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ÉTUDE DE LA STABILITÉ THERMIQUE D'UN POLY(OXY ARYL SULFONYL ARYLÈNE) UTILISÉ COMME PHASE STATIONNAIRE EN CHROMATOGRAPHIE GAZ–LIQUIDE

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(Reçu le 25 juin 1981)

SUMMARY

Thermal stability of a poly(oxy aryl sulfonyl arylene) used as stationary phase in gas–liquid chromatography

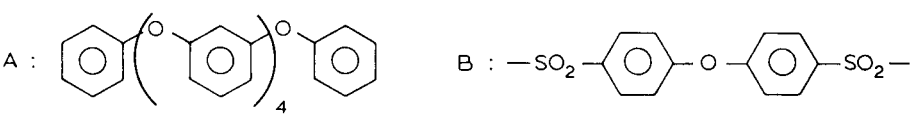
Poly(oxy aryl sulfonyl arylene) is used as a gas chromatographic stationary phase under the trade name PS 179. Its thermal stability depends largely on the nature of the support used. The supports studied are in order of increasing catalytic effect on thermal decomposition: dendritic sodium chloride, Chromosorb W AW HMDS, Chromosorb G NAW, Chromosorb G AW DMCS and Chromosorb P NAW.

Thermal degradation results both in weight loss and change of the chemical nature of the liquid phase, with loss of SO_2 groups and formation of a heavy, reticulated gum. Absolute and relative retention volumes as well as efficiency are markedly affected.

INTRODUCTION

La plupart des phases stationnaires polaires disponibles pour la chromatographie en phase gazeuse (polyesters, silicones, nitriles etc.) ne sont utilisables qu'à des températures bien inférieures à 250°C. Inversement les phases thermostables connues sont peu polaires.

Or le poly(oxy aryl sulfonyl arylène) de formule générale $AB(AB)_nA$, avec:



synthétisé en 1974 par Mathews *et al.*[1] est décrit comme un bon thermostable, le

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critère de stabilité retenu étant sa faible volatilité[2], modérement polaire, utilisable en chromatographie gaz–liquide entre 200 et 400°C[1,2], ce qui en fait une phase d'un grand intérêt. Cependant les publications qui traitent de ses propriétés sont de caractère appliqué[1–6] et non des études systématiques. Ces dernières ont été rendues possibles par l'apparition d'un produit commercial, le PS 179 (Applied Science Labs., State College, PA, États-Unis). Ce travail a été entrepris afin de préciser les limites de stabilité thermique des phases stationnaires obtenues en imprégnant des supports chromatographiques avec du PS 179.

La notion de stabilité thermique d'une phase est encore confuse et le plus souvent subjective. Cependant Keller[7] et Thizon *et al.*[8] en ont donné des définitions: les principaux critères retenus sont la tension de vapeur et la perte de masse de la phase stationnaire ainsi que la stabilité des paramètres relatifs à la retention et à l'efficacité de la colonne. La durée de vie utile d'une colonne peut ainsi être reliée à la perte de poids de la phase liquide qu'elle contient[8].

Dans ce travail nous avons déterminé la variation en fonction du temps des propriétés chromatographiques d'une colonne de PS 179 (rétention, polarité, efficacité) ainsi que de la masse de phase stationnaire qu'elle contient et nous avons étudié l'influence de la nature du support sur la stabilité thermique du PS 179.

PARTIE EXPÉRIMENTALE

Produit

Nous avons utilisé le PS 179 fourni par Applied Science Laboratories et comme support du chlorure de sodium dendritique (Morton Salt) et différents types de Chromosorb (Johns Manville).

Chromatographie gaz–liquide

Les mesures ont été effectuées à l'aide d'un chromatographe Girdel 75 CD/PT (Girdel, Suresnes, France) muni d'un détecteur à ionisation de flamme, relié à un enregistreur Sefram Servotrace (Sefram, Paris, France).

Les différentes colonnes utilisées sont en acier inoxydable, ont 2 m de longueur, 2.17 mm de diamètre intérieur, sont remplies de support imprégné de PS 179 par une méthode classique[8]. Le Chromosorb passivé a été obtenu par traitement au carbonate de baryum[9].

Les caractéristiques des colonnes étudiées sont données dans le Tableau I.

Le vieillissement des colonnes a été étudié en fonction de la température et du temps en les soumettant à un programme de vieillissement simple, précisé dans la légende de chaque figure. Ce programme comporte des paliers, généralement de plusieurs centaines d'heures, à des températures croissantes de 220 à 350°C. Des programmes en dents de scie n'ont pas été effectués. Au cours du vieillissement les mesures des temps de rétention réduits, t'_R, de divers composés et de leur efficacités sont effectuées après avoir ramené les colonnes à 220°C. La durée de ces analyses, quelques heures, n'est pas prise en compte dans le calcul de la durée du séjour à 220°C. Aux changements de palier de température les colonnes sont refroidies, pesées puis réchauffées après purge soigneuse de l'air.

Après la fin du programme de vieillissement les colonnes ont été pesées, vidées

TABLEAU I

CARACTÉRISTIQUES DES DIFFÉRENTES COLONNES ÉTUDIÉES

	Chromosorb G NAW	*Chromosorb G AW DMCS*	*Chromosorb W AW HMDS*	*Chromosorb P NAW*	*NaCl*
Masse de support imprégné introduit dans la colonne (g)	5.02	5.05	(1) 2.15 (2) 2.19	3.80	8.21
Taux d'imprégnation (%, en masse)	4	4	(1) 4 (2) 8	6	1.5
Masse de PS 179 (mg)	201	202	(1) 86 (2) 175	228	123
Efficacité (nombre de plateaux théoriques par mètre de colonnes)	515*	234*	(1) 187* (2) 160*	1227*	26**

* N calculé à 220°C, après conditionnement de la colonne, avec linolénate de méthyle.
** N calculé à 220°C, après conditionnement de la colonne, avec acénaphthène.

de leur contenu et la phase stationnaire désorbée de son support par extraction au dichlorométhane. Des extractions ultérieures au tetrahydrofurane chauffé à reflux n'ont pas permis de récupérer de produits supplémentaires.

Les fractions désorbées ont été soumises à différentes analyses: chromatographie par perméation de gel, analyse élémentaire.

Chromatographie par perméation de gel

Différentes colonnes ont été utilisées sur un appareil monté au laboratoire: (a) P_1 (colonne Cheminert) de 1090 mm de longueur, 25.4 mm de diamètre intérieur, avec un volume de lit de 497 cm^3, remplie de gel gonflable d'un copolymère styrène–divinyl benzène Bio-Beads SX_1 dont les masses d'exclusion sont 600–14000; volume d'exclusion de la colonne 134.7 cm^3; élution par le tetrahydrofurane, débit 64 cm^3/h. (b) P_2, composée de 4 colonnes en série de mêmes dimensions: longueur 300 mm, diamètre intérieur 7.8 mm, remplies de micro-Styragel (Waters Assoc., Milford, MS, États-Unis) dont les masses d'exclusion sont 500, supérieure à 20,000,000.

La détection est faite par spectroscopie UV avec un spectrophotomètre Cary 17 (trajet optique, 1 mm, λ = 260 mm) (Varian, Palo Alto, CA, États-Unis).

Analyse chimique

Les analyses chimiques élémentaires ont été effectuées par le service de microanalyse du CNRS à Solaize.

Analyse thermogravimétrique

Les analyses thermogravimétriques ont été réalisées avec un analyseur thermogravimètrique DuPont 951 (DuPont, Wilmington, DE, États-Unis) couplé avec un enregistreur DuPont 990. Vitesse de programmation 10°C/min, nacelle en platine, balayage à l'azote U, débit de l'ordre de 27 ml/min. Des analyses thermogravimè-

triques témoins ont été réalisées, dans des conditions identiques avec les supports non imprégnés.

RÉSULTATS

Dans le cas d'une phase hydrocarbure la perte de phase de la colonne et la diminution des temps de rétention de composés hydrocarbonés sont parallèles[8] de sorte que la simple mesure des poids des colonnes est suffisante pour caractériser leur durée de vie. Dans le cas présent où la phase polaire est susceptible de se dégrader en changeant de nature chimique, la situation est plus complexe, les retentions relatives et indices de rétention pouvant varier d'une manière imprévue. Nous avons donc étudié successivement tous ces paramètres.

Stabilité des propriétés chromatographiques

Le paramètre retenu pour cette étude est le temps de rétention réduit t'_R de différents solutés polaires et apolaires: alcanes ayant de 18 à 30 atomes de carbone, acénaphtène, phénanthrène, oléate, linoléate et linolénate de méthyle.

On constate d'abord, d'une manière générale que les temps de rétention ne sont pas stables dans les premières 20–40 h lorsque l'on étudie une nouvelle colonne. Au bout de ce laps de temps ils deviennent bien reproductibles.

On constate d'après les Figs. 1–5 que la stabilité du PS 179 est fortement in-

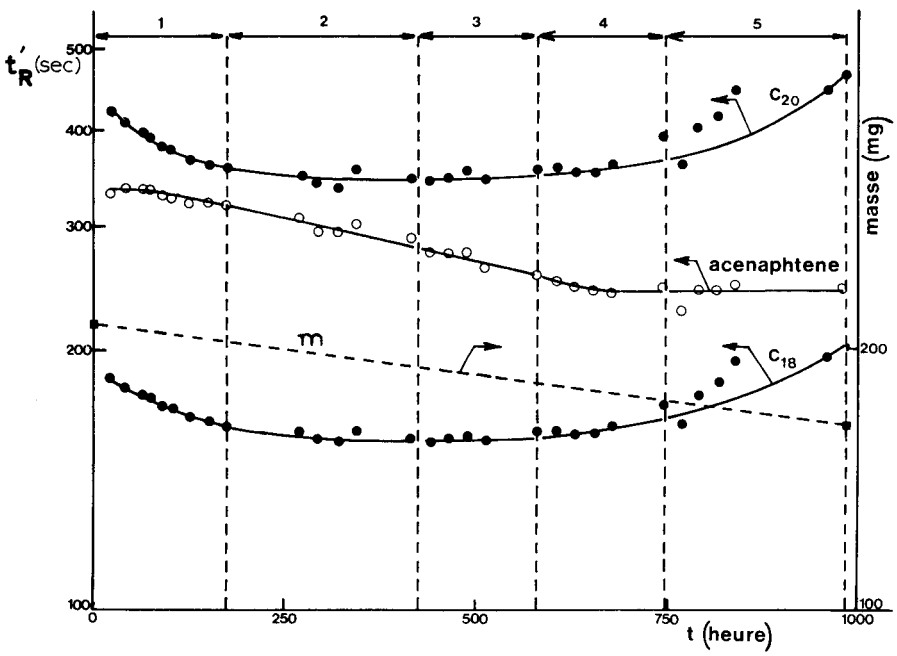

Fig. 1. Variation du temps de rétention réduit, t'_R (sec) de l'octadécane, de l'eicosane et de l'acénaphthène en fonction du temps. Variation concomittante de la masse de phase stationnaire (échelle de droite). Phase stationnaire: Chromosorb P NAW passivé, imprégné à 6% de PS 179. Température de mesure 220°C. Programme de vieillissement: paliers isothermes de durée variable: 1, $0 < t < 174$ h $T = 220$°C; 2, 174 h $< t <$ 414 h $T = 250$°C; 3, 414 h $< t <$ 580 h $T = 280$°C; 4, 580 h $< t <$ 744 h $T = 300$°C; 5, 744 h $< t <$ 984 h $T = 320$°C.

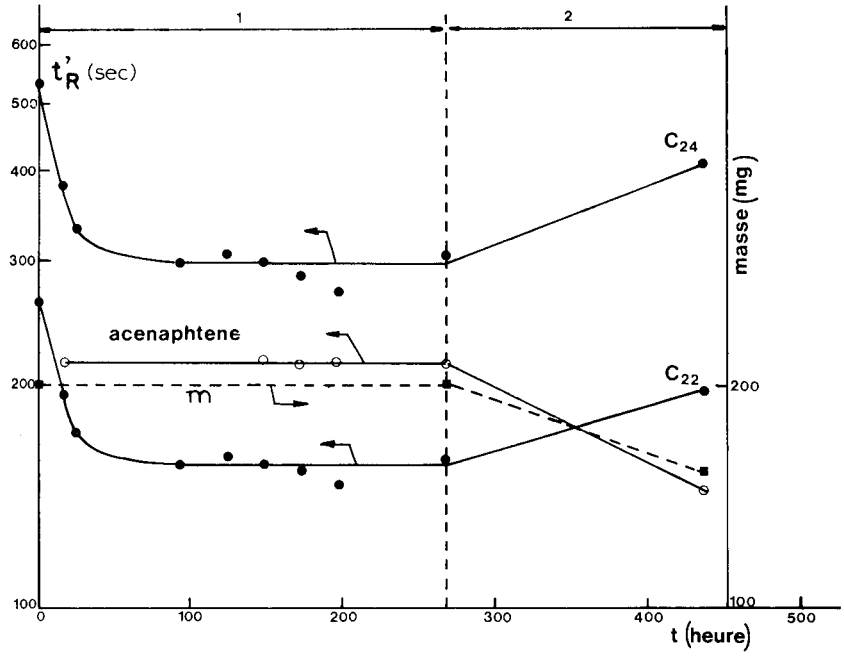

Fig. 2. Variation du temps de rétention réduit, t'_R (sec) du docosane, du tétracosane et de l'acénaphtène en fonction du temps. Variation concomittante de la masse de phase stationnaire (échelle de droite). Phase stationnaire: Chromosorb G NAW imprégné à 4 % de PS 179. Température de mesure 220°C. Programme de vieillissement: deux paliers isothermes: 1, $0 < t < 268$ h $T = 220$°C; 2, 268 h $< t <$ 436 h $T = 325$°C.

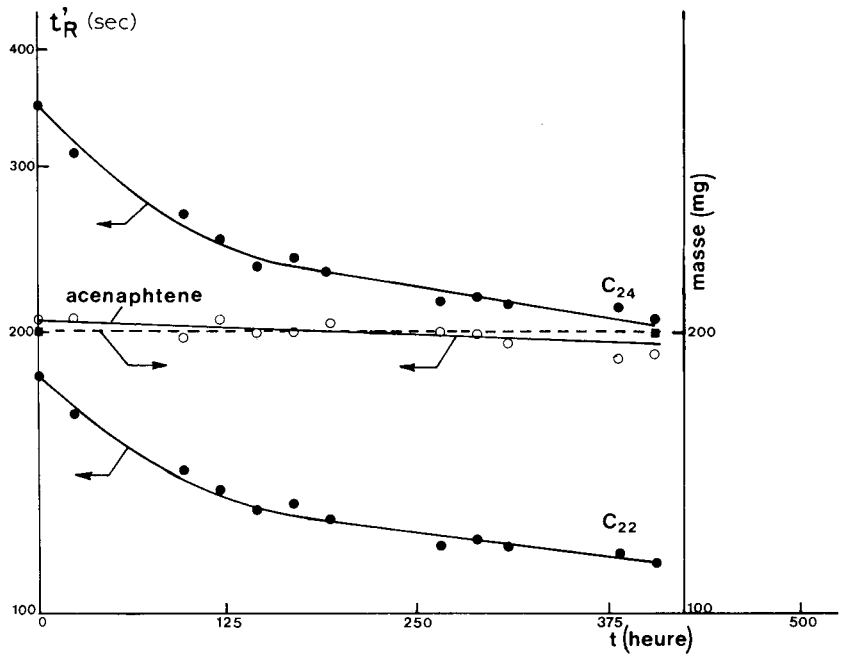

Fig. 3. Variation du temps de rétention réduit, t'_R (sec) du docosane, du tétracosane et de l'acénaphtène en fonction du temps. Variation concomittante de la masse de phase stationnaire (échelle de droite). Phase stationnaire: Chromosorb G AW DMCS imprégné à 4 % de PS 179. Température de mesure 220°C. Durant toute l'expérience la colonne a été maintenue à 220°C.

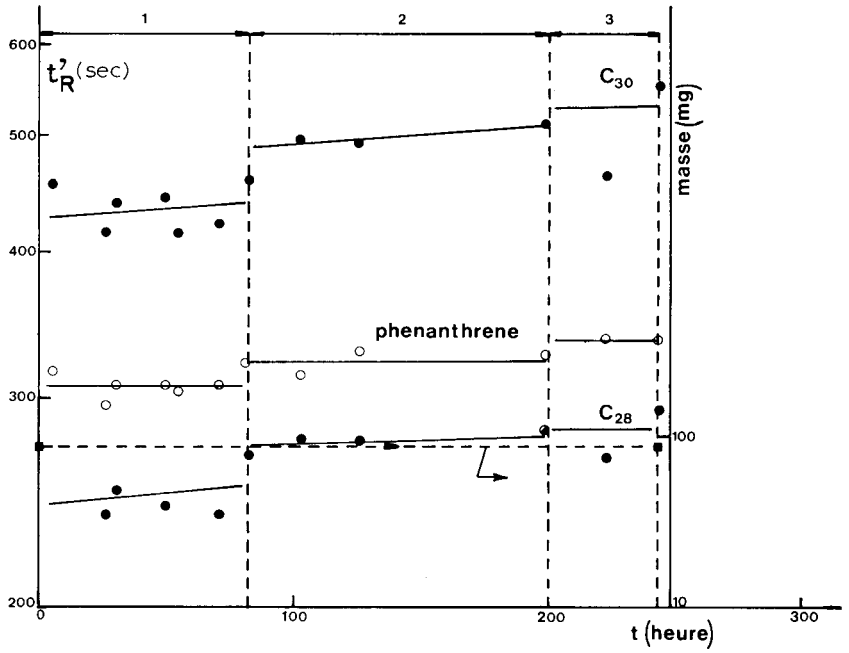

Fig. 4. Variation du temps de rétention réduit t'_R (sec) de l'octocosane, du triacontane et du phénanthrène en fonction du temps. Variation concomittante de la masse de phase stationnaire (échelle de droite). Phase stationnaire: Chromosorb W AW HMDS imprégné à 4% de PS179. Température de mesure 220°C. Programme de vieillissement: paliers isothermes de durée variable: 1, $0 < t < 82$ h $T = 220$°C; 2, 82 h $< t <$ 198 h $T = 250$°C; 3, 198 h $< t <$ 243 h $T = 280$°C.

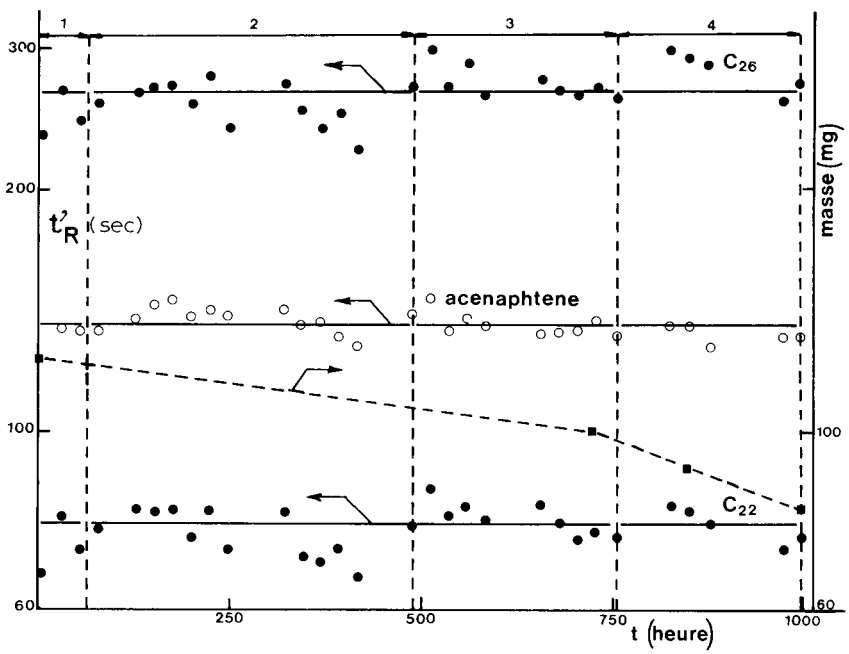

Fig. 5. Variation du temps de rétention réduit t'_R (sec) du docosane de l'hexacosane et de l'acénaphtène en fonction du temps. Variation concomittante de la masse de phase stationnaire (échelle de droite). Phase stationnaire: NaCl dendritique imprégné à 1.5% de PS 179. Température de mesure 220°C. Programme de vieillissement: 1, $0 < t < 63$ h la colonne a été chauffée alternativement à 220 et 280°C; 2, 63 h $< t <$ 487 h $T = 300$°C; 3, 487 h $< t <$ 753 h $T = 320$°C; 4, 753 h $< t <$ 998 h $T = 350$°C.

fluencée par la nature du support utilisé. On distingue trois comportements différents suivant le support imprégné:

(a) sur les Chromosorb P NAW passivé, G NAW, G AW DMCS, (Figs. 1 à 3) on observe une variation assez différente du t'_R des *n*-alcanes (solutés apolaires) et de l'acénaphtène (soluté polaire) au cours du vieillissement des colonnes. A basse température, au début du traitement, la rétention de tous les corps diminue. La variation est moindre pour l'acénaphtène. A haute température la rétention des alcanes augmente. La perte de masse est modérée mais mesurable. On note que ces variations sont moins importantes lorsque le support est lavé à l'acide et silanisé (Figs. 2 et 3).

(b) sur Chromosorb W AW HMDS (Fig. 4) on observe une légère augmentation des t'_R au cours du vieillissement de la colonne. Cependant ce phénomène est moins accentué pour le phénanthrène que pour les *n*-alcanes. La perte de phase est négligeable.

(c) sur NaCl dendritique (Fig. 5): les t'_R des *n*-alcanes et de l'acénaphtène sont assez stables, pratiquement constants. Les écarts observés sont attribués aux imprécisions expérimentales dûes à la médiocrité de l'efficacité de la colonne. La perte de masse de la colonne n'est pas négligeable au dessus de 300°C.

Ces variations du temps de rétention réduit t'_R entrainent des variations parfois importantes du facteur de capacité de la colonne k', et surtout des indices de rétention. Comme le temps mort de la colonne reste constant au cours de tous les traitements de vieillissement, k' est proportionnel à t'_R. On voit que ses variations ne reflètent que très imparfaitement celles de la teneur en phase liquide de la colonne.

L'indice de rétention[10] devrait rester constant si la phase liquide se décomposait sans changer de propriétés. On observe deux comportements différents suivant le support imprégné. Sur Chromosorb P NAW passivé, G NAW et probablement G AW DMCS les indices de retention de l'acénaphtène et de l'acénaphtylène passent par un maximum au cours du programme de vieillissement. Cela résulte de ce que les temps de rétention diminuent beaucoup plus vite au début pour les alcanes que pour les composés aromatiques, puis augmentent plus vite également. La variation d'indice la plus importante que nous ayons noté, pour l'acénaphtène, est de 140 unités sur Chromosorb G NAW; elle est de 80 unités seulement sur Chromosorb P NAW passivé.

D'autre part nous avons remarqué que la variation de l'indice de rétention d'un soluté aromatique ne semble pas être liée aux propriétés chimiques de celui-ci car les variations observées sont comparables pour l'acénaphtène, l'acénaphtylène, la quinoléine et le 2–4 dimethyl phenol sur Chromosorb P NAW passivé.

Au contraire sur NaCl dendritique et sur Chromosorb W AW HMDS les indices de rétention de l'acénaphtène et de l'acénaphtylène ainsi que ceux de nombreux autres composés demeurent quasiment constants.

Enfin le nombre de plateaux théoriques (mesure de l'efficacité) diminue considérablement au cours du vieillissement pour les colonnes réalisées avec les Chromosorb G et P NAW passivé (*cf.* Fig. 6), parallélement à l'augmentation de la rétention des *n*-alcanes. L'efficacité des colonnes de Chromosorb W et de chlorure de sodium dendritique, cette dernière très mauvaise, varie sensiblement moins.

Ces résultats mettent en évidence l'influence considérable du support sur la stabilité des propriétés chromatographiques du PS 179. On remarque que les supports Chromosorb W AW HMDS et NaCl dendritique ont une influence moindre sur l'instabilité du PS 179; ce résultat est en accord avec des travaux antérieurs[5,8].

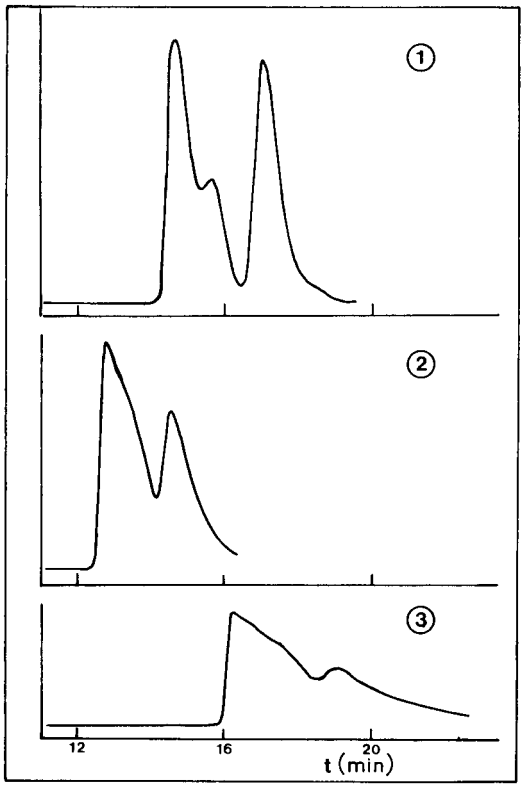

Fig. 6. Évolution du chromatogramme d'un mélange d'oléate de méthyle, de linoléate de méthyl et de linolénate de méthyle au cours du vieillissement d'une colonne. Phase stationnaire: Chromosorb P NAW passivé, imprégné à 6% de PS 179. Température d'élution 220°C. Le programme de vieillissement de la colonne est donné Fig. 1. 1, Après 65 h de vieillissement; 2, après 652 h de vieillissement; 3, après 984 h de vieillissement.

Étude thermogravimétrique

L'analyse thermogravimétrique nous permet de comparer la stabilité thermique du PS 179 pur non dispersé sur un support, à celle du PS 179 dispersé sur différents supports (*cf.* Fig. 7 et Tableau II).

Le PS 179 pur se comporte comme Mathews *et al.*[1] et Schwartz *et al.*[2] l'ont décrit mais dès qu'il est dispersé sur un support sa stabilité diminue et les températures T_0 et T_M sont plus ou moins abaissées selon la nature du support, le chlorure de sodium et le Chromosorb W AW HMDS ayant l'influence la plus faible. Ces résultats sont assez bien reliés à l'activité chimique du support: le chlorure de sodium ne porte aucun groupe chimique acide ou basique à la différence des Chromosorb P, G et W qui ont sur leur surface des atomes de Al, B et Si liés à des atomes d'oxygène et des groupes OH, constituant des sites fortement acides ou basiques selon le cas et donc ayant des propriétés catalytiques éventuelles. La réaction de silanisation fait disparaître la plupart des groupes OH et gêne l'accès à la surface en créant une couche dense de groupes Si $(CH_3)_3$. Elle désactive donc beaucoup cette surface.

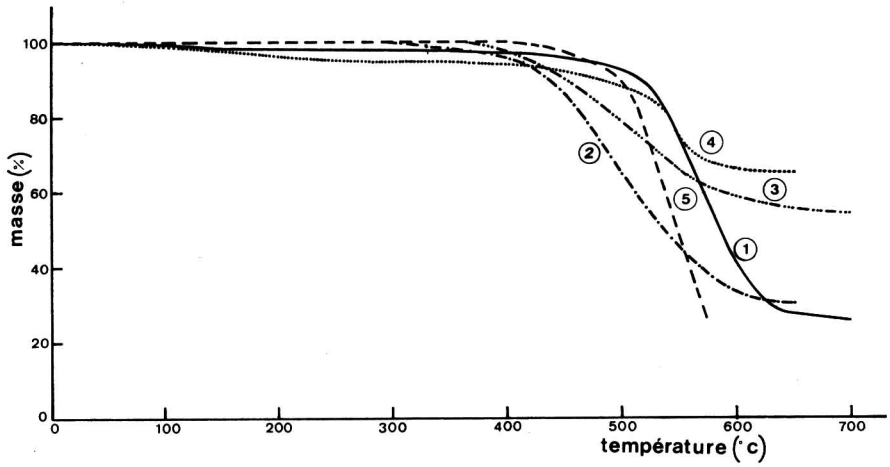

Fig. 7. Thermogrammes de PS 179: masse résiduelle de l'échantillon (%) en fonction de la température, programmée à 10°C/min. 1, Pur (masse de l'échantillon 5.4 mg); 2, dépôt à 4% sur Chromosorb G NAW (masse de l'échantillon 12.2 mg); 3, dépôt à 6% sur Chromosorb P NAW passivé (masse de l'échantillon 19.1 mg); 4, dépôt à 8% sur Chromosorb W AW HMDS (masse de l'échantillon 18.6 mg); 5, dépôt à 1.5% sur NaCl dendritique (masse de l'échantillon 14.8 mg).

'ABLEAU II

ÉSULTATS DE L'ANALYSE THERMOGRAVIMÈTRIQUE

'roduits	*Masse de PS 179 (mg)*	T_M★ *(°C)*	T_0★ *(°C)*	V_M *en % de perte de masse/sec*	*Perte de masse du PS 179 accompagnant la décomposition (%)*
S 179 non imprégné	5.40	570	515	0.12_5	74
'hromosorb G NAW imprégné à 4% de PS 179	0.49	500	450	0.12_5	70
'hromosorb P NAW passivé imprégné à 6% de PS 179	1.15	500	410	0.04	46
'hromosorb W AW HMDS imprégné à 8% de PS 179	1.49	545	490	0.07	36
laCl dendritique imprégné à 1.5% de PS 179	0.22	550	500	0.17	≈100

★ Chaque thermogramme (*cf.* Fig. 7) est caractérisé par l'équation de la tangente au point d'inflexion: pente de la tangente: "vitesse" V_M; abscisse du point d'inflexion, température T_M; abscisse à l'origine (masse initiale ou 100%): température T_0.

Analyse comparative du PS 179 avant et après avoir fonctionné comme phase stationnaire en chromatographie gaz–liquide

Les résultats obtenus lors de l'étude chromatographique et en particulier l'existence d'un important résidue fixé au support, ne peuvent pas s'expliquer uniquement par la perte de masse de la phase stationnaire. Nous avons donc été amené à procéder à différentes analyses du PS 179 ayant fonctionné comme phase stationnaire.

Désorption du PS 179 de son support. Dans aucun cas nous ne pouvons extraire par un solvant la totalité de la phase stationnaire résiduelle (Tableau III). L'extraction

TABLEAU III

RÉSIDU DE PS 179 SUR LE SUPPORT APRÈS FONCTIONNEMENT DES DIFFÉRENTES COLONNES

	Chromosorb G NAW (cf. Fig. 2)	*Chromosorb W AW HMDS (cf. Fig. 4)*	*Chromosorb P NAW passivé (cf. Fig. 1)*	*NaCl dendritique (cf. Fig. 5)*
Quantité de PS 179 restant sur le support après fonctionnement (% de la quantité déposé)	78	(1) 100 (2) 100	75	65
Fraction de PS 179 récupérable par désorption au CH_2Cl_2 (% du résidu)	30	(1) 79 (2) 69	12	87

au tétrahydrofuranne ne permet pas d'extraire l'insoluble au dichloromethane. Le produit non extractible, récupéré après dissolution du NaCl dendritique dans l'eau est insoluble dans le tétrahydrofuranne. L'analyse thermogravimétrique du Chromosorb P NAW passivé après extraction du résidu montre que le produit insoluble est thermiquement plus stable que le PS 179 lui-même (augmentation de T_0 et T_M).

