steroids with a Δ^4-3-keto-group gave a blue-violet colour with this reagent. Progesterone and other 20-keto-21-deoxysteroids, *e.g.* 16-dehydroprogesterone, 11-ketoprogesterone and 17α-hydroxyprogesterone react with a blue-grey colour, while ketolic and dihydroxy-ketolic steroids give a pink colour. By means of this reaction 2 to 3 μg of cortexone could be detected.

The GORNALL AND ZIMMERMANN reactions[21,66] were found to take place in much the same manner on chromatoplates as on paper chromatograms[10]. The GORNALL reaction shows greater sensitivity with polar steroids, while the ZIMMERMANN reaction is found to be more sensitive with the less polar 3-ketosteroids.

D. Side-chain reactions

(*1*) *17,21-Dihydroxy-20-ketosteroids.* These steroids can be detected by the PORTER–SILBER reaction[50], using phenylhydrazine–sulphuric acid reagent as described by SILBER AND PORTER[57]. After spraying, the plates may be observed after one hour if left at room temperature, or in a few minutes if heated to 60°. 17,21-Dihydroxy-20-ketosteroids give a dihydrazone as the end product with an E_{max} at about 400 mμ. However, 21-aldo-20-ketosteroids[58], 21-hydroxy-20-keto-16-dehydropregnene- and 16,21-dihydroxy-20-ketopregnane-steroids[37] also give a positive reaction.

The entire group of 17α,21-dihydroxy-20-ketosteroids tested give a positive PORTER–SILBER reaction with a sensitivity of 1 μg, with the exception of 16α-hydroxycortisol, 16α-hydroxycortisone and 16α-hydroxy-compound "S". These exceptions are in agreement with the observation of BERNSTEIN AND SILBER[5] on the reactivity of triamcinolone and may perhaps be explained by the fact that these compounds are unable to form the 16-dehydro-derivative which is an intermediate in the formation of the PORTER-SILBER chromogen[62].

Steroids such as corticosterone, *epi*-corticosterone, cortexone, aldosterone, 19-hydroxycortexone and others that do not possess the dihydroxyketone structure, and the above 16-hydroxysteroids give a very weak positive reaction following prolonged heating. This reaction, which was described for corticosterone and 11-dehydrocorticosterone on paper by NEHER AND WETTSTEIN[43] is probably due to the formation of the 3-phenylhydrazone of the Δ^4-3-keto-steroids, which shows an E_{max} between 340 and 360 mμ[50].

16α-Hydroxycortexone gave a negative reaction when the procedure was carried out as described above, but when observed again after several hours, the reaction had become positive.

Steroids with a 20-hydroxy group may give a positive reaction if the plates are heated or observed after prolonged exposure at room temperature. The colour produced differs, however, from the typical PORTER–SILBER reactions as follows: 20α- and 20β-hydroxy-pregn-4-en-3-ones: yellow-orange; 20β-hydroxypregna-4,6-dien-3-one: brown-orange; 20-dihydro-compound "S": olive; Reichstein's compound "E": dark blue-grey; Reichstein's compound *epi*-"E": pale grey-violet; and Reichstein's compound *epi*-"U": pale rose. The colours may result from a reaction between the steroid and the sulphuric acid of the reagent.

(*2*) *17-Deoxy-α-ketolic steroids.* These steroids could be detected after oxidation to their corresponding glyoxals by means of cupric acetate[30].

The plates were sprayed with a 0.01 *M* methanolic cupric acetate solution and left at room temperature overnight. Subsequently they were sprayed in the same manner as described for the PORTER–SILBER reaction above.

Of the six 17-deoxy-α-ketolic steroids tested in the course of this work, cortexone, corticosterone and *epi*-corticosterone give positive reactions which are as strong as those of 17α,21-dihydroxy-20-ketosteroids. 19-Hydroxycortexone, 16α-hydroxycortexone and aldosterone, however, give a very weak positive reaction. The colour appears almost immediately after spraying the plates.

The reaction of LEWBART AND MATTOX[30] appears to be a good method for the identification of corticosterone, *epi*-corticosterone and cortexone on silica gel plates. Since the glyoxal derivatives of 17,21-dihydroxy-20-ketosteroids give an almost instantaneous PORTER–SILBER reaction (E_{max} 425–450 mμ), this oxidation method

may also be employed for the identification of these steroids as suggested by BIRMINGHAM[6]. This is especially useful when 17,21-dihydroxy-20-keto- and 17-deoxy-20,21-ketolic steroids are present together.

(3) *Formaldehydogenic steroids.* These were detected by the method of SCHWARTZ[53] as described for paper chromatography by PAN[48]. The plates were sprayed with a 1 % solution of potassium periodate in 70 % (v/v) ethanol, and after 10 min at room temperature, were sprayed again before they were dry with a methanolic solution containing 15 % ammonium acetate, 1 % acetic acid and 1 % 2,4-pentanedione. After 15 to 20 min formaldehydogenic steroids show a yellow spot in daylight, yellow-green in U.V. light (252 mμ lamp). The sensitivity is to 1–2 μg. Thirteen formaldehydogenic steroids with a 20,21-ketolic, 17,20,21-trihydroxy- and 17,21-dihydroxy-20-keto-structure have been shown to be positive in the SCHWARTZ reaction with a sensitivity of 2 μg: corticosterone, *epi*-corticosterone, cortisol, *epi*-cortisol, 16α-hydroxycortisone, 16α-hydroxycortisol, 16α-hydroxy-compound "S", cortisone, 6α-hydroxycorticosterone, 11-dehydrocorticosterone, Reichstein's compound "S", cortexone and 16α-hydroxycortexone. No 20,21-glycol-steroid was, however, tested. In U.V. light the yellow-green colour permits the detection of less than 1 μg.

Various non-formaldehydogenic steroids with 20-hydroxy-21-deoxy- and 20-keto-21-deoxy-structure were negative in this test.

(4) *21-Deoxy-20-ketosteroids.* These were revealed *in situ* by the nitroprusside reaction of FEIGL[18] for methyl ketones as applied to steroid methyl ketones by PAN[47].

This method is suitable for thin-layer chromatography when the plates are protected by a filter-paper (Whatman No. 2) prior to the application of the methanolic nitroprusside paste reagent.

Amounts of 5 to 6 μg of 21-deoxy-20-ketosteroids, such as progesterone, 6-dehydro-, 17α-hydroxy-, 6β-hydroxy-, 11α-hydroxy- and 11β-hydroxyprogesterone give a bright violet spot after 60 min at room temperature. This can be observed on the uncovered surface of the plate. 16-Dehydroprogesterone and the 21-hydroxysteroids proved to be negative in this reaction.

(5) *Vanillin–phosphoric acid reagent*[13]. This reagent was used for the identification of *17-hydroxy-20-keto-21-deoxysteroids* as suggested by MCALEER AND KOZLOWSKI[38]. The plates were sprayed with a 2 % solution of vanillin in phosphoric acid (85 %) and then heated for 20 min at 90–95°. A bright orange colour indicates the presence of the above steroids.

The results of the application of the CHABROL reaction to Δ^4-3-keto-C_{21}-steroids on thin-layer chromatograms are summarized in Table I. Some steroids develop a colour after 15 min heating at 95–100°.

Using 1 μg amounts, pregna-4,17(20)-dien-3-one, 20β-hydroxypregna-4,6-dien-3-one, 20α- and 20β-hydroxypregn-4-en-3-one give a lilac-grey colour, 20-dihydro-compound "S" and Reichstein's compound *epi*-"E" a violet-grey, Reichstein's compound "E" a pale violet-blue and Reichstein's compound "U" a carmine-brown colour.

In 1 μg amounts 17α-hydroxy- and 11β,17α-dihydroxyprogesterone develop the characteristic orange colour described for 17α-hydroxy-20-keto-21-deoxysteroids, while 17α-hydroxyprogesterone could be detected in 0.5 μg amounts.

Reichstein's compound "U", 11β-hydroxyprogesterone, corticosterone and 6β-

TABLE I

APPLICATION OF CONCENTRATED PHOSPHORIC ACID AND VANILLIN–PHOSPHORIC ACID REAGENTS TO THE DETECTION OF THIRTY-SIX Δ^4-3-KETOPREGNANE-STEROIDS

Amount of steroid: 8–10 μg

Steroid	*Conc. phosphoric acid (at least 85%)*		*Vanillin–phosphoric acid reagents*	
	Daylight	*U.V. light*	*15 min*	*30 min*
P^4 3 one	—	—	—	lc.gy.
$P^{4,17(20)}$ 3 one	ce.	y.gr.	gy.lc.	bh.lc.*
20α ol P^4 3 one	ce.	—	gy.lc.	bh.lc.*
20β ol P^4 3 one	ce.	—	gy.lc.	bh.lc.*
20β ol $P^{4,6}$ 3 one	ce.	—	gy.lc.	bh.lc.*
$17\alpha,20\beta,21$ ol P^4 3 one	p.gy.br.	y.or.	v.gy.	bkh.ol.*
$11\beta,17\alpha,20\beta,21$ ol P^4 3 one	gr.gy.	—	bl.v.	pu.v.*
$11\alpha,17\alpha,20\beta,21$ ol P^4 3 one	p.pu.	—	bl.v.	pu.v.*
$17\alpha,20\beta,21$ ol P^4 3,11 one	—	—	p.sm.	br.
$17\alpha,20\alpha,21$ ol P^4 3,11 one	or.r.-or.br.	y.or.	c.bh.	c.br.*
P^4 3,20 one	—	—	—	bl.gy.
$P^{4,6}$ 3,20 one	—	—	—	p.or.r.
$P^{4,16}$ 3,20 one	—	—	—	p.ol.gy.
6β ol P^4 3,20 one	—	—	—	p.y.br.
11β ol P^4 3,20 one	ce.	—	p.or.r.	br.ol.
11α ol P^4 3,20 one	—	—	—	p.gy.br.
16α ol P^4 3,20 one	—	—	—	gy.
17α ol P^4 3,20 one	p.br.	r.	or.	m.*
21 ol P^4 3,20 one	ol.br.	y.or.	—	bl.gy.
$11\beta,17\alpha$ ol P^4 3,20 one	gh.y	y.	or.	or.bh.
$11\beta,21$ ol P^4 3,20 one	gy.br.	p.bu.gr.	p.or.r.	gy.br.
$11\alpha,21$ ol P^4 3,20 one	—	p.bu.gr.	—	y.br.
$16\alpha,21$ ol P^4 3,20 one	lc.re.	pk.	—	gy.
$17\alpha,21$ ol P^4 3,20 one	lc.re.	pk.	—	lc.gy.
19,21 ol P^4 3,20 one	—	gh.bl.	p.pu.	br.lc.
$11\beta,21$ ol 18 al P^4 3,20 one	—	—	—	p.gy.br.
$6\beta,11\beta,21$ ol P^4 3,20 one	—	—	p.pu.	p.v.
$11\beta,17\alpha,21$ ol P^4 3,20 one	—	—	—	bf.
$11\alpha,17\alpha,21$ ol P^4 3,20 one	—	p.bu.gr.	—	gy.br.
$16\alpha,17\alpha,21$ ol P^4 3,20 one	—	—	—	gy.
$11\beta,16\alpha,17\alpha,21$ ol P^4 3,20 one	p.br.	gh.y.	p.ol.y.	bh.ol.
P^4 3,11,20 one	p.gy.br.	p.or.	—	gy.br.
21 ol P^4 3,11,20 one	—	—	—	bf.
$17\alpha,21$ ol P^4 3,11,20 one	ol.	gh.y.	—	br.ol.
$6\beta,17\alpha,21$ ol P^4 3,11,20 one	—	—	p.sm.	lc.re.
$16\alpha,17\alpha,21$ ol P^4 3,11,20 one	—	—	—	y.

* Colour developed for 1 μg of steroid.

Abbreviations:

bf.	= buff	c.	= carmine	m.	= minium	r.	= red
bh.	= brownish	ce.	= cream	ol.	= olive	re.	= rose
bl.	= blue	gh.	= greenish	or.	= orange	sm.	= salmon
br.	= brown	gr.	= green	p.	= pale	v.	= violet
bu.	= bluish	gy.	= grey	pk.	= pink	y.	= yellow
bkh.	= blackish	lc.	= lilac	pu.	= purple		

hydroxycortisone also show this orange colour, but with a much lower sensitivity. Pregna-4,17(20)-dien-3-one and the three steroids with a 20-hydroxy-21-deoxy-structure tested, developed a yellow fluorescence under U.V. light early on in this reaction. This disappeared on heating.

The CHABROL reaction, described for steroids of the cholane series, is regarded[1, 27]

as a specific reaction for cholic acid depending upon the concentration of phosphoric acid in the reagent. The specificity of this reaction for 7α-hydroxycholane-steroids was established by CHABROL *et al.*[13], CHARONNAT AND GAUTHIER[14] and HÄUSSLER[22, 23] and later confirmed by CERRI AND SPIALTINI[12]; apocholic acid—a bile acid with an 8(14)-unsaturated bond—reacts as cholic acid[51]. Later MCALEER AND KOZLOWSKI[38] described the reaction as specific for 17-hydroxy-20-keto-21-deoxysteroids.

In our experience, however, other C_{21}-deoxysteroids react with a sensitivity similar to the extremely sensitive orange colour noticed by MCALEER AND KOZLOWSKI. They have, however, different absorption maxima. Structurally different steroids react just as strongly, for example: 7α-hydroxy-oestradiol (celadon-green), 20-dihydro-compound "S" (black-olive) and 17β-hydroxy-5α-androstane-3,7-dione (yellow-orange). Since some steroids react with concentrated phosphoric acid, the colour reaction of a steroid with the vanillin–phosphoric acid reaction must be compared with that produced with phosphoric acid prior to evaluation.

E. Non-specific reactions for steroids

(*1*) *Phosphoric acid reaction.* The chromatoplates were sprayed with concentrated phosphoric acid (85 %) and heated at 90–95° for 15 min. Colours were observed in daylight and U.V. light. With many of the steroids studied here, concentrated phosphoric acid develops colours in daylight or in U.V. light, some of the reactions being quite specific. Reichstein's compound "S" and 16α-hydroxycortexone, for instance, appear as lilac-rose spots in daylight, which turn to pink under U.V. light. The fluorescence in U.V. light of 20 steroids tested are indicated in Table I.

Dilute phosphoric acid solution[43], concentrated phosphoric acid[20, 15] or so-called 100 % phosphoric acid[45] are employed to detect steroids of the pregnane series. However, for Δ^4-3-keto-C_{21}-steroids this reaction is not as sensitive as for others, such as alcoholic pregnane-steroids, oestrogens, Δ^5-3β- or Δ^5-7-hydroxysteroids.

(*2*) *Sulphuric acid reaction.* This reaction was carried out as previously described[33]. The colours developed are not specific, but in U.V. light some fluorescence can be noted: red for cortexone and Reichstein's compound "S", orange for 19-hydroxy- and 17α,19-dihydroxycortexone, green for corticosterone, *epi*-corticosterone, *epi*-cortisol, pregna-4,17(20)-dien-3-one, 20α- and 20β-hydroxypregn-4-en-3-one; and yellow for 20β-hydroxypregna-4,6-dien-3-one and compounds "E" and *epi*-"E" of Reichstein.

(*3*) *Liebermann-Burchard reaction.* This was carried out as described for paper chromatograms by NEHER AND WETTSTEIN[43]. The grey-brown or brown colours developed after 15 min heating at 85–90° are not specific. Under U.V. light, the spots (15–20 μg) develop the following fluorescence: *bluish-green* for cortisone, 16α-hydroxycortisone, cortisol, 16α-hydroxycortisol, *epi*-corticosterone, 11-dehydrocorticosterone, aldosterone, 16α-hydroxy-compound "S", progesterone, 6-dehydro-, 6α-hydroxy-, 11α-hydroxy- and 16α-hydroxy-progesterone; *yellow-green or greenish-yellow* for compound "U" (Reichstein), *epi*-cortisol, 11-keto-progesterone, 16-dehydroprogesterone, 19-hydroxy- and 17α,19-dihydroxy-cortexone; *yellow* for Reichstein's compound *epi*-"E", cortexone, 16α-hydroxycortexone, pregna-4,17(20)-dien-3-one, 20β-hydroxypregna-4,6-dien-3-one and epimeric 20-hydroxypregn-4-en-3-one; *yellow-brown* or *brown* for corticosterone and 17α-hydroxyprogesterone, and *orange* for Reichstein's compounds "E" and "S".

TABLE II

COLOUR REACTIONS OF THIRTY-SEVEN Δ^4-3-KETO-C_{21}-STEROIDS ON THIN-LAYER CHROMATOGRAMS AFTER TREATMENT WITH ANISALDEHYDE–SULPHURIC ACID AND VANILLIN–SULPHURIC ACID REAGENTS

Amount of steroid: 4–5 μg. The colours developed at 95–100°. The time required for the colour to be observed for anisaldehyde–sulphuric acid reagent is indicated in parentheses; whenever no time is noted for vanillin–sulphuric acid reagent this signifies 12–15 min.

Steroid	*Reagent*	
	Anisaldehyde-sulphuric acid	*Vanillin-sulphuric acid*
P^4 3 one	y.or.(10) – or.br.(12–15) – cr.(16–18)	or.
$P^{4,17(20)}$ 3 one	gy.lc.(12) – bh.v.(15)	bl.gy.
20β ol P^4 3 one	bf.(5–6) – dk.v.bl.(10–12)	bl.gy.(8) – dk.bl.gy.(15)
20α ol P^4 3 one	dk.v.bl.(10–12)	bl.gy.
20β ol $P^{4,6}$ 3 one	pk.(10–12) – or.br.(16) (U.V. = or.)	p.v.(8–10) – v.gy.(15)
17α,20β,21 P^4 3 one	p.v.(10–12) – gy.v.(16)*	gy.v.(5–6) – pu.v.
11β,17α,20β,21 ol P^4 3 one	or.br.(5–6) – gy.v.(10–12)	dk.rh.lc.(5–6) – bh.v.
11α,17α,20β,21 ol P^4 3 one	or.br.(5–6) – gy.v.(10–12)	dk.rh.lc.(5–6) – bh.v.
17α,20α,21 ol P^4 3,11 one	p.sm.(5–6) – br.r.(10–12)*	pk.(5–6) – br.lc.
17α,20β,21 ol P^4 3,11 one	sm.(5–6) – br.lc.(10–12)	pk.(5–6) – r.lc.
P^4 3,20 one	y.(5–6) – y.br.(10–12) – c.br.(18)	or.y.(10–12) – br.(15)
$P^{4,6}$ 3,20 one	sm.(5–6) – r.or.(12–15) (U.V. = or.)	or.br.(15)
$P^{4,16}$ 3,20 one	y.or.(5–6) – y.bh.(12) – c.r. (16)	or.(10–12) – r.br.(15)
6β ol P^4 3,20 one	or.(6) – r.br.(10–12) (U.V. = y.)	or.y.(5–6) – c.br.
11β ol P^4 3,20 one	y.or.(10–12) – or.br.(16) (U.V. = y.)	or.y.(5–6) – c.br.
11α ol P^4 3,20 one	y.or.(10–12) – c.br.(16) (U.V. = y.)	or.br.
16α ol P^4 3,20 one	or.(5–6) – y.br.(12) – r.br.(16) (U.V. = y.)	y.br.
17α ol P^4 3,20 one	y.or.(10–12) – br.(12) – br.v.(16) (U.V. = y.)	p.r.(5–6) – br.r.(12–15)
21 ol P^4 3,20 one	gr.gy.(10–12) – br.(16)	br.r.
11β,17α ol P^4 3,20 one	y.br.(12)	r.
11β,21 ol P^4 3,20 one	bl.gy.(12) – r.br.(16)	br.r.
11α,21 ol P^4 3,20 one	bl.gy.(12) – bh.r.(16)	br.r.
16α,21 ol P^4 3,20 one	y.br.(10–12) – r.br.(16)	y.br.
17α,21 ol P^4 3,20 one	lc.br.(12)	r.br.
19,21 ol P^4 3,20 one	ol.(5–6) – s.bl.(10–12)*	pk(5–6) – br.lc.
11β,21 ol 18 al P^4 3,20 one	sm.(5–6) – c.r.(10–12)*	or.(5–6) – r.br.
6β,11β,21 ol P^4 3,20 one	c.(5–6) – pu.(10–12)*	or.y.(5–6) – bg.v.*
11β,17α,21 ol P^4 3,20 one	r.or.(10–12) – c.br.(16)	br.r.
11α,17α,21 ol P^4 3,20 one	r.or.(10–12) – br.lc.(16)	br.r.
16α,17α,21 ol P^4 3,20 one	dl.y.(5–6) – ol.(10–12) – p.or.r.(16) (U.V. = y.)	r.br.
17α,19,21 ol P^4 3,20 one	p.sm.(5–6) – br.r.(10–12)*	pk.(5–6) – br.lc.
16α,11β,17α,21 ol P^4 3,20 one	y.(5–6) – ol.y.(10–12) – or.br.(16) (U.V. = y.)	r.br.
P^4 3,11,20 one	y.or.(10–12) – r.br.(16)	or.
21α ol P^4 3,11,20 one	c.br.(12)	br.r.
17α,21 ol P^4 3,11,20 one	or.(5–6) – c.r.(12)	br.r.
6β,17α,21 ol P^4 3,11,20 one	or.(5–6)dk.re.lc.(10–12)	sm.(5–6) – br.lc.
16α,17α,21 ol P^4 3,11,20 one	r.or.(5–6) – or.br.(10–12) – br.r.(16) (U.V. = or.)	bh.r.

* Colour developed with 1.5–2.0 μg of steroid

Abbreviations:

bf.	= buff	dk.	= dark	ol.	= olive	sm.	= salmon
bg.	= bright	dl.	= dull	or.	= orange	sy.	= straw-yellow
bh.	= brownish	gh.	= greenish	p.	= pale	s.gr.	= sea-green
bl.	= blue	gr.	= green	pk.	= pink	s.bl.	= sea-blue
br.	= brown	gy.	= grey	pu.	= purple	v.	= violet
bu.	= bluish	lc.	= lilac	r.	= red	y.	= yellow
c.	= carmine	lg.	= light	re.	= rose		
ce.	= cream	oc.	= ochre	rh.	= reddish		

(4) *Antimony trichloride reaction.* This reaction was carried out as described in a previous paper[34]. It is less sensitive for Δ^4-3-keto-C_{21}-steroids than for Δ^5-3β-hydroxysteroids or oestrogens, and for a large number of the steroids tested, 20 μg developed no colour whatsoever or only a non-specific grey or brownish one.

There are, however, some exceptions: grey-lilac or pale violet spots (U.V. pale orange) are obtained with pregna-4,17(20)-dien-3-one, 20β-hydroxypregna-4,6-dien-3-one and epimeric 20-hydroxypregn-4-en-3-one; and brownish red (U.V. red-orange) spots are noted with 20-dihydro-compound "S" and Reichstein's compound "E".

(5) *Sulphuric acid and aromatic aldehydes.* The sulphuric acid–aromatic aldehyde reaction was carried out as previously described[34]. The results of the application of this reagent to thirty steroids utilising anisaldehyde, vanillin, benzaldehyde, salicylaldehyde and *p*-dimethylaminobenzaldehyde as the aromatic aldehyde are shown in Tables II and III.

In this reaction, the colours developed are dependent upon temperature, time of heating and intensity of spraying. The described colours can be obtained only when the stated experimental conditions are observed. The change of colours during and after heating make the use of standards for simultaneous reactions advisable. A freshly prepared 1 % solution of the aromatic aldehyde gives the best results. If higher concentrations are used, a deep background coloration occurs, as described for paper chromatography[26].

Even though a direct relationship between the structure of the steroid and the colour obtained cannot be determined, many steroids with similar structural groups show similar colours. 16-Hydroxysteroids and many hydroxy-derivatives of progesterone react with all the aldehydes tested, a yellow or yellow-brown colour being obtained.

The 11-ketosteroids tested, compound A, cortisone, Reichstein's compounds "E" and *epi*-"E" and 11-ketoprogesterone, appear as red or carmine spots with anisaldehyde.

The colours of 20α-hydroxy- and 20β-hydroxy-pregn-4-en-3-one and pregna-4,17(20)-dien-3-one are similar with the five aldehydes: blue-grey (vanillin), violet (anisaldehyde) or carmine-brown (benzaldehyde, salicylaldehyde or *p*-dimethylaminobenzaldehyde). The colours developed with steroids containing a side-chain with the 17,20,21-trihydroxy-structure, such as Reichstein's 20-dihydro-substance "S" and Reichstein's compounds "E" and *epi*-"E" are also similar in each of the five reactions.

The red colour developed with vanillin for 17α-hydroxy- and 11β,17α-dihydroxyprogesterone is typical, as is the pink colour, changing to brown-lilac, obtained for 19-hydroxy- and 17α,19-dihydroxy-deoxycorticosterone, with the same aldehyde. Just as typical is the green-grey colour which appears when deoxycorticosterone is treated with the anisaldehyde–sulphuric acid reagent.

The three 6β-hydroxysteroids tested, 6β-hydroxyprogesterone, 6β-hydroxycorticosterone and 6β-hydroxycortisone, develop similar colours with each of the aldehydes employed.

When *p*-dimethylaminobenzaldehyde is used as the aromatic aldehyde, the majority of the progesterone and deoxycorticosterone hydroxy-derivatives develop a non-specific or a yellow-brown colour with a yellow or orange U.V. fluorescence. With *p*-dimethylaminobenzaldehyde 16α-, 17α- and 19-hydroxy-deoxycorticosterones,

TABLE III

COLOUR REACTIONS OF THIRTY-SEVEN Δ^4-3-KETO-C_{21}-STEROIDS ON THIN-LAYER CHROMATOGRAMS AFTER TREATMENT WITH SULPHURIC ACID–AROMATIC ALDEHYDE REAGENTS

Amount of steroid: 4–5 μg. The colours are recorded in daylight and U.V. light after 12–15 min heating at 95–100°. For abbreviations see Table II.

Steroid	*Aldehyde*					
	Benzaldehyde		*Salicylaldehyde*		*p-Dimethylaminobenzaldehyde*	
	Daylight	*U.V. light*	*Daylight*	*U.V. light*	*Daylight*	*U.V. light*
P^4 3 one	dl.y.	—	or.y.	p.or.r.	y.or.	y.
$P^{4,17(20)}$ 3 one	p.pk.(5–6)* – c.br.	y.	c.br.	ol.gr.	r.br.(5–6) – c.br.	or.
20β ol P^4 3 one	p.br.lc.(5–6) – c.br.	y.	c.br.	ol.gr.	y.br.(5–6) – c.br.	or.
20α ol P^4 3 one	p.br.lc.(5–6) – c.br.	y.	c.br.	ol.gr.	y.br.(5–6) – c.br.	or.
20β ol $P^{4,6}$ 3 one	v.	r.br.	r.br.	y.br.	ol.br.	y.
17α,20β,21α P^4 3 one	p.pu.(8) – p.v.	bf.	lc.(3) – p.v.	or.	dl.pu.(5–6) – or.br.	or.
11β,17α,20β,21 ol P^4 3 one	pk.(5–6) – gy.lc.	r.or.	lc.pu.(5–6) – d.gy.bl.	y.gr.	lg.sm.(5–6) – y.br.	or.
11α,17α,20β,21 ol P^4 3 one	pk.(5–6) – gy.lc.	or.	lc.pu.(5–6) – d.gy.bl.	y.gr.	bf.(5–6) – y.br.	or.
17α,20α,21 ol P^4 3,11 one	sy.(8) – gy.br.	—	y.gr.(5–6) – s.gr.	gr.	y.gr.(5–6) – y.ol.	bf.
17α,20β,21 ol P^4 3,11 one	sy.	or.	ol.	y.gr.	y.	y.
P^4 3,20 one	dl.y.	dl.y.	dl.y.	dl.y.	y.or.	y.
$P^{4,6}$ 3,20 one	dl.y.	y.	gh.y.	s.gr.	gh.y.(8) – ol.y.	gh.y.
$P^{4,16}$ 3,20 one	dl.y.	dl.y.	or.y.	dl.y.	y.or.	y.
6β ol P^4 3,20 one	y.or.	y.or.	y.gr.(5–6) – y.ol.	lg.bl.	y.(5–6) – or.br.	or.
[illegible]	[illegible]	[illegible]	[illegible]		y.or.	y.or.

11α ol P4 3,20 one	y.or.	y.	y.	y.gr.	y.or.	y.or.
16α ol P4 3,20 one	p.br.	p.or.	y.	lg.bl.	p.y.(5–6) – ol.y.	y.
17α ol P4 3,20 one	gh.y.	y.or.	y.gr.	gr.	or.y.(5–6) – or.br.	or.
21 ol P4 3,20 one	br.ol.	y.	lc.gy.	gy.bu.	y.br.	or.
11β,17α ol P4 3,20 one	br.ol.	or.	y.ol.	or.	y.or.	sm.
11β,21 ol P4 3,20 one	br.ol.	y.	br.ol.	y.or.	y.br.	or.
11α,21 ol P4 3,20 one	br.ol.	y.	br.ol.	or.	y.br.	or.
16α,21 ol P4 3,20 one	br.ol.	y.	y.ol.	y.gr.	ol.	y.or.
17α,21 ol P4 3,20 one	dk.gr.gy.	or.br.	ol.	dl.y.	ol.	y.or.
19,21 ol P4 3,20 one	sg.(8) – gy.br.	—	ol.(5–6)	lg.bl.	y.gr.(5–6) – y.ol.	bf.
11β,21 ol 18 al P4 3,20 one	sy.	or.	y.(8) – oc.	p.gh.bl.	y.gr.	y.
6β,11β,21 ol P4 3,20 one	p.br.(8) – y.br.	—	y.gr.(5–6)** – s.gr.	p.gh.bl.	y.(5–6) – y.or.**	or.
11β,17α,21 ol P4 3,20 one	br.ol.	y.gr.	ol.	y.or.	y.br.	or.
11α,17α,21 ol P4 3,20 one	br.ol.	y.gr.	ol.	y.or.	y.br.	or.
16α,17α,21 ol P4 3,20 one	p.br.	p.or.	sy.	ce.	y.gr.	y.
17α,19,21 ol P4 3,20 one	sy.(8) – gy.br.	—	y.gr.(5–6)	gr.	y.gr.(5–6) – y. ol.	bf.
11β,16α,17α,21 ol P4 3,20 one	p.br.	p.or.	y.br.	or.	y.gr.	y.or.
P4 3,11,20 one	y.	dl.y.	or.y.	dl.y.	y.or.	y.
21 ol P4 3,11,20 one	ol.y.	y.bh.	ol.y.	dl.y.	y.or.	y.or.
17α,21 ol P4 3,11,20 one	ol.y.	y.bh.	ol.y.	dl.y.	y.or.	y.or.
6β,17α,21 ol P4 3,11,20 one	bf.	r.or.	ol.y.	p.gh.bl.	y.(8) – p.sm.	y.or.
16α,17α,21 ol P4 3,11,20 one	p.br.	p.or.	bf.	y.	y.gr.	y.

* Time required (in minutes) for colour development, whenever this differs from that given above.
** Colour developed with 1.5–2.0 μg of steroid.

however, appear as yellow spots, and 16α-hydroxy-derivatives of Reichstein's compound "S", cortisone and cortisol as yellow-green spots.

The sulphuric acid–aromatic acid reaction can be used to characterize progesterone and 6-dehydroprogesterone, when anisaldehyde or benzaldehyde is employed. In contradistinction to progesterone and 16-dehydroprogesterone, which give a feeble reaction with benzaldehyde, 6-dehydroprogesterone appears as a greenish yellow spot, sea-green under U.V. light. When anisaldehyde is used, the red-orange spot with orange fluorescence from 6-dehydroprogesterone can also be differentiated from the yellow or yellow-orange spot of progesterone and 16-dehydroprogesterone.

With the anisaldehyde–sulphuric acid reaction it was found best to observe the spots in daylight; on the other hand, very good fluorescent spots occurred when salicylaldehyde was employed as the aromatic aldehyde.

(*6*) *Mylius reaction*[41]. The reaction with iodine–potassium iodide (Lugol solution) was carried out according to BURTON *et al.*[9]: the plates were sprayed with a 0.3 % iodine solution in a 0.5 % aqueous solution of potassium iodide. After the original colour was observed the plates were resprayed with ether[39], which modifies the reaction of many steroids.

TABLE IV

RESULTS OF THE APPLICATION OF THE MYLIUS REACTION (IODINE–POTASSIUM IODIDE) TO THIRTY-SEVE Δ^4-3-KETO-C_{21}-STEROIDS BEFORE (I) AND AFTER (II) ETHER TREATMENT

Amount of steroid: 8–10 μg

Steroid	*I*	*II*	*Steroid*	*I*	*II*
P^4 3 one	y.	y.	11β,17α ol P^4 3,20 one	—	—
$P^{4,17(20)}$ 3 one	y.	y.	11β,21 ol P^4 3,20 one	y.	y.
20β ol P^4 3 one	y.	y.	11α,21 ol P^4 3,20 one	bl.[d]	bl.
20α ol P^4 3 one	br.oc.	br.oc.	16α,21 ol P^4 3,20 one	lg.v.gy.	bl.
20β ol $P^{4,6}$ 3 one	or.	or.	17α,21 ol P^4 3,20 one	y.	or.br.
17α,20β,21 ol P^4 3 one	y.	y.	19,21 ol P^4 3,20 one	—	p.y.br.
11β,17α,20β 21 ol P^4 3 one	—	y.	11β,21 ol 18 al P^4 3,20 one	y.	y.
11α,17α,20β 21 ol P^4 3 one	—	y.	6β,11β,21 ol P^4 3,20 one	y.	y.
17α,20β,21 ol P^4 3,11 one	y.	y.[b]	11β,17α,21 ol P^4 3,20 one	y.[e]	bl.
17α,20α,21 ol P^4 3,11 one	y.	y.[b]	11α,17α,21 ol P^4 3,20 one	y.	y.
P^4 3,20 one	y.	bl.	16α,17α,21 ol P^4 3,20 one	—	—
$P^{4,6}$ 3,20 one	y.	y.	17α,19,21 ol P^4 3,20 one	—	y.
$P^{4,16}$ 3,20 one	y.or.	r.br.	11β,16α,17α,21 ol P^4 3,20 one	—	—
6β ol P^4 3,20 one	y.	y.	P^4 3,11,20 one	y.	r.br.
11β ol P^4 3,20 one	y.	y.	21 ol P^4 3,11,20 one	y.	y.
11α ol P^4 3,20 one	bl.	bl.	17α,21 ol P^4 3,11,20 one	bl.	bl.
16α ol P^4 3,20 one	lg.v.bl.	br.oc.	6β,17α,21 ol P^4 3,11,20 one	—	—
17α ol P^4 3,20 one	or.br.[a]	or.br.[a]	16α,17α,21 ol P^4 3,11,20 one	—[f]	—
21 ol P^4 3,20 one	y.	br.oc.[c]			

[a] With 15 μg steroid: blue.
[b] Increased sensitivity.
[c] With 50 μg steroid: red-orange; 75 μg: blue.
[d] After 5 min the blue colour disappears.
[e] After longer spraying: yellow changes to blue.
[f] With 20 μg steroid: feeble blue; 30 μg: blue.

Abbreviations:

bl. = blue	br.oc. = brown ochre	lg.v.bl. = light violet blue
or. = orange	or.br. = orange brown	p.y.br. = pale yellow brown
r.br. = red brown	y. = yellow	y.or. = yellow orange.

The results of the application of this reaction to thirty-seven steroids are listed in Table IV. Some colours are not stable and the sensitivity varies from one steroid to another. The blue colour of *epi*-corticosterone disappears in 5 min and the yellow colour obtained with cortisol may change into blue after prolonged spraying with Lugol's solution without ether treatment.

According to SZENT-GYÖRGYI[61] the blue colour obtained with some steroids is due to a charge transfer, in which the complex (I_3^-), formed by the interaction of I_2 and KI, is an electron donor, and the steroids act as acceptors.

In this work a reaction is stated to be positive whenever a colour develops owing to the presence of a steroid. This is the same interpretation as used previously by KRITCHEVSKY AND CALVIN[28] and HOHENSEE AND HÜTTENRAUCH[25], who did not restrict the positive reaction to the classical blue colour obtained by MYLIUS[41] with cholic acid.

The sensitivity of this method is in our hands not in complete agreement with results previously reported using paper chromatography. For instance 17α-hydroxyprogesterone was detectable on plates at the level of 15 μg, while ZAFFARONI AND BURTON[64] and FUJISAKI *et al.*[19] could detect a much smaller quantity on paper. On the other hand 8 μg of 16α-hydroxyprogesterone could easily be spotted, although MCALEER AND KOZLOWSKI[39] could not detect less than 15 μg of this substance on chromatograms. In general the use of ether does not increase the sensitivity of the colour reactions on plates, even for those steroids for which ether had been shown to be an important enhancing factor on paper. On a thin-layer, for example, 75 μg of cortexone are necessary to obtain a blue colour, following spraying with ether.

The detection of cortisone on chromatoplates at the level of 15 μg confirms previous reports by ZAFFARONI *et al.*[65] and BASSIL AND BOSCOTT[3]. However, the use of iodine vapours[29] is not recommended for plate chromatograms, in agreement with the results previously reported by MATHEWS *et al.*[36].

DISCUSSION

For the development of spots on paper chromatograms of Δ^4-3-keto-C_{21}-steroids a large number of reactions has been available for a long time (see *e.g.* refs. 52, 56 and 42).

Some of the reactions presented here were especially developed for thin-layer chromatography, in order to increase the sensitivity or to make their application on chromatoplates possible.

Many of these reactions have been previously applied for a small number of Δ^4-3-ketopregnane-steroids only. The extension of these reactions to a large number of steroids under similar conditions permits an adequate study of their specificity as well as their easy application for identification of steroids.

Impurities sometimes make the application of a colour reaction on chromatoplates for the identification of steroids derived from biological extracts difficult.

A suitable method for purification of the steroids before their identification by means of the colour reactions here proposed consists of elution of the area which includes the substance, followed by the microsublimation of the steroid according to the technique of BREUER AND KASSAU[8]. After sublimation and prior to identification, the steroid must be rechromatographed to prove that it has not changed during the operation.

The R_F values in the eight solvent systems developed for Δ^4-3-keto-C_{21}-steroids, the application of the functional and individual colour reactions here described and the formation of derivatives, such as hydrazones, acetates, 17-keto-C_{19}-steroids and etiocholanic acids, also separable by thin-layer chromatography (unpublished results), constitute a good approach to steroid identification.

ACKNOWLEDGEMENTS

The author wishes to thank Doc. Dr. E. DICZFALUSY, head of the Hormone Laboratory, Karolinska Sjukhuset, Stockholm, for stimulating discussions, helpful advice and generous support in this work. For valuable discussions the author is also indebted to Dr. R. WILSON, Toronto, Canada.

The author gratefully acknowledges gifts of steroids from Dr. S. BERNSTEIN (American Cyanamid Co., Pearl River, U.S.A.), Prof. H. BREUER (Chirurgische Universitätsklinik Bonn-Venusberg, Germany), Dr. O. A. DE BRUIN (N.V. Philips-Duphar, Weesp, The Netherlands), Dr. J. JOSKA (Academy of Sciences, Prague, Czechoslovakia), Prof. K. JUNKMANN (Schering A.G., Berlin, Germany), Prof. W. KLYNE (Medical Research Council, London, Great Britain), Dipl. Eng. I. KÖNYVES (A.B. Leo, Hälsingborg, Sweden), Dr. R. NEHER, (Ciba Ltd. Basel, Switzerland), Prof. J. SCHMIDT-THOMÉ (Farbwerke Hoechst A.G., Frankfurt a.M.-Hoechst, Germany) and Prof. J. ZANDER (Köln University, Germany).

SUMMARY

The application of thirty-four reactions for the characterization of thirty-seven Δ^4-3-keto-C_{21}-steroids *in situ*, on thin-layer chromatograms, is described.

The methods involve the detection of (a) α,β-unsaturated ketosteroids; (b) reducing corticosteroids; (c) ketonic steroids; (d) 17α,21-dihydroxy-20-ketosteroids; (e) 17-deoxy-α-ketolic steroids; (f) formaldehydogenic steroids; (g) 21-deoxy-20-ketosteroids; (h) 17-hydroxy-20-keto-21-deoxysteroids and (i) individual steroids (no specific general reactions for steroids).

The sensitivity, specificity, optimal conditions and as far as possible, the reaction mechanisms are discussed. In some cases a comparison is made between the results of paper and thin-layer chromatography.

REFERENCES

1 Y. ABE, *J. Biochem. (Tokyo)*, 25 (1937) 181.
2 D. ABELSON AND P. K. BONDY, *Anal. Chem.*, 28 (1956) 1922.
3 G. T. BASSIL AND R. J. BOSCOTT, *Biochem. J.*, 48 (1951) xlviii.
4 H. S. BENNET, *Proc. Soc. Exptl. Biol. Med.*, 42 (1939) 768.
5 S. BERNSTEIN AND R. H. SILBER, in discussion to S. BERNSTEIN, *Recent Progr. Hormone Res.*, 14 (1958) 1.
6 M. K. BIRMINGHAM, *Nature*, 184 (1959) B.A. 67.
7 A. BODANSKY AND J. KOLLONITSCH, *Nature*, 175 (1955) 729.
8 H. BREUER AND E. KASSAU, *Acta Endocrinol., Suppl.*, 51 (1960) 1113.
9 R. B. BURTON, A. ZAFFARONI AND E. H. KEUTMANN, *J. Biol. Chem.*, 188 (1951) 763.
10 I. E. BUSH, *The Chromatography of Steroids*, Pergamon Press, Oxford, 1961.
11 V. ČERNY, *3rd. Conf. Inst. Chem., Prague, Dec. 21st, 1951*, quoted by Ž. PROCHÁZKA, *Chem. Listy*, 47 (1953) 718.
12 O. CERRI AND A. SPIALTINI, *Boll. Chim. Farm.*, 96 (1957) 193.

[13] E. CHABROL, R. CHARONNAT, J. COTTET AND P. BLONDE, *Compt. Rend. Soc. Biol.*, 115 (1934) 834.
[14] R. CHARONNAT AND B. GAUTHIER, *Compt. Rend.*, 223 (1946) 1009.
[15] R. I. COX, *Nature*, 181 (1958) 638.
[16] H. DANNENBERG, *Abhandl. Preuss. Akad. Wiss., Math.-Naturw. Kl.*, 21 (1939) 3.
[17] L. DORFMAN, *Chem. Rev.*, 53 (1953) 47.
[18] F. FEIGL, *Spot Tests in Organic Analysis*, 5th Ed., Elsevier, Amsterdam, 1956.
[19] M. FUJISAKI, Y. ARAI, T. KON AND M. ITOH, *Folia Endocrinol. Japon.*, 29 (1953) 148.
[20] S. GOLDBERG AND M. FINKELSTEIN, *Bull. Res. Council Israel*, A5 (1956) 310.
[21] A. G. GORNALL AND M. P. MACDONALD, *J. Biol. Chem.*, 201 (1953) 279.
[22] E. P. HÄUSSLER, *Z. Physiol. Chem.*, 290 (1952) 155.
[23] E. P. HÄUSSLER, *Z. Physiol. Chem.*, 291 (1952) 168.
[24] R. D. H. HEARD AND H. SOBEL, *J. Biol. Chem.*, 165 (1946) 687.
[25] F. HOHENSEE AND R. HÜTTENRAUCH, *Z. Physiol. Chem.*, 310 (1958) 19.
[26] S. KATZ, *Arch. Biochem. Biophys.*, 91 (1960) 54.
[27] S. KAWAGUCHI, *J. Biochem. (Tokyo)*, 28 (1938) 445.
[28] D. KRITCHEVSKY AND M. CALVIN, *J. Am. Chem. Soc.*, 72 (1950) 4330.
[29] D. KRITCHEVSKY AND M. R. KIRK, *Arch. Biochem. Biophys.*, 35 (1952) 346.
[30] M. L. LEWBART AND V. R. MATTOX, *Anal. Chem.*, 33 (1961) 559.
[31] B. P. LISBOA, *Acta Endocrinol.*, 43 (1963) 47.
[32] B. P. LISBOA, *J. Chromatog.*, 13 (1964) 391.
[33] B. P. LISBOA AND E. DICZFALUSY, *Acta Endocrinol.*, 40 (1962) 60.
[34] B. P. LISBOA AND E. DICZFALUSY, *Acta Endocrinol.*, 43 (1963) 545.
[35] J. M. MANARO AND A. ZYGMUNTOWICZ, *Endocrinology*, 48 (1951) 114.
[36] J. S. MATTHEWS, V. A. L. PEREDA AND P. A. AGUILERA, *J. Chromatog.*, 9 (1962) 331.
[37] V. R. MATTOX, H. L. MASON AND A. ALBERT, *Proc. Mayo Clinic*, 28 (1953) 569.
[38] W. J. MCALEER AND M. A. KOZLOWSKI, *Arch. Biochem. Biophys.*, 62 (1956) 196.
[39] W. J. MCALEER AND M. A. KOZLOWSKI, *Arch. Biochem. Biophys.*, 66 (1957) 125.
[40] C. MONDER AND A. WHITE, *Endocrinology*, 68 (1961) 159.
[41] F. MYLIUS, *Z. Physiol. Chem.*, 11 (1887) 306.
[42] R. NEHER, *J. Chromatog.*, 1 (1958) 205.
[43] R. NEHER AND A. WETTSTEIN, *Helv. Chim. Acta*, 34 (1951) 2278.
[44] N. NELSON, *J. Biol. Chem.*, 153 (1944) 375.
[45] W. J. NOWACZINSKI AND P. R. STEYERMARK, *Can. J. Biochem. Physiol.*, 34 (1956) 592.
[46] I. OSTROMISSLENSKY, *J. Russ. Phys. Chem. Soc.*, 12 (1909) 731.
[47] S. C. PAN, *J. Chromatog.*, 8 (1962) 449.
[48] S. C. PAN, *J. Chromatog.*, 9 (1962) 81.
[49] V. PELCOVÁ, unpublished results, quoted by O. SIBLÍKOVÁ, M. ČERNÁ AND I. M. HAIS, *Cesk. Farm.*, 11 (1962) 187.
[50] C. C. PORTER AND R. H. SILBER, *J. Biol. Chem.*, 185 (1950) 201.
[51] E. L. PRATT AND H. B. CORBITT, *Anal. Chem.*, 24 (1952) 1665.
[52] L. M. REINEKE, *Anal. Chem.*, 28 (1956) 1853.
[53] D. P. SCHWARTZ, *Anal. Chem.*, 30 (1958) 1855.
[54] V. SCHWARZ, *Nature*, 169 (1952) 506.
[55] G. M. SHULL, J. L. SARDINAS AND R. C. NUBEL, *Arch. Biochem. Biophys.*, 37 (1952) 186.
[56] O. SIBLÍKOVÁ AND I. M. HAIS, *Cesk. Farm.*, 7 (1958) 1.
[57] R. H. SILBER AND C. C. PORTER, *J. Biol. Chem.*, 210 (1954) 923.
[58] R. H. SILBER AND C. C. PORTER, *Methods Biochem. Anal.*, 4 (1957) 139.
[59] N. R. STEPHENSON, *Can. J. Biochem. Physiol.*, 37 (1959) 391.
[60] R. STUPNICKI AND E. STUPNICKA, *J. Chromatog.*, 9 (1962) 235.
[61] A. SZENT-GYÖRGYI, *Life Sciences*, (1963) 112.
[62] A. WALSER AND H. P. SCHLUNKE, *Experientia*, 15 (1959) 71.
[63] A. WERNER, *Ber.*, 42 (1909) 4324.
[64] A. ZAFFARONI AND R. B. BURTON, *J. Biol. Chem.*, 193 (1951) 749.
[65] A. ZAFFARONI, R. B. BURTON AND E. H. KEUTMANN, *Science*, 111 (1950) 6.
[66] W. Z. ZIMMERMANN, *Z. Physiol. Chem.*, 233 (1935) 257.

DÜNNSCHICHTCHROMATOGRAPHISCHE TRENNUNG VON INDOLDERIVATEN IN NEUTRALEN FLIESSMITTELN

GÜNTER BALLIN

Botanisches Institut der Universität Rostock (Deutschland)

(Eingegangen den 21. Januar 1964)

Die Anwendung papierchromatographischer und -elektrophoretischer Methoden in der Auxinforschung ermöglichte die Isolierung und Identifizierung zahlreicher Indolverbindungen in den Extrakten aus pflanzlichen Geweben. In der Literatur häufen sich aber die Angaben über die Instabilität von Indolderivaten und besonders von nativen Komplexen mit einer indolischen Komponente[1].

Die bis jetzt publizierten Ergebnisse zur Dünnschichtchromatographie (DC) beweisen die gute Eignung dieser Methode zur Trennung indolischer Verbindungen[2–6]. Verwendet werden jedoch vorwiegend saure oder alkalische Fliessmittel, obwohl auch von der DC bekannt ist, dass z.B. Indol-3-essigsäure in sauren und Indol-3-acetonitril in alkalischen Fliessmitteln zersetzlich sind[3]. Hingewiesen sei auch auf die bei DC mögliche Bildung von Indol-3-acetamid als Artefakt, wie es bei papierchromatographischer Trennung in ammoniakalischen Laufmitteln der Fall ist[7].

Diese Befunde liessen uns nach geeigneten neutralen Fliessmitteln mit guten Trenneigenschaften suchen. Es sollten Fliessmittel entwickelt werden, die 1. eine Trennung saurer und nichtsaurer Indolderivate im gleichen Gemisch, 2. eine Trennung nichtsaurer Indolderivate und 3. eine Trennung saurer Indolderivate ermöglichen.

EXPERIMENTELLER TEIL

Als Trägerplatten dienten 9 × 12 cm grosse Photoplatten, auf welche die Sorptionsschicht in einer Dicke von 250 μm mit einem Streichgerät* unter Verwendung von "Kieselgel G für Dünnschichtchromatographie"** nach der Vorschrift von STAHL[6] aufgebracht wurde. Die vorgetrockneten Platten wurden 30 Min. bei 110° getrocknet und bis zur Verwendung in einem Exsikkator über Blaugel aufbewahrt.

Die Entfernung der Startpunkte von der Unterkante der Platte betrug 15 mm. Chromatographiert wurde aufsteigend in abgedunkelten Trennkammern mit 50 ml eines frisch bereiteten Fliessmittels bei Kammersättigung. Die Trennstrecke wurde stets auf 10 cm begrenzt.

Folgende Indolderivate wurden in Methanol gelöst und je 0.5 μg einzeln oder als Gemisch mit einer Mikropipette aufgetragen:

1. Saure Indolderivate: Indol-3-carboxylsäure (ICS), Indol-3-essigsäure (IES), Indol-3-propionsäure (IPS), Indol-3-buttersäure (IBS).

2. Nichtsaure Indolderivate: Indol-3-acetamid (IAAm), Indol-3-äthanol =

* Hersteller: CAMAG, Muttenz/Schweiz.

** E. Merck AG, Darmstadt.

Tryptophol (Try), Indol-3-aldehyd (IA), Indol-3-acetonitril (IAN), Indol-3-essigsäureäthylester (IES-äth).

Der Nachweis der Indolverbindungen erfolgte auf der trockenen Platte durch Besprühen mit dem Reagens nach VAN URK und anschliessender Oxydation mit Königswasserdämpfen[3]. IA wurde mit dem Reagens nach VAN ECK[8] sichtbar gemacht.

Die Fliessmittel setzten sich aus Komponenten zusammen, die sich ohne Anwendung höherer Temperaturen durch Trocknen der entwickelten Platten an der Luft entfernen liessen. Untersucht wurden 2 Fliessmittelsysteme in abgestuft veränderter Zusammensetzung:

System A: Chloroform–Tetrachlorkohlenstoff–Methanol.

System B: Chloroform–Äthanol.

Als drittes Fliessmittel wurde Äthylacetat–Isopropylalkohol–Wasser (65:24:11) verwendet.

Die Ergebnisse mit System A und B sind in den Fig. 1 und 2 graphisch dargestellt. Farbreaktionen der Indolderivate sowie deren Laufzeiten und R_F-Werte in den geeignetsten Fliessmitteln und in Äthylacetat–Isopropylalkohol–Wasser (65:24:11) können der Tabelle I entnommen werden. Alle Werte sind Mittelwerte aus 4–6 Versuchen.

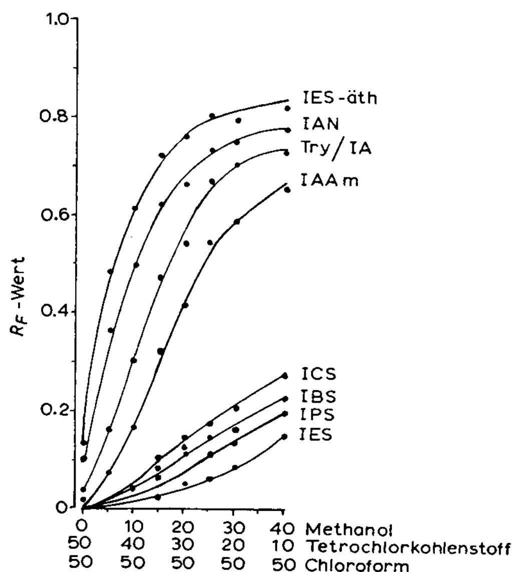


Fig. 1. Abhängigkeit der R_F-Werte von 9 Indolderivaten vom Verhältnis Tetrachlorkohlenstoff: Methanol in Fliessmitteln mit konstantem Gehalt an Chloroform (Abkürzungen siehe S. 152–3).

ERGEBNISSE UND DISKUSSION

Fliessmittelsystem A: Chloroform–Tetrachlorkohlenstoff–Methanol (Fig. 1)

Mit geringem Methanol-Anteil eignet sich dieses Gemisch gut zur Ab- und Auftrennung nichtsaurer Indolderivate, besonders in folgender Zusammensetzung: Chloroform–Tetrachlorkohlenstoff–Methanol (50:40:10). In diesem Fliessmittel bleiben die sauren Indolverbindungen am Startpunkt, die nichtsauren werden gut getrennt. Eine Ver-

ringerung des Gehaltes an Tetrachlorkohlenstoff zugunsten von Methanol vermindert die guten Trenneigenschaften dieses Systems für nichtsaure Verbindungen und schränkt die effektive Trennstrecke ein.

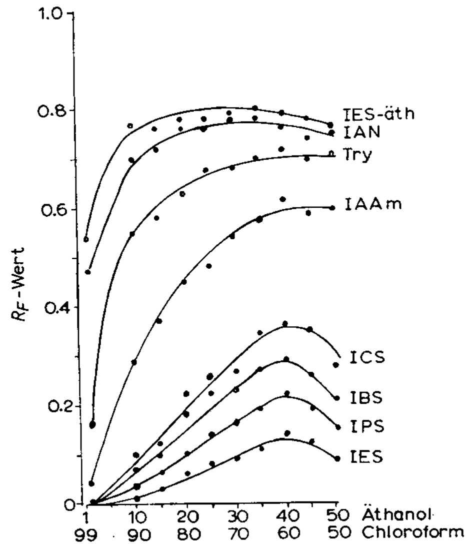


Fig. 2. Abhängigkeit der R_F-Werte von 8 Indolderivaten vom Verhältnis Chloroform: Äthanol (96 %ig) (Abkürzungen siehe S. 152–3).

In der Zusammensetzung 50:25:25 trennt dieses Gemisch saure und nichtsaure Indolderivate noch befriedigend von- und untereinander. Eine weitere Erhöhung des Methanolgehalts führt zwar zu höheren R_F-Werten der sauren Verbindungen, gleichzeitig aber zu grösseren diffusen Flecken. Damit verschlechtern sich Trennung und untere Nachweisgrenze.

In diesem Fliessmittelsystem verhalten sich IA und Try chromatographisch gleich, lassen sich aber durch geeigneten Nachweis (IA färbt sich mit VAN ECK-Reagens sofort intensiv gelb) identifizieren.

Fliessmittelsystem B: Chloroform–Äthanol (Fig. 2)

Über die Einsatzmöglichkeiten dieses Systems für bestimmte Trennungsaufgaben entscheidet der Äthanolgehalt. GMELIN UND VIRTANEN[2] verwendeten zur Identifizierung der Spaltprodukte des Glucobrassicins die Kombination 99:1.

Davon ausgehend wurde in eigenen Versuchen die Polarität des Systems durch Erhöhung des Äthanolgehalts gesteigert und versucht, es zur Trennung sowohl der sauren als auch der nichtsauren Verbindungen heranzuziehen.

Mit geringem Äthanolgehalt (bis 10 %) eignet es sich gut zur Trennung der nichtsauren Indolderivate, wobei auch IAN und IES-äth recht gut getrennt vorliegen. Mit zunehmender Polarität des Gemisches verschlechtert sich wie bei Fliessmittelsystem A die Trennung der nichtsauren Verbindungen und verbessert sich die der sauren. Letztere werden am besten durch das Fliessmittel Chloroform–Äthanol (60:40) getrennt. Weiterer Zusatz von Äthanol verursacht niedrigere R_F-Werte.

Fliessmittel: Äthylacetat–Isopropylalkohol–Wasser (65:24:11)

Die Trennung der Indolsäuren war in den bisher beschriebenen Fliessmitteln unbefriedigend, besonders wegen der relativ niedrigen R_F-Werte von IES. Bei der DC pflanzlicher Extrakte verbleiben Verunreinigungen vorwiegend in der Nähe des Startpunktes und können den IES-Nachweis stören.

Aus zahlreichen, weniger geeigneten Gemischen wurde das bereits für die Trennung von Phenolcarbonsäuren[9] verwendete Fliessmittel Äthylacetat–Isopropylalkohol–Wasser (65:24:11) ausgewählt (Tabelle I).

Von den in der Tabelle I angeführten Fliessmitteln eignen sich die Gemische I und II nur zur Trennung der nichtsauren Indolderivate. Zur Trennung der sauren Verbindungen kann Fliessmittel III eingesetzt werden. Die Fliessmittel IV und V ermöglichen die gleichzeitige Trennung saurer und nichtsaurer Indolstoffe. In allen untersuchten Fliessmitteln bleibt das Fleckenmuster, d.h. die Reihenfolge der Flecken, der Indolderivate gleich (Fig. 1, 2; Tabelle I).

TABELLE I

R_F-WERTE VON INDOLDERIVATEN IN 5 FLIESSMITTELN, DURCHSCHNITTLICHE LAUFZEITEN UND FARBREAKTIONEN MIT VAN URK- BZW. VAN ECK-REAGENS

(Aufgetragene Menge: 0.5 µg; Adsorptionsschicht: Kieselgel G, 250 µm; Kammersättigung; Trennstrecke: 10 cm)

Substanz	*Fliessmittel**					*Farbreaktionen Van Urk*
	I	*II*	*III*	*IV*	*V*	
IES	0.00	0.01	0.29	0.06	0.11	blau-violett
IPS	0.01	0.03	0.37	0.10	0.19	blau
IBS	0.02	0.07	0.44	0.14	0.27	blau
ICS	0.04	0.10	0.57	0.17	0.34	rot
IAAm	0.16	0.29	0.59	0.54	0.57	blau-violett
Try	0.30	0.55	0.70	0.67	0.70	blau-grau (gelber Rand)
IA	0.29			0.68		gelb**
IAN	0.49	0.70	0.75	0.73	0.78	grau
IES-äth	0.61	0.77	0.75	0.80	0.80	violett
Laufzeit (Min.)	25	30	50	30	35	

* Fliessmittel I: Chloroform–Tetrachlorkohlenstoff–Methanol (50:40:10).
II: Chloroform–Äthanol (96%ig) (90:10).
III: Äthylacetat–Isopropanol–Wasser (65:24:11).
IV: Chloroform–Tetrachlorkohlenstoff–Methanol (50:25:25).
V: Chloroform–Äthanol (96%ig) (65:35).
** Sofort erscheinender, intensiver Fleck mit VAN ECK-Reagens.

Durch geeignete Kombination ihrer Bestandteile lassen sich mit diesen neutralen Fliessmitteln spezielle Trennaufgaben lösen. In vielen Fällen wird sich bei Anwendung geeigneter Gemische die in der Auxinforschung übliche Fraktionierung der pflanzlichen Extrakte erübrigen.

In einer weiteren Arbeit wird über den Nachweis und die quantitative Bestimmung zellstreckungsfördernder Indolverbindungen mittels biologischer Testverfahren im Anschluss an die DC berichtet werden[10].

ZUSAMMENFASSUNG

Saure und nichtsaure Indolderivate wurden dünnschichtchromatographisch getrennt unter Verwendung neutraler Fliessmittel. Die R_F-Werte verschiedener Verbindungen in 5 Fliessmitteln werden angegeben.

SUMMARY

Acidic and non-acidic indole derivatives have been separated by means of thin-layer chromatography using neutral solvents. The R_F values of various compounds in 5 solvents are summarized.

LITERATUR

1 M. Kutácek, J. Nováková und M. Valenta, *Flora (Jena)*, 153 (1963) 54.
2 R. Gmelin und A. I. Virtanen, *Ann. Acad. Sci. Fennicae, Ser. A II*, Nr. 107 (1961).
3 E. Stahl und H. Kaldewey, *Z. Physiol. Chem.*, 323 (1961) 182.
4 T. Diamantstein und H. Ehrhart, *Z. Physiol. Chem.*, 326 (1961) 131.
5 H. Kaldewey, persönliche Mitteilung.
6 E. Stahl, *Dünnschicht-Chromatographie*, Springer, Berlin, Göttingen, Heidelberg, 1962.
7 M. H. Zenk, *Planta*, 58 (1962) 668.
8 H. F. Linskens, *Papierchromatographie in der Botanik*, Springer, 2. Aufl., Berlin, Göttingen, Heidelberg, 1959.
9 J. Halmekoski, *Suomen Kemistilehti*, 35 (1962) 39 (zit. bei [6]).
10 G. Ballin, in Vorbereitung.

SEPARATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY THIN-LAYER CHROMATOGRAPHY ON IMPREGNATED LAYERS

ARNE BERG AND JØRGEN LAM

Department of Organic Chemistry, Chemical Institute, University of Aarhus (Denmark)

(Received February 21st, 1964)

INTRODUCTION

The separation of polycyclic aromatic hydrocarbons from complex mixtures as encountered, for example, in tobacco smoke, air pollution studies[1,2], and in pyrolysis experiments[3] has been achieved mainly by chromatographic means, usually column or paper methods.

A small number of studies have been carried out on known mixtures of hydrocarbons using paper chromatography[4-7]. Quite good separations have been accomplished, but the method suffers, among other things, from non-reliability of R_F values unless the conditions for running the chromatograms are controlled very strictly. The chief difficulty is due to the necessity of using specially impregnated (acetylated) paper, the reproducibility of the grade being rather poor.

The possibility of finding a more easily accessible (and much less expensive) chromatographic adsorbent prompted the use of thin-layer chromatography (TLC). Although the reproducibility of R_F values by this method is not better than in paper chromatography, other advantages (shorter running time, sharper spots and a broader spectrum of spray reagents, for instance) connected with TLC, made this method promising for the present purpose.

Since this work was started two papers have appeared dealing with the problem. WIELAND, LÜBEN AND DETERMANN[8] succeeded in separating a mixture of hydrocarbons* on a plate covered with *acetylated* cellulose powder. The chromatogram was developed with a methanol–ether–water mixture. Only very few details are given.

In a more detailed study KUCHARCZYK, FOHL AND VYMĚTAL[9] used silica gel and alumina as adsorbents. The R_F values (with *n*-hexane and with carbon tetrachloride as solvents) of a number of hydrocarbons (and some heterocyclic compounds) are reported, as well as their colours in ultraviolet light and when sprayed with tetracyanoethylene and with formaldehyde sulphuric acid reagent. It is emphasized, however, that the R_F values depend very much on experimental conditions. As to the condensed polycyclic hydrocarbons examined the separation, judged by the reported R_F values, is poor.

In the present study we have examined a number of polycyclic aromatic hydro-

---

* Anthracene, phenanthrene, fluoranthene, pyrene, 1,2-benzanthrene, chrysene, perylene and 3,4-benzopyrene. Phenanthrene and pyrene were not separated.

carbons on plates covered with alumina or silica gel, and a variety of developing solvents have been tried.

Following the idea that a complexing reagent, when mixed (in relatively small amounts) with the adsorbent, might cause a further separation due to different complexing power toward different hydrocarbons, we have studied the effect of various admixtures.

EXPERIMENTAL

Hydrocarbons

The hydrocarbons used are summarized in Table I. Except for numbers 12, 14 and 19, supplied by Aldrich Chemical Co., U.S.A., No. 2 from Heyl and Co., Germany, and No. 5 and 10, which were synthesized in this laboratory, the rest of the compounds was supplied by L. Light & Co., Great Britain. The purity of most of these substances, purified if necessary, was indicated by non-resolvability in a chromatographic test. Anthracene was scintillator grade. Coronene, chrysene and 3,4-benzofluoranthene were rather impure. They were all purified by column chromatography.

Test solutions were prepared in benzene, the concentration of polycyclic hydrocarbon being 0.5 %. When this concentration could not be obtained saturated solutions were used (see Table I). The solutions were kept in darkness.

TABLE I

	Hydrocarbon[a]	Fluorescent spots[b]				Coloured spots[b]	
		Al	*Sil*	*Caf Sil*	*DMF Al*	*TNF Sil*	*TNF Al*
1	Anthracene	v	v	v	v	v	r-br
2	Pyrene	g	g	v	g	br	br
3	Chrysene[c]	bl-v	v	v	v	y-br	y-br
4	3,4-Benzofluoranthene	bl	bl	bl	bl	y	y-br
5	3,4-Benzopyrene[d]	y	y	bl(v)	y	y-gr	gr-g
6	Perylene[c,e]	bl(br)	bl(br)	bl-g	bl(y)	y	y-g
7	1,12-Benzoperylene	y	y-g	v	y-g	bl-gr	br
8	Coronene[c]	v	v	v	v	br	r-br
9	Fluoranthene	bl	l.bl	bl			
10	1,2-Benzanthracene	l.bl	l.bl	v			
11	1,2-5,6-Dibenzanthracene[c]	bl-g	bl	v			
12	1,2-3,4-Dibenzanthracene	bl	bl	v			
13	1,2-3,4-Dibenzopyrene[d]	bl	y	y			
14	1,2-4,5-Dibenzopyrene[c,d]	g	bl	v			
15	3,4-9,10-Dibenzopyrene[c,d]	br	y-br	bl-v			
16	Fluorene	—	—	—		y	
17	Acenaphthylene	—	—	—		y	
18	Phenanthrene	—	—	—		y	
19	Triphenylene	—	—	—		y	
20	1,2-Benzopyrene[c,d]	bl	bl	v		br	
21	2,3-Benzanthracene	—	g	g		v	

[a] Hydrocarbon mixtures used were as follows: I = 1–3; II = 1–8; III = 9–15; IV = 16–21.

[b] Abbreviations: Al = alumina; Sil = silica gel; Caf = caffeine; DMF = dimethylformamide; TNF = 2,4,7-trinitrofluorenone; bl = blue; br = brown; g = green; gr = grey; l = light; r = red; v = violet; y = yellow. A dash indicates that the spot is non-fluorescent.

[c] A saturated solution (in benzene) was used for spotting the plates.

[d] Concerning the numbering of positions in pyrene see ref. 11.

[e] In most cases a perylene spot in U.V. light was bright blue with a distinct brown or yellow-brown centre region.

Unless otherwise indicated 1 μl of sample solution was used for spotting the plate. The spotting was done by means of a micropipette of the constriction type (total volume 1 μl).

Solutions containing more than one hydrocarbon (test mixtures) were prepared by mixing equal volumes of the solutions of the pure substances, and as many microliters were applied to the plate as the number of hydrocarbons in the mixture. A 2 μl pipette was used. The test mixtures used are indicated in Table I.

For quantitative work (extraction and estimation by U.V.-spectroscopy) greater amounts of hydrocarbons are needed*.

Plates

Glass plates, 20 × 20 cm, were used. The adsorbent layers were prepared by means of a Mutter-Hofstetter device** and the thickness of the layer was in all but a few instances 0.25–0.30 mm. Prescribed procedures (see *e.g.*, ref. 10) were followed. After air-drying for half an hour the plates were activated at 150° for 3 h (alumina) or at 120° for 2 h (silica gel). The plates were stored in a desiccator over silica gel. Plates that were not heat-activated, *i.e.* simply dried in air for 24 h, were also used.

Adsorbents

The adsorbents used were the following: Alumina (Aluminiumoxyd-G, Merck) and silica gel (Kieselgel-G, Merck, nach Stahl).

Impregnated layers

The following substances were used for impregnating the layers: picric acid, styphnic acid, 2,4,7-trinitrofluorenone, caffeine, urea, dimethylformamide, and silver nitrate.

The active layers were prepared as follows: for the three first-named compounds the one in question was dissolved in 1 or 2 ml of acetone or alcohol, and the solution added to the amount of water to be used for preparing the layer in the usual way. In this way the complexing reagent was evenly distributed on the adsorbent.

The amounts of these three nitro compounds used per unit area of plate were about equivalent to (or twice that of) the amounts of hydrocarbons per unit area of the separated spots. This means that, according to the amounts of hydrocarbons used (see above), each plate was loaded with 7.5 mg (or 15 mg) of the reagent.

Caffeine (0.5 g per plate), urea (0.5 g per plate) or silver nitrate (2.5 g per plate) were dissolved in the amount of water necessary for preparing the plates. It was necessary to dissolve caffeine at 50–60° taking advantage of the largely enhanced solubility of caffeine at increased temperature. In order to avoid recrystallization of the caffeine when suspending the adsorbent in the solution, the adsorbent too was preheated to the same temperature. The silver nitrate plates were dried and stored in the dark. The amount of caffeine corresponded to a molar ratio of caffeine to hydrocarbon of roughly 100 to 1.

For the preparation of dimethylformamide-impregnated plates, heat activated alumina-covered plates were dipped for 5 min in an ethereal solution of the reagent (0.5, 5 or 10%), and then, after air-drying for half an hour, they were dried for a further five minutes at 70°. Silica gel layers loosened from the glass when treated in this way.

* Work on this line is in progress and will be published soon.
** Camag & Co., Muttenz, Switzerland.

Solvents

Pure solvents as well as solvent mixtures were tried. Except for benzene, the solvents used were not analytical grade or specified for chromatographic use, and were in most cases chemically pure laboratory reagents, which, if necessary, were further purified. Ethyl ether was freed from peroxides. Light petroleum, the bulk solvent used, was a fraction with b.p. 40–50°. All the solvents used were non-fluorescent in ultraviolet light. Per cents indicated for mixed solvents are by volume.

Development

The distance travelled by the solvent front was 13 cm in all cases, ascending technique being used. The development tank (9 × 22 × 23 cm) was lined with filter paper on one side. The running time varied between 30 and 50 min. All experiments were run at room temperature, 22° ± 1°. Very often, repeated developments were performed on a plate. The plate was dried for a short time in a stream of air (room temperature) before the next run.

Detection and identification

The spots on the developed chromatograms were located by inspection in ultraviolet light (3660 Å). It is interesting to note that the fluorescence colours on caffeine-impregnated silica gel plates are somewhat different from (see Table I) and much more brilliant than the colours on ordinary plates. Equally interesting is the fact, that the spots, when developed on such plates, are distinctly sharper than usual.

When nitro compounds are added to the active layer, the fluorescence in ultraviolet light disappears. With 2,4,7-trinitrofluorenone the spots of the hydrocarbon–trinitrofluorenone complexes become visible. The fluorescence is not quenched on plates impregnated with dimethylformamide or with silver nitrate.

Spraying with conc. sulphuric acid or nitric acid followed by heating in an oven to 180–200°, as well as contact with iodine vapour, has been used for detection.

RESULTS AND DISCUSSION

Table I records the fluorescence colours in ultraviolet light and the colours in daylight of the hydrocarbons on different types of layers. It should be emphasized that the fluorescence colours depend somewhat on such factors as concentration of hydrocarbon in the spot, possible impurities, and whether the plate is wetted by the solvent or not. The colours indicated apply to a plate dried in air.

In Figs. 1–3 a series of representative chromatograms are recorded. For each the following details are given: hydrocarbon mixture (II, III or IV); type of layer; mode of impregnation and activation of layer; developer; number of repeated runs on the plate using the same developer; mode of detection (ultraviolet light (U.V.), and colour of complex (CC)).

Developers, size of samples

Some indication for the selection of suitable developers was obtained from a preliminary study involving the three hydrocarbons anthracene (1), pyrene (2), and 3,4-benzopyrene (5). Only ordinary alumina or silica gel plates, activated as described, were used for this purpose.

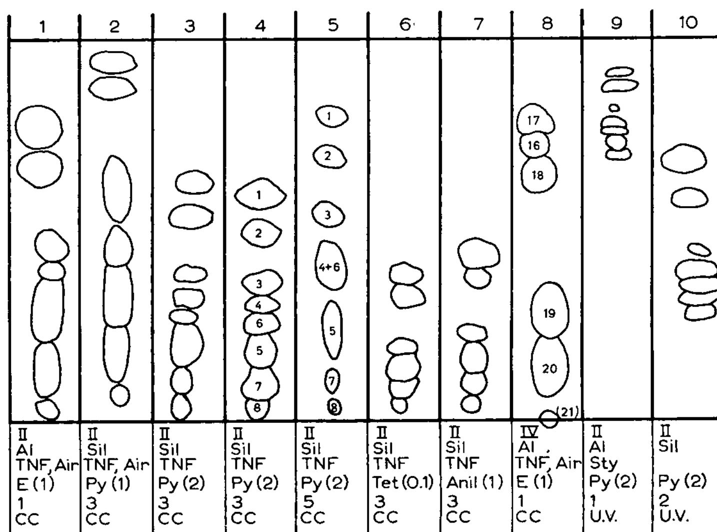


Fig. 1. Effect of 2,4,7-trinitrofluorenone (TNF). Symbols: II and IV = hydrocarbon mixtures II and IV; Sil = silica gel G; Al = alumina G; Sty = styphnic acid; Air = plate air-dried only; E = ether; Py = pyridine; Tet = tetralin; Anil = aniline. Bracketed figures indicate per cent (by volume) of the named component in a mixture with light petroleum. Figures in the fifth row indicate number of runs on a plate. Order of appearance of hydrocarbons II given in chromatograms 4 and 5 apply to the other chromatograms, too. CC = detection by colour of complex.

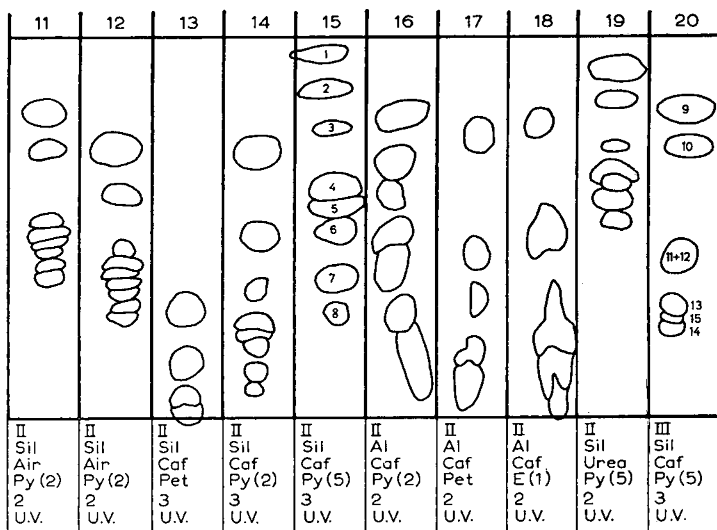


Fig. 2. Effect of caffeine (Caf). Symbols: II and III = hydrocarbon mixtures II and III; Pet = light petroleum; U.V. = detection by fluorescence in ultraviolet light. For the other abbreviations see legend to Fig. 1. Order of appearance of hydrocarbons II given in Expt. 15 apply to the other chromatograms, too.

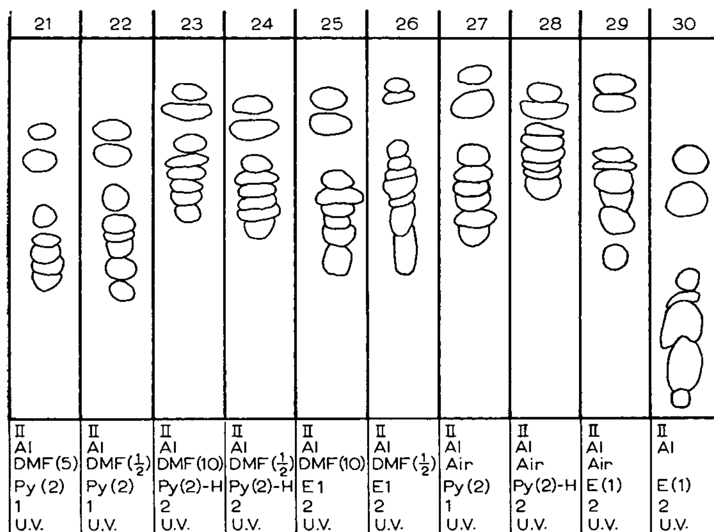


Fig. 3. Effect of N,N-dimethylformamide (DMF, bracketed figures indicate concentration of DMF in the ethereal solution used for impregnation) and of air-drying (Air). Symbols: H means that *n*-heptane was used instead of light petroleum. For the other abbreviations see legends to Figs. 1 and 2. Order of appearance of hydrocarbons II in all chromatograms is as indicated in Expt. 15 (Fig. 2).

The eluting power in a series of pure solvents was found to follow the order usually called the eluotropic series[10]. Only aliphatic and alicyclic hydrocarbons showed any promising separating effects whereas other solvents, including chlorinated aliphatic hydrocarbons, were found to be quite unsatisfactory. This is in contrast to the findings of KUCHARCZYK, FOHL AND VYMĚTAL[9] who used chloromethanes. The reported R_F values of the polycyclic aromatics are, however, not very different from each other.

Light petroleum was found to be the most suitable pure solvent, especially when used on alumina plates. But with more complex hydrocarbon mixtures it failed to separate the slower running hydrocarbons.

Much better results were obtained with mixed developers with light petroleum as the bulk solvent. Polar solvents, in small amounts, modified the developer to give fairly good separations. Pyridine (2–5 %), ether (1 %) and acetic acid (0.04 %) were found most useful. Ether and acetic acid were especially so on alumina plates whereas pyridine worked better on silica gel. A variety of other polar and some non-polar solvents were also possible additives, *e.g.* aniline and tetralin (Expt. 6 and 7, Fig. 1).

Adsorbents and impregnating agents

The obvious idea that a complexing agent, when admixed with the adsorbent, may influence the mobility of the substances to be separated, has been utilized in very few instances[10] and then only silver nitrate and boric acid have been used as complexing agents. Separation of some *cis–trans* isomers, for instance, has been thus achieved.

Nitro compounds. With polycyclic aromatics, the agents of choice would seem to be such well-known substances as picric acid and styphnic acid, 2,4,7-trinitro-

fluorenone, *sym*-trinitrobenzene, and tetracyanoethylene. Whereas the two last-mentioned compounds still have to be tried, the three others are included in this study. Picric acid is valueless, but styphnic acid showed some effect (Expt. 9, Fig. 1), and trinitrofluorenone proved to be excellent (Fig. 1). Since the complexes formed are coloured, the possibility of direct detection of the spots is offered by this method. This of course is of special value for non-fluorescent hydrocarbons. Detection, undoubtedly, can be difficult in some instances due to faintly coloured complexes. Identification of the different hydrocarbons cannot be done by colours alone, but must be supplemented by R_F values or, preferentially, by running pure test substances on the same plate. Use of the last-mentioned technique is appropriate for a number of reasons. First, R_F values in thin-layer chromatography are often not very well-defined due to difficulties in reproducing experimental conditions exactly, this being the case when complexing agents are admixed with the adsorbent. Furthermore it was noticed repeatedly, though not always, that hydrocarbons with only slightly different R_F values, when separating from a mixture, move faster than when running alone. This is probably due to overloading of the starting spot containing the mixture. Secondly, R_F values refer to a single run on a plate, whereas in this study it has been found advantageous to repeat a run on a plate two, three or even more times. Yet, the reproducibility of experiments is satisfactory, as illustrated by experiments 3 and 4 in Fig. 1. These considerations apply to other types of plates too (Expt. 11 and 12, Fig. 2). The effect of repeated runs is illustrated by Expt. 4 and 5 (Fig. 1).

Caffeine. It is well known that association colloids which, in aqueous solution, form micelles at a critical concentration exert a solubilizing effect on polycyclic aromatic hydrocarbons[12]. The same is true for lactic and butyric acid[13], though with these substances it is not necessarily due to micelle formation. The solubilizing effect exerted on the aromatics by purines and related substances in aqueous solution is evidently caused by complex formation. The ability of caffeine in this respect is outstanding. This effect was first noticed by BROCK, DRUCKREY AND HAMPERL[14] and later studied thoroughly by WEIL-MALHERBE[15] and by BOYLAND AND GREEN[16].

It is now found that caffeine, used in admixture with thin-layer adsorbents (silica gel), has a pronounced influence on the separation of mixtures of polycyclic aromatic hydrocarbons. Probably complex formation is the cause in this case too. As far as the polar character of the aqueous solvent is of importance for the complex formation, this possibility has its counterpart in chromatography in the polarity of the adsorbent. As can be seen from experiments 10, 11, 14, 15 and 16, 17, 18 (Figs. 1 and 2), the effect is most evident on silica gel plates.

The brilliancy of the fluorescence colours on caffeine-impregnated plates can possibly be explained as a result of diminished transparency of the silica gel layer, light-scattering in the layer causing increased secondary excitation of the hydrocarbons. The observed shifts in colours are quite understandable on account of complex formation.

BOYLAND AND GREEN[16] found that urea was without effect on the solubilization of polycyclic aromatic hydrocarbons, in agreement with our finding that urea was of no value for the separation of these substances (compare Expt. 10 (Fig. 1), 11 and 19 (Fig. 2)).

Attempts to utilize the solubilization effect by developing the chromatograms with aqueous solutions of caffeine, with or without added pyridine, failed. On alumina

layers the hydrocarbon spots move rapidly, but unfortunately with severe tailing and very poor separation. On silica gel, on the contrary, the spots move very slowly being accelerated with increasing concentration of caffeine and still more so by addition of pyridine. In the last case tailing becomes pronounced and at any rate no separation is obtained.

Silver nitrate. The ability of the silver ion to bind to π-electron structures has been demonstrated by fractional extraction of benzene homologues with aqueous solutions of silver nitrate. The principle, as stated above[10], has proved valuable in some instances in thin-layer chromatography on silica gel plates, and the possibility of applying it to the problem at hand was examined. Separations, however, were not satisfactory.

Dimethylformamide. The effect of impregnating alumina layers with dimethylformamide is seen from Fig. 3, experiments 25 and 30. The spots move faster than on unimpregnated plates, but separation is almost the same. Furthermore, there is no significant difference between the developers used (experiments 21 to 26), and the concentration of the solution of dimethylformamide used to prepare the plates seems to be of no importance. An interesting observation can be made by comparing experiment 28 with numbers 23 and 24, and experiment 29 with numbers 25 and 26. These chromatograms show that drying of the alumina layers in air, without subsequent activation by heat, has almost the same effect as impregnating activated layers with dimethylformamide. In experiment 27 the spots have moved a little further than in numbers 21 and 22, but otherwise these chromatograms are very much alike.

Often chromatography on adsorbents impregnated with dimethylformamide or similar substances is regarded as purely underlying the partition principle, although it is argued, too, that adsorption may play a rôle. This evidently must be the case with polycyclic aromatic hydrocarbons in view of the observed similar effects of water and dimethylformamide and since partition between two components, one of which is water, is not very likely without participation of the alumina to which the water is adsorbed.

R_F values

Tabling of reliable R_F values and use of such figures demands a precisely standardized technique which must be strictly followed. As this is not necessary in order to obtain good separations, as discussed above, no list of R_F values will be given here. We believe it safe to say that too many R_F values recorded in the literature are of limited value for identification purposes beyond being merely suggestive. Qualitatively, a general order of appearance of the different hydrocarbons in a chromatogram can be stated. From top to bottom, the order for the most frequently used mixture (II) almost invariably is as follows: anthracene (1), pyrene (2), chrysene (3), 3,4-benzofluoranthene (4), 3,4-benzopyrene (5), perylene (6), 1,12-benzoperylene (7), and coronene (8). This order is maintained even when other adsorbents or developers are used except for (5) and (6), which are interchanged on layers impregnated with TNF (see Expt. 4, 5 (Fig. 1) and 15 (Fig. 2).

For the two other mixtures the order, as far as they have been examined, is (only numbers are given) for III: 9, 10, 11, 12 (or 12, 11), 13, 14 (or 14, 13), and 15; and for IV: 16, 17 (or 17, 16), 19, 20, and 21. The alternative orders are connected with the lack of degree of separation of such closely related hydrocarbons as 11 and 12

and similarly 13, 14 and 15. Whereas the three last-mentioned ones can be distinguished from each other, by their fluorescence, in the chromatogram (Expt. 20, Fig. 2), this is not the case with 11 and 12. With mixture IV the best results were obtained on TNF-impregnated plates (Expt. 8, Fig. 1). As our experimental evidence, concerning the hydrocarbons of III and IV, is rather scarce only the two chromatograms mentioned are shown in this paper.

In some of the chromatograms, although separation has occurred, there are more spots lying very close together. Certainly such spots do not represent pure hydrocarbons, but separation is sufficient to secure, in many instances, the identity of the main component in a spot, when relative position is considered and some colour test is applied and, preferentially, comparison with test substances is made.

Two-dimensional development

This technique has been used in order to increase the number of consecutive runs on a plate using the same developer. No change in development conditions is introduced by this procedure, but well developed chromatograms are obtained. A single experiment is recorded showing the successful separation of ten spots from a mixture of eleven hydrocarbons (Fig. 4).

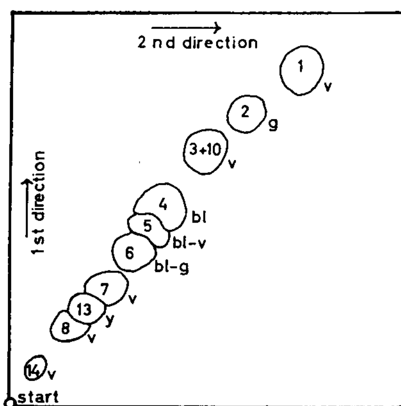


Fig. 4. Development in two directions in a separation of eleven hydrocarbons including test mixture II and hydrocarbons 10, 13, and 14. Adsorbent, caffeine on silicagel. Developer, pyridine (5%) in light petroleum. Two runs in each direction. Numbers in spots indicate hydrocarbons identified by fluorescence colours and distances travelled. Abbreviations: bl = blue; g = green; v = violet; y = yellow.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Statens almindelige Videnskabsfond, Denmark, for a grant to both of them. We gratefully thank Prof. HAKON LUND for working facilities placed at our disposal. For technical assistance our thanks are due to Mrs. VIBEKE JENSEN.

SUMMARY

Thin-layer chromatographic separation on alumina and on silica gel of mixtures of polycyclic aromatic hydrocarbons is promoted by modifying the adsorbent with

small amounts of complex forming substances. 2,4,7-Trinitrofluorenone on alumina and caffeine on silica gel are very efficient. Mixtures of light petroleum with small amounts of polar solvents serve well as developers.

NOTE ADDED IN PROOF

The use of some nitro compounds as complexing agents in TLC is reported in a note by FRANCK-NEUMANN AND JÖSSANG[17].

REFERENCES

1 J. BONNET AND S. NEUKOMM, *Oncologia*, 10 (1957) 124.
2 B. L. VAN DUUREN, *J. Natl. Cancer Inst.*, 21 (1958) 1.
3 J. LAM, *Acta Pathol. Microbiol. Scand.*, 39 (1956) 198; *ibid.* 45 (1959) 237.
4 D. S. TARBELL, E. G. BROOKER, A. VANTERPOOL, W. CONWAY, C. J. CLAUS AND T. J. HALL, *J. Am. Chem. Soc.*, 77 (1955) 767.
5 T. WIELAND AND W. KRACHT, *Angew. Chem.*, 69 (1957) 172.
6 F. MICHEL AND W. SCHMINKE, *Angew. Chem.*, 69 (1957) 334.
7 T. M. SPOTSWOOD, *J. Chromatog.*, 2 (1959) 90; *ibid.* 3 (1960) 101.
8 TH. WIELAND, G. LÜBEN AND H. DETERMANN, *Experientia*, 18 (1962) 430.
9 N. KUCHARCZYK, J. FOHL AND J. VYMĚTAL, *J. Chromatog.*, 11 (1963) 55.
10 E. V. TRUTER, *Thin Film Chromatography*, Cleaver Hume Press, London, 1963.
11 H. VOLLMANN, H. BECKER, M. CORELL AND H. STREECK, *Ann.*, 531 (1937) 1.
12 P. EKWALL AND K. SETÄLÄ, *Acta Chem. Scand.*, 2 (1948) 733.
13 P. EKWALL AND L. SJÖBLOM, *Acta Chem. Scand.*, 6 (1952) 96.
14 N. BROCK, H. DRUCKREY AND H. HAMPERL, *Arch. Exptl. Pathol. Pharmakol.*, 189 (1938) 709.
15 H. WEIL-MALHERBE, *Biochem. J.*, 40 (1946) 351.
16 E. BOYLAND AND B. GREEN, *Brit. J. Cancer*, 16 (1962) 347.
17 M. FRANCK-NEUMANN AND P. JÖSSANG, *J. Chromatog.*, 14 (1964) 280.

THIN-LAYER CHROMATOGRAPHY OF PROTEINS ON SEPHADEX G-100 AND G-200

C. J. O. R. MORRIS

Department of Experimental Biochemistry, The London Hospital Medical College, London (Great Britain)

(Received March 5th, 1964)

Gel filtration[1] and thin-layer chromatography[2] are now both established analytical techniques. The advantages of thin-layer chromatography, speed and adaptability to very small samples, make it very attractive for the fractionation of proteins on the ultramicro scale. HOFMANN[3] has described the chromatography of several proteins on thin layers of hydroxyl-apatite, while DETERMANN[4] and JOHANSSON AND RYMO[5] have described thin-layer methods using the cross-linked dextran gel filtration medium Sephadex (Pharmacia, Uppsala, Sweden). The method of DETERMANN[4] was developed for the tightly cross-linked dextran gel Sephadex G-25, and was not suitable for the chromatographic separation of proteins. The method of JOHANSSON AND RYMO[5] was used with both Sephadex G-25 and G-75, and in the latter case could be used for the chromatography of small proteins.

The availability of the loosely cross-linked bead-form materials Sephadex G-100 and G-200 has extended the upper molecular weight limit for the successful chromatography of proteins up to at least 180,000. The application of these materials in the thin-layer chromatography of proteins has necessitated the development of a special technique described in detail in this paper.

EXPERIMENTAL

Materials

Sephadex G-100 (water regain 10, batch No. TO 1992) and G-200 (water regain 20, batch No. 224C) both with particle size $<$ 400 mesh were obtained from Pharmacia A.B., Uppsala, Sweden through the courtesy of Dr. B. GELOTTE.

Proteins

Bovine serum albumin (BSA)	Crystalline, Armour & Co. England.
Bovine γ-globulin (γ)	Armour & Co. England.
α-Chymotrypsin (Chy)	3 × crystallized, Seravac Laboratories, Colnbrook, England.
Cytochrome *c* (Cyt)	Salt-free, Seravac Laboratories.
Haemoglobin (Hb)	Human carboxy-, prepared from washed red cells, stored at $-15°$.
Lysozyme (Lys)	4 × crystallized, gift from Dr. J. R. MARRACK.
Myoglobin (Myo)	Crystalline, whale, Seravac Laboratories.
Ovalbumin (Ova)	Crystalline, Light & Co. Colnbrook, England.

Ovomucoid (Ovm)	Crystalline, Light & Co.
Pepsin (Pep)	2 × crystallized, Armour & Co.
Ribonuclease (Rib)	Crystalline, B. D. H. Ltd., Poole, England.
Thyroglobulin (Thy)	Light & Co.
Trypsin (Try)	2 × crystallized, Seravac Laboratories.

Method

Gel suspensions were prepared by thoroughly mixing 6 g of Sephadex G-100 or 4 g of Sephadex G-200 with 100 ml of the solvent in a beaker. The concentrations given are critical, and should be strictly adhered to, although it is possible that different optimum concentrations may be necessary with different batches of Sephadex. Care should be taken to ensure that no aggregates are present in the final gel suspension. The quantities given are sufficient for six 10 × 20 cm plates. The gel suspension should be stored in the covered beaker for at least 48 h to allow swelling of the dextran gel to proceed to completion. 10 × 20 cm glass plates are thoroughly cleaned with detergent and distilled water before use, and the dry plates are coated with a 0.9 mm thick layer of Sephadex by means of a Camag thin-layer spreader (Camag A.G., Muttenz, Switzerland). The plates are immediately transferred to a closed vessel containing a dish of the solvent, and stored in the horizontal position for at least 18 h before use. This pre-equilibration process markedly improves the reproducibility of the results. The plates are then mounted horizontally, preferably with illumination of the translucent gel layer from underneath, and 0.5–1.0 μl of the test solution containing 1–20 μg of protein applied as a series of spots about 1.5 cm apart in a line 3 cm from a short edge of the plate. A micro-pipette or preferably a micrometer syringe may be used for the application. The size of the sample zone should not exceed 3 mm, and especial care should be taken to ensure that the soft gel layer is not damaged during the application.

Development is carried out in the apparatus shown in Fig. 1.

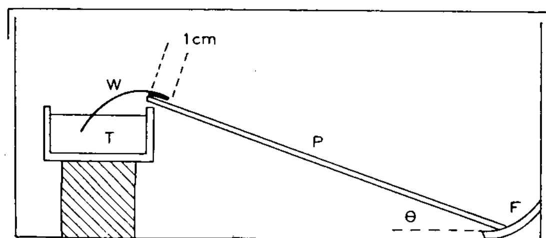


Fig. 1. Apparatus for thin-layer chromatography of proteins.

Solvent (0.5 *M* NaCl in the studies reported here) is led to the plate P by means of the Whatman No. 3 MM filter paper wick W. Excess liquid is prevented from accumulating at the bottom of the plate by the filter paper pad F which is moistened to ensure good contact with the gel layer. The wick W should overlap about 1 cm onto the gel layer and the gel surface at the upper edge of the plate should be 1–1.5 cm above the level of the solvent in the trough T. The angle θ should be about 10° for Sephadex G-100 plates and about 20° for G-200 plates. Human CO-haemoglobin should migrate about 70 mm in 4–5 h under optimum operating conditions. Excessively fast develop-

ment leads to streaking of the zones, but development can be continued up to 8 h or more without apparent loss in resolution. Excessively slow migration rates are however usually an indication of incorrectly prepared plates.

When development is considered to be complete (haemoglobin, cytochrome *c* or myoglobin may be used as markers), the plate is removed from the development chamber, and supported horizontally after careful removal of the wick W. A 10 × 20 cm piece of Whatman No. 3 MM filter paper is then applied progressively to the gel surface, starting near the line of initial zones and proceeding to the other end of the plate. Care must be taken to avoid trapping air bubbles between the gel and the paper. This is facilitated if the smoother side of the paper is downwards, and if the paper is rolled on to the gel layer. Schleicher & Schüll No. 2043b paper appears to give slightly better prints with Sephadex G-100 plates but is unsuitable for G-200 plates owing to its lower water capacity. The position of the line of initial zones is then marked on the paper and the covered plate transferred to an oven for drying at 80–90° for 30 min.

The protein zones may be located on the dried plate by any appropriate staining technique. Staining in 0.2 % Ponceau S in 10 % aqueous acetic acid for 30 min, followed by extensive washing with water to remove excess dye, has been used in most of the present work. 1 % Naphthalene Black 12B (E. Gurr & Co., London, England) in methanol–water–glacial acetic acid (50:40:10), or 0.01 % Nigrosine (E. Gurr & Co.) in the same solvent, followed by washing with the mixed solvent, have been found to be useful where a greater sensitivity is required. During the early stages of the washing process the paper prints become detached from the gel plates so that they may finally be washed and dried separately.

Column chromatography

96 × 1.1 cm columns of Sephadex G-100 (batch No. TO 33) or Sephadex G-200 (batch No. TO 41) (both 100–400 mesh), were packed by the sedimentation method recommended by the manufacturers. 2–8 mg samples of the test proteins in 0.5 ml of 0.5 *M* NaCl were applied to the column and development continued with the same solvent at a flow rate of 2–2.5 ml/h/cm². The column effluent was monitored at 257 mμ by means of an LKB Uvicord recording absorptiometer, and the retention volumes (V_e) of the test proteins obtained directly from the positions of the recorded zone maxima. The value of the gel internal volume (V_i) was obtained from the retention volume of water, the electrical conductance of the column effluent being monitored with a Radiometer type CDM 2 conductance meter and an LKB type 6520 A recorder. The column void volume (V_0) was estimated from the retention volumes of α-macroglobulin or thyroglobulin, which were identical on both the Sephadex G-100 and G-200 columns. Partition coefficients (K_d) were calculated from the retention:

$$K_d = (V_e - V_0)/V_i \qquad (1)$$

RESULTS

Typical thin-layer chromatograms of cytochrome *c*, ovalbumin and thyroglobulin on Sephadex G-100, and lysozyme, haemoglobin and γ-globulin on Sephadex G-200, individually and in mixtures are shown in Figs. 2 and 3 respectively. Fig. 4 shows a thin-layer chromatogram of haemoglobin, bovine serum albumin, γ-globulin and a

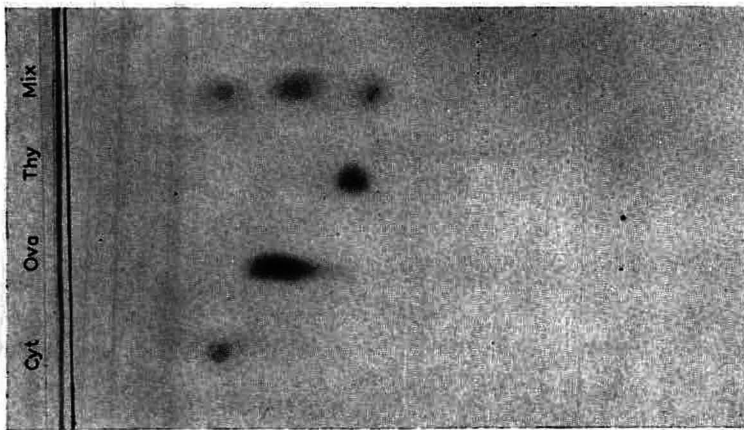


Fig. 2. Thin-layer chromatogram of cytochrome *c* (Cyt), ovalbumin (Ova) and thyroglobulin (Thy) on Sephadex G-100–0.5 *M* NaCl.

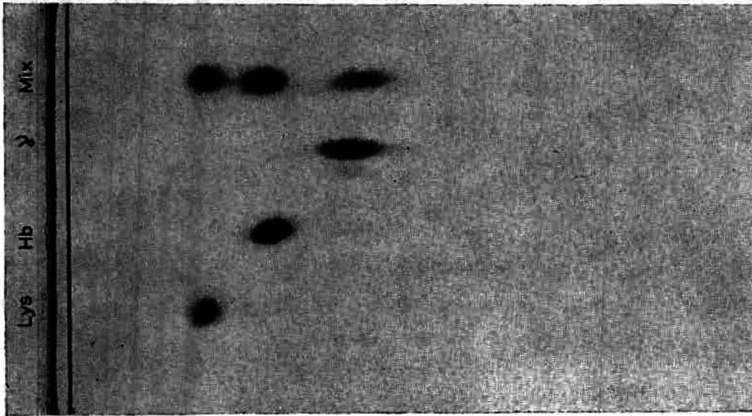


Fig. 3. Thin-layer chromatogram of lysozyme (Lys), haemoglobin (Hb) and γ-globulin (γ) on Sephadex G-200–0.5 *M* NaCl.

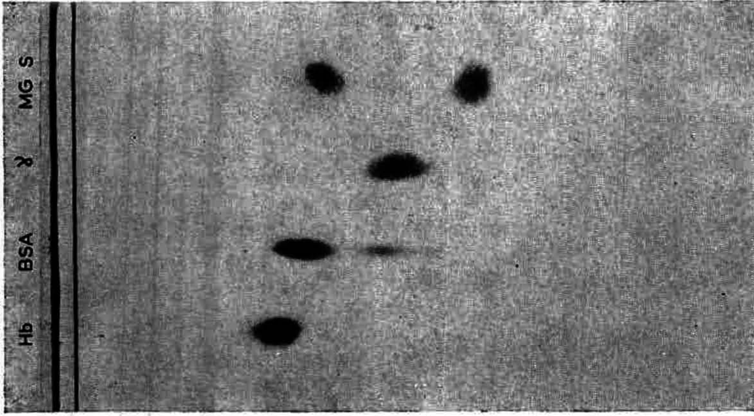


Fig. 4. Thin-layer chromatogram of haemoglobin (Hb), bovine serum albumin (BSA), γ-globulin (γ) and a macroglobulinaemia serum (MG S) on Sephadex G-200 –0.5*M* NaCl.

macroglobulinaemia serum on Sephadex G-200. The fast-moving abnormal macroglobulin component is clearly evident (see FLODIN[6]), which suggests that the thin-layer method may be of diagnostic value in this condition.

Since the position of the liquid front cannot be located in the present method, the migration velocities of the various test proteins have been compared with that of haemoglobin on thin-layer plates of Sephadex G-100 and G-200. An R_{Hb} value is then defined by:

$$R_{Hb} = d_p/d_{Hb} \quad (2)$$

where d_P and d_{Hb} are the distances traversed by the test protein and by haemoglobin respectively during the development of the chromatogram. R_{Hb} values for twelve proteins on G-100 and G-200 based on the mean of 9–13 determinations, are collected in Table I, together with the molecular weights of the proteins.

TABLE I

R_{Hb} VALUES OF PROTEINS IN THE SYSTEMS SEPHADEX G-100–0.5 M NaCl AND SEPHADEX G-200–0.5 M NaCl

Protein	*Molecular weight* $\times 10^{-3}$	R_{Hb}	
		G-100	*G-200*
Cytochrome *c*	13.0	0.68	0.74
Ribonuclease	13.6	0.68	0.74
Lysozyme	14.5	0.65	0.70
Myoglobin	16.9	0.79	0.80
α-Chymotrypsin	22.5	0.87	0.87
Trypsin	23.8	0.83	0.86
Ovomucoid	27.0	0.94	1.03
Pepsin	35.0	0.99	1.04
Ovalbumin	45.0	1.03	1.04
Haemoglobin	68.0	1.00	1.00
Bovine serum albumin	65.0	1.14	1.22
Bovine γ-globulin	180.0	1.28	1.54
Thyroglobulin	650.0	1.33	1.83
Macroglobulins	1,000	—	1.86

DISCUSSION

The chromatogram prints illustrated in Figs. 2 and 3 show the method gives compact, well-defined symmetrical zones with relatively little tailing. Investigation of a series of concentrations of a single protein have shown that R_{Hb} values are concentration independent. The resolving power of the method is remarkably high, and provided that the load is restricted to about 10 μg of any individual protein, substances differing in R_{Hb} by about 0.1 can be completely resolved in a 10 cm migration. The resolving power of the 20 cm plate is thus at least equal to that of a 120 cm column (compare WHITAKER[7]). This high resolving power is undoubtedly due to the small particle size (< 400 mesh) and to the low linear flow rates (1.4–1.6 cm/h) used. Attempts to use the ordinary chromatographic grades of Sephadex (100–400 mesh) gave inferior results, probably due to the greater size heterogeneity of the gel particles. Similarly higher flow rates gave impaired resolution. Since diffusion of the solute zones does not appear to be a limiting factor, plates 50–100 cm long could probably

be used with advantage for difficult separations. The maximum load which can be used without zone distortion appears to be about 20–30 μg of most proteins. The minimum is set only by the limitations of the methods of zone detection.

The relations between R_{Hb} and K_d values for the test proteins on Sephadex G-100 and G-200 are shown in Figs. 5 and 6 respectively. It can be seen that for both systems linear relations exist, so that for Sephadex G-100:

$$K_d^{G100} = 1.35 - 1.0\ R_{Hb}^{G100} \quad (3)$$

while for Sephadex G-200:

$$K_d^{G200} = 1.35 - 0.73\ R_{Hb}^{G200} \quad (4)$$

These equations allow the K_d values for proteins on preparative scale gel filtration columns to be predicted from preliminary experiments with 2–10 μg of material taking about 4 h for completion. Equations (3) and (4) are of course only strictly valid for the solvent used in these experiments.

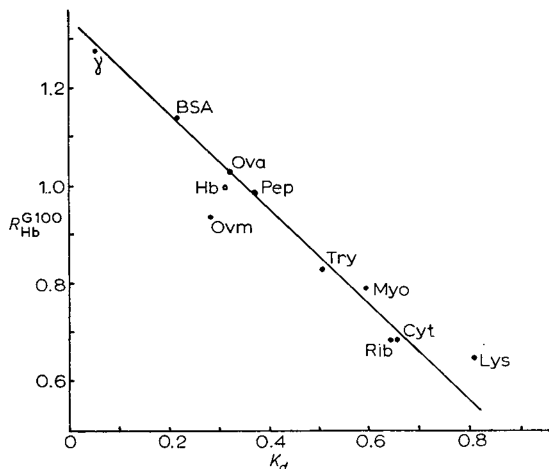


Fig. 5. K_d^{G100} as a function of R_{Hb}^{G100}.

Lysozyme, haemoglobin and ovomucoid depart from the linear relations on both Sephadex G-100 and G-200, although the deviations are less in the latter case. These proteins also show abnormal K_d values in relation to their molecular weights (see below), and their departure from equations (3) and (4) are probably due to minor differences in the Sephadex preparations used for the column and for the thin-layer experiments.

WHITAKER[7] has shown that V_e/V_0 on Sephadex G-100 columns is directly proportional to the logarithms of the molecular weights (M) for a number of proteins, including several of those used in the present work. A similar relation between R_{Hb} and log M should also hold in view of equations (3) and (4). The plot of R_{Hb}^{G100} values against log M was in fact non-linear, although all the experimental points lay on a smooth curve. The plot of R_{Hb}^{G200} against log M was however linear, obeying equation (5):

$$\log M = 1.47\ R_{Hb}^{G200} + 3.0 \quad (5)$$

and is shown in Fig. 7. The reason for this discrepancy is unknown.

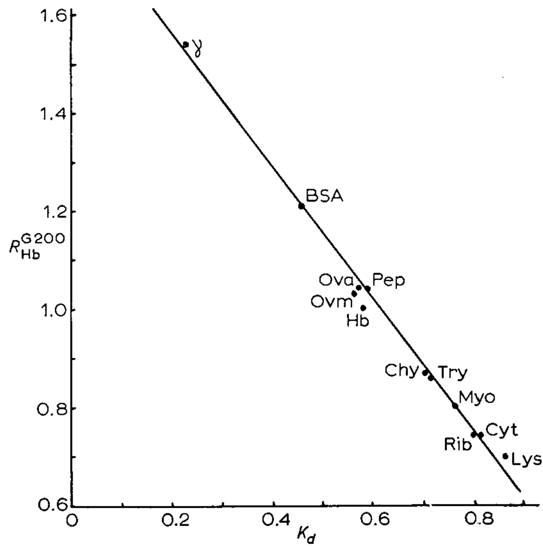


Fig. 6. K_d^{G200} as a function of R_{Hb}^{G200}.

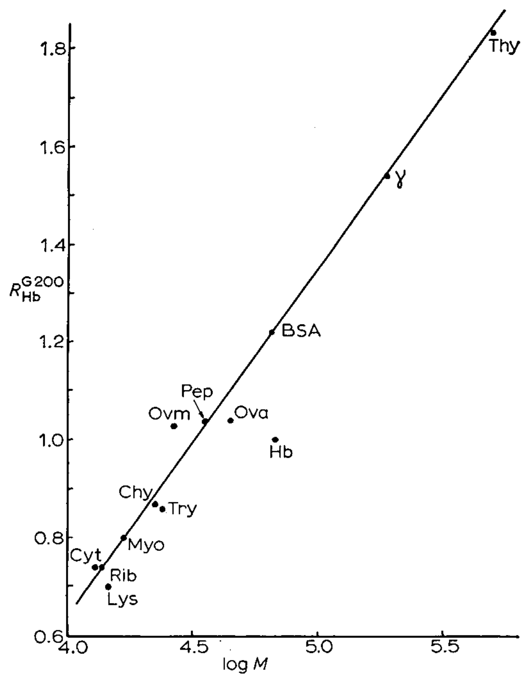


Fig. 7. Log M as a function of R_{Hb}^{G200}.

Lysozyme, haemoglobin and ovomucoid again behave anomalously. The relatively slow migration of lysozyme, probably due to an ion-exchange retardation of this very basic protein has been observed repeatedly[7–9]. Haemoglobin behaves as though it had a molecular weight of 33,000, approximately one-half of the value obtained by sedimentation–diffusion in solution and from X-ray crystallographic data. This anomaly had also been observed previously by ANDREWS[10], on agar, by WHITAKER[7] on Sephadex G-100, and by FAWCETT AND MORRIS[11] on polyacrylamide gel columns. These observations on different gel media make it unlikely that the anomaly is due to ion-exchange or adsorption retardation, while the magnitude of the effect suggests that under certain conditions haemoglobin may dissociate in solution into subunits comprised of a single α and a single β chain with a molecular weight of about 34,000. Alternatively the anomaly may be due to an equilibrium in solution between the 68,000 molecule and the α and β chains, as in the case of α-chymotrypsin investigated by WINZOR AND SCHERAGA[12]. The anomalous behaviour of ovomucoid which has an R_{Hb} value corresponding to a molecular weight of 37,000 has also been observed by WHITAKER[7].

The general validity of equation (5) however allows an estimate of the molecular weight of a protein to be made with an amount of the order of 1 μg by thin-layer chromatography on Sephadex G-200. It may be possible to lower this limit by an order of magnitude if more sensitive methods of zone location can be devised. Preliminary experiments in this laboratory have shown that the Sephadex gel layer can be overlaid with a thin uniform agar layer so that it may be possible to employ the techniques of immuno-diffusion[13] or specific enzymic reaction[14] for the location of protein zones with a great increase both in sensitivity and specificity.

ACKNOWLEDGEMENTS

The author is indebted to Dr. BERTIL GELOTTE and to Pharmacia A.B., Uppsala, Sweden, for gifts of Sephadex, and to the Central Research Fund of the University of London for the purchase of an LKB Uvicord absorptiometer.

SUMMARY

1. A detailed description is given of a technique for the thin-layer chromatography of 1–20 μg amounts of proteins on the cross-linked gel filtration media Sephadex G-100 and G-200.

2. A close correlation has been obtained between the results of the chromatography of twelve test proteins on chromatographic columns and on thin-layer plates.

3. The relation between the molecular weights of proteins and their behaviour on Sephadex gels is discussed and a method for the estimation of protein molecular weights from the results of thin-layer chromatography is derived.

NOTE ADDED IN PROOF

Since this paper was submitted for publication, ANDREWS[15] and JOHANSSON AND RYMO[16] have described methods for thin-layer chromatography of proteins on Sephadex G-100 and G-200.

REFERENCES

1 J. PORATH AND P. FLODIN, *Nature*, 183 (1959) 1657.
2 E. STAHL, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin, 1962.
3 A. F. HOFMANN, *Biochim. Biophys. Acta*, 60 (1962) 458.
4 H. DETERMANN, *Experientia*, 18 (1962) 430.
5 B. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 16 (1962) 2067.
6 P. FLODIN, *Dextran Gels and Their Application in Gel Filtration*, Pharmacia, Uppsala, 1962.
7 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
8 J. PORATH, *Biochim. Biophys. Acta*, 39 (1960) 193.
9 F. MIRANDA, H. ROCHAT AND S. LISSITZKY, *J. Chromatog.*, 7 (1962) 142.
10 P. ANDREWS, *Nature*, 196 (1962) 36.
11 J. S. FAWCETT AND C. J. O. R. MORRIS, to be published.
12 D. J. WINZOR AND H. A. SCHERAGA, *Biochemistry*, 2 (1963) 1263.
13 O. OUCHTERLONY, *Acta Pathol. Microbiol. Scand.*, 26 (1949) 507.
14 R. L. HUNTER AND C. L. MARKERT, *J. Histochem. Cytochem.*, 7 (1959) 42.
15 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
16 B. JOHANSSON AND L. RYMO, *Acta Chem. Scand.*, 18 (1964) 217.

PAPER CHROMATOGRAPHY–ANTHRONE DETERMINATION OF SUGARS*

S. G. SUNDERWIRTH, GARY G. OLSON** AND GESTUR JOHNSON
Department of Chemistry, Colorado State University, Fort Collins, Colo. (U.S.A.)

(Received February 3rd, 1964)

INTRODUCTION

Paper chromatography has been used as a tool in the determination of sugars since the original publication on the subject by PARTRIDGE AND WESTALL[1] appeared in 1948. In many cases the sugars have been located on the paper and determined without elution using photometric[2], reflectance[3,4] or visual[5] measurements. Another technique which has been used is to separate the sugars on paper, locate the sugar spots by means of a color reagent, elute the spots and measure the color spectrophotometrically. Among the reagents which have been used to develop the color using this technique are *p*-anisidine hydrochloride[6] and aniline hydrogen phthalate[7]. PHILLIPPU[8] and SHALLENBERGER AND MOORES[9] have described methods which involve location of sugars on the chromatogram, elution of the unsprayed spots, development of the color with suitable reagents and spectrophotometric analysis of this color.

The anthrone reagent has been used extensively in carbohydrate analysis since the original report by DREYWOOD[10] in 1946. This reagent is a general reagent for carbohydrates and is usually used for the determination of total sugars. However, WISE *et al.*[11] have shown that the anthrone reagent may be used at 50° for the determination of total fructose. Since sugars may be quantitatively eluted from paper using water[12], it would seem that a procedure utilizing paper chromatographic separation followed by elution and anthrone analysis would be a rapid and reliable method for the determination of sugars. Some work centered around these procedures has been reported by DIMLER *et al.*[13], WHISTLER AND HICKSON[14], PAVLINOVA[15] and TAKI[16]. In this paper the standardization of a procedure using paper chromatographic separation of glucose, fructose and sucrose and their subsequent elution and analysis employing the ROE anthrone reagent[17] is reported. The use of this method in the determination of these sugars in plant tissues is also reported.

EXPERIMENTAL

Apparatus

The chromatograms were developed by ascending and descending techniques in pyrex chromatographic jars (8½ × 18 and 12 × 24 in. respectively). The chromatographic paper used was Whatman No. 1 filter paper, which was shown to contain no water extractable substances that interfere with the anthrone reagent.

* Approved by the Director, Colorado Agricultural Experiment Station, as Scientific Series Publication No. 905.
** Present address: Department of Chemistry, Oregon State University, Corvallis, Oregon.

The color formed with the anthrone reagent was read in an Evelyn colorimeter (Rubicon Co., Philadelphia, Pa.) using a 620 mμ filter.

To obtain reproducible spotting volumes, a microsyringe pipet control and micropipets (Hamilton Co., Whittier, Calif.) were employed.

Reagents and solutions

Three stock solutions containing 200 mg of glucose, fructose and sucrose respectively per 10 ml of 80 % ethanol were prepared. A fourth stock solution containing 200 mg each of glucose, fructose and sucrose per 10 ml of 80 % ethanol was prepared.

The developing solvents were those suggested by DURSO AND MUELLER[18]. Ethyl acetate, acetic acid and water were used in the ratios (v/v) 6:3:2, 5:3:2 and 4:3:2. The solvent was freshly prepared once a week.

The spray reagent used was prepared by dissolving 1.0 g of *p*-anisidine hydrochloride in 10 ml of methanol and diluting to 100 ml with 1-butanol. To this solution was added 1.0 g of sodium hydrosulfite. After shaking for several minutes, the mixture was filtered and stored at 4°. The reagent became somewhat colored but was suitable for use for several months.

The anthrone reagent[17] was prepared in the following manner: concentrated sulfuric acid (333 ml) was slowly added to 140 ml of distilled water. Care was taken to prevent the temperature from rising above 110°. Five grams of thiourea (Baker analyzed) was then dissolved in this solution. After the solution had cooled to 90°, 0.25 g of anthrone (Mathieson Chemical Company) was added and the resulting solution stored at 4°. This reagent remained usable for 4 weeks.

Procedure

The solutions containing the sugars in 80 % ethanol were spotted on the paper using the microsyringe pipet control and micropipets. For maximum separation, the spots should not contain more than 300 μg of any sugar. In the analysis of unknown solutions, 10 μl of the fourth stock solution was spotted as a separate spot to serve as an internal standard for glucose, fructose and sucrose. This solution was also spotted on both ends of the chromatogram to serve as end strips in locating the sugars. After air drying, the chromatograms were developed by either descending or ascending techniques.

The spots were located in the following manner: both end strips were cut from the paper and sprayed with *p*-anisidine hydrochloride solution. After drying for 5 min, the strips were held about 3 in. above a hot plate until the color appeared.

Using these end strips as guides, the unsprayed portion of the paper was cut into squares each containing one of the separated sugars. These squares were cut into smaller pieces and placed into test tubes. The sugars were extracted with 5 ml of water (about 1.0 ml of water is used for each 20–50 μg of sugar). During the extraction process (about 1 h) the tubes were shaken every 7–10 min.

To remove the cellulose fibers from the mixture the following procedure was used: filter paper (9 cm Whatman No. 1) was folded into funnels and 50 ml of distilled water was run through the paper in order to remove loose fibers. After the filter papers had completely dried in the funnels (24 h at room temperature or 1 h at 100°), the mixtures containing the extracted sugars and paper strips were filtered through the

previously washed and dried filter papers. Usually a large number of filter papers were washed and dried and kept ready for use.

The analysis was carried out using 1 ml of the filtrate which should contain 20–50 µg of sugar. One ml of the filtrate was added to 10 ml of anthrone reagent in a test tube. The tubes were capped with rubber caps, shaken and then heated in boiling water (95°) for exactly 15 min. After cooling rapidly to room temperature (a cold water bath may be used), the samples were transferred to colorimeter tubes and read in the Evelyn colorimeter using a 620 mµ filter. A blank containing 1.0 ml of distilled water in 10 ml of anthrone reagent was run with each set of tubes. The spots resulting from the stock solution of glucose, fructose and sucrose were used as the standards.

In order to check the amount of recovery of the sugars from the paper, two sets of ten analyses were run. One set consisted of chromatographed sugars containing 200 µg of each sugar. Extraction of these sugars, after chromatography and elution with 5.0 ml of water, gave a solution which would contain 40 µg of sugar per ml if complete extractions occurred. These were run against unchromatographed solutions of the sugars obtained by diluting the stock solutions so that the concentrations were 40 µg/ml. Both sets were treated exactly the same and were run simultaneously.

The extracts of plant tissue (dried, pulverized corn seedlings were used in this investigation) were obtained by extraction of a known amount of tissue (1.0 g) with a known volume (10 ml) of 80% ethanol. After overnight extraction on a shaker at room temperature, the mixture was filtered and the filtrate used directly.

A check on the recovery of sugars from plant material was carried out. In this experiment, known amounts of the sugars were added to spots containing the plant extracts. The spots were developed and analyzed in the usual way and the results compared with those from the spots which are not spiked with additional sugars. A chromatographed standard was also run in these recovery experiments.

RESULTS

The best separation of a mixture of glucose, fructose and sucrose occurred using descending techniques. The best solvent system used was ethyl acetate–acetic acid–water (6:3:2). Using this solvent system and descending techniques, samples containing in excess of 200 µg of each sugar were easily separated. On the other hand, using ascending techniques, reliable results could not be obtained using more than 100 µg of each sugar.

The reproducibility of the descending method is excellent as is shown in Table I. Since the samples were usually run on different days employing a different anthrone reagent, or one of different age, the optical density was not directly proportional to the amount of sugar between different runs. That is, the optical density for 40 µg of glucose analyzed on one day may not be twice that for 20 µg of glucose run on a different day. A chromatographed standard should be run with each analysis to compensate for differences in anthrone reagent and any differences in handling.

The percentage recovery of the sugars from paper is shown in Table II. The recovery of the separated sugars was quite good except for sucrose. However, the value of 84% recovery was not considered to be critical since a chromatographed standard of about the same concentration as the unknown was always run. This spot then served as a standard for the colorimetric analysis.

TABLE I

REPRODUCIBILITY OF THE PAPER CHROMATOGRAPHY–ANTHRONE DETERMINATION OF SUGARS USING THE DESCENDING TECHNIQUE

Sugar	*µg/spot*	*Optical density*	
		*Mean**	*Std. dev.*
Fructose	100	0.1215	0.0113
	200	0.311	0.014
Glucose	100	0.1006	0.0049
	200	0.1700	0.0040
Sucrose	100	0.1193	0.0042
	200	0.2518	0.0041

* Results of 12 analyses. A different anthrone reagent or one of different age was used for each set of 12 analyses. Therefore, the optical density was not directly proportional to the amount of sugar between different runs.

TABLE II

RECOVERY OF SUGARS FROM PAPER

Sugar (200 µg/spot)	*Trial*	*Source*	*Optical density*		*Recovery (%)*
			*Mean**	*Std. dev.*	
Fructose	1	Stock solution	0.301	0.005	
		Eluted from paper	0.312	0.010	103.6
Fructose	2	Stock solution	0.2722	0.0035	
		Eluted from paper	0.2907	0.0116	106.8
Glucose	1	Stock solution	0.1968	0.0043	
		Eluted from paper	0.2096	0.0036	106.5
Glucose	2	Stock solution	0.1997	0.0027	
		Eluted from paper	0.2151	0.0090	107.7
Sucrose	1	Stock solution	0.2359	0.0047	
		Eluted from paper	0.1966	0.0022	83.3
Sucrose	2	Stock solution	0.2455	0.0047	
		Eluted from paper	0.2001	0.0064	84.0

* Results of 10 analyses. Different anthrone reagents were used in each trial. Therefore, the optical density varied between trials.

TABLE III

COMPARISON OF THE PAPER CHROMATOGRAPHY–ANTHRONE METHOD WITH THE MODIFIED GLUCOSE-OXIDASE METHOD IN THE ANALYSIS OF CORN SEEDLINGS

Sugar	*Sugar content (mg/g)*		*Recovery data*	
	Paper chromatography–anthrone method	*Glucose-oxidase method*	*Sugar added (µg)*	*Sugar recovered (µg)*
Fructose	73.2	75.8	100	98.9
Glucose	50.5	48.0	100	94.8
Sucrose	225.6	230.1	100	111.6

The method was applied to analysis of glucose, fructose and sucrose in dried corn seedlings. The results obtained were compared with those obtained from the same samples using a modified glucose-oxidase method[19]. Table III shows that the paper

chromatography–anthrone method compares favorably with the modified glucose-oxidase method. In the analyses of plant extracts it is necessary to use chromatographed standards and to run triplicates of the plant extracts. It is not necessary to run an internal standard on each chromatogram. The spots from many chromatograms may be analyzed using the internal standards from another chromatogram, provided all samples and standards are analyzed simultaneously using the same anthrone reagent.

Table III also shows that sugars added to plant extracts may be recovered in good yields. Since the recovery of the chromatographed standard was taken at 100 %, the recovery of sucrose appears to be greater than 100 %. This is not critical if the internal standard contains approximately the same amount of sucrose as the material analyzed. A preliminary analysis to determine the approximate amount of sugar in the unknown should be carried out before more exact analyses are run. In the case of glucose and fructose this is not so important since the recovery of these sugars from paper has been demonstrated to be approximately 100 % (Table II).

The method, when used according to the described technique, gives reliable results. It is possible to analyze a large number of samples rapidly using very simple apparatus. Once the technique has been mastered, 20 samples per 24 h period may be analyzed for glucose, fructose and sucrose. The method can be readily adjusted to include any sugars which can be separated by paper chromatography.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the Faculty Improvement Committee of Colorado State University.

SUMMARY

Mixtures of glucose, fructose and sucrose were successfully analyzed by a method employing separation on paper, elution and colorimetric determination with the anthrone reagent. Since the anthrone reagent is employed as the colorimetric reagent, prior hydrolysis of sucrose is not necessary. The method, which requires very simple apparatus, may be used for the rapid and accurate determination of sugars in plant materials.

REFERENCES

1 S. M. PARTRIDGE AND R. G. WESTALL, *Biochem. J.*, 42 (1948) 238.
2 E. F. MCFARREN, K. BRAND AND H. R. RUTKOWSKI, *Anal. Chem.*, 23 (1951) 1146.
3 J. E. JEFFREY, E. V. PARTLOW AND W. J. POLGLASE, *Anal. Chem.*, 32 (1960) 1774.
4 R. M. MCCREADY AND E. A. MCCOMB, *Anal. Chem.*, 26 (1954) 1645.
5 K. HOLZER AND K. SCHMIDT, *Sci. Pharm.*, 24 (1956) 24; *C.A.*, 50 (1956) 8934d.
6 J. B. PRIDHAM, *Anal. Chem.*, 28 (1956) 1967.
7 C. M. WILSON, *Anal. Chem.*, 31 (1959) 1199.
8 A. J. PHILLIPPU, *Anal. Chem.*, 31 (1959) 1615.
9 R. S. SHALLENBERGER AND R. G. MOORES, *Anal. Chem.*, 29 (1957) 27.
10 R. DREYWOOD, *Ind. Eng. Chem., Anal. Ed.*, 18 (1946) 499.
11 C. S. WISE, R. J. DIMLER, H. A. DAVIS AND C. E. RIST, *Anal. Chem.*, 27 (1955) 33.
12 R. A. LAIDLAW AND S. G. REID, *Nature*, 166 (1950) 476.
13 R. J. DIMLER, W. C. SCHAEFER, C. S. WISE AND C. E. RIST, *Anal. Chem.*, 24 (1952) 1411.
14 R. L. WHISTLER AND J. L. HICKSON, *Anal. Chem.*, 27 (1955) 1514.
15 O. A. PAVLINOVA, *Fiziol. Rast.*, 4 (1959) 98; *C.A.*, 52 (1958) 12040c.
16 M. TAKI, *Agr. Biol. Chem. (Tokyo)*, 26 (1962) 1; *C.A.*, 56 (1962) 12292i.
17 J. H. ROE, *J. Biol. Chem.*, 212 (1955) 335.
18 D. F. DURSO AND W. A. MUELLER, *Anal. Chem.*, 28 (1956) 1366.
19 G. JOHNSON, C. B. LAMBERT, D. K. JOHNSON AND S. G. SUNDERWIRTH, *J. Agr. Food Chem.*, 12 (1964) 216.

AN IMPROVED METHOD FOR THE DETECTION OF CAERULOPLASMIN*

C. C. CURTAIN

*The Baker Medical Research Institute, Melbourne (Australia)***

(Received December 31st, 1963)

URIEL[1] showed that the copper-binding protein of blood, caeruloplasmin, could be detected and estimated by its ability to oxidise *p*-phenylenediamine, forming a dark quinonoid polymer. In the course of investigations into the heterogeneity of caeruloplasmin[2] in the populations of New Guinea, using the microtechniques of protein chromatography on DEAE cellulose paper[3] and acrylamide-gel electrophoresis[4] it became necessary to improve the sensitivity of the *p*-phenylenediamine oxidation method because of the low concentration of some of the minor components resolved in the acrylamide gel.

FISCHER[5] first described the formation of dyes by the oxidative condensation by silver halide in an exposed photographic plate of *p*-phenylenediamine with compounds containing active methylene or methine groups. This reaction is the basis of nearly all modern photographic colour processes and a very wide range of *p*-phenylenediamine derivatives and coupling agents have been described[6].

This paper describes a method of intensifying the colour produced by the action of caeruloplasmin on *p*-phenylenediamine by coupling with 2-cyano-acetylcumarone, a colour photographic coupling agent.

EXPERIMENTAL

Materials

DEAE cellulose paper (Whatman DE 20) was obtained from Messrs. Reeve Angel (London).

Acrylamide containing 5 % (w/w) N,N-methylenebisacrylamide was obtained as "AM-9 Chemical Grout" from Cyanamid (Australia).

p-Phenylenediamine was prepared by twice recrystallising B.D.H. reagent grade *p*-phenylenediamine from alcohol.

2-Cyano-acetylcumarone was a gift of Dr. N. LEWIS, Director of Research Laboratories, Kodak (Australasia) Ltd., Abbotsford, Victoria.

Caeruloplasmin was prepared by chromatography of Cohn fraction IV of human plasma on hydroxylapatite according to the method of BROMAN[7]. Both of the deep blue fractions obtained by this method were pooled.

Serum was prepared from blood which was obtained by venipuncture from nor-

* The expenses of this investigation were defrayed in part by a Grant from the National Health and Medical Research Council, Canberra, Australia.

** Postal address: Commercial Road, Prahran S. 1., Victoria, Australia.

mal human donors and patients attending the Alfred Hospital, Melbourne, Victoria.

Buffers. The following buffers were prepared from analytical reagent grade salts according to CLARK[8].

pH 5.0, 0.05 *M* sodium acetate; pH 5.5, 0.05 *M* sodium acetate; pH 6.0, 0.05 *M* sodium phosphate; pH 6.5, 0.05 *M* sodium phosphate; pH 7.0, 0.05 *M* sodium phosphate; pH 7.5, 0.05 *M* sodium phosphate; pH 8.0, 0.05 *M* sodium phosphate.

Methods

Spot testing for intensity of colour development was carried out by pipetting 0.01 ml of serum or 0.01 ml of caeruloplasmin solution onto a piece of Whatman No. 1 filter paper. The paper was then incubated in the test solution at 37° for the desired period. The papers were dried in a current of air at 37° and the density of colour determined in an E.E.L. chromatography scanner.

Ion exchange paper chromatography was carried out on a Whatman DE20 paper, using the methods described previously[3].

Acrylamide gel electrophoresis was carried out in gels prepared by dissolving 6 g of AM-9 and 0.5 g of ammonium persulphate in 100 ml of pH 5, 0.05 *M* sodium acetate buffer, then adding 0.5 ml of dimethylaminopropionitrile. The solutions were poured into moulds identical to those used by SMITHIES[9] for two-dimensional filter paper–starch-gel electrophoresis. Electrophoresis in the gels was carried out under the same conditions as used for starch-gels by SMITHIES[9].

RESULTS

Conditions for development of maximum colour intensity

The effect of pH. The spot test was carried out with the reagents dissolved in the pH 5.0, 6.0, 7.0, 8.0 buffers. The concentrations used were 0.05 g of *p*-phenylenediamine and 0.02 g of coupler per 100 ml, the incubation time was 30 min. The pH of maximum intensification in the presence of coupler appeared to be the same as for the develop-

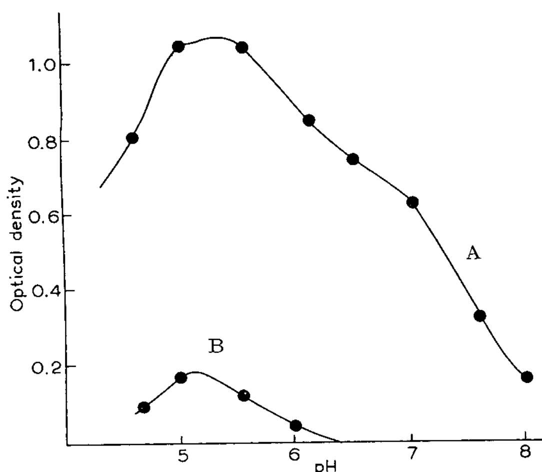


Fig. 1. The effect of varying pH on the colour produced by the oxidation of *p*-phenylenediamine by serum in the presence (A) and absence (B) of 2-cyano-acetylcumarone.

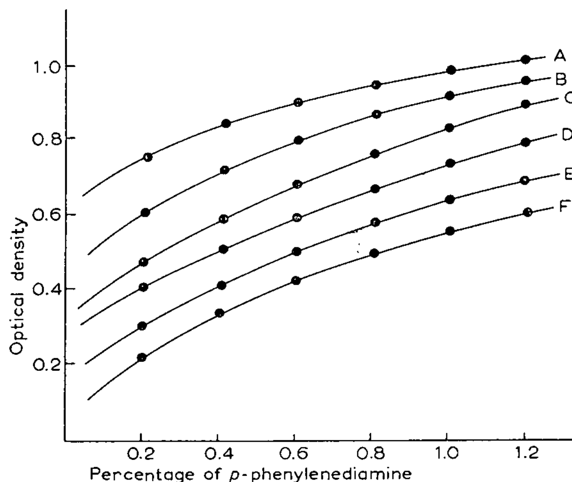


Fig. 2. The effect of varying *p*-phenylenediamine and coupler concentrations on the colour produced in the presence of serum at pH 5.5. (A) 0.2 % coupler; (B) 0.16 % coupler; (C) 0.12 % coupler; (D) 0.08 % coupler; (E) 0.04 % coupler; (F) 0.02 % coupler.

ment of the *p*-phenylenediamine colour alone as determined by the paper spot test (pH 5.5). The intensity in the presence of coupler at pH 5.5 appeared to be approximately 6 times that obtained with *p*-phenylenediamine alone (Fig. 1). After 4 h incubation this ratio fell to 3 to 1 in favour of the coupled reaction.

The effect of reagent concentration. The *p*-phenylenediamine and coupler concentrations could be varied over a wide range, at pH 5.5, without a great effect on the colour as estimated by the paper spot test (Fig. 2). As standards, a 0.1 % (w/v) *p*-phenylenediamine and a 0.2 % (w/v) coupler solution in pH 5.5, 0.05 *M* acetate buffer were adopted.

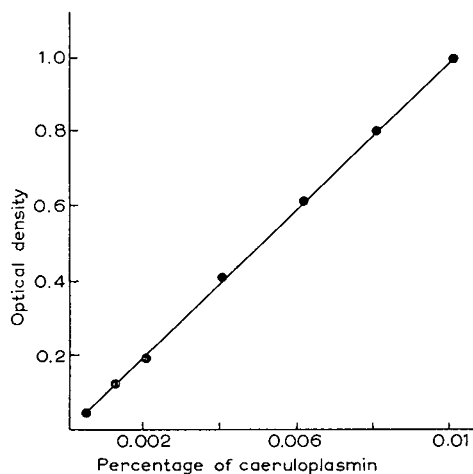


Fig. 3. The effect of the concentration of purified caeruloplasmin on the colour produced by the oxidation of *p*-phenylenediamine in the presence of coupler.

The effect of caeruloplasmin concentration. The spot test was applied to purified caeruloplasmin in a concentration ranging from 0.001 % (w/v) to 0.01 % (w/v). The incubation time was 30 min. A linear relationship was found between the concentration of caeruloplasmin and the density of the colour produced at pH 6.0 (Fig. 3). The density of colour produced by each spot was of the same order as that produced by a spot of serum containing the same concentration of caeruloplasmin as determined by the method of URIEL[1].

Detection of caeruloplasmin variants by two-dimensional ion-exchange paper chromatography and acrylamide gel electrophoresis

Duplicate 0.1 ml samples of serum were submitted to ion-exchange paper chromatography under the conditions described previously[3]. One chromatographic strip was developed in the *p*-phenylenediamine coupler mixture. Using this as a guide the caeruloplasmin-containing area was cut from the other strip and placed in a slit cut in the acrylamide gel. Electrophoresis was carried out at 4° for 6 h at a potential gradient of 7 V cm^{-1}. At the end of the run the gel was removed from the tray, sliced into two halves as described by SMITHIES[9] for starch-gels, and one half incubated for 10 min in the standard *p*-phenylenediamine–coupler mixture and the other half in *p*-phenylenediamine solution alone. The two halves were then compared. In all cases enhanced staining of the minor caeruloplasmin components was observed. In some cases these components were so weak as to be unstained by *p*-phenylenediamine alone. A pair of such patterns is shown in Fig. 4.

DISCUSSION

Compared with conventional methods of staining proteins after zone electrophoresis or chromatography, stains based upon enzymatic activity possess one serious disadvantage. For conventional staining the proteins are immobilised in the support (by heat denaturation or protein precipitants) but with the enzymatic stains the proteins must be left in the native state and are free to diffuse. Hence the time required to effect reasonable staining is important in preserving the original resolution of the pattern. Even a modest increase in sensitivity is worthwhile, therefore, if it shortens the time of staining. The other important aspect of increased sensitivity is the ability to use reduced sample volumes and micromethods. This is most important in surveys of primitive populations where a great many different tests must be carried out on small single samples of blood, owing to the difficulty of collecting and transporting specimens.

The particular coupling agent used, 2-cyano-acetylcumarone, was selected by a number of criteria. Unlike the amine couplers it seemed unlikely to have any chelating properties which could affect the copper of the caeruloplasmin, nor did it have any strongly charged groups which could lead to protein binding, either inactivating the caeruloplasmin or making the coupler unavailable. Finally its structure indicated that condensation could take place at other than alkaline pH values. A full review of the chemistry of the coupling agents used in photographic colour development has been given by VITTUM AND WEISSBERGER[6].

Fig. 4. Patterns produced by two-dimensional ion-exchange paper chromatography and acrylamide gel electrophoresis of serum after staining with (a) the standard *p*-phenylenediamine-coupler solution and (b) in the presence of 0.1 % (w/v) *p*-phenylenediamine in pH 5.5, 0.05 *M* acetate buffer.

SUMMARY

An improved method for the detection of caeruloplasmin is based upon the intensification in the presence of a coupling reagent, 2-cyano-acetylcumarone, of the colour produced by its oxidation of *p*-phenylenediamine. The colour produced was approximately 6 times denser than that produced in the presence of *p*-phenylenediamine alone. The method has the advantage that shorter incubation times can be used, thus preserving resolution by minimising diffusion after zone electrophoresis.

REFERENCES

[1] J. URIEL, *Bull. Soc. Chim. Biol.*, 39, Suppl. 1 (1957) 105.
[2] A. G. MORELL AND I. H. SCHEINBERG, *Science*, 131 (1960) 930.
[3] C. C. CURTAIN, *Nature*, 191 (1961) 1269.
[4] S. RAYMOND AND L. WEINTRAUB, *Science*, 130 (1959) 711.
[5] R. FISCHER, *Ger. Pat.* No. 253 335 (1912).
[6] P. W. VITTUM AND A. WEISSBERGER, *J. Phot. Sci.*, 2 (1954) 81.
[7] L. BROMAN, *Nature*, 182 (1958) 1655.
[8] W. M. CLARK, *The Determination of Hydrogen Ions*, 3rd Ed., The Williams and Wilkins Co., Baltimore, 1928.
[9] O. SMITHIES, *Biochem. J.*, 71 (1959) 585.

A MODIFIED DISC ELECTROPHORETIC METHOD FOR ANIMAL BLOOD SERUM PROTEINS*

K. ANANTH NARAYAN, SUHASINI NARAYAN AND FRED A. KUMMEROW

The Burnsides Research Laboratory, University of Illinois, Urbana, Ill. (U.S.A.)

(Received January 30th, 1964)

Recent advances in zone electrophoretic methods have provided a fund of knowledge on protein components of animal and vegetable tissues, hitherto unavailable. In particular, the disc electrophoretic method developed by ORNSTEIN AND DAVIS[1] has routinely demonstrated over 20 components with human serum as compared with 5 by conventional Tiselius electrophoresis. In connection with rat experiments in progress in our laboratory, it was found necessary to determine electrophoretically all the serum protein components using as small a quantity of blood as possible.

In the standard disc electrophoretic procedure, the sample is polymerized in a large pore size acrylamide gel. Frequently the polymerization of this sample gel is inhibited and could give rise to erroneous results. In the present communication, micro quantities of blood were drawn from the animals and their serum electrophoretic pattern determined using a modified disc electrophoretic procedure which avoids use of sample gel as the anti-convection medium. In order to indicate the versatility of the method, the technique has been extended to four other small animals (chick, mouse, guinea pig and rabbit) and, to our knowledge, disc electrophoretic patterns of their sera have not been previously reported.

MATERIALS AND METHODS

Collection of blood and serum preparation

Micro quantities of blood were collected in hematocrit capillary tubes from either animals or human beings making use of a lancet prick. The tail was pricked in the case of the rat and the mouse, the ear in the case of the rabbit and guinea pig and the pectoral region in the case of the chick. The tubes were sealed with citrocaps and the blood immediately centrifuged for 15 min in an International clinical centrifuge. The serum was separated from the sedimented material by cutting the capillary tube and the serum quantitatively transferred to a glass vial and "solution B-sucrose" solution was added (0.23 ml per cm of serum in the capillary tube as measured with a cm scale).

Calibration of the capillary tubes

The tubes used were those distributed by Aloe Scientific Company. Several tubes

* This work was supported by research grant no. CA 01932-11 from the National Institute of Health, U.S. Public Health Service, Department of Health, Education & Welfare.

chosen at random were calibrated using mercury. The volume per cm of the capillary tube was 7.4 μl $\pm$ 0.3 where 0.3 is the sample standard deviation.

Electrophoretic procedure

The procedure was the same as that described by ORNSTEIN AND DAVIS[1], except for the replacement of "solution B-sucrose" solution instead of the upper gel solution as the anti-convection medium. The spacer gel was gently overlaid with the sample solution and inserted into the upper reservoir with care. The current used was usually 2.5 mA/tube and the time of run was usually 35 min for animal sera and 55 min for human sera.

"Solution B-sucrose" solution

This solution was prepared by mixing 10 ml of solution B[1], 30 ml of distilled water and 40 ml of 20 % (w/v) sucrose solution.

RESULTS AND DISCUSSION

Although there are electrophoretic methods which make use of small quantities of serum, there are no suitable methods available which describe use of micro quantities of blood as the starting material. Generally a relatively large quantity of the blood is obtained from an animal and allowed to clot for a period of 2 to 4 h and the serum obtained by centrifugation. In all the electrophoretic runs described below, only 7 to 40 μl of blood were collected in capillary tubes and the serum obtained by centrifuging immediately for 15 min as described earlier. BARRETO[2] has recently developed a method for the preparation of lambda quantities of serum for paper electrophoresis. However, his method uses larger quantities of blood, may involve heat denaturation of the labile proteins, and requires transfer of serum from the capillary tube to a pipette for quantitative measurement.

The results obtained with human sera are shown in Figs. 1-4, and those for the various animal sera in Figs. 5 and 6. Figs. 5 and 6 represent normal blood serum electrophoretic patterns of five common laboratory animals as follows: A 227-1 and 2 albino rat; A 237-1 and 2 chick (crossbreed); A 285-5 and 6 mouse; 291-1 and 2 guinea pig; 291-5 and 6 rabbit. All samples were run in duplicate and the reproducibility will be apparent from the photographs. A tentative identification of the bands for human serum has been made by ORNSTEIN AND DAVIS[1]. It was suggested that the leading faint band was the prealbumin and was followed by the heavy albumin band, the three postalbumins, the transferrin, the region of haptoglobins and "7 S" γ-globulins, slow α_2-macroglobulin and β-lipoprotein. In the case of starch gel, the bands have been identified by SMITHIES for human serum[3] and by BEATON *et al.* for rat serum[4]. On this basis, the following tentative identification is suggested for rat serum protein bands. A faint prealbumin was followed by the albumin band, three to four postalbumins, one or two transferrins, fast α_1- and α_2-globulins and slow α_1- and α_2-globulins, and finally a faint β-lipoprotein band. A total of 14 bands was seen in the gels and can be compared with a similar number obtained by BEATON *et al.* using two-dimensional filter paper-starch gel electrophoresis. Possibly the identification suggested here may also be extended to the other animal serum proteins. The number of components seen in the gels themselves under the present experimental conditions

for the various sera were: Human 23; rat 14; chick 11; mice 11; guinea pig 11; rabbit 11. In the case of mice, using starch gel electrophoresis CONS AND GLASS[5] report that 11 bands were observed. The quantity of serum used by them was about 16 times the quantity used in the present experiments.

In order to illustrate the reproducibility of the present method, human blood serum from a single individual was used in one experiment (Fig. 1). The time of run was 7 min shorter than normal and hence the fine haptoglobin and γ-globulin bands were not resolved. However, the reproducibility of the method can be inferred from the albumin and transferrin bands.

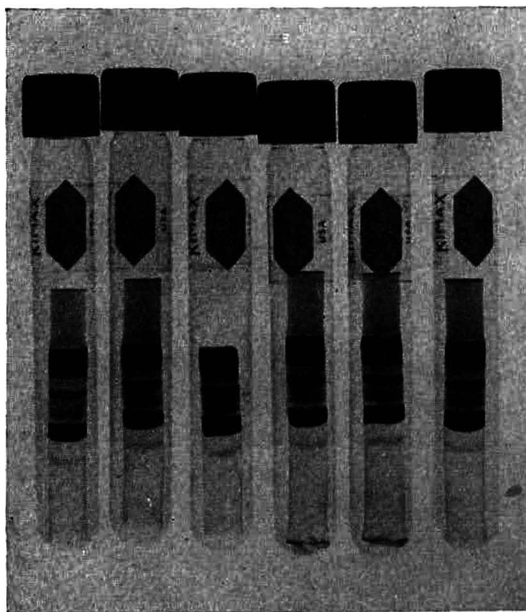


Fig. 1. Human blood serum electrophoretic patterns. D 17-1, 4, 5, 6, 7, 8 human serum, identical samples, using "solution B-sucrose", 3.9 μl, 48 min at 2.5 mA.

In our earlier experiments conducted according to the procedure of ORNSTEIN AND DAVIS where the sample was polymerized in the sample gel, we encountered problems of improper sample gel polymerization, intense background staining in the γ-globulin region and a certain amount of protein precipitation at the sample gel–spacer gel interface. Fig. 2 depicts photographs of runs obtained with identical amounts of human serum taken from a single individual using standard disc electrophoretic conditions but in which the sample gel failed to polymerize completely. It was evident that there was poor reproducibility under these conditions. Although the reason why these sample gels did not polymerize was not known, the following factors are known to inhibit polymerization: slight hemolysis, protein concentration, aging of upper gel solution, traces of acids and increased amounts of specific proteins such as globulins.

Improper sample gel polymerization appeared to be a common problem for many laboratories and was referred to at a recent conference on gel electrophoresis[6]. Further-

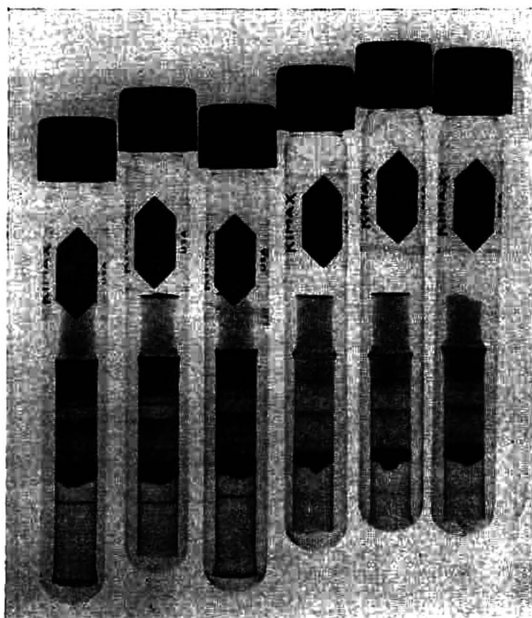


Fig. 2. Human blood serum electrophoretic patterns. D 29-1, 3, 4, D 31-1, 2, 3, human serum identical samples using incompletely polymerized sample gel, 3.9 μl, 24 min at 5 mA.

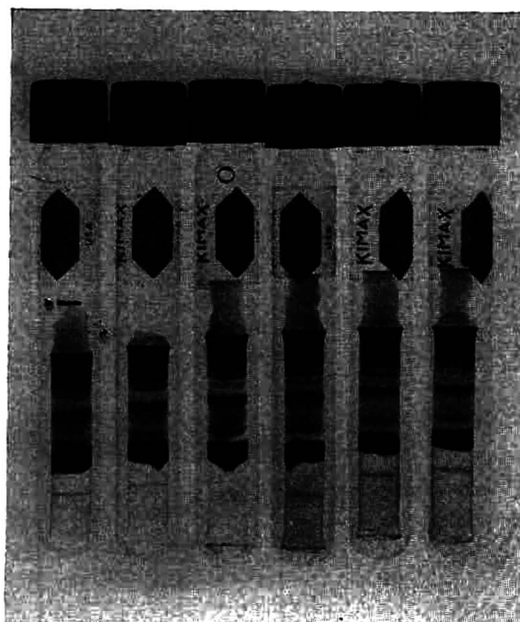


Fig. 3. Human blood serum electrophoretic patterns. D 34-1 and 2 human serum 3.5 μl using sample gel, 26 min at 5 mA. D 21-1 and 2 identical sample 3.9 μl using solution B-sucrose, 115 min at 1.25 mA. D 42-4 human serum stored at $-18°$ for one week, 2 μl, 110 min at 1.25 mA. D 42-6 identical sample stored at 3° for one week, 2 μl, 110 min at 1.25 mA.

more it has been observed by HEIDEMAN[7] that albumin could not be recovered quantitatively in the main gel under standard disc electrophoretic conditions using sample gel. It therefore appeared to us that it would be desirable to eliminate this polymerization step by diluting the sample with solution B-sucrose and by layering it over the spacer gel. Fig. 3 D 34-1, 34-2 and D 21-1 and 21-2 provide a comparison of two procedures, the first two are a typical run using completely polymerized sample gel and the conditions described by ORNSTEIN AND DAVIS, and the latter two using solution B-sucrose solution and the present experimental conditions. It was evident that the latter patterns were better resolved and showed more definition. This may be explained as being due to partial denaturation of γ-globulins caused by the heat generated during the run D 34. D 34-1 and 34-2 may also be compared with D 42-4 and 42-6. The latter were run at 1.25 mA/tube for 110 min and at a decreased sample concentration of 2 μl per tube. While this procedure decreased the background stain it also produced increased spreading of the bands due to diffusion.

Fig. 3, D 42-4 and 6 depict differences in the serum protein pattern upon storage in a freezer (—18°) and at 3° for one week. Similar changes were observed in a subsequent run with samples of serum stored for three weeks (Fig. 4, D 51-2 and D 51-7

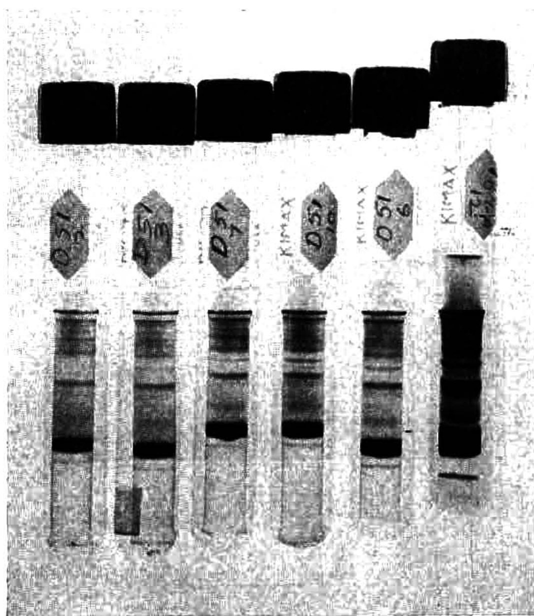


Fig. 4. Human blood serum electrophoretic patterns. D 51-2 and 7 human serum stored at —18° for 3 weeks, 1.9 μl, 55 min at 2.5 mA. D 51-3 and 10 identical sample stored at 3° for 3 weeks, 1.9 μl, 55 min at 2.5 mA. D 51-6 identical sample diluted with solution B-sucrose and stored at —18° for 3 weeks, 1.9 μl, 55 min at 2.5 mA. 189A-12 human serum sample 7 μl, 56 min at 2.5 mA.

frozen sample *vs.* D 51-3 and D 51-10, 3° sample). These were run at the lower concentration but at a higher current (2.5 mA) in order to reduce spreading. In general disappearance of some of the globulin bands was followed by appearance of new fine bands. These changes are less pronounced in the photographs but are clearly visible in

the gels. Although some of the bands in Fig. 3, D 21 are sharper by comparison because of higher concentration, the finer bands are masked due to intense background stain. This stain cannot be removed either electrophoretically or by extensive washing and may represent unresolved migrating protein components or labile protein denatured by heat, pH or small ionic strength changes. This effect was minimized by using the lowest possible serum concentration and lowest possible current (Fig. 4, D 51). Since it is difficult to store small quantities of serum as such, it was considered worthwhile to study the effect of storage of serum diluted in "solution B-sucrose" solution. D 51-6 represents a sample which was kept frozen for 3 weeks in this manner. The electrophoretic pattern was observed to be similar to that of undiluted serum frozen as such. The present method does not restrict one to low concentrations of samples as in the case of sample gel. Fig. 4 189A-12 represents a sample of human serum run at a concentration of about 7 μl.

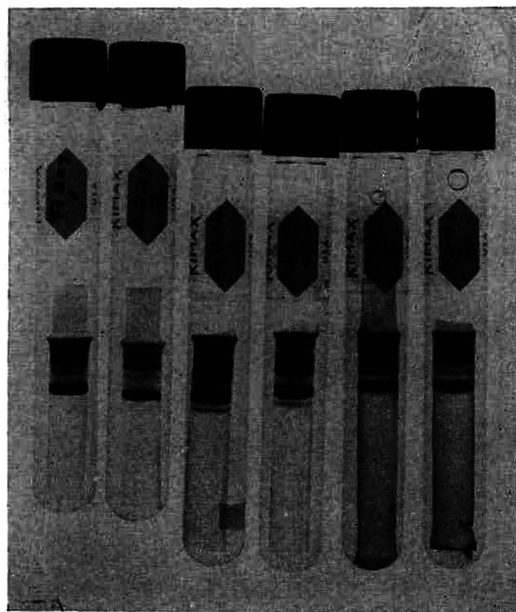


Fig. 5. Animal blood serum electrophoretic patterns. A 227-1 and 2 rat serum 2.4 μl, 30 min at 2.5 mA. A 237-1 and 2 chick serum 2.4 μl, 30 min at 2.5 mA. A 285-5 and 6 mouse serum 3 μl, 30 min at 2.5 mA.

Although quantitative aspects of the gel patterns have not been discussed here, preliminary work using Model E Microdensitometer have indicated reproducibility of the patterns under present experimental conditions. Thus with this technique only 4 μl of *whole blood* need to be drawn from either man or animal to obtain a complete electrophoretic blood serum picture. This amount may be compared with 20-50 μl of *serum* required for normal starch gel electrophoresis. The method was observed to be rapid, more convenient and simpler than the normal clotting method which not only requires greater quantities of blood but may partially hemolyse and interfere with sample gel polymerization. Finally it permits periodic examinations of the blood

serum protein pattern and may therefore find application in clinical work and in animal experimentation.

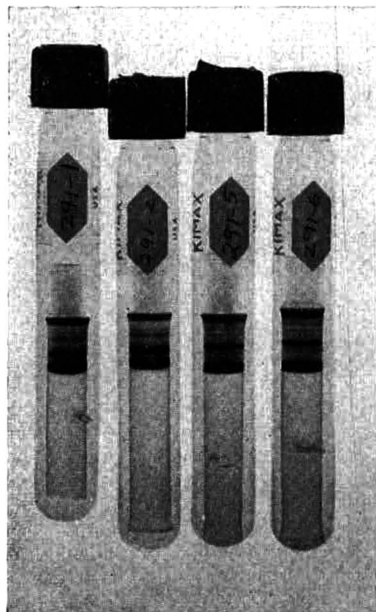


Fig. 6. Animal blood serum electrophoretic patterns. 291-1 and 2 guinea pig serum 3.6 µl, 35 min at 2.5 mA. 291-5 and 6 rabbit serum 3.6 µl, 35 min at 2.5 mA.

SUMMARY

A modified disc electrophoretic method has been developed and the serum electrophoretic patterns of chick, rat, mouse, guinea pig and rabbit have been obtained from micro quantities of blood. The modification involved use of sucrose solution rather than the large pore gel as the anti-convection medium and thus eliminated the problem of incomplete sample gel polymerization. The method described required as little as 4 µl of whole blood for a complete run, was more convenient and rapid than the usual methods and may find application in animal experimentation and in clinical work.

REFERENCES

1 L. Ornstein and B. J. Davis, *Disc Electrophoresis*, preprinted by Distillation Products Industries, Eastman Kodak Co, 1962.
2 R. C. R. Barreto, *J. Chromatog.*, 6 (1961) 278.
3 O. Smithies, *Advan. Protein Chem.*, 14 (1959) 65.
4 G. H. Beaton, A. E. Selby and A. M. Wright, *J. Biol. Chem.*, 236 (1961) 2001.
5 J. M. Cons and L. E. Glass, *Proc. Soc. Exptl. Biol. Med.*, 113 (1963) 893.
6 *Conf. Gel Electrophoresis*, The New York Academy of Sciences, New York, Dec. 2-3, 1963.
7 M. L. Heidman, Jr., *Conf. Gel Electrophoresis*, The New York Academy of Sciences, New York, Dec. 2-3, 1963.

DIE TRENNUNG DER PHENOLSÄUREN MIT HILFE DER HOCHSPANNUNGSELEKTROPHORESE

A. STURM JR. UND H. W. SCHEJA
I. Medizinische Klinik der Medizinischen Akademie, Düsseldorf (Deutschland)*

(Eingegangen den 28. Februar 1964)

Phenolsäuren sind aromatische Carbonsäuren, die am Benzolring statt eines oder mehrerer Wasserstoffatome Hydroxylgruppen tragen. Untersuchungen in den letzten Jahren über die angeborenen Stoffwechselanomalien, insbesondere über die Phenylketonurie und Alkaptonurie, über die Umwandlung und Entgiftung aromatischer Aminosäuren[33] und über die Ursache psychischer und neurologischer Veränderungen Leberkranker[13-15] haben es wahrscheinlich gemacht, dass die Phenolsäuren eine wesentliche pathophysiologische Bedeutung besitzen können. Eingehende Untersuchungen über die physiologische Schwankungsbreite des menschlichen Phenolsäuremusters und über dessen Veränderungen bei verschiedenen Krankheitsbildern stossen jedoch immer wieder auf die Schwierigkeit, mehrere Phenolsäuren in einem Arbeitsgang qualitativ und quantitativ zu erfassen. Mit Hilfe der Hochspannungselektrophorese war es uns möglich, 15 bis 20 Phenolsäuren auf dem Pherogramm exakt zu trennen. Über das Ergebnis dieser Untersuchungen soll im folgenden berichtet werden.

METHODIK

Apparatur

Die Untersuchungen wurden mit Hochspannungselektrophorese-Apparaturen mit flüssigen und mit festen Wärmeaustauschern durchgeführt. Als Elektrophoresegerät mit flüssigem Wärmeaustausch wurde die von MICHL[21, 22] beschriebene, von KICKHÖFEN UND WESTPHAL[17, 18] zur Trennung von Peptiden und Aminosäuren und von HEILMEYER und Mitarbeiter[16] zur Trennung des enteiweissten Serums modifizierte Apparatur verwandt. Als inertes Medium benutzten wir, wie früher angegeben[27], statt des Hexans Heptan. Zur Hochspannungselektrophorese mit festem Wärmeaustauscher wurde der Pherograph Original Frankfurt nach WIELAND UND PFLEIDERER[31, 32] verwandt**.

Elektrolyt

Pyridin–Eisessig–Wasser im Verhältnis 1:10:89. Das pH dieses Gemisches beträgt 3.6.

Papier

Schleicher und Schüll 2043b Mgl. Papiergrösse: 45 × 2.5 cm für die Elektrophorese

* Direktor: Prof. Dr. F. GROSSE-BROCKHOFF.
** Hersteller: Fa. L. Hormuth (Inh.: W. E. Vetter), Heidelberg-Wiesloch, Deutschland.

mit flüssigem Wärmeaustausch und 40 × 32 cm für die Elektrophorese mit festem Wärmeaustausch.

Testlösungen

Der grösste Teil der Testsubstanzen konnte käuflich erworben werden. Die *m*-Hydroxyhippursäure, die *o*-Hydroxyhippursäure und das Vanilloylglycin wurden nach Vorschrift[23] nach Einnahme der entsprechenden Benzoesäuren bzw. von Vanillinsäure und Glycin aus dem Urin extrahiert. Die Testsubstanzen wurden in 60–98 %-igem Äthylalkohol p.a. aufgelöst. Bei einer Auftragsmenge von 0.02 ml auf den Papierstreifen erwies sich eine Konzentration von 0.5–2.0 mg Testsubstanz/ml Äthylalkohol am geeignetsten.

Färbereagentien

(a) *p*-Nitroanilin-Reagenz nach Vorschrift von VON STUDNITZ[24]: 1 Teil 0.1 %ige *p*-Nitroanilinlösung, 1 Teil 0.2 %ige Natriumnitritlösung, 2 Teile 10 %ige Kaliumkarbonatlösung.

(b) Aroylglycin-Reagenz nach Angaben von SMITH[23]: 1 Teil 5 %ige *p*-Dimethylaminobenzaldehydlösung in Essigsäureanhydrid, 4 Teile Aceton p.a.

(c) Eisenchlorid-Reagenz nach Vorschrift von SMITH[23]: 2 %ige Eisen(III)-Chlorid-Lösung.

Durchführung der Elektrophorese

0.02 ml der Äthylalkohol-Testsubstanzlösung wurden mit einer Pipette in Form eines feinen Striches senkrecht zur Laufrichtung auf das mit der angegebenen Elektrolytlösung gut angefeuchtete Papier aufgetragen. Bei Anwendung der Elektrophorese-Apparatur mit flüssigem Wärmeaustausch liegt der Auftragsort 8 cm von der Kathode entfernt. Trennzeit und Voltstärke: 30 min bei 1400 V, anschliessend 60 min bei 2200 V, anschliessend 90 min bei 2700 V (= 30–60 V/cm). Der mittlere Stromdurchgang beträgt hierbei 10–45 mA. Bei Verwendung der Apparatur nach WIELAND UND PFLEIDERER liegt der Auftragsort 13 cm von der Kathode entfernt. Die Trennzeit beträgt 420 min bei einer Spannung von 2100 V (= 50 V/cm). Der mittlere Stromdurchgang schwankt hierbei zwischen 40 und 80 mA.

Nach Beendigung der elektrophoretischen Trennung wurde das Papier im Trockenschrank 30 min bei 60° getrocknet.

ERGEBNISSE

Auf den in Fig. 1a dargestellten Elektrophoresestreifen waren 16 Testsubstanzen aufgetragen und die elektrophoretische Trennung mit der Apparatur nach MICHL in der beschriebenen Art durchgeführt worden. Anschliessend wurde der Streifen mit *p*-Nitroanilin-Reagenz besprüht. Wie die Abbildung zeigt, stellen sich 14 der aufgetragenen 16 Substanzen als scharf begrenzte Banden gut dar: In Richtung Kathode — vom Auftragsort aus gesehen — in blau-grauer Farbe das DL-Dihydroxyphenylalanin (Nr. 1), in Richtung Anode — ebenfalls vom Auftragsort aus gesehen — in dunkelroter Farbe die 5-Hydroxyindolessigsäure[25] (Nr. 2), in roter Farbe die *p*-Hydroxybenzoesäure (Nr. 3), in grau-brauner Farbe die 3,4-Dihydroxyphenylessigsäure (Nr. 4), in lila-blauer Farbe die *p*-Hydroxyphenylessigsäure (Nr. 5), in

gelber Farbe die α-Resorcylsäure (Nr. 6), in violett-brauner Farbe die *o*-Hydroxyphenylessigsäure (Nr. 7), in roter Farbe die *m*-Hydroxybenzoesäure (Nr. 8), in lila Farbe das Vanilloylglycin (Nr. 9), in hellroter Farbe die *m*-Hydroxyhippursäure (Nr. 10), in orangener Farbe die *o*-Hydroxyhippursäure (Nr. 11), in grau-blauer Farbe die 3,4-Dihydroxymandelsäure (Nr. 12), in blau-violetter Farbe die Vanillinmandelsäure (Nr. 13) und in lila-blauer Farbe die Xanthurensäure (Nr. 14).

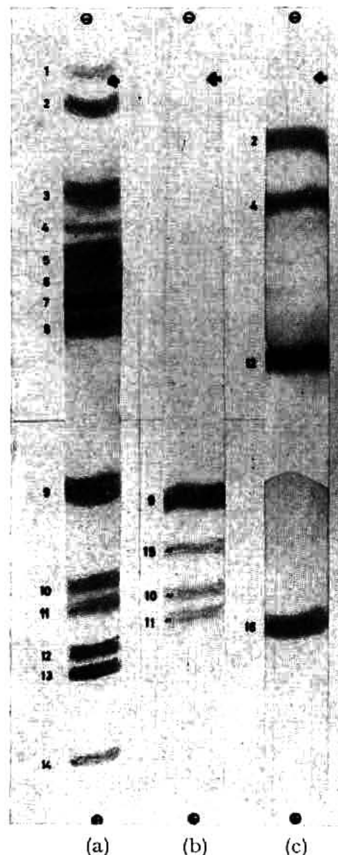


Fig. 1. Phenolsäuren-Hochspannungspherogramm (→: Auftragsort). (a) Spray mit *p*-Nitroanilin-Reagenz. Trennung bei 30–60 V/cm. Trennzeit: 180 min. (b) Spray mit Aroylglycin-Reagenz. Trennung bei 30–60 V/cm. Trennzeit: 180 min. (c) Spray mit Eisenchlorid-Reagenz. Trennung bei 30–60 V/cm. Trennzeit: 90 min. Weitere Erklärungen siehe Text.

Zwei der aufgetragenen 16 Testsubstanzen stellen sich auf dem Elektrophoresestreifen der Fig. 1a nicht dar: die *p*-Hydroxyhippursäure und die Phenylbrenztraubensäure. Bei Anfärbung des Elektrophoresestreifens mit Aroylglycin kommt die *p*-Hydroxyhippursäure (Nr. 15) — wie in Fig. 1b dargestellt — zwischen dem Vanilloylglycin (Nr. 9) und der *m*-Hydroxyhippursäure (Nr. 10) in dunkelgelber Farbe gut zur Darstelling. Als weitere Fraktion ist bei Anfärbung mit Aroylglycin die *o*-Hydroxyhippursäure sichtbar (Nr. 11).

Die Phenylbrenztraubensäure hingegen "wandert" bei der angegebenen Trenndauer und Voltstärke aus dem Streifen heraus in den Puffer hinein. Zur Darstellung dieser Fraktion ist daher eine kürzere Laufzeit und niedrigere Spannung (30 min bei 1400 V, anschliessend 60 min bei 2200 V) — bei gleichem Auftragsort — notwendig. Unter diesen Bedingungen ist auch die Phenylbrenztraubensäure (Nr. 16) nach Anfärbung mit Eisenchlorid, am weitesten anodisch liegend, als grau-blaue Bande gut sichtbar (siehe Fig. 1 c). Wie die Fig. 1 c weiter zeigt, stellen sich auf dem gleichen Streifen nach Anfärbung mit Eisen(III)-Chlorid auch die 5-Hydroxyindolessigsäure, die 3,4-Dihydroxyphenylessigsäure und die 3,4-Dihydroxymandelsäure gut dar.

Vier Phenolsäuren, die Homovanillinsäure, die Ferulasäure, die Vanillinsäure[26] und die 2-(4-Hydroxyazobenzoe)benzoesäure sind in dem als inertes Medium verwandten organischen Lösungsmittel Heptan teilweise löslich. Um Substanzverluste bei der Auftrennung zu vermeiden, wurden diese Säuren mit einem Pherographen mit festem Wärmeaustausch getrennt. Fig. 2 zeigt einen aus dem Papierbogen ausgeschnittenen Elektrophoresestreifen nach Auftrennung der genannten vier Test-

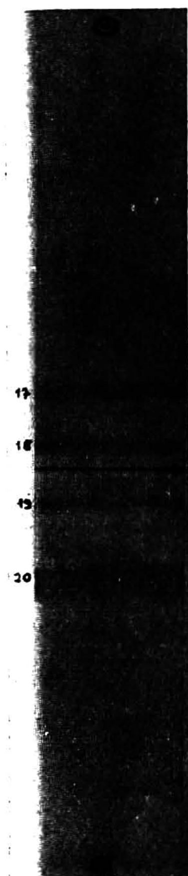


Fig. 2. Phenolsäuren-Hochspannungspherogramm (→: Auftragsort). Spray mit *p*-Nitroanilin-Reagenz. Trennung bei 50 V/cm. Trennzeit: 420 min. Weitere Erklärungen siehe Text.

substanzen: auf der kathodischen Seite stellt sich nach Spray mit *p*-Nitroanilin-Reagenz die Vanillinsäure (Nr. 17) in blau-violetter Farbe und die Ferulasäure (Nr. 18) in blau-grüner Farbe, auf der anodischen Seite die Homovanillinsäure (Nr. 19) in dunkelgrau-blauer und die 2-(4-Hydroxyazobenzoe)benzoesäure (Nr. 20) in gelb-brauner Farbe gut dar.

BESPRECHUNG DER ERGEBNISSE

Obwohl die Phenolsäuren, wie eingangs darauf hingewiesen, eine wesentliche pathophysiologische Bedeutung besitzen können, wurden eingehendere Untersuchungen über ihre Ausscheidung unter physiologischen und pathologischen Bedingungen bisher nur von wenigen Autoren durchgeführt.

