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THÉORIE DE LA RÉTENTION EN CHROMATOGRAPHIE EN PHASE GAZEUSE RÉALISÉE AVEC UN GRADIENT LONGITUDINAL POSITIF DE TEMPÉRATURE DONT LA VITESSE DE CROISSANCE EST CONSTANTE ·

MAURICE COUDERT ET JEAN-MAURICE VERGNAUD
Université d'Alger, Département Chimie, Faculté des Sciences, Alger (Algérie)

(Reçu le 13 Juillet 1970; manuscrit modifié reçu le 9 septembre 1970)

SUMMARY

Retention in gas chromatography obtained with a longitudinal temperature gradient with a constant growth rate

A new method of gas chromatography is described: chromatography with a longitudinal temperature gradient, which is a linear function of time. The inlet column temperature is constant. A theory of retention of solutes is proposed. The role of various parameters is specified: column length, rate of growth of temperature gradient, inlet column temperature.

INTRODUCTION

Comme nous l'avions fait précédemment[1] il parait utile tout d'abord de différentier notre méthode de la "Chromathermography" dont la traduction française est devenue "chromatographie à gradient de température". En effet, hors la ressemblance de la dénomination qui peut d'ailleurs preter à confusion, ces deux méthodes sont tout à fait différentes par l'appareillage, par le principe et la théorie, et bien entendu par les résultats.

La méthode de la "Chromatographie à gradient de température" est très bien décrite dans le "Manuel pratique de chromatographie en phase gazeuse" publié sous la direction de TRANCHANT[2]. Ainsi, développée expérimentalement par TURKEL'TAUB[3] et ZHUKHOVITSKII *et al.*[4,5], et de façon théorique par OHLINE[6], cette méthode consiste à faire progresser un four le long de la colonne dans le sens du gaz vecteur avec une vitesse déterminée; ce four entraine avec lui les solutés dont la vitesse d'élution est suffisamment élevée.

Nous avons présenté précédemment[1,7] la "Chromatographie en phase gazeuse avec gradient longitudinal de température établi le long de la colonne". Avec cette méthode, la température est constante en fonction du temps, et varie le long de la colonne d'une façon linéaire selon un gradient longitudinal constant.

Les résultats attrayants obtenus nous ont amené à persévérer dans cette voie, et nous proposons une méthode nouvelle dérivée de la première. En effet, dans cette

nouvelle méthode, nous conserverons le principe de l'utilisation du gradient longitudinal de température le long de la colonne, mais la valeur de ce gradient n'est plus constante, et croit au contraire de façon linéaire avec le temps.

Après avoir élaboré la théorie de la rétention des solutés élués, nous déterminerons le rôle joué vis à vis de l'élution par les trois paramètres: vitesse de croissance du gradient longitudinal de température, longueur de la colonne, température à l'entrée de la colonne.

APPAREILLAGE ET CONDITIONS OPÉRATOIRES

L'appareil utilisé a été décrit antérieurement[2,3]. Chromatographe F 7 (Perkin-Elmer) avec catharomètres. Colonne acier inoxydable: longueur 2 m, diamètre intérieur et extérieur respectivement 3 et 4 mm. Liquide stationnaire contenant 2.5% de caoutchouc silicone SE-52; gaz vecteur, hélium avec un débit de 40 ml/min.

Le gradient longitudinal de température a été réalisé à l'aide d'un fil chauffant Rhodorsil® de 110 Ω et de 440 cm de longueur, enroulé autour de la colonne de telle sorte que le nombre de spires par unité de longueur de colonne varie le long de celle-ci selon une progression arithmétique. À la sortie de la colonne, nous avons choisi une longueur de 3.5 cm de fil chauffant par cm de colonne, et la raison de la progression est égale à 0.012 cm de fil chauffant par cm de colonne.

Le problème posé par la réalisation de l'élévation du gradient de température de façon linéaire avec le temps a pu être résolu en alimentant la résistance avec une tension dont la valeur croit avec le temps selon une loi déterminée expérimentalement. Nous avons pu ainsi vérifier d'une part qu'à chaque instant la température varie linéairement le long de la colonne, et d'autre part qu'en chaque point de la colonne la température croit de façon linéaire avec le temps. Afin de maintenir constante la température à l'entrée de la colonne, nous avons dû refroidir légèrement le four afin d'évacuer les calories excédentaires.

Les calculs des temps de rétention ont été éffectués avec une machine IBM 1620 en utilisant la méthode Runge–Kutta.

Les solutés choisis sont les alcanes normaux compris entre l'hexane et le dodécane, et leur pureté est voisine de 99.9%.

ÉTUDE THÉORIQUE

Nous formulons trois hypothèses qui sont d'ailleurs admises en chromatographie à température programmée.

L'équilibre thermique est réalisé à chaque instant au cours de la programmation du gradient de température.

L'équilibre thermodynamique du soluté entre les deux phases est atteint, grâce à un transfert de masse élevé.

La vitesse linéaire du gaz vecteur est constante le long de la colonne, le gradient de pression de ce gaz étant très faible.

L'équation classique représentant la vitesse de propagation du gaz vecteur est:

$$\frac{dx}{dt} = V_{gv} \cdot R_F \qquad (1)$$

dans laquelle:

V_{gv} = la vitesse linéaire du gaz vecteur à l'abscisse x;

R_F = le facteur de rétention du soluté considéré.

En utilisant[3] la troisième hypothèse, et en se rappelant que le facteur R_F est égal au rapport du temps de rétention du gaz vecteur par le temps de rétention isotherme du soluté, la relation (1) peut s'écrire:

$$\frac{\mathrm{d}x}{\mathrm{d}t} = \frac{L}{t_{rT}} \tag{2}$$

dans laquelle:

L = la longueur de la colonne;

t_{rT} = le temps de rétention du soluté élué de façon isotherme à la température T avec la colonne de longueur L.

Avec notre méthode, la valeur du gradient longitudinal de température croit linéairement avec le temps. La température est donc une fonction des deux variables indépendantes: abscisse x le long de la colonne, et temps t, et sa variation peut se représenter par la différentielle totale:

$$\mathrm{d}T = \left(\frac{\partial T}{\partial x}\right)_t \cdot \mathrm{d}x + \left(\frac{\partial T}{\partial t}\right)_x \cdot \mathrm{d}t \tag{3}$$

Or, la valeur du gradient longitudinal de température au temps t est égale à:

$$\left(\frac{\partial T}{\partial x}\right)_t = b \cdot t \tag{4}$$

b = la vitesse de croissance du gradient longitudinal de température, qui s'exprime en °C/m·sec.

De plus, en un point de la colonne d'abscisse x, la vitesse linéaire d'élévation de la température est égale à:

$$\left(\frac{\partial T}{\partial t}\right)_x = b \cdot x \tag{5}$$

On obtient évidemment la même valeur b dans les équations (4) et (5), et ceci se démontre aisément en utilisant les propriérés des différentielles totales.

La combinaison des équations (3), (4) et (5) permet de définir la valeur de la température T_{xt} de la colonne à l'abscisse x et au temps t en fonction des autres paramètres:

$$T_{xt} = T_{00} + b \cdot t \cdot x \tag{6}$$

en appelant T_{00} la température à l'entrée de la colonne, et qui demeure constante.

En remplaçant dans l'équation (3) les dérivées partielles par leur valeur explicitée dans les relations (4) et (5), le temps t par sa valeur tirée de la relation (6), et la différentielle $\mathrm{d}t$ par sa valeur obtenue dans l'équation (2), on obtient l'équation différentielle fondamentale:

$$\frac{\mathrm{d}T}{\mathrm{d}x} = \frac{b}{L} \cdot t_{rT} \cdot x + \frac{T_{xt} - T_{00}}{x} \tag{7}$$

Cette équation (7) ne peut être intégrée, mais elle a été résolue numériquement avec l'aide d'un ordinateur numérique, et pour la valeur L de x, on obtient ainsi la température de rétention du soluté T_r.

Le temps de rétention t_r du soluté élué avec cette méthode se calcule ensuite aisément en transformant l'équation (6):

$$t_r = \frac{T_r - T_{00}}{b \cdot L} \tag{8}$$

VARIATION DU TEMPS DE RÉTENTION EN FONCTION DES VALEURS DONNÉES AUX PARAMÈTRES

Nous examinerons successivement, vis à vis du temps de rétention, l'importance du rôle joué par les trois paramètres que sont: la longueur de la colonne, la vitesse de croissance du gradient, et la température à l'entrée de la colonne.

Influence de la longueur de la colonne sur les temps de rétention

Pour réaliser cette étude, nous avons travaillé successivement avec plusieurs colonnes de longueurs différentes, en maintenant constantes les valeurs données aux

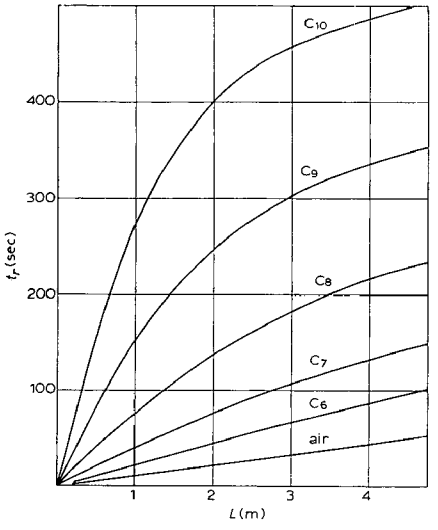

Fig. 1. Variation du temps de rétention des solutés avec la longueur de la colonne. $T_{00} = 70°$; $b = 0.05°/m \cdot sec$ air; C_6 = *n*-hexane; C_7 = *n*-heptane; C_8 = *n*-octane; C_9 = *n*-nonane; C_{10} = *n*-décane.

autres paramètres: $T_{00} = 70°$, $b = 0.05°/m \cdot sec$. Les valeurs des temps de rétention ont été d'autre part calculées en utilisant les équations (7) et (8), avec l'aide de l'ordinateur.

Nous avons pu alors représenter sur la Fig. 1, la variation du temps de rétention des alcanes normaux compris entre l'hexane et le décane, en fonction de la longueur de la colonne. Un examen de cette courbe permet de formuler plusieurs conclusions intéressantes. D'une part, fait évident avec les hypothèses formulées, le temps de rétention de l'air est proportionnel à la longueur de la colonne. D'autre part, l'hexane

et l'heptane ont leur temps de rétention sensiblement proportionnel à la longueur. Par contre, il n'en est plus de même pour le nonane et le décane dont la croissance du temps de rétention avec la longueur est bien plus faible, et ceci est particulièrement net pour le décane.

Ainsi, un allongement de la longueur de la colonne provoque un allongement du temps de rétention différent selon la nature des solutés, et cet allongement du temps est d'autant plus faible que le point d'ébullition du soluté est élevé. Cette remarque est intéressante, car l'emploi d'une colonne de longueur suffisante pour réaliser une séparation déterminée, ne provoque pas un allongement considérable du temps de rétention des derniers solutés.

Influence de la vitesse de croissance du gradient longitudinal de température sur le temps de rétention

Nous avons représenté sur la Fig. 2 la variation du temps de rétention des mêmes alcanes, avec la valeur de la vitesse de croissance du gradient, les valeurs des autres paramètres étant maintenues constantes: $L = 2$ m, $T_{00} = 70°$.

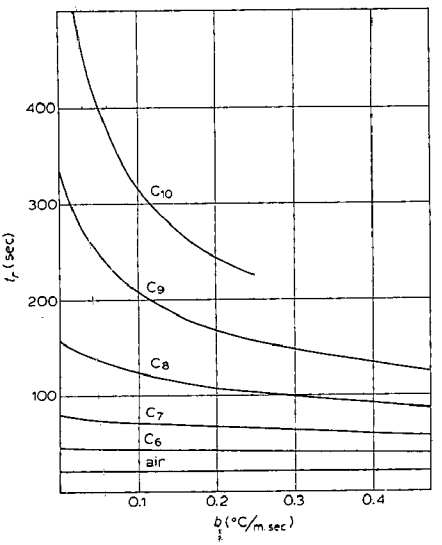

Fig. 2. Variation du temps de rétention des solutés avec la valeur de la vitesse de croissance du gradient. $L = 2$ m; $T_{00} = 70°$.

À la valeur nulle de b, il correspond évidemment la chromatographie isotherme réalisée à 70°. La Fig. 2 permet donc d'apprécier le gain de temps permis par notre méthode sur la chromatographie isotherme. De plus, nous constatons que le temps de rétention de l'air et des premiers solutés comme l'hexane et l'heptane, diminue très peu lorsque b croit de 0 à 0.5°/m·sec. Par contre, les derniers solutés élués, comme le nonane et le décane voient leur temps de rétention diminuer de façon considérable lorsque b croit.

Ainsi, l'emploi d'une vitesse de croissance du gradient élevée provoque un resserrement des pics des solutés, sans cependant réduire de façon génante le temps

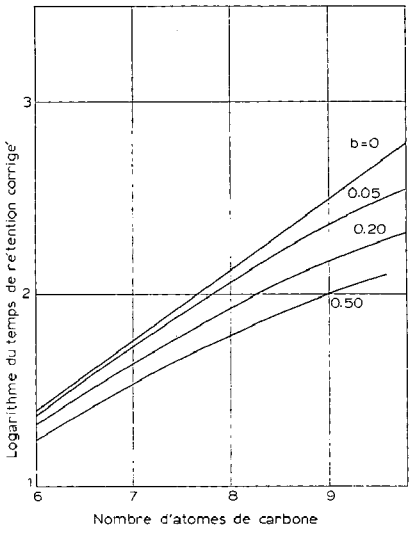

Fig. 3. Variation du logarithme du temps de rétention corrigé des alcanes en fonction de leur nombre d'atomes de carbone, pour différentes valeurs de b. $b = 0$; $b = 0.05°/m \cdot sec$; $b = 0.2$; $b = 0.5$.

de rétention des premiers solutés. Nous pouvons d'ailleurs préciser cette conclusion en examinant la Fig. 3 sur laquelle nous avons représenter la variation du temps de rétention en fonction du nombre d'atomes de carbone des alcanes, pour des valeurs de la vitesse b comprises entre 0 et $0.5°/m \cdot sec$.

Influence de la température T_{00} sur le temps de rétention

Nous avons représenté sur la Fig. 4, la variation du temps de rétention des

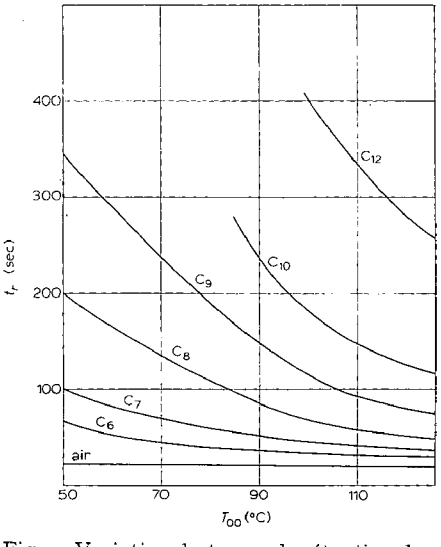

Fig. 4. Variation du temps de rétention des solutés en fonction de la valeur de T_{00}, avec la méthode préconisée. $L = 2$ m; $b = 0.05$ °/m · sec.

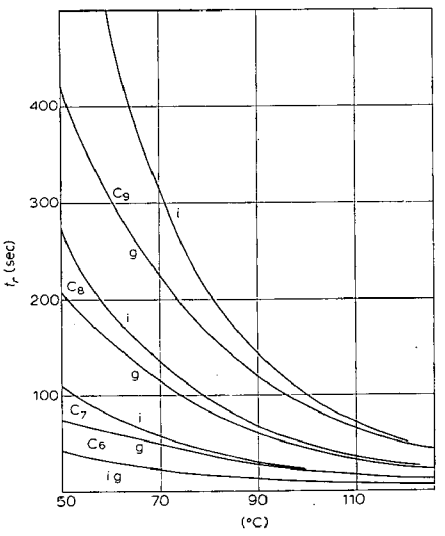

Fig. 5. Variation du temps de rétention des solutés en fonction de la valeur de T_{00}, pour différents solutés. i = Chromatographie isotherme; g = Chromatographie avec gradient variant avec le temps, $L = 2$ m; $b = 0.05°/m \cdot sec$.

solutés avec la valeur donnée à T_{00}, en maintenant constantes les valeurs des autres paramètres: $L = 2$ m et $b = 0.05°/m \cdot sec$.

Plusieurs constatations peuvent être formulées. D'une part, le temps de rétention de l'air varie très peu avec la température T_{00}. Par contre, le temps de rétention des alcanes diminue d'une façon considérable lorsque l'on élève la valeur de T_{00}. De plus, on peut constater que cette réduction des temps de rétention est sensiblement le même pour tous les alcanes. En effet, le rapport du temps de rétention corrigé obtenu à 50°, par le temps de rétention corrigé correspondant à 100°, est compris entre 3.3 et 3.7 pour tous les alcanes considérés.

Nous pouvons comparer sur la Fig. 5 la variation du temps de rétention des solutés avec la valeur de T_{00}, obtenue d'une part en chromatographie isotherme et d'autre part avec la méthode préconisée. Ainsi, nous pouvons constater que le gain de temps de rétention permis par notre méthode sur la chromatographie isotherme est d'autant plus appréciable que la valeur de T_{00} est basse, et que la condensation en carbone du soluté est grande.

CONCLUSIONS

Après avoir élaboré la théorie de la propagation des solutés élués en chromatographie avec gradient longitudinal de température dont la valeur croit avec le temps, il nous a été possible de déterminer la valeur du temps de rétention obtenu dans des conditions opératoires particulières. Il nous a ensuite été possible de vérifier la concordance des valeurs des temps de rétention calculées et mesurées expérimentalement. Et enfin, nous avons pu préciser l'importance du rôle joué vis à vis du temps de rétention des solutés, par les différents paramètres que sont: la longueur de la colonne, la vitesse de croissance du gradient longitudinal de température, et la température à

l'entrée de la colonne. Pour chaque cas, nous avons comparé les valeurs des temps de rétention obtenus en chromatographie isotherme avec celles obtenues avec la méthode préconisée, afin d'apprécier le gain de temps qu'elle permet.

RÉSUMÉ

Une méthode nouvelle de chromatographie en phase gazeuse est présentée: la chromatographie avec gradient longitudinal de température dont la valeur du gradient croit linéairement avec le temps. La température à l'entrée de la colonne est constante, et elle est moins élevée que celle de la sortie. Une théorie de la propagation du soluté est élaborée, et l'équation obtenue relie le temps de rétention aux différents paramètres: longueur de la colonne, vitesse de croissance du gradient, et température à l'entrée de la colonne. Le rôle joué par ces trois paramètres vis à vis du temps de rétention des solutés est précisé.

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QUANTITATIVE DETERMINATION OF CARBOHYDRATES IN CELLULOSIC MATERIALS BY GAS–LIQUID CHROMATOGRAPHY

AUTOMATIC INTEGRATION OF ALDITOL ACETATE PEAK AREAS

EERO SJÖSTRÖM AND SIRKKA JUSLIN
Technical University of Helsinki, Laboratory of Wood Chemistry, Otaniemi (Finland)

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SUMMARY

Automatic integration, based on the voltage-to-frequency conversion principle, has been systematically studied to improve further the gas chromatographic method which was developed earlier for the quantitative analysis of wood and pulp carbohydrates. Mixtures containing various amounts of alditol acetates (L-arabinitol, D-xylitol, D-mannitol, D-galactitol, and D-glucitol) were injected into the gas chromatograph and the output data were then evaluated by integrator. The precision of the analysis was found to be good even in the case of some overlapping of the peaks. However, for reaching high accuracy, excellent chromatographic separation is a prerequisite.

INTRODUCTION

Gas chromatography of monosaccharides as fully acetylated alditol derivatives is a very useful procedure which can be applied to the quantitative determination of carbohydrates in wood and pulp after total hydrolysis. In our earlier work[1] the peak area measurements were made manually, *i.e.* by weighing the cut chromatograms. In the present work automatic integration of the peak areas was studied in order to improve further the reliability of the method as well as to minimise the time required for the analysis.

Several integrator types are commercially available for the evaluation of gas chromatograms. However, the instruments based on the electronic voltage-to-frequency conversion technique are considered most reliable[2]. Such integrators have been earlier studied by JOHNSON *et al.*[3] with respect to the reproducibility and linear dynamic range of integration. Mixtures consisting of acrylonitrile, *n*-propanol, and dioxane were used. Also, BAUMANN AND TAO[4] studied the effect of slope sensitivity, filtering and baseline correction rate on accuracy.

Integrators based on the voltage-to-frequency conversion principle were used also in the present work. In addition to the reproducibility of integration, factors influencing the analytical accuracy were subjected to a systematic study. The mixtures

used consisted of fully acetylated pentitols (L-arabinitol, D-xylitol) and hexitols (D-glucitol, D-mannitol, D-galactitol).

EXPERIMENTAL

A Perkin-Elmer gas chromatograph Model 900 equipped with dual columns and flame ionisation detector, was used. The 1/8 in. stainless-steel columns (2 m) were filled with packing material (2.3 g) consisting of a mixture of 2.5% Silicone oil XF-1150 and 2% ethylene glycol succinate on 100–120 mesh Chromosorb W-H.P. The plate number, measured from the mannitol hexaacetate peak, was about 2100.

Nitrogen was used as the carrier gas (30 ml/min). The gas flow rates to the detectors were adjusted to 55 ml/min for the hydrogen and 300 ml/min for the oxygen–nitrogen gas mixture (20% O_2, 80% N_2). The injection port was maintained at 300°, the detectors at 220°, and the columns at 175°.

For the measurements of the peak areas an Infotronics integrator Model CRS-100 was mainly used with following adjustments: baseline tracking up 6 μV/min, down 200 μV/min; slope sensitivity, 2; peak rate, 3 sec/peak; input noise rejection, 3; shoulder printout off; digital filtering, 2000 counts; false trip reject on; count rate, 1000 counts/mV.

Some experiments were made using a Hewlett-Packard integrator, Model 3370 A. The adjustments were the following: noise suppression max.; slope sensitivity up 0.01 mV/min, down 0.01 mV/min; peak Σ level, 1000 mV; shoulder control, front off, rear 1000 mV; baseline reset delay 0, during the third peak the switch was turned to ∞ position for the rest of the run.

Various mixtures containing accurately weighed amounts (10–100 mg) of alditol acetates, prepared as described earlier[1], were dissolved in pyridine (1–2 ml). The amount injected was *ca.* 0.5 μl.

RESULTS AND DISCUSSION

The five alditol acetates were not all separated quite completely in the column

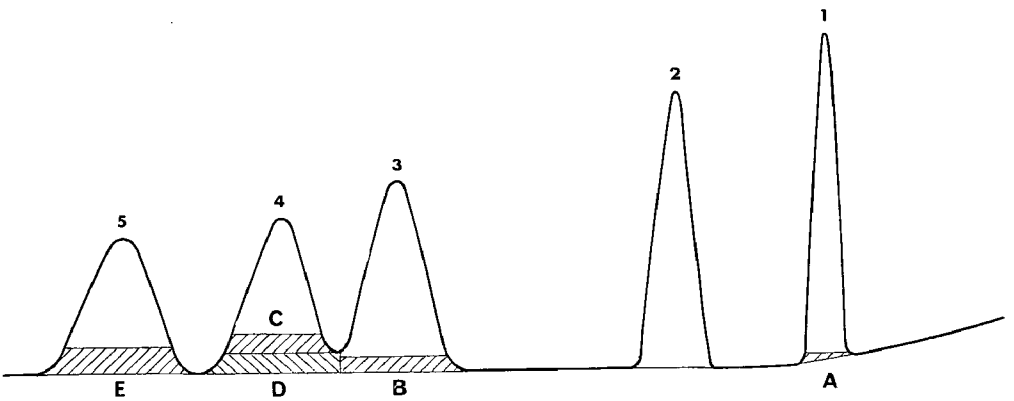

Fig. 1. Schematic chromatogram illustrating the unintegrated parts (shaded areas). For clearness, the errors have been exaggerated. 1 = Arabinitol pentaacetate; 2 = Xylitol pentaacetate; 3 = Mannitol hexaacetate; 4 = Galactitol hexaacetate; 5 = Glucitol hexaacetate.

used. As appears from Fig. 1, mannitol and galactitol hexaacetate peaks overlap to a certain extent. Therefore, when all five alditol acetates are present, the resulting chromatogram is "non-ideal". Three five-component mixtures containing varying proportions of alditol acetates were introduced into the gas chromatograph and the injection of each mixture was repeated ten times. The alditol acetate composition was then calculated on the basis of the pulses counted by the integrator. The results obtained with the Infotronics integrator are summarised in Table I.

As can be seen the precision of the analysis is comparatively good but the accuracy is unsatisfactory. This is obviously connected with several factors, *cf.* Fig. 1. One source of error is the fact that the peak corresponding to arabinitol pentaacetate

TABLE I

ANALYSIS OF ALDITOL ACETATE MIXTURES—INFOTRONICS INTEGRATOR

Alditol acetate	*Calc.*[a] *y*	*Found*[b] *x*	*S.D.*	*Range*
Arabinitol	6.58	6.97	0.16	0.53
Xylitol	8.89	9.24	0.17	0.56
Mannitol	6.53	7.21	0.08	0.28
Galactitol	6.53	6.43	0.08	0.31
Glucitol	71.48	70.15	0.25	0.17
Arabinitol	10.05	10.38	0.11	0.36
Xylitol	10.25	10.39	0.34	0.39
Mannitol	10.21	10.72	0.19	0.16
Galactitol	19.89	19.75	0.16	0.20
Glucitol	49.60	48.76	0.42	0.48
Arabinitol	19.21	19.65	0.24	0.74
Xylitol	16.95	16.99	0.17	0.46
Mannitol	23.08	23.06	0.26	0.97
Galactitol	20.63	20.45	0.11	0.28
Glucitol	20.12	19.85	0.24	0.74

[a] The percent composition of the alditol acetate mixture.
[b] These figures refer to the average percent composition calculated on the basis of 10 runs.

is very close to the solvent (pyridine) front. The integrator automatically follows the baseline until integration begins; integration is referred to the level reached at the beginning of a peak. The baseline is continuously sloping during the integration of the arabinitol peak, and hence, the shaded area A in Fig. 1 remains unintegrated. Further, the last three peaks are relatively gently sloping. The voltage change from the detectors, *i.e.* the slope of the chromatogram primarily determines the starting point of integration. Because of the insufficiently fast change, a certain rise in the baseline level occurs. Therefore, the areas marked with B, C, and E in Fig. 1 remain unintegrated. This could be avoided by using the highest slope sensitivity value (position 1 in Infotronics CRS-100 integrator). However, this high sensitivity caused vagueness in the termination of integration and hence was not advantageous. Finally, peaks Nos. 3 and 4 (mannitol and galactitol hexaacetates) are not separated completely. Since the peaks are relatively gently sloping there is sufficient time for the

TABLE II

ANALYSIS OF ALDITOL ACETATE MIXTURES—HEWLETT-PACKARD INTEGRATOR

Alditol acetate	*Calc.*[a] *y*	*Found*[b] *x*	*S.D.*	*Range*
Arabinitol	6.74	7.50	0.11	0.31
Xylitol	7.09	7.55	0.08	0.22
Mannitol	7.11	8.03	0.04	0.14
Galactitol	8.04	8.04	0.17	0.63
Glucitol	71.02	68.87	0.24	0.65
Arabinitol	10.40	10.68	0.11	0.35
Xylitol	9.94	10.18	0.29	0.81
Mannitol	10.62	10.88	0.11	0.39
Galactitol	20.16	19.71	0.20	0.64
Glucitol	48.89	48.57	0.43	1.14
Arabinitol	20.01	20.27	0.45	1.71
Xylitol	19.70	19.92	0.29	0.88
Mannitol	20.05	19.68	0.20	0.57
Galactitol	20.01	19.84	0.17	0.49
Glucitol	20.22	20.29	0.43	1.28

[a] The percent composition of the alditol acetate mixture.
[b] These figures refer to the average percent composition calculated on the basis of 10 runs.

integrator to change the baseline level after trailing edge of peak No. 3. The area marked with D is therefore not integrated.

Additional experiments were made using the Hewlett-Packard integrator. This instrument makes it possible to adjust the positive and negative slope detector sensitivities separately. However, the most sensitive positions (0.01 mV/min) gave the best results. Similar chromatographic conditions as in previous runs were used although the column characteristics were somewhat different. As can be seen from Table II, the precision and accuracy do not significantly differ from the previous runs.

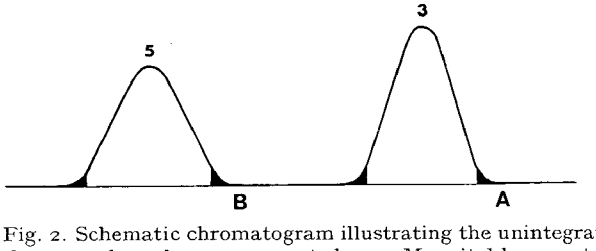

Fig. 2. Schematic chromatogram illustrating the unintegrated parts (shaded areas). For clearness, the errors have been exaggerated. 3 = Mannitol hexaacetate; 5 = Glucitol hexaacetate.

To approach as ideal chromatographic conditions as possible mixtures containing only three components (xylitol, mannitol, and glucitol) were studied. In addition, baseline rise during peaks 3 and 5 (mannitol and glucitol) was prevented manually so as to correspond to the levels marked by A and B in Fig. 2. The results from these runs are reproduced in Table III. It appears that both the precision and the accuracy are fairly good.

TABLE III

ANALYSIS OF ALDITOL ACETATE MIXTURES—INFOTRONICS INTEGRATOR

Alditol acetate	*Calc.*[a] *y*	*Found*[b] *x*	*S.D.*	*Range*
Xylitol	5.01	4.88	0.08	0.30
Mannitol	10.10	10.11	0.08	0.26
Glucitol	84.89	85.01	0.13	0.39
Xylitol	9.90	9.81	0.17	0.47
Mannitol	83.29	83.35	0.16	0.43
Glucitol	6.81	6.85	0.15	0.46
Xylitol	19.93	19.96	0.14	0.48
Mannitol	30.32	30.39	0.05	0.16
Glucitol	49.75	49.65	0.13	0.35
Xylitol	32.78	33.05	0.22	0.58
Mannitol	33.30	33.18	0.12	0.45
Glucitol	33.92	33.78	0.14	0.38
Xylitol	33.63	34.04	0.28	0.81
Mannitol	33.08	32.93	0.14	0.33
Glucitol	33.29	33.03	0.16	0.48
Xylitol	40.36	40.41	0.11	0.26
Mannitol	49.79	49.74	0.07	0.20
Glucitol	9.85	9.86	0.06	0.15
Xylitol	70.12	70.36	0.08	0.24
Mannitol	5.42	5.37	0.04	0.14
Glucitol	24.46	24.27	0.06	0.20

[a] The percent composition of the alditol acetate mixture.
[b] These figures refer to the average percent composition calculated on the basis of 10 runs.

The figures in Table IV were further examined comparing the found values (x) with the calculated values (y). For this purpose the constants of the best line ($y = a + bx$) and the scatter were determined. When comparing a and s_a values in different cases it can be concluded that for 95% probability limit the constant a does not significantly differ from zero, which means that there is no systematic error. It can

TABLE IV

ESTIMATION OF ANALYTICAL ACCURACY[a]

Alditol acetate	*Intercept*		*Slope*	
	a	s_a[b]	b	s_b[b]
Xylitol	0.07	0.12	0.994	0.003
Mannitol	0.06	0.08	0.999	0.002
Glucitol	0.12	0.10	0.999	0.002

[a] These figures were calculated on the basis of data in Table III and they refer to the best line ($y = a + bx$) within the corresponding concentration range of each alditol acetate.
[b] Standard deviation.

be concluded that constant b does not significantly deviate from 1.000. This value which corresponds to the "response factor" is identical for the three studied alditol acetates.

Finally, on the basis of the above results it is obvious that the studied electronic integrators are very suitable for the analysis of alditol acetate mixtures. However, for high analytical accuracy a sufficient chromatographic separation is a prerequisite. Work is now in progress for further improvement of the column packing material.

ACKNOWLEDGEMENTS

We wish to thank the National Research Council for Technical Sciences for financial support. Thanks are also due to Mr. P. LAININEN, M.Sc., for valuable discussions, and Miss ANNELI EKLUND, M.Sc., for some preliminary work. We are grateful to Hewlett-Packard Cy, Helsinki, Finland, for the loan of the integrator.

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CHROM. 5035

A GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF HALOPERIDOL IN HUMAN PLASMA

INA A. ZINGALES
Research Laboratory, Cleveland State Hospital, Cleveland, Ohio 44105 (U.S.A.)*

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SUMMARY

A gas chromatographic method for the qualitative and quantitative determination of haloperidol in plasma at therapeutic levels has been developed.

The procedure involves extraction of the drug from alkalinized plasma into *n*-heptane, successive concentration into aqueous and organic solvents, and separation on a gas–liquid chromatograph equipped with an electron capture detector.

The method has been applied to plasma samples from patients receiving 9 to 15 mg of haloperidol *pro die*. Plasma levels encountered after oral doses of 3 to 5 mg varied from zero to 10 ng of haloperidol per ml of plasma.

INTRODUCTION

Haloperidol, 4-[4-(*p*-chlorophenyl)-4-hydroxypiperidino]-4′-fluorobutyrophenone, has been used for more than a decade in the treatment of acute and chronic psychoses.

There is an extensive literature concerning the pharmacology and clinical properties of the drug, but little information is available on its excretion, metabolism and distribution in tissues. A number of studies relating to the chemistry of the drug have been published[1–4]. DEMOEN[2] describes the physical properties and analytical procedures for the assay of haloperidol in dosage forms. Colorimetric methods for the determination of the drug in pure forms or in pharmaceutical preparations are based on color reaction with *m*-dinitrobenzene[5] or 3,5-dinitrobenzoic acid[6]. An analytical procedure developed by SOEP[7] involves the extraction of the drug from urine of rat, paper chromatography, and determination of the fluorine content of the spot. The color complex formed with methyl orange is the basis of a method applied by DEMOEN[2] to urine and feces of laboratory animals.

The excretion and metabolism of the drug have been studied in rats after the administration of tritium labeled haloperidol[8]. According to the authors, oxidative N-dealkylation represents the major metabolic pathway. BRAUN *et al.*[9] studied the distribution of haloperidol in tissues. Their results show that, 3 h after its adminis-

* William L. Grover, M.D., Superintendent.

tration, approximately 15% of the tritium labeled dose was present in the tissues. Most of the radioactivity was concentrated in the liver. Concentration in blood varied between an average of 0.38% of the administered dose at 1 h and 0.59% at 5 h.

None of these methods are applicable to the analysis of blood samples following administration of therapeutic doses.

The increasing interest in the use of haloperidol in mental diseases prompted the present investigation. It is confined to the description of an analytical procedure for the determination of unmetabolized haloperidol in the plasma of patients receiving doses ranging between 9 and 15 mg *pro die*.

Gas chromatography utilizing the affinity of the electron capture detector to halogenated substances was, for its sensitivity and selectivity, the technique of choice.

EXPERIMENTAL

Reagents

Only analytical grade reagents were used. Stock solutions were prepared by dissolving 5 mg of haloperidol in 100 ml of *n*-heptane containing 1.5% isoamyl alcohol. Under refrigeration these solutions were stable for several weeks. Working standard solutions contained 1 to 40 ng of haloperidol per microliter.

Material

Blood samples were collected in the morning 1 to 3 h after oral administration of 3 to 5 mg doses. Only samples from patients receiving 9 to 15 mg of the drug *pro die* were analyzed. They had been in haloperidol therapy at various dosages for at least two weeks prior to sampling.

Extraction procedure

A sample of 10 ml of plasma made alkaline by the addition of 1 ml of 2.5 *N* sodium hydroxide, was extracted with 20 ml of *n*-heptane containing 1.5% isoamyl alcohol by shaking mechanically for 15 min. After centrifugation at 3,000 r.p.m., 18 ml of the organic phase were transferred to a 30-ml glass test tube containing 5 ml of 0.1 *N* hydrochloric acid. The tube was mechanically shaken for 10 min. Following centrifugation, the acidic aqueous layer was made alkaline by adding 1 ml of 1 *N* sodium hydroxide and then extracted with 5 ml of the *n*-heptane–isoamyl alcohol mixture. After centrifugation, 4.8 ml of the organic phase were transferred to a 5-ml glass centrifuge test tube and evaporated to dryness under nitrogen. The residue was dissolved in 25–50 μl of *n*-heptane–isoamyl alcohol; 3 to 10 μl of this were injected into the chromatograph.

Aliquots of the reference solutions containing 1 to 50 ng of haloperidol per microliter were transferred to a centrifuge test tube. The solvent was evaporated under nitrogen and the residue dissolved in 100 μl of 0.001 *N* hydrochloric acid. Ten milliliters of distilled water, or haloperidol free plasma or urine were added and the extraction continued as described above.

Gas chromatography

A Beckman GC-4 gas chromatograph equipped with an electron capture detector, and a Beckman 10 in. potentiometric recorder were used in this work. The

ordinate scale was expanded for increase in electrometer sensitivity as suggested by the manifacturer. Column, temperatures, flow rates and electrical settings are described below. The column was conditioned at 320° for 48 h with a helium flow rate of 25 ml/min.

Conditions for chromatography

Column: glass, 4 ft., ¼ in. O.D., 2 mm I.D.; 2% OV-1 on Chromosorb W HP 80–100 mesh.

Temperatures: inlet lines 300°; column oven 210°; detector and detector lines 320°.

Flow rates: helium, carrier gas 55 ml/min; helium, discharge gas 100 ml/min; carbon dioxide 3 ml/min.

Detector electrical settings: polarizing voltage 675 duodial, bias voltage 520 duodial, source current 7 mA.

Electrometer settings: range 100, attenuation 1024, suppression current off (background current equivalent to 80% full scale); range 100, attenuation settings from 512 to 128, suppression current on (for scale expansion).

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, haloperidol has a

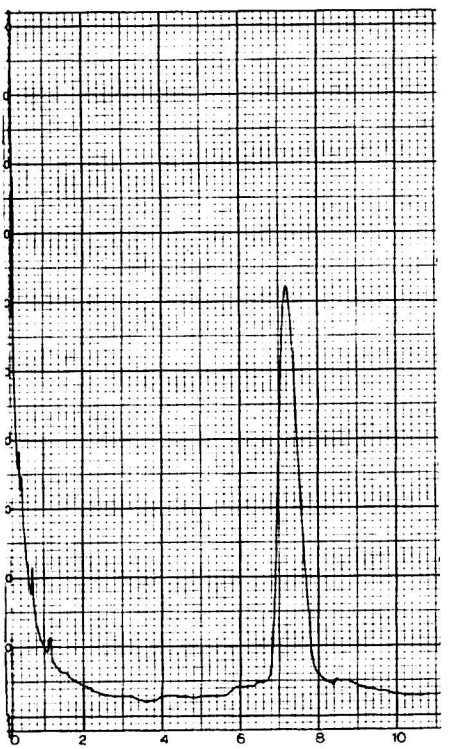

Fig. 1. GLC traces of a standard solution of haloperidol in *n*-heptane–isoamyl alcohol. Injected 20 ng.

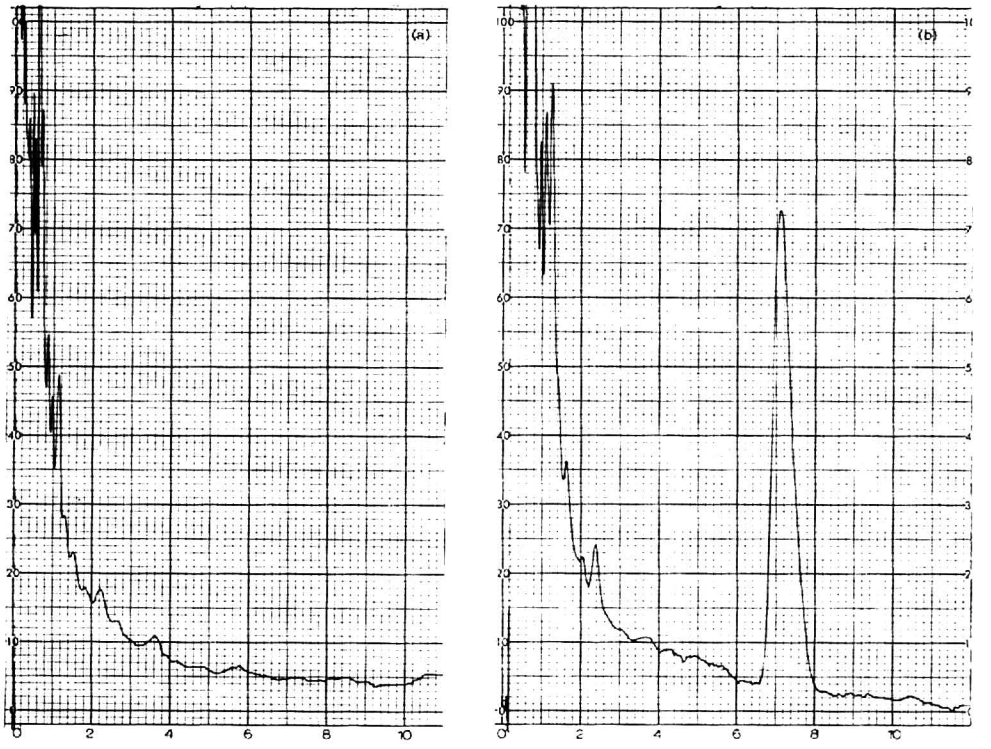

Fig. 2. GLC traces of :(a) an extract from 10 ml of blank plasma and (b) an extract from 10 ml of the same sample to which 250 ng of haloperidol had been added. Injected 30 ng; recovery 86.3%.

TABLE I

RECOVERY OF HALOPERIDOL FROM WATER, URINE AND PLASMA

Sample	*Haloperidol added ng/10 ml*	*No. of determinations*	*Percent recovery ± S.D.*
Water	50	10	98.9 ± 3.1
	100	13	99.8 ± 2.5
	250	5	101.4 ± 2.9
	500	5	100.1 ± 2.3
Urine	50	8	95.4 ± 4.3
	100	10	98.2 ± 4.3
	250	10	99.1 ± 2.9
	500	8	101.3 ± 4.2
Plasma	50	12	85.8 ± 3.9
	100	11	84.9 ± 3.2
	250	7	85.3 ± 2.8
	500	5	88.1 ± 3.8

retention time of approximately 7 min. Fig. 1 shows the chromatogram obtained from a standard solution of haloperidol in *n*-heptane–isoamyl alcohol. Fig. 2 represents chromatograms from an extract of 10 ml of blank plasma and 10 ml of the same sample to which 250 ng of haloperidol had been added. No interfering peaks in the same region of haloperidol have been observed in the chromatograms obtained from several blank plasma samples analyzed.

To verify the adequacy of the extraction procedure, amounts of haloperidol from 50 to 500 ng were added to 10 ml of distilled water or drug free urine or plasma. The results of these determinations are shown in Table I.

Concentrations of haloperidol in the extracts were measured by disc integration readings. The response of the detector to injections of 5 to 500 ng is a linear function of the concentration (Fig. 3); however, because of variations in the sensitivity of the detector, haloperidol concentrations in the sample analyzed were not calculated from a graph of this relationship. Extracts of blank plasma samples to which appropriate amounts of haloperidol had been added, were prepared each time analyses of unknown samples were performed. Aliquots of these extracts were injected immediately before and after each chromatographic analysis of the unknown. Variations in the sensitivity of the detector were significantly reduced by increasing the source current to 12 mA for 10 min after running 4 or 5 samples from biological material.

The procedure described has been applied to samples of plasma from patients

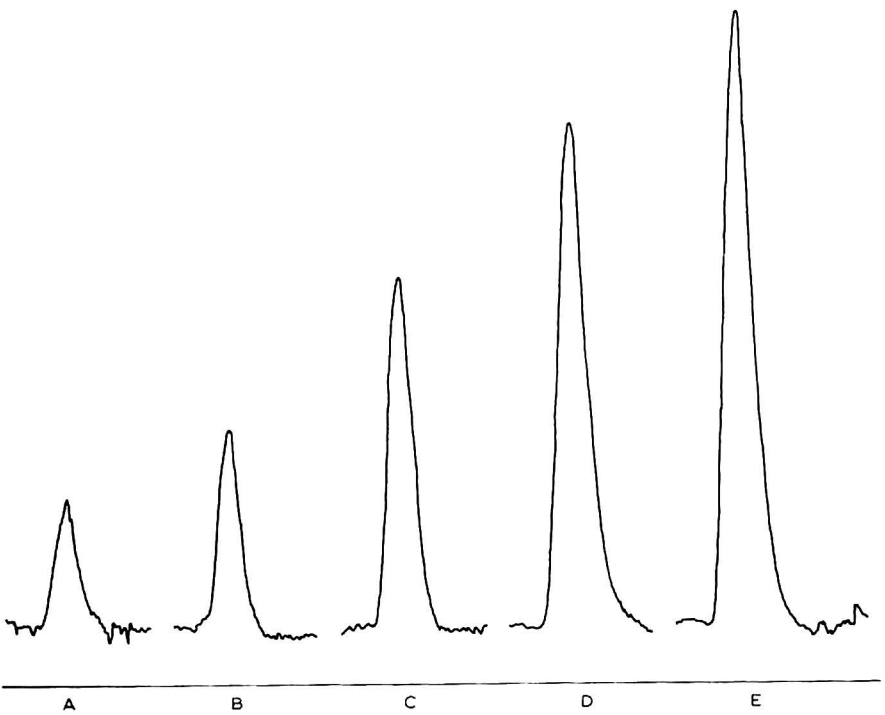

Fig. 3. Peaks obtained from injections of 5 ng (A) to 25 ng (E) of haloperidol in *n*-heptane–isoamyl alcohol. 5 ng can be quantitatively determined with accuracy.

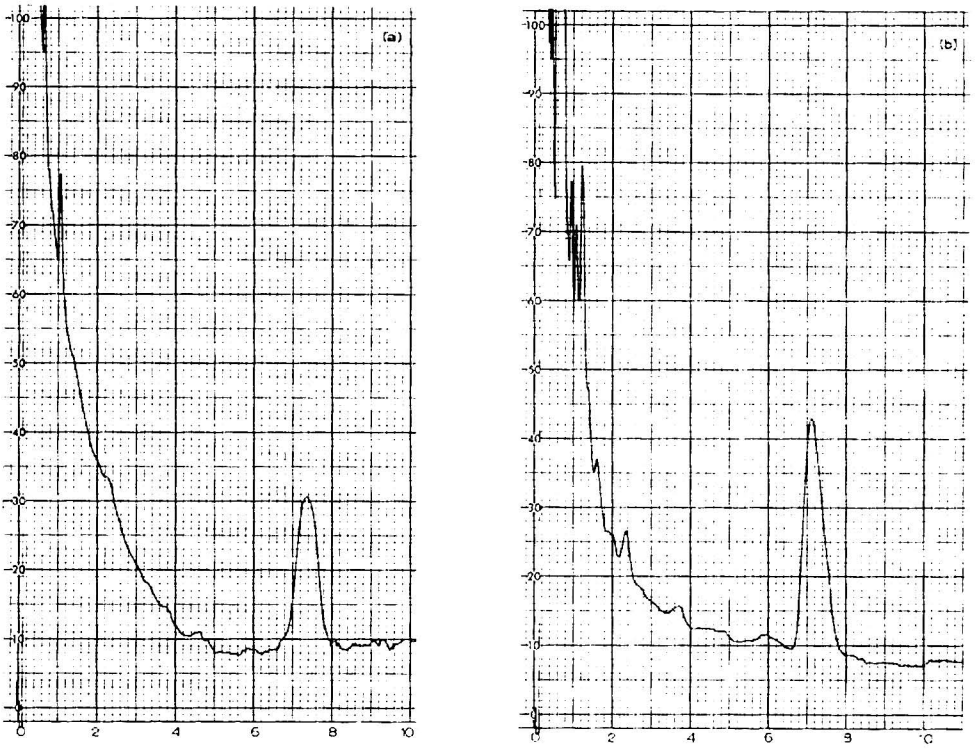

Fig. 4. GLC traces of extracts from plasma samples from two patients receiving: (a) 9 mg and (b) 10 mg of haloperidol *pro die*. No other medication was administered.

TABLE II

DATA OF CASES STUDIED

Patient No.	*Mg of haloperidol pro die*	*Other drugs administered*	*Times of collection after morning doses*	*Plasma levels (ng/ml)*
1	3 *t.i.d.*	–	120 min	2.3
2	3 *t.i.d.*	Benztropine	125 min	traces
3	3 *t.i.d.*	Chlorpromazine, trifluoperazine	60 min	negative
4	5 *b.i.d.*		120 min	3.2
5	4 *t.i.d.*	Chlorpromazine, trifluoperazine, benztropine	80 min	negative
6	4 *t.i.d.*	Imipramine, benztropine	180 min	2.2
7	4 *t.i.d.*	Chlorpromazine, fluphenazine, benztropine	120 min	traces
8	4 *t.i.d.*	Chlorpromazine, diphenhydramine	180 min	2.8
9	4 *t.i.d.*	Diazepam, diphenhydramine, benztropine	150 min	3.3
10	5 *t.i.d.*	Tolbutamide, diphenylhydantoin	120 min	4.1
11	5 *t.i.d.*	Amitriptyline, benztropine	80 min	traces
12	5 *t.i.d.*	Imipramine, benztropine	170 min	2.7
13	5 *t.i.d.*	Chlorpromazine, benztropine	160 min	10.0
14	5 *t.i.d.*	Thioridazine, diphenylhydantoin	90 min	2.9

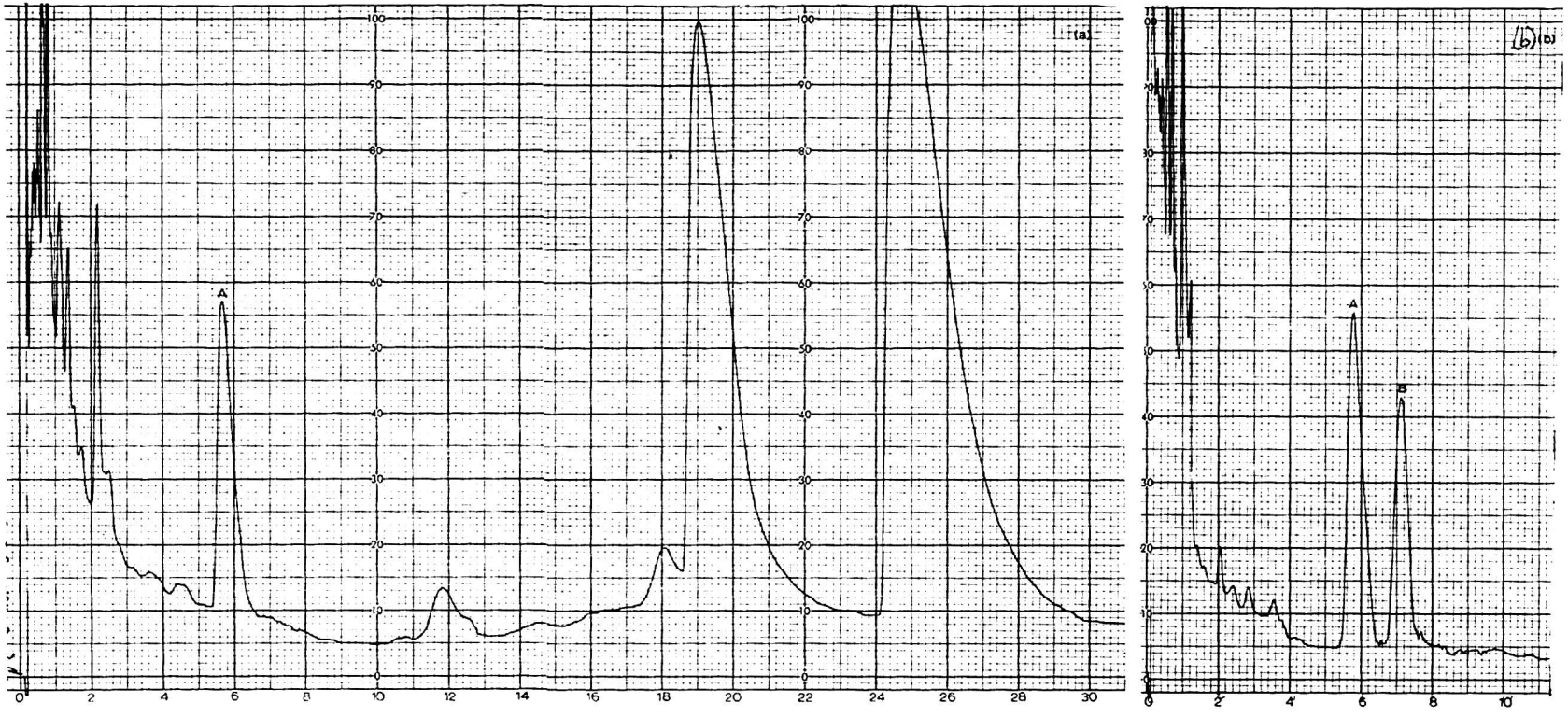

Fig. 5. GLC traces of: (a) an extract from plasma of a patient receiving only chlorpromazine and (b) an extract from another aliquot of the same sample to which 200 ng of haloperidol had been added. Peak A corresponds to a metabolite of chlorpromazine; peak B to haloperidol.

in haloperidol therapy. As mentioned previously, these patients were receiving various dosages of the drug for at least two weeks prior to sampling. At the time of collection of the samples, daily dosages ranged from 9 to 15 mg. Only 2 of the 14 samples analyzed were from patients receiving only haloperidol. Fig. 4 shows typical chromatograms from the extracts of these two samples. The remainder of the patients were receiving, in addition to haloperidol, a variety of other drugs. Doses of haloperidol, drugs administered, times of sample collection and plasma levels detected are given in Table II.

In order to ascertain the specificity of the method for the detection of haloperidol, plasma samples from patients receiving imipramine, amitriptyline, protriptyline, chlordiazepoxide, diazepam, trifluoperazine, chlorpromazine or thioridazine were extracted and chromatographed under the same conditions described for haloperidol. With the exception of chlorpromazine, the chromatograms obtained from these extracts showed no peaks in the haloperidol region. Fig. 5 represents a chromatogram from the extract of a plasma sample from a patient receiving only chlorpromazine (600 mg *pro die*). Under the chromatographic conditions used, chlorpromazine, mono- and di-demethyl chlorpromazine have retention times of approximately 2 min; peak A in Fig. 5 (a and b) has the same retention time as chlorpromazine sulfoxide (approximately 6 min). In order to ascertain conclusively that this

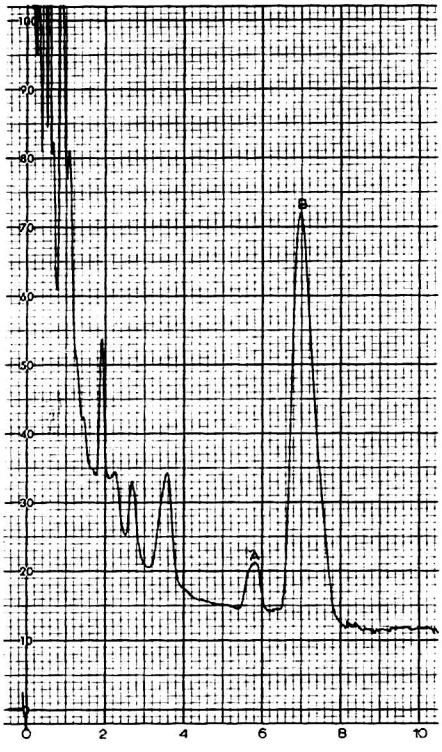

Fig. 6. GLC trace of an extract from plasma of a patient receiving 15 mg of haloperidol and 200 mg of chlorpromazine *pro die*. Peaks A and B as in Fig. 5.

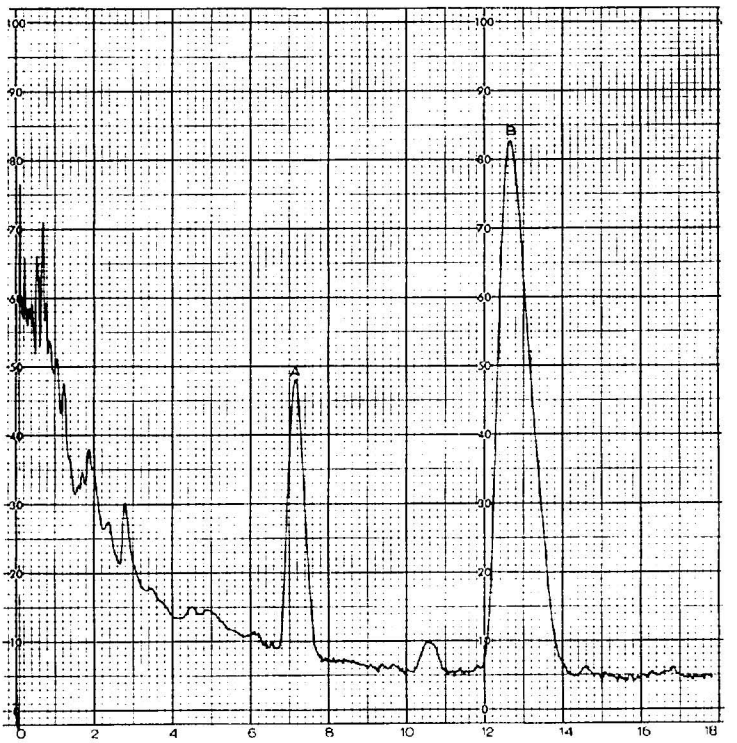

Fig. 7. GLC trace of an extract from urine of a patient receiving only haloperidol. Peak A has the same retention time as haloperidol. Peak B could represent a metabolite of haloperidol.

chlorpromazine metabolite could not interfere with the detection of haloperidol when the two drugs are administered together, 200 ng of haloperidol were added to another portion of the same plasma sample. The sample was then extracted and an aliquot of the extract injected into the chromatograph. Fig. 5 (b) shows the separation of the two peaks. Fig. 6 represents an extract of a plasma sample from a patient receiving 15 mg of haloperidol and 200 mg of chlorpromazine *pro die*.

When the analyses of plasma samples resulted negative for haloperidol, samples of urine from the same subjects were analyzed according to the procedure described, in order to exclude the possibility that the drug had not been ingested. A positive urine analysis indicated that the plasma level in the patient was too low to be detected by the method described. Fig. 7 represents a typical chromatogram from one of these extracts. Peak B could correspond to a haloperidol metabolite, as haloperidol was the only medication administered to the patient during the previous five months (3–9 mg *pro die*). Extracts from blank urine present no interfering peaks in the same region.

As shown by the results obtained, there are considerable variations in the concentration of haloperidol in plasma of patients maintained in similar dosage schedules. The highest plasma level (998 ng %) among the samples analyzed was detected repeatedly in plasma samples from a patient whose response to the therapy was considered by the ward physician to be very poor. Thus, within the limits of the number of samples analyzed, evidence exists that the response of the patient to halo-

peridol therapy is not necessarily related to high plasma levels of the free unmetabolized drug. Preliminary experiments in this laboratory show that the drug is bound to plasma proteins in detectable amounts. The possibility of biotransformation of the drug into pharmacologically active metabolite(s), undetected by the procedure described, must also be considered.

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CHROM. 5050

GAS–LIQUID CHROMATOGRAPHY OF GOSSYPOL*

MICHAEL A. McCLURE
Department of Plant Pathology, University of Arizona, Tucson, Ariz. (U.S.A.)

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SUMMARY

Extraneous peaks resulting from the gas–liquid chromatography of gossypol trimethylsilyl derivatives are shown to be the products of incomplete silylation of the gossypol molecule. A comparison of methods for the treatment of cotton root extracts prior to chromatography is presented and the quantitative determination of gossypol as its trimethylsilyl ether is discussed.

INTRODUCTION

Gossypol (2,2'-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene) and related pigments are naturally occurring substances found primarily in specialized glands of the cotton plant. The toxicity of gossypol to a wide variety of organisms has been investigated and its role in disease resistance demonstrated[1]. As part of a program concerning the biochemical nature of cotton resistance to nematodes it was of interest, therefore, to determine the gossypol content of cotton roots of various cotton varieties. A recent publication[2] has dealt with the gas–liquid chromatographic (GLC) determination of gossypol. According to this report, the highly reactive silyl donor N,O-bis(trimethylsilyl) acetamide (BSA) was a suitable agent for the preparation of the trimethylsilyl derivative of gossypol. Upon GLC, however, such a derivative yielded two shoulder peaks which the authors attributed to the occurrence of gossypol in three tautomeric forms. Evidence was cited in support of this proposal, that purification of the TMS-gossypol derivative by column or thin-layer chromatography did not remove the shoulder peaks. It was also stated that silylation in different solvents reduced the shoulder peaks in certain instances and varied individual peak size in others. Tetrahydrofuran (THF) was a solvent reported to result in the production of at least two shoulder peaks.

During the course of the present investigation it was noted that the occurrence of shoulder peaks was related to the ratio of BSA to gossypol and that a single peak could be produced by the addition of adequate amounts of BSA to the reaction mixture. This report presents evidence which indicates that the extraneous peaks arise from incomplete silylation of the gossypol molecule. A procedure for the partial purification of gossypol prior to silylation is also given and the quantitative determination of gossypol in cotton roots discussed.

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METHODS AND MATERIALS

Gossypol acetic acid[3], a primary reference standard, containing 89.62% gossypol by weight was dissociated by solution in dilute sodium carbonate and the gossypol recovered according to the method of PONS *et al.*[4]. The resulting product was used without further purification.

GLC analyses were performed on a Varian Aerograph chromatograph equipped with a flame ionization detector. A 46 × 0.21 cm (I.D.) stainless-steel column packed with 3% SE-52 on 100–120 mesh Gas-Chrom Q (Applied Science, State College, Pa.) was operated isothermally at 250 or 255° with a carrier (nitrogen) flow of 46 ml/min. Injections were made directly on the column, with the injector portion of the column maintained at 275°.

Standard solutions for quantitative analysis and determination of detector response (Fig. 1) were prepared by dissolving quantities of gossypol in carbon disulfide, sufficient to provide a known volume of solution upon addition of the BSA. For

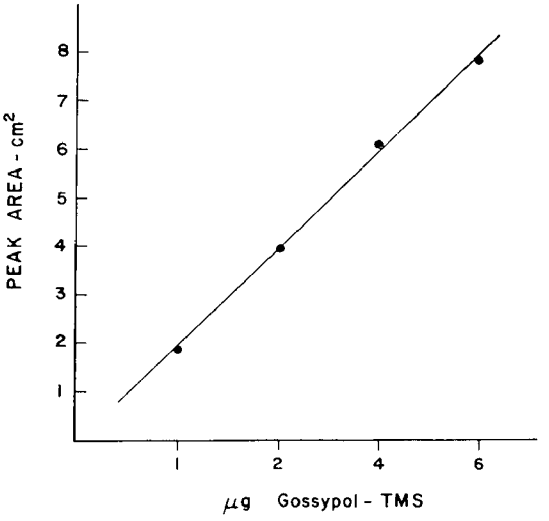


Fig. 1. Detector response and calibration curve for gossypol-TMS.

example, to make a solution containing 1 mg/ml of gossypol, 1 ml of CS_2 containing 1 mg of gossypol was evaporated under N_2 in a tared glass vial and the residue redissolved in 0.6 ml of carbon disulfide. To this was added 0.4 ml BSA (Pierce Chemical Company, Rockford, Ill.), the vial flushed with N_2, sealed and held at 50° for 30 min. One microliter aliquots were taken directly from the reaction mixture for injection.

Peak areas were estimated by triangulation, and standard curves prepared by plotting peak area against weight or molarity of gossypol. Unknown quantities of gossypol were determined by measuring peak areas and comparing them directly with those of the standard curve. A new standard curve was prepared for each analysis although a deviation of less than 3% was noted between analyses.

IR spectra were obtained in CS_2 on a Perkin-Elmer InfraCord equipped with NaCl micro-cavity cells (Barnes Engineering, Stamford, Conn.).

EXPERIMENTAL

The influence of BSA concentration on the production of extraneous peaks was determined by weighing 10 mg quantities of gossypol into tared glass vials and adding 1 ml of THF and 15–360 μl of BSA. The vials were capped with teflon-lined lids, shaken and held at 50° for 20 min prior to analysis.

Alternatively, 10 mg of gossypol were reacted with 30 μl of BSA in 1 ml of THF and the mixture incubated at 40°. At regular intervals, following addition of the BSA, 1 μl samples were subjected to GLC analysis.

Partially silylated gossypol was prepared by dissolving 120 mg of gossypol in 2 ml of THF and 125 μl of BSA. After standing at room temperature for 30 min, the THF and BSA were removed at 80° under high vacuum. The last traces of BSA were removed by dissolving the product in 5 ml of anhydrous carbon tetrachloride which was then removed under vacuum at 80°. This process was repeated three times and the resulting residue taken up in a small amount of carbon tetrachloride. Unsilylated gossypol was removed by sweep-distillation at 275° and the partially silylated material collected in glass U-tubes at −80°. The product obtained by this procedure had a GLC retention time corresponding to that of peak c in Fig. 2, and a GLC purity of 95%. Gossypol-TMS corresponding to peak a (Fig. 2) was obtained in 99.5% purity in a fashion similar to that just described except that the reaction was carried out in carbon disulfide, 1 ml of BSA added and the reaction mixture, under N_2, heated at 40° for 30 min in a sealed glass vial.

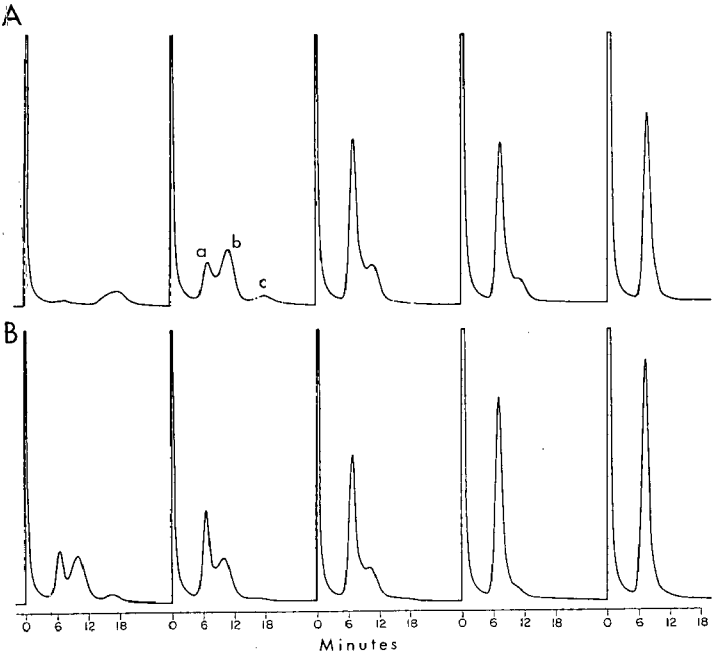


Fig. 2. (A) Chromatograms produced following the reaction of gossypol with 15, 30, 60, 120 and 360 μl of BSA. (B) Chromatograms produced at time intervals of 0, 30, 60, 180 and 300 min following the addition of 30 μl of BSA to 10 mg of gossypol in 1 ml of THF.

Plant root materials were prepared for GLC analysis of gossypol following a cleanup procedure suggested by BELL[5]. Fresh roots (30–50 g) were mascerated in a high-speed blendor in 300 ml of re-distilled acetone. The extract was filtered once through a Whatman No. 2 filter, prewashed with acetone, and the filtrate rinsed with an additional 200 ml of acetone. The acetone and most of the water were then removed under vacuum at 50° and the residue dissolved in 200 ml of ether. The ether solution was washed once with 100 ml of water at pH 6.5 and extracted with 200 ml of 0.5% sodium borate. After a single extraction of the borate phase with 100 ml of ether, it was acidified to pH 2 with 6 *N* HCl and re-extracted with 3 portions (100 ml) of ether. Traces of HCl were removed by washing the combined ether extracts once with 200 ml of water, drying over anhydrous sodium sulfate and evaporating to dryness at 30° under vacuum. The residue was silylated as described above for standard gossypol and 1 μl aliquots of the reaction mixture injected directly.

In an effort to reduce sample preparation to a minimum, crude acetone extracts were dried as before and the residue was silylated in CS_2 without further purification. Relative recovery of gossypol from cotton roots was estimated for both techniques by supplementing 5 g quantities of fresh roots with 5 mg of authentic gossypol. Four separate analyses were performed for each method using unsupplemented crude extracts as controls.

RESULTS

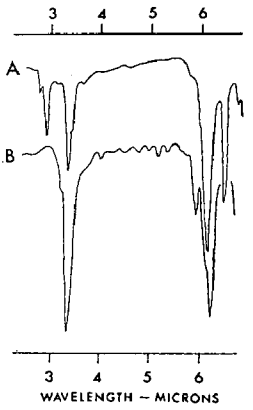
BSA concentration

Concentrations of BSA less than 36 μl per 1 mg of gossypol resulted in the production of more than one GLC peak (Fig. 2A). When 10 mg of gossypol were treated with 15 μl of BSA, a chromatogram was produced in which the major component (peak c) had a retention time of 2.5 relative to gossypol-TMS (peak a). At 30 μl of BSA to 10 mg of gossypol, the major component (peak b) had a retention time of 1.8 in relation to gossypol-TMS. Of the total peak area, 35% was represented by gossypol-TMS (peak a) and less than 10% by peak c. Increasing concentrations of BSA increased the area under peak a and reduced peaks b and c, accordingly. Since the total peak area at the highest BSA concentration exceeded that at 30 μl BSA by almost 20%, it was assumed that a portion of the gossypol in the latter case remained unsilylated or insufficiently silylated to pass through the GLC column.

Sampling at increasing time intervals following the introduction of a minimal amount of BSA produced a series of chromatograms (Fig. 2B) similar to those obtained by adding increasing amounts of BSA to the reaction mixture. In addition to gossypol-TMS, injections made soon after the addition of BSA resulted in the appearance of peaks b and c. When the reaction period was increased, the area under these peaks decreased accompanied by a proportionate increase in peak a (gossypol-TMS).

Infrared analyses

IR spectra (Fig. 3A) of material comprising peak c showed absorption bands at 2.8 and 6.2 μ corresponding to the oscillations of free phenolic hydroxyl groups and carbonyl groups respectively. The presence of the band at 2.8 μ is thus indicative of the incomplete silylation of the gossypol molecule since such bands do not exist in the



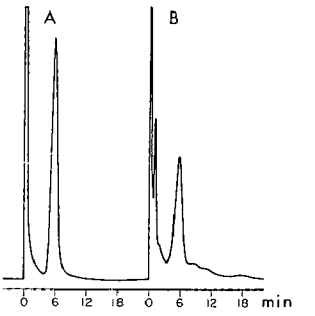


Fig. 3. IR spectra of (A) partially silylated gossypol (peak c) and (B) gossypol-TMS (peak a).

Fig. 4. Chromatograms of gossypol-TMS extracted from cotton roots. (A) borate purified prior to silylation, (B) silylation of crude extract.

spectra of gossypol hexamethyl ethers. Accordingly, spectra of fully silylated gossypol (Fig. 3B) showed no absorption at 2.8 μ (OH) and increased absorption at 3.42 μ (CH_3). Partial silylation of gossypol also resulted in the appearance of a strong absorbtion band at 6.4 μ which could not be explained on the basis of the aldehydic structure of gossypol or its TMS derivative.

Cotton roots

Acetone extracts of cotton roots subjected to borate–ether purification gave rise to a single symmetrical peak upon GLC of the TMS reaction mixture (Fig. 4A). Non-purified extracts also produced a well-defined gossypol-TMS peak, accompanied, however, by an elevated baseline and several ill-defined peaks (Fig. 4B). It was not determined whether these additional peaks arose from incomplete silylation of the native gossypol or if they represented other compounds of sufficient volatility to pass through the GLC column. Heating the reaction mixture at 40° for 3 h did not reduce their proportion relative to gossypol-TMS. Relative recoveries of gossypol from cotton roots by silylation of the crude acetone extract and extracts partially purified by treatment with 0.5% sodium borate are presented in Table I. It was noted that in order to silylate completely gossypol in supplemented acetone extracts, it was necessary to add BSA in excess of the amount considered adequate for purified samples. Less than 120 μl per mg gossypol resulted in the appearance of shoulder peaks corresponding to peaks b and c in Fig. 2.

DISCUSSION

The occurrence of gossypol and certain of its derivatives in one or more tautomeric forms has been proposed on the basis of IR and chemical studies[6]. These studies have shown that at least some derivatives exist primarily in one form. Thus, while the dimethyl ether of gossypol is said to have the aldehyde tautomeric form, the hexamethyl ether of gossypol is thought to exist in the quinoid form and hexaacetates in

the hemiacetal form. In previous GLC studies it has been suggested that the appearance of more than one peak following injection of the TMS derivative supports the occurrence of such tautomers. The present investigation shows that at least one of the peaks, formed when THF is used as the reaction solvent, results from incomplete silylation of the free phenolic hydroxyl groups. No free phenolic hydroxyl stretching absorption in the IR spectrum was observed when gossypol was silylated under optimum conditions. If it were possible to separate, by GLC, all of the products of incomplete silylation, more than three peaks might be expected. The asymmetry of peak a (Fig. 2) suggests the presence of additional peaks incompletely resolved under the conditions of analysis. Gossypol showed less of a tendency to form partially silylated products in CS_2 than in THF. Furthermore, GLC flame ionization detectors are relatively insensitive to carbon disulfide with the result that less interference from the injection solvent can be expected. For these reasons, carbon disulfide is a more suitable solvent than THF for the silylation of gossypol. Should THF be the solvent of choice, however, it is necessary to use at least 40 μl of BSA per mg of gossypol to effect complete silylation.

Partial purification of the acetone extracts with a solution of sodium borate resulted in 40–56% losses of the gossypol added to fresh cotton roots. Failure to recover higher percentages can be explained in part by the presence, in cotton roots, of powerful emulsifiers. These substances make it difficult to obtain good two-phase separations of gossypol-containing organic solvents washed with aqueous solutions. The addition of methanol serves to break these emulsions, but also may cause gossypol to be partially distributed in the discarded phase.

Although silylation of crude acetone extracts of cotton roots resulted in an increased base line and the appearance of additional peaks (Fig. 4B) (probably not gossypol-TMS related), considerably higher recoveries of added gossypol were observed (Table I). Also, reduced sample manipulation resulted in greater analytical precision. For cotton roots, this procedure is preferred.

TABLE I

RECOVERY OF GOSSYPOL FROM COTTON ROOTS

$$\% \text{ recovery} = \frac{\text{wt. of gossypol recovered} - \text{wt. of indigenous gossypol}}{\text{wt. of gossypol added}} \times 100.$$

Expt. No.	*Recovery (%)*	
	Acetone extract	*Borate treated extract*
1	92.4	44.0
2	92.8	59.4
3	91.8	54.6
4	92.4	59.6
Mean ± S.E.	92.4 ± 0.2	54.4 ± 3.7

ACKNOWLEDGEMENT

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CHROM. 5080

A STUDY OF FURFURYL ALCOHOL RESIN COMPONENTS BY GEL PERMEATION CHROMATOGRAPHY

S. B. WALLON, J. B. BARR AND B. A. PETRO
Union Carbide Corporation, Carbon Products Division, P.O. Box 6116, Cleveland, Ohio 44101 (U.S.A.)

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SUMMARY

Furfuryl alcohol resins contain a broad spectrum of compounds. A resin containing a high proportion of low molecular weight constituents has been prepared, and twelve compounds, five of which were previously unreported, have been separated from the resin and characterized. Both qualitative and quantitative gel permeation chromatographic data were obtained for a number of these compounds, and calibration curves have been prepared from the data. The gel permeation chromatographic technique was also used to study the reaction products formed by several of these compounds.

INTRODUCTION

Furfuryl alcohol polymerizes when catalyzed by acidic catalysts, amines, or γ-alumina to yield a complex liquid mixture of polyfurfuryl compounds which ultimately condense to a chemically inert solid[1–3]. The rate and degree of polymerization can be controlled by regulation of the catalyst concentration, temperature, and reaction time.

The composition of liquid furfuryl alcohol resins, the condensation process, and the structure of thermoset resins have been studied extensively, but few investigators have applied chromatographic techniques to them. The first chromatographic study was conducted by SHONO *et al.*, who utilized paper chromatography[4–6]. These workers studied the effect of hydrogen ion concentration on the condensation rate and reported that the decrease in pH which occurs during the polymerization of furfuryl alcohol could be ascribed to the formation of levulinic acid. SHONO *et al.* also identified furfuryl alcohol, 5-furfurylfurfuryl alcohol, 5-(5-furfurylfurfuryl)furfuryl alcohol, difurfuryl ether, difurylmethane, 2,5-bis(hydroxymethyl)furan, and bis(5-hydroxymethyl-2-furyl)methane in resin systems prepared in their laboratory.

A second chromatographic study was conducted by BOQUIST *et al.*, who employed the gas chromatographic technique[7–9]. These investigators concluded that

the pyrolytic degradation of furfuryl alcohol resins occurs in three steps: (1) dehydration and decarboxylation, (2) carbon–carbon scission and disproportionation, and (3) dehydrogenation.

While studying the polymerization of furfuryl alcohol, WEWERKA utilized gel permeation chromatography (GPC) to analyze resins produced by using various catalysts[10–11]. He concluded that the molecular weight distribution of the resins ranged from approx. 5,000 down to 100. WEWERKA also found that resins catalyzed with γ-alumina exhibited GPC peaks shifted to lower elution volumes than those catalyzed by other methods.

A study of furfuryl alcohol resins was initiated in this laboratory to separate and characterize individual components which could function as model compounds representative of various fractions of the resins. In the course of this work, twelve compounds were isolated from furfuryl alcohol resins; five of these were reported for the first time[12]. Gel permeation chromatography was used extensively for determination of sample purity and, occasionally, for separations.

EXPERIMENTAL

Furfuryl alcohol resins were prepared in the laboratory with sulfuric acid catalyst and distilled into four fractions with boiling ranges of (A) 25–90°, (B) 90–110°, (C) 110–140°, and (D) 145° and higher. Individual components were then separated from the fractions by utilizing distillation, extraction, and various chromatographic techniques.

Furfuryl alcohol (I), difurylmethane (2,2′-methylenedifuran (IV)), and difurfuryl ether (2,2′-(oxydimethylene)difuran (VIII)) were isolated from fraction A. Fraction B contained 5-furfurylfurfuryl alcohol (II), 2,5-bis(5-furfuryl)furan (V), 5-furfuryl-2,2′-(oxydimethylene)difuran (IX), 2-methyl-5-(5-furfurylfurfuryl)furan (X), and 1,6-dihydroxy-2,2,6-trimethyl-4-heptanone (XII). Three compounds, 2,2′-methylenebis(5-furfurylfuran) (VI), 2,5-bis(5-furfurylfurfuryl)furan (VII), and 2-methyl-5-(5-furfurylfurfurylfurfuryl)furan (XI), were separated from fraction C. Fraction D yielded 2,2′-methylenebis(5-furfurylfuran) (VI) and 5-(5-furfurylfurfuryl)-furfuryl alcohol (III). Chemical formulas and numerical designations are given in Table I.

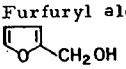
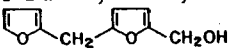
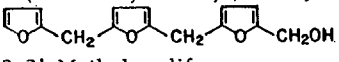
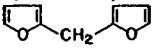
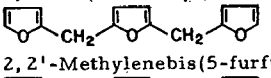
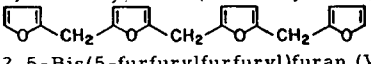
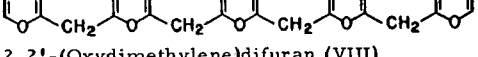
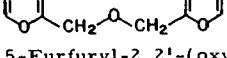
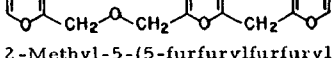
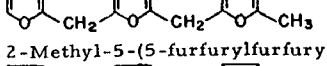
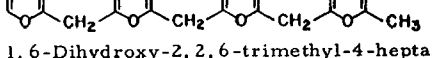
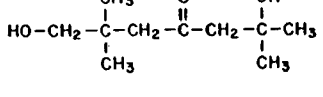
Details of resin preparation and fractionation, separation techniques, and the identification of compounds have been previously reported[12].

Gel permeation chromatography (GPC)

A Waters Associates Model 200 chromatograph was used to obtain gel permeation chromatograms. This instrument was equipped with five 4-ft. × 3/8-in. Styragel columns which had maximum permeabilities of 500, 60, 45, 45, and 45 Å. A 5 ml siphon was used to collect fractions. The detector employed was a differential refractometer. Toluene at 80° was used as the eluent, and the sample flow rate was maintained at 1 ml/min. Initial experiments were performed with tetrahydrofuran and toluene at various temperatures to determine the optimum operating conditions. Sample concentrations varied from 0.2 wt.% to 6.0 wt.% for the pure compounds and up to 10 wt.% for the polymer. Material which did not dissolve in toluene at 80° was removed by filtration before the solution was injected onto the column. Injection

TABLE I

FURFURYL ALCOHOL RESIN COMPONENTS

No.	*Compound*	*GPC peak Δ (ml)*
I	Furfuryl alcohol 	-17.0
II	5-Furfurylfurfuryl alcohol 	4.5
III	5-(5-Furfurylfurfuryl)furfuryl alcohol 	21.0
IV	2,2'-Methylenedifuran 	8.0
V	* 2,5-Bis(5-furfuryl)furan 	24.0
VI	2,2'-Methylenebis(5-furfurylfuran) 	36.0
VII	2,5-Bis(5-furfurylfurfuryl)furan (VII) 	46.0
VIII	2,2'-(Oxydimethylene)difuran (VIII) 	13.5
IX	* 5-Furfuryl-2,2'-(oxydimethylene)difuran 	29.0
X	* 2-Methyl-5-(5-furfurylfurfuryl)furan 	29.0
XI	* 2-Methyl-5-(5-furfurylfurfurylfurfuryl)furan 	41.0
XII	* 1,6-Dihydroxy-2,2,6-trimethyl-4-heptanone 	24.5

* New compounds.

times varied from 45 to 120 sec, depending on sample size. Refractometer response was linear, and resolution remained constant for the various sample sizes and injection times used.

Heat treatment of the polymer and components

Representatives of the major classes of compounds (alcohols, ethers, and polyfurfuryl furans) encountered in furfuryl alcohol resins were heated, and the reaction products were examined by GPC. In some experiments, the compounds were heated in air; in others, samples were degassed in vacuum ($\sim 10^{-5}$ Torr) and sealed in Pyrex

ampoules under vacuum or in argon. The samples were heated at 150 to 200° for times varying up to 24 h until some polymerization had occurred, but heating was discontinued before the sample had thermoset.

DISCUSSION

GPC of resin components

Gel permeation chromatography was used extensively in our work not only for separations but also to determine sample purity; samples which appear to be pure by other methods may contain sufficient impurities to show additional GPC peaks.

The normal calibration of the instrument was made using the polypropylene glycol standards supplied by Waters Associates and linear alkanes from C_1 to C_{36}. These compounds covered the linear portion of the curve as well as the nonlinear regions at the maximum and minimum pore volumes. As the pure compounds were isolated and their structures determined, an unsuccessful attempt was made to fit these compounds on the calibration curve. The hydroxymethyl substituted compounds were retained on the column considerably longer than the unsubstituted compounds. This effect was also observed for water, which eluted approximately seventy milliliters after nitrogen. The methyl substituted compounds eluted slightly earlier than expected based upon the elution time of the unsubstituted compounds. The unsubstituted compounds and the ethers formed a third curve. Apparently, the end group (methyl, hydrogen, or hydroxymethyl), in addition to size, is an important factor affecting the elution volume. This variation in elution of the three different types of compounds resulted in three calibration curves.

Calibration of the gel permeation chromatograph was carried out by using the twelve resin components and two additional compounds, 3-methyl furan and furan. The latter are not found in the resin but were included because they are the simplest members of their respective series. Some of the resin components were obtained only in mixtures; however, peak resolution and intensity were satisfactory in each case to distinguish the calibrating compound from its impurities. After the instrument was calibrated with the known compounds, the identification of new compounds could be verified from their positions on the calibration curves. In some cases, initial identification was made from the GPC curves and verified by other methods. The calibration curves are presented in Fig. 1.

Positions of GPC peaks are reported in Table I. The peak positions are expressed as the difference (Δ) between the peak position of the sample and that of the dissolved nitrogen, which provides a convenient internal standard since it appears in each chromatogram. This method was chosen because the Δ values are not sensitive to any changes in instrumentation or column packing. Compounds I and XII peak in the opposite direction from all others because the refractive index of their solutions is lower than that of the reference.

Fig. 2 shows a chromatogram of Varcum 8251, a commercial furfuryl alcohol resin (Reichhold Chemical Corporation), with its identifiable components labeled. The broad peak at approx. 115 ml corresponds with the higher molecular weight constituents which elute from the column first. Extrapolation of the front of this peak on the calibration curves indicates that this material has a molecular weight of ~1,000. Approximately five percent of the resin is insoluble in hot toluene. The

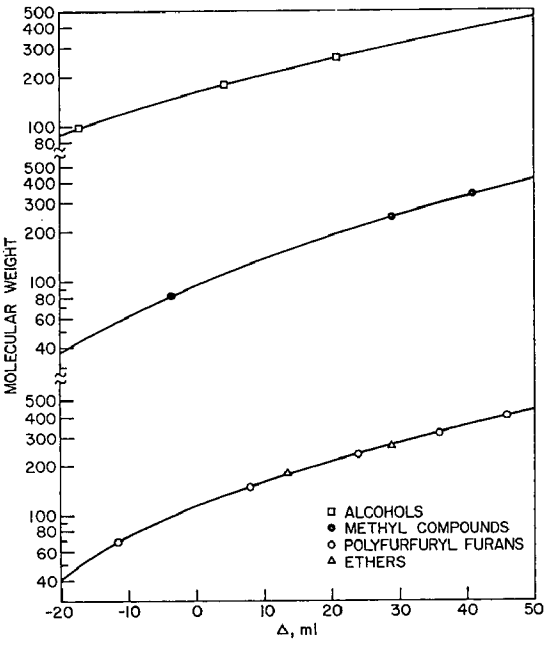


Fig. 1. Calibration curves for gel permeation chromatograph.

infrared spectrum of this fraction indicates that it contains higher molecular weight homologs of the identified components. The resin is completely soluble in tetrahydrofuran, and similar extrapolation of the GPC curves in this solvent also indicated a maximum separated molecular weight of 1,000. The positions of the maximum breaks in the calibration curves are not known; these extrapolations are based on data available at this time.

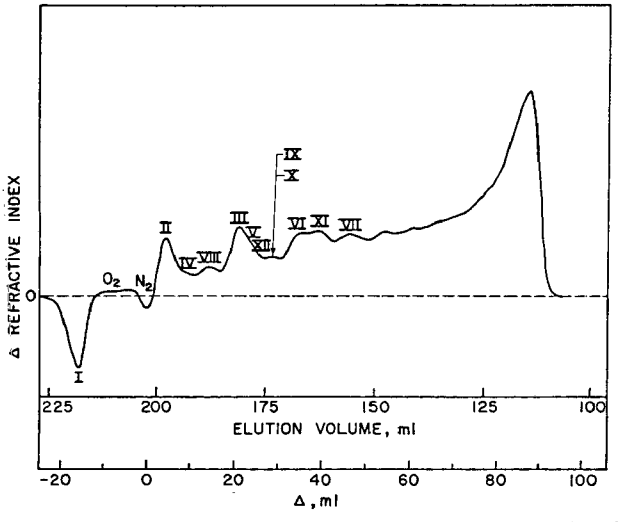


Fig. 2. Gel permeation chromatogram of Varcum 8251. For key see Table I.

WEWERKA has reported separation of materials up to 5,000 molecular weight by using polyglycols as calibration standards[10,11]. Since a separate calibration curve is necessary for each type of compound in a given solvent, a curve should be drawn through only the points corresponding with the furfuryl alcohol compounds of WEWERKA's data[10]; then, a maximum separated molecular weight of 1,000 will be indicated at 80 ml (18 counts). That value is in agreement with the data reported herein.

The resins prepared in the laboratory contained more of the lower molecular weight components than are usually present in commercial resins. These components elute at 160–200 ml. Some of the experimental resin constituents show distinct peaks; however, for the most part, the absorptions overlap, and the resolution is poor. Fig. 3 shows chromatograms of the fractions of an experimental resin separated by distillation with a vigreux column. The chromatograms clearly show the composition of each distillation range.

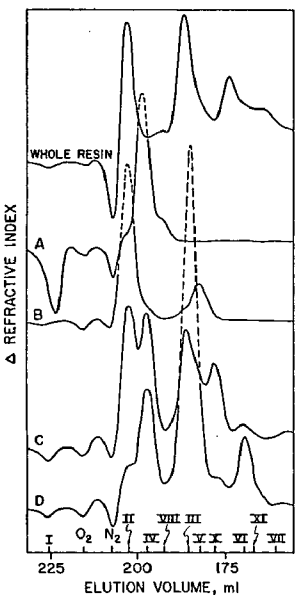


Fig. 3. Gel permeation chromatograms of furfuryl alcohol resin fractions. For key see Table I.

Quantitative data were obtained for six compounds in an effort to determine the amounts of these components in a given resin. Curves were obtained for a minimum of three concentrations of each compound. The calibrating compounds, which exhibited well-defined elution peaks, were chosen from those shown in Table I and included the alcohols (I, II, and III), difurylmethane (IV), 2,5-bis(5-furfuryl)furan (V), and difurfuryl ether (VIII). The calibration data are summarized in Fig. 4 as least squares plots of concentration *vs.* peak area (100 cm^2 = 0.8784 g).

One of the goals of the project was to develop some means of monitoring the composition of the resin without resorting to separation processes. As a quantitative tool, the GPC approach showed potential as a time-saving technique. A scan of the

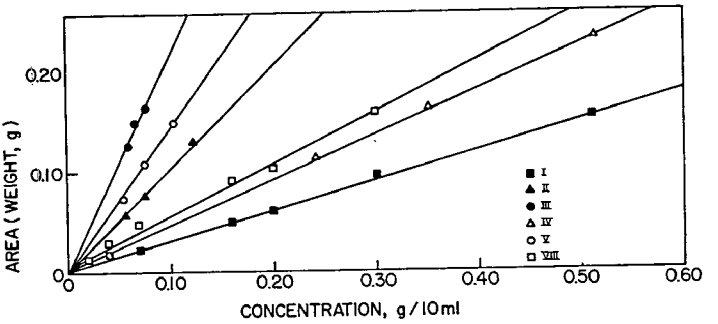


Fig. 4. Quantitative calibration curves for gel permeation chromatograph. For key see Table I.

whole resin shows several peaks which could be identified from the data obtained for the pure compounds; however, the chromatogram is very complex, and the overlapping of peaks, as well as the presence of both negative and positive peaks, precludes even a crude quantitative analysis of the resin. A composite of the GPC curves of the individual compounds is shown in Fig. 5B. A curve for a resin prepared in the laboratory is presented in Fig. 5A for comparison.

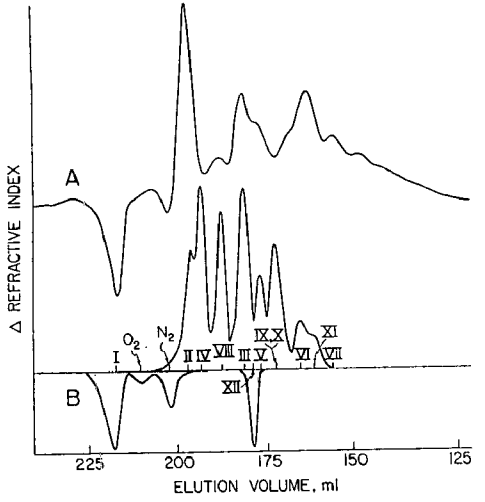


Fig. 5. Composite gel permeation chromatogram. For key see Table I.

Heat treatment of furfuryl compounds

The polymerization of furfuryl alcohol has been studied extensively, and researchers agree that the rate of resinification depends on reaction temperature and on pH; it is also influenced by oxygen and numerous other catalysts. The basic mode of polymerization is the intermolecular condensation of the hydroxymethyl group of one molecule and the α-hydrogen of another furan ring. Water is eliminated in the process:

Repeated condensation leads to a linear arrangement of furan rings connected with methylene links and terminated with a hydroxymethyl group. Several competing reactions occur which produce difurfuryl ether (dehydration reaction), difurylmethane (formaldehyde elimination), and levulinic acid.

Some investigators have pointed out that the polymerization mechanism is more complex than is indicated by this simple scheme, particularly when the curing is carried out in air[13]. Atmospheric oxygen attacks the furan ring, and the condensation route is different from that followed by the acid-catalyzed reaction. In practice, a combination of oxygen and acid catalysis is generally encountered.

To determine the origin of some of the new compounds isolated during this study, several resin components representing the major classes of compounds present in the resin were heat-treated, and the products were subjected to GPC analysis. Furfuryl alcohol polymerized readily in evacuated ampoules without a catalyst, but other alcoholic components are more stable and required the presence of air. Maleic anhydride catalyst (1.65 wt. %) was added to the remaining compounds.

Furfuryl alcohol (I) and the alcohol dimer (II) condensed to give products having chromatograms similar to that of Varcum. The contributions of the methyl compounds, (X) and (XI), were particularly evident in the products from compounds II and III. The chromatogram of products from difurylmethane (IV) was strikingly different. The latter exhibited well-defined major peaks of the ethers, (VIII) and (IX), and of the tetramer (VI). Heat treatment of the trimer (V) for 24 h produced only a single product peak which was identified as pentamer (VII). Difurfuryl ether (VIII), when heated for the same period, decomposed extensively and produced a tan solid which was highly insoluble. The infrared spectrum of the solid showed absorptions characteristic of carbonyl, hydroxyl, and ether groups.

The gel permeation chromatographic analyses clearly show some of the complexities of the polymerization. In addition to furfuryl alcohol and its homologs, other intermediate compounds can undergo rearrangement and condensation reactions. Difurylmethane is a source of ethers as well as higher unsubstituted homologs, whereas difurfuryl ether decomposes, producing a complex solid material. The methyl substituted polyfurfuryl compounds were not observed in the products from compounds other than furfuryl alcohol and the dimer alcohol.

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CALIBRATION OF TIGHTLY CROSS-LINKED GEL FILTRATION MEDIA FOR DETERMINATION OF THE SIZE OF LOW MOLECULAR WEIGHT, NON-INTERACTING SOLUTES*

J. MAX GOODSON, V. DISTEFANO** AND J. C. SMITH

Departments of Dental Research and Pharmacology and Toxicology, University of Rochester, Rochester, N.Y. (U.S.A.)

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SUMMARY

A method for calibration of tightly cross-linked gel filtration chromatography media based on the elution characteristics of a series of selected sugars and alcohols is described. The elution position of all molecules is determined by the column effluent refractive index change and is correlated with both the molecular weight and the unhydrated radius estimated from molecular models.

INTRODUCTION

Gel chromatography has been employed for estimation of the size of high molecular weight compounds. Calibration is achieved by observing the elution position of macromolecules of known size[1]. In a similar manner, the size of small molecules may be determined by gel filtration chromatography on tightly cross-linked media. However, special consideration must be given to the choice of calibrating molecules and the method of determining their size.

EXPERIMENTAL

Materials and methods

The gels (Sephadex G-10, G-15*** and Bio-Rad P-2§) were prepared by swelling in distilled water for at least 24 h. Columns*** (1.5 × 90 cm) were packed by sedimentation taking care to exclude bubbles or discontinuities in the gel bed. The eluent, 0.15 *M* NaCl in 0.01 *N* acetic acid (pH 3.3), was allowed to flow at 10 ml/h for at least 24 h at room temperature prior to use. The gel bed was then adjusted to

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** Present address: Departments of Oral Biology and Pharmacology, University of California, San Francisco Campus, San Francisco, Calif., U.S.A.

*** Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

§ Bio-Rad Laboratories, Richmond, Calif.

a height of 85 cm (total bed volume 150 cm^3) by removal of excess gel. All determinations were made at room temperature.

Hydrochloric acid-treated gels were prepared as follows: 150 cm^3 of expanded Sephadex G-15 or G-10 was mixed with 150 ml of 1 *N* or 6 *N* HCl and placed in a boiling water bath for 2 h with intermittent stirring. The gel was then allowed to settle and the supernatant decanted.

Hydrochloric acid was removed by repeated washes with distilled water followed by vacuum filtration. The gel was extracted with two 150 ml portions of chloroform–methanol (1:1) and one 150 ml portion of diethyl ether using vacuum filtrations between each extraction. The gel was dried in a hood for 24 h and heated in an oven at 100° for 2 h. The resulting xerogel was expanded in distilled water and packed in chromatographic columns as before.

Chromatography

The following calibrating molecules were employed: Blue Dextran 2000 (Pharmacia Fine Chemicals), stachyose, and raffinose (Sigma Chemical Co.), maltose, and propylene glycol (Eastman Organic Chemicals), glycerol (Baker Chemical Co.), ethylene glycol (Fisher Scientific Co.), methyl alcohol (Mallinckrodt Chemical Works), and 99.84 atom percent deuterium oxide (Volk Radiochemical Co.). The elution position of chromatographed molecules was determined from the effluent refractive index*. Fig. 1 indicates that the refractive index change of sugars and alcohols with

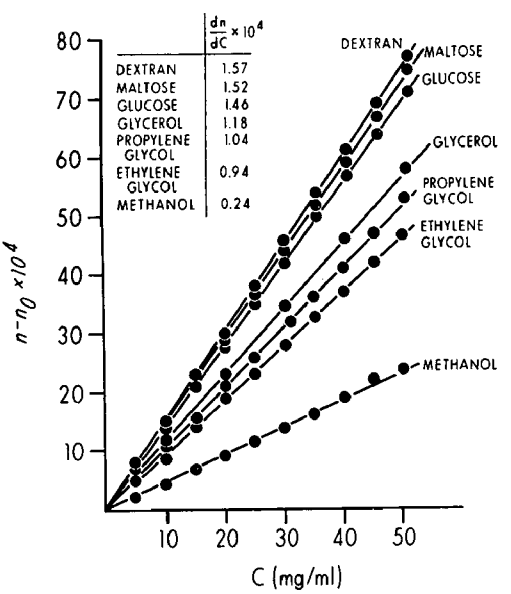

Fig. 1. Refractive index characteristics of sugars and alcohols. The refractive index change from that of the column eluent ($n-n_0$) for sugars and alcohols of various concentrations (C(mg/ml)) is linear over the concentration range $C \leqslant 50$ mg/ml. The characteristic refractive index change (dn/dC) varies with the molecular size. The data were taken from a compilation by WOLF AND BROWN[2].

* E-C Apparatus Corp., Philadelphia, Pa. Refractive index column monitor Model EC 211.

concentration (dn/dc) was relatively linear over the range $C \leq 50$ mg/ml. The refractive index monitor response was adjusted for a full-scale deflection of 35×10^{-4} refractive index units.

Elution data are presented in terms of the distribution coefficient (K_D) defined by the equation:

$$K_D = \frac{V_e - V_0}{V_i} \tag{1}$$

where V_e is the observed elution volume of the chromatographed substance, V_0 is the void volume or volume external to the gel mesh network and V_i is the accessible volume within the gel mesh.

On Sephadex gels, the void volume (V_0) was measured by the elution volume of Blue Dextran*, a high molecular weight (2×10^6 daltons) polysaccharide which is completely excluded from the gel pores. Since polyacrylamide gels bind Blue Dextran, void volume determinations were made with bovine serum albumin**.

The internal volume (V_i) was determined from the elution position of deuterium oxide. The lower refractive index[3] of deuterium oxide ($^2H_2{}^{16}O$) compared with protium oxide ($^1H_2{}^{16}O$) resulted in a negative refractive index peak which co-chromatographed with tritium oxide ($^3H_2{}^{16}O$). Internal volume (V_i) was computed as the difference between the elution volume of deuterium oxide and the void volume indicator. Resolution in terms of zone broadening was determined by measuring the standard deviation of the elution peak (ref. 1, p. 70).

Unhydrated radii of probe molecules

The molecular sieving characteristics of tightly cross-linked gels were determined from the elution volume of stachyose, raffinose, maltose, glucose, glycerol, propylene glycol, ethylene glycol and methanol. Unhydrated radii were measured from CPK*** molecular models by the method of GOLDSTEIN AND SOLOMON[4]. Geometric mean radii in Å were computed from three mutually perpendicular diameters (d_1, d_2, d_3 in cm) on molecular models in their most extended and collapsed configurations as:

$$r_g = \frac{(d_1 d_2 d_3)^{\frac{1}{3}}}{(2)(1.25)} \tag{2}$$

The unhydrated radius of a given molecule is taken as the arithmetic mean of the largest and smallest geometric mean radii determined by this method.

$$r = \frac{r_{g(\text{max.})} + r_{g(\text{min.})}}{2} \tag{3}$$

The unhydrated radius (r) was related to the molecular weight (M_W) by a function of the form[6,7]:

$$r = K\,(M_W)^n \tag{4}$$

where n and K are constants to be evaluated.

* Pharmacia Fine Chemicals, Piscataway, N.J.

** Bovine serum albumin, Fraction V, Pentex, Inc., Kankakee, Ill.

*** Corey-Pauling-Koltun space-filling models; Schwarz Bioresearch, Inc., Orangeburg, N.Y. Scale factor = 1.25 cm/Å.

TABLE I

MOLECULAR WEIGHTS AND UNHYDRATED RADII OF SELECTED SUGARS AND ALCOHOLS USED FOR COLUMN CALIBRATION

The maximum radii ($r_{g(max.)}$) and minimum radii ($r_{g(min.)}$) computed by eqn. 2 from measurements on molecular models were used to determine the unhydrated radii (r) by eqn. 3.

	M_w	$r_{g(max.)}$(Å)	$r_{g(min.)}$(Å)	r (Å)
Stachyose	667	7.30	5.69	5.78[a]
Raffinose	505	5.73	4.83	5.28
Maltose	342	4.28	5.01	4.64
Glucose	180	3.67	3.46	3.56
Glycerol	92	3.05	2.72	2.89
Propylene glycol	76	2.99	2.70	2.85
Ethylene glycol	62	2.76	2.24	2.50
Methanol	32	2.12	2.14	2.13
Deuterium oxide	20	—	—	1.53

[a] Determined by the method of Robinson and Stokes (ref. 7, p. 124).

Consideration of gel filtration models

Four mathematical models were tested for their ability to predict distribution coefficients (K_D) from molecular size (see DISCUSSION). Of these, the equations

$$K_D = a - b\,[\log_{10} r] \quad \text{and;} \tag{5}$$

$$K_D = A - B\,[\log_{10} M_W] \tag{6}$$

were used to represent the elution characteristics of test molecules.

RESULTS

Unhydrated radii of sugars and alcohols selected for column calibration

The molecular models constructed for measurement of unhydrated radii are illustrated in Fig. 2. Radii computed from eqns. 2 and 3 are listed in Table I. The radius determination for stachyose was less precise due to the flexibility of the molecular model and is indicated in Table I by the relatively large difference between the measured maximum ($r_{g(max.)} = 7.30$) and minimum ($r_{g(min.)} = 5.69$) radii. For this oligosaccharide, a radius approximation was made by extrapolation (ROBINSON AND STOKES (ref. 7, p. 124)). The unhydrated radius estimate thus obtained was 5.78 Å.

Correspondence of these molecular radii to eqn. 4 was tested by a least-squares fit of the data from Table I. The resulting equation, $r = 0.65\ (M_W)^{0.336}$ predicted the radii of this series from their molecular weights with an average error of 2.2% (Fig. 3).

Elution properties of selected calibration molecules

Elution profiles of the selected sugars and alcohols on four different columns are illustrated in Fig. 4. Concentrations of the test molecules were selected to give

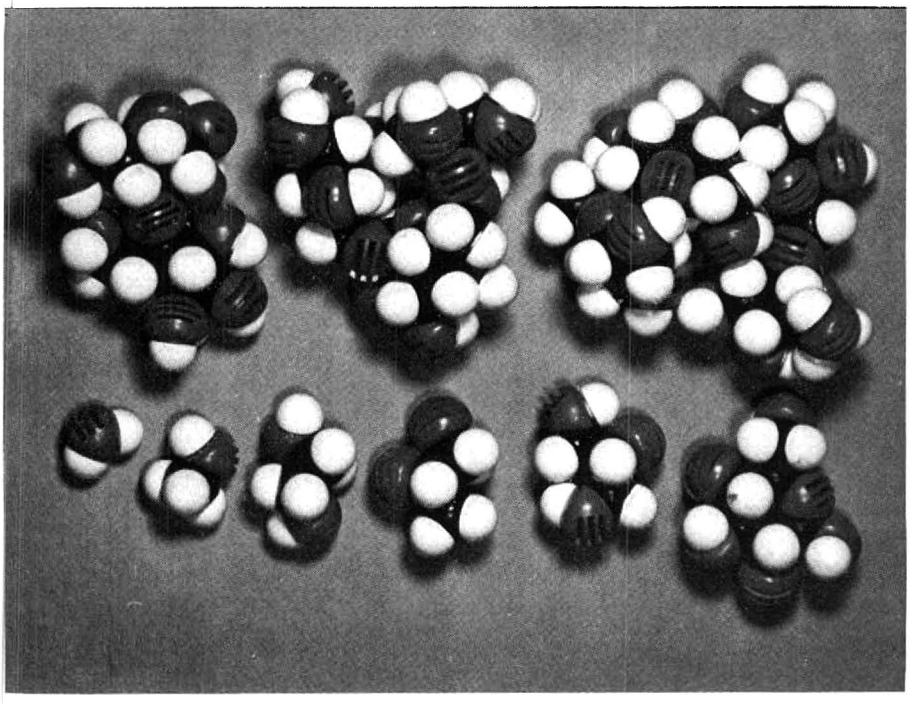

Fig. 2. CPK molecular models used to determine the unhydrated radius of calibrating molecules. In the order of their increasing size, the models represent water, methanol, ethylene glycol, propylene glycol, glycerol, and glucose (bottom row); maltose, raffinose, and stachyose (top row).

All sugars were constructed in the D form with the following configurations. Glucose: β-glucopyranose; maltose: β-glucopyranose-(1-4)-α-glucopyranose; raffinose: β-fructofuranose-(1-6)-α-galactopyranose-(1-6)-α-glucopyranose; stachyose: α-galactopyranose-(1-6)-α-galactopyranose--(1-4)-α-glucopyranose-(1-2)-fructufuranose.

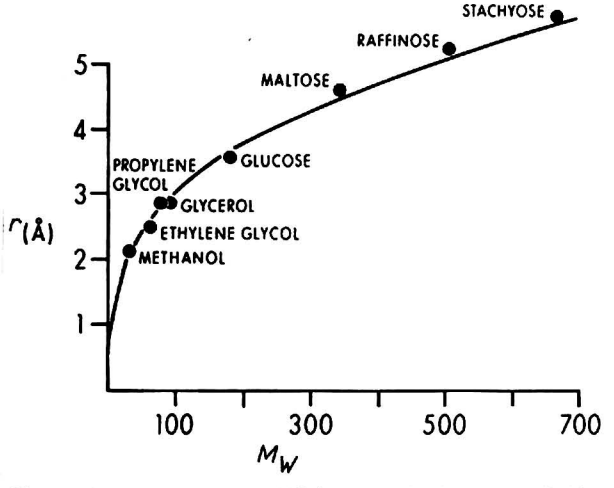

Fig. 3. Molecular weights (M_w) and unhydrated radii (r) of molecules selected for column calibration. The solid line is the function $r = 0.65\ (M_w)^{0.336}$, a least-squares fit equation from the data of Table I.

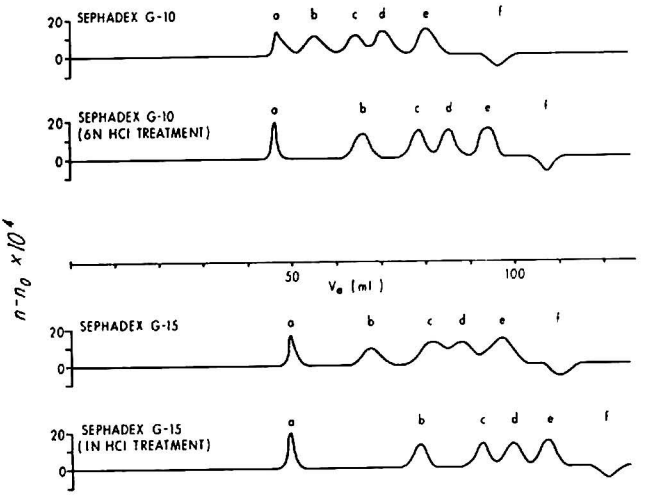

Fig. 4. Characteristic elution patterns of a mixture of test molecules on 1.5 × 85 cm Sephadex G-10 and G-15 columns. The elution volume (V_e) of each molecule was determined from the maximum refractive index change (n-n_0) of the column effluent. The samples contained: (a) Blue Dextran, 1 mg; (b) stachyose, 50 mg; (c) maltose, 50 mg; (d) glucose, 50 mg; (e) ethylene glycol, 100 μl; and (f) deuterium oxide, 900 μl; and NaCl 9 mg (eluent concentration). Of the other calibrating compounds the amounts used were: raffinose, 50 mg; glycerol, 50 μl; propylene glycol, 50 μl and methanol, 200 μl.

approximately equal maximal refractive index deflections. The results from multiple chromatographic runs utilizing the series of test molecules on each of the gel filtration media are shown in Table II. These K_D values were fitted by least squares to eqns. 5 and 6 utilizing the radii and molecular weights in Table I. The coefficients a, b, A and B of Table III define equations which describe the elution properties of the test molecules on each of the gel filtration media studied. Extrapolation of the functions to $K_D = 0$ gives an estimate of the largest molecular size which will just diffuse into the gel pores. Similarly, extrapolation to $K_D = 1$ provides an estimate of the largest molecular size that will diffuse completely into the internal volume of the gel.

DISCUSSION

The elution properties of a series of sugars and alcohols were studied on three commercially available, tightly cross-linked gel filtration media and three acid-treated dextran gels. The acid-treated Sephadex G-10 and Sephadex G-15 showed a significant improvement in chromatographic resolution. This improvement is illustrated by increased internal volumes (V_i) and smaller elution peak standard deviations ($\bar{\sigma}$). As previously reported[17], acid treatment resulted in an enlargement of the external pore aperture.

Bio-Gel P-2, the most tightly cross-linked acrylamide gel commercially available, exhibited a much larger pore size than the dextrans, Sephadex G-10 and G-15. Thus the acrylamide gel was found to be less suitable for the separation of small molecules and to possess molecular sieving characteristics similar to those of Sephadex G-25.

TABLE II

CHROMATOGRAPHIC PARAMETERS DESCRIBING THE CHARACTERISTIC ELUTION OF TEST MOLECULES ON TIGHTLY CROSS-LINKED GEL PREPARATIONS

The void volume (V_0) was taken as the elution volume of Blue Dextran and internal volume (V_i) was computed from the elution volume of deuterium oxide (N determinations). The distribution coefficients (K_D) of test molecules were computed from eqn. 1. Multiple determinations of K_D for the same molecule indicated an average standard deviation of 0.027. The mean standard deviation, $\bar{\sigma}$, of the elution peak determined from the peak width is a measure of zone broadening.

	V_0 (ml) ± S.D.	V_i (ml) ± S.D.	N	K_D								$\bar{\sigma}$ ± S.D. (N)
				Stachyose	*Raffinose*	*Maltose*	*Glucose*	*Glycerol*	*Propylene glycol*	*Ethylene glycol*	*Methanol*	
Sephadex G-10	46.6 ± 0.8	51.0 ± 1.6	3	0.17	0.24	0.35	0.48	0.60	0.67	0.67	0.80	2.17±0.23(14)
Sephadex G-10 1*N* HCl	45.5 ± 0.9	50.1 ± 0.6	3	0.13	0.21	0.32	0.53	0.58	0.67	0.67	0.80	1.83±0.16 (12)
Sephadex G-10 6*N* HCl	46.4 ± 2.3	61.4 ± 3.5	3	0.31	0.43	0.52	0.63	0.76	0.78	0.77	0.96	1.53±0.27 (11)
Sephadex G-15	48.6 ± 0.8	63.0 ± 1.9	5	0.31	0.39	0.52	0.63	0.72	0.76	0.75	0.87	2.42±0.37 (13)
Sephadex G-15 1*N* HCl	48.8 ± 1.1	72.7 ± 1.7	3	0.41	0.49	0.60	0.70	0.77	0.79	0.80	0.87	1.89±0.31 (14)
Bio-Gel P-2	46.8 ± 0.9	69.5 ± 11.0	6	0.48	0.63	0.65	0.82	0.89	0.85	0.88	0.86	2.23±0.80 (14)

TABLE III

EQUATION COEFFICIENTS, STATISTICAL MEASURES OF VARIATION AND EXTRAPOLATED VALUES DESCRIBING THE RELATIONSHIP BETWEEN ELUTION POSITION AND MOLECULAR SIZE OF TEST COMPOUNDS CHROMATOGRAPHED ON TIGHTLY CROSS-LINKED GEL MEDIA

The coefficients a and b relate to eqn. 5 and the coefficients A and B relate to eqn. 6 where K_D is the distribution coefficient, r is the average geometric mean radius, and M_W is the molecular weight of the chromatographed test molecules. σK_D is the standard error of observed diffusion constant values from the least-squares fitted equation. $\%E_r$ and $\%E_{M_W}$ are the average percent error in radius and molecular weight, respectively, for predicted K_D values from those observed experimentally.

Evaluations of each model at $K_D = 0$ gives the radius ($r(K_D = 0)$) and molecular weight ($M_W(K_D = 0)$) of a hypothetical molecule which is marginally excluded. Evaluation of each model at $K_D = 1$ gives the radius ($r(K_D = 1)$) and molecular weight ($M_W(K_D = 1)$) of the smallest molecule participating in chromatographic process.

	Equation coefficients				*Statistical measures of variation*				*Extrapolated radius and molecular weight*			
	(5)		*(6)*		*(5)*		*(6)*		*(5, in Å)*		*(6, in Daltons)*	
	a	b	A	B	σK_D	$\%E_r$	σK_D	$\%E_{M_W}$	$r(K_D=0)$	$r(K_D=1)$	$M_W(K_D=0)$	$M_W(K_D=1)$
Sephadex G-10	1.274	1.43	1.545	0.480	0.027	3.6	0.022	8.5	7.8	1.6	1,660	14
Sephadex G-10 1*N* HCl	1.322	1.53	1.618	0.517	0.040	4.5	0.041	11.3	7.3	1.6	1,340	16
Sephadex G-10 6*N* HCl	1.387	1.37	1.645	0.458	0.043	4.8	0.033	12.8	10.4	1.9	3,900	26
Sephadex G-15	1.298	1.25	1.537	0.421	0.037	5.1	0.037	15.6	10.9	1.7	4,500	19
Sephadex G-15 1*N* HCl	1.247	1.05	1.451	0.354	0.035	5.8	0.040	18.3	15.6	1.7	12,600	19
Bio-Gel P-2	1.320	1.03	1.532	0.355	0.066	11.3	0.080	39.8	18.9	2.0	20,800	32

The sugars and alcohols used in this study as calibration molecules were selected for their availability in high purity and their lack of demonstrable gel interaction. Certain molecular species were categorically excluded as test molecules because of their chemical similarity to molecules which have been demonstrated to exhibit weak gel interactions. Thus, low molecular weight aldehydes, such as glyceraldehyde and glycolaldehyde, were excluded because of an observed interaction of acetaldehyde with dextran gels. Similarly, high molecular weight aliphatic alcohols were excluded on the basis of gel interaction with *n*-butanol[10]. Inorganic electrolytes were not used because of their extensive hydration[11,12].

The use of molecular radius in this study depends on the validity of the measurement procedure described. This procedure has been used with success by GOLDSTEIN AND SOLOMON[4] to estimate the pore size of erythrocyte membranes. The measurement procedure assumes that the "true" molecular configuration lies between the two extreme configurations: $r_{g(\max.)}$ and $r_{g(\min.)}$. The CPK models are designed to approximate the space-filling characteristics of molecules from accepted values of bond angles and Van der Waal's radii of constituent atoms[5]. A principal disadvantage of the estimation of molecular size from CPK models is the degree of subjective variability possible in a single measurement which is contingent on the molecular configuration and measurement axes chosen. However, the algebraic mean between the maximum and minimum radius values observed from multiple determinations decreases subjective variability and establishes limits within which the true radius must lie.

Other methods have been used to estimate the radii of small molecules[6,7]. The radius of a sphere of equal weight and density[6] is a commonly used measure of unhydrated radius. However, the use of solid or liquid densities of pure substances in this computation is questionable. The solid or liquid density of a pure substance reflects the efficiency of packing and the intermolecular forces existing in pure substances rather than the effective density of a substance dissolved in a relatively large amount of water. Molecular radii calculated by this method exhibited poorer correlations with both molecular weight and column chromatographic elution volumes. Determination of Stokes' radii[7] from the diffusion coefficient or electrophoretic mobility requires an empirical correction for molecules with radii of less than 5 Å. The exact form of the correction is uncertain and its application to small neutral molecules is questionable[11].

Radius measurements from molecular models constitute a simple and direct method which circumvents the problem of density while incorporating the effect of molecular configuration. It has been demonstrated, here, that this measurement produces an internally consistent set of radius values which behave in the manner expected for approximately spherical molecules.

An evaluation of the correspondence of elution data to theoretical models described by eqns. 5 and 6 indicated (Table III) that under the conditions described they could be used to predict the observed diffusion constant values (K_D) within a standard deviation of approximately 0.04 from either the radius measured on molecular models or the known molecular weight. Conversely, eqn. 5 could be used to predict the measured molecular radius within an average error of about 5% and eqn. 6 could be used to predict the molecular weight within an average error of about 10%. The inferior correspondence of Bio-Gel P-2 data to these models arises largely

from the small fraction of the total gel sieving range covered by the test molecules.

Other theoretical models tested included that of ACKERS[8]:

$$K_D = \left(1 - \frac{r}{K_1}\right)^2 \left[1 - 2.104\left(\frac{r}{K_1}\right) + 2.09\left(\frac{r}{K_1}\right)^3 - 0.95\left(\frac{r}{K_1}\right)^5\right] \quad (7)$$

and that of PORATH[9]:

$$K_D = K_2\left(1 - \frac{2r}{K_3}\right)^3 \quad (8)$$

where K_1, K_2 and K_3 are fitted parameters. The model of ACKERS, based on the assumption of restricted molecular diffusion in a cylindrical pore gave poor correspondence to the data of Table II. The basic assumption of elution dependency on diffusion rate has been criticized[1], since the elution volume of a molecule is independent of the column flow rate. In addition, the poor correlation found in this study indicated a lack of general applicability of this model to predict elution values on dextran media with high cross-linking ratios. The Porath model, though accurately predicting elution positions, was found to give large values for the marginally excluded molecule ($K_D = 0$) and failed to predict the observed exclusion of inulin on Sephadex G-15. For these reasons, eqns. 5 and 6 were found to be superior to the other mathematical models tested.

Extrapolation to unity distribution coefficient gave the smallest molecular size which could participate in the chromatographic process. The diffusion constant of deuterium oxide was defined as unity, and therefore was eliminated in the curve fitting procedure so that extrapolation constituted an unbiased estimate of the internal chromatographic limit. Table III indicated that the chromatographic process on both Sephadex G-10 and G-15 extended to a limiting value imposed by the dimensions of water. With the Sephadex gels, extrapolation to unity distribution coefficient ($r(K_D = 1)$, $M_W(K_D = 1)$, Table III) gave molecular weight values close to that of monomeric water and radius values close to that measured on molecular models of monomeric water (1.53 Å). This result has an important corollary. Under the conditions of the experiment, deuterium oxide and tritiated water do not exhibit statistically significant anomolous behavior on Sephadex G-10 and G-15 in relation to the sugars and alcohols used as test molecules, indicating that these labeled water species behave as monomers.

The use of isotopically labeled water for the determination of internal volume has been criticized by some authors[10,12]. MORSI AND STERLING[13] have demonstrated that purified starch completely exchanges hydroxyl hydrogen with deuterium oxide after one hour of equilibration. MARSDEN[10] has suggested that a correction for the distribution constant determined by tritiated water should be used to account for this phenomenon. A value of 5.9% was given for Sephadex G-25 based on its epichlorhydrin cross-linking ratio. Cross-linking ratios for Sephadex G-10 and G-15 have not been determined[14]. However, the close correspondence of the observed elution values of deuterium oxide and tritiated water with that expected theoretically indicates that if this exchange occurs the effect does not significantly alter the elution properties of isotopically labeled water in this chromatographic system.

The question of the form in which water exists within dextran gel pores has been raised by YOZA AND OHASHI[15]. There is a large literature concerned with the

existence of polymeric aggregates of water in certain physical and biological systems[16]. The large size of water aggregates within the gel pores would result in an earlier apparent elution. Close correspondence of the internal chromatographic limit to the dimensions of monomeric water is consistent with the hypothesis that water exists in a completely dissociated form within the gel pore.

Since the processes of hydrogen exchange and water aggregation have opposing effects, it cannot be ruled out that both phenomena occur simultaneously, resulting in a cancellation of their chromatographic results. Neither can the possibility be ruled out that all test molecules are significantly hydrated in solution resulting in an uniform additive increase in radius. However, if hydration of these molecules occurs, the degree of hydration must be small. ROBINSON AND STOKES (ref. 7, p. 306) have suggested that glycerol may move with one molecule of water. GOLDSTEIN AND SOLOMON[4] has commented that the glycols move through solution more slowly than expected, although he was unable to differentiate between chemical hydration effects and hydrodynamic drag. In the absence of evidence for significant hydration of sugars and alcohols in solution, it may be concluded that the radii measured for the calibrating molecules are internally consistent and reasonable estimates of their effective molecular sizes.

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CHROM. 5054

CHROMATOGRAPHY OF INORGANIC IONS ON THIN LAYERS OF PROTEIN

P. R. BRADY AND R. M. HOSKINSON
C.S.I.R.O. Division of Textile Industry, Belmont, Victoria 3216 (Australia)

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SUMMARY

The preparation of thin layers of natural and chemically modified keratin from wool cortical cells is described. The R_F values of a number of cations and anions on these thin layers have been measured. The migration of these ions is interpreted in terms of ion exchange with the protein substrate.

INTRODUCTION

There has been considerable interest in the ion-exchange chromatography of inorganic ions on both inorganic and synthetic organic substrates of various types. The purpose of this work is to describe the chromatography of a number of cations and anions on thin layers of a naturally occurring protein, wool keratin, and to demonstrate the way in which the chromatographic behaviour of this material may be altered by chemical modification.

In its natural state, wool is a complex protein which contains a number of chemically reactive groups; these include hydroxyl, amino, amide, carboxyl, sulphydryl and disulphide groups. The amino groups are derived from arginine, lysine and histidine and the carboxyl groups from glutamic and aspartic acids. By titration and analysis, wool has been shown to have acid and base binding capacities both of about 0.8 mequiv./g[1]. These capacities compare favourably with those of many synthetic ion-exchange resins and it might be expected that wool keratin should exhibit the properties of an amphoteric (weak acid, weak base) ion exchanger. Wool has in fact been shown to have ion-exchange capability[2] but because of its fibrous form, it has not been especially convenient or effective for use in chromatographic procedures. Recently, wool has been prepared in a form more suitable for chromatography by rendering fibres into component cortical cells; these are spindle shaped cell residues (approx. 100 × 4 μm) consisting almost entirely of insoluble protein and thin layers and columns have been prepared from them[3]. Since wool can be readily esterified and deaminated, it is also possible to prepare thin layers of cortical cells which exhibit predominantly weak base or weak acid ion-exchange capability.

Previously, cations and anions have been separated on thin layers of resin

granules bound with cellulose and calcium sulphate[4] and on paper that had been impregnated with finely powdered ion-exchange resins[5]. Inorganic exchangers, such as insoluble salts of the tetravelent metals zirconium and titanium, have also been used both as thin layers[6] and impregnated papers[7]. Again, paper containing absorbed liquid anion exchangers has been employed for the separation of cations[8]. While unmodified cellulose is of little value as an ion exchanger because of its low capacity[9] considerable use has been made of modified cellulose containing, in particular, strong acid or basic groups. The only work specifically on weak exchangers appears to be that reported by WIELAND AND BERG[10] and LEDERER[11] who have studied the migration of cations on carboxylic exchange papers.

EXPERIMENTAL

Preparation of cortical cells

The following enzymic digestion is essentially that of BLACKBURN[12]. Commercially scoured wool of 64s quality (60 g) was digested at 65° for 3 h in an aqueous solution (2 l), containing sodium bisulphite (20 g) and papain (20 g), which had been adjusted to pH 6.5 by addition of 1 *M* sodium hydroxide solution. Prolonged digestion led to an increased proportion of small particles which when incorporated into layers, markedly decreased the rate of development of chromatograms, particularly with aqueous solvents. After digestion of the fibres, cortical cells were recovered by filtration and then washed with distilled water (3 × 500 ml). The cells were then suspended in distilled water (300 ml) and the pH of the solution was adjusted to 3.0 by the careful addition of 6 *M* hydrochloric acid. The cells were heated in this suspension at 75° for 30 min, filtered, washed and stored in distilled water containing a little chloroform to inhibit growth of micro-organisms. The acidification and washing procedures were required to remove sodium bisulphite.

Chemical modification of cortical cells

Esterification. Oven-dried (100°, 1 h) cortical cells (9 g) were stirred in methanol (200 ml) containing concentrated hydrochloric acid (2 ml) and heated under reflux for 8 h. The cells were collected on a sintered glass filter and suspended in distilled water (100 ml). The pH of this slurry was adjusted to 6.5 by the addition of 1 *M* sodium hydroxide solution and checked from time to time during 0.5 h. The cells were then collected, rinsed with methanol and stored under methanol.

Deamination. Moist cortical cells (5 g) were suspended in 3.7 *M* sodium nitrate solution (165 ml) containing glacial acetic acid (35 ml) and stored in a lightly stoppered flask at room temperature for 24 h. The cells were then distinctly yellow. They were collected by filtration, washed with distilled water (4 × 300 ml), filtered and formed into layers immediately.

Preparation and properties of thin layers

Moist cortical cells (14 g) were suspended in 100 ml of an ethanol–water mixture by high speed homogenisation. For unmodified cells, an ethanol–water proportion of 1:1 was used whilst for esterified and deaminated cells it was 7:3. The composition of these mixtures had a considerable influence on the viscosity of the resulting slurry and this ultimately affected the ease of layer formation. After standing for a

few minutes to allow bubbles to escape, the slurry was layered on to glass plates using the Desaga* apparatus set at 0.4 mm. The layers were allowed to dry at room temperature for at least 16 h. When dry, the layers were extremely robust and rigidly adhered to the glass plates. A micrograph of the surface of a cortical cell layer taken with a scanning electron microscope is shown in Fig. 1. It can be seen that many cortical cells have fragmented during digestion and that subsequent intertwining of filamentous structures in drying accounts for the excellent cohesion of the layers.

Keratin layers could be removed from glass surfaces and recovered intact after brief immersion (1–2 min) of the plate on which they were formed in hot water (70°). After drying, the separated keratin film had a papery texture and retained its characteristic strength and flexibility. Fig. 2 shows such a keratin film.

Pretreatment of layers

Each layer was wet out and allowed to stand for at least 4 h in the solution

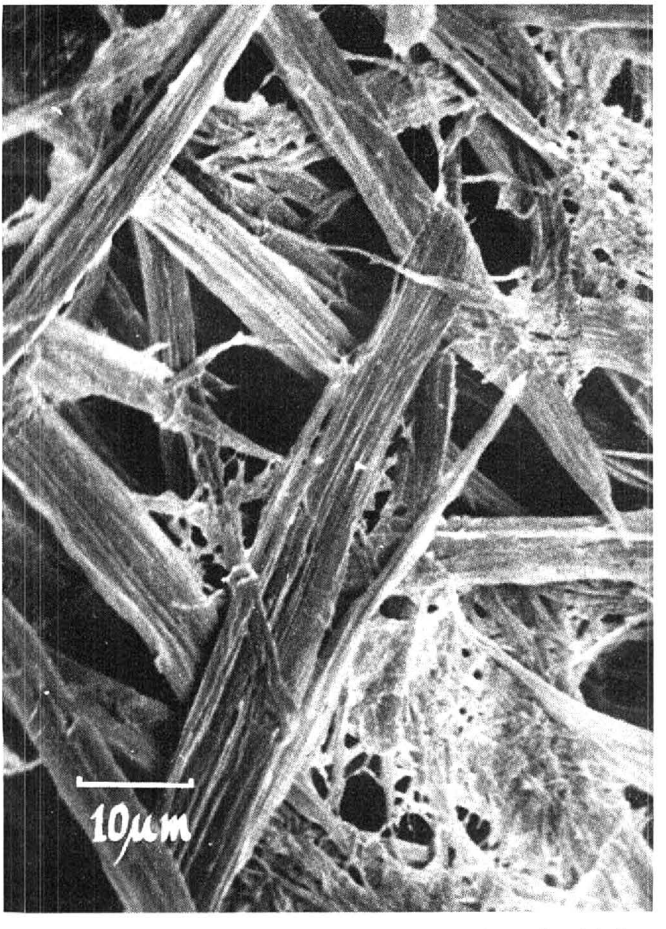

Fig. 1. Scanning electron micrograph of the surface of a thin layer of wool cortical cells.