Ces observations suggérent une réticulation partielle du PS 179 sur le support.

Les fractions de résidu récupérées ont été soumises à différentes analyses, chromatographie par perméation de gel et analyse chimique.

Chromatographie par perméation de gel. La Fig. 8 donne la répartition des masses moléculaires du PS 179 d'origine. Un étalonnage préalable nous a permis d'attribuer une composition AB $(AB)_nA$ à chaque pic.

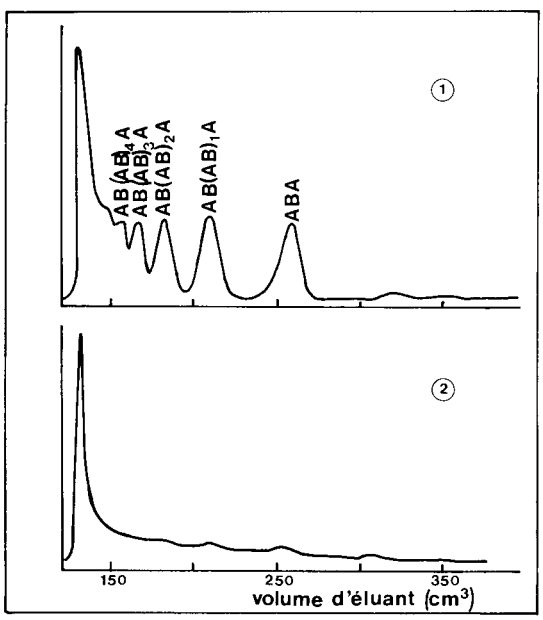

Fig. 8. Fractionnement d'échantillons de PS 179 par chromatographie par perméation de gel sur la colonne P_1. 1, Échantillon de PS 179 pur, non traité; 2, échantillon de PS 179 extrait d'une colonne préparée avec du Chromosorb P NAW passivé imprégné à 6 % de PS 179 et ayant travaillé 984 h (*cf.* programme Fig. 1).

L'étude des chromatogrammes obtenus pour les fractions extraites après différents traitements montre que pour un vieillissement de la colonne de l'ordre de 240 h à une température inférieure à 300°C avec le Chromosorb W AW HMDS comme support il n'y a pas de modification appréciable du PS 179.

Par contre pour un vieillissement d'une durée de l'ordre de 1000 h il y a modification sensible de la distribution des masses:

(a) sur NaCl dendritique (*cf.* colonne décrite Fig. 5), le rapport des concentrations faibles masses moléculaires/fortes masses moléculaires diminue nettement, mais il ne semble pas qu'il s'agisse d'un phénomène de simple évaporation car l'aire du pic ABA diminue moins vite que l'aire du pic ABABA.

(b) sur Chromosorb P NAW passivé (*cf.* colonne décrite Fig. 1) on observe une disparition des fractions de faibles masses (*cf.* Fig. 8) et l'apparition de macromolécules de plus fortes masses (*cf.* Fig. 9), pouvant atteindre plusieurs dizaines de milliers.

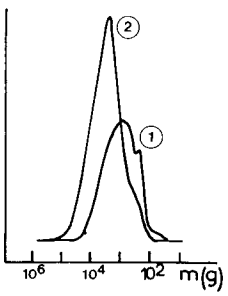

Fig. 9. Fractionnement des mêmes échantillons de PS 179 que Fig. 8, par chromatographie par perméation de gel sur la colonne P_2. *m*, Masse molaire apparente résultant d'un étalonnage par le polystyrene.

TABLEAU IV

ANALYSE ÉLÉMENTAIRE DU PS 179 APRÈS FONCTIONNEMENT COMME PHASE STATIONNAIRE

Produits	*Nombre atomes C/nombre atomes S*
PS 179 original	27.5–28.5
PS 179 désorbé du NaCl dendritique au CH_2Cl_2	29.3
Fraction insoluble de PS 179 recueillie après dissolution du NaCl dendritique dans l'eau	23.5
PS 179 désorbé du Chromosorb G NAW	31.5–31.1
Valeurs théoriques pour	
ABA	42
ABABA	33
$AB(AB)_2A$	30
$AB(AB)_3A$	28.5

TABLEAU V

RÉCAPITULATIF DES RÉSULTATS OBTENUS

	Supports				
	Chromosorb P NAW passivé	*Chromosorb G NAW*	*Chromosorb G AW DMCS*	*Chromosorb W AW HMDS*	*NaCl dendritique*
Temps de travail (h)	984	436	400	(1) 243 (2) ≈ 50	998
Température de travail (°C)	$220 < T < 320$	$220 < T < 325$	220	(1) $220 \leqslant T \leqslant 280$ (2) $220 \leqslant T < 300$	$220 \leqslant T \leqslant 350$
t'_R, *n*-alcanes					
t'_R aromatique					
Indice de rétention					
k'		Linolénate de méthyle		Phénanthrène	Acénaphtène

Efficacité	(1) Efficacité décroît regulièrement de P NAW à NaCl				
(1) à l'origine					
(2) après vieillissement	(2) ↘	↘	→	→	→
ATG					
T_0 et T_M	T_0 et T_M croissent régulièrement de P NAW à NaCl				
Surface spécifique (m^2/g)	≈6	0.5	≈0.5	≈1	⩽0.5
Perte de masse (%)	25	22	0	(1) 0	35
		0 pour 268 h de travail à 220 C		(2) 0	
Taux de réticulation du résidu (%)	88	70		(1) 21 (2) 31	13
Perte de S (%)		8.5–12.5			3–6
Chromatographie par perméation de gel; résultats	Perte des M de faibles masses apparition de M lourdes	Perte des M de faibles masses		Pas de modification appréciable	Perte des macromolécules de faibles masses

Analyse chimique

Le PS 179 désorbé au dichloromethane du NaCl dendritique ou du Chromosorb G NAW s'est appauvri en soufre par rapport au produit initial (*cf.* Tableau IV). Cette perte de soufre peut s'expliquer par un processus de dégradation connu des polysulfones[11]: rupture au niveau de la liaison de plus faible énergie (C–S) puis perte de SO_2. Au contraire le résidu insoluble est plus riche en soufre, mais nous n'avons pu en faire une analyse fonctionelle. Il faut noter que le résultat concernant le résidu insoluble sur NaCl ne doit pas s'étendre aux autres résidus, pour lesquels nous n'avons aucune donnée.

DISCUSSION

Les résultats décrits ci-dessus sont rassemblés dans le Tableau V. Les différentes analyses effectuées mettent en évidence pour un vieillissement des colonnes de l'ordre de 1000 h à des températures allant de 220 à 350°C: une réticulation partielle de la phase sur le support (Tableaux II et V), une perte des macromolécules de faibles masses (Fig. 8), la formation de macromolécules de plus fortes masses (Fig. 9) et une perte de soufre (Tableaux IV et V) pour les fractions extractibles.

Ceci suggère une évolution de la phase stationnaire suivant au moins deux schémas possibles: (1) évaporation lente des macromolécules de faibles masses; (2) réaction de coupures, perte de SO_2 suivie de recombinaison conduisant à une ramification (augmentation de la masse moléculaire) puis à une réticulation. Ce second schéma est très probablement catalysé par les sites fortement acides ou basiques que l'on trouve à la surface des supports silico-aluminates, surtout s'ils sont non traités. Sur NaCl dendritique le schéma 1 serait prépondérant par rapport au schéma 2 (peu de réticulation sur ce support, Tableau V). Par contre sur Chromosorb P NAW passivé et G NAW le schéma 2 serait prépondérant. Sur Chromosorb W AW HMDS il est difficile de conclure car les colonnes ont peu vieillies et à des températures inférieures à 300°C. Toutefois le taux de réticulation, sur ce support, n'est pas négligeable (Tableau V).

L'évolution des paramètres chromatographiques en fonction du vieillissement des colonnes, est liée à la décomposition du PS 179. Au début la réticulation du produit à la surface du support se traduit par une diminution des temps de rétention corrigés. Pour un vieillissement plus avancé, la dégradation du PS 179 est défavorable à la rétention des solutés aromatiques polaires probablement par suite de la réduction des intéractions moléculaires pour ces familles, à la suite de la perte de groupes SO_2 mais favorable à la rétention des *n*-alcanes (Tableau V): la disparition des centres actifs polaires (perte de SO_2) pourrait en effet augmenter la solubilité des alcanes dans un liquide devenu sensiblement moins polaire, ceci malgré la perte de masse et la réticulation.

L'étude des propriétés chromatographiques du résidu montre en effet qu'il a un comportement très peu polaire, presque comparable à celui du SE 30, donc sensiblement moins polaire que le polyphenyléther (*cf.* Tableau II). Ainsi avec une partie du support résiduel de la colonne 2, soit environ 1.7 g de Chromosorb W AW HMDS imprégné d'environ 2.5% d'un résidu non extractible, avons nous rempli une colonne de 1.5 m. Les mesures sont peu précises en raison du faible temps de rétention corrigé consécutif à la faible quantité de phase résiduelle, mais l'indice de rétention

du phénanthrène est voisin de celui observé sur SE-30 (1850). La séparation des esters méthyliques des acides gras de l'huile de lin est semblable à celle obtenu sur SE-30. Ces résultats confirment le schèma de dégradation proposé.

CONCLUSION

La décomposition du PS 179 est fonction du temps, de la température et de la nature du support qui joue un rôle catalytique. Elle se traduit par une perte de masse et une modification chimique importante. Les caractéristiques d'une colonne sont donc tributaires du degré d'avancement de la décomposition et de la nature du support à un degré considérable.

Si nous ne considérons comme critère de stabilité que la perte de masse de la phase, le classement des différentes colonnes est délicat. Chaque colonne a subi un vieillissement différent (de 50 à 1000 h de travail à des températures allant de 220 à 350°C).

Pour les colonnes réalisées avec le Chromosorb P NAW passivé et NaCl dendritique, l'extrapolation des résultats obtenus permet de prévoir une perte de 50% de la masse initiale de PS 179 en moins de 2000 h à 300°C environ. Cette durée correspond à une vie pratique de la colonne de près d'un an si l'on considère une utilisation de 8 h par jour, 5 jours par semaine. Le programme choisi, une très lente programmation de température, n'est cependant pas forcément équivalent aux quelques milliers d'analyses en température programmée qui seraient effectuées pendant ce temps.

Vis à vis des propriétés chromatographiques les colonnes réalisées avec le Chromosorb W AW HMDS et NaCl dendritique sont plus stables que celles réalisées avec les Chromosorb G et P NAW passivé, mais ces colonnes sont nettement moins efficaces au moins à 220°C, l'efficacité s'améliorant lorsque la température s'élève.

Les résultats obtenus nous permettent de retenir pour des études ultérieures les supports suivants: Chromosorb W AW HMDS pour une utilisation limitée à température inférieure à 300°C, NaCl dendritique pour une utilisation prolongée à température supérieure à 300°C.

Il ne parait pas de toutes manières que des colonnes préparées avec cette phase puissent être utilisées pendant une longue durée à une température supérieure à 300°C, quel que soit le support, sans que les données de rétention ne varient très notablement. Même le chauffage accidentel d'une colonne au dessus de 300°C peut avoir des conséquences importantes sur ce plan.

Il est fort possible que les résultats obtenus soient en partie ou en totalité dus à une oxydation lente ou à une décomposition catalysée par l'oxygène. Nous n'avons pas pris de précautions particulières sur ce point de sorte que nos résultats sont typiques de ceux obtenus dans la plupart des laboratoires et nous avons probablement eu tort. Il est certain que des traces d'oxygène dans le gaz vecteur ont souvent une très néfaste influence sur la stabilité thermique des phases[8].

RÉSUMÉ

La stabilité thermique des poly(oxy aryl sulfonyl arylène) utilisés comme phase stationnaire en chromatographie gaz–liquide dépend beaucoup de l'influence du sup-

port, dès 220°C dans certains cas. Le chlorure de sodium dendritique et le Chromosorb W AW HMDS ont une influence moindre que les Chromosorb G NAW, G AW DMCS et P NAW passivé.

La dégradation thermique se traduit par une perte de masse et une modification chimique de la phase. Ces deux facteurs entrainent une variation plus ou moins importante des propriétés chromatographiques (retention, polarité, efficacité).

Seuls des supports très inertes comme NaCl dendritique permettent l'utilisation prolongée du PS 179 à haute température (300°C). Malheureusement les colonnes réalisées avec ce support sont d'efficacité très médiocre.

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ÉTUDE DE LA POLARITÉ D'UN POLY(OXY ARYL SULFONYL ARYLÈNE) UTILISÉ COMME PHASE STATIONNAIRE EN CHROMATOGRAPHIE GAZ–LIQUIDE

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SUMMARY

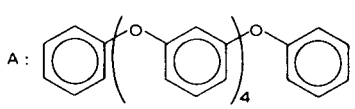
Polarity of a poly(oxy aryl sulfonyl arylene) used as stationary phase in gas–liquid chromatography

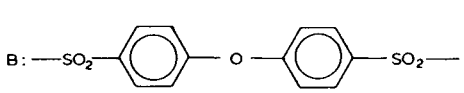
Retention indices of polynuclear hydrocarbons, phenols and aza-arenes on poly(oxy aryl sulfonyl arylene) were measured at different temperatures. Comparison between these data and the retention indices of the same compounds on SE-30, polyphenylether (6 rings), OV-17, Carbowax 20M, shows the polarity of this stationary phase (PS 179) to be similar to that of Carbowax 20M.

The large polarity and the thermal stability of PS 179 permits the analysis of complex mixtures of heavy aromatic hydrocarbons and related compounds.

INTRODUCTION

L'objet de ce travail est l'étude de la polarité d'un polycondensat appartenant à la famille des poly(oxy aryl sulfonyl arylène), le PS 179, de formule $AB(AB)_nA$ où:





Il est utilisé comme phase stationnaire en chromatographie gaz–liquide à des températures supérieures à 200°C.

Cette phase se distingue des autres phases stationnaires thermostables usuelles (polysiloxanes, polycarborane siloxanes) par son caractère assez fortement polaire. Elle pourrait donc être très utile pour l'analyse de composés polaires de faible tension de vapeur.

Parmi les travaux mettant en évidence la polarité du PS 179[1–5] on peut citer l'analyse de lubrifiants synthétiques, de pesticides et des esters méthyliques des acides

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stéraniques de la bile. La polarité de cette phase est difficile à mesurer et à comparer à celles de corps plus classiques car le PS 179 ne peut être utilisé à des températures inférieures à 200°C et donc les constantes de McReynolds[6] ne peuvent être mesurées car les étalons correspondants ne sont pas assez retenus. Schwartz *et al.*[3] ont suggéré l'emploi d'autres étalons[5] pour classer cette phase. Mais, à notre connaissance, aucune étude systématique de la polarité du PS 179 n'a été effectuée. Nous avons cependant montré dans un travail antérieur l'influence marquante de la nature du support sur la dégradation thermique du PS 179, laquelle s'accompagne d'une variation plus ou moins sensible selon les supports de la polarité.

L'analyse des hydrocarbures aromatiques polycycliques non fonctionnels et fonctionnels (composés polaires, de faible volatilité, très stables thermiquement) nous a semblé bien adaptée à l'étude de la polarité du PS 179, d'autant plus que l'analyse de mélanges complexes de ces molécules est un problème d'actualité (analyse des polluants atmosphériques, fractions lourdes des pétroles etc.) et plusieurs auteurs ont mentionné la nécessité de posséder une gamme de colonnes de polarité différente, stables à température élevée[7–9].

Dans une première partie nous étudions l'influence du support sur la polarité du PS 179. Nous avons pour cela utilisé des colonnes récemment préparées et peu vieillies, en particulier n'ayant séjourné que très brièvement à une température supérieure à celle où sont faites les mesures. Ce travail nous conduit au choix d'un support.

Dans la seconde partie nous comparons les indices de rétention de différents hydrocarbures aromatiques mesurés sur PS 179, SE-30 (polydimethyl siloxane), polyphenyl ether à 6 noyaux [oxyde de bis(*m*-phenoxy phenoxy) phenyle], OV-17 (polyphenyl methyl siloxane), Carbowax 20M [poly(oxy ethylène)]. Les indices de rétention[10] dépendent des intéractions moléculaires entre solutés et phase stationnaire. Leur variation traduit l'intensité relative de celles-ci pour différentes phases stationnaires et donc peut servir à caractériser ce que l'on appelle d'une manière peu précise la polarité de la phase, polarité qui traduit d'une manière complexe l'aptitude à donner ou céder des électrons, la polarisabilité et le moment dipolaire des différents groupes de la molécule de phase fixe. Ce travail n'entend pas éclaircir la notion de polarité mais caractériser les propriétés d'une phase.

PARTIE EXPÉRIMENTALE

Les mesures ont été effectuées à l'aide d'un chromatographe Girdel 75 CD/PT (Girdel, Suresnes, France) muni d'un détecteur à ionisation de flamme relié à un enregistreur Sefram Servotrace (Sefram, Paris, France).

Les différentes colonnes réalisées sont en acier inoxydable ont 2 m de longueur, 2.17 mm de diamètre intérieur et sont remplies de support imprégné par la méthode classique.

La phase liquide utilisée est le PS 179 (Applied Science Labs., State College, PA, États-Unis). Sa distribution moléculaire a été caractérisée par chromatographie par perméation de gel[5].

Pour la première partie les supports utilisés sont: le Chromosorb P NAW passivé imprégné à 6%, le Chromosorb G NAW imprégné à 4%, le Chromosorb G AW DMCS imprégné à 4% et la NaCl dendritique imprégné à 1.5%.

Dans la seconde partie le support utilisé est le Chromosorb W AW HMDS

imprégné à 8 % de phase stationnaire (4 % dans le cas du SE-30) afin que les temps de rétention réduits ne soient pas inférieurs à 25 sec.

Les temps de rétention isothermes des composés étudiés n'ont pas évolué pendant le laps de temps nécessaire à cette étude (48 h entre 220 et 300°C). Après fonctionnement de la colonne aucune perte de masse n'a été enregistrée. La colonne a été vidée de son contenu et la phase liquide a été extraite du support avec du dichlorométhane. L'extrait représente 69 % du PS 179 originellement déposé sur le support. Il a été analysé par chromatographie par perméation de gel; on ne note aucune modification par rapport au PS 179 d'origine. Le résidu non extractible (31 % en poids de la phase liquide d'origine) apparait très vite par traitement thermique[5]. Son influence sur les propriétés chromatographiques de la phase est inconnue et les conditions de sa formation peuvent expliquer des résultats peu reproductibles. Les temps de transit de l'inerte (t_m) ont été mesurés avec le méthane.

Le soluté est en solution à 1 % dans le tetrahydrofurane. La quantité de solution

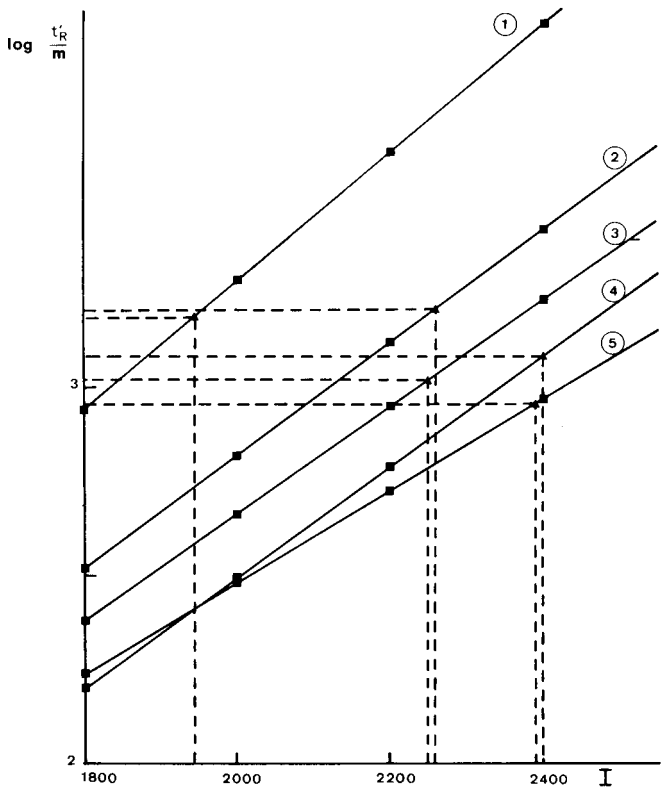

Fig. 1. Influence de la nature du support sur la détermination de l'indice de rétention de l'acénaphtène. Courbes 1 à 5, variations du logarithme du temps de rétention absolu (t'_R/m) des *n*-alcanes avec le nombre d'atomes de carbone. 1 = PS 179 déposé à 6 % sur Chromosorb P NAW passivé; 2 = PS 179 déposé à 4 % sur Chromosorb G NAW; 3 = PS 179 déposé à 4 % sur Chromosorb G AW DMCS; 4 = PS 179 déposé à 1.5 % sur NaCl dendritique; 5 = PS 179 déposé à 4 % sur Chromosorb W AW HMDS. ■, *n*-Alcane; ▲, acénaphthène. Exemple: sur 1 l'acénaphtène est élué entre l'octadécane et l'eicosane, sur 2 entre le docosane et le tétracosane. Lignes horizontales: temps de rétention absolue de l'acénaphthène. Lignes verticales: indices de rétention correspondant.

TABLEAU I

INFLUENCE DU SUPPORT SUR LES TEMPS DE RÉTENTION RÉDUIT t'_R RAMENÉS À L'UNITÉ DE MASSE DE PHASE STATIONNAIRE, ET LES INDICES DE RÉTENTION

Température 220°C. Colonne non vieillie (moins de 48 h à 220°C).

	Chromosorb P NAW passivé	*Chromosorb G NAW*	*Chromosorb G AW DMCS*	*Chromosorb W AW HMDS*	*NaCl dendritique*
t'_R/m pour l'octadécane (sec g^{-1})	881	330	237	170	158
t'_R/m pour l'acénaphtène (sec g^{-1})	1513	1584	1047	912	1216
Indice de rétention de l'acénaphtène	1940	2260	2249	2390	2400
Surface spécifique (m^2 g^{-1})	≈ 6	0.5	≈ 0.5	≈ 1	$\ll 0.5$

injectée est de 0.15 μl. Pour déterminer l'indice de rétention d'un soluté, il est injecté avec les *n*-alcanes qui l'encadrent.

Influence du support

Résultats. La Fig. 1 montre que le temps de rétention d'un soluté, ici l'acènaphtène, dépend de la nature du support. Les variations du temps de rétention d'un support à l'autre sont beaucoup plus importantes pour les *n*-alcanes (solutés apolaires) que pour l'acénaphtène (soluté polaire). D'où des variations importantes de l'indice de rétention de l'acénaphtène avec la nature du support (Tableau I) malgré une variation assez faible du temps de rétention de l'acénaphtène lui-même.

Discussion. Ces résultats peuvent s'expliquer par une contribution à la rétention de l'adsorption à l'interface gaz–liquide[11–14]. En effet si seule la solubilité des corps étudiés dans la phase liquide expliquait leur rétention la grandeur t'_R/m, proportionelle au volume de rétention spécifique devrait être constante pour chaque corps, indépendamment du support. Il n'en est rien.

L'adsorption à l'interface est fonction de la nature du support, de la nature du soluté et de l'aire de l'interface gas–liquide (donc de la surface spécifique du support et du taux d'imprégnation).

La contribution à la rétention est ici plus importante pour les *n*-alcanes que pour l'acénaphtène: le PS 179 solvant fortement aromatique, polaire, dissout moins les *n*-alcanes, composés apolaires, qui sont exclus de la solution et donc adsorbés à l'interface gaz–liquide, que les composés aromatiques.

Par ailleurs il faut noter que les supports traités par silanisation ont une énergie de surface et donc une mouillabilité bien moindres que les supports non traités. Il est donc fort probable que les supports silanisés ne sont pas complètement mouillés par le PS 179 et donc que l'interface gaz–liquide a une aire inférieure à celle sur support non traité, ce qui explique la très faible rétention de l'octadécane sur ces supports.

L'interprétation des écarts observés Tableau I pour la rétention de l'acénaphtène (soluté aromatique polaire) est plus délicate. Toutefois, dans le cas de l'étude de la rétention du benzène sur le chloronaphthalène, les résultats obtenus par Martin[11] mettent en évidence une contribution non négligeable de l'adsorption à l'interface gaz–liquide.

Les résultats obtenus (Fig. 1 et Tableau I) mettent en évidence l'influence de la nature du support sur l'indice de rétention d'un soluté donné: l'indice de rétention dépend à la fois de la rétention du soluté et de celle des alcanes et se trouve donc assez sensible aux effets de surface. L'indice de rétention n'a de sens que si l'on précise la nature du support et le taux d'imprégnation. Par contre la rétention relative de composés aromatiques est moins affectée. Les rétentions relatives du 2,4-diméthylphénol et de la quinoléine à l'acénaphtène sont respectivement de 0.30 et 0.55 sur Chromosorb W AW HMDS et de 0.33 et 0.53 sur Chromosorb P NAW, une variation qui n'excède guère les erreurs expérimentales.

Choix du support. Nos précèdents travaux[5] ont mis en évidence les variations de différents paramètres chromatographiques (dont l'indice de rétention) au cours du temps, lors du vieillissement thermique des colonnes. Mais nous avons constaté que les indices de rétention mesurés avec le Chromosorb W AW HMDS et NaCl dendritique sont quasiment identiques. D'autre part les colonnes réalisées avec le Chromosorb W AW HMDS sont plus efficaces que celles réalisées avec le NaCl dendritique. Ces différentes propriétés sont présentées Fig. 2.

Nous avons donc retenu pour l'étude de la polarité du PS 179, le Chromosorb W AW HMDS.

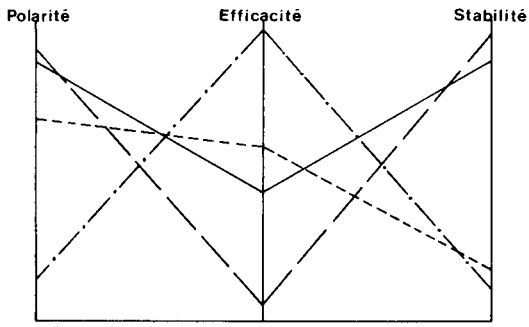

Fig. 2. Comparaison qualitative du comportement du PS 179 déposé sur différents supports. ———, Chromosorb W AW HMDS; – – – –, Chromosorb G NAW; – –, NaCl dendritique; – · –, Chromosorb P NAW passivé.

Comparaison du PS 179 avec une phase apolaire le SE 30

Les indices de rétention des hydrocarbures aromatiques non fonctionnels, hydroxylés et azotés, mesurés sur PS 179 et sur diverses phases classiques sont rassemblés dans les Tablaux II, III et IV.

Polarité du PS 179. La Fig. 3 représente les variations des indices de rétention des différents solutés étudiés en fonction de leur température d'ébullition. Par définition la courbe des *n*-alcanes (courbe 1) est la même pour toutes les phases. La position d'un point correspondant à un soluté sur une phase donnée permet d'apprécier la polarité de cette phase.

Sur SE-30, phase apolaire, les hydrocarbures aromatiques non fonctionnels dont la température d'ébullition est inférieure à 300°C suivent d'assez près la courbe des *n*-alcanes; ceux dont la température d'ébullition est supérieure à 300°C et les dérivés fonctionnels sont très légèrement moins retenus; ils se situent en effet au dessous de cette courbe.

Sur PS 179 les hydrocarbures aromatiques non fonctionnels sont tous très au dessus de la courbe des *n*-alcanes, le déplacement allant de 700 pour le naphtalène à 1500 pour le pyrène et 2300 pour le perylène.

TABLEAU II

INDICES DE RÉTENTION D'HYDROCARBURES AROMATIQUE NON FONCTIONNELS

La précision des mesures sur les indices de rétention a été évaluée à ± 5 unités.

Hydrocarbures aromatiques	*PS 179*		*SE-30**		*OV-17***		*Polyphényl éther 6 noyaux*		*Carbowax 20M*	
	T (°C)	*I*	*T (°C)*	*I*	*T (°C)*	*I*	*T (°C)*	*I*	*T (°C)*	*I*
Naphtalène	220	1968	200	1250	230	1499	220	1586	220	1844
Méthyl-2-naphtalène	220	2074	220	1356	230	1595	220	1689	220	1941
Méthyl-1-naphtalène	220	2118	220	1375	230	1628	220	1719	220	1979
Biphenyle	220	2179	220	1420	230	1628	220	1783	220	2056
Acénaphtène	220	2391	220	1540	230	1833	220	1929	220	2216
Acénaphtylène	220	2418	220	1511	230	1808	220	1919	220	2259
Dibenzofurane	220	2464	220	1573	230	1859			220	2308
Fluorène	240	2639	240	1659	230	1935			220	2374
Phénanthrène	240	3008	240	1850	230	2171				
Anthracène	240	3023	240	1863	230	2179				
Fluoranthène	260	3558	240	2110	230	2462				
Pyrène	260	3668	240	2162	230	2529				
1,2-Benzofluorène	280	3855	240	2249	230	2607				
2,3-Benzofluorène	280	3870	240	2254	230	2621				
1,2-Benzanthracène	280	4261	240	2448						
Chrysène	280	4294	260	2504						
Triphenylène	280	4299	260	2495						
Benzo[α]pyrène	300	5000***	260	2844						
Perylène	300	5120***	260	2850						

* Ces résultats concordent bien avec ceux publiés sur OV-101 déposé à 5% sur Gas-Chrom Q[9].

** Résultats publiés[9].

*** Précision ± 20 unités.

TABLEAU III

INDICES DE RÉTENTION D'HYDROCARBURES AROMATIQUES HYDROXYLES

La précision des mesures des indices de rétention a été évaluée à: ±5 unités sur PS 179; ±7 unités sur SE-30; ±9 unités sur polyphényl éther 6 noyaux; ±3 unités sur Carbowax 20M.

Composés	*PS 179*		*SE-30*		*Polyphényl éther 6 noyaux*		*Polyphényl éther**		*Carbowax 20M*	
	T (°C)	*I*	*T (°C)*	*I*	*T (°C)*	*I*	*T (°C)*	*I*	*T (°C)*	*I*
o-Cresol	220	1900	220	1048	220	1433	160	1354	220	2004
m-Cresol	220	1958	220	1075	220	1464	160	1386	220	2093
p-Cresol	220	1979	220	1073	220	1457	160	1385	220	2089
2,4-Diméthyl phénol	220	1979	220	1162	220	1518	160	1456	220	2087
2,5-Diméthyl phénol	220	1988	220	1161	220	1507	160	1453	220	2072
3,5-Diméthyl phénol	220	2056	220	1179	220	1550	160	1489	220	2174
3,4-Diméthyl phénol	220	2131	220	1209	220	1572	160	1530	220	2233
α-Naphthol	240	2863	200	1517			160	1944		
β-Naphthol	240	2916	200	1531			160	1969		

* Mesures effectuées par Buryan et Macàk[15] taux d'imprégnation 5% sur Chromaton N AW HMDS.

TABLEAU IV

INDICES DE RÉTENTION D'HYDROCARBURES AROMATIQUES AZOTÉS

La précision des mesures sur les indices de rétention est la même que pour les phénols (Tableau III).