CLOTTEN UND CLOTTEN[8] trennten mit Hilfe der Hochspannungselektrophorese mit flüssigem Wärmeaustausch Phenolsäuren im Urin auf. Ihre bisher mitgeteilten Ergebnisse beschränkten sich jedoch auf einzelne wenige Phenolsäuren und sind noch unvollständig. Bei den üblichen, vornehmlich papierchromatographischen und

Fig. 3. Phenolsäuren-Hochspannungspherogramm des Urins. Spray mit *p*-Nitroanilin-Reagenz. Trennung bei 30–60 V/cm. Trennzeit: 110 min.

elektrophoretischen Bestimmungsmethoden[1–7, 9–15, 19, 20, 23, 28–30] überlagern sich die einzelnen Phenolsäurefraktionen auf dem Papier häufig, sodass eine exakte Bestimmung nicht durchgeführt werden kann. Mit Hilfe der Hochspannungselektrophorese ist es möglich, einen grossen Teil der Phenolsäuren relativ rasch in schmale, scharf von einander abgegrenzte einzelne Fraktionen zu trennen. Das Hochspannungspherogramm der Phenolsäuren gestattet so nicht nur einen raschen, gut orientierenden Überblick über das Phenolsäuremuster des Untersuchten, sondern es ermöglicht auch eine quantitative Bestimmung der meisten Phenolsäuren, da sich diese auf dem Pherogramm nur selten überlagern und so exakt einzeln eluiert werden können. Insbesondere bei Fragestellungen und Untersuchungen, die die Erfassung aller ausgeschiedenen Phenolsäuren erfordern, dürfte die hochspannungselektrophoretische Trennmethode besonders geeignet sein, bzw. als ideale Ergänzungsmethode für andere Untersuchungsmethoden dienen. In Fig. 3 sei als Beispiel für die klinische Anwendung der beschriebenen Methode das Phenolsäuren-Urinpherogramm eines Patienten demonstriert. Die Abbildung zeigt, dass sich die Phenolsäuren des Urins mit Hilfe der Hochspannungselektrophorese gut darstellen und trennen lassen. Über die Einzelheiten der Identifizierung dieses Urinpherogrammes wird an anderer Stelle berichtet werden.

DANK

Einige Testsubstanzen wurden uns liebenswürdigerweise von Herrn Prof. Dr. O. KRAUPP, Pharmakologisches Institut der Universität Wien, zur Verfügung gestellt, wofür wir sehr herzlich danken. Herrn Prof. Dr. F. HARTMANN, Direktor der Medizinischen Universitäts-Poliklinik Marburg und seinem Mitarbeiter, Herrn Dr. RUGE dürfen wir für wertvolle Hinweise bei der Beschaffung einiger Testsubstanzen danken.

ZUSAMMENFASSUNG

Es wird über eine Methode berichtet, die es gestattet, mit Hilfe von hohen Spannungsgefällen 15 bis 20 Phenolsäuren in einzelne Fraktionen zu trennen. Die Möglichkeiten der Methodik werden diskutiert.

SUMMARY

A high-voltage electrophoretic method is described by which it is possible to separate mixtures of 15–20 phenolic acids into distinct fractions. The applications of the method are discussed.

LITERATUR

1 M. D. ARMSTRONG, K. N. F. SHAW AND P. E. WALL, *J. Biol. Chem.*, 218 (1956) 293.
2 E. C. BATE-SMITH UND R. G. WESTALL, *Biochim. Biophys. Acta*, 4 (1950) 427.
3 H. BICKEL UND F. SOUCHON, *Arch. Kinderheilkunde*, (1955) Beiheft No. 31.
4 R. J. BOSCOTT UND C. W. COOKE, *Quart. J. Med.*, 23 (1954) 307.
5 R. J. BOSCOTT UND B. H. KIRMAN, *Biochem. J.*, 60 (1955) 4.
6 E. BOYLAND UND D. C. WILLIAMS, *Biochem. J.*, 64 (1956) 578.
7 K. G. BRAY, W. V. THORPE AND K. WHITE, *Biochem. J.*, 46 (1950) 271.
8 R. CLOTTEN UND A. CLOTTEN, *Hochspannungselektrophorese*, Georg Thieme Verlag, Stuttgart, 1962.
9 F. CRAMER, *Papierchromatographie*, Verlag Chemie GmbH, Weinheim/Bergstrasse, 4. Aufl., 1958.
10 C. E. DALGLIESH, *J. Clin. Pathol.*, 8 (1955) 73.

[11] M. EFRON, *High voltage paperelectrophoresis*, in I. SMITH, *Chromatographic and Electrophoretic Techniques*, W. Heinemann Medical Books Ltd., London, 1960.
[12] I. M. HAIS UND K. MACEK, *Handbuch der Papierchromatographie*, VEB Gustav Fischer, Jena, 1960.
[13] H. HARTMANN, *Gastroenterologia*, Suppl. 95 (1961) 182.
[14] H. HARTMANN, *Klin. Wochschr.*, 39 (1961) 273.
[15] H. HARTMANN UND W. RUGE, *Deut. Arch. Klin. Med.*, 208 (1962) 298.
[16] L. HEILMEYER, R. CLOTTEN, J. SANO, A. STURM JR. UND A. LIPP, *Klin. Wochschr.*, 32 (1954) 831.
[17] B. KICKHÖFEN UND O. WESTPHAL, *Z. Naturforsch.*, 7b (1952) 655.
[18] B. KICKHÖFEN UND O. WESTPHAL, *Z. Naturforsch.*, 7b (1952) 659.
[19] D. KLEIN UND J. M. CHERNAIK, *Clin. Chem.*, 7 (1961) 257.
[20] O. KRAUPP, H. STORMANN, H. BERNHEIMER UND H. OBENAUS, *Klin. Wochschr.*, 37 (1959) 76.
[21] H. MICHL, *Monatsh. Chem.*, 82 (1951) 489.
[22] H. MICHL, *Monatsh. Chem.*, 83 (1952) 737.
[23] I. SMITH, *Chromatographic and Electrophoretic Techniques*, W. Heinemann Medical Books Ltd., London, 1960.
[24] W. VON STUDNITZ, *Scand. J. Clin. Lab. Invest.*, 12, Suppl. 48 (1960).
[25] A. STURM JR., *Clin. Chim. Acta*, 7 (1962) 714.
[26] A. STURM JR., *Deut. Med. Wochschr.*, 88 (1963) 1000.
[27] A. STURM JR., *Klin. Wochschr.*, 39 (1961) 365.
[28] T. SWAIN, *Biochem. J.*, 53 (1953) 200.
[29] S. L. TOMPSETT, *J. Pharm. Pharmacol.*, 13 (1961) 747.
[30] S. L. TOMPSETT, *Clin. Chim. Acta*, 3 (1958) 149.
[31] TH. WIELAND UND G. PFLEIDERER, *Angew. Chem.*, 67 (1955) 257.
[32] TH. WIELAND AND G. PFLEIDERER, *Angew. Chem.*, 69 (1957) 199.
[33] R. T. WILLIAMS. Biological oxidation of aromatic rings, *Biochem. Soc. Symp.*, No. 5 (1950); zit. nach K. DIMROTH, *Einfache isocyclische Verbindungen*, aus HOPPE-SEYLER-THIERFELDER, *Handbuch der physiologisch- und pathologisch-chemischen Analyse*, Springer, Berlin, 1955.

ANION EXCHANGE CHROMATOGRAPHY OF TRANSITION METALS IN TARTRATE MEDIUM*

GERALD P. MORIE AND THOMAS R. SWEET,
Department of Chemistry, The Ohio State University, Columbus, Ohio (U.S.A.)

(Received January 27th, 1964)

INTRODUCTION

Tartrate medium has been used for a number of anion exchange studies[1-3]. Recently the separation of manganese, cobalt, zinc, copper and iron in tartrate medium was reported[4]. It was also found that the tartrate system was convenient for the quantitative separation of iron(II) and iron(III)[5].

In the present work, equilibrium studies were made on the tartrate complexes of chromium, molybdenum, nickel, silver, cadmium and mercury. On the basis of the data obtained for these metals and the data for manganese, cobalt, zinc and iron[4], a number of column separations were developed.

EXPERIMENTAL

Reagents and apparatus

Dowex 2X-8-anion exchange resins were converted to the tartrate form using *d*-tartaric acid obtained from J. T. Baker Chemical Company, Phillipsburgh, N.J. Batch determinations were made with 50–100 mesh resin since it was more convenient for this work than the finer mesh resin. Mesh size 200–400 was selected for column operation because it reaches equilibrium faster. A comparison of the distribution coefficients of chromium on the two mesh size resins was made and no significant difference was found.

The isotopes used in this investigation were chromium-51, manganese-54, iron-59, cobalt-60, nickel-63, zinc-65, molybdenum-99, silver-110, cadmium-115 and mercury-187. All of the above isotopes except manganese-54 were obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn. The manganese-54 was obtained from Nuclear Science and Engineering Corporation, Pittsburg, Pa.

Nearly all isotopes were obtained as either the chloride or nitrate salt and could be used without modification. However, molybdenum-99 was obtained as ammonium molybdate in ammonium hydroxide solution. It was necessary to convert this to molybdic acid in order to form a complex ion with tartaric acid[6]. This was accomplished by heating the active ammonium molybdate at 800° F for one hour as described by KILLEFFER[7]. Molybdic acid solutions were made slightly basic to prevent precipitation as the isopoly acid.

---

* Taken in part from the M.Sc. Thesis of GERALD P. MORIE, The Ohio State University, Columbus, Ohio, 1963.

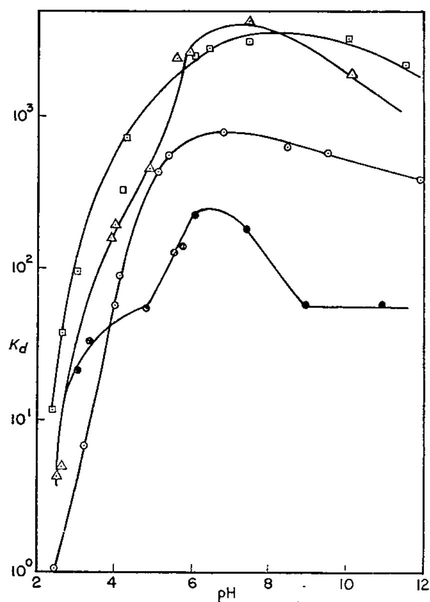


Fig. 1. Chromium.

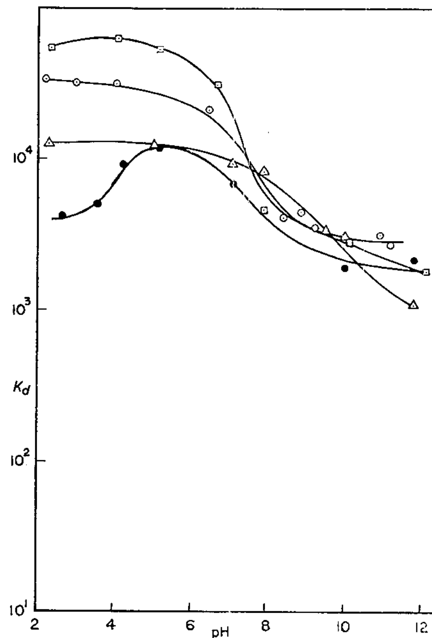


Fig. 2. Molybdenum.

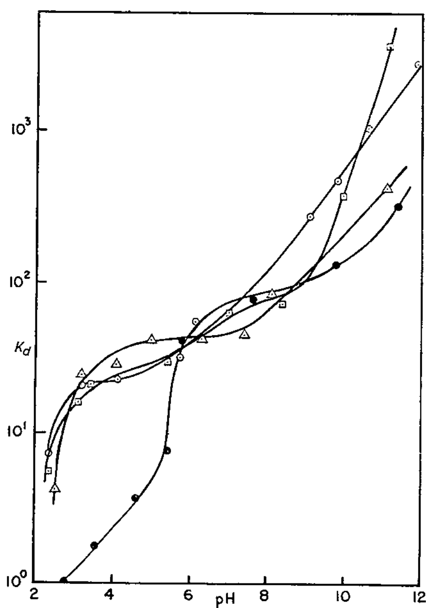


Fig. 3. Nickel.

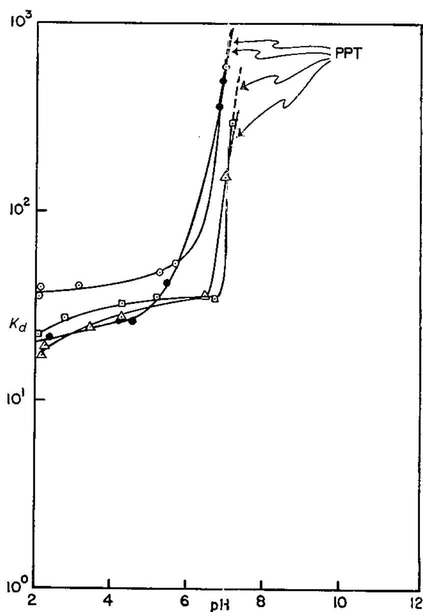


Fig. 4. Silver.

Figs. 1–4. Distribution coefficients for metals. Effect of pH and concentration. ⊙ – in $8.5 \cdot 10^{-2}$ *M* tartrate; ⊡ – in $4.25 \cdot 10^{-2}$ *M* tartrate; △ – in $2.12 \cdot 10^{-2}$ *M* tartrate; ● – in $2.12 \cdot 10^{-3}$ *M* tartrate.

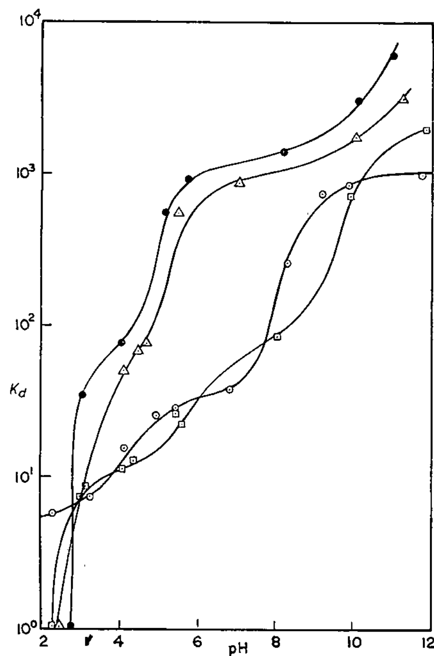


Fig. 5. Cadmium.

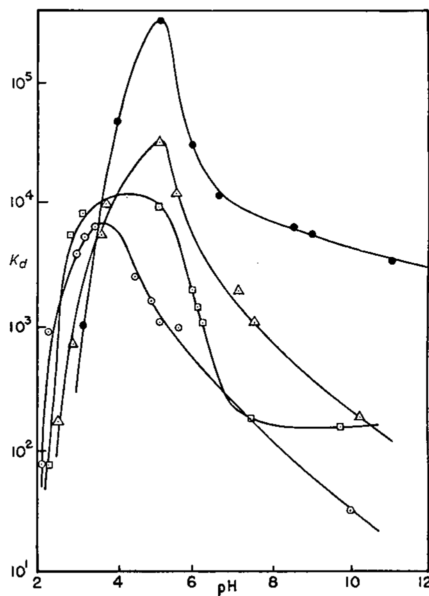


Fig. 6. Mercury.

Figs. 5–6. Distribution coefficients for metals. Effect of pH and concentration. ⊙ – in $8.5 \cdot 10^{-2}$ *M* tartrate; ⊡ – in $4.25 \cdot 10^{-2}$ *M* tartrate; ⚠ – in $2.12 \cdot 10^{-2}$ *M* tartrate; ● – in $2.12 \cdot 10^{-3}$ *M* tartrate.

Nickel-63 activity was measured with a windowless gas flow counter since it is a weak beta emitter of 0.067 MeV. The instrument employed was a Nuclear Chicago Model D-47 gas flow detector equipped with a time interval printer.

All other apparatus and reagents used were described in a previous publication[4].

Procedure

Distribution coefficients. The distribution coefficients for $4.25 \cdot 10^{-4}$ *M* solutions as a function of pH and tartrate concentration were determined by the batch equilibration method described in an earlier communication[4].

After the solution and the resin had reached equilibrium in the batch determinations, aliquots were withdrawn by pipet and the activity was measured. Five ml samples of all solutions except nickel were placed in vials for scintillation counting. Since nickel-63 is a weak β emitter, 1 ml portions of it were withdrawn and placed in planchets and allowed to dry. Activity measurements for these samples were obtained using a windowless gas flow counter.

The pH of the remaining solution was measured and recorded as the equilibrium pH. The activity of the 5 or 1 ml portion was compared to the activity of an equal portion of the original solution to obtain the activity on the resin. Cadmium-115 and molybdenum-99 have relatively short half lives and it was necessary to correct for the decay of these isotopes.

The distribution coefficient, K_d, for each metal was calculated using the following formula:

$$K_d = \frac{\text{c.p.m. in resin phase per gram of resin}}{\text{c.p.m. in solution phase per ml of solution}}$$

Column separations

After preparing a resin bed of the desired depth in a 0.8 cm I.D. column, the resin was brought to the pH and tartrate concentration necessary for the separation. This was accomplished by passing a tartrate solution of the proper pH through the column until the effluent pH matched the eluent pH.

After preparation of the column, a 2 ml sample containing the radioactive metals to be separated was introduced at the top of the column with a pipet. After this portion had passed into the resin, a 125 ml separatory funnel containing the eluting agent was fitted to the top of the column and the flow was started. The effluent was allowed to flow into a fraction collector which collected 2 ml portions. With the exception of nickel-63, these 2 ml portions were viewed qualitatively with a 200 channel pulse height analyzer and were counted on a R.I.D.L. scintillation counter. Nickel was detected by adding 5 drops of concentrated sodium hydroxide and 5 drops of dimethylglyoxime to each 2 ml portion of the effluent and the solutions were observed for a red precipitate. Concentrated hydrochloric acid was then added to dissolve the precipitate and the solutions were diluted to 3 ml. A 1 ml portion was taken from each vial, placed on a planchet, dried, and counted on a windowless flow counter.

RESULTS AND DISCUSSION

The data resulting from the batch equilibrations are shown in Figs. 1–6. These distribu-

TABLE I

CONDITIONS FOR SEPARATIONS

Separation	*Column*	*Sample*	*Flow rate*	*Eluting agent*
Mn, Co, Ni, Fe, Mo	8.0 cm long, treated with $8.5 \cdot 10^{-2}$ *M* tartaric acid at pH 4.0	9 μg Mn, 10 μg Fe, 10 μg Co, 16 μg Mo, 10 μg Ni in $8.5 \cdot 10^{-2}$ *M* tartrate at pH 4.0	3 drops/min	$8.5 \cdot 10^{-2}$ *M* tartrate solution at pH 4.0 for Mn and Co; tartaric acid in 0.1 *M* HCl for Fe; 3 *M* NaOH for Mo
Cr, Ni, Fe, Mo	5.0 cm long, treated with $8.5 \cdot 10^{-2}$ *M* tartaric acid at at pH 3.5	11 μg Cr, 12 μg Ni, 12 μg Fe, 20 μg Mo in $8.5 \cdot 10^{-2}$ *M* tartrate at pH 3.5	4 drops/min	$8.5 \cdot 10^{-2}$ *M* tartrate at pH 3.5 for Cr and Ni; $8.5 \cdot 10^{-2}$ *M* tartaric acid in 0.1 *M* HCl for Fe; 3 *M* NaOH for Mo
Mn, Cr, Fe, Mo	3.5 cm long, treated with $4.24 \cdot 10^{-2}$ *M* tartaric acid at pH 3.0	12 μg Mn, 11 μg Cr, 12 μg Fe, 20 μg Mo in $4.25 \cdot 10^{-2}$ *M* tartrate at pH 3.0	5–6 drops/min	$4.25 \cdot 10^{-2}$ *M* tartrate at pH 3.0; 1 *M* HCl for Fe; 3 *M* NaOH for Mo
Zn, Cd, Hg	4.5 cm long, treated with 100 ml 3 *M* HNO_3, then $8.5 \cdot 10^{-2}$ *M* tartrate at pH 2.5	18 μg Zn, 32 μg Cd, 57 μg Hg in $8.5 \cdot 10^{-2}$ *M* tartrate at pH 2.5	4 drops/min	$8.5 \cdot 10^{-2}$ *M* tartrate at pH 2.5; 1 *M* HNO_3 for Hg

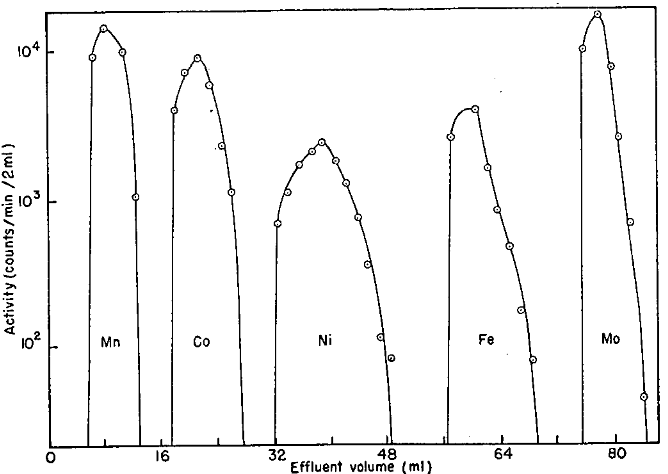


Fig. 7. The separation of manganese, cobalt, nickel, iron and molybdenum.

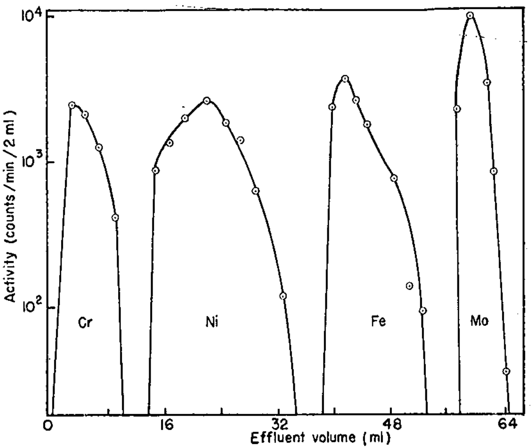


Fig. 8. The separation of chromium, nickel, iron and molybdenum.

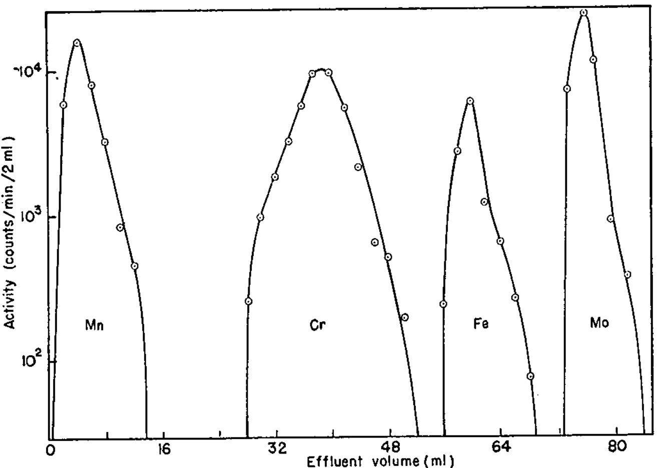


Fig. 9. The separation of manganese, chromium, iron and molybdenum.

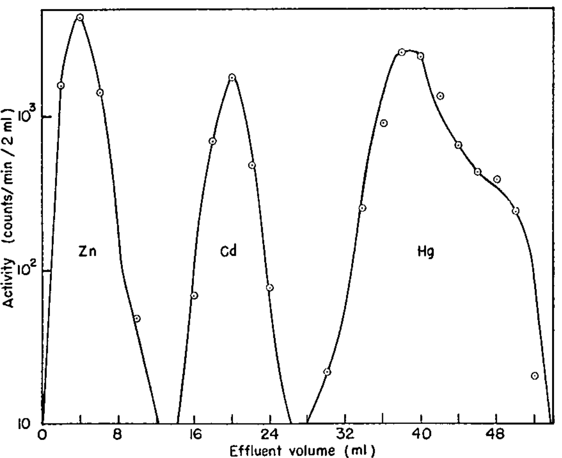


Fig. 10. The separation of zinc, cadmium and mercury.

tion coefficients and those reported in an earlier publication[4], were used to determine conditions for several column separations. Four of these are shown in Figs. 7–10. The conditions for these separations are given in Table I.

SUMMARY

The tartrate complexes of chromium, molybdenum, nickel, silver, cadmium and mercury were studied by the batch equilibration method on Dowex 2X-8 anion exchange resin. From the distribution coefficients obtained for the above metals and for manganese, cobalt, zinc and iron, several column separations were developed. Radioisotopes were used in the batch equilibration studies and in the chromatographic separations.

REFERENCES

1 R. A. GLASS, *J. Am. Chem. Soc.*, 77 (1955) 807.
2 A. P. KRESHKOV AND E. N. SOYUSHKINA, *Khromatogr. ee Teoriya i Primenenie, Akad. Nauk SSSR, Otd. Khim. Nauk, Tr. Vses. Soveshch., Moscow, 1958,* (publ. 1960) p. 367; *C.A.*, 55 (1961) 19,600.
3 D. I. RYABCHIKOV, A. N. ERMAKOV, V. R. BELYAEVA AND I. N. MAROV, *Zh. Neorgan. Khim.*, 5 (1960) 1051; *C.A.*, 55 (1961) 3269.
4 G. F. PITSTICK, T. R. SWEET AND G. P. MORIE, *Anal. Chem.*, 35 (1963) 995.
5 G. P. MORIE AND T. R. SWEET, *Anal. Chem.*, 36 (1964) 140.
6 P. SOUCHAY, *Bull. Soc. Chim. France*, 16 (1949) 122.
7 D. H. KILLEFFER, *Molybdenum Chemistry*, Interscience, New York, 1952.

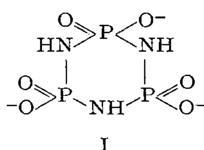
CHROMATOGRAPHIC STUDIES ON THE HYDROLYSIS OF PHOSPHORUS COMPOUNDS

PART X. INVESTIGATIONS INTO THE HYDROLYSIS OF AMIDO- AND IMIDO-PHOSPHATES

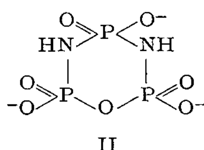
F. H. POLLARD, G. NICKLESS AND A. M. BIGWOOD
Department of Chemistry, The University, Bristol (Great Britain)

(Received February 24th, 1964)

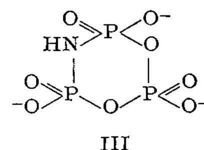
During the course of investigations into the chemistry of imidometaphosphates[1-4], such as trimetaphosphimate (I), diimidotrimetaphosphate (II), monoimidotrimetaphosphate (III), tetrametaphosphimate (IV) and pentametaphosphimate (V) anions,



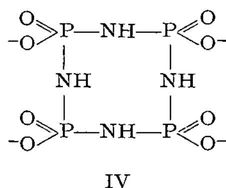
I



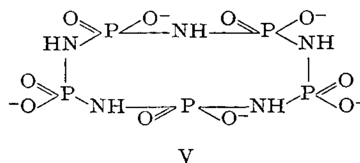
II



III

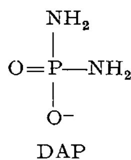


IV

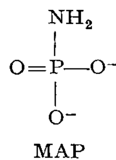


V

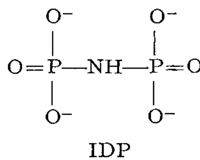
a study of the linear phosphorus-nitrogen acids was carried out. The investigation was thought necessary in an attempt to interpret the behaviour of the higher complex ring acids in solution, in terms of the characteristics shown by the simpler acids, namely, diamidophosphate (DAP), monoamidophosphate (MAP), monoimidodiphosphate (IDP), and diimidotriphosphate (DITP).



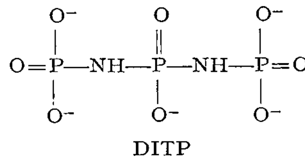
DAP



MAP



IDP



DITP

All linear imidophosphates ultimately degrade in acid solution to monoamidophosphate MAP and orthophosphate[5,6]. Therefore, it was necessary to develop a

suitable means of separating and identifying DAP, MAP, and orthophosphate (MP). Anion-exchange chromatography using a gradient elution technique with chloride solutions has been developed to separate the ring imidophosphates completely[3,4]. Investigations into the possible similar use of this technique in this case subsequently proved successful not only for the separation of amidophosphates, but also for the linear imidophosphates. This technique was used to elucidate the hydrolytic degradations of MAP, IDP and DITP.

EXPERIMENTAL AND RESULTS

Resin. Dowex 1 × 8, mesh size 100–200 B.S.S.
Column. 50 cm × 0.9 cm diameter.
Eluant. 0.075 *M* potassium chloride (buffered to pH 11.4), passed through the column for 200 ml, followed by 0.60 *M* potassium chloride at pH 5.0 dropping into 1 l of 0.075 *M* potassium chloride at pH 7.8.
Temperature. The column was operated at 1° using a circulating water-jacket.
Analysis of eluant. By collecting 10 ml fractions using a syphon fraction collector, and subsequent treatment by the standard colorimetric method for determination of phosphorus by the phosphovanadomolybdate complex[4].

In some cases, when absolute quantitative results were not required, analysis was carried out using a Technicon autoanalyser system, a procedure which has been described in great detail elsewhere[7]. Basically the column eluant pumped at 48.0 ml/h was hydrolysed to convert all phosphorus species to phosphate with a sequence of 7.5 *N* H_2SO_4 for 15 min at 95°, and 10 *N* H_2SO_4 for 30 min at 95°. The orthophosphate so made was subsequently determined as the phosphomolybdenum blue colour using hydrazine sulphate as reducing solution. The final colour flowed continuously through a colorimeter coupled to a potentiometric recorder.

The type of separation achieved using such a system is given diagrammatically in Fig. 1.

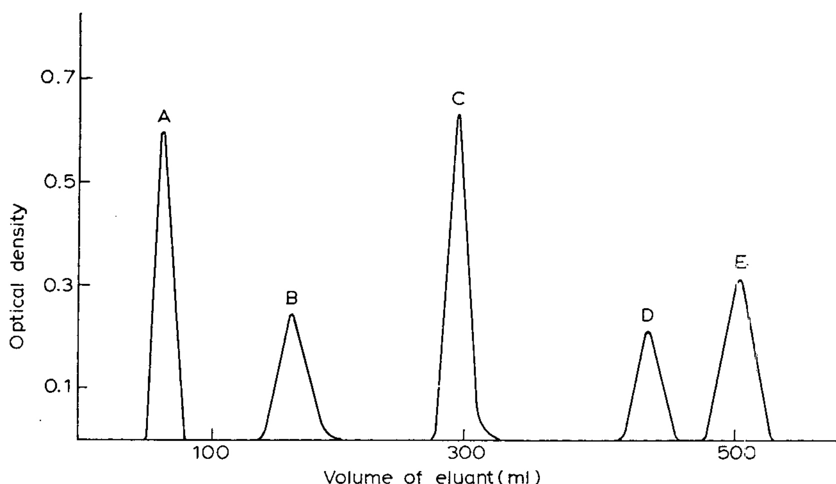


Fig. 1. Separation of amido- and imidophosphates. A = Diamidophosphate. B = Monoamidophosphate. C = Orthophosphate. D = Imidodiphosphate. E = Diimidotriphosphate.

Hydrolysis of amidophosphate

Sodium MAP (supplied by Albright and Wilson Ltd.) was analysed for orthophosphate content and was found to be 99.6 % pure. This was thought to be pure enough for further study.

0.10 g samples of sodium MAP were dissolved in 10 ml of acetate–hydrochloric acid buffer pH 4.40 suspended in a water bath at 40.0°. Aliquots of this solution were removed at different time intervals and delivered into 5.0 ml ice-cold 0.1 N caustic soda solution. 1.0 ml of this solution was transferred to the anion-exchange column and the elution procedure carried out.

Results. From the graph of log a/b (where a = total phosphorus concentration at time t = 0, and b = total amidophosphate concentration present at time t min) against t (min) was produced a linear relationship for a first order reaction with a velocity constant, k, of $3.60 \cdot 10^{-3}$ min^{-1} (see Fig. 2). During the course of the hydrolysis, small

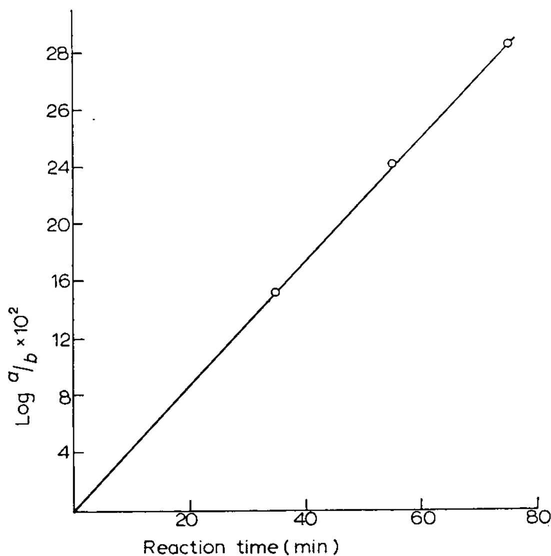


Fig. 2. First order hydrolysis of monoamidophosphate.

quantities of pyrophosphate were detected, but they were neglected in the elucidation reaction kinetics. A typical elution pattern of a reaction mixture is shown in Fig. 3.

Hydrolysis of monoimidodiphosphate

Sodium IDP was prepared and purified by HERBERT of these laboratories[8]. Previous investigations into the hydrolytic degradation of the imidodiphosphate anion[5,6,9] have indicated the possibility of the replacement of the imido-linkage by oxygen to yield the pyrophosphate anion as well as fission of the imido-link to yield MAP and orthophosphate.

Separation of IDP and pyrophosphate (DP) proved extremely difficult by anion exchange, and a much weaker gradient of the chloride eluant was used.

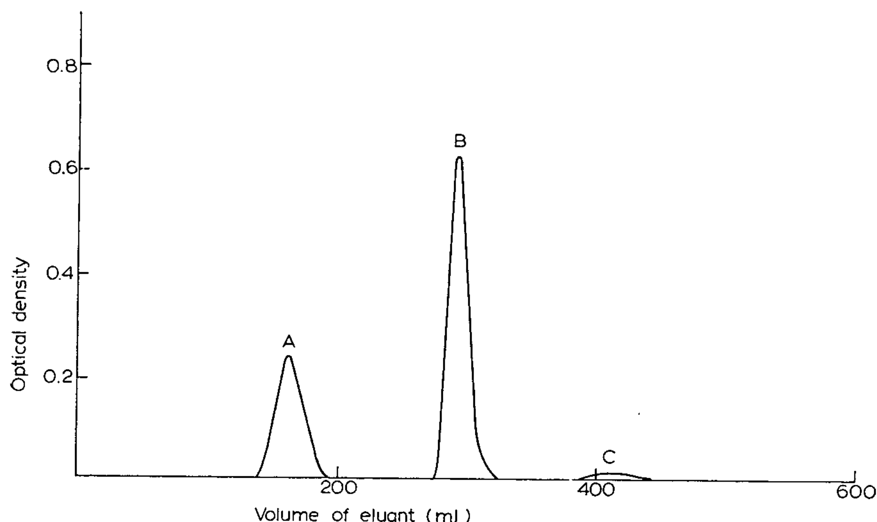


Fig. 3. Elution pattern for the hydrolysis of monoamidophosphate. A = Monoamidophosphate. B = Orthophosphate. C = Pyrophosphate.

Details for the separation of MAP, MP, IDP, and DP, were as given below, and as shown in Fig. 4.

Column. Dowex 1 × 8, 100–200 mesh.

Eluants. 0.075 M KCl (at pH 11.4) for 200 ml, then 0.25 M KCl (at pH 7.8) dropping into 1 l of 0.075 M KCl (at pH 5.0).

Temperature. The column was run at *ca*. 1°.

The hydrolysis of imidodiphosphate was carried out under exactly similar conditions of acidity and temperature as for MAP, and the same procedure used for the analysis.

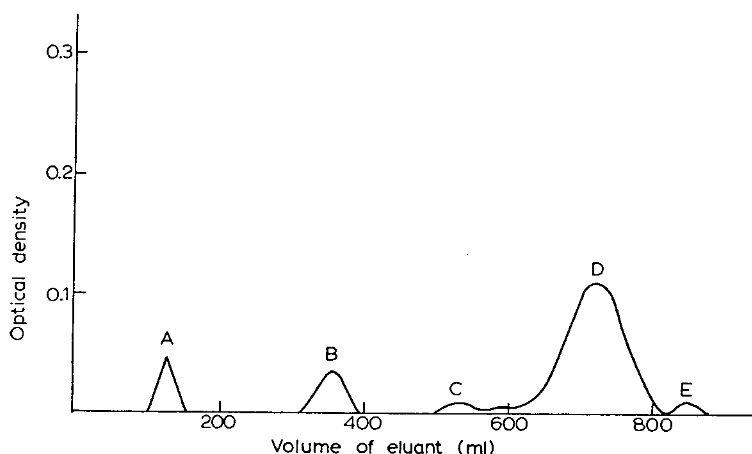


Fig. 4. Elution pattern for the hydrolysis of imidodiphosphate. A = Monoamidophosphate. B = Orthophosphate. C = Forward tailing (decomposition) of D. D = Imidodiphosphate. E = Pyrophosphate.

Results. Sodium IDP was found to hydrolyse very rapidly at pH 4.40, and 40.0° to MAP and orthophosphate. Pyrophosphate was detected, but only in small quantities of the order of 5 %.

The first order reaction velocity constant was evaluated to approximately $1 \cdot 10^{-1}$ min^{-1}, and is shown in Fig. 5.

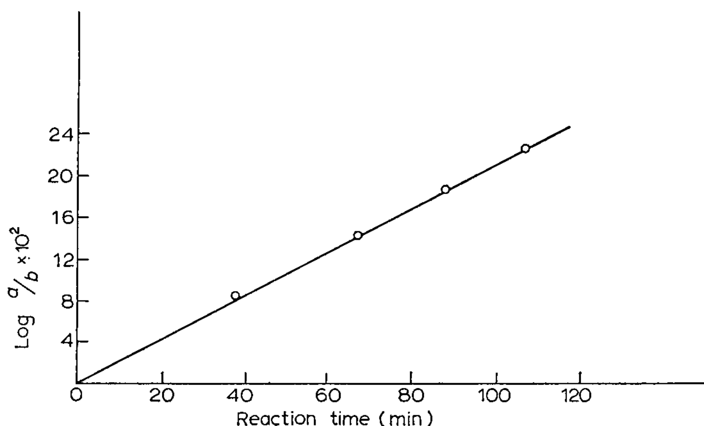


Fig. 5. First order hydrolysis of imidodiphosphate.

Hydrolysis of diimidotriphosphate

Using the separation conditions given in relation to Fig. 1, the hydrolysis of sodium DITP[8] (prepared and purified in these laboratories by P. A. HERBERT) was investigated at 40.0° and pH 4.40. Analysis of the reaction products was carried out using the Autoanalyser system, and a typical trace for a reaction time of 90 min is given in Fig. 6.

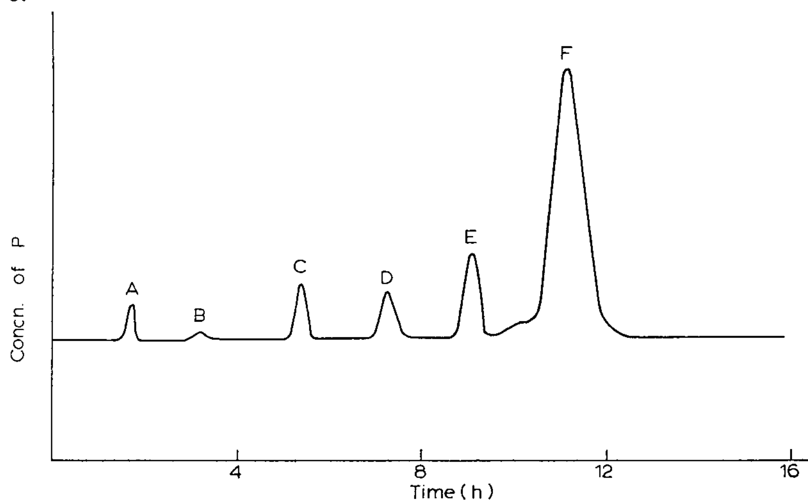


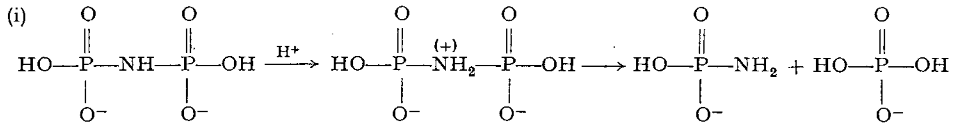
Fig. 6. Elution pattern for the hydrolysis of diimidotriphosphate. A = Diamidophosphate. B= Monoamidophosphate. C = Orthophosphate. D = Unknown species (see text). E = Imidodiphosphate. F = Diimidotriphosphate.

The reaction velocity constant could not be evaluated to any certain degree of accuracy, but the system is being examined in greater detail.

DISCUSSION

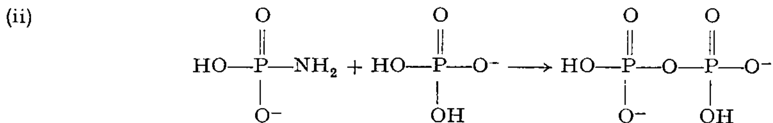
The study of the hydrolytic breakdown of IDP and DITP shows a definite pattern of behaviour for linear imidophosphates in general. IDP breaks down on hydrolysis to amidophosphate, orthophosphate and small quantities of pyrophosphate. The breakdown of the imido linkage therefore occurs primarily by protonation of the -NH-group,

(i)



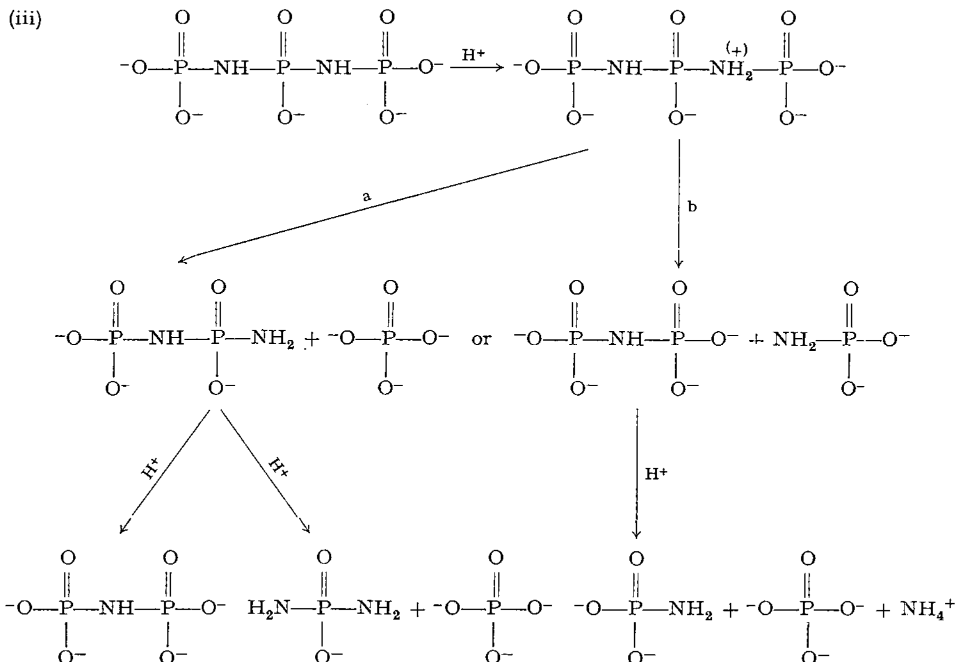
and condensation of amido and orthophosphate occurs to yield pyrophosphate.

(ii)



Similarly, DITP hydrolysis by the same mechanism of protonation of one -NH-linkage, and subsequent fission to yield amidoimidodiphosphate and orthophosphate (iiia), or imidodiphosphate and amidophosphate (iiib).

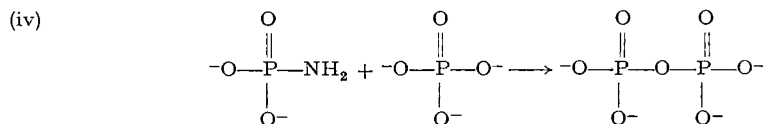
(iii)



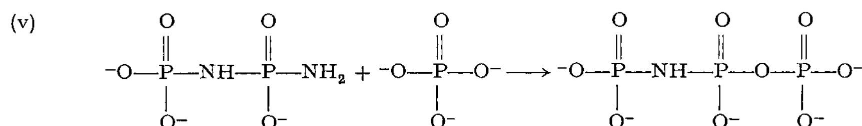
Analysis of the hydrolysis products of the DITP anion (Fig. 6) shows the presence of diamidophosphate and an unknown species (D) which by inference from its faster retention time with reference to imidodiphosphate must be amidodiphosphate. The main reaction path, therefore, seems to be (iiia) and not (iiib).

With the presence of amidophosphate in solutions, small quantities of condensation products are almost certainly formed:

(iv)



(v)



but unfortunately no positive establishment of their existence was found.

Attention must finally be drawn to the stability of monoamidophosphate ($3.60 \cdot 10^{-3}$ min^{-1}) with reference to the stability of monoimidodiphosphate ($1 \cdot 10^{-1}$ min^{-1}) under identical conditions. Further studies into the variation of reaction velocity constant with pH are now being carried out, and details will be published later.

ACKNOWLEDGEMENT

Grateful acknowledgement is made to the Department of Scientific and Industrial Research for a Maintenance Grant to one of us (A.M.B.) during the period of this research.

SUMMARY

A study of the hydrolytic degradation of the linear phosphorus-nitrogen acids is outlined and discussed in an attempt to interpret the behaviour of more complex ring imidophosphate acids in solution.

REFERENCES

1 F. H. Pollard, G. Nickless and R. W. Warrender, *J. Chromatog.*, 9 (1962) 493.
2 F. H. Pollard, G. Nickless and R. W. Warrender, *J. Chromatog.*, 9 (1962) 513.
3 F. H. Pollard, G. Nickless and A. M. Bigwood, *J. Chromatog.*, 11 (1963) 534.
4 F. H. Pollard, G. Nickless and A. M. Bigwood, *J. Chromatog.*, 12 (1963) 527.
5 A. Narath, F. H. Lohmann and O. T. Quimby, *J. Am. Chem. Soc.*, 78 (1956) 4493.
6 O. T. Quimby, A. Narath and F. H. Lohmann, *J. Am. Chem. Soc.*, 82 (1960) 1099.
7 F. H. Pollard, G. Nickless, D. E. Rogers and M. T. Rothwell, *J. Chromatog.*, in press.
8 P. A. Herbert, *B.Sc. Thesis*, Bristol, 1962.
9 N. L. Nielson, R. R. Ferguson and W. S. Coakley, *J. Am. Chem. Soc.*, 83 (1961) 133.

Short Communication

Separation of S_8, S_7NH, and S_4N_4 by adsorption chromatography

Sulfur, heptasulfur imide and tetrasulfur tetranitride are products of the reaction of ammonia with S_2Cl_2 and are formed in various reactions involving sulfur–nitrogen compounds[1]. Characterization of these reactions has been delayed because of the difficulty of quantitatively analyzing the product mixtures. As might be expected from the fact that the three substances have similar molecular structures (puckered eight-membered rings), they have similar solubilities in organic solvents and are difficult to separate from one another. Various investigators[2–5] have mentioned the use of adsorption chromatography in the separation of S_8 from S_7NH and related materials, but no details have been given. We describe here a method involving elution with benzene from alumina for the separation of S_4N_4 from S_7NH and S_8, and a method involving elution with carbon tetrachloride from silica gel for the separation of S_7NH from S_8.

Experimental

Commercial alumina (M. Woelm-Eschwege, acid, activity grade 1) and silica gel (J. T. Baker Chemical Co., "suitable for chromatographic use") were used. When required, "dried" alumina and silica gel were prepared by heating for 12 h at 150° and 200°, respectively. The solvents were dried over P_2O_5 and distilled. The column beds were 18 cm long and 2.54 cm in diameter; a flowrate of 1 ml/min was used. Known mixtures of S_8, S_7NH and S_4N_4 were prepared by weighing out the pure materials; the chromatographic fractions were evaporated to dryness and weighed. Data are presented in Table I.

The melting points of S_4N_4 and S_7NH (187–187.5° and 113.5°, respectively) are good criteria of purity; the purity of S_8 may be ascertained from its infrared spectrum, which should show no bands in the NaCl region. Eluate containing S_4N_4 is readily recognized by its orange color. Eluate containing S_7NH is colorless, but may be identified by the purple-violet color which forms on treating a small portion with an equal volume of a 10 % solution of KOH in anhydrous methanol. Similar treatment of eluate containing only S_8 gives no color, but, as is also the case with S_7NH, a yellow color forms on heating the mixture.

Discussion

Tetrasulfur tetranitride is held very tenaciously by alumina and silica gel; benzene (a good solvent for S_4N_4) was used for its elution. When columns of either undried alumina or undried silica gel were used, very poor recoveries of S_4N_4 were achieved (see Table I), and non-elutable sulfur compounds were retained in the columns. We believe that S_4N_4 undergoes hydrolysis on the undried adsorbents to form various sulfur oxyacids which are insoluble in benzene. About 90 % recovery of S_4N_4 was

TABLE I

CHROMATOGRAPHIC SEPARATION OF KNOWN MIXTURES OF S_8, S_7NH AND S_4N_4

	S_8 (g)		S_7NH (g)	S_4N_4 (g)
Non-dried alumina				
Taken	0.2352		0.0105	0.1242
Recovered	0.2345		0.0092	0.0882
Dried alumina				
Taken	0.1765		0.0227	0.3311
Recovered		0.1952		0.3106
Non-dried silica gel				
Taken	0.1906		0.2015	0.1283
Recovered	0.1900		0.2051	0.0394
Dried silica gel				
Taken	0.1770		0.1900	0.2002
Recovered		0.3652		0.1821

achieved using dried silica gel, and about 95 % recovery was achieved using dried alumina (R_F 0.15); we recommend use of the latter adsorbent for the separation of S_4N_4 from S_7NH and S_8.

Both S_7NH and S_8 are weakly held by alumina and silica gel; the relatively poor solvent carbon tetrachloride was used for the elution of these compounds. When columns of either dried alumina or dried silica gel were used, both S_7NH and S_8 came out together with the solvent front. When undried adsorbents were used, the S_7NH was eluted in a well-separated band (R_F 0.15) after the S_8. We believe the S_7NH is held by hydrogen bonding to the water of hydration of the undried adsorbent. Both S_7NH and S_8 were consistently recovered in 98–102 % yield with undried silica gel columns, and we recommend use of the latter adsorbent for the separation of these compounds.

This research was supported in part by the U.S. Atomic Energy Commission.

Department of Chemistry, University of California, Berkeley, Calif., and Inorganic Materials Research Division, Lawrence Radiation Laboratory, Berkeley, Calif. (U.S.A.)

M. VILLENA-BLANCO
W. L. JOLLY

1 M. GOEHRING, *Ergebnisse und Probleme der Chemie der Schwefelstickstoffverbindungen*, Akademie-Verlag, Berlin, 1957.
2 J. WEISS, *Angew. Chem.*, 71 (1959) 246.
3 H. G. HEAL, *J. Chem. Soc.*, (1962) 4442.
4 P. TAVS, H.-J. SCHULZE-STEINEN AND J. E. COLCHESTER, *J. Chem. Soc.*, (1963) 2555.
5 J. BUCKLEY AND H. G. HEAL, *J. Inorg. Nucl. Chem.*, 25 (1963) 321.

Received July 24th, 1964

Notes

Gaschromatographische Trennung der bei der Hydroformylierung von *n*-Octenen gebildeten C_9-Alkohole

Die bei der Hydroformylierung von *n*-Octenen entstehenden Gemische isomerer C_9-Alkohole konnten gaschromatographisch unter Verwendung einer mit Polypropylenglykol belegten Makro-Golaysäule, Typ 4 G(R) der Firma Perkin-Elmer, Frankfurt/M., quantitativ analysiert werden.

Die Arbeitsbedingungen waren: Makro-Golaysäule aus Kupfer, Länge 100 m, Innendurchmesser 1 mm, stationäre Phase Polypropylenglykol, Kolonnentemperatur 124°, Einlasstemperatur 220°, Trägergas Helium, Durchflussmenge ca. 9 cm³/Min. Verwendet wurde das Gerät 116 E der Firma Perkin-Elmer mit einer Wärmeleitähigkeitsmesszelle.

Tabelle I enthält die unter den genannten Bedingungen erhaltenen Retentions-

TABELLE I

ERGEBNIS DER GASCHROMATOGRAPHISCHEN ANALYSE ZWEIER TESTGEMISCHE ISOMERER C_9-ALKOHOLE

C_9-Alkohole	Retentions-zeit (Min.)	Test I (Gew.-%)		Test II (Gew.-%)	
		Einwaage	gef.	Einwaage	gef.
2-Propyl-*n*-hexanol-1	88	10.7	11	24.0	25
2-Äthyl-*n*-Heptanol-1	95	18.9	19	26.4	25
2-Methyl-*n*-octanol-1	99	33.2	34	24.8	26
n-Nonanol-1	128	37.2	36	24.8	24

zeiten und die Ergebnisse der Analysen zweier Testgemische der auf präparativem Weg dargestellten C_9-Alkohole.

Die 2-alkylverzweigten C_9-Alkohole wurden durch Reduktion der über eine Malonestersynthese leicht zugänglichen entsprechenden *n*-Octancarbonsäuren mit Lithiumaluminiumhydrid rein dargestellt. Über Einzelheiten wird an anderer Stelle berichtet werden.

Institut für Technische Chemie der Technischen Hochschule Aachen (Deutschland)

ELISABETH BENDEL
GUDULA MAHR
BERNHARD FELL
MOHAMED F. EL DAOUSHY

Eingegangen den 21. April 1964

Ein Gasprobengeber zum Betrieb bei erhöhter Temperatur

Mit seiner "microcatalytic chromatographic technique" schuf EMMETT[1-3] eine heute oft als Pulsmethode bezeichnete einfache Möglichkeit zur Untersuchung der Produktverteilung heterogen katalytischer Reaktionen mit Hilfe der Gaschromatographie. Zur Bestimmung von Reaktionsordnungen und Aktivierungsenergien ist jedoch ein kontinuierlich arbeitender Reaktor vorzuziehen, insbesondere, da sich dann die Katalysatoraktivität besser konstant halten lässt. Wir verwendeten einen an anderer Stelle[4] beschriebenen differentiell arbeitenden Strömungsreaktor in Verbindung mit einem Gaschromatographen wie das Schema Fig. 1 zeigt.

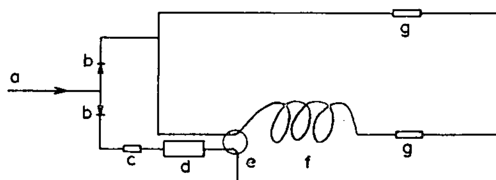


Fig. 1. Schema des Gaschromatographen. (a) Trägergas; (b) Ventile; (c) Verdampfer; (d) Reaktor; (e) Probengeber; (f) Kolonne; (g) Detektor.

Arbeitet man mit bei Zimmertemperatur kondensierbaren Substraten, die in einem Verdampfer dem Trägergas beigegeben werden, so müssen alle Leitungen und Apparateteile auf dem Weg zur Kolonne beheizt werden. Dabei stösst man insbesondere auf Schwierigkeiten bei der Schmierung und Beheizung des Gasprobengebers. Bei Verwendung eines Gasprobengebers der Firma Beckman Instruments Inc. und Schmierung mit Silikonfetten bzw. Molykote traten schon bei Betriebstemperaturen unter 100° nach kürzester Zeit Undichtigkeiten auf, die sich in Sprüngen der Nullinie bei der Probengabe und in unreproduzierbarer Anzeige bemerkbar machten. Da in kommerziellen Geräten die Gasprobengeber im allgemeinen auf Zimmertemperatur gehalten werden, und ohne grösseren Umbau eine Beheizung

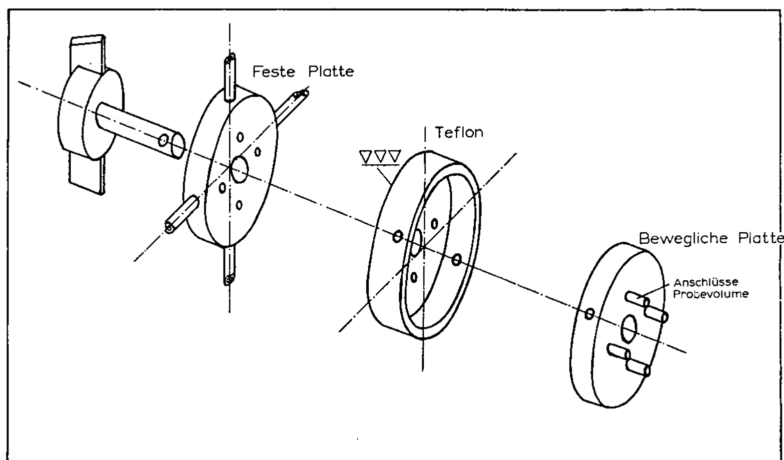


Fig. 2. Probengeber.

schwierig ist, haben wir einen Gaschromatographen aufgebaut, bei dem der Gasprobengeber direkt in den Kolonnenraum gesetzt wurde. Zur Abdichtung des verwendeten Beckman-Probengebers wurde zwischen die beiden gegen einander drehbaren Stahlflächen eine 4 mm starke Teflonscheibe eingeführt (Fig. 2). Diese wurde durch einen Stift mit der beweglichen Scheibe fest verbunden und diente so als selbstschmierende Dichtung. Die Oberflächen der Teflonscheibe wurden zunächst mit Poliertonerde von Hand und dann auf einer rotierenden, mit Seide bespannten Platte fein poliert. Nach dieser Behandlung war der Probengeber auch bei Betriebstemperaturen bis 300° absolut dicht. Um die polierte Teflonfläche nicht frühzeitig durch zu hohe mechanische Beanspruchung zu verletzen, sollte sie nicht mit zu hohem Druck gegen die Stahlfläche gepresst werden.

Auf ähnliche Weise lassen sich auch an kommerziellen Geräten Gasprobengeber, ev. durch Einbau in einen gesonderten Thermostatenraum beheizen.

Wir danken Herrn Prof. Dr. G.-M. SCHWAB für sein stetes Interesse, der Deutschen Forschungsgemeinschaft und dem Fonds der Chemie für finanzielle Unterstützung.

Physikalisch-Chemisches Institut der Universität München (Deutschland)

HELMUT KNÖZINGER
LUTZ KUDLA

1 R. J. KOKES, H. TOBIN, JR. UND P. H. EMMETT, *J. Am. Chem. Soc.*, 77 (1955) 5860.
2 W. K. HALL UND P. H. EMMETT, *J. Am. Chem. Soc.*, 79 (1957) 2091.
3 P. H. EMMETT, *Advan. Catalysis*, 9 (1957) 645.
4 G.-M. SCHWAB UND H. KNÖZINGER, *Z. Physik. Chem. (Frankfurt)*, [NF] 37 (1963) 230.

Eingegangen den 18. März 1964

J. Chromatog., 16 (1964) 217–218

Derivatographische Bestimmung der höchsten Anwendungstemperatur der Trennflüssigkeit bei der Verteilungschromatographie

Zu den verschiedenen verteilungschromatographischen Aufgaben werden zahlreiche Trennflüssigkeiten angewandt. Bei der Benützung dieser Trennflüssigkeiten ist es wichtig die Höchsttemperatur zu kennen bei welcher sie noch gebraucht werden dürfen. Über diese Temperaturen der einzelnen Flüssigkeiten sind unterschiedliche Angaben in der Literatur zu finden[1–6]. Auch die eindeutigen Angaben sind mit gewisser Vorsicht in der Praxis zu benützen da nach unseren Erfahrungen häufig schon weit unter der angegebenen Temperatur beträchtliche Mengen der Trennflüssigkeit aus der Säule entweichen, wodurch einerseits die Funktion des Detektors beeinträchtigt, andererseits die Kapazität der Säule in unberechenbarer Weise beeinflusst würde. Unter solchen Veränderungen lassen sich Messungen nicht mit Sicherheit reproduzieren. Ähnliche Erfahrungen wurden schon von zahlreichen Forschern gemacht[7].

Es ist zwar wahr, dass bei beliebiger Flüchtigkeit den Verdampfungsverlusten

der Verteilungsflüssigkeit vorgebeugt werden kann, indem das Schleppgas an der Eintrittsstelle durch eine mit der Trennflüssigkeit gefüllte und bei der Temperatur der Kolonne gehaltene Wascheinrichting geleitet und so mit den Dämpfen der Trennflüssigkeit gesättigt wird[8]. Diese Bedingung kann jedoch bei analytischen Aufgaben nur schwer erfüllt werden.

Zur Bestimmung der höchsten Anwendungstemperatur benützten wir — uns auf die im unseren Institut seit Jahren ausgeführten thermoanalytischen Forschungen und Literaturangaben[9–11] stützend — zuerst eine aus gewöhnlicher luftgebremster analytischer Waage bereitete Thermowaage[12] dann den Derivatographen (Typ. Paulik-Paulik-Erdey, MOM, Budapest). Beide Instrumente waren zur Lösung der Aufgabe geeignet. Der Derivatograph besitzt aber ausserdem den Vorteil, dass darin die Wechselwirkungen von Trennflüssigkeit und von anderen Parametern (Träger, Schleppgas usw.) ebenfalls genau studiert werden können, da gleichzeitig mit der TG-Kurve auch die DTG- und DTA-Kurven* aufgenommen werden.

Der Derivatograph wurde auch zur Kontrolle der benützten bzw. der im Handel erhältlichen Kolonnen herangezogen. Die Messungen zeigten, dass die anfänglich 20% betragende Trennflüssigkeit nach 50–60 gaschromatographischen Analysen auf 9–11% sinkt. Die handelsüblichen Kolonnen erhielten im allgemeinen statt der angegebenen 20% Befeuchtung nur 15–17%.

Auf Grund der derivatographischen Untersuchungen wird die Höchsttemperatur der Anwendbarkeit von verschiedenen Trennflüssigkeiten im folgenden gegeben (Tabelle I).

TABELLE I

DIE HÖCHSTTEMPERATUR DER ANWENDBARKEIT VON TRENNFLÜSSIGKEITEN

Trennflüssigkeit	*Höchsttemperatur (°C)*
Polyäthylenglykol 400	90
Dibutylphthalat	100
Dinonylphthalat	120
Bernsteinsäurepolyester	150
Carbowax 4000	150
DC Silikonöl 200	150
DC Silikonöl 550	200
Diäthylenglykol-succinat	200
Silikon Vakuumfett	280
Apiezon L	280

Aus der Reihe der aufgenommenen Thermogramme ist in Fig. 1 als Beispiel die thermogravimetrische Kurve des DC Silikonöl 550 auf 1,000 g bezogen dargestellt.

Da die Empfindlichkeit der gaschromatographischen Detektoren wesentlich empfindlicher als die der Thermowaage bzw. des Derivatographen ist wurde als obere Temperatur der Trennflüssigkeit der Temperaturwert genommen, bei welchem die Abweichung von der Gerade an der thermogravimetrischen Kurve beginnt. Die von uns erhaltenen Werte waren in der Regel niedriger als die Literaturangaben, die gaschromatographische Analysen bezeugten jedoch die Richtigkeit unserer Angaben.

* TG- = thermogravimetrische; DTG- = differential-thermogravimetrische; DTA- = differential-thermoanalytische.

Im Laufe der weiteren derivatographischen Untersuchungen wurde die Wirkung der stofflichen Art der Trägersubstanz (Ziegelpulver, Silikagel, Celite, Aluminiumoxyd) der Korngrösse (0.1–1.0 mm) der Befeuchtung (5.0–30 %) der stofflichen Art des Schleppgases (Stickstoff, Kohlendioxyd, Argon), der Strömungsgeschwindigkeit (0–6.0 l/h) weiterhin der Aufheizungsgeschwindigkeit (3.0–20.0°/min) auf die obere Temperaturgrenze der Anwendbarkeit untersucht.

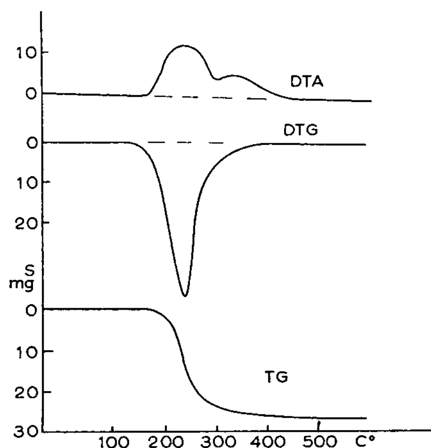


Fig. 1. Die derivatographische Kurven von DC Silikonöl 550.

Es wurde festgestellt, dass diese Parameter die obere Temperaturgrenze nicht beeinflussen, obzwar WILLIAMS[13] Unterschiede in der Grenztemperatur von auf Chromosorb-P und Teflonträger aufgetragene Silikon E 301 Trennflüssigkeit beobachtet zu haben behauptet.

Wir danken Prof. L. ERDEY für seine Ratschläge bei den Versuchen.

Institut für Allgemeine Chemie, Technische Universität Budapest (Ungarn)

J. TAKÁCS
J. BALLA
L. MÁZOR

1 R. KAISER, *Gas-Chromatographie*, Akad. Verlagsges., Leipzig, 1960, p. 194.
2 R. KAISER, *Chromatographie in der Gasphase*, 3 (1962) 40.
3 E. BAYER, *Gas Chromatography*, Elsevier, Amsterdam, 1961, p. 28.
4 A. B. LITTLEWOOD, *Gas Chromatography*, Academic Press, New York, 1962, p. 92.
5 S. D. NOGARE UND R. S. JUVET, *Gas–Liquid Chromatography*, John Wiley, New York, 1962, p. 121.
6 S. T. PRESTON, *J. Gas Chromatog.*, 1, No. 3 (1963) 8.
7 L. SZEPESY, *Gázkromatográfia*, Müszaki Könyvkiadó, Budapest, 1963, p. 80.
8 G. SCHAY, *A gázkromatográfia alapjai*, Akadémia Kiadó, Budapest, 1961, p. 202.
9 S. D. NOGARE UND J. C. HARDEN, *Anal. Chem.*, 31 (1959) 1829.
10 S. D. NOGARE UND C. E. BENNETT, *Anal. Chem.*, 30 (1958) 1157.
11 S. D. NOGARE UND W. E. LANGLOIS, *Anal. Chem.*, 32 (1960) 767.
12 L. ERDEY, F. PAULIK UND J. PAULIK, *Magy. Tud. Akad., Kem. Tud. Oszt. Kozlemen.*, 7 (1956) 55.
13 A. F. WILLIAMS, *Gas Chromatography*, Butterworths, Washington, 1960, p. 210.

Eingegangen den 16. März 1964

Analysis of acetylacetone by means of gas chromatography

Since there is no method available in the literature[1–6] which is selective or sensitive enough for the analysis of acetylacetone, we have developed a gas chromatographic method for this purpose and have carried out gas chromatography on a crude, a purified and a pure acetylacetone sample. The investigations were carried out on a Carlo Erba Fraktovap Modell C type gas chromatograph. The following experimental conditions were found to be best:

Detector: thermistor; temperature 175.0°; bridge current 20 mA; sensitivity 3–50 %, depending on the amount of the sample.

Sample: 0.2–10.0 μl.

Column: spiral of stainless steel; length 1800 mm; internal diameter 5 mm; packing 20 % silicone oil 550 on Celite (with particle diameter of 0.2 mm).

Temperature of the evaporator: 230°.

Carrier gas: hydrogen; flow rate 20 ml/min; pressure 0.13 kg/cm^2.

Temperature of the thermostat: 175.0°.

Compensograph: sensitivity 2.5 mV; paper speed 1.25 cm/min.

Fig. 1 shows the chromatogram of the crude acetylacetone. Using the internal standard method for qualitative identification and quantitative evaluation the presence of the constituents listed in Table I was established.

TABLE I

RESULTS OF GAS CHROMATOGRAPHIC ANALYSIS OF CRUDE ACETYLACETONE

No.	*Constituent*	*Retention time (min)*	*Retention volume (ml)*
1	Acetoacetic ester (contamination)	1.28	25.60
2	Acetone	1.84	36.80
3	Diacetone alcohol	2.00	40.00
4	Acetic acid	2.16	43.20
5	Ethyl acetate	2.32	46.40
6	Water	3.02	60.40
7	Acetylacetone	3.92	78.40
8	Phorone	6.48	129.60
9	Acetyl-acetoacetic ester	10.55	211.00

In the acetylacetone purified by distillation only the following constituents were found: (1) Acetoacetic ester (contamination), (2) diacetone alcohol, (3) acetic acid, (4) acetylacetone, and (5) phorone, while the pure acetylacetone contained only the following trace impurities: (1) acetoacetic ester (contamination), (2) diacetone alcohol, and (3) acetic acid.

Comparing the data obtained from the analysis of the crude, purified and pure acetylacetone respectively, it is clear that similar mixtures were analysed, only the number and the amount of the contaminants differing in the samples.

An interesting phenomenon has been observed with chromatography of pure acetoacetic ester, when seven peaks were observed (see Fig. 2). After many measurements we established that the first peak is caused by contaminations, while the others are due to the acetoacetic ester. They are probably the consequence of equilibrium

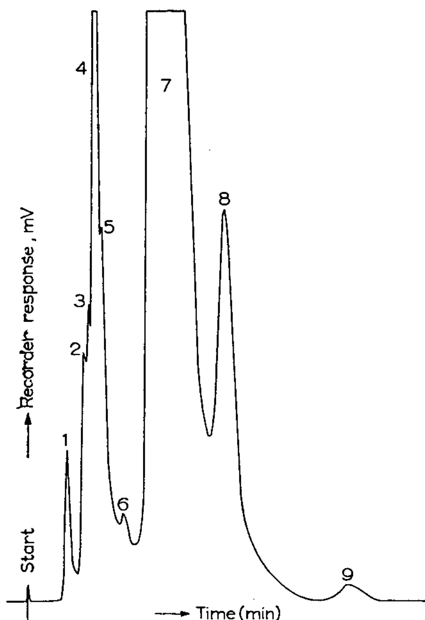


Fig. 1. Chromatogram of purified acetylacetone.

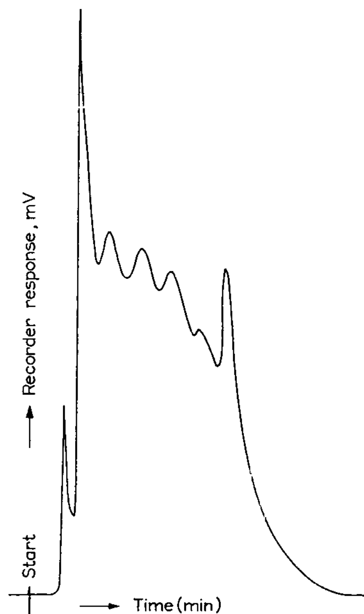


Fig. 2. Chromatogram of pure acetoacetic ester.

positions of keto-enol rearrangement and internal transformations of acetoacetic ester during analysis. Experiments to elucidate this phenomenon are in progress and results of these studies will be published later.

We should like to express our thanks to Prof. LÁSZLÓ ERDEY for his valuable help and advice.

Institute for General Chemistry, Technical College, Budapest (Hungary)

L. MÁZOR
J. TAKÁCS
J. BALLA

1 P. W. WEST, B. SEN, B. R. SANT, K. L. MALLIK AND J. G. SEN GUPTA, *J. Chromatog.*, 6 (1961) 220.
2 F. FEIGL, V. ANGER AND G. FISCHER, *Mikrochim. Acta*, (1962) 878.
3 M. BARBIER, L. P. VINOGRADOVA AND S. J. ZAV'YALOV, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1961) 162.
4 A. P. ALTSHULLER AND I. R. COHEN, *Anal. Chim. Acta*, 24 (1961) 61.
5 W. J. BLAEDEL AND D. L. PETITJEAN, *Anal. Chem.*, 30 (1958) 1958.
6 C. D. HODGMAN (Editor), *Handbook of Chemistry and Physics*, 40th Ed., Chem. Rubber Publ. Co., Cleveland, Ohio, 1958–1959, p. 2436.

Received March 16th, 1964

Estimation of glycerol and diglycerol in the presence of each other

Polymerisation of glycerol results in a series of compounds containing two or more glycerol residues. Commercial diglycerols contain usually, beside a small quantity of glycerol, substantial amounts of dimeric polymer and varying amounts of higher polymers. These mixtures may be analysed by periodate oxidation combined with the determination of hydroxyl values but as in most indirect methods the errors may be considerable. Further complications arise from the possible existence of three isomers of diglycerol *i.e.* α,α'-, α,β'- and β,β'-diglycerols which consume 2, 1 and 0 moles of periodate respectively, although an appreciable formation of the β,β'-isomer is unlikely. When faced with the need of estimating diglycerol in the presence of glycerol, the amount of available material precluding the application of indirect chemical methods, a chromatographic separation of the two compounds was attempted. Paper chromatography with solvent systems such as butanol–acetic acid–water, chloroform–ethanol and others failed to give satisfactory separation but gas–liquid chromatography proved feasible. The present communication reports the identification and quantitative determination of glycerol and diglycerol in the form of acetates using an argon ionisation detector.

Experimental

Materials. Commercial glycerol triacetate was purified by distillation *in vacuo.* Diglycerol tetra-acetate was prepared by acetylation of a commercial diglycerol which contained on the basis of periodate analysis approximately 53 % of α,α'-dimer, 45 % of α,β'-dimer and 2 % of glycerol. The acetylation was carried out by refluxing 1 g of diglycerol with 25 g of acetic anhydride for 2 h and removing the excess of the acetylating agent *in vacuo* at 100°. The product was dissolved in ethyl ether and washed with water. The ethereal solution was dried with anhydrous sodium sulphate and the ether was evaporated *in vacuo.* The product was purified by passing 0.2 g lots through a preparative chromatograph (Wilkens Aerograph Model A-700) using a column packed with celite containing 20 % Apiezon L. Subsequent chromatographic analyses showed it to be free of glycerol triacetate.

A fractionally distilled and repeatedly crystallised methyl stearate m.p. 39.5–41° was used as reference compound for the determination of the relative retention times of glycerol and diglycerol acetates.

Apparatus. A chromatograph constructed in this Laboratory and fitted with an Argon ionising detector[1] and operating at 207° was used in this work. The columns were 240 cm long and had 6.5 mm I.D. The selection of a suitable packing required prolonged experimenting. Liquid phases of high polarity such as polydiethylene-glycol adipate on celite adsorbed diglycerol tetraacetate entirely. Columns with 5–10 % Apiezon L on celite effected a good separation of glycerol and diglycerol acetates but the dimer was partly adsorbed. Satisfactory results were obtained with silicone high vacuum grease and silicone rubber gum SE-30 on glass beads. Glass beads of 0.177 mm diameter (A.S.T.M. Grade 80) were coated with 0.25 % of silicone high vacuum grease purified as described by NELSON AND MILUN[2]. The same grade of glass beads was coated with 0.5 % of silicone rubber gum SE-30.

Analysis of synthetic mixtures and relative retention times. The separation of three known mixtures of glycerol triacetate and diglycerol tetraacetate is shown in Fig. 1

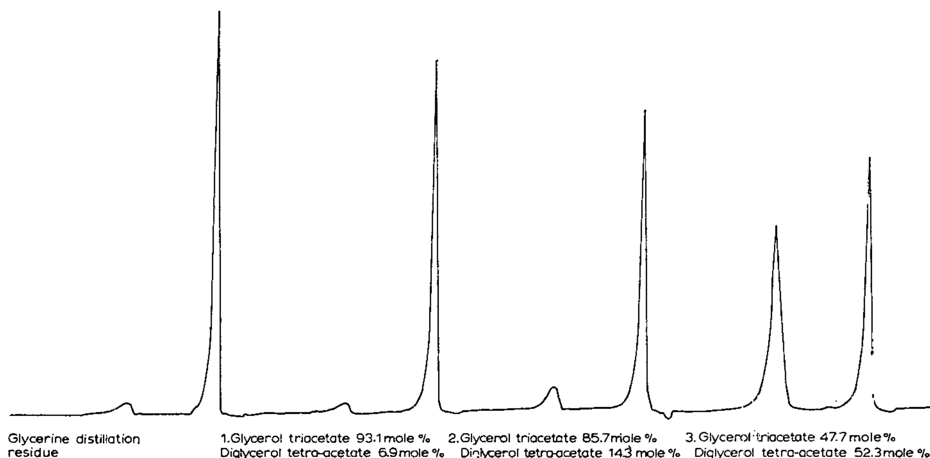


Fig. 1. Chromatograms of known binary mixtures of glycerol triacetate and diglycerol tetraacetate and of an acetylated glycerine distillation residue.

and the differences between the amounts weighed and found are listed in Table I. As could be expected, resolution of the two isomeric diglycerol tetraacetates undoubtedly present in the mixture did not take place.

The relative retention times of glycerol triacetate, diglycerol tetraacetate and methyl stearate are shown in Table II. The separation of methyl stearate from both acetates appeared excellent but the values for it, found in mixtures of known composition, were greatly in excess of the amounts taken, possibly owing to different molar responses.

Some applications of the method. A sample of glycerine residue from a commercial glycerine distilling plant—glycerine "foots"—containing approximately 12 % glycerol, 24 % sodium chloride and free alkali and 64 % water was analysed for its diglycerol content. After removing most of the water *in vacuo* the sample was taken up in acetic acid and filtered. Acetic acid was evaporated *in vacuo*, the residue was acetyl-

TABLE I

CHROMATOGRAPHIC DETERMINATION OF DIGLYCEROL TETRAACETATE IN KNOWN BINARY MIXTURES OF GLYCEROL AND DIGLYCEROL ACETATES AND IN GLYCERINE DISTILLATION RESIDUE

Glassbeads coated with 0.25 % silicone grease and 0.5 % SE-30 respectively.

Mixture	*Diglycerol tetraacetate (mole %)*		
	Taken	*Found**	
		Silicone grease	*SE-30*
1	6.9	5.9 ± 0.5	7.2 ± 0.9
2	14.3	13.8 ± 0.6	14.2 ± 1.3
3	52.3	51.9 ± 0.8	52.4 ± 0.8
Glycerine dist. residue	—	—	8.1 ± 0.7

* Based on four chromatograms in each case.

TABLE II

RELATIVE RETENTION TIME OF GLYCEROL TRIACETATE, DIGLYCEROL TETRAACETATE AND METHYL STEARATE

Compound	*Silicone grease*	*SE-30*
Glycerol triacetate	0.081	0.067
Diglycerol tetraacetate	0.516	0.486
Methyl stearate	1.000	1.000

ated and purified as described in the preparation of diglycerol tetraacetate and chromatographed (see Fig. 1 and Table I).

To estimate the diglycerol content in products such as commercial monoglycerides or other glyceride mixtures 0.1–0.5 g of the sample is saponified with ethanolic or methanolic potassium hydroxide and after removing most of the alcohol *in vacuo* the mixture is acidified with aqueous acetic acid and the liberated fatty acids are extracted with chloroform. The aqueous layer is heated *in vacuo* below 100° to evaporate most of the water and the residue acetylated and purified as previously described. By saponifying and re-acetylating a known mixture of glycerol triacetate and diglycerol tetraacetate it was found that the composition of the mixture remained unchanged, thus proving the stability of diglycerol under the above mentioned conditions.

A simultaneous estimation of glycerol, diglycerol and fatty acids in glyceride mixtures could be effected by applying a method suggested by HORROCKS AND CORNWELL[3] according to which glycerides are subjected to hydrogenolysis with lithium aluminium hydride followed by acetylation and gas–liquid chromatography. However, owing to the limited resolving power of the columns suited for diglycerol estimation this procedure is applicable only to simple glyceride mixtures containing essentially saturated fatty acids.

Fats Research Division,
Department of Scientific and Industrial Research,
Wellington (New Zealand)

L. HARTMAN

1 J. E. LOVELOCK, A. T. JAMES AND E. A. PIPER, *Ann. N.Y. Acad. Sci.*, 72 (1959) 720.
2 J. NELSON AND A. MILUN, *Chem. Ind. (London)*, (1960) 663.
3 L. A. HORROCKS AND D. G. CORNWELL, *J. Lipid Res.*, 3 (1962) 165.

Received April 23rd, 1964

Carboxymethylcellulose: a binder for thin-layer chromatography of lipids and indoles*

We have found carboxylmethylcellulose (CMC) to be an effective binder with silicic acid for thin-layer chromatography (TLC) of fatty acid derivatives and indoles.

Cellulose and its derivatives have been used as adsorbents in TLC but to our knowledge they have not been recommended as binders for silicic acid. For instance, RANDERATH[1-3] studied nucleic acid derivatives on pure cellulose powder with and without plaster of Paris as a binder. TEICHERT *et al.*[4] separated alkaloids using pure cellulose impregnated with formamide and WOLLENWEBER[5,6] has used cellulose powder with plaster of Paris for chromatography of synthetic food coloring matter and amino acids. Brinkmann Instruments, Inc. distributes carboxymethylcellulose and other cellulose derivatives with and without a $CaSO_4$ binder.

The converse utility of cellulose derivatives as binders themselves appears to have escaped notice.

Materials and methods

The adsorbent is prepared as follows: silicic acid (Mallinckrodt 100 mesh No. 2847) is slurried with 3 N HCl for approximately 10 min, rinsed with distilled water until the slurry reaches pH 4.5 then rinsed with acetone and dried overnight at 105°. The silicic acid is sieved and that grade passing through a No. 325 mesh (44 μ) screen is used for the adsorbent. 28.5 g washed silicic acid is mixed with 1.5 g carboxymethylcellulose (No. 70 Premium, Low Viscosity grade) in 60 ml warm distilled water. This will make eight 200 × 200 mm plates with the apparatus for application purchased from C. Desaga, Heidelberg, Germany. The plates are air dried overnight then activated before use at 100° for 30 min.

Silica Gel G** was prepared by adding 30 g of dry gel to 80 ml distilled water.

All thin-layer chromatograms were run in tanks at a uniform temperature of 26°.

Results

Fig. 1 illustrates the chromatographic qualities of Silica Gel G and silicic acid–CMC. Both develop adequate separations of the mono-, di- and triglycerides, methyl esters and alcohols. However, even with larger quantities, the hydrocarbon n-octadecane is not observed on plates prepared with Silica Gel G and it takes twice as long to get similar results with the Silica Gel G.

The neutral and acidic indoles ran similarly on both adsorbents in the modified aqueous system, isopropanol–ammonia–water (100:10:5)[7]. Fig. 2 indicates the differences when they are run in 2-butanone–hexane (18:82)[8]. It is interesting to note here, that indole-3-acetic, indole-3-pyruvic, indole-3-butyric and indole-3-propionic acid begin to separate on the CMC plate but remain at the origin on the Silica Gel G plate.

When the acidic indoles are run under the same conditions in 2-butanone–hexane (25:75) they move from the origin with different R_F values yet still remain at the origin with Silica Gel G.

Silica Gel G was not an adequate adsorbent when using KCl (20 % w/v in water)[9]

* This investigation was supported in whole by Public Health Service Research Grant GM-06921 from the National Institutes of Health.

** Brinkmann Instruments Inc., Great Neck, L.I., N.Y.

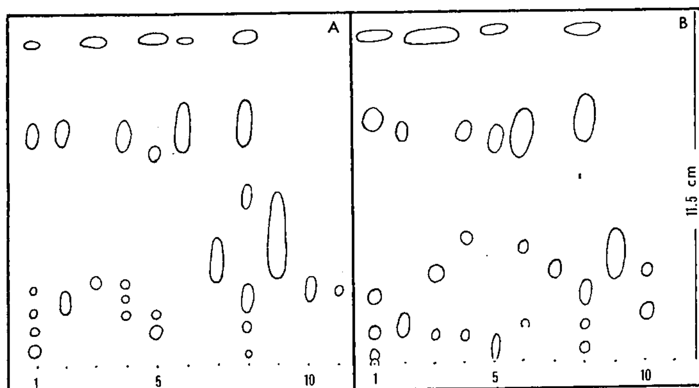


Fig. 1. Ascending chromatography of commercial grades of fatty acid derivatives. A. Silicic acid–CMC. B. Silica Gel G. (1) Methyl palmitate; (2) Palmitic diglycerides; (3) Methyl palmitoleate; (4) Tripalmitin; (5) Methyl stearate; (6) Tristearin; (7) Stearyl alcohol; (8) Triolein; (9) Oleyl alcohol; (10) Cholesterol; (11) *n*-Octadecane; Solvent: pet. ether–ethyl ether–acetic acid (98:10:1). Time: A, 30 min; B, 60 min. Chromogenic reagent: 2',7'-Dichlorofluorescein.

as a solvent because the layer blistered. Fig. 3 indicates the performance of silicic acid–CMC in this solvent which took 15 min to run as opposed to 45 min with Silica Gel G. The results of this separation indicate that this may be a useful solvent system for two-dimensional thin-layer chromatography of indoles.

Discussion

Carboxymethylcellulose, 70 Premium Low Viscosity, was chosen for its ready solubility in water, physiological inertness and binding effectiveness. A range of other grades are available[10] and advantage might be taken of their properties for use with other adsorbents and separations.

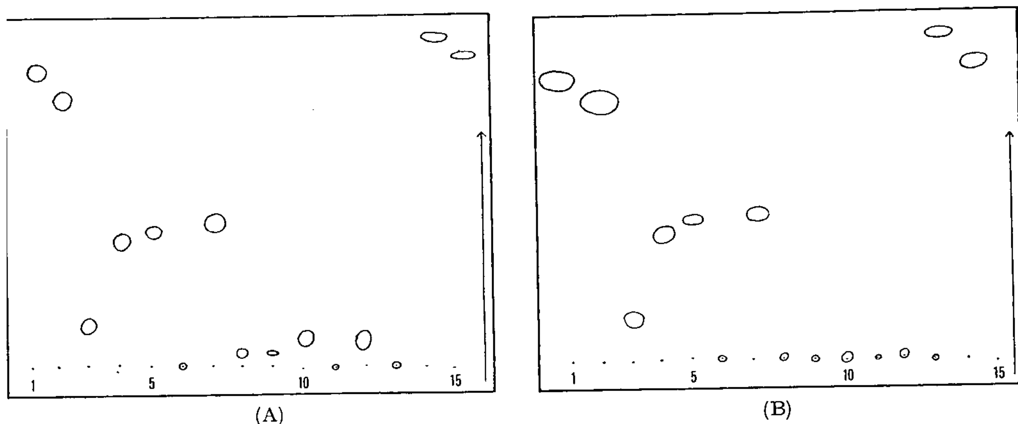


Fig. 2. Ascending chromatography on silicic acid–CMC. A. Silicic acid–CMC. B. Silica Gel G. (1) Skatole; (2) Indole; (3) Tryptophol; (4) Indole-3-acetonitrile; (5) Indole-3-carbinol; (6) Indole-3-acetamide; (7) Ethyl indole-3-acetate; (8) Indole-3-acetic acid; (9) Indole-3-pyruvic acid; (10) Indole-3-butyric acid; (11) Indole-3-lactic acid; (12) Indole-3-propionic acid; (13) Indole glycolic acid; (14) 1,2-Dimethylindole; (15) 2,3-Dimethylindole. Solvent: 2-butanone–hexane (18:82). Time: A, 30 min; B, 45 min. Chromogenic reagent: Ehrlich's reagent.

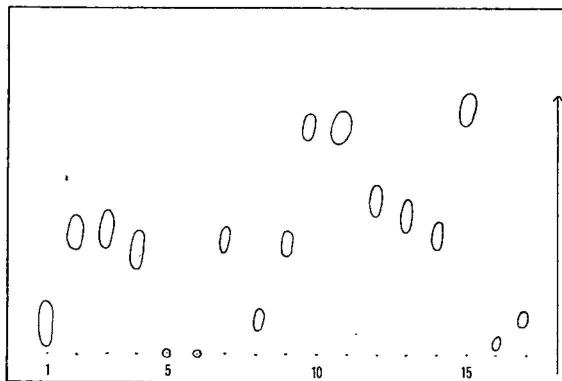


Fig. 3. Ascending chromatography of indoles on silicic acid–CMC. (1) Skatole; (2) Indole; (3) Tryptophol; (4) Indole-3-acetonitrile; (5) 2,3-Dimethylindole; (6) 1,2-Dimethylindole; (7) Indole-3-acetamide; (8) Ethyl indole-3-acetate; (9) Indole-3-acetic acid; (10) Indole-3-pyruvic acid; (11) Tryptamine-HCl; (12) Serotonin (5-Hydroxytryptamine); (13) 5-Methoxytryptamine; (14) 5-Methyltryptamine; (15) 5-Hydroxytryptophan; (16) N,N-Diethyltryptamine; (17) N,N-Dimethyltryptamine. Solvent: KCl 20% w/v in water. Time: 15 min. Chromogenic reagent: Ehrlich's reagent.

We have found that in all cases the running time for silicic acid–CMC is about half that for Silica Gel G. The resolution with some solvent systems is better with this adsorbent than with Silica Gel G. The binding properties are such that the very smooth, thin layer strongly adheres to the plate, is not the least bit powdery, and may be written on with pencil without breaking the layer.

It is not possible to use concentrated H_2SO_4 as a chromogenic reagent for fatty acid analysis with CMC, but the following reagents do give distinctive color reactions for the lipids: 2,7 dichlorofluorescein[11], α-cyclodextrin[11], Rhodamine 6G[12] and iodine vapour[11]. Ehrlich's reagent[7] is effective for the indoles.

Acknowledgements

Carboxymethylcellulose was a gift of the Hercules Powder Co., and palmitic acid diglycerides a gift from Distillation Products Industries.

Department of Biology, Yale University, New Haven, Conn. (U.S.A.)

JEAN B. OBREITER
BRUCE B. STOWE

1 K. RANDERATH, *Nature*, 194 (1962) 768.
2 K. RANDERATH, *Biochem. Biophys. Res. Commun.*, 6 (1961/1962) 452.
3 K. RANDERATH, *Thin-layer Chromatography*, Academic Press Inc., New York, 1963, pp. 33–36.
4 K. TEICHERT, E. MUTSCHLER AND H. ROCHELMEYER, *Z. Anal. Chem.*, 181 (1961) 325.
5 P. WOLLENWEBER, *J. Chromatog.*, 7 (1962) 557.
6 P. WOLLENWEBER, *J. Chromatog.*, 9 (1962) 369.
7 B. B. STOWE AND K. V. THIMANN, *Arch. Biochem. Biophys.*, 51 (1954) 501.
8 R. E. KAPLAN, unpublished solvent system.
9 C. E. DALGLIESH, *Biochem. J.*, 64 (1956) 481.
10 *Hercules Cellulose Gum (CMC)*, Copyright 1951, by Hercules Powder Co., Wilmington, Delaware.
11 D. C. MALINS, *J. Am. Oil Chemists' Soc.*, 37 (1960) 576.
12 R. F. WITTER, G. V. MARINETTI AND A. MORRISON, *Arch. Biochem. Biophys.*, 68 (1957) 15.

Received May 4th, 1964

The separation and identification of some unusual coumarin derivatives by thin-layer chromatography on silica gel*

In view of an interest in coumarin derivatives as possible inhibitors of certain enzymic reactions in the human erythrocyte[1], it seemed necessary to have available a method by which these compounds could be separated and identified. Although paper chromatography[2,3] and thin layer chromatography[4,5] techniques have already been reported for the separation and identification of some coumarin derivatives, it was considered advantageous to develop methods utilizing the latter technique for a large number of these derivatives in various solvent systems.

The present report, therefore, describes methods which can be used to separate and identify a number of coumarin derivatives.

Experimental

Glass plates 20 cm by 20 cm were used. Distilled water (60 ml) was added to a flask containing 30 g of silica gel G and the flask was shaken vigorously for 90 sec. A layer 0.25 mm thick was applied to five glass plates using an applicator (Unoplan Leveler obtained from Shandon Scientific Company, Ltd, London N.W. 10, England). The plates stood for 10 min at room temperature, and were then heated in an air oven for 10 min at 110–112°; they were subsequently turned so that the final drying process (taking 50 min) occurred with the plates in the vertical position. The prepared plates were cooled and stored in a desiccator until used.

The coumarin derivatives, dissolved in acetone, were applied in 2 μg quantities 2 cm from the lower edge of the plate. Development was carried out in three separate systems: System I: acetone–2,2,4-trimethylpentane–water (100:40:1); System II: acetone–ethyl acetate–petroleum ether–water (100:100:33:3.5); System III: 4-methyl-2-pentanone–acetone–petroleum ether (60:20:20).

The petroleum ether (30–60°) was obtained from the J. T. Baker Chemical Company, the pentanone and pentane derivatives from Eastman.

The system utilized was placed in the bottom of a rectangular tank (Shandon Scientific Co., Ltd.) to a height of 1 cm. Two absorbent disposable wicks (20 × 25 cm) were placed on opposite inside walls of the tank and saturated with the developing solvent. After inserting the plate, the tank was sealed with two layers of masking tape. The plates were removed when the solvent system had ascended to a distance of approximately 3 cm from the upper edge of the plate.

Migration velocities for systems I, II and III were 30, 30 and 34 cm per hour. Compounds were detected by the use of a shortwave ultraviolet Minerallight Lamp (Ultra-Violet Products, Inc., San Gabriel, Calif.), which emitted at approximately 2537 Å. Those compounds which did not fluoresce were detected by a 1% solution of potassium permanganate. A yellow color on a violet background was observed after heating for 10 min at 110°. Melting points were determined with a Koffler micro melting point apparatus.

Results and discussion

Twelve of the compounds were coumarin derivatives, two were indanediones. The R_F

* Supported in part by Public Health Service Grants No. HD-00370 and HD-00361, National Institute of Child Health and Development.

TABLE I

THIN-LAYER CHROMATOGRAPHY OF SOME COUMARIN DERIVATIVES

Compound	*m.p.*	*I*		*II*		*III*		*Fluor.*	$KMnO_4$
		R_F	R_S	R_F	R_S	R_F	R_S		
Coumarin	67–68	0.80	1.0	0.90	1.0	0.73	1.0	——	++
4-Hydroxycoumarin	212–213	0.26	0.33	0.31	0.34	0.12	0.16	++	++
7-Hydroxycoumarin	225–227	0.74	0.93	0.77	0.86	0.71	0.97	++	++
3,3′-Methylene-bis-(4-hydroxycoumarin)	289–292	0.28	0.35	0.46	0.51	0.15	0.21	++	++
3,3′-Thio-bis-(4-hydroxycoumarin)	292–294	0.17	0.21	0.32	0.36	0.12	0.16	+	+
3,3′-Methylene-bis-(4-hydroxy-7-methoxycoumarin)	270–273	0.24	0.30	0.37	0.41	0.14	0.19	+	——
3,3′-Methylene-bis-(4-propionoxycoumarin)	243–245	0.80	1.0	0.85	0.94	0.81	1.11	+	——
3,3′-Ethylidene-bis-(4-hydroxycoumarin)	171–173	0.44	0.55	0.63	0.70	0.44	0.60	++	++
β,β-Di-(4-hydroxy-3-coumarin)-ethyl chloride	255–258	0.15	0.19	0.16	0.18	0.05	0.07	++	+++
3,3′-Propylidene-bis-(4-hydroxycoumarin)	143–145	0.44	0.55	0.56	0.62	0.44	0.60	++	+
3,3′-Butylidene-bis-(4-hydroxycoumarin)	126–128	0.48	0.60	0.68	0.76	0.42	0.58	++	+
3,3′-Benzylidene-bis-(4-hydroxycoumarin)	230–232	0.44	0.55	0.66	0.73	0.54	0.74	++	——
4-Hydroxycoumarin-3-carboxylic acid ethyl ester	139–140	0.14	0.18	0.15	0.17	0.04	0.06	+++	+++
2-Benzohydryl-1,3-indanedione	129–130	0.20	0.25	0.0	—	0.09	0.12	——	+
1-(1,3-Diketo-2-indanyl)-1-phenyl-2-nitropropane	—	0.24	0.30	0.87	0.97	0.09	0.12	——	+

and R_S values together with the magnitude of fluorescence or color development and melting points are shown in Table I. The R_S value is defined as the ratio of the distance moved from the origin by a derivative to the distance moved by coumarin itself.

Of the systems examined I and II were approximately equal in separating the coumarin derivatives. System III, on the other hand, had the greatest migration velocity and effected the separation of some derivatives which were not resolved with I and II.

The combination of fluorescence with ultraviolet light and potassium permanganate spray proved to be satisfactory for visualization of the coumarin derivatives. All the chromatographic spots were detected by one of the two methods and most compounds were detected by both.

Acknowledgements

The authors are grateful to Herschel Porter, Eli Lilly and Company, Indianapolis, Ind., for providing some of the compounds used.

Nebraska Psychiatric Institute and Department of Biochemistry, University of Nebraska College of Medicine, Omaha, Nebr. (U.S.A.)

JOHN H. COPENHAVER
MICHAEL J. CARVER

1 M. J. CARVER AND W. L. RYAN, *Proc. Soc. Exptl. Biol. Med.*, 104 (1960) 710.
2 L. REIO, *J. Chromatog.*, 1 (1958) 338.
3 J. B. HARBORNE, *Biochem. J.*, 74 (1960) 270.
4 R. A. BERNHARD, *Nature*, 182 (1958) 1171.
5 E. SUNDT AND A. SACCARDI, *Food Technol.*, 16 (1962) 89.

Received March 12th, 1964

J. Chromatog., 16 (1964) 229–231

Thin-film chromatography of some azo-dyestuffs

During a study of the metal complexes of heterocyclic azo-dyestuffs, it became necessary to check the purity of the azo-dyestuffs in order to ascertain whether a single product is formed on coupling the diazotate with the phenol. Thin-film chromatography has proved most successful for this purpose.

Thin-film plates were prepared in the usual way using Merck Silica Gel G, to which starch was added to aid binding properties. The plates were activated by drying in an oven at 110° for 20 min.

Many solvents were used to separate the dyestuffs, and those of composition listed below were most successful:

Solvent I: 50 ml 40–60° petroleum ether, 50 ml diethyl ether and 5 ml absolute ethanol.

Solvent II: 60 ml *n*-butanol, 20 ml absolute ethanol and 20 ml of 2 *N* aqueous ammonium hydroxide.

TABLE I

R_F VALUES OF HETEROCYCLIC AZO-DYESTUFFS

Compound (abbreviation)	*R_F values (± 0.05)**		
	Solvent I	*Solvent II*	*Solvent III*
4-(2′-Thiazolylazo)-resorcinol (TAR)	0.50	0.80	0.80
4-(2′-Benzothiazolylazo)-resorcinol (BTAR)	0.20	0.90	0.95
1-(2′-Thiazolylazo)-2-naphthol (TAN)	0.70	0.85	0.90
1-(2′-Benzothiazolylazo)-2-naphthol (BTAN)	0.75	0.90	0.95
2-(2′-Thiazolylazo)-*p*-cresol (TAC)	0.80	0.85	0.90
4-(2′-Thiazolylazo)-phenol (TAP)	0.55	0.75	0.85
2-(2′-Thiazolylazo)-phloroglucinol (TAPh)	0	0.50	0.60(l) 0.80(vs)
2-(2′-Thiazolylazo)-pyrogallol (TAPyg)	0	0–0.60 (str)	0.80–0.95 (str)
4-(2′-Pyridylazo)-resorcinol (PAR)	0.25 (str)	0.70	0.80
1-(2′-Pyridylazo)-2-naphthol (PAN)	0.68	0.95	0.99
4-(Phenylazo)-resorcinol (BAR)	0.70	0.65(l) 0.95(s)	0.90(l) 0.97(s)
Phenylazo-*m*-cresol (BAmC)	0.75	0.90	0.90
Benzene-*p*-sulphonic acid-azo-resorcinol (BAR-S)	0	0.40	0.60(vs) 0.70(l)
Benzene-*p*-sulphonic acid-azo-*m*-cresol (BAC-S)	0	0.65	0.70
2-(Salicylideneamino)-pyridine (SAMPy)	0.95	0.85	1.0
2,6-bis-(salicylideneamino)-pyridine (diSAMPy)	0	0.50–0.95 (str)	0.75–1.0 (str)
2-(2′-Hydroxyphenyliminomethyl)-pyridine (POAP)	0.60	0.80–0.95(str)	0.95
2,6-Bis-(2′-hydroxyphenyliminomethyl)-pyridine (BPOP)	0.15	0.95(str)	0.95
1-Hydroxy-(2′-naphthylideneamino)-pyridine (NAMPy)	0.70	0.95	0.98
2-(2′-Thiazolylazo)-phenol-4-sulphonic acid (TAP-S)	0	0.90	0.90
2-(2′-Thiazolylazo)-1-naphthol-4-sulphonic acid (TAN α4S)	0	0.95(l) 0.45(s)	0.90(l) 0.60(s) 0.50(vs)
(2′-Thiazolylazo)-1-naphthol-5-sulphonic acid (TAN α5S)	0	0.55	0.65
1-(2′-Thiazolylazo)-2-naphthol-6-sulphonic acid (TAN β6S)	0	0.55(l) 0.70(s) 0.80(vs)	0.70(l) 0.90(s)
1-(2′-Thiazolylazo)-2-naphthol-7-sulphonic acid (TAN β7S)	0	0.65	0.70
1-(2′-Thiazolylazo)-2-naphthol-8-sulphonic acid (TAN β8S)	0	0.55–0.75 (str)	0.70
(2′-Thiazolylazo)-1-naphthol-3,6-disulphonic acid (TAN α36S)	0	0.25(l) 0.40(s) 0.37(vs)	0.40(s) 0.50(l) 0.70(s)
1-(2′-Thiazolylazo)-2-naphthol-3,6-disulphonic acid (TAN β36S)	0	0.40	0.50
1-(2′-Thiazolylazo)-2-naphthol-6,8-disulphonic acid (TAN β68S)	0	0–0.40 (str)	0.40(l) 0.55(s) 0.70(vs)
Eriochrome Black T	0	0.45–0.60 (str)	0.80

* Terms in parentheses refer to type of spot; l = large; s = small; vs = very small; str — streaky.

Solvent III: 40 ml isopropanol, 30 ml methyl ethyl ketone and 30 ml 0.880 ammonia solution.

The R_F values of the dyestuffs tested in each of these solvents is given in Table I. Nearly all the compounds showed up as coloured spots, but the intensity of these was increased by spraying the plates with an ethanolic solution of cupric acetate (which forms strongly coloured complexes with most of these dyestuffs).

Solvent I was unsuccessful for azo-dyestuffs soluble in water, since they were virtually insoluble in the non-polar organic solvents. The effectiveness of solvents II and III is shown by the fact that several of the compounds tested contained impurities which separated as very small spots on the plate.

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

F. H. POLLARD
G. NICKLESS
T. J. SAMUELSON
R. G. ANDERSON

Received February 24th, 1964

Thin-layer chromatography of 4-dimethylaminoazobenzene and some of its metabolites

The potent rat hepatocarcinogen, 4-dimethylaminoazobenzene (DAB), is metabolised by rat liver to a variety of products. 4-Monomethylaminoazobenzene (MAB), 4-aminoazobenzene (AB), 4'-hydroxy-4-dimethylaminoazobenzene (OH-DAB) and possibly 4'-hydroxy-4-monomethylaminoazobenzene (OH-MAB) have been identified amongst those products which retain the azo group[1]. 4'-Hydroxy-4-aminoazobenzene (OH-AB) was not identified. In this work, the dyes were separated by a rather involved procedure by which the non-hydroxylated dyes were chromatographed on an alumina column and the hydroxylated dyes on a column of Hyflo Super Cel.

In the course of a reinvestigation of the metabolism of DAB we developed a simple and rapid method by means of which DAB, MAB, AB and their 4'-hydroxy-derivatives may be separated and identified.

Silica gel G (E. Merck, AG.) was vigorously shaken with 2 parts by weight of distilled water for 1 1/2 min and applied by means of the Shandon Unoplan Leveler to glass plates (20 cm × 20 cm) to give a layer 250 μ thick. Brief drying at room temperature was followed by activation at 100°/40 min after which the plates were kept over $CaCl_2$ in a desiccator.

The dyes (1 μg or less) were applied either singly or in admixture in 5 μl of methanol solution along a base-line 3 cm from the lower edge of the plate. The chromatograms were developed with a chloroform (reagent grade washed with water, dried over Na_2SO_4, redistilled and stabilised by addition of methanol to 1%)–methanol mixture (95:5). The solvent front was allowed to ascend 10 cm above

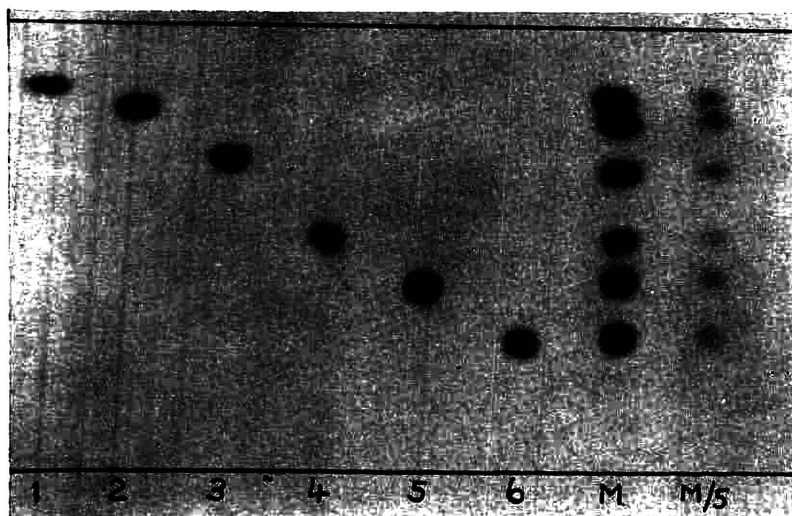


Fig. 1. Thin-layer chromatogram. 1 = DAB; 2 = MAB; 3 = AB; 4 = OH-DAB; 5 = OH-MAB; 6 = OH-AB; 1 μg of each dye. M = mixture consisting of 1 μg of each dye. M/5 = mixture consisting of 0.2 μg of each dye.

the base-line (about 20 min) when the plates were removed from the tank, allowed to dry and then exposed to concentrated HCl fumes for 5 min.

The spots produced were well formed and of characteristic colour and reproducible R_F values (Table I, Fig. 1). The colours of the hydroxy-dyes changed about

TABLE I

R_F VALUES AND COLOURS OF DYES

Dye	*Colours of spots*		*R_F values*
	Immediately	*After 10 min*	
DAB	rose red	rose red	0.85
MAB	orange red	orange red	0.79
AB	orange	orange	0.69
OH-DAB	brown	purple	0.52
OH-MAB	rust-red	purple	0.42
OH-AB	orange	purple red	0.30

10 min after the removal of the plates from the acid vapour; all of the spots faded over night. Mixtures containing all six dyes were readily separated and visualised when each was present in the range 1 μg -0.025 μg.

Cancer Research Unit,
University of Sheffield (Great Britain)

J. C. TOPHAM
J. W. WESTROP

[1] G. C. MUELLER AND J. A. MILLER, *J. Biol. Chem.*, 176 (1948) 535.

Received April 27th, 1964

Thin-layer chromatographic separation of close positional isomers of hydroxy fatty acids, alcohols and esters

Various isomeric 18-carbon hydroxy compounds were analysed by thin-layer chromatography (TLC) on 270 μ layers of silica gel G. These compounds included the hydroxy products derived from formoxylation of petroselinic acid and subsequent hydrolysis, which would lead to a mixture of mainly 6- and 7-hydroxystearic acids; products similarly obtained from oleic acid, *viz.* chiefly 9- and 10-hydroxystearic acids; 12-hydroxystearic acid (hydrogenated ricinoleic acid); and 18-hydroxystearic acid (hydrogenated kamlolenic acid). Alcohols corresponding to these products were prepared by lithium aluminium hydride reduction, and corresponding methyl esters by esterification with methanol–sulphuric acid. The solvent system employed was diethyl ether–light petroleum in the proportion (v/v) of 40:60 for acids and alcohols, and 25:75 for methyl esters. Acetic or formic acid (2 %) was added to both systems. Chromic acid charring was used for detection.

The $R_F \times 100$ values are shown in Table I.

TABLE I

R_F VALUES OF HYDROXYSTEARIC ACIDS, ALCOHOLS AND ESTERS

Hydroxystearic isomer	*Number of spots*	$R_F \times 100$		
		Acids	*Alcohols*	*Esters*
6-, 7-, (8-?)	3	36, 39, 43	22,26, 30	32, 37, 40
9-, 10-	2	45, 50	31, 35	45, 50
12-	1	56	40	55
18-	1	40	28	37

Confirmation of the identity of the 6- and 7-, and 9- and 10-hydroxystearic acids obtained by the formoxylation–hydrolysis process was provided by catalytic hydrogenation of 6,7- and 9,10-epoxystearic acids to the hydroxy products, when mixtures of compounds with the same R_F values as those in Table I were obtained. The third component in the 6- and 7-hydroxy mixture, judging from the relative position, is likely to be the 8-hydroxy isomer. Thus formoxylation of petroselinic acid resulted in three, and of oleic acid in two, major positional isomers.

The main interest of the work, however, is that isomeric hydroxy fatty acids, in which only the position of the hydroxyl group differs, are resolved by TLC, indicating that the polarity and adsorption characteristics of the molecule are altered even by a slight change in the position of the substituent hydroxyl. When the alcohols are all secondary, the R_F values increase gradually as the hydroxyl group recedes from the terminal carboxyl group. When the hydroxy group occupies a terminal position to yield a primary alcohol, the R_F value is out of alignment and considerably lower, indicating unusual characteristics.

Acids, alcohols and esters are all readily resolved, the ester separations being marginally better.

Separation of the formoxylation reaction products of erucic acid by this method revealed 13- and 14-hydroxydocosanoic acids. The procedure is also being used routinely in this laboratory to monitor the reaction products obtained by the formoxylation of unsaturated acids and the catalytic hydrogenation of epoxy fatty acids.

Regional Research Laboratory,
Hyderabad (India)

R. SUBBARAO
K. T. ACHAYA

Received March 11th, 1964

J. Chromatog., 16 (1964) 235–236

Mise en évidence de quelques dérivés de la phénothiazine par chromatographie en couche mince

Diverses méthodes ont été proposées pour la séparation et l'identification des principaux dérivés de la phénothiazine, que ce soit par chromatographie sur papier[1–4], chromatographie en couche mince[5,6] ou encore par chromatographie en phase gazeuse[7,8].

Nous avons tenté d'appliquer à la couche mince, le système de solvants proposé par NADEAU ET SOBOLEWSKI[2] pour la séparation des phénothiazines sur papier, en utilisant comme support de la poudre de cellulose.

Expérimentation

(a) *Préparation des plaques.* Dix grammes de poudre de cellulose (Camag) sont mis en suspension dans 60 ml d'eau distillée. La suspension homogène de poudre de cellulose est ensuite répandue à l'aide du matériel Desaga, sur des plaques de verre de 20 × 20 cm et celles-ci sont séchées durant 60 min, à l'étuve à 100°.

(b) *Solution de référence.* Nous utilisons des solutions alcooliques renfermant 1 mg de dérivés phénothiazines par ml et nous déposons 10 à 20 μg de ces dérivés sur la plaque de chromatographie.

(c) *Phase mobile.* Nous utilisons comme phase mobile, une solution aqueuse à 5 % de sulfate ammonique saturé par de l'alcool isobutylique.

(d) *Durée de migration.* Si l'on a soin de tapisser les parois de la cuve à l'aide d'une feuille de papier chromatographique ordinaire, ce qui assure une saturation plus rapide et plus constante de celle-ci, la durée de migration du solvant, pour une hauteur de 14 cm, est de 2 heures maximum.

Résultats

Le Tableau I donne les valeurs de R_F trouvées et les colorations obtenues avec les différents révélateurs expérimentés.

Conclusions

La chromatographie en couche mince sur poudre de cellulose et l'utilisation, pour la

TABLEAU I

VALEURS DE R_F ET IDENTIFICATION DES DÉRIVÉS DE LA PHÉNOTHIAZINE

	R_F	Détection des spots*		
		A	B	C
Moditen®	0.03	rose pâle	brun-jaune-incolore	violet
Trilafon®	0.06	rose	brun-jaune-incolore	violet
Stemetil®	0.13	rose	brun-jaune-incolore	violet
Largactil®	0.23	rose	brun-jaune-incolore	violet
Majeptil®	0.25	rose	brun-jaune-incolore	violet
Nozinam®	0.33	bleu-violet	brun-jaune-incolore	violet
Theralene®	0.34	rose	rose-brun-incolore	violet
Lispamol®	0.41	rose	brun-jaune-incolore	violet
Phenergan®	0.54	rose	brun-jaune-incolore	violet
Multergan®	0.64	rose	violet-brun-jaune	violet

* A = F.N.P. (FORREST): chlorure ferrique 5 % 5 ml, acide perchlorique 20 % 45 ml, acide nitrique 50 % 50 ml.
B = Vapeurs d'iode.
C = Iodoplatinate (R. HILF): solution iodure de potassium 10 % 45 ml, solution chlorure. de platine 5 % 5 ml, eau distillée 100 ml.

révélation, de réactifs différents, constituent un moyen aisé et rapide de mise en évidence des principaux dérivés de la phénothiazine.

Laboratoire de Toxicologie, Faculté de Médecine, Université de Liège (Belgique)

A. NOIRFALISE
M. H. GROSJEAN

1 R. FISCHER ET N. OTTERBECK, *Sci. Pharm.*, 26 (1958) 184.
2 G. NADEAU ET G. SOBOLEWSKI, *J. Chromatog.*, 2 (1959) 544.
3 T. H. LIN, L. W. REYNOLDS, I. M. RONDISH ET E. J. VAN LOON, *Proc. Soc. Exptl. Biol. Med.*, 102 (1959) 602.
4 D. A. EAGLESON, *Am. J. Clin. Pathol.*, 39, No. 6 (1963) 648.
5 J. BÄUMLER ET S. RIPPSTEIN, *Pharm. Acta Helv.*, 36 (1961) 382.
6 I. SUNSHINE ET E. ROSE, *Clin. Chem.*, 8, No. 4 (1962) 421.
7 M. W. ANDERS ET G. J. MANNERING, *J. Chromatog.*, 7 (1962) 258.
8 W. J. A. VANDENHEUVEL, E. O. A. HAAHTI ET E. C. HORNING, *Clin. Chem.*, 8, No. 4 (1962) 351.

Reçu le 27 avril 1964

J. Chromatog., 16 (1964) 236–237

Trennung der wichtigsten Opium-Alkaloide durch Dünnschichtchromatographie

Die Vielseitigkeit der Dünnschichtchromatographie veranlasste viele Forschungsgruppen die analytische Trennung der Opium-Alkaloide durch diese einfache Technik zu versuchen.

Die Trennung des Morphins, Codeins und Thebains kann in verschiedenen Lösungsmittelsystemen durchgeführt werden[1-8]. Der Nachteil dieser Methoden liegt darin, dass in diesen Fällen die weiteren zwei Hauptalkaloide, Papaverin und Narkotin, nicht oder nur durch sehr geringe R_F-Wertdifferenzen voneinander getrennt

werden. In den Lösungsmittelgemischen aber, die die Trennung des Papaverins von Narkotin ermöglichen, werden Morphin, Codein und Thebain voneinander nicht scharf genug getrennt.

Zur Überwindung der Schwierigkeiten der Dünnschichtchromatographie der Opium-Alkaloide wurden in der Literatur mehrere zweidimensionalen Verfahren veröffentlicht. Durch zweidimensionale Chromatographie mit zwei verschiedenen Lösungsmittelsystemen konnten z.B. KAMP und Mitarbeiter[9] und POETHKE UND KINZE[10] die fünf Hauptalkaloide des Opiums voneinander einwandfrei trennen.

Unter Berücksichtigung der verschiedenen Nachteile des zweidimensionalen Verfahrens versuchten wir das Problem durch eindimensionale Dünnschichtchromatophie zu lösen. Es gelang uns ein solches Lösungsmittelsystem zu entwickeln, welches die scharfe Trennung der 5 wichtigsten Opium-Alkaloide voneinander durch wesentliche Differenzen der R_F-Werte ermöglicht (siehe Tabelle I).

TABELLE I

R_F-WERTE*

Morphin	0.12
Codein	0.26
Thebain	0.45
Papaverin	0.59
Narkotin	0.74

* Mittelwerte aus 80 Versuchen.

Zur Ausführung der Versuche diente die Grundausrüstung nach STAHL der Firma Desaga (Heidelberg). Die Schichten aus Kieselgel-G (für Dünnschichtchromatographie, Merck) auf 20 × 20 cm Glasplatten wurden nach Angaben den der Grundausrüstung beigefügten Vorschriften gestrichen und anschliessend aktiviert. Chromatographiert wurden je 2 μg Mengen der einzelnen Alkaloide (= 2 μl 0.1 %iger Lösungen), bzw. 1 μl von Tinctura Opii.

Als Lösungsmittel wurde ein Gemisch aus Xylol–Methyläthylketon–Methanol–Diäthylamin (20:20:3:1, v/v) angewendet. Nach Entwicklung der Chromatogramme (Laufstrecke: 13.5 cm, Laufzeit: etwa 25 Min) wurde das Lösungsmittel bei 50° vollständig verflüchtigt. Als Anfärbereagenz diente modifiziertes Dragendorff Reagenz.

Staatliches Institut für Pharmazie, Budapest (Ungarn)

I. BAYER

1 A. MARIANI UND O. MARIANI-MARELLI, *Rend. Ist. Super. Sanita*, 22 (1959) 79.
2 K. TEICHERT, E. MUTSCHLER UND H. ROCHELMEYER, *Deut. Apotheker-Ztg.*, 100 (1960) 477.
3 D. NEUBAUER UND K. MOTHES, *Planta Med.*, 9 (1961) 466.
4 J. A. C. VAN PINXTEREN UND M. E. VERLOOP, *Pharm. Weekblad*, 97 (1962) 1.
5 W. POETHKE UND W. KINZE, *Pharm. Zentralhalle*, 101 (1962) 685.
6 W. E. TSCHITSCHIRO, *Aptechn. Delo*, 12 (1963) 36.
7 K. IKRAM, G. A. MIANA UND M. ISLAM, *J. Chromatog.*, 11 (1963) 260.
8 N. J. MARY UND E. BROCHMANN HANSEN, *Lloydia*, 26 (1963) 205.
9 W. KAMP, W. F. M. ONDERBERG UND W. A. VAN SETERS, *Pharm. Weekblad*, 98 (1963) 993.
10 W. POETHKE UND W. KINZE, *Pharm. Zentralhalle*, 102 (1963) 692.

Eingegangen den 6. April 1964

Thin-layer chromatography of cinchona alkaloids

I. Separation and identification of vinyl-bases and their dihydro-derivatives

Various authors[1-11] have attempted to establish conditions for the separation of cinchona alkaloids by thin-layer chromatography. Their results, which were not always satisfactory, chiefly relate to the separation of the four principal bases of this group: quinine, quinidine, cinchonine and cinchonidine, or to their detection in a mixture with other alkaloids in compounded drugs or other preparations.

Until recently, the separation of mixtures of the diastereoisomers of these alkaloids was beset with overwhelming experimental difficulties. Nor was it possible to separate distinctly the vinyl-bases of cinchona alkaloids and their dihydro-derivatives.

In recent years, such methods as paper electrophoresis[12], gas chromatography[13] and thin-layer chromatography[14-16] have been applied with the aim of achieving such separations. The last mentioned method has been the most successful, since a mixture of the four cinchona bases[17] could be separated satisfactorily.

The aim of this paper is to describe in detail a method for the rapid separation of standard dihydro-derivatives from the various vinyl cinchona alkaloids.

Experimental

Materials. Standards of vinyl-bases and their dihydro-derivatives were obtained by preparative purification of commercial raw materials containing alkaloids of the cinchona bark. By means of repeated recrystallisation of the bases and their salts from several solvents, other vinyl alkaloids of the same group were removed from the mixture. In order to eliminate the dihydro-derivatives, the alkaloids were subjected to threefold purification according to THRON AND DIRSCHERL[18]. The method is based on the difference in solubility of the dihydro-derivative and of the addition compound of mercuric acetate with the vinyl alkaloids in aqueous ammonia.

Apparatus and reagents. The glass plates were coated with an adsorbent layer using a modification* of the Research Specialties Co.** applicator[19].

Adsorbent: Kieselgel G for thin-layer chromatography, Merck No. 7731.

Solvent: Chloroform p.a., methanol p.a. redistilled, diethylamine, twice purified.

Reagent: Dragendorff's reagent modified by MUNIER AND MACHEBOEUF[20].

Procedure. The adsorbent layer was prepared by Stahl's method. 35 g Kieselgel G, 60 ml of water and 10 ml 0.1 N sodium hydroxide were thoroughly mixed in a mortar. The slurry was poured on to the plates (100 × 200 mm) to a standard thickness of 0.3 mm, dried in air until opaque and then for 2.5 h at 50°, and finally activated for 30 min at 110°. The chromatoplates were developed by the ascending technique in a round chamber with ground lid. The chamber was lined with paper moistened with solvent. An 0.5 % butanol solution of the substances was applied to the starting line, which was 15 mm from the edge of the plates. A volume of either 0.45 μl (2.2 μg) was applied by means of a micropipette or 0.48 μl (2.4 μg) with a calibrated platinum wire loop, at intervals of about 1-1.5 cm. Immersion of the plate in the developing solvent was to a depth of 5 mm. The solvent system consisted of chloroform, methanol and

* Device with calibrated appliance for adjusting layer thickness.

** 200 South Garrad Blvd., Richmond, Calif., U.S.A.

diethylamine (80:20:1). The time required to reach the front (10 cm) was about 25–26 min. After removing the solvent with a stream of hot air, the plates were observed in U.V. light and the positions of the fluorescent spots determined. The plates were then sprayed with Dragendorff's reagent, which yielded orange spots.

Discussion and results

From our experimental results and those of others, optimum conditions for the separation of cinchona alkaloids are obtained with kieselgel G adsorbent made alkaline with NaOH and when a solvent system containing diethylamine is used.

Preliminary experiments with solvent systems containing different amounts of diethylamine showed that the best separation was obtained using the solvent system chloroform–methanol–diethylamine (80:20:1). The development time was about 25 min.

The solvent systems chloroform–acetone–diethylamine (5:4:1)[15] and chloroform–methanol–diethylamine (80:20:0.2)[17] failed to give a satisfactory separation of the cinchona hydrobases and vinyl alkaloids under the conditions used here.

Satisfactory reproducibility of R_F values to within 0.03, for 8 alkaloids, was achieved (Table I). The $\Delta R_F \times 100$ values for the four mixtures differ, amounting to 7 for quinine and dihydroquinine, 9 for quinidine and dihydroquinidine, 10 for

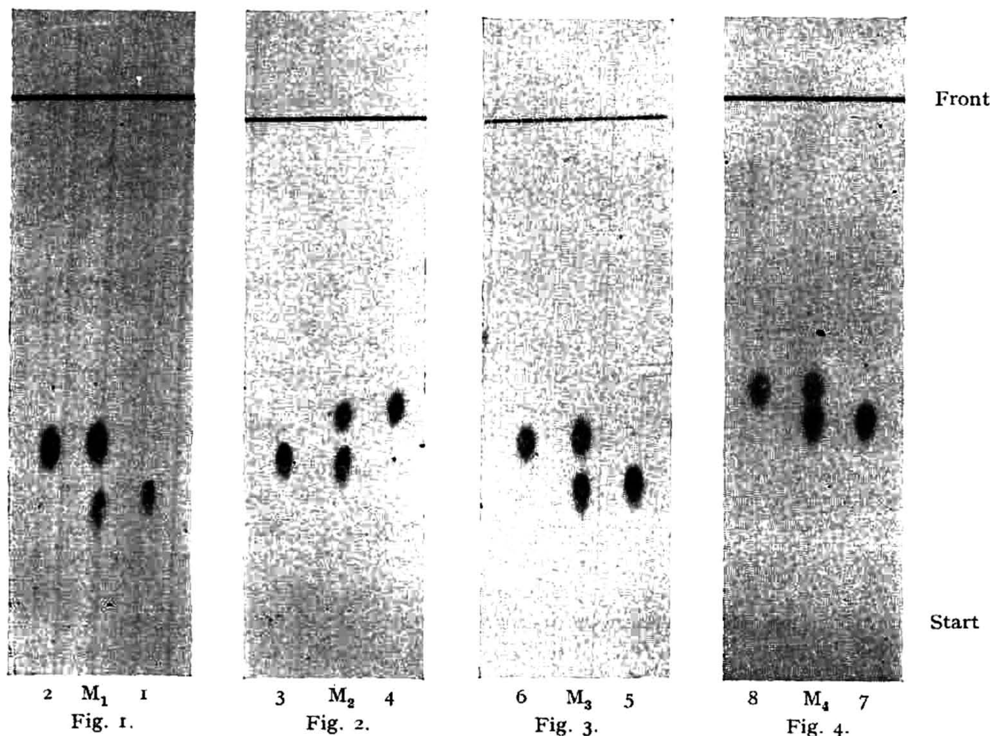


Fig. 1. Fig. 2. Fig. 3. Fig. 4.

Figs. 1–4. Solvent: chloroform–methanol–diethylamine (80:20:1). Kieselgel G Merck 7731. M_1 = mixture of quinine and dihydroquinine; M_2 = mixture of quinidine and dihydroquinidine; M_3 = mixture of cinchonidine and dihydrocinchonidine; M_4 = mixture of cinchonine and dihydrocinchonine. For explanation of the numbers, see Table I.

TABLE I

RANGE OF R_F VALUES FOR VARIOUS CINCHONA ALKALOIDS

Kieselgel G Merck 7731. Solvent: chloroform–methanol–diethylamine (80:20:1).

*No.**	*Alkaloids*	*R_F values*
1	Dihydroquinine	0.38–0.41
2	Quinine	0.45–0.47
3	Dihydroquinidine	0.36–0.39
4	Quinidine	0.46–0.48
5	Dihydrocinchonidine	0.29–0.32
6	Cinchonidine	0.39–0.41
7	Dihydrocinchonine	0.28–0.31
8	Cinchonine	0.38–0.41

* See also Figs. 1–4.

cinchonidine and dihydrocinchonidine, and 13 for cinchonine and dihydrocinchonine (Table II).

Figs. 1–4 show the central parts of developed chromatograms for which the R_F values are reproducible to within 0.01 (Table II), and where the values of $\Delta R_F \times 100$ for the mixtures are equal or almost equal to the average for the whole chromatogram (Table III).

Our experimental conditions were not greatly different from Stahl's standard conditions; this contributed considerably towards the reproducibility of our results. From the chromatograms shown, it is seen that at the concentrations applied sharp oval spots of sufficient intensity are obtained on development.

Alkaloids such as quinine and quinidine exhibit fluorescence with well-determined centre of intensity on illumination with a U.V. lamp; unlike paper chromatograms, these remain visible after spraying with the developing reagent. The coloured spots in the layer neutralized with acetic acid vapour were permanent over many months.

TABLE II

$\Delta R_F \times 100$ OF VINYL AND DIHYDROBASES OF CINCHONA ALKALOIDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Numeration of alkaloids according to that of Table I and Figs. 1–4. Kieselgel G Merck 7731. Solvent system: chloroform–methanol–diethylamine (80:20:1).

No.	*R_F*			*$\Delta R_F \times 100$*	*Limits of R_F reproducibility*
	Vinyl bases	*Mixture*	*Dihydrobase*		
1		0.40	0.41		0.01
2	0.47	0.47		7	0.00
3	0.37	0.37			0.00
4		0.46	0.47	9	0.01
5		0.31	0.32		0.01
6	0.40	0.41		10	0.01
7		0.28	0.30		0.02
8	0.40	0.41		13	0.01

TABLE III

R_F AND $\Delta R_F \times 100$ OF CINCHONA ALKALOIDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Numeration of alkaloids as in Table I and Figs. 1–4. Kieselgel G Merck 7731. Solvent system: chloroform–methanol–diethylamine (80:20:1). Solvent front: 10 cm. Time of run: 25 min.

No.		*R_F values for the mixtures over entire chromatogram*			*$\Delta R_F \times 100$ for the mixtures*		
1	M_1	0.38	0.40	0.40	7	7	7
2		0.45	0.47	0.47			
3	M_2	0.38	0.37	0.36	8	9	10
4		0.46	0.46	0.46			
5	M_3	0.29	0.31	0.30	10	10	10
6		0.39	0.41	0.40			
7	M_4	0.27	0.28	0.30	11	13	11
8		0.38	0.41	0.41			

Results of further work on the separation of four hydrobases and eight alkaloids will be published later.

Department of General Chemistry, Higher School of Economics,
Institute of Cultivation, Fertilization and Soil Science,
Poznań (Poland)

A. SUSZKO-PURZYCKA
W. TRZEBNY

1 D. J. LUSSMAN, E. R. KIRCH AND G. L. WEBSTER, *J. Am. Pharm. Assoc.*, Sci. Ed., 40 (1951) 368; *J. Pharm. Pharmacol.*, 4 (1952) 129.
2 R. MUNIER, M. MACHEBOEUF AND N. CHERRIER, *Bull. Soc. Chim. Biol.*, 34 (1952) 204.
3 P. DE MOERLOOSE, *Pharm. Tijdschr. Belg.*, 29 (1952) 117; 30 (1953) 5; *Pharm. Weekbl.*, 89 (1954) 541.
4 P. CASTILLE, *Pharm. Weekbl.*, 89 (1954) 1.
5 J. REICHELT, *Pharmazie*, 11 (1956) 718.
6 J. REICHELT AND M. SARSUNOVA, *Pharmazie*, 13 (1958) 21.
7 T. BORKOWSKI AND A. DŁUZNIEWSKA, *Dissertationes Pharm.*, 15 (1963) 95.
8 Z. MARGASIŃSKI, A. SZYMAŃSKA AND L. WASILEWSKA, *Acta Polon. Pharm.*, 12 (1955) 65.
9 F. ADAMANIS, W. MIZGALSKI AND D. MALEJKA, *Acta Polon. Pharm.*, 16 (1959) 485.
10 F. ADAMANIS AND D. MALEJKA, *Bull. Soc. Amis Sci. Lettres Poznań, Ser. C*, 10 (1960) 77.
11 L. SYPER, *Dissertationes Pharm.*, 15 (1963) 411.
12 L. A. WILLIAMS, Y. M. BRUSOCK AND B. ZAK, *Anal. Chem.*, 32 (1960) 1883.
13 H. A. LLOYD, H. M. FALES, P. F. HIGHET, W. J. A. VANDENHEUVEL AND W. C. WILDMAN, *J. Am. Chem. Soc.*, 82 (1960) 3791.
14 K. H. MÜLLER AND H. HONERLAGEN, *Arch. Pharm.*, 293 (1960) 202.
15 D. WALDI, K. SCHNATZKERZ AND F. MUNTER, *J. Chromatog.*, 6 (1961) 61.
16 J. BÄUMLER AND S. RIPPSTEIN, *Pharm. Acta Helv.*, 36 (1961) 382.
17 R. VAN SEVEREN, *J. Pharm. Belg.*, [N.S.] 17 (1962) 40.
18 H. THRON AND W. DIRSCHERL, *Ann.*, 515 (1935) 252.
19 H. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.
20 R. MUNIER AND M. MACHEBOEUF, *Bull. Soc. Chim. Biol.*, 33 (1951) 846.

Received April 14th, 1964

A procedure for the direct reading of fluorescent spots on thin-layer chromatography plates using the Turner fluorometer

Thin-layer chromatography has proved useful for the qualitative identification of several carbohydrates isolated from tobacco products. In particular, rapid methods have been developed for the identification of erythrose, galactose and galacturonic acid. For our needs the thin-layer procedure had certain advantages over conventional paper techniques.

In conjunction with analytical studies, a rather novel use of the Turner Model III Fluorometer* was developed. This involved the direct reading of fluorescent carbohydrate spots on TLC plates of special design. The unique point of this procedure involved the use of a flexible stainless steel plate as a support for the thin-layer medium. Both the steel plate and the thin-layer medium had the ability to bend to the configuration of the rotating drum in the chromatography door of the fluorometer. Fluorescent spots on the plates thus could be read directly; this technique coupled the advantages of separation by thin-layer chromatography with quantitative analyses by an instrument heretofore used almost exclusively with paper chromatograms. The technique should have application to substances other than carbohydrates.

Experimental and results

The chromatography plates were fashioned from 0.005 in. thick stainless steel shim stock, cut in 2 in. × $8^1/_2$ in. strips and roughened on one surface by rubbing with a coarse grade of emery cloth (Norton Abrasive No. 36). This rough surface facilitated the adhesion of a coating material consisting of a blend of 18 g of Merck (Stahl) Silica gel G with $CaSO_4$, 27 g of Johns-Manville Celite and 125 ml of water. The slurry was applied to the plates in the manner described by LEES AND DEMURIA[1], dried and activated by heating for 1 h at 110° and used the same day. While the silica gel–Celite support was preferred because of its low fluorescent background, some success was obtained with other coating agents namely, Macherey-Nagel and Co. MN Cellulose 300 and finely ground Whatman No. 1 paper (5 μ particle size) with $CaSO_4$ binder. It was necessary to consider the interactions of solvents, binders and supports because of the possible effect in quenching fluorescence or producing extraneous fluorescent products.

Concentrated aqueous carbohydrate solutions were spotted on the plates in 0.1 μl amounts and at levels ranging from 1 to 20 μg for each sugar. After air drying, the plates were placed in a Mitchell chamber[2] for ascending single or double development; usually for periods of 1–2 h at room temperature. The solvent system, *n*-butanol–acetic acid–diethyl ether–water (9:6:3:1) was quite satisfactory for the separation of erythrose. Galacturonic acid, galactose, arabinose and ribose were separated routinely with either an ethyl acetate–pyridine–water (2:1:2) or a *n*-butanol–acetic acid–water (4:1:2) system.

After air drying, the plates were sprayed with an alcoholic solution of *p*-aminohippuric acid and heated for 8 min at 140° (as described by SATTLER AND ZERBAN[3]) to form fluorescent compounds of the carbohydrates. The plates were scanned in the fluorometer usually within 2 h after development.

* G. K. Turner Associates, 2524 Pulgas Avenue, Palo Alto, Calif., U.S.A.

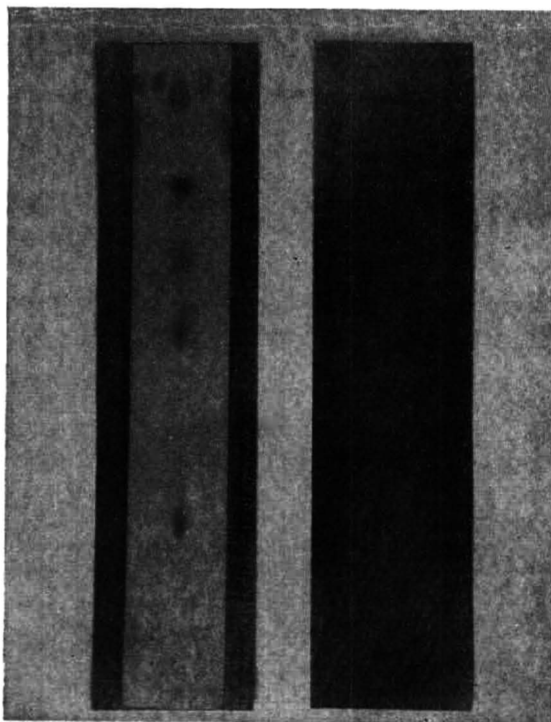


Fig. 1. Flexible stainless steel TLC plates (descriptions in text).

Fig. 2. Flexible TLC plate attached to rotating drum of fluorometer. At the left another chromatogram awaits reading.

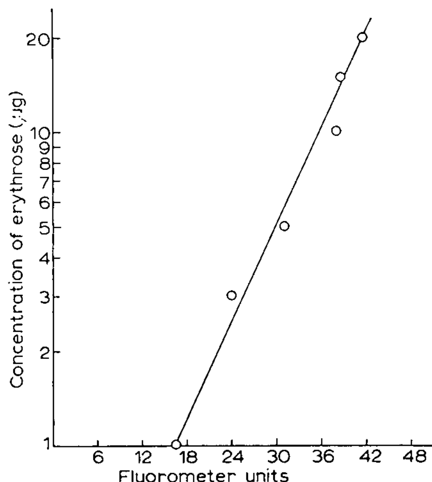


Fig. 3. Calibration curve for erythrose.

The plates were secured to the rotating drum of the chromatography door by the use of small pieces of masking or adhesive tape. An oblong mask was selected which outlined a reading area of 3 × 25 mm on the plates. The scale of the instrument was zeroed at a blank portion of the chromatogram, and the fluorescent intensity of the carbohydrate zones was measured by scanning at 0.5 cm intervals along the entire length of the chromatogram. It was found that a light intensity opening of 30 × with a 7–37 primary filter and a 2A–12 secondary filter with a 2 ND neutral wedge sufficed for most of the tests. On a weight basis, erythrose was the most intensely fluorescing carbohydrate studied under these conditions, and a sizeable range of the fluorometer scale was utilized for concentrations between 1 and 20 μg. Standard calibration curves were prepared each time a sugar was tested.

In Fig. 1 appear, from left to right, a completed chromatogram and an uncoated stainless steel plate. The chromatogram illustrates in ascending order: galacturonic acid, galactose, arabinose and ribose. The topmost spots are impurities which migrate with the solvent system.

Fig. 2 illustrates the Turner fluorometer with a flexible chromatogram affixed to the rotating drum of the chromatography door. To the left, leaning on the instrument, is another chromatogram, shown only to demonstrate the relative rigidity of the stainless steel plates. A calibration curve for erythrose is shown in Fig. 3. The log of the carbohydrate concentration is plotted *versus* fluorometer units. A fairly linear plot is obtained over a range of from 1 to 20 μg.

Research Laboratory, General Cigar Co., Inc.,
Lancaster, Pa. (U.S.A.)

W. M. CONNORS
W. K. BOAK

1 T. M. LEES AND P. J. DEMURIA, *J. Chromatog.*, 8 (1962) 108.
2 L. C. MITCHELL, *J. Assoc. Offic. Agr. Chemists*, 40 (1957) 999.
3 L. SATTLER AND F. W. ZERBAN, *Anal. Chem.*, 44 (1952) 1127.

Received April 13th, 1964

Über das chromatographische Verhalten einiger diastereomerer Diole

Für die Produktanalyse geplanter kinetischer Untersuchungen über die Hydrolyse aromatisch substituierter Epoxide musste ein Verfahren gefunden werden, diastereomere Diole zu trennen. Es handelt sich dabei um die Substanzpaare *threo-* und *erythro-*1-Phenyl-2-methylglykol, *meso-* und *racem.*-Diphenylglykol und *threo-* und *erythro-*1,2-Diphenylpropandiol-1,2.

Über die Trennung von Diolen an Al_2O_3 und das Wesen des Trenneffektes liegen Angaben in der Literatur vor[1,2]. Wir fanden bei der Dünnschichtchromatographie an Fasertonerde[3], dass diese Methode für quantitative Zwecke wenig brauchbar ist, da die Diole zur Schwanzbildung neigen.

Die Verwendung von Cellulose (Papier), an der der Trenneffekt durch einen Verteilungsvorgang hervorgerufen wird[4], brachte gute Ergebnisse. Dabei ist zu beachten, dass die Reihenfolge der Isomeren umgekehrt ist wie bei der Adsorptionschromatographie[5] (siehe Tabelle I).

Über die Trennung zyklischer *cis-trans*-isomerer Diole liegen Angaben in der Literatur vor[6]. Hier sind die Voraussetzungen für eine Trennung allerdings wesentlich günstiger, da die Konformationen durch die Struktur mindestens teilweise festgelegt sind.

Mit den in der Papierchromatographie üblichen Lösungsmitteln (Alkohol, Säuren, Amine) gelingt es nicht, die hier behandelten diastereomeren Diole zu trennen. Der Grund dafür ist, dass diese Lösungsmittel selbst als Donator oder Acceptor bei der Bildung von Wasserstoffbrücken fungieren können. Entstehen intermolekulare Lösungsmittel–Diol-Brücken, so kann sich die intramolekulare Brückenbindung der Diole infolge der Konkurrenz nur noch schwach ausbilden. Da der Trenneffekt gerade auf dem Vorliegen der intramolekularen Brücke beruht, kann er durch die genannten Lösungsmittel verkleinert oder aufgehoben werden. Der Trenneffekt ist bei den hier untersuchten Diolen recht empfindlich gegen solche Einflüsse. Schon in Chloroform und Benzol, die nur relativ schwache Wasserstoffbrücken bilden, ist die Trennung schlecht. Im Gegensatz dazu setzt sich in anderen Fällen (z.B. *o-* und *m-*Oxybenzoesäure) der Einfluss der intramolekularen Wasserstoffbrücken durch. Diese sind aber bei den hier untersuchten offenkettigen Diolen zu schwach[7], um gegen die Konkurrenz der intermolekularen Brücken bestehen zu können.

Aus den Ergebnissen chromatographischer Versuche kann man bei isomeren Verbindungen durchaus auf die Konformation rückschliessen. Z.B. gelang es im Gegensatz zu den Hydrobenzoinen nicht, die α-Methylhydrobenzoine an Papier zu trennen. Offensichtlich liegt das daran, dass die Energieniveaus der Konformeren durch den Methylsubstituenten nivelliert worden sind.

Aus den Gruppenwechselwirkungen (Fig. 1) kann man ablesen, dass die α-methylierte Verbindung in der *threo*-Reihe schwerer, in der *erythro*-Reihe leichter in die Nahestellung der OH-Gruppen einschwenken sollte als das unmethylierte Diol. Damit muss der Trenneffekt an Papier verschlechtert werden, was mit der Beobachtung übereinstimmt.

Experimentelles

Dünnschichtchromatographie. Es wurde mit Fasertonerde als Adsorbens gearbeitet. Die Schichten wurden aufgegossen, da reproduzierbare Schichtdicken nicht erforder-

TABELLE I

ERGEBNISSE DER CHROMATOGRAPHISCHEN VERSUCHE

*Substanz**	*Adsorbens*	*Lösungsmittel*	R_F	*Schwanzbildung bis* R_F	R_F *threo* / R_F *erythro*
threo-Phmg	Fte	Me	0.55	0.0	0.81
erythro-Phmg			0.68		
threo-Phmg	Fte	Me–Ac (1:1)	0.47	0.0	0.73
erythro-Phmg			0.64		
threo-Phmg	Papier	Tetra, W ges.	0.18		1.28
erythro-Phmg			0.14		
threo-Phmg	Papier	Bzl, W ges.	0.48		1.10
erythro-Phmg			0.43		
threo-Phmg	Papier	Chl, W ges.	0.80		1.00
erythro-Phmg			0.80		
threo-Phmg	Papier	Bzl–A (9:1)	0.85		1.00
erythro-Phmg			0.85		
racem.-Dphg	Fte	Me–Ac (1:1)	0.70	0.4	0.91
meso-Dphg			0.77		
racem.-Dphg	Papier	Pä–Bzl–Me–W (3:1:2:2)	0.56		1.31
meso-Dphg			0.43		
racem.-Dphg	Papier	Tetra, W ges.	0.96		1.02
meso-Dphg			0.94		
racem.-Dphg	Papier	Bzl, W ges.	0.85		1.10
meso-Dphg			0.77		
racem.-Dphg	Papier	Chl, W ges.	0.93		1.00
meso-Dphg			0.93		
threo-MDphg	Fte	Me–Ac (1:1)	0.69	0.5	0.93
erythro-MDphg			0.75		
cis-Cpd	Fte	Me–Ac (1:1)	0.49	0.13	0.72
trans-Cpd			0.68		
cis-Cpd	Gips	Tetra	0.14	0.0	
trans-Cpd			0.25		

* Phmg = Phenylmethylglykol
Dphg = Diphenylglykol
MDphg = Methyldiphenylglykol
Cpd = Cyclopentandiol
Fte = Fasertonerde
Me = Methanol

Ac = Aceton
W = Wasser
Bzl = Benzol
Chl = Chloroform
A = Alkohol
Pä = Petroläther (30–50°)

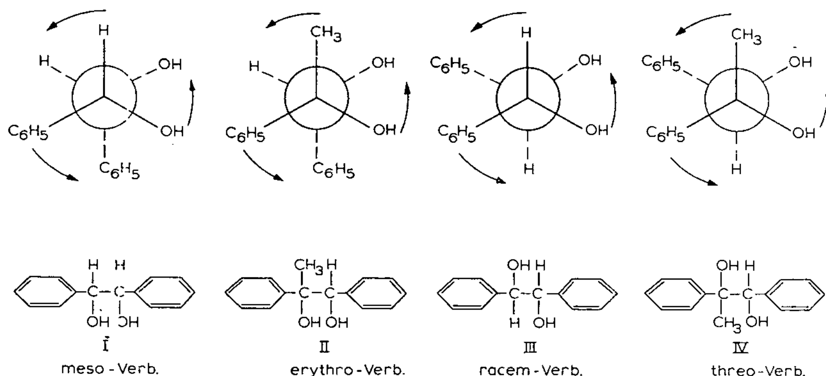


Fig. 1. Vergleich der Gruppenwechselwirkung von diastereomeren Hydrobenzoinen und α-Methylhydrobenzoinen.

lich waren und zu dicke Schichten bröckeln. Nachweis der Diole: Ammoniakalische $AgNO_3$-Lösung.

Papierchromatographie. Schleicher & Schüll-Papier 2043b wurde benutzt. Nur die absteigende Methode gab brauchbare Ergebnisse (Kammersättigung!). Detektion: Perjodat-Benzidin.

Institut für Pflanzenchemie der Technischen Universität Dresden, Tharandt (D.D.R.)

FRIEDRICH FISCHER
HELMUT KOCH

1 M. SVOBODA UND J. SICHER, *Collection Czech. Chem. Commun.*, 20 (1955) 1452.
2 L. F. FIESER, *J. Chem. Educ.*, 31 (1954) 291.
3 S. HUNECK, *J. Chromatog.*, 7 (1962) 561.
4 I. M. HAIS UND K. MACEK, *Handbuch der Papierchromatographie*, VEB Gustav Fischer Verlag, Jena, 1958, S. 71.
5 E. L. ELIEL, *Stereochemistry of Carbon Compounds*, McGraw-Hill, London, 1962, S. 132.
6 C. W. J. BROOKS UND L. YOUNG, *Biochem. J.*, 63 (1956) 264.
7 L. P. KUHN, *J. Am. Chem. Soc.*, 76 (1954) 4323.

Eingegangen den 8. April 1964

J. Chromatog., 16 (1964) 246–248

Paper chromatography of oligogalacturonides*

Partial hydrolysis of polygalacturonic acid yields galacturonic acid and a homologous series of oligogalacturonides. The smaller oligogalacturonides have been separated by both paper[1–4] and anion-exchange[5,6] chromatography. This paper describes the separation of oligogalacturonides, with the degree of polymerization as high as 12. The relationship between chromatogram mobility and molecular size for this group of oligosaccharides is discussed.

* Journal paper No. J. 4822 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 1267. Supported in part by funds provided by Regional Project NC-27.

A partial enzymatic hydrolyzate of pectin was prepared in the following manner. Fifty grams of pectin were dissolved in 3 l hot water and, after cooling, 2 g pectinase (Nutritional Biochemicals Corp.) were added. The solution was incubated at room temperature until the viscosity decreased to that of water. The solution was then rapidly brought to boiling to inactivate the pectinase. After cooling, an extract of alfalfa (source of pectinesterase; 75 g alfalfa herbage blended in 150 ml 5 % saline) was added, and the solution was incubated for 24 h, with the addition of N NaOH to maintain the pH at 6.5. The solution was boiled again, and the process was repeated with a fresh extract of alfalfa. The oligogalacturonides were precipitated by the addition of 4 volumes 95 % ethanol, and lyophilized.

Chromatographic separations were made on Whatman No. 3 MM paper, using the descending technique. A Chromatocab (Research Specialties Co., Model A125), which accommodates 18 × 22-in. sheets, was used in a room maintained at 30°. The system (solvent I), ethyl acetate–acetic acid–water (10:5:6 v/v) effectively separated the smaller oligogalacturonides. Increasing the proportion of water in solvent I increased the mobilities of all the acids, with the greatest increases for the longer analogues.

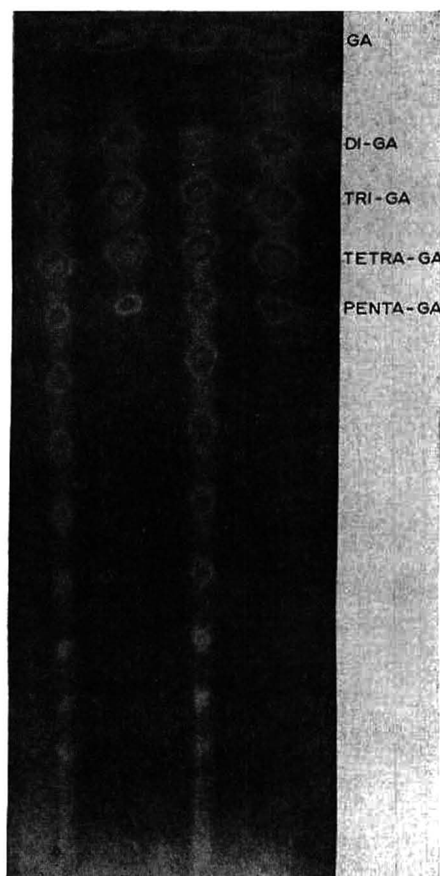


Fig. 1. Paper chromatogram of oligogalacturonides developed in ethyl acetate–acetic acid–water (10:5:9 v/v) and dipped in a mixed indicator solution.

Since the spots were not only shifted farther but also compressed on the sheet, a modification of solvent I, ethyl acetate–acetic acid–water (10:5:9 v/v) (solvent II), was used to separate the oligogalacturonides.

Fig. 1. shows the resolution of an enzymatic hydrolyzate of pectin, using solvent II with a multiple development technique. This chromatogram was obtained by five developments, each 12 h long. The reference compounds, galacturonic acid, di-, tri-, tetra- and penta-galacturonic acid, were prepared and purified by chromatography on Dowex-1 (formate). The oligogalacturonides appeared as red spots on a dark green background, when treated with a mixed indicator solution of 50 mg thymol blue, 250 mg methyl red and 600 mg bromthymol blue in 1 l 95 % ethanol. The pH of the dip solution was adjusted by the addition of N NaOH until a blue-green color was attained. Residual acetic acid on the chromatograms was removed by brief autoclaving and thoroughly drying prior to dipping. Separation of oligogalacturonides with the degree of polymerization as high as 12 was achieved with relative ease. However, it was considerably more difficult to separate the higher analogues.

The effect of the degree of polymerization on oligogalacturonide mobility is shown in Fig. 2. The R_{GA} (mobility relative to galacturonic acid) values represent data obtained from a series of chromatograms developed simultaneously by the continuous descending technique. Development time for individual chromatograms was varied from 15 to 75 h. When galacturonic acid moved off the sheet, R_{GA} was calculated from the mobility for tetra-galacturonic acid for which the R_{GA} value was well established. A plot of the logarithm of the R_{GA} values against the degree of polymerization disclosed a simple correlation between the mobility and the molecular size of the oligo-

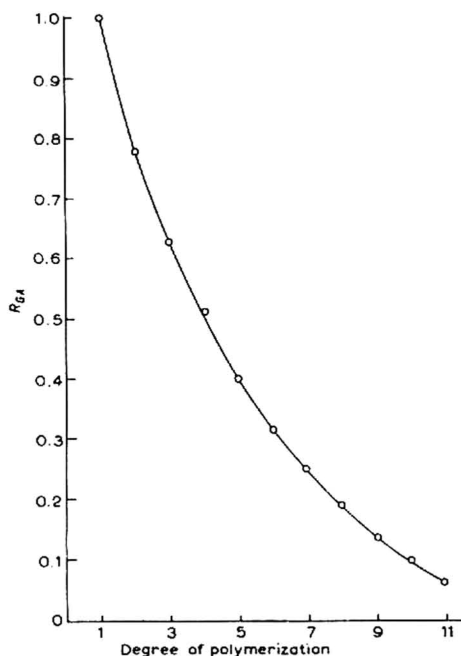


Fig. 2. Effect of the degree of polymerization on the migration of oligogalacturonides on paper chromatograms developed in solvent II.

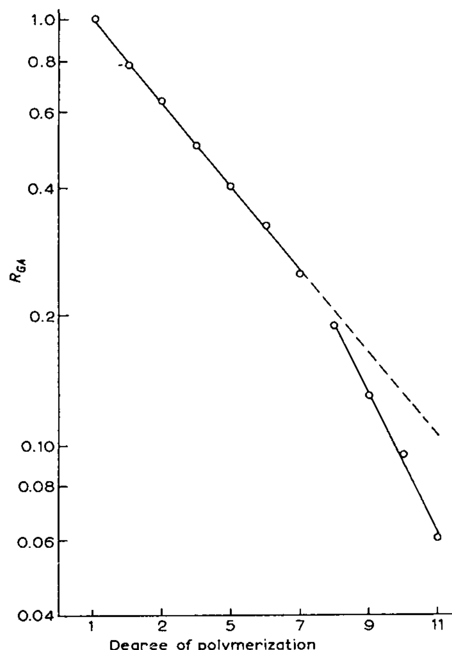


Fig. 3. A logarithmic plot of the R_{GA} values for oligogalacturonides in solvent II.

galacturonides as large as the heptamer (Fig. 3). As the degree of polymerization exceeded 7, a straight line with increased slope was obtained.

The relationship between chromatogram mobilities and molecular size of homologous oligosaccharide series has been reported[7,8]. On plotting the logarithm of R_F values against the molecular size for amylo-oligosaccharides, a straight line was obtained which included all except the lowest members of the series. Linear correlations were obtained for a number of homologous series by plotting molecular size against the logarithm of a partition function α', defined by the equation

$$\alpha' = R_F / (1 - R_F).$$

When a similar plot was made for our data, a straight line was obtained only for the oligogalacturonides possessing a degree of polymerization between 4 and 8.

Department of Biochemistry and Biophysics,
Iowa State University, Ames, Iowa (U.S.A.)

RUSSELL PRESSEY
R. S. ALLEN

1 H. J. PHAFF AND B. S. LUH, *Arch. Biochem. Biophys.*, 36 (1952) 231.
2 R. M. MCCREADY AND C. G. SEEGMILLER, *Arch. Biochem. Biophys.*, 50 (1954) 440.
3 A. L. DEMAIN AND H. J. PHAFF, *Arch. Biochem. Biophys.*, 51 (1954) 114.
4 D. E. WRIGHT, *Arch. Biochem. Biophys.*, 86 (1959) 251.
5 R. DERUNGS AND H. DEUEL, *Helv. Chim. Acta*, 37 (1954) 657.
6 J. ASHBY, J. BROOKS AND W. W. REID, *Chem. Ind. (London)*, (1955) 360.
7 D. FRENCH AND D. W. KNAPP, *J. Biol. Chem.*, 187 (1950) 463.
8 D. FRENCH AND G. M. WILD, *J. Am. Chem. Soc.*, 75 (1953) 2612.

Received March 24th, 1964

Detection of non-reducing carbohydrate with orcinol in phosphoric acid

During work on the fractionation of mucoproteins and their degradation products, it has often been necessary to detect small amounts of combined (non-reducing) carbohydrate. Methods using periodate depend on the availability of pairs of free vicinal hydroxyl groups, and the only really general type of procedure is to use a reagent such as orcinol in a strong acid such as sulphuric acid, which breaks down the polysaccharide[1-3]. This means, of course, that spots or bands have to be eluted from paper before the reaction can be applied. In the modification described here, sulphuric acid is replaced by phosphoric acid and the reaction is done on spot tiles instead of in test tubes. This makes it much more sensitive (down to 0.2 μg), and simple enough to carry out on a large number of samples without undue labour; at the same time, rough quantitation can still be achieved.

When a reagent containing sulphuric acid is used on a spot tile, charring occurs as the acid becomes concentrated by evaporation of water during the heating period. This is avoided by replacing sulphuric with phosphoric acid. The reagent used contained orcinol (100 μg per ml) in 50% (v/v) phosphoric acid; it was stored at 2° and made up fresh weekly. Spot tiles containing twelve cavities per tile were used. In order to maintain the liquid in the form of compact droplets as the volume decreased by evaporation, the tiles were given a hydrophobic surface by rinsing with dimethyldichlorosilane (5% v/v) in benzene and allowing to air-dry. To ensure scrupulous cleanness, they were rinsed with water immediately before use, excess water being merely shaken off.

Sample volumes ranged from 10 to 100 μl; since colour development takes place only when excess water has evaporated off, the colour attained is independent of the initial volume. To each sample, one small drop (20 μl) of orcinol reagent was added. Mixing was effected by circular horizontal movement of the tiles, which were then placed for one hour in an oven at 105°. Blanks became pale yellow, whereas a reddish-brown colour developed in the presence of carbohydrate. Within the range 0.2 to 1.6 μg mannose or galactose, the range of tones formed was well differentiated, and casual inspection sufficed for estimation of the amounts present within a factor of two, standards for comparison being run concurrently with the unknowns. With care, estimation within closer limits could be achieved.

Colour development began after about half an hour at 105°; colours did not change markedly if the heating period was prolonged to two hours. At 95°, colour development took more than an hour. At 120°, grey colours were formed, less suitable for visual estimation than the range from pale yellow to deep reddish-brown obtained at 105°.

The colours given by equal amounts of mannose and galactose were indistinguishable; about twice as much glucose or soluble starch, and about eight times as much N-acetylneuraminic acid, were required to produce equivalent colours. Glucosamine hydrochloride in amounts up to 8 μg gave no observable colour, but with 16 μg a faint grey was obtained. Ovomucoid and a bovine plasma mucoprotein fraction gave colours consistent with their hexose contents. Interference by proteins is due, partly at least, to their tryptophan contents. Thus bovine insulin, which is free of tryptophan, gave only a yellow colour in amounts up to 120 μg; 10 μg bovine chymotrypsinogen (5% tryptophan), on the other hand, gave a pale grey colour.

With tryptophan itself, in amounts above about 1 μg, a pale purple colour was obtained; the same colour was formed with phosphoric acid only, in the absence of orcinol. Such interference by tryptophan seems to be the main difference in specificity between this reaction and the test tube reaction with orcinol in sulphuric acid.

The method has been used in this laboratory by Mr. J. G. BEELEY on fractions obtained by paper electrophoresis after proteolytic degradation of ovomucoid. Cuts of paper (1.6 cm^2) were eluted centrifugally with 100 μl water, and 20 μl samples tested as above. Paper blanks were not appreciable. The method could also be used for rapid testing of fractions obtained by column chromatography.

Department of Chemistry, The University, Manchester (Great Britain) F. R. JEVONS

1 M. SÖRENSEN AND G. HAUGAARD, *Biochem. Z.*, 260 (1933) 247.
2 R. J. WINZLER, in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 2, Interscience, New York, 1955, p. 290.
3 F. K. HARTLEY AND F. R. JEVONS, *Biochem. J.*, 84 (1962) 134.

Received March 20th, 1964

Colorimetric identification following tritium recovery from paper*

To assure quantitative recovery of tritium-labeled compounds from paper chromatograms, it is often necessary to elute the material from the paper rather than using scanning techniques. However, various identifying color reagents cannot be used on the developed chromatogram if the eluates are to be counted in a liquid scintillation spectrometer. We have encountered this situation in the isolation of cardiac glycoside metabolites. Glycoside color reagents such as picric acid, *m*-dinitrobenzene, antimony trichloride, and trichloracetic acid markedly quench counts and/or decrease our ability to extract the materials from the developed chromatogram. We have therefore resorted to cutting the developed chromatogram into thin strips throughout its length. Each section is quantitatively extracted, counted and corrected for quench by internal standardization. The picric acid color reagent, which quenches counts about 99%, is then added to each counting vial to verify the location of detectable amounts of carrier compounds used as markers. Investigators may find this color identification after quantitative recovery useful if they are similarly limited by quenching reagents and the counting solution is compatible with color development following radioassay.

Department of Pharmacology, College of Medicine, State University of Iowa, Iowa City, Iowa (U.S.A.) J. L. SPRATT

Received March 17th, 1964

* Supported by USPHS Research Grant GM-09784.

Paper chromatography of some catecholamines and related compounds

McGeer and Clark[1] have recently described the paper chromatography of some catecholamines and related compounds in twenty one solvent systems; in general, four solvents are used for the paper chromatographic separation of catecholamines, *i.e.* water-saturated phenol[2], phenol-0.1 *N* HCl[3], *n*-butanol saturated with hydrochloric acid[4], and *n*-butanol–acetic acid–water (4:1:5)[5]. These solvents require 12–24 h for a satisfactory development of the chromatogram and as a result of the prolonged exposure, oxidation of the catecholamines to the corresponding red aminochromes is usually observed on the paper; the extent to which this oxidation occurs can be reduced by flushing the tank with carbon dioxide, nitrogen or sulphur dioxide[6] prior to development. A further complication in the interpretation of chromatograms of catecholamines may result from the presence of double-spots which are sometimes produced during the chromatography of pure substances[7]. Shepherd and West[8,9] have reported that double-spots are formed when adrenaline and related compounds are chromatographed in the presence of trichloroacetic acid; the author has also observed the presence of double-spots (in addition to that due to the oxidation product) when pure samples of salts (tartrate or hydrochloride) of some of these amines are chromatographed in *n*-butanol–HCl or *n*-butanol–acetic acid–water solvents.

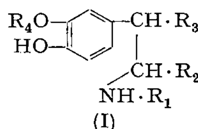
Another investigation[10] in this laboratory required a solvent suitable for the paper chromatography of mixtures containing adrenaline, which required a relatively short development time and gave chromatograms free from any ambiguity due to the artifacts mentioned above. A solvent system found to meet these requirements was methanol (160 ml)–water (40 ml)–quinoline (8 ml), which gave a satisfactory resolution of most of the mixtures examined within 4–5 h; no oxidation of the catecholamines to aminochromes was observed on the chromatograms despite the basic nature of the solvent, presumably due to the relative short development period. The usefulness of the methanol–water–quinoline solvent for the chromatography of catecholamines and some related amines was examined (see Table I). Good separations of the following groups of amines were obtained: (1) noradrenaline, adrenaline and N-isopropylnoradrenaline, (2) adrenaline and its methyl or ethyl ether and (3) metanephrine and normetanephrine. 3-Hydroxytyrosine (dopa) had an R_F value that was sufficiently low to permit its separation from any of the other amines used.

Experimental

The paper chromatographic examination of the amines shown in Table I was carried out in 1 l cylinders at room temperature, using the ascending technique, on Whatman No. 1 paper with methanol (160 ml)–water (40 ml)–quinoline (8 ml) as the developing solvent. A total rise of about 30 cm, taking 4–5 h, was employed. In addition to the catecholamines listed in Table I, the R_F values of two other amines were determined; these were phenylephrine, 1-(3-hydroxyphenyl)-2-methylaminoethanol (R_F = 0.68) and methoxamine, 2-amino-1-(2,5-dimethoxyphenyl)-1-propanol (R_F = 0.74). The catecholamines were detected by spraying the developed chromatograms with 1% potassium ferricyanide followed by exposure to ammonia. Metanephrine and normetanephrine (orange spots) and phenylephrine (yellow spot) were visualized with diazotised sulphanilic acid, and methoxamine (violet spot) with ninhydrin.

TABLE I

PAPER CHROMATOGRAPHY OF SOME PHENOLIC AMINES* IN METHANOL (160 ml)–WATER (40 ml)–QUINOLINE (8 ml)



Amine (I)	R_1	R_2	R_3	R_4	R_F value
Noradrenaline	H	H	OH	H	0.66
Adrenaline	CH_3	H	OH	H	0.72
N-Ethylnoradrenaline	C_2H_5	H	OH	H	0.75
N-Isopropylnoradrenaline	iso-C_3H_7	H	OH	H	0.82
Epinine	CH_3	H	H	H	0.69
Adrenaline methyl ether	CH_3	H	OCH_3	H	0.77
Adrenaline ethyl ether	CH_3	H	OC_2H_5	H	0.84
Noradrenaline methyl ether	H	H	OCH_3	H	0.72
Dopamine	H	H	H	H	0.65
Dopa	H	COOH	H	H	0.42
2-Methyladrenaline	H	CH_3	OH	H	0.77
Metanephrine	CH_3	H	OH	CH_3	0.76
Normetanephrine	H	H	OH	CH_3	0.69

* R_F values for some related amines are given in the experimental section.

Acknowledgements

The author is grateful to Dr. R. A. HEACOCK, of this laboratory, for valuable discussions throughout the course of this investigation, which was supported by grants from the Government of Saskatchewan (Department of Public Health) and the Department of National Health and Welfare (Ottawa). The author is grateful to Burroughs-Wellcome and Co. (Canada) Ltd. for a generous gift of epinine hydrochloride and Mrs. B. D. SCOTT for the preparation of several other compounds used in this investigation.

Psychiatric Research Unit, University Hospital, Saskatoon, Saskatchewan (Canada)

G. L. MATTOK

1 E. G. MCGEER AND W. G. CLARK, *J. Chromatog.*, 14 (1964) 107.
2 W. O. JAMES, *Nature*, 161 (1948) 851.
3 M. GOLDSTEIN AND I. ABELIN, *Helv. Chim. Acta*, 39 (1956) 158.
4 U. HAMBERG AND U. S. VON EULER, *Acta Chem. Scand.*, 4 (1950) 1185.
5 R. W. SCHAYER, *J. Biol. Chem.*, 189 (1951) 301.
6 T. B. B. CRAWFORD, *Biochem. J.*, 48 (1951) 203.
7 H. G. CASSIDY, in A. WEISSBERGER, *Technique of Organic Chemistry*, Vol. X, Interscience Publishers Inc., New York, 1957, p. 205.
8 D. M. SHEPHERD AND G. B. WEST, *Nature*, 169 (1952) 797.
9 G. B. WEST, *J. Pharm. Pharmacol.*, 11 (1959) 595.
10 G. L. MATTOK AND R. A. HEACOCK, *Can. J. Chem.*, 42 (1964) 1401.

Received May 11th, 1964

Ein Allgemeinverfahren zur Fleckenfärbung bei der Papierchromatographie von Vulkanisations-Beschleunigern

Bei der Papierchromatographie von Vulkanisations-Beschleunigern werden einzelne Gruppenreagenzien zum Anfärben von Flecken eingesetzt, die auf Beschleuniger bestimmter Gruppen zurückzuführen sind. Es liegt z.Z. noch kein einziges Reagens vor, das imstande wäre, die von sämtlichen oder zumindest den meisten Beschleunigern herrührenden Flecken anzufärben. Zur Anfärbung der Flecken versuchten wir das Papier abwechselnd mit N-Bromsuccinimid- bzw. Fluoresceinlösung zu behandeln. Mit dem Originalreagens von COOK[1] (N-Bromsuccinimid, in Methylchloroform aufgelöst) fielen die Ergebnisse unserer Versuche unbefriedigend aus, denn nur wenige der angewandten Beschleuniger ergaben deutlich gefärbte Flecken. Da das N-Bromsuccinimid je nach der Polarität des Lösungsmittels ein unterschiedliches Verhalten aufweist, entschlossen wir uns, weitere Lösungsmittel heranzuziehen: Tetrachlorkohlenstoff, Chloroform, Methylalkohol, Äthylalkohol, Aceton und Essigsäure. Die besten Ergebnisse erzielten wir mit nachstehendem Verfahren.

Reagenzien: (1) Man löst 0.5 g N-Bromsuccinimid in 100 ml Eisessig. Im Dunkeln ist die Lösung mehrere Tage haltbar. Blassgelbe Färbung (freies Brom!) setzt die Empfindlichkeit nicht herab (2). Auflösung von 0.01 g Fluorescein in 100 ml Äthylalkohol. Die Lösung ist längere Zeit haltbar.

Das Chromatogramm wird nach vollständiger Beseitigung der Lösungsmittel zunächst mit fein zerstäubter N-Bromsuccinimid- und gleich darauf mit Fluoresceinlösung besprüht. Nach dem Trocknen beobachtet man beim Tages- und im U.V.-Licht. Die meisten Beschleuniger bewirken gelbgefärbte Flecken, die im Dunkeln grünlich schimmern; der Hintergrund ist rosenrot, im U.V.-Licht hingegen orangefarben. Die scharfen Umrisse der Flecken im rosenroten Grund des Papiers sind zum Teil von der Papierart abhängig. Am deutlichsten treten die Flecken hervor am Papier Schleicher & Schüll 2045 b gl und 2040 b m bzw. Niederschlag FN 12, FN 13 und FN 17.