* C. Desaga, G.m.b.H., Hauptstrasse 60, Heidelberg, G.F.R.

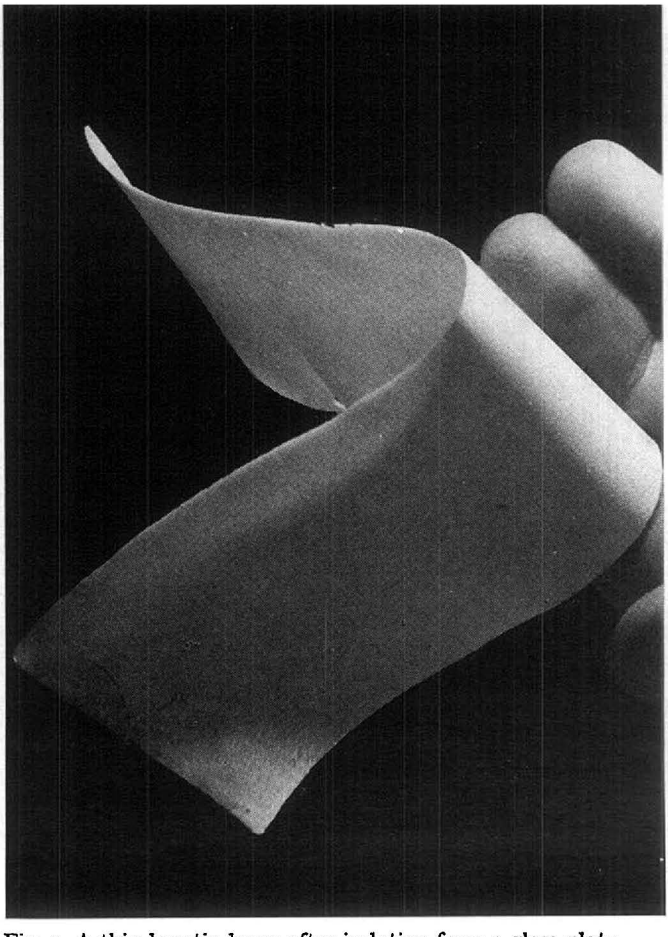

Fig. 2. A thin keratin layer after isolation from a glass plate.

(1000 ml) which was subsequently used for chromatographic elution. In this manner the pH of the keratin layer was equilibrated with that of the eluant. In order to minimise differences of pH of the cortical cells and the solutions, which may arise by the Donnan effect[13], the ionic strength of all the solutions used was at least 0.2. The internal cohesion of the layers prevented any disintegration during this soaking treatment. After equilibration, plates were rinsed by dipping once in distilled water and then allowed to drain and dry at room temperature. When dry, the layers were trimmed by removing a strip of cells around the edges of the plates and, as required, the layers were divided into a number of narrow segments simply by scoring channels in the layers with a narrow chisel. Substances could be chromatographed on individual segments of the layer. Layers were sufficiently robust for the starting points to be marked with a soft lead pencil.

Solutions of cations and anions

The concentration of salt in spotting solutions of the cations and anions was

20 mg/ml. Chlorides or nitrates of cations and sodium or potassium salts of anions were used. All chemicals were AR grade.

Development and visualisation of chromatograms

After spotting with the salt solution (1 μl), the chromatograms were developed in the ascending mode using normal techniques. After development, the liquid front was marked in pencil, the chromatograms were dried in air and the ions visualised by means of spray reagents. Not all reagents in common use are suitable for keratin layers. Those found successful are listed in Table I. Particular applications are given

TABLE I

SPRAY REAGENTS SUITABLE FOR USE ON KERATIN LAYERS

Reagent	*Composition*[a]
1	1 *M* Ammonia solution saturated with hydrogen sulphide
2	0.5% 8-Hydroxyquinoline in 60% aqueous ethanol. Layers were exposed to ammonia vapour after spraying and examined under UV light
3	0.2% Quinalizarine in ethanol
4	0.2% Aqueous aluminon
5	5% Pyrogallol in 5 *M* aqueous ammonia
6	0.1% Diphenylcarbazide in ethanol
7	0.4% Ammonium molybdate in 8% nitric acid followed by (8) after drying
8	1% Stannous chloride in 10% hydrochloric acid
9	1% Aqueous ferrous sulphate
10	1% Aqueous ferric nitrate
11	5% Potassium iodide in 2 *M* hydrochloric acid
12	Fresh aqueous solution containing 3% silver nitrate and 0.3% fluorescein. After spraying spots were developed by exposure to UV light

[a] All percentages are w/v.

in Tables II and III. Most ions were run singly on strips and it was possible to spray each strip individually by masking the rest of the plate. Substances requiring different visualising reagents could therefore be chromatographed on the same plate.

Unless otherwise stated, the ions migrated as discrete spots. The R_F values quoted in the tables are arithmetic means of at least three different determinations. In no case was the total variation in R_F greater than $\pm$ 4%.

RESULTS AND DISCUSSION

Cations

The R_F values of a number of cations obtained with phthalate buffers on normal and deaminated wool plates are shown in Table II. On unmodified keratin, all the metal ions were found to be more mobile at pH 4 than at pH 5. This is consistent with ion exchange of cations on carboxylate groups in the keratin. The pK_a of carboxyl groups in wool is of the order of 4[1] and it follows that at pH 4 about 50% of these groups will be protonated and unavailable for cation exchange. Therefore the proportion of cations bound at any time will be less than at pH 5 when all carboxyl groups are ionised. As R_F values are proportional to the ratio of concentrations

TABLE II

R_F VALUES OF CATIONS ON THIN KERATIN LAYERS

For spray reagents see Table I.
The solvent buffers were: (A) phthalate buffer, pH 4.0, ionic strength 0.2; (B) phthalate buffer, pH 5.0, ionic strength 0.2; (C) 0.2 *M* KNO_3 adjusted to pH 5.0 with nitric acid.

Cation	*Spray reagent*	*R_F values*			
		Unmodified keratin			*Deaminated keratin*
		A	*B*	*C*	*B*
Ag^+	1	×[a]	×	0.03T	—
Pb^{2+}	1	×	×	0.04T	—
Hg^{2+}	1	×	×	0.00	—
Fe^{3+}	1	×	×	0.00	—
Cu^{2+}	1	0.09T	0.04T[b]	0.05T	—
Cd^{2+}	1	0.59	0.49	0.22	0.16
Co^{2+}	1	0.72	0.63	0.47	0.22
Ni^{2+}	1	0.78	0.62	0.43	0.28
Mn^{2+}	2	0.84	0.74	0.49	0.32
Zn^{2+}	2	0.64	0.40	0.28	0.17
Cr^{3+}	2	0.47	0.30	0.20	—
Mg^{2+}	3	0.91	0.90	0.80	0.56
Al^{3+}	4	0.52	0.28	0.15	0.06T
Ca^{2+}	5	0.90	0.82	0.65	0.44
Sr^{2+}	5	0.89	0.82	0.63	0.44
Ba^{2+}	5	0.84	0.80	0.64	0.39
Sn^{4+}	6	0.05	0.04	0.02	—

[a] × Indicates that these cations precipitate in phthalate buffer.
[b] T Indicates tailing of spots.

of species free in solution to those bound on the exchanger[14] higher R_F values would be expected at pH 4 than at pH 5.

Several cations precipitated in the phthalate buffer, so R_F values were also measured in 0.2 *M* KNO_3 solution that had been adjusted to pH 5 by addition of dilute nitric acid. The R_F values of all cations that were mobile in phthalate buffer were found to be lower in the presence of potassium nitrate although their relative order of migration was not appreciably affected.

The migration of many of the metal ions was similar to that observed by LEDERER[11] on carboxymethyl cellulose paper (Whatman CM 50). This is illustrated in Fig. 3 in which the R_F values of the cations in phthalate buffer at pH 5.0 on unmodified keratin layers are plotted against LEDERER's values for the same metal ions in acetate buffer at pH 4.75 (0.5 *M* acetic acid, 0.5 *M* sodium acetate). A linear correlation between the R_F values on the two types of substrate is evident for all but Hg^{2+}, Ag^+, Cu^{2+} and the alkaline earth metals. Both Hg^{2+} and Ag^+ were strongly retained by keratin probably because they reacted with cystine disulphide bonds[15] which are plentiful in the cells*. Because of its tailing, the Cu^{2+} ion would also appear to react

* Native wool keratin has a sulphydryl content of about 35 μmoles SH (as cysteine) per g dry wool but this value can be increased to 500 μmoles SH per g by treatment with appropriate reducing agents. Measurements of the sulphydryl and disulphide content of the cells by the polarographic method of LEACH[17] indicated that they contained 80 μmoles of cysteine per g and 420 μmoles of cystine per g.

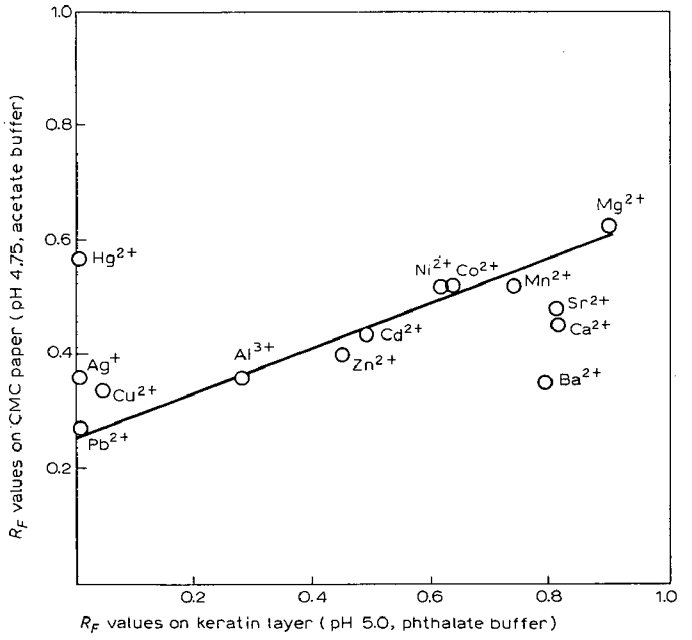

Fig. 3. Comparison of R_F values of metal ions on carboxymethyl cellulose paper in acetate buffer at pH 4.75 and on keratin in phthalate buffer at pH 5.0.

with wool to some extent. Ca^{2+}, Sr^{2+} and Ba^{2+} were not as strongly retained by keratin as by carboxymethyl cellulose and were not well resolved.

As can be seen from Table II, cations were more strongly retained by deaminated than by native keratin. The positively charged groups normally present in wool at values of pH less than 9 are removed by deamination leaving only carboxylate groups to interact with the cations.

It is important to bear in mind that because the buffer solutions contained concentrations of Na^+ and K^+ far in excess of the chromatographed ions the latter must have possessed greater affinity for keratin than either Na^+ or K^+; otherwise the chromatographed ions would have run up in the solvent front. In fact, DELMENICO AND PETERS[16] have shown that the sodium ion has little or no measurable affinity for wool and this is presumably true of the potassium ion.

Anions

The hydrogen phthalate anion was quite strongly bound on keratin layers because, of the anions examined in phthalate buffers, all but the dichromate anion (R_F 0.31) migrated close to the liquid front. The chromatography of anions was therefore studied only in the presence of chloride ion which has been shown to have little affinity for wool[16].

The R_F values of a number of anions on unmodified keratin at two different pH's are shown in Table III. The R_F values at pH 5.0 are in every case greater than at pH 2.5. At pH 5.0 the anions are adsorbed on the cationic protonated amino groups

TABLE III

R_F VALUES OF ANIONS ON THIN KERATIN LAYERS

For spray reagents see Table I.
The solvent buffers were: (A) HCl–KCl buffer, pH 2.5, ionic strength 0.2; (B) 0.2 *M* KCl adjusted to pH 5.0 with HCl.

Anion	*Spray reagent*	*R_F values*		
		Unmodified keratin		*Esterified keratin*
		A	*B*	*B*
$H_2PO_4^-$	7	0.72	0.85	0.78
$H_2P_2O_7$	7	0.30	0.81	0.45
$H_2PO_3^-$	7	0.65	0.90	0.76
$H_2PO_2^-$	7	0.65	0.88	0.73
SeO_3^{2-}	8	0.15T[a]	0.92	—
AsO_2^-	1	0.89	0.78	0.87
$Fe(CN)_6^{3-}$	9	0.05	1.0	0.07
$Fe(CN)_6^{4-}$	10	0.06	1.0	0.08
IO_3^-	11	0.15T	—	—
BrO_3^-	11	0.45	0.87	0.56
$Cr_2O_7^{2-}$	2	0.05	0.35	0.04
CNS^-	10	0.18	0.52	0.18
I^-	12	0.25	0.62	0.26
Br^-	12	0.39	0.78	0.41

[a] T Indicates tailing of spots.

but the concentration which can be bound is less than at pH 2.5 when the carboxylate groups are protonated and uncharged.

The R_F values of anions on unmodified wool at low pH are similar to those on esterified wool at pH 5.0 as shown in Table III. In both cases the carboxyl groups are uncharged and the keratin behaves exclusively as an anion exchanger.

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CHROM. 5055

CHROMATOGRAPHY OF DINITROPHENYLAMINO ACIDS AND HETEROCYCLIC BASES ON THIN LAYERS OF PROTEIN

P. R. BRADY AND R. M. HOSKINSON
C.S.I.R.O., Division of Textile Industry, Belmont, Victoria 3216 (Australia)

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SUMMARY

The properties of thin layers of protein prepared from wool cortical cells are further investigated. Thin-layer chromatographic separations of dinitrophenylamino acids and of certain heterocyclic compounds (substituted purines and pyrimidines) are described.

INTRODUCTION

Recently[1] we reported that wool cortical cells, the product of enzymic digestion of keratin fibres, could be used to form thin layers of protein that were suitable for chromatography. The potential of such layers in resolving certain types of dyestuff was outlined. Wool protein has free side-chain amino, carboxyl, hydroxyl, disulphide and sulphydryl groups[2]; it could be expected that chemical modification of these would cause subtle variations in chromatographic behaviour. This expectation has been confirmed[3] in studies of the ion-exchange properties of both native and modified keratin. The present study of dinitrophenyl (DNP) amino acids and heterocyclic bases (purines and pyrimidines) was undertaken to extend the range of substances chromatographed on thin layers of protein and to further compare the properties of unmodified and methyl-esterified cortical cells.

Thin-layer chromatographic (TLC) procedures on other supports have been described for DNP amino acids[4–9] and heterocyclic bases derived from nucleic acids[10–14].

EXPERIMENTAL

Materials

Cortical cells were isolated, methyl-esterified and formed into thin layers as described[3]. The dinitrophenylamino acids were obtained from the Sigma Chemical Company.

Chromatographic procedure

All layers were eluted in the relevant solvent and then air-dried at least once, before chromatographing the test substances. This procedure ensured the removal

of impurities (especially from layers of unmodified cells) that migrated as a broad band in the solvent front. Failure to observe this precaution resulted in poor resolution and irregular solvent fronts. All development was effected in closed tanks containing wicks to provide solvent-saturated vapour. Depending on their solubility, the DNP amino acids were applied from either aqueous or ether solutions. Preliminary spotting of the plates with ethanol facilitated subsequent application of aqueous solutions.

The DNP amino acids were detected either visually or, particularly with esterified cells where background absorption was low, by inspection under UV light (254 nm or 366 nm sources). The purines and pyrimidines were detected only with the UV lamp. The R_F values were measured after a single development of 12 cm.

RESULTS AND DISCUSSION

Dinitrophenylamino acids

Originally we had hoped to study the chromatography of amino acids on the protein support and to obtain data on amino acid interactions with protein. However, it was found that visualisation of the amino acids on cortical cell layers was subject to interfering reactions between the support and common chromogenic reagents. Consequently the coloured and readily observable DNP amino acids were employed. Of several solvents described for the TLC of DNP amino acids[4] only the base-containing toluene system (S_3 here) was found suitable for use with cortical cell layers. In this work an additional acidic (S_1) and a basic (S_2) solvent were established.

Table I records the R_F values of some DNP amino acids on layers prepared from unmodified and methyl-esterified cortical cells with various solvent systems. On both substrates the range of R_F values suggests that these solvents would be suitable for analytical purposes. With the exception of N-DNP-L-arginine, N,S-di-DNP-L-cysteine, N,N'-di-DNP-L-ornithine and N,O-di-DNP-L-tyrosine, the DNP amino acids migrated in approximately the same order irrespective of differences in support and developing solvent. Nevertheless, with S_1, R_F values were significantly higher on esterified cells indicating decreased affinity of the DNP amino acids for the modified support.

Solvent S_3 has been widely used in the thin-layer chromatography of DNP amino acids on silica and its use with methyl-esterified keratin layers permits comparison of the two types of layer. The R_F values on the modified keratin were lower than published[4] values for silica chromatography, although the relative order of migration was essentially the same. Significant exceptions were N-DNP-L-tryptophan and N,N'-di-DNP-L-histidine. Relatively, the former compound was more firmly retained and the latter more rapidly eluted on keratin than on silica. It is noteworthy that the aromatic amino acid derivatives N-DNP-L-tryptophan and N-DNP-L-phenylalanine which were poorly separated on silica gel[4] were better resolved on the protein layer.

In comparing the relative effects of the acidic (S_1) and basic (S_2) solvents, used with unmodified cortical cells, several patterns were observed:

(*i*) Most compounds had R_F values which were slightly higher (uniformly about 10%) in S_2 than those in S_1.

(*ii*) The bis-DNP derivatives (N,S-di-DNP-L-cysteine; N,N'-di-DNP-L-histi-

TABLE I

R_F VALUES OF SOME DINITROPHENYLAMINO ACIDS ON THIN PROTEIN LAYERS

R_F values are means of at least four determinations. Solvent systems are: S_1 = *n*-butanol–water–glacial acetic acid (30:20:10); S_2 = *tert.*-amyl alcohol–0.88 ammonia (50:10); S_3 = toluene–pyridine–ethylene chlorhydrin–0.88 ammonia (100:30:60:60), this is the well known "toluene system" which separates into two phases. Only the upper phase was used. Pretreatment of the layers with the lower phase did not influence the R_F values subsequently obtained with the top phase.

DNP amino acid	*R_F values × 100*			
	Unmodified cortical cells		*Methyl-esterified cortical cells*	
	S_1	S_2	S_1	S_3
N-DNP-D,L-α-amino-*n*-butyric acid	49	58	70	33
N-DNP-D,L-α-aminocaprylic acid	87	84	87	57
N-DNP-L-arginine	92	28	86	20
N-DNP-L-asparagine	23	30	42	8
N-DNP-L-aspartic acid	14	7	34	1
N-DNP-L-cysteic acid	1	4	1	1
N,S-di-DNP-L-cysteine	9	72	16	42
N,N′-di-DNP-L-cystine	1	s[b]	1	s[b]
N-DNP-D,L-glutamic acid	24	10	49	2
N-DNP-L-glutamine	26	32	44	9
N-DNP-glycine	27	38	48	19
N,N′-di-DNP-L-histidine[a]	47	66	84	40
N-DNP-L-isoleucine	71	69	83	37
N,N′-di-DNP-L-lysine	38	67	48	52
N-DNP-D,L-methionine	44	65	60	36
N-DNP-D,L-norleucine	64	74	82	41
N-DNP-D,L-norvaline	59	68	76	39
N,N′-di-DNP-L-ornithine[a]	29	55	40	46
N-DNP-L-phenylalanine	43	61	67	39
N-DNP-L-proline	45	41	69	24
N-DNP-L-threonine	35	47	56	18
N-DNP-L-tryptophan	45	58	58	25
N,O-di-DNP-L-tyrosine	40	82	48	64
N-DNP-L-valine	62	67	80	34

[a] These substances tended to elongate with solvent S_1 and unmodified cells.
[b] "s" indicates that this substance streaked badly.

dine; N,N′-di-DNP-L-lysine and N,O-di-DNP-L-tyrosine) all had significantly higher R_F values in the basic solvent (S_2). Apparently this behaviour is due to increased lipophilic properties conferred by a second dinitrophenyl ring.

(*iii*) The aspartic and glutamic acid derivatives had slightly lower R_F values in S_2. With the exception of N,N′-di-DNP-L-cystine (see *v*) these were the only dicarboxylic acids studied.

(*iv*) N-DNP-L-arginine was the only compound which showed a large decrease in R_F value in the basic solvent (S_2). Unlike most of the other DNP amino acids, the arginine derivative has a free basic group; its guanidino function, in the protonated form, can associate with keratin carboxylate anions and, presumably, it is this effect which causes the lower R_F value in S_2.

(*v*) N,N′-di-DNP-L-cystine was immobile in S_1, and streaked badly in S_2. This effect was not due to poor solubility in S_1 or S_2 and it contrasts with N,S-di-DNP-L-

cysteine which chromatographed normally. The possibility that this disulphide has reacted chemically with the sulphydryl groups of the protein substrate requires examination.

Bidimensional separations

Protein layers can be used to effect bidimensional separations. For this purpose, unmodified cell layers were most suitable because they gave the sharpest resolution of compounds. Of the two solvents studied with unmodified cells, S_1 provided the most compact spots and was therefore used first (normal to the layering direction), followed by S_2. Fig. 1 represents a tracing of an actual bidimensional resolution of

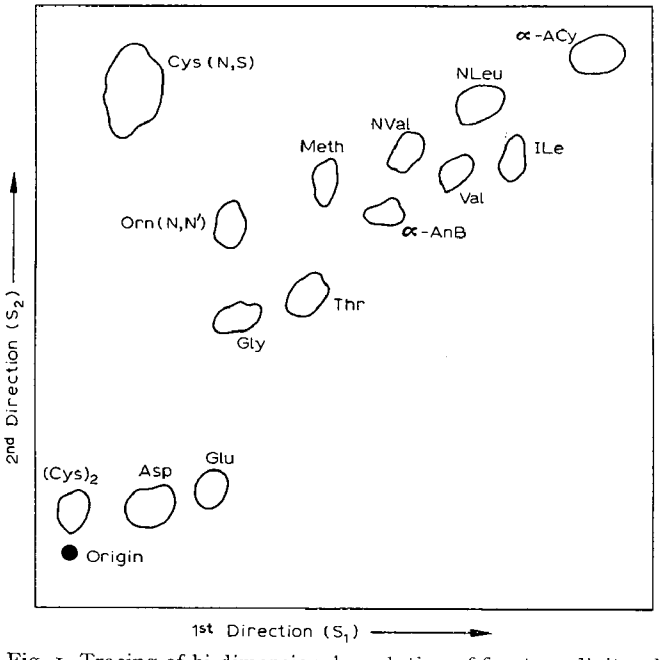

Fig. 1. Tracing of bi-dimensional resolution of fourteen dinitrophenylamino acids. 1st dimension (normal to layering direction), solvent S_1; 2nd dimension, solvent S_2.

fourteen selected compounds with single developments of 10 cm in each direction. It is clear that satisfactory finger-printing was obtained on the layers.

Pyrimidines and purines

Because layers of methyl-esterified cortical cells are slightly fluorescent, detection of pyrimidines and purines under UV light is more satisfactory than on layers of untreated cells. Esterified layers were therefore selected for chromatography of heterocyclic compounds and Table II records the R_F values of a number of pyrimidine and purine derivatives. The substances chosen are commonly encountered as synthetic intermediates. With both types of heterocyclic compound a neutral solvent (S_4) was found which provided satisfactory resolution. The acidic solvent (S_5) which resolved the pyrimidine derivatives proved ineffective for the separation of purines.

TABLE II

R_F VALUES OF PYRIMIDINES AND PURINES ON METHYL-ESTERIFIED PROTEIN LAYERS

R_F values are means of three determinations. Solvent systems are :S_4 = *n*-butanol saturated at 25° with water; S_5 = *n*-butanol–water–glacial acetic acid (70:20:10).

Compound	*R_F values × 100*	
	S_4	S_5
Pyrimidine		
4-Amino-5-bromo-	83	91
4-Amino-5-bromo-2-hydroxy-	57	74
4-Amino-2,6-dihydroxy-5-nitro-	3	34
4-Amino-2-mercapto-	55	67
4,5-Diamino-	66	77
4,5-Diamino-2,6-dimercapto-	10	45
4,5-Diamino-6-hydroxy-	57	70
4,5-Diamino-6-hydroxy-2-mercapto-	28	61
4,5-Diamino-6-mercapto-	51	67
4,5-Diamino-6-methylmercapto-	77	85
4,6-Dihydroxy-5-nitro-	4	10
2-Hydroxy-4-mercapto-	63	72
Purine		
2-Amino-	74	
6-Amino-	59	
2-Hydroxy-	34	
6-Mercapto-	60	
8-Mercapto-	68	
Purine	81	
8-Azaadenine	52	

Even though S_5 is, generally, a non-ionising solvent, to properly understand the significance of the R_F values of the pyrimidines in acidic media, information about the formation of ionic species is required. Data of this kind are not available, nevertheless, some comments on the influence of chemical structure on R_F are possible. In the 4,5-diaminopyrimidine series, further substitution with groups capable of forming hydrogen bonds (OH and SH) produced compounds whose R_F values were lowered; the reduction depended on the type and number of substituting groups. Conversely, a decrease in the number of substituent groups capable of forming hydrogen bonds (as in the replacement of the 5-amino function of 4,5-diamino pyrimidine with bromine) had the effect of increasing the R_F value. The introduction of a methylmercapto function, which presumably decreased hydrophilic character also resulted in an increased R_F value. These trends were apparent with both solvents although the acidic system (S_5) furnished higher R_F values than the neutral system (S_4). The two highly polar dihydroxy-5-nitropyrimidines migrated to an useful degree with system (S_5) only. The substituted purines chromatographed in a manner similar to the pyrimidines. The introduction of hydrogen-bonding groups (OH, SH, NH_2) produced derivatives whose R_F values were lower than the parent compounds.

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CHROM. 5037

PROBLEMS IN WATER ANALYSIS FOR PESTICIDE RESIDUES*

A. BEVENUE, T. W. KELLEY AND J. W. HYLIN
Department of Agricultural Biochemistry, University of Hawaii, Honolulu, Hawaii 96822 (U.S.A.)

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SUMMARY

Organic solvents, glassware, plastic ware, cellulose extraction thimbles, filter paper, and silica gels may contribute contaminants to water samples which may interfere with the subsequent gas chromatographic analysis of the samples for pesticides in the parts per billion range. Prior to their use, heat treatment of the glassware and the silica gels is recommended to eliminate contaminants contributed by these materials. Plastic ware and filter paper should not be included in the analytical procedure.

INTRODUCTION

The examination of waters for pesticides has included samples of potable waters, fresh water streams, lakes, rivers, the oceans, and even sewage outfall areas. Without doubt, there will be non-pesticide chemicals in some samples that will possess analytical characteristics similar to some pesticides when they are examined by electron capture gas chromatography. In addition, false data may be acquired from extraneous sources during the manipulation of the samples in the analytical laboratory which will not be eliminated by confirmatory techniques, such as thin-layer chromatography (TLC), unless certain precautionary measures are taken prior to the analysis of the samples.

This report reviews some of these problems and also brings to the reader's attention several areas of analytical interferences which, to our knowledge, have not been specifically mentioned heretofore in the literature. The experienced analyst may be aware of these problems. However, with the increased interest in environmental studies coupled with the required establishment of many new laboratories possibly staffed with personnel inexperienced in trace analysis techniques, this report may aid the analyst in avoiding some unforeseen problems in the analysis of waters for pesticides.

When large samples of water (five or more gallons) are extracted for analysis and the extract is concentrated to a small volume, the suspected pesticide(s) in the water may be confirmed by TLC and spray reagent techniques if the pesticide residue

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in the extract is in the microgram range. However, the trend toward the use of small grab samples (one gallon or less) and the desire to find and report residues in the part per trillion, or fraction thereof, range eliminates the possibility of using the spray technique for verification, because of the detectability limits of the stain reagent. Under the latter conditions, the area of the developed TLC plate containing the suspected pesticide is eluted with a suitable organic solvent and the concentrated extract therefrom is again subjected to gas chromatographic analysis. Extraneous interferences are magnified on the recorder chart unless (1) special precautions are taken with the organic solvents, the glassware, and other equipment used in the analytical procedure and (2) the thin-layer adsorbent is completely free from organic contaminants.

EXPERIMENTAL

Organic solvents

Organic solvents of "reagent grade" quality cannot be used for pesticide residue analysis in the nanogram–picogram range, because of contaminants in the solvent which will be magnified on the gas chromatograph recorder chart when the final concentrated extract is applied to the gas chromatograph. It is inexcusable to use such reagents in the analytical procedure since high-purity solvents are now commercially available. If necessary, reagent-grade solvents should be redistilled in an all-glass system; however, the redistilled reagent should be checked before use.

Glassware and other equipment used prior to TLC and GC analysis of the water sample

LAMAR *et al.*[1] recommended heating all glassware, except volumetric ware, overnight at 300° prior to use with water samples. The volumetric glassware was cleaned with a solution of sodium dichromate in concentrated sulfuric acid. They also warned against the use of rubber, cork, or plastic stoppers for water sample containers. The Federal Water Pollution Control Administration water analysis manual[2] recommended heating the glassware at 400° if the type and size of glassware permitted such drastic treatment. AMOS[3] reported variable results—some satisfactory, some poor—when glassware was soaked in acid or base solutions or detergent solutions.

Plastic tubing used in vacuum equipment to remove sections of the silica powder from TLC plates have contributed organic contaminants to the powder[3,4].

Soxhlet extraction thimbles (Whatman cellulose) contain substances which will produce pseudo-pesticide peaks on the gas chromatogram unless the thimbles are solvent-extracted prior to use[3,5].

The following additional precautionary measures are suggested based on studies conducted in our laboratory. The glass jars used for developing the TLC plates may not tolerate the stress of heat treatment; therefore, sodium dichromate–sulfuric acid solution should be applied to the interior walls of the jar, followed by rinsing with water, acetone, and hexane. Whatman filter paper sheets are commonly used as liners in the chromatographic tank to saturate the interior of the tank with the vapors of the developing solvent. This practice cannot be tolerated in water analysis confirmatory work, because the paper may contaminate the developing solvent with organic materials which will be transferred to the TLC silica gel plate and finally to the concentrated eluted extract. Although the separation of certain groups of chlori-

nated pesticides may not be as efficient without the use of the paper liner, the eluted fractions from the TLC plates will give satisfactory results on the gas chromatograph for confirmatory analyses.

The syringes used for gas chromatograph samplings must be scrupulously clean and may require copious sequential washes with alcohol, acetone, and hexane accomplished by passing the solvents through the barrel of the syringe with the aid of a vacuum pump or water aspirator apparatus.

The inclusion of any glassware which contains ground-glass sections, such as glass-stoppered centrifuge tubes or volumetric flasks, will add to the analytical problem. Heating the glassware will not remove the contaminants from the ground, glass sections. Lengthy periods of washing with copious amounts of solvents may clean the ground-glass areas, but the procedure is impractical. If this type of glassware must be used, the contents of the container should not be poured from one container to another; the transfer should be made preferably with clean, heat-treated, disposable glass pipettes.

Four cleaning procedures for glassware were examined, each of which consisted of the same initial preparation as follows: Silica gel (0.1 ml dry volume), known to contain organic contaminants, was added to each of a series of centrifuge tubes (Kontes No. 410550, 5 ml capacity) and also to each of a series of Chromaflex sample tubes (Kontes No. 422560, 2 ml capacity); only 0.01 ml dry volume of gel was added to each sample tube. Hexane (0.5 ml) was added to each tube; the contents were coated on the interior walls of the tube by means of agitation on a vortex mixer. The contents of each tube was discarded. With preknowledge of the organic contaminant content of the silica gel, the above-mentioned amounts of gel were added to each tube to approximate the amount of gel that would be scraped from a TLC plate for further study and which would also approximate the amount of background contamination observed on the gas chromatograph recorder chart. Each tube was then washed with tap water and a nylon-bristle brush to remove adhering particles of gel from the walls of the tubes. Each tube was then rinsed with copious amounts of distilled water. The four subsequent cleaning procedures with sets of the above-mentioned contaminated tubes are outlined in Table I. Auxiliary glassware used throughout the analytical procedure was cleaned in a similar manner.

Subsequent to the cleaning procedures outlined in Table I, 0.5 ml of redistilled

TABLE I

RINSING SOLUTIONS IN GLASSWARE CLEANING PROCEDURES

Method			
1	*2*	*3*	*4*
Ethanol[a]	Dichromate–H_2SO_4[b]	Acetone	Dichromate–H_2SO_4[b]
Acetone	Tap water		Tap water
Hexane	Distilled water		Distilled water
	Acetone		Acetone
Air-dry	Air-dry	Air-dry	Air-dry
		Heat[c]	Heat[c]

[a] Glassware rinsed three times with each solvent in order of the listed sequence.
[b] Glass was soaked for 16 h in a solution of sodium dichromate–concentrated sulfuric acid.
[c] Glass was heated in an air oven for 16 h at 200°.

hexane was added to each centrifuge tube by means of a heat-treated disposable pipette, to avoid contacting the ground glass area of the tube with the solvent. The tube was then agitated for about 30 sec on a vortex mixer to wash the wall of the tube with the solvent, again being careful not to wet the ground glass area of the tube. The hexane was transferred, by pipette, from the centrifuge tube to the Chromaflex sample tube. The procedure was repeated four times, combining all hexane fractions in the sample tube. The hexane content of the sample tube was concentrated to about 0.02 ml with the aid of a stream of filtered nitrogen, and aliquots of the solution were applied to the gas chromatograph to check the efficiency of the glass cleaning procedure.

Referring to Table I, method No. 1 will not completely remove contaminants from the glassware. Methods 2, 3, and 4 will remove all contaminants and any one of the three procedures can be recommended. However, because of its relative simplicity, method No. 3 is preferred. It is apparent that organic solvents alone will not remove firmly bonded organic contaminants from glass; the more drastic treatment with an oxidizing reagent and a concentrated mineral acid and/or heat are prerequisites for contaminant-free glass equipment.

SCHAFER *et al.*[6] studies on pesticides in "drinking" waters described a stirring bar mechanism to mix thoroughly hexane and the water sample in gallon jugs for the extraction of the pesticides. Using this technique, it has been our experience that if Teflon magnetic stirring bars are used, the water sample will be grossly contaminated if the Teflon bars have been in previous contact with plant extracts or other biological media. If one contemplates using this mixing technique in water analyses, only new Teflon bars should be used and they should be screened for possible contamination properties prior to use.

Silica gel adsorbents

The transition in the past 10–15 years from the milligram range to the nanogram–picogram range in chemical analysis techniques must be considered in the following discussion on silica gel adsorbents. MILLER AND KIRCHNER[7] noted that silicic acid adsorbents contained as much as 100 mg of a yellow oily material in 100 g of adsorbent which was soluble in ethyl acetate or acetone and which, if present, interfered with UV and fluorescein tests on the chromatograms. STANLEY *et al.*[8] prewashed silica gel TLC plates with petroleum ether, followed by a continuous wash for 4 to 16 h with ethyl alcohol, to remove organic materials that would interfere with diphenyl analysis in citrus fruits. This type of cleanup for the silica gel was apparent-sufficient for the measurement of milligram quantities of diphenyl by a spectrophotometric procedure. BOWYER *et al.*[9] extracted silicic acid with a chloroform–methanol (2:1) mixture to remove lipid contaminants prior to using the silicic acid for the analysis of fatty acids, also in the milligram range. BROWN AND BENJAMIN[10] noted that organic contaminants in commercially available silica gels obscured the desired spots on the acid-sprayed chromatogram and recommended washing the plates with a mixture of diethyl ether–methanol (20:80). AMOS[3] extracted various grades of silica gel with acetone and obtained residues of dark brown oils in amounts ranging from 1.0 to 39 mg per 100 g of gel. KOVACS[11] washed silica gel plates with distilled water prior to use, to remove "chlorides" that would interfere with the $AgNO_3$ spray reagent subsequently used for the detection of pesticides at the 0.10 μg

level. Later, KOVACS[12] discontinued the use of silica gel for pesticide residue analysis and replaced the gel with the adsorbent aluminum oxide, because of the high levels of "chlorinated" impurities in the silica gel. SMITH AND EICHELBERGER[13] used silica gel, as purchased, for the separation of pesticides extracted from a water sample; the amount of each pesticide applied to the TLC plates was about 0.2 mg. Sections of the developed silica gel plates were eluted for gas chromatography confirmatory analysis. Although the suspected pesticides were "confirmed" by this procedure, other unknown components sensitive to the electron capture detector were noted on the gas chromatogram. GEISS *et al.*[4] noted that silica gel contained organic contaminants, and the problem was aggravated by the use of plastic tubing which also contributed volatile contaminants to the gel when the tubing was used in a suction technique for the removal of silica gel sections from the developed plate for further analysis.

In our experimental studies, five different commercially available silica gels, with and without calcium sulfate binder, were found to be contaminated with organic materials which would confuse the interpretation of the gas chromatographic data. Some of the commercial gels were received in plastic bottles, and some were received in aluminum bottles. The contaminants from the gels packed in the aluminum containers, with plastic caps, were less than the amounts found in the plastic-packed gels, but great enough to cause interpretative problems with the analytical data. Experi-

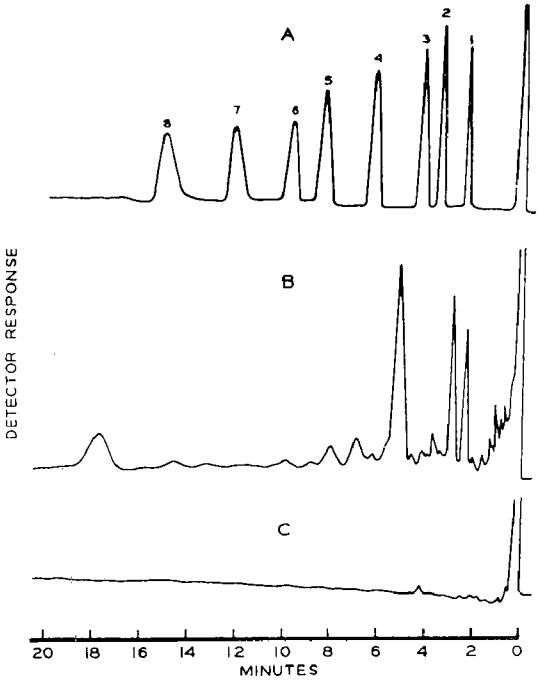

Fig. 1. Gas chromatograph curves obtained from a 1/8 in. × 5 ft. glass column containing 4% SE-30–6% QF-1 silicones on Chromosorb W, HP, 80/100 mesh; column temperature 180°; electron capture detector. (A) Chlorinated pesticide standards: Peaks 1 and 6 are Lindane and Dieldrin, respectively, each 0.3 ng. Peaks 2, 3, 4, 5, 7, 8 are Heptachlor, Aldrin, Heptachlor epoxide, DDE, DDD, DDT, respectively, each 0.6 ng. (B) Contaminants extracted from silica gel prior to heat treatment. (C) Extract of heat-treated silica gel.

ments showed that the plastic containers were at least a partial source of the gel contamination, which confirmed the observations of GEISS *et al.*[4].

Heat treatment of the silica gels at 300° for 16 h effectively removed the contaminants (See Fig. 1); this treatment did not affect the TLC properties of the gels. A less convenient but effective procedure for the removal of contaminants from silica gels is the Soxhlet extraction of the gel with redistilled chloroform for 3 h, followed by extraction with redistilled hexane for 4 h. The Soxhlet cellulose thimbles, if used as extraction containers, must be prewashed in a similar manner.

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CHROM. 5079

THIN-LAYER CHROMATOGRAPHY OF DIESTERS AND SOME MONOESTERS OF PHOSPHORIC ACID

JØRGEN STENERSEN
Norwegian Plant Protection Institute, 1432 Vollebekk (Norway)

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SUMMARY

Several monoesters and diesters of phosphoric acid and phosphorothioic acid are separated in two chromatographic systems which are described. A spray reagent, DQC (2,6-dibromobenzoequinone-4-chloroimide, 1%, in acetic acid), gives a yellow color with thiolo-sulfur and sulfhydryl groups, a red color with thiono-sulfur and a brown color with thioureas. The identity of phosphorothioate esters is often changed due to isomerization reactions. This has led to erroneous conclusions in previous reports concerning the color obtained with the DQC spray and the identity of the product formed by demethylation of the insecticide bromophos.

INTRODUCTION

In a recent publication[1], a simple procedure for the separation of some insecticide degradation products was reported. Scrutinizing the reference substances—many of them obtained from producers of insecticides—demonstrated, however, that their identities are not always in accordance with their label. This is mainly due to isomerization reactions.

In the present study a revised list of R_F values of compounds run in the acetonitrile–water system, and the color developed with the spray reagent (DQC) used for sulfur-containing phosphates, is given, and the isomerization and esterification reactions are discussed. Another chromatographic system, more suitable for the monoesters of phosphoric acid, is reported. The DQC spray has been tried for other sulfur-containing compounds.

EXPERIMENTAL

Substances for chromatography

AUTENRIETH AND HILDEBRAND[2] synthesized phenyl phosphorothionate by hydrolysis of phenyl phosphorothiodichloride in ethanolic alkali. The NMR spectrum of the product formed by this method in our laboratory showed the presence of phenoxy and ethoxy groups in a 1:1 proportion. The product actually formed was O-ethyl O-phenyl phosphorothionate. The use of other alcohols as solvents led to

other monoalkyl monophenyl phosphorothionates. In the same way, *p*-nitrophenyl phosphorothiodichloride (synthesized according to TOLKMITH[3]), gave O-alkyl O-*p*-nitrophenyl phosphorothionates.

Many O-alkyl O-aryl phosphorothionates could therefore easily be synthesized by this method. Small samples for TLC were prepared by mixing 10 mg aryl phosphorothiodichloride with 0.5 ml alcohol and 0.5 ml 0.2 *M* NaOH. The mixtures were heated at 37° overnight and 1 μl samples were directly chromatographed. The O-aryl phosphorothionates were synthesized in the same way, using acetone instead of alcohol in the reaction mixture. Also, when 2-propanol was used, small amounts of monoesters were formed together with isopropyl aryl phosphorothionate diester. No confirmation by NMR was, however, performed for these monoesters because the reaction products were unstable and difficult to crystallize. Under similar reaction conditions, $(C_6H_5O)_2PSCl$ and $(CH_3O)_2PSCl$ were hydrolysed to give $(C_6H_5O)_2PSONa$ and $(C_2H_5O)_2PSONa$, respectively.

Dialkylesters were prepared according to methods described earlier[4,5]. $C_2H_5S(C_2H_5O)PO_2NH_4$ and $C_2H_5S(C_6H_5O)PO_2Na$ were prepared from the O-ethyl compounds by isomerization. They were heated (as dry powder) in a Pyrex tube on the bunsen flame for 2 min. The isomerization of $(CH_3O)_2PSONH_4$ to $CH_3S(CH_3O)PO_2NH_4$ also occurred spontaneously if kept refrigerated, but the sodium and potassium dimethyl phosphorothionate were stable.

Salts of S-methyl O-aryl phosphorothioates were prepared by refluxing equimolar amounts of KI, NaI or LiCl with O,O'-dimethyl O''-aryl phosphorothionates in butanone. Isomerization and demethylation take place as described by HILGETAG *et al.*[6]. Methyl aryl phosphates and dimethyl phosphate were synthesized by the same method.

The other substances used were purchased or obtained as gifts from different producers of insecticides. If doubtful, their identities were checked by NMR.

Chromatography

Layers (0.25 mm) of Silica Gel G were activated by heating at 110° for 1 h. No precautions were taken to prevent deactivation. Air drying was sufficient when the humidity of the air was moderate. 0.20-mm layers of microcrystalline cellulose (Sigmacell, Type 19) were prepared by blending 15 g with 85 ml water in a Servall Omnimixer at about 10000 r.p.m. for 1 min. Before spraying on glass plates, the mixture was de-aerated by applying vacuum. The plates were air-dried before use.

Technical acetonitrile (Merck) was used without any purification. The standard mixture used was 88% acetonitrile + 12% water, but other mixtures were also tried, using pure water or 1.2 *N* HCl[7]. Chromatograms on cellulose were developed in water–propionic acid–benzene (25:62:25)[8].

Water solutions, 0.5% w/v of the esters and 0.1% w/v of the inorganic phosphates were made, and 1-μl aliquots were chromatographed.

Spray reagents

The sulfur-containing compounds were detected by spraying with 1% DQC (2,6-dibromobenzoequinone-4-chloroimide) in acetic acid[1]. The reagent was tried for other sulfur-containing compounds in spot tests. Phosphate was detected by the very sensitive method described previously[1], using Chrystall Violett in the second spray.

RESULTS AND DISCUSSION

The acetonitrile–water system is excellent for separating a wide variety of diesters of phosphoric- and phosphorothioic acid. The R_F values are given in Table I.

TABLE I

R_F VALUES OF PHOSPHATE AND PHOSPHOROTHIOATE ESTERS

(I) Layer, Silica Gel G; development, acetonitrile–water (88:12). (II) Layer, microcrystalline cellulose; development, water–propionic acid–benzene (25:62:25).

Substance	*I*	*II*
Inorganic phosphate and monoesters		
Na_3PO_4	0.0	0.2
Na_3PSO_3	0.10	0.2
$CH_3OPO_3Na_2$	0.01	0.36
$C_6H_5OPO_3Na_2$	0.03	0.50
4-NO_2-$C_6H_4OPO_3Na_2$	0.0	0.46
β-naphthyl-OPO_3HNa	0.0	0.57
β-glycerophosphate	0.0	0.25
$CH_3OPSO_2Na_2$	0.03	0.30
$C_6H_5OPSO_2Na_2$	0.06	0.41
p-NO_2-$C_6H_4OPSO_2Na$	0.10	0.38
Phosphate diesters		
$(CH_3O)_2PO_2Na$	0.10	0.43
$(C_2H_5O)_2PO_2N$	0.25	—
$(C_4H_9O)_2PO_2H$	0.25	0.54
CH_3O(4-NO_2-C_6H_4O)PO_2Na	0.35	0.58
CH_3O(4-Br-2,5-Cl_2-C_6H_3O)PO_2Na	0.51	0.72
CH_3O(CHCl=C(2,4,5-Cl_3-C_6H_2)O)PO_2Na	0.57	0.87
$(C_6H_5O)_2PO_2Na$	0.55	0.67
(4-NO_2-C_6H_4O)$_2PO_2Na$	0.72	0.60
Thiolo diesters		
$CH_3S(CH_3O)PO_2Na$	0.18	0.45
C_2H_5S $(C_2H_5O)PO_2NH_4$	0.22	0.65
$CH_3S(CH_3O)P(O)NH_2$	0.70	0.82
$CH_3S(C_6H_5O)PO_2Na$	—	0.68
$C_2H_5S(C_6H_5O)PO_2Na$	0.42	—
CH_3S(4-NO_2-C_6H_4O)PO_2Na	0.54	0.59
CH_3S(4-NO_2-3-CH_3-C_6H_3O)PO_2Na	0.54	0.65
CH_3S(4-Br-2,5-Cl_2-C_6H_2O)PO_2Na	0.60	0.83
C_2H_5S(4-Br-2,5-Cl_2-C_6H_2O)PO_2Na	0.62	0.86
Thiono diesters		
$(CH_3O)_2PSONa$	0.38	0.39
$(C_2H_5O)_2PSONa$	0.48	0.55
$C_2H_5O(C_2H_5)PSONa$	0.34	0.82
$(CH_3O)_2PS_2K$	0.74	0.30
$CH_3O(C_6H_5O)PSONa$	0.63	0.53
$C_2H_5O(C_6H_5O)PSONa$	0.65	0.58
iso-$C_3H_7O(C_6H_5O)PSONa$	0.67	0.62
$(C_6H_5O)_2PSONa$	0.71	0.64
CH_3O(4-NO_2-C_6H_4O)$PSONa$	0.70	0.53
C_2H_5O(4-NO_2-C_6H_4O)$PSONa$	0.70	0.58
iso-C_3H_7O(4-NO_2-C_6H_4O)$PSONa$	0.71	0.60
CH_3O(4-NO_2-3-CH_3-C_6H_3O)$PSONa$	0.72	0.59
CH_3O(4-Br-2,5-Cl_2-C_6H_2O)$PSONa$	0.75	0.80
2,4,5-Cl_3-$C_6H_2O(C_2H_5)PSONa$	0.69	0.82

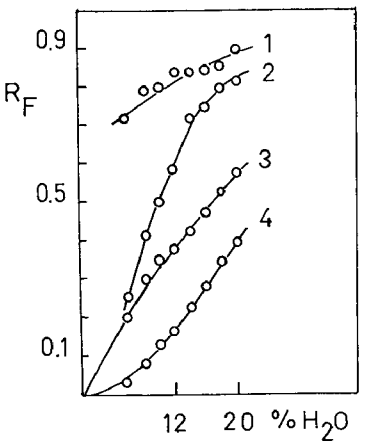

Fig. 1. The variation of R_F values, with the water content of acetonitrile–water mixtures used as the mobile phase. 1 = $CH_3O(4\text{-}Br\text{-}2,5\text{-}Cl_2\text{-}C_6H_2O)PSONa$; 2 = $CH_3S(4\text{-}Br\text{-}2,5\text{-}Cl_2C_6H_2O)PO_2Na$; 3 = $(CH_3O)_2PSONa$; 4 = $CH_3S(CH_3O)PO_2Na$.

O-alkyl and S-alkyl phosphorothioates are separated from each other and from the phosphate esters. JAGLAN AND GUNTHER[7] have used the system for separating the S- and the O-isomers of desmethyl methyl parathion. While HCl was added to their system, it was not found necessary to do so in the present study, and many of the other compounds were not separated in the acetonitrile–HCl system. The conflicting results may be due to the different procedures for activating plates or the different amounts of water present in the acetonitrile. The R_F values are highly influenced by the water content, as demonstrated in Fig. 1.

Many of the salts were available with different cations (sodium, potassium, lithium, ammonium or tetramethyl ammonium), but the R_F values did not seem to be significantly influenced by the cation. Therefore only the sodium salts are presented in Table I except when they were not available.

As it appears from their R_F values, the phosphomonoesters are not very well separated with acetonitrile. They are, however, well separated in water–propionic acid–benzene on microcrystalline cellulose.

This system is also suitable for many diesters and has quite different separation properties from the acetonitrile–water system, as thiono-sulfur seems to lower the R_F values compared to the corresponding esters with or without thiolo-sulfur. The time of development is about $2\frac{1}{2}$ h. Organophosphorus compounds are not so easily destroyed on an organic layer—a necessity when a detection method based upon color development with inorganic phosphate is used.

The spray reagent DQC gave a *yellow* color with S-alkyl phosphorothioates and a *reddish* color with O-alkyl phosphorothionates. Sulfhydryl groups, as in mercaptoethanol, glutathione (reduced and oxidized), cystein, cystine, etc., also gave *yellow* colors. The sensitivity of mercaptoethanol was about 0.2 μg, and that of glutathione was about 1 μg. Substituted thioureas gave a *brown* color. Therefore DQC may also be a sensitive spray for other sulfur-containing compounds. S-acetyl thiocholine and S-methyl glutathione gave *no* coloration.

These results concerning R_F values and color developments clearly indicates

that one of the products of bromophos (O,O'-dimethyl O''-4-bromo-2,5-dichlorophenyl phosphorothionate) metabolism has previously been erroneously identified as bis-desmethyl bromophos[9,10]. The reference substance labeled "des-methyl bromophos" was actually the S-methyl derivative ($CH_3S(4\text{-}Br\text{-}2,5\text{-}Cl_2\text{-}C_6H_2O)PO_2Na$), and those labeled O-aryl phosphorothionate monoesters were actually O-alkyl O-aryl phosphorothionates in these reports[9,10]. These results prove that *one* methyl group of bromophos is split off, in agreement with the work of PLAPP AND CASIDA[11,12].

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THE COMPOSITION OF THE UNSATURATED PHENOLIC COMPONENTS OF ANACARDIC ACID

J. H. P. TYMAN AND N. JACOBS
Department of Chemistry, Brunel University, London W3 (Great Britain)

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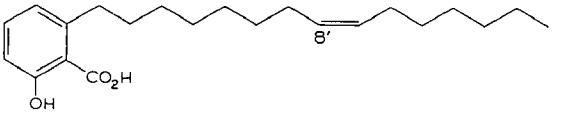
SUMMARY

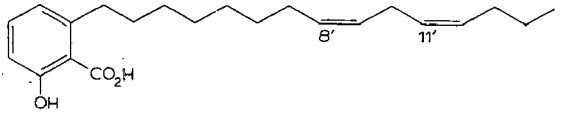
The monoene, diene, and triene components of anacardic acid have been quantitatively separated by argentation–thin-layer chromatography, and determined by ultraviolet spectrophotometry and gravimetry. Some progress has been made in the direct determination of the composition without separation of the component acids by use of nuclear magnetic resonance spectroscopy.

INTRODUCTION

The unsaturated components of anacardic acid*, the principal constituent of cashew nut-shell liquid (*Anarcardium occidentale*)[1–4], have been examined indirectly through chromatography of cardanol methyl ether[5], obtained through decarboxylation and methylation, and by low temperature acetone crystallisation[6] of anacardic acid itself. These qualitative studies have revealed the existence of 8′-monoene (I),



(I)



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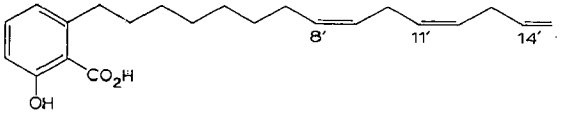

(III)

* The name anacardic acid although originally retained for the main component of cashew nut-shell liquid has more recently been used for 2-hydroxy-6-alkylbenzoic acids where the alkyl group (C_{11} and higher) has the relevant saturation and/or unsaturation.

8',11'-diene (II), and 8',11',14'-triene unsaturation (III) in a pentadecyl side chain. Recently, small proportions of a component having a saturated tridecyl side chain and four components arising from a heptadecyl side chain have been described[7] in cashew nut-shell liquid from an unstated source.

In the course of a quantitative investigation of the composition of cashew nut-shell liquid by a spectrophotometric method[8], qualitative observations on the unsaturated ingredients of the individual phenols were made by the argentation–TLC method[9–11]. The present paper describes work, completed in 1966, on the quantitative separation of the monoene, diene, and triene components of anacardic acid and more recent spectroscopic work; the method was not so successful when applied to the cardanol, cardol, and 4-methylcardol constituents of cashew nut-shell liquid and these results will be described subsequently.

A method for the determination of the composition of purified anacardic acid was sought to obviate the chromatographic separation of the component acids. Although infrared (IR) absorption and mass spectrometry were extremely useful procedures when applied qualitatively for confirming the presence of certain structural features and determination of molecular weights respectively there were difficulties in their quantitative application. NMR spectroscopy was, however, more useful in this latter respect provided the class of the component was qualitatively known. Further work is required to improve the procedure.

Quantitative information on the distribution of unsaturation in natural cashew nut-shell liquid phenols and in the decarboxylated material is of interest both with regard to the biosynthesis of these materials and to possible side reactions occurring in the industrial decarboxylation process.

EXPERIMENTAL

Analytical (8 × 10 cm) and preparative TLC plates (20 × 20 cm) were prepared in the usual way with the applicator set to 0.250 mm and 1 mm, respectively. Silver nitrate-impregnated plates (% $AgNO_3$, w/w on Silica Gel G), prepared by incorporation of the former in the slurry, were stored in glass containers in the dark. All plates were activated in an oven at 110° for 1 h and then cooled to ambient temperature. All operations were carried out in the absence of direct sunlight. TLC applications and solvent evaporations were carried out in an atmosphere of nitrogen. For recording TLC results, analytical plates were visualised with 50% w/w aqueous H_2SO_4 by charring at 150°.

UV absorption spectra were recorded automatically on an Optica (Great Britain) and a Perkin Elmer Model 137 spectrophotometer and manually on a Unicam SP 500 instrument. Methanol (A.R. grade) was used throughout. All optical density measurements were the average of two readings.

Cashew nuts (*Anacardium occidentale*) were of Tanganyikan origin.

Separation of cashew nut-shell liquid (CNSL) components

Disintegrated cashew nut-shells separated from the kernel by fracturing at −70° (solid CO_2) were extracted with diethyl ether for 24 h with occasional shaking at ambient temperature. After filtration the solid residue was comminuted in a blender and re-extracted with diethyl ether. The solvent in the combined filtrates was

evaporated at ambient temperature under reduced pressure in the presence of nitrogen to avoid decarboxylation, until the residue was constant in weight.

CNSL (0.1957 g) in chloroform (2 ml) was distributed along the baseline of two preparative plates and developed in 200 ml of diethyl ether–petroleum ether (40–60°) (50:150) containing 10 ml aqueous ammonia (0.880 S.G.) until the solvent front was 1 cm from the top of the plate. After removal of the organic solvents in a stream of nitrogen, the plates were developed in diethyl ether (150 ml) containing aqueous ammonia (0.880; 10 ml) until the solvent front had travelled 8 cm from the baseline, dried and sprayed with 0.01% ethanolic Rhodamine 6G. Under UV light seven bands of increasing R_F value were discernible as follows: (1) baseline material, ammonium anacardate, (2) anacardic acid, (3) cardol, (4) unknown minor ingredient, (5) 4-methylcardol, (6) cardanol, (7) unknown minor ingredients. Bands (1) and (2) were combined, eluted with methanol (25 ml) for 24 h, filtered, the solid washed with methanol (2 × 25 ml) and diethyl ether (2 × 25 ml) and after evaporation at room temperature of the combined filtrates, the residual material in chloroform (3 ml) was purified further on a preparative plate by development in 200 ml of diethyl ether–petroleum ether (40–60°) (50:150) containing 95% formic acid (1.5 ml). Anacardic acid formed an intense band near the solvent point, while traces of cardol were left nearer the baseline, and was recovered as before. The Rhodamine 6G indicator was removed by washing an ethereal solution of the evaporated product with 0.01 *N* aqueous sulphuric acid (6 × 15 ml) followed by water (6 × 15 ml). The dried solution ($MgSO_4 \cdot H_2O$) was evaporated and residual material which was homogeneous was stored in chloroform (concentration, approx. 15 mg/ml).

Separation of the unsaturated components of anacardic acid on silver nitrate-impregnated Silica Gel G

Owing to the relatively low ε value of anacardic acid at λ_{max} 308 nm it was necessary to use larger amounts than could easily be obtained on the analytical scale.

TABLE I

R_F VALUES OF THE COMPONENTS OF ANACARDIC ACID

Solvent system No.	*Solvent proportions*			*R_F values*		
	Petroleum ether	*Diethyl ether*	*Formic acid*	*Monoene*	*Diene*	*Triene*
1	50	—	1		all at baseline	
2	50	—	3		all at baseline	
3	50	10	1		poor separation	
4	50	10	3		poor separation	
5	50	20	3	0.69	0.51	0.33
6	50	20	5	0.69	0.52	0.33
7	50	30	3	0.90	0.75	0.59

The use of this wavelength precludes interference from non-phenolic material. Plates prepared with 10% silver nitrate were less effective on the preparative than on the analytical scale whereas 20% silver nitrate gave almost as good results at both levels and was accordingly used. Reproducible R_F values were not always achieved. Table I

summarises the values (on plates having 20% silver nitrate) found with different proportions of petroleum ether (40–60°), diethyl ether and formic acid.

Ethyl acetate–chloroform mixtures were not so useful and acetic acid or mono-, di, or trichloroacetic acids (to avoid possible reducing action) were not as effective as formic acid. Solvent system (5) was generally used throughout. For quantitative work, Rhodamine 6G was unsatisfactory for detection of separated components, as it faded rapidly due to the formic acid present. Dichlorofluorescein and 3,5-dihydroxypyrene-8,10-disulphonic acid were both suitable for visualisation but the former could only be removed by water washing from petroleum solutions, whereas diethyl ether was generally the preferred solvent for fractions. Water was an effective indicator since the bands showed up as hydrophobic areas.

Quantitative experiments

Anacardic acid in chloroform solution (1 ml) was applied to a 20 × 20 cm preparative plate and developed with solvent system (5). After evaporation of nearly all the solvent in a nitrogen atmosphere, the bands were visualised by means of pyrene–sulphonic acid. The solvent front, the three main bands, and the baseline were each eluted with methanol (25 ml) during 2 h with intermittent shaking. After filtration and washing the silica gel with methanol (5 × 10 ml), the combined filtrates were evaporated nearly to dryness at ambient temperature *in vacuo* and the concentrate was extracted with ether. The small amount of insoluble material (Ag anacardate) was warmed with a small volume of dilute nitric acid and re-extracted with ether and the combined ethereal solutions were washed with water until neutral. The residual material left after evaporation of the ether was dissolved and made up to 25 ml in methanol. The optical densities of the pure solutions were measured at 308–309 nm. Material from the solvent front and from the baseline showed no absorption

TABLE II

UV ABSORPTION OF THE COMPONENTS OF ANACARDIC ACID FROM CASHEW NUT-SHELL LIQUID

Optical density observed			*Optical density corrected*		*Total optical density*	*Total optical density (%)*		
Triene (T)	*Diene (D)*	*Monoene (M)*	*Diene*	*Monoene*	*(T + D + M)*	*Triene*	*Diene*	*Monoene*
0.581	0.180	0.498	0.181	0.504	1.266	45.9	14.3	39.8
0.995	0.408	0.842	0.410	0.852	2.257	44.1	18.2	37.8
0.987	0.356	0.830	0.357	0.840	2.184	45.2	16.4	38.5

maximum, while the monoene, diene, and triene showed clear maxima. Examined on $AgNO_3$ analytical plates each fraction gave a single spot of different R_F value, while on unimpregnated plates all the components had the same R_F value corresponding to that of the fully hydrogenated material.

Minor variations in the work-up procedure in or after elution were made, such as the direct use of ether and dilute nitric acid or elution with methanol containing

hydrochloric acid to remove silver nitrate, but none of these altered the optical density found. The results of three experiments are summarised in Table II. (Since only the molar extinction coefficients are uniform for the mono, di and triene the optical densities were corrected using molecular weights 346, 344 for the monoene, diene, respectively and c, concentration = optical density × mol. wt./molar extinction coefficient × 10. The correction is in fact trivial.)

The average result was 45.1% triene, 16.3% diene and 38.7% monoene.

A negligible proportion of the saturated component was present. Elution of a progressive series of strips from above the monoene band to the top of the plate failed to yield any material having a maximum absorption at 308 nm. It was considered that the saturated component could be present with cardol in band (3) of the initial separation but careful purification failed to yield any such material.

Separation and estimation of component acids by gravimetry

A total of eleven plates impregnated with 20% silver nitrate was developed and the combined bands eluted giving the triene 0.0889 g (44.1%), the diene 0.0352 g (17.4%) and the monoene 0.0774 g (38.4%) affording a good measure of agreement with the spectroscopic method. The physical properties of the separated components agreed with those described[6]. The monoene was a solid, m.p. approx. 50°. The diene solidified on cooling to 0° and had a melting point just above ambient temperature, while the triene was an oil which only solidified on cooling with acetone–carbon dioxide.

The monoene was the only sample which could be analysed. Found: C, 76.20%; H, 9.83%; calculated for $C_{22}H_{34}O_3$; C, 76.25%; H, 9.89%.

Spectroscopic characterisation of the components of anacardic acid

IR absorption. All three unsaturated materials showed characteristic weak absorption in the region 700–710 cm^{-1}, characteristic of *cis* C–H (out-of-plane bend) although this was partly obscured by the $(CH_2)_n$ rocking frequency. In all cases the much stronger *trans* C–H absorption at 965–975 cm^{-1} was absent (for reference purposes oleic and elaidic acids were used). Although the triene showed strong absorption at 900 cm^{-1} due to the terminal $C=CH_2$ group its determination in anacardic acid was rendered difficult by overlapping adjacent bands from the monoene and the diene.

UV light irradiation. Irradiation of the monoene, diene and triene in petroleum ether (40–60°) solutions in silica cells during 24 h caused rapid isomerisation in the presence of 1% iodine to the all *trans* configurations in each case with strong absorption at 965 cm^{-1}. In the absence of iodine, isomerisation was considerably slower. The whole process with or without iodine could be monitored by TLC. In the absence of a catalyst the diene and triene showed two additional spots of higher R_F value than the starting material corresponding, it is thought, to *cis/trans* and *trans/cis* configuration of the 8′ and 11′ double bonds.

Mass spectra. The parent ions for the saturated monoene, diene and triene components were at *m/e* 348, 346, 344 and 342 corresponding to the molecular formulae $C_{22}H_{36-n}O_3$, where n = 0, 2, 4, 6, respectively. Higher masses corresponding to possible C_{17} components were present in traces.

Proton magnetic resonance spectra. Spectra were initially run on a Varian T60 and subsequently on an HA100 instrument in order to obtain better resolution between benzylic protons and those adjacent to two double bonds. Carbon tetra-

TABLE III

NMR ABSORPTION OF ANACARDIC ACID AND ITS HYDROGENATION PRODUCT

s = singlet; d = doublet; t = triplet; q = quarter; m = multiplet.

Type of group		*τ value*	*Type of splitting*
Anacardic acid			
OH, CO_2H	(internally bonded)	−0.92	broad s
HAr	(two types)	2.63–2.8, 3.16–3.35	m
—HC = CH—	(olefinic)	4.6–4.79	m
CH_2 = C	(olefinic)	4.93–5.13	m
—CH_2—Ar	(benzylic)	6.94–7.11	t
—CH=CH—CH_2—CH=CH—	(methylenic 2 double bonds)	7.17–7.27	m
—CH_2—CH=CH—	(methylenic 1 double bond)	7.82–8.14	m
—$(CH_2)_2$	(methylenic chain)	8.2—8.98	double s
—CH_3	(methyl)	9.0–9.3	t
6-Pentadecylsalicylic acid			
3HAr	(3 aromatic H)	2.54–2.72, 3.04–3.34	m
—CH_2Ar	(2-benzylic H)	6.92–7.18	t
—$(CH_2)_2$—	(2 methylenic H)	8.2–8.9	s
—CH_3	(3 methyl H)	9.04–9.22	t

chloride was the preferred solvent as deuterochloroform contained sufficient chloroform to interfere with the integration in the region of aromatic protons.

The spectra of the monoene, diene, triene, and the saturated component obtained by hydrogenation were first obtained. Bands were identified by the use of appropriate reference compounds such as 6-methylsalicyclic acid, salicylic acid, eugenol, oleic acid, elaidic acid (and their esters) and by application of the Shoolery rules. It was found desirable to use freshly prepared specimens free of autoxidised products. The spectrum of a freshly prepared sample of anacardic acid was next determined and bands due to the individual components identified. The values for anacardic acid and its hydrogenation product are listed in Table III. In the monoene the coupling constants (4.6 c.p.s.) and sharpness of the peaks due to –CH=CH– protons indicated that *cis* and none of the *trans* isomer was present. The complex olefinic peaks in the diene and triene precluded similar observations but the IR absorption of these two components indicates the *cis* configuration of the double bonds. From scale expansion of the integration the average relative areas (from three runs) were 78 for the –CH=CH– protons and 20 for $=CH_2$ protons. By comparison (see DISCUSSION) of the theoretical values from different mixtures of the monoene, diene and triene, the composition of anacardic acid itself was found by trial and error to approximate to 45% triene, 15% diene and 40% monoene.

DISCUSSION

Anacardic acid from cashew nut-shell liquid was separated on silver nitrate-impregnated silica gel plates and no material remained at the baseline through silver salt formation or autoxidation. Reduction was observed with cardanol and cardol and

its absence with the acid may be possibly due to the internal bonding of the –OH and $-CO_2H$ groups. The method separates the components of the acid according to the degree of unsaturation but not chain length. (This, however, in the case of the present CNSL appeared to be substantially C_{15}.)

The lack of IR absorption at 965 cm^{-1} indicated that the unsaturated components were present in the *cis* form[12]. The configuration of the constituent acids has previously been inferred from experiments carried out with cardanol methyl ether. Upon irradiation of anacardic acid with UV light under mild conditions the monoene was partly transformed into the *trans* form while the diene and triene gave, it was believed, the partly isomerised *cis*-8′, *trans*-11′ and *trans*-8′, *cis*-11′ products of higher R_F values than starting material but lower than the all *trans* product. The existence of partly *trans* found in the natural product would in fact have rendered the argentation–TLC separation almost impossible due to overlapping bands.

The NMR spectra of the component acids and of the saturated acid afforded confirmation of certain groupings. The coupling constant (4.6 c.p.s.) in the triplet attributable to the –CH=CH– protons of the monoene is similar to the value for oleic acid and larger than that for elaidic (3.6 c.p.s.) and is consistent with a *cis* configuration. The spectrum for the triene clearly indicated the presence of $=CH_2$ protons. Although at higher τ values it was not easy to ascribe exactly to given groups the areas found from integration, in the region of the olefinic protons the areas due to –CH=CH– and $=CH_2$ groups could be distinguished. A trial and error fitting procedure has indicated a composition in agreement with the spectrophotometric method. By the additional use of another part of the spectrum a more exact mathematical solution should be possible. The region where the methylenic protons of the $-CH=CH-CH_2-CH=CH-$ group and the benzyl group (a common structural feature for all the component acids) overlap would in theory be suitable. In practice some difficulty was experienced in obtaining reproducible areas from integration and further work is required. (Nevertheless an NMR procedure appeared to be the only one for determining the composition of anacardic acid without preliminary separation of the component acids.)

No monoethenoid fatty acids were found in CNSL although GELLERMAN AND SCHLENK[7] have reported their occurrence. In a typical chromatogram on analytical plates with solvent 5, oleic acid (R_F 0.54) and elaidic acid (R_F 0.61) were well separated from the monoene (R_F 0.66), and the diene (R_F 0.47). The presence of monoethenoid acids, although non-absorbing at the wavelength used for determining the component anacardic acids, would have led to a discrepancy between the gravimetric and spectrophotometric methods. The agreement between the two procedures was however, satisfactory.

There are wide differences in the compositional figures found by PAUL AND YEDDANAPALLI[6], by GELLERMAN AND SCHLENK[7] and the present authors. The acetone crystallisation technique would not be expected to give sharp separation of the component acids and the figures found for anacardic acid of Indian origin namely, 4% saturated, 37% monoene, 44% diene, and 15% triene, are not likely to be accurate. The acids isolated were characterised by C/H analysis. The theoretical values for the monoene and the triene differ by only 0.89% for C and 1.16% for H, while the C_{16} and C_{18} monoethenoid fatty acids have an almost identical %C with %H slightly higher.

The relative proportion of component acids found by GELLERMAN AND SCHLENK[7], 0.9% saturated, 14.3% monoene, 20% diene, and 38.4% triene (the remainder comprising unsaturated fatty acids and traces of C_{13} and C_{17} anacardic acids) are lower in the case of the monoene and diene than those found in the present work. The method of extraction of the cashew nut-shell liquid used was not described and the nuts were from an unstated source.

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CHROM. 5077

THIN-LAYER PARTITION CHROMATOGRAPHY OF STEROIDS USING VOLATILE STATIONARY PHASES

DAVID J. WATSON AND DELPHINE BARTOSIK*

The Worcester Foundation for Experimental Biology, Inc., 222 Maple Avenue, Shrewsbury, Mass. 01545 (U.S.A.)

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SUMMARY

A simple technique for using Bush-type (volatile stationary phase) chromatographic systems on thin-layer plates composed of Silica Gel GF is described. The R_F values of several steroids of biological interest are presented for both aqueous methanolic and aqueous acetic acid systems. Suggestions for the modification of paper partition chromatography systems for use with silica gel plates are also presented.

INTRODUCTION

Progress in the development of solvent systems suitable for the separation, by adsorption thin-layer chromatography, has been extensive. Many such systems for separating steroids of the estrane[1,2], androstane[3–5] and pregnane[6–9] series have been published. Nevertheless, there is a practical need for partition chromatography, in that certain steroid pairs commonly found in biological materials can be separated only with difficulty by adsorption chromatography. Common examples include progesterone and androstenedione, pregnenolone and dehydroepiandrosterone, and 20α- and 20β-dihydroprogesterone.

Although many workers have used reversed-phase partition chromatography on thin-layer plates for the resolution of lipid and sterol mixtures[10], VAEDTKE AND GAJEWSKA[11] were the first to report the use of thin-layer partition chromatography for the separation of steroids. These workers used Zaffaroni-type systems in which impregnation of the plate with the liquid stationary phase is required. To our knowledge, the use of thin-layer partition chromatography using Bush-type solvent systems has not been achieved. We should therefore like to present a simple, reproducible technique for using volatile stationary phase partition chromatography systems on Silica Gel GF plates and to present examples of its usefulness.

* Present address: Department of Obstetrics and Gynecology, New York Medical College, Fifth Avenue at 106th Street, New York, N.Y. 10029, U.S.A.

MATERIALS AND METHODS

Preparation of tank

A Brinkman rectangular TLC tank (Cat. No. 25-10-22) is lined with Whatman filter paper (two thicknesses of No. 1 or one thickness of No. 3MM). A trough is placed in the bottom of the tank which should be wide enough to hold a 20 cm plate. We have used a trough and support obtained for paper chromatography tanks (Will Scientific Co., Cat. Nos. 8430 and R 8429). The lid of the tank should have a hole, approximately $\frac{1}{2}$ in. in diameter, fitted with a cork. The lid is sealed on with a film of starch–glycerine paste[12] and held firmly with two five-pound lead weights, in order to minimize evaporation of solvent from the atmosphere of the tank. The tank is prepared by saturating the paper liner with 200 ml of stationary phase, which forms a layer in the bottom, and 75 ml of mobile phase which overlays the stationary phase. The atmosphere should be allowed to equilibrate for at least 1 h.

Preparation of plates

Thin-layer plates are prepared with Mallinckrodt Silica Gel TLC-7 GF, 30 g in 60 ml of water, and are spread in the customary way to make five plates, 20 cm × 20 cm × 250 μ. The plates are left to dry at room temperature for about 30 min, and then dried in an oven at 100° for 2 h. We have confirmed the report of HEFTMANN[13] that plates which have been left to dry for 24 h at room temperature perform as well as those dried at 100°. This eliminates the need for taking special precautions for storage of plates. If Merck (Brinkman) Silica Gel GF is used, the R_F values are about half of those obtained with Mallinckrodt silica gel. The radical changes in solvent composition thus required make it desirable to use the Mallinckrodt product. The reason for this difference is not apparent, but since it is also found when comparing customary adsorption TLC solvent systems for the two products, there is reason to suspect it arises from differences in the preparation of the two silica gels[14].

Preparation of solvent systems

Biphasic solvent systems are prepared in the manner for paper partition chromatography, by mixing the appropriate solvents in the indicated ratios in a separatory funnel and allowing the two layers to separate. The two phases thus formed are stored in glass bottles with glass stoppers.

Conditions of chromatography

The samples and standards are spotted on the plates exactly as for adsorption TLC. The plate is then placed in the trough, and left to equilibrate for 2 h at room temperature. A funnel with a plastic tube attached is then used to add 40 ml of the mobile phase directly into the trough, through the hole in the lid. When removing the funnel, one should avoid dripping solvent onto the plate. Development requires 30–60 min, depending on the solvent system used. After the solvent has risen to the desired height (usually 15 cm from the origin) the plate is removed and dried in the fume hood. The trough can be removed and the tank re-used at least two more times.

Chromatographic systems used

Abbreviations used in solvent system nomenclature are taken from BUSH[12].

L/80: Cyclohexane–methanol–water (100:80:20)

LT81/80: Cyclohexane–toluene–methanol–water (89:11:80:20)
LT51/80: Cyclohexane–toluene–methanol–water (83:17:80:20)
LT41/80: Cyclohexane–toluene–methanol–water (80:20:80:20)
LT21/80: Cyclohexane–toluene–methanol–water (67:33:80:20)
L/A85: Cyclohexane–acetic acid–water (100:85:15)
LT21/A80: Cyclohexane–toluene–acetic acid–water (67:33:80:20)
LT41/A85: Cyclohexane–toluene–acetic acid–water (80:20:85:15)
LT41/A80: Cyclohexane–toluene–acetic acid–water (80:20:80:20)
LT31/F80: Cyclohexane–toluene–90% formic acid–water (80:20:89:11)

The use of cyclohexane rather than *n*-hexane, light petroleum etc. is recommended for the reason given by BUSH[12], namely, that ΔR_M values are sometimes increased when using a cyclic hydrocarbon as a major component of the mobile phase.

Similarly we recommend the use of toluene rather than benzene because $\Delta R_{M(r)}$ values are frequently slightly greater with toluene. The toxicity of toluene is also appreciably less than benzene.

EXPERIMENTAL

The R_F and R_M values for a number of common steroids of biological interest are presented in Tables I and II, for methanolic and acetic or formic acid systems, respectively.

The R_F values presented in Tables I and II are, in all cases, slightly less than those reported for similar biphasic solvent systems used for paper partition chromatography (PPC)[12,15,16]. Some of the ΔR_M values due to modification of structure ($\Delta R_{M(r)}$ values) obtained with thin-layer partition chromatography (TLPC) are presented in Table III. These values are quite similar to those reported for PPC[12,15,16], suggesting that partition rather than adsorption effects predominate.

TABLE I

R_F AND R_M VALUES FOR SOME COMMON STEROIDS IN AQUEOUS METHANOLIC SYSTEMS

	L/80		*LT81/80*		*LT41/80*		*LT21/80*	
	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
Progesterone	0.49	0.017	0.62	−0.213	0.71	−0.388	0.76	−0.501
Androstenedione	0.22	0.550	0.32	0.327	0.47	0.052	0.57	−0.122
Pregnenolone	0.32	0.327	0.40	0.176	0.53	−0.052	0.60	−0.176
Dehydroepiandrosterone	0.15	0.753	0.20	0.602	0.31	0.348	0.37	0.231
20β-Dihydroprogesterone	0.22	0.550	0.31	0.348	0.43	0.122	0.52	−0.035
20α-Dihydroprogesterone	0.14	0.788	0.22	0.550	0.32	0.327	0.40	0.176
7-Oxo-cholesterol	0.89	−0.908	0.95	—	0.94	—	0.94	—
Cholesterol	S.F.	—	S.F.	—	S.F.	—	S.F.	—

Systematic names, abbreviations and trivial names used refer to the following chemical substances: progesterone (P^4) = pregn-4-ene-3,20-dione; androstenedione (A^4) = androst-4-ene-3,17-dione; pregnenolone (P^5) = pregn-5-en-3β-ol-20-one; dehydroepiandrosterone (DHEA) = androst-5-en-3β-ol-17-one; 20β-dihydroprogesterone (20β-P^4) = pregn-4-en-20β-ol-3-one; 20α-dihydroprogesterone (20α-P^4) = pregn-4-en-20α-ol-3-one; cholesterol (chol.) = cholest-5-en-3β-ol; 7-oxo-cholesterol (7-oxo-chol.) = cholest-5-en-3β-ol-7-one.

TABLE II

R_F AND R_M VALUES FOR SOME COMMON STEROIDS IN AQUEOUS ACETIC AND FORMIC ACID SYSTEMS

	L/A85		*LT41/A85*		*LT21/A80*		*LT31/F80*	
	R_F	R_M	R_F	R_M	R_F	R_M	R_F	R_M
Progesterone	0.13	0.825	0.41	0.158	0.50	0.00	0.25	0.477
Androstenedione	0.05	1.131	0.21	0.575	0.31	0.347	0.08	1.061
Pregnenolone	0.19	0.629	0.49	0.017	0.55	−0.087	0.44	0.104
Dehydroepiandrosterone	0.09	1.033	0.31	0.347	0.37	0.231	0.17	0.688
20β-Dihydroprogesterone	0.08	1.058	0.32	0.327	0.41	0.158	0.15	0.753
20α-Dihydroprogesterone	0.05	1.210	0.24	0.501	0.35	0.268	0.11	0.908
7-Oxocholesterol	0.35	0.268	0.61	−0.194	nd[a]	—	0.53	−0.052
Cholesterol	0.73	−0.432	0.84	−0.720	nd	—	0.83	−0.689

[a] No detection.

It is of special interest to note that the $\Delta R_{M(r)}$ (Δ^4-3-one→Δ^5-3β-ol), whether for the C_{21} steroids or the C_{19} steroids, changes from a positive value for the aqueous methanolic systems, to a negative value for the aqueous acidic systems. This observation has also been noted for PC systems[12] and is of particular analytical value. For example, for practical purposes, pregnenolone has a slower mobility than progesterone in aqueous methanolic systems, but a faster mobility in aqueous acidic systems.

TABLE III

$\Delta R_{M(r)}$ FOR THREE STRUCTURAL CHANGES

	20α-OH → 20β-OH	*Δ^4-3-one → Δ^5-3β-ol*	*$C_{19} \rightarrow C_{21}$*
L/80	−0.23	+0.25	−0.50
LT81/80	−0.20	+0.34	−0.49
LT51/80	−0.20	+0.34	−0.49
LT41/80	−0.20	+0.35	−0.44
LT31/80	−0.21	+0.36	−0.38
LT21/80	−0.22	+0.34	−0.39
L/A85	−0.15	−0.15	−0.36
LT41/A85	−0.17	−0.18	−0.37
LT21/A80	−0.11	−0.10	−0.33
LT31/F80	−0.16	−0.37	−0.58

DISCUSSION

The advantages of TLC as compared to PC are multiple and need not be belabored. The establishment of a simple technique for using Bush-type solvents in thin-layer partition chromatography (TLPC) enables the investigator to utilize the advantage of partition chromatography without sacrificing the ease of TLC.

A theoretical problem of practical concern in adsorption chromatography is that compounds differing in the number of carbon atoms cannot be easily separated.

One approach has been to use low-polar solvents with repeated development[17], and STÁRKA AND HAMPL[18] have described mathematical formulae by which the number of developments for optimal separation could be calculated. Earlier workers have used the Zaffaroni type systems in which the stationary phase is impregnated onto the supporting silica gel. A disadvantage of such systems is the difficulty of removing the stationary phase after development of the chromatogram. This difficulty is eliminated by using the Bush-type, volatile, stationary phase as described in this report.

The aqueous methanolic and the aqueous acetic acid systems for which data are presented in Tables I and II, respectively, represent a small part of the total range of systems reported for use with PC. The polarity of these systems is suitable for the polarity of the steroids presented as examples. The separation of more polar steroids would necessitate the use of more polar solvent systems.

As a first approximation, paper partition chromatography (PPC) systems may be used with this technique (TLPC). However, we have noted that a slight decrease in the methanol concentration of the stationary phase (*e.g.*, L/85→L/80) is usually necessary in order to get similar R_F values in PPC and TLPC, respectively. Alternatively, the polarity of the mobile phase may be decreased (*e.g.*, LT21/80→LT41/80).

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CHROM. 5048

THIN-LAYER AND PAPER CHROMATOGRAPHY OF STEROIDAL β-D-GLUCOPYRANOSIDES, β-D-GLUCOPYRANOSIDURONIC ACIDS, AND DERIVATIVES

JOHN J. SCHNEIDER
Department of Medicine, Jefferson Medical College, Philadelphia, Pa. 19107 (U.S.A.)

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SUMMARY

A number of related steroidal β-D-glucopyranoside peracetates, free β-D-glucopyranosides, and β-D-glucopyranosiduronic acids, chiefly in the androstane and pregnane series, were chromatographed on thin layers of silica gel. In all three classes R_F differences between members of axial–equatorial pairs were small and, contrary to rule, the equatorially-oriented member frequently was the more mobile. It was not possible to separate mixtures of free glucosides and glucosiduronic acids. Thin-layer chromatography on silica gel remains, however, an excellent method for detecting simple free glucosides.

Partition paper chromatography of the free glucosides and glucosiduronic acids gave far better results: reasonable R_F differences were obtained for all axial–equatorial pairs, the axially-oriented member of a given pair was the more mobile in all cases, and mixtures of free glucosides and glucosiduronic acids were separated without difficulty.

INTRODUCTION

In an earlier study[1] sixteen anomeric pairs of steroidal tri-O-acetyl-D-glucopyranosiduronic methyl esters, seven pairs of D-glucopyranoside tetraacetates, and four pairs of 2′-acetamido-2′-deoxy-tri-O-acetyl-D-glucopyranosides, together with the free steroids (aglycons) from which they were derived, were chromatographed on thin layers of silica gel. It was observed that the α member was more mobile than its anomer in all cases except one, and that the mobility of a given derivative* relative to the corresponding aglycon was variable, but determined in part by the nature of the aglycon and the carbohydrate moiety.

Subsequently an additional fourteen glucoside peracetates and the corresponding free glucosides were prepared[2] as well as the free glucosiduronic acids derived

* The general term "derivative" refers both to β-D-glucopyranoside peracetates and tri-O-acetyl-β-D-glucopyranosiduronic methyl esters. The trivial terms "glucoside" and "glucosiduronic acid" represent in the text conjugates which are formally β-D-glucopyranosides and β-D-glucopyranosiduronic acids, respectively.

from a number of glucosiduronic methyl ester tri-O-acetates synthesized earlier[3]. This completed the preparation of four series of steroidal conjugates, consisting of glucosides, glucosiduronic acids and their respective derivatives, which utilized as far as possible the same group of aglycons (chiefly androstanes and pregnanes) within each series. The present report considers the thin-layer chromatography of the glucoside peracetates, free glucosides and glucosiduronic acids, as well as the partition paper chromatography of the latter two classes.

METHODS

Thin-layer chromatography was carried out at $25 \pm 2°$ by the ascending method on Silica Gel IB-F sheets. Systems (Table I) suitable for the acetylated

TABLE I

COMPOSITION OF CHROMATOGRAPHIC SYSTEMS

System No.	*Composition*
1	Ethyl acetate, 10 ml, diluted to 25 ml with isooctane (2,2,4-trimethylpentane)
2	Ethyl acetate, 12 ml, diluted to 25 ml with isooctane
3	Ethanol, 2.5 ml, diluted to 25 ml with chloroform
4	Ethanol, 3 ml, diluted to 25 ml with chloroform
5	Ethanol, 1.5 ml, acetic acid, 3 ml, diluted to 25 ml with chloroform
6	Ethanol, 2 ml, acetic acid, 3 ml, diluted to 25 ml with chloroform
7	Ethyl acetate–toluene–methanol–water (50:150:70:130)
8	Ethyl acetate–toluene–methanol–water (25:175:80:120)
9	Ethyl acetate–toluene–acetic acid–water (50:150:60:140)
10	Ethyl acetate–toluene–acetic acid–water (30:170:60:140)
11	Isooctane–toluene–methanol–water (170:30:170:30)
12	Isooctane–methanol–water (200:180:20)
13	Toluene–*tert.*-butanol–acetic acid–water (185:30:60:140)

glucosides consist of ethyl acetate–isooctane mixtures[1]; R_F values with such systems usually can be reproduced to within 0.1 R_F unit. Free glucosides are best chromatographed with ethanol–chloroform systems; the glucosides move as well-defined, round components, but R_F values are more difficult to reproduce, due probably to temperature changes within the jar as a result of initiating chromatography too soon after adding the solvent. The 3-component ethanol–chloroform–acetic acid type of system is considered only fairly satisfactory for the chromatography of free glucosiduronic acids; the components are ovoid rather than round, and the solvent front is invariably irregular. In all cases the conjugates were detected by spraying the dried plate with a 20% solution of *p*-toluenesulfonic acid in ethanol followed by heating at *ca.* 120° for 3 to 5 min. This converts them into products which fluoresce moderately to strongly under a lamp emitting maximally at around 360 nm. Compounds bearing the 3β-hydroxy-5-ene system (for example derivative 1) provide visible colors appreciably sooner than their saturated counterparts. Some, but not all, aglycon-conjugate pairs give fluorogens which differ in color.

Paper chromatography employed 19 × 60 cm sheets of Whatman No. 1 paper and was carried out in the descending manner. The considerable polarity of the free

conjugates limited the choice of systems, but two types proved satisfactory, namely one employing ethyl acetate–toluene–aqueous methanol (or aqueous acetic acid) and a second, based on toluene–*tert.*-butanol–aqueous methanol (or aqueous acetic acid). The conjugates were detected by dipping the dried paper in a 10% solution of phosphomolybdic acid in ethanol and heating at *ca.* 90°. However, many of the saturated conjugates give so faint a color as to indicate the need for a more sensitive reagent.

RESULTS AND DISCUSSION

Table II gives R_F values for fourteen glucoside peracetates and the corresponding aglycons when chromatographed on thin layers of silica gel. For purposes of discussion, the compounds may be divided into pairs (2, 3; 4, 5; 7, 8; 9, 10; 11, 12)

TABLE II

THIN-LAYER CHROMATOGRAPHY OF STEROID β-D-GLUCOPYRANOSIDE PERACETATES AND AGLYCONS

Numbers in Tables II–IV are not assigned to individual compounds, but serve as guides between text and tables. Aglycons (free steroids) are given formal names in this and other tables in order better to show steric relationships, and because some lack generally accepted trivial names. The axial (*a*) or equatorial (*e*) orientation of the C-3 hydroxyl group is indicated in parentheses after the name.

No.	*Aglycon*	*System No.*	R_F *values*	
			Derivative	*Aglycon*
1	3β-Hydroxyandrost-5-en-17-one	1	0.16	0.20
2	3α-Hydroxy-5α-androstan-17-one (*a*)	1	0.18	0.24
3	3β-Hydroxy-5α-androstan-17-one (*e*)	1	0.17	0.20
4	3α-Hydroxy-5β-androstan-17-one (*e*)	1	0.18	0.15
5	3β-Hydroxy-5β-androstan-17-one (*a*)	1	0.17	0.24
6	3β-Hydroxypregn-5-en-20-one	1	0.19	0.25
7	3α-Hydroxy-5α-pregnan-20-one (*a*)	1	0.20	0.27
8	3β-Hydroxy-5α-pregnan-20-one (*e*)	1	0.20	0.22
9	3α-Hydroxy-5β-pregnan-20-one (*e*)	1	0.21	0.19
10	3β-Hydroxy-5β-pregnan-20-one (*a*)	1	0.20	0.28
11	3α,17-Dihydroxy-5β-pregnan-20-one (*e*)	2	0.20	0.12
12	3β,17-Dihydroxy-5β-pregnan-20-one (*a*)	2	0.17	0.20
13[a]	Androst-5-ene-3β,17β-diol	1	0.22	0.12
14	Androst-5-ene-3β,17β-diol	1	0.22	0.12

[a] Conjugates 13 and 14 are, respectively, the 3β-yl- and 17β-yl-β-D-glucopyranoside pentaacetates of the diol.

the members of which differ only with respect to the orientation of the C-3 hydroxyl group of the aglycon (equatorial (*e*), in the plane of the ring, or axial (*a*), out of the plane of the ring). As a general (but not invariable) rule in steroid chromatography, that member of a given pair which bears the axially-oriented hydroxyl group is the more mobile[4]. It can be seen from the table that the R_F values derived from the aglycons uniformly adhere to this rule. However, the derivatives do not (one equivocal and two exceptional results in five pairs). Very similar relationships can be seen in Tables II and IV of the earlier study[1] which involved principally the thin-layer chromatography of steroidal glucosiduronic acid derivatives. It is concluded that the

TABLE III

THIN-LAYER CHROMATOGRAPHY OF STEROID β-D-GLUCOPYRANOSIDES AND β-D-GLUCOPYRANOSIDURONIC ACIDS

No.	*Aglycon*	*Glucoside*		*Glucosiduronic acid*	
		System No.	R_F	*System No.*	R_F
15	3β-Hydroxyandrost-5-en-17-one	3	0.17	5	0.15
16	3α-Hydroxy-5α-androstan-17-one (*a*)	3	0.19	5	0.18
17	3β-Hydroxy-5α-androstan-17-one (*e*)	3	0.16	5	0.15
18	3α-Hydroxy-5β-androstan-17-one (*e*)	3	0.18	5	0.14
19	3β-Hydroxy-5β-androstan-17-one (*a*)	3	0.17	5	0.16
20	3β-Hydroxypregn-5-en-20-one	3	0.13	5	0.16
21	3α-Hydroxy-5α-pregnan-20-one (*a*)	3	0.18	5	0.23
22	3β-Hydroxy-5α-pregnan-20-one (*e*)	3	0.13	5	0.18
23	3α-Hydroxy-5β-pregnan-20-one (*e*)	3	0.15	5	0.19
24	3β-Hydroxy-5β-pregnan-20-one (*a*)	3	0.14	—	—
25	3α,17-Dihydroxy-5β-pregnan-20-one (*e*)	4	0.14	6	0.13
26	3β,17-Dihydroxy-5β-pregnan-20-one (*a*)	4	0.20	—	—
27	(22S,25S)-Spirost-5-en-3β-ol	3	0.17	5	0.23
28	(22S,25S)-5α-Spirostan-3β-ol	3	0.17	5	0.23
29	Cholest-5-en-3β-ol	3	0.17	5	0.23
30	5α-Cholestan-3β-ol	3	0.17	5	0.23
31[a]	Androst-5-ene-3β,17β-diol	4	0.15	6	0.17
32	Androst-5-ene-3β,17β-diol	4	0.15	6	0.17

[a] Conjugates 31 and 32 are, respectively, the 3β-yl- and 17β-yl-β-D-glucopyranosides (or the 3β-yl- and 17β-yl-β-D-glucopyranosiduronic acids) of the diol.

axial–equatorial rule cannot be extended to compounds bearing large, polyfunctional groups at C-3 under the conditions prevailing in adsorption chromatography.

It is also to be noted that R_F differences in axial–equatorial pairs are distinctly smaller in derivatives than in the corresponding aglycons. Although these differences can be reproduced (as opposed to R_F values), it is clear that it would be very difficult to separate, as on a column, any of the pairs in this table.

The mobility of a given aglycon relative to the corresponding derivative is variable and unpredictable except in those cases where the aglycon bears an additional functional hydroxyl group (as in pairs 13 and 14), or where the carbohydrate moiety is inherently more polar, as in 2′-acetamido-2′-deoxy-tri-O-acetyl-α(and β)-D-glucopyranosides (see Table V in ref. 1). This is a point of practical importance because such aglycon–derivative mixtures are encountered in the synthesis of glycosides by the Koenigs-Knorr method. It cannot be assumed that substrates bearing a single functional hydroxyl group will conveniently precede or follow the product in the course of column chromatography*.

The R_F values in Table III were derived from eighteen free glucosides and, in most cases, the corresponding glucosiduronic acids. These results roughly parallel those noted in Table II and demonstrate that separations between axial–equatorial or other pairs are not improved by substituting free conjugates for their derivatives.

* As indicated earlier[1] inseparable aglycon–derivative mixtures can be resolved by acetylation followed by re-chromatography. However, it is inadvisable to acetylate the primary reaction mixture prior to its initial column chromatography.

TABLE IV

PAPER CHROMATOGRAPHY OF STEROID β-D-GLUCOPYRANOSIDES, β-D-GLUCOPYRANOSIDURONIC ACIDS AND AGLYCONS

No.	*Aglycon*	*Glucoside*		*Glucosiduronic acid*		*Aglycon*	
		System No.	R_F	*System No.*	R_F	*System No.*	R_F
33	3β-Hydroxyandrost-5-en-17-one	7	0.14	9	0.12	11	0.21
34	3α-Hydroxy-5α-androstan-17-one (*a*)	7	0.25	9	0.21	11	0.38
35	3β-Hydroxy-5α-androstan-17-one (*e*)	7	0.17	9	0.15	11	0.29
36	3α-Hydroxy-5β-androstan-17-one (*e*)	7	0.18	9	0.17	11	0.33
37	3β-Hydroxy-5β-androstan-17-one (*a*)	7	0.23	9	0.21	11	0.40
38	3β-Hydroxypregn-5-en-20-one	8	0.11	10	0.11	12	0.25
39	3α-Hydroxy-5α-pregnan-20-one (*a*)	8	0.22	10	0.22	12	0.40
40	3β-Hydroxy-5α-pregnan-20-one (*e*)	8	0.14	10	0.12	12	0.26
41	3α-Hydroxy-5β-pregnan-20-one (*e*)	8	0.16	10	0.14	12	0.26
42	3β-Hydroxy-5β-pregnan-20-one (*a*)	8	0.19	—	—	12	0.31
43	(22S,25S)-Spirost-5-en-3β-ol	8	0.17	10	0.17		
44	(22S,25S)-5α-Spirostan-3β-ol	8	0.18	10	0.19		
45	Cholest-5-en-3β-ol	8	0.21	10	0.21		
46	5α-Cholestan-3β-ol	8	0.21	10	0.24		

However, thin-layer chromatography in ethanol–chloroform systems remains an excellent method for detecting glucosides (as in extracts from biological systems), and the chief utility of Table III is therefore to record representative R_F values in this type of system*.

It was noted in Table II that the epimeric diol glucoside pentaacetates 13 and 14 were not separated, and a recent attempt to distinguish between the corresponding glucosiduronic acid derivatives was equally unsuccessful. The values in Table III (31 and 32) show, not surprisingly, that the free conjugates are equally difficult to separate. It seems reasonable to conclude in this case that a given carbohydrate moiety provides the same contribution to polarity at either the C-3 or C-17 sites.

The complete lack of separation between members of pairs 27, 28 and 29, 30 is difficult to understand when it is considered that the structural difference in each case (the 3β-hydroxy-5-ene system *vs.* the 3β-hydroxy-A/B-*trans* (5α) arrangement) is common also to pairs 15, 16 and 20, 21 which are to a degree separated. These results suggest that the large side chains of the spirostan(ene) and cholestan(ene) conjugates interfere with those adsorption/elution processes on which separation depends.

Finally, it was not possible to separate mixtures of free glucosides and glucosiduronic acids by thin-layer chromatography in ethanol–chloroform–acetic acid systems. It was observed earlier[1] that their derivatives have virtually identical R_F values in neutral systems.

* As an aid in detecting glucosides under circumstances where only the aglycon is available, each pair was simultaneously chromatographed in ethanol–chloroform systems so adjusted as to provide R_F (glucoside) values of about 0.1. $\Delta R_{M(\text{glucosidation})}$ values calculated from these data varied from 0.095 to 1.16 and averaged 1.08.

It is to be noted that ethanol–chloroform systems are limited in application to weakly or moderately polar glucosides, that is monosides in which the aglycon bears at most three hydroxyl groups. Our experience to date relative to adsorption chromatography suggests that, once detected, the glucoside is best recovered by acetylation followed by chromatography on silica gel using an ethyl acetate–isooctane or similar system. Ref. 5 furnishes an example.

Table IV presents the results of a study in which suitable* free glucosides, glucosiduronic acids and the corresponding aglycons were chromatographed on paper using similar systems. The results are superior to those obtained using thin-layer chromatography. Considered in terms of axial–equatorial pairs within the conjugates proper, R_F differences average 0.06; this does not approach the averaged value of 0.09 obtained in the case of the aglycons, but is sufficiently large to assure separations on partition-type columns in most cases. Secondly, the axial–equatorial relative mobility rule is adhered to by conjugates and aglycons alike. Finally, true separations of glucoside–glucosiduronic acid mixtures can be obtained. For example, R_F values for the 20-ketopregnanes 38–41 in system 13 are: 0.15, 0.08; 0.28, 0.16; 0.19, 0.10; 0.19, 0.10 (R_F values for the uronic acids are the second in each pair).

It was stated over a decade ago (and in rather lofty terms considering the limited evidence available) that "the polar conjugating group does not cancel those influences which the (remaining) functional groups in the steroid nucleus or side chain have on partitioning processes. Thus in appropriate systems dehydroisoandrosterone, etiocholanolone and androsterone glucuronides move in the same order and are separated about as well as the corresponding free compounds"[6]. This generalization expresses the net observed effect of various factors, including the configuration of the substituent at C-3 of the steroid moiety, on relative mobilities within a given series. The paper chromatographic results in Table IV substantiate this proposition, but it is equally clear, from the relative R_F values in Tables II and III, that it is invalid under the conditions prevailing in adsorption chromatography.

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CHROM. 5053

PAPER ELECTROPHORESIS OF BINARY MIXTURES OF COPPER(II) HISTIDINATE AND COPPER(II) COMPLEXES OF OTHER AMINO ACIDS

THE NATURE OF THE "THIRD SPOT"

J. L. FRAHN
Division of Nutritional Biochemistry, C.S.I.R.O., Adelaide, South Australia 5000 (Australia)

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SUMMARY

The supernumerary (third) spots which separate when binary mixtures of copper(II) complexes of histidine and some other amino acids are subjected to paper electrophoresis were re-examined. It is concluded that, in the early stages of electrophoresis, the spots consist of mixed-ligand complexes composed of copper(II), histidine and other amino acid and that, as electrophoresis proceeds, the complexes undergo two different reactions, simultaneously. A portion of them re-equilibrates to form homogeneous copper(II) complexes of each of their constituent amino acids, while the remainder decomposes to the relatively stable 1:1 histidine–copper(II) complex and the other amino acid which is liberated as the free, uncomplexed ion. This interpretation is based on electrophoresis in mildly alkaline electrolytes of mixtures containing copper(II) histidinate and the copper(II) complex of either an acidic or a basic amino acid.

INTRODUCTION

WIELAND AND FISCHER[1] have reported the formation of a "third spot" during the paper electrophoretic separation of a mixture of the copper(II) complexes of histidine and lysine, and suggested that the spot consisted of the mixed-ligand complex, His–Cu(II)–Lys*. More recently, JURSÍK AND PETRŮ[2] found that a third spot also formed when mixtures of copper(II) complexes of L-histidine and other basic amino acids (L-ornithine and L-arginine) were subjected to electrophoresis, and they attributed its formation to a coulombic attraction between a free carboxylate group of the His_2–Cu(II) complex and the free ω-amino groups of the copper(II) complexes of the basic amino acids. Although much evidence now exists for the presence of free carboxylate groups in copper(II)–histidine complexes[3,4], it seems unlikely that an

* The complexes are conveniently formulated in this way using the following abbreviations: His = DL-histidine; Lys = L-lysine; Cyst = L-cysteic acid; Arg = L-arginine; Orn = DL-ornithine; Glu = L-glutamic acid; Asp = DL-aspartic acid.

ion-pair resulting from the proposed coulombic attraction would survive the process of electrophoresis as an isolated system in an indifferent electrolyte. It is true, however, that ion-pair formation has been demonstrated by paper electrophoresis[5–8], but only when one of the ions involved constituted part of a background electrolyte.

A third spot also formed during the electrophoresis of a mixture of the copper(II) complexes of histidine and glutamic acid[1] and, in the present work, His_2–Cu(II) was found to behave similarly in the presence of the copper(II) complexes of other acidic amino acids (DL-aspartic and L-cysteic acids)*. Clearly, ion-pair formation involving a free carboxylate group of the His_2–Cu(II) complex cannot be invoked to explain the supernumerary spots formed in the presence of the acidic amino acids. The results of the present work can be interpreted on the basis of the original suggestion of WIELAND AND FISCHER[1]. The third spot forming in each case appears to be due, primarily, to a mixed-ligand complex composed of copper(II), histidine and the other amino acid, but the complex re-equilibrates partially during electrophoresis to form homogeneous copper(II) complexes of each of its constituent amino acids. Simultaneously, a portion of it appears to decompose to the His-Cu(II) 1:1 complex and the other amino acid which is liberated as a free, uncomplexed ion. The latter reaction may occur predominantly during the separation of mixtures of the copper(II) complexes of histidine and the basic amino acids.

EXPERIMENTAL

Materials

Commercial samples of the amino acids, DL-histidine monohydrochloride, DL-ornithine monohydrobromide and DL-aspartic acid (B.D.H.), L-glutamic acid (Sigma Chemical Company), L-arginine monohydrochloride (L. Light & Co.), L-lysine monohydrochloride (E. Merck, Darmstadt) and L-cysteic acid (Calbiochem, Inc.) each gave a single spot on paper electrophoresis in borate buffer (pH 9.2). Cupric acetate (B.D.H.) was of analytical reagent grade.

Preparation of solutions of the copper(II) complexes. Solutions (0.05 *M*) of the 2:1 amino acid–copper(II) complexes were prepared by dissolving the calculated quantities of cupric acetate and an amino acid in a little water, adding 1 *N* NaOH to pH 9, and diluting appropriately with water.

Each of the required binary mixtures was prepared by dissolving calculated quantities of cupric acetate, histidine hydrochloride and another of the above selection of amino acids in a little water and bringing the solution to pH $\simeq$ 9 by the addition of 1 *N* NaOH. Sufficient water was then added to make the solutions 0.1 *M* with respect to each amino acid and to copper(II). Amino acid–copper(II) 2:1 complexes were thus formed in solution, each in 0.05 *M* concentration.

A solution containing the His-Cu(II) 1:1 complex (0.1 *M* with respect to copper(II) and to histidine) was prepared by dissolving the calculated quantities of

* The third spot separating from these mixtures is not related to the supernumerary, copper-positive spot which separates from copper(II) salts of copper(II) complexes of the acidic amino acids[2] *e.g.* Cu(II)[Glu_2-Cu(II)]. In these compounds, the amino acid and copper(II) are present in 1:1 ratio, but only half the copper(II) is actually combined as the complex. The work described here was conducted with mixtures containing the acidic amino acids and copper(II) in 2:1 ratio and the supernumerary spots which separated from appropriate mixtures contained both copper(II) and amino acids.

cupric acetate and histidine hydrochloride in about 3/4 of the necessary volume of water. The addition of 1 *N* NaOH to pH $\simeq$ 7.5 caused a slight precipitate to appear which would not redissolve on shaking or by gentle warming. Water was then added to make the solution up to the required volume.

The continued addition of 1 *N* NaOH to such a solution (pH $\simeq$ 7.5) was accompanied, at first, by the appearance of a heavy precipitate. This redissolved completely on shaking, to give an intense blue-green solution when pH values a little in excess of 11 were reached (*cf.* ref. 9).

Both of the solutions (pH $\simeq$ 7.5 and pH $>$ 11) were used to demonstrate the electrophoretic properties of the His–Cu(II) 1:1 complex and gave comparable results when borate buffer was used as the electrolyte.

Electrolytes

The following electrolytes were used: (a) sodium borate buffer (pH 9.2) containing 0.2 gram-atom of boron per litre[10]; (b) acetate buffer (pH 4.6) containing 6.39 g $CH_3COONa \cdot 3H_2O$ and 3.2 g glacial acetic acid in 1 l of water. The solution was 0.1 *M* with respect to total acetate.

Spray reagents

The spray reagents used were: (A) ninhydrin (0.2 g) was dissolved in 96 ml *n*-butanol previously saturated with water, and 4 ml glacial acetic acid was added to the solution; (B) rubeanic acid (0.1 g) dissolved in 100 ml methanol.

Apparatus

Paper electrophoresis was conducted in the enclosed-strip apparatus described previously[11] using Whatman No. 4 paper in strips 13.5 $\times$ 61 cm, with 45 cm under pressure and cooled. Mains water at 18° was circulated through the coils of the cooling-plate and maintained the temperature of the paper at about 20°.

Procedures

General. Samples (0.5 μl) of the copper(II) complexes and other solutions were transferred by means of a platinum loop to papers impregnated with the borate electrolyte, and equilibrated for 15 min by enclosure within the apparatus. Caffeine was used as marker for zero migration without serious error[12], and rates of migration of test compounds were calculated relative to that of nitrobenzene-*p*-sulphonate applied to the same paper (M_N values[10]).

Electrophoresis was normally allowed to proceed for 1 h at about 21 V/cm. (Nitrobenzene-*p*-sulphonate moved 11.3 cm under these conditions.)

The papers were dried in the oven at 100°. Caffeine, nitrobenzene-*p*-sulphonate and copper(II) were located as dark blue spots under a Hanovia "Chromatolite" ultraviolet lamp.

The location of copper(II) was confirmed by spraying papers with rubeanic acid; the amino acids were detected with the ninhydrin reagent. Histidine in low concentrations on papers was difficult to detect in the presence of copper(II) unless the complex was first destroyed. The complex was effectively destroyed and free histidine liberated by brief exposure of partially dried papers to H_2S gas. Alternatively, papers were treated first with rubeanic acid, redried, sprayed with the ninhydrin reagent and then heated at 100° to develop the colour.

Procedure A. Identification of the contents of the spots and streaks which separate when a mixture of copper(II) complexes of DL-*histidine and* L-*cysteic acid is subjected to paper electrophoresis in borate buffer.* A solution containing a mixture of the copper-(II) complexes of histidine and cysteic acid was applied to the starting-line of a paper impregnated with the borate buffer, and electrophoresis was conducted for 30 min. The paper was partially dried in the oven, exposed to H_2S gas to precipitate copper(II) as CuS and liberate the amino acids. The pherogram was then dried completely, and one lane cut from it was lightly sprayed with the sodium acetate electrolyte (pH 4.6) and laid transversely across the middle of a fresh paper previously impregnated with the sodium acetate electrolyte and inserted in another apparatus. The assembly is represented in Fig. 2 where the lane removed from the borate pherogram is represented by the narrow strip, the original anode lying beyond the end marked "A". For reference, a solution containing histidine and cysteic acid (0.05 *M* with respect to each) was applied to the point marked "X", and electrophoresis was carried out for 25 min at 21 V/cm.

The second separation thus took place in a direction at right angles to the first. The paper, dried and treated with the ninhydrin reagent, showed that the reference mixture of amino acids had separated cleanly, cysteic acid migrating toward the new anode and histidine toward the cathode. The amino acids present in the three spots, as well as in the interconnecting streaks, on the lane of the original (borate) pherogram were thus readily identified. The spots and streaks still evident on the lane of the original pherogram are due to CuS precipitated on the cellulose fibres during the exposure of the pherogram to H_2S gas.

Procedure B. Formation of His_2*–Cu(II) by* in situ *reaction of the contents of the separated third spot with free* DL-*histidine applied directly to the pherogram.* A paper strip (which resulted in the pherogram reproduced in Fig. 3) was prepared for the experiment by drawing across it the line Y 2.4 cm to the anode side of line X which was to serve as the starting-line. The paper was impregnated with borate electrolyte and inserted in the apparatus; samples of the mixture of the copper(II) complexes of histidine and cysteic acid were applied to line X in lanes 1, 4, 5, 6, 7 and 9, and electrophoresis was conducted for 45 min. It had been ascertained, by means of a previous experiment, that the centres of the separated third spots would then coincide with the points at which line Y intersected the respective lanes. Electrophoresis was interrupted at this stage while the following solutions were applied to line Y in the lanes specified: histidine (0.05 *M*) in lanes 2, 4 and 6; histidine (0.01 *M*) in lanes 5 and 7; and the His_2–Cu(II) complex (0.05 *M*) in lanes 3 and 8.

Electrophoresis was resumed for 45 min after which the paper was partially dried and cut longitudinally into two strips containing, respectively, lanes 1–5 and lanes 6–9. The former was exposed to H_2S gas, completely dried, and treated with the ninhydrin reagent. The dried strip containing lanes 6–9 was sprayed with rubeanic acid.

RESULTS AND DISCUSSION

The phenomenon under review was found by WIELAND AND FISCHER[1] to occur during electrophoresis in sodium acetate electrolyte (0.1 *M*; pH 7.5), and JURSÍK AND PETRŮ[2] subsequently used the same electrolyte for their work, reporting that

the phenomenon was observed only at pH 7.5 and depended upon the composition of the buffer used. Sodium acetate solutions in this pH range have little or no buffering capacity, however. Hydroxyl ions present, for example, in 0.5 μl of 0.1 N NaOH solution applied to papers impregnated with the electrolyte (pH 7.5) survived as discrete spots or streaks after electrophoresis for more than 1 h, and it is believed that hydroxyl ions contained in the more alkaline test solutions used in the present work caused distortion of some pherograms, making interpretation of the results difficult. Furthermore, some amino acids such as histidine could be made to streak toward one or the other electrode according to the pH of the applied solutions. The poor buffering capacity of the sodium acetate electrolyte was also evident when a mixture of histidine and lysine containing copper(II) equivalent to only one half of the total amino acids present was subjected to electrophoresis. The appearance of the third spot was found to be dependent on the pH of the applied solutions. Only those solutions in excess of about pH 8.5 yielded the third spot. JURSÍK AND PETRŮ[2]

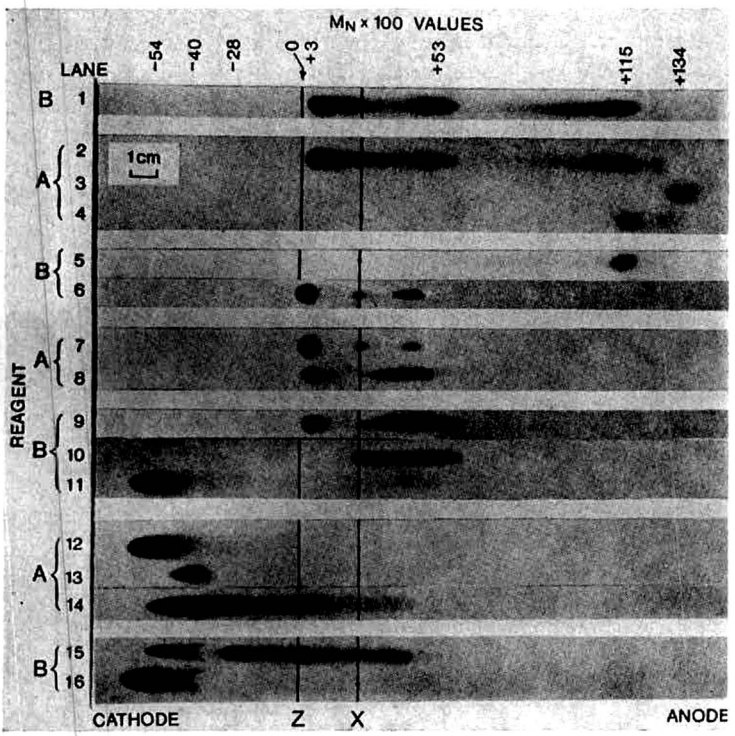


Fig. 1. Results of the paper electrophoresis of some amino acid–copper(II) complexes and mixtures thereof in borate buffer for 1 h at 20 V/cm and 20°. Solutions (0.05 M) of the following were applied to the starting line, X, as specified. Lanes 1,2: mixture of His_2–Cu(II) and $Cyst_2$–Cu(II); lane 3: L-cysteic acid; lanes 4,5: $Cyst_2$–Cu(II); lanes 6,7: His_2–Cu(II); lanes 8,9: the preparation, of pH > 11, of His–Cu(II) (1:1); lane 10: cupric acetate; lanes 11,12: Arg_2–Cu(II); lane 13: L-arginine; lanes 14,15: mixture of His_2–Cu(II) and Arg_2–Cu(II); lane 16: Arg_2–Cu(II) (0.05 M) containing free L-arginine (0.05 M). The dried papers were cut into strips and sprayed, as indicated, with either reagent A (ninhydrin) or reagent B (rubeanic acid) for the detection of amino acids and copper(II). The extent of the electroendosmotic flow, using caffeine as marker, is indicated by line Z. M_N values[10] express mobilities relative to the nitrobenzene-p-sulphonate ion (not shown on the pherograms).

reported that a third spot separated from a mixture of copper(II) histidinate and lysine but not from a mixture of copper(II) lysinate and histidine. The pH value of the latter was not specified, but it is possible that this puzzling result is explained by the above finding.

Other electrolytes with better buffering properties were therefore sought and, contrary to the experience of JURSÍK AND PETRČ[2], a third spot was found to separate from appropriate mixtures of the amino acid–copper(II) complexes at pH values considerably in excess of 7.5 and in electrolytes other than sodium acetate. Carbamation of the amino acids caused complications however, when carbonate electrolytes were used[11,13], but carbamation does not occur in the presence of the borate buffer (pH 9.2) described in the EXPERIMENTAL section, and this was used for all the separations of copper(II) complexes reported here. Similar borate buffers were selected by SZWAJ AND KAŃSKI[14] for use in a related study.

The results of the electrophoresis of some copper(II)–amino acid complexes and mixtures thereof are shown in Fig. 1. The effect of the presence of $Cyst_2$–Cu(II) on the electrophoresis of His_2–Cu(II) (lanes 1 and 2) is typical of the complexes of other acidic amino acids (glutamic and aspartic acids), the third spot being anionic, in each case, and lying between the spots representing His_2–Cu(II) and the complex of the other amino acid*. The pherogram resulting from the separation of mixtures of the copper(II) complexes of arginine and histidine (lanes 14 and 15) exemplifies those obtained when the complexes of other basic amino acids replace that of arginine in the mixture, the third spot now being of intermediate cationic mobility. The

TABLE I

RELATIVE RATES OF MIGRATION OF THE AMINO ACIDS, THEIR COPPER(II) COMPLEXES, AND THE "THIRD SPOTS" WHICH SEPARATE FROM APPROPRIATE MIXTURES OF THE COMPLEXES

Compounds were detected after paper electrophoresis in sodium borate electrolyte (pH 9.2) at 21 V/cm and 20° for 1 h.

Pure compounds	$M_N \times 100$[a]	*Mixtures*	*"Third spot"* $M_N \times 100$
DL-Histidine	+ 26		
His_2–Cu(II)	+ 3		
His–Cu(II) (1:1)	+ 46		
L-Cysteic acid	+134		
$Cyst_2$–Cu(II)	+115	His_2–Cu(II) + $Cyst_2$–Cu(II)	+53
L-Glutamic acid	+ 92		
Glu_2–Cu(II)	+101	His_2–Cu(II) + Glu_2–Cu(II)	+50
DL-Aspartic acid	+ 99		
Asp_2–Cu(II)	+105	His_2–Cu(II) + Asp_2–Cu(II)	+51
L-Arginine	− 40		
Arg_2–Cu(II)	− 54	His_2–Cu(II) + Arg_2–Cu(II)	−28
DL-Ornithine	− 30		
Orn_2–Cu(II)	− 54	His_2–Cu(II) + Orn_2–Cu(II)	−33
L-Lysine	− 48		
Lys_2–Cu(II)	− 67	His_2–Cu(II) + Lys_2–Cu(II)	−37

[a] M_N values[10] express mobilities relative to the nitrobenzene-*p*-sulphonate ion, which moved 11.3 cm. Positive values represent anionic, negative values cationic mobilities.

* A third spot also separates from a mixture of copper(II) citrate and His_2–Cu(II) showing that the phenomenon is not limited to mixtures containing only amino acid ligands.

mobilities (as $M_N \times 100$ values) of the ionic species studied are given in Table I, positive values representing anionic mobilities, negative values cationic mobilities. The mobilities included in Fig. 1 serve to identify the respective spots.

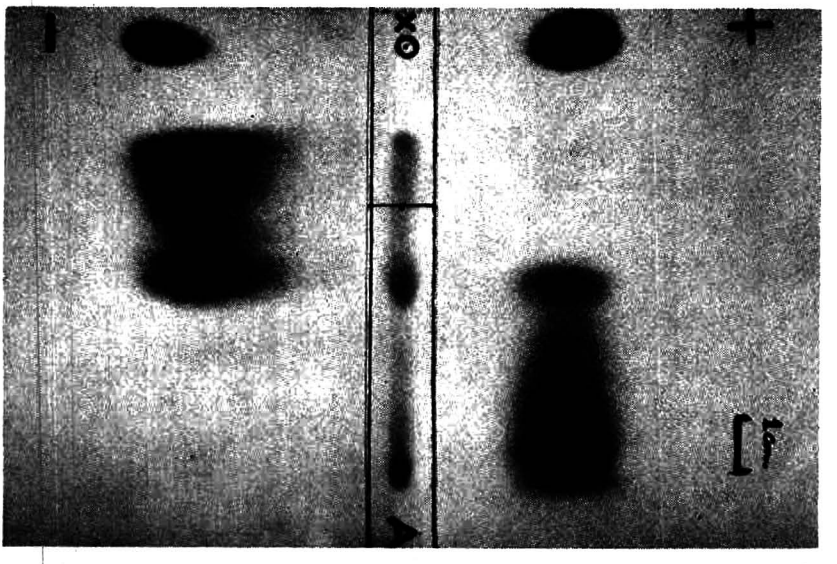


Fig. 2. Identification of the contents of the spots and streaks which separate when a mixture of the copper(II) complexes of DL-histidine and L-cysteic acid is subjected to paper electrophoresis in borate buffer for 30 min (procedure A, EXPERIMENTAL section). The lane removed from the borate pherogram is represented by the narrow strip, the original anode lying beyond the end marked "A". The spots and streaks still evident on the lane of the original pherogram are due to CuS precipitated there during the exposure of the paper to H_2S gas. The fastest (most anionic spot) of the borate pherogram is shown to contain copper(II) and cysteic acid, the slowest spot, copper(II) and histidine, and the intermediate (third) spot, a mixture of copper(II), histidine and cysteic acid. The streaks are shown to contain histidine (to the cathode side of the third spot) and cysteic acid (to the anode side) and both streaks also contain copper(II).

During the earlier stages of electrophoresis, the third spot separating from any mixture is connected on each side by streaks to the spots representing the copper(II) complex of histidine and of the other amino acid. When the other amino acid is cysteic acid, the streaks contain histidine (to the cathode side of the third spot) and cysteic acid (to the anode side) and both streaks also contain copper(II). The third spot contains a mixture of histidine, cysteic acid and copper(II). These facts were demonstrated by means of procedure A, outlined in the EXPERIMENTAL section, and the results of a typical experiment are reproduced in Fig. 2. In contrast to the streaks which form when a mixture of the complexes is subjected to electrophoresis, the complexes of cysteic acid and histidine, applied separately, migrate as compact spots as shown in Fig. 1, lanes 4, 5, 6 and 7. (A little free cysteic acid is seen to have separated, however, from the $Cyst_2$–Cu(II) complex.)

The above facts can be interpreted on the assumption that, in the early stages of electrophoresis, the third spot consists, at least partially, of a mixed-ligand complex of the type originally proposed by WIELAND AND FISCHER[1], and that this complex (for example, His–Cu(II)–Cyst) rearranges progressively, according to equilibrium (1).

$$2\ \text{His–Cu(II)–Cyst} \rightleftharpoons \text{His}_2\text{–Cu(II)} + \text{Cyst}_2\text{–Cu(II)} \quad (1)$$

The products of the rearrangement, the homogeneous amino acid–copper(II) complexes, then migrate away from the third spot with characteristic mobilities, giving rise to the observed streaks. As the electrophoresis proceeds, it is observed, again by means of procedure A, that the cysteic acid content of the third spot diminishes at a greater rate than that of histidine until, after 45 min, only histidine and copper(II) remain as detectable components of the third spot. (This now gives the slate-grey ninhydrin reaction typical of histidine as detected under our conditions.) This observation is not due to a greater sensitivity of histidine than cysteic acid to the ninhydrin reagent. In fact, the limits of detectability of these amino acids are almost identical, being equivalent to about 0.7 nmole of each per cm^2 of spot area.

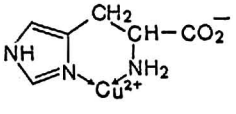
Although the composition of the third spot changes in this way during the course of electrophoresis, its mobility (M_N *ca.* 0.5) remains constant within the limits of experimental error. Evidently, the histidine and copper(II) which remain as the only detectable components of the third spot are not combined as the usual 2:1 complex, the mobility of which is quite different (*viz.*, M_N 0.03).

It is reasonable to assume that the elongated anionic spot which separates from mixtures containing histidine and copper(II) in equimolecular ratio (Fig. 1, lanes 8 and 9) is due to the His–Cu(II) 1:1 complex, and it has been designated as such in Table I. The complex is partially adsorbed by the cellulose support but the mobility, M_N 0.46, corresponding to the head of the elongated spot, probably approaches the true value for its mobility. This is comparable, therefore, with the mobility of the above third spot as well as with those separating from mixtures containing the copper(II) complexes of the other acidic amino acids (see Table I). The contents of the third spot separating from each of these mixtures tend to streak, indicating that they too, are partially adsorbed by the cellulose support. It is therefore suggested that, in the later stages of the electrophoretic separations, the third spot consists of the His–Cu(II) 1:1 complex and that the rearrangement described above (equilibrium (1)) occurs concurrently with the partial decomposition of the mixed-ligand complex represented by equilibrium (2).

$$\text{His–Cu(II)–Cyst} \rightleftharpoons \text{His–Cu(II)} + \text{Cyst} \quad (2)$$

MALEY AND MELLOR[15] have noted that copper(II) complexes much more strongly with histidine than with some other amino acids. It is not surprising, therefore, that the decomposition of the mixed-ligand complex, His–Cu(II)–Cyst involves the dissociation of the cysteic acid rather than the histidine moiety. The cysteic acid thus progressively liberated migrates rapidly toward the anode and ultimately leaves a rather stable residue of the His–Cu(II) 1:1 complex as the sole constituent of the third spot. This survives electrophoresis as a definite spot at the head of a faint streak for prolonged periods and is readily detectable after runs of 3 h or more.

The structure below is one of several which have been proposed for the 1:1 com-



plex and it has been shown to be a relatively stable species[16–19]. It has a formal net charge of +1, yet it behaves as an anion (Fig. 1, lanes 8 and 9). LEBERMAN AND RABIN[16,18] have postulated from their titration data that the 1:1 copper (II)complex takes up an hydroxyl ion from solution to form a neutral (zwitter-ionic) species. It appears, under the present conditions, to take up at least two hydroxyl ions from the alkaline electrolyte to acquire a net negative charge. It thus resembles the free cupric ion which is also anionic in the borate buffer, as indicated by the streak shown in Fig. 1, lane 10. This behaviour, typical of the cupric ion in other mildly alkaline electrolytes, is probably due to the ionisation of an aquo–metal complex of copper(II) (ref. 20) or to the presence of cuprate or bicuprate ions in the alkaline medium[21].

PERRIN[17] has concluded that the His–Cu(II) 1:1 complex is present in significant amounts in neutral and alkaline solutions containing the normal 2:1 complex and this is confirmed by the results shown in Fig. 1, lanes 6 and 7. The 1:1 complex present in the His_2–Cu(II) preparation has survived the process of electrophoresis to separate as a small, somewhat elongated anionic spot of characteristic mobility. YOSHINO AND MAKI[22] have made similar observations which led them also to suggest

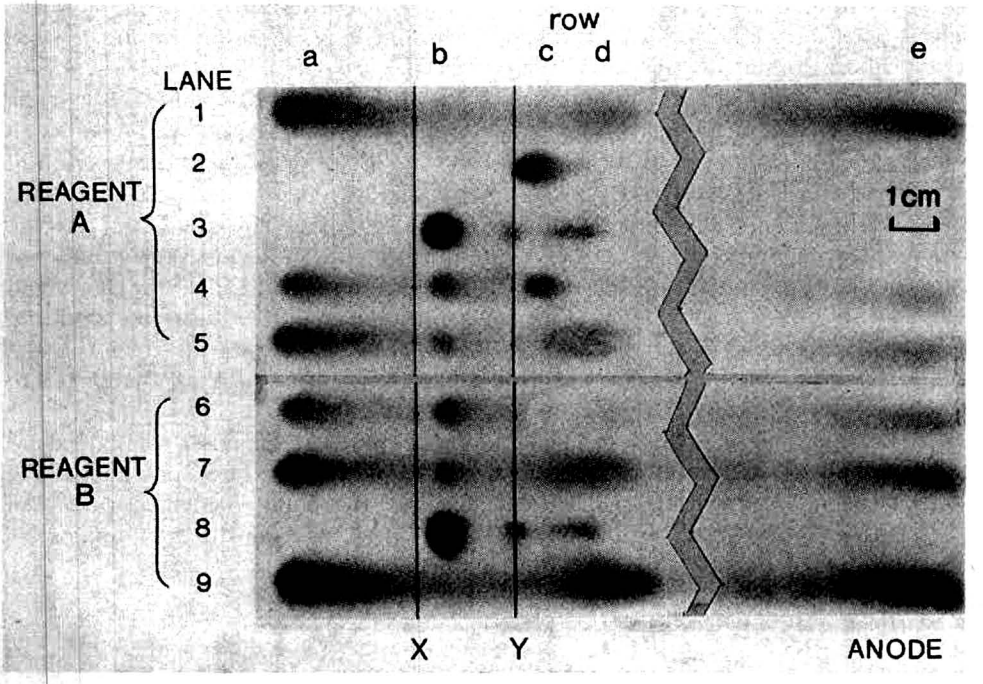


Fig. 3. Formation of His_2–Cu(II) by *in situ* reaction of the contents of the separated third spot with free DL-histidine applied directly to the pherogram (procedure B, EXPERIMENTAL section). A solution containing the mixture, His_2–Cu(II) and $Cyst_2$–Cu(II), was applied to the starting-line, X, in lanes 1, 4, 5, 6, 7 and 9, and subjected to electrophoresis for 45 min. The experiment was interrupted and the following solutions applied to line Y in the lanes specified: histidine (0.05 *M*) in lanes 2, 4 and 6, histidine (0.01 *M*) in lanes 5 and 7, and His_2–Cu(II) (0.05 *M*) in lanes 3 and 8. Electrophoresis was resumed for 45 min after which the paper was divided into two strips containing, respectively, lanes 1–5 and lanes 6–9. The amino acids were located in lanes 1–5 with reagent A (ninhydrin) and copper(II) in lanes 6–9 using reagent B (rubeanic acid).

that the 1:1 complex, co-ordinating with two hydroxyl ions, is present in alkaline solutions of the 2:1 complex.