Composés	*PS 179*		*SE-30*		*Polyphényl éther 6 noyaux*		*Carbowax 20M*	
	T (°C)	*I*	*T (°C)*	*I*	*T (°C)*	*I*	*T (°C)*	*I*
Quinoléine	220	2182	220	1306	220	1719	220	2023*
Isoquinoléine	220	2241	220	1334	220	1762	220	2057
Indole	220	2409	220	1324	220	1774	220	2449
Acridine	260	3222	240	1869				
Carbazole	260	3475	240	1877				

* Poulson[16] a publié pour l'indice de rétention de la quinoléine 2005 sur Chromosorb G imprégné à 5% de Carbowax.

Les dérivés fonctionnels sont encore plus retenus. La différence est plus faible lorsque le noyau aromatique est substitué par une chaine alkyle. On note même une inversion de l'ordre d'élution de l'acénaphtène et de l'acénaphtylène (*cf.* Tableau II et Fig. 4).

Ces observations mettent bien en évidence le caractère polaire du PS 179 et surtout son affinité pour les composés aromatiques et son faible pouvoir solvant des alcanes. L'emploi des "indices de rétention aromatiques" imaginés par Lee *et al.*[17] permettrait certainement d'obtenir des résultats d'un emploi plus pratique.

Sélectivité du PS 179. Pour les hydrocarbures aromatiques non fonctionnels étudiés les Figs. 4, 5 et 6 mettent en correspondance les temps de rétention mesurés sur PS 179 d'une part, sur SE-30 d'autre part. Lorsque la droite passant par les points représentant deux composés a une pente 1, la sélectivité des 2 phases pour ces

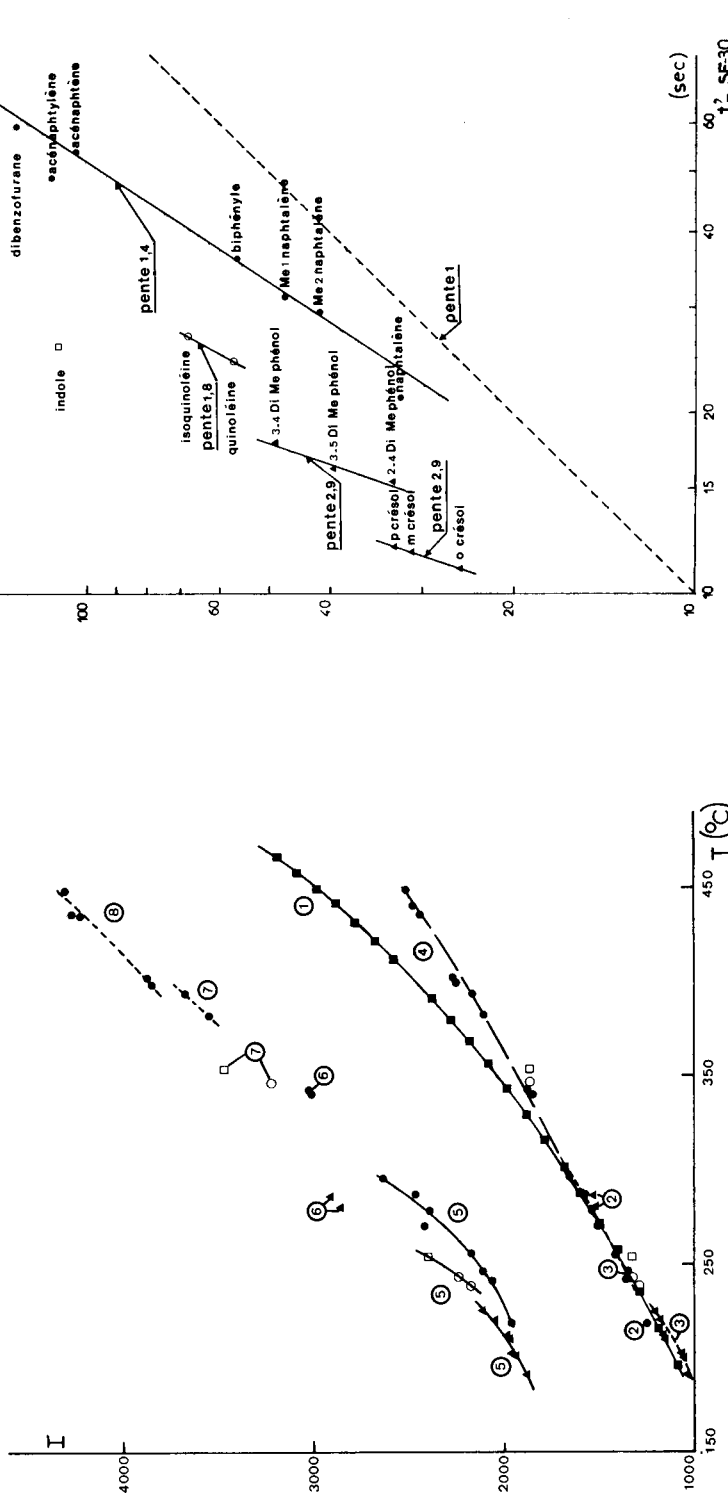

Fig. 3. Variation de l'indice de rétention de différents solutés en fonction de leur température d'ébullition. 1 = Courbe des *n*-alcanes (référence); 2 = SE-30 à 200°C; 3 = SE-30 à 220°C; 4 = SE-30 à 240°C; 5 = PS 179 à 220°C; 6 = PS 179 à 240°C; 7 = PS 179 à 260°C; 8 = PS 179 à 280°C. ■, *n*-Alcanes; ●, hydrocarbures aromatiques simples; ▲, phénols; composés aromatiques polynucléaires ayant un noyau du type pyridine (○) ou du type pyrolle (□).

Fig. 4. Sélectivité relative de PS 179 et SE-30 vis à vis de composés aromatiques de température d'ébullition inférieure à 300°C. Température de mesure: 220°C.

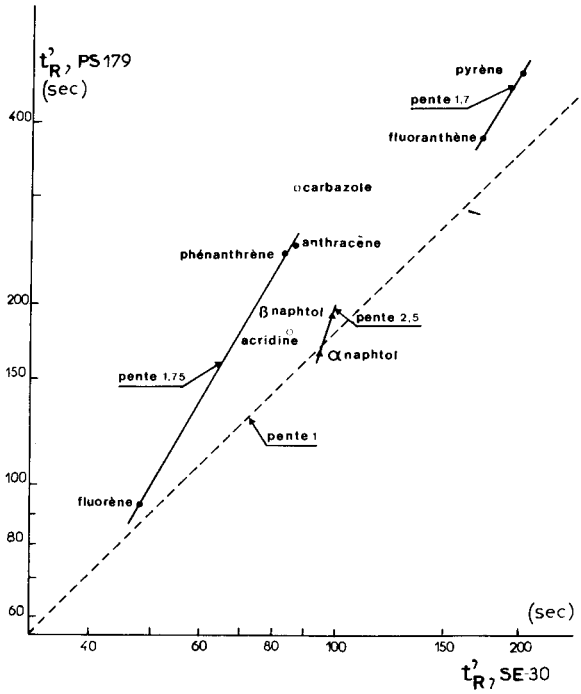

Fig. 5. Sélectivité relative de PS 179 et SE-30 vis à vis de composés aromatiques de température d'ébullition comprises entre 280 et 400°C. Température de mesure (°C):

Composé aromatique	*PS 179*	*SE-30*
Fluorène, phénanthrène, anthracène	240	240
Fluoranthène, pyrène, acridine, carbazole	260	240
α-Naphtol, β-naphtol	240	200

corps est identique. Bien sur seuls les points mesurés à la même température pour une phase donnée peuvent être utilisés.

Pour les hydrocarbures aromatiques non fonctionnels le PS 179 est d'une façon génèrale plus sélectif que le SE-30. Pour la séparation d'isomères ayant une tension de vapeur et une polarité voisines, le PS 179 est plus sélectif que le SE-30 dans la plupart des cas, par exemple pour les méthyl-naphthalènes, crésols, xylénols, quinoléines (Fig. 4), les naphtols (Fig. 5) et le couple pérylène–benzopyrène (Fig. 6). Aucune amélioration de la sélectivité n'est observée pour le couple anthracène–phénanthrène (Fig. 5) ou les benzofluorènes 1,2 et 2,3 (Fig. 6). Par contre le PS 179 est moins sélectif que le SE-30 vis à vis de la famille benzanthracène, chrysène, triphénylène (*cf.* Fig. 6). Nous avons trop peu de données sur ce point pour suggérer une règle générale. On remarquera cependant que ces trois corps sont des benzophénanthrènes.

Comparaison du PS 179 avec d'autres phases

Polarité. L'examen des Tableaux II, III, IV et de la Fig. 7 montre que le PS 179 est une phase plus polaire que l'OV-17 (une méthyl phényl silicone) et que le

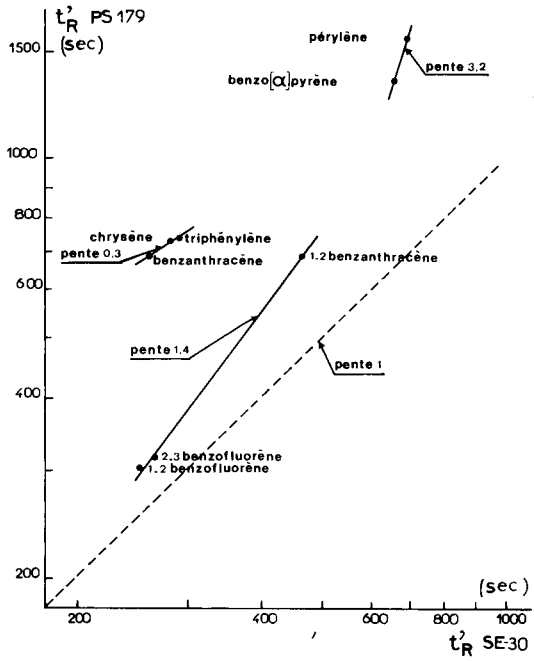

Fig. 6. Sélectivité relative de PS 179 et SE 30 vis à vis de composés aromatiques de température d'ébullition comprises entre 400 et 500°C. Température de mesure (°C):

Composé aromatique	*PS 179*	*SE-30*
1,2-Benzofluorène, 2,3-Benzofluorène, benzanthracène	280	240
Chrysène, triphenylène	280	260
Benzo[α]pyréne, pérylène	300	260

polyphényl éther à 6 noyaux. Ce résultat est important car cet éther entre dans la composition du PS 179 où plusieurs molécules de polyphényléther sont pontées. L'accroissement de polarité du PS 179 provient donc de l'introduction du groupement $-O_2S-C_6H_4-O-C_6H_4-SO_2-$ et en particulier des groupements sulfonyles.

La polarité du PS 179 est voisine de celle du Carbowax 20M. Le PS 179 qui a un caractère aromatique prononcé retient plus les hydrocarbures aromatiques que le Carbowax 20M. Par contre le Carbowax 20M retient plus les composés susceptibles de former des liaisons hydrogène, celles-ci apparaissent plus facilement avec le groupe éther alkylique du Carbowax 20M qu'avec le groupe éther aromatique du PS 179.

Sélectivité. Le PS 179 est plus sélectif que le polyphényl éther 6 noyaux et le SE-30 vis à vis des xylénols, naphtols et quinoléines (Fig. 8). La sélectivité du PS 179 est comparable à celle du Carbowax 20M vis à vis des xylénols (Fig. 8).

Pour les hydrocarbures aromatiques azotés le PS 179 est plus sélectif que le Carbowax 20M vis à vis des quinoléines mais moins sélectif vis à vis du couple quinoléine–indole. Il en est de même pour l'acridine et le carbazole (Fig. 8). La sélectivité du Carbowax 20M apparaît ainsi liée à la possibilité de former des liaisons hydrogènes avec le groupe éther, celle du PS 179 aux intéractions polaires des groupes SO_2 et aux intéractions entre systèmes aromatiques.

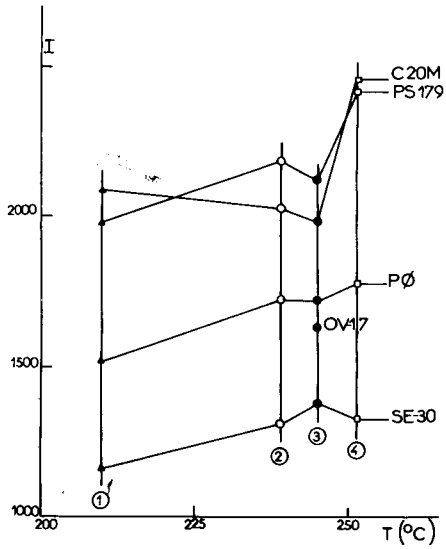

Fig. 7. Comparaison des indices de rétention de différents composés aromatiques sur PS 179, Carbowax 20M (C 20M), polyphényl éther 6 noyaux (PØ), SE-30 et OV-17. 1 = 2,4-Diméthyl phénol; 2 = quinoléine; 3 = méthyl-1 naphtalène; 4 = indole.

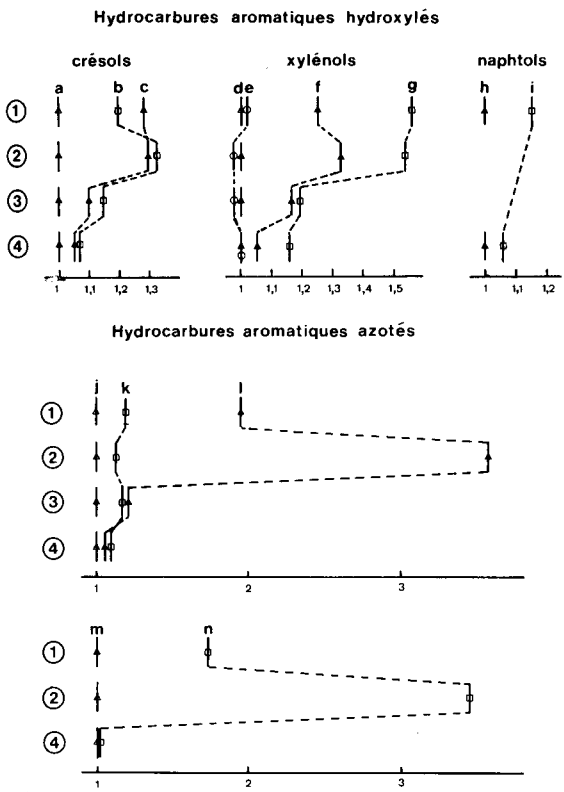

Fig. 8. Temps de rétention relatif de différents composés aromatiques mesurés sur: 1, PS 179; 2, Carbowax 20M; 3, polyphenyl éther 6 noyaux; 4, SE-30. Composés étudiés: a = *o*-crésol; b = *m*-crésol; c = *p*-crésol; d = 2,4-dimethyl phénol; e = 2,5-dimethyl phénol; f = 3,5-dimethyl phénol; g = 3,4-dimethyl phénol; h = α-naphtol; i = β-naphtol; j = quinoléine; k = isoquinoléine; l = indole; m = acridine; n = carbazole. Les références sont successivement les composés a, d, h, j, m.

CONCLUSION

L'indice de rétention d'un soluté donné sur PS 179 est fonction du support utilisé et du traitement thermique préalable subi par la colonne. Les colonnes réalisées avec le Chromosorb W AW HMDS sont plus stables que celles réalisées avec le Chromosorb P NAW passivé, G NAW et G AW DMCS et plus efficaces que celles réalisées avec NaCl dendritique[5]. Le Chromosorb W AW HMDS a donc été retenu pour l'étude des propriétés du PS 179. Il nous paraît important de souligner la nécessité d'utiliser un support peu actif, de surface spécifique assez faible, silanisé et de ne pas chauffer excessivement les colonnes. L'emploi du carbone donnerait probablement de bons résultats. Dans ces conditions la polarité du PS 179 est supérieure à celle du SE-30, du polyphényl éther 6 noyaux, de l'OV-17 et voisine de celle du Carbowax 20M.

Pour les composés aromatiques considérés le PS 179 est, en général, plus sélectif que le SE-30 et la polyphényl éther 6 noyaux. Le résultat de la comparison du PS 179 avec le Carbowax 20M depend des familles étudiées. Ces deux phases ont des polarités voisines mais seul le PS 179 permet l'analyse des hydrocarbures aromatiques de faible tension de vapeur car il est beaucoup plus stable thermiquement.

RÉSUMÉ

L'indice de retention de composés analysés par chromatographie sur PS 179 [poly(oxy aryl sulfonyl arylène)] dépend du support utilisé.

Parmi les supports étudiés nous avons choisi le Chromosorb W AW HMDS pour déterminer les indices de rétention d'hydrocarbures aromatiques polycycliques (2 à 5 noyaux) non fonctionnels et fonctionnels (hydroxylés, azotés).

La comparaison de ces indices avec ceux mesurés sur SE-30, polyphenyl ether 6 noyaux, OV-17, Carbowax 20M montre que la polarité du PS 179 est supérieure à celle du SE-30, du polyphenyl ether 6 noyaux et de l'OV-17 et voisine de celle du Carbowax 20M.

La polarité et la stabilité thermique du PS 179 permettent l'analyse de dérivés de masse moléculaire élevée des hydrocarbures aromatiques.

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HOCHLEISTUNGS-GASCHROMATOGRAPHIE AN FLÜSSIGKRISTALL-GLASKAPILLAREN

III. TRENNUNG VON ISOMEREN KOHLENWASSERSTOFFEN AN CHOLESTERINISCHER MESOPHASE

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(Eingegangen am 23. Juni 1981)

SUMMARY

High-resolution gas chromatography with liquid crystal glass capillaries. III. Separation of hydrocarbon isomers on cholesteric mesophase

High-performance glass capillary columns with cholesteryl butyrate as stationary phase were applied for separations of isomeric alkylbenzenes and *n*-alkenes in cholesteric, crystalline, isotropic and supercooled range. Structure–retention correlations, retention indices, dI/dT values and homomorphy factors are discussed.

The cholesteric modification shows a higher selectivity for *trans–cis*-isomeric *n*-alkenes, than nematic and smectic mesophases or common stationary phases.

EINLEITUNG

Nach Untersuchungen an nematischen und smektischen Modifikationen[1,2] wurde das Retentionsverhalten isomerer Kohlenwasserstoffe an einer cholesterinischen Mesophase gemessen. Solche Phasen wurden erstmals von Dewar und Schroeder[3], Barrall *et al.*[4] sowie von Kelker und Winterscheidt[5] in gepackten Säulen gaschromatographisch untersucht. Von Heath *et al.*[6] wurde Cholesterylcinnamat in Glasskapillaren zur Isomerentrennung eingesetzt. Insgesamt wurden cholesterinische Phasen wie auch smektische Flüssigkristalle in der Gaschromatographie bisher wenig verwendet.

Cholesterinische Flüssigkristalle sind eine Variante der nematischen Struktur, bei der sich die Vorzugsrichtung der einzelnen Ebenen von Schicht zu Schicht um einen bestimmten Winkel ändert. Daraus resultiert eine verdrillte Struktur, die be-

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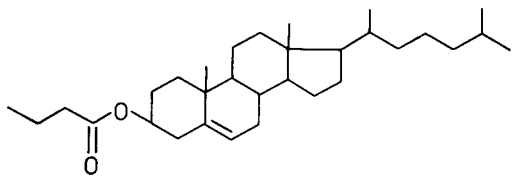
sondere Löseeigenschaften erwarten lässt. Der Drehsinn der Verdrillung hängt vom Vorliegen des *d*- oder *l*-Enantiomeren ab, die Ganghöhe der Helix ist temperaturabhängig. Die cholesterinische Mesophase tritt nur bei optisch aktiven Verbindungen auf und ist der nematischen Struktur energetisch nahezu gleichwertig.

In der vorliegenden Arbeit wurde der Einfluss der cholesterinischen Struktur auf die Selektivität der Trennung von isomeren Kohlenwasserstoffen untersucht und mit den Löseeigenschaften nematischer und smektischer Phasen verglichen.

EXPERIMENTELLES

Als stationäre Phase verwendeten wir Cholesterylbutyrat (CHOB) der Firma E. Merck (Darmstadt, B.R.D.), das mit einem cholesterinischen Bereich von 102–113°C angegeben wird[7]:



Kalorimetrische Messungen an CHOB mit einem Perkin-Elmer DSC-2 Differential-Scanning-Kalorimeter ergaben einen Schmelzbereich von 98.2–101.6°C für das erste Aufschmelzen, danach einen Schmelzpunkt von 99.3°C und einen Klärpunkt von 11.4°C. Der cholesterinische Bereich ist bis 90°C unterkühlbar.

Es wurden die bereits an nematischen und smektischen Phasen verwendeten Mischungen aller 40 isomeren *n*-Alkene C_{10}–C_{13} zusammen mit den korrespondierenden *n*-Alkanen sowie der C_7–C_{11}-*n*-Alkylbenzene einschliesslich der isomeren Xylene als Substrate eingesetzt.

Für die Messungen verwendeten wir Gaschromatographen Carlo Erba GI 452 und Perkin-Elmer F 11 mit Flammenionisationsdetektor, als Trägergas diente Wasserstoff (Säulenvordruck 0.12 MPa und lineare Geschwindigkeit, $\bar{u}$ = 34 cm/sec). Als Trennsäule wurde eine Glaskapillare von 58 m × 0.25 mm I.D. verwendet, deren Art der Vorbehandlung bereits beschrieben wurde[1].

Die Trennleistung der Flüssigkristallkapillare betrug für *cis*-2-Tridecen bei einem Kapazitätsverhältnis von k = 11,7 und 110°C n = 122,000 theoretische und N = 104,000 effektive Böden.

Das Retentionsverhalten wurde im kristallinen, cholesterinischen, isotropen sowie im unterkühlten cholesterinischen Bereich gemessen.

ERGEBNISSE UND DISKUSSION

Trennung von Alkylbenzenen

Im Gegensatz zu den Ergebnissen der nematischen und smektischen Phasen zeigte die untersuchte cholesterinische Mesophase trotz vergleichbar hoher Effektivität nur im unterkühlten Bereich kurz oberhalb der Kristallisation (92–90°C) für *p*- und *m*-Xylene eine Trennung mit einem Trennfaktor α = 1.013 bei 90°C (Fig. 1), was mit Ergebnissen der anderen cholesterinischen Phasen in Übereinstimmung steht[3,5,8].

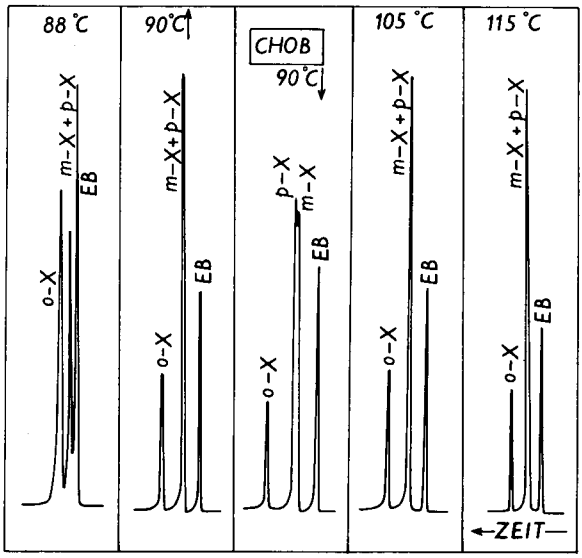


Fig. 1. Trennung von isomeren C_8-Alkylbenzenen an CHOB in Abhängigkeit von der Säulentemperatur ((Heizung↑, Kühlung↓). EB = Ethylbenzen; *o*-, *m*-, *p*-X = *o*-, *m*-, *p*-Xylen.

Die *p*-/*m*-Selektivität von CHOB ist kleiner als die von 4-*n*-Pentylacetophenon (O-4-*n*-Octyloxybenzoyloxim) (OBO)[1] und 5-*n*-Heptyl-2-(4-*n*-nonyloxyphenyl)pyrimidin (NPP)[2] und nur ausreichend für eine Verschiebung der Retention des *p*-Xylens zur Koelution mit *m*-Xylen.

Zum Vergleich der δI-Werte für *o*-Xylen–Ethylbenzen an OBO und NPP sind die δI-Werte für die gemessenen Bereiche an CHOB in Tabelle I dargestellt.

Trennung von isomeren n-Alkenen C_{10}–C_{13}

In Fig. 2–6 sind die Chromatogramme der Trennungen von isomeren C_{10}–C_{13}-*n*-Alkenen sowie der korrespondierenden *n*-Alkane nach Heizung und Kühlung der stationären Phase zu sehen. Dabei wurden die besten Trennungen im unterkühlten Mesophasenbereich gefunden. Es konnten alle 40 isomeren *n*-Alkene mit Ausnahme der *trans*-5–*trans*-6-Dodecene und der *trans*-4–*trans*-5-Undecene und -Decene getrennt werden. Trennungen der letzteren gelangen bereits an nematischen und smektischen Modifikationen bei tieferen Temperaturen mit Ausnahme der *trans*-4–*trans*-5-Decene.

TABELLE I

δI-WERTE FÜR *p*- UND *m*-XYLENE UND *o*-XYLEN-ETHYLBENZEN AN CHOB IN ABHÄNGIGKEIT VON DER SÄULENTEMPERATUR

Heizung↑, Kühlung↓.

	88°C↑	90°C↑	101°C↑	105°C↑	115°C↑	90°C↓
p- und *m*-Xylene	0	0	0	0	0	1.6
o-X–EB	38.2	39.6	45.1	45.8	46.2	43.9

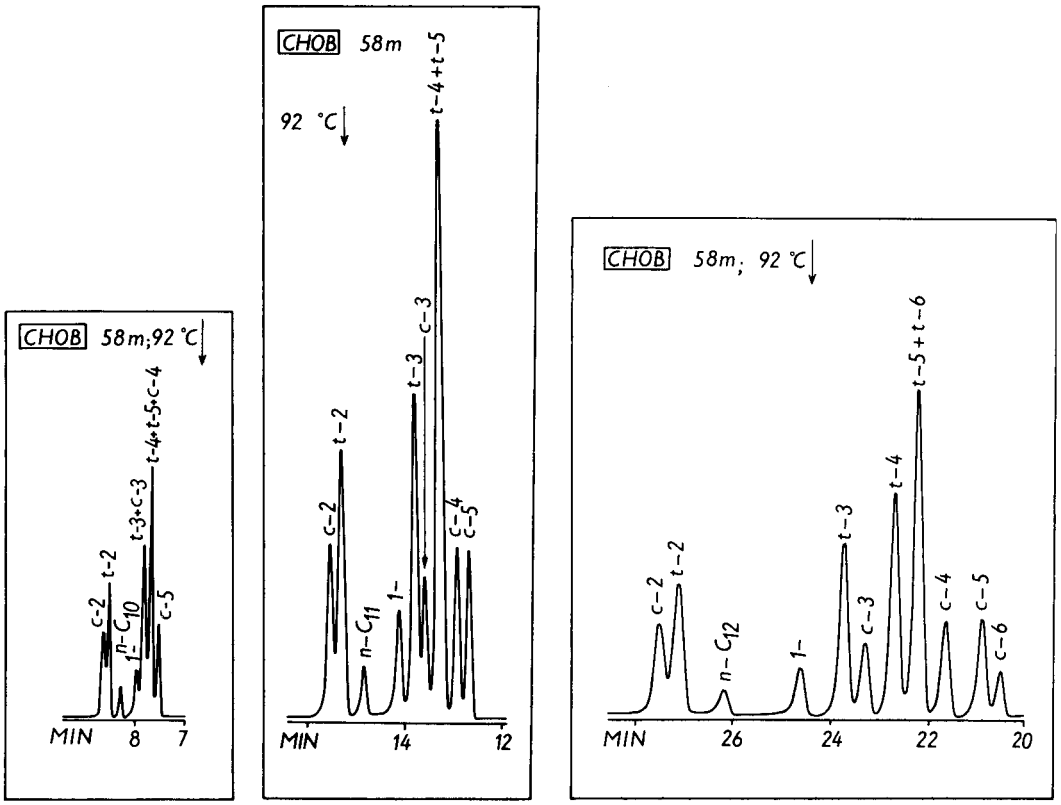


Fig. 2. Trennung von isomeren *n*-Decenen an CHOB bei 92°C (Kühlung). *c-* = *cis*-, *t-* = *trans*-Isomere; 1- = 1-Alken; *n*-C_{10} = *n*-Decan.

Fig. 3. Trennung von isomeren *n*-Undecenen an CHOB bei 92°C (Kühlung).

Fig. 4. Trennung von isomeren *n*-Dodecenen an CHOB bei 92°C (Kühlung).

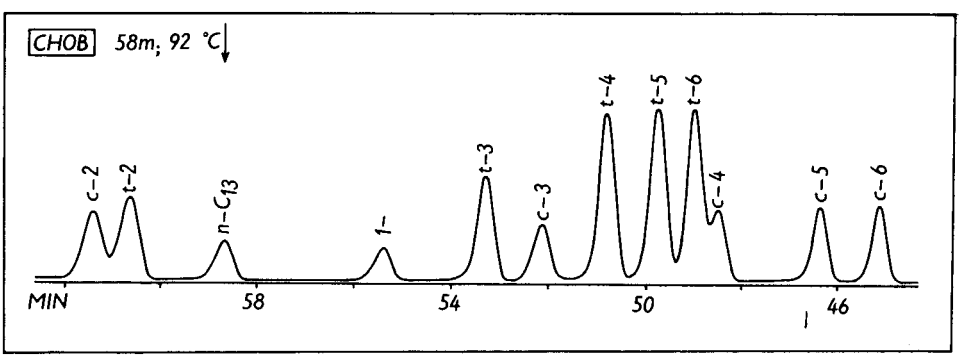


Fig. 5. Trennung von isomeren *n*-Tridecenen an CHOB bei 92°C (Kühlung).

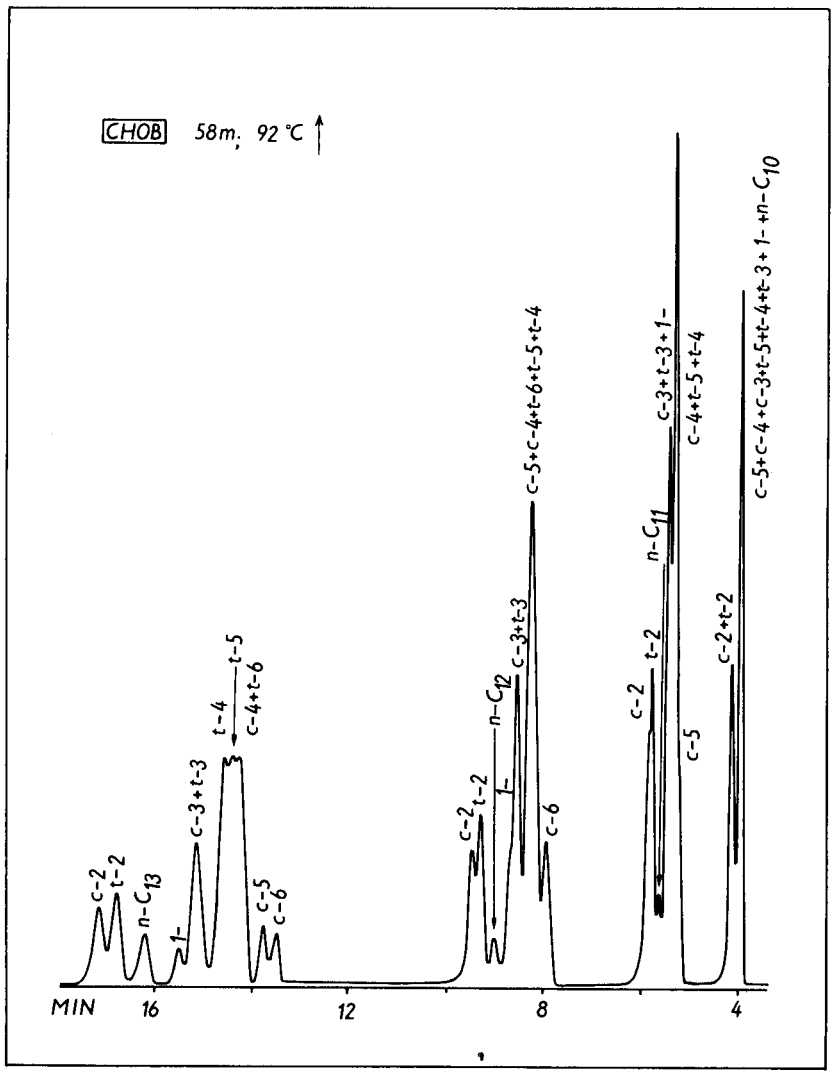


Fig. 6. Trennung von isomeren *n*-Alkenen C_{10}–C_{13} an CHOB bei 92°C (Heizung).