Einzelne Beschleuniger liefern unterschiedlich gefärbte Flecken, was die Erkennung erleichtert. Tabelle I gibt Aufschluss über die von uns geprüften, in der Praxis meistverwendeten Beschleuniger der verschiedenen Gruppen.

TABELLE I

Beschleuniger	*Handelsbezeichnung*	*Fleckenfärbung*	
		in sichtbarem Licht	*in U.V.-Licht*
Thiazole			
Merkaptobenzthiazol	Captax Vulcacit Mercapto Thiotax Vulcafor MBT	gelb	grün
Dibenzothiazolyldisulfid	Altax Vulkacit DM Vulcafor MBTC Thiofide	orange (blass)	braun
Zink-Merkaptobenzthiazol	Bantex Vulkacit ZM	blassgelb	grünlichbraun
Merkaptobenzimidazol	Antioxydant MB	gelb	dunkelgrün

(Fortsetzung, S. 257)

TABELLE (*Fortsetzung*)

Beschleuniger	*Handelsbezeichnung*	*Fleckenfärbung*	
		in sichtbarem Licht	*in U.V.-Licht*
Guanidine			
Diphenylguanidin	Vulcafor DPG DPG Vulkacit D	rot	rot
Di-*o*-tolylguanidin	Vulcafor DOTG	rot	rot
Diphenylguanidinphthalat	Guantal	rot	rot
Thiurame			
Tetramethylthiuramdisulfid	Vulkacit Thiuram Vulcafor TMT Thiurad	gelb	blaugrün
Tetramethylthiurammonosulfid	Vulkacit Thiuram MS Vulcafor MS Monothiurad	gelb	blaugrün
Tetramethylthiuramdisulfid	Vulcafor TET Ethyl Thiurad	gelb	blaugrün
Dithiocarb amate			
Natrium-Dimethyldithiocarbamat	—	gelb	gelbgrün
Natrium-Diäthyldithiocarbamat	Vulcafor SDC	gelb	gelbgrün
Natrium-Cyclohexyldithiocarbamat	Vulkacit WL	gelb	gelb
Zink-Dimethyldithiocarbamat	Vulkacit L Methasan	gelb	grün
Zink-Diäthyldithiocarbamat	Vulkacit LDA Vulcafor ZDC Ethasan	gelb	hellgrün
Zink-Dibutyldithiocarbamat	Vulkacit LDB	gelb	hellgrün
Piperidin-pentamethylendithio-carbamat	Vulkacit P Uskoritel 552	gelb	hellgelb
Zink-Äthylphenyldithiocarbamat	Vulkacit Pextra N Vulcafor ZEP	gelb	grün
Diäthylammoniumdiäthyl-dithio-carbamat	Vulcafor DDCN	gelb	grün
Cyclohexyläthylammoniumdithio-carbamat	Vulkacit 774	gelbrot	orange
Xanthogenate			
Natrium-Isopropylxanthat	Vulcafor SPX	gelb	grün
Zink-Isopropylxanthat	Vulcafor ZIX	gelb	grün
Sulfenamide			
Benzothiazolylsulfendiäthylamid	Vulkacit AZ	gelb	grün
Benzothiazolylsulfencyclohexyl-amid	Vulkacit CZ Santocure Vulcafor HBS	gelb	grün
2-(4-Morpholinyl-merkapto)-benzthiazol	Santocure MOR	gelb	grün
N-Butyl-2-benzthiazolsulfenamid	Santocure NS	gelb	grün
Aldehydamine			
Butyraldehydanilin	Vulcafor BA	gelb	grau
Hexymethylentetramin	Vulkacit H	rosenrot	orange

Das beschriebene Verfahren kann auch als Tüpfelreaktion zur Erkennung mancher Beschleuniger Verwendung finden. Die Empfindlichkeit der Reaktion ist hierbei recht verschieden; so lassen sich z.B. 2–5 γ Thiurame und Dithiocarbamate, hingegen 20–50 γ Beschleuniger der übrigen Gruppen sicher nachweisen.

Für erwiesene Unterstützung bei der experimentellen Arbeit schulden wir Frau V. ANTONOVA Dank und Anerkennung.

Institut für Organische Chemie,
Bulgarische Akademie der Wissenschaften,
Sofia (Bulgarien)

A. POPOV
V. GĂDEVA

[1] I. M. HAIS UND K. MACEK, *Handbuch der Papierchromatographie*, VEB Gustav Fischer Verlag, Jena, 1958, S. 670.

Eingegangen den 1. April 1964

J. Chromatog., 16 (1964) 256–258

Separation of Al, Ga, In and Tl by reversed-phase chromatography on papers treated with di-(2-ethylhexyl) orthophosphoric acid

The potentiality and selectivity of reversed-phase partition chromatography with paper treated with di-(2-ethylhexyl) orthophosphoric acid (HDEHP) in inorganic separations has been applied in this laboratory to the rare earths[1] and to the alkali metals and alkaline earths[2,3].

During the systematic study on the chromatographic behaviour of a large number of cations on HDEHP-treated papers, as a function of HCl molarity in the eluent, the possibility of a good separation of Al, Ga, In and Tl became apparent.

Recently, the interest in such elements has considerably increased in connection with new fields of research and technology such as nuclear energy, space communication and semiconductors. It was therefore considered worthwhile to investigate the behaviour of these elements in reversed phase chromatography with HDEHP-treated papers.

The separation of Al, Ga, In and Tl by classical paper chromatography has been attempted by various authors using alcohol–HCl mixtures[4,5,12] or phenol–alcohol–HCl mixtures[6] as eluents.

In the present investigation a good separation of the four elements was obtained on Whatman No. 1 paper pre-treated with 0.1 *M* HDEHP-cyclohexane solutions and eluted with 1 *M* or 8 *M* HCl, these two molarities being selected on the basis of the systematic study referred to above. Preparation of the paper and general procedure are described in a previous work[3]. Chromatography was by the ascending technique at room temperature ($23° \pm 1°$) in tightly closed large jars, with 7×46 cm paper strips cut perpendicular to the machine direction.

A stock solution containing $5 \cdot 10^{-6}$ equivalents/ml was prepared for each element and to avoid hydrolysis was brought to different HCl molarities. The molarities of HCl of the respective stock solutions, together with the original substance and Supplier, are as follows: $AlCl_3$ (C. Erba-Milano), 10^{-3} *M* HCl; Ga metal (BDH-London), 0.05 *M* HCl; $InCl_3$ (BDH), $4 \cdot 10^{-3}$ *M* HCl; TlCl (C. Erba), 0.2 *M* HCl. The aluminium and indium solutions were directly prepared by dissolution of the chlorides, that of gallium by HF treatment of the metal and conversion to chloride with HCl, and that of thallium (III) by oxidation of the TlCl solution with *aqua regia*, followed by ether extraction from a 6 *M* HCl solution, precipitation with 0.1 *M* NaOH and final purification, according to the method reported by HORROCKS AND VOIGT[7].

Three spots, about 0.05 ml each, were deposited on every paper strip, one on the central axis of the sheet and the other two equally spaced on either side. The three spots were on a starting line 25 mm from the end of the strip. The central spot contained the four elements and the two lateral ones a group of two or three of the four elements. After elution, the strip was cut longitudinally to obtain three narrow strips. The following procedure was then adopted for the different spots since, for the greatest sensitivity, aluminium and indium are best detected by spraying with a 0.1 % alcoholic morin solution and gallium and thallium with an analogous quercetin solution.

A preliminary check was made for the position of the spots on the two lateral strips, and then the central one was developed with the appropriate solution by spraying the respective positions.

The experimental results obtained by elution of 0.1 *M* HDEHP-treated paper with 1 *M* and 8 *M* HCl are shown in Table I, where the R_F values of the four elements are reported. The R_F values for untreated paper eluted under identical conditions are also reported in this table.

TABLE I

COMPARISON OF R_F VALUES OF Al, In, Tl AND Ga ON TREATED AND UNTREATED PAPER AT DIFFERENT ACIDITIES

Element	R_F			
	1 M HCl eluent		*8 M HCl eluent*	
	HDEHP paper	*Untreated paper*	*HDEHP paper*	*Untreated paper*
Al^{3+}	0.00	0.95	0.78	0.79
In^{3+}	0.32	0.84	0.89	0.90
Tl^{3+} and Tl^{+}	0.78	0.80	0.17	0.74
Ga^{3+}	0.92	0.95	0.04	0.65

From these data, together with the diagrammatic sketch of chromatograms shown in Fig. 1, it appears that a neat separation of the four elements is possible with HDEHP-treated paper and that a separation of In, Tl and Ga is possible on normal cellulose paper, whilst Al gives a spot which is close to that of Tl. The results obtained with untreated paper confirm the trend found by LEDERER AND OSSICINI[8] with Whatman No. 1 paper eluted with HCl, even though their experimental conditions were not exactly the same.

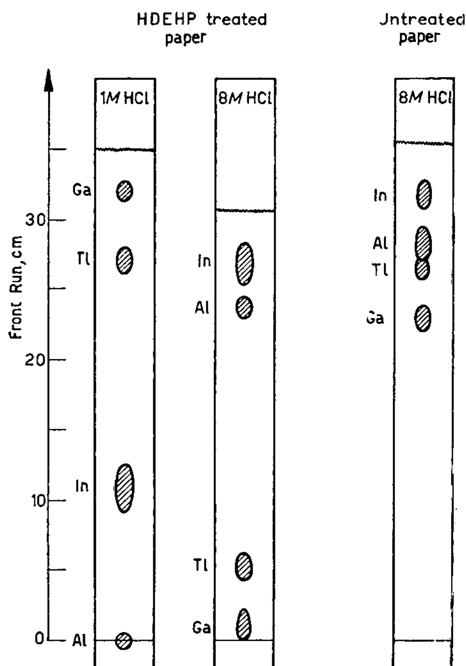


Fig. 1. Diagram of chromatography of Al–Ga–In–Tl on HDEHP-treated paper and on untreated paper.

By comparing the results for HDEHP-paper and untreated paper, with the same eluent, the following conclusions can be drawn:

(i) Aluminium and indium mainly undergo cation exchange with HDEHP. This can be seen from the fact that with 1 *M* HCl they are strongly retained only on the treated paper, whilst with 8 *M* HCl their R_F's are almost identical on both treated and untreated papers;

(ii) The behaviour of thallium and gallium cannot be accounted for by a cation exchange mechanism on HDEHP since at the low acidity they are not adsorbed on HDEHP-paper but are appreciably retained at the high acidity. The formation of anionic chloro-complexes of these two elements, such as $TlCl_4^-$ and $GaCl_4^-$, would suggest an anionic mechanism of adsorption, but this is not likely to occur in the system investigated in this work. On the other hand, experimental evidence exists that such anionic chloro-complexes are strongly adsorbed on cationic resins as Dowex-50[9] and are sensibly retained (low R_F's) by paper treated with a neutral extractant such as tri-*n*-octylphosphine oxide[10].

In Fig. 1 the sequence of elution of the four elements is shown as Ga–Tl–In–Al with 1 *M* HCl and In–Al–Tl–Ga with 8 *M* HCl. In this laboratory, the behaviour of these elements in reversed-phase chromatography on paper and on columns of cellulose powder treated with the same extractant has been shown to be equivalent[11]. Therefore separation of the four elements for purification purposes can be attained with columns made of cellulose powder treated with HDEHP. In the latter case, the possibility of influencing the elution order by altering the acidity of the eluent is a

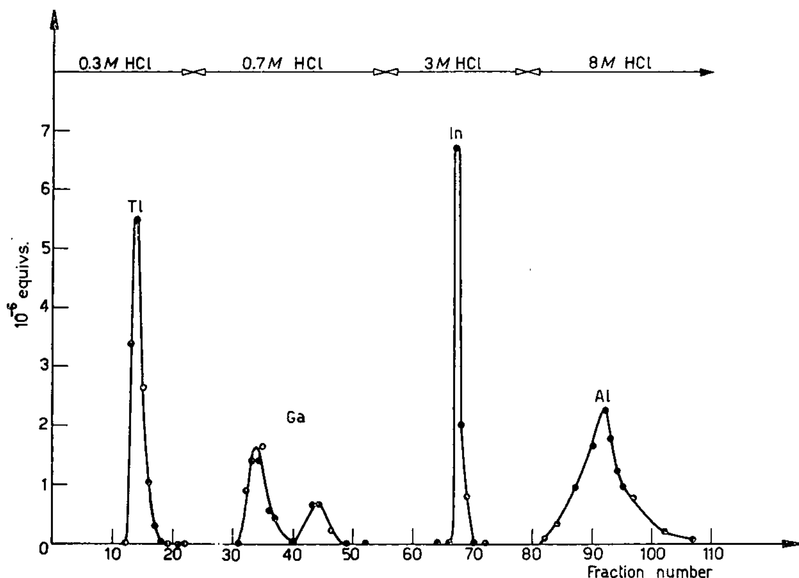


Fig. 2. Separation of Tl–Ga–In–Al (10^{-6} moles each) with HCl on 0.1 M HDEHP cellulose powder (bed 13 mm × 250 mm). Fraction volume 2 ml. Flow-rate of eluent 0.5 ml/min.

great advantage, since it is convenient to select conditions whereby the macro-component is eluted from the column whilst the trace impurities are retained.

To check one of the possible applications of the present investigation to column chromatography, a column (13 mm × 250 mm high) was prepared containing cellulose powder (Whatman No. 1) treated with a 0.1 M HDEHP–cyclohexane solution. As shown in Fig. 2 the elution order with HCl having different molarities was Tl–Ga–In–Al. A volume of about 1.5 ml of feed solution 0.01 M in HCl, containing $5 \cdot 10^{-6}$ moles of each of the elements, was deposited on to the bed which was eluted at room temperature (24 ± 1°) at a flow-rate of 0.5 ml/min.

Laboratori C.I.S.E., Segrate, Milan (Italy) *

E. CERRAI
G. GHERSINI

1 E. CERRAI AND C. TESTA, *J. Chromatog.*, 8 (1962) 232.
2 E. CERRAI AND G. GHERSINI, *J. Chromatog.*, 13 (1964) 211.
3 E. CERRAI AND G. GHERSINI, *J. Chromatog.*, 15 (1964) 236.
4 T. NASCUTIU, *Acad. Rep. Populare Romine, Studii Cercetari Chim.*, 9 (1961) 719; *C.A.*, 57 (1962) 11.
5 S. FISEL, I. GABE AND M. PONI, *Acad. Rep. Populare Romine, Filiala Iasi, Studii Cercetari Stiint. Chim.*, 13 (1962) 33; *C.A.*, 59 (1963) 1065.
6 R. J. MAGEE AND I. A. P. SCOTT, *Talanta*, 3 (1959) 131.
7 D. L. HORROCKS AND A. F. VOIGT, *J. Am. Chem. Soc.*, 79 (1957) 2441.
8 M. LEDERER AND L. OSSICINI, *J. Chromatog.*, 13 (1964) 188.
9 K. A. KRAUS, D. C. MICHELSON AND F. NELSON, *J. Am. Chem. Soc.*, 81 (1959) 3204.
10 E. CERRAI AND C. TESTA, *J. Chromatog.*, 7 (1962) 112.
11 E. CERRAI, C. TESTA AND C. TRIULZI, *Energia Nucl. (Milan)*, 9 (1962) 193.
12 R. A. GUEDES DE CARVALHO, *Rev. Port. Quim.*, 1, No. 2 (1958) 77.

Received March 16th, 1964

* Address: C.I.S.E., Casella Postale 3986, Milano, Italy.

The detection and chromatography on paper of boric acid, sodium tetraborate and benzene boronic acid and the use of chlorogenic and caffeic acids to detect the ions of B, W, Mo and Ge

Investigation of the function of boron in higher plants (for which it is an essential element) led to the following study of the paper chromatography of boric acid, sodium tetraborate and benzene boronic acid, all of which stimulate the growth of flax when they are added to a boron-free nutrient culture solution in the concentration range $5 \cdot 10^{-8}$ to $1 \cdot 10^{-6}$ M. At, and above, $1 \cdot 10^{-4}$ M, benzene boronic acid is toxic to growth, causing the initiation of adventitious buds on the hypocotyl and the suppression of lateral root growth.

Detection on paper

These three B compounds all have one or more pairs of hydroxyl groups which cause them to complex or esterify with other stereochemically complementary organic polyols, such as carbohydrates[1,2] polyphenols[3,4] and coumarins[5]. Where complexes are formed with compounds, such as 3,4-dihydroxycinnamic acids which already fluoresce in or absorb U.V. light, the formation of the borate–hydroxyphenol complex causes an increase in the wavelength of maximum absorption ($\lambda_{\text{U.V.}}$ max.)[4]. There is also a change in the intensity and quality of the U.V.-induced fluorescence, compared to that of the phenol alone. This latter property has been used here to detect these hydroxy-boron compounds on paper.

The compounds were spotted on to filter paper which had been wetted with 0.1 M NaOH and subsequently dried. The paper was then sprayed with a chlorogenic [3-(3,4-dihydroxycinnamoyl)-quinic] acid solution (0.1% w/v, in acetone) and dried. It was then placed under a U.V. lamp (Engelhard Hanovia, with Chance glass OX_1 filter, max. emission 3660 Å). Where the paper was impregnated with one of the boron compounds, the background fluorescence was greatly increased and altered in colour. By this method it is possible to detect 1 μg (in a spot 5 mm in diameter) of the three compounds used. The pre-existing U.V. fluorescence of benzene boronic acid was enhanced by a factor of approximately 10, and altered in colour from a dull blue to a bright light blue. Boric acid and sodium tetraborate themselves have no U.V. fluorescence until sprayed with chlorogenic acid. It seems possible that B could be quantitatively assayed by this method, using U.V. fluorescence spectrophotometry.

This method has been extended to detect, on paper, the metallic ions of Ge, W and Mo, the hydroxides of which complex in solution with polyols in a manner similar to the ions derived from H_3BO_3[6]. Table I lists the daylight and U.V. fluorescence colours of these metallic compounds on filter paper, when sprayed with either chlorogenic or caffeic (3,4-dihydroxycinnamic) acid solutions.

These results indicate that certain polyphenols (such as caffeic and chlorogenic acids) can be detected and identified on paper, and possibly quantitatively estimated in solution, by the choice of suitable metallic reagents. The converse is also true: certain hydrated metallic ions give distinctive shifts in U.V. fluorescence when complexed with a range of already fluorescent polyphenols, used as "spot" reagents.

The induction of U.V. fluorescence, or of changes in inherent U.V. fluorescence, by boric acid when mixed in solution with fluorescein or cochineal has been described

TABLE I

THE DAYLIGHT AND U.V. FLUORESCENCE COLOURS OF B, W, Mo AND Ge COMPOUNDS WHEN SPOTTED ONTO FILTER PAPER, AND SPRAYED WITH EITHER CHLOROGENIC OR CAFFEIC ACID SOLUTIONS

Solution spotted onto filter paper		*Spray used*			
Compound used		*Caffeic acid*	*Chlorogenic acid*	*Caffeic acid*	*Chlorogenic acid*
		Colour of spot			
Solute	*Solvent*	*In daylight*		*Under U.V. light*	
—	—	—	very pale yellow	bright blue	bright blue-green
H_3BO_3	0.1 *M* NaOH	—	—	bright blue	bright blue
Na_2WO_4	0.1 *M* NaOH	pale yellow	very pale yellow	brown	bright yellow
H_2MoO_4	0.1 *M* NaOH	intense brown	—	intense black	black
GeO_2	0.1 *M* NaOH	—	very pale yellow	light grey	bright light yellow

(from earlier papers) by RADLEY AND GRANT[11] in 1939. They also mention the effects of the salts of Be, Al and Mo upon the fluorescence colours of certain compounds[11]. Since the advent of paper chromatography, the use of inherent U.V. fluorescence is one of the many standard methods for the detection of substances on chromatograms. U.V. fluorescence has been *induced* by forming complexes with 8-hydroxyquinoline[7], and immobile fluorescent compounds have been introduced into the matrix of chromatographic columns[8]. KIRCHNER *et al.*[9] used the quenching of U.V. fluorescence to detect terpenes on chromatographic columns. BAKER AND COLLIS[10] appreciated the possibility of using a *specific* fluorescent reagent (a derivative of 7-hydroxycoumarin), which they synthesised, to detect hydroxy and amino compounds on chromatograms, but they do not appear either to have exploited this, or to have specifically used the shift in U.V. fluorescence for the purposes of detection and identification. The results of the experiments described in this paper indicate that this property can be exploited further by the *systematic* choice of fluorescent reagents or U.V. fluorescence-modifying metallic ions.

The chromatography of boric acid, benzene boronic acid and sodium tetraborate

These three boron compounds were run, by upwards displacement, on No. 1 Whatman paper in six solvents. The R_F values of each compound in each solvent are given in Table II. The compounds were detected by spraying successively with 0.1 *M* NaOH and chlorogenic acid.

TABLE II

THE R_F VALUES OF BORIC ACID, BENZENE BORONIC ACID AND SODIUM TETRABORATE IN SIX SOLVENTS

No.	*Solvent*	R_F		
		H_3BO_3	$Na_2B_4O_7$	$PhB(OH)_2$
1	H_2O	0.87	0.87	0.72
2	Ethanol	0.0	0.0	1.0
3	Ethanol–water (80:20)	0.65	0.16	0.80
4	Butan-1-ol–acetic acid–water (4:1:5, by vol.) (top layer)	0.47	0.25; 0.50	0.90
5	Acetic acid (1 % solution)	0.71	(not detected)	0.75
6	Benzene	0.0	0.0	(streaked)

It is apparent that, of the solvents used, No. 4 was perhaps the most satisfactory, although with sodium tetraborate it induced a double spot. This may be attributed to dissociation from the tetraborate to give some monoborate ion.

It is thus evident that the biologically active boron compounds here used may be successfully separated by paper chromatography, and also detected in microgram quantities on paper. In addition, the method of detection appears to have a more general application to other metallic hydroxides.

Acknowledgements

I am grateful to Prof. H. E. STREET for encouragement and support, to Dr. KEVIN GALLAGHER for criticism, and also to the Royal Society and the Nuffield Foundation for the tenure of a Commonwealth Bursary. The benzene boronic acid was a gift from Borax Consolidated Ltd.

The Botany Department, University College of Swansea, Swansea (Great Britain) T. F. NEALES*

1 H. S. ISBELL, J. F. BREWSTER, N. B. HOLT AND H. L. FRUSH, *J. Res. Natl. Bur. Std.*, 40 (1948 129.
2 E. J. BOURNE, E. M. LEES AND H. WEIGEL, *J. Chromatog.*, 11 (1963) 253.
3 L. JURD, *J. Chromatog.*, 4 (1960) 369.
4 J. B. HARBORNE, *Biochem. J.*, 84 (1962) 100.
5 T. SWAIN, *Biochem. J.*, 53 (1953) 200.
6 H. WEIGEL, *Advan. Carbohydrate Chem.*, 18 (1963) 61.
7 F. H. POLLARD AND J. F. W. McOMIE, *Chromatographic Methods for Inorganic Analysis*, Butterworths, London, 1953.
8 H. BROCKMANN AND F. VOLPERS, *Chem. Ber.*, 80 (1947) 77.
9 J. G. KIRCHNER, J. M. MILLER AND G. J. KELLER, *Anal. Chem.*, 23 (1951) 420.
10 W. BAKER AND C. B. COLLIS, *J. Chem. Soc.*, (1949) S12.
11 J. A. RADLEY AND J. GRANT, *Fluorescence Analysis in Ultra-violet Light*, Chapman and Hall, London, 1939, Chap. 8.

Received March 20th, 1964

* Present address: The Botany Department, Melbourne University, Parkville, N. 2, Victoria, Australia.

Chromatography on starch columns

MOORE AND STEIN[1] showed that phenylalanine, tyrosine and tryptophan could be separated on a column of starch using 0.1 N hydrochloric acid as the developing solvent, and suggested that the retardation was due to adsorption. Other workers have employed starch columns developed with immiscible solvents for the chromatography of the iodotyrosines[2] and purine–pyrimidine mixtures[3]. Using a starch (Morning Star Nicol, Inc., N.Y.) column and 0.1 N hydrochloric acid phenylalanine, tyrosine, monoiodotyrosine, tryptophan and diiodotyrosine have been easily and quantitatively resolved (Fig. 1).

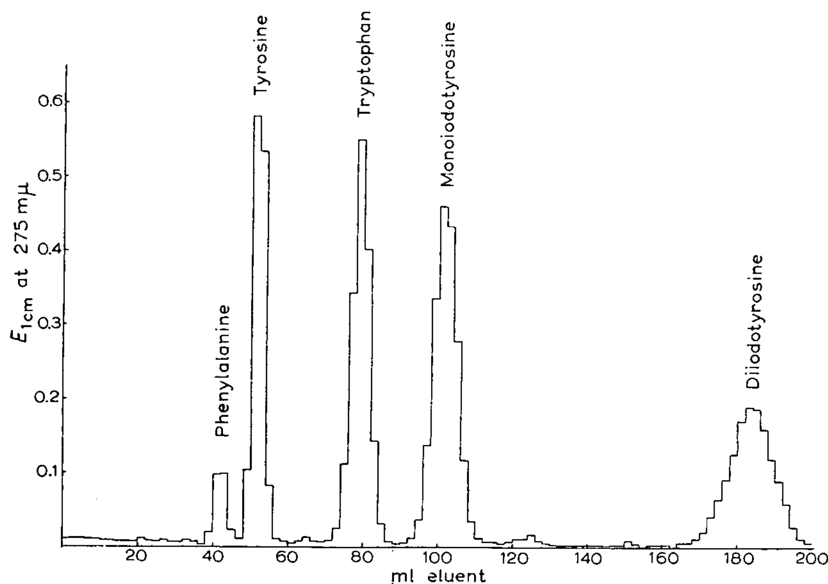


Fig. 1. Separation on a starch column 45.3 × 1.2 cm.

Other mixtures have also been separated by means of this system (Table I). It is interesting to note that on changing the developing solution from 0.1 *N* hydrochloric acid to 0.025 *N* sodium borate, pH 9.3, the retention of tryptophan was unaffected but that of diiodotyrosine was reduced to give a peak coincident with that of tyrosine (column 4). Table I also summarizes the results obtained with purines and pyrimidines.

TABLE I

Column	*Size (cm)*	*Eluting medium*	*Compound and retention volume (ml)*
1	45.3 × 1.2	0.1 *N* HCl	Phenylalanine (42), tyrosine (51), tryptophan (79), monoiodotyrosine (101), diiodotyrosine (184)
2	50.1 × 1.2	0.1 *N* HCl	Tyrosine (56), dihydroxyphenylalanine (61)
3	21.0 × 1.2	0.1 *N* HCl	Tryptophan(36), 5-hydroxytryptophan(42)
4	21.5 × 1.2	0.025 *N* borate	Tyrosine and diiodotyrosine(24), tryptophan(36)
5	21.5 × 1.2	0.025 *N* borate	Xanthurenic acid(12), kynurenic acid(15)
6	50.1 × 1.2	0.025 *N* borate	Nicotinic acid(44), tryptophan(87)
7	56.5 × 1.2	0.1 *N* HCl	Thymidine(39), cytosine(48), adenine(65), guanine(73)
8	56.5 × 1.2	0.1 *N* HCl	Uric acid(73).

Department of Experimental Biochemistry,
The London Hospital Medical College, London (Great Britain)

T. H. Farmer

1 S. Moore and W. Stein, *J. Biol. Chem.*, 178 (1949) 53.
2 I. N. Rosenberg, *J. Clin. Endocrinol. Metab.*, 11 (1951) 1063.
3 M. M. Daly and A. E. Mirsky, *J. Biol. Chem.*, 179 (1949) 981.

Received April 2nd, 1964

Book Reviews

Paper Chromatography, a Comprehensive Treatise, edited by I. M. HAIS AND K. MACEK, Publishing House of the Czechoslovak Academy of Sciences, Prague, and Academic Press, London and New York, 1963, 955 pp., price Kcs 110.— or £ 9.10.0.

Handbuch der Papierchromatographie, herausgegeben von I. M. HAIS UND K. MACEK, Band I, *Grundlagen und Technik,* zweite Auflage, VEB Gustav Fischer Verlag, Jena, 1963, 1069 pp., price DM 72.40.

Handbuch der Papierchromatographie, herausgegeben von I. M. HAIS UND K. MACEK, Band III, *Bibliographie 1957–1960 und Anwendungen,* VEB Gustav Fischer Verlag, Jena, 1963, 700 pp., price DM 40.90.

The comprehensive treatise of paper chromatography by HAIS AND MACEK consists of three volumes: volume 1 reviews the principles and applications of paper chromatography, volume 2 lists the bibliography from the beginning till 1957 and volume 3 contains the bibliography from 1957 till 1960.

The present review deals with the first English and second German editions of Volume 1 and the first German edition of Volume 3. We would like to mention that there are also Czech, Roumanian, Hungarian and Russian editions of this work.

The English edition was translated under the editorship of Prof. C. B. COULSON, Dr. L. G. PLASKETT, Dr. D. W. RIBBONS and Dr. R. A. WALL. The methods discussed for the various substances are only selections of course but are extremely well chosen and richly illustrated with graphs showing R_F values and coloured plates. One evident short-coming of such a comprehensive work is that only exceptionally papers later than 1961 are mentioned in the text. There are about 100 pages of "practical notes" consisting of the exact procedures for the preparation of reagents, the preparation of impregnated filter papers and selected quantitative methods.

The reviewer feels that this treatise is the most important that has appeared of late and will be invaluable for many years to come. It has found a permanent place on his desk, where it is being consulted regularly by numerous workers. The authors and editors should be congratulated for having performed such a difficult task in such an excellent manner.

M. LEDERER (Rome)

Infra-red Spectroscopy and Molecular Structure, An Outline of the Principles, edited by M. DAVIES, Elsevier Publishing Co., Amsterdam, 1963, xiii + 468 pp., price Dfl. 37.50, 75 s, DM. 42.—.

This valuable book "is intended to provide an introduction to the principles and practice of those aspects of infra-red spectroscopy which are of major interest in the study of molecular structure and molecular behaviour". The international assemblage of individual chapter authors, thirteen in all, each writing authoritatively on the basic principles of his topic (rather than on a survey of recent advances), marks this book as a necessary addition to the library of anyone concerned with the theory and/or practice of infra-red spectroscopy, and that is meant to include, particularly, advanced students just beginning in the field. There is not to be found a great deal that is new, but in this one volume is found an excellent collection of treatments of the diverse aspects of the total subject.

The thirteen chapters include: Introductory Survey (21 pp.), M. DAVIES; Instrumentation and General Experimental Methods (62 pp.), A. E. MARTIN, a survey of all facets of modern spectrometers, accessories, and experimental techniques; Low Frequency Infra-red Spectroscopy (25 pp.), G. R. WILKINSON; The Infra-red Spectra of Simple Molecules (54 pp.), W. J. JONES, excellent one-chapter survey; Force Constant Calculations for Small Molecules (30 pp.), I. M. MILLS; Raman Spectroscopy (26 pp.), J. C. EVANS; Characteristic Features in the Spectra of Organic Molecules (43 pp.), D. HADŽI; Infra-red Spectra of Solids: Dichroism and Polymers (40 pp.), S. KRIMM; Inorganic Applications of Infra-red Spectroscopy (33 pp.), E. A. V. EBSWORTH, of great value in spite of the appearance of NAKAMOTO's fine book; Quantitative Intensity Studies and Dipole Moment Derivatives (31 pp.), J. OVEREND; The Methods and Results of Dispersion Studies (27 pp.), J. FAHRENFORT; Hydrogen Bonding and Solvent Effects (35 pp.), H. E. HALLAM; and Infra-red Emission Spectra (17 pp.), W. C. PRICE.

Whereas the skimpy index leaves much to be desired, the many references to be found at the chapter ends are up-to-date and very useful. A very thoughtful feature of the book is the placement at the bottom of every other page of the page numbers where the references can be found. The book is well-bound, the paper of high quality and the figures are clear and well-drawn.

There is good balance in the book between principles and practice. There is not the overbearance of mathematics often found in such books, and adequate references to the details of group theory and wave mechanics are provided for those unfamiliar with these mathematical languages. To the phrase "something for everyone", which comes to mind with this book, might be added "a lot for many", and these appear to be quite general and fair appraisals. This reviewer makes no pretense to be able to judge scientifically the worth and accuracy of all of the varied topics covered, but for those topics in which he is competent to judge, the verdict must be "well done".

J. SELBIN (Baton Rouge)

Chromatography in Geology, by ARTHUR S. RITCHIE, Elsevier Publishing Company, Amsterdam, 1964, viii + 185 pages, price 50 s.

Although many books on chromatography and its applications have appeared recently, this is the first which attempts to introduce this technique to the geologist. The volume, of 185 p., is divided into two roughly equal parts, the first dealing with the principles and techniques and the second with the applications of chromatography in geology.

The author has succeeded in presenting both the theory and techniques in a very simplified, concise and clear form, suitable not only for geologists but also for anybody working in inorganic chemistry and desiring to learn about the usefulness of chromatography as an aid to solving his problems.

The second part contains one chapter on the analysis of natural waters, soils, locks and ores and another on the identification of metal ions in minerals. In the ratter there are numerous tables with reagents for the detection of the more common ions and a scheme of identification of metal ions by R_F values in a number of chosen solvents; this scheme is applied to the study of about 150 minerals.

The literature cited is not excessive but more than adequate.

On the whole the author has completely succeeded in his purpose and the book can be recommended as an excellent laboratory handbook.

G. GRASSINI (Rome)

Complexation in Analytical Chemistry, by ANDERS RINGBOM, Vol. XVI of the series *Chemical Analysis*, Interscience, New York, 1963, 395 pages, price 113 s.

Complexation equilibria and their analytical applications are given here an approximate treatment at a level intermediate between that of a complete general and rigorous analytical treatise, and that of a handbook of analytical procedures.

Although in principle such an approach might seem questionable, it turns out actually to be a useful link between the purely theoretical approach and the purely applicative one. This book will be particularly useful to analysts who wish to rationalise routine analytical prescriptions in complexometry and to explain newer literature developments in complexometric procedures.

Basic concepts such as the relationship between thermodynamic and non-thermodynamic equilibrium constants, conditional constants and their relationship to general constants are introduced in a clear manner and their importance adequately stressed.

A valuable extensive tabulation of critically selected values of stability constants is given at the end of the volume.

C. FURLANI (Trieste)

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

ANSELMI, S.: (Chromatography in the analysis of fatty substances). *Riv. Ital. Sostanze Grasse*, 38 (1961) 433–435; *C.A.*, 60 (1964) 1939g.

2. FUNDAMENTALS, THEORY AND GENERAL

WEISS, B., ROSSI, G. V. AND REBER, L. A.: A mathematical basis for the solvent reversal technique of paper chromatography. *Nature*, 197 (1963) 280–282.

3. TECHNIQUES I

ACKERMANN, G. AND ASSMUS, G.: Beitrag zur quantitativen Auswertung von Papierchromatogrammen durch Remissionsmessungen. *Z. Anal. Chem.*, 200 (1964) 418–427.

OGNYANOV, I.: Detection of unsaturated compounds. *Compt. Rend. Acad. Bulgare Sci.*, 16, No. 2 (1963) 161–163; *C.A.*, 60 (1964) 1121e — paper and thin-layer chromatography.

4. TECHNIQUES II

Systemation analysis, automation and preparative-scale paper chromatography

REIO, L.: The paper chromatographic identification of aromatic compounds related to metabolites of fungal and mammalian origin. *Svensk Kem. Tidskr.*, 76, No. 5 (1964) 1-25 — systematic analysis of about 900 compounds; recording of data on punched cards for IBM computer.

6. ALCOHOLS

MACEK, K., VANĚČEK, S. AND BEČVÁŘOVÁ, H.: Chromatographische Bestimmung einiger Zwischenprodukte der Ephedrinsynthese. *Collection Czech. Chem. Commun.*, 29 (1964) 311–315 — paper and gas chromatography of alcohols, ketones and aldehydes.

7. PHENOLS

POSPÍŠIL, J. AND TAIMR, L.: Chromatographisches Verhalten der *tert.*-Butyl- und *tert.*-Octylgruppen enthaltenden Hydrochinon-Derivate. *Collection Czech. Chem. Commun.*, 29 (1964) 374–380 — R_F values of 30 phenols; relation between structure and chromatographic behaviour.

SHIBATA, Y., KAI, Y. AND KONDO, T.: (Phenolic constituents from *Cryptomeria japonica* wood. II. Quantitative determination methods of the phenols). *Nippon Mokuzai Gakkasishi*, 9 (1963) 142–145; *C.A.*, 60 (1964) 1626e.

10. CARBOHYDRATES

BICHSEL, S. E. AND JOHNSON, I. R.: An improved paper chromatography method for the determination of raffinose and kestose in beet root samples. *J. Am. Soc. Sugar Beet Technologists*, 12 (1963) 449–454; *C.A.*, 60 (1964) 1913b.

COMMERFORD, J. D., VAN DUZEE, G. T. AND SCALLET, B. L.: Macro paper chromatography of corn starch hydrolyzates. *Cereal Chem.*, 40 (1963) 482–486; *C.A.*, 60 (1964) 1914e.

HORNUFF, G. VON AND RICHTER, H.: Studien über chemische Zusammensetzung der Baumwollfasern verschiedener Anbaugebiete. *Faserforsch. Textiltech.*, 15 (1964) 115–126; 165–179.

OHKUMA, S.: (Spray methods for the detection of 2-amino-2-deoxyhexoses and their N-acetyl derivatives on paper chromatograms). *Proc. Japan Acad.*, 39 (1963) 400–405; *C.A.*, 60 (1964) 24b.

RICHTER, M. AND STROH, H. H.: Die papierchromatographische Fraktionierung der Stärkekohlenhydrate. *Stärke*, 14 (1962) 415–424; from *Ernährungsforsch.*, 9 (1964) 92.

YOSHIKAWA, M.: Enzymatic decomposition of alginate obtained from *Laminaria* and *Undaria* algae. *Hyogo Noka Daigaku Kenkyu Hokoku, Nogei-kagaku Hen*, 5 (1961) 94–96; *C.A.*, 60 (1964) 1915g.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BLUNDSTONE, H. A. W.: Paper chromatography of organic acids. *Nature*, 197 (1963) 377.

BROCKELT, G. AND POHLOUDEK-FABINI, R.: (Chemistry and physiology of metabolically important acids. XV. Indirect polarographic determination of α-oxo acids in the form of their quinoxalines). *Sci. Pharm.*, 31 (1963) 94–105; *C.A.*, 60 (1964) 1584c.

CORTÉS, I. M. AND DE BETHENCOURT, C. D.: (Die chromatographische und photometrische Bestimmung von organischen Säuren und Farbstoffen in Wein). *Chim. Anal. (Paris)*, 44 (1962) 527–532; from *Z. Anal. Chem.*, 200 (1964) 148 — quantitative analysis *in situ*.

HIRAYAMA, O.: Chromatography of lipids and its biochemical applications. IV. A simple method for analysis of triglycerides by paper chromatography. *Nippon Nogeikagaku Kaishi*, 35 (1961) 367–371; *C.A.*, 60 (1964) 1941c.

HIRAYAMA, O.: Chromatography of lipids and its biochemical applications. V. Paper chromatography of fatty acid, mono-, di- and triglyceride mixtures. *Nippon Nogeikagaku Kaishi*, 35 (1961) 372–376; *C.A.*, 60 (1964) 1941e.

HIRAYAMA, O.: Chromatography of lipids and its biochemical applications. VII. Paper chromatographic analysis of component glycerides in soybean oil and stillingia oil. *Nippon Nogeikagaku Kaishi*, 35 (1961) 441–444; *C.A.*, 60 (1964) 1941h.

KAUFMANN, H. P. AND CHOWDHURY, D. K.: (Catalytic hydrogenation of organic compounds on paper for chromatographic analysis). *Lab. Sci. (Milan)*, 11 (1963) 67–72; *C.A.*, 60 (1964) 7a.

KWAPNIEWSKI, Z. AND SLIWIOK, J.: (Detection of higher fatty acids on paper chromatograms). *Chem. Anal. (Warsaw)*, 9 (1964) 119–121.

LOESCHCKE, V. AND FRANCKSEN, H.: Trichocarpin, ein neues als Resistenzfaktor bedeutsames Phenolglykosid aus Pappelrinde. *Naturwissenschaften*, 51 (1964) 140.

PAPKE, E. AND POHLOUDEK-FABINI, R.: Papierchromatographische und papierelektrophoretische Verteilung der Ketosäure-2,4-dinitrophenylhydrazone und Versuche zur Ausarbeitung einer Methode zur Bestimmung von Ketosäuren aus Frischpflanzenmaterial. *Pharmazie*, 19 (1964) 27–33.

SMITH, P.: The determination of metabolites of catecholamines by chromatographic techniques. In VARLEY H. AND GOWENLOCK, A. H. (Editors): *The Clinical Chemistry of Monoamines West-European Symposia on Clinical Chemistry*, Vol. 2, Elsevier, Amsterdam, 1963, pp. 31–38.

SUCKER, H.: (The analysis of the fatty acids of suppository bases and emulsifying agents by paper chromatography of acylhydroxamic acids). *Deut. Apotheker-Z.*, 101 (1961) 441–443; *C.A.*, 60 (1964) 366h.

VERESHCHAGIN, A. G. AND GANIEVA, M.: (Lipid metabolism in maturing and germinating cottonseed and the effect of gamma-irradiation on this process). *Biokhimiya*, 29 (1964) 288–299.

13. STEROIDS

LAMBERT, M. AND PENNINGTON, G. W.: 6β-Hydroxycortisol(4-pregnane-6β,11β,17α,21-tetrol-3,20-dione) in Liquor Amnii. *Nature*, 197 (1963) 391–392.

14. STEROID GLYCOSIDES

LUTYNSKI, E. W.: (Colorimetric and chromatographic determination of lanatoside C content in solutions for oral and parental use). *Dissertationes Pharm.*, 15 (1963) 205–213; *C.A.*, 60 (1964) 371a.

15. TERPENE DERIVATIVES

TSCHESCHE, R., DUPHORN, I. AND SNATZKE, G.: (Triterpenes. IX. Constitution of cincholic acid and the acidic triterpene glycosides of cinchona bark). *Ann.*, 667 (1963) 151–163; *C.A.*, 60 (1964) 1802f — paper and thin-layer chromatography.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

DREWRY, J.: Examination of detergents by paper chromatography. Part II. *Analyst*, 89 (1964) 75–76.

KURDUBOV, YU. F., REBROVA, N. B. AND BARAMBOIM, N. B.: (Determination of vulcanization accelerators by paper chromatography). *Nauchn. Tr. Mosk. Tekhnol. Inst. Legkoi Prom.*, No. 24 (1962) 49–53; *C.A.*, 60 (1964) 3171e.

NAKAHARA, T., DEHARA, M. AND HIYAMA, H.: (Separation and identification of reaction products of *m*-phenylenediamine and ethylene chlorohydrin). *Kagaku To Kogyo (Osaka)*, 37, No. 1 (1963) 42–44; *C.A.*, 60 (1964) 1120e.

ROBINSON, R.: The clinical chemistry of phaeochromocytomas. In VARLEY, H. AND GOWENLOCK, A. H. (Editors): *The Clinical Chemistry of Monoamines, West-European Symposia on Clinical Chemistry*, Vol. 2, Elsevier, Amsterdam, 1963, pp. 63–70; 102.

SUH, G. D.: Identification of (rubber vulcanizate) antioxidants. *Ta Han Hua Hsueh Hui-Chih*, 6 (1962) 99–103; *C.A.*, 60 (1964) 1907c.

18. AMINO ACIDS

KOLOUŠEK, J., JIRÁČEK, V. AND ČIŽINSKÝ, B.: (Air-driven ultracentrifuge for investigations of free amino acids, peptides, amides and free ammonia in brain and liver). *Sb. Lékař.*, 66, No. 6 (1964) 171–177.

PAVLAS, P.: (Chromatographic and electrophoretic identification of α-aminoadipic acid and colamine in sugar juices). *Listy Cukrovar.*, 79 (1963) 209–213; *C.A.*, 60 (1964) 1911h.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

ARMSTRONG, D. A. J. AND MILLS, G. L.: Chemical characterization of kinins of human plasma. *Nature*, 197 (1963) 490.

KRUTILINA, A. I., VENKSTERN, T. V. AND BAEV, A. A.: (A mapping procedure for ribonuclease hydrolysates of sRNA's). *Biokhimiya*, 29 (1964) 333–337 — two-dimensional paper chromatography.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BERÁNEK, J. AND PITHA, J.: Nucleic acid components and their analogues. XLVI. Some derivatives of 6-azacytidine and 6-azauridine. *Collection Czech. Chem. Commun.*, 29 (1964) 625–634 — R_F values of 22 compounds.

CHLÁDEK, S. AND SMRT, J.: Oligonucleotidic compounds. VIII. Synthesis of adenylyl-(5' → 3')-uridine, adenylyl-(5' → 3')-cytidine, guanylyl-(5' → 3')-uridine, guanylyl-(5' → 3')-cytidine, cytidylyl-(5' → 3')-cytidine, adenylyl-(5' → 3')-uridylyl-(5' → 3')-cytidine and related compounds. *Collection Czech. Chem. Commun.*, 29 (1964) 214–233 — R_F values of 9 adenosine derivatives, 10 guanosine derivatives and 8 oligonucleotides.

CONLAY, J. J.: Effect of ionizing radiation on adenine in aerated and de-aerated aqueous solutions. *Nature*, 197 (1963) 555–557.

LOHRMANN, R., LAGOWSKI, J. M. AND FORREST, H. S.: 3-Ribosyluric acid. Part III. Unambiguous syntheses of 3-ribosyluric acid and related compounds. *J. Chem. Soc.*, (1964) 451–459.

LOTZ, M., FALLON, H. J. AND SMITH, Jr., L. H.: Excretion of orotic acid and orotidine in heterozygotes of congenital orotic aciduria. *Nature*, 197 (1963) 194–195.

SHESTAKOV, V. G., SHABAROVA, Z. A. AND PROKOFEV, M. A.: (Synthesis of the methyl ester of P^1-(adenosine-5')-diphospho-(P^2 → N)-phenylalanine). *Biokhimiya*, 29 (1964) 300–311.

ŽEMLIČKA, J., SMRT, J. AND ŠORM, F.: Nucleic acid components and their analogues. XLVIII. Synthesis and structure of nitrogen mustard derivatives of cytidine and 6-azacytidine. *Collection Czech. Chem. Commun.*, 29 (1964) 635–644.

22. ALKALOIDS

MANDAK, M., STRUHAR, M. AND KLUCAROVA, H.: (The determination of tropane alkaloids in drugs). *Farm. Obzor*, 32 (1963) 97–105; *C.A.*, 60 (1964) 367g.

PANKIEWICZ, H.: (Colorimetric and chromatographic estimation of *Lobelia* infusions. Their durability by the method of accelerated aging). *Farm. Polska*, 19 (1963) 234–238; *C.A.*, 60 (1964) 371b.

PARIS, R. R. AND ROUSSELET, R.: (Paper chromatography and electrophoresis in the control of alkaloids in drugs. Possibilities and reasons for mistakes). *Farm. Aikakauslehti*, 72 (1963) 261–269; *C.A.*, 60 (1964) 367b.

PLATEK, J.: (Studies on domestic tabacco. III. The main alkaloids and their occurrence in some commercial varieties). *Chem. Anal. (Warsaw)*, 2 (1964) 107–112.

ŠTROUF, O. AND TROJÁNEK, J.: On alkaloids. X. The structure of vincine. *Collection Czech. Chem. Commun.*, 29 (1964) 447–456.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

GRANDBERG, I. I., TABAK, S. V., FAIZOVA, G. K. AND KOST, A. N.: (Pyrazoles. XXXVII. Chromatographic separation of aminopyrazoles). *Zh. Obshch. Khim.*, 33 (1963) 2585–2586; *C.A.*, 60 (1964) 515c — paper and thin-layer chromatography.

HOLLIMAN, F. G., JEFFERY, B. A. AND BROCK, D. J. H.: Phenazines. III. Synthesis of 7-aminophenazine-1-, 7-aminophenazine-2-, and 8-aminophenazine-2-carboxylic acids. *Tetrahedron*, 19 (1963) 1841–1848; *C.A.*, 60 (1964) 1751b.

JEPSON, J. B.: The formation and metabolism of hydroxyindoles. In VARLEY, H. AND GOWENLOCK, A. H. (Editors): *The Clinical Chemistry of Monoamines, West-European Symposia on Clinical Chemistry*, Vol. 2, Elsevier, Amsterdam, 1963, pp. 107–115.

KOMOTO, M.: The reaction products of glucose and ammonia. V. Isolation and identification of 4(5)-(2-hydroxyethyl)-imidazole. *Nippon Nogeikagaku Kaishi*, 36 (1962) 461–463; *C.A.*, 60 (1964) 2332d.

KOMOTO, M.: The reaction products of glucose and ammonia. VI. Isolation and identification of 4(5)-(2,3,4-trihydroxybutyl)-imidazole and presumption of 4(5)-(2,3-dihydroxypropyl)-imidazole. *Nippon Nogeikagaku Kaishi*, 36 (1962) 464–468; *C.A.*, 60 (1964) 2332e.
KOMOTO, M.: The reaction products of glucose and ammonia. VII. Relation between paper chromatograms and the chemical structures of imidazole compounds. *Nippon Nogeikagaku Kaishi*, 36 (1962) 541–546; *C.A.*, 60 (1964) 2332g.
KOMOTO, M.: The reaction products of glucose and ammonia. VIII. Mechanism of the decomposition of glucose in aqueous ammonia solution, deduced mainly from the imidazole compounds produced. *Nippon Nogeikagaku Kaishi*, 36 (1962) 546–551; *C.A.*, 60 (1964) 2332h.
SANDLER, M.: The determination of hydroxyindoles in biological materials. In VARLEY, H. AND GOWENLOCK, A. H. (Editors): *The Clinical Chemistry of Monoamines, West-European Symposia on Clinical Chemistry*, Vol. 2, Elsevier, Amsterdam, 1963, pp. 116–130.

24. ORGANIC SULPHUR COMPOUNDS

ANONYMOUS: Changes in official methods of analysis made at the 77th annual meeting, October 14–17, 1963. *J. Assoc. Offic. Agr. Chemists*, 47 (1964) 194–195.
STATHER, F., REICH, G. AND STEINHARDT, R.: (Constitution and tanning power of a condensation product of 2-naphtholsulphonic acids, *p*-cresol and formaldehyde). *Ges. Abhandl. Deut. Lederinsts. Freiberg/Sa.*, No. 18 (1962, publ. 1963) 108–123; *C.A.*, 60 (1964) 776a.
THOMPSON, J. F., ARNOLD, W. N. AND MORRIS, C. J.: A sensitive qualitative test for sulphoxides on paper chromatograms. *Nature*, 197 (1963) 380–381 — starch, sodium iodide and HCl.
WEISBURGER, E. K. AND BOYD, R. E.: Fluorenylcysteines. *J. Chem. Soc.*, (1964) 515–518 — paper and thin-layer chromatography.

25. ORGANIC PHOSPHORUS COMPOUNDS

ROWEN, R.: Purification and characterization of an induced plasma lipoprotein that inhibits streptolysin O. *Proc. Soc. Exptl. Biol. Med.*, 114 (1963) 183.
SHVETS, V. I., VOLKOVA, L. V., RYZHENKOVA, S. F., LUKASHENKO, E. E. AND PREOBRAZHENSKIĬ, N. A.: (Synthesis of symmetrical and unsymmetrical α-cephalins). *Zh. Obshch. Khim.*, 33 (1963) 2876–2879; *C.A.*, 60 (1964) 1579c.
TSIZIN, YU. S. AND PREOBRAZHENSKII, N. A.: (Complex lipids. Synthesis of 2-aminoethyl DL-2-amino-2-carboxyethyl phosphate). *Zh. Obshch. Khim.*, 33 (1963) 2873–2876; *C.A.*, 60 (1964) 1579a.
WOGGEN, H., SPRANGER, D. AND ACKERMANN, H.: (Beitrag zur Chromatographie von Thiophosphorsäureestern, insbesondere Tinox). *Nahrung*, 7 (1963) 612–618; from *Ernährungsforsch.*, 9 (1964) 99.

26. METALLO-ORGANIC COMPOUNDS

WILLIAMS, D. J. AND PRICE, J. W.: The paper chromatography of some organo-tin compounds. Part II. Reversed-phase systems. *Analyst*, 89 (1964) 220–222 — R_F values of 19 compounds.

27. VITAMINS

LOHRMANN, R. AND FORREST, H. S.: Synthesis of 8-substituted glucosylpteridines related to known naturally occurring materials. *J. Chem. Soc.*, (1964) 460–465.

28. ANTIBIOTICS

SCHMIDT-KASTNER, G. AND SCHMID, J.: (Alveomycin, an antibiotic from actinomycetin). *Med. Chem., Abhandl. Med.-Chem. Forschungsstaetten Farbenfabriken Bayer*, 7 (1963) 528–539; *C.A.*, 60 (1964) 1542f.

29. INSECTICIDES AND OTHER PESTICIDES

KEENAN, G. I., LISK, D. J. AND O'BRIEN, D. R.: Note on *p*-dimethylaminobenzaldehyde as a sensitive chromogenic agent for detection of 4-dimethylamino-*m*-tolyl methylcarbamate on paper. *J. Assoc. Offic. Agr. Chemists*, 47 (1964) 28.

30. SYNTHETIC AND NATURAL DYES

ALLAN, Z. J. AND PODSTATA, J.: Aromatische Diazo- und Azoverbindungen. LVII. Reaktionen von Ammoniumhydrogensulfit mit benzolischen Aminoazofarbstoffen und mit Nitrobenzol. *Collection Czech. Chem. Commun.*, 29 (1964) 752–775.
BOOTH, V. H. AND RUSSEL, G. E.: Specific lipids produced by sugar beet infected with yellowing viruses. *Nature*, 197 (1963) 1328–1329 — two-dimensional chromatography; (I) $ZnCO_3$, (II) paraffin.

BROWN, J. C.: Further applications of chromatography in dyeing and finishing. *J. Soc. Dyers Colourists*, 80 (1964) 185–195 — paper and thin-layer chromatography.
CALDERBANK, A., CAMERON, D. W., CROMARTIE, R. I. T., HAMIED, Y. K., HASLAM, E., KINGSTON, P. G. I., LORD TODD AND WATKINS, J. C.: Colouring matters of the Aphididae. Part XX. The structure of the xanthoaphins and chrysoaphins. *J. Chem. Soc.*, (1964) 80–89.
PLÁ DELFINA, J. M. AND MOREU, J. M.: (Rapid chromatographic method for the determination of the position of sulphonic groups in some azo dyes used in foodstufs, pharmaceuticals and cosmetics). *Galenica Acta (Madrid)*, 16 (1963) 265–277.
STEINMÜLLER, H.: Ursachenermittlung der Farbstreifigkeit bei Viskoseseidengeweben und Gewirken und Wege zur Behebung in der Textilveredlung. *Deut. Textiltech.*, 14 (1964) 32–35, 91–93.
THEIDL, H.: Zur Stereochemie optischer Aufheller vom Stilben-Typ. *Melliand Textilber.*, 45 (1964) 514–519.

31. PLASTICS AND THEIR INTERMEDIATES

GUDE, A.: (Papierchromatographische Identifizierung von PVC-Weichmachern). *Kunststoffe*, 52 (1962) 679–680; from *Z. Anal. Chem.*, 201 (1964) 317.

32. PHARMACEUTICAL APPLICATIONS

COLO, A., CARDINI, C. AND MARIANI, A.: (Control of pharmaceutical products containing cynarin). *Farm. Aikakauslehti*, 72 (1963) 286–292; *C.A.*, 60 (1964) 375b.
CURRY, A. S.: The toxicology of monoamine oxidase inhibitors and tranquillisers. In VARLEY, H. AND GOWENLOCK, A. H. (Editors): *The Clinical Chemistry of Monoamines, West-European Symposia on Clinical Chemistry*, Vol. 2, Elsevier, Amsterdam, 1963, pp. 205–214.
MARGARIDA, M., BRAGA, F. AND BALTAZAR, J.: (Detection and determination of components in an injectable spasmolytic solution). *Rev. Port. Farm.*, 13 (1963) 145–150; *C.A.*, 60 (1964) 368d.

33. INORGANIC SUBSTANCES

HU, Z.-T. AND SHI, S.-CH.: Separation of V, Mo and W by paper chromatography. *Hua Hsueh Tung Pao*, (1963) 312–313, 320; *C.A.*, 60 (1964) 6d.
SAGORTSCHEV, B., BOZADŽIEVA, L. AND MITROPOLITSKA, E.: (Die chromatographische Trennung von Eisen(III) und Chrom(III) mit anschliessender photometrischer bzw. titrimetrischer Bestimmung). *Dokl. Bulgar. Akad. Nauk*, 15 (1962) 483–486; from *Z. Anal. Chem.*, 201 (1964) 235.

34. RADIOACTIVE COMPOUNDS

DIMITRIADOU, A., TURNER, P. C. R. AND FRASER, T. R.: Activation analysis of paper chromatograms for iodine ($^{127}I \rightarrow {}^{128}I$). *Nature*, 197 (1963) 446–449.
EVANS, E. A. AND STANFORD, F. G.: Decomposition of tritium-labelled organic compounds. *Nature*, 197 (1963) 551–555.
GARDINER, J. E.: A radioactive marking ink. *Nature*, 197 (1963) 414.
RACHINSKIĬ, V. V.: (Composition of photosynthetic products in different plant species). *Vestn. Sel'skokhoz. Nauki, Vses. Akad. Sel'skokhoz. Nauk*, No. 3 (1964) 20–22.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

GIACOBAZZI, C. AND GIBERTINI, G.: (Die Anwendung der Dünnschichtchromatographie in der Pharmazie). *Boll. Chim. Farm.*, 101 (1962) 490–496; from *Z. Anal. Chem.*, 201 (1964) 388.
ISHIKAWA, M.: Thin layer chromatography. Experimental procedure. *Kagaku No Ryoiki*, 17 (1963) 179–189; *C.A.*, 60 (1964) 7c.
MIMA, H.: Thin layer chromatography without binders. *Kagaku No Ryoiki*, 17 (1963) 189–196; *C.A.*, 60 (1964) 7c.

2. FUNDAMENTALS, THEORY AND GENERAL

MUNTER, F.: (Systematic thin-layer chromatography: a working hypothesis). *Chemiker-Ztg.*, 87 (1963) 656–659; *C.A.*, 60 (1964) 7f.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

MATSUSHITA, H., SUZUKI, Y. AND SAKABE, H.: Separation and determination of polycyclic aromatic hydrocarbons by thin layer chromatography. *Bull. Chem. Soc. Japan*, 36 (1963) 1371; *C.A.*, 60 (1964) 26g.

6. ALCOHOLS

KNAPPE, E., PETERI, D. AND ROHDEWALD, I.: Dünnschichtchromatographische Identifizierung technisch wichtiger Polyalkohole. *Z. Anal. Chem.*, 199 (1964) 270–276.

7. PHENOLS

KNAPPE, E. AND ROHDEWALD, I.: Die dünnschichtchromatographische Identifizierung der niederen Phenole über ihre Kupplungsprodukte mit Echtrotsalz Al. *Z. Anal. Chem.*, 200 (1964) 9–14.

KRAUS, L. AND DUPÁKOVÁ, D.: Der derzeitige Stand der Bewertung von Arbutindrogen. *Pharmazie*, 19 (1964) 41–45 — polyamide layers.

SMITH, G. A. L. AND SULLIVAN, P. J.: Determination of the steam-volatile phenols present in cigarette-smoke condensate. Part II. Determination of phenol, cresols and guaiacol by thin-layer chromatography. *Analyst*, 89 (1964) 312–318 — kieselguhr G impregnated with formamide; phenols developed as azo compounds.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

MACMILLAN, J. AND SUTER, P. J.: Thin layer chromatography of the gibberellins. *Nature*, 197 (1963) 790.

9. OXO COMPOUNDS

BADINGS, H. T. AND WASSINK, J. G.: Separation and identification of aliphatic aldehydes and ketones by thin-layer chromatography of the 2,4-dinitrophenylhydrazones. *Neth. Milk Dairy J.*, 17 (1963) 132–149; *C.A.*, 60 (1964) 26d.

MEHLITZ, A., GIERSCHNER, K. AND MINAS, T.: (Thin-layer chromatographic separation of 2,4-dinitrophenylhydrazones. Differentiation of 2,4-dinitrophenylhydrazones of saturated aldehydes and ketones as well as of unsaturated carbonyl compounds by means of a colour reaction with potassium ferricyanide). *Chemiker-Ztg.*, 87 (1963) 573–576; *C.A.*, 60 (1964) 1118e.

10. CARBOHYDRATES

CARRUTHERS, A., DUTTON, J. V., OLDFIELD, J. F. T., ELLIOTT, C. W., HEANEY, R. K. AND TEAGUE, H. J.: Estimation of sugars in beet molasses. I and II. *Intern. Sugar J.*, 65 (1963) 234–237; 266–270; *C.A.*, 60 (1964) 1913e.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BARRETT, C. B., DALLAS, M. S. J. AND PADLEY, F. B.: The quantitative analysis of triglyceride mixtures by thin-layer chromatography on silica impregnated with silver nitrate. *J. Am. Oil Chemists' Soc.*, 40 (1963) 580–584; *C.A.*, 60 (1964) 771d.

ENG, L. F., LEE, Y. L., HAYMAN, R. B. AND GERSTL, B.: Separation and isolation of methyl esters and dimethyl acetals formed from brain lipids. *J. Lipid Res.*, 5 (1964) 128–130.

GRÉEN, K. AND SAMUELSSON, B.: Prostaglandins and related factors. XIX. Thin-layer chromatography of prostaglandins. *J. Lipid Res.*, 5 (1964) 117–120.

KAUFMANN, H. P. AND WESSELS, H.: Die Struktur der Triglyceride: Theorien und Bestimmungsmethoden. *Fette, Seifen, Anstrichmittel*, 66 (1964) 13–20.

KINOSHITA, S.: Analysis of sucrose fatty acid esters. I. Qualitative analysis of sucrose fatty acid esters by thin-layer chromatography. *Kogyo Kagaku Zasshi*, 66 (1963) 450–455; *C.A.*, 60 (1964) 3207h.

KINOSHITA, S. AND OYAMA, M.: Analysis of sucrose fatty acid esters. II. Quantitative analysis of sucrose fatty acid esters by thin-layer chromatography. *Kogyo Kagaku Zasshi*, 66 (1963) 455–458; *C.A.*, 60 (1964) 3208b.

KISHIMOTO, Y. AND RADIN, N. S.: Structures of the 2-hydroxy unsaturated fatty acids of pig brain sphingolipids. *J. Lipid Res.*, 5 (1964) 94–97.

SAMBASIVARAO, K. AND MCCLUER, R. H.: Lipid components of gangliosides. *J. Lipid Res.*, 5 (1964) 103–108.

SCHMID, E., ZICHA, L. AND KRAUTHEIM, J.: Thin-layer chromatography in the diagnosis of phaeochromocytoma and malignant argentaffinoma. In VARLEY, H. AND GOWENLOCK, A. H. (Editors): *The Clinical Chemistry of Monoamines. West-European Symposia on Clinical Chemistry*, Vol. 2, Elsevier, Amsterdam, 1963, pp. 97–98.

13. STEROIDS

HARA, S.: Thin-layer chromatography of steroids. *Kagaku No Ryoiki*, 17 (1963) 196–206; *C.A.*, 60 (1964) 7c.

WOLFMAN, L. AND SACHS, B. A.: Separation of cholesterol and desmosterol by thin-layer chromatography. *J. Lipid Res.*, 5 (1964) 127–128 — silica impregnated with undecane.

15. TERPENE DERIVATIVES

VORBRUEGGEN, H., PAKRASHI, S. C. AND DJERASSI, C.: (Terpenoids. LIV. Studies on Indian medicinal plants. 7. Arborinol, a new triterpene type). *Ann.*, 668 (1963) 57–76; *C.A.*, 60 (1964) 567c.

18. AMINO ACIDS

PATTERSON, S. J. AND CLEMENTS, R. L.: The application of paper and thin-layer chromatography to the identification of thyroxine in a feeding-stuffs additive. *Analyst*, 89 (1964) 328–331 — cellulose layers with starch.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

KUTHAN, J., JANEČKOVÁ, E. AND HAVEL, M.: Über Dihydropyridine. V. Zur Bildung der Isomeren 1,2- und 1,4-Dihydroderivate bei der Reaktion von Methylmagnesiumjodid mit 3,5-Dicyanopyridin und 3,5-Dicyan-2-methylpyridin. *Collection Czech. Chem. Commun.*, 29 (1964) 143–151.

24. ORGANIC SULPHUR COMPOUNDS

O'BRIEN, J. S., FILLERUP, D. L. AND MEAD, J. F.: Brain lipids. I. Quantification and fatty acid composition of cerebroside sulfate in human cerebral gray and white matter. *J. Lipid Res.*, 5 (1964) 109–116 — thin-layer and paper chromatography.

25. ORGANIC PHOSPHORUS COMPOUNDS

DITTMER, J. C. AND LESTER, R. L.: A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.*, 5 (1964) 126–127.

LANDS, W. E. M. AND HART, P.: Metabolism of glycerolipids. V. Metabolism of phosphatidic acid. *J. Lipid Res.*, 5 (1964) 81–87.

OBOL'NIKOVA, E. A. AND SAMOKHVALOV, G. I.: (Synthesis of polyenes. XX. Chemical behaviour of the acetals of 4-bromo- and 4-hydroxypentanals). *Zh. Obshch. Khim.*, 33 (1963) 1860–1864; *C.A.*, 60 (1964) 543e.

28. ANTIBIOTICS

IKEKAWA, T., IWAMI, F., AKITA, E. AND UMEZAWA, H.: Application of thin-layer chromatography for separation and identification of antibiotics. *Penishirin Sono Ta Koseibusshitsu*, A 16, No. 1 (1963) 56–57; *C.A.*, 60 (1964) 1539c.

30. SYNTHETIC AND NATURAL DYES

BOLLIGER, H. R., KÖNIG, A. AND SCHWIETER, U.: Beitrag zur Dünnschicht-Chromatographie der Carotine. *Chimia (Aarau)*, 18 (1964) 136 — activated "light magnesium oxide" layers.

SALO, T. AND SALMINEN, K.: (Nachweis der synthetischen Lebensmittelfarbstoffe). *Suomen Kemistilehti*, B 35 (1962) 146–151; from *Z. Anal. Chem.*, 200 (1964) 160.

STAHL, E.: Dünnschicht-Chromatographie von Carotin- und Carotinoidgemischen. In STAHL, E., BOLLIGER, H. R. AND LEHNERT, L. (Editors): *Carotine und Carotinoide*, D. Steinkopff Verlag, Darmstadt, 1963, pp. 129–134.

SYNODINOS, E., KOTAKIS, G. AND KOKKOTI-KOTAKI, E.: (Separation of synthetic dyes by thin-layer chromatography). *Chim. Chronika (Athens, Greece)*, 28, No. 8 (1963) 77–79; *C.A.*, 60 (1964) 1089e.

32. PHARMACEUTICAL APPLICATIONS

GELDMACHER-MALLINCKRODT, M. AND LAUTENBACH, L.: Nachweis des Schlafmittels Revonal Merck. *Arch. Toxikol.*, 20 (1963) 31–37; from *Z. Anal. Chem.*, 201 (1964) 396.

JASPERSEN-SCHIEB, R. AND FLÜCK, H.: (Identitäts- und Reinheitsprüfungen von ätherischen Ölen mittels Dünnschicht-Chromatographie). *Boll. Chim. Farm.*, 101 (1962) 512–518; from *Z. Anal. Chem.*, 201 (1964) 66.

33. INORGANIC SUBSTANCES

HAMMERSCHMIDT, H. AND MUELLER, M.: (Identification of paper fillers and coating materials by use of thin-layer chromatography). *Papier*, 17 (1963) 448–450; *C.A.*, 60 (1964) 1925h.

Gas Chromatography

1. REVIEWS AND BOOKS

ANVAER, B. I. AND OKHOTNIKOV, B. P.: (Gas chromatography in the analysis of inorganic substances. A review). *Zh. Anal. Khim.*, 19 (1964) 484–498 — an excellent review with 212 references.

BRANDENBERGER, H.: Forensic chemistry today. *Chimia (Aarau)*, 17 (1963) 249–257.

HOOBS, A. P.: Gas analysis. *Anal. Chem.*, 36, No. 5 (1964) 130R–139R — 217 references, partly GC.

HORNSTEIN, I. AND CROWE, P. F.: Meat flavor — a review. *J. Gas Chromatog.*, 2 (1964) 128–131 — 30 papers are reviewed.

SCHOMBURG, G.: Qualitative Identifizierung mit Hilfe der Gaschromatographie. *Z. Anal. Chem.*, 200 (1964) 360–377 — a discussion in review form (20 references) with original measurements.

BUZON, J., GUICHARD, N., GUIOCHON, G., LEBBE, J. AND PREVOT, A. In TRANCHANT, J. (Editor): *Manuel Pratique de Chromatographie en Phase Gazeuse*, Masson et Cie, Paris, 1964, 231 pp.

2. FUNDAMENTALS, THEORY AND GENERAL

2a. Gas–liquid systems

BARR, J. K. AND SAWYER, D. T.: Functional dependence of the liquid-phase mass transfer term in gas chromatography. *147th Am. Chem. Soc. Meeting, Philadelphia, Pa., April 6–10, 1964, Div. Anal. Chem.*, Abstr. No. 8.

HARRIS, W. E. AND HABGOOD, H. W.: Some temperature effects in gas chromatography. *Talanta*, 11 (1964) 115–128 — flow conditions cannot be chosen at any one temperature to give maximum efficiency for all solutes.

KELLER, R. A. AND STEWART, G. H.: Mixed solvents in gas liquid chromatography. *Anal. Chem.*, 36 (1964) 1186–1191 — elution of a solute with two liquids may be accomplished either by using two columns in sequence, each containing a single liquid, or by means of mixed packings each holding a single liquid, or by using a packing holding a solution of the two liquids; the reasons that the three methods are not equivalent should be sought in kinetic aspects and not in thermodynamic aspects.

2b. Gas–solid systems

GIDDINGS, J. C.: Theory of gas–solid chromatography. Potential for analytical use and the study of surface kinetics. *Anal. Chem.*, 36 (1964) 1170–1175 — the kinetic mass transfer term C_k is in the order of 10^{-7} sec for common packed columns with uniform adsorptive sites; GSC gives real advantages in terms of column efficiency and high speed analysis, and real potential for the measurement of surface kinetics.

2c. Thermodynamics and theoretical relationships

GIDDINGS, J. C.: Role of column pressure drop in gas chromatography resolution. *Anal. Chem.*, 36 (1964) 741–744 — theoretically the magnitude of the pressure drop has a secondary role compared to other variables such as column length, flow velocity and average pressure.

GORDON, S. M., KRIGE, G. J. AND PRETORIUS, V.: Theoretical prediction of the stationary phase contribution to the plate height in chromatography. *Anal. Chem.*, 36 (1964) 750–853 — the C_L term is not influenced so strongly by the amount of stationary phase as may be expected from the "film" model.

HENLY, R. S., ROSE, A. AND SWEENY, R. P.: Equilibrium considerations of finite solute concentrations in gas liquid chromatography. *Anal. Chem.*, 36 (1964) 744–750 — an equation for calculation of K for binary or multi-phase chromatographic systems is derived and discussed.

KRIGE, G. J. AND PRETORIUS, V.: The theory of preparative linear gas chromatography. The basic equation in frontal analysis. *J. Gas Chromatog.*, 2 (1964) 115–120 — theoretical explanations without using or referring to the numerous basic papers by G. SCHAY (Budapest) and his school.

STERNBERG, J. C.: Effect of pressure gradient on chromatographic column efficiency. *Anal. Chem.*, 36 (1964) 921–922 — the number of plates obtainable per time unit under otherwise equal conditions varies inversely with pressure.

2d. General

ASHLEY, Jr., J. W., HILDEBRAND, G. P. AND REILLEY, C. N.: Gas chromatographic response to plug-shaped sample inputs: calculation of plate height from response curve. *Anal. Chem.*, 36 (1964) 1369–1371.

HILDEBRAND, G. P.: Response as a function of sample input profile and the use of combination columns in gas chromatography. *Dissertation Abstr.*, 24 (1964) 3077–3078.

MERRITT, C., Jr., WALSH, J. T., ROBERTSON, D. H. AND MCCARTY, A. I.: Qualitative gas chromatographic analysis by means of retention volume constants — behavior of isomers. *J. Gas Chromatog.*, 2 (1964) 125–127 — the ratio of retention volumina on two stationary phases is the same for the CH_3-group in fatty acid esters series.

RACKOW, B.: Die molekulare Information als Entwicklungsgrad eines technisch-analytischen Verfahrens, nachgewiesen am Gaschromatographen. *Z. Chem.*, 4 (1964) 155.

SCOTT, R. P. V. AND GRANT, D. W.: Measurement of elution peaks in gas–liquid chromatography. *Analyst*, 89 (1964) 179–184 — peak height × peak width at half-peak height is the most precise method.

3. TECHNIQUES I

3a. Detectors

ABEL, K.: An improved gas-tight micro cross-section ionization detector. *Anal. Chem.*, 36 (1964) 954–955 — detailed design and electric scheme of a dual chamber detector.

CAMIN, D. L., KING, R. W. AND SHAWHAN, S. D.: Capillary gas chromatography using micro-volume thermal conductivity detectors. *Anal. Chem.*, 36 (1964) 1175–1178 — detector gives signal for less than 10^{-10} g of solute and is useful for 0.02 in. I.D. capillary columns up to 250°.

GUILLAUME, M. AND WARIN, R.: Radiometric detector of high sensitivity for gas chromatographic eluates. *Bull. Soc. Chim. Belges*, 72 (1963) 686–698 — sensitivity is 1000 d.p.m. for ^{14}C (CH_3OH) and 15,000 d.p.m. for ^{3}H ($C_2H_5C^3H_3$).

GUIOCHON, G.: Étude de la variation du facteur de réponse des catharomètres avec le débit du gaz vecteur. *J. Chromatog.*, 14 (1964) 378–386 — minimum detectable concentration of solute in the carrier gas is the best means of characterising the sensitivity of a detector responding to concentration.

HILL, S. W. AND NEWELL, H. A.: Effect of nitrous oxide and carbon dioxide on the sensitivity of a "small" argon detector for use in gas chromatography. *Nature*, 201 (1964) 1215–1216 — N_2O increases the sensitivity.

JOHNSON, D. E., WEICHLEIN, R. G., MILLER, J. D. AND BURCHFIELD, H. P.: Use of the microcoulometric gas chromatograph in drug metabolism studies. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. Bio-22.

RYHAGE, R.: Use of a mass spectrometer as a detector and analyzer for effluents emerging from high temperature gas liquid chromatography columns. *Anal. Chem.*, 36 (1964) 759–764 — the emerged substance/helium ratio is increased in two coupled molecule separators before entrance in the mass spectrometer and about 10% of ionized matter is scanned at m/e 12 to 500 in 1 to 2 sec.

YAMANE, M.: Photoionization detector for gas chromatography. I. Detection of inorganic gases. *J. Chromatog.*, 14 (1964) 355–367 — design of the detector and its working properties are given; He as carrier gas.

3b. Column performance and filling studies

HALÁSZ, I. AND HORVÁTH, C.: Micro beads coated with a porous thin layer as column packing in gas chromatography. Some properties of graphited carbon black as the stationary phase. *Anal. Chem.*, 36 (1964) 1178–1186 — description of the preparation of porous layer glass beads and discussion of properties of powdered solids.

JONES, Jr., W. C.: Selection of solvents for separation of isomers by gas chromatography. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. A-8.

KARGER, B. L. AND COOKE, W. D.: Effect of column length on resolution under normalized time conditions. *Anal. Chem.*, 36 (1964) 985–991 — short columns can give better resolution in shorter time and at lower temperature than very long columns.

KARGER, B. L. AND COOKE, W. D.: Effect of particle size and average velocity on resolution under normalized time conditions. *Anal. Chem.*, 36 (1964) 991–995 — in case of small k', coarse particles give better resolution than fine particles; operation at a velocity above $u_{opt.}$ gives a better resolution without loss in retention time.

KUNG, J. T. AND RAMAGNOLI, R. J.: Gas liquid chromatographic analysis of high-boiling polar compounds utilizing nichrome helices as the support material. *Anal. Chem.*, 36 (1964) 1161–1162 — compounds with b.p. higher than 200° on SE-30 by PTGC at 70–200°.

LYSYJ, I. AND NEWTON, P. R.: A new technique for the preparation of coated glass-bead column for gas chromatography. *Anal. Chem.*, 36 (1964) 949–950 — a vacuum applied alternately on inlet and outlet of glass-bead column with stationary phase solution.

MORTIMER, J. V. AND GENT, P. L.: The use of organo-clays as gas chromatographic stationary phases. *Anal. Chem.*, 36 (1964) 754–756 — Bentone 34–silicone oil MS-550 (1:1) is examined for separation of C_6–C_9 aromatics at different temperatures (xylenes, dichlorobenzenes, phenols).

TAKEUCHI, T. AND SETSUDA, T.: (Study on the interaction between the analyzed reagents, the liquid phase and the solid support in lightly loaded column gas chromatography). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 1799–1801.

VANDENHEUVEL, W. J. A., GARDINER, W. L. AND HORNING, E. C.: Properties of gas chromatographic column packings prepared with polyvinylpyrrolidone-treated supports. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. A-10 — for sterol analysis.

3c. Apparatus, accessories and materials for GC

BEROZA, M.: Pressure-drop leak detector for gas chromatographs equipped with molecular sieve traps. *J. Gas Chromatog.*, 2 (1964) 138.

FORSS, D. A., BAZINET, M. L. AND SWIFT, S. M.: Sampling devices for gas chromatography. *J. Gas Chromatog.*, 2 (1964) 134–135 — designs are given.

HERBENER, R. E.: A demonstration device for gas chromatography. *J. Chem. Educ.*, 41 (1964) 162.

KARASEK, F. W.: Chromatographic analysis. *U.S. Pat.*, 3,121,321 (Febr. 2, 1964) — two columns, the first of which is purged, while the second serves as analysing column.

LOWRY, R. R.: Solid sample injector for gas liquid chromatography. *Anal. Chem.*, 36 (1964) 1407–1408 — design description, but only a few literature references to this subject of research.

PAGLIS, J. P. AND RITZENTHALER, B. A.: Gas chromatography. *U.S. Pat.*, 3,120,749 (Febr. 11, 1964) — a pressure controller maintains a constant pressure drop across an orifice at the outlet of the column.

ŘÍHA, F.: (Apparatus for following and controlling polymerization processes). *Chem. Prumysl*, 14/39 (1964) 249–252 — a process gas chromatograph and transistored integrator with digital output is described.

STOLL, E. H., METCALFE, L. D., NUDING, N. E., HARTSUCH, G. P. AND SHAPIRO, S. H.: Process gas chromatography of fatty acid distillation streams. II. Design and construction of the process chromatograph. *55th Annual Meeting Am. Oil Chemists' Soc., New Orleans, La., April 19–22, 1964*, Abstr. No. 65; from *J. Am. Oil Chemists' Soc.*, 41, No. 3 (1964) 36 — injection at 300°; thermal conductivity; free fatty acids.

VIRUS, W.: Probegeber und Dosierungssysteme in der Gas-Chromatographie. *Brennstoff-Chem.*, 44, No. 12 (1963) W139 – W141 — construction details are given.

WATSON, J. T. AND BIEMANN, K.: High-resolution mass spectra of compounds emerging from a gas chromatograph. *Anal. Chem.*, 36 (1964) 1135–1137 — detail of all-glass pressure reduction system.

4. TECHNIQUES II

4a. Preparative-scale GC

DENEKAS, M. O., DUNTON, M. L., DANIEL, N. R. AND KOONS, C. B.: Use of preparative gas chromatography, modified F.I.A. apparatus, and a unique chemical reaction for analysis of individual hydrocarbons in crude oil. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. OG-3.

STREIBL, M. AND KONEČNÝ, K.: (Simple apparatus for preparative gas chromatography). *Chem. Listy*, 58 (1964) 580–583.

4c. High-speed GC

AYERS, B. O. AND RAIBLE, C. J.: The attainment of high-speed chromatographic separations and implementation for process control. *147th Am. Chem. Soc. Meeting, Philadelphia, Pa., April 6–10, 1964, Div. Anal. Chem.*, Abstr. No. 7.

4d. Special microtechniques

HOFF, J. E. AND FEIT, E. D.: New technique for functional group analysis in gas chromatography. Syringe reaction. *Anal. Chem.*, 36 (1964) 1002–1008 — 10^{-5}–10^{-8} g of solute per ml of carrier gas is brought into contact with classification reagents in the syringe used to inject the sample into the gas chromatograph.

ROOT, J. W., LEE, E. K. C. AND ROWLAND, F. S.: Isotopic molecules: separation by recycle-gas chromatography. *Science*, 143 (1964) 676–678 — separation was achieved of CH_4–CD_4, *n*-C_4H_{10}–*n*-C_4D_{10}, *cyclo*-C_4H_7T–C_4H_8 by cycling on two columns in series (one detector between them); column fillings: molecular sieve 5 A and safrol.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

KUDRYAVTSEVA, N. A. AND TARASOV, A. I.: (Chromatographic investigation of gaseous hydrocarbons dissolved in petroleum). *Khim. i Tekhnol. Topliv i Masel*, No. 5 (1964) 32–36.

POLLARD, S. A.: Determination of 1-butene-3-yne in C_4 hydrocarbons by gas liquid chromatography. *Anal. Chem.*, 36 (1964) 999–1002 — retention data for 12 C_2–C_4 hydrocarbons on β,β'-oxydipropionitrile and dimethylsulfolane at 40°.

5b. Other hydrocarbons

BARRAL, II, E. M. AND BAUMANN, F.: Gas chromatographic analysis of normal and branched chain hydrocarbons in the range C_7 to C_{20} using molecular sieve. *147th Am. Chem. Soc. Meeting, Philadelphia, Pa., April 6–10, 1964, Div. Anal. Chem.*, Abstr. No. 45.

CARNES, W. J.: Composition of straight chain alkylbenzenes by gas chromatography. *Anal. Chem.*, 36 (1964) 1197–1200 — C_9–C_{14} linear alkylbenzenes on Apiezon L, SE-30 and DC-550 in 150 ft., 0.01 in. I.D. capillary columns; the best liquid is DC-550 at 120–170° by PTGC 1.5°/min.

FREY, H. M.: Thermal unimolecular isomerizations of substituted cyclobutenes. Part 3. 3-Methylcyclobutene. *Trans. Faraday Soc.*, 60 (1964) 83–87 — retention data on β,β'-oxydipropionitrile at 0° and 19°.

GÄUMANN, T.: Strahlungschemie der Kohlenwasserstoffe. 9. Mitt. Benzol. *Helv. Chim. Acta*, 46 (1963) 2873–2885 — on Emulphor-O, DC-710 and SE-30.

HALÁSZ, I., HEINE, E., HORVATH, C. AND STERNAGEL, H. G.: Gaschromatographische Analyse von C_1–C_7 Kohlenwasserstoffgemischen mit "Festschicht" und "gepackten" Kapillarkolonnen. *Brennstoff-Chem.*, 44 (1963) 387–389 — on graphite CK3 and Al_2O_3 of 73 m^2/g.

PETROCELLI, J. A., PUZNIAK, T. J. AND CLARK, R. O.: Process gas chromatographic distillation analyzer. *Anal. Chem.*, 36 (1964) 1008–1011 — PTGC dual column for rational evaluation of Engler distillation analysis data.

PHILIPPE, R. J., MOORE, H., HONEYCUTT, R. G. AND RUTH, J. M.: Some hydrocarbons of the gas phase of cigarette smoke. *Anal. Chem.*, 36 (1964) 859–865 — C_1–C_6 hydrocarbons separated on alumina or alumina coated with β,β'-oxydipropionitrile.

SAUERLAND, H. D.: Eine gaschromatographische Bestimmung von Phenanthren, Anthracen und Carbazol. *Brennstoff-Chem.*, 45 (1964) 55–56 — on $CaCl_2$ by PTGC, up to 1 % of phenanthrene in anthracene and *vice versa*.

VERSINO, B., GEISS, F. AND BARBERO, G.: Verwendung von Bentone-haltigen Trennsäulen für die Gaschromatographie von Polyphenylgemischen. *Z. Anal. Chem.*, 201 (1964) 20–29 — Bentone 34/silicone grease is advocated for analysis of polyphenyls up to quinquaphenyls and dibromodiphenyl and bromoterphenyls at temperatures up to 300°.

5c. Halogen derivatives of hydrocarbons

DE LA MARE, P. B. D., JOHNSON, E. A. AND LOMAS, J. S.: The kinetics and mechanism of aromatic halogen substitution. Part XVII. Chlorination of 9,10-dihydrophenanthrene. *J. Chem. Soc.*, (1963) 5973–5978 — retention data of chlorophenanthrenes on Apiezon M at 197°.

DEWAR, M. J. S. AND FAHEY, R. C.: Electrophilic addition to olefins. III. The stereochemistry of addition of deuterium bromide to 1-phenylpropene. *J. Am. Chem. Soc.*, 85 (1963) 3645–3648 — retention data of stereoisomers on DEGS at 150°.

JACOBS, E. S.: The gas chromatographic determination of halopropane in blood. *Anesthesia Analgesia, Current Res.*, 43 (1964) 177–185 — on DC-710 at 40° after drying on P_2O_5 powder column.

THEYE, R. A.: Chromatographic analysis of expired air containing halothane. *Anesthesiology*, 25 (1964) 75–79.

THOMAS, A. F., PALLUY, E., WILLHALM, B. AND STOLL, M.: La déshydratation du trichlorométhyl-2-butanol-2. *Helv. Chim. Acta*, 46 (1963) 2089–2097.

TOBEY, S. W. AND WEST, R.: Hexachlorocyclopropane. *J. Am. Chem. Soc.*, 86 (1964) 56–61 — retention data on DC-11 and tricresyl phosphate at 155–165°.

WOOTTON, J. C. AND SOURCHENE, W. L.: Toxic factors in fat. A contribution to the knowledge of the structure of two hydropericardium-producing factors from a toxic fat. *J. Agr. Food Chem.*, 12 (1964) 94–98 — retention data of hexachlorohexahydrophenanthrene isomers on silicone grease at 179°.

6. ALCOHOLS

SHELTON, J. R. AND GILDE, H. G.: Reaction of free radicals with olefins. Thermal decomposition of *tert.*-butyl peracetate in the presence of 4-vinylcyclohexene. *J. Org. Chem.*, 29 (1964) 482–485 — retention data of isomeric ethylcyclohexanols on Carbowax at 100°.

SMITH, E. D., JOHNSON, J. L. AND OATHOUT, J. M.: Measurement and use of substrate and partition liquid selectivities in gas chromatography. II. Ethanol–methanol separations. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. A-12.

7. PHENOLS

CROSBY, D. G.: Metabolites of 2,4-dichlorophenoxyacetic acid (2,4-D) in bean plants. *J. Agr. Food Chem.*, 12 (1964) 3–6 — retention data of 2,4-dichlorophenol (and 2,4-D) and derivatives on DC-11 and SE-30 at 175–202°, 215–225° and 255–260°.

FREEDMAN, R. W. AND CROITORU, P. P.: Quantitative gas liquid chromatography of phenols by complete trimethylsilylation of hindered phenols in presence of acidic oxides. *Anal. Chem.*, 36 (1964) 1389–1390 — refluxing with hexamethyldisilazane, TiO_2 as catalyst and Na_2SO_4 as drying agent, at 200°, followed by chromatography on dioctyl sebacate at 125°; capillary column 150 ft., 0.01 in. I.D.

GILL, H. H.: Quantitative analysis of Bisphenol A by gas liquid chromatography. *Anal. Chem.*, 36 (1964) 1201–1203 — on LAC-2R-446 at 240°.

RUDOLFI, T. A., SHCHERDRINA, M. M., LUSHCHIK, V. I. AND LASKINA, E. D.: (Gas–liquid chromatography and infra-red spectra of isomeric allyl guetols and allyl guaiacols). *Zh. Anal. Khim.*, 19 (1964) 619–621 — retention data on methyl phenyl silicone grease PMFS-2 and DEGA at 174°.

SHULGIN, A. T.: Separation and analysis of methylated phenols as their trifluoroacetate ester derivatives. *Anal. Chem.*, 36 (1964) 920–921 — retention data of 20 C_6–C_{11} phenols on silicone fluid 710 and tris-*o*-phenylphenyl phosphate at 75° and 108°.

9. OXO COMPOUNDS

RALLS, J. W.: Higher recoveries of carbonyl compounds in flash exchange gas chromatography of 2,4-dinitrophenylhydrazones. *Anal. Chem.*, 36 (1964) 949 — $NaHCO_3$ in the bottom of the exchange tube increases the recovery.

SAMBASIVARAO, K. AND MCCLUER, R. H.: Lipid components of gangliosides. *J. Lipid Res.*, 5 (1964) 103–108 — retention data of saturated and unsaturated C_{16}–C_{18} aldehydes on LAC-728 and PEGS at 200° and 180°.

10. CARBOHYDRATES

WELLS, W. W., KATAGI, T., BENTLEY, R. AND SWEELEY, C. C.: Gas chromatography of sugar phosphates. *Biochim. Biophys. Acta*, 82 (1964) 408–411.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BILLS, D. B., KHATRI, L. L. AND DAY, E. A.: Method for the determination of the free fatty acids of milk fat. *J. Dairy Sci.*, (1963) 1342–1347 — free fatty acids are concentrated on an anion exchange resin, converted to methyl esters; retention data on LAC-3R-728 at 94° (C_4–C_3) or 194° (C_{10}–C_{18}).

BUTTERY, R. C., LUNDIN, R. E., MCFADDEN, W. H., JAHNSEN, V. J. AND KEALY, M. P.: Volatile hop constituents. Identification of methyl dec-4-enoate and methyl deca-4,8-dienoate. *Chem. Ind. (London)*, (1963) 1981–1982 — isolated by GLC on silicone SF-96 (100), PEGS and Carbowax 20M.

HORNING, E. C., ARRENS, E. H., LIPSKY, S. R., MATTSON, F. H., MEAD, J. F., TURNER, D. A. AND GOLDWATER, W. H.: Quantitative analysis of fatty acids by gas chromatography. *J. Lipid Res.*, 5 (1964) 20–27.

KORYLNYK, W. AND METZLER, E. A.: Composition of lipids from lima beans and certain other beans. *J. Sci. Food Agr.*, 14 (1963) 841–844 — retention data of C_{14}–C_{18} fatty acid on DEGS or silicone at 213°.

KRYGLOV, E. A., VAĬSBERG, K. M. AND ABRAMOVICH, Z. I.: (Investigation of individual composition of synthetic fatty acids from petroleum paraffins). *Khim. i Tekhnol. Topliv i Masel*, No. 5 (1964) 36–38 — on polymethylphenyl siloxane grease at 240°.

KUKSIS, A.: Direct gas chromatographic fractionation of mixed neutral lipids of natural origin. *Can. J. Biochem.*, 42 (1964) 419–430 — on SE-30 at 330–350°.

MAGNE, F. C.: Analysis of cyclopropanoid and cyclopropenoid acids in fats and oils. *55th Annual Meeting Am. Oil Chemists' Soc., New Orleans, La., April 19–22, 1964*, Abstr. No. 20; from *J. Am. Oil Chemists' Soc.*, 41 (1964) 16.

METCALFE, L. D. AND STOLL, E. H.: The process gas chromatography of fatty acid distillation streams. I. Analytical chemistry. *55th Annual Meeting Am. Oil Chemists' Soc., New Orleans, La., April 19–22, 1964*, Abstr. No. 64; from *J. Am. Oil Chemists' Soc.*, 41 (1964) 33 — free fatty acids on H_3PO_4 + polyester column.

OKUI, S., UCHIYAMA, M. AND MIZUGAKI, M.: Metabolism of hydroxy fatty acids. II. Intermediates of the oxidative breakdown of ricinoleic acid by genus *Candida*. *J. Biochem.*, 54 (1963) 536–540 — retention data of 5 hydroxy-fatty acids on DEGS at 200°.

PONS, JR., W. A. AND FRAMPTON, V. L.: Precision and accuracy — gas liquid chromatographic analysis of C_{14}–C_{18} fatty acid methyl esters. *55th Annual Meeting Am. Oil Chemists' Soc., New Orleans, La., April 19–22, 1964*, Abstr. No. 75; from *J. Am. Oil Chemists' Soc.*, 41 (1964) 34 — mass response to thermal conductivity detector decreases with increasing molecular weight in the case of saturated C_{14}–C_{18} esters or with unsaturation in that of C_{18} unsaturated esters.

ROGOZINSKI, M.: A rapid quantitative esterification technique for carboxylic acids. *J. Gas Chromatog.*, 2 (1964) 136–137 — a methanol–sulphuric acid esterification procedure is described.
TAKEUCHI, T. AND SUZUKI, Y.: (Change of the constituents of unsaturated fatty acids in rape-seed oil during oxidation). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 1855–1857 — retention data on PEG and silicone grease.
WOOD, R. D., RAJU, P. K. AND REISER, R.: Gas–liquid chromatography of trimethylsilyl derivatives of monoglycerides and hydroxy-fatty acids. *55th Annual Meeting Am. Oil. Chemists' Soc., New Orleans, La., April 19–22, 1964*, Abstr. No. 76 — C_8–C_{18} fatty acids.

13. STEROIDS

BAILEY, E.: The use of gas–liquid chromatography in the assay of some corticosteroids in urine. *J. Endocrinol.*, 28 (1964) 131–138 — on neopentyl glycol adipate at 230°.
KITTINGER, G. W.: Quantitative gas chromatography of 17-desoxycorticosteroids and other steroids produced by the rat adrenal. *Steroids*, 3 (1964) 21–42 — retention data on SE-30 at 245°.
KUKSIS, A.: Gas chromatographic fractionation of natural steryl ester mixtures. *Can. J. Biochem.*, 42 (1964) 407–417.
TOUCHSTONE, J. C., VARON, H. H. AND MURAWEC, T.: Conjugated and free estriol in *Corpus luteum* of human pregnancy. *Biochemistry*, 3 (1964) 126–129 — oestriols on QF-1 at 230°.

15. TERPENE DERIVATIVES

ANDERSON, J., BOSVIK, R. AND SYDOW, E. VON: The composition of the essential oil of black-currant leaves (*Ribes nigrum* L.). *J. Sci. Food Agr.*, 14 (1963) 834–840 — retention data of 17 terpenes on DC-200 and LAC-446 at 110° and 90°.
HUNTER, G. L. K. AND BRODGEN, Jr., W. B.: A rapid method for isolation and identification of sesquiterpene hydrocarbons in cold-pressed grapefruit oil. *Anal. Chem.*, 36 (1964) 1122–1123 — GC fractions are identified by time-of-flight mass spectrography and I.R. spectrography.
HUNTER, G. L. K. AND BROGDEN, Jr., W. B.: 2,4-*p*-Menthadiene. A new monoterpene from Valencia orange oil. *J. Org. Chem.*, 29 (1964) 498–499 — on Carbowax 20M at 145°.
ICONOMOU, N., VALKANAS, G. AND BÜCHI, J.: Die Anwendung der Gaschromatographie in der Reinheitsprüfung von Arzneistoffen. Mitt. 11. Qualitative und quantitative gaschromatographische Bestimmung der Terpenkohlenwasserstoffe in dem Terpentinöl. *Pharm. Acta Helv.*, 38 (1963) 875–886 — retention data of 15 terpenes on Carbowax 1500 and di-isodecyl phthalate at 100° and 162°.
KOLŠEK, J. AND MATIČIČ, M.: Beitrag zur gaschromatographischen Analyse des Lavendelöls. *J. Chromatog.*, 14 (1964) 331–339 — retention data of 3 terpenes on 8 stationary phases at 110–120°.
KOVÁTS, Sz., E.: Zur Kenntnis ätherischer Öle. 4-Mitt. Zur Kenntnis des sog. "destillierten" Limetten-Öls (*Citrus medica L.*, var. *acida*, Brandis; *Citrus aurantifolia*, Swingle). *Helv. Chim. Acta*, 46 (1963) 2705–2731 — Apiezon L and Emulphor-O.
TAIRA, T.: (Report on the by-products of alcoholic fermentation by gas chromatography. Part III. Occurrence of geraniol and methylcarbinol in sweet potato and cane molasses fusel oil). *J. Agr. Chem. Soc. Japan*, 37 (1963) 630–631.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

CRAIG, J. C., MARY, N. Y. AND ROY, S. K.: Application of gas chromatography to the detection of *tert.*-amine oxides. *Anal. Chem.*, 36 (1964) 1142–1143.
GUTENMANN, W. H. AND LISK, D. J.: Electron affinity residue determination of CIPC, monuron, diuron and linuron by direct hydrolysis and bromination. *J. Agr. Food Chem.*, 12 (1964) 46–48 — retention data of brominated anilines on DC silicone grease (precipitated from ethyl acetate solution) at 200°.
HENBEST, H. B. AND STRATFORD, M. J. W.: Amine oxidation. Part VIII. The reaction of tri-*n*-butylamine with ozone. *J. Chem. Soc.*, (1964) 711–714 — retention data of di- and tributylamine, dibutylformamide, dibutylbutyramide and derivatives on silicone oil at 100° and 140°.
LINDSTEDT, S.: Gas chromatography of adrenalin, noradrenalin and related amines as their trimethylsilyl ethers. *Clin. Chim. Acta*, 9 (1964) 309–310 — retention data of 8 derivatives of QF-1 at 115–125°.
NELSON, D. F. AND KIRK, P. L.: Identification of the pyrolyzates of substituted barbituric acids by gas chromatography. *Anal. Chem.*, 36 (1964) 875–878 — nitriles are shown to be the main characteristic pyrolysis products.
PETROVA, M. P. AND DOLGINA, A. I.: (Analysis of methylamine and ammonia mixtures by a method of gas–liquid partition chromatography). *Zh. Anal. Khim.*, 19 (1964) 239–242.

PRATT, G. L. AND PURNELL, J. H.: Gas-phase reactions of ethyl radicals with nitric oxide. *Trans. Faraday Soc.*, 60 (1964) 371–377 — acetaldoxime and methyl cyanide on PEG-600.

18. AMINO ACIDS

CRUICKSHANK, P. A. AND SHEENAN, J. C.: Gas chromatographic analysis of amino acids as N-trifluoroacetylamino acid methyl esters. *Anal. Chem.*, 36 (1964) 1191–1197 — separation of 21 amino acid derivatives on neopentyl glycol succinate by PTGC from 65–95° (1.5°/min), 95–140° (2°/min), 140–210° (4°/min) and 210° (isothermal) in 2 hours.

SMITH, E. D., RIDDICK, E. B. AND SHEPPARD, H.: Gas chromatographic analysis of amino acid derivatives. II. The methyl esteramines. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. Bio-21.

SMITH, E. D., RIDDICK, E. B. AND SLATEN, B. L.: Gas chromatographic analysis of amino acid derivatives. I. The *n*-trifluoroacetyl methyl esters. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. Bio-20.

26. METALLO-ORGANIC COMPOUNDS

FRANC, J. AND DVOŘÁČEK, J.: Structuranalyse siliciumorganischer Verbindungen mit Hilfe der Gaschromatographie. *J. Chromatog.*, 14 (1964) 340–347 — combined pyrolysis–GC technique.

SNEGOVA, A. D., MARKOV, L. K. AND PONOMARENKO, V. A.: (Use of gas–liquid chromatography for the analysis of halogen-containing organosilicone and organogermanium compounds). *Zh. Anal. Khim.*, 19 (1964) 610–614 — retention data on silicone grease (methyl phenyl) PFMS-4 and fluoro-silicone grease FS at 120–155°.

WURST, M.: Analyse von Organosiliciumverbindungen. III. Trennung und Bestimmung linearer und cyclischer Polydimethylsiloxane mittels Gaschromatographie. *Collection Czech. Chem. Commun.*, 29 (1964) 1458–1465 — retention data and correlations of hexamethyldisiloxane to hexadecamethylheptasiloxane and cyclic analogs on elastomer (Lukopren M) at 150°, 165°, 180° and 195°.

YAMAKAWA, K., TANIKAWA, K. AND ARAKAWA, K.: Organometallic compounds. II. Gas chromatography of metal acetylacetonates. *Chem. Pharm. Bull. (Tokyo)*, 11 (1963) 1405–1408 — retention data on SE-30 and SE-52 at 100–200°.

27. VITAMINS

DUNAGIN, Jr., P. E. AND OLSON, J. A.: Gas liquid chromatography of retinol (vitamin A) derivatives. *Anal. Chem.*, 36 (1964) 756–759 — on low coated SE-30 column at max. 150°.

SCHUDEL, P., MAYER, H., RUEGG, R. AND ISLER, O.: Über die Chemie des Vitamins E. 5. Mitt. Die Synthese von rac. *all-trans*-ζ_1- und ε-Tocopherol. *Helv. Chim. Acta*, 46 (1963) 2517–2526.

YANOTOVSKY, M. T. S.: (Gas–liquid chromatography of the intermediate compounds in the synthesis of vitamin A). *Zh. Anal. Khim.*, 19 (1964) 262–263.

29. INSECTICIDES AND OTHER PESTICIDES

EGAN, H., HAMMOND, E. W. AND THOMSON, J.: The analysis of organo-phosphorus pesticide residue by gas chromatography. *Analyst*, 89 (1964) 175–178.

DE FAUBERT MAUNDER, M. J., EGAN, H. AND ROBURN, J.: Some practical aspects of the determination of chlorinated pesticides by electron-capture gas chromatography. *Analyst*, 89 (1964) 157–167 — a diagram of the electron-capture detector is given.

GOULDEN, R., GOODWIN, E. S. AND DAVIES, L.: Improvement of identification in the gas–liquid chromatographic analysis of agricultural samples for residues of some chlorinated pesticides. I. Improvement of resolution on single columns and application of the multicolumn "spectro-chromatogram". *Analyst*, 88 (1963) 941–950.

GOULDEN, R., GOODWIN, E. S. AND DAVIES, L.: Improvement of identification in the gas–liquid chromatographic analysis of agricultural samples for residues of some chlorinated pesticides. II. A halogen-sensitive detector in complementary or alternative use to an electron-capture ionisation detector. *Analyst*, 88 (1963) 951–958.

KAHN, L. AND WAYMAN, C. H.: Apparatus for continuous extraction of nonpolar compounds from water applied to determination of chlorinated pesticides and intermediates. *Anal. Chem.*, 36 (1964) 1340–1343 — GC analysis after extraction.

RALLS, J. W. AND CORTES, A.: Determination of Sevin in green beans by bromination and electron capture gas chromatography. *J. Gas Chromatog.*, 2 (1964) 132–133 — bromine derivatives of 1-naphthyl N-methylcarbamate on DC-11 at 145° in the p.p.b. range.

31. PLASTICS AND THEIR INTERMEDIATES

CROTEN, B.: Application of pyrolysis–gas chromatography to polymer characterization. *Anal. Chem.*, 36 (1964) 1206–1212 — more than 150 polymers were identified qualitatively, while ester types in cellulosics, and styrene content in SBR vulcanisates were estimated quantitatively.

NORMAN, C. W., WILKINSON, L. B. AND BUETTNER, J. P.: The determination of residual monomers in latex by gas chromatography. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. A-13.

STEVENS, M. P. AND PERCIVAL, D. F.: Gas chromatographic determination of free phenol and free formaldehyde in phenolic resins. *Anal. Chem.*, 36 (1964) 1023–1024 — resin solids are precipitated from the caustic solution and the remaining water solution is analyzed directly for formaldehyde and extracted with ether for determination of phenol.

32. PHARMACEUTICAL APPLICATIONS

NIEDERMAYER, A. O.: Determination of phenylacetic acid in penicillin fermentation media by means of gas chromatography. *Anal. Chem.*, 36 (1964) 938–939 — on DEGA + 2% H_3PO_4 at 200°.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

ABEL, K.: Determination of argon in the presence of oxygen and other atmospheric gases by adsorption chromatography. *Anal. Chem.*, 36 (1964) 953–954 — oxygen is converted on Pt-catalyst to water; the water is then allowed to react with CaC_2 to give acetylene, which is measured gas chromatographically in one single analysis on molecular sieve.

BOURKE, P. J., DAWSON, R. W. AND DENTON, W. H.: Detection of volume parts per million of permanent gases in helium. *J. Chromatog.*, 14 (1964) 387–404 — technique for analysing 0.02 v.p.m. CO_2 to 0.5 v.p.m. H_2 in helium stream.

DRUZHININ, F. G. AND MITYANIN, V. P.: (Automatic analysis of blast-furnace gases by means of gas chromatographic method). *Zavodsk. Lab.*, 30 (1964) 531–533 — automation of CO and H_2 analysis on XT-2 chromatograph with air as carrier gas.

JONES, K. AND HALFORD, P.: Separation and determination of argon and oxygen in high-purity nitrogen streams by gas chromatography. *Nature*, 202 (1964) 1003–1004 — Ar + O_2 on molecular sieve 5 A column and Ar after elimination of O_2 on a parallel molecular sieve 5 A + Pd column; at the same retention times the difference in the peak heights gives the content directly.

LEE, J., SUDWORTH, G. B. AND GIBSON, J.: A gas-chromatographic apparatus for the analysis of mine gases. *Analyst*, 89 (1964) 103–114 — katharometer equipped GC apparatus with transistorised voltage and current stabilisers; errors less than ± 5% rel. at concentrations of 0.1–1.1 vol. % of H_2, CH_4, O_2 and N_2; complete scheme given.

33b. Volatile inorganic compounds

BIGHI, C.: Microdetermination of dithiocarbamates by gas chromatography. *J. Chromatog.*, 14 (1964) 348–354 — CS_2 from acid decomposition was condensed at —196° and chromatographed.

BURSON, K. R. AND TARVER, D. A.: Determination of phosphine in hydrogen. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. In-10 — PH_3 on charcoal with argon as carrier gas.

34. RADIOACTIVE COMPOUNDS

BEZARD, J., BOUCROT, P. AND CLEMENT, G.: Collecte d'esters d'acides gras marqués au tritium et au carbone-14 élués par chromatographie gaz–liquide. *J. Chromatog.*, 14 (1964) 368–377 — the results were the same for both types of labelled fatty acids.

SGOUTAS, D. S. AND KUMMEROW, F. A.: Chemical synthesis of tritium-labeled linoleic acid. *Biochemistry*, 3 (1964) 406–411 — on DEGS at 190°.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

ARAKI, S. AND KATOH, T.: (Analysis of high-boiling air pollutants by gas chromatography). *Japan Analyst*, 12 (1963) 1027–1032 — 0.02 p.p.m. quantities are detected after concentration from 40 l air samples on silicone grease column; furfural, nitrobenzene, phenol, cresols, etc.

ASHIGAKI, A. AND KASHIRO, Y.: (Studies on the synthesis of pyridine from furfural. I. Reaction products obtained by ammonolytic cleavage of tetrahydrofuran derivatives). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 1886–1890 — retention data of N-containing compounds (pyridines, nitriles, etc.) on PEG-6000 at 120°.

DITTMER, D. C.: Reduction of carbonyl group by a model for a coenzyme. *J. Am. Chem. Soc.*, 86 (1964) 91–96 — retention data of chlorinated alcohols and acetones on DC-550 and Carbowax 20M at 150–213°.

DRISCOLL, J. L., MARTIN, H. F. AND GUDZINOWICZ, B. J.: A gas chromatographic method for the quantitative analysis of some urinary metabolites of chlorpromazine. *J. Gas Chromatog.*, 2 (1964) 109–114 — on SE-30 at 270°.

JOHNSTONE, R. A. W. AND QUAN, P. M.: The phytadienes and norphytene, and their relation to some components of cigarette smoke. *J. Chem. Soc.*, (1963) 5706–5713 — retention data of phytadienes and C_{15}–C_{17} fatty acids on polyester-like stationary phases.

LANTOS, C. P., MCNIVEN, N. L., ICHII, S., BEDOUKIAN, P. Z. AND DORFMAN, R. I.: Determination of isocaproic acid and isocaproic aldehyde in incubation media using gas chromatography. *Steroids*, 3 (1964) 43–54 — on terephthalic acid polyester with Carbowax 4000 (1:2 mol) at 132° (acid) and 53° (aldehyde).

LEONARD, R. H., KUBITZ, K. A. AND ROCKWELL, J. N.: Gas chromatographic analysis of rosin for adduct and dimer content. *55th Annual Meeting, Am. Oil Chemists' Soc., New Orleans, La., April 19–22, 1964*, Abstr. No. 63; from *J. Am. Oil Chemists' Soc.*, 41 (1964) 33 — on SE-30 coated on silanised Chromosorb W.

MCCARTY, A. I., WYMAN, H. AND PALMER, J. K.: Gas chromatographic identification of banana fruit volatile. *J. Gas Chromatog.*, 2 (1964) 121–124 — identification of more than 17 compounds (-ols, -als, esters); retention data on Carbowax 4000 and UCON Polar at 65°, 72° and 92°.

RHOADES, J. W. AND MILLAR, J. D.: Gas chromatographic method for the comparative analysis of fruit flavors. *19th Southwest Regional Meeting, Am. Chem. Soc., Houston, Tex., Dec. 1963*, Abstr. No. A-14.

SCHWECKE, W. M. AND NELSON, J. H.: Determination of antioxidants in certain food products and packing materials by gas chromatography. *J. Agr. Food Chem.*, 12 (1964) 86–89 — BHA, BHT and di-BHA on SE-30 at 150°.

SHIBATA, K. AND MATSUDA, S.: (Components of the distillation residue in the manufacturing process of 2-ethylhexanol by aldol condensation). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 66 (1963) 1822–1827 — retention data of corresponding acids and alcohols at 140°, 160° and 180°.

WASZECIAK, P. AND NADEAU, H. G.: Reaction of ethers with acetyl chloride and the identification of products by gas chromatography. *Anal. Chem.*, 36 (1964) 764–767.

J. Chromatog., 16 (1964) 269–284

ERRATA

J. Chromatog., 15 (1964) 119, 120

Legend to Fig. 1,
line 4: "Flow rate = 2 ml/min." should read "Flow rate = 0.2 ml/min."

Legend to Fig. 2,
line 14: "Flow rate = 2 ml/min." should read "Flow rate = 0.2 ml/min."

line 20: "Flow rate = 2 ml/min." should read "Flow rate = 0.2 ml/min."

line 25: "Flow rate = 4 ml/min." should read "Flow rate = 0.4 ml/min."

J. Chromatog., 15 (1964) 530

Table X. The top line of the table heading should read as follows: *Polythionates present* ($S_nO_6^{2-}$)

QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS OF TERNARY MIXTURES OF BENZYL CHLORIDE, BENZAL CHLORIDE AND BENZOTRICHLORIDE

H. G. HARING AND J. KROON
N.V. Chemische Fabriek "Naarden", Naarden-Bussum (The Netherlands)

(Received March 12th, 1964)

INTRODUCTION

Gas–liquid chromatography (GLC) is a very sensitive and selective technique, which enables us (1) to separate components of a mixture and (2) to obtain qualitative or even quantitative data about the relative amounts of the components present in the sample studied.

Since the gas chromatographic literature reveals that no general relationship exists between amount of component injected and peak height or peak area produced, for accurate quantitative analysis by GLC, the instrument used has to be calibrated with predetermined amounts of the compounds to be analysed or with mixtures of known composition. In certain cases though, a simple relationship may be found. For example, in a study of the liquid-phase photochlorination of toluene it has been found that, when using a Perkin Elmer Vapor Fractometer 116 with a thermistor detector and equipped with type C column (silicone oil), peak area ratios of the side-chain chlorinated compounds proved to be numerically equal to mole ratios[1]. However, when platinum wire thermal conductivity cells are used instead of thermistors, the peak area ratios distinctly differ from those of the mole ratios given in the synthetic mixtures.

In this case, for the purpose of routine analysis, calibration curves have been determined, for the conversion of peak area ratios into mole ratios, or weight percentages, of the components in binary mixtures. For the analysis of ternary mixtures, a nomographic method is described using two binary calibration curves.

APPARATUS AND PROCEDURE

The gas chromatographic instrument used was a Perkin-Elmer Fractometer 116, equipped with platinum wire thermal conductivity cells and a column, type "C" (silicone oil). Hydrogen was the carrier gas, flow rate 70 ml/min at a pressure of 0.4 atm., column temperature 160°.

Fig. 1 shows an example of a chromatogram thus obtained. The relative retention times are 1.00 for benzyl chloride, 1.85 for benzal chloride, and 2.72 for benzotrichloride.

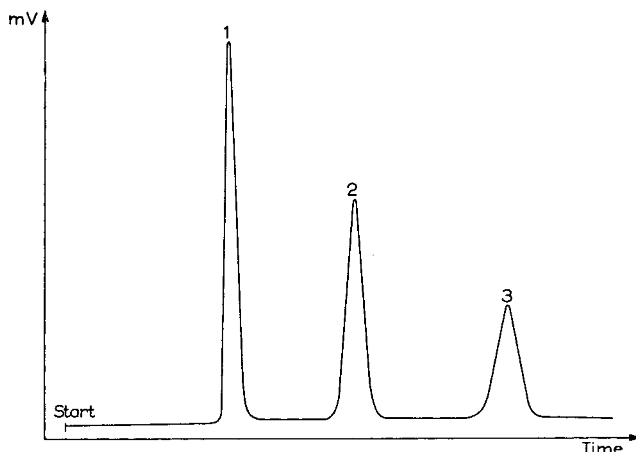


Fig. 1. Gas chromatogram of a mixture of benzyl chloride (1), benzal chloride (2) and benzotrichloride (3).

INSTRUMENT CALIBRATION

The gas chromatograph was calibrated by analysing a series of synthetic mixtures, composed of accurately weighed amounts of the pure components, benzyl chloride, benzal chloride and benzotrichloride.

A series of binary mixtures of benzyl chloride and benzal chloride was first analysed, then a series of mixtures of benzal chloride and benzotrichloride, and finally a series of ternary mixtures obtained by adding known amounts of benzyl chloride to the benzal chloride–benzotrichloride mixtures.

The data obtained with the binary mixtures were used for construction of calibration curves, the results with the ternary mixtures served as an experimental check of the nomographic method for converting peak area percentages into weight percentages (see below).

For checking the reproducibility of the results each sample was analysed immediately after composing it, as well as after a few days, samples having been kept cool and stored in closed bottles.

Peak area determination

Several methods for determination of the peak area (see *e.g.* ref. 2) are known. In our case, the most accurate and least subjective method proved to be the calculation of the peak height times the peak width at half height. A method consisting of dividing the peak area into a number of triangles, the areas of which were subsequently summed, proved to be particularly tedious and subjective (differences of 8 % between various analysts occurring).

RESULTS AND DISCUSSION

Calibration curves for binary mixtures

Table I summarizes the data obtained from the two series of binary mixtures used for calibration, where it can be seen that the peak area percentages found are approxi-

TABLE I

ANALYSIS OF BINARY MIXTURES

Benzyl chloride/Benzal chloride			*Benzal chloride/Benzotrichloride*		
Wt. % intake	*Mole % calcd.*	*Peak area % found*	*Wt. % intake*	*Mole % calcd.*	*Peak area % found*
0.0*	0.0	0.0	0.0**	0.0	0.0
0.88	1.14	0.9	8.0	9.6	8.4
1.96	2.50	1.8	20.1	23.6	21.7
4.71	6.0	4.6	35.0	39.6	38.0
9.10	11.4	9.8	50.2	55.4	53.3
12.1	15.1	11.2	69.9	73.9	73.6
30.0	35.6	33.2	90.1	91.7	92.7
50.4	56.6	55.8	92.5	93.7	94.5
53.2	59.4	59.4	95.0	95.9	96.7
70.6	75.5	75.1	97.5	97.9	98.5
89.6	91.7	92.5			
89.9	92.0	91.7			
95.1	96.0	97.4			
98.0	98.4	98.9			
98.1	98.5	98.9			

* 100% Benzal chloride.
** 100% Benzotrichloride.

mately equal to the calculated mole percentages, the largest deviation being 4%! More accurate results can be obtained, however, by using *calibration curves* as shown in Figs. 2 and 3. These curves were constructed by plotting percentages of total peak area against weight percentages given, for benzyl chloride in mixtures with benzal chloride (Fig. 2) and for benzal chloride in mixtures with benzotrichloride (Fig. 3).

Peak area percentages found when analysing binary mixtures of unknown composition can thus be readily converted into weight percentages.

Reproducibility of the data

Table II shows the data obtained when analysing samples in duplicate, with a time interval of a few days. The reproducibility of the peak area percentages proves to be very good: a mean deviation of only 0.4–0.5 % is shown to occur.

The total peak area is less reproducible, however; the standard deviation amounts to about 2.5 %. This means that an accurate quantitative analysis cannot be based on the determination of a single peak area in comparison with the peak area of the pure component in a calibration chromatogram.

Analysis of ternary mixtures

In the case of ternary mixtures, the relationship between peak area percentage and corresponding weight percentage cannot be obtained from a simple two-dimensional calibration graph, but must be represented by a three-dimensional diagram. This diagram will consist of a right triangular prism with ternary composition diagrams as base and top surfaces, and binary plots of peak area percentages *versus* weight percentages as side surfaces.

This space model would not, however, be suitable for analytical purposes, but it can be "unfolded" into a simplified nomograph shown in Fig. 4.

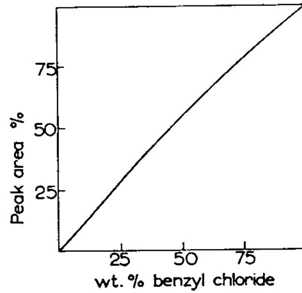


Fig. 2. Calibration graph for converting percentages of total peak area into weight percentages of the components of binary mixtures. Benzyl chloride with benzal chloride.

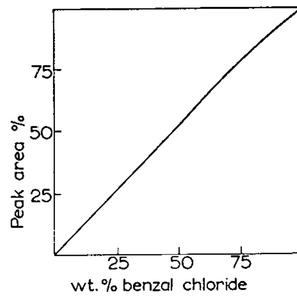


Fig. 3. Calibration graph for converting percentages of total peak area into weight percentages of the components of binary mixtures. Benzal chloride with benzotrichloride.

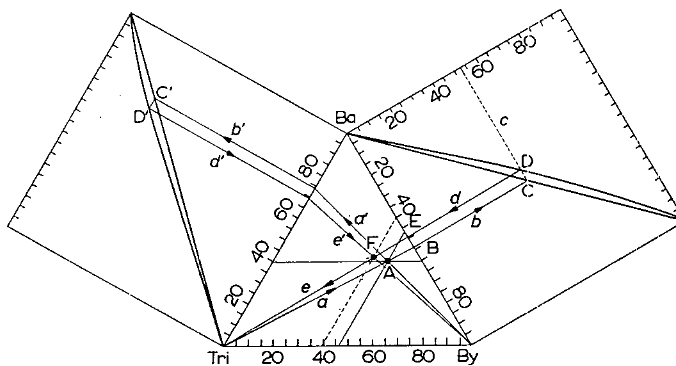


Fig. 4. Nomograph for converting percentages of total peak area into weight percentages of the components of ternary mixtures.

TABLE II

COMPARISON OF DUPLICATE ANALYSES

Benzyl chloride/Benzal chloride			*Benzal chloride/Benzotrichloride*		
Wt. % intake	*% Peak area*	*Total peak area*	*Wt. % intake*	*% Peak area*	*Total peak area*
			8.0	7.9	671
0.88	0.92	783		8.9	683
	0.85	761	20.1	21.2	669
4.71	4.6	799		22.2	668
	4.6	772	35.0	37.2	657
9.1	9.6	792		38.5	658
	10.0	771	50.2	54.8	678
30.0	33.2	763		54.8	671
	33.2	771	69.9	73.8	688
50.4	56.4	768		73.4	696
	55.4	751	90.1	92.8	674
70.6	75.9	389*		92.7	704
	74.3	379	92.5	94.4	686
89.9	91.5	393		94.6	689
	91.9	368	95.0	96.9	757
95.1	97.5	380		96.4	698
	97.4	372	97.5	98.5	725
				98.5	708
Mean-deviation	0.4	2.1 %		0.5	2.5 %

* At reduced sensitivity.

The nomograph consists of a triangular composition diagram and two binary calibration graphs, by which means the ternary system can be split up into two binary systems. A graphical artifice is furthermore introduced by drawing the diagonal in the binary calibration graphs, which enables one to use the triangular diagram for plotting both peak area percentages and weight percentages on the same axis.

As an example, a peak area percentage of 53.5 on the ordinate of the right-hand calibration graph of Fig. 4 may—via line *c*, point C and line *b*—now be represented by a point B = 53.5 % on the absciss of the graph. The weight percentage corresponding to the 53.5 % of total peak area mentioned, represented by point D according to the calibration graph, then can be read—via line *d*— from the same absciss, in point E = 48.2 wt %.

Using the nomograph

An example of how to convert the peak area percentages into weight percentages of the components of the ternary mixture analysed is given below; one proceeds as follows:

1. Let the peak area percentages be: 46.1 % for benzyl chloride (By), 40.0 % for benzal chloride (Ba) and 13.9 % for benzotrichloride (Tri). This "composition" will then be represented by point A in the triangular diagram of Fig. 4.

2. Now draw a line *a* from angular point Tri through A till it intersects side By–Ba in B. Line *a* represents the locus of all "compositions" having the same By/Ba peak area ratio, irrespective of the percentage of Tri; point of intersection B gives a

measure for this ratio, *viz.* 53.5 % By to 46.5 % Ba. This result may be checked arithmetically as follows:

$$\frac{46.1}{46.1 + 40.0} \cdot 100\,\% = 53.5\,\% \text{ and } \frac{40.0}{46.1 + 40.0} \cdot 100\,\% = 46.5\,\%.$$

3. When normal graph paper has been used for the square diagrams the next step is to follow the millimeter division 53.5, line *b*, till it intersects the diagonal in point C.

4. Draw a line *c* from point C, parallel to the absciss By–Ba; line *c* intersects the calibration curve in D.

5. Then follow line *d*, parallel to *b*, till it intersects the absciss By–Ba in point E = 48.2. Point of intersection E now presents a measure for the weight percentage ratio of By and Ba, irrespective of the percentage of Tri; this ratio is found here to be 48.2 wt % By to 51.8 wt % Ba.

6. Finally draw line *e* from point E to angular point Tri; line *e* will be the locus of all ternary compositions having the By/Ba ratio mentioned. Somewhere on this locus *e* the composition required will be situated.

7. In order to find the unknown point on line *e*, a *second locus e'* may now be constructed by repeating the above procedure using the binary calibration graph Ba–Tri; locus *e'* will represent weight percentage compositions having a Ba/Tri ratio corresponding to the initially given ternary "peak area composition" A.

The construction is presented in Fig. 4 by lines *a'*, *b'*, *c'*, *d'*, and *e'*:

Locus *a'* represents peak area compositions having a Ba/Tri ratio of:

$$\frac{40.0}{40.0 + 13.9} \cdot 100\,\% = 74.2\,\% \text{ Ba to } \frac{13.9}{40.0 + 13.9} \cdot 100\,\% = 25.8\,\% \text{ Tri.}$$

After conversion into weight percentages locus *e'* represents sample compositions given by point E', corresponding to a Ba/Tri ratio of 70.0 wt % Ba to 30.0 wt % Tri.

8. The point of intersection of the loci *e* and *e'*, point F, finally represents the ternary composition of the sample analysed, *viz.* 39.4 % By, 42.3 % Ba and 18.3 % Tri.

This result may be arithmetically checked as follows: From the calibration graphs have been derived locus *e*:

By:Ba = 48.2:51.8, and locus *e'*:

Ba:Tri = 70.0:30.0.

Hence: By:Ba:Tri = 48.2:51.8:(30.0·51.8/70.0) = 48.2:51.8:22.2.

By reducing these values, the sum of which is 122.2, to a total of 100 % (*i.e.* dividing each by 1.222) the weight percentage composition of the mixture analysed is found: By:Ba:Tri = 39.4 %:42.3 %:18.3 %.

Experimental check of the method

Table III presents the data for a series of synthetic ternary mixtures of benzyl chloride (By), benzal chloride (Ba) and benzotrichloride (Tri). These mixtures have been analysed by GLC using the above mentioned technique for converting percentages of total peak area into weight percentages.

TABLE III

ANALYSIS OF A NUMBER OF TERNARY MIXTURES OF PREDETERMINED COMPOSITION, FOR CHECKING THE METHOD

No.	*Wt. % by intake*			*% of total peak area*			*Wt. % found*		
	By	*Ba*	*Tri*	*By*	*Ba*	*Tri*	*By*	*Ba*	*Tri*
1	17.9	80.0	2.1	20.0	78.3	1.7	18.7	79.6	1.7
2	16.6	79.2	4.2	17.2	80.3	2.5	16.9	79.3	3.8
3	16.6	77.1	6.3	19.0	76.5	4.5	17.5	76.4	6.1
4	16.6	75.2	8.2	19.3	74.2	6.5	17.8	73.3	8.9
5	16.6	6.7	76.7	21.3	5.9	72.8	17.0	5.8	77.2
6	16.7	58.2	25.1	19.4	61.0	19.6	16.8	59.5	23.7
7	16.7	41.6	41.7	19.8	44.5	35.7	17.0	43.0	40.0
8	16.7	29.1	54.2	20.6	36.1	48.3	16.6	30.8	52.6
9	16.7	16.8	66.5	20.5	17.1	62.4	16.7	16.7	66.6
10	41.0	57.5	1.5	44.6	54.9	0.5	40.1	58.9	1.0
11	40.0	57.0	3.0	43.8	54.8	1.4	39.7	57.2	3.1
12	40.0	55.5	4.5	43.5	54.1	2.4	38.5	56.9	4.6
13	40.0	54.1	5.9	45.2	51.0	3.8	40.1	54.5	5.4
14	40.1	4.8	55.1	46.5	4.5	49.0	40.0	4.8	55.2
15	40.2	41.8	18.0	44.6	41.0	14.4	39.0	42.5	18.5
16	40.1	30.0	29.9	45.8	29.5	24.7	39.4	31.0	29.6
17	40.0	21.0	39.0	48.2	20.4	31.4	40.2	21.2	38.6
18	40.0	12.1	47.9	47.4	11.0	41.6	41.3	12.3	46.4
	Mean deviation in %						0.6	0.8	0.6
	Maximum deviation in %						1.5	1.9	1.7

The mixtures have all been analysed in duplicate, which showed a very good reproducibility: the mean difference between duplicate analyses proved to be less than 1 %. For the sake of simplicity in Table III values are mentioned for one series only.

A comparison of the weight percentages taken with the weight percentages found by analysis shows that the mean deviation is less than 1 %, with a maximum deviation of less than 2 %. This shows the nomographic method to be distinctly more accurate than the original approximation method which puts peak area percentages equal to mole percentages.

Note. The nomographic method described above is particularly suitable for routine analysis of large numbers of samples. In the case of a very limited number of analyses it may be advantageous to use only the arithmetical check method in combination with the two binary calibration graphs.

SUMMARY

A nomographic method is described for the quantitative gas chromatographic analysis of ternary mixtures of benzyl chloride, benzal chloride and benzotrichloride. The nomograph converts percentages of total peak area on the gas chromatogram into

weight percentages of the components of the sample. The nomograph is constructed by combining a triangular composition diagram with two binary calibration graphs. The latter graphs have been experimentally determined by gas chromatographic analysis of two series of synthetic binary mixtures. An experimental check shows the method to be accurate to within 1–2 wt. %.

REFERENCES

1 H. G. HARING AND H. W. KNOL, *Chem. Process. Eng.*, 45 (1964).
2 J. F. K. HUBER, *Chem. Weekblad*, 59 (1963) 445.

GAS CHROMATOGRAPHIC SEPARATION OF BROMO- AND CHLOROPYRIDINES

C. A. LANDHEER
Laboratory of Organic Chemistry of the Agricultural University, Wageningen (The Netherlands)

(Received March 19th, 1964)

In the course of investigations, carried out in this laboratory on the bromination and mercuration of pyridine and pyridine-N-oxide[1-4], rather complicated reaction products were obtained, which for analysis were converted into mixtures of the corresponding bromopyridines. An attempt was made to separate these substances quantitatively by gas-liquid chromatography (GLC). In this paper a description is given of a suitable method for the chromatographic determination of the compounds mentioned.

At first, only a column (A) was used at 165° with a filling of Chromosorb W coated with tritolyl phosphate (weight ratio 100:20), the carrier gas being hydrogen. With this column it was possible to separate all mono- and dibromopyridines, except 3-bromopyridine and 4-bromopyridine, which have the same retention volumes.

Therefore we changed to a procedure based on the fact that 4-bromopyridine polymerizes to non-volatile pyridyl pyridinium bromide when heated. Mixtures containing both bromopyridines were chromatographed before and after keeping them for 3 h at 100–110°. Since in the first analysis both 3- and 4-bromopyridine were evolved from the column and during the second one only 3-bromopyridine emerged the amounts of both isomers could be estimated. Results were not completely satisfactory, however, because 4-bromopyridine partly reacted with 3-bromopyridine when heated, yielding 4-pyridyl 3-bromopyridinium bromide. Therefore, we investigated whether other stationary phases would realize a direct separation of 3-bromo- and 4-bromopyridine. Experiments were carried out with Apiezon L, polyethylene-glycol, diglycerol, phenanthrene, polyglycol glutarate and Tide. Whereas Apiezon, diglycerol and phenanthrene effected no separation of 4-bromo and 3-bromopyridine at all, polyethylene glycol and polyglycol glutarate showed separation factors of 1.04 and 1.08 respectively, indicating a possible separation on high efficiency columns. The best result, however, was obtained with the commercial detergent Tide.

Tide is the trademark for a special all-purpose detergent made by Procter and Gamble (U.S.A.). Its composition according to DECORA AND DINNEEN[5] is: lauryl sulphate (sodium salt, 12 %), alkyl arylsulphonate (sodium salt, 5 %), sodium sulphate (15 %), higher molecular phosphates (45 %), silicates (9 %), lauryl alcohol (1.5 %), water (11 %), carboxymethyl-cellulose (sodium salt, 1 %), optical dyes.

The use of Tide in GLC was introduced by GOHLKE AND MCLAFFERTY[6]. In 1959, DESTY AND HARBOURN[7] gave details for its use as a packing in general purpose columns. DECORA AND DINNEEN[5,8] extracted Tide with petroleum ether and used the porous

residue as a stationary phase support. They studied the behaviour of 10 stationary phases in combination with this support for the separation of a mixture of 14 pyridine homologues. Tide as such was used by PORCARO AND JOHNSTON[9] for the separation of the isomeric amyl alcohols and by MATTHEWS *et al.*[10] in large scale columns for the separation of C_8-aldehydes. SANDLER AND STORM[11] extracted the surface active agent of Tide with petroleum ether and impregnated kieselguhr with the Tide extracts for the determination of formaldehyde.

We found that a column filled with Chromosorb impregnated with a petroleum ether extract of Tide, achieved complete separation of 3- and 4-bromopyridine at 100°, 3-bromopyridine again being eluted first. The peak of 4-bromopyridine was very asymmetric with a sharp front and tailed. Usually this is an indication of adsorption of the compound in question by the column material. It was confirmed that adsorption occurs in this case, by the fact that the retention time of 4-bromopyridine decreases with increasing amounts of sample. The separation factor of 4- and 3-bromopyridine varied from 1.1 to 1.2.

The tailing of the 4-bromopyridine peak also interferes with the separation of this compound from 2-bromopyridine, which elutes after the 4-isomer. We succeeded in suppressing the undesirable tailing of the 4-bromopyridine by coating the Chromosorb with soda before impregnating it with Tide extract according to the procedure of GOLDING AND TOWNSEND[12] for the separation of mono-, di- and trimethylpyridines. Table I shows the separation factors obtained when using the column filled with this stationary phase (B).

TABLE I

SEPARATION FACTORS FOR BROMOPYRIDINES

Column B: length 200 cm, diameter 0.4 cm; filling 9.2 g of a mixture of Chromosorb W, soda and Tide extract (weight ratio = 100:6.3:21.3).

Separation factors	*100°*	*80°*
R_v 4-bromopyridine/R_v 3-bromopyridine	1.15	1.17
R_v 2-bromopyridine/R_v 4-bromopyridine	1.11	1.05–1.10
R_v (= retention volume) of 3-bromopyridine	1010 ml	2090 ml

The separation of 2-bromo and 4-bromopyridine was still incomplete, however, due to some residual tailing of the 4-bromopyridine peak. An attempt was made to increase the separation factor of 2-bromo- and 4-bromopyridine by using a short pre-column (length 30 cm, diameter 0.4 cm; filling: 1.6 g of Chromosorb coated with soda and Polywachs 2000 (weight ratio = 100:6:20)). Now the separation of these isomers was good (factor = 1.52), but that of 4- and 3- bromopyridine was incomplete again (separation factor 1.07 at 100°; R_v 3-bromopyridine = 1380 ml). A better result was obtained when the pre-column (C) was filled with 1.5 g of a mixture of Chromosorb, soda, Tide extract and Polywachs 2000 (weight ratio = 78.3:5.0; 15.2:1.5). Table II shows the separation factors obtained with columns B + C and their dependence on the column temperature.

With increasing temperature the 4-bromopyridine peak tends to shift towards the 3-bromopyridine peak. This temperature effect is another indication that 4-bromopyridine is much more strongly adsorbed by the column filling than 3-bromo-

TABLE II

SEPARATION FACTORS FOR BROMOPYRIDINES
Columns B + C

Separation factors	*100°*	*90°*	*80°*
R_v 4-bromopyridine/R_v 3-bromopyridine	1.12	1.15	1.17[5]
R_v 2-bromopyridine/R_v 4-bromopyridine	1.18	1.18	1.17
R_v of 3-bromopyridine	1250 ml	1710 ml	2500 ml

pyridine, adsorption occurring to a lesser degree at higher temperatures. As well as the temperature effect, the asymmetric form of the 4-bromopyridine peak on column (A) and the large shift of the peak top to smaller retention volumes with increasing sample amounts, there is the effect of the soda which is known to suppress adsorption, on the peak form of 4-bromopyridine. Thus it may be concluded that some kind of selective adsorption must be responsible for the separation of 3-bromo- and 4-bromopyridine when Tide extract is used as stationary phase.

According to DECORA AND DINNEEN[5], the sodium salts of lauryl sulphate and of a dodecyl phenylsulphonate are the chief organic components of Tide. In order to establish a stationary phase more precisely which allows the separation of 3- and 4-bromopyridine, four columns containing the following fillings were tried:

1. Chromosorb W (83 %), lauryl sulphate (sodium salt, 17 %).
2. Chromosorb W (83 %), dodecyl phenylsulphonate (sodium salt, 17 %).
3. Chromosorb W (83 %), lauryl sulphate (sodium salt 12 %).
4. Inorganic residue of the Tide extraction (85 %), lauryl sulphate (sodium salt, 15 %) and dodecyl phenylsulphonate (sodium salt, 5 %).

None of these columns gave a separation of 3- and 4-bromopyridine as effected by the Tide extract, possibly due to the fact that the structures of the surface active agents used in our experiment differ from those of the Tide components. Thus, for the quantitative analysis of mixtures of bromopyridines, application of the Tide-column B + column C must be recommended, together with a second analysis using the tritolyl phosphate column (A), which enables a better separation of the dibromopyridines.

The chromatographic analysis of mixtures of monochloropyridines has also been studied. Analogous phenomena were encountered here. The columns with the Tide extract filling again gave the best separation of the three isomers, 4-chloropyridine showing a less symmetric peak and eluting between the 3- and 2-isomers. Also the temperature effects, though somewhat smaller, corresponded with those observed in the experiments with the monobromopyridines.

EXPERIMENTAL

A Becker gas chromatograph (Delft, the Netherlands) with a katharometer as detector was used.

Preparation of Tide extract

The detergent was extracted in a Soxhlet apparatus (after drying at 120°) with

ห้องสมุด กรมวิทยาศาสตร์

petroleum ether (boiling range: 60–80°). The petroleum ether solution was evaporated to dryness on a steam bath, whereupon the residue was heated for some hours at 120°, yielding a semi solid.

Impregnation of Chromosorb with soda

30 g of Chromosorb was allowed to stand in 150 ml of a 13.5 % aqueous solution of soda crystals ($Na_2CO_3 \cdot 10H_2O$) for some hours. Then the liquid was sucked off and the solid matter dried at 120° to constant weight (31.9 g), 6 % soda being left on the Chromosorb.

Impregnation of Chromosorb with the stationary phase

Amounts of the stationary phases, usually dissolved in chloroform, were added to the Chromosorb in the desired ratios. The slurries were evaporated to dryness on a steam bath with gentle stirring with a feather, whereupon the powders obtained were dried at 120° and screened to 60–100 mesh.

Columns

These are copper tubes, internal diameter 0.4 cm.

Column A. Length: 200 cm; filling: 8.0 g of Chromosorb + tritolyl phosphate (weight ratio 100:20).

Column B. Length: 200 cm; filling: 9.2 g of a mixture of Chromosorb W, soda and "Tide" extract (weight ratio 100:6.3:21.3).

Column C. Length: 30 cm; filling: 1.5 g of a mixture of Chromosorb W, soda, "Tide" extract and Polywachs 2000 (weight ratio 100:6.3:19.3:1.93).

Carrier gas

Hydrogen was used, the flow rate varying from 60 to 120 ml/min.

The retention volumes given in the text are all uncorrected.

ACKNOWLEDGEMENT

I am indebted to Prof. Dr. H. J. DEN HERTOG, Director of this Laboratory for reading the manuscript.

SUMMARY

A method is described for quantitative analysis of mixtures of mono- and dibromopyridines or mono- and dichloropyridines by gas chromatography.

The procedure consists of two subsequent chromatographic separations. In the first one a column containing tritolyl phosphate on Chromosorb is used; it enables the separation of all components except the 3- and 4-halogenopyridines which have the same retention volume. A second separation is carried out with a column containing a filling developed on the basis of an extract of the commercial detergent Tide, which gives an excellent analysis of the monohalogeno pyridines.

REFERENCES

1 M. VAN AMMERS AND H. J. DEN HERTOG, *Rec. Trav. Chim.*, 81 (1962) 124.
2 H. J. DEN HERTOG, L. V.D. DOES AND C. A. LANDHEER, *Rec. Trav. Chim.*, 81 (1962) 864.

[3] H. C. VAN DER PLAS, H. J. DEN HERTOG, M. VAN AMMERS AND B. HAASE, *Tetrahedon Letters*, No. 1 (1961) 32.
[4] M. VAN AMMERS, H. J. DEN HERTOG AND B. HAASE, *Tetrahedron*, 18 (1962) 227.
[5] A. W. DECORA AND G. J. DINNEEN, *Anal. Chem.*, 32 (1960) 164.
[6] R. S. GOHLKE AND F. W. MCLAFFERTY, *Communication at the 129th Meeting Am. Chem. Soc., Dallas, Texas, April 1955*.
[7] D. H. DESTY AND C. L. A. HARBOURN, *Anal. Chem.*, 31 (1959) 1965.
[8] A. W. DECORA AND G. J. DINNEEN, *ISA Proc. Anal. Instr. Div. 2nd Intern. Gas Chrom. Symp., Lansing, Mich., June 1959*, preprints, Vol. 2, p. 12.
[9] P. J. PORCARO AND V. D. JOHNSTON, *Anal. Chem.*, 33 (1961) 361.
[10] J. S. MATTHEWS, F. H. BUROW AND R. E. SNYDER, *Anal. Chem.*, 32 (1960) 691.
[11] S. SANDLER AND R. STORM, *Anal. Chem.*, 32 (1960) 1890.
[12] W. E. GOLDING AND C. A. TOWNSEND, *Chem. Ind. (London)*, 48 (1960) 1476.

FLAME IONIZATION DETECTOR RESPONSE FOR THE CARBONYL CARBON ATOM IN THE CARBOXYL GROUP OF FATTY ACIDS AND ESTERS

R. G. ACKMAN AND J. C. SIPOS
Fisheries Research Board of Canada, Technological Research Laboratory, Halifax, Nova Scotia (Canada)

(Received February 14th, 1964)

INTRODUCTION

On the basis of observations that carbonyl carbon atoms, including those in a number of esters of aliphatic acids, gave no effective response in flame ionization detectors[1,2], it has been assumed that the carboxyl carbon atoms in the fatty acids themselves may not make an effective contribution to molecular response in this type of gas chromatography detector. Since quantitative analysis of fatty acid mixtures has been difficult it is only recently that this assumption has been questioned by KABOT AND ETTRE[3]. The experimental conditions of these authors involved the use of inert supports to prevent adsorption of acids in packed columns, or of Golay columns.

The present study compares the data of KABOT AND ETTRE[3] with other data (ACKMAN AND BURGHER[4]) obtained by a different technique involving addition of formic acid vapor to the carrier gas to suppress adsorption on columns prepared with conventional supports, and extends the application of the latter technique to some higher acids. The conclusions reached indicate that in the higher fatty acids the carboxyl carbonyl carbon atom may give up to a full carbon atom response on a relative weight basis. A limited study of the molar responses for various esters of fatty acids indicates that the deficiency in response for the lower esters, excepting formates, is 1.5 carbon atoms, and for the higher esters falls to 1 carbon atom. Formate esters of the lower alcohols apparently do not have as great a deficiency in molar response.

EXPERIMENTAL

In acid studies the Wilkens Hi-FY type flame ionization detector, oven, recorder and integrator were those previously described[4]. The column primarily employed was of stainless steel tubing, 6 ft. in length and $^1/_8$ in. O.D., packed with 5 % neopentyl glycol adipate polyester on 60–80 mesh Gas-Pack F (a Teflon impregnated support obtained from Chemical Research Services, Inc., Addison, Ill., U.S.A.). Operating conditions for valeric and higher acids were: column temperature 180°, injection port temperature 250°, carrier gas (helium) at 10 p.s.i., with air and hydrogen constant at normal flow rates for this detector. Analyses of certain lower acids were carried out under similar conditions at a column temperature of 140°. Formic acid vapor was added to the carrier gas in these studies.

Analyses of esters were carried out with the same column at appropriate column temperatures and carrier gas flow rates, and also on a column of stainless steel tubing, 8 ft. in length and $^1/_8$ in. O.D., packed with 10 % SE-30 silicone polymer on Gas-Pack F support. Other studies were carried out with a Barber-Colman model 10, fitted with a model 5121 flame ionization detector. Columns were of glass, 6 ft. in length and $^1/_8$ in. I.D., packed with either 15.5 % EGSS-Y organosilicone polyester (Applied Science Laboratories, State College, Pa., U.S.A.) on 100–120 mesh Gas-Chrom P support, or 5 % SE-30 on 70–80 mesh Anakrom ABS support. Appropriate operating conditions for the higher esters were: column temperature 100°, injection port temperature 280°, carrier gas (argon) at 4 to 8 p.s.i. For lower esters and hydrocarbons the respective temperatures were 60° and 120°, with carrier gas at 2 to 4 p.s.i. Air and hydrogen flow rates were constant in all experiments and normal for this detector. A Minneapolis-Honeywell 5 mV recorder fitted with a Disc Instruments, Inc., ball and disc integrator was used with this apparatus.

All injections were performed with Hamilton No. 7001-N microsyringes. Samples for the Wilkens apparatus were normally run as 10 % solutions in carbon disulphide, the syringe being rinsed and partly filled with 0.0003 ml of solvent, then with approximately 0.0003 ml of solution, and the plunger withdrawn a further 0.0001 ml before puncture of the injection septum. This procedure gave better reproducibility than straightforward injection of solutions of sample. Samples for the Barber-Colman apparatus were injected, on occasion, by a similar procedure using carbon disulphide or neohexane as a solvent where appropriate, but in cases of more volatile materials injection of the sample mixture could be effected by normal procedure, since this detector is less susceptible to overload. All analyses carried out on the two machines were in satisfactory agreement. Sample mixtures were in all cases of two materials only, with proportions such that both peaks gave nearly full scale response. No attenuation changes were made during analyses.

The fatty acids employed were prepared in this laboratory, as were the higher esters, with purities exceeding 99%. The hydrocarbons and lower esters employed were reagent grade and gave only one significant peak on gas chromatography.

RESULTS AND DISCUSSION

Acids

The previous data of ACKMAN AND BURGHER[4], and of KABOT AND ETTRE[3] (Table I, literature columns) were converted so that the highest common acid (valeric) was taken as 100 response units, with other acids in proportion for equal weights. These data were plotted in a fashion similar to that of Fig. 1, as relative response against the weight percent "active" carbon atoms. This was initially done on the assumption that the carboxyl group made no contribution to response in the flame ionization detector. The experimental curve started above the theoretical line and then fell below it, indicating that the assumption that the carboxyl group gave no response must be incorrect. Accordingly the data were recalculated on the basis that for the higher fatty acids the carboxyl group might give a full response. In this case, as indicated in Fig. 1, both sets of data approached the theoretical line satisfactorily.

It was considered desirable to establish the minimum chain length at which the deficiency in contribution of the carboxyl group carbonyl carbon atom becomes significant and the molar responses for valeric and caproic acids, relative to pelargonic

TABLE I

AVERAGE RELATIVE RESPONSES FOR EQUAL WEIGHTS OF FATTY ACIDS

Fatty acid	*Ackman and Burgher*[4]			*Kabot and Ettre*[3]				
	Literature[a]	*Relative to*		*Literature*		*Relative to*		
		$C_5 = 100$	$C_{10} = 119$			$C_5 = 100$	$C_{12} = 122$	
	(c)	*(c)*	*(c)*	*(c)*	*(d)*	*(c)*	*(c)*	*(d)*
Acetic (C_2)	47	48	46	48	—	53	52	—
Propionic (C_3)	74	75	73	69	63	77	75	69
Butyric (C_4)	90	91	88	86	85	96	94	93
Isobutyric (C_4)	—	89[b]	84[b]	—	—	—	—	—
Valeric (C_5)	99	100	97	90	92	100	98	100
Valeric (C_5)	—	100[b]	95[b]	—	—	—	—	—
Isovaleric (C_5)	100	101	98	—	—	—	—	—
Caproic (C_6)	105	106	103	—	—	—	—	—
Caproic (C_6)	—	109[b]	103[b]	—	—	—	—	—
Enanthic (C_7)	—	—	—	100	100	111	109	109
Caprylic (C_8)	—	—	—	104	—	116	113	—
Pelargonic (C_9)	—	123[b]	116[b]	—	—	—	—	—
Capric (C_{10})	—	126[b]	119[b]	?	—	?	?	—
Lauric (C_{12})	—	—	—	112	—	125	122	—

a Average of Tween and NPGA results.
b Present study.
c Packed column.
d Golay column.

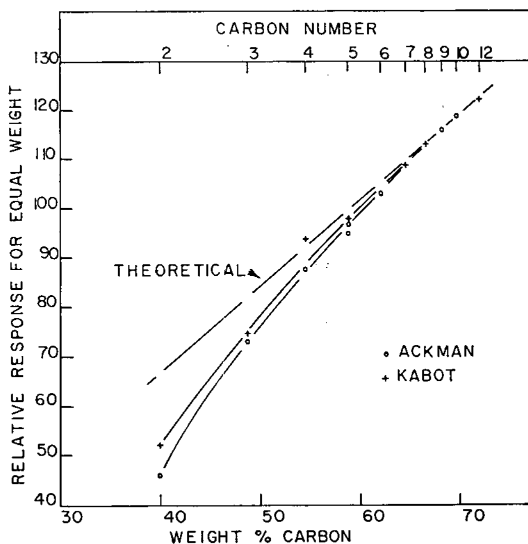


Fig. 1. Plot of relative response for equal weights of acids against weight percent of all carbon atoms in the molecule.

and capric acids, were established from a large number of results employing the described operating conditions with formic acid vapor in the carrier gas. These data are given in Table II, with the average relative molar responses for valeric and caproic

acids being 472 and 584, respectively, when the higher acids are assigned values based on a response of 100 units per carbon atom. The molar response data were then converted to response for equal weights and correlated with the previous data through caproic acid, the combined data then being expressed in proportion to the highest acid (capric) assuming the latter to give the full theoretical response for all ten carbon atoms. Similarly the data of KABOT AND ETTRE[3] were converted to a full response for lauric acid. The plots of the two sets of results are shown in Fig. 1, where despite some scattered points there is fair agreement between the two sets of data, and both sets of

TABLE II

AVERAGE MOLAR RESPONSES FOR SOME ACIDS RELATIVE TO OTHER ACIDS AS DETERMINED IN THE PRESENT STUDY

Fatty acid	*Molar response relative to*		
	Caproic = 584	*Pelargonic = 900*	*Capric = 1000*
Isobutyric (C_4)	359	—	—
Valeric (C_5)	—	475	469
Caproic (C_6)	—	583	586

data indicate as suggested by the results of these authors that within probable experimental error the relative weight response of fatty acids is theoretical for the full number of carbon atoms in the chain, including the carboxyl group carbonyl carbon atom, for fatty acids with more than six or seven carbon atoms.

A previous suggestion[5] that for these higher chain lengths equal weights of acids give nearly equal response is only a fair approximation since the presence of two oxygen atoms gives a more substantial rate of change in relative weight percent "active" carbon with increments of methylene groups in the case of the acids than in hydrocarbons of corresponding chain lengths[6]. The same considerations apply to analyses of esters of aliphatic acids[7] (*cf.* ref. 8). Correction factors[3,6,7] are therefore necessary for precise results even with the higher acids and their esters.

On a molar response basis the deficiency in response for the carboxyl carbonyl atom for valeric acid indicates that the example selected for comment by KABOT AND ETTRE[3], the relative responses of valeric, pelargonic and capric acids, cannot give an integral multiple for either of the higher acids (Table II). In view of other differences in instrumentation it is unlikely that the small differences in the results are due to the presence of formic acid vapor in the carrier gas, although an apparent case of suppression of ionization has been discussed elsewhere[9]. The reason for the deficiency in carbonyl carbon atom response in the lower fatty acids is not apparent, since there is no evidence for an alternating value such as might be associated with an inductive effect from the hydrocarbon chain Although previous reports[10] (*cf.* ref. 5) suggested that isovaleric and isobutyric acids might have markedly higher relative responses than the corresponding normal acids, the results obtained in the present study and elsewhere suggest that iso-acids give substantially similar results. In the present comparison of the relative responses of isobutyric acid with that of normal butyric acid from a previous study it should be noted that a similar bias is shown by the normal valeric acids from the two sets of data, suggesting that a slight systematic error accounts for the lower relative response given for isobutyric acid (Table II). This

view is in agreement with results for normal butanol and isobutanol, and for normal butyl acetate and isobutyl acetate[5] (*cf.* ref. 1), where both forms have substantially the same response. This is an indication that further investigation of the "cracking" of these molecules in the flame of the detector is required. KABOT AND ETTRE[3] have suggested that the polar functional group affects the response of the adjacent methylene carbon atom, an effect neutralized by the lengthening hydrocarbon chain in the higher acids.

It must be emphasized that these findings in regard to acids refer to the *relative* responses *among* the various acids. The molar or weight responses in relation to other materials such as hydrocarbons, as pointed out for acids by KABOT AND ETTRE[3], may be quite different, depending on the different fundamental polyatomic groups characteristic of each class of organic homologues (*cf.* refs. 1 and 2).

Methyl esters

A few ester analyses of materials analogous in chain length with the higher acids studied were initially run on the Wilkens apparatus with the polyester column in order to verify that no systematic error was involved in the latter analyses. The expected integral molar responses (for one carbon atom = 100 response units), based on the number of "active" carbon atoms as the total less the carboxyl group, as indicated by PERKINS *et al.*[2], could not be obtained for pairs of esters such as methyl caproate and methyl caprate (Table III). These anomalous responses were found to be

TABLE III

AVERAGE RELATIVE MOLAR RESPONSE OF ESTERS RELATIVE TO OTHER ESTERS

Compound investigated	*Reference material*	*Reference molar response*	*Compound molar response*	*Carbon atom deficiency*	*Column*
Methyl valerate	Methyl pelargonate	900	450	1.5	Pol
Methyl valerate	Methyl caprate	1000	446	1.5	Sil
Ethyl valerate	Methyl pelargonate	900	561	1.4	Pol
Methyl caproate	Methyl pelargonate	900	560	1.4	Pol
Methyl caproate	Methyl pelargonate	900	572	1.3	Sil
Methyl caproate	Methyl caprate	1000	558	1.4	Pol

independent of operating variables, injection procedure, and the presence or absence of formic acid vapor in the carrier gas. Similar results were obtained with the Barber-Colman apparatus.

Accordingly a variety of esters were investigated to obtain responses relative to two hydrocarbons. To extend the range of analyses both polyester and silicone columns were employed in the two different types of apparatus. This also permitted varying the order of appearance of components in hydrocarbon–ester mixtures. The results are given in Table IV.

The assumption of integral (100 units per carbon atom) reference relative responses for heptane and decane (Table IV) does not seriously affect comparison of data in relation to the two hydrocarbons, since the differences in weight percent "active" carbon for these materials is small. This difference may be somewhat larger in the case of methyl pelargonate and methyl caprate (Table III) and is probably

associated with the generally slightly higher responses for the lower esters relative to methyl pelargonate.

The data in Tables III and IV indicate that in methyl and ethyl esters of the shorter chain acids, excepting formic, the net deficiency in carbon atom response is normally 1.4 to 1.5 carbon atoms. This declines as the fatty acid chain lengths exceed 8 carbon atoms to a deficiency of 1 carbon atom for acids with 10 or more carbon atoms.

TABLE IV

AVERAGE RELATIVE MOLAR RESPONSES OF ESTERS RELATIVE TO HYDROCARBONS

Compound investigated	*Reference material*	*Reference molar response*	*Compound molar response*	*Carbon atom deficiency*	*Column*
Heptane	Decane	1000	703	—	Pol
Heptane	Decane	1000	694	—	Sil
Methyl formate	Heptane	700	87	1.1	Sil
Ethyl formate	Heptane	700	179	1.2	Sil
Propyl formate	Heptane	700	284	1.2	Sil
Methyl acetate	Heptane	700	157	1.5	Sil
Ethyl acetate	Heptane	700	252	1.5	Sil
Methyl propionate	Heptane	700	249	1.5	Sil
Propyl propionate	Heptane	700	463	1.4	Sil
Methyl butyrate	Heptane	700	355	1.4	Pol
Ethyl valerate	Heptane	700	550	1.5	Pol
Ethyl valerate	Heptane	700	549	1.5	Sil
Methyl caproate	Decane	1000	563	1.4	Pol
Methyl caprylate	Decane	1000	779	1.2	Sil
Methyl pelargonate	Decane	1000	898	1.0	Sil
Methyl caprate	Decane	1000	995	1.0	Sil

These results could be interpreted to mean that the alcohol portion of the molecule is split off to give an alcohol with a response deficiency of 0.5 carbon atom[2,6], and that in the lower acids the carboxyl carbonyl carbon atom also has a deficiency of 1 carbon atom. In the esters of higher acids either a different scission of the ester linkage may take place, giving a hydrocarbon residue in lieu of the alcohol, or the carboxyl group carbon atom begins to have a significant response parallelling that for the higher fatty acids.

The formate esters appear to be somewhat different in character, although this has not been investigated for those esters containing longer chain alcohols. The formates examined by PERKINS *et al.*[2] appeared consistent excepting for methyl formate. The lower value for this ester in relation to the two other formate esters examined (Table IV) may therefore be a property of this unique ester. The intermediate net carbon atom deficiency for the formate esters suggests that in the cracking of the molecules a proportion of hydrocarbons are formed from and in addition to the alcohols, since formic acid itself gives no response[4,11]. Thus the molar response results for methyl, ethyl and propyl formates, 87, 179 and 284, respectively, may be compared on the same basis to the results reported for the corresponding alcohols[1,5], 75, 170 and 256, respectively. The comparison seems valid since the result for ethyl acetate in the present study is 252 as against 253 from the same literature source. It should, however, be noted that these relative responses for methanol and ethanol are somewhat higher than suggested by PERKINS *et al.*[2,6].

The comparative responses for these esters, relative to hydrocarbons, were obtained by a different procedure from that of PERKINS *et al.*[2]. The latter authors used an open tube and separate injections of materials with calibrated syringes. The binary molar response results for ethyl, propyl and isobutyl acetates obtained by STERNBERG *et al.*[1] by another procedure, are respectively 253, 375 and 476, relative to heptane as 700 units per mole, and are in moderate agreement with the present results. Differences in the relative volatility of the materials injected did not seriously affect the present results, since similar relative response values were obtained with or without the addition of a more volatile solvent such as carbon disulphide. Sample sizes were varied in each analysis to check for detector overload which might occur with the first peak. The absence of this effect was also demonstrated by the similarity of results when peak order was reversed in certain analyses by changing from polyester to silicone columns. For methyl formate and methyl acetate, relative to heptane, smaller samples did give greater response for the ester relative to heptane. The peak height ratios did not change in such analyses and the greater response for these two esters was observed to be due to a relatively greater tailing of the ester peak. This suggests that a fixed amount of the ester sample may be temporarily adsorbed on the support or in the rubber connectors of the Barber-Colman columns. At high attenuations this tailing material would not be recorded or integrated.

Polycarboxylic acids were not investigated, but KUKSIS AND VISHWAKARMA[12] have observed in column analyses that the responses for some of the Krebs cycle acid esters, possessing in some cases hydroxyl and carbonyl functional groups in addition to more than one carboxyl group, were apparently proportional to the *total* number of carbon atoms present when compared with octadecane.

SUMMARY

The response of the carboxyl group in free fatty acids, although markedly less than the equivalent response for a methylene carbon atom in the lower fatty acids, approaches this value in acids with six or more carbon atoms. The relative responses for these higher fatty acids are then proportional to the relative weight percent carbon content on a weight basis.

In esters a complex molar response pattern is evident, apparently due to scission of the ester linkage with the lower acids to give an alcohol with a response of half a methylene carbon atom, and an acid or carboxyl group fragment with little or no response. In the methyl esters of saturated fatty acids with nine or more carbon atoms the net loss in response falls to the equivalent of one methylene carbon atom and the relative response is then proportional to the relative weight percent carbon content based on the number of carbon atoms in the fatty acid chain. Formate esters apparently give slightly higher responses than other esters of the same net number of carbon atoms. This suggests that the differences observed in the various esters may be due in the cases of those esters with higher responses to formation of a hydrocarbon in lieu of an alcohol during the initial scission of the ester group linkages. Alternatively in the esters other than formates the carboxyl group may give an increasing response as the fatty acid chain length increases.

REFERENCES

1 J. C. STERNBERG, W. S. GALLAWAY AND D. T. L. JONES, in N. BRENNER, J. E. CALLEN AND M. D. WEISS (Editors), *Gas Chromatography, Proc. 3rd Intern. Symp.*, Academic Press, New York, 1962, p. 231–267.
2 G. PERKINS, Jr., G. M. ROUAYHEB, L. D. LIVELY AND W. C. HAMILTON, in N. BRENNER, J. E. CALLEN AND M. D. WEISS (Editors), *Gas Chromatography, Proc. 3rd Intern. Symp.*, Academic Press, New York, p. 269–285.
3 F. J. KABOT AND L. S. ETTRE, *J. Gas Chromatog.*, 1, No. 10 (1963) 7.
4 R. G. ACKMAN AND R. D. BURGHER, *Anal. Chem.*, 35 (1963) 647.
5 L. S. ETTRE, *J. Chromatog.*, 8 (1962) 525.
6 G. PERKINS, Jr., R. E. LARAMY AND L. D. LIVELY, *Anal. Chem.*, 35 (1963) 360.
7 R. G. ACKMAN AND J. C. SIPOS, *J. Am. Oil Chemists' Soc.*, 41 (1964) 377.
8 L. S. ETTRE AND F. J. KABOT, *J. Chromatog.*, 11 (1963) 114.
9 R. G. ACKMAN AND J. C. SIPOS, *J. Chromatog.*, 13 (1964) 337.
10 E. EMERY AND W. E. KOERNER, *Anal. Chem.*, 33 (1961) 146.
11 J. E. LOVELOCK, *Anal. Chem.*, 33 (1961) 162.
12 A. KUKSIS AND P. VISHWAKARMA, *Can. J. Biochem. Physiol.*, 41 (1963) 2353.

GAS-LIQUID CHROMATOGRAPHY OF VOLATILE FATTY ACIDS FROM FORMIC ACID TO VALERIC ACID

I. CARBOXYLIC ACIDS AS STATIONARY PHASES

R. B. JACKSON

Fodder Conservation Section, C.S.I.R.O., Highett, Victoria (Australia)

(Received March 23rd, 1964)

In their classical paper on gas-liquid chromatography JAMES AND MARTIN[1] described the separation and micro-estimation of volatile fatty acids, using columns of Celite impregnated with a mixture of a silicone oil and stearic acid.

In spite of the excellent performance and stability of these columns reported by JAMES AND MARTIN, other workers have since observed a rapid loss of efficiency when they have been used at temperatures in the region of 135°. Some workers have attributed the loss of efficiency to bleeding of stearic acid from the column[2,3]. To prevent this, MCINNES[2] and BOER[3] replaced stearic acid with the less volatile behenic acid, but this did not prolong the life of the columns to any appreciable extent. In this laboratory too, columns containing silicone oil-stearic acid and silicone oil-behenic acid as liquid phases have become useless after running at 135° for 16 h.

If the loss of efficiency were due to some change in the liquid phase, it seemed more likely that the silicone oil would be involved rather than the behenic acid, since behenic acid is unlikely to undergo any chemical change at 135° in an atmosphere of nitrogen, whereas lack of information about the composition, stability and purity of the silicone oil leaves some doubt concerning its behaviour. Furthermore there appeared to be no reason why behenic acid alone should not be satisfactory as a liquid phase.

This communication reports some of the observations which have been made in this laboratory on the use of behenic acid and sebacic acid as liquid phases for the separation of C_1 to C_5 saturated monocarboxylic acids.

EXPERIMENTAL

Apparatus and materials

The instrument used in this work was an F & M Model 300 gas chromatograph (F & M Scientific Corporation) which employs a hot-wire thermal-conductivity detector. The detector was operated at 200° with a filament current of 150 mA. Acid-washed Chromosorb W, 80-100 mesh (purchased from F & M Scientific Corporation) was used as the solid support. Behenic acid (Hopkin & Williams) was recrystallized twice from chloroform and sebacic acid (British Drug Houses) was recrystallized twice from acetone. The stainless steel columns were 122 cm long and 4 mm internal diameter. The column packings were prepared by dissolving the liquid phase

in acetone, adding acid-washed Chromosorb W (80–100 mesh) and removing the acetone at reduced pressure in a rotary evaporator. When the acetone had been removed, the temperature was raised above the melting point of the liquid phase and maintained for about 10 min to ensure even distribution of the liquid phase. The columns were conditioned at 135° for 16 h with a carrier gas flow rate of 5 ml/min. 1.5 μl samples of a mixture of approximately equal weights of formic, acetic, propionic, isobutyric, *n*-butyric, isovaleric and *n*-valeric acids were used for testing column performance.

RESULTS

Fig. 1 shows a typical separation of a mixture of C_1 to C_5 acids on a column packed with 20 % (w/w) behenic acid on acid-washed Chromosorb W. This column showed no loss of efficiency when operated at 135° for a week with a carrier gas flow of 30 ml/min. At 115° a complete separation of formic from acetic acid and of isobutyric from *n*-butyric acid was obtained. In an attempt to improve the symmetry of the formic

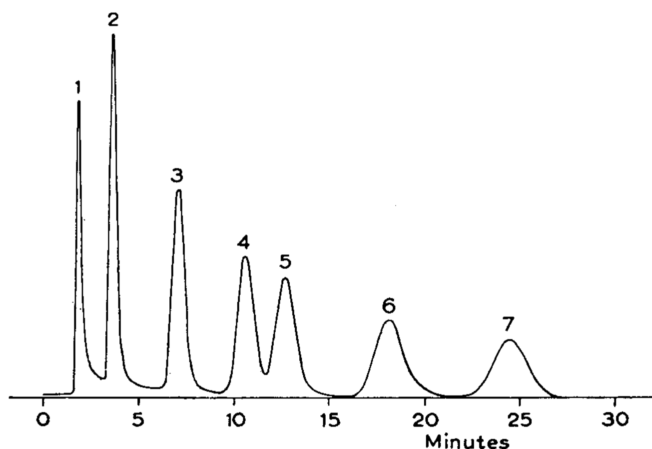


Fig. 1. Separation of a mixture of C_1 to C_5 acids on a column of 20 % (w/w) behenic acid on acid-washed Chromosorb W. Column temperature 135°. Helium flow rate 30 ml/min. Attenuation × 8. Peaks: 1 = formic acid; 2 = acetic acid; 3 = propionic acid; 4 = isobutyric acid; 5 = *n*-butyric acid; 6 = isovaleric acid; 7 = *n*-valeric acid.

acid peak, 4 % (w/w) orthophosphoric acid[1] was added to acid-washed Chromosorb W before the behenic acid. A typical separation is shown in Fig. 2. Although the tailing of formic acid was slightly reduced by adding phosphoric acid to the stationary phase, a new peak (Fig. 2, peak 1) appeared before formic acid. This was probably due to decomposition of formic acid as it only appeared when formic acid was present in the sample.

The success achieved with behenic acid prompted the investigation of sebacic acid as a liquid phase. 20 % (w/w) sebacic acid was deposited on acid-washed Chromosorb W.

The retention times on the sebacic acid column were more than double those on the behenic acid column when both columns were operated at 135° and a flow-rate of 30 ml/min. This was probably due to the sebacic acid column containing about twice

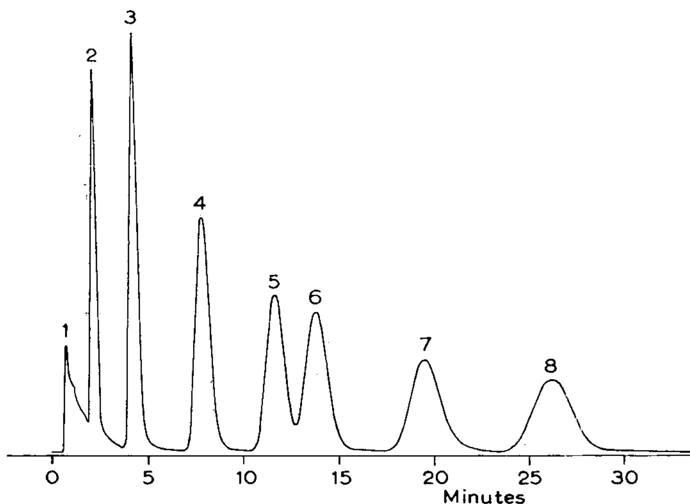


Fig. 2. Separation of a mixture of C_1 to C_5 acids on a column of 20% (w/w) behenic and 4% (w/w) orthophosphoric acids on acid-washed Chromosorb W. Column temperature 135°. Helium flow-rate 30 ml/min. Attenuation × 8. Peaks: 1 = formic acid decomposition product; 2 = formic acid; 3 = acetic acid; 4 = propionic acid; 5 = isobutyric acid; 6 = *n*-butyric acid; 7 = isovaleric acid; 8 = *n*-valeric acid.

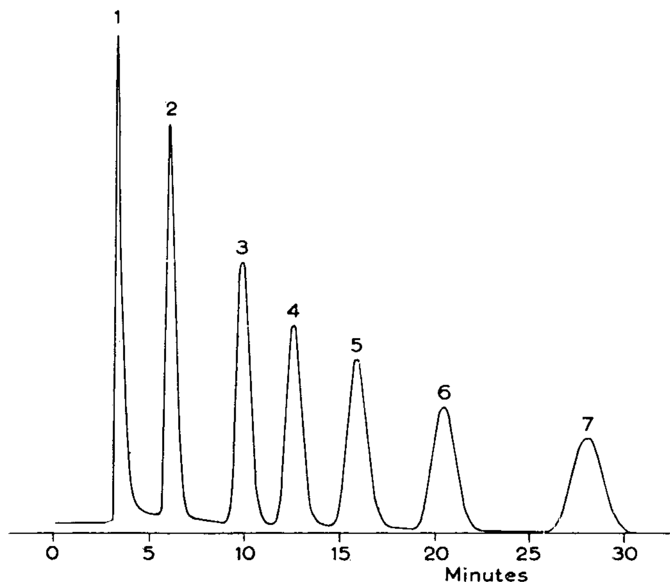


Fig. 3. Separation of a mixture of C_1 to C_5 acids on a column of 20% (w/w) sebacic acid on acid-washed Chromosorb W. Column temperature 135°. Helium flow-rate 60 ml/min. Attenuation × 4. Peaks: 1 = formic acid; 2 = acetic acid; 3 = propionic acid; 4 = isobutyric acid; 5 = *n*-butyric acid; 6 = isovaleric acid; 7 = *n*-valeric acid.

the number of carboxyl groups in the behenic acid column, thus allowing greater association in the liquid phase. When the flow rate was increased to give retention times roughly comparable with those on the behenic acid column, the separation shown in Fig. 3 was obtained. It is apparent that the sebacic acid column gave a better separation of formic from acetic acid and also of isobutyric from *n*-butyric acid, than did the behenic acid column.