Confirmation of the identity of the third spot was obtained from the results of procedure B, described in the EXPERIMENTAL section. The initial period of 45 min of electrophoresis brought about the separation of the third spot from the mixture of copper(II) complexes of histidine and cysteic acid which was applied to the starting-line, X, of the pherogram (Fig. 3) in the lanes specified. (It will be recalled that the results of procedure A, discussed above, showed that electrophoresis for a period of 45 min sufficed to yield a third spot which was entirely devoid of cysteic acid and contained only copper(II) and histidine.) The centres of the separated third spots, at the end of the initial period of electrophoresis, coincided with the points at which line Y intersects the respective lanes. The third spot contents were, therefore, effectively mixed with free histidine by interrupting the electrophoresis at this stage and applying the histidine solutions (0.05 *M* and 0.01 *M*) to line Y, as indicated. The resumption of electrophoresis for 45 min then resulted in the pherogram reproduced in Fig. 3 which, to facilitate discussion, is subdivided into the transverse rows a, b, c, d, and e, each containing spots of identical mobility. Rows a and e contain, respectively, His_2–Cu(II) and $Cyst_2$–Cu(II) and row d contains the third spot which separates from the mixture in the normal course of events, as shown in "control" lanes 1 and 9. Lanes 4, 5, 6 and 7 in which histidine was superimposed on the third spot at line Y contain a spot (row b) the mobility (M_X 0.03) of which is identical with that of His_2–Cu(II). Furthermore, its reactions to ninhydrin and rubeanic acid are identical with the reactions of the 2:1 complex; the comparison was made with samples of the latter applied to line Y (lanes 3 and 8) prior to the second period of electrophoresis. The spot in row b was therefore attributed to the 2:1 complex formed by *in situ* reaction on the pherogram between the contents of the third spot and the added histidine. Moreover, the spot in row b represents the only detectable product of the reaction, other spots (row d, lanes 5 and 7) being due to third spot contents which remained in excess after reaction with the more dilute (0.01 *M*) histidine solution. The spot (row c, lane 4) represents excess histidine applied to the third spot as the 0.05 *M* solution. (The movement of free histidine, applied to line Y and subjected to the second period of electrophoresis, is shown in lane 2.)

These findings are consistent with the conclusion that the His–Cu(II) 1:1 complex gradually accumulates as the main constituent of the third spot during the course of the electrophoretic separations. The spot contents would be expected to react with free histidine, according to equilibrium (3), to form the 2:1 complex, as observed.

$$\text{His–Cu(II)} + \text{His} \rightleftharpoons \text{His}_2\text{–Cu(II)} \qquad (3)$$

Electrophoresis of a mixture of the copper(II) complexes of arginine and histidine results in the separation of four spots (Fig. 1, lanes 14 and 15). The most anionic of these is perhaps more accurately described as the head of an undifferentiated streak. This extends back to the spot containing the His_2–Cu(II) complex, and the entire streak was shown, by a method similar to procedure B, to consist of the 1:1 complex. Some of this complex probably forms in the original mixture by a reaction analogous to that represented by equilibrium (2) and/or according to equilibrium (4).

$$Arg_2\text{–}Cu(II) + His_2\text{–}Cu(II) \rightleftharpoons 2His\text{–}Cu(II) + 2Arg \quad (4)$$

The formation of the 1:1 complex is thus accompanied by the liberation of free arginine, the presence of which was deduced from the following observations.

Unlike the copper(II) complexes of the acidic amino acids, the cationic copper complexes of arginine and other basic amino acids form comet-shaped spots and have a strong tendency to streak, as shown in lanes 11 and 12. Streaking of copper(II) does not occur, however, when the arginine complex is subjected to electrophoresis in the presence of free arginine. Copper(II) is then detected as a somewhat elongated spot with a concave trailing edge, as shown in lane 16, the characteristic shape of the spot being due to free arginine complexing with residual, streaked copper(II) and "sweeping" it along the lane in the wake of the main spot of the complex. (Lane 13 shows free arginine migrating as a compact spot rather less rapidly than its copper(II) complex.) The most cationic spot separating from the mixture of copper(II) complexes of arginine and histidine is due to the arginine complex and, when sprayed with rubeanic acid, it displays a concave trailing edge similar in appearance to the spot in lane 16. The spot adopts this characteristic shape even during the early stages of the separation, suggesting the presence of free arginine in the original mixture as applied. Although the space between this spot and the third spot is almost devoid of copper(II) at all times during the experiment, the amount of free arginine in the space gradually increases, indicating that only arginine, and none or little of its copper(II) complex, is liberated from the third spot. Inspection of lane 14 shows the liberated arginine as a node within the streak between the third spot and the Arg_2–Cu(II) spot. Assuming again that the third spot consists of a mixed-ligand complex, in this case His–Cu(II)–Arg, the reaction would be represented by equilibrium (5), analogous to equilibrium (2).

$$His\text{–}Cu(II)\text{–}Arg \rightleftharpoons His\text{–}Cu(II) + Arg \quad (5)$$

Being anionic, the His–Cu(II) 1:1 complex simultaneously formed during the experiment migrates back toward the origin, accounting for the heavy streak connecting the third spot with that due to the His_2–Cu(II) 2:1 complex. The contents of the third spot are thus gradually lost to this anionic streak as well as to the growing cationic spot of arginine, and when electrophoresis has proceeded for 90 min the third spot is no longer recognisable as such. It is then represented only by a tapering tail to the anionic streak.

Prolonged electrophoresis of mixtures containing the copper(II) complexes of basic amino acids thus results in the disappearance of the third spot*. It therefore differs from the rather stable third spot which separates from mixtures of the complexes of histidine and the acidic amino acids, the persistence of the spot in the latter case being aided by the fact that the His–Cu(II) 1:1 complex and the mixed-ligand complexes containing acidic amino acids are all anions of similar mobility.

* A mixture containing the copper(II) complex of ornithine, however, yields a much more stable third spot than those containing complexes of the other basic amino acids. The cationic mobility of the mixed-ligand complex, His–Cu(II)–Orn, which constitutes this third spot, is about the same as that of free ornithine; their M_N values are −0.33 and −0.3, respectively. Migrating in the presence of free amino acid, the mixed-ligand complex tends to be stabilized against decomposition of the kind represented in equilibrium (5).

Experimental evidence for the existence of mixed-ligand complexes of copper(II) containing two different amino acids has also been obtained by SARKAR and coworkers[23–25], and it is noteworthy that these complexes contain histidine as a common component. Other workers have obtained similar complexes in crystalline form[26]. Complexes of copper(II) containing mixed chelating ligands, only one of which is an amino acid, and yet others in which neither ligand is an amino acid, are well known and many of these also have been obtained in crystalline form[27,28].

In our experience, mixed-ligand copper(II) complexes containing histidine are unstable in aqueous medium and attempts to isolate them result in their decomposition according to the equilibria defined above. Their ultimate decomposition to the His–Cu(II) 1:1 complex is most easily demonstrated, under our conditions of electrophoresis, with those containing the acidic amino acids. Under other conditions of electrophoresis, however, it may be possible to obtain a stable spot consisting solely of the His–Cu(II) 1:1 complex from a mixed-ligand complex containing a basic amino acid, and this appears to have been achieved by JURSÍK AND PETRŮ[2]. Their results are consistent, therefore, with the present concept of the nature of the supernumerary spots and of the equilibria existing in the mixtures which give rise to them.

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ZONENFORMEN BEI DER IONOPHORESE

W. PREETZ UND H. HOMBORG
Institut für Analytische Chemie und Radiochemie der Universität des Saarlandes, 66 Saarbrücken (B.R.D.)

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SUMMARY

Formation of zones during ionophoresis

The formation of nonsymmetrical zone profiles during ionophoresis on carrier material as well as in free solution can be explained on the basis of concentration-dependent equilibria. The tailing which can be observed in paper ionophoresis is caused by the adsorptive interaction between the carrier and the ions which are to be separated. It can be almost eliminated by addition of polyvalent large ions to the basic electrolyte or to the test-solution. By this method the quantitative evaluation of the pherograms is made easier.

EINLEITUNG

Die Zonenionophorese auf Trägermedien, vorwiegend auf Papier, in Gelen und bestimmten Dünnschichten hat sich infolge des geringeren apparativen Aufwands in der Praxis wesentlich stärker durchgesetzt als die historisch ältere Methode der freien Ionophorese[1,2]. Der Hauptvorteil eines porösen Trägermediums beruht auf seiner strömungsstabilisierenden Wirkung, wodurch Konvektionsstörungen weitgehend ausgeschaltet werden. Wegen der grossen Phasengrenzflächen flüssig—fest treten jedoch im Gegensatz zur freien Ionophorese eine Anzahl Wechselwirkungen auf, die die Wanderungsgeschwindigkeit geladener Teilchen beeinflussen.

Ist dafür gesorgt, dass auf einem elektrolytgetränkten Träger an allen Orten annähernd gleiche Ionenstärke und Leitfähigkeit herrschen, so wirken sich die meisten Einflüsse nach Betrag und Richtung auf alle Teilchen derselben Art im gleichen Masse aus. Das gilt für den Umwegfaktor nach KUNKEL UND TISELIUS[3], durch den die Beweglichkeiten aller Ionen herabgesetzt werden, ebenso, wie für den "barrier-effect" nach MACDONALD[4], durch den vor allem grosse Ionen gebremst werden. Auch Sog und Endosmose verursachen eine systematische Änderung der effektiven Wanderungsgeschwindigkeiten. Die genannten Effekte sind innerhalb ziemlich weiter Grenzen nicht von der Konzentration der zu trennenden Ionen abhängig und verursachen daher keine Veränderung der vorgegebenen Form der Zonen während der ionophoretischen Trennung. Vielmehr beobachtet man allgemein eine symmetrische Verbreite-

rung der Zonen durch Diffusion, die in erster Näherung umgekehrt proportional der Wurzel aus der Trenndauer ist[5].

Im folgenden werden Einflüsse besprochen, die zur Ausbildung von charakteristischen unsymmetrischen Zonenprofilen während der Trennung führen. Die Ursachen dafür werden diskutiert und experimentelle Massnahmen zur Erreichung einer besseren Trennschärfe beschrieben.

CHARAKTERISTISCHE ZONENPROFILE

Nach dem Auftragen einer Substanz auf einen Träger in Form einer Zone, d.h. in einer Linie von mehreren cm Länge, ist die Konzentrationsverteilung durch eine steile Gausskurve gegeben, Fig. 1a. Diese Verteilungskurve verliert während der

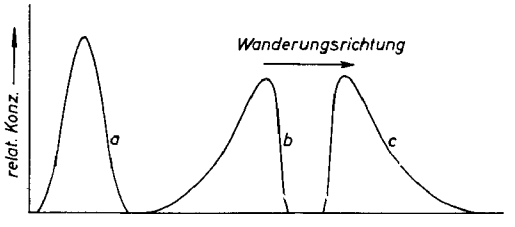

Fig. 1. Konzentrationsprofile bei der Zonenionophorese. (a) Zone am Start. (b) Zone bei Adsorption am Träger. (c) Zone ohne Adsorption am Träger bzw. in freier Lösung.

Ionophorese ihre symmetrische Gestalt, wenn die Beweglichkeit der zu trennenden Ionen innerhalb der Zone von ihrer örtlichen Konzentration abhängt, d.h. wenn die Teilchen des höher konzentrierten mittleren Teils schneller oder langsamer wandern als diejenigen, die sich an der verdünnteren Vorder- oder Rückfront befinden. Das ist immer der Fall, wenn die Ionenwanderung durch konzentrationsabhängige Gleichgewichte beeinflusst wird oder eine Änderung in der Ionenstärke eintritt. Vor allem Dissoziations-, Hydrolyse-, Hydratations- und Komplexgleichgewichte sowie Adsorptionsvorgänge haben zur Folge, dass statt der Beweglichkeit u nur die kleinere Nettobeweglichkeit u_n beobachtet wird[2].

Dissoziationsgleichgewichte

Für den Fall unvollständiger Dissoziation gilt:

$$u_n = u \cdot \alpha \qquad (1)$$

α ist der Dissoziationsgrad, der nach dem Ostwaldschen Verdünnungsgesetz von der Konzentration der beteiligten Ionen abhängt. Demnach besitzen wegen des höheren Dissoziationsgrades in den Bereichen der verdünnteren Flanken die dort befindlichen Ionen eine höhere Nettobeweglichkeit als im konzentrierteren Mittelteil der Zone. Die Folge davon ist, dass bei der Wanderung im elektrischen Feld die Ionen der Vorderfront dem Schwerpunkt der Zone vorauseilen, während die Teilchen der Rückfront mit gleichfalls höherer Geschwindigkeit in den konzentrierten Teil der Zone hineinwandern. Es bildet sich das Konzentrationsprofil der Fig. 1c heraus. Die Zone zeigt eine sehr scharfe konzentrierte Rückfront, während die Flanke in Richtung der Wanderung kometen- oder schweifförmig ausläuft.

Das gleiche Zonenprofil ergibt sich, wenn die Ionenstärke in der Zone grösser als im umgebenden Grundelektrolyten ist, weil auch dann die effektive Ionenbeweglichkeit nach den Flanken hin zunimmt.

Das in Fig. 1c dargestellte Zonenprofil wird vor allem bei der Ionophorese in freier Lösung beobachtet. Auf Trägern findet man es nur, wenn keine Adsorption eintritt. Eine Umkehrung in die Form der Fig. 1b ergibt sich für freie Lösungen dann, wenn bei der Sekundärdissoziation schwacher Komplexe Teilchen mit kleinerer Ladungszahl entstehen. Das Auftreten dieser Form auf Trägern weist immer auf adsorptive Wechselwirkungen hin.

Adsorption und chromatographischer Effekt

Die üblicherweise verwendeten Trägermedien, insbesondere auch Papier, vermögen eine aufgetragene Substanz in geringem Umfang zu adsorbieren. Die festgehaltene Menge richtet sich nach einem Gleichgewicht, das durch eine Adsorptionsisotherme beschrieben werden kann[6]. Vom hochkonzentrierten mittleren Teil einer zunächst symmetrischen Zone wird ein prozentual kleinerer Anteil der Substanz adsorbiert als von den verdünnteren Flanken. Die effektive Beweglichkeit ist deshalb in der Mitte höher als an den Seiten. Die Folge ist die Ausbildung des Zonenprofils der Fig. 1b mit einer scharfen konzentrierten Vorderfront und einer schweifartigen Rückfront. Besonders ausgeprägt ist diese adsorptive Wechselwirkung bei grossen organischen Ionen, z.B. Methylenblau[6]. Aber auch bei schweren anorganischen Ionen, wie den Hexahalogenokomplexen der Platinelemente, ist sie deutlich zu beobachten. Bei kleinen einfach geladenen Ionen wie K^+ und Cl^- ist die Adsorption klein, so dass bei ihnen meistens das Profil der Fig. 1c auftritt.

Nur in einigen speziellen Fällen ermöglicht die durch teilweise Adsorption bedingte Verlangsamung bestimmter Ionen, die auch als chromatographischer Effekt bezeichnet wird, die Trennung sehr ähnlicher Ionen. Während z.B. Cu^{2+} and Cd^{2+} bei der freien Ionophorese etwa gleich schnell wandern, gelingt die Trennung auf Papier sehr einfach, weil Cu^{2+} stark gebremst wird[7].

In der Mehrzahl der Fälle verursacht die Schweifbildung durch Adsorption am Träger Schwierigkeiten, insofern als die Bestimmung der Wanderungsgeschwindigkeit erschwert und zusätzlich zur Diffusion eine Zonenverbreiterung hervorgerufen wird. Darunter leidet vor allem die Trennschärfe. Aber auch die Isolierung einzelner Substanzen durch Elution lässt sich bei scheifartig ausmündenden Zonen nur schlecht durchführen. Noch schwieriger ist die quantitative Auswertung solcher Pherogramme, z.B. durch photometrische oder, im Falle markierter Verbindungen, mit Hilfe radiometrischer Methoden.

Überladene Zonen

Eine Zone ist überladen, wenn die Leitfähigkeit der aufgetragenen Analysenlösung wesentlich grösser als die des Grundelektrolyten ist. Die wichtigste Bedingung für die ungestörte Zonenionophorese, nämlich gleichmässiger Spannungsabfall bzw. gleiche Feldstärke über die gesamte Wanderungsstrecke, ist dann nicht mehr erfüllt, sondern stellt sich während des Versuches erst ein. Dabei bilden sich, wie LEGOUX[8] durch radiometrische Verfolgung der Wanderung einfacher Kationen feststellte, unsymmetrische Zonenprofile aus.

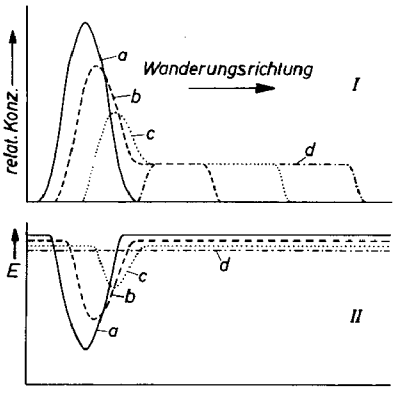

Fig. 2. Veränderung der Gestalt überladener Zonen während der Ionophorese (I). (II) Feldstärkeverlauf. Zeit: o = a < b < c < d.

Die zeitliche Veränderung der Form einer überladenen Zone und des dazugehörigen Feldstärkeverlaufes ist in Fig. 2 schematisch dargestellt.

Die Feldstärke E ist umgekehrt proportional zur Leitfähigkeit κ, wie folgende Gleichung zeigt:

$$E = \frac{1}{\kappa} \cdot \frac{I}{q} \tag{2}$$

q = Querschnitt, I = Stromstärke.

Am Auftragungsort der Analysenlösung grosser Leitfähigkeit stellt sich bei Anlegen der Spannung eine geringe Feldstärke ein, Kurve a der Fig. 2, II. Vor allem auf die Teilchen des hochkonzentrierten Zentrums der Zone wirkt nur eine geringe Feldstärke ein, die Wanderungsgeschwindigkeit ist entsprechend klein. Die an der vorderen verdünnteren Flanke befindlichen Ionen werden dagegen schnell hinweggeführt. Dabei bilden sich unsymmetrische Zonenformen aus, Kurven b und c der Fig. 2, I, die der von Fig. 1c ähneln. Mit fortschreitender Zeit verbreitert sich die ursprünglich schmale Zone unter Verdünnung immer mehr. Es stellt sich ein Konzentrationsplateau ein, dessen Höhe von der Leitfähigkeit des Grundelektrolyten abhängt. Erst wenn die Einstellung gleichmässiger Feldstärke eingetreten ist, Kurve d der Fig. 2, II, wandert die gesamte, sehr breite Zone mit konstanter Geschwindigkeit und unterliegt dann den im ersten Teil des Kapitels beschriebenen Wechselwirkungen mit dem Träger.

Durch überladene Zonen wird, bedingt durch die zwangsläufig eintretende Verbreiterung, die Trennschärfe stets beeinträchtigt. Sehr scharfe Zonen bilden sich gleich zu Beginn der Trennung aus, wenn im Vergleich zum Grundelektrolyten verdünnte Lösungen aufgebracht werden, weil dann in Umkehrung des beschriebenen Vorgangs eine Aufkonzentrierung und Fokussierung erfolgt.

Prinzipiell sind jedoch diese durch Konzentrations- und Feldstärkeinhomogenitäten hervorgerufenen Veränderungen der Zonen gegenüber den im vorangehenden besprochenen Effekten zu unterscheiden.

EXPERIMENTELLES

Neben kontinuierlichen trägerfreien Methoden[9,10] hat auch die diskontinuierliche Zonenionophorese auf Trägern für die Darstellung von Gemischtligandkomplexen, z.B. der Reihen $[OsCl_xJ_{6-x}]^{2-}$ und $[OsBr_xJ_{6-x}]^{2-}$ ($x = 0$–6), Bedeutung[11]. Besonders bei schnell hydrolysierenden Verbindungen, wie sie die jodidreichen Komplexe darstellen, ist sie unentbehrlich.

Die Trennungen mit Hilfe der Hochspannungspapierionophorese werden in einer früher beschriebenen Apparatur[12] unter folgenden Bedingungen durchgeführt:

Trägermaterial:	Filterpapier, B mgl, 90 × 5 cm (Fa. Schleicher und Schüll)
Probegemisch:	*ca.* 100 μg Komplexsalze in 1–2 Tropfen Wasser gelöst
Elektrodenabstand:	80 cm
Spannung:	5000 V
Stromstärke:	*ca.* 15 mA/Streifen
Feldstärke:	60 V/cm
Trennzeit:	*ca.* 30 min
Kühlbadtemperatur:	−5°
Grundelektrolyt:	verschiedene gleicher Ionenstärke

Liegen in der Trennlösung alle Gemischtligandkomplexe einer Reihe nebeneinander vor, so findet man sieben etwa äquidistante Zonen. Je nach Art des verwendeten Grundelektrolyten und bestimmter adsorbierbarer Zusätze zur Trennlösung ergeben sich unterschiedliche Zonenprofile.

Trennung in Gegenwart eines nicht adsorbierbaren Grundelektrolyten

Als nicht adsorbierbarer Grundelektrolyt dient eine Lösung, die je 0.2 *M* an CH_3COOK und CH_3COOH ist. Verwendet man dieses Puffergemisch als Fliessmittel bei der aufsteigenden Chromatographie, so ergeben sich für die Hexahalogenokomplexe von Os(IV) R_F-Werte zwischen 0.7 und 0.8. Die Rückfronten zeigen Schweifbildung.

Bei der Papierionophorese bildet der leichteste, am schnellsten wandernde Komplex ($x = 6$) eine Zone mit sehr scharfer Vorderfront. Die Konzentrationsverteilung der mittleren Zonen ist annähernd symmetrisch. Die letzte Zone läuft kometenartig aus, Fig. 3.

Auch wenn nur einige gemischte Komplexionen vorliegen ergeben sich diese typischen Zonenprofile für die am schnellsten und langsamsten wandernden Zonen. Die Abweichung von der symmetrischen Verteilungskurve wird ausser bei den End-

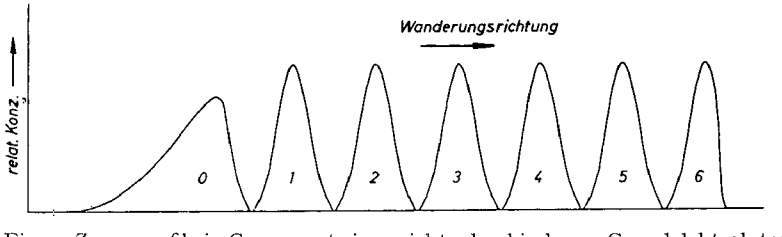

Fig. 3. Zonenprofile in Gegenwart eines nicht adsorbierbaren Grundelektrolyten. Grundelektrolyt: 0.2 *M* CH_3COOK + 0.2 *M* CH_3COOH. Komplexgemisch: $[OsCl_xJ_{6-x}]_{6-}$; ($x = 0$–6).

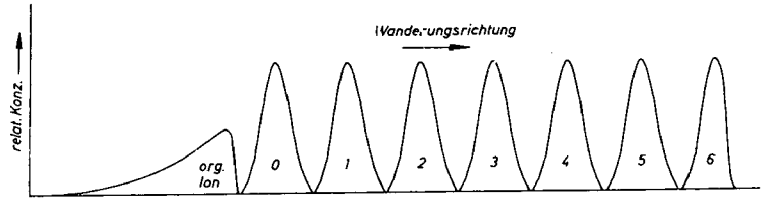

Fig. 4. Zonenprofile in Gegenwart adsorbierbarer Ionen im Probegemisch. Grundelektrolyt und Komplexgemisch wie bei Fig. 3. Organisches Ion: 2-Naphthylamin-6.8-disulfonat bzw. 2-Naphthol-3,6-disulfonat.

gliedern auch für die übrigen beobachtet, wenn die Probe nicht zonen- sondern punktförmig aufgetragen wird.

Trennung bei Zugabe eines adsorbierbaren Ions zur Probelösung

Die im vorangehenden beschriebene Versuchsdurchführung wird nur insofern geändert, als der wässrigen Lösung der Komplexe eine kleine Menge eines Salzes zugesetzt wird, dessen grosses mehrfach geladenes Anion eine etwas geringere Beweglichkeit besitzt als das langsamste Komplexion. Es eignen sich z.B. die Dinatriumsalze der 2-Naphthylamin-6,8-disulfonsäure bzw. der 2-Naphthol-3,6-disulfonsäure. Bei der Ionophorese erhält man die durch Fig. 4 schematisch wiedergegebenen Zonenprofile.

Gegenüber der Fig. 3 fällt auf, dass jetzt auch die letzte Komplexzone ($x = 0$) eine symmetrische Konzentrationsverteilung aufweist. Das organische "Hilfsion", das im UV-Licht sichtbar wird, weist dagegen extreme Schweifbildung auf, die sich bis fast an die Startlinie erstreckt.

Trennung in Gegenwart eines adsorbierbaren Grundelektrolyten

Das Acetat des bisher verwendeten Grundelektrolyten wird unter Konstanthaltung der Ionenstärke und des pH-Wertes zu einem Viertel bis zur Hälfte durch die Dinatriumsalze der erwähnten Disulfonsäuren ersetzt. In Gegenwart der gut an Papier adsorbierbaren organischen Ionen werden die Zonenprofile der Fig. 5 beobachtet.

Auch jetzt ergibt sich für die letzte Zone eine annähernd symmetrische Konzentrationsverteilung, zumindest ist die Schweifbildung im Vergleich zu Fig. 3 stark verringert. Die am schnellsten wandernde Zone weist dagegen eine kometenartige

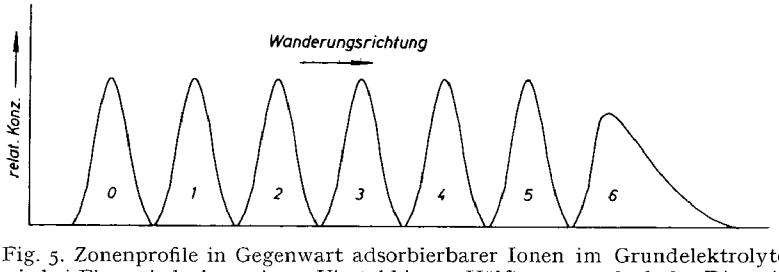

Fig. 5. Zonenprofile in Gegenwart adsorbierbarer Ionen im Grundelektrolyten. Grundelektrolyt wie bei Fig. 3, jedoch zu einem Viertel bis zur Hälfte ersetzt duch das Dinatriumsalz von 2-Naphthylamin-6,8-disulfonsäure bzw. 2-Naphthol-3,6-disulfonsäure. Komplexgemisch wie bei Fig. 3.

Vorderfront auf. Bei gleicher Trenndauer sind die von den einzelnen Zonen zurückgelegten Wanderungsstrecken bei Anwesenheit der adsorbierbaren organischen Ionen im Grundelektrolyten um etwa 10% grösser als in reiner Acetatlösung.

DISKUSSION DER ERGEBNISSE

Die bei der Ionophorese auf Trägermedien auftretenden charakteristischen Zonenprofile lassen sich durch die Wahl des Grundelektrolyten beeinflussen. Die adsorptive Wechselwirkung zwischen dem Träger und den wandernden Ionen ist um so ausgeprägter, je grösser und höher geladen diese sind.

Verwendet man als Grundelektrolyten eine Lösung, die nur kleine einfach geladene Ionen enthält, z.B. einen Acetatpuffer, so ist die Wechselwirkung mit dem Papier gering, und es werden vorzugsweise die zu trennenden, zweifach geladenen und grösseren Komplexionen adsorbiert. Das führt zum kometenartigen Ausmünden der Rückfront. Folgen mehrere Zonen sehr dicht auf einander, so schieben sie sich vor einander her, d.h. die zurückbleibenden Ionen der voranwandernden Zone werden durch die der nachfolgenden Zone desorbiert. Die Konzentrationsverteilung bleibt symmetrisch.

Setzt man dem Grundelektrolyten grosse mehrfach geladene Ionen zu, so blockieren diese weitgehend die Adsorptionszentren. Die Zonenprofile gleichen denen in freier Lösung. Die Ausschaltung der Adsorption der zu trennenden Ionen äussert sich auch in der Zunahme der Wanderungsgeschwindigkeit. Durch die Gegenwart gut adsorbierbarer Teilchen lässt sich demnach die Umkehrung der Zonenprofile von der Gestalt b in c der Fig. 1 erreichen.

Um symmetrische, gut auswertbare Zonen zu erhalten, genügt erfahrungsgemäss der Zusatz einiger Prozent der organischen Salze zum Grundelektrolyten. Ist ihre Anwesenheit für nachfolgende Untersuchungen störend, so empfiehlt sich ein kleiner Zusatz solcher gut adsorbierbarer Ionen zum Probegemisch, deren Beweglichkeit etwas geringer als die der Ionen der interessierenden Zone ist. Wie in Fig. 4 dargestellt, werden die Komplexionen der letzten Zone durch das auf das Papier aufziehende organische Ion verdrängt, so dass sich ein symmetrisches Zonenprofil ausbildet.

DANK

Wir danken dem Direktor des Instituts, Herrn Professor Dr. E. BLASIUS, für die Förderung unserer Arbeit.

ZUSAMMENFASSUNG

Die Ausbildung unsymmetrischer Zonenprofile während der Ionophorese auf Trägern und in freier Lösung wird durch konzentrationsabhängige Gleichgewichte erklärt. Die bei der Papierionophorese zu beobachtende Schweifbildung beruht auf der adsorptiven Wechselwirkung zwischen dem Träger und den zu trennenden Ionen. Sie lässt sich durch Zugabe mehrfach geladener grosser Ionen zum Grundelektrolyten bzw. zur Probelösung weitgehend ausschalten. Die quantitative Auswertung der Pherogramme wird dadurch erleichtert.

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CHROM. 5069

THE EFFECT OF CONCENTRATION UPON THE CHROMATOGRAPHIC BEHAVIOUR OF TECHNETIUM IN CONCENTRATED HYDROCHLORIC ACID

M. JEAN WILLIAMS AND T. DEEGAN
Liverpool Regional Cardiac Centre, Sefton General Hospital, Liverpool (Great Britain)

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SUMMARY

The chromatographic behaviour of various concentrations of technetium in 11.4 *M* HCl was studied on Whatman No. 3MM and DE-81 papers with 0.6 *M* and 1.2 *M* HCl, respectively, as developing solvents. Within the concentration range, 0.5–2.0 mg per ml, results were similar to those already documented. In addition, two further species were detected, one appearing at concentrations above 2 mg per ml, and the other at concentrations below 0.1 mg per ml. The possible structures of these two species are discussed.

INTRODUCTION

Previous investigations[1,2] into the reaction between Tc(VII), as pertechnetate, and concentrated HCl have employed cellulose or ion-exchange paper chromatography to define the intermediates present. During a study involving reduction of trace quantities of [^{99m}Tc]pertechnetate in similar conditions, anomalous results were obtained with the same methods.

This paper reports the effect of concentration changes upon the reaction of Tc(VII) and concentrated HCl in terms of the chromatographic behaviour of the species involved.

EXPERIMENTAL

The technetium used in this study was a solution of ammonium [^{99}Tc]pertechnetate, which was adjusted with distilled water to a concentration of 10 mg ^{99}Tc per ml. In addition, [^{99m}Tc]pertechnetate, obtained by elution of a [^{99}Mo]molybdate generator with 0.15 *M* NaCl, was used to define chromatographic zones during analysis. (All isotopic materials were obtained from the Radiochemical Centre, Amersham, Bucks.)

Ascending chromatography was performed on 2.5 cm × 25 cm strips of both Whatman No. 3MM and DE-81 papers using 0.6 *M* and 1.2 *M* HCl, respectively, as developing solvents. Solutions for analysis were prepared by addition of approxi-

mately 30 μCi of [^{99m}Tc]pertechnetate to aliquots of the standard pertechnetate solution. After evaporation to dryness in vacuo, the residues were reconstituted in 11.4 *M* HCl (analytical grade) to give the concentration range investigated. The standard application to each strip was 5 μl. Development with No. 3MM papers was for 16–18 cm and with DE-81 papers, 12–14 cm. After drying, each strip was cut into 5 mm sections, the ^{99m}Tc-activities of which were determined in a well-type scintillation counter (Nuclear Enterprises Ltd., Beenham, Berks.). The activities of the individual sections were expressed as percentages of the total activity of the chromatogram.

RESULTS

The R_F values of the Tc(VII) and Tc(IV) states, as TcO_4^- and $TcCl_6^{2-}$, were determined for the two systems in preliminary experiments. The R_F value of the Tc(V) state was derived from the findings of the study. Comparison with the published data of SHUKLA[2] is presented in Table I.

TABLE I

R_F VALUES OF THE VARIOUS Tc SPECIES

	Present study	*Shukla (ref. 2)*
No. 3MM		
Tc (IV)	0.87	0.87
Tc (V)	0.18	0.18
Tc (VII)	0.70	0.68
DE-81		
Tc (IV)	0.10	0.10
Tc (V)	0.46	0.48
Tc (VII)	0.48	0.47

In general, the concentrations of the solutions were not defined in previous investigations of the reaction between Tc(VII) and concentrated HCl[1,2]. The range covered in the present study was from trace quantities ([^{99m}Tc]pertechnetate only) to 10 mg per ml. The results of the chromatographic analyses of the various solutions showed different effects dependent upon whether the concentration was greater or less than 0.5 mg per ml.

Concentration range: 0.5–10.0 mg per ml

The results of analyses of samples taken from solutions within 30 min of constitution are shown in Fig. 1.

On No. 3MM paper, the main species was the slow running Tc(V); some Tc(VII) was also detected in the R_F 0.7 region, and at higher concentrations a further species was apparent beyond R_F 0.8.

On DE-81 paper, most of the activity was detected in the area shared by Tc(V) and Tc(VII). Again, at higher concentrations a fast moving species with an R_F value greater than 0.6 was detected. The absence of appreciable activity in the low R_F region

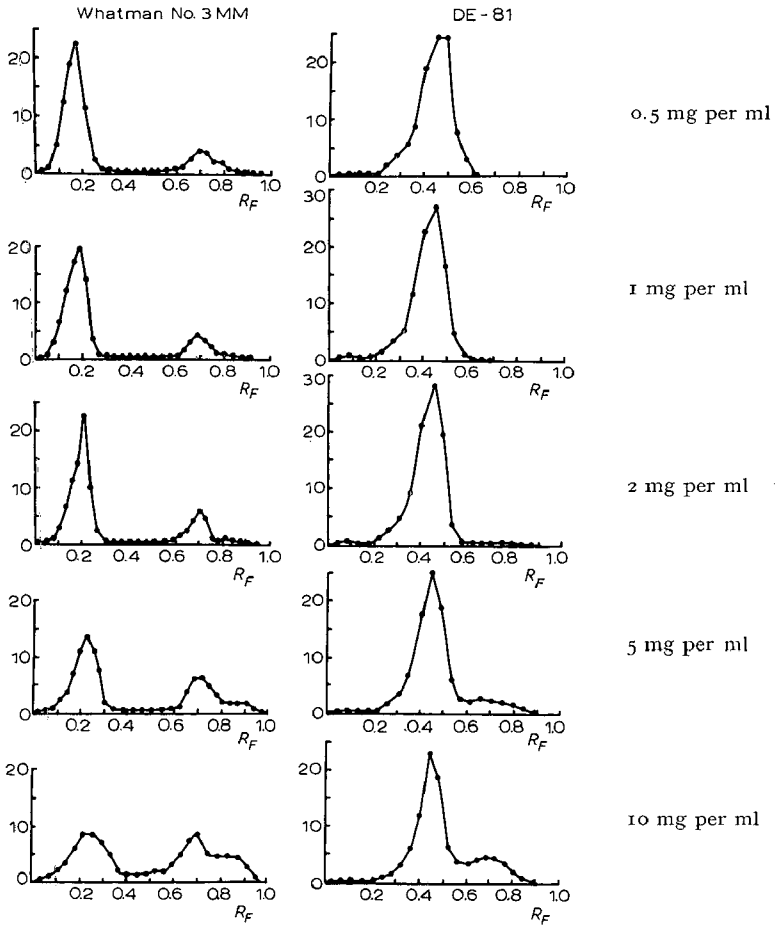

Fig. 1. Chromatograms of 0.5–10 mg per ml solutions, 30 min after constitution, on Whatman No. 3MM and DE-81 papers with 0.6 *M* and 1.2 *M* HCl, respectively, as developing solvents. Ordinates: percentage total chromatogram activity.

suggested that Tc(IV) was not present in quantity and could not have contributed much to the fast moving species on No. 3MM paper.

Repetition of the analyses with samples taken after 24 h reaction showed only small changes. On No. 3MM paper, a decrease in the amount of Tc(V) was apparent together with an increase in the activity in the R_F 0.7 region. As, judged by the amount of slow running material on DE-81 paper, the contribution of Tc(IV) was only increased minimally, it must be presumed that a greater quantity of Tc(VII) was present in the reaction mixture after 24 h.

By assessment of the areas under the peaks in the simultaneous chromatograms, coupled with the R_F values of the individual species in the two systems, it was possible to determine the proportions of each component in the reaction mixture at the various concentrations (Fig. 2). After 30 min the conversion of Tc(VII) to Tc(V) became less as the concentration of technetium increased and was accompanied by an

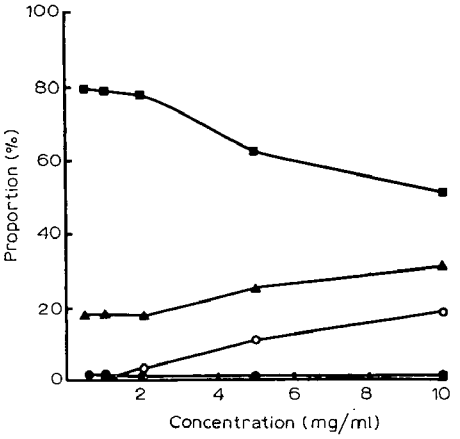

Fig. 2. Proportions of each component in the concentration range, 0.5–10 mg Tc per ml. Tc (IV) ●—●, Tc (V) ■—■, Tc (VII) ▲—▲, fast moving component (F) ○—○.

increasing amount of the fast moving component, F, at levels above 2 mg per ml. Throughout, the amount of Tc(IV) was very small.

Concentration range: less than 0.5 mg per ml

Samples taken within 30 min of constitution gave the results shown in Fig. 3

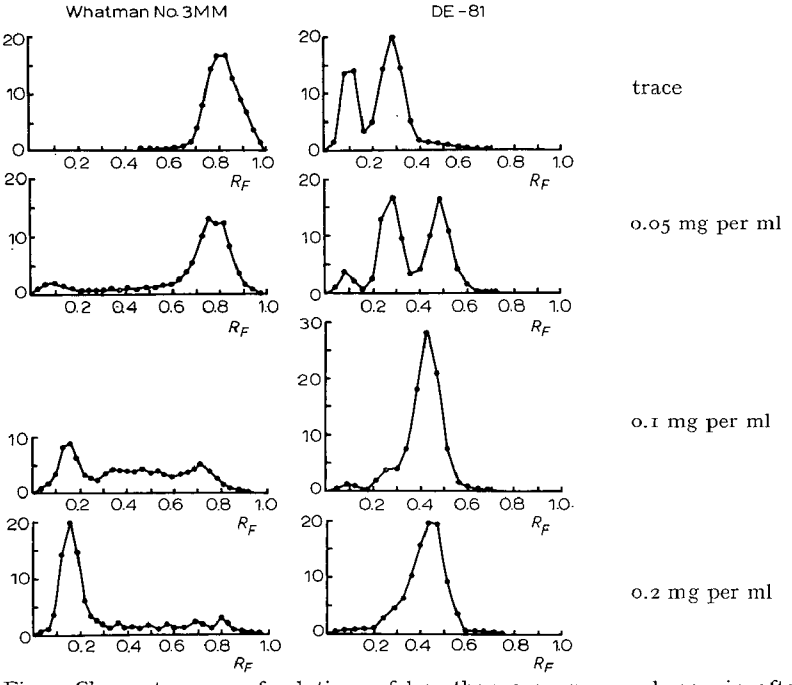

Fig. 3. Chromatograms of solutions of less than 0.5 mg per ml, 30 min after constitution, on Whatman No. 3MM and DE-81 papers with 0.6 *M* and 1.2 *M* HCl, respectively, as developing solvents. Ordinates: percentage total chromatogram activity.

for this range. In general, as the concentration decreased there was a movement of activity towards the solvent front on No. 3MM paper and towards the origin on the DE-81 paper.

At concentrations of 0.1 and 0.2 mg per ml. the discrete separations seen previously in analyses on No. 3MM paper became confused. Apart from the Tc(V) peak no other species was clearly defined. It is probable that the indeterminate area between R_F 0.3 and 0.8 resulted from secondary reactions on the paper since no parallel effects were observed in the DE-81 system. Here, as previously, the bulk of the activity was in the region of the common R_F of Tc(V) and Tc(VII) but the amount of material between R_F 0.2 and 0.3 produced a more definite shoulder to the peak at these concentrations. As previously, little change in distribution was apparent after 24 h.

When the concentration was reduced further to 0.05 mg per ml, marked changes appeared on both papers. On No. 3MM paper there was overall displacement of activity towards the solvent front with a peak at R_F 0.77. The Tc(V) peak was no longer a major feature and the trail between R_F 0.3 and 0.6 was much diminished. In the DE-81 system, the shoulder observed at higher concentrations emerged as a discrete peak at R_F 0.28 between the positions of Tc(IV) and Tc(V).

For the first time analyses after 24 h showed altered distributions (Fig. 4). On No. 3MM paper, the Tc(V) peak disappeared and all activity was concentrated in a double peak at R_F's 0.84 and 0.90. The Tc(V) peak was also absent on DE-81 paper

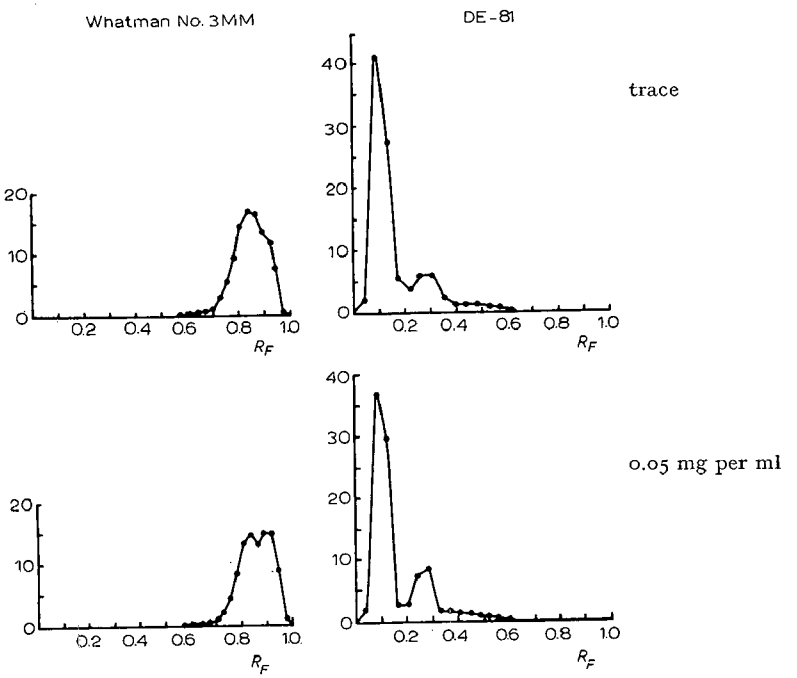

Fig. 4. Chromatograms of trace and 0.05 mg per ml solutions, 24 h after constitution, on Whatman No. 3MM and DE-81 papers with 0.6 *M* and 1.2 *M* HCl, respectively, as developing solvents. Ordinates: percentage total chromatogram activity.

and the activity at R_F 0.28 was reduced in quantity. The major component was the Tc(IV) peak.

The analyses of trace concentrations on No. 3MM paper after 30 min reaction showed a single wide peak with a maximum R_F value of 0.80. Two peaks were again evident on DE-81 paper at R_F's 0.10 and 0.28. No Tc(V) peak was detected in either system. After 24 h the results facsimilated those at 0.05 mg per ml; the peak maximum on No. 3MM paper had moved towards the solvent front whilst on DE-81 paper the Tc(IV) peak increased at the expense of the component at R_F 0.28.

DISCUSSION

Within the concentration range, 0.5–2.0 mg per ml, the distribution and type of species detected were similar to those designated previously as Tc(IV), Tc(V), and Tc(VII)[1,2]. Outside this range two further components were apparent, namely at higher concentrations one which was fast moving in both systems and at lower concentrations one which was fast moving on No. 3MM paper but rather slow moving on DE-81 paper.

From a spectroscopic investigation of the reaction between Tc(VII) and concentrated HCl, BUSEY[3] proposed that the most likely form of Tc(V) is $TcOCl_4^-$, but amongst alternatives possible are $TcOCl_3$ and $TcOCl_5^{2-}$. A further species detected when the reaction mixture was irradiated with sunlight was assigned the structure $TcCl_6^-$.

In the range above 2 mg per ml, no appreciable changes in the proportions in the reaction mixture were observed between analyses after 30 min and after 24 h, which suggested that stable equilibrium was quickly established. The differences in the relative distributions at the various concentrations involved only Tc(V), Tc(VII) and the fast moving component, F. The smallness of the contribution of Tc(IV) implied a slow rate of secondary reduction of Tc(V).

BUSEY[3] failed to detect any intermediate state between Tc(VII) and Tc(V) in the reaction which suggests that F is probably an alternative species to the usual $TcOCl_4^-$. In view of the chromatographic properties the most likely form is $TcOCl_3$, the neutral structure of which would imply a high R_F value on the anion-exchange paper. It is conjectural whether F occurs as an intermediate in concentrations less than 2 mg per ml, or arises at higher concentrations from the decreasing Cl^-/Tc ratio as the amount of nuclide increases.

In the low concentration range the main changes were the decrease in activity in the Tc(V) regions, culminating in the total absence at trace levels, and the changed distributions after 24 h. The latter, however, may have been relative rather than absolute since the same rate of secondary reduction would result in a more significant change in proportions in this range.

Another species was evident from the emergence of a peak at R_F 0.28 on DE-81 paper. Hydrolysis products with similar R_F values on anion-exchange papers occur in solutions of Tc(IV) in 1.2 *M* HCl[1]. The greater molarity of the present reaction conditions together with the increased amount of Tc(IV) after 24 h precludes this structure for the new species. HARPER *et al.*[4] has stated that Tc(V) is the valency necessary for ligand formation with such entities as albumin and WILLIAMS[5] has successfully combined trace quantities of ^{99m}Tc with this protein at efficiences of 90%

after reduction of Tc(VII) with concentrated HCl. This data suggests that the new component is a further alternative of Tc(V). The chromatographic properties point to two possibilities, $TcCl_6^-$ or $TcOCl_5^{2-}$, both of which would have relatively slow movement on DE-81 paper.

Summarising, it is considered that reduction of Tc(VII) to Tc(V) occurred throughout the range investigated but the nature of the pentavalent form present in the reaction mixture altered with concentration.

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Notes

CHROM. 5064

Gas chromatographic separation of esters of fluoro analogs of citric acid cycle intermediates

As an approach to the study of the rate limiting role of citric acid cycle enzymes in tissue metabolism, specific fluoro carboxylic acid enzyme inhibitors were synthesized[1], and their effect on isolated enzymes and multienzyme systems investigated[2]. Application of fluoro carboxylic acid enzyme inhibitors as metabolic probes in organ systems and in the whole animal requires analytical methods capable of detecting these fluoro acids in various tissues, which contain also the physiological nonfluorinated carboxylic acid enzyme substrates. In view of the recent progress in the gas chromatographic analysis of the citric acid cycle esters[3], the results presented in this paper show that gas chromatography may offer a feasible solution to this analytical problem.

Materials

Ethyl 3-fluorolactate[4,5], diethyl fluorosuccinate, 3-fluoro-2-oxoglutarate, diethyl 3-fluoro-2-hydroxyglutarate, triethyl 2-fluoroisocitrate[4], diethyl 2-fluoroglutarate, triethyl fluoropropane-1,1,3-tricarboxylate[6] and fluorocitrate[7] were synthesized according to previously developed methods. The syntheses of fluoromalonate[8] diethyl fluorooxalacetate[9] and diethyl 3-fluoromalate[10] were carried out by procedures published by other workers.

Diethyl 2-oxoglutarate, triethyl citrate and triethyl isocitrate were prepared by the addition of ethanol to a mixture of the carboxylic acids in trifluoroacetic anhydride[11]. Other esters (see Table I) were obtained commercially. Before analysis on the DEGS column, the ester samples were first purified by preparative gas chromatography on a 20-ft. SE-30 column.

Apparatus. The Varian Aerograph Model 700 was used, with thermal conductivity detection. A column (12 ft. × 1/8 in. O.D.) containing diethylene glycol succinate (DEGS), 12% on Chromosorb W, was operated at a helium flow rate of 67 ml/min (50 p.s.i.). A column (5 ft. × 1/4 in. O.D.) containing silicone polymer (SE-30) 20% on Chromosorb W, was operated at a flow rate of 150 ml/min (20 p.s.i.) Individual esters were preparatively purified on a column (20 ft. × 3/8 in. O.D.) of SE-30, 30% on Chromosorb W. The input temperature was 225°. Injected samples contained 0.1 to 0.5 μl of each ester.

RESULTS AND DISCUSSION

Succesful separation of esters of carboxylic acids and their fluoro homologs was obtained with a DEGS column. Retention times were measured for each ester at three column temperatures 25° apart in the range 100–225°. Nearly straight lines were obtained by plotting the logarithm of the retention time against the reciprocal of the temperature. The retention times were related to diethyl succinate as summarized

TABLE I

RELATIVE RETENTION TIMES OF ETHYL ESTERS OF CARBOXYLIC ACIDS AND CORRESPONDING FLUORO ANALOGS ON DEGS COLUMNS AT 125°, 150°, 175° AND 200°

Ethyl ester	*Temp. (°C)*	*Relative retention time*[a]	*Fluoro analog*	*Relative retention time*	*Ratio*[b]
Lactic	125	0.28	3-fluoro	0.66	2.3
	150	0.36		0.77	2.1
	175	0.50		0.66	1.3
Malonic	125	0.65	fluoro	1.0	1.5
	150	0.66		1.0	1.5
	175	0.77		0.87	1.1
Succinic	125	1.0	fluoro	1.5	1.5
	150	1.0		1.5	1.5
	175	1.0		1.2	1.2
Glutaric	125	1.4	2-fluoro	2.6	1.9
	150	1.4		2.3	1.6
	175	1.3		1.8	1.4
	200	1.3		1.6	1.2
Oxalacetic	150	2.5	fluoro	3.0	1.2
	175	dec.		2.3	
	200	dec.		2.1	
Malic	150	3.9	3-fluoro	3.0	0.77
	175	3.1		2.4	0.77
	200	2.7		2.1	0.78
2-Oxoglutaric	150	3.9	3-fluoro	3.2	0.78
	175	3.7		3.2	0.86
	200	3.0		2.7	0.90
3-Hydroxyglutaric	150	3.2	2-fluoro	3.7	1.2
	175	4.4		4.8	1.2
	200	3.5		4.6	1.3
Propane tricarboxylic	150	4.5	fluoro	4.6	1.0
	175	5.9		6.7	1.2
	200	4.2		5.0	1.2
Citric	175	13.0	2-fluoro	18.0	1.4
	200	8.7		11.4	1.3
Isocitric	175	dec.	2-fluoro	18.0	
	200	dec.		19.4	

[a] Diethyl succinate = 1.00.
[b] The ratio of fluoro to nonfluoro compound retention times.

in Table I. For mixtures of each ester with its fluoro analog, the ratio of retention times is summarized in the last column.

In most cases the nonfluoro ester emerged from the column before the corresponding fluoro ester. The fluoro ester emerged first in the cases of diethyl malate and diethyl oxoglutarate.

Extraneous peaks were observed at 125° for all esters of keto acids. At 175°, triethyl isocitrate decomposed completely and fluoroisocitrate was partially decomposed. In general, the fluoro analogs appeared to be more thermally stable than the nonfluoro esters.

Trimethylsilyl[12] and trimethylsilyl oximino derivatives[13] were prepared from the hydroxy and keto esters, but gas chromatographic separation of these derivatives of fluoro analogs from the corresponding nonfluoro ester was not successful.

Esters of hydroxy and keto carboxylic acids and their fluoro homologs were

successfully separated by isothermal gas chromatography on a DEGS column, as the basis of an analytical procedure for the study of tissue distribution and *in vivo* metabolic effects of fluoro inhibitors.

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Departments of Pharmacology and
Biochemistry and Biophysics and
The Cardiovascular Research Institute,
School of Medicine,
University of California, San Francisco
Calif. 94122 (U.S.A.)

ROBERT J. DUMMEL
ERNEST KUN*

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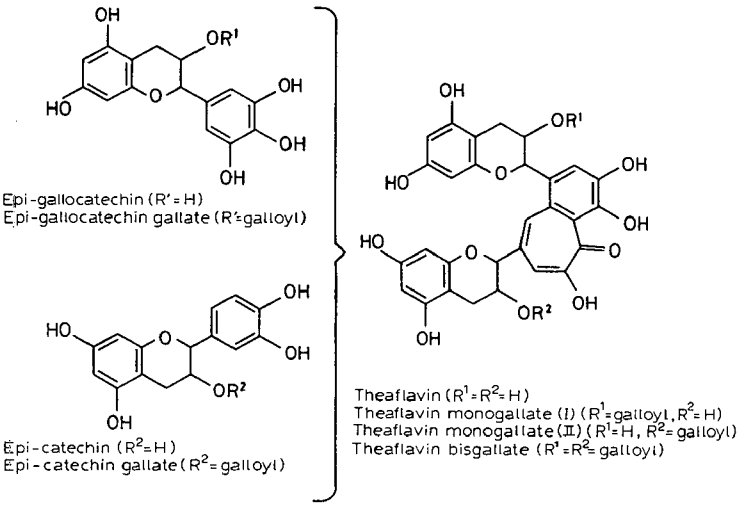
CHROM. 5071

The separation of theaflavins on Sephadex LH-20

Theaflavins, orange-red pigments with an important contribution to the appearance and "mouthfeel" of black tea liquors[1], are formed during tea manufacture by the oxidative condensation of epi-catechin, epi-gallocatechin and their galloyl esters (Fig. 1)[2,3]. They may be detected in tea extracts by column chromatography on



Sephadex LH-20 in 60% aqueous acetone[4], although this method gives no separation of the individual theaflavin species. It has now been found that separation of theaflavin, theaflavin monogallates and theaflavin bisgallate may be achieved by adsorption chromatography on Sephadex LH-20 in 35% aqueous acetone. Apart from a brief recent mention[5] this is the first published demonstration of the existence of the bisgalloyl ester of theaflavin in black tea.

Preparative method

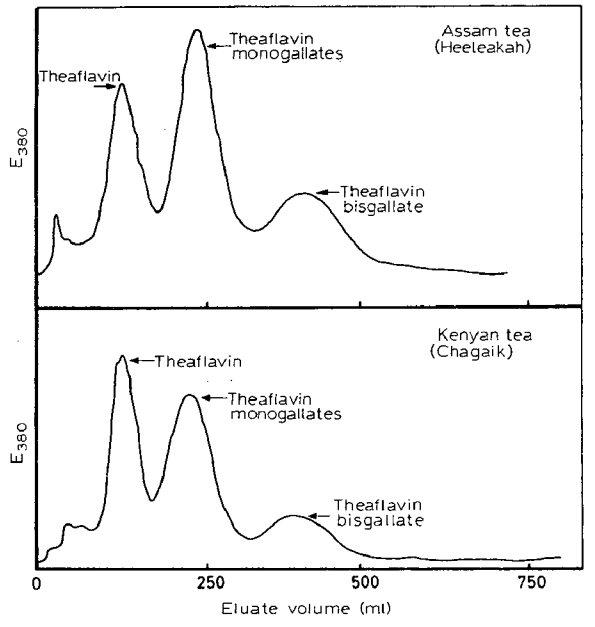
A slurry of Sephadex LH-20 (Pharmacia Ltd.) in 35% aqueous acetone was packed in a Whatman glass chromatography column of 2.54 cm × 30 cm (Reeve Angel Ltd.) and equilibrated with the same solvent. A crude theaflavin sample was prepared by extracting a chloroform-washed aqueous tea brew with ethyl acetate, washing briefly with 2.5% aqueous sodium bicarbonate solution followed by distilled water, and removing the ethyl acetate by rotary evaporation. The crude extract was dissolved in a water-*tert.*-butanol mixture, from which it was freeze dried, and 500 mg of solid was dissolved in a small volume of 35% acetone and applied to the Sephadex column.

Elution at the natural flow rate (0.5 ml/min) was continued for approx. 24 h. Initially, three purple bands were eluted (probably corresponding to decomposition

products of theaflavins) followed by two yellow bands (which paper chromatography revealed to occupy similar positions to the spots Q and Z of ROBERTS *et al.*[6] or the spots F and G of VUATAZ AND BRANDENBERGER[7]). The theaflavins remained on the column and moved only slowly, separating into three orange bands. By the end of the 24 h period the first orange band had been eluted, and the remaining two orange bands were collected by extruding the column, sectioning it, and extracting the Sephadex with 60% acetone. All three samples, after concentrating and freeze-drying, migrated as single spots on two-dimensional paper chromatography with 2-butanol–acetic acid–water (14:1:5) and acetic acid (2%) as solvents.

Analytical method

A smaller Whatman column (1.0 cm × 10 cm) was packed with Sephadex LH-20 in 35% acetone and equilibrated. A flow rate of 1 ml/min was maintained by a micropump (F. A. Hughes & Co.) and 20 mg of crude extract was applied to the column. The eluate was monitored at 380 nm by a Vitatron flow-through photometer unit (Fisons Scientific Instruments Ltd.) and displayed on a logarithmic-scale recorder. Typical traces from standard extracts of different teas are shown in Fig. 2.



Results and discussion

The identity of the theaflavin fractions was established by comparison with authentic synthetic samples of theaflavin, the isomeric monogallates and theaflavin bisgallate prepared by ferricyanide oxidation of the relevant flavanols[8]. Paper chromatography was in agreement with these conclusions.

The choice of 35% acetone as eluting solvent seemed to be optimum between satisfactory resolution and reasonable speed of operation. The resolution was noticeably improved, however, when the column was operated at 4° rather than at room

temperature. Further attempts to separate the monogallates by using more dilute acetone solutions as eluents have failed. This would seem to indicate that adsorption by hydrogen bonding to the phenolic and carbonyl oxygens is probably a major factor responsible for the separation[9], with a contribution also due to London forces which are proportional to molecular volume [10].

The analytical method is of use in comparing different tea samples. Thus Fig. 2 depicts a comparison between single estate teas from Assam and Kenya and indicates a marked difference between the degree of esterification of the theaflavin fraction in these two samples.

Brooke Bond Liebig Research Laboratories, A. G. H. LEA
Blounts Court, Sonning Common, Reading, Berks. D. J. CRISPIN
(Great Britain)

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CHROM. 5074

A rapid method for relative quantitation of lipid classes separated by thin-layer chromatography

No adequate procedures have been available for rapid and reproducible quantitation of large numbers of lipid samples. For this reason many areas amenable to lipid studies remain unexplored and the role of lipids in many biochemical and physiological responses is not well understood.

Methods for extracting and purifying lipid samples[1,2], and for separating these samples by thin-layer chromatography (TLC) into the individual classes of neutral and phospholipids have existed for some time[3,4]. Recent developments in this area have produced somewhat more efficient methods for individual lipid class separations[5–7]. One of the limiting steps in lipid analysis is quantitation of the lipid classes after TLC separation. Numerous analytical procedures have been developed in an attempt to accomplish this task[8–17]. Most of these procedures are both slow and tedious as they require the isolation of the lipid class from the separation media for subsequent analysis. Of the procedures developed for quantitation of lipids on intact chromatograms, the char-densitometric technique appears to be applicable to quantitation of multiple samples. Other char techniques attempted in our laboratory were either not useful for phospholipids[15] or useful for lower quantities of material[16]. The char-densitometric procedure, described below, was designed to analyze changes in per cent distribution of serum lipid classes. This procedure allows for rapid and reliable analysis of multiple samples on a thin-layer chromatogram with good reproducibility between different chromatograms.

Experimental

Materials and methods. Individual lipid standards and artificial mixes were obtained from Supelco, Inc., Bellefonte, Penn., U.S.A. Chromatographic spray bottles were obtained from Arthur H. Thomas Company, Philadelphia, Penn., U.S.A. A Model SD-3000 dual-beam spectrodensitometer, Model SDC-300 density computer, was obtained from Schoeffel Instrument Company, Westwood, N.J., U.S.A. A Model CRS-100 digital electronic integrator and associated paper readout was obtained from Infotronics Corp., Houston, Texas, U.S.A.

Thin-layer chromatograms were prepared and developed as previously reported[4,5]. Visualization of the developed chromatograms was accomplished by spraying the plates with an aqueous 20% ammonium bisulfate solution[14] until they appeared translucent. The translucent chromatograms were transferred to a hot plate and charred at 170°. Even distribution of heat on the glass plates was accomplished by placing a 25 × 25 × 1 cm aluminum sheet between the hot plate surface and the thin-layer chromatogram, by maintaining the charring plates in a draft free enclosure, and by placing the chromatogram on the metal sheet at ambient temperature and heating to char temperature at 5°/min. Chromatograms were held at char-temperature for 1 h before the hot plates were turned off and the entire system slowly cooled to ambient temperature over a 30-min time period. All charred plates are photographed for reference purposes.

The charred lipid spots on the chromatograms were scanned on the dual-beam

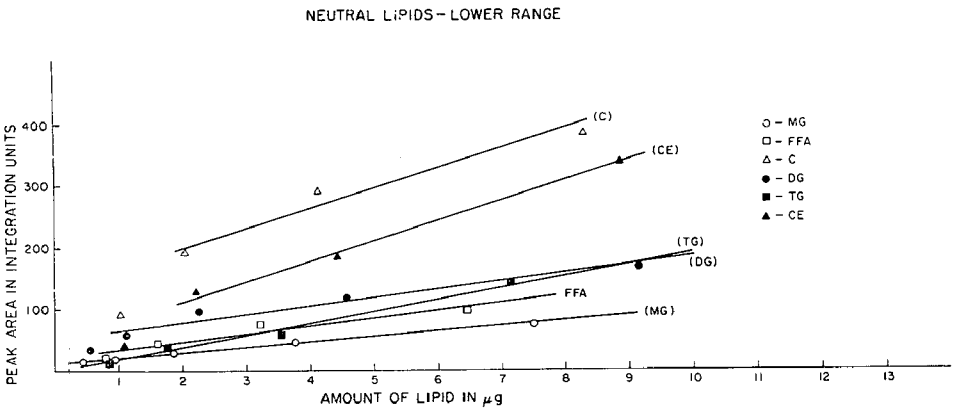

Fig. 1. Lower range (0–10 μg) for neutral lipids. Six neutral lipids classes with μgrams lipid spotted *vs.* peak area in integration units. The fatty acid on all neutral lipids was palmitic acid. MG, monopalmitin; FFA, palmitic acid; C, cholesterol; DG, dipalmitin; TG, tripalmitin; CE, cholesterol palmitate.

spectrodensitometer at a wavelength high enough to avoid absorbance by glass. Alternate lanes were spotted providing an adjacent empty lane for background correction by the densitometer. The plates were automatically scanned using transmittance techniques available with the densitometer. As the scan progresses, peaks corresponding to the optical density of the particular spot are recorded on a linear output chart and the peak areas simultaneously integrated by an electronic integrator. Comparison of the chromatogram picture, the strip chart peak record, and

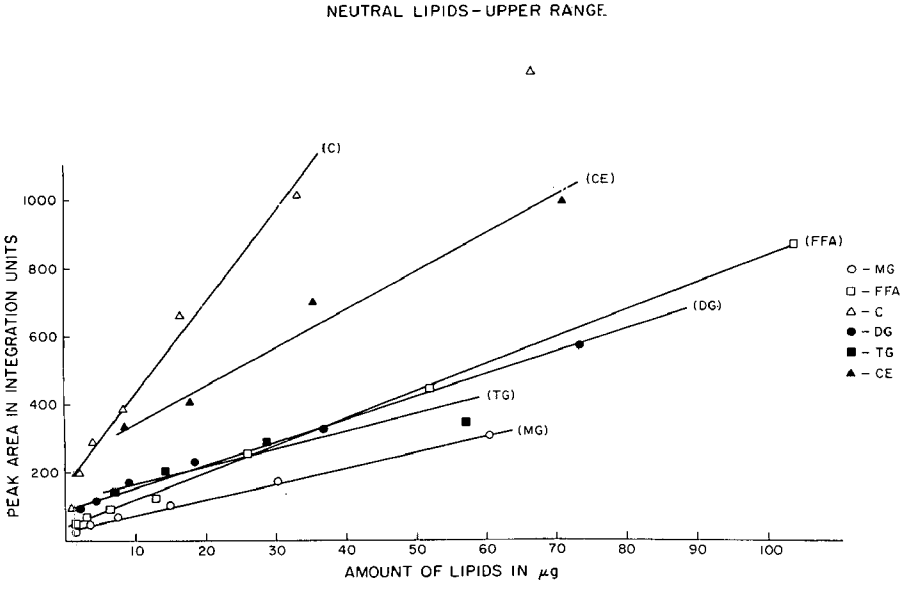

Fig. 2. Upper range (10–100 μg) for neutral lipids. Six neutral lipid classes with μgrams lipid spotted *vs.* peak area in integration units. The fatty acid on all neutral lipids was palmitic acid. MG, monopalmitin; FFA, palmitic acid; C, cholesterol; DG, dipalmitin; TG, tripalmitin; CE, cholesterol palmitate.

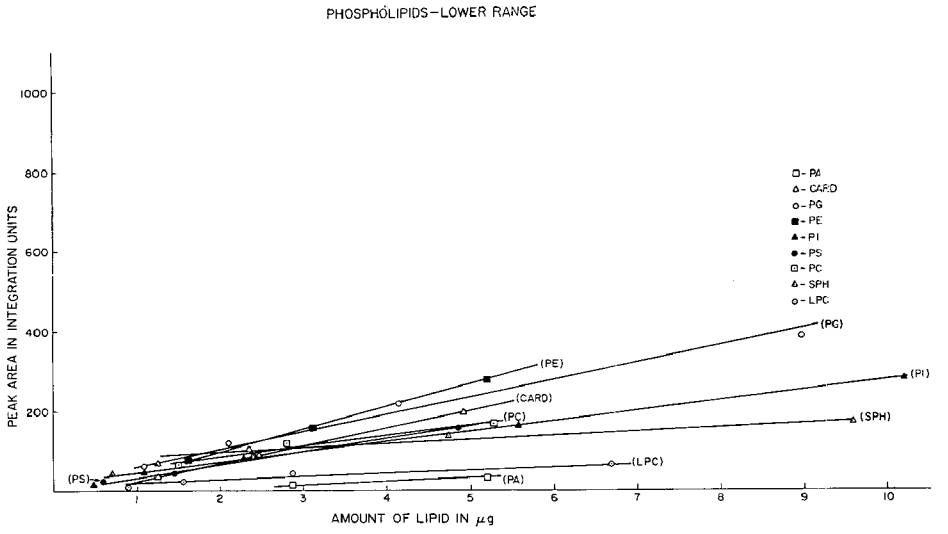

Fig. 3. Lower range (0–10 μg) for phospholipids. Nine phospholipid classes with μg lipid spotted *vs.* peak area in integration units. PA, phosphatidic acid; CARD, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

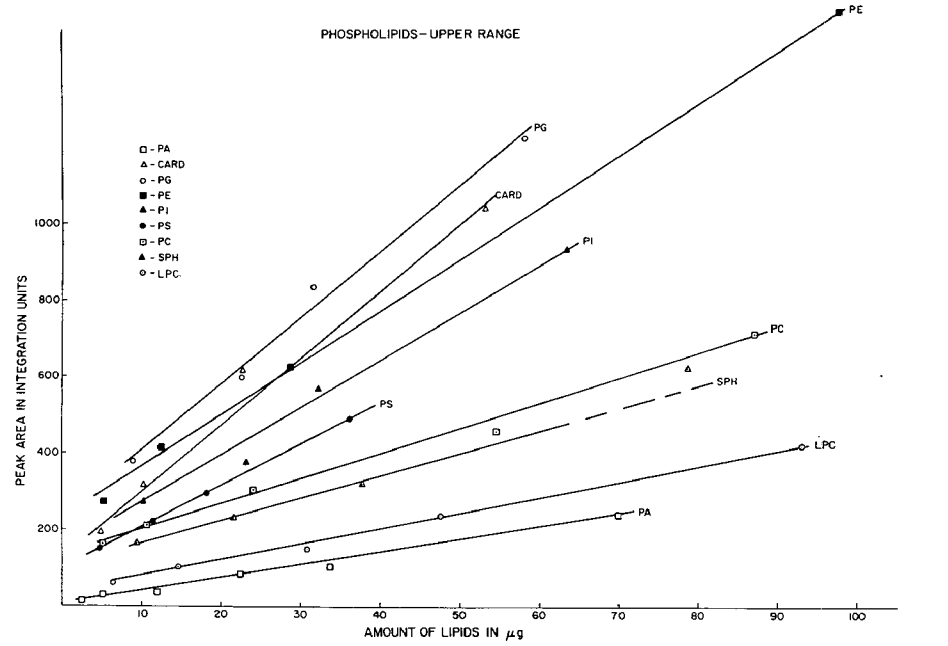

Fig. 4. Upper range (10–100 μg) for phospholipids. Nine phospholipid classes with μg lipid spotted *vs.* peak area in integration units. PA, phosphatidic acid; CARD, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

the integrator output provides the data required for the relative quantitation. The results are expressed as a relative per cent distribution within the spotted lane.

Results and discussion. Figs. 1–4 demonstrate the response of this procedure to increasing amounts of spotted and developed lipid material for each lipid class investigated. The demonstrated linear response region is the same from plate to plate although slight differences in the slope of the responses may occur between different plates. For most lipids the entire region tested was linear, in comparison to a rather restricted range as reported by FEWSTER *et al.*[16]. Table I shows the reproducibility, from lane to lane on the same plate and from plate to plate obtained by this method with artificial mixtures of both neutral and phospholipids. These data demonstrate the overall precision and reliability of this procedure.

This procedure provides a method for determining the relative per cent distribution of a complex lipid sample. While this procedure, in itself, does not determine

TABLE I

VARIABILITY IN QUANTITATION OF ARTIFICIAL LIPID MIXTURES BY THE CHAR-DENSITOMETRIC TECHNIQUE

	Phospholipids								
	LPC	*SPH*	*PC*	*PS*	*PI*	*PE*	*PG*	*CARD*	*PA*
	Lane to lane								
Relative % composition	9.5	22.4	28.8	3.4	9.5	12.9	6.3	3.0	3.2
S.D.	0.62	0.81	1.72	0.76	0.78	0.14	0.19	0.47	0.53
	Plate to plate								
Relative % composition	10.5	24.8	31.3	2.7	7.4	11.9	5.8	2.3	2.2
S.D.	0.80	1.79	1.52	0.99	1.08	0.53	0.41	0.58	0.59
	Neutral lipids								
	MG	*FFA*	*C*	*DG*	*TG*	*CE*			
	Lane to lane								
Relative % composition	3.7	12.0	29.0	3.4	18.3	33.4			
S.D.	0.36	1.25	1.51	0.25	1.41	1.15			
	Plate to plate								
Relative % composition	3.5	11.7	28.1	3.3	19.0	34.1			
S.D.	0.92	3.12	2.08	0.69	2.51	3.13			

the absolute amount of material present, it is readily applicable in instances where comparisons of lipid class distribution between samples derived from the same or similar origins are desired. Using this procedure eight samples can be analyzed on one chromatogram and 10–15 chromatograms can be quantitated per day with relative ease. Samples are generally analyzed in duplicate or triplicate in our laboratory and standard mixes spotted in the two outside lanes. The use of a dual-beam densitometer compensates for variation in the thickness of the plate coating. As reported by FEWSTER *et al.*, it is best to quantitate plates within a few hours after charring[16].

The char technique used in this procedure, as described by BOROWSKI AND ZIMIŃSKI[18] appears to give better results than those using sulfuric acid or sulfuric

acid plus an oxidizing agent. One advantage is that a much cleaner plate background is obtained which is more applicable to densitometric quantitation. It also gives good results for phospholipids in contrast to the method of BIEZENSKI *et al.*[15]. Finally, the range of response is wider than that reported by FEWSTER *et al.*[16].

As several reports suggest, the char technique yields different slopes of integration units plotted versus the amount of lipid applied in μmole or μg. The use of relative per cent distribution rather than absolute quantitation is useful for screening samples obtained from similar sources, for example, serum or tissue. The procedure can be used for determining large changes in a particular lipid class with reliability. Absolute quantitation could be achieved by comparison to known amounts of lipid applied to the same plate as suggested by FEWSTER *et al.*[16]. Relative per cent distribution measurement of lipid classes appears to provide useful data in assessing major changes in lipid class distribution.

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Physiological Sciences Department,
Naval Medical Research Institute,
Bethesda, Md. 20014 (U.S.A.)

GEORGE M. ADAMS
TERRY L. SALLEE

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CHROM. 5038

Dünnschichtchromatographischer Nachweis von 2-Desoxy-polyolen

Für den dünnschichtchromatographischen Nachweis der 2-Desoxy-aldosen hat sich die Chinaldin-Reaktion bewährt[1,2]. Wir fanden, dass diese für α-Methylenaldehyde spezifische Reaktion[3] auch zum Nachweis von 2-Desoxy-sorbit, 2-Desoxy-dulcit und 2-Desoxy-ribit geeignet ist, wenn man die 2-Desoxy-polyole auf dem entwickelten Chromatogramm durch Perjodatoxydation in eine reaktionsfähige Verbindung, die aus theoretischen Gründen mit β-Hydroxy-propionaldehyd identisch sein dürfte, überführt.

Zur Oxydation der 2-Desoxy-polyole wird die Platte nach Abdunsten des Fliessmittels mit 0.1% Perjodsäure in 20% wässriger *o*-Phosphorsäure besprüht und 15 Min bei 50° belassen. Die überschüssige Perjodsäure wird mittels SO_2 zerstört. Nach anschliessendem Besprühen mit dem Diaminobenzoesäure-Reagens (1 g 3,5-Diaminobenzoesäure·2HCl in 85 ml 10% *o*-Phosphorsäure) wird die Platte 15 Min bei 100° erhitzt. 2-Desoxy-polyole erscheinen im kurzwelligen UV-Licht ($\lambda = 254$ nm) als grünfluoreszierende Flecken auf dunklem Untergrund. Die Erfassungsgrenze liegt bei 0.1 μg am entwickelten Chromatogramm.

2-Desoxy-aldosen reagieren unter den angegebenen Bedingungen nicht, sodass durch direkte und nach Perjodatoxydation durchgeführte Chinaldin-Reaktion zwi-

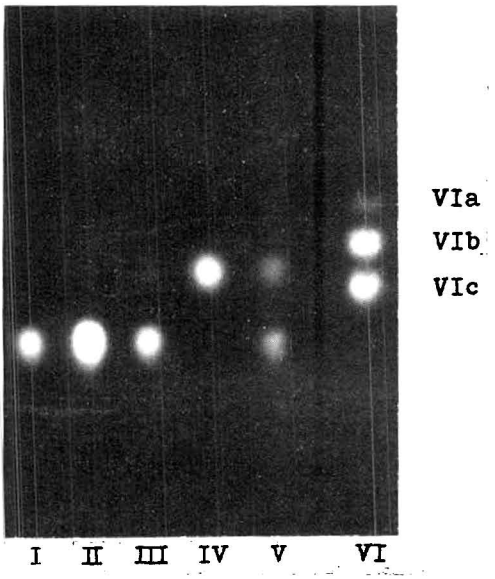

Fig. 1. Dünnschichtchromatographische Trennung und Anfärbung von 2-Desoxy-polyolen und 2-Desoxy-aldosen. Sorptionsmittel: Kieselgel G; Fliessmittel: Äthylacetat–Propanol–2–Wasser (65:23:12); Färbung der 2-Desoxy-polyole (I–IV) vgl. Text, Färbung der 2-Desoxy-aldosen (VI) mit dem 3,5-Diaminobenzoesäure-Reagens ohne Perjodatoxydation[1]. I = 2-Desoxy-sorbit, 0.5 μg; II = Extrakt aus Tabakpflanzen, vgl. Text; III = 2-Desoxy-dulcit, 0.5 μg; IV = 2-Desoxy-ribit, 0.5 μg; V = 2-Desoxy-sorbit, 2-Desoxy-dulcit, 2-Desoxy-ribit, je 0.1 μg; VIa = 2-Desoxy-ribose, VIb = 2-Desoxy-glucose, VIc = 2-Desoxy-galaktose. Photographie im UV-Licht unter Verwendung eines Schott'schen Glasfilters GG 14[4].

schen diesen und 2-Desoxy-polyolen differenziert werden kann (Fig. 1). Erwartungsgemäss gaben Polyole, Aldosen, Ketosen und Disaccharide keinen positiven Ausfall der Reaktion. Auch 6-Desoxy-hexosen reagieren nicht, sodass das Nachweisverfahren für 2-Desoxy-polyole spezifisch ist.

Ein Anwendungsbeispiel ist in Position II des Dünnschichtchromatogramms widergegeben (Fig. 1), wo ein Extrakt aus Tabakpflanzen aufgetragen wurde. Diese waren mit 2-Desoxy-glucose inkubiert worden, wobei die 2-Desoxy-aldose zum entsprechenden Zuckeralkohol reduziert wurde (WEIDEMANN, unveröffentlicht).

Chemisches Institut der
Städtischen Krankenanstalten,
85 Nürnberg (B.R.D.)

G. WEIDEMANN

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J. Chromatog., 54 (1971) 141–142

Book reviews

CHROM. 4957

Thermal Characterisation Techniques, edited by PHILIP E. SLADE, JR. AND LLOYD T. JENKINS, Marcel Dekker, New York, 1970, 371 pp., price £8.18, $18.75.

This, the second volume in a series entitled *Techniques and Methods of Polymer Evaluation*, appears some time after the third volume, *Characterisation and Analysis of Polymers by Gas Chromatography*. It contains six chapters of which only one is likely to be of direct interest to readers of the Journal of Chromatography; this is a contribution by BRAUER on pyrolysis–gas chromatographic techniques for polymer identification. Unfortunately the aforementioned volume 3 of the series deals rather more extensively and successfully with the same subject and the reader seeking information in this field alone is recommended to read it rather than the book now being reviewed.

BRAUER's contribution is a diligent summary of the literature through to 1969, but is generally lacking in comment or critique. The author does, however, allow himself to draw attention to the need for standardisation of PGC techniques, and he may like to know that the British Gas Chromatography Discussion Group is currently working on just such a programme. The organisation of the chapter is not very satisfactory; some 30 pages are devoted to a rambling account of "qualitative identification" including a description of the various pyrolysis techniques and multitudinous examples of polymer identification. One is left with the feeling the author has put the chapter together from the literature with scissors and adhesive tape but without much thought or imagination—in short, a reasonably complete but pedestrian account.

The book, as a whole, should appeal to the materials scientist studying polymers. The other chapters it contains cover differential scanning calorimetry, stress-strain temperature relations in high polymers, torsional braid analysis, thermal conductivity of polymers, and electrothermal analysis of polymers. Accounts of the theory and technique of these, for the most part rather unfamiliar, subjects are well supported by practical examples of their use in studying polymer behaviour. The book represents a good single source of information on these thermal methods and is attractively laid out and printed in the manner one is coming to expect from Marcel Dekker. The price, however, does seem rather high.

S. G. PERRY
Esso Research Centre,
Abingdon, Berks. (Great Britain)

CHROM. 5006

An Introduction to Isozyme Techniques, by GEORGE J. BREWER, Academic Press, New York and London, 1970, xii + 186 pp., price $ 11.

The considerable interest shown in the multiple molecular forms of enzymes (isozymes or isoenzymes) by scientists in a wide range of disciplines has stimulated a distinguished worker in the field, Dr. GEORGE BREWER, to collect together a number of reliable techniques used in his laboratory. Many of the research workers concerned, physiologists, geneticists, zoologists, botanists, microbiologists and physicians, may have little experience of biochemical methods, and it is to these groups that this book is especially directed, though biochemists will find much of interest in its pages. The author points out that the methods described may not necessarily be the best available, but he and his co-workers have used them satisfactorily. Some indeed have been developed or improved by Dr. BREWER and his colleagues.

Most attention is devoted to starch gel electrophoresis, but electrophoresis on other media, particularly acrylamide gel, is also described. Full details of the apparatus required, gel preparation, sample application, electrophoretic conditions and staining methods are given, and a chapter is devoted to sample preparation. In view of the practical importance of the genetically determined anomalies of red-cell enzymes, the reviewer was pleased to find a good account of the preparation of haemolysates for electrophoresis. The treatment of leukocytes, cell cultures, serum, soft tissues, Drosophila, plant tissues and microorganisms is also discussed.

Specific staining techniques for about forty different enzymes are given in detail. These include glucose 6-phosphate dehydrogenase, phosphohexose isomerase, lactate dehydrogenase, acid and alkaline phosphatase, fumarase, aldolase, hexokinase, esterase and carbonic anhydrase. There is some repetition in this section, but as the book is clearly intended for use at the bench, this feature is desirable since it adds to the convenience of the user. Particularly valuable is the inclusion of the author's comments after the description of each staining procedure.

The practical section is followed by a brief but critical review of the applications of isozyme separations in clinical medicine; organ, tissue and intracellular differentiation; genetic variation; and ontogeny. The last chapter (by C. F. SING) is devoted to an analysis of electrophoretic variation, including data acquisition and processing.

The author has maintained a critical approach throughout and makes frequent reference to unsolved problems. The book abounds with helpful hints, it is well-written and the selected bibliography provides a convenient entry to the enormous literature on the subject. Though there is no mention of the chromatographic methods which have found important applications in the study of the heterogeneity of esterases and transaminases, and discussion of the "nothing dehydrogenase" phenomenon is confined to a brief reference in the section on alcohol dehydrogenase, the book can unreservedly be recommended to anyone about to commence work in this fascinating field.

Charing Cross Hospital Medical School,
London (Great Britain)

J. H. WILKINSON

CHROM. 5046

SEPARATION OF FLUOROBROMOBENZENES BY GAS–LIQUID CHROMATOGRAPHY

ALBERTINE E. HABBOUSH AND ADNAN H. TAMEESH
College of Science, University of Baghdad (Iraq)
(Received September 16th, 1970)

SUMMARY

Fluorobromobenzenes were separated on squalane, silicone oil, polyethylene glycol (1500), polyethylene glycol succinate, polyoxyethylene sorbitan monostearate, di-*n*-decyl phthalate and polypropylene glycol. Specific retention volumes, band impurities, and electron polarisabilities were calculated and reported.

INTRODUCTION

There has been no quantitative separation and study of fluorobromobenzenes by gas–liquid chromatography. HABBOUSH AND NORMAN[1] could resolve these isomers on dinonyl phthalate, polyethylene glycol stearic acid, and tritolyl phosphate, but with band impurity not lower than 10^{-3}.

In the present work, a complete analysis of fluorobromobenzene isomers was made on squalane, silicone oil, polyethylene glycol (1500), polyethylene glycol succinate, polyoxyethylene sorbitan monostearate, di-*n*-decyl phthalate and polypropylene glycol. The specific retention volumes, separation factors, and band impurities were calculated. The forces which could affect the separations and elution orders of these isomers were discussed.

EXPERIMENTAL AND RESULTS

Materials and methods

Apparatus. A Perkin-Elmer Model 451 fractometer equipped with a thermistor-type thermal conductivity detector was used. The recorder was a 2.5 mV Honeywell Brown Electronic unit. Nitrogen was used as the carrier gas. The average column temperature was controlled to within $\pm 0.2°$.

Columns. These were specially ordered from Perkin Elmer, Great Britain. The columns used were stainless-steel tubing (2 m long and 1/4 in. O.D.) bent into a U shape and surrounded by asbestos sleeves to ensure uniform heating. The chemical composition of the liquid phases used are shown in Table I. Packing specifications of all columns used were identical. The weight of pure liquid phase per column was 3.40 g and the liquid on support was 20 % w/w. These were certified in this laboratory

TABLE I

THE CHEMICAL COMPOSITIONS OF THE LIQUID PHASES USED[4]

Chemical name	*Formula*	*Density 20°C (g/ml)*	*Mol. wt.*	*MOT*[a] *(°C)*
Polyethylene glycol	$(CH_2)_4(OH)_2O(OCH_2CH_2)_n$	1.152	1500	225
Polypropylene glycol (Ucon-LB-550-x)	$(CH_2)_6(OH)_2O(O(CH_2)_3)n$	0.984[b]	550	200
Di-*n*-decyl phthalate	$C_6H_4[COO(CH_2)_9CH_3]_2$	0.960	446.68	200
Polyethylene glycol succinate	$[O(CH_2CH_2O)_2COCH_2CH_2CO-]_n$	—	$(188.2)_n$	225
Polyoxyethylene sorbitan monostearate	—	—	—	160
2,6,10,15,19,23-Hexamethyl-tetracosane (squalane)	$C_{30}H_{62}$	0.829	422.5	160
Silicone oil (MS 200/50)	$(CH_3)_3Si-[OSi(CH_3)_2]_n-O-Si(CH_3)_3$	0.971	—	200

[a] Maximum operating temperature when they were packed in columns.
[b] Density at 100°F.

using a solvent extraction technique. The solid support was 60–80 mesh Chromosorb P.

Materials. Pure *o-*, *m-*, and *p*-fluorobromobenzenes were obtained from T. J. Sas & Son Ltd., London.

Sampling. Equal weights of the isomers were blended in a 3-ml cylinderical pyrex glass cell made in this laboratory. Sample sizes ranged from 0.5 to 1 μl. The injections were done using a 10-μl Hamilton 1 in. fixed needle syringe. An optimum flow rate of 20 ml/min at NTP was encountered throughout the investigations. The average inlet pressures was 6 ± 1 p.s.i. The specific retention volumes, V_g^o, were calculated following the well-known procedure given by LITTLEWOOD *et al.*[2]. The retention volume for the air peak was taken to be zero. Specific retention volumes for

TABLE II

SPECIFIC RETENTION VOLUMES, V_g^o (ml/g) FOR FLUOROBROMOBENZENES AT DIFFERENT TEMPERATURES

Phase	*Temperature (°C)*	*Fluorobromobenzene isomer*		
		Meta	*Para*	*Ortho*
Polypropylene glycol	140	174	193	225
Di-*n*-decyl phthalate	120	307	354	448
Squalane	120	429	474	577
	140	200	223	295
Silicone oil	120	195	204	235
	140	132	139	160
	180	64	64	73
Polyethylene glycol	100	47	52	60
	120	38	38	44
Polyethylene glycol succinate	100	147	173	211
	140	115	134	159
	180	88	102	121
Polyoxyethylene sorbitan monostearate	100	135	158	195
	120	77	89	108
	140	52	59	70

TABLE III

COLUMN EFFICIENCIES FOR FLUOROBROMOBENZENES AT DIFFERENT COLUMN TEMPERATURES[a]

Fluorobromobenzene isomer	n	α	η	n	α	η
	Polypropylene glycol (140°C)			*Di-n-decyl phthalate (120°C)*		
Meta	2830			2350		
		1.11	6×10^{-3}		1.15	5×10^{-4}
Para	2530			2110		
		1.16	1×10^{-4}		1.27	1×10^{-6}
Ortho	2820			2170		
	Squalane (120°C)			*Silicone oil (120°C)*		
Meta	2280			3730		
		1.11	4×10^{-9}		1.04	8×10^{-2}
Para	2620			3440		
		1.22	1×10^{-7}		1.15	2×10^{-6}
Ortho	2500			3900		
	Polyethylene glycol (100°C)			*Polyethylene glycol succinate (100°C)*		
Meta	120			2130		
		1.1	10^{-1}		1.18	5×10^{-5}
Para	325			2130		
		1.15	10^{-1}		1.22	6×10^{-6}
Ortho	680			2360		
	Polyoxyethylene sorbitan monostearate (100°C)					
Meta	2200					
		1.17	8×10^{-5}			
Para	2200					
		1.23	5×10^{-7}			
Ortho	2700					

[a] When a set of isomers was examined at more than one temperature, values of n, α, and η are recorded for the temperature at which resolution was more satisfactory.

fluorobromobenzene isomers at different column temperatures on the liquid phases examined are listed in Table II. The number of theoretical plates, n, was calculated as recommended by JOHNSON AND STROSS[3]. Table III gives the number of theoretical plates, separation factors and fractional band impurities for the isomers.

Electron polarisabilities per unit volume, α_e^v, were calculated using the Clausius–Mosotti equation:

$$\alpha_e^v = 3(n^2 - 1)/4\pi N(n^2 + 2)$$

Where n is the refractive index and N is Avogadro's number.

Refractive indices of fluorobromobenzenes at 30°C were measured in this laboratory using an Ape refractometer. Dipole moments were calculated as given by SMITH[5]. These physical constants are tabulated in Table IV.

Fluorobromobenzenes were selectively resolved on polyoxyethylene sorbitan monostearate, polyethylene glycol succinate, and di-*n*-decyl phthalate (as seen from Fig. 1 and Table III).

TABLE IV

SOME PHYSICAL CONSTANTS OF FLUOROBROMOBENZENE ISOMERS

Fluorobromobenzene isomer	$n_D^{30°}$	*E.P.* ($cm^3 \times 10^{26}$)	*B.p.*[a] (°C)	*D.M.* (*Debye*)
Ortho	1.5294	12.227	151–2	2.56
Meta	1.5229	12.100	149–51/764 mm	1.48
Para	1.5243	12.129	153.5/756 mm	0.08

[a] Ref. 6.

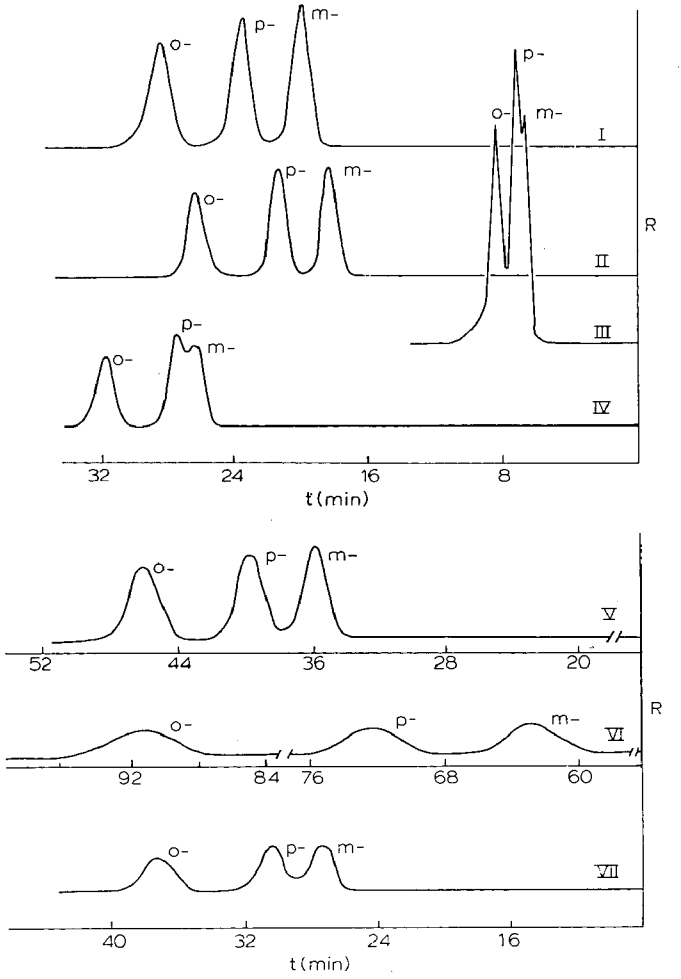

Fig. 1. Chromatograms showing the separation of fluorobromobenzene isomers on: I = Polyethylene glycol succinate at 100°C; II = polyoxyethylenesorbitan monostearate at 100°C; III = polyethylene glycol at 100°C; IV = silicone oil at 120°C; V = polypropylene glycol at 140°C; VI = di-*n*-decyl phthalate at 120°C; VII = squalane at 140°C. *R* is the recorder deflection, and *t* is the retention time in min.

DISCUSSION

The order of elution of fluorobromobenzenes on all the columns was *meta*, *para*, and then *ortho*. This order is not the order of the increase of the calculated dipole moments of these isomers. It seems that the polarisation of the *p*-isomer is higher than the corresponding *m*-isomer, *i.e.* the *p*-isomer would exert higher polarity than the calculated polarity, this being due to the polarising effect of the solvent (or the induced dipoles to the solvent). The *ortho* derivative has an effectively larger dipole moment, leading to a stronger interaction with the solvent. The calculated electron polarisabilities per unit volume for these isomers support the above mechanism (see Table IV).

The most selective liquid phases for the separation and quantitative determination of these isomers are the polyglycols. When an ester group (succinate, stearate or phthalate) is introduced to the structure of a polyglycol-type liquid phase, the selectivity is much increased (as seen in Table III, Fig. 1). The carbonyl groups present in such structures may act as good polarising groups leading to better resolutions.

The results obtained in this work can be utilised successfully in the quantitative determinations and in following the kinetics of the reactions involving these isomers.

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CHROM. 5057

GAS CHROMATOGRAPHIC SEPARATION OF DIASTEREOISOMERIC ALKYL METHYLPHOSPHONOFLUORIDATES AND RELATED COMPOUNDS

A. VERWEIJ, E. BURGHARDT AND A. W. KOONINGS

Chemical Laboratory of the National Defence Research Organization TNO, Rijswijk, Z.H. (The Netherlands)

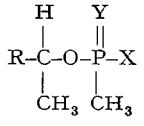
(Received August 13th, 1970)

SUMMARY

A gas chromatographic study was performed concerning the structure–separation relationship of diastereoisomeric alkyl methylphosphonofluoridates and related compounds. Relative retention values (r), determined on two stationary phases of different polarity, were used as a measure for the separation of the stereoisomeric forms. It was demonstrated that both steric and electronic interactions influence the separation. The P–O–C ester linkage and the P–F bond proved to be important in the differential interaction between the diastereoisomers and the stationary phases.

INTRODUCTION

The results of several investigations concerning the chromatographic separation of diastereoisomeric carboxylic esters[1–5], amides[2,6] and alcohols[7] have been published. Comparable studies dealing with organophosphorus compounds are unknown to us. We wish to report on the structure–separation relationship of diastereoisomeric alkyl methylphosphonofluoridates and related compounds with the general formula



in which R = C_nH_{2n+1}, C_nH_{2n-1}, C_nH_{2n-3} or $(CH_3)_2NCH_2$, Y = O or S and X = F, H or OCH_3. The gas chromatographic analyses were performed on two columns of different polarity. In order to gain insight into the factors determining the separation of the stereoisomeric forms, the effects of structural variations at both chiral centres were examined. The relative retention[8] (r) derived from the adjusted retention times of the diastereoisomers of the respective compounds was used as a measure for their separation.

In general when two compounds are analysed under identical gas chromatographic conditions, r is a function of vapour pressures and solubilities in the stationary phase. When comparing r values obtained on columns of different polarity, used at

the same temperature, mainly solubility effects have to be considered. In this way it is possible to investigate the contribution of electronic interactions to the separation.

EXPERIMENTAL

Chromatographic procedure

The gas chromatographic data were obtained at 110° on two glass columns (2.7–3 m × 3–4 mm I.D.). The apolar column was packed with 25 % (w/w) DC-550 silicone oil, the polar one with 25 % (w/w) Triton X-305, both coated on Chromosorb W-AW, 80–100 mesh. The columns were housed in a Varian Aerograph, Model 1520-3B, equipped with two flame ionisation detectors. The carrier gas was nitrogen at a flow rate of 20 ml/min. Injection blocks and detectors were maintained at 195°. Samples ($\leqslant$ 0.1 μl) were introduced by on-column injection.

Using Chromosorb as support material, a decomposition of 2,4-dimethylpentylmethylphosphinic fluoride was observed. Therefore the compound was analysed on silanised glass columns, which were packed with teflon powder (40–60 mesh) as an inert support material. The coating (5 % w/w) and packing procedures used were according to KIRKLAND[9]. This change in support material did not influence the results as was derived from control experiments.

The adjusted retention times collected in Table II were obtained from representative chromatograms. The median relative retention values given were calculated according to the statistical method of DEAN AND DIXON[10]. The relative standard deviation is about 0.5 %.

Materials

The methods used for the synthesis of the organophosphorus compounds and some physical constants are summarised in Table I. The purity of the compounds proved to be satisfactory as was checked by the gas chromatographic procedure described above. Infrared spectroscopy was used to identify the eluted peaks of representatives of the different types of compounds. Separate peaks ascribed to diastereoisomeric forms gave identical infrared spectra.

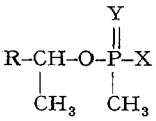
RESULTS AND DISCUSSION

$R = C_nH_{2n+1}$

As can be seen from Table II the relative retentions (r) obtained for the members of the homologous series of *sec.*-alkyl methylphosphonofluoridates (compounds 1–5) increase on lengthening the chain of the alkyl group R. The increase is observed on both the polar and apolar columns. When comparing compounds with the same carbon chain length (compounds 2, 7 and 8) an increase in the bulk size at the γ-carbon atom gave an increase in r. Another series (compounds 1, 6 and 9) shows a shorter carbon chain length but similar branching at the β-carbon atom. In this case a considerably smaller effect was found; only for compound 9 a resolution is observed. These results indicate that the resolution is determined by the length and the branching of the carbon chain R as well as the distance of the branching from the chiral carbon atom.

TABLE I

PHYSICAL CONSTANTS AND METHODS OF SYNTHESIS OF DIASTEREOISOMERIC ORGANOPHOSPHORUS COMPOUNDS WITH THE GENERAL FORMULA



Compound No.	*R*	*Y*	*X*	*b.p./mm*	n_D^{25}	*Synthesised according to Ref.*
1	CH_3CH_2	O	F	47°/4.5	1.3912	11[a]
2	$CH_3(CH_2)_2$	O	F	73°/10	1.3966	11
3	$CH_3(CH_2)_3$	O	F	65°/3	1.4058	11
4	$CH_3(CH_2)_4$	O	F	64°/1	1.4090	11
5	$CH_3(CH_2)_5$	O	F	79°/0.7	1.4131	11
6	$(CH_3)_2CH$	O	F	53°/2	1.3968	11[a]
7	$(CH_3)_2CHCH_2$	O	F	73°/7	1.4010	11[a]
8	$(CH_3)_3CCH_2$	O	F	66°/3	1.4071	11[a]
9	$(CH_3)_3C$	O	F	57°/4	1.4045	11
10	$CH_3(CH_2)_5$	S	F	56°/0.3	1.4453	12
11	$(CH_3)_2CHCH_2$	S	F	54°/3	1.4390	12
12	$CH_3(CH_2)_5$	O	H	68°/0.01	—	13
13	$(CH_3)_2CHCH_2$	O	OCH_3	36°/0.05	—	[b]
14	$CH_2=CH$	O	F	decomp.	—	11[a]
15	$HC\equiv C$	O	F	64°/4	1.4117	11[a]
16	$CH_3C\equiv C$	O	F	47°/0.1	1.4255	11[a]
17	$(CH_3)_2NCH_2$	O	F	55°/0.5	—	11[a]

[a] Modified by the addition of 1 mole triethylamine to the reaction mixture.
[b] Synthesised from compound No. 7 and sodium methylate.

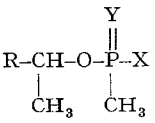
The importance of the distance between the chiral centres was investigated by moving the alcoholic chiral centre from the α- to the β-carbon atom. 2-Methylpentyl methylphosphonofluoridate, $[CH_3(CH_2)_2CH(CH_3)CH_2O]CH_3P(O)F$, did not separate on both columns contrary to the obvious separations of compound 3. From this it may be concluded that the distance between the chiral centres is critical. An increase in the distance will result in a larger conformational mobility giving an unsatisfactory difference in interaction between the diastereoisomers and the stationary phase.

$R = C_nH_{2n-1}$ or C_nH_{2n-3}

An unsaturated group R is present in compounds 14–16. When comparing compounds 1, 14 and 15 having identical carbon chain lengths the increase in the r value runs parallel with the degree of unsaturation. On lengthening the carbon chain with one carbon atom (compare compounds 15 and 16) the r values decrease contrary to the results obtained for the saturated compounds. Nevertheless the r values of 16 exceed those of the comparable saturated compound 2. It follows that the relatively large r values for 15 are probably due to the influence of the C ≡ C function as well as to the presence of the acetylenic hydrogen atom.

TABLE II

RETENTION TIMES AND RELATIVE RETENTION VALUES (r) OF DIASTEREOISOMERIC ORGANOPHOSPHORUS COMPOUNDS[a] WITH THE GENERAL FORMULA



Compound No.	R	Y	X	Polar column			Apolar column		
				Adj. ret. (min)		r	Adj. ret. (min)		r
				peak 1	peak 2		peak 1	peak 2	
1	CH_3CH_2	O	F	11.6	11.6	1.00	9.0	9.0	1.00
2	$CH_3(CH_2)_2$	O	F	20.5	20.0	1.02[c]	15.8	15.8	1.00
3	$CH_3(CH_2)_3$	O	F	39.5	41.8	1.058	32.0	33.5	1.047
4	$CH_3(CH_2)_4$	O	F	74.5	80.7	1.083	56.6	60.4	1.068
5	$CH_3(CH_2)_5$	O	F	111.0	121.0	1.089	102.0	110.0	1.073
6	$(CH_3)_2CH$	O	F	14.1	14.1	1.00	16.4	16.4	1.00
7	$(CH_3)_2CHCH_2$	O	F	26.9	28.9	1.074	22.6	23.8	1.055
8	$(CH_3)_3CCH_2$	O	F	25.2	28.4	1.131	26.3	28.4	1.087
9	$(CH_3)_3C$	O	F	29.7	31.2	1.05[c]	22.7	23.4	1.03[c]
10	$CH_3(CH_2)_5$	S	F	84.7	92.8	1.094	116.0	125.0	1.077
11	$(CH_3)_2CHCH_2$	S	F	23.3	24.9	1.065	25.4	26.6	1.049
12	$CH_3(CH_2)_5$	O	H	359.0	378.0	1.052	231.0	239.0	1.036
13	$(CH_3)_2CHCH_2$	O	OCH_3	82.9	82.9	1.00	64.3	64.3	1.00
14	$CH_2=CH$	O	F	16.2	16.7	1.03	8.5	8.5	1.00
15	$HC\equiv C$	O	F	34.5	43.7	1.262	8.8	10.3	1.174
16	$CH_3C\equiv C$	O	F	71.1	83.7	1.178	27.7	31.2	1.125
17	$(CH_3)_2NCH_2$	O	F	8.8	10.1	1.155[b]	26.4	29.2	1.106

[a] Gas chromatographic conditions as outlined in the experimental part. Compounds 1, 2, 6 and 8 were analysed on columns 2.7 m × 4 mm I.D., the other compounds on columns 3 m × 3 mm I.D.

[b] For the polar column (90 cm × 3 mm I.D.) 10% liquid loading was used.

[c] Because of largely overlapping peaks a curve resolver (Du Pont de Nemours 310) was used.

$R = (CH_3)_2CHCH_2$ versus $(CH_3)_2NCH_2$

Replacing an apolar $CH(CH_3)_2$ group by an isosteric polar $N(CH_3)_2$ group (compare compounds 7 and 17) an increase in the r value on both columns is observed. This result supports the forementioned conclusion concerning the importance of electronic effects.

$Y = O$ versus S

From the retention data collected in Table II (compare compounds 5 with 10 and 7 with 11), it can be seen that the retention time on the apolar column increases when P=S is substituted for P=O. On this column the vapour pressure differences will prevail showing separations which parallel molecular weights. On the polar column the reversed sequence is observed. In this case electronic interactions especially between the oxygen compounds and the stationary phase will predominate. However, the r values change very little on both columns when P=O is replaced by P=S. It follows that this substitution has only a minor effect on the separation of the diastereoisomers under consideration.

X = F versus H or OCH_3

The contribution of the P–F function to the r values can be estimated when comparing the data of the compounds 5 with 12 and 7 with 13. Replacement of the fluorine atom by a hydrogen atom (compare 5 and 12) will result in a small change in steric requirements since the Van der Waals radii are ~1.4 Å and ~1.2 Å, respectively. As far as steric reasons are concerned comparable r values would be expected. Nevertheless, the r values decrease considerably on both columns when the P–H* is substituted for a P–F. The decreased difference in interactions of the diastereoisomers with the stationary phase contrasts with the increased electronic interactions as reflected by the higher retention times.

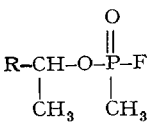
Substituting the P–OCH_3 for a P–F function (compare compounds 7 and 13) it can be seen from Table II that the separation is completely lost. This result may be ascribed to the lower polarity of the P–OCH_3 group and the presence of a second ester group at the phosphorus atom which diminishes the functional chirality.

From these results it can be concluded that the P–F function plays an essential role in the separation of the diastereoisomeric alkyl methylphosphonofluoridates.

P–O–C versus P–C–C

In order to examine the contribution of the ester linkage to the separation, the P–O–C group was replaced by the isosteric P–C–C group (compare 7 with 2,4-dimethylpentylmethylphosphinic fluoride**). The introduction of the P–C–C group causes a diminished functional chirality in analogy with the forementioned reasoning concerning the P–OCH_3 substitution for P–F. On both columns the phosphinic fluoride gave no perceptible separation. It follows that the P–O–C ester linkage is important for the separation of diastereoisomeric phosphonofluoridates. A similar result was found by KARGER *et al.*[1] for diastereoisomeric carboxylic esters. They regarded the ester function as an important zone in the differential interaction with the stationary phase.

The forementioned results concerning the structure–separation relationship of diastereoisomeric alkyl methylphosphonofluoridates with the general formula



on polar and apolar gas chromatographic columns can be summarised as follows: The relative retention r

(a) increases on lengthening as well as on branching of the carbon chain R;

(b) increases considerably by replacement of the alkyl group R by a function of increased polarity such as a vinyl, an acetylenic or dialkylamino group;

(c) decreases to $r = 1.00$ by moving the chiral centre from the α-carbon to the β-carbon atom in the alkoxy group;

* Infrared vapour spectra of compound 12 did not indicate the presence of a P–OH bond.

** $[(CH_3)_2CHCH_2CH(CH_3)CH_2]CH_3P(O)F$. Gas chromatographic conditions were changed as outlined in the experimental part. Synthesis according to Ref. 14.

(d) shows a small change when substituting P=S for P=O;

(e) decreases to $r = 1.00$ when the P–O–C ester linkage is replaced by a P–C–C grouping;

(f) decreases when the P–F function is replaced by a P–H or P–O–CH_3 group;

(g) increases when substituting a polar column for an apolar column.

It may be concluded that both steric and electronic interactions are important for the separation of diastereoisomeric alkyl methylphosphonofluoridates.

ACKNOWLEDGEMENT

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GAS CHROMATOGRAPHY OF ALKYL IODIDES

II. INFLUENCE OF STRUCTURE ON RETENTION TIME AND SENSITIVITY TO ELECTRON CAPTURE DETECTOR*

GIANRICO CASTELLO AND GIUSEPPINA D'AMATO

Istituto di Chimica Industriale, Universitá di Genova, Centro Nazionale Chimica Macromolecole, Sez. V, Genova (Italy)

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SUMMARY

Several linear and branched iodoalkanes were synthesised and analysed by gas chromatography with tricresylphosphate columns and an electron capture detector.

The behaviour of retention times and retention indices was examined as a function of the number of carbon atoms, the position of the iodine in the molecule, and the number and position of branchings.

Also the sensitivity of the electron capture detector to alkyl iodides was studied, and the capture coefficient, the absolute molar response and the relative molar response were determined.

While all these values depend on several parameters, the relative molar response depends only on standing current, and therefore can be easily used for standardisation.

INTRODUCTION

In a previous paper[1], the gas chromatographic (GC) analysis of alkyl iodides was performed using tricresylphosphate columns, and a regular behaviour of retention times and sensitivities to electron capture detector (ECD) was found for the homologous series of 1-iodoalkanes.

The linear dependence of log t_a (where t_a is the adjusted retention time) (ref. 2) on the number of carbon atoms in the linear molecule, allowed the introduction of retention indices relative to 1-iodoalkanes (I_{ni}) as means of identification for all the branched-chain alkyl iodides. These retention indices are quite similar to the retention indices proposed by KOVÁTS[3].

The values of I_{ni} for some branched iodoalkanes, as determined in the work cited[1], have only shown that branched-chain and 2-iodo compounds have a lower retention time than 1-iodoalkanes.

A greater number of branched and linear iodoalkanes were synthesised and

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analysed in the present work in order to investigate the correlations between structure, retention time and sensitivity.

EXPERIMENTAL

In addition to the products cited in ref. 1, several products were synthesised and are listed in Table I. They were generally prepared from the corresponding alcohol, by treatment with constant boiling point hydriodic acid (57 %) (ref. 4). Only 3-iodo-2,2,4-trimethylpentane was obtained from the alcohol by reaction with purified red phosphorus and iodine[5].

Some products, whose syntheses were impossible due to the lack of the corresponding starting compound, were obtained by irradiation of the congruent hydrocarbon with γ rays in the presence of iodine.

TABLE I

PHYSICAL CONSTANTS OF SYNTHESISED AND ANALYSED COMPOUNDS

Unless otherwise specified, boiling points are at 760 Torr, index of refraction and density at 20°C.

No.	*Alkyl iodides*	*Boiling point* (°C)	*Index of refraction*	*Density* (g/ml)
1	2-Iodopropane	89.5	1.5026	1.70
2	2-Iodobutane	120	1.4991	1.5984
3	2-Iodopentane	144–145	1.4954	1.53
4	3-Iodopentane	145–146	1.4967	1.52
5	2-Iodohexane	90–91 (70 Torr)	1.4911 (25°C)	1.4269 (15°C)
6	3-Iodohexane	110 (70 Torr)	1.4933	1.45
7	2-Iodoheptane	100 (50 Torr)	1.4827	1.304
8	3-Iodoheptane	88.9 (30 Torr)	1.4901	1.3675 (25°C)
9	4-Iodoheptane	185	1.4915	1.39
10	1-Iodo-2-methylbutane	148	1.4977	1.53
11	1-Iodo-3-methylbutane	147	1.4939	1.51
12	2-Iodo-2-methylbutane	124.3–125.5	1.50	1.49
13	2-Iodo-3-methylbutane	141	1.4965	1.5026
14	1-Iodo-2-methylpentane	72 (22 Torr)	1.4938	1.44
15	1-Iodo-3-methylpentane	170	1.4925	1.46
16	1-Iodo-4-methylpentane	173.2	1.4892	1.4283
17	2-Iodo-2-methylpentane	142	1.4940	1.41
18	2-Iodo-3-methylpentane	83 (65 Torr)	1.484	1.451 (18°C)
19	2-Iodo-4-methylpentane	160	1.4900	1.4713
20	3-Iodo-2-methylpentane	147	1.477	1.31
21	3-Iodo-3-methylpentane	120 (vacuum)	1.4930	1.37
22	1-Iodo-2-ethylbutane	—	1.4975	1.46
23	1-Iodo-2,2-dimethylbutane	110 (vacuum)	1.4960	1.45
24	1-Iodo-3,3-dimethylbutane	162	1.4315	1.34
25	2-Iodo-3,3-dimethylbutane	145	1.4973	1.42
26	1-Iodo-2,3-dimethylbutane[a]	—	—	—
27	2-Iodo-2,3-dimethylbutane	145	1.4895	1.39
28	2-Iodo-2,4-dimethylpentane	140–142	1.4690	1.08
29	1-Iodo-2,2,4-trimethylpentane	131	1.4770	1.22
30	3-Iodo-2,2,4-trimethylpentane	126	1.4894	1.23
31	1-Iodo-2,4,4-trimethylpentane[a]	—	—	—
32	4-Iodo-2-methyl-7-ethylnonane	170	1.4726	1.0805
33	4-Iodo-2,6,8-trimethylnonane	140 (vacuum)	1.4590	1.0201
34	2,2,7,7-tetramethyl-iodooctane[a]	—	—	—

[a] Prepared by γ-irradiation (see text).

The analyses were made using a Varian Aerograph Model 500-D electrometer and a separate column oven Model 550-B, to allow for better temperature control. A 3 m × 1/8 in. glass column, filled with 15 % tricresylphosphate on DMCS-treated Chromosorb W (80–110 mesh) was used. The detector was an electron capture of the concentric type, containing 250 mCi of titanium tritide; the polarising voltage was 90 V d.c. Ultrapure nitrogen was used as carrier gas. The sample, about 1 μl of iodoalkane solution in purified *n*-hexane, was directly injected on-column to minimise decomposition.

RESULTS

In Table II are reported the adjusted retention times (t_a), obtained by subtracting the gas hold-up time t_a from the total retention time t_r, and the retention indices relative to the linear 1-iodoalkanes (I_{ni}).

TABLE II

ADJUSTED RETENTION TIMES AND RETENTION INDICES RELATIVES TO *n*-IODIDES OF SOME ISOMERIC ALKYL IODIDES

No.		t_a (*min*)	I_{ni}	δI_{ni}	δI_{ni}%
1	2-Iodopropane	1.84	245	55	18.33
2	2-Iodobutane	3.93	359.5	40.5	10.12
3	2-Iodopentane	7.00	444	66	13.20
4	3-Iodopentane	7.73	458	42	8.40
5	2-Iodohexane	13.53	540	60	10.00
6	3-Iodohexane	13.58	540.5	59.5	9.92
7	2-Iodoheptane	29.55	636	64	9.14
8	3-Iodoheptane	29.05	633.5	66.5	9.50
9	4-Iodoheptane	26.44	619.5	81.5	11.64
10	1-Iodo-2-methylbutane	8.22	467	33	6.60
11	1-Iodo-3-methylbutane	7.33	451	49	9.80
12	2-Iodo-2-methylbutane	7.04	445	55	11.00
13	2-Iodo-3-methylbutane	6.99	444	56	11.20
14	1-Iodo-2-methylpentane	15.12	556	44	7.33
15	1-Iodo-3-methylpentane	15.60	561	39	6.50
16	1-Iodo-4-methylpentane	15.42	559	41	6.83
17	2-Iodo-2-methylpentane	13.21	538	62	10.33
18	2-Iodo-3-methylpentane	13.67	541.5	58.5	9.75
19	2-Iodo-4-methylpentane	13.23	537	63	10.50
20	3-Iodo-2-methylpentane	13.17	536	64	10.67
21	3-iodo-3-methylpentane	13.72	542	58	9.67
22	1-Iodo-2-ethylbutane	15.15	557	43	7.17

The behaviour of I_{ni} *vs.* the number of carbon atoms for straight-chain iodoalkanes is reported in Fig. 1. The plot is obviously linear for 1-iodoalkanes, but it is interesting to observe that also 2- and 3-iodoalkanes show a similar relationship. As only one 4-iodoalkane was synthesised, it was not possible to determine the corresponding function.

The following equation for 1-, 2- and 3-iodoalkanes can therefore be written:

$$I_{ni} = A_0 + A_1 n$$

using the least square method, the coefficient A_0 and A_1 were calculated and are reported in Table III.

Behaviour of 2-iodoalkanes parallels that of 1-iodoalkanes, while 3-iodoalkanes have a minor slope. It is probable that by increasing the length of the chain the slope of 3-iodoalkanes will increase.

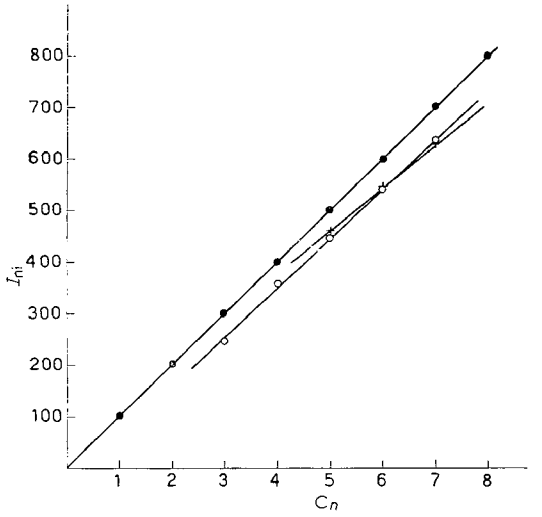

Fig. 1. Retention indices relative to *n*-iodoalkanes as a function of the number of carbon atoms for 1-iodo, 2-iodo and 3-iodo straight-chain alkanes. ●, 1-Iodoalkanes; ○, 2-iodoalkanes; +, 3-iodoalkanes.

TABLE III

COEFFICIENT OF EQUATION $I_{ni} = A_0 + A_1 n$ CALCULATED BY THE LEAST-SQUARE METHOD FROM DATA OF TABLE II AND FIG. 1. FOR STRAIGHT-CHAIN IODOALKANES

	A_0	A_1
1 Iodo	0	100
2 Iodo	−50.38	98.42
3 Iodo	+33.33	85.75

If the iodine is substituted on one of the carbon atoms internal to the chain, a smaller I_{ni} than that of the 1-iodoalkanes is therefore obtained. Differences between the various internal positions are of minor importance, as can be seen from Fig. 2, where the I_{ni} of straight-chain iodoalkanes is plotted *vs.* the position of iodine in the molecule. When iodine is at the end of the chain, the I_{ni} is obviously a multiple of 100. When iodine is connected to a methylenic carbon atom, the decrease of I_{ni} is approximately the same, independent of the length of chain and of the position. This behaviour of I_{ni} for straight-chain iodides is very similar to that of boiling points. By changing the iodine position in branched-chain compounds, as 2-methyl-iodobutanes, 2-methyl-iodopentanes and 3-methyl-iodopentanes, a similar behaviour can be seen.

In Table II are reported, as δI_{ni} and δI_{ni} %, the differences between the I_{ni} of the linear 1-iodoalkanes (by definition equal to multiples of 100) and those of each isomeric compound, respectively, in index units and in percent values. If the iodine atom is substituted on the first carbon atom of the chain, we can observe a δI_{ni} % of about 7 %. If the iodine is internal to the chain, it produces a larger decrease of I_{ni}. In fact, the average δI_{ni} % is about 11 % for 2-iodo-2-methylbutane and 2-iodo-3-methylbutane, 10.5 % for 2-methyl-iodopentanes and 9.7 % for 3-methyl-iodopentanes with iodine in positions 2 and 3. From these values it seems that the decrease in the retention time is relatively more important for short chains and larger if the methyl group is substituted in position 2.

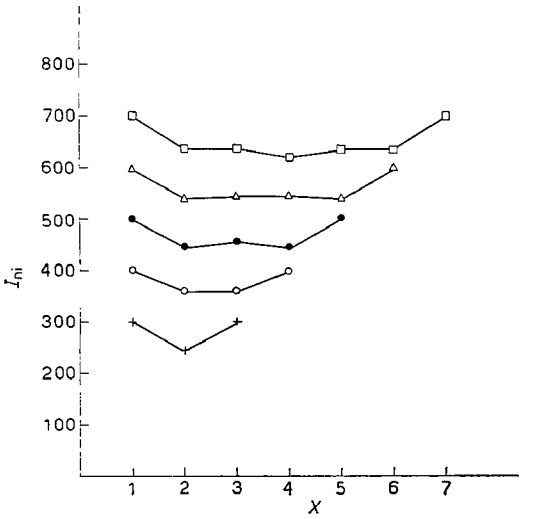

Fig. 2. I_{ni} of straight-chain iodoalkanes as a function of the position of iodine in the molecule. □, x-Iodoheptanes; △, x-iodohexanes; ●, x-iodopentanes; ○, x-iodobutanes; +, x-iodopropanes.

It is now interesting to investigate the effect of the different positions of the methyl group in compounds with the same position of the iodine. In the three parts of Fig. 3 are plotted the values of I_{ni} for alkyl iodides with iodine in positions 1, 2 and 3, respectively. Values on the abscissa indicate the methyl group position. One should observe that, to compare products with the same basic structure, 2-iodo-*n*-alkanes may be considered as 1-iodo-1-methylalkanes, 3-iodoalkanes as 2-iodo-1-methyl alkanes, etc. For example, to see the effects of methyl substitution on the various carbon atoms of the 1-iodopentane, we can consider the 2-iodohexane as the first member of the series of general formula 1-iodo-x-methylpentane, etc. The compounds without methyl substitution are therefore plotted at abscissa zero (*i.e.*, as an example, 1-iodohexane, 2-iodohexane and 3-iodohexane for the three parts of Fig. 3).

In Table IV are reported the retention indices I_{ni} of various compounds with a branched skeleton. The increase in branching causes a decrease in I_{ni}. This effect increases for compounds with a higher number of carbon atoms, but the small number of possible isomers analysed does not allow a general law to be deduced. Not withstanding this, the behaviour of C_{12} methyl-branched iodoalkanes is interesting; di-

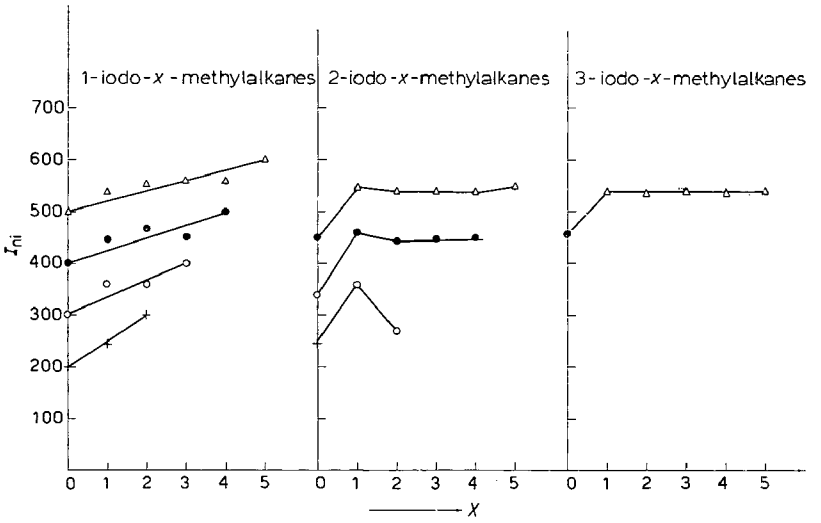

Fig. 3. I_{ni} of branched-chain iodoalkanes as a function of the different positions of iodine and methyl group. Number of carbon atoms in basic chain: + = 2; ○ = 3; ● = 4; △ = 5.

TABLE IV

RETENTION INDICES I_{ni} OF BRANCHED-CHAIN ALKYL IODIDES

No.		I_{ni}	δI_{ni}	δI_{ni}%	$\delta I_{ni(branch)}$
23	1-Iodo-2,2-dimethylbutane	540	60	10.00	30
24	1-Iodo-3,3-dimethylbutane	496	104	17.33	49.5
25	2-Iodo-3,3-dimethylbutane	501	99	16.50	52
26	1-Iodo-2,3-dimethylbutane[a]	538	62	10.33	31
27	2-Iodo-2,3-dimethylbutane	501	99	16.50	49
28	2-Iodo-2,4-dimethylpentane	604	96	13.71	48
29	1-Iodo-2,2,4-trimethylpentane	665	135	16.87	45
30	3-Iodo-2,2,4-trimethylpentane	662	138	17.25	46
31	1-Iodo-2,4,4-trimethylpentane[a]	695	105	13.12	35
32	4-Iodo-2-methyl-7-ethylnonane	964	236	19.67	118
33	4-Iodo-2,6,8-trimethylnonane	858	342	28.50	114
34	2,2,7,7-Tetramethyl-iodo-octane[a]	700	500	41.66	125

[a] Prepared by γ-irradiation (see text).

viding δI_{ni} by the number of branches, a constant value is obtained. It seems that for compounds with a great number of carbon atoms, the effect of iodine is small compared to that of the chain branching and that every new branch produces the same decrease in retention time. From the experimental data reported above, a general trend for the separation of alkyl iodides on tricresylphosphate columns can be obtained: the retention time decreases when the branching of the molecule increases and when the iodine is substituted on carbon atoms internal to the molecule.

On the basis of these observation, a reasonable explanation of the separation

mechanism can be the fact that tricresylphosphate is a polar stationary phase and that the great retention time of alkyl iodides is due to their dipole moment. The separation between isomeric alkyl iodides with small differences in the boiling points is probably due to different dipole moments.

SENSITIVITY

The sensitivity as a function of structure can be studied using as measuring unit the K (capture coefficient) (refs. 6–8) defined by:

$$K = \frac{I_b - I_c}{I_c} \frac{(V + a)}{c} \tag{1}$$

where I_b is the standing current, as measured from the difference between the detector signals with and without applied field, that takes into account the reduction due to column bleeding;

I_c is the decreased current observed on the introduction of a capturing species into the detector;

$I_b - I_c$ is therefore the detector signal;

c is the instantaneous concentration of the capturing species;

V is the voltage of the field applied to the detector;

a is a constant characteristic of the detector used and is equal to the ratio L/K_i between the leakage of electrons from the reaction volume and the electron mobility under electrical field.

According to ZLATKIS AND PETTITT[8] the K can be calculated with the following practical formula:

$$K = \left(\frac{I_b - I_c}{I_c}\right) (W/2) \frac{F}{ZM} \tag{2}$$

where:

$(W/2)$ is the width of the chromatographic peak at half height (in seconds);

F is the gas flow (in ml/sec);

Z is the sample size;

M is the molar concentration of the sample injected.

The temperature dependence of the capture coefficient K gives information about the mechanism taking place in the detector. According to WENTWORTH AND CHEN[6] four paths for the mechanism of electron attachment phenomena to alkyl iodides RI can be written:

$$e^- + RI \rightleftharpoons RI^- \tag{A}$$

$$e^- + RI \rightarrow R^\cdot + I^- \tag{B}$$

$$e^- + RI \rightarrow RI^- \begin{cases} \nearrow R^\cdot + I^- & \text{(C)} \\ \searrow R^- + I^\cdot & \text{(D)} \end{cases}$$

Mechanism A represents attachment by a nondissociative mechanism to form a stable negative ion. B and C represent attachment with immediate or following

dissociation. Mechanism C is assumed to have lower activation energy. Mechanism D can be excluded, as it is unlikely with alkyl iodides. The choice between mechanisms A and B or C may be made taking into account the fact that mechanism A must theoretically show an increase of ln $KT^{3/2}$ *versus* $1/T$, while mechanisms B and C must show a decrease or a constant behaviour.

In Fig. 4 and in Table V the values of ln $KT^{3/2}$ in function of $1/T$ for some alkyl iodides, representative of various skeletal structures, are reported. With the exception of 2-iodo-2-methylalkanes, one can observe a decrease in or a constant behaviour of these values, which seem to confirm a reaction of the B or C type. These mechanisms are highly probable, due to the fact that alkyl iodides tend to cleave at the carbon–iodine bond, with formation of an I^- and an alkyl radical $R^\cdot$.

The observed increase of ln $KT^{3/2}$ *vs.* $1/T$ for 2-iodo-2-methylalkanes seems to

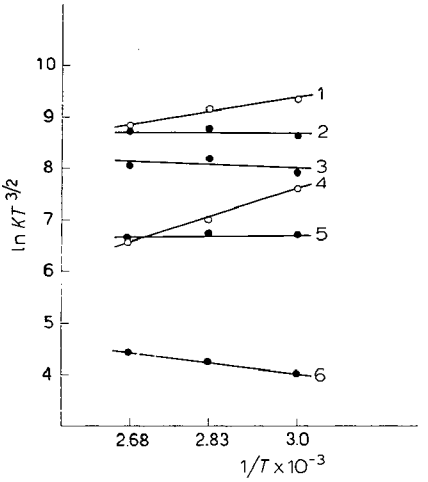

Fig. 4. Values of ln $KT^{3/2}$ in function of the reciprocal of absolute temperature. 1 = 2-Iodo-2-methylpropane; 2 = 1-iodo-2-methylpropane; 3 = 1-iodopropane; 4 = 2-iodo-2-methylbutane; 5 = 1-iodo-3,3-dimethylbutane; 6 = 2-iodopropane.