Die Retentionsindizes der C_{10}–C_{13}-*n*-Alkene an CHOB wurden in Tabelle II zusammengestellt.

Die Peaks wurden mit Hilfe der Struktur–Retentions-Korrelationen identifiziert[9]. Fig. 7 zeigt die Abhängigkeit der Homomorphie-Faktoren von der C-Zahl für die homologen Reihen der *n*-Alkene. Die an nematischen und smektischen Phasen gemessene Alternation ist an CHOB nicht klar erkennbar.

Die nach C-Zahl, Geometrie des Moleküls und Lage der Doppelbindung geordneten dI/dT-Werte sind in Tabelle III zusammengestellt. In jedem Fall sind diese Werte für *cis*-Isomere grösser als für korrespondierende *trans*-Isomere und ihre Differenzen sind praktisch gleich gross: $\delta(dI/dT) = 0.1$ Index-Einheiten (I.E.)/°C.

TABELLE II

RETENTIONSINDIZES DER *n*-DECENE, *n*-UNDECENE, *n*-DODECENE UND *n*-TRIDECENE, GEMESSEN AN CHOB BEI 101, 105 und 115°C (HEIZUNG) UND 92°C (KÜHLUNG) UND DIE d*I*/d*T*-WERTE FÜR DEN BEREICH 105–110°C

n-Alkene	I_{101}^{CHOB}	I_{105}^{CHOB}	I_{115}^{CHOB}	I_{92}^{CHOB}	dI^{CHOB}/dT
cis-5-Decen	982.8	984.6	987.3	980.6	–
cis-4-Decen	985.8	986.4	987.3	984.5	–
trans-5-Decen	985.8	986.4	987.3	984.5	–
trans-4-Decen	985.8	986.4	987.3	984.5	–
cis-3-Decen	989.8	990.2	991.2	988.3	–
trans-3-Decen	989.8	990.2	991.2	988.3	–
1-Decen	992.7	993.4	993.8	991.9	–
trans-2-Decen	1005.7	1006.0	1006.8	1005.0	–
cis-2-Decen	1009.1	1009.7	1011.6	1007.6	–
cis-5-Undecen	1076.8	1078.5	1080.4	1074.5	0.30
cis-4-Undecen	1079.8	1081.4	1083.5	1077.6	0.31
trans-5-Undecen	1083.0	1084.1	1085.2	1082.0	0.21
trans-4-Undecen	1083.0	1084.1	1085.2	1082.0	0.21
cis-3-Undecen	1087.7	1089.5	1090.4	1086.0	0.25
trans-3-Undecen	1088.9	1089.5	1090.4	1088.1	0.15
1-Undecen	1092.2	1093.0	1093.4	1092.1	0.11
trans-2-Undecen	1105.2	1105.7	1106.0	1104.8	0.13
cis-2-Undecen	1108.1	1109.3	1110.5	1107.1	0.21
cis-6-Dodecen	1171.1	1173.0	1175.2	1168.3	0.33
cis-5-Dodecen	1173.3	1174.7	1177.2	1170.6	0.37
cis-4-Dodecen	1177.8	1179.2	1182.0	1175.2	0.38
trans-6-Dodecen	1180.5	1181.0	1182.8	1178.6	0.27
trans-5-Dodecen	1180.5	1181.0	1182.8	1178.6	0.27
trans-4-Dodecen	1182.8	1183.2	1184.5	1181.4	0.20
cis-3-Dodecen	1187.0	1188.3	1189.8	1184.8	0.26
trans-3-Dodecen	1188.5	1188.6	1189.8	1187.3	0.19
1-Dodecen	1192.8	1193.1	1193.6	1192.1	0.08
trans-2-Dodecen	1205.4	1205.5	1206.0	1204.7	0.08
cis-2-Dodecen	1208.2	1208.8	1210.0	1206.5	0.20
cis-6-Tridecen	1266.8	1268.6	1271.3	1263.7	0.35
cis-5-Tridecen	1270.4	1272.1	1274.7	1267.4	0.34
cis-4-Tridecen	1276.7	1277.8	1279.4	1273.6	0.24
trans-6-Tridecen	1276.7	1277.8	1279.4	1275.0	0.24
trans-5-Tridecen	1278.9	1279.9	1281.4	1277.1	0.22
trans-4-Tridecen	1281.5	1282.2	1283.4	1280.0	0.16
cis-3-Tridecen	1285.8	1287.1	1289.1	1283.7	0.31
trans-3-Tridecen	1287.7	1288.4	1289.1	1286.7	0.13
1-Tridecen	1292.7	1293.1	1293.8	1292.1	0.06
trans-2-Tridecen	1305.1	1305.5	1305.9	1304.5	0.06
cis-2-Tridecen	1307.8	1308.8	1310.0	1306.2	0.17

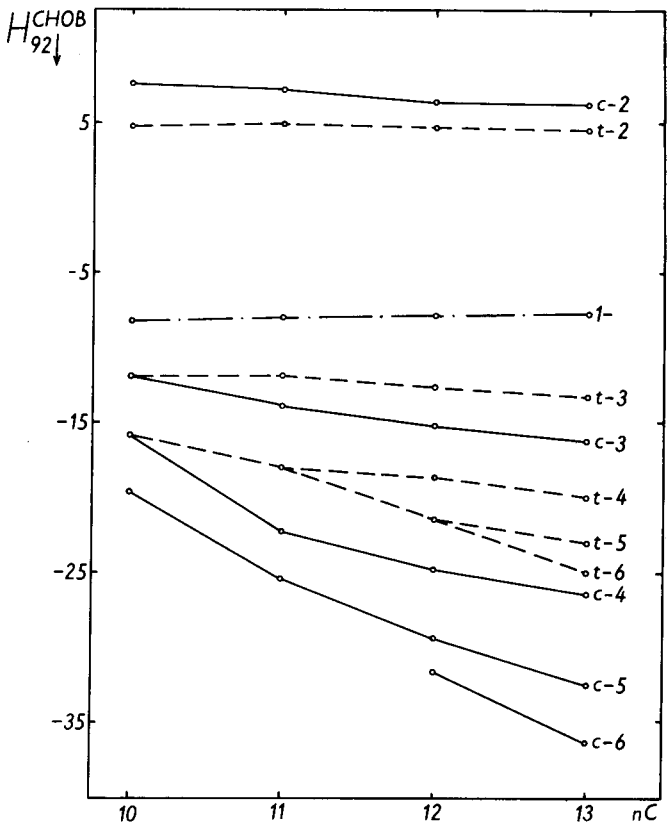


Fig. 7. Homomorphiefaktoren H_{92}^{CHOB} von C_{10}–C_{13}-*n*-Alkenen an CHOB bei 92°C (Kühlung).

TABELLE III

ABHÄNGIGKEIT DER d*I*/d*T*-WERTE VON DER ZAHL DER KOHLENSTOFFATOME, DER MOLEKÜLGEOMETRIE UND DER LAGE DER DOPPELBINDUNG FÜR *n*-ALKENE C_{11}–C_{13} AN CHOB

Alkene	C_{11}	C_{12}	C_{13}
trans-2-	0.13	0.08	0.06
trans-3-	0.15	0.19	0.13
trans-4-	0.21	0.20	0.16
trans-5-	0.21	0.27	0.22
trans-6-	–	0.27	0.24
cis-2-	0.21	0.20	0.17
cis-3-	0.25	0.26	0.31
cis-4-	0.31	0.38	0.24
cis-5-	0.30	0.37	0.34
cis-6-	–	0.33	0.35
1-	0.11	0.08	0.06

TABELLE IV

TRENNFAKTOREN α FÜR BENACHBARTE LAGEISOMERE VON *n*-TRIDECENEN AN CHOB (HEIZUNG UND KÜHLUNG)

Alkene	*Heizung (°C)*					*Kühlung (°C)*				
	88	*92*	*101*	*105*	*115*	*88*	*90*	*92*	*101*	*105*
cis-2–*cis*-3	1.165	1.164	1.173	1.167	1.154	1.176	1.189	1.187	1.173	1.168
cis-3–*cis*-4	1.078	1.079	1.067	1.068	1.068	1.084	1.081	1.080	1.069	1.066
cis-4–*cis*-5	1.040	1.041	1.048	1.042	1.032	1.035	1.049	1.048	1.046	1.045
cis-5–*cis*-6	1.026	1.025	1.026	1.025	1.023	1.027	1.029	1.029	1.027	1.025
trans-2–*trans*-3	1.133	1.134	1.135	1.130	1.122	1.135	1.146	1.145	1.136	1.130
trans-3–*trans*-4	1.050	1.048	1.046	1.045	1.039	1.049	1.054	1.052	1.047	1.044
trans-4–*trans*-5	1.013	1.014	1.019	1.016	1.014	1.017	1.023	1.023	1.018	1.016
trans-5–*trans*-6	1.015	1.014	1.015	1.015	1.014	1.016	1.017	1.016	1.016	1.015

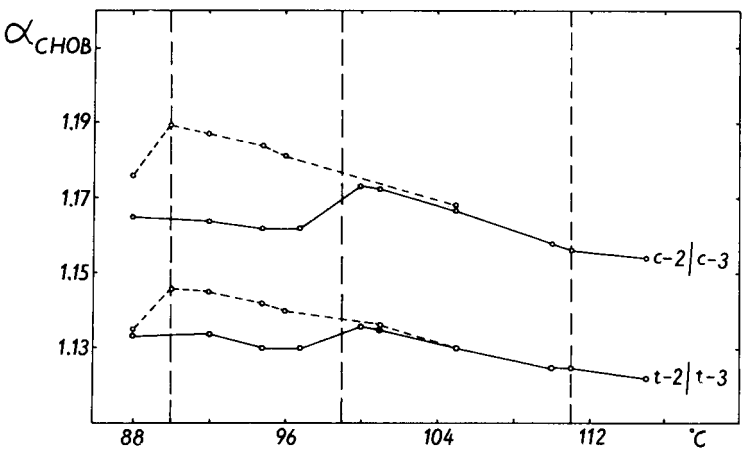


Fig. 8. Temperaturabhängigkeit der Trennfaktoren α für *cis*-2–*cis*-3- und *trans*-2–*trans*-3-Tridecene an CHOB. —— = Heizung, ------- = Kühlung.

TABELLE V

TRENNFAKTOREN α FÜR BENACHBARTE LAGEISOMERE VON *n*-TRIDECENEN AN CHOB, OBO UND SQUALAN (SQ) BEI 90°C

Tridecene	α_{90}^{CHOB}	α_{90}^{OBO}	α_{90}^{SQ}
cis-6–*cis*-5	1.029	1.024	1.021
cis-5–*cis*-4	1.049	1.041	1.038
cis-4–*cis*-3	1.081	1.070	1.048
cis-3–*cis*-2	1.189	1.186	1.143
trans-6–*trans*-5	1.017	1.012	1.018
trans-5–*trans*-4	1.023	1.016	1.004
trans-4–*trans*-3	1.054	1.050	1.040
trans-3–*trans*-2	1.146	1.145	1.097

TABELLE VI

TRENNFAKTOREN α FÜR GEOMETRISCHE ISOMERE VON *n*-TRIDECENEN AN CHOB, OBO UND SQUALAN (SQ) BEI 90°C

Tridecene	α_{90}^{CHOB}	α_{90}^{OBO}	α_{90}^{SQ}
trans-6–*cis*-6	1.092	1.066	1.052
trans-5–*cis*-5	1.077	1.054	1.048
trans-4–*cis*-4	1.052	1.029	1.014
trans-3–*cis*-3	1.026	1.009	1.007
trans-2–*cis*-2	0.988	0.974	0.966

Die Trennfaktoren α (t'_{R_2}/t'_{R_1}) für benachbarte Lageisomere zeigt Tabelle IV, die Abhängigkeit der α-Werte von Heizung und Kühlung der stationären Phase für *cis*-2–*cis*-3- und *trans*-2–*trans*-3-Tridecene ist in Fig. 8 dargestellt.

Der Vergleich der Trennfaktoren von CHOB mit nematischer Phase (OBO)[1] und üblicher stationärer Phase (Squalan)[8] bei 90°C ist in Tabelle V zu sehen. Für geometrische Isomere zeigt CHOB grössere α-Werte im Vergleich mit OBO und Squalan als Folge der grösseren Löslichkeit für *trans*-Isomere (Tabelle VI).

SCHLUSSFOLGERUNGEN

Die untersuchte cholesterinische Mesophase zeigte im Vergleich mit nematischen und smektischen Flüssigkristallen eine geringere Selektivität für *p*- und *m*-Xylene. Für lageisomere *n*-Alkene ist die Selektivität vergleichbar, für geometrische Isomere ist sie jedoch grösser als bei nematischen und smektischen Phasen.

Die Trennungen auf CHOB, OBO und NPP sind ähnlich aufgrund der vergleichbaren Effektivität der Kapillaren und der Optimierung der Trenntemperatur.

Struktur–Retentions-Korrelationen an Homorphie-Faktoren und dI/dT-Werten ermöglichten die Charakterisierung der isomeren *n*-Alkene.

DANK

Die Autoren danken Herrn Dr. A. Wiegeleben (Halle) für die Durchführung der kalorimetrischen Messungen.

ZUSAMMENFASSUNG

Hochleistungs-Glaskapillaren mit Cholesterylbutyrat als stationärer Phase wurden im cholesterinischen, kristallinen, isotropen sowie im unterkühlten Bereich zur Trennung von isomeren Alkylbenzenen und *n*-Alkenen eingesetzt. Struktur–Retentions-Korrelationen, Retentionsindizes, dI/dT-Werten und Homomorphie-Faktoren wurden diskutiert.

Die cholesterinische Modifikation zeigte im Vergleich mit nematischen und smektischen Mesophasen sowie mit üblichen stationären Phasen eine grössere Selektivität für *trans*–*cis*-isomere *n*-Alkene.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF INORGANIC PLATINUM(II) COMPLEXES USING SOLVENT-GENERATED ANION EXCHANGERS

II. THE EFFECT OF ELECTROLYTES ON SOLUTE RETENTION

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SUMMARY

The chromatographic properties of bivalent neutral platinum complexes on solvent-generated anion exchange columns were investigated using cisplatin (*cis*-dichlorodiammineplatinum; a clinically useful anti-neoplastic agent) as a model compound. Solute retention was controlled by the addition of electrolytes to totally aqueous mobile phases. The effect of salts on retention was rationalized in terms of the Stern–Gouy–Chapman theory of electrical double layers and the application of solvophobic theory. The presence of bromide or nitrate in the mobile phase decreased retention, apparently by decreasing the thermodynamic activity of the cationic sites in the stationary phase due to the creation of an inner Helmholtz plane in the electrical double layer. At low concentration, citrate and acetate (at pHs where the carboxylate is appreciably ionized) caused increased retention of cisplatin owing to the effect of the added salt on the surface tension of the mobile phase as rationalized by solvophobic theory. At higher concentrations of citrate, retention of the solute decreased owing to the secondary contribution of electrostatic effects. Retention was explained in terms of ion–dipole interactions reinforced by hydrophobic contributions.

INTRODUCTION

Interest in the clinical analysis of neutral bivalent platinum species has been stimulated by laboratory and clinical evidence demonstrating the dramatic anti-neoplastic activity of cisplatin, [*cis*-dichlorodiammineplatinum(II)], $[Pt(NH_3)_2Cl_2]^0$ (CDDP), toward many solid malignancies which are refractory to other drug therapy[1]. Such analysis requires a chromatographic component since these compounds need to be separated from biological matrices and are highly reactive toward nucleophiles both in aqueous solution and in biological fluid[1].

Most clinically applicable methods for cisplatin are non-selective and respond to total platinum in the sample without regard to the ligands co-ordinated to the metal. Basolo *et al.*[2] first described separation of cisplatin from other platinum com-

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plexes on a cellulose support and observed that retention was increased with increasing concentration of organic modifier in the mobile phase. More recently, we have reported that cisplatin is retained on chemically bonded and solvent-generated anion exchange columns[3–5]. In both instances, retention is primarily due to ion–dipole interactions. Whereas the addition of organic modifier to the chemically bonded system increased the capacity ratio (k') of cisplatin, organic modifiers facilitated elution of the solute from the solvent-generated exchanger. This difference in behavior was attributed to the effect of organic modifiers on the activity coefficients of the solute and cationic surfactant in the stationary phase. However, on solvent-generated exchangers, the maximum value of k' obtainable only approached unity.

The addition of electrolytes to the mobile phase produced profound effects on the chromatographic behavior of cisplatin on the solvent-generated and the chemically bonded anion exchange system, at similar salt concentrations. The potential for controlling the chromatographic properties of bivalent platinum compounds on solvent-generated anion exchange columns by the manipulation of electrolyte composition and concentration in purely aqueous mobile phases is the subject of the present study. The opportunity for carrying out such separations in the absence of organic modifiers is particularly attractive because these systems are being interfaced with detectors whose performance is compromised by the presence of organic solvents in the mobile phase.

EXPERIMENTAL

High-performance liquid chromatography (HPLC)

The liquid chromatograph consisted of an Altex Model 110A pump (Altex, Berkeley, CA, U.S.A.) and an Altex Model 210 injector fitted with a 20-μl loop. The eluent composition was monitored with an Altex Model 156 differential refractometer and an Altex Model 153 UV detector (280 nm) connected in series. The detector outputs were recorded on a dual pen potentiometric chart recorder. A flow-rate of 1.0 ml min^{-1} was used throughout.

A μBondapak C_{18} column (10 μm; 300 × 3.9 mm I.D.) was obtained from Waters Assoc. (Milford, MA, U.S.A.) and an ODS Hypersil column (Shandon & Southern, Sewickley, PA, U.S.A.; 5 μm, 100 × 4.6 mm I.D.) was slurry packed according to the method described by Bristow *et al.*[6]. The column temperature was maintained at (30 ± 0.1)°C as described previously[5]. Solute capacity ratios, k', were calculated[5] using deuterium oxide for the determination of t_0.

Solvent-generated anion exchangers were prepared by the adsorption of hexadecyltrimethylammonium bromide (HTAB) onto the hydrophobic stationary phases[5]. After the column had been pre-loaded, stability was maintained by the addition of 10^{-4} mol dm^{-3} HTAB to the mobile phase.

Materials

HTAB was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without further treatment. Distilled water was used throughout. All other chemicals were of at least reagent grade and used as received from various sources.

Crystalline samples of cisplatin and *trans*-dichlorodiammineplatinum(II) were obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Stable solu-

tions of these solutes were prepared in 0.1 mol dm^{-3} sodium chloride. Aqueous solutions containing a mixture of *cis*-$[Pt(NH_3)_2BrCl]^0$ and *cis*-$[Pt(NH_3)_2Br_2]^0$ were prepared *in situ* by incubating cisplatin in 1 mol dm^{-3} sodium bromide for *ca.* 2 h at 30°C. Similarly, an aqueous solution containing a mixture of *cis*-$[Pt(NH_3)_2Cl(H_2O)]^+$ and *cis*-$[Pt(NH_3)_2(H_2O)]^{2+}$, was prepared by incubating cisplatin in pure water.

Kinetic studies

The degradation of cisplatin in aqueous solutions containing various anions (as their sodium salts) was investigated at 30°C. The initial concentration of cisplatin was either 0.1 or 0.2 mg ml^{-1}. The effect of nucleophile type and concentration was studied over the following ranges: bromide, 0.01–1.00 mol dm^{-3}; nitrate, 0.01–1.00 mol dm^{-3}; acetate 0.05–0.30 mol dm^{-3}; citrate 0.01–0.80 mol dm^{-3}. In the cases of acetate and citrate, the effects of pH were also studied over the range 2.8–6.0. Cisplatin disappearance was monitored by HPLC with UV detection comparing the peak heights of the drug with standard solutions prepared in 0.1 mol dm^{-3} sodium chloride.

RESULTS

Effect of monovalent ions

The effect of the addition of the monovalent anions, nitrate, bromide and acetate (as their sodium salts) on the retention of cisplatin was investigated over the concentration range 0–0.1 mol dm^{-3}. In the case of acetate, the effect of pH was also determined.

Fig. 1 shows the relationship between the reciprocal of the capacity ratio of cisplatin, the concentration of added salt, and, in the case of acetate, the effect of pH. The addition of sodium nitrate or sodium bromide to the mobile phase resulted in a decrease in the retention of cisplatin. This effect was more pronounced for nitrate than for bromide. For both these salts, retention decreased more rapidly between 0 and 0.02 mol dm^{-3} added salt than at higher concentrations. In the case of added acetate buffer (pK_a 4.76), retention was dependent upon both pH and concentration. At pH 7.0, cisplatin retention increased with increasing concentration of acetate buffer. At pH 5.00, the retention of cisplatin increased slightly between 0 and 0.02 mol dm^{-3} acetate and then remained constant at higher concentrations; whereas at pH 3.5, cisplatin retention decreased with increasing buffer concentration.

Effect of multivalent ions

The effect of citrate (pK_a 3.13, 4.76 and 5.40) on the retention of cisplatin was investigated as a function of buffer concentration and pH, and the results are shown in Fig. 2. The addition of low concentrations of citrate ($0–5 \cdot 10^{-3}$ mol dm^{-3}) produced a dramatic enhancement of the retention of cisplatin, and this effect increased with increasing pH. The retention of the drug decreased gradually with increasing concentration of citrate over the range $5 \cdot 10^{-3}$ to $1 \cdot 10^{-1}$ mol dm^{-3}.

The addition of the dibasic anion, sulphate, produced a similar enhancement of the retention of cisplatin. With addition of 10^{-1} mol dm^{-3} sodium sulphate, cisplatin eluted with a capacity ratio of 3.22. However, inclusion of sodium sulphate in the

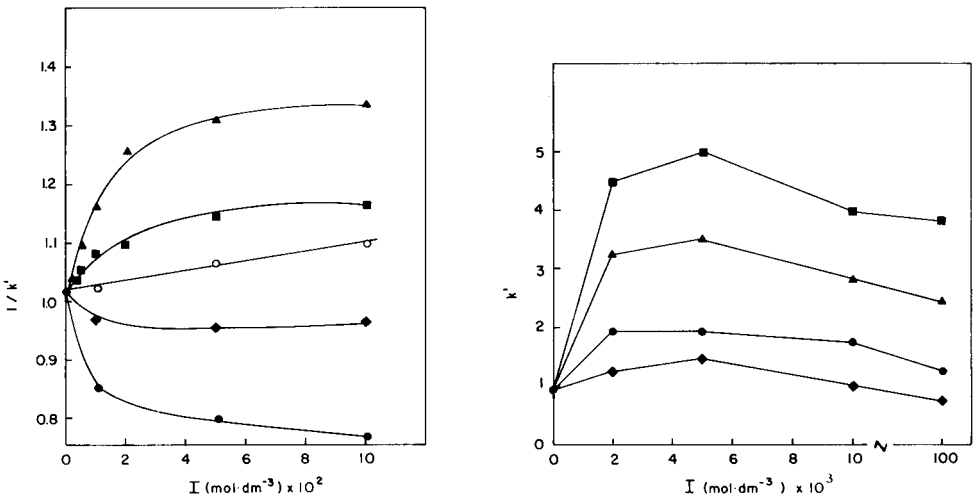

Fig. 1. The effect of salt concentration (I) on the capacity ratio (k') of cisplatin. Stationary phases, μBondapak C_{18} loaded with 1.31 μmol m^{-2} HTAB; mobile phase, 10^{-4} mmol dm^{-3} HTAB in water plus sodium bromide (■), sodium nitrate (▲) or sodium acetate–acetic acid buffers at pH 7.0 (●), 5.0 (◆) and 3.5 (○); temperature, 30 ± 0.1°C; solute concentration, 1.0 mg ml^{-1}.

Fig. 2. The effect of citrate buffer concentration (I) on the capacity ratio (k') of cisplatin. Stationary phases, μBondapak C_{18} loaded with 1.31 μmol m^{-2} HTAB; mobile phases, 10^{-4} μmol dm^{-3} HTAB in water plus citrate buffer at pH 7.0 (■), 5.5 (▲), 4.0 (●) and 3.00 (◆); temperature, 30 ± 0.1°C; solute concentration, 1 mg ml^{-1}.

mobile phase was associated with gradually increasing column back-pressure on repeated injection of cisplatin. This column blockage precluded further investigations into the effects of sulphate.

DISCUSSION

By measurement of breakthrough times a monolayer of 1.31 μmol m^{-2} HTAB was found on the stationary phase (μBondapak C_{18}), in equilibrium with a mobile phase of 10^{-4} mol dm^{-3} HTAB. Such systems are capable of retaining anionic solutes as a result of electrostatic interactions[8–10] and are often termed "solvent-generated" anion exchangers. Such systems are also useful in the resolution of neutral inorganic solutes such as cisplatin[5] with a high degree of functional group selectivity (Table I). Evidence has been presented[5] suggesting that retention of neutral platinum-(II) species arises from ion–dipole interactions between solute and the cationic stationary phase. Further evidence for ion–dipole mediated retention is provided here since the apolar transplatinum is less well retained than the more polar *cis*-isomer in these systems (Table I). However, the elution order of the three halogenated complexes ($[Pt(NH_3)_2Cl_2]^0$, $[Pt(NH_3)_2BrCl]^0$ and $[Pt(NH_3)_2Br_2]^0$ suggests that these ion–dipole interactions are reinforced by a hydrophobic effect[11]. Clearly, electrostatic repulsive forces are responsible for the poor retention and the lack of separation of the two aquated species $[Pt(NH_3)_2Cl(H_2O)]^+$ and $[Pt(NH_3)_2(H_2O)_2]^{2+}$. The increased HTAB uptake observed on the column used in this study is consistent with

TABLE I

THE CAPACITY RATIOS, k', OF SOME PLATINUM(II) COMPLEXES

Stationary phase, μBondapak C_{18} + 1.31 μmol m^{-2} HTAB; mobile phase, 10^{-4} mol dm^{-3} HTAB in water; temperature, 30 ± 0.1°C.

Solute	k'*
cis-$[Pt(NH_3)_2(H_2O)_2]^{2+}$	0.10
cis-$[Pt(NH_3)_2Cl(H_2O)]^{+}$	0.10
trans-$[Pt(NH_3)_2Cl_2]^0$	0.30
cis-$[Pt(NH_3)_2Cl_2]^0$ (CDDP)	0.98
cis-$[Pt(NH_3)_2BrCl]^0$	1.35
cis-$[Pt(NH_3)_2Br_2]^0$	2.50

* $k' = (t_{CDDP} - t_0)\, t_0^{-1}$; t_0 determined with ^2H_2O.

batch-to-batch variation between columns and accounts for the higher retention of cisplatin compared with that seen previously[5].

Retention model for cisplatin

The effects of adding salt to the mobile phase on the retention of ionic solutes in ion exchange systems is well documented (*e.g.*, ref. 12). These effects may be described in terms of competition between the solute ions and mobile phase ions for the oppositely charged stationary phase ligands. Thus, increasing the ionic strength of the mobile phase decreases the retention of ionic solutes such that their capacity ratios are reciprocally related to the activity of the competing ions in the mobile phase[12].

It is clear from Figs. 1 and 2 that the modifying influence of added anions on the retention of cisplatin arises from at least two effects. One effect apparently arises from the influence of the anions on the electronic character of the stationary phase, which can be rationalized in terms of the Stern–Gouy–Chapman (SGC) theory of electrical double layers[13]. A second "salting out" effect arises from the influence of added salt on the mobile phase surface tension, which may be explained by the application of solvophobic theory[14–16].

According to the SGC theory, the positively charged stationary phase would have an associated electrical double layer of anions which preserves electrical neutrality. Within this double layer, the electrical potential decays linearly between the surface and the outer Helmholtz plane (OHP) and nearly exponentially between the OHP and the bulk mobile phase. Increasing the electrolyte concentration may result in a concomitant increase in the anion concentration in the double layer, followed by increased adsorption of quaternary ammonium ions to maintain electrical neutrality. These effects, however, appear to play a minor role in the present system, since the rapid column equilibration times observed on changing the salt concentration were inconsistent with further adsorption of the cationic surfactant.

A second consequence of an increase in the ionic concentration of the mobile phase is specific adsorption of dehydrated anions producing a inner Helmholtz plane (IHP). Adsorption of anions at the IHP results in ion-pair formation[17] and a reduction in the thermodynamic activity of the sorbed cationic surfactant. This postulation

is consistent with the findings of Cantwell and Puon[17] and provides the most likely explanation for the decrease in retention of cisplatin with increasing salt concentration.

Basolo *et al.*[2] have shown previously that increased ionic strength reduces the interaction of cisplatin with polar adsorbents such as cellulose as a result of effects of the ionic cloud of the solute. Thus, it may be concluded that increases in the ion concentration in the stationary phase may reduce retention in the present system owing to a reduction in the activity coefficients of both the solute and quaternary ammonium groups in the stationary phase.

A priori, increases in the size, charge and polarizability of the anionic species present in the two chromatographic phases of the solvent-generated anion exchanger would be expected to decrease the retention of cisplatin. This was not observed in the cases of acetate and citrate, which produced complex changes in retention. Increased citrate concentration produced an initial increase in retention to $5 \cdot 10^{-3}$ mol dm^{-3} citrate with a decrease in retention at higher concentrations. The form of the relationship between k' and salt concentration did not change with pH, but decreased in magnitude with decreasing pH, presumably owing to a reduction in the charge on the citrate ion. At pH 3.0, the retention of cisplatin was relatively unaffected by citrate concentration, indicating that un-ionized citric acid has little effect on cisplatin retention.

At higher pH, acetate caused a slight enhancement of the retention of cisplatin, as opposed to the decrease observed with bromide and nitrate. However, at low pH, the presence of undissociated acetic acid produced a decrease in retention. This latter effect probably arises from the ability of acetic acid to behave as an organic modifier, in a fashion analogous to methanol, which has been shown to reduce the retention of cisplatin in solvent-generated anion exchange systems[5]. At pH 5.0, the retention of cisplatin was relatively independent of acetate concentration since the protonated and unprotonated species are present in approximately equal proportions and the two effects tended to cancel.