The stabilities of the packings described above appear to be quite satisfactory. The behenic and sebacic acid columns have been run continuously at temperatures between 135° and 150° with a carrier gas flow-rate of 30 ml/min for more than a week without any apparent loss of resolving power.

DISCUSSION

The behenic and sebacic acid packings described above, appear to be superior to packings which have been described previously for the separation of C_1–C_5 volatile fatty acids. Polyester type stationary phases have been widely used for separating mixtures of volatile fatty acids, but the retention time of formic acid on these columns usually lies somewhere between those of acetic and propionic acids and seriously overlaps the peak of either or both of these acids. Similar results are obtained with Carbowax and Tween 80 as liquid phases. The stationary phase of dioctyl sebacate containing 15 % of sebacic acid described by RAUPP[4], gives excellent separations of the C_1 to C_5 acids, but the separation of formic from acetic acid is inferior to those given by behenic and sebacic acids.

Behenic acid and sebacic acid can only be used as stationary phases at temperatures above their melting points, namely, 80° and 133° respectively. This limitation may preclude their use in some circumstances. In this connection it would be of interest to investigate the use of high molecular weight unsaturated acids as liquid phases, since these have much lower melting points than the corresponding saturated acids. The excellent separations obtained with behenic acid as a liquid phase suggest that the instability of the silicone oil–stearic acid phase may be associated with the silicone oil; an investigation of this problem is in progress in this laboratory and will be the subject of a latter communication.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian Dairy Produce Board.

SUMMARY

The use of behenic acid and sebacic acid as liquid phases for the gas–liquid chromatography of C_1 to C_5 fatty acids has been described. Complete resolution of all normal and iso-acids was obtained on 122 cm columns containing sebacic acid as the liquid phase at 135°. With behenic acid as the liquid phase the separations of formic from acetic acid and isobutyric from *n*-butyric acid were not quite complete at 135°, however, complete separation was obtained at 115°.

Both the behenic and sebacic acid columns showed satisfactory stability at 135°.

REFERENCES

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 A. G. McInnes, in D. H. Desty (Editor), *Vapour Phase Chromatography*, Butterworths, London, 1956, p. 304.
3 H. Boer, *World Petrol. Congr. 4th, Rome, 1955*, Section V/A, Paper 1.
4 G. P. Raupp, *Angew. Chem.*, 71 (1959) 284.

EINIGE KRITISCHE BETRACHTUNGEN ZUR PHOTOMETRISCHEN AUSWERTUNG VON DÜNNSCHICHTPLATTEN

R. KLAUS

*Kontroll-Laboratorium der Firma E. Merck AG**, *Darmstadt (Deutschland)*

(Eingegangen den 4. März 1964)

Mit der fortschreitenden Anwendung der Dünnschichtchromatographie als routinemässigem Analysenverfahren taucht immer wieder die Frage der photometrischen Auswertung auf. Wir bemühen uns daher seit einigen Monaten, geeignete Methoden sowohl für angefärbte als auch für fluoreszierende Dünnschichtchromatogramme zu finden. Neuerdings wurden die Untersuchungen auf fluoreszierende Platten mit Kieselgel HF 254 ausgedehnt.

Wir liessen uns bei den Versuchen von dem Gedanken leiten, die uns zur Verfügung stehenden Geräte ohne wesentliche Eingriffe in ihren Aufbau auf ihre eventuelle Verwendung zu prüfen. In Frage kamen hierfür Remissionsmessungen mit einem lichtelektrischen Remissionsmessgerät der Firme Zeiss, Durchlässigkeits- einschliesslich Extinktions- bzw. Fluoreszenzmessungen mit einem Spektralphotometer PMQ II der Firma Zeiss und letztlich Durchlässigkeitsmessungen mit einem Spektrenauswertgerät der Optischen Werke, Jena. Letzteres konnte in die Untersuchungen bisher noch nicht einbezogen werden, da uns die hierzu erforderliche Optik zur Vergrösserung des Gesichtsfeldes noch nicht zur Verfügung steht.

DURCHLÄSSIGKEITSMESSUNGEN VON PLATTEN MIT ANGEFÄRBTEN FLECKEN

Auf Grund der Erfahrungen, die wir bei den Untersuchungen zur Auswertung fluoreszierender Papierchromatogramme[1] mit dem Spektralphotometer PMQ II der Firma Zeiss gemacht haben, versuchten wir zunächst, dieses zur Photometrierung von Platten mit angefärbten Flecken heranzuziehen.

Versuchsanordnung

Die Versuchsanordnung ist aus Fig. 1 und 2 zu ersehen. Die zu analysierende Platte steht in einem vertikalen Rahmen zwischen Leuchte und Monochromator. Dieser Rahmen kann mit Hilfe der Schraube S_1 in vertikaler Richtung bewegt werden. Der Motor M transportiert über die Kupplung K die Platte in horizontaler Richtung senkrecht zu der optischen Achse. Mit Schraube S_2 der Kupplung kann diese Bewegung von Hand ausgeführt werden. Zur Photometrierung der zu analysierenden Flecke wird die Lage der Platte durch Drehen der Schraube S_1 so weit verändert, dass erstere sich in Höhe der optischen Achse befinden. Durch Einschalten des automatischen Plattenvorschubes werden die Flecke an einem Vorspalt vorbeibewegt. Ein an das Anzeige-

* Direktor: Prof. Dr. K. G. KREBS.

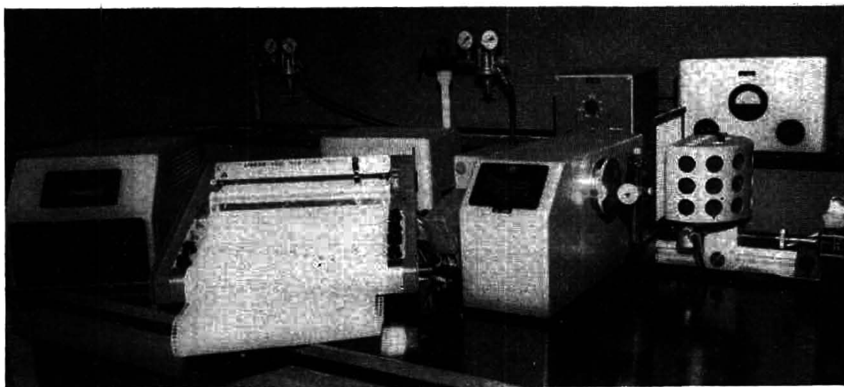


Fig. 1. Gesamtansicht der Messanordnung für Durchlässigkeits- bzw. Extinktionsmessungen.

gerät angeschlossener Kompensationsschreiber registriert die der jeweiligen Zone zuzuschreibenden Messwerte.

Beziehungen zwischen Fleckform und photometrischen Grössen bei Durchlässigkeitsmessungen

Bei der visuellen Auswertung von Dünnschichtplatten beobachtet man u. U., dass der Fleck der zu bestimmenden Komponente gegenüber den Vergleichsflecken mehr oder weniger deformiert ist. Wir versuchten daher, zunächst auf rechnerischem Wege den Einfluss der Fleckform auf das Messergebnis zu klären, und insbesondere den durch eine zusätzliche Deformation des Analysenflecks möglichen Fehler abzuschätzen. Wir be-

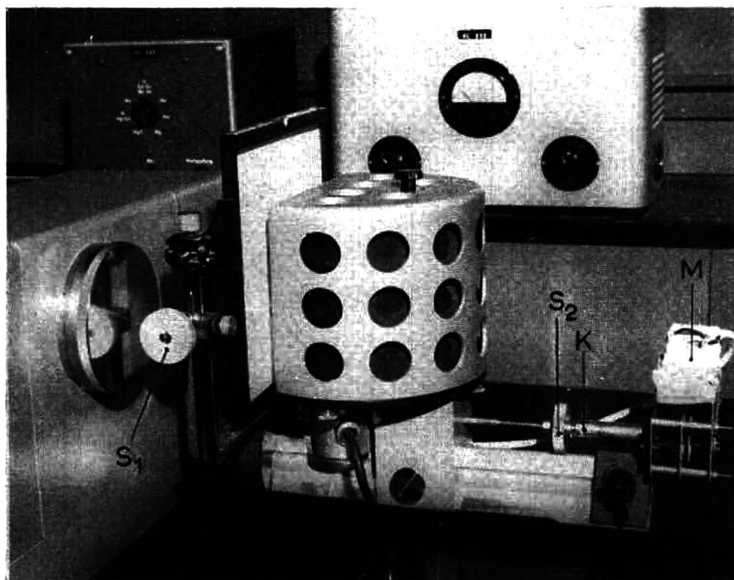


Fig. 2. Zusatzeinrichting für Durchlässigkeits- bzw. Extinktionsmessungen.

handelten bewusst die Analyse unter Berücksichtigung der angedeuteten Deformation als Allgemeinfall, der den Sonderfall einer nicht auftretenden Deformation einschliesst.

Geht man von einer konstanten Messfläche f aus (Fig. 3), auf der sich ein Fleck

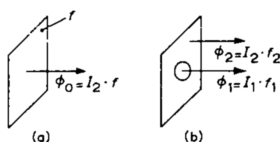


Fig. 3. Messfläche f. (a) Ohne Fleck. (b) Mit Fleck.

beliebiger Grösse aber konstanter Farbdichte befindet, so lässt sich unter Berücksichtigung der von den einzelnen Zonen durchgelassenen Lichtströme ϕ eine Aussage über die Gesamtdurchlässigkeit $D_{\lambda\Sigma}$ von f bei einer vorgegebenen Wellenlänge λ machen:

$$D_{\lambda\Sigma} = \frac{\phi_1 + \phi_2}{\phi_0} \quad \text{mit} \quad \begin{aligned} \phi_0 &= I_2 \cdot f \\ \phi_1 &= I_1 \cdot f_1 \\ \phi_2 &= I_2 \cdot f_2 \\ f &= f_1 + f_2 \end{aligned}$$

wobei I_1 und I_2 die jeweiligen durchgelassenen Intensitäten bedeuten (Fig. 3).

Setzt man diese Grössen in die Gleichung für $D_{\lambda\Sigma}$ ein, so erhält man nach einigen Umformungen folgende Beziehung:

$$D_{\lambda\Sigma} = 1 - \frac{f_1}{f} (1 - D_\lambda)$$

$D_{\lambda\Sigma}$ bedeutet hierbei die der gesamten Fläche f zuzuschreibende Durchlässigkeit bezogen auf eine gleich grosse Stelle der Platte ohne Fleck (= 100 %), $D_\lambda = I_1/I_0$ ist die Durchlässigkeit des homogen angenommenen Fleckes ebenfalls auf die gleiche Plattenstelle bezogen.

Variiert man in dieser Gleichung f_1 mit D_λ als Parameter, so erhält man den in Fig. 4 wiedergegebenen Zusammenhang $D_{\lambda\Sigma}$ = Funkt.(f_1). Aus dieser so ermittelten Kurvenschar lassen sich nun die Bedingungen ableiten, unter denen sich für verschiedene Flecke die gleichen Gesamtdurchlässigkeiten ergeben.

Der Mehrdeutigkeit der Funktion entsprechend bestimmt man für $D_{\lambda\Sigma}$ = konst. die jeweiligen Wertepaare f_1 und D_λ und trägt D_λ in Abhängigkeit von f_1 graphisch

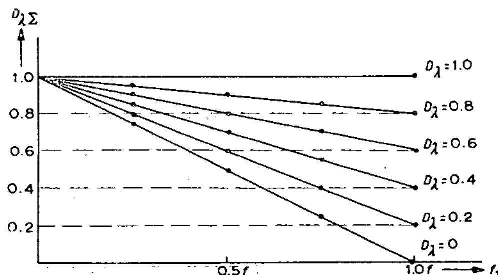


Fig. 4. Abhängigkeit der Gesamtdurchlässigkeit $D_{\lambda\Sigma}$ von der Fleckfläche f_1 mit D_λ als Parameter.

auf. Fig. 5 zeigt diese Kurven für drei von uns angenommene Werte $D_{\lambda\Sigma}$. Die der Forderung $D_{\lambda\Sigma}$ = konst. genügenden Durchlässigkeiten D_λ nehmen mit abnehmender Fleckgrösse sehr stark ab und nähern sich bei einer von der jeweiligen, vorgegebenen Durchlässigkeit $D_{\lambda\Sigma}$ abhängenden Fläche f_1 dem Wert 0.

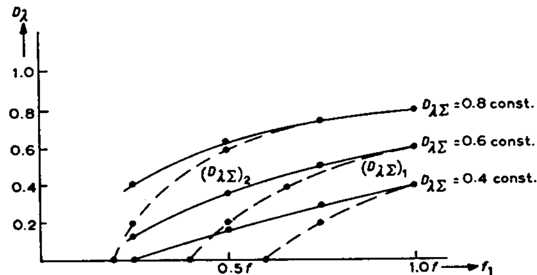


Fig. 5. ---- Fleckdurchlässigkeiten D_λ als Funktion von f_1 für $D_{\lambda\Sigma}$ = konst. ——— Fleckdurchlässigkeiten D_λ bei jeweils konstant aufgetragenen Substanzmengen als Funktion von f_1 und einer angenommenen Gültigkeit des Lambert-Beerschen Gesetzes.

Während die einleitend untersuchte Funktion einen Zusammenhang herstellt zwischen den einzelnen, die Gesamtdurchlässigkeit der Messfläche bestimmenden Faktoren, sollen die folgenden Untersuchungen über das Verhalten der Grössen $D_{\lambda\Sigma}$, f_1 und D_λ bei Veränderung der Fleckbelegung und einer angenommenen Gültigkeit des Lambert-Beerschen Gesetzes Aufschluss geben. Ausgangspunkt der Überlegungen ist ein Fleck, dessen Gesamtdurchlässigkeit $D_{\lambda\Sigma}$ bei der Fleckgrösse von $f_1 = f$, $(D_{\lambda\Sigma})_1$ betragen soll. Setzt man, wie bereits erwähnt, die Gültigkeit des Lambert-Beerschen Gesetzes voraus, so müsste bei einer Verkleinerung der Fleckgrösse von $f_1 = f$ auf $f_1 = {}^1/_2 f$ bei konstant aufgetragener Substanzmenge entsprechend der auf das Doppelte gesteigerten Belegung die Extinktion E "im Fleck" sich verdoppeln. Die der Extinktion E zuzuordnende Grösse D_λ wird in $D_{\lambda\Sigma}$ eingesetzt: man erhält die Gesamtdurchlässigkeit des Fleckes $(D_{\lambda\Sigma})_2$. Führt man eine analoge Überlegung mit weiteren Wertepaaren durch, so folgt hieraus die in Fig. 5 durchgezogen gezeichnete Kurvenschar. Gegenüber den nach der Gleichung $D_{\lambda\Sigma} = 1 - f_1 (1 - D_\lambda)/f$ = konst. ermittelten Kurven ergeben sich, wie Fig. 5 zeigt, zum Teil erhebliche Abweichungen, die bereits eine Abhängigkeit des Messwertes von der Fleckgrösse erkennen lassen.

Die Zusammenhänge werden deutlicher, wenn man wie in Fig. 6 die Abhängigkeit der für drei angenommene Konzentrationen C_1, C_2 und C_3 erhaltenen Gesamtdurch-

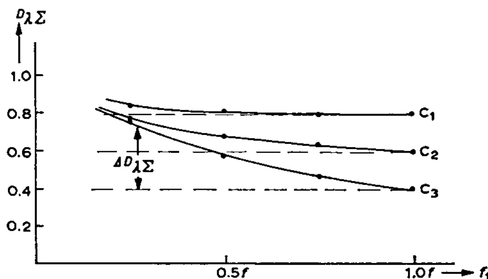


Fig. 6. Definition der Grösse $\Delta D_{\lambda\Sigma}$; Abhängigkeit von f_1 und aufgetragener Substanzmenge.

lässigkeiten $D_{\lambda\Sigma}$ von der Fleckgrösse unter Berücksichtigung des Lambert–Beerschen Gesetzes graphisch aufträgt. Errechnet man nun die Durchlässigkeitsdifferenzen zwischen den so erhaltenen Kurven und den entsprechenden Isotransmittances, so gibt diese Grösse $\Delta D_{\lambda\Sigma}$ eine Antwort auf die eingangs gestellte Frage.

Trägt man bei einer chromatographischen Analyse steigende Mengen der Eichsubstanz auf, so nehmen Farbdichte und je nach der angewandten Arbeitstechnik auch die Fleckgrösse mehr oder weniger zu. Hieraus ergibt sich, wie aus Fig. 6 zu ersehen ist, dass sich die Grösse $\Delta D_{\lambda\Sigma}$, der die Bedeutung einer methodischen Abweichung zukommt, mit steigender Konzentration laufend verändert, was zu einer Verzerrung der Eichkurve führt. Beim Auftragen konstanter Volumina ist diese nur konzentrationsbedingt. Treten sekundäre Veränderungen des Analysenfleckes nicht ein, so ergibt eine unter diesem Gesichtspunkt durchgeführte Analyse daher ein durchaus brauchbares Ergebnis. Trägt man dagegen zum Erstellen der Eichkurve steigende Volumina auf, so tritt eine von dem jeweiligen Volumen abhängige Beeinflussung der Messwerte ein, die eine quantitative Bestimmung fraglich werden lässt.

Darüberhinaus führt, wie bereits angedeutet, eine durch sekundäre Einflüsse hervorgerufene Deformierung des Analysenflecks zu einer Verfälschung des Messergebnisses, die um so grösser ist, je kleiner das Verhältnis f_1/f und je grösser die Farbdichte des Fleckes ist.

Fasst man die im vorstehenden gemachten Ausführungen zusammen, so lassen sich für Durchlässigkeits- bzw. Extinktionsmessungen folgende günstigste Analysenbedingungen angeben:

(a) Fleckausdehnung gross;

(b) Durchlässigkeit D_λ im Fleck gross;

(c) Deformation des Fleckes gering.

Das Einhalten dieser Bedingungen dürfte bei vielen Analysen aber nur schwer durchzuführen sein. Für diese Fälle weisen wir auf die beiden folgenden Korrekturmöglichkeiten hin:

(a) Im Falle annähernd linear verlaufender Eichkurven kann die Zusatzmethode mit nachfolgender Extrapolation angewendet werden.

(b) Eine gute Annäherung dürfte auch der nach der Gleichung $C = C_0 \cdot \Delta/\Delta'$ berechnete Wert ergeben. C_0 bedeutet hierbei den aus der Standardkurve abgelesenen Wert, Δ ein bekannter Zusatz zur Analysensubstanz, Δ' der aus der Eichkurve ermittelte Zusatz.

Versuche mit Testflecken

Von einigen unter Benutzung eines Extinktionsschreibers durchgeführten Versuchen mit Testflecken sei nun im folgenden berichtet:

Fig. 7 zeigt ein photographisches Positiv einer mit einer Fuchsinlösung hergestellten Testreihe (a). Die Auftropftechnik wurde dabei so gewählt, dass mit steigender

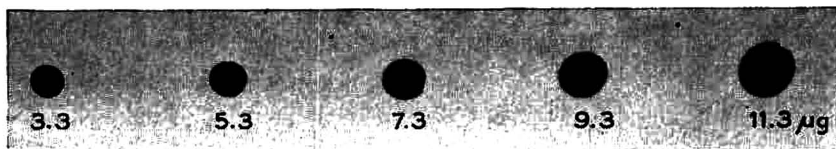


Fig. 7. Fuchsin – Testreihe (a).

aufgetropfter Substanzmenge die Fleckgrösse ebenfalls zunimmt. Nach Planimetrierung der Extinktionsortskurven (Fig. 8) erhält man den im Fig. 9 graphisch dargestellten Zusammenhang (Fall a).

Parallel hierzu wurde eine weitere Testreihe (b) mit demselben Konzentrationsbereich untersucht. Die Versuchsbedingungen wurden aber so abgeändert, dass die Ausdehnung der Testflecke konstant und ausserdem dem grössten Fleck der Reihe (a)

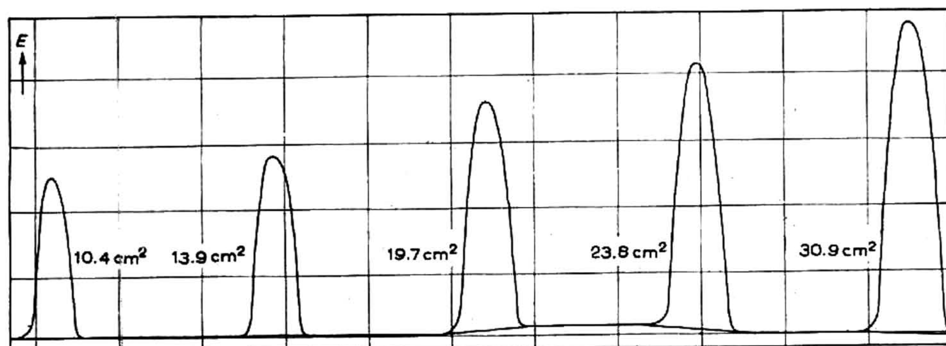


Fig. 8. Extinktionsortskurven der in Fig. 7 dargestellten Testreihe.

flächengleich sind. Die aus der Reihe (b) resultierende Kurve (Fig. 9) liefert nun erheblich höhere Extinktionsintegrale. Dies kann als eine Bestätigung der bereits abgeleiteten Beziehungen zwischen Fleckform und Messwert angesehen werden.

Mit zwei weiteren Testreihen wurden analoge Untersuchungen in einem gegenüber (a) bzw. (b) verkleinerten Konzentrationsbereich angestellt. Während bei Reihe (c) die Flecke wieder eine konstante Grösse hatten (flächengleich mit b), nahm diese bei Reihe (d) mit steigender Konzentration (analog a) zu. Es bestand also wieder Flächengleichheit der Flecke (c) mit dem grössten Fleck (d). Die Messergebnisse sind in Fig. 10 festgehalten. Auch hier zeigt sich ein dem Fig. 9 entsprechendes Verhalten.

Es liegt nun nahe, die Kurven (c) und (d) in (a) und (b) einzuordnen. Während sich die Werte der Flecke mit konstanter Ausdehnung zu einer gemeinsamen Kurve zusammenfassen lassen, ist eine Kombination der Kurven (a) und (d) nicht möglich.

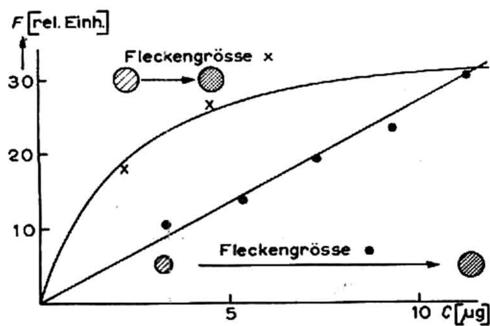


Fig. 9. Integrierte Extinktionsortskurven als Funktion der aufgetragenen Substanzmenge, ● = Fall (a), × = Fall (b).

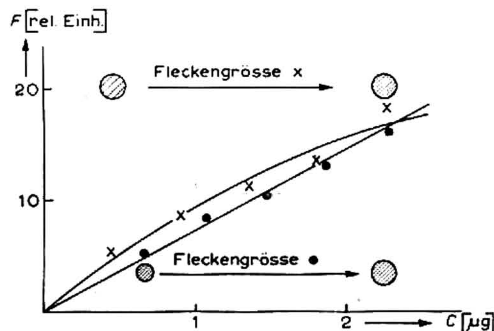


Fig. 10. Analog Fig. 9, × = Fall (c), ● = Fall (d).

Die bereits definierte Abweichung $\Delta D_{\lambda\Sigma}$ des höchsten Konzentrationspunktes der Reihe (d) unterscheidet sich von dem des niedrigsten Punktes der Reihe (a) beträchtlich. Die in den beiden Bildern dargestellten Versuchsreihen bestätigen aber die Aussage, dass der Einfluss der Fleckform auf das Messergebnis um so geringer wird, je geringer die Farbdichte im Fleck, d. h. je grösser D_λ ist.

VERSUCH ZUR AUSWERTUNG DURCH REMISSIONSMESSUNGEN

Wie einleitend angedeutet, versuchten wir, neben dem bereits beschriebenen Verfahren die Remissionsmethode auf ihre Brauchbarkeit zur Auswertung von Dünnschichtplatten mit angefärbten Flecken zu prüfen.

Der experimentelle Teil der Arbeiten wurde mit einem lichtelektrischen Remissionsphotometer der Firma Zeiss durchgeführt. Um eine Beschädigung der Schichtseite, die beim Anlegen der Platte an die Messöffnung entstehen kann, zu vermeiden, wurde die Platte zuvor mit Neatan besprüht.

Auch hier hielten wir es für angebracht, zunächst die Frage nach der Abhängigkeit des Messwertes von der Fleckform als primär zu klären.

Der Gesamtremissionsgrad eines festgelegten Flächenelementes f mit einem Fleck der Ausdehnung f_1 und Remissionsgrad R_λ (bezogen auf eine Plattenblindstelle) errechnet sich:

$$R_{\lambda\Sigma} = 1 - \frac{f_1}{f}(1 - R_\lambda)$$

Die Diskussion dieser Gleichung, auf die im einzelnen hier nicht eingegangen werden soll, führt nun zu einem den Durchlässigkeitsuntersuchungen analogen Ergebnis. Wir beschränken uns deshalb darauf, von den in diesem Zusammenhang durchgeführten Messreihen auf eine charakteristische hinzuweisen.

Fig. 11 zeigt die Abhängigkeit einer durch die Gleichung $1/h = R_{0\lambda\Sigma}/R_{\lambda\Sigma}$ definierten Grösse von dem aufgetragenen Volumen und damit indirekt auch von der Fläche f_1 bei konstanter Substanzmenge (Fuchsin). Mit wachsender Fleckausdehnung nimmt der Gesamtremissionsgrad $R_{\lambda\Sigma}$ laufend ab, d. h. der Wert von $1/h$ zu.

Dies steht vollkommen im Einklang zu der bei den Durchlässigkeitsmessungen gefundenen Abhängigkeit $D_{\lambda\Sigma}$ = Funktion $(f_1)_{C=\text{konst.}}$.

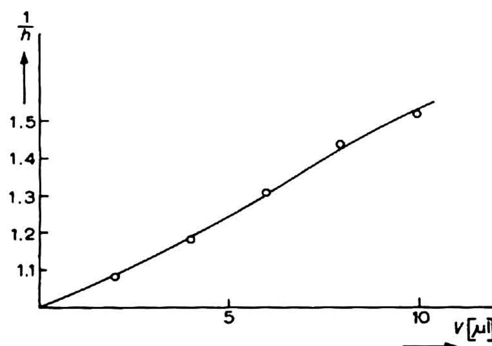


Fig. 11. Abhängigkeit der Grösse $1/h$ von dem aufgetragenen Volumen bei konstanter Substanzmenge.

Die quantitative Beurteiling einer auf einer Dünnschichtplatte aufgetragenen Messreihe verlangt daher auch bei dieser Methode im Falle einer unerwünschten Deformation des zu analysierenden Flecks die Anwendung zusätzlicher Korrekturverfahren.

AUSWERTUNG VON PLATTEN MIT FLUORESZIERENDEN FLECKEN

Versuchsanordnung

Die Versuche zur photometrischen Auswertung von fluoreszierenden Flecken wurden ebenfalls mit einem Spektralphotometer PMQ II der Firma Zeiss durchgeführt. Ausgehend von der Tatsache, dass der Umlenkspiegel des Lampengehäuses in dem Monochromator abgebildet wird[2], änderten wir die in Fig. 2 gezeigte Zusatzeinrichtung derart ab, dass die Dünnschichtplatte an den Ort des Umlenkspiegels gebracht wird.

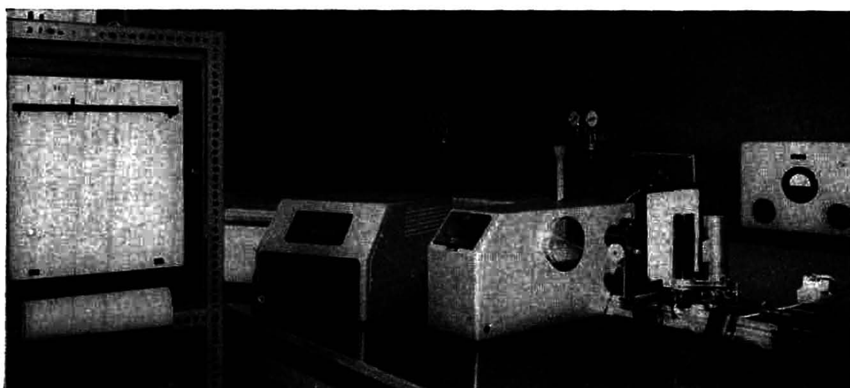


Fig. 12. Gesamtansicht der Messanordnung für Fluoreszenzmessungen.

Zur Anregung bestrahlen zwei HgS-Lampen unter einem Winkel von 45° zur Plattennormalen den auf die optische Achse justierten Fleck. Als Flächenbegrenzung kann vor der Platte wahlweise eine Spalt- oder Kreisblende montiert werden, deren Abmessungen der Grösse des Umlenkspiegels angepasst sein müssen. Fluoreszierende Flecke mit dem Umlenkspiegel überschreitende Grössenverhältnisse können mit einer Zwischenabbildung ausgewertet werden. Der Plattenvorschub wird wieder von dem Motor M durchgeführt. Die Registrierung der Fluoreszenzintensitäten erfolgt mit einem an das Anzeigegerät angeschlossenen Kompensationsschreiber. Fig. 12 zeigt die gesamte Messanordnung, Fig. 13 die Zusatzeinrichtung.

Zusammenhang zwischen photometrischen Grössen und Fleckparametern

Der bei entsprechender Bestrahlung von einer fluoreszierenden Fläche in der Zeiteinheit ausgesandte Lichtstrom errechnet sich aus der Gleichung:

$$\phi = J \cdot \omega$$

Hierbei bedeuten J die Strahlungsstärke des Senders und ω den Raumwinkel in

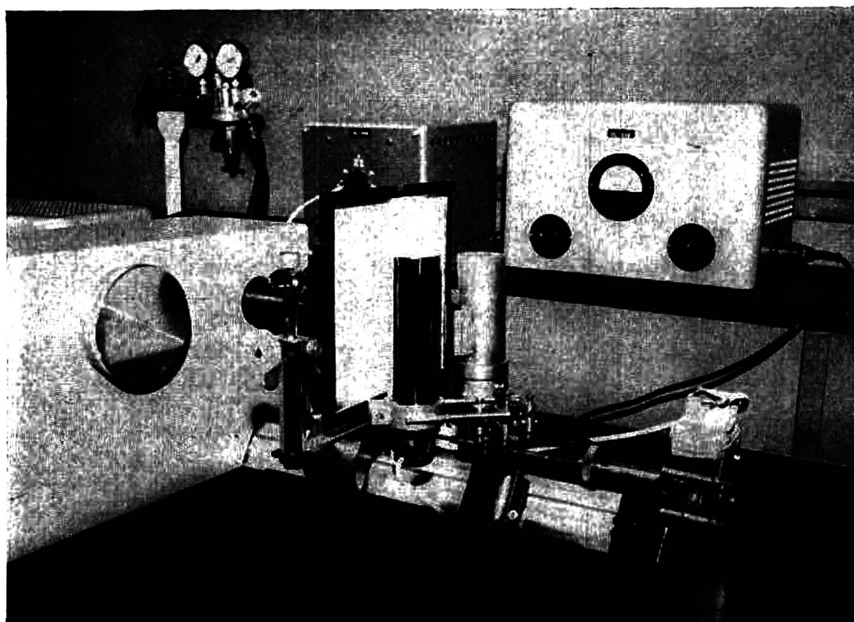


Fig. 13. Zusatzeinrichtung für Fluoreszenzmessungen.

den die Ausstrahlung stattfindet. Geht man nun von konstanten geometrischen Abbildungsverhältnissen aus, so ist ω = konst. (k_1) für verschiedene Flecke. d. h.

$$\phi_\lambda = k_1 \cdot J = s_\lambda \cdot f_1 \cdot k_1 \cdot k_2$$

k_2 bedeutet hier eine dem Emergenzwinkel proportionale Grösse, f_1 Fläche, s_λ Strahlungsdichte des Senders. Zwischen dem ausgesandten Lichtstrom ϕ_λ und dem am Photometer abgelesenen Fluoreszenzwert F_λ besteht andererseits die Beziehung:

$$F_\lambda = k_3 \cdot \phi_\lambda$$

Bringt man beide Gleichungen zueinander in Beziehung, so erhält man schliesslich:

$$F_\lambda = k \cdot s_\lambda \cdot f_1$$

Da es sich bei chromatographischen Analysen jeweils um Messungen mit Vergleichsflecken handelt, ist die Kenntnis der absoluten Grösse der Werte k nicht erforderlich. Somit lässt sich folgende Beziehung aufstellen:

$$F_\lambda \sim s_\lambda \cdot f_1$$

Dieser Ausdruck verbindet die photometrischen Grössen F_λ und s_λ mit der Fläche f_1 und führt zu den beiden Sonderfällen:

$$\text{(a) } s_\lambda = \text{konst.} \rightarrow F_\lambda \sim f_1$$

$$\text{(b) } f_1 = \text{konst.} \rightarrow F_\lambda \sim s_\lambda$$

In beiden Fällen ergibt die Rechnung einen linearen Zusammenhang zwischen dem Messwert und der jeweiligen unabhängig Veränderlichen.

Zur Prüfung der Richtigkeit dieser Aussagen, vornehmlich aber zur Abschätzung der Grenzen der in (b) aufgestellten Beziehung in Verbindung mit der aufgetragenen Substanzmenge, sei auf die beiden folgenden Versuche hingewiesen:

Ausgehend von der Beziehung $F \sim s_\lambda \cdot f_1$ wurde bei dem ersten Versuch $s_\lambda =$ konst. gesetzt und f_1 variiert durch Veränderung der aufgetragenen Volumina. Wie bereits in einer früheren Arbeit berichtet wurde, ergab sich erwartungsgemäss in gewissen Grenzen ein linearer Zusammenhang zwischen gemessener Fluoreszenzintensität F_λ und Fläche f_1.

Fig. 14. Fluorescein-Na – Testreihe.

Der zweite Versuch, der anschliessend beschrieben wird, geht von einer konstanten Fläche f_1 aus, bei Variation von s_λ. Man erhält diese Testreihe, indem man konstante Volumina verschieden konzentrierter Substanzlösungen (in diesem Falle Fluorescein-Na) aufträgt. Fig. 14 zeigt ein photographisches Positiv des betreffenden Plattenausschnittes.

Die Registrierung der fluoreszierenden Flecke wurde nach zwei verschiedenen Methoden durchgeführt. Im ersten Fall wurde mit einer Spaltblende als Vorspalt vor der Dünnschichtplatte gearbeitet. Diese Technik hat den Nachteil, dass die Gesamtfluoreszenz jedes Fleckes erst durch Integration der Fluoreszenzortskurven (die so erhaltene Grösse sei mit $F_{\lambda\Sigma}$ bezeichnet) erhalten wird.

Fig. 15 zeigt die registrierten Fluoreszenzortskurven, Fig. 16 Kurve × die integrierten Fluoreszenzortskurven in Abhängigkeit von der Konzentration.

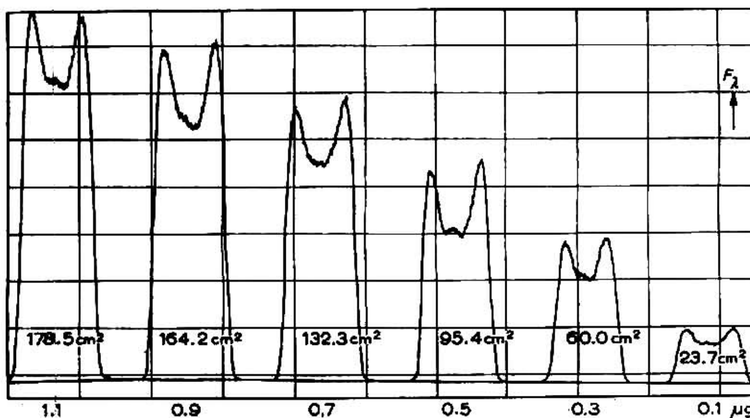


Fig. 15. Mit Spaltblende aufgenommene Fluoreszenzortskurven der Testreihe von Fig. 14.

Für die zweite Möglichkeit der Auswertung wurde eine Kreisblende, die den eingangs erwähnten Bedingungen genügt, gewählt. Die Peakhöhe ist hier ein direktes Mass für die der Gesamtfläche zuzuschreibenden Fluoreszenz.

Fig. 17 zeigt die registrierten Fluoreszenzkurven, Fig. 16 Kurve ● die Abhängigkeit der Peakhöhe von der aufgetragenen Substanzhöhe.

Ein Vergleich der beiden in Fig. 16 dargestellten Kurven lässt eine nur relativ geringe Abweichung erkennen. Bei grösseren Konzentrationen ($\sim$ 1 μg) zeichnet sich

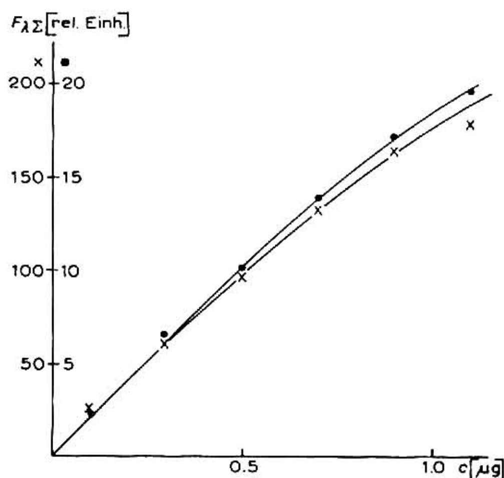


Fig. 16. Gesamtfluoreszenz $F_{\lambda\Sigma}$ als Funktion der aufgetragenen Substanzmenge C (Fluorescein-Na) ● = Registrierung mit Kreisblende, × = Registrierung mit Spaltblende.

eine beginnende konkave Krümmung der Kurven ab, die auf eine Veränderung der Eindringtiefe der anregenden Strahlung bei zu grossen Flächenbelegungen zurückzuführen sein dürfte.

Die bei diesem Versuch zu klärende Frage nach der Abhängigkeit der Gesamtfluoreszenz von der Leuchtdichte s_λ unter Berücksichtigung der aufgetragenen Substanzmenge bei konstanter Fläche f_1 kann somit wie folgt beantwortet werden:

Für *nicht zu grosse Konzentrationen* besteht zwischen der gemessenen Fluoreszenz und der Konzentration ein angenähert linearer Zusammenhang. Dies weist aber andererseits, da f_1 = konst., auf eine lineare Abhängigkeit zwischen der Konzentration und der Leuchtdichte s_λ hin.

Fasst man die in den beiden Versuchen erhaltenen Ergebnisse zusammen, so ergibt sich eine lineare Abhängigkeit der gemessenen Gesamtfluoreszenz von der aufgetragenen Substanzmenge einerseits bei konstanter Leuchtdichte, andererseits bei konstanter Fläche aber nicht zu grossen Leuchtdichten. Mit diesen Aussagen lässt sich nun die auch hier interessierende Frage nach dem Zusammenhang zwischen Fleckform und Messergebnis beantworten. Eine durch sekundäre Einflüsse hervorgerufene Deformation des Fleckes dürfte unter normalen Bedingungen das Messergebnis nicht beeinflussen, zusätzliche Korrekturen sind demnach im allgemeinen nicht erforderlich.

Die Gültigkeit dieser Aussagen wird durch den im Fig. 18 angedeuteten Versuch mit mehreren Fluorescein-Na-Testflecken bestätigt.

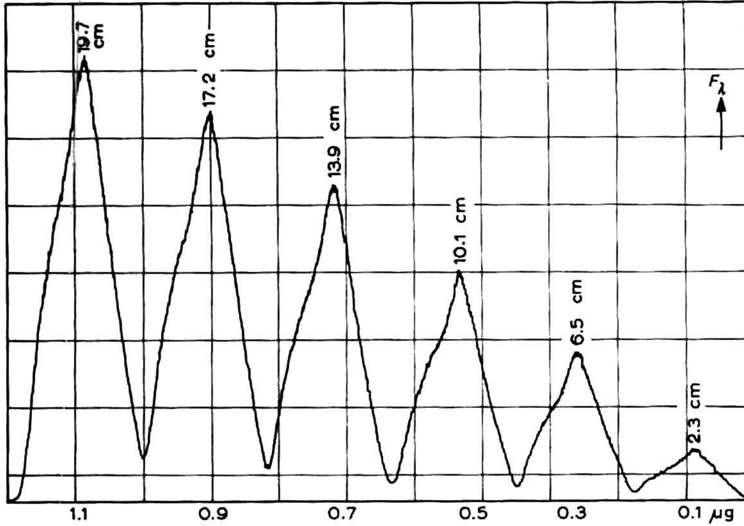


Fig. 17. Mit Kreisblende aufgenommene Fluoreszenzkurven der Testreihe von Fig. 14.

Im folgende sei nun ein Fluoreszenzversuch beschrieben, der einerseits die Vorteile der photometrischen Auswertung zeigt, andererseits aber auch die Mängel erkennen lässt, die der Vorbereitung der Platte zum Teil noch anhaften.

Auf einer Dünnschichtplatte wurde die zu analysierende Substanz (St 155) einschliesslich steigender Mengen der Vergleichssubstanz aufgetragen und entsprechend behandelt. Das Zustandekommen der Fluoreszenz verlangt nun in diesem Falle, dass man die Platte kurzzeitig einer höheren Temperatur (*ca.* 120°) aussetzt. Die auf diese Weise erhaltenen fluoreszierenden Flecke wurden mit der beschriebenen Anordnung (unter Verwendung der Kreisblende) registriert (Fig. 19) und durch Messung der Peakhöhe ausgewertet.

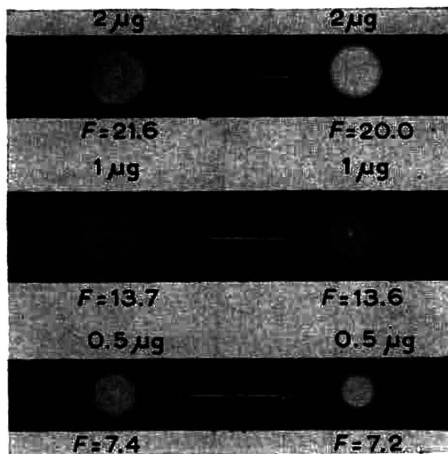


Fig. 18. Zusammenhang zwischen Fluoreszenz und Fleckfläche bei drei verschiedenen aufgetragenen Mengen Fluorescein-Na.

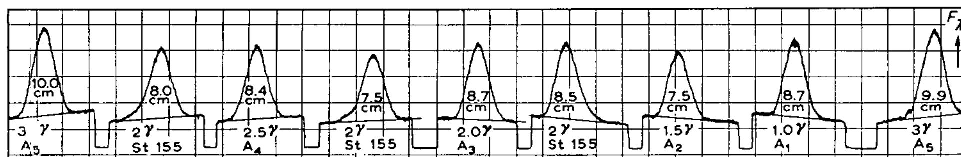


Fig. 19. Fluoreszenzkurven einer Testsubstanz einschliesslich Vergleiche A_1–A_5.

Für die zu analysierende Substanz, die dreimal aufgetragen wurde, ergaben sich Peakhöhen von 8.5 cm/7.5 cm/8.0 cm, also Werte mit nicht unbeachtlichen Streuungen. Ähnlich liegen die Verhältnisse bei den Vergleichsflecken A_1–A_5. Die Streuungen, wahrscheinlich hervorgerufen durch eine nicht konstante Temperaturverteilung im Heizraum, lassen hier sogar das Aufstellen einer Eichkurve nicht ratsam erscheinen. Zur Reproduzierbarkeitsprüfung wurde der Vergleichsfleck mit der höchsten Konzentration (A_5) mehrere Male ausgewertet. Aus den beiden im Bild ersichtlichen Fluoreszenzkurven lassen sich Peakhöhen von 9.9 cm bzw. 10.0 cm ersehen.

Wenn auch bei der Mehrzahl der chromatographischen Analysen aufgrund einer einfacher zu kontrollierenden Plattenbehandlung mit geringeren Streuungen gerechnet werden kann, so sollten diese dennoch nicht unberücksichtigt bleiben.

Der Vorteil der photometrischen Auswertung besteht nun gerade darin, dass, im Gegensatz zu der visuellen Beurteilung, die Flecke in ihrer Gesamtheit für das Endergebnis bestimmend sind, sei es durch Mittelbildung der Messwerte der zu analysierenden Flecke oder durch die Festlegung einer aus den einzelnen streuenden Standardwerten ermittelten Eichkurve.

AUSWERTUNG VON DÜNNSCHICHTPLATTEN BEI VERWENDUNG VON KIESELGEL HF 254 ALS SCHICHTMATERIAL

In zunehmendem Masse finden in letzter Zeit Leuchtstoffplatten Anwendung, die unter dem Einfluss kurzwelliger U.V.-Strahlung, in diesem Falle der Hg-Linien $\lambda \sim 254$ nm, eine grüngelbe Fluoreszenz ergeben[3]. Trägt man auf die Platte nun Substanzen auf, die im Bereich $\lambda \sim 254$ nm zwar eine Absorption aufweisen, diese aber nicht in eine Fluoreszenzstrahlung transformieren, so resultiert hieraus eine Verringerung der Fluoreszenz des Leuchtstoffes am Ort des absorbierenden Stoffes.

Die von uns durchgeführten Versuche sollten auch hier klären, welcher Zusammenhang zwischen der Messgrösse einerseits und aufgetragener Substanzmenge andererseits besteht und im besonderen welche Bedeutung hierbei der jeweiligen Fleckgrösse zukommt. Hierüber sei im folgenden berichtet.

Versuchsanordnung

Es wurde mit der in Fig. 13 beschriebenen Versuchsanordnung unter Verwendung einer Kreisblende gearbeitet. Die Anregung erfolgte durch die beiden HgS-Lampen mit entsprechenden Filtern UG 5 der Firma Schott und Gen, Mainz.

Messungen von Testflecken

Fig. 20 zeigt drei willkürlich angenommene Testflächen, auf denen die Substanzmengen C_0 (= 0), C_1 und C_2 aufgetragen sein sollen. Ordnet man diesen Testflächen die

Gesamtfluoreszenzen $F_{0\Sigma}$, $F_{1\Sigma}$ und $F_{2\Sigma}$ zu, so wäre eine fallende Charakteristik zu erwarten. Errechnet man dagegen aus den Werten $F_{C\Sigma}$ die auf die Fläche mit $C = 0$ bezogene Fluoreszenzverminderung, indem man die Differenzen

$$\Delta F_{1\Sigma} = F_{0\Sigma} - F_{1\Sigma}$$

$$\Delta F_{2\Sigma} = F_{0\Sigma} - F_{2\Sigma}$$

bildet, so erhält man für die Funktion $\Delta F_{C\Sigma} =$ Funkt. (C) mit C steigende Werte für $\Delta F_{C\Sigma}$.

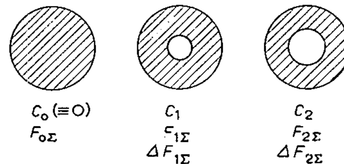


Fig. 20. Testflächen zur Definition der Grösse $\Delta F_{C\Sigma}$.

In den folgenden Untersuchungen wird die so definierte Grösse $\Delta F_{C\Sigma}$ als für die Analyse charakteristische Grösse eingesetzt. Es wurden die beiden folgenden Testreihen hergestellt:

Bei Reihe (a) wurden steigende Volumina einer Coffein-Standardlösung derart aufgetragen, dass Flecke mit 1–10 μg Coffein entstanden. Die mit Substanz belegten Zonen nahmen bei diesem Versuch mit wachsender aufgetragener Menge zu.

Bei dem Versuch (b) wurden konstante Volumina von Coffeinlösungen steigender Konzentration ebenfalls im Bereich 1–10 μg aufgetragen. Es bildeten sich mit Coffein belegte Zonen annähernd konstanter Ausdehnung. Die Fleckgrösse entsprach dabei etwa dem grössten Fleck der Reihe (a). Die Fig. 21 und 22 zeigen die registrierten Fluoreszenzkurven der beiden Testreihen.

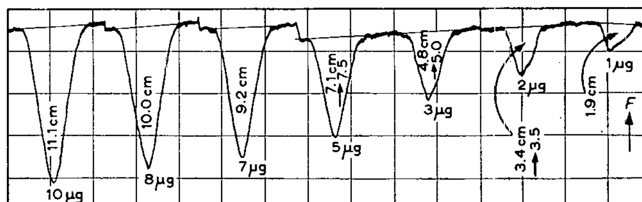


Fig. 21. Mit Kreisblende registrierte Fluoreszenzkurven von steigend aufgetragenen Coffein-Mengen. Versuch (a), zunehmende Fleckgrösse.

Auf Fig. 21 ist für die im Bereich 1–5 μg liegenden Peaks deutlich der nicht konstante Untergrund, d. h. in diesem Falle der Fluoreszenzintensität $F_{0\Sigma}$ zu erkennen. Zur Vermeidung rechnerischer Untergrundkorrekturen ist daher gegebenenfalls eine Veränderung einer der Geräteparameter derart erforderlich, dass sich jeweils konstante Werte $F_{0\Sigma}$ ergeben. Die aus den Messergebnissen resultierenden Kurven zeigt Fig. 23.

Während für den Punkt $C = 10$ μg für beide Reihen entsprechend der übereinstimmenden Fleckgrösse die gleiche Fluoreszenzverminderung $\Delta F_{C\Sigma}$ ermittelt wurde, ergaben sich fur $C < 10$ μg nicht unerhebliche Differenzen.

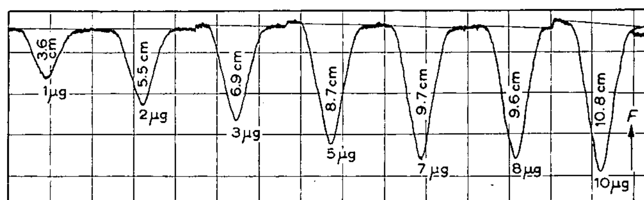


Fig. 22. Analog Fig. 21. Versuch (b), annähernd konstante Fleckgrösse.

Es zeichnet sich ein, wenn auch nicht in diesem Ausmass, dem im Kapitel angefärbte Dünnschichtplatten entsprechender Sachverhalt ab: mit abnehmender Fleckgrösse verringert sich bei jeweils konstant aufgetragenen Substanzmengen der Wert der Fluoreszenzverminderung.

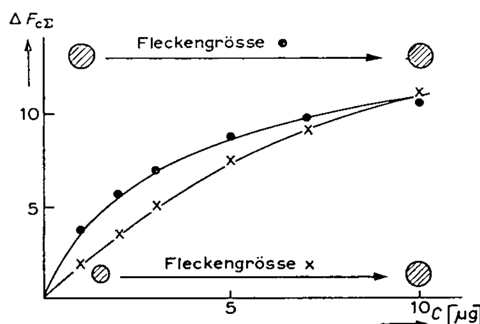


Fig. 23. Zusammenhang zwischen Fluoreszenzverminderung und aufgetragener Substanzmenge (Coffein) bei: (a) steigender Fleckgrösse, ×; (b) konstanter Fleckgrösse,

ZUSAMMENFASSUNG

In den vorstehenden Ausführungen werden die Versuche beschrieben, welche zur photometrischen Auswertung von angefärbten bzw. fluoreszierenden Dünnschichtplatten nach verschiedenen Methoden angestellt wurden.

Ferner wird die Beeinflussung des Messwertes von der jeweiligen Fleckform sowohl rechnerisch als auch experimentell behandelt und insbesondere auf eine Verfälschung des Analysenergebnisses bei Nichtbeachtung von zusätzlich auftretenden Fleckdeformationen hingewiesen. Es werden zwei mögliche Korrekturverfahren erwähnt, über die gesondert zu einem späteren Zeitpunkt berichtet werden soll.

SUMMARY

A description is given of the photometric evaluation of coloured and fluorescent spots on thin-layer plates and of the different methods employed.

The influence of the actual shape of the spot on the values obtained is examined, both experimentally and mathematically. It is pointed out that erroneous results can be obtained by disregarding deformation of the spots. Two possible correction procedures are mentioned, but these will be reported separately at a later date.

LITERATUR

[1] W. Esselborn und R. Klaus, *Pharm. Ztg.*, 105 (1960) 34.
[2] G. Zeiss, *Gerätebeschreibung zum Spektralphotometer PMQ II.*
[3] E. Stahl, *Dünnschicht-Chromatographie*, Springer, Berlin, 1962, S. 57; *Präparate Merck für di Dünnschicht-Chromatographie nach Stahl*, E. Merck AG, 1962.

THIN-LAYER TECHNIQUES FOR MAKING PEPTIDE MAPS*

W. J. RITSCHARD

Department of Muscle Research, Institute of Biological and Medical Sciences, Retina Foundation, Boston, Mass. (U.S.A.)

(Received March 9th, 1964)

Peptide maps, or "fingerprints" have proved very useful in characterizing proteins by the pattern of peptides resulting from the application of chromatography and electrophoresis to a proteolytic digest. This technique was first described by INGRAM[1], and later improved by KATZ, DREYER AND ANFINSEN[2]. An outstanding example of the power of this method is the identification of a single amino acid as being responsible for the difference between normal and sickle cell anemia human hemoglobin[1].

BRENNER and co-workers[3,4] successfully applied thin-layer chromatography to amino acids and their derivatives. Thin-layer electrophoresis was previously used by HONEGGER[5] and by PASTUSKA AND TRINKS[6] for separating amines, amino acids, and organic acids. Because of the heat produced during electrophoresis, potentials of not more than 500 V were applied.

These results led us to the utilization of the combination of thin-layer chromatography and electrophoresis for the two-dimensional separation of peptides. Modifications of the chromatographic equipment and procedures used by BRENNER *et al.* were made. In the electrophoretic step we used 950 to 1.000 V, but additional cooling of the plates has been found absolutely necessary. The advantages of the thin layer technique lie in its rapidity and in the small amounts of material needed.

METHODS

Digestion of proteins

Digests of several proteins were prepared, including protamine and myosin which are cited as illustrations in this paper. The most convenient procedure is that of KATZ *et al.*[2], in which a volatile buffer is used. Trypsin and chymotrypsin are the most specific enzymes employed, but others may also be used. Trypsin and chymotrypsin were purchased from Worthington Biochemical Corporation, protamine sulfate from Nutritional Biochemicals Corporation. Myosin was prepared as previously described[7].

Thin-layer plates

Silica gel G proved to be the best carrier material for successive chromatography and electrophoresis. Surfaces coated with this material have good mechanical properties and allow excellent separations with distribution patterns similar to those found with paper sheets. Thin-layer plates are prepared according to BRENNER *et al.*[3],

* This work was supported by grants from the National Heart Institute (H-5949), the Muscular Dystrophy Associations of America, Inc., the Life Insurance Medical Research Fund, the American Heart Association, Inc., and the National Science Foundation.

the best coating being obtained with a mixture of 20 g silica gel G in 80 ml water*. The mixture is shaken for 1 min in an Erlenmeyer flask closed with a rubber stopper. The plates, having standard sizes of 200 × 50 mm or 200 × 200 mm, are dried overnight at room temperature since the use of plates dried in an oven leads to poorly reproducible results.

Solvent systems

In order to find the optimal solvent mixture of those listed in Table I, preliminary chromatographic runs with 200 mm × 50 mm thin layer plates are made. Cylindrical polyethylene jars, 210 mm × 90 mm, lined with filter paper soaked with the solvent

TABLE I

SOLVENT SYSTEMS FOR CHROMATOGRAPHY OF ENZYME DIGESTS OF PROTEINS

System	*Volume ratio*
A. Neutral systems	
1. 96% Ethanol–water	70:30
2. *n*-Propanol–water	70:30
B. Basic systems	
1. 96% Ethanol–34% ammonium hydroxide	70:30
2. *n*-Propanol–34% ammonium hydroxide	70:30
3. Chloroform–methanol–34% ammonium hydroxide	40:40:20
C. Acidic systems	
1. 96% Ethanol–water–acetic acid	70:20:10
2. *n*-Propanol–water–acetic acid	70:20:10
3. *n*-Butanol–water–acetic acid	80:20:20

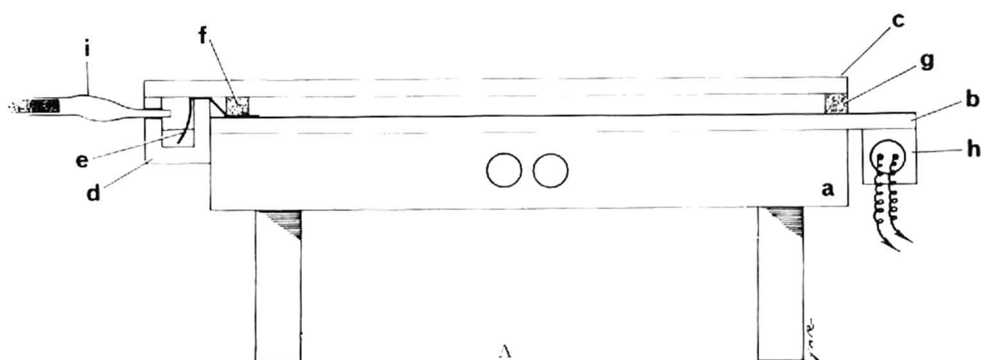
mixture and covered with glass lids are used. At the end of the run, which takes between 1 and 2 h, the plates are dried at 100° for 10 min and developed with ninhydrin spray. Chromatography, with the chosen solvent, for preparing the peptide maps is then carried out on 200 mm × 200 mm plates.

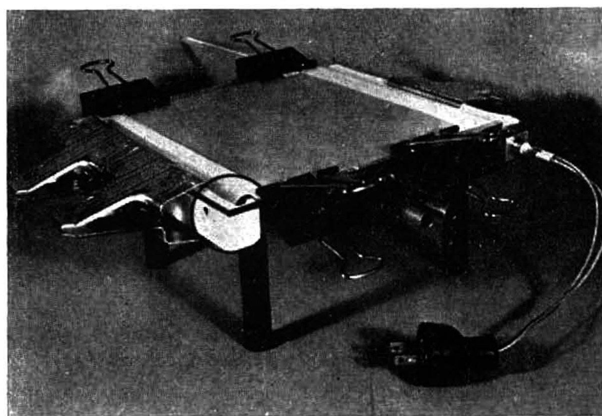
Modified Brenner-Niederwieser chamber (B-N chamber)

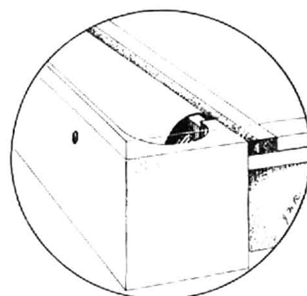
When longer runs are necessary for adequate separations, a chamber similar to that described by BRENNER AND NIEDERWIESER[8] is used, with some modifications. Their technique basically consists of a continuous flow of the solvent over the thin layer, upon which a second glass plate is placed as a cover. The solvent evaporates at the open end of the chamber. We have increased the distance between the two plates in order to prevent the filling of the space between the two plates with solvent condensed on the upper plate. A more rapid flow of the solvent is obtained with the use of an electric heating element at the edge of the chamber opposite the trough (Fig. 1).

A solvent trough of the dimensions given by BRENNER AND NIEDERWIESER, but made of high density polyethylene instead of stainless steel, is used. The edge over which the paper connection(wick) between the trough and the thin layer plate passes is 0.5 mm lower than the opposite edge. Whatman No. 4 paper is used for the wick in order to provide fast flow of the solvent, and it has a simpler (rectangular 175 mm ×

* The thin-layer equipment of the Brinkmann Instruments, Inc., Westbury, N. Y., was used.







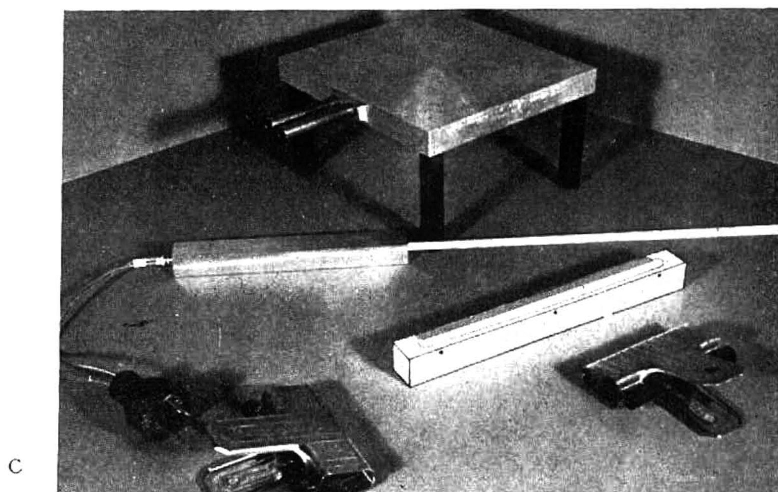


Fig. 1. Modified B-N chamber. A. Schematic drawing. (a) Aluminum platform with cooling coil inside. (b) Coated thin-layer plate. (c) Cover glass plate. (d) Solvent trough. (e) Paper connection (175 mm × 30 mm). (f) Teflon strip (200 mm × 50 mm × 30 mm). (g) Small teflon spacers (5 mm × 5 mm × 3 mm). (h) Heating element. (i) Pipette with polyethylene cathether. B. Assembled chamber with detailed view of the solvent trough. C. Disassembled chamber: Aluminum platform (185 mm × 175 mm). Heating element (185 mm × 20 mm × 15 mm) with inserted thermometer. Modified solvent trough.

30 mm) shape than in the original technique and is not folded. A teflon (200 mm × 5 mm × 3 mm) strip is fitted over the paper connection in order to obtain the proper distance between the two plates (3 mm). Two small teflon spacers (5 mm × 5 mm × 3 mm) are used between the two glass plates at the other end of the chamber. Both sides are sealed with scotch tape in order to prevent lateral evaporation. Cooling is provided by placing the chamber on an aluminum platform containing a cooling coil through which tap water is circulated. The heating element consists of an aluminum bar equipped with a heating cartridge* and a thermometer. The temperature is adjusted with a powerstat.

Assembling the chamber

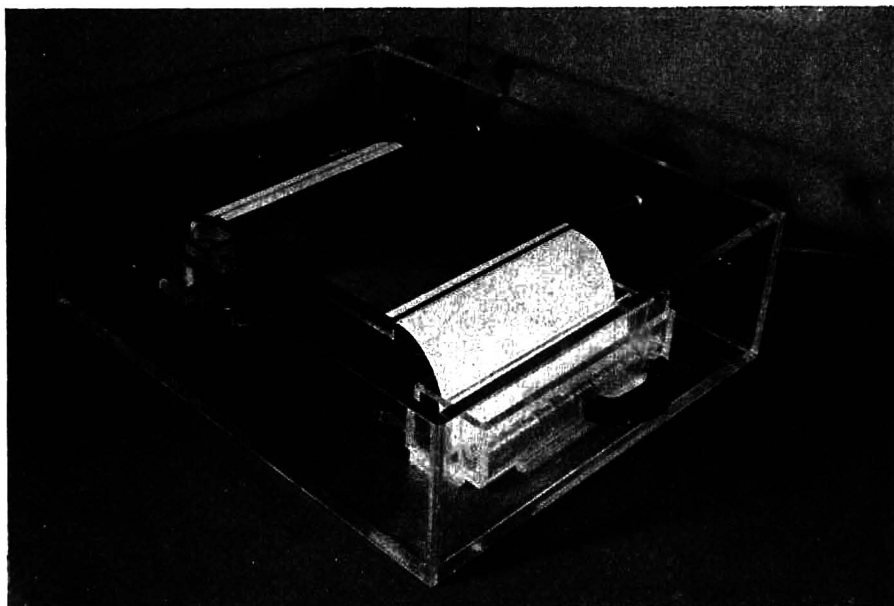
The chamber is assembled in an inverted position. The cover glass is placed on the aluminum platform and the open surface of the solvent trough with its wick place is put opening downward on the plate and clamped into position. The teflon strip is laid under the free end of the wick parallel to the solvent trough, and the other two teflon spacers are arranged at the opposite corners of the plate. Then the silica gel surface of the thin-layer plate is placed on the spacers. The two plates are clamped together along one edge and pressed together by hand along the opposite edge while being sealed with scotch tape. The taped edge is then clamped and the other edge similarly taped and clamped. The entire chamber is then inverted on the aluminum platform, as shown in Fig. 1. The heating element is clamped to the thin-layer plate, the cooling water turned on, and the temperature of the heating element adjusted to 100°. The solvent mixture, 20 ml, is added through a small hole in the trough using a pipette with a polyethylene catheter on the tip. Because the opening of the trough is not covered by the paper wick, in contrast to BRENNER's technique, the evel of the solvent can be monitored during the run and additional solvent may be ntroduced if necessary.

Electrophoresis

The electrophoresis chamber shown in Fig. 2 consists of two electrode vessels with platinum electrodes, a platform (190 mm × 190 mm) the thin-layer plate and cover plate. Connections of Whatman No. 3 paper link the electrode vessels to the thin-layer plate, and the cover plate is held in position with clamps. To avoid corrosion, all metal surfaces are coated with a clear acrylic paint. A power supply with an output of 0-1000 V and 0-200 mA is used**. Preliminary electrophoretic runs are made as follows: various amounts of the peptide mixture are spotted on a line in the middle of a 200 mm × 200 mm thin-layer plate, allowing at least 10 mm between spots. Buffer is evenly sprayed on the plate, which is then laid on the aluminum platform.

The electrode vessels are then joined by the paper bridges to the silica gel layer, the glass cover plate is brought into position, and the two plates are clamped. Tap water provides sufficient cooling permitting the application of 950–1000 V and currents of 30–80 mA. After a 30 min run, the plate is removed, dried at 100° and sprayed with ninhydrin reagent. These preliminary results decide the optimal sample concentration and time of the run. The direction of peptide migration during preliminary electrophoresis determines whether the spots for preparing the peptide maps should be

* C35 standard Hotwatt Heating Cartridge, Hotwatt, Inc., Danvers, Mass.
** Power supply 3-1009, Buchler Instruments, Inc., Fort Lee, N. J.



A

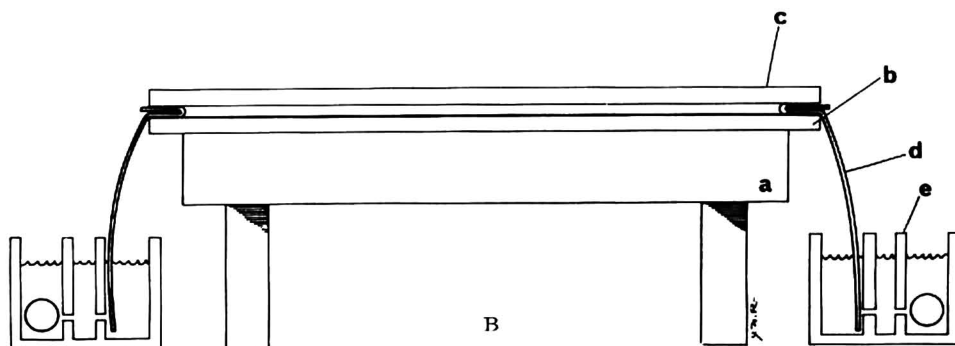


Fig. 2. Electrophoresis chamber. A. Assembled chamber with electrical connections and rubber tubings leading to the cooling coil. B. Schematic drawing. (a) Aluminum platform (190 mm × 190 mm). (b) Coated thin-layer plate. (c) Cover plate. (d) Paper connection. (e) Electrode vessel.

applied at a corner or at the middle of an edge of the plate. Usually migration occurs towards the cathode, permitting corner application for chromatography. A good buffer for electrophoresis consists of 1 ml of pyridine plus 10 ml of glacial acetic acid made up to 500 ml, pH 3.5. Ammonium acetate and ammonium carbonate buffers at low ionic strength are also satisfactory.

Peptide maps

Peptide maps are prepared on thin-layer plates (200 mm × 200 mm) by successive chromatography and electrophoresis as described above. The preliminary chromatographic runs indicated the choice of solvent and also suggested whether ascending

chromatography or the B-N chamber would be more feasible. Ascending chromatography, when feasible, saves considerable time, requiring 1–2 h as opposed to 10–15 h in the B-N chamber. Sample application is made with a micropipette. The amount of peptide mixture applied varies from 0.05 to 0.5 mg. To obtain highly concentrated deposits, repeated application can be made and the spots, not to exceed 4 mm in diameter, are dried with a hair dryer (excessive heat will damage the coating of the plate). Following chromatography, the plate is heated for 10 min at 100°, after cooling sprayed with the selected buffer, and subjected to electrophoresis in the direction perpendicular to that in which the chromatography was carried out. With the pyridine–acetic acid buffer at 950–1000 V and 30 mA. electrophoresis is completed in 1 h. The plate is then dried at 100° and the peptide spots developed with ninhydrin, or by using the chlorine *o*-tolidine test[9, 10]. The locations of the spots should be marked with a pencil immediately after applying the reagent because the color diffuses and fades rapidly.

RESULTS AND DISCUSSION

Fig. 3 shows a peptide map of a tryptic digest of protamine. Tryptic digests of myosin yield a much larger number of peptides, of which more than 60 were separated on a thin-layer plate (Fig. 4).

The thin-layer techniques we have described for making peptide maps result in a saving of time as compared to more conventional methods, and also permit the use of smaller amounts of proteolytic digest and relatively simple equipment. In contrast, peptide maps on paper require complicated apparatus: large chromatography jars, high voltage power supplies, and electrophoresis chambers with organic solvents as cooling liquids. In cases of difficult peptide separation, the B-N chamber with the modifications described above is recommended. However, by selecting an appropriate solvent system most separations can utilize ascending chromatography and thus

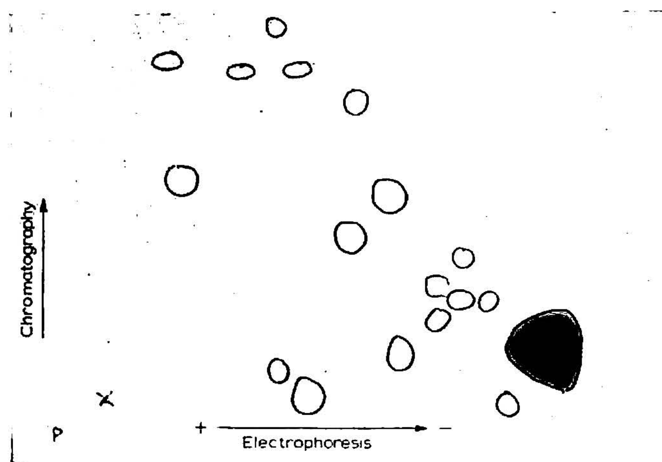


Fig. 3. Peptide map of the tryptic digest of protamine sulphate. Enzyme to substrate ratio = 1:100. Buffer: 0.1 *M* ammonium carbonate, pH 8.5. Incubation time: 24 h. First dimension: chromatography. Solvent system: Chloroform–methanol–ammonium hydroxide (40:40:20). Time: 60 min. Second dimension: electrophoresis. Buffer: pyridine–glacial acetic acid–water (1:10:489 ml). Current: 980 V, 30 mA. Time: 1 h.

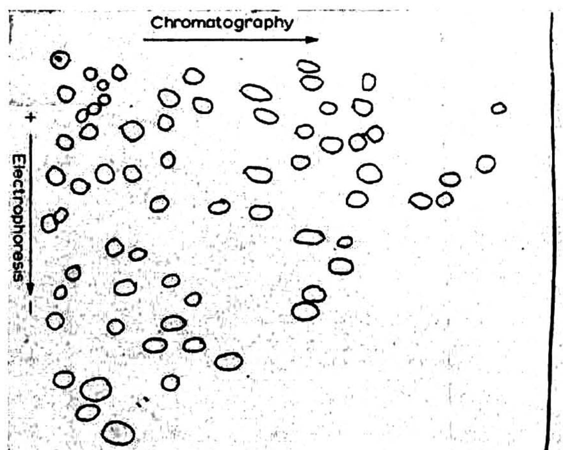


Fig. 4. Peptide maps of the tryptic digest of myosin. Same experimental conditions as in Fig. 3.

allow faster runs. Under such conditions, eight peptide maps can easily be prepared in a day. These techniques might be useful not only for structural analysis of proteins but also for checking the purity of fractions obtained by preparative column chromatography of peptide mixtures.

ACKNOWLEDGEMENTS

Dr. J. GERGELY's interest and generous support is gratefully acknowledged. The author wishes to thank Mrs. L. SESTANJ for skilful assistance and Dr. C. PAINE and Dr. F. ROTHSTEIN for valuable discussions.

The modified B-N chamber and the electrophoresis chamber were built in the Retina Foundation workshop under the supervision of Mr. R. THOMPSON.

SUMMARY

A method for preparing peptide maps is described which makes use of thin layer techniques instead of employing paper sheets. In short preliminary runs, the optimal experimental conditions (solvent systems, buffers, time, and sample concentrations) are explored and optimal conditions chosen for the preparation of the peptide maps. Chromatography of about two hours is followed by electrophoresis at 950–1000 V. The peptide mixture is applied in amounts of 0.05 to 0.5 mg per peptide map. Eight peptide maps are easily prepared per day.

REFERENCES

1 V. M. INGRAM, *Nature*, 178 (1956) 792.
2 A. M. KATZ, W. J. DREYER AND C. B. ANFINSEN, *J. Biol. Chem.*, 234 (1959) 2897.
3 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 16 (1960) 378.
4 A. R. FAHMY, A. NIEDERWIESER, G. PATAKI AND M. BRENNER, *Helv. Chim. Acta*, 44 (1961) 2022.
5 C. G. HONEGGER, *Helv. Chim. Acta*, 44 (1961) 173.
6 G. PASTUSKA AND H. TRINKS, *Chemiker Ztg.*, 85 (1961) 535.
7 J. GERGELY, M. GOUVEA AND D. KARIBIAN, *J. Biol. Chem.*, 212 (1955) 165.
8 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 17 (1961) 237.
9 F. REINDEL AND W. HOPPE, *Chem. Ber.*, 87 (1954) 1103.
10 E. STAHL, *Dünnschicht-Chromatographie*, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1962, p. 501.

THIN-LAYER CHROMATOGRAPHY IN THE STUDY OF ESTER SULPHATES

F. S. WUSTEMAN, K. S. DODGSON, A. G. LLOYD, F. A. ROSE AND N. TUDBALL
Department of Biochemistry, University College, Cardiff (Great Britain)

(Received March 26th, 1964)

It is generally recognised that thin-layer chromatography is superior to that on filter paper for the study of weakly polar substances. As yet, however, this rapid and sensitive technique has been relatively little used for the separation of organic molecules bearing strongly ionised groups.

In connection with studies on the biochemistry of a wide variety of sulphate esters, paper chromatography and electrophoresis are used routinely in these laboratories to establish the homogeneity of starting materials and to follow their transformations in biological systems. Frequent use is made of ^{35}S-labelled sulphate compounds which are then detected by autoradiography[1,2] or automatic strip-scanning[3].

This communication describes the application of thin-layer techniques to the separation of a variety of alkyl, aryl, and steroid sulphuric acid esters.

EXPERIMENTAL

Materials

Isomeric nitrocatechol sulphates were prepared by a modification of the method of SMITH[4] and other phenolic sulphate esters by a modification of the procedure of BURKHARDT AND LAPWORTH[5].

Sulphate esters of alcohols and hydroxylated amino acids were prepared according to LLOYD, TUDBALL AND DODGSON[6]. L-Tyrosine O-sulphate and related *para*-substituted phenolic sulphates and their parent compounds were obtained as described by FLANAGAN[7].

The N-sulphates of L-serine and bis(2-hydroxyethyl)amine were prepared by the method of WARNER AND COLEMAN[8] and L-cysteine S-sulphate by a modification of the method of CLARK[9].

Estriol sulphate and estradiol mono- and disulphates were gifts from A.B. Leo, Hälsingborg, Sweden; shark bile scymnol sulphate was provided by Professor G. A. D. HASLEWOOD whilst other steroid sulphates were either commercial preparations or were synthesised as described by ROY[10].

All sulphate esters were crystallised as sodium or potassium salts.

Solvents

Solvent A. Benzene–ethyl methyl ketone–ethanol–water, 3:3:3:1 (v/v).
Solvent B. 2-Propanol–chloroform–methanol–water, 10:10:5:2 (v/v).
Solvent C. 2-Propanol–chloroform–methanol–10 *N* ammonia, 10:10:5:2 (v/v).
Solvent D. 1-Butanol–acetic acid–water, 3:1:1 (v/v).

Spray reagents

Steroids were detected by spraying the dried plate with 50 % aqueous sulphuric acid and heating at 140° until coloured spots appeared[11]. Though the final colour obtained is characteristic for the steroid nucleus (for instance, grey-blue for cholesterol and dehydroepiandrosterone sulphates, orange for estrogen sulphates) the individual compounds listed vary in the ease with which the colours appear and in the intermediate shades obtained. In cases where identifications are not possible on the basis of R_F values alone comparisons of the colours produced by this spray with unknown and reference compounds should be of value in the characterisation of steroid sulphates.

Amino acids and their O-sulphate esters were rendered visible by a spray of 0.5 % ninhydrin in ethanol.

Para-substituted phenols related to L-tyrosine and their corresponding ester sulphates were detected by a modification of the spray for *para*-alkylated phenols on paper as described by TOMPSETT[12]. The dry plate was sprayed lightly with a 0.3 % solution of 1-nitroso-2-naphthol in acetone, followed by a solution of nitrous acid prepared by mixing equal volumes of ice-cold aqueous solutions of sulphuric acid (10 %) and sodium nitrite (5 %). Heating at 140° gives a purplish-red colour with *para*-alkylated phenols and (more slowly) their sulphate esters while most other phenol derivatives give a sandy-red colour.

Many phenolic sulphates, in particular those bearing a nitro substituent, could be located as dark areas when the plate was viewed under ultraviolet light.

Any compound not readily detected by one of the above methods was made visible by means of a spray prepared by dissolving potassium permanganate (0.5 g) in sulphuric acid (15 ml)[13]. Greater contrast was obtained, where necessary, by overspraying with an aqueous solution of a redox indicator (barium diphenylamine sulphonate, British Drug Houses Ltd.).

Preparation of plates

A slurry of Silica Gel G (Merck and Co.) (30 g) in water (60 ml) was applied to 5 × 20 cm and 20 × 20 cm glass plates using a Unoplan Leveller (Shandon & Co.) with the spreader set at 250 μ. The plates were dried at 110° for 45 min and stored over anhydrous silica gel.

Chromatographic procedure

Loads of 3–5 μg were applied in 1 μl aliquots of aqueous solution. Some classes of compounds required lower (steroid sulphates) or higher (alkyl sulphates) loads due to the differing sensitivity of the spray reagents.

Plates were developed by the ascending technique over a distance of 10 cm in a pre-equilibrated tank lined with filter paper, or by the use of a saturation chamber as described by DAVIES[14].

Though R_F values on thin-layer plates are reproducible if conditions are carefully controlled (for instance, unvarying room temperature and maintenance of a constant distance between the origin and solvent level in the reservoir—see ref. 15) it was found more convenient, for routine runs, to measure mobilities relative to those of reference compounds. Thus the mean R_F values of L-tyrosine (solvent D), phenyl sulphate (solvents A and B) and dehydroepiandrosterone-3β-sulphate (solvents

A and C) were calculated from at least five independent determinations. The mobilities of all other compounds were then corrected with reference to one of these three standards and absolute R_F values calculated accordingly.

The separation of ester sulphates of phenols, aliphatic alcohols and steroids

The results of this study are summarised in Tables I to V.

TABLE I

R_F VALUES OF L-TYROSINE, RELATED *p*-HYDROXYPHENYL DERIVATIVES, AND THEIR ESTER SULPHATES

Compound	*R_F of parent compound*		*R_F of ester sulphate*	
	Solvent D	*Solvent A*	*Solvent D*	*Solvent A*
L-Tyrosine	0.58	0.15	0.32	0.05
4-Hydroxyphenylpyruvate	0.65, 0.45	0.13	0.25	0.06
4-Hydroxyphenylacetate	1.00	0.31	0.61	0.08
4-Hydroxyphenylpropionate	1.00	0.42	0.72	0.10
4-Hydroxyphenylacrylate	1.00	0.41	0.78	0.08
4-Hydroxybenzoate	1.00	0.52	0.76	0.07
4-Hydroxybenzaldehyde	1.00	0.88	0.75	0.42
Methyl L-tyrosine	0.65	0.74	—	—
Ethyl L-tyrosine	0.66	0.80	—	—

TABLE II

R_F VALUES OF HYDROXYLATED AMINO ACIDS, THEIR ESTER SULPHATES, AND MISCELLANEOUS RELATED COMPOUNDS

Ester sulphate	*R_F in solvent D*	
	Substance	*Parent compound*
L-Serine O-sulphate	0.21	0.30
L-Serine N-sulphate	0.25	0.30
L-Threonine O-sulphate	0.23	0.34
L-Hydroxyproline O-sulphate	0.22	0.28
L-Cysteine S-sulphate	0.28	0.43
Glycollate O-sulphate	0.27	0.54
Bis(2-hydroxyethyl)amine N-sulphate	0.28	—
Singirin	0.35	—

TABLE III

R_F VALUES OF ESTER SULPHATES OF ALIPHATIC ALCOHOLS IN SOLVENT B

Compound	*R_F*	*Compound*	*R_F*
Methyl sulphate	0.31	Ethyl sulphate	0.38
Cyclopentyl sulphate	0.45	Cyclohexyl sulphate	0.53
n-Hexyl sulphate	0.56	Propanediol monosulphate	0.11

TABLE IV

R_F VALUES OF SUBSTITUTED PHENOL SULPHATES

Ester sulphate of	*R_F in solvent*	
	A	*B*
Phenol	0.46	0.45
2-Chlorophenol	0.48	0.47
3-Chlorophenol	0.50	0.49
4-Chlorophenol	0.50	0.48
2-Methylphenol	0.47	0.47
3-Methylphenol	0.47	0.51
4-Methylphenol	0.47	0.49
2-Methoxyphenol	0.40	0.41
3-Methoxyphenol	0.45	0.46
4-Methoxyphenol	0.45	0.44
4-Hydroxy-3-nitrophenol	0.46	0.48
4-Hydroxy-2-nitrophenol	0.60	0.53
2-Hydroxy-5-nitrophenol	0.51	0.41
2,3-Dichlorophenol	0.54	0.53
2,4-Dichlorophenol	0.54	0.53
3-Nitrophenol	0.53	0.50
4-Nitrophenol	0.57	0.52
2-Hydroxy-4-chlorophenol	0.56	0.47

TABLE V

R_F VALUES OF STEROID SULPHATES

Substance	*R_F in solvent*	
	A	*C*
Cholesterol-3β-sulphate	0.58	0.67
Cortisone-21-sulphate	0.44	0.58
Dehydroepiandrosterone-3β-sulphate	0.49	0.66
Estradiol-3-sulphate	0.48	0.60
Estradiol-17-sulphate	0.46	0.60
Estradiol-3,17-disulphate	0.16	0.27
Estriol-3-sulphate	0.35	0.48
Estrone-3-sulphate	0.54	0.71
Parent steroids	0.85–0.95	1.00
Scymnol sulphate	0.29	0.35

In general ester sulphates of weakly polar compounds can be resolved in solvent A (solvents B and C give closely similar results) while those with other polar groups in the molecule require a conventional 1-butanol–acetic acid–water mixture of which solvent D is a typical example.

DISCUSSION

Though the R_F values of only a selection of alkyl, aryl and steroid ester sulphates are quoted here it is clear that silica gel can usefully replace filter paper as the supporting medium for chromatographic studies on compounds of this type. It also seems likely

that the same can be said for S- and N-sulphates since the examples studied here [those of L-cysteine, L-serine and bis(2-hydroxyethyl)amine] had mobilities of the same order as those of similar O-sulphates.

Since it is often desirable to locate the ester sulphate and its parent compound on the same chromatogram, the spray reagents chosen were ones which either split-off the sulphate group instantaneously or reacted with another part of the molecule. The introduction of an ester sulphate group has a profound effect on the chromatographic mobility of all compounds except those (such as sugars) which are highly polar themselves. This means that, in solvents which resolve ester sulphates, the parent unsulphated compounds usually move at, or near, the solvent front.

Isomeric monosubstituted phenolic sulphates are poorly resolved while the parent phenols[16] can be separated readily on thin-layers of Silica Gel G. This can be attributed to the effect on R_F values of interactions between the hydroxyl group and other substituents on the phenolic nucleus, particularly those in the *ortho* position[16]. Such interactions are impossible for the highly ionised, chemically-saturated sulphate group. Though mixtures of such isomers are unlikely to be met in practice, they can be resolved using specific colour reagents (for instance, the nitrosonaphthol spray for various substituted phenol sulphates) or by removing the ester sulphate group by acid hydrolysis and identifying the parent compound. Many workers have already studied the resolution of free steroids[17] and amino acids[18] by means of thin-layer chromatography.

Apart from the greater speed and sensitivity common to all thin-layer work, the systems described here possess several advantages not found in those currently used for the separation of sulphuric acid esters. As already mentioned, the inert support allows the use of vigorous sprays which detect both sulphate esters and parent compounds at the same time. The spots can also be scraped off and are easily eluted for isolation or spectral examination of the compound. Since ascending development takes between 35 min (solvent A) and 150 min (solvent D) and the plates are easily handled, routine two-dimensional separations are quite feasible, so allowing detailed resolution of complex mixtures. Chromatography can be combined with electrophoresis in a second dimension[19], a technique which is particularly valuable in the case of strongly acidic compounds like ester sulphates since the electrophoretic mobility of compounds bearing weaker acid groups can be reduced or completely suppressed by working at a low pH. Electrophoresis and chromatography are complementary in this respect since inorganic sulphate has negligible mobility in the solvent systems quoted here.

The use of an inorganic supporting medium promises to be of particular value in studies where spots labelled with (^{35}S) are to be located since such spots are better defined than on paper and can be located either by autoradiography or by automatic scanning of the glass plate[19].

ACKNOWLEDGEMENTS

This work was supported by a grant (No. A-1982) to K.S.D. from the U.S. Public Health Service.

SUMMARY

Thin-layer chromatography on silica gel has been applied to the separation of sulphate

esters of alkyl, aryl and steroid hydroxy compounds from each other and from their parent unsulphated compounds. The value of this technique in studies on the biochemistry of sulphate esters is discussed.

REFERENCES

1 T. YAGI, *Biochim. Biophys. Acta*, 82 (1964) 170.
2 J. B. ADAMS, *Biochim. Biophys. Acta*, 71 (1963) 243.
3 K. S. DODGSON, A. G. LLOYD AND N. TUDBALL, *Biochem. J.*, 79 (1961) 111.
4 J. N. SMITH, *J. Chem. Soc.*, (1951) 2861.
5 G. N. BURKHARDT AND A. LAPWORTH, *J. Chem. Soc.*, (1926) 684.
6 A. G. LLOYD, N. TUDBALL AND K. S. DODGSON, *Biochim. Biophys. Acta*, 52 (1961) 413.
7 T. H. FLANAGAN, *Ph. D. Thesis*, University of Wales, 1963.
8 D. T. WARNER AND L. L. COLEMAN, *J. Org. Chem.*, 23 (1958) 1133.
9 H. T. CLARK, *J. Biol. Chem.*, 97 (1932) 235.
10 A. B. ROY, *Biochem. J.*, 62 (1956) 41.
11 R. D. BENNETT AND E. HEFTMANN, *J. Chromatog.*, 9 (1962) 348.
12 S. L. TOMPSETT, *Clin. Chim. Acta*, 3 (1958) 149.
13 H. ERTEL AND L. HORNER, *J. Chromatog.*, 7 (1962) 268.
14 B. H. DAVIES, *J. Chromatog.*, 10 (1963) 518.
15 M. BRENNER, A. NIEDERWIESER, G. PATAKI AND A. R. FAHMY, *Experientia*, 18 (1962) 101.
16 K. RANDERATH, *Thin-Layer Chromatography*, Academic Press, London, 1963, p. 177.
17 K. RANDERATH, *Thin-Layer Chromatography*, Academic Press, London, 1963, p. 111.
18 M. BRENNER AND A. NIEDERWIESER, *Experientia*, 16 (1960) 378.
19 K. RANDERATH, *Thin-Layer Chromatography*, Academic Press, London, 1963, p. 65.

MÉTHODES DE DÉTECTION DES STÉROÏDES CONJUGUÉS SÉPARÉS PAR CHROMATOGRAPHIE EN COUCHE MINCE

O. CRÉPY, O. JUDAS ET B. LACHESE

Laboratoire de Chimie biologique, Faculté de Médecine, Paris (France) *

(Reçu le 16 mars 1964)

Dans ce travail nous présentons une adaptation à la chromatographie en couche mince de deux procédés de détection des sulfates et des glucosiduronates de stéroïdes utilisés en chromatographie sur papier[1-2].

Nous décrivons deux exemples de leur application à l'étude des stéroïdes conjugués: le premier concerne la séparation des glucosiduronates de 5β-pregnane 3α-yl 20α-ol et de 5β-pregnane 3α-yl 20-one et de leurs isomères en 5α; le second se rapporte à l'identification des sulfates de stéroïdes en C_{19}.

DÉTECTION DES GLUCOSIDURONATES

Cette technique est basée sur la combinaison des glucosiduronates avec le 1(2 pyridyl-azo)2-naphtol et la formation avec le cobalt d'un chélate coloré.

Réactifs utilisés

(1) Solution de 1(2-pyridyl-azo)2-naphtol ou P.A.N. à 0.4 g % dans l'éthanol. Ce réactif est dilué extemporanément par 4 volumes de dichlorométhane.

(2) Solution de nitrate de cobalt à 0.8 % dans l'eau bidistillée.

(3) Solution tampon d'acétate de Na/acide acétique 2 *M*, de pH 4.6, préparée avec de l'eau bidistillée et de l'acide acétique exempt de traces de fer.

(4) A 8 ml de la solution (2) on ajoute 4 ml de la solution (3) et on complète a 100 ml avec de l'eau bidistillée.

Mode opératoire

La solution (1) (P.A.N.) est pulvérisée sur la plaque jusqu'à ce que toute celle-ci soit d'un jaune uniforme. Quand la plaque est sèche, la solution (4) est pulvérisée à son tour. Les taches de glucosiduronates apparaissent en violet sur fond jaune. Ces taches changent d'aspect rapidement. Cependant quand la plaque est sèche elles réapparaissent plus faiblement en verdâtre sur fond vert plus clair. Il est possible avec ce procédé, de déceler 1 à 2 μg de glucosiduronates.

En chromatographie sur papier, il est indispensable d'enlever l'excès de P.A.N. en traitant le chromatogramme par le chlorhydrate de phénylhydrazine[1]. Il n'est pas nécessaire de le faire avec la chromatographie sur plaques.

Par contre, toute trace de métaux susceptibles de former des chélates avec le

* Laboratoire de Chimie biologique (Prof. M. F. JAYLE), 45 rue des Saints-Pères, Paris-6e.

P.A.N. doit être prohibée. C'est pourquoi il faut utiliser de l'eau bidistillée pour la préparation des plaques. Il faut également que les solvants soient très purs et que l'acide acétique soit exempt de fer. (Nous utilisons à cet usage l'acide acétique glacial pour analyses Merck.)

DÉTECTION DES SULFATES DE STEROÏDES

Cette réaction est basée sur la formation d'un complexe coloré, entre le bleu de méthylène et les estersulfates.

Mode opératoire

25 mg de bleu de méthylène sont écrasés dans un mortier, en ajoutant à plusieurs reprises de l'acide sulfurique $N/20$ et en décantant la solution dans une fiole de 100 ml jusqu'à ce que tout le bleu de méthylène soit dissous. La fiole est complètée au trait de jauge avec la solution d'acide sulfurique et gardée à l'abri de la lumière. Au moment de l'emploi, on dilue le réactif avec volume égal d'acétone pure. Le réactif est pulvérisé sur la plaque jusqu'à ce que celle-ci soit uniformément bleue. Les taches apparaissent rapidement avec des couleurs variants suivant les estersulfates. Ces couleurs sont stables pendant plusieurs heures, elles s'atténuent ensuite.

Il arrive exceptionnellement que certains sulfates comme le sulfate de *p*-crésol se révèlent difficilement. On peut cependant les mettre en évidence en plaçant la plaque dans une cuve contenant du chloroforme. Si on laisse celui-ci monter par capillarité, il entraîne avec lui le complexe estersulfates/bleu de méthylène et laisse à sa place une tache blanche.

L'addition d'acétone au réactif limite la diffusion des taches et accélère son évaporation. Elle permet d'obtenir une pulvérisation plus homogène, les gouttelettes étant beaucoup plus fines, en raison de la diminution de la tension superficielle du liquide. Le fond bleu ciel est plus uni et les taches sont plus nettes. Le sulfate de Na présent dans le réactif de Vlitos habituel a été supprimé car ce sel est précipité par l'acétone.

Par ce procédé, il est possible de déceler 1 à 2 μg d'estersulfate.

APPLICATION

Premier exemple

Séparation des glucosiduronates de 5β-pregnane 3α-yl 20α-ol, 5β-pregnane 3α-yl 20-one, 5α-pregnane 3β-yl 20α-ol et 5α-pregnane 3β-yl 20-one, par chromatographie de partage en deux dimensions.

Support utilisé. Mélange 9/1 de Kieselguhr G/Kieselgel G. La poudre délayée dans deux fois son poids d'eau bidistillée est étalée sur la lame de verre selon la méthode classique de STAHL; épaisseur: 0.25 mm.

Les plaques sont chauffées à 105° pendant 30 min et gardées dans un exsiccateur.

Système de solvants utilisé. Toluène–butanol tert.–acide acétique–eau (82:18:30:70).

Comme il s'agit d'une chromatographie de partage il est nécessaire que la plaque soit en équilibre avec les deux phases du mélange. Pour cette raison, le papier Whatman qui borde les parois de la petite cuve rectangulaire est imprégné de la phase aqueuse et plusieurs béchers contenant l'une et l'autre des phases sont placés au fond de la cuve.

Après avoir déposé en 1 tache de 3 mm de diamètre l'extrait contenant les 4 glucosiduronates, à 1.5 cm du bord gauche et au bas de la plaque, celle-ci est placée dans la cuve. On laisse l'équilibre s'établir pendant $1^1/_2$ h, puis on introduit la phase organique du solvant. Lorsque celui-ci a atteint environ 15 cm la plaque est sèchée à l'air et replacée dans la cuve en la faisant tourner de 45°. On attend de nouveau $1^1/_2$ h avant de verser la phase mobile qui monte de nouveau jusqu'à environ 15 cm. Après séchage de la plaque, celle-ci est révélée par le réactif au P.A.N.

La Fig. 1 illustre les résultats obtenus. On voit que par cette technique les 4 glucosiduronates sont bien séparés.

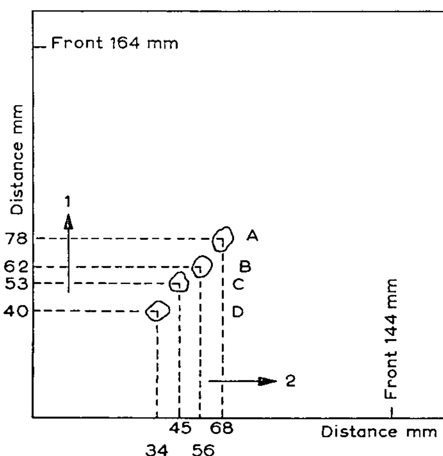


Fig. 1. Séparation par chromatographie en deux dimensions des 4 glucosiduronates suivants.

	Glucosiduronate de	R_F (1)	R_F (2)
(A)	5α-prégnane 3α-yl 20-one	0.47	0.47
(B)	5β-prégnane 3α-yl 20-one	0.38	0.38
(C)	5α-prégnane 3α-yl 20-ol	0.32	0.31
(D)	5β-prégnane 3α-yl 20-ol	0.24	0.24

Deuxième exemple

Chromatographie de partage et détection par le réactif au bleu de méthylène, du sulfate de *p*-crésol et de 5 estersulfates de stéroïdes en C_{19}.

Snpport utilisé. Mélange de Kieselguhr G/Kieselgel G préparé comme dans le cas précédent. Les proportions sont: (1) 19/1 et (2) 9/1.

Système de solvants. Mélange de acétate de butyle–toluène–NH_4OH 4*N*–méthanol dans les proportions (1) 85:35:50:70 et (2) 110:90:120:160.

Les plaques sont mises en équilibre avec les deux phases du solvant comme dans le cas précédent ou selon la variante suivante: deux cuves sont préparées; dans la première, la phase aqueuse se trouve dans le fond de la cuve et imprégne aussi le papier Whatman adhérant aux parois. Une série de petits béchers sont remplis de phase organique. Après dépôt des extraits sur la plaque celle-ci est placée dans la cuve,

sur des petits rouleaux de verre qui la maintiennent au-dessus de la surface du liquide. Dans la deuxième cuve, la phase organique se trouve au fond et imprègne aussi le papier entourant les parois, la phase aqueuse est contenue dans une série de petits béchers.

Après 2 h de séjour dans la première cuve, la plaque est retirée et plongée immédiatement dans la deuxième cuve. Le solvant organique monte par capillarité jusqu'à 15 cm. La plaque est alors séchée à l'air et révélée par le réactif au bleu de méthylène. Les résultats sont indiqués dans le Tableau I.

TABLEAU I

CHROMATOGRAPHIE DES DIFFÉRENTS SULFATES DE STEROÏDES EN C_{19}

	Migration en cm		*Révélation par le réactif au bleu de méthylène*
	*Syst. 1**	*Syst. 2**	*Couleur des taches*
Sulfate de *p*-crésol	5.1	3.2	± bleu
Sulfate d'androsta-5-ene 3β-yl 17-one (déhydroepiandrostérone)	8.5	5.0	rose vif
Sulfate de 5α-androstane 3α-yl 17-one (androstérone)	8.2	5.1	bleu outre-mer
Sulfate de 5α-androstane 3β-yl 17-one (épiandrostérone)	8.7	5.5	rose orangé
Sulfate de androst-5-ane 3β-yl 20β-ol (androstenediol)	6.1	3.8	rose vif
Sulfate de 5α-androstane 3α-yl 20β-diol (androstanediol)	7.6	4.0	violet

* *Solvants*: Systeme 1: Acétate de butyle–toluène–NH_4OH 4 *N*–méthanol (85:35:50:70). Système 2: Acétate de butyle–toluène–NH_4OH 4 *N*–méthanol (110:90:120:160).

Les migrations des sulfates d'androstérone, de déhydroépiandrostérone et d'épiandrostérone sont très voisines mais les couleurs obtenues avec ces stéroïdes sont différentes. Les 3 stéroïdes possédant un hydroxyle en 3β donnent des couleurs rose vif ou orangé, les 3α-hydroxystéroïdes donnent une coloration bleue ou violette.

RÉSUMÉ

Description de deux procédés de révélation des stéroïdes conjugués adaptés à la chromatographie en couche mince, le premier utilisant le réactif au pyridyl-azo-naphtol pour la révélation des glucosiduronates, le deuxième, le réactif au bleu de méthylène pour celle des estersulfates.

Leur application à la séparation chromatographique des glucosiduronates de 5α- et 5β-prégnane 3α-yl 20α-ol et de 5α et 5β-pregnane 3α-yl 20-one et à celle de cinq estersulfates en C_{19} est discutée.

SUMMARY

Two procedures for the detection of conjugated steroids on thin-layer chromatograms are described. In the first, pyridyl-azo-naphthol is used to reveal glucosiduronates, and in the second methylene blue reagent for sulphates. The application of these reagents to the chromatographic separation of the glucosiduronates of 5α- and 5β-pregnan-3α-yl-20α-ols and 5α- and 5β-pregnan-3α-yl-20-ones, as well as to that of five C_{19} sulphates, is discussed.

BIBLIOGRAPHIE

1 O. CREPY, B. LACHESE ET O. JUDAS, *Rev. Franc. Etudes Clin. Biol.*, 6 (1961) 601.
2 O. CREPY ET O. JUDAS, *Rev. Franc. Etudes Clin. Biol.*, 5 (1960) 284.

SEPARATION AND DETERMINATION OF AMINOANTHRAQUINONES BY MEANS OF THIN-LAYER CHROMATOGRAPHY

JAROSLAV FRANC AND MARIE HÁJKOVÁ
Research Institute for Organic Syntheses, Pardubice-Rybitví (Czechoslovakia)

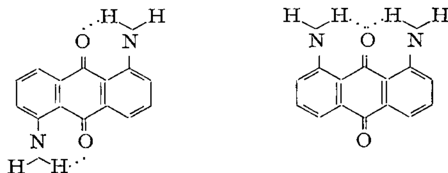
(Received March 16th, 1964)

The 1,5- and 1,8-dinitroanthraquinones prepared by nitrating anthraquinone are important intermediates in the production of dyes. It is known that in this nitration process other isomers are also produced, namely 1,6-, 1,7-, 2,6- and 2,7-, together with 1- and 2-nitroanthraquinone. The qualitative separation of all these isomers has been reported earlier[1]. Petroleum-impregnated chromatographic paper was used for the separation, the R_F value of the 1,8-isomer was about 0.8, while the 1,5-isomer remained at the start.

In practice, however, this procedure is unsuitable owing to the fact that it is never known whether or not a number of other substances are remaining at the start together with the 1,5-isomer and thus escaping quantitative determination. Also, when the evaluation is done by colorimetric measurement directly on the paper, the determination of substances remaining at the start is subject to a large error.

Since the corresponding amino-derivatives are also used as intermediates in the preparation of dyes, a separation of various isomers in this form has been attempted.

The separation of the amino-derivatives, however, presents a certain difficulty. Whereas the dinitro-derivatives have widely differing R_F values due to large differences in their dipole moments, 1,5- and 1,8-diaminoanthraquinone are very difficult to separate because they have only a relatively low difference in their dipole moments which is practically totally removed by the formation of inner hydrogen bonds:



For this reason the separation of these isomers by means of paper chromatography was not successful. However, it was possible to achieve a separation which was good enough for quantitative evaluation by means of thin-layer chromatography. We have also succeeded in separating 1-aminoanthraquinone, which sometimes accompanies the diaminoanthraquinone derivatives mentioned in smaller or larger amounts, together with other isomers, such as the 1,7- and 1,6-isomers.

The quantitative evaluation of individual components is carried out by means of an instrument for the automatic evaluation of paper chromatograms in reflected light.

EXPERIMENTAL

The separation is carried out by means of chromatography on a thin layer of Al_2O_3, using glass plates of 4 × 15 cm size. The reason for selecting this size is that it is the maximum size for the automatic evaluation instrument used; the layer thickness is 1.5 mm.

Technical Al_2O_3 of grain size less than 100 DIN is used. The Al_2O_3 is first neutralised with HCl and after washing with water activated for 2 h at 350°.

Deactivation is carried out in a desiccator, which is first evacuated for one hour in order to remove dimethylformamide from the sample, and then water is placed in the desiccator and left there for $^1/_2$ h. The activity of aluminum oxide prepared in this way corresponds to activity III according to Brockmann. The aminoanthraquinone sample is dissolved in dimethylformamide to make an 0.2 % solution, which is then diluted with dimethylformamide to 0.04 %. 0.02 ml of this solution (*i.e.* a total of 8 μg) is placed on the start of the chromatogram. The developing solvent is a cyclohexane–ether mixture (1:1).

After separation, the solvent is allowed to evaporate and the intensity of the spots, with the chromatogram in the horizontal position, is measured by means of the Extinktions-Registriergerät with Integrator ERI-10 (Zeiss, Jena), which can work with transmitted as well as reflected light. It is possible to measure spots even in a freely poured layer of Al_2O_3. A suitable filter is used for the measurement (for aminoanthraquinones it is yellow-green, with an absorption maximum at about 510 mμ). Fig. 1 shows a typical result for a mixture of diaminoanthraquinones.

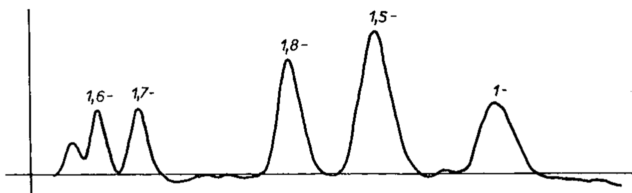


Fig. 1. Densitometer recording after chromatographic separation of various aminoanthraquinone isomers.

Since it has been found that at the concentrations used there is a linear relationship between the area of the spots and the concentration, it is sufficient to determine the ratio of the areas of individual pure components mixed in the same weight ratio.

The magnitude of the coefficient of variation, which has a mean value of ± 6 %, has been determined by statistical evaluation of results obtained for synthetic mixtures.

RESULTS AND DISCUSSION

In the course of our work several thousand determinations have been carried out and thus considerable experience with the method has been gained. It was found that it is very important to work with a suitable activity, or otherwise the spots will be smudged and overlap due to the relatively close R_F values of 1,5- and 1,8-diaminoanthraquinone.

Originally, the Al_2O_3 was deactivated by simply exposing the layer to atmospheric moisture, which was measured by means of a hygrometer. This method, however, was not suitable, since at 95% relative humidity deactivation took 4 h, and at 75 % took $1^1/_2$days. The deactivation is therefore carried out in a moist chamber. This also protects aluminum oxide from contamination by other substances in the laboratory atmosphere, which might interfere with the separation process. It is also possible to evacuate the desiccator first and thus remove dimethylformamide, whose presence interferes in the separation. The aluminum oxide must not be basic.

Owing to the high volatility of one of the components of the developing mixture it is necessary to work as far as possible at temperatures around 20°.

Quantitative evaluation was possible with a Zeiss densitometer, which measures the chromatograms in a horizontal position and can work with transmitted as well as reflected light. It was designed originally for electropherograms, and we believe there has been no previous report of its application to thin-layer chromatograms.

The mixtures most frequently analysed contained the following components: 1,5-, 1,8-, 1,6- and 1,7-diaminoanthraquinone and 1-aminoanthraquinone. The relatively high coefficient of variation is caused by the lack of precision of the determination of 1-aminoanthraquinone and 1,7-diaminoanthraquinone. In the case of 1-aminoanthraquinone this is caused by the continuity of the aluminum oxide layer being slightly disturbed during the process of development, so that behind the 1-aminoanthraquinone the zero line is slightly distorted. 1,7-Diaminoanthraquinone is close to the starting line, where impurities, probably hydroxyanthraquinones or amino-hydroxyanthraquinones, are always present and interfere in the determination of the zero line of this isomer. 2-Aminoanthraquinone is usually also present, but mostly in small amounts, and is difficult to separate from 1,8-diaminoanthraquinone.

One advantage of this aminoanthraquinone separation is that owing to the fact that these substances are self-coloured, it is unnecessary to use any further means of detection of the spots. After removal of the plate from the development chamber the colour is relatively intense, but weakens after evaporation of the solvent; after that, however, it does not change for more than 24 h.

Although the precision of the determination is not very great, the error may be considerably decreased by repeated determinations. It is, furthermore, the only method for the determination of the 1,5- and 1,8-isomers in the presence of each other. It would be advantageous if the chromatographic separation could be performed on larger plates, but unfortunately we are limited by the dimensions of the densitometer. To increase precision, especially where the ratios of the components are unfavourable, it is recommended that a synthetic mixture, whose composition corresponds approximately to that of the sample, is analysed at the same time. Thus it is possible to determine which of the components causes the greatest error.

The time for the complete analysis is relatively short, the chromatographic separation taking about 10 min, and evaluation on the densitometer about 2.5 min. It is unnecessary to state that all operations must be performed with the utmost care, since this is an ultramicroanalysis.

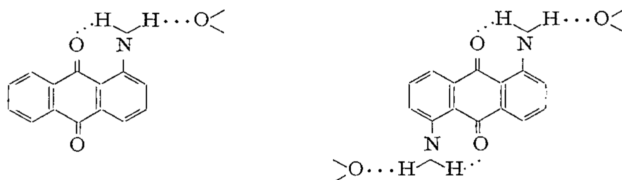
From Table I it would seem that the separation process is mainly controlled by the formation of hydrogen bonds. For example, 1-aminoanthraquinone has a high R_F value, since it has an internal hydrogen bond, and thus cannot form an intermolecular bond with the immobile phase; 2-aminoanthraquinone, on the other hand, has a far

TABLE I

R_F VALUES OF AMINOANTHRAQUINONES

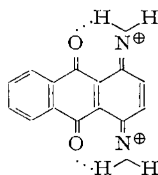
Anthraquinone	R_F	*Colour of spot*	*Number of hydrogen bonds (O···H–N)*
1-Amino-	0.62	orange	1
2-Amino-	0.23	yellow	0
1,2-Diamino-	0.065	violet	1
1,4-Diamino-	0.10	violet	2
1,5-Diamino-	0.46	orange-red	2
1,8-Diamino-	0.35	red-violet	2
2,6-Diamino-	0.00	orange-brown	0
1,6-Diamino	0.08	red	1
1,7-Diamino	0.14	orange	1

lower R_F value ($R_F = 0.39$), due to the absence of an internal hydrogen bond. The case of the diaminoanthraquinones is similar, the isomers which have two internal hydrogen bonds having higher R_F values than those which have only one, and these again have higher R_F values than isomers which have no internal hydrogen bond. 1-Aminoanthraquinone still, however, has a higher R_F value than the diaminoanthraquinones which have two internal hydrogen bonds, owing to the fact that only one hydrogen atom of the $-NH_2$ group forms a chelate bond:



Thus, the diaminoanthraquinones are more strongly bound to the stationary phase, in this case to water.

In the case of diaminoanthraquinones which have two internal hydrogen bonds, it is possible, up to a certain measure, to assess the energy of this bond from the R_F values. The 1,5-isomer has the highest R_F value, because the energy of both bonds is equal. In the 1,8-isomer, both protons form a chelate bond with the same oxygen, resulting in the fact that the second bond is weaker due to electrons being exhausted by the first bond, so that a part of this bond participates in the intermolecular bond with the immobile phase. It is interesting to compare the 1,4- and 1,5-isomers, whose R_F values ought to be equal. In fact, the R_F value of the 1,4-isomer is lower. This would seem to show that the second hydrogen bond is not formed, or if it is formed then the energy of the intermolecular hydrogen bond is far greater than would correspond to the structure:



The colour of the individual isomers is also related to the number of internal hydrogen bonds. It seems that the colour is darker with a larger number of internal hydrogen bonds. Comparison is also possible with respect to substitution in the 1- and 2-positions, since 1-substituted isomers are darker.

Another point of interest is the chromatographic equivalent of the hydrogen bond for this system, *viz.* aluminum oxide saturated with water and a cyclohexane–ether mobile phase. Considering that the energy of the hydrogen bond in 1-aminoanthraquinone is 4.8 kcal/mole (ref. 2), we obtain for the chromatographic equivalent of the hydrogen bond a value of $R_E = 0.65$ kcal/mole, the same value as was calculated for most separations (with an immobile aqueous phase; $R_E = 0.59 \pm 0.05$ kcal/mole) in paper chromatography.

SUMMARY

A procedure for the separation and determination of mono- and di-aminoanthraquinones has been developed. The separation is carried out on a thin layer of poured granular Al_2O_3, and the colorimetric evaluation of spots carried out with a Zeiss Type ERI-10 instrument. The precision of the method is $\pm$ 6%.

REFERENCES

1 J. FRANC, *Chem. Listy*, 49 (1955) 872; *Collection Czech. Chem. Commun.*, 20 (1955) 1384.
2 J. FRANC, *Chem. Listy*, 52 (1958) 13; *Collection Czech. Chem. Commun.*, 24 (1959) 250.

THIN-FILM ELECTROPHORESIS

PART I. THE ELECTROPHORETIC BEHAVIOUR OF COAL-TAR FOOD COLOURS ON PAPER AND THIN FILMS

W. J. CRIDDLE, G. J. MOODY AND J. D. R. THOMAS
Department of Chemistry, Welsh College of Advanced Technology, Cardiff (Great Britain)

(Received March 2nd, 1964)

Over the last decade a large number of papers have dealt with the paper chromatographic behaviour of food colours. The introduction of *The Colouring Matter in Food Regulations*, 1957[1] resulted in attempts to standardise detection methods and this culminated in the publication, in 1960, of a monograph[2] on the separation and identification of the food colours permitted in the United Kingdom. While the majority of the papers published use R_F values as the means of identification, YANUKA *et al.*[3] maintain that it is difficult to obtain reproducible results for absolute R_F values. They have described an identification method based on a characteristic curve composed of eight spots, instead of on a single R_F value. The curve is obtained by simultaneously running eight chromatograms of the colour under investigation at eight different pH values in one single solvent system.

The separation and identification of food colours by thin-layer chromatographic techniques was first reported over ten years ago[4,5], but this and subsequent work[6-9] appears to have been confined to alumina.

With regard to electrophoretic studies on food colours, these have usually been carried out on paper[10-12], although the use of cellulose acetate membrane[12] and thin film supports[13] have recently been described.

The rapid progress in thin-film chromatography over the last few years has prompted the use of such materials as alumina, kieselguhr and silica gel as supporting adsorbents for electrophoretic studies of amines and amino acids[14,15], periodate and iodate[16], and phenols and phenol carboxylic acids[17]. Apart from a preliminary report[13], no other work appears to have been published on the electrophoretic behaviour of food colours on thin films.

Except for Oil Yellow GG, Oil Yellow XP, Naphthol Yellow S and Ponceau 3R all the permitted coal-tar food colours[1] have been subjected to electrophoresis in six different electrolytes of widely varying pH values on the thin-film materials, kieselguhr (Shandon), alumina G (Merck) and silica gel G (Merck), as well as on Whatman No. 1 filter paper.

EXPERIMENTAL

Preparations of electrolyte solutions

Acetic acid (N) solution, ammonium hydroxide (0.1 N) solution and the buffer solutions of pH 4.0, 6.0 and 8.0 were prepared in the manner previously described[10]. The buffer solution of pH 9.2 is a 0.05 M aqueous solution of borax.

Preparation of the colour solutions

Aqueous solutions (0.1 % w/v) of the appropriate powdered colour were used throughout.

Apparatus

The thin films were prepared from a slurry of the appropriate adsorbent in water (30 g adsorbent to 60 ml water) with a Shandon "Unoplan" Leveller and Spreader in the usual way and dried at 105°.

The Baird and Tatlock Constant Current/Constant Voltage Electrophoresis Apparatus was used for the experiments.

Procedure

For electrophoresis on thin films it was found convenient to score the dry film into strips by means of a scriber. The scored plate (20 cm × 17.5 cm) was then placed across the bridge of the horizontal electrophoresis tank and contact made between the film and each electrolyte compartment by means of a filter paper wick previously soaked with the electrolyte solution under study and with one edge resting along the full width of the plate of film. The lid was then placed on the electrophoresis tank and the film allowed to become saturated with electrolyte solution by means of capillary action through the wicks. Even though the electrolyte fronts normally took only 10 to 15 min. to meet, a period of one hour was allowed to elapse before applying the test solution. This time interval ensured that migration of the colours due to capillary rise of the electrolyte was minimal, as shown by preliminary experiments with colour spotted at various points on the film between the electrode compartments.

Despite the apparent delay of one hour in preparing plates, it was considered advantageous to use this procedure rather than to prepare the plate directly from a slurry of the thin-film adsorbent made from the electrolyte solution. In this alternative procedure, the film would have to be spread on one plate at a time.

A further variation in the procedure involving spraying of the thin-film plate with the appropriate electrolyte gave rise to non-reproducible results.

For electrophoresis on paper, strips of Whatman No. 1 filter paper, previously soaked in electrolyte solution, were placed across the glass ribbed support (at right angles to the ribs) provided with the Baird and Tatlock horizontal tank.

The colour solution was streaked on the cathode end of the support in each case and electrophoresis allowed to proceed for a timed period at a constant potential.

RESULTS

The colours were subjected to electrophoresis singly for a period of one hour at a constant potential of 200 V. The various mobilities were found to be reproducible and are summarised in Table I. The recorded mobilities represent migration towards the anode (positive electrode) in each case except when the mobility is preceded by a negative sign.

While the results for electrophoresis on Whatman No. 1 paper quoted in Table I are those obtained for the paper lying across the ribs of the glass bridge of the electrophoresis tank, it is interesting to note that different migration distances were obtained when the paper was allowed to lie on a smooth glass surface during electrophoresis.

TABLE I

MIGRATION DISTANCES (mm) OF COAL TAR FOOD DYES ON PAPER AND THIN-FILM SUPPORTS AFTER ELECTROPHORESIS AT 200 V FOR ONE HOUR

Dye	*Molecular weight*	*Number of ionisable sodium atoms*	*Support*	*Electrolyte solution*					
				N acetic acid	*4.0*	*6.0*	*8.0*	*9.2*	*0.1 N ammonia*
Ponceau 4R	604	3	P	47^{F}	$36^{T\ \&\ F}$	$28^{T\ \&\ F}$	16^{T}	41	79
			K	39	37	44	41	56	29
			A	28	44	53	46	53	32
			S	41	39	47	39	52	26^{F}
Amaranth	604	3	P	19^{F}	15	8	7	15	46^{T}
			K	35	32	46	42	58	31
			A	27^{T}	33^{T}	48^{T}	$37^{T\ \&\ F}$	$51^{S\ F}$	$19^{T\ \&\ F}$
			S	42	40	47	39	47	23^{F}
Fast Red E	502	2	P	11	9^{T}	5^{T}	3	4	21^{T}
			K	41	33	45	34	55	24
			A	$21^{T\ \&\ F}$	5	5	$26^{T\ \&\ F}$	$24^{T\ \&\ F}$	1
			S	37	38	42^{T}	32	47	$21^{SF,ST}$
Carmoisine	502	2	P	14	8	5ST	3ST	18	38^{T}
			K	34	32	38	34	53	29
			A	23^{T}	31^{T}	47^{T}	34	$42^{T\ \&\ F}$	27
			S	38	34	34	34	44	21^{F}
Black PN	866	4	P	6^{T}	2^{T}	1	1	4	21^{T}
			K	38	33	45	44	50	16
			A	1	4	1	2	3^{T}	0
			S	35	38	42^{T}	39	48	23^{SF}
Ponceau SX	480	2	P	21^{T}	12^{T}	7^{T}	4^{T}	26^{T}	59^{T}
			K	32	34	38	36	52	27
			A	25ST	25ST	43^{T}	37	$39^{T\ \&\ F}$	24ST
			S	36	33	36	35	46	20^{F}
Ponceau MX	480	2	P	18^{T}	9ST	5^{T}	2^{T}	18ST	30^{T}
			K	39	32	42^{T}	35	51	21
			A	26ST, 17ST	22^{T}	$43^{T\ \&\ F}$	$28^{T\ \&\ F}$	$34^{T\ \&\ F}$	$21^{T\ \&\ F}$
			S	34	35	37^{T}	34	44	19^{SF}, ST

(continued on p. 353)

TABLE I (*continued*)

Dye	Molecular weight	Number of ionisable sodium atoms	Support	Electrolyte solution: N acetic acid	4.0	6.0	8.0	9.2	0.1 N ammonia
Orange G	452	2	P	44	30	26^{F}	14^{F}	42	67^{T}
			K	35	33	38	36	46	20
			A	33	43	52	34	47	27
			S	40	34	34	36	44	18^{F}
Orange RN	350	1	P	11	7	5^{T}	2	7	12
			K	25	23	26	27	40	12
			A	24	12^{T}	47^{F}, 32^{F}	20	$28^{T\,\&\,F}$	16^{T}
			S	23	21, 32^{faint}	19	25ST	$24^{T\,\&\,F}$	10
Red 2G	509	2	P	22	21	13ST	8	13	50^{T}
			K	34	27	32	31	46	19
			A	31	43	49	31	47	27
			S	33	31	30	23	40	15^{F}
Sunset Yellow FCF	452	2	P	22	17^{T}	17ST	9	19	45^{T}
			K	35	30	35	37	52	30
			A	34	32^{T}	50ST	36	51	28
			S	40	38	43	35	44	$18^{ST\,\&\,SF}$
Red 6B	566	2	P	13	10^{T}	4^{T}	1	10	34^{T}
			K	35	27	36ST	32	49	18
			A	27	32	45	34	45	25^{SF}
			S	32	32ST	34	33	42	$15^{ST\,\&\,SF}$
Red 10B	467	2	P	23^{T}	13	9ST	5ST	17	28
			K	45	31	41	30	49	20
			A	35	40	52	33	49	32
			S	36	34	39	33	43	20^{SF}
Chocolate Brown HT	652	2	P	$25^{T\,\&\,F}$	$30^{T\,\&\,F}$	$12^{T\,\&\,F}$	$10^{T\,\&\,F}$	$43^{T\,\&\,F}$	$65^{T\,\&\,F}$
			K	$21^{T\,\&\,F}$	$24^{T\,\&\,F}$	faded	18^{F}	$53^{T\,\&\,F}$	13^{F}
			A	0	0	0	0	0	0
			S	26^{T}	19^{F}	13^{F}	28^{F}	faded	$16^{T\,\&\,F}$

(*continued on p. 354*)

TABLE I (*continued*)

Dye	*Molecular weight*	*Number of ionisable sodium atoms*	*Support*	*Electrolyte solution*					
				N acetic acid	*4.0*	*6.0*	*8.0*	*9.2*	*0.1 N ammonia*
Yellow RFS	432	2	P	37	30	41ST	24	29	68
			K	42	30	42	29	50	23
			A	38	47	50	35	51	31
			S	41	39	43	29	47	18^{F}
Yellow RY	418	2	P	42	27	20ST	7^{T}	32	61^{T}
			K	40	36	44	40	54	29
			A	34	37	53	40	51	24
			S	42	40	45	39	51	28^{F}
Brown FK	520 } 328 }	{ 2 { 1		Brown Yellow	Brown Yellow	Brown Yellow	Brown Yellow	Brown Yellow	Brown Yellow
			P	4 2	3 —	1 3	1 4	2 7	4 11
			K	12^{F} —4	3 —	22 —10	36 16	48 36	20 11
			A	1 14	14 25	47 41	36 28	40 30	27 24
			S	21^{T} 2	28^{F} 8	faded 6	29 21	41 25	19^{F} $11^{T \& F}$
Blue VRS	566	1	P	12	17	22ST	22^{T}	4	26
			K	4	7	6	—1	24	8
			A	21	18	26	12	25ST	16
			S	2	1	1	1	1	0
Violet BNP	720	1	P	12^{T}	12^{T}	8^{T}	4^{T}	14^{T}	19^{T}
			K	2	2	1	7	33^{T}	$6^{T \& SF}$
			A	7^{T}	0	8^{T}	$8^{T \& F}$	6ST	5^{T}
			S	3	1	1	0	1	1
Green S	576	1	P	18ST	19	5^{T}	11	25^{T}	42ST
			K	10	16	10	20^{T}	35^{F}	9
			A	24	8	32	24	24	18
			S	6	4	2	8	3	1
Red FB	583	2	P	0	0	0	0	0	1
			K	27	23^{T}	27^{T}	1	42	19
			A	0	0	1	1	2	2
			S	26^{T}	$17^{T \& F}$	13^{T}	17^{T}	$19^{T \& F}$	10^{T}

(*continued on p. 355*)

TABLE I (*continued*)

Dye	*Molecular weight*	*Number of ionisable sodium atoms*	*Support*	*Electrolyte solution*					
				N acetic acid	*4.0*	*6.0*	*8.0*	*9.2*	*0.1 N ammonia*
Yellow 2G	568	2	P	48	36	22	25	43	79
			K	32	30	31	34	54	25
			A	35	45	53	42	43	31
			S	38	32	36	34	41	19^{F}
Tartrazine	534	3	P	32	38	25	14	40	70
			K	33	36	51	49	59	35
			A	39	42	49	49	52	35
			S	44	43	47	41	47	$21^{ST \& F}$
Indigo Carmine	466	2	P	43^{T}	18ST	11ST	6^{T}	21	36
			K	40	33	44	33	52	22
			A	34	26^{F}	42^{F}	$35^{T \& F}$	48	30^{F}
			S	37	36	44	35	47	22ST
Chocolate Brown FB	558 516	1 1		Brown Yellow	Brown Yellow	Brown Yellow	Brown Yellow	Brown Yellow	Brown Yellow
			P	4 2	1 0	1 3	0 —	2 9	3 9
			K	15 −7	20 0	25 −10	36 20	43 33	20 8
			A	0 20	13 29	46 41	28 20	38 32	19 15
			S	0 —	7 —	4 —	32 24	40 25	13 —
Erythrosine BS	876	2	P	0	1	1	1	2	5
			K	0	0	0	30^{T}	37	20
			A	0	10	$38^{T \& F}$	$24^{T \& F}$	26	20^{T}
			S	0	$6^{T \& F}$	10^{T}	19ST	16^{T}	$8^{ST \& F}$

T = tailed; F = faded; ST = slightly tailed; SF = slightly faded; P = Whatman No. 1 paper; K = kieselguhr; A = alumina G; S = silicagel G.

Some examples of observed distances (in mm) under these different conditions using acetic acid (N) as electrolyte are, Tartrazine 32 and 47, Sunset Yellow FCF 22 and 31, Carmoisine 14 and 16, and Amaranth 19 and 31, the latter figure in each case being for the electrophoresis with the paper lying on the smooth glass surface.

Fig. 1 illustrates the application of kieselguhr as a supporting medium for the electrophoretic separation of food colours.

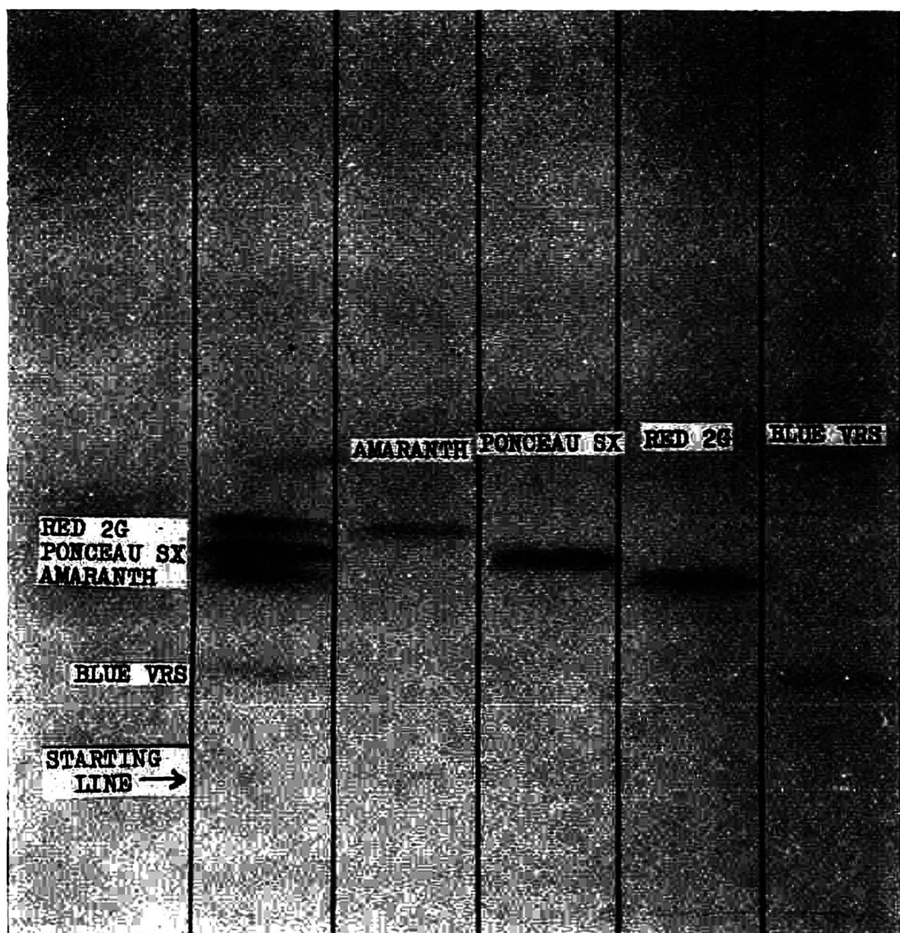


Fig. 1. Typical separation of food colours (45 min) on kieselguhr (pH = 9.2).

DISCUSSION

The mobility of a charged ion under the influence of an electric field depends upon a variety of factors. Some of these are characteristic of the environment, while others depend on the charge and structure of the ions under investigation.

In the present investigation the environmental factors have been varied with respect to the pH of the electrolyte and the supporting medium. In maintaining a constant voltage of 200 V throughout the series of experiments, it is appreciated that

varying the electrolyte and supporting medium results in current variation. Every precaution was taken to standardise the other environmental variables, as indicated by the procedural factors with regard to the ribbed glass bridge in the experiments using paper and the saturation of the thin film with electrolyte in the remaining experiments.

The different mobilities obtained for the paper lying across the ribbed glass plate compared with those obtained with the paper lying on a smooth glass surface may be attributed, at least partially, to what MORI AND KIMURA[18] define as "dark current", that is, the current flowing in areas other than the supporting medium. These authors have also shown that apparatus used as well as different grades of paper also affect mobility. In view of differences between various commercial electrophoresis units, especially tanks, it is difficult to make a comparison of results previously reported[10-12].

The nearest approach to thin-film electrophoresis of food colours so far reported is the study using cellulose acetate strips[12]. On this support of minimal adsorptive power, it was found that the mobility was greater for colours with the higher charge, while for colours of the same charge, the mobility decreased with increasing molecular weight[12]. These workers claim that the use of cellulose acetate eliminates tailing, but it has been found[12,19] that this support very readily permits the isolation of isomeric colours often present in the commercial products. For example, in a typical run, Erythrosine BS gives rise to as many as four bands[19]. While this may be advantageous in production control, it can be a source of difficulty to the analyst concerned only with identifying the major constituents present.

Even on a smooth non-adsorptive support such as cellulose acetate, two colours out of eleven studied did not comply with the expected migration pattern on the basis of charge and molecular weight[12]. The presence of phenolic hydroxyl groups in the molecule is suggested as one of the factors responsible for these discrepancies[12]. This might well be true, for attention has already been drawn to the fact that methyl groups in different positions in Ponceau MX can under certain conditions be responsible for causing the colour to separate into two bands on electrophoresis[10]. That such discrepancies readily occur is apparent by inspection of the mobilities quoted in Table I for Chocolate Brown FB and Brown FK.

It is clear, therefore, that great care must be taken in drawing conclusions from electrophoretic studies on such complex molecules as the food colours. However, some general trends are apparent and a comparison of the mobilities of certain groups and pairs of colours is interesting.

Generally speaking, the mobility is greater for these colours with the higher number of dissociable sodium ions. Two notable exceptions are Red FB and Black PN on both paper and alumina under all conditions of pH, although the mobility of Black PN in ammonia (0.1 *N*) on the paper support is appreciable.

Colours of the naphthyl-azo-naphthol derivatives have approximately the same mobilities as those of the phenyl-azo-naphthol group.

Migration distances for the middle pH ranges, that is, pH 4.0, 6.0 and 8.0 are rather less on the paper support than under more acid or alkaline conditions. This trend is not apparent for the thin film supports.

Owing to the different effects of substituents on the phenolic hydroxyl and amino groups in the molecule and often the different position occupied by these groups

themselves, care has to be taken over comparisons in the mobilities of the different colours and attention will be given to only a few cases from Table I.

The phenyl-azo-2-naphthol derivatives have similar migration distances to those of the phenyl-azo-1-naphthol group. With the former group, Orange G with its extra sulphonic acid group over Orange RN always possesses the higher mobility. The same is true of Sunset Yellow FCF. In this latter case, the extra sulphonic acid group is on the *p*-phenyl position as against the 8-naphthol position in Orange G, but this factor does not influence the migration velocity in the case of the thin-film supports. On the paper support, however, Sunset Yellow FCF shows a reduced mobility when compared with Orange G.

Of the phenyl-azo-1-naphthol derivatives, comparison may be made between Red 10B and Red 2G which are identical except for the 8-naphthol position. This carries an amino group in the former case and an acetamido group in Red 2G. As expected, the mobility is similar for all supports at all conditions of pH. There are, however, sufficient differences in mobilities in a few cases to permit separation of the two colours, that is, except where the support is alumina.

The tri-aryl methanol anhydride colours do not show any enthusiasm for migration on silica gel films, but kieselguhr and alumina show several conditions of pH for separating the three colours.

Except where the migration was minimal, that is, on alumina, the behaviour of Chocolate Brown HT was unsatisfactory on all supports and under all conditions of pH in that extensive tailing always occurred. The only conclusion that can be drawn from studies on this colour is that, except on alumina, its isolation by electrophoresis would be extremely difficult.

Finally, mention must be made about the quality of the electropherograms in relation to those obtained on paper and cellulose acetate strips. A strong criticism of paper electrophoresis of food colours as an analytical tool is the tailing that occurs during separation[10,12]. This difficulty is due to its strong adsorptive capacity and the characteristic is to some extent true of alumina. Resolution is much sharper for the other two supports, particularly for kieselguhr and tailing does not often occur. Of course, this is to be expected since kieselguhr has little adsorptive capacity, but a disappointing feature, as indeed with all the thin-film supports, is that the range of mobilities is not as great as that obtained on paper. However, the much sharper resolution more than compensates for this shortcoming (Fig. 1).

ACKNOWLEDGEMENT

The authors are indebted to L. J. Ponting and Son Ltd., Hexham, Northumberland for the supply of food colours.

SUMMARY

The electrophoretic behaviour of the coal-tar food colours permitted in the United Kingdom, except for Oil Yellow GG, Oil Yellow XP, Naphthol Yellow S and Ponceau 3R has been examined in six different electrolytes of widely differing pH values. The supports used consisted of Whatman No. 1 paper, kieselguhr, alumina and silica gel. The results, which illustrate the high degree of resolution obtained using thin film supports, are presented and discussed.

REFERENCES

1 *The Colouring Matter in Food Regulations, 1957*, H. M. Stationery Office, London, 1957.
2 *Separation and Identification of Food Colours Permitted by the Colouring Matters in Food Regulations, 1957*, Association of Public Analysts, London, 1960.
3 Y. Yanuka, Y. Shalon, E. Weissenberg and I. Nir-Grosfeld, *Analyst*, 87 (1962) 791.
4 M. Mottier and M. Potterat, *Mitt. Gebiete Lebensm. Hyg.*, 43 (1952) 123.
5 M. Mottier and M. Potterat, *Mitt. Gebiete Lebensm. Hyg.*, 44 (1953) 192.
6 M. Mottier and M. Potterat, *Anal. Chim. Acta*, 13 (1955) 46.
7 M. Mottier, *Mitt. Gebiete Lebensm. Hyg.*, 47 (1956) 372.
8 J. Davideck, J. Pokorny and G. Janicek, *Z. Lebensm. Untersuch. Forsch.*, 116 (1962) 13.
9 A. Montag, *Z. Lebensm. Untersuch. Forsch.*, 116 (1962) 413.
10 J. Crossley and J. D. R. Thomas, *Analyst*, 83 (1958) 462.
11 P. P. Legard, *Ann. Fals. Fraudes*, 52 (1959) 5.
12 M. H. Anwar, S. Norman, B. Anwar and P. Laplaca, *J. Chem. Educ.*, 40 (1963) 537.
13 W. J. Criddle, G. J. Moody and J. D. R. Thomas, *Nature*, 202 (1964) 1327.
14 C. G. Honegger, *Helv. Chim. Acta*, 44 (1961) 173.
15 E. V. Truter, *Thin-Film Chromatography*, Cleaver-Hume Press, London, 1963, p. 89.
16 F. Dobici and G. Grassini, *J. Chromatog.*, 10 (1963) 98.
17 G. Pastuska and H. Trinks, *Chemiker-Ztg.*, 85 (1961) 535.
18 I. Mori and M. Kimura, *J. Pharm. Soc. Japan*, 74 (1954) 179, 181.
19 J. D. R. Thomas, unpublished work.

COMPLEXES BETWEEN POLYHYDROXY-COMPOUNDS AND INORGANIC OXY-ACIDS

VI. PAPER ELECTROPHORESIS IN STANNATE SOLUTION*

E. M. LEES AND H. WEIGEL

Chemistry Department, Royal Holloway College, University of London, Englefield Green, Surrey (Great Britain)

(Received March 6th, 1964)

Polyols are known to form complexes with the anions of several inorganic oxy-acids, *e.g.* borate[2,3], arsenite[3], germanate[4,5], antimonate[6], molybdate[7], and tungstate[7]. Such complexes form the basis for paper electrophoresis of carbohydrates and related compounds. In many cases their electrophoretic mobilities have been correlated with the structures of both the inorganic complexing agents and the polyols. We now report the paper electrophoretic behaviour of polyols in stannate solution.

EXPERIMENTAL

Electrophoresis was carried out on 10 cm wide sheets of Whatman No. 3MM filter paper. The electrolyte was a solution of sodium stannate in water (2 %, pH 11.5). Compounds were detected with acetone–silver nitrate–ethanolic sodium hydroxide[8]. D-Glucitol was used as a standard for comparison of rates of migration, and hydroxymethylfurfural as a non-migrating marker for correction of electro-osmosis. Migration rates in stannate solution are thus expressed as $M_s(Sn)$ values. Under the conditions used, D-glucitol had a mobility (u) of 14.3×10^{-5} cm^2 V^{-1} sec^{-1}.

RESULTS AND DISCUSSION

In all cases examined the mobilities (u) of polyols in stannate solution are appreciably higher than in 0.1 N sodium hydroxide[3]. Moreover, the sequence of mobilities of the polyols in stannate solution is markedly different from that in 0.1 N sodium hydroxide. Thus, migration in stannate solution of the compounds examined is due primarily to complex formation rather than ionisation of hydroxyl groups, and the method can be regarded as complementary to those in other electrolytes[7].

Table I shows that only two hydroxyl groups are required for complex formation. It is interesting that the mobility of *erythro*-2,3-butanediol is about half that of the *threo*-isomer. On the other hand, the mobility of *erythro*-2,3-butanediol in borate[3], arsenite[3], and sulpho-benzeneboronic acid[9] is *ca.* 25, 18, and 0 %, respectively, of that of *threo*-2,3-butanediol. Neither of these isomers migrates in germanate solution[10]. From the Sn–O distances[11] in sodium stannate, $Na_2Sn(OH)_6$, and potassium stannate,

* For Part V see ref. 1.

TABLE I

$M_s(Sn)$ VALUES OF POLYHYDROXY COMPOUNDS

Polyhydroxy compounds	$M_S(Sn) \times 10^2$
Acyclic compounds	
1,2-Ethanediol	1
1,2-Propanediol	1
erythro-2,3-Butanediol	2
threo-2,3-Butanediol	4
Glycerol	23
Erythritol	57
L-Threitol	62
D-Arabinitol	95
1-deoxy-	58
D-Lyxitol, 1-deoxy-	78
Ribitol	72
Xylitol	100
1-deoxy-D-	88
Allitol	88
D-Altritol	95
1-deoxy-	80
1,6-dideoxy-	45
Galactitol	99
1-deoxy-L-	87
1,6-dideoxy-	72
D-Glucitol	100
1-deoxy-	89
3-O-methyl-	30
L-Gulitol	
1-deoxy-	94
3-O-methyl-	85
D-*arabino*-Hexitol	
2-deoxy-	48
3-deoxy-	24
D-*ribo*-Hexitol, 3-deoxy-	24
D-Mannitol	93
1-deoxy-	94
1,2-di-O-methyl-	66
2-O-methyl-	88
L-Mannitol, 1,6-dideoxy-	67
Cyclitols	
*Allo*inositol	100
(+)-Inositol	55
*Epi*inositol	101
*Muco*inositol	67
*Myo*inositol	42
*Scyllo*inositol	50
Aldoses and derivatives	
DL-Glyceraldehyde	94
D-Erythrose	107
L-Threose	103
D-Arabinose	84
methyl α-pyranoside	48
D-Lyxose	115
methyl α-pyranoside	53
D-Ribose	104
methyl β-pyranoside	104

(continued on p. 362)

TABLE I (*continued*)

Polyhydroxy compounds	$M_S(Sn) \times 10^2$
D-*erythro*-Pentose	
2-deoxy-	24
1,2-dideoxy-	19
D-Xylose	81
methyl α-furanoside	3
D-Altrose, 1,6-anhydro-β-pyranose	80
D-Galactose	78
1,6-anhydro-β-pyranose	81
methyl β-pyranoside	43
L-Galactose, 6-deoxy- (L-fucose)	69
D-Glucose	63
1,6-anhydro-β-pyranose	0
6-deoxy-	63
3-O-methyl-	78
4-O-methyl-	44
methyl α-pyranoside	28
D-Gulose	107
1,6-anhydro-β-pyranose	77
6-deoxy-	105
D-*arabino*-Hexose, 2-deoxy-	31
D-*lyxo*-Hexose, 2-deoxy-	23
D-*ribo*-Hexose, 2-deoxy-	52
D-Mannose	100
1,6-anhydro-β-pyranose	96
3,4-di-O-methyl-	71
methyl α-pyranoside	41
L-Mannose, 6-deoxy- (L-rhamnose)	100
Ketoses and derivatives	
D-Fructose	91
1-O-methyl-	80
D-*erythro*-Hexulose, 3-deoxy-	60
L-Sorbose	94
Disaccharides	
α,α-Trehalose	11
Sophorose	57
Nigerose	57
Laminaribiose	75
Maltose	65
Cellobiose	62
Isomaltose	58
Gentiobiose	65

$K_2Sn(OH)_6$, an average O–O distance in $Sn(OH)_6^{2-}$ ions of 2.77 Å can be calculated. This is probably great enough to allow the formation of a non-planar 5-membered ring (I, from *threo*-2,3-butanediol). In this event, the complex of *erythro*-2,3-butanediol (II) will be more stable (relative to that of the *threo*-isomer) than those formed from the other oxy-acid anions.

The order of mobility of acyclic polyols of identical molecular size is, with the exception of 1-deoxy-D-mannitol, related to the number of *threo*-1,2-diol groupings in each. This is not unexpected as, in the planar zig-zag conformation of these compounds, the O–O distance in *threo*-1,2-diol groups (2.82 Å) is close to that in the

$Sn(OH)_6^{2-}$ ion. Thus, the contribution to mobility arising from *threo*-disposed adjacent hydroxyl groups is larger than that from *erythro*-1,2-diol groupings.

In the series of aldopentoses and -hexoses the largest contribution to mobility seems to arise from *cis*-1,2-diol groupings of their pyranose forms, although the O–O distances in the chair conformation of *cis*- and *trans*-1,2-diols of six-membered ring compounds are identical (2.82 Å). This is in agreement with the differences in reactivity of 1,2-cyclohexanediols observed in other cyclisation reactions[12]. Substitution in or of one of the *cis*-disposed adjacent hydroxyl groups reduces the mobility (*e.g.* D-mannose, 2-deoxy-D-*arabino*-hexose, D-ribose; 2-deoxy-D-*erythro*-pentose). By virtue of the α,β-equilibrium all pyranoses can possess a *cis*-1,2-diol grouping. Thus, in all cases examined, except D-ribose, glycoside formation also reduces the mobility. It is noteworthy that D-ribose is thought to exist, in aqueous solution, almost entirely in its β-pyranose form[13], which possesses the same number of *cis*-1,2-diol groupings as its methyl pyranoside.

The complex-forming 1,6-anhydro-β-pyranoses have higher $M_s(Sn)$ values than expected (*cf.* 1,6-anhydro-β-D-galactopyranose, methyl β-D-galactopyranoside; 1,6-anhydro-β-D-mannopyranose, methyl α-D-mannopyranoside). It is probable that, owing to the formation of the 1,6-anhydro-ring, their *cis*-related, adjacent hydroxyl groups have moved into a spatial disposition even more favourable for complex formation.

Of the 1,6-anhydro-β-pyranoses examined the glucose derivative is the only compound which did not migrate during electrophoresis in stannate solution. Its pyranose ring can, theoretically, adopt the 1C and 3B conformations (REEVES' nomenclature[14]), possessing, respectively, axially and equatorially disposed hydroxyl

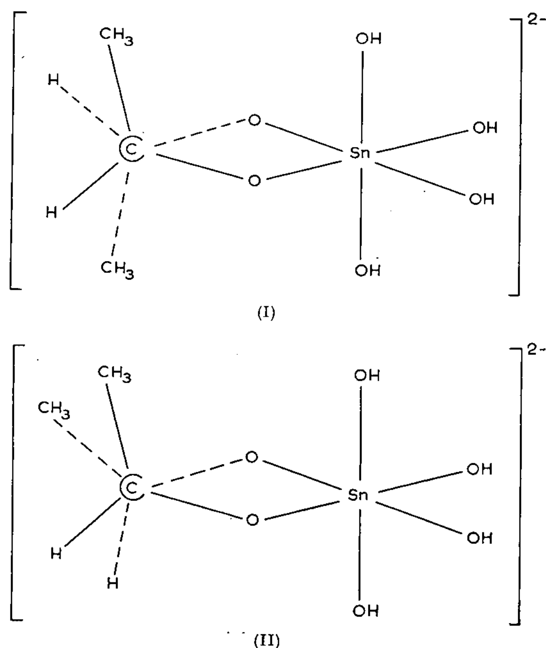


Fig. 1.

groups. The fact that a 1(*eq*),2(*eq*)-diol grouping can complex with stannate (*cf.* methyl α-D-glucopyranoside, D-glucose, 6-deoxy-D-glucose) suggests that the pyranose ring in 1,6-anhydro-β-D-glucopyranose exists in the 1 C conformation.

The cyclitols possessing four *cis*-1,2-diol groupings (*epi*inositol and *allo*inositol) have higher mobilities than those possessing only two (*muco-*, *dextro-*, and *myo*inositol). However, *scyllo*inositol, which possesses only *trans*-arranged hydroxyl groups, migrates faster than *myo*inositol. A tridentate structure, as proposed for borate complexes of certain cyclitols[15], would not account for this effect unless two stannate ions could combine with *scyllo*inositol, when all hydroxyl groups are axially disposed.

Tin and germanium are members of the same group in the periodic table of elements and form the same type of anion, *i.e.* $Sn(OH)_6^{2-}$ and $Ge(OH)_6^{2-}$. The sequences of electrophoretic mobilities of polyols in stannate and germanate[4] solutions show certain similarities, *e.g.* in the series of aldohexoses, methyl pyranosides of aldohexoses, and cyclitols. However, the same similarities are not observed in the series of aldopentoses and acyclic polyols. The investigation has also shown that stannate forms complexes with a greater range of polyols than do molybdate and tungstate[7], which require, for complex formation, very specific structural features.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. R. KUHN, Prof. T. REICHSTEIN and Dr. N. K. RICHTMYER for kindly providing some of the specimens, to the Department of Scientific and Industrial Research for the award of a Scholarship (to E.M.L.), and to Prof. E. J. BOURNE for helpful discussions.

SUMMARY

Paper electrophoresis in stannate solution has shown that stannate forms complexes with several acyclic and cyclic polyols. *Threo*-1,2-diol groups in acyclic compounds complex more strongly than the corresponding *erythro* groupings. With six-membered ring compounds the largest contribution to mobility arises from *cis*-1,2-diol groups, although *trans*-1,2-diol groups can complex. The paper electrophoretic mobilities of the polyols are discussed from the view-point of the conformations of the polyols and the structure of the stannate ion.

REFERENCES

1 H. J. F. ANGUS, E. J. BOURNE AND H. WEIGEL, *J. Chem. Soc.*, in press.
2 A. B. FOSTER, *Advan. Carbohydrate Chem.*, 12 (1957) 81.
3 J. L. FRAHN AND J. A. MILLS, *Australian J. Chem.*, 12 (1959) 65.
4 B. LINDBERG AND B. SWAN, *Acta Chem. Scand.*, 14 (1960) 1043.
5 W. J. POPIEL, *Chem. Ind. (London)*, (1961) 434.
6 F. SEARLE AND H. WEIGEL, unpublished results.
7 H. WEIGEL, *Advan. Carbohydrate Chem.*, 18 (1963) 61.
8 W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444.
9 P. J. GAREGG AND B. LINDBERG, *Acta Chem. Scand.*, 15 (1961) 1913.
10 H. J. F. ANGUS AND H. WEIGEL, unpublished results.
11 C. O. BJÖRLING, *Arkiv Kemi, Mineral. Geol.*, 15, No. 2 (1941).
12 D. H. R. BARTON AND R. C. COOKSON, *Quart. Rev. (London)*, 10 (1956) 44.
13 G. R. BARKER, private communication.
14 R. E. REEVES, *Advan. Carbohydrate Chem.*, 6 (1951) 107.
15 S. J. ANGYAL AND D. J. MCHUGH, *J. Chem. Soc.*, (1957) 1423.

CHROMATOGRAPHIC BEHAVIOUR OF LIGHT RARE EARTHS ON ANION-EXCHANGE PAPER IN NITRATE-METHANOL SYSTEMS

E. CERRAI AND C. TRIULZI
*Laboratori C.I.S.E., Segrate, Milan (Italy)**

(Received March 11th, 1964)

The use of inorganic acid–alcohol media for adsorption of inorganic ions on anion-exchange resins has been described earlier in the literature.

Owing to the tendency shown by several elements to form negatively charged chloro-complexes which are more or less adsorbed by strong anion-exchange resins, chloride–alcohol systems are the ones that have been most extensively investigated. The application of such chloride–alcohol media to anion-exchange separations was reported by WILKINS AND SMITH[1] as well as by FRITZ AND PIETRZYK[2].

KORKISCH and co-workers[3] have recently shown that in a nitric acid medium the adsorption of rare earths on a strong-base anion-exchange resin can be considerably enhanced by the presence of an aliphatic alcohol in the acidic nitrate solution. Generally, at the same acidity, the higher the alcohol concentration the higher is the adsorption of the element. Furthermore, a certain selectivity between the different rare earths in the methanol–HNO_3 system has been shown to occur (especially in the low atom number group) by FARIS AND WARTON[4], which is practically absent when pure nitric acid is used as the eluent.

In the present work the possibility was investigated of exploiting such selective adsorption for a chromatographic separation of light rare earths on paper.

In analogy with the adsorption of rare earths from nitrate–nitric acid solutions on strong anionic resins, the chromatography of some lanthanides on paper treated with a liquid anion exchanger has already been studied in this laboratory[5]. Therefore an attempt was first made to use paper strips treated with a solution of tri-*n*-octylamine (TNOA)–cyclohexane as the chromatographic support in conjunction with an eluent formed by a methanol–nitric acid mixture. Unfortunately, the TNOA-treated paper strips, which normally behave like a thin layer of an anionic resin with pure inorganic eluents, could not be applied to chromatographic elution with mixed solvents. Some preliminary experiments, in which the R_F of europium was determined on a 0.1 M TNOA-treated paper eluted with 80 % vol. methanol–HNO_3 mixtures, showed that the spots were irregular and that part of the amine was eluted from the paper by the methanol thus giving higher R_F's than were obtained with untreated paper. A comparison of such values is given in Table I.

Furthermore, liquid–liquid extraction experiments with radioactive tracer europium had shown that no extraction of the element occurred in the organic phase

* Address: C.I.S.E., Casella Postale 3986, Milano, Italy.

TABLE I

COMPARISON BETWEEN R_F OF EUROPIUM ON UNTREATED WHATMAN NO. 1 STRIPS AND THAT ON THE SAME PAPER PRE-TREATED WITH 0.1 M TNOA–CYCLOHEXANE

Ascending elution with 80% vol. methanol–HNO_3 mixtures.

Nominal HNO_3 molarity	*1.2*	*1.8*	*2.4*	*3.0*	*Observations*
Untreated paper	0.53	0.56	0.54	0.59	Regular spots
TNOA-treated paper	0.79	0.72	0.76	0.73	Irregular spots

(0.35 M TNOA–cyclohexane) from a mixture of 3 M HNO_3–methanol also with a high alcohol content.

It was thus concluded that unlike the case of nitrate–HNO_3 systems, TNOA-treated papers were of no use for chromatography of rare earths with methanol–HNO_3 eluents.

Therefore a support more like an anion-exchange resin, with an inherently greater stability, was chosen, namely, Whatman diethylaminoethyl-cellulose paper DE-20, which is a cellulose anion exchanger where the amine groups are chemically bonded to the cellulose matrix.

A systematic study of HCl–alcohol systems for inorganic chromatography on cellulose ion exchangers (among them DE-20) was recently reported by LEDERER AND MOSCATELLI[6]. Diethylaminoethyl-cellulose paper was also applied by ARNOLD AND RITCHIE[7] to the separation of rare earths in citrate medium.

EXPERIMENTAL

Reagents, equipment and procedure

The rare earths (nitrates and oxides) were supplied by Fluka (Switzerland) and Light's (London). The required amount was dissolved in the minimum volume of conc. HNO_3 and then carefully dried. Afterwards, 0.15 M HNO_3 was added to obtain solutions containing about 3 mg of each element per ml. The following rare earths were used: La(57), Ce(58), Pr(59), Nd(60), Sm(62), Eu(63), Gd(64), Tb(65), Dy(66), Ho(67), Er(68), Yb(70) and Lu(71). Some preliminary experiments showed that a satisfactory selectivity was only attainable for light rare earths and thus the detailed investigation was confined to the group from La to Gd.

The spots were detected with a 1% solution of 8-hydroxyquinoline dissolved in a 50/50 (vol.) water–ethanol solution. Experiments on ion-exchange paper (DE-20, Whatman) were carried out on 107 × 225 mm sheets in which fourteen 85 mm long, 13 mm wide parallel strips had been cut. Some reference experiments were performed with Whatman No. 1 CRL/1 type pure cellulose sheets, formed into twelve strips.

Ascending elution with methanol–HNO_3 mixtures was adopted and a tightly closed jar (18 cm × 10 cm diam.) was used fitted with a support for a chromatographic sheet folded to form a cylinder.

Spots of 0.01 to 0.02 ml of solution (30 to 60 μg of the element) were deposited on the paper with a micropipette. In multiple chromatograms, a period of about 30 min was required for the front to move about 8.5 cm and with single (2 × 20 cm) strips about 60 min for a 16 cm run.

The anion-exchange paper is normally supplied in the free base form and results depend on whether or not the paper has been previously treated and, in the latter case, on the way of treatment. Prior to investigating any other item an appropriate method of treatment was checked.

Selection of the pre-treatment procedure of DE-20 paper

The method used for pre-treatment of DE-20 paper appeared to influence both selectivity within the light rare-earth group and general behaviour of the chromatograms, such as spot shape and front regularity. As an index of selectivity, for each sheet obtained with a different treatment method, it was decided to compare results represented by the relationship of the R_F's as functions of the atomic number (eluent: 3 M HNO_3–80 % vol. methanol) with the theoretical relationship which can be expected with the highest selectivity for rare earths from La to Gd. Since the relationship between experimental R_F's and atomic number was found sufficiently linear for these rare earths, the theoretical correlation of maximum selectivity is obviously represented by a straight line which passes through $R_F = 0$ for La(57) and $R_F = 1$ for Gd(64) thus having a null intercept and a 0.143 slope.

As for the general appearance of the chromatograms it was decided to reject cases in which spots were badly shaped and the front line seriously affected.

Eight possibilities were explored, they are:

(1) Paper in the free base form as supplied;

(2) Paper eluted with 0.1 M NaOH (12 h), then with 0.1 M $NaNO_3$ (12 h) and finally washed 2 min in distilled water;

(3) Paper eluted with 0.3 M HNO_3 (30 min) and washed as above;

(4) *Idem* with 0.3 M $NaNO_3$ (30 min) and washed;

(5) *Idem* with 0.3 M NaOH (30 min) and washed;

(6) Paper washed in 1 M HNO_3 (15 sec), repeated with fresh 1 M HNO_3 (15 sec) and final washing in distilled water for 30 sec;

(7) As (6), but using 1 M $NaNO_3$ for the two washings;

(8) As (6), but using 1 M NaOH for the two washings.

Results are given in Table II, in which the intercepts and slopes of the relationship between experimental R_F's and atomic numbers are reported for rare earths from La

TABLE II

EFFECT OF PRE-TREATMENT OF DE-20 ON EXPERIMENTAL RESULTS IN ELUTION OF RARE EARTHS La TO Gd WITH 3 M HNO_3–80% VOL. METHANOL MIXTURES

CRL/1 pure cellulose paper after the same treatment is given for comparison.

Treatment	*Intercept*		*Slope*		*Observations*
	DE-20	*CRL/1*	*DE-20*	*CRL/1*	
1	0.13	0.67	0.010	0	Regular spots, irregular solvent front
2	0.16	0.67	0.052	0	Paper became very brittle, poor reproducibility
3	0.12	0.65	0.065	0	Regular spots and solvent front
4	0.14	0.70	0.062	0	Regular spots, slightly irregular solvent front
5	0.13	0.72	0.066	0	Regular spots, very irregular front
6	0.10	0.59	0.054	0	Very regular spots and solvent front
7	0.12	0.68	0.058	0	Regular spots, irregular front
8	0.18	0.68	0.056	0	Very irregular spots and solvent front

to Gd eluted with 3 M HNO_3–80 % vol. methanol on DE-20 paper. Results for elutions on Whatman No. 1, CRL/1 pure cellulose paper after the same kind of pre-treatment are also given for comparison.

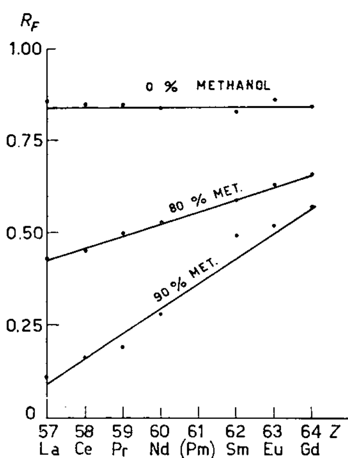
From the results given in Table II it can be concluded that in the HNO_3–methanol system pure cellulose paper does not exhibit selectivity for the different rare earths (slope = 0) and R_F values range from 0.59 to 0.72 depending upon the kind of treatment. The anion-exchange paper DE-20 generally has sufficient selectivity for the different rare earths of the La to Gd group, but the reliability of the chromatograms depends on the pre-treatment. From these results three kinds of treatment were shown to be sufficiently satisfactory, namely numbers 3, 4 and 6. Due to the rapidity of treatment and regularity of results, method 6 was chosen for the work described throughout this paper. This consists of two 15 sec washes with 1 M HNO_3 and a 30 sec wash in water, followed by drying in a current of warm air, and will be referred to as the "standard pre-treatment of paper".

Effect of HNO_3 molarity and of methanol concentration

The effect of the nominal molarity of HNO_3 and concentration of methanol in the eluent was investigated by measuring the R_F values on DE-20 paper as a function of atomic numbers of the rare earths from La to Gd for different molarities of HNO_3.

Elutions on DE-20 paper, previously treated by the standard method, were performed with HNO_3 of the following molarities 0.6, 1.2, 2.4 and 3. The plots of R_F *vs.* atomic number are reported in Figs. 1–4. In each plot a family of curves indicates results at different methanol percentages. Percentage of alcohol and nitric acid molarity are both referred to the total volume of the eluent mixture.

In the four figures, it is shown that pure nitric acid, whatever its molarity from 0.6 to 3 M, yields high R_F values (*i.e.* poor adsorption of the elements) without any selectivity between the different rare earths (horizontal straight line). The presence of



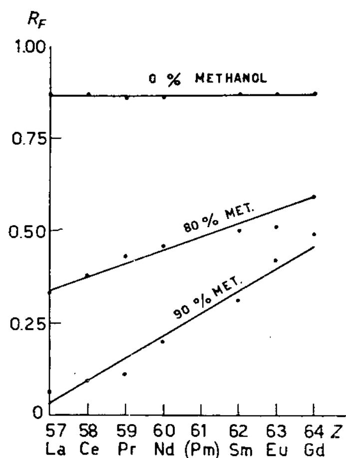
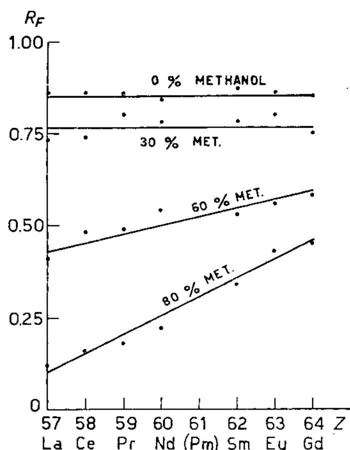


Fig. 1. Plots of R_F *vs.* atomic number of light rare earths: elution of DE-20 paper with 0.6 M HNO_3 containing methanol at the percentage indicated on the plots.

Fig. 2. Plots of R_F *vs.* atomic number of light rare earths: elution of DE-20 paper with 1.2 M HNO_3 containing methanol at the percentage indicated on the plots.



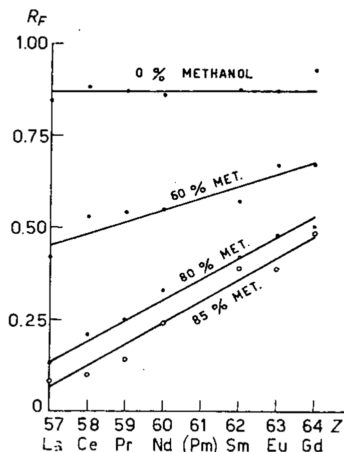
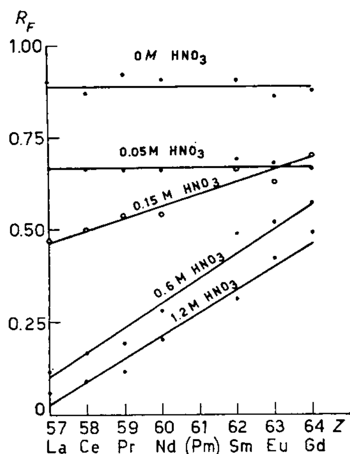


Fig. 3. Plots of R_F *vs.* atomic number of light rare earths: elution of DE-20 paper with 2.4 *M* HNO_3 containing methanol at the percentage indicated on the plots.

Fig. 4. Plots of R_F *vs.* atomic number of light rare earths: elution of DE-20 paper with 3.0 *M* HNO_3 containing methanol at the percentage indicated on the plots.

methanol increases both adsorption and selectivity which are higher the higher the alcohol percentage. In conditions in which selectivity occurs the elements are less adsorbed the higher the atomic number. In the range of a high alcohol percentage, an increase of the acid molarity is generally beneficial as shown in Figs. 5 and 6 in which the plots of R_F *vs.* atomic number are reported respectively for 90 % and 95 % alcohol at different HNO_3 molarities below 1.2 and 0.6 *M*. In each of the two plots both



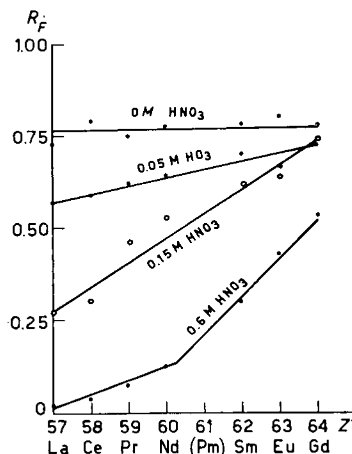
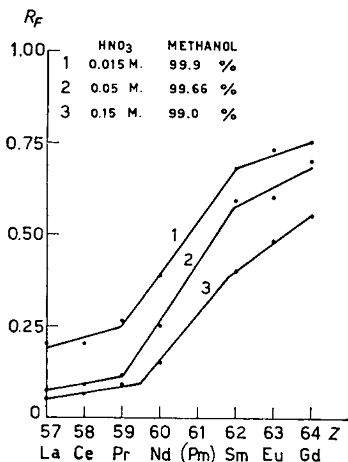


Fig. 5. Plots of R_F *vs.* atomic number of light rare earths: elution of DE-20 paper with HNO_3 of the molarities quoted containing 90 % methanol.

Fig. 6. Plots of R_F *vs.* atomic number of light rare earths: elution of DE-20 paper with HNO_3 of the molarities quoted containing 95 % methanol.

selectivity and adsorption increase with the acid molarity. Due to the improvement attained on changing from 90 % to 95 % methanol–HNO_3 elutions, as shown by a comparison of plots on Fig. 5 with those on Fig. 6, the range of very high alcohol concentrations was further investigated. Thus, elutions with more than 99 % alcohol and very small amounts of 15 M HNO_3 were performed. On the basis of the amount of acid added and of the total volume of the mixture, the molarities of HNO_3 were 0.015, 0.05 and 0.15 M. Results are given in Fig. 7 for mixtures of methanol–HNO_3 with methanol 99 % and above. The shape of the plots shows that although selectivity is generally satisfactory, a linear function of R_F *vs.* Z is not followed at such high alcohol concentrations so that selectivity is higher for the elements in the middle of the light lanthanide group.

To investigate the effect of the presence of water in the methanol eluent, some experiments were performed by using acid-free methanol–water mixtures from 100 % to 0 % in alcohol. The results in Fig. 8 show that both adsorption and selectivity only



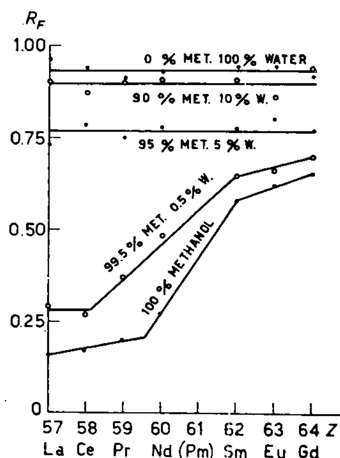


Fig. 7. Plots of R_F *vs.* atomic number of light rare earths: elution of DE-20 paper with HNO_3–methanol mixtures of composition indicated.

Fig. 8. Plots of R_F *vs.* atomic number of light rare earths: elution of DE-20 paper with water–methanol mixtures of composition indicated.

become considerable at higher methanol concentration. In fact the plot differs from a horizontal straight line only above 95 % methanol. As already shown in Fig. 7 for high methanol concentrations in HNO_3 the plot is not a straight line but presents a higher selectivity for the elements in the middle of the light lanthanide group. The elution with 100 % methanol gave slightly tailed spots. The similarity between plots of high methanol concentration either with water or with nitric acid suggests that the effect is mainly due to the presence of alcohol provided that the anion-exchange paper is pre-treated to assume the nitrate form (standard procedure), after which paper presents a pH equal to 1. It was also shown that elutions with 99.9 % methanol–water on untreated DE-20 (free hydroxyl base form) gave consistently zero R_F as expected from the pH of paper which is approximately 10, precipitation of hydroxides at the point of application being most likely.

The quantity $1/R_F - 1$ can be considered proportional to the adsorption coefficient and hence the behaviour of DE-20 paper evaluated. The results for two representative rare earths, cerium and samarium, have been collected and plotted in terms of $1/R_F - 1$ against methanol percentage in the alcohol–acid mixture (see Figs. 9 and 10). The increase of adsorption with the increase of both alcohol concentration and

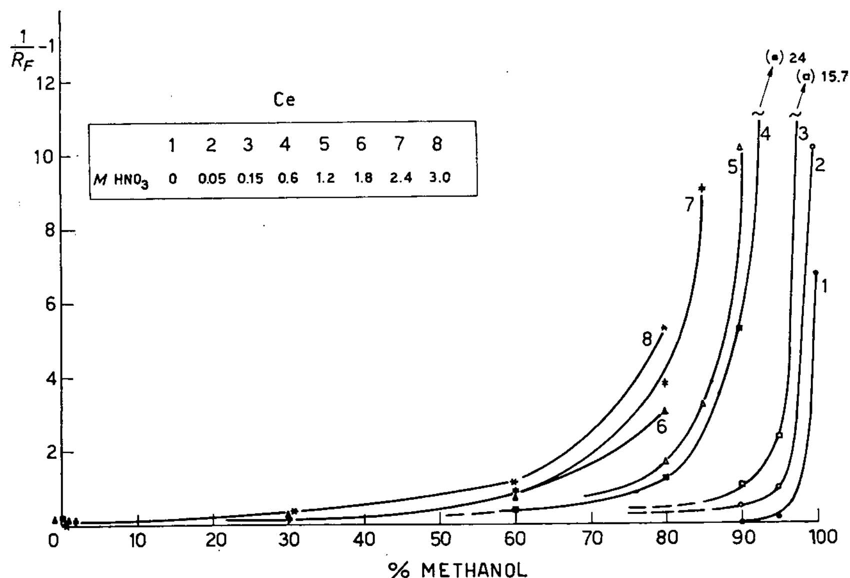


Fig. 9. Effect of methanol percentage (vol.) in the eluent at the quoted HNO_3 molarities on the adsorption of cerium on DE-20 paper.