TABLE V

VALUES OF ln $KT^{3/2}$ FOR VARIOUS ALKYL IODIDES AT DIFFERENT TEMPERATURES (COLUMN *a*, *b*, *c*)
After 10 days of aging of the standard solution (column d), and difference between values of columns c and d.

	a	*b*	*c*	*d*	*c–d*
	333°K	*353°K*	*373°K*	*373°K*	*373°K*
1-Iodopropane	7.981	8.188	8.027	8.024	0.003
2-Iodopropane	4.002	4.255	4.340	4.241	0.099
Diiodomethane	—	—	5.878	5.839	0.039
1-Iodo-2-methylpropane	8.602	8.786	8.699	8.707	0.008
2-Iodo-2-methylpropane	9.338	9.154	8.776	8.694	0.082
2-Iodo-2-methylbutane	7.636	6.992	6.640	6.540	0.100
1-Iodo-3,3-dimethylbutane	6.693	6.716	6.640	6.630	0.010
1-Iodo-2,2,4-trimethylpentane	—	—	5.336	5.336	0.000

indicate a mechanism of type A. This is improbable because the tendency to formation of I^- and $R^\cdot$ with mechanism B or C increases, going from primary to secondary and tertiary alkyl iodides. To explain the observed increase, one must take into account the fact that the compounds with iodine on secondary and tertiary carbon atoms show little stability during synthesis and storage. An increase of $\ln KT^{3/2}$ *vs.* $1/T$ may only show that at higher temperatures a greater decomposition takes place. The greater slope of the 2-iodo-2-methylbutane plot is in accordance to the fact that this compound is less stable than 2-iodo-2-methylpropane. To confirm this fact, in the last column of Table V are reported the values obtained from analysis of the same standard solution (about 1 p.p.m. per compound) used for the analysis reported in the other columns, after 10 days of storing at 0°C.

Temperature was the same (373°K) as in the third column. While other compounds give quite constant values, the decrease for 2-iodoalkanes and for diodomethane can be easily observed. Note that, as previously observed, the decomposition of very dilute solutions (from 0.5 to 2.5 p.p.m.) is slow in comparison with these of pure compounds. The decomposition with time of products different from 1-iodoalkanes makes the quantitative analysis very difficult, as it is impossible to store for a long period the synthesised standards.

The absolute molar responses (AMR), suggested in the previous work[1], can be used for quantitative work if analytical parameters are the same, as the AMR were found to be characteristic of every compound. If any of the analytical parameters (temperature, column length, percent of stationary phase) will change, the molar

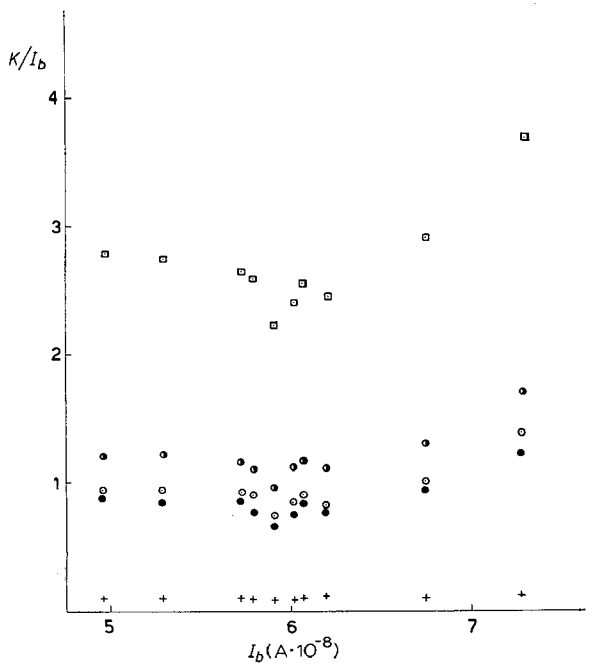

Fig. 5. Variation of ratio K/I_b in function of I_b during a long time of detector use. ⊙, 1-Iodopropane; +, 2-iodopropane; ◑, 1-iodo-2-methylpropane; ⊡, 2-iodo-2-methylpropane; ●, 1-iodo-3,3-dimethylbutane.

response relative (RMR) to 1-iodopropane can be used. Only one condition must be satisfied in this case: the standing current I_b must be constant, as RMR values depend on it.

Theoretically, the best way to know the sensitivity for each compond is the knowledge of its capture coefficient K. In fact, from Eqn. 1, taking into account that c is an instantaneous concentration and that the time that it takes for the sample to pass through the detector is inversely proportional to the gas flow rate through the detector, determination of sample quantity should be made from knowledge of I_b, I_c and K. Unfortunately, the variation of the standing current during detector life depends on many factors[7] whose effect is competitive. While temperature, pressure,

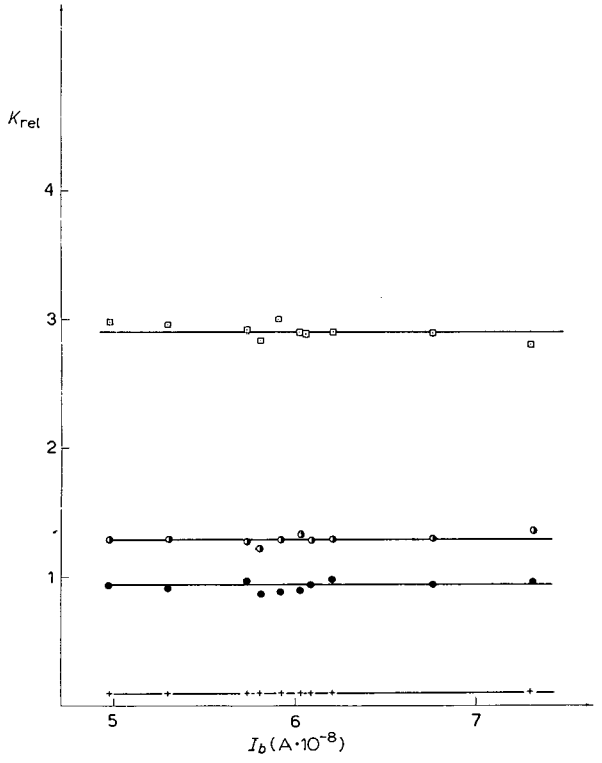

Fig. 6. Capture coefficient relative to 1-iodopropane (K_{rel}) in function of the standing current I_b. +, 2-Iodopropane; ◑, 1-iodo-2-methylpropane; ●, 1-iodo-3,3-dimethylbutane; □, 2-iodo-2-methylpropane.

flow can be easily maintained constant, column bleeding and contamination cannot be controlled. Therefore, values of K have non-regular behaviour in function of the standing current. In Fig. 6 are reported the values of K/I_b *vs.* I_b obtained during a long time (about 1 month) of detector use. Variation of I_b due to contamination causes non-linear variation of K, also when temperature, pressure and flow are identical. A constant value is obtained if, instead of K, one takes as a measure of sensitivity the K_{rel}, ratio between the K of every compound and the K of 1-iodopropane (Fig. 6).

TABLE VI

CAPTURE COEFFICIENT RELATIVE TO 1-IODOPROPANE (K_{rel}) AND RELATIVE STANDARD DEVIATION

Alkyl iodide	K_{rel}	$\bar{\sigma}_{rel}$
2-Iodopropane	0.096	±4.0%
1-Iodo-2-methylpropane	1.30	±3.7%
2-Iodo-2-methylpropane	2.89	±2.5%
1-Iodo-3,3-dimethylbutane	0.93	±5.0%

In Table VI are reported the values of K_{rel} for the compounds of Figs. 6 and 7, and the corresponding σ_{rel}. The constancy of these values is satisfactory. K_{rel} can therefore be used as a measure of the sensitivity to the various compounds, and the values obtained from freshly prepared standard solutions can be applied to quantitative analysis, thus avoiding the difficulty due to decomposition. In fact, addition of a stable compound (1-iodopropane) as internal standard will allow a rapid calculation of the amount of other iodides present in the mixture. The values of Table VI are indicative, as it is clear that the reported values of K_{rel} cannot be used by other workers with different types of detectors or different columns and that a series of K_{rel} must be determined *una tantum* with the parameters selected for the analysis.

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A SEARCH FOR ORGANICS IN HYDROLYSATES OF LUNAR FINES*

CHARLES W. GEHRKE**, ROBERT W. ZUMWALT***, WALTER A. AUE§, DAVID L. STALLING§§ AND JAY J. RASH§§§
University of Missouri, Columbia, Mo. 65201 (U.S.A.)
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SUMMARY

Lunar fines from the Apollo 11 and 12 missions have been analyzed for amino acids and a wide range of other derivatizable organic compounds by gas–liquid chromatography (GLC). A minimum of 4–6 p.p.b. of *each* amino acid would have been detected, but there were no indications of the presence of amino acids or other organics in the samples. However, a number of GLC peaks were found by the same methodology and proved to be dimethylpolysiloxanes by mass spectrometry (MS). They also appeared in the analysis of two meteorites and the terrestrial materials peridotite and basalt, while these compounds were not detected in numerous chemically related samples. It was established through various experimental approaches, that substances of unknown structure present in the above named samples caused the breakdown and volatilization of silicone traces in GLC and GLC–MS units. These substance(s) were stable to pyrolysis at 1000° in air, soluble in organic solvents, and similar to or stronger than hydrochloric acid in their capability to liberate siloxanes. Nine major and five minor elements present in the Lunar samples were not responsible for the chromatographic peaks.

INTRODUCTION

In our first paper[1] on the analysis of hydrochloric acid hydrolysates of the Apollo 11 lunar fines, we described the search for amino acids with a highly sensitive gas–liquid chromatographic (GLC) method[2]. The N-trifluoroacetyl *n*-butyl ester derivatives were formed by successive esterification with *n*-butanol–HCl and subsequent acylation with trifluoroacetic anhydride. Amino acids, if present, were less than 4

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** Professor and research associate in Space Sciences, University of Missouri, Columbia, and co-investigator on returned Lunar samples, Apollo's 11 and 12.

*** National Science Foundation Predoctoral Fellow, University of Missouri, Columbia.

§ Associate Professor and research associate in space sciences, University of Missouri, Columbia.

§§ Chief chemist, Fish Pesticide Research Laboratory, U.S. Bureau of Sport Fisheries and Wildlife, Columbia, Mo.

§§§ Research assistant, University of Missouri, Columbia.

p.p.b., however, the chromatograms showed that other types of organic material were present.

Since numerous blanks gave no indication of similar materials, we concluded that they were not artifacts of the analytical method. Combined gas chromatography–high and low resolution mass spectrometry (GLC–MS) showed all major peaks to contain the dimethylpolysiloxane structure, $[R(OSi[CH_3]_2)_n]^+$.

In view of the extensive use of silicones on earth and in the space program, contamination of the Apollo 11 sample was considered highly likely. Concerted studies conducted over an 8 month period were made to elucidate the source of this material. Earlier analyses[1] showed clearly that the lunar sample was *not* contaminated with silicones. Furthermore, while all terrestrial materials then studied concurrently with the Apollo 11 sample failed to yield any of the siloxane peaks, another extraterrestrial material — Pueblito de Allende meteorite — gave essentially the same chromatographic peaks as the lunar fines.

This present paper describes our efforts to define the substances which produced the prominent siloxane peaks in GLC and to determine their origin. Three working hypotheses were considered: (a) siloxanes occur in extraterrestrial materials, (b) siloxane precursors occur in extraterrestrial materials and form polymethylsiloxanes in one or more steps of the analytical procedure employed, and, (c) some inorganic substance(s) that are present in extraterrestrial material (but not in blanks) cause siloxanes that are always present in traces in the GLC–MS instrument systems, to break into smaller fragments and show up on the chromatograms.

To test these hypotheses, the analytical procedure was modified in several key aspects to define the chemistry necessary for obtaining the siloxanes. At various points in the method, pyrolysis at 1000° was used to destroy any organic material present, and esterification was conducted with both deuterated and fluorinated alcohols to label the compounds for later analysis by mass spectrometry. Also, an earlier study had shown, that it was impossible to obtain chromatographic peaks (similar to those generated by the lunar material) from commercial methyl and phenyl polysiloxanes by hydrolysis, esterification and/or acylation procedures.

However, all gas chromatographs and associated instrumentation contain traces of silicones. Indeed, even in the unlikely event that silicone-coated (or silylated) supports and substrates had never been used, silicone septa and O-rings would have introduced some contamination into the GC and MS systems. Since silicones are nonvolatile and usually remain stationary, they are not detected in chromatograms, thus, hardly anyone notices their presence. In earlier studies it was shown that such silicones could be detected both by flame ionization and by mass spectrometry, when hydrochloric acid or hydrochloric acid–alcohol mixtures were injected onto polyester chromatographic columns. These silicones were stripped by the acid reagent from the instrumental system or packing. Although this procedure damages most column materials when frequently exercised, we used it to completely free the system of the last traces of silicones prior to analyses by GLC–MS for final confirmation of silicones in the samples.

In investigating the three possible theories for observing dimethylpolysiloxanes, a number of other terrestrial and extraterrestrial samples were chosen (for obvious reasons) and analyzed: Apollo 12 fines, Pueblito de Allende and Murray meteorites, obsidian, peridotite, tektite, silicon carbide, silicic acid and two samples of basalt.

To evaluate possible effects of the high concentrations of inorganic material on the derivatization and GLC analysis of organic compounds of various functionality, the 1 *N* HCl hydrolysate of lunar fines was spiked with an alcohol, an amino acid, an amine, a fatty acid, and a dicarboxylic acid.

EXPERIMENTAL

A. Apparatus and reagents

The gas chromatographs and high resolution GC–MS system used in this study included the same basic instruments described in the previous investigations by GEHRKE *et al.*[1] on Apollo 11 samples at the Ames Research Center. In addition, a GLC–low resolution MS system incorporating a Loenco GC, a Llewellyn membrane interface, and a CEC Model 491 mass spectrometer was also used. Studies at the University of Missouri, Columbia incorporated a Packard 7300 series gas chromatograph interfaced through a Watson-Biemann type molecular separator to a CEC Model 110-B high resolution mass spectrometer which was used to obtain a "siloxane free" analytical instrumental system.

The amino acid standards, *n*-butanol, anhydrous HCl, trifluoroacetic anhydride, and dichloromethane were of the same reagent quality as in ref. 1.

A stock solution was prepared containing *n*-octanol, *n*-hexylamine, valine, lauric acid, and adipic acid at a concentration of *ca.* 15 mg of each compound in 50 ml of *n*-butanol–3 *N* HCl. Ethyl acetate was obtained from Mallinckrodt Chemical Works, and was of "Nanograde" purity. The silicic acid was purchased from Bio-Rad, Inc., and was a Bio-Sil HA, Control No. 6383, 325 mesh. Silicon carbide was obtained from K&K Laboratories, Catalog No. 18268. The micro reaction vials were obtained from Analytical Biochemistry Laboratories, Columbia, Mo.

B. Chromatographic columns

Both 1 m × 4 mm I.D. and 2.5 m × 2 mm I.D. glass columns of OV-17 and ethylene glycol adipate (EGA) chromatographic phases were prepared in the manner reported earlier[1]. Also, a 300 ft. capillary column coated with OV-17 was used for introduction of the sample into the CEC Model 110-B mass spectrometer.

In an effort to obtain chromatographic columns entirely free of silicone contamination, four new glass columns were exhaustively washed with methanolic KOH, chromic acid cleaning solution, acetone, *n*-butanol–3 *N* HCl, and benzene. The "empty glass columns" were checked for organics by placing the columns in the GLC–MS system, and monitoring the effluent after the repeated injection of solvents, including *n*-butanol–3 *N* HCl. The columns were then removed from the instrument, filled with 80/100 mesh A.W. Chromosorb W, and checked again for siloxanes. They were again removed, the Chromosorb coated with 0.65 % EGA, and again placed in the GC oven for conditioning and analysis. The level of siloxane contamination present was determined by GLC–MS using both oscillographic recording and photoplate detection. Also, the *transfer lines* and *molecular separator* were checked for siloxane contamination by introducing *n*-butanol–3 *N* HCl directly into the transfer line.

C. Hydrolysis of samples (with 1 N HCl)

The following samples (1 to 2 g each) were hydrolyzed under reflux for *ca.* 15 h with (5 to 10 ml) 1 *N* HCl. The Apollo 12 lunar fines (1.2 g) were extracted with triple

distilled water (6 ml) under reflux for 15 h prior to the 1 *N* HCl extraction procedure.

Apollo 12 Lunar Fines	ARC 12023.04
Basalt	NASA 040270
Basalt	NASA 080669
Pueblito de Allende Meteorite	NASA 31-4A
Murray Meteorite	NASA 040870
Tektite	NASA 040770
Obsidian	NASA 041170
Peridotite	NASA 043070
Silicic acid	
Silicon carbide	
Sand monitor	

The sand monitor was received from the Lunar Receiving Laboratory, Houston, Texas, for the purpose of evaluating possible contaminants that might have originated in that facility.

The acidic hydrolysates were transferred to centrifuge tubes and centrifuged at *ca.* 2700 r.p.m. for 3 to 4 min. The clear solutions were decanted for further sample treatment and analysis.

D. Analytical method[2]

Aliquots of the acidic hydrolysates from (C), equivalent to 0.25 to 1.0 g of the samples, were placed in 10-ml beakers and evaporated to *ca.* 0.5 to 1 ml under an infrared (IR) lamp, then transferred to the pear-shaped reaction vials. Alternatively, an aliquot of the 1 *N* HCl hydrolysate was extracted with ethyl acetate (3 × with 5 ml (a)), leaving the bulk of inorganic salts in the 1 *N* HCl phase. Then both the ethyl acetate and acidic phases were evaporated and taken through the esterification and acylation steps. The evaporations were completed in the micro reaction vials after washing the 10 ml beakers with 1 ml aliquots of 1 *N* HCl.

After completion of evaporation, samples were allowed to cool, *n*-butanol–3 *N* HCl was then added (200 μl/0.25 g of sample), the vials were closed with teflon-lined caps, followed by ultrasonic mixing for 30–60 sec, then esterified at 100° for 30 min. The *n*-butanol was removed by evaporation under an IR lamp, 100 μl of a dichloromethane–trifluoroacetic anhydride mixture (0.5 μl TFAA/100 μl CH_2Cl_2) were added, then the closed vial was heated at 100° for 10 min. The "derivatized" samples were then analyzed by GLC, GLC–MS (high resolution) and direct probe-MS. All other details for a "nanogram method for amino acids" by ZUMWALT AND GEHRKE are described elsewhere[2].

E. Other experiments

A recovery experiment of the five compounds with various functional groups was carried out by adding *ca.* 1 μg of each compound to 0.5 g equiv. of the 1 *N* HCl hydrolysate of Apollo 11 lunar fines. The sample was then derivatized in accordance with the analytical method described above, and analyzed by GLC.

Labeling experiments were conducted using 1 *N* DCl in D_2O as the hydrolysis medium, and both C_2D_5OD–3 *N* DCl and $C_3F_7CD_2OD$–3 *N* HCl were used in place of the *n*-butanol–3 *N* HCl. Pyrolysis experiments of the acidic hydrolysates were carried out in platinum crucibles in an electric muffle furnace at 1000°. Direct der-

ivatization of 1 mg amounts of OV-17 substrate with *n*-butanol–3 *N* HCl was made by heating at 100°, 30 min, evaporating the alcohol, then adding CH_2Cl_2 prior to GLC analysis. Also, a parallel OV-17 sample was analyzed in this manner by pyrolysis at 975° for 15 h followed by derivatization. In addition, each reagent used in the derivatization was investigated with a basalt sample hydrolysate to determine its

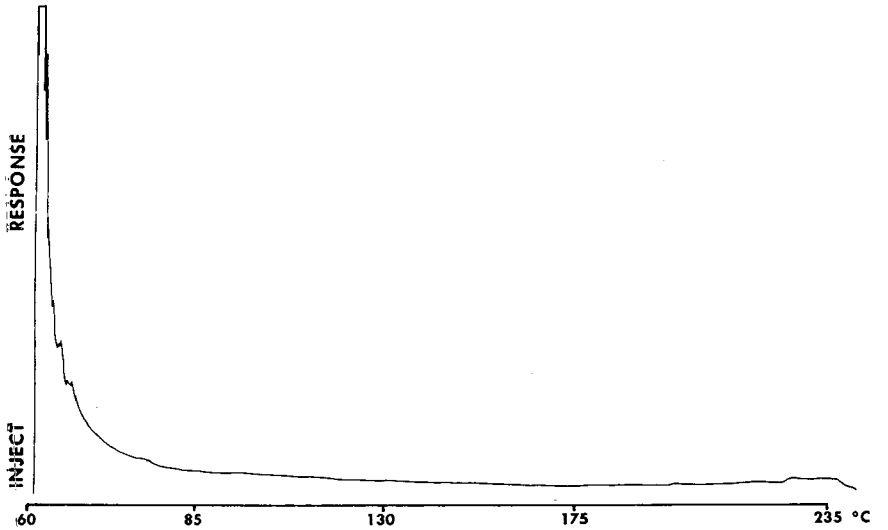

Fig. 1. Reagent blank. 25 ml of ethyl acetate; final volume: 100 μl; injected: 6 μl; attenuation: 8×10^{-11} a.f.s.; initial temperature: 60°; initial hold: 4 min; program rate: 4°/min; final temperature: 235°; column: 1.0 w/w% OV-1 on 80–100 mesh H.P. Chromosorb G, 1 m × 4 mm I.D. glass.

contribution to the formation of siloxanes. In addition, a study was designed to determine the possible effects of some metal chlorides on the chromatographic system. Mixtures of chloride salts of Fe(III), Ti(II), Al(III), Mg(II), Na(I), Cr(III), Cu(II), Rb(I), Zn(II), K(I), Co(II), Cd(II), Ca(II), and Ni(II) were taken through the entire derivatization method then analyzed on an OV-17 column.

RESULTS AND DISCUSSION

The chromatographic results from the initial analyses of the 1 *N* HCl extracts of the Apollo 11 lunar fines and PDA meteorite prompted further study of the meteorite prior to the Apollo 12 investigations. Typical chromatograms obtained for the complete reagent blank and PDA sample are presented in Figs. 1 and 2. The prominent chromatographic peaks at retention temperatures of 69, 77, 99, and 109° show ion fragments typical of methylpolysiloxanes with the general formula $[R(OSi[CH_3]_2)_n]^+$ as reported earlier[1].

The Apollo 12 lunar fines then were studied using the same analytical methods, with the exceptions as presented in the EXPERIMENTAL section. Fig. 3 shows the chromatogram obtained from the ethyl acetate extract of the Apollo 12 1 *N* HCl hydrolysate. The chromatogram obtained from the remaining aqueous HCl phase of Apollo 12 is presented in Fig. 4; the same major mass spectrometric peaks were found.

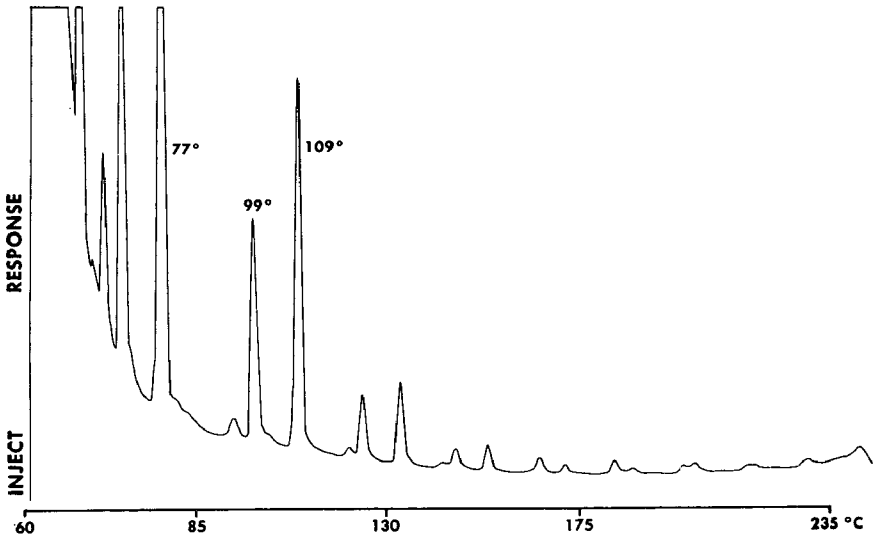

Fig. 2. Ethyl acetate extract of 1 *N* HCl hydrolysate of PDA meteorite. Sample: 0.3 g equiv., 3 ml of 1 *N* HCl; 25 ml of ethyl acetate; final volume: 100 μl; injected: 6 μl; attenuation: 8×10^{-11} a.f.s.; initial temperature: 60°; initial hold: 4 min; program rate: 4°/min; final temperature: 235°; column: 1.0 w/w% OV-1 on 80–100 mesh H.P. Chromosorb G, 1 m × 4 mm I.D. glass.

Both samples were again studied by GLC–MS, and the peaks were characterized as siloxanes. Corresponding reagent blanks revealed no evidence of the siloxane peaks as seen in Fig. 5.

Extensive analyses conducted on a tektite, an obsidian sample, silicic acid,

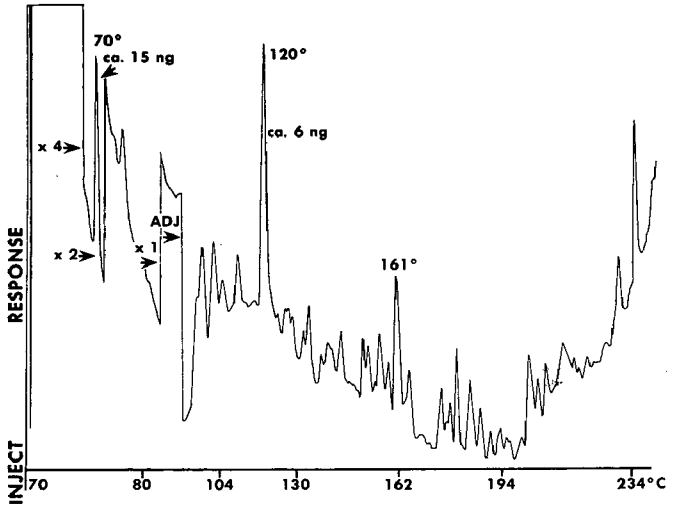

Fig. 3. Apollo 12 lunar fines, ethyl acetate extract of 1 *N* HCl hydrolysate. 1 g equiv.; final volume: 100 μl; injected: 5 μl; attenuation: 4×10^{-12} a.f.s. = × 1; initial temperature: 70°; initial hold: 6 min; program rate: 4°/min; final temperature: 235°; column: 1.0 w/w% OV-17 on 80–100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

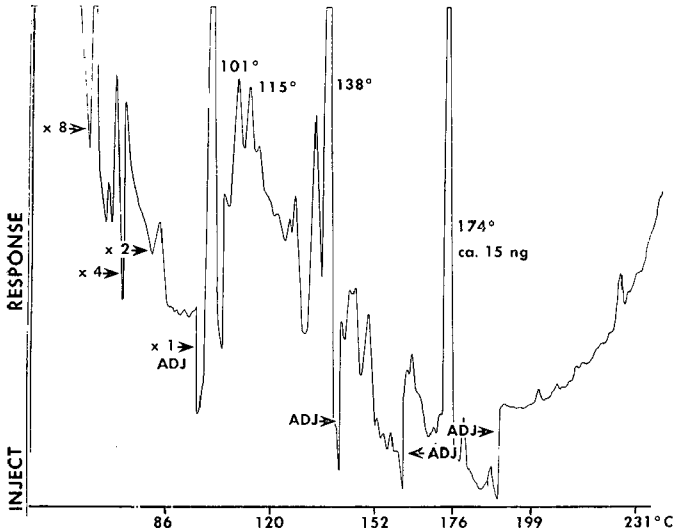

Fig. 4. Apollo 12 lunar fines 1 *N* HCl hydrolysate (after ethyl acetate extraction). 1 g equiv.; final volume: 250 μl; injected: 5 μl: attenuation: 4×10^{-12} a.f.s. = × 1; initial temperature: 70°; initial hold: 6 min; program rate: 4°/min; column: 1.0 w/w% OV-17 on 80–100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

sand monitor from the Houston Lunar Laboratory, and silicon carbide resulted in no significant chromatographic peaks.

Although every effort has been made to select as "blank" samples which simulate lunar materials in physical or chemical properties, it should perhaps be stressed at this point that no true "blanks" of extraterrestrial materials exist.

The situation would, of course, be different if the compounds of interest were known and the lunar sample itself could be used for spiking experiments.

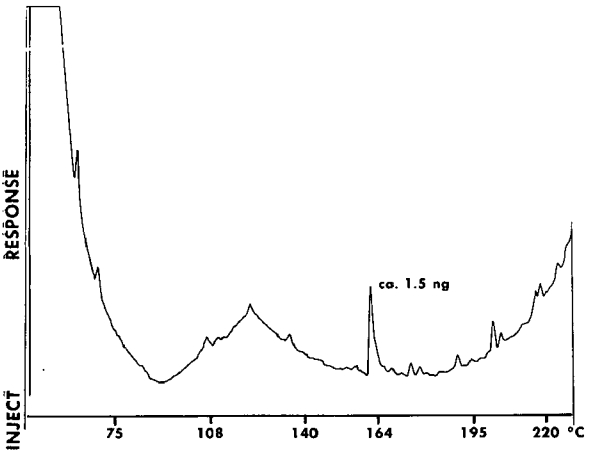

Fig. 5. Reagent blank. Ethyl acetate extract of 5 ml of 1 *N* HCl; final volume: 100 μl; injected: 5 μl: attenuation: 4×10^{-12} a.f.s.; initial temperature: 70°; initial hold: 6 min; program rate: 4°/min; final temperature: 228°; column: 1 w/w% OV-17 on 80–100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

Previous studies of the 1 *N* HCl extract of a basalt sample had not generated chromatographic peaks when a 1 h hydrolysis was used[1]. However, when the hydrolysis time was prolonged to 15 h, chromatograms as seen in Figs. 6 and 7 were obtained

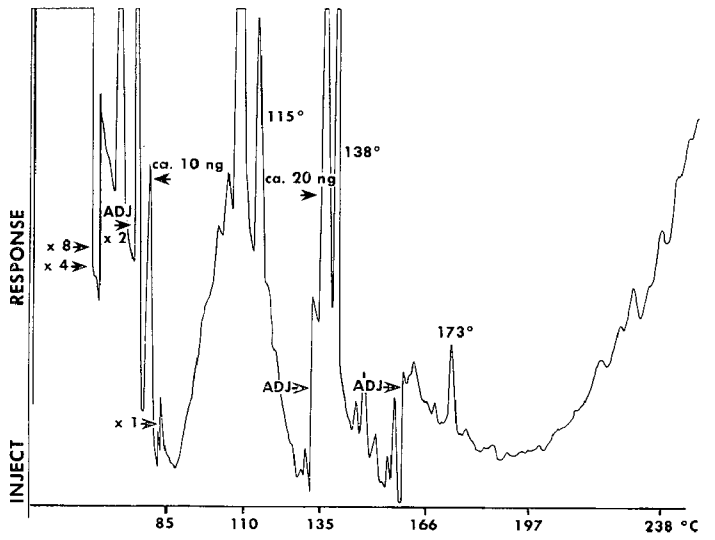

Fig. 6. Basalt; ethyl acetate extract of 1 *N* HCl hydrolysate; 1 g equiv.; final volume: 100 μl; injected: 5 μl; attenuation: 4×10^{-12} a.f.s. = 1; initial temperature: 70°; initial hold: 6 min; program rate: 4°/min; final temperature: 245°; column: 1 w/w% OV-17 on 80–100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

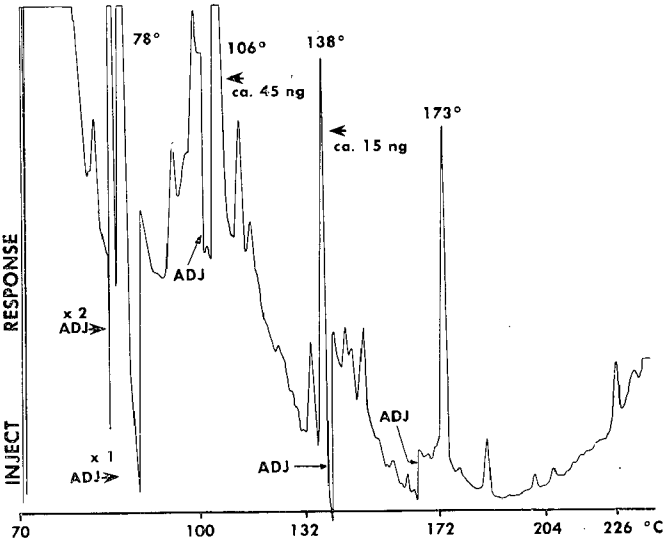

Fig. 7. Basalt 1 *N* HCl hydrolysate (after ethyl acetate extraction); 1 g equiv.; final volume: 250 μl; injected: 5 μl; attenuation: 8×10^{-12} a.f.s. = × 1; initial temperature: 70°; initial hold: 6 min; program rate: 4°/min; final temperature: 228°; column: 1.0 w/w% OV-17 on 80–100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

for the ethyl acetate and HCl fractions, respectively. After the many negative results obtained from a variety of geological and other material, this was the first time that a *terrestrial* sample had given rise to chromatographic peaks similar to those found for the lunar and meteorite samples. Again, GLC–MS identified these peaks as siloxanes; similar to those found in the Apollo 11, 12, and PDA experiments. Analyses were repeatedly performed on two basalt specimens to confirm these results.

Concurrent studies on the Murray meteorite also resulted in the generation of pronounced chromatographic peaks as seen in Fig. 8. Some of these peaks were also confirmed to be siloxanes by GLC–MS.

It is evident from the chromatograms presented that peaks observed in the above experiments represented quantities of siloxanes approaching 100 ng/peak in some instances, thus differing markedly from the reagent blanks and "blank" samples which did not exhibit this unusual characteristic.

The analysis of the five component performance mixture of *n*-octanol, *n*-hexylamine, valine, lauric acid, and adipic acid revealed each of these types of compounds were susceptible to GLC detection under the derivatization and chromatographic conditions used. The analysis of this standard mixture is presented in Fig. 9. Fig. 10 presents a chromatogram obtained on analysis of the 1 *N* HCl hydrolysate of lunar fines *spiked* with the standard mixture. *n*-Hexylamine, valine, and lauric acid were recovered, indicating these types of compounds would have been derivatizable and therefore amenable to detection by GLC–MS, were compounds of these types present in the lunar fines. *n*-Octanol and adipic acid were not recovered, with loss of *n*-octanol

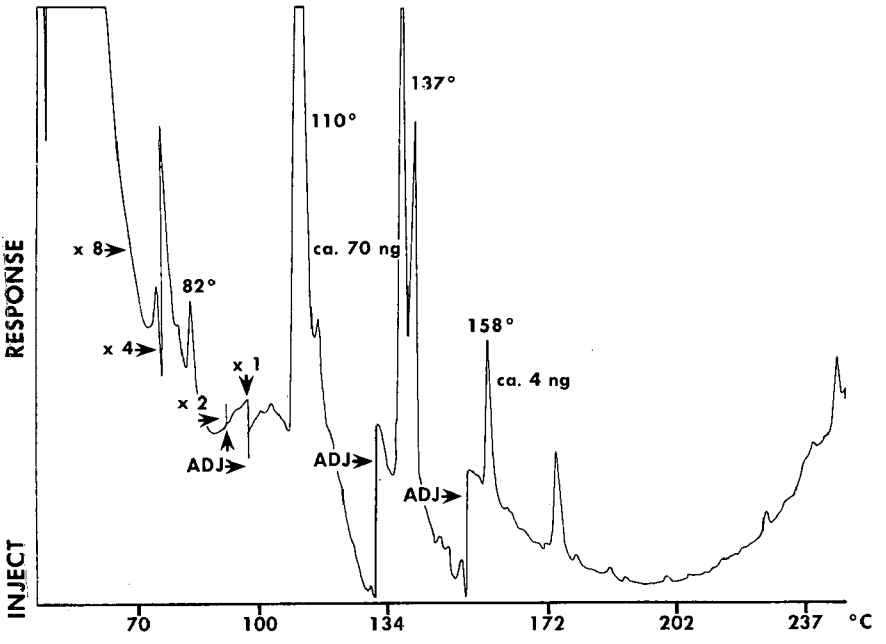

Fig. 8. Murray meteorite 1 *N* HCl hydrolysate (after ethyl acetate extraction). Final volume: 250 μl; injected: 5 μl; initial temperature: 70°; initial hold: 6 min; program rate: 4°/min; final temperature: 245°; attenuation: 8×10^{-12} a.f.s. = × 1; column: 1.0 w/w% OV-17 on 80–100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

occurring during the evaporation steps of the derivatization procedure. The subsequent pyrolysis experiments were then devised to trace the origin of the siloxane chromatographic peaks. The acidic basalt extract, which had yielded prominent GLC peaks before, showed the same peaks after pyrolysis at 700° for 15 h. It was considered highly unlikely that organic molecules could have remained intact at this temperature. However, the results were reconfirmed in another experiment using a pyrolysis temperature of 1100° for 15 h. These experiments clearly demonstrated that chromatographic siloxane peaks still occurred after subjecting the sample to conditions which should have *totally destroyed* any siloxanes indigenous to the sample.

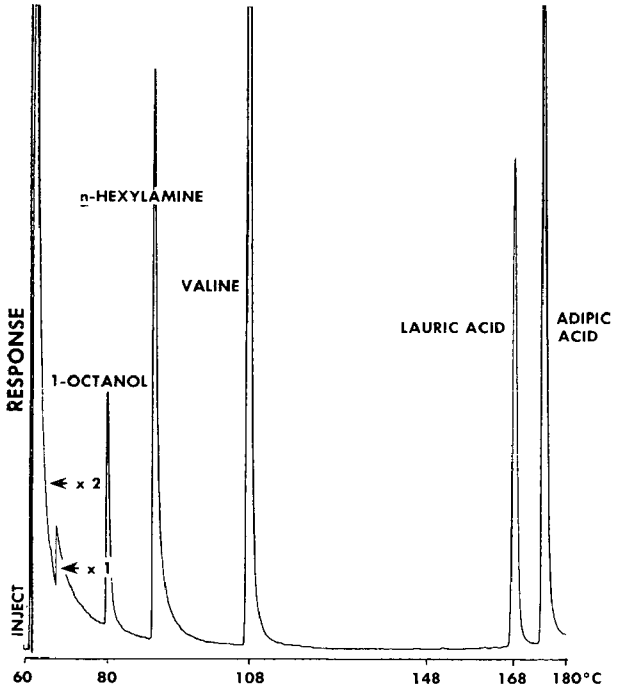

Fig. 9. Performance and chromatographic standard. Derivatization and GLC analysis of a five-component mixture. Sample injected: *ca.* 1 μg of each; attenuation: 2×10^{-10} a.f.s. = × 1; initial temperature: 60°; program rate: 6°/min; final temperature: 180°; injection port: 280°; 3.0 w/w% OV-17 on 80–100 mesh H.P. Chromosorb W, 1.5 m × 4 mm I.D. glass.

To exclude the possibility that the siloxanes were the product of an unusual synthetic process occurring during sample preparation, labeling experiments were conducted with fluorine and deuterium containing alcohols. Neither experiment resulted in an apparent mass shift of the siloxane fragments studied by GLC–MS. The obvious conclusion, therefore, was that the siloxanes were not being synthesized in the analytical method, nor were they indigenous to the samples. Rather, they may have originated in the gas chromatographic system, as the result of a particular characteristic of, or material(s) contained in and unique to the sample itself.

At this point in time, peridotite, a granitoid igneous rock composed of ferromagnesium minerals, principally chrysolite, $(Fe,Mg_2)SiO_4$, was included in the stud-

ies. Chromatographic peaks, again identified as siloxanes on analysis of this material were observed.

This finally substantiated the earlier findings on basalt, that the appearance of siloxane peaks was not exclusively limited to extraterrestrial materials.

With some of the possibilities for siloxane formation excluded, a comprehensive study was now undertaken to demonstrate that the siloxanes originated indeed from the GLC–MS instrumental analytical system itself.

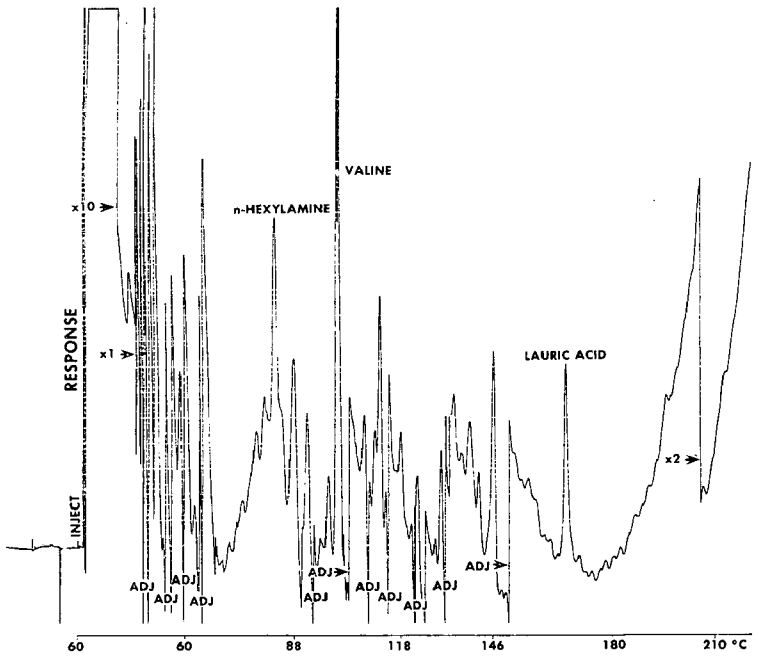

Fig. 10. Apollo 11 1 *N* HCl hydrolysate five component mixture *spike*; sample: 0.5 g equiv. of lunar fines, *spiked* with *ca.* 1 μg of each; 200 μl *n*-butanol–3 *N* HCl; 100 μl TFAA–CH_2Cl_2 (0.5 μl/100 μl); final volume: 100 μl; injected: 5 μl; initial temperature: 60°; initial hold: 6 min; program rate: 4°/min; final temperature: 210°; attenuation: 1 × 10^{-11} a.f.s. = × 1; injection port: 280°; column: 1.0 w/w% OV-17 on 80–100 mesh H.P. Chromosorb G, 2.5 m × 2 mm I.D. glass.

High resolution–direct probe mass spectrometry of the derivatized hydrolysate of the basalt sample using photoplate detection was used to bypass the GLC system. No evidence of siloxanes was observed. However, introduction of the sample by direct probe is a less efficient method of sample introduction than by GLC. Therefore, the GLC–MS system had to *be freed* of any siloxanes which had a reasonable chance to influence the results. At the trace levels detectable by MS and GLC, this task was formidable. It would not have been possible, in fact, to be confident in the success of the cleaning procedures without the earlier information from solvent studies which had shown that gas chromatographic or silicone columns would generate siloxane peaks upon injection of hydrochloric acid (3 *N*) or HCl dissolved in *n*-butanol. Obviously, it is not a common practice to inject hydrochloric acid into a gas chromatograph. Nor had butanol–3 *N* HCl (which is used in the analytical procedure) ever

been injected — as demonstrated by the repeated blanks. The butanol–HCl used in the analytical method had been totally removed in the evaporation. Thus, *n*-butanol–3 *N* HCl was chosen to evaluate the GLC–MS combination with regard to the preparation of an instrumental system entirely free from silicone contamination.

After rigorously cleaning all GLC parts that would come in contact with an injected sample, a new, cleaned *empty* glass column was placed in the instrument. On injection of *n*-butanol–3 *N* HCl and C_2D_5OD–3 *N* in DCL, traces of siloxanes were detected mass spectrometrically, but diminished on repeated injections. This technique was continued until a chromatographic column of 0.65 w/w % EGA on 80/100 mesh A.W. Chromosorb W exhibited either "extremely low" or "undetectable levels" of siloxane contamination. This "silicone free" instrumental system was then used to study carefully the acidic extracts of the basalt and PDA meteorite. Deuterated reagents were used to prepare both the basalt and PDA sample hydrolysates using 1 *N* DCl in D_2O, and C_2D_5OD–3 *N* in DCl. On analysis of these samples by GLC–MS using the EGA column, no significant chromatographic peaks were observed, nor were siloxanes apparent in the MS oscillographic recording, although prominent peaks were produced when the same samples were injected on an OV-17 column. However, some siloxane fragments were noted by the more sensitive photoplate detection when the labeled derivatized basalt and PDA samples were chromatographed on the EGA system, but again no shift in mass number was observed, ruling out completely the possibility of a synthesis process involving $-CD_3$ during sample preparation or chromatography.

The next step was to reconfirm the findings of the earlier pyrolysis experiments using both an OV-17 column and the "silicone free" EGA column–MS system. Both basalt and PDA sample hydrolysates were analyzed, another aliquot of the hydrolysate was then pyrolyzed at 975° for 15 h, derivatized, then analyzed again. The analyses with the OV-17 column before and after pyrolysis were substantially the same, with prominent chromatographic peaks being generated in each instance. However, the analyses conducted with the "silicone free" EGA GLC–MS system showed only *low levels* of siloxane fragments, and in general these did not appreciably decrease after pyrolysis. In only one instance were the siloxanes completely undetectable by MS due to the absence of even traces of these compounds in the instrumental system. It is therefore apparent that some samples, the extraterrestrial Apollo's 11 and 12, PDA and Murray meteorites, basalt and peridotite, themselves possess a unique chemistry which liberates siloxanes from the instrumental GLC–MS system. The behavior of these samples thus contrasts sharply with that of all the other terrestrial samples (except basalt and peridotite) taken through the same analytical-instrumental methodology (tektite, obsidian, silicic acid, SiC, sand monitor, quartz crystals, and method blanks).

Further studies confirmed that siloxanes formed in the derivatization from OV-17 and butanol–3 *N* HCl, were not stable as expected to pyrolysis at 1000°; no chromatographic peaks were obtained.

Some concluding experiments were designed to determine if a particular element or ion in the sample was responsible for the generation of the siloxane peaks. The metal chlorides listed in Section E were taken through the derivatization and GLC methods, but failed to produce significant GLC chromatographic peaks. Further studies to define unequivocally the substance(s) responsible for the formation of the

gas chromatographic siloxane peaks were regarded as beyond the scope of these investigations.

CONCLUSIONS

Our earlier investigations on Apollo 11 lunar fines, PDA meteorite, and several terrestrial samples by a multistep GLC method designed to detect amino acids, had shown that extraterrestrial samples cause methylsiloxane peaks in the chromatograms. This investigation on Apollo 12 and many other samples traced the origin of these siloxanes. Three working hypotheses had been formulated (a) siloxanes occur in extraterrestrial material, (b) siloxane precursors occur in extraterrestrial material and form polymethylsiloxanes in one or more steps of the analytical procedure employed, and, (c) some unidentified substance(s) that are present in extraterrestrial material (but not in blanks) cause siloxanes that are always present in traces in the GLC–MS instrument systems, to break into smaller fragments and show up on the chromatograms.

It was established through use of labeled reagents, pyrolysis at 1000°, use of "siloxane free" substrates, supports and "siloxane free" GLC–MS instruments, and a number of other experiments, that exhaustive acid hydrolysis (1 *N* HCl for 15 h) yield substance(s) from the samples. These substances are stable to pyrolysis at 1000°, soluble in organic solvents, and liberate organosiloxanes from the ubiquitous traces of silicone ever present in analytical instrumentation. In all of our experiments, major attention and importance was always focused on using representative blanks. However, in experiments on extraterrestrial material, as lunar or meteorite samples, a true blank does not exist. The effect of the sample itself cannot be excluded nor simulated.

It was strikingly apparent that the extraterrestrial samples (Apollo's 11 and 12, PDA and Murray meteorites) and terrestrial basalt and peridotite possess a unique chemistry or contain unidentified substance(s) which free siloxanes from the instrumental GLC–MS system. This unique behavior of these samples contrasts sharply with that of all the other terrestrial samples taken through the same analytical-instrumental methodology, *i.e.*, tektite, obsidian, silicic acid, SiC, sand monitor, quartz crystals, reagent method blanks, and OV-17 hydrolyzed, derivatized and pyrolyzed.

To determine if a particular major or minor element or ion in the samples were responsible for the generation of siloxane peaks, experiments were designed to investigate the effect of Fe(III), Ti(II), Al(III), K(I), Mg(II), Ca(II), Na(I), Cr(III), Cu(II), and trace elements, Ni(II), Zn(II), Co(II), Cd(II), and Rb(I). All failed to give significant GLC peaks above background.

Of the three "working hypotheses" considered to explain the source of the methylsiloxane peaks, our experiments have shown conclusively one to be correct. Unidentified substances present in Apollo's 11 and 12, PDA and Murray meteorites, basalt, and peridotite cause the emergence of siloxanes from the GLC–MS instrumentation analytical systems. The chemical nature of the substance(s) present in these samples responsible for the formation of siloxane peaks remains an interesting problem.

GLC–MS results from the Apollo's 11 and 12 and meteorite studies clearly point out that the analysis for trace organics in inorganic matrices, in particular analysis of

extraterrestrial samples, presents a complex and extremely difficult problem. A true reagent-sample blank is not available, and the conclusions can only be drawn after a rigorously safe-guarded experimental design with cross checking at every step. Apollo's 11 and 12 experiments demonstrate that chromatographic peaks can be generated by materials in the samples whose nature is unknown. Organic solvent extracts may contain substance(s) that form GLC peaks from chromatographic substrates, supports, reagents, or the trace organics present in the instrumental system itself.

Therefore, the fact that samples show prominent GLC peaks, whereas various blanks do not, could have been extremely misleading in this and other investigations in the nanogram sensitivity range. It is to be expected that similar possibilities for erroneous data interpretation will be encountered from the analysis of other extraterrestrial samples, where the ultimate in GLC–MS-sensitivities are being used.

In summary this study has resulted in several conclusions: (1) The lunar material does not contain any appreciable amounts of organic material (2 to 20 p.p.b. level) which would have been detected directly or as derivatives under these experimental conditions. The gas chromatographic techniques used would have shown a very wide range of organic structures ($-NH_2$, –OH, –COOH, –SH, etc.).

(2) Several extraterrestrial — and two of many analyzed terrestrial — materials contain substances which lead to the liberation of siloxane peaks from the silicone traces in GLC instrumentation. A common denominator of these samples may perhaps be the fact, that all of them are igneous materials, formed at high temperature under anaerobic conditions, which contain iron magnesium silicates.

(3) From a general point of view in regard to trace analysis of organic compounds in complex inorganic matrices, there exist severe possibilities for error inherent in GLC–MS instrumentation at sensitivities in the nanogram range. These possibilities have generally gone unnoticed.

(4) The current controversy: tektite, terrestrial or extraterrestrial? may be enriched by an indication pointing to its terrestrial origin. All extraterrestrial materials examined up to now caused prominent siloxane peaks, whereas tektite failed to do likewise.

SPECIAL ACKNOWLEDGEMENT

The analytical research described in this paper was conducted under the auspices of the Exobiology Division's Chemical Evolution Branch of NASA's Ames Research Center, Moffett Field, California. The initial discovery of polysiloxanes associated with extraterrestrial samples was possible only through the use of the Ames ultraclean laboratory designed for the analysis of lunar materials, and NASA's gas chromatograph–high resolution mass spectrometer system interfaced to a high-speed computer. For their guidance in our studies and their help in obtaining terrestrial and extraterrestrial materials of geochemical relevance, the authors want to express their appreciation and gratitude to Dr. CYRIL PONNAMPERUMA, the scientific leader of a Consortium of Investigators; Dr. KEITH KVENVOLDEN, who managed the intricate interrelationship of people and machines; and Dr. ALLAN DUFFIELD, who steered the initial mass spectrometric experiments.

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CHROM. 5109

A GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF THE RATIO OF GITOGENIN AND DIGITOGENIN IN MIXTURES

P. S. COWLEY, F. J. EVANS* AND R. F. A. GINMAN
The School of Pharmacy, Brighton Polytechnic, Lewes Road, Moulsecoomb, Brighton BN2 4GJ (Great Britain)
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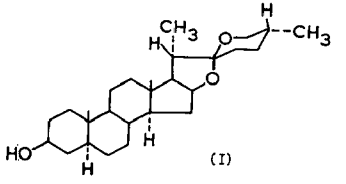
SUMMARY

A quantitative gas–liquid chromatographic method, applicable to microgram amounts, for the determination of the ratio of gitogenin and digitogenin in mixtures is described. The method involves making known mixtures of each sapogenin with tigogenin, preparing the trimethylsilyl ethers and measuring the peak area ratios of these ethers after separation by gas–liquid chromatography. Optimum conditions for the preparation of the ethers is described. The method has been applied to the determination of gitogenin and digitogenin in extracts of *Digitalis purpurea* seeds, it has also been used to determine the purity of commercial digitonin.

INTRODUCTION

The separation of the polyhydroxy sapogenins, gitogenin (II) and digitogenin (III), by chromatographic means has been achieved only with difficulty and the separation factors are low[1–4]. Thin-layer chromatographic (TLC) methods give good separation only after acetate formation[5]. Gas–liquid chromatography (GLC) of several groups of sapogenins has been attempted[6]. This paper describes a quantitative method,



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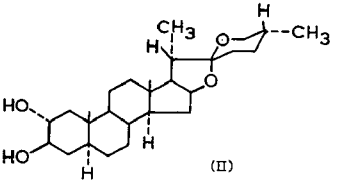

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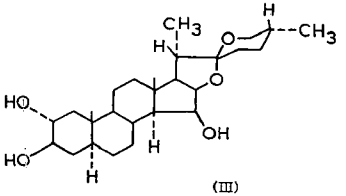

(III)

* Present address: Department of Pharmacognosy, School of Pharmacy, University of London, Brunswick Square, London W.C.1, Great Britain.

applicable to microgram amounts, for the separation of gitogenin and digitogenin. The method is based on the determination of the peak area ratios of the trimethylsilyl (TMS) ethers of these compounds following GLC separation of mixtures with tigogenin (I). Optimum conditions for the preparation of the ethers are described. The method has been applied to the determination of gitogenin and digitogenin in extracts of the seeds of *Digitalis purpurea* L., and the purity of commercial digitonin has also been determined by the method.

EXPERIMENTAL

Reagents

Trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS) were obtained from Aldrich Chemical Co.; pyridine and tetrahydrofuran from B.D.H. Ltd., these last two were redistilled and stored over sodium wire prior to use. The Silica Gel G was obtained from E.Merck.

Apparatus

A Marryat gas chromatograph was used with a flame ionisation detector and modified in this laboratory to take glass columns with on column injection.

Columns

Four glass columns were used during the development of this method. The packing materials were prepared by the method of HORNING *et al.*[7]. Column 1, 6 ft. long by 1/8 in. diameter, was packed with Chromosorb W (100–120 mesh) AW-DMCS treated obtained from Johns Manville Products, and coated with 1.5 % QF-1 silicone oil from Loenco Inc. Column 2 was of the same size and packed with the same support but coated with 1 % neopentyl glycol succinate from Perkin-Elmer. Column 3 was also similar but coated with 1.2 % SE-30 silicone gum rubber from the General Electric Co. The fourth column was 9 ft. long but otherwise similar to the third column.

Reference sapogenins

Tigogenin was obtained from Koch-Light Ltd., digitonin from Halewood Chemicals Ltd.; the gitogenin and digitogenin were gifts kindly sent by Prof. Reichstein of Basle University. The purity of these compounds was checked by TLC using air-dried Silica Gel G in 250 μ layers. The plates were developed with chloroform–acetone (9:1) for a distance of 10 cm, they were then sprayed with 10 % antimony trichloride in benzene and heated for 10 min at 120°. The digitogenin proved to be pure with an R_F value of 0.44. The tigogenin and gitogenin, with R_F values of 0.85 and 0.45, respectively, both contained small amounts of impurities which showed as faint spots with R_F values of 0.70 (tigogenin) and 0.22 (gitogenin). These compounds were purified by preparative layer chromatography (PLC) on Silica Gel G layers of 500 μ thickness and using the same solvent system as before. The zones corresponding to the tigogenin and gitogenin were removed and the sapogenins eluted with 250 ml of chloroform–methanol (8:2). The solvent was removed by distillation under reduced pressure.

The purity of the digitonin was checked by the following method. A 500 mg sample was refluxed with 40 ml of hydrochloric acid–water–methanol (1:1:1) for 2 h.

After cooling 20 ml of 1 % ammonium sulphate were added and the mixture extracted with 60 ml of chloroform–methanol (8:2) in 10 ml portions. The combined extracts were dried over anhydrous sodium sulphate and then evaporated to dryness by distillation under reduced pressure. The residue was taken up in the minimum quantity of chloroform–methanol (8:2) and a small sample chromatographed on Silica Gel G using dichloromethane–methanol (97:3) as developer. After spraying and heating, as previously described, two spots appeared, the main one corresponding to digitogenin and the other to gitogenin. The remainder of the residue in chloroform–methanol was purified by PLC as described for the tigogenin and gitogenin.

Isolation of the sapogenins from the seeds of D. purpurea

About 10 g of seeds were comminuted in an electric mill and then homogenised with 50 ml of 50 % methanol in a Waring blendor for 10 min. The mixture was filtered and the residue extracted for 2 h in a Soxhlet with 60 ml of methanol. The filtrate and the extract were combined and diluted to 200 ml with 0.5 % ammonium sulphate solution. The lipids were removed from this by extraction with 200 ml of petroleum ether–diethyl ether (3:2), and the saponins were extracted with 200 ml of chloroform–methanol (3:2). This saponin extract was dried over anhydrous sodium sulphate and the solvent removed by distillation under reduced pressure. The residue was hydrolysed and purified by PLC as described for the digitonin, the sapogenins thus obtained were then ready for analysis by GLC.

Silylation methods

About 1.0 to 1.5 mg of the reference compounds and of the mixtures were dissolved in 0.5 ml of one of the silylation mixtures (Table I) in a stoppered bottle. The reaction was allowed to proceed for 2 h at 60° and from 2 to 8 μl were used for the GLC analysis.

TABLE I

SILYLATION REACTION MIXTURES

Mixture	*Composition of mixture*			
	Tetrahydrofuran (ml)	*HMDS (ml)*	*Pyridine (ml)*	*TMCS*
1	0.80	0.40	0.20	20 μl
2	0.80	0.40	0.40	20 μl
3	0.80	0.40	0.80	20 μl
4	0.50	0.40	1.00	0.10 ml
5	1.00	0.40	nil	0.10 ml
6	1.00	0.40	0.05	0.10 ml

The GLC analysis

The retention data for the purified reference compounds and for their TMS ethers, with respect to the four columns, are given in Tables II and III. Column 4 was found to be the best for quantitative work as shown by Fig. 1. The response ratios, using column 4, of gitogenin and digitogenin with respect to tigogenin, all as their TMS ethers, are given in Tables IV and V.

TABLE II

RETENTION DATA ON GLC COLUMN 4

Oven temperature, 260°; nitrogen gas flow, 35 ml/min.

Substance	*Retention time (min)*	*Relative retention time, tigogenin TMS ether = 1*
Tigogenin	19.0	0.76
Tigogenin TMS ether	25.0	1.00
Gitogenin	34.0	1.36
Gitogenin TMS ether	38.5	1.54
Digitogenin	48.0	1.92
Digitogenin TMS ether	46.5	1.86
	54.0	2.16
	61.0	2.44
	64.0	2.56

TABLE III

RETENTION DATA ON COLUMNS 1 AND 2

Column 1: oven temperature, 220°; nitrogen gas flow, 65 ml/min. Column 2: oven temperature, 240°; nitrogen gas flow, 60 ml/min.

Substance	*Retention time (min)*		*Relative retention time, tigogenin TMS ether = 1*	
	Column 1	*Column 2*	*Column 1*	*Column 2*
Tigogenin TMS ether	3.0	8.5	1.0	1.0
Gitogenin TMS ether	6.3	13.26	2.1	1.56
Digitogenin TMS ether	7.02	15.10	2.34	1.78

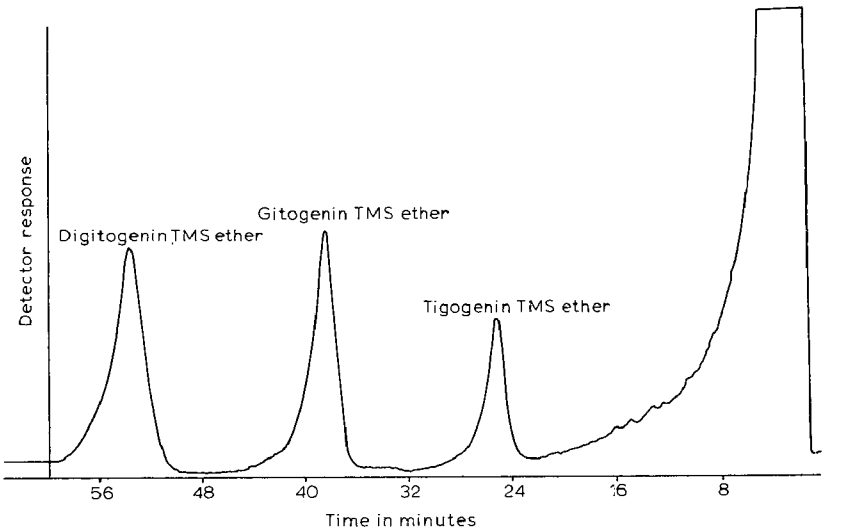

Fig. 1. Gas–liquid chromatogram showing separation of the trimethylsilyl ethers of tigogenin, gitogenin and digitogenin on column 4 at 260° and a nitrogen gas flow of 35 ml/min. Silylation using mixture 6.

TABLE IV

RATIOS OF GITOGENIN TO TIGOGENIN

Weight ratio gitogenin–tigogenin	*Mean area ratio gitogenin TMS ether–tigogenin TMS ether*	*Area ratio/weight ratio*
0.5313	0.5700	1.07284
0.5046	0.5204	1.03139
Mean area ratio/weight ratio		1.052
N (overall number of determinations)		16
% Standard error		4.61

TABLE V

RATIOS OF DIGITOGENIN TO TIGOGENIN

Weight ratio digitogenin–tigogenin	*Mean area ratio digitogenin TMS ether–tigogenin TMS ether*	*Area ratio/weight ratio*
1.4100	1.6091	1.1410
1.6550	1.8157	1.0970
0.3780	0.4120	1.1030
0.5533	0.6319	1.1421
Mean area ratio/weight ratio		1.121
N (overall number of determinations)		34
% Standard error		4.74

RESULTS AND DISCUSSION

Silylation studies

The effect of pyridine concentration and of the time of heating on the formation of the sapogenin TMS ethers at two concentration levels of TMCS have been investigated using GLC column 4. Table I gives the composition of the silylating mixtures used.

It was found that tigogenin and gitogenin, in all the mixtures except number 5, gave only single peaks with retention times of 25.0 and 38.5 min, respectively. When there was no pyridine present, as in mixture 5, neither tigogenin nor gitogenin were fully silylated since they each gave an additional peak with retention times of 19.0 and 34.0 min, respectively, corresponding to the unreacted parent sapogenin. If the time of heating was shortened as found with mixture 6 to less than 1 h, the same two peaks for each compound were produced. From these results it is concluded that full silylation of tigogenin and gitogenin can be facilitated by the presence of a trace of pyridine and with a reaction time of 2 h. It appears that the hydroxy groups are readily available for silylation.

In contrast to this the composition of the silylating mixture and the time of reaction appear to be more critical for the silylation of digitogenin. By increasing the concentration of pyridine, as in mixtures 1 to 3, the number of peaks produced by digitogenin diminished from four, with retention times of 46.5, 54.0, 61.0 and

64.0 min, to two, with retention times of 46.5 and 54.0 min. The relative sizes of the peaks also changed, the peaks at 61.0 and 64.0 min were smaller with mixture 2 than with mixture 1, whilst with mixture 3 the peak at 46.5 min increased in size and the one at 54.0 min decreased. On increasing the amount of TMCS in the silylating fluid, as in mixture 4, only two peaks were obtained, with retention times of 46.5 and 54.0 min. When pyridine was absent, as in mixture 5, peaks at 48.0 and 54.0 min were obtained.

The time of heating also affected the number and the relative sizes of the peaks. If digitogenin was heated for only 10 min in mixture 4 then only the peak at 54.0 min was obtained, however if the time is extended to 45 min an extra peak at 46.5 min appeared and if the time is further extended to several hours only the peak at 46.5 min was present. Prolonged heating in mixture 3 also gave only one peak at 46.5 min, but prolonged heating in mixture 5, pyridine absent, yielded a single peak at 54.0 min.

From a comparison of the relative retention times of the TMS ethers it is suggested that the peak at 54.0 min corresponds to the fully silylated digitogenin, *i.e.* all three hydroxy groups are silylated. It is also suggested that the peak at 46.5 min is the disilyl ether with the 2- and 3-hydroxy groups silylated. These suggestions are supported by comparing the relative retention times of monohydroxy-tigogenin, dihydroxy-gitogenin and trihydroxy-digitogenin. Too high a concentration of pyridine leads to the production of decomposition products[8], as shown by the peaks at 61.0 and 64.0 min. An examination of the stereochemistry of digitogenin shows that the hydroxy groups on carbons 2 and 3 may be silylated comparatively easily compared with the more sterically hindered hydroxy group on carbon 15. This may explain why conditions for the silylation of digitogenin are more critical than for tigogenin and gitogenin.

Response ratios of gitogenin TMS ether and of digitogenin TMS ether to tigogenin TMS ether

The TMS ethers of tigogenin and digitogenin were prepared using mixture 6. Varying amounts of these ethers were injected and the peak areas measured by the product of the height and of the width at half height. The linear dose response of these compounds is shown in Fig. 2. Weights in excess of 10 μg per peak resulted in some tailing with loss of accuracy in measuring.

Mixtures of gitogenin with tigogenin and of digitogenin with tigogenin were prepared and silylated using mixture 6. These reacted mixtures were stable during one week and from 1 to 5 μl were used for the GLC analysis. At least three analyses were performed on each mixture and the peaks areas calculated as before, the results are shown in Tables IV and V. The linearity of the peak area ratio to the weight ratio, in the case of the digitogenin–tigogenin mixtures, is shown in Fig. 3. The peak area ratio/weight ratio for the gitogenin–tigogenin mixtures is 1.052 and for the digitogenin–tigogenin mixtures it is 1.121. The increase in these ratios, namely 0.052 and 0.069, respectively, each represents an increase in molecular weight of a trimethylsilyl group. From the similarity of these two increases we conclude that they are true response ratios in the flame detector.

The method described in this paper enables the ratio of gitogenin and digitogenin to be determined in mixtures. The percentage standard error of the method is about 4% and it is applicable to microgram quantities. We used the method to analyse

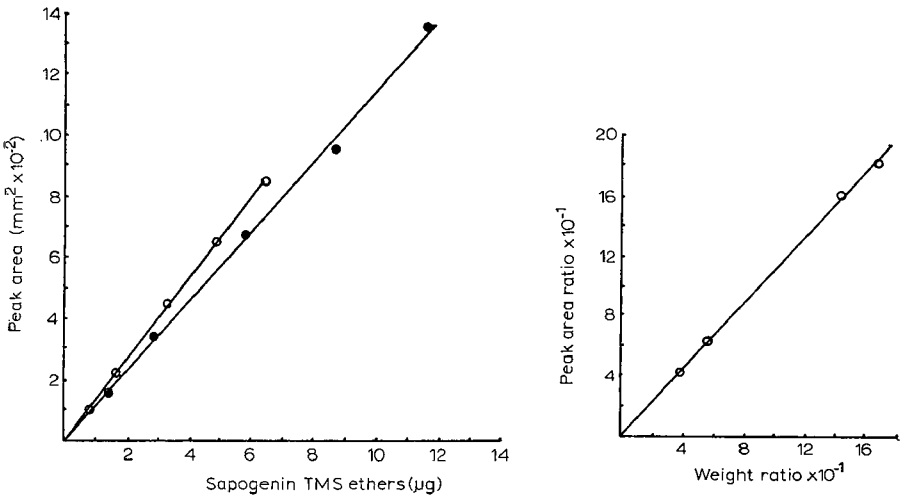

Fig. 2. Response ratio of sapogenin TMS ethers in the flame ionisation detector. ○, Digitogenin TMS ether; ●, tigogenin TMS ether.

Fig. 3. Response ratio of digitogenin TMS ether to tigogenin TMS ether.

a commercial sample of digitonin and found that it contained 11.1 % of gitogenin, showing that commercial digitonin may not be used as a pure source of digitogenin. The method was also used to analyse the ratio of sapogenins extracted from the seeds of *D. purpurea*, this was found to be 74.0 % of digitogenin to 26.0 % of gitogenin. We did not find tigogenin in the extract although its presence has been reported[9].

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CHROM. 5084

COMPARATIVE GAS CHROMATOGRAPHIC STUDIES OT CORTICOSTEROID BORONATES

C. J. W. BROOKS AND D. J. HARVEY
Department of Chemistry, University of Glasgow, Glasgow W.2 (Great Britain)
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SUMMARY

Cyclic esters formed by reaction of corticosteroids with methyl-, *n*-butyl-, *tert.*-butyl-, cyclohexyl- and phenylboronic acid have been studied, and found to be satisfactory derivatives for analytical gas chromatography. The composition of the esters has been confirmed by mass spectrometry: molecular ions were observed for all but one of the 75 boronates investigated. The problem of achieving stoichiometric reactions for analytical use has been examined.

INTRODUCTION

Previous work[1,2] has shown that corticosteroids can be effectively stabilised for gas chromatography in the form of cyclic esters formed with phenylboronic acid or *n*-butylboronic acid. Analogous derivatives have now been prepared with methylboronic, *tert.*-butylboronic and cyclohexylboronic acid. Methylboronates are notable for the small increment in molecular weight attending their formation: they accordingly have short retention times, and give easily measurable mass spectra. It was envisaged that the bulky *tert.*-butyl group might confer greater stability towards hydrolysis, but such an effect has not been observed: however, the retention times of *tert.*-butylboronates are conveniently short because of the steric properties of the *tert.*-butyl group.

In this paper, gas chromatographic data are surveyed for cyclic boronates of a variety of corticosteroids. The potential applications of boronic acids as reagents for corticosteroids are discussed.

The use of methylboronic and *tert.*-butylboronic acid has been briefly reported in a preliminary note[3].

EXPERIMENTAL

Materials

Steroids were obtained from commercial suppliers. Methylboronic acid was prepared (by Mr. D. S. STEVENSON) by the method described by MCCUSKER *et al.*[4]; *tert.*-butylboronic acid was prepared by a variation of the method of SNYDER *et al.*[5],

in which a fractionating column was used[4] to reduce losses during the isolation of the product. This acid was sensitive to air and was best handled under nitrogen, but it could be stored either dry or in solution in ethyl acetate at 0° without appreciable decomposition. All evaporations were carried out under vacuum or in a stream of nitrogen.

Chromatographic methods

Gas–liquid chromatography (GLC) was carried out with a Perkin-Elmer F-11 chromatograph using 6 ft. glass columns, 4 mm I.D., packed with 1 % OV-17 on Gas-Chrom Q, 100–120 mesh. Gas chromatography–mass spectrometry (GC–MS) was conducted with an LKB 9000 instrument using 3 or 6 ft. glass columns, 4 mm I.D., packed with 1 % OV-17 on Gas-Chrom Q, 100–120 mesh. The ionising voltage was 70 eV, accelerating voltage 3.5 kV, and electron multiplier voltage 3.1 kV. Tabulated mass spectra are to be submitted to the Mass Spectrometry Data Centre (A.W.R.E., Aldermaston, Great Britain). Thin-layer chromatography (TLC) of corticosteroid boronates was carried out using "ChromaR sheet 500" (Mallinckrodt) with chloroform as mobile phase.

Preparation of boronate esters

In the standard procedure, the steroid (10 μmole) and the appropriate boronic acid (10 μmole) were dissolved in ethyl acetate (1 ml) and the mixture was kept at room temperature for 5 min. Under these conditions, 17α,20-diols, 20,21-diols and 17α,20,21-triols were fully converted to boronates as indicated by TLC, and the reaction mixtures gave single peaks when examined by GLC. The products from 17,21-dihydroxy-20-ketones were mainly cyclic boronates as judged by GLC, but small peaks due to loss of the side-chain were present: these were considerably reduced by the addition of a slight excess (10 %) of the boronic acid. A larger excess of reagent could be tolerated where other hydroxyl groups were absent. Yields from the 20,21-ketols were much lower, but could be improved by the use of up to 3 molar equivalents of boronic acid.

Further transformations of cyclic boronates

Trimethylsilyl ethers of 3α,17α,20-trihydroxysteroid 17α,20-boronates and 3α,11β, 20,21-tetrahydroxysteroid 20,21-boronates. The cyclic boronate isolated by evaporation of the solution prepared as above was dissolved in dry pyridine (0.1 ml). Hexamethyldisilazane (HMDS; 0.1 ml) and trimethylchlorosilane (TMCS; trace) were added and the mixture was kept at room temperature for 5 min. The pyridine and reagents were removed by evaporation, and the residue was extracted with cyclohexane (1 ml). Samples (1 μl) of the solution were examined by GLC and GC–MS. The mass spectra of the products confirmed the presence of the trimethylsilyl ether group and the boronate ring. Strong peaks at *m/e* 73 and 75 have been disregarded in assigning base peak which for the purpose of the present paper are defined as the most intense peaks above *m/e* 80.

The following base peaks, molecular ions (intensities as % of base peak, in parentheses), and retention indices* were observed:

* Retention indices cited in the experimental section were determined on columns with OV-17 stationary phase, by programmed temperature gas chromatography, from 230° at 2°/min.

5β-pregnane-3α,17α,20α-triol 17,20-methylboronate 3-trimethylsilyl ether, base peak *m/e* 215, M^+ 432 (15 %); $I = 2840$;

5β-pregnane-3α,17α,20β-triol 17,20-methylboronate 3-trimethylsilyl ether, base peak *m/e* 215, M^+ 432 (11 %), $I = 2805$;

5β-pregnane-3α,17α,20β-triol 17,20-*tert.*-butylboronate 3-trimethylsilyl ether, base peak *m/e* 215, M^+ 474 (14 %); $I = 2845$;

5β-pregnane-3α,11β,20α,21-tetrol 20,21-*tert.*-butylboronate 3-trimethylsilyl ether*, base peak *m/e* 382, M^+ 490 (1.5 %); $I = 3465$;

5β-pregnane-3α,11β,20β,21-tetrol 20,21-*tert.*-butylboronate 3-trimethylsilyl ether*, base peak *m/e* 382, M^+ 490 (1.5 %); $I = 3400$.

Acetates of diol and triol boronates. The hydroxysteroid cyclic boronate (10 μmole) was dissolved in dry pyridine (0.5 ml) and a large excess (0.1 ml) of acetic anhydride was added. The mixture was left overnight at room temperature, and the reagents were removed by evaporation. The product was taken up in ethyl acetate (1 ml) and samples (1 μl) were examined by GLC. The presence of the acetate and the boronate group was confirmed by mass spectrometry. The following base peaks, molecular ions and retention indices were observed:

5β-pregnane-3α,17α,20α-triol 17,20-methylboronate 3-acetate, base peak, *m/e* 342, M^+ 402 (5 %); $I = 3085$;

17α,20β,21-trihydroxypregn-4-en-3-one *n*-butylboronate monoacetate[2], base peak *m/e* 43, M^+ 456 (20 %) $I = 3925$.

20-O-Methyloxime 17,21-n-butylboronate of 3α,17α,21-trihydroxy-5β-pregnan-20-one ("Tetrahydro S"). The formation of 3α,17α,21-trihydroxy-5β-pregnan-20-one 17α,21-*n*-butylboronate 20-O-methyloxime from 3α,17α,21-trihydroxy-5β-pregnan-20-one 17α,21-*n*-butylboronate has been described previously[2]. This compound has now been prepared from 3α,17α,21-trihydroxy-5β-pregnan-20-one 20-O-methyloxime. A mixture of 3α,17α,21-trihydroxy-5β-pregnan-20-one (3.4 mg) and O-methylhydroxylamine hydrochloride (5 mg) in dry pyridine (1.0 ml) was kept at 60° overnight, cooled and diluted with water. The product was extracted from the mixture with ethyl acetate, washed with water and dried with 5 Å molecular sieve. The solvents were evaporated, and the residue was dissolved in ethyl acetate (1.0 ml) together with *n*-butylboronic acid. The product was characterised by GC–MS: base peak *m/e* 43, M^+ 445 (34 %); $I = 3455$.

3α,17α,21-Trihydroxy-5β-pregnan-20-one 17,21-n-butylboronate 20-O-methyloxime 3-trimethylsilyl ether. The preparation of 3α,17α,21-trihydroxy-5β-pregnan-20-one 17α,21-*n*-butylboronate 20-O-methyloxime 3α-trimethylsilyl ether by the action of N,O-bistrimethylsilylacetamide (BSA) on the *n*-butylboronate O-methyloxime has been described previously[2]. The ether has also been prepared using N-trimethylsilyl-diethylamine in place of BSA. This reaction sequence gave similar results when an excess (3 molar proportions altogether) of *n*-butylboronic acid was used in the first step. Base peak *m/e* 397, M^+ 517 (25 %); $I = 3300$.

Relative stability of various cyclic boronates towards solvolysis

Examples have been given of the stability of *n*-butylboronates derived from

* Mass spectral data for these compounds were recorded with an LKB 9000 instrument in the Institute for Lipid Research, Baylor College of Medicine, Houston, Texas, U.S.A. We thank Dr. M. G. HORNING for providing this facility.

17,20-diols[1], 20,21-diols and 17,20,21-triols[2], in the presence of reagents for acetylation or trimethylsilylation. Selective removal of the boronate grouping by solvolysis with propane-1,3-diol has also been achieved. We have compared the effect of propane-1,3-diol on a series of boronates.

Propane-1,3-diol (20 μg) in ethyl acetate (20 μl) was added to the cyclic boronate in ethyl acetate (20 μl of solution prepared by the standard procedure). Samples (2 μl) were examined at intervals by GLC, with results cited in Table I.

TABLE I

EFFECT OF PROPANE 1,3-DIOL ON CORTICOSTEROID BORONATES IN ETHYL ACETATE SOLUTION

Degree of hydrolysis: (A) Hydrolysis complete in samples taken after 1 min; (B) hydrolysis complete in samples taken after 12 min; (C) partial hydrolysis after 1 day; (D) little or no hydrolysis after 1 day.

Steroid	*Degree of hydrolysis of boronates*		
	Methyl	*tert.-Butyl*	*n-Butyl*
17α,20α-Diol	C	D	C
17α,20β-Diol	D	D	D
20,21-Diol	C	C	C
17α,20α,21-Triol	D	D	D
17α,21-Diol-20-one (3 examples)	A	B	B
20,21-Ketol	A	A	A

Formation of boronates in the presence of excess reagent

Effect of solvent

Methylboronic acid (0.6 mg, 10 μmole) in ethyl acetate (0.1 ml) was added to a solution of the steroid (10 μmole) in ethyl acetate (1 ml). After 5 min a sample of this solution (1 μl) was examined by GLC. Additional methylboronic acid (1.0 mg) was added to the solution, and further samples (1 μl) were examined by GLC. This was repeated with *n*-butylboronic acid and *tert.*-butylboronic acid in each of the following solvents: pyridine, ether, hexane, cyclohexane, dimethylformamide, dioxan and acetone. With steroids containing free hydroxyl groups at positions 3, 11 or 20, marked reduction in peak height and increased tailing were invariably observed in the presence of an excess of the boronic acid.

Derivative formation

TMS ethers. The cyclic boronate, prepared in the presence of an excess of the boronic acid in pyridine, was treated with HMDS and TMCS as described above. The product was extracted with cyclohexane and samples (1 μl) were examined by GLC. Satisfactory peaks corresponding to boronate trimethylsilyl ethers were obtained from 17,20-diols, 20,21-diols and 17,20,21-triols, but 17,21-dihydroxy-20-ketones and 20,21-ketols gave mixtures of products with loss of the boronate grouping. Treatment with BSA instead of HMDS and TMCS gave similar results.

Acetates. Preparation of acetates was successful for boronates of side-chain diols and triols, but again hydrolysis of the boronate ester occurred with the 17,21-dihydroxy-20-oxosteroid boronates and 20,21-ketols.

Attempted displacement of acyclic boronates by reagents forming cyclic boronates

2-Hydroxycyclohexanone or ephedrine[2] was added in excess to a solution of

3α,17α,21-trihydroxy-5β-pregnan-20-one 17α,21-*n*-butylboronate prepared in the presence of an excess of *n*-butylboronic acid, and samples (1 μl) of the mixture were examined by GLC. Again partial hydrolysis of the 17,21-dihydroxy-20-oxosteroid cyclic boronate occurred. Similar results were obtained with 3β, 21-dihydroxypregn-5-en-20-one *n*-butylboronate.

RESULTS AND DISCUSSION

The five boronic acids studied were generally similar in their reactions with the corticosteroids. However, there were distinct differences in the reactivity of the several types of corticosteroid, and in the properties of the derived esters, as already observed with *n*-butylboronates[1]. 17,20-Diols, 20,21-diols, and especially 17,20,21-triols yielded stable esters which resisted hydrolysis and could be submitted to thin-layer chromatography. 17,21-Dihydroxy-20-ketones gave esters which were easily solvolysed by propane-1,3-diol: in this respect *tert.*-butylboronates were only marginally more stable than methylboronates. The reaction times for ester formation were generally short; up to 5 min at room temperature was sufficient for the formation of all the boronates studied, and most reactions appeared to be complete within 1 min. Under these conditions, single peaks were produced by the boronates of the 17,20-diols, 20,21-diols, and 17,20,21-triols, on admixture of equimolar proportions of the steroid and boronic acid. A slight excess of the boronic acid was needed to produce a single peak with the 17α,21-dihydroxy-20-ketones; with equimolar proportions, a small peak due to the 17-oxosteroid (produced by thermal decomposition of the unreacted steroid)[6] was always present (Fig. 1). The formation of cyclic boronates of 20,21-ketols was incomplete even when several molar proportions of reagent were present: moreover, the use of an excess of boronic acid was impracticable for corticosteroids containing hydroxyl groups additional to those in the side-chain.

Gas chromatographic properties

The derivatives reported here gave satisfactory gas chromatographic peaks, with stabilisation of the corticosteroid side-chains, except for the ketol boronates, which showed evidence of partial decomposition. Retention indices are summarised in Table II. The comparatively short retention times of methyl- and *tert.*-butylboronates are evident. They are further illustrated in Fig. 2, which shows the separation of various boronates of cortisone, and in Fig. 3, which depicts the separation of methylboronates of a range of corticosteroids.

The cyclic boronates of 17β-side-chain diols and triols were sufficiently stable for the formation of derivatives of unreacted hydroxyl and carbonyl groups to be achieved. Solvolysis of the boronate ring was, however, observed with many of the cyclic boronates of the 17α,21-dihydroxy-20-ketones and 20,21-ketols.

Boronates of 17,20-diols, 20,21-diols and 17,20,21-triols were stable towards silylating reagents, and where the steroid boronate still contained free hydroxyl groups, the fully derivatised compound could be obtained. Consequent improvement in GLC peak shape, compared with that of the hydroxysteroid boronate, was frequently observed. The mass spectra of these compounds confirmed the presence of both the boronate ring and the trimethylsilyl groups.

Acetates of hydroxysteroid boronates could also be prepared by the use of

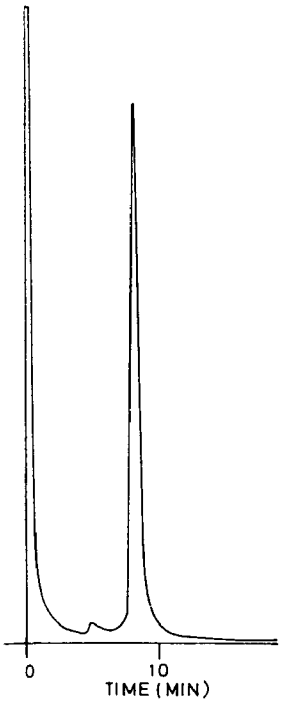

Fig. 1. Isothermal (250°) trace of 3α,17α,21-trihydroxy-5β-pregnan-20-one 17,21-methylboronate prepared by admixture of equimolar proportions of the steroid and methylboronic acid. The sample injected (in 1 μl of ethyl acetate) represents 0.01 μmole of steroid. The small peak preceding the main peak is due to 3α-hydroxy-5β-androstan-17-one.

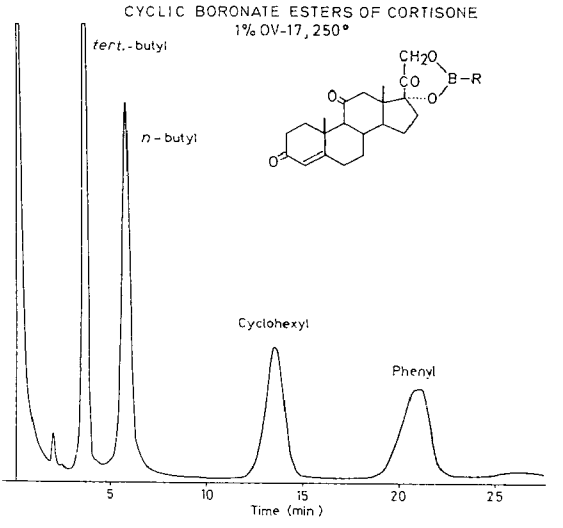

Fig. 2. Isothermal (250°) separation of the *tert.*-butyl-, *n*-butyl-, cyclohexyl-, and phenylboronates of cortisone on 1% OV-17 (6 ft.). The sample injected (in 4 μl of ethyl acetate) represents 0.01 μmole of each steroid. The methylboronate has a retention index very similar to that of the *tert.*-butylboronate and is not separated under these conditions.

acetic anhydride in pyridine, but neither trimethylsilyl ethers nor acetates of the 17α,21-dihydroxy-20-ketone or 20,21-ketol boronates could be obtained without considerable solvolysis of the boronate rings.

The carbonyl group of cyclic boronates derived from 17,21-dihydroxy-20-oxo-steroids has been shown[2] to form an O-methyloxime derivative without destruction of the boronate ester. We have now shown that the resulting methyloxime boronate can react further with silylating reagents, with retention of the boronate ring, in contrast to the compounds where the 20-oxo group is not protected. Somewhat improved yields were noted when the methyloxime group was introduced before the boronate. Of the reagents investigated for the silylation of these derivatives, BSA and N-trimethylsilyl-diethylamine appeared to be the most satisfactory.

Mass spectrometric properties

The most valuable feature of the mass spectra of corticosteroid boronates is the general prominence of molecular ions (Table II), or of the ions of *m/e* (M-18) where free hydroxyl groups were present. In most cases, fragment ions containing

TABLE II

GAS CHROMATOGRAPHIC AND MASS SPECTROMETRIC DATA FOR CORTICOSTEROID BORONATES

Abbreviated nomenclature: P = pregnane; ^{4}P = pregn-4-ene; ^{5}P = pregn-5-ene.

	Steroid	*Boronate type*	*Retention index*	*Mass spectrometric data*			
				M^+		$(M-18)^+$	
				m/e	*% of base peak*	*m/e*	*% of base peak*
1	5β-P-3α,17α,20α-triol	Methyl	3010[a]	360	13	342	100
		tert.-Butyl	3050[a]	402	18	384	100
		n-Butyl	3265	402	8	384	53
		Cyclohexyl	3590[b]	428	11	410	100
		Phenyl	3775[b]	422	26	404	100
2	5β-P-3α,17α,20β-triol	Methyl	2970[a]	360	10	342	100
		tert.-Butyl	3010[a]	402	12	384	100
		n-Butyl	3265	402	5	384	56
		Phenyl	3775[b]	(not recorded)			
3	5β-P-3α,11β,17α,20β-tetrol	Methyl	3255[b]	376	1	358	48
		tert.-Butyl	3270[b]	418	1	400	46
		n-Butyl	3480	418	2	400	48
4	^{4}P-20β,21-diol-3-one	Methyl	3380[b]	356	18	338	1
		tert.-Butyl	3520[b]	398	12	380	1
		n-Butyl	3680	398	6	380	1
		Cyclohexyl	4030[b]	424	56	406	4
		Phenyl	4330[b]	418	10	400	1
5	5α-P-3α,11β,20α,21-tetrol	Methyl	3460[b]	376	4	358	70
		tert.-Butyl	3600[b]	418	5	400	72
6	5α-P-3α,11β,20β,21-tetrol	Methyl	3470[b]	376	2	358	59
		tert.-Butyl	3565[a]	418	1	400	63
7	^{4}P-17α,20α,21-triol-3-one	Methyl	3595[b]	372	30	354	11
		tert.-Butyl	3650[b]	414	23	396	10
		n-Butyl		414	35	396	13
		Cyclohexyl	4205[b]	(not recorded)			
8	^{4}P-17α,20β,21-triol-3-one	Methyl	3620[b]	372	26	354	8
		tert.-Butyl	3650[b]	414	25	396	7
		n-Butyl	3835[b]	414	100	396	23
		Cyclohexyl	4080[b]	440	48	422	9
		Phenyl	4345[b]	434	40	416	5
9	5α-P-3β,11β,17α,20β,21-pentol	Methyl	3650[b]	392	1	374	12
		tert.-Butyl	3715[b]	(434)	0	416	16
10	5β-P-3α,17α,20α,21-tetrol-11-one	Methyl	3490[a]	390	25	372	56
		tert.-Butyl	3570[a]	432	30	414	80
		n-Butyl	3800	432	95	414	100
11	^{4}P-17α,21-diol-3,20-dione	Methyl	3360[b]	370	73	352	2
		tert.-Butyl	3400[b]	412	71	394	2
		n-Butyl	3580	412	74	394	2
		Cyclohexyl	3965[b]	438	34	420	2
		Phenyl	4120[b]	432	41	414	1
12	5β-P-17α,21-diol-3,20-dione	Methyl	3175[a]	372	45	354	4
		tert.-Butyl	3215[a]	414	25	396	3
		n-Butyl	3400	414	46	396	2
		Cyclohexyl	3710[b]	440	16	422	2
		Phenyl	3885[b]	434	62	416	2

(*Continued on page 200*)

TABLE II (*continued*)

	Steroid	*Boronate type*	*Retention index*	*Mass spectrometric data*			
				M^+		$(M-18)^+$	
				m/e	*% of base peak*	*m/e*	*% of base peak*
13	5β-P-3α,17α,21-triol-20-one	Methyl	3180[b]	374	4	356	27
		tert.-Butyl	3220[b]	416	4	398	25
		n-Butyl	3345	416	8	398	39
14	[4]P-17α,21-diol-3,11,20-trione	Methyl	3450[b]	384	73	366	2
		tert.-Butyl	3490[b]	426	73	408	2
		n-Butyl	3660	426	47	(408)	0
		Cyclohexyl	4065[b]	452	50	434	2
		Phenyl	4230[b]	446	55	428	1
15	5β-P-17α,21-diol-3,11,20-trione	Methyl	3300[b]	386	35	368	1
		tert.-Butyl	3305[b]	428	18	410	1
		n-Butyl	3600	428	21	410	1
		Cyclohexyl	3825[b]	454	11	436	1
		Phenyl	3990[b]	448	36	430	1
16	5β-P-3α,17α,21-triol-11,20-dione	Methyl	3270[b]	388	10	370	48
		tert.-Butyl	3270[a]	430	11	412	49
		n-Butyl	3465	430	13	412	42
17	[4]P-11β,17α,21-triol-3,20-dione	Methyl	3630[b]	386	31	368	38
		tert.-Butyl	3660[b]	428	35	410	39
		n-Butyl	3890[b]	428	43	410	47
18	5β-P-3α,11β,17α,21-tetrol-20-one	Methyl	3360[a]	390	1	372	15
		tert.-Butyl	3400[a]	432	1	414	12
		n-Butyl	3605	432	5	414	10
19	[4]P-21-ol-3,20-dione	Methyl	3310[b]	354	19	(336)	0
		tert.-Butyl	3480[b]	396	37	(378)	0
20	5α-P-21-ol-3,20-dione	Methyl	3220[b]	356	14	(338)	0
		tert.-Butyl	3380[b]	398	24	(380)	0
21	[5]P-3β,21-diol-20-one	Methyl	3150[b]	356	25	338	8
		tert.-Butyl	3325	398	22	380	8
		n-Butyl	3470[b]	398	66	380	12
		Phenyl	3850[b]	418	10	400	12

[a] 240° } otherwise, measured by programmed temperature gas chromatography, 200–280°.
[b] 250° }

boron were produced which were partially characteristic of the corticosteroid side-chain involved. However, except for the boronates of 17,20-diols and 20,21-ketols, such ions were not usually among the most abundant. Fig. 4 shows typical mass spectra, recorded for the methylboronate and *tert.*-butylboronate of 5β-dihydro-S (17α,21-dihydroxy-5β-pregnane-3,20-dione). Both derivatives give the same base peak, resulting from scission of ring D. The *tert.*-butylboronate is notable for the strong peak arising through loss of the *tert.*-butyl group. Further details of the observed mass spectral fragmentations will be discussed in a separate communication.

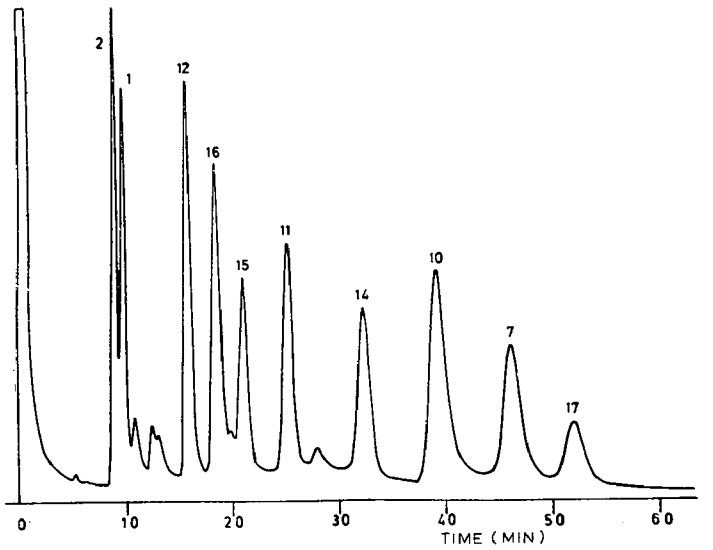

Fig. 3. Isothermal (230°) separation of the methylboronates of ten corticosteroids (approx. 1 μg of each) on 1% OV-17 (12 ft.). The numbers of the peaks refer to the structures given in Table II. The three small peaks between peaks 1 and 12 are due to 17-oxosteroids produced by thermal decomposition of unreacted 17α,21-dihydroxy-20-oxosteroids (see RESULTS AND DISCUSSION), and the peak between Nos. 11 and 14 was caused by an unidentified impurity present in No. 17.

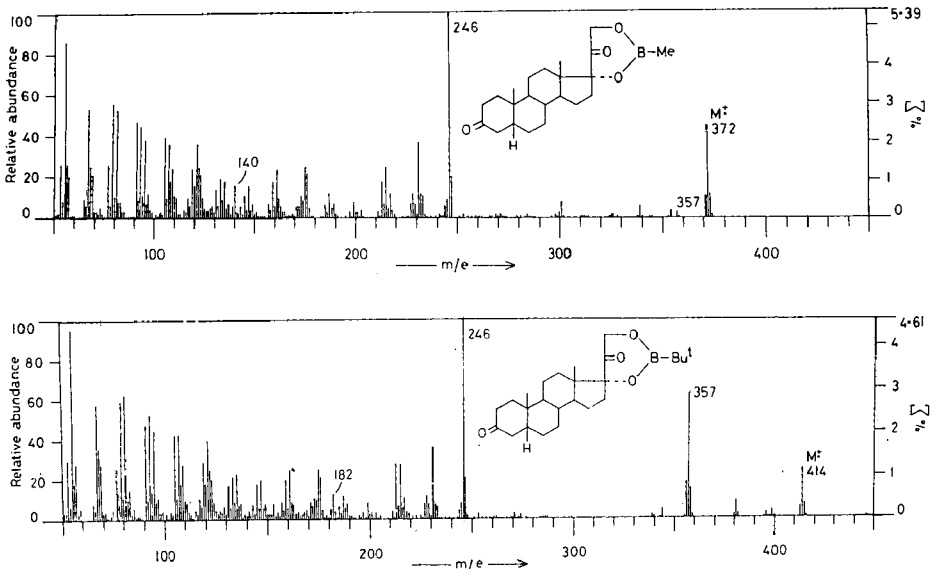

Fig. 4. Mass spectra of the methyl- and *tert.*-butylboronate of 5β-dihydro S (17α,21-dihydroxy-5β-pregnane-3,20-dione).

Structure of cyclic boronates formed from 17,20,21-triols

The boronates formed by 17,20,21-triols have been tentatively regarded as six-membered esters involving the 17- and 21-hydroxyl groups[2]. Such a structure is similar to that established for the two boronate rings in galactitol bis-phenylboronate[7]. On the other hand, a five-membered structure has been proposed[7–9] for glycerol phenylboronate. We have not yet been able to assign firmly the structures of the boronates of 17,20,21-triols. The dioxaborinane form is consistent with the smooth oxidation of the *tert.*-butylboronates of both 17α,20α,21- and 17α,20β,21-trihydroxypregn-4-en-3-one with dimethylsulphoxide–acetic anhydride[10,11] to the corresponding ester of 17α,21-dihydroxypregn-4-ene-3,20-dione; this was identified by its retention index and mass spectrum. (Attempts to effect the reverse reaction, by reduction of the steroidal dihydroxyacetone boronate with sodium borohydride, were unsuccessful, because of solvolysis of the boronate ester.) The stability of the triol boronates is also most easily understood in terms of a six-membered formulation in which the 20-oxygen atom co-ordinates with the boron atom[7]. Certain of the mass spectrometric data appear to indicate a five-membered ester structure, but the possibility that this arises during fragmentation, perhaps via the co-ordinated structure proposed, is considered reasonable. We are continuing investigations of this structural problem.

Structure of cyclic boronates derived from 20,21-ketols

These are presumed to involve the enol form of the 20-oxo group, as shown by a marked reduction of the carbonyl absorption in the infrared: the remaining carbonyl peak was produced by unreacted ketol. The proton magnetic resonance spectrum of a solution prepared from 21-hydroxy-5α-pregnane-3,20-dione and 3 molar equivalents of methylboronic acid showed no signal at the position expected for an olefinic proton, indicating a Δ^{17} rather than Δ^{20} structure for these derivatives.

Analytical formation of boronate esters in the presence of excess of reagent

In order to apply boronic acids for the gas-phase characterisation of natural steroids, it would be convenient, even if not essential, to use the reagents in excess. Unreacted boronic acids are readily eluted during gas chromatography, in the form of their trimeric anhydrides (boroxines), so that the excess of reagent presents no direct problem. The reagent may, however, interact with isolated hydroxyl groups, yielding esters of low volatility which impair the gas chromatographic analysis. We have examined two approaches to this problem.

Improvement in the selectivity of reaction. It was envisaged that a suitable choice of reaction medium, and selection of the most effective boronic acid, might permit satisfactory formation of the cyclic ester without concomitant reaction of isolated hydroxyl groups. Variation of the solvent, however, produced little or no observable effect on the reaction as far as the formation of interfering acyclic boronates was concerned. Ethyl acetate, cyclohexane, hexane, ether, dioxane, N,N-dimethylformamide, acetone and pyridine were investigated, but drastic reductions in peak heights were observed in all cases as soon as the boronic acid was added in excess. Moreover, this effect was observed for all the boronic acids studied.

Use of a second reagent. The aim here was to remove the relatively unstable acyclic boronate groups without affecting the cyclic esters. Various silylating and

acylating reagents were found to displace the interfering acyclic boronate groups, restoring well-shaped GLC peaks for cyclic boronates formed from 17,20-diols, 20,21-diols and 17,20,21-triols. This is illustrated in Fig. 5. Fig. 5a is a gas chromatographic trace of 5β-pregnane-3α,17α,20β-diol 17,20-*n*-butylboronate, prepared by mixing the reagents in equimolar proportions. An aliquot taken after addition of more *n*-butylboronic acid shows a sharp decrease in peak height, with substantial tailing (Fig. 5b). Addition of HMDS–TMCS leads to trimethylsilylation of the 3α-hydroxyl group with displacement of the interfering boronic acid, and restoration of a satisfactory peak (Fig. 5c), more symmetrical than that of the boronate of the free triol (Fig. 5a).

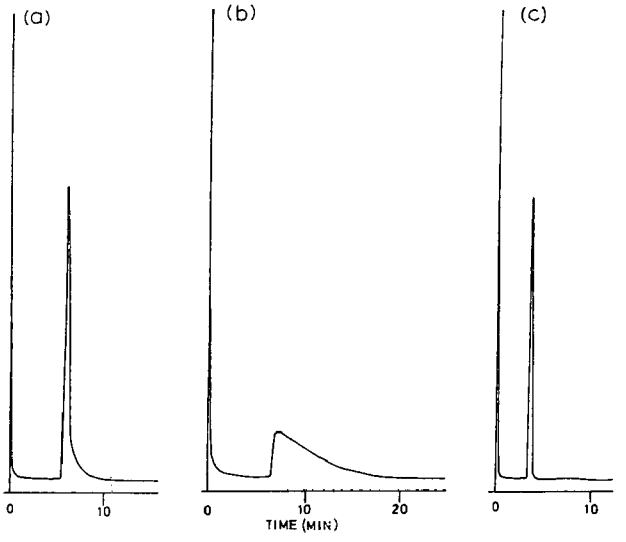

Fig. 5. (a) GLC trace of 5β-pregnane-3α,17α,20β-triol 17,20-*n*-butylboronate (0.01 μmole) obtained on 1% OV-17 at 230°. (b) The effect of adding an excess of *n*-butylboronic acid to (a). (c) The effect of trimethylsilylation of the 3α-hydroxyl group: this displaces the acyclic boronate group present in (b) without affecting the cyclic ester (17, 20). The aliquot used represents 0.005 μmole of steroid.

Use of these reagents with dihydroxyacetone or ketol boronates apparently caused solvolysis of the boronate ring, unless, in the case of the dihydroxyacetone, the 20-oxo fraction was first protected (*e.g.* as its O-methyloxime). Such a three-stage reaction is clearly inconvenient for analytical use.

We have also examined the effect of adding an excess of a reagent of low molecular weight, designed to form a cyclic boronate less stable than the desired steroidal boronate, but more stable than the interfering acyclic boronate. 2-Hydroxycyclohexanone and ephedrine[2] were investigated as possible reagents, but were ineffective. Their use also resulted in cleavage of the 17α,21-dihydroxy-20-ketosteroid and 20,21-ketol boronates.

CONCLUSIONS

Although boronic acids show promise as reagents for the gas-phase characterisation of corticosteroids, their use in quantities in excess of the amount necessary

for complete formation of the cyclic ester still presents a problem where isolated hydroxyl groups are present. This problem may be satisfactorily overcome for cyclic boronates of 17,20-diols, 20,21-diols and 17,20,21-triols by the additional formation of trimethylsilyl or acetyl derivatives of these groups, resulting in compounds showing excellent GLC properties. Boronates of 17α,21-dihydroxy-20-oxosteroids and of 20,21-ketols are too unstable to be treated in this way. Accordingly, boronates are convenient for characterisation of 17α,21-dihydroxy-20-oxosteroids only where the latter have been isolated in almost pure form and in approximately known amount. The low molecular weight increment accompanying the formation of methylboronates makes these esters particularly suitable for the characterisation of polyhydroxysteroids and other compounds of high molecular weight containing suitably-disposed hydroxyl groups.

ACKNOWLEDGEMENTS

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CHROM. 5098

GAS–LIQUID CHROMATOGRAPHY OF N-ACYL AMINO ACID ALKYL ESTERS*

S.-C. J. FU AND D. S. H. MAK

University Chemistry Research Laboratory and Department of Chemistry, The Chinese University of Hong Kong (Hong Kong)

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SUMMARY

Columns of polar and non-polar nature were chosen for studies of N-acyl amino acid alkyl esters in which systematic changes of the acyl and alkyl ester were made. Procedures for N-acylation of amino acids followed by esterification were found to give clean preparations of acyl amino acid esters, the identity and purity of which were established. The N-*n*-butyryl *n*-propyl esters of twenty-four amino acids were subjected to gas–liquid chromatography. By pairing the two columns, all the amino acid derivatives give distinct peaks, except arginine, ornithine and citrulline. The elution patterns on the polar and non-polar columns are similar but not identical.

Using glycine as a standard unit, N-acylglycine methyl esters, N-acetylglycine alkyl esters and N-acylglycine alkyl esters have been chromatographed on both columns at isothermal temperatures. The effect on retention time shown by the N-acyl group is of the same magnitude as that shown by the alkyl ester. The plot of log retention time *vs.* number of carbons of substituent X was found to be linear. The sum of the slopes of N-acylglycine methyl ester and of the N-acetylglycine alkyl ester equals the slope of N-acylglycine alkyl ester, which demonstrates that the effects of substituent X are additive.

INTRODUCTION

N-Acyl amino acid esters have been used in gas–liquid chromatography (GLC) for amino acid analysis[1–8]. The amino acid derivatives investigated were chosen on the basis of their volatility and ease in conversion from the amino acids. In the literature, a large proportion of communications are in the form of short notes or preliminary reports. The derivatives converted as well as the experimental conditions have varied widely. Furthermore, many investigations appear to be variations on a general theme with little discrimination in selection of derivatives or in design of experiments. It has been found impossible in this laboratory to correlate the effects of weight, size, geometric shape, polarity, etc. of the N-acyl and O-ester substituents of amino acids in relation to the patterns of the chromatogram.

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N-Acyl and O-alkyl ester substitutions on aliphatic amino acid have been chosen in this investigation on account of their versatility in structural variation, all of which nevertheless are of the basic structure as below.

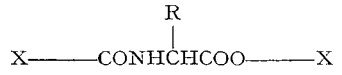

Fig. 1.

Series of N-acyl amino acid alkyl esters have been chromatographed in both polar and non-polar columns. The identical substituents X by design, on both ends of the molecule, assure simplicity in evaluating and calculating the effect on the chromatogram.

This communication consists of studies on series of amino acids with systematic change of N-acyl and O-alkyl ester substituents. The shape of the N-acyl amino acid esters approaches the form of a dumb-bell as the size of X increases. Experiments have also been performed on N-acyl amino acid alkyl esters of unequal X substituents.

It is hoped that this investigation may contribute further understanding to the relationship of substituents on amino acids and their GLC patterns. This study and further extension may provide more rationale for selection of derivatives for analysis of amino acids in the most effective manner and perhaps also in the shortest possible time.

EXPERIMENTAL

Reagents and materials

All amino acids were from Takara Kohsan Co., Ltd., Tokyo, Japan, and were of pure grade with no other amino acid or nucleic acid detectable chromatographically in a 30 μg sample. DL-α-Aminobutyric acid (DL-butyrine)* was from Sigma Co., St. Louis, Mo., U.S.A. Their purity was confirmed in this laboratory by paper chromatography in two solvent systems: water–formic acid–2-butanol (150:30:20, v/v) and 2-butanol–3 % ammonium hydroxide (150:60, v/v).

Compressed gases were from Hong Kong Oxygen & Acetylene Co., Ltd., Hong Kong; N_2 99.9991 %, H_2 99.5 % and air with oil and hydrocarbons filtered.

Instrument and columns

The instrument used was an F&M Model 5750, Hewlett and Packard Co. gas chromatograph with dual coil columns, dual flame ionisation detectors and dual-pen recorder. Column A was a 1.83 m (6 ft.) × 3.2 mm (1/8 in.) O.D. stainless steel tube, with 10 % Carbowax 20M on Chromosorb W, 60–70 mesh, acid washed, dimethylchlorosilane treated, preconditioned for 10 h at 225°. Column B was a 1.83 m (6 ft.) × 3.2 mm (1/8 in.) O.D. stainless steel tube, 1 % silicone gum GE XE-60, on Chromosorb W, 60–70 mesh AW, DMCS, preconditioned as for column A. The column was later coated with hexamethyldisilazane by injecting 10 μl of 10 % of the silylation reagent in anhydrous hexane and again preconditioned at 225° for 1 h. The gas flow rates were 28 ml/min, 32 ml/min, and 296 ml/min (20 mm, 30 mm, and 100 mm on the gauges of the instrument) for H_2, N_2, and air, respectively.

* Butyrine for α-amino-*n*-butyric acid was first suggested by GREENSTEIN AND FU[9]. The term was adopted by GREENSTEIN AND WINITZ[10].

Preparation of N-acyl amino acid esters

(I) Esterification of amino acid followed by N-acylation

Amino acid, 1 mg, was suspended in 2 ml of the appropriate alcohol and the reaction mixture was saturated with anhydrous hydrogen chloride for 5 min. The excess hydrogen chloride and alcohol were removed under reduced pressure in a Flash evaporator. The residual oil, sometimes crystallized, of amino acid ester hydrochloride was treated with 1 ml of acid anhydride at 25–30° for 10 min with vigorous stirring. The reaction mixture was again evaporated under reduced pressure to remove the excess reagent, etc. The resulting sirup of the N-acyl amino acid ester was dissolved in either benzene or the alcohol for chromatographic studies.

(II) *N-Acylation of amino acids followed by esterification*

N-Acyl amino acids. (a) To 1 mg of amino acid in 1 ml of water, 1 ml of acid anhydride was added, and mixed with vigorous stirring first at room temperature and then at 30° for a total of 10–15 min. The resulting white crystalline N-acyl amino acid precipitated. The aliphatic acid formed and water were removed under reduced pressure in a Flash evaporator. The white crystals were treated three times each with 2 ml of benzene and evaporated under reduced pressure to remove the trace amounts of impurities and water. This procedure was effective only for acetic, propionic and butyric anhydrides.

(b) In a 5-ml centrifuge tube, 1 mg of amino acid, 1 ml of acid anhydride (or acid chloride) of a higher aliphatic acid and 1 ml of 10 % sodium hydroxide were mixed with vigorous stirring at 5° and then at room temperature. The oil drops dissolved in 10–15 min indicating completion of the reaction. 1 mole equivalent of 6 *N* hydrochloric acid was added to neutralize the sodium hydroxide. The crystallized N-acyl amino acid was isolated by centrifugation. The N-acyl amino acid obtained was dried over P_2O_5 and solid sodium hydroxide in a vacuum desiccator and used for subsequent esterification. As in the Schoetten-Baumann reaction, acetic and propionic chlorides cannot be used.

Esterification of N-acyl amino acids. (c) To the dried N-acyl amino acid residue, 2 ml of the appropriate alcohol and 0.5 ml of benzene were added in the presence of approximately 1 mg of Amberlite IR-120(H) which was first thoroughly washed successively with abs. ethanol and benzene. This reaction mixture was heated under reflux for 10 min. The resin was separated from the solution by filtration and was washed three times each with 1 ml of benzene. The washings were combined with the filtrate. The excess of the alcohol and benzene were removed under reduced pressure in a Flash evaporator. The residue, N-acyl amino acid ester, remaining in the flask was dissolved in either 1 ml of benzene or 1 ml of the alcohol. The solution was immediately used for chromatographic studies.

(d) Alternatively, the dried N-acyl amino acid residue could be esterified in 2 ml of the alcohol by passing a stream of dry hydrogen chloride for 5–10 min or until the acyl amino acid dissolved. The reaction mixture was then heated under reflux for another 10 min. The excess alcohol and hydrogen chloride were removed by evaporation in a Flash evaporator under reduced pressure. The resulting sirup was dissolved as described in (c).

(III) Isolation of N-acyl amino acids and N-acyl amino acid esters

In some instances, pure N-acyl amino acid or N-acyl amino acid ester were isolated. The preparative procedures used were the same as given above, except 0.1–0.5 g of amino acid was used. Some of the known compounds prepared were used as reference to verify the conversion procedures and as standards for quantitative estimation of N-acyl amino acid esters. The compounds given below are new compounds which were identified and analyzed.

N-*n*-Valerylglycine was prepared from glycine and valeric anhydride by procedure IIb; m.p. 81–81.5° (benzene). Anal.*: calcd. for $C_7H_{13}NO_3$: C, 52.81; H, 8.23. Found: C, 53.02; H, 7.99.

N-*n*-Valerylglycine methyl ester was prepared from N-valerylglycine and abs. methanol by procedure IId; b.p.$_{2.8}$ 120°. Anal.: calcd. for $C_8H_{15}NO_3$: C, 55.49; H, 8.73. Found: C, 55.82; H, 8.84.

N-Enanthylglycine was prepared from glycine and enanthic chloride by procedure IIb; m.p. 88° (H_2O). Anal.: calcd. for $C_9H_{17}NO_3$: C, 57.75; H, 9.16. Found: C, 58.10; H, 9.16.

N-Enanthylglycine methyl ester was prepared from N-enanthylglycine and abs. methanol by procedure IId; b.p.$_{3.5}$ 160°. Anal.: calcd. for $C_{10}H_{19}NO_3$: C, 59.68; H, 9.51. Found: C, 59.91; H, 9.32.

Chromatographic procedure

Starting from 1 mg of the amino acid, the benzene or the alcohol solution of the N-acyl amino acid ester prepared was diluted to 1 ml in a calibrated volumetric tube. It gave a concentration of approximately 1×10^{-5} mole/ml, *i.e.*, 1×10^{-8} mole per μl or 1 μg/μl, assuming the molecular weight of amino acid is 100. A Hamilton syringe No. 701N, 10 μl or No. 75, 5 μl was used. Either 0.5 μl or 1.0 μl of the solution was injected into the columns. For higher dilution, the N-acyl amino acid ester was diluted to 5 to 30 ml in order to measure at various concentrations. The injection was never less than 0.5 μl. The N-acyl amino acid alkyl esters were chromatographed individually and as a mixture under isothermal and programmed conditions.

RESULTS AND DISCUSSION

Columns

Commercially packed columns were chosen for this investigation on account of availability and minimum variation in column characteristics due to packing, composition of stationary and liquid phase, etc. Column A of 10 % Carbowax 20M liquid phase and column B of 1 % silicone gum rubber liquid phase were used. In Fig. 2, the chromatogram of N-isobutyryl amino acid isopropyl esters was used as an illustrative example. The retention time of the acyl esters eluted from column B was 3 min and offered relatively good separation except that glycine and valine appeared as one peak. Column A, however, showed good separation of all the esters under the same conditions.

Both columns were tested for recognizable signals at minimum amount of the acyl ester. The observable minimum quantity was 0.01 μg on both columns but the

* The elemental analyses were performed by the Microanalytical Laboratory, University of Singapore.

noise level was too high to be useful without a signal analyzer/averager. The noise to signal ratio was 1:2.5. However, 0.02 μg would give desirable peaks in height and shape for normal measurement. Measurements were made in most cases at concentrations of 0.1–5 μg. The column performance in terms of plate number (N) was also determined. A modification[11] of the equation suggested by JAMES AND MARTIN[12] was used for calculations. The average N calculated for column B was 1000 at isothermal temperatures 150° and 165°. When the column was operated at higher temperatures, the resolution and column behavior became increasingly poor. At 200°, the average N dropped as low as 256. However, column A showed an average N of 1207 and 1091 for 165° and 200°, respectively.

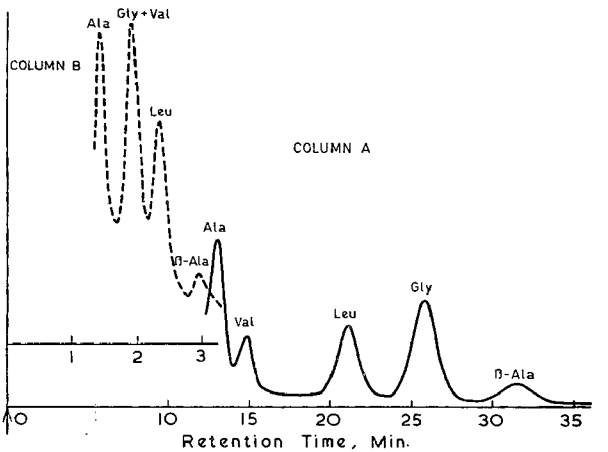

Fig. 2. Gas–liquid chromatograms of N-isobutyryl amino acid isopropyl esters. Sample: A mixture of approx. 1 μg each of the amino acids converted, in 1 μl benzene. Conditions: isothermal at 165°, inj. port 230° and det. 210°. (———), column A: attenuation 2 × 10^2; chart speed 0.25 in./min. (– – – –), column B: attenuation 8 × 10^2; chart speed 1 in./min.

N-Acyl amino acid alkyl esters

The conversion of amino acid to its N-acyl amino acid ester generally was accomplished by esterification of the amino acid in alcohol in the presence of a hydrogen halide. The amino acid ester was then N-acylated by reacting with excess of acid anhydride or acid chloride. However, the total percentage of conversion was not quantitative. Particularly, in this investigation, when higher aliphatic acid anhydrides or chlorides were used, the removal of aliphatic acid formed and the excess acid anhydride or chloride, by distillation *in vacuo*, was found too difficult. Decomposition often took place even at very low pressure.

The N-acylation of the amino acid followed by esterification had to be investigated, from which procedure II was established. The esterification of N-acyl amino acid with alcohol in the presence of Amberlite IR-120 (H) or hydrogen chloride was found to be quantitative. The removal of alcohol was accomplished by distillation at comparatively low temperature. Acyl esters which showed two or three components when prepared by procedure I were obtained in GLC pure forms. The esters converted by procedure IIc or d were compared with the corresponding authentic samples of N-acyl esters at the same concentration. Their retention time, peak height and peak

shape on the chromatogram were indistinguishable in all aspects. Since the esterifications proved to be quantitative, it was possible to test the percentage conversion of the N-acylation step. If the resulting N-acyl amino acid ester was not quantitative, the percentage yield must be due to the N-acylation procedure employed. Some of the standard N-acyl amino acid esters prepared were isolated and purified by micro fractional distillation or recrystallization. In some cases, elementary analyses were performed to ascertain identity and purity. These standard N-acyl amino acid esters have been stored at 0° in the dark for more than a year without deterioration. These pure samples were also used as standards to determine minor variations of new columns. In no instance was N-diacylation of the amino acid observed in any of the procedures described.

GLC studies

The aliphatic amino acids: glycine, alanine, butyrine*, valine, leucine and β-alanine were chosen on account of their gradual increase in size of the side chain R group on the amino acid structure, as shown in Fig. 1. Symmetric substitutions on the N and C terminals of the amino acid were made; for example, the CH_3 of the acetyl and the CH_3 of the methyl ester, in N-acetyl amino acid methyl ester, which straddled

$$\begin{array}{l} \\ \\ \text{R} \\ | \end{array}$$

the –CONHCHCOO– unit, assumed a form of symmetry; as did the C_2H_5 of *n*-propionyl and the C_2H_5 of the ethyl ester in N-*n*-propionyl amino acid ethyl ester, etc.

The N-acylating groups were from acetyl to enanthyl and the esters were from methyl to *n*-hexyl. Among all amino acid derivatives studied, the N-butyryl amino acid propyl esters give the most desirable pattern. Further, 24 amino acids were chromatographed in both column A and B, shown in Table I. Although column B showed very fast elution of the six aliphatic amino acids, it gave desirable retention times and patterns for acidic, basic and some heterocyclic amino acids whose retention times on column A would be too long to be practical for routine analytical use. Their elution patterns were further improved at temperature-programmed conditions. The non-polar column apparently offered the most advantages for amino acids of complex structure. By pairing these two columns, all the amino acids gave distinct peaks, except arginine, ornithine and citrulline. The order of elution on the polar and non-polar columns was not identical but there was a common elution pattern.

Effect of substituents on the N and C terminals of amino acids

The effects of N-acyl and alkyl ester substituents were studied individually and in combination. Glycine was used as a standard unit in the acyl alkyl esters. N-Acylglycine methyl esters, N-acetylglycine alkyl esters and N-acylglycine alkyl esters were chromatographed on both columns at isothermal temperatures. The effect of the N-acyl group on the retention time is shown in Table II. On changing the ester group from methyl to *n*-hexyl on acetylglycine, the retention time was systematically prolonged, as shown in Table III. Although the effect of the alkyl ester appeared to be slightly greater than that of the N-acyl, the difference is so slight that they may actually be considered of the same order of magnitude. The combined effects of N-acyl

* Butyrine was eluted from the columns with valine in most cases. Therefore, it was not used as a component when a mixture of amino acids was converted for chromatographic studies.

TABLE I

RETENTION TIME (min) OF *n*-BUTYRYL AMINO ACID *n*-PROPYL ESTERS

Temperatures: injection port 230°, detector 210°.

Column A			*Column B*		
Amino acid	*Isothermal 165°*	*Programmed 100–185° (4°/min)*	*Isothermal 150°*[a]	*Isothermal 165°*	*Programmed 100–180° (4°/min)*[a]
Ala	36.6	30.0	6.3	2.6	13.9
But[b]	40.6	31.3	6.8	3.1	14.5
Val	41.0	31.3	7.8	3.5	14.9
isoLeu	51.6	40.6	10.2	4.4	16.6
Leu	61.2	37.6	10.4	4.8	16.9
Gly	67.2	41.4	7.8	3.6	15.4
β-Ala	82.0	47.4	11.7	5.0	17.2
Pro	109.6	67.4	17.7	7.2	18.9
γ-But[c]	[d]	97.0	26.6	10.6	21.1
Thr	[d]	140.4	38.0	14.0	24.4
Asp	[d]	[d]	41.6	17.0	26.6
Ser	[d]	[d]	47.2	18.2	25.6
Met			60.0		29.6
Phe			66.0		28.8
Cys			96.4		34.8
Glu			105.2		33.7
Hypro			149.6		46.0
Lys			[d]		21.9
Tyr			[d]		22.2
His			[d]		33.6[e]
Try			[d]		53.6[e]
Arg			[d]		[d]
Orn			[d]		[d]
Cit			[d]		[d]

[a] Injection port 240° and detector 220°.
[b] See footnote on p. 210.
[c] γ-Aminobutyric acid, *cf.*[b].
[d] No peak appeared in additional 120 min.
[e] Programmed column temperature 100–230 (4°/min), injection port 270° and detector 250°.

TABLE II

RETENTION TIME (min) OF N-ACYLGLYCINE METHYL ESTERS

Temperatures: injection port 230°, detector 210°.

N-Acyl group	*Column A*		*Column B*	*Ester group*
	165°	*200°*	*165°*	
Acetyl	28.8	8.8	1.5	Methyl
Propionyl	30.1	9.5	1.6	Methyl
n-Butyryl	39.5	11.9	2.0	Methyl
n-Valeryl	57.8	16.8	2.8	Methyl
n-Caproyl	83.6	21.4	4.4	Methyl
Enanthyl	117.4	31.0	6.4	Methyl

TABLE III

RETENTION TIME (min) OF N-ACETYLGLYCINE ALKYL ESTERS

Temperatures: injection port 230°, detector 210°.

N-Acyl group	*Column A*		*Column B*	*Ester group*
	165°	*200°*	*165°*	
Acetyl	28.8	8.8	1.5	Methyl
Acetyl	31.9	9.0	1.6	Ethyl
Acetyl	44.6	12.2	2.3	*n*-Propyl
Acetyl	66.4	16.8	3.2	*n*-Butyl
Acetyl	96.0	23.4	4.4	*n*-Amyl
Acetyl	148.0	35.0	6.2	*n*-Hexyl

TABLE IV

RETENTION TIME (min) OF N-ACYLGLYCINE ALKYL ESTERS

Temperatures: injection port 230°, detector 210°.

N-Acyl group	*Column A*		*Column B*	*Ester group*
	165°	*200°*	*165°*	
Acetyl	28.8	8.8	1.5	Methyl
Propionyl	32.2	10.2	1.9	Ethyl
n-Butyryl	67.2	18.1	3.6	*n*-Propyl
n-Valeryl	145.4	34.8	7.2	*n*-Butyl
n-Caproyl	—	58.8	14.0	*n*-Amyl
Enanthyl	—	118.2	29.4	*n*-Hexyl

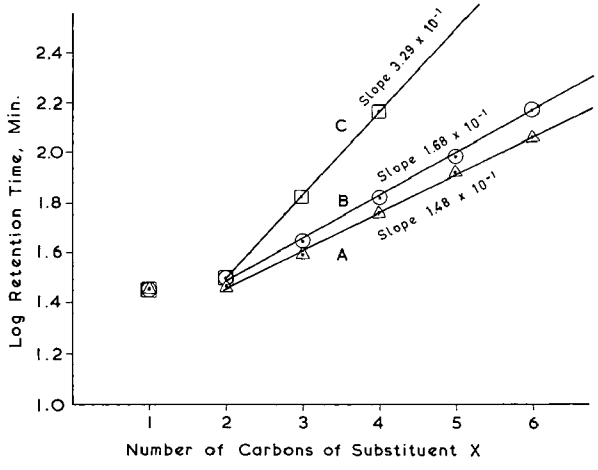

Fig. 3. Log retention time *vs.* number of carbons of substituent X on column A, isothermal at 165°. A (△—△), X–CONHCH$_2$COO–CH$_3$, B (⊙—⊙), CH$_3$–CONHCH$_2$COO–X, C (⊡---⊡), X–CONHCH$_2$COO–X.

and alkyl esters on the retention time are shown in Table IV. When the data in the three tables were plotted, log retention time *vs.* number of carbons of the substituents X (see Fig. 1), a linear relationship was revealed. It was particularly noteworthy that the slope of N-acylglycine methyl esters plus the slope of N-acetylglycine alkyl esters equaled the slope of N-acylglycine alkyl esters, in all cases, which demonstrated that the effects of the substituents X were additive; an example is shown in Fig. 3.

Substituents X used were all normal (straight chain) aliphatic groups. The retention time was found to be proportionally prolonged with increasing size of X. The size increment of X accompanying the increase in molecular weight is a factor which should not be overlooked. In order to distinguish between these two effects, the chromatograms were made of N-isobutyryl amino acid isopropyl esters which are the structural isomers of N-*n*-butyryl amino acid *n*-propyl esters. If the molecular weight increment was the dominating factor affecting the retention time, the corresponding isomers should have nearly identical GLC patterns and retention times. The fact was that the retention time of the N-isobutyryl amino acid isopropyl esters shown in Table V, which was approximately one third that of the *n*-isomers, shown in Table I, was found to fall near their corresponding N-acetyl amino acid methyl esters, shown in Table VI. This indicates that the shape of the substituent X rather than other factors dominated the GLC patterns and retention times.

TABLE V

RETENTION TIME (min) OF N-ISOBUTYRYL AMINO ACID ISOPROPYL ESTERS

Temperatures: injection port 230° and detector 210°.

Amino acid	*Column A*		*Column B*
	165°[a]	*200°*	*165°*[a]
Ala	13.2	4.5	1.5
Val	15.3	5.1	1.9
But	15.6	5.0	1.3
Leu	21.3	6.6	2.3
Gly	26.0	7.8	1.9
β-Ala	31.8	9.6	2.8

[a] See also Fig. 2.

TABLE VI

RETENTION TIME (min) OF N-ACETYL AMINO ACID METHYL ESTERS

Temperatures: injection port 230°, detector 210°.

Amino acid	*Column A*		*Column B*
	165°	*200°*	*165°*
Ala	15.2	5.0	1.2
Val	17.8	5.8	1.5
But	18.2	5.9	1.5
Leu	26.2	8.2	2.1
Gly	28.0		1.6
β-Ala	34.0	10.3	2.1

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CHROM. 5070

ADSORPTION ON NEUTRAL POLYSTYRENE RESIN

A SIMPLE METHOD FOR EXTRACTION OF 2,4-DINITROPHENYL DERIVATIVES FROM AQUEOUS SOLUTION AND FOR DECOLORATION OF PROTEIN HYDROLYSATES*

A. NIEDERWIESER
Chemisches Labor der Universitäts-Kinderklinik, CH-8032 Zürich (Switzerland)
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SUMMARY

DNP-Derivatives of amino acids, peptides, and hexosamines, as well as dinitrophenylhydrazones are strongly adsorbed on Porapak Q from aqueous solutions. Adsorption of DNP-amino acids is strongest at low pH values and shows, in low salt concentrations, a minimum near pH 9. Addition of salts strongly increases their adsorption also in alkaline medium. The effect of "salting out on adsorbent" cannot be described by the general Setschenow equation and is influenced by the nature of the salt. In 1 *M* solutions of alkali halogenides at pH 8.8, the salting-out effect (DNP-cysteic acid on Porapak Q) is influenced much more by different anions than by different cations: $F > Cl > Br \gg J$ and $Li > Na \geqslant K \geqslant Cs$.

The adsorption isotherm of DNP-cysteic acid in 0.1 *N* HCl–0.1 *M* NaCl is nonlinear (linear capacity less than 0.6 %). The "logarithmic capacity" determined from the Freundlich isotherm is only about 1%, the total capacity being about 1 mmole/g Porapak Q. This nonlinearity suggests a heterogeneity of the adsorbent surface.

Adsorption of free phenylalanine is weak and practically not influenced by pH and salt concentration.

Procedures are described for adsorption of DNP-derivatives from acid hydrolysates and from dinitrophenylation mixtures. Application for decoloration of urine hydrolysates and extraction of other nonpolar compounds (steroids, dyes) is suggested.