It is likely that the salting-out effects responsible for increased retention observed with citrate, sulphate and acetate arise from a hydrophobic effect due to the influence of salt on mobile phase surface tension. Further evidence of a hydrophobic effect influencing the retention of platinum(II) complexes is shown by the elution order of the brominated solutes (Table I). Replacement of one or both of the chloro groups in cisplatin by the more hydrophobic bromo group results in increased retention. Furthermore, the reduced retention of transplatinum, which presents a smaller hydrophobic surface than cisplatin, may be due to solvophobic effects as well as differences in dipole moments.

Horváth and co-workers[15,16] have applied solvophobic theory[14] to reversed-phase HPLC systems employing secondary equilibria. They have shown that the retention of solutes in these systems may be described by an equation of the form:

$$\ln k' = k_s + k_p + k_h(\Delta A) \qquad (1)$$

which is a summation of all possible solute–solvent–stationary phase interactions that may contribute to the retention of the solute. The term k_s depends only on the properties of the mobile and stationary phases (*i.e.*, it is solute independent); k_p in this

system describes the ion–dipole interactions between cisplatin and the cationic stationary phase. The third term, $k_h(\Delta A)$, is a measure of the hydrophobic interactions, since k_h is given by $\gamma(RT)^{-1}$ where γ is the surface tension of the mobile phase, and ΔA is the decrease in hydrophobic surface area on binding of the solute to the stationary phase[15,16].

The addition of salt to the mobile phase results in an increase in surface tension[15,18] according to

$$\gamma = \gamma_0 + \tau m \tag{2}$$

where γ_0 is the surface tension of the mobile phase in the absence of salt, m is the molal salt concentration, and τ is a constant related to the nature of the added salt. Combining eqns. 1 and 2 gives eqn. 3

$$\ln k = k_s + k_p + (\gamma_0 + m\tau)(\Delta A)\,(RT)^{-1} \tag{3}$$

which predicts a linear relationship between $\ln k'$ and τ at a fixed salt concentration. Table II shows the relationship between the capacity ratio of cisplatin at a fixed salt concentration and the values[18] of τ, which is given by

$$\ln k' = 0.85\tau - 1.23 \qquad r = 0.998 \qquad n = 4 \tag{3a}$$

TABLE II

THE RELATIONSHIP BETWEEN THE CAPACITY RATIO, k', OF CISPLATIN AT A FIXED ELECTROLYTE CONCENTRATION IN THE MOBILE PHASE AND THE τ VALUES OF THE ADDED SALTS

Electrolyte	k'*	τ**
Sodium nitrate	0.74	1.06
Sodium bromide	0.86	1.32
Acetate buffer (pH 7.0)	1.31	***
Sodium sulphate	3.22	2.73
Citrate buffer	3.97	3.12

* Stationary phase, μBondapak + 1.31 μmol m^{-2} HTAB; mobile phase, 10^{-4} mol dm^{-3} HTAB + 0.1 mol dm^{-3} electrolyte; temperature, 30 C.

** See eqn. 3 in ref. 18.

*** Not reported in the literature.

These results indicate that the complex effects of salts on the retention of cisplatin in solvent-generated anion exchangers arise from two effects, as described by eqn. 3. Increasing in the ionic concentration of the mobile phase results in a reduction in the thermodynamic activity of the cationic binding sites leading to a decrease in the contributions of k_p and k_s (eqn. 3) and decreased retention of cisplatin. However, addition of salt to the mobile phase may also lead to an enhancement of retention owing to its influence on the hydrophobic term, $k_h(\Delta A)$.

For salts with a large value of τ (*e.g.*, trisodium citrate), the retention of cis-

platin is governed by the hydrophobic term, $k_h(\Delta A)$. Conversely, the terms k_p and k_s dominate the effect of salts with small values of τ (*e.g.*, sodium nitrate). Although no values of τ have been reported for sodium acetate and the mono- and bivalent sodium citrate, the data presented by Melander and Horvath[18] suggest that τ values decrease with decreasing charge and ionic size. This would account for the decrease in retention of cisplatin with decreasing pH of the citrate buffer in the mobile phase and the lower retention observed for acetate compared with citrate.

Stability of cisplatin

The rate of degradation of cisplatin from aqueous solution (Fig. 3) was limited by its rate of aquation, and thus was independent of pH and the presence of citrate, acetate and nitrate[4,19]. Conversely, in the presence of bromide, degradation shows a first-order dependency on bromide concentration (second-order rate constant, k_2 = 0.56 h^{-1} mol^{-1} dm^3 at 30°C)[20,21] as well as proceeding via aquation, *i.e.*, $k_{obs} = k_2[Br^-] + k_1$ (where k_1 is the intrinsic rate constant due to aquation). Thus, within the elution times of cisplatin under all mobile phase conditions, less than 5% cisplatin was degraded on the column.

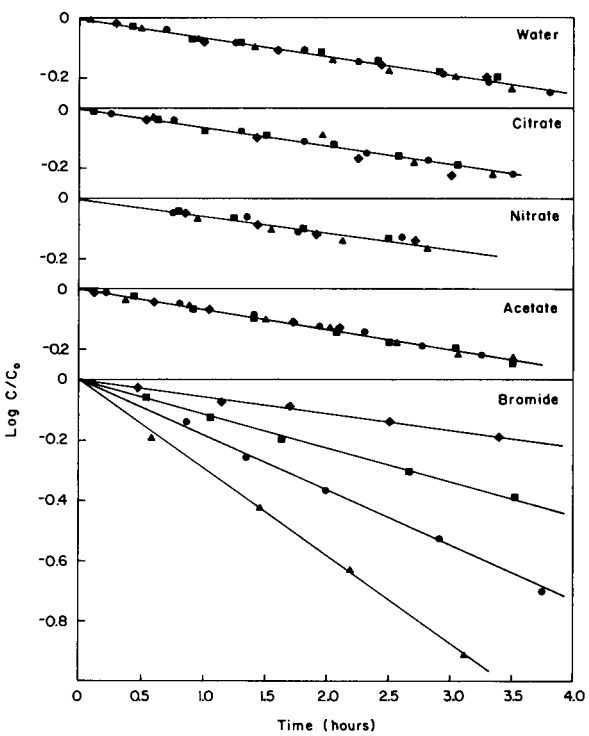

Fig. 3. Degradation of cisplatin with time from aqueous solutions containing various anionic species (at 30°C). Citrate: ■, 0.01 mol dm^{-3}, pH 2.57; ●, 0.10 mol dm^{-3}, pH 5.74; ◆, 0.01 mol dm^{-3}, pH 4.16; ▲, 0.80 mol dm^{-3}, pH 2.78. Nitrate: ■, 0.01 mol dm^{-3}; ●, 0.10 mol dm^{-3}; ◆, 0.50 mol dm^{-3}; ▲, 1.00 mol dm^{-3}. Acetate: ●, 0.05 mol dm^{-3}, pH 4.76; ▲, 0.10 mol dm^{-3}, pH 3.62; ◆, 0.20 mol dm^{-3}, pH 2.84; ■, 0.30 mol dm^{-3}, pH 5.75. Bromide: ◆, 0.01 mol dm^{-3}; ■, 0.30 mol dm^{-3}; ●, 0.60 mol dm^{-3}; ▲, 1.00 mol dm^{-3}. For reaction studied in pure water, symbols represent each of four separate determinations. The concentration of cisplatin is expressed in terms of the fraction of the initial concentration, C/C_0.

The HPLC systems developed for the assays of cisplatin were capable of separating the degradation products of cisplatin formed during the stability studies. In most cases it was possible to monitor the rate of production of these degradation products as well as the rate of loss of cisplatin. Some of the assays were performed on a short (100 mm) column packed with ODS Hypersil, which produced identical separations to those obtained on μBondapak C_{18} but offered significant advantages in terms of improved peak shapes and shortened analysis times (Fig. 4).

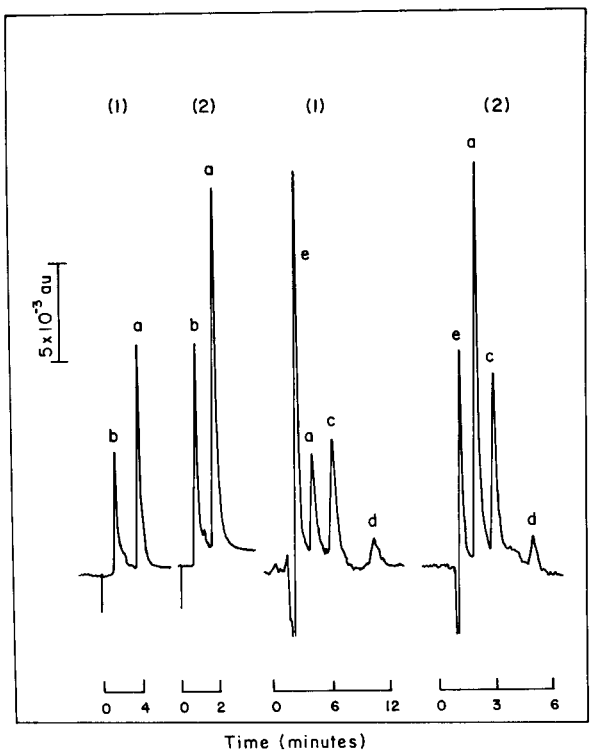

Fig. 4. Separation of platinum(II) complexes on solvent-generated anion exchangers, supported by μBondapak C_{18} (1) and ODS Hypersil (2). Mobile phase, 10^{-4} mol dm^{-3} HTAB in water. Stationary phases: 1, μBondapak C_{18} + HTAB (10 μm; 300 × 3.9 mm I.D.); 2, ODS Hypersil + HTAB (5 μm; 100 × 4.6 mm I.D.). Temperature, 30°C. Flow-rate, 1.0 ml min^{-1}. Peaks: a = $[Pt(NH_3)_2Cl_2]^0$; b = $[Pt(NH_3)_2Cl(H_2O)]^+$ and $[Pt(NH_3)_2(H_2O_2]^{2+}$; c = $[Pt(NH_3)_2ClBr]^0$; d = $[Pt(NH_3)_2Br_2]^0$; and, e = solvent.

In conclusion, bivalent platinum complexes are well separated on solvent-generated anion exchangers, and the mechanism may be described in terms of ion–dipole interactions in the stationary phase reinforced by a hydrophobic effect, and retention may be manipulated by controlling the composition and concentration of electrolyte in the mobile phase.

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REVERSED-PHASE LIQUID COLUMN CHROMATOGRAPHY OF PYRIMIDINE AND PURINE DERIVATIVES

I. UNBUFFERED BINARY AQUEOUS ORGANIC MOBILE PHASES ON OCTADECYLSILICA

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SUMMARY

High-performance liquid chromatographic separation of representative pyrimidine and purine derivatives on LiChrosorb RP-18 packing with plain, salt-free aqueous organic mobile phases has been studied. Methanol, acetonitrile, dioxan and tetrahydrofuran were used as the organic modifiers. Quantitative data are given for the dependence of the retention parameters on the mobile phase composition. The different organic modifiers are shown to produce significant changes in selectivity for particular types of compounds.

INTRODUCTION

Reversed-phase liquid chromatography (RPLC) has become a well established method for the separation of pyrimidine and purine derivatives, particularly of the biochemically important nucleosides and nucleobases. Typically, *n*-alkyl (mostly *n*-octadecyl) chemically bonded siliceous supports are used as the stationary phase, while aqueous buffers (acidic to neutral) containing an organic modifier, such as methanol[1–7] or acetonitrile[8–10], serve as the eluent. Unbuffered, salt-free eluents have only occasionally been used: aqueous methanol[11–13], aqueous acetonitrile[14,15] and pure water[11].

As the pyrimidine and purine compounds of interest may contain a large variety of ionic and polar functional groups, it is desirable to be able to manipulate their ionic states, and, hence, additions of buffering or ion-interaction reagents to the eluent may be necessary to create suitable conditions for a specific chromatographic separation. Nevertheless, a more thorough investigation of unbuffered mobile phase systems seems to be useful for two reasons. First, a knowledge of the retention characteristics of such simple systems should serve as the basis for a better understanding of some features of the more complicated ionic and/or ionogenic eluents; secondly, these simple systems are preferable in preparative application.

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The aim of the present work was to investigate the retention behaviour of typical pyrimidine and purine compounds on octadecylsilica with neutral binary aqueous organic mixtures. Four different organic modifiers have been examined: methanol and acetonitrile, because of their widespread use and general utility in RPLC; and tetrahydrofuran and *p*-dioxan, two solvents potentially capable of specific selective interactions[16,17] but hitherto unexplored in the chromatography of nucleic acid constituents.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment comprised the following: a Milton Roy Model 396-57 minipump with Model 709 pulse dampener (Laboratory Data Control, Riviera Beach, FL, U.S.A.); a home-made septum injector permitting syringe injection onto the top of the column packing; a 250 × 4.2 mm I.D. stainless-steel column (LiChroma tubing; Applied Science Labs., State College, PA, U.S.A.); either a fixed-wavelength UV detector (Model 1203 UV III monitor, Laboratory Data Control) operated at 254 nm or a differential refractometer (Model RIDK 101; LaboratorníPřístroje, Prague, Czechoslovakia) and a 5-mV potentiometric recorder (Model EZ 13, Laboratorní Přístroje). The column was packed with LiChrosorb RP-18, mean particle size, d_p = 10 μm (E. Merck, Darmstadt, G.F.R.), using a home-made slurry packing apparatus with chloroform at pressure of a 50 MPa. The measurements were made at 20°C.

The eluents were prepared from deionized water and methanol p.a., dioxan p.a. (Lachema, Brno, Czechoslovakia) or tetrahydrofuran p.a. and acetonitrile p.a. (VEB Laborchemie, Apolda, G.D.R.). The compositions of the binary mixtures, denoted by C, are given as % (v/v), *i.e.*, numerically, as the volume, in ml, of the organic solvent which was mixed with (100 − C) ml water.

The commercially available pyrimidines and purines were purchased either from Lachema or from Sigma (St. Louis, MO, U.S.A.). Other derivatives were prepared in this Institute. The samples were dissolved in methanol–water (1:1) to yield concentrations of 50–200 μg/ml, and were injected into the column in 0.5–2.0 μl volumes, using SGE Type B syringes (Scientific Glass, Melbourne, Australia).

The retention times, t_R, were measured as the distance between the injection point and the peak maximum on the chromatogram. Retention (capacity) factors, k, were evaluated from the relationship:

$$k = (t_R - t_M)/t_M$$

Each k value was obtained from at least four measurements. The mobile phase hold-up time, t_M, was determined from injections of ^2H_2O, as was originally proposed by Karch *et al.*[18]. Table I lists values of V_M, the hold-up volume of the column (dead volume), calculated from t_M and the appropriate volumetric flow-rate, together with the total column porosities, $\varepsilon = V_M/V_c$, where V_c is the volume of the empty column (3.46 ml in this case).

As is seen, differing V_M values were obtained for different mobile phase compositions. This is in general agreement with recent findings of McCormick and Karger[19], who also have given explanations for such phenomena. The marked decrease in

TABLE I

DEAD VOLUME, V_M, AND POROSITY OF THE LICHROSORB RP-18 COLUMN

Eluent	V_M *(ml)*	ε
Methanol–water (20:80, v/v)	2.32	0.67
Methanol–water (10:90, v/v)	2.34	0.68
Methanol–water (2:98, v/v)	2.34	0.68
Acetonitrile–water (8:92, v/v)	2.08	0.60
Acetonitrile–water (2:98, v/v)	2.12	0.61
Dioxan–water (2:98, v/v)	2.05	0.59
Tetrahydrofuran–water (2:98, v/v)	2.08	0.60
Water	1.68	0.49

V_M in pure water is most striking; again, however, similar observations have been made previously[19,20].

RESULTS AND DISCUSSION

When chromatographed on LiChrosorb RP-18 with aqueous methanol, acetonitrile, tetrahydrofuran or dioxane, the various pyrimidine and purine derivatives fell into three groups.

(1) Compounds such as orotic acid, orotidine, barbituric acid, uric acid and 4,6-dihydroxypyrimidine emerged as sharp, narrow peaks with elution times shorter than the mobile phase hold-up time, t_M, *i.e.*, they were excluded from at least part of the intraparticle void volume. These compounds possess pK_a values between 5.7 (uric acid) and 2.1 (orotic acid) (*cf.*, refs. 21 and 22) and, hence, must be present, wholly or predominantly, as anions in the neutral mobile phase. Exclusion of ionized species from *n*-alkyl bonded siliceous packings is known[23–25] and could be eventually exploited as a means for group separations.

(2) Some amino derivatives displayed a distinct tendency to tailing. This is attributed, in accordance with the literature[17,26], to heterogeneous interactions involving both the alkyl chains and the free silanol groups of the packing. Typical values of the asymmetry factor, A_s (defined as the rear to front bandwidth ratio at 10% peak height), were 4–5 for adenine (the worst case) and 2.5–3 for cytosine. More symmetrical peak shapes were observed with the corresponding ribosides and deoxyribosides, and reasonable chromatograms were obtained for such substances. Nevertheless, the retention data were influenced by the concentrations of the solutes (even in the nanogram to microgram range investigated) and for this reason cytosine and adenine derivatives will not be included in the present evaluation.

(3) The other compounds, mostly derivatives of uracil and various oxopurines, gave symmetrical or nearly symmetrical peaks with A_s values of 1.1–1.2. They are listed (together with the abbreviations used) in Table II. It is noted that several substances with amino functionalities belong to this group, such as guanine, 5-aminouracil and 4-amino-6-hydroxypyrimidine. There is no obvious explanation for the different behaviour of these amino compounds compared to that of the adenine and cytosine group mentioned above. Their pK_a values[21,22] do not differ greatly

TABLE II

VALUES OF THE INTERCEPTS, ln k_0, AND SLOPES, A, OF EQN. 1 FOR PYRIMIDINES AND PURIN ON LICHROSORB RP-18

Compound	*Methanol–water**		*Acetonitrile–water***		*Dioxan–water****		*Tetrahydrofuran water****	
	ln k_0	*A*	*ln* k_0	*A*	*ln* k_0	*A*	*ln* k_0	*A*
2-Hydroxypyrimidine	−0.48	0.079	−0.49	0.230	−0.36	0.240	−0.66	0.240
2-Hydroxy-5-methylpyrimidine	0.86	0.094	0.82	0.258	0.71	0.310	0.10	0.280
Uracil (Ura)	−0.29	0.074	−0.29	0.228	−0.14	0.251	−0.41	0.341
Uridine (Urd)	0.77	0.122	0.85	0.326	0.76	0.381	0.34	0.479
2′-Deoxyuridine (dUrd)	1.35	0.127	1.37	0.346	1.13	0.387	0.72	0.490
1-β-D-Arabinofuranosyluracil	1.30	0.132	1.31	0.372	1.05	0.373	9.65	0.481
2,2′-Anhydro-1-β-D-arabinofuranosyluracil	−0.56	0.105	−0.45	0.244	−0.61	0.261	−0.84	0.401
Thymine (Thy)	1.07	0.090	1.08	0.255	1.08	0.313	0.71	0.410
5-Methyluridine	1.84	0.131	1.85	0.356	1.71	0.427	1.24	0.511
Thymidine (dThd)	2.52	0.151	2.54	0.395	2.12	0.410	1.72	0.590
1-Methylthymine (m^1Thy)	2.25	0.124	2.22	0.308	1.86	0.360	1.22	0.515
5-Hydroxymethyluracil	−0.20	0.097	−0.24	0.264	−0.15	0.297	−0.47	0.390
4-Amino-6-hydroxypyrimidine	−0.41	0.075	−0.36	0.240	−0.21	0.313	−0.46	0.360
4-Amino-6-hydroxy-1-β-D-Rbf-pyrimidine§	1.09	0.137	1.09	0.362	0.82	0.367	0.52	0.519
5-Aminouracil (n^5Ura)	−1.08	0.058	−1.01	0.162	−0.10	0.027	§§	
2-Hydroxypurine	−0.05	0.106	−0.09	0.304	−0.17	0.320	−0.65	0.295
Hypoxanthine (Hyp)	0.52	0.099	0.47	0.294	0.38	0.327	0.06	0.381
Inosine (Ino)	1.88	0.158	1.96	0.446	1.39	0.457	0.95	0.640
1-Methylinosine (m^1Ino)	2.79	0.181	2.83	0.464	2.13	0.520	1.56	0.770
Xanthine (Xan)	0.78	0.108	0.70	0.308	0.65	0.303	0.40	0.439
Xanthosine (Xao)	—	—	—	—	1.90	0.450	1.31	0.620
Guanine (Gua)	0.65	0.107	0.62	0.303	0.92	0.153	0.80	0.060
Guanosine (Guo)	1.98	0.151	2.02	0.422	1.60	0.450	1.12	0.519
2′-Deoxyguanosine (dGuo)	2.24	0.150	2.30	0.434	1.86	0.470	1.49	0.560
1-Methylguanosine (m^1Guo)	2.76	0.161	2.86	0.456	2.46	0.533	1.82	0.653
Isoguanosine	1.72	0.153	1.77	0.402	2.45	0.403	2.03	0.360

* 2–20% (v/v) methanol.
** 1–6% (v/v) acetonitrile.
*** 1–4% (v/v) dioxan or tetrahydrofuran.
§ Rbf = Ribofuranosyl.
§§ Non-linear.

(cytosine, 4.4; adenine, 4.2; guanine, 3.2; 4-amino-6-hydroxypyrimidine, 1.4) and all should exist almost wholly as neutral molecules at pH 7. Tentatively, it is suggested that the heterogenous interactions become operative only with molecules of a certain functionality and a certain rigid molecular shape —the "template effect" as discussed by Knox and Pryde[27].

Methanol–water

Experimental ln k values are plotted against C in Fig. 1 for several representative solutes. Although the ln k *versus* C relationship is generally non-linear[28], the present data for the methanol–water system obey a linear relationship over the whole

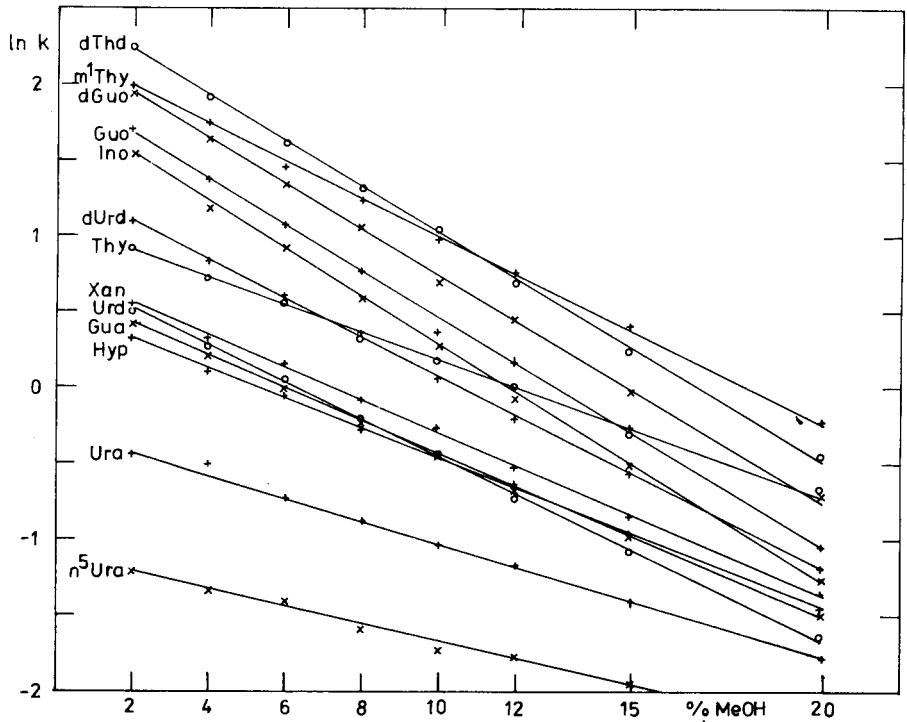


Fig. 1. Plot of ln k values in the methanol–water system against volume percent of methanol (MeOH).

composition range from 20 to 2% methanol, with a high correlation coefficient (typically, $r = 0.98$)

$$\ln k = \ln k_0 - A\,C \tag{1}$$

where A denotes the slope and $\ln k_0$ the intercept of the lines. Values of $\ln k_0$ and A are summarized in Table II.

The dependence of k on the mobile phase composition displays characteristic features for certain compound types. Thus, values of A are very similar for compounds differing only in one functional group (*cf.*, *e.g.*, 2-hydroxypurine, Xan, Hyp and Gua) or in the sugar moiety (Urd, dUrd, arabinosyluracil). On the other hand, nucleosides have systematically higher A values than their parent bases (Urd *versus* Ura, Ino *versus* Hyp, etc.) and this can lead to significant changes in selectivity or even to reversals of the elution order (Thy and dUrd, m¹Thy and dThd, etc.).

It is interesting to compare these results with the comprehensive set of data, of Brown and coworkers[1,2,29]. In spite of the fact that the latter authors used mixtures of acidic phosphate buffers with methanol (and, moreover, different types of octadecylsilica), the overall trends are very similar for solutes common to both sets of data, *i.e.*, mainly uracils and oxopurines. As an example, Fig. 1 of the present study cann be directly compared with Fig. 1 of ref. 29: the relative positions of the lines for Ura, Urd, Hyp, Xan, Ino and Guo are almost the same. It appears that chromato-

graphy of such compounds is scarcely affected by the presence of the buffering salts.

The chromatographic measurements were commenced with higher concentrations of methanol and then proceeded gradually to eluents richer in water; the retention times naturally rose in accordance. On changing from 2% methanol to pure water as eluent the retentions initially increased further and tended to values roughly corresponding to the linear extrapolation of the ln k *versus* C relationship, as established with different contents of methanol. However, as more water passed through the column, the retention times began to fall again and stabilized only after long column equilibration, requiring at least 150–200 column volumes. The steady-state retention times in pure water were even lower than those obtained in 10% methanol and the corresponding k values [calculated with the appropriate (low) value of V_M, *cf.*, Table I] bore no relation to the extrapolated k_0 values. It must be emphasized with regard to Table II that ln k_0 represents merely the intercept of the ln k *versus* C relationship for binary mixtures in the indicated range of compositions and is by no means a measure of the retention in pure water.

The phenomena associated with changing from a mobile phases containing an organic modifier to pure water (and *vice versa*) were quite reporducible provided that the long equilibration times necessary were taken into account. Very similar observations have recently been reported by Scott and Simpson[30], who also noticed the anomalously low retentive characteristics of LiChrosorb RP-18 in water and presented a plausible explanation; they classify LiChrosorb RP-18 as a "brush"-type reversed-phase packing, having free hydrocarbon chains which probably interact between themselves when no organic additive is present in the aqueous mobile phase.

Acetonitrile–water

The ln k values obtained were plotted against the acetonitrile content of the mobile phase and some typical relationships are depicted in Fig. 2. As acetonitrile is a stronger eluent than methanol in RPLC, the useful concentration range is narrower than in the case of the methanol modifier, but even in this range the dependences show a definite curvature. Nevertheless, linear interpolation is fully justified between 6 and 1% acetonitrile, and, hence, the parameters of eqn. 1 have been calculated and are given in Table II for this region. As in the previous case, the ln k_0 values can be regarded only as a mathematical aid in interpreting the retention data, but it may be noted that, for most solutes, they are closely similar for methanol–water and for acetonitrile–water. A full coincidence, of course, would be expected if the underlying relationship, as derived from measurements in the binary mixtures, were valid also when the common component of the two binaries became the single constituent of the mobile phase.

Compared to methanol–water, the slopes (A values) are generally greater for acetonitrile–water, but the differences between individual solutes are similar, the rate of change of retention with the volume fraction of the modifier being more pronounced for nucleosides than for the bases; again, both separation factors and elution orders are affected in this way.

p-Dioxan–water and tetrahydrofuran–water

The results for these two systems will be treated together, as they have common features. For selected solutes, the ln k *versus* C relationships are plotted in Figs. 3 and

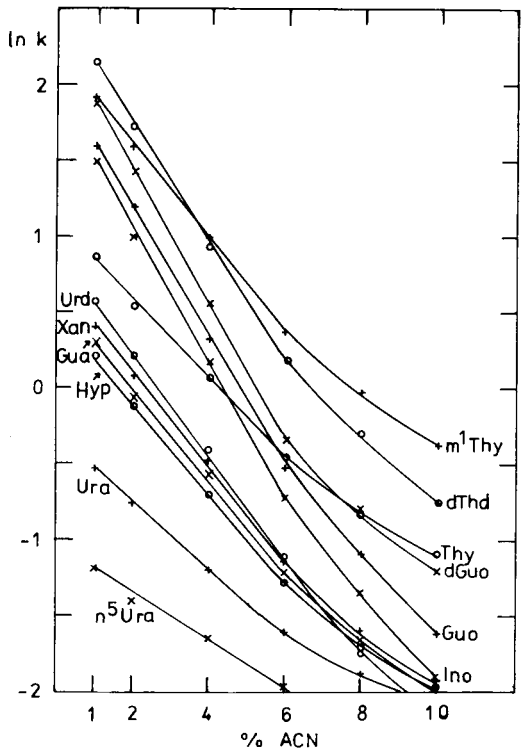
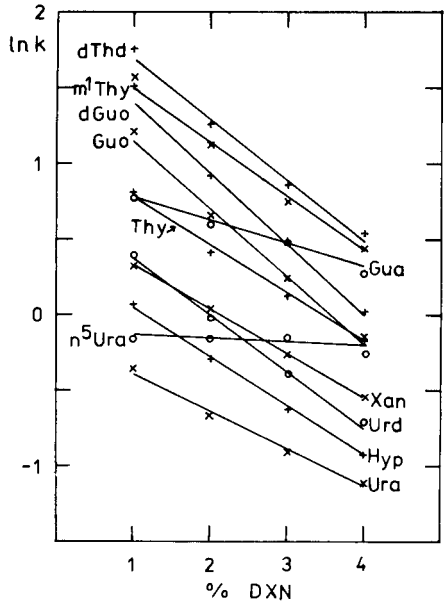


Fig. 2. Plot of ln k values in the acetonitrile–water system against volume percent of acetonitrile (ACN).

4; in Table II, the parameters of the linear interpolation are given for all compounds. The course of the ln k dependence shows a definite curvature even in the restricted composition range available for practical separations; the linear correlation according to eqn. 1 must be regarded only as an approximation. According to Schoenmakers *et al.*[28], the departures from linearity of the ln k *versus* C dependence are always more pronounced for organic modifiers less polar than methanol.

Some similarities between the dioxan–water and tetrahydrofuran–water systems and the two binaries discussed previously are readily apparent from the Figures or can be deduced from Table II, in particular the relative magnitudes of the slopes for simple bases and nucleosides. However, in other respects there are significant differences. Isoguanosine, the retention of which was always lower than that of guanosine (Guo) in methanol–water and acetonitrile–water, becomes one of the most retarded solutes and emerges far behind Guo. The most striking changes apply to Gua and n^5Ura, not only is the retention markedly enhanced over the whole composition range, but, moreover, the character of the composition dependence is altered. For n^5Ura the degree of retention is almost invariable in dioxane–water, whereas with the tetrahydrofuran–water system the retention decreases with increasing water content.