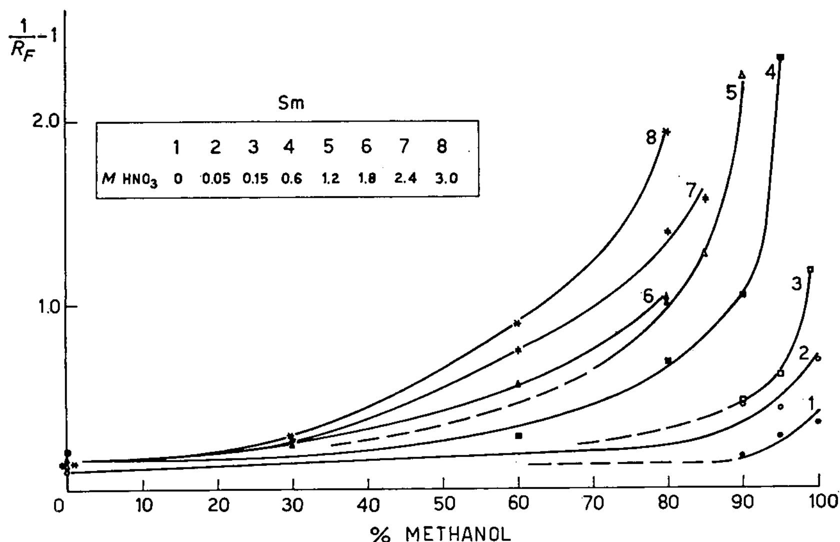


Fig. 10. Effect of methanol percentage (vol.) in the eluent at the quoted HNO_3 molarities on the adsorption of samarium on DE-20 paper.

HNO_3 molarity is clearly apparent. A comparison between the two figures shows that under the same conditions samarium is less adsorbed than cerium as expected from the atomic number, in fact the $1/R_F - 1$ scale for cerium is 5 times greater than that for samarium.

Chromatography on pure cellulose paper

To determine the effect of pure cellulose on the overall phenomenon, a series of experiments were carried out with normal CRL/1 Whatman paper after pre-treatments analogous to those for DE-20 and with the same eluents. As expected, pure cellulose paper showed a lower adsorption of the rare earths than that of the anion-exchange paper, such adsorption being slightly dependent on pre-treatment method. Since no appreciable difference in R_F occurs for the different rare earths on CRL/1, the average R_F values ranged between 0.65 and 0.74 depending on the pre-treatment method. The lower value 0.65 was obtained with the standard pre-treatment procedure already described for DE-20.

A series of experiments were carried out with CRL/1 paper after standard pre-treatment and R_F's measured as functions of both methanol concentration and HNO_3 molarity. Due to the absence of a significant selectivity within the different rare earths in this case, the average R_F values were considered, although experiments had been carried out with every element of the group cited above. The collected experimental results in Table III show that the behaviour on pure cellulose paper is substantially

TABLE III

AVERAGE R_F VALUES OF LIGHT RARE EARTHS ON CRL/1 CELLULOSE PAPER AS FUNCTIONS OF METHANOL PERCENTAGE AND HNO_3 MOLARITY IN THE ELUENT

CH_3OH %	*M* HNO_3								
	0	0.015	0.05	0.15	0.6	1.2	1.8	2.4	3.0
0	—	—	0.86	—	0.84	0.84	0.84	0.84	0.84
30	—	—	—	—	—	—	0.75	—	0.82
60	—	—	—	—	0.71	—	0.66	0.62	0.64
70	—	—	—	—	0.66	—	0.63	—	—
80	—	—	—	—	0.66	0.53	0.63	0.54	0.59
85	—	—	—	—	0.68	0.60	0.73	0.57	—
90	—	—	0.68	0.60	0.70	0.63	—	—	—
95	0.78	—	0.66	0.68	0.72	—	—	—	—
99	—	—	—	0.67	—	—	—	—	—
99.66	—	—	0.68	—	—	—	—	—	—
99.90	—	0.74	—	—	—	—	—	—	—

different from that on an anion-exchange paper since, as noted before, not only selectivity is negligible but also the effect of HNO_3 molarity on adsorption is very limited. The methanol concentration appears to influence the adsorption to some extent and maximum adsorption (minimum R_F) occurs around 80 %.

The effect of temperature on the R_F value

The elution temperature between 0° and 50° was shown to have little effect on the R_F value.

TABLE IV

EFFECT OF TEMPERATURE ON R_F

Rare earth	R_F		
	0°	*20°*	*50°*
Cerium	0.62	0.63	0.69
Samarium	0.65	0.66	0.70

Cerium and samarium were eluted with 60% methanol–1.8 M HNO_3 at 0°, 20° and 50°. As shown in Table IV the effect is practically negligible between 0° and 20° and only becomes a little more significant between 20° and 50°.

The separation of rare earths on DE-20 paper

After the experiments referred to above, conditions for the separation of two or more rare earths from a mixture were selected. Elutions were made by ascending chromatography in closed atmosphere at 20° ± 2° with 2 × 20 cm strips. Results, which are in sufficient agreement with predictions from initial experiments are reported in Table V. As an example the diagrams of separations of La–Nd–Sm and La–Sm–Gd are given in Fig. 11.

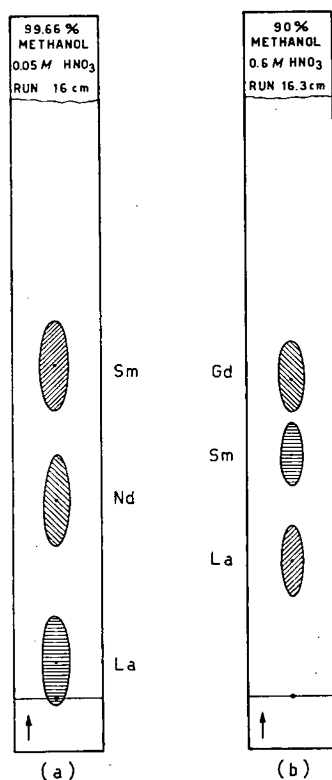


Fig. 11. Diagram of chromatographic separations of Sm–Nd–La (a) and of Gd–Sm–La (b) on DE-20 paper. Ascending elution with eluent indicated in the figure.

TABLE V

SOME ASCENDING CHROMATOGRAMS OBTAINED WITH 2 × 20 cm DE-20 STRIPS UNDER DIFFERENT CONDITIONS

Separations	*Run (cm)*	*Eluent*		R_F
		Nominal M HNO_3	% CH_3OH	
Ce–Nd–Gd	14.6	2.4	80	Ce = 0.27; Nd = 0.40; Gd = 0.54
La–Sm–Gd*	16.3	0.6	90	La = 0.23; Sm = 0.40; Gd = 0.53
Ce–Nd–Eu	15.6	1.2	90	Ce = 0.13; Nd = 0.29; Eu = 0.46
Ce–Pr	16.5	0.15	95	Ce = 0.24; Pr = 0.38
Ce–Nd–Sm	17.4	0.15	99	Ce = 0.06; Nd = 0.21; Sm = 0.40
Pr–Sm–Gd	17.2	0.15	99	Pr = 0.12; Sm = 0.46; Gd = 0.60
La–Nd–Sm*	16.0	0.05	99.66	La = 0.06; Nd = 0.33; Sm = 0.56
La–Nd–Gd	15.2	0.15	99.9	La = 0.17; Nd = 0.37; Gd = 0.61
Ce–Nd–Sm	16.0	0.15	99.9	Ce = 0.11; Nd = 0.41; Sm = 0.60

* See Fig. 11.

CONCLUSIONS

The chromatographic separation of rare earths belonging to the La to Gd group on anion-exchange paper (DE-20) has been experimentally shown to be possible when HNO_3–methanol mixtures are used as eluents.

Other conditions being equal, the R_F value is an increasing function of the atomic number when elution is performed with a mixture containing a high percentage of alcohol. In this case the separative effect is high enough to permit separation of adjacent pairs of lanthanides.

It must be pointed out that, by comparison, much larger separation effects are exhibited by papers treated with the cation exchanger di-(2-ethylhexyl)-orthophosphoric acid (HDEHP) eluted with HCl, as reported in a previous work[8]. Besides the somewhat lower selectivity of the anion-exchange paper with respect to the liquid cation-exchange-treated paper, it must be pointed out that the latter paper can be applied to the whole lanthanide group whereas the DE-20 paper eluted with HNO_3–methanol mixtures is effective with the light rare earth group only. As for the general aspects, whilst with the anion-exchange paper the adsorption decreases with atomic number, with the HDEHP-treated paper it increases with atomic number.

The anion-exchange paper adsorbs each rare earth more strongly the higher the methanol concentration. Such an effect was also found by LEDERER *et al.*[9] in the elution of several elements, among them lanthanum on a quaternary ammonium resin loaded paper (Amberlite SB-2) with HNO_3–ethanol mixtures.

In spite of the considerable experimental evidence that in this case both adsorption and selectivity depend upon the presence of diethylaminoethyl-cellulose groups on the paper, since pure cellulose paper behaves in a totally different way, such phenomena cannot be completely attributed to an anion-exchange process. In fact experiments performed under the same conditions of HNO_3 molarity and methanol content with liquid–liquid extraction with the liquid anionic exchanger tri-*n*-octylamine, gave no evidence of any adsorption of the element into the organic phase, whereas it occurs with solid anion-exchange resins[3].

ACKNOWLEDGEMENT

The authors gratefully acknowledge the collaboration of Mr. ANTONIO ALBINI in the laboratory work.

SUMMARY

The chromatographic behaviour of rare earths from La to Gd has been investigated in HNO_3–methanol systems on the anion-exchange paper DE-20. The possibility of separation of two or more rare earths from each other has been shown as a consequence of the favourable effect of high methanol concentrations on selectivity. A comparison was also made with results obtained by elution under the same conditions on pure cellulose CRL/1 paper. This paper possessed only limited adsorption properties and did not show any selectivity toward different rare earths.

The fact that in the liquid–liquid extraction system with the liquid anion exchanger tri-*n*-octylamine no rare earth adsorption occurs into the organic phase suggests that the adsorption and selectivity shown by the anion-exchange paper is not attributable to a pure anion-exchange process.

REFERENCES

1 D. H. WILKINS AND G. E. SMITH, *Talanta*, 8 (1961) 138.
2 J. S. FRITZ AND D. J. PIETRZYK, *Talanta*, 8 (1961) 143.
3 J. KORKISCH, I. HAZAN AND G. ARRHENIUS, *Talanta*, 10 (1963) 865.
4 J. P. FARIS AND J. W. WARTON, *Anal. Chem.*, 34 (1962) 1077.
5 C. TESTA, *Anal. Chem.*, 34 (1962) 1556.
6 M. LEDERER AND V. MOSCATELLI, *J. Chromatog.*, 13 (1964) 194.
7 R. ARNOLD AND J. F. RITCHIE, *J. Chromatog.*, 10 (1963) 205.
8 E. CERRAI AND C. TESTA, *J. Chromatog.*, 8 (1962) 232.
9 M. LEDERER, V. MOSCATELLI AND C. PADIGLIONE, *J. Chromatog.*, 10 (1963) 82.

REVERSED-PHASE PARTITION CHROMATOGRAPHY WITH DI-(2-ETHYLHEXYL) ORTHOPHOSPHORIC ACID AS THE STATIONARY PHASE

PART I. SEPARATION OF RARE EARTHS

R. J. SOCHACKA AND S. SIEKIERSKI

Department of Radiochemistry, Institute of Nuclear Research, Warsaw (Poland)

(Received March 25th, 1964)

It has been shown in previous papers from this Department that reversed-phase partition chromatography with tributyl phosphate (TBP) as the stationary phase can be applied to the separation of rare earths[1,2] and other ions[3–8]. Although the separation of adjacent rare earths including such a difficult separable pair as Eu–Gd is very satisfactory even on a 10 cm long column at room temperature, the application of TBP for that purpose has, nevertheless, two disadvantages. The first is the very high concentration of nitric acid, which is required for the separation of light rare earths and the second is the rather small mean separation factor. Although the latter is small the separation of adjacent rare earths is possible when in tracer amounts, because of the very small height equivalent of the theoretical plate (0.2 mm). An extracting agent with a higher separation factor would nevertheless be desirable especially in the case of widely differing amounts of the rare earths to be separated. The need for a better extracting agent than TBP was soon recognised by many authors, and in the past three years several papers have appeared[9–16] dealing with the application of di-(2-ethylhexyl) orthophosphoric acid (HDEHP) to reversed phase partition chromatography. But in the opinion of the present authors the advantages of the higher separation factor of HDEHP were nearly offset by the use of columns of very small separating power. The height of the plate of the columns used by PIERCE AND PECK[9,10] is 2 mm at 60° and at a flow rate of 0.5 $ml \cdot cm^{-2} \cdot min^{-1}$. This value is about 10 times greater than that for columns with TBP retained on siliconized kieselguhr and operated at room temperature. The height of the plate of the columns used by CERRAI, TESTA AND TRIULZI[12] is 4 mm at 45° and decreases to 1 mm at 75°. These results seem to indicate that Corvic, cellulose powder and Kel-F are not such good supporting materials for the stationary phase as siliconized kieselguhr. Consequently, the separation of some adjacent rare earths (Ce–Pr–Nd, Eu–Gd, Dy–Ho, Yb–Lu) was not complete when these supporting materials were used, even at elevated temperatures and with the use of the gradient elution technique. On the other hand, good separation of all rare earths at 70° by the gradient elution technique was reported by WINCHESTER[15], who used siliconized kieselguhr as supporting material.

Work on the application of HDEHP retained on kieselguhr to the separation

of rare earths has been also carried out in this Department. This paper and another which follows, present the main results obtained with special emphasis on several new factors that have not been reported by previous authors.

EXPERIMENTAL

Column material and column preparation

Kieselguhr "Hyflo Supercel" was used as the solid support for HDEHP. The kieselguhr was fractionated by the sedimentation method. After drying, each fraction of the kieselguhr was treated with vapours of dimethyldichlorosilane and dried again for 1–2 h at 200°. Adequate treatment of the kieselguhr with dimethyldichlorosilane is essential for obtaining columns with good separating properties. Columns can be prepared by one of two methods. In the first method, the silicone treated kieselguhr is introduced in small portions into a glass tube and gently pressed with a glass rod after each portion. Afterwards an appropriate amount of HDEHP, alone or in a diluent, is introduced and the column eluted with dilute acid under pressure to remove air. In the second method the silicone-treated kieselguhr is mixed with HDEHP and with a volatile solvent, *e.g.*, hexane. Nearly all the solvent is then evaporated at room temperature. The rest of the solvent is removed under reduced pressure. The dry powder obtained in this way is put into the column as described in the first method. There is no difference in the quality of columns obtained by the two methods, nevertheless the second method is to be preferred, since removal of air from the column is less troublesome. Unless otherwise stated the length of the bed was 10 cm and the diameter about 3 mm. Columns of such dimensions contained about 0.40 g of kieselguhr.

Stability of the columns and reproducibility of the results

The stability of the columns prepared by one of the two methods described above depends mainly on the content of HDEHP retained on the support. Columns used in the course of this work, containing 10 % of HDEHP (relative to the weight of kieselguhr) were perfectly stable and did not change their properties even after more than 20 runs.

The position of a peak in runs on the same column with low HDEHP content was found to vary by about 3 %.

The quality of a column is best measured by H—the effective height of the theoretical plate (later referred to simply as the height of the plate). To determine H, the number of plates was calculated from the equation given by GLUECKAUF[17]:

$$N = \frac{8V^2_{max}}{W^2}$$

where V_{max} is the volume of the eluate at the peak maximum and W is the width of the elution peak at 1/e of the maximum solute concentration. The height of the plate for the columns prepared from different batches of the same fraction of column material was found to vary by less than 15 % at constant flow rate and temperature. The mean value calculated from the elution of europium with HNO_3 at a flow rate of 0.75 $ml \cdot cm^{-2} \cdot min^{-1}$ is about 0.33 mm for the specific fraction of kieselguhr used in the course of this work.

Reagent and radiotracers

HDEHP, supplied by Light and Co, was carefully purified by the method described by STEWART AND CRANDALL[18]. The content of the monoderivative was less than 0.2 %.

Most of the radioisotopes used were prepared by irradiation of appropriate targets in the Polish reactor "EWA". Some radioisotopes were obtained from the Radiochemical Centre, Amersham.

Unless otherwise stated the amount of rare-earths carrier was below 0.001 mg.

Elution

Elutions were carried out at room temperature using acids of appropriate concentration pre-equilibrated with HDEHP. Drops of the eluate were collected, dried and their activity assayed by means of an end window G.M. counter. The flow rate was 0.75 $ml \cdot cm^{-2} \cdot min^{-1}$ or about 2 drops/min.

The volume of the sample introduced into the column was about 0.03 ml.

RESULTS AND DISCUSSION

The effect of the grain size, amount of HDEHP and of diluent on the height of the plate

The height of the plate depends on the grain size of the kieselguhr. In the present study the fraction which settled within 10 min in a 20 cm high beaker was used. Using this fraction columns could be easily prepared where the height of the plate is about 0.33 mm. This value was thought to be fairly satisfactory for work with HDEHP because of its high separation factor. It is nevertheless possible to prepare columns with a plate height of less than 0.2 mm. For the latter purpose the fraction of kieselguhr settling between 20 and 30 min should be used.

The height of the plate is affected by the amount of HDEHP retained on the kieselguhr in the column. This is illustrated by the data quoted in Table I. The height

TABLE I

THE EFFECT OF THE AMOUNT OF HDEHP ON THE HEIGHT OF THE PLATE

mg of HDEHP per 1 g of kieselguhr	*50*	*65*	*75*	*100*	*150*	*200*	*250*	*500*
H in mm	0.34	0.33	0.33	0.35	0.59	0.96	1.0	1.95

of the plate was calculated from the position and width of the europium peak. Elution was carried out with HCl at a flow rate of 0.75 $ml \cdot cm^{-2} \cdot min^{-1}$. An increase in the amount of HDEHP from 5 % to 10 % (relative to the amount of kieselguhr) does not change the height of the plate. A further increase from 10 % to 50 % increases the height of the plate several times. Unless otherwise indicated the ratio of HDEHP to kieselguhr chosen for further work was 1:10.

Some experiments were carried out on the columns with solutions of HDEHP in kerosene or TBP as diluent. The presence of a diluent does not improve the height of the plate.

Separation of microamounts of rare earths

Typical examples of the separation of tracer amounts of rare earths in HNO_3 as eluting agent are shown in Figs. 1, 2, 3 and 4. The values of the separation factors (β) for adjacent rare earths in HNO_3 and HCl are quoted in Table II. The separation factor is low in both these acids for the following pairs: Ce–Pr, Pr–Nd, Eu–Gd,

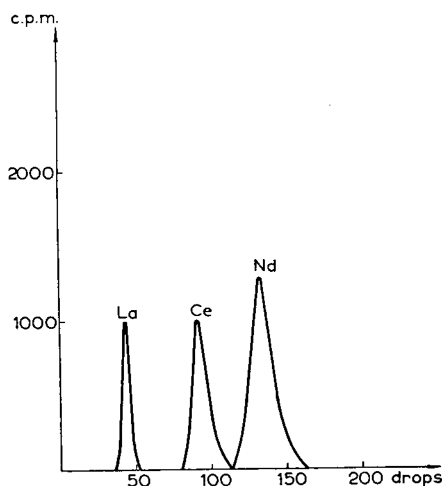


Fig. 1. Separation of La–Ce–Nd with 0.21 M HNO

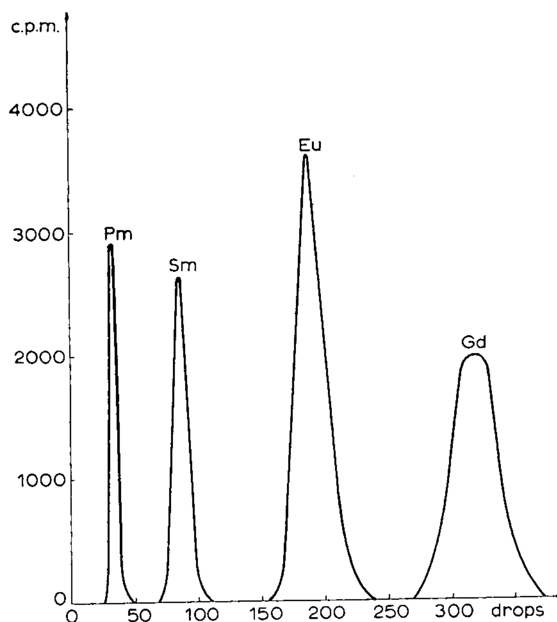


Fig. 2. Separation of Pm–Sm–Eu–Gd with 0.39 M HNO_3.

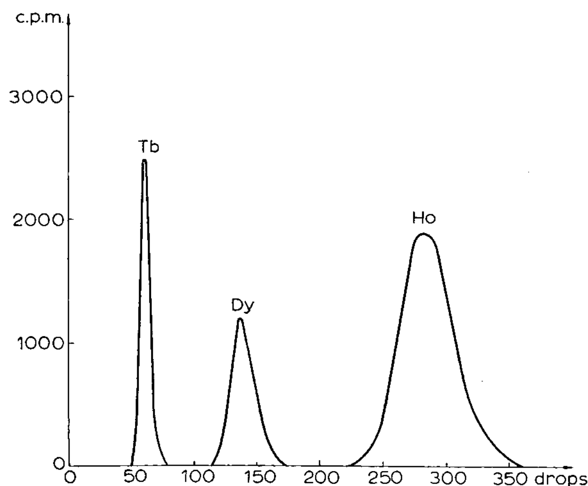


Fig. 3. Separation of Tb–Dy–Ho with 1.16 M HNO_3.

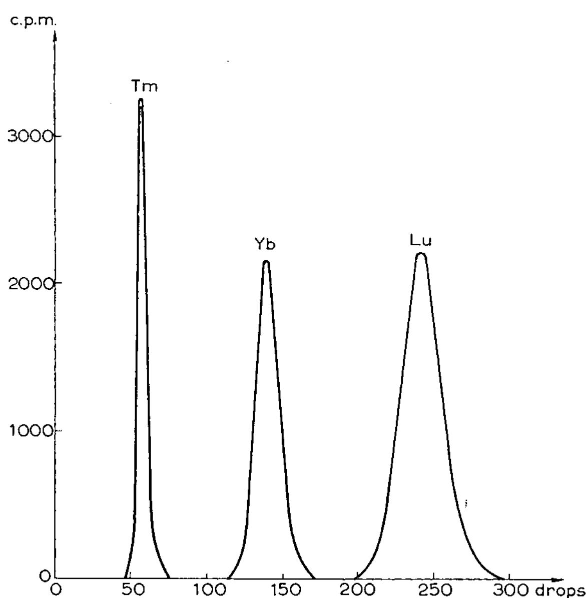


Fig. 4. Separation of Tm–Yb–Lu with 5.12 M HNO_3.

Dy–Ho and Yb–Lu. Contrary to the results reported by PIERCE AND PECK no difficulty was found in separating the last three pairs. We were not able to separate Ce–Pr–Nd using 10 cm long columns and HNO_3 as eluting agent, but with HCl as eluting agent the separation proved quite satisfactory (Fig. 5). Generally, the separation factor for the light rare earths is a little more favorable in HCl than in HNO_3.

TABLE II

SEPARATION FACTORS FOR ADJACENT RARE EARTHS

	La–Ce	*Ce–Pr*	*Pr–Nd*	*Nd–Pm*	*Pm–Sm*	*Sm–Eu*	*Eu–Gd*	*Gd–Tb*	*Tb–Dy*	*Dy–Ho*	*Ho–Er*	*Er–Tm*	*Tm–Yb*	*Yb–Lu*
HCl	2.8	1.5	1.3	2.7	3.2	2.2	1.5	5.0	2.6	2.1	2.8	3.4	2.8	1.9
HNO_3	2.7	*Ce–Nd* 1.55		2.1	2.7	2.1	1.7	5.5	3.0	2.2	2.7	3.5	3.1	1.9

It is difficult to give a reasonable explanation for this difference, since according to PEPPARD *et al.*[19], the light rare earths are only very slightly complexed at low concentrations of both these acids. The mean (geometric) separation factor is 2.41 in both HCl and HNO_3. This value is substantially greater than those reported by PIERCE, PECK AND HOBBS[16] for HCl and $HClO_4$ as eluants. The probable reason for the higher separation factor reported in the present work is the lower temperature at which the experiments were performed. That an increase in temperature decreases the separation factor was shown by CERRAI, TESTA AND TRIULZI[12].

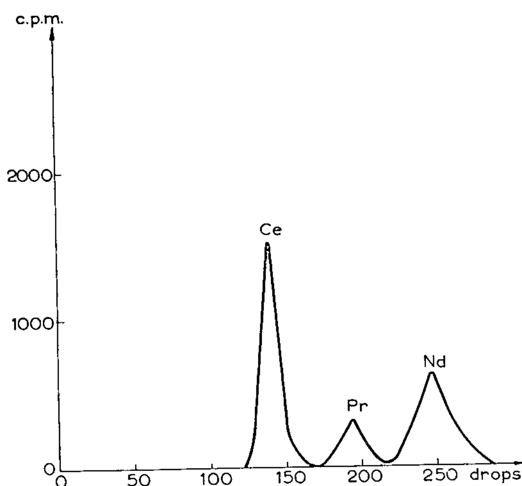


Fig. 5. Separation of Ce–Pr–Nd with 0.14 *M* HCl.

The separation factors in HCl and HNO_3 do not differ substantially for the heavy rare earths, but a new effect can be observed which rules out HCl as an eluant at room temperature. It can be seen that the height of the plate determined from the position and width of the peak of a heavy rare earth element is several times higher with HCl than with HNO_3 as eluant. The effect of the acid is best illustrated by comparison of Figs. 4 and 6. The peaks of Tm and Lu eluted with HCl are so broad that there is no space between them for Yb. This effect of acid was probably not noticed by earlier authors working on the same systems because it disappears at higher temperatures. The effect of acid, temperature and flow rate on the height of the plate will be discussed in detail in Part II of this series.

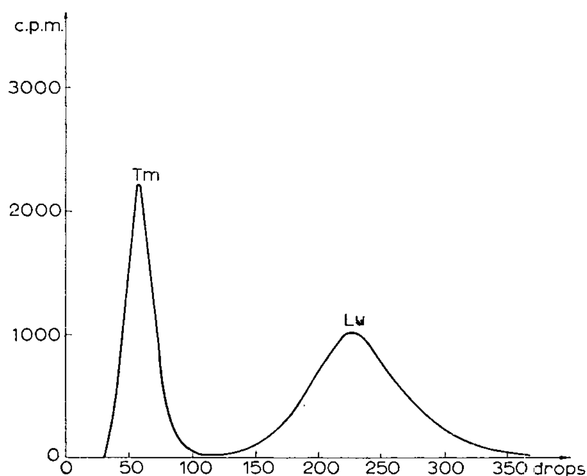


Fig. 6. Elution of Tm and Lu with 2.95 *M* HCl.

Separation of microamounts of Tb from macroamounts of Er

The amounts of the rare earths used in all previous experiments were below 0.001 mg. For many practical purposes such as isotope production, or studying nuclear reactions, the separation of microamounts of one element from a macroamount of another element which was used as a target is more important than the separation of tracer amounts of elements. To study the applicability of HDEHP retained on the kieselguhr to this problem the pair of elements Tb–Er was chosen. In the series of experiments the amount of Tb was kept constant (below 0.001 mg) and the amount of Er was

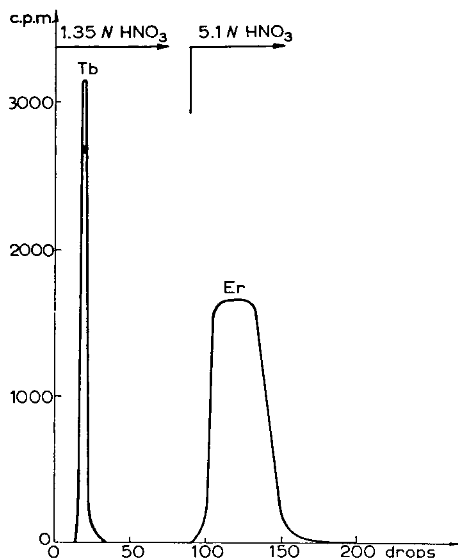


Fig. 7. Separation of microamounts of Tb from 22.6 mg of Er.

TABLE III

SEPARATION OF MICROAMOUNTS OF Tb FROM MACROAMOUNTS OF Er

Column	*Length of the bed (mm)*	*Diameter of the tube (mm)*	*Amount of kieselguhr (g)*	*Amount of HDEHP (g)*	*Concn. of Er (M/l)*	*Max. amount of Er (mg)*	*Molar ratio HDEHP/Er*	*Mequiv. of Er/1 g of HDEHP*	*Mequiv. of Er/1 g of column material*
I	90	2.9	0.350	0.142	0.27	22.6	3.24	2.86	0.83
II	100	7.0	1.76	0.709	0.19	110	3.32	2.78	0.80

gradually increased. In this manner the maximum amount of Er which does not affect the separation was determined. HNO_3 of low concentration was employed for the elution, so that as much as possible of the theoretical column exchange capacity was available. Only after the Tb was completely eluted was more concentrated acid applied to speed up the elution of Er. Two columns differing in size were used. Both of them contained 40 % HDEHP. The results are shown in Table III and in Figs. 7 and 8.

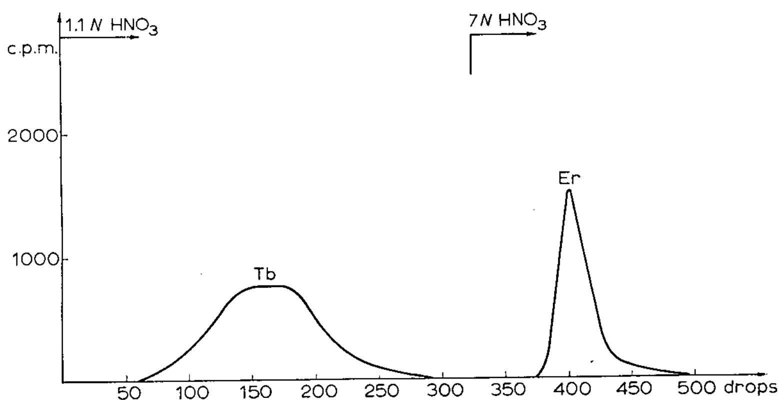


Fig. 8. Separation of microamounts of Tb from 110 mg of Er.

CONCLUSIONS

The ratio of HDEHP to Me in the extracted complex is equal to 3 with a high loading of the organic phase.

When the separation factor between two rare earths is $\geqslant 17$ (the value of β for the Tb–Er pair), practically the whole exchange capacity of the column can be made available without affecting the separation of the microamount of the lighter element from the macroamount of heavier.

There is a linear proportionality between the maximum amount of the rare earth elements retained on the column and the amount of HDEHP. This makes it possible to predict suitable dimensions for a column, according to the amount of the macrocomponent in the mixture.

SUMMARY

Reversed phase partition chromatography with HDEHP retained on kieselguhr as the stationary phase was applied to the separation of rare earths. The influence of the amount of HDEHP on the height of the plate has been studied. Up to 10 % of HDEHP (with respect to the amount of kieselguhr) the height of the plate is about 0.33 mm which makes possible very clear separations of adjacent rare earths at room temperature. No gradient elution technique was found necessary for the separation. The height of the plate can be decreased to less than 0.2 mm by using a specially selected fraction of kieselguhr.

Microamounts of Tb were separated from macroamounts of Er. The amount of Er retained on the column and separated from Tb was found to be proportional to the amount of HDEHP.

It was found that the width of the elution peak of heavy rare earths is much greater with HCl than with HNO_3 as eluting agent.

REFERENCES

1 S. Siekierski and I. Fidelis, *J. Chromatog.*, 4 (1960) 60.
2 I. Fidelis and S. Siekierski, *J. Chromatog.*, 5 (1961) 161.
3 S. Siekierski and B. Kotlińska, *At. Energ. (USSR)*, 7 (1959) 160.
4 R. Gwóźdź and S. Siekierski, *Nukleonika*, 5 (1960) 671.
5 I. Fidelis, R. Gwóźdź and S. Siekierski, *Nukleonika*, 8 (1963) 245.
6 I. Fidelis, R. Gwóźdź and S. Siekierski, *Nukleonika*, 8 (1963) 319.
7 I. Fidelis, R. Gwóźdź and S. Siekierski, *Nukleonika*, 8 (1963) 327.
8 S. Siekierski and R. Sochacka, *Inst. Nucl. Res., Warsaw, Rept.*, 262/V (1961).
9 T. B. Pierce and P. F. Peck, *Nature*, 194 (1962) 84.
10 T. B. Pierce and P. F. Peck, *Nature*, 195 (1962) 597.
11 E. Cerrai, C. Testa and C. Triulzi, *Energia Nucl. (Milan)*, 9 (1962) 193.
12 E. Cerrai, C. Testa and C. Triulzi, *Energia Nucl. (Milan)*, 9 (1962) 377.
13 J. W. Winchester, *J. Chromatog.*, 10 (1963) 502.
14 E. Cerrai and C. Testa, *J. Inorg. Nucl. Chem.*, 25 (1963) 1045.
15 T. B. Pierce and R. S. Hobbs, *J. Chromatog.*, 12 (1963) 74.
16 T. B. Pierce, P. F. Peck and R. S. Hobbs, *J. Chromatog.*, 12 (1963) 81.
17 E. Glueckauf, *Trans. Faraday Soc.*, 51 (1955) 34.
18 D. C. Stewart and H. W. Crandall, *J. Am. Chem. Soc.*, 73 (1951) 1377.
19 D. F. Peppard, G. W. Mason and I. Hucher, *J. Inorg. Nucl. Chem.*, 24 (1962) 881.

REVERSED-PHASE PARTITION CHROMATOGRAPHY WITH DI-(2-ETHYLHEXYL) ORTHOPHOSPHORIC ACID AS THE STATIONARY PHASE

PART II. FACTORS AFFECTING THE HEIGHT OF THE PLATE

S. SIEKIERSKI AND R. J. SOCHACKA
Department of Radiochemistry, Institute of Nuclear Research, Warsaw (Poland)

(Received March 25th, 1964)

In Part I of this series[1], results were reported of the use of di-(2-ethylhexyl) orthophosphoric acid (HDEHP) retained on kieselguhr for the separation of rare earths. The aim of the present work is the detailed study of the factors affecting the height of the plate in reversed phase partition chromatography with HDEHP as extracting agent.

EXPERIMENTAL

Column material and methods of preparation were the same as previously described[1]. The inner diameter of the columns was 3 mm and the length of the bed was 10 cm in all experiments. The weight ratio of HDEHP to kieselguhr was 1:10. For runs at elevated temperatures columns were fitted with water jackets fed from a thermostat. Heating up to 70° did not change the mechanical properties of the bed, although some bubble formation was observed.

RESULTS AND DISCUSSION

The effect of flow rate and temperature on the height of the plate

It is generally assumed that the effective height of the plate (H) is the sum of three independent components:

$$H = H_O + H_D + H_T$$

where the finite thickness of the grains is responsible for H_O, the longitudinal diffusion for H_D and some slow step in the mass transfer between the two phases for H_T. Provided the flow rate (v) is not too slow H_O is independent of flow rate, whereas H_D decreases and H_T increases with increasing flow rate. At constant flow rate, an increase in temperature causes the value of H_D to increase and that of H_T to decrease. Study of the effect of temperature and flow rate should provide evidence on which component is the most important in determining the overall height of the plate. Experiments on the effect of flow rate and temperature were performed for Eu, Tb and Tm in HNO_3 and HCl. The results obtained are shown in Figs 1, 2, 3, 4, 5 and 6. The main conclusions are as follows:

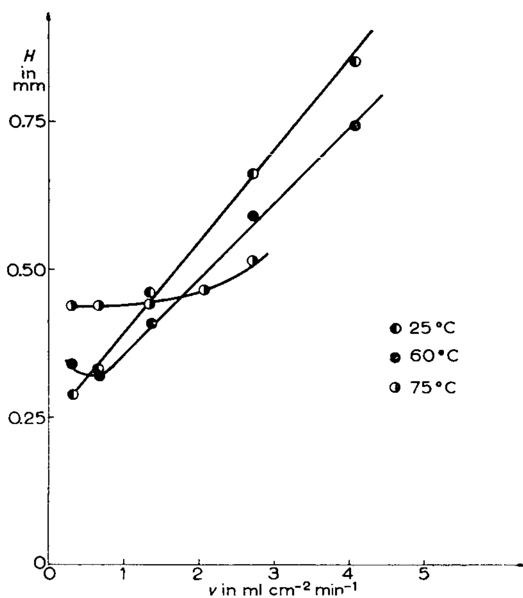


Fig. 1. The effect of flow rate and temperature on the height of the plate. Elution of Eu with 0.36 M HNO_3.

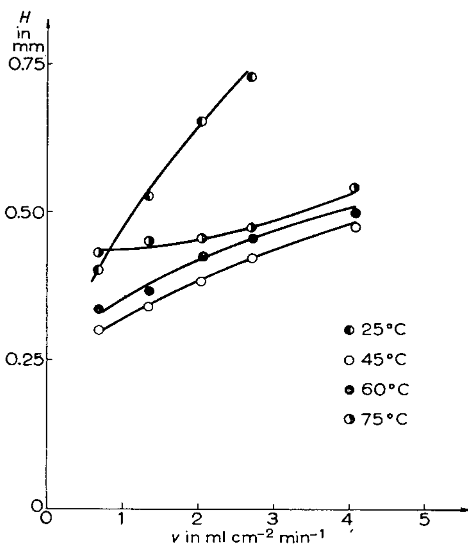


Fig. 2. The effect of flow rate and temperature on the height of the plate. Elution of Eu with 0.36 M HCl.

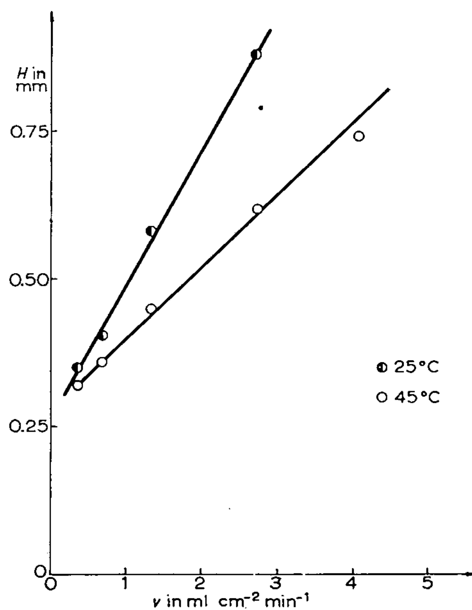


Fig. 3. The effect of flow rate and temperature on the height of the plate. Elution of Tb with 0.86 M HNO_3.

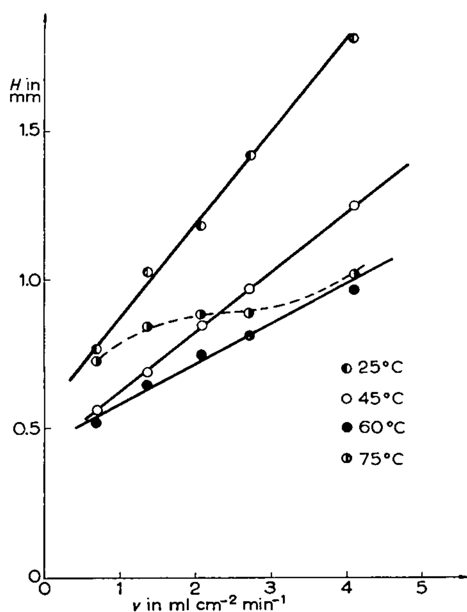


Fig. 4. The effect of flow rate and temperature on the height of the plate. Elution of Tb with 0.86 M HCl.

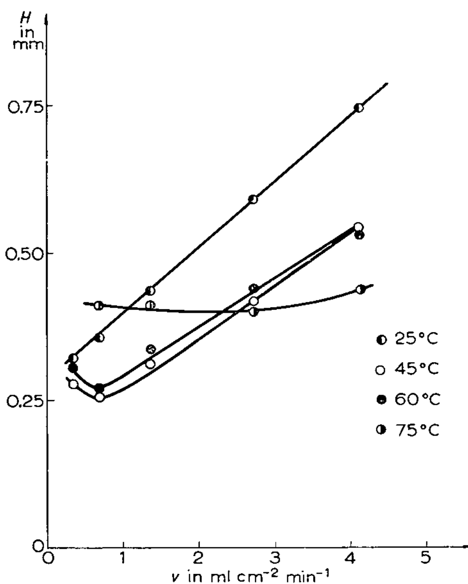


Fig. 5. The effect of flow rate and temperature on the height of the plate. Elution of Tm with 4.52 M HNO_3.

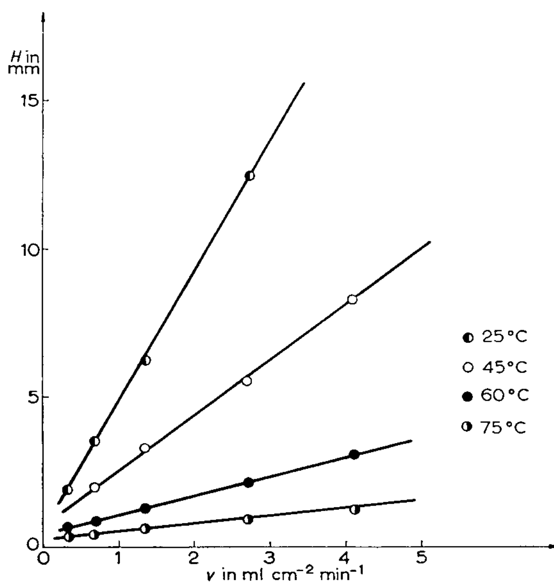


Fig. 6. The effect of flow rate and temperature on the height of the plate. Elution of Tm with 4.50 M HCl.

1. Elution of Eu, Tb, Tm with HNO_3 and elution of Eu with HCl. These systems differ in some details but have several important features in common:

The height of the plate is relatively low.

The height of the plate increases approximately linearly with flow rate at 25°, 45° and 60° but the slope of the line is rather small. At 75° the height of the plate is practically independent of flow rate. In the case of Eu, Tb and Tm in HNO_3 there is some indication of a minimum in the $H = f(v)$ curve at very low flow rates.

The influence of temperature on the height of the plate is small. An increase in temperature from 25° to about 45° at constant flow rate in most cases decreases the height of the plate. There is little change in H between 45° and 60°, whilst a change in temperature from 60° to 75° decreases the height of the plate at high flow rates and increases it at low flow rates.

The results show that at 25°, 45° and 60° the total height of the plate is the sum of two factors only: H_O and H_T. The contribution of each of these factors depends on flow rate. The longitudinal diffusion component contributes substantially only at 75° and to a very much smaller extent at 60° and 45° at very low flow rates. Since $H_T \rightarrow 0$ when $v \rightarrow 0$ it should be possible to determine H_O by extrapolation, provided the temperature is sufficiently low so that the contribution of H_D can be neglected. The value of H_O obtained by extrapolation from different plots is very near to 0.25 mm. According to GIDDINGS[2] $H_O = 2ar_O$, where r_O is the radius of the grain and a is the so-called packing constant: $1 < a < 6$. Since the r_O of the grains of kieselguhr used in this work is about 0.0075 mm, one gets a value for $a \approx 17$ for the packing constant. This is rather a high value indicating that either the grain size is larger because of aggregation, or that there is very uneven flow due to channelling.

2. Elution of Tm with HCl. The height of the plate was found to be very great, especially at low temperatures, and it increases linearly with flow rate at all temperatures and is much more pronounced than in previous systems.

A change in temperature from 25° to 75° very markedly decreases the height of the plate at each flow rate. The influence of temperature explains why earlier workers did not observe the effect of the eluting acid since most of their work was carried out at elevated temperature.

The results for Tm in HCl indicate that the overall height of the plate is determined only by H_T except at very high temperatures and very low flow rates. The extrapolated value of H_O lies between 0.25 and 0.5 mm, which is very near to that obtained in previous cases.

3. Elution of Tb with HCl. The behaviour of Tb in HCl is intermediate to that of Tb or Eu in HNO_3 and that of Tm in HCl. The total height of the plate is the sum of H_O and H_T. Generally $H_T > H_O$. The extrapolated value of H_O is about 0.5 mm. The longitudinal diffusion contributes to the total height of the plate only at 75° and with low flow rates.

The study of the effect of flow rate and temperature on H has revealed that at low and moderate temperatures and not too low flow rates the total height of the plate is chiefly determined by H_T. The different behaviour of heavy rare earths in HCl and HNO_3 as eluting agents must therefore be caused by different values of H_T. The value of H_T is determined by the slowest stage in the mass transfer between the two phases. In most cases this slow stage is the diffusion process, but it can also be a comparatively slow chemical reaction, accompanying the mass transfer. The com-

parison of the height of the plates for Tm in HNO_3 and HCl at 25° and $v = 3$ $ml \cdot cm^{-2} \cdot min^{-1}$ shows that H is about 22 times greater in HCl. Such an enormous difference in plate heights cannot be caused by the difference in diffusion coefficients of the Tm-containing species in the aqueous or organic phases. The inevitable conclusion is that the high value of H in HCl for all the rare earths starting from Tb is caused by some slow chemical stage in the formation or dissociation of the extractable complex.

The effect of electrolyte on the height of the plate

The results of changing flow rate and temperature have shown that the high value of H for Tm in HCl is caused by some slow stage in the overall process of transfer between the mobile and stationary phases. To study this effect in more detail, first the value of H in HCl and HNO_3 was determined, as a function of the atomic number (Z) of the rare earth element. The results, including measurements for some rare earths eluted with H_2SO_4 and $HClO_4$, are shown in Table I from which the following conclusions can be drawn:

In the case of HNO_3 as eluent the height of the plate does not depend on Z. H is somewhat higher in $HClO_4$ than in HNO_3 and also seems to be independent of Z.

In the case of HCl as eluent, H is independent of Z for the light rare earths and is practically the same as in HNO_3. H increases several times between Gd and Er and remains approximately constant for the last four rare earths. The behaviour of rare earths in H_2SO_4 seems to be similar to that in HCl.

The four acids studied can be thus divided into two groups. The first group includes nitric and perchloric acids, the second group includes hydrochloric and sulphuric acids. These two groups differ in the height of the plate for heavy rare earths. It can be argued that an increase in H is caused by the increase in concentration of the acid needed to elute heavy rare earths, and not by the change in chemical properties with increasing Z. This possibility was ruled out by an experiment in which Eu was eluted with a solution containing 0.38 M HCl and 2.36 M NaCl. The value of H found in this experiment was 0.38 mm, only slightly different from that in the solution of low chloride content. In a solution of approximately the same chloride content (2.3 M HCl), the height of the plate for Er is about 4 mm. It should also be noted that when Eu and Tb were eluted with HCl of the same concentration the values of H nevertheless differed fairly appreciably (see Table I). Since the increase in H starts at Tb, it is possible that this is a half-filled shell effect. Such effects are frequently encountered in the chemistry of rare earths. But this possibility is ruled out owing to the behaviour of yttrium, which is characterised by a very high value of H.

A priori, two explanations can be offered for the strange behaviour of the heavy rare earths in HCl and H_2SO_4. According to the first, the complexes of these rare earths with HDEHP differ in their composition when extracted from acids belonging to different groups. This possibility is excluded by the experiments carried out by PEPPARD, MASON AND HUCHER[3] and PIERCE, PECK AND HOBBS[4]. These experiments proved that inorganic anions such as Cl^-, NO_3^- and ClO_4^- are absent in the species which are extracted into the organic phase. This must be also true for sulphates since the elution peak of a rare earth element in sulphuric acid lies between those for nitric and perchloric acids.

According to the second explanation the high value of H for the heavy rare earths

TABLE I

THE DEPENDENCE OF THE HEIGHT OF THE PLATE ON THE ATOMIC NUMBER OF THE RARE EARTH ELEMENTS

H in mm; flow rate v = 0.7 $ml \cdot cm^{-2} \cdot min^{-1}$; room temperature.

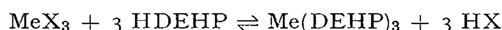
	La	*Ce*	*Pr*	*Nd*	*Pm*	*Sm*	*Eu*	*Gd*	*Tb*	*Dy*	*Ho*	*Er*	*Tm*	*Yb*	*Lu*	*Y*
HNO_3	0.31	0.33	—	0.33	0.32	0.32	0.34	0.31	0.33	0.39	0.36	0.36	0.32	0.33	0.32	—
HCl	0.30	0.29	0.34	0.35	0.35	0.36	0.33	0.31	0.78	1.4	1.7	4.0	3.6	6.0	4.4	6.0
$HClO_4$	—	0.36	—	—	—	—	0.40	—	0.45	—	—	0.50	0.45	—	—	—
H_2SO_4	—	0.37	—	—	0.46	—	0.45	—	0.75	—	—	5.0	7.5	—	—	—

in HCl and H_2SO_4 may be caused by different degrees of complexing which, in turn, may result in some sluggishness in establishing extraction equilibrium. To study this possibility the ratio of the mean molal stoichiometric activity coefficients ($\gamma_\pm$) of the trace amounts of rare earths salts in 1:1 electrolytes (HNO_3, HCl, $HClO_4$) was determined. Since $\gamma_\pm$ is calculated under assumption of complete dissociation of a salt, its value reflects any association which can take place in a solution (low value of $\gamma_\pm$ means high association). From the extraction equilibrium the following equations can be easily derived:

$$D = A \frac{m^3_E}{m^3_H} \cdot \frac{\gamma^4_{\pm MX3}}{\gamma^3_{\pm HX}} \text{ or } \gamma^4_{\pm MX_3} = D \frac{m^3_H \cdot \gamma^3_{\pm HX}}{A \cdot m^3_E}$$

where D = the extraction coefficient, m_E = the molality of extracting agent, m_H = the molality of hydrogen ions, $\gamma_\pm$ = the mean molal stoichiometric activity coefficient. MX_3 denotes a rare earth salt, and HX an acid, and A is a constant.

The following equation was assumed to represent the extraction process:



$$MeX_3 + 3\ HDEHP \rightleftharpoons Me(DEHP)_3 + 3\ HX$$

Since the value of D can be easily determined from the position of the peak, and m_H, m_E and $\gamma_{\pm HX}$ are known, the ratio of the activity coefficients of the salts can be calculated. Results are shown in Tables II, III, IV and V. The main conclusions are as follows:

The association between cation and anion is somewhat lower for Eu, Tb and Er in hydrochloric than in nitric acid of the same concentration, and is much lower in

TABLE II

THE ASSOCIATION OF EUROPIUM SALTS IN VARIOUS ACIDS

Flow rate v = 0.7 ml·cm^{-2}·min^{-1}; room temperature.

	$HClO_4$	HCl	HNO_3	H_2SO_4
Molality of acid m	0.87	0.85	0.86	—
Position of the peak in free volumes V_{max}-V_0	1.66	0.91	0.82	1.4
Activity coefficient of acid $\gamma_{\pm HX}$	0.80	0.79	0.72	—
Ratio of the activity coefficients of salts $\gamma^4_{\pm Eu(ClO_4)_3} : \gamma^4_{\pm EuCl_3} : \gamma^4_{\pm Eu(NO_3)_3}$	1.0	0.53	0.36	—
Height of the plate H in mm	0.40	0.33	0.33	0.45

TABLE III

THE ASSOCIATION OF TERBIUM SALTS IN VARIOUS ACIDS

Flow rate v = 0.7 ml·cm^{-2}·min^{-1}; room temperature.

	$HClO_4$	HCl	HNO_3	H_2SO_4
Molality of acid m	0.87	0.85	0.86	—
Position of the peak in free volumes V_{max}-V_0	13.3	6.8	6.9	11.5
Activity coefficient of acid $\gamma_{\pm HX}$	0.80	0.79	0.72	—
Ratio of the activity coefficients of salts $\gamma^4_{\pm Tb(ClO_4)_3} : \gamma^4_{\pm TbCl_3} : \gamma^4_{\pm Tb(NO_3)_3}$	1.0	0.49	0.38	—
Height of the plate H in mm	0.45	0.78	0.33	0.75

TABLE IV

THE ASSOCIATION OF ERBIUM SALTS IN VARIOUS ACIDS

Flow rate $v = 0.7$ ml·cm^{-2}·min^{-1}; room temperature.

	$HClO_4$	HCl	HNO_3	H_2SO_4
Molality of acid m	2.44	2.30	2.35	—
Positions of the peak in free volumes V_{max}-V_0	7.23	1.84	3.65	4.88
Activity coefficient of acid $\gamma_{\pm HX}$	1.21	1.09	0.83	—
Ratio of the activity coefficients of salts $\gamma^4_{\pm}$ Er(ClO$_4$)$_3$: $\gamma^4_{\pm}$ ErCl$_3$: $\gamma^4_{\pm}$ Er(NO$_3$)$_3$	1.0	0.18	0.16	—
Height of the plate H in mm	0.50	4.0	0.36	5.0

perchloric acid. This conclusion is in full agreement with the results of PEPPARD[3] who also found that NO_3^- and Cl^- ions have approximately the same complexing properties toward rare earths in dilute solutions.

The association between the Tm cation and the anions in concentrated solutions of the acids decreases in the order: $HNO_3 > HCl > HClO_4$.

The comparison of the ratio of activity coefficients with the height of the plate clearly indicates the absence of any correlation between the degree of association and the value of H. This conclusion remains true also when H_2SO_4 is taken into account.

TABLE V

THE ASSOCIATION OF THULIUM SALTS IN VARIOUS ACIDS

Flow rate $v = 0.7$ ml·cm^{-2}·min^{-1}; room temperature.

	$HClO_4$	HCl	HNO_3	N_2SO_4
Molality of acid m	5.65	5.05	5.22	—
Position of the peak in free volumes V_{max}-V_0	3.15	0.94	1.72	2.33
Activity coefficient of acid $\gamma_{\pm HX}$	4.08	2.43	1.10	—
Ratio of the activity coefficient of salts $\gamma^4_{\pm}$ Tm(ClO$_4$)$_3$: $\gamma^4_{\pm}$ TmCl$_3$: $\gamma^4_{\pm}$ Tm(NO$_3$)$_3$	1.0	0.06	0.01	—
Height of the plate H in mm	0.45	3.6	0.32	7.5

It was not possible to compare the degree of association in H_2SO_4 with other acids on the basis of activity coefficients, because H_2SO_4 is a 2:1 electrolyte, but there is little doubt that the SO_4^{2-} anion is a relatively strong complexing agent towards rare earths. Assuming that the complex-forming tendency of H_2SO_4 is higher or approximately the same as that of HNO_3 one gets the following order for the increasing association in the aqueous phase: $HClO_4 < HCl < HNO_3 < H_2SO_4$. The order of the plate height is quite different: $H_{HNO_3} < H_{HClO_4} < H_{HCl} \approx H_{H_2SO_4}$.

The height of the plate must nevertheless be related in some manner to the complex formation in the aqueous phase, although it is not related to the degree of association. This conclusion is supported by experiments with mixtures of electrolytes. Table VI shows the effect of increasing concentration of $NaNO_3$ on the height of the plate for Tb in 0.75 M HCl. The addition of NO_3^- ions which are somewhat stronger complexing agent than Cl^- ions shifts the equilibrium toward formation of $[TbNO_3]^{2-}$ and, as a result, the plate height decreases. A reversed effect is observed when NaCl

is added to the weakly complexing perchloric acid. A marked decrease in the height of the plate of Tb is also observed when 1.8 *M* $NaNO_3$ is added to 0.9 *M* H_2SO_4.

The results discussed so far have eliminated two possible explanations for the column behaviour of the heavy rare earths, these are: different composition of the extractable species, and different degree of the complexing in the aqueous phase, depending on the acid used for elution. It was also established that this behaviour is in some way connected with the complex formation in the aqueous phase. In the authors' opinion there is no contradiction between these two statements, since the height of the plate is a kinetic effect resulting from the kinetic stability of the complex.

TABLE VI

THE EFFECT OF THE CONCENTRATION OF $NaNO_3$ ON THE PLATE HEIGHT OF TERBIUM IN 0.75 *M* HCl

Flow rate v = 0.7 ml·cm^{-2}·min^{-1}; room temperature.

$NaNO_3$ M	0	0.1	0.25	0.5	0.75	2.0
H in mm	0.8	0.7	0.61	0.49	0.41	0.36

This stability is not always related to the thermodynamic stability. It is difficult to formulate a satisfactory explanation for the behaviour of the heavy rare earths in various acids on the basis of existing experimental material, but it is possible to make some suggestions. To do this two additional points should be taken into account. First, it should be noted that the light rare earths are only slightly complexed at low concentrations of mineral acids (this follows from the stability constants determined by PEPPARD and coworkers[3]), and, at the same time, the values of the height of the plate for the light rare earths are low. This means that the process of column extraction in which the hydrated cations of the light rare earths are involved is a fast process. The second point is that the column extraction of light and heavy rare earths from perchloric acid is also a comparatively fast process since the value of H is rather low for $HClO_4$, although not as low as for HNO_3. Now, it is generally assumed that the ClO_4^- anion is a weak complexing agent and that cations retain their hydration layer in perchlorate solutions. It was shown for instance that the association between the Ce^{3+} cation and the ClO_4^- anion is caused by hydrogen bonding between water molecules in the first coordination shell of the cation and the ClO_4^- anion in the second coordination shell[5]. These facts would suggest that the column extraction equilibrium may be established quickly even for the heavy rare earth cations, provided the inner coordination shell of the cation is occupied by water molecules only. If this explanation is correct the NO_3^- anions should be also coordinated in the second coordination layer, although the complexes formed by nitrates are relatively strong. On the contrary the Cl^- and SO_4^{2-} anions would be able to replace water molecules and enter the inner coordination shell of the cation. These two types of complexes differ for some unknown reason in their kinetic stability which results in the different height of the plate. Clearly, further and independent experiments are necessary to decide whether the difference in the hydration, or other differences in the structure between the various complexes are responsible for the observed difference in the height of the plate. Work with this aim in view is in progress.

ACKNOWLEDGEMENTS

We wish to thank Miss J. Szydłowska and Mrs. K. Niedziółka for their technical assistance.

SUMMARY

The effect has been studied of the atomic number of the lanthanide, of the eluting acid, the flow rate and temperature on the height of the plate in reversed phase partition chromatography with HDEHP as the stationary phase. It has been shown that the height of the plate is chiefly determined by the mass transfer between the aqueous and organic phases. In the case of elution of the heavy rare earths with HCl and H_2SO_4 the mass transfer is a slow process because of some slow chemical stage in the overall process of extraction.

REFERENCES

1 R. J. Sochacka and S. Siekierski, *J. Chromatog.*, 16 (1964) 376.
2 J. C. Giddings, *Nature*, 184 (1959) 357.
3 D. F. Peppard, G. W. Mason and I. Hucher, *J. Inorg. Nucl. Chem.*, 24 (1962) 881.
4 T. B. Pierce, P. F. Peck and R. S. Hobbs, *J. Chromatog.*, 12 (1963) 81.
5 L. J. Heidt and J. Berestecki, *J. Am. Chem. Soc.*, 77 (1955) 2049.

ANION EXCHANGE BEHAVIOR OF RARE EARTH ELEMENTS IN POTASSIUM SULFATE MEDIUM

HIROSHI HAMAGUCHI, ATSUHIRO OHUCHI,
NAOKI ONUMA AND ROKURO KURODA

Department of Chemistry, Tokyo Kyoiku University, Koishikawa, Tokyo (Japan)

(Received April 3rd, 1964)

Until recently, the most effective developments in ion exchange separation of rare earth elements have been made when cation exchangers and eluent systems consisting of organic complexing agents have been used. More recently, considerable effort has been made to exploit the use of anion exchange systems with inorganic eluents. Although strong base anion exchange resins do not adsorb the rare earths to any significant extent from mineral acid solutions[1–4], appreciable adsorption has been reported from other media. Pronounced uptake of rare earths from sodium triphosphate solution, adjusted to pH 1 to 9, on a strongly basic anion exchanger has been observed by SUBBARAMAN *et al.*[5]. The rare earths, in concentrated nitrate solutions, have also been found to adsorb sufficiently to permit the effective separation of complex mixtures of rare earth tracers[6]. Information has also been presented concerning the use of lithium chloride[7], sulfite[8], carbonate[9–11], thiocyanate[12] and sulfite, nitrite and thiosulfate[6] media.

Potassium sulfate does not appear to have attracted any attention for the separation of rare earths. Indeed, very little systematic work has been done with the rare earths in the anion exchange resin–sulfate or sulfuric acid systems. BUNNEY *et al.*[2] stated that yttrium, cerium and americium do not show any significant adsorption by Dowex 2 resin at any sulfuric acid concentration level. SAITO AND SEKINE[9] reported that a trace amount of ^{90}Y is slightly adsorbed on a strong-base anion exchange resin from ammonium sulfate and sulfuric acid systems. EDGE[13] has examined briefly the possible separation of some rare earths in dilute sulfuric acid solutions containing ethanol. However, little prospect of an effective anion exchange separation was expected for the neighbouring rare earths Nd and Pr. Thus, the more extensive investigation of the behavior of the whole series of rare earth elements in the sulfate medium, which would provide the basis for a better understanding of this system, appeared to be warranted.

EXPERIMENTAL

Resin

Anion exchanger, Dowex 1, X-8, 200 to 400 mesh, sulfate form. A commercial analytical grade Dowex resin was converted to the sulfate form before use. The converted resin was air-dried and stored in a large desiccator over a saturated solution of potassium bromide.

Columns

Conventional glass tubes of 10 mm internal diameter, 600 mm long, packed at the

bottom with glass wool were used. The columns were filled with a slurry of 43 g of the dried resin and the top of the column was connected to a 500 ml dropping funnel through a rubber stopper. A bed height of 570 mm is usually obtained.

Stock solution of metals

Stock solutions of the rare earths were prepared by dissolving the appropriate amount of the respective oxides (99.9 % purity) in a small amount of 3 *M* sulfuric acid, evaporating to dryness and dissolving in 100 ml of deionized water to give approximately 2.5 mg rare earth metal per ml solution. A stock solution of cerium(III) was prepared by treating ceric oxide with small amount of 3 *M* sulfuric acid, with occasional addition of an excess of hydrogen peroxide to reduce cerium(IV) to the tervalent state, and fuming to dryness. The residue was dissolved in water and diluted to a definite volume to give a concentration of 2.5 mg Ce per ml. The strength of the stock solutions was determined by titration with 0.001 *M* EDTA, standardized previously with a zinc metal of primary standard using Xylenol Orange as indicator.

Column procedure

Before starting the elution, the column should be pretreated with a sufficient volume of 0.01 *M* potassium sulfate solution, of the same concentration as used for the elution. About 5 ml of a sample solution, adjusted to 0.01 *M* in potassium sulfate, is allowed to run into the resin bed at room temperature. Sufficient time, usually 2 h must be allowed for the sample solution to run down the column. The weakly adsorbed rare earths are then eluted with 0.01 *M* potassium sulfate solution at a flow rate of 10 ml per 60 $\pm$ 10 min. The effluent is collected in 10 ml fractions and each fraction titrated with 0.001 *M* EDTA as described above.

To determine the void space of the column 0.01 *M* potassium sulfate solution containing carrier-free ^{134}Cs was fed into the top of the column and allowed to pass through the column. The break-through point of the ^{134}Cs was detected by measurement of the activity of 1 ml fractions of the effluent with a well type gamma scintillation counter. After the activity of the effluent reached a constant level, the column was rinsed with 0.01 *M* potassium sulfate solution until the effluent fraction showed no sign of ^{134}Cs activity. The two volumes, *viz.* that required for the break-through and that for the removal of the activity, were recorded. The two values thus obtained agreed well and gave 22.8 ml for the column used.

RESULTS AND DISCUSSION

To investigate the column elution behavior of individual rare earths in the potassium sulfate medium *ca.* 4.8 mg of each rare earth was adsorbed on the head of the column and eluted with 0.01 *M* potassium sulfate solution. Cumulative elution curves are plotted in Fig. 1.

It can be seen that there are considerable differences in the points of both break-through and complete elution among the individual rare earths, which should provide a basis for possible separations of some rare earth elements. The values of the respective distribution coefficients, K_d, for the rare earth elements, were calculated with the following equation:

$$K_d = \frac{U_{\max} - v_0}{M}$$

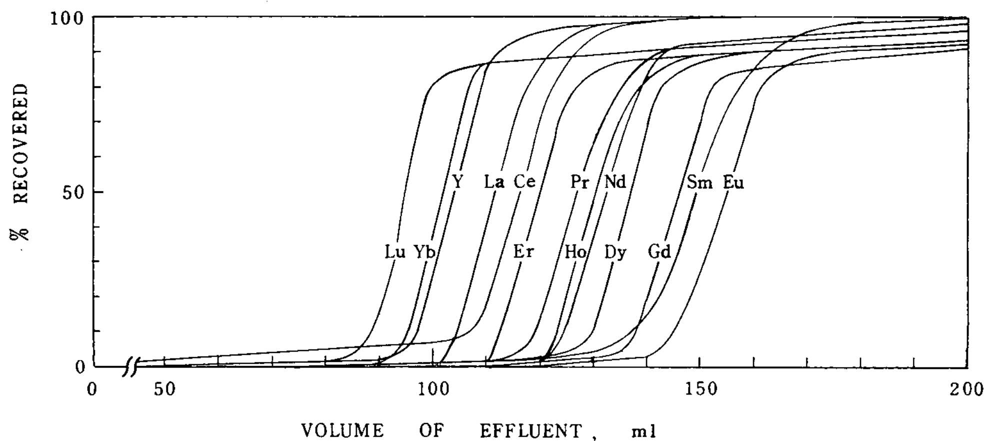


Fig. 1. Cumulative elution curves for the rare earth elements.

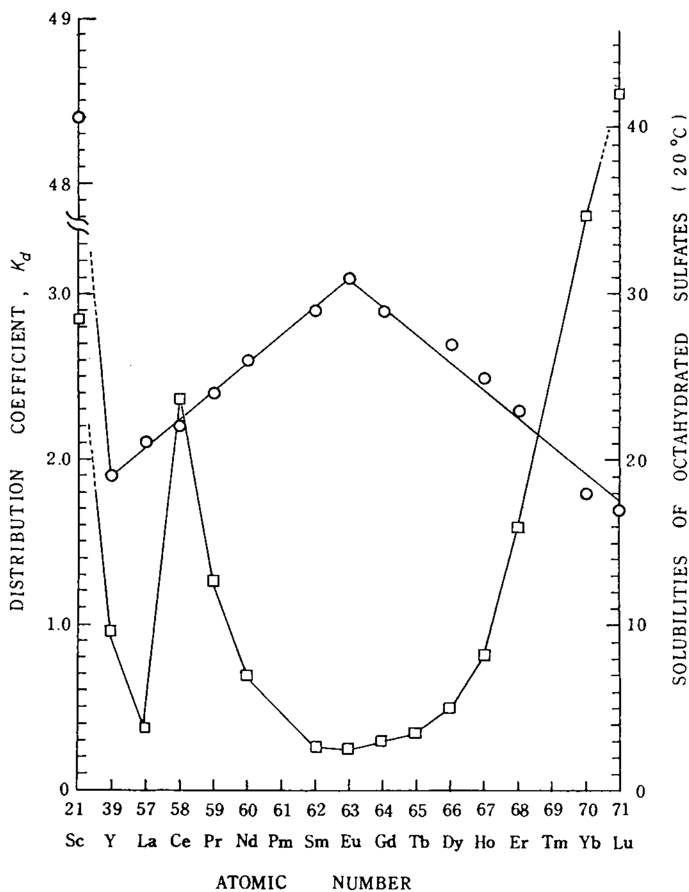


Fig. 2. Distribution coefficients of the lanthanides as a function of the atomic number. □ : solubility data; ○ : distribution coefficient data.

where U_{max} is the volume of the effluent when the concentration of respective rare earth reaches its maximum elution, U_0 the void space of the column, and M the weight of the dried resin in the column. K_d values thus calculated for 0.01 M potassium sulfate medium are plotted in Fig. 2 as a function of atomic number.

Dependence of K_d on the concentration of potassium sulfate was determined by a batch method for europium and scandium using the carrier-free radioactive isotopes ^{154}Eu ($T_{1/2}$ = 16 years) and ^{46}Sc ($T_{1/2}$ = 85 days), respectively. The result is shown in Fig. 3.

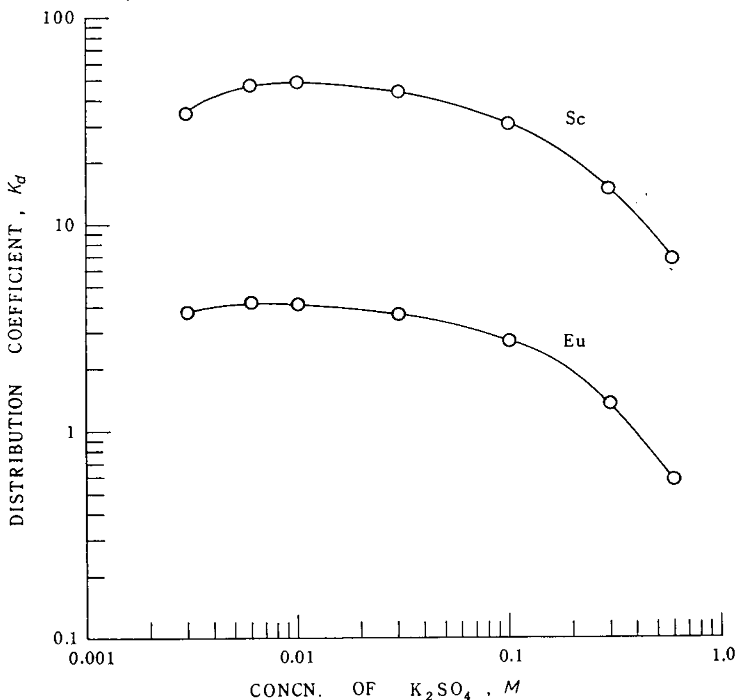


Fig. 3. Distribution coefficients of Eu and Sc with Dowex 1, X-8, at different concentrations of potassium sulfate.

As can be seen the distribution coefficient is not linear with respect to the increase of potassium sulfate concentration, but shows an adsorption maximum at about 0.01 M potassium sulfate concentration. Such a relation of the distribution coefficient to the concentration of sulfate ion seems to suggest that the complexes participating in the anion exchange mechanism in sulfate medium are quite involved, and that there are cationic and anionic species of sulfato complexes in the concentration range tested here. The maximum uptake of the sulfato complex of scandium or rare earth elements appears at the sulfate concentration where a neutral complex predominates in the solution[14]. At lower concentrations of sulfate the Donnan exclusion becomes less effective with increasing concentration of sulfate, and the uptake of the sulfato complex increases regularly up to the maximum, while at higher concentrations the rare earths are largely in an anionic form so that the uptake should decrease with increasing

concentration of sulfate. This can be explained, easily, because the higher complexes occupy more fixed ionic groups per atom in the resin phase and are in competition with an increasing number of free sulfate ions.

As seen in Fig. 2 the distribution coefficient curve for lanthanides indicates a maximum in the vicinity of atomic number 63, europium. Similarly, the sequence of elution does not vary monotonically from lanthanum to lutetium; the retention volumes increase in accordance with the increase of the atomic number for the cerium group, while they decrease in the same direction for the yttrium group.

The position of yttrium in the elution sequence of rare earths in sulfate medium is of interest. As indicated in Fig. 2 the distribution coefficient of yttrium lies between that of thulium and ytterbium. Thus yttrium can be assigned an apparent atomic number of 69.5. This does not agree with the apparent atomic number ascribed to yttrium from the elution sequence in anion exchanger–EDTA system[15].

We would like to mention for comparison that the behavior of yttrium varies widely in cation exchange systems[16] with organic eluents; for example, yttrium elutes between Dy–Tb with EDTA, near Nd with DTPA, near Pr with HEDTA, near Eu with citrate at 10–20° and Dy–Ho with citrate at 87–100°, near Ho–Dy with lactate, and near Dy–Ho with thiocyanate[17].

It is also of interest to note that there is an apparent correlation between the values of the distribution coefficients and the solubilities of octahydrated sulfates[18] of the lanthanide elements (see Fig. 2). The solubility of sulfates of the type, $Ln_2(SO_4)_3 \cdot 8H_2O$, first diminishes within the cerium group, reaches a minimum at europium, and then increases with increase of atomic number. However, such correlation does not hold for scandium and lanthanum.

A similar correlation was found for the behavior of lanthanide elements in the system Amberlite IRA-400–aqueous solution of disodium ethylenediaminetetraacetate[15]. The distribution coefficients increase regularly within the cerium group, reaching a maximum at europium, and then decrease on going to higher atomic numbers. On the other hand the solubility of the complex salts, NaLnY, varies conversely showing a minimum in the vicinity of Sm–Eu. A similar relationship between the solubility of rare earth double salts and the adsorption by an anion exchange resin from nitrate solution has been observed by DANON[19]. Based on these observations one may conclude that the maximum affinity to the anion exchange resin corresponds to the minimum solubility. The different solubilities of the sulfates of the lanthanide elements in an alkali metal sulfate solution have been explained by the formation of complex compounds of the type $Me_3[Ln(SO_4)_3]$ or $Me[Ln(SO_4)_2]$, where Me = Na^+, K^+, NH_4^+ (ref. 16). The minumum solubility in the vicinity of Eu–Sm suggests that the possibility of predominance of neutral sulfato complexes of europium and samarium is greater relative to the other lanthanide elements, in the sulfate solution and that this will necessarily result in a greater uptake of europium and samarium on the anion exchanger, as stated before.

Separation of rare earths

The separation of all the rare earths cannot be achieved by a single elution with potassium sulfate as eluent, because the distribution coefficients for the rare earths do not vary in a simple way with increasing atomic number. However, the present anion exchange separation can easily be preceded by a double alkali sulfate precipitation,

which will, if properly handled, separate the light (La to Gd) and heavy (Tb to Lu) rare earths, according to temperature and the particular alkali sulfate employed for the precipitation. In the present anion exchange procedure, the conditions are favorable for the separation of certain selected pairs or groups of rare earths. It is obviously important in obtaining good separation of two elements that the separation factor, which is defined by the ratio of their distribution coefficients, $\alpha_2^1 = K_{d1}/K_{d2}$ should be large. In the sulfate medium described here, the separation factor for adjacent pairs of rare earths is not so high as that observed in other media coupled with cation exchangers, and shows a rather uniform value, averaging 1.08. The separation factor for the pairs, in which the atomic number differs by n, can approximately be given by $(1.08)^n$. For the pairs Ho–Y and Dy–Y, which are generally difficult to separate, α_Y^{Ho} and α_Y^{Dy} are 1.33 and 1.41, respectively, which is, in general, higher than the factors obtained for the cation exchanger–α-hydroxyisobutyrate system ($\alpha_{Ho}^{Y} = 1.56$, $\alpha_Y^{Dy} = 1.04$ at 87°) and lactate system ($\alpha_{Ho}^{Y} = 1.21$, $\alpha_Y^{Dy} = 1.14$ at 90°).

In Fig. 4 typical elution curves are shown for several pairs or groups of rare earth elements. Assuming the normal distribution for the elution curves of neodymium and europium, we calculated the total number of theoretical plates in the column employed, and obtained 341 for neodymium and 314 for europium by the procedure given by BEUKENKAMP *et al.*[20]. The effective height of the theoretical plate (EHTP) was estimated as 0.17 cm for neodymium and 0.18 cm for europium. Actually, one might expect the EHTP to be of the order of the diameter of the resin particles (about 0.07 mm for 200 mesh) under optimum conditions. The values obtained are about twenty times higher than the practical particle size. However, because of packing irregularities,

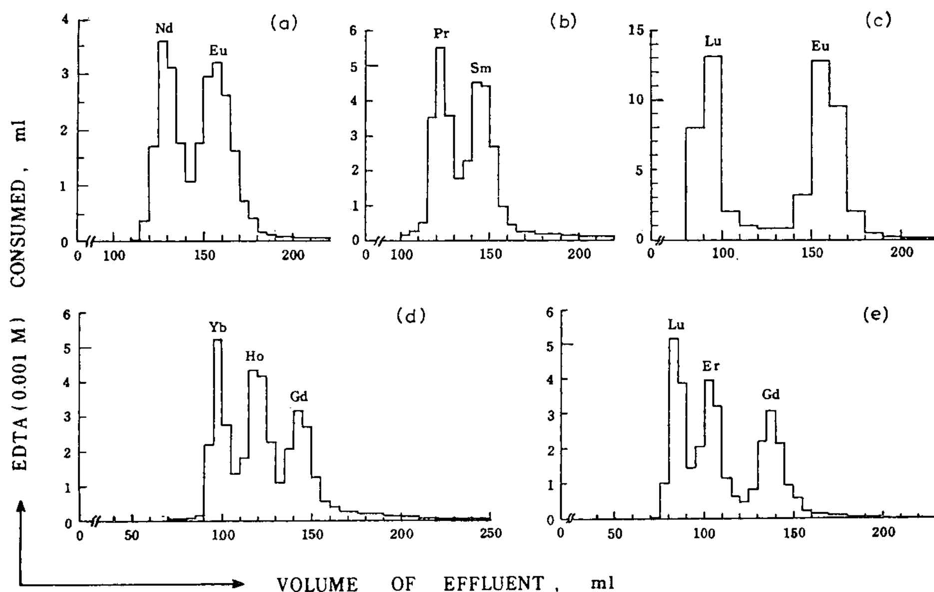


Fig. 4. Separation of selected pairs or groups of rare earth elements by elution with potassium sulfate solution. (a) Separation of neodymium and europium. (b) Separation of praseodymium and samarium. (c) Separation of lutetium and europium. (d) Separation of ytterbium, holmium and gadolinium. (e) Separation of lutetium, erbium and gadolinium.

channeling effects, and the need for extremely slow flow rates, ideal conditions are rarely realized in practice. Even in the excellent rare earth separations of KETELLE AND BOYD[21] the EHTP was approximately 0.1 cm[22], which is comparable to those obtained in the present separations. The total number of theoretical plates which are necessary to obtain a quantitative separation of neodymium and europium is $1.1 \cdot 10^3$, which corresponds to a column height of about 1.9 m. Likewise, a column height of about 8 m is required to separate quantitatively the adjacent pair, holmium and erbium. It is noteworthy that the rare earth-like element scandium has a distinctly high distribution coefficient compared to the rare earth elements (see Fig. 3). This should provide a promising procedure for the separation of scandium from other elements, because plenty of elements, as well as rare earths, have slight or no adsorption on anion exchange resins from sulfate or sulfuric acid medium.

SUMMARY

The anion exchange behavior of rare earth elements as well as scandium and yttrium in potassium sulfate medium was investigated. Rare earths and yttrium showed slight adsorption on the strongly basic anion exchange resin Dowex 1, X-8, from dilute potassium sulfate solution, while scandium was rather strongly adsorbed on the resin. The distribution coefficients of the rare earth elements were found to increase in a regular way from lanthanum to europium, reaching a maximum at europium and then decreasing with increasing atomic number. Although the system could not effect the separation of the whole of the rare earth elements in a single elution, the possibility of achieving effective chromatographic separation of selected pairs or groups of rare earth elements on a column, 57 cm long, was demonstrated.

REFERENCES

[1] R. F. BUCHANAN AND J. P. FARIS, *Conf. Use of Radioisotopes*, in *Phys. Sci. and Industry, Rept.*, RICC/173, Copenhagen, Denmark, 1960.
[2] L. R. BUNNEY, N. E. BALLOU, J. PASCUAL AND S. FOTI, *Anal. Chem.*, 31 (1959) 324.
[3] F. ICHIKAWA, *Bull. Chem. Soc. Japan*, 34 (1961) 183.
[4] K. A. KRAUS AND F. NELSON, *Am. Soc. Testing Mater., Spec. Tech. Publ.*, No. 195, 1958, p. 27.
[5] P. R. SUBBARAMAN, K. S. RAJAN AND J. GUPTA, *Z. Anorg. Allgem. Chem.*, 301 (1959) 261.
[6] Y. MARCUS AND F. NELSON, *J. Phys. Chem.*, 63 (1959) 77.
[7] E. K. HULET, R. G. GUTMACHER AND M. S. COOPS, *J. Inorg. Nucl. Chem.*, 17 (1961) 350.
[8] R. C. VICKERY, *J. Chem. Soc.*, (1955) 2360.
[9] N. SAITO AND T. SEKINE, *Nature*, 180 (1957) 753.
[10] S. MISUMI AND T. TAKETATSU, *J. Inorg. Nucl. Chem.*, 20 (1961) 127.
[11] T. TAKETATSU, *Talanta*, 10 (1963) 1077.
[12] J. P. SURLS, JR. AND G. R. CHOPPIN, *J. Inorg. Nucl. Chem.*, 4 (1957) 62.
[13] R. A. EDGE, *J. Chromatog.*, 6 (1961) 452.
[14] F. HELFFERICH, *Ion Exchange*, McGraw-Hill Book Co., New York, 1958.
[15] J. MINCZEWSKI AND R. DYBCZYŃSKI, *J. Chromatog.*, 7 (1962) 98.
[16] P. C. STEVENSON AND W. E. NERVIK, *The Radiochemistry of the Rare Earths, Scandium, Yttrium, and Actinium*, U.S. Atomic Energy Comm., NAS–NS 3020, 1961.
[17] H. HAMAGUCHI, R. KURODA AND N. ONUMA, *Talanta*, 10 (1963) 120.
[18] K. S. JACKSON AND G. RIENÄCKER, *J. Chem. Soc.*, (1930) 1687.
[19] J. DANON, *J. Inorg. Nucl. Chem.*, 7 (1958) 422.
[20] J. BEUKENKAMP, W. RIEMAN, III, AND S. LINDENBAUM, *Anal. Chem.*, 26 (1954) 505.
[21] B. H. KETELLE, AND G. E. BOYD, *J. Am. Chem. Soc.*, 69 (1947) 2800.
[22] J. H. YOE AND H. J. KOCH, *Trace Analysis*, J. Wiley & Sons, New York, 1957.

ION EXCHANGE PROCEDURES

V. SEPARATION OF BARIUM AND RADIUM*,**

FREDERICK NELSON
Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. (U.S.A.)

(Received March 18th, 1964)

In a separations scheme under development at this laboratory, barium and radium occur together in a subgroup separated from all the other elements. With the present procedure, barium and radium, at low concentrations, may be separated from each other. It is a modification of the method of DUYCKAERTS AND LEJEUNE[2]; the elements are absorbed on a small column of Dowex 50 cation exchange resin and then sequentially eluted with solutions of the di-ammonium salt of ethylenediaminetetraacetic acid [$(NH_4)_2H_2EDTA$] of appropriate pH.

DISCUSSION

Satisfactory column separations of barium and radium have been demonstrated with cation exchange resins and ammonium citrate[3,4] or lactate[5] solutions as eluents. These separations are better than those obtained with non-complexing mineral acids such as HCl[5]. However, fairly concentrated citrate or lactate solutions are used and this introduces complications when removal of excess solute is needed, *e.g.*, as in subsequent determination of radium by α-counting, where essentially residue-free "plates" are desirable.

With EDTA solutions of appropriate pH, the separation factor, $D_v(\text{Ra})/D_v(\text{Ba})$, is significantly larger than for citrate or lactate.[2] In addition, because of the high stability of their EDTA complexes, barium and radium can be eluted with relatively dilute EDTA solutions (*e.g.* 0.01 *M*).

Optimum conditions of pH for adsorption and elution of barium and radium (at low concentration) were established by a series of column experiments with 0.01 *M* $(NH_4)_2H_2EDTA$ solutions adjusted to the desired pH with concentrated NH_3 (14 *M*). The di-ammonium EDTA salt was used rather than the more available di-sodium salt because it can be completely removed by "flaming". Small columns (0.28 cm^2 × 3 cm) of Dowex 50-X8 (–400 mesh) in the ammonium-form were used. Elution positions were determined radiometrically, using ^{133}Ba and ^{226}Ra as tracers. The Po, Pb and Bi decay products of ^{226}Ra were removed immediately before use by passing a 2 *M* HCl solution of the tracer through a small column of Dowex-1 anion exchange resin. The HCl

* This document is based on work performed for the U.S. Atomic Energy Commission at the Oak Ridge National Laboratory, operated by Union Carbide Corporation.
** For Part IV, see ref. 1.

solution was evaporated to dryness and the residue containing ^{226}Ra was taken up in an appropriate EDTA solution.

The results are shown in Fig. 1, a plot of log D_v *vs.* pH, where D_v is amount per ml bed/amount per ml solution. Barium and radium are strongly adsorbed at low pH. Adsorbability of both elements decreases rapidly with increasing pH; the functions are arbitrarily plotted as straight lines. Barium may rapidly be eluted while retaining radium near pH = 8.8 where D_v(Ba) = *ca.* 1.2 and D_v(Ra) = *ca.* 9; radium may be eluted at pH $\geqslant$ 10.0 where D_v becomes < 1.

Our results may be compared with column experiments of DUYCKAERTS AND LEJEUNE[2]. From their data for 0.01 *M* EDTA solutions at pH 9, we compute a separation factor, D_v(Ra)/D_v(Ba) = *ca.* 7 which is in reasonably good agreement with our value, D_v(Ra)/D_v(Ba) = *ca.* 7.5. This rather favorable separation factor is not surprising. In the absence of complexing agents, radium is more strongly adsorbed by the resin than barium; additional improvement in separability results when EDTA solutions are employed as eluents since barium is more strongly complexed than radium by this reagent[6].

TYPICAL SEPARATION

A typical separation of barium and radium is shown in Fig. 2. For this separation, a 0.4 ml aliquot containing ^{133}Ba and ^{226}Ra in 0.01 *M* EDTA (adjusted to pH 8.8 with NH_3) was added to a 0.28 cm^2 $\times$ 3 cm column of Dowex 50-X8, -400 mesh resin in

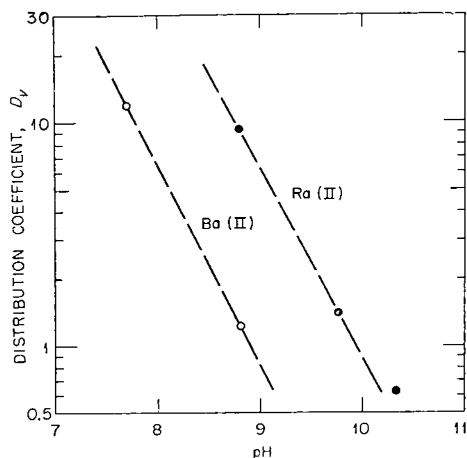


Fig. 1. Adsorption of barium and radium from 0.01 *M* EDTA solutions (Dowex 50-X8, NH+ form, 25°).

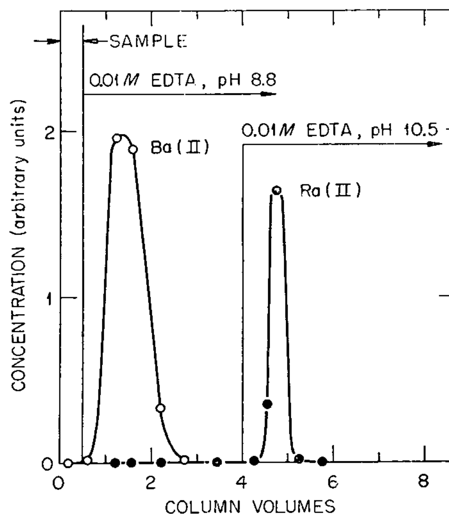


Fig. 2. Separation of barium and radium (Dowex 50-X8, 3 cm $\times$ 0.28 cm^3, 25°).

the NH+-form. The column had been pretreated with the same EDTA solution; on continued elution with this solution, Ba was removed in a sharp band with peak concentration near 2 column volumes. Radium was removed in a sharp band with 0.01 *M* EDTA at pH 10.5.

PROCEDURE

(a) *Materials and reagents*

Resin. Dowex 50-X8 (-400 mesh), hydrogen-form. The resin is converted to the NH_4^+-form by treating it in a column with 10 column volumes (c.v.) of 1 *M* NH_4Cl. After conversion, excess NH_4Cl is washed from the bed with *ca.* 3 c.v. of distilled water. The resin is stored in water.

Apparatus. A section of plastic tubing 0.6 cm inside diameter and 12 cm in length is used to prepare the column. The tubing is pulled out to a tip at one end and a porous Teflon plug inserted to retain the resin. Additional apparatus are plastic test tubes, Teflon evaporating dishes, plastic transfer pipettes and syringes.

Column. Resin bed: 0.28 cm^2 × 3 cm; column volume – 0.85 ml.

Flow rate: *ca.* 0.6 cm/min.

Temperature: 25°.

Effluent volumes (column volumes – c.v.):

Ba fraction: 4 c.v. (3.4 ml) of Solution II;

Ra fraction: 2 c.v. (1.7 ml) of Solution III.

Solutions. (I) 1 *M* NH_4Cl;

(II) 0.01 *M* $(NH_4)_2H_2EDTA$ – adjusted to pH 8.8 with conc. NH_3;

(III) 0.01 *M* $(NH_4)_2H_2EDTA$ – adjusted to pH 10.5 with conc. NH_3.

Note: If the di-ammonium EDTA salt is not available, solutions II and III may be prepared from the di-sodium salt by cation exchange as illustrated in the following example:

A 10 ml aliquot of 0.10 *M* Na_2H_2 EDTA solution is passed into a 2.0 cm^2 × 5 cm column of water-washed Dowex 50-X8 in the NH_4^+-form. The column is washed with *ca.* 25 ml of distilled water; the effluent contains $(NH_4)_2H_2EDTA$ and has a pH of *ca.* 4.7. Water is added to give the desired EDTA concentration and then concentrated NH_3 (dropwise) is added to give the desired pH.

(b) *Sample preparation*

The sample containing Ba and Ra in HNO_3 is evaporated in a Teflon beaker to near dryness and the residue is taken up in *ca.* 0.4 ml of Solution II.

(c) *Column operation*

Resin as a slurry in water is added to the plastic column until a resin bed 3 cm in length (*ca.* 0.85 ml) is formed. The bed is pretreated with 2 c.v. of Solution II and the sample is added. Flow rate is controlled by air pressure to about 0.6 cm/min. When the sample has passed into the bed, 0.4 ml of 0.01 *M* EDTA at pH 8.8 is added as wash, followed by an additional 3 c.v. (2.5 ml) of the same solution. This removes Ba. The column is then treated with 2 c.v. (1.7 ml) of 0.01 *M* EDTA at pH 10.5 to remove Ra. The column may be regenerated with *ca.* 4 c.v. of water.

The total column operation time is about 30 min.

SUMMARY

A cation exchange procedure is described for separating barium and radium. Separation is achieved with EDTA solutions of controlled pH.

REFERENCES

[1] T. Murase, E. L. Lind and F. Nelson, *J. Chromatog.*, 14 (1964) 478.
[2] G. Duyckaerts and R. Lejeune, *J. Chromatog.*, 3 (1960) 61.
[3] E. R. Tompkins, *J. Am. Chem. Soc.*, 70 (1948) 3520.
[4] W. H. Power, H. W. Kirby, W. C. McGluggage, G. D. Nelson and J. H. Payne, Jr., *Anal. Chem.*, 31 (1959) 1077.
[5] G. M. Milton and W. E. Grummit, *Can. J. Chem.*, 35 (1957) 541.
[6] F. Nelson, R. A. Day and K. A. Kraus, *J. Inorg. Nucl. Chem.*, 15 (1960) 140.

Notes

Isolation and quantitative determination of pseudouridine in urine by anion-exchange chromatography*

A convenient method for the isolation of pseudouridine from urine was recently described by COHN *et al.*[1]. Urine was freed from cations and anions by successive passage through columns of strong-acid and weak-base ion-exchange resins. The neutral substances, including pseudouridine, which pass through both these columns were then applied in an alkaline borate solution to a strong-base ion-exchange resin and the uncharged or mono-ionic compounds including uracil, were eluted with a sodium borate-ammonium bicarbonate buffer. Pseudouridine which under these conditions exists as a doubly charged borate complex was subsequently eluted with ammonium bicarbonate. Removal of the buffer by evaporation and treatment with strong-acid resin furnished pseudouridine. However, this procedure in the hands of the checkers led to a product still contaminated with borate, the last traces of which had to be eliminated by paper chromatography.

This communication describes a simpler procedure which has been used extensively in our laboratories for preparative as well as analytical purposes. Pseudouridine is obtained from urine by the use of only two resin columns and a single, completely volatile buffer-system which avoids the danger of contamination with borate or other inorganic ions. Another advantageous feature of this method is the possibility of isolating components other than pseudouridine which are obtained in this fractionation, free of inorganic ions for purification and study (see below).

In a typical preparative experiment 3185 ml normal urine (24 h collection) was passed through a column (7.5 × 11 cm) of Dowex 50-X8 (H-form). The column was eluted with 4000 ml of water and the combined effluents were adjusted to pH 5–5.3 by stirring with an appropriate quantity of Dowex 3-X4 (free base) for 30–45 min. Approximately 400 g wet resin was required in this experiment. The mixture was then filtered and the resin washed with four 250 ml portions of water. The filtrate and washings were evaporated to dryness under reduced pressure and temperature. The residue was redissolved in 100 ml *N* ammonia and the solution applied to a column (3.2 × 32 cm) of Dowex 2-X8 (bicarbonate-form) which had been equilibrated previously with 400 ml *N* ammonia. The column was then eluted with 0.02 *M* triethylammonium bicarbonate buffer at pH 9.5. The absorption of the eluate at 253 mμ was monitored with a recording U.V. flow-photometer and collected in 15 ml fractions on a fraction collector at a flow rate of 3 ml/min. A large peak consisting mainly of N-methyl-2-pyridone-5-carboxamide emerged almost immediately. It was followed by three distinct peaks of varying size. In some urines screened in our laboratories peaks

* Contribution No. 3124 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, Calif.

No. 3 and 4 (see Fig. 1) were insignificant or absent. A preliminary examination of the fractions represented by these peaks has shown them to be a complex mixture of substances. The main components, however, appear to be peptides attached to a moiety with strong absorption in the 260 mμ region. A more detailed description of these interesting, as yet unidentified, metabolites will be presented in a separate report.

Pseudouridine began to emerge when approximately 5000 ml buffer had passed through the column and was collected in the next 2500 ml. Uracil (peak 6 in Fig. 1),

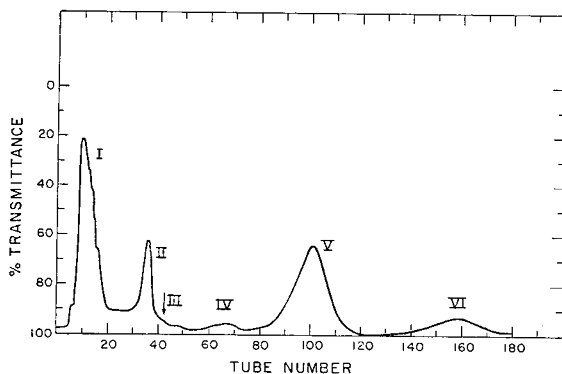


Fig. 1. Chromatographic analysis of pseudouridine in normal urine (8 mg creatinine/analysis) on a 0.8 × 20 cm column of Dowex 2-X8 (bicarbonate form). Elution with 0.025 *M* triethylammonium bicarbonate, pH 9.5. Flow rate: 1 ml/min, 4 ml/tube.

identified by its λ_{max} and 260/280 mμ ratios at pH 2 and 12, was not eluted until about 9500 ml buffer had passed through the column. The eluate containing the pseudouridine was evaporated to dryness under reduced pressure and temperature and the residue was distilled 3 to 4 times with 15–20 ml of water in order to remove the last traces of buffer. The crude pseudouridine thus obtained was dried in a desiccator over phosphorous pentoxide for 12 h and extracted with two 2 ml portions of boiling 95 % ethanol. The extracts were filtered and allowed to cool. The first extract which had an amber color yielded 13.6 mg pseudouridine with a diffuse m.p. at about 190°. The second, colorless extract gave 8.7 mg product with m.p. 213–215°. These two crops when pooled and recrystallized from 3 ml methanol furnished 14.4 mg crystalline pseudouridine, m.p. 221–222°; λ_{max} 262 mμ; ε_{max} 7980; A 290/260:0.066 at pH 2, and λ_{max} 287 mμ; ε_{max} 7960; A 290/260:2.26 at pH 12. The product migrated as a single component in isopropanol–acetic acid–water (60:30:10), R_F 0.29; *n*-butanol-water (86:14), R_F 0.11; 3-methyl-1-butyn-3-ol–water (75:25), R_F 0.32; 3-methyl-1-pentyn-3-ol saturated with *N* ammonia, R_F 0.12 and acetonitrile–water–formic acid (70:25:5), R_F 0.36. The solid which remained after the first two extractions with 95 % ethanol was dissolved in 5.2 ml boiling methanol and filtered. On cooling 20.25 mg crystalline pseudouridine was obtained, m.p. 220–221°; λ_{max} 262 mμ; ε_{max} 7580; A 290/260:0.069 at pH 2, and λ_{max} 287 mμ; ε_{max} 7530; A 290/260 2.20 at pH 12. The product was chromatographically homogeneous in the above solvent systems. The mother liquors yielded a further 8 mg pseudouridine with m.p. 219–222°. Total yield of crystalline pseudouridine: 42.4 mg. Slight modifications

of this isolation procedure may be necessary to take into account variation in the level of urinary pseudouridine in different individuals and samples.

In the analytical version of this procedure a quantity of urine corresponding to 10 mg creatinine was passed through a Dowex 50 column (0.8 × 10 cm) followed by 450 ml of water. The effluents were neutralized with Dowex 3 resin to pH 5 and filtered. The resin was washed with four 25 ml portions of water and the pooled filtrates were evaporated to dryness. The residue was redissolved in 10 ml *N* ammonia and 8 ml of the solution was chromatographed on a column (0.8 × 20 cm) of Dowex 2 (bicarbonate-form). Triethylammonium bicarbonate (0.025 *M*) of pH 9.5 was used for elution and fractions of 4 ml were collected at a flow rate of 1 ml/min. Fig. 1 illustrates the excellent separation obtained with this system. The tubes containing pseudouridine were pooled and evaporated to dryness. The residue was redissolved in 3 ml of water and a portion representing approximately 1.4–3 optical density units was further purified by paper chromatography (duplicate spots) on Whatman No. 1 paper with methylbutynol–water (75:25) as a solvent. This eliminates U.V. absorption originating from impurities in the buffer system and resin. Pseudouridine was located on the paper under U.V. light, the absorbing spots were cut out and eluted with 3 ml glycine buffer (pH 2) each and the nucleoside determined spectrophotometrically by its absorption at 262 mμ. For calculations ε_{max} 7980 was used. A blank was prepared by identical treatment of a similar area of the chromatogram free of pseudouridine. The reproducibility of this assay in 6 duplicate runs was $\pm$ 5%. In three experiments in which authentic pseudouridine was added to urine 85%, 89% and 90% were recovered by this method. Approximately two thirds of the loss occurred during the paperchromatography step and about one third during the other operations of the procedure.

N-Methyl-2-pyridone-5-carboxamide (peak I) was determined quantitatively by evaporation of the eluate to dryness, passage of the redissolved residue through a composite column consisting of successive sections (0.8 × 5 cm each) of Dowex 50 (H-form) and Dowex 20 (OH-form) and spectrophotometric assay of the effluent at 258 mμ[2].

Acknowledgement

This work was supported by a grant from the National Institutes of Health, Public Health Service.

Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, Calif. (U.S.A.)

HEINRICH RINDERKNECHT
VICTORIA MA

1 W. E. COHN, V. KURKOW AND W. CHAMBERS, *Biochem. Prep.*, 10 (1963) 135.
2 J. M. PRICE, *J. Biol. Chem.*, 211 (1954) 117.

Received May 25th, 1964

Effect of the phosphorylation state of thymidine derivatives on Sephadex K_d values*

It has been demonstrated[1-3] that gel filtration chromatography on Sephadex G-25, G-50 and G-75 will separate bases, nucleosides, nucleotides, oligonucleotides and polynucleotides from each other. Each variety of Sephadex has its specific range of maximum usefulness, and no one variety is effective over the whole range of molecular sizes.

During studies with the reaction reported by SCHRAMM *et al.*[4] for polymerizing 5'-thymidylic acid, products were isolated[5] containing more than one phosphorus per nucleoside and therefore not belonging to the above series. These products were examined for size by passage through Sephadex and were found to have elution positions corresponding to greater sizes than predicted from their molecular weights.

To explore this observation further in a systematic manner, thymidine and eighteen of its derivatives varying in size and state of phosphorylation have been comparatively chromatographed on Sephadex G-25 and G-50 columns (Table I).

TABLE I

COMPILATION OF COLUMN CHARACTERISTICS

Sephadex type	*Column dimensions (cm)*	*Flow rate (ml/min)*	V_i *(ml)*	V_o *(ml)*
G-25	1.0 × 175	0.70	67.6	60.3
G-50	1.0 × 170	0.30	87.7	53.4
G-75	2.3 × 36	0.70	96.0	50.9

A few of the compounds have also been studied on a G-75 column. The results are reported in Table II using K_d units derived from eqn. (1)[6]:

$$K_d = \frac{V_e - V_o}{V_i} \tag{1}$$

where V_e, V_o and V_i are the elution volume, outside volume and inside volume, respectively. For G-25 and G-50, V_o was the V_e of S-RNA. With G-75, DNA was used. For all three columns, V_i was determined[9] as the V_e for tritium water minus V_o. Thymine had $K_d = 1.13$ on G-25 and 1.12 on G-50. The eluant was 0.005 M triethylammonium bicarbonate.

The data from the decrease in K_d values with increasing size along each homologous series were fitted successfully to an exponential drop in K_d with linear increase in the number of thymidine units per molecule. From these expressions and from the specific G-25 K_d for $p(tp)_2$, molecular weights were calculated for the $K_d = 0.04$ member of six of the series (Table II).

It is seen that larger G-numbered Sephadexes can resolve higher molecular weight compounds. In using Sephadex as a chromatographic medium to assay the

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

TABLE II

THYMIDINE DERIVATIVES ON SEPHADEX

Terminal phosphorus	*Sephadex type*	*Compounds*[a] *and K_d values*						*Mol. wt. for K_d = 0.04*
None[b]		t	tpt	$t(pt)_2$	$t(pt)_3$	$t(pt)_5$	$t(pt)_9$	
	G-25	0.99	0.51	0.34	0.12	0.04	0.01	1,800
	G-50	0.98	0.61	0.54	0.30	0.14	0.05	3,000
5′-Phosphate[c]		pt	$(pt)_2$	$(pt)_3$	$(pt)_4$	$(pt)_6$	$(pt)_{10}$	
	G-25	0.31	0.13	0.06	0.02	—	—	1,100
	G-50	0.50	0.30	0.22	0.13	0.06	—	2,150
	G-75	0.80	0.65	0.53	0.44	0.30	0.15	5,200
3′-Phosphate[d]		tp	$(tp)_2$					
	G-25	0.32	0.12					—
	G-50	0.47	0.30					
3′,5′-Diphosphate[d]		ptp	$p(tp)_2$					
	G-25	0.07	0.04					700
	G-50	0.22	0.18					—

[a] Thymidine is represented by t and esterified phosphate by p such that pt is 5′-thymidylic acid, tp is 3′-thymidylic acid, and ptp is thymidine 3′,5′-diphosphate. Degree of linear oligomerization is represented by the subscripts.

[b] The last four compounds were synthesized from 5′-phosphates by cleavage with *E. coli* alkaline phosphatase.

[c] Prepared according to KHORANA AND VIZSOLYI[7].

[d] Prepared according to KHORANA[8].

degree of chemical polymerization of 5′-thymidylic acid, G-25 would be a poor choice since no clear distinction could be made between a tetramer and any higher polymer. Conversely, G-75 should give useful size information up to the eicosamer.

It is also evident from the data that comparison on one type of Sephadex of the different states of terminal phosphorylation yields the order *none* > *5′* (*or 3′*) > *3′,5′* for the molecular weight at any K_d value. An explanation might be that phosphate ester groups are associated with vicinal water molecules which are tightly enough bound to give the effect of a higher molecular weight in solution.

Acknowledgements

The authors wish to express their thanks to D. G. OTT, D. L. WILLIAMS and V. N. KERR, who synthesized many of the compounds.

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

F. N. HAYES
E. HANSBURY
V. E. MITCHELL

1 B. GELOTTE, *J. Chromatog.*, 3 (1960) 330.
2 B. GELOTTE, *Naturwiss.*, 48 (1961) 554.
3 S. ZADRAŽIL, Z. ŠORMOVÁ AND F. ŠORM, *Collection Czech. Chem. Commun.*, 26 (1961) 2643.
4 G. SCHRAMM, H. GRÖTSCH AND W. POLLMANN, *Angew. Chem.* (*Intern. Ed. Engl.*), 1 (1962) 1.
5 F. N. HAYES AND E. HANSBURY, *J. Am. Chem. Soc.*, 86 (1964).

6 P. FLODIN, *Dextran Gels and Their Applications in Gel Filtration*, Meijels Bokindustri, Halmstad, 1962.
7 H. G. KHORANA AND J. P. VIZSOLYI, *J. Am. Chem. Soc.*, 83 (1961) 675.
8 H. G. KHORANA, *Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest*, John Wiley and Sons, Inc., New York, N.Y., 1961.
9 P. FLODIN, *Dextran Gels and Their Applications in Gel Filtration*, Meijels Bokindustri, Halmstad, 1962, p. 48.

Received May 8th, 1964

J. Chromatog., 16 (1964) 410–412

A simple device for applying solutions to non-bound preparative thin-layer chromatographic plates

MOTTIER AND POTTERAT'S[1] method of covering glass plates with dry adsorbent has been used by MISTRYUKOV[2,3], ČERNÝ *et al.*[4] and VACÍKOVÁ *et al.*[5] to make preparative thin-layer chromatographic plates. The preparation of non-bound plates is less cumbersome and quicker than that of preparative bound plates. No special applicator is required to spread the adsorbent, and no need exists for the somewhat lengthy drying procedure[6] which is required to avoid cracking of bound plates of sufficient thickness for separations on a preparative scale.

The most tedious operation in the use of non-bound plates has been the application of the substrate solution. Micropipettes have been used commonly, but with these it is difficult to apply the solution in a uniform thin line. MORGAN'S[7] device for obtaining a series of spots by a row of capillary tubes was tried in this laboratory, but the tubes tended to become clogged with adsorbent. RITTER AND MEYER[8] have reported an apparatus with which the solution is placed onto the adsorbent from a hypodermic needle as the plate is moved slowly back and forth. This procedure was not satisfactory in our hands. A device consisting essentially of a wedge has been described[9] for the application of microliter quantities of solution to electrophoresis paper. A simpler and readily assembled wedge of greater capacity is described in this note.

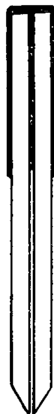


Fig. 1. Side view of the applicator.

The device consists of two $^1/_8$-inch-thick glass plates (19 cm × 5 cm) separated by spacers placed at intervals between the plates. The plates are held together in several places by strips of masking tape. The bottom edges of the two plates are beveled so that a wedge is produced (Fig. 1).

The applicator is clamped on a ring stand in a level stationary position, and the solution is placed in a small trough (length, 20 cm; height, 1.2 cm; top width, 1.0 cm) which is then placed under the applicator and moved up to bring the wedge into contact with the surface of the solution. In this manner some of the solution is drawn up between the plates by capillary action. A device of the dimensions described, with spacers consisting each of 16 layers of household aluminum foil, will take up approximately 1 ml of benzene solution. The charged applicator is leveled again carefully, and the chromatographic plate is raised by a laboratory jack to establish contact with the wedge. The adsorbent draws out the solution from the space between the glass plates without significantly damaging the layer. A thin uniform line of material is thus deposited. Development with solvent then gives relatively narrow bands which are straight and uniform. This method has proved quite satisfactory in resolving on a single plate a mixture containing up to 100 mg of material and allowing the processing of 2 g of mixture in one day.

U.S. Army Chemical Research and Development Laboratories, Edgewood Arsenal, Md. (U.S.A.) L. B. FARMER

1 M. MOTTIER AND M. POTTERAT, *Anal. Chim. Acta*, 13 (1955) 46.
2 E. A. MISTRYUKOV, *Collection Czechoslov. Chem. Commun.*, 26 (1961) 2071.
3 E. A. MISTRYUKOV, *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1961) 1406.
4 V. ČERNÝ, J. JOSKA AND L. LÁBLER, *Collection Czechoslov. Chem. Communs.*, 26 (1961) 1658.
5 A. VACÍKOVÁ, V. FELT AND J. MALÍKOVÁ, *J. Chromatog.*, 9 (1962) 301.
6 P. DAUVILLIER, *J. Chromatog.*, 11 (1963) 405.
7 M. E. MORGAN, *J. Chromatog.*, 9 (1962) 379.
8 F. J. RITTER AND G. M. MEYER, *Nature*, 193 (1962) 941.
9 G. E. W. WOLSTENHOLME AND E. C. P. MILLAR (Editors), *Ciba Foundation Symposium on Paper Electrophoresis*, Little, Brown and Company, Boston, Mass., 1956, p. 191.

Received May 1st, 1964

Dünnschichtchromatographie von Tropin, Pseudotropin und Tropinon

Die papierchromatographische Trennung eines Gemisches von Tropin, Pseudotropin und Tropinon bereitet nach unseren Erfahrungen erhebliche Schwierigkeiten. Fliessmittel-Systeme wie *n*-Propanol–Wasser (3:1), *n*-Butanol (mit Wasser gesättigt), *n*-Butanol–Eisessig (10:1, mit Wasser gesättigt), *sek.*-Butanol–Salzsäure (100:2, mit Wasser gesättigt) lieferten weder auf unbehandeltem noch auf imprägniertem Papier (Schleicher und Schüll 2043b, Borat-Puffer, pH = 7.8) befriedigende Resultate. Dagegen erzielten wir mit Hilfe der Dünnschichtchromatographie eine einwandfreie Trennung der drei Substanzen.

5 g Kieselgel G nach STAHL (Merck, Darmstadt) wurden mit 12 ml Wasser angerührt und als Paste mit dem Streichgerät gleichmässig auf Glasplatten (20 cm × 20 cm) verteilt. Die Platten trockneten wir 2 Stunden lang bei 110° im Schrank. Je 20–50 μg der drei Basen, in Chloroform oder Äthanol gelöst, wurden auf jeden Startpunkt aufgetragen. Von allen geprüften Fliessmittel-Systemen erwies sich ein Gemisch von Äthanol und 25%igem Ammoniak (8:2) als besonders geeignet. Zur Detektion der Alkaloide benutzten wir ein nach MUNIER modifiziertes DRAGENDORFF-Reagens. Die Basen erscheinen dabei als charakteristisch violett-lila Flecken.

R_F-Werte (Fig. 1):

Tropin	0.26
Pseudotropin	0.44
Tropinon	0.75

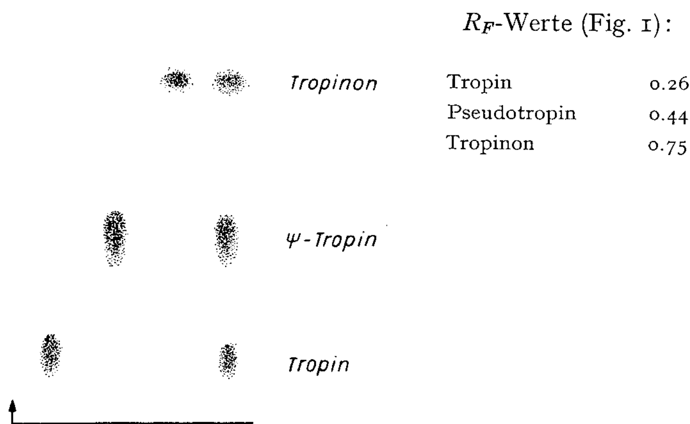


Fig. 1.

Akademie-Institut für Biochemie der Pflanzen, Halle, Saale (Deutschland)

D. NEUMANN
H.-B. SCHRÖTER

Eingegangen am 22. Mai 1964

Sodium carbonate–sodium bicarbonate buffer for the electrophoretic separation of nucleotides and phosphoric esters

Several alkaline buffers have been used for the electrophoretic separation of nucleotides. Sodium borate (0.05 M) pH 9.2, and a few organic buffers have been tried with limited success. Phosphate buffer (0.05 M) pH 7.5 gives good separations but has the obvious disadvantage of interfering with the detection of phosphate containing compounds[1].

A buffer of sodium carbonate–sodium bicarbonate (0.25 M) pH 9.2 (ref. 2) has been found to give fast and clear separations as shown in Table I. Electrophoresis was carried out with Whatman No. 1 paper and the apparatus of MARKHAM AND SMITH[3] for 2 h at 600 V (15 V/cm) and 30–60 mA (2–4 mA/cm).

TABLE I

MOBILITY OF VARIOUS COMPOUNDS

Compound	M_{P_i}	*Compound*	M_{P_i}
AMP-morpholidate	0.27	GDP	0.67
Cyclic 3′, 5′-AMP	0.29	CDP	0.67
ADP-maltose	0.33	ADP-3-phosphoglyceric acid	0.68
Diadenosine-diphosphate	0.33	UMP	0.75
ADP-glucose	0.40	Pyrophosphate	0.82
AMP-5′	0.49	Dihydroxyacetone-phosphate	0.84
CMP	0.57	UDP	0.85
ADP	0.58	2,3-Diphosphoglyceric acid	0.92
ATP	0.58	2-Phosphoglyceric acid	0.97
ADP-glyceric acid	0.60	3-Phosphoglyceric acid	1.02
UDP-glucose	0.60	Phospho-enol pyruvic acid	1.04
Galactose-1-phosphate	0.61	2,3-Cyclic phosphoglyceric acid	1.14
Glucose-1-phosphate	0.61		
Mannose-1-phosphate	0.61		
Xylose-1-phosphate	0.64		

As shown in Fig. 1, good separations were obtained only at high ionic strength. This is in contrast to the results of STRANSKÝ[4] with citrate buffer at pH 4.8.

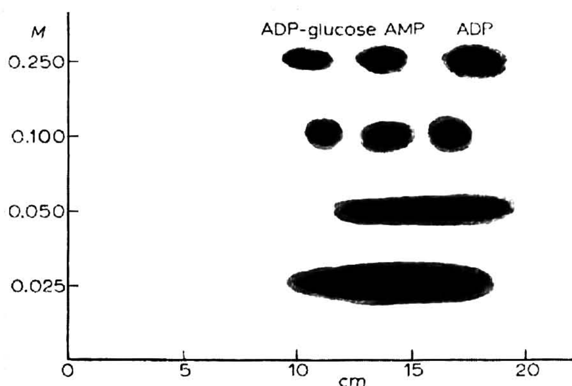


Fig. 1. Electrophoretic separation of adenine nucleotides in sodium carbonate–sodium bicarbonate buffers of different ionic strength (pH 9.2).

Compounds which are rather alkali-labile are not decomposed by the buffer. Thus UDP-glucose shows no sign of decomposition within 3–4 h. The buffer solution can be stored for months without alteration and can be recommended after considerable experience.

This investigation was supported in part by a research grant (No. GM 03442) from the National Institutes of Health, U.S. Public Health Service, by the Rockefeller Foundation and by the Consejo Nacional de Investigaciones Científicas y Técnicas (República Argentina).

Instituto de Investigaciones Bioquímicas "Fundación Campomar" and Facultad de Ciencias Exactas y Naturales, Obligado 2490, Buenos Aires 28 (Argentina)

EDUARDO RECONDO*
I. RUTH J. GONÇALVES**
MARCELO DANKERT

1 S. BURROWS, F. S. M. GRYLLS AND J. S. HARRISON, *Nature*, 170 (1952) 800.
2 G. E. DELORY AND E. J. KING, *Biochem. J.*, 39 (1945) 245.
3 R. MARKHAM AND J. D. SMITH, *Nature*, 168 (1951) 406.
4 Z. STRÁNSKÝ, *J. Chromatog.*, 10 (1963) 456.

Received May 15th, 1964

* Career investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (República Argentina).
** Post-Graduate Fellow of the Govêrno do Estado de Rio Grande do Sul (Brasil).

J. Chromatog., 16 (1964) 415–416

Identification of dialkyl peroxides by paper chromatography

It has been shown that good separation of alkyl hydroperoxides can be obtained by paper chromatography[1] though, as yet, no satisfactory method for the separation of dialkyl peroxides has been reported. The following separation was devised in order to characterise dialkyl peroxides produced in the radiation induced oxidation of hydrocarbons but may well be of value in other work.

Experimental procedure

The apparatus used was similar to that described by CARTLIDGE AND TIPPER[1]. Whatman No. 3 chromatography paper was treated with 5 vol. % solution of silicone oil (Hopkin and Williams MS 1107) in 80–100° petroleum ether and dried in an oven at 110° for 1 h. Samples were applied and the paper was sandwiched between glass plates which had previously been treated with the silicone solution. Chromatograms were run using a solution of water in methanol as moving phase, the rate of movement of the solvent front being approximately 7 cm/h. They were then developed by spraying with a solution of ferrous thiocyanate[2] and left to stand for 20–30 min when the presence of peroxides was indicated by red spots. It was possible to detect 100 μg of peroxide.

TABLE I

R_F VALUES OF DIALKYL PEROXIDES

(Stationary phase: silicone from 5 % solution; moving phase: water–methanol)

Compound	R_F	
	10 % H_2O in moving phase	*15 % H_2O in moving phase*
Di-*n*-hexyl peroxide	0.43	0.21
Di-cyclohexyl peroxide	—	0.44
Di-*n*-pentyl peroxide	0.58	0.35
Di-*n*-butyl peroxide	0.70	0.55
Alkyl hydroperoxides	1.0	1.0

Results are shown in Table I. The dialkyl peroxides used were prepared by the method of WILLIAMS AND MOSHER[3].

It will be seen that a good separation of peroxides may be achieved. The R_F values are somewhat dependent on the experimental conditions and in establishing the identity of an unknown, it was found preferable to run control samples of known peroxides with the unknown.

Acknowledgement

Thanks are due to Dr. C. F. H. TIPPER for helpful advice during the course of this work.

Donnan Laboratories, The University, Liverpool (Great Britain)

G. DOBSON
G. HUGHES

1 J. CARTLIDGE AND C. F. H. TIPPER, *Anal. Chim. Acta*, 22 (1960) 106.
2 M. J. ABRAHAM, A. G. DAVIES, D. R. LLEWELLYN AND E. M. THAIN, *Anal. Chim. Acta*, 17 (1957) 499.
3 A. R. WILLIAMS AND H. S. MOSHER, *J. Am. Chem. Soc.*, 76 (1954) 2984.

Received May 19th, 1964

Book Review

Gas Chromatography, edited by LEWIS FOWLER, Academic Press, New York–London, 1963, 270 pp.

This volume contains the proceedings of the Fourth International Symposium on Gas Chromatography held in June 1963 at Michigan State University under the auspices of the Analysis Instrumentation Division of the Instrument Society of America.

It consists of a number of invited papers and research reports produced by people with a broad range of interests, and thus various aspects of gas chromatography have been treated.

Topics such as the theory of packed column efficiency, aspects of thermodynamics of solutions studied through gas–liquid chromatography, reduced pressure gas chromatography and lunar gas chromatography, just to mention some of the more representative topics, are extensively treated; the discussion held at the symposium is also reported.

The purpose of this symposium was the stimulation of ideas and the dissemination of information about gas chromatography, and there is no doubt that this aim has been achieved. Anyone, be he experienced or a novice, will certainly profit by going through this interesting volume.

A. LIBERTI (Naples)

Announcement

SEMINAR ON FATTY ACIDS

A Seminar on Fatty Acids will be held at the Regional Research Laboratory, Hyderabad-9, India, during the first week of February 1965. The seminar will be conducted in two broad sessions—academic and industrial. In the academic session research papers will be presented covering the latest advances in the field of fatty acids and their derivatives. The industrial session will consider various aspects of manufacture and utilization of fatty acids, as well as their standards and specifications. Further particulars may be obtained from the Director of the laboratory.

Bibliography Section

Paper Chromatography

1. REVIEWS AND BOOKS

HEFTMANN, E.: Chromatography. *Anal. Chem.*, 36 (1964) 14R–35R — a review of papers on paper, column and thin-layer chromatography published in 1961–1963; 1766 references.

2. FUNDAMENTALS, THEORY AND GENERAL

MICHAL, J. AND ACKERMANN, G.: Zur Entmischung der Lösungsmittel bei der chromatographischen Trennung. I. Selektive Sorption des Fliessmittels auf Cellulose und Entstehung der 2. Front in der Papierchromatographie. *Talanta*, 11 (1964) 441–449.

MICHAL, J. AND ACKERMANN, G.: Zur Entmischung der Lösungsmittel bei der chromatographischen Trennung. II. Selektive Sorption der Mischungen Alkohol–Wasser und Alkohol–Wasser–Säure an der Cellulosesäule. *Talanta*, 11 (1964) 451–459.

PEJŠA, K., DOBÍHALOVÁ, L., FIDLER, Z. AND DVOŘÁK, J.: Countercurrent electrophoresis on paper. IX. Comparison of electrophoretic, chromatographic and static distribution isotherms. *J. Chromatog.*, 14 (1964) 464–468.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

DENTI, E., LUBOZ, M. P. AND MASSAGLIA, A.: Paper chromatography of polyphenyls. Quantitative determination of diphenyl in the presence of polyphenyls. *J. Chromatog.*, 14 (1964) 539–542 — in the form of sulphonation products.

MUKAI, M., TEBBENS, B. D. AND THOMAS, J.: Multidimensional chromatography of arenes produced during combustion. *Anal. Chem.*, 36 (1964) 1126–1130.

6. ALCOHOLS

BURGOS, J., HEMMING, F. W., PENNOCK, J. F. AND MORTON, R. A.: Dolichol: a naturally-occurring C_{100} isoprenoid alcohol. *Biochem. J.*, 88 (1963) 470–482.

TUSTANOWSKI, S., NOWICKI, R., NOWICKA, I. AND ZIELINSKI, A. Z.: (Detection of diglycerol in the presence of glycerol by paper chromatography). *Chem. Anal. (Warsaw)*, 9 (1964) 623–624.

ULBRICH, V. AND DLASK, V.: Identifizierung von Glycidyläthern. Trennung und Identifizierung von α-Alkyl(aryl)-äthern des Glycerins mit Hilfe der Papierchromatographie. *J. Chromatog.*, 14 (1964) 432–438.

7. PHENOLS

ERNST, W. AND BÄR, F.: Die Umwandlung des 2,4-Dinitro-6-*sec.*-butylphenols und seiner Ester im tierischen Organismus. *Arzneimittel-Forsch.*, 14 (1964) 81–84.

GUMPRECHT, D. L.: Paper chromatography of phenylphenols. *Anal. Chem.*, 36 (1964) 1154–1155.

ZWIMPFER, G. AND BÜCHI, J.: Die papierchromatographische Prüfung der Filix-Phlorglucide und der Rohfilicine. *Pharm. Acta Helv.*, 39 (1964) 327–336.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

DI MODICA, G. AND TIRA, S.: (Chromatographic comparison of flavonoid pigments from *Inulae*). *Ann. Chim. (Rome)*, 53 (1963) 764–773; *C.A.*, 59 (1963) 11885b.

9. OXO COMPOUNDS

BURTON, J. S. AND STEVENS, R.: Chemistry of hop constituents. XVIII. Hulupinic acid. *J. Chem. Soc.*, (1964) 952–955 — Whatman No. DE-20 paper.

FORSS, D. A. AND STARK, W.: Paper chromatography of the 2,4-dinitrophenylhydrazones of alk-1-en-3-ones. *Anal. Chem.*, 36 (1964) 941–942.

KHORANA, M. L. AND SANGHAVI, M. M.: Two new glucosides from *Cassia angustifolia* pods. *J. Pharm. Sci.*, 53 (1964) 110–112.

LEMLI, J., DEQUEKER, R. AND CUVEELE, J.: (Anthraquinone drugs. I. The presence of dianthrones in rhubarb root). *Pharm. Weekblad*, 98 (1963) 500–502; *C.A.*, 59 (1963) 9085f.

LEMLI, J., DEQUEKER, R. AND CUVEELE, J.: (Anthraquinone drugs. II. The presence of rheindianthrone in the roots of *Rheum palmatum*). *Pharm. Weekblad*, 98 (1963) 529–533; *C.A.*, 59 (1963) 9085g.

POHLOUDEK-FABINI, R. AND GÖTZKERITZ, D.: (Papierchromatographisch-spektrophotometrische Bestimmung von Carvon als Reinsubstanz oder in ätherischen Ölen). *Nahrung*, 7 (1963) 122–138; from *Z. Anal. Chem.*, 202 (1964) 385.

POLACZEK, L., KUSZCZAK, H. AND FISCHHOF, K.: (Method of determination of ethyl flavono-7 hydroxyacetate ("Recordil") and some possible impurities from its synthesis). *Chem. Anal. (Warsaw)*, 9 (1964) 275–281.

10. CARBOHYDRATES

BERTONE, E.: (Determination of sugars in urine by the method of paper chromatography). *Minerva Med.*, 54 (1963) 975–981; *C.A.*, 59 (1963) 11866b.

BIHARI-VARGA, M.: A method for the chromatographic determination of carbohydrates. *Acta Chim. Acad. Sci. Hung.*, 38 (1963) 55–56; *C.A.*, 59 (1963) 15584b — spraying with $CuSO_4$, cutting out and iodometric estimation.

BRDARIC, R., MIHOLJCIC, M. AND JADRIC, S.: (Paper chromatography of products formed by the action of salivary and pancreatic amylase of albino rats on starch). *Arch. Farm. (Belgrade)*, 13, No. 2 (1963) 93–97; *C.A.*, 59 (1963) 11811g.

EGOROVA, V. D.: (The determination of monosaccharides in bacterial cells by paper chromatography). *Lab. Delo*, 9, No. 4 (1963) 42–44; *C.A.*, 59 (1963) 11865h.

ELDREDGE, N. T., READ, G. AND CUTTING, W.: Sialic acids in the brain and tissues of various animals. *Med. Exptl.*, 8 (1963) 265–277; *C.A.*, 59 (1963) 15670g.

MUKERJEE, H. AND SRI RAM, J.: A new solvent system for paper chromatographic separation of glucuronic and galacturonic acids. *J. Chromatog.*, 14 (1964) 551–552.

PALLAVICINI, J. C., GABRIEL, O., DI SANT'AGNESE, P. A. AND BUSKIRK, E. R.: Isolation and characterization of carbohydrate–protein complexes from human sweat. *Ann. N.Y. Acad. Sci.*, 106, Art. 2 (1963) 330–338; *C.A.*, 59 (1963) 9161c.

ROBYT, J. AND FRENCH, D.: Purification and action pattern of an amylase from *Bacillus polymyxa*. *Arch. Biochem. Biophys.*, 104 (1964) 338–345.

RUKHLYADEVA, A. P., SEMIKINA, L. V. AND CHEREDNICHENKO, V. S.: (Quantitative chromatographic determination of carbohydrates). *Tr. Tsentr. Nauchn.-Issled. Inst. Spirt. i Likero-Vodochn. Prom.*, No. 13 (1962) 14–18; *C.A.*, 59 (1963) 9066e — determination with anthrone reagent after elution.

UENO, T.: Semichemical soy sauce. VI. Identification and determination of sugars by multiple paper chromatography. *Nippon Nogeikagaku Kaishi*, 34 (1960) 1039–1042; *C.A.*, 59 (1963) 9246f.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

ALEXANDROWICZ, J.: (Determination of lower (C_1–C_6) and higher (C_8–C_{18}) fatty acids in culture fluids of anaerobically growing sporogenic *Clostridium*). *Med. Doswiadczalna Mikrobiol.*, 15 (1963) 149–158; *C.A.*, 59 (1963) 9102c.

APARICIO, M.: (Separation of the glycerides by fractional crystallization and paper chromatography in order to characterize butter). *Proc. 16th Intern. Dairy Congr., Copenhagen, 1962, Sect. B*, pp. 5–10; *C.A.*, 59 (1963) 15860a.

BAJOR, G. F. AND CLARK, W. G.: Ion-exchange resin paper for separation of the acidic urinary metabolites norepinephrine-2-^{14}C in human subjects. *J. Chromatog.*, 14 (1964) 447–450 — Dowex-1 impregnated paper.

GARCIA FERNANDEZ, J. C. AND LANUCARA, E. N.: (Chromatographic technique for the investigation of monochloroacetic acid in wines and ciders). *Rev. Asoc. Bioquim. Arg.*, 28 (1962) 179–182; *C.A.*, 59 (1963) 12129c.

GNEDKOV, P. A.: (Chromatographic investigation of organic acids in extracts of Crassulaceae). *Farmatsevt. Zh. (Kiev)*, 18 (1963) 27–31; *C.A.*, 59 (1963) 14292c.

KAZANSKAYA, L. N. AND BEZRUCHENKO, L. P.: (Identification of non-volatile organic acids in bread baking products by chromatography). *Khlebopekar. i Konditer. Prom.*, 7, No. 7 (1963) 10–14; *C.A.*, 59 (1963) 12085e.

KIRCHMEIER, O.: (Paper-chromatographic determination of the quality of silages). *Z. Tierphysiol., Tierernaehr. Futtermittelk.*, 18 (1963) 111–114; *C.A.*, 59 (1963) 15866d.

SAVORY, E.: The detection of carboxylic acids on paper chromatograms by means of the dimethylglyoxime–nickel biuret reaction. *J. Chromatog.*, 14 (1964) 549–550.

UDAGAWA, K. AND KINOSHITA, S.: A colorimetric determination of gibberellin A_3. II. Specificity of the de ermination. *Nippon Nogeikagaku Kaishi*, 35 (1961) 224–228; *C.A.*, 59 (1963) 14505c.

WEBER, M. A., HOAGLAND, A. N., KLEIN, J. AND LEWIS, K.: Biosynthesis of α-ketoadipic acid by extracts of baker's yeast. *Arch. Biochem. Biophys.*, 104 (1964) 257–266.

13. STEROIDS

GOWER, D. B.: Chromatographic separation of C_{19}-16-dehydro-steroids. *J. Chromatog.*, 14 (1964) 424–431 — silica gel thin layer and silicic acid impregnated paper.

HELLSTRÖM, K. AND LINDSTEDT, S.: Cholic-acid turnover and biliary bile-acid composition in humans with abnormal thyroid function. *J. Lab. Clin. Med.*, 63 (1964) 666–679.

KECSKES, L., MUTSCHLER, F., THAN, E. AND FARKAS, I.: (The isolation of estrone, 17-estradiol and estriol from human ovaries by paper chromatography). *Acta Endocrinol.*, 39 (1962) 483–490; *C.A.*, 59 (1963) 9034h.

KORNEL, L.: A new method for elution of conjugated steroids from paper strips. *Anal. Chem.*, 36 (1964) 443–444.

KUSHINSKI, S. AND DEMETRIOU, J. A.: Analysis of urinary metabolites of 17β-estradiol-4-C^{14}. A rapid paper chromatographic method for obtaining a profile of urinary metabolites. *Steroids*, 2 (1963) 253–270; *C.A.*, 59 (1963) 15557c — R_F values of 32 oestrogens.

MAHESH, V. B. AND HERRMANN, W.: Isolation of estrone and 11-hydroxyestrone from a feminizing adrenal carcinoma. *Steroids*, 1 (1963) 51–61; *C.A.*, 59 (1963) 9035c.

STÁRKA, L. AND HAMPL, R.: Die Isolation des 7α-Hydroxydehydroepiandrosteronsulfates aus dem menschlichen Plasma. *Naturwiss.*, 51 (1964) 164.

TALMAGE, J. M., PENNER, M. H. AND GELLER, M.: Quingestrone — determination of minute quantities of decomposition products by paper chromatography. *J. Pharm. Sci.*, 53 (1964) 76–79.

TAMM, J., VOIGT, K. D. AND VOLKWEIN, U.: Water-soluble steroid conjugates. I. Extraction, separation and estimation. *Steroids*, 2 (1963) 271–277; *C.A.*, 59 (1963) 15558d — R_F and ΔR_{Ms} values of 15 substances.

14. STEROID GLYCOSIDES

KRASSO, A. F., WEISS, E. AND REICHSTEIN, T.: Die Cardenolide von *Beaumontia grandiflora* Wallich. *Pharm. Acta Helv.*, 39 (1964) 168–179.

15. TERPENE DERIVATIVES

RICHTER, G. AND MUSCHOLL, P.: Papierchromatographie ätherischer Öle. I. Ein neues Verfahren zur direkten papierchromatographischen Trennung ätherischer Öle auf imprägniertem Papier. *J. Chromatog.*, 14 (1964) 439–446 — advantages of paraformaldehyde-treated paper are stressed.

16. NITRO AND NITROSO COMPOUNDS

WAGNER, G. AND PISCHEL, H.: Über Synthese und Spaltung von Nitromercaptopyridin- und Nitromercaptobenzol-S-glucosiden. *Pharmazie*, 19 (1964) 197–200.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

LEPPANEN, V. V. E. AND OKA, M.: Metabolism of tryptophan in cancer of various sites. *Ann. Med. Exptl. Biol. Fenniae (Helsinki)*, 41 (1963) 123–137; *C.A.*, 59 (1963) 14405e.

OATES, J. A., NIRENBERG, P. Z., JEPSON, J. B., SJOERDSMA, A. AND UDENFRIEND, S.: Conversion of phenylalanine to phenethylamine in patients with phenylketonuria. *Proc. Soc. Exptl. Biol. Med.*, 112 (1963) 1078–1081.

OHKUMA, S. AND NIINUMA, K.: Chromatographic detection of spermine and choline in human semen. *Proc. Japan Acad.*, 39 (1963) 136–140; *C.A.*, 59 (1963) 11866e.

ROBERTS, D. J.: Some possible causes of pharmacological activity in blank eluates following the separation of sympathomimetic catecholamines by paper chromatography. *J. Pharm. Pharmacol.*, 16 (1964) 313–322.

18. AMINO ACIDS

BARUA, R. K. AND BHUYAN, K.: Identification of S-methylcysteine sulfoxide by paper chromatography, in the presence of other sulfur-containing amino acids. *Current Sci. (India)*, 32 (1963) 353–354; *C.A.*, 59 (1963) 15583d.

DE LUCA, F., PINCHERA, A., DELLABARBA, D., MENZIGER, G., DI GIROLAMO, M. AND CRAMAROSSA, L.: (Chromatographic study of thyroid hormones in plasma, urine and faeces in man after ^{131}I administration). *Folia Endocrinol. (Pisa)*, 15 (1962) 786–803; *C.A.*, 59 (1963) 9036h.

HÄKKINEN, H. M., KULONEN, E. AND WALLGREN, H.: The effect of ethanol and electrical stimulation on the amino acid metabolism of rat-brain-cortex slices *in vitro*, *Biochem. J.*, 88 (1963) 488–498.

HARTEL, J. AND PLEUMEEKERS, A. J. G.: A quantitative chromatographic determination of cysteic acid in amino acid mixtures on ion exchange papers. *Anal. Chem.*, 36 (1964) 1021–1022 — spectrophotometry after elution.

JOSEPH, K. T. AND RAJENDRAN, K. R.: Quantitative estimation of proline (in protein hydrolyzates) by paper chromatography. *Leather Sci.*, 10 (1963) 70–71; *C.A.*, 59 (1963) 9065g — elution of ninhydrin-coloured spot and photometry.

KARPOV, V. L.: (The isolation of ^{14}C-labelled amino acids by ion exchange and paper chromatography). *Vestn. Leningr. Univ.*, 18, No. 9, *Ser. Biol.*, No. 2 (1963) 108–114; *C.A.*, 59 (1963) 11867b.

KHARAT'YAN, A. M.: (Paper-chromatographic study of some blood serum and urine amino acids in patients having Botkin's disease). *Sb. Nauchn. Tr. Tashkentsk. Gos. Med. Inst.*, 17 (1961) 22–26; *C.A.*, 59 (1963) 15748g.

LOEFER, J. B. AND SCHERBAUM, O. H.: Free amino acids in Tetrahymenidae. *J. Protozool.*, 10 (1963) 275–279; *C.A.*, 59 (1963) 11916h.

MORREALE DE ESCOBAR, G., LLORENTE, P., JOLIN, T. AND ESCOBAR DEL REY, F.: The "transient instability" of thyroxine and its biochemical applications. *Biochem. J.*, 88 (1963) 526–530.

SCHARPENSEEL, H. W. AND KRAUSSE, R.: (Radiochromatographic studies on the turnover of sulphate, and of the S-amino acids cystine and methionine in soil and humic acid). *Z. Pflanzenernaehr. Dueng. Bodenk.*, 101 (1963) 11–23; *C.A.*, 59 (1963) 9267e.

YANG, S. F. AND MILLER, G. W.: Biochemical studies on the effect of fluoride on higher plants. 1. Metabolism of carbohydrates, organic acids and amino acids. *Biochem. J.*, 88 (1963) 505–509.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

FUJIWARA, T. AND COULSON, C. B.: Further studies on milk-whey glycopeptides and the products of lysine–lactose browning systems. *Biochem. J.*, 88 (1963) 61P–62P.

20. PROTEINS

DEYL, Z. AND ROSMUS, J.: Paper chromatography of the denaturation and first degradation products of collagen. *J. Chromatog.*, 14 (1964) 537–539 — mixtures of alcohol–acetic acid–water.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BERGNER, H.: (Paper-chromatographic separation of 2',3'-ribomononucleotides, with special consideration of the possibilities for quantitative microanalytical errors). *Z. Med. Labortech.*, 3 (1962) 305–322; *C.A.*, 59 (1963) 9066f.

BOBRANSKI, B. AND SYPER, L.: Metabolism of 5-allyl-5-(β-hydroxypropyl)-barbituric acid in the human body. *Arch. Immunol. Terapii Doswiadczalnej*, 9 (1961) 579–591; *C.A.*, 59 (1963) 12041e.

BROWN, E. G.: Purine and pyrimidine derivatives in mature pea seeds. *Biochem. J.*, 88 (1963) 498–504.

HAINES, J. A., REESE, C. B. AND LORD TODD: The methylation of nucleosides and mononucleotides with diazomethane. *J. Chem. Soc.*, (1964) 1406–1412.

JACOBSON, K. B.: Chromatographic separation of nucleotides and nucleosides. *J. Chromatog.*, 14 (1964) 542–543 — on DEAE-cellulose paper and plain paper.

KURS'KII, M. D.: (Study by paper chromatography of the content of adenosine triphosphate (ATP) and products of its metabolism in the brain of animals). *Ukr. Biokhim. Zh.*, 35 (1963) 535–541; *C.A.*, 59 (1963) 14375d.

22. ALKALOIDS

GUTORSKA, A.: (Separation and identification of nicotinic and isonicotinic acids and amides by paper chromatography). *Chem. Anal. (Warsaw)*, 9 (1964) 597–600.

HEARST, P. J.: Greenheart alkaloids. II. Isolation and characterization of seven alkaloids. *J. Org. Chem.*, 29 (1964) 466–470.

IVANOV, N.: (Study of alkaloids in Bulgarian cigarets and smoke by means of paper chromatography). *Compt. Rend. Acad. Bulgare Sci.*, 12 (1959) 317–320; *C.A.*, 59 (1963) 15616d.

KAPADIA, G. J., BALDWIN, H. H. AND SHAH, N. J.: Paper chromatography and identification of *Magnolia acuminata* L. alkaloids. *J. Pharm. Pharmacol.*, 16 (1964) 283–284.

PIERZCHALSKI, T. AND PIECHNIK, Z.: (Quantitative determination with cationite paper of several glycoalkaloids occurring simultaneously in potatoes). *Chem. Anal. (Warsaw)*, 9 (1964) 283–289.
PLATEK, J.: (Paper chromatographic method for rapid detection of the main alkaloids in tobacco). *Chem. Anal. (Warsaw)*, 9 (1964) 261–266.
SYHORA, K., ČEKAN, Z., HEŘMÁNEK, S. AND TROJÁNEK, J.: Steroid derivatives. XVIII. Isolation of alkaloids of the solasodine type from Solanaceae. *Planta Med.*, 10 (1962) 318–326; *C.A.*, 59 (1963) 15598e.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

JANNES, J., LEPPANEN, V. V. E. AND OKA, M.: Tryptophan metabolites in the urine and liver metastases in carcinoid syndrome. *Ann. Med. Exptl. Biol. Fenniae (Helsinki)*, 41 (1963) 115–122; *C.A.*, 59 (1963) 14405d.
KOSAKA, K., IWAHARA, M. AND KONDO, T.: Direct-reacting bilirubin. *Proc. 8th Intern. Congr. Hematol., Tokyo, 1960*, Vol. 2 (Publ. 1962), pp. 1247–1250; *C.A.*, 59 (1963) 15595f.
MAGGIORE, Q.: (Quantitative paper chromatography of conjugated bilirubin in various biological fluids). *Rass. Fisiopatol. Clin. Terap. (Pisa)*, 35 (1963) 74–79; *C.A.*, 59 (1963) 11866g.
SILVESTRINI, B., CATANESE, B., CORSI, G. AND RIDOLFI, P.: The urinary metabolites of 5-(2-diethylaminoethyl)-3-phenyl-1,2,4-oxadiazole. *J. Pharm. Pharmacol.*, 16 (1964) 38–42.
YASUDA, H.: Microdetermination of the acrid substance in *Eutrema wasabi* by paper chromatography. *Nippon Nogeikagaku Kaishi*, 34 (1960) 725–728; *C.A.*, 59 (1963) 9246c.

24. ORGANIC SULPHUR COMPOUNDS

AEBI, H., LAUBER, E., LEHNER, H. AND MICHAELIS, W.: Zur Kenntnis der Verteilung in den Organen und der Ausscheidung von 9-[(N-Methyl-^{14}C-3-piperidyl)-methyl]-thioxanthen-hydrochlorid (Methixen). *Arzneimittel-Forsch.*, 14 (1964) 92–95.
COYNE, C. M. AND MAW, C. A.: The paper chromatography of aliphatic sulphonates. *J. Chromatog.*, 14 (1964) 552–555 — 15 substances, 7 solvent systems and 5 detection methods are described.
FOLKARD, A. R. AND JOYCE, A. E.: Collection and identification of thiols and disulfides. *J. Sci. Food Agr.*, 14 (1963) 510–514; *C.A.*, 59 (1963) 14278e.
VIGNOLI, L., GOUEZO, F. AND MOREL, M. C.: (Revealing spots of N-substituted phenothiazines, and their urinary metabolites on paper chromatograms). *Bull. Soc. Pharm. Marseille*, 11, No. 44 (1962) 53–56; *C.A.*, 59 (1963) 9065e.
WAGNER, G. AND BÖHME, S.: Über die Darstellung optisch aktiver Hydroxyphenylalkyl-sulfoxide. *Arch. Pharm.*, 297 (1964) 257–267.

25. ORGANIC PHOSPHORUS COMPOUNDS

ISHIKAWA, T.: Paper chromatography of sucrose esters. *Kogyo Kagaku Zasshi*, 66 (1963) 715–717; *C.A.*, 59 (1963) 15583a.
JOHNSTON, J. M. AND BEARDEN, J. H.: Phosphatidic acids as intermediates in fatty acid absorption. *Proc. 6th Intern. Conf. Biochem. Lipids, Marseilles, 1960*, (Publ. 1961), pp. 172–178; *C.A.*, 59 (1963) 14388f.
ROSE, H. G.: Studies on the molecular structure of rat liver cardiolipin. *Biochim. Biophys. Acta*, 84 (1964) 109–127.
TURPINI, R., MOSSA, A. AND CIPOLLI, P. L.: (Glass-paper chromatography as a means of detecting cerebral and serum phospholipids). *Boll. Soc. Ital. Biol. Sper.*, 37 (1961) 1315–1317; *C.A.*, 59 (1963) 11864b.

26. METALLO-ORGANIC COMPOUNDS

HILL-COTTINGHAM, D. G. AND LLOYD-JONES, C. P.: Analysis of iron-chelates in plant extracts. II. Ferric ethylenediamine-bis(*o*-hydroxyphenylacetic acid). *J. Sci. Food Agr.*, 14 (1963) 171–175; *C.A.*, 59 (1963) 11865e.

27. VITAMINS

FÜRTIG, W. AND POHLOUDEK-FABINI, R.: Spezifischer Nachweis und quantitative Bestimmung der Dehydroascorbinsäure nach papierchromatographischer Verteilung. *Pharmazie*, 19 (1964) 209–215.
HERRMANN, J. AND ANDRAE, W.: (Oxidative decomposition products of L-ascorbic acid. I. Paper chromatographic detection). *Nahrung*, 7 (1963) 243–255; *C.A.*, 59 (1963) 14269d.
LOSITO, R. AND MILLAR, G. J.: Preparative separation of vitamins K_1 and K_3 from vitamins $K_{2(30)}$ and $K_{2(35)}$ by column chromatography. *J. Chromatog.*, 14 (1964) 496–499.

MIKI, T., KIKUCHI, N. AND SAHASHI, Y.: Paper chromatographic estimation of L-ascorbic acid and D-araboascorbic acid. *J. Vitaminol.* (*Kyoto*), 8 (1962) 279–285; *C.A.*, 59 (1963) 11865g.

SVOBODOVÁ-LÉBLOVÁ, S., KOŠTIŘ, J. V. AND HAIS, I. M.: Paper partition chromatography of riboflavin decomposition products. The action of some reducing and oxidizing agents on riboflavin solutions. *J. Chromatog.*, 14 (1964) 451–455.

VLITOS, A. J. AND CUTLER, H. G.: The natural auxins of the sugar cane. A paper-chromatographic separation of the growth factors present in true seed. *Proc. Brit. West Indies Sugar Technologists, 1960*, pp. 113–127; *C.A.*, 59 (1963) 11891a.

28. ANTIBIOTICS

AMERICAN CYANAMID CO. (by COSULICH, D. B., PATRICK, J. B. AND WILLIAMS, R. P.): Antibiotics. *French Pat.* M 1661 (Febr. 18, 1963); *C.A.*, 59 (1963) 12132c.

GUPTA, V. S. AND RAO, P. L. N.: Antibiotic principles of *Garcinia morella*. V. Reverse-phase paper chromatography of constituents of gambose resin and seed coat, and isolation and characterization of γ- and δ-guttiferinic acids and guttiferins. *Indian J. Chem. Soc.*, 1 (1963) 259–266; *C.A.*, 59 (1963) 14293b.

KHOKHLOV, A. S. AND RESHETOV, P. D.: Chromatography of streptothricins on carboxymethylcellulose. *J. Chromatog.*, 14 (1964) 495–496.

29. INSECTICIDES AND OTHER PESTICIDES

DUTT, M. C. AND SEOW, P. H.: New spray reagents for the detection of thiophosphate insecticides on paper chromatograms. *J. Agr. Food Chem.*, 11 (1963) 467; *C.A.*, 59 (1963) 15580f — Metanil Yellow, Yellow RFS, and methyl orange.

KORANSKY, W., PORTIG, J. AND MUENCH, G.: (Absorption, distribution and elimination of α- and γ-benzene hexachloride). *Arch. Exptl. Pathol. Pharmakol.*, 244 (1963) 564–575; *C.A.* 59 (1963) 9227h.

30. SYNTHETIC AND NATURAL DYES

BECK, F.: (Die Wertbestimmung saurer Diazofarben, ihren Gehalt an Feuchtigkeit und Natriumchlorid und insbesondere ihr papierchromatograpisches Verhalten). *Stain Technol.*, 38 (1963) 165–171; from *Z. Anal. Chem.*, 203 (1964) 147.

CALZOLARI, C., COASSINI, L. AND LOKAR, L.: (Partition paper chromatography of food dyes). *Rass. Chim.*, 15, No. 2 (1963) 49–60; *C.A.*, 59 (1963) 9238f.

LATINÁK, J.: Chromatographie der optischen Aufhellmittel auf Basis der 4,4′-Diaminostilben-2,2′-disulfosäure. *J. Chromatog.*, 14 (1964) 482–484.

POPOVA, I. A.: (The investigation of plastid pigments by means of paper chromatography). *Tr. Botan. Inst. Akad. Nauk SSSR, Ser. 4, Eksperim. Botan.*, No. 16 (1963) 154–164; *C.A.*, 59 (1963) 14295h — colorimetry after elution from paper.

SAAKOV, V. S.: (Method of preparing pure xanthophylls). *Botan. Zh.*, 48 (1963) 554–557; *C.A.*, 59 (1963) 9015d.

SADINI, V.: (Identification of extraneous food dyes in dairy products). *Proc. 16th Intern. Dairy Congr., Copenhagen, 1962, Sect. C.*, pp. 474–486; *C.A.*, 59 (1963) 15850h — R_F values of 20 coal tar dyes.

WOLLENWEBER, P.: (Trennung von synthetischen Lebensmittelfarbstoffen). *Mitt. Bg. GdCh. Fachgr. Lebensmittelchem. Gerichtl. Chem.*, 17 (1963) 67–70; from *Z. Anal. Chem.*, 203 (1963) 63.

32. PHARMACEUTICAL APPLICATIONS

LEYBOLD, K. AND STAUDINGER, H.: (Hydroxylation of phenylbutazone and imipramine by rabbit liver microsomes). *Z. Ges. Exptl. Med.*, 136 (1962) 78–85; *C.A.*, 59 (1963) 12043g — paper and thin-layer chromatography.

LIN, S.-C. C. AND WAY, E. L.: Use of paper chromatographic techniques on urine for evaluating narcotic usage by the nalorphine pupil test. *J. Forensic Sci.*, 8 (1963) 209–219; *C.A.*, 59 (1963) 15584d.

RENGEI, B.: (Paper chromatography in toxicological examination). *Morphol. Igazsagugi Orvosi Szemle*, 1 (1961) 130–135; *C.A.*, 59 (1963) 15844a.

ZARNACK, J. AND PFEIFER, S.: Dünnschichtchromatographie in Unterricht und Praxis der Arzneianalyse. I. Methodik: Analgetica, Antipyretica, Purine, Sulfonamide, Alkaloide und analoge Synthetica. *Pharmazie*, 19 (1964) 216–224.

33. INORGANIC SUBSTANCES

DATTA, S. K. AND SAHA, S. N.: Paper chromatographic separation of thorium, zirconium and uranium. *Z. Anal. Chem.*, 202 (1963) 332–339.

MÖLLER, H. G. AND ZELLER, N.: Glyoxal-bis-(2-hydroxyanil), ein Reagens für papierchromatographisch getrennte Kationen. *J. Chromatog.*, 14 (1964) 560–564 — examination of 34 ions.

O'LAUGHLIN, J. W. AND BANKS, CH. V.: Separation of various cations by reversed-phase partition chromatography using neutral organophosphorus compounds. *Anal. Chem.*, 36 (1964) 1222–1229.

PRÁŠILOVÁ, J. AND ŠEBESTA, F.: The chromatography of some cations on paper impregnated with ammonium phosphotungstate. *J. Chromatog.*, 14 (1964) 555–560 — ^{86}Rb, ^{134}Cs, ^{89}Sr and ^{91}Y.

SZCZĚPANIAK, W.: (Diphenylcarbazide resin. II. Quantitative determination of microgram quantities of chromium on paper impregnated with the ion exchanger Sel-K5). *Chem. Anal. (Warsaw)*, 9 (1964) 481–485.

34. RADIOACTIVE COMPOUNDS

HIRSCH, P.: (CO_2-fixation by hydrogen bacteria. II. Chromatographic evidence of early fixation products). *Arch. Mikrobiol.*, 46 (1963) 53–78; *C.A.*, 59 (1963) 14317b.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

KHAFAGY, S. M.: (Characterization and assay of gazarin, bitter principle of fruits of *Daucus carota* var. *boissieri*). *Svensk Farm. Tidskr.*, 67 (1963) 349–354; *C.A.*, 59 (1963) 9016h — paper and thin-layer chromatography.

Thin-layer Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

BRODASKY, T. F.: Reproducibility of R_F and correlation of chromatographic patterns on paper and thin layer plates. *Anal. Chem.*, 36 (1964) 996–999.

3. TECHNIQUES I

ABBOTT, D. C. AND THOMSON, J.: Wedge-layer chromatography for pesticide residue clean-up. *Chem. Ind. (London)*, (1964) 481.

BROWN, T. L. AND BENJAMIN, J.: Useful thin-layer chromatography techniques. *Anal. Chem.*, 36 (1964) 446–447 — prewashing of silica plates; photography of plates.

HUETTENRAUCH, R., KLOTZ, L. AND MUELLER, W.: (Thin-layer chromatography on ion-exchange resins). *Z. Chem.*, 3 (1963) 193; *C.A.*, 59 (1963) 14278e — vitamin B complex on Wofatit CP 300.

KAPADIA, G. J. AND RAO, G. S.: Circular thin-layer chromatography of tetracyclines. *J. Pharm. Sci.*, 53 (1964) 223–224.

TAKITANI, S. AND MATSUDA, K.: Simple electro-motive applicator for thin-layer chromatography. *Japan Analyst*, 13 (1964) 562–563.

WREN, J. J. AND SZCZEPANOWSKA, A. D.: Chromatography of lipids in presence of an antioxidant, 4-methyl-2,6-di-*tert.*-butylphenol. *J. Chromatog.*, 14 (1964) 405–410.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

SAWICKI, E., STANLEY, T. R., PFAFF, J. D. AND ELBERT, W. C.: Thin-layer chromatographic separation of benzo(*a*)pyrene and benzo(*k*)fluoranthene from airborne particulates. *Chemist Analyst*, 53 (1964) 6–8.

6. ALCOHOLS

WEKELL, J. C., HOULE, C. R. AND MALINS, D. C.: A method for the isolation of mono- and dihydric alcohols from complex mixtures. *J. Chromatog.*, 14 (1964) 529–531 — alcohols are fractionated in the form of nitrates.

7. PHENOLS

SLONAKER, D. F. AND SIEVERS, D. C.: Identification of trace quantities of antioxidants in polyethylene. *Anal. Chem.*, 36 (1964) 1130–1132.

WENKERT, E., LOESER, E.-M., MAHAPATRA, S. N., SCHENKER, F. AND WILSON, E. M.: Wheat bran phenols. *J. Org. Chem.*, 29 (1964) 435–439.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

MENSSEN, H. G. AND HONERLAGEN, H.: Ein exakter Aescin-Nachweis mit Hilfe der Dünnschichtchromatographie. *Mitt. Deut. Pharm. Ges.*, 34 (1964) 97–98.

VULF'SON, N. S., ZARETSKII, V. I. AND CHETVERIKOVA, L. S.: (Thin-layer chromatography of naturally-occurring coumarins and furocoumarins). *Izv. Akad. Nauk SSSR, Ser. Khim.*, (1963) 1503–1505; *C.A.*, 59 (1963) 15584c.

9. OXO COMPOUNDS

COBB, W. Y.: Separation of 2,4-dinitrophenylosazones of vicinal dicarbonyls into classes by thin-layer chromatography. *J. Chromatog.*, 14 (1964) 512–513 — Sea Sorb 43 with silica gel or celite and plaster of Paris.

ZAMOJSKI, A. AND ZAMOJSKA, F.: (Identification of volatile aliphatic aldehydes and ketones in the form of 2,4-dinitrophenylhydrazones by thin-layer chromatography). *Chem. Anal. (Warsaw)*, 9 (1964) 589–596.

10. CARBOHYDRATES

FUJIWARA, T. AND COULSON, C. B.: Glycopeptide components of bovine milk whey with possible anti-mycobacterial activity. *Biochem. J.*, 88 (1963) 61P — alumina plates.

GRASSHOF, H.: Dünnschicht-chromatographische Bestimmungen von Zuckern und Zuckeralkoholen auf Magnesiumsilikat. *J. Chromatog.*, 14 (1964) 513–515 — replacement of part of the water in the propanol–water system by a primary amine reduces the R_F values of ketoses and non-reducing compounds, but not of aldoses.

SHASHA, B. AND WHISTLER, R. L.: Celite–starch for thin-layer chromatography. *J. Chromatog.*, 14 (1964) 532–533 — applied for amino acids, sugars, methyl glycosides, purines, pyrimidines, nucleosides and nucleotides.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

COPIUS-PEEREBOOM, J. W. AND BEEKES, H. W.: Thin-layer chromatography of preserving agents. *J. Chromatog.*, 14 (1964) 417–423 — on cellulose, silica gel and kieselgur, kieselgel paper.

DE VRIES, B. AND JURRIENS, G.: Determination of triglyceride composition by horizontal thin-layer chromatography. *J. Chromatog.*, 14 (1964) 525–526.

JONES, D. F., MACMILLAN, J. AND RADLEY, M.: (Identification of gibberellic acid in immature barley). *Brauwissenschaft*, 16 (1963) 316–317; *C.A.*, 59 (1963) 15603d.

KAGAWA, T., FUKINBARA, T. AND SUMIKI, Y.: Thin-layer chromatography of gibberellins. *Agr. Biol. Chem. (Tokyo)*, 27 (1963) 598–599; *C.A.*, 59 (1963) 14503h.

KOREY, S. R. AND GONATAS, J.: Separation of human brain gangliosides. *Life Sci.*, 2 (1963) 296–302; *C.A.*, 59 (1963) 15675f.

LANEELLE, G.: (Mycolic acids of *Mycobacterium paratuberculosis*; fractionation by thin-layer chromatography). *Compt. Rend.*, 257 (1963) 781–783; *C.A.*, 59 (1963) 9100h.

NELSON, J. H., GLASS, R. L. AND GEDDES, W. F.: Silicic acid chromatography of wheat lipids. *Cereal Chem.*, 40 (1963) 337–343; *C.A.*, 59 (1963) 12082d.

RINK, M. AND HERRMANN, S.: Dünnschichtchromatographische Trennung von Ketocarbonsäuren. *J. Chromatog.*, 14 (1964) 523–524 — products of the reaction with 4-oxothiazolidine-2-thione are separated on acetyl-cellulose.

WAGNER, H. AND FRIEDRICH, H.: Über das Vorkommen einer Octadecatetraensäure in der Samen einiger Boraginaceen. *Naturwissenschaften*, 51 (1964) 164.

WAGNER, H. AND POHL, P.: Zur Kenntnis der Polyenfettsäuren von Meeresalgen. *Naturwissenschaften*, 51 (1964) 163–164.

13. STEROIDS

AUDRIN, P., FOSSARD, F. C., BOURGOIN, CH., JUNG, L. AND MORAND, P.: (Application of thin-layer chromatography in identifying and estimating hormones. II. Separation and determination of urinary aldosterone). *Rev. Franc. Etudes Clin. Biol.*, 8 (1963) 507–512; *C.A.*, 59 (1963) 14256b.

BANG, H. O.: A simplified method for the quantitative determination of pregnanediol in urine. *J. Chromatog.*, 14 (1964) 520–523.

IKAN, R., HAREL, S., KASHMAN, J. AND BERGMANN, E. D.: The separation of sterols and corresponding stanols by thin-layer chromatography. *J. Chromatog.*, 14 (1964) 504–506.

JACOBSOHN, G. M.: Quantitation of estrone, estradiol and estriol on thin-layer chromatograms by a photogrammetric procedure. *Anal. Chem.*, 36 (1964) 275–279.

O'DORCHAI, R., FLANAGAN, P. J. AND THOMSON, J. B.: Steroids. I. 4α-Methylergostan. *J. Chem. Soc.*, (1964) 1142–1147 — detection with chlorosulphonic acid–acetic acid mixture.

OERTEL, G. W., TORNERO, M. C. AND GROOT, K.: Thin-layer chromatography of steroid conjugates. *J. Chromatog.*, 14 (1964) 509–511 — ion-exchange celluloses.

SAMUEL, P., URIVETZKY, M. AND KALEY, G.: Separation and radioassay of fecal cholesterol and coprosterol using thin-layer chromatography. *J. Chromatog.*, 14 (1964) 508–509.

TAKEUCHI, M.: Analysis of steroids. I. Analysis of steroid hormones by thin-layer chromatography. *Chem. Pharm. Bull. (Tokyo)*, 11 (1963) 1183–1188; *C.A.*, 59 (1963) 15558h — relationship between the adsorptivity and functional groups.

TISHLER, F. AND BRODY, S. M.: Methandrostenolone — mechanism of hydrochloric acid induced fluorescence. *J. Pharm. Sci.*, 53 (1964) 161–164 — preparative TLC.

VECSEI (WEISZ), P., KEMÉNY, V. AND GÖRGÉNYI, A.: Separation of corticosteroids by thin-layer chromatography on silica gel plates containing tetrazolium blue. *J. Chromatog.*, 14 (1964) 506–507.

14. STEROID GLYCOSIDES

MATSUMOTO, N.: Analysis of steroids. II. Analysis of steroid sapogenins by thin-layer chromatography. *Chem. Pharm. Bull. (Tokyo)*, 11 (1963) 1189–1192; *C.A.*, 59 (1963) 15559a — R_F values of 20 steroid sapogenins.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

BENASSI, C. A., VERONESE, F. M. AND GINI, E.: Thin-layer chromatography of metabolic derivatives of tryptophan. *J. Chromatog.*, 14 (1964) 517–519 — polyamide layers.

NEURATH, G. AND DOERK, E.: Identifizierung und quantitative Bestimmung einzelner primärer und sekundärer Amine aus Gemischen als 4′-Nitro-azobenzolcarbonsäure-(4)-amide. *Chem. Ber.*, 97 (1964) 172–178 — two-dimensional TLC on silica gel G.

18. AMINO ACIDS

EULER, H. VON, HASSELQUIST, H. AND LIMNELL, I.: (Thin-layer chromatographic experiments). *Arkiv Kemi*, 21 (1963) 259–264; *C.A.*, 59 (1963) 15706g.

MASSAGLIA, A. AND ROSA, U.: Separation of ^{131}I-labelled monoiodotyrosine and diiodotyrosine by thin-layer chromatography. *J. Chromatog.*, 14 (1964) 516–517.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

SAHLI, M. AND OESCH, M.: Beitrag zur Dünnschichtchromatographie von Barbituraten. *J. Chromatog.*, 14 (1964) 526–529 — 18 barbiturates and hydantoins on silica gel.

22. ALKALOIDS

MCLAUGHLIN, J. L., GOYAN, J. E. AND PAUL, A. G.: Thin-layer chromatography of ergot alkaloids. *J. Pharm. Sci.*, 53 (1964) 306–310 — on silica gel G.

OSWALD, N. AND FLÜCK, H.: Getrennte Bestimmung von *Alkaloideta* mittels Dünnschichtchromatographie. 1. Mitt. Methode zur getrennten Bestimmung der Tropinalkaloide in Solanaceendrogen und der *Cinchona*-Alkaloide. *Pharm. Acta Helv.*, 39 (1964) 293–304.

PENNA-HERREROS, A.: Chromatographic separation of morphine, normorphine and nalorphine. *J. Chromatog.*, 14 (1964) 536.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

BENDER, D. F., SAWICKI, E. AND WILSON, R. M.: Fluorescent detection and spectrophotofluorometric characterization and estimation of carbazoles and polynuclear carbazoles separated by thin-layer chromatography. *Anal. Chem.*, 36 (1964) 1011–1017.

PECHTOLD, F.: Untersuchung über den oxydativen Abbau von Derivaten des 4-Aminophenazons. *Arzneimittel-Forsch.*, 14 (1964) 258–259.

24. ORGANIC SULPHUR COMPOUNDS

LEHNER, H., LAUENER, H. AND SCHMUTZ, J.: Zum Metabolismus von 9-[(N-Methyl-3-piperidyl)-methyl]-thioxanthen·HCl. *Arzneimittel-Forsch.*, 14 (1964) 89–91.

25. ORGANIC PHOSPHORUS COMPOUNDS

ARAKI, E.: Thin-layer chromatography of total serum lipids. *Nisshin Igaku*, 50 (1963) 85–91; *C.A.*, 59 (1963) 11866d.

BURTON, R. M. AND GIBBONS, J. M.: Lipid composition of a rat-brain synaptic-vesicle fraction. *Biochim. Biophys. Acta*, 84 (1964) 220–223.

HORROCKS, L. A.: Thin-layer chromatography of brain phospholipids. *J. Am. Oil Chemists' Soc.*, 40 (1963) 235–236; *C.A.*, 59 (1963) 11864e.

27. VITAMINS

JOHNSON, D. B. AND GOODWIN, T. W.: α-Hydroxyethylthiamine in plant tissues. *Biochem. J.*, 88 (1963) 62P–63P.

29. INSECTICIDES AND OTHER PESTICIDES

GELDMACHER-MALLINCKRODT, M.: (Detection of Systox (Demeton) and Meta-Systox as complexes with heavy metals). *Deut. Z. Ges. Gerichtl. Med.*, 54 (1963) 90; *C.A.*, 59 (1963) 15873e.

30. SYNTHETIC AND NATURAL DYES

BATTERHAM, T. J. AND WEISS, U.: The structure of elsinochrome A. *Proc. Chem. Soc.*, (1963) 89–90; *C.A.*, 59 (1963) 11802e.

GASPARIČ, J. AND CEE, A.: Chromatographische Trennung substantiver Farbstoffe. *J. Chromatog.*, 14 (1964) 484–486 — on silica gel G.

PEYRON, L.: (Fluorescent substances present in the oleiferous cells of citrus fruits). *Compt. Rend.*, 257 (1963) 235–238; *C.A.*, 59 (1963) 9082g.

WHELAN, F. J. AND PLAA, G. L.: The application of thin-layer chromatography to sulfobromophthalein metabolism studies. *Toxicol. Appl. Pharmacol.*, 5 (1963) 457–463; *C.A.*, 59 (1963) 15778e.

32. PHARMACEUTICAL APPLICATIONS

BRUD, W. AND DANIEWSKI, W.: (The thin-layer chromatography as an industrial analytical method. Determination of anaesthesine in ethyl *p*-glycosyl-aminobenzoate and of resorcinol and resorcinol diacetate in the products of resorcinol monoacetate synthesis). *Chem. Anal. (Warsaw)*, 9 (1964) 267–273.

33. INORGANIC SUBSTANCES

SIECHOWSKI, J.: (An attempt at quantitative determination of chromic acid by thin-layer chromatography). *Chem. Anal. (Warsaw)*, 9 (1964) 391–392.

TAKITANI, S., FUKUOKA, N., IWASAKI, Y. AND HASEGAWA, H.: Total analysis of metallic ions by thin-layer chromatography. 2. *Japan Analyst*, 13 (1964) 469–471 — combination of the NH_4HS method and TLC for systematic analysis of cations.

ZABIN, B. A. AND ROLLINS, C. B.: Inorganic ion exchangers for thin-layer chromatography. *J. Chromatog.*, 14 (1964) 534–535 — 8 cations on zirconium phosphate (H^+–form) and hydrous zirconium oxide (NH_4^+- and HCl-form).

Gas Chromatography

1. REVIEWS AND BOOKS

ABEL, K. AND BOBLE, F. W.: Three absolute gas chromatography detection methods and their potential for clinical analysis. *Trans. N.Y. Acad. Sci.*, 26, Ser. II, No. 2 (1963) 159–181.

DAL NOGARE, S.: Summary of the second International Houston Symposium on "Advances in Gas Chromatography", *J. Gas Chromatog.*, 2 (1964) 189–191 — a review of the symposium held in Houston, Tex., March 23–26, 1964.

GAS CHROMATOGRAPHY DIRECTORY. *J. Gas Chromatog.*, 2 (1964) 202–206 — a compilation of companies active in the field of gas chromatography: GC apparatus, accessories, supplements, services and literature sources; practically no information from East European countries such as Czechoslovakia, USSR, East Germany, etc.

INGHELBRECHT, M.: Analyse du gaz par la méthode chromatographique. *Rev. Gen. Gaz*, 85, No. 3–4 (1963) 59–60.

LITTLEWOOD, A. B.: Informal symposium of the Gas Chromatography Discussion Group. Process monitoring and control. *J. Gas Chromatog.*, 2 (1964) 186–188 — a review of 3 papers (HAWKES, J. C., PINE, C. S. F. and NOEBELS, A. J.), read at the London Meeting, April 10th, 1964.

ZHUKHOVITSKIĬ, A. A. AND ANVAER, B. I.: (Gas chromatography). *Zh. Vses. Khim. Obshchestva im. D. I. Mendeleeva*, 9 (1964) 186–195.

2. FUNDAMENTALS, THEORY AND GENERAL

2a. Gas–liquid systems

GIDDINGS, J. C.: The theoretical plate as a measure of column efficiency. *J. Gas Chromatog.*, 2 (1964) 167–169 — in addition to plate height, it is necessary to know the relative velocity difference and other parameters; plate height should be considered as a significant but not complete expression of the column resolution power.

GIDDINGS, J. C. AND SCHETTLER, P. D.: Measurement and interpretation of the C terms of gas chromatography. *Anal. Chem.*, 36 (1964) 1483–1489 — two methods for the experimental isolation of gas and liquid contributions, C_g and C_l, are developed and applied.

KNOX, J. H. AND MCLAREN, L.: A new method for measuring gaseous diffusion coefficients and obstructive factors. *Anal. Chem.*, 36 (1964) 1477–1482 — equation for determining D_g and γ by a technique in which the sharp band of chromatographed gas is forced to stay for a known time in order to spread by diffusion) in the column during GC analysis.

SCOTT, R. P. W.: Pressure changes during passage of a solute through a theoretical plate. *Anal. Chem.*, 36 (1964) 1455–1461 — the solute causes a pressure change which sharpens the front and tailing; it is significant especially in preparative GC.

2b. Gas–solid systems

HANSEN, R. S., MURPHY, J. A. AND MCGEE, T. C.: Gas chromatographic measurement of gas–solid interaction potentials and solid surfaces areas. *Trans. Faraday Soc.*, 60 (1964) 597–603 — GSC retention volumes at high temperatures are closely related to the adsorption.