INTRODUCTION

Liquid–liquid extraction procedures of small samples are time-consuming and difficult to automize for routine analysis. An alternative exists in adsorption on a column packed solid phase. Such columns can be handled much easier in series and avoid difficulties with emulsions which often cause delays in liquid–liquid extraction. Adsorption on charcoal is a well-known means for removing organic materials from

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aqueous solution. However, its adsorptive properties are insufficiently reproducible. Even small molecules, *e.g.* 2,4-dinitrophenylamino acids, may be bound irreversibly.

In a previous publication[1] we described a method for recognizing peptide patterns in human urine. Part of this method is the adsorption of DNP-peptides from aqueous solution on Porapak Q®. The observation that the adsorption of DNP-cysteic acid was not well reproducible led me to investigate the adsorption behavior of 2,4-dinitrophenyl (DNP) derivatives more thoroughly. The results will be described here.

Porapak Q® is a porous co-polymer of styrene and ethylvinylbenzene cross-linked with divinylbenzene*. The yellowish-white beads are of strong physical structure and available in controlled particle size and high internal porosity (specific area about 500 m^2/g). The material has found wide application as the stationary phase in gas chromatography[2,3], in chromatography with high-pressure gases or supercritical fluids[4], and in TLC of aromatic hydrocarbons[5] as suggested by JANÁK[6,7].

Recently, another neutral polystyrene resin, Amberlite XAD-2, was applied for the extraction of steroids and steroid glucuronides from aqueous solution[8,9], a technique first described by BRADLOW[8]. Unfortunately, this material is at present only available in relatively coarse beads.

MATERIALS AND PROCEDURES

Porapak Q®, 150–200 mesh, (Waters Associates, Framingham, Mass., U.S.A.) was swelled in ethanol and washed thoroughly with water and buffer.

All solvents and materials were of highest purity grade available. DNP-Derivatives of protein amino acids were purchased from Serva Entwicklungslabor, Heidelberg (G.F.R.); the DNP-derivatives of citrulline, cysteic acid, galactosamine, glucosamine, dipeptides, and tripeptides were synthesized using standard techniques[1,10,11] and thin-layer chromatography for purity control.

For equilibrium studies 300 mg of swelled Porapak Q was filled in a glass column of 10 mm I.D., provided with a glass disk, and was washed with buffer. 10 ml of sample solution were repeatedly (10 times) run through the column. The extinction of the solution was measured before and after equilibration, with or without previous dilution. The result was corrected for outer column volume (correction factor $f = 1.05$).

EXPERIMENTAL

Basic conditions for adsorption on neutral polystyrene resin

Adsorbent. Porapak Q must be wetted in ethanol for at least 15 min and the ethanol must then be removed completely by several water washings. A resin which was not wetted with an organic solvent does not adsorb at all. The ethanol-treated resin may be stored in distilled water for at least two weeks without significant change in its adsorption properties**.

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* Porapak Q contains keto groups. See under *Recovery*.

** 300 mg of Porapak Q pretreated with ethanol and stored in water for 16 days was washed with 0.2 *M* phosphate buffer (pH 7) and equilibrated with 0.3 mg of 2,4-DNP-cysteic acid sodium salt in 10 ml of the same buffer. The resin adsorbed 49% of the DNP-derivative, compared with 50% when the resin was washed with ethanol and buffer immediately before the experiment.

Swelling in a nonpolar solvent like xylene followed by short ethanol and long water washings does not change the adsorption capacity.

Solvent. The sample solution and the washings must be aqueous and should not contain organic solvents. Depending on the substances to be adsorbed, a few percent of ethanol or acetone may be permissible but the adsorption capacity of the resin may decrease considerably. On the other hand, a low pH and high salt concentration favor the adsorption of dinitrophenyl derivatives, as will be seen below.

Effect of pH

The influence of pH on the adsorption of several 2,4-dinitrophenyl derivatives and of phenylalanine on Porapak Q is shown in Fig. 1. Adsorption is strongest at low pH values. While the influence of pH on the adsorption of the free amino acid phenylalanine is rather small, all dinitrophenyl derivatives investigated show a clear adsorption minimum near pH 9 in 0.1 *M* buffer solutions. The amphoteric DNP-arginine, containing a strongly basic guanidino group, is adsorbed strongest, the sulfonic acid DNP-cysteic acid is bound weakest. Surprisingly, even DNP-cysteic acid can also be adsorbed quantitatively in alkaline medium on a short column provided a high salt concentration is applied. There is a second adsorption maximum near pH 11 in 1 *M* carbonate buffer as indicated in Fig. 1. It is interesting to note that BRADLOW[8] found no influence of pH on the adsorption of steroids on Amberlite XAD-2.

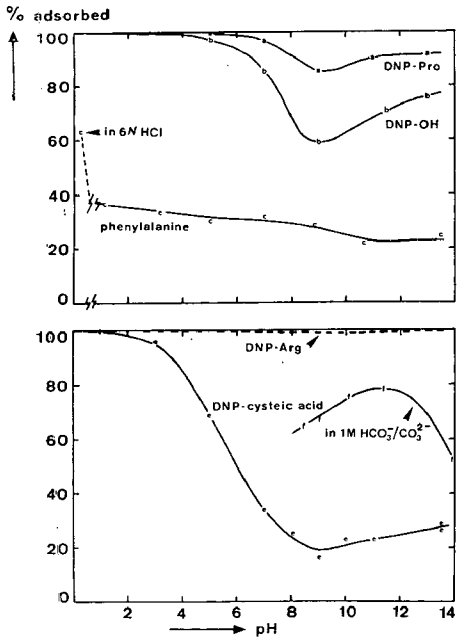


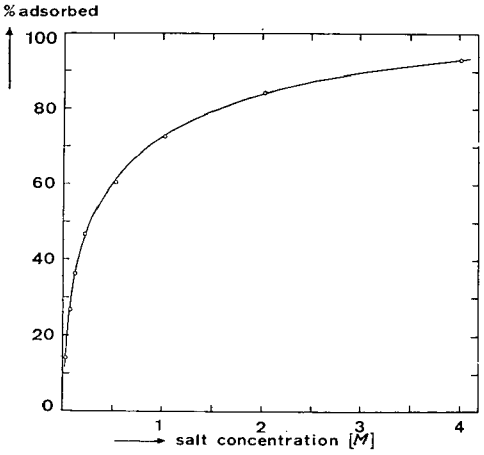
Fig. 1. Effect of pH on the adsorption of phenylalanine and DNP-derivatives on Porapak Q in 0.1 *M* (and 1 *M*) buffers. Note the weak influence of pH on the adsorption of phenylalanine and the strong on the adsorption of DNP-derivatives with a minimum near pH 9 in 0.1 *M* buffers and a further maximum near pH 11 in 1 *M* carbonate buffer. Arg: arginine; DNP-OH: 2,4-dinitrophenol. 0.1 *M* buffers used: 0.1 *N* HCl (pH 1); 0.1 *M* sodium citrate–HCl (pH 2–5); 0.1 *M* sodium–potassium phosphate (pH 6–8); 0.1 *M* glycine–KOH (pH 9–14).

Effect of salts and non-electrolytes

A very pronounced influence of salt concentration on the adsorption of dinitrophenyl derivatives on Porapak Q was found. This is shown in Fig. 2 for DNP-cysteic acid at pH 8.8. The effect of "salting out onto adsorbent" was shown for all DNP-derivatives investigated but not for the free amino acid phenylalanine. The general phenomenon of salting out (for a discussion see, *e.g.*, ref. 12) usually can be described by the Setschenow equation

$$\log \frac{S_0}{S} = \log \frac{f}{f_0} = k_s C_{\text{salt}}$$

where S_0 and S, f_0 and f refer to the solubility and the activity coefficient of the substance in water and salt solution, respectively, k_s is the salting out constant, and C_{salt} means salt concentration. This relation is based, among others, on the fact that the activity coefficient of a pure solid is constant. The presence of an adsorbent and the fact of a nonlinear adsorption isotherm (see below) alters the situation in so far as the activity coefficient of the adsorbed substance is no more constant but depends on the substance concentration. Hence, a plot of the logarithm of the solubility against the



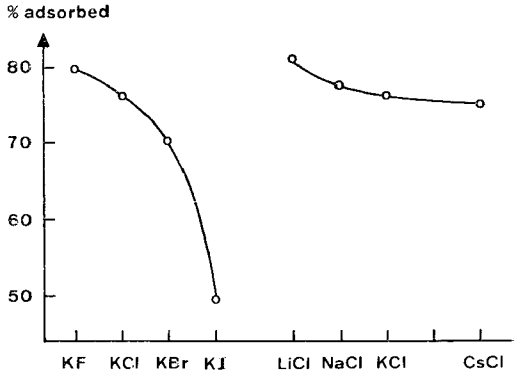


Fig. 2. Effect of sodium chloride concentration on the adsorption of DNP-cysteic acid (0.3 mg) on Porapak Q (300 mg) in 10 ml of 0.02 *M* glycine–KOH buffer pH 8.8 at 25°.

Fig. 3. Effect of 1 *M* potassium halides and of 1 *M* alkali chlorides on the adsorption of DNP-cysteic acid (0.3 mg) on Porapak Q (300 mg) in 10 ml of 0.05 *M* glycine–KOH buffer pH 8.8 at 22°.

salt concentration should not give a straight line. In fact, no straight line was obtained in the example described above (DNP-cysteic acid in sodium chloride solutions at pH 8.8 in presence of Porapak Q).

As may be expected, the nature of the salt is of some influence on the salting-out effect also in the presence of an adsorbent. A comparison of the alkali halogenides, applied as 1 *M* solutions at pH 8.8, shows that the nature of the cation is of minor influence (Fig. 3). The small lithium ion effects a somewhat stronger adsorption of DNP-cysteic acid than the other alkali cations. Similarly, the small fluoride anion

causes a stronger adsorption than the other halogenides; its effectiveness in comparison to the large iodide is striking. Obviously, an inversed influence is effected by anions and cations. This is suggested by Fig. 3, comparing the convex shaped curve of potassium halogenides (at the left) with the concave shaped curve of alkali chlorides.

Non-electrolytes do not favor adsorption of DNP-derivatives. As shown in Table I, 1 *M* solutions of glucose, urea, and especially of sucrose are able to desorb DNP-cysteic acid to some extent. As mentioned already, organic solvents such as alcohols and acetone, drastically decrease the adsorption of DNP-derivatives.

It is generally accepted that nonelectrostatic attraction from hydrophobic or van der Waals–London dispersion forces provides the driving forces for the binding of large ions to polymers. This is of importance especially if the solute is bearing a large hydrophobic group attached asymmetrically to the charged group, as in many DNP-derivatives, similar to detergents. However, in order to avoid the temptation to construct a *post facto* rationalization, it should not be tried to explain the above few observations in terms of hydrophobic forces, hydration energy, internal pressure of the solution, water structure, etc. Much more data would be necessary.

TABLE I

EFFECT OF POLAR NON-ELECTROLYTES AND SALTS ON ADSORPTION OF DNP-CYSTEIC ACID ON PORAPAK Q IN 0.05 *M* GLYCINE–KOH BUFFER pH 8.8 AT 22°

Concentration of additive	*DNP-cysteic acid adsorbed*[a] (%)
1 *M* Sucrose	12.0
1 *M* Urea	15.2
1 *M* Glucose	20.6
none	26.2
1 *M* Glycine buffer pH 8.8[b]	45.3
1 *M* NaCl	75.2

[a] 300 mg Porapak Q was equilibrated with 10 ml of buffer solution containing 0.3 mg DNP-cysteic acid sodium salt and additives tabulated.

[b] 0.1 *M* potassium glycinate–0.9 *M* glycine. In 0.1 *M* NaCl, about 40% would be adsorbed.

Adsorption isotherm at low pH

The adsorption of DNP-cysteic acid was measured as a function of sample concentration in 0.1 *N* HCl containing 0.1 *M* NaCl. The isotherm obtained is convex and similar to the Langmuir type. This would mean that monolayer adsorption rather than multilayer adsorption occurs. However, a bilogarithmic plot (Freundlich isotherm, see Fig. 4) shows a straight line only over a relatively small concentration range — up to about 12 μmole·g^{-1} — but with a remarkable steep slope ($m = 0.77$). A steep slope should mean high adsorption strength. Apparently the adsorbent surface is heterogeneous. Assuming a total adsorbent capacity of 10^3 μmole·g^{-1}, the adsorbing sites responsible for the lower linear part of the Freundlich isotherm (Fig. 4) adsorbe only about 1% of this amount ("logarithmic capacity").

Isotherm linearity and linear capacity generally decrease for more strongly adsorbed compounds and for heterogeneous adsorbents. In the system investigated the linear capacity (10% deviation from straight line in a lin–lin plot, see Fig. 5)

amounts to about 6 μmole·g^{-1}, or about 0.6 % of the total capacity, and thus is very small. This means that chromatography under such conditions would be unfavorable because of the tenacious tailing which is to be expected, and probably would lead to incomplete elution of the bands.

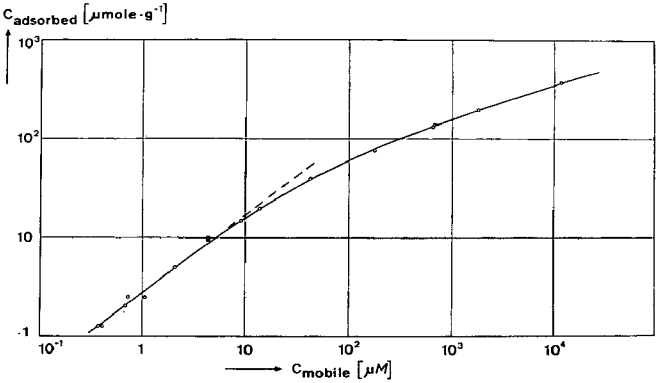


Fig. 4. Freundlich isotherm of DNP-cysteic acid on Porapak Q in 0.1 *N* HCl–0.1 *M* NaCl at 26°. The "logarithmic capacity" is only about 1% of the total capacity. The curve suggests surface heterogeneity of the adsorbent.

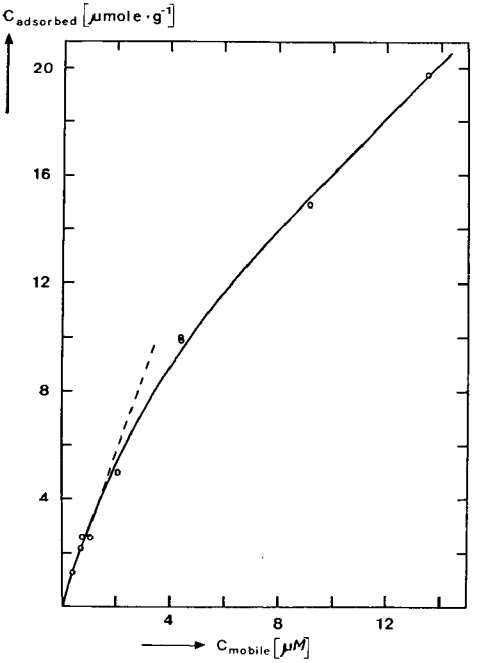


Fig. 5. Adsorption isotherm of DNP-cysteic acid in 0.1 *N* HCl–0.1 *M* NaCl at 26°. Note the non-linearity.

Adsorption capacity

As is evident from Figs. 1 and 2 and Table I, the adsorption capacity for DNP-derivatives is a function of pH and of the concentration of salts and other components

in the solvent. From the Freundlich isotherm (Fig. 4) a remarkably high theoretical adsorption capacity of about 1 mmole DNP-cysteic acid per g Porapak Q (in 0.1 *N* HCl containing 0.1 *M* NaCl at 25°) can be calculated. In practice, however, a somewhat lower value is to be expected. For instance, 0.5 mmole DNP-cysteic acid per g resin could be quantitatively adsorbed on a column of 10 × 80 mm in 6 *N* HCl, and 0.4 mmole DNP-arginine per g resin in 0.1 *N* HCl.

Recovery

All DNP-derivatives investigated (except dinitrophenylhydrazine, see below) are eluted quantitatively (> 97 %) with acetone–water (80:20). Occasionally, however, traces of previously adsorbed samples can be detected in washings following desorption (memory effect). Therefore, storage of the resin in acetone–water and washing of the resin prior to use is recommended.

Dinitrophenylhydrazine, applied in acid solution, binds irreversibly to some extent. It reacts with keto groups which are present in Porapak Q and which can be demonstrated also by IR-spectroscopy. To avoid this irreversible binding, the keto groups of the polymer can be reduced to the alcohol by sodium borohydride in methanol or to the hydrocarbon by hydrazine hydrate–sodium ethylate in ethylene glycol at 190°. Amberlite XAD-2 is free of keto groups.

Cleaning of the resin

A resin contaminated by impurities, *e.g.* from a urine hydrolysate, is purified by washing the column with the strong detergent RBS-25* followed by washings with water and ethanol. This treatment does not change the adsorption properties of the resin. However, contamination by a denaturated protein may not be removed in all cases. For instance, cytochrome C, equilibrated with Porapak Q, could not be removed completely. As mentioned above, also dinitrophenylhydrazine, applied in 0.1 *N* HCl, binds irreversibly to Porapak Q.

APPLICATIONS

Adsorption of DNP-derivatives from acid hydrolysate

In a total hydrolysate of DNP-peptide or DNP-protein, free amino acids and DNP-amino acids usually are subjected to different methods of analysis. Therefore, separation of DNP-amino acids from the free amino acids is advantageous, especially if only low amounts are available. The following procedure may be used, scaled down for small amounts, if necessary. 2 ml hydrolysate containing up to 70 μmole DNP-amino acid in 6 *N* HCl are diluted tenfold with distilled water and slowly filtered through a small column filled with 300 mg Porapak Q, equilibrated with 0.1 *N* HCl. The resin is washed with 15 ml of 0.1 *N* HCl (or 5 % acetic acid). The combined eluates (fraction 1) containing the free amino acids are evaporated in vacuo. The DNP-derivatives adsorbed on Porapak Q are then eluted with 10 ml of acetone–water (80:20) and evaporated to dryness in a stream of nitrogen at 50° (fraction 2). As shown in Table II, all amino acids are found quantitatively in fraction 1. The only exception is phenylalanine**, which is recovered to 80 % in fraction 1, the other 20 % being eluted

* Available from Carl Roth, OHG, Karlsruhe, G.F.R.

** Tryptophan would also be adsorbed[14], but is destroyed in the foregoing acid hydrolysis.

TABLE II

ADSORPTION OF DNP-DERIVATIVES FROM ACID HYDROLYSATES

Percentage recovery of free amino acids after filtration through Porapak Q in 0.6 *N* HCl and washing with 0.1 *N* HCl (fraction 1) and 50% ethanol (fraction 2), respectively. Fraction 2 may contain DNP-derivatives. The test solution contained 2.5 μmole of each amino acid in 2 ml of 6 *N* HCl. Other details see text.

Amino acid	*Fraction 1*	*Fraction 2*
Asp	105	0
Thr	104	0
Ser	100	0
Pro	100	0
Glu	100	0
Gly	99	0
Ala	98	0
Val	95	0
$(Cys)_2$	101	0
Met	102	0
Ile	104	0
Leu	98	0
Tyr	95	0
Phe	80	17
Lys	105	0
His	99	0
Arg	98	0

in fraction 2 with the DNP-amino acids. It is interesting that phenylalanine is also strongly adsorbed on the basic ion-exchanger Zerolit H (containing a polystyrene matrix) under conditions used for separation of free amino acids and DNP-amino acids[13]. The above observation (Table II) is a direct indication that the adsorption of phenylalanine on such ion-exchangers is caused also by the matrix itself, *i.e.* by polystyrene.

Decoloration of urine hydrolysates

Decomposition products formed during acid hydrolysis of urine may interfere with assays of polar constituents based on colorimetry. The hydrolysate is easily decolorized by filtering through a small column of Porapak Q, pretreated with 0.1 *N* HCl. The column is washed afterwards with 0.1 *N* HCl or 5 % acetic acid. Aromatic compounds may be retained partially. In this case, the elution procedure described above (adsorption of DNP-derivatives from acid hydrolysates) is recommended. In any case it will be necessary to check and correct for losses and dilution in the standardized procedure. The resin may be cleaned with RBS-25, as described above.

Adsorption of DNP-amino acids and DNP-peptides after dinitrophenylation

As indicated in Fig. 1, DNP-derivatives can be adsorbed in alkaline medium on Porapak Q in a solvent of high salt concentration. Hence, it is possible to avoid the time-consuming extraction of DNP-derivatives after dinitrophenylation and to get one single fraction. In general, the conventional separation of "ether-soluble" and "acid-soluble" DNP-derivatives is not advisable because di-DNP-histidine and many DNP-peptides are spread over both fractions.

Adsorption from alkaline solution. To the reaction mixture, containing up to 100 μmole of DNP-derivatives and less than 10 % ethanol, 1 volume of 2 *M* NaCl is added. The mixture is filtered slowly through 1 g Porapak Q in a column, prewashed with 1 *M* NaCl. Then the resin is washed successively with 5 ml of 1 *M* NaCl, 2 × 5 ml of 1 *N* HCl, and 2 × 5 ml of 5 % acetic acid. The first 5-ml portion of the 1 *N* HCl is used previously to rinse the reaction vessel and then is transferred to the column. The DNP-derivatives are eluted with 10–20 ml of acetone–water (80:20). Again, small portions of this solvent are employed previously for rinsing the reaction vessel, and these washings are transferred quantitatively to the column.

Adsorption from acid solution. If the dinitrophenylation has been carried out with a large excess of fluorodinitrobenzene, it may be advantageous to remove first the excess reagent by ether extraction in alkaline medium. After removal of the remaining ether from the aqueous phase under *vacuo*, the solution is adjusted to pH $\leqslant$ 2 with conc. HCl and filtered through the Porapak Q column. Vessel and Porapak are washed with 2 × 5 ml of 5 % acetic acid. The vessel is then carefully rinsed with small portions of acetone–water (80:20), these solution are transferred to the column, and the DNP-derivatives are eluted with acetone–water (80:20), totalling about 10–20 ml.

Further applications

Porapak Q was also shown to adsorb easily dinitrophenylhexosamines, dinitrophenylhydrazones, estrogens, and dyes from aqueous solutions. It is to be expected that the simple adsorption on neutral polystyrene resin may replace the liquid–liquid extraction of still other substances bearing a large nonpolar group.

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CHROM. 5063

THE USE OF A BACTERIAL L-AMINO ACID DECARBOXYLASE FOR THE CONTROL OF THE DEGREE OF RACEMIZATION OF AMINO ACID MIXTURES OBTAINED FROM PROTEIN HYDROLYSATES

ANGELO MONDINO, GIANNI BONGIOVANNI, LUCIA ROSSI AND CARLO VILLA
Istituto di Ricerche Antoine Marxer S.p.A., Ivrea, 10015 (Italy)
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SUMMARY

A method for the simultaneous determination of D-forms of glutamic acid, lysine and arginine in protein amino acid mixtures is described. It is based upon the use of the acetone powder of an *E. coli* strain (containing L-amino acid decarboxylases which are active on these three amino acids at the same time), in conjunction with the ion-exchange amino acid chromatography. Since it has been observed that in the acid hydrolysis of proteins for the industrial preparation of amino acid mixtures, when glutamic acid, lysine and arginine are not undergoing racemization, all the other amino acids are also free of D-isomers, the authors propose that this method can be adopted for quality control of this process.

INTRODUCTION

New methods for the production of amino acid mixtures on an industrial scale, starting from acid hydrolysates of proteins of different sources, by means of a high yield ion-exchange procedure, have been recently achieved[1]. These amino acid mixtures, according to their composition (which is a function of the amino acid profile of the starting protein) may find wide application in the pharmaceutical field as well as in the nutrition of man and aninals. Moreover, this procedure, which can be applied to both homogeneous proteins and to proteinaceous materials also containing other constituents, can be considered as a valid means for better utilization of protein wastes.

The first step of the process is acid hydrolysis of the protein. According to the nature of the starting material, the concentration of sulfuric acid can be 3–9 *N*, whereas the protein in the hydrolysis mixture can reach the concentration of 25 % (p/v). The hydrolysis, which can be carried on either at atmospheric pressure or under high pressure, must obviously proceed as far as possible in order to get the highest possible final yield of amino acids, avoiding, on the other hand, the formation of D-stereoisomers which do not have any biological value.

Consequently, to obtain optimal results by hydrolysis of a new protein and for quality control of the amino acid mixtures produced by a working plant, the avai-

lability of a rapid and easy analytical method for the determination of the possible degree of racemization is necessary.

In our laboratories it was repeatedly observed (for different proteins and proteinaceous materials) that, if the hydrolysis conditions were not able to cause an appreciable racemization of glutamic acid, lysine and arginine, D-forms of the remainder amino acids were also not detectable. For the determination of the possible D-isomer of aspartic acid, proline, alanine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine, the amino acid mixtures were submitted to the activity of D-amino acid oxidase obtained from pig kidney, according to the methods reported by BOULANGER AND OSTEUX[2], MUELLER[3] and DIXON AND KLEPPE[4–6]. For the determination of D-glutamic acid, D-lysine, D-histidine and D-arginine, which were badly oxidized by this enzyme, the amino acid mixtures were incubated with specific L-amino acid decarboxilases of bacterial origin, as reported by GALE[7]. In both cases before and after the enzymatic treatment, the amino acids of the mixtures were determined by ion-exchange chromatography, in order to evaluate the percentage of D-forms eventually present. When using the D-amino acid oxidase as a crude acetone powder (which is preferable to the purified enzyme for its better stability), the determination of L-amino acids, present in considerable amount in the crude preparation, was to be made.

At this point it has to be mentioned that in a recent paper in which a high sensitivity method for the determination of D-amino and L-amino acids as DL- and LL-dipeptides is described, MANNING AND MOORE[8] report that in the acid hydrolysis of ribonuclease (even if carried on in a very different way and for other purposes), glutamic acid forms the D-isomer in a higher percentage (4.2 %) than threonine and serine (0.2 %). They report also that in the acid hydrolysis of synthetic bradykinin, the serine residue racemizes at a lesser extent (0.45 %) than arginine (1.7 %). Contrarily, as NEUBERGER[9] reported in 1948, it was retained that these hydroxyamino acids, owing to the hydroxyl group in β-position, were particularly prone to racemization under acidic treatment.

On the basis of these experimental data and particularly after our observations which were previously mentioned, it could be assumed that the control of the acid hydrolysis conditions for the industrial manufacturing of the amino acid mixtures is feasible by following merely the racemization degree of glutamic acid, lysine and arginine.

Since in our laboratories a strain of *E. coli* particularly rich in many L-amino acid decarboxylases has been isolated, it was possible to set up a simple and rapid method, which in conjunction with the amino acid column chromatography, allows the simultaneous determination of these three amino acids.

In this paper the preparation of the enzyme as well as the preparation of the substrate and the analytical procedure are reported. The precision of the method and the degree of sensitivity are also discussed.

MATERIALS

A strain of *E. coli* isolated in our laboratory was employed. The stock culture of the microorganism is carried on DIFCO Bacto Nutrient Agar slants. In order to obtain the inoculum for the final culture, the organism is inoculated into a few

millilitres of DIFCO Bacto Nutrient Broth and incubated for 20–24 h at 37°. The culture medium consists of 3 % DIFCO Bacto Casitone and 3 % glucose.

L-Glutamic acid, L-lysine·HCl, L-arginine, D-glutamic acid, D-lysine·HCl, D-arginine·HCl, DL-glutamic acid, DL-lysine and DL-arginine·HCl were employed. All these amino acids were purchased from Fluka.

Phosphate–citrate buffer (pH 5.2) was prepared by adding 46.6 ml of a 0.1 *M* solution of citric acid (19.2 g of citric acid per 1000 ml) to 53.6 ml of a 0.2 *M* solution of sodium orthophosphate mono acid (35.6 g of $Na_2HPO_4 \cdot 2H_2O$ per 1000 ml).

The following amino acid solutions in buffer (pH 5.2) were employed: 1 μmole of L-glutamic acid per ml; 1 μmole of L-lysine per ml; 1 μmole of L-arginine per ml; a mixture of 1 μmole of L-glutamic acid, 1 μmole of L-lysine·HCl and 1 μmole of L-arginine per ml; a mixture of 1 μmole of D-glutamic acid, 1 μmole of D-lysine·HCl and 1 μmole of D-arginine·HCl per ml; a mixture of 1 μmole of DL-glutamic acid, 1 μmole of DL-lysine and 1 μmole of DL-arginine·HCl per ml.

A solution containing 6 % sulfosalicylic acid was employed as deproteinating agent. An amino acid analyzer "Aminolyzer", manufactured by Optica Co., Milan, was used.

METHODS

1000 ml of sterile culture medium are inoculated with *E. coli* and incubated in a thermostat at 37° for 24 h. Then the cell suspension is distributed into 250-ml centrifuge bottles and centrifuged for 15 min at 4000 r.p.m.; the supernatant liquid is decanted and all the sediments, resuspended in water, are gathered into one centrifuge bottle and centrifuged down again. The cells are then suspended with 5 ml of water, then 25 ml of acetone are added at room temperature to this suspension, which is filtered under very moderate vacuum, employing a rapid-flow filter paper. Just before the precipitate is dry on the filter, it is washed with 100 ml of acetone, followed by 100 ml of ether. When all the ether is evaporated, the powder is recovered from the filter and put into a desiccator over phosphoric anhydride. Under these conditions, its activity lasts more than 2 months. From 1 l of culture, the powder yield is about 400 mg.

An amount of this powder is suspended in 1 ml of phosphate–citrate buffer. 1 ml of the amino acid solution in the same buffer is added and then the mixture is agitated for 40 min at room temperature. The reaction mixture is now centrifuged for 15 min at 4000 r.p.m.; 1 ml of the supernatant fluid is transferred into another centrifuge tube and 1 ml of the 6 % sulfosalicylic acid solution is added. After a few seconds of shaking by hand, the tube is centrifuged at 10000 r.p.m. for 10 min. 0.4 ml of the supernatant solution may then be employed for the analysis of the amino acids on each column, by using the analytical system described by MONDINO in a previous paper[10]. In order to eliminate completely the overlapping of lysine and histidine peaks, the temperature of the short column for the separation of the basic amino acids has been lowered to 48°.

Since in our analytical system, as reported previously[10–14], the peak evaluation is feasible by simply measuring the height of the peaks over the baseline in mm, from the ratio between the height of the peaks of the treated sample chromatogram and the height of the peaks of the chromatogram obtained for the untreated amino acid solution, the percentage of D-isomer possibly formed can be evaluated.

RESULTS AND DISCUSSION

Since it can be presumed that the L-glutamic acid, L-lysine and L-arginine decarboxilases are not present in the powder of our *E. coli* strain all at the same concentration level and that the optimum pH is not the same for the three enzymes, it has been necessary to check the enzymatic activity of the powder on the three amino acids separately at the same pH, with the same incubation time. According to the methodology previously described, the solutions containing 1 μmole of the single L-amino acids (L-glutamic acid, L-lysine and L-arginine) have been tested by addition of different amounts of powder. In this way the least amounts of powder necessary for obtaining the complete decarboxylation of 1 μmole of amino acid have been found. They are as follows. For the L-glutamic acid the least quantity is between 45 and 50 mg, depending upon the preparation. For the L-lysine it is between 35 and 40 mg and for the L-arginine it is less than 10 mg. In Fig. 1, the titration curves are reported. The complete decarboxylation of the solution containing the mixture of L-glutamic acid, L-lysine and L-arginine at the concentration level of 1 μmole/ml of each amino acid, was consequently achieved by employing 50 mg of acetone powder for a 40-min incubation time. The amino acid chromatogram obtained for this solution after incubation was in fact totally lacking glutamic acid, lysine and arginine. When these three amino acids are to be checked in the reciprocal presence, at different concentration levels, the amount of powder has to be chosen taking into consideration the curves reported in Fig. 1.

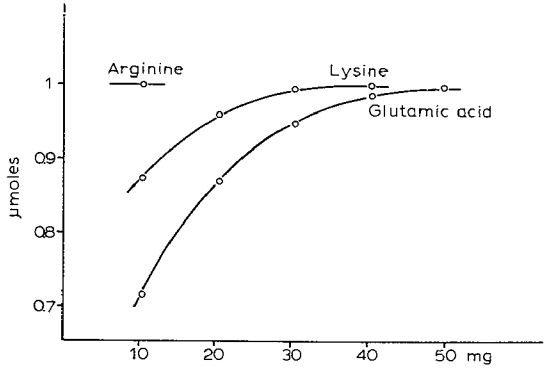

Fig. 1. Titration curves of the enzymatic activity of the three decarboxylases present in the acetone powder. On the abscissae the mg of the crude acetone powder employed are reported.

In order to check the total enzymatic inactivity of the amino acid decarboxylases produced by our strain of *E. coli* toward the D-isomers, the solutions containing 1 μmole/ml of the single D-amino acids (D-glutamic acid, D-lysine and D-arginine) have been tested according to the methodology described above. The results of this experiment, repeated three times, confirmed that these amino decarboxylases are active only toward the L-forms. Having obtained a very strict coincidence in the height of the peaks both in the chromatograms of the samples which were treated with the enzyme, incubated and deproteinated and in the chromatograms of the blanks to which only a sulfosalicylic acid solution was added, it can be stated that during the de-

proteination process no loss of glutamic acid, lysine and arginine takes place for adsorption or other causes.

In Table I are reported the results of an experiment, which was performed in order to check the degree of precision of the determination of D-isomers. The solution containing a mixture of DL-glutamic acid, DL-lysine and DL-arginine at the concentration level of 1 μmole/ml of buffer (pH 5.2) has been incubated with the acetone powder according to the method previously described. The experiment was repeated 6 times, and every time a chromatogram was run. The peak heights of the remaining D-forms of glutamic acid, lysine and arginine are reported and their values, statistically evaluated, confirm a precision degree better than $\pm 1.6\,\%$.

TABLE I

RESULTS OF THE CHROMATOGRAMS OF A MIXTURE OF DL-ISOMERS AFTER TREATMENT WITH THE *E. coli* ACETONE POWDER

The peak heights of the remaining D-isomers are expressed in mm.

Chromatogram	*1*	*2*	*3*	*4*	*5*	*6*	*S.D.*	*C.V.*
D-Glutamic acid	57.5	56.0	55.0	56.5	56.0	57.0	±0.87	±1.55
D-Lysine	99.5	97.5	97.0	99.0	97.7	99.5	±1.21	±1.23
D-Arginine	33.0	33.0	33.5	34.5	33.0	34.0	±0.49	±1.47

As far as the sensitivity is concerned, it can be said that it is strictly depending upon the degree of sensitivity offered by the method of amino acid chromatography employed. Moreover, a way of enhancing the analytical sensitivity for these three amino acids (which in this case are usually present in a very small amount), consists of charging a larger sample on the chromatographic column, even with the chance of having all the other amino acid peaks off scale. Consequently, a method of amino acid analysis has to be adopted in which the peaks of glutamic acid, lysine and arginine are well separated down to the baseline and do not overlap at all with the neighboring peaks. This occurs obviously when a complete mixture of amino acids is submitted to the action of the powder. As a matter of fact the overloading on the column of all the other amino acids, which are not decarboxylated, will cause a broadening of their peaks that can interfere with the small peaks corresponding to the D-forms, if any, of glutamic acid, lysine and arginine. Operating in this way and with the help of the sensitivity range expander which can be found on our apparatus[10], two parts of D-glutamic acid and two parts of D-lysine can be detected in the presence of 1000 parts of the L-isomers; concerning D-arginine the sensitivity is somewhat lower, owing to the form of this peak.

For readers desiring to try the method described, agar slants of the *E. coli* employed can be obtained from the authors.

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CHROM. 5097

SÉPARATION DES OLIGOPEPTIDES ET DES ACIDES AMINÉS PAR CHROMATOGRAPHIE SUR RÉSINE ÉCHANGEUSE D'IONS, CHELEX X-100

JACQUES BOISSEAU ET PIERRE JOUAN

Laboratoire de Neurobiologie Moléculaire, Équipe associée au C.N.R.S. No. 119, Faculté des Sciences, Rennes (France)

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SUMMARY

Separation of oligopeptides and amino acids by chromatography on the ion-exchange resin, Chelex X-100.

An improved method is described for the separation of peptides and α-amino acids using the Chelex X-100 exchanger in the Cu^{2+} form. After equilibration of the resin with ammonia solution, pH 10.3, acidic, and neutral peptides, and acidic amino acids are eluted with water. The neutral amino acids and basic peptides are eluted with 1.5 *M* ammonium hydroxide. Tryptophan, histidine and arginine emerge with 6 *M* ammonium hydroxide. All fractions are obtained free of Cu^{2+}.

INTRODUCTION

La séparation des peptides de faible poids moléculaire des acides aminés reste un problème difficile à résoudre. L'isolement d'un peptide à partir d'un milieu biologique complexe, en vue de la détermination de sa structure primaire, par exemple, nécessite la supression de toute contamination par des acides aminés libres. Cette purification est rendue malaisée car les oligopeptides et les acides aminés présentent des caractères voisins de solubilité, de poids moléculaire et de charge électrique.

La propriété qu'ont les acides aminés et les peptides de former des chélates avec des ions métalliques a permis l'élaboration de diverses techniques. FAZAKERLEY ET BEST[1] ont obtenu, les premiers, une telle séparation à partir d'une colonne de gel de Séphadex G-25 chargé d'ions cuivriques et équilibré à pH 11 par un tampon salin. Les peptides ne sont pas retenus sur cette colonne à l'inverse des acides aminés qui sont élués par une solution d'acide chlorhydrique 0.1 *N*. Après cette chromatographie, les corps sont obtenus sous forme de complexes cuivriques ce qui nécessite l'élimination du cuivre pour leur étude ultérieure. Cette méthode est très limitée par la faible capacité de fixation du gel et par l'utilisation du tampon salin. TOMMEL *et al.*[2,3] séparent les peptides et les acides aminés sur des colonnes de DEAE et de TEAE-cellulose à

l'aide de tampons volatiles. Les composés sont complexés au préalable par le cuivre. Les acides aminés, non retenus sur ces colonnes, sont entrainés par une solution tampon d'acétate de collidine à pH 8 et les peptides sont élués par des solutions d'acidité croissante. Les produits doivent finalement être débarrassés du cuivre complexant. Au laboratoire, une technique a été mise au point qui permet une bonne séparation des dipeptides neutres des acides aminés, grâce à une colonne de DEAE-Séphadex équilibrée à pH 8.5 par une solution tampon d'acétate de collidine. L'élution est réalisée par des solutions d'acidité croissante.

BUIST ET O'BRIEN[4] ont repris la technique de FAZAKERLEY ET BEST en remplaçant le Séphadex par une résine Chelex X-100. Cette résine est un copolymère styrène–divinylbenzène muni de groupements fonctionnels iminodiacétates. Elle présente vis à vis des ions métalliques de transition divalents une affinité supérieure à celle des échangeurs monofonctionnels. Fixé sur la résine, l'ion métallique garde la possibilité de contracter des liaisons de coordination avec des composés qui seront déplacés ultérieurement par un autre coordinant. Cette résine Chelex X-100, saturée par du cuivre, a déjà été utilisée par plusieurs auteurs, pour la fixation sélective des acides aminés à partir de l'eau de mer[5] et pour le fractionnement de composés nucléiques[6]. La technique de séparation des oligopeptides et des acides aminés par une telle résine est basée sur les propriétés complexantes du cuivre vis à vis de ces deux groupes de composés et sur les différences de stabilité et de charge électrique existant entre les complexes cuivriques des peptides, anioniques, et des acides aminés, cationiques ou neutres.

Nous avons essayé d'améliorer ces méthodes pour rendre cette technique préparative plus rentable. Nous avons utilisé des éluants aqueux non salins et volatiles et une colonne de résine Chelex X-100 qui, en plus de sa forte capacité, permet d'obtenir des acides aminés et des peptides libres de toute trace de cuivre complexant.

MATÉRIELS ET MÉTHODES

Préparation de la résine

Cinquante grammes de résine Chelex X-100 (200–400 mesh) (BioRad, Richmond, Calif., U.S.A.) sont complexés au cours d'une agitation magnétique de 15 h dans 250 ml de $CuCl_2$ 1 *M*. La résine est lavée sur filtre de verre fritté par 20 à 30 l d'eau distillée. On vérifie que le filtrat ne contient plus la moindre trace de cuivre par l'addition de diéthyldithiocarbamate de sodium en solution méthanolique (3.5 g pour 100 ml). Tout le cuivre libre étant éliminé, la résine est agitée pendant 15 h dans 500 ml d'ammoniaque 3 *M*. Elle est à nouveau lavée sur filtre de verre fritté avec de l'eau distillée jusqu'à ce que le pH du filtrat atteigne une valeur de 10.3 ± 0.1.

Remplissage de la colonne

Avant de remplir la colonne à chromatographie (hauteur: 27 cm; diamètre: 1.5 cm) avec de la résine ainsi complexée, on introduit de la résine non complexée, uniquement traitée par de l'ammoniaque 3 *M*. On obtient, au bas de la colonne, un dépot de 1 cm de hauteur capable de fixer le cuivre éventuellement déplacé de la résine complexée par les divers éluants. La colonne est ensuite remplie par de la résine complexée. Le débit de cette colonne est de 20 ml par heure.

Conduite de la chromatographie

Lorsque les solutions renfermant les constituants à séparer ont pénétré dans la résine, l'élution est réalisée successivement par 450 ml d'eau distillée, 450 ml d'ammoniaque 1.5 *M* (pH 11.8) et 350 ml d'ammoniaque 6 *M* (pH 12.5). Les éluats sont recueillis par fractions de 10 ml. Les substances présentes dans les différents éluats sont repérés en lumière ultraviolette à 280 mμ et à 230 mμ en l'absence de toute absorption spécifique. Les fractions obtenues sont lyophilisées et stockées au congélateur avant l'analyse de leurs contenus. On peut régénérer la résine en faisant passer de l'eau distillée sur la colonne jusqu'à ce que le pH de l'éluat présente une valeur de 10.3 ± 0.1. Il est toutefois plus rapide de retirer la résine de la colonne, de l'équilibrer à pH 10.3 en la lavant par de l'eau distillée sur filtre de verre fritté. Cette résine ainsi traîtée est utilisable plusieurs mois.

Composés étudiés

On a déposé dans 5 ml d'eau 5 μmoles des corps suivants: les 18 acides aminés naturels, la cystine, la 4-hydroxyproline et divers peptides (glycyl–alanine, glycyl–tyrosine, glycyl–tryptophanne, glycyl–histidine, glycyl–lysine, carnosine, glutathion, prolyl–glycyl–phénylalanine).

Comme application de cette méthode à un milieu biologique, nous avons utilisé un extrait de glandes pinéales (Epiphyses) de mouton, préparé selon une technique décrite précédemment[7,8]. Une poudre lyophilisée d'épiphyses deshydratées par l'acétone est extraite par percolation par du butanol contenant successivement 5 % puis 10 % HCl 0.1 *N*. Après purification et délipidation de l'extrait, les composés de faible poids moléculaire sont séparés par filtration sur gel de Séphadex G-25 équilibré dans une solution tampon d'acétate de pyridine 0.05 *M* à pH 5. Cette fraction, lyophilisée, est reprise par de l'eau distillée et déposée sur la colonne de résine Chelex X-100.

Analyse des fractions séparées

Les composés contenus dans les diverses fractions sont identifiées par plusieurs systèmes de chromatographie et d'électrophorèse sur papier: électrophorèse à pH 3.9 dans le système pyridine–acide acétique–eau (30:100:4870) à 400 V pendant 4 h sur papier Whatman No. 1[9]; électrophorèse à pH 1.8 dans de l'acide acétique 5 *M*, à 400 V pendant 10 h, sur papier Whatman No. 1; électrophorèse à pH 11.7 dans de l'ammoniaque 1 *M* à 400 V pendant 15 h sur papier Whatman No. 1[9]; chromatographie descendante dans le système butanol–acide acétique–eau (4:1:5) à 30° pendant 10 h sur papier Whatman No. 1[10].

Après leur migration, les acides aminés et les peptides sont colorés par une solution butanolique acidifiée de ninhydrine à 0.25 g pour 100 ml[11]. La proline est révélée par une solution butanolique acide d'isatine à 0.2 g pour 100 ml[12], la tyrosine par une solution alcoolique d'α-nitroso-β-naphtol à 0.1 g pour 100 ml[13], le tryptophanne par de la *p*-diméthylaminocinnamaldéhyde à 2 g pour 100 ml dans un mélange à parties égales d'éthanol et d'acide chlorhydrique 6 *N*[14].

Méthodes de dosage quantitatif

Le taux de récupération des acides aminés élués est déterminé par le dosage à la ninhydrine[11], celui des peptides par la méthode de O. LOWRY *et al.*[15].

RÉSULTATS

Séparation des composés standard

L'élution par l'eau permet d'obtenir deux fractions: la première présente un maximum d'absorption à 230 mμ au 35^e ml et elle contient les acides aspartique et glutamique, le glutathion, la glycyl–alanine, la glycyl–tyrosine et la prolyl–glycyl–phénylalanine (Fig. 1). La deuxième fraction, dont l'absorption à 230 mμ est maximum au 125^e ml, renferme le glycyl–tryptophanne (Fig. 1).

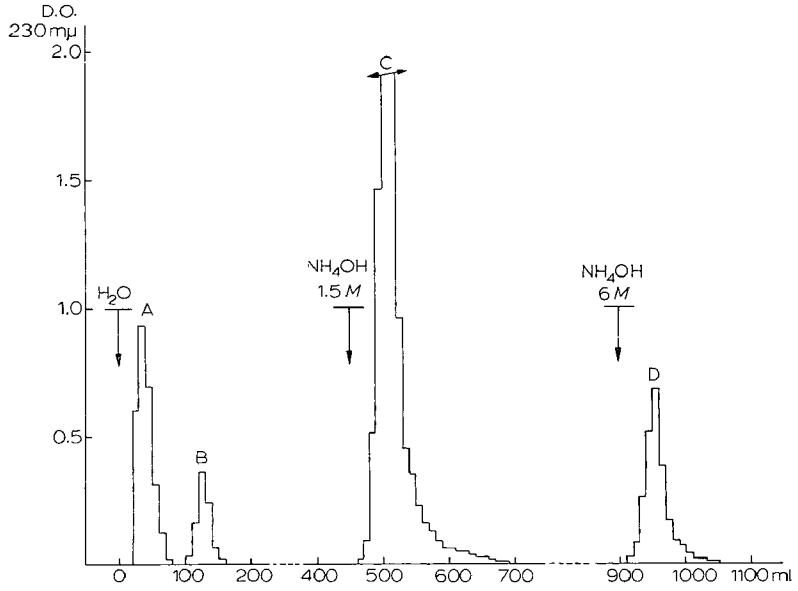

Fig. 1. Séparation de 20 acides aminés naturels et de 9 oligopeptides sur résine Chelex X-100, complexée par des ions cuivriques et équilibrée à pH 10.3. Colonne de 27 cm × 1.5 cm. Fraction A: élution des acides aspartique et glutamique, des peptides: glutathion, glycyl–alanine, glycyl–tyrosine, prolyl–glycyl–phénylalanine. Fraction B: élution du glycyl–tryptophanne. Fraction C: élution des acides aminés neutres (sauf le tryptophanne), de la lysine et des peptides: glycyl–histidine, glycyl–lysine, carnosine.

L'élution par l'ammoniaque 1.5 *M* entraîne une fraction dont le maximum d'absorption à 230 mμ se situe au 510^e ml. Elle contient tous les acides aminés neutres sauf le tryptophanne, la lysine et les peptides basiques tels la carnosine, la glycyl–histidine, la glycyl–lysine (Fig. 1).

L'élution par l'ammoniaque 6 *M* permet d'obtenir une fraction dont le maximum d'absorption à 230 mμ se situe au 995^e ml. Elle contient le tryptophanne, l'histidine et l'arginine (Fig. 1).

Le pourcentage de récupération a été déterminé avec un constituant de chaque éluat. Il est de 99.6 % pour le glycyl–tryptophanne (éluat aqueux), de 100 % pour la tyrosine (éluat NH_4OH 1.5 *M*), de 98.5 % pour le tryptophanne (éluat NH_4OH 6 *M*).

Application de la technique au fractionnement d'un extrait épiphysaire

L'éluat aqueux entraîne trois fractions dont les maxima d'absorption à 230 mμ

se situent respectivement aux 40e, 245e et 375e ml. Trois autres sont éluées par l'ammoniaque 1.5 *M*. Leurs maxima d'absorption se situent aux 510e, 625e et 770e ml. La dernière élution par l'ammoniaque 6 *M* entraîne deux fractions dont les maxima d'absorption se situent aux 935e et 975e ml (Fig. 2).

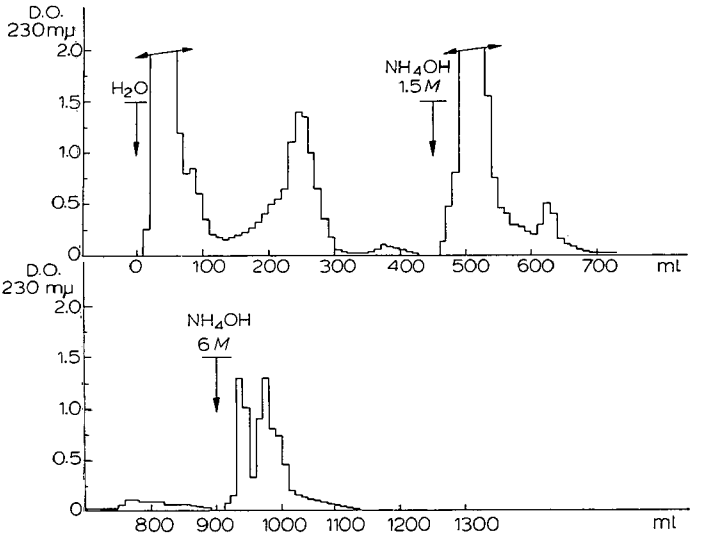

Fig. 2. Fractionnement d'un extrait de glandes pinéales sur une colonne (27 cm × 1.5 cm) de résine Chelex X-100 complexée par des ions cuivriques et équilibrée à pH 10.3.

Le pourcentage de récupération des composés fractionnés est du même ordre que pour les corps témoins. Pour un extrait de glandes pinéales de moutons dont le matériel ninhydrine et LOWRY positif a été dosé, les résultats, exprimés respectivement en mg de leucine et en mg de tyrosine sont indiqués dans le Tableau I.

TABLEAU I

POURCENTAGE DE RÉCUPÉRATION DU MATÉRIEL NINHYDRINE (EXPRIMÉ EN mg DE LEUCINE) ET LOWRY POSITIF (EXPRIMÉ EN mg DE TYROSINE) D'UN EXTRAIT DE GLANDES PINÉALES DE MOUTON

	Leucine (*mg*)	*Tyrosine* (*mg*)
Quantité déposée	96.4	9.77
Eluat H_2O	12.54	3.39
Eluat NH_4OH 1.5 *M*	85.60	5.77
Eluat NH_4OH 6 *M*	1.56	1.12
Total récupéré	99.70	10.28
Pourcentage de récupération	103.4%	105%

Des résultats supplémentaires peuvent être obtenus avec cette technique

Il suffit d'équilibrer la colonne de résine à un pH moins basique, 8.5 par exemple. On peut, dans ce cas, améliorer sensiblement la séparation des corps élués par l'eau

(Fig. 3). Les trois peptides, glutathion, glycyl–alanine et glycyl–tyrosine, sont séparés convenablement alors qu'ils étaient élués ensemble lorsque la résine était équilibrée à pH 10.3.

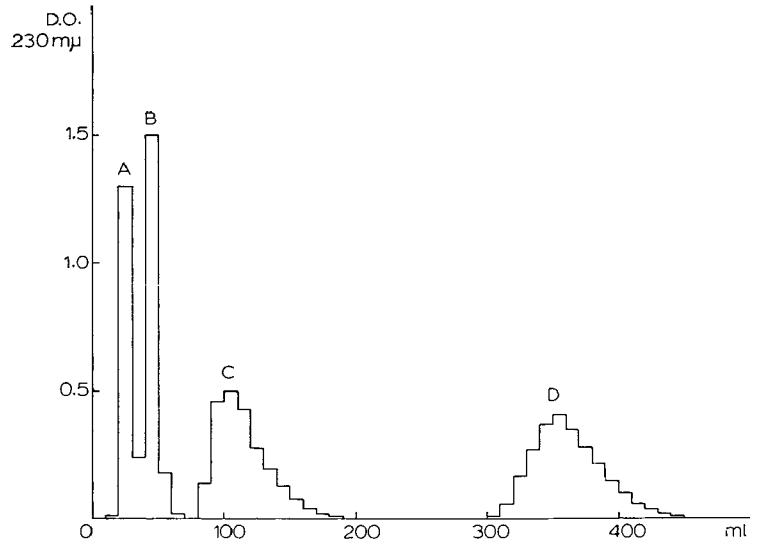

Fig. 3. Séparation des peptides, contenus dans l'éluat aqueux, sur résine Chelex X-100 complexée par des ions cuivriques et équilibrée à pH 8.5. Fraction A: élution du glutathion. Fraction B: élution de la glycyl–alanine. Fraction C: élution de la glycyl–tyrosine. Fraction D: élution du glycyl–tryptophanne.

DISCUSSION

Les analyses réalisées à partir des composés témoins montrent que les peptides acides et neutres, élués par l'eau, sont séparés des acides aminés neutres et basiques, retenus sur la colonne. Ces peptides peuvent être, en plus l'objet d'un fractionnement si la résine complexée est équilibrée à pH 8.5 au lieu de pH 10.3.

Nous n'avons pas pu obtenir une séparation complète entre les acides aminés et les peptides car les acides aminés acides sont entraînés avec les peptides acides et neutres alors que les peptides basiques sont élués par l'ammoniaque 1.5 *M* avec l'ensemble des acides aminés neutres. Ces inconvénients ont déjà été signalés dans les travaux précédents[2,3,4]. Cette séparation peut être achevée ultérieurement par des techniques électrophorétiques simples: à pH 3.9, par exemple, on sépare les acides aminés acides des peptides neutres d'une part, les peptides basiques des acides aminés neutres d'autre part. L'analyse de l'extrait de glandes pinéales de mouton a permis de détecter, dans l'éluat aqueux, quatorze peptides dont l'étude structurale est en cours.

Par rapport aux travaux antérieurs[5,6], cette méthode présente les améliorations suivantes qui la rendent d'un emploi très facile: à la différence du Séphadex cuivrique[1], la résine Chelex complexée permet, grâce à sa capacité élevée, de traîter des quantités importantes d'extrait. Cet intérêt n'est pas négligeable si on considère la

grande disproportion habituelle entre les quantités de peptides et d'acides aminés présentes dans un milieu biologique.

L'isolement des peptides est une étape qui précède généralement une étude de structure. Il ne faut donc pas risquer de dénaturer ces composés par des conditions de travail trop drastiques. Les peptides acides et neutres sont obtenus à un pH inférieur à 10.3, dans un éluant de très faible force ionique, préférable à une solution de pH 11, presque saturée en tétraborate de sodium[1,4].

Notre analyse est assez rapide car, si elle dure en tout 48 h, les peptides acides et neutres sont obtenus au bout d'une vingtaine d'heures.

Pour simplifier au maximum cette technique préparative, nous avons préféré à des tampons salins, de forte concentration[1,4], des éluants faciles à éliminer comme l'eau et l'ammoniaque 1.5 *M* et 6 *M*.

L'utilisation d'une résine chargée en cuivre dispense de complexer au préalable les composés à séparer par agitation dans une solution cuivrique[2,3]. Par ailleurs, les produits récupérés dans les éluats sont libres de toute trace de cuivre complexant, ce qui évite les manipulations longues et délicates pour son élimination[1–4].

Enfin la résine Chelex complexée, facilement régénérée pour une nouvelle analyse, est utilisable plusieurs mois.

RESUMÉ

A l'aide d'une résine échangeuse de cations Chelex X-100, complexée par du cuivre, il est possible de séparer des acides aminés la plupart des peptides de faible poids moléculaire.

Cette résine est équilibrée à pH 10.3 par de l'ammoniaque. Les peptides acides et neutres sont élués par de l'eau, les acides aminés neutres et basiques par des solutions d'ammoniaque.

Toutes ces fractions sont obtenues exemptes de cuivre. Les peptides acides et neutres sont contaminés par les acides aminés acides et les peptides basiques par les acides aminés neutres. Des séparations électrophorétiques simples permettent de remédier à ces inconvénients.

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CHROM. 5049

SOME ASPECTS OF FRACTIONATION OF DNA ON AN IR-120 Al^{3+} COLUMN

III. EFFECT OF THE METHOD OF DEPROTEINISATION ON THE CHROMATOGRAPHIC PROFILES OF DNA

R. M. KOTHARI
Department of Zoology, University of Poona, Poona-7 (India)
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SUMMARY

The effect of the method of deproteinisation on the chromatographic behaviour of DNA using an IR-120 Al^{3+} column is being studied. For this purpose, DNA's deproteinised by four different methods were chromatographed and their profiles were studied. Although the method of deproteinisation did not seem to influence significantly the DNA fractionation, retention itself was affected. DNA isolated by the chloroform–amyl alcohol method and that by the guanidine hydrochloride method were completely retainable without any treatment. DNA isolated by the phenol–fluoride method was not retainable but became significantly retainable only after Mg^{2+} equilibration under finite conditions, indicating an important role of Mg^{2+} for retention. Similar behaviour was also shown by DNA isolated by the SDS method. After retention was achieved, the elution profiles of DNA, isolated by four different methods, were similar with a little variation in percent elution in different fractions.

INTRODUCTION

It is reported[1,2] that the chromatographic, physical, or chemical properties of DNA isolated by different methods from a given tissue of a given source may not be identical. KIT[3] studied the chromatographic profiles of DNA isolated from the same source and the same tissue but by different methods and found that the profiles are quite similar. It was, therefore, thought worthwhile to study if the method of isolation and deproteinisation (methodology) has any effect on the chromatographic profiles of DNA, using an IR-120 Al^{3+} column.

EXPERIMENTAL AND RESULTS

IR-120 Al^{3+} column

10 g of dry regenerated Amberlite IR-120 (Na^+ form), polystyrene sulphonate, equilibrated with 0.2 *M* aluminium chloride solution gave the IR-120 Al^{3+} column[4,5]. Glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) was percolated through the

column till the pH of the influent and effluent were the same. This column was then used for fractionating DNA's deproteinised by different methods[6–9].

Deoxyribonucleic acids

DNA samples were isolated from buffalo liver using the following methods:

(1) the chloroform–amyl alcohol method of SEVAG *et al.*[6];

(2) the method using 10 *M* guanidine hydrochloride of VOLKIN and CARTER[7];

(3) the 5 % sodium dodecyl sulphate (SDS) method of KAY *et al.*[8];

(4) the phenol–fluoride method of KIRBY[9].

DNA samples isolated by different deproteinising methods were native and fairly pure (90–95 %). They were associated with varying amounts (1–10 %) of residual protein and were devoid of RNA. The nativity and purity were examined as discussed earlier[4].

Procedure

DNA's, thus isolated, were dissolved in glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) to get homogeneous solutions. The solutions were loaded on different IR-120 Al^{3+} columns, the columns were washed with 3 bed volumes of the above buffer to strip off any loosely retained DNA, and wherever adsorption occurred, DNA's from the columns were eluted with different eluting agents in the usual sequence[4]. Fractions, each 25 ml, were collected and assayed for DNA content by BURTON's reaction[10]. (The details of the experimental procedure have been discussed earlier[4].) Percent retention of DNA and percent elution of the total adsorbed DNA are given in Table I.

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF DNA'S ISOLATED BY DIFFERENT METHODS OF DEPROTEINISATION FROM BUFFALO LIVER, ON AN IR-120 Al^{3+} COLUMN

DNA isolated by	*Percent retention*	*Percent elution*	*Profiles*
Chloroform–amyl alcohol	100	100	Typical seven fractions (F_1–F_7)
10 *M* guanidine hydrochloride	90	100	Typical seven fractions (F_1–F_7)
5% SDS	Nil	—	Typical seven fractions (F_1–F_7)
Phenol–fluoride	Nil	—	Typical seven fractions (F_1–F_7)

It is clear from Table I that DNA isolated by the methods of SEVAG *et al.*[6], of VOLKIN AND CARTER[7] are 100 and 90 % retainable, respectively, and, on elution, the typical 7 fractions (F_1–F_7) were obtained, the fractionation being based upon differences in base composition of the fractions eluted[4]. DNA's isolated by the method of KAY *et al.*[8] and by KIRBY[9] are not retainable.

The series of following experiments were performed to know if the residual SDS, or phenol and/or fluoride impurities in the nonretained DNA were responsible for nonretention on an IR-120 Al^{3+} column.

DNA isolated by the SDS method

DNA isolated by the SDS method was extensively dialysed against cold 0.14 *M*

saline and chromatographed on an IR-120 Al^{3+} column and was found to be non-retainable. The effluate DNA without and with Mg^{2+} equilibration (0.001 *M* Mg^{2+} at 4° for 24 h) was chromatographed on fresh IR-120 Al^{3+} columns and the chromatographic behaviour was studied.

DNA isolated by Kirby's method

DNA isolated by KIRBY's method was chromatographed on an IR-120 Al^{3+} column after extensive dialysis against cold 0.14 *M* saline.

DNA isolated by Sevag's method and treated with phenol

DNA isolated by SEVAG's method was treated with phenol in the absence of sodium fluoride and was chromatographed on an IR-120 Al^{3+} column. Another such sample, extensively washed with cold ether till the etherial supernatant gave a negative test for phenol, indicating a complete removal of phenol, was also chromatographed on the column.

DNA isolated by Sevag's method and treated with fluoride

DNA isolated by SEVAG's method was treated with 0.3 *M* sodium fluoride in the absence of phenol. It was extensively dialysed against glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) at 4° till the dialysed residue showed a negative test for fluoride, indicating a complete removal of fluoride ions. Dialysed DNA, thus obtained, was chromatographed on an IR-120 Al^{3+} column before and after equilibration with 0.001 *M* Mg^{2+} at 4° for 24 h.

DNA isolated by Sevag's method and simultaneously treated with phenol and fluoride

DNA isolated by SEVAG's method was simultaneously treated with phenol and 0.3 *M* sodium fluoride and freed from phenol as well as sodium fluoride by the treatments given above. The sample was then chromatographed on an IR-120 Al^{3+} column and the profiles were studied.

The percent retention of DNA and percent elution of the total adsorbed DNA (subjected to additional treatments) are given in Table II. Fig. 1 shows the percent

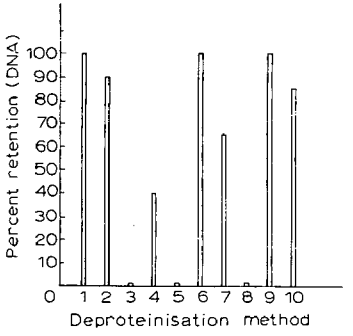

Fig. 1. Percent retention of buffalo liver DNA deproteinised by different methods on IR-120 Al^{3+} column. 1 = SEVAG's method; 2 = VOLKIN AND CARTER's method; 3 = KAY AND DOUNCE's method; 4 = KAY AND DOUNCE's method + Mg^{2+}; 5 = KIRBY's method; 6 = KIRBY's method + Mg^{2+}; 7 = SEVAG's method + phenol; 8 = SEVAG's method + NaF; 9 = SEVAG's method + NaF + Mg^{2+}; 10 = SEVAG's method + phenol + NaF.

TABLE II

RETAINABILITY OF DIFFERENTLY DEPROTEINISED BUFFALO LIVER DNA (SUBJECTED TO ADDITIONAL TREATMENTS) ON AN IR-120 Al^{3+} COLUMN

Deproteinisation method	*Additional treatment*	*Percent retention*	*Percent elution*	*Profiles*
SDS	Extensive dialysis	Nil	—	—
Effluate from above after passage through IR-120 Al^{3+}	Nil	Nil	—	—
Sample as above	0.001 *M* Mg^{2+}, 4°, 24 h	40	100	0.5 *M* saline and 1% EDTA elutable fractions only
KIRBY	Extensive dialysis	Nil	—	—
SEVAG	Phenol, without ether washings	70	100	0.5 *M* saline, 1% EDTA and water elutable fractions only
SEVAG	Phenol, with ether washings	66	100	0.5 *M* saline, 1% EDTA and water elutable fractions only
SEVAG	0.3 *M* NaF, extensive dialysis	Nil	—	—
SEVAG + 0.3 *M* NaF, extensive dialysis	0.001 *M* Mg^{2+}, 4°, 24 h	100	100	Typical seven fractions (F_1–F_7)
SEVAG	Phenol–0.3 *M* NaF, extensive dialysis	85–90	100	Typical seven fractions (F_1–F_7)

retention of DNA samples deproteinised by different methods with and without additional treatments (combined results of Tables I and II).

DISCUSSION

Method of deproteinisation and retainability

It has been noted that DNA isolated by SEVAG's method is 100% retainable, by VOLKIN AND CARTER's method 90% retainable, by KAY's and by KIRBY's methods completely nonretainable on the IR-120 Al^{3+} column. In the latter two cases, DNA is made retainable to the extent of 40 and 100%, respectively, after Mg^{2+} equilibration.

Nonretainability of DNA isolated by the SDS method

DNA isolated by the SDS method was nonretainable as such and it remained so even after extensive dialysis to remove residual SDS. The nonretainability of DNA may be explained as follows.

During the isolation of DNA, complex formation between DNA and SDS may have taken place wherein SDS occupied the same "loci" of DNA necessary for its retention on the IR-120 Al^{3+} column, and such "loci" of DNA being "capped" by SDS, no retention was possible.

It might also be that SDS may have formed a complex with resin-Al^{3+} and DNA free of SDS impurity may have come out in the effluate. Therefore, the effluate DNA was again loaded on a fresh IR-120 Al^{3+} column, but DNA was still nonretainable. The possibility that SDS may have "poisoned" the column also does not hold true because DNA isolated by SEVAG's method was 100% retainable on the same column.

It is known that SDS deproteinises deoxyribonucleoprotein (DNP) forming DNA and a denatured protein from one end[8]. If the deproteinisation is incomplete, part of the protein may still be associated with DNA in a denatured state. Retention of β-amylase and some studies[11] on phosphate metabolising enzyme on an IR-120 Al^{3+} column have shown that denatured proteins are not retained on the column. The residual denatured protein impurity along with DNA may exist as a "coating" on the DNA fibre as exists with certain viral RNA's and result in nonretainability of DNA.

Retainability of DNA isolated by the SDS method in the presence of Mg^{2+}

The effluate DNA, isolated by the SDS method, when equilibrated with Mg^{2+} and chromatographed, was found to be 40 % retainable. Mg^{2+} equilibration was tried without any specific reason except that Mg^{2+} is known to maintain a finite three dimensional conformation in the case of RNA and is a cation commonly found in biological systems. Demineralisation due to the SDS method may result in the nonretainability of DNA which may be rendered partially retainable again by Mg^{2+} equilibration.

Behaviour of DNA isolated by Kirby's method

As DNA isolated by KIRBY's method[9] employing phenol and 0.3 *M* fluoride was found nonretainable, it was thought that residual phenol and/or fluoride impurities may be inhibitory to the adsorption of DNA on the IR-120 Al^{3+} column. Sodium fluoride was however found to aid in the adsorption of DNA isolated from normal somatic tissue, on a Dowex-50 Hg^{2+} column[5]. As the results looked contradictory, care was taken to remove phenol by extensive ether washings and fluoride by dialysis. Still DNA was nonretainable on the IR-120 Al^{3+} column.

Behaviour of DNA isolated by Sevag's method and treated with phenol

It was found that DNA isolated by SEVAG's method and treated with phenol in the absence of fluoride was 66 % retainable after removal of traces of phenol and 70 % retainable in the presence of traces of phenol, indicating that nonretainability of part of the DNA isolated by KIRBY's method was apparently not due to the residual phenol impurity.

Behaviour of DNA isolated by Sevag's method and treated with fluoride

It was found that DNA isolated by SEVAG's method which is retainable on the IR-120 Al^{3+} column becomes nonretainable after fluoride treatment in the absence of phenol, even though last traces of fluoride were removed by extensive dialysis. It is unlikely that fluoride causes any damage, *viz.* depolymerisation or denaturation to the DNA rendering it nonretainable. This nonretainable DNA is rendered 100 % retainable by Mg^{2+} equilibration, indicating that the nonretainability on the IR-120 Al^{3+} column is due to some effect by fluoride which is nullified by Mg^{2+}. It is unlikely that Mg^{2+} undertakes "repairs" of DNA resulting from depolymerisation or denaturation making it retainable. Earlier studies[12,13] showed that the denatured DNA is completely retainable on the IR-120 Al^{3+} column.

Use of sodium fluoride in KIRBY's method may be removing Mg^{2+} by chelation. Mg^{2+} is also known to favour certain conformations of DNA and to aggregate nucleic

acids. Removal of Mg^{2+} as a complex possibly results in the deformation of the DNA structure, ultimately making it nonretainable.

Disaggregation due to fluoride treatment and aggregation due to Mg^{2+} equilibration may also be the probable phenomenon. Aggregation and disaggregation may be involving structural alterations, incidentally resulting in retainability and nonretainability of DNA, respectively.

It is difficult to explain why DNA, isolated primarily by SEVAG's method and then treated with KIRBY's phenol–fluoride deproteinising phase simultaneously, is completely retainable on the IR-120 Al^{3+} column, while DNA isolated directly by KIRBY's method is not retainable under an identical set of experimental conditions.

As use of different methods leaves different amounts of residual proteins, the effect of protein association on the chromatographic profiles of DNA on the IR-120 Al^{3+} column was studied and it was noted[14] that protein association has no major effect on the chromatographic behaviour of DNA.

These results, on the whole, indicate that the method of deproteinisation has a role in the retention of DNA on the IR-120 Al^{3+} column, although it does not affect the profiles, once adsorbed. The role of Mg^{2+} is also thought provoking.

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CHROM. 5068

THE NECESSITY OF ELUTION AND IDENTIFICATION OF DRUGS INDICATED BY THIN-LAYER CHROMATOGRAPHY

RICHARD J. COUMBIS, CHARLES C. FULTON, JOSEPH P. CALISE AND CARY RODRIGUEZ

Office of The State Medical Examiner of New Jersey, Newark, N.J. 07107 (U.S.A.)

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SUMMARY

The existence of drugs found in human urine by extraction and thin-layer chromatography require further verification, not only because the great number of drugs now in use complicates identification from R_F values but also because the R_F values obtained from the crude extracts that are chromatographed are inexact and inconsist. Thus chromatography is essential for separation but insufficient for identification. The procedure for extraction and thin-layer chromatography, as well as the procedure for elution, is given. Elution from the thin-layer plate after spraying has previously posed some difficulties, but a simple shake-out from a basic slurry has now been found very satisfactory. Tests for final identification are then given. Color and microcrystal tests, aided when necessary by UV readings, are discussed for morphine, quinine, methadone, propoxyphene, chlorpromazine, nicotine, trimethobenzamide and procaine, on the basis of actual findings in urine samples.

INTRODUCTION

In recent years the necessity for analysis of drugs in human urine has expanded enormously. The most popular method of distinguishing the extracted basic drugs is thin-layer chromatography (TLC); however, the outstanding feature of this method is that the drug's identity requires verification.

First, hundreds of basic drugs cannot possibly be distinguished, one from other, simply by the positions (R_F) of their spots on the chromatogram in a short space of 10 cm, or even in a much longer distance. Only about 20 different positions really can be distinguished from each other on the usual thin-layer plate. The habit of reporting R_F values to hundreds, as 0.63 for example, conceals this fact, in part, only too well; but even if such R_F values were exact, they would account for only 100 positions. The overstated precision leads to laxity in interpreting R_F positions. The appearance of the spots helps somewhat, but not enough for any certainty; the spots, sprayed with platinic iodide, yield different colors for different substances, to a certain extent, which provides additional distinctions between the values. However, the colors are chiefly due to the color of the reagent and mostly have only a limited gamut. Therefor the identity at a particular R_F position cannot be settled decisively.

Secondly, because the crude extracts are actually chromatographed, the R_F value of a particular substance varies considerably in different cases, as does the color of its spot. Some analysts, even when searching chiefly for morphine, extract all the bases together without any real purification, and then spot them together with extractable impurities on one plate. Even with better extractions, the analyst soon finds much variability in the chromatographic results. He therefore begins regarding almost any spot similar to the right color and in the vicinity of the proper R_F value as representing what he is looking for. This assumption is unharmful if a real identification is made thereafter, but not if a mistake goes unnoticed perhaps until the analyst is called to account for his work.

The great drawback is the practice of accepting any sort of "positive" result as an affirmation of the existence of the substance that one is expecting or looking for. When an additional or "confirmatory" test is made, it must be very specific, not a reaction that will elicit a "positive" response from hundreds of compounds — for example, a chemical reduction reaction for the "confirmation" of morphine. Most confirmatory tests made by spraying with a different chromatographic reagent are in this general category. An additional disadvantage is that a response may be due to two or more substances in the same spot. Multiple spraying, in itself a confession of the fallacy of identification made after spraying once, may enhance the guesswork but not provide a real identification. Sometimes chromatographic results are reported only as "positive" and not as R_F values that can be confirmed.

In 1954, MANNERING *et al.*[4] reported on their careful identification of morphine in urine samples by paper chromatography, verified by color and microcrystal tests. From 1103 urine samples run, morphine was identified beyond doubt in 412. Of 601 morphine-negative samples, 36 gave spots at the R_F position for morphine, but most of these spots were from the wrong extract or were off-color or very feeble. The eluates of five morphine-positive spots verified, after derivation from the proper extract, from the R_F value and by the color of the iodoplatinate spot, did not give positive color tests with H_2SO_4 reagents and were not morphine. The authors conclude that while this error represents but a small percentage of the total determinations, "it points out the error that could result from drawing final conclusions from the chromatographic data alone". TLC is not likely to be more accurate now considering the great increase in the number of drugs since 1954 and sometimes the lack of a separate extraction before chromatography.

TLC or some other form of chromatography is usually quite indispensable for separation of constituents in a mixture and for good indication of what substances are present, but the results obtained from TLC of crude extracts should not be mistaken for real identification of the substances present. The examination of human urine for addictive drugs is not primarily a statistical matter but one of individual concern, and chemists should make completely certain that no mistakes can be attributed to the chemistry used.

EXPERIMENTAL

Extraction and TLC

Urine is extracted first with ether from acid, then with chloroform from an alkaline solution, then reacidified, hydrolyzed, adjusted to pH 8.5, and re-extracted

with chloroform–isopropanol (primarily for morphine). The two basic extracts from pH 11.0 and pH 8.5 are chromatographed separately. They are spotted, together with appropriate standards, on commercial thin-layer plates, 250-μ thick, and developed with a solvent of ethyl acetate–ammonium hydroxide–methanol (85:5:10) or ethanol–acetic acid–water (240:120:40), air dried and then sprayed lightly with 10% acetic acid, placed under UV light to check for fluorescent areas which are outlined with a pencil. The plate is then air dried to remove excess acetic acid and subsequently sprayed with platinic iodide solution. The sprayed plates are air dried and allowed to stand for 1 h or more, and a spot to be further investigated or verified for a particular substance is scraped into a beaker. Weak morphine spots have been observed to develop even after 4–18 h at room temperature.

The details of the foregoing procedure may be varied. The present article is concerned primarily with the elution and its relation to verification. We do recommend, however, that the three separate extractions be made carefully, and attempts to simplify the procedure too much will only cause difficulties and doubts later.

Elution

In a previous article[1] a simple methanol elution of a thin-layer spot was given. Microcrystal tests were then applied to the eluates. This has been useful (particularly for verifying morphine) but some objectionable features were soon recognized. Methanol elution is somewhat tricky for recovery of the drug and, in any case, methanol dissolves iodide from the spray which interferes with many of the best color and crystal tests. From paper chromatograms, elutions are made with a small volume of borax–Na_2SO_3 solution and 25 ml of chloroform which recovers the free base. This is far more satisfactory. Various attempts have been made to elute similarly the thin-layer spots, but the results seem to be unsatisfactory.

Recently, prolonged trouble with the eluates of methadone spots, even with the control spots, prompted a new attack on this problem. The results were so successful that it is somewhat difficult to understand exactly what the obstacles were. The methanol elution is not really as simple as it seems. The scraped material is digested with only 1 or 2 ml of methanol for a few moments while warming or heating in the steam bath; then the methanol is filtered into a 5-ml beaker. This treatment is usually repeated twice, and the filter is finally washed down with a little methanol. The separatory funnel extraction now proposed is not more difficult, even for routine, if a sufficient supply of clean, small separatories is kept at hand and is not particularly novel chemically. However, we feel that others should be advised of the successful elutions from TLC plates and reminded of the importance of verification of TLC spots.

The difference in the eluates is enormous. If the substance causing the spot is uncertain, the eluate may be first run in quite dilute HCl, or in alcohol, on the UV spectrophotometer, and the solution then again evaporated. If the substance is suspected, chemical tests may be applied without using an intervening UV procedure. Both color and crystal rests may be used, if the amount (judged by size and intensity of the spot) is more than minimal. If it is nearly minimal, the one best crystal test is generally sufficient verification of the identity, when successful, if the previous chromatographic indication was good.

Elution procedure

As a routine procedure in our laboratory, we transfer the scrapings of an out-

lined TLC spot into a 5- or 10-ml beaker, digest it with 5 ml of ammoniacal solution A or B, and transfer the suspension into a 120-ml separatory funnel. Scrapings remaining in the beaker are resuspended with an additional 5 ml of ammoniacal solution A or B and combined with the previous suspension. A second beaker, preferably an 8-ml "hollow stopper", polyethylene beaker properly labeled (case number, pH of extraction and R_F) is filled with the solvent of choice (ether, chloroform or chloroform–isopropanol) and poured into the separatory funnel. Contents are shaken for about 1 min. The organic layer is filtered into the properly labeled polyethylene beaker and evaporated to dryness. Specimens are now suitable for UV spectrophotometry and chemical color and microcrystal tests.

Reagents

Ammoniacal solution A is used primarily in association with TLC spots suspected of being morphine, morphine-like drugs, or weak bases. It is prepared by adding 0.82 ml of conc. ammonium hydroxide A.R. (58 %) to 400 ml of distilled water. One gram of sodium carbonate is added and brought to a volume of 500 ml. pH of solution should be checked and verified to be 8.5 to 8.8

Ammoniacal solution B is used for other basic drugs. It is prepared by adding 10 ml of conc. ammonium hydroxide A.R. (58 %) to 500 ml of distilled water. pH should be checked and verified to be 11.0.

Ether (ethyl ether anhydrous A.R.) is the solvent of choice for methadone and quinine. Chloroform (ACS) is a general solvent for all basic compounds. Chloroform–isopropanol (3:1) is used for morphine.

Identification (verification tests)

With the base reseparated by elution with solvent extraction, any test sufficiently sensitive may be applied. For numerous basic drugs, suitable crystal and color tests have already been given[2,3]. The best tests for some of the most important drugs will be reviewed here. The eluate is dissolved in about 0.02 ml of 2 % acetic acid. Little droplets of the solution are taken for tests.

To test for morphine, a little droplet of solution is evaporated on a spot plate and tested with the Ferreira reagent (conc. molybdate in $H_2SO_4 \cdot SO_3$) (ref. 2). With morphine, this gives an intense dark purple color which fades after a few minutes. This is exceedingly sensitive, and if the purple color is not produced, morphine is not present. If the test is positive, final proof of morphine is obtained by testing another droplet of the solution (or dilution of it, if the presence of much morphine is indicated) with aq. K_2HgI_4, observing the result microscopically[1,2]. K_2CdI_4 may also be used. Other substances have been found in spots at the R_F position of morphine, particularly one temporarily designated as Unknown N.J. No. 1, the amount of which may be fairly large. With I–KI reagent M-2, it gives sizable dark red isotropic grains. Sometimes morphine is present along with the other substance; sometimes not. With the methanol elution, the convenient color test could not be used. So far, we have identified morphine with certainty in more than a 100 specimens of urine collected from addicts.

Quinine is one of the most obvious substances on the chromatogram, since its spots are fluorescent. If an ammoniacal solvent has been used, the dried plate may first be sprayed with diluted acetic acid, the fluorescence observed and the plate dried

again, then sprayed with platinic iodide solution. This is not as objectional as spraying with diluted sulfuric acid, which dries down to concentrated acid capable of causing many changes in substances. However, these are certainly pitfalls in assuming a fluorescent spot, anywhere in the neighborhood, to be quinine. It might be quinidine, which in general will react just like quinine, except to the microcrystal tests; in fact what was supposed to be quinine in toxicologic cases has several times been found to be quinidine at the Chief Medical Examiner's in New York City. The quinine is most readily proved with H_2PtBr_6 in (4 + 1) HBr. More subject to interference with impurities, but even more sensitive, is the I–KI reagent Q-6. If the amount is not too small, I–KI reagent C-3 may be used for herapathite crystals[2]. This is certainly more proof than necessary.

Methadone travels very near the solvent front with the chromatographic solvent mixture that has been chiefly used here. Numerous impurities and several common drugs collect at the solvent front. Addicts admitted to the Drug Abuse Clinic here are receiving methadone. This makes methadone a logical suspect for a spot near the solvent front, but it has been possible to prove it only in a minority of cases of noticeable spots. One of the "impurities" may be a metabolite of methadone, but in several cases, the spot is definitely due to a different drug; there has been confusion with propoxyphene and chlorpromazine. In two other cases, a definite test was obtained, but the substance was not identified. The eluted methadone may be identified by testing the dry substance (the deposit on a microscope slide left by evaporation of a droplet of solution) with I–KI reagent Q-6 (ref. 1). The X-crystals are highly characteristic. If the amount is not too small, a good color test can be obtained with Mandelin reagent (vanadate in H_2SO_4) (ref. 2).

The base propoxyphene also travels near the solvent front with the chromatographic solvent used most here, and has been confused with methadone although the colors of the spots of the pure bases are different. Crystal tests for propoxyphene are difficult unless it is very pure or present in substantial quantity. It may be proved by three very sensitive reactions having different colors: Ferreira reagent, black; H_2SO_4 reagent C-2, purple-violet changing to black; and M/20 reagent (Marquis reagent diluted 20 times with H_2SO_4), purple[2].

Propoxyphene sometimes occurs at a lower R_F. The principal metabolic change is the loss of one methyl group from the nitrogen, thus probably not affecting the color tests which depend on another part of the molecule. However, a large spot of R_F 0.65, when rechromatographed, went up to R_F 0.93, showing that it was actually unchanged propoxyphene. The eluate gave the proper color tests. It is obvious that a spot due to propoxyphene may be taken for something else if identification is based chiefly on the R_F value or position of the spot. In any case, the substance should not be called dextropropoxyphene (Darvon) unless it is actually proved to be the dextro isomer, since levopropoxyphene (Novrad) is on the market. An isomer can be distinguished by microcrystal tests if recovered pure enough and if controls are available to form the racemate[2]. The method is due to the work of CLARKE.

One supposed methadone spot was identified as certainly a phenothiazine and almost certainly chlorpromazine by $HAuCl_4$ in HOAc-4 (1 + 1) H_2SO_4 added with coverglass to the dry substance. This test could not have been used on an iodide-containing methanol eluate. With the eluted substance, and likewise with known chlorpromazine, it gave a red color and dichroic crystals, colorless to pink. A few

other phenothiazine drugs are somewhat similar, but a good chlorpromazine result can be distinguished. In the particular case, there was insufficient sample for an additional test.

Nicotine is a common substance in the urine of smokers, often in surprisingly large amounts. The nicotine spots are usually not of interest except that the analyst must be reasonably sure that they are due to nicotine and not to a drug that may appear at the same position, with or without nicotine. Therefore, at times, the nicotine should be identified beyond doubt. Nicotine may be tested by volatilizing it either into a reagent drop or into a drop of dilute HCl, which is thereafter tested[2]. However, if elution is made with solvent extraction, the eluate may be advantageously tested directly and the tests will show if a spot is entirely or chiefly something else (but some degree of impurity of the nicotine is to be expected). The eluate is dissolved in a small drop of (2 + 1) HOAc, a droplet is transferred to a plain slide, and two successive dilutions are made on the slide with (2 + 1) HOAc. To the most dilute droplet, a droplet of $HAuBr_4$ in HOAc–(2 + 3)H_2SO_4 is added, and is allowed to stand without a coverglass. The nicotine bromaurate crystals are unmistakable[2]. If there is not any satisfactory result, the next stronger solution or finally the most concentrated of the three droplets is tested. If the most dilute solution shows the nicotine, the dry deposits of the next may be tested with I–KI reagent M-2, with a coverglass, and a fairly concentrated deposit with I–KI reagent N-2 or $HAuBr_4$ in (2 + 1) HOAc[2].

Trimethobenzamide is a drug recently identified here for the first time in a urine sample. In this case, we were told that amongst 3 or 4 substances mentioned as previously taken, a newly admitted addict was said to have been using Tigan (trimethobenzamide). The UV curve of the eluted thin-layer spot was seen to correspond to this substance. This finding was confirmed by 3 different color tests: with conc. molybdate in H_2SO_3, purple changing to violet; with nitrite in H_2SO_4, persistent purple; with conc. HNO_3, quick development of a purple color which changes to brown, then to yellow. Molybdate in fuming H_2SO_4 gives still a fourth distinct color test. No microcrystal test has yet been found (Reagents, 2).

The eluate of a completely unknown spot was recognized as procaine by a test with $HAuBr_4$ in HOAc-3 (2 + 3) H_2SO_4, added with coverglass to the dry substance, which gave large serrate red blades. The round forms in the aqueous test with H_2PtCl_6 were also obtained. These tests probably would not even have been tried on the methanol eluate because both these reagents react with iodide. Procaine has since been found in three other cases, and the UV curve has also been used in the identification. The subjects were newly admitted to the Drug Abuse Clinic. Procaine is the major adulterant of cocaine and a minor adulterant of heroin, but in these cases, its origin is not known to us. It is, of course, sometimes injected by a dentist. The case history, in one case, suggests the possibility that addicts were sold procaine and told it was Numorphan.

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CHROM. 5089

AN INVESTIGATION OF FLAVONES AS FLUOROGENIC SPRAY REAGENTS FOR ORGANIC COMPOUNDS ON A CELLULOSE MATRIX*

PART I. GENERAL DISCUSSION OF THE METHOD

V. MALLET AND R. W. FREI
Department of Chemistry, Dalhousie University, Halifax, Nova Scotia (Canada)
(Received September 16th, 1970)

SUMMARY

Ten flavones were investigated in this study as possible fluorogenic sprays for polar organic compounds separated on cellulose thin-layer chromatograms. Baygon (2-isopropoxyphenyl-N-methylcarbamate) was chosen as the test system since the authors are primarily interested in pesticide analysis. Intensely yellow spots were observed on slightly yellow fluorescing backgrounds with solutions of fisetin, robinetin and flavonol, which are all flavonols with unsubstituted 5-positions. An attempt has been made to explain this fact.

The fluorescence phenomenon is believed to be attributable to an enhancement effect on the fluorescence of the flavones caused by the relatively high polarity of the adsorbed organic molecule in the chromatographic spot.

The fluorescence spectra of fisetin and background are shown and visual observation of the high stability of the fluorescence suggests the possible use of flavones for quantitative studies of pesticides.

INTRODUCTION

Only few methods for the analysis of pesticides by *in situ* fluorometry on thin-layer and paper chromatograms have appeared in the literature. Chlorinated insecticide spots, for instance, can be rendered fluorescent after the chromatogram is sprayed with N-methylcarbazole or Rhodamine B[1]. Buquinolate (ethyl 4-hydroxy-6,7-diisobutoxy-3-quinoline carboxylate) was determined in chicken feeds by BORFITZ *et al.*[2] who used a chromatographic–fluorometric method. Fluorescent whiteners, known as Calcofluors, have been applied successfully to the visualisation of carbamates, uracils and ureas[3].

A procedure for the fluorescent determination of phosphate esters on paper chromatograms was suggested by GORDON *et al.*[4] and later applied by RAGAB[5] to pesticides on silica gel layers. This method involved bromination followed by spraying with ferric chloride and finally with 2-(*o*-hydroxyphenyl)benzoxazole to produce

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fluorescent spots for a number of organophosphate insecticides. The method was later improved by FREI and co-workers[6] who made use of a combination of new reagents and salt solutions. Recently, an *in situ* fluorometric method for the quantitative determination of Sevin was developed[7]. In this method Sevin was hydrolysed with sodium hydroxide directly on the plate to the highly fluorescent anion of α-naphthol.

In the present study, flavones were investigated as fluorogenic sprays for the visualisation of pesticides on cellulose layers. The chemistry of flavones has been extensively studied and is well presented in a book by GEISSMAN[8]. These compounds have been used as analytical chelating agents for the determination of cations and some anions[9]. Many of the chelates were observed to be fluorescent. The only instance where a flavone has been applied to the detection of organic materials was reported by TADEMA AND BATELAAN for phenols and nitrophenols[10]. Fluorescence quenching was used.

Flavones were chosen for this investigation because of their ability to be almost non-fluorescent in a non-polar medium and extremely fluorescent in a polar one. It was thought initially that if such compounds were sprayed on a surface of low polarity such as cellulose, the background would remain weakly or non-fluorescent. The presence of a polar substance on the surface would enhance the fluorescence of the spray, hence enabling its detection as a spot.

In this particular work on pesticides, the insecticidal carbamate Baygon was chosen as the test sample since carbamates are polar compounds and also because of the great need for a fluorescent method suitable for their analysis.

EXPERIMENTAL

Chemicals and apparatus

The flavones fisetin, kaempferol, quercetin, morin, and rutin were purchased from Fluka A.G., Chemische Fabrik, Buchs S.G., Switzerland; chrysin, apigenin and acacetin from Aldrich Chemical Co., Inc., Milwaukee, Wisc., U.S.A.; robinetin-aglucone from Koch-Light Laboratories, Colnbrook, Bucks., Great Britain; and flavonol from Eastman Organic Chemicals, Distillation Product Industries, Rochester 3, N.Y., U.S.A.

Cellulose powder (MN-300, Macherey, Nagel and Co., Duren, G.F.R.) was used. A sample of Baygon was provided by Chemagro Corporation, Kansas City, Mo., U.S.A. It was recrystallised before use from 50 % ethanol. All the chemicals utilised in this study were of reagent grade quality and the solvents were distilled before use.

The fluorescence spectra were recorded with an Aminco-Bowman Spectrophotofluorometer with TLC attachment. A 230-W mercury UV lamp, Hanovia type 16106, was employed for visual observation of the fluorescence.

General procedure

Prior to use, the cellulose powder was washed twice in a mixture of isopropanol–ammonium hydroxide–water (6:3:1) and once in isopropanol alone, then dried in an oven at 105° for 8 h. The plates were then prepared (250 μ thickness) with a Desaga TLC applicator. A mixture of 15 g of cellulose in 85 ml of water homogenised in a blender was applied. After drying in air, the plates were eluted in ether to ensure the removal of any remaining organic impurities and dried in air immediately before use.

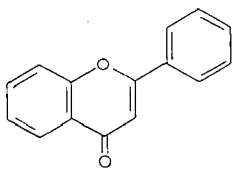
A standard solution of Baygon was prepared 1000 p.p.m. (1 mg/ml) in isopropanol and 1 μl of the pesticide solution was spotted with a 10-μl Hamilton microsyringe. Then the plate was sprayed lightly with a 0.05 % solution of the flavone in isopropanol, dried in air and observed under a long wavelength UV light source.

A TLC separation of Baygon was also carried out following a procedure proposed by RAGAB[11]. A cellulose plate was spotted with 1 μg of the pesticide, then sprayed with a 5 % solution of mineral oil in hexane (v/v), and eluted 10 cm in a solution of acetone–water, (50:50). The plate was dried and sprayed lightly with a 0.05 % solution of fisetin in isopropanol.

For the recording of fluorescence spectra, the plate was placed face down on the TLC attachment of the instrument. The spot was positioned visually in the light path and excitation and emission peaks recorded in the usual fashion.

RESULTS AND DISCUSSION

The structure of flavone is shown below (I). The flavone molecule may contain hydroxy groups at all the positions in the rings, except 1- and 4-, and is highly conjugated; a condition necessary for fluorescence.



(I)

The flavones utilised in this study are listed in Table I. Flavonol, fisetin, and robinetin are 3-hydroxyflavones; kaempferol, quercetin, and morin are 3,5-dihydroxyflavones; chrysin, apigenin, and acacetin are 5-hydroxyflavones; and rutin is a 3-O-glucoside of quercetin.

TABLE I

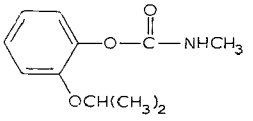
EVALUATION OF FLAVONES

Common name	*Chemical name*	*Fluorescence of background*	*Fluorescence of pesticide spot*[a]
Flavonol	3-hydroxyflavone	slightly yellow	yellow
Fisetin	3,3′,4′,7-tetrahydroxyflavone	slightly yellow	yellow
Robinetin	3,3′,4′,5′,7-pentahydroxyflavone	slightly yellow	yellow
Kaempferol	3,4′,5,7-tetrahydroxyflavone	none[b]	none
Quercetin	3,3′,4′,5,7-pentahydroxyflavone	none[b]	none
Morin	2′,3,4′,5,7-pentahydroxyflavone	none[b]	none
Chrysin	5,7-dihydroxyflavone	none	none
Apigenin	4′,5,7-trihydroxyflavone	none	none
Acacetin	5,7-dihydroxy-4′-methoxyflavone	none	none
Rutin	quercetin-3-rutinoside	none	none

[a] Baygon (1 μg) was spotted on a cellulose plate, sprayed with a 0.05 % solution (isopropanol) of the flavone.

[b] In the cases of morin, quercetin, and kaempferol, the background becomes slightly fluorescent with a large excess of the spray.

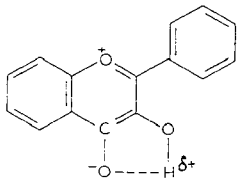
Table I summarizes the results obtained when a 0.05 % solution of the flavone is sprayed on a cellulose thin-layer plate. Only the 3-hydroxyflavones gave fluorescence of the background. The 3,5-dihydroxyflavones and the nonflavonols did not produce any fluorescence when sprayed lightly on the plate, although kaempferol, quercetin, and morin showed a slight fluorescence when sprayed in excess. In the cases of flavonol, robinetin, and fisetin, an intense yellow fluorescence developed on the plate where 1 μg of Baygon (II) had been spotted. Thus, this phenomenon is specific to the three 3-hydroxyflavones with the unsubstituted 5-position.



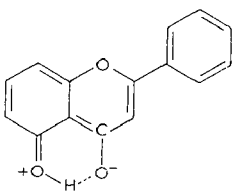
(II)

It is well known that flavonols (3-hydroxyflavones) are extremely fluorescent in a medium of high dielectric constant ($HClO_4$) or mild basicity[12] (NH_4OH), while others that do not possess the 3-hydroxy group, are not. A basic difference exists between 3-, and 5-hydroxyflavones. A number of studies by partition chromatography[13,14], IR[15], UV, and visible absorption[16], and NMR[12] have been carried out on the nature of the hydroxyl groups in the molecule and they all indicate that the 5-hydroxyflavone is much more hydrogen bonded than the 3-hydroxy species. According to SIMPSON AND GARDEN[13], the hydrogen bonding between the carbonyl and the 3-hydroxy group differs from the chelation of the 5-hydroxy group in that there is no mechanism in the former structure for the transfer of electrons from the hydroxyl to the carbonyl group.

It was suggested that for 3-hydroxyflavones (III) an electrostatic attraction is established between the hydrogen atom carrying a fractional positive charge, arising from the -I effect of the hydroxy-oxygen atom, and the carbonyl-oxygen atom. In the case of 5-hydroxyflavones (IV), resonance throughout the molecule permits the complete separation of charges between the carbonyl and hydroxyl groups thus strengthening the hydrogen bonding.



III



(IV)

From the IR studies[14] it was shown that introduction of a 5-hydroxy group into a 3-hydroxyflavone causes the absorption due to the O–H stretching to be intensified and shifted indicating a decrease in the overall hydrogen bonding. The fact that structures (III) and (IV) are cross-conjugated, *i.e.*, the two chelate systems are formed by mutually opposing mechanisms, would explain that the simultaneous chelation of 3- and 5-hydroxy groups is weaker than expected.

These resonance structures (III and IV) help to explain why 5-hydroxyflavones are not fluorescent while 3-hydroxyflavones are. In structure (IV), the "*n*" electrons of the oxygen atom in the pyrone ring are not delocalised as is the case for structure (III) and it is well known that these electrons can cause quenching or at least a reduction of the total fluorescence when present in a molecule[17]. Therefore it is not surprising that when both 3- and 5-hydroxy groups are present in the flavone molecule, as is the case with morin, the total fluorescence observed is less than that given when only the 3-hydroxy group is present. This is supported by the fact that only fisetin, robinetin, and flavonol gave positive results (Table I).

Although 3,5-dihydroxyflavones can be considered non-fluorescent on cellulose which is a weakly polar adsorbent, it seems logical to assume that they would become increasingly fluorescent on more polar surfaces with a shift in equilibrium to the five-membered ring structure (III). This has been partly confirmed in another study where significant fluorescence was observed on silica gel and alumina layers after spraying lightly with morin or other similar compounds.

Other effects which may be of importance are the pH of the plate, which differs somewhat on various chromatographic adsorbents, and also possibly a special kind of interaction (chemisorption) between substrate and spray reagent. Some fluorescence has been observed, for example, on silica gel plates after exposure to ammonia or bromine vapours and subsequent spraying. This new aspect is currently being investigated. The other factor of importance may be the difference in the hydrogen bonding between the spray reagent and the cellulose, which contains many hydroxyl groups, as compared to silica gel or alumina. Not much is known about these interactions and they are generally classified under the term chemisorption.

It was observed that 1 μg of Baygon could also very easily be detected on the plate with the fisetin spray after it had been sprayed with mineral oil and eluted in 50 % acetone[11], by reversed-phase chromatography. It was noticed, however, that in order to keep the background fluorescence to a minimum, freshly prepared cellulose plates had to be used and precleaning of the cellulose powder was essential. A number of eluting solvents, such as chloroform, hexane, carbon tetrachloride, pyridine, *n*-butanol, *n*-octanol, benzene, ethanol, 25 % acetic acid, ether, acetone, water, hydrochloric acid (1 %), and ammonia (1 %) were also investigated and no interference with the actual fluorescence phenomenon was observed.

The fluorescence spectrum of the fisetin background and that of the highly fluorescent Baygon spot are shown in Fig. 1. The excitation and emission wavelengths for the fluorescent spot are identical with those of the background, but of considerably increased intensity. Thus as had been expected, a fluorescence enhancement of the flavone is brought about by the pesticide. A similar phenomenon is also observed in solution if a few drops of fisetin are added to about 10 ml of a 1000 p.p.m. solution of Baygon. An intensive fluorescence results in comparison with the blank.

The fluorescence spectra of flavonol and robinetin were also recorded, with excitation and emission maxima for the flavonol background and spot being at 360 and 528 nm, and for the robinetin background and spot at 375 and 535 nm, respectively. In the case of flavonol, the background of the plate is strongly fluorescent, thus not very practical for analytical use, while robinetin behaves similarly to fisetin.

The neutral pH and the low polarity of the cellulose surface seem important criteria for reducing the fluorescence of the background. With further investigations

it may be possible to reduce background fluorescence by using a more purified form of cellulose or another adsorbent. Fluorescing agents other than flavones could also be investigated.

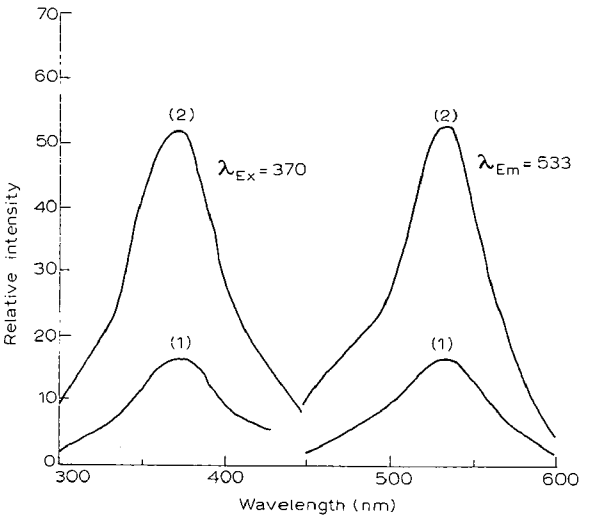


Fig. 1. Emission and excitation spectra of fisetin on cellulose layers. Conditions: (1) background; Meter Multiplier — 0.03; (2) Baygon spot; Meter Multiplier — 0.01.

CONCLUSIONS

The principle on the basis of which flavones are functioning as fluorogenic reagents, namely, the fluorescence enhancement of certain reagents due to an increase in polarity of the surrounding medium, opens a wide new field of investigation. Obviously, there should be a large number of other reagents that may behave similar to flavones and variations in the choice of chromatographic adsorbents will also yield interesting results as is shown by a similar study currently being carried out on silica gel.

While pesticides are the main interest to us in this investigation, further studies of the method are suggesting that the number of organic compounds that could be determined by this same approach is unlimited. The limitations of the resulting analytical methods will rest primarily with the efficiency of the chromatographic separation procedures.

The visually observed stability of the fluorescence produced depends solely on the reagent and is usually such that *in situ* quantitative determination of compounds on chromatograms seems feasible. Quantitative work is also supported by the fact that all the compounds would fluoresce at the same wavelength which would facilitate scanning procedures. Studies along those lines are currently in progress.

ACKNOWLEDGEMENTS

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CHROM. 5059

EXPERIMENTAL DETERMINATION OF ELUTION REQUIREMENTS IN DISPLACEMENT ION EXCHANGE*

J. E. POWELL, H. R. BURKHOLDER AND K. GONDA**

Institute for Atomic Research and Department of Chemistry, Iowa State University, Ames, Iowa 50010 (U.S.A.)

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SUMMARY

It is clear that the progress of separation of any number of lanthanons by displacement ion-exchange elution can be predicted by expressions derived from the basic equilibria involved. The computation process can be reversed so that separation factors are obtained from boundary movement diagrams.

INTRODUCTION

Separation theory pertinent to resolution of lanthanide (and other) mixtures *via* chelate formation and displacement cation-chromatography has evolved gradually from an approach suggested by POWELL AND SPEDDING in 1959[1]. Since then, noteworthy contributions have been made regarding the treatment of ternary[2,3] and more general multicomponent mixtures[4,5]. The treatment herein is intended to paraphrase the general elegant development of HELFFERICH AND JAMES[5], and to illustrate that not only can boundary movement diagrams be constructed (and elution requirements be determined) from a prior knowledge of mixture composition and separation factors but, conversely, that practical separation factors can be established from experimentally determined boundary movements.

THEORY

Imagine a quantity of multicomponent mixture of lanthanides deposited non-selectively on a cation-exchange bed, forming a loaded zone in which the mole fraction $\overline{X}_1$ of each component is uniform throughout. The charge is to be eluted under conditions promoting displacement chromatographic elution, *i.e.*, so that small increments of the material sorbed in the load zone are continually displaced from the stationary phase at the rear (upstream boundary) of the zone, percolated in dilute solution in

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** Present address: Tokai Works, Power Reactor and Nuclear Fuel Development Corporation, Tokai, Ibaraki, 319-11, Japan.

the mobile aqueous phase through the rest of the sorbed mixture, and redeposited on the stationary phase at the front (downstream edge) of the load zone. Such behavior is promoted by employing a dilute solution of ammonium salt of a strongly chelating anion (such as the EDTA or HEDTA anion) as the eluant and having the cation-exchange bed ahead of the load zone charged with a cationic species that forms a more stable complex with the chelating agent than does any component of the mixture being separated. If the chelatant is selective in the degree to which it complexes individual lanthanons (and/or the resin itself manifests selectivity), the system exhibits a series of separation factors (α_i^1) between the first (most easily eluted) lanthanon and the other components of the mixture. The individual separation factors relative to component 1 may be represented generally as

$$\alpha_i^1 = (X_1/X_i)/(\overline{X_1}/\overline{X_i}) \qquad (i = 1 \text{ through } n) \tag{1}$$

where X_i and $\overline{X_i}$ are the mole fractions of the ith component existing in the solution and resin phases, respectively, when the components of the two phases are at equilibrium. From this definition of the separation factors involved, and the stipulation that $X_1 + X_2 + \ldots + X_n = 1$,

$$\frac{X_1\overline{X_1}}{\alpha_1^1\overline{X_1}} + \frac{X_1\overline{X_2}}{\alpha_2^1\overline{X_1}} + \ldots + \frac{X_1\overline{X_n}}{\alpha_n^1\overline{X_1}} = 1 = (X_1/\overline{X_1}) \sum_{i=1}^{n} (\overline{X_i}/\alpha_i^1) \tag{2}$$

Since $(X_1/\overline{X_1})$ equals $\alpha_j^1(X_j/\overline{X_j})$ under equilibrium conditions, it is seen that the mole fraction of any solution component at equilibrium with a sorbed mixture is defined by the expression

$$X_j = \overline{X_j} \Big/ \left(\alpha_j^1 \sum_{i=1}^{n} (\overline{X_i}/\alpha_i^1)\right) \tag{3}$$

Under these conditions, from the moment elution is initiated until the load is displaced sufficiently to effect disengagement of components 1 and n, two series of concentration subzones (plateaus) of decreasing multiplicity develop (one series downstream and one upstream from the diminishing residual n-component zone). Each developing subzone, as well as the diminishing zone of original mixture, is defined at its extremities by recognizable boundaries (actually narrow concentration gradient regions between concentration plateaus) associated with successive disappearance, one at a time, of individual components (n through 1 downstream, and 1 through n upstream). That is, until components 1 and n disengage from each other, the individual chromatographic subzones (from the downstream end to the upstream end of the load zone) contain the components 1; 1,2; 1,2,3; ...; 1,2,3, ..., n; 2,3,4, ..., n; ...; $n-1$, n; n, respectively.

The rate of movement of the boundary which separates the front of the n-component subzone from the rear of the downstream $(n-1)$-component subzone (devoid of component n) is determined by the circumstance that the n-component zone contains $\overline{X_n}$ moles of component n per mole of mixture, and the fact that equilibrium considerations dictate a net transport, rearward across the n-component zone, amounting to $(\overline{X_n} - X_n)$ moles of component n per mole of load displacement. It is obvious from this that the rate at which this boundary progresses down the system

amounts to $(1 - (\overline{X}_n - X_n)/\overline{X}_n)$ or simply $(X_n/\overline{X}_n)$ moles per mole of load displaced by elution. By similar lines of reasoning, one concludes that the upstream boundary of the n-component residual zone progresses downstream on the resin bed system at a rate of $(1 + (X_1 - \overline{X}_1)/\overline{X}_1)$ or $(X_1/\overline{X}_1)$ moles per mole of load displacement. In fact, it is clear that each boundary between consecutive subzone mixtures of decreasing multiplicity (both downstream and upstream) moves at a rate (moles per mole of load displacement) fixed simply by the ratio of mole fractions (in solution and on the resin) of the singular component which is missing on one side of the concentration gradient region which comprises the boundary. The composition of each uniform mixture comprising a downstream subzone, therefore, depends directly on the composition of the mixture of next higher multiplicity situated immediately upstream.

Let d be the integral order number for the position of a particular subzone mixture (numbered downstream from the residual n-component mixture), so that $n - d$ denotes both the multiplicity of the mixture and the sequential elution order number of the singular component (present only on the upstream side of the gradient region dividing the d and $d + 1$ subzones). Then the mole fractions ($_{d+1}X_j$ and $_{d+1}\overline{X}_j$) in solution and on the resin of the jth component present in the $d + 1$ subzone are related to the mole fractions ($_dX_j$ and $_d\overline{X}_j$) of that element and the mole fractions ($_dX_{n-d}$ and $_d\overline{X}_{n-d}$) of the slowest-moving component (n — d) present in the d subzones as follows:

$$(_{d+1}X_j - {}_dX_j)/(_{d+1}\overline{X}_j - {}_d\overline{X}_j) = {}_dX_{n-d}/{}_d\overline{X}_{n-d} \tag{4}$$

Since requirements for equilibrium (eqn. 3) are such that

$$_dX_j = {}_d\overline{X}_j \Big/ \left(\alpha_j^1 \sum_{i=1}^{n-d} ({}_d\overline{X}_i/\alpha_i^1)\right) \tag{5}$$

$$_dX_{n-d} = {}_d\overline{X}_{n-d} \Big/ \left(\alpha_{n-d}^1 \sum_{i=1}^{n-d} ({}_d\overline{X}_i/\alpha_i^1)\right) \tag{6}$$

$$_{d+1}X_j = {}_{d+1}\overline{X}_j \Big/ \left(\alpha_j^1 \sum_{i=1}^{n-d-1} ({}_{d+1}\overline{X}_i/\alpha_i^1)\right) \tag{7}$$

it is readily deduced that

$$_{d+1}\overline{X}_j = \frac{{}_d\overline{X}_j(1 - \alpha_{n-d}^j)}{1 - \alpha_{n-d}^j(D/D')} \tag{8}$$

and since $\sum_{j=1}^{n-d-1} ({}_{d+1}\overline{X}_j) = 1$, that

$$\sum_{j=1}^{n-d-1} \frac{{}_d\overline{X}_j(1 - \alpha_{n-d}^j)}{1 - \alpha_{n-d}^j(D/D')} = 1 \tag{9}$$

where D equals $\sum_{i=1}^{n-d} ({}_d\overline{X}_i/\alpha_i^1)$, a predetermined quantity, and D' equals $\sum_{i=1}^{n-d-1} ({}_d\overline{X}_i/\alpha_i^1)$, an unknown involving the (as yet undetermined) mole fractions of the n–d–1 components in the $d + 1$ subzone.

After expanding the condensed eqn. 9 and substituting the various known ${}_d\overline{X}_j$ and α^j_{n-d} values, as well as the (from these) predetermined D term, D' is found by solving the resulting polynomial (quadratic when $n-d-1$ equals 2, cubic when $n-d-1$ equals 3, etc.). Since $D < D' < 1$, the polynomial in D' is readily obtained by successive approximations utilizing an appropriately programmed electronic computer. After finding D', it is a simple matter to establish the individual ${}_{d+1}\overline{X}_j$ values by means of eqn. 8. It is thus seen to be a straightforward although laborious, algebraic exercise to compute the compositions (mole fractions of each component) of the uniform mixtures in every downstream subzone, one after the other, from a knowledge of the initial composition of the n-component residual mixture from which subzones are successively generated.

The situation upstream from the n-component residual mixture is very similar to that existing downstream. The composition of each subzone is generated by equilibrium and transport phenomena from the preceding subzone containing one more component. If u is used to denote the order number upstream from the n-component mixture of a particular subzone, so that $u+1$ describes the sequential elution order number of the fastest moving component in that subzone, the mole fractions (${}_{u+1}X_j$ and ${}_{u+1}\overline{X}_j$) of component j in the next $(u+1)$ subzone are related to the mole fractions (${}_uX_j$ and ${}_u\overline{X}_j$) of that element and to the mole fractions (${}_uX_{u+1}$ and ${}_u\overline{X}_{u+1}$) of the fastest moving component of the u subzone, as follows:

$$({}_{u+1}X_j - {}_uX_j)/({}_{u+1}\overline{X}_j - {}_u\overline{X}_j) = {}_uX_{u+1}/{}_u\overline{X}_{u+1} \tag{10}$$

where equilibrium considerations dictate that

$${}_uX_j = {}_u\overline{X}_j \Big/ \left(\alpha_j^{u+1} \sum_{i=u+1}^{n} ({}_u\overline{X}_1/\alpha_i^{u+1})\right) \tag{11}$$

$${}_uX_{u+1} = {}_u\overline{X}_{u+1} \Big/ \left(\sum_{i=u+1}^{n} ({}_u\overline{X}_i/\alpha_i^{u+1})\right) \tag{12}$$

$${}_{u+1}X_j = {}_{u+1}\overline{X}_j \Big/ \left(\alpha_j^{u+2} \sum_{i=u+2}^{n} ({}_{u+1}\overline{X}_i/\alpha_i^{u+2})\right) \tag{13}$$

Thus, for adjacent subzones, u and $u+1$ (numbered upstream), prior to the time that components 1 and n disengage from each other,

$${}_{u+1}\overline{X}_j = \frac{{}_u\overline{X}_j(1 - 1/\alpha_j^{u+1})}{1 - U/(\alpha_j^{u+2}U')} \tag{14}$$

and

$$\sum_{j=u+2}^{n} \frac{{}_u\overline{X}_j(1 - 1/\alpha_j^{u+1})}{1 - U/(\alpha_i^{u+2}U')} = 1 \tag{15}$$

where U is the known quantity $\sum_{i=u+1}^{n} ({}_u\overline{X}_1/\alpha_i^{u+1})$ and U' is the unknown $\sum_{i=u+2}^{n} ({}_{u+1}\overline{X}_i/\alpha_i^{u+2})$. The polynomial in U' obtained by expanding eqn. 15 is solved for U' in exactly the same way that the polynomial from eqn. 9 was solved for D'. Then the values of ${}_{u+1}\overline{X}_j$ are obtained from eqn. 14.

After components 1 and n have disengaged, a second generation subzone (comprised of elements 2 though n — 1) begins to form from the first generation upstream and downstream subzones (comprised of components 2 through n and 1 though n — 1, respectively). As other fast moving and slow moving components disengage, additional subzones form and disappear until the original mixture is separated into n unicomponent regions. The pattern and parentage of individual subzones is indicated in Fig. 1. It is seen that many of the subzones indicated form at the expense of two parent subzones; however, one needs only to know the composition of one parent to

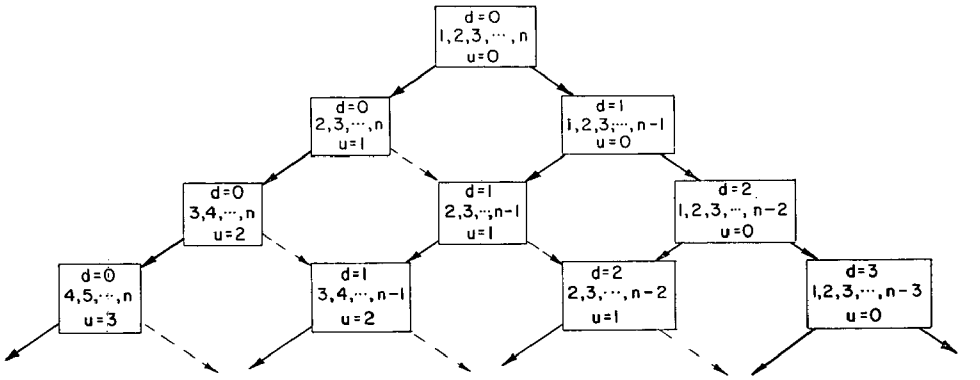

Fig. 1. Hierarchy of plateaus and subzones in an ion-exchange displacement separation.

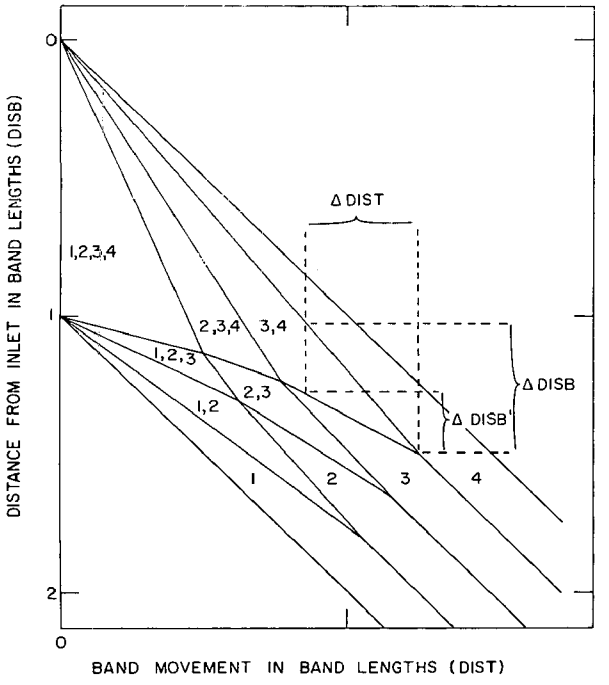

Fig. 2. Boundary movement diagram for a four element separation.

ascertain the composition of a dependent zone. That is, just as the composition of each mixture along the upper lefthand diagonal (Fig. 1) may be computed, one after the other, from the composition of the original mixture, the composition of each mixture along subsequent left-sloping sets can be determined from the composition of less and less complex mixtures computed down the upper righthand diagonal (see computational sequences indicated by the arrows in Fig. 1).

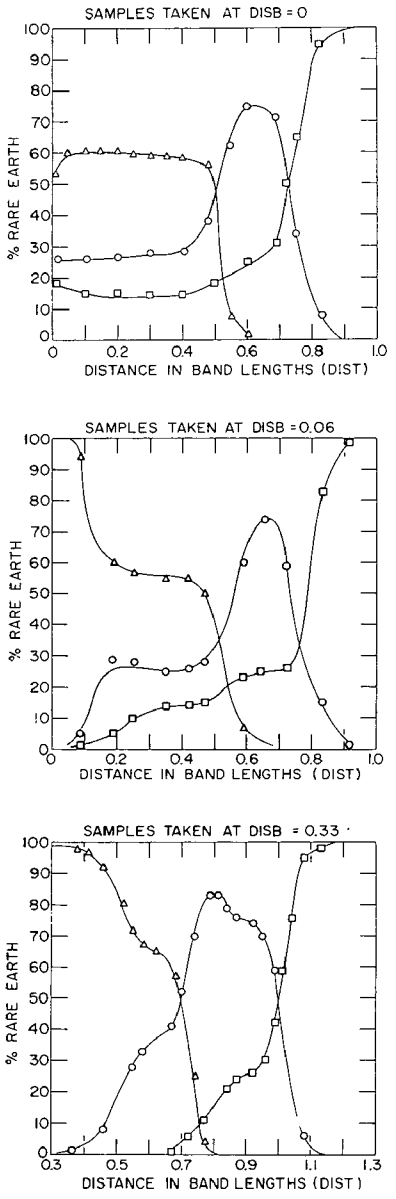

Fig. 3. Successive band profiles of a thulium, ytterbium, erbium separation.

Knowledge of the various $(X_j/\overline{X}_j)$ values corresponding to specific subzone mixtures enables one to construct boundary movement diagrams (point-slope-intersection plots) in which line segment intersections determine the elution distances required to achieve disengagement of specific pairs. Conversely, if the individual separation factors are not known in advance, boundary movement diagrams can be constructed by interpreting analytical profiles taken at a number of fixed points along the bed system during the elution of a mixture. These diagrams can then be used to assess the various α_k^j values operative during the elution (see Fig. 2). The slopes (ΔDISB/ΔDIST) and (ΔDISB'/ΔDIST) of pairs of converging line segments

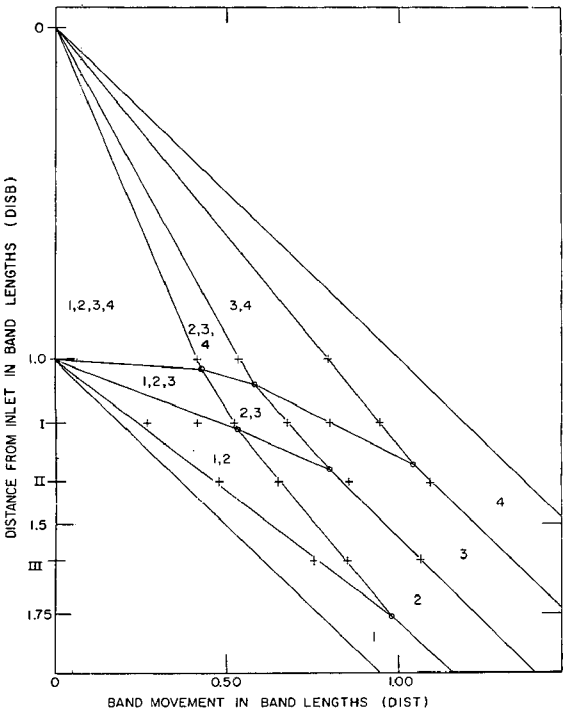

Fig. 4. Boundary movement diagram for an erbium, holmium, dysprosium, terbium separation.

(which represent rates of progress of the upstream and downstream boundaries of diminishing mixtures) define the ratios $(X_j/\overline{X}_j)$ and $(X_k/\overline{X}_k)$, where the subscripts j and k denote the elution order numbers of the unique components (present in the subzone under consideration but absent from the next upstream and next downstream subzones, respectively). Consequently,

$$\alpha_k^j = (X_j/\overline{X}_j)/(X_k/\overline{X}_k) = \Delta\text{DISB}/\Delta\text{DISB}' \tag{16}$$

EXPERIMENTAL

The cation-exchange beds used in this work were comprised of 40–50 mesh spheres of Dowex 50 X8 packed in 2-in. I.D. Pyrex glass columns, fitted with Saran

cloth bed supports, neoprene gaskets and Plexiglass end closures. The bed systems generally consisted of a load zone section followed by a series of shorter appendages allow convenient withdrawal of analytical samples from the aqueous phase at predetermined points along the system as the elution progressed. In all experiments the eluant was 0.014 *M* ammonium EDTA solution adjusted to pH 8.4. The retaining bed (in the short appendages) was initially charged with a Cu^{2+}–H^+ mixture by passing a 1 *M* $CuSO_4$–1 M H_2SO_4 mixture through the appendages. The aqueous phase samples were analyzed spectrophotometrically.

Experiment I

The load zone section was loaded to saturation with an excess of Er–Tm–Yb chloride mixture and flushed with distilled water in a backwash operation to insure uniform distribution of the components within the load zone. A series of Cu^{2+}–H^+

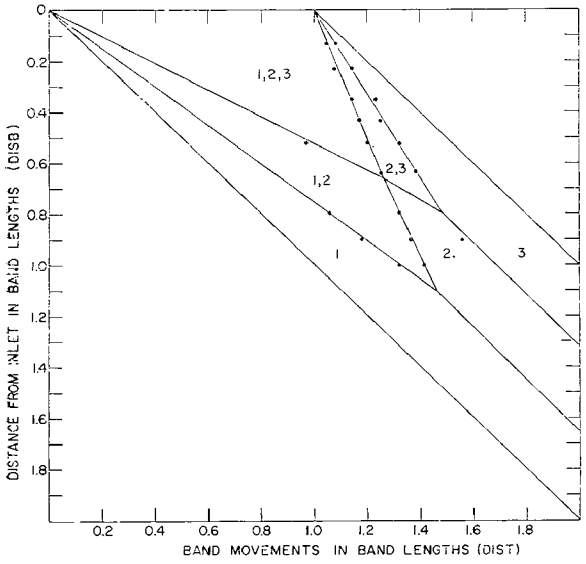

Fig. 5. Boundary movement diagram for a prechelated thulium, ytterbium, erbium separation.

beds (0.06, 0.33, 0.51, 0.68 and 1.0 times the capacity of the load zone) was appended to the load zone section, and elution was initiated at *ca.* 25° and a flow rate of 25 ml per min. Samples of the eluate passing between column segments were withdrawn periodically and analyzed carefully. Fig. 3 shows compositions observed at typical sampling points as a function of elution progress. From such plots it was ascertained when (in terms of band lengths) each subzone boundary had progressed a specified distance.

Experiment II

An Er–Ho–Dy–Tb mixture was loaded and eluted as in the first experiment and profiled at points designated by O, I, II, and III along the ordinate of Fig. 4.

Experiment III

Equilibrated solutions of erbium EDTA, thulium EDTA and ytterbium EDTA obtained from a set of previous elutions were mixed and passed onto a system of retaining beds followed by ammonium EDTA eluant. The mixture was sampled periodically in transit at points corresponding to 0.13, 0.23, 0.35, 0.43, 0.52, 0.64, 0.80, 0.90 and 1.00 band lengths to construct Fig. 5.

RESULTS AND DISCUSSION

The boundary movement diagrams obtained experimentally were used to establish separation factors and the results are compared in Table I to separation factors computed solely from literature values of lanthanide EDTA stability constants[6,7]. It is seen that agreement between the practical results and the predicted values (in parenthesis) is fair in most instances. Hence this approach provides means for studying chelate formation under conditions not suitable for potentiometric and polarographic measurements. For example, separation factors have been established in this way at elevated temperature and used to confirm a predicted change in dentate character of the HEDTA ligand[8].

TABLE I

COMPARISON OF PRACTICAL AND THEORETICAL SEPARATION FACTORS

Experiment	*R.E. pair*	*Separation factor*
I	Yb–Tm	2.8 (1.8)
	Tm–Er	2.3 (3.1)
	Yb–Er	6.2 (5.6)
II	Er–Ho	1.6 (1.8)
	Ho–Dy	2.7 (2.6)
	Dy–Tb	2.4 (2.3)
	Er–Dy	4.4 (4.7)
	Er–Tb	10.7 (10.1)
	Ho–Tb	6.7 (5.9)
III	Yb–Tm	2.9 (1.8)
	Tm–Er	1.9 (3.1)
	Yb–Er	5.6 (5.6)

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CHROM. 5075

INFLUENCE OF TEMPERATURE ON THE ION-EXCHANGE PROPERTIES OF STANNIC ARSENATE

SEPARATION OF Pb^{2+}, UO_2^{2+} AND Cr^{3+} FROM NUMEROUS METAL IONS

MOHSIN QURESHI, H. S. RATHORE AND RAJENDRA KUMAR
Chemical Laboratories, Aligarh Muslim University, Aligarh, U.P. (India)
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SUMMARY

The amorphous precipitates (stannic arsenate) obtained by mixing the 0.1 *M* aqueous solutions of stannic chloride and sodium arsenate in the volume ratio of 1:1 have been dried at different temperatures (40–600°). The ion-exchange properties, the solubility and the IR studies of the dried samples have been investigated. The sorption studies on the sample dried at 40° have been made in detail by raising the temperature (from 20 to 95°) of the equilibrating solution and its columns have been used for the separation of Pb^{2+}, UO_2^{2+} and Cr^{3+} from numerous metal ions.

INTRODUCTION

Stannic arsenate[1,2] has promising cation-exchange properties, an outstanding thermal stability, high uptake of certain ions and good resistance against water and dilute acids. However, it is necessary to study its ion-exchange properties at higher temperatures so that its usefulness at these temperatures may be elucidated. Unfortunately very few studies have been made of the effect of temperature on the ion-exchange equilibrium[3]. We therefore summarise in this report the adsorption studies on five samples dried at different temperatures and on one sample using the equilibrating solution at higher temperatures.

EXPERIMENTAL

Materials

Stannic chloride crystals (B & A) and sodium arsenate heptahydrate (Poland) were used. All other reagents were of analytical grade.

Apparatus

An electric temperature-controlled SICO shaker, Philips X-ray unit and Bausch and Lomb spectronic 20 colorimeter were used for shaking, X-ray studies and spectro-

photometric measurements, respectively. A Perkin-Elmer Model 137 spectrophotometer was used for IR studies.

Method of preparation

Stannic arsenate was prepared by mixing the aqueous solutions of 0.10 *M* stannic chloride and sodium arsenate (1:1) at room temperature. In order to study the effect of drying temperatures, the precipitate so obtained was dried at different temperatures as given in Table I. The dried products were broken in demineralised water, converted to the hydrogen form as usual and then finally dried at 40°. For sorption studies, 50–150 mesh exchanger particles were used. Determination of chemical composition, solubility and ion-exchange capacity of stannic arsenate was made as reported previously[1,2].

Distribution coefficient

The K_D values for metal ions were determined as reported earlier[2]. In this case the total volume of equilibrating solution was 50 ml instead of 100 ml. Uranyl ions were determined spectrophotometrically with hydrogen peroxide[4] and other ions by titration with 0.002 *M* EDTA solution. The K_D values at different temperatures were ascertained by shaking at that temperature till equilibrium was achieved. In some cases the system was removed from the shaker and then allowed to attain room temperature before the final cation concentration was obtained. In other cases, the equilibrating solution was separated from the solid exchanger by decantation at the same temperature at which the study was performed, and then the final cation concentration was found in the separated liquid. The K_D values obtained by these two methods are recorded in Table III under Q and Q*, respectively. In both these cases the loading was 6% of the cation-exchange capacity owing to the high uptake of cations by stannic arsenate and to facilitate comparison of changes in K_D values.

Preparation of the column

For separation studies 1.50 g of the exchanger in the hydrogen form were placed in a 30 × 0.39 cm (I.D.) glass column with glass wool support. The column was washed with demineralised water till the washing was neutral. Loading the cations in the column was done as usual[2].

Measurement of IR absorption spectra

IR spectra of the samples of stannic arsenate in the hydrogen form dried at 40, 200 and 400° were measured with a standard KBr disk technique.

RESULTS

Properties

Almost all the properties of sample 1 are the same as reported in our earlier papers[1,2] on other samples of stannic arsenate. No appreciable decomposition, hydrolysis or change in appearance of exchanger particles was observed during the operations at elevated temperatures.

The ion-exchange capacity for K^+ of all the samples is found as given in Table I.