Whereas in methanol–water and acetonitrile–water, guanine always emerged before guanosine, the elution order is reversed with the tetrahydrofuran and dioxan modifiers (although, with the latter, only at $C > 2.5\%$). This is probably the first



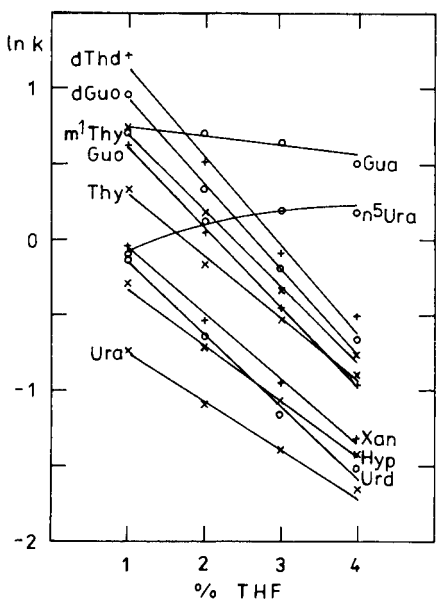


Fig. 3. Plot of ln k values in the dioxane–water system against volume percent of dioxan (DXN).

Fig. 4. Plot of ln k values in the tetrahydrofuran–water system against volume percent of tetrahydrofuran (THF).

known case, in RPLC on octadecylsilica, of a ribonucleoside eluting faster than the parent base. Brown and Grushka[31] have discussed the peculiarity of the typical RPLC retention order of nucleoside/base pairs and attributed the higher retention factors of nucleosides to their ability to form hydrophobic aggregates in the mobile phase. The present result for Gua and Guo seems indirectly to corroborate this; if the "normal" elution order (nucleoside after base) can be altered by a change in the mobile phase composition, then it must be related to mobile-phase interactions and cannot be considered as an inherent property of the alkylsilica stationary phase.

Comparison of the four modifiers

Besides the retention shifts, pointed out in the preceding paragraphs, there are some further differences between the modifiers that affect separation selectivities and are significant enough to have practical importance. In order to compare methanol, acetonitrile, dioxan and tetrahydrofuran it is necessary to normalize the conditions with respect to the water content, as this primarily determines the elution strength. Here, normalization has been done arbitrarily, from the practical point of view. The retention of the thymidine/deoxyuridine pair was taken as the basis for comparison since the separation factor ($\alpha = k_2/k_1$) for these compounds was at least altered by the nature of the organic modifier. In Table III, k values of the pyrimidine and purine derivatives are compared for four different systems, each containing a different modifier and a different water content, but all resulting in $k \approx 3$ for thymidine. (The values were obtained by linear interpolation according to eqn. 1.)

Pertinent information can be easily obtained from Table III. For example, the

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JES OF THE RETENTION FACTORS, k, IN MOBILE PHASES OF COMPARABLE STRENGTHS FOR MIDINES AND PURINES ON LICHROSORB RP-18

ound	*Methanol–water (9.5:90.5, v/v)*	*Acetonitrile–water (3.5:96.5, v/v)*	*Dioxan–water (2.5:97.5, v/v)*	*Tetrahydrofuran–water (1.0:99.0, v/v)*
lroxypyrimidine	0.29	0.27	0.38	0.41
lroxy-5-methylpyrimidine	0.96	0.91	0.93	0.83
l (Ura)	0.37	0.34	0.46	0.47
ıe (Urd)	0.68	0.74	0.83	0.87
ɔxyuridine (dUrd)	1.15	1.17	1.17	1.26
Arabinofuranosyluracil	1.04	1.00	1.12	1.18
.nhydro-1-β-D-arabinofurano-ıracil	0.21	0.27	0.28	0.29
ine (Thy)	1.23	1.21	1.35	1.35
hyluridine	1.79	1.82	1.90	2.08
idine (dThd)	2.97	3.16	3.00	3.09
hylthymine (m^1Thy)	2.89	3.13	2.61	2.03
lroxymethyluracil	0.33	0.31	0.40	0.42
ino-6-hydroxypyrimidine	0.33	0.30	0.37	0.44
ino-6-hydroxy-1-β-D-Rbf-imidine	0.81	0.83	0.90	1.00
inouracil (n^5Ura)	0.20	0.20	0.85	0.98
lroxypurine	0.35	0.31	0.38	0.39
xanthine (Hyp)	0.66	0.57	0.64	0.73
ıe (Ino)	1.46	1.49	1.28	1.36
thylinosine (m^1Ino)	2.92	3.32	2.29	2.20
ıine (Xan)	0.78	0.68	0.89	0.96
ıosine (Xao)	—	—	2.18	1.99
ine (Gua)	0.69	0.64	1.72	2.09
osine (Guo)	1.72	1.72	1.60	1.82
oxyguanosine (dGuo)	2.25	2.16	1.97	2.53
thylguanosine (m^1Guo)	3.42	3.52	3.06	3.22
anosine	1.31	1.43	4.22	5.31

biochemically important quadruplet Hyp, Xan, Gua and Urd emerge very close together in methanol–water eluents, buffered[1,29] or unbuffered. Their relative retentions have been recalculated in terms of the separation factors (α values) and are given in Table IV. The reference compound ($\alpha = 1$) was chosen individually for each system so as clearly to display the pairs most difficult to resolve. Two such pairs, with $\alpha = 1.03$, occur in the methanol–water system (Hyp/Urd and Urd/Gua), whereas in acetonitrile–water all four solutes are more separated. In dioxan–water and tetrahydrofuran–water, owing to the selective retardation of Gua, the separation can be enhanced further.

The work reported here has been done on a single type of octadecylsilica. Nevertheless, preliminary experience with another type of reversed-phase packing indicates that the selectivity changes in the separation of nucleic acid components through the choice of the organic modifier have more general validity.

TABLE IV

SEPARATION FACTORS, α, FOR HYPOXANTHINE, XANTHINE, GUANINE AND URIDINE ON LICHROSORB RP-18 IN DIFFERENT MOBILE PHASES (SEE TEXT)

Compound	*Methanol–water (9.5:90.5, v/v)*	*Acetonitrile–water (3.5:96.5, v/v)*	*Dioxan–water (2.5:97.5, v/v)*	*Tetrahydrofuran–water (1.0:99.0, v/v)*
Hyp	0.97	0.89	0.77	0.84
Xan	1.14	1.06	1.07	1.10
Gua	1.03	1	2.07	2.40
Urd	1	1.16	1	1

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CONTINUOUS-FLOW SCANNING OF SELECTED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PEAK COMPONENTS BY MICROPROCESSOR CONTROL

APPLICATION TO ANALYSIS OF EXTRACTS FROM HUMAN LYMPHOCYTES

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SUMMARY

Continuous-flow wavelength scanning of compounds separated by high-performance liquid chromatography is achieved through the use of fixed and variable-wavelength micro ultraviolet detectors connected in series but separated by a low-pressure three-way valve. Activation of the valve allows entrapment of selected peaks in the variable-wavelength detector without interfering with the response of the fixed-wavelength detector which is utilized for peak quantitation. A microprocessor program is employed to maintain control and accuracy during the scanning sequence. Good correlation was found between ultraviolet spectra of standards obtained on a conventional spectrometer and those on separated peaks. This system allows the identification and quantification of picomole amounts of peaks separated during one analysis of a biological sample.

INTRODUCTION

The high sensitivity and resolution of complex mixtures of biological origin by high-performance liquid chromatography (HPLC) generates a problem in the identification of separated compounds. Most methods of chemical analysis, such as nuclear magnetic resonance or infrared spectroscopy, or elemental analysis, require large sample sizes (*i.e.*, milligram amounts), amounts that are far above separations obtainable by analytical HPLC. The use of mass spectroscopy, while highly sensitive for sample identification, requires a volatile sample. Comparison of retention time(s) with known standards is useful, but not sufficient by itself as an absolute means of identification. Compounds with similar chromatographic properties may co-elute, and precise identification requires the comparison of retention times under different chromatographic conditions. Thus, for most compounds of biological interest, the

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use of spectroscopic characterization provides a simple on-line means of HPLC recognition. Absorbance ratios have been widely used as an aid in peak identification[1–6]; however, optimal utilization requires prior knowledge of the absorbance properties of the components in the mixture which is being separated. For samples of biological origin, the exact nature of the individual compounds is not always known. The more refined technique of stopped-flow scanning allows examination of the entire spectrum of a chromatographic peak using a variable-wavelength HPLC detector[5,7,8]. While this method is highly useful when examining an unknown compound, a major drawback is the requirement to stop chromatographic flow during the duration of the wavelength scan. The start/stop of flow makes peak quantitation unreliable and requires a second injection for this purpose[9]. To aid in the combined identification–quantification when limited amounts of material are available for analysis, a method is described below which allows for peak scanning(s) during continuous chromatographic flow to one of two detectors connected in tandem to the outflow of a HPLC column. Microprocessor control is utilized to provide an automatic scanning sequence. The application of this technique will be shown for the analysis of an extract of human lymphocytes applied to a strong anion exchanger microparticulate HPLC column.

METHODS

Apparatus

A Spectra Physics 8000A liquid chromatograph (Santa Clara, CA, U.S.A.) equipped with a two-channel data system was used for all separations. On-line detection was performed using a Spectra Physics Model 8310 fixed-wavelength (254 nm) detector and a Schoeffel Model 770 spectroflow variable-wavelength detector equipped with SFA 339 wavelength drive and MM 700 Memory Module (Kratos, Westwood, NJ, U.S.A.). A low-pressure three-way valve (No. 1-43-900, General Valve Corp., East Hanover, NJ, U.S.A.) was connected between the two detectors to divert the flow during the peak trapping in the variable-wavelength detector. The output of the variable-wavelength detector was connected to a Houston single-channel Omniscribe recorder equipped with an event marker and remote control switch for the chart speed (Houston Instrument Co., Austin, TX, U.S.A.), and to an analogue-to-digital converter (Spectra Physics). Spectra from standards were obtained on a Beckman Acta II spectrophotometer at a scan rate of 100 nm/min. Optoisolators were employed to serve as an electronic buffer between the relays activating the various events during the wavelength scanning sequence and microprocessor controlled switches (Fig. 1). A constant voltage d.c. power supply was used for this circuit. A detailed schematic is shown in Fig. 2. Electronic components were obtained from Radio Shack.

Chemicals and supplies

The mobile phase was prepared from reagent grade monobasic potassium phosphate (Sigma, St. Louis, MO, U.S.A.) and potassium chloride (Mallinkrodt, Louisville, KY, U.S.A. (the phosphate was purified according to the method of Shmukler[10]. The water was double-distilled and deionized. Prior to use, the buffers were filtered through a 0.2-μm filter (Sartorius, Haywood, CA, U.S.A.). Degassing was

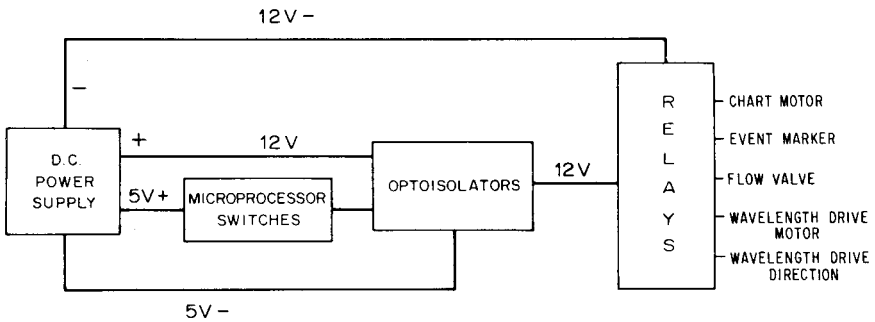

Fig. 1. Accessory control unit of microprocessor controlled continuous-flow peak scanning. A regulated d.c. power supply is utilized to provide 12 V for the relays and 5 V for the optoisolator microprocessor switching circuitry. The activation of the various relays whose control function is listed above is achieved via a two-step switching process: the microprocessor switch applies power to an optoisolator which in turn provides power to a given relay.

achieved via a helium purging system (Spectra Physics). Ribonucleotide standards were obtained from Sigma.

Chromatographic columns

A Whatman Partisil 10 SAX (25 × 0.46 cm I.D.) column was used for analytical separation (Whatman, Clifton, NJ, U.S.A.). A guard column (7 × 0.2 cm I.D.) filled with Pellicular Anion Exchanger (Whatman) was used to protect the analytical column. A precolumn (25 × 0.46 cm I.D.) filled with 37–53-μm silica (Whatman) was utilized to saturate the mobile phase with dissolved silica and served to extend the lifetime of the analytical column.

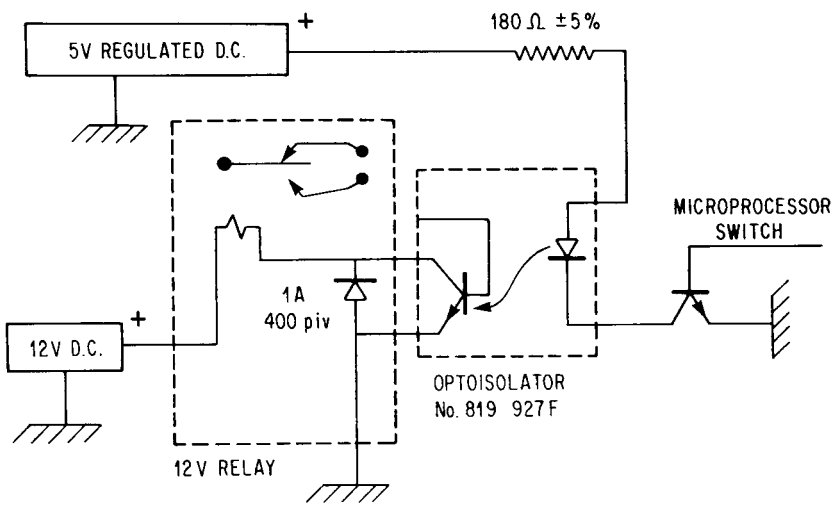

Fig. 2. Electronic circuitry of microprocessor–optoisolator 12-V relay switch. The closing of the normally open state of a microprocessor switch provides ground to the optoisolator circuitry allowing the 12 V d.c. to flow to the control relay. This three-step process allows for a rapid switching sequence as well as for protection of the microprocessor from back power surges from the relay coil.

Biological material

Informed consent for obtaining peripheral blood from donors was according to the provisions of the Helsinki Conference. Lymphocytes were purified as previously described[11]. Cell pellets containing 2–30 $\times$ 10^6 cells were extracted using 60% methanol essentially as reported by Donofrio *et al.*[12]. For each 10^7 cells, 200 μl of 60% methanol were added for extraction of the cell pellet. Extracts were taken to dryness using a Savant Speed Vac Concentrator (Savant, Hicksville, NY, U.S.A.), hydrated with water, and stored frozen at -70°C until subjected to HPLC analysis.

Chromatographic conditions

The separation conditions were a modification of the method of McKeag and Brown[13]. The elution conditions were as follows: temperature, 40°C; flow-rate 1.5 ml/min from 0 to 40 min, 3.0 ml/min from 40 to 52 min, 2 ml/min from 52 to 72 min; buffers, (A) 0.007 *M* KH_2PO_4, 0.007 *M* KCl, pH 4.0, (B) 0.25 *M* KH_2PO_4, 0.5 *M* KCl, pH 5.0; mobile phase, 0–5 min isocratic A, 5–40 min linear gradient A $\rightarrow$ B; 40–52 min isocratic B, 52–67 min linear B $\rightarrow$ A, 67–72 min isocratic A.

HPLC ultraviolet scanning

Ultraviolet (UV) spectra of trapped peaks were obtained at high sensitivity ranges of the Model 770 variable-wavelength detector (0.04–0.01 a.uf.s.). To allow distortion free spectra, a background spectrum was stored using buffer A on the 0.1 a.u. memory range of the MM 700 background subtract accessory. Since the steps followed for preparation of the mobile phase resulted in no significant difference in the absorbance properties of buffers A and B, buffer A was used as the blank for all spectral scans. Spectra were stored/scanned at a rate of 100 nm/min.

Program operation

The subroutine controlling the operation of the function relays is a simple eleven-step procedure that can easily be adapted to any microprocessor system which can be programmed and which contains switched on/off transistors. The flow chart of this program is contained in Fig. 3.

RESULTS

Fig. 4 shows a flow diagram of the chromatographic system employed for continuous-flow scanning. Prior to the trapping of a chromatographic peak, the outflow from the analytical column passes through detector 1 (for quantitation at 254 nm) to detector 2 and out to waste. For UV scanning of a selected peak, the detector response is followed either by the pen deflection of recorder 2 or by the digital signal on the data channel. Upon observation of the top of the peak, activation of the three-way valve diverts the flow from detector 2 to waste. The 1-m height of the detector 2 waste outlet prevents diffusion outflow of the trapped peak. Once the desired beginning wavelength is set, the scanning program sequences shown in the flow chart in Fig. 3 are started via a microprocessor command. A typical wavelength scan sequence from 215 to 350 nm takes *ca.* 1.5 min. Duplicate or triplicate scans showed identical patterns (data not shown). Flow to the second detector is re-established when power is removed from the flow valve. No pertubation to detector 1 was

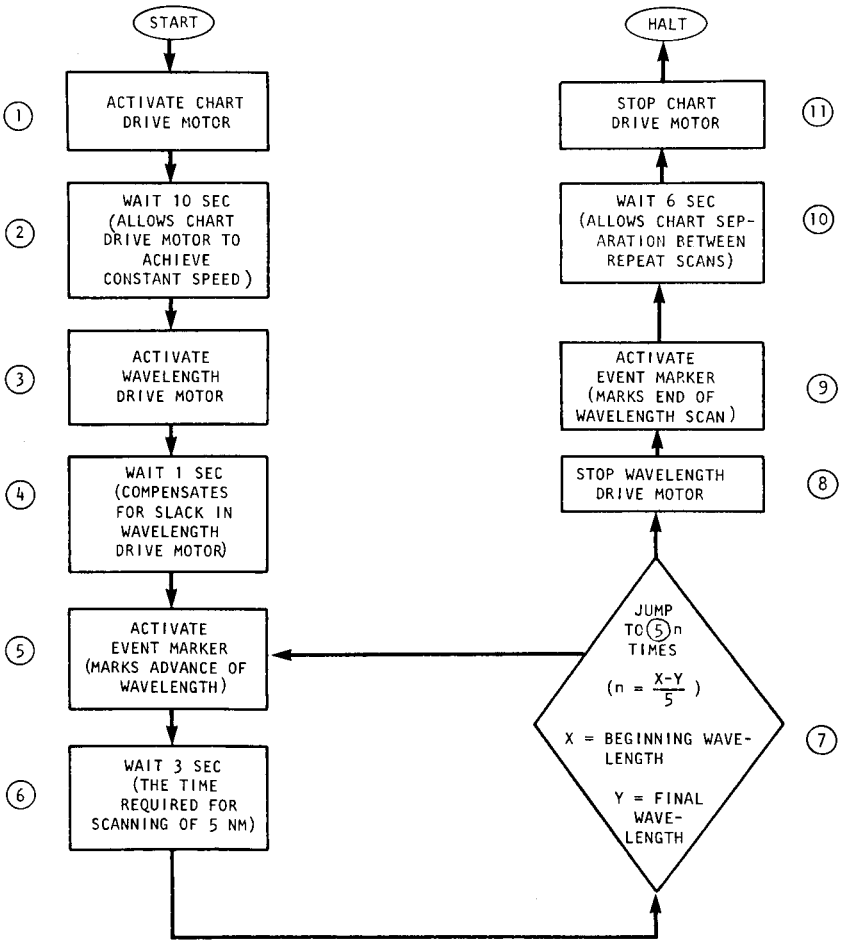

Fig. 3. Flow chart of program controlling automatic scanning sequence with wavelength notation.

observed upon the activation of the three-way valve. A new peak trapping and scan sequence could be begun once a new baseline was established.

Fig. 5 shows an elution profile obtained from an extract of human lymphocytes. The peak identifications were based on retention times of standards and verified by wavelength scanning and 280/254 nm ratios. Selected peaks sequentially scanned in the UV region during the course of one chromatographic separation are shown in Fig. 6 with spectral scans of reference standards. With the exception of the far-UV portion of the spectra, where differential O_2 absorption and variations in the monochromaters of the two instruments are more apparent, the agreement between scans is evident. The data obtained with IMP (Figs. 5 and 6B) show that closely resolved peaks can be subjected to continuous-flow UV scanning with satisfactory results.

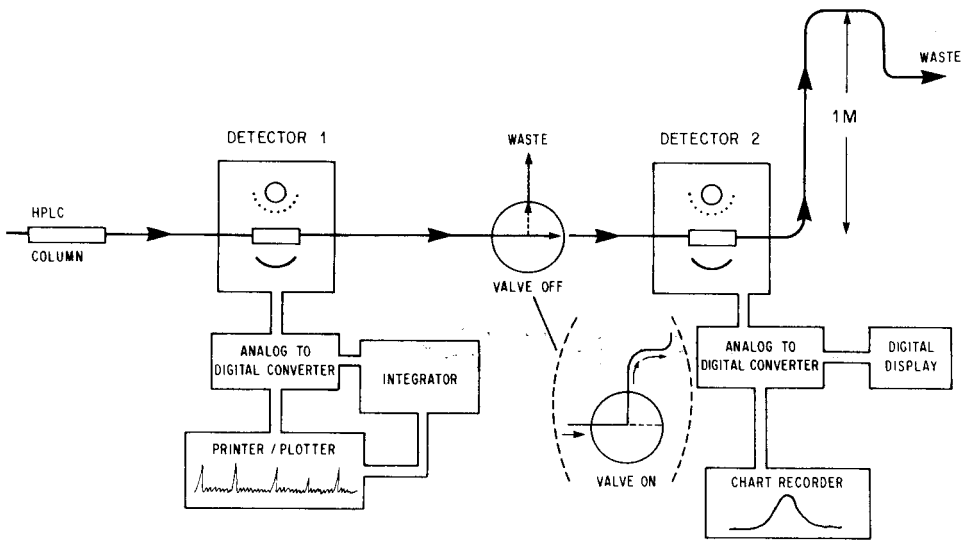

Fig. 4. Post-column elution flow diagram. The flow from an analytical HPLC column is allowed to sequentially pass through two detectors. The three-way valve between detector 1 and detector 2, in its inactivated state, allows the flow to pass directly to detector 2, and out through a 1-m high waste outlet. When current (110 vac) is applied to the valve, flow is diverted from detector 2 to waste leaving material trapped in the second detector. The 1-m height of the waste outlet of detector 2 coupled with the "on" configuration of the three-way valve prevents any diffusion of the trapped sample out of detector 2.

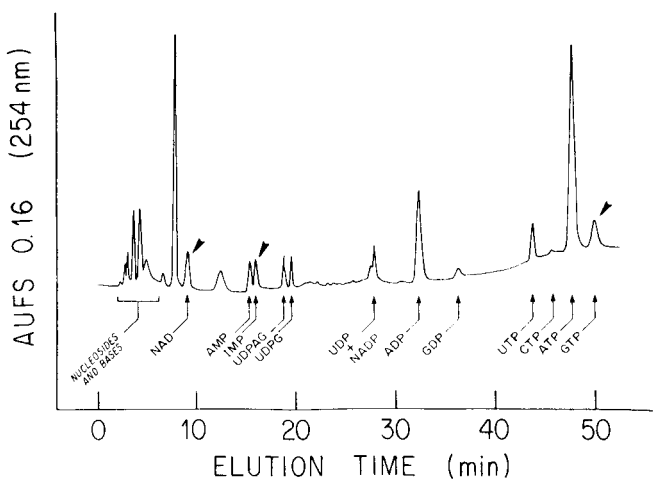

Fig. 5. Separation of nucleotides on Partisil 10 SAX from an extract prepared from 3×10^6 human lymphocytes. Chromatographic conditions are as described in the Methods. The marked peaks were subjected to peak trapping in the variable-wavelength detector during the same chromatographic separation.

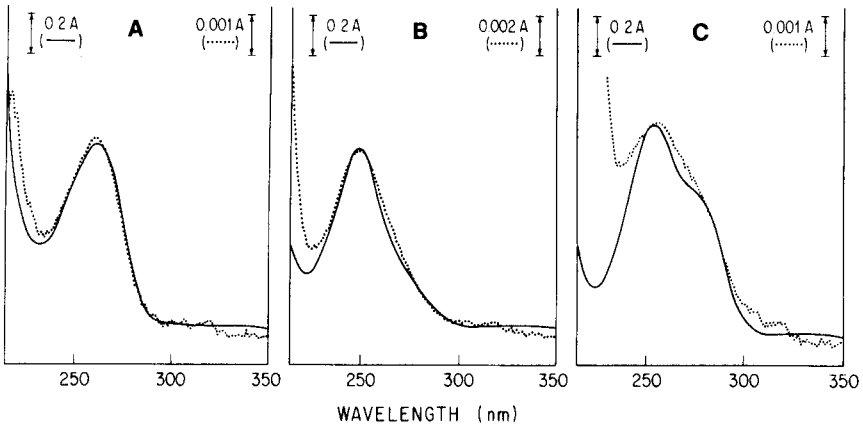

Fig. 6. UV wavelength scans of trapped HPLC peaks by the continuous-flow scanning technique (. . .) and reference compounds (—) by a standard UV spectrometer. A = NAD; B = IMP; C = GTP. Scanning range, from 215–350 nm; absorbance range as indicated.

DISCUSSION

A method is described which allows for the multiple use of HPLC separations where quantitation and peak identification by wavelength scanning on the same sample can be achieved. This technique, which makes use of two detectors, results in no interference in the chromatography as can result with stopflow scanning, and is of value particularly when limited material is available for analysis. Previous work has demonstrated the applicability of stopped-flow wavelength scanning to aid in the identification of a variety of components separated by HPLC[5,7,8,14]. A method for simultaneous multiwavelength detection has been described, but its utilization is limited by the cost of a rapid scan spectrometer and the complexity of the data processing and control systems[15]. The system employed in this study allows flexibility in the number of scans obtainable and the wavelength range of the scan, and is simple to set up.

Microprocessor control was utilized to carry out a repeatable sequence throughout the spectral scan along with user-supplied commands for peak trapping and initiation of the UV scan sequence. While a fully automated UV scan sequence was not carried out, owing to minor variations in retention times that occur with an ion exchange column, the consistent separations obtainable with a reversed-phase column would be ideally suited for this purpose. Conversely, trapped peak scans via manual control are possible, although considerable sacrifice in the consistency and reproducibility of the recorded spectra results. The system described in this paper was developed around the capabilities of a liquid chromatograph with a self-contained microprocessor having the ability of accepting a program controlling transistorized switching. The program and electronics detailed in the Figs. 1–3 can easily be adapted to any of a number of microprocessor controllers and data systems that are currently available for connection to an existing HPLC system (Spectra Physics SP4000, SP4100, Digital MINC 11, Waters Model 720 Controller). The use of a separate regulated power supply was required to assure the reproducibility in the operation of the transistorized switching circuits.

The ability to trap a peak in the small volume employed by most commercial HPLC detectors (*ca.* 10 μl) in a pathlength of 0.5–1 cm allows a sensitivity as low as 100 pmoles for wavelength characterization on typical compounds (*e.g.*, with $E_{mm} \approx 10$). The equivalent characterization using the cell configurations and sensitivity available with regular UV spectrometers on an HPLC separated component, could only be attempted with material that is collected from preparative scale HPLC separations.

The examination of purified preparations from samples of biological origin often leads to small yields of material for HPLC analysis as a result of the purification process. While not as detailed as other spectroscopic measurements, UV spectra do provide a highly sensitive, non-destructive means for identification of a compound, particularly when coupled with other information such as retention times or co-chromatography with reference compounds[14]. The technique of continuous-flow UV scanning has been shown to be ideally suited to identify and measure small, closely spaced chromatographic peaks. For maximum sensitivity and accurate interpretation of data, appropriate background corrections, whether manual or electronic, are required for each spectral scan.

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CONCENTRATION AND ISOLATION OF ORGANIC ACIDS ON GRAPHITIZED CARBON BLACK

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SUMMARY

The application of graphitized carbon black to the extraction of traces of organic acids has been studied. It is demonstrated that the recovery of these substances cannot be carried out without considering the nature of these compounds and their molecular structure. The amount of adsorbent and the kind of eluent required were deduced respectively from the breakthrough values and from distribution ratios.

INTRODUCTION

The importance of the determination of organic substances present at low concentration levels in natural and waste waters and in aqueous biological media has resulted in the development of numerous analytical methods using a solid matrix for the recovery of organic substances[1–12].

Any sorbent matrix must be evaluated with regard to its sorption and desorption properties, for the examined sample and substances to be recovered. As reported previously[13], the parameters to be examined during adsorption are: pH, flow-rate, salinity, breakthrough curve, particle size and column geometry; during the subsequent desorption, the flow-rate of the chosen eluent mixture must be controlled. Knowledge of the adsorption ratio (solid matrix–liquid system) helps in the choice of the solvent or solvent mixture to be used in the desorption stage.

Previously, graphitized carbon black (GCB) has been used in gas chromatography (GC)[14,15,23], in high-performance liquid chromatography[16,17], for the recovery of some organic substances from water[18–20] and for sample enrichment in traps in air pollution analysis[21]. In a recent study[19] it was shown that GCB may be a good adsorbent also for organic acids. The present work examines in more in detail the system water, GCB and some organic acids and its possible applications.

EXPERIMENTAL

Materials and reagents

The organic chemicals were purchased from E. Merck, Carlo Erba and Riedel de Haen.

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Concentrated stock solutions were prepared by weight by dissolving the pure substances in a water-soluble organic solvent, a maximum of 100 μl of organic solution per litre of water being employed for aqueous samples.

All the solvents used were either spectograde or analytical grade. The analytical grade solvents were further purified by fractional distillation whenever blank GC determinations suggested the presence of impurities detectable by a flame ionization detector.

Adsorption columns

Glass columns (0.7 cm I.D.), equipped with a PTFE stopcock, were packed with graphitized carbon black (100, 250 or 1000 mg). The adsorbent was held in place with small plugs of silanized glass wool. The graphitized carbon black (GCB) (Carbopack B; Supelco, Bellefonte, PA, U.S.A.) is characterized by a surface area of 100 m^2/g, 80–100 mesh.

Apparatus

A Dani 3900 gas chromatograph equipped with a flame ionization detector (FID) was used. All the standard mixtures and extracts were chromatographed using a porous-layer open tubular (PLOT) glass column (13 m × 0.3 mm I.D.) precoated with kaolin and coated dynamically with free fatty acid phase (FFAP)[22].

Analytical procedures

Adsorption isotherms. Solutions of various compounds ranging in concentration from 50 to 200 μg/l at pH 4 were used. Aliquots (250 ml) of these solutions were stirred at 21 ± 1°C with a weighed amount (250 mg) of GCB for 24 h, sufficient to reach the equilibrium as shown by preliminary experiments. After equilibration the liquid phase was filtered and extracted three times with 20-ml portions of methylene chloride. The combined extracts were dried with granular anhydrous sodium sulphate (5 g) and, after addition of internal standard, were concentrated by a rotary evaporator and analyzed by GC.

Breakthrough plots. The influence of the pH, flow-rate and ionic strength on the adsorption were studied. One litre of water "at different pH values" (pH range 2–11.5), containing 100 μg of each compound, was passed at a flow-rate of 240 ml/h through a glass column packed with 100 mg GCB. Equal amounts of this solution and of the percolate were extracted with three 50-ml portions of methylene chloride. The organic phase, after concentration in a rotary evaporator, was examined by GC.