KISELEV, A. V., NIKITIN, YU. S., PETROVA, R. S., SHCHERBAKOVA, K. D. AND YASHIN, YA. I.: Effect of pore size of silica gels on the separation of hydrocarbons. *Anal. Chem.*, 36 (1964) 1526–1533 — adsorption heat measurements are compared and GC applications are discussed.

PAPA, L. J.: Analysis of mixtures by differential reaction rates and gas–solid adsorption in gas chromatography. *Dissertation Abstr.*, 24 (1964) 3078 — more active type of sites cover about 23% of total surface area.

2c. Thermodynamics and theoretical relationships

KOVATS, E.: Gas chromatographic characterization of organic substances in the retention index system. *2nd Intern. Symp. Advances Gas Chromatography, Houston, Tex., March 23–26, 1964.*

LITTLEWOOD, A. B.: The specific retention of monofunctional organic solutes in monofunctional hexadecyl derivatives. *Anal. Chem.*, 36 (1964) 1441–1451 — regularities in V_g are observed and applied for the calculation of retention behaviour, with errors less than 10%.

2d. General

ACKMAN, R. G.: Fundamental groups in the response of flame ionization detectors to oxygenated aliphatic hydrocarbons. *J. Gas Chromatog.*, 2 (1964) 173–179 — molar response seems to be calculable from fundamental groups formed by initial thermal breakdown of the molecule in the flame.

GUIOCHON, G.: Influence of the apparatus on the apparent efficiency of columns in gas chromatography. *J. Gas Chromatog.*, 2 (1964) 139–145 — a theoretical discussion of the contributions of the apparatus to the zone spreading.

HOUGHTON, G.: The additivity of rate and diffusion phenomena in continuous chromatography. *J. Chromatog.*, 15 (1964) 5–8 — theory can also be applied to GC.

MATUKUMA, A.: (Retention index of methyl-paraffins in gas chromatography and comparison with "boiling point index"). *J. Chem. Soc. Japan, Pure Chem. Sect.*, 84 (1963) 774–779 — I_R on squalane is larger than on Apiezon and PEG and differs for derivatives having the methyl group in positions 2 or 3.

SIMSON, R. E. AND MICALLEF, J. A.: Hand-sorted punched-card system for gas-chromatographic data. *Chem. Ind. (London)*, (1964) 743–745.

ZHUKHOVITSKIĬ, A. A., TURKEL'TAUB, N. M., SHVARTSMAN, V. P. AND SHLYAKOV, A. F.: (Diffuse spreading of the fronts and the calculation of the composition mixtures in chromatography without carrier gas). *Dokl. Akad. Nauk SSSR*, 156 (1964) 654–657.

3. TECHNIQUES I

3a. Detectors

DIJKSTRA, A., FABRIE, C. C. M., KATEMAN, G., LAMBOO, C. J. AND THISSEN, J. A. L.: A recording conductometer for the determination of small amounts of carbon dioxide and its use in combination with the combustion technique in gas chromatography. *J. Gas Chromatog.*, 2 (1964) 180–183 — measuring of conductivity of NaOH solution absorbing CO_2; four-electrode cell and new absorption vessel are described in detail.

KARMEN, A.: Specific detection of halogens and phosphorus by flame ionization. *Anal. Chem.*, 36 (1964) 1416–1421 — combustion products of halogen- and phosphorus-containing compounds react with Na on a wire mesh that has been treated with NaOH and which is heated in a hydrogen flame, thus increasing the ionization stream; nanogram quantities can be detected.

LOVELOCK, J. E., SHOEMAKE, G. R. AND ZLATKIS, A.: Improved ionization cross-section detectors. *Anal. Chem.*, 36 (1964) 1410–1415 — the small volume (8 μl) gives very high sensitivity; these detectors are advocated for interplanetary explorations.

3b. Column performance and filling studies

GUILLEMIN, C. L. AND AURICOURT, F.: Choice of carrier gas for the gas density balance. *J. Gas Chromatog.*, 2 (1964) 156–159 — SF_6 as carrier gas gives higher sensitivity (comparable with katharometer) and wt. % data for permanent and related gases.

HAWKES, S. J. AND MOONEY, E. F.: Temperature limitations of stationary phases in gas chromatography. *Anal. Chem.*, 36 (1964) 1473–1477 — the volatilities and viscosities of 32 stationary phases are given.

KISELEV, V. A. AND YASHIN, YA. I.: (Influence of the structure of silica gels on the separation of hydrocarbons by gas chromatography). *Neftekhimiya*, 4 (1964) 494–500 — the Van Deemter curve becomes flatter with increasing pore diameter and its minimum is displaced to higher gas velocities.

LECHNER-DE CHÂTEL, A.: (Study of various gas-chromatographic carriers in the determination of octachlorocyclopentene). *Magy. Kem. Folyóirat*, 70 (1964) 113–115.

PARCHER, J. F. AND URONE, P.: An improved solution coating technique for gas chromatographic supports. *J. Gas Chromatog.*, 2 (1964) 184–185 — an empirical technique for coating from 1–10 wt. % is described.

ROGOZINSKI, M.: Subtraction gas chromatography of labile halogen compounds. *J. Gas Chromatog.*, 2 (1964) 163 — Versamide 900 irreversibly sorbs α-bromo-fatty acid esters.

SCHNEIDER, W., BRUDERRECK, H. AND HALASZ, I.: Gas chromatographic separation of hydrocarbons (C_1 to C_8) by carbon number using packed capillary columns. *Anal. Chem.*, 36 (1964) 1533–1540 — CK3 graphitized carbon black (0.15–0.22 mm) coated with 0.4 wt. % of squalane and packed in 2 mm. I.D. glass capillary.

STERNBERG, J. C. AND POULSON, R. E.: The particle-to-column diameter ratio effect on band spreading. *Anal. Chem.*, 36 (1964) 1492–1502 — a small turbulence effect contributes to the decrease of resolution at higher velocities.

SZYMANSKI, H. A.: Molecular sieve as solid support. *J. Gas Chromatog.*, 2 (1964) 154–155 — separation is achieved on sieves coated with 10% of $C_{12}H_{26}$ or with compounds to be chromatographed.

3c. Apparatus, accessories and materials for GC

BLOMSTRAND, R. AND GÜRTLER, J.: A method for the introduction of submicrogram samples into a gas chromatograph. *Acta Chem. Scand.*, 18 (1964) 276–278 — evaporation from a small Pt spiral heated to a sufficiently high temperature.

GRANT, D. W.: Automatic capillary gas chromatography and sampling of distillation products. *Anal. Chem.*, 36 (1964) 1519–1522 — design of sampling valve operated up to 200°.

JARRELL, J. E. AND ALLISON, A. W.: Automatic liquid sample injector for gas chromatography. *J. Gas Chromatog.*, 2 (1964) 192–193 — variation 2%.

4. TECHNIQUES II

4a. Preparative-scale GC

GORDON, S. M. AND PRETORIUS, V.: Theory of preparative linear chromatography by elution development. The basic equations. *J. Gas Chromatog.*, 2 (1964) 196–201 — theoretical basis is given for the fact that, under all circumstances, there is an optimum value for the sample inlet volume.

TARAMASSO, M. AND DINELLI, D.: Preparative scale gas chromatography by a laboratory rotating unit. Part III. Isomers separation. *J. Gas Chromatog.*, 2 (1964) 150–153 — 100 columns, 1.2 m long, 6 mm I.D. are applied to the separation of some hydrocarbons as halogen derivatives.

VLEUGELS, J. L. P. AND POSTHUMUS, J.: Fraction cutter for use in preparative gas chromatography. *J. Gas Chromatog.*, 2 (1964) 172.

4c. High-speed GC

VIDGERGAUZ, M. S. AND ANDREEV, L. V.: (Gas chromatography on small diameter columns). *Neftekhimyia*, 4 (1964) 507–509 — application of capillary columns (I.D. less than 1 mm) to high-speed analysis.

4d. Special microtechniques

ALISHOEV, V. R. AND BEREZKIN, V. G.: (Development of chromatographic columns using a moving sorbent). *Dokl. Akad. Nauk SSSR* 155 (1964) 876–879 — moving sorbent gives shorter times of analysis and sharper zones for high-boiling compounds too.

BEROZA, M. AND ACREE, JR., F.: A new technique for determining chemical structure by gas chromatography. *J. Assoc. Offic. Agr. Chemists*, 47 (1964) 1–14 — Pd catalyst on diatomaceous earth as precolumn is applied with good results.

CLARKE, S. A.: Pressure-programmed gas–liquid partition chromatography. *Nature*, 202 (1964) 1106 — the same practical results as by PTGC are obtained; no cooling-off period necessary; reproducible programming of pressure is easier; $\log P = kt + \log C$ and $k = (\log F - \log C)/T$ where C, F and P are the inlet column pressures at the start, finish and an intermediate time t, of a programme of duration T.

JANÁK, J.: Multi-dimensional chromatography using different developing methods. *J. Chromatog.*, 15 (1964) 15–28 — theory and practice of a new type of two-dimensional chromatography in which one dimension is made by GC, the second one by thin-layer or paper chromatography; small quantities from the gas chromatogram are eluted on to the start-line of the continuously moving thin layer; application of the method is described.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5a. Gaseous hydrocarbons

MIYAKE, H. AND MITOOKA, M.: (Gas chromatographic analysis of C_1–C_4 hydrocarbons using a mixed stationary liquid of high polarity). *J. Chem. Soc. Japan, Pure Chem. Sect.*, 84 (1963) 923–928 — dibutyl maleate + β,β'-oxydipropionitrile at 50°.

PLŠKO, Š. AND FILÁK, J.: (Determination of low hydrocarbon content in partially stabilized petroleums). *Ropa Uhlie*, 6 (1964) 188–191.

TSITSISHVILI, G. V., ANDRONIKASHVILI, G. A., TSHUMBURIDZE, T. A. AND KORIDZE, Z. I.: (Chromatographic separation of C_1–C_4 hydrocarbon gases on zeolites of X-type with different content of calcium). *Dokl. Akad. Nauk SSSR.*, 156 (1964) 932–936 — a study of adsorption and catalytic properties.

5b. Other hydrocarbons

ALEKSANDROV, A. N., DEMENTIEVA, M. I., FEDCHENKO, G. S., SKOP, S. L. AND TYSOVSKIĬ, G. I.: (Analysis of vinyltoluene by means of mass spectrometric and gas chromatographic methods). *Khim. i Tekhnol. Topliv i Masel*, No. 6 (1964) 64–67 — retention data of 16 aromatics on 3 stationary phases at 165°.

ATTRILL, J. E.: Analysis of pyrolysis products of biphenyl. *AEC Report ORNL-3537*, Nov. 17 (1963) 117–118.

HEINZE, H. O. AND ECKHARDT, F.: Beurteilung von Ölen mit Hilfe von ultrarotspektroskopischen und gaschromatographischen Untersuchungen. *Brennstoff-Chem.*, 45 (1964) 84–88 — on PEG in capillary columns at 170°.

HORTON, A. D. AND BOTTS, J. L.: Gas chromatography as applied to nuclear technology. II. Analysis of the hydrolysis products of uranium carbides and thorium carbides. *Nucl. Sci. Eng.*, 18 (1964) 97–105 — retention data of C_2–C_8 hydrocarbons liberated by hydrolysis and chromatographed on squalane, tricresyl phosphate, di-2-ethylhexyl sebacate and silica gel by PTGC 25–125°.

KREKEL, G. AND STEINBRECHER, F.: Untersuchung von Benzolvorerzeugnis nach konventionellen Verfahren und mittels Gaschromatographie. *Brennstoff-Chem.*, 45 (1964) 81–84 — aromatics on PEG at 150°.

5c. Halogen derivatives of hydrocarbons

DOUGHERTY, T. J.: Structure of vinyl fluoride–trifluoromethyl iodide telomers. *J. Am. Chem. Soc.*, 86 (1964) 460–463 — by PTGC on Carbowax 1500 at 90–220°, 4.6°/min.

MALINOWSKA, K.: (Analysis of halogen derivatives of C_1–C_6 hydrocarbons by gas chromatography). *Chem. Anal. (Warsaw)*, 9 (1964) 585–588 — on E-301 at 130°.

6. ALCOHOLS

BALAKHONTSEVA, V. N. AND POLTININA, R. M.: (Determination of glycols by a method of gas–liquid chromatography). *Zh. Anal. Khim.*, 29 (1964) 757–760 — on PEG-10M.

7. PHENOLS

SMITH, G. A. L. AND KING, D. A.: Separation and identification of the steam volatile phenols present in cigarette smoke condensate by capillary column gas liquid chromatography. *Chem. Ind. (London)*, (1964) 540–541 — retention data for 16 phenols as methyl ethers and acetates by capillary GC on 2,4-xylenyl phosphate at 140° and 100°.

SMITH, J. R., NORMAN, R. O. C. AND RADDA, G. K.: Quantitative determination of isomeric phenols. *J. Gas Chromatog.*, 2 (1964) 146–149 — optimal data for separation of phenols and derivatives (fluoro-, chloro-, methoxy-, nitro- and cyano-) are given; low coating of glass beads with DEGA is recommended.

TAKEUCHI, T. AND KATO, N.: (Determination of a small amount of each isomer in 1- or 2-naphthol by gas–liquid chromtography). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 67 (1964) 305–308 (English summary p. A19).

9. OXO COMPOUNDS

BRILL, W. F. AND INDICTOR, N.: Reaction of *tert.*-butyl hydroperoxide with olefins. *J. Org. Chem.*, 29 (1964) 710–713 — retention data of *cis*- and *trans*-epoxides of 4-methyl-2-pentenes on diisodecyl phthalate at 130°.

NIINIVAARA, R. P., POHJA, M. S. AND KOMULAINEN, S. E.: Some aspects about using bacterial pure cultures in the manufacture of fermented sausage. *Food Technol.*, 18, No. 2 (1964) 25–31 — GC study of carbonyl fractions on PEG at 175°.

ŠINGLIAR, M., BRIDA, J. AND SPIŠSKÝ, V.: (Some analytical problems of products of oxo-synthesis). *Chem. Zvesti*, 18 (1964) 527–532 — retention data of C_4–C_5 aldehydes (and some alcohols) on PEG-400 at 140°.

10. CARBOHYDRATES

SWEELEY, C. C. AND WALKER, B.: Studies on the analysis of carbohydrates in glycolipids and gangliosides by gas chromatography. *Anal. Chem.*, 36 (1964) 1461–1466 — after methanolysis, analysis on SE-30 on silanized diatomite.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

BADAMI, R. C. AND GUNDSTONE, F. D.: Vegetable oils. XIII. The component acids of isano (boleko) oil. *J. Sci. Food Agr.*, 14 (1963) 863–866 — retention data of saturated, olefinic and acetylenic fatty acid esters on Apiezon L at 200°.

BINDER, R. G., APPLEWHITE, T. H., DIAMOND, M. J. AND GOLDBLATT, L. A.: Chromatographic analysis of seed oils. II. Fatty acid composition of *Dimorphotheca oil*. *J. Am. Oil Chemists' Soc.*, 41 (1964) 108–111 — C_{14}–C_{22} fatty acids on DEGS and Apiezon L at 200° and 250°.

CASON, J., SANGE, G. L., MILLER, W. T. AND WEISS, A.: Multibranched higher saturated acids from tubercle bacillus. *Tetrahedron*, 20 (1964) 91–106 — retention data of specific fatty acids on silicone grease at 225–278°.

CROSSLEY, A. AND THOMAS, A.: Keeping properties of edible oils. III. Identification of trace material adsorbed from peanut oil by chromatography on alumina. *J. Am. Oil Chemists' Soc.*, 41 (1964) 95–100 — retention data of methyl esters of oxygenated fatty acids on polyvinyl acetate column.

DANIELS, N. W. R., FRAPE, D. L., EGGITT, P. W. R. AND COPPOK, J. B. M.: Studies on the lipids of flour. II. Chemical and toxicological studies on the lipids of chlorine-treated cake flour. *J. Sci. Food Agr.*, 14 (1963) 883–893 — retention data on PEGo-phthalate and PEGA at 190°.

GOUW, T. H. AND VLUGTER, J. C.: Physical properties of fatty acid methyl esters. I. Density and molar volume. *J. Am. Oil Chemists' Soc.*, 41 (1964) 142–146 — on PEGA and Apiezon L.

KINGSBURY, K. J. AND MORGAN, D. M.: Analysis of the fatty acids of normal human depot fat by gas liquid chromatography. *Biochem. J.*, 90 (1964) 140–147 — C_8–C_{23} and branched C_{13}–C_{24} fatty acids on PEGS, polyvinyl acetate and Apiezon L at 185°, 175–180° and 195°.

KUCK, J. C., PONS, Jr., W. A. AND FRAMPTON, V. L.: Physical and chemical properties of alumina bleached cottonseed oil. *J. Am. Oil Chemists' Soc.*, 41 (1964) 101–104 — on DEGS at 190°.

MABROUK, A. F., DUTTON, H. J. AND COWAN, J. C.: Homogeneous catalytic hydrogenation of sorbic acid with pentacyanocobaltate. II. *J. Am. Oil Chemists' Soc.*, 41 (1964) 153–158 — C_6–C_7 acids on DEGS at 120°.

MERCURI, O., CARRAZONI, N. E. AND BRENNER, R. R.: Methyl oct-*cis*-2-enoate. Its synthesis, GLC behavior, and infrared spectra. *J. Am. Oil Chemists' Soc.*, 41 (1964) 89–92 — retention data of *cis*- and *trans*-isomers of -enoate in comparison with -anoate and -ynoate on Apiezon N and PEGA at 100°.

O'BRIEN, J. S., FILLERUP, D. L. AND MEAD, J. F.: Brain lipids. I. Quantification and fatty acid composition of cerebroside sulfate in human cerebral gray and white matter. *J. Lipid Res.*, 5 (1964) 109–116 — retention data of fatty acids (mainly 24:0 and 24:1) on Apiezon L at 235° and DEG at 180°.

O'BRIEN, J. S. AND ROUSER, G.: Analysis of hydroxy fatty acids by gas–liquid chromatography. *Anal. Biochem.*, 7 (1964) 288–296 — retention data of free and acetylated 2- to 16-hydroxy-fatty acids on EGS at 185° and on Apiezon L at 235°.

ORSUKI, A. AND HANYA, T.: (Gas chromatographic determination of lower fatty acids in polluted water). *J. Chem. Soc. Japan, Pure Chem. Sect.*, 84 (1963) 798–802 — after isolation through steam distillation and absorption in NaOH solution on PEGS at 120°.

PREISS, B. AND BLOCH, K.: ω-Oxidation of long chain fatty acids in rat liver. *J. Biol. Chem.*, 239 (1964) 85–88 — retention data of hydroxy- and keto-acids on XE-60 and DEGS at 212° and 198°.

SCOTT, T. W., WARD, P. F. V. AND DAWSON, R. M. C.: Formation and metabolism of phenyl-substituted fatty acids in ruminant. *Biochem. J.*, 90 (1964) 12–25 — retention data of phenyl-fatty acid methyl esters on Apiezon L and PEGA.

SUBBARAM, M. R.: Separation of saturated and unsaturated fatty acid esters of cholesterol by gas–liquid chromatography. *J. Chromatog.*, 15 (1964) 79–80.

SUBBARAM, M. R. AND YOUNGS, C. G.: Isomerization of mono ethenoid acids during hydrogenation. *J. Am. Oil Chemists' Soc.*, 41 (1964) 150–152 — by PTGC on Apiezon L at 100–250°, 5.6°/min.

13. STEROIDS

BROOKS, S. C. AND GODEFROI, V. C.: Quantitative collection of microamounts of steroids from gas–liquid chromatography. *Anal. Biochem.*, 7 (1964) 135–146 — a detailed study for the 1–200 μg range; quantitative collection in U-tube immersed in liquid nitrogen.

CHAMBERLAIN, J. AND THOMAS, G. H.: Characterization of 20-oxosteroids by gas chromatography. *Anal. Biochem.*, 8 (1964) 104–115 — retention data of 9 steroids on QF-1-0065 at 250° and TLC separation of some overlapping peaks.

CREECH, B. G.: Separation and determination of ketosteroids, pregnandiol and pregnantriol on one column. *J. Gas Chromatog.*, 2 (1964) 194–195 — as trimethylsilyl ethers at 210–215°.

HAMILTON, R. J., VANDENHEUVEL, W. J. A. AND HORNING, E. C.: An extension of the "steroid number" concept to relationships between the structure of steroids. *Biochim. Biophys. Acta*, 70 (1963) 679–687 — many retention data on SE-30, NGS and QF-1 at 222° and 232°.

KNIGHTS, B. A.: Gas chromatographic analysis of plant sterols. Part I. Characterisation of sterol double bonds using the ΔR_M function. *J. Gas Chromatog.*, 2 (1964) 160–162 — GC analysis of alcohols (as trimethylsilyl ethers or trifluoroacetates) resulting from hydroboration of sterols; relative retention and ΔR_{Mr} data on 4 stationary phases for 9 and 10 sterols.

KROMAN, H. S., KING, M. O. AND BENDER, S. R.: A method for the gas chromatographic separation of estrogens employing a solid injection system. *J. Chromatog.*, 15 (1964) 92–94 — on QF-1 at 252°.

NAIR, P. P., SARLOS, I. S., SOLOMON, D. AND TURNER, D. A.: Simultaneous separation of 17-ketosteroids and estrogens by biphase gas chromatography. *Anal. Biochem.*, 7 (1964) 96–102 — retention data of 10 steroids as trimethylsilyl ethers or trifluoroacetates on SE-30 and NGS at 197–205°.

LUUKKAINEN, T. AND ADLERCREUTZ, H.: Gas chromatography of methylated estrogens and application of the method to the analysis of human late pregnancy bile. *Biochim. Biophys. Acta*, 70 (1964) 700–703 — retention data of methyl ethers of steroids on XE-60, QF-1 and SE-30 at 212°, 180° and 190°.

TOUCHSTONE, J. C.: Routine quantitative gas chromatography of urinary estriol. *J. Gas Chromatog.*, 2 (1964) 170–171 — on QF-1-0065 at 255° with an argon detector.

WOTIZ, H. H. AND CHATTORAJ, S. C.: Methods of estrogen determination in low and high titre urines using thin-layer and gas chromatography. *Anal. Chem.*, 36 (1964) 1466–1472 — TLC separation in 4 groups and GLC quantification.

15. TERPENE DERIVATIVES

BARON, C. AND MAUME, B.: Les menthoglycoles. *Parfum., Cosmet., Savons*, 6 (1963) 361–368 — stereoisomers on Reoplex 400 at 175°.

COREY, E. J., MITRA, R. B. AND UDA, H.: Total synthesis of *d,l*-caryophyllene and *d,l*-isocaryophyllene. *J. Am. Chem. Soc.*, 86 (1964) 485–492 — on nitrile-silicone and fluoro-silicone grease at 125–180°.

HELLEYR, R. O., KEYZER, H. AND MCKERN, H. H. G.: The volatile oils of the genus *Eucalyptus* (family *Myrtaceae*). III. The leaf oil of *E. crenulata* Blakely and de Benzeville. *Australian J. Chem.*, 17 (1964) 283–285 — methoxypolyethylene glycol 750 plus picric acid as stationary phase.

IKEDA, R. M. AND SPITLER, E. M.: Composition of citrus oils. Isolation, identification and gas chromatographic estimation of some esters and alcohols of lemon oil. *J. Agr. Food Chem.*, 12 (1964) 114–117 — retention data of 11 terpenes on polydiethanolamine succinate at 139°.

KESTERSON, J. W. AND HENDRICKSON, R.: A comparison of red and white grapefruit oils. *Am. Perfumer Cosmet.*, 79 (1964) 34–36 — retention data on Carbowax 20M and DEGS at 150°.

LUKEŠ, V. AND KOMERS, R.: On terpenes. CLXIII. Gas chromatography of sesquiterpenic hydrocarbons. *Collection Czech. Chem. Commun.*, 29 (1964) 1598–1603 — retention data of 21 C_{15} terpenes (from acyclic to tricyclic) on Apiezon L at 204°, PEGA at 180°, PEG-4000 at 174° and tetrakis-O-(2-cyanoethyl)-pentaerythritol.

SYDOW, E. VON.: Mass spectrometry of terpenes. II. Monoterpene alcohols. *Acta Chem. Scand.*, 17 (1963) 2504–2512 — purity determination on sucrose acetate isobutyrate and N,N,N′N′-tetrakis-(2-hydroxypropyl)-ethylenediamine at 100° and 110°.

16. NITRO AND NITROSO COMPOUNDS

HOFFMANN, A. K., FELDMAN, A. M., GELBLUM, E. AND HODGSON, W. G.: Mechanism of the formation of di-*tert.*-butylnitroxide from *tert.*-nitrobutane and sodium metal. *J. Am. Chem. Soc.*, 86 (1964) 639–646 — retention data for nitro- and nitroso-butanes, butyl nitrate and nitroxide and other compounds on diisodecyl phthalate and silicone grease at 110° and 118°.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

ANDERSONS, A. AND SHYMANSKAYA, M.: (Analysis of ethanolamine and piperazine mixtures by gas–liquid chromatography). *Izv. Akad. Nauk Latv. SSR, Ser. Khim.*, (1963) 525–529.

SANDBERG, D. H., BOCK, S. A. AND TURNER, D. A.: Quantitative measurement of Thalidomide by gas–liquid chromatography. *Anal. Biochem.*, 7 (1964) 129–132 — on XE-60 at 230°, QF-1 and DEGS at 185°.

18. AMINO ACIDS

VITT, S. V., SAPOROVSKAYA, M. B. AND BELIKOV, V. M.: (Analysis of amino acids by capillary chromatography). *Izv. Akad. Nauk SSSR, Otd. Khim. Nauk*, (1964) 947–958 — N-trifluoroacetyl methyl esters on Apiezon L; complete separation of diastereo isomers of isoleucine and threonine as N-trifluoroacetyl *n*-butyl esters at 140°.

22. ALKALOIDS

BROCHMANN-HANSEN, E. AND SVENDSEN, A. B.: Quantitative determination of morphine in opium by gas–liquid chromatography. *J. Pharm. Sci.*, 52 (1963) 1134–1136 — retention data of morphine, laudonosine and tetraphenylethylene on PEG-9000 + SE-30 (0.1% + 4.0%) at 183°.

24. ORGANIC SULPHUR COMPOUNDS

MILLIGAN, B., RIVETT, D. E. AND SAVIGE, W. E.: Photolysis of dialkyl sulfides, disulfides and trisulfides. *Australian J. Chem.*, 16 (1963) 1020–1029 — retention data of sulphides (S_1–S_4) on silicone rubber or DC-550 at 125° and 200°.

OAKS, D. M., HARTMANN, H. AND DIMINCK, K. P.: Analysis of sulphur compounds with electron capture/hydrogen flame dual channel gas chromatography. *Anal. Chem.*, 36 (1964) 1560–1565 — a single column stream (Carbowax 20M, 143°) is split and led to two detectors and registered on a two-pen recorder.

26. METALLO-ORGANIC COMPOUNDS

BANK, H. M., SAAM, J. C. AND SPEIER, J. L.: The addition of silicon hydrides to olefinic double bonds. IX. Addition of *sym.*-tetramethyldisiloxane to hexene-1, -2, and -3. *J. Org. Chem.*, 29 (1964) 792–794 — retention data of 3 hexyltrimethylsilanes on liquid paraffin at 41°.

29. INSECTICIDES AND OTHER PESTICIDES

BACHE, C. A., GUTENMANN, W. H. AND LISK, D. L.: Detection of Amiben in tomatoes by electron affinity gas chromatography. *J. Agr. Food Chem.*, 12 (1964) 185–187 — retention data of 3-amino-2,5-dichlorobenzoic acid on DC silicone grease at 200°; estimation in the 0.02–1 p.p.m. range.

BECKMAN, H. AND BEVENUE, A.: Microcoulometric gas chromatographic analysis of grapes and cottonseed for chlorobenzilate residues. *J. Agr. Food Chem.*, 12 (1964) 184–185 — on DC-11 at 260° and on SE-30 by PTGS at 100–210°, 15°/min.

LICHTENSTEIN, E. P., MORGAN, D. G. AND MUELLER, C. H.: Naturally occurring insecticides in cruciferous crops. *J. Agr. Food Chem.*, 12 (1964) 158–161 — retention data of 2-phenylethyl isothiocyanate on SE-30/NGA at 152°.

PENNELL, J. T., MISKUS, R. AND CRAIG, R.: The use of gas chromatography for the quantitative determination of microamounts of insecticide picked up by mosquitoes. *Bull. World Health Organ.*, 30 (1964) 91–95 — dieldrin on SE-30 at 180°.

VAN MIDDELEM, C. H. AND WAITES, R. E.: Gas chromatographic and colorimetric measurement of dimethoate residue. *J. Agr. Food Chem.*, 12 (1964) 178–182 — on SE-30 at 175°.

31. PLASTICS AND THEIR INTERMEDIATES

DRIENOVSKÝ, P. AND KYSEL', O.: (Pyrolysis of atactical polypropylene). *Chem. Zvesti*, 18 (1964) 512–526 — up to 700° the main product is propylene, at higher temperatures methane and ethylene; hydrogen as carrier gas.

DUNDON, J. P.: A study of cross-linking using vapor-phase chromatography. *Textile Res. J.*, 34 (1964) 340–346.

33. INORGANIC SUBSTANCES

33a. Permanent and rare gases

ABRAMOV, V. N., FISAK, V. I.: (Analysis of combustion products of methane–air mixtures by gas chromatography). *Zavodsk. Lab.*, 30 (1964) 675 — CH_4, CO and CO_2 in 3 min up to 10^{-3}%.

AĬNSHTEIN, S. A., ANVAER, B. I. AND TURKEL'TAUB, N. M.: (Use of gas–liquid chromatography for separation of some inorganic gases). *Zavodsk. Lab.*, 30 (1964) 665–671 —separation of O_2 and N_2 on isobutyl alcohol and isooctane at —78° (no mention is made of the very similar observations of DESTY in 1959); retention data for N_2,CO,O_2,CH_4 at —78° and CH_4,CO_2, HCl, H_2S and Cl_2 on hexadecane and butyrolactone at 20°.

ATTRILL, J. E.: Process chromatography for engineering tests. *AEC Report ORNL-3537*, Nov. 17 (1963) 47 — mixture of H_2, CO, CO_2 and $O_2 + N_2 + CH_4$.

KRICHEVSKY, M. I., ROGOSA, M. AND BISHOP, F. S.: Gas chromatographic analysis of hydrogen–carbon dioxide mixtures. *Anal. Biochem.*, 7 (1964) 350–356 — on molecular sieve 5A by PTGC.

MOSKVINA, A. A., KUZNECOVA, L. V., DOBYCHIN, S. L. AND ROZOVA, M. I.: (Microelemental analysis by means of gas chromatography. Determination of carbon, hydrogen and nitrogen inorganic compounds). *Zh. Anal. Khim.*, 29 (1964) 749–753 — after combustion with CuO and reduction of NO_x with Cu on triethanolamine at 98° (N_2, CO_2 and H_2O).

PANSON, A. G. AND ADAMS, L. M.: Complete gas chromatographic analysis of hydrogen in fixed gases and hydrocarbons using one detector and helium as gas carrier. *J. Gas Chromatog.*, 2 (1964) 164–166. — optimum conditions with cross-section detector: 0.25 ml sample size, detector voltage 8 V, molecular sieve 13X at 40° and He 85 ml/min.

PETKOVIČ, L. V., KOSANIČ, M. M. AND DRAGANIČ, I. G.: (Determination of CO_2, H_2 and O_2 in aqueous solutions by gas chromatography). *Bull. Inst. Nucl. Sci. "Boris Kidrich" (Belgrade)*, 15 (1964) 9–15.

33b. Volatile inorganic compounds

DEVYATYKH, G. G., ZORIN, R. D., AMEL'GENKO, A. M., LYAMANOV, S. B. AND EZHELEVA, A. E.: (Chromatographic analysis of mixtures of some volatile inorganic hydrides). *Dokl. Akad. Nauk SSSR*, 156 (1964) 1105–1108 — hydrides of elements of Groups IV–VI on DC-702 and two Soviet silicone greases VKZH-94B and PFMS-4; detection through hydrogen liberated in quartz reactor before katharometer.

34. RADIOACTIVE COMPOUNDS

TADMOR, J.: Application of isotopic exchange in gas chromatography. *Anal. Chem.*, 36 (1964) 1565–1573 — exchange of ^{36}Cl between chlorine of metal chloride and that sorbed on the uncoated solid phase.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

ATTAWAY, J. A., WOLFORD, R. W. AND ALBERDING, G. E.: Identification of alcohols and volatile organic acids from natural orange essence. *J. Agr. Food Chem.*, 12 (1964) 118–121 — C_1–C_9 alcohols, terpene alcohols and C_2–C_8 acids on Carbowax 20M.

BAKER, R. A.: Chromatographic evaluation of activated carbon. *J. Am. Water Works Assoc.*, 56 (1964) 92–98 — retention data of *n*-butanol, *n*-amyl acetate and water on UCON LB-550X at 100°.

BECHER, P. AND BIRKMEIER, R. L.: The determination of hydrophile–lipophile balance by gas–liquid chromatography. *J. Am. Oil Chemists' Soc.*, 41 (1964) 169–172 — by means of the retention ratio of polar and non-polar compounds the polarity of surface-active agents (used as stationary phase) is measured.

BINGHAM, R. J.: Gas chromatographic studies on the volatiles of sterilized concentrated milk. *Dissertation Abstr.*, 24 (1964) 3523.

CONNELL, D. W.: Volatile flavoring constituents of the pineapple. *Australian J. Chem.*, 17 (1964) 130–140 — retention data of C_1–C_5 alcohols, C_2–C_8 fatty acid esters and thioesters on di-2-ethylhexyl sebacate, PEG and diisodecyl phthalate at 170°.

ENGELHARDT, J. AND FEJES, P.: (Gas chromatographic analysis of mixtures containing cyclohexanol, cyclohexanone and phenol). *Magy. Kem. Folyoirat*, 70 (1964) 171–174 — retention data on PEG-4000 at 150° and 180° with acetonylacetone as internal standard.

KWEI, T. K. AND ARNHEIM, W. N.: Solubility of nonpolar gases in polymers: some new considerations. *J. Polymer Sci., Part A, Gen. Papers*, 2 (1964) 1873–1878 — applicable for sorption of small molecules in many stationary phases.

LIN TE-TSEN: (Application of gas chromatography in elemental analysis of organic compounds). *Chemistry (Huaxue Tongban)*, No. 2 (1964) 1–7.

NESTLER, H. AND SYCH, G.: Untersuchungen zur Reinheitsbestimmung von Monoäthylenglykol beim Grisutenherstellungsprozess. *Chem. Tech. (Berlin)*, 16 (1964) 283–287 — retention data of different compounds on 10 stationary phases at 165–207°.

THE NITRATION OF FLUOROTOLUENES AND FLUOROXYLENES. PRODUCT ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY

N. ICONOMOU AND G. VALKANAS

Institute of Pharmacy and Institute of Organic Chemical Technology, Swiss Federal Institute of Technology, Zurich (Switzerland)

(Received April 10th, 1964)

INTRODUCTION

In the nitration of fluorotoluenes[1] and fluoroxylenes[2] the introduction of the nitro group permits a separation of the isomers by fractional distillation. A stable b.p. after repeated fractionations was taken as proof of purity, and such fractions were identified by conversion to derivatives of known characteristics.

The strong directing power of fluorine and the fact that the fluorinated and the corresponding fluorine-free aromatic hydrocarbons have nearly identical b.p.[4], permitted the assumption that: (a) if one compound is produced in greater amount the nitro group will be positioned *para* to fluorine, and (b) the b.p. sequence in fluoronitrotoluenes and -xylenes will be the same as the b.p. sequence of the fluorine-free nitrotoluenes and -xylenes[2]. It would seem, therefore, that gas–liquid chromatography offers an accurate and rapid method for the study of these reaction products.

An efficient silicone oil, DC-200, column was found best for the separation of the nitration products of fluorotoluenes and -xylenes. Apiezon L columns have been recently used for the separation of mono-[5] and dinitrotoluene[6] isomers, while for the separation of the chloronitrobenzene isomers polar columns are recommended[7]. The behaviour of fluoronitrotoluenes and -xylenes is more like that of the nitrotoluenes than the chloronitrobenzenes since the fluorinated isomers have differences in b.p. similar to the fluorine-free isomers. Separation efficiencies in the non-polar columns are measured by b.p. differences[8].

EXPERIMENTAL AND RESULTS

The nitration of the fluorotoluenes was performed with fuming nitric acid at —10° as described by SCHIEMANN[1]. The nitration of the fluoroxylenes, except for greater cooling, was achieved under similar conditions[2]. The fluorotoluenes and -xylenes were prepared, by means of the Schiemann fluoroborate process[9], from the corresponding amines of 99 % claimed purity (puriss., "Fluka", Switzerland), which was confirmed by preparing derivatives reported in the literature[10].

The crude product of the nitration reaction was fractionated and the mononitrated products were collected (fractions boiling 95–130°/14 mm). These in all cases gave a satisfactory analysis (C, H, F, N) and gas chromatographic results identical to the ones obtained for the crude reaction product. Dinitration did not occur in detectable amounts under the conditions employed.

The gas chromatographic analysis gave a very good separation and the elution sequence was identical to the b.p. order. Compounds like 2-fluoro-3-nitrotoluene and 2-fluoro-5-nitrotoluene which give the same fluorine-free nitrotoluene could not be separated, so that such pairs appeared in one chromatographic peak. In evaluating the chromatograms obtained, percentages of peak areas were taken for percentages of composition. Analyses of known mixtures have shown that a molar response correction coefficient is not required.

All fluoroxylenes except fluoro-*p*-xylene gave chromatographic peaks for a number of fluoronitroxylenes equal to the places free for substitution. Fluoro-*p*-xylene gave up to 94 % 2-fluoro-5-nitro-1,4-xylene together with two by-products as a shoulder of the main peak. *o*-Fluorotoluene gave three and the *meta*-isomer two peaks for mononitration products. *p*-Fluorotoluene and 4-fluoro-1,2-xylene could not be nitrated under the conditions described and gave nitroxylenols and nitrocresols instead[1,2].

Infrared spectral analysis was used to identify the different isomers, the spectra of which in the low frequency region, 900–750 cm^{-1}, are characteristic. For the main components samples could be obtained gas chromatographically pure by distillation and then recrystallisation from methanol[2]. Minor reaction products were collected after gas chromatographic separation.

DISCUSSION

Of the four expected chromatographic peaks for the 2-fluoronitrotoluenes only three were obtained (Fig. 1-A), the pair 2-fluoro-3-nitrotoluene and 2-fluoro-5-nitrotoluene appearing together. The amounts of isomer were calculated by means of the partial rate factors of nitration of fluorobenzene[11], which should be a good approximation. The products I, II and III (see Table I) were isolated by fractional distillation and characterised as described previously[3], but the composition of 6 % of I, 84 % of II and 10 % of III given in the literature and the fact that the fourth isomer (IV) was not mentioned disagrees with our results.

The nitration of *m*-fluorotoluene should give three isomeric products, all of which were identified and characterised[1]. The main product (V) and the third isomer (VII) have nearly the same b.p. and appear together in the chromatogram (Fig. 1-B). This was confirmed by I.R. analysis of the product of the chromatographic peak where additional absorption bands due to 1-, 2-, 3- substitution were easily recognised. To calculate the percentage of substitution in VII it was assumed that steric effects are not important in the approach of the nitronium ion to the two positions *ortho* to fluorine, as toluene nitrated under similar conditions[12] gave 41 % *ortho*- and 59 % *para*-nitrotoluene. Therefore in this case it would be expected that the yield of VII would be 70 % of (VI) or 16 % of the nitration products (literature data[1] give a composition of 7.5 % for (VI), 83 % for (V) and 4.5 % for (VII)).

The study of the nitration of fluoroxylenes is simpler in that a smaller number of isomers is expected and the differences in b.p. are greater. Thus, gas chromatography permits a good separation of all isomers (Figs. 1-C, 1-D and 1-E), the identification of which has been described[2] and later confirmed by I.R. analysis.

3-Fluoro-6-nitro-1,2-xylene (VIII) is the main product of the nitration of 3-fluoro-1,2-xylene appearing first in the chromatogram (Fig. 1-C). The isomer with the

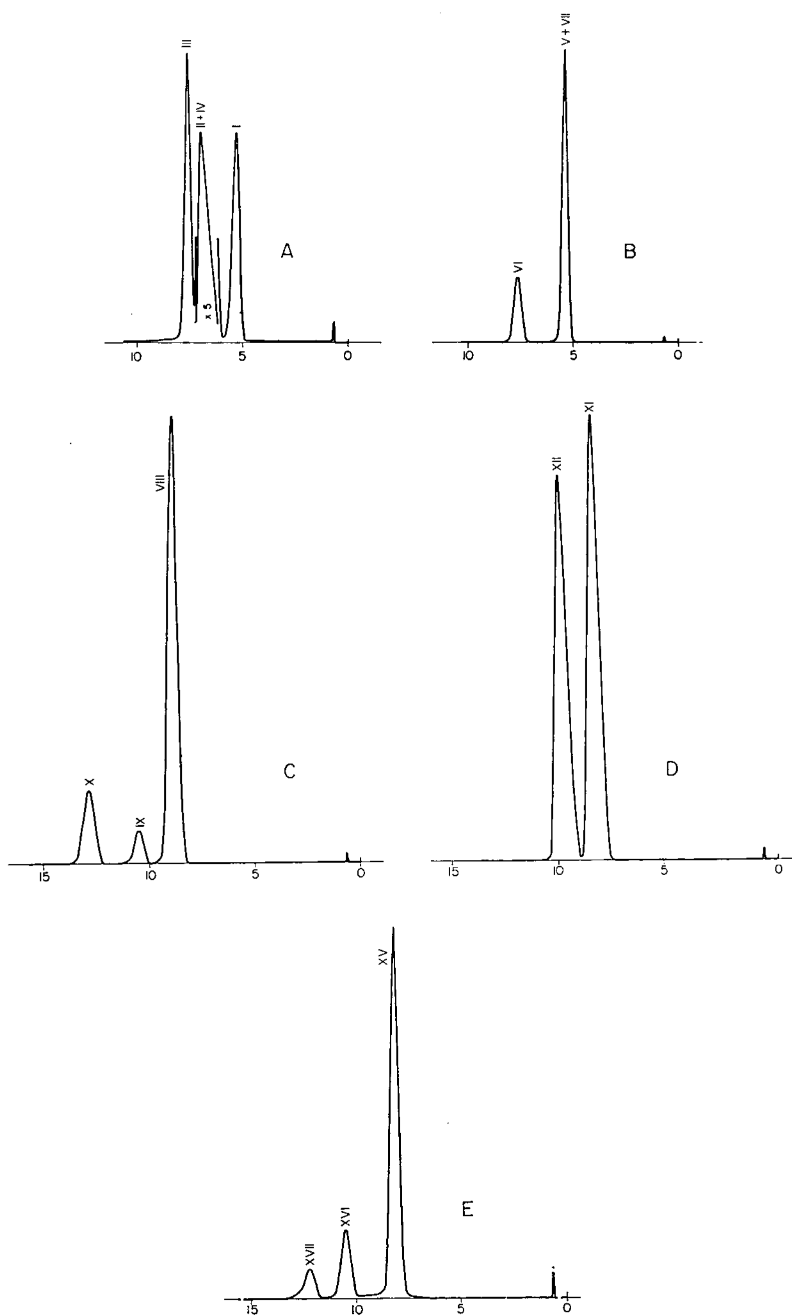


Fig. 1. Analysis of the nitration products of A: *o*-fluorotoluene; B: *m*-fluorotoluene; C: 3-fluoro-1,2-xylene; D: 2-fluoro-1,3-xylene; E: 4-fluoro-1,3-xylene. Conditions for chromatography: Chromatograph: Perkin-Elmer Fraktometer Model 116; column dimensions: 200 × 0.635 cm O.D.; solid support: Chromosorb R (60/80); stationary phase: Silicone Oil DC-200 (20:80); temperature: 191°; carrier gas: helium at 86 ml/min; detector: thermistor at 8 mV; recorder: 2.5 mV;1 sec; 0.5 cm/min.

TABLE I

ISOMER DISTRIBUTION IN THE NITRATION OF FLUOROTOLUENES AND FLUOROXYLENES AND PHYSICAL PROPERTIES OF FLUORONITROTOLUENES AND FLUORONITROXYLENES

	Compound	%	*b.p.*[b] (°*C*/*mm*)	*m.p.*[b] (*C*°)
	o-Fluorotoluene			
I	2-Fluoro-6-nitro-toluene	10.2	97–97.2°/11	—2
II	2-Fluoro-5-nitro-toluene	68.0	99.4–99.6°/13	41.5
III	2-Fluoro-4-nitro-toluene	12.2		
IV	2-Fluoro-3-nitro-toluene	9.6[a]		
	m-Fluorotoluene			
V	3-Fluoro-6-nitro-toluene	61.2	97–98°/10	27–28
VI	3-Fluoro-4-nitro-toluene	22.8	—	53.2
VII	3-Fluoro-2-nitro-toluene	16.0[a]	92.4–92.8°/12	17.5–18
	3-Fluoro-1,2-xylene			
VIII	3-Fluoro-6-nitro-1,2-xylene	75.8	109–110°/14	36–37
IX	3-Fluoro-5-nitro-1,2-xylene	6.1		
X	3-Fluoro-4-nitro-1,2-xylene	18.1	126–127°/14	
	2-Fluoro-1,3-xylene			
XI	2-Fluoro-4-nitro-1,3-xyiene	52.6	107–108°/14	
XII	2-Fluoro-5-nitro-1,3-xylene	47.4	111–112°/14	42–43
	5-Fluoro-1,3-xylene			
XIII	5-Fluoro-2-nitro-1,3-xylene	61.2	93–94°/14	51–52
XIV	5-Fluoro-4-nitro-1,3-xylene	38.8	102–103°/14	50–51
	4-Fluoro-1,3-xylene			
XV	4-Fluoro-6-nitro-1,3-xylene	75.5	108–109^{c}/14	
XVI	4-Fluoro-2-nitro-1,3-xylene	16.3		
XVII	4-Fluoro-5-nitro-1,3-xylene	8.2		
	2-Fluoro-1,4-xylene			
XVIII	2-Fluoro-5-nitro-1,4-xylene	93.7	107–107.5°/14	50–51

[a] Calculated.
[b] The physical constants for fluoronitrotoluenes and fluoronitroxylenes are taken from refs. 1 and 2, respectively.

higher retention time gave an infrared spectrum indicating two adjacent hydrogen atoms, which is the case with (X).

The two symmetrical fluoro-1,3-xylenes, 2-fluoro-1,3-xylene and 5-fluoro-1,3-xylene each gave rise to two isomeric nitration products, which were well separated and easily characterised. In the case of nitration of 2-fluoro-1,3-xylene the substitution *para* to fluorine is a little less than double the partial rate of substitution *meta* to fluorine, though in the case of 5-fluoro-1,3-xylene substitution in the position *para* to fluorine is larger, this owing to the unusually low reactivity of positions *ortho* to fluorine[13].

The nitration of 4-fluoro-1,3-xylene gave on gas chromatographic analysis (Fig. 1-E) three fluoro-nitroxylenes to which structures could be assigned. The main product of substitution is also the isomer with the lower b.p.; it gave an infrared spectrum for two adjacent hydrogen atoms consistent with the 4-fluoro-6-nitro-1,3-xylene structure (XV). Of the two other isomers 4-fluoro-2-nitro-1,3-xylene (XVI) appears second in the chromatogram while the minor component 4-fluoro-5-nitro-1,3-xylene (XVII) appears third. In interpreting these results we should take into account that the nitration of 4-fluoro-1,3-xylene also gives a 6–8 % yield of nitroxylenols[2].

2-Fluoro-1,4-xylene gave 93.7 % nitration *para* to fluorine. Upon pouring the crude nitration reaction product on ice 2-fluoro-5-nitro-1,4-xylene (XVIII) crystallises in fine needles. The other two minor products expected appeared as a shoulder in the chromatogram and they could not be further separated for identification purposes.

Out-of-plane bending –CH absorption is reported to move towards higher frequencies in the nitrated aromatic compounds[14]. However, in all cases it has been demonstrated that absorption in the low frequency region below 900 cm^{-1} is not seriously affected by the order of substitution[14]. This was found to hold here in that fluoronitroxylenes of different order in substitution, such as 2-fluoro-5-nitro-1,4-xylene, 2-fluoro-5-nitro-1,3-xylene, and 3-fluoro-6-nitro-1,4-xylene, gave rise to well located characteristic absorptions due to isolated hydrogen atoms at 890–900 cm^{-1}. The spectra of 2-fluoro-5-nitro-1,4-xylene and 4-fluoro-6-nitro-1,3-xylene are representative of the infrared spectra obtained (Figs. 2 and 3).

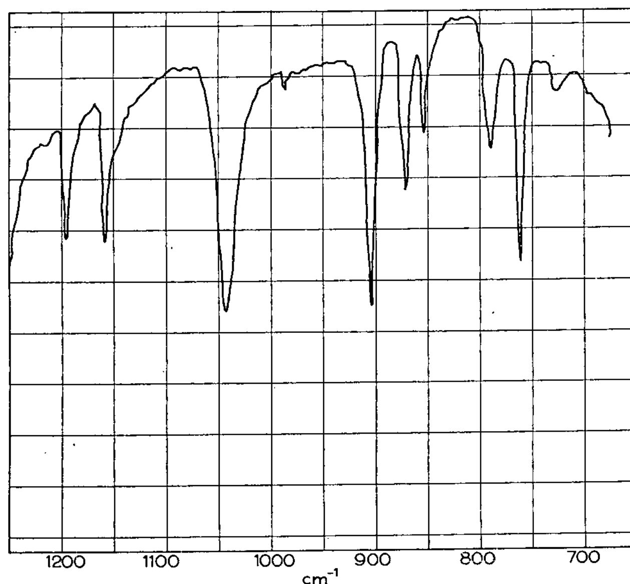


Fig. 2. Infrared spectrum in the low frequency region (1250–650 cm^{-1}) of 2-fluoro-5-nitro-1,4-xylene.

The same consistency was also found in the 1-, 2-, 3-, 4-substitution cases. A strong band due to two adjacent hydrogen atoms for three isomeric 1-, 2-, 3-, 4-substituted fluoronitroxylenes appears between 823 and 827 cm^{-1}, namely, 3-fluoro-6-

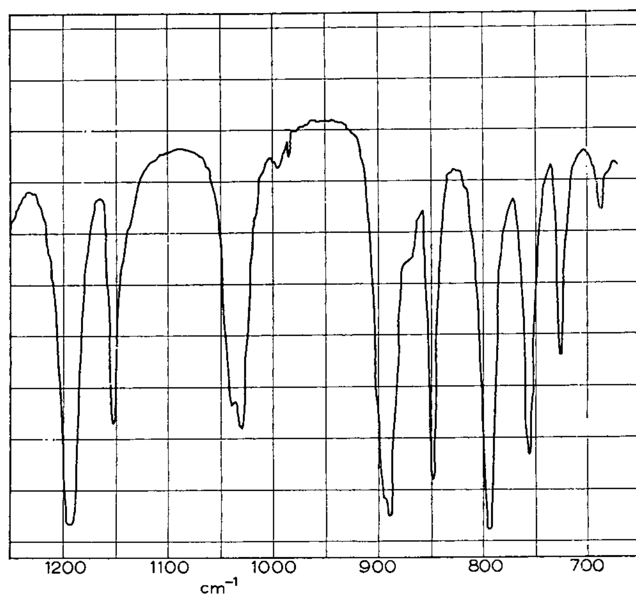


Fig. 3. Infrared spectrum in the low frequency region (1250–650 cm^{-1}) of 4-fluoro-6-nitro-1,3-xylene.

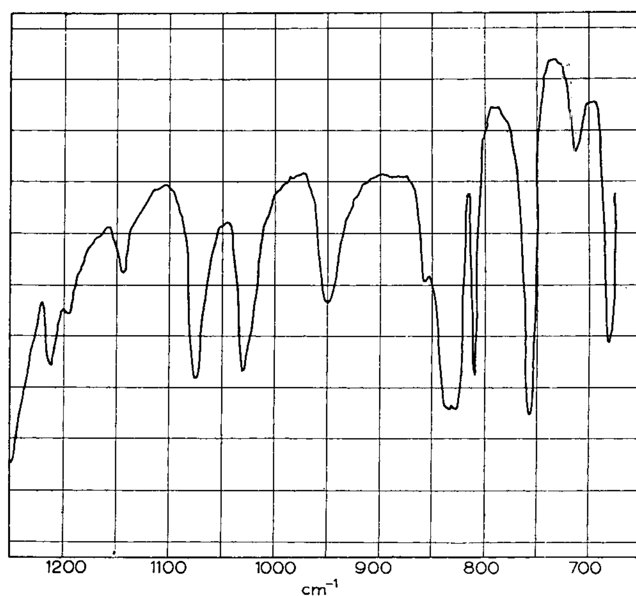


Fig. 4. Infrared spectrum in the low frequency region (1250–650 cm^{-1}) of 3-fluoro-6-nitro-1,2-xylene.

nitro-1,2-xylene (823 cm^{-1}), 3-fluoro-4-nitro-1,2-xylene (826 cm^{-1}) and 2-fluoro-4-nitro-1,3-xylene (827 cm^{-1}). Fig. 4 gives the infrared spectrum of 3-fluoro-6-nitro-1,2-xylene as representative of the class.

SUMMARY

The distribution of products in the nitration of fluorotoluenes and fluoroxylenes is determined by gas–liquid chromatography on a silicone oil DC-200 column. The nitration of the four isomeric fluoroxylenes and two isomeric fluorotoluenes studied was found to give high percentages of products with the nitro-group *para* to fluorine but they were not as high as previously reported by other methods of analysis.

REFERENCES

1 G. SCHIEMANN, *Ber.*, 62 (1929) 1794.
2 G. VALKANAS, *J. Chem. Soc.*, (1963) 5554.
3 H. SUSCHITZKY, *J. Chem. Soc.*, (1955) 4026.
4 G. C. FINGER, F. H. REED AND J. L. FINNERTY, *J. Am. Chem. Soc.*, 73 (1951) 153.
5 J. R. KNOWLES, R. O. C. NORMAN AND G. K. RADDA, *J. Chem. Soc.*, (1960) 4885.
6 J. S. PARSONS, S. M. TSANG, M. P. DIGIAIMO, R. FEINLAND AND R. A. L. PAYLOR, *Anal. Chem.*, 33 (1961) 1858.
7 K. J. BOMBAUGH, *Anal. Chem.*, 33 (1961) 29.
8 M. H. KLOUWEN AND R. TER HEIDE, *J. Chromatog.*, 7 (1962) 297.
9 A. ROE, *Org. Reactions*, 5 (1949) 193.
10 M. YOKOYAMA, *Helv. Chim. Acta*, 12 (1929) 771; H. STEPHEN, W. F. SHORT AND G. GLADDING, *J. Chem. Soc.*, 117 (1920) 526; H. L. HALLER, E. Q. ADAMS AND E. T. WHERRY, *J. Am. Chem. Soc.*, 42 (1920) 1842.
11 A. F. HOLLEMAN, *Chem. Rev.*, 1 (1924) 187.
12 C. K. INGOLD, A. LAPWORTH, E. ROTHSTEIN AND D. WARD, *J. Chem. Soc.*, (1931) 1959.
13 P. B. D. DE LA MARE AND J. H. RIDD, *Aromatic Substitution, Nitration and Halogenation*, Academic Press, Inc., New York, 1959, p. 85.
14 L. J. BELLAMY, *The Infrared Spectra of Complex Molecules*, John Wiley & Sons, Inc., New York, 1960, p. 79–80.

PLATE HEIGHT IN COILED COLUMNS*

J. CALVIN GIDDINGS

Department of Chemistry, University of Utah, Salt Lake City, Utah (U.S.A.)

(Received April 14th, 1964)

Under a title similar to the above, MAISEN AND HARDING[1] have criticized certain mathematical approximations used by the writer[2] in obtaining the plate height of coiled and bent columns. With one exception, an oversight corrected in the subsequent literature[3,4], the approximations are integral steps in the generalized nonequilibrium theory[3,5-8], for which coiled columns are only a special case. Thus their criticism can be discussed in part by reference to this broad theory of chromatography. Before discussing details, we may cite the following evidence in support of the generalized nonequilibrium theory.

1. This theory agrees exactly with the results of GOLAY[9], KHAN[10] and JONES[11] for uniform films of liquids (as on a capillary wall).

2. Exact agreement is found with GOLAY[9] AND KHAN[10] on the plate-height contribution of the flowing gas in a capillary column.

3. The results are identical to an independent method[12] used to study diffusion in ion-exchange beads.

4. Other methods[13-16] confirm the validity of this theory in the study of single-step kinetic processes.

5. Experimental results with a diffusion tube[17] (for measuring gaseous diffusion coefficients) have confirmed the theory to within 5 % accuracy.

6. Experimental results with glass-bead columns[18] have confirmed the theory within a 20-50 % margin on plate height.

7. Experimental results on capillary columns[19,20] verify the theory in a general way, particularly in regard to the gas phase processes.

The approximations of the generalized nonequilibrium theory are made necessary by the extreme complexity of the dynamics of chromatography. It is not a theory for the exact mathematician who would refuse to proceed at the 95 % level just because the other 5 % could not be handled rigorously. In sacrificing a few percent accuracy, highly simplified results are obtained. The exactness in most cases probably well exceeds the limit of experimental accuracy, thus making the theory perfectly adequate for developing the concepts of chromatography in conjunction with experimental work.

The generalized nonequilibrium theory is based on certain judicious approximations which are related to the fundamental nature of chromatographic processes. The near-equilibrium approximation is the main one. With a few exceptions in preparative work, column efficiency would degenerate almost totally if the near-equilibrium ap-

* This work was supported by research grant No. GM 10851-07 from the National Institutes of Health, Public Health Service.

proximation were not valid. Thus for any effective column the equilibrium departure term, ε, is small compared to unity and can be ignored under the proper circumstances.

The magnitude of ε is determined by equating the accumulation rate of solute, s, based on longitudinal flow and diffusion with that based on lateral diffusion. In the former case the overall concentration is important and ε terms may obviously be ignored. In the latter case nonequilibrium is the sole driving force and ε must be retained. The final error is the order of ε, *i.e.*, about 1–5 %*. The argument of MATSEN AND HARDING, that while ε is small, so are the lateral diffusion effects, is not entirely clear. The value of ε is not being compared to some undefined effects whose dimensions were not stated. It is, instead, being compared to unity.

A second question raised was with respect to the overall mass-balance equation, their equation (4). It is contended that equation (5) should be employed. Now equation (4) is used to obtain the s related to longitudinal flow and diffusion, and we shall be satisfied if our relative error in this term is no larger than ε. As a first step, employing their eqn. (4), the concentration, c, is written as c^* $(1 + \varepsilon)$ and ε is ignored. As a second step the flow-velocity profile is approximated as in the original paper, eqn. (1), *i.e.*, $v = v_0$ $(1 - r \sin \theta/R_0)$, and this is substituted into their eqn. (4). (The maximum error in this approximation, if the ratio of coil to column radius is 10, is only 1 %.) The integration of this equation leads to a result identical to our own, *i.e.*, to eqn. (4). In saying that our equation is applicable only in "trivial cases", the authors are therefore implying that any result with a 1–5 % approximation is trivial. This view is especially erroneous in chromatography where many effects are still uncertain by an order of magnitude.

MATSEN AND HARDING conclude that the two steps just discussed are responsible for a final s equation (their eqn. (6)) which is unrealistic because it is independent of lateral diffusivity. The fact that s (the accumulation rate for solute at a given point) is independent of lateral diffusivity under near-equilibrium conditions is, however, extremely clear on physical grounds. The quantity s is governed by the deficiency or excess (with respect to the mean) of solute flowing into a given region (longitudinal diffusion may be ignored for the sake of this argument). If an excess flows into a particular region as a result of a high flow rate (and thus high flow transport), this will be parceled out to neighboring regions until mutual equilibrium is *nearly* reached. The amount transferred, indicated by the s term, will be governed almost entirely by the excesses and deficiencies, not by the diffusivities. The latter will simply change the concentration difference which is the driving force for diffusion. This difference, compared to the mean concentration, is only the order of ε in magnitude, and is thus unimportant for mass-conservation purposes. (We may think of the accumulation term as resulting from a diffusive flux, which, in one dimension, may be written as $J = -D$ $\partial c/\partial x$. Since J is essentially constant, as shown above, a change in D leads to a change in the gradient, $\partial c/\partial x$. MATSEN AND HARDING apparently did not consider the change in $\partial c/\partial x$, and thus assumed that J must change, contrary to the physical nature of all near-equilibrium processes.)

Finally MATSEN AND HARDING conclude that the use of two different material flux expressions, their eqns. (7) and (8), is incorrect. In fact, however, these two

* For the analytical column used in the original paper, assuming a length of 2 m, the extreme maximum of ε, a distance σ from the peak center, is only 0.03. The minimum is zero. On the average in this case, ε can be ignored with respect to unity with only a 1–2 % error.

equations differ by only a small fraction, and their use is to be determined by the role they play in the development of the theory. These roles are distinctly different, each involving a different order of approximation, and their respective uses are entirely proper within this framework. They cannot both be right, as stated by MATSEN AND HARDING, but they can both be valid approximations to be used where appropriate.

For the most part the objections to the original theory are based on a few of the approximations made. No proof is given that these approximations are incorrect. Instead, it is stated that such approximations would be more safely reserved for the final equations. By the authors' own admission, however, the final equations cannot even be obtained without first making the approximations. Thus they must limit themselves to such special cases as that of zero diffusivity, the equations for which would be valid only for preparative columns approaching infinite diameter. The necessity to proceed with reasonable assumptions will always be an integral part of chromatographic theory. That the assumptions of this theory are valid is shown by the foregoing arguments and confirmed by the extensive agreement with other theories and data as mentioned at the beginning.

The only valid criticism of the earlier coiled-column equation is related to the equivalence of the length of flow paths. This error has long been recognized by this writer, and was corrected in the literature early in 1963[3,4]. The correction[4] is a simple matter, not requiring the explicit use of toroidal coordinates as suggested. The functional form of the original equation was correct but a numerical correction factor of 4 must be applied. The true plate-height equation is:

$$H = \frac{7 \, v r_0^4}{12 R_0^2 \, \gamma D_g}$$

where v is flow velocity, r_0 is tube radius, R_0 is coil radius, D_g is gaseous diffusion coefficient and γ is the obstruction factor for molecular diffusion in the gas.

SUMMARY

The main approximations used by the author in an earlier treatment of coiled columns are justified. This refutes the recent arguments of MATSEN AND HARDING[1] on this matter. A corrected equation for plate height in coiled columns is given.

REFERENCES

1 J. M. MATSEN AND J. W. HARDING, *J. Chromatog.*, 12 (1963) 145.
2 J. C. GIDDINGS, *J. Chromatog.*, 3 (1960) 520.
3 J. C. GIDDINGS, *Anal. Chem.*, 35 (1963) 439.
4 J. C. GIDDINGS, *J. Gas Chromatog.*, 1, No. 4 (1963) 38.
5 J. C. GIDDINGS, *J. Chem. Phys.*, 31 (1959) 1462.
6 J. C. GIDDINGS, *J. Chromatog.*, 3 (1960) 443.
7 J. C. GIDDINGS, *J. Chromatog.*, 5 (1961) 46.
8 J. C. GIDDINGS, *J. Phys. Chem.*, 68 (1964) 184.
9 M. J. E. GOLAY, in D. H. DESTY (Editor), *Gas Chromatography 1958*, Academic Press, New York, 1958, p. 36.
10 M. A. KHAN, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, Washington, 1962, p. 3.
11 W. L. JONES, *Anal. Chem.*, 33 (1961) 829.
12 D. C. BOGUE, *Anal. Chem.*, 32 (1960) 1777.
13 J. C. GIDDINGS, *J. Chem. Phys.*, 26 (1957) 1755.

[14] J. C. GIDDINGS, *J. Chromatog.*, 2 (1959) 44.
[15] P. C. SCHOLTEN AND K. J. MYSELS, *J. Chem. Phys.*, 35 (1961) 1845.
[16] T. A. BAK AND W. G. KAUMAN, *Trans. Faraday Soc.*, 55 (1959) 1109.
[17] J. C. GIDDINGS AND S. L. SEAGER, *Ind. Eng. Chem., Fundamentals*, 1 (1962) 277.
[18] J. C. GIDDINGS, K. L. MALLIK AND M. EIKELBERGER, *Anal. Chem.*, 34 (1962) 1026.
[19] D. H. DESTY AND A. GOLDUP, in R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, Washington, 1960, p. 162.
[20] R. P. W. SCOTT AND G. S. HAZELDEAN, in R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, Washington, 1960, p. 162.

EXPOSURE OF AN ISOTOPE EFFECT BY ^{3}H-LABELED FATTY ACIDS ON SILICA–SILVER NITRATE CHROMATOGRAPHY

D. S. SGOUTAS AND F. A. KUMMEROW

The Burnsides Research Laboratory, University of Illinois, Urbana, Ill. (U.S.A.)

(Received May 19th, 1964)

When tritium-labeled methyl linoleate[1] was eluted from a silica–silver nitrate column in our laboratory, it was apparent that the radioactive peak in the effluent did not precisely coincide with the methyl ester peak as located by spectrophotometric measurements but followed it closely. The two curves were identically shaped; the difference in resolution seemed to be due to a slower movement of the tritiated molecules. Similar isotope fractionation effects were observed upon chromatography of methyl esters of oleic or linoleic acid doubly labeled with tritium and carbon-14. Molecules labeled with tritium at the active centers of unsaturation showed a higher retention time on silica–silver nitrate columns than molecules labeled with carbon-14 at the carboxyl group.

EXPERIMENTAL

The chromatographic method of DE VRIES[2] was adapted. Silicic acid (100 g) was mixed with a silver nitrate solution (100 g in 200 ml water) dried in a tray at 120° for 16 h and passed through a 140 mesh sieve. For most experiments a column 150 mm in length and 12 mm in diameter was packed with 10 g of adsorbent. On one occasion a longer column under pressure was employed and in another a series of columns with varying amounts of silver nitrate per g of adsorbent was used. In each experiment a charge of approximately 70 mg of methyl ester was applied to the column, the eluting solvent was always benzene in light petroleum ether (b.p. 40–60°). The flow rate was about 1 ml/2 min and fractions of 1 ml were collected. The methyl linoleate concentration was determined spectrophotometrically[3] with the aid of a Cary Model 11 M recording spectrophotometer.

For radioactivity measurements 0.1 to 0.5 ml from each fraction was evaporated under nitrogen and taken up in 15 ml of a solution consisting of 3 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(phenyloxazolyl)-benzene per liter of toluene. A Packard Tricarb, automatic Model 314-EX scintillation spectrometer was used. Samples containing the two radioisotopes, ^{3}H and ^{14}C were counted by the discriminator ratio method[4] as modified by KABARA *et al.*[5]. The level of both isotopes was so chosen that count rates exceeded a 20:1 count/background ratio in order to eliminate the necessity of reckoning with the background in the ^{3}H/^{14}C ratio determination.

Linoleic acid labeled with tritium at the 9, 10, 12 and 13 positions was prepared as reported earlier[1]. [1-^{14}C]-Linoleic acid was purchased from California Corporation of Biochemical Research. In contrast to our tritium labeled linoleic acid the purchased

[1-^{14}C]-linoleic acid contained a considerable amount of *trans* isomers. For the purpose of this study, removal of *trans* isomers was necessary because homogeneity (regarding the geometry of the double bonds) of the labeled compounds was imperative. Since silica impregnated with silver nitrate resolves compounds which differ either in the number or geometry of double bonds, the presence of *trans* isomers would result in additional peaks. Thus, methyl [1-^{14}C]-linoleate was purified by chromatography on a silica-silver nitrate column using 20 ml of the eluting solvents in the following sequence: 35, 40, 45, 50 and 55 % benzene in petroleum ether and finally 70 ml benzene. Aliquots of the eluant fractions were counted and alternatively characterized by thin-layer chromatography on silicic acid–silver nitrate plates[1] and by infrared analysis*. The elution curve gave three components; the last to elute was almost 98 % *cis, cis* 18-diene and represented 86 % of the total material.

A mixture of [9, 10, 12, 13-^{3}H]-linoleic and [1-^{14}C]-linoleic acids was completely hydrogenated[6] to give stearic acid labeled in an identical manner as the original linoleic acids. Preparative gas phase chromatography was used for the purification of the methyl stearate[7].

Oleic acid labeled with ^{14}C at the carboxyl group and with ^{3}H at 9, 10, 12 and 13 positions was similarly prepared by controlled hydrogenation[8]. The product of this hydrogenation was subjected to preparative gas phase chromatography and the 18-monoene peak was isolated. Geometric and positional isomers of the 18-monoene formed during the hydrogenation were removed by silica–silver nitrate column chromatography[2]. The eluting solvent was 28 % benzene in petroleum ether. Periodate permanganate oxidation at the double bond indicated that 90 % of the 18-monoene had the double bond at the 9 position. Methyl esters of all the fatty acids were formed by reaction with diazomethane[9].

RESULTS AND DISCUSSION

The isotope effect is shown in Fig. 1A and B. In this experiment, the sample of methyl linoleate (75 mg) had a total activity of 0.35 μC. The column was 150 mm long (Fig. 1A) and the elution was carried out with 20 ml each of 40, 45, 50 and 55 % benzene in petroleum ether and finally with 70 ml benzene. It is apparent that the presence of tritium in the olefinic positions caused a slower movement of the methyl linoleate through the column. If it is assumed that both curves representing the concentration and the radioactivity distribution were gaussian with the same standard deviation σ and different means (m_1 and m_2) a semilogarithmic plot of the specific activity S *versus* fraction number n would result in a straight line and the relationship could be described by the formula[10]:

$$\ln S = \frac{m_1 - m_2}{\sigma^2} n + \frac{m_2^2 - m_1^2}{2\,\sigma^2}$$

Our plot shows a linear relationship between the logarithm of specific activity and the fraction number for that part of the chromatogram where the two curves overlap each other. A rapid increase of the specific activity with decreasing fraction number

* For the determination of *trans* double bonds by infrared spectroscopy, samples were prepared by grinding 30 mg KBr with 0.1 ml of ester solution in a mortar. Discs of 5 mm diameter were pressed in an evacuated die and examined in a Beckman IR-7 infrared spectrophotometer.

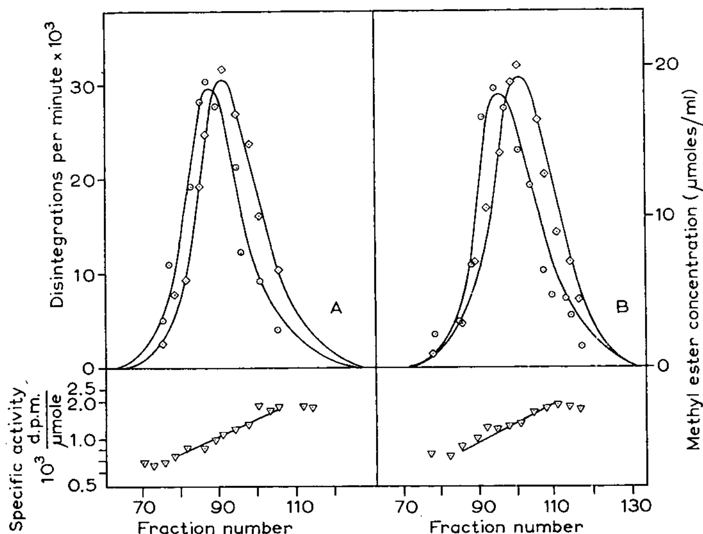


Fig. 1. Effluent curves from the column chromatography of tritium labeled methyl linoleate on silica–silver nitrate column (A = 150 × 12 mm; B = 230 × 12 mm); ⊙, methyl ester concentration as determined by spectrophotometric measurement; ◇, methyl ester activity as determined by ^3H counting; ▽, calculated specific activity.

made it difficult to extrapolate the specific activities of earlier fractions for which spectrophotometric measurements gave a relatively larger error. The slope of the line is a measure of the resolution of the labeled and the unlabeled compound. When a 230 mm long column was employed, the slope was increased indicating a higher resolution (Fig. 1B). On the other hand, the region of the linear relationship between ln S and n became shorter and the error at the leading and trailing edges of the peak concentration became larger.

When methyl [1-^{14}C]-linoleate was passed through a similar column under identical experimental conditions, the activity change was only 1.8 %, no greater than the limits of errors of the experiment. However, when a mixture of methyl [1-^{14}C]-linoleate and methyl [9, 10, 12, 13-^3H]-linoleate (ratio of $^3H/^{14}C = 3.25$) was chromatographed and the ratio of the two radioisotopes was determined in each fraction of the collected effluent a steady increase of the ratio in favor of tritium was observed indicating a faster movement of the carbon-14 labeled molecules. The logarithm of the ratio of the isotopes plotted against the fraction number resulted in a slope identical with that in Fig. 1.

In order to exclude the possibility that the observed isotope effect was due to some radioactive contamination, we tested many fractions by gas chromatography and thin-layer chromatography using the isotope dilution technique. In all cases the radioactivity coincided with the methyl linoleate peak or spot and there was no indication that artifacts could have been responsible for the differences. There are two possible explanations which should be considered for this phenomenon. The first involves the possibility of an increase in mass by the tritium, which appears to be unlikely, since theory predicts that replacement of one or two hydrogen atoms by tritium causes only a change of 2 or 3 % in mass. This increase in mass would not seem sufficient to cause

the observed differences in chromatographic behavior. The second involves the possibility that tritium exerts an effect upon the ability of the olefinic linkage to form coordination complexes with the silver ion.

It seems relevant to speculate whether the presence of tritium at the olefinic bonds may relate to changes in the affinity of the olefinic bonds to form complexes. This possibility seems to be quite reasonable in view of general agreement that resolution on a silica–silver nitrate column is attributable to the formation of these complexes. Indeed, if the effect reflects a difference in the coordination products, the slope which is a measure of the degree of resolution should depend upon the number of double bonds affiliated with tritium and present in the vehicle molecule. In addition the magnitude of the isotope effect should be related to the concentration of silver ions in the adsorbent. In order to test these points the double-label isotope ratio technique was employed. The assumption was made that the carbon-14 labeled methyl esters carrying the label in a position remote from the reaction center have a similar behavior on the column to the unlabeled esters and thus, the carbon-14 could serve as an internal reference standard. This assumption was supported by the fact previously established that methyl [1-^{14}C]-linoleate behaved identically to the unlabeled compound.

Methyl esters of the following specifically labeled fatty acids were chromatographed in the amounts indicated (the figures showing μmoles, μC of ^{3}H and the ratio of ^{3}H/^{14}C respectively): [1-^{14}C]-stearic and [9, 10-^{3}H]-stearic acid 200, 0.38, 1.20; [1-^{14}C]-oleic and [9, 10, 12, 13-^{3}H]-oleic acid 210, 0.41, 1.24. For each methyl ester two effluent radioactivity curves were determined, one based on carbon-14 and the other on tritium.

If we consider the carbon-14 curve as representing the actual concentration, the tritium activity curve shows an apparent concentration in those areas where partial resolution occurred. The ratio of the apparent concentration to the actual concen-

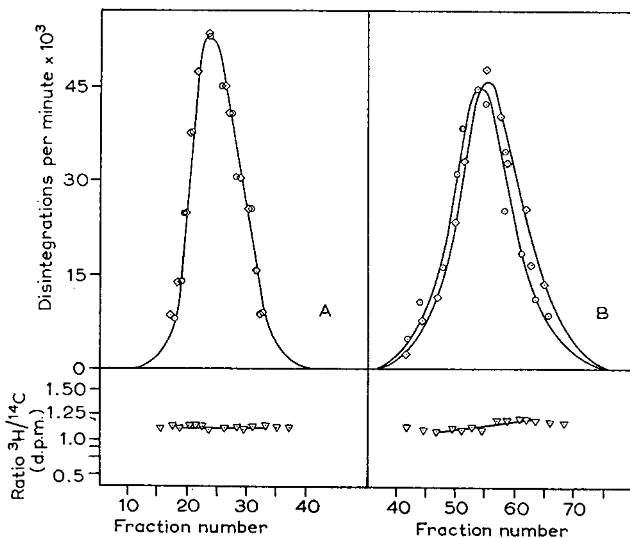


Fig. 2. Effluent curves from the column chromatography of double labeled methyl stearate (A) and methyl oleate (B): ⊙, ^{14}C activity as determined by ^{14}C counting; ◇, ^{3}H activity as determined by ^{3}H counting; ▽, ratio of ^{3}H/^{14}C.

tration which is identical to the ratio of $^{3}H/^{14}C$ provided a measure of relative specific activity in successive fractions. Fig. 2 represents the pairs of curves. As anticipated, there was no effect in the case of methyl stearate while resolution of doubly labeled methyl oleate was apparent. The value obtained for the slope of methyl oleate was approximately one third of the observed value for methyl linoleate. These slopes can be used only for qualitative comparisons. Further evidence in support of our interpretation of the observed phenomenon is given in Table I. Here, the slope constant representing the degree of resolution of methyl linoleate varied directly with the concentration of the silver nitrate solution used for the preparation of the adsorbent.

TABLE I

SLOPE OF CURVE RELATING THE LOGARITHM OF SPECIFIC ACTIVITY OF TRITIUM LABELED METHYL LINOLEATE TO FRACTION NUMBER

Grams of silver nitrate per 100 ml water per 100 g silicic acid	*Slope constant value*
0	—
25	0.32
50	0.40
75	0.42
100	0.46

It therefore appears certain that tritium exerts its effect on the formation and breaking of the silver ion double bond complexes which comprise the transition structure during sorption and desorption.

Isotope effects based on chromatographic behavior have been reported for a number of chemical systems. The use of column chromatography[10–12] and gas chromatography[13] in a repeated cycling operation has been proposed for the separation and enrichment of isotope mixtures. In most cases it was shown that the effect depended upon the isotope occupying a particular position in the molecule[12,14]. From that particular position the isotope could affect electronic effects (inductive effects, resonance, ionization) which had a direct bearing upon the chromatographic behaviour of the entire molecule. For tritium-labeled linoleic acid, tritium could exist in a stable form at either the 9, 10, 12 or 13 (or any combination) position and its presence in those positions would exert an effect on the formation of the silver–olefin equilibrium constants. Recently, CVETANOVIC, DUNCAN AND FALCONER[15] have published the gas chromatographic results of the effect of progressive deuteration on the retention volumes of olefinic hydrocarbons on silver nitrate–ethylene glycol firebrick columns. They have shown a reasonably additive effect of considerable magnitude attributable to deuterium isotope effects on the silver–olefin equilibrium constants.

Such phenomena may not be readily evident and it is the excellent resolving power of modern chromatographic techniques that reveals them. Nevertheless, if overlooked or ignored it may result in a serious flaw in the evaluation and interpretation of experimental data, whenever radioactive tracers are used in combination with chromatography.

ACKNOWLEDGEMENT

This study was supported by a grant from the Special Industry Board of the National Dairy Council. Thanks are due to Mr. MING FANG for his technical assistance.

SUMMARY

A tritium isotope effect was observed during the chromatography of tritiated unsaturated fatty acids as their methyl esters on silica–silver nitrate columns. The results are interpreted in terms of the coordination complexes formed between olefinic bonds and silver ions.

REFERENCES

1 D. S. Sgoutas and F. A. Kummerow, *Biochemistry*, 3 (1964) 406.
2 B. de Vries, *J. Am. Oil Chemists' Soc.*, 40 (1963) 184.
3 S. F. Herb and R. W. Riemenschneider, *Anal. Chem.*, 25 (1953) 953.
4 G. T. Okita, J. J. Kabara, F. Richardson and G. V. Leroy, *Nucleonics*, 15 (1957) 111.
5 J. J. Kabara, N. R. Spafford, M. A. McKendry and N. L. Freeman, in Seymour Rothchild, (Editor), *Advances in Tracer Methodology*, Vol. 1, Plenum Press, New York, 1963, p. 76.
6 W. J. Gensler and J. J. Bruno, *J. Org. Chem.*, 28 (1963) 1254.
7 A. K. Hajra and N. S. Radin, *J. Lipid Res.*, 3 (1962) 131.
8 R. O. Feuge, E. R. Cousins, S. P. Fore, E. F. Dupre and R. T. O'Connor, *J. Am. Oil Chemists' Soc.*, 30 (1953) 454.
9 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1962) 1412.
10 K. A. Piez and H. Eagle, *Science*, 122 (1955) 968.
11 F. H. Spedding, J. E. Powell and H. J. Svec, *J. Am. Chem. Soc.*, 77 (1955) 1393.
12 H. Gottschling and E. Freese, *Nature*, 196 (1962) 829.
13 J. W. Root, E. K. C. Lee and F. S. Rowland, *Science*, 143 (1964) 676.
14 K. A. Piez and H. Eagle, *J. Am. Chem. Soc.*, 78 (1956) 5285.
15 R. J. Cvetanovic, E. J. Duncan and W. E. Falconer, *Can. J. Chem.*, 41 (1963) 2095.

THE ANALYSIS OF ARYLAMINES AND PHENOLS IN OXIDATION-TYPE HAIR DYES BY PAPER CHROMATOGRAPHY

R. B. SMYTH AND G. G. McKEOWN

Food and Drug Directorate,
Department of National Health and Welfare,
Ottawa, Ontario (Canada)

(Received April 20th, 1964)

In the course of examining various types of cosmetic preparations in this laboratory, the need for a simple method for the separation and identification of the colouring agents of oxidation-type (*p*-phenylenediamine) hair dyes became evident. Although specific procedures exist[1] for many of the major hair dye components, no overall analytical method for colouring agents has been reported. The colouring agents most commonly used are the phenylenediamines, diaminotoluenes, nitrophenylenediamines, aminophenols, nitroaminophenols, and di- and trihydroxybenzenes. Many compounds are in use and therefore paper partition chromatography, with its simplicity and high separating power, was the logical choice as the analytical technique to be used in this work.

Little has been published on the paper chromatographic separation of compounds of this type. The sole publication specifically devoted to colouring agents of hair dyes was by IACOBELLI-Turi[2]. This worker accomplished the separation of *p*-phenylenediamine, 2,5-diaminotoluene, *p*-aminophenol, resorcinol and pyrogallol, and also studied the behaviour of these compounds toward a variety of detecting reagents. ŠIMEK[3] and also PANNELL AND LUVALLE[4] have chromatographed photographic developers and some of these compounds are the same as those used as colouring agents of hair dyes. SUNDT[5] has studied the three isomeric aminophenols but, under his chromatographic conditions, these compounds failed to move from the starting line. BATE-SMITH[6] has reported the separation of the isomeric dihydroxybenzenes and pyrogallol.

This paper is concerned with the paper partition chromatography of 29 compounds, all of which have been used as colouring agents of hair dyes. A mixture of *n*-butanol, ethanol and water containing acetic acid and sodium sulphite was used as the developing solvent. This solvent system gave a good distribution of R_F values. All compounds could be identified by their R_F values together with their behaviour toward the detecting reagents used in this work.

EXPERIMENTAL

1. Materials

All of the reference compounds were obtained from chemical supply houses and were of the best quality that was available. These materials were used without further purification.

2. Pretreatment of hair dye samples prior to spotting chromatograms

When the hair dye preparation was a liquid and completely miscible with water, a 1–2 ml portion was diluted with an equal volume of water and the diluted solution was spotted directly on the chromatograms.

For a preparation that was a liquid, but not completely miscible with water, a 2 ml portion was diluted with 20 ml of *n*-hexane in a separatory funnel. This mixture was then shaken gently with 10 ml of dilute aqueous acetic acid (1:9). When the two phases had separated, the lower aqueous acid layer was run off and used to spot the chromatograms.

For a hair dye preparation that was either a dry solid or a paste, approximately 1 g was stirred vigorously with 5 ml of dilute aqueous acetic acid (1:19). The resulting mixture was filtered through glass wool and the filtrate was spotted on the chromatograms.

3. Detecting reagents

Ammoniacal silver nitrate. Two grams of silver nitrate were dissolved in 100 ml of distilled water. To this solution was added concentrated aqueous ammonia, dropwise with vigorous swirling, until the precipitate that formed initially just redissolved. An excess of 1 ml of aqueous ammonia was then added.

p-Dimethylaminobenzaldehyde. To a solution containing 1 g of *p*-dimethylaminobenzaldehyde in 100 ml of absolute ethanol was added 1 ml of concentrated hydrochloric acid.

Sodium 1,2-naphthoquinone-4-sulphonate. A solution containing 0.5 g of sodium 1,2-naphthoquinone-4-sulphonate in 95 ml of distilled water was mixed with 5 ml of glacial acetic acid. If undissolved matter was present, it was removed by filtration through glass wool.

4. Paper chromatography

The paper chromatograms were developed in a cylindrical glass tank (24 in. high and 12 in. in diameter) with a plate glass cover. Within the tank was a stainless steel rack that supported glass troughs positioned for downward flow of the developing solvent. The paper used for the chromatograms was Whatman No. 3 MM cut into strips 6×22 in. A starting line was drawn about 3 in. from one end of each paper and marked at intervals of $^3/_4$ in. in order to position the spottings. Standard solutions were prepared by dissolving approximately 25 mg of the amine or phenol reference compound in 5 ml of a (1:19) solution of glacial acetic acid in water. When working with the amines, fresh solutions were prepared each day because these compounds degraded rapidly when in solution.

Solutions of the hair dye samples were prepared as described previously and spotted along with *p*-phenylenediamine and other appropriate reference compounds on three papers, one for each of the detecting reagents. A 5 λ volume of an unknown or a standard solution usually produced a satisfactory chromatogram. The spots were dried and the three chromatograms were developed simultaneously. The developing solvent was the upper phase of a mixture of 500 ml of *n*-butanol, 100 ml of ethanol, 400 ml of distilled water, 10 ml of glacial acetic acid, and 10 g of anhydrous sodium sulphite. The chromatograms were developed until the solvent front had travelled 10–12 in. from the starting line; this required 4–5 h. The chromatograms were then

ห้องสมุด กรมวิทยาศาสตร์

removed from the developing tank and dried in air for about 30 min. Each paper was then sprayed with one of the three detecting reagents. No heating of the chromatograms was required for colour development. After allowing at least 30 min for full development of the spots, the chromatograms were evaluated. Table I lists the R_F values of the amines and phenols which were studied and data on their behaviour toward the three detecting reagents. Nitro compounds, being yellow in colour, usually could be seen on the chromatograms before spraying with the detecting reagents.

TABLE I

CHROMATOGRAPHIC DATA FOR SOME ARYLAMINES AND PHENOLS USED AS COLOURING AGENTS IN OXIDATION-TYPE HAIR DYES

Solvent: *n*-Butanol–ethanol–water–acetic acid–sodium sulphite (50:10:40:1:1, upper layer).
Paper: Whatman No. 3-MM.
Detection: D_1 = Ammoniacal silver nitrate.
D_2 = Ethanolic *p*-dimethylaminobenzaldehyde plus hydrochloric acid.
D_3 = Sodium 1,2-naphthoquinone-4-sulphonate plus acetic acid.

Compound	R_F	D_1*	*Colour***	
			D_2	D_3
2-Amino-1-phenol-4-sulphonic acid	0.08	5 sec	Y	O
2,4-Diaminophenol	0.34	5 sec	B-O	Bl-P
p-Phenylenediamine	0.36	5 sec	R	R-P
m-Phenylenediamine	0.46	30 min	O-Y	P
4-Methoxy-*m*-phenylenediamine	0.49	5 min	O	P
2,5-Diaminotoluene	0.49	5 min	R	P
2,4-Diaminotoluene	0.58	30 min	Y	P
p-Aminophenol	0.59	5 sec	Y	P
p-Methylaminophenol	0.62	5 sec	Y	P
o-Phenylenediamine	0.63	5 min	O	P
2-Nitro-*p*-phenylenediamine	0.64	5 sec	B	P
p-Diethylaminoaniline	0.68	5 sec	Y	P
4,4′-Diaminodiphenylmethane	0.68	30 min	Y	R
m-Aminophenol	0.71	5 min	G-Y	P
p-Anisidine	0.72	5 min	Y	P
4-Nitro-*o*-phenylenediamine	0.73	5 sec	B-Y	B
Picramic acid	0.75	5 sec	O	P
Pyrogallol	0.75	5 sec	P	P
o-Aminophenol	0.77	5 sec	Y	O
N-Phenyl-*p*-phenylenediamine	0.79	5 sec	B-R	Bl
4-Amino-2-nitrophenol	0.82	5 sec	Y	P
4-Chloro-*o*-phenylenediamine	0.83	5 min	O-Y	B-R
Hydroquinone	0.86	5 sec	neg.	neg.
Catechol	0.87	5 sec	neg.	neg.
2-Amino-4-nitrophenol	0.88	5 sec.	Y	O
o-Anisidine	0.89	30 min	Y	R
Resorcinol	0.90	5 min	P	neg.
1,5-Naphthalenediol	0.91	5 sec	G	neg.
p,p′-Methylene-bis-(N,N-dimethyl-aniline)	0.92	30 min	Y	G

* Approximate time required for development of spot.
** Y = yellow; B = brown; O = orange; R = red; G = green; P = purple; Bl = blue; neg. = negative.

RESULTS AND DISCUSSION

Oxidation-type hair dyes are usually aqueous solutions containing 1–5 % of a mixture of colouring agents. In addition ammonia, surfactants, hair conditioners, perfume

and sodium sulphite may be present. These other materials did not interfere with the identification of the colouring agents. Products of this type were found to be quite viscous and direct spotting usually did not produce satisfactory chromatograms. This appeared to be due to the physical nature of these preparations in that they did not readily "wet" and penetrate the paper on spotting. For most products, this difficulty was overcome by the simple expediency of diluting the sample with water. For those liquid samples that produced two phases when mixed with water, the alternative extraction procedure using dilute acetic acid gave satisfactory chromatograms without significant losses of colouring agents. Paste and solid samples presented no problem in that the colouring agents could readily be extracted with dilute acetic acid. All of the commercial preparations that were examined in this laboratory gave satisfactory chromatograms after application of the appropriate pretreatment procedure.

Twenty-nine compounds were investigated in this work and it was found that not all of the possible combinations could be separated with the single developing solvent. Investigation of the use of several developing solvents to achieve a unique R_F value for each compound met with little success. In general, the developing solvents separated these compounds in the same order but gave different ranges of R_F values. The alternative of employing several detecting reagents in order to identify compounds having similar R_F values proved to be quite successful. However, there were a few cases where compounds having the same R_F values could be identified only if they were present singly.

The chromatographic procedure used in this work requires few comments. No conditioning or equilibration of the chromatographic paper was necessary prior to development. In the initial stages of this work, the organic phase of *n*-butanol–ethanol–water (5:1:4) was chosen as developing solvent on the basis that it produced a wide range of R_F values. However, extensive streaking of the spots occurred with this solvent. In addition, there was a loss of sensitivity toward the detecting reagents which was related to the development time of the chromatograms. In some instances, a compound could be detected easily after 1 h of development but after 4–5 h the compound could not be detected on the chromatogram. Probably, this loss of sensitivity was caused by air oxidation of the compounds. The addition of a small quantity of acetic acid to the developing solvent mixture eliminated the streaking and gave well-defined spots. The amount of acetic acid added was minimal because large amounts of the acid were found to be difficult to remove from the developed chromatograms and reduced the sensitivity of the silver nitrate detecting reagent. The addition of sodium sulphite reduced losses from oxidation during development of the chromatograms and greatly improved the sensitivity of the method. It was observed that R_F values began to change after using a given batch of developing solvent for several days. With freshly prepared solvent, the reproducibility of R_F values was excellent.

The detecting reagents used in this work were selected after investigating a wide variety of potential reagents. Those selected produced permanent spots, reacted with all or most of the reference compounds, and showed good sensitivity. The limit of detection was determined under actual chromatographic conditions, using four of the reference compounds. These were *p*-phenylenediamine, 2,5-diaminotoluene, 2-nitro-*p*-phenylenediamine and pyrogallol. Identical results were obtained with all four

compounds. The limit of detection using *p*-dimethylaminobenzaldehyde was 0.1 γ and for the other reagents the limit was 1 γ. *p*-Dimethylaminobenzaldehyde gave a variety of colours and this was very useful for identification of compounds with similar R_F values. In order to take full advantage of the potential of this detecting reagent, it was necessary to allow sufficient time after spraying the chromatograms for complete development of the colours. Many spots that initially were bright yellow, changed to off-shades of yellow or orange on standing and this was accompanied by an increase in the intensity of the colour. The colours listed in Table I for this reagent are those observed after about 30 min.

Ammoniacal silver nitrate reacted with all of the compounds under investigation and showed a high degree of sensitivity. With this reagent, all compounds produced a brown or black colour. The variation in colour seemed to be more dependent on the concentration of material in the spot than on its identity. It was important that the paper was dried thoroughly to remove acetic acid before spraying with this reagent, otherwise there was a loss of sensitivity. The time required for development of spots by this reagent was also of value for identification although this varied with the amount of the compound in the spot. With this reagent, the background darkened rapidly in the presence of sunlight and eventually the spots were obliterated. However, this process was not so rapid as to interfere with the detection of those compounds that were slow to form spots provided that the sprayed chromatogram was located away from direct sunlight. When it was desired to preserve chromatograms treated with this reagent, the sprayed chromatogram was immersed in 5% aqueous sodium thiosulphate solution and then washed with water. This treatment resulted in some reduction in intensity of the spots.

The use of sodium 1,2-naphthoquinone-4-sulphonate as a detecting reagent was based on work reported by SCHMIDT[7]. The reagent reacted with all compounds bearing an amino group but the range of colours produced was rather limited. This reagent was used mainly to distinguish between arylamines and polyhydric phenols and also to provide confirmation of identification.

The polyhydric phenols presented the greatest difficulties in identification, particularly hydroquinone and catechol. These two compounds were not separated by the developing solvent and behaved in an identical manner toward the detecting reagents. However, these compounds could be distinguished by developing an additional chromatogram and spraying it with aqueous 1% ferric chloride solution; hydroquinone produced little or no colour whereas catechol gave a black spot. Pyrogallol produced a black spot with this reagent and resorcinol and 1,5-naphthalenediol both gave pale brown spots.

Several dozen hair dye preparations were analyzed using this method. The colouring agents identified in these products were as follows: *o*-phenylenediamine, *p*-phenylenediamine, 2,4-diaminotoluene, 2,5-diaminotoluene, N-phenyl-*p*-phenylenediamine, *o*-aminophenol, *m*-aminophenol, *p*-aminophenol, *p*-anisidine, 2-nitro-*p*-phenylenediamine, 4-nitro-*o*-phenylenediamine, 4-amino-2-nitrophenol, catechol, resorcinol and pyrogallol. Only those preparations producing grey or black shades contained a single colouring agent and this was either *p*-phenylenediamine or 2,5-diaminotoluene. All of the reddish shades contained at least one nitro compound. Most of the preparations contained a complex mixture of colouring agents having 6–12 components.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. M. SILEIKA and Mr. R. A. GRAHAM, of the Food and Drug Directorate Laboratory in Toronto, for suggesting the use of sodium 1,2-naphthoquinone-4-sulphonate as a detecting reagent and also for performing part of the investigational work that was done on this reagent.

SUMMARY

A paper partition chromatographic procedure is described for the separation and identification of aryldiamines, aminophenols and polyhydric phenols used as colouring agents of oxidation-type hair dyes. Twenty-nine compounds were chromatographed on Whatman No. 3 MM paper using, as developing solvent, the top layer of a mixture of 500 ml *n*-butanol, 100 ml ethanol, 400 ml water, 10 ml acetic acid and 10 g sodium sulphite. Good separations of most combinations of compounds were achieved. Where resolutions could not be accomplished, positive identifications could be made through the use of several detecting reagents. The application of this procedure to the analysis of colouring agents of hair dye preparations is described.

REFERENCES

1 S. H. NEWBURGER, *A Manual of Cosmetic Analysis*, Association of Official Agricultural Chemists Inc., Washington, D.C., 1962, p. 76.
2 C. IACOBELLI-TURI, *Rend. Ist. Super. Sanita*, 19 (1956) 461.
3 J. ŠIMEK, *Chem. Průmysl*, 10 (1960) 403.
4 J. H. PANNELL AND J. E. LUVALLE, *Anal. Chem.*, 25 (1953) 1566.
5 E. SUNDT, *J. Chromatog.*, 6 (1961) 475.
7 E. C. BATE-SMITH, *Biochem. Soc. Symp. (Cambridge, Engl.)*, 3 (1949) 62.
6 E. G. SCHMIDT, *Ind. Eng. Chem., Anal. Ed.*, 11 (1939) 99.

DIRECT SPECTROPHOTOMETRIC EXAMINATION OF 2,4-DINITROPHENYLHYDRAZONES ON PAPER CHROMATOGRAMS

D. A. FORSS, P. R. EDWARDS, B. J. SUTHERLAND AND R. BIRTWISTLE

Division of Dairy Research and
Division of Mathematical Statistics, C.S.I.R.O.,
Melbourne (Australia)

(Received April 27th, 1964)

INTRODUCTION

Carbonyl compounds, including the recently detected vinyl ketones[1,2], are important in the flavours of foods and have been widely studied through their 2,4-dinitrophenylhydrazones. A common first step in the characterization of the 2,4-dinitrophenylhydrazones following separation by paper chromatography is their broad classification according to light absorption maxima (λ_{max}). This has usually been determined in solution after elution from the paper. However, the direct measurement of λ_{max} on paper chromatographic spots is considerably more rapid and sensitive. The λ_{max} of paper chromatographic spots is often reported[3] but this is of little value unless the λ_{max} of several reference compounds for the particular paper chromatographic system is known. The λ_{max} of the 2,4-dinitrophenylhydrazones of *n*-alkanals, *n*-alk-2-enals, *n*-alka-2,4-dienals and *n*-alkan-2-ones on phenoxyethanol paper chromatograms[4] and of *n*-nonanal, *n*-non-2-enal and *n*-undecan-2-one on paraffin oil paper chromatograms[5] have been reported, but for these and other classes of carbonyls, and for other chromatographic systems, additional data is needed.

The spectra of 2,4-dinitrophenylhydrazones of homologous series of *n*-alkanals, *n*-alk-2-enals, *n*-alka-2,4-dienals, *n*-alka-2,6-dienals, *n*-alkan-2-ones, *n*-alk-1-en-3-ones (vinyl ketones) and *n*-alk-3-en-2-ones were measured by transmission through paper and are reported here.

EXPERIMENTAL

Four paper chromatographic systems were studied. In three of the systems the papers were impregnated: with vaseline (GADDIS AND ELLIS[6]), paraffin oil (KLEIN AND DE JONG[5]) and phenoxyethanol (LYNN, STEELE AND STAPLE[7]). Acetylated paper was used in a fourth system described by FORSS AND RAMSHAW[8]. The first two systems are "reversed phase" and the last two systems are "normal phase". The R_F data of the 2,4-dinitrophenylhydrazones are reported separately[9].

Rectangular strips of paper containing single spots of the 2,4-dinitrophenylhydrazones were held in a special carriage by a spring steel clip and their light absorption measured in a Beckman DK2 spectrophotometer after 1 day and 7 days from completion of the chromatogram.

The spectral data for the C_{2-14} *n*-alkanal, $C_{4-11,16}$ *n*-alk-2-enal, $C_{6-12,14,16,18}$ *n*-alka-2,4-dienal, C_{3-13} *n*-alkan-2-one and C_{4-10} *n*-alk-1-en-3-one 2,4-dinitrophenylhydrazones were examined*. The following assessments were made and are recorded in Tables I–IV.

(a) Whether there were any significant differences between the carbon numbers and if so whether any trend existed. (Where this trend was found to be significant it was always negative, *i.e.* λ_{max} decreased as the carbon number increased.)

(b) Where trends were found their magnitudes at 1 and 7 days were compared.

(c) The mean values of λ_{max} for all carbon numbers at 1 and 7 days were compared to see if there was any change between times of measurement. The significance level of this comparison is shown after the bracket linking the two means where:

n.s. denotes "not significant";

s. denotes "significant at the 5 % probability level";

h.s. denotes "significant at the 1 % probability level".

In addition to the above the standard error of the individual means and the coefficients of variation (C.V.) are tabled.

The data for the 2,4-dinitrophenylhydrazones of methanal, propenal and penta-2,4-dienal which are anomalous, and that for the $C_{9,10}$ *n*-alka-2,6-dienals and $C_{6,7,10}$ *n*-alk-3-en-2-ones are found in Table V.

COMPARISON OF THE FOUR SYSTEMS

The value of the procedures depends on the consistency of the λ_{max} values obtained within each class of 2,4-dinitrophenylhydrazones, on the extent of differentiation between the classes, and on reasonable stability with time.

TABLE I

LIGHT ABSORPTION DATA – VASELINE SYSTEM OF GADDIS AND ELLIS

Compound as 2,4-dinitrophenylhydrazone	*Time (days)*	*Trend with carbon No.*	*Mean*		*S.D.*	*S.E. of mean*	*C.V. (%)*
Alkan-2-ones	1	Negative	362.2	n.s.	1.86	0.51	0.5
	7	Negative	362.0		2.15	0.66	0.6
Alka-2,4-dienals	1	Nil	397.4	h.s.	2.81	0.60	0.7
	7	Nil	394.0		2.22	0.51	0.6
Alkanals	1	Negative	361.1	n.s.	2.39	0.71	0.7
	7	Negative	361.3		1.88	0.55	0.5
Alk-2-enals	1	Negative	380.8	n.s.	2.21	0.58	0.6
	7	Negative	379.0		2.20	0.73	0.6
Alk-1-en-3-ones	1	Negative	373.6	n.s.	3.08	1.18	0.8
	7	Negative	372.8		2.90	1.17	0.8

Vaseline system of GADDIS AND ELLIS (*Table I*)

The data for this system give consistent results with a C.V. of the order of 0.6 %. Apart from the 2,4-dienals, there is a significant trend with carbon number which is

* The lower carbon number alkanal, alk-2-enal, alka-2,4-dienal and alkan-2-one hydrazones could not be studied by the methods of KLEIN AND DE JONG, and GADDIS AND ELLIS (see ref. 9).

of the same order at both times of measurement. In addition, there is no appreciable change in the means from 1 to 7 days.

The 2,4-dienals depart from the above pattern in all but C.V.; there is little variability between carbon numbers and relatively high variability between replications. GADDIS AND ELLIS[6] suggested that such variability might be due to the greater instability of the 2,4-dienal 2,4-dinitrophenylhydrazones.

With this system it would be difficult to distinguish the *n*-alkan-2-one from the *n*-alkanal 2,4-dinitrophenylhydrazones but the other three classes could be distinguished from them and from one another.

Paraffin oil system of KLEIN AND DE JONG (*Table II*)

The data are less consistent than with the vaseline system. The variability is acceptably uniform and corresponds to a C.V. of the order of 0.5 %. However, only 3 of the 10 series of observations show a significant trend with carbon number. (This trend also occurred with the *n*-alk-3-en-2-ones). This trend occurred only on measurements performed at one day and could relate to differential effects along the paper arising from the slower drying of the chromatogram developed with dioxane–water (4:1). However, it was not observed in the vaseline system which also uses an aqueous developing solvent, methanol–water (9:1).

TABLE II

LIGHT ABSORPTION DATA – PARAFFIN OIL SYSTEM OF KLEIN AND DE JONG

Compound as 2,4-dinitrophenylhydrazone	*Time (days)*	*Trend with carbon No.*	*Mean*		*S.D.*	*S.E. of mean*	*C.V. (%)*
Alkan-2-ones	1	Negative	356.7	h.s.	2.59	0.79	0.7
	7	Nil	361.8		1.20	0.40	0.3
Alka-2,4-dienals	1	Nil	388.8	h.s.	3.20	0.73	0.8
	7	Nil	385.6		1.40	0.53	0.4
Alkanals	1	Negative	355.5	h.s.	1.81	0.59	0.5
	7	Nil	360.2		0.83	0.28	0.2
Alk-2-enals	1	Nil	374.1	h.s.	1.87	0.52	0.5
	7	Nil	377.3		0.82	0.33	0.2
Alk-1-en-3-ones	1	Negative	367.6	n.s.	3.34	1.40	0.9
	7	Nil	371.8		2.59	1.16	0.7

In three of the classes there was a highly significant increase in λ_{max} from the first to the seventh day, but the dienals showed a decrease and the *n*-alk-1-en-3-ones did not change.

It would appear that this method is likely to be less reliable than the vaseline system.

Our results at 7 days for *n*-alkanals (360 mμ), *n*-alkan-2-ones (362 mμ) and *n*-alk-2-enals (377 mμ) agree with the figures of KLEIN AND DE JONG for *n*-nonanal (358 mμ), *n*-undecan-2-one (362 mμ) and *n*-non-2-enal (376 mμ).

The same differentiation between classes observed with the vaseline system is also obtained here; this is slightly affected by the greater change of λ_{max} with time.

Phenoxyethanol system of LYNN *et al.* (*Table III*)

This method gives reasonably consistent results. The variability is fairly uniform and corresponds to a C.V. of the order of 0.4%. In 2 of the 10 sets of data there is a significant trend with carbon number. In all cases there is a significant trend with time, the values of λ_{max} being lower at 7 days than at 1 day. The data agree well with that of NONAKA *et al.*[4].

This was the only system which permitted differentiation of the 5 classes.

TABLE III

LIGHT ABSORPTION DATA – PHENOXYETHANOL SYSTEM OF LYNN *et al.*

Compound as 2,4-dinitrophenylhydrazone	*Time (days)*	*Trend with carbon No.*	*Mean*		*S.D.*	*S.E. of mean*	*C.V. (%)*
Alkan-2-ones	1	Nil	374.6	s.	1.09	0.21	0.3
	7	Negative	373.1		1.74	0.44	0.5
Alka-2,4-dienals	1	Nil	405.0	h.s.	2.49	0.48	0.6
	7	Nil	402.2		0.46	0.16	0.1
Alkanals	1	Nil	370.5	h.s.	1.34	0.29	0.4
	7	Negative	368.2		1.66	0.42	0.4
Alk-2-enals	1	Nil	388.9	h.s.	0.79	0.19	0.2
	7	Nil	387.3		0.65	0.17	0.2
Alk-1-en-3-ones	1	Nil	383.8	s.	1.61	0.45	0.4
	7	Nil	382.4		1.59	0.54	0.4

Acetylated paper system of FORSS AND RAMSHAW (*Table IV*)

This method gave the most consistent results. The variability is quite consistent with a C.V. of the order of 0.4%. In no case is there any trend with carbon number and in no case is there any significant difference between the readings at 1 day and 7 days.

TABLE IV

LIGHT ABSORPTION DATA – ACETYLATED PAPER SYSTEM OF FORSS AND RAMSHAW

Compound as 2,4-dinitrophenylhydrazone	*Time (days)*	*Trend with carbon No.*	*Mean*		*S.D.*	*S.E. of mean*	*C.V. (%)*
Alkan-2-ones	1	Nil	367.1	n.s.	1.25	0.29	0.3
	7	Nil	366.9		0.76	0.22	0.2
Alka-2,4-dienals	1	Nil	393.0	n.s.	1.47	0.38	0.4
	7	Nil	392.8		1.48	0.36	0.4
Alkanals	1	Nil	362.2	n.s.	1.30	0.28	0.4
	7	Nil	362.8		1.33	0.31	0.4
Alk-2-enals	1	Nil	377.7	n.s.	1.35	0.32	0.4
	7	Nil	377.9		1.03	0.26	0.3
Alk-1-en-3-ones	1	Nil	375.4	n.s.	1.95	0.47	0.5
	7	Nil	375.8		2.28	0.76	0.6

TABLE V

LIGHT ABSORPTION DATA (MEAN AND STANDARD DEVIATION IN mμ) OF MISCELLANEOUS COMPOUNDS

Compound as 2,4-dinitrophenylhydrazone	*Vaseline system of Gaddis and Ellis*		*Paraffin oil system of Klein and De Jong*		*Phenoxyethanol system of Lynn et al.*		*Acetylated paper system of Forss and Ramshaw*	
	1 day	*7 days*	*1 day*	*7 days*	*1 day*	*7 days*	*1 day*	*7 days*
Methanal					361.1, 0.64	362.6, 1.06	358.0, 1.73	357.8, 1.92
Propenal					379.2, 1.17	381.1, —*	370.8, 2.05	372.1, 1.27
Penta-2,4-dienal					397.2, 1.09	396.3, 0.75	386.7, 1.94	387.9, 1.45
Nona-2,6-dienal	381.2, 3.19	379.7, 2.81	376.5, 0.84	376.7, 1.21	387.0, —*	385.0, —*	376.8, 0.71	376.8, 1.17
Deca-2,6-dienal	380.5, —*	379.7, 0.58	376.2, 0.45	376.6, 0.89	387.0, —*	385.7, —*	377.4, 0.55	377.6, 0.55
Hex-3-en-2-one	386.3, 3.06	385.0, 1.00	378.2, 2.05	378.7, 1.03	390.2, 0.96	389.7, 1.15	379.0, 0.71	379.5, 1.00
Hept-3-en-2-one	383.7, 3.79	381.0, 1.00	375.8, 2.49	377.0, —*	390.5, 0.57	390.0, 1.73	379.8, 0.84	380.5, 0.57
Dec-3-en-2-one	377.0, 2.10	376.5, 1.38	372.2, 1.86	375.6, 1.14	390.7, 1.03	389.5, 1.29	379.4, 0.97	379.4, 0.55

* With the available data no adequate estimate of standard deviation could be made.

The *n*-alkanals, *n*-alkan-2-ones and *n*-alka-2,4-dienals could be readily distinguished from each other and from the *n*-alk-2-enals and *n*-alk-1-en-3-ones which themselves could not be distinguished.

Miscellaneous compounds on the four systems (Table V)

The propenal and penta-2,4-dienal derivatives could only be studied in the two "normal systems" using phenoxyethanol and acetylated paper. With both systems propenal would be difficult to distinguish from the *n*-alk-1-en-3-ones (vinyl ketones) but penta-2,4-dienal would not be confused with any of the other hydrazones. However, in all four systems, it would not be possible to distinguish by light absorption the *n*-alk-2-enals, *n*-alka-2,6-dienals and *n*-alk-3-en-2-ones.

The λ_{max} of compounds listed in Table V were unaffected by storage for 7 days except that the *n*-alk-3-en-2-ones on the paraffin oil system showed a decrease of λ_{max} with increase in carbon number at one day, but this difference became less on storage.

GENERAL DISCUSSION

It is difficult to explain the decrease in λ_{max} with increase in carbon number which occurred in the three systems using paper impregnated with an involatile organic material. With the "normal phase" phenoxyethanol system it occurred only with the *n*-alkanals and *n*-alkanones (at 7 days) which may be related to the relative instability on some chromatographic systems of the saturated 2,4-dinitrophenylhydrazones[10]. With the two "reversed phase" systems using vaseline and paraffin oil the movement of compounds is inversely related to the carbon number and the phenomenon might be due to the developing solvents drying the non-polar stationary phase near the solvent front.

The speed and sensitivity of the direct spectrophotometric examination of 2,4-dinitrophenylhydrazones make this method very attractive. The variability is probably greater than that encountered when 2,4-dinitrophenylhydrazones are dissolved in solvents such as ethanol or chloroform but would be of the same order as when 2,4-dinitrophenylhydrazones are extracted from paper chromatograms. The peaks obtained with 2,4-dinitrophenylhydrazones are flattish and the measurement of λ_{max} depends more than usual on the subjective judgement of the person making the measurements.

ACKNOWLEDGEMENTS

We wish to thank Dr. ADAM GADDIS, U.S. Department of Agriculture, Beltsville, Md. and Mr. ELDON PIPPEN, U.S. Department of Agriculture, Albany, Calif. for their critical comments on this paper.

SUMMARY

The light absorption maxima of the 2,4-dinitrophenylhydrazones of seven classes of aliphatic carbonyl compounds were measured by transmission on spots obtained from four paper chromatographic systems. Differentiation between classes by these measurements is discussed.

REFERENCES

[1] W. Stark and D. A. Forss, *J. Dairy Res.*, 29 (1962) 173.
[2] D. A. Forss, E. H. Ramshaw and W. Stark, *J. Am. Oil Chemists' Soc.*, 39 (1962) 308.
[3] A. Crossley, T. D. Heyes and B. J. F. Hudson, *J. Am. Oil Chemists' Soc.*, 39 (1962) 9.
[4] M. Nonaka, E. L. Pippen and G. F. Bailey, *Anal. Chem.*, 31 (1959) 875.
[5] F. Klein and K. de Jong, *Rec. Trav. Chim.*, 75 (1956) 1285.
[6] A. M. Gaddis and R. Ellis, *Anal. Chem.*, 31 (1959) 870.
[7] W. S. Lynn, Jr., L. A. Steele and E. Staple, *Anal. Chem.*, 28 (1956) 132.
[8] D. A. Forss and E. H. Ramshaw, *J. Chromatog.*, 10 (1963) 268.
[9] D. A. Forss and W. Stark, *Anal. Chem.*, 36 (1964) 941.
[10] D. A. Forss and E. A. Dunstone, *Australian J. Chem.*, 10 (1957) 506.

THIN-LAYER CHROMATOGRAPHY OF ORGANOPHOSPHORUS PESTICIDES AND ACIDS ON MICROCHROMATOPLATES*

C. W. STANLEY
Midwest Research Institute, Kansas City, Mo. (U.S.A.)

(Received March 31st, 1964)

INTRODUCTION

While thin-layer chromatography (TLC) has been applied to a wide variety of separations in the past few years, it has been applied relatively little to organophosphorus pesticides or to organophosphorus acids. WALKER AND BEROZA[1] have summarized the literature on pesticides and have presented data on 62 chlorinated or organophosphorus pesticides in three solvents (chloroform, benzene, and hexane) and in mixed solvent systems consisting of each of these solvents with ethyl ether, ethyl acetate, acetone, methanol, and acetic acid. KOVACS[2] extended WALKER AND BEROZA's studies and obtained better resolution of chlorinated pesticides and BLINN[3] has applied TLC to the determination of the pesticide phorate residue. All of these studies have been conducted with standard glass plates, 20 cm in length.

The substitution of microscope slides for the standard plates provides an economical, rapid TLC method. These microchromatoplates may be prepared by dipping the plates into a suspension of the solid phase[4], by spraying[5,6], by spreading with a commercially available applicator[7], or by spreading with an easily constructed applicator[8]. The microchromatoplates require less preparation time than standard plates since no preliminary cleaning of the plate is required and the slides may be discarded after use. These microchromatoplates require less solvent and a shorter time for development than the standard plates. Therefore, these plates are ideal for the screening of solvents to establish the requisite conditions for any desired separation. The R_F values obtained on these microchromatoplates differ somewhat from those obtained on the standard plates but resolution of two components can be demonstrated.

EXPERIMENTAL

Apparatus

Standard TLC plates were prepared with the Desaga/Brinkmann Model S-II Adjustable Applicator**. Desaga developing tanks** were used for these standard plates.

The microchromatoplates were prepared with essentially the same apparatus as that described by WASICKY[8] and shown in Fig. 1. The applicator is made of brass, with dimensions as given by WASICKY. The base plate, made of brass bar stock, is 92 cm long and 3.5 cm wide. Along the back edge and the left end, there is a 0.9 cm

---

* Presented in part at the 15th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 1964.

** Brinkmann Instruments, Inc., Westbury, N.Y.

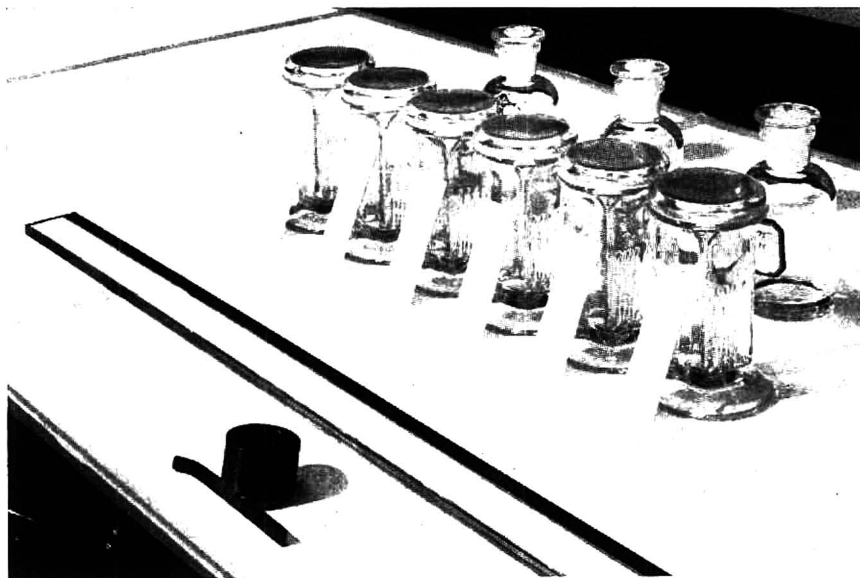


Fig. 1. Applicator and developing jars for microchromatoplates.

wide retaining ledge, raised 1 mm above the base. As this ledge is thinner than the 1.2 mm microscope slides, the applicator rides on the surface of the slides during the slurry spreading. Standard microscope slides*, 25.4 mm × 76.2 mm, were used without preliminary cleaning. Coplin staining jars** were used as developing tanks (see Fig. 1).

Chemicals

The solid phases, Silica Gel G, Silica Gel H, Aluminum Oxide G, and Kieselguhr G (Brinkmann Instruments, Inc.) were used as solid phases. The developing solvents were of reagent grade and were used without further purification. The pesticides were obtained as analytical standards through the courtesy of various producers and were used as received. Organophosphorus acids were either purchased from chemical supply houses or were furnished by Victor Chemical Division of Stauffer Chemical Company, Chicago, Ill. A stock solution of each compound was prepared at a concentration of 1 mg/ml by dissolving a weighed quantity in a solvent. Benzene was used as the solvent for all compounds except for some acids, for which a mixed benzene-acetone solvent (4:1) was used.

Preparation of plates

The standard plates were prepared with a nominal thickness of 0.25 mm according to instructions supplied with the Desaga Applicator.

In the preparation of the microchromatoplates, 12 microscope slides were placed end to end against the retaining ledge on the applicator base plate. For Silica Gel G,

* Catalog No. M6125, Protecta-Slide, S/P. Scientific Products, Division of American Hospital Supply Corporation, Evanston, Ill.
** Catalog No. S7660, Scientific Products.

Kieselguhr G, and Aluminum Oxide G, 6 g of powder were weighed into a 125 ml, glass-stoppered, Erlenmeyer flask and 12 ml of distilled water were added. The flask was shaken vigorously for 30 sec, and the slurry was poured into the applicator which was resting at the right-hand end of the row of slides. As soon as the slurry began to run out of the slot at the back of the applicator, the applicator was pushed as smoothly as possible along the row of slides to spread the layer of solid phase onto the slides. After the plates had set for a few minutes, the slides were removed from the base plate and were dried at 110° in an oven. Silica Gel H was spread in the same fashion except that 6 g of powder were mixed with 14.5 ml of water. The plates coated with Silica Gel H were allowed to air dry for at least 4 h after which they were dried in the oven. The ratio of solid phase to water was fairly critical with all the solid phases which were used and particularly with Silica Gel H. The usual precautions with the "G" phases, which contain a calcium sulfate binder, were followed. Since the water content of the solid phase might influence R_F values with nonaqueous solvents, plates were used as soon as possible after being removed from the drying oven. With aqueous developing solvents the water content of the solid phase was not critical.

Development of chromatograms

Two samples were spotted with either a 10 μl or a 50 μl Microliter Syringe* on a microchromatoplate at 1 cm from one end of the plate. The pesticides were usually 10 μl in volume; the acids were 10, 20, or 50 μl in volume. A mark was placed 5 cm above the point of sample application to note the end of solvent travel. The end of the microchromatoplate on which the sample had been spotted was immersed to a depth of 0.5 cm in 3 ml of solvent in a Coplin jar, and the chromatogram was permitted to develop at room temperature (about 24°). The time required for development varied with the volatility of the solvent; for example, benzene required 9 min for a solvent travel of 5 cm.

Chromogenic reagents

The chromogenic reagents which were sprayed onto the plates for sample detection were:

Iodine. Dissolve 0.5 g of iodine in 100 ml of ethanol[9]. This chromogenic reagent gives spots with many organophosphorus compounds. It is also always used before Fluorescein or 4-MUB.

FCSSA. (a) Dissolve 0.1 g of ferric chloride hexahydrate in 1 ml of 1 N hydrochloric acid and dilute the solution to 100 ml with 80 % ethanol; this solution is stable for about 2 weeks. (b) Dissolve 1 g of sulfosalicylic acid in 100 ml of 80 % ethanol[10]. Spray first with (a), then with (b). This chromogenic reagent or the Hanes reagent was used for detection of alkylphosphoric acids.

IOP. Dissolve 1 g of platinic chloride in 10 ml or water and mix this solution with 10 g potassium iodide dissolved in 250 ml of water. To prepare the spray solution, dilute one volume of this stock solution with six volumes of water[10].

$AgNO_3$. Dissolve 1.7 g of silver nitrate in 5 ml of water, add 10 ml of 2-phenoxyethanol, and dilute the solution to 200 ml with acetone[1]. Spray the plate with the

* Hamilton Company, Whittier, Calif.

$AgNO_3$ spray reagent and then expose the plate to 3660 Å ultraviolet light for about 10 min.

Fluorescein. Prepare a 0.01 *M* solution in 0.01 *N* sodium hydroxide. Dilute this stock solution 3 to 100 with ethanol for spraying[11]. The plate is sprayed first with Iodine spray reagent, any visible spots are recorded, and then the plate is resprayed with Fluorescein. Some compounds will produce spots visible under room light; others will produce spots visible under either 2547 Å or 3660 Å ultraviolet light.

4-MUB. Dissolve 0.075 g of 4-methylumbelliferone in 100 ml of ethanol–water (1:1) and add 10 ml of 0.1 *N* ammonium hydroxide[12]. The plate is first sprayed with Iodine spray reagent, any visible spots are recorded, and then the plate is resprayed with 4-MUB. The plate is observed under either 2547 Å or 3660 Å ultraviolet light.

Hanes reagent. Dissolve 0.5 g of ammonium molybdate tetrahydrate in 5 ml of water and add 1.5 ml of hydrochloric acid and 2.5 ml of perchloric acid. Cool the solution and dilute it to 50 ml with acetone[13]. This solution gives better results if aged for a day and is stable for about three weeks. After the reagent is sprayed on the plate, expose the plate first to an infrared heat lamp at a distance of 30 cm for 2 min, then to a 3660 Å ultraviolet light for 7 min.

These spray reagents were adapted from the many sprays used in paper chromatography. Their lower limit of detection was not determined; most will detect at least 1 μg of the compound spotted on a TLC plate. About 0.5 ml of each solution will cover a single plate when a 10 ml tube sprayer* is used with compressed air. Some sprays were applied consecutively; for example, a plate might be sprayed with Iodine, then with either Fluorescein or 4-MUB, and finally with $AgNO_3$.

When volatile organic solvents were used as developers, a suitable chromogenic spray could readily be found. However, when acidic or basic aqueous solvents were used as developers, some reagents would give a good color test when the sample was spotted directly on a TLC plate; after the sample had been developed with the solvent, there was no color produced upon spraying. The alkylphosphoric acids gave the most trouble in this respect.

DISCUSSION AND RESULTS

Pesticides

Thirty-one organophosphorus pesticides (given with chemical names in Table I) were studied on Silica Gel G in an eluotropic series of six solvents. The results are summarized in Table II. If streaking occurred, the R_F values are the leading and trailing edges of the spot. When a single R_F value is given, the width of the spot is about $\pm$0.03. Many of these pesticides contain isomers, manufacturing impurities, or decomposition products; these pesticides give multiple spots with one or more developing solvents. The nonpolar solvent, cyclohexane, moved appreciably only Merphos, which is a phosphite. Although no solvent separated all pesticides, benzene produced the best general separation.

Four pesticides and two acids were studied on 20 cm plates, with a solvent travel of 15 cm. The R_F values obtained on microchromatoplates for a solvent travel of 5 cm and on standard plates for a solvent travel of 15 cm are given in Table III. The results show that R_F values are lower for the longer solvent travel. R_F values

* Catalog No. 2C-50, Microchemical Specialties Co., Berkeley, Calif.

TABLE I

CHEMICAL NAMES OF ORGANOPHOSPHORUS PESTICIDES

Pesticide	*Name*
Betasan[a]	N-[2-(O,O-Diisopropylthiophosphoryl)ethyl]benzenesulfonamide
Ciodrin[b]	O,O-Dimethyl O-[1-methyl-2-(1-phenylcarbethoxy)vinyl] phosphate
DDVP[c]	O,O-Dimethyl O-2,2-dichlorovinyl phosphate
DEF[c]	S,S,S-Tributyl phosphorotrithioate
Delnav[d]	2,3-*p*-Dioxane S,S-bis(O,O-diethyl phosphorodithioate)
Demeton[c]	O,O-Diethyl O(or S)-2-(ethylthio)ethyl phosphorothioate
Diazinon[e]	O,O-Diethyl O-(2-isopropyl 6-methyl-4-pyrimidyl) phosphorothioate
Dicapthon[f]	O,O-Dimethyl O-(2-chloro-4-nitrophenyl) phosphorothioate
Dimethoate[f]	O,O-Dimethyl S-(N-methylcarbamoyl)methyl phosphorodithioate
Di-Syston[c]	O,O-Diethyl S-2-(ethylthio)ethyl phosphorodithioate
EPN[g]	O-Ethyl O-*p*-nitrophenyl phenylphosphonothioate
Malathion[f,h]	O,O-Dimethyl S-[1,2-bis(ethoxycarbonyl)ethyl] phosphorodithioate
Merphos[i]	S,S,S-Tributyl phosphorotrithioite
Methyl Demeton[c]	O,O-Dimethyl O(or S)-(2-ethylthio)ethyl phosphorothioate
Methyl Parathion[f,h]	O,O-Dimethyl O-*p*-nitrophenyl phosphorothioate
Methyl Trithion[a]	O,O-Dimethyl S-(*p*-chlorophenylthiomethyl) phosphorodithioate
Naled[j]	O,O-Dimethyl 1,2-dibromo-2,2-dichloroethyl phosphate
OMPA[k]	Octamethylpyrophosphoramide; Bis-N,N,N′, N′-tetramethyl-phosphorodiamidic anhydride
Para-Oxon[f]	O,O-Diethyl O-*p*-nitrophenyl phosphate
Parathion[l]	O,O-Diethyl O-*p*-nitrophenyl phosphorothioate
Phorate[f]	O,O-Diethyl S-ethylthiomethyl phosphorodithioate
Phosdrin[b]	O,O-Dimethyl O-(2-carbomethoxy-1-methylvinyl) phosphate
Phosphamidon[j]	O,O-Dimethyl O-[2-chloro-2-(N,N-diethylcarbamoyl)-1-methylvinyl] phosphate
Phosfon[i]	Tributyl 2,4-dichlorobenzylphosphonium chloride
Ronnel[m]	O,O-Dimethyl O-(2,4,5-trichlorophenyl) phosphorothioate
Ruelene[m]	O-(4-*tert.*-Butyl-2-chlorophenyl) O-methyl N-methyl phosphoramidate
TEPP[l]	Tetraethyl pyrophosphate; Bis-O,O-diethyl phosphoric anhydride
Trichlorfon[c]	O,O-Dimethyl (1-hydroxy-2,2,2-trichloroethyl) phosphonate
Trithion[a]	O,O-Diethyl S-(*p*-chlorophenylthiomethyl) phosphorodithioate
VC-13[i]	O,O-Diethyl O-(2,4-dichlorophenyl) phosphorothioate
Zinophos[f]	O,O-Diethyl O-(2-pyrazinyl) phosphorothioate

Obtained from:
[a] Stauffer Chemical Co., Richmond, Calif.
[b] Shell Chemical Co., New York, N.Y.
[c] Chemagro Corp., Kansas City, Mo.
[d] Hercules Powder Co., Wilmington, Del.
[e] Geigy Chemical Corp., Yonkers, N.Y.
[f] American Cyanamid Co., Princeton, N.J.
[g] E. I. du Pont de Nemours & Co., Wilmington, Del.
[h] Thompson-Hayward Chemical Co., Kansas City, Mo.
[i] Virginia-Carolina Chemical Co., Richmond, Va.
[j] California Chemical Co., Richmond, Calif.
[k] Pennsalt Chemicals Corp., Tacoma, Wash.
[l] American Potash & Chemical Corp., Los Angeles, Calif.
[m] Dow Chemical Co., Midland, Mich.

reported by WALKER AND BEROZA[1] for the four pesticides with a solvent travel of about 10 cm are shown for comparison. Their R_F values are intermediate between the 5 cm and the 15 cm solvent travel for most of the pesticides. PEIFER[4] stated that the polarity of the developing solvent necessary to obtain the same R_F value is partially dependent upon the distance traveled by the solvent front but he presented no comparative data.

TABLE II

R_F VALUES FOR PESTICIDES ON MICROCHROMATOPLATES

Solvent travel: 5 cm. Solid phase: Silica Gel G.

Pesticide	*Solvent*						*Chromogenic reagent*
	Cyclohexane	*Benzene*	*Acetone*	*Ethyl acetate*	*Isopropanol*	*Methanol*	
Betasan[a] (Disan[a])	0	0.03	1.0	1.0	0.95	1.0	IOP
Ciodrin[a]	0	0 0.05	0.96	0.66 0.75	0.77	0.94	Iodine
DDVP (Dichlorovos)	0	0	0 0.93	0 0.73	0 0.75	0 0.82	$AgNO_3$
DEF[a]	0 0–0.12[b]	0 0.04 1.0	0 1.0	0 1.0	0.93	1.0	IOP
Delnav[a]	0	0 0.33	0 1.0	0 1.0	0 0.88	0.96	Iodine
Demeton (Systox)	0	0 0.54	0 0.95 1.0	0 0.77 0.98	0.85 0.90	0.87	Iodine
Diazinon[a]	0	0.08	1.0	1.0	0.83	0.92	$AgNO_3$
Dicapthon	0	0.64	1.0	1.0	0.89	0.95	$AgNO_3$
Demethoate (Cygon[a])	0	0	0.88	0.39	0.77	0.92	Iodine
Di-Syston[a]	0	0.68	1.0	1.0	0.92	1.0	Iodine
EPN	0	0.64–0.74[b]	1.0	1.0	0.87	0.86–1.0[b]	$AgNO_3$
Malathion	0	0 0.07	0 1.0	0 0.94	0 0.88	0.86 0.94	Iodine
Merphos[a]	0 0.06–0.16[b] 0.22–0.34[b]	0 0.09 1.0	1.0	1.0	0.92	1.0	IOP
Methyl Demeton (Meta-Systox[a])	0	0	0 0.93	0 0.66	0.04 0.74–0.86[b]	0.91	Iodine
Methyl Parathion	0	0.55	1.0	1.0	0.89	0.96	Iodine
Methyl Trithion[a]	0	0.68–0.78[b]	1.0	1.0	0.84–0.94[b]	1.0	IOP
Naled (Dibrom[a])	0	0 0.08	0 0.96	0 0.85	0 0.80	0 0.90	$AgNO_3$
OMPA (Schradan)	0	0	0.04	0.01	0.19–0.36[b]	0.76	Iodine
Para-Oxon	0	0	0.94	0.77	0.79	0.96	Iodine
Parathion	0	0.60	0.98	0.98	0.87	0.89	Iodine
Phorate (Thimet[a])	0	0 0.68	0 1.0	0 1.0	0.89	1.0	Iodine
Phosdrin[a]	0	0	0.88	0.45 0.59 0.82	0.68	0.85	$AgNO_3$
Phosphamidon	0	0	0 0.82	0 0.26	0.02 0.60	0.09 0.85	Iodine
Phosfon[a]	0	0	0	0	0–0.38[b]	0.18–0.84[b] 0.87	Iodine
Ronnel	0	0.86	1.0	0.98	0.83	0.96	Hanes reagent
Ruelene[a]	0	0	0.86	0.43	0.79	0.93	Iodine
TEPP, 40% (Tetron-100[a])	0	0	0 0.81	0 0.35	0.07 0.84	0.92	Hanes reagent
Trichlorfon (Dipterex[a])	0	0	0.88 0.98	1.0	0.82 0.97	0.88 0.98	$AgNO_3$
Trithion[a]	0	0.76–0.86[b]	1.0	1.0	0.92	1.0	IOP
VC-13[a]	0	0.86	1.0	1.0	0.90	1.0	Hanes reagent
Zinophos	0	0–0.14	0.96	0.90	0.71–0.83[b]	0.80–0.96[b]	IOP

[a] Trade mark.
[b] Streaking.

TABLE III

R_F VALUE AS A FUNCTION OF LENGTH OF SOLVENT TRAVEL

Solid phase: Silica Gel G.
Solvent system: a = Benzene
b = *n*-Propanol–*n*-butanol–2 *N* ammonium hydroxide (50:20:30, v/v).
c = *n*-Butanol–acetic acid–water (60:20:20, v/v).

Compound	*Solvent travel*			*Solvent system*
	5 cm	*15 cm*	*10 cm*[1]	
Demeton	0	0	0	a
	0.54	0.39	0.43	
Malathion	0	0	0.08	a
	0.07	0.05		
Methyl Parathion	0.55	0.37	0.41	a
Parathion	0.60	0.52	0.47	a
Chloromethylphosphonic acid	0.06–0.15	0.12		b
Chloromethylphosphonic acid	0.30–0.42	0.29		c
Phenylphosphonic acid	0	0		b
Phenylphosphonic acid	0.44–0.58	0.39		c

The repeatability of R_F values for a solvent travel of 5 cm is shown in Table IV. Excellent agreement was obtained between two runs for both demeton and para-oxon. The samples in Run 2 had been sprayed with the Iodine spray reagent before the chromatogram was developed. This may account for the absence of the component at $R_F = 0$ for demeton. The component at this R_F in Run 1 gave a much fainter spot than the other two components and is probably the sulfoxide.

TABLE IV

REPEATABILITY OF R_F VALUES

Solvent travel: 5 cm. Solid phase: Silica Gel G.

Pesticide	*Solvent*	*R_F values*	
		Run 1	*Run 2*
Demeton	Acetone	0	—
		0.95	0.93
		1.0	1.0
Para-Oxon	Ethyl acetate	0.77	0.75

Organophosphorus acids

The organophosphorus acids require more polar solvents than the neutral esters such as the pesticides. The solvent systems selected for trial were based on those systems used in paper chromatography of phospholipids[13], amino acids[14], and organophosphorus acids[15,16]. Preliminary results on the TLC for some alkylphosphoric and organophosphonic acids are summarized in Table V. These solvent systems are not satisfactory since considerable streaking was evident.

Table III shows that the R_F values for standard plates tend to be somewhat lower than R_F values with the microchromatoplates for the acids as well as for the neutral esters.

TABLE V

R_F VALUES FOR ORGANOPHOSPHORUS ACIDS

Solvent travel: 5 cm. Solid phase: Silica Gel G, except alkylphosphoric acids in solvent 4 are on Silica Gel H.
Solvent systems: 1 = 2-Propanol–water–conc. ammonium hydroxide (75:24:1, v/v),
2 = *n*-Propanol–*n*-butanol–2 *N* ammonium hydroxide (50:20:30, v/v),
3 = 2-Propanol–conc. ammonium hydroxide (75:25, v/v),
4 = *n*-Butanol–formic acid–water (60:20:20, v/v),
5 = *n*-Butanol–acetic acid–water (60:20:20, v/v),
6 = *n*-Butanol–acetic acid–water (80:20:100, v/v); use the top phase.

Acid	*Solvent system*					
	1	*2*	*3*	*4*	*5*	*6*
Monomethylphosphoric	0	0.04	0–0.12[a]	0		
Dimethylphosphoric	0.45	0.39	0.37	0.68–0.78[a]		
Monoethylphosphoric	0	0.09	0.03	0	0.28–0.46[a]	
Diethylphosphoric	0.60	0.50	0.50	0.74–0.90[a]	0.28–0.46[a]	
Monoisopropylphosphoric	0.08	0–0.18[a]	0	0		
Diisopropylphosphoric	0.74	0.64	0.57	0.74–0.92[a]		
Monobutylphosphoric	0–0.14[a]	0–0.26[a]	0.03	0		
Dibutylphosphoric	0.73	0.61	0.59	0.74–0.94[a]		
Chloromethylphosphonic	0–0.16[a]	0.06–0.15[a]	0	0.54	0.30–0.42[a]	0.10–0.24[a]
Phenylphosphonic	0–0.20[a]	0	0	0.24–0.61[a]	0.44–0.58[a]	0.24–0.46[a]

[a] Streaking

Table VI shows that the solid phase had little effect on the R_F value for the organophosphorus acids when Silica Gel H and Aluminum Oxide G were substituted for Silica Gel G. Preliminary work with Kieselguhr G had indicated little advantage for this solid phase over other solid phases.

Suitable chromogenic agents for organophosphorus acids have been difficult to find. Several agents gave good tests for samples spotted on TLC plates; however, after the plates had been developed, the agents produced poor spots or none. The reasons for this behavior have not been elucidated; the residual solvent may interfere or the free acid may have been converted to a salt. This behavior is still being studied.

TABLE VI

EFFECT OF SOLID PHASE ON ACID R_F VALUES

Solvent system: Isopropanol–conc. ammonium hydroxide (75:25, v/v).

Acid	*Solid phase*		
	Silica Gel G	*Silica Gel H*	*Aluminum Oxide G*
Monoisopropylphosphoric	0	0.03	0
Diisopropylphosphoric	0.57	0.59	0.57
Monobutylphosphoric	0.03	0.07	0
Dibutylphosphoric	0.64	0.65	0.51

SUMMARY

R_F values are given for 31 organophosphorus pesticides in six solvents on microchromatoplates. The use of microscope slides as supports for thin-layer chromatog-

raphy adsorbents is described and results obtained for four pesticides and four acids on the microchromatoplates are compared with those obtained with standard thin-layer chromatography plates. Preliminary results on the thin-layer chromatography of alkylphosphoric and organophosphonic acids are presented.

REFERENCES

1 K. C. WALKER AND M. BEROZA, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 250.
2 M. F. KOVACS, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 884.
3 R. C. BLINN, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 952.
4 J. J. PEIFER, *Mikrochim. Acta*, 3 (1962) 529.
5 I. BEKERSKY, *Anal. Chem.*, 35 (1963) 261.
6 K. MORITA AND F. HARUTA, *J. Chromatog.*, 12 (1963) 412.
7 A. F. HOFFMAN, *Anal. Biochem.*, 3 (1962) 145.
8 R. WASICKY, *Anal. Chem.*, 34 (1962) 1346.
9 H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 38 (1961) 708.
10 H. F. MACRAE AND W. P. MCKINLEY, *J. Agr. Food Chem.*, 11 (1963) 174.
11 W. P. MCKINLEY AND J. H. MAHON, *J. Assoc. Offic. Agr. Chemists*, 42 (1959) 725.
12 N. FADERL, *Mitt. Gebiete Lebensm. Hyg.*, 53 (1962) 154.
13 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
14 R. S. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, New York, 1958.
15 H. A. MOULE AND S. GREENFIELD, *J. Chromatog.*, 11 (1963) 77.
16 F. W. PLAPP AND J. E. CASIDA, *Anal. Chem.*, 30 (1958) 1622.

IDENTIFICATION ET FRACTIONNEMENT DES PRINCIPAUX INSECTICIDES ORGANO-PHOSPHORÉS PAR CHROMATOGRAPHIE EN COUCHE MINCE

M. SALAMÉ
Laboratoire de Toxicologie, Faculté de Médecine, Université de Liège (Belgique)

(Reçu le 27. avril 1964)

Différents auteurs se sont préoccupés de séparer les insecticides organo-phosphorés par chromatographie. Les premiers utilisèrent la chromatographie sur colonne (LAWS ET WEBLEY[1]), d'autres, la chromatographie sur papier en une ou deux dimensions (MITCHELL[2], MCKINLEY ET READ[3], LE MOAN[4]). Plus récemment, BÄUMLER ET RIPPSTEIN[5] proposèrent la technique de chromatographie en couche mince pour la séparation de sept organo-phosphorés soufrés.

Dans cette publication, nous donnons les résultats d'une étude systématique des mobilités relatives d'une série d'insecticides organo-phosphorés traités en chromatographie en couche mince par différents éluants. Cette étude permet de définir un procédé d'identification efficace et rapide de chacun des insecticides retenus par l'utilisation de deux systèmes choisis.

CONDITIONS ET RÉSULTATS EXPÉRIMENTAUX

(1) *Insecticides*

Notre expérimentation a porté sur dix insecticides organo-phosphorés choisis parmi les plus utilisés, savoir: un phosphate (le D.D.V.P. ou O-2,2-dichlorovinyl O,O-diméthyl phosphate); quatre thiophosphates (le Parathion ou E 605 ou O,O-diéthyl O-*p*-nitrophényl thiophosphate, le Diazinon ou O,O-diéthyl O-(2-isopropyl-6-méthyl-4-pyrimidyl thiophosphate, le Demeton-Methyl ou E 154 ou O,O-diméthyl 2-(éthylthio)-éthyl thiophosphate, le Mercapto-Phos ou S 1752 ou O,O-diméthyl O-4-(méthylmercapto)-3-méthylphényl thiophosphate), cinq dithiophosphates (le Malathion ou O,O-diméthyl S-dicarboéthoxyéthyl dithiophosphate, le Rogor (Dimethoate) ou O,O-diméthyl S-carbamylméthyl dithiophosphate, le E 1582 ou Azinphos Méthyl ou O,O-diméthyl S-(4-oxobenzotriazino-3-méthyl) dithiophosphate, le E 1513 ou Azinphos Éthyl ou O,O-diéthyl S-(4-oxobenzotriazino-3-méthyl) dithiophosphate, le F.A.C. ou diéthyl-S-isopropyl-mercapto-méthyl dithiophosphate).

(2) *Technique utilisée*

La technique générale de préparation des plaques est celle employée par STAHL[6]. La polarité élevée des esters phosphoriques nécessite l'emploi d'un substrat lui-même assez polaire. Avec la cellulose, par exemple, tous les produits se retrouvent à front de solvant. Les meilleurs résultats sont obtenus avec le silicagel.

TABLEAU I

R_F DES INSECTICIDES ORGANO-PHOSPHORÉS DANS DIFFÉRENTS SYSTÈMES ÉLUANTS

Insecticide	*R_F dans système*															
	Ea_1	Ea_2	Ea_3	Ec_1	Ec_2	Ec_3	Eae_1	Eac_2	Ect_1	Eet_2	Em_1	Em_2	Em_3	Bc_4	Bae_3	Bae_4
Diazinon	0.55	0.75	0.98	0	0.19	0.66	0.77	0.86	0.37	0.60	0.43	0.66	0.70	0.25	0.36	0.60
E 605 Parathion	0.58	0.70	0.94	0.22	0.60	0.94	0.90	0.87	0.77	0.85	0.63	0.82	0.75	0.75	0.90	0.92
S 1752 Mercapto-Phos	0.55	0.66	0.93	0.35	0.66	0.90	0.85	0.91	0.66	0.85	0.76	0.85	0.80	0.90	0.88	0.90
Malathion	0.34	0.55	0.98	0	0.22	0.83	0.73	0.89	0.30	0.52	0.33	0.64	0.45	0.35	0.46	0.70
E 1513 Azinphos Ethyl	0.21	0.42	0.95	0	0.09	0.85	0.52	0.85	0.18	0.35	0.25	0.27	0.32	0.36	0.36	0.60
E 1582 Azinphos Methyl	0.18	0.37	0.92	0	0.15	0.75	0.43	0.80	0.15	0.30	0.16	0.25	0.25	0.30	0.30	0.50
F.A.C.	0.12	0.35	0.96	0	0.10	0.25	0.30	0.61	0.05	0.20	0.05	0.10	0.14	0.08	0.08	0.16
E 154 Demethon Methyl	0.12	0.32	0.91	0	0.09	0.24	0.26	0.45	0.06	0.20	0.10	0.14	0.15	0.10	0.07	0.15
D.D.V.P.	0.18	0.30	0.98	0	0.36	0.37	0.21	0.60	0.13	0.24	0.15	0.17	0.18	0.34	0.31	0.34
Rogor	0.03	0.15	0.75	0	0	0.05	0.01	0.12	0	0.09	0.02	0.03	0.10	0.02	0	0.05

Ea_1 = éther de pétrole–acétone (90:10).
Ea_2 = éther de pétrole–acétone (75:25).
Ea_3 = éther de pétrole–acétone (50:50).
Ec_1 = éther de pétrole–chloroforme (90:10).
Ec_2 = éther de pétrole–chloroforme (50:50).
Ec_3 = éther de pétrole–chloroforme (10:90).
Eae_1 = éther de pétrole–acétate d'éthyle (75:25).
Eae_2 = éther de pétrole–acétate d'éthyle (50:50).
Eet_1 = éther de pétrole–éthanol (97.5:2.5).
Eet_2 = éther de pétrole–éthanol (95:5).
Em_1 = éther de pétrole–méthanol (98:2).
Em_2 = éther de pétrole–méthanol (95:5).
Em_3 = éther de pétrole–méthanol (90:10).
Bc_4 = benzène–chloroforme (50:50).
Bae_3 = benzène–acétate d'éthyle (95:5).
Bae_4 = benzène–acétate d'éthyle (90:10).

Le pouvoir éluant étant soumis à plusieurs variables, la classification de JACQUES ET MATHIEU[7] a servi à l'établissement d'une série de systèmes d'élution. L'éther de pétrole retenu comme solvant le moins polaire, fut enrichi avec différents solvants de polarité croissante tels le chloroforme, l'acétone, l'éthanol, l'acétate d'éthyle et le méthanol. Une autre série fut obtenue en enrichissant dans les mêmes proportions, du benzène avec les mêmes solvants (Tableau I). En remplaçant l'éther de pétrole par l'hexane, on a obtenu des résultats sensiblement parallèles.

(3) *Techniques de révélation*

Les méthodes employées pour la révélation des organo-phosphorés en chromatographie sur papier, donnent également de bons résultats en couche mince, plusieurs procédés sont décrits par ZWEIG[8]. Les deux systèmes suivants nous paraissent les plus intéressants.

(i) La méthode de MACRAE ET MCKINLEY[9] décrite par WADE ET MORGAN[10]. Elle permet de révéler un grand nombre d'organo-phosphorés (phosphates et thiophosphates).

La plaque est soumise aux vapeurs de brome pendant une dizaine de minutes puis vaporisée par une solution à 0.1 % de $FeCl_3$ dans l'éthanol à 80 %, on laisse sécher 15 min. Les ions ferriques non complexés sont mis en évidence par une solution à 1 % d'acide sulfosalicylique dans l'éthanol à 80 %. On obtient des taches blanches sur un fond mauve. La sensibilité est de l'ordre de 5 μg.

(ii) Le chlorure de palladium à 0.5 % en solution chlorhydrique permet de révéler jusqu'à 2 μg d'un ester organo-phosphoré soufré, on observe des taches jaune foncé sur fond blanc. Il ne révèle le Parathion qu'à la concentration de 10 μg. Cependant, on peut arriver à révéler 2 μg de ce produit en traitant ensuite la plaque, par du KOH alcoolique à 15 %.

RÉSULTATS

De l'ensemble des systèmes d'éluants étudiés, 16 systèmes ont été retenus. Les mobilités relatives de dix esters phosphoriques choisis sont rassemblées dans le Tableau I. Aucun système ne permet de séparer valablement et en une seule opération, les dix produits étudiés.

La comparaison des différents R_F obtenus dans les différents systèmes montre toutefois, qu'il est possible au moyen de deux chromatographies successives, d'identifier de façon spécifique, un quelconque des produits étudiés. Un premier système, constitué par le mélange éther de pétrole–acétone (75:25) (Ea_2) permet de classer les esters en deux catégories: ceux à R_F supérieur à 0.55 et ceux inférieurs à 0.42 par ordre de R_F décroissant. Un second système choisi dans le Tableau II, en fonction des résultats obtenus lors de la première chromatographie, permet d'identifier à coup sûr, le ou les produits en cause.

On peut constater que le nombre d'atomes de soufre ou sa position dans la molécule n'ont aucune influence sur la mobilité de l'ester phosphorique. C'est la polarité de la molécule seule qui conditionne le R_F de chaque système solvant utilisé.

RÉSUMÉ

La mobilité d'une dizaine d'esters organo-phosphorés choisis parmi les plus utilisés a été étudiée en chromatographie en couche mince par différents éluants. Deux chro-

TABLEAU II

SYSTÈMES ÉLUANTS POUR L'IDENTIFICATION DES INSECTICIDES ORGANO-PHOSPHORÉS

Insecticide	*Système*															
	Ea_1	Ea_2	Ea_3	Ec_1	Ec_2	Ec_3	Eae_1	Eae_2	Eet_1	Eet_2	Em_1	Em_2	Em_3	Bc_4	Bae_3	Bae_4
Diazinon		R			X	T			X		X	R	X	X		
E 605 Parathion		R		X	X				X		X	R		X		
S 1752 Mercapto-Phos		R		X					X		X	R	X	X		
Malathion	T	R							X	X	X	R	X	X		
E 1513 Azinphos Ethyl		R					X	X		X		R				X
E 1582 Azinphos Methyl		R				X	X	X				R				X
F.A.C.		R				X		T				R				X
E 154 Demethon Methyl		R					X	X				R				
D.D.V.P.		R			T							R				
Rogor		R	T					X				R				

* T: Système permettant d'isoler le produit de tous les autres.
X: Système permettant d'isoler le produit d'un ou de plusieurs autres présentant des R_F voisins.
R: Système de référence.

matographies successives permettent de manière simple et rapide de détecter et d'identifier avec sensibilité et précision, l'un des pesticides étudiés.

SUMMARY

The mobilities of ten of the most common organo-phosphoric esters in thin-layer chromatography have been studied using different solvent systems. Two successive chromatograms allow a rapid and specific identification of each of the studied pesticides.

BIBLIOGRAPHIE

1 E. Q. LAWS ET D. J. WEBLEY, *Analyst*, 86 (1961) 249.
2 L. C. MITCHELL, *J. Assoc. Offic. Agr. Chemists*, 43 (1960) 810.
3 W. P. MCKINLEY ET S I. READ, *J. Assoc. Offic. Agr. Chemists*, 45 (1962) 467.
4 G. LE MOAN, *Ann. Fals. Expert. Chim.*, 55 (1962) 63.
5 J. BÄUMLER ET S. RIPPSTEIN, *Helv. Chim. Acta*, 44 (1961) 1162.
6 E. STAHL, *Chemiker-Ztg.*, 82 (1958) 323.
7 J. JACQUES ET J. P. MATHIEU, *Bull. Soc. Chim. France*, (1946) 94.
8 G. ZWEIG (Editeur), *Analytical Methods for Pesticides, Plant Growth Regulators and Food Additives*, Vol. 1, Academic Press, New-York, 1963.
9 H. F. MACRAE ET W. P. MCKINLEY, *J. Assoc. Offic. Agr. Chemists*, 44 (1961) 207.
10 H. E. WADE ET D. M. MORGAN, *Nature*, 171 (1953) 529.

SOME OBSERVATIONS ON THE THIN-LAYER CHROMATOGRAPHY OF ORGANO-CHLORINE PESTICIDES

D. C. ABBOTT, H. EGAN AND J. THOMSON

Department of Scientific and Industrial Research, Laboratory of the Government Chemist, Cornwall House, London (Great Britain)*

(Received April 14th, 1964)

Thin-layer chromatography has grown rapidly in importance in recent years and is now accepted as a quick and efficient technique for the detection and determination of compounds of many differing types, both organic and inorganic. However, its use in the field of pesticide residue analysis appears to have been rather limited. Starch-bound silica gel plates have been used[1] for the separation of aldrin, dieldrin and endrin, while more detailed studies have been made by PETROWITZ[2] and KOVACS[3]. WALKER AND BEROZA[4] included 16 organo-chlorine compounds among the 61 pesticides they examined by 19 solvent systems on silica-gel plates but few of them were sufficiently resolved to be of any positive assistance in the identification of residues, particularly where several pesticides occur together. Quite good separation of six organo-chlorine insecticides has been claimed[5], using activated-alumina (200–220°) plates with hexane as mobile solvent. More recently loose-layer chromatography has been used[6] to separate organo-chlorine pesticides into two groups prior to gas–liquid chromatographic determination.

In this laboratory, thin-layer chromatography has been used for the separation and identification of some herbicides[7]; this work has now been extended to cover several organo-chlorine insecticides and some of the metabolites of DDT. Several separatory systems have been devised which are intended for use for the confirmation of the provisional identifications provided by gas–liquid chromatography[8,9], or paper chromatography[10,11], of pesticide residues present in animal or vegetable tissue.

EXPERIMENTAL

Apparatus

A Desaga thin-layer spreading apparatus (obtainable from Camlab Ltd., Cambridge) was used.

Materials

Silica gel G, alumina G, and kieselguhr G (all obtainable from E. Merck, Darmstadt) were used throughout, either singly or in admixture.

Pesticide solutions

Solutions, 5 mg per ml, of the pesticides examined were made in ethyl acetate.

* Stamford Street, London, S.E. 1.

Chromatographic technique

The carrier plates (20 cm × 20 cm) were layered (250 μ thick) in the usual way[3], dried and activated by heating in an air oven at 120° for 2 h. Activated plates were then stored over silica gel until used, preferably within 36 h of preparation. The pesticide solutions were spotted with a 1 μl micro-pipette at 1 cm intervals along an origin line 1.5 cm from one edge of the plate and parallel to it; a second line was marked 15 cm from the origin. Each plate was developed by ascending chromatography in a suitable tank[10], under conditions of equilibrium, until the solvent front reached the second line, generally between 45 min and 2 h from commencement. The plate was then removed from the tank and dried at 100° for 10 min prior to spot development by one of the systems described below.

Spot development systems

Several types of spot indicators have been investigated. The "chromogenic reagent" used by MITCHELL[11], silver nitrate with 2-phenoxyethanol in ethanol, has been found to be less satisfactory for use with thin-layer plates than it is for paper chromatograms[10]. Owing to the rapid darkening of the background, the shapes of the spots were rather poorly defined and small amounts of pesticides were readily overlooked owing to the faintness of the spot.

The use of a simple ethanolic solution of silver nitrate (0.5 % w/v) as a spray reagent, followed by irradiation with germicidal ultraviolet light for 10 min, has proved very satisfactory, well-defined black-brown spots being obtained on an off-white background which is slow to darken. Because of its simplicity, this system has been used for most of the work described here, although several other promising indicator systems have also been examined as shown below. This silver nitrate indicator may also be used semi-quantitatively; 0.5 μg or less of most of the pesticides was readily observed although α- and γ-BHC required longer irradiation (30 min).

The combination of a pH indicator material with silver nitrate, both with and without ultraviolet irradiation, has given some colourful plates; in each case the developed plates were sprayed with 0.5 % ethanolic silver nitrate, dried, sprayed with indicator (0.2 % ethanolic) and dried again before irradiation. Bromocresol green gave yellow spots on a green-blue background after irradiation, the spots becoming a deep orange colour on heating. Yellow spots on blue were shown by bromocresol purple but the spots faded to off-white on irradiation and gave poor definition. Spraying with bromothymol blue yielded yellow spots on a white basis, the spots darkening on irradiation. Methyl orange was not very suitable, giving pale-pink spots on a white background.

The best indicator of this type that we have examined comprises a mixture of bromophenol blue with silver nitrate; a similar system has been used by WOOD[12] for the marking of chloride and bromide spots on paper. Following spraying with 0.5 % ethanolic silver nitrate, the plates were dried for 5 min at 100° and then sprayed with a solution of bromophenol blue (0.2 %) and silver nitrate (0.15 %) in a mixture of equal volumes of ethanol and ethyl acetate. On further drying at 100° for 10 min the pesticides were marked as bright yellow well-defined spots superimposed upon a deep blue background; irradiation with ultraviolet light had no effect on this coloration, which was stable for at least a week.

Some thin-layer plates have also been prepared in which silver nitrate has been

"built-in" either during layering or by spray application after layering in the usual way. Addition of silver nitrate to the layer-mix gives rise to rapid gelation; this has been offset by reducing the mixing-time, prior to pouring the mix into the spreader, by one quarter. No alteration of R_F values was observed on using these plates.

Separatory systems investigated

The bulk of the work described here has been carried out on silica gel, with some use of alumina and silica gel–alumina mixtures. Preliminary studies showed that kieselguhr was unlikely to be satisfactory, most of the pesticides migrating with the solvent front or else streaking badly. In general the mobile solvents used were of one of three types: (a) light hydrocarbon solvents; (b) light hydrocarbon solvent together with liquid paraffin or silicone oil; (c) solvents similar to (b) together with dioxane. The R_F values obtained in this study are listed in Table I.

TABLE I

R_F VALUES OF PESTICIDES USING VARIOUS SEPARATORY SYSTEMS

Compound	$R_F \times 100$ *in system*														
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
Aldrin	88	98	73	58	69	67	70	79	64	62	67	98	82	95	70
α-BHC	—	69	—	—	43	37	—	59	28	29	52	92	63	87	—
γ-BHC	—	58	—	—	37	27	—	47	18	19	46	94	55	78	—
p,p′-DDE	87	98	87	74	62	61	68	73	57	56	65	98	78	95	65
o,p′-DDT	71	90	74	50	58	54	62	71	46	48	59	97	73	89	50
p,p′-DDT	72	91	78	52	54	49	60	69	39	40	57	98	69	89	42
de-HCl-*p,p′*-TDE	85	98	88	67	62	61	72	76	53	51	49	98	75	93	53
p,p′-Dichlorobenzophenone	—	—	—	—	48	45	53	67	27	26	59	92	55	31	14
Dieldrin	69	58	53	30	48	41	46	63	48	54	65	88	52	37	12
Endosulfan A	—	—	—	—	52	47	63	61	35	31	58	94	64	65	17
Endosulfan B	—	—	—	—	—	—	—	—	—	—	12	86	9	4	2
Endrin	—	—	—	—	52	42	58	65	26	26	49	88	61	51	13
Heptachlor	82	98	69	48	62	61	65	73	53	52	65	88	78	95	58
Heptachlor epoxide	—	—	—	—	—	—	—	—	—	—	39	88	57	49	17
Methoxychlor	—	—	—	28	36	27	30	45	10	13	—	—	—	—	—
p,p′-TDE	66	77	58	67	46	33	45	59	26	28	52	92	57	71	25

Key to systems

System No.	*Plate*	*Mobile solvent*
1	Silica gel–alumina (1:1)	Cyclohexane–liq. paraffin (20%)
2	Silica gel–alumina (1:1)	Cyclohexane–silicone oil (8%)
3	Silica gel	Cyclohexane–*n*-hexane (1:1)
4	Silica gel	Cyclohexane–benzene (1:1)–liq. paraffin (10%)
5	Silica gel	Cyclohexane–liq. paraffin (20%)–dioxane (10%)
6	Silica gel	Cyclohexane–liq. paraffin (20%)–dioxane (5%)
7	Silica gel	Cyclohexane–liq. paraffin (10%)–dioxane (3.5%)
8	Silica gel	Cyclohexane–liq. paraffin (5%)–dioxane (2%)
9	Silica gel	Pet. ether (40–60°)–liq. paraffin (20%)
10	Silica gel	Pet. ether–liq. paraffin (10%)
11	Silica gel	Pet. ether–liq. paraffin (5%)–dioxane (1%)
12	Kieselguhr	as 11
13	Alumina	as 11
14	Alumina	*n*-Hexane
15	Silica gel	*n*-Hexane

Development of 1:1, silica gel–alumina plates with cyclohexane containing 20% v/v of liquid paraffin showed some promise. Replacement of the paraffin with a silicone oil (8%) gave good separation of *p,p'*-DDE, dieldrin and *p,p'*-TDE from each other, but did not resolve aldrin, *p,p'*-DDE, dehydrochlorinated *p,p'*-TDE and heptachlor, all of which travelled very close to the solvent front. Better separations were obtained when silica gel plates were developed in cyclohexane–*n*-hexane or cyclohexane–benzene–liquid paraffin; this system gave particularly good separation of a mixture containing aldrin, *p,p'*-DDE, dehydrochlorinated *p,p'*-TDE, dieldrin and heptachlor.

The conjunction of various proportions of liquid paraffin and dioxane with cyclohexane as the mobile phase has been studied with silica gel plates. From Table I it may be seen that by suitable choice of the appropriate mixture of the three components of the mobile phase separations, of α- and γ-BHC or of dieldrin and *p,p'*-TDE for example, may be improved. Replacement of cyclohexane with petroleum ether (40–60°) in systems of this type has given other useful separations, *e.g.*, aldrin–heptachlor (system 9), *o,p'*-DDT–*p,p'*-DDT (system 10) and endosulfan A–endosulfan B (system 11).

Kieselguhr was again shown to be of little use with solvents of this type but alumina showed more promise than had previously been achieved with this material. The use of an alumina plate with *n*-hexane alone as mobile phase was found to be very suitable for the separation of endrin from *p,p'*-DDT and of dieldrin from *p,p'*-DDE; using this system up to nine separate spots could be obtained from a single mixture. Application of the same solvent to silica gel plates proved similarly useful, good resolution being obtained of those compounds whose R_F values were rather high on the alumina plate, *i.e.*, aldrin, heptachlor, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT and endosulfan A.

TABLE II

SYSTEM OF CHOICE FOR A GIVEN SEPARATION

Numbers refer to systems detailed in the key to Table I.

	Aldrin	α-BHC	γ-BHC	*p,p'*-DDE	*o,p'*-DDT	*p,p'*-DDT	de-HCl-*p,p'*-TDE	Dieldrin	Endosulfan A	Endrin	Heptachlor	Heptachlor epoxide
α-BHC	9											
γ-BHC	9	8										
p,p'-DDE	4	9	9									
o,p'-DDT	9	2	2	4								
p,p'-DDT	9	2	2	15	15							
de-HCl-*p,p'*-TDE	15	9	2	11	4	9						
Dieldrin	15	10	14	14	14	14	14					
Endosulfan A	15	14	9	15	15	15	15	14				
Endrin	15	14	14	15	15	14	14	10	14			
Heptachlor	15	2	2	15	1	15	4	14	15	15		
Heptachlor epoxide	15	14	14	15	14	14	14	11	11	11	14	
p,p'-TDE	15	14	2	15	15	3	3	14	7	14	15	14

From the results given in Table I the system for the best separation of any given pair of the compounds may be chosen. Table II lists the selected systems. From Table II it is obvious that a few systems are of general utility. Six such systems are listed in Table III, together with a selection of resolvable pesticides.

As can be seen from these tables, by choice of the appropriate thin-layer system,

TABLE III

COMPOUNDS RESOLVED BY PREFERRED SYSTEMS

System No. (Table I)	*Resolved compounds*
2	α-BHC, γ-BHC, *p,p'*-DDE, *p,p'*-DDT, *p,p'*-TDE
4	Aldrin, *p,p'*-DDE, dehydrochlorinated *p,p'*-TDE, dieldrin, heptachlor
9	Aldrin, α-BHC, γ-BHC, *p,p'*-DDT, heptachlor, methoxychlor
11	Dieldrin, *p,p'*-DDT, endrin, heptachlor epoxide, endosulfan B
14	α-BHC, γ-BHC, dichlorobenzophenone, dieldrin, endosulfan A, endosulfan B, heptachlor, heptachlor epoxide, *p,p'*-TDE
15	Aldrin, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT, dieldrin, endosulfan B, heptachlor, heptachlor epoxide, *p,p'*-TDE

it is possible to separate all of the pesticides examined one from another. The resolution and identification of the components of mixtures of pesticide residues is also possible. The procedures described have been successfully applied to extracts of vegetable and animal origin which have been "cleaned-up" by a N,N-dimethylformamide partition process[13].

Effect of temperature of development on R_F value

Differing views have been expressed in the literature as to the extent of the effect of temperature upon development times and R_F values in thin-layer chromatography. STAHL *et al.*[14], studying essential oils on silica gel plates developed in a hexane–acetic acid mixture, found no alteration in running time on changing the temperature from 20° to either 4° or 28°. The effects of insecure closure of the tank and variation in the depth of immersion in the mobile phase were much more important. Similarly BRENNER *et al.*[15] found that raising the temperature from 18° to 38° had virtually no effect on the R_F values of a number of amino acids developed with a phenol–water mixture, although the reproducibility of these values became poorer.

MULLER AND HONERLAGEN[16], however, in their study of the chromatography of cinchona bark alkaloids with a mixed kerosine–diethylamine–acetone solvent, found that the R_F values were strongly temperature dependent; they advised the use of 25° as being the most convenient for their purpose. HARTHON[17] also found that the control of the development temperature was critical if constant R_F values of nitramine-explosives were to be obtained. To guard against small variations in R_F he used a reference compound on every silica gel plate developed in a petroleum ether–acetone mixture.

The R_F values of the pesticides given in Table I were obtained at room temperatures (15–20°). Attempts have now been made to show the effects of development at different temperatures ranging from —20° (deep-freeze cabinet) to 40° (warm water-bath). Silica gel plates were developed in *n*-hexane for 40 min, a metal tank being used for the highest and lowest temperatures. Spots were marked by the silver nitrate–ultraviolet irradiation process described. For results obtained see Table IV.

It is clear that the R_F values of all of these compounds were temperature dependent to some extent. Compounds with R_F values above 0.40 at room temperature showed the greatest variation. Endrin, dieldrin and endosulfan A, with R_F values

TABLE IV

VARIATION IN R_F WITH DEVELOPMENT TEMPERATURE

Compound	$R_F \times 100$ *at temperature*					
	—20°	*0°*	*10°*	*20°*	*30°*	*40°*
Aldrin	33	55	68	77	85	90
p,p'-DDE	27	49	60	70	80	90
o,p'-DDT	20	37	50	58	68	77
p,p'-DDT	17	31	40	48	58	70
de-HCl-*p,p'*-TDE	21	42	48	60	70	79
Dieldrin	1	7	10	12	12	12
Endosulfan A	6	14	16	17	17	17
Endosulfan B	2	2	3	4	4	4
Endrin	2	9	10	11	12	12
Heptachlor	22	45	55	65	75	86
Heptachlor epoxide	4	13	15	17	18	19
p,p'-TDE	12	21	25	32	38	42

below 0.20 at room temperature, showed almost no variation at higher temperatures but gave a decided drop in R_F value at —20°. *p,p'*-TDE held an intermediate position in all respects.

For practical purposes chromatography at 0° has some uses. Development is more rapid and consequently more compact spots are obtained, thus improving the resolution obtained between pesticides of close R_F values, *e.g.* dieldrin and heptachlor epoxide or heptachlor and *p,p'*-DDE. For development at the higher temperature it was essential to pre-heat the prepared plate to the required temperature before placing it in the chromatographic tank; without this precaution immediate condensation of the solvent occurred on to the plate.

Documentation of thin-layer plates

During this study the need arose for a simple and rapid method for recording the chromatograms obtained in a permanent form. Preservation of the actual chromatogram either on or off[18] the plate was discounted on the grounds of the cost of the carrier plates, the fragile nature of the layer and the impermanence of the developed image. Photographic methods[19], while excellent for coloured compounds and the preparation of slides for projection, involve expensive equipment and lengthy processing; commercial photo-copying apparatus is also expensive although suitable[20].

Attention has recently been given to the use of photosensitive papers[21,22] for documentation purposes. A dry process has been advocated by EISENBERG[23], using a direct positive Diazo blueprint paper to produce positive replicates of chromatograms. Diazo papers readily available to us appeared to be somewhat slower than that used by EISENBERG and his exposure time, 10 min, was already inconveniently long. However, a study of this method involving several grades of ammonia-process paper and different lighting systems has led to the evolution of the procedure detailed below. The papers may be handled openly under normal laboratory lighting conditions without loss in sensitivity. The final prints are permanent and are readily filed for storage. Although the use of overrun photoflood bulbs is advised, ordinary 60 W or 100 W bulbs can be used provided the exposure time is increased accordingly.

Documentation method

Circumscribe the spots on the developed plate down to the glass with a soft-lead pencil or scrape them completely from the plate. (Fluorescent spots may be so marked under suitable irradiation and weak spots are suitably defined against an opal illuminated background.) Similarly mark the spot origin and the solvent front, remove the excised material by blowing gently. Place the marked plate face downward on a sheet of the Diazo paper slightly longer than the carrier plate. "Ammonax" 8.M.13 Positive Diazo Paper, Hall-Harding Ltd., London and "Densblack" Ammonia Process Paper, Type 45, 9X, E. N. Mason and Sons Ltd., Colchester, have been found suitable. The former blue paper is about three times as fast as the "Densblack" but the greater contrast given by the black background of this paper gives better definition.