TABLE I

PROPERTIES OF STANNIC ARSENATE

Sample No.	*Drying temperature*		*Colour*	*Ion-exchange capacity (mequiv./g)*
	Initial (°C)	*Final (°C)*		
1	40	40	White	1.30
2	100	40	White	1.44
3	200	40	Dirty white	1.25
4	400	40	Bluish black	0.95
5	600	40	Bright bluish black	0.52

Sample 1 has a Sn–As ratio of 1:1. Its solubility in some common solvents is given in Table II.

TABLE II

SOLUBILITY OF STANNIC ARSENATE IN g/l $\times 10^{-3}$ AT $33 \pm 1°$

Sample No.	*Water*		*4 M HNO_3*		*4 M HCl*	
	Sn	*As*	*Sn*	*As*	*Sn*	*As*
1	1.30	55.00	52.00	62.00	Dissolves completely	
2	2.80	50.00	53.00	68.75	Dissolves completely	
3	2.90	50.00	61.00	68.75	500.00	625.00
4	2.30	57.50	56.00	93.75	Dissolves completely	
5	2.30	60.00	50.00	106.20	Dissolves completely	

The hydrogen form of the dry exchanger is shown to be amorphous by the powder method of X-ray analysis. The results of the sorption studies are summarised in Tables III–V and in Fig. 2.

Separations

Separation of Pb^{2+} from Zn^{2+} and Mn^{2+}. Since the K_D value for lead is appreciably higher than for other cations it can be separated from Zn^{2+} and Mn^{2+}; Zn^{2+} and Mn^{2+} are eluted by 1 *M* NH_4NO_3 quantitatively and Pb^{2+} with 1 *M* NH_4NO_3 in 0.5 *M* HNO_3. The results are given in Table VI.

Separation of UO_2^{2+} from numerous metal ions. Uranyl is adsorbed on the top of the column in the form of a yellow band. Dispersion of the yellow band is not observed on elution with water, 1 *M* NH_4NO_3 or 1 *M* NH_4NO_3 in 0.02 *M* HNO_3. However, metal ions other than uranyl can be eluted completely from the column using the above eluants (Table VII).

Separation of Cr^{3+} from numerous metal ions. On applying Cr^{3+} solution to the stannic arsenate column, a blue band forms on the top which is difficult to elute. The blue band persists and cannot be eluted with $NaNO_3$, NH_4NO_3, NaCl, NH_4Cl, and NH_4CNS solutions; dil. HCl, dil. HCl and NH_4Cl mixture, dil. HNO_3, dil. HNO_3 and NH_4NO_3 mixture, dil. H_2SO_4, dil. H_2SO_4 and H_2O_2 mixture. Fe^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+} are eluted quantitatively with 1 *M* NH_4NO_3 solution.

TABLE III

DISTRIBUTION COEFFICIENT OF METAL IONS UNDER A RANGE OF ELEVATED TEMPERATURES

Cations	K_D *values*[a] *(ml/g at pH 2) at different temperatures (°C)*						
	20°	*40°*	*60°*	*80°*		*95°*	
	Q	*Q*	*Q*	*Q*	*Q**	*Q*	*Q**
Mg^{2+}	22.18	20.00	31.30	29.23	34.40	28.40	34.40
Ca^{2+}	45.00	40.00	43.00	45.00	51.00	48.10	48.10
Sr^{2+}	54.28	50.00	50.00	54.29	58.80	63.10	54.30
Ba^{2+}	165.10	147.25	136.50	126.66	136.50	126.66	147.30
Zn^{2+}	80.50	80.50	90.85	102.42	180.50	108.70	178.30
Cd^{2+}	168.33	180.00	192.72	206.67	330.00	239.00	329.20
Fe^{3+}	T.A.[b]	T.A.	T.A.	T.A.	T.A.	T.A.	T.A.
Ni^{2+}	33.48	51.30	54.66	64.00	88.10	69.75	88.10
Co^{2+}	58.04	75.10	102.50	102.50	116.00	102.50	116.00
Al^{3+}	235.00	783.00	793.00	1240.00	1240.00	1240.00	2580.00
Ga^{3+}	280.00	122.00	2540.00	2540.00	2540.00	2540.00	2540.00
In^{3+}	810.00	T.A.	T.A.	T.A.	T.A.	T.A.	T.A.
Pb^{2+}	T.A.	T.A.	T.A.	T.A.	T.A.	T.A.	T.A.
Mn^{2+}	71.00	90.00	92.00	101.18	144.50	107.30	137.58
Cu^{2+}	220.00	273.50	320.00	380.00	740.00	473.00	740.00
Cr^{3+}	T.A.	T.A.	T.A.	T.A.	T.A.	T.A.	T.A.

[a] K_D in column Q: without quenching the ion-exchange reaction; K_D in column Q*: on quenching the ion-exchange reaction.
[b] T.A. = Total adsorbed.

TABLE IV

VARIATIONS OF K_D VALUES WITH TEMPERATURE FOR TRANSITION METAL IONS

Cations	$dK_D/dt \times 10^{-2}$			
	20°	*40°*	*60°*	*75°*
Zn^{2+}	00.00	51.80	54.60	37.60
Cd^{2+}	58.40	60.97	63.90	94.20
Ni^{2+}	87.30	52.05	50.27	47.88
Co^{2+}	85.30	112.50	74.10	59.28
Mn^{2+}	95.00	50.30	51.63	48.40
Cu^{2+}	267.50	250.00	266.00	337.30

IR spectra. The results of IR spectra are shown in Fig. 1. The spectra show four absorption bands: the first between 3700 and 2600 cm^{-1} with a maximum at 3500 cm^{-1}, the second between 1800 and 1500 cm^{-1} with maximum at 1625, the third between 1150 and 750 cm^{-1} with maximum at 840 cm^{-1} and the fourth between 650 and 400 cm^{-1} with maximum at 490 cm^{-1}. The first band is characteristic of interstitial water (or free water) and hydroxyl groups, the second of interstitial water, the third of $HAsO_4^{2-}$ groups and the fourth of the Sn–O bond.

DISCUSSION

The results show that stannic arsenate is specific for the separation of Pb^{2+}, Fe^{3+}, Cr^{2+} and UO_2^{2+} from numerous metal ions. Quantitative separations are thus

TABLE V

DISTRIBUTION COEFFICIENTS OF METAL IONS ON STANNIC ARSENATE SAMPLES DRIED AT DIFFERENT TEMPERATURES AT 33 ± 1°

Cations	*K_D values (ml/g at pH 2) (Sample No.)*				
	1	*2*	*3*	*4*	*5*
UO_2^{2+}	900.00	900.00	566.66	900.00	900.00
Mg^{2+}	98.46	75.20	17.30	43.50	51.80
Ca^{2+}	138.10	94.90	31.20	74.70	74.70
Sr^{2+}	152.20	136.90	34.66	123.00	102.00
Ba^{2+}	240.00	213.8	94.34	191.50	176.00
Zn^{2+}	317.00	284.90	92.30	263.10	263.10
Cd^{2+}	861.30	716.30	122.20	380.00	380.00
Hg^{2+}	52.90	47.60	7.00	7.00	9.64
Fe^{3+}	T.A.[a]	T.A.	T.A.	T.A.	T.A.
Ni^{2+}	149.40	162.10	34.36	118.40	101.50
Co^{2+}	202.50	227.50	51.36	168.40	168.40
Al^{3+}	1 900.00	4 900.00	1 900.00	1 900.00	1 900.00
Ga^{3+}	4 900.00	4 900.00	4 900.00	4 900.00	4 900.00
In^{3+}	13 600.00	13 600.00	5 380.00	5 380.00	5 380.00
Pb^{2+}	T.A.	T.A.	T.A.	T.A.	T.A.
Mn^{2+}	288.00	265.80	60.90	169.80	156.10
Cu^{2+}	760.00	746.60	416.00	1 190.00	916.00
Cr^{3+}	T.A.	T.A.	T.A.	T.A.	T.A.

[a]T.A. = Total adsorbed

TABLE VI

SEPARATION OF Pb^{2+} FROM Zn^{2+} AND Mn^{2+} ON STANNIC ARSENATE (SAMPLE NO.2) COLUMNS

Sample No.	*Mixture separated*	*Eluants*	*Volume of effluent (ml)*	*Taken (mg)*	*Found (mg)*
1	Zn^{2+}	1 *M* NH_4NO_3	60	2.03	2.03
	Pb^{2+}	1 *M* NH_4NO_3 in 0.5 *M* HNO_3	40	3.110	3.110
2	Mn^{2+}	1 *M* NH_4NO_3	60	0.8237	0.8190
	Pb^{2+}	1 *M* NH_4NO_3 in 0.5 *M* HNO_3	40	3.110	3.110

possible of Pb^{2+} from Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} (Table VI) and of UO_2^{2+} from Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+} and Sr^{2+}. The irreversible adsorption of Cr^{3+} suggests the formation of a chromium arsenate.

Table III shows that the K_D values after cooling the system to room temperature are lower than those determined by quenching the ion-exchange reaction at the temperature under study. This shows the reversibility of the ion-exchange reaction with respect to temperature. There is no appreciable change in the K_D values of the alkaline earth and trivalent metal ions on raising the temperatures of equilibrating solution from 20–95°. However the increase in K_D values of divalent transition metal ions such as Zn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} is significant at elevated temperatures. Since the enthalpy change in the ion-exchange reaction is small, it appears that the

TABLE VII

SEPARATION OF URANYL FROM Mn^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, Cu^{2+} AND Fe^{2+} ON STANNIC ARSENATE (SAMPLE NO. 2) COLUMNS

Sample No.	*Mixture separated*	*Eluants*	*Volume of effluent (ml)*	*Taken (mg)*	*Found (mg)*
1	Mn^{2+}	1 *M* NH_4NO_3	60	0.8237	0.8190
	UO_2^{2+}	0.50 *M* HNO_3	60	3.857	3.753
2	Ca^{2+}	1 *M* NH_4NO_3	60	0.6010	0.5873
	UO_2^{2+}	0.50 *M* HNO_3	60	3.857	3.753
3	Mg^{2+}	1 *M* NH_4NO_3	60	0.3646	0.3711
	UO_2^{2+}	0.50 *M* HNO_3	60	3.857	3.904
4	Sr^{2+}	1 *M* NH_4NO_3 in 0.02 *M* HNO_3	60	1.322	1.322
	UO_2^{2+}	0.50 *M* HNO_3	60	3.857	3.805
5	Cu^{2+}	1 *M* NH_4NO_3 in 0.02 *M* HNO_3	60	0.9533	0.9696
	UO_2^{2+}	0.50 *M* HNO_3	60	3.857	3.857
6	Fe^{2+}	1 *M* NH_4NO_3	60	1.325	1.307
	UO_2^{2+}	0.50 *M* HNO_3	60	3.857	3.904

reaction following ion-exchange in these cases involves considerable enthalpy change[5]. Therefore the change in K_D values with respect to temperature has been calculated for the 6 metal ions mentioned (Table IV). The plots of dK_D/dt *versus* dt (Fig. 2) show a gradual increase in the adsorption of Cu^{2+} and Cd^{2+} with an increase in temperature, while the adsorption of Co^{2+}, Ni^{2+}, Mn^{2+} and Zn^{2+} first increases and then finally decreases on increasing the temperature of the system.

The explanation of these changes seems to be quite complicated due to continuous changes in hydrated radii of ions, the structure of exchanger, dielectric constant and the pH of the equilibrating solution with variations in temperature (20–95°). A few points may, however, be stressed[3]. In almost all cases $Qt/Q95° < 1$ except for Mg^{2+} and Ba^{2+}. These exceptions may be due to specific interactions[3]. The data recorded in Table V show that the K_D values of metal ions on stannic arsenate decrease[6]

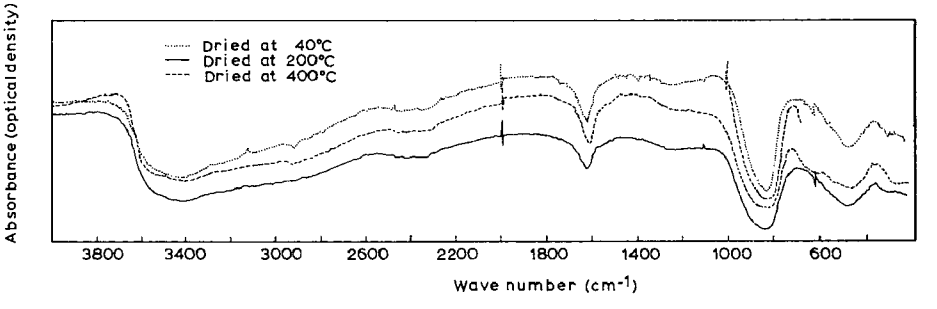

Fig. 1. Infrared absorption spectra of stannic arsenate dried at different temperatures by KBr disk method.

as the drying temperature increases. However, the K_D values of sample 3 dried at 200° are smaller than of other samples because stannic arsenate loses interstitial water molecules up to 300°, and after this temperature condensation starts. Removal of interstitial water molecules increases the degree of crosslinking while the condensation decreases the concentration of exchangable hydrogen ions in the sorbent phase and therefore causes a lower sorption of cations. Furthermore the degree of crosslinking in sample 3 (dried at 200°) is maximal, therefore the lower values of distribution coefficients are obtained on the sample mentioned.

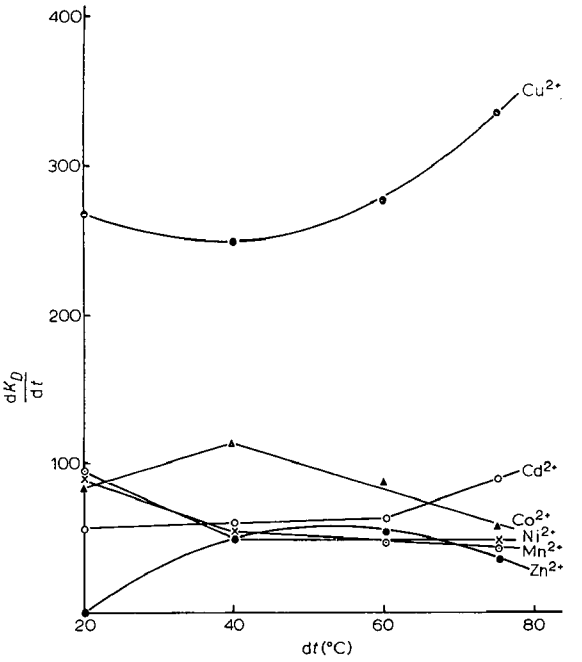

Fig. 2. Change in K_D value with respect to temperature of divalent transition metal ions.

All the colour changes in stannic arsenate at higher temperatures are due to the formation of tin and arsenic oxides. Since similar colour changes are observed when a mixture of stannic chloride and sodium arsenate was heated at higher temperatures (400–800°). However, there is no change in colour when sodium stannate and sodium arsenate were heated separately, while a remarkable change in colour (bluish black) was visualised when stannic hydroxide was heated at temperatures mentioned above.

A comparison of IR spectra of stannic arsenate dried at different temperatures as well as compared to the IR spectra of zirconium phosphate[7] and antimonic acid[8] shows that all the three samples contain interstitial water molecules, replaceable hydrogen ions in the form of OH^- group, As–O and Sn–O bonds. Therefore on the basis of IR analysis and the properties observed, a general formula for this product may be postulated as $SnO\ (AsO_3OH)\ nH_2O$. The above conclusions do not contradict the results of thermogravimetric and X-ray analysis reported in our earlier paper[1]. The good performance of stannic arsenate at elevated temperatures and its thermal

stability makes it promising for the ion-exchange work at higher temperatures such as purification of high-pressure cooling water of pressurised water-cooled reactors.

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Notes

CHROM. 5056

Gas chromatographic separation of methylphenols on tricresylphosphates using open tubular columns

Gas chromatography is widely employed for analysis of mixtures of phenol, cresols and xylenols used as starting materials for phenol–formaldehyde polycondensates as well as for analysis of final resins by pyrolysis obtaining phenols of related composition as the raw material used[1–3]. Various types of liquid phases have been proposed for gas chromatographic analysis of methylphenols. Phosphates, especially tri(2,4-xylenyl)phosphate and tricresylphosphate belong to the most selective liquid phases for methylphenols[1,2,4–7]. The relatively good separation of some methylphenols on tricresylphosphate as the liquid phase[8,9] prompted us to study the selectivity of pure triortho- (TOCP), trimeta- (TMCP), and tripara-cresylphosphate (TPCP) towards phenol, cresols and xylenols using open tubular columns.

Experimental

Apparatus. A Fractovap Model GI (Carlo Erba, Milan) gas chromatograph equipped with a flame ionisation detector was used for these studies. Nitrogen was used as the carrier gas. The stainless-steel open tubular columns were 20 m long, and had an I.D. of 0.01 in.

Chemicals. Methylphenols were obtained commercially from Caolite and Chem. Products, London, and from Aldrich Chem. Comp., Wisc. Individual tricresylphosphates were prepared from pure cresols (in the purity of 98–99 %, by gas chromatography) with the reaction of $POCl_3$.

Column preparation. The plug method was used. Columns were coated with a solution (0.3–0.5 ml) of the liquid phase (95 mg) and orthophosphoric acid (5 mg, 85 w/w) in acetone (1.0 ml). Prior to use the columns were conditioned at 120° for 3 h under carrier gas flow.

Results and discussion

The net retention times of phenol, cresols and xylenols relative to 3-methylphenol on TOCP, TMCP and TPCP at a temperature of 120° are given in Table I. As could be expected from the literature, 2,6-dimethylphenol and phenol, 3- and 4-methylphenol, and 2,4- and 2,5-dimethylphenol are the critical pairs of the 10-component mixture. It is evident from Table I that the column with TPCP allows the best separation for closely related compounds. Only poor resolution for all pairs was obtained on TOCP. The pair of 2,6-dimethylphenol and phenol is best resolved on the column with TMCP while 2,4- and 2,5-dimethylphenol is only poorly resolved on this column.

The elution order of phenol and 2,6-dimethylphenol (steric hindrance) indicates

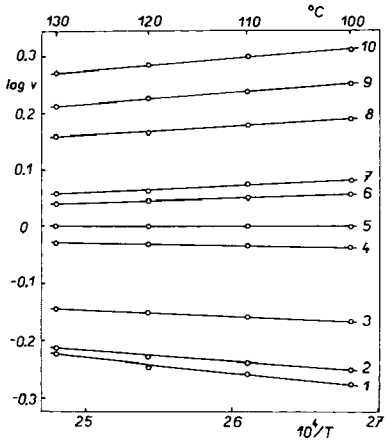
TABLE I

THE NET RETENTION RELATIVE TIMES OF PHENOL, CRESOLS AND XYLENOLS AT 120°

No.	*Compound*	*TOCP*	*TMCP*	*TPCP*	*TPP*
1	2,6-Dimethylphenol	0.57	0.54	0.55	0.56
2	Phenol	0.59	0.60	0.58	0.61
3	2-Methylphenol	0.71	0.71	0.69	0.69
4	4-Methylphenol	0.93	0.93	0.92	0.94
5	3-Methylphenol	1.00	1.00	1.00	1.00
6	2,4-Dimethylphenol	1.11	1.10	1.10	1.07
7	2,5-Dimethylphenol	1.16	1.15	1.16	1.11
8	2,3-Dimethylphenol	1.46	1.43	1.45	1.41
9	3,5-Dimethylphenol	1.68	1.65	1.71	1.65
10	3,4-Dimethylphenol	1.92	1.92	1.94	1.92

that hydrogen bonds between phenols and the liquid phase play a part in the separation. The stronger the hydrogen bond the better the separation of this pair on polar liquid phases. It seems from this observation that the relative polarity of the liquid phases increases in order of TOCP, TPCP and TMCP, which is in agreement with the polarity effects of the methyl group in benzene nuclei. (The relatively poorer hydrogen bond on TOCP towards TPCP can be attributed to sterical hindrance.) For comparison, in Table I are also included the net retention times on triphenylphosphate (TPP). The elution sequence of the other phenols is in the order of their boiling points. The effect of the operating temperature on relative retention times at temperatures of 100, 110, 120 and 130° is illustrated in Figs. 1–3. As can be seen, the temperature changes only slightly affect the separations.

A chromatogram of a mixture of phenol, cresols and xylenols using TPCP as



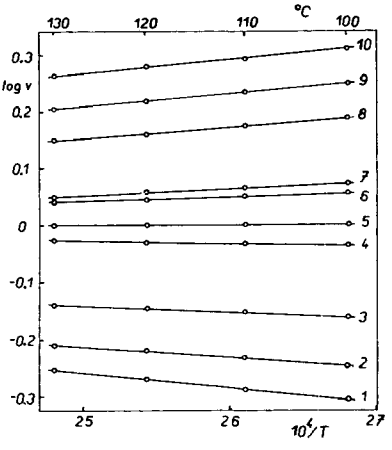
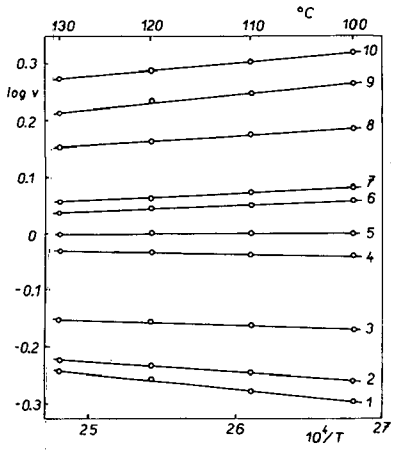


Fig. 1. Variation of the logarithm of relative retention × log v of phenol and methylphenols on TOCP column with change of temperatures (No. identification in Table I).

Fig. 2. Variation of the logarithm of relative retention × log v of phenol and methylphenols on TMCP column with change of temperatures (No. identification in Table I).



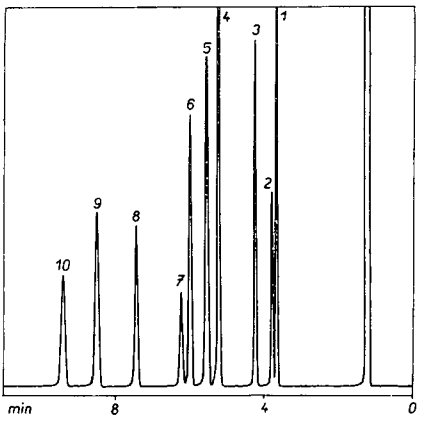


Fig. 3. Variation of the logarithm of relative retention × log v of phenol and methylphenols on TPCP column with change of temperatures (No. identification in Table I).

Fig. 4. Gas chromatogram of phenol and methylphenols on TPCP liquid phase at 130° (See peak names, Table I).

the liquid phase is illustrated in Fig. 4. The high efficiency of the open tubular column and the good selectivity of the liquid phase used allowed a complete separation of the 10 compounds in less than 10 min. This column had a good thermal stability up to 135°. The undesirable effect of the metallic wall of the column usually resulting in tailing was eliminated by addition of phosphoric acid to the liquid phase[10].

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Institute of Chemistry, Komensky University, Bratislava (Czechoslovakia) JÁN HRIVŇÁK

Research Institute of Agrochemical Technology, Bratislava (Czechoslovakia) EMANUEL BEŠKA

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CHROM. 5062

Gas chromatographic assay of phenylbutazone in biological fluids

The quantitative estimation of phenylbutazone (I) in biological fluids is very difficult since only a small amount of the drug is present. The presence of its metabolites [*i.e.*, 1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-*n*-butylpyrazolidine (II) and 1,2-diphenyl-3,5- dioxo-4-(3-hydroxybutil)-pyrazolidine (III)][1,2], as well as other matter that interferes with the assay, complicates the quantitative estimation. The various methods consisting of selective extractions of I followed by a spectrophotometric determination[2–5] are clearly unsatisfactory for the specificity.

Our attempts to overcome the drawbacks mentioned above by using paper or thin-layer chromatography were unsatisfactory, due to some decomposition observed for I just after it was applied. This phenomenon is quite similar to that reported by MACEK[6] for a number of substances of the pyrazolone type. While seeking an alternate method which would be more sensitive and specific, we turned our attention to achieving by gas chromatography the quantitation of I in biological fluids. Promazine was chosen as an internal standard because its retention time was found to be very close to that of I (15 and 17.5 min, respectively, under our working conditions).

Experimental

Equipment and working conditions. A Pye 25 Series 104 gas chromatograph fitted with a dual flame ionisation detector head and coiled Pyrex glass columns (150 × 0.4 cm) was used. The columns were packed with 2.5% silicone rubber SE-30 on 100–120 mesh Gas-Chrom Q. The working conditions were: column temperature, 195°; nitrogen flow, 40 ml/min; hydrogen flow, 40 ml/min; air flow, 400 ml/min.

Assay. A mixture of 1–200 μg of I, 10 μg–2 mg of II and 10 μg–2 mg of III was dissolved in 0.1 ml of acetone and was then added to 1 ml of rat serum or urine. The sample was then acidified with 2 ml of 1 *N* HCl and extracted by shaking for 30 min with 20 ml of highly pure *n*-heptane. The organic phase was separated, dried over

TABLE I

RECOVERY OF PHENYLBUTAZONE FROM RAT SERUM

Sample	*Phenylbutazone added (μg)*	*1-Phenyl-2-p-hydroxyphenyl-3,5-dioxo-4-n-butyl-pyrazolidine added (μg)*	*1,2-Diphenyl-3,5-dioxo-4-(3-hydroxybutyl)-pyrazolidine added (μg)*	*Phenylbutazone found (μg)*	*Δ %*
1	1.25	11.98	10.75	1.31	+4.80
2	1.13	11.76	10.51	1.18	+4.43
3	50.85	500.71	499.31	49.65	−2.36
4	50.05	501.07	502.25	50.50	+0.90
5	101.10	1002.05	998.50	102.50	+1.39
6	99.68	999.57	1000.35	100.52	+0.84
7	149.95	1495.75	1501.57	148.00	−1.30
8	150.38	1502.37	1498.35	154.15	+2.51
9	201.28	2002.25	1997.75	203.75	−1.23
10	200.15	2001.50	2003.24	197.50	−1.32

TABLE II

RECOVERY OF PHENYLBUTAZONE FROM RAT URINE

Sample	*Phenylbutazone added* (μg)	*1-Phenyl-2-p-hydroxyphenyl-3,5-dioxo-4-n-butyl-pyrazolidine added* (μg)	*1,2-Diphenyl-3,5-dioxo-4-(3-hydroxybutyl)-pyrazolidine added* (μg)	*Phenylbutazone found* (μg)	Δ%
1	1.15	10.85	10.58	1.21	+5.22
2	1.23	11.50	10.75	1.29	+4.88
3	51.25	505.30	500.60	53.15	+3.70
4	50.37	498.50	501.51	52.00	+3.13
5	99.81	1001.00	1005.77	95.60	−4.22
6	100.75	999.75	995.60	99.75	−0.99
7	151.37	1510.55	1500.43	154.15	−1.84
8	150.55	1505.00	1502.37	152.20	−1.10
9	200.00	2005.57	2008.17	196.50	+1.75
10	198.75	1995.68	1999.46	197.30	−0.73

Na_2SO_4, and filtered; 15 ml of this extract were then evaporated to dryness, taking care that the temperature did not exceed 20°. The residue was taken up in 1 ml of standard solution (1–200 μg of promazine dissolved in 1 ml of carbon disulfide in a ground-glass stoppered tube) and the new solution was evaporated to dryness at room temperature in a drier connected to a vacuum pump. The new residue was dissolved in 50–100 μl of carbon disulfide and 3–6 μl of this solution were injected into the gas chromatograph.

Results and discussion

Tables I and II report, respectively, the results of the gas chromatographic assay of I in rat serum and urine after addition of a mixture of I, II and III. It can be seen that the standard error did not exceed 5%. The sensitivity of this technique was 1 μg of I per ml of serum or urine. The other substances which are normally extracted from these fluids with *n*-heptane did not interfere with the assay. The metabolites of I were somewhat extracted from *n*-heptane, but they were not eluted from the gas chromatographic columns under our working conditions.

Research Laboratories, R. PEREGO
Istituto De Angeli, E. MARTINELLI
Via Serio, 15-I 20139 *Milan (Italy)* P. C. VANONI

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CHROM. 5058

Dünnschichtchromatographisch-enzymatischer und gaschromatographischer Nachweis von 4,4'-Dichlorbenzophenon und seinen Abbauprodukten

Die Chlorkohlenwasserstoff-Insektizide stellen wegen ihrer grossen Persistenz ein ernstes Problem für die Umwelt dar, nicht zuletzt deswegen, weil das stabilste Endprodukt der Abbaukette ebenso unbekannt ist wie dessen Wirkung. GEIKE[1] konnte zeigen, dass Chlorkohlenwasserstoffe auf Dünnschichtplatten die Rinderleber-Esterase beeinflussen und nach UV-Bestrahlung in relativ starke Esterasehemmer übergehen. Weiter konnte gezeigt werden, dass die Chlorkohlenwasserstoff-Insektizide nach UV-Bestrahlung Trypsin hemmen[2]. In allen diesen Fällen entstehen zahlreiche, bisher nichtidentifizierte Bestrahlungsprodukte. Obwohl die anderen Chlorkohlenwasserstoffe sicher nicht weniger schädlich sind als DDT, ist gerade dieser Wirkstoff in die allgemeine Diskussion geraten — wahrscheinlich weil in letzter Zeit über seine Wirkung alarmierende Nachrichten publiziert wurden.

Nach UV-Bestrahlung entstehen nicht nur eine Reihe von Esterase-[1] und Trypsin-hemmenden[2] Substanzen aus DDT, sondern es wird auch die Lactoperoxydase gehemmt und das Absorptionsspektrum verändert[3]. Arbeiten von CHISHOLM *et al.*[4] und FLECK[5] zeigten, dass unter dem Einfluss von UV- und Sonnenlicht aus DDT unter anderem 4,4'-Dichlorbenzophenon entsteht, doch liegen bisher keine Daten über seine Wirkung auf Enzyme vor. Vorliegende Arbeit untersucht daraufhin auf dünnschichtchromatographischer Basis die Wirkung dieser Substanz und möglicher Abbauprodukte auf verschiedene Enzyme.

4,4'-Dichlorbenzophenon (Eastman Organic Chemicals, Rochester) wurde in einer Konzentration von 10 und 1 mg/ml Aceton gelöst. Mengen von 100 bis 4 μg Substanz wurden auf Kieselgel G-Platten[1] aufgetragen und in Cyclohexan–Aceton (10:4) chromatographiert. Nach dem Entwickeln wurde zunächst mit Puffer und anschliessend mit Rinderleber-Esterase[1], Trypsin[2] (E.C. 3.4.4.4 — Merck, Darmstadt) oder saurer Phosphatase (E.C. 3.1.3.2 — Boehringer; 2 U/mg mit 1 mg/ml Na-Acetat-Puffer pH 4.8 bei 5–7 ml/Platte) besprüht und eine halbe Stunde inkubiert. Danach wurde das jeweilige Substrat gesprüht (im Falle der Phosphatase 10 mg/ml Nitrophenylphosphat bei 5–7 ml/Platte). Die UV-Bestrahlung erfolgte nach oder vor und nach dem Entwickeln, wie an anderer Stelle beschrieben[1,2], wobei in diesem Falle das Besprühen mit Puffer stets unmittelbar vor der Enzymapplikation erfolgt.

Weder mit Trypsin noch mit saurer Phosphatase, die von einer Reihe von Chlorkohlenwasserstoff-Insektiziden und deren UV-Bestrahlungsprodukten gehemmt wird[6], konnte unter Normalbedingungen oder nach UV-Bestrahlung eine Hemmung durch 4,4'-Dichlorbenzophenon festgestellt werden. Von den drei untersuchten Enzymen wird lediglich die Rinderleber-Esterase beeinflusst. Wie aus der Tabelle I hervorgeht, sind unter Normalbedingungen zwei Hemmflecke auf der Platte festzustellen, die nicht mit dem durch Rhodamin B nachgewiesenen 4,4'-Dichlorbenzophenon übereinstimmen. Von diesen beiden Hemmflecken ist der untere erheblich kleiner und etwas schwächer. Im Gaschromatogramm können bei Applikation von 1 μg neben dem 4,4'-Dichlorbenzophenon sogar drei Substanzen nachgewiesen werden, von denen die stärkste etwa 45 %, die zweite etwa 12 % und die dritte schliesslich knapp 4 % des 4,4'-Dichlorbenzophenonpeaks ausmachte. Werden 10 μg in den Gas-

TABELLE I

WIRKUNG VON 4,4'-DICHLORBENZOPHENON UND ZWEIER ANDERER SUBSTANZEN NACH VERSCHIEDENEN VORBEHANDLUNGEN AUF RINDERLEBER-ESTERASE

Nachweisgrenze bei 4 μg Auftragmenge.

Substanz	*Ohne Vorbehandlung*	*UV nach dem Entwickeln*	*UV vor und nach dem Entwickeln*	*hR_F-Werte nach UV*	*hR_F-Werte Rhodamin B*
4,4'-Dichlorbenzophenon	—	+ +	+ +	70	70
Substanz X-1	+ +	+ +	+ +	14.6	—
Substanz X-2	+	+	+	11	—

chromatographen eingespritzt, so nehmen vornehmlich die erst- und zweitgenannte Substanz stark ab und sind bei 20 bzw. 50 μg Einspritzmenge nicht mehr zu finden, während die dritte Fremdsubstanz erhalten bleibt. Diese Erscheinung, dass der Anteil der Abbauprodukte bei grösseren Einspritzmengen prozentual abnimmt, ist in der Gaschromatographie von Pflanzenschutzmitteln gelegentlich festzustellen[7], ein völliges Verschwinden konnte bisher jedoch nicht beobachtet werden*. Alle drei Begleitsubstanzen lassen sich nicht mit Rhodamin B nachweisen. Erst nach UV-Bestrahlung erscheint neben den ursprünglichen Hemmflecken, an deren Intensität sich nichts ändert, auch 4,4'-Dichlorbenzophenon als Hemmfleck. Die Nachweisgrenze liegt bei einer Auftragmenge von 4 μg auf die Dünnschichtplatten. Legt man die Anteile zugrunde, die sich aus dem Gaschromatogramm mit 1 μg Substanz ergeben, dann liegt der Nachweis für die Abbauprodukte bei etwa 1,1 bzw. 0,3 μg, wobei angenommen wurde, dass den beiden gefundenen Hemmflecken die zwei neben dem 4,4'-Dichlorbenzophenon auftretenden Hauptpeaks im Gaschromatogramm zuzuordnen sind. UV-Bestrahlung vor und nach dem Entwickeln führt gegenüber den Ergebnissen, wo nur nach dem Entwickeln bestrahlt wurde, zu keiner Änderung in der Hemmintensität der vorhandenen oder zum Auftreten neuer Flecke.

Aufgrund der vorliegenden Ergebnisse erscheint es unwahrscheinlich, dass es sich bei den unter Normalbedingungen auftretenden Esterasehemmern um Verunreinigungen von der Synthese des 4,4'-Dichlorbenzophenons her handelt, da die untersuchte Substanz aufgrund der Angaben der Hersteller keine grösseren Verunreinigungen besitzt und nach Bestrahlung mit UV-Licht ebenfalls in Esterasehemmer übergeht. Eine Identifizierung dieser Abbauprodukte wird für die Zukunft angestrebt, um dann mit reinen Substanzen eingehendere Untersuchungen über ihre Wirkung auf Enzyme durchführen zu können.

Biologische Bundesanstalt für Land- und Forstwirtschaft, F. GEIKE
Institut für Pflanzenschutzmittelforschung, D1 Berlin 33 (B.R.D.)

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* Bei einer acht Wochen später durchgeführten gaschromatographischen Untersuchung der gleichen Lösungen liess sich ausser 4,4'-Dichlorobenzophenon keine weitere Substanz nachweisen. Die erhebliche Schwanzbildung des Lösungsmittel- und 4,4'-Dichlorbenzophenonpeaks, die sogar über die früheren Substanzpeaks hinausreicht, spricht jedoch für einen weiteren Abbau.

CHROM 5078

Improvement of the starch–iodide method for detection of imides and other NH-containing compounds on thin-layer chromatograms

The best methods currently available for the chromatographic detection of ninhydrin-negative nitrogenous compounds such as imides, amides and cyclic peptides involve chlorination followed by detection of the N-chlorinated areas by spraying with a chlorine-sensitive chromogenic reagent, after removal of the background chlorine by aeration. A chromogenic reagent widely used with paper chromatograms consists of a solution containing potassium iodide and starch[1–3], but this reagent has not proved sufficiently sensitive for some of the more micro-scale TLC systems now in use. Also since it is a somewhat viscous aqueous solution it is difficult to spray it evenly and to avoid distortion of delicate silica gel thin layers. These defects greatly impair definition and render the method unreliable. A solution of benzidine in 50 % methanol has proved far more sensitive[4,5] and reliable as a chromogenic spray, particularly for thin-layer chromatograms and it has been particularly valuable for the detection of the cyclic imides produced from pyrrole rings in the micro-degradation technique of RÜDIGER developed for the micro-scale investigation of tetrapyrrole structure[6–8]. However, benzidine is a potent carcinogen and its use as a spray reagent constitutes a particularly insidious health hazard unless complex and very inconvenient precautions are observed.

In our efforts to find a safe and satisfactory replacement for the benzidine spray, we returned eventually to the iodide–starch reagent and found that a simple modification makes it almost as effective as benzidine. Starch is simply included in the thin-layer material from the beginning so that the plates, after chromatography, chlorination and aeration, need only be treated with potassium iodide. This can be sprayed on in 75 % acetone much more finely and evenly than the iodide–starch solution so that definition is improved to the same level as that obtainable with the benzidine spray. Furthermore, the acetone solution does not disrupt silica gel thin layers, these being in any case far more stable in the presence of the added starch which acts as an effective binder. The level of sensitivity approaches, thought it still does not quite equal, that obtainable with benzidine and has proved completely satisfactory for use in conjunction with RÜDIGER's microdegradation technique and the various refinements thereof. The presence of the starch in the Silica Gel G layers does not affect chromatography of the pyrrole-derived imides in the solvent systems normally used for their separation[6–8], and the technique of performing the chromic acid degradation of microgram quantities of tetrapyrroles directly on the starting point on the thin-layer chromatograms prior to chromatography[6,7] is also unaffected.

The modification also leads to improved detection limits and much better definition with other compounds, such as peptides, on thin-layer chromatograms and the presence of starch in the thin layers does not seem to be detrimental to their chromatography, although this should be checked out for each chromatographic system before use.

Experimental details

A 0.5 % solution of soluble starch is made in the usual manner by mixing the starch with a little cold water, stirring in boiling water and filtering the cooled solution.

The adsorbent for the thin-layer plates is slurried in this starch solution rather than in water, but the plates are otherwise made in the usual manner except that they should not be heated above 90° during activation. Silica Gel G layers are activated at 90° for at least 1 h. As mentioned above, such layers are much firmer and easier to work with than layers made without starch.

Chromatographic procedures are carried out as usual. After chromatography, care must be taken to thoroughly evaporate off any positive-reacting components of the chromatographic system such as ammonia. Any polar components, particularly water, must also be thoroughly dried off, otherwise sensitivity will be impaired.

Detection is achieved by first chlorinating with 1% *tert.*-butyl hypochlorite in cyclohexane sprayed evenly over the chromatogram. (As reported by MAZUR *et al.*[3] for paper chromatograms, chlorination with this reagent is more convenient and results in better sensitivity than chlorination with chlorine gas.) The excess hypochlorite is removed by aerating the chromatogram in a stream of cool dry air (*e.g.* in a partly open fume cupboard). This usually takes 15–30 min and can be monitored by spotting aliquots of the subsequent iodide spray onto previously assigned blank regions of the chromatogram to check the background. When this is negative or very low, the chromatogram is immediately sprayed with a fresh 1% solution of analytical grade potassium iodide in acetone–water (3:1). Positive substances immediately appear as brown spots. The intensity of the spots can be increased if desired by allowing the acetone to evaporate off briefly and then moistening the chromatogram evenly with a finely atomised spray of water acidified with a little hydrochloric acid. The spots then assume the blue-black hue characteristic of the starch–iodine complex in aqueous solution.

In order to achieve maximum sensitivity and definition it is necessary to control or monitor the aeration step carefully to ensure that the background is adequately cleared but that aeration is not prolonged unnecessarily beyond that point, as this results in excessive breakdown of the labile N-chloro groups. Such breakdown mainly takes place by hydrolysis, so that sensitivity is also dependent on careful initial removal of any moisture from chromatograms before spraying and on the dryness of the air during aeration. It is essential to use a fresh solution of iodine-free potassium iodide for the visualization spray, otherwise a heavy background is obtained.

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Department of Biochemistry, University College, Galway (Ireland)

S. D. KILLILEA
P. O'CARRA

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CHROM. 5107

Activity stain for urocanase and histidase on polyacrylamide gel

Recently methods were given for the location of histidase (L-histidine ammonia-lyase, EC 4.3.1.3) and urocanase on polyacrylamide gel. To detect histidase, KLEE[1] absorbed histidine on paper which he placed on the gel. This paper was removed and analyzed for urocanate. Histidase and urocanase have also been detected by treating the gel with the substrate, and photographing the gel under an ultraviolet (UV) light[2]. Another technique for the identification of urocanase was based on the reaction of imidazolepropionate, the product of the urocanase reaction, with 2,6-dichlorophenolindophenol. This method reveals a transparent band on a blue background[2]. GORDON[3] summarizes several methods for observation of enzyme activity on gels, but he does not list any activity stain which utilizes the UV quenching ability of substrates or products.

This report presents a method for location of histidase and urocanase directly on the gel. Urocanate, the substrate or product of these enzymes, absorbs UV light and appears as a dark area on an eosin-treated gel in contrast to the fluorescent yellow area found in the absence of urocanate. A similar procedure might be adapted to detect other enzymes with a substrate or product which absorbs UV light. A preliminary report of this technique has been given[4].

Materials and methods

Pseudomonas putida A.3.12 was grown as previously described[5]. Cells were frozen in 1 g pellets, disrupted in a Hughes press and suspended in two times their volume in 0.01 *M* phosphate buffer, pH 7.0. The crude extract was obtained by centrifugation at 4°.

Crude extracts (3 mg of protein) were subjected to electrophoresis on polyacrylamide gel slabs by the method previously described[6]. The gels were stained when required with 1% Aniline Blue Black in 7% acetic acid to detect about 12 to 16 protein bands (Fig. 1B). In this method, the extract can be placed in a band at the origin, and after electrophoresis various portions of the gel slab can be stained for protein or enzyme activity, or left unstained and portions corresponding to bands excised.

Experimental

The position of histidase and urocanase in the polyacrylamide gel was located with an activity stain. To locate urocanase, a portion of the gel slab (10 × 55 mm) representing a single channel (150 μg of protein, crude extract) was excised from the larger slab and 1 ml of 0.04 *M* urocanate was applied on the surface of the gel. After 10 min at room temperature, the excess urocanate was washed off with distilled water. The gel was then flooded with 15 ml of 0.1% eosin and decanted after 5 min. When viewed under UV light, the gel had a subdued, darkened color because the fluorescence of eosin was quenched by the absorbance of urocanate (maximum 277 nm). At the position of urocanase a yellow band appeared because of the fluorescence of eosin easily visible in the absence of urocanate. The exact location of the band was observed in a Chromatovue cabinet with the 254 nm light source. By the time the

band became intense enough to photograph (Fig. 1C) it had become more diffuse.

Two histidase bands (fast, F; slow, S) were revealed when 1 ml of 0.04 *M* L-histidine was used in place of urocanate and eosin was applied before instead of after the substrate. At the locations of histidase activity dark bands appeared against a bright yellow background due to the UV absorption of the product, urocan-

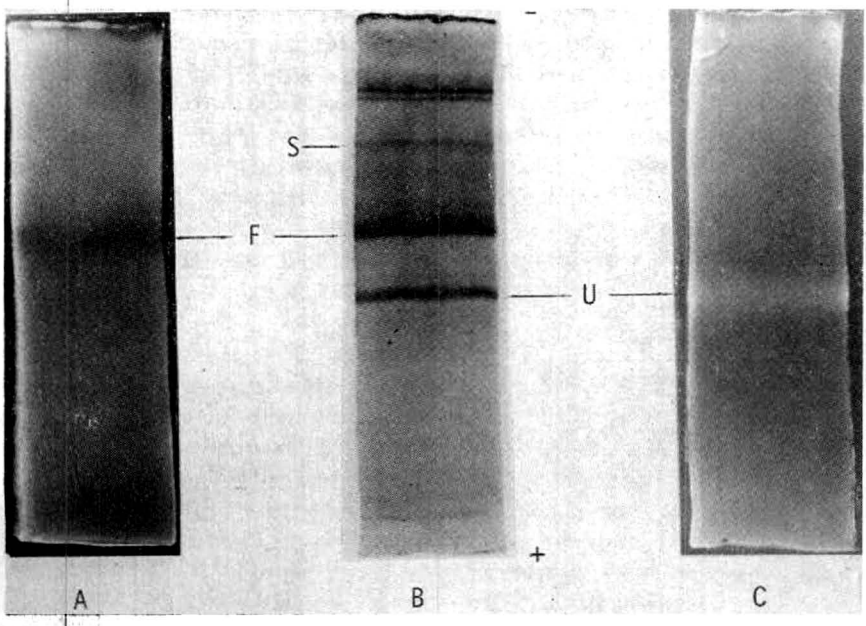

Fig. 1. Detection of histidase and urocanase bands after gel electrophoresis. (A) This photograph under UV light shows the dark band of histidase-F observed with the activity stain for histidase after 5 min. (B) This photograph under white light shows protein bands on a gel stained with Aniline Blue Black. (C) This photograph under UV light shows the bright (yellow) urocanase band observed with the activity stain for urocanase after 5 min. The sample on each portion of gel contained about 150 μg of protein. All three gel portions came from a single electrophoresis run. F, Histidase-F; S, histidase-S; U, urocanase.

ate. Fig. 1A is a photograph of a typical activity stain for histidase-F after 5 min incubation. Histidase-S (Fig. 1) was visible to the eye after 30–60 min but could not be easily photographed.

Both enzymes were revealed by longer incubation and careful observation. Fig. 2 is a photograph of an eosin-treated portion of gel which was incubated with L-histidine for 1 h. Both histidase bands and the urocanase band were visible. During 1 h incubation, the histidase converted histidine to urocanate (black background) which diffused through the gel. At the location of urocanase, this urocanate disappeared and a bright area appeared on the gel. Thus, it was possible to locate both enzymes with a single activity stain although the longer time required for a photograph makes the bands appear diffuse. The bands can be located precisely by observation at short intervals and by marking the gel with a slit when the bands first appear. When crude extract was heated (80°, 15 min) the urocanase and the histidase-S bands were no longer seen. Therefore, the electrophoresis was carried out at 4° as a precaution.

The gels were cut into strips, eluted and the enzymatic activity tested by

spectrophotometric and chromatographic methods. Once the enzymes were located by the activity stains on polyacrylamide gel, the bands from neighboring unstained gel were excised, eluted[6] and assayed on a spectrophotometer[5,6]. Activities ($\Delta A/5$ min at 277 nm) of histidase-F, histidase-S and control bands were 0.120, 0.015, and 0.0, respectively, from a portion of gel containing approximately 150 μg protein. Excised bands of the enzymes were also tested for activity by paper chromatography[5].

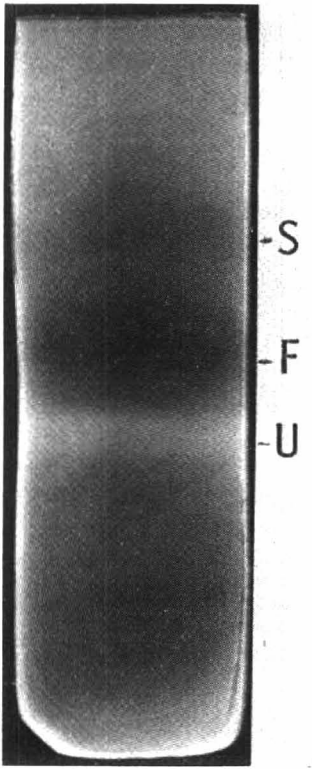

Fig. 2. Simultaneous detection of histidase and urocanase. This photograph under UV light shows the dark bands of histidase-F and histidase-S (slightly visible) and the bright band of urocanase (U) after 1 h incubation with L-histidine and eosin. The sample on this portion of gel contained 150 μg of protein.

Both methods confirmed that the activity stain detected protein bands with urocanase or histidase activity. Control protein bands which were negative to the activity stains had no enzyme activity by these methods. Analysis by either procedure showed that histidase-S has a much lower activity than histidase-F. Based on the relative intensity of the bands (F and S) after staining with Aniline Blue Black, it appears that the band histidase-S contains much less protein than histidase-F.

Discussion

This activity stain gives a rapid direct visible detection of active enzymes without the necessity of a photograph or other manipulations. It is possible to detect enzymes either in small amounts (approximately 3 μg of protein) or with low

activity by longer incubation with the substrate and it is covenient to compare directly samples in adjacent channels run under identical conditions. The evidence for two electrophoretically distinct forms of histidase in a crude extract is based on the two bands appearing after the histidase activity stain, the standard spectrophotometric assay of unstained bands after elution, and the conversion of histidine to urocanate when gel portions from unstained bands are incubated with histidine. There is a progressive conversion of histidine to a product which corresponds to urocanate in R_F value, color reaction to diazotized sulfanilic acid and UV quenching ability on paper chromatograms.

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Bacteriology Research Laboratory,
Veterans Administration Hospital,
Iowa City, Iowa 52240 (U.S.A.)

DENNIS E. ROTH
DANIEL H. HUG

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CHROM. 5044

A reaction for identification of Al^{3+}, Ga^{3+}, In^{3+}, and Tl^{+} separated by paper chromatography

Many reactions have been used for the identification of the periodic group III-A elements on paper chromatograms[1–4] but most of them are not common to the whole group of ions. In the present work, a reagent is proposed which forms colored complexes with Al^{3+}, Ga^{3+}, In^{3+} and Tl^{+}, allowing detection of all the above cations separated on a paper chromatogram.

Oxidized hematoxylin has been investigated by one of us[5] in the chromatographic identification of Ge(IV). The same reagent has now been found to give colored spots with all the ions mentioned above. Work involving the detection of group III-A elements in ores can be simplified by the use of this single detectant after separation by paper chromatography.

Experimental

Samples. Solutions of the individual ions were prepared from the pure metal by dissolving in HNO_3 and diluting with distilled water to obtain a concentration of 1 mg/ml.

Reagent. For the chromatographic identification of the ions, a solution of oxidized hematoxylin prepared according to NEWCOMBE *et al.*[6] was used.

Procedure. Aliquots (5–10 μl) of the different ionic solutions and their mixture were spotted on strips of Whatman No. 1 paper and the analysis was performed by the ascending technic. Seven different compositions of the solvent system isobutanol–HCl–water, described in Table I, were tested in order to find the best conditions for the complete separation of the cations (Table II). The organic upper layer was always used. Also the systems methyl ethyl ketone (C_4H_8O)–2 % HCl (10:90), 0.1 *M* in NH_4Cl; C_4H_8O–2 % HCl (20:80), 0.1 *M* in NH_4Cl; and C_4H_8O–2 % HCl (30:70), 0.1 *M* in NH_4Cl were tried.

TABLE I

COMPOSITION OF ISOBUTANOL–HCl–WATER SOLVENT SYSTEMS AND THE MOLARITY OF HCl IN THE ORGANIC PHASE

Solvent	*Isobutanol*	*HCl*	*Water*	*HCl molarity in the organic phase*
1	65	15	120	0.266
2	75	15	105	0.342
3	90	15	95	0.386
4	70	20	110	0.443
5	80	20	100	0.450
6	70	25	105	0.543
7	70	30	100	0.788

TABLE II

R_F VALUES OF PERIODIC GROUP III-A IONS WITH ISOBUTANOL–HCl–WATER SOLVENTS

Ion	*Solvent*						
	1	*2*	*3*	*4*	*5*	*6*	*7*
Al^{3+}	0.02	0.02	0.02	0.04	0.04	0.03	0.01
Ga^{3+}	0.01	0.03	0.03	0.15	0.08	0.08	0.20
In^{3+}	0.13	0.16	0.14	0.23	0.25	0.24	0.12
Tl^{+}[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a] Tl^+ does not migrate because it is very little soluble in dilute HCl.

After each run the paper was dried overnight in a stream of air at room temperature, then sprayed with the reagent and immediately after exposed to NH_3 vapors in order to eliminate the last traces of acid from the solvent. The group III-A cations were revealed as intense violet or pink-violet spots.

Decreasing amounts of the samples were tested in order to find the detection limit, which was 0.6 γ for Ga^{3+}, 0.7 γ for Al^{3+}, 3.3 γ for In^{3+}, and 3.0 γ for Tl^+.

Results and discussion

The best separation was achieved with the organic phase of the mixture isobutanol–HCl–water (70:20:110) for which the corresponding R_F's are given in Table III. Tailing was observed with all four cations in the solvents containing methyl ethyl ketone. On the other hand, compact spots were obtained in all the different isobutanol systems used.

TABLE III

R_F VALUES OF IONS WITH THE SOLVENT SYSTEM ISOBUTANOL–HCl–WATER (70:20:110), SOLVENT No. 4

Ion	R_F	*Ion*	R_F
Al^{3+}	0.04	Fe^{3+}	0.11
Ga^{3+}	0.15	Sn^{2+}	1.0
In^{3+}	0.23	Sn^{4+}	0.92
Tl^{+}	0.00	Sb^{3+}	1.0
Zr^{4+}	0.01	Bi^{3+}	0.58
		Ge(IV)	0.35

Interference by other elements was investigated. Among 25 cations, Fe^{3+}, Sn^{2+}, Sn^{4+}, Sb^{3+}, Bi^{3+} and Ge(IV) form colored complexes with hematoxylin, but they do not interfere in the chromatographic identification of periodic group III-A ions, since they have R_F values quite different from these, as can be seen in Table III. The only real interference is Zr^{4+}, which presents the same behavior as Tl^{+} ($R_F = 0.01$) and should, therefore, be absent from the solution.

Dr. E. F. R. FRAGA, from the Instituto de Física, U.F.R.G.S., is thanked for providing samples of pure indium and thallium.

Instituto de Química,
Escola de Engenharia Universidade Federal do Rio Grande do Sul,
Pôrto Alegre (Brasil)

YEDA P. DICK
VERA M. SALIM

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Book Reviews

CHROM. 5012

Ion Exchange in Analytical Chemistry, by WILLIAM RIEMAN, III AND HAROLD F. WALTON, Pergamon Press, Oxford, New York, Toronto, Sydney and Braunschweig, 1970, 294 + xiv pp., price £6.10.0, $17.50.

"This book is to provide analytical chemists with a broad survey of the role that ion exchange can and should play in a chemical analysis." While it can be argued that this is preaching to the converted, the book, nevertheless, has a pre-requisite for its readers, a careful perusal of the types of ion exchanger and of their preparation, structure and properties including the kinetics of ion exchange. The authors have succeeded in their aim, although not everyone will be happy with the many mathematical relationships used in the text for developing the theme. However, the qualitative explanations are well done and the mathematically disinclined need have no qualms about turning to this book. If criticism is to be made, it is that the mathematical treatment could be fuller, but this could only have been at the expense of size and the book is already too expensive to appear on many individual bookshelves.

The various applications of ion exchange, both chromatographic and non-chromatographic, are elegantly presented, but without much experimental detail for specific applications, although this is well-compensated by a wide (but not exhaustive) reference list and name index. With regard to the theory of ion-exchange chromatography, the plate equilibrium theory is emphasised over the mass-transfer theory as being "more helpful to the analyst who wishes to calculate from the data of a few elutions the concentration and pH of eluent that will give the most efficient separation of a given mixture". Here again the reviewer feels the treatment might have been fuller.

The penultimate chapter is devoted to less common exchangers; this might have been more adventurous with an eye in the crystal ball directed to their future, but perhaps the authors are wise to temper valour with discretion! The final chapter on the study of complex ions earns its keep in a book which recognises the important role of ion-exchange chromatography (over a third of the text is devoted to chromatography) which is itself so dependent on complexation for its successes.

In conclusion, this well-produced, pleasantly-jacketed book is of value to all those interested in separations.

University of Wales Institute of Science and Technology, Cardiff (Great Britain) J. D. R. THOMAS

CHROM. 5104

Infrared Vapour Spectra, by D. WELTI, Heyden, London, 1970, 222 pages, price £8.5, $21.00, D.M. 77.00.

"The object of this publication is to emphasise that vapour spectra can be used for structural determinations as easily as solution liquid or solid spectra." "It is important, therefore, to convince both spectroscopists and gas chromatographers who do their own spectroscopic determinations, that vapour spectra can be just as useful as, and complementary to, the spectra obtained from other states." "The book is intended to be of use both as a reference to analytical spectroscopists carrying out routine structural identifications, and as a stimulant to this people who are developing the techniques for directly combining gas chromatography and infrared spectroscopy." These three quotations from the preface state the author's aims in writing this book; how does he set-about the task?

There are two chapters dealing with the structural use of vapour spectra: the major one covers frequency correlations and is limited to eight main classes of compounds (alcohols, phenols, carboxylic acids, amines, amides, esters and lactones, aldehydes, and ketones), and the other is a short explanation of how and what information is obtainable from band contours. The structural applications covered in this section have a general usefulness that is not limited to the identification of gas chromatographic fractions in particular.

For this latter purpose there are two chapters describing the experimental techniques necessary for obtaining vapour spectra. Although the discussion of sample-handling involves factors of general interest and importance in vapour spectra measurement it is slanted towards the requirements of measuring spectra of chromatographic amounts of material and hence concentrates on cell efficiency and temperature considerations. The other chapter, dealing with experimental techniques, is exclusively devoted to the practical link-up of the chromatograph and the spectrometer and covers the alternative approaches of trapping (with and without interrupted elution) and leisurely scanning, or continuous elution in conjunction with fast scanning.

The third section of the book accounts for over two-thirds of the total contents and consists of three hundred spectra (recorded from 4000–625 cm^{-1}) and an index to the two thousand-odd vapour spectra in commercial reference collections, in the literature, and in the present book. The spectra are sufficiently large to be of real use for comparison purposes, and the practical conditions employed are stated alongside each spectrum.

There is a lot of information in what is a relatively short text. A useful feature of the chapter on frequency correlations is the pairing of a vapour phase spectrum with a condensed phase spectrum to illustrate the spectral changes for each class of compound; and there is a very useful discussion of the systematic differences and the factors responsible for them. The entries in the tables of frequency correlations are admitted to be based on a very limited number of compounds in each class.

Those who are actively concerned with combining gas chromatography and infrared spectroscopy should find plenty of practical information that is not otherwise easily accessible. It is based on, and benefits from, the author's experience in this field. His assessment of the requirements for a GC–IR combination more successful

than is available at present constitutes a particularly welcome conclusion to the comprehensive description of approaches that have been tried in recent years.

As an addition to what is already available in the way of vapour spectra most of the three hundred presented here are of organic compounds in the range C_5 and higher. To some extent, therefore, the present compilation does partially rectify the lack of vapour spectra of compounds other than simple ones of low molecular weight, but obviously it still leaves a large gap and obviously it does not pretend to be sufficient by itself as a source of reference spectra.

The shortest chapter in the book is that on the use of vapour phase band contours. It is probably too abbreviated for those to whom it is intended to serve as an introduction. It would have been improved by including some actual illustrations of gas phase contours.

Finally two minor points: some entries in the Index *e.g.* "temperature" are more cryptic than useful, and, in the chemical class spectral index, some entries under "amines" are misplaced. There are a few instances where the text or the argument is ambiguous, but these are not sufficiently important to itemise, and the factual mistakes noticed are either trivial or obvious.

To sum up; Mr. WELTI has achieved his purpose very successfully and this book should prove useful to those chemists for whom it is intended, always provided that they can afford it.

Esso Research Centre,
Abingdon, Berks. (Great Britain)

H. SPEDDING

CHROM. 5103

Undergraduate Instrumental Analysis, J. W. ROBINSON, Marcel Dekker, New York, 1970, 379 pages, price $ 11.5, £ 5-5.

This book attempts to survey instrumental analysis and was written as an introductory course for undergraduate students. Without experience of student reaction a full assessment is not possible; however, comments on balance of material and expected reactions may be helpful. The selection of techniques for discussion in such a test is difficult and the author has by and large produced a balanced coverage. Each chapter contains basic priciples of the particular technique or apparatus, applications in current practice, a short bibliography, suggested experiments (outline aims) and a series of problems both numerical and descriptive. The discussions of applications are an excellent and worthwhile feature and will be appreciated by both teachers and taught. Chapters cover reliability of results, sampling, introduction to spectroscopy, nuclear magnetic resonance, infrared absorption, ultraviolet spectroscopy, atomic absorption spectroscopy, colorimetry and polarimetry, flame photometry, emission spectroscopy, X-ray spectroscopy, chromatography, thermal analysis, mass spectroscopy and electrochemistry. If chapters 7 and 8 were interposed a more logical

sequence would arise. Of particular interest to readers of this journal is the chapter on chromatography, covered in 40 pages, half of which is devoted to gas chromatography; the definitions and discussion of retention data and their use are sparse. Regretable omissions are electrophoresis and molecular-sieve chromatography both of which should find a place in undergraduate courses. Other omissions are enzyme and kinetic methods in general. The numerous diagrams are in general good but the relative photometric error — transmittance curve is inaccurate. In future editions it is hoped that S.I. units will be adopted throughout and fewer typographical errors remain. Despite these criticisms the book will be a useful and readable addition to undergraduate libraries.

Loughborough University of Technology, D. THORBURN BURNS
Loughborough, (Great Britain)

CHROM. 5102

Determination of Steroid Hormones, by I. F. SOMMERVILLE, Radiochemical Centre, Amersham, 1970, 48 pages.

This 48 page booklet is one of a series presumably intended as introductory guides to workers not conversant with the use of radioisotopes in a specified field. In this publication, Dr. SOMMERVILLE has attempted to cram a litre of information into a 100 ml cylinder and has very nearly succeeded. The book contains 19 figures and 168 references as well as a fairly comprehensive survey of steroid methodology of the last ten years Some self-indulgence is apparent. Many of the figures and references are of Dr. SOMMERVILLE's own work but these also serve to illustrate his wide experience of his subject. However, criticism must be levelled at some of the examples quoted. For instance, the dubious practice of adding large quantities of radioactive isotopes as tracers to samples for GLC analysis (*e.g.* 0.1 μg [^{14}C]progesterone to 10 ml of plasma) may please the Amersham Sales Department but few others. Also, the presentation of the gas chromatogram of standard steroid heptafluorobutyrates looks cosy but what the biochemist wants to see is what the chromatograms from biological material look like.

In compensation the writing is clear and objective with a first-rate introduction outlining the historical aspects of the subject. The schematic diagrams are well presented and the section on competitive binding *versus* gas chromatographic methods is pertinent and timely. The two full page diagrams of conjectural steroid metabolic pathways must be considered a luxury for a volume of this size when the important technique of radio-immunoassay is barely mentioned. The most useful feature of the book will be as a signpost to those unfamiliar with steroid methodology, an achievement which justifies its intention.

Imperial Cancer Research Fund, Lincoln's Inn Fields, B. S. THOMAS
London (Great Britain)

CHROM. 4988

Cellulose Acetate Electrophoresis — Techniques and Applications, by H. P. CHIN, Ann Arbor-Humphrey Science Publishers, London, 1970, 139 pp., price £6.

This book contains thirteen short, scissors and paste chapters which appear to sum up the author's knowledge of the subject. The first chapter is something of a tour-de-force in that the author reviews the history of electrophoresis — including three references in the last century — and yet fails to make any reference to KOHN, who invented the technique and whose papers have been extensively quoted. The second chapter is the usual well-known digression into the mathematical theory of electrophoresis, which, at least as far as the author is concerned, is of no value practically as it is never mentioned again. Three chapters on equipment, techniques and evaluation follow but with insufficient data for a beginner — and who else would want this book? The next six chapters ramble vaguely through serum proteins, lipoproteins, hemoglobins and enzymes. In chapter twelve, the inventor of the technique finally breaks through. Entitled "Immunodiffusion and immunoelectrophoresis", this chapter appears to be a poor précis of KOHN's own work including his photographs. The price of this book is quite excessive, indeed whatever its price it would be too much.

Courtauld Institute of Biochemistry,
London (Great Britain) I. SMITH

CHROM. 5105

The Determination of Hydrazino–Hydrazide Groups, H. E. MALONE, Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig, 1970, 391 pages, price 140 sh., $18.75.

This is volume 5 in the excellent series of monographs on Organic Functional Group Analysis being jointly edited by Professor R. BELCHER and DR. D. M. W. ANDERSON. It presents a comprehensive survey of analytical methods used for the determination of hydrazine, substituted hydrazines, semicarbazide, and derivatives formed by them alone and in admixture. The principle techniques covered are titrimetric, spectrophotometric and electroanalytical but a small section on chromatographic methods is also included. In addition, the last chapter deals with the use of hydrazines as analytical reagents. Throughout the volume, numerous procedures are given in considerable detail even though some of them must by now be considered of historical interest only. The lack of critical appraisal is unfortunate but understandable in view of the wide range of procedures described and a more serious criticism lies in the fact that coverage of the modern literature appears to be somewhat patchy. Nevertheless, it is the first time that such a comprehensive treatment of these methods has been produced and the book will be invaluable to research workers using these compounds.

University of Strathclyde,
Glasgow (Great Britain) J. M. OTTAWAY

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See also PC section.

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10. CARBOHYDRATES

10a. Mono and oligosaccharides; structural studies

See PC section.

11. ORGANIC ACIDS AND LIPIDS

11a. Organic acids and simple esters

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See also PC section.

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See PC section.

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10. CARBOHYDRATES

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10b. Polysaccharides, mucopolysaccharides and lipopolysaccharides

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19. PEPTIDES; CHEMICAL STRUCTURE OF PROTEINS

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CHROM. 5085

THE CALCULATION OF AREA FACTORS WITHOUT THE USE OF PURE COMPONENTS OF ANALYSED MIXTURES

ALEKSANDER JANIK
Institute of Heavy Organic Synthesis, Blachownia Sl. (Poland)

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SUMMARY

The method of linear relationship described enables one to calculate the area factors related to a standard or to a component of an analysed mixture when the detector responds linearly to the concentration of components in the carrier gas and when mixtures with linearly independent concentrations of components are available.

INTRODUCTION

Quantitative analysis by gas chromatography of mixtures of organic compounds having different molecular weights and chemical characteristics is a complex task[1]. It is difficult to obtain good resolution of all the components in the mixture and to find the area factor values which express the relationship between the component concentration in the carrier gas and the detector signal measured as the peak areas or their heights (height factor). A procedure that has been recommended is to use calibration mixtures nearly identical, qualitatively and quantitatively, to the mixture to be analysed, but this method is tedious and time consuming and cannot be applied for routine analysis, especially of mixtures that are quantitatively different. In addition, for this procedure, one must have all the components of the mixture to be analysed as chromatographically pure compounds—a difficult requirement.

The aim of this paper is to demonstrate the calculation of area factors of all the components of the analysed mixture, when no components are available as chromatographically pure compounds.

THEORETICAL

Let us make the following assumptions: (1) The detector signal measured as the peak area (or peak height) is proportional to the concentration of the component. (2) The quantities of the samples injected into the column are chosen so that concentrations of all the components remain within the linear region of the detector. (3) A

stock of m mixtures of unknown quantitative composition is available, and it contains at least as many mixtures with a linearly independent concentration as there are components, n, in the analysed mixtures. Thus m must be greater than or equal to n. By this method, the qualitative composition can be unknown, peaks being designated by numbers. (4) All components are shown as peaks on the chromatogram and no component is adsorbed in the column.

Procedure 1

A definite amount (by weight, volume or other unit of measure) of the standard which must be chromatographically pure and whose peak must not interfere with those of the components is added to each stock solution. From the chromatogram of the mixture analysed with standard the data for the following equation is obtained.

$$k_s P_s + \sum_{i=1}^{n} k_i P_i = Q + Q_s \tag{1}$$

where k is the area factor of the standard s or the component i; P is the peak area of the standard s or the component i; $Q = \sum_{i=1}^{n} Q_i$ is the corrected area of all components; $Q_s = k_s P_s$ is the corrected area of the standard. Because

$$\frac{Q}{Q_s} = \frac{C}{C_s} \tag{2}$$

eqn. 1 can be written in the form

$$\sum_{i=1}^{n} \frac{k_i}{k_s} \cdot P_i = \frac{C}{C_s} \cdot P_s \tag{3}$$

where

C is the weight of the sample analysed, and

C_s is the weight of standard added.

For each mixture we obtain eqn. 3 and a set of such equations can be solved by the conventional method when $m = n$ or by the least-squares procedure when $m > n$. When calculated in that way, the area factors k_i/k_s are used for calculating appropriate percentage concentrations, X, of all components in each mixture analysed, using the formula

$$X_i = \frac{\frac{k_i}{k_s} \cdot P_i}{\sum_{i=1}^{n} \frac{k_i}{k_s} \cdot P_i} \cdot 100 \tag{4}$$

Procedure 2

Any component in the mixture analysed, denoted by k, which is available in a chromatographically pure state can be used as a standard. In this case from the chromatogram of the analysed mixture, we have

$$\sum_{i=1}^{n} k_i P_i = Q \tag{5}$$

and from the chromatogram of a mixture with a definite amount of component k we obtain the equation

$$\sum_{i=1}^{n} k_i P_i' = Q$$

The area of the peak of component k, P_k', must be related to the amount of the analysed mixture, from which the first chromatogram was obtained. This is calculated by

$$P_{kk} = P_k' \frac{\sum_{\substack{i=1 \\ i \neq k}}^{n} P_i}{\sum_{\substack{i=1 \\ i \neq k}}^{n} P_i'} \tag{6}$$

Then the following equation is true:

$$\sum_{\substack{i=1 \\ i \neq k}}^{n} k_i P_i + k_k P_{kk} = Q + Q_{kk} - Q_k \tag{7}$$

Because

$$Q_{kk} - Q_k = k_k(P_{kk} - P_k) \tag{8}$$

and

$$\frac{Q}{Q_{kk} - Q_k} = \frac{C}{C_{kk}} \tag{9}$$

then

$$\sum_{\substack{i=1 \\ i \neq k}}^{n} \frac{k_i}{k_k} \cdot P_i = \frac{C}{C_{kk}}(P_{kk} - P_k) - P_k \tag{10}$$

where C_{kk} is the weight of component k added.

Taking $k_k = 1$, a set of equations such as eqn. 10 enables one to calculate the area factors k_i by a conventional or least-squares procedure. The percentage concentrations of all components can be calculated from the formula

$$X_i = \frac{k_i P_i}{\sum_{i=1}^{n} k_i P_i} \cdot 100 \tag{11}$$

Procedure 3

This procedure is the simplest one but only can be applied if strictly the same amount of analysed mixture (*e.g.* by volume) is injected into the chromatograph. Then the area factors can be calculated from a set of equations in the following form.

$$\sum_{i=1}^{n} k_i P_i = 1 \tag{12}$$

The percentage concentration of all components can be calculated from eqn. 11. The percentage concentrations are related, of course, to appropriate units of measure (weight, volume, etc.)

DISCUSSION

It is obvious that the results obtained by these procedures will be more accurate if the conditions for analysis satisfy the above assumptions. Accuracy can be judged statistically by estimating pure error and lack of fit[2]. If the lack of fit is significant, there are two situations possible. Firstly, one or more components, whose concentrations are not constant in all the mixtures, are adsorbed in the column. Secondly, the assumption that the detector signal is a linear function of the concentration of the component is false. Then the relation between concentration or corrected area and the area recorded by the detector may be better expressed by the formula

$$Q_i = k_i P_i + k_{ii} P_i^2 \tag{13}$$

In the second case, good results can be obtained by procedure 1 if (a) the standard can be added in quantities that remain in the linear range of the detector or (b) if constant amounts of standard are added to all the mixtures and if their peaks have identical or nearly the same areas.

Then the following equation holds.

$$\sum_{i=1}^{n} \left(\frac{k_i}{k_s} P_i + \frac{k_{ii}}{k_s} P_i \right) = \frac{C}{C_s} P_s \tag{14}$$

To calculate k_i/k_s and k_{ii}/k_s, an appropriate stock of analysed mixtures, containing at least n mixtures with linear independent concentrations, should be available. Others mixtures can be produced by combining two other mixtures.

The percentage concentrations of all the components can be calculated from the equation

$$X_i = \frac{\frac{k_i}{k_s} P_i + \frac{k_{ii}}{k_s} P_i^2}{\sum_{i=1}^{n} \left(\frac{k_i}{k_s} P_i + \frac{k_{ii}}{k_s} P_i^2 \right)} \cdot 100 \tag{15}$$

Requirement b that has been given above is practically the same as that for procedure 3—that identical amounts of each sample must be injected into the chromatograph. Area factors can be calculated from the set of equations

$$\sum_{i=1}^{n} (k_i P_i + k_{ii} P_i^2) = 1 \tag{16}$$

The percentage concentrations are calculated from an equation similar to eqn. 15.

All procedures that have been discussed require a stock of mixtures with linearly independent concentrations. If the concentration of any component slightly differs throughout the stock or if the concentrations of the components in different mixtures are almost linearly dependent, it is advantageous to have at least one or more chroma-

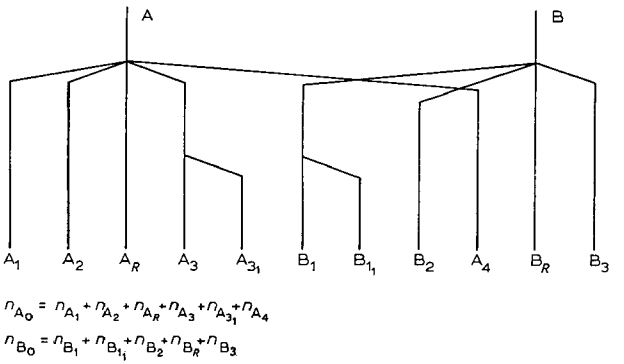

Fig. 1. The spectra of products of substances A_0 and B_0.

tographically pure components or to have only components of the analysed mixtures as impurities. These components can be mixed with analysed mixtures to make their concentrations more linearly independent.

The number of mixtures needed for calculation of area factors can be reduced if we can accept some assumptions concerning some components and create conditions which characterise their sums. This may be explained, for example, by quantitative analysis of a mixture which contains products and residues after having undergone chemical reaction, *e.g.* over a catalyst (Fig. 1).

As substances A and B do not yield the same products (the spectra of their products are independent), the sum of the products and residues of A in moles equals the number of moles of A present in the starting mixture; the same is true for B. In this way, the molar area factors are separately calculated for products and residues of A and B, and the required number of analysed mixtures with linearly independent concentrations of components may be reduced from 11 to 5.

Such an approach to the quantitative analysis of mixtures enables one, firstly, to reduce the number of mixtures needed for calculating area factors and the number of unknowns in sets of equations, and, secondly, to check some assumptions concerning the reaction mechanism.

Using the computer for calculation of area factors and of concentration of components and using at least the necessary number of analysed mixtures, the area factors and concentration of components may be estimated with higher accuracy for each consecutive analysed mixture.

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CHROM. 5086

QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS WITH AND WITHOUT PURE COMPONENTS OF ANALYSED MIXTURES

ALEKSANDER JANIK AND JACEK HETPER
Institute of Heavy Organic Synthesis, Blachownia Sl. (Poland)

(Received October 6th, 1970)

SUMMARY

Multicomponent mixtures of acetone, methanol, propanol and butanol were analysed using calibration mixtures, constant area factors and a linear relationship. The latter is simple to apply and, compared with the former methods, gives satisfactory accuracy.

INTRODUCTION

No general gas chromatographic method for quantitative analysis exists due to the small range in which the detector responds linearly to the concentration of components and to the differences in area factor values of compounds. Thus it was recommended that calibration mixtures having compositions nearly identical qualitatively and quantitatively to those of analysed mixtures (method of calibration mixtures, CM)[1] be prepared. This method, although very accurate, is tedious and time consuming. Therefore, for routine analysis, the area factors are calculated from one or more calibration mixtures and the mean is used to calculate the percentage concentration of components of analysed mixtures (method of constant area factors, CAF)[1]. If all the components of the mixtures are not available as chromatographically pure substances or if one or more components are adsorbed in the column, then an external standard to which concentrations can be related is used. The concentrations can be normalised only if all the components of an analysed mixture are available. The disadvantage of all these methods is that it is necessary to have all the components of the analysed mixture in which one is interested. Calculation of area factors while having no pure components has been previously described (method of linear relationship, LR)[2].

The aim of this paper is to show the usefulness and to compare the accuracy of the LR method with that obtained by the methods of calibration mixtures and constant area factors in the case of internal normalisation (CMN and CAFN, respectively) and in the case of relation of concentrations to external standard (CMS and CAFS, respectively).

EXPERIMENTAL

Materials

Acetone A.R. grade (Chemical Works Oświęcim); methanol A.R. grade (Chemical Works Oświęcim); *n*-propanol A.R. grade (Isopharm); *n*-butanol A.R. grade (Isopharm); polyethylene glycol M.W. 1000 (Shell); and Chromosorb W (Johns-Manville Prod. Corp.) were used.

TABLE I

COMPOSITION OF MIXTURES AND PEAK AREAS

Sample no.	*Injection*	*Weight m_i (g)*				*Sample weight*	*Areas P_i of peaks (cm²)*			
		Acetone (i = 1)	*Methanol (i = 2)*	*n-Propanol (i = 3)*	*n-Butanol (i = 4)*	*m*[a]	*Acetone (i = 1)*	*Methanol (i = 2)*	*n-Propanol (i = 3)*	*n-Butanol (i = 4)*
1	a	2.2914	2.3678	2.4510	2.3841	7.1102	1.33	1.03	1.82	1.91
	b						1.17	0.92	1.56	1.69
	d						1.68	1.13	2.24	2.37
	e						1.66	1.16	2.18	2.31
	c	—	—	—	1.4278	5.6928	1.34	1.09	1.86	4.03
2	a	6.8668	0.6394	0.1510	2.6760	7.6572	3.31	0.31	0.13	2.02
	b						3.32	0.30	0.08	1.88
	c	—	—	—	1.8206	4.8871	2.61	0.22	0.12	3.59
3	a	0.1706	0.1498	7.7120	2.6142	8.0324	0.12	0.08	4.70	1.68
	b						0.14	0.09	5.88	2.28
	c	—	—	—	1.9953	5.0182	0.09	0.07	3.77	3.81
4	a	1.5600	2.3886	4.0242	2.6288	7.9728	0.80	1.02	2.59	2.01
	b						0.80	0.95	2.60	1.90
	c	—	—	—	1.5945	5.0333	0.66	0.82	2.20	3.53
5	a	5.4758	0.8406	1.5932	2.7080	7.9096	3.65	0.36	1.20	2.17
	b						2.62	0.27	0.75	1.60
	c	—	—	—	1.6199	5.0292	3.77	0.33	1.22	5.35
6	a	1.9756	3.8292	2.0204	1.3520	7.8252	1.52	1.91	1.82	1.40
	b						1.35	1.70	1.62	1.15
	c	—	—	—	0.6571	4.4584	1.32	1.72	1.68	2.47
7	a	0.7963	5.5102	1.6118	2.6349	7.9183	0.51	2.53	1.36	2.51
	b						0.55	2.39	1.30	2.30
	c	—	—	—	1.7888	5.1452	0.38	1.92	1.00	4.38
8	a	3.8051	1.6541	2.4046	2.6294	7.8638	2.98	0.84	2.28	2.82
	b						2.38	0.59	1.95	2.31
	c	—	—	—	1.5760	5.1120	1.85	0.53	1.52	4.03
9	a	0.1811	7.0755	0.6580	2.6212	7.9146	0.18	3.12	0.61	2.36
	b						0.34	2.86	0.47	2.07
	c	—	—	—	1.9401	5.1378	0.23	2.25	0.57	4.37
10	a	1.9986	0.3766	5.7171	2.5697	8.0923	1.44	0.16	4.90	2.48
	b						1.39	0.15	4.64	2.35
	c	—	—	—	1.8603	5.1981	1.00	0.10	3.48	4.45

[a] m equals Σm_i for injections a, b, d, e and to weight of second part of sample for injection c.

TABLE II

QUANTITATIVE COMPOSITION OF 3-COMPONENT MIXTURES [$n = 3$]

Mixture no.	*Component*	*Percentage concentration present x_{pi} or calculated x_{ai}*					
		Present	*CMN*	*CMS*	*CAFN* $k_{14} = 1.36$ $k_{24} = 1.96$ $k_{34} = 1.10$	*CAFS* $k_{14} = 1.36$ $k_{24} = 1.96$ $k_{34} = 1.10$	*LR (procedure 1)* $k_{14} = 1.37$ $k_{24} = 1.97$ $k_{34} = 1.11$
1	1	32.2	32.1	32.4	31.0	31.7	31.1
	2	33.3	32.7	33.0	34.6	35.4	34.6
	3	34.5	35.2	35.6	34.4	35.1	34.3
2	1	89.6	88.3	83.2	85.8	78.0	85.8
	2	8.4	8.5	8.0	11.5	10.5	11.5
	3	2.0	3.2	3.0	2.7	2.5	2.7
3	1	2.1	2.3	2.5	3.0	3.2	3.0
	2	1.9	2.0	2.2	2.8	3.0	2.9
	3	96.0	95.7	104.0	94.2	100.0	94.1
4	1	19.6	19.2	18.5	18.3	17.8	18.4
	2	29.9	31.5	30.3	33.7	32.7	33.6
	3	50.5	49.3	47.5	48.0	46.6	48.0
5	1	69.3	67.6	71.1	71.1	78.3	71.2
	2	10.6	9.9	10.4	10.0	11.1	10.1
	3	20.1	22.5	23.7	18.9	20.8	18.7
6	1	25.2	25.2	23.3	26.5	25.5	26.5
	2	48.9	49.0	45.2	47.9	46.2	47.9
	3	25.9	25.8	23.9	25.6	24.7	25.6
7	1	10.0	8.9	8.5	9.7	9.2	9.7
	2	69.6	70.6	67.5	69.4	65.7	69.4
	3	20.4	20.5	19.6	20.9	19.8	20.9
8	1	48.4	48.0	49.7	49.3	48.2	49.5
	2	21.0	23.7	24.5	20.1	19.6	20.0
	3	30.6	28.3	29.3	30.6	29.8	30.5
9	1	2.3	1.1	1.1	3.5	3.4	3.5
	2	89.4	89.0	85.5	87.0	85.6	87.0
	3	8.3	9.9	9.5	9.5	9.4	9.5
10	1	24.7	24.4	24.3	25.6	25.0	25.6
	2	4.7	4.7	4.7	4.1	4.0	4.1
	3	70.6	70.9	70.6	70.3	68.8	70.3

Apparatus

A laboratory-constructed chromatograph with a flame ionisation detector and 0–2 mV recorder was employed. The column (2 m × 4 mm I.D.) was packed with 15% polyethylene glycol on Chromosorb W. Temperature of the column was 100° and of the injector 120°. Carrier gases were nitrogen (50 ml/min), hydrogen (40 ml/min) and air (1000 ml/min). The samples diluted with water (1:300) were injected in equal amounts (0.4 μl) with a Hamilton syringe. Peak areas were calculated as products of the height and width at half height.

Sample preparation

The samples (Table I) prepared by weighing the appropriate amounts of components were placed in stoppered conical flasks. The mixtures thus obtained were divided into two parts. The first part was treated once as an analysed mixture (injection a) and the second as a calibration mixture (injection b). Mixture 1 was

TABLE III

QUANTITATIVE COMPOSITION OF 4-COMPONENT MIXTURES [$n = 4$]

Mixture no.	*Component*	*Percentage concentration present x_{pi} or calculated x_{ai}*			
		Present	*CMN*	*CAFN* $k_{14} = 1.36$ $k_{24} = 1.96$ $k_{34} = 1.10$	*LR (procedure 2)* $k_{14} = 1.37$ $k_{24} = 1.89$ $k_{34} = 1.20$
1	1	24.1	24.1	23.5	23.2
	2	25.0	24.5	25.8	24.8
	3	25.8	26.5	25.9	27.7
	4	25.1	24.9	24.8	24.3
2	1	66.4	64.2	61.9	62.1
	2	6.2	6.3	8.4	8.1
	3	1.5	2.4	1.9	2.2
	4	25.9	27.1	27.8	27.6
3	1	1.6	1.7	2.2	2.1
	2	1.4	1.6	2.2	2.1
	3	72.4	73.7	72.1	73.8
	4	24.6	23.0	23.5	22.0
4	1	14.7	14.3	13.8	13.5
	2	22.5	23.5	25.1	23.5
	3	38.0	36.8	35.8	38.3
	4	24.8	25.4	25.3	24.7
5	1	51.6	51.0	54.1	53.9
	2	7.9	7.5	7.8	7.3
	3	15.0	17.0	14.4	15.5
	4	25.5	24.5	23.7	23.3
6	1	21.5	21.3	22.5	22.5
	2	41.6	41.1	40.6	38.9
	3	22.1	21.8	21.7	23.5
	4	14.8	15.8	15.2	15.1
7	1	7.5	6.6	7.1	7.3
	2	52.2	52.4	51.4	49.7
	3	15.3	15.1	15.5	16.9
	4	25.0	25.9	26.0	26.1
8	1	36.3	36.2	36.8	36.4
	2	15.7	17.9	14.9	15.2
	3	22.9	21.4	22.7	24.3
	4	25.1	24.5	25.6	25.1
9	1	1.7	0.8	2.7	2.7
	2	67.2	66.3	65.1	63.8
	3	6.2	7.3	7.1	7.9
	4	24.9	25.6	25.1	25.6
10	1	18.7	18.5	19.3	18.5
	2	3.6	3.6	3.1	2.8
	3	53.6	53.8	53.1	55.3
	4	24.1	24.1	24.5	23.4

injected additionally twice for calculating CAF (injection d and e). The second part had been weighed again and a known amount of butanol was added (mixture c).

RESULTS

The data given in Table I were calculated as for a 3-component mixture composed of acetone, methanol and propanol and butanol as external standard (Table II) or as for a 4-component mixture of acetone, methanol, propanol and butanol (Table

TABLE IV

THE VARIANCES OF METHODS

No.	*z*	*n = 3*					*n = 4*		
		CMN	*CMS*	*CAFN*	*CAFS*	*LR (procedure 1)*	*CMN*	*CAFN*	*LN (procedure 2)*
1	10	1.12	2.60	1.60	3.31	1.61	0.94	1.31	1.58
2	9	1.13	2.44	1.20	2.62	1.42	0.88	1.05	1.53
3	9	1.13	2.44	1.41	2.46	1.54	0.88	0.95	0.96

III). The following expressions and equations were used for calculating values included in Tables II and III.

Present concentration

$$x_{pi} = \frac{m_{bi}}{\sum_{i=1}^{n} m_{bi}} \cdot 100 \tag{1}$$

CMN

$$x_{ai} = \frac{m_{bi} \cdot \frac{P_{ai}}{P_{bi}}}{\sum_{i=1}^{n} m_{bi} \cdot \frac{P_{ai}}{P_{bi}}} \cdot 100 \tag{2}$$

CMS

$$x_{ai} = \frac{m_{bi} \cdot m_{as} \cdot P_{bs} \cdot P_{ai}}{m_{bs} \cdot m_{a} \cdot P_{bi} \cdot P_{as}} \cdot 100 \tag{3}$$

CAFN

$$x_{ai} = \frac{k_{ij} \cdot P_{ai}}{\sum_{i=1}^{n} k_{ij} \cdot P_{ai}} \cdot 100 \tag{4}$$

$$k_{ij} = \frac{m_{bi} \cdot P_{bj}}{m_{bj} \cdot P_{bi}} \tag{5}$$

CAFS

$$x_{ai} = \frac{k_{is} \cdot P_{ai}}{P_{as}} \cdot \frac{m_{as}}{m_{a}} \cdot 100 \tag{6}$$

$$k_{is} = \frac{m_{bi} \cdot P_{bs}}{m_{bs} \cdot P_{bi}} \tag{7}$$

k_{ij} and k_{is} were calculated for b = b, d, e injections of sample 1 and the mean value was taken for further calculations.

LR

The area factors were calculated according to procedure 1 (ref. 2) for a 3-component mixture:

$$\begin{cases} \sum_{i=1}^{3} k_{is} \cdot P_{ai} = \dfrac{m_a}{m_{as}} \cdot P_{as} \\ \sum_{i=1}^{3} k_{is} \cdot P_{bi} = \dfrac{m_b}{m_{bs}} \cdot P_{bs} \end{cases} \tag{8}$$

and according to procedure 2 (ref. 2) a for 4-component mixture:

$$\begin{cases} \sum_{i=1}^{3} k_{ij} \cdot P_{ai} = \dfrac{m_c}{m_{cj}} \cdot (P_{kj} - P_{aj}) - P_{aj} \\ \sum_{i=1}^{3} k_{ij} \cdot P_{bi} = \dfrac{m_c}{m_{cj}} \cdot (P_{kj} - P_{bj}) - P_{bj} \end{cases} \tag{9}$$

$$P_{kj} = \frac{\sum_{i=1}^{3} P_{bi}}{\sum_{i=1}^{3} P_{ci}} \cdot P_{cj} \quad \text{or} \quad P_{kj} = \frac{\sum_{i=1}^{3} P_{ai}}{\sum_{i=1}^{3} P_{ci}} \cdot P_{cj} \tag{10}$$

x_{ai} was calculated according to eqn. 4, taking $j = s = 4$, $k_{jj} = 1$ where

m_a, m_b, m_c are total weight of sample a, b, c;

m_{ai}, m_{bi}, m_{ci} are weights of component i in sample a, b, c;

x_{pi}, x_{ai} are actual and calculated concentrations of component i;

P_{ai}, P_{bi}, P_{ci} are peak areas of component i of a, b, c injection;

k_{is}, k_{ij} are area factors related to the external standard or j component of mixture.

From the results collected in Tables II and III, the variances of each method were calculated from equation

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n \cdot z} (x_{pi} - x_{ai})^2}{n \cdot z - 1}} \tag{11}$$

where

n is the number of components in the mixture;

z is the number of analysed mixtures.

DISCUSSION

For this work we chose to use a laboratory-made chromatograph whose performance is not perfect. We attempted to make all laboratory work routine but not sophisticated. For the mixtures, we used components which could be mixed together to give maximal differences in the area factor values. We hope that all this makes our results reliable, even for gas chromatographic laboratories, that are not well equipped.

As seen from the values in the first row of Table IV, the methods which yield concentrations related to the external standard are less accurate; the method of constant area factors and method of linear relationship are much more accurate. The most accurate seems to be the method of calibration mixtures. We believe that the method is slightly overrated because the calibration mixtures were identical with analysed ones.

The revision of data collected in Tables II and III reveals that mixture 2 exhibits the greatest deviation from the true value because it contained nearly pure acetone which had evaporated during the handling. This mixture was eliminated, the area factors for LR were recalculated and the new variances of all methods are given in the second row of Table IV. The variances were compared with the Fisher test and it was found that at the 95% level differences in variances are not significant between CMN, CAFN and LR (procedure 1) (for 3-component mixtures) but are significant between CMN and CAFN from one side and LR (procedure 2) from the second (for 4-component mixtures).

In the third row variances calculated according to the following equation are given.

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n \cdot z} (x_{ai} - x_{bi})^2}{n \cdot z - 1}}$$

where x_{ai}, x_{bi} are percentage concentration of component i calculated from a and b injections, which characterise the reliability of instrument recordings and area measurements. Comparison of the values in the second and third rows indicates that these last errors are the greatest part of the total errors of each method. This probably explains why LR (procedure 2) is less accurate if we notice that P_{ki} was calculated according to eqn. 10 and the left side of eqn. 9 are operations on the areas of peaks.

The method of linear relationship requires no components of the analysed mixture as chromatographically pure compounds. It is simple to carry out and its accuracy is as satisfactory as that of comparative methods.

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CHROM. 5100

IMPROVED METHODS FOR THE ESTIMATION BY GAS–LIQUID CHROMATOGRAPHY OF LIGNIN DEGRADATION PRODUCTS FROM PLANTS

R. D. HARTLEY
Grassland Research Institute, Hurley, Maidenhead, Berks. (Great Britain)

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SUMMARY

A method is described for the gas–liquid chromatographic separation and estimation of the following compounds obtained by the saponification of ester linkages present in plant lignins: *p*-coumaric acid, ferulic acid and vanillin. A similar method is described for the separation and quantitative estimation of the following compounds produced by alkaline nitrobenzene or alkaline cupric oxide oxidation of plant lignin: *p*-hydroxybenzaldehyde, vanillin, syringaldehyde, *p*-hydroxyacetophenone, acetovanillone and acetosyringone. *p*-Coumaric and ferulic acids were identified in the oxidation products of grass lignin. As little as 1 μg of a compound could be quantitatively estimated by these methods.

INTRODUCTION

The presence of lignin in the cell-walls of plant materials is of considerable interest in studies concerned with ruminant nutrition as lignin is known to affect detrimentally the nutritional quality of herbages[1]. As a herbage ages, the changes which occur in the cell-walls in relation to nutritional quality are not well defined, and for this reason further characterisation of plant lignins is required. HIGUCHI *et al.*[2,3] have recently established the presence of ester linkages between the main portion of the lignin (the lignin core) of grasses and *p*-coumaric acid (PCA) and ferulic acid (FA), using gas–liquid chromatography (GLC) with Apiezon N as stationary phase. Several workers[4–6] have also used GLC with Apiezon N or SE-30 stationary phases for the estimation of some or all of the following six lignin oxidation products: *p*-hydroxybenzaldehyde (PHB), vanillin (V), syringaldehyde (S), *p*-hydroxyacetophenone (PHAP), acetovanillone (AV) and acetosyringone (AS). For our purposes, methods were required which would separate mixtures of the six lignin oxidation products and also resolve mixtures of PCA, FA and sinapic acid (SA) and enable as little as 1 μg of a compound to be estimated. SA was included in the mixture of acids as it is closely related to PCA and FA and could be present in lignin degradation products. Apiezon N and SE-30 stationary phases gave poor resolution of these

mixtures due to the production of broad peaks and of tailing. In agreement with BRAND[6], peak areas for the six oxidation products could not be estimated due to poor separation.

This paper describes improved GLC methods using cyclohexane dimethanol succinate (CDMS) as stationary phase both for the estimation of compounds obtained by saponification of the ester linkages present in lignins of plants and for the estimation of major degradation products produced by oxidation of lignin core materials.

EXPERIMENTAL

GLC conditions

Separation of PCA, FA, SA and V. A Pye Series 104 Chromatograph with a flame ionisation detector was employed with a 2.75 m glass column (I.D. 4 mm) containing 10% CDMS on 80–100 mesh Diatomite C'Q'. The rate of flow of argon carrier gas was 100 ml/min and the inlet pressure 4.1×10^5 Nm^{-2} (gauge). The column oven temperature was maintained at 190° and the detector oven at 210°. An inlet heater was not employed, the samples being injected directly into the column packing using an 11 cm needle. A Kent Chromalog 3 integrator was employed to determine peak areas for quantitative work.

Separation of PHB, V, S, PHAP, AV and AS. The above conditions were repeated using a flow rate of argon carrier gas of 110 ml/min, a column oven temperature maintained at 220° and a detector oven temperature of 230°.

Quantitative calibration

A reference solution containing 1.00 mg/ml each of chromatographically pure PHB (Hopkin & Williams), V (Hopkin & Williams), S (Koch–Light), PHAP (Koch–Light), AV (Koch–Light) and AS (Ralph N. Emanuel) was prepared using acetone and a calibration graph of peak area against weight was constructed for each compound in the range 1–8 μg. A linear relationship was found in each case. The method was found to be highly reproducible, for example, four consecutive samples of reference V (4 μg each time) gave integrator readings in the ratio of 1.00:1.02:1.05:1.01.

The quantitative estimation of PCA, FA and SA was complicated by variation in peak area from run to run due to differing levels of absorption on the column. To minimise these effects, a similar sample containing chromatographically pure PCA (Koch–Light), FA (Koch–Light) and SA (Koch–Light) was injected both before and after a plant extract. The amount of a particular acid in the plant extract was estimated from a comparison of the integrated value with the integrated values of the reference samples. The analysis was repeated if the results from the two reference samples differed by more than 10%. The reproducibility of the method and the absorption effect can be seen in the following example where four consecutive samples of FA (4 μg each time) were chromatographed. The integrator readings were in the ratio of 1.00:1.03:1.13:1.16.

Material used for degradation studies

The following materials were prepared by freeze-drying:

Sample 1. Mature leaf blade fraction of Italian ryegrass (*Lolium multiflorum*), var. RVP, harvested after tillering had occurred but before ear emergence.

Sample 2. Whole shoot fraction of perennial ryegrass (*Lolium perenne*), var. S. 24, harvested at the end of the flowering stage.

Their ash-free lignin contents, determined by the VAN SOEST procedure[7], were: sample 1, 0.79%; sample 2, 3.45%.

Preparation of cell-wall material

This fraction was prepared from dry grass material (0.5–1.0 g) by the method of VAN SOEST AND WINE[8] using boiling neutral detergent solution to remove cell contents; no sodium sulphite was used and the filtered neutral detergent fibre (NDF) fraction was thoroughly washed with hot water and air-dried.

Sodium hydroxide treatment of cell-wall material

The NDF fractions (0.2–0.5 g) were treated with 1 *N* NaOH (20 ml per sample) in stainless-steel tubes (25 ml capacity) fitted with screw caps. The mixtures were thoroughly shaken at 20 ± 2° for 24 h. Each reaction mixture was filtered (No. 1 porosity glass sinter), washed with water (20 ml) and the filtrate acidified to pH 2.5 with concentrated HCl. Solid NaCl was added to saturate the solution, and the mixture extracted with peroxide-free ether (3 × 60 ml). The combined ethereal extracts were dried (anhydrous Na_2SO_4) and the solvent evaporated at 30° *in vacuo*. The residue was dissolved in acetone (1.0–2.0 ml) and analysed by GLC (5 μl samples injected).

A second fraction was obtained by a further extraction of the acidified solution with ether (50 ml). GLC analysis of the residue showed the presence of only trace amounts of saponification products.

A further saponification was carried out on plant material which had been alkali-treated; no further PCA, FA or V could be detected.

With each series of plant samples being analysed by the saponification method, a blank experiment was carried out using reagents without plant material to ensure that there was no interference from reagent impurities.

Oxidation of the lignin "cores" of grasses

Two methods were employed; in both cases blank experiments were again carried out.

1. Alkaline nitrobenzene oxidation. The alkali-extracted NDF fractions were oxidised with nitrobenzene (1.0 ml) and 2 *N* NaOH solution (10 ml) in screw-capped stainless-steel tubes (25 ml capacity) at 160 ± 2° for 3 h; the tubes were heated and thoroughly shaken in a rotating aluminium block. Each reaction mixture was filtered (No. 1 porosity glass sinter), washed with water (10 ml) and then with peroxide-free ether (20 ml). The filtrate and washings were extracted with ether (2 × 50 ml) and the ethereal layers rejected. The aqueous layer was acidified to pH 2.5 with concentrated HCl and solid NaCl was added to saturate the solution which was then extracted with ether (2 × 60 ml). The combined ethereal solutions were dried (anhydrous Na_2SO_4) and the solvent evaporated. The residue was dissolved in acetone (0.5 ml) and PHB, V, S, PHAP, AV, AS, PCA and FA estimated by the GLC techniques previously described (5 μl injected).

2. Alkaline cupric oxide oxidation. The alkali-extracted NDF fractions, $CuSO_4 \cdot 5H_2O$ (1.7 g) and 3*N* NaOH (10 ml) were heated at 180 ± 2° for 2.5 h with constant

shaking by the above technique. The reaction mixtures were centrifuged at 10,000 *g* for 10 min in a refrigerated centrifuge. The supernatant solution and centrifuged washings (2 × 10 ml water) were combined, acidified, extracted and analysed as described above.

In both of the above oxidations, a further ether extraction gave only negligible quantities of the eight compounds being analysed. Similar results were obtained when cell-wall residues from each oxidation were re-oxidised.

After several GLC determinations of degradation products from the saponification or oxidation procedures, it was necessary to remove the non-volatile material which accumulated at the injection point. This was carried out by the replacement of the top 12 cm of column packing.

Thin-layer chromatographic (TLC) separation of PCA, FA and V present in lignin saponification and oxidation products

The following solvent systems were employed using Silica Gel G[9] with Fast Blue Salt B[9], alkaline potassium permanganate[9], and 2,4-dinitrophenylhydrazine[10] as reagents:

Toluene–methanol–acetic acid (79:14:7). R_F values: PCA, 0.52; FA, 0.58; V, 0.64.

Ethyl acetate. R_F values: PCA, 0.16; FA, 0.07; V, 0.79.

Hexane–amyl alcohol–acetic acid (100:16:0.25)[9]. R_F values: PCA, 0.59; FA, 0.40; V, 0.40.

Combined GLC–mass spectrometric (MS) analysis of compounds present in lignin saponification and oxidation products

For the identification of PCA, FA and V in lignin saponification products, the above GLC conditions were employed except that the column oven was maintained at 210° and the carrier gas (helium) flow rate reduced to 50 ml/min. Similar conditions were employed for the GLC–MS identification of the oxidation products of lignin core materials, except that the column oven temperature was increased to 225°.

Mass spectra were recorded at 70 eV with the source at 160° (A.E.I. MS9 Instrument).

RESULTS AND DISCUSSION

In order to carry out studies on lignins present in plant cell-walls, it was first necessary to remove the cell contents. Various solvents have been used for this purpose. In exploratory work, we have compared the neutral detergent method[8] with the ethanol–benzene method which has been frequently employed[2–6]. Both methods gave similar results but the former was preferred as it was more rapid and did not involve the use of a highly toxic solvent.

Saponification of the ester linkages present in the lignins of plant cell-walls was carried out at 20° by a method similar to that of HIGUCHI *et al.*[2]. Continuous ether-extraction after saponification and acidification was however found to be unnecessary.

The GLC method described in this paper was used to examine the saponification products. The results for grass sample 1, together with a reference chromatogram, are shown in Figs. 1 and 2. The PCA, FA and V identified were the only com-

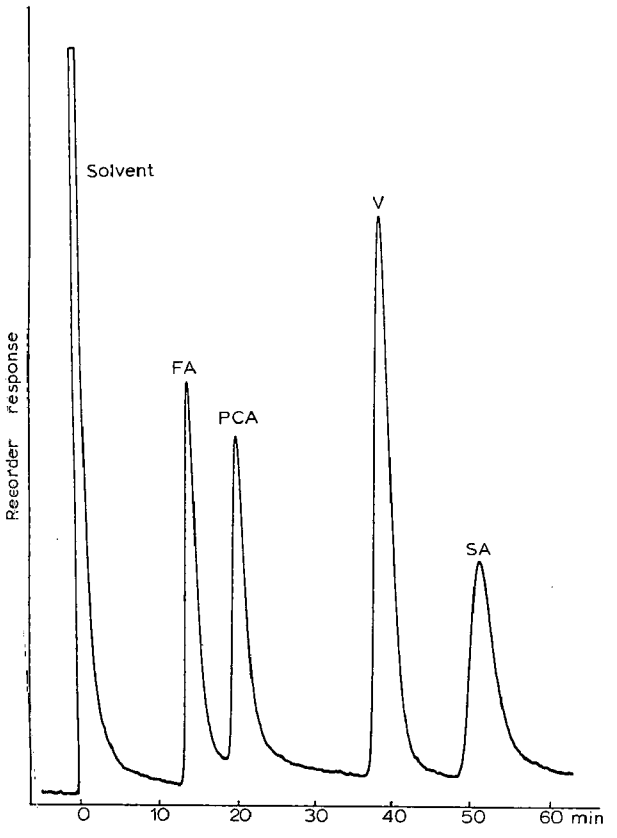

Fig. 1. Separation (3.0 μg of each) of *p*-coumaric acid (PCA), ferulic acid (FA), sinapic acid (SA) and vanillin (V) by GLC (conditions in text).

pounds detected in the saponification products from either of the two grass samples. Further evidence for the presence of these three compounds was obtained by TLC using three different solvent systems. Confirmation of the presence of PCA, FA and V was obtained by comparison of the GLC–MS analysis of the saponification products with a similar analysis of a reference mixture of the three compounds. The mass spectrum of V was characterised by strong parent (M) and M—1 peaks. PCA and FA did not give parent peaks but the base peak in each case was M—44, corresponding to loss of carbon dioxide. A strong peak (M—59) was also present in the spectrum of FA corresponding to further loss of a methyl group.

The two grass samples yielded only trace amounts of V, representing less than 3% of the total yield of the three compounds. The total amount of these substances obtained from grass sample 1 represented 34.9% of ash-free lignin as determined by the VAN SOEST procedure[7], or 0.28% of the original freeze-dried grass. The percentage of ester groupings was very high in this leaf blade material compared with the amounts obtained by HIGUCHI *et al.*[2] from stem-materials of various grasses. The ratio of FA/PCA was 6.5 in the blade material in contrast to ratios of between 0.31 and 0.74

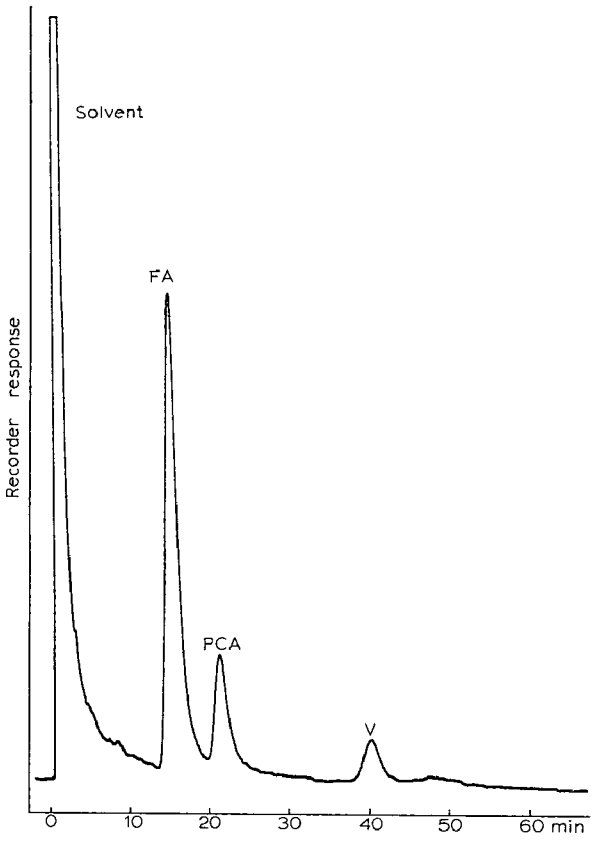

Fig. 2. Separation of the saponification products from cell-wall material from grass sample 1 by GLC (conditions in text).

TABLE I

YIELDS OF ALKALINE NITROBENZENE AND CUPRIC OXIDE OXIDATION PRODUCTS OF ALKALI-EXTRACTED CELL-WALL MATERIAL FROM GRASS SAMPLES 1 AND 2

Compound	*Yield*[a]		
	Alkaline nitrobenzene oxidation		*Alkaline cupric oxide oxidation*
	Sample 1	*Sample 2*	*Sample 2*
p-Hydroxybenzaldehyde (PHB)	zero	0.15	0.17
Vanillin (V)	1.15	4.50	2.85
Syringaldehyde (S)	0.16	3.02	1.51
p-Hydroxyacetophenone (PHAP)	zero	0.05	0.03
Acetovanillone (AV)	0.03	0.09	0.47
Acetosyringone (AS)	zero	0.58	0.58
p-Coumaric acid (PCA)	0.03	0.30	0.15
Ferulic acid (FA)	0.71	0.60	0.30
Total	2.08	9.29	6.06

[a] Per cent of ash-free lignin (determined by the VAN SOEST method[7]).

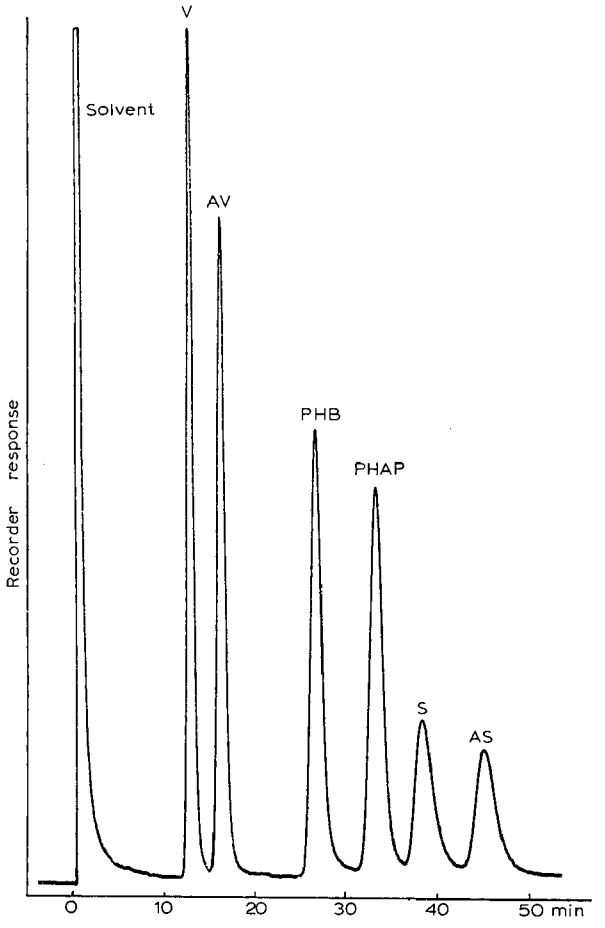

Fig. 3. Separation (1.0 μg of each) of *p*-hydroxybenzaldehyde (PHB), vanillin (V), syringaldehyde (S), *p*-hydroxyacetophenone (PHAP), acetovanillone (AV) and acetosyringone (AS) by GLC (conditions in text).

in HIGUCHI's stem materials. Grass sample 2, which was composed of stem, leaf and senescent material, gave only 11.7% (based on ash-free lignin) of saponification products and the FA/PCA ratio was 1.0.

The absence of SA in the saponification products, even though S was obtained by lignin oxidation, was in agreement with other results from grasses[2,11], wheat[12], maize[13] and sugar cane[14].

The possibility that V obtained by sodium hydroxide-treatment of lignins was being produced from liberated FA by air oxidation was investigated by submitting FA to the usual saponification procedure. No V was detected. Hence it seems likely that V is linked as an ester grouping either to the lignin core or to other peripheral groups.

Although there is good evidence[2,3] that, in some cell-wall fractions of grasses, all the PCA and at least some of the FA are present as lignin ester groups, further

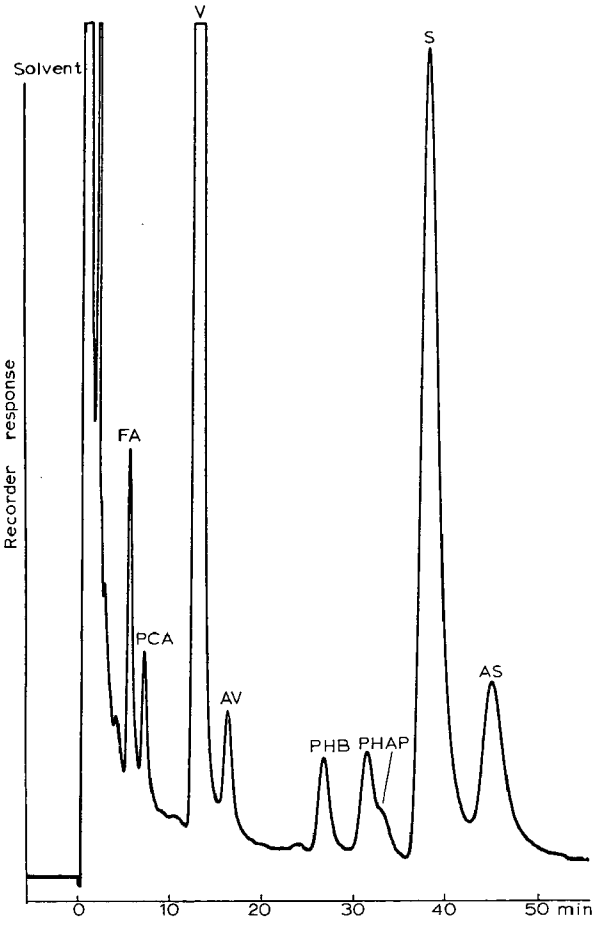

Fig. 4. Separation of the alkaline nitrobenzene oxidation products from alkali-extracted cell-wall material from grass sample 2 by GLC (conditions in text).

work is required to find out if, in some circumstances, PCA, FA and V can also arise from other sources in cell-wall materials.

The lignin core materials left after removal of the lignin ester groupings by sodium hydroxide-treatment, were examined by degradative oxidation followed by GLC determination of the products. The alkaline nitrobenzene[5] and alkaline cupric oxide[6] methods were employed with certain modifications. The reaction mixtures were continuously shaken and the oxidation products extracted with ether. Continuous solvent extraction was found to be unnecessary. The results for grass sample 2, together with a reference GLC separation of PHB, V, S, PHAP, AV and AS, are illustrated in Figs. 3–5 and summarised in Table I. Both methods of oxidation gave rise to two compounds which had the same retention times as PCA and FA on the CDMS column at either 190° or 220°. Further evidence for the presence of PCA and FA was obtained by TLC using Silica Gel G and three different solvent systems.

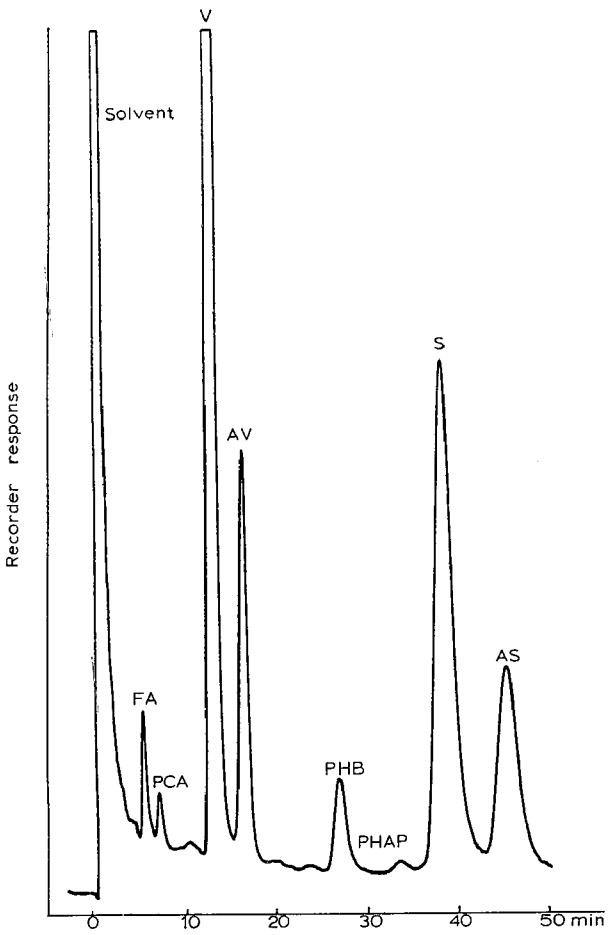

Fig. 5. Separation of the alkaline cupric oxide oxidation products from alkali-extracted cell-wall material from grass sample 2 by GLC (conditions in text).

Confirmation of the presence of all the major oxidation products was obtained by GLC–MS analysis and comparison with a similar analysis of a reference mixture containing the suspected compounds. PCA, FA and V gave similar mass spectra to those reported above. PHB and S were identified by their strong parent and M—1 peaks. AS and AV also gave strong parent peaks but the base peaks were M—15 due to loss of CH_3. The presence of PCA and FA in lignin oxidation products from maize and wood has been reported earlier[13,15] but this is apparently the first report of their occurrence in the products from grass lignin.

As shown in Table I, V and S were the main oxidation products, and the V/S ratio for the nitrobenzene oxidation was 1.49 compared with 1.89 for the cupric oxide reaction. Appreciable amounts of the corresponding ketones were obtained by both methods except that nitrobenzene gave only small quantities of AV. When alkali-treated grass sample 1 was subjected to alkaline nitrobenzene oxidation, V was again

the major product and, as expected, the V/S ratio (7.2) was considerably higher than for sample 2 which contained older plant material.

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CHROM. 5099

THE MEASUREMENT BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF OESTRA-1,3,5,-TRIENE-3,15α,16α,17β-TETROL (OESTETROL) IN PREGNANCY URINE

R. W. KELLY

Medical Research Council, Clinical Endocrinology Unit, 2, Forrest Road, Edinburgh, EH1 2QW (Great Britain)

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SUMMARY

A method is reported for the rapid quantitative determination of oestra-1,3,5 (10)-triene-3,15α,16α,17β-tetrol (oestetrol) in pregnancy urine. The technique uses a gas chromatograph coupled to a mass spectrometer detector. The mass spectrometer is tuned to monitor the *m/e* 191 ion produced during the fragmentation of oestetrol tetra(trimethylsilyl) ether by electron bombardment. The signal from the oestetrol is compared with the signal from a known mass of a synthetic standard, 4-methyl oestra-1,3,5(10)-triene-1,15α,16α,17β-tetrol, which fragments similarly to oestetrol. The synthetic standard is added to a hydrolysed urine sample before extraction to correct for losses during extraction. Because of the specificity of this technique, no purification is necessary other than the extraction of phenolic steroids. The precision is 6% and a linear response of the detector is observed over the range of oestetrol concentrations normally met. The technique is sensitive enough to allow measurement of oestetrol from the tenth week of pregnancy. One nanogram of oestetrol can be detected with a signal-to-noise ratio of 6:1 and the limit of measurement is five nanograms of oestetrol in a sample injected into the gas chromatograph.

INTRODUCTION

The levels of oestra-1,3,5-triene-3,15α,16α,17β-tetrol (oestetrol) in pregnancy urine are of interest because this steroid has been shown to be predominantly foetal in origin[1,2] and consequently the levels may reflect the stage of foetal development. Although oestriol can be measured in unpurified pregnancy urine extracts by gas chromatography (GC) with conventional detectors, oestetrol cannot be measured satisfactorily by such methods because of the lower concentration of this steroid.

GC methods for oestetrol measurement published so far[3,4] incorporate liquid phase chromatography. This is necessary with flame ionisation detectors to obtain sufficient specificity and adequate sensitivity. In the present GC method, no liquid phase chromatography stages are used. Specificity is provided by the use of a mass spectrometer (MS) as a highly specific detector for the gas chromatograph. This

technique allows measurement of ng quantities of oestetrol in unpurified extracts of phenolic compounds from pregnancy urine.

When a mass spectrometer is tuned to monitor one mass-to-charge ratio and, as such, is used as a selective detector for a gas chromatograph, high detector sensitivities can be obtained. These sensitivities are of a much higher order than the normal sensitivity of a gas chromatograph–mass spectrometer (GC–MS) combination when it is used for qualitative analysis. This increase is obtained by using wider slit widths, lower band width amplifiers and the best ion source parameters and magnet position for the ion under study. Moreover, in addition to its high sensitivity, the mass spectrometric detector also possesses an intrinsically high selectivity. When this selectivity of the mass spectrometer is coupled with the separating efficiency of GC, the discriminating ability is such that impure samples can be analysed for specific components in the presence of hundredfold excesses of closely related compounds.

Such ion-specific analysis can suitably be applied to the measurement of oestetrol because the mass spectrum of the tetra(trimethylsilyl) ether derivative of oestetrol has a strong peak at *m/e* 191 (ref. 5).

Although combined GC–MS, when used in the ion-specific mode, is a sensitive and selective technique, the problem is the quantitation, or more specifically, the correlation of the signal generated with the mass of a particular compound injected into the gas chromatograph. Apart from the normal losses or uncertainty in the injection of liquid samples into the gas chromatograph and the possible losses of polar compounds on the column, the mass spectrometric detector introduces varying signal-to-mass response for every varying voltage or current which influences the intensity, focussing or measurement of the ion beam. Although such factors, which could affect the signal to mass response, are stabilised as far as possible, absolute measurements based on signal-to-mass ratio are unreliable and cannot at present form a basis for precise measurement. However, comparison of the mass spectrometer signal from two nearly identical compounds eluting from a gas chromatography column close to each other, should be a reliable method of measuring the mass of one of them if the mass of the other is known, for in such a case the only variations in detector sensitivity which will affect the result are those occurring between the elution of the two peaks. This approach gives excellent results in electron capture detection[6], but in the case of the specific ion detector the requirements for the internal standard are more stringent: the standard must possess the same signal generating group as the compound to be measured, yet differ in some way which allows gas chromatographic separation. This problem is complicated because the signal generating group in this connection is often poorly defined, because competing fragmentations (possibly originating many carbon atoms distant) involving the signal generating group, will influence the signal-to-mass response. In this measurement of oestetrol a reference compound (4-methyloestra-1,3,5(10)triene-1,15α,16α,17β-tetrol) was synthesised; this compound has the same B, C and D ring configuration as oestetrol and has a phenolic hydroxyl group in the "A" ring but the A ring is rearranged. Because the local environment is the same during the origin of the *m/e* 191 ion in both the natural and reference compounds, the signal per mole response should be the same in each case.

MATERIALS, METHODS AND INSTRUMENTATION

Materials

Ethyl acetate, benzene and ether were analytical grade and fresh but otherwise unpurified. Bis(trimethylsilyl) acetamide and trimethylchlorosilane (Pierce Chemical Company) were redistilled and stored in sealed ampoules before use. The synthetic oestetrol was prepared from 1-hydroxy-4-methyloestra-1,3,5(10)trien-17-one which was prepared by acetolysis (dienone–phenol rearrangement) of androsta-1,4-diene-3,17-dione (ref. 7). The Δ^{15} unsaturation was introduced by a standard method[8] and the Δ^{15}-17-ketone was reduced and hydroxylated according to the method of FISHMAN AND GUZIK[9,10]. The tetrol was purified by thin-layer chromatography (TLC) and crystallised from aqueous methanol, m.p. 153–155°. The orientation of the 15 and 16 hydroxyls in the tetrol isolated was inferred from the fact that it was the major product of the hydroxylation, and the more polar of the two tetrols formed. The structure was confirmed by mass spectrometry. A standard solution of the synthetic oestetrol was prepared in 50% aqueous ethanol of a concentration equal to approximately 20 μg per ml and this solution was stored at 0° in $\frac{1}{2}$ ml sealed glass ampoules which were opened as required. Authentic crystalline oestetrol was obtained as a gift from Professor S. SOLOMON.

Methods and instrumentation

Hydrolysis and extraction of urine. Aliquots of urine (5 ml) were diluted to 10 ml with 0.1 *N* acetate buffer and 50 μl *Helix pomatia* extract (5,000 Roy units sulphatase, 40,000 Fishman units glucuronidase) were added. The mixture was incubated for 24 h at 37°, then a further 50 μl of *Helix Pomatia* extract were added and the incubation was repeated. 25 μl of the synthetic standard solution was added to the hydrolysed urine which was saturated with salt and extracted with 20 ml ether–ethyl acetate (2:1), the organic extract was washed with 5 ml saturated carbonate solution pH 10.5 (ref. 11), then washed with 20 ml 0.5 *N* sodium hydroxide. The alkaline extract was removed, acidified, saturated with sodium chloride and re-extracted with 20 ml ether–ethyl acetate. The organic layer was washed with 5 ml saturated sodium bicarbonate solution, further saturated with sodium chloride, dried with magnesium sulphate and evaporated to dryness on a rotary evaporator. The residue was dissolved in 250 μl of ethanol and 50 μl aliquots were taken for analysis, these were evaporated under vacuum at the bottom of a 50 mm long tube of 2 mm I.D. 20 μl of a 20% solution of bis(trimethylsilyl) acetamide in benzene and 5 μl of a 20% solution of trimethyl chlorosilane were added and the tube was sealed in a flame. The tubes were broken open for analysis after a minimum of 2 h and 2–3 μl aliquots were injected into the GC–MS combination, allowing most of the solvent to be sucked through a capillary in the outlet of the chromatograph. Alternatively, the accelerating voltage was dropped to 2 kV during the emergence of the solvent.

Gas chromatograph–mass spectrometer. The gas chromatograph was a Hewlett Packard Model 402 equipped with U shaped columns. Helium was used as carrier gas. The columns were 5 ft. long, 2 mm I.D., packed with $\frac{1}{2}$% OV-1 on 100/120 mesh "Gas-Chrom Q" with a resolution of 400 plates per foot, columns were fitted between an injection mount and an auxillary detector position, the fitting at this position has inserted through it a stainless-steel capillary through which the effluent can be sucked

(by an auxillary diaphragm pump) during the emergence of the solvent. The effluent from the gas chromatograph is transferred via a Swagelok coupling to a resistively heated metal capillary leading to a WATSON–BIEMAN separator[12]. The separator was silanised *in situ* using dimethyl diacetoxysilane in the vapour phase. The enriched sample from the separator is fed through a glass line into an Associated Electrical Industries MS 12 mass spectrometer mounted on vibration-free concrete. Typical conditions during a specific ion analysis were as follows:

Temperature: gas chromatograph oven 250°, gas chromatograph detector block 250°, steel transfer capillary 240°, molecular separator 240°, re-entrant tube and glass lines 230°, source block 230°.

Pressures: 50 lb./sq. in. helium pressure at column inlet, 20 lb./sq. in. at column outlet, 1 Torr at outside of glass frit, 1×10^{-5} Torr in source housing and 1×10^{-7} Torr in analyser.

Voltage and currents: Accelerating voltage 8 kV, trap current 100 μA, ionising voltage 20 eV, electron multiplier voltage 1.8 kV. The source slit width was 0.010 in., analyser slit width was 0.015 in. Magnet current was allowed to stabilise over a period of about 1 h prior to ion specific analysis. Magnet current was adjusted (with the help of a mass marker attachment) to give maximum signal on the collector meter from the small peak at *m/e* 191, present in the mass spectrum of the bleed from the OV-1 column. The signal output was then switched to the recorder ready for ion specific analysis.

The mass spectrometer head amplifier was modified to incorporate a 1000 MΩ resistance in the grid leak of the first valve; this allows lower electron multiplier voltages to be used. Signal passed to the main signal amplifier through the bandwidth filter set at 1 c.p.s. and the output from the signal amplifier was fed through a 10:1 voltage divider network and a further resistance capacitance filter to a pen recorder (Rikadenki Model B341X-A) with input selection at 100 mV. The output was fed simultaneously through a 100:1 voltage divider to a "Chromalog 2" electronic integrator (Kent instruments), set for 1 V maximum input. Signal from the total ion current monitor was fed to a second channel of the pen recorder. Areas under peaks were determined either from peak height measurements or from the integral values from the electronic integrator.

RESULTS AND DISCUSSION

Hydrolysis

Enzyme hydrolysis was used because acid hydrolysis sometimes gave appreciable concentrations of epioestriol as an artifact and although epioestriol gives only a weak 191 ion at 20 eV, the peak overlaps that of the synthetic oestetrol. Enzyme hydrolysis was carried out for 48 h, but reducing this time to 24 h results in very little loss of accuracy.

Instrument stability

The use of a mass spectrometer to monitor one mass-to-charge ratio requires of the instrument a high stability. The particular instrumentation used in this study was sufficiently stable to allow specific ion analysis at high sensitivity throughout a working day, with the exception of 1 h at the start during which the source supplies

settle and after which final tuning of the ion beam is carried out. During a period of specific ion analysis the magnet current is left continuously at the required value and no change is necessary from day to day.

Suitability of internal standard

The suitability of the internal standard has to be considered from the aspect of the correction for extraction losses and the quantitation in the final measurement. During the extraction of a urine, the synthetic oestetrol does not behave identically to the natural oestetrol. The relative behaviour of the synthetic and natural steroid was studied by incomplete extraction followed by re-extraction at various stages in the normal procedure. During extraction with sodium hydroxide the ratio of synthetic to natural in the re-extracted sample was the same as in the original extraction, indicating that losses at this stage are fully compensated for. However, in the ether–ethyl acetate extractions there is preferential extraction of the synthetic tetrol and thus if the recoveries are not high some innaccuracy will be introduced at these stages. The overall recovery of oestetrol is 70% and consequently the preferential extraction of the synthetic standard will introduce some error, although all accidental spillages and losses during the alkali extraction stage will be allowed for.

The recovery could be improved by using repeated extractions at each stage but the accuracy using the present procedure is sufficient for the purpose of the analysis. Moreover, because of the very high polarity of the oestetrol the exhaustive extractions necessary to get high recoveries would add more contamination to the extract.

The requirement of the internal standard during the mass spectral measurement is that it should give the same ion current per mole as does oestetrol. From a theoretical consideration, the standard used should nearly satisfy this condition. It is not certain by exactly which mechanism the m/e 191 ion ($C_7H_{19}O_2Si_2$) is produced, but it is probable that it arises through silyl migration and a ring enlarged intermediate[13]. In any case the origin of the ion at 20 eV is confined to the D ring. The production of the 191 ion could only be affected by long range effects or by prior ionisation elsewhere in the molecule. The long range effects are unlikely, due to the number of intervening carbon atoms and prior ionisation is kept to a minimum by using a low ionising voltage. The mass spectra of oestetrol and synthetic oestetrol are shown in Fig. 1. The signal to mass responses of the oestetrol and synthetic oestetrol are very similar, the ratio being 1.0 ± 0.1. The variation is long term and depends on the instrumental conditions. Because of this long term variation, standard mixtures are analysed every day.