For evaluating the "influence of flow", similar experiments using *p*-chloro-*m*-cresol and 2,3-dichlorophenol were carried out at pH 4 at various flow-rates in the range 80–800 ml/h with the help of a water pump. The percolate was collected and examined as above after the passage of 1000, 1250, 1500, 1750 and 2000 ml.

Finally, experiments were carried out at pH 4, with a flow-rate of 240 ml/h using water containing different quantities of NaCl and 100 μg/l each of *o*-chloro-*m*-cresol and 2,3-dichlorophenol in order to evaluate the effect of the "ionic strength".

The results of the experiments carried out with GCB packed columns (250 and 1000 mg) under optimum operating conditions (see later) were used to draw the breakthrough curves of all the compounds.

Recovery of acidic substances. The desorption of acidic substances adsorbed on

1000-mg columns of GCB was studied by passing through the column a 50-ml volume of a large number of organic solvents at a selected flow-rate of 60 ml/h and by analyzing the organic phase. Before elution, a nitrogen flow was briefly forced through the column in order to remove traces of water. The solvent mixture was selected after determination of the adsorption ratios $K = C_S/C_L$ (Table II). These values were obtained by shaking for 8 h at 25 ± 1°C the suspension obtained upon adding known amounts of a stock solution corresponding to 15 μg of the examined compounds and 500 mg GCB to 100 ml of each solvent. After equilibrium the suspension was filtered through a sintered glass filter and the organic phase, after addition of internal standard, was concentrated and analyzed chromatographically.

Samples

To check the efficiency of the method, two applications were examined: recovery of organic acid from human urine and phenols from river water.

Human urine. A 50–100-ml volume of sample (pH 4) was passed through a column (0.7 mm I.D.) packed with 250 mg GCB at a flow-rate of 240 ml/h. The adsorbed substances were recovered with 25 ml of benzene–methanol (2:1).

River water. One litre of sample (pH 4) was passed through the column packed with 1000 mg GCB at a flow-rate of 240 ml/h. The adsorbed substances were recovered with 75 ml of benzene–methanol (3:1).

RESULTS AND DISCUSSION

Fig. 1 shows the adsorption isotherms of some of the examined compounds. The isotherms of 2,3,4,6-tetrachlorophenol, myristic acid, stearic acid and benzoic acid are not reported, because in these cases the partition between the solid and the liquid phases lies completely towards the solid phase.

The isotherms show that in the examined concentration range there is a linear

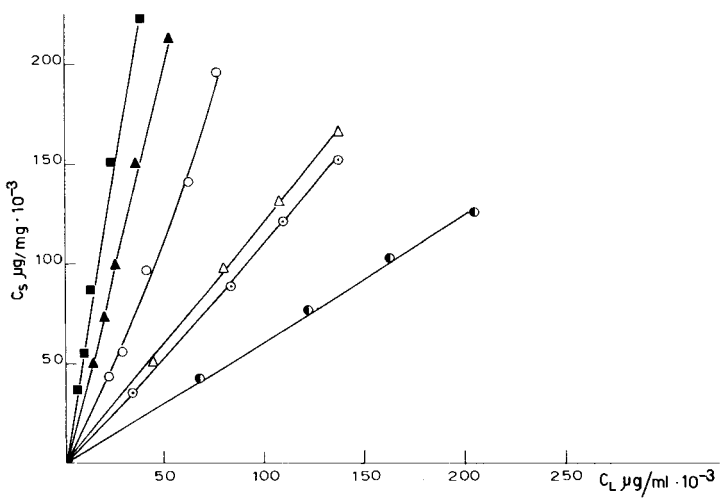

Fig. 1. Adsorption isotherms: ◐, heptanoic acid; ⊙, octanoic acid; △, *p*-chloro-*m*-cresol; ○, 2,3-dichlorophenol; ▲, 2,4,6-trichlorophenol; ■, decanoic acid.

TABLE I

ADSORPTION (%) OF MODEL ORGANIC COMPOUNDS FROM AQUEOUS SOLUTION (1000 ml) ON 100 mg OF GCB AT DIFFERENT pH VALUES

The concentration of each compound was 100 μg/l.

Compound	*pH*				
	2	*4*	*6*	*7.5*	*11.5*
Heptanoic acid	8	10	11	9	5
Octanoic acid	39	50	32	14	8
Decanoic acid	100	100	98	71	20
Myristic acid	100	100	100	74	21
Benzoic acid	98	100	98	70	18
2-Chlorophenol	34	48	30	10	6
p-Chloro-*m*-cresol	70	78	77	70	10
2,3-Dichlorophenol	89	91	92	80	7
2,4,6-Trichlorophenol	100	100	100	86	21
2,3,4,6-Tetrachlorophenol	100	100	98	88	20

relationship between the amount adsorbed on the solid and the aqueous concentration. Adsorption seems to increase with a decrease of the water solubility of the various compounds; therefore, for the fatty acids the adsorption increases with increasing chain length.

In Table I the percentages of different compounds adsorbed on GCB at different pH values is reported. The results show that in the pH range 3–6 there is no marked variation in adsorption because the studied compounds are mainly in the molecular form. At pH >7 the adsorption on GCB is very low because most of the compounds are in the salt form. A value of pH 4 was used in all further experiments.

The breakthrough plots were obtained for *p*-chloro-*m*-cresol and 2,3-dichlorophenol at three different flow-rates: 80, 240 and 800 ml/h (Fig. 2a, b). These experiments show that the collection efficiency increases with decreasing flow-rate in the range 800–240 ml/h. When the flow-rate is lower than 240 ml/h the variation in the collection efficiency is negligible. A flow-rate 240 ml/h was used in all further experiments.

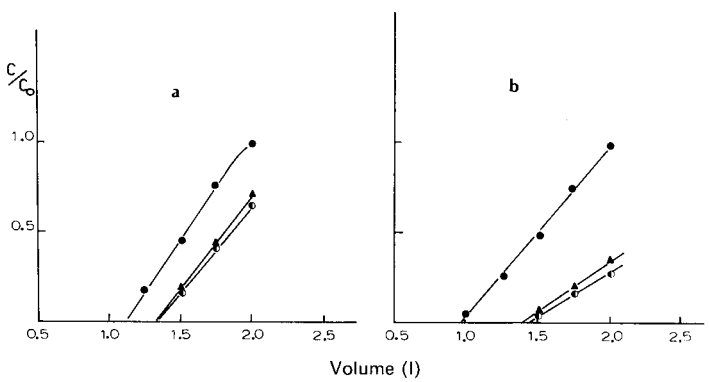

Fig. 2. Breakthrough curves at flow-rates 800 ml/h (●), 250 ml/h (▲) and 80 ml/h (◐). a, *p*-Chloro-*m*-cresol; b, 3,4-dichlorophenol.

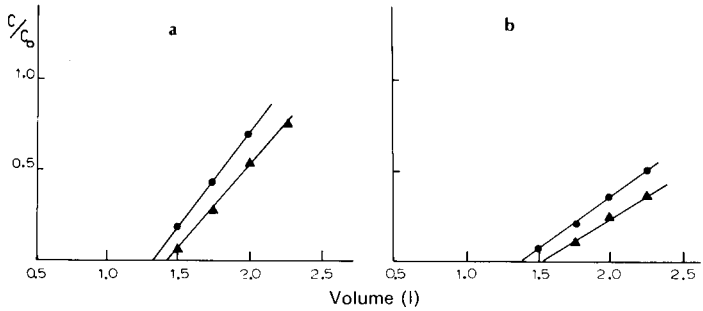

Fig. 3. Breakthrough curves at 30 g/l NaCl (●) and 0.1 g/l NaCl (▲). a, *p*-Chloro-*m*-cresol; b, 3,4-dichlorophenol.

The ionic strength effect on the collection efficiency was evaluated by using water solutions containing different quantities of NaCl. The plots for the above two compounds are reported in Fig. 3a, b. The NaCl (30 g/l) present in the water sample causes moderate variations of breakthrough volumes.

With the optimum operating conditions, the breakthrough curves of all the compounds examined were obtained (Fig. 4) using a glass column packed with 250 mg GCB. Curves for the compounds that after the passage of 2000 ml were not still present in the effluent are not shown. *m*-Cresol has the lowest retention volume.

We can optimize the size of the column using a model substance, selected in regard to the analytical problem that is to be studied. From Figs. 4 and 5 it is seen that an increase of four-fold in the length of the column and in the quantity of GCB (1000 mg instead of 250 mg) results in about a three-fold increase in the breakthrough volume.

The "GCB–solvent distribution ratios", obtained as

$$K = \frac{C_S}{C_L} = \frac{\mu\text{g of compound per g of solid phase}}{\mu\text{g of compound per ml of solvent}}$$

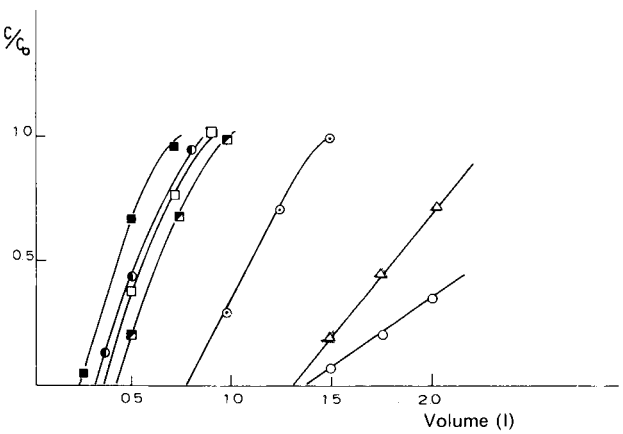

Fig. 4. Breakthrough curves of *m*-cresol (■), heptanoic acid (◐), *o*-cresol (□), 2-chlorophenol (◩), octanoic acid (○), *p*-chloro-*m*-cresol (△) and 2,3-dichlorophenol (○) on GCB column (250 mg).

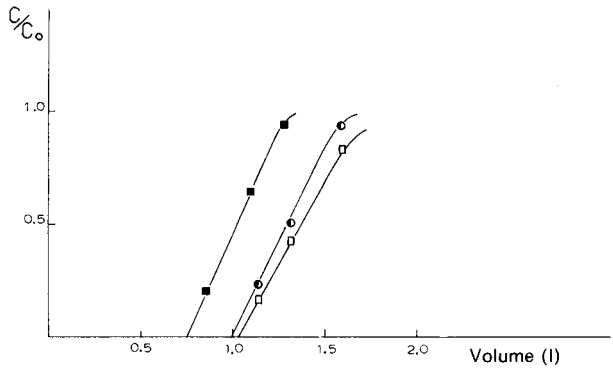

Fig. 5. Breakthrough curves of *m*-cresol (■), heptanoic acid) and *o*-cresol (□) on GCB column (1000 mg).

are reported in Table II. Benzene and methanol give the best distribution ratios for the recovery of the examined compounds from GCB; hence these substances can be recovered with benzene–methanol mixtures.

Table III shows that, while the recovery values with methanol agree with the distribution ratios, this is not the case with benzene. In fact even if the distribution ratios are favourable to benzene, the percentage recoveries are about 10–20%. However, if benzene–methanol mixtures are used, the results are in accord with the distribution ratio values. The disagreement in the case of benzene might be due to the water which coats the GCB and prevents benzene from coming into contact with the

TABLE II

ADSORPTION COEFFICIENTS IN GCB–LIQUID SYSTEMS

Amounts: GCB, 500 mg; liquid system, 100 ml; compound, 15 μg. A = Light petroleum (b.p. 40–60°C); B = diethyl ether; C = benzene; D = dichloromethane; E = methanol.

Compound	*Liquid system*				
	A	*B*	*C*	*D*	*E*
2-Chlorophenol	244	88	0	0	33
o-Cresol	82	44	0	0	47
m-Cresol	60	67	6	4	8
p-Chloro-*m*-cresol	276	265	13	10	22
2,3-Dichlorophenol	108	94	13	17	35
β-Naphthol	976	225	0	0	33
2,4,5-Trichlorophenol	467	244	22	17	38
2,4,6-Trichlorophenol	265	244	33	0	63
2,3,4,6-Tetrachlorophenol	445	225	27	22	137
m-tert.-Butylphenol	139	200	0	10	15
Heptanoic acid	27	41	30	22	0
Octanoic acid	33	53	27	27	0
Decanoic acid	33	44	17	38	0
Myristic acid	157	133	38	177	16
Stearic acid	371	139	47	326	58
Benzoic acid	340	464	67	164	100

TABLE III

RECOVERY OF MODEL ORGANIC COMPOUNDS FROM 1000 mg OF GCB USING DIFFERENT ELUENTS

A = 50 ml benzene; B = 50 ml benzene–methanol (2:1); C = 50 ml benzene–methanol (1:1); D = 50 ml benzene–methanol (1:2); E = 50 ml benzene–methanol (1:3); F = 50 ml methanol.

Compound	*Recovery (%)*					
	Eluent					
	A	*B*	*C*	*D*	*E*	*F*
2-Chlorophenol	28	88	74	92	91	71
o-Cresol	26	55	51	60	78	100
m-Cresol	19	78	84	90	96	91
p-Chloro-*m*-cresol	20	49	74	82	91	90
2,3-Dichlorophenol	25	50	81	92	90	89
β-Naphthol	0	57	98	102	100	29
2,4,5-Trichlorophenol	9	65	90	98	101	80
2,4,6-Trichlorophenol	8	48	61	79	95	101
2,3,4,6-Tetrachlorophenol	6	76	88	91	103	76
m-tert.-Butylphenol	6	22	33	55	84	101
Heptanoic acid	29	89	87	94	88	99
Octanoic acid	18	85	84	88	87	100
Decanoic acid	12	75	70	77	81	92
Myristic acid	16	85	76	79	65	63
Stearic acid	18	95	79	81	78	20
Benzoic acid	0	98	87	71	61	81

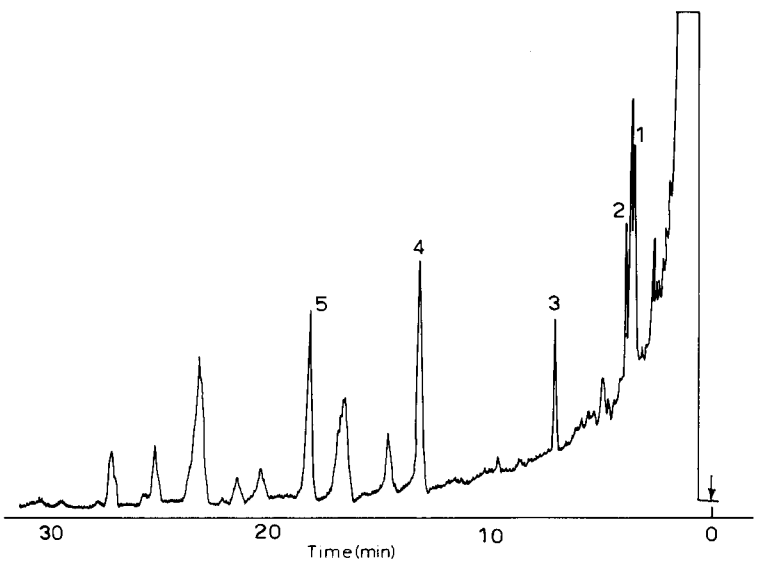

Fig. 6. Chromatogram obtained from human urine. Peaks: 1 = benzoic acid; 2 = lauric acid; 3 = myristic acid; 4 = palmitic acid; 5 = heptadecanoic acid (internal standard).

solid surface, whereas the presence of a small quantity of methanol may be sufficient to prevent the shielding of the water.

If we examine, for example, the behaviour of β-naphthol, whose distribution coefficient in methanol is 33, we find that is recovered only partially by this solvent, and in negligible quantity by benzene, even if the distribution ratio is 0. When a benzene–methanol mixture is passed through the column, the percentage recovery reaches 100%.

For the analyses of human urine and river water the amount of GCB and the volume of eluent were deduced respectively from the breakthrough values and from Table III. Fig. 6 shows a gas chromatogram obtained from human urine on a FFAP capillary column at 200°C with a carrier gas (hydrogen) flow-rate, $\bar{u}$, of 45 cm/sec. The concentrations obtained for the identified components were: benzoic acid, 10 μg/l; lauric acid, 6 μg/l; myristic acid, 14 μg/l and palmitic acid, 29 μg/l.

Fig. 7 shows the chromatogram obtained from river water by using the same column but different operating conditions: temperature, 190°C; carrier gas (hydrogen) flow-rate, $\bar{u}$ = 36 cm/sec. Four peaks were identified, two phenols and two fatty acids. The nature of the substances was confirmed by treating the samples with 0.1 *N* NaOH, followed by extraction with methylene chloride and chromatography of the extract. The identified peaks did not appear in the resulting chromatograms.

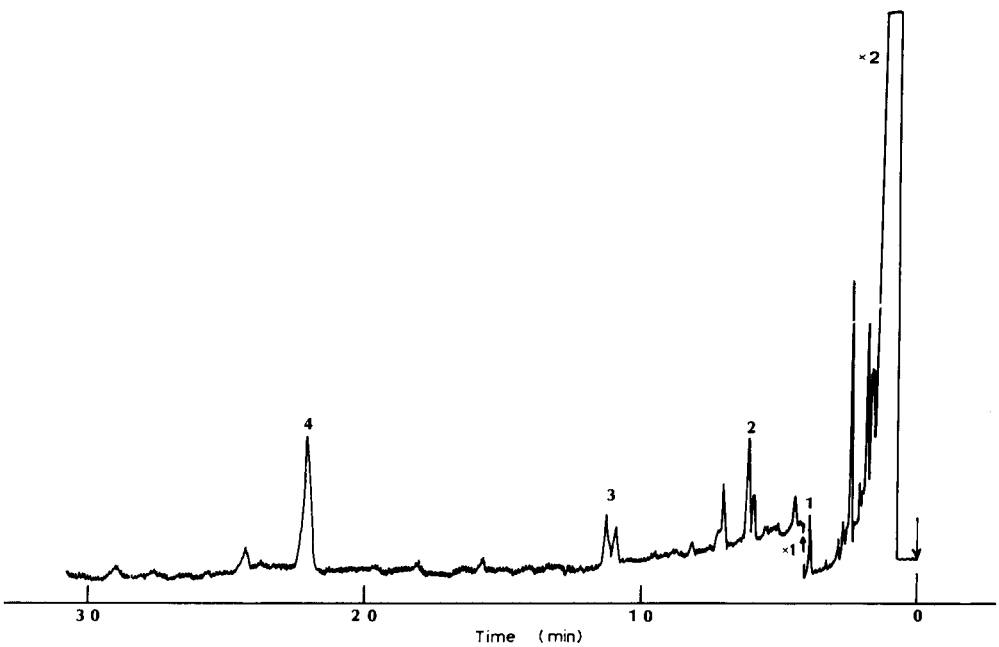

Fig. Chromatogram obtained from river water. Peaks: 1 = 2,4,6-trichlorophenol; 2 = *p*-chloro-*m*-cresol; 3 = myristic acid; 4 = palmitic acid.

CONCLUSIONS

The results show clearly that the recovery of organic acids by means of col-

umns of GCB adsorbent cannot be carried out without considering the nature of the substances to be recovered and their molecular structure.

In fact the organic acids differ considerably in solubility and in K_a values. Therefore the amount of carbon, the volume of the sample, the volume and composition of the eluent must be determined for each application.

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USE OF PERIODATE AND METHYLAMINE FOR THE QUANTITATION OF INTRACELLULAR 5-FLUORO-2′-DEOXYURIDINE-5′-MONOPHOSPHATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography was used for the quantitation of [^{3}H]fluorouracil and metabolites in L1210 cells with or without pre-treatment with methotrexate. Ribonucleotide pools were evaluated on a chemically bonded anion-exchange column. Deoxynucleotide pools were determined following periodate and methylamine treatment which eliminated the ribonucleotides (>99.9% complete) and allowed for a rapid quantitation of the acid-soluble 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) pools. FdUMP levels as low as 10 fmol/10^6 cells were detectable by polystyrene anion-exchange chromatography. Confirmation of these FdUMP values was performed by rechromatography on a reversed-phase ion-pair column. The use of periodate and methylamine as a means for the reliable elimination of the ribonucleotides will allow accurate isolation of deoxynucleotides by high-performance liquid chromatography.

INTRODUCTION

5-Fluorouracil is a clinically useful drug for treating carcinoma of the breast and gastrointestinal tract. We have previously reported that when methotrexate (MTX) precedes fluorouracil (FUra) there is synergistic cytotoxicity of L1210 cells[1]. The therapeutic and toxic effects of FUra are presumed to be mediated from the inhibition of thymidylate synthetase (E.C. 2.7.4.6) by FdUMP and/or the effect of FUTP incorporated into RNA[2].

Possible pathways leading to FdUMP formation include the transfer to FUra of the phosphoribosyl moiety from 5-phosphoribosyl-1-pyrophosphate by orotate phosphoribosyltransferase (E.C. 2.4.2.10). The FUMP formed can then be phosphorylated to FUDP, reduced to FdUDP and finally converted into FdUMP. An alternative mechanism uses uridine (E.C. 2.4.2.3) or thymidine phosphorylase (E.C. 2.4.2.4) to transfer a ribose or deoxyribose to FUra to form the ribonucleoside or deoxyribonucleoside derivative which can be phosphorylated by uridine-cytidine kinase (E.C. 2.7.2.48) or thymidine kinase (E.C. 2.7.1.21) to FUMP or FdUMP, respectively.

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A high-performance liquid chromatographic (HPLC) method was developed specifically to evaluate FdUMP. Thin-layer[3] and column chromatography[4–6] have been used to evaluate metabolic *de novo* pyrimidine and purine pathways, but poor resolution, inadequate sensitivity and the time required to perform these separations render these methods unacceptable for rapid and repetitive determinations. A very sensitive competitive ligand-binding assay for measuring FdUMP levels in cell culture has been developed by Moran *et al.*[7] and Murinson *et al.*[8]. Garrett and Santi[9] recently reported a rapid and sensitive method for evaluating deoxynucleoside triphosphates in cell extracts following destruction of the ribonucleotides by periodate and methylamine, which was then used by Washtien and Santi[10] for the evaluation of FdUMP levels in cell extracts previously exposed to [^{3}H]FUra. The chemical degradation of ribonucleotides as a result of periodate and methylamine treatment has been elegantly described by Khym[11], Brown and Read[12] and Rammler[13], and has been utilized by others for the evaluation of ^{32}P-labelled deoxynucleotides[14,15]. Ritter and Bruce[16] did not use methylamine with the periodate-treated cell extracts, and reported variable and inconsistent resolution of deoxynucleotides because of interfering ribonucleotides.

Our study was designed to define the precise methodology by which multiple cell extracts could be prepared for rapid and reproducible analysis of both ribonucleotides and deoxynucleotides by HPLC. A second objective was to determine by several chromatographic methods that the radioactivity eluting in the deoxynucleotide region of cells treated with [^{3}H]FUra was indeed FdUMP and that the procedure could be quantitative in the fmol/10^6 cell range, which is *ca.* 1000-fold more sensitive than previous methods using HPLC and [^{3}H]FUra.

MATERIALS AND METHODS

Chemicals

[2-^{14}C]FUMP (45 mCi/mmol), [6-^{3}H]FdUMP (25 Ci/mmol), [6-^{3}H]FUMP (20 Ci/mmol), [2-^{14}C]FdUMP (50 mCi/mmol) and [6-^{3}H]FUra (25 Ci/mmol) were obtained from Morevak Biochemicals (City of Industry, CA, U.S.A.) and greater than 95% pure by HPLC. UMP, UDP, UTP, FdUrd, UDP-Glc, FUra and FUrd were purchased from Sigma (St. Louis, MO, U.S.A.) FdUMP was purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.). Aquasol Liquid Scintillant was purchased from New England Nuclear (Boston, MA, U.S.A.). Certified ACS sodium phosphate monobasic monohydrate, ammonium acetate anhydrous, sodium acetate anhydrous and glacial acetic acid were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Phosphoric acid (85%), sodium hydroxide, and ammonium hydroxide were purchased from Mallinckrodt (St. Louis, MO, U.S.A.). ACS certified HCl, sodium periodate and methylamine ($CH_3NH_2 \cdot HCl$) were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Methanol and UV grade acetonitrile were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Tetra-*n*-butylammonium bromide, 99%, was purchased from Aldrich (Milwaukee, WI, U.S.A.).

A flow chart of the methodology for analysis of cells is shown in Fig. 1. Detailed explanation of each aspect of the analysis is given in the following paragraphs.

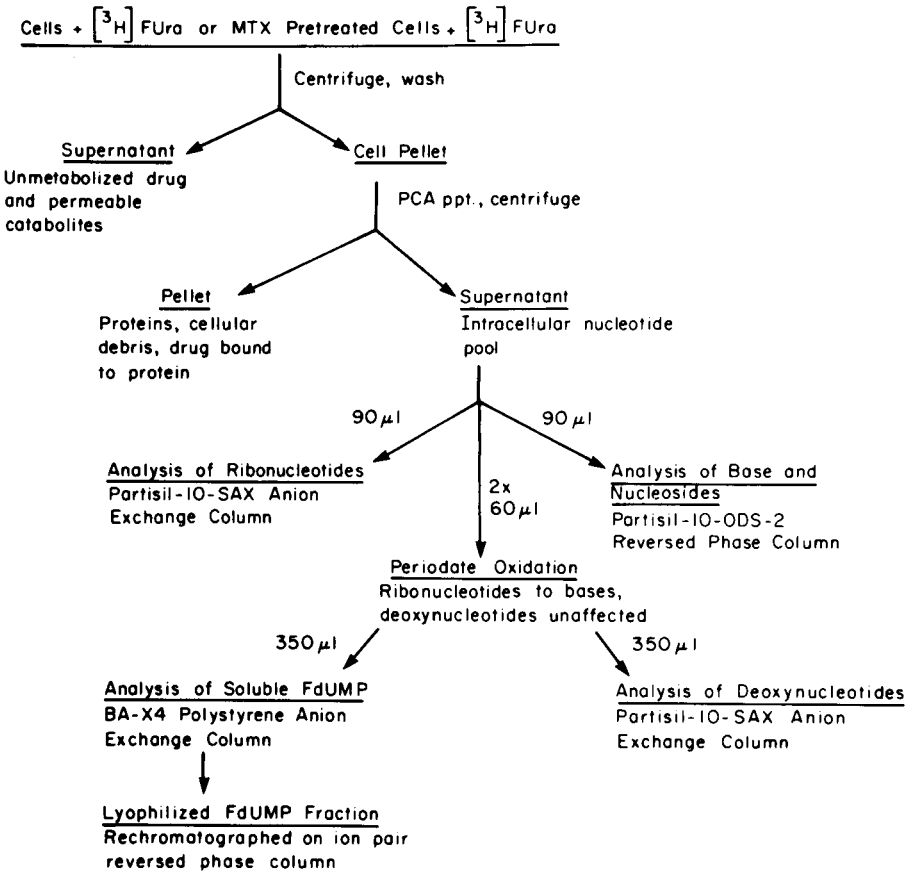

Fig. 1. Schematic representation of the preparation and evaluation of fluoropyrimidine nucleotides.

HPLC mobile phase preparation

Solutions (1 mol/l) of sodium phosphate and sodium acetate were screened for minimum ultraviolet (UV) absorbance at 254 nm (1-cm path length). Acceptable lots of phosphate and acetate had an absorbance of less than 0.02 and 0.005 O.D, respectively. All buffers were made up daily in deionized distilled water and filtered through a 0.2-μm metrical filter (Gelman GA-8; Gelman, Ann Arbor, MI, U.S.A.) and degassed by sonication prior to use.

Tissue culture

L1210 cells were grown as suspension cultures in Fischer's medium supplemented with 10% horse serum. A 50-ml volume of the cell suspension at logarithmic growth (*ca.* 2×10^5 cells/ml) were exposed for 3 h to 10 μ*M* MTX, while another group of cells served as controls. Following the MTX exposure the cells were exposed to 1 μ*M* [6-^{3}H]FUra (25 Ci/mmol) for 1, 3 and 6 h. A second group of cells was concentrated to a 5-ml suspension by centrifugation (1000 *g* for 4 min) before adding [6-^{3}H]FUra for 1 h to reduce the amount of radiolabel used. After the exposure to

[6-^{3}H]FUra, cell pellets were prepared by centrifugation and cellular extracts obtained. The concentration of L1210 cells for 1 h has previously been shown to have no effect on cellular metabolism as determined by viability studies performed in soft agar[17].

Preparation of cell extract

The cell suspensions were centrifuged at 2000 rpm in a Sorval RC-58 centrifuge (DuPont Instruments, Newtown, CT, U.S.A.) at 4°C. The cell pellet was then washed with ice-cold PBS (4°C) and centrifuged as before. The supernatant was decanted before lysing the cells by adding ice cold 0.5 *M* $HClO_4$ (500 μl). The $HClO_4$ precipitate was removed by centrifugation at 2000 rpm for 2 min, leaving a clear supernatant which contained the acid-soluble nucleotides. The pellet of cellular debris was then digested (1 *N* KOH) and the radioactivity determined. Only 10% of the total intracellular radioactivity was present in this acid-precipitable cell fraction; 90% was associated with the intracellular supernatant.

Periodate oxidation

A 60-μl volume of the KOH-neutralized $HClO_4$ L1210 cell extract was transferred to a 400-μl polypropylene test-tube (Sarstedt, G.F.R.). A 150-μl volume of 12 m*M* sodium periodate, which was prepared daily[18], was mixed with the 60-μl sample aliquot and incubated at 37°C for 30 min. A 60-μl volume of 0.4 *M* $CH_3NH_2 \cdot HCl$, pH 7.5, and 120 μl of 0.01 *M* NaOH were then added to the periodate-treated sample, and the mixture was incubated at 37°C for 15 min to complete base cleavage. A total of five reaction tubes were prepared per sample. Two reaction mixtures were analyzed by HPLC for deoxynucleotides on a Partisil 10 SAX and BAX4 column. A third mixture was used for rechromatographic analysis as described below. The final two samples were used for determining recoveries of radioisotopes from the two analytical columns. A 10-μl volume of unlabelled FdUMP at 1 m*M* was added to one reaction mixture being analyzed on the BAX4 column; 10 μl of FdUMP, UMP, UDP-Glc, UDP and UTP at 1 m*M* were added to the other reaction mixture being analyzed on the Partisil 10 SAX column. The slight separation between FUMP and FdUMP on the BAX4 column served as an internal check on the effectivenees of the periodate oxidation. All samples were kept on an ice bath prior to HPLC analysis; 350-μl aliquots (52.5 μl of actual sample) were injected onto each of the analytical columns.

Special equipment

All gradient chromatographic analyses were performed on an Altex Model 332 liquid chromatograph using two Altex Model 153 UV detectors connected in tandem to monitor 254 and 280 nm. Isocratic chromatographic analyses were run on a modularly constructed liquid chromatograph comprised of the following components: a dual-piston reciprocating pump (Model 100, Altex, Berkeley, CA, U.S.A.); a manual loop injector (Model 7120, Rheodyne, Berkeley, CA, U.S.A.); two fixed-wavelength UV detectors set for 254 and 280 nm, and a dual-pen chart recorder (Model 385, Linear Instruments, Wheaton, MD, U.S.A.). Temperature control was achieved with a circulating water bath (Blue M Electric, Blue Island, IL, U.S.A.) and a glass water jacket (Rainin Instruments, Woburn, MA, U.S.A.).