Illuminate from above with two 275 W photoflood bulbs mounted in a 25 cm × 25 cm × 20 cm reflector placed centrally over the plate, the bulbs being about 10 cm above the glass surface. After 5 sec ("Ammonax") or 15 sec ("Densblack") remove the illumination source and suspend the exposed paper in an atmosphere of ammonia; a paper-chromatographic tank[10] is very suitable for this purpose. The marked spots appear as white rings or circles on the blue or black background almost immediately.

Permission to publish this paper has been given by the Government Chemist, Department of Scientific and Industrial Research.

SUMMARY

The thin-layer chromatography of several organo-chlorine insecticides and related compounds has been studied using fifteen separatory systems. Various indicators have been used and the effect of temperature upon R_F value is described. The separations obtained support other methods in establishing the identity of pesticide residues. A simple method for the documentation of thin-layer plates is also included.

REFERENCES

1 J. Yamamura and T. Niwaguchi, *Proc. Japan Acad.*, 38 (1962) 129.
2 H.-J. Petrowitz, *Chemiker Ztg.*, 85 (1961) 867.
3 M. F. Kovacs, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 884.
4 K. C. Walker and M. Beroza, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 250.
5 J. Baumler and S. Rippstein, *Helv. Chim. Acta*, 44 (1961) 1162.
6 A. Taylor and B. Fishwick, *Lab. Pract.*, 13 (1964) 525.
7 D. C. Abbott, H. Egan, E. W. Hammond and J. Thomson, *Analyst*, 89 (1964) 480.
8 E. S. Goodwin, R. Goulden and J. G. Reynolds, *Analyst*, 86 (1961) 697.
9 A. Taylor, *Analyst*, 87 (1962) 824.
10 W. H. Evans, *Analyst*, 87 (1962) 569.
11 L. C. Mitchell, *J. Assoc. Offic. Agr. Chemists*, 41 (1958) 781.
12 T. Wood, *Nature*, 176 (1955) 175.
13 M. J. de Faubert Maunder, H. Egan, E. W. Godly, E. W. Hammond, J. Roburn and J. Thomson, *Analyst*, 89 (1964) 168.
14 E. Stahl, G. Schröter, G. Kraft and R. Renz, *Pharmazie*, 11 (1956) 633.
15 M. Brenner, A. Niederwieser, G. Pataki and A. P. Fahmy, *Experientia*, 18 (1962) 101.
16 K. H. Muller and H. Honerlagen, *Mitt. Deutsch. Pharm. Ges.*, 30 (1960) 202.
17 J. G. L. Harthon, *Acta Chem. Scand.*, 15 (1961) 1401.
18 R. L. Squibb, *Nature*, 198 (1963) 317.
19 T. L. Brown and J. Benjamin, *Anal. Chem.*, 36 (1964) 446.
20 J. Hilton and W. B. Hall, *J. Chromatog.*, 7 (1962) 266.
21 H. Sprenger, *Z. Anal. Chem.*, 199 (1964) 338.
22 H. T. Gordon, *Science*, 128 (1958) 414.
23 F. Eisenberg, *J. Chromatog.*, 9 (1962) 390.

IDENTIFICATION OF ETHYL CENTRALITE-NITROGEN TETROXIDE REACTION PRODUCTS*

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

(Received May 8th, 1964)

INTRODUCTION

In the course of an investigation to evaluate the effective capacity of various stabilizers to react with nitrogen tetroxide[1], it was found that very few qualitative methods were available to identify the reaction products. The ethyl centralite–nitrogen tetroxide (EC–N_2O_4) reaction was of particular interest to us, since ethyl centralite (EC) is one of the more commonly used stabilizers in nitrocellulose formulations.

SHROEDER *et al.* describe a column chromatographic method of separating and estimating the EC derivatives in smokeless powder[2]; however, this technique was found to be inadequate for our purpose. Other quantitative methods devised for the determination of a specific EC derivative were inapplicable for identification work.

This article presents a two-dimensional thin-layer chromatographic method for the separation and identification of twenty-five ethyl centralite nitration products. The method is applied to the analysis of the EC–N_2O_4 reaction products.

REAGENTS AND EQUIPMENT

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

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

Reagent grade solvents. Petroleum ether from Merck & Co., Inc., with boiling range from 30–60°.

Spray reagents. 0.003 % dichlorofluorescein in ethanol; 0.25 % *p*-diethylaminobenzaldehyde (*p*-DEAB) and 0.2 *N* HCl in absolute ethanol.

An Agla micrometer syringe or capillary melting point tube used as a sample applicator.

Kensco short wavelength ultraviolet light source.

EXPERIMENTAL

Preparation of thin layer

Modification of STAHL'S technique for thin layer preparation was necessary in order to incorporate the zinc dust into the silica gel G, a procedure described in an earlier

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

publication[3]. A slurry mixture was prepared by adding 30 g of silica gel G and 1 g of zinc dust to 65 ml of rapidly stirred water. Coating was accomplished by pulling the applicator with a Bodine speed reductor motor at a constant speed over five glass plates. The silica gel G–Zn plates were activated at 120° overnight before use.

R_G measurement

With twenty-five authentic samples on hand, the migration rate of each compound with respect to ethyl centralite (reference compound) was measured in ten different solvents. From cross-plots of the R_G values, 1,2-dichloroethane (solvent I) and ethyl acetate–petroleum ether (25:75) (solvent II) were found to be the most efficient solvent pair. Fig. 1 represents a two-dimensional plot of the R_G values as measured in solvents I and II. Only 4-nitrophenol was found to tail on migration.

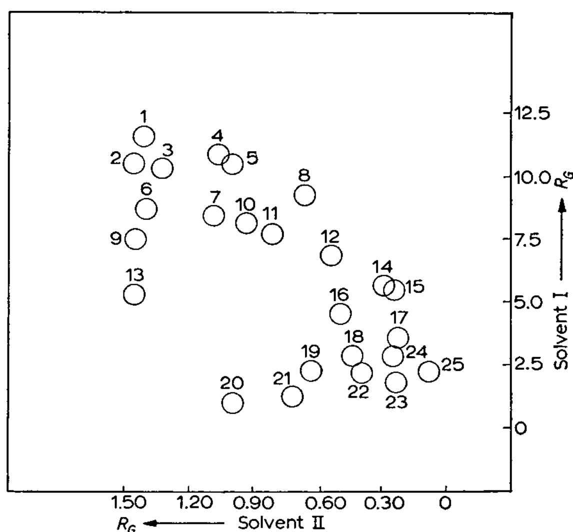


Fig. 1. R_G measurements of nitro ethyl centralites and related compounds. (1) nitrobenzene; (2) 2-nitroEA; (3) 2,4,6-trinitroEA; (4) N,2,4,6-tetranitroEA; (5) 1,3-dinitrobenzene; (6) 1,3,5-trinitrobenzene; (7) N-nitroso-4-nitroEA; (8) 2,4-dinitroEA; (9) N-nitrosoEA; (10) 2-nitroaniline; (11) 4-nitroEA; (12) N-nitroso-2-nitroEA; (13) EA; (14) 4-nitroaniline; (15) 2,4-dinitroaniline; (16) 2,2′,4,4′-tetranitroEC; (17) 2,4,4′-trinitroEC; (18) 2,4′-dinitroEC; (19) 4-nitroEC; (20) EC; (21) 2-nitroEC; (22) 2,2′-dinitroEC; (23) 4-nitrophenol; (24) 4,4′-dinitroEC; (25) 2,4-dinitroEC.

Procedure

An acetone solution containing 0.5–1 mg of the EC–N_2O_4 reaction products is transferred to a point approximately 1.5 in. from the lower right-hand corner of an activated silica gel G/Zn plate. The sample is chromatographed in 250 ml of solvent I for an hour. After exposure of the plate to the atmosphere for 10 min, the sample is rechromatographed at 90° to the direction of flow of solvent I in an equal volume of solvent II for an additional hour. The separated products are located by spraying the plate with dichlorofluorescein and viewing the chromatoplate under a short wavelength ultraviolet light source. The location of EC is marked, and the plate is resprayed with *p*-DEAB. All the nitroso, nitro, and partially substituted amino compounds develop immediately.

RESULTS AND DISCUSSION

Fig. 1 indicates that twenty-four compounds can be separated and identified. The combined properties of both solvents tend to separate the nitro-derivatives of ethylaniline (EA) and EC into two groups; the upper-half of the chromatogram contains the nitroEA compounds, and the lower-half contains the nitroEC compounds. In the case of the nitroEC derivatives, solvent I separates these compounds according to the degree of nitration. This fact, coupled with chemical tests, was extremely useful in identifying the dinitroEC isomers that could not be prepared in sufficient quantities for elemental analyses.

A twenty-five component mixture was chromatographed by the described procedure. Fig. 2 is a tracing of the developed chromatogram. The components are found in the locations predicted from Fig. 1, but are somewhat closer together. This is attributed to the influence of each compound on the migration rate of the others. The

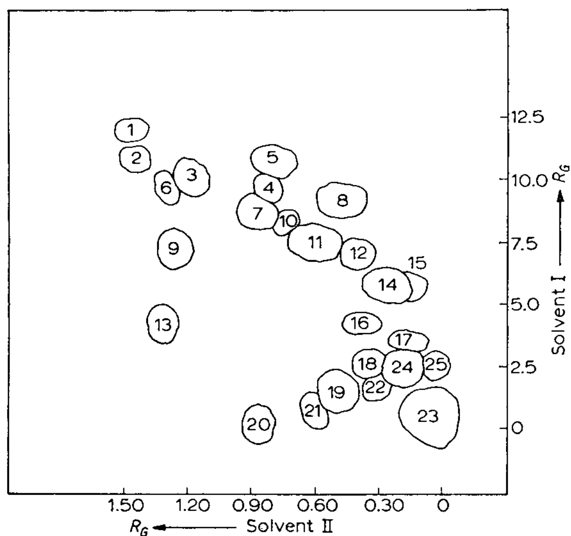


Fig. 2. Two-dimensional thin-layer chromatography of synthetic mixture. Compounds as identified in Fig. 1 caption.

4-nitro- and 2,4-dinitroanilines overlap; however, the *p*-DEAB reagent develops the spots red and brown, respectively, to make identification possible. Therefore, from the location and the color of the developed spots, all the compounds included in this work can be identified.

Samples of EC–N_2O_4 reaction products aged for different lengths of time were analyzed. The results are listed in Table I in the approximate order of decreasing concentration.

The complexity of the reaction products is evident. The dinitroEC derivatives are the major products. The presence of aniline, benzene, and phenolic compounds is an indication that degradation of EC and/or its nitro derivatives has occurred. This behavior of EC is in contrast to the diphenylamine (DPA) stabilizer; the DPA molecule remains intact during the nitrosation and/or nitration process. As one would expect,

TABLE I

IDENTIFICATION OF $EC–N_2O_4$ REACTION PRODUCTS

Sample	*Identified products*
(A) $EC–N_2O_4$ reaction products (aged 1 day)	4,4′-dinitroEC = 2,4′-dinitroEC > 2,2′-dinitroEC > N-nitroso-4-nitroEA > 2,4-dinitroEC > unknown > 4-nitrophenol > traces of N-nitroso-2-nitroEA 2,2′,4,4′-tetranitroEC 2,4-dinitroEA 4-nitroaniline 2,4,4′-trinitroEC nitrobenzene
(B) $EC–N_2O_4$ reaction products (aged 1 month)	4,4′-dinitroEC = 2,4′-dinitroEC > 2,2′-dinitroEC > N-nitroso-4-nitroEA = 4-nitrophenol > 2,4-dinitroEC > unknown > 2,2′,4,4′-tetranitroEC > 4-nitroaniline = 2,4-dinitroEA > traces of N-nitroso-2-nitroEA nitrobenzene 2,4,4′-trinitroEC
(C) $EC–N_2O_4$ reaction products (aged 6 months)	4,4′-dinitroEC = 2,4′-dinitroEC > 2,2′-dinitroEC > N-nitroso-4-nitroEA > 4-nitrophenol > 2,4-dinitroEC > 2,2′,4,4′-tetranitroEC > 2-nitroEA ⩾ 4-nitroEA = 2,4-dinitroEA > N-nitroso-2-nitroEA > N-2,4,6-tetranitroEA > N-nitrosoEA = 4-nitroaniline > traces of nitrobenzene, 2,4,4′-trinitroEC, and 4 unknowns

the degradation of $EC–N_2O_4$ reaction products increases with age, as shown in the 6 months-old sample.

It is disconcerting to find unidentified compounds in the reaction products. For example, the unknown located below the N-nitroso-4-nitroEA spot (see Fig. 1) in samples A and B could not be readily isolated to determine its identity; however, from the location of the spot, it is thought to be one of the isomers of dinitroaniline.

The degree of completion of the EC–N_2O_4 reaction was also determined. It was found that 99% of the EC reacted with N_2O_4 under the conditions used to study stabilizer capacity toward N_2O_4. A similar result was found for the DPA–N_2O_4 reaction, as reported in an earlier publication[1].

By comparing the major reaction products of EC and DPA, it is possible to explain why DPA is a more efficient scavenger of N_2O_4 than EC. The predominant products of the EC–N_2O_4 reaction are the dinitroEC compounds, whereas the DPA–N_2O_4 reaction yields mainly the N-nitroso-dinitro-, dinitro-, and trinitroDPA derivatives. Therefore, the higher nitrosated and nitrated DPA derivatives account for DPA having a higher capacity toward N_2O_4 than EC.

The need for two spray reagents is obvious. Ethyl centralite cannot be detected with *p*-DEAB because it has no nitro group. On the other hand, dichlorofluorescein will detect in the neighborhood of 5 μg of EC, but will not distinguish overlapping spots such as the 4-nitro- and 2,4-dinitroanilines. With the *p*-DEAB reagent, all the nitroso, nitro, and partially substituted amino compounds can be detected in amounts of 1–4 μg.

Approximately 70% of the authentic samples were not available commercially; therefore, they were prepared or isolated from the nitration products of EC. The 2-nitro-N-ethylaniline (2-nitroEA) was prepared by condensing 1-chloro-2-nitrobenzene with ethylamine. The 4-nitroEA was obtained through the hydrolysis of N-ethyl-4-nitroacetanilide with KOH. By using standard nitrosation procedures, the corresponding nitroso-nitro compounds were synthesized. The remaining nitroEA derivatives were isolated from the nitration products of EC via thin layer chromatography.

The nitroEC derivatives were the most difficult samples to obtain. The 2-nitroEC and 4-nitroEC required the preparation of nitrocarbanilyl chloride intermediates which were then condensed with EA to obtain the desired compounds[4]. The 4,4'-dinitro-, trinitro-, and tetranitroEC derivatives were isolated from the nitration products of EC. The 2,2'-dinitro-, 2,4'-dinitro-, and 2,4-dinitroEC derivatives were separated from the EC–N_2O_4 reaction products only in minute quantities. With the exception of these three dinitroEC isomers, all samples were purified by thin-layer chromatography and characterized by m.p. and elemental analysis.

The identity of the three dinitroEC compounds was established through chemical and chromatographic methods. Under suitable conditions, acid hydrolysis of these compounds should yield the EA derivatives. Accordingly, a known sample (2,4,4'-trinitroEC) was hydrolyzed in concentrated sulfuric acid at 80° for 10 min. After the solution was neutralized, and the hydrolyzed products extracted with methylene chloride, thin-layer chromatography revealed 4-nitroEA, 2,4-dinitroEA and a small amount of unhydrolyzed trinitroEC in the extract. Under similar conditions of acid hydrolysis, followed by thin-layer chromatographic analysis, 2,2'-dinitro-, 2,4'-dinitro- and 2,4-dinitroEC showed the expected EA derivatives, proving conclusively the identity of the parent compounds.

ACKNOWLEDGEMENTS

The author wishes to thank H. E. UNGNADE for the preparation of nitrated ethyl centralite and N-ethylaniline samples, and M. J. NARANJO for the elemental analyses.

SUMMARY

A two-dimensional thin-layer chromatographic method for the separation and identification of the numerous products produced in the nitration of ethyl centralite is described. The method is applied to the analysis of ethyl centralite–nitrogen tetroxide reaction products. Two spray reagents used in conjunction with the silica gel G/Zn plate offer a sensitive detection method for each of the compounds.

REFERENCES

1 S. K. Yasuda, *Explosivstoffe*, in the press.
2 W. A. Shroeder, M. K. Wilson, C. Green, P. E. Wilcox, R. S. Mills, and K. N. Trueblood, *Ind. Eng. Chem.*, 42 (1950) 539.
3 S. K. Yasuda, *J. Chromatog.*, 13 (1964) 78.
4 P. E. Wilcox and W. A. Shroeder, *J. Org. Chem.*, 15 (1950) 944.

MULTI-DIMENSIONAL CHROMATOGRAPHY USING DIFFERENT DEVELOPING METHODS

II. MICROSCOPIC AND COLORIMETRIC INVESTIGATION OF COMPOUNDS SEPARATED BY GAS CHROMATOGRAPHY IN SUBMICRO-QUANTITIES

JAROSLAV JANÁK

Laboratory of Gas Analysis of the Czechoslovak Academy of Science, Brno (Č.S.S.R.)

(Received July 7th, 1964)

In a previous communication[1] a new two-dimensional method of chromatography was described, in which the gas chromatogram is continuously developed on to the start-line of a thin layer of adsorbent or sheet of chromatographic paper, which is moved under the outlet orifice of the gas chromatographic column in a certain program. In this paper, the case will be discussed where instead of a plate with a thin layer of adsorbent (or a sheet of paper) a simple plate is used, made of glass or other material. In addition it is pointed out that it is possible to use crystallographic or colorimetric methods for identification of nanogram to picogram quantities of matter, using microscopy techniques. Only two earlier papers[2,3] dealing with a similar matter in another way and in the milligram range can be cited.

EXPERIMENTAL AND DISCUSSION OF RESULTS

Sublimated chromatograms

The technique described previously[1] was used. High boiling gas chromatographic fractions were condensed on a thick glass plate, while for lower boiling substances a jacketed metallic plate of the same size which was cooled with an internally circulating liquid (water, brine) was used. The investigation was carried out with tar substances having boiling points above 200°. Samples of 2–10 mg of mixture were injected, at a column temperature of 280°; the distance between the column capillary outlet and the glass plate was 1–2 mm; carrier gas velocity was 1 ml/sec. Gas chromatographic fractions leaving the column were condensed as a sublimate on the glass plate, well developed zones consisting of characteristic microcrystals of the substances being formed. The shape of the crystal zones is the same as is known from the recording of the gas chromatographic peaks. In Fig. 1 a sublimatogram of the mixture of biphenyl, acenaphthene, fluorene and phenanthrene is shown. The width of the sublimated zones was 3–4 mm, the diameter of the GC-column orifice being 1.5 mm. Each zone begins and ends with a crystalline bloom while the center of the zone generally consists of well developed microcrystals. The form and type of the microcrystals is characteristic. In Figs. 2–4 a series of photomicrographs of sharp and typical tran-

Fig. 1. Sublimatogram of a mixture of diphenyl, acenaphthene, fluorene, phenanthrene (photograph taken in the plane of the surface of the supporting glass plate with lateral illumination).

Fig. 2. Start line of the acenaphthene zone (magnified 50 ×).

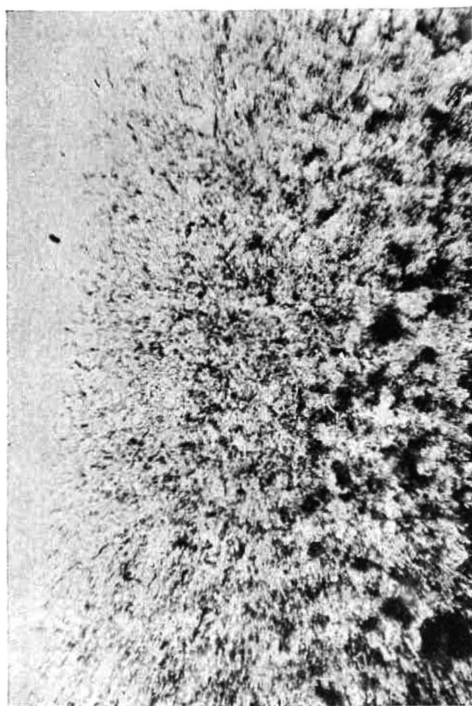


Fig. 3. Start line of the fluorene zone (magnified 50 ×).

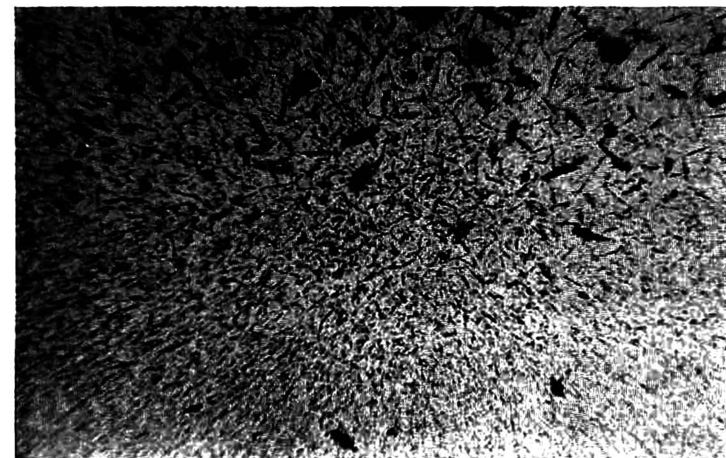


Fig. 4. Start line of the carbazole zone (magnified 50 ×).

Fig. 5. Microcrystals of acenaphthene (actual size 10^{-1}–10^{-2} mm).

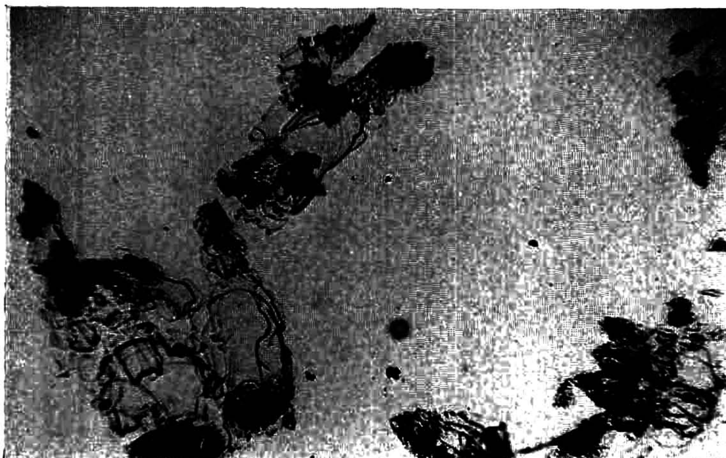


Fig. 6. Microcrystals of fluorene (actual size 10^{-1}–10^{-2} mm).

Fig. 7. Glass rod with ball for the transfer of microcrystals (thickness of the rod 0.10 mm, ball diameter 0.15 mm).

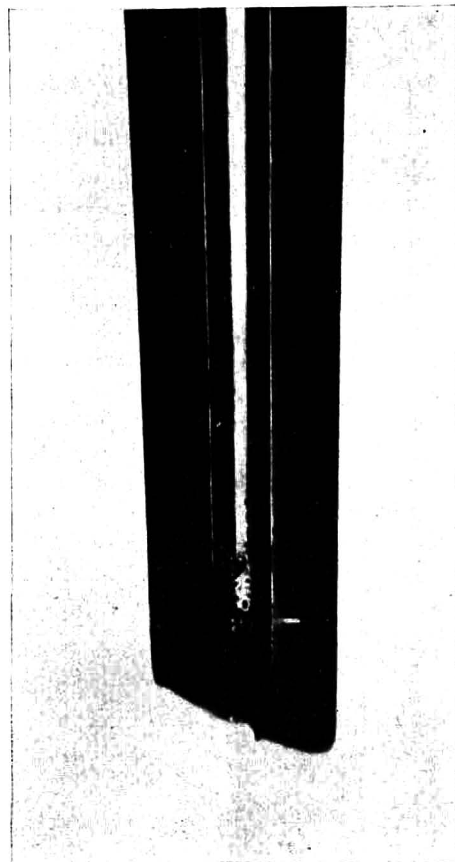


Fig. 8. Glass micropipette (internal diameter 0.07 mm, smooth tip with an angle of 45–60°).

sitions of gas-chromatographic fractions are shown. In Fig. 2 the start of the acenaphthene zone can be seen, beginning with characteristic needles. In Figs. 3 and 4 the edges of fluorene and carbazole zones are illustrated. The growth and increase of the thickness of the crystal layer and their different arrangement are apparent.

Further microscopic examination showed the crystal structure in detail. Fig. 5 shows a characteristic microcrystal of acenaphthene. It is usually a tree-like needle with oblique growth, angle almost exactly 30° and 210°. The actual size is 10^{-1} to 10^{-2} mm; the weight is of the order of 10^{-2} micrograms. It is evident that its character is quite different from that of the fluorene crystals (Fig. 6), whose ill defined leaflets can be described on the basis of planes of symmetry as 001. By comparison carbazole crystals (see Fig. 9) also develop a tree-like structure, whose needles grow at an angle of 85° and 265° on the supporting axis.

In the case of a mixed zone, when overlapping of chromatographic zones occurs, sharply differentiated eutectics or mixtures of well developed crystals of pure substances are formed.

Handling of crystals and colorimetric investigation

Figs. 7 and 8 show the main devices for handling small quantities of substances. A glass rod (Fig. 7) 0.05–0.10 mm thick, ending in a small ball, maximum diameter 0.2 mm, serves to the transport of crystals weighing few tens of nanograms. On handling the rod is fastened in two fixed points, the motion of its end being controlled by means of a micrometer screw while being observed under a microscope with 100-fold magnification. For the transport of solvents and solutions a thin glass capillary (Fig. 8) with outer diameter of 0.1–0.2 mm and inner diameter of 0.07–0.15 mm has proved to be useful. It has to have a smooth tip, the best angle being 35° to 60°.

The use of the device and the method are shown in an example. A crystal weighing approximately 10^{-2} micrograms with a volume of *ca.* 10^{-5} mm^3 was placed near a grain of silica gel with a volume of *ca.* 10^{-3} mm^3 by means of the rod (see Fig. 9). The crystal was dissolved by adding *ca.* 0.1 mm^3 benzene (*ca.* 5-10 mm of the benzene column inside the micropipette). The dissolution is carried out in the following way: the tip of the pipette is placed in contact with the silica gel grain near the crystal. A droplet of solvent wets the silica gel, spreads out, reaches the crystal and dissolves it. The benzene, together with the solution, is all spontaneously absorbed, due to capillary forces, by the silica gel grain. Evaporation of the benzene, taking 5–10 sec, leaves the dissolved matter of the crystal within the grain.

Fig. 9. Carbazole crystal transferred among the silica gel grains (the approximate diameter of a grain is 0.15 mm).

Fig. 10. Colour intensity representation of the carbazole–(TCE) complex in black and white ($20 \cdot 10^{-9}$ g of carbazole).

TABLE I

COLOURS OF TETRACYANOETHYLENE COMPLEXES WITH SOME AROMATIC AND HETEROCYCLIC COMPOUNDS

Compounds	*Colour*
Hydrocarbons	
2,3,6-Trimethylnaphthalene	blue
1-Phenylnaphthalene	grey
2-Methylfluorene	blue-grey
3:4-Benzofluorene	green
9,10-Dihydroanthracene	orange
1,2,3,4-Tetrahydroanthracene	blue-violet
1:2-Benzanthracene	blue-green*
3-Methylphenanthrene	violet
3,6-Dimethylphenanthrene	violet
1:2-Benzopyrene	grey-green
3:4-Benzopyrene	grey-blue
1,2,3,4-Tetrahydrofluoranthene	blue-violet
Benzo-[*m,n,o*]-fluoranthene	violet
3:4-Benzofluoranthene	violet
Perylene	yellow
O-compounds	
Phenol	orange-brown
2-Methylphenol	brown
3-Methylphenol	brown
4-Methylphenol	violet
2,3-Dimethylphenol	brown
2,4-Dimethylphenol	violet-brown
2,5-Dimethylphenol	violet-brown
2,6-Dimethylphenol	brown
3,4-Dimethylphenol	blue-violet
3,5-Dimethylphenol	brown
2-Ethylphenol	brown
3-Ethylphenol	brown
4-Ethylphenol	violet
2-Methyl-4-ethylphenol	black-violet
2-Ethyl-4-methylphenol	blue-violet
3,4-Diethylphenol	brown
2-Ethyl-5-*n*-propylphenol	violet-brown
2-Ethyl-5-*n*-butylphenol	brown-violet
3-*n*-Propylphenol	red brown
3-*n*-Butylphenol	brown
2,2'-Dihydroxybiphenyl	red-brown
2-Methylbiphenylene oxide	brown
1,8-Dimethylbiphenylene oxide	red-violet
Brazan	faint grey-violet
2-Hydroxybiphenylene oxide	blue-violet
2-Hydroxyfluorene	blue-green
4-Phenanthrol	red
N-compounds	
2,3-Dimethylcarbazole	sky blue
2-Methylacridine	yellow
4-Azafluorene	yellow
2-Azafluoranthene	yellow
1-Azapyrene	yellow
1-Azacarbazole	yellow
7-Azaindole	yellow
S-compounds	
6:7-Benzothionaphthene	blue-violet

* Colour disappears within a few seconds.

The process presents the following interesting possibilities. The concentration of material within the grain is approximately 1:10^2. The visibility limit of the most coloured complexes of aromatic hydrocarbons with tetracyanoethylene (TCE)[1,4] on a thin layer of silica gel is 1–2 μg in an area of *ca.* 120 mm^2 and a thickness of 0.9 mm, *i.e.* a volume of *ca.* 100 mm^3. The visibility limit in silica gel is therefore of the order of 1:10^5. As the concentration of carbazole in the grain is of the order of 1:10^2, it exceeds the visibility limit of this coloured complex by 3 orders.

If 0.05–0.1 mm^3 of a benzene solution of tetracyanoethylene is added in the same way, an intense colour due to the formation of the complex within the grain is seen, even if a quantity of less than 20 nanograms is treated. In Fig. 10 a black and white photograph of a silica gel grain, from Fig. 9, containing the coloured complex (ultramarine blue) is shown.

A series of coloured pictures (Figs. 11–14) shows the colours obtained with nano- to picogram quantities of various materials (acridine, carbazole, 2-methylcarbazole, and phenanthrene) in silica gel. In Table I colours of the tetracyanoethylene complexes with a larger number of aromatic and heterocyclic substances which can be used in the investigation of the composition of tar fractions are listed. Table I completes the data given in a previous paper[1] for about 100 compounds.

It will be seen that classical colorimetric methods can be applied to the identification of substances in concentrations that can be only detected by ionization methods at present. It is shown that by means of this technique it is possible to investigate colorimetrically even gas chromatographic fractions obtained from a capillary chromatograph. This has not been considered possible up to now.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Dr. M. SPURNÝ (Botanic Institute, Department of Biological and Film Studies, Czechoslovak Academy of Science, Brno) for his advice and assistance in taking photographs, to Dr. H. OBERKOBUSCH (Gesellschaft für Teerverwertung, Duisburg, West Germany) for his gift of some of the pure model substances used, and to Dr. I. KLIMEŠ for his technical assistance.

SUMMARY

Many classical colorimetric and crystallographic methods can be applied to the identification of nano- to picogram quantities of compounds obtained after separation by gas chromatography, if the compounds are examined under a microscope.

Techniques of handling crystals are described. The method is useful for the investigation of capillary gas chromatographic effluents.

REFERENCES

1 J. JANÁK, *J. Chromatog.*, 15 (1964) 15.
2 K. YOSHIMURA, *Japan Analyst*, 11 (1962) 397.
3 B. CASU AND L. CAVALLOTTI, *Anal. Chem.*, 34 (1962) 1514.
4 P. V. PEURYFOY, S. G. SLAYMAKER AND M. NAGER, *Anal. Chem.*, 31 (1959) 1740.

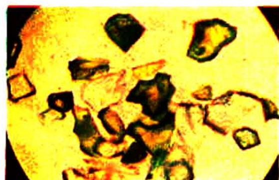


Fig. 11. Colour of the acridine–TCE complex (10^{-7} g of acridine).

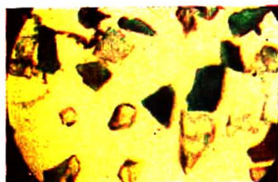


Fig. 12. Colour of the carbazole–TCE complex (10^{-9} g of carbazole).

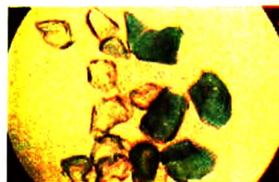


Fig. 13. Colour of the 2-methylcarbazole–TCE complex (10^{-9} g of methylcarbazole).

Fig. 14. Colour of the phenanthrene–TCE complex ($50 \cdot 10^{-12}$ g of phenanthrene).

The coloured plates were printed by Grafia, ČSSR.

QUANTITATIVE ANALYSIS OF SULFONAMIDE MIXTURES BY THIN-LAYER CHROMATOGRAPHY

T. BIĆAN-FIŠTER AND V. KAJGANOVIĆ

Institute for the Control of Drugs, Zagreb (Yugoslavia)

(Received April 3rd, 1964)

In a recent paper[1] thin-layer chromatography (TLC) was applied to the separation of 12 commonly used sulfonamides. The purpose of this paper is to provide by means of TLC a rapid separation and quantitative determination of some commercial sulfonamide mixtures.

Procedures for the assay of sulfonamide mixtures in the U.S.P. and the N.F. are based on the separation of sulfonamides by paper chromatography and the colorimetric determination of the extracted sulfonamides by the BRATTON–MARSHALL reaction[2]. A total of at least 14 paper strips are needed for the analysis of one triple sulfa preparation and many operations are involved. It should not be forgotten that not only does the large tank used require a long equilibration period but this is followed by 18 h development time.

Several authors have recently proposed procedures for the colorimetric determination of sulfonamide mixtures after a somewhat simplified paper chromatographic separation[3-6]. MARZYS[7] developed a spectrophotometric method for sulfonamide mixtures, while OLIVARI[8] determines sulfonamide mixtures directly on the chromatogram. However, all these procedures are rather tedious and time consuming for routine work and the application of TLC to this kind of analysis seemed very tempting.

The proposed procedure uses simple solvent systems for the separation of the most commonly used sulfonamide mixtures. After extraction from the adsorbent each sulfonamide is determined colorimetrically by means of the BRATTON–MARSHALL reaction[2].

The procedure can be applied to the determination of mixtures containing sulfathiazole, sulfamerazine, sulfamethazine; sulfacetamide, sulfamerazine and sulfadiazine (U.S.P., N.F.); and sulfadiazine, sulfamerazine and sulfamethazine (U.S.P.).

EXPERIMENTAL

Apparatus

Thin-layer chromatography apparatus No. 600, Desaga, Heidelberg.

Agla micrometer syringe, Burroughs Welcome & Co., London.

Solvents and solvent systems

Chloroform–methanol (90 ml + 10 ml).

Chloroform–methanol–ammonia solution (25%) (90 ml + 15 ml + 2.4 ml).

Ether.

Reagents

Kieselgel G for thin-layer chromatography, Merck No. 136021.

Acid sodium nitrite reagent; a freshly prepared 0.1 % solution in 0.1 *N* HCl.

N-1-Naphthylethylenediamine dihydrochloride reagent: 0.1 % solution in distilled water.

Ammonium sulfamate reagent: 0.5 % solution in distilled water.

Sodium nitrite reagent: a freshly prepared 0.1 % solution in distilled water.

All reagents used were of p.a. purity grade.

Reference substances

Sulfacetamide, sulfadiazine, sulfamerazine, sulfamethazine and sulfathiazole. Purity grade: U.S.P. and B.P., respectively.

Procedure

Tablets. Twenty tablets were weighed and finely powdered. A quantity of the powder equivalent to one sulfonamide tablet was taken and transferred to a 50 ml volumetric flask. 40 ml of a mixture of 50 ml 70 % ethanol and 2 ml 25 % ammonia solution were added to the flask which was shaken for 15 min, then made up to the mark with the solvent mixture and centrifuged (15 min at 2,500 r.p.m.). Kieselgel G coated plates (20 × 20 cm) were prepared by the technique proposed by STAHL[9] and activated by drying in an oven for 60 min at 105°. Three times 3 μl spots were applied along the starting line as 1 cm horizontal lines of the extracts of the sulfonamide mixture to be assayed and 3 μl spots of the corresponding standard solutions were similarly applied with a microsyringe and the chromatogram was run with the corresponding solvent system. When the solvent reached the front (*ca.* 45 min) the plate was dried in an oven for 10 min at 105° in the cases where the solvent mixture containing ammonia had been used. The spots were located by spraying only faintly with acid sodium nitrite reagent followed by the N-1-naphthylethylenediamine reagent and the spots of each separated sulfonamide were scraped off into a 25 ml glass stoppered flask, to which was added 5 ml 0.1 *N* HCl. Each flask was shaken well for about 20 min, centrifuged and 3 ml aliquots of each supernatant sample and standard solution were pipetted into a 25 ml flask. 1 ml sodium nitrite reagent was added to each flask followed after 3 min by 1 ml ammonium sulfamate reagent. After a further 2 min 1 ml N-1-naphthylethylenediamine reagent was added. After standing for 15 min the absorbancies of the sample and standard solution were determined at 545 mμ, relative to a reagent blank.

Weight of single sulfonamide (g) per tablet:

$$\frac{E_s \times \text{mg standard in 3 ml} \times 27.77}{E_{st}} \times \frac{\text{Mean weight of tablets}}{\text{Weight of samples}}.$$

Suppositories. Ten suppositories were weighed and pressed into a homogeneous mass. A quantity of the mass corresponding to *ca.* 250 mg total sulfonamides was weighed out and extracted by shaking in a separatory funnel with 100 ml ether, 10 ml water and 2 ml ammonia solution (25 %). After allowing the layers to separate the aqueous layer was drained into a 25 ml volumetric flask, and the ether layer washed in the separatory funnel several times with a few ml of water, the washings

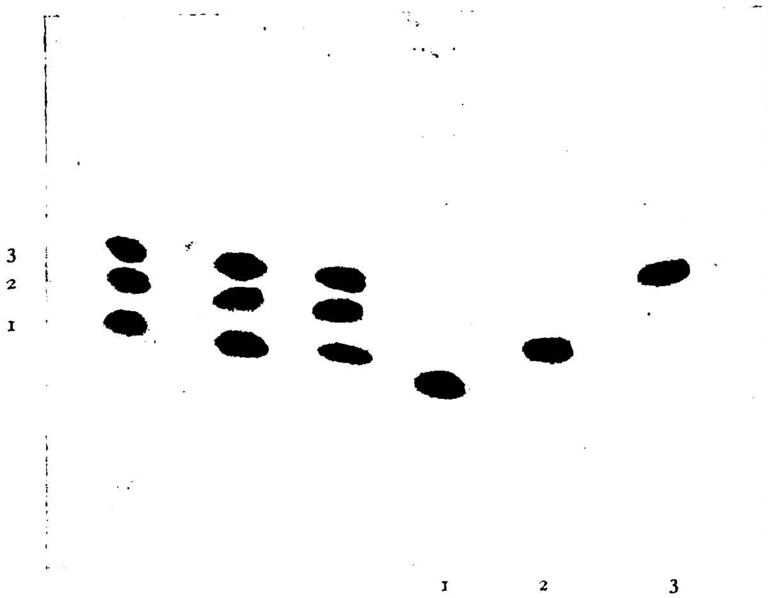


Fig. 1. Chromatographic separation of sulfathiazole (1), sulfadiazine (2) and sulfamerazine (3).

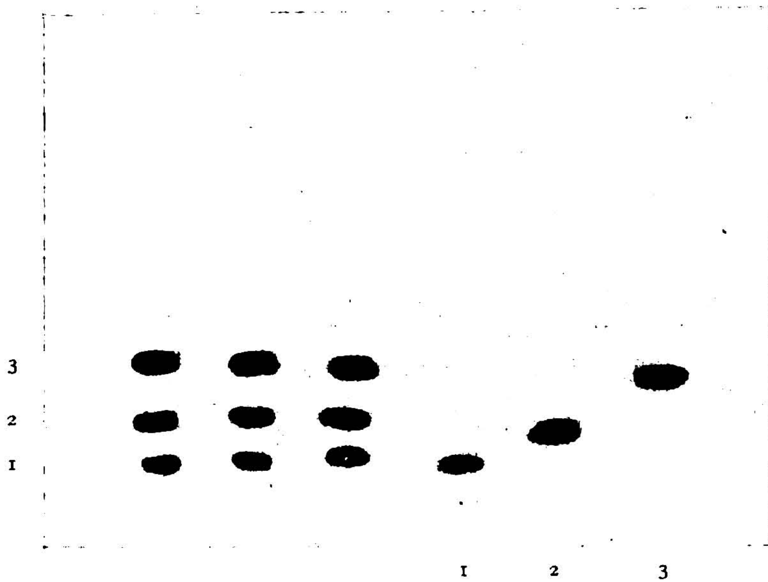


Fig. 2. Chromatographic separation of sulfacetamide (1), sulfadiazine (2) and sulfamerazine (3).

being added to the solution already in the flask which was then filled up to the mark with water. Portions of 3 µl of the sulfonamide mixture and the corresponding standard solutions were applied to the Kieselgel G plate which was then treated as described above for tablets.

Weight of single sulfonamide (g) per suppository:

$$\frac{E_s \times \text{mg standard in 3 ml} \times 27.77}{E_{st}} \times \frac{\text{Mean weight of suppository}}{\text{Weight of sample}} .$$

Suspension. A quantity of the suspension corresponding to about 500 mg of total sulfonamides, was weighed accurately into a 50 ml volumetric flask to which was added 40 ml of the ethanol–ammonia solution mixture (50:2), and after mixing well the volume was adjusted to the mark with the same solvent mixture. After centrifuging, 3 µl portions of the supernatant of the sulfonamide mixture to be analysed and the corresponding standard solutions were applied to the plate. The method is then the same as for tablets.

Weight of single sulfonamide (g) per g of suspension sample:

$$\frac{E_s \times \text{mg standard in 3 ml} \times 27.77}{E_{st}} .$$

DISCUSSION AND RESULTS

Although ether proved to be a very good solvent for qualitative purposes[1], for quantitative separations we preferred other solvents. From the results obtained it appeared that the volatility of ether diminished the accuracy of the method and at the same time affected the reproducibility of results. The solvent systems chloroform–methanol and chloroform–methanol–ammonia, which had given promising results in the qualitative separation of some sulfonamide mixtures[1], were tried for this reason. A perfect quantitative separation of sulfathiazole, sulfamerazine and sulfadiazine was obtained with the chloroform–methanol (90:10) mixture (Fig. 1), whereas for the other two sulfonamide mixtures, *viz.* sulfacetamide, sulfamerazine and sulfamethazine, and sulfadiazine, sulfamerazine and sulfamethazine, the solvent system chloroform–methanol–ammonia was found to be better. This solvent system was most stable and gave a high resolution of the sulfonamides when 90 ml chloroform were well mixed with 15 ml methanol and 2.4 ml ammonia solution (25 %) (Figs. 2 and 3). Separation of the mixture sulfathiazole, sulfadiazine, sulfamethazine and sulfamerazine was best with ether alone (Fig. 4).

The determination of the separated and extracted sulfonamides was first carried out on the basis of U.V. absorption. Although such technique has been employed previously[10] our results were rather discouraging as we encountered several difficulties such as high absorbancy of the blank and solubility of the Kieselgel G in the hydrophilic solvent used for the extraction of the sulfonamides, which gave rise to colloidal solutions. For these reasons we considered that a colorimetric procedure would be much more favorable for this technique and the BRATTON–MARSHALL reaction was adopted.

Experiments were carried out with standard mixtures of sulfonamides each

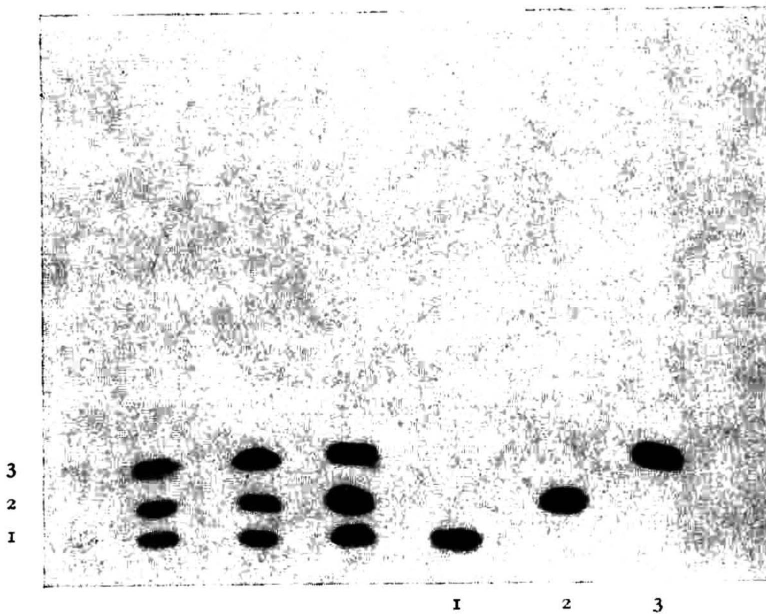


Fig. 3. Chromatographic separation of sulfadiazine (1), sulfamerazine (2) and sulfamethazine (3).

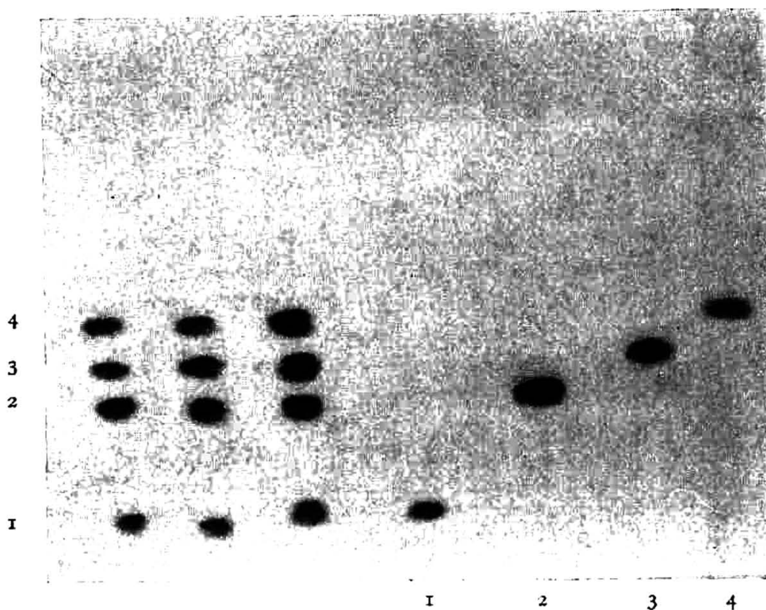


Fig. 4. Chromatographic separation of sulfathiazole (1), sulfadiazine (2), sulfamerazine (3) and sulfamethazine (4).

TABLE I

ANALYSIS OF STANDARD MIXTURES I, II AND III

Analysis No.	*μg found*			*μg found*			*μg found*		
	Sulfadiazine 6.60 μg added	*Sulfamerazine 6.60 μg added*	*Sulfathiazole 4.78 μg added*	*Sulfadiazine 10.02 μg added*	*Sulfamerazine 10.02 μg added*	*Sulfamethazine 10.02 μg added*	*Sulfacetamide 9.9 μg added*	*Sulfamerazine 9.9 μg added*	*Sulfadiazine 9.9 μg added*
1	6.27	4.54	6.31	9.46	10.46	10.17	9.52	9.84	9.33
2	6.20	4.53	6.69	10.28	9.49	9.92	9.78	9.16	10.30
3	6.03	4.94	6.74	10.12	10.09	9.41	10.39	10.19	9.84
4	6.33	4.58	6.55	10.12	10.15	10.06	9.27	9.77	9.28
5	6.69	4.78	6.48	9.99	9.55	10.24	10.14	10.39	10.24
6	6.85	4.91	6.62	9.98	9.99	10.05	10.45	9.83	10.42
7	6.08	4.32	6.30				9.19	9.55	9.33
8	6.32	4.40	7.04				9.67	9.13	10.20
9	6.50	4.85					9.55	9.95	10.00
Mean value	6.36	4.65	6.60	9.99	9.95	9.97	9.78	9.97	9.88
Standard deviation ($P = 0.05$)	±0.22%	±0.22%	±0.21%	±0.28%	±0.40%	±0.32%	±0.47%	±0.32%	±0.34%
Limits of error	±3.4%	±3.8%	±3.3%	±2.8%	±4.1%	±3.2%	±3.8%	±3.2%	±3.4%

TABLE II

ANALYSIS OF COMMERCIAL MULTIPLE PHARMACEUTICAL PREPARATIONS

Preparation and concentration		*Found mg*	*% recovery*
Tablets	*mg*		
Sulfadiazine	167	174.0	104.2
Sulfamerazine	167	169.3	101.4
Sulfamethazine	167	168.1	100.7
Sulfadiazine	185	187.4	101.3
Sulfamerazine	130	133.4	102.6
Sulfathiazole	185	187.4	101.3
Sulfathiazole	125	125.0	100.0
Sulfadiazine	125	126.8	101.44
Sulfamethazine	125	127.2	101.8
Sulfamerazine	125	126.8	101.5
Suspension	*g/100 ml*		
Sulfadiazine	3.33	3.32	99.70
Sulfamerazine	3.33	3.66	110.0
Sulfamethazine	3.33	3.21	96.39
Suppositories	*mg*		
Sulfadiazine	167	159.3	95.4
Sulfamethazine	167	158.9	95.2
Sulfamerazine	167	162.8	97.5

containing equal parts of the constituent compounds which are common in commercial pharmaceutical preparations. Standard mixtures were:

I. Sulfadiazine 186 mg; sulfamerazine 130 mg and sulfathiazole 185 mg.

II. Sulfadiazine, sulfamerazine and sulfamethazine each 167 mg.

III. Sulfacetamide, sulfamerazine and sulfadiazine each 167 mg.

These mixtures were analysed by the proposed method by two analysts and the limits of error for each sulfonamide was determined. Results are given in Table I.

Analyses were also made of available commercial multiple sulfonamide pharmaceutical preparations. The results obtained indicate both good checks and satisfactory adherence to the labeled declarations for each component compound (Table II).

ACKNOWLEDGEMENT

The authors wish to thank Mrs. A. HARAPIN for valuable technical assistance in the course of this work.

SUMMARY

A simple method for the thin-layer chromatographic separation and determination of some commonly used sulfonamide mixtures is proposed.

After separation on plates coated with Kieselgel G each sulfonamide is extracted and determined colorimetrically by means of the BRATTON–MARSHALL reaction. The total time of the analysis is less than 5 h. Statistical analysis of the results obtained with standard mixtures gave very good results.

REFERENCES

1 T. BIĆAN-FIŠTER AND V. KAJGANOVIĆ, *J. Chromatog.*, 11 (1963) 492.
2 A. C. BRATTON AND E. K. MARSHALL, Jr., *J. Biol. Chem.*, 128 (1939) 537.
3 N. MAIENTHAL, J. CAROL AND F. M. KUNZE, *J. Assoc. Offic. Agr. Chem.*, 44 (1961) 313.
4 M. TH. VAN DER VENNE AND J. B. T'SIOBBEL, *Chromatographie Symposium II, 1962*, Soc. Belge Sci. Pharm., Brussels, 1963, p. 191.
5 F. M. KUNZE AND L. ESPINOZA, *J. Assoc. Offic. Agr. Chem.*, 46 (1963) 899.
6 G. THOMAS, J. RANSY, P. ROLAND AND A. VAN DEN BULCKE, *J. Pharm. Belg.*, [N.S.] 5 (1950) 263.
7 A. MARZYS, *Analyst*, 86 (1961) 460.
8 G. OLIVARI, *Boll. Chim. Farm.*, 97 (1958) 552.
9 E. STAHL, *Dünnschichtchromatographie*, Springer, Berlin, 1962.
10 H. GÄNSHIRT, *Arch. Pharm.*, 296 (1963) 129.

DIE QUANTITATIVE BESTIMMUNG VON Δ^4-17β-HYDROXYÖSTREN-DERIVATEN UND DEREN ANWENDUNG ZUR STABILITÄTSPRÜFUNG VON TABLETTEN-ZUBEREITUNGEN

HERBERT G. GÄNSHIRT UND J. POLDERMAN

Pharmazeutische Forschungslaboratorien, N.V. Organon, Oss (Die Niederlande)

(Eingegangen den 23. April 1964)

In jüngster Zeit haben Δ^4-17β-Hydroxyöstren-Derivate in Form von Arzneimitteln grosse Beachtung gefunden. Über die Stabilitätsprüfung verschiedener Östrenol-Tablettenzubereitungen liegt bereits eine qualitative Untersuchung von FOKKENS UND POLDERMAN[1] vor. Mit Hilfe der Dünnschichtchromatographie stellten die Autoren fest, dass die Östrenole und ihre Arzneiformen in Abhängigkeit von der 17α-Substitution unterschiedliche Stabilität aufweisen. Durch Zufügen entsprechender Stabilisatoren konnte, so weit aus den qualitativen Untersuchungen zu schliessen war, die Haltbarkeit wesentlich verbessert werden.

Es erschien uns nun interessant, diese qualitativen Befunde mit einer geeigneten quantitativen Methode eingehend zu prüfen. Da bei der Umsetzung der Östrenole zahlreiche Sekundärprodukte auftreten, war es naheliegend eine Methode zu suchen, womit das noch vorhandene unveränderte Östrenol bestimmt werden konnte. Wir benutzten dazu das von den genannten Autoren verwendete dünnschichtchromatographische Verfahren zum Abtrennen des noch vorhandenen Östrenols von seinen Umsetzungsprodukten und arbeiteten eine Methode zur quantitativen Bestimmung der Östrenolflecke aus.

Verschiedene Möglichkeiten wurden in Betracht gezogen, zum Beispiel die Auswertung der Fleckenfläche wie es von PURDY UND TRUTER[2-4], sowie von anderen Autoren[5] angegeben wurde und die kolorimetrische Bestimmung der Östrenole mit einem geeigneten Reagenz nach Lokalisieren auf dem Chromatogramm und Extraktion in Anlehnung an eine Methode wie sie z.B. früher für die Analyse von Gallensäure beschrieben wurde[6,7].

EXPERIMENTELLER TEIL

Verwendete Geräte

(1) Perkin-Elmer 4000 A, Spectracord, Spektrophotometer.

(2) Ultraviolett-Strahlungsquelle zum Nachweis der Flecke: Ultra-Violett Equipment, C-3, Black Light Eastern Corp., Bayside, N.Y.

(3) Schablone zur Herstellung der Sorptionsschichten: Unoplan®-Leveller No. 2810, Shandon Scientific Company Ltd., 65 Pound Lane, London N.W. 10.

(4) Streichgerät zur Herstellung der Sorptionsschichten: Selbsthergestelltes Gerät für 250 μ Schichtdicke.

(5) Glastrennkammern der Fa. Desaga, Hauptstr. 60, Heidelberg.

(6) λ-Pipetten.

Reagentien

Kieselgel, G, Kieselgel H und Kieselgel HF (Merck, Darmstadt).

Heptan, normal (Merck, Darmstadt, No. 4365).

Aceton, p.a. (Merck, Darmstadt).

Methylenchlorid, frisch destilliert. (Mit diesem Methylenchlorid hergestellter Blindwert darf sich nicht anfärben).

Schwefelsäure 95–97 %, p.a. (Merck, Darmstadt).

Verdünnte Schwefelsäure: Schwefelsäure $^7/_3$ = Schwefelsäure–Wasser (70:30, V/V).

Verdünnte Schwefelsäure: Schwefelsäure $^8/_2$ = Schwefelsäure–Wasser (80:20, V/V).

Schwefelsäure in Äthanol: Schwefelsäure–Äthanol (1:99, V/V).

Essigsäure 96 %, p.a. (Merck, Darmstadt, No. 90061).

Vanillin-Reagenz: 400 mg Vanillin in 10 ml Eisessig auflösen.

Durchführung der Untersuchungen

(a) Auswertung der Fleckenflächen nach Besprühen mit Schwefelsäure

Um möglichst homogene Sorptionsschichten herstellen zu können, wurde die Unoplan®-Schablone, die einen ebenen Übergang der Trägerglasplatten auch bei unterschiedlicher Dicke gewährleistet, verwendet. Als Sorptionsmaterial diente ohne Gipszusatz hergestelltes Kieselgel H bzw. HF, dessen Suspension die Konsistenz beim Stehen nicht verändert. Das Mengenverhältnis Kieselgel–Wasser wurde wie allgemein üblich[8] gewählt. Das Sorptionsmaterial wurde mit einem "Waring-Blender" vollkommen homogen suspendiert und mit einem Streichgerät so gleichmässig wie möglich mit einer Schichtdicke von 250 μ ausgestrichen. Das von der Lieferfirma der Unoplan Schablone bezogene Streichgerät befriedigte nicht. Es ist der Schablone nicht genau angepasst und ermöglicht keine sichere Führung. Daher wurde ein selbst hergestelltes, die Vorteile der Schablone ausnutzendes Streichgerät verwendet. Das Desaga* Streichgerät konnte auf Grund seiner Abmessungen nicht in Verbindung mit der Unoplan-Schlabone eingesetzt werden. Beim Auftragen der Standardlösungen wurden die Hinweise von PURDY UND TRUTER[3,4] befolgt. Es wurden Standardlösungen verschiedener Konzentration hergestellt und immer dasselbe Volume (10 μl), in Form eines Tropfens, mit einer geeichten λ-Pipette, 1.5 cm vom unteren Plattenrand entfernt, aufgebracht.

Nach Einwirken des Fliessmittels — Heptan–Aceton (60:30) — über eine Trennstrecke von 10 cm und Abdampfen des Lösungsmittelgemisches an der Luft, wurde mit Schwefelsäure in äthanolischer Lösung möglichst gleichmässig besprüht und 10 Min. auf 100° erhitzt. Unter U.V.-Licht (366 mμ) wurden die gelb fluoreszierenden Flecke sorgfältig mit einer Nadel aufgezeichnet und der Fleckenumriss mit Hilfe von

* Fa. Desaga, Heidelberg, D.B.R., Hauptstr. 60.

Transparentpapier auf gutes Schreibpapier kopiert, ausgeschnitten und die Papierfläche gewogen. Die Daten einer solchen Untersuchung mit Äthylöstrenol sind in Tabelle I wiedergegeben.

TABELLE I

AUSWERTUNG DER FLECKENFLÄCHE VON ÄTHYLÖSTRENOL*

Aufgebrachte Menge w (μg)	5	10	20	40	80
log w	0.7	1.0	1.3	1.6	1.9
Papiergewicht der kopierten Flecke (mg)	4.72	6.08	8.15	9.40	10.97
Fleckenfläche f (mm^2)	72	94	126	145	169
$\sqrt{f}$	8.5	9.7	11.2	12.0	13.0
S_{rel}, % (bei 4 Bestimmungen)	7.5	7.5	4.8	5.2	3.6

* Die angegebenen Werte sind Mittelwerte von 4 Bestimmungen. 10 mm Fleckenfläche entsprachen 0.65 mg Papiergewicht.

(*b*) *Durchführung der kolorimetrischen Bestimmung nach Chromatographie, Lokalisierung mit Joddampf und anschliessender Extraktion der Östrenolflecke*

Chromatographie. Die Herstellung der Kieselgel-Sorptionsschicht ist hier nicht kritisch. Sowohl Kieselgel G, wie auch H können verwendet werden. Die in Tabelle III angegebene Menge Standard und die in Tabelle V angeführte Menge Tablettenextrakt wird 1.5 cm vom unteren Plattenrand entfernt mit Hilfe von λ-Pipetten aufgetragen. Fliessmittel, Heptan–Aceton (60:30) über eine Trennstrecke von 10 cm einwirken lassen. Nach Abdampfen des Fliessmittels an der Luft, Platte in Jodatmosphäre stellen. Die nach 15–30 Sek. erscheinenden Steroidflecke mit einer Nadel anzeichnen. Anschliessend die Platte solange im Dunkeln bei Zimmertemperatur aufbewahren, bis die braune Anfärbung der Flecke nicht mehr zu sehen ist. Dann die angezeichneten Flecke abkratzen und in 5 ml. Zentrifugengläser mit eingeschliffenen Glasstopfen überführen. In derselben Weise einen leeren Kieselgelfleck als Blindwert I von der Platte abkratzen und verarbeiten.

Kolorimetrische Bestimmung. In alle Zentrifugengläser, die in Tabelle III angeführte Menge Methylenchlorid pipettieren, 3 Min. kräftig schütteln und zentrifugieren. Von der klaren überstehenden Lösung 1 ml in Reagenzgläser pipettieren. In ein weiteres Reagenzglas 1 ml Methylenchlorid abmessen (Blindwert II). Lösungsmittel auf dem Wasserbad unter Verwendung von Wasserstrahl-Vakuum abdampfen. Da die Östrenole lichtempfindlich sind, schnell weiter arbeiten. Wird die Vanillin–Schwefelsäure-Reaktion benutzt, so gibt man zum Rückstand 0.5 ml Vanillin-Reagenz und vorsichtig, so dass zunächst keine Mischung stattfindet 3.5 ml Schwefelsäure $^7/_3$. Anschliessend wird gut umgeschüttelt. Wird die Schwefelsäurereaktion verwendet, so gibt man zum Rückstand 0.5 ml Eisessig und vorsichtig, so dass zunächst keine Mischung stattfindet 3.5 ml Schwefelsäure $^8/_2$. Anschliessend wird wieder gut umgeschüttelt. Nach Aufbewahren der Reaktionsgemische unter den in Tabelle III angeführten Bedingungen misst man in 1 cm-Küvetten bei den angegebenen Wellenlängen unter Verwendung des Methylenchloridblindwertes (Blindwert II). Der Kieselgelblindwert (Blindwert I) darf gegen den Methylenchloridblindwert gemessen, keine Extinktion aufweisen.

Um bei Stabilitätsuntersuchungen den Analysenfehler möglichst nieder zu halten (vgl. Tabelle IV) ist es zweckmässig pro Bestimmung jeweils 3 Standard-

und 3 Tablettenextraktflecke auf eine Platte aufzubringen. Die erhaltenen Extinktionswerte für Standard und Tablettenextrakt werden dann gemittelt und daraus der Gehalt pro Tablette berechnet.

DISKUSSION DER ERGEBNISSE

Bei dem Versuch die Fleckenflächen chromatographierter Standardmengen der Östrenole quantitativ auszuwerten, fanden wir auch im vorliegenden Fall die von PURDY UND TRUTER[3] angegebene lineare Abhängigkeit zwischen der Quadratwurzel der Fleckenfläche und dem Logarithmus der aufgebrachten Menge. Die Reproduzierbarkeit des Verfahrens war jedoch wie aus dem in Tabelle I wiedergegebenen Beispiel zu ersehen ist, auch bei Verwendung möglichst homogener Sorptionsschichten und Aufbringen der Steroidlösungen unter standardisierten Bedingungen nicht befriedigend. Da aus den pharmazeutischen Zubereitungsformen teilweise noch Hilfsstoffe mit extrahiert werden, welche die Fleckenform im Chromatogramm verändern, konnte diese Methode trotz ihres geringen Arbeitsaufwandes nicht für unsere quantitativen Stabilitätsprüfungen verwendet werden. Die Bestimmung der an die Östrenolflecke in Jodatmosphäre addierbaren beziehungsweise adsorbierten Jodmenge durch Kolorimetrie oder Mikrotitration schien eine weitere analytische Möglichkeit zu sein. Eine entsprechende Methode für die Papierchromatographie wurde kürzlich von DITTRICH[9] beschrieben. Über unsere Ergebnisse bei der Auswertung von Dünnschichtchromatogrammen soll nach Abschluss der Untersuchungen an anderer Stelle berichtet werden.

Günstige Resultate erhielten wir mit der dritten in Erwägung gezogenen Methode. Die Östrenolflecke wurden mit Joddampf lokalisiert, extrahiert und anschliessend mit Schwefelsäure bzw. mit Vanillin–Schwefelsäure kolorimetrisch bestimmt. Der Vorteil dieser Methode ist nicht allein die gute Reproduzierbarkeit, sondern auch die relativ spezifische Anfärbung in Abhängigkeit von der C-17α-Substitution. Aus Tabelle II

TABELLE II

EXTINKTIONSMAXIMA DER ÖSTRENOLE BEIM ANFÄRBEN MIT SCHWEFELSÄURE UND VANILLIN–SCHWEFELSÄURE UNTER GLEICHEN BEDINGUNGEN

Östrenol		*Schwefelsäure*		*Vanillin–Schwefelsäure*	
		nm	*E**	*nm*	*E***
17-Äthyl		420	0.046	470	0.835
17-Allyl	I	422	0.321	450	0.310
	II			540	0.260
17-Äthinyl	I	375	1.699	520	0.149
	II	515	0.276		
6-Methyl-17-äthinyl	I	375	1.523	520	0.180
	II	508	0.403		
17-Propargyl		380	0.125	540	0.125

* 100 μg, 20 Min., Zimmertemperatur.
** 50 μg, 1 St., 40°.

geht hervor, dass 17-Allyl-, 17-Äthinyl- und 6-Methyl-17-äthinyl-östrenol mit Schwefelsäure Anfärbungen ergeben, die im sichtbaren Bereich des Spektrums ein

TABELLE III

DATEN ZU DER QUANTITATIVEN ANALYSENVORSCHRIFT FUR DIE ÖSTRENOLE

	Östrenol				
	17-Äthyl	*17-Allyl*	*17-Äthinyl*	*6-Methyl-17-äthinyl*	*17-Propargyl*
Konzentration der verwendeten Standardlösung Methylenchlorid (μg/ml)	2	5	5	5	2.5
Auf die Platte aufgebrachte Mengen, μg (μl)	60 (30)	200 (40)	200 (40)	100 (20)	100 (40)
Verwendete Volumen Methylenchlorid für die Extraktion der Flecke aus dem Kieselgel (ml)	3	2	2	2	2
Vom Extrakt jeweils 1 ml für die Farbreaktion eingesetzt, entsprechend folgenden Mengen Östrenol (μg)	20	100	100	50	50
Reagenz für die kolorimetrische Bestimmung	Vanillin–Schwefelsäure	Schwefelsäure	Schwefelsäure	Schwefelsäure	Vanillin–Schwefelsäure
Reaktionsbedingungen und Messwellenlänge (nm)	1 Std., 40° 470	1 Std., 40° 422	20 Min., Z.T.* 515	20 Min., Z.T. 508	1 Std., 60° 545
Für die angegebenen Mengen Östrenol im Mittel gefundene Extinktion, 1 cm Schichtdicke	0.324	0.390	0.276	0.202	(0.366)
Entsprechend Tabelle IV bestimmte relative Standardabweichungen S_{rel} (%)	3.2	4.0	3.2	4.3	nicht bestimmt
Linearitätsprüfungen (Mittel von je 3 Bestimmungen)	20 μg, E 0.324 30 μg, E 0.500 40 μg, E 0.677	50 μg, E 0.195 100 μg, E 0.390 200 μg, E 0.781	100 μg, E 0.276 200 μg, E 0.545 300 μg, E 0.810	50 μg, E 0.202 100 μg, E 0.403 125 μg, E 0.509	nicht bestimmt
Im Mittel nach Chromatographie wiedergefundene Menge, im Vergleich mit direkt bestimmten Standardmengen (%)	95	95	94	96	90

* Z.T. = Zimmertemperatur.

Maximum besitzen, die 17-Äthyl- und Propargylverbindung nicht. Die Äthylverbindung weist dagegen mit Vanillin-Schwefelsäure ein Spektrum mit einem im sichtbaren Bereich liegenden, gut auswertbaren Maximum auf. Propargylöstrenol zeigt auch mit diesem Reagenz kein ausgeprägtes Spektrum. Um diese Verbindung quantitativ bestimmen zu können, mussten daher die Reaktionsbedingungen auf 1 St., 60° abgeändert werden. Bei keinem der untersuchten Steroide wurde das Spektrum durch die Lokalisierung mit Joddampf verändert. Auch andere Autoren, die Joddampf zum Auffinden von getrennten Substanzen verwendeten, sahen keine Abweichungen bei der nachfolgenden U.V.-spektrophotometrischen Bestimmung[10,11] oder der kolorimetrischen Auswertung im sichtbaren Bereich[12]. Nur bei der Analyse von Triglyceriden wurde bisher über Schwierigkeiten berichtet. Durch Jodierung der mehrfach ungesättigten Fettsäuren trat ein Verlust derselben bei anschliessender Gaschromatographie auf[13]. Die Lokalisation der Östrenole war auch mit Sorptionsschichten, die 0.02–0.03 % 3,5-Dihydroxypyren-8,10-disulfonsäure Natriumsalz enthielten, unter U.V.-Licht möglich, so wie es von TSCHESCHE und Mitarbeitern beschrieben wurde[14]. Die für die Östrenolanalyse verwendeten Fliess- und Extraktionsmittel lösen die genannte Substanz nicht aus der Schicht.

Einzelheiten über die Ausführung der Schwefelsäuremethoden sind in Tabelle III zusammengefasst. Die Reproduzierbarkeit innerhalb der Platten wurde als relative

TABELLE IV

REPRODUZIERBARKEIT DER ANALYSE VON ÄTHYLÖSTRENOL INNERHALB DER PLATTEN

Platte	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	x_9	x_{10}
$E \times 1000$	314	337	319	328	324	347	310	328	323	337
	319	338	293	319	335	337	328	308	310	326
	337	335	323	337	324	319	321	308	307	326
$\bar{x}$	323	337	312	328	328	334	320	315	313	330
$\Sigma(x_i - \bar{x})^2$	293	5	530	162	81	404	165	268	145	81

$$S = \sqrt{\frac{\Sigma(x_{i_1} - \bar{x})^2 + \Sigma(x_{i_2} - \bar{x})^2 + \cdots + \Sigma(x_{iM} - \bar{x}M)}{N - M}} = \sqrt{107} = \pm\ 10.4$$

$$S_{rel} = \frac{S \times 100}{W} = \frac{10.4 \times 100}{324} = \pm\ 3.2\,\%$$

$\bar{x}$ = Mittelwert der Platte; x_i = Einzelner Messwert; N = Zahl der Messungen = 30; M = Zahl der Platten = 10; W = Mittelwert aller Extinktionen; S = Standardabweichung, absolut; S_{rel} = Standardabweichung, relativ.

Standardabweichung berechnet, so wie es am Beispiel von Äthylöstrenol in Tabelle IV genau wiedergegeben ist. Es wurden Fehler von ±3.2 bis ±4.3 % gefunden. Die Grössenordnung dieser Fehler ist dieselbe wie sie früher auf dem Steroidsektor von anderen Autoren bei der kolorimetrischen Bestimmung von Gallensäuren nach dünnschichtchromatographischer Trennung[6,7] und bei der U.V.-spectroskopischen Analyse von 6-Chlor-17α-hydroxypregna-4,6-dien-3,20-dionacetat[15] gefunden wurde. Der

TABELLE V

HALTBARKEITSPRÜFUNGEN VON ÖSTRENOL-TABLETTEN

*Präparat**	*Stabilisator*	*Besondere Vermerke*	*Östrenol wiedergefunden (mg)***						
			0-Zeit	*3 W Kühlschrank*	*6 W 37°*	*12 W 37°*	*3 W 45°*	*6 W 45°*	*12 W 45°*
Orgabolin®-Tabletten	—	—	—	2.05	—	0.06	0.19	0.05	—
(2.1 mg Äthylöstrenol pro 200 mg Tablette)	—	mit 0.4 mg Paraffin pro Tablette	—	1.94	—	0.41	1.94	0.40	—
	α-Tocopherol	—	—	2.00	—	1.85	1.92	1.89	—
Gestanon®-Tabletten	Komplexon	Magnesiumstearat[a]	4.79	—	4.90	4.86	4.44	4.36	4.50
(5 mg Allylöstrenol pro 250 mg Tablette)	Komplexon	Magnesiumstearat[b]	4.74	—	4.89	4.67	5.10	4.56	4.53
	Komplexon	Magnesiumstearat[c]	4.90	—	4.52	4.93	4.22	4.15	4.47
	Komplexon	Magnesiumstearat[d]	4.78	—	4.68	4.97	4.69	4.71	4.56
	Komplexon	Magnesiumstearat[e]	4.68	—	4.58	4.69	4.41	4.48	4.49
Lyndiol®-Tabletten	α-Tocopherol	Muster a	—	—	—	—	4.70	4.91	—
(5 mg Äthinylöstrenol pro 100 mg Tablette)	α-Tocopherol	Muster b	—	—	—	—	4.65	5.11	—

* Extraktionsmethode: Orgabolin®-Tabletten, 200 mg Tablettenpulver + 2 ml Methylenchlorid, 5 Min. schütteln, zentrifugieren; 60 μl auftragen.
Gestanon®-Tabletten, 250 mg Tablettenpulver + 1 ml Methylenchlorid, 5 Min. schütteln, zentrifugieren; 40 μl auftragen.
Lyndiol®-Tabletten, 80 mg Tablettenpulver + 1 ml Methylenchlorid, 5 Min. schütteln, zentrifugieren; 50 μl auftragen.

** W = Wochen; — = nicht durchgeführt.

von MATTHEWS und Mitarbeitern[10] bei der U.V.-spektrophotometrischen Bestimmung von Testosteron und 4-Chlor-17α-hydroxy-progesteron nach Dünnschichtchromatographie gefundene Fehler ist dagegen sehr klein. Jedoch wurden von diesen Autoren zu wenig Analysen durchgeführt, um die Resultate mit den oben genannten Ergebnissen vergleichen zu können. Wie aus Tabelle III hervorgeht, wurde bei den vorliegenden Untersuchungen auch innerhalb eines relativ grossen Konzentrationsbereiches Linearität zwischen eingesetzter Östrenolmenge und Messwert gefunden. Beim Vergleich der Messwerte von nicht chromatographierten und chromatographierten Östrenol-Standardmengen wurden mit Ausnahme von Propargylöstrenol nach einmaligem Extrahieren im Mittel 95 % der eingesetzten Substanzmenge wiedergefunden (Tabelle III). Wie die Linearitätsuntersuchungen zeigten, ist die zurückgewonnene Menge im untersuchten Bereich bei Verwendung gleicher Volumina Extraktionsmittel nicht von der eingesetzten Substanzmenge abhängig. Auf wiederholtes Extrahieren wurde daher verzichtet, um das Verfahren möglichst einfach zu gestalten.

Mit dieser Methode wurden die in Tabelle V angeführten Tablettenzubereitungsformen untersucht. Aus den beschleunigten Haltbarkeitsprüfungen der Äthylöstrenoltabletten (Orgabolin®-Tabletten) geht hervor, dass die früher von FOKKENS UND POLDERMAN[1] angegebene Stabilisierung des oxydationsempfindlichen Äthylöstrenols in Tablettenform mit α-Tocopherol, wie die jetzt durchgeführten quantitativen Untersuchungen zeigen, ausserordentlich wirksam ist. Der in den ersten Wochen der Stabilitätsprüfung auftretende Schutzeffekt, den geringe, den Tabletten zugesetzte, Paraffinmengen ausüben, ist bisher noch nicht geklärt. Bei den Allylöstrenol enthaltenden Tabletten (Gestanon®-Tabletten) wurde auf Grund qualitativer chromatographischer Befunde eine Abhängigkeit der Stabilität von der verwendeten Magnesiumstearatqualität erwartet. Diese Vermutung konnte jedoch durch die quantitativen Analysen, wie aus Tabelle V hervorgeht, nicht bestätigt werden. Vielmehr waren alle geprüften Muster weitgehend stabil. Lediglich bei längerer Einwirkung höherer Temperatur (3, 6 und 12 Wochen, 45°) wurde ein Absinken des Mittelwertes aller Muster beobachtet. Auch das in Lyndiol®-Tabletten als Wirkstoffkomponente enthaltene Äthinylöstrenol war wie Tabelle V zeigt in zwei mit Tocopherol stabilisierten Mustern gut haltbar.

Nach Prüfung einer grossen Zahl von Tablettenmustern können wir sagen, dass die beschriebene quantitative Methode die qualitativen, chromatographischen Befunde wertvoll ergänzt. Bei der chromatographischen Prüfung werden manchmal geringe Mengen von Umsetzungsprodukten erkannt, die bei der quantitativen Bestimmung des noch vorhandenen Östrenols nicht erfasst werden können. Geht der Abbau jedoch über 5 bis 10 % hinaus, so kann nur durch quantitative Bestimmung des noch vorhandenen, unveränderten Östrenols die weitere Zersetzung verfolgt werden.

DANK

Für die sorgfältige Durchführung der zahlreichen Analysen danken wir Frl. B. VAN BOEKEL und Frl. J. MASTWIJK.

ZUSAMMENFASSUNG

Für die Stabilitätsprüfung von Δ^4-17β-Hydroxyöstren-Derivaten in Tabletten wurde eine quantitative Methode ausgearbeitet. Die Östrenole werden mit Hilfe der Dünnschichtchromatographie von Umsetzungsprodukten und Begleitstoffen abgetrennt. Nach Lokalisation mit Joddampf werden die Steroidflecke aus der Sorptionsschicht extrahiert, das Lösungsmittel abgedampft und anschliessend mit Schwefelsäure oder Vanillin–Schwefelsäure spektrophotometrisch bestimmt. Mit dieser Methode wurden Tabletten geprüft, welche 17α-Äthyl-, 17α-Allyl- oder 17α-Äthinylöstrenol enthielten. Am Beispiel der Äthylöstrenol-Tabletten ist zu sehen, dass in Abhängigkeit von Tablettenhilfsstoffen und zugesetzten Stabilisatoren grosse Haltbarkeitsunterschiede auftreten. Jedoch zeigten die verschiedenen Östrenole in den geprüften im Handel befindlichen Präparaten durch die darin befindlichen Stabilisatoren gute Haltbarkeit.

SUMMARY

A method for the quantitative determination of Δ^4-17β-hydroxyestrene derivatives has been worked out. The estrenols are first separated by thin-layer chromatography and after localisation with iodine vapour, the steroid spots are extracted. The residue from the evaporated extract is then determined by a colorimetric method, using sulphuric acid or vanillin–sulphuric acid as reagent. With this method the stability of tablets containing 17α-ethyl-, 17α-allyl- or 17α-ethinylestrenol has been tested. As has been shown with ethylestrenol tablets, there are large differences in stability depending on the excipients and the chosen stabiliser. However, the stability of the estrenol tablets tested, which were stabilised with different suitable additives, was found to be quite good.

LITERATUR

1 J. Fokkens und J. Polderman, *Pharm. Weekbl.*, 96 (1961) 657.
2 S. J. Purdy und E. V. Truter, *Chem. Ind.* (*London*), (1962) 506.
3 S. J. Purdy und E. V. Truter, *Analyst*, 87 (1962) 802.
4 E. V. Truter, *Thin Film Chromatography*, Cleaver-Hume Press Ltd., London, 1963, S. 112.
5 J. Aurenge, M. Degeorges und J. Normand, *Bull. Soc. Chim. France*, (1963) 1732.
6 H. G. Gänshirt, F. W. Koss und K. Morianz, *Arzneimittel-Forsch.*, 10 (1960) 943.
7 E. Stahl, *Dünnschichtchromatographie*, Springer Verlag, Berlin, 1962, S. 47.
8 K. Randerath, *Dünnschichtchromatographie*, Verlag Chemie, Weinheim, Bergstr., 1962, S. 29.
9 S. Dittrich, *J. Chromatog.*, 12 (1963) 47.
10 J. S. Matthews, A. L. Pereda V. und A. Aguilera, P., *J. Chromatog.*, 9 (1962) 331.
11 H. G. Gänshirt, *Arch. Pharm.*, 296 (1963) 129.
12 E. Vioque und R. T. Holman, *J. Am. Oil Chemists' Soc.*, 39 (1962) 63.
13 M. Z. Nichaman, C. C. Sweeley, N. M. Oldham und R. E. Olson, *J. Lipid Res.*, 4 (1963) 484.
14 R. Tschesche, G. Biernoth und G. Wulff, *J. Chromatog.*, 12 (1963) 342.
15 H. L. Bird, H. F. Brickley, J. P. Comer, P. E. Hartsaw und M. L. Johnson, *Anal. Chem.*, 35 (1963) 346.

THIN-LAYER CHROMATOGRAPHY OF DEOXYRIBONUCLEIC ACID ON ECTEOLA CELLULOSE

R. D. BAUER AND K. D. MARTIN

Department of Chemistry, California State College at Long Beach, Long Beach, Calif. (U.S.A.)

(Received April 23rd, 1964)

INTRODUCTION

Thin-layer chromatography has found wide application in the past few years, with notable success in the separation and detection of nucleic acid derivatives including deoxyribooligonucleotides[1]. Recently, JOSEFSSON[2] reported a semi-quantitative micro-determination of nucleic acid derivatives on cellulose powder using thin-layer adsorbent techniques. However, studies have been lacking in the application of this technique to the nucleic acids themselves. Since ECTEOLA cellulose has been found to be a good support for the column chromatography of deoxyribonucleic acid (DNA)[3], this seemed to be the support of choice for thin-layer chromatography. ROSENKRANZ AND BENDICH[4] showed a correlation between the chromatographic migration using ECTEOLA as a column support and the sedimentation coefficient of DNA. This technique has shown wide application in ascertaining the molecular state of the nucleic acid. Also, these workers have reported a correlation between the migration of DNA on paper and the sedimentation coefficient[5].

This study shows how ECTEOLA-cellulose may be used as a support for the convenient thin-layer chromatography of DNA. A correlation between the thin-layer and column techniques using the same chromatographic support is made here. The results of these chromatographic techniques can also be related to the sedimentation coefficients as obtained by ultracentrifugation.

EXPERIMENTAL

Calf thymus DNA (sodium salt), as obtained from Worthington Biochemical Corporation, Freehold, New Jersey, was used in this study.

Heat treatment

A solution of DNA (2 mg/ml of distilled water) was heated for varying amounts of time ranging from five minutes to one hour and then rapidly cooled in an ice bath. At all conditions other than heat treatment the DNA was dissolved in 0.01 *M* phosphate buffer, pH 7.

Column chromatography

Fractionation of unheated and heated DNA was carried out on columns of ECTEOLA (capacity 0.39 mequiv./g, lot No. 1079, obtained from Brown Company, Berlin, New

Hampshire) using a gradient elution system as outlined by BENDICH *et al.*[3]. Heated and control DNA samples were chromatographed on parallel columns fed by a common two mixing chamber system. This was used so that ready comparison of the elution patterns could be obtained. The flow was controlled by pumping the solvents through the columns using a peristaltic pump (obtained from Harvard Apparatus Company, Inc. Dover, Mass.) at a rate of 4 ml per hour. Ten ml fractions were collected and the absorbance of each fraction at 260 mμ was determined using a Beckman DU Spectrophotometer. A complete analysis of a DNA sample by this method usually requires approximately 20 days.

Ultracentrifugal analysis

Solutions of heated and control DNA in 0.01 *M* phosphate buffer, pH 7, were analyzed at 59,780 r.p.m. in a Spinco Model E ultracentrifuge equipped with ultraviolet optics. The samples were diluted to a constant absorbance of 0.70 optical density. Average sedimentation coefficients were calculated for each sample after the ultracentrifuge patterns were converted into plots of concentration *versus* distance by use of a Spinco model R Analytrol equipped with a microanalyzer attachment.

Thin layer chromatography

Serva ECTEOLA-TLC prepared for thin layer chromatography (obtained from Gallard-Schlesinger Chem. Mfg. Co., Garden City, N.Y.) was used. Eight grams of the ECTEOLA-TLC were suspended in 50 ml of distilled water and then applied to 8 $\times$ 8 in. glass plates with Research Specialities Company equipment. It was found desirable to sieve the dry resin through a 61 μ sieve prior to use. The plates were dried at room temperature. A solution of 1.0 *M* NH_3 in 2 *M* NaCl and 0.01 *M* phosphate (pH 11.0) was found to be the solvent of choice. This is the same solvent used in the next to the last step in the column fractionation procedure. This has also been reported as the solvent of choice in paper chromatography of nucleic acids[5]. The plates were allowed to equilibrate overnight in the presence of the solvent prior to use. It was found necessary to actually prewet the plates prior to the DNA application by allowing the solvent to rise 1.5 cm above the application point. The DNA samples were applied to this prewet plate by means of a micropipet. The DNA failed to migrate to any detectable extent without this prior moistening. The plates were then developed by allowing migration of the solvent to a level about 14 cm beyond the point of application. This usually required about 90 min. All chromatography was carried out at room temperature. After drying the plates, the DNA spots were detected and marked under ultraviolet light. Although some tailing was almost always noted, the rates of migration could easily be estimated using the most dense portion of the center of the ultraviolet-absorbing areas. This value is reported as the R_F* in this study.

RESULTS AND DISCUSSION

The results of the column chromatography of the control and 60 min heat treated DNA are shown in Fig. 1. These results are consistent with those given by ROSENKRANZ AND BENDICH[6] for the heating of DNA in water. The loss of the more slowly eluted fractions indicate some loss of the higher molecular weight fractions of the DNA upon heat treatment. This heat degradation can also be seen in results of the thin-layer

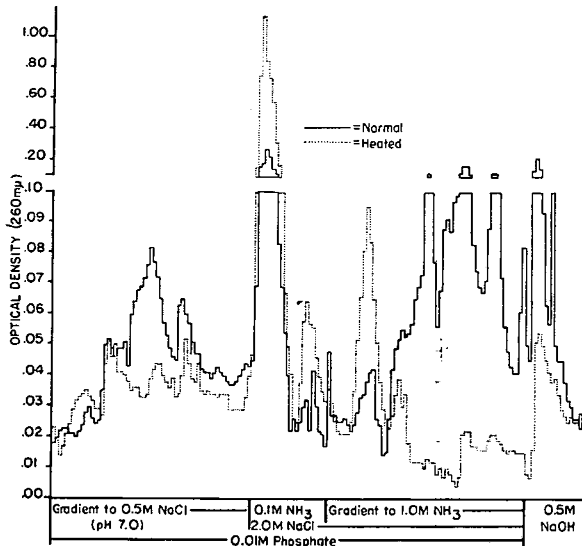


Fig. 1. Chromatographic profiles of native and heated (1 hour) solutions of calf thymus DNA using ECTEOLA cellulose.

chromatography (TLC) in Table I. The increases in heating time, to a maximum of 60 min in this study, yields a degraded DNA sample with a progressive increase in R_F* values. Native DNA showed no observed migration. The increased rate of migration can be correlated with the shift to a greater amount of more easily eluted fraction as seen in the column elution profiles of Fig. 1. The profile displacement in the column chromatography and some of the spreading observed in the ultraviolet absorbing areas which contributes to the rather broad range of the R_F* values (Table I) in the TLC is indicative of the heterogeneous nature of the DNA molecules.

TABLE I

EFFECTS OF HEATING WATER SOLUTIONS OF DNA ON R_F* VALUES OF TLC AND SEDIMENTATION COEFFICIENTS

Time of heating (min)	*Sedimentation values (ave.)*	*ECTEOLA cellulose thin-layer chromatography*	
		R_F*	*No. of samples*
0	24.2	0	24
5	12.1	0.46 ± 0.05	11
10	11.5	0.53 ± 0.12	10
15	10.1	0.54 ± 0.05	10
30	6.4	0.61 ± 0.10	12
60	5.3	0.76 ± 0.10	15

It should be re-emphasized that without the prewet procedure in the TLC no satisfactory migration could be observed for any of the DNA samples. This effect must be due to the irreversible binding of the DNA to the dry ECTEOLA cellulose. This observation is perhaps not unexpected since column chromatography is always

carried out under conditions in which the support is moistened with the initial solvent.

Table I also shows the effects of heating on the average sedimentation coefficient of the DNA. The marked change in the $S_{ave.}$ of 24.2 to 5.3 upon heating for 60 min. is reflected in the R_F* change from essentially zero, or no observable migration under these conditions, to a value of 0.76. The relationship between the calculated sedimentation coefficients and the R_F* values for the various heat degraded DNA samples is shown in Fig. 2. The changes observed in the rates of migration using TLC must then

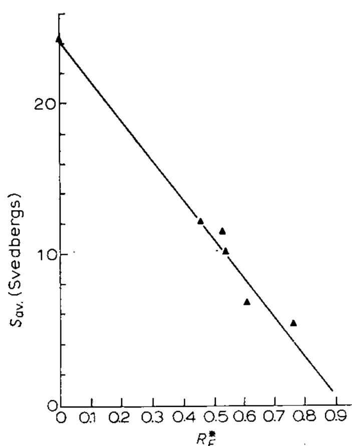


Fig. 2. Correlation between average sedimentation coefficients and R_F* values from thin-layer chromatography of a series of heat-degraded calf thymus DNA samples.

be related to changes in the physicochemical properties of the DNA as demonstrated by ultracentrifugation studies. These data are quite consistent with those correlating sedimentation rates with both column chromatography[4] and paper chromatography[5]. Thus, sedimentation values of DNA samples can be quickly and simply approximated from TLC studies.

Thin-layer chromatography has also been applied to a variety of other nucleic acid samples including commercially available herring sperm DNA and yeast RNA. An average R_F* value of 0.77 was obtained for the herring sperm DNA. The yeast RNA showed a major spot with an R_F* value of 0.81 and three much lighter spots with lower R_F* values. The differences between the lack of migration of the highly polymerized calf thymus DNA and the rapid migration of these samples reflects the ability of this system to discriminate among macromolecules. In this way, TLC might well be applied as a convenient technique in the preliminary separation of a number of physicochemically distinct nucleic acid samples.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. D. OMIECZYNSKI of the Long Beach Veterans Administration Hospital for carrying out the work on the ultracentrifuge. This investigation was supported by research grant A-5169 from The National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

SUMMARY

These studies indicate that convenient thin-layer chromatography techniques may be applied to the separation of high molecular weight nucleic acid samples. ECTEOLA-cellulose was used as a support and 1.0 M NH_3 in 2 M NaCl and 0.01 M phosphate was the solvent. This study correlates the R_F* values of DNA using thin-layer chromatography with the behavior of DNA in studies of chromatography as well as ultracentrifugation. Progressive degradation of the DNA by heat is accompanied by an increase in R_F* values.

REFERENCES

1 K. RANDERATH, *Thin-Layer Chromatography*, Academic Press, New York, 1963.
2 L. JOSEFSSON, *Biochim. Biophys. Acta*, 72 (1963) 133.
3 A. BENDICH, H. B. PAHL, G. C. KORNGOLD, H. S. ROSENKRANZ AND J. R. FRESCO, *J. Am. Chem. Soc.*, 80 (1958) 3949.
4 H. S. ROSENKRANZ AND A. BENDICH, *J. Am. Chem. Soc.*, 81 (1959) 902.
5 A. BENDICH AND H. S. ROSENKRANZ, *Arch. Biochem. Biophys.*, 94 (1961) 417.
6 H. S. ROSENKRANZ AND A. BENDICH, *J. Am. Chem. Soc.*, 81 (1959) 6255.

THE SOLUTION CHEMISTRY AND CHROMATOGRAPHIC BEHAVIOUR OF TECHNETIUM IN AQUEOUS HCl AND HBr

L. OSSICINI, F. SARACINO AND M. LEDERER

Laboratorio di Cromatografia del C.N.R., Istituto di Chimica Generale ed Inorganica, Rome (Italy)

(Received April 22nd, 1964)

INTRODUCTION

The literature on the solution chemistry and chromatography of technetium has been reviewed exhaustively in a number of recent articles[1-3] and several studies have been carried out on the behaviour of pertechnetate in HCl solutions. For example, reduction to $TcCl_6^{2-}$ was reported under various conditions and the formation of an unstable intermediate observed.

There seems to be no work so far on the solution chemistry of pertechnetate in HBr solutions, $Tc^{IV}Br_6^{2-}$ being usually prepared from $Tc^{IV}Cl_6^{2-}$ by evaporation with HBr.

The adsorption of pertechnetate on ion exchangers has been studied under various conditions and cationic character was even ascribed to pertechnetate owing to adsorption on cation exchangers.

In our recent work with ion exchange papers we have been able to distinguish between adsorption effects on resins and "true" ion exchange involving the charged groups on the resins and thought that data from ion exchange papers could explain the behaviour of pertechnetate better than column studies. This work is described in the first part of this paper.

While studying the adsorption of various species of Tc on cellulose paper we observed that the unstable intermediate (mentioned above) which seems to be pentavalent Tc is strongly adsorbed from dilute acid solutions and may thus be identified and studied in the presence of other Tc species.

Using this strong adsorption on cellulose paper of Tc(V) in combination with chromatography on cellulose anion exchange paper we were able to study the species present in HCl and HBr solutions. The solution chemistry of Tc in HCl could be amplified considerably and the behaviour in HBr observed. Finally the hydrolysis of $TcCl_6^{2-}$ and $TcBr_6^{2-}$ was investigated in dilute HCl and HBr (respectively). It was possible to show that $TcCl_6^{2-}$ is much more stable to hydrolysis than $TcBr_6^{2-}$.

EXPERIMENTAL

Chromatograms were all carried out by ascending development with small volume jars. Ion exchange papers were all first washed twice with acid (usually 2 *N* HCl) and distilled water and then converted to the required form by equilibrating for 30 min with a normal solution of the required cation or anion.

The technetium used in this work was a solution of ammonium pertechnetate (62.8 mg in 2 ml) supplied by the Radiochemical Centre, Amersham, England. This should be entirely the isotope mass 99 of half life $2.12 \cdot 10^5$ years and was found free of other radioelements by chromatography in various systems.

Two detection methods were used. A coloured spot was obtained by spraying with a solution of $SnCl_2$ and KCNS in 2 *N* HCl, the actual colour obtained depending on the other acids present on the paper, as well as the original valency of the Tc and the time of drying. The reagent also yields a coloured spot with perrhenate. Alternatively radioactive scans of 5 mm sections of the chromatograms were performed with a beta end-window counter. This has the advantage of giving quantitative results but has the disadvantage of not distinguishing closely moving bands which are readily perceived with the colour reagent.

The papers impregnated with inorganic exchangers were prepared by impregnating the paper with solutions of $Al(NO_3)_3$, $Fe(NO_3)_3$ and $ZrOCl_2$, respectively, and exposing these to an atmosphere of NH_3 followed by extensive washing with distilled water and drying in air.

In the work on the solution chemistry of Tc in HCl and HBr only two ion exchange papers were used, the strongly basic cellulose exchange paper of Macherey, Nagel & Co. (containing quaternary ammonium groups, capacity 0.3–0.4 mequiv./g) and the strongly acid cellulose exchange paper of Macherey, Nagel & Co. (containing sulphonic groups, capacity 0.4–0.7 mequiv./g). These were chosen in order to obtain separations of variously charged ions with only the adsorption properties of cellulose in addition to ion exchange effects. As will be seen in section (ii) this permitted the study of reversible complexing of Tc(V).

RESULTS

(i) *The behaviour of pertechnetate and perrhenate on various exchange papers*

Figure 1 shows the R_F values of perrhenate and pertechnetate on sulphonic resin paper, cellulose paper and quaternary ammonium resin paper.

It has already been shown by solvent extraction and paper chromatography with organic solvents that both $HReO_4$ and $HTcO_4$ strongly favour the non-aqueous phase and hence it is not surprising that they also adsorb strongly on the resin network of sulphonic as well as basic resins. This adsorption is highest with HCl and H_2SO_4 and lowest with HNO_3 and $HClO_4$. The separations obtained with perchlorate elution were explained so far by considering the competition between the strongly retained ClO_4^- ions for ReO_4^- and TcO_4^- ions. They may, however, equally well be explained by the competition of poorly hydrated ClO_4^- for adsorption sites on the organic network. The latter view is supported by the lower adsorption on cellulose paper in $HClO_4$ and HNO_3 as compared with HCl and H_2SO_4.

In the systems shown in Fig. 1 good separations were obtained either in moderately concentrated $HClO_4$, HNO_3 or in 8 *N* HCl. The latter is not likely to be due to a partial reduction in HCl as we could show that no reduction occurs in aqueous solution (*i.e.* in absence of the resin) when pertechnetate is allowed to stand in 8 *N* HCl for a time similar to that for development (*i.e.* about 1 h). This larger difference between the TcO_4^- and ReO_4^- spots in HCl is noteworthy as the difference in most other systems is almost constant.

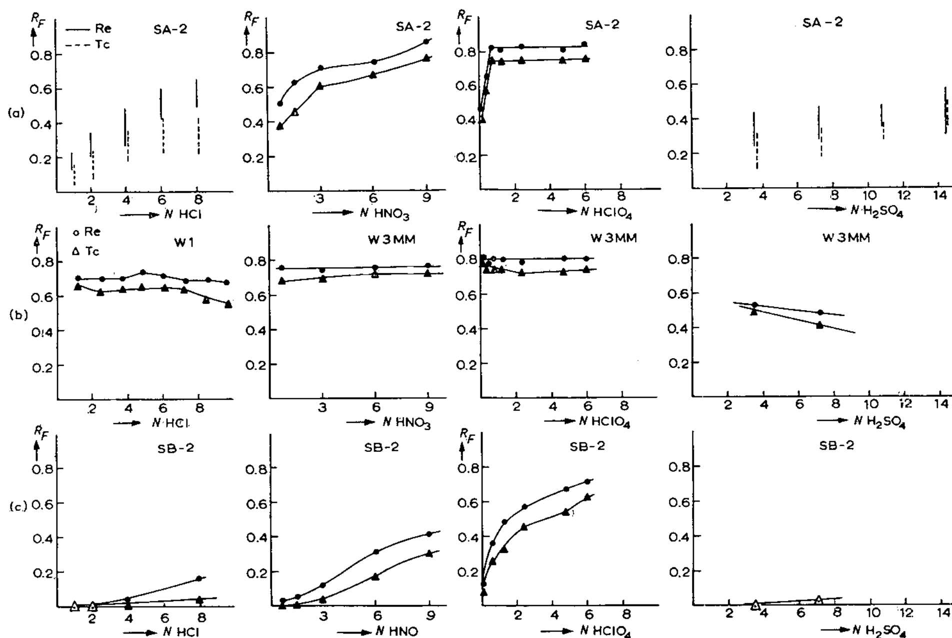


Fig. 1. R_F values of TcO_4^- and ReO_4^- plotted against the concentration of the eluant. (a) On sulphonic resin paper Amberlite SA-2; from left to right HCl, HNO_3, $HClO_4$ and H_2SO_4; (b) on Whatman No. 3MM cellulose paper, sequence as in (a); (c) on quaternary ammonium resin paper Amberlite SB-2, sequence as in (a) ▲ = TcO_4^-; ● = ReO_4^-.

The salting-out effect on various resin and cellulose exchangers with $(NH_4)_2SO_4$ was studied as shown in Fig. 2. Here the differences in R_F values between Tc and Re seem to be almost constant while the adsorption is considerably increased with increased ammonium sulphate concentrations. This rather strong salting-out effect, which is opposite to the mass-action effect which may be expected in ion exchange, again shows that adsorption other than actual ion exchange plays a considerable role in the fixation of pertechnetate on to resins.

The adsorption on some inorganic oxides is shown in Fig. 3. Here papers impregnated with aluminium oxide, ferric oxide and zirconium oxide are compared with pure cellulose paper.

With various concentrations of neutral LiCl no differences between cellulose with and without inorganic oxides were noted and it may be concluded that adsorption in solutions more than 1 N in LiCl is negligible.

(ii) The chromatographic behaviour of Tc(V)

An unstable intermediate has been observed when TcO_4^- was reduced to $TcCl_6^{2-}$ in 6–8 M HCl at 100°[4]. According to CROUTHAMEL[5] this intermediate is most likely to be Tc(V) and while the present work does nothing to confirm this view we shall assume that this is correct and refer to the intermediate compound as Tc(V). In subsequent headings of this paper its stability in HCl and HBr will be discussed. For the studies described here solutions in HCl and HBr were used, in all of which high concentrations of Tc(V) were found during kinetic studies.

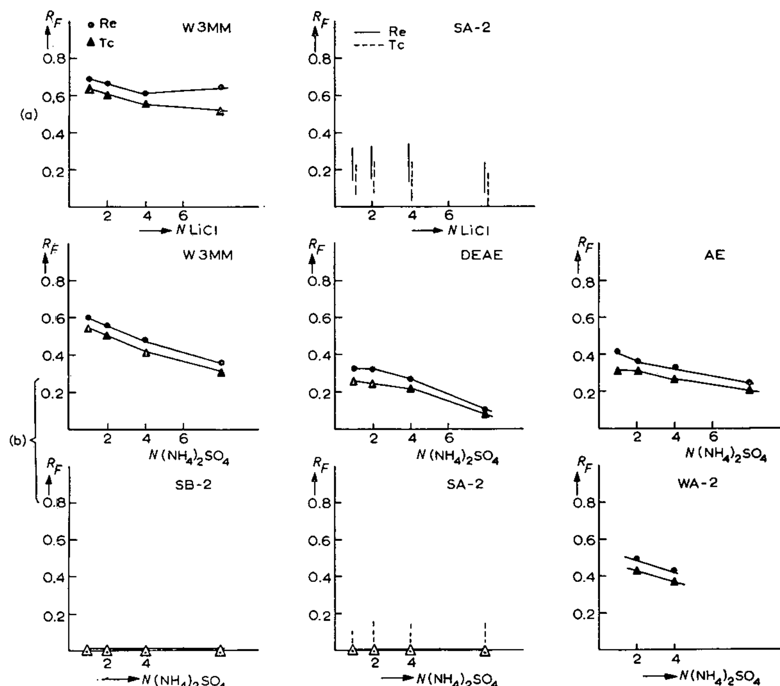


Fig. 2. R_F values of TcO_4^- and ReO_4^- plotted against the concentration of the eluant. (a) With LiCl on Whatman No. 3MM and Amberlite SA-2 paper; (b) with $(NH_4)_2SO_4$ on Whatman No. 3MM, Whatman DEAE paper, Whatman aminoethylcellulose paper, Amberlite SA-2 paper and Amberlite WA-2 paper. ▲ = TcO_4^-; ● = ReO_4^-.

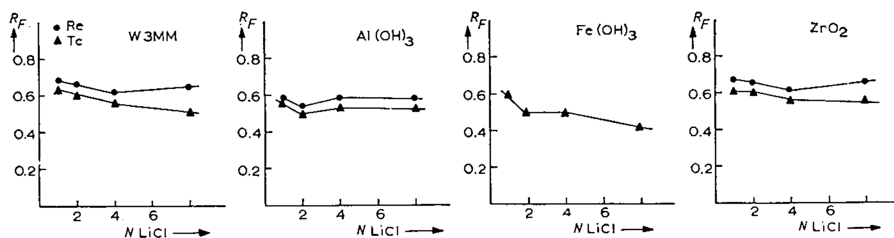


Fig. 3. R_F values of TcO_4^- and ReO_4^- on cellulose paper impregnated with inorganic oxides with LiCl as eluant. From left to right: cellulose paper, $Al(OH)_3$ paper, $Fe(OH)_3$ paper and ZrO_2 paper.

Fig. 4 shows the R_F values of Tc(V) in HCl, HBr and $HClO_4$. The increase in R_F values with the concentration of HCl and HBr would suggest complex formation with halide ions especially as such an increase does not occur with $HClO_4$. Thus the present results may be explained assuming that a neutral oxyhalide is present at low acid concentrations which may complex with halides.

If the R_F values on cellulose paper are correlated with those on the Macherey, Nagel strong anion exchange cellulose paper it is evident that a maximum of anionic behaviour is exhibited at about 1.8 N HBr and 1.2 N HCl (Fig. 4c).

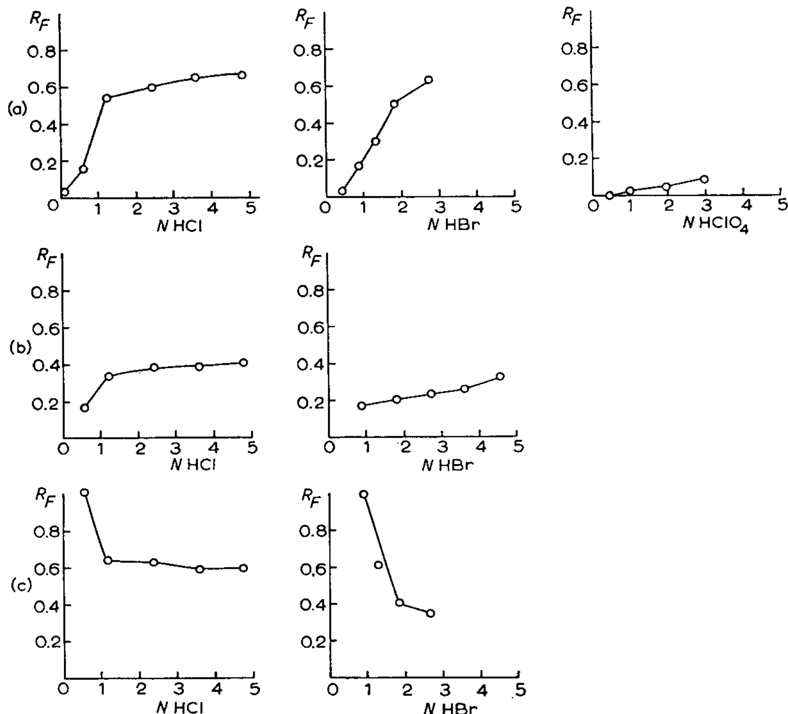


Fig. 4. R_F values of Tc(V). (a) On Whatman No. 3MM paper with HCl, HBr and $HClO_4$ as eluants; (b) on Macherey Nagel strong anion exchange cellulose paper with HCl and HBr as eluants; (c) R_F values on the anion exchange paper "corrected" for adsorption on the cellulose, *i.e.* R_F anion exchange paper/R_F cellulose paper.

The strong adsorption on cellulose paper from dilute acids is a unique feature of Tc(V), which permits its detection in the presence of TcO_4^- as well as all of the Tc(IV) species so far studied which all travel well above R_F 0.5. Tc(V) when sprayed with $SnCl_2$-KCNS reagent instantaneously yields a colour like Tc(VII) while Tc(IV) only appears on standing.

(iii) The reaction of pertechnetate with conc. HCl at room temperature

Fig. 5 shows chromatograms of ammonium pertechnetate dissolved in conc. HCl developed with 0.6 *N* HCl on Whatman No. 3MM paper after 0, 1, 6, 24 and 48 h. Even after 48 h the preponderant species present is the slow-moving Tc(V). The solution turns yellow immediately on contact indicating that a small amount of Tc(IV) is already forming. While chromatograms on the anion exchange paper should have revealed the presence of Tc(VII), if still existent, there was no noticeable spot even at zero time. As the fast peak first decreases and then again increases after 1 hour we believe that a trace (*i.e.* less than 5 %) of Tc(VII) was perhaps present at the beginning but was already reduced after 1 h.

It seems thus that solutions in which about 80 % of the total Tc is present as Tc(V) may be prepared simply by dissolving pertechnetate in cold HCl, and these solutions are stable for at least 48 h.

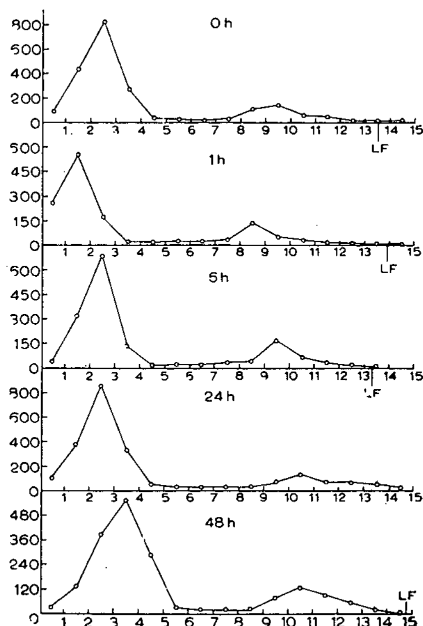


Fig. 5. The reaction of TcO_4^- with conc. HCl at room temperature. Chromatograms on Whatman No. 3MM paper developed with 0.6 N HCl and scanned for radioactivity. Ordinates: activity in counts/30 sec per cm. Abscissae: length of the chromatogram in cm. The point of application is on the left. The slow peak is Tc(V), the small fast peak all species of either Tc(VII) or Tc(IV). LF = liquid front.

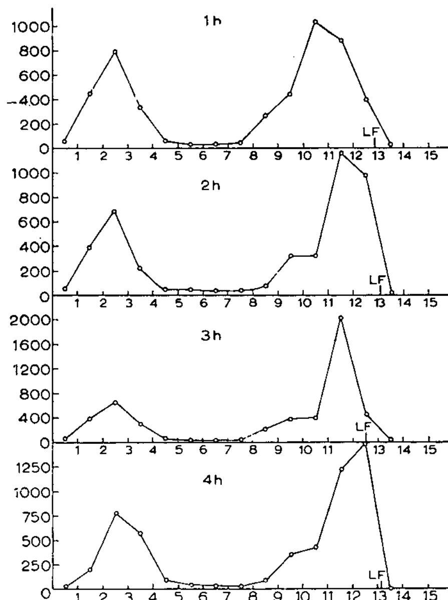


Fig. 6. Reaction of TcO_4^- with conc. HCl at 100°. Chromatograms on Whatman No. 3MM paper developed with 0.6 N HCl and scanned for radioactivity. Ordinates: activity in counts/30 sec per per cm. Abscissae: length of chromatogram in cm. The point of application is on the left. LF = liquid front.

Preliminary experiments in 8 *N* HCl showed that while no reduction of pertechnetate could be detected in one hour there was slow reduction. After 48 h the main species present is still Tc(VII) but both Tc(V) and Tc(IV) could also be detected.

During the work with HCl solutions we also tried to chromatograph on cellulose paper using 0.12 *N* HCl as solvent. After the strips had been dried and scanned for chromatography, the amounts did not correspond to those developed with higher concentrations of HCl. We believe that some Tc volatilised from the paper either during development or on drying. It is not clear which species becomes volatile under these conditions, and we intend to do further work on this problem.

(iv) The reaction of pertechnetate with conc. HCl at 100°

About 1 mg of ammonium pertechnetate was carefully dried in a vacuum desiccator and mixed with 0.25 ml conc. HCl in a micro-test tube and heated on a steam bath. Drops were withdrawn at intervals and chromatographed with 5 % HCl on Whatman No. 3MM paper. The chromatograms were scanned for radioactivity and the scans shown in Fig. 6 were obtained. It could be shown by chromatography on anion exchange paper that no pertechnetate was present after 30 min heating which was of course expected as it is reduced immediately even in the cold in conc. HCl to Tc(V). It is remarkable, however, that Tc(V) is present even after heating for 4 h.

These experiments were carried out three times and the amount of Tc(V) after 30 min was about 50 % and after 1 h about 30 %. The amount of Tc(V) seems to increase again after 3 h but this is due to the formation of crystals of $(NH_4)_2TcCl_6$ and not to a reoxidation of the Tc(IV) already formed.

Some reviews[1,2] quoting GERLIT[6] have stated that TcO_4^- is reduced in conc. HCl in one hour in the cold to $TcCl_6^{2-}$. This is of course not correct, nor was this claimed by GERLIT[6] who merely mentioned that TcO_4^- is completely reduced in one hour without mentioning specifically the valency of the reduction product.

(v) The reaction of pertechnetate with HBr at room temperature

As there seems to be no mention of the solution chemistry of pertechnetate in HBr in the literature so far, we have investigated this reaction in greater detail with various concentrations of HBr.

Fig. 7 shows the chromatograms obtained with Whatman No. 3MM paper using 0.9 *N* HBr as solvent.

In conc. HBr, Tc(V) does not exist any more after 24 h, while it is almost completely reduced in 80 % HBr only after 72 h, and in 60 % HBr begins to form after about 24 h. In up to 40 % HBr pertechnetate is not reduced even after 72 h.

In some HBr solutions Tc(IV), Tc(V) and Tc(VII) can exist in presence of each other and all 3 valencies may be separated on the anion exchange paper as shown in Fig. 8.

Tc(IV) separates here into two slow moving spots of which the slower seems to be $TcBr_6^{2-}$ and the faster $TcBr_5H_2O^-$ as will be shown in section (vii).

(vi) The reaction of pertechnetate with conc. HBr at 100°

Dry ammonium pertechnetate (about 1 mg) was dissolved in 0.25 ml of conc. HBr (Carlo Erba) and heated on a steam bath. After 30 min a sample was withdrawn and could be shown to contain only the $TcBr_6^{2-}$ ion by chromatography on anion exchange paper and Whatman No. 3MM paper.

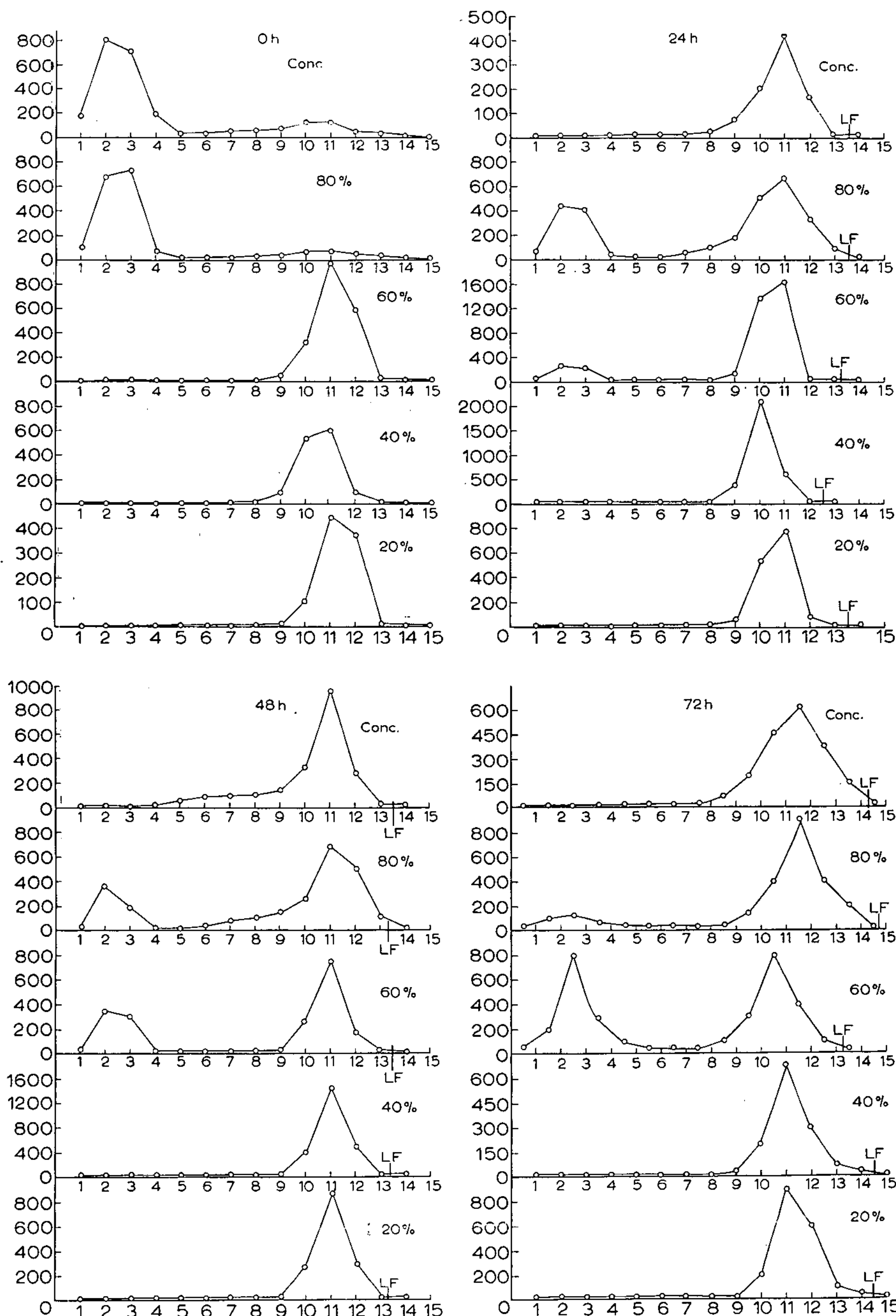


Fig. 7. The reaction of TcO_4^- with HBr at room temperature. Chromatograms on Whatman No. 3MM paper developed with 0.9 *N* HBr as solvent. Representation as in Figs. 5 and 6. From top to bottom: conc. HBr, 80 % HBr, 60 % HBr, 40 % HBr and 20 % HBr. On cellulose paper Tc(V) is separated from all Tc(IV) and Tc(VII) species. LF = liquid front.

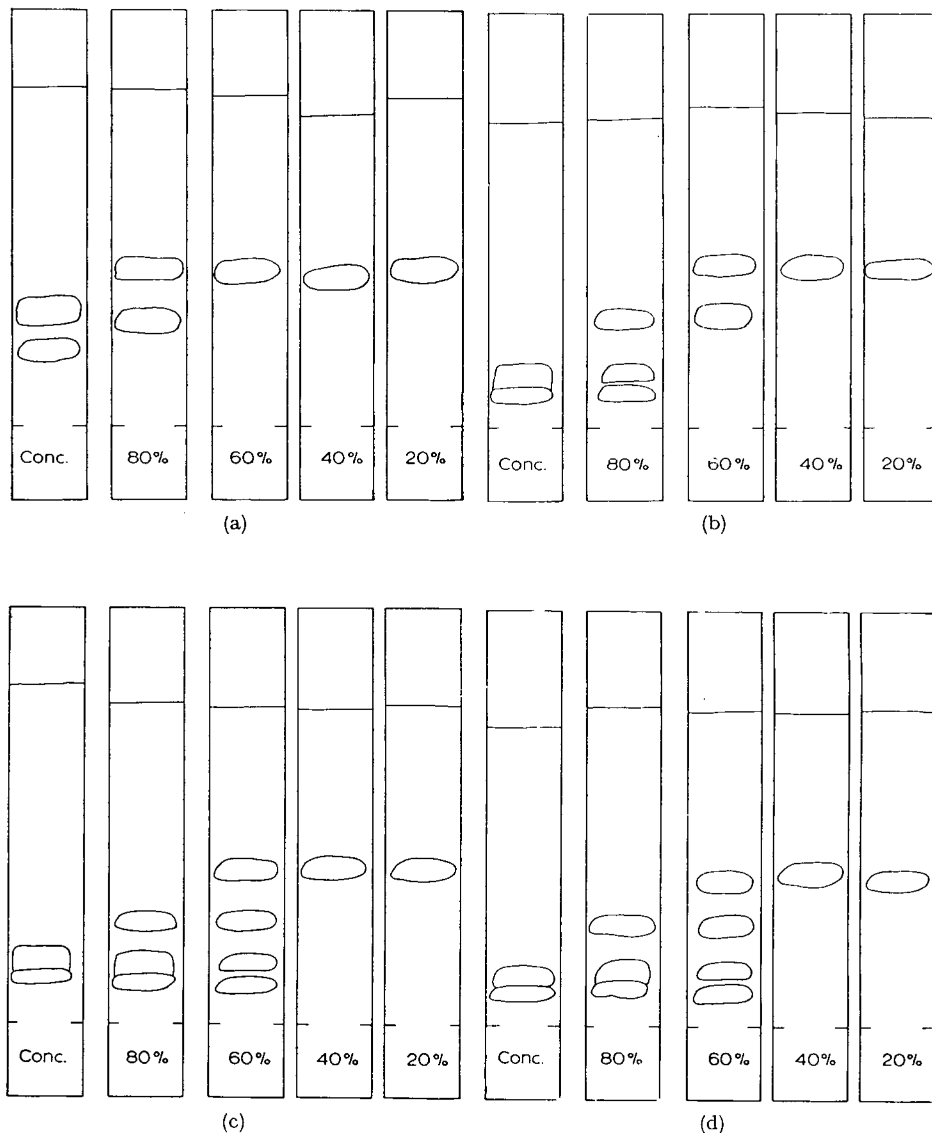


Fig. 8. The reaction of TcO_4^- with HBr at room temperature. Chromatograms on Macherey, Nagel strong anion exchange cellulose paper with 1.8 N HBr as solvent. Designs of chromatograms after spraying with $SnCl_2$–KNCS reagent (a) at 0 h, from left to right conc. HBr, 80% HBr, 60% HBr, 40% HBr and 20% HBr; (b) after 24 h; (c) after 48 h; and (d) after 72 h. The spot R_F 0.49 is TcO_4^-, the spot R_F 0.32 is Tc(V) while the two slow moving spots are species of Tc(IV).

(vii) The hydrolysis of $TcCl_6^{2-}$ *and* $TcBr_6^{2-}$

During the study of the reaction of pertechnetate with HBr as well as in some HCl solutions the intensely coloured halogeno Tc(IV) complexes were found to separate into two adjacent spots on chromatography with anion exchange paper.

It is already well known that both $TcCl_6^{2-}$ and $TcBr_6^{2-}$ undergo hydrolysis in aqueous solutions[7] and hence we suspected that the extra spot formed is due to a hydrolysis product. The study of the hydrolysis of $TcCl_6^{2-}$ and $TcBr_6^{2-}$ described below confirmed this and gave a general idea of the number of hydrolysed species which can be expected in dilute acid solutions.

(*a*) *The hydrolysis of* $TcBr_6^{2-}$ *in 0.9 N HBr at room temperature.* H_2TcBr_6 was prepared by heating pertechnetate with conc. HBr and diluting to 0.9 *N*. A chromatogram of this residue dissolved in 10 % HBr yielded one prominent peak of R_F 0.08 and traces only of other species when chromatographed with 20 % HBr on anion exchange paper. Fig. 9 shows scans of the radioactivity of chromatograms run after

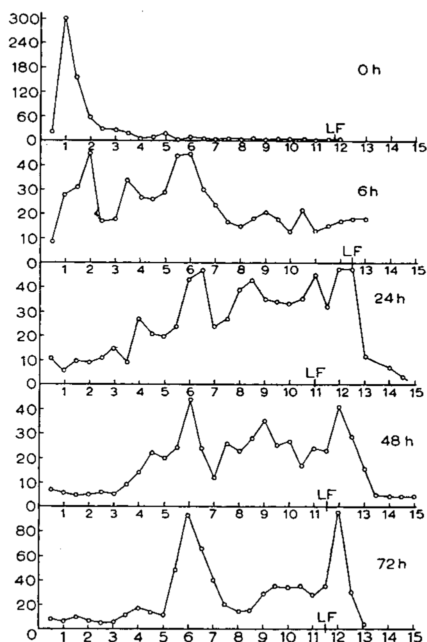


Fig. 9. Hydrolysis of $TcBr_6^{2-}$. Radioactivity scans of chromatograms on Macherey, Nagel strong anion exchange cellulose paper with 1.8 *N* HBr. Ordinates: activity in counts/30 sec per cm. Abscissae: length of the chromatogram in cm. From top to bottom: $TcBr_6^{2-}$ immediately after dissolution in 0.9 *N* HBr, after 6 h, after 24 h, after 48 h and after 72 h.

allowing the solution to stand for various times at room temperature. After 6 h two additional peaks (R_F 0.27 and 0.46) are prominent as well as a high background (elongated spot or series of spots) reaching up to the liquid front. After 24 h the initial spot of $TcBr_6^{2-}$ has practically disappeared and after 72 h also the spot of R_F 0.27 becomes small and there is a pronounced peak at the liquid front.

If it may be assumed that only aquo-bromo-complexes are present in 10 % aqueous HBr then the spot at R_F 0.27 is most likely the monoaquo-pentabromo technetate (IV) and the spot at R_F 0.46 the diaquo-tetrabromo-technetate (IV). The high activity in the upper portion of the paper would suggest the presence of neutral and cationic Tc(IV) species. These solutions were also tested for absence of Tc(V) by chromatography

with cellulose paper and no slow-moving species could be detected. The radioactivity scans shown here were made on chromatograms which were first sprayed with the reagent. The visible spots which were revealed had a compactness which is not given by the radioactive scans. The three slow moving spots were well recognised on all chromatograms while the upper portion of the bands gave indifferent colourations and hence the radioactivity scans are only reported here.

(*b*) *The hydrolysis of* $TcCl_6^{2-}$ *in 1.2 N HCl*. Ammonium hexachlorotechnetate (IV) was prepared according to DALZIEL *et al.*[8] by reducing ammonium pertechnetate with KI in conc. HCl and repeatedly evaporating with conc. HCl.

The yellow compound so obtained was dissolved in 1.2 N HCl and yielded on anion exchange paper, with 1.2 N HCl as solvent, one single spot R_F 0.07 with a slight forward comet.

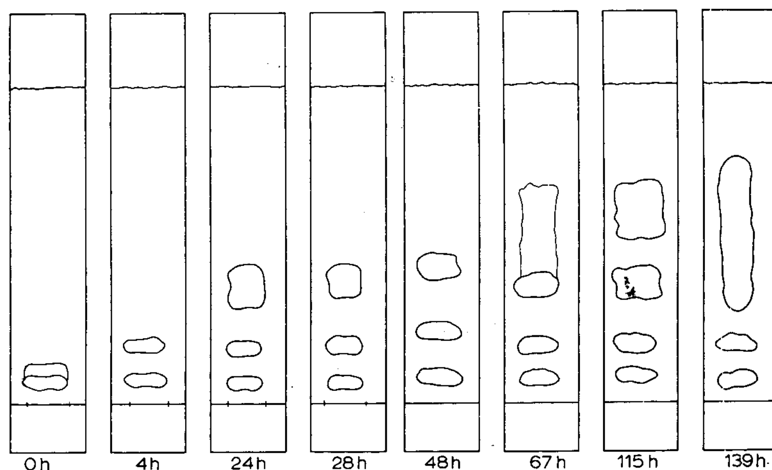


Fig. 10. Hydrolysis of $TcCl_6^{2-}$. Schematic drawings of chromatograms on Macherey, Nagel strong anion exchange cellulose paper developed with 1.2 N HCl. From left to right: $TcCl_6^{2-}$ immediately after dissolution in 1.2 N HCl and after 4 h, 24 h, 28 h, 48 h, 67 h, 115 h and 139 h.

On standing for various lengths of time at room temperature a number of faster moving spots are formed as shown in Fig. 10. If it may again be assumed that only mononuclear aquo-chloro-Tc (IV) complexes are formed then the spot of R_F 0.18 seems to be the monoaquo-pentachloro-technetate(IV) and the spot of R_F 0.39 the diaquo-tetrachloro-technetate(IV). Other spots are only formed after 67 h.

If Figs. 9 and 10 are compared it is evident that $TcCl_6^{2-}$ is much more stable to hydrolysis than $TcBr_6^{2-}$, the first still being preponderant after 139 h and the latter being absent after 24 h.

SCHWOCHAU[3] mentions that TcF_6^{2-} is much more stable to hydrolysis than the other halotechnetates(IV). The order of stability to hydrolysis may thus be established as F > Cl > Br.

(*viii*) *The reaction of* TcO_2 *with dilute acids*

In studying the hydrolysis of hexahalotechnetates(IV) the presence of either cationic or neutral species was observed in aged solutions. We thus decided to attempt the

preparation of solutions with predominantly cationic species by dissolving TcO_2 in dilute acids.

These attempts were not crowned with success. TcO_2 proved to be practically insoluble in dilute HCl or HBr and only dissolved on prolonged heating on the water bath. The resulting solutions contained anionic species as well as cationic ones.

(*a*) *Reaction of TcO_2 with 0.9 N HBr.* TcO_2 was suspended in 10% HBr and heated on the water bath as no reaction took place at room temperature. Almost complete dissolution took about 5 h. Samples were withdrawn earlier and chromatographed as shown in Fig. 11. The activity at R_F 0 is due to undissolved suspended TcO_2. When all has gone into solution the main activity seems to be due to the mono-aquo-pentabromo-technetate(IV) species.

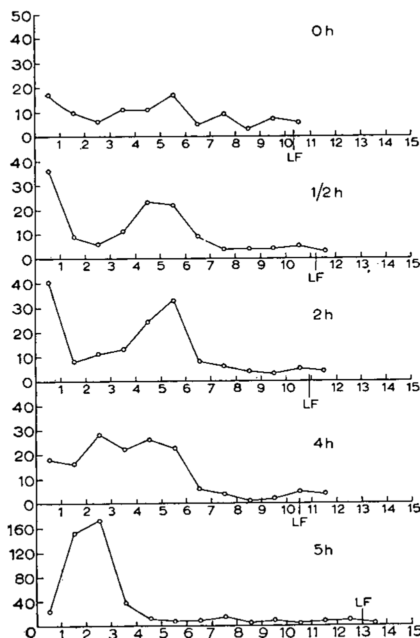


Fig. 11.

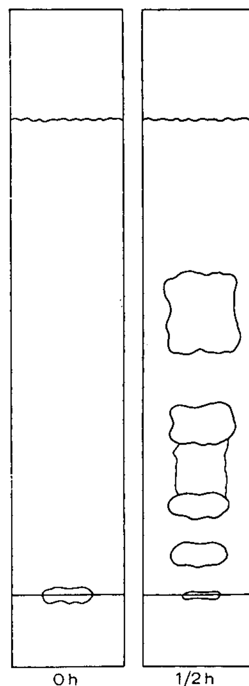


Fig. 12.

Fig. 11. Dissolution of TcO_2 in 0.9 N HBr. Radioactivity scans of chromatograms on Macherey, Nagel strong anion exchange cellulose paper developed with 1.8 N HBr. Ordinates: activity in counts/30 sec per cm. Abscissae: length of the chromatogram in cm. From top to bottom: immediately after suspending TcO_2 in HBr (supernatant is placed on paper), after 1/2 h, 2 h, 4 h and 5 h on the steam bath.

Fig. 12. Dissolution of TcO_2 in 1.2 N HCl. Schematic drawing of chromatograms on Macherey, Nagel strong anion exchange cellulose paper developed with 1.2 N HCl. From left to right: immediately after suspending TcO_2 in HCl and after 30 min on the steam bath. The spot at R_F 0 is undissolved TcO_2.

(*b*) *Reaction of TcO_2 with 1.2 N HCl.* Fig. 12 shows the chromatograms after 0 and 30 min of heating on a steam bath. First only suspended TcO_2 is found at R_F 0 and after 30 min there is an equilibrium mixture with several anionic species as well as fast moving species.

(ix) The reaction between Tc(VII) and Tc(IV) in dilute HCl solution

One remarkable feature of the solution chemistry of technetium is that in certain solutions three valencies (VII), (V) and (IV) can coexist for considerable periods of time. We thought it would be interesting to study the reaction between Tc(VII) and Tc(IV) in dilute HCl, in which both these species are stable, so as to have some idea of the speed of this reaction and of its possible influence on the chromatographic results obtained.

Pertechnetate and $TcCl_6^{2-}$ in a ratio of about 3:2 were dissolved in 1.2 *N* HCl. A chromatogram of this solution immediately after mixing gave only two fast-moving peaks, the faster due to $TcCl_6^{2-}$ and the slower due to TcO_4^-. After 24 h no measurable amounts of Tc(V) had yet been formed, however, the separation of Tc(VII) and Tc(IV) had become indistinct presumably owing to hydrolysis of the latter. After 43 h there is a small distinct peak of Tc(V) representing about 9 % of the total activity. This peak increases only to 12 % after 67 h. The chromatograms are shown in Fig. 13.

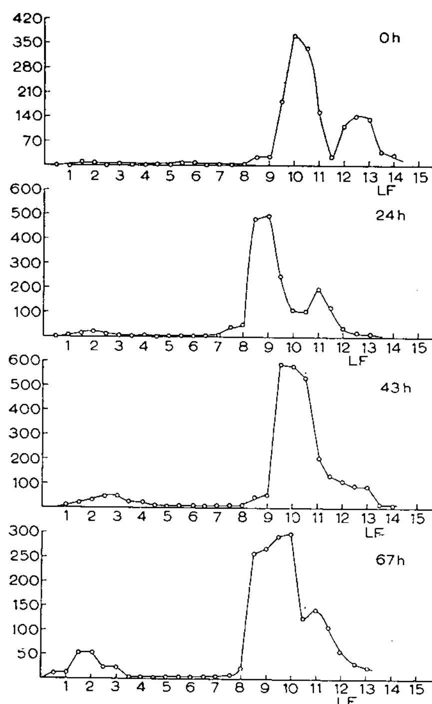


Fig. 13. The reaction between Tc(VII) and Tc(IV) in 1.2 *N* HCl. Radioactivity scans of chromatograms developed with 0.6 *N* HCl on Whatman No. 3MM paper. Ordinates: activity in counts per 30 sec per cm. Abscissae: length of the chromatogram in cm. From top to bottom: immediately after mixing, after 24 h, after 43 h and after 67 h.

DISCUSSION

The present work can be considered as a preliminary chromatographic investigation of the solution chemistry of technetium in HCl and HBr.

On several points further work suggested itself, such as the quantitative study

of the kinetics of the reduction of Tc(VII) in HCl and HBr. Both the long half-life of the radionuclide ^{99}Tc and the paper chromatographic techniques developed here lend themselves admirably to this purpose, and we intend to take this up in the near future.

Amongst the properties of technetium which were not well defined previously we would like to mention the rather unexpectedly high stability of Tc(V) in HCl solutions and the slow reaction between Tc(VII) and Tc(IV) in dilute HCl solution as well as the coexistence of the three different valencies in various concentrations of HCl and HBr.

The hydrolysis of hexahalotechnetates(IV) revealed a number of either neutral or cationic species which could not be investigated in detail by anion exchange paper chromatography. The work on the rather similar rhodium complexes (for a review see ref. 9) would suggest that these could be better resolved by paper electrophoresis and this will also be investigated in due course.

SUMMARY

The solution chemistry of technetium was studied in HCl and HBr employing paper chromatography and ion exchange paper chromatography. Techniques for the identification of TcO_4^-, Tc(V) and various Tc(IV) species were worked out.

REFERENCES

1 E. ANDERS, *Ann. Rev. Nucl. Sci.*, 9 (1959) 203.
2 E. ANDERS, *The Radiochemistry of Technetium*, Nuclear Science Series, National Academy of Sciences, National Research Council, Washington, D.C., 1960.
3 K. SCHWOCHAU, *Angew. Chem.*, 76 (1964) 9.
4 E. ALPEROVITCH, *Doctoral Thesis*, Columbia University, New York, 1954; quoted in ref. 1.
5 E. C. CROUTHAMEL, *Anal. Chem.*, 29 (1957) 1756.
6 J. B. GERLIT, *Proc. Intern. Conf. Peaceful Uses of Atomic Anergy, Geneva, 1955*, 7 (1956) 145.
7 G. E. BOYD, *J. Chem. Educ.*, 36 (1959) 3.
8 J. DALZIEL, N. S. GILL, R. S. NYHOLM AND R. D. PEACOCK, *J. Chem. Soc.*, (1958) 4012.
9 E. BLASIUS, *Metodi di Separazione nella Chimica Inorganica*, Corsi e Seminari di Chimica, Vol. 2, C.N.R., Rome, 1963, p. 141.

ION EXCHANGE PROCEDURES

VI. CATION EXCHANGE OF Ac(III) AND Fr(I) IN HCl AND $HClO_4$ SOLUTIONS; ISOLATION OF ^{227}Ac FROM ^{231}Pa AND ^{227}Ac DAUGHTERS*,**

FREDERICK NELSON

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

(Received May 13th, 1964)

In a previous paper[2] cation exchange adsorption functions of most elements were reported for a wide range of HCl and $HClO_4$ concentration; Ac(III) and Fr(I) were not included since, at the time, we did not have tracers of these elements on hand. Recently we obtained*** a few mg of ^{231}Pa, which decays to 22 y ^{227}Ac and 22 m ^{223}Fr (AcK), and examined the adsorption behavior of these elements. The present paper reports the results and describes a cation exchange procedure for isolating ^{227}Ac from milligram amounts of ^{231}Pa and from members of the ^{227}Ac decay chain, particularly 18.2 d ^{227}Th, 11.7 d ^{223}Ra, 36 m ^{211}Pb, and 2.16 m ^{211}Bi.

RESULTS AND DISCUSSION

1. Adsorption functions of francium (I) and actinium (III) (Fig. 1)

Adsorption of Fr(I) from 1 to 12 *M* HCl and $HClO_4$ solutions was measured by the band elution method. Small aliquots of purified ^{227}Ac (in secular equilibrium with ^{223}Fr) in appropriate HCl or $HClO_4$ solutions were added to 0.28 cm^2 $\times$ 3 cm columns of Dowex 50-X4 and then eluted. The actinium tracer was purified before use as described in Section 2. The elution band for Fr(I) was identified by decay rate measurements and gamma spectroscopy.

Adsorbability of Fr(I) in 1 to 12 *M* HCl and 1 to 12 *M* $HClO_4$ is low, D_v (amount per liter bed/amount per liter solution) is 5.7 and 3.0 for 1 *M* HCl and 1 *M* $HClO_4$, respectively; at higher HCl and $HClO_4$ concentrations, adsorption decreases and eventually becomes negligible. The adsorption behavior of Fr(I) in HCl and $HClO_4$ solutions is, as expected, very similar to that of Cs(I)[2].

Adsorption of Ac(III) from HCl and $HClO_4$ solutions was measured by the column method when adsorbability was low ($D_v < 10$) and by batch equilibration when $D_v >$ 10; the solutions were assayed by β-counting. The ^{227}Ac was purified immediately before equilibration and short equilibration times (*ca.* 12 h) were used so that errors from growth of β-emitting daughter activities were minimized.

---

* Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

** For Part V see ref. 1.

*** The author is indebted to Dr. A. CHETHAM-STRODE of the Oak Ridge National Laboratory Chemistry Division for kindly supplying the ^{231}Pa.

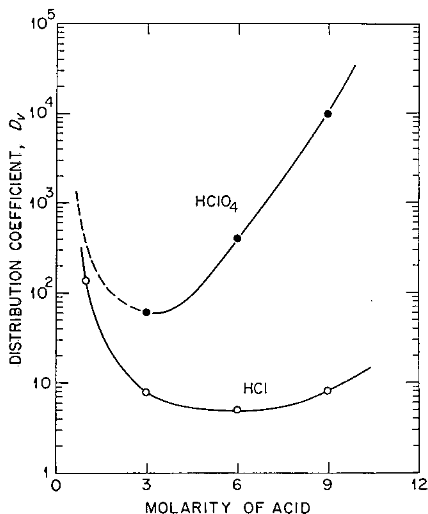


Fig. 1. Adsorption of Ac(III) from HCl and $HClO_4$ solutions (Dowex 50-X4, 25°).

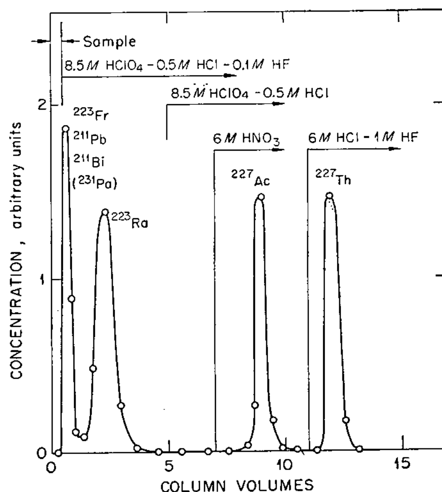


Fig. 2. Isolation of ^{227}Ac by cation exchange (Dowex 50-X4, -400 mesh, 50°).

The results are shown in Fig. 1, a semi-log plot of D_v *versus* molarity of acid. In HCl, adsorbability decreases with increasing acid concentration to a shallow minimum, $D_v = 5$, near 6 M HCl, and then increases slowly; these data confirm the results of DIAMOND, STREET AND SEABORG[3] with a similar Dowex 50 resin. In $HClO_4$, Ac(III) is strongly adsorbed at all $HClO_4$ concentrations; adsorbability increases rapidly with increasing M $HClO_4$ from a minimum adsorption, $D_v = 60$, near 3 M $HClO_4$ to $D_v = 10^4$ near 9 M $HClO_4$.

2. *Isolation of ^{227}Ac*

About 3 mg of ^{231}Pa as the hydroxide was dissolved in 1 ml of concentrated HF in a Teflon evaporating dish and the solution was evaporated to a volume of *ca.* 0.5 ml. The solution was taken up in 1 ml of 0.5 M HCl–0.5 M HF, warmed, and added to a 0.28 cm^2 $\times$ 5 cm column of Dowex 50-X4 (-400 mesh) which had been pretreated with 0.5 M HCl–0.5 M HF. On elution with 3 column volumes of the same solvent, Pa passed through the column, presumably as a negatively-charged fluoride complex, while Ac(III) and the decay products, ^{223}Ra and ^{227}Th, were adsorbed. The column was treated with 6 M HCl–1 M HF; this medium removes Ra(II) and Ac(III) as well as Th(IV).[4] Approximately 10 column volumes are necessary for complete elution of Ac(III). Subsequent work showed that 6 M HNO_3 would have been a better eluting agent for Ac(III), as further described below.

The Ac fraction was placed in a Teflon evaporating dish; one ml of concentrated $HClO_4$ was added, and the solution evaporated to a volume of *ca.* 0.1 ml. The residue was taken up in 0.5 ml of warm 8.5 M $HClO_4$–0.5 M HCl–0.1 M HF and added to a 0.28 cm^2 $\times$ 4 cm column of Dowex 50-X4 (-400 mesh) in a water jacket at 50°. The eluting solution contained HF to assure complexing and elution of possible residual ^{231}Pa; HCl was present to assure rapid elution of Pb and Bi daughters as chloride complexes[2]. In separate equilibration experiments at 25° it was established that

$D_v = 510$ for Ac(III) in 8.5 M $HClO_4$–0.5 M HCl–0.1 M HF, indicating that addition of small amounts of HCl or HF do not seriously reduce the adsorbability of Ac(III) at high M $HClO_4$. On elution with the acid mixture, ^{223}Fr, ^{211}Pb and ^{211}Bi appeared, as expected, in the first column volume of effluent while ^{223}Ra eluted in a sharp band near 2.3 column volumes as shown in Fig. 2. The Ra elution band was corrected for ^{223}Fr activity which continuously eluted and "contaminated" the effluent.

The ^{227}Ac was removed in a sharp band with 6 M HNO_3, and ^{227}Th, with 6 M HCl–1 M HF. Before removing actinium, the column was treated with 2 column volumes of 8.5 M $HClO_4$–0.5 M HCl to remove HF, thus avoiding possible partial elution of Th(IV) during elution of ^{227}Ac with 6 M HNO_3. The separated ^{227}Ac was analysed immediately by β and α counting (pulse analysis). The ratio of β to α counts and the α-spectrum were as expected and indicated the actinium was radiochemically pure for all practical purposes.

The method described here is similar, in principle, to the cation exchange method of CABELL[5] in which selective elution of the decay products, Fr, Pb, Bi and Ra is accomplished with 2 M HCl and 3 M HNO_3 solutions; Ac is eluted with 6 M HNO_3 as in our method. Use of a concentrated $HClO_4$–HCl medium appears preferable for the initial adsorption step since Ac(III) is much more strongly adsorbed from this medium, relative to Pb, Bi and Ra, than from HCl or HNO_3 solutions.

ACKNOWLEDGEMENTS

The author is indebted to Mrs. M. DAVIS for carrying out the required β- and α-analyses, to Dr. J. HALPERIN for valuable assistance in interpretation of α-pulse analysis data, and to Dr. K. A. KRAUS for helpful comments.

SUMMARY

The cation exchange behavior of Ac(III) and Fr(I) in HCl and $HClO_4$ solutions is described and a method for isolating ^{227}Ac from its daughters and from ^{231}Pa is presented.

REFERENCES

1 F. NELSON, *J. Chromatog.*, 16 (1964) 403.
2 F. NELSON, T. MURASE AND K. A. KRAUS, *J. Chromatog.*, 13 (1964) 503.
3 R. M. DIAMOND, K. STREET, JR. AND G. T. SEABORG, *J. Am. Chem. Soc.*, 76 (1954) 1461.
4 T. MURASE, E. L. LIND AND F. NELSON, *J. Chromatog.*, 14 (1964) 478.
5 M. J. CABELL, *Can. J. Chem.*, 37 (1959) 1094.

Short Communication

Anwendung der Dünnschichtchromatographie zur Sequenzanalyse von Peptiden

3. Mitt. Zerstörungsfreie Revelation von Aminosäuren auf Dünnschichtchromatogrammen*

Zum Nachweis der freien Aminosäuren wird in der Dünnschichtchromatographie allgemein die Ninhydrin-Reaktion verwendet[1]. Dieses Vorgehen lässt jedoch eine Elution und Rechromatographie nicht zu, obschon dies insbesondere bei der Untersuchung von komplexem biologischen Material von Bedeutung wäre.

Im Zusammenhang mit Untersuchungen über die Schichtqualität und Aminosäuretrennung, haben wir darauf hingewiesen, dass zur zerstörungsfreien Revelation von Aminosäuren 2,4-Dinitrofluorbenzol und Phenylisothiocyanat geeignete Reagenzien sind[2]. In der Zwischenzeit benutzten BAXTER *et al.*[3] Trinitrobenzol-1-sulfonsäure zum Nachweis von ^{14}C-Aminosäuren auf Papierchromatogrammen.

In der vorliegenden Arbeit möchten wir über die zerstörungsfreie Revelation der Aminosäuren mittels 2,4-Dinitrofluorbenzol (DNFB) berichten.

Chromatographie

30 g Kieselgel-G (Merck, Darmstadt, Deutschland) + 60 ml Wasser bzw. 10 g Cellulosepulver D (CAMAG, Muttenz, Bl., Schweiz) + 65 ml Wasser werden nach STAHL[4] bei einer Schichtdickeneinstellung von 0.25 mm auf 20 × 20 mm Glasplatten aufgebracht. Man lässt die Platten über Nacht an der Luft trocknen. Die Chromatographie erfolgt aufsteigend nach den Angaben von BRENNER *et al.*[5] mit *n*-Propylalkohol–Wasser (7:3, v/v) als Fliessmittel. Die aufgetragene Aminosäure beträgt 5×10^{-2} μM in 10% Isopropylalkohol (Aminoacids Standard Solutions, Shandon, London, Great Britain).

Revelation

Die getrocknete Platte wird mit einer Pufferlösung[6] (8.4 g $NaHCO_3$ + Wasser + 2.5 ml 1 *N* NaOH + Wasser ad 100 ml) und mit einer 10% (g/v) methanolischen DNFB-Lösung[6] besprüht.

Die Schicht wird an den beiden Plattenrändern in einer Breite von je 5 mm abgestreift. Auf die blanken Rändern legt man zwei Polyäthylenstreifen passender Breite. Man bedeckt nun die Schicht mit einer zweiten Glasplatte und erwärmt im Dunkeln 1 Std. auf 40°. Die Trägerplatte wird abgekühlt und in ein Ätherbad gelegt. Nach 10 min trocknet man kurz und zeichnet die Flecken an. Die Platte kann auch photographiert werden. Die Markierung der Flecken bzw. die Photographie muss rasch

* 2. Mitteilung: G. PATAKI, *Helv. Chim. Acta*, im Druck.

erfolgen, da die Platte mit der Zeit eine gelbe Farbe annimmt und die Flecken verschwinden.

Die Abbildung zeigt die Tageslichtphotographie eines Chromatogramms. Mit der beschriebenen Methode kann man etwa 10^{-2} bis 10^{-3} μM Aminosäure nachweisen. Die Kapazität von Kieselgel und von Cellulose ist unter unseren Versuchsbedingungen etwa gleich gross; auf Cellulose-Schichten beobachtet man jedoch bei grösseren Auftragmengen eine grössere Neigung zur "Schwanzbildung".

Unsere Arbeitsweise eröffnet einige interessante Möglichkeiten bei der Dünnschichtchromatographie von Aminosäuren. Man kann z.B. ein unbekanntes Aminosäuregemisch eindimensional chromatographieren und nach Revelation mit DNFB mit einem geeigneten Fliessmittel in der zweiten Dimension entwickeln. Hierbei

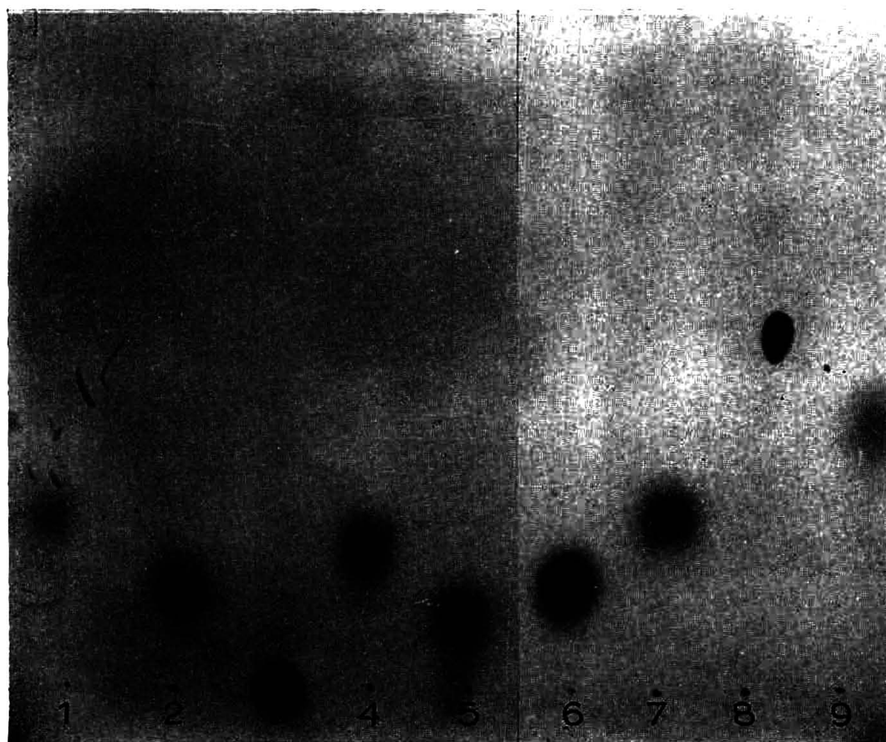


Fig. 1. Tageslichtphotographie eines Chromatogramms von freien Aminosäuren auf Kieselgel-G mit *n*-Propylalkohol–Wasser (7:3, v/v) als Fliessmittel. Revelation mit Dinitrofluorbenzol. 1 = Alanin, 2 = Asparaginsäure, 3 = Arginin, 4 = Glycin, 5 = Histidin, 6 = Prolin, 7 = Serin, 8 = Tyrosin, 9 = Valin.

werden selbstverständlich die Dinitrophenyl-Aminosäuren in der zweiten Dimension chromatographiert. Ein Vergleich des Fleckenmusters mit demjenigen eines "normalen" zweidimensionalen Chromatogramms erhöht die Sicherheit der Identifizierung. Die mit DNFB angefärbten Aminosäuren können selbstverständlich eluiert und rechromatographiert werden. Dieses Vorgehen dürfte auch zur quantitativen Aminosäurebestimmung geeignet sein.

Abschliessend sei bemerkt, dass die beschriebene Methode auch zur zerstörungsfreien Revelation von Peptiden angewandt werden kann. In Verbindung mit der "Dünnschicht-Fingerprint"-Technik nach WIELAND UND GEORGOPOULOS[7] erleichtert sie die Sequenzanalyse von Peptiden.

Laboratorium der Universitäts-Frauenklinik**, Basel (Schweiz)*

GYÖRGY PATAKI

1 M. BRENNER, A. NIEDERWIESER UND G. PATAKI, in E. STAHL (Herausgeber), *Dünnschichtchromatographie*, Springer, Berlin, 1962.
2 G. PATAKI, *J. Chromatog.*, im Druck.
3 C. F. BAXTER UND I. SENONER, *Anal. Biochem.*, 7 (1961) 55.
4 E. STAHL, in E. STAHL (Herausgeber), *Dünnschichtchromatographie*, Springer, Berlin, 1962.
5 M. BRENNER, A. NIEDERWIESER, G. PATAKI UND A. R. FAHMY, *Experientia*, 18 (1962) 101.
6 D. WALZ, A. R. FAHMY, G. PATAKI, A. NIEDERWIESER UND M. BRENNER, *Experientia*, 19 (1963) 213.
7 TH. WIELAND UND D. GEORGOPOULOS, *Biochem. Z.*, im Druck.

Eingegangen den 17. September 1964

* Leiter: Dr. M. KELLER.
** Direktor: Prof. Dr. TH. KOLLER.

Notes

Non-ideality of the gas phase in frontal analysis

The activity coefficient at infinite dilution f_2^∞ of a volatile solute in an involatile solvent determined by gas–liquid chromatography is:

$$f_2^\infty = \frac{n_1^L RT}{p_2^0 V^G} \quad (1)$$

where n_1^L is the number of moles of solvent in the column,
p_2^0 is the vapour pressure of the solute at the column temperature T,
V^G is the true retention volume.

Equation (1) is derived by assuming, amongst other things, that the gas phase is ideal and that the activity coefficient is pressure independent. However, corrections must be made for the invalidity of these assumptions if activity coefficients measured by gas–liquid chromatography are to agree with static results[1–3].

These corrections will be more important if the carrier gas is a mixture of an inert gas (by this is meant a gas that is virtually insoluble in the involatile solvent) and the solute, *e.g.* an elution peak is superimposed on the plateau obtained in frontal analysis. These "plateau" experiments would give the activity coefficient as a function of solute concentration thus allowing one to test solution theories in a concentration region which is inaccessible to static techniques.

In this note an equation is derived which allows for the non-ideality of the gas phase and the pressure dependence of the activity coefficient. As a model for the thermodynamic effects in a column it is assumed that this is equivalent to a static system under the same conditions as the column, with the volume of the gas phase equal to the true retention volume and with equal amounts of solute n_2 in both phases. This model readily gives eqn. (1) and the derivation of the full equation is a simple problem in thermodynamics[4] which will now be outlined.

The activity coefficient is defined unambiguously in terms of the absolute activities, λ, at zero pressure by:

$$\lambda_2^L(0) = \lambda_2^0(0)xf_2(0) \tag{2}$$

where x is the mole fraction of the solute in the solvent.

The absolute activities at zero pressure may be expressed in terms of their values at a particular low pressure by using the fact that the volumes of liquids are pressure independent. Thus eqn. (2) becomes:

$$\ln\lambda_2^L(\bar{P}) = \ln\lambda_2^0(p_2^0)xf_2(0) + \frac{\bar{P}\bar{v}_2}{RT} - \frac{p_2^0 v_2^0}{RT} \tag{3}$$

where $\bar{P}$ is the pressure of the static system,

v_2^0 is the molar volume of the solute,

$\bar{v}_2$ is the partial molar volume of the solute in the solvent.

The absolute activities of the solute in the gas phase for slightly imperfect gases at low pressure are:

$$\ln\lambda_2^G(\bar{P}) = \ln\frac{y\bar{P}}{\varphi} + \frac{\bar{P}}{RT}\left\{B_{22} - (1-y)^2(B_{11} - 2B_{12} + B_{22})\right\} \tag{4}$$

$$\ln\lambda_2^G(p_2^0) = \ln\frac{p_2^0}{\varphi} + \frac{p_2^0 B_{22}}{RT} \tag{5}$$

where B_{ij} is the appropriate second virial coefficient,

y is the mole fraction of the solute in the gas phase,

φ is the internal partition function of the solute and is independent of its environment.

Equations (3) and (4) are related by the condition for equilibrium, *i.e.* equality of absolute activities in both phases. This together with eqn. (5), the equation of state for the binary gas mixture:

$$\bar{P}V^G = (n_1^G + n_2)\,[RT + \bar{P}\{(1-y)^2 B_{11} + 2y(1-y)B_{12} + y^2 B_{22}\}] \tag{6}$$

and some manipulation leads to the full equation:

$$\ln f_2(0) = \ln\frac{n_1^L + n_2}{p_2^0}\,\frac{RT}{V^G}\left[1 + \frac{\bar{P}}{RT}\left\{(1-y)^2B_{11} + 2y(1-y)B_{12} + y^2B_{22}\right\}\right]$$
$$+ \frac{\bar{P}}{RT}\left\{B_{22} - \bar{v}_2 - (1-y)^2(B_{11} - 2B_{12} + B_{22})\right\} - \frac{p_2^0}{RT}\left\{B_{22} - v_2^0\right\} \tag{7}$$

In elution gas–liquid chromatography the solute concentration is vanishingly small so that eqn. (7) reduces to:

$$\ln f_2^{\infty}(0) = \ln \frac{n_1^L RT}{p^0 V^G} + \frac{\bar{P}}{RT}\left\{2\,B_{12} - \bar{v}_2\right\} - \frac{p_2^0}{RT}\left\{B_{22} - v_2^0\right\} \quad (8)$$

if $\ln[1 + \bar{P}B_{11}/RT]$ is expanded and only first order terms retained. This equation has been derived previously and has been tested by measuring the pressure dependence of the k' factor[3,5].

In frontal analysis, however, the solute concentration cannot be neglected and eqn. (7) should be used to correct for the non-ideality of the gas phase. Although this equation is complicated it may be simplified by expanding the logarithm of the term in square brackets and neglecting all but first order terms. This assumption will normally be valid so that eqn. (7) becomes:

$$\ln f_2(0) = \ln \frac{n_1^L + n_2}{p_2^0}\frac{RT}{V^G} + \frac{\bar{P}}{RT}\left\{2\,yB_{22} + 2(1-y)B_{12} - \bar{v}_2\right\} - \frac{p_2^0}{RT}\left\{B_{22} - v_2^0\right\} \quad (9)$$

In many cases the volume of mixing is small so that the partial molar volume may be replaced by the molar volume. Thus for 0.1 mole fraction of n-octane in nitrogen at 30° the coefficient of $\bar{P}$ in eqn. (9) is -0.0591 atm^{-1} and the constant term is 0.0058 but at infinite dilution the coefficient is only -0.0254 atm^{-1}. These calculations show that, as expected, the correction terms for the non-ideality of the gas phase may be large and if accurate values of activity coefficients are to be obtained these corrections should be made.

Finally the measurement of the pressure dependence of the k' factor may be used to measure the second virial coefficient of the solute in the same way that elution gas–liquid chromatography gives the mixed second virial coefficient.

Acknowledgement

I should like to thank the D.S.I.R. for the award of a studentship.

University Chemical Laboratory, Cambridge (Great Britain) G. R. LUCKHURST

1 E. R. ADLARD, M. A. KHAN AND B. T. WHITHAM, in R. P. W. SCOTT (Editor), *Gas Chromatography 1960*, Butterworths, London, 1960, p. 251.
2 D. H. EVERETT AND C. T. H. STODDART, *Trans. Faraday Soc.*, 57 (1961) 746.
3 D. H. DESTY, A. GOLDUP, G. R. LUCKHURST AND W. T. SWANTON, in M. VAN SWAAY (Editor), *Gas Chromatography 1962*, Butterworths, London, 1962, p. 67.
4 E. A. GUGGENHEIM, *Thermodynamics*, 3rd Ed., North Holland Publishing Company, Amsterdam, 1957, Chap. 5.
5 D. C. LOCKE AND W. W. BRANDT, in L. FOWLER (Editor), *Gas Chromatography*, Academic Press, New York, London, 1963, p. 55.

Received May 4th, 1964

Studies with β-phenethylbiguanide

1. Microdetection colorimetric tests

As a part of studies on the human metabolism of β-phenethylbiguanide (β-PEBG), an oral hypoglycemic agent, several location reagents have been found to be useful for detection of the unaltered compound on paper chromatograms in the microgram and submicrogram range. The observations support the view that β-PEBG behaves as a monosubstituted guanidine in these color tests.

Experimental

Three reagents giving positive color tests with β-PEBG are the pentacyanoaquoferriate (PCF), Sakaguchi, and α-naphthol–diacetyl reagents. These reagents were prepared as previously described[1], except the bromine liquid, 0.3 ml, was dissolved in 100 ml of 2 % NaOH in water for preparation of the Sakaguchi reagent. The α-naphthol and 2,3-butanedione (diacetyl) were Eastman Grade (Eastman Kodak Co.), and other chemicals were A.R. grade obtained from J. T. Baker Chemical Co. or The Matheson Company, Inc. Tests were performed with β-PEBG free base and with various salts of β-PEBG including the monohydrochloride, the dihydrochloride, and the sulfate. The sensitivity limitations were obtained by spotting the appropriate amount of β-PEBG on unwashed Whatman No. 2 chromatography paper and performing the color test using a dipping technique without prior development of the chromatogram.

Pertinent data concerning these color tests together with structural representations of β-PEBG are illustrated in Fig. 1. The color produced with the α-naphthol–diacetyl reagent appears to be the most sensitive, with a detection limitation of 0.05 μg β-PEBG compared to 0.25 μg obtained with either the PCF or Sakaguchi

	PENTACYANOAQUOFERRIATE REAGENT	SAKAGUCHI REAGENT	α-NAPHTHOL-DIACETYL REAGENT
COLOR OF SPOT	PINK	ORANGE	PURPLE
DETECTION LIMITS	0.25 μg	0.25 μg	0.05 μg
TIME OF DEVELOPMENT	IMMEDIATE ROOM TEMPERATURE	IMMEDIATE ROOM TEMPERATURE	3 MINUTES 100° C
TYPE OF GUANIDINE AND TEST GIVEN (+) POSITIVE (-) NEGATIVE	MONOSUBSTITUTED (+) N,N-DISUBSTITUTED (+) N,N'-DISUBSTITUTED (-)	MONOSUBSTITUTED (+) N,N-DISUBSTITUTED (-) N,N'-DISUBSTITUTED (-)	MONOSUBSTITUTED (+) N,N-DISUBSTITUTED (+) N,N'-DISUBSTITUTED (-)
STRUCTURAL REPRESENTATIONS OF β-PEBG	$C_6H_5-CH_2-CH_2-NH-C(=NH)-NH-C(=NH)-NH_2$ (R) MONOSUBSTITUTED GUANIDINE	$C_6H_5-CH_2-CH_2-NH-C(=NH)-NH-C(=NH)-NH_2$ (R_1, R_2) N,N'-DISUBSTITUTED GUANIDINE	

Fig. 1. Summary of colorimetric microdetection tests.

reagents. Following paper chromatography as little as 1 μg β-PEBG has been detected with these reagents. The detection limitations of smaller amounts of β-PEBG following chromatography have not been investigated.

No differences have been noted between the color tests given by β-PEBG free base and the salts mentioned. The alkali in these color tests would be expected to convert these salts to the free base[2] by proton removal. Therefore, it appears likely the free base is produced as an intermediary in reactions involving the salts investigated.

These location reagents have been reported to be useful for distinguishing structurally dissimilar guanidines, and to give positive color reactions with certain monosubstituted guanidinium compounds, but not with N,N'-disubstituted guanidines[1]. β-PEBG can be represented structurally as a monosubstituted or an N,N'-disubstituted guanidine. Since β-PEBG gives a positive test with all three reagents, the data have been interpreted as indicating β-PEBG behaves as a monosubstituted guanidine in these color tests. None of the colorimetric reactions mentioned is specific for β-PEBG, and detection requires prior purification of β-PEBG from other guanidinium compounds.

Acknowledgements

The authors are indebted to Dr. HARVEY S. SADOW, U.S. Vitamin and Pharmaceutical Co. who kindly furnished samples of β-PEBG and a grant-in-aid. This work was also supported in part by a Training Grant No. Tl AM 5230, National Institute of Arthritis and Metabolic Disease, USPHS.

Division of Diabetes and Metabolism, Department of Medicine, University of Oregon Medical School, Portland, Oreg. (U.S.A.)

RICHARD E. BAILEY
DAVID A. DURFEE

1 I. SMITH, *Chromatographic and Electrophoretic Techniques*, Interscience, New York, 1960, p. 226.
2 S. L. SHAPIRO, V. A. PARRINO AND L. FREEDMAN, *J. Am. Chem. Soc.*, 81 (1959) 2220.

Received May 29th, 1964

J. Chromatog., 16 (1964) 546–547

A new technique for preparative paper chromatography

Preparative partition-chromatography techniques have been reviewed in several recent treatises on chromatography[1–3]. Many techniques that depend upon increasing the mass of the stationary phase are cumbersome and tedious or have low capacity[4–10]. The continuous methods[11,12], which require elaborate, synchronous mechanical equipment, are not used extensively although commercial equipment has been available.

The technique outlined in this report requires only readily available laboratory supplies and equipment. It has high, adjustable capacity and resolving power, simplicity, convenience, and versatility that facilitates multi-directional fractionations and the use of glass fiber, cellulose acetate, ion-exchange or any partition media available in tape form.

Fig. 1 shows a diagrammatic cross-section of the system. It is composed of a roll of commercial filter paper tape (A) from which a central portion has been unwound to accommodate a segment of the same filter paper tape (B) which is impregnated with a

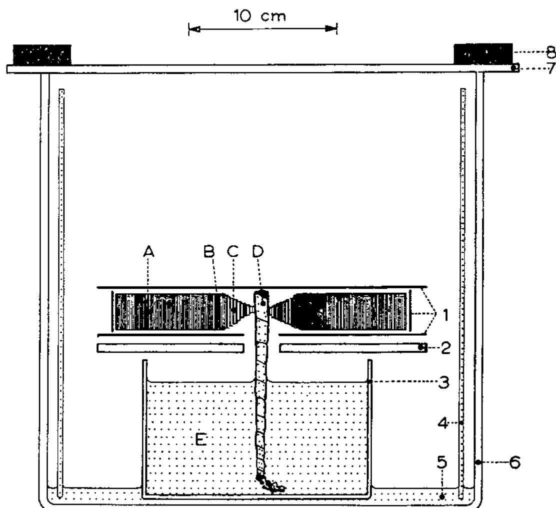


Fig. 1. Diagram of cross-section of assembly for preparative paper chromatography. A = filter-paper-tape roll; B = origin containing mixed solutes; C = distributor; D = wick; E = solvent reservoir; 1 = fluorocarbon-plastic film; 2 = glass plate; 3 = pyrex dish; 4 = blotting paper; 5 = solvent for chamber saturation; 6 = pyrex jar; 7 = transparent plastic cover; 8 = weight.

solution of mixed solutes and dried. The impregnated tape serves as the origin and is irrigated by a distributor (C) wound from progressively wider filter paper strips to which solvent is fed by a cylindrical filter paper tape wick (D) containing a core of cotton fibers. The wick dips into the irrigating solvent (E). All surfaces except the wick are covered with non-wettable plastic film. A glass plate with a hole in the middle to accommodate the wick supports the tape roll over a dish of irrigating solvent. The assembly is housed in a covered glass jar containing a small amount of solvent and lined with blotter paper to minimize solvent evaporation from the chromatographic column. The origin is marked conveniently by rubbing a pencil lead along both edges of the dried solute-impregnated tape segment. The chromatogram is developed by radial, horizontal migration of solvent and solutes through and between adjacent layers of tape. If necessary, irrigation may be interrupted briefly to measure solvent or solute boundaries. Non-uniform radial or lateral solvent flow to top or bottom surfaces can be adjusted by changing the distributor configuration by gentle pressure or by manipulating the wick. Loci of colorless solutes can be located at any time by removing the plastic cover temporarily, pressing a disc of filter paper on the horizontal surface of the roll with the aid of a soft rubber roller, and staining the imprint with the reagents commonly employed for paper chromatograms. Imprints upon glass-fiber paper can be stained with corrosive reagents. Colorless solutes can also be located directly on the horizontal surfaces by applying small amounts of test reagents from a toothpick or micro-pipet, or by placing a thin strip of filter paper impregnated with reagent between the origin and solvent boundary. Normally, direct staining does not

interfere with further chromatographic development because only a small surface is involved. A radioactivity detector can be employed to locate radioactive zones on the original chromatogram or on imprints. A radiochromatogram strip-scanner can continuously inspect paper tape when the completed chromatogram is unwound and dried.

When the solvent has migrated to within a few millimeters of the edge of the roll, the wick is removed and final imprints are obtained. The distributor is replaced with a one-hole rubber stopper containing an axial rod that is used as an arbor for unwinding the roll. Imprints or surface markings serve as guides when the tape is unwound and segmented to recover resolved substances. Each segment can be extracted for the isolation of individual constituents or rechromatographed directly on a new assembly.

The rate of solvent migration depends upon the size and shape of the wick and distributor, the width of the paper tape, and the solvent characteristics. The capacity and resolving power of the column increases with increasing width of the tape and radius of the roll. A 4-cm × 200-meter roll of Whatman No. 1 chromatography paper* having a radius of 10 cm and a mass of 700 g was employed to separate 1.5 g each of L-proline and L-leucine.