Theoretically the Bieman separator will discriminate between the natural and synthetic oestetrols in favour of the higher molecular weight synthetic standard. In practice, since the molecular weight difference is small and the effect is partly offset by the theoretical increase in the signal per mole response of the lower molecular weight compound, no such effect is observed.

Precision

The precision of this application of ion specific analysis to the measurement of oestetrol in pregnancy urine is represented by a standard deviation of 6% (36 paired samples); this figure was obtained by duplicate determinations of steroid levels in

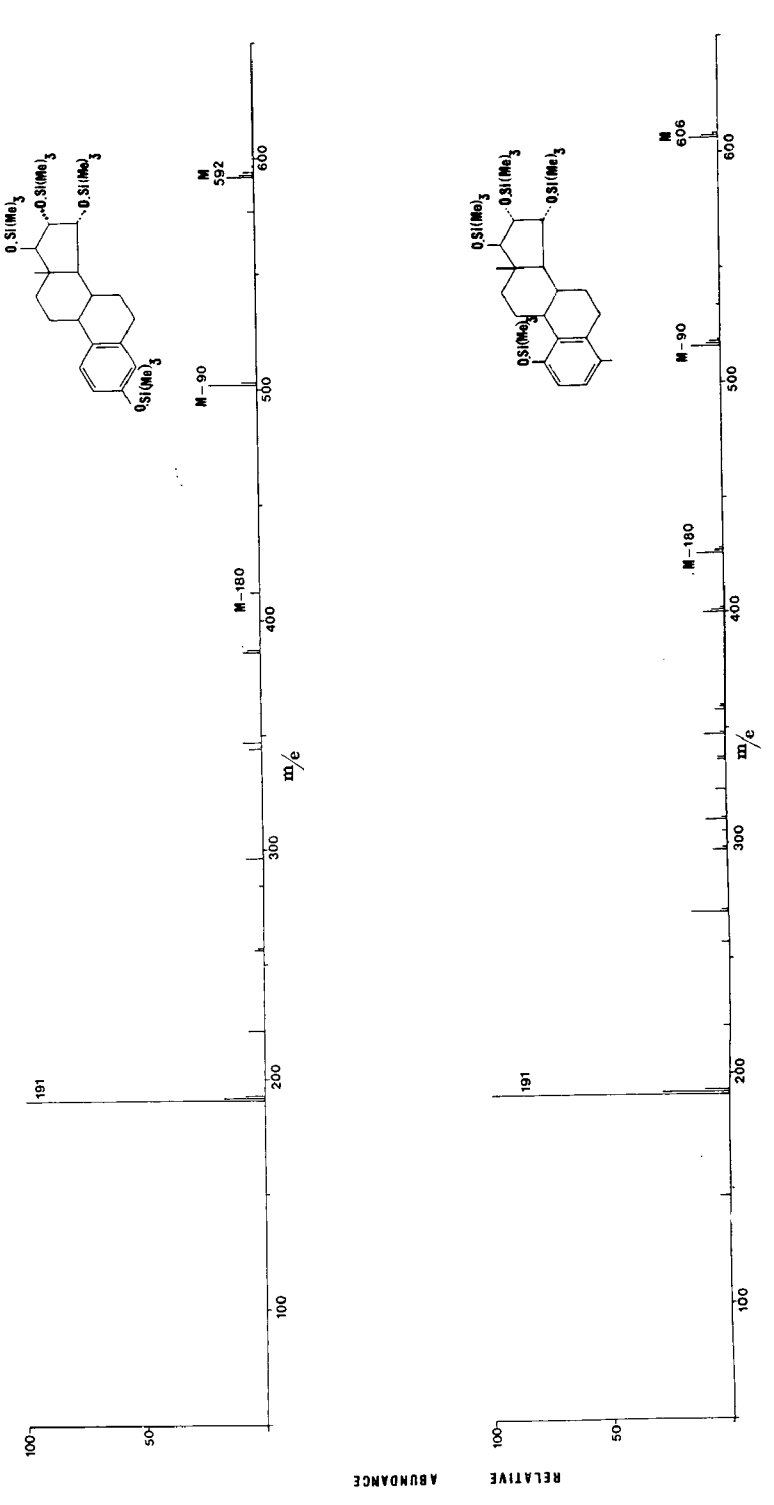

Fig. 1. Mass spectra of 3,15α,16α,17β-tetrahydroxyoestra-1,3,5(10)-triene tetra(trimethylsilyl) ether and 4-methyl-1,15α,16α,17β-tetrahydroxyoestra-1,3,5(10)-triene tetra(trimethylsilyl) ether.[22] Energy of bombarding electrons, 20 eV.

urine samples by the method of SNEDECOR[14]. This precision value represents not only the variation in the final analytical determination but also the variation due to losses in extraction. The variation in the ion specific analysis of a urine extract is represented by the precision of duplicate determinations of the oestetrol to synthetic oestetrol ratio in the same extract samples; this gives a value of the standard deviation of 3% (38 samples). The difference in these observed standard deviations gives some idea of the imprecision introduced by the extraction technique.

Using the technique presented here the ion specific analysis of a urine sample is less precise than the similar analysis of pure standards; one reason for this is the difficulty in getting accurate area measurements from the relatively complicated traces of the urine samples. This difficulty could be overcome by adding a further purification stage, but because the internal standard could not be used throughout the procedure, the overall precision would in fact be lower. The precision of the ion specific analysis for pure samples using electronic integration for quantitation is 2.5% (21 samples). This more closely represents the precision of the actual measuring technique and might be more nearly attained in samples where more readily extractable and more easily recovered compounds are to be measured.

Specificity

The conditions which must be met before a compound can interfere with the measurement of oestetrol are that the compound must have both a relatively strong peak at *m/e* 191 and also an elution volume nearly equal to either oestetrol or the synthetic internal standard. No compound has yet satisfied these criteria fully. The

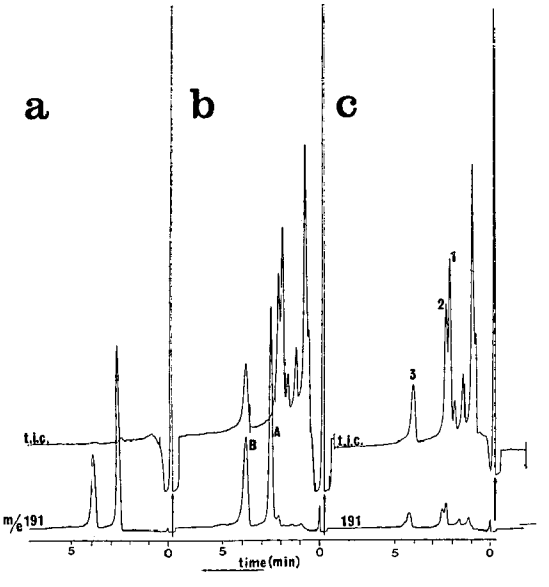

Fig. 2. *m/e* 191 specific trace (lower) and total ion current trace (upper) of standard mixtures of steroids. (c) Mixtures of steroid silyl ethers. Peak 1 = oestriol, peak 2 = 17-epioestriol, peak 3 = cholesterol (2 μg of each component). (b) Mixture of steroids as in (c) but 1 μg of each component with 20 ng synthetic oestetrol (A) and 10 ng of natural oestetrol (B); t.i.c. monitor sensitivity 2 × that in (c). (a) Mixture of oestetrols alone. Conditions as in text.

specificity is enhanced by working with a low ionising voltage; dropping the ionising voltage from 25 eV to 20 eV results in only a small decrease in the sensitivity of the detector to oestetrol and the standard, but greatly reduces the sensitivity of the detector to other compounds. Monitoring the 191 ion at 20 eV the signal to mass response is 10,000 times greater for oestetrol than for oestriol. The relative responses of the 191 specific trace and the total ion current (t.i.c.) monitor are demonstrated (Fig. 2) for the synthetic and natural oestetrol and for one hundred times the weight of various C_{18} and other steroids. The trace b shows that the ng amounts of oestetrol are masked by the other compounds almost completely on the total ion current trace, but are readily measurable on the selective 191 trace. Thus a further element of specificity is introduced if the ratio of the response from the selective detector to the response from the t.i.c. detector is considered.

Accuracy

The accuracy is largely dependant on the relative behaviour of oestetrol and the internal standard. As has been mentioned, the ratio of response of the selective detector to the two is 1.0 $\pm$ 0.1. This ratio was checked during ± 2 V variations in ionising voltage, $\pm 5\%$ variations in the photomultiplier voltage (compensating for the overall sensitivity change by altering recorder gain) and during small variations in ion repeller voltage (again keeping overall sensitivity constant by altering recorder gain). No significant change in the ratio was observed. It is probable, however, that the small changes in the ratio that do occur are partly due to chromatographic conditions. Partial loss due to catalytic activity and adsorbtion will be experienced by both the natural oestetrol and the synthetic, but, because the natural oestetrol has an appreciably longer retention time, greater losses occur for this compound. Certainly with small samples of oestetrol (less than five ng) the ratio of synthetic to natural becomes unreliable and it is reasonable to assume that losses at this level are becoming significant.

The accuracy is connected with the specificity and if the natural and synthetic oestetrols are not separated from interfering material, accuracy will suffer. There is no evidence for heterogeneity of the peaks and mass spectra at various points in the peaks indicates homogeneity. Fig. 3 shows a typical 32 week pregnancy urine with and without the internal standard showing that there is no peak masked by the standard. To check whether any peak is masked by the oestetrol, an extract of pregnancy urine was run on TLC; the area corresponding to oestetrol was scraped off. The remainder was eluted and analysed by ion specific analysis. No significant peak was present at the oestetrol position.

The linearity of response of the mass spectrometric detector was studied over the range of oestetrol concentrations met during analysis of normal pregnancy urines. Different volumes (10–250 μl) of a standard solution of crystalline oestetrol (16.9 μg per ml) were added to 5 ml aliquots of male urine which contained no oestetrol. The concentrations of the samples were equivalent to urines of 34 μg–1 mg oestetrol per litre. Twenty-two samples were individually extracted and each was measured in duplicate. The urines were processed using the normal procedure. The ratio of the signal per mole response of synthetic to natural oestetrol in a standard mixture run on the same days as the analysis was 1.00 and using this value a linear plot (Fig. 4) is obtained with a correlation coefficient of 0.997. This demonstrates the linearity of

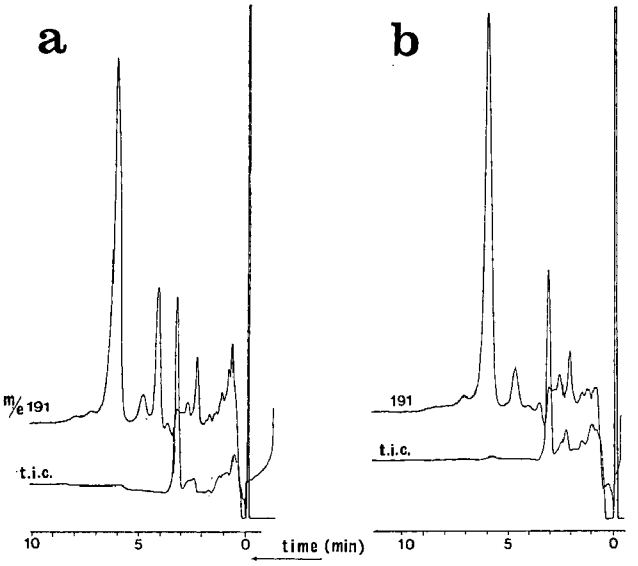

Fig. 3. (a) Silylated extract of 32 week pregnancy urine with synthetic oestetrol added; (b) as for (a) but no synthetic oestetrol added. The large peak on the t.i.c. trace is oestriol trimethylsilyl ether.

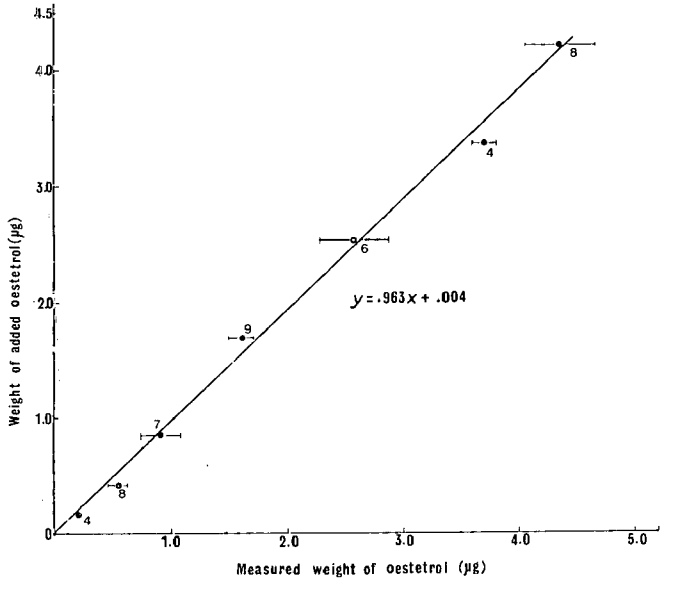

Fig. 4. Plot of added oestetrol against amount measured, linear regression analysis gives amount added (μg) as 0.963 times amount measured plus 0.004 μg. Numbers of samples are given beside the points.

the detector over the range used. Pregnancy urines can be kept within this range by using 1.0 ml aliquots of late pregnancy urine, this brings a further advantage, that the urine can be diluted tenfold with buffer to minimise the effects of enzyme inhibitors.

Sensitivity

The limit of determination is five ng per sample. Below this value the standard deviation begins to rise, presumably due to losses on the column, molecular separator and inlet lines. One ng of oestetrol can readily be detected with a signal to noise ratio of 6:1.

The sensitivity limit in the determination of oestetrol which enters the ionisation chamber is set by the bleed from the silicon polymer stationary phase. There is a significant bleed peak at *m/e* 191 which helps in tuning in the instrument to peak 191 but although the constant component of the signal from the bleed can be compensated for electrically, the noise which is introduced limits the sensitivity of the detector. The sensitivity is sufficient to measure oestetrol levels in urine samples from the 24th week of pregnancy. Oestetrol levels from the 10th week of pregnancy can be measured by using larger aliquots of urine.

Practicability

The selectivity of the specific ion measurement allows the purification of samples to be cut to a minimum. The extraction of the phenolic steroids and the formation of silyl ethers are all that is necessary. A technician can extract 12 samples at a time and can take 24 samples (12 in duplicate) a day through the extraction and silylation procedure. The limitation on the speed of measurement of the silylated extracts is the retention time of oestetrol trimethylsilyl ether. This retention time can be cut to 5 min, and under these conditions upwards of 40 samples (20 in duplicate) can be analysed in one day.

Conclusion

The high specificity of the mass spectrometer as a detector allows the measurement of small amounts of oestetrol in relatively impure pregnancy urine extracts. The high specificity is accompanied by a high sensitivity and the precision of the technique is moderate. Both sensitivity and precision could be greatly improved with instrumental advances.

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CHROM. 5082

USE OF THE RESONANCE PRINCIPLE IN THE PERMITTIVITY DETECTORS FOR LIQUID CHROMATOGRAPHY

STANISLAV HADERKA

Institute of Instrumental Analytical Chemistry, Czechoslovak Academy of Sciences, Brno (Czechoslovakia)

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SUMMARY

Theoretical analysis of the resonance principle has been carried out from the point of view of its use for detection in liquid chromatography. Equations have been derived for the response of the detector to either the real or imaginary component of complex permittivity of the binary mixture: carrier liquid–analyzed component. It is shown that the response on either of the components is a linear function of concentration. The use is advantageous for a liquid with a very low tgδ and dielectric constant for the carrier.

INTRODUCTION

The detectors hitherto employed in liquid chromatography were recently evaluated by HUBER[1]. Whereas the other parts of the channel in a liquid chromatograph have attained remarkable parameters, the detector appears to be the weakest link of the chain at the present time. The development of the analytical utilization of liquid chromatography will depend to a considerable extent on the improvement of these detectors.

For the time being, there is no detector in liquid chromatography that could represent, with respect to the versatility of utilization, the detectors used in gas chromatography, and it seems that this situation will exist also in the near future. Therefore, new detection principles are searched for. One of them is the change of the permittivity brought about by the presence of the component under analysis in the carrier liquid and, consequently, the change in the parameters of the measuring condenser. The methods for measuring both components of the complex permittivity have been given considerable attention[2] owing to the direct relationship between the above parameters and the dipole moments as well as the relaxation constants of molecules in the alternating electric field. Some possibilities of utilizing the principle of the change of the real component of the complex permittivity, ε', in transforming the capacitance change into a frequency change and in measuring the capacity change with the aid of a.c. bridges of the Wheatstone type, are quoted in the literature[3]. The

great influence of the changes of capacitance and losses on the amplitude of the voltage or current in a resonant electrical circuit with low losses around the resonance offers further possibilities for the development of a detector with a very high sensitivity towards changes in the permittivity.

The aim of this paper is to carry out a theoretical analysis of the resonance principle with respect to the utilization of the latter for the detection in liquid chromatography and to the determination of the possibilities the resonance detector is capable to afford.

PRINCIPLE OF THE DETECTOR

If a voltage of constant amplitude U_g is applied to a series resonance circuit composed of fixed inductance L, variable capacity C, and resistor R, then, at a constant frequency, it holds[4] for the dependence of the voltage at the condenser terminals, U_c, on the tuning capacity C:

$$\frac{U_c}{U_r} = \frac{1}{\sqrt{1 + Q^2(\Delta C/C_r)^2}} \qquad (1)$$

where U_c is the effective value of the voltage across the condenser at the capacity C, U_r stands for the same quantity, but at a resonance capacity C_r, Q is the quality

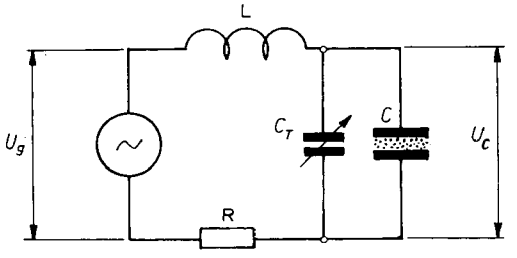

Fig. 1. Schematic of the detecting circuit in the use of the resonance principle.

factor of the resonance circuit, and $\Delta C/C_r$ is the relative capacity increment; $\Delta C/C_r = (C - C_r)/C_r = (C/C_r) - 1$. A graphical illustration of eqn. 1 for the capacity values $C \geqq C_r$ is given in Fig. 2.

Let us suppose the use of a carrier liquid with a "dielectric constant", ε'_c, smaller than ε'_x of the analyzed component and having a loss tangent δ_c very close to that of the detected component $\mathrm{tg}\delta_x$. In this case, it is necessary to detune by the trimming condenser C_T the resonant circuit in such way that the zero point P_1 is located on the upper outset of the linear part of the resonance curve when the carrier liquid flows through the measuring condenser C. Then, if the analyzed component with $\varepsilon'_x > \varepsilon'_c$ occurs in the carrier liquid, the resultant real part of the permittivity, ε'_m, is larger than that of the carrier liquid, so that the capacity of the measuring condenser increases proportionally to the concentration of the component and the voltage U_c on the condenser terminals falls. The voltage across the condenser is measured by a diode electronic voltmeter, in which the background voltage, $U_{c(0)}$ may be easily backed off, so that the slide-back recorder connected to the terminals of

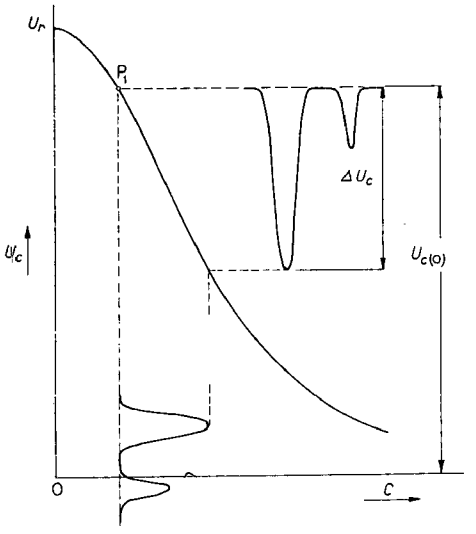

Fig. 2. Zero-point setting of the resonance detector.

the measuring instrument of the diode voltmeter will provide a conventional chromatographic record (Fig. 2).

DYNAMIC RANGE

According to eqn. 1, the slope of a resonance curve is given by:

$$y' = -Q^2x/\sqrt{(1 + Q^2x^2)^3} \tag{2}$$

The greatest slope is attained in the point of inflexion, $P_{inf}(x_0,y_0)$, given by the condition:

$$y'' = 0 \tag{3}$$

from which it follows for the coordinates of the latter:

$$x_0 = 1/Q\sqrt{2}, \quad y_0 = 0.8165 \tag{4, 5}$$

It is apparent from eqn. 5 that the inflexion point occurs at a constant relative height, regardless of the magnitude of the quality factor Q. It follows from eqn. 2 for the slope at the point of inflexion:

$$y_0' = -Q^2x_0/\sqrt{(1 + Q^2x_0^2)^3} = -0.3848\,Q \tag{6}$$

The dynamic range of linearity is defined as the concentration region in which the differential sensitivity is equal to the integral sensitivity[5] or where the difference between both types of sensitivity does not exceed certain limits; LOVELOCK[6] recommends a tolerance of 3%. The tangent line in the point of inflexion is given by the equation:

$$y - 0.8165 = -0.3848\,[x - (1/Q\sqrt{2})] \tag{7}$$

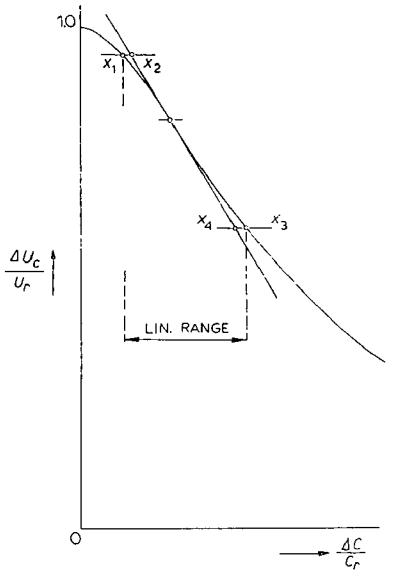

Fig. 3. Dynamic range of the resonance detector.

It holds for the point with the coordinate x_1 (Fig. 3), on the resonance curve above the point of inflexion, in which the deviation from the straight line drawn through the point of inflexion equals 3%:

$$x_1 = 0.97\, x_2 \tag{8}$$

When substituting y and x in eqn. 7 by the respective quantities from eqns. 1 and 8, one obtains for x_1 the equation:

$$0.1574\, Q^4 x_1^4 - 0.8637\, Q^3 x_1^3 + 1.3423\, Q^2 x_1^2 - 0.8637\, Q x_1 + 0.185 = 0 \tag{9}$$

On introducing a new variable by $z_1 = Q x_1$, one obtains for the root of the above equation, using the *regula falsi* method:

$$x_1 = 0.413/Q \tag{10}$$

The end point x_3, below the point of inflexion, is given by:

$$x_3 = 1.03\, x_4 \tag{11}$$

A procedure analogous to that used in case of the point x_1 leads to the following equation for the point x_3:

$$0.1396\, Q^4 x_3^4 - 0.8134\, Q^3 x_3^3 + 1.3245\, Q^2 x_3^2 - 0.8134\, Q x_3 + 0.1849 = 0 \tag{12}$$

and the coordinate of the point x_3 is given by:

$$x_3 = 1.201/Q \tag{13}$$

Hence, the linearity region characterized by the 3% deviation corresponds to the following limiting capacities:

$$x_3 - x_1 = (\Delta C_3 - \Delta C_1)/C_r = 0.788/Q \tag{14}$$

The capacity of the measuring condenser is necessarily composed of two components: a variable capacity C_x, the magnitude of which varies with the ε' of the liquid, and a constant capacity C_k, formed by the fixed dielectric determining the mutual position of the electrodes and providing the closure of the condenser cavity. Hence, it holds for the total capacity:

$$C = C_k + C_x = C_k + \varepsilon_0 \varepsilon' A/d = C_k + \varepsilon' C_0 \tag{15}$$

where ε_0 is the permittivity of the void space ($10^{-9}/9 \times 4\pi$ Farad/m), ε' is the relative real component of the complex permittivity, A is the cross-section of the dielectric, and d is the thickness of the dielectric.

If a carrier liquid with the real part ε'_c flows through the measuring condenser, it is necessary that the zero point be located at the place corresponding to the coordinate x_1. In order to set precisely this point, it is necessary to use the auxiliary trimming condenser C_T, as has already been quoted (Fig. 1). It apparently holds for the relative detuning of the capacity to the points x_1 and x_3:

$$\frac{\Delta C_1}{C_r} = \frac{\varepsilon'_c C_0 + C_T + C_k}{\varepsilon'_c C_0 + C_k} \tag{16}$$

$$\frac{\Delta C_3}{C_r} = \frac{\varepsilon'_m C_0 + C_T + C_k}{\varepsilon'_c C_0 + C_k} \tag{17}$$

where ε'_c, ε'_m are the real parts of the relative complex permittivity of the carrier, and of the mixture, respectively. On comparing the above equations with eqn. 14, there results the following general relation for the dynamic linearity range:

$$\frac{(\varepsilon'_m - \varepsilon'_c)C_0}{C_k + \varepsilon'_c C_0} = \frac{\varepsilon'_m - \varepsilon'_c}{(C_k/C_0) + \varepsilon'_c} = 0.788/Q \tag{18}$$

Thus, the relative real component of the permittivity of the carrier liquid with an admixture of the analyzed component may attain, within the dynamic linearity range, a maximum value given by:

$$\varepsilon'_m = 0.788\, Q^{-1}\left(\frac{C_k}{C_0} + \varepsilon'_c\right) + \varepsilon'_c \tag{19}$$

Now, let us investigate the limits in which the concentration of the substance under analysis, m_x, may vary without the deviation from the linear course exceeding the above 3%.

The additive physical quantity is the polarization[7]. However, it may be assumed for the range of low concentrations that the linear additivity relation applies also to the real permittivity component so that the relation between the concentration of the analytical substance, m_x, with the permittivity ε'_x, the concentration of the carrier liquid, m_c, having the permittivity ε'_c, and the resultant permittivity ε'_m may be described by the linear equation:

$$\varepsilon'_m = m_c \varepsilon'_c + m_x \varepsilon'_x \tag{20}$$

where the concentrations m_x and m_c are expressed in volume fractions.

Thus, we obtain for a binary system:

$$\varepsilon'_m = (1 - m_x)\,\varepsilon'_c + m_x\varepsilon'_x \tag{21}$$

and the real component of the resultant permittivity is given by:

$$\varepsilon'_m = m_x(\varepsilon'_x - \varepsilon'_c) + \varepsilon'_c = 0.788\,Q^{-1}\left(\frac{C_k}{C_0} + \varepsilon'_c\right) + \varepsilon'_c \tag{22}$$

The maximum concentration of the analyzed component is defined by:

$$m_{x(\max.)} = 0.788\,\frac{\frac{C_k}{C_0} + \varepsilon'_c}{Q(\varepsilon'_x - \varepsilon'_c)} \tag{23}$$

SENSITIVITY TO THE CHANGE OF ε'

In chromatography, the sensitivity of the concentration detectors has been defined as the response in mV to 1 mg of the analyzed substance contained in 1 ml of the column effluent. Since the concentration is expressed by volume fractions in our case, we shall define the sensitivity as the response in mV to 1 μl of the substance in 1 ml of the effluent. The sensitivity values expressed according to this definition will differ inappreciably from those based on the original "response to weight per volume" definition.

Since the d.c. voltage $U_{c(0)}$ at the point x_1 is supposed to be backed off (Fig. 2), the absolute value of the d.c. response is equal to the difference between the amplitudes of the high frequency voltage U_c across the condenser at the points x_3 and x_1:

$$U_{c(1)} - U_{c(3)} = U_r\left(\frac{1}{\sqrt{1 + Q^2x_1^2}} - \frac{1}{\sqrt{1 + Q^2x_3^2}}\right) \tag{24}$$

which yields on substituting for Qx_1 and Qx_3 from eqns. 10 and 13:

$$U_{c(1)} - U_{c(3)} = 0.284\,U_r \tag{25}$$

With regard to the definition suggested, it may then be written for the sensitivity to the change of the real component of permittivity:

$$S_{\varepsilon'} = \frac{0.284\,U_r}{m_{x(\max.)}} = 0.36\,U_rQ\,\frac{\varepsilon'_x - \varepsilon'_c}{\frac{C_k}{C_0} + \varepsilon'_c} \tag{26}$$

Since it holds[4]:

$$Q = U_r/U_g \tag{27}$$

where U_g is the driving generator voltage, one may write the following practically advantageous formula for the sensitivity $S\varepsilon'$:

$$S_{\varepsilon'} = 0.36\,U_gQ^2\,\frac{\varepsilon'_x - \varepsilon'_c}{\frac{C_k}{C_0} + \varepsilon'_c} \tag{28}$$

Example:

$$C_0 = 40 \text{ pF}, C_k = 10 \text{ pF}, \varepsilon'_x = 4, \varepsilon'_c = 2, Q = 200, U_g = 0.1 \text{ V}.$$

$$S_{\varepsilon'} = 0.36 \times 0.1 \times 200^2 \times \frac{(4-2)}{0.25+2} = 1280 \frac{\text{mV}}{\mu\text{l/ml}}$$

The concentration dynamic range extends from zero to:

$$m_{x(\max.)} = 0.788 \frac{\frac{10}{40} + 2}{200\,(4-2)} = 4.433 \times 10^{-3}$$

i.e., within $0 - 4.433\ \mu$l/ml.

EFFECT OF ε'' ON THE DETECTOR RESPONSE

We have supposed in our considerations until now that the loss tangent, tgδ, of the carrier liquid is approximately the same as that of the components under detection, so that the resultant loss tangent, tgδ_m, remains practically unchanged when changing the concentration. The detector then reacts linearly by a voltage change on the concentration m_x of the component detected, within the linearity concentration range.

It is apparent from eqn. 1 that the detector response is a function of the capacity change, $\Delta C/C_r$, and of the quality factor Q. If the quality factor Q is considered to be a variable quantity and assuming that $\Delta C/C_r$ is constant, the relations derived will hold true analogously for the response to the presence of a substance the "dielectric constant" of which is approximately equal to the ε'_c of the carrier liquid, but whose losses are higher than that of the carrier liquid. The resonance detector may be highly sensitive even to very small loss changes, where the other methods, *e.g.* bridge ones, show already low sensitivity.

It follows from the first Maxwell equation for the electric current density J in the condenser:

$$\vec{J} = g\vec{E} + \varepsilon^*_a \frac{\partial \vec{E}}{\partial t} \tag{29}$$

where g is the conductivity of the dielectric, E is the electric field intensity, and ε^*_a is the absolute complex permittivity, given by:

$$\varepsilon^*_a = \varepsilon'_a - j\,\varepsilon''_a \tag{30}$$

After substituting for ε^*_a in eqn. 29 and multiplying the whole equation by the dielectric cross-section A, and assuming a homogeneous electric field and isotropic dielectric (liquid), one gets for the current in the condenser with the dielectric thickness d:

$$\vec{I} = g\frac{A}{d}\vec{U}_c + \omega\varepsilon''_a \frac{A}{d}\vec{U}_c + j\omega\,\varepsilon_0\varepsilon'\frac{A}{d}\vec{U}_c = G_g\vec{U}_c + G_{\varepsilon''}\vec{U}_c + j\omega C\vec{U}_c \tag{31}$$

It follows from the above equation that the behavior of a real condenser with liquid showing dielectric losses and a non-zero conductivity may be modelled by an

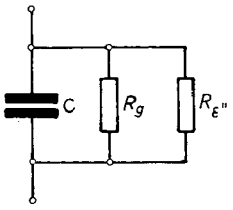

Fig. 4. Equivalent circuit for the condenser with a liquid with losses.

equivalent electric circuit in which resistances equal to the inverse values of the respective conductances are connected in parallel to an ideal condenser without loss (Fig. 4):

$$R_g = \frac{1}{G_g} = \frac{d}{gA} \tag{32}$$

$$R_{\varepsilon''} = \frac{1}{G_{\varepsilon''}} = \frac{d}{\omega\,\varepsilon''_a A} = \frac{d}{\omega\,\varepsilon_0 \varepsilon'' A} \tag{33}$$

It holds for the reciprocal value of the quality factor[4]:

$$\frac{1}{Q} = \frac{1}{Q_L} + \frac{1}{Q_c} = \frac{1}{Q_L} + \frac{G}{\omega_r C_r} \tag{34}$$

where

Q_L is the quality factor of the inductive coil,
Q_c is the quality factor of the measuring condenser,
ω_r is the angular resonance frequency,
C_r is the resonance capacity value, and
G is the total parallel conductance of the condenser.

The conductivity component in eqn. 32 may be included into losses, which yields, after substituting from eqn. 33 into eqn. 34, the equation that interprets the effect of the imaginary component of the complex permittivity on the resultant quality factor of the measuring resonant circuit:

$$\frac{1}{Q} = \frac{1}{Q_L} + \frac{\varepsilon_0 \varepsilon'' A}{C_r d} \tag{35}$$

In the case of the flow of pure carrier liquid with ε'_c the circuit is supposed to be in resonance. Hence, there holds for the resonance capacity:

$$C_r = \varepsilon_0 \varepsilon'_c \frac{A}{d} = \varepsilon'_c C_0 \tag{36}$$

so that it holds for the resultant quality factor at the flow of pure carrier liquid:

$$\frac{1}{Q} = \frac{1}{Q_L} + \frac{\varepsilon''_c}{\varepsilon'_c} = \frac{1}{Q_L} + \mathrm{tg}\delta_c \tag{37}$$

If a detected component is present having the parameters ε'_x, ε''_x, it will analogously hold for the resultant quality factor:

$$\frac{1}{Q_m} = \frac{1}{Q_L} + \mathrm{tg}\delta_m = \frac{1}{Q_L} + \frac{\varepsilon''_m}{\varepsilon'_m} \tag{38}$$

Provided a linear relationship holds between the concentration expressed in volume fractions, m, and the imaginary component of the complex permittivity, ε'', for low concentrations, analogously to eqn. 20, one gets for the resultant real part of the imaginary component of the permittivity of a binary mixture:

$$\varepsilon''_m = m_c\varepsilon''_c + m_x\varepsilon''_x = (1 - m_x)\,\varepsilon''_c + m_x\varepsilon''_x \tag{39}$$

and, after substituting into eqn. 38,

$$\frac{1}{Q_m} = \frac{1}{Q_L} + \frac{\varepsilon''_c + m_x(\varepsilon''_x - \varepsilon''_c)}{\varepsilon'_m} = \frac{1}{Q_L} + m_x\frac{\varepsilon''_x}{\varepsilon'_m} + \mathrm{tg}\delta_c - m_x\,\mathrm{tg}\delta_c \tag{40}$$

which yields for the quality factor of the mixture with the component under detection:

$$Q_m = \frac{Q}{m_x\,Q\left(\frac{\varepsilon''_x}{\varepsilon'_m} - \mathrm{tg}\delta_c\right) + 1} \tag{41}$$

As it is supposed that $\varepsilon'_x \doteq \varepsilon'_c$, the capacity remains constant during detection, which corresponds to the zero point at the flow of pure carrier:

$$\frac{\Delta C_1}{C_r} = 0.413\,Q^{-1} \tag{42}$$

but the quality factor falls from the initial value Q to Q_m due to the presence of the substance detected and it holds:

$$Q_m\frac{\Delta C_1}{C_r} = 0.413\frac{Q_m}{Q} \tag{43}$$

The detector response to the effect of ε''_x will be:

$$\Delta U_c = U_{c(1)} - U_{c(2)} = \frac{U_gQ}{\sqrt{1 + 0.413^2}} - \frac{U_gQ_m}{\sqrt{1 + 0.413^2Q^2_m/Q^2}} \tag{44}$$

From eqn. 41 it follows:

$$\frac{Q_m}{Q} = \frac{1}{m_xQ\left(\frac{\varepsilon''_x}{\varepsilon'_c} - \mathrm{tg}\delta_c\right) + 1} \doteq \frac{1}{m_xQ\,(\mathrm{tg}\delta_x - \mathrm{tg}\delta_c) + 1} \tag{45}$$

and substituting into eqn. 44 one gets for the response to ε''_x:

$$\Delta U_c \doteq U_gQ\left(0.924 - \frac{1}{\sqrt{[m_xQ\,(\mathrm{tg}\delta_x - \mathrm{tg}\delta_c) + 1]^2 + 0.413^2}}\right) \tag{46}$$

Fig. 5 shows the plots of the courses of the function $\Delta U_c = f_{\varepsilon''}(m_x)$ for the case of $Q = 200$, $U_g = 0.1$ V, and $\mathrm{tg}\delta_c = 10^{-3}$. Plots A and B have been calculated for the $\mathrm{tg}\delta_x$ of 2×10^{-3} and 11×10^{-3}, respectively.

The functions $\Delta U_c = f_{\varepsilon'}(m_x)$ and $\Delta U_c = f_{\varepsilon''}(m_x)$ are linear within low concentration limits. Owing to the law of superposition, the resultant response is also linear, being the sum of the response to ε'_x and ε''_x.

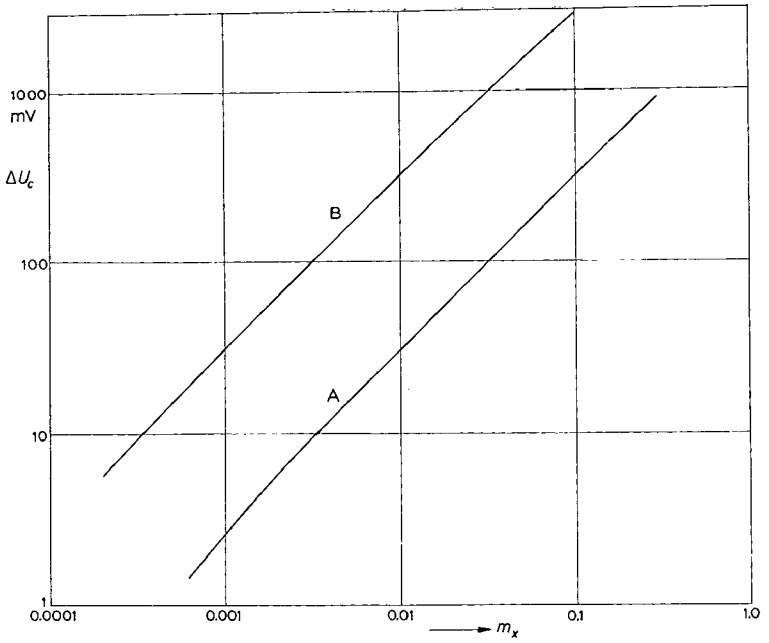

Fig. 5. Dependence of the response brought about by ε''_x on concentration for different $\mathrm{tg}\delta_x$.

CONCLUSION

A theoretical analysis of the basic characteristics of the LC permittivity detector was performed in which the principle of resonance has been used. In the detection of liquid components having $\varepsilon'_x > \varepsilon'_c$ and approximately the same loss tangent, $\mathrm{tg}\delta_x \doteq \mathrm{tg}\delta_c$, the predominating component of the response is the voltage drop across the condenser due to the capacity change. In the opposite case, where $\varepsilon'_x \doteq \varepsilon'_c$, but $\mathrm{tg}\delta_x > \mathrm{tg}\delta_c$, there predominates in the response the voltage drop across the condenser caused by the decrease of the quality factor Q of the resonant circuit. As both responses are approximately linear in the limits of low concentrations, the resultant response is the sum of both partial responses.

It is always advantageous to choose a non-conductive substance having lowest possible dielectric losses as the carrier liquid in employing the resonance principle.

In respect to the present-day state of the engineering in the resonance methods, when it is possible to attain $Q = 1000$ with both coaxial resonators as well as in circuits with lumped parameters[8], the resonance principle of detection presents remarkable possibilities.

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CHROM. 5106

ANALYSIS OF SUCROSE ESTERS OF LONG-CHAIN FATTY ACIDS ON SEPHADEX LH-20

KAZUO KONISHI, HIROH INOUE AND NORIO TANIGUCHI

Industrial Research Laboratories, Kao Soap Co. Ltd., 1334 Minatoyakushubata, Wakayama-shi (Japan)

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SUMMARY

The sucrose esters of long-chain fatty acids were separated into their components by gel chromatography on Sephadex LH-20. The conditions for gel chromatography were investigated and adjusted as follows: column, 2.0 × 230 cm; column temperature, 50°; developing solvent, DMF; flow rate, 60 ml/h; sample size, 50 mg. Mono-, di-, tri- and higher sucrose esters, sucrose and methyl esters of fatty acids were separated. Reproducibility was satisfactory. The time required for a single determination was about 12 h.

INTRODUCTION

Sucrose esters, nonionic surfactants, formed by reaction of sucrose with methyl esters of long-chain fatty acids are usually mixtures of sucrose, methyl esters of fatty acids, mono-, di-, tri- and higher sucrose esters. Many analytical methods[1–8] have been studied to examine the conditions necessary for production and quality control. However, most of these methods were thin-layer (TLC) and paper chromatography (PC), and they were, if anything, insufficient for accurate quantitative determination and for collection of the components, pure samples of which are needed for further studies of physico-chemical properties of the nonionic surfactant.

Gel chromatography in which separation is based on molecular size has been widely applied in biochemistry and in synthetic polymer chemistry. In recent publications[9,10], gel chromatography has frequently been used in studying oligomers and small molecules and is useful for analysis of non-volatile substances that cannot be determined by gas chromatography. Thus this technique appears to be promising for separation of sucrose, mono-, di-, tri- and higher sucrose esters from sucrose esters of long-chain fatty acids.

The present paper describes the separation and determination of sucrose esters of long-chain fatty acids by gel chromatography on Sephadex LH-20.

EXPERIMENTAL

Apparatus

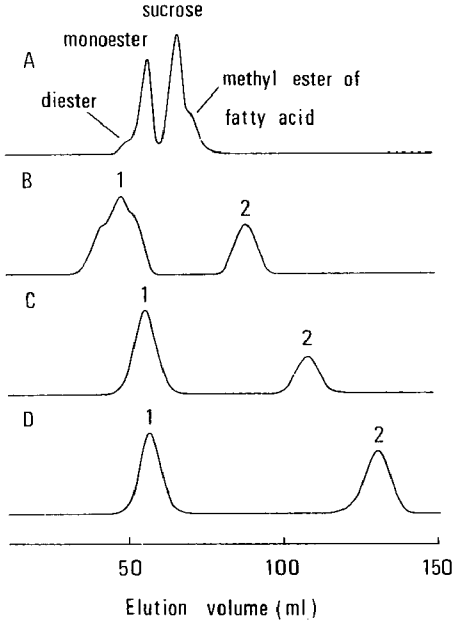
A glass column (2.0 × 230 cm) equipped with a jacket to control the column temperature was used. A differential refractometer (Waters Associates, Model R-4) was employed as a detector and the elution chromatogram was recorded automatically by a recorder. The flow rate was controlled with a pump (Japan Electron Optics Laboratory Co., Ltd., Model JLC-P2). A polyethylene tube (1.8 mm O.D.) was used as a connection between each apparatus.

Reagent and samples

For selecting a developing solvent, analytical grade dimethylformamide (DMF) and distilled water were used. Sephadex LH-20 gel (Pharmacia Fine Chemicals) was used as a column substrate. Commercially available sucrose esters of long-chain fatty acids were used as samples.

PROCEDURE

Sephadex LH-20 (175 g) was allowed to swell for 24 h in contact with DMF and



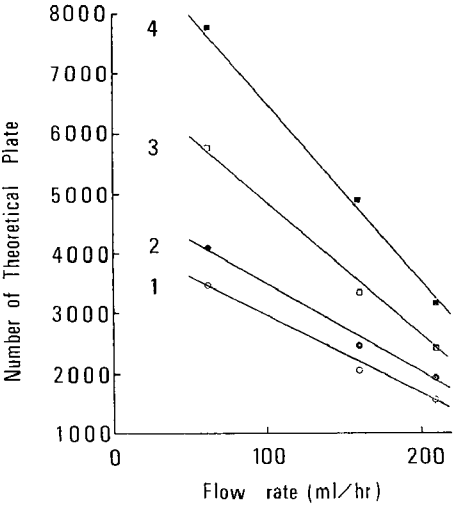


Fig. 1. Gel chromatograms obtained during preliminary tests for developing solvents. (A) Sephadex LH-20–DMF; (B) Sephadex LH-20–water; (C) Sephadex G-25–water; (D) Sephadex G-50–water. Chromatographic conditions: sample size, 50 mg; column size, 1.5 × 100 cm; column temperature, *ca.* 25[a]. 1, ester part; 2, sucrose.

Fig. 2. The relationship between flow rate and number of theoretical plates. (1) sucrose diester; (2) sucrose monoester; (3) sucrose; (4) methyl ester of fatty acid. Chromatographic conditions: sample size, 50 mg; column size, 2.0 × 230 cm; solvent, DMF; column temperature, 43°.

then was carefully poured into the glass column. Thereafter, the gel bed was allowed to settle for 24 h while DMF flowed through the column. The column temperature was maintained at 50° and the flow rate was controlled at 60 ml/h by a pump. 50 mg of a sample were diluted to 1 ml with DMF and carefully applied on top of the gel bed. The chromatogram was recorded automatically with a differential refractometer and a recorder. Each component fractionated was identified by its IR spectrum and the molecular weight was determined using a vapour-pressure osmometer. Peak resolution, R, was calculated as in a previous paper[10].

RESULTS AND DISCUSSION

The chromatographic conditions such as developing solvents, samples size, column temperature and flow rate were investigated. The R was calculated by using the monoester, diester and sucrose peaks.

Developing solvent

The developing solvents, about twelve, generally used in gel chromatography on Sephadex LH-20 cannot all be employed in analyses of sucrose esters of long-chain fatty acids because part of the sucrose esters is insoluble in methanol, ethanol, isopropanol, *n*-butanol, dioxane, acetone, ethyl acetate, tetrahydrofuran, chloroform and toluene. Dimethylformamide (DMF) and distilled water are good solvents for sucrose esters and can also be employed in gel chromatography on Sephadex LH-20 as developing solvents. The results are shown in Fig. 1. In this case, in order to shorten the length of the experiment, a glass column (1.5 × 100 cm) was used because sufficiently good results were obtained to choose the developing solvent.

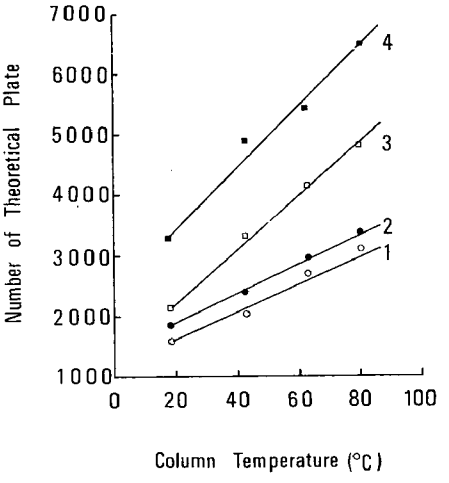
When DMF was employed, the separation of each component of sucrose esters was satisfactory, while the resolution of sucrose esters was inadequate using distilled water as developing solvent. However, using distilled water, the separation between sucrose esters and sucrose was very good, especially on Sephadex G-25 or G-50 instead of Sephadex LH-20. Therefore, in order to remove sucrose from sucrose esters, distilled water is suitable for a developing solvent. DMF, in which the sucrose esters readily dissolved and with which the peak resolution R was best, was selected as the developing solvent.

Flow rate

The effect of flow rate, from 40 to 210 ml/h, on the separation was investigated using DMF and a sample, and the number of theoretical plates was plotted as presented in Fig. 2. The lower was the flow rate, the better was the separation. From these results, the flow rate was fixed at 60 ml/h.

Column temperature

The effect of column temperature on the separation was investigated from 20 to 80° (Fig. 3). As shown, the higher was the temperature, the better was the separation within the experimental range. It is considered that diffusion of solute into the gel increases with rising temperature and consequently that the equilibrium rate of partition is improved at each theoretical plate. Thus the column temperature was fixed at 50°.



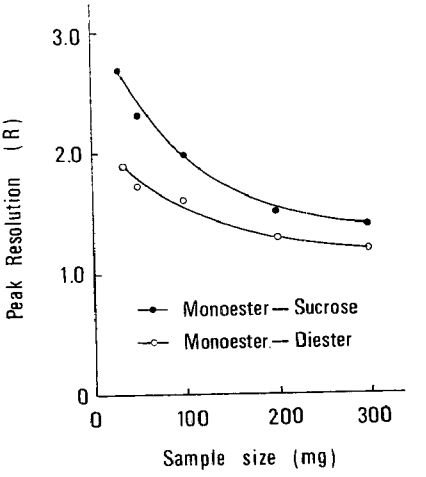


Fig. 3. The relationship between column temperature and number of theoretical plates. (1) sucrose diester; (2) sucrose monoester; (3) sucrose; (4) methyl ester of fatty acid. Chromatographic conditions: sample size, 50 mg; column size, 2.0 × 230 cm; solvent, DMF; flow rate, 156–174 ml/h.

Fig. 4. The relationship between sample size and peak resolution. Chromatographic conditions: column size, 2.0 × 230 cm; solvent, DMF; flow rate, 60 ml/h; column temperature, 50°.

Sample size

The relationship between peak resolution and sample size, from 30 to 300 mg/ml of DMF, was studied (Fig. 4). 30–100 mg of sample gave the best separation; however, because of the requirements of the recorder, a sample size of 50 mg was selected. For quantitative analysis, a 300-mg sample was selected for convenience.

TABLE I

ANALYTICAL RESULTS AND REPRODUCIBILITY

Chromatographic conditions: sample size, 50 mg; solvent, DMF; column size, 2.0 × 230 cm; flow rate, 60 ml/h; column temperature, 50°.

Sucrose ester	*Monoester*	*Diester*	*Triester*	*Tetra-ester*	*Penta-ester*	*Sucrose*	*Others*
1	70.7	18.6	2.4			8.3	
	68.4	19.2	2.7			9.6	
	67.7	18.9	2.8			10.5	
	69.4	18.3	3.0			9.3	
	69.0	17.9	2.2			10.8	
	68.1	19.0	2.6			10.3	
	70.0	17.5	1.9			10.6	
Average	69.0	18.5	2.5			9.9	
$\sqrt{V}$	1.1	0.62	0.38			0.89	
2	28.3	5.3	0.1			57.4	8.9
3	50.5	21.5	3.7			20.6	3.7
4	69.6	27.6	2.7				
5	13.6	14.2	6.6	2.4	1.6	61.7	

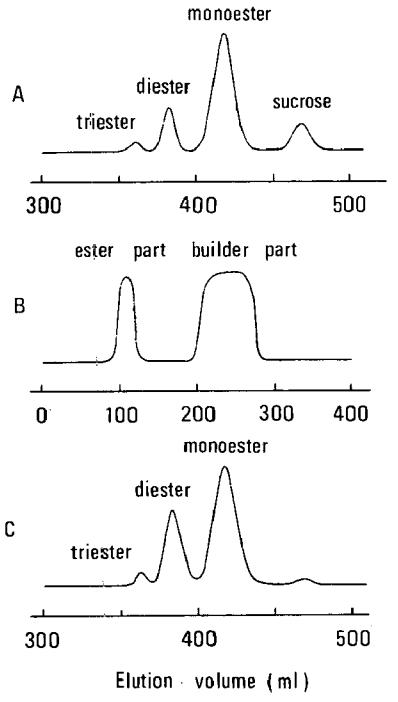


Fig. 5. Typical gel chromatogram. (A) sucrose esters; (B) a washing-up preparation; (C) sucrose ester part of a washing-up preparation. Chromatographic conditions: column size, 2.0 × 230 cm (A and C), 2.5 × 42 cm (B); solvent, DMF (A and C), water (B); Sephadex, LH-20 (A and C), G-25 (B); sample size, 50 mg (A, B, and C); flow rate, 60 ml/h (A and C), 235 ml/h (B); column temperature, 50° (A and C), 24° (B).

Gel chromatogram

A typical gel chromatogram obtained under the above-mentioned conditions is shown in Fig. 5A. It appears that the separation of mono-, di-, tri- and higher sucrose esters, sucrose, and methyl esters of fatty acids is satisfactory. Commercially available sucrose esters were analysed and the results are presented in Table I. The reproducibility which was satisfactory is also summarised in Table I.

Practical application of the method

The method described above was applied to the analysis of a washing-up preparation, in which sucrose esters of long-chain fatty acids are present as detergent. Because the washing-up preparation is a mixture of detergents and several kinds of builders, phosphate, phosphoric acid and low-molecular-weight organic sulphonates, etc., these materials must first be removed from the sucrose esters. Thus, gel chromatography using a Sephadex G-25–water system was carried out. The gel chromatogram obtained by this method is shown in Fig. 5B in which it appears that the separation between sucrose esters and the builders is satisfactory. However, in this case, sucrose in the sample was included in the builders. Part of the sucrose esters was collected quantitatively and the fraction was dried at 50°. Then sucrose esters thus obtained were chromatographed using Sephadex LH-20–DMF as described under EX-

PERIMENTAL. The gel chromatogram is shown in Fig. 5C which shows that each component of the sucrose esters is separated as well as when only sucrose esters are chromatographed, except that no sucrose peak appears in the chromatogram.

In analysing sucrose esters of long-chain fatty acids, in some respects, gel chromatography on Sephadex LH-20 is considerably more effective than TLC or PC. For example, in gel chromatography, conversion of sample to derivatives is not necessary, compounds are eluted more rapidly than by other chromatographic techniques, for a given flow rate, and the column has no tendency to prolong retention of samples and may be repeatedly used. Also, reproducibility is satisfactory when chromatographic conditions are constant, and components separate very conveniently. However, when the carbon number distribution of alkyl groups of a sample is large, this analytical method could not be applied to the sample because the resolution of each component of sucrose esters is unsatisfactory.

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COMPARISON BETWEEN COLUMN CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY IN THE DETERMINATION OF THE MOLECULAR-WEIGHT DISTRIBUTION OF POLYETHYLENE GLYCOL DERIVATIVES

C. CALZOLARI, L. FAVRETTO AND B. STANCHER
Istituto di Merceologia, Università di Trieste, Trieste (Italy)

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SUMMARY

The molecular-weight distribution of the polyethylene glycol derivatives $Ph \cdot S(CH_2CH_2O)_nCH_2CH_2S \cdot Ph$ (where n is the degree of polymerisation) was determined by column chromatography combined with ultraviolet absorption spectroscopy, and by thin-layer chromatography combined with direct photometric evaluation after visualisation with iodine vapour. The second method was found to be less precise than the first but had a comparable accuracy and permitted the separation of oligomers between $n = 6$ and $n = 19$.

INTRODUCTION

Determination of the molecular-weight distribution (MWD) plays an important part in the quality control of polyethylene glycol (PEG). Thin-layer chromatography (TLC) combined with direct photometric evaluation of the spots after visualisation with iodine vapour has recently been proposed as a rapid method for this determination[1,2].

Although the response of some compounds to iodine vapour varies with the degree of polymerisation n, the resulting systematic error can be corrected by means of a correction factor, found by examining the response of pure oligomers to iodine or by comparing the MWD with one obtained by an absolute method such as column chromatography (CC) combined with UV spectrophotometry[3]. This second method involves more work but is particularly promising in the fractionation of polyethylene glycols with chromophoric groups because—owing to the high peak capacity—gradient elution permits the separation of a great number of components[4].

The present aim was to assess the accuracy and the precision of TLC in this field by comparing it with CC. The work was done on compounds with the general formula $Ph \cdot S(CH_2CH_2O)_nCH_2CH_2S \cdot Ph$ ($n = 1$–19), the molecular absorption coefficient of these compounds being sufficiently high to enable one to analyse 1–2 mg of the oligomer mixture by CC with gradient elution.

EXPERIMENTAL

Commercial PEG with a number-average molecular weight of 600 was converted into the above-mentioned derivative, and the latter was purified[5]. In addition, pure compounds with $n = 1$–9 were prepared from synthetic homogeneous ethylene glycol oligomers. The corresponding dichlorides were repeatedly distilled at reduced pressure, then reacted with sodium thiophenoxide, and the products were purified by CC (the monomer also by vacuum distillation). The purity was checked by temperature-programmed GLC[5], IR and UV absorption spectroscopy, and NMR spectroscopy. The IR spectrophotometric analysis was done on a liquid sample with a Perkin-Elmer Infracord, and the NMR spectroscopic analysis with a Jeol C-60 instrument, the sample having been dissolved in deuterated chloroform (50%, v/v). The characteristics of these standards are shown in Table I.

TABLE I

DATA FOR THE PURE INDIVIDUAL MEMBERS OF THE SERIES $Ph \cdot S(CH_2CH_2O)_nCH_2CH_2S \cdot Ph$ USED AS STANDARDS

The molar absorption coefficient in 95% ethanol at 253.5 nm and 20° is $\varepsilon = 15\,600\ l \cdot cm^{-1} \cdot mole^{-1}$.

n	*Degree of purity (% peak area)*	*Oligomers present as impurities (% peak area)*	*Elution temperature (°C)*	$n_{589\ nm}^{20°}$
1	99.9[a]	—	156	1.6169
2	99.9	—	173	1.5928
3	99.9	—	188	1.5782
4	99.5	0.5 ($n = 3$)	205	1.5671
5	99.9	—	221	1.5581
6	98.0	0.8 ($n = 5$) 1.2 ($n = 7$)	237	1.5508
7	99.0	1.0 ($n = 6$)	253	1.5438
8	97.0	0.5 $n = 6$ 2.5 $n = 7$	269	1.5390
9	96.2	0.2 ($n = 7$) 3.6 ($n = 8$)	284	1.5359

[a] Boiling point: 171–173° at 0.03 mm Hg.

The chromatographic column had an internal diameter of 5.0 mm, was thermostatically controlled at 20.0 ± 0.1°, and was connected to an Erba Fotocromacon AS-35 fraction collector, preventing losses by evaporation. The adsorbent column had a height of about 27 cm and an interstitial volume V_0 of 6.5 ml; it was prepared by sedimenting a suspension of 3 g of the adsorbent in 10 ml of the starting eluent. A nitrogen pressure of about 500 mm Hg upstream of the column ensured an eluent flow rate of 0.7–0.8 ml/min. About 2 mg of the sample was placed on a stationary-phase disk not thicker than 1 mm at the top of the column with the aid of a teflon capillary connected to a 10-μ Hamilton microsyringe.

The 2.00-ml fractions were evaporated in a drying cabinet at 40° and 2 mm Hg; the residue was redissolved in 2.00 ml of 95% ethanol, and the solutions were subjected to spectrophotometric determination at 253.5 nm in 1-cm cells, the results being used to obtain the elution pattern in the form of a histogram. Eluate fractions

containing the same compound were combined, diluted with ethanol to a known volume, and quantitatively analysed by spectrophotometry. The same absorption coefficient ($\varepsilon = 15\,600\ l \cdot cm^{-1} \cdot mole^{-1}$) was taken for all the compounds so that the molar fraction was proportional to the spectrophotometric absorption for all the members of the polymer mixture.

The degree of polymerization of the above fractions was found by comparing the latter with pure standards with the aid of TLC and GLC, carried out on the residues left behind after the evaporation of the ethanolic solutions. Compounds with $n = 2$–5 could not be separated by CC and were separated and estimated by GLC.

The silica gel used as the adsorbent in CC was obtained by screening commercial silicic acid (Mallinckrodt AR); it had a particle size of 0.035–0.050 mm, permitting better resolution than the granulometric fractions used before[4]. The eluents were mixtures of methylene chloride (Carlo Erba, freshly distilled over P_2O_5) and acetone (Erba chromatographic reagent).

To reduce the retention volumes V_R (in ml), the adsorbent, maximally activated by heating for 15 h at 180 ± 5°, was partially deactivated with 3% (w/w) water or by silanisation (addition of 3% trimethylchlorosilane) as described previously[4]. The degree of activation was found by the following two methods:

(a) Determination of the distribution coefficient K (in ml/g) at 20° for the compounds with $n = 4$–7, fractionated with a 95:5 (v/v) mixture of methylene chloride and acetone; $K = (V_R - V_0)/W$, where W is the weight of the adsorbent in g);

(b) Determination of the adsorption isotherm of the compounds with $n = 4$–6 in the presence of the same eluent at 20° by spectrophotometric measurement of the oligomer concentration in the liquid phase before and after the establishment of equilibrium with the adsorbent[6].

For TLC, optical-grade glass plates (20 × 2.5 cm) were coated with 20–40 μ Silica Gel G (Merck) by means of an applicator with a fixed 200-μ slit. 40 μg of the oligomeric mixture, or not more than 5 μg of each pure oligomer in a chloroform solution, were applied to the plate with a 10-μl Hamilton microsyringe mounted on a micrometer support, the sample spots having a diameter of not more than 3 mm. The plates were developed vertically to a run of 17.0 cm in a cylindrical Desaga tank (20 × 5 cm), equilibrated at 20 ± 1°, the developer being a 90:10 (v/v) mixture of butanone and water. Other details of the process have been described before[2].

The eluted spots were visualised with iodine vapour in the course of 1 h, then protected with a glass plate and subjected to photometric evaluation with a Joyce Loebl Chromoscan set for measuring the transmission. This was done by scanning in the elution direction at $\lambda = 430$ nm (glass filter), with 5 × 1, 6 × 1, and 7 × 1 mm slits. The recorder response, linearised in absorbance in the range of 0–1 A, permitted the determination of the integrated absorbance of peaks, whose area was found planimetrically.

RESULTS AND DISCUSSION

Column chromatography

Fig. 1 shows log K *vs.* n in the case of deactivated and maximally activated

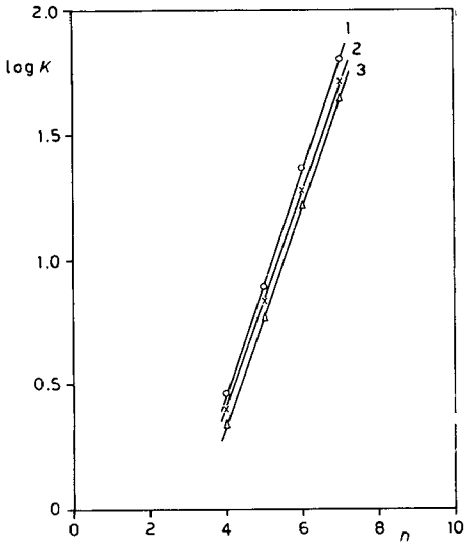


Fig. 1. Variation of log K with the degree of polymerisation (n) of compounds $Ph \cdot S(CH_2CH_2O)_n CH_2CH_2S \cdot Ph$ in the case of silica gel adsorbents with various degrees of activation, and a 95:5 (v/v) mixture of methylene chloride and acetone as eluent. 1, maximally activated adsorbent; 2, adsorbent deactivated with 3% (w/w) water; 3, adsorbent deactivated with 3% (w/w) trimethylchlorosilane (TMCS).

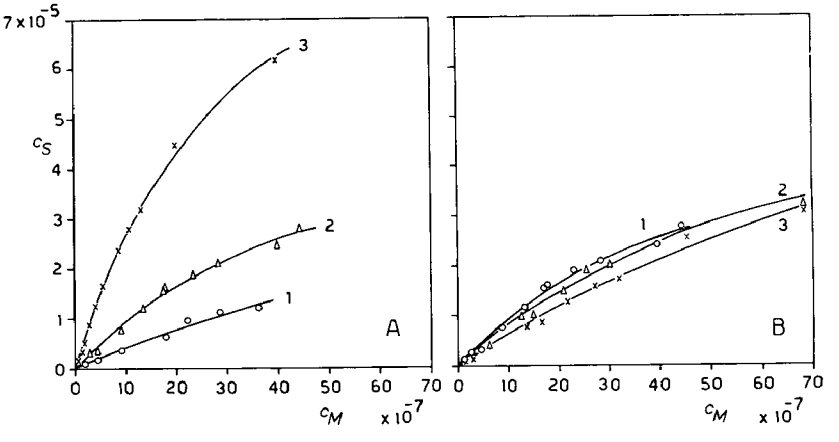


Fig. 2. Adsorption isotherms of pure members of the series $Ph \cdot S(CH_2CH_2O)_n CH_2CH_2S \cdot Ph$ at 20°, the liquid phase being a 95:5 (v/v) mixture of methylene chloride and acetone. Diagram A: $n = 4$ (curve 1), $n = 5$ (curve 2), and $n = 6$ (curve 3); adsorbent: maximally activated silica gel. Diagram B: $n = 5$; adsorbent: maximally activated silica gel (curve 1), silica gel deactivated with 3% water (curve 2) and silica gel deactivated with 3% trimethylchlorosilane (curve 3). c_M is the equilibrium concentration of the adsorbate in the liquid phase (mole/ml), and c_S is the specific adsorption (mole/g). The continuous curves connecting the experimental points were obtained by least squares on the assumption that the Langmuir isotherm is obeyed.

TABLE II

VALUES OF THE CONSTANTS A AND B IN THE LANGMUIR ISOTHERM OF $Ph \cdot S(CH_2CH_2O)_nCH_2CH_2S \cdot Ph$ COMPOUNDS AT 20°

The liquid phase is a 95:5 (v/v) mixture of methylene chloride and acetone.

n	*Degree of activation of the silica gel adsorbent*	A ($ml \cdot g^{-1}$)	B ($ml \cdot mole^{-1}$)
4	Maximum	4.24	5.46×10^4
5	Maximum	11.2	1.82×10^5
	Deactivated with 3% water	9.30	1.32×10^5
	Deactivated with 3% TMCS	6.71	6.27×10^4
6	Maximum	33.7	2.87×10^5

adsorbents. In accordance with MARTIN's equation[7], the adsorption energy is seen to vary linearly with the degree of polymerisation of the adsorbate in all three cases.

Fig. 2 shows the adsorption isotherms for the various systems at 20°. The experimental values were connected into continuous curves on the assumption that the Langmuir isotherm is obeyed, *i.e.* that $c_S = Ac_M/(1 + Bc_M)$, where c_S is the

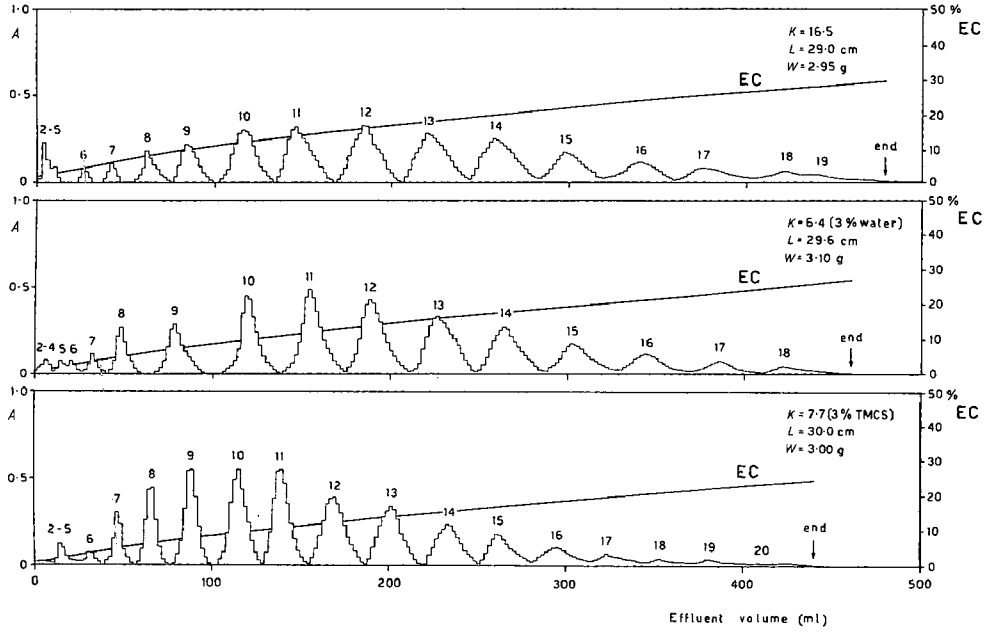


Fig. 3. Column chromatograms obtained for $Ph \cdot S(CH_2CH_2O)_nCH_2CH_2S \cdot Ph$ by gradient elution and spectrophotometric evaluation. Adsorbents: maximally activated silica gel (top), silica gel deactivated with 3% water (middle), and silica gel deactivated with 3% trimethylchlorosilane (bottom). Eluent: mixtures of methylene chloride and acetone. The eluent composition at the top of the adsorbent column is given by a straight line in conjunction with the ordinate on the right, which shows the amount of acetone in the mixture (%, v/v). A = spectrophotometric absorbance at 253.5 nm; L = height of the adsorbent column (cm), and W = weight of the adsorbent column (g). The degree of activation of the silica gel is expressed by the distribution coefficient K of naphthalene, eluted with n-pentane. The number over each peak is the corresponding degree of polymerisation n.

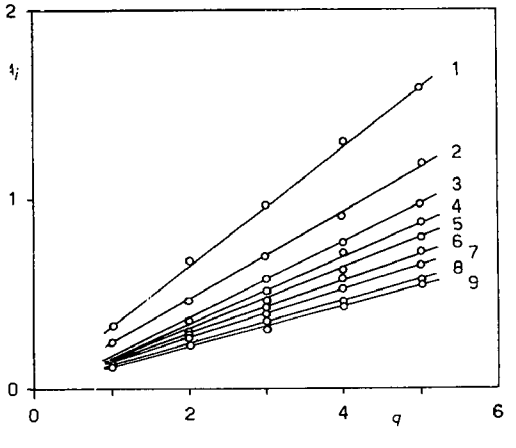
specific adsorption (in moles of adsorbate per g of adsorbent), c_M is the equilibrium concentration (in moles per ml of liquid phase), and A and B are constants whose values are found by the method of least squares (*cf.* Table II). As c_M approaches zero, A comes to represent the slope of the linear isotherm. Deactivation of the adsorbent reduces both A and B, and thus increases the c_M range in which the isotherm is approximately linear. Log A increases linearly with n, the line being parallel to that obtained by plotting the chromatographic log K values against the n values. The silanised adsorbent seems to be best for the compounds under consideration.

Fig. 3 shows some elution patterns obtained in the gradient elution for a PEG preparation with $\bar{n} = 12.2$. The partially deactivated adsorbents are seen to have permitted a better separation of the high-n compounds.

Thin-layer chromatography

As may be seen from Fig. 4, there is a linear relationship for $n = 1$–9 between the sample size (in μg) and the integrated absorbance of the peak, measured planimetrically on the same plate after visualisation with iodine vapour. Such straight lines are obtained for various visualisation times. In the case of one and the same plate, the ratio between the slopes of the straight-line calibration curves for two compounds is constant and approaches unity for $n > 7$.

In Fig. 5 r is the ratio between the slope of the calibration curve for a compound with a polymerisation degree n and the slope of the calibration curve for the nonamer, this second slope being taken as 1. The correction factors to be applied to the integrated absorbance of compounds with $n < 9$ are as follows (the value of n is indicated in parentheses): 0.35 (1), 0.46 (2), 0.57 (3), 0.63 (4), 0.70 (5), 0.76 (6), 0.87 (7), and 0.95 (8). These correction factors are calculated as $1/r$ in each case. It has



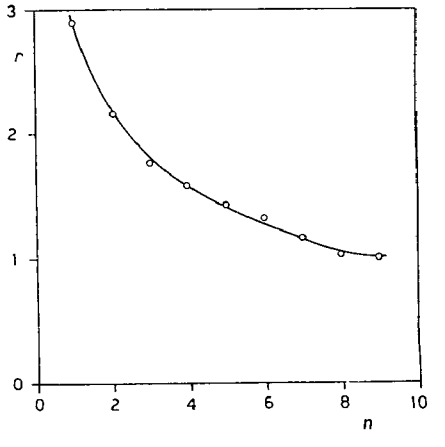


Fig. 4. Relationship between the amount of sample q (μg) placed on the plate and the integrated absorbance A_i (in arbitrary units), measured planimetrically.

Fig. 5. Ratio r between the slope of the calibration curve for a compound with a degree of polymerization n and the slope of the calibration curve for the nonamer (r is always the mean of three measurements).

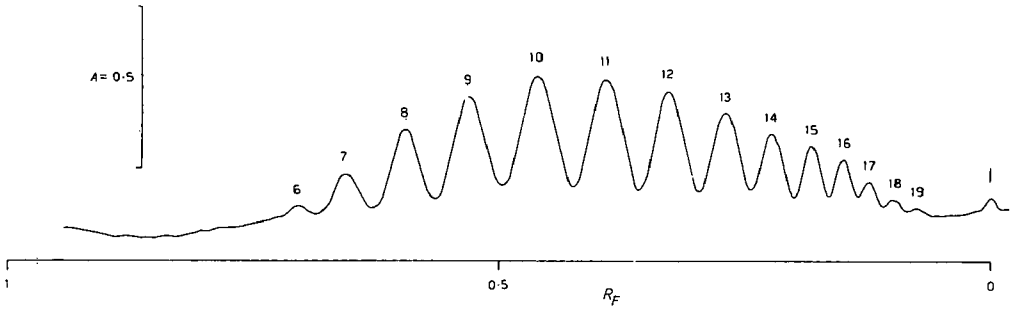


Fig. 6. Thin-layer chromatogram of PEG derivatives with $\bar{n} = 12.2$. Single elution with a 90:10 (v/v) mixture of butanone and water at 20° (A = absorbance).

been assumed that the compounds with $n > 9$ respond to the iodine vapour similarly to the nonamer.

Fig. 6 shows a chromatogram obtained by the single elution of a mixed PEG with $\bar{n} = 12.2$. The resolution of the individual compounds is seen to be practically complete up to $n = 19$.

TABLE III

DISTRIBUTION OF THE DEGREE OF POLYMERISATION FOR A $Ph \cdot S(CH_2CH_2O)_nCH_2CH_2S \cdot Ph$ PREPARATION WITH $\bar{n} = 12.2$, AS OBTAINED BY CC (NUMBER OF DETERMINATIONS $N = 2$) AND TLC ($N = 3$).

$\bar{x}_n$ is the molar fraction (mean of N determinations), and s is the mean-square deviation. The variation coefficients (in %) are given in brackets. The last line shows the calculated number-average degree of polymerisation $\bar{n}_{calc.} = \Sigma \bar{x}_n n$.

n	$\bar{x}_n \pm s$			
	Column chromatography ($N = 2$)			*Thin-layer chromatography ($N = 3$)*
	0% water	*3% water*	*3% TMCS*	
2[a]	0.0026 ± 0.0001	0.0037 ± 0.0001	0.0042 ± 0.0002	— —
3[a]	0.0037 ± 0.0001	0.0049 ± 0.0001	0.0057 ± 0.0002	— —
4[a]	0.0044 ± 0.0003	0.0064 ± 0.0002	0.0074 ± 0.0001	— —
5[a]	0.0164 ± 0.0006	0.0190 ± 0.0004	0.0111 ± 0.0003	— —
6	0.0499 ± 0.0006	0.0437 ± 0.0005	0.0404 ± 0.0001	0.0073 ± 0.0015
7	0.0848 ± 0.0006	0.0716 ± 0.0030	0.0715 ± 0.0009	0.0406 ± 0.0069
8	0.1163 ± 0.0005 (0.5)	0.1079 ± 0.0007 (0.6)	0.1039 ± 0.0002 (0.2)	0.1022 ± 0.0082 (8.0)
9	0.1525 ± 0.0007 (0.5)	0.1436 ± 0.0006 (0.4)	0.1269 ± 0.0009 (0.7)	0.1525 ± 0.0136 (8.9)
10	0.1558 ± 0.0010 (0.6)	0.1458 ± 0.0022 (1.5)	0.1362 ± 0.0003 (0.2)	0.1737 ± 0.0100 (5.7)
11	0.1416 ± 0.0009 (0.6)	0.1333 ± 0.0007 (0.5)	0.1322 ± 0.0002 (0.1)	0.1691 ± 0.0105 (6.2)
12	0.1155 ± 0.0003 (0.3)	0.1125 ± 0.0004 (0.4)	0.1159 ± 0.0007 (0.6)	0.1299 ± 0.0052 (4.0)
13	0.0812 ± 0.0009 (1.1)	0.0707 ± 0.0100 (14.1)	0.0859 ± 0.0009 (1.0)	0.0994 ± 0.0136 (13.6)
14	0.0425 ± 0.0003	0.0533 ± 0.0003	0.0600 ± 0.0003	0.0592 ± 0.0130
15	0.0174 ± 0.0008	0.0374 ± 0.0003	0.0455 ± 0.0005	0.0314 ± 0.0067
16	0.0087 ± 0.0001	0.0234 ± 0.0157	0.0283 ± 0.0003	0.0215 ± 0.0032
17	0.0044 ± 0.0002	0.0089 ± 0.0006	0.0148 ± 0.0005	0.0091 ± 0.0014
18	0.0023 ± 0.0002	0.0039 ± 0.0003	0.0076 ± 0.0002	0.0042 ± 0.0012
19	trace —	— —	0.0025 ± 0.0001	0.0019 ± 0.0006
20	— —	— —	trace —	— —
$\bar{n}_{calc.}$	9.98	10.31	10.56	10.88

[a] Values obtained by GLC.

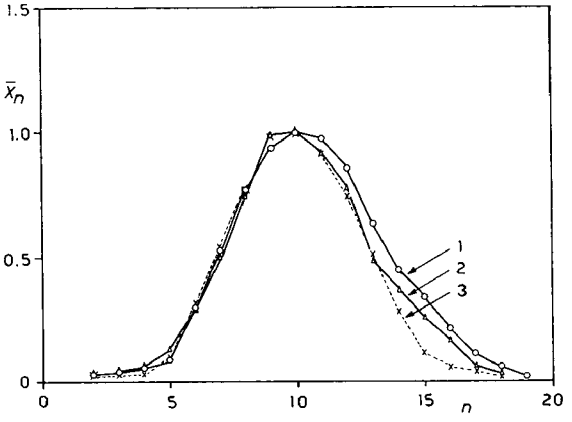


Fig. 7. Distribution of the degree of polymerisation in a $Ph \cdot S(CH_2CH_2O)_nCH_2CH_2S \cdot Ph$ preparation, as obtained by CC with maximally activated silica gel (curve 3), silica gel deactivated with 3% TMCS (curve 1), and silica gel deactivated with 3% water (curve 2). $\bar{x}_n$ is the molar fraction (mean of two determinations); $\bar{x}_{10}$ was taken as 1.

Comparison between CC and TLC

The data in Table III show that CC is more precise than TLC. In particular, CC with an adsorbent deactivated by silanisation permits the determination of the individual compounds in the mixture with a variation coefficient of about 0.5%, the corresponding value in the case of TLC being about 8%. Furthermore, the results obtained by CC with such an adsorbent are also more accurate than those obtained by CC with the other adsorbents examined; the progressive underestimation

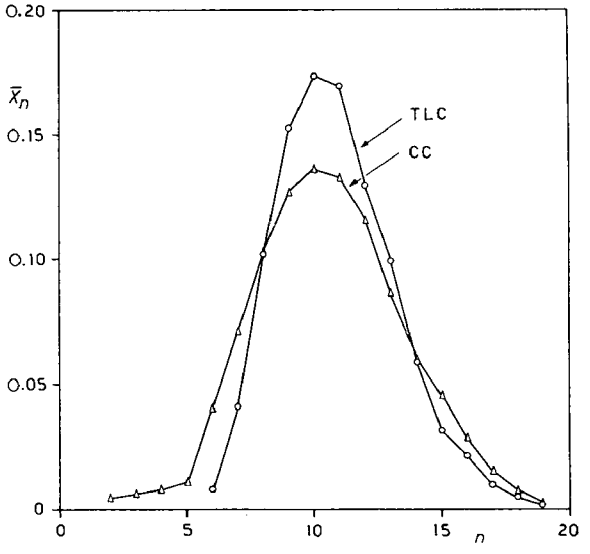


Fig. 8. Distribution of the degree of polymerisation in PEG derivatives with $\bar{n} = 12.2$, as obtained by CC and by TLC. $\bar{x}_n$ is the molar fraction (*cf.* Table III).

of high-n compounds, seen in the three distribution curves calculated with $\bar{x}_{10} = 1$ (*cf.* Fig. 7), is least pronounced with the silanised adsorbent and most pronounced with the maximally activated one. Though less precise, TLC has an accuracy comparable to that of CC, and it offers the means of rapid analysis of samples even smaller than 0.1 mg. Fig. 8 shows that the two distribution curves are of the same type, and the calculated $\bar{n}$ values agree in the two cases, even though TLC does not respond sensitively enough to compounds with $n<6$.

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CHROM. 5088

THIN-LAYER CHROMATOGRAPHY ON SILICA GEL AS A METHOD FOR ASSIGNING THE RELATIVE CONFIGURATIONS TO SOME ALIPHATIC DIASTEREOMERIC COMPOUNDS

M. PALAMAREVA AND M. HAIMOVA
Department of Chemistry, University of Sofia, Sofia 26 (Bulgaria)
AND
J. STEFANOVSKY, L. VITEVA AND B. KURTEV
Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia (Bulgaria)

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SUMMARY

Separation of 37 diastereomeric pairs of compounds from the group Ar–CH(X)–CH(Y)–Ar′ (X and Y are polar groups; Ar and Ar′ are phenyl or *m*-, *p*-alkoxy substituted phenyl groups) is achieved by thin-layer chromatography on silica gel. In all cases the erythro-isomers have higher R_F values than do the corresponding threo-isomers, irrespective of the polarity of the developing solvents as well as of the formation of an intramolecular hydrogen bond between X and Y. The phenomena are explained on the basis of the preferred conformations of the diastereomers. Thin-layer chromatography on silica gel is proposed as a method for assigning the relative configurations of diastereomeric pairs of compounds of the above type.

INTRODUCTION

Separation of aliphatic diastereomeric compounds with two asymmetric carbon atoms has been achieved by thin-layer chromatography (TLC) on silica gel[1,2], boric acid-impregnated silica gel[3,4], Fasertonerde (alumina with fibre structure)[5] and cellulose[2]. Only in two of the papers quoted above do the authors correlate the chromatographic behaviour of the diastereomeric compounds with their relative configurations. On examining the separation of the methyl esters of diastereomeric fatty acids of higher molecular weights with two adjacent hydroxyl groups by TLC on boric acid-impregnated silica gel, MORRIS[3] has found that the threo-isomers have higher R_F values than do the erythro-isomers. He has assumed that the threo-compounds complex much more readily and, since the complexes are less polar than the original diols, these migrate much faster than the erythro-isomers. DREFAHL *et al.*[4] have separated diastereomeric aliphatic amino alcohols by TLC on silica gel, impregnated with boric acid, and have established that the diastereomer occurring, hydrogen bonding intramolecularly to a lesser degree, has a higher R_F value. This

has been explained by these authors with the assumption that the solvent in the developing system, itself capable of intermolecular hydrogen bonding, interacts more strongly with the isomer having less intramolecular hydrogen bonding. Furthermore, the difference in the extent of complex formation between boric acid and the diastereomeric amino alcohols has been pointed out as a contributing factor. Thus, the above two explanations for the difference in TLC behaviour of diastereomeric aliphatic compounds are based only on studies of compounds with intramolecular hydrogen bonding from the type HO...HO and NH_2...HO and with boric acid-impregnated silica gel as adsorbent.

In recent years many diastereomeric pairs of compounds, derivatives of 1,2-diarylethanes with two asymmetrical carbon atoms, were synthesised in our laboratory for various purposes. Their relative configurations were assigned which permitted us to investigate the separation by TLC on silica gel of a substantial number of diastereomeric pairs of compounds belonging to the group of Ar-CH(X)-CH(Y)-Ar' (X and Y are polar groups; Ar and Ar' are phenyl groups or *m*-, *p*-alkoxylated ones), as well as to establish whether a correlation exists between chromatographic behaviour and relative configurations of the compounds studied.

EXPERIMENTAL

The adsorbents used for thin-layer plates were Silica Gel DG (Riedel de Haen, 30 g and 80 ml of distilled water) and cellulose powder (Schuchardt, 20 g, 1 g of gypsum and 85 ml of water). The slurry was spread with an apparatus according to Stahl with a coating thickness of approximately 250 μ. The coated plates were air dried and kept in a dust-free chamber. The samples were applied 1.5 cm from the edge of the plate with a 2-cm distance between them. The length of run was 10 cm. The chromatograms were developed at room temperature in a glass chamber saturated with the solvent system. The cases of multiple development are indicated in Table I. Both Dragendorff's reagent followed by spraying with ether–iodine (for the basic compounds) and sulphuric acid were used for detection. The solvent systems used were A = ether–heptane (2:1); B = heptane–ethyl acetate–methanol–ammonia (12:10:1.5:1, the upper layer); C = ether–petroleum ether (2:1), D = ether–heptane (1:5); E = benzene–ethyl acetate–ethanol–ammonia (50:40:5:5, the upper layer); F = ether–heptane (1:1); G = ether; H = ether–petroleum ether (1:1); I = ether–acetone (3:2); J = ether–acetone (4:1); K = ether–acetone (9:1); L = ether–acetone (7:3); M = ether–methanol (17:3); N = ether–methanol (1:1); O = ethyl acetate–methanol–acetic acid (16:4:2); P = benzene–methanol–acetic acid (60:6:1); and Q = benzene–methanol–acetic acid (30:1:1). Whatman No. 1 paper was used for paper chromatography (PC) with ascending development of the chromatograms.

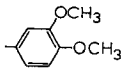
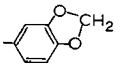
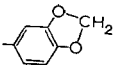
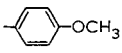
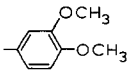
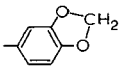
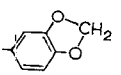
Methyl esters of threo- and erythro-3-hydroxy-2,3-diphenylpropionic acids (23 and 24, Table I) were prepared by methylation with diazomethane of the hydroxy acids 73 and 74, respectively[6].

RESULTS AND DISCUSSION

The results from the separation by TLC on silica gel of the diastereomeric pairs of compounds of the type mentioned above prepared by us and other authors

TABLE I

R_F VALUES OF THE DIASTEREOMERIC PAIRS OF Ar–CH(X)–CH(Y)–Ar'

Ar	*Ar'*	*X*	*Y*	*Configuration*	*Reference*	*Compound No.*	R_F	*Solvent system*
Ph	Ph	NH_2	$COOCH_3$	threo	7,8	1	0.30	A developed twice
				erythro		2	0.39	
	Ph	NH_2	$COOCH_3$	threo	9	3	0.48	B developed twice
				erythro		4	0.55	
	Ph	NH_2	$COOCH_3$	threo	9	5	0.27	A
				erythro		6	0.41	
Ph		NH_2	$COOCH_3$	threo	9	7	0.69	B developed twice
				erythro		8	0.76	
Ph	Ph	$NHCH_3$	$COOCH_3$	threo	10	9	0.39	A
				erythro		10	0.55	
	Ph	$NHCH_3$	$COOCH_3$	threo	10	11	0.48	A–B (1:1)
				erythro		12	0.60	
	Ph	$NHCH_3$	$COOCH_3$	threo	10	13	0.27	A–B (1:1)
				erythro		14	0.39	
	Ph	$NHCH_3$	$COOCH_3$	threo	10	15	0.31	A
				erythro		16	0.51	
Ph	Ph	$N(CH_3)_2$	$COOCH_3$	threo	10	17	0.34	C
				erythro		18	0.42	
Ph	Ph	NHPh	$COOCH_3$	threo	11	19	0.27	D[a] developed twice
				erythro		20	0.34	
Ph	Ph	$NHCONH_2$	$COOCH_3$	threo	12	21	0.25	E developed twice
				erythro		22	0.30	
Ph	Ph	OH	$COOCH_3$	threo	—	23	0.51	F
				erythro		24	0.59	
Ph		OH	$COOCH_3$	threo	13	25	0.38	B–H (1:5)
				erythro		26	0.47	

[a] Resolved by M. MLADENOVA.

(continued on p. 386)

TABLE I *(continued)*

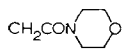
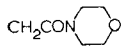
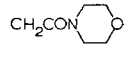
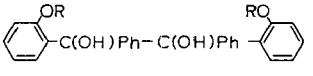
Ar	*Ar'*	*X*	*Y*	*Configuration*	*Reference*	*Compound No.*	R_F	*Solvent system*
Ph	Ph	$CON(CH_3)_2$	CH_2COOCH_3	threo	14	27	0.52	G
				erythro		28	0.68	
Ph	Ph	$CON(C_2H_5)_2$	CH_2COOCH_3	threo	14	29	0.32	H
				erythro		30	0.39	
Ph	Ph		CH_2COOCH_3	threo	14	31	0.43	G
				erythro		32	0.52	
Ph	Ph	$CON(CH_3)_2$	$CH_2COOC_2H_5$	threo	14	33	0.60	G
				erythro		34	0.70	
Ph	Ph	$CON(C_2H_5)_2$	$CH_2COOC_2H_5$	threo	14	35	0.40	H
				erythro		36	0.46	
Ph	Ph		$CH_2COOC_2H_5$	threo	14	37	0.52	G
				erythro		38	0.58	
Ph	Ph	$CON(CH_3)_2$	$CH_2CON(CH_3)_2$	threo	15	39	0.61	I
				erythro		40	0.70	
Ph	Ph	$CON(CH_3)_2$	$CH_2CON(C_2H_5)_2$	threo	15	41	0.49	I
				erythro		42	0.66	
Ph	Ph	$CON(CH_3)_2$		threo	15	43	0.45	I
				erythro		44	0.60	
Ph	Ph	$CON(C_2H_5)_2$	$CH_2CON(CH_3)_2$	threo	15	45	0.52	J
				erythro		46	0.58	
Ph	Ph	$CON(C_2H_5)_2$	$CH_2CON(C_2H_5)_2$	threo	15	47	0.31	G
				erythro		48	0.43	
Ph	Ph	$CON(C_2H_5)_2$		threo	15	49	0.32	K
				erythro		50	0.43	
Ph	Ph		$CH_2CON(CH_3)_2$	threo	15	51	0.32	J
				erythro		52	0.44	
Ph	Ph		$CH_2CON(C_2H_5)_2$	threo	15	53	0.59	J
				erythro		54	0.70	
Ph	Ph			threo	15	55	0.38	L
				erythro		56	0.54	

TABLE I *(continued)*

Ar	*Ar'*	*X*	*Y*	*Configuration*	*Reference*	*Compound No.*	R_F	*Solvent system*
Ph	Ph	NH_2	CH_2OH	threo	7	57	0.44	M
				erythro		58	0.54	
Ph	Ph	$NHCH_3$	CH_2OH	threo	16	59	0.45	M
				erythro		60	0.57	
Ph	Ph	$CH_2N(CH_3)_2$	CH_2CH_2OH	threo	17	61	0.38	N
				erythro		62	0.53	
Ph	Ph	$CH_2N(C_2H_5)_2$	CH_2CH_2OH	threo	17	63	0.35	N
				erythro		64	0.52	
Ph	Ph		CH_2CH_2OH	threo	17	65	0.15	G
				erythro		66	0.46	
Ph	Ph	CH_2OH	CH_2CH_2OH	threo	17	67	0.64	G
				erythro		68	0.85	
Ph	Ph	$NHCH_3$	COOH	threo	10	69	0.46	O developed twice
				erythro		70	0.52	
Ph	Ph	$NHCONH_2$	COOH	threo	12	71	0.15	P developed three times
				erythro		72	0.21	
Ph	Ph	OH	COOH	threo	18	73	0.22	Q
				erythro		74	0.31	

are given in Table I. The relative configurations of the compounds studied are known (see the references).

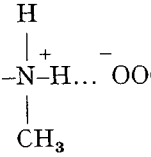
The most obvious conclusion drawn from the data (Table I) is that in all cases of separation of the 37 diastereomeric pairs of compounds studied, the erythro-isomers possess higher R_F values than do the corresponding threo-isomers, irrespective of whether intramolecular hydrogen bonding between X and Y exists and of the polarity of the solvent system used (see systems D and O). It is worth noting that TLC on silica gel of 6 diastereomeric pairs of compounds (from the group



R is an alkoxyl group) has shown higher R_F values for the *ms*-forms than for the

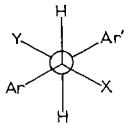
corresponding D,L-isomers[1].

Intramolecular hydrogen bonding is observed only in some of the diastereomeric pairs of compounds investigated—in compounds 57–66 of the type N...HO, in 67, 68 of the type HO...HO. A zwitterion structure and intramolecular hydrogen bonding

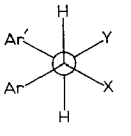


of the type is assumed for the diastereomeric pair of 69, 70 (compare with ref. 2). Obviously a generally valid explanation, similar to that given by the authors cited above[3,4], in our case is impossible.

Although the variety of X and Y in the compounds studies is rather large, and therefore we are dealing with different classes of organic compounds, some common relationships even if only qualitative have been established for the behaviour of the individual compounds of the two steric series. For instance, in the cases in which N...HO intramolecular hydrogen bonding leading to the formation of a six-membered ring (57–60) is possible, it is predominant in the threo-isomers[7,16]; on formation of an eight-membered ring (61–66) the hydrogen bond is stronger again in the threo-isomers[17]. In the compounds with $COOCH_3$ and $CON(CH_3)_2$ groups, the signals for the methyl protons of these groups appear in the erythro-isomers at a higher field[10,13–15,19]. A simple relationship has been found between absolute configuration and sign of rotation in the optically active compounds[20]. Characteristic differences have also been established in the chemical behaviour of such compounds. For example, some cases of *cis*-cyclisation have been investigated. When the ring closure occurs by including a unit between X and Y, the threo-isomers show higher reactivity[7,12,21,22]. Further, if the open-chained compounds are optically active, then a larger change of the molecular rotation due to the ring closure has been observed in the erythro-series[23–25]. When a ring is closed by including a unit between X and Ar', the erythro-isomers show higher stereospecificity[26]. All these differences in behaviour are readily explained by the preferred conformations of the compounds studied. Investigation of the NMR spectra[10,13,19,27] of the latter has shown that it is a general tendency for the preferred conformations of the compounds from the erythro- and threo-series to be of the type A and B, respectively. The antiperiplanar arrangement of the methine hydrogen atoms is characteristic for A and B. Alternatively, X and Y as well as Ar and Ar' in the erythro-series are antiperiplanar,



A(erythro)



B(threo)

while in the threo-series they are synclinal. Thus the two steric series in this particular case appear as *series of conformational similarity*.

Detailed investigation both of the adsorption of each of the compounds studied on silica gel and of its solvation by the solvent systems used would be rather a

labour-consuming problem. However, the variety of X and Y (strongly polar in some cases and weakly polar in others) as well as the variety of the developing solvents on the one hand, when one and the same result is obtained ($R_{F\,(erythro)} > R_{F\,(threo)}$) on the other hand, indicate that definite factors are predominant. Here again we consider that the preferred conformations of the compounds are decisive.

Apparently the interaction between the most polar groups and the silica gel adsorbent will be the strongest. This is supported by the fact that the more polar X and Y are, the more polar the developing solvent must be. Hence, the threo-isomers should be more strongly adsorbed than the erythro-isomers for the following reasons. When the two most polar substituents are in steric proximity they can *both* react (incl. specifically[28]) with the surface of the adsorbent. These groups are close to each other in the preferred conformations of the threo-isomers. Actually, conformations with close X and Y groups are possible and also are present in the erythro-series. However, these conformations, because of additional steric hindrance, are less stable and hence their adsorbates should also be less stable. Therefore, it is reasonable to expect that in all cases the threo-isomers should exhibit a higher tendency of adsorption and respectively a lower R_F value. The influence of the interaction between the substance adsorbed and the developing solvent should also be in the same direction. Depending on the solvents used, either X and Y or aryl groups will solvate more strongly. Solvation of the groups increases their effective volume. Therefore, when the two strongest solvating groups are synclinal, they will mutually hinder their solvation. Hence, conformation A preferred in the erythro-series, is more favourable here. *Vice versa*, such a conformation is less favourable in the threo-series. Certainly this is a case of no great differences since with acyclic compounds the energy differences between the conformers are not great but nevertheless great enough for realising the resolution of 37 diastereomeric pairs of compounds on silica gel. We consider the importance of the adjacent aryl groups mainly as a factor causing the preferred conformations indicated above of the compounds studied and not as groups favourably interacting with the surface of the adsorbent.

The compounds with intramolecular hydrogen bonding of the type N...HO (57–66) and HO...HO (67, 68) show the same order of retention of the diastereomeric compounds by TLC on silica gel as it is in the compounds lacking such a bond. This fact can be used as a proof of our assumption that these bonds are cleaved by the action of the silica gel. Therefore, in these cases as in the ones discussed above, the threo-isomers will adsorb more strongly and solvate more weakly. This assumption is entirely reasonable considering that the silica gel hydroxyl groups are more acidic than the alcohol hydroxyl groups[29]. The results found by FISCHER AND KOCH[5] on chromatographic separation of aliphatic diastereomeric diols by TLC on Fasertonerde and by PC may also be used to support the same assumption. These authors have established in the case of TLC on Fasertonerde that the erythro- respective *ms*-isomer has a higher R_F value than the threo- respective D,L-isomer, while in the case of PC of the same compounds the order of retention of the diastereomeric compounds in the chromatograms is reversed. We consider that on Fasertonerde (a strong polar adsobent) cleavage of the intramolecular hydrogen bond of the type HO...HO takes place whereas on cellulose such a cleavage cannot occur. That is why the order of retention of the diastereomeric compounds is different in both cases. We cannot refer to our attempts to separate 59 from 60 and 67 from 68 by

TLC on cellulose and by PC, since on developing the chromatograms even with the least polar solvents (benzene, petroleum ether, hexane, heptane and others) the substances merely migrate to the front without any detectable separation of the diastereomeric compounds.

Our investigations on assignment of the relative configurations of other acyclic as well as cyclic diastereomeric compounds on the basis of their chromatographic behaviour will continue in the future.

CONCLUSION

We propose that TLC on silica gel be used as a method for assignment of the relative configurations of diastereomeric pairs of compounds of the type Ar–CH(X)–CH(Y)–Ar', where X and Y are polar groups and Ar and Ar' are phenyl or *m*-, *p*-alkoxylated phenyl groups. Erythro configuration should be assigned to the diastereomer with a higher R_F value while threo-configuration to that with a lower R_F value, irrespective of whether an intramolecular hydrogen bond of the type stated above between X and Y is formed.

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CHROM. 5115

SEPARATION AND IDENTIFICATION OF FOOD COLOURS

I. IDENTIFICATION OF SYNTHETIC WATER SOLUBLE FOOD COLOURS USING THIN-LAYER CHROMATOGRAPHY

R. A. HOODLESS, K. G. PITMAN, T. E. STEWART, J. THOMSON AND J. E. ARNOLD*

Department of Trade and Industry, Laboratory of the Government Chemist, London S.E.1 (Great Britain)

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SUMMARY

A thin-layer chromatographic method is described for the separation and identification of forty-nine synthetic food colours which are used in food products or which have been used. R_F and R_X (with respect to Orange G) values are tabulated and a scheme for the rapid identification of the components of a mixture of dyes is proposed.

INTRODUCTION

Colouring matters in food

Processed foods are often coloured to retain the appearance of the original material and to provide a more appealing product. Foodstuffs may be coloured by (a) synthetic organic dyestuffs, (b) inorganic pigments and (c) natural colouring materials obtained from vegetable and animal sources. Synthetic organic dyestuffs are generally used. However, no two countries in the world have identical lists of permitted food colours because there are differences of opinion about the toxicity of the various food colours. Consequently it is possible that foodstuffs may be imported into a country which forbids the colouring matters present in the products. A method has been developed for the identification of synthetic food colours using thin-layer chromatography. The dyes covered by the method are those which are permitted in countries, who are members of the Codex Alimentarius Commission, or dyes which have been used in the past but are now considered too harmful for use in foodstuffs. These dyes are listed in Table I together with their colour index number and the countries in which they are permitted.

A number of thin-layer chromatographic separations of water soluble dyes used in food have been described but most of these deal with only a limited number of dyes; normally those permitted in one country only or a group of dyes of similar

* Borough polytechnic.

TABLE I

FOOD DYES PERMITTED IN VARIOUS COUNTRIES

Colour	*Colour index No. (1956)*	*Africa*	*America*		*Asia*					*Australia*		*Europe*												
		South Africa	*Canada*	*U.S.A.*	*India*	*Israel*	*Japan*	*Russia*	*Turkey*	*Australia*	*New Zealand*	*Austria*	*Czechoslovakia*	*Denmark*	*E.E.C. countries (six)*	*Finland*	*Norway*	*Poland*	*Portugal*	*Spain*	*Sweden*	*Switzerland*	*Great Britain*	*Yugoslavia*
Amaranth	16185	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythrosine	45430	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+		+	+	+	+	+
Ponceau 4R	16255	+			+	+	+			+	+	+	+	+	+	+	+	+		+	+	+	+	+
Citrus Red No. 2	12156		+	+																	+			
Orange I	14600																							
Quinoline Yellow	47005											+		+	+					+	+			+
Sunset Yellow FCF	15935	+	+	+	+	+			+	+	+	+	+	+	+	+	+	+		+	+	+	+	+
Tartrazine	19140	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+
Fast Green FCF	42053	+	+	+			+									+	+				+	+		
Green S	44090										+			+	+								+	
Brilliant Blue FCF	42090	+	+	+					+	+														+
Indanthrene Blue	69800									+		+		+	+		+			+	+	+		+
Indigo Carmine	73015	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+		+	
Patent Blue V	42051														+									
Carmoisine	14720	+			+	+			+	+	+	+	+	+	+	+	+	+		+	+	+	+	+
Scarlet GN	14815									+		+		+	+	+	+	+		+	+	+		+
Ponceau 6R	16290									+		+	+	+	+	+	+			+	+			
Fast Red E	16045				+								+	+		+	+			+	+	+	+	
Ponceau SX	14700											+		+										
Red FB	14780				+		+				+	+											+	
Red 6B	18055				+		+					+		+		+	+						+	
Ponceau MX (Ponceau 2R)	16150													+									+	
Red 2G	18050	+												+									+	

Sudan Red G	12150						+							+								+
Red 10B	17200											+									+	
Orange GGN	15980							+		+		+	+	+	+				+			
Orange RN	15970	+							+			+									+	
Orange G	16230	+										+									+	
Acid Yellow																						
(Fast Yellow AB)	13015							+		+		+	+	+	+	+		+	+	+		+
Chrysoin S	14270									+			+	+								
Oil Yellow GG (Sudan G)	11720	+													+				+		+	+
Oil Yellow XP	12740											+									+	
Yellow 2G	18965											+									+	
Guinea Green B	42085					+											+					
Light Green SF Yellowish	42095					+																
Blue VRS	42045				+	+			+	+		+		+								
Violet BNP	—								+			+									+	
Violet 6B																						
(Benzyl Violet 4B)	42640		+	+										+	+							
Brown FK	—							+	+			+									+	
Chocolate Brown FB	—							+	+			+									+	
Chocolate Brown HT	20285							+	+			+									+	
Black PN																						
(Brilliant Black BN)	28440				+			+		+	+	+	+	+	+	+			+	+	+	+
Black 7984	27755												+	+							+	
Ponceau 3R	16155																+					
Bordeaux B	16180	+																				
Eosine	45380					+																
Naphthol Yellow S	10316																+					
Violet 5BN	42650	+																				
Auramine	41000																					
Butter Yellow	11020																					
Chrysoidine	11270																					
Acid Magenta	42510																					
Methyl Violet	42535																					
Oil Orange E	12055																					
Oil Orange SS	12100																					
Oil Orange XO	12140																					
Oil Yellow AB	11380																					
Oil Yellow OB	11390																					
Rhodamine B	45170																					

TABLE II

CODES FOR DYES CHROMATOGRAPHED IN SOLVENTS 1, 2, 3, AND 4

Code	*Possible identity of dye*	*Code*	*Possible identity of dye*	*Code*	*Possible identity of dye*
AAAA	Blue VRS	DADC	Orange RN	EBDE	Carmoisine
	Brilliant Blue	DBCA	Naphthol Yellow S	EBEB	Quinoline Yellow
	Light Green Yellowish	DBCB	Orange GGN	EBEC	Quinoline Yellow
			Sunset Yellow	ECCA	Orange RN
	Patent Blue V	DBCC	Orange GGN	ECCB	Fast Red E
AAAB	Brilliant Blue		Sunset Yellow	ECCC	Fast Red E
	Fast Green	DCCA	Orange RN		Indigo Carmine
	Green S	DCCB	Orange GGN		Orange RN
	Light Green Yellowish		Sunset Yellow		Red 10 B
		DCCC	Acid Yellow	ECCD	Carmoisine
	Patent Blue V		Orange GGN		Indigo Carmine
	Yellow 2G		Orange RN		Red 10 B
AAAC	Yellow 2G		Sunset Yellow	ECCE	Carmoisine
AABA	Scarlet GN	DCCD	Acid Yellow		Red 10 B
AACA	Scarlet GN		Orange GGN	ECDA	Orange RN
ABAA	Brilliant Blue		Sunset Yellow	ECDB	Fast Red E
	Light Green	DCDA	Orange RN	ECDC	Bordeaux B
	Yellowish	DCDC	Orange RN		Fast Red E
ABAB	Brilliant Blue	DCDD	Red 6B		Indigo Carmine
	Light Green	DCDE	Red 6B		Orange RN
	Yellowish	DCED	Red 6B		Ponceau 3R
ABAC	Acid Magenta	DCEE	Red 6B		Ponceau MX
ABAD	Acid Magenta	DDDD	Amaranth		Ponceau SX
ACAC	Acid Magenta		Red 6B		Red 10 B
ACAD	Acid Magenta	DDDE	Red 6B	ECDD	Carmoisine
ACCD	Tartrazine	DDED	Red 6B		Indigo Carmine
ADCD	Ponceau 6R	DDEE	Red 6B		Ponceau SX
	Tartrazine	DEDD	Red 6B		Red 6B
ADCE	Ponceau 6R	DEDE	Red 6B		Red 10 B
AECD	Ponceau 6R	DEED	Red 6B	ECDE	Carmoisine
AECE	Ponceau 6R	DEEE	Red 6B		Red 6B
BAAA	Guinea Green B	EAAA	Auramine		Red 10 B
	Violet 5BN		Methyl Violet	ECEC	Bordeaux B
	Violet BNP		Rhodamine B		Ponceau 3R
BBBB	Orange G		Violet 6B		Ponceau MX
BCBC	Ponceau 4R	EABA	Auramine		Ponceau SX
BCCC	Ponceau 4R		Methyl Violet	ECED	Ponceau SX
BCCD	Tartrazine		Rhodamine B		Red 6B
BDCD	Tartrazine	EACA	Auramine	ECEE	Red 6B
CAAA	Guinea Green B		Eosine	EDCC	Indigo Carmine
	Violet BNP		Erythrosine		Red 10 B
	Violet 5BN		Chrysoidine	EDCD	Indigo Carmine
	Violet 6B		Orange I		Red 10 B
CACB	Chrysoin S		Orange RN	EDCE	Red 10 B
CACC	Chrysoin S	EACB	Chrysoidine	EDDC	Indigo Carmine
CBCA	Naphthol Yellow S		Eosine		Red 10 B
CBCB	Orange GGN		Erythrosine	EDDD	Indigo Carmine
	Sunset Yellow		Orange I		Red 6B
CBCC	Orange GGN	EACC	Chrysoin S		Red 10 B
	Sunset Yellow		Orange RN	EDDE	Red 6B
CCBC	Ponceau 4R	EADA	Chrysoidine		Red 10 B
CCCB	Acid Yellow		Orange I	EDED	Black 7984
	Orange GGN		Orange RN		Black PN
	Sunset Yellow	EADB	Orange I		Red 6B
CCCC	Acid Yellow		Quinoline Yellow	EDEE	Black 7984
	Orange GGN	EADC	Orange RN		Black PN

TABLE II *(continued)*

Code	*Possible identity of dye*	*Code*	*Possible identity of dye*	*Code*	*Possible identity of dye*
	Ponceau 4R		Quinoline Yellow		Red 6B
	Red 2G	EAEB	Quinoline Yellow		Red FB
	Sunset Yellow	EAEC	Quinoline Yellow	EEDD	Red 6B
CCCD	Acid Yellow	EBCB	Fast Red E	EEDE	Red 6B
CCDC	Red 2G	EBCC	Fast Red E	EEED	Black 7984
DAAA	Rhodamine B	EBCD	Carmoisine		Black PN
	Violet 6B	EBCE	Carmoisine		Red 6B
DABA	Rhodamine B	EBDB	Fast Red E	EEEE	Black 7984
DACA	Orange RN		Quinoline Yellow		Black PN
DACB	Chrysoin S	EBDC	Fast Red E		Red 6B
DACC	Chrysoin S		Quinoline Yellow		Red FB
	Orange RN	EBDD	Carmoisine		Indanthrene Blue
DADA	Orange RN				

colour[1–13]. Cellulose and silica gel appeared to be the two most promising adsorbents for the separation of the water soluble dyes and so we have used only these two adsorbents with a variety of development solvents as listed in Table III.

A scheme for the quick identification of a colour or mixture of colours is proposed which is not dependent on the measurement of R_F values. This consists of running the dye or mixture of dyes in four solvents on thin-layer plates coated with cellulose with two standard dyes and then giving the dyes a code depending on where they travel to in relation to the two standard dyes. This code is compared with the list of codes given in Table II thereby giving an initial identification of the dyes. The identity of the food colour is then confirmed by running in solvents together with spots of the suspected food colours.

TABLE III

CHROMATOGRAPHIC SOLVENTS USED IN THE THIN-LAYER CHROMATOGRAPHIC SEPARATION OF THE DYES

Solvents 1–10 are used with cellulose plates; solvents 11–15 are used with silica gel plates.

Solvent No.	*Composition*	*Reference*
1	Trisodium citrate (2 g), water (85 ml), 0.88 ammonia (15 ml)	1
2	*tert.*-Butanol–propanoic acid–water (50:12:38)	1
3	Trisodium citrate (2 g), hexamine (5 g), water (50 ml), methanol (50 ml)	3
4	2-Methyl propan-1-ol–water–ethanol–0.88 ammonia (25:25:50:2)	2
5	Propan-1-ol–ethyl acetate–water (6:1:3)	1
6	Butan-1-ol–water–glacial acetic acid (20:12:10)	—
7	Hydrochloric acid, S.G. 1.18–water (23:77)	—
8	Butan-1-ol–water–pyridine–ethanol (4:4:2:2)	—
9	Ethyl methyl ketone–acetone–water–0.88 ammonia (70:30:30:0.5)	—
10	Butan-1-ol–water–ethanol–quinoline (4:4:3:2)	—
11	Propan-2-ol–0.88 ammonia (4:1)	2
12	Propan-2-ol–0.88 ammonia (85:15)	—
13	Methanol–chloroform–water–quinoline (4:2:2:2)	—
14	Methanol–chloroform–quinoline (4:4:2)	—
15	Propan-2-ol–chloroform–water–diethylamine (50:25:20:15)	—

MATERIALS AND METHODS

Apparatus

Thin-layer chromatographic apparatus for the preparation of thin layers 0.25 mm thick on 200 × 200 mm glass plates. Chromatographic development tanks. 5 μl pipettes *e.g.* Microcap disposable pipettes.

Reagents

Cellulose powder. Microcrystalline cellulose, available from Applied Science Laboratories Inc. Prepare plates as follows: Shake 20 g cellulose powder with 60 ml methanol for 3 min and blend at high speed for 30 sec. Spread onto plates and air-dry or dry in an oven at 80°.

Silica Gel G. Available from E. Merck. Prepare plates as follows: Shake 30 g Silica Gel G with 60 ml water for 1 to 2 min. Spread onto plates and, after the layer has set, activate the plates by heating to 105° for 1 h.

Reference dye solutions. 0.1 % in water.

Chromatographic solvents. See Table III. All solvent mixtures should be freshly prepared.

Procedure

Place two spots of 1–2 μl of the dye solution onto each of four cellulose plates at a distance of at least 20 mm from the edge and bottom of the plate. Also spot on the plates 1–2 μl of a solution of Orange G and a solution of Amaranth as reference spots and place a spot of a mixture of Orange G and Amaranth on top of one of the sample spots. Dry the spots by placing the plates in an oven at 105° for 5–10 min. Develop the cooled plates in solvents 1, 2, 3 and 4 for a length of run of about 150 mm at room temperature. Remove the plates from the tanks and allow them to air dry. When the plates are dry rule lines across so as to divide the plates into the following sections: code A: spots travelling above Orange G; code B: spots travelling with Orange G; code C: spots travelling below Orange G but above Amaranth; code D: spots travelling with Amaranth; code E: spots travelling below Amaranth.

Check whether the sample has affected the development characteristics of Orange G and Amaranth and if so make allowance for this when dividing the plate into sections. Observe which section the spots from the sample solution appear in for each plate and write down all possible composite codes for each spot by listing the code individual letters in the order—solvent 1, solvent 2, solvent 3, solvent 4. Compare the codes with the list given in Table II and hence obtain a preliminary identification of the dyes. When two or more spots are similar in colour, cross code the dyes so that all possible dyes are obtained from Table II. Also if a dye is visible in one solvent but not in another then this indicates that the dye is masked by another dye and so all codes for spots in that solvent must be used in constructing the composite codes. A further identification of the dyes may be obtained by calculating the R_F and R_X (with respect to Orange G) values and referring to the Tables IV–VII. This will eliminate some of the dyes obtained from Table II. All R_F and R_X values have been calculated by measuring to the leading edge of the spots.

The identification of the sample dye is then confirmed by chromatography on a plate with standard spots of the suspected colours using suitable solvents. Spots of

TABLE IV

R_F AND R_X (WITH RESPECT TO ORANGE G) VALUES FOR RED DYES

Colour	*Colour index No.*	*Approximate R_F values*									*Approximate R_X values*								
		Solvent No.									*Solvent No.*								
		1	*2*	*3*	*4*	*5*	*6*	*8*	*9*	*11*	*1*	*2*	*3*	*4*	*5*	*6*	*8*	*9*	*11*
Amaranth	16185	0.6	0.3	0.5	0.6	0.4	0.2	0.6	0.4	0.4	0.8	0.4	0.5	0.8	0.7	0.4	0.8	0.4	0,9
Bordeaux B	16180	0.2	0.6	0.4	0.6	0.5	0.6	0.7	1.0	0.4	0.3	0.9	0.4	0.8	0.9	1.0	1.0	1.0	0.9
Carmoisine	14720	0.3	0.7	0.6	0.5	0.7	0.6	0.8	0.9	0.4	0.4	1.1	0.6	0.7	1.1	1.0	1.1	0.9	0.9
Eosine	45380	0.2	1.0	0.7	0.8	1.0	1.0	0.9	1.0	0.6	0.3	1.5	0.7	1.1	1.6	1.7	1.3	1.0	1.4
Erythrosine	45430	0.1	1.0	0.7	0.9	1.0	1.0	0.9	1.0	0.7	0.2	1.5	0.7	1.2	1.6	1.7	1.3	1.0	1.6
Fast Red E	16045	0.4	0.7	0.6	0.7	0.6	0.5	0.8	1.0	0.4	0.6	1.0	0.6	1.0	1.0	0.9	1.1	1.0	0.9
Ponceau 3R	16155	0.2	0.6	0.4	0.6	0.5	0.5	0.8	0.9	0.3	0.3	0.9	0.4	0.8	0.7	0.9	1.1	0.9	0.7
Ponceau 4R	16255	0.7	0.5	0.9	0.6	0.4	0.3	0.7	0.6	0.2	1.0	0.6	1.0	0.8	0.7	0.5	1.0	0.6	0.4
Ponceau 6R	16290	0.8	0.2	0.8	0.4	0.3	0.1	0.6	0.2	0.1	1.1	0.2	0.8	0.5	0.4	0.2	0.8	0.2	0.2
Ponceau MX	16150	0.2	0.7	0.5	0.6	0.5	0.5	0.8	0.9	0.4	0.3	0.9	0.5	0.8	0.9	0.9	1.1	0.9	0.8
Ponceau SX	14700	0.4	0.7	0.5	0.6	0.5	0.5	0.8	0.9	0.4	0.6	0.9	0.5	0.8	0.9	0.9	1.1	0.9	0.8
Red 2G	18050	0.6	0.6	0.7	0.6	0.4	0.5	0.7	0.9	0.4	0.8	0.8	0.7	0.8	0.7	0.9	1.0	0.9	0.9
Red 6B	18055	0.4	0.3	0.4	0.5	0.4	0.2	0.6	0.5	0.4	0.6	0.4	0.4	0.7	0.7	0.4	0.8	0.5	0.9
Red 10B	17200	0.2	0.5	0.6	0.6	0.4	0.3	0.7	0.8	0.4	0.3	0.6	0.6	0.8	0.7	0.5	1.0	0.8	0.9
Red FB	14780	0.0	0.3	0.1	0.2	0.4	0.2	0.7	0.4	0.6	0.0	0.4	0.1	0.3	0.7	0.4	1.0	0.4	1.3
Rhodamine B	45170	0.5	1.0	0.9	1.0	1.0	1.0	0.9	1.0	0.8	0.7	1.5	1.0	1.3	1.6	1.7	1.4	1.0	1.8
Scarlet GN	14815	0.9	0.7	0.9	0.8	0.8	0.6	0.8	1.0	0.5	1.1	0.9	1.0	1.1	1.2	1.0	1.1	1.0	1.2

TABLE V

R_F AND R_X (WITH RESPECT TO ORANGE G) VALUES FOR YELLOW AND ORANGE DYES

Colour	*Colour index No.*	*Approximate R_F values*									*Approximate R_X values*								
		Solvent No.									*Solvent No.*								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*11*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*11*
Auramine	41000	0.3	1.0	0.9	1.0	0.9	1.0	not visible	0.8	0.8	0.4	1.4	1.0	1.6	1.4	1.9	not visible	1.3	1.8
Acid Yellow	13015	0.7	0.6	0.9	0.6	0.6	0.5	0.7	0.7	0.4	0.8	0.9	1.0	0.9	0.9	0.9	1.1	1.0	1.0
Chrysoidine	11270	0.1	0.8	0.7	0.9	0.8	0.9	0.1	0.9	0.8	0.2	1.2	0.7	1.6	1.2	1.7	0.2	1.4	1.8
Chrysoin S	14270	0.5	0.8	0.8	0.6	0.9	0.7	0.4	0.8	0.5	0.6	1.2	0.9	0.9	1.3	1.4	0.7	1.2	1.0
Naphthol Yellow S	10316	0.6	0.7	0.8	0.7	0.7	0.6	not visible	0.7	0.5	0.7	1.0	0.9	1.1	1.1	1.1	not visible	1.1	1.0
Orange G	16230	0.8	0.7	0.9	0.6	0.7	0.5	0.7	0.7	0.4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Orange GGN	15980	0.6	0.7	0.8	0.6	0.6	0.5	0.2	0.7	0.4	0.8	1.0	0.9	1.0	1.0	1.0	0.3	1.0	1.0
Orange I	14600	0.4	0.8	0.8	0.7	0.9	0.7	0.1	0.8	0.5	0.6	1.2	0.9	1.1	1.3	1.4	0.2	1.2	1.2
Orange RN	15970	0.4, 0.5	0.9	0.7	0.6, 0.8	0.6, 0.9	0.5, 0.8	0.1	0.7, 0.8	0.7	0.5, 0.7	1.2	0.8	0.9, 1.3	0.8, 1.4	0.9, 1.5	0.2	1.0, 1.2	1.6
Quinoline Yellow	47005	0.1, 0.3	0.7	0.4	0.6	0.7	0.6	0.1	0.6, 0.7	0.7	0.1, 0.4	1.0	0.5	1.0	1.1	1.2	0.1	0.9, 1.0	1.6
Sunset Yellow	15985	0.6	0.7	0.8	0.6	0.6	0.5	0.2	0.7	0.4	0.7	0.9	0.9	1.0	0.9	1.0	0.4	1.0	1.0
Tartrazine	19140	0.8	0.4	0.8	0.4	0.5	0.3	0.4	0.5	0.3	1.0	0.6	0.8	0.6	0.7	0.6	0.6	0.7	0.6
Yellow 2G	18965	0.9	0.8	1.0	0.6	0.8	0.6	0.9	0.7	0.4	1.1	1.2	1.1	1.0	1.2	1.2	1.5	1.0	1.0

TABLE VI

R_F AND R_X (WITH RESPECT TO ORANGE G) VALUES FOR BROWN AND VIOLET DYES

Colour	*Colour index No.*	*Approximate R_F values*								*Approximate R_X values*							
		Solvent No.								*Solvent No.*							
		1	*2*	*3*	*4*	*5*	*11*	*14*	*15*	*1*	*2*	*3*	*4*	*5*	*11*	*14*	*15*
Brown FK	—	streak	streak	streak	0.6	streak	0.7, 0.8	streak	0.4, 0.5	streak	streak	streak	0.9	streak	1.1, 1.3	streak	0.6, 0.7
Chocolate Brown FB	—	streak	streak	streak	streak	streak	0.0	0.0	small streak	streak	streak	streak	streak	streak	0.0	0.0	small streak
Chocolate Brown HT	20285	streak	streak	streak	streak	streak	0.0	0.0	long streak	streak	streak	streak	streak	streak	0.0	0.0	long streak
Acid Magenta	42685	1.0	0.4, 0.6	0.9	0.6	0.5, 0.6	0.2	not visible	not visible	1.4	0.7, 1.0	1.1	0.8	0.8, 0.9	0.3	not visible	not visible
Methyl Violet	42535	streak	1.0	0.8, 0.9	1.0	1.0	0.8, 0.9	0.8	0.7	streak	1.5	1.0, 1.1	1.4	1.6	1.3, 1.4	1.9	1.1
Violet BNP	—	0.7	0.8	0.9	0.9	0.9	0.5	0.4	0.5	1.0	1.3	1.1	1.3	1.4	0.7	1.1	0.7
Violet 5BN	42650	0.7	0.8	0.9	0.9	0.9	0.5	0.4	0.5	1.0	1.3	1.1	1.3	1.4	0.7	1.1	0.7
Violet 6B	42640	0.6	0.8	1.0	1.0	0.9	0.5, 0.6, 0.7	0.4, 0.5	0.6	0.8	1.3	1.2	1.4	1.4	0.8, 0.9, 1.0	1.1, 1.2	0.9

TABLE VII

R_F AND R_X (WITH RESPECT TO ORANGE G) VALUES FOR GREEN, BLUE AND BLACK DYES

Colour	*Colour index No.*	*Approximate R_F values*									*Approximate R_X values*								
		Solvent No.									*Solvent No.*								
		1	*2*	*3*	*4*	*5*	*11*	*12*	*13*	*10*	*1*	*2*	*3*	*4*	*5*	*11*	*12*	*13*	*10*
Fast Green FCF	42053	0.9	0.8	1.0	0.8	0.8	0.1	0.1	0.8	0.8	1.2	1.1	1.1	1.0	1.1	0.5	0.2	1.0	1.0
Green S	44090	0.9	0.8	0.9	0.8	0.8	0.1	0.1	0.7	0.8	1.2	1.1	1.0	1.0	1.2	0.4	0.2	0.9	1.0
Guinea Green B	42085	0.7	0.9	1.0	1.0	0.9	0.3	0.3	0.8	0.9	0.9	1.2	1.1	1.3	1.3	1.1	1.4	1.0	1.2
Light Green Yellowish	42095	0.9	0.8	1.0	0.9	0.8	0.3	0.1	0.8	0.8	1.1	1.1	1.1	1.2	1.1	0.9	0.5	1.0	1.0
Blue VRS	42045	0.9	0.9	1.0	0.9	0.9	0.3	0.2	0.8	0.9	1.1	1.2	1.1	1.2	1.3	1.1	1.0	1.0	1.2
Brilliant Blue FCF	42090	0.9	0.8	1.0	0.9	0.8	0.3	0.2	0.8	0.8	1.1	1.1	1.1	1.2	1.1	1.0	0.7	1.0	1.0
Indanthrene Blue[a]	69800	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Indigo Carmine	73015	0.2, 0.3	0.4	0.5	0.6	0.5	0.3	0.2	0.8	0.7	0.3, 0.4	0.6	0.6	0.8	0.7	1.0	1.0	1.0	0.9
Patent Blue V	42051	0.9	0.9	1.0	0.9	0.9	0.1	0.0	0.8	0.9	1.2	1.2	1.1	1.1	1.3	0.2	0.0	1.0	1.2
Black 7984	27755	0.2	0.3	0.2	0.4	0.4	0.1	0.0	0.7	0.5	0.2	0.4	0.2	0.6	0.5	0.4	0.0	0.9	0.7
Black PN	28440	0.4	0.3	0.2	0.4	0.4	0.1	0.0	0.7	0.7	0.4	0.4	0.2	0.6	0.5	0.4	0.0	0.9	0.9

[a] Indanthrene Blue is insoluble in water and most organic solvents.

the sample solution are also overspotted with spots of the suspected dyes. The unknown dye is identified by giving a single spot with the correct standard while all the other standards give rise to double spots.

If the sample contains several dyes, more than one solvent may be necessary for complete confirmation of the dyes.

DISCUSSION

In constructing the table of codes for the dyes, slight variations in the development characteristics of the dyes have been taken into account so that some dyes occur under a number of different codes. Brown FK, Chocolate Brown FB and Chocolate Brown HT have not been included in this table as they streak in the solvents used. If the standard dyes, Orange G and Amaranth, run very differently when overspotted on the sample from when they are spotted separately on the plate then the spots in the sample should be coded twice, once using the standards in the sample to divide up the plate and once using the standard spotted separately to divide up the plate. By this means all possible dyes will be obtained, but a number of these will be rejected on the basis of colour and R_F value. However, do not discount dyes which could give rise to the colour of the spot, *e.g.* an orange coloured spot may be a red and yellow dye superimposed.

As most problems arise from the possibility of a red and yellow dye being together in the mixture, the separation of the reds, oranges and yellows are set out in Table VIII. All R_F and R_X values have been calculated by measuring to the leading edge of a spot as this was found to be more reliable for spots which tail. When confirming the identity of a dye by running it with standard dyes it is useful to observe

TABLE VIII

SEPARATION OF REDS, ORANGES AND YELLOWS IN SOLVENTS 1, 2, 3 AND 4
Dyes in italics are completely separated from the others.

Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4* $R_F = 1.0$
Yellow 2G	Eosine	*Yellow 2G*	Rhodamine B
Scarlet GN	Erythrosine	*Rhodamine B*	Auramine
Tartrazine	Rhodamine B	*Auramine*	
Ponceau 6R		*Orange G*	Chrysoidine
Orange G	*Auramine*		Orange RN
Ponceau 4R	*Orange RN*	Scarlet GN	
		Acid Yellow	*Ethyrhrosine*
Acid Yellow	Chrysoin S	Chrysoin S	Eosine
Red 2G	Chrysoidine	Naphthol Yellow S	Scarlet GN
Orange GGN	Orange I		Naphthol Yellow S
Sunset Yellow		Eosine	Orange I
Naphthol Yellow S	*Yellow 2G*	Erythrosine	
		Ponceau 4R	Orange G
Orange RN	Scarlet GN	Ponceau 6R	Yellow 2G
Amaranth	Naphthol Yellow S	Orange GGN	
Chrysoin S		Orange I	Fast Red E
	Orange G	Orange RN	Acid Yellow
Orange RN	Carmoisine	Sunset Yellow	Chrysoin S
Orange I	Quinoline Yellow	Tartrazine	Orange GGN
Rhodamine B			Quinoline Yellow
Red 6B	Fast Red E		Sunset Yellow
Ponceau SX	Orange GGN	Amaranth	
Fast Red E	Sunset Yellow	Carmoisine	Bordeaux B
		Fast Red E	Ponceau 3R
Carmoisine	Bordeaux B	Ponceau SX	Ponceau 4R
Bordeaux B	Ponceau 3R	Red 2G	Ponceau MX
Auramine	Ponceau MX	Red 10 B	Ponceau SX
Quinoline Yellow	Ponceau SX	Chrysoidine	Red 2G
	Acid Yellow		Red 10 B
Eosine	Orange RN	Bordeaux B	Orange RN
Ponceau 3R		Ponceau 3R	
Ponceau MX	Ponceau 4R	Ponceau MX	Amaranth
Red 10 B	Red 2G	Quinoline Yellow	Carmoisine
Erythrosine			Tartrazine
	Red 10 B		
Chrysoidine	Tartrazine	*Red 6B*	Ponceau 6R
Quinoline Yellow			Red 6B
	Amaranth	*Red FB*	
Red FB	Ponceau 6R		
	Red 6B		*Red FB*
	Red FB		

$R_F = 0$

the plate under UV light of 254 nm and 350 nm as some of the dyes fluoresce.

The following mixtures of dyes could not be separated in any of the solvents tried: Chocolate Brown HT and Chocolate Brown FB, Ponceau 3R and Ponceau MX, Violet 5BN and Violet BNP.

Chocolate Brown HT can be tentatively distinguished from Chocolate Brown FB by running in solvent 15 on silica gel. Chocolate Brown FB produces a small streak from the spotting line whereas Chocolate Brown HT produces two spots and a streak from the spotting line. The two spots travel higher than the streak from Chocolate Brown FB.

ALDRED[6] has reported that Violet 5BN and Violet BNP can be separated on silica gel using a mixture of 2-methyl propan-1-ol, ethanol and water as developing solvent. When this system was tried we did not obtain a separation of the samples of Violet 5BN and Violet BNP which we were using. Some of these dyes may be broken down during extraction from the foodstuffs, or in the foodstuff itself, and the decomposition products may affect the separation of the dyes. Work is in progress on these aspects to see how they will affect the identification scheme and further publications of the results of this work will follow.

Violet 5BN is permitted only in South Africa and Violet BNP is permitted only in Denmark, New Zealand and the United Kingdom. Consequently the need to separate these two dyes should not arise very often. However, they can be distinguished by their IR spectra. No work has been carried out on extraction of these dyes from foodstuffs and it is realised that co-extractives may affect the running characteristics of various dyes but by overspotting the sample with the suspected dyes in the final confirmation any irregularities should not affect the identification of the dyestuff.

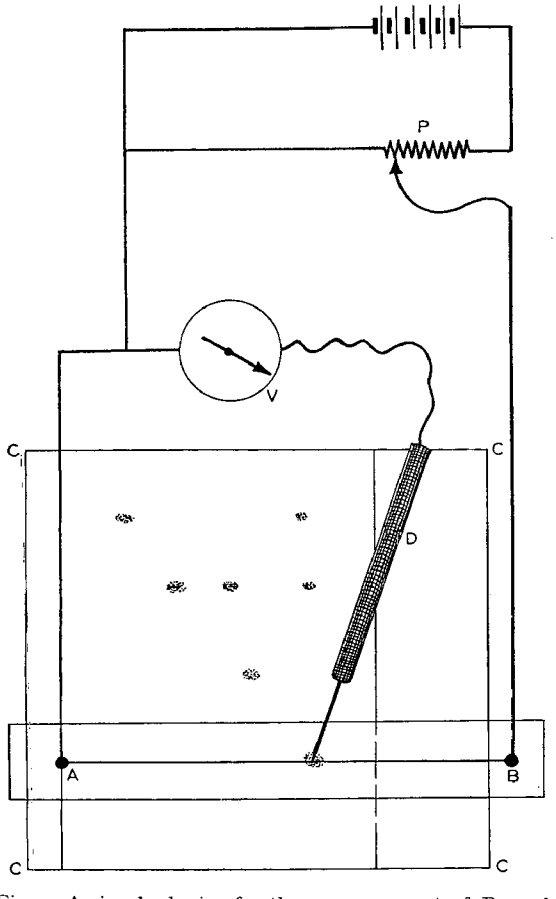

Fig. 1. A simple device for the measurement of R_F values. A — B, resistance wire mounted on perspex; C, a developed thin-layer plate; P, 1 kΩ potentiometer; V, 2.5 V. f.s.d. voltmeter; D, contact probe.

R_F measurement

To relieve the tedium of measuring a large number of R_F values a simple electrical device was constructed. The device consists of a perspex template which slides over the thin-layer plate. The template has a length of resistance wire stretched between two terminals and a sliding contact for making contact with the resistance wire. The resistance wire is made part of a simple potentiometer circuit as shown in Fig. 1. The template is placed over the thin-layer plate and adjusted so that terminal "A" is over the spotting line. The sliding contact is moved to the solvent front or the standard spot, if R_X values are required, and the potentiometer "P" adjusted so that the voltmeter reads 1.0 units. The sliding contact is then moved over the spot whose R_F or R_X value is required and the voltmeter reading noted. The template is then moved along keeping it in contact with the bottom edge of the plate until it is over the next spot.

Documentation of chromatograms

Copies of the thin-layer chromatograms were made by a simple blue print type procedure. The spots on the plates are scribed round with a needle and then placed coated surface down on a piece of "Blackline" paper. (Blackline paper for ammonia development, ZY5M, obtainable from Mason Ltd., Colchester.) The back of the plate is illuminated by means of photoflood bulbs for approximately 40 sec. The plate is removed and the paper is suspended in a tank containing a few millilitres of 0.88 ammonia solution for about 1 min. The print obtained consists of black spots ringed with a white line.

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THE ELECTROOSMOTIC FLOW IN AGAROSE GELS AND THE VALUE OF AGAROSE AS STABILIZING AGENT IN GEL ELECTROFOCUSING

RUDOLF QUAST

Hygiene-Institut der Philipps-Universität, Marburg a.d.Lahn (G.F.R.)

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SUMMARY

All twelve commercial preparations of agarose tested for electroosmosis showed electroosmotic flow. Considerable differences were found between the gels. Preliminary experiments suggest that it is possible to reduce electroosmosis of a certain batch by treating it with anion-exchange resin. Measurement of the conductivity of an agarose solution or gel is of no help when searching for a product with low electroosmosis. Recommendations for the application of agarose and polyacrylamide in gel electrofocusing are based on the theory of electroosmosis, on the properties of the gels, and on the practical aspects of isoelectric focusing.

INTRODUCTION

Isoelectric focusing in gels (gel electrofocusing) increasingly attracts the attention of experimental scientists working in the field of molecular biochemistry. Several investigators who used agarose as an anticonvective agent[1–3] described electroosmotic effects which differed, depending on the use of Ampholine* for pH gradient 3–10 or Ampholine for a gradient having a narrower range. In order to assess the usefulness of agarose as a supporting substance in gel electrofocusing experiments, the electroosmotic behavior of different preparations of agarose was determined in a series of electrophoresis experiments in which dextran with three different molecular weights served as marker substances[4,5].

EXPERIMENTAL

Various brands of agarose were employed, as shown in Table I. With the exception of Sepharose 6B, all samples were used without treatment prior to dissolving for the preparation of gel slabs. Sepharose 6B was dialyzed against distilled water for more than 24 h in the cold to remove the preservative; it was then partially dehydrated with absolute ethanol and finally dried at 50°. All agaroses were used as 1% (w/v) gels.

* Trade mark of LKB-Producter, Bromma, Sweden.

TABLE I

AGAROSE PREPARATIONS USED FOR THE DETERMINATION OF THE ELECTROOSMOTIC VELOCITY

Manufacturer	*Product*	*Lot No.*
BioRad Laboratories (Richmond, Calif.)	Agarose Powder	Contr. No. 5102
Calbiochem	Agarose B Grade	801450
EGA-Chemie KG. Steinheim		[a]
Koch-Light Lab. Ltd.	Agarose pure	42652
L'Industrie Biologique Française	Agarose F.F.	3018
Mann Research Lab. (N.Y.)	Agarose special grade	U 2184
Pharmacia (Uppsala)	Sepharose 6B	7639
Seakem (Marine Colloid Inc.)		752107
Serva (Heidelberg)	Agarose, reinst	11397
British Drug Houses Ltd.	Agarose for electrophoresis	Prod. No. 33006
Paines and Byrne Ltd.	"Meath"-Agarose	538-431
Behringwerke (Marburg)	Agarose Oreo	F 2217

[a] Ordered as product of Aldrich Chemical Co., Inc.

Dextran (Serva, Heidelberg) having three different molecular weight ranges was used: Dextran 15–20, mol. wt. 15000–20000; Dextran 40, mol. wt. 40000; and Dextran 500, mol. wt. 500000. Each dextran sample was dissolved as a 62.5 mg/ml solution in the buffer used for electrophoresis (0.005 *M* Sörensen phosphate, pH 7.0).

Conductivity measurements in agarose solutions and gels were carried out with a Philips conductivity meter, Type PW 9501, and a conductivity cell, PW 9512/00, having a cell constant of 1.27 cm^{-1}. The conductivity cell was kept at constant temperature with a Lauda thermostat, type K2D. A precision pH meter (Knick, Berlin, Type 350) and a combined glass electrode (Schott & Gen.) were used for pH determinations.

Electrophoresis experiments were done at constant voltage and nearly constant temperature—variation less than 2° during a run—employing the electrophoresis chamber of Vitatron Instruments described by WIEME[4]. The blocks on which the agarose gel slabs rested consisted of 7.5% (w/v) photopolymerized polyacrylamide gel. Octane from petroleum, b.p. 120–130°, which surrounded the gels during the run served as the cooling liquid. It was used instead of pentane because of its lower vapor pressure. The octane in turn was cooled by an ice trough on top and by a metal coil through which cold water (4°) was circulated. The whole electrophoresis chamber rested on a magnetic stirring table which effected rapid mixing of the octane. The gels were prepared according to WIEME (ref. 4, p. 69) on microscope slides. The thickness of the gel layer was 0.8 ± 0.1 mm. After ageing the gel slides for at least 24 h in the refrigerator in order to reduce possible variations of electroosmotic properties (ref. 4, p. 69) they were cut out of the layer in the petri dish. Three sample slits were punched 27.5 ± 1 mm from the anodal end of the slide and at exactly right angles to the direction of the field. This was done with an LKB punch equipped with three 6-mm-long razor blades, 2 mm apart from each other. The slits were filled with sample solution by using capillary pipettes with finely drawn tips, each slide receiving all three types of dextran. After electrophoresis for a defined time interval and with a

potential gradient of 15.4 V/cm over the length of the gel, the slides were removed from the chamber and immersed in a dextran-precipitating solution of acetic acid, ethanol, and water (5:70:25, v/v) (ref. 4, pp. 76 and 99). They were then scanned by means of a Vitatron UFD photometer and densitometer. Two 0.2-mm slit apertures in front of the photomultiplier tube, type RCA IP 22, provided for high linear resolution. A wavelength of 401 nm was selected by an interference filter. The ratio of densitometer belt movement (1.72 cm/min) to paper speed of the Vitatron UR 100 recorder (15 cm/min) enlarged the distance by a factor of 8.72. This facilitated measurement of the distance travelled by dextran during the run. From these measurements as well as from the time of the run and from the voltage drop over the length of the gel, the electroosmotic velocity in the agarose gel at unit field strength was calculated.

RESULTS

The conductivity of the agarose solutions in water decreased linearly with temperature with no deviation from linearity in the temperature range where gel formation occurred. Conductivity at 20° and electroosmotic velocity of the same agarose preparation did not correlate, which indicates that solutions of commercial agaroses in distilled water contain measurable quantities of low-molecular-weight ions.

The variation coefficient of repeated measurements of the electroosmotic flow from the same slide, *i.e.* positioning the slide on the densitometer belt, recording the absorbance *vs.* the length of the gel, measuring the distance between the start peak and the peak of absorbance of the dextran precipitate, and calculating the electroosmotic velocity of the agarose from these data, was less than 10%. Using Student's t-test, no difference in electroosmotic flow in a preparation caused by the position of the gel slide in the electrophoresis chamber during the run was indicated which, however, might be due to the small size of the sample ($n = 4$). Also, by use of the same test a difference between the mobility of Dextran 15–20 and Dextran 40 could not be found. Therefore, all calculations of electroosmotic velocity in the agarose gels were based on the distances covered by both Dextran 15–20 and Dextran 40. The values for Dextran 500 had to be omitted because they were consistently lower. The reason for this difference in mobility may be due to the markedly higher viscosity of the Dextran 500 solution resulting in reduced electroosmotic velocity and to the restriction of free diffusion of the Dextran 500 molecules caused by steric hindrance in the gel.

Considerable variations in electroosmotic velocity were found between different agarose preparations. Table II shows mean value and standard deviation of the electroosmotic velocity of each of the 12 agarose samples. They may be grouped into those with electroosmotic velocities between 1.5 and 2.0, those with velocities between 2.5 and 3.0, and those with values higher than 4×10^{-5} $cm^2 \cdot V^{-1} \cdot sec^{-1}$. Whether these differences were caused by different preparation procedures is not known. From the nature of the polysaccharide mixture of agar as described by ARAKI[6], the ionic groups remaining in the agarose gel matrix are probably carboxylic or sulfonic acid residues. In order to reduce the electroosmotic velocity, agarose in solution was treated with anion-exchange resin at a temperature above its gelling point. In a typical experiment of this kind, 1 g of agarose was dissolved in 100 ml of distilled

TABLE II

MEAN VALUES AND STANDARD DEVIATIONS OF THE ELECTROOSMOTIC VELOCITY IN 1% (w/v) AGAROSE GELS

Phosphate buffer, 0.005 moles/l, pH 7.0; 15.4 V/cm, 2.5–4°.

Agarose	*Electroosmotic velocity* (10^{-5} $cm^2 \cdot V^{-1} \cdot sec^{-1}$)
BDH	1.56 ± 0.12
EGA (Aldrich)	1.76 ± 0.25
Koch-Light	1.78 ± 0.14
Mann	1.89 ± 0.09
Pharmacia	2.48 ± 0.21
BioRad Lab.	2.59 ± 0.12
Seakem	2.70 ± 0.16
Behringwerke	2.85 ± 0.25
L'Industrie Biologique	2.94 ± 0.19
Paines and Byrne	3.27 ± 0.25
Serva	4.19 ± 0.26
Calbiochem	6.12 ± 0.19

water by boiling. 1 g of BioRad AG 1-X1 anion-exchange resin (50–100 mesh, chloride form) was added, and the mixture was kept at 50° overnight with repeated shaking. After sedimenting of the resin particles, the supernatant was decanted and cooled to gel formation. The gel was cut, frozen, and crushed in a mortar. Part of the water was removed by the addition of absolute ethanol, and the gel particles were dried at 50°. The electroosmotic velocity of such a preparation was markedly reduced, as compared with a gel prepared from the original material (see Table III).

DISCUSSION AND CONCLUSIONS

According to ARAKI[6] agarose is a polymer of D-galactose and 3,6-anhydro-L-galactose. In aqueous solutions and at pH values usually encountered in electrophoresis or isoelectric focusing experiments, the presence of charged groups may not be expected in such polymer molecules, and, hence, it must be assumed that commercially available agarose preparations are contaminated to varying degrees by ionizable macromolecules, *e.g.* agaropectin, which are the cause of electroosmotic flow in the gel.

The influence of the electroosmotic flow on the resolution obtainable in gel

TABLE III

REDUCTION OF ELECTROOSMOTIC VELOCITY IN 1% (w/v) AGAROSE GEL BY TREATMENT WITH ANION-EXCHANGE RESIN

Experimental conditions same as in Table II.

Agarose	*Electroosmotic velocity* (10^{-5} $cm^2 \cdot V^{-1} \cdot sec^{-1}$)
BioRad Lab.	2.59 ± 0.12
BioRad exchanged	1.50 ± 0.14

electrofocusing experiments may be characterized as follows: the faster the electroosmotic flow within the gel, the greater the difference between the actual position of a zone of macromolecules and its isoelectric point on the pH gradient. This is due to the fact that the ampholytes (mol. wt. about 500) which form the pH gradient return to their respective isoelectric positions much faster after having been carried away by the electroosmotic flow than the macromolecules which are perhaps retarded by the gel if they have a molecular weight of more than 10^5 to 10^6. The latter will always lag behind their isoelectric position with a distance depending on their diffusion coefficients and the slope of their titration curves in the vicinity of their isoelectric points. Thus, the requirements for the condensation of the macromolecules in zones of Gaussian concentration distribution, namely constant conductivity and constant pH within a single zone[7] are not met, and, hence, a blurring of the bands occurs which has a diminishing effect on the resolution of neighbouring bands[8].

In order to facilitate the discussion on the magnitude of the electroosmotic flow in gels and the factors influencing it, some physico-chemical facts will be recalled. For simplification, gels are treated as capillary systems. The electroosmotic flow in capillaries of a radius much greater than $1/\kappa$, which denotes the radius of the ion cloud over the electrical charges which are fixed to the wall of the capillary, may be described by eqn. 1 which is derived from the Helmholtz–Smoluchowski equation (ref. 9, p. 406):

$$\frac{dV}{dt} \equiv D = \frac{I\varepsilon\zeta}{4\pi\eta_0\kappa'} \tag{1}$$

where I stands for the electrical current, ε the field strength, ζ the electrokinetic potential φ at the surface of shear[10], η_0 the viscosity of the liquid, and κ' its conductivity. In small-pore capillary systems with radii below 50 nm, deviations from the Helmholtz–Smoluchowski equation were observed[9]. In such systems, the term ζ of eqn. 1 becomes meaningless because the radius of the capillary is of the order of the radius of the ion cloud, $r \leqq 1/\kappa$, which implies that the definition of the surface of shear does not hold. The electroosmotic flow in such capillaries is described by eqn. 2 (ref. 9):

$$\frac{dV}{dt} \equiv D = \frac{IFc_{R'}}{8\eta_0\kappa''} \cdot r^2 \tag{2}$$

where F is the product of Avogadro's number and elementary charge, $c_{R'}$ the concentration of counterions, and κ'' the conductivity of the fluid within the capillaries.

The question now arises as to the conclusions that may be drawn from this knowledge on electroosmotic flow and to the difference which exists between both types of capillary systems with respect to the application of gels as stabilizing agents in isoelectric focusing experiments. The dependence of the electroosmotic flow, D, on the square of the radius of the capillary, r^2, in the small-pore capillary system (eqn. 2) is obvious. This makes it necessary to calculate the dimensions of the radius of the capillaries and of the ion clouds, $1/\kappa$, in the different types of gels, in order to know which model applies to the gel system under study.

The average pore radius for a 1% agar gel (Ionagar No. 2) has been determined as 90–120 nm (ref. 4, p. 117). Similar values may be calculated from data for the

selectivity of agarose gel beads for gel filtration provided by the manufacturers. The radius of the ion cloud, $1/\kappa$, is given by KORTÜM (ref. 9, p. 171) as 3.93 nm and 1.24 nm for an aqueous solution of a 1,3-salt at 25°, and for concentrations of 10^{-3} moles/l and 10^{-2} moles/l, respectively. From these data, it appears probable that eqn. 1 applies to the conditions of the electrophoretic experiments reported here. In electrofocusing experiments, the conductance of the column decreases towards the end of the run. The only free ions present in the solution of the pH gradient during the final stages of an experiment are the zwitterionic ampholytes and the focused macromolecules. Both types of molecules are multivalent ions in aqueous solutions. The average concentration of ampholytes is usually of the order of 10^{-1} to 10^{-2} moles/l, while the macromolecules are much less concentrated. With increasing valence of the ionic species and increasing concentration of the ions, the radius of the ion cloud, $1/\kappa$, decreases. It seems, therefore, reasonable to assume that eqn. 1 holds for electrofocusing experiments in large-pore gels, *e.g.* gels with an agarose concentration up to 1% (w/v). However, since nothing is known about the exact pore size distribution within the gels, deviations of the electroosmotic flow from the one expected from eqn. 1 cannot be excluded because capillaries with much smaller pore sizes than the average may be present.

DAVIES[11] has demonstrated considerable differences of conductivity along density-stabilized pH gradients which were formed by electrofocusing Ampholines of various pH ranges. In experiments with agarose as stabilizing agent, this phenomenon leads to differences of electroosmotic flow (*cf.* eqn. 1) in a gel, and hence also of electroosmotic pressure, as soon as the pH gradient is established. Under certain experimental conditions this may cause the gel to rupture. Another effect which has been observed by CATSIMPOOLAS[2] may be explained in this way. The anionic groups fixed in the gel matrix have protolytic dissociation constants which most probably lie in the range of 10^{-2} to 10^{-4}. Therefore, the acidic side of the pH gradient, pH 3–4, may exert a great influence on the degree of dissociation of such groups.

Decreasing the number of fixed charges by lowering the degree of dissociation leads to a drop in electrokinetic potential at the surface of shear, ζ, which in turn results in a reduction of electroosmotic flow in the anodic part of the gel. Since the direction of the electroosmotic flow is towards the cathode, this, in turn, leads to dehydration and to shrinkage of the anodic part of the gel. Data supporting this interpretation were presented by WALDMAN-MEYER[5] for paper, and by RAACKE[12] for starch as supporting media in electrophoresis experiments.

Electroosmosis may also occur in polyacrylamide gels[13]. Although I have not observed electroosmotic flow in control experiments with photopolymerized polyacrylamide gel in the system described above, it has been encountered during gel electrofocusing experiments with polyacrylamide by VESTERBERG (*cf.* ref. 14, p. 4) and POGACAR[15]. The average pore size in polyacrylamide gel of 7.5% (w/v) concentration is estimated as being 50 nm[16,17]. Thus it seems probable that a considerable number of pores are of the size for which eqn. 2 applies, *i.e.* $r \leqq 1/\kappa$. Since the electroosmotic flow is proportional to the square of the radius of the capillaries (see eqn. 2), it is mandatory to prepare homogeneous gels for gel electrofocusing in polyacrylamide.

Although polyacrylamide gels have been used in most experiments of gel electrofocusing, agarose may have certain advantages. For instance, dilute agarose gels may be pumped through capillary tubing, agarose gel is transparent in the UV

range[4,18] without special treatment as is necessary for polyacrylamide gel[19,20], and agarose does not seem to interact chemically with sample molecules, again in contrast to polyacrylamide which affects enzyme activity[21,22], interferon, poliovirus, and insulin[23] probably by interaction with residues of the persulfate catalyst system. Furthermore, agarose is nontoxic while the monomer solutions for polyacrylamide gels are very skin- and neuro-toxic. From these considerations, recommendations for gel electrofocusing experiments are as follows:

(1) Low concentrations of especially purified gel substances should be used, *e.g.* recrystallized acrylamide monomer or agarose treated with anion-exchange resin.

(2) The ampholyte concentration should be high in order to reduce electroosmotic flow.

(3) The anodic solution should not be a strong acid when using agarose gel (*cf.* ref. 2).

(4) Precipitates within the gel should be strictly avoided (*cf.* ref. 24).

(5) Increasing the viscosity of the liquid in the gel by adding neutral, chemically inert substances, *e.g.* sucrose, may be of help in reducing electroosmotic flow.

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CHROM. 5087

CHROMATOGRAPHIC BEHAVIOUR AND STRUCTURAL UNITS OF CONDENSED PHOSPHATES

II. INFLUENCES OF DEVELOPING SOLVENTS

TAKAYA IIDA AND TAKEO YAMABE

The Institute of Industrial Science, The University of Tokyo, 22-1, Roppongi 7-chome, Minato-ku, Tokyo 106 (Japan)

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SUMMARY

Influences of the components (acidic solvents: water, ammonia water, trichloroacetic acid, and acetic acid; basic solvents: water and ammonia water) and pH values of developing solvents on R_F values, the ratio of the thickness of the mobile phase and the stationary phase, and free energy necessary to transport the structural units of condensed phosphates from the stationary phase to the mobile phase were examined. They are greatly influenced by the water content and pH value. The influences of the other components are slight.

INTRODUCTION

We reported in a previous paper[1] that the relationships between R_M values and the structural units (the middle PO_4 group, the end PO_4 group, and the single [orthophosphate] PO_4 group) of condensed phosphates were obtained and that the ratio of the thickness of the mobile phase to the stationary phase and the free energy necessary to transport 1 mole of each structural unit from the stationary to the mobile phase can be calculated by the use of these relationships.

Herein, we rewrite these relationships

for the metaphosphate ion consisting of m phosphorus atoms

$$R_{M\,(m\text{-meta})} = -\log (A_M/A_S) + m\Delta\mu_{\text{middle}}/2.3RT \quad (1)$$

for the pyrophosphate ion

$$R_{M(\text{pyro})} = -\log (A_M/A_S) + 2\Delta\mu_{\text{end}}/2.3RT \quad (2)$$

for the orthophosphate ion

$$R_{M\,(\text{ortho})} = -\log (A_M/A_S) + \Delta\mu_{\text{ortho}}/2.3RT \quad (3)$$

for the polyphosphate ion consisting n phosphorus atoms

$$R_{M\,(n\text{-poly})} = -\log (A_M/A_S) + 2\Delta\mu_{\text{end}}'/2.3RT + (n-2)\Delta\mu_{\text{middle}}'/2.3RT \quad (4)$$

where (A_M/A_S) is the ratio of the thickness of the mobile phase to the stationary phase, and $\Delta\mu_{\text{middle}}$, $\Delta\mu_{\text{end}}$, $\Delta\mu_{\text{ortho}}$, $\Delta\mu_{\text{middle}}'$ and $\Delta\mu_{\text{end}}'$ are the free energy neces-

sary to transport 1 mole of a middle group of metaphosphate, an end group of pyrophosphate, and the ortho group, middle and end groups of polyphosphate, respectively.

In general, developing solvents used in paper chromatography (PC) and thin-layer chromatography (TLC) of condensed phosphates are mixed solutions of some alcohols and water. They are roughly divided into acidic and basic solvents, and trichloroacetic acid, ammonia water, etc., are added to the mixed solutions. Acidic solvents are suitable for the separation of ortho-, pyro-, and polyphosphates and basic solvents are suitable for the separation of metaphosphates.

EBEL[2] described the influence of the water content in the acidic and basic solvents on R_F values and the influence of the addition of a small quantity of ammonia water to the acidic solvents on R_F values. THILO AND FELDMANN[3] described those influences and the influence of pH values of the solvents on R_F values. In this paper, we studied the influences of the water content and the other components and pH values of the solvents on R_F values, A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$. We did not examine the influences of $\Delta\mu_{middle}'$ and $\Delta\mu_{end}'$ because there seem to be mutual interactions between the middle and end groups of the polyphosphates.

The following influences are examined in this paper. (a) acidic solvents: water content, quantity of ammonia water, quantity of trichloroacetic acid, quantity of

TABLE I

DEVELOPING SOLVENTS

Solvent	*Ammonia water (28%, ml)*	*Trichloro-acetic acid (g)*	*Acetic acid (glacial, ml)*	*Water content[a] (%)*	*Alcohol[b]*	*pH[c]*
A1-30	0.25	5.0	1.0	30	iP	1.62
A1-40	0.25	5.0	1.0	40	iP	1.47
A1-50	0.25	5.0	1.0	50	iP	1.33
A2-0.50	0.50	5.0	1.0	40	iP	1.67
A2-0.75	0.75	5.0	1.0	40	iP	1.82
A2-1.00	1.00	5.0	1.0	40	iP	2.03
A2-1.25	1.25	5.0	1.0	40	iP	2.37
A2-1.50	1.50	5.0	1.0	40	iP	3.33
A3-1	0.25	1.0	1.0	40	iP	2.80
A3-3	0.25	3.0	1.0	40	iP	1.70
A4-2	0.25	5.0	2.0	40	iP	1.50
A4-3	0.25	5.0	3.0	40	iP	1.47
A5-0.20	0.20	2.0	1.0	40	iP	1.89
A5-1	1.0	2.0	1.0	40	iP	5.59
A5-2	2.0	2.0	1.0	40	iP	8.33
A5-4	4.0	2.0	1.0	40	iP	9.20
B1-30	1.0			30	iP–iB	—
B1-40	1.0			40	iP–iB	11.39
B1-50	1.0			50	iP–iB	—
B2-2	2.0			40	iP–iB	11.45
B2-4	4.0			40	iP–iB	11.86

[a] Aqueous solutions (30, 40 or 50 ml) containing 3 components (acidic solvents) or 1 component (basic solvents) were diluted to 100 ml with alcohols.

[b] iP = isopropyl alcohol; iP–iB = isopropyl–isobutyl alcohol (1:1).

[c] pH = reading of the pH meter (glass electrode).

acetic acid; (b) basic solvents: water content, quantity of ammonia water; and (c) pH values of solvents.

EXPERIMENTAL

Developing solvents

The compositions of developing solvents are shown in Table I. pH values are also given.

Thin layers

In 60 ml of water were suspended 15 g of Avicel (FMC Corporation). Thin-layer plates were prepared as usual (250 mμ).

Samples of phosphates

A solution of mixed metaphosphates was prepared according to THILO AND SCHULKE[4]. Solutions of sodium salts of ortho-, pyro-, tripoly-, trimeta-, tetrameta-[5], and hexameta-[6] phosphates were used as standards.

Procedure

After the solutions of the phosphates had been spotted, the thin-layer plate was allowed to equilibrate with the vapour of the developing solvent for 2 h; the phosphates were then developed in a saturation chamber (ascending method). The temperature was maintained at 5° in an air bath (Coolnics, Komatsu-Yamato). When the solvent had travelled 10 cm from the application point of the phosphates, the plate was taken out and dried in air. The phosphates were then hydrolysed with an aqueous nitric acid solution (1:1) and visualised with ammonium molybdate and stannous chloride[7,8].

RESULTS AND DISCUSSION

R_F values obtained and A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ calculated are listed in Table II.

Acidic solvents

Water content (A1-30, A1-40, and A1-50). The plots of R_F value *vs.* water content, of R_M value *vs.* degree of condensation, and of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* water content are shown in Figs. 1, 2, and 3. In proportion as the water content increases, the R_F value increases by a great margin. A_M/A_S increases by a great margin, $\Delta\mu_{ortho}$ also increases, $\Delta\mu_{middle}$ slightly decreases and $\Delta\mu_{end}$ is almost constant. It is thought that the R_F value is greatly influenced by the water content and that this depends on the great increase of A_M/A_S.

Quantity of ammonia water (A1-40, A2-0.50, A2-0.75, A2-1.00, A2-1.25, and A2-1.50). The plots of R_F value *vs.* quantity of ammonia water, and of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* quantity of ammonia water are shown in Figs. 4 and 5. In proportion as the quantity of ammonia water increases, the R_F values of pyro- and metaphosphates slightly decrease, but the R_F value of orthophosphate is

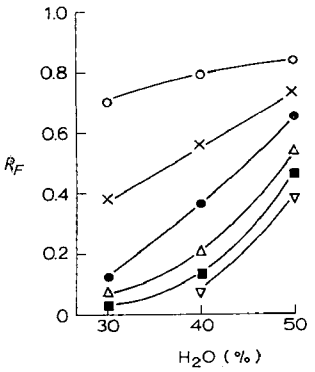
TABLE II

R_F VALUES OBTAINED AND A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, AND $\Delta\mu_{ortho}$ CALCULATED

Phosphates	*A1-30*	*A1-40*	*A1-50*	*A2-0.50*	*A2-0.75*	*A2-1.00*	*A2-1.25*	*A2-1.50*	*A3-1*	*A3-3*	*A4-2*
Ortho (O)	0.70	0.80	0.83	0.80	0.78	0.78	0.78	0.78	0.78	0.81	0.81
Pyro (Py)	0.38	0.56	0.73	0.54	0.47	0.43	0.38	0.36	0.55	0.61	0.61
Tripoly (TrP)	0.17	0.44	0.64	0.38	0.29	0.26	0.21	0.20	0.46	0.45	0.50
Trimeta (TrM)	0.12	0.36	0.65	0.30	0.22	0.19	0.17	0.18	0.34	0.37	0.38
Tetrameta (TtM)	0.06	0.20	0.54	0.16	0.12	0.09	0.09	0.09	0.19	0.24	0.21
Pentameta (PeM)	0.03	0.13	0.46	0.10	0.07	0.05	0.05	0.05	0.14	0.16	0.15
Hexameta (HexM)	0.01	0.07	0.38	0.06	0.03	0.02	0.02	0.02	0.07	0.08	0.08
Heptameta (HepM)		0.02	0.30	0.02	0.02				0.02[a]	0.03[a]	
Octameta (OctM)			0.22								
Nonameta (NonM)			0.15								
Decameta (DecM)			0.11								
A_M/A_S	1.26	3.03	5.81	2.45	2.52	2.54	2.04	2.28	3.10	3.90	3.75
$\Delta\mu_{middle}$	410	328	211	341	400	440	417	429	339	345	347
$\Delta\mu_{end}$	198	245	211	202	338	335	333	387	257	252	242
$\Delta\mu_{ortho}$ (cal/mole)	−345	−115	96	−271	−190	−185	−305	−244	− 74	− 50	− 70

Phosphates	*A4-3*	*A5-0.50*	*A5-1*	*A5-2*	*A5-4*	*B1-30*	*B1-40*	*B1-50*	*B2-2*	*B2-4*
Ortho (O)	0.82	0.80	0.39	0.14	0.13	0.13	0.30	0.66	0.29	0.28
Pyro (Py)	0.60	0.54	0.17	0.04	0.04	0.05	0.20	0.47	0.15	0.15
Tripoly (TrP)	0.48	0.40	0.08	0.02	0.02	0.04	0.21	—	0.17	0.18
Trimeta (TrM)	0.38	0.37	0.21	0.25	0.26	0.23	0.59	0.86	0.58	0.57
Tetrameta (TtM)	0.23	0.21	0.11	0.13	0.13	0.11	0.48	0.80	0.44	0.45
Pentameta (PeM)	0.14	0.13	0.05	0.09	0.08	0.06	0.39	0.76	0.36	0.36
Hexameta (HexM)	0.08	0.07	0.02	0.04	0.04	0.03	0.32	0.71	0.28	0.29
Heptameta (HepM)				0.02[a]	0.02[a]		0.25	0.66	0.24	
Octameta (OctM)										
Nonameta (NonM)										
Decameta (DecM)										
A_M/A_S	4.14	4.24	3.61	2.18	2.67	2.65	4.04	13.20	3.70	4.10
$\Delta\mu_{middle}$	358	502	473	368	383	413	199	155	202	216
$\Delta\mu_{end}$	281	360	794	1120	1150	1084	769	747	842	870
$\Delta\mu_{ortho}$ (cal/mole)	− 53	33	958	1490	1590	1592	1241	1060	1219	1302

[a] Removed from the least squares calculation.



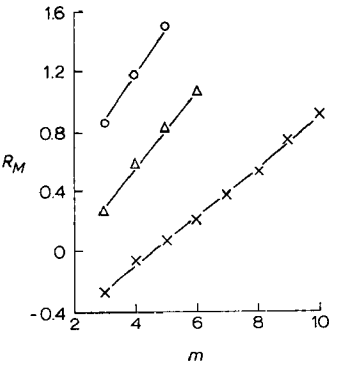


Fig. 1. Plots of R_F *vs.* water content. ○, O; ×, Py; ●, TrM; △, TtM; ■, PeM; ▽, HexM.

Fig. 2. Plots of R_M *vs.* degree of condensation (m). ○, 30%; △, 40%; ×, 50%.

almost identical. A_M/A_S and $\Delta\mu_{ortho}$ are almost the same, but $\Delta\mu_{middle}$ and $\Delta\mu_{end}$ seem to increase slightly.

Quantity of trichloroacetic acid and acetic acid (A3-1, A3-3, and A1-40, and A1-40, A4-2, and A4-3). The plots of R_F value *vs.* quantity of trichloroacetic acid and acetic acid, of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* quantity of trichloroacetic acid and acetic acid are shown in Figs. 6, 7, 8, and 9. Even if the quantity of trichloro-

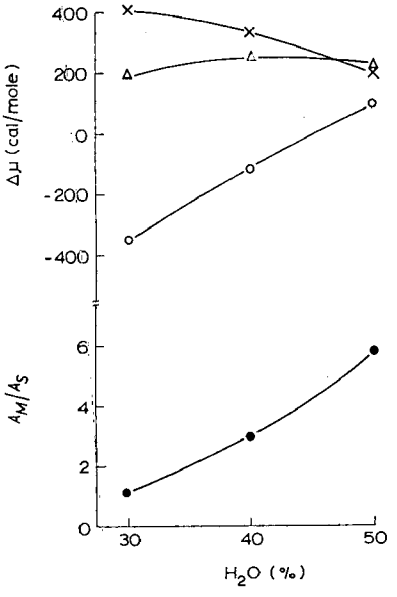
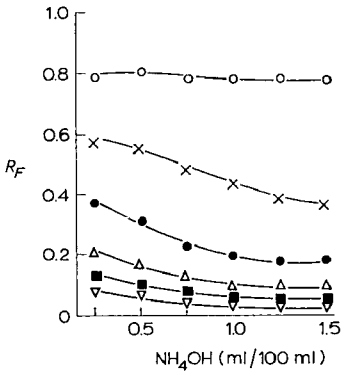


Fig. 3. Plots of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* water content. ×, middle; △, end; ○, ortho; ◐, A_M/A_S.



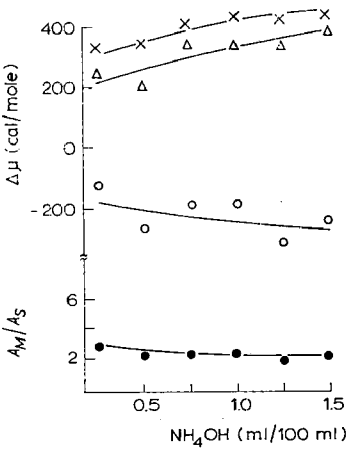


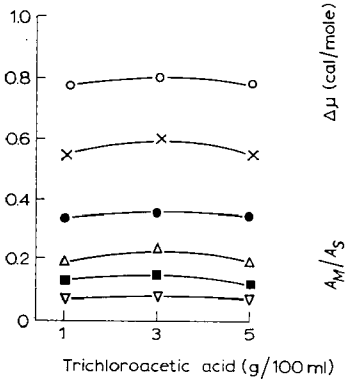
Fig. 4. Plots of R_F *vs.* quantity of ammonia water. ○, O; ×, Py; ●, TrM; △, TtM; ■, PeM; ▽, HexM.

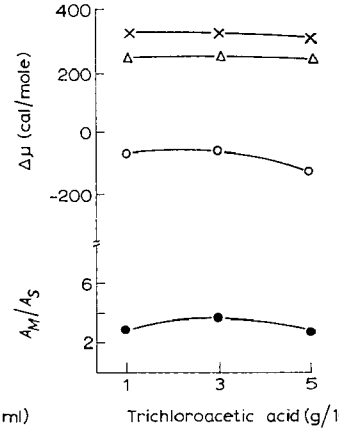
Fig. 5. Plots of A_M/A_S, $\Delta\mu_{\text{middle}}$, $\Delta\mu_{\text{end}}$, and $\Delta\mu_{\text{ortho}}$ *vs.* quantity of ammonia water. ×, middle; △, end; ○, ortho; ●, A_M/A_S.

acetic acid and acetic acid is changed, R_F, A_M/A_S, $\Delta\mu_{\text{middle}}$, $\Delta\mu_{\text{end}}$, and $\Delta\mu_{\text{ortho}}$ are almost constant.

Basic solvents (B1-30, B1-40, and B1-50)

Water content (B1-30, B1-40, and B1-50). The plots of R_F value *vs.* water content, of R_M value *vs.* degree of condensation, and of A_M/A_S, $\Delta\mu_{\text{middle}}$, $\Delta\mu_{\text{end}}$, and $\Delta\mu_{\text{ortho}}$ *vs.* water content are shown in Figs. 10, 11, and 12. As in the case of the acidic





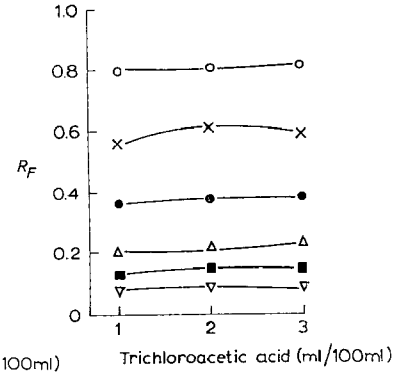


Fig. 6. Plots of R_F *vs.* quantity of trichloroacetic acid. ○, O; ×, Py; ●, TrM; △, TtM; ■, PeM; ▽, HexM.

Fig. 7. Plots of A_M/A_S, $\Delta\mu_{\text{middle}}$, $\Delta\mu_{\text{end}}$, and $\Delta\mu_{\text{ortho}}$ *vs.* quantity of trichloroacetic acid. ×, middle; △, end; ○, ortho; ●, A_M/A_S.

Fig. 8. Plots of R_F *vs.* quantity of acetic acid. ○, O; ×, Py; ●, TrM; △, TtM; ■, PeM; ▽, HexM.

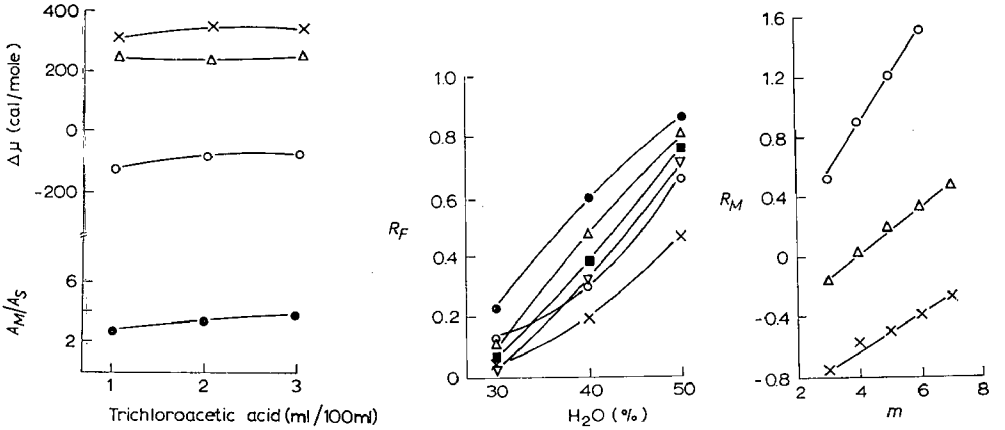


Fig. 9. Plots of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* quantity of acetic acid. ×, middle; △, end; ○, ortho; ●, A_M/A_S.

Fig. 10. Plots of R_F *vs.* water content. ○, O; ×, Py; ●, TrM; △, TtM; ■, PeM; ▽, HexM.

Fig. 11. Plots of R_M *vs.* degree of condensation (m). ○, 30%; △, 40%; ×, 50%.

solvent, the R_F value increases by a great margin, in proportion to the increase of the water content. A_M/A_S increases by a great margin, but $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ decrease slightly.

Quantity of ammonia water (B1-40, B2-2, and B2-4). The plots of R_F value *vs.* quantity of ammonia water, and of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* quantity

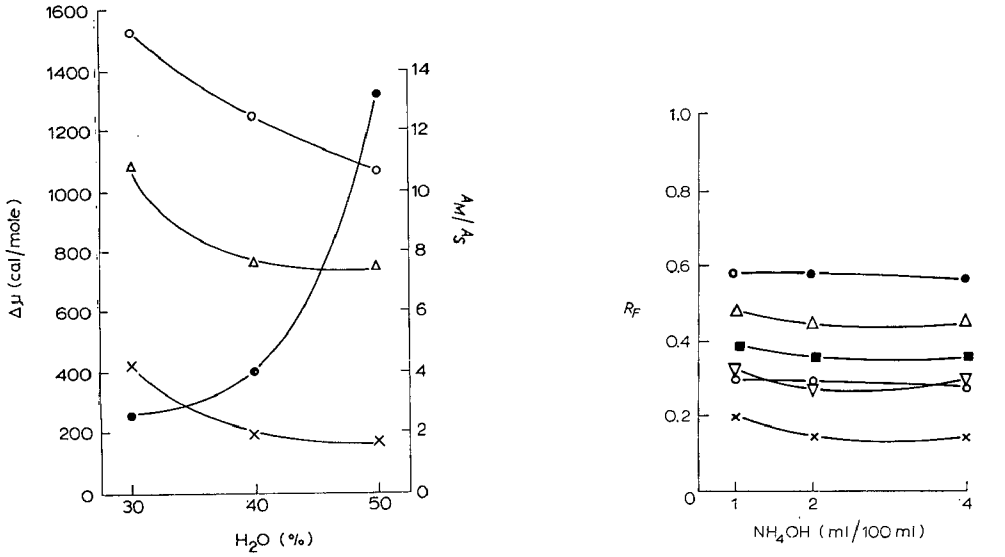


Fig. 12. Plots of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* water content. ×, middle; △, end; ○, ortho; ●, A_M/A_S.

Fig. 13. Plots of R_F *vs.* quantity of ammonia water. ○, O; ×, Py; ●, TrM; △, TtM; ■, PeM; ▽, HexM.

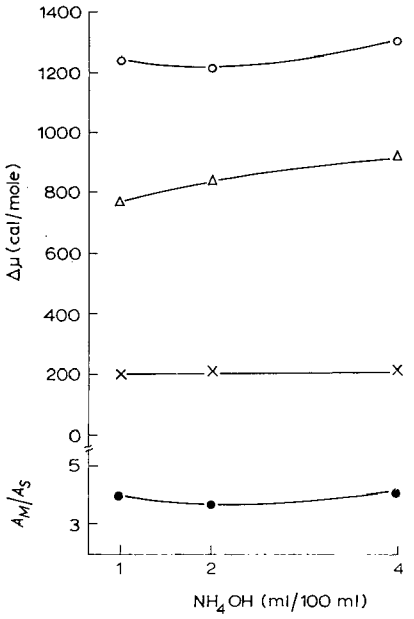
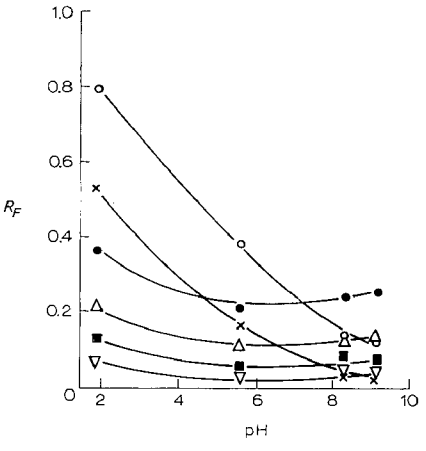


Fig. 14. Plots of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* quantity of ammonia water. ×, middle; △, end; ○, ortho; ●, A_M/A_S.



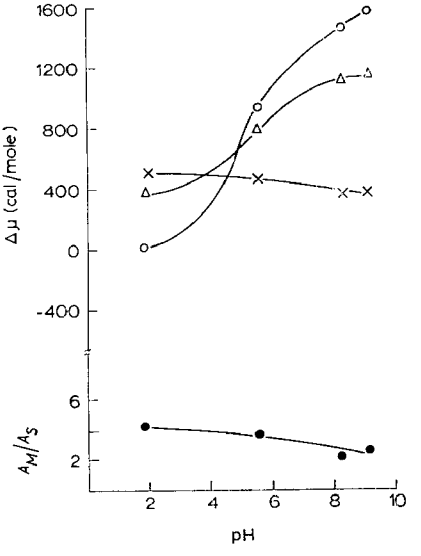


Fig. 15. Plots of R_F *vs.* pH of solvent. ○, O; ×, Py; ●, TrM; △, TtM; ■, PeM; ▽, HexM.

Fig. 16. Plots of A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ *vs.* pH of solvent. ×, middle; △, end; ○, ortho; ●, A_M/A_S.

of ammonia water are shown in Figs. 13 and 14. Even if the quantity of ammonia water is changed, R_F, A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, and $\Delta\mu_{ortho}$ are almost constant. This is not the case with the acidic solvent.

pH value of solvent (A5-0.20, A5-1, A5-2, and A5-4). The plots of R_F value *vs.* pH of the developing solvent, and A_M/A_S, $\Delta\mu_{middle}$, $\Delta\mu_{end}$, $\Delta\mu_{ortho}$ *vs.* pH of the developing solvent are shown in Figs. 15 and 16. When the pH of the developing solvent is increased, the R_F values of ortho- and pyrophosphates decrease by a large margin and the R_F of metaphosphates decreases slightly. A_M/A_S and $\Delta\mu_{middle}$ decrease slightly, but $\Delta\mu_{end}$ and $\Delta\mu_{ortho}$ increase by a large margin. As shown in Fig. 5, even if the quantity of ammonia water is increased, $\Delta\mu_{end}$ increases slightly and $\Delta\mu_{ortho}$ is almost constant as long as the pH change is not too large. Thus it is thought that the increase of $\Delta\mu_{end}$ and $\Delta\mu_{ortho}$ depends on the pH increase of the developing solvent, not on the increase of the quantity of ammonia water. The dependence of the R_F value of phosphates on the pH of the developing solvent is similar to that in the case of paper chromatography of metal ions (ortho- and pyrophosphates—Cu^{2+}, Ba^{2+}, and Pb^{2+}, metaphosphates—$HgCl_2$)[9].

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Notes

CHROM. 5076

The use of ammonia as the carrier gas in gas–liquid chromatography

In the course of our work on gas chromatographic separation of some amines, it was found that ammonia is of special interest as a carrier gas not only for chromatography of amines* but for a number of other compounds as well.

Experimental

All separations were performed on a homemade all-glass capillary chromatograph with a flame ionisation detector (FID). Capillaries were of borosilicate glass (0.25 or 0.40 mm I.D., 30–80 m in length) coated with the liquid phase as described[2]. Carbowax 4000 and Soviet methyl, phenyl and β-cyanoethyl polysiloxanes were tested as liquid phases[3]. Ammonia was fed directly into the gas system from a 1-l tank with a needle valve. The inlet pressure was 0.3–1.5 atm. Excess ammonia escaping through the splitting system (split ratio, 1:50) was captured by the water pump suction.

Results and discussion

FID sensitivity. An interesting property of ammonia as the carrier gas is that it enhances the sensitivity of the FID to an order of magnitude almost comparable with nitrogen. The baseline, at the same time, is exellent**.

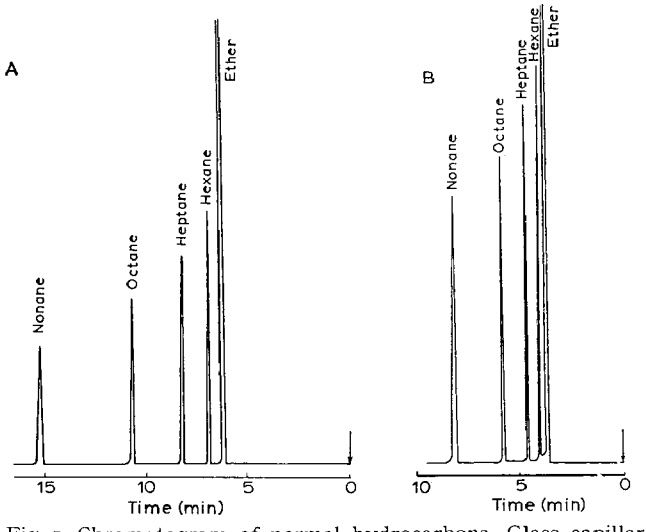
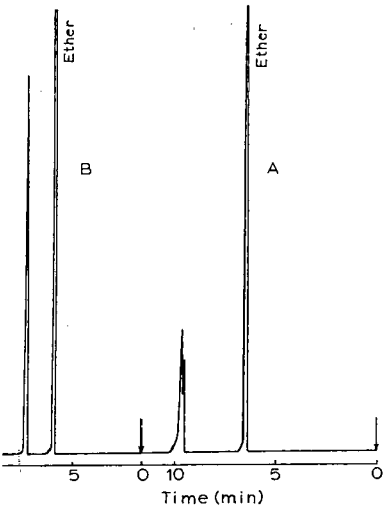


Fig. 1. Chromatogram of normal hydrocarbons. Glass capillary column (40 m, 0.25 mm I.D.). Liquid phase: phenyl polysiloxane. Temperature: 85°. Inlet pressure: 0.6 atm. (A) Carrier gas: nitrogen. (B) Carrier gas: ammonia.

* Ammonia (in a mixture with nitrogen) was recommended for converting hydrochlorides of the amino acid esters into free bases directly in the evaporator[1].

** When switching over from nitrogen to ammonia, the detector current increases slightly (see ref. 1).



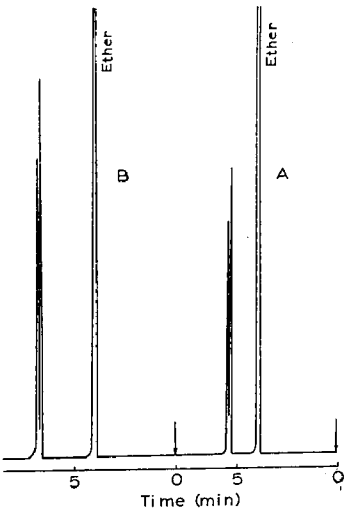


Fig. 2. Chromatogram of *cis*- and *trans*-1,3,5-trimethylpiperidines. (A) Carrier gas: nitrogen. (B) Carrier gas: ammonia. Inlet pressure: 0.6 atm. Temperature: 120°. Column: see legend to Fig. 1.

Fig. 3. Chromatogram of *cis*- and *trans*-N-methylperhydropyridine (A) and *cis*- and *trans*-N-methyldecahydroquinoline (B). Carrier gas: ammonia. Inlet pressure: 0.6 atm. Temperature: 160°. Column: see legend to Fig. 1.

Retention times. As may be seen from Fig. 1 A, B, for normal paraffins ammonia yielded almost half the retention times that nitrogen did. At the same time, the actual separation of these mixtures and the shape of their peaks do not alter when the carrier gas is changed. For other compounds (*e.g.* see Fig. 2), separation may change if the carrier gas is changed.

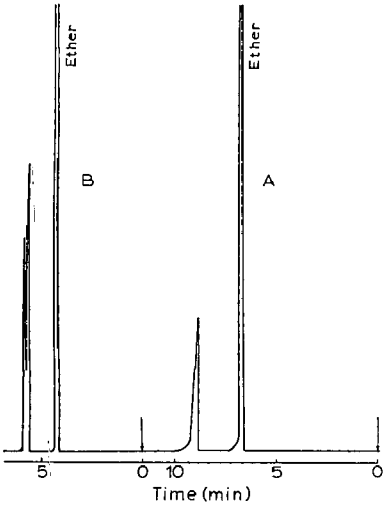


Fig. 4. Chromatogram of *cis*- and *trans*-3,5-dimethylpiperidines. (A) Carrier gas: nitrogen. (B) Carrier gas: ammonia. Inlet pressure: 0.6 atm. Temperature: 125°. Column: see legend to Fig. 1.

Thermal stability of liquid phases. With ammonia as carrier gas no excessive bleeding was noticed with the polyether and polysiloxane liquid phases studied. Some polysiloxane capillary columns were run for a long time (several months) at temperatures up to 200–250° with no signs of deterioration.

Chromatography of amines. In Figs. 3 and 4 are shown some chromatograms of amines obtained with nitrogen and ammonia as carrier gases. These chromatograms indicate the substantial reduction of tailing and, in some cases, remarkable increase in separation (see Fig. 4). Here the tail-reducing property of ammonia only on polysiloxane liquid phases deserves special comment. On polyethylene glycol*, ammonia has no obvious effect on peak shapes except for shortening the retention times. Thus, tail-reduction seems to be connected with the competitive interaction of amines and ammonia with silicone atoms of the liquid phase.

The results of this preliminary research show that ammonia as the carrier gas may be advantageously used for chromatography of amines and other compounds non-reactive towards ammonia.

N.D. Zelinsky Institute of Organic Chemistry, E. L. ILKOVA
U.S.S.R. Academy of Sciences, Moscow (U.S.S.R.) E. A. MISTRYUKOV

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* Coated on KOH-treated glass columns.

CHROM. 5112

A modified gradient elution procedure for single column amino acid analysis*

Amino acid analysis of connective tissue proteins poses problems not encountered with most protein hydrolysates. The difficulty arises in the separation of hydroxyproline from aspartic acid, and separation of hydroxylysine, lysinonorleucine and the desmosines from other basic amino acids. Of the current procedures in the literature[1-4], only the method of MILLER AND PIEZ[1] resolves these amino acids in a length of time suitable for routine analysis. However, the ninhydrin reagent commonly used with standard amino acid analysis is not compatible with their buffer system. Amino acid color yields are only 25% of maximum at the start of the run, with quite a significant increase in the base line toward the end of the run.

Recently a rapid procedure of programmed analysis has been described which gives good separation of the amino acids in collagen in less than 4 h[5]. This procedure is not practical for most of the standard analyzers, as it requires four buffer changes and three temperature changes.

This paper describes a rapid method that resolves all the amino acids arising from connective tissue proteins on a single column, with no temperature change, and utilizing the same buffers and reagents used in the standard two-column method[6,7].

Methods

A Beckman Model 116 amino acid analyzer with the starting buffer line connected to a nine chambered Varigrad gradient device was used in these experiments. The long column, 0.9 × 69 cm, was packed to a column height of 50 cm with UR-30 resin. Flow rates were 70 ml/h for the buffer and 30 ml/h for the ninhydrin reagent. Water jacket temperature was maintained at 56° throughout the run.

The ninhydrin reagent, the 0.20 *N* sodium citrate buffer, pH 3.25 and the 0.35 *N* sodium citrate buffer, pH 5.25, were prepared as described in the Beckman manual. The pH 2.91 buffer was prepared the same as the pH 3.25 buffer except two percent of the water was replaced with *n*-propanol and the pH adjusted with concentrated HCl. Forty-three grams of sodium chloride were added per liter of pH 5.25 buffer to bring the final sodium concentration to 1.08 *N*.

The gradient was prepared as shown in Table I.

The column was equilibrated with the pH 2.91 buffer prior to the start of the analysis. After application of the sample, the starting buffer line was connected to the Varigrad and the run started. A change to the pH 5.25 buffer was made after 130 min.

Results and discussion

Fig. 1 shows a chromatogram of a synthetic mixture of amino acids. Peak retention times for amino acids not shown in the chromatogram are: cysteic acid, 28 min; 3-hydroxyproline, 43 min; methionine sulfoxides, 51 min; isodesmosine, 175 min; lysinonorleucine, 190 min; and ornithine, 201 min.

If the buffers were not free from contaminating ammonia, a shoulder remained after the ammonia peak, and resulted in an increased baseline through histidine

* Supported in part by grants SM-13112 and GM 977 from the U.S. Public Health Service, E105J from the American Cancer Society, and Institutional Grant GRS No. 287.

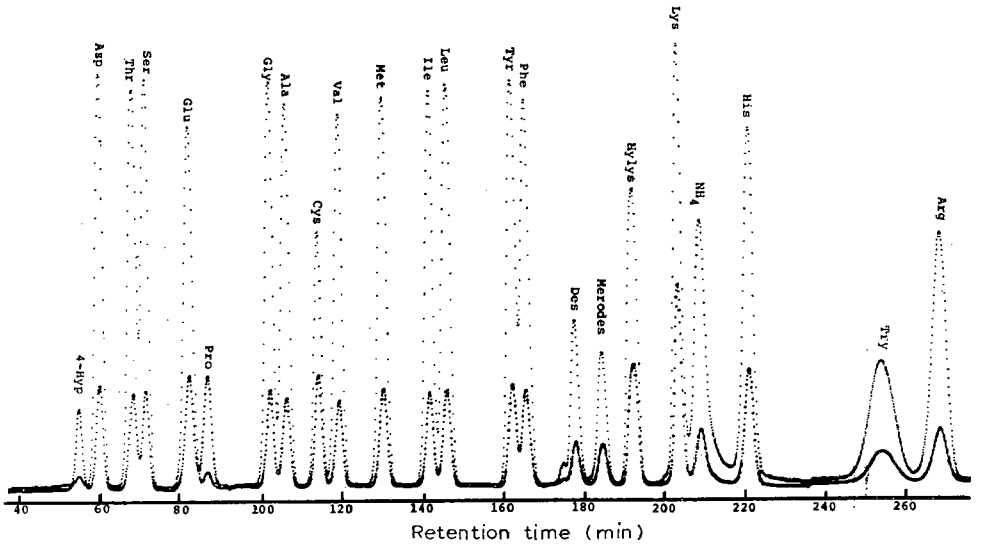

Fig. 1. Chromatogram of a synthetic mixture of amino acids using the procedure described in the text.

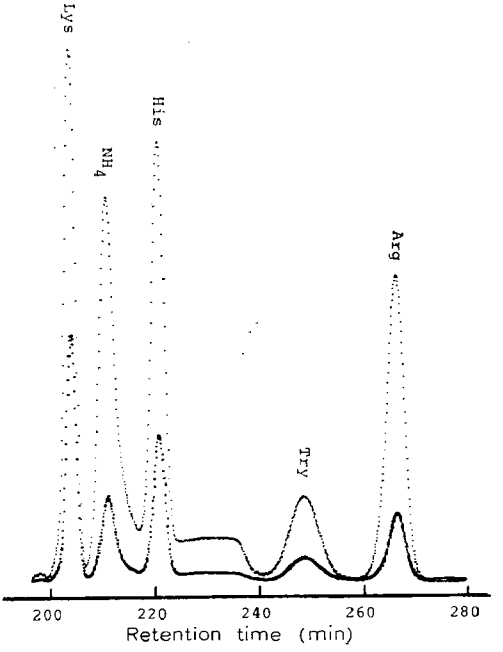

Fig. 2. Latter part of a gradient elution chromatogram, illustrating the ammonia plateau that appears when the pH 2.91 buffer contains ammonia.

TABLE I

PREPARATION OF THE VARIGRAD GRADIENT

Chamber	*pH 2.91 (ml)*	*pH 3.25 (ml)*	*0.4 N Na-citrate (ml)*
1	36	—	—
2	36	—	—
3	—	36	—
4	—	30	6
5	—	26	10
6	—	18	18
7	—	6	30
8	—	—	36
9	—	—	36

(Fig. 2). Unless the ammonia was present in large amounts, however, this did not affect the resolution or accuracy of the analysis. This shoulder could be eliminated by redistilling over sulfuric acid the water that was used to make the pH 2.91 buffer.

The low pH of the equilibrating buffer was required for the separation of hydroxyproline from aspartic acid. This separation could also be accomplished by lowering the temperature, but inadequate resolution and broadening of peaks resulted. Increasing the normality of the pH 5.25 buffer as little as 0.03 *N* in sodium resulted in tryptophan and arginine being eluted together.

Washing the column with NaOH after each analysis is not necessary with most protein hydrolysates. The buffer line is cleared with the pH 2.91 buffer and the column equilibrated. The entire process of making the gradient and equilibrating the column to start a new run requires about 20 min.

Department of Pathology, University of Colorado, School of Medicine, Denver, Colo. 80220 (U.S.A.)

B. C. STARCHER
L. Y. WENGER
L. D. JOHNSON

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CHROM. 5113

Detection of several non-protein amino acids in the presence of protein amino acids

Since there are indications in the literature of possible confusion of some non-protein amino acids with protein amino acids, we wish to present evidence that the following non-protein amino acids are detectable and simultaneously distinguishable from protein amino acids utilizing a Beckman amino acid analyzer: α-amino butyric acid, β-alanine, taurine, norvaline, sarcosine, norleucine, homocystine, betaine, hydroxyproline, and L,L-α,ε-diaminopimelic acid. Urea was also run. We wish to present new data for *cis*-Δ^4-dehydrolysine, and 3,5-diaminohexanoic acid which appear close to lysine in the amino acid analysis chromatogram.

Experimental

a. *Materials*. The Beckman Model 120 amino acid analyzer was used in these studies. Basic column buffer pH 5.25 and column length 20 cm. Neutral-acidic column length 50 cm, pH 3.25, followed by pH 4.30. The temperature was 55.5°. Flow rate: 1 ml/min. The following is a list of compounds and the companies from which they were purchased: D,L-norvaline from K & K Laboratories, D,L-methionine from Mann Research Laboratories, D,L-homocystine, L-lysine, valine, threonine, L-histidine·HCl, β-alanine, urea, leucine, α-amino butyric acid, glutamic acid, taurine, betaine·HCl, sarcosine·HCl, hydroxyproline, and phenylalanine from Nutritional Biochemicals Corporation.

It is a pleasure to thank Dr. LIN TSAI at Dr. Thressa Stadtman's Laboratory at the National Institutes of Health for the 3,5-diamino hexanoic acid·2 HCl which was used as a standard in these studies.

cis-Δ^4-Dehydrolysine was prepared according to the literature[1]. L,L-α,ε-Diaminopimelic acid was prepared according to the literature[2]. The N-succinyl-L,L-α,ε-diaminopimelic acid from which the L,L-α,ε-diaminopimelic acid was prepared was a gift from CHARLES GILVARG. It was hydroloyzed and the resulting L,L-α,ε-diaminopimelic acid was isolated on a Dowex-50 Column, similar to work described previously[2].

b. *Chromatography*. Solutions of the standard compounds were made so that 1–60 mμ moles of each amino acid were applied to the appropriate "basic" or "neutral-acidic" columns after adjusting the pH of the amino acid solutions to pH 2. Chromatograms of the compounds were run singly as well as in mixtures.

Chromatograms of these compounds were also run at the Worthington Biochemical Corporation under the direction of Dr. A. L. BAKER and Mr. V. WORTHINGTON.

Results

The color constants for the non-protein amino acids as well as protein amino acid controls are indicated in Table I. Fig. 1 shows the elution profiles of the non-protein amino acids as well as the protein amino acids which are closest to them.

Thus, we especially note the separation between norvaline, L,L-α,ε-diaminopimelic acid, isoleucine, methionine, betaine, and leucine; homocystine and β-alanine; 3,5-diamino hexanoic acid, *cis*-Δ^4-dehydrolysine, and lysine.

TABLE I

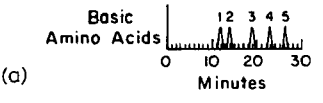
AMINO ACID ANALYZER COLOR CONSTANTS FOR AMINO ACIDS

The color constant is calculated with the use of the equation $Hw = CD$, where H is the height of the peak measured with the use of the Beckman expanded absorbance scale (4–5 mV over 10 in. span,) w is the number of dots in the peak above the halfheight, D is the number of μmoles of amino acid applied to the column, and C is the color constant. Phenylalanine or lysine may be considered standards for comparison to the literature. The 5700 Å data was used except for hydroxyproline and proline for which 4400 Å data was used .The conditions for the chromatographic runs are described in the text and are typical for the model of amino acid analyzer used.

Amino acid	*Color constant*
Homocystine	69
β-Alanine	11
Methionine	48
Urea	4
Taurine	25
Betaine	15
Sarcosine	5
Hydroxyproline	7
Phenylalanine	62
α-Amino-*n*-butyric acid	72
L,L-α,ε-Diaminopimelic acid	42
Isoleucine	21
Lysine	65
cis-Δ^4-Dehydrolysine	49
3,5-Diaminohexanoic acid	15
Norvaline	57
Norleucine	45

Discussion

Data showing the positions of 147 compounds in amino acid chromatograms have appeared in the literature[3] including non-protein amino acids such as *meso*-α,ε-diaminopimelic acid, norleucine, homocystine, hydroxyproline, taurine, urea, sarcosine, and β-alanine. However, not all of these compounds have been run concur-



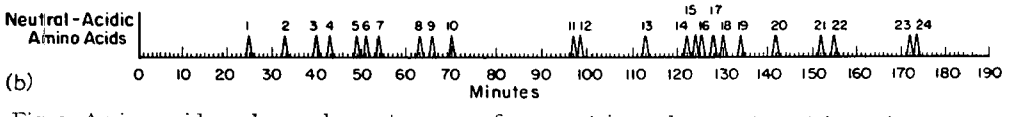


Fig. 1. Amino acid analyzer chromatograms of nor-protein and nearest protein amino acids. The distances between peaks are measured between apexes. Conditions are described in the text. (a) 1 = Tryptophan, 2 = 3,5 diaminohexanoic acid, 3 = lysine, 4 = *cis*-Δ^4-dehydrolysine, 5 = histidine. (b) 1 = Cysteic acid, 2 = urea, 3 = taurine, 4 = aspartic acid, 5 = methionine sulfone, 6 = hydroxyproline, 7 = threonine, 8 = glutamic acid, 9 = sarcosine, 10 = proline, 11 = α-amino-*n*-butyric acid, 12 = cystine, 13 = valine, 14 = betaine, 15 = methionine, 16 = L,L-α,ε-diamino pimelic acid, 17 = norvaline, 18 = isoleucine, 19 = leucine, 20 = norleucine, 21 = tyrosine, 22 = phenylalanine, 23 = homocystine, 24 = β-alanine.

rently. Often, compounds in the presence of different sets of compounds have been found to have non-reproducible chromatographic patterns. However, we have found that it is possible to reproducibly separate fifteen non-protein amino acids in the presence of the fourteen chromatographically nearest protein amino acids. There was reproducibility of time of appearance of the apexes of the amino acid analyzer peaks for several different runs of different sets of all these compounds as well as for when they were all run concurrently. We also determined the positions of several new amino acids whose positions have not previously been determined.

Leucine and norleucine have been shown to be separable[4,5]. However, difficulties were found in the separation of methionine and norvaline, and L,L- and *meso*-mixtures of α,ε-diaminopimelic acid, and also isoleucine and leucine[6,7].

Homocystine has been chromatographed[6–8] using several conditions. Different extents of separation were obtained depending on whether 50 cm or 150 cm columns were used. In one instance[6] homocystine was not readily separable from phenylalanine and tyrosine and in another instance[8] homocystine was not separable from β-alanine. However, the figure indicates that chromatographic separations are possible amongst all these compounds.

The diamino acids 3,5-diaminohexanoic acid[9] and *cis*-Δ^4-dehydrolysine[1] resemble lysine in structure. Whereas difficulty may have been expected in their chromatographic separation, in fact, they are distinguishable as shown.

It is a pleasure to thank Professor CARL SAGAN for encouragement and productive discussion during these studies. Thanks also go to Professor E. RACKER for use of an amino acid analyzer and to Professor DAVID B. WILSON and Mr. MICHAEL KANDRACH for assistance during its use. This work was supported by N.A.S.A. Grant NGR 33-010-101. The author wishes to thank CARVEL FLOOD and MARYE WANLASS for typing.

Laboratory for Planetary Studies,
Center for Radiophysics and Space Research,
Cornell University, Ithaca, N.Y. 14850 (U.S.A.)

PAUL SHAPSHAK

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CHROM. 5114

Behaviour of dehydroisoandrosterone, testosterone and their conjugates on DEAE-Sephadex

Recent publications have shown the value of DEAE-Sephadex chromatography in separating several types of phenolic steroid conjugates[1-4]. The present report concerns the behaviour of two neutral C_{19} steroids and their conjugates in simple NaCl concentration gradients on DEAE-Sephadex columns.

Experimental

Reagents. DEAE-Sephadex (A-25) of medium porosity was purchased from Pharmacia (Canada) Ltd., Montreal, Quebec, and packed to yield columns of 58 × 1 cm as described elsewhere[4].

Steroids and conjugates. [7-^{3}H]Dehydroisoandrosterone (DHA) of specific activity (S.A.) 1.6 Ci/mmole was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. and [4-^{14}C]testosterone (T) of S.A. 29.2 Ci/mole was purchased from Radiochemical Centre, Amersham, Bucks., Great Britain (now Amersham-Searle). The conjugates were purchased from New England Nuclear Corp. These were [7-^{3}H]dehydroisoandrosteron-3-yl-β-D-glucopyranosiduronate (DHAG) of S.A. 10 Ci/mmole, the NH_4^+ salt of [7-^{3}H]dehydroisoandrosteron-3-yl-sulphate (DHAS) of S.A. 10 Ci/mmole, [1,2-^{3}H]testosteron-17-yl-β-D-glucopyranosiduronate (TG) of S.A. 50 Ci/mmole, and the NH_4^+ salt of [7-^{3}H]testosteron-17-yl-sulphate (TS) of S.A. 8 Ci/mmole.

Methods. Aqueous solutions of various mixtures of the above labelled compounds, containing a drop of methanol to facilitate solution of the free steroids, were applied to DEAE-Sephadex columns which were developed with linear concentration gradients of NaCl in H_2O as described elsewhere[2,4]. The mixing vessel in each case contained 400 ml H_2O and the donor vessel contained 400 ml of either 0.2, 0.3, 0.4 or 0.8 *M* NaCl. 5-ml fractions of eluate were collected and radioactivity was determined by liquid scintillation spectrometry[5].

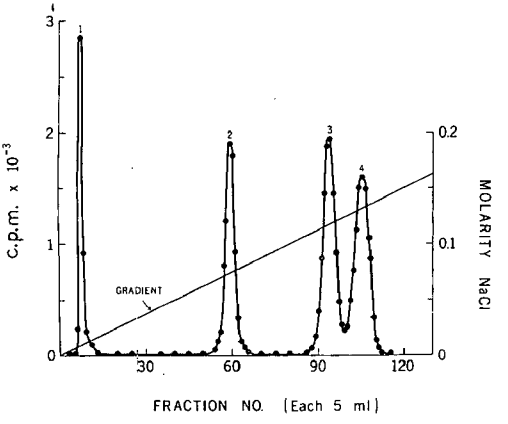

Fig. 1. DEAE-Sephadex chromatography in a linear gradient (0–0.2 *M* NaCl) of: peak 1, DHA or T; peak 2, DHAG or TG; peak 3, TS; peak 4, DHAS.

Results and discussion

In none of the gradients could DHA be separated from T nor could DHAG be separated from TG. However, the free steroids were easily separable from the glucosiduronates in all gradients. The sulphates, in turn, were well separated from the free steroids and glucosiduronates and, furthermore, DHAS and TS were separable from each other to varying extents in the different gradients. The greatest degree of separation between the two sulphates occurred in the 0–0.2 *M* NaCl gradient (Fig. 1) and in the 0–0.3 *M* gradient. The chromatographic mobilities of DHA and T were independent of the gradient employed but, as expected, behaviour of the conjugates was dependent on salt concentration. The sulphates of DHA and T were eluted in positions similar to certain oestrogen monoglucosiduronates[2,4] and considerably before oestrogen monosulphates[2].

It is of interest to note that when, in one experiment, employing the 0–0.2 *M* NaCl gradient, labelled oestrone and 17β-oestradiol were chromatographed along with ^{3}H-DHA, the oestrogens were eluted between fractions 12 and 20 while DHA appeared between fractions 6 and 11. It would appear from this and from the behaviour of the conjugates that the presence of a phenolic group allows a greater retention by the DEAE-Sephadex (see ref. 4).

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University Medical Clinic,
The Montreal General Hospital,
Montreal, Quebec (Canada)

R. HOBKIRK
S. DAVIDSON

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Chromatography of plant constituents on crosslinked dextrans in phenol–acetic acid–water mixtures

Chromatography in phenol–acetic acid–water (PAW) mixtures on Sephadex* of the G-series (dextran crosslinked with epichlorhydrin) has proved useful for fractionating polypeptides according to their primary-valence molecular weight, without complications from association of subunits or retardation by aromatic amino acid residues[1,2]. However, the Sephadexes of lower degree of crosslinking (G->75) do not swell suitably in PAW, so that this technique has not proved useful for polypeptides above mol. wt. 5,000. The lipophilic O-hydroxypropylated LH-Sephadex[3] seemed to offer hope for extending upwards the range of molecular weight which could be handled. At present, only LH-20 is commercially available. The late Dr. B. GELOTTE (of AB Pharmacia, Uppsala) kindly provided some experimentally prepared higher members of the LH series for us to test in PAW. These showed promise with proteins in the lower molecular-weight range (5,000–20,000). However, we found considerable sorptive retention of phaeophytin by LH-Sephadexes in PAW, whereas with G-Sephadexes phaeophytin behaves normally (see below). This deterred us from using LH-Sephadexes for fractionating peptides and proteins of leaf extracts in PAW (*cf.* refs. 4, 5). Recently PUSZTAI AND WATT[6,7] have found Biogel P-100** (crosslinked polyacrylamide) suitable for handling a wide molecular-weight range of peptides and proteins in PAW.

More recently, we have become interested in fractionating those neutral and weakly acidic leaf constituents which do not migrate electrophoretically in PAW[5,8]. Most of these behaved as low-molecular-weight substances on Sephadex G-75*** (see ref. 5). We accordingly did model experiments with a number of typical plant constituents to assess whether Sephadex G-25 in PAW would give a further fractionation according to molecular weight. The results (Fig. 1) show that, whereas the amino acid and lipid derivatives studied, as well as phaeophytin, behaved more or less 'normally', there was appreciable sorptive retention of polyphenolic and polycarboxylic compounds.

Similar sorption has been noted by other authors[9–23] with various Sephadexes used in aqueous solvents, and has been to some extent mitigated by addition of organic solvents. We had hoped that the high concentrations of phenolic and carboxylic groups in PAW would antagonize such sorption, but have, on the whole, been disappointed.

Materials studied

These are listed in the legend to Fig. 1. The phaeophytin arose spontaneously in PAW from a mixture of chlorophylls *a* and *b* (kindly provided by Dr. R. HILL, F.R.S.). N-acetyl-DL-alloisoleucine[24], N,N'-dibenzoyl-DL-lysine[25] and N-2,4-dini-

* AB Pharmacia, Uppsala, Sweden.

** Bio-Rad Laboratories, Richmond, Calif., U.S.A.

*** Recoveries of bound amino acids after fractionation were good. The excess recoveries mentioned[5] have now been traced to contamination of the water used for evaporation of the phenol, and did not arise from the Sephadex.

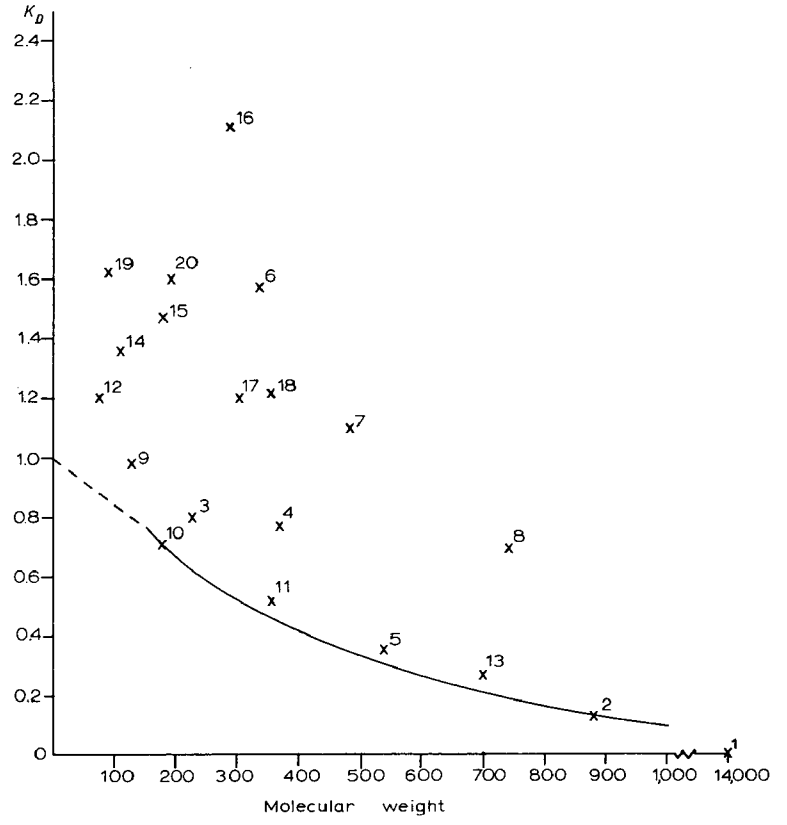

Fig. 1. Relation of K_D with molecular weight for: 1 = cytochrome *c*; 2 = phaeophytin; 3 = N-2,4-dinitrophenylethanolamine; 4 = N$^{\alpha}$-2,4-dinitrophenyl-L-tryptophan; 5 = β-carotene; 6 = quercetin; 7 = quercitrin; 8 = robinin; 9 = L-pyrrolidonecarboxylic acid; 10 = N-acetyl-DL-alloisoleucine; 11 = N,N'-dibenzoyl-DL-lysine; 12 = glycine; 13 = phosphatidylethanolamine; 14 = pyrocatechol; 15 = caffeic acid; 16 = catechin; 17 = nordihydroguaiaretic acid; 18 = chlorogenic acid; 19 = oxalic acid; 20 = citric acid. The curve represents the function: $K_D = 2.60 - 0.84 \log_{10}$ (mol. wt.) for mol. wts. 150–1000. The distance of each point above the curve serves as a measure of the sorption of each substance by the gel matrix.

trophenylethanolamine[26] were synthesised in our laboratory. The others were obtained commercially.

Procedure

Gel beds (9.4 cm^3) were prepared[4] using Sephadex G-25 (fine: Lot No 2726 or To 4150) in phenol–acetic acid–water (1:1:1, w/v/v). The elution volumes (V_e) for the coloured compounds (1–8) were estimated by eye for emergence of the centres of the zones. For the colourless compounds, small fractions were collected and the zone centres located by spray tests of aliquots of these spotted on filter paper as follows: RYDON AND SMITH reaction[27] (9); 0.1% (w/v) triketohydrindene hydrate in water-saturated butan-1-ol (12, 13); 0.4% (w/v) ferric chloride in dilute aqueous HCl (14–18); 0.04% (w/v) Bromocresol Green in 95% (v/v) aqueous ethanol (19, 20). Com-

pounds 10 and 11 gave poor RYDON AND SMITH reaction, and were located by evaporating aliquots of the fractions to dryness and titrating (Bromothymol Blue) against 0.01 N $Ba(OH)_2$.

The distribution coefficient (K_D) for each compound was calculated using eqn. A[28].

$$K_D = \frac{V_e - V_0}{V_i} \tag{A}$$

where V_0 is the void volume (elution volume for cytochrome c) and V_i the volume of solvent within the grains. V_i was calculated from eqn. B.

$$V_i = V_t - V_0 - a\,v_g \tag{B}$$

where V_t is the total bed volume, a is the wt. of dry Sephadex used (g) and v_g the partial specific volume of the Sephadex, assumed to be 0.6 $cm^3 \cdot g^{-1}$ (see ref. 29).

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Agricultural Research Council's Food Research Institute, Colney Lane, Norwich, NOR 70F (Great Britain)

W. M. LAIRD
R. L. M. SYNGE

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CHROM 5108

Identification of organic compounds *

LXXIV. Paper electrophoresis of acid anthraquinone dyes

In a previous communication of this series[1], we studied the paper chromatographic behaviour of acid anthraquinone dyes and found the optimum conditions for their separation and identification. The present paper deals with the application of paper electrophoresis to the same problem. The suitability of paper electrophoresis or thin-layer electrophoresis respectively, to the separation and identification of different types of acid dyes has been studied by several authors[2–8], but no special attention has been directed to a systematic study of anthraquinone dyes. It was our endeavour to find the optimum experimental conditions for the paper electrophoretic separation of these dyes and to study the relationship between the electrophoretic behaviour of these dyes and their structure which could be taken advantage of in elucidating the structure of unknown dyes.

Experimental

Dyes. All dyes under investigation were either commercial products of known structure or pure model compounds from our collection of standards. They were applied in the form of 0.5–1% solutions in aqueous pyridine.

Apparatus. The apparatus used was of the type with a free hanging paper strip in a moist chamber designed by the Czechoslovak Academy of Sciences.

Paper. Whatman No. 3 MM paper strips (27 × 35 cm) were used throughout all experiments.

Electrolytes. For buffer solution I (pH 9) 518 ml of 0.1 *M* KH_2PO_4 in 50% ethanol were mixed with 482 ml of 0.1 *N* sodium hydroxide in 50% ethanol. For buffer solution II (pH 4), 350 ml of 0.1 *M* Na_2HPO_4 in 50% ethanol were mixed with 750 ml of 0.1 *M* citric acid in 50% ethanol. The potential was 10 V/cm and the developing time was 5–6 h.

Results and discussion

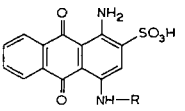
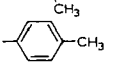
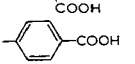
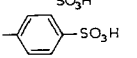
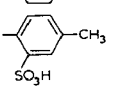
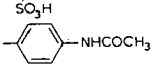
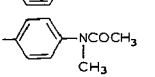
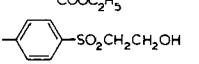
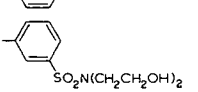
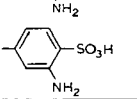
During our preliminary experiments carried out in both alkaline and acid media, the alkaline medium was found to be more suitable. When using aqueous solutions of electrolytes, however, we observed the formation of elongated streaks. The addition of ethanol to the electrolyte solution caused a considerable decrease of the mobility of the dyes, but the spots became very sharp. The positive influence of the presence of an organic solvent in the electrolyte solution on the quality of electropherograms of acid dyes has been observed by several authors. Ethanol[6], propylene glycol[4], and formamide[9] have been used.

Optimum results have been obtained using the phosphate buffer (pH 9) containing 50% ethanol. The electrophoretic mobilities (relative to compound I) of the compounds under investigation are summarised in Table I. The migration distance of compound I was 55–60 mm within 6 h. The difference of 0.2 in the relative mobilities was sufficient for the distinct separation of two compounds. The compounds in Table I can be divided into five groups according to their structures: I–XXI,

* Part LXXIII: Sborník VŠChT, Pardubice, in press.

TABLE I

ELECTROPHORETIC MOBILITIES OF ACID ANTHRAQUINONE DYES RELATIVE TO COMPOUND I
Electrolyte: buffer solution, pH 9; potential, 10 V/cm; time, 6 h.

Compound		*Mobility*
I–XX		
I	R = H	1.00
II	R = 	0.91
III	R = 	0.99
IV	R = 	1.04
V	R = 	1.04
VI	R = 	1.04
VII	R = 	1.46
VIII	R = 	1.60
IX	R = 	1.51
X	R = 	1.66
XI	R = 	1.66
XII	R = 	1.66
XIII	R = 	1.65
XIV	R = 	0.70
XV	R = 	0.82
XVI	R = 	0.86
XVII	R = 	0.82
XVIII	R = 	0.72
XIX	R = 	0.86
XX	R = 	1.51

(continued on p. 438)

TABLE I *(continued)*

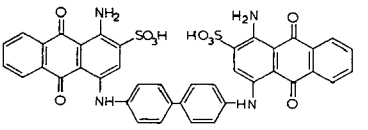
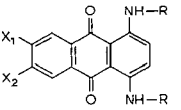
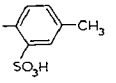
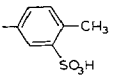
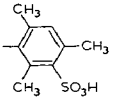
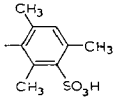
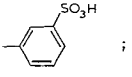
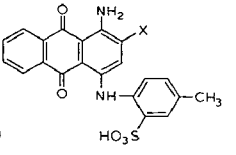
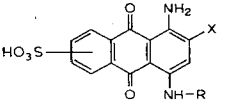
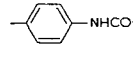
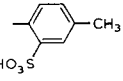
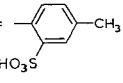
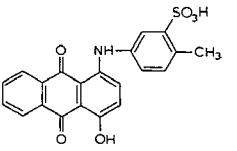
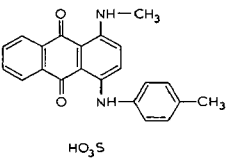
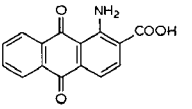
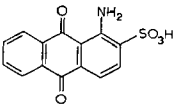
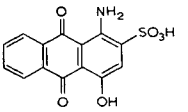
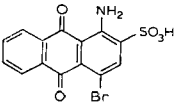
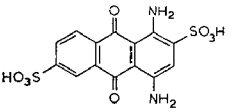
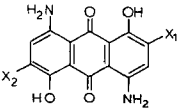
XXI		0.22
XXII–XXVI		
XXII	R =  ; $X_1 = X_2 = H$	1.32
XXIII	R =  ; $X_1 = X_2 = H$	1.34
XXIV	R =  ; $X_1 = X_2 = H$	1.38
XXV	R =  ; $X_1 = X_2 = Cl$	1.49
XXVI	R =  ; $X_1 = H$; $X_2 = SO_3H$	1.83
XXVII–XXVIII	 XXVII X = CH_3 XXVIII X = Br	0.73
XXIX–XXXI		0.75
XXIX	R =  ; X = SO_3H	1.08 1.22
XXX	R =  ; X = CH_3	1.22 1.42
XXXI	R =  ; X = Br	1.24 1.44

TABLE I *(continued)*

XXXII		0.98
XXXIII		0.83
XXXIV		1.09
XXXV		1.22
XXXVI		1.13
XXXVII		1.17
XXXVIII		1.65
XXXIX-XLI		
	XXXIX $X_1 = X_2 = H$	0.00
	XL $X_1 = SO_3H$; $X_2 = H$	0.62
	XLI $X_1 = X_2 = SO_3H$	0.86

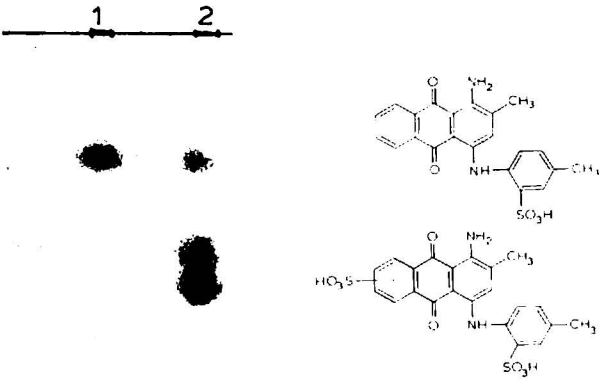


Fig. 1. Electropherogram of acid anthraquinone dyes. 1 = Kiton Fast Blue CR (CI Acid Blue 47); 2 = Acilan Fast Blue REX (CI Acid Blue 49). Electrolyte, buffer solution I (pH 9); Potential, 10 V/cm; time, 6 h.

XXII–XXVI, XXVII–XXXI, XXXII–XXXVIII, and XXXIX–XLI. The mobility of the individual compounds within each group is dependent predominantly on the number of sulpho and carboxy groups, the presence of each of these groups causing a considerable increase in mobility. The substitution of the first member of each group by other functional groups is usually of only slight influence. If the buffer solution II (pH 4) is used, the carboxy groups of the dyes VII–IX containing one sulpho and one carboxy group are no more dissociated which results in a considerable decrease of the mobility of the dyes. Their mobility is close to that of the monosulfo derivatives under these conditions. Thus, the detection of the carboxy group in a dye of unknown structure is possible by comparing its electrophoretic behaviour at pH 5 and 9. The most successful result of our study has been the distinct separation of the isomeric 2,6- and 2,7-disulpho derivatives (XXIX–XXXI) demonstrated in Fig. 1. This fact is in accordance with the observation of FRANC[10] on the resolution of the isomeric 2,6- and 2,7-anthraquinone disulphonic acids in alkaline medium, the 2,7-isomer showing faster mobility. Using paper chromatography neither the isomeric acids[11] nor the isomeric dyes[1] could have been resolved.

The results obtained have shown that efficient separation of acid anthraquinone dyes can be achieved by paper electrophoresis in alkaline medium using buffer solutions containing ethanol, particularly in cases when mixtures of dyes after sulphonation containing derivatives of different sulphonation degree and isomeric disulpho derivatives are to be analysed. The differentiation of carboxy groups and sulpho groups is also possible.

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Research Institute of Organic Syntheses, Pardubice-Rybitví (Czechoslovakia)

J. GASPARIČ

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Separation of phenolic and indolic acids from untreated urine

The author has described methods[1–4] for chromatographing crude or unprocessed samples. These have been based on the following principles:

(1) Using multiple runs, it is possible to get compact spots, even in the presence of contaminants. The routine type of multiple run, however, does not favour the separation of substances having high R_F values[5]. This drawback is overcome by using multiple runs of increasing running times[4]. Selection of the solvent is important as the influence of the contaminants in distorting the separations varies with the solvent.

(2) In the case of aromatic, heterocyclic and less polar aliphatic compounds, it is possible to find solvents which will separate these compounds well from water-soluble contaminants. Once separated, the compounds are not influenced by the contaminants in subsequent runs[1,2]. If the spots are elongated in the first run, another run in the same direction results extremely well in compact spots[2].

(3) If a sample is applied on the paper in the form of a large rectangular spot, the concentration of the contaminants per unit area is rather small and the compounds may separate out as compact bands during a run. These bands can be made to form compacted spots before the actual two-dimensional separation[3].

The method described in this paper also demonstrates the practicability of the principles outlined above. In this method, urine corresponding to 0.8 mg of creatinine is applied, as such, on Whatman 3MM paper for chromatographic separation of phenolic and indolic acids, avoiding the entire extraction step.

Experimental

Urine corresponding to 0.8 mg of creatinine is applied as a 5.5 × 1.0 cm rectangular spot in one corner of a sheet (34 × 28 cm) of filter paper (Whatman 3MM). The 5.5-cm long edge of the spot lies parallel to the 34-cm long edge of the paper and 1.5 cm distant from it. The 1-cm long edge of the spot is 2.5 cm away from the 28-cm long edge of the paper.

In order to spread out phenolic and indolic acids from the rectangular spot, 3 consecutive ascending runs are employed along the breadth of the paper in the solvent system ether–xylene–formic acid–methanol–water (600:200:80:10:6). In each of the 3 runs, the solvent rises (in 10 min) to a line drawn 8 cm from and parallel to the upper edge of the rectangular spot. This helps to separate phenolic and indolic acids as compact bands from aliphatics and salts which remain behind in the rectangular spot.

Compacting the bands into spots. For phenolic acid chromatograms, compacting the bands into spots is done using 95% ethanol in an ascending run (45 min) in an uncovered chromatographic chamber placed under a fan. The run is made along the length of the paper, after cutting away the spot rectangle from the paper. The solvent front slowly advances to and stays in line with the upper 1-cm edge of the rectangular hole. For this run, an all-glass chamber (40 (height) × 21 × 16 cm) is being used in this laboratory. A petri dish containing 50 ml of 95% ethanol is placed 28 cm above the bottom of the chamber. Ordinary filter paper is supported along the walls of the chamber and is immersed in ethanol which keeps the filter paper soaked as well as ensures that the chamber is saturated with vapour between the compacting runs. This

prevents (during the compacting run) too much evaporation of alcohol from the solvent in the dish or from that ascending on the paper. Use of the fan may be required to stop the ascent of solvent at the desired height.

For indolic acids, the solvent system ethyl methyl ketone–pyridine–water (25:5:20) is used instead of 95% ethanol. The migration of this solvent cannot be stopped at the desired height under the conditions mentioned above, but the rate of ascent can be reduced considerably by using the fan. 20 to 30 min may be needed

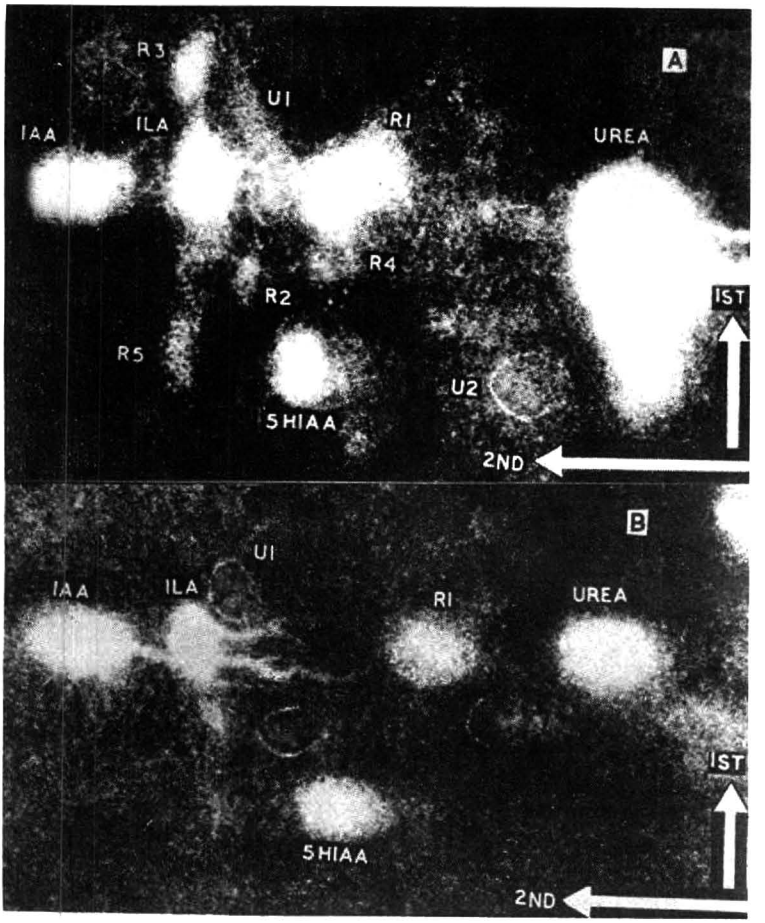

Fig. 1. Segments of two-dimensional (ascending) chromatograms for urinary indolic acids on Whatman 3MM paper. (A) Chromatogram prepared according to the present method by spotting urine corresponding to 0.8 mg of creatinine, as such, on paper. (B) Chromatogram from extract[6] (same extract as for phenolic acids), corresponding to 0.8 mg of creatinine, of the same urine sample as used for the above chromatogram. Ehrlich reagent is used for staining the two chromatograms. The solvent systems are isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water–ammonia (4:2:2:2:1) and ether–xylene–formic acid (85%)–methanol–water (500:300:80:10:6), for the first and the second runs, respectively. Abbreviations for spots are as follows: IAA = indolylacetic acid; ILA = indolyllactic acid; 5 HIAA = 5-hydroxyindolylacetic acid; U1 and U2 = unidentified indolic compounds (stain purple-blue with Ehrlich reagent); R1, R2, R3, R4, R5 = unidentified compounds (stain red with Ehrlich reagent).

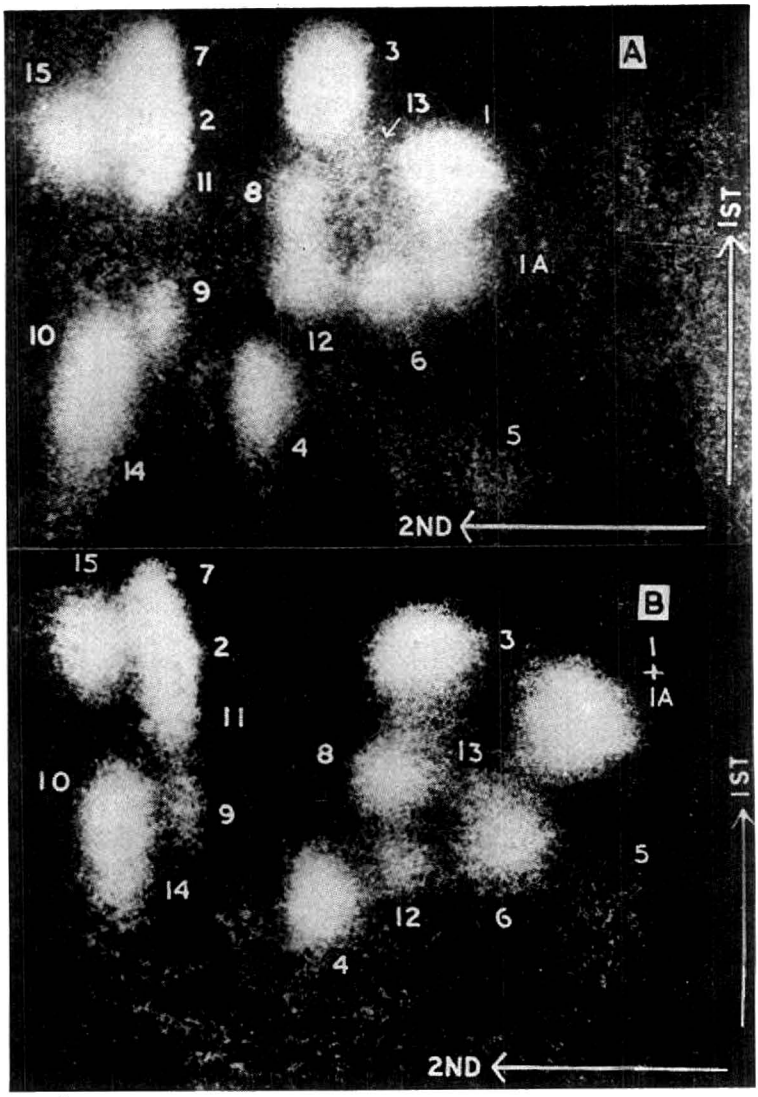

Fig. 2. Segments of two-dimensional (ascending) chromatograms for urinary phenolic acids on Whatman 3MM paper. (A) Chromatogram prepared according to the present method by spotting urine corresponding to 0.8 mg of creatinine, as such, on paper. (B) Chromatogram for extract[6] corresponding to 0.8 mg of creatinine, of the same urine sample as used for the above chromatogram. *p*-Nitroaniline reagent is used to stain the two chromatograms. Solvents are isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water–ammonia (4:2:2:2:1) and ether–xylene–formic acid (85%)–methanol–water (500:300:80:10:6), for the first and the second runs, respectively. Numbers indicate phenolic acid as follows: 1 = *m*-hydroxyhippuric acid; 1 A = unidentified spot (stains red); 2 = *p*-hydroxyphenylacetic acid; 3 = *m*-hydroxyphenylhydracrylic acid; 4 = unidentified spot (stains orange-yellow); 5 = *p*-hydroxyhippuric acid; 6 = 3-methoxy-4-hydroxymandelic acid; 7 = *m*-hydroxyphenylacetic acid; 8 = unidentified spot (stains orange-yellow); 9 = *o*-hydroxyhippuric acid; 10 = *p*-hydroxybenzoic acid; 11 = homovanillic acid; 12 = 5-hydroxyindolylacetic acid; 13 = 3-methoxy-4-hydroxyphenylhydracrylic acid; 14 = vanillic acid; 15 = *m*-hydroxybenzoic acid.

for the solvent to ascend to the desired height. In this case, therefore, two (30 min each) or three (20 min each) consecutive runs are needed to compact indolic acid bands into spots.

Runs for separating the spots. The paper is then subjected to 2 consecutive runs (1 h, 15 h) in the solvent system isopropyl alcohol–*n*-butyl alcohol–*tert.*-butyl alcohol–water–ammonia (4:2:2:2:1), in the same direction as that of the compacting run. Before these runs, it is advisable to cut away a parallel strip of the paper 5 cm from the lower edge. The lower segment, not containing phenolic or indolic acids, is discarded.

The final run (3 h) in the solvent system ether–xylene–formic acid (85%)–methanol–water (500:300:80:10:6), is at right angles to the direction of the latter run.

Phenolic and indolic acids are revealed by staining the chromatograms with *p*-nitraniline and Ehrlich reagent, respectively[6].

Results and discussion

Good separations obtained by the present method (see Figs. 1 and 2) demonstrate the practicability of the principles discussed in the Introductory section. These principles have thus been used for chromatography of amino acids[1,3,4], phenolic amines[2], phenolic acids and indolic acids, and the scope of application to other fields is ample in separations both on paper and on thin layers.

Recoveries of different phenolic and indolic acids in the present procedure are comparable to those obtained in the conventional procedures (Figs. 1 and 2). When indolic acid chromatograms are stained with the Ehrlich reagent, some unidentified red spots are seen besides the known indolic acid spots[7]. These red spots are more abundant in chromatograms obtained by the present method. Phenolic acid chromatograms obtained by the present procedure, when compared with the conventional ones (prepared by chromatographing an extract[6]), the following features are noted:

(1) By the present method there is better separation of spots in the first migration (Fig. 2). The spot of *p*-hydroxyhippuric acid is well separated from other spots and the spot of 3-methoxy-4-hydroxyphenylhydracrylic acid is well separated from that of 3-methoxy-4-hydroxymandelic acid.

(2) An unidentified spot (1 A in Fig. 2) is well separated from the spot of *m*-hydroxy hippuric acid. The two spots overlap in the conventional chromatograms.

This study is partly supported by a research grant from the Indian Council of Medical Research. Mr. GROVER AND Mr. RAVINDER are thanked for preparing the illustrations in this paper.

Department of Physiology, Medical College, Rohtak (India) A. S. SAINI

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CHROM. 5096

Thin-layer chromatography of mycophenolic acid and related compounds

Mycophenolic acid is an antibiotic that can be isolated from penicillium cultures[1,2]. Recent reports of antiviral and antitumor properties[3] of mycophenolic acid have renewed interest in the compound. Such interest has led to the total synthesis of mycophenolic acid by BIRCH AND WRIGHT[4] and to the development of a quantitative gas chromatographic assay by GAINER AND WESSELMAN[5]. Several reports are in the literature which include the chromatography of mycophenolic acid[6–9], but much of the work has involved general screening of antibiotics and has not been specific for mycophenolic acid. WILLIAMS *et al.*[10] were the first to report thin-layer chromatographic (TLC) data for mycophenolic acid along with their work with paper chromatography (PC).

This paper reports the results of extensive evaluation of TLC systems for mycophenolic acid. In addition, compounds related to mycophenolic acid are included in this study.

Experimental

Chemicals. All chemicals were of reagent grade and were used without further purification.

Developing systems. All solvents were mixed on a v/v basis immediately before being put into the developing chamber.

Spray reagent. A 1% solution of $FeCl_3 \cdot 6H_2O$ in methanol (w/v) was used.

Equipment. Pre-coated 250-μ Silica Gel F_{254} and pre-coated 0.1-mm Cellulose Powder MN 300 F_{254} thin-layer plates were supplied by Brinkmann Instruments, Inc. Micropipets (Microcaps®) from Drummond Scientific Company were used for making sample applications.

TABLE I

SOLVENT SYSTEMS FOR TLC ON SILICA GEL PLATES

No.	*Solvent systems*	*Development time (min)*
1	Chloroform–methanol (9:1)	52
2	Benzene–ethyl acetate–acetic acid (80:20:5)	85
3	*n*-Pentylacetate–*n*-propyl alcohol–acetic acid–water (4:2:1:1)	142
4	Benzene–*n*-propyl alcohol–acetic acid (9:6:1)	98
5	Acetonitrile–water (4:1)	53
6	Chloroform–acetic acid (20:1)	55
7	Benzene–acetic acid (20:1)	48
8	Ethyl acetate–acetic acid (20:1)	54
9	Ethyl ether–acetic acid (20:1)	72
10	Methylene chloride–acetic acid (20:1)	55
11	Petroleum ether–ethyl ether–acetic acid (80:30:5)[a]	50
12	Petroleum ether–ethyl ether–acetic acid (80:30:5)[b]	170

[a] Plate developed three times in this system.
[b] Plate developed once; no liner was used in the development tank.

Procedure. The TLC chamber was lined with Whatman No. 1 filter paper and allowed to equilibrate with the developing solvents overnight in order to achieve complete equilibrium before use, unless otherwise stated. A sample of each compound was dissolved in methanol at a concentration of 10 mg/ml, and 10 μl of this solution were spotted on a thin-layer plate, using a micropipet. The plate was developed at room temperature, allowing the solvent front to move 15 cm from the point of application and then removed from the chamber and dried at room temperature. The plate was then visualized under a UV lamp in both the short (254 nm) and long (366 nm) wavelength regions unless otherwise specified.

Results and discussion

Seven different solvent systems were examined for use in the chromatography of monosodium mycophenolate on cellulose-coated thin-layer plates. The results of this investigation indicated that mycophenolic acid and related compounds cannot be satisfactorily chromatographed on cellulose if simple solvent systems are employed.

The TLC results obtained by using silica gel plates are quite meaningful. Table I shows the solvent systems utilized, along with corresponding development

TABLE II

$R_F \times 100$ VALUES ON SILICA GEL PLATES IN VARIOUS SOLVENT SYSTEMS

Solvent systems are as expressed in Table I. Compounds: A = Mycophenolic acid; B = ethyl mycophenolate; C = methyl mycophenolate; D = normycophenolic acid; E = cyclic acid hydrolysis product with the assigned name 3,4-dihydro-5-methoxy-2,6-dimethyl-9(7H)-oxo-2H-furo <3,4-h>benzopyran-2-propionic acid.

Solvent system	*Compound*				
	A	*B*	*C*	*D*	*E*
1	27	70	70	17	17
2	45	68	64	40	25
3	67	71	71	65	57
4	77	81	81	76	70
5	53	79	79	47	47
6	34	49	47	29	16
7	11	21	20	9	2
8	55	65	64	51	45
9	67	83	80	63	39
10	43	54	54	38	25
11	43	67	58	34	11
12	41	65	56	32	10

times. In Table II, the R_F values determined for the compounds studied are listed, along with the appropriate solvent systems. Monosodium mycophenolate and disodium mycophenolate behaved similarly to mycophenolic acid in all solvent systems investigated.

The solvent system which gives the best overall separation of the compounds studied was utilized in developing the plate photographed in Fig. 1. Ferric chloride is specific for phenolic hydroxy groups, so the lack of color for the spot in lane E after spraying the plate with ferric chloride was a good indication that acid hydrolysis of mycophenolic acid resulted in cyclization involving the hydroxy group. The fact that

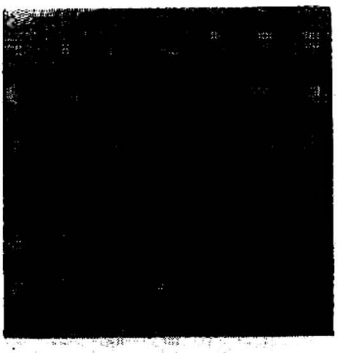

Fig. 1. Thin-layer chromatogram of mycophenolic acid and related compounds. Solvent system No. 12, Table I. Lanes correspond to compounds A–E, Table II.

the two related esters, ethyl mycophenolate and methyl mycophenolate, can be quantitatively separated from one another by TLC gives credence to the method.

All of the systems investigated are satisfactory for identification of mycophenolic acid by comparing R_F values of main spots in sample lanes with the R_F value of the spot in the reference standard lane. However, some of the systems are suitable for following stability of mycophenolic acid in addition to control.

Analytical Chemical Development Department, F. E. GAINER
Eli Lilly and Company, R. L. HUSSEY
Indianapolis 46206, Ind. (U.S.A.)

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CHROM. 5093

Chromatographic techniques using liquid anion exchangers

IV. HF systems*

Reversed-phase extraction chromatography on supports impregnated with liquid anion exchangers is used with solutions of many inorganic acids as eluants. Most frequently, paper and thin-layer techniques are employed, but with HF systems only paper chromatographic (PC) results have been published[1]. In the present communication, we report on thin-layer chromatography (TLC) with HF-containing eluants. Four exchangers were selected to represent primary (Primene), secondary (Amberlite LA-2) and tertiary (Alamine) amines, and quaternary ammonium salts (Aliquat). Elution was performed with 0.1–20 *N* aq. HF solutions.

For reversed-phase TLC, glass plates are normally covered with silica gel impregnated with a liquid exchanger, but silica gel and glass are unsuitable for fluoride systems. It was found that cellulose powder on polyvinyl chloride sheets is a very useful support for this class of separations.

Materials and methods

Aliquat 336 is a methyl-tri-*n*-alkylammonium chloride with an average of 27–33 carbon atoms and a mean mol. wt. of 475; Alamine 336 is a C_8–C_{10} straight-chain alkylamine with a tertiary amine content of minimal 90% and a mean mol.wt. of 392. Both exchangers are manufactured by General Mills (Kankakee, Ill., U.S.A.). Amberlite LA-2 is a dodecyltrialkylmethylamine with a mean mol. wt. of 374; Primene JM-T is a mixture of a relatively large number of trialkylmethylamines (18–24 carbon atoms); the middle fractions having a mean mol. wt. of 300–310 were isolated by repeated vacuum distillation[2]. The latter two amines are supplied by Rohm and Haas (Philadelphia, Pa., U.S.A.).

A 0.1 *M* solution of the amines in chloroform is equilibrated with an equal volume of 1 *N* HF; Aliquat is converted into the fluoride salt by shaking its (0.1 *M*) solution with an equal volume of a 0.3 *M* AgF + 0.7 *N* HF solution. It is of interest to note that repeated equilibration with 1 *N* HF does not lead to quantitative conversion of the Aliquat chloride. Use of an Aliquat (F, Cl) mixture so obtained in chromatography causes the occurrence of too high R_F values and streaking of the spots, especially at low HF normalities. 100 ml of the solution containing liquid anion exchanger are thoroughly mixed with 30 g of cellulose powder (Avicel TG 104, F. M. C., Marcus Hooke, Pa., U.S.A.). PVC sheets (0.5 mm thickness) are cut into plates of appropriate size (7 × 2.5 cm) and these are dipped into the cellulose slurry. Superfluous material is wiped off the back and a small margin is made along the edges. 2–6 spots are applied to each plate. Sample solutions are prepared by dissolving commercially available oxides or salts in 1 *N* HF. (With the metal ions, care was taken to avoid the presence of strongly complexing foreign anions.)

Ascending chromatography is carried out for a 3.0-cm run, in perspex jars. Developing times as a rule are between 3 and 5 min. The (impregnated) cellulose adheres well to the PVC carrier material. As the only exception, Primene-treated cel-

* For part III of this series, see ref. 3.

lulose sometimes flakes off when developing with 0–2 *N* HF. Details regarding the experimental technique and the visualisation procedures may be found in refs. 3 and 4.

Results and discussion

Of the 28 ions studied, Mn(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and In(III) show slight sorption ($R_F > 0.8$) over the whole HF concentration range investigated. These results agree with those obtained by PRZESZLAKOWSKI[1] on paper impregnated with anion exchanger and by FARIS[5] in a study of the adsorption of elements from aqueous HF solutions by a strong base resin exchanger. It may be assumed, therefore, that the ions in question do not form anionic metal–fluoro complexes or only form very weak ones.

For all other ions, R_F *vs.* *N* HF plots are presented in Fig. 1. Streaking of the spots occasionally occurs at low HF concentrations; Ti(IV) always forms streaking spots below 1 *N* HF. The combined data, which generally show good agreement with

TABLE I

QUALITATIVE SEPARATIONS IN LIQUID ANION EXCHANGER–HF SYSTEMS

Ions to be separated				*Exchangers*	*N HF*
Mo	As	Ge		Primene	4
B	Ti	Al		LA-2	3
Nb	W	Fe		LA-2	12
Re	W	Mo		LA-2	6–8
U	V	Fe		LA-2; Alamine	4
Ta	W	V		LA-2; Alamine; Aliquat	8–12
Re	Te	V	Se	LA-2; Alamine; Aliquat	4–6

the paper chromatographic results of PRZESZLAKOWSKI[1], indicate that all four exchanger–HF systems investigated may well be employed in practice. Several interesting separations may be read from the figure. Examples are given in Table I.

It is interesting to note that for all ions investigated, sorption decreases with an increase of the aqueous HF concentration. The conclusion holds for both liquid and resin anion exchangers. This characteristic sorption *vs.* concentration curve has been explained by the formation of extractable anionic fluoro complexes at relatively low aqueous HF concentrations and non-extractable higher metal–fluoro complexes at higher acid concentrations (*cf.* ref. 6). However, for a more plausible explanation, we can point to the competitive extraction of hydrofluoric acid itself.

As is well known from several liquid–liquid extraction studies with tertiary amines[7,8], HF is easily extracted beyond the amount of acid necessary to neutralise the amine; ratios of $R_3N:HF = 1:2–4$ are obtained for 1–10 *N* HF. The extraction of excess acid may be interpreted in terms of the formation of HF_2^-, and subsequently higher hydrogen-bonded anions. Research in our laboratory has demonstrated that the same phenomenon occurs with primary and secondary amines, and also with quaternary ammonium salts; *i.e.*, we may write (R = alkyl; R′ = alkyl or H):

$$RR'_2N_{org.} + HF_{aq.} \rightleftharpoons RR'_2NH^+F^-_{org.} \quad (1)$$

for the neutralisation of the amines, and

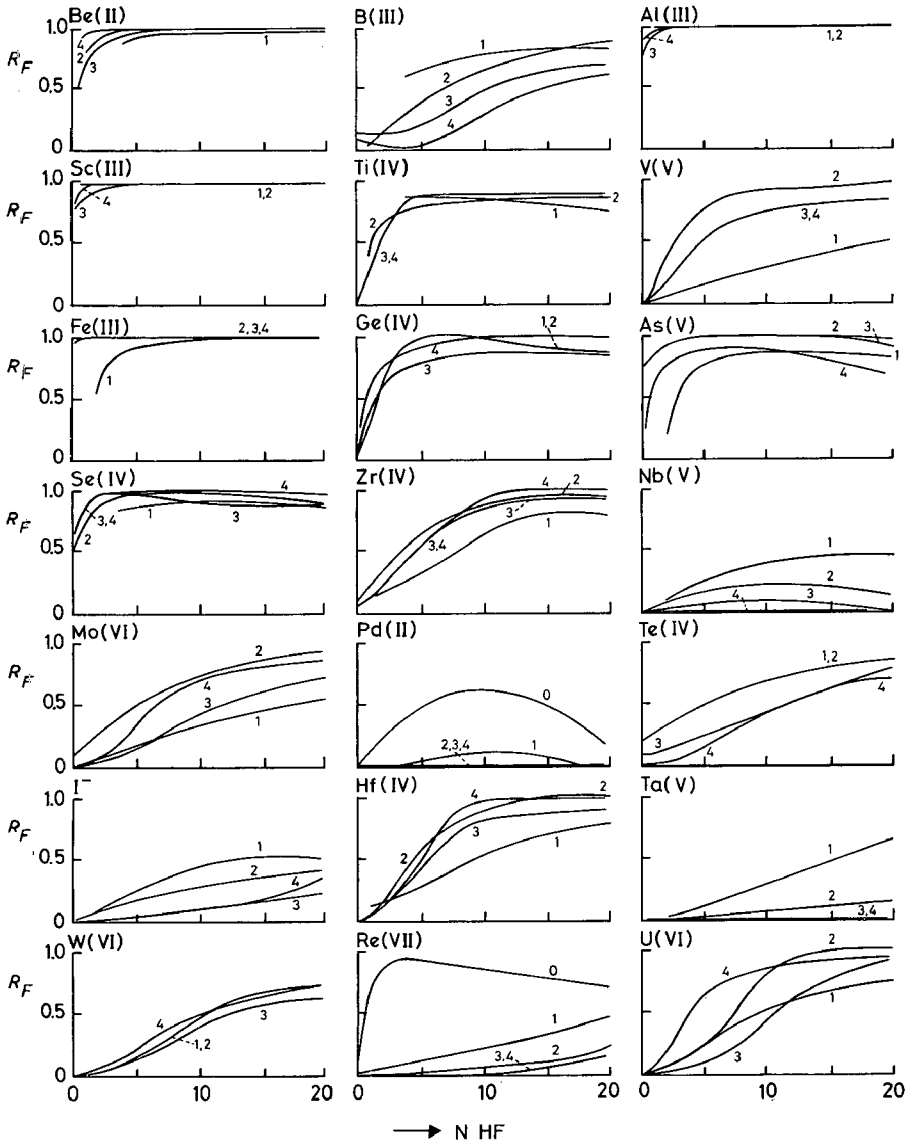

Fig. 1. R_F *vs.* N HF spectra for 21 ions, using cellulose impregnated with Primene (1), Amberlite LA-2 (2), Alamine 336 (3) or Aliquat 336 (4), and cellulose not treated with an anion-exchanger (0).

$$RR'_3N^+F^-_{org.} + n\ HF_{aq.} \rightleftharpoons RR'_3N^+H_nF_{n+1}^-{}_{org.} \quad (2)$$

for the uptake of excess HF by both alkylamine·HF and quaternary ammonium fluoride salts.

The $H_nF_{n+1}^-$ entity is more readily extractable than the relatively small, simple F^- anion, and so competes much better than the latter with the extraction of the metal–fluoro, etc. anions. The formation of $H_nF_{n+1}^-$ will thus considerably decrease the sorption of the elements investigated.

An analogous phenomenon, though to a much lesser extent, occurs in chloride systems. Here, the formation and preferential extraction of HCl_2^- at high aqueous HCl concentrations also causes the desorption of many metal ions, as is well known from both chromatography and extraction[9].

In order to determine the effect of phenomena such as hydrolysis and adsorption to the support, chromatography is also carried out on non-impregnated cellulose powder. R_F values of 0.7–0.8 or higher, consistently found for all ions with the exception of Pd(II) and Re(VII) (see Fig. 1), show that the above-mentioned effects hardly play a role. In conclusion, for the elements collected in Fig. 1, sorption is predominantly determined by anion exchange. Thus, the equilibria governing the reversed-phase extraction chromatography in fluoride systems may be represented by:

$$p\ RR'_3N^+H_nF_{n+1}^-{}_{org.} + p\ H^+_{aq.} + X^{p-}_{aq.} \rightleftharpoons (RR'_3N^+)_pX^{p-}_{org.} + p(n+1)\ HF_{aq.} \quad (3)$$

with $n \geqslant 0$; X^{p-} represents metal–fluoro anions such as FeF_6^{3-} and TaF_7^{2-}, and anions of various other types such as *e.g.* I^- and ReO_4^-.

As a last point of interest, we may discuss the sorption strength of the four liquid anion exchangers. For all ions tested the sorption on Alamine-treated cellulose is higher than on Amberlite-impregnated cellulose, although the differences are sometimes very small. This observation confirms the results of PRZESZLAKOWSKI[1], who finds the order of sorption tertiary (TnOA) > secondary (Amberlite LA-2). However, somewhat surprisingly, with the quaternary exchanger Aliquat and the primary amine Primene, controversial results are obtained. As can be observed from the figures for some ions, *e.g.* B(III), Nb(V), Te(IV), Ta(V), Re(VII) and I^-, the order tertiary $\simeq$ quaternary > secondary > primary, commonly found with monobasic inorganic acids[3,10], is obtained. However, with others, *e.g.* V(V), Fe(III), Mo(VI), Zr(IV) and Hf(IV), the order of increasing sorption is properly speaking reversed: primary > secondary $\simeq$ tertiary $\simeq$ quaternary. Lastly, for a few ions, *e.g.* Ti(IV), Ge(IV), W(VI) and U(VI), a distinct sorption sequence of the exchangers cannot be given at all.

Since only a few systematic investigations have been carried out on the extraction of metal ions from fluoride solutions, it is not easy to draw parallels, if existent, between chromatography and extraction. It can be noted, however, that in the case of Fe(III), SHEVCHUK *et al.*[11] have observed that the extraction efficiency of primary amines clearly surpasses that of all other exchangers tested. This is possibly due to steric factors; since three amine·H^+ cations must be attached to the FeF_6^{3-} anion, the sorption/extraction by means of primary amines will be favoured over that involving more bulky, *i.e.* secondary, tertiary or quaternary, exchangers. This hypo-

thesis has been stated earlier to explain the good extractability of highly charged complex anions by primary amines in *e.g.* cyanide and oxalate[12,13], sulphate[14], and phosphate[15] systems, and found valid for both extraction and chromatography. The present chromatographic experiments confirm the hypothesis both by yielding the "normal" sorption sequence for the mononegative anions BF_4^-, ReO_4^- and I^-, and the reversed order for FeF_6^{3-}. However, in the absence of further data on the nature of the extracted species and in lack of a complete understanding of the factors causing the occurrence of the normal sorption sequence with singly and doubly charged anions, it cannot be concluded yet that steric effects are the only cause of the (partial) reversal of the normal order of sorption strength.

Free Reformed University,
Chemistry Laboratory,
Amsterdam (The Netherlands)

U. A. TH. BRINKMAN
P. J. J. STERRENBURG
G. DE VRIES

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Book Reviews

CHROM. 5033

Chromatographische und mikroskopische Analyse von Drogen, edited by E. STAHL, Gustav Fischer Verlag, Stuttgart, 1970, 184 pp., price D.M. 28,—.

The long experience of the editor and his co-workers in his laboratory in both pharmacognosy and thin-layer chromatography has resulted in a very practical guide for the identification and evaluation of botanical drugs. The book carries the sub-title *A practical supplement to the European pharmacopœias* and this is undoubtedly true. Chapter II, the main part of the book, gives a lot of valuable practical information which cannot be found in the present pharmacopœias: chemical structure of the active components, clear drawings and description of the characteristic parts to be seen under the microscope and an accurate outline for chromatographic analysis. Although photographs are not given, chromatograms are reproduced as coloured drawings giving both the colours of the spots and that of the background. With the aid of a set of sixty different colours the reproductions look quite satisfactory. Exact R_F values are not given but are given as guide values in the form of "R_F brackets", *i.e.* 30–40, 75–80, etc. Also relative humidities are given under which these guide values can be expected (50% for all experiments described). Although very correct in principle, this can give rise to confusion as no explanation has been given of the effect of the relative humidity on the separation. Furthermore, it cannot be expected that all users of the book will be able to perform their work at 50% R.H. It would have been better to state the humidity ranges in which the given migrations can be obtained. It is remarkable that reference is made to a limited number of European pharmacopœias only, namely those from Germany, Austria, Switzerland, France, Great Britain and the new European pharmacopœia.

The book starts with short introductory paragraphs on the methodology of TLC, TAS-techniques, organoleptic examination and microscope analysis, 36 pages in total. Although clearly written in general, the value of this material is somewhat doubtful: It is not necessary for the worker in the field, whereas the unexperienced reader will need more information to obtain a satisfactory understanding of the technique and to avoid oversimplifications. A few literature references would have been useful.

The final chapter contains a list of reagents for both microscopy and chromatographic detection.

The book is well produced in a handy ring binding, is easily readable and gives valuable information for a reasonable price. It will certainly find its way among pharmacognosists.

Groningen University,
Groningen (The Netherlands)

R. A. DE ZEEUW

CHROM. 5065

Instrumental Analysis Manual, Modern Experiments for the Laboratory, by G. G. GUILBAULT AND L. G. HARGIS, Marcel Dekker Inc., New York, 1970, 444 pp., price $ 7.75.

This soft-covered book is basically a laboratory manual containing fifty experiments covering the major instrumental analytical techniques, of a suitable level for students up to graduate standard. These experiments are designed to be carried out in a 3- or 4-hour laboratory session, and lists of apparatus and reagents required for each experiment are given, although the finer points of procedure and instrument operation are not included.

The experiments are classified into six parts, *viz.* molecular and atomic spectroscopy (including electron spin resonance but not mass spectrometry), electrochemical methods, separation methods, radiochemical methods, miscellaneous methods (including mass spectrometry), and a section on electronics and their application to instrumental analysis.

Manuals with experiments in this field are not very common and this book will appeal to colleges which include a formal analytical course as part of their chemistry syllabus, and who have usually to design their own experiments. It is particularly useful for its coverage of the newer techniques such as specific ion electrodes, kinetic–catalytic methods and atomic absorption spectrometry, where "cook book" experiments are not readily available.

The section on separation techniques gives four experiments for gas chromatography which are representative of current practical usage, but, surprisingly, a description of flame ionisation detectors is not included, whereas katharometers and electron capture detectors are described. There are two experiments on ion-exchange separations and one each on electrophoresis, thin-layer chromatography and gel filtration.

In addition to the experiments, each technique is prefaced by an explanatory chapter which attempts to present the theory and principles in a few pages, and it is these sections which are the least satisfactory aspect of the book as they are too short to be effective, and it would be reasonable to assume that the student would not be attempting the experiment if he had not previously been acquainted with the theory. The inclusion of a section on electronics might also be thought superfluous in a manual of this type, although a knowledge of electronics capabilities is undeniably useful to the instrumental analyst.

Esso Research Centre,
Abingdon, Berks. (Great Britain)

D. T. COKER

CHROM. 5066

Separation Methods in Organic Chemistry and Biochemistry, by FRANK J. WOLF, Academic Press, New York and London, 1969, 237 pp., price $ 11.50, 107/- s.

This book is concerned with the problems involved in the separation of substances by liquid–liquid and liquid–solid systems in common use in both organic chemistry and biochemistry. The author rightly claims that one of his major objects is to provide perspectives for the commonly used methods, and indications for their use. That Dr. WOLF has achieved this object is not in doubt. By skilful elucidation of the theoretical principles involved in group-separation procedures, liquid–liquid partition, ion-exchange, gel permeation and adsorption chromatography, the practical examples presented to supplement these principles are almost superfluous. Nevertheless, they are welcome in the most didactic way possible.

The layout of the book is sensible. Group separations are distinguished from fractionation procedures in a philosophical sense. For instance, the latter are necessary when low separation factors are encountered and this is admirably illustrated by the use of simple countercurrent distribution. This "food for thought" makes the reader aware of the potentialities of this rather neglected technique and of its possible application as a rapid purification procedure prior to other forms of analysis, *e.g.* gas chromatography.

The usefulness of the theoretical plate height concept of column efficiency is emphasised, particularly in low resolution methods such as separation of macromolecules by gel permeation chromatography. Factors influencing resolution such as flow rate, temperature, sample application, aggregation, etc., are well discussed together with a similar chapter on the use of ion exchange.

The other useful part of the book is the appendix, particularly the table of dielectric constants of solvents presented as an analogous guide to their polarity characteristics and, also, the comprehensive tables of properties of ion-exchange resins. References and indices are a model of presentation. This book fulfils a need in the current science literature and it is difficult to see how it could be bettered. It is a work that will be kept, not on the shelf, but on the laboratory bench.

Department of Clinical Endocrinology,
Imperial Cancer Research Fund,
London (Great Britain)

B. S. THOMAS

Proceedings of the 3rd Analytical Chemical Conference, Budapest, August 1970, edited by I. BUZÁS, Akadémiai Kiadó, Budapest, 1970, 318 pp. (Volume 1) plus 459 pp. (Volume 2), price $ 21.60.

These two soft-covered volumes contain papers presented at the conference; they contain no discussions and appear to be preprints. Volume 1 is devoted to "separation methods", Volume 2 to "organic analysis and thermal analysis". A total of approximately 100 papers, mainly by Eastern European authors, are included.

Author Index

Subject Index

Errata

J. Chromatog., 52 (1970) 227–235

page 230, Fig. 1, last peak, "3-4" should read "1-1".

J. Chromatog., 53 (1970) 453–467

page 457, eqn. 26 should read $\chi = \frac{BA}{\eta L}$.