A typical separation of a 3-component mixture containing 600 mg of amino acids illustrates the utility of the technique. The distributor was prepared from a standard 50 × 50-cm sheet of Whatman No. 1 filter paper. Strips 50 cm long and varying in widths from 0.5 to 2.5 cm were wound progressively around a 0.25-in. wood dowel so that the vertical thickness of the coil increased regularly with increasing radius as shown in Figs. 1 and 2. The distributor radius was 2.6 cm when the vertical thickness reached 2.5 cm. A mixture of 500 mg each of DL-valine, L-proline and L-arginine (as the monohydrochloride) was dissolved in 10 ml of water. The solution was transferred to a large watch glass and absorbed completely into two strips of 2.5-cm wide, Whatman No. 1 chromatography paper tape having a total length of 208 cm. The air-dried paper strips contained 7.2 mg of amino acid mixture per centimeter of tape length. An 83.5-cm strip of this tape containing 200 mg of each amino acid was marked along both edges to establish the position and width of the "origin." The strip was wound around the distributor, to extend the latter's radius by 1.3 mm. The central part of a 2.5-cm thick × 10-cm radius (370 g) roll of Whatman No. 1 chromatography paper was unwound to provide a 5.5-cm I.D. opening. With the aid of a few additional tape windings, the distributor-origin assembly was fitted snugly into the opening. Care was taken to align the helical winding with that of the roll. Slight counter-rotation of the distributor assembly helped to get an intimate fit between the two parts and subsequently a smooth, uniform solvent flow. A 1.1 × 10-cm, slightly conical, cylindrical wick was formed by pushing out the central section of a 10-turn coil of the same paper tape. The wick was mounted in the central opening of the distributor, with the apex of the truncated cone hanging about 7.5 cm below the lower horizontal surface of the roll. Absorbent cotton was stuffed into the hollow wick to increase solvent flow to the distributor. A 2.5-cm wide strip of fluorocarbon plastic tape was wound around the edge of the assembly to keep the tape from unrolling and to reduce evaporation from the solvent front. A thin sheet of the same plastic film was placed over the upper surface, and a similar sheet with a 2.5-cm central perforation was placed under the lower surface. An 0.5 × 23-cm glass plate with a 2.5-cm diameter hole in the middle sup-

* Reference to a company or product 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.

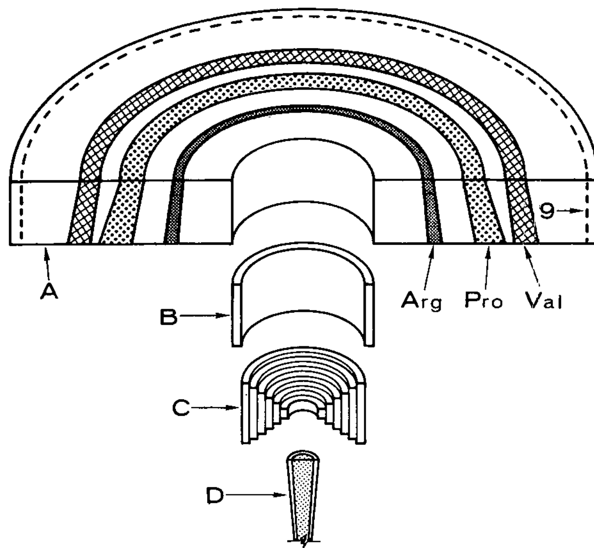


Fig. 2. Cutaway isometric projection of tape-roll chromatogram, based on imprints prepared from upper and lower surfaces. Symbols A, B, C, D are the same as in Fig. 1; 9 = solvent boundary.

ported the assembly over a pyrex dish containing about 1.5 l of irrigating solvent. The solvent mixture of *tert.*-butanol–85 % formic acid–water in the ratio 69.5:4.0:26.5 migrates slowly but separates amino acid mixtures effectively[13]. The entire assembly was housed in a 30 × 30-cm cylindrical pyrex jar, which was lined with solvent-impregnated blotting paper and sealed with a transparent plastic plate undercoated with silicone grease. At 21° the solvent moved 7.0 cm beyond the origin in five days. The wick was removed and imprints of the upper and lower surfaces were prepared on 24-cm filter paper discs. The imprints were immersed for a few seconds in a modified[13] ninhydrin dip reagent[14]. Intense colors appeared within 20 min at ambient temperature. Patterns on the two imprints were compared to estimate the distribution of the amino acids within the roll (Fig. 2). The tape was unwound on a lattice-rod reel and cut into three sections corresponding to the zones of the three amino acids. Each segment was wound into a separate coil, formed into a cone by extending the center section, and placed on a 9.0-cm I.D. Buchner funnel. The cones were extracted over a 24-hour period by dropwise addition of about 1 l of water from a separatory funnel. Each eluate was evaporated to dryness under reduced pressure. Yields of crude products were approximately 80–90 %. The valine contained a little proline because the irrigating solvent had migrated slightly faster on the lower part of the roll (Fig. 2). Arginine and proline were chromatographically pure although slightly yellow. Extended soaking of the paper tape cones did not remove all of the amino acids, especially if the tapes had dried thoroughly before extraction. Ninhydrin reacted on tapes that had been extracted for several hours in a Soxhlet extractor, indicating residual amino acids or partial interaction with the filter paper.

Some modifications of the basic procedure have been tried. The chromatographic process can be accelerated by reversing the wick so that it protrudes above the surface of the roll and irrigating it by an automatic, metered, solvent feed device mounted

above the assembly. However, separations are less satisfactory when the column is loaded heavily and equilibrium is not attained between the partitioning phases.

A mixture of dyes was separated by use of two types of two-directional procedures. After preliminary fractionation on a tape roll, a section of tape containing unresolved dyes was placed between a new distributor and tape roll and rechromatographed directly with a second solvent system to complete the separation. The second technique involved sectioning, drying, and rewinding of the unresolved zone into a tight coil. It was mounted between identical coils of fresh tape having the same diameter as the rewound segment. The assembly was placed in a dish of a second solvent that covered about two-thirds of the lower coil. Weights placed upon the upper-most coil improved surface contact. The assembly was covered by a glass jar. Ascending migration of solvent carried one of the unresolved dyes into the upper coil effecting two-directional, three-dimensional chromatography.

Eluting components from the periphery and collecting fractions in an automatic fraction collector might be expected to improve separations. The plastic strip around the edge of the column could be replaced with a filterpaper skirt having a serrated lower edge which would permit drop formation and collection in a funnel leading to a fraction collector. A similar arrangement with the skirt and wick immersed in separate buffer reservoirs containing electrodes attached to a power supply might provide a convenient system for preparative electrophoresis of mixtures that are not readily separated by partition chromatography.

*Fruit and Vegetable Chemistry Laboratory**,
Pasadena, Calif. (U.S.A.)

LOUIS B. ROCKLAND

1 R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press, New York, 1958, pp. 43–48.
2 E. HEFTMAN, *Chromatography*, Reinhold, New York, 1961, pp. 152–154.
3 E. LEDERER AND M. LEDERER, *Chromatography*, 2nd Ed., Elsevier, Amsterdam, 1957, pp. 136–137.
4 E. VON ARX AND R. NEHER, *Helv. Chim. Acta*, 39 (1956) 1664.
5 H. H. BROWNELL, J. G. HAMILTON AND A. A. CASSELMAN, *Anal. Chem.*, 29 (1957) 550.
6 C. E. DANIELSON, *Arkiv Kemi*, 5 (1953) 173.
7 L. HAGDAHL AND C. E. DANIELSON, *Nature*, 174 (1954) 1062.
8 H. K. MITCHELL AND F. A. HASKINS, *Science*, 110 (1949) 278.
9 W. L. PORTER, *Anal. Chem.*, 23 (1951) 412
10 L. ZECHMEISTER, *Science*, 113 (1951) 35.
11 J. SOLMS, *Helv. Chim. Acta*, 38 (1955) 1127.
12 H. SVENSSON, C. E. AGRELL, S. N. DEHLÉN AND L. HAGDAHL, *Science Tools*, 2 (1955) 17.
13 L. B. ROCKLAND AND B. NOBE, *J. Agr. Food Chem.*, in press.
14 L. B. ROCKLAND AND J. C. UNDERWOOD, *Anal. Chem.*, 26 (1954) 1557.

Received May 25th, 1964

---

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

Apparatus for quantitative application of samples as streaks in paper and thin-layer chromatography

A device for quantitative and uniform application of samples as a streak to thin-layer chromatographic plates was described recently[1]; it is suitable also for paper chromatography[2]. A simple carriage (Fig. 1) enables an "Agla" micrometer syringe (Burroughs Wellcome & Co., London) to be used for the same purpose.

The apparatus consists of a platform supported by four rubber-treaded wheels. The wheels on each side nearly touch one another; their size is not critical but a diameter of 4 cm is suitable. The "Agla" micrometer syringe, already loaded with the solution to be applied, is placed so that the knurled portion of the head of the micrometer screw gauge is between the two left-hand wheels and is supported by them.

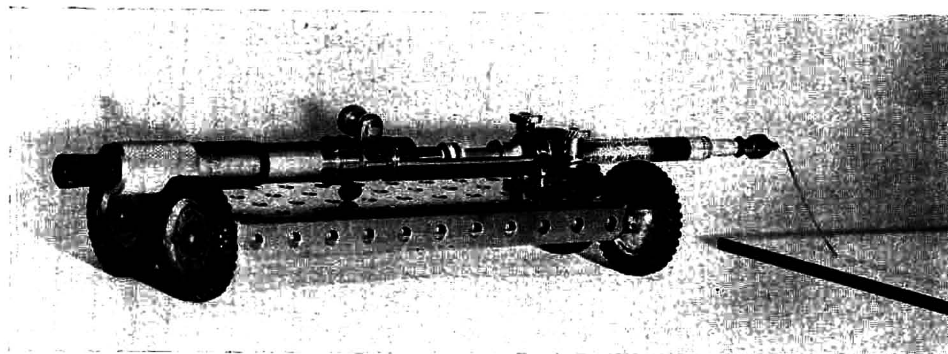


Fig. 1.

The V-shaped part of the syringe holder rests on two screws attached to the right-hand side of the platform, so that the syringe is prevented from touching the right-hand wheels, over which it projects. The screws, which bear against the rounded part of the syringe holder, also prevent it from moving laterally. When the device is pushed away from the operator, in a direction parallel to the baseline of the chromatogram, the left-hand wheels drive the knurled head and solution is ejected from the syringe. 2 μl are delivered for each centimetre travelled by the apparatus; this amount, which is convenient, could be increased or decreased by attaching a cylinder of appropriate diameter to the head of the micrometer screw gauge.

The solution is transferred to the baseline of paper chromatograms via a right-angled glass needle (Burroughs Wellcome & Co., London) fixed to the syringe nozzle. The needle slants back towards the operator and the torque in the system keeps its tip in contact with the paper in spite of any irregularities in the surface. For thin-layer chromatoplates, whose surface is easily damaged, pressure at the tip must be lessened. By allowing the locking screw of the micrometer screw gauge to bear against the platform, as shown in Fig. 1, torque is eliminated. Light contact with the chromatoplate is then obtained by attaching polyethylene capillary tubing (0.4 mm bore, Portland Plastics Ltd., Hythe, Kent) to the glass, or a metal, right-angled needle, as described by MILLETT *et al.*[1]. Under these conditions, silica-gel G and cellulose layers are not seriously damaged.

The apparatus depicted was made in a few minutes from "Meccano" units. It should prove suitable also for preparative paper and thin-layer chromatography.

The author is grateful to Mr. D. WILSON for help in simplifying the apparatus, and to Mr. F. D. COWLAND for the photograph.

Rothamsted Experimental Station, Harpenden, Herts. (Great Britain) M. F. BACON

1 M. A. MILLETT, W. E. MOORE AND J. F. SAEMAN, *Anal. Chem.*, 36 (1964) 491.
2 S. W. MCKIBBINS, J. F. HARRIS AND J. F. SAEMAN, *J. Chromatog.*, 5 (1961) 207.

Received June 10th, 1964

J. Chromatog., 16 (1964) 552–553

Zur Dünnschichtchromatographie von Carbobenzoxy-Aminosäuren

Die Dünnschichtchromatographie eignet sich vorzüglich zur Trennung und zur Charakterisierung von Aminosäuren und von Aminosäurederivaten (für Zusammenfassung vgl. Zit. 1–3).

EHRHARD UND CRAMER[4] sowie RINIKER[5] chromatographierten Carbobenzoxy-(CbO)-Aminosäuren, CbO-Peptide und CbO-Peptidester auf Kieselgel G-Schichten und fanden, dass die freien Aminoverbindungen von den entsprechenden CbO-Derivaten abgetrennt werden können. Demnach kann die Bildung der CbO-Verbindungen und auch die Abspaltung der CbO-Gruppe dünnschichtchromatographisch verfolgt werden.

TABELLE I

R_F-WERTE* UND R_F-STREUUNGEN VON CARBOBENZOXY-AMINOSÄUREN

CbO-Aminosäure	R_F	*SB***	S_{R_F}***
Ala	0.665	0.63–0.69	0.020
β-Ala	0.650	0.61–0.68	0.028
Asp	0.540	0.51–0.57	0.023
Asp (NH_2)	0.640	0.62–0.66	0.016
Arg	0.490	0.46–0.51	0.017
(NO_2)-Arg	0.690	0.67–0.71	0.018
Glu	0.585	0.55–0.61	0.020
Gly	0.660	0.62–0.70	0.029
(ε-CbO)-Lys	0.460	0.44–0.48	0.015
Met	0.700	0.67–0.73	0.020
di-CbO-Orn	0.725	0.70–0.76	0.020
Phe	0.715	0.68–0.75	0.026
Pro	0.670	0.63–0.70	0.029
Ser	0.675	0.64–0.71	0.026
Tyr	0.720	0.69–0.76	0.025
Val	0.695	0.65–0.72	0.031

* Mittelwerte aus je 6 Bestimmungen.
** Schwankungsbreite.
*** Standardabweichung.

TABELLE II

R_F-WERTE* UND R_F-STREUUNGEN VON AMINOSÄUREN

Aminosäure	R_F	SB**	S_{RF}***
Ala	0.390	0.37–0.41	0.017
Asp	0.465	0.43–0.49	0.022
Arg	0.030	0.02–0.04	0.003
Glu	0.550	0.52–0.57	0.019
Gly	0.350	0.32–0.37	0.019
Lys	0.020	—	—
Met	0.510	0.48–0.54	0.020
Phe	0.550	0.53–0.57	0.016
Pro	0.280	0.25–0.31	0.019
Ser	0.400	0.38–0.43	0.018
Tyr	0.570	0.54–0.60	0.020
Val	0.465	0.43–0.50	0.029

* Mittelwerte aus je 6 Bestimmungen.
** Schwankungsbreite.
*** Standardabweichung.

Die genannten Autoren[4,5] haben nur einige CbO-Aminosäuren untersucht. Wir beschäftigen uns in der vorliegenden Arbeit mit der Chromatographie von 16 CbO-Aminosäuren.

Experimentelles

Zur Schichtbereitung und zur Chromatographie verwendeten wir die "Grundausrüstung zur Dünnschichtchromatographie" der Fa. Desaga (Heidelberg/Deutschland).

Die Chromatographie erfolgte nach früheren Angaben[6] auf 0.25 mm starken Kieselgel-G-Schichten. Zur Schichtbereitung vgl. BRENNER *et al.*[6].

Als Fliessmittel benutzten wir Äthylalkohol abs. puriss.–dest. Wasser (7:3 v/v). Die CbO-Aminosäuren wurden in Essigester, die freien Aminosäuren 0.1 N HCl gelöst. Auftragmenge: 1 μg in 1 μl.

Zur Revelation diente die Chlor/Tolidin-Reaktion[7] und Ninhydrin.

TABELLE III

VERGLEICH DER R_F-WERTE VON CARBOBENZOXY-AMINOSÄUREN MIT DEN R_F-WERTEN VON AMINOSÄUREN

Verbindungspaar (CbO-Aminosäure/ Aminosäure)	ΔR_F
Ala	0.275
Asp	0.075
Arg	0.460
Glu	0.035
Gly	0.310
Lys	0.440
Met	0.190
Phe	0.165
Pro	0.390
Ser	0.275
Tyr	0.150
Val	0.230

Ergebnisse

Um die Trennbarkeit der CbO-Aminosäuren von den freien Aminosäuren beurteilen zu können, haben wir CbO-Aminosäuren und einige freie Aminosäuren gleichzeitig chromatographiert. Die Tabellen I und II enthalten die R_F-Werte. Angegeben sind auch die R_F-Schwankungen[8] und die Standardabweichungen[6].

Mit Hilfe der Chlor–Tolidin-Reaktion lassen sich noch Mengen von 0.5 μg erkennen[7]. Mann kann also noch eine Verunreinigung von ca. 0.2 % erfassen.

In der Tabelle III sind die R_F-Unterschiede (ΔR_F) zwischen CbO-Aminosäuren und dem entsprechenden freien Aminosäuren angegeben. Abgesehen von CbO-Glutaminsäure und Glutaminsäure gelingt die Trennung leicht.

Laboratorium der Universitäts-Frauenklinik*** GYÖRGY PATAKI
Basel (Schweiz)

1 M. BRENNER, A. NIEDERWIESER UND G. PATAKI, in E. STAHL (Herausgeber), *Dünnschicht-chromatographie*, Springer, Berlin, 1962, S. 463 ff.
2 M. JUTISZ UND P. DE LA LLOSA, *Bull. Soc. Chim. France*, (1963) 2913.
3 G. PATAKI, *Z. Klin. Chem.*, im Druck.
4 E. EHRHARDT UND F. CRAMER, *J. Chromatog.*, 7 (1962) 405.
5 R. RINIKER, zit. in E. STAHL (Herausgeber), *Dünnschichtchromatographie*, Springer, Berlin, S. 424.
6 M. BRENNER, A. NIEDERWIESER, G. PATAKI UND A. R. FAHMY, *Experientia*, 18 (1962) 101.
7 G. PATAKI, *J. Chromatog.*, 12 (1963) 541.
8 R. POHLOUDEK-FABINI UND H. WOLLMANN, *Pharmazie*, 15 (1960) 590.

Eingegangen den 5. Juni 1964

* Leiter: Dr. M. KELLER.
** Direktor: Prof. Dr. TH. KOLLER.

Technique for detection of unsaturated lipid spots on thin-layer chromatography plates using indicators bleached with bromine

A number of chromogenic agents have been used for detection of lipid spots on thin-layer chromatography (TLC) plates. Among the most commonly used are U.V., dichlorofluorescein, H_2SO_4 and iodine[1–3]. A comprehensive list of chromogenic agents has been compiled by BOBBIT[4].

When using bromocresol green by the method of KIRCHNER[1] to detect terpenes and lipids in this laboratory it was found that the contrast between the spot of an unsaturated lipid and the background could be increased by bleaching the background with bromine vapor. It was further found that a number of other pH indicators could be used to detect lipid spots by this technique.

Experimental. Approximately 50 μg of oleic acid in organic solvent was spotted on TLC plates of Biosil A30-60 silicic acid (BioRad Laboratories). The plates were sprayed with 0.05 to 0.3 % solution of the indicator in methanol and then exposed to bromine. The appearance of the plates was observed before and after exposure to bromine.

TABLE I

INDICATORS FOUND TO BE SENSITIVE TO BROMINE VAPOR

	Without bromine		*Bleached with bromine*	
	Color of spot	*Color of background*	*Color of spot*	*Color of background*
p-Amino-azobenzene	Y	Y	S	Y
Congo red*	R	R	B	Gy
2',4'-Dichlorofluorescein*	Y	S	Y	S
2,4-Dinitrophenylhydrazine	Y	Y	Y	C
Gentian violet* (Crystal violet)	B	V	B	Y
Methyl orange*	R	O	R	C
Methyl red	O	S	P	C
Methyl violet	B	V	B	Gy
Orange I*	O	O	O	C
Orange II	O	O	O	C
Pan	O	Y	O	C
p-Phenylazophenol	Y	Y	O	C
Phenol red*	Y	Y	P	Y
Quinalizarin	O	V**	O	C
Rhodamine B	P	R	P	C
Sudan red	R	R	P	C
Sudan yellow	O	O	O	C
Superchrome Black TS (Eastman)	V	V	V	C

B = blue; C = colorless; Gy = grey; O = orange; P = pink; Pu = purple; R = red; S = salmon; V = violet.
* Indicates good contrast.
** Orange at low pH. Violet at high pH.

Indicators which gave good contrast upon bleaching with bromine are listed in Table I. Indicators which primarily act by detecting difference in pH between the spot and the TLC adsorbent are listed in Table II and indicators and dyes which gave poor contrast are listed in Table III.

It appears that only unsaturated lipids, which absorb bromine, give increased

TABLE II

INDICATORS WHICH ARE SENSITIVE PRIMARILY TO pH

Chromogenic agent	*pH range and color*	
Alizarin yellow R	Y 10–12 R	
Bromcresol green	Y 3.8–5.6 B	
Bromthymol blue	Y 6.0–7.6 B	Background bleached colorless with bromine
Dichlorophenolsulfonphthalein (chlorophenol red)	Y 4.8–6.7 R	
Malachite green	Y 0–2 B 11.4–13 C	
Sodium alizarin sulfonate	Y 3.8–5 Pu	Background bleached colorless with bromine

* For abbreviations, see Table I.

contrast between the spot and background by this technique. This suggests that the technique can be used to differentiate between degrees of saturation.

Increased contrast between the spot and the background may also be obtained by washing out the background color with a solvent which does not displace the

TABLE III

INDICATORS AND DYES WHICH WERE UNSATISFACTORY AS CHROMOGENIC AGENTS

Chromogenic agent	*Color*
Azobenzene	Y
β-Naphthol	C
Cochineal	C
Methylene blue	B
Nigrosin	V
o-Nitroaniline	Y
4-*o*-Tolylazo-*o*-toluidine	Y
Phenolphthalein	C
Sodium indigo sulfonate	B
Thymolphthalein	C
Titan yellow (Clayton yellow)	Y

* For abbreviations, see Table I.

spots, *e.g.*, a silicic acid TLC chromatogram of lipids which had been sprayed with dichlorofluorescein was developed again with water. The dichlorofluorescein followed the solvent front except for the dyed lipid spots which remained stationary and were clearly visible on a white background.

BioRad Laboratories, Richmond, Calif. (U.S.A.)

CHARLES B. ROLLINS*
ROY D. WOOD

1 J. G. KIRCHNER, J. N. MILLER AND G. J. KELLER, *Anal. Chem.*, 23 (1951) 420.
2 D. C. MALINS AND H. K. MANGOLD, *J. Am. Oil Chemists' Soc.*, 37 (1960) 576.
3 H. K. MANGOLD AND C. MALINS, *J. Am. Oil Chemists' Soc.*, 37 (1960) 383.
4 J. M. BOBBIT, *Thin Layer Chromatography*, Reinhold, New York, 1963.

Received May 29th, 1964

* Present address: Science Labs., 1452 Drake, San Pablo, Calif., U.S.A.

Fluorometric identification of sub-microgram amounts of morphine and related compounds on thin-layer chromatographs

Recently KUPFERBERG *et al.*[1] developed a sensitive fluorometric assay for morphine in biologic fluids. The method is dependent upon oxidation of morphine by potassium ferricyanide to pseudomorphine, which is highly fluorescent. The present report describes the adaptation of this procedure for the separation and detection of minute quantities of morphine and other narcotic analgesics utilizing thin-layer chromatography.

Materials and methods

Chromatographic plates were prepared using a slurry of silica gel G (25 g in 50 ml H_2O) adjusted to pH 8.5 with 5.0 *N* NaOH. The slurry was applied with a 250 μ spreader (Brinkman) and dried at 98° for 1 h. Standard solutions of morphine, *n*-allylnormorphine (Nalline), normorphine, 6-monoacetylmorphine, diacetyl-morphine (heroin), dihydromorphine, meperidine (Demerol), and methadone were prepared by dissolving 1 mg of the base in absolute alcohol.

The standards were applied to the plates in quantities of 0.020 ml containing 0.1 to 10 μg of the compound. The plates were placed in a chamber saturated with methanol, *n*-butanol, benzene and water (60:15:10:15) (COCHIN AND DALY[2]). After the solvent ascended 10 cm the plates were removed, dried *in vacuo*, and sprayed with a solution containing 57 mg of potassium ferricyanide and 7.8 mg potassium ferrocyanide per 100 ml distilled water. The fluorescent spots were maximally developed in 10 min and appeared blue under ultraviolet light ("Mineral Light", Model V-41 with cobalt filter, Ultraviolet Products).

Results

The technique described effectively separated a mixture of morphine, normorphiine, dihydromorphine, 6-monoacetylmorphine and *n*-allylnormorphine (Table I). Heron, codeine, meperidine and methadone could not be identified since they failed to yield fluorescent spots. The R_F values of the substances giving a positive test are listed in Table I. Although the values varied slightly from plate to plate, on any one plate results on the same compound were highly reproducible for amounts ranging from 0.1 to 10 μg.

Although fluorescent spots were observed after developed plates were exposed to air, maximal sensitivity was achieved only by drying the plates *in vacuo* and then

TABLE I

R_F VALUES OF COMPOUNDS SEPARATED ON SILICA GEL G AT pH 8.5 AND IDENTIFIED BY FLUORESCENCE FOLLOWING OXIDATION BY POTASSIUM FERRICYANIDE

Compound	R_F
Normorphine	0.15
Dihydromorphine	0.25
Morphine	0.33
6-Monoacetylmorphine	0.55
n-Allyl-normorphine	0.85

spraying with the ferri-ferrocyanide reagent. The smallest amount of any one compound that could be identified was 0.1 μg.

Discussion

The phenanthrene nucleus of morphine with a free phenolic hydroxyl appears necessary for the formation of the fluorophore as evidenced by the positive test given by normorphine, dihydromorphine, morphine, 6-monoacetylmorphine and nalorphine. Narcotics without these structural prerequisites such as meperidine, methadone, diacetylmorphine and codeine did not fluoresce when treated with the ferri-ferrocyanide reagent.

The formation of fluorescent spots as a result of the oxidation of morphine and other phenanthrene narcotic agents by potassium ferricyanide is controlled by various critical factors. The most important of these is the pH of the silica gel G. If the pH is greater than 9.0, the fluorescence formation is rapid and of short duration; a pH of 8.0 or less produces a fluorescence which is stable but of submaximal intensity.

The second factor which must be controlled is the quantity of potassium ferricyanide used. Too much oxidant causes rapid and excessive oxidation of the phenanthrene narcotic agents; too little oxidant produces less than maximal fluorescence. The ratio of potassium ferri- and ferrocyanide at the concentrations described was found to give optimal stable fluorescence. The method as presented here should find wide application for identifying morphine surrogates with the phenolic phenanthrene moiety. It has been used previously to detect and identify morphine in plasma extracts[1]. The procedure should also allow for similar separation and identification of *n*-allylnormorphine, normorphine, 6-monoacetylmorphine, and dihydromorphine in tissue extracts.

Acknowledgements

This study was supported in part by USPHS grants 5 Tl NB 5270 05 and RG 1839. We are grateful to Dr. T. K. ADLER AND Dr. S. C. LIN for their valuable suggestions.

Department of Pharmacology, Schools of Medicine and Pharmacy, University of California, San Francisco Medical Center, San Francisco, Calif. (U.S.A.)

HARVEY J. KUPFERBERG*
ALAN BURKHALTER
E. LEONG WAY

1 H. J. KUPFERBERG, A. BURKHALTER AND E. L. WAY, *J. Pharmacol.*, in press.
2 J. COCHIN AND J. W. DALY, *Experientia*, 18 (1962) 294.
3 E. L. WAY, J. W. KEMP, J. M. YOUNG AND D. R. GRASSETTI, *J. Pharmacol.*, 129 (1960) 144.

Received June 5th, 1964

* Present address: Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda 14, Md., U.S.A.

Book Review

Chromatographic Methods, by R. STOCK AND C. B. F. RICE, Chapman and Hall, London, 1963, 206 pages, 72 figs., 21 tables, price 40s.

This is an introduction to chromatography which not only gives the new-comer a good idea of what chromatography is and which method is suited for his particular purpose, but also enables him, by giving full descriptions of the recommended techniques and a careful selection of model experiments, to start work on his own. The outline is subordinated to this practical purpose: After brief introductory information on the basic concepts and terms, three main sections follow: on columns with liquid mobile phases, on sheets (paper and thin-layer chromatography, zone electrophoresis) and on gas chromatography. The authors, from the College of Technology, Liverpool, seem to have considerable teaching experience in specialized chromatography courses; this may have led to limitation to subjects that can be thoroughly explained within the allotted time or space and to omission of others. In contrast to other elementary texts, the biochemical and biological applications are not given prominence. The separation of proteins on columns with ion-exchange cellulose derivatives or gel filtration on molecular sieves are thus not referred to at all and applications of zone electrophoresis for proteins are only just mentioned. It may be a matter of opinion whether in the model experiments it is justified to put so much emphasis on inorganic applications: 10 out of a total of 18 experiments. Yet it cannot be denied that chromatography should be given more attention in practical classes of elementary inorganic chemistry, and this text may help in this direction. Theoretical considerations are not given, with the exception of gas chromatography, but the authors are aware of the theoretical basis and are careful not to introduce erroneous concepts for the sake of didactic simplicity. Nevertheless, one may wonder whether a dichotomy of adsorption and partition chromatography would leave proper place for classification of ion-exchange chromatography. The book is not simply a compilation and didactic rearrangement of existing texts, but many original ideas and observations are also introduced. For instance, the excellent discussion on the establishment of the stationary and mobile phase in paper chromatography (p. 65) is very useful and not only of theoretical interest. It is a matter of opinion whether the absorption of the organic component of the solvent mixture by a paper made water-repellent should be called true reversed phase chromatography in contrast to impregnation of paper with oils. A misprint was noted in the list of contents: the omission of the page number of Chapter 5.

This handy book will be useful to teachers and students of higher secondary schools and their technical equivalents, especially for introducing gas chromatography, but the reviewer has even successfully used it as an aid in individual post-graduate training.

I. M. HAIS (Hradec Králové)

Bibliography Section

Paper Chromatography

2. FUNDAMENTALS, THEORY AND GENERAL

SOCZEWIŃSKI, E. AND WAKSMUNDSKI, A.: On the relation between the R_M coefficient and hydrogen ion concentration in buffered paper chromatography. *Bull. Acad. Polon. Sci., Ser. Sci. Chim.*, 9 (1961) 445–449.

WAKSMUNDSKI, A., SOCZEWIŃSKI, E. AND JUSIAK, L.: (The effect of the chemical nature of buffers upon the R_F = f(pH) curves of organic electrolytes in buffered paper chromatography). *Ann. Univ. Mariae Curie-Sklodowska, Lublin-Polonia, Sect. AA*, 15, No. 1 (1960) 1–11.

3. TECHNIQUES I

ČELAP, M. B. AND JANJIĆ, T. J.: Determination of micro amounts of elements using the ring oven method with preliminary chromatographic separation. *Mikrochim. Acta*, (1963) 313–315.

DEYL, Z., ROSMUS, J. AND PAVLÍČEK, M.: Chromatographie im Fliehkraftfeld. XI. Chromatographie mit geometrischer Begrenzung der mobilen Phase. *Mikrochim. Acta*, (1963) 390–394.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

RONDIA, D.: (The analysis of polycyclic hydrocarbons in air). *Arch. Belges Méd. Sociale, Hyg., Méd. Travail Méd. Légale*, 21, No. 2 (1963) 73–98; *C.A.*, 60 (1964) 2247g.

7. PHENOLS

CLARK, I. T.: Chromatographic separation of phenols on ion-exchange paper. *J. Chromatog.*, 15 (1964) 65–69.

KAPADIA, G. J. AND ZALUCKY, T. B.: Chromogenic activity of carbonyl-substituted resorcinols with Ehrlich reagent. *J. Chromatog.*, 15 (1964) 76–79.

WAGENBRETH, A. N.: (Phenol of the leaves of various grafts of the genus *Pyrus* in the period of vegetative growth). *Arch. Gartenbau*, 11 (1963) 339–361; *C.A.*, 60 (1964) 4462c.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

GILL, S.: (Chemical composition of *Trifolium arvense*. II. Paper chromatography of polyphenolic compounds). *Dissertationes Pharm.*, 15 (1963) 241–249; *C.A.*, 60 (1964) 2043g.

REZNIK, H. AND NEUHAEUSEL, R.: (Colorless anthocyanin in submerged aquatic plants). *Z. Botan.*, 47 (1959) 471–489; *C.A.*, 60 (1964) 2042h.

9. OXO COMPOUNDS

ZIEGLER, D. M. AND PETTIT, F. H.: Formation of an intermediate N-oxide in the oxidative demethylation of N,N-dimethylaniline catalyzed by liver microsomes. *Biochem. Biophys. Res. Commun.*, 15 (1964) 188.

10. CARBOHYDRATES

BOSE, S. M.: Methods for determining the nature of linkages of certain constituents of the carbohydrate moiety to the protein core in skin mucoid. *Proc. Symp. Aspects Protein Structure, Madras, 1963*, pp. 357–367; *C.A.*, 60 (1964) 4386b.

CASSIA, B. AND FERLITO, S.: (Identification of some sugars in urine by paper chromatography. I. Techniques). *Boll. Soc. Ital. Biol. Sper.*, 39 (1963) 335–338; *C.A.*, 60 (1964) 4438c.

HORNUNG, M.: Paper chromatography of pneumococcal cell-wall hydrolyzates containing glucosamine, galactosamine, muramic acid, and peptides. *J. Bacteriol.*, 86 (1963) 1345–1346; *C.A.*, 60 (1964) 4439f.

JINDRA, A., FELKLOVÁ, M., JIRÁČEK, V. AND BÖSWART, J.: Variations of sugar and morphine content in *Papaver somniferum* L. during ontogenesis. *Planta Med.*, 12 (1964) 205–210.

KOTO, K., MATSUI, S. AND SAWAE, S.: (Determination of raffinose by the chromatographic elution method). *Seito Gijutsu Kenkyukaishi*, 11 (1962) 20–27; *C.A.*, 60 (1964) 4322d.

PITTERA, A., CASSIA, B. AND FERLITO, S.: (Chromatographic identification of several sugars in normal human urine). *Arch. Studio Fisiopatol. Clin. Ricambio*, 27, No. 2 (1963) 85–96; *C.A.*, 60 (1964) 4441h.

SECOR, G. E. AND WHITE, L. M.: Reversed-phase paper chromatography of some substituted hydrazine derivatives of monosaccharides and related compounds. *J. Chromatog.*, 15 (1964) 111–115.

VAUGHAN, J. M. AND DICKEY, E.E.: Oxidation of a sprucewood glucomannan with lead tetraacetate. *J. Org. Chem.*, 29 (1964) 715–718.

YOSIZAWA, Z.: Deamination of the partially N-deacetylated acid mucopolysaccharides. *Tohoku J. Exptl. Med.*, 80 (1963) 26–31; *C.A.*, 60 (1964) 1970d.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

ANGHILERI, L. J.: Paper chromatography of I^{131}-labeled oleic acid and I^{131}-labeled triolein. *Intern. J. Appl. Radiation Isotopes*, 15 (1964) 95–96.

BENTLEY, R. AND THIESSEN, C. P.: Biosynthesis of tropolones in *Penicillium stipitatum*. I. The isolation, separation, and assay of stipitatonic and stipitatic acid. *J. Biol. Chem.*, 238 (1963) 1880–1888.

BOBRANSKI, B. AND SYPER, L.: Determination of α-allyl-γ-valerolactone in physiological body fluids. *Arch. Immunol. Therap. Exptl.*, 11 (1963) 127–133; *C.A.*, 60 (1964) 2197a.

DUBOVSKÝ, J. AND PETRASEK, J.: (Quantitative estimation of important catechol amine metabolites by paper chromatography). *Vnitrni Lekar.*, 9 (1963) 503–508; *C.A.*, 60 (1964) 2029e.

DUNCOMBE, W. G.: The colorimetric micro-determination of long-chain fatty acids. *Biochem. J.*, 88 (1963) 7–10 — spectrophotometric determination of copper soaps; variable paper blank.

FIJOLKA, P., RADOWITZ, W. AND RUNGE, F.: (Paper chromatographic analysis of dicarboxylic acids and glycols of polyester resins). *Plaste Kautschuk*, 10 (1963) 521–525; *C.A.*, 60 (1964) 3492g.

GÖDICKE, W. AND BROSOWSKI, K. H.: Die Isolierung der 3-Methoxy-4-hydroxy-mandelsäure aus dem Urin unter Verwendung der Keilstreifenmethode. *J. Chromatog.*, 15 (1964) 88–89.

IMAI, K., REEVES, H. C. AND AJL, S. J.: *n*-Propyl malate synthetase. *J. Biol. Chem.*, 238 (1963) 3193–3198.

PODKOWIENSKA, H.: (Developing of organic acids on paper). *Prace Zakresu Towaroznawst. Chem. Wyzsza Szkola Ekon. Poznan. Zeszyty Nauk, Ser. I*, No. 10 (1963) 95–98; *C.A.*, 60 (1964) 4442d — iodine vapours.

RAAFAT, M. A., VERBEKE, R. AND PEETERS, G.: The metabolism of propionate in the isolated cow's udder. *Biochem. J.*, 88 (1963) 155–159.

ROSS, P. AND WOOTON, I. D. P.: Glycine conjugation and toxicity of phenolic acids. *Clin. Chim. Acta*, 9 (1964) 434–440 — R_F values of 9 substituted hippuric acids.

SAFTA, M.: (Determination of lactic acid in ensiled corn by means of paper chromatography). *Acad. Rep. Populare Romine, Studii Cercetari Biochim.*, 6 (1963) 285–288; *C.A.*, 60 (1964) 2259e.

SOLDATENKOV, S. V. AND MAZUROVA, T. A.: (Analysis of organic acids of plants using ion-exchange resins and paper chromatography). *Metodika Kolichestv. Bumazhn. Khromatog. Sakharov, Organ. Kislot i Aminokislot u Rast. Sb.*, (1962) 27–42; *C.A.*, 60 (1964) 4440c.

13. STEROIDS

BIRCHALL, K., CATHRO, D. M., FORSYTH, C. C. AND MITCHELL, F. L.: A method for the separation and estimation of neutral steroids in the urine of newborn infants. *J. Endocrinol.*, 27 (1963) 31–51; *C.A.*, 60 (1964) 4410f.

GOEBEL, P.: (Paper chromatographic separation and quantitative determination of estrogen in urine). *Verhandl. Deut. Ges. Inn. Med.*, 67 (1961, publ. 1962) 333–338; *C.A.*, 60 (1964) 4411a.

LEWBART, M. L. AND MATTOX, V. R.: The mechanism of the Porter–Silber reaction. I. Rearrangement of the dihydroxyacetone group of steroids. *J. Org. Chem.*, 29 (1964) 513–521.

STANČÁKOVÁ, A.: Quantitative Analyse der Cortisol- und Corticosteron-Metaboliten mittels der Elutionspapierstreifen-Chromatographie. *Clin. Chim. Acta*, 10 (1964) 55–61.

STÁRKA, L. AND MATYS, Z.: (Normal excretion of 17-keto steroids in urine). *Endokrinologie*, 44 (1963) 156–163; *C.A.*, 60 (1964) 2155a.

STITCH, S. R., OAKEY, R. E. AND ECCLES, S. S.: The biosynthesis and metabolism of oestrogens in the rat. 1. Conversion of [4-^{14}C]-testosterone into [^{14}C]-oestradiol-17β by the rat ovary. *Biochem. J.*, 88 (1963) 70–76.

15. TERPENE DERIVATIVES

RICHTER, G. AND MUSCHOLL, P.: Papierchromatographie ätherischer Öle. II. Die densitometrische Bestimmung ungesättigter Terpen-Alkohole und -Ester in ätherischen Ölen. *J. Chromatog.*, 15 (1964) 39–49.

STEVENSON, J., HEMMING, F. W. AND MORTON, R. A.: The intracellular distribution of solanesol and plastoquinone in green leaves of the tabacco plant. *Biochem. J.*, 88 (1963) 52–56.

16. NITRO AND NITROSO COMPOUNDS

GASPARIČ, J.: An anomalous chromatographic behaviour of some di- and trinitrophenols. *J. Chromatog.*, 15 (1964) 83–85.

KITAHARA, S., ITO, A. AND HIYAMA, H.: Separation and determination of isomers (mainly of dye intermediates) by paper chromatography. III. Separation and determination of impurities in 1-nitronaphthalene. *Kagaku To Kogyo (Osaka)*, 37 (1963) 31–37; *C.A.*, 60 (1964) 4279c.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

COWARD, R. F., SMITH, P. AND WILSON, O. S.: Paper chromatography of phenolic derivatives of phenylethylamine and some other diazo-reactive bases in urine. *Clin. Chim. Acta*, 9 (1964) 381–391 — R_F values of 38 compounds mostly phenylethylamine derivatives.

DUBEY, S. S.: Isolation, identification and hydrolysis of lombricine from Indian earthworms. *Indian J. Chem.*, 1 (1963) 453–454; *C.A.*, 60 (1964) 4505h.

KOWALCZYK-KANIEWSKA, T.: (Colorimetric determination of 3-aminophenol in sodium 4-aminosalicylate). *Acta Polon. Pharm.*, 19 (1962) 341–344; *C.A.*, 60 (1964) 3492b.

PERRY, T. L., SHAW, K. N. F., WALKER, D. AND REDLICH, D.: Urinary excretion of amines in normal children. *Pediatrics*, 30 (1962) 576–584.

ROBERTS, D. J.: Some possible causes of pharmacological activity in blank eluates following the separation of sympathomimetic catecholamines by paper chromatography. *J. Pharm. Pharmacol.*, 16 (1964) 313–322.

SAX, S. M., HARBISON, P. L., SAX, M. AND BAUGHMAN, R. H.: A study of malignolipin picrate. *J. Biol. Chem.*, 238 (1963) 3817–3819.

SEN, N. P. AND MCGEER, P. L.: 4-Methoxyphenylethylamine and 3,4-dimethoxyphenylethylamine in human urine. *Biochem. Biophys. Res. Commun.*, 14 (1964) 227.

18. AMINO ACIDS

EL-HEFNAWI, H. AND EL-HAWARY, M. F. S.: Chromatographic studies of amino acids in serums and urines of patients with xeroderma pigmentosum and their normal relatives. *Brit. J. Dermatol.*, 75 (1963) 235–244; *C.A.*, 60 (1964) 2168h.

GOLDSTONE, A. AND ADAMS, E.: Further metabolic reactions of γ-hydroxyglutamate: amidation to γ-hydroxyglutamine; possible reduction to hydroxyproline. *Biochem. Biophys. Res. Commun.*, 16 (1964) 71.

MACRI, I., AVANZINO, G. L. AND IVALDI, G.: (Chromatographic identification of acylaminoacylhydroxamic acid derivatives). *Boll. Soc. Ital. Biol. Sper.*, 38 (1962) 1535–1536; *C.A.*, 60 (1964) 3493h.

MARTIN, G. C. AND WILLIAMS, M. W.: A column and paper chromatographic method for separation of N-dimethylamino-succinamic acid in plant extracts. *J. Chromatog.*, 15 (1964) 95–97.

MÜLLER, K., SKRUBE, H. AND SPITZY, H.: Quantitative Untersuchungen über die papierchromatographische Wanderung Schilddrüsenhormone. *Mikrochim. Acta*, (1963) 297–304.

MUND, CH. AND VENNER, H.: Zur Technik des Nachweises von α,ε-Diaminopimelinsäure. *Naturwissenschaften*, 51 (1964) 298–299.

RICHARDSON, K. E. AND TOLBERT, N. E.: Condensation of glycine with phenol during paper chromatography. *J. Chromatog.*, 15 (1964) 50–56 — artifact formation attributed to the presence of formaldehyde.

ROTHSTEIN, M. AND MAYOH, H.: Glycine synthesis and isocitrate lyase in the nematode *Caenorhabditis briggsae*. *Biochem. Biophys. Res. Commun.*, 14 (1963) 43.

19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS AND PEPTIDES

COOKE, J. P., ANFINSEN, C. B. AND SELA, M.: The identification of unreactive amino groups in ribonuclease and their significance to enzymatic activity. *J. Biol. Chem.*, 238 (1963) 2034–2039.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

BIGGS, H. G. AND DOUMAS, B.: Oxidative catabolism of thymine. *J. Biol. Chem.*, 238 (1963) 2470–2471.

CARDINI, C. AND QUERCIA, V.: A simple uni-dimensional separation of nucleosides. *J. Chromatog.*, 15 (1964) 86–87 — ion-exchange cellulose.

DAVILA, C. AND LEDOUX, L.: (Chromatography of deoxyribonucleic acids (DNA) on diethylaminoethyl cellulose paper). *2nd Chromatog. Symp., Brussels, 1962*, pp. 87–96; *C.A.*, 60 (1964) 4438h.

DOCTOR, B. P., CONNELLY, C. M., RUSHIZKY, G. W. AND SOBER, H. A.: Studies on the chemical structure of yeast amino acids acceptor ribonucleic acids. *J. Biol. Chem.*, 238 (1963) 3985–3990.
EL'PINER, I. E. AND SOKOL'SKAYA, A. V.: (Physico-chemical transformations of pyrimidine and purine bases in the field of ultra-sonic waves, accompanied by the formation of a series of fluorescent substances). *Dokl. Akad. Nauk SSSR*, 153 (1963) 200–203.
IVES, D. H., MORSE, JR., P. A. AND POTTER, V. R.: Feedback inhibition of thymidine kinase by thymidine triphosphate. *J. Biol. Chem.*, 238 (1963) 1467–1474 — on DEAE-cellulose paper.
MCCORMICK, D. B.: Specificity of flavin adenine dinucleotide pyrophosphorylase for flavin phosphates and nucleoside triphosphates. *Biochem. Biophys. Res. Commun.*, 14 (1964) 493.
RABINOWITZ, M. AND GOLDBERG, I. H.: Glycogen synthesis and other reactions of pseudouridine diphosphate glucose. *J. Biol. Chem.*, 238 (1963) 1801–1806.
RYABOVA, T. S., GLEBOV, R. N., SHABAROVA, Z. A. AND PROKOFEV, M. A.: (Synthesis of N-adenylyl-5'-phenylalanine methyl ether by the carbodiimide method). *Dokl. Akad. Nauk SSSR*, 153 (1963) 363–365.
STANFORD, F. G.: R_F values of some monosubstituted purines. *J. Chromatog., Chromatog. Data*, 15 (1964) D12.
VERACHTERT, H., BASS, S. T. AND HANSEN, R. G.: Enzymic synthesis of inosine 5'-diphosphate glucose and inosine 5'-diphosphate mannose. *Biochem. Biophys. Res. Commun.*, 15 (1964) 158.
VOLK, A. W. AND ASHWELL, G.: Enzymatic formation of TDP-3-acetamido-3,6-dideoxy hexose. *Biochem. Biophys. Res. Commun.*, 12 (1963) 116.

22. ALKALOIDS

NOWACKI, E.: (Chromatographic method of qualitative determination of lupin alkaloids in single seeds). *Roczniki Nauk Rolniczych, Ser. A*, 88 (1963) 135–141; *C.A.*, 60 (1964) 4440e.
SCHWARTZ, S. L. AND MCKENNIS, JR., H.: Studies on the degradation of the pyrrolidine ring of (—)-nicotine *in vivo*. Formation of γ-(3-pyridyl)-γ-oxobutyric acid. *J. Biol. Chem.*, 238 (1963) 1807–1812.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

BAER, H.: (The mode of action of fusaric acid). *Phytopathol. Z.*, 48 (1963) 149–177; *C.A.*, 60 (1964) 4497a.
HULTQUIST, D. E. AND MORRISON, M.: Lactoperoxidase. 1. The prosthetic group of lactoperoxidase. *J. Biol. Chem.*, 238 (1963) 2843–2846 — R_F values of porphyrin methyl esters.
KRANTZ, S. B., GALLIEN-LARTIGUE, O. AND GOLDWASSER, E.: The effect of erythropoietin upon heme synthesis by marrow cells *in vitro*. *J. Biol. Chem.*, 238 (1963) 4085–4090 — on siliconized paper.

24. ORGANIC SULPHUR COMPOUNDS

VAN DER VENNE, M. TH. AND T'SIOBBEL, J. B.: (R_F values (also thin layers) of sulphamides). *Chromatographie Symposium II, 1962, Soc. Belge Sci. Pharm., Bruxelles, 1963*, p. 196; from *J. Chromatog., Chromatog. Data*, 15 (1964) D10–D11 — R_F values of 34 sulphonamides.

25. ORGANIC PHOSPHORUS COMPOUNDS

ANSELL, G. B. AND SPANNER, S.: The occurrence of a long-chain ether analogue of phosphatidylethanolamine in brain tissue. *Biochem. J.*, 88 (1963) 56–64 — starch added to prevent powdering of silicic acid.
NATHENSON, S. G. AND STROMINGER, J. L.: Enzymatic synthesis of N-acetylglucosaminylribitol linkages in teichoic acid from *Staphylococcus aureus*, strain Copenhagen. *J. Biol. Chem.*, 238 (1963) 3161–3169.

26. METALLO-ORGANIC COMPOUNDS

ADLOFF-BACHER, M. AND ADLOFF, J. P.: Sur le marquage du triphényl-bismuth par le radiobismuth: étude chromatographique. *J. Chromatog.*, 15 (1964) 70–75.

27. VITAMINS

PLAUT, G. W. E.: Studies on the nature of the enzymic conversion of 6,7-dimethyl-8-ribityllumazine to riboflavin. *J. Biol. Chem.*, 238 (1963) 2225–2243 — R_F values of 16 compounds.
WINESTOCK, C. H., AOGAICHI, T. AND PLAUT, G. W. E.: The substrate specificity of riboflavin synthetase. *J. Biol. Chem.*, 238 (1963) 2866–2874 — R_F values of 5'-deoxyribityl and ribityl derivatives of flavin and 6,7-dimethyllumazine.

28. ANTIBIOTICS

ROUSSELET, R. AND PARIS, R.: Sur une méthode rapide de contrôle des solutés aqueux de chloramphénicol par chromatographie sur papier. *Ann. Pharm. Franc.*, 22 (1964) 249–255.

29. INSECTICIDES AND OTHER PESTICIDES

BOGGS, H. M.: Procedures for residues of dinitro compounds. *J. Assoc. Offic. Agr. Chem.*, 47 (1964) 346–348.

ONLEY, J. H.: Rapid method for chlorinated pesticide residues in fluid milk. *J. Assoc. Offic. Agr. Chem.*, 47 (1964) 317–321.

YIP, G.: Paper chromatographic analysis of chlorophenoxy acids and esters in wheat. *J. Assoc. Offic. Agr. Chem.*, 47 (1964) 343–345.

30. SYNTHETIC AND NATURAL DYES

KANEDA, K.: Bilirubinoid azo pigments. I. Paper chromatography of bilirubinoid azo pigments. *Okayama Igakkai Zasshi*, 75 (1963) 115–124; *C.A.*, 60 (1964) 1976d.

ŠRÁMEK, J.: Paper chromatography of dyes. IV. Paper chromatography of water-soluble dyes. *J. Chromatog.*, 15 (1964) 57–64.

32. PHARMACEUTICAL APPLICATIONS

ŠKRABÁNEK, P., CHUNDELA, B., HYNIE, I. AND KÁCL, K.: (Isolation and detection of Bromural in toxicology). *Deut. Z. Ges. Gerichtl. Med.*, 54 (1963) 217–226; *C.A.*, 60 (1964) 4441f.

THOMPSETT, S. L.: Note on the detection of hexoestrol, stilboestrol, dienoestrol and the *p*-hydroxy metabolites of phenobarbitone and phenytoin in urine. *J. Pharm. Pharmacol.*, 16 (1964) 207–208.

33. INORGANIC SUBSTANCES

BHATNAGAR, R. P. AND POONIA, N. S.: Paper chromatographic separation of some less familiar transition metals using a solvent containing chloroform. *Anal. Chim. Acta*, 30 (1964) 211–212.

BHATNAGAR, R. P. AND POONIA, N. S.: Paper chromatographic separation of Ag, Pb, Hg and Tl using a solvent containing chloroform. *Anal. Chim. Acta*, 30 (1964) 310.

HAYEK, E. AND DALLA TORRE, H.: Chromatographische Abtrennung des Urans aus wässriger Lösung an Papier und Zellulose. *Mikrochim. Acta*, (1963) 1078–1081.

HEININGER, JR., C. AND LANZAFAME, F. M.: High speed separation of the light rare earth on centrifugally accelerated ion-exchange paper. *Anal. Chim. Acta*, 30 (1964) 148–154.

KÜBLER, W.: Nachweis und quantitative Bestimmung von Phosphaten in Waschmitteln mit Hilfe der Papierchromatographie. *Mitt. Gebiete Lebensm. Hyg.*, 54 (1963) 478–492.

LEDERER, M. AND SARACINO, F.: Chromatography on ion-exchange papers. XV. The adsorption of metal ions on cation exchangers from perchloric acid solutions. *J. Chromatog.*, 15 (1964) 80–82 — resin-loaded and substituted cellulose papers; influence of $HClO_4$ concentration on 20 ions.

MOGHISSI, A.: R_F values of iodide, iodate and tellurite. *J. Chromatog., Chromatog. Data*, 15 (1964) D2.

PFEIL, E.: (Paper chromatographic separation of common cations). *2nd Chromatog. Symp., Brussels, 1962*, pp. 203–206; *C.A.*, 60 (1964) 3468g.

POLLARD, F. H., NICKLESS, G. AND JENKINS, H.: New reagents specially designed for use in inorganic chromatography. *Anal. Chem., Proc. Intern. Symp. Birmingham Univ., Birmingham (Engl.), 1962*, (1963) 160–164; *C.A.*, 60 (1964) 3467b.

ROTHBART, H. L., WEYMOUTH, H. W. AND RIEMAN, III, W.: Separation of the oligophosphates. *Talanta*, 11 (1964) 33–41; *C.A.*, 60 (1964) 3469c.

SHERMA, J. AND CLINE, CH. W.: Anion-exchange paper chromatography of metal ions. *Anal. Chim. Acta*, 30 (1964) 139–147.

34. RADIOACTIVE COMPOUNDS

ANGHILERI, L. J.: A chromatographic study of the stability of iodine-131 labeled sodium *o*-iodohippurate. *J. Nucl. Med.*, 4 (1963) 155–158.

DOBBS, H. E.: The use of a windowless gas-flow counter for detecting weak β-emitters on paper chromatograms. *J. Chromatog.*, 15 (1964) 29–38.

FERRARI, G., PASSERA, C. AND BENEDETTI, E.: (Mechanism of amino acid synthesis in plants. II. Rapid assimilation in *Chlorella vulgaris* of $NaHCO_3$ carbon into the guanidyl group of arginine). *Ric. Sci., Rend.*, B3 (1963) 189–192; *C.A.*, 60 (1964) 2073g.

SANTOI, J. AND KOVACS, V.: (Chromatographic determination of amino acids in biological liquids by the internal standard methods). *Lab. Delo*, 9, No. 10 (1963) 41–42; *C.A.*, 60 (1964) 2036e.

WISS, O. AND BRUBACHER, G.: The occurrence of thiamine metabolites in rat liver after administration of ^{35}S-labelled thiamine. *Ann. N.Y. Acad. Sci.*, 98, Art. 2 (1962) 508–515; *C.A.*, 60 (1964) 4556d.

Thin-layer Chromatography

1. REVIEWS AND BOOKS

Long, A. G.: Thin-layer chromatography. *Ind. Chemist*, 39 (1963) 465–468; *C.A.*, 60 (1964) 3463d.

Waldi, D.: (Thin-layer chromatography in medical laboratories). *Aerztl. Lab.*, 9 (1963) 221–227; *C.A.*, 60 (1964) 2030b.

3. TECHNIQUES I

Bancher, E., Scherz, H. and Prey, V.: Die Kleinplattenmethode in der Dünnschichtchromatographie. *Mikrochim. Acta*, (1963) 712–719.

Berger, J. A., Meyniel, G., Blanquet, P. and Petit, J.: (Thin-layer chromatography with several layers side by side on the same plate). *Compt. Rend.*, 257 (1963) 1534–1537; *C.A.*, 60 (1964) 3469a.

Connolly, J. P., Flanagan, P. J., Dorchai, R. Ó. and Thomson, J. B.: Sample application in preparative thin-layer chromatography. *J. Chromatog.*, 15 (1964) 105–106.

Janák, J.: Gas chromatography as a sampling procedure for thin-layer or paper chromatography. *J. Gas Chromatog.*, 1, No. 10 (1963) 20–23; *C.A.*, 60 (1964) 3470e.

Janák, J.: Multi-dimensional chromatography using different developing methods. *J. Chromatog.*, 15 (1964) 15–28 — effluent from the orifice of the gas chromatograph is applied on thin layers; 28 tar hydrocarbons detected with tetracyano-ethylene, R_F values in 3 systems tabulated; reagent sprayed on by means of a wetted tooth-brush rubbed on a metal sieve.

4. TECHNIQUES II

Systematic analysis, automation and preparative-scale thin-layer chromatography

Seikel, M. K., Millett, M. A. and Saeman, J. F.: Notes on semimicro preparative thin-layer chromatography. *J. Chromatog.*, 15 (1964) 115–118.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

Suryaraman, M. G. and Cave, W. T.: Detection of some aliphatic saturated long-chain hydrocarbon derivatives by thin-layer chromatography. *Anal. Chim. Acta*, 30 (1964) 96–100.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

Nybom, N.: Thin-layer chromatographic analysis of anthocyanidins. *Physiol. Plantarum*, 17 (1964) 157–164 — cellulose layers.

Reisch, J., Bornfleth, H. and Rheinbay, J.: (Analysis of drug mixtures. III. Thin-layer chromatography of some 4-hydroxycoumarin anticoagulants). *Pharm. Ztg.-Nachr.*, 108 (1963) 1183–1184; *C.A.*, 60 (1964) 3947e.

10. CARBOHYDRATES

Blumenfeld, O. O., Paz, M. A., Gallop, P. M. and Seifter, S.: The nature, quantity and mode of attachment of hexoses in ichthyocol. *J. Biol. Chem.*, 238 (1963) 3835–3839 — TLC of 2,4-dinitrophenylosazone derivatives.

De Souza, N. O. and Panek, A.: Detection of saccharification products by thin-layer chromatography. *J. Chromatog.*, 15 (1964) 103–105.

Diemair, W. and Kölbel, R.: Über den Nachweis und die Bestimmung von Dextrinen. II. Chromatographische Methoden. *Z. Lebensm.-Untersuch.-Forsch.*, 124 (1964) 157–179 — thin-layer, paper and column chromatography.

Garbutt, J. L.: Inexpensive adsorbents for thin-layer chromatography of carbohydrates. *J. Chromatog.*, 15 (1964) 90–92 — celite filter aids.

Wiedenhof, N.: Thin-layer chromatography of cyclodextrins and some other sugars using microchromatoplates. *J. Chromatog.*, 15 (1964) 100–102 — on silicic acid or alusil.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

Gensler, W. J., Leeding, M. V. and Rao A. Somasekar: Synthesis and configurational significance of 3-(O-methyl)-neoepipicropodophyllin. *J. Org. Chem.*, 29 (1964) 1594–1597.

Lynes, A.: The analysis of straight-chain (n-C_1–C_9) carboxylic acids by a thin-layer chromatographic method. *J. Chromatog.*, 15 (1964) 108–110.

Samuelsson, B.: Isolation and identification of prostaglandins from human seminal plasma. 18. Prostaglandins and related factors. *J. Biol. Chem.*, 238 (1963) 3229–3234.

13. STEROIDS

OZON, R.: (*In vivo* analysis of estrogen metabolism in the course of sexual differentiation in *Pleurodeles waltlii*). *Compt. Rend.*, 257 (1963) 2332–2335; *C.A.*, 60 (1964) 4514d.

14. STEROID GLYCOSIDES

ZELNIK, R., ZITI, L. M. AND GUIMARÃES, C. V.: A chromatographic study of the bufadienolides isolated from the venom of the parotid glands of *Bufo paracnemis* Lutz 1925. *J. Chromatog.*, 15 (1964) 9–14.

15. TERPENE DERIVATIVES

PINAR, M. AND SCHMID, H.: (3′-Methoxylimaspermine, limapodine, 3′-methoxylimapodine and tubotaiwine from *Aspidosperma limae*). *Ann.*, 668 (1963) 97–104; *C.A.*, 60 (1964) 4203e.

18. AMINO ACIDS

BARON, D. N. AND ECONOMIDIS, J.: Thin-layer chromatography of amino acids and sugars. *J. Clin. Pathol.*, 16 (1963) 484–486; *C.A.*, 60 (1964) 4444f.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

KELLEHER, J. AND ROLLASON, J. G.: A rapid method for partial identification of barbiturates in blood using thin layer chromatography on microscopic slides. *Clin. Chim. Acta*, 10 (1964) 92–94.

REISCH, J., BORNFLETH, H. AND RHEINBAY, J.: (Analysis of drug mixtures. II. Separation of drugs by ion exchangers and determination by thin-layer chromatography). *Pharm. Ztg.-Nachr.*, 108 (1963) 1182–1183; *C.A.*, 60 (1964) 3947d.

22. ALKALOIDS

ADAM, G. AND SCHREIBER, K.: (*Solanum* alkaloids. XXVI. Preparative separation of stereoisomeric iminocholestanes and other steroids by means of thin-layer chromatography by using iodine as indifferent tracer). *Z. Chem.*, 3 (1963) 100–102; *C.A.*, 60 (1964) 4443a.

GROEGER, D.: (The occurrence of ergolene derivatives in *Ipomoea* species). *Flora (Jena)*, 153 (1963) 373–382; *C.A.*, 60 (1964) 2043e.

25. ORGANIC PHOSPHORUS COMPOUNDS

ALLEN, C. F., GOOD, P., DAVIS, H. F. AND FOWLER, S. D.: Plant and chloroplast lipids. I. Separation and composition of major spinach lipids. *Biochem. Biophys. Res. Commun.*, 15 (1964) 424–430 — thin-layer and paper chromatography.

BAER, E. AND RAO, G. V.: Phosphatidyl-2-amino-2-methylpropanols. I. Synthesis of dimyristoyl- and distearoyl-L-α-glycerylphosphoryl-2-amino-2-methylpropanol. *J. Biol. Chem.*, 238 (1963) 1941–1946.

CURRI, S. B. AND NINFO, V.: (Histochemistry of phospholipids. I. Preparation and thin-layer chromatography of phospholipids as an experimental model). *Riv. Anat. Patol. Oncol.*, 23 (1963) 479–506; *C.A.*, 60 (1964) 4441d.

JATZKEWITZ, H.: The role of cerebroside sulfuric esters in leukodystrophy and a new method for the quantitative ultramicro determination of brain sphingolipids. *Brain Lipids Lipoproteins Leucodystrophies, Proc. Neurochem. Symp., Rome, 1961* (publ. 1963), pp. 147–152; *C.A.*, 60 (1964) 2182e.

PHILIPPART, M. AND MENKES, J.: Isolation and characterization of the main splenic glycolipids in Gaucher's diseases: evidence for the site of metabolic block. *Biochem. Biophys. Res. Commun.*, 15 (1964) 551.

27. VITAMINS

SKINNER, W. A.: Vitamin E oxidation with free radical initiators. Azobis-isobutyronitrile. *Biochem. Biophys. Res. Commun.*, 15 (1964) 469.

WEBER, F. AND WISS, O.: (Metabolism of vitamin E in the rat). *Helv. Physiol. Pharmacol. Acta*, 21 (1963) 131–141; *C.A.*, 60 (1964) 2098b.

29. INSECTICIDES AND OTHER PESTICIDES

BACHE, C.: Note to the estimation of ambien in tomatoes by thin-layer chromatography. *J. Assoc. Offic. Agr. Chem.*, 47 (1964) 355.

MORLEY, H. V. AND CHIBA, M.: Thin-layer chromatography for chlorinated pesticide residue analysis without cleanup. *J. Assoc. Offic. Agr. Chem.*, 47 (1964) 306–310.

ONLEY, J. H.: Rapid method for chlorinated pesticide residues in fluid milk. *J. Assoc. Offic. Agr. Chem.*, 47 (1964) 317–321.

30. SYNTHETIC AND NATURAL DYES

DOI, Y.: Analysis of Derris root preparations by thin-layer chromatography. *Kagaku Keisatsu Kenkyusho Hokoku*, 16 (1963) 51–57; *C.A.*, 60 (1964) 4441c.

32. PHARMACEUTICAL APPLICATIONS

FUWA, T., KIDO, T. AND TANAKA, H.: (Drug analysis by thin-layer chromatography. II. Sulfa drugs). *Yakuzaigaku*, 23 (1963) 102–105; *C.A.*, 60 (1964) 3951g.
STIER, A.: Zur Frage der Stabilität von Halothan (2-Brom-2-chlor-1,1,1-trifluoräthan) in Stoffwechsel. *Naturwissenschaften*, 51 (1964) 65.

33. INORGANIC SUBSTANCES

BAUDLER, M. AND STUHLMANN, F.: Zur Dünnschichtchromatographie von Mono- und Diphosphorsäuren. *Naturwissenschaften*, 51 (1964) 57–58.
BÄUMLER, J. AND RIPPSTEIN, S.: Zur Mikroquecksilberbestimmung. *Mitt. Gebiete Lebensm. Hyg.*, 54 (1963) 472–478.
MARKL, P. AND HECHT, F.: Dünnschichtchromatographie anorganischer Ionen. I. Trennung von Kationen. *Mikrochim. Acta*, (1963) 889–894.
MARKL, P. AND HECHT, F.: Dünnschichtchromatographie anorganischer Ionen. II. *Mikrochim. Acta*, (1963) 970–975.

34. RADIOACTIVE COMPOUNDS

DAVIES, W. H., MERCER, E. I. AND GOODWIN, T. W.: Some observations on the plant sulpholipid. *Biochem. J.*, 88 (1963) 63P — exposure for autoradiography shorter than for a paper chromatogram.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

VAN DER HEIDE, R. F.: Dünnschichtchromatographische Analyse organischer Stabilisatoren in Hart-PVC. *Z. Lebensm.-Untersuch.-Forsch.*, 124 (1964) 198–200 — R_F values of 14 compounds.

Gas Chromatography

1. REVIEWS AND BOOKS

APPLEGARTH, D. A.: The gas–liquid chromatography of carbohydrate derivatives. *Can. Pulp Paper Ind.*, 16, No. 7 (1963) 34–37 — a review of 31 papers.
D'OYLY-WATKINS, C.: Gas chromatography. *Nature*, 203 (1964) 129–131 — detailed review of 3 papers read at an informal symposium of the GC Discussion Group on April 7, 1964.
GOLOVNYA, R. V., MIRONOV, G. A. AND SOKOLOV, S. D.: (Chemistry of flavours of food products). *Usp. Khim.*, 33 (1964) 816–817 — 299 references, many of them dealing with GC-analysis.

2. FUNDAMENTALS, THEORY AND GENERAL

2a. Gas–liquid systems

BARR, J. K. AND SAWYER, D. T.: Studies of the liquid phase mass-transfer term in gas chromatography. *Anal. Chem.*, 36 (1964) 1753–1759 — dependence of C_l on carrier gas is verified.
GLUECKAUF, E.: Theory of chromatography. Part 13. Behaviour of wide bands in a chromatographic column. *Trans. Faraday Soc.*, 60 (1964) 729–736 — an equation predicts maximum band width of the feed which still gives sufficient separation in a given column.
LITTLEWOOD, A. B.: The dispersion of chromatographic zones due to uneven flow in packed columns. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 90–105 — zone dispersion in common columns is caused mainly by uneven flow of carrier gas.

2b. Gas–solid systems

EVERETT, D. H.: Interaction of gases and vapours with solids. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, p. XIV — aspects of the current theories of adsorption of molecules from the gaseous phase on solids.

GALE, R. L. AND BEEBE, R. A.: Determination of heats of adsorption on carbon black and bone mineral by chromatography using the eluted pulse technique. *J. Phys. Chem.*, 68 (1964) 555–567 — equations relate the surface coverage along the column to the form of elution curve; many retention data are given.

KISELEV, A. V.: Intermolecular interactions during adsorption and gas chromatography on solids. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 118–128 — adsorbents are divided into 4 classes according to the type of interaction force.

2c. Thermodynamics and theoretical relationships

DÖRING, C. E. AND HAUTHAL, H. G.: Gaschromatographische Bestimmung von Reaktionsgleichgewichten am Beispiel des Isomerisierungsgleichgewichtes der Hexadiene-(2,4). *J. Prakt. Chem.*, [4] 24 (1964) 27–37 — all stereoisomers on bis-(propionitrile) thioether + methyl cyanoacetate.

GOLAY, M. J. E.: The separation of molecules. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, p. X — facts concerning the entropy of gas mixtures are discussed.

ROWLINSON, J. S.: Molecular interactions in liquids and solutions. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, p. XIII — the nature and strength of the intermolecular forces is discussed in connection with GC-processes.

2d. General

CONDAL-BOSCH, L.: Some problems of quantitative analysis in gas chromatography. *J. Chem. Educ.*, 41, No. 4 (1964) A235 — A259.

GIDDINGS, J. C.: A critical evaluation of the theory of gas chromatography. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, p. XI — a review of the current theoretical standing of GC and a critical discussion of the major unsolved problems.

INSTITUTE OF PETROLEUM GAS CHROMATOGRAPHY DISCUSSION GROUP DATA SUB-COMMITTEE: Recommendations for the publication of retention data. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 44–50 — the form in which retention data should preferably be published is given.

MARTIN, A. J. P.: Chromatography in relation to other separation techniques. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, p. IX — fundamental requirements of separation processes are considered.

RATUSKÝ, J. AND BAŠTÁŘ, L.: Effect of hydrogen bonds on the relative retention during gas–liquid chromatography with β-cyanoethyl polyvalent alcohols. *Chem. Ind. (London)*, (1964) 579–580 — discussion of dipole moments of *o*-, *m*- and *p*-isomers of 13 different benzene derivatives and their relative retention data.

SCOTT, R. P. W.: New horizons in column performance. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, p. XII — performance is considered in terms of analysis time, resolution and column loading.

3. TECHNIQUES I

3a. Detectors

BANNER, A. E., ELLIOTT, R. M. AND KELLY, W.: Use of the mass spectrometer for detection and identification of capillary column effluents. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 20–26 — rapid scanning (0.5–300 sec) mass spectrometer of high resolution (min. slits 2500; 0.004 in. slits 1000; for MS 20–200 in 1 sec, 200) used as detector.

BRADLEY, L. L. T. AND FALCONER, W. E.: A simple flame ionization detector cell. *J. Sci. Instr.*, 40 (1963) 606 — construction details given; output with propylene 3 $\mu A \cdot mg^{-1} \cdot sec$.

GÓRSKI, A. AND ROUSSEAU, J.: (Investigation of suitability of rheometer as detector for gas chromatography). *Prace Central. Inst. Ochrony Pracy*, 13, No. 39 (1963) 96–108 — hydrogen as carrier gas, working temperature 120°.

HAAHTI, E., NIKKARI, T. AND KÄRKKÄINEN, J.: Continuous gas-phase detection of lipids in effluents of (liquid) chromatographic columns. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 27–33 — based on the difference in volatility of the solvent and the eluted material followed by FID.

JAMES, A. T., RAVENHILL, J. R. AND SCOTT, R. P. W.: A general method for the monitoring of effluents from non-aqueous liquid chromatographic systems. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 34–43 — a moving wire on which is coated a small fraction of the eluted (liquid) moving phase; after solvent evaporation this is cracked and the products are detected by argon ionization.

KING, JR., W. H.: Piezoelectric sorption detector. *Anal. Chem.*, 36 (1964) 1735–1739 — quartz plate of 1.27 cm diameter and 7.3 mils thick, vibration 9 Mc, response time 0.05 sec, selective sensitivity 10^{-9} g, coating with different GC substrates.

PARSALL, B. R. AND GHOSH, S. K.: Ionisation methods of mine gas analysis. *Colliery Eng.*, 40 (1963) 374–377, 417–422 — a discussion of β-ionization detectors.

SMITH, V. N. AND FIDIAM, J. F.: Electron drift-velocity detector for gas chromatography. *Anal. Chem.*, 36 (1964) 1739–1744 — parallel-plate ionization chamber with tritium source and argon as carrier gas; negative pulse duration is selected so that traces of permanent gases increase the electron drift-velocity; detection limit about 10^{-10} g/sec.

3b. Column performance and filling studies

ALTENAU, A. G. AND ROGERS, L. B.: Characterization of selected heavy-metal salts as adsorbents for gas chromatography. *Anal. Chem.*, 36 (1964) 1726–1732 — crystals of $CuSO_4 \cdot 5H_2O$, $3CdSO_4 \cdot 8H_2O$ and $MgCl_2 \cdot 6H_2O$, $Cu(Py)_4(NO_3)_2$ and $Cu(Py)_4SO_4$ and $Cu(NH_3)_4(NO_3)_2$ after careful elimination of water, pyridine or ammonia give adsorbents with small surface and high selectivity; retention data of many groups of compounds are given and working parameters of adsorbents are discussed.

HALÁSZ, I., HARTMANN, K. AND HEINE, E.: Column types in gas chromatography. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 51–63 — column efficiency is described by the performance parameter (atm·sec) which is proportional to the analysis time and to the pressure drop; six groups of GC columns are classified.

KIRKLAND, J. J.: Some modified gas chromatographic adsorbents and supports. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 146–157 — fibrillar boehmit as selective adsorbent and Teflon-6 and Teflon-coated support material are studied.

RUDENKO, B. A., POTAPOVA, L. G. AND KUCHEROV, V. F.: (The use of natural fats as liquid stationary phases in gas-liquid chromatography). *Zh. Anal. Khim.*, 19 (1964) 802–809 — relative retention data of hydrocarbons and halogen derivatives on 10 different fats; stability was found to be sufficient up to 220° without stabilization and up to 240–260° with stabilization; katharometer.

SCOTT, C. S. AND PHILLIPS, C. S. G.: New potentialities in gas–solid chromatography. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 129–145 — modification of alumina gives high selectivity and symmetrical peaks; gas–solid displacement chromatography gives a concentration effect of $1:10^8$ and permits separation of 50 g samples.

SMITH, E. D., JOHNSON, J. L. AND OATHOUT, J. M.: Study of solid support and partition liquid interactions in gas chromatographic separation of ethanol–methanol mixtures. *Anal. Chem.*, 36 (1964) 1750–1753 — 15 partitioning liquids and 4 support materials were studied.

3c. Apparatus, accessories and materials for GC

ABEL, K. AND NOBLE, F. W.: Application of an analog computer in analytical chromatographic and electrophoretic separations. *Anal. Chem.*, 36 (1964) 1855–1856 — Gaussian curves are projected by means of an oscilloscope (one or more function generator channels) on the actual chromatogram which has overlapping peaks, and these are then corrected for position and height of the peaks; much experience seems to be necessary in order to decrease the amount of artefacts.

BAŠTÁŘ, L.: (Sampling device with a micropipette for gas chromatograph Chrom.-I). *Chem. Listy*, 58 (1964) 683–685 — device that is very similar to the Tenney-Harries pipette.

BEDNAS, M. E. AND RUSSELL, D. S.: Transfer cell for gas chromatography. *Can. J. Chem.*, 42 (1964) 1249–1250.

CERESIA, G. B. AND BRUSCH, C. A.: A practical, inexpensive gas chromatograph. *Am. J. Pharm. Educ.*, 23 (1964) 194–198.

SNELSON, F. L.: A simplified peak trapping technique for gas chromatography. *Chem. Ind. (London)*, (1964) 575–576 — an inexpensive electrostatic precipitator; designs are given.

VITT, S. V., BONDAREV, V. B. AND POLININ, V. L.: (Separation of mixtures of close-boiling components on a capillary chromatograph with flame-ionization detection). *Izv. Akad. Nauk SSSR*, (1964) 1145–1150 — construction details; 150,000 plates on 55 m tube length, 0.25 mm I.D.; copper.

4. TECHNIQUES II

4a. Preparative-scale GC

HIGGINS, G. M. C. AND SMITH, J. F.: The theory and design of preparative scale columns. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 65–89 — overloading phenomena are studied experimentally and discussed.

4b. Programmed temperature GC

HUPE, K. P. AND BAYER, E.: Temperature distribution in temperature programmed columns. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 106–117 — temperature gradient of about 20° is predicted for columns of 3.8 cm I.D.

4d. Special microtechniques

FRITZ, G. AND KSINSIK, D.: Zur Bestimmung von Molekulargewichten bei der gaschromatographischen Trennung. *Z. Anorg. Allgem. Chem.*, 325 (1963) 3–7 — determination in a single run by gas density determination with thermal conductivity measurement.

ZHUKHOVITSKIĬ, A. A.: Some new gas chromatographic separation techniques. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 3–11 — an experimental presentation of so-called step, vacancy, differential, iteration chromatography, and chromatography without carrier gas is given.

5. HYDROCARBONS AND HALOGEN DERIVATIVES

5b. Other hydrocarbons

DAWSON, JR., H. J.: Detection of traces of polynuclear aromatics in hydrocarbons by gas chromatography. *Anal. Chem.*, 36 (1964) 1852–1853 — by means of an electron capture detector used simultaneously with a FID, in white oil by PTGC on SE-30.

HOFMANN, M., LEIBNITZ, E. AND KÖNNECKE, H. G.: Über die gas-chromatographische Trennung der C_6 bis C_8 Aromaten und ihre quantitative Bestimmung neben nichtaromatischen Kohlenwasserstoffen. *Chem. Tech. (Berlin)*, 16 (1964) 414–420 — retention data on 7,8-benzoquinoline, α,β-dinaphthyl sulphide, ethylene glycol-bis-propionitrile and 3,5-dinitrobenzoic acid *n*-propyl ester at 80°.

OLAH, G. A., FLOOD, S. H., KUHN, S. J., MOFFATT, M. E. AND OVERCHUCK, N. A.: Aromatic substitution. XVI. Friedel-Crafts isopropylation of benzene and methylbenzenes with isopropyl bromide and propylene. *J. Am. Chem. Soc.*, 86 (1964) 1046–1054 — retention data on propylene glycol at 100°, and on a mixture of *m*-bis-(*m*-phenoxyphenoxy)-benzene and Apiezon L at 80°.

OLAH, G. A., FLOOD, S. H. AND MOFFATT, M. E.: Aromatic substitution. XVIII. Friedel-Crafts *tert.*-butylation of benzene and methylbenzenes with *tert.*-butyl bromide and isobutylene. *J. Am. Chem. Soc.*, 86 (1964) 1060–1064 — retention data on Ucon LB 550-X (PPG) at 150°.

SHTOF, M. D., KHOMUNKO, V. L. AND USTENKO, V. I.: (Chromatographic analysis of low-boiling hydrocarbons of petroleum). *Khim. i Tekhnol. Topliv i Masel*, No. 7 (1964) 65–67 — on paraffin oil by PTGC, 30–115°, 3.3°/min.

5c. Halogen derivatives of hydrocarbons

EVANS, D. W. S.: Rapid elimination of water in the gas-chromatographic determination of chloroform in aqueous solutions. *Analyst*, 89 (1964) 295–296 — descriptions of devices.

GALIMBA, F. B., WILSON, W. E. AND SAMUEL, A. H.: The radiation-induced chlorination of toluene by stannic chloride. *J. Phys. Chem.*, 68 (1964) 647–651 — retention data on five-ring polymetaphenyl ether at 80° and 85°.

LECHNER, L. AND SOMOGYI, A.: Nachweis und quantitative Bestimmung von chlorierten Cyclopentadienderivaten durch Gaschromatographie und UV-Spectrophotometrie. *Talanta*, 11 (1964) 987–992 — on Apiezon N at 165°.

MAILEN, J. C., REED, III, T. M. AND YOUNG, J. A.: Thiourea as substrate for gas chromatography of fluorocarbons. *Anal. Chem.*, 36 (1964) 1883 — retention data of 8 alkanes and alkenes at 25°; GSC separation without tailing; He as carrier gas.

OLAH, G. A., KUHN, S. J., FLOOD, S. H. AND HARDIE, B. A.: Aromatic substitution. XIV. Ferric chloride catalyzed bromination of benzene and alkyl benzenes with bromine in nitromethane solution. *J. Am. Chem. Soc.*, 86 (1964) 1029–1044 — retention data on Ucon LB 550-X (PPG) at 100° and 150°, and on a mixture of *m*-bis-(*m*-phenoxyphenoxy)-benzene and Apiezon L at 60°.

OLAH, G. A., KUHN, S. J., FLOOD, S. H. AND HARDIE, B. A.: Aromatic substitution. XV. Ferric chloride catalyzed bromination of halobenzenes in nitromethane solution. *J. Am. Chem. Soc.*, 86 (1964) 1044–1046 — retention data on Ucon LB 550-X (PPG) at 150° and 180°, and on polypropylene at 100°.

OLAH, G. A., KUHN, S. J. AND HARDIE, B. A.: Aromatic substitution. XVII. Ferric chloride and aluminium chloride catalyzed chlorination of benzene, alkyl benzenes, and halobenzenes. *J. Am. Chem. Soc.*, 86 (1964) 1055–1060 — retention data on Ucon LB 550-X (PPC) at 110° and 150°.

OLAH, G. A., FLOOD, S. H. AND MOFFATT, M. E.: Aromatic substitution. XIX. Friedel-Crafts isopropylation and *tert.*-butylation of halobenzenes. *J. Am. Chem. Soc.*, 86 (1964) 1065–1066 — retention data on PPG at 40°, 60°, 100° and 110°.

7. PHENOLS

FREEDMAN, R. W.: Quantitative analysis of low-boiling phenols by capillary column separation of trimethylsilyl ethers. *Anal. Chem.*, 36 (1964) 1880–1881 — retention data of 15 phenols on 2,4-xylenyl phosphate at 125° (300 ft, 0.02 in. I.D.).

RUDOLFI, T. A., SHARAPOVA, R. I. AND LUSHCHIK, V. I.: (Gas chromatography of cresol isomers). *Zh. Anal. Khim.*, 19 (1964) 903–905 — separation and retention data of all isomers on dimenthyl phosphate at 133°.

8. SUBSTANCES CONTAINING HETEROCYCLIC OXYGEN

DESILVA, J. A. F., SCHWARTZ, M. A., STEFANOVIC, V. AND KAPLAN, J.: The determination of blood Diezepam (Valium®) by gas–liquid chromatography. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 493, Abstr. No. 2348 — after hydrolysis 2-methylamino-5-chlorobenzophenone was chromatographed.

LIBERMAN, A. L., BRAGIN, O. V. AND VASINA, T. V.: Catalytic dehydrocyclization of diethyl ether to yield a five-membered heterocyclic system. *Izv. Akad. Nauk SSSR*, (1964) 1352–1354.

PARKS, O. W., SCHWARTZ, D. P. AND KEENEY, M.: Identification of *o*-aminoacetophenone as a flavour compound in stale dry milk. *Nature*, 202 (1964) 185–187 — retention data on Apiezon L and DEGA at 125° and 148°.

9. OXO COMPOUNDS

SMOLKOVÁ, E., KOLOUŠKOVÁ, V. AND FELTL, L.: Gaschromatographische Studie von Formaldehydlösungen. *Z. Anal. Chem.*, 202 (1964) 262–272.

11. ORGANIC ACIDS AND SIMPLE LIPIDS

CARRUTHER, C.: The fatty acid composition of the sterol-wax ester fraction of mouse epidermis in various stages of growth. *Acta Dermato-Venereol.*, 44 (1964) 76–81 — on EGA and Apiezon M at 195° and 210°.

CÁRTONI, G. P., LIBERTI, A., PALLOTTA, U. AND PALOMBARI, R.: (Gas chromatographic determination of elaidic acid in fats by bromination). *Riv. Ital. Sostanze Grasse*, 40 (1963) 653–659 — retention data on DEGS at 180°.

CLAYTON, D. J.: Use of gas chromatography for determining the volatile fatty acids in silage. *U.A.A.S. Quart. Rev.*, 14, No. 60 (1963) 156–161 — a titration detector.

ETÉMADI, A. H.: Techniques microanalytiques d'étude de structure d'esters α-ramifiés β-hydroxylés. Chromatographie en phase vapeur et spectrométrie de masse. *Bull. Soc. Chim. France*, (1964) 1537–1541 — pyrolytic GC; fatty acids and aldehydes.

GERSON, T., MCINTOSH, J. E. A. AND SHORLAND, F. B.: The determination of methyl esters of polyunsaturated acids by gas–liquid chromatography. *Biochem. J.*, 91 (1964) 11 C — C_{20}–C_{22} acids on DEGA at 185°.

GODDIJN, J. P., VAN PRAAG, M. AND HARDON, H. J.: Quantitative gaschromatographische Bestimmung der Benzoesäure, deren Abkömmlinge und der Sorbinsäure. *Z. Lebensm.-Untersuch.-Forsch.*, 123 (1963) 300–305 — retention data on SE-30 at 170°.

HOFFMAN, N. E., BARBORIAK, J. J. AND HARDMAN, H. F.: Gas chromatographic analysis of lactic acid. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 493, Abstr. No. 2347 — on Carbowax 20M at 70°.

HORVAT, R. J., LANE, W. G., NG, H. AND SHEPHERD, A. D.: Saturated hydrocarbons from autoxidizing methyl linoleate. *Nature*, 203 (1964) 523–524 — C_1–C_5 on molecular sieve 5A at 150°, C_4–C_5 on Apiezon M and SF-96 (50) at 100° or 32°.

KELLOGG, H. M., BROCHMANN-HANSSEN, E. AND SVENDSEN, A. B.: Gas chromatography of esters of plant acids and their identification in plant materials. *J. Pharm. Sci.*, 53 (1964) 420–423 — retention data of methyl and ethyl esters of di- and tricarboxylic acids on SE-30 (55°), Apiezon L (122°), PEGS (Craig ester) (182°).

LESTER, D.: Determination of acetic acid in blood and other tissue by vacuum distillation and gas liquid chromatography. *Anal. Chem.*, 36 (1964) 1810–1812 — GLC of distillate on Carbowax 20M–terephthalic acid–Fluoropak glass column; carrier gas saturated with formic acid; flame ionization.

LOUGH, A. K.: Use of methanol containing boron trifluoride for the esterification of unsaturated fatty acids. *Nature*, 202 (1964) 795 — no methoxy substitution was found when esterification is carried out with 12.5% BF_3 for 2 min.
LUGAY, J. C. AND JULIANO, B. O.: Fatty acid composition of rice lipids by gas-liquid chromatography. *J. Am. Oil Chemists' Soc.*, 41 (1964) 273–275 — retention data on DEGS at 203°.
OKUI, S., UCHIYAMA, M., SATO, R. AND MIZUGAKI, M.: Metabolism of hydroxy acids in depot fat after feeding of ricinoleic acid. *J. Biochem.*, 55 (1964) 81–86 — on DEGS at 200°.
OSTWALD, R. AND SHANNON, A.: Composition of tissue lipids and anaemia of guinea pigs in response to dietary cholesterol. *Biochem. J.*, 91 (1964) 146–154 — retention data of methyl esters of fatty acids on DEGA at 190–200°.
PETTERSSON, Y.: Fettsäurezusammensetzung vegetabiler Öle unter besonderer Berücksichtigung der Polyensäuren. *Z. Lebensm.-Untersuch.-Forsch.*, 124, No. 1 (1963) 14–17.
SINK, J. D., WATKINS, J. L., ZIEGLER, J. H. AND MILLER, R. C.: Analysis of fat deposition in swine by gas liquid chromatography. *J. Animal Sci.*, 23 (1964) 121–125 — on PEGS at 180°.
SMITH, JR., C. R., HAGEMANN, J. W. AND WOLFF, J. A.: The occurrence of 6,9,12,15-octadecatetranoic acid in *Echium plantagineum* seed oil. *J. Am. Oil Chemists' Soc.*, 41 (1964) 290–291 — C_{18} monoene to C_{18} tetraene fatty acids were found.
TULLOCH, A. P. AND CRAIG, B. M.: Determination of double bond position in unsaturated triglycerides by analysis of the oxidation products by gas liquid chromatography. *J. Am. Oil Chemists' Soc.*, 41 (1964) 322–326 — mono- and dibasic acids on silicone and ethylene glycol phthalate at 205°.
WILSON, R. H., DOTY, V. AND RENCZ, K.: Gas chromatographic analysis of high molecular weight fatty acid methyl esters using the technique of relative molar response. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 174, Abstr. No. 439.
ZIELINSKI, JR., W. L.: Evaluation of the internal standard method for the quantitative estimation of oil polymer content by gas chromatography. *J. Am. Oil Chemists' Soc.*, 41 (1964) 249–251.

13. STEROIDS

BLONDIN, G. A., KULKARNI, B. D. AND NES, W. R.: The characterization of the 5,7-peroxides of $\Delta^{5,7}$-steroids by gas liquid chromatography. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 173, Abstr. No. 438.
CHAMBERLAIN, J., KNIGHTS, B. A. AND THOMAS, G. H.: A system of analysis by gas chromatography of 17α- and 17β-pregnane-3,20-diols and their identification as metabolites of progesterone in man, the monkey, rabbit and guinea-pig. *J. Endocrinol.*, 28 (1964) 235–246 — on QF-1, SE-30, NPGA at 250°, 250° and 225°.
FRANCE, J. T., MCNIVEN, N. L. AND DORFMAN, R. I.: Determination of micro quantities of androsterone, aetiocholanolone, and dehydroepiandrosterone by gas-liquid chromatography. *Acta Endocrinol., Suppl.*, 90 (1964) 71–80 — retention data of trimethylsilyl ethers on XE-60 at 225°.
KIRSCHNER, M. A. AND LIPSETT, M. B.: The analysis of urinary steroids using gas-liquid chromatography. *Steroids*, 3 (1964) 277–294 — retention data on SE-30, XE-60 at 195–215°.
KUKSIS, A. AND HUANG, T. C.: A gas chromatographic study of the *in vivo* conversion of plant sterols to derivatives of coprostanol. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 553, Abstr. No. 2710.
SOLOMON, D., NAIR, P. P., YOUSEM, H. AND TURNER, D. A.: Use of biphase gas chromatography in sex steroid analysis. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 553, Abstr. No. 2709 — as trimethylsilyl and trifluoroacetyl ether derivatives.
THOMAS, G. H.: Urinary metabolites of labelled androstenedione in normal and adrenalectomized guinea pigs. *Nature*, 202 (1964) 260–261 — retention data on QF-1 at 250°.
YOUSEM, H. L. AND STRUMMER, D.: Simple gas chromatographic method for estimation of urinary estriol in pregnant women. *Am. J. Obstet. Gynecol.*, 88 (1964) 375–380 — on QF-1 at 180–200°.

15. TERPENE DERIVATIVES

ARAI, A., YAO, C. C. AND ICHIKIZAKI, I.: The gas chromatographic separation and characterization of δ-cyclogeraniolene and α-cyclogeraniolene formed from isophorone by Wolff–Kirschner reduction. *Bull. Chem. Soc. Japan*, 36 (1963) 1432–1434 — retentoin data on Apiezon J and PEG-6000 at 123° and 136°.
HEFENDEHL, F. W.: Die Abscheidung von Terpenalkoholen durch Reaktions-Gaschromatographie mit Borsäure. *Naturwiss.*, 51 (1964) 138–139.
IGNATOVA, L. A., TOLSTIKOV, G. A., LISHTVANOVA, L. N. AND GORYAEV, M. I.: (Chemical composition of essential oil of the juniper (*Juniperus semiglobosa* Rgl.)). *Zh. Prikl. Khim.*, 37 (1964) 1389–1391 — α-thujone and related terpenes on lanolin at 110°.

NIGAM, I. C. AND LEVI, L.: Essential oils and their constituents. XIX. Detection of new trace components in oil of rosewood. *Perfumery Essent. Oil Record*, 54 (1963) 814–816 — retention data of 12 terpenes on PPGSebacate at 150° and on Reoplex 400 at 110°, 150° and 170°.

OHTA, Y., NISHIMURA, K. AND HIROSE, Y.: Studies on the monoterpene fraction of geranium oil from *Pelargonium roum* Bourbon. *Agr. Biol. Chem. (Tokyo)*, 28 (1964) 5–9 — on Carbowax 1500 at 110°, 140° and 150°.

RUDLOFF, E. VON: Gas–liquid chromatography of terpenes. Part X. The volatile oils of the leaves of Sitka and Engelman spruce. *Can. J. Chem.*, 42 (1964) 1057–1062 — on PEGA, Carbowax 20M, Apiezon N, QF-1, etc.

SATO, A. AND RUDLOFF, E. VON: The heartwood extractives of *Pinus resinosa* ait. *Can. J. Chem.*, 42 (1964) 635–640 — terpene alcohols and acids on PEG-20M by PTGC 65–200°.

SHIMIZU, M., UCHIMARU, F. AND OHTA, G.: Studies on the constituents of rice-bran oil. VI. Detection of cycloartanol by gas chromatography. *Chem. Pharm. Bull. (Tokyo)*, 12 (1964) 74–76 — retention data on SE-30 at 240°.

16. NITRO AND NITROSO COMPOUNDS

OLAH, G. A. AND KUHN, S. J.: Aromatic substitution. XX. Intact and dealkylating nitration of propylated and butylated alkylbenzenes with nitronium tetrafluoroborate. *J. Am. Chem. Soc.*, 86 (1964) 1067–1070 — retention data on Ucon LB 550-X (PPG) at 190°.

17. AMINES, AMIDES AND RELATED NITROGEN COMPOUNDS

LEW, H. Y.: Analysis of detergent mixtures containing amine oxides. *J. Am. Oil Chemists' Soc.*, 41 (1964) 297–300 — on Apiezon L by PTGC 180–280°, 4°/min.

MILLER, B.: The mechanism of the rearrangement of *p*-quinamines to 4-aminodiphenyl ethers. *J. Am. Chem. Soc.*, 86 (1964) 1127–1135 — retention data on silicone grease at 250°.

SEVERIN, M. AND RENARD, M.: (Study by vapour phase chromatography of sodium hypochlorite action on amino acids). *Riv. Ital. Sostanze Grasse*, 40 (1963) 649–652 — retention data of C_2–C_5 nitriles on DC-550 at 60°.

18. AMINO ACIDS

HAGEN, P. AND BLACK, W.: A method for the quantitative determination of the composition of a mixture of 19 amino acids by gas chromatography of their N-trifluoroacetyl methyl esters. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 371, Abstr. No. 1622 — amino acids are treated with a methanolic solution of thionyl chloride and then with trifluoroacetic anhydride; separation on Carbowax 20M and 1540.

21. PURINES, PYRIMIDINES, NUCLEOSIDES, NUCLEOTIDES, NUCLEIC ACIDS, BARBITURATES

MACGEE, J.: Purines and pyrimidines by gas liquid chromatography. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 531, Abstr. No. 2575 — on SE-30 and DEGS at 170°.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

HOLMSTEDT, B., VANDENHEUVEL, W. J. A., GARDINER, W. L. AND HORNING, E. C.: Separation and identification of tryptamine-related indole bases by gas chromatographic methods. *Anal. Biochem.*, 8 (1964) 151–157 — retention data of free and substituted (as trimethylsilyl ethers) derivatives on F-60 and EGSS-Z at 182°, and on NGS at 216° and 227° relative to anthracene.

24. ORGANIC SULPHUR COMPOUNDS

DAY, E. A., LINDSAY, R. A. AND FORSS, D. A.: Dimethyl sulfide and the flavor of butter. *J. Dairy Sci.*, 47 (1964) 197–199 — on Ucon non-polar at 25°.

PROFFT, E. AND SOLF, G.: Über Chlorthiophene. *J. Prakt. Chem.*, [4] 24 (1964) 38–73.

27. VITAMINS

BUCANA, C., NAIR, P. P. AND TURNER, D. A.: Determination of vitamin D by gas liquid chromatography. *Federation Proc.*, 23, No. 2 (1964) Pt 1, p. 394, Abstr. No. 1759 — on SE-52; epimerisation of vitamin D_2 and D_3 takes place but remains reproducible when the whole amount of vitamins is changed.

28. ANTIBIOTICS

IKEKAWA, N., KAGAWA, T. AND SUMIKI, Y.: Determination of nine gibberellins by gas and thin layer chromatography. Biochemistry of *Bakanae fungus*. *Proc. Japan Acad.*, 39 (1963) 507–512 — retention data of methyl esters on SE-30 and QF-1-0065 at 207° and 220°.

29. INSECTICIDES AND OTHER PESTICIDES

GUTENMANN, W. H. AND LISK, D. J.: Gas chromatographic method for Silvex. *J. Am. Water Works Assoc.*, 56 (1964) 189–190 — on silicone grease at 200°.

31. PLASTICS AND THEIR INTERMEDIATES

PARSON, J. S.: Bracket method for molecular weight determination of pyrolysis products using gas chromatography with a gas density detector. *Anal. Chem.*, 36 (1964) 1849–1852 — use of carrier gases with molecular weights greater or less than that of the unknown makes (more accurate) interpolation possible.

TAKEUCHI, T. AND KAKUGO, M.: (Analysis of high polymers by pyrolysis method using a commercial pipette for gas chromatograph). *J. Chem. Soc. Japan, Ind. Chem. Sect.*, 67 (1964) 308–312 — retention data of pyrolytic products of methyl methacrylate–methylacrylate copolymers.

WILKINSON, L. B., NORMAN, C. W. AND BUETTNER, J. P.: Determination of residual monomers in latex by gas chromatography. *Anal. Chem.*, 36 (1964) 1759–1762 — latex is dissolved in a suitable solvent and the solution injected (acrylonitrile–butyl acrylate in acetic anhydride, styrene–2-ethylhexyl acrylate in dimethoxypropane and vinyl chloride–vinylidene chloride latexes in cyclohexanone).

33. INORGANIC SUBSTANCES

33b. Volatile inorganic compounds

SALVADEO, A., PEZZAGNO, C., CAPODAGLIO, E. AND ANDREUZZI, P.: (Measurement of pulmonary capillary flow by rebreathing of nitrous oxide and gas chromatographic analysis. Results obtained in normal subjects). *Folia Cardiol.*, 22 (1963) 275–287 — N_2, N_2O, CO_2 etc. on silica gel and molecular sieve 5A.

34. RADIOACTIVE COMPOUNDS

LIBERTI, A., CARTONI, G. P. AND BRUNER, F.: Isotope effect in gas chromatography. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 158–168 — C_6H_6–C_6D_6 and C_6H_{12}–C_6D_{12} separation ratios are measured by GC and compared with vapour pressure measurements.

35. MISCELLANEOUS COMPOUNDS AND COMPLEX MIXTURES

ALLEN, C. F. H. AND HAPP, G. P.: The thermal reversibility of the Michael reaction. I. Nitriles. *Can. J. Chem.*, 42 (1964) 641–649 — retention data of nitriles and ketones.

ALLEN, C. F. H. AND HAPP, G. P.: The thermal reversibility of the Michael reaction. II. Nitroketones and related compounds. *Can. J. Chem.*, 42 (1964) 650–654 — retention data of some ketones and furan derivatives on SE-30 at 200°.

ALLEN, C. F. H. AND HAPP, G. P.: The thermal reversibility of the Michael reaction. III. 1,5-Diketones and adducts bearing acidic groups. *Can. J. Chem.*, 42 (1964) 655–661 — retention data of some nitriles on DC-200 at 218–219°.

ATTAWAY, J. A. AND WOLFORD, R. W.: Isolation and identification of volatile flavour components in recovered orange essence using gas chromatography. *5th Intern. Symp. Gas Chromatography 1964, Sept. 8–10, Brighton, Gr. Brit., Preprintbook*, Inst. Petroleum, London, 1964, pp. 12–19 — by combination of different GC and other separation techniques more than 50 compounds of different structure are identified.

BUTTERY, R. G., BLACK, D. R., KEALY, M. P. AND MCFADDEN, W. H.: Volatile hop esters. *Nature*, 202 (1964) 701–702 — capillary PTGC, 50–200°, on Tween 20; mainly fatty acid esters are identified.

CAPELLA, P., FEDELL, E., CIRIMELE, M., LANZANI, A. AND JACINI, G.: (Minor constituents of vegetable oils. Separation and examination of triterpene alcohols and sterols). *Riv. Ital. Sostanze Grasse*, 40 (1963) 660–665 — on SE-30 at 230°.

GUEDJ, R. AND JULLIEN, J.: Sur l'identification et l'évaluation quantitative des produits obtenus dans l'oxydation ménagée du cyclohexane en phase vapour. *Bull. Soc. Chim. France*, (1964) 1501–1506 — aldehydes, cycloketones, hydrocarbons etc. on Ucon polar 50-HB-2000 at 160°.

KROGER, M. AND PATTON, S.: Gas chromatography of cheese volatiles. *J. Dairy Sci.*, 47 (1964) 296–297 — brief report of the more detailed thesis of the first author.

MATSON, A., SMITH, A. H. AND PAUL, A. G.: Analysis of the fixed oil of *Clitocybe illudens* Schw., *J. Pharm. Sci.*, 53 (1964) 460–461 — different fatty acids and sterols on DEGS at 205° and on SE-30 at 222°.

NISHIMURA, K. AND HIROSE, Y.: The aroma constituents of "Kogyoky" apple. *Agr. Biol. Chem. (Tokyo)*, 28 (1964) 1–4 — on PEG and silicone grease at 130°.

OGORODNIKOV, S. K., RABOVSKAYA, R. V., KOROL', N. G. AND PRESMAN, B. I.: (Investigation of azeotropism in binar systems containing perfluoroethylamine with C_5 and C_6 hydrocarbons). *Zh. Prikl. Khim.*, 37 (1964) 1597–1601 — on dinonyl phthalate at 50–60°.

JOURNAL OF CHROMATOGRAPHY VOL. 16 (1964)

AUTHOR INDEX

ABBOTT, D. C., H. EGAN AND J. THOMSON. 481
ACHAYA, K. T., see ROOMI, M. W.
——, see SUBBARAO, R.
ACKMAN, R. G. AND J. C. SIPOS. 298
ALLEN, R. S., see PRESSEY, R.
ANDERSON, R. G., see POLLARD, F. H.
AUBEAU, R., L. CHAMPEIX AND J. REISS. 7
BACON, M. F. 552
BAILEY, R. E. AND D. A. DURFEE. . . . 546
BALLA, J., see MÁZOR, L.
——, see TAKÁCS, J.
BALLIN, G. 152
BAUER, R. D. AND K. D. MARTIN. . . . 519
BAYER, I. 237
BENDEL, E., G. MAHR, B. FELL AND M. F. EL DAOUSHY 216
BERG, A. AND J. LAM. 157
BHANDARI, P. R.. 130
BIĆAN-FIŠTER, T. AND V. KAJGANOVIĆ. . 503
BIGWOOD, A. M., see POLLARD, F. H.
BIRTWISTLE, R., see FORSS, D. A.
BOAK, W. K., see CONNORS, W. M.
BOMBAUGH, K. J. AND J. N. LITTLE. . . 47
BÜCHI, J., see ICONOMOU, N.
BURKHALTER, A., see H. J. KUPFERBERG
CARVER, M. J., see COPENHAVER, J. H.
CERRAI, E. AND G. GHERSINI. 258
—— AND C. TRIULZI 365
CHAMPEIX, L., see AUBEAU, R.
CONNORS, W. M. AND W. K. BOAK. . . . 243
COPENHAVER, J. H. AND M. J. CARVER . . 229
CRÉPY, O., O. JUDAS AND B. LACHESE. . 340
CRIDDLE, W. J., G. J. MOODY AND J. D. R. THOMAS. 350
CURTAIN, C. C.. 181
DANKERT, M., see RECONDO, E.
DENOLIN-DEWAERSEGGER, L., see VAN BINST, G.
DOBSON, G. AND G. HUGHES. 416
DODGSON, K. S., see WUSTEMAN, F. S.
DURFEE, D. A., see BAILEY, R. E.
EDWARDS, P. R., see FORSS, D. A.
EGAN, H., see ABBOTT, D. C.
EL DAOUSHY, M. F., see BENDEL, E.
FARMER, L. B. 412
FARMER, T. H.. 264
FELL, B., see BENDEL, E.
FISCHER, F. AND H. KOCH. 246
FORSS, D. A., P. R. EDWARDS, B. J. SUTHERLAND AND R. BIRTWISTLE . 460
FRANC, J. AND M. HÁJKOVÁ. 345
FRIEDRICH, K. AND K. STAMMBACH. . . 22
GĂDEVA, V., see POPOV, A.
GÄNSHIRT, H. G. AND J. POLDERMAN . . 510
GHERSINI, G., see CERRAI, E.
GIDDINGS, J. C. 444
GONÇALVES, I. R. J., see RECONDO, E.
GROSJEAN, M. H., see NOIRFALISE, A.
HÁJKOVÁ, M., see FRANC, J.
HAMAGUCHI, H., A. OHUCHI, N. ONUMA AND R. KURODA. 396
HANSBURY, E., see HAYES, F. N.
HARING, H. G. AND J. KROON 285
HARTMAN, L. 223
HAYES, F. N., E. HANSBURY AND V. E. MITCHELL 410
HUGHES, G., see DOBSON, G.
ICONOMOU, N. AND G. VALKANAS . . . 437
——, ——, AND J. BÜCHI 29
JACKSON, R. B. 306
JANÁK, J. 494
JEVONS, F. R. 252
JOHNSON, G., see SUNDERWIRTH, S. G.
JOLLY, W. L., see VILLENA-BLANCO, M.
JUDAS, O., see CRÉPY, O.
KAJGANOVIĆ, V., see BIĆAN-FIŠTER, T.
KELLER, R. A., see WEINBERG, B. B.
KLAUS, R.. 311
KNÖZINGER, H. AND L. KUDLA. 217
—— AND H. SPANNHEIMER. 1
KOCH, H., see FISCHER, F.
KONOPKA, W., see REINHARD, E.
KROON, J., see HARING, H. G.
KUDLA, L., see KNÖZINGER, H.
KUMMEROW, F. A., see NARAYAN, K. A.
——, see SGOUTAS, D. S.
KUPFERBERG, H. J., A. BURKHALTER AND E. L. WAY. 558
KURODA, R., see HAMAGUCHI, H.
LACHESE, B., see CRÉPY, O.
LAM, J., see BERG, A.
LANDHEER, C. A.. 293
LAURENT, E. P., see LAURENT, T. C.
LAURENT, T. C. AND E. P. LAURENT. . . 89
LEDERER, M., see OSSICINI, L.
LEES, E. M. AND H. WEIGEL 360
LISBOA, B. P. 136
LITTLE, J. N., see BOMBAUGH, K. J.
LLOYD, A. G., see WUSTEMAN, F. S.
LUCKHURST, G. R. 543
MA, V., see RINDERKNECHT, H.
MCKEOWN, G. G., see SMYTH, R. B.
MAHR, G., see BENDEL, E.
MARTIN, K. D., see BAUER, R. D.
MARTIN, R. H., see VAN BINST, G.
MATTOK, G. L.. 254
MÁZOR, L., J. TAKÁCS AND J. BALLA. . . 221
——, see TAKÁCS, J.
MITCHELL, V. E., see HAYES, F. N.
MOODY, G. J., see CRIDDLE, W. J.
MORIE, G. P. AND T. R. SWEET. 201
MORRIS, C. J. O. R.. 167
NARAYAN, K. A., S. NARAYAN AND F. A. KUMMEROW. 187

NARAYAN, S., see NARAYAN, K. A.
NEALES, T. F. 262
NELSON, F. 403, 538
NEUMANN, D. AND H.-B. SCHRÖTER. . . 414
NICKLESS, G., see POLLARD, F. H.
NOIRFALISE, A. AND M. H. GROSJEAN . . 236
OBREITER, J. B. AND B. B. STOWE . . . 226
OHUCHI, A., see HAMAGUCHI, H.
OLSON, G. G., see SUNDERWIRTH, S. G.
ONUMA, N., see HAMAGUCHI, H.
OSSICINI, L., F. SARACINO AND M. LEDERER. 524
PATAKI, G. 541, 553
POLDERMAN, J., see GÄNSHIRT, H. G.
POLLARD, F. H., G. NICKLESS AND A. M. BIGWOOD. 207
——, ——, T. J. SAMUELSON AND R. G. ANDERSON 231
POPOV, A. AND V. GĂDEVA. 256
PRESSEY, R. AND R. S. ALLEN 248
RANDERATH, E., see RANDERATH, K.
RANDERATH, K. AND E. RANDERATH 111, 126
RECONDO, E., I. R. J. GONÇALVES AND M. DANKERT. 415
REINHARD, E., W. KONOPKA AND R. SACHER 99
REISS, J., see AUBEAU, R.
RINDERKNECHT, H. AND V. MA. 407
RITSCHARD, W. J. 327
ROCKLAND, L. B.. 547
ROLLINS, C. B. AND R. D. WOOD. . . . 555
ROOMI, M. W., M. R. SUBBARAM AND K. T. ACHAYA. 106
ROSE, F. A., see WUSTEMAN, F. S.
SACHER, R., see REINHARD, E.
SALAMÉ, M. 476
SAMUELSON, T. J., see POLLARD, F. H.
SARACINO, F., see OSSICINI, L.
SCHEJA, H. W., see STURM, Jr., A.
SCHRÖTER, H.-B., see NEUMANN, D.
SGOUTAS, D. S. AND F. A. KUMMEROW. . 448
SIEKIERSKI, S. AND R. J. SOCHACKA . . 385
——, see SOCHACKA, R. J.
SIPOS, J. C., see ACKMAN, R. G.
SMYTH, R. B. AND G. G. MCKEOWN. . . 454
SNYDER, L. R.. 55
SOCHACKA, R. J. AND S. SIEKIERSKI . . 376
——, see SIEKIERSKI, S.
SPANNHEIMER, H., see KNÖZINGER, H.
SPRATT, J. L. 253
STAMMBACH, K., see FRIEDRICH, K.
STANLEY, C. W. 467
STOWE, B. B., see OBREITER, J. B.
STURM, Jr., A. AND H. W. SCHEJA . . . 194
SUBBARAM, M. R., see ROOMI, M. W.
SUBBARAO, R. AND K. T. ACHAYA . . . 235
SUNDERWIRTH, S. G., G. G. OLSON AND G. JOHNSON. 176
SUSZKO-PURZYCKA, A. AND W. TRZEBNY. 239
SUTHERLAND, B. J., see FORSS, D. A.
SWEET, T. R., see MORIE, G. P.
TAKÁCS, J., J. BALLA AND L. MÁZOR . . 218
——, see MÁZOR, L.
THOMAS, J. D. R., see CRIDDLE, W. J.
THOMSON, J., see ABBOTT, D. C.
TOPHAM, J. C. AND J. W. WESTROP. . . 233
TRIULZI, C., see CERRAI, E.
TRZEBNY, W., see SUSZKO-PURZYCKA, A.
TUDBALL, N., see WUSTEMAN, F. S.
VALKANAS, G., see ICONOMOU, N.
VAN BINST, G., L. DENOLIN-DEWAERSEGGER AND R. H. MARTIN 34
VILLENA-BLANCO, M. AND W. L. JOLLY . 214
WAY, E. L., see KUPFERBERG, H. J.
WEIGEL, H., see LEES, E. M.
WEINBERG, B. B. AND R. A. KELLER . . 40
WESTROP, J. W., see TOPHAM, J. C.
WOOD, R. D., see ROLLINS, C. B.
WUSTEMAN, F. S., K. S. DODGSON, A. G. LLOYD, F. A. ROSE AND N. TUDBALL 334
YASUDA, S. K.. 488

JOURNAL OF CHROMATOGRAPHY VOL. 16 (1964)

SUBJECT INDEX

Acetylacetone, analysis by gas chromatography . . . 221
Acetylenic compounds, fatty, separation by thin-layer chromatography . . . 106
Acids, carboxylic, as stationary phases in the gas–liquid chromatography of volatile fatty acids . . . 306
——, fatty, see Fatty acids
——, phenolic separation by high-voltage electrophoresis . . . 194
Actinium-227, isolation from its daughters and from ^{231}Pa . . . 538
Activity coefficient in gas–liquid chromatography, non-ideality of the gas phase in frontal analysis . . . 543
Adsorption heats, determination by means of gas chromatography . . . 1
Alcohols, C_9, obtained on hydroformylation of *n*-octenes, separation by gas chromatography . . . 216
——, diastereomeric diols, chromatographic behaviour . . . 246
——, ester sulphates, thin-layer chromatography . . . 336
——, fatty, hydroxy derivatives, separation of close positional isomers by thin-layer chromatography . . . 235
Aldehydes, 2,4-dinitrophenylhydrazones, direct spectrophotometric examination on paper chromatograms . . . 460
Alkaloids, cinchona, separation by thin-layer chromatography . . . 239
——, indole, determination of their pyrolysis products by gas chromatography . . . 34
——, opium, separation by thin-layer chromatography . . . 237
Aluminium, separation from Ga, In and Tl by chromatography on papers treated with di-(2-ethylhexyl) orthophosphoric acid . . . 258
Amido-phosphates, chromatographic studies on their hydrolysis . . . 207
Amines, aryl derivatives, in oxidation-type hair dyes, analysis by paper chromatography . . . 454
——, phenolic, paper chromatography . . 254
Amino acids, carbobenzoxy derivatives, thin-layer chromatography . . . 553
——, chromatography on starch columns. 264
——, detection on thin-layer chromatograms . . . 541
——, hydroxylated, ester sulphates, thin-layer chromatography . . . 336
——, separation by a new technique for preparative paper chromatography . . . 547
Aminoanthraquinones, separation and determination by thin-layer chromatography . . . 345
Anion-exchange chromatography, behaviour of rare earth elements in potassium sulphate medium . . . 396
——, isolation and quantitative determination of pseudo-uridine in urine . . 407
——, of nucleotides on poly-(ethylene-imine)-cellulose thin layers . . 111, 126
——, of transition metals in tartrate medium . . . 201
Anion-exchange paper, chromatographic behaviour of light rare earths on . . 365
Apparatus, for applying solutions to non-bound preparative thin-layer chromatographic plates . . . 412
—— for quantitative application of samples as streaks in paper and thin-layer chromatography . . . 552
——, sample injection system for gas chromatography for use at higher temperatures . . . 217
Applicator, for applying solutions to non-bound preparative thin-layer chromatographic plates . . . 412
—— for use in gas chromatography at higher temperatures . . . 217
—— for use in paper and thin-layer chromatography . . . 552
Azo-dyestuffs, thin-film chromatography . . . 231, 233
Barium, separation from radium by an ion-exchange procedure . . . 403
Benzal chloride, quantitative gas chromatographic analysis in mixtures with benzyl chloride and benzotrichloride 285
Benzeneboronic acid, detection and chromatography on paper using chlorogenic acid . . . 262
Benzotrichloride, quantitative gas chromatographic analysis in mixtures with benzyl chloride and benzal chloride . . . 285
Benzyl chloride, quantitative gas chromatographic analysis in mixtures with benzal chloride and benzotrichloride . . . 285
Boric acid, paper chromatography and detection using chlorogenic and caffeic acids . . . 262
Boron compounds, detection and chromatography on paper of boric acid, sodium tetraborate and benzeneboronic acid, using chlorogenic and caffeic acids . . . 262
Bromopyridines, gas chromatography . . 293

Caeruloplasmin, detection by means of an improved method 181
Caffeic acid, use in paper chromatography for the detection of ions of B, W, Mo and Ge 262
Carbobenzoxy-amino acids, thin-layer chromatography 553
Carbohydrates, determination of sugars by paper chromatography using anthrone as colorimetric reagent . . 176
——, non-reducing, detection with orcinol in phosphoric acid 252
——, paper chromatography of oligo-galacturonides 248
——, phosphates, separation by electrophoresis using sodium carbonate–sodium bicarbonate buffer 415
——, thin-layer chromatography, a procedure for the direct reading of fluorescent spots using the Turner fluorometer 243
Carboxymethylcellulose as a binder for thin-layer chromatography of lipids and indoles 226
Catecholamines and related compounds, paper chromatography 254
Cation exchange, see Ion exchange
Cellulose, changes in the immobile aqueous phase during chromatograms formed with absolute ethanol . . . 40
Chlorine, organic compounds, pesticides, thin-layer chromatography 481
Chlorogenic acid, use in paper chromatography for the detection of the ions of B, W, Mo and Ge 262
Chloropyridines, separation by gas chromatography 293
Cinchona alkaloids, separation by thin-layer chromatography 239
Coal-tar food colours, electrophoretic behaviour on paper and thin films . . 350
Column chromatography, changes in the immobile aqueous phase of cellulose during chromatograms formed with absolute ethanol 40
Complexes between polyhydroxy compounds and inorganic oxy-acids, paper electrophoresis 360
Coumarin derivatives, separation and identification by thin-layer chromatography on silica gel 229
Deoxyribonucleic acid, thin-layer chromatography on ECTEOLA cellulose 519
Dialkyl peroxides, identification by paper chromatography 416
Di-(2-ethylhexyl) orthophosphoric acid, as the stationary phase in reversed-phase partition chromatography 376, 385
——, use for the treatment of paper in the reversed-phase chromatography of Al, Ga, In and Tl 258
Diglycerol, estimation in the presence of glycerol 223
4-Dimethylaminoazobenzene and its metabolites, thin-layer chromatography 233
2,4-Dinitrophenylhydrazones, direct spectrophotometric examination on paper chromatograms 460
Diols, diastereoisomers, chromatographic behaviour 246
Disc electrophoretic method for animal blood serum proteins 187
Dyes, azo compounds, thin-film chromatography 231, 233
——, coal-tar food colours, electrophoretic behaviour on paper and thin films 350
——, hair, oxidation-type, analysis of arylamines and phenols by paper chromatography 454
Eluents, relationship between their composition and elution strength . . . 55
Enthalpy of adsorption, determination by gas chromatography 1
Esters, fatty, flame ionization detector response for the carbonyl carbon atom in the carboxyl group 298
——, ——, hydroxy derivatives, separation of close positional isomers by thin-layer chromatography 235
——, sulphates studied by thin-layer chromatography 334, 340
Ethyl centralite, products of its reaction with nitrogen tetroxide identified by thin-layer chromatography . . . 488
Ethylenic compounds, fatty, separation by thin-layer chromatography . . . 106
Fatty acetylenic, ethylenic and saturated compounds, separation by thin-layer chromatography 106
Fatty acids, alcohols and esters, hydroxy derivatives, separation of close positional isomers by thin-layer chromatography 235
—— and esters, flame ionization detector response for the carbonyl carbon atom in the carboxyl group 298
——, thin-layer chromatography 106
——, tritium-labelled, an isotope effect during chromatography on silica–silver nitrate columns 448
——, volatile, gas–liquid chromatography, using carboxylic acids as stationary phases 306
Flame ionization detector response for the carbonyl carbon atom in the carboxyl group of fatty acids and esters. 298
Flavonoids of hops, identification by thin-layer chromatography 130
Fluorotoluenes, nitration studied by gas–liquid chromatography 437
Fluoroxylenes, nitration studied by gas–liquid chromatography 437
Food colours, coal-tar, electrophoretic behaviour on paper and thin films . 350
Francium, cation exchange in HCl and $HClO_4$ solutions 538

Frontal analysis in gas–liquid chromatography, non-ideality of the gas phase. 543
Gallium, separation from Al, In and Tl by chromatography on papers treated with di-(2-ethylhexyl) orthophosphoric acid 258
Gas chromatography, microscopic and colorimetric investigation of compounds separated by 494
——, sample injection system for use at higher temperatures 217
——, use for the determination of heats of adsorption 1
Gas–liquid chromatography, non-ideality of the gas phase in frontal analysis . 543
Gases, determination of traces of water vapour in gases by gas chromatography 7
Gel filtration, effect of the phosphorylation state of thymidine derivatives on Sephadex K_d values 410
——, studied by means of an electrical analogy 89
——, thin-layer chromatography of proteins on Sephadex 167
Germanium ions, detection in paper chromatography by means of chlorogenic and caffeic acids. 262
Gibberellins, separation in plant extracts by thin-layer chromatography and horizontal column chromatography. 99
Glycerol, estimation in the presence of diglycerol. 223
Guanidines, microdetection colorimetric tests. 546
Gum turpentines of *Pinus* species, determination of their composition by gas–liquid chromatography 29
Hair dyes, oxidation-type, analysis of arylamines and phenols by paper chromatography. 454
Heat of adsorption, determination by gas chromatography. 1
Heptasulphur imide, separation from S_8 and S_4N_4 by adsorption chromatography 214
Herbicides, triazines, determination of their vapour pressures by gas chromatography. 22
High-voltage electrophoresis, separation of phenolic acids by 194
Hops, identification of flavonoids of *Humulus lupulus* Linne by thin-layer chromatography. 130
Humulus lupulus Linne flavonoids, identification by thin-layer chromatography 130
Hydrocarbons, polycyclic aromatic, separation by thin-layer chromatography on impregnated layers. . . . 157
Hydroxy fatty acids, alcohols and esters, separation of close positional isomers by thin-layer chromatography 235
Δ^4-17β-Hydroxyoestrene derivatives, quantitative determination by thin-layer chromatography 510
Imido-phosphates, chromatographic studies on their hydrolysis 207
Indium, separation from Al, Ga and Tl by chromatography on papers treated with di-(2-ethylhexyl) orthophosphoric acid 258
Indoles, alkaloids, determination of their pyrolysis products by gas chromatography 34
——, thin-layer chromatography using carboxymethylcellulose as binder. . 226
——, thin-layer chromatography in neutral solvents 152
Inorganic ions, anion-exchange behaviour of rare earth elements in potassium sulphate medium 396
——, anion-exchange chromatography of transition metals in tartrate medium. 201
——, cation exchange of Ac(III) and Fr(I) in HCl and $HClO_4$ solutions 538
——, chromatographic behaviour of light rare earths on anion-exchange paper in nitrate–methanol systems. . . . 365
——, detection of B, W, Mo and Ge in paper chromatography by means of chlorogenic and caffeic acids 262
——, separation of Al, Ga, In and Tl by chromatography on papers treated with di-(2-ethylhexyl) orthophosphoric acid 258
——, separation of Ba and Ra by an ion-exchange procedure 403
——, separation of rare earths by reversed-phase partition chromatography with di-(2-ethylhexyl) orthophosphoric acid as the stationary phase 376, 385
——, the solution chemistry and chromatographic behaviour of technetium in aqueous HCl and HBr 524
Insecticides, organo-chlorine, thin-layer chromatography. 481
——, organo-phosphorus, separation and identification by thin-layer chromatography. 476
——, ——, thin-layer chromatography on microchromatoplates. 467
Ion-exchange chromatography, cation exchange of Ac(III) and Fr(I) in HCl and $HClO_4$ solutions 538
——, separation of barium and radium 403
——, separation of nucleotides on poly-(ethyleneimine)-cellulose thin layers 111, 126
——, solution chemistry of technetium studied in HCl and HBr using ion exchange paper 524
——, see also Anion-exchange chromatography

Ketones, 2,4-dinitrophenylhydrazones, direct spectrophotometric examination on paper chromatograms . . . 460
Linear elution adsorption chromatography, strong eluents and alumina, the basis of eluent strength 55
Lipids, thin-layer chromatography using carboxymethylcellulose as binder. . 226
——, unsaturated, detection on thin-layer chromatography plates using indicators bleached with bromine . . . 555
Liquid–liquid chromatography with a recording detector. 47
Molybdenum ions, detection in paper chromatography by means of chlorogenic and caffeic acids. 262
Morphine and related compounds, fluorometric identification of submicrogram amounts on thin-layer chromatographs 558
Multi-dimensional chromatography using different developing methods . . . 494
Nitro compounds, aromatic, identification of ethyl centralite–nitrogen tetroxide reaction products 488
——, ——, identification of nitration products of fluorotoluenes and fluoroxylenes by gas–liquid chromatography 437
Nucleic acids, thin-layer chromatography of deoxyribonucleic acid on ECTEOLA cellulose 519
Nucleotides, ion-exchange chromatography on poly-(ethyleneimine)-cellulose thin-layers 111
——, resolution of complex mixtures by two-dimensional anion-exchange thin-layer chromatography 126
——, electrophoresis using sodium carbonate–sodium bicarbonate buffer 415
n-Octenes, gas chromatography of the C_9 alcohols obtained on hydroformylation. 216
Oligogalacturonides, paper chromatography 248
Opium alkaloids, separation by thin-layer chromatography 237
Organic compounds, microscopic and colorimetric investigation after separation by gas chromatography . . . 494
Organo-chlorine pesticides, thin-layer chromatography. 481
Organo-phosphorus pesticides, thin-layer chromatography 467, 476
Δ^4-3-Oxo-C_{21}-steroids, characterization on thin-layer chromatograms by *in situ* colour reactions 136
Oxy-acids, inorganic, complexes with polyhydroxy-compounds, paper electrophoresis 360
Paper chromatography, changes in the immobile aqueous phase of cellulose during chromatograms with absolute ethanol. 40
Paper chromatography, preparative, a new technique. 547
Partition chromatography, determination of the highest temperature at which partition solvents may be used. . . 218
Peptide maps, method of preparation using thin-layer techniques 327
Peptides, sequence analysis by means of thin-layer chromatography 541
Peroxides, dialkyl, identification by paper chromatography. 416
Pesticides, organo-chlorine, thin-layer chromatography. 481
——, organo-phosphorus separation and identification by thin-layer chromatography 476
——, ——, thin-layer chromatography on microchromatoplates. 467
β-Phenethylbiguanide, microdetection colorimetric tests 546
Phenolic acids, separation by high-voltage electrophoresis. 194
Phenols, ester sulphates, thin-layer chromatography. 336
——, in oxidation-type hair dyes, analysis by paper chromatography. 454
Phenothiazine derivatives, thin-layer chromatography. 236
Phosphoric esters, separation by electrophoresis using sodium carbonate–sodium bicarbonate buffer. 415
Phosphorus compounds, inorganic, chromatographic studies on their hydrolysis 207
——, organic, thin-layer chromatography 467, 476
Pinus gum turpentines, determination of their composition by gas–liquid chromatography. 29
Plate height in coiled columns. 444
Plate height in reversed-phase partition chromatography with di-(2-ethylhexyl) orthophosphoric acid as the stationary phase. 385
Poly-(ethyleneimine)-cellulose, use in the ion-exchange chromatography of nucleotides 111, 126
Polyhydroxy-compounds, paper electrophoresis in stannate solution. . . . 360
Protactinium-231, cation exchange in HCl and $HClO_4$ solutions 538
Proteins, animal blood serum, a modified disc electrophoretic method . . 187
——, detection of caeruloplasmin. . . . 181
——, thin-layer chromatography on Sephadex. 167
——, thin-layer techniques for making peptide maps 327
Pseudotropine, separation from tropine and tropinone by thin-layer chromatography. 414
Pseudouridine, isolation from urine and quantitative determination by anion-exchange chromatography. . 407

Purines, chromatography on starch columns 264
Pyridines, bromo- and chloro-derivatives, separation by gas chromatography. 293
Pyrimidines, chromatography on starch columns 264
Radium, separation from barium by an ion-exchange procedure 403
Rare earths, anion-exchange behaviour in potassium sulphate medium. . . . 396
——, chromatographic behaviour on anion-exchange paper in nitrate–methanol systems 365
——, separation by reversed-phase partition chromatography, with di-(2-ethylhexyl) orthophosphoric acid as the stationary phase 376, 385
Sample applicator, for applying solutions to non-bound preparative thin-layer chromatographic plates . . . 412
—— for gas chromatography for use at higher temperatures 217
—— for use in paper and thin-layer chromatography. 552
Sephadex, K_d values, effect of the phosphorylation state of thymidine derivatives on. 410
——, thin-layer chromatography of proteins on 167
Solvents for use in partition chromatography, determination of the highest temperature at which they may be used 218
Starch as adsorbent in column chromatography 264
Steroids, conjugated, thin-layer chromatography. 337, 340
——, Δ^4-17β-hydroxyoestrene derivatives, quantitative determination by thin-layer chromatography 510
——, Δ^4-3-oxo-C_{21}-steroids, characterization on thin-layer chromatograms by *in situ* colour reactions. 136
——, sulphates, thin-layer chromatography 337, 340
Sugars, determination by paper chromatography, using anthrone as colorimetric reagent. 176
——, see also Carbohydrates
Sulphate esters studied by thin-layer chromatography. 334, 340
Sulphonamides, quantitative analysis by thin-layer chromatography 503
Sulphur, separation from S_7NH and S_4N_4 by adsorption chromatography . . 214
Surfactants, separation by liquid–liquid chromatography with a recording detector 53
Technetium, solution chemistry and chromatographic behaviour in aqueous HCl and HBr 524
Terpenes of gum turpentines of *Pinus* species, determination by gas–liquid chromatography 29
Tetrasulphur tetranitride, separation from S_8 and S_7NH by adsorption chromatography 214
Thallium, separation from Al, Ga and In by chromatography on papers treated with di-(2-ethylhexyl) orthophosphoric acid 258
Theory, gel filtration studied by means of an electrical analogy 89
——, linear elution adsorption chromatography, the basis of eluent strength 55
——, non-ideality of the gas phase in frontal analysis 543
——, plate height in coiled columns . . . 444
Thin-layer chromatography, apparatus for quantitative application of samples as streaks. 552
——, a critical examination of the photometric evaluation of chromatoplates 311
——, a procedure for the direct reading of fluorescent spots using the Turner fluorometer. 243
——, simple device for applying solutions to non-bound preparative plates . . 412
Thymidine derivatives, effect of the phosphorylation state on Sephadex K_d values 410
Transition metals, anion-exchange chromatography in tartrate medium . . 201
Triazine herbicides, determination of their vapour pressures by gas chromatography. 22
Tritium isotope effect during chromatography of tritium-labelled fatty acids on silica–silver nitrate columns. . . 448
Tritium-labelled compounds, colorimetric identification following recovery from paper 253
Tropine, separation from pseudotropine and tropinone by thin-layer chromatography. 414
Tropinone, separation from tropine and pseudotropine by thin-layer chromatography. 414
Tungsten ions, detection in paper chromatography by means of chlorogenic and caffeic acids 262
Turpentines of *Pinus* species, determination of their composition by gas–liquid chromatography 29
Tyrosine derivatives, chromatography on starch columns 264
Vapour pressures, small, determination by gas chromatography. 22
Vulcanization accelerators, a general colour reagent for use in paper chromatography. 256
Water vapour, determination of traces in a gas by gas chromatography . . . 7

CHROMATOGRAPHIC DATA

SUPPLEMENT TO THE
JOURNAL OF CHROMATOGRAPHY
VOL. 16 (1964)

EDITORS:
C. G. CASINOVI (Rome, (Italy)
C. B. COULSON (Legon, Ghana)
M. LEDERER (Rome, Italy)

ELSEVIER PUBLISHING COMPANY
AMSTERDAM

All rights reserved

ELSEVIER PUBLISHING COMPANY

PRINTED IN THE NETHERLANDS BY

DRUKKERIJ MEIJER N.V., WORMERVEER

TABLE 1

R_F VALUES (RELATIVE) OF HEXITOLS

(H. G. BRITTON, *Biochem. J.*, 85 (1962) 402)

Solvents: S_1 = Amyl alcohol–pyridine–water (4:3:2, by vol.) (E. KAWERAU, *Chromatog. Methods*, 1 (1956) No. 2).
S_2 = Butan-1-ol–acetic acid–water (4:1:5, by vol.).
S_3 = Phenol–water (5:2, w/v).
S_4 = Butan-2-one–acetic acid–satd. aq. boric acid soln. (9:1:1, by vol.) (W. R. REES AND T. REYNOLDS, *Nature*, 181 (1958) 767).

Paper: Whatman No. 1.

Detection: D_1 = Trevelyan reagent (W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444 as modified by I. SMITH, *Chromatographic Techniques*, Heinemann Medical Books, London, 1960, p. 250), for S1, S2, S3, reducing and non-reducing carbohydrates.
D_2 = Britton reagent: 40% HF–acetone (1:40, v/v) (H. G. BRITTON, *Biochem. J.*, 73 (1959) 19 P), for S_4, in presence of borate.
D_3 = Periodate–benzidine reagent (J. A. CIFONELLI AND F. SMITH, *Anal. Chem.*, 26 (1954) 1132), for S_4, in presence of borate.
D_4 = Aniline–diphenylamine reagent (I. SMITH, *loc. cit.*).
D_5 = Naphthoresorcinol reagent (I. SMITH, *loc. cit.*).
D_4 and D_5 are used for reducing sugars.

Compound	$R_{Sorbitol}$*			
	S_1	S_2	S_4	S_4
Allitol	1.15	1.13	1.0	1.03
Dulcitol	1.03	1.02	0.97	0.81
Iditol	0.92	1.97	1.0	0.91
Mannitol	1.08	1.07	0.97	0.84
Talitol	1.10	1.09	1.02	0.96

* $R_{Sorbitol} = R_F$ compound/R_F sorbitol.

TABLE 2

ELECTROPHORETIC MOBILITIES OF VARIOUS ALDITOL DERIVATIVES

(M. L. WOLFROM, J. R. VERCELLOTTI AND D. HORTON, *J. Org. Chem.*, 29 (1964) 540)

Electrolyte: Molybdate solution, pH 5 (E. J. BOURNE, D. H. HUTSON AND H. WEIGEL, *Chem. Ind. (London)*, (1959) 1047; *J. Chem. Soc.*, (1960) 4252; (1961) 35).

Compound	*Linkage*	*Relative mobility*
D-Glucitol		1.0
Sophoritol	β-D-(1 → 2)	0.9
Nigeritol	α-D-(1 → 3)	0.0
Laminaribiitol	β-D-(1 → 3)	0.0
Maltitol	α-D-(1 → 4)	0.4
Cellobiitol	β-D-(1 → 4)	0.4
Isomaltitol	α-D-(1 → 6)	0.8
Gentiobiitol	β-D-(1 → 6)	0.8
Melibiitol	α-D-(1 → 6)	0.8

TABLE 3

R_F VALUES OF SOME SUGARS

(J. M. VAUGHAN AND E. E. DICKEY, *J. Org. Chem.*, 29 (1964) 715)

Solvents: S_1 = Ethyl acetate–pyridine–water (8:2:1).
S_2 = Ethyl acetate–acetic acid–water (9:2:2).
Paper: Schleicher and Schüll 598.

Compound	R_F	R_C*	
	S_1	S_1	S_2
Glyoxal	0.7–0.9	—	—
Erythrose	0.65	—	—
Mannose	0.23	—	—
Glucose	0.18	—	—
4-O-β-D-Glucopyranosyl-D-mannose	—	1.90	1.42
Mannobiose	—	1.05	1.21
Cellobiose	—	1.00	1.00
4-O-β-Mannopyranosyl-D-glucose	—	0.57	0.85

* R_F value relative to that of cellobiose.

TABLE 4

R_F VALUES OF NINHYDRIN-POSITIVE COMPOUNDS OF *Lathyrus* SPP.

(E. A. BELL, *Biochem. J.*, 83 (1962) 225)

Solvents: S_1 = Butan-1-ol–acetic acid–water (60:15:25, by vol.).
S_2 = 200 ml phenol–water (4:1, w/v) and 1 ml NH_4OH (sp. gr. 0.88) before use.
S_3 = 200 ml phenol–ethanol–water (120:40:40, w/v/v) and 1 ml NH_4OH (sp. gr. 0.88) before use.
S_4 = Ethanol–NH_4OH (sp. gr. 0.88)–water (18:1:1, by vol.).
S_5 = Methanol–water–pyridine (40:10:2, by vol.).
S_6 = Lutidine–water (132:60, v/v).
S_7 = Butan-1-ol–pyridine–water (1:1:1, by vol.) (I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. 1, 2nd Ed., Heinemann Medical Books, London, 1960, p. 84).
Paper: Whatman No. 1 (descending).
Time of run: 40 h (S_1, S_6); 17 h (S_2–S_6,S_7).
Detection: Ninhydrin reagent (0.2 % w/v ninhydrin in aq. 95 % acetone), colours developed at 80°.

Compound	$R_F \times 100$*						
	S_1	S_2	S_3	S_4	S_5	S_6	S_7
Arginine	16	91	66	8	17	7	15
Lathyrine	17	83	60	7	35	15	29
Homoarginine	20	94	76	12	19	10	17
Glutamic acid	25	19	11	4	—	7	22
Aspartic acid	18	13	6	2	—	6	19
Alanine	30**	57	43	33**	57	23**	37**
β-(γ-Glutamylamino)-propionitrile***	30	83	64	24	53	34	43

* Approximate values.
** These values for alanine are quoted from I. SMITH, *loc. cit.*, the other values in the column are calculated from $R_{Alanine}$ values.
*** Tentative identification (*cf.* G. F. MCKAY, J. J. LALICH, E. D. SCHILLING AND F. M. STRONG, *Arch. Biochem. Biophys.*, 52 (1954) 313; E. D. SCHILLING AND F. M. STRONG, *J. Am. Chem. Soc.*, 76 (1954) 3848).
— = not given.

TABLE 5

ELECTROPHORETIC MOBILITIES OF (POLY)CARBAMATES OF (POLY)AMINO COMPOUNDS

(J. L. FRAHN AND J. A. MILLS, *Australian J. Chem.*, 17 (1964) 256)

Electrolyte: 0.1 *N* NaOH.
Potential: 20 V/cm for 30 min.
Temperature: 18–20°.
Mobility: M_{DS} = Mobility relative to that of naphthalene-2,5-disulphonic acid.

Amino compound	*Amino groups*	$M_{DS} \times 100$	
		Parent compound	*Carbamate*
(*a*) *Simple, saturated amines*			
3-Aminopropan-1-ol	1	0	61
Benzylamine	1	4	55
Ethylenediamine	2	0	62, 106
meso-Pentane-2,4-diamine	2	0	49, 81
2-Methylpentane-2,4-diamine	2	0	44, —
2,2-Bis-(aminomethyl)-propane-1,3-diamine	4	0	42, 75, 97, 115
Diethylenetriamine	3	0	46, 81, 108
Triethylenetetramine	4	0	36, 67, 94, 109*
Piperazine	2	0	52, 97*
(*b*) *Amino acids*			
Glycine	1	80	121
DL-Alanine	1	67	100
β-Aminopropionic acid	1	67	100
β-Aminoisobutyric acid	1	58	90
cis-3-Aminocyclohexanecarboxylic acid	1	53	84
DL-Glutamic acid	1	98	115
L-Cystine	2	82	106, 117
Taurine	1	77	113
L-Ornithine	2	54	86, 106
Azetidine-2-carboxylic acid	1	69	105
L-Proline	1	59	91
Piperidine-3(and 4)-carboxylic acid	1	55	90
Sarcosine	1	69	116
N-Methyl-L-tyrosine	1	75	92*
(*c*) *Aromatic amino compounds*			
Aniline	1	0	62
o-Toluidine (and *p*-toluidine)	1	0	54
p-Anisidine	1	0	53
p-Dimethylaminoaniline	1	0	46*
m-Phenylenediamine	2	0	51*, —
p-Phenylenediamine	2	0	51, —
(*d*) *Miscellaneous compounds*			
Hydroxylamine	1	5	84
Hydrazine	2	0	82, 116
Phenylhydrazine	2	0	54, —
D-Glucosamine	1	33	61

— = failure to detect an expected carbamate spot.
* faint spot.

TABLE 6

ELECTROPHORETIC MOBILITIES OF SOME DITHIOCARBAMATES

(J. L. FRAHN AND J. A. MILLS, *Australian J. Chem.*, 17 (1964) 256)

Electrolyte: 0.1 N NaOH.
Potential: 20 V/cm for 30 min.
Temperature: 18–20°.
Mobility: M_{DS} = Mobility relative to that of naphthalene-2,5-disulphonic acid.

Amino compound	$M_{DS} \times 100$	
	Parent compound	*Dithio-carbamate*
Ethylenediamine	0	75, 124
Piperidine	0	66
Piperazine	0	64, —
Glycine	81	149
α-Aminoisobutyric acid	66	110
L-Proline	59	104

— = failure to detect an expected dithiocarbamate spot.

TABLE 7

R_F VALUES OF SOME PTERIDINE DERIVATIVES

(R. LOHRMANN AND H. S. FORREST, *J. Chem. Soc.*, (1964) 460)

Solvents: S_1 = n-Propanol–1 % aq. NH_3 (2:1).
S_2 = n-Butanol–30 % aq. acetic acid (2:1).
S_3 = 3 % Ammonium chloride.
S_4 = 4 % Sodium citrate.

Compound	R_F			
	S_1	S_2	S_3	S_4
2-Amino-3,4,7,8-tetrahydro-4,7-dioxo-pteridine derivative				
6-Carboxy-8-methyl-	0.17	0.20	0.58	0.64
6-Methyl-8-phenyl-	0.65	0.67	0.58	0.57
8-(2′-Hydroxyethyl)-6-methyl-	0.50	0.43	0.53	0.53
6-Carboxy-8-(2′-hydroxyethyl)-	0.19	0.19	0.63	0.68
6-Carboxy-8-(2′-hydroxyethyl)-ethyl ester	0.64	0.50	0.62	0.55
8-D-Glucosyl-	0.37	0.09	0.73	0.74
8-D-Glucosyl-6-methyl-	0.43	0.15	0.74	0.74
6-Carboxy-8-D-glucosyl-	0.17	0.08	0.80	0.82
1,2,3,4,7,8-Hexahydro-2,4,7-trioxo-pteridine derivative				
8-D-Glucosyl-	0.40	0.08	0.74	0.64
8-D-Glucosyl-6-methyl-	0.45	0.15	0.76	0.68

TABLE 8

R_F VALUES OF SOME IMIDAZOLE DERIVATIVES

(T. VITALI AND G. BERTACCINI, *Gazz. Chim. Ital.*, 94 (1964) 296)

Solvents: S_1 = *n*-Butanol–33% methylamine (8:3).
S_2 = *n*-Butanol–acetic acid–water (4:1:5).
Paper: Whatman No. 1.

Compound	R_F	
	S_1	S_2
Histamine	0.63	0.41*
N′-Methyl-histamine	0.84	0.26*
N′,N′-Dimethyl-histamine	0.85	0.24*
Spinaceamine	0.60	0.22*
6-Methyl-spinaceamine	0.78	0.23*
L-Histidine	0.17	0.20*
Spinacine	0.16	0.23*
6-Methyl-spinacine	0.22	0.25*
Histaminol	0.67	0.51
Imidazole-4-acetic acid	0.22	0.41
Ethyl imidazole-4-acetate	0.60	0.68
Imidazole-4-acetonitrile	0.71	0.53
Trimethyl-2-(4-imidazolyl)-ethyl-ammonium chloride	0.15	0.19

* Head of the trail formed.

TABLE 9

R_F VALUES (THIN-LAYER) OF SOME IMIDAZOLE DERIVATIVES

(T. VITALI AND G. BERTACCINI, *Gazz. Chim. Ital.*, 94 (1964) 296)

Solvents: S_1 = Benzene–ethanol–methylamine (10:8:2).
S_2 = Methanol–water (7:3).
Thin layer: Kieselgel G (Merck) not activated (with solvent S_1).
Kieselgel G (Merck) buffered with 0.2 *M* phosphate, pH 6.8, not activated (with solvent S_2).

Compound	R_F	
	S_1	S_2
Histamine	0.54	0.42*
N′-Methyl-histamine	0.77	—
N′,N′-Dimethyl-histamine	0.78	0.25*
Spinaceamine	0.59	0.44*
6-Methyl-spinaceamine	0.77	0.20*
L-Histidine	0.09*	0.42
Spinacine	0.10*	0.53
6-Methyl-spinacine	0.11*	0.48
Histaminol	0.63	—
Imidazole-4-acetic acid	0.11	—
Ethyl imidazole-4-acetate	0.80	—
Imidazole-4-acetonitrile	0.72	—
Trimethyl-2-(4-imidazolyl)-ethyl-ammonium chloride	0.0	—

* Head of the trail formed.

TABLE 10

R_F VALUES OF SOME THYMIDINE DERIVATIVES

(T. M. JACOB AND H. G. KHORANA, *J. Am. Chem. Soc.*, 86 (1964) 1630)

Solvent: Isopropanol–conc. ammonia–water (7:1:2, v/v).
Paper: Whatman No. 1 or 3 MM.

Compound	R_F
Thymidine-5′-phosphate	0.09
P^1,P^2-Di-(thymidine-5′)-pyrophosphate	0.15
Thymidylyl-(3′→5′)-thymidine	0.38
Thymidine	0.66
3′-O-(*p*-Toluenesulfonyl)-thymidine	0.87
3′-O-(Mesitylenesulfonyl)-thymidine	0.87

TABLE 11

R_F VALUES OF SOME NUCLEOSIDES AND NUCLEOTIDES

(A. BALLIO, S. RUSSI AND G. SERLUPI-CRESCENZI, *Gazz. Chim. Ital.*, 94 (1964) 156)

Solvents: S_1 = 1 *M* ammonium acetate, pH 3.8–ethanol (30:75).
S_2 = Isobutyric acid–1 *N* ammonia (100:60).
S_3 = *n*-Propanol (20 ml)–0.1 *M* phosphate buffer, pH 6.8 (1000 ml) and ammonium sulphate (600 g).
S_4 = Isoamyl alcohol–disodium phosphate (5%) (biphasic).
Paper: Whatman No. 1, acid washed.
Temperature: 25°.

Compound	R_F			
	S_1*	S_2	S_3	S_4
Inosine	1.01	0.53	0.31	0.68
Adenosine	1.00	0.84	0.089	0.50
Cytidine-5′-diphosphato-ethanolamine	0.68	0.44	0.68	0.88
Nicotinamide-adenine-dinucleotide	0.22	0.54	0.23	0.71
Cytidine-5′-monophosphate	0.72	0.49	0.69	0.83
Nicotinic acid	1.27	0.80	0.35	0.77
Adenosine-5′-monophosphate	0.63	0.67	0.23	0.75
Adeninosuccinic acid	0.98	0.61	0.22	0.80
Nicotinic acid-adenine-dinucleotide	0.29	0.38	0.18	0.77
Adenosinosuccinic acid	0.99	0.55	0.25	0.85
Guanosine-5′-monophosphate	0.61	0.40	0.34	0.81
Uridine-5′-monophosphate	0.82	0.35	0.69	0.87
Adenosine-5′-diphosphatoribose	0.56	0.34	0.24	0.83
Adenosine-5′-diphosphate	0.48	0.44	0.28	0.79
Adenylosuccinic acid	0.69	0.34	0.34	0.98
Guanosine-5′-diphosphato-mannose	0.34	0.20	0.40	0.86
Uridine-5′-diphosphato-acetylglucosamine	0.61	0.18	0.62	0.92
Uridine-5′-diphosphato-glucose	0.48	0.16	0.75	0.92
Uridine-5′-diphosphato-galactose	0.48	0.16	0.75	0.92
Uridine-5′-diphosphate	0.55	0.21	0.96	0.93
Uridine-5′-diphosphato-glucuronic acid	0.62	0.19	0.76	0.98
Adenosine-5′-triphosphate	0.22	0.38	0.28	0.97
Uridine-5′-triphosphate	0.42	0.13	0.77	—

* R_F value relative to that of adenosine.

TABLE 12

R_F VALUES OF SOME NUCLEOTIDES AND RELATED COMPOUNDS

(G. I. DRUMMOND, M. W. GILGAN, E. J. REINER AND M. SMITH, *J. Am. Chem. Soc.*, 86 (1964) 1626)

Solvent: Isopropanol–concentrated ammonia–water (7:1:2).
Paper: Whatman No. 40.

Compound	R_F
Deoxyadenosine-5′-phosphate	0.17
Deoxyadenosine-3′,5′-phosphate	0.43
Deoxyadenosine	0.59
Adenine	0.49
Deoxycytidine-5′-phosphate	0.14
Deoxycytidine-3′,5′-phosphate	0.43
Deoxycytidine	0.61
Cytosine	0.52
Deoxyguanosine-5′-phosphate	0.06
Deoxyguanosine-3′,5′-phosphate	0.25
Deoxyguanosine	0.40
Guanine	0.27
Thymidine-5′-phosphate	0.19
Thymidine-3′,5′-phosphate	0.52
Thymidine	0.71
Thymine	0.69

TABLE 13

R_F VALUES OF SOME NICOTINIC ACID DERIVATIVES

(A. BALLIO AND S. RUSSI, *Gazz. Chim. Ital.*, 94 (1964) 237)

Solvents: S_1 = 95% ethanol–1 *M* ammonium acetate, pH 3.8 (75:30).
S_2 = Isobutyric acid–1 *N* ammonium hydroxide (100:60).
Paper: Whatman No. 1, acid washed.
Temperature: 25°.

Compound	R_F	
	S_1*	S_2
Nicotinic acid mononucleotide	0.48	0.40
Nicotinamide	1.03	0.89
Nicotinamide riboside	0.81	0.72
Nicotinamide mononucleotide	0.48	0.55
Nicotinamide adenine dinucleotide	0.20	0.54
Nicotinamide adenine dinucleotide phosphate	0.05	0.40
Nicotinic acid	1.00	0.79
Nicotinic acid adenine dinucleotide	0.16	0.42

* R_F value relative to that of nicotinic acid.

TABLE 14

R_F VALUES (RELATIVE) OF COMPOUNDS RELATED TO THE SYNTHESIS OF 5,8-DIDEAZA-5,6,7,8-TETRAHYDROFOLIC ACID

(J. I. DEGRAW, L. GOODMAN AND B. R. BAKER, *J. Org. Chem.*, 26 (1961) 1156)

Solvents: S_1 = Butan-1-ol–acetic acid–water (5:2:3) (A. E. BENDER, *Biochem. J.*, 48 (1951) XV).
S_2 = N,N-Dimethylformamide–water (6:4).
S_3 = Butan-1-ol–ethanol–12 *N* NH_4OH–water (4:1:1:4) (*cf.* E. L. HIRST, L. HOUGH AND J. K. N. JONES, *J. Chem. Soc.*, (1949) 928).
S_4 = Butan-1-ol–acetic acid–water (4:1:5) (R. L. M. SYNGE, *Biochem. J.*, 48 (1951) 429).
S_5 = Benzene–methanol–water (2:6:1) (T. WIELAND AND W. KRACHT, *Angew. Chem.*, 69 (1957) 172).
S_6 = 5% aq. Na_2HPO_4 (C. E. CARTER, *J. Am. Chem. Soc.*, 72 (1950) 1835).
Paper: Whatman No. 1 (descending).
Detection: U.V. light.

Compound	R_{Ad}*					
	S_1	S_2	S_3	S_4	S_5	S_6
2-Amino-5,6,7,8-tetrahydro-4-hydroxy-6-quinazoline-carboxaldehyde dimethyl acetal	1.29					1.68
2-Amino-5,6,7,8-tetrahydro-4-hydroxy-6-quinazoline-carboxaldehyde (I)	1.14					
2-Acetamido-5,6,7,8-tetrahydro-4-hydroxy-6-quinazoline-carboxaldehyde dimethyl acetal					1.83	
2-Acetamido-5,6,7,8-tetrahydro-4-hydroxy-6-quinazoline-carboxaldehyde				1.46	1.32	
Phenylhydrazone of (I)	1.60				1.66	
p-[(2-Acetamido-5,6,7,8-tetrahydro-4-hydroxy-6-quinazolinyl)-methylamino]-benzoic acid		1.25	0.43			
p-[(5,6,7,8-Tetrahydro-4-hydroxy-6-quinazolinyl)-methylamino]-benzoic acid			0.22			
p-{N-[(2-Acetamido-5,6,7,8-tetrahydro-4-hydroxy-6-quinazolinyl)-methyl]-2,2,2-trifluoroacetamido}-benzoic acid					1.67	
N-{*p*-[(2-Amino-5,6,7,8-tetrahydro-4-hydroxy-6-quinazolinyl)-methylamino]-benzoyl}-glutamic acid (5,8-dideaza-5,6,7,8-tetrahydrofolic acid)	1.17					
Glutamic acid	0.30					

* R_{Ad} = R_F compound/R_F adenine.

TABLE 15

R_F VALUES OF SOME ORGANO-TIN COMPOUNDS IN REVERSED-PHASE PAPER CHROMATOGRAPHY

(D. J. WILLIAMS AND J. W. PRICE, *Analyst*, 89 (1964) 220)

Solvents: S_1 = Methanol–*N* hydrochloric acid (3:1), stationary phase: dinonyl phthalate.
S_2 = Methanol–*N* hydrochloric acid (1:1), stationary phase: dinonyl phthalate.
S_3 = Methanol–*N* hydrochloric acid (1:3), stationary phase: dinonyl phthalate.
S_4 = Methanol–*N* hydrochloric acid (1:1), stationary phase: tritolyl phosphate.
S_5 = 2,2,4-Trimethylpentane–acetic acid (92.5:7.5), stationary phase: 2-phenoxyethanol.
Paper: Whatman No. 4.
Temperature: 20°.

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Me_2SnCl_2	0.96	0.96	0.96	0.96	0.02
Me_3SnCl	—	—	—	0.80*	—
$EtSnCl_3$	0.96	0.96	0.91	—	0.00
Et_2SnCl_2	0.96	0.93	0.88	0.64	0.13**
Et_3SnOH	0.83	0.58	0.29	0.29	0.44
Pr_2SnCl_2	0.81	0.58	0.32	0.20	0.38***
$(Pr_3Sn)_2O$	0.44	0.06	0.00	0.03	0.76
$BuSnCl_3$	0.96	0.96	0.88	—	0.00
Bu_2SnCl_2	0.65	0.24	0.06	0.06	0.49
Bu_3SnCl	0.21	0.00	0.00	—	0.84
$(Bu_3Sn)_2O$	—	—	—	0.00	—
Bu_4Sn	0.00	0.00	0.00	—	0.96
Hex_2Sn (laurate)$_2$	0.22	0.00	0.00	—	0.69
Oct_2SnCl_2	0.04	0.00	0.00	—	0.85
Oct_3SnCl	0.00	0.00	0.00	—	0.95
Oct_4Sn	0.00	0.00	0.00	—	0.96
Et_2 lauryl SnOAc	0.05	0.00	0.00	—	—
$PhSnCl_3$	0.96	0.93	0.84	—	0.00
Ph_2SnCl_2	0.82	0.32	0.14	—	0.10
Ph_3SnCl	0.28	0.01	0.00	—	0.27
Ph_4Sn	0.00	0.00	0.00	—	†

* Rather diffuse spot.
** Elongated spot.
*** Tailing.
† Streaks extending for the length of the paper.

TABLE 16

R_F VALUES OF BIOGENIC AMINES

(D. L. VAN RHEENEN, *Rec. Trav. Chim.*, 82 (1963) 225)

Solvents: S_1 = Phenol saturated with water.
S_2 = Butan-1-ol–acetic acid–water (4:1:5).
S_3 = Ethylene glycol monomethyl ether–propionic acid–water (70:15:15), saturated with sodium citrate.

Compound	R_F		
	S_1	S_2	S_3
Methylamine	0.42	0.21	0.50
Dimethylamine	0.73	0.23	0.54
Ethylamine	0.62	0.31	0.60
Diethylamine	0.90	0.44	0.75
Propylamine	0.71	0.43	0.66
Butylamine	0.80	0.54	0.72
Amylamine	0.83	0.67	0.74
β-Phenylethylamine	0.84	0.67	0.73
Tyramine	0.68	0.50	0.68
1,3-Diaminopropane	0.08	0.08	0.22
Putrescine	0.18	0.08	0.29
Cadaverine	0.29	0.08	0.38
Spermine	0.12	0.07	0.09
Spermidine	0.14	0.08	0.17
Ethanolamine	0.37	0.19	0.50
1-Aminopropanol-2	0.51	0.27	0.60
Agmatine	0.40	0.13	0.40
Lysine	0.18	0.10	0.25
β-Alanine	0.39	0.20	0.44
γ-Aminobutyric acid	0.47	0.25	0.56
Taurine	0.20	0.13	0.36
Kynurenine	0.77	—	—
Glutamine	0.40	0.11	0.28
Citrulline	0.47	—	—
3-Aminopropanol-1	0.53	0.22	0.53
4-Aminobutanol-1	0.64	0.25	0.60
Tryptamine	0.81	0.62	0.70
Histamine	0.29	0.09	0.28
ε-Aminocaproic acid	0.66	0.41	0.67
Carnosine	0.39	0.09	0.19
Norephedrine	0.82	0.64	0.76
Histidine	0.22	0.08	0.19
Arginine	0.29	0.08	0.30

TABLE 17

PAPER ELECTROPHORESIS OF BIOGENIC AMINES

(D. L. VAN RHEENEN, *Rec. Trav. Chim.*, 82 (1963) 225)

Electrolyte: Citric acid buffer, pH 3.7.
Potential: 1980 V.
Temperature: —6°.

Compound	R_{MA}*	*Compound*	R_{MA}*
Methylamine	1.00	Agmatine	0.68
Dimethylamine	0.88	Lysine	0.40
Ethylamine	0.79	β-Alanine	0.32
Diethylamine	0.61	γ-Aminobutyric acid	0.40
Propylamine	0.68	Taurine	0.10
Butylamine	0.60	Kynurenine	0.11
Amylamine	0.54	Glutamine	0.09
β-Phenylethylamine	0.49	Citrulline	0.08
Tyramine	0.41	3-Aminopropanol-1	0.62
1,3-Diaminopropane	0.82	4-Aminobutanol-1	0.56
Putrescine	0.80	Tryptamine	0.35
Cadaverine	0.76	Histamine	0.70
Spermine	0.49	ε-Aminocaproic acid	0.39
Spermidine	0.61	Carnosine	0.37
Ethanolamine	0.70	Norephedrine	0.43
1-Aminopropanol-2	0.61	Histidine	0.37
		Arginine	0.36

* $R_{MA} = \frac{\text{distance travelled by compound}}{\text{distance travelled by methylamine}}$

TABLE 18

R_F VALUES OF ETHYLAMINE SALTS OF SOME BUTYRIC ACID DERIVATIVES

(A. R. MATTOCKS, *J. Chem. Soc.*, (1964) 1918)

Solvent: Butan-1-ol–ethylamine–water (R. M. MANGANELLI AND F. B. BROFAZI, *Anal. Chem.*, 29 (1957) 1441).
Paper: Whatman No. 1.

Ethylamine salt of	R_F
Acetic acid	0.18
α-Hydroxy-α-methyl-butyric acid	0.35
α-Acetoxy-α-methyl-butyric acid	0.50
α-Isobutyroxy-α-methyl-butyric acid	0.72

TABLE 19

R_F VALUES OF OXIDIZED GLUTATHIONE, ITS HOMOLOGUE, THEIR DERIVATIVES AND SOME AMINO ACIDS

(P. R. CARNEGIE, *Biochem. J.*, 89 (1963) 471)

Solvent: Butan-1-ol–acetic acid–water (4:1:5) (aged for 24 h).
Paper: Whatman No. 1.
Temperature: 28°.

Compound	R_{Ala}*
Glutathione (GSSG)	0.22
HomoGSSG	0.30
GSH	0.80**
HomoGSH	0.91**
GSO_3H	0.30
$HomoGSO_3H$	0.35
GSH N-ethylmaleimide adduct	1.12
HomoGSH N-ethylmaleimide adduct	1.33
Aspartic acid	0.66
Glutamic acid	0.85
α-Aminoadipic acid	1.00
Cystine	0.25
Cysteic acid	0.36
Cysteine N-ethylmaleimide adduct	1.50

* R_F for alanine taken as 0.30.
** Streak.

TABLE 20

ELECTROPHORETIC MOBILITIES OF OXIDIZED GLUTATHIONE, ITS HOMOLOGUE, THEIR DERIVATIVES AND SOME AMINO ACIDS

(P. R. CARNEGIE, *Biochem. J.*, 89 (1963) 471)

Electrolyte: Pyridine acetate, pH 4.0 (W. GRASSMANN, K. HANNIG AND M. PLÖCKL, *Z. Physiol. Chem.*, 255 (1955) 258).
Potential: 23 V/cm for 45 min.
Temperature: 4°.
Paper: Whatman No. 3 MM.

Compound	*Anionic mobility** ($10^5\mu$) ($cm^2 \cdot V^{-1} \cdot sec^{-1}$)
Glutathione (GSSG)	6.3
HomoGSSG	3.0
GSH	4.8
HomoGSH	2.6
GSO_3H	13.0
$HomoGSO_3H$	9.5
GSH N-ethylmaleimide adduct	4.5
HomoGSH N-ethylmaleimide adduct	2.3
Aspartic acid	8.5
Glutamic acid	4.6
α-Aminoadipic acid	3.5
Cystine	—1.3
Cysteic acid	15.0
Cysteine N-ethylmaleimide adduct	—1.3

* Values corrected for endosmosis, using N-2,4-dinitrophenylethanolamine as marker.

TABLE 21

R_F VALUES (THIN LAYER) OF CAROTENOIDS

(K. EGGER, *Chromatographie Symposium II—1962*, Société Belge des Sciences Pharmaceutiques, Bruxelles, 1963, p. 75)

Solvent: Mixtures of acetone and 95 % ethanol as shown in the figure.
Thin layer: Kieselguhr impregnated with paraffin oil.

Substances:

1	Lutein	7	Zeaxanthin dimethyl ether
2	Canthaxanthin	8	Torularhodin methyl ester
3	Cryptoxanthin	9	Astaxanthin dipalmitate
4	β-Apo-8′-carotinal	10	β-Carotene
5	Lutein monopalmitate	11	Lutein dipalmitate
6	Echinenone		

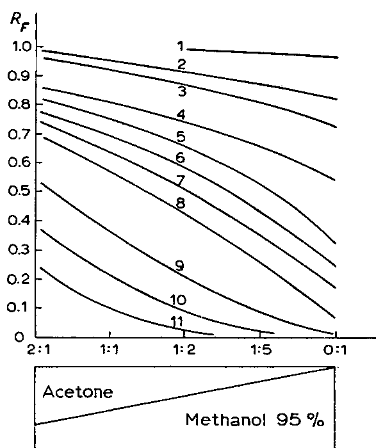


TABLE 22

R_F VALUES OF SOME RETRONECINE DERIVATIVES

(A. R. MATTOCKS, *J. Chem. Soc.*, (1964) 1918)

Solvent: Butan-1-ol–acetic acid–water (H. C. CROWLEY AND C. C. J. CULVENOR, *Australian J. Chem.*, 12 (1959) 694).
Paper: Whatman No. 1, buffered with 0.1 *M* sodium acetate.

Compound	R_F
1-Chloromethyl-7β-(α-hydroxy-α-methylbutyroxy)-5,6,7,7a-tetrahydropyrrolizine	0.88
7β-Acetoxy-1-chloromethyl-5,6,7,7a-tetrahydropyrrolizine	0.67
Monochlororetronecine	0.53

TABLE 23

R_F VALUES OF *Phylica rogersii* PILLANS ALKALOIDS

(R. R. ARNDT AND W. H. BAARSCHERS, *J. Chem. Soc.*, (1964) 2244)

Solvents: S_1 = Chloroform–benzene–formamide (6:4:5) (upper phase).
S_2 = Cellosolve–toluene–buffer (prepared by adding 9.5 ml of 0.2 *M* sodium acetate to 90.5 ml of 0.2 *M* acetic acid) (5:5:1).
S_3 = Butan-1-ol–acetic acid–water (16:3:10) (upper phase).
Paper: Whatman No. 1 (impregnated with a 40% solution of formamide in methanol for S_1).

Compound	R_F		
	S_1	S_2	S_3
Reticuline	—	—	0.57
N-Methyl-laurotetanine	1.00	—	—
N-Methyl-laurotetanine ethyl ether methiodide	—	0.55	0.57
Rogersine	0.80	—	—
Rogersine methiodide	—	0.42	0.50
O-Ethyl rogersine tartrate	—	0.56	0.61

TABLE 24

R_F VALUES OF *Virgilia oroboides* ALKALOIDS

(G. C. GERRANS AND J. HARLEY-MASON, *J. Chem. Soc.*, (1964) 2202)

Solvents: S_1 = Butanol–water–36% hydrochloric acid (50:17:7.5) (filter disc).
S_2 = Butanol–water–glacial acetic acid (50:17:2) (filter disc).
S_3 = Butanol–glacial acetic acid–water (4:1:5) (ascending).
Paper: Not specified.

Compound	R_F		
	S_1	S_2	S_3
Calpurnine	0.87	0.59	0.72
Epilupinine	0.79	0.51	0.65
Virgiline	0.61	0.37	0.57
13-Hydroxylupanine	0.52	0.35	0.52
Cytisine	0.35	0.19	0.38

TABLE 25

R_F VALUES OF SUBSTITUTED QUINIC ACIDS

(E. HASLAM, G. K. MAKINSON, M. O. NAUMANN AND J. CUNNINGHAM, *J. Chem. Soc.*, (1964) 2137)

Solvents: S_1 = 6% aqueous acetic acid.
S_2 = Butan-2-ol–acetic acid–water (14:1:5).
Temperature: 20° ± 2°.

Compound	R_F	
	S_1	S_2
Chlorogenic acid	0.60	0.70
Neochlorogenic acid	0.65	0.62
1-O-*p*-Coumaroylquinic acid	0.78	0.75
5-O-*p*-Coumaroyl-1-O-ethoxycarbonylquinic acid	0.87	0.88
5-O-*p*-Coumaroylquinic acid	0.75	0.72
4-O-*p*-Coumaroylquinic acid	0.68	0.75
5-O-Caffeoylquinic acid	0.63	0.62

TABLE 26

R_F VALUES OF SOME ELLAGIC ACID DERIVATIVES

(B. P. MOORE, *Australian J. Chem.*, 17 (1964) 901)

Solvents: S_1 = 0.1% aq. ammonia.
S_2 = Propan-1-ol–1% aq. ammonia (2:1).
S_3 = Butan-1-ol–1% aq. ammonia (1:1), upper phase.
Paper: Whatman No. 1.

Compound	R_F		
	S_1	S_2	S_3
2,2'-Dicarboxy-4,6,4',6'-tetrahydroxydiphenyl-dilactone	0.10	0.42	0.09
3,4,3'-Tri-O-methylellagic acid	0.18	0.78	0.45
3,3'-Di-O-methylellagic acid	0.19	0.41	0.04

CHROMATOGRAPHIC DATA VOL. 16 (1964)

AUTHOR INDEX

ARNDT, R. R. AND W. H. BAARSCHERS . D14
BAARSCHERS, W. H., see ARNDT, R. R.
BAKER, B. R., see DEGRAW, J. I.
BALLIO, A. AND S. RUSSI D7
——, ——, AND G. SERLUPI-CRESCENZI. . D6
BELL, E. A. D2
BERTACCINI, G., see VITALI, T.
BRITTON, H. G. D1
CARNEGIE, P. R. D12
CUNNINGHAM, J., see HASLAM, E.
DEGRAW, J. I., L. GOODMAN AND B. R. BAKER D8
DICKEY, E. E., see VAUGHAN, J. M.
DRUMMOND, G. I., M. W. GILGAN, E. J. REINER AND M. SMITH D7
EGGER, K. D13
FORREST, H. S., see LOHRMANN, R.
FRAHN, J. L. AND J. A. MILLS . . D3, D4
GERRANS, G. C. AND J. HARLEY-MASON. . D14
GILGAN, M. W., see DRUMMOND, G. I.
GOODMAN, L., see DEGRAW, J. I.
HARLEY-MASON, J., see GERRANS, G. C.
HASLAM, E., G. K. MAKINSON, M. O. NAUMANN AND J. CUNNINGHAM . . D15
HORTON, D., see WOLFROM, M. L.
JACOB, T. M. AND H. G. KHORANA . . . D6
KHORANA, H. G., see JACOB, T. M.
LOHRMANN, R. AND H. S. FORREST. . . D4
MAKINSON, G. K., see HASLAM, E.
MATTOCKS, A. R. D11, D13
MILLS, J. A., see FRAHN, J. L.
MOORE, B. P. D15
NAUMANN, M. O., see HASLAM, E.
PRICE, J. W., see WILLIAMS, D. J.
REINER, E. J., see DRUMMOND, G. I.
RUSSI, S., see BALLIO, A.
SERLUPI-CRESCENZI, G., see BALLIO, A.
SMITH, M., see DRUMMOND, G. I.
VAN RHEENEN, D. L. D10, D11
VAUGHAN, J. M. AND E. E. DICKEY. . . D2
VERCELLOTTI, J. R., see WOLFROM, M. L.
VITALI, T. AND G. BERTACCINI D5
WILLIAMS, D. J. AND J. W. PRICE . . . D9
WOLFROM, M. L., J. R. VERCELLOTTI AND D. HORTON D1

CHROMATOGRAPHIC DATA VOL. 16 (1964)

SUBJECT INDEX

Electromigration data
- Alditol derivatives D1
- Amino acids . . . D3, D4, D11, D12
- ——, derivatives. D3, D4
- Amino compounds . . . D3, D4, D11
- ——, carbamates D3
- ——, dithiocarbamates. D4
- Glutathione and derivatives. . . . D12
- Homoglutathione and derivatives . D12

R_F values, etc
- Alkaloids of *Phylica rogersii* pillans . D14
- —— of *Virgilia oroboides* D14
- Amines. D10
- Amino acids D2, D10, D12
- Butyric acid derivatives, ethylamine salts D11
- Carotenoids. D13
- 5,8-Dideaza-5,6,7,8-tetrahydrofolic acid and related compounds . D8
- Ellagic acid derivatives D15
- Glutathione and derivatives. . . . D12

R_F values, etc. (*continued*)
- Hexitols D1
- Homoglutathione and derivatives . D12
- Imidazole derivatives. D5
- *Lathyrus* spp., ninhydrin-positive compounds from D2
- Nicotinic acid derivatives. D7
- Ninhydrin-positive compounds from *Lathyrus* spp D2
- Nucleosides. D6
- Nucleotides and related compounds D6, D7
- Organo-tin compounds D9
- *Phylica rogersii* pillans alkaloids . . D14
- Pteridine derivatives. D4
- Quinazoline derivatives. D8
- Quinic acids, substituted D15
- Retronecine derivatives. D13
- Sugars D2
- Thymidine derivatives D6
- Tin, organic compounds D9
- *Virgilia oroboides* alkaloids D14

CHROMATOGRAPHIC DATA

VOL. 16 (1964)