Measurement of radioactive effluent

Effluent from the Partisil 10 SAX and BAX4 polystyrene anion-exchange columns was collected in 0.9- and 1.0-ml fractions, respectively. After 0.9 ml of deionized-distilled water was combined with each fraction from the Partisil 10 SAX column, 10 ml of Aquasol were added. A similar amount of Aquasol was added directly to each fraction from the BAX4 column. Effluent from the LiChrosorb C_{18} and the Partisil ODS-2 column was collected in 0.5- and 1.0-ml fractions, respectively, and 5 ml of Aquasol were added to each fraction.

The vials were shaken vigorously to produce a clear homogenous solution before determining the radioactive content. The counting efficiencies were 25 ± 5% for ^{3}H and 75 ± 5% for ^{14}C and remained constant throughout the various gradient profiles. The recovery of radioisotope from the analytical column was determined by placing an identical volume of chromatographed sample in a tube of column effluent generated by the fraction collector.

The radiolabelled fractions were counted on a Beckman LS7000 liquid scintillation counter (Irvine, CA, U.S.A.) interfaced to a 48K Horizon I North Star minicomputer (Berkeley, CA, U.S.A.). This enabled data to be printed on a Model 43 teletype and stored on a mini floppy disk to be plotted later and integrated using software written in BASIC.

Rechromatography of soluble FdUMP on ion-pair reversed-phase chromatography

In order to characterize the FdUMP peak from the BAX4 polystyrene anion-exchange column, rechromatography of this region on a 250 × 4.6 mm I.D. LiChrosorb C_{18} 5 μm column maintained at 50°C was employed. A solution of 5 m*M* sodium phosphate, monobasic, 5 m*M* tetra-*n*-butylammonium bromide and 10% methanol was titrated to pH 6.8 with 1.0 *N* NaOH and used to elute the column at 1.0 ml/min.

The FdUMP region from the BAX4 column was collected into a 25-ml centrifuge tube and lyophilized twice. For the second lyophilization, 5.0 ml of deionized distilled water were added. The sides of the tube were rinsed with 2.0 ml of methanol and blown to dryness with nitrogen. The residue was finally dissolved in 100 μl of mobile phase and injected onto the column.

RESULTS

Limits of detection

The limit of detection for each of the chromatographic systems was based on radioactivity in three vials. With a maximum background equal to 50 cpm, 300 cpm was readily detectable and was arbitrarily chosen to represent detectable radioactivity. The limits of FdUMP detection for each of the chromatographic systems was 10–15 fmol/10^6 cells.

Periodate oxidation

In examining optimal conditions for periodate oxidation, 1 m*M* solutions of UMP, UDP and UTP in KOH neutralized $HClO_4$ L1210 cells extracts, representing *ca.* 10^6 cells/ml, were degraded under several concentrations of periodate, methylamine and sodium hydroxide. The optimal pH was 7.5. The addition of sodium hydroxide was necessary to maintain the pH at 7.5 during the reaction and therefore

effect a quantitative conversion of ribonucleotides. Reaction times were also optimized to produce a quantitative chemical degradation of ribonucleotides in the presence of deoxyribonucleotides in the cell extract. Ultimate conditions showed cleavage of

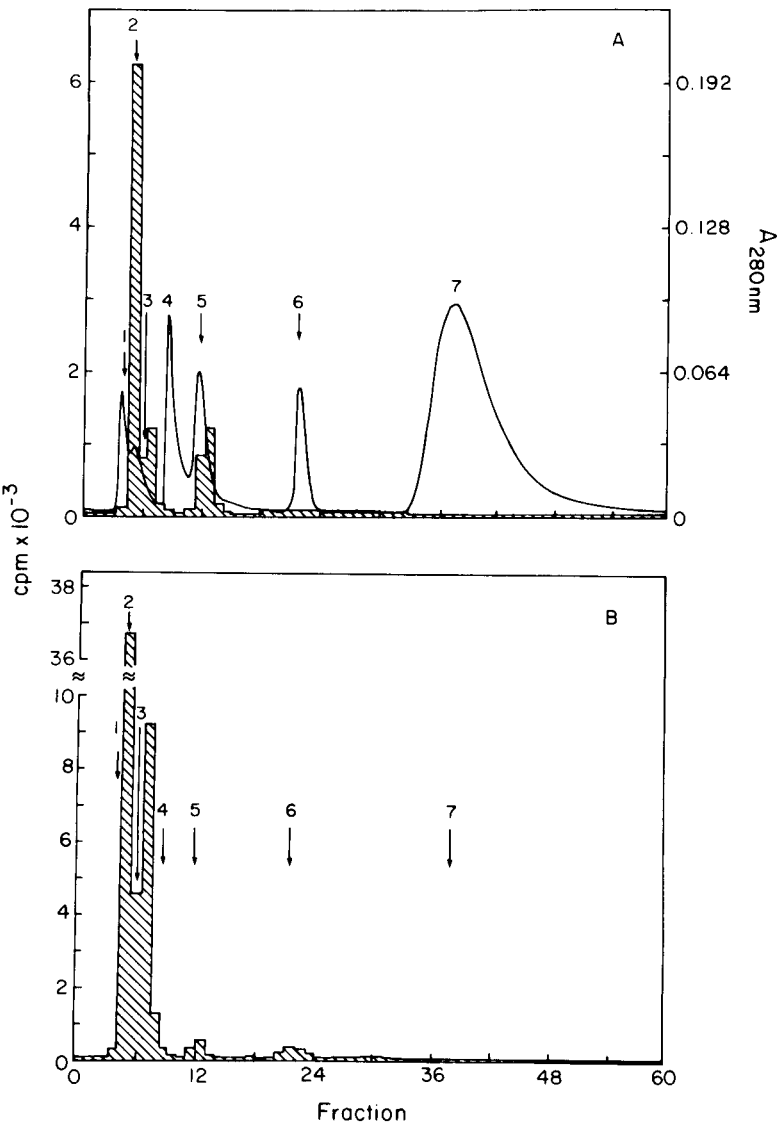

Fig. 2. Separation of nucleosides and bases was performed using a Whatman 250 × 4.6 mm I.D. Partisil ODS-2 10 μm reversed-phase column. KOH-neutralized $HClO_4$ cell extract (90 μl) was injected onto this column and eluted isocratically at ambient temperature at a flow-rate of 0.7 ml/min with 0.15 *M* sodium acetate, pH 5.5. (A) L1210 cells treated for 1 h with 1 μ*M* [6-^{3}H]FUra (20 Ci/mmol). (B) Cell extracts of MTX (10 μ*M*) pre-treated cells followed by 1 h of [6-^{3}H]FUra treatment. Cold markers of UTP, UDP-Glc, UDP, FUMP, FdUMP, FUra, FUrd and FdUrd (represented by peaks 1–7, respectively) were coinjected with cell extracts, and radiochromatograms were plotted. Endogenous cellular components are not visible at this wavelength and sensitivity.

ribonucleotide [2-^{14}C]FUMP (45 mCi/mmol) was always at least 99.9 % quantitative. Similar results were achieved with [6-^{3}H]FUMP, (20 Ci/mmol). The final conditions agree closely with those outlined by Rammler[13]. Appropriate controls were carried out to ensure that under the reaction conditions employed, the chemical integrity of the deoxynucleotide species was maintained. [6-^{3}H]FdUMP (20 mCi/mmol) was less than 0.1 % degraded. Similar results were obtained with [2-^{14}C]FdUMP (50 mCi/mmol). Non-radiolabelled dUDP and dUTP were not affected with periodate,while UDP and UTP were completely degraded as monitored by UV at 254 and 280 nm (0.04 a.u.f.s.).

Lyophilization

Lyophilization of [6-^{3}H]FdUMP (20 Ci/mmol) as a control demonstrated that 90 % was recovered with no radioactivity appearing in any region except that marked by FdUMP.

Chromatograpy

Several investigators have used reversed-phase chromatography to examine nucleoside profiles in biological fluids[20–22]. In this study a modification of these reversed-phase techniques was used to elute nucleosides, nucleotides and deoxynucleosides of FUra isocratically. Resolution between drug and nucleoside metabolites was achieved after optimizing flow-rate, pH and ionic strength on the Partisil ODS-2 column (Fig. 2). All nucleotide metabolites eluted within the first 9 min and were well resolved from drugs and nucleosides.

Chromatographic systems for nucleotide separation using gradient elution on anion-exchange columns are also available[23,24]. Baseline separation was achieved between mono-, di- and triphosphate nucleotides of FUra on the Partisil 10 SAX column (Fig. 3). All nucleosides and drugs eluted in the void volume. Again FdUMP and FUMP remained unresolved.

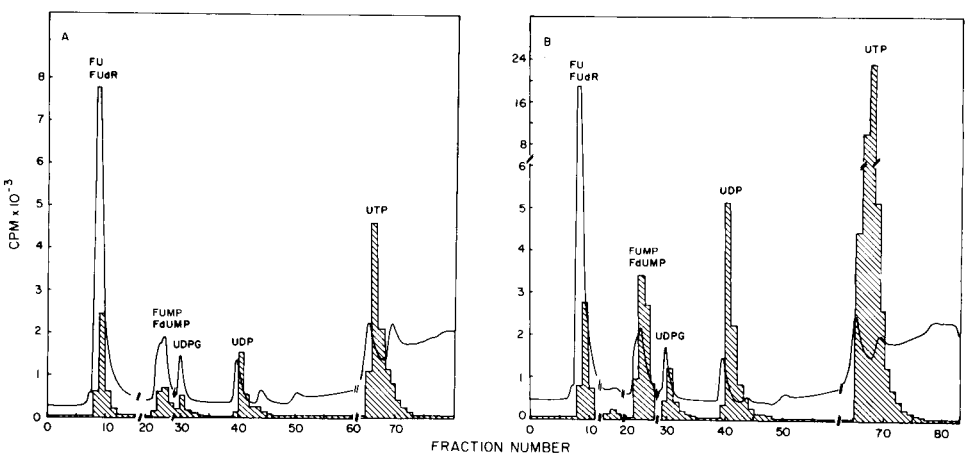

Fig. 3. KOH-neutralized cell extract (90 μl) was mixed with 10 μl of 1 m*M* UMP, FdUMP, UDP, UDP-Glc and UTP as cold markers and injected onto a Whatman 10 SAX 250 × 4.6 mm I.D. anion-exchange column. A linear gradient profile was run from 0.02 to 0.75 *M* sodium phosphate, pH 3.3, over 40 min. Ambient column temperatures were used with a flow-rate of 1.8 ml/min. A and B show the separation of control and MTX pre-treated cells each exposed for 1 h to FUra. The radioactivity co-eluting with UDPG, UDP and UTP represent FUDPG, FUDP and FUTP.

A BAX4 polystyrene anion-exchange column at elevated pH and temperature was used to obtain the desired resolution between FdUMP pools and the void volume radioactivity which was associated with chemically degraded ribonucleotides (Fig. 4).

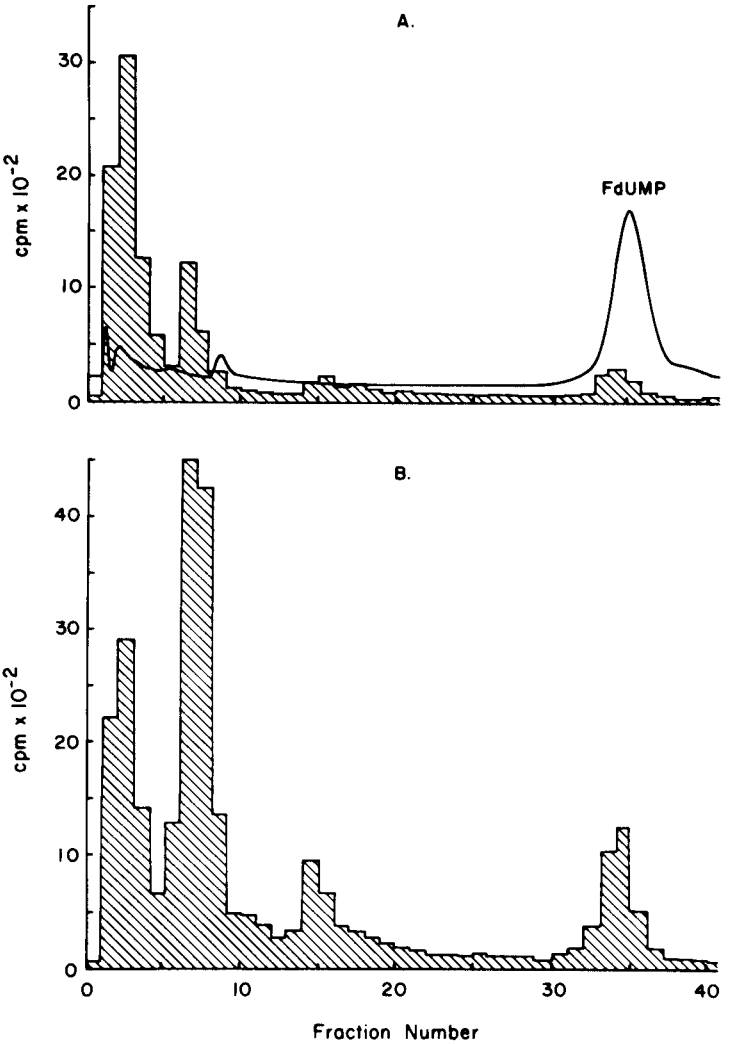

Fig. 4. Separation on the BAX4 polystyrene column of the periodate- and methylamine-treated acid-soluble extract of L1210 cell suspensions, unexposed (A) and exposed (B) to 10 μM MTX for 3 h before adding 1 μM [6-^{3}H]FUra (25 Ci/mmol) for 1 h. Cold FdUMP was used as an unlabelled marker. The analytical column was prepared by slurry-packing a 316 stainless-steel tube (10 cm × 4.6 mm I.D.; Analabs, North Haven, CT, U.S.A.) with 3 g of 4% cross-linked polystyrene anion-exchange resin, *ca.* 10 μm particle size, BAX4 (James B. Benson, Reno, NV, U.S.A.) suspended in 15.0 ml of 0.4 *M* ammonium acetate, pH 7.0. A flow-rate of 1.0 ml/min was maintained during the packing process with pressures not exceeding 1000 p.s.i. Conditions for elution of the periodate oxidized sample were as follows: temperature, 50°C; flow-rate, 1.0 ml/min; 0.4 *M* ammonium acetate buffer, pH 7.0, run isocratically for 6.8 min followed by a linear gradient from 0.4 *M* to 0.6 *M* ammonium acetate, pH 7.0, for 25.0 min. Initial composition was resumed over a 2.0 min period with 15 min re-equilibration prior to the next injection. Regeneration became necessary usually if back-pressure exceeded 1000 p.s.i. for the above chromatographic conditions and was performed by a previously described procedure[19].

This idea is an extension of the chromatography developed by Khym and Cohn[5]. The separation of the FdUMP region from the void volume was much improved compared with analysis on a Partisil 10 SAX column.

Ion-pair reversed-phase chromatography with the LiChrosorb C_{18} 10 μm column provided further evidence for the existence of FdUMP. Washtien and Santi[10] employed a LiChrosorb C_{18} 5-μm column at ambient temperatures to achieve separation between FUMP and FdUMP. By elevating the temperature of the column to 50°C and using 10% methanol in the mobile phase, the column efficiency improved, yielding reasonable retention times and effectively separating FdUMP from FUMP. This chromatographic method clearly demonstrated that the collected radioactive peak in the FdUMP region from the BAX4 column was quantitatively associated with FdUMP. Table I summarizes the results of these chromatographic systems.

The ATP/ADP ratios for all cell extracts analyzed were 5:1, indicating little hydrolysis at the time of analysis and a good energy balance (data not shown). Although there was a five-fold increase in FUTP levels compared to controls observed for cells pre-treated with MTX, periodate oxidation of these samples followed by analysis of the reaction mixture on the BAX4 polystyrene anion-exchange chromatography system revealed there was no radioactivity remaining in the triphosphate region. There was, however, an approximately 5-fold increase in FdUMP levels of MTX pre-treated samples (114 fmol/10^6 cells) over controls (22 fmol/10^6 cells). The effect of MTX on FUra metabolism has been reported in detail elsewhere[25].

DISCUSSION

The destruction and elimination of ribonucleotides by periodate oxidation is a method that will allow the subsequent separation and quantification of deoxyribonucleotides by HPLC, specifically FdUMP from the fluoropyrimidine ribonucleotides. This method may be applicable for the investigation of any intracellular deoxynucleotide pool or other drugs which could be metabolized to deoxyribonucleotide derivatives. An example of the use of this method was demonstrated in L1210 cells pre-treated with MTX in which a 5-fold increase in FdUMP was documented (22 fmol/10^6 cells *vs.* 114 fmol/10^6 cells). Washtien and Santi[10] found 80 fmol of the complex of FdUMP–5,10-methylenetetrahydrofolate–thymidylate synthetase per 10^6 cells (L1210) after incubating with 0.25 μM [6-^{3}H]FUra for 22 h. These investigators were able to detect only intracellular levels of free FdUMP after heat treatment of the sample, which dissociated this enzyme complex. A stable ternary complex has been demonstrated[26,27] between FdUMP, thymidylate synthetase and 5,10-methylenetetrahydrofolate when prepared in acid as in our studies. The formation of this covalent complex offers a tenable explanation for differences in FdUMP levels found in MTX pre-treated cells and control cells. MTX, which inhibits dihydrofolate reductase, would result in low levels of the co-factor 5,10-methylenetetrahydrofolate, which could therefore reduce the amount of the stable covalent complex formed. Hence cells pre-treated with MTX would show high levels of unbound intracellular FdUMP. The control samples, which would have higher levels of 5,10-methylenetetrahydrofolate, might be expected to form a stable protein complex with FdUMP, which could conceivably precipitate during $HClO_4$ extraction and therefore result in less free intracellular FdUMP. We are currently using high-performance gel filtration chro-

TABLE I

RETENTION DATA FOR HPLC

Values in minutes.

Column	*Chromatographic system*	*FUra*	*FUrd*	*FdUrd*	*FdUMP*	*FUMP*	*UDP-Glc*	*UDP*	*UTP*
Partisil SAX	Chemically bonded anion-exchange	4.8	4.8	4.8	11.0	–	18.0	23.0	33.0
BAX4	Polystyrene anion-exchange	7.2	6.5	8.6	32.2	36	24.2	–	–
LiChrosorb C_{18}	Ion-pair reversed-phase	4.8	–	–	40.6	33.7	–	–	–
Partisil ODS-2	Reversed-phase	11.2	22.6	37.8	8.7	6.0	4.0	4.2	4.0

matography for isolating this ternary complex in control and MTX-treated cells to evaluate this possibility[28].

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ABBREVIATIONS

FUra	= 5-fluorouracil
Furd	= 5-fluorouridine
FdUrd	= 5- fluoro-2′-deoxyuridine
FdUMP	= 5-fluoro-2′-deoxyuridine-5′-monophosphate
FUMP	= 5-fluorouridine-5′-monophosphate
FUDP	= 5-fluorouridine-5′-diphosphate
FUTP	= 5-fluorouridine-5′-triphosphate
UMP	= uridine-5′-monophosphate
UDP	= uridine-5′-diphosphate
UTP	= uridine-5′-triphosphate
HPLC	= high-performance liquid chromatography
MTX	= methotrexate
dUDP	= 2′-deoxyuridine-5′-diphosphate
dUTP	= 2′-deoxyuridine-5′-triphosphate
FdUDP	= 5-fluoro-2′-deoxyuridine-5′-diphosphate
FdUTP	= 5-fluoro-2′-deoxyuridine-5′-triphosphate
FUDP-Glc	= 5-fluoro-uridine-5′-diphosphoglucose
UDP-Glc	= uridine-5′-diphosphoglucose

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THE DETERMINATION OF O,S,S-TRIMETHYLPHOSPHORODITHIOATE IN THE PLASMA AND VARIOUS TISSUES OF RATS USING HIGH-RESOLUTION GAS CHROMATOGRAPHY WITH NITROGEN–PHOSPHORUS DETECTION

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(Received May 15th, 1981)

SUMMARY

A method has been developed for the determination of O,S,S-trimethylphosphorodithioate in the plasma, lung, liver, brain and thymus of rats using high-resolution gas chromatography. The organophosphorus compound was extracted from the biological sample with ethyl acetate and analysed on a Carbowax 20M fused-silica capillary column with a nitrogen-phosphorus specific detector. O,S,S-Triethylphosphorodithioate was used as an internal standard added to the sample before extraction. The sensitivity of the method allowed the compound to be measured in 0.1-ml aliquots of plasma or in 20-mg wet weight of tissue down to a level of 5 ng/sample. The method has been applied to a pharmacokinetic study in the rat after an oral or intravenous dosage with 25 mg/kg of O,S,S-trimethylphosphorodithioate.

INTRODUCTION

O,S,S-Trimethylphosphorodithioate (OSS-Me) is one of a number of minor impurities found in technical formulations of malathion[1,2] which has been shown to potentiate the toxicity of this insecticide in the rat[1–3]. By inhibiting rat liver carboxylesterase it prevents the normal metabolism of malathion and results in the increased formation of a toxic metabolite malaoxon[4]. When administered on its own to rats at an LD_{50} dose OSS-Me causes two distinct toxic effects. Initially, over the first 24 h, it produces a typical cholinergic response from which the animal recovers. A second phase of toxicity is evident between days 3 and 6, which is related to an effect on the lung. The LD_{50} of this compound is related to this second phase of toxicity[5].

As a contribution to a better understanding of the toxicity of OSS-Me in the rat, a study of the distribution and kinetic behaviour of this organophosphorus compound has been made. For the purpose of this study an assay procedure was required which would allow measurements to be made at low levels in small samples of plasma or tissue. For this reason we have developed a method based on the use of high-

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resolution capillary gas chromatography with a sensitive and selective thermionic nitrogen–phosphorus detector (NPD).

MATERIALS AND METHODS

Reagents

OSS-Me and O,S,S-triethylphosphorodithioate (OSS-Et), prepared by the method of Aldridge *et al.*[3], were obtained from Dr. J. W. Miles, Bureau of Tropical Diseases, Centre for Disease Control, Atlanta, GA, U.S.A. The purity of these compounds was checked by capillary gas chromatography with flame ionization detection and was found to be greater than 98%. Standard stock solutions (1 mg/ml) prepared in ethyl acetate were stable for several months when stored at 4°C. Ethyl acetate (BDH, Poole, Great Britain) was of Analar grade and was used without further purification.

Animal studies

Female LAC Porton derived Wistar rats (body weight 170–200 g; 8–10 weeks old) were used throughout. OSS-Me was dissolved in glycerol formal (Fluorochem, Glossop, Great Britain) to obtain a dosage volume of 1 ml/kg, and this was administered either intravenously or orally at a dosage of 25 mg/kg. Oral dosing was carried out by oesophogeal intubation and intravenous dosing by injection into a lateral tail vein following prewarming of the tail at 48°C.

At the pre-determined times rats were bled out from the heart under deep ether anaesthesia and tissue obtained for analysis. Blood samples collected in heparinized capillary tubes were centrifuged immediately after collection and the plasma stored at −5°C until analysed. Tissues were removed from the rat within 1–2 min of bleeding out and were immediately frozen in liquid nitrogen. The frozen tissues were weighed, and homogenized in ice-cold distilled water using a Ystral X10/20 homogenizer to give a 20% homogenate.

Sample preparation

To a 0.1-ml aliquot of rat plasma or tissue homogenate, contained in a 1.5-ml polypropylene microcentrifuge tube (Alpha Laboratories, Eastleigh, Hants, Great Britain), was added 10 μl of the internal standard solution of OSS-Et in ethyl acetate (50 μg/ml). The sample was extracted with 500 μl of ethyl acetate by Vortex mixing for 30 sec, followed by centrifugation for 2 min at 2000 *g*. The solvent layer was then transferred, using a Pasteur pipette, into a similar centrifuge tube for analysis by gas chromatography.

Gas–liquid chromatography

Analyses were carried out on a Packard Becker 419 gas chromatograph equipped with dual flame ionization detectors. One of these detectors was replaced by a Perkin-Elmer alkali-bead NPD. The existing liquid injection system on this instrument was modified for the split–splitless injection of samples into a capillary column.

The gas chromatographic separation was made using a fused-silica capillary (20 m × 0.3 mm I.D.) coated in the authors' laboratory with Carbowax 20M by the static coating procedure to give a film thickness of 0.4 μm. The flexibility of fused

silica allowed the end of the column to be located just below the tip of the detector jet. No make-up gas was used to the detector. Helium was employed as carrier gas with a flow-rate, measured at 180°C, of 6 ml/min. The column was operated isothermally at an oven temperature of 180°C. The NPD was operated at an air flow-rate of 120 ml/min and a hydrogen flow-rate of 4 ml/min. The rubidium silicate bead temperature was adjusted according to the sensitivity required for the analysis. The amplifier attenuation was 8×10^{-11} A and the recorder was set at 20 mV. The 1-μl samples were injected using a split ratio of 10:1.

Calibration curves

Standard calibration curves were prepared by analysing 0.1-ml aliquots of blank rat plasma or tissue homogenates containing known amounts (100, 400, 600, 800, 1000, 1600 and 2000 ng) of OSS-Me and 500 ng of the internal standard (OSS-Et). The peak height ratios OSS-Me:OSS-Et were plotted against the concentration of OSS-Me. The concentration of OSS-Me in the unknown samples was derived from regression equations obtained from the standard curve. When the concentration levels were expected to be less than 500 ng/ml sample, one-tenth of the above amounts of OSS-Me and internal standard were used for the calibration curve.

RESULTS AND DISCUSSION

The alkali-bead NPD, when operated in the nitrogen–phosphorus mode, is a very stable and reliable detector which exhibits a very high sensitivity of detection for many organophosphorus compounds[6,7]. When used with a capillary column, as little as 1 pg of OSS-Me and OSS-Et can be detected with a signal-to-noise ratio of 20:1. The selectivity of this detection system allowed OSS-Me and the added internal standard OSS-Et to be determined in ethyl acetate extracts of plasma and tissue homogenates at very low levels. No concentration or purification of the solvent extract was found to be necessary.

Fig. 1 shows a typical trace from the analysis of a 0.1-ml plasma sample from a rat following an oral dose of 25 mg/kg OSS-Me. Similar contaminant-free tracings

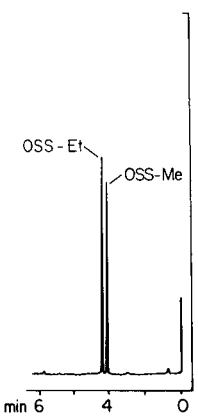

Fig. 1. Gas chromatogram of an extract from a rat plasma sample containing 4 μg of OSS-Me per ml of plasma. For the gas chromatographic conditions see text.

were obtained from tissue samples. The analysis of blank rat plasma and tissue homogenates gave very few background peaks, none of which interfered with the measurement of the peak derived from either OSS-Me or the internal standard.

A representative calibration curve for quantitation in plasma is shown in Fig. 2. Good linearity was obtained over this concentration range and over the lower range between 100 ng/ml and 2 μg/ml.

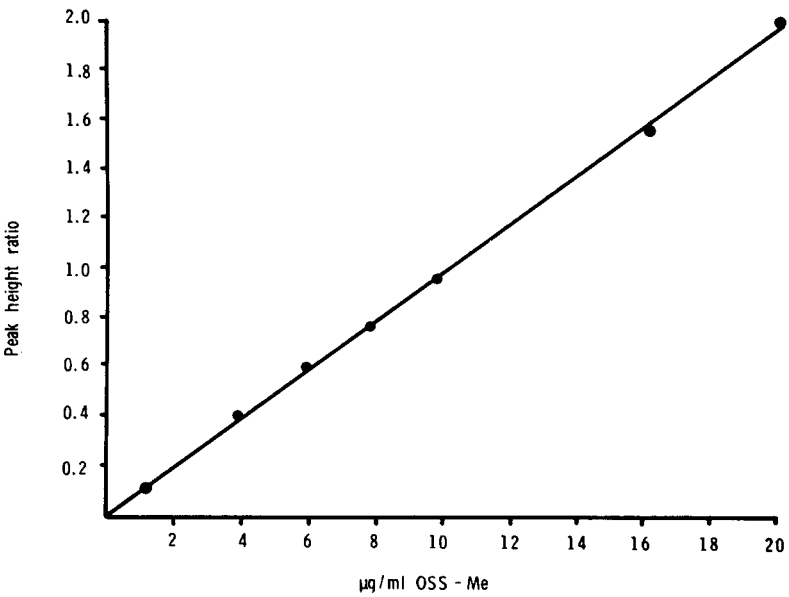

Fig. 2. Calibration curve for the analysis of OSS-Me in plasma using OSS-Et as internal standard.

A single extraction with ethyl acetate gave a high recovery of both OSS-Me and the internal standard. Using OSS-Et as an internal standard the mean absolute recovery of OSS-Me from spiked rat plasma was 87.5% (S.D. ± 4.3%) and from a tissue homogenate 89.6% (S.D. ± 3.4%). The accuracy of the method was determined from recovery experiments of authentic OSS-Me added to blank plasma and lung samples at concentrations of 4, 8 and 16 μg/ml plasma or tissue homogenate (1 g of wet weight tissue homogenate made up to 5 ml with distilled water). The precision of the method was determined from replicate analyses at these same concentrations. The results from these analyses are given in Table I.

The sensitivity of the method can, if required, be improved by reducing the volume of ethyl acetate used for extraction and also by injecting the extract into the capillary column using the splitless rather than the split injection technique. The lower limit of detection is in the order of 50 ng/ml plasma or 250 ng/g tissue.

A number of rat plasma samples were analysed immediately after collection and then following their storage at 4°C for two weeks. No significant difference in the results was obtained. Extracted samples were also stable when stored under these same conditions. Tissues were immediately immersed in liquid nitrogen after their removal from the animal to prevent possible enzyme degradation. After homogenization, these samples were also stable when stored at 4°C.

TABLE I

PRECISION AND ACCURACY OF THE METHOD FROM THE REPLICATE ANALYSES OF OSS-Me ADDED TO BLANK PLASMA AND LUNG HOMOGENATE SAMPLES

Each value is the mean of six determinations.

Amount added (*μg/ml*)	*Plasma*		*Tissue*	
	Amount found (*μg/ml* ± *S.D.*)	*Recovery* (*%* ± *S.D.*)	*Amount found* (*μg/ml* ± *S.D.*)	*Recovery* (*%* ± *S.D.*)
0.4	0.43 ± 0.01	108.3 ± 2.36	0.42 ± 0.03	105.0 ± 6.27
0.8	0.84 ± 0.02	105.4 ± 2.76	0.83 ± 0.06	103.7 ± 7.60
1.6	1.58 ± 0.05	98.9 ± 3.26	1.56 ± 0.07	97.7 ± 4.41

This method has been applied to a study of the pharmacokinetics in the rat after oral and intravenous injection of OSS-Me. Following oral administration, peak concentrations of the organophosphorus compound were found in the plasma between 30 min and 1 h after dosage. The concentration fell to approximately one-tenth of these levels 5 h later, but measurable amounts were still present after 24 h (see Fig. 3). In the liver, lung, brain and thymus the concentration–time profile was similar, showing that the compound was evenly distributed throughout the animal.

After intravenous injection the plasma and tissue concentrations at 30 min corresponded to those observed 60 min after oral administration, but from then on the disappearance curves were similar. The only exception was liver, where peak levels after intravenous dosing were *ca.* 90% lower. The calculated half-life values were *ca.* 60 min. More details from these kinetic studies will be given in a future publication.

In conclusion, the method described in this publication has proved to be ac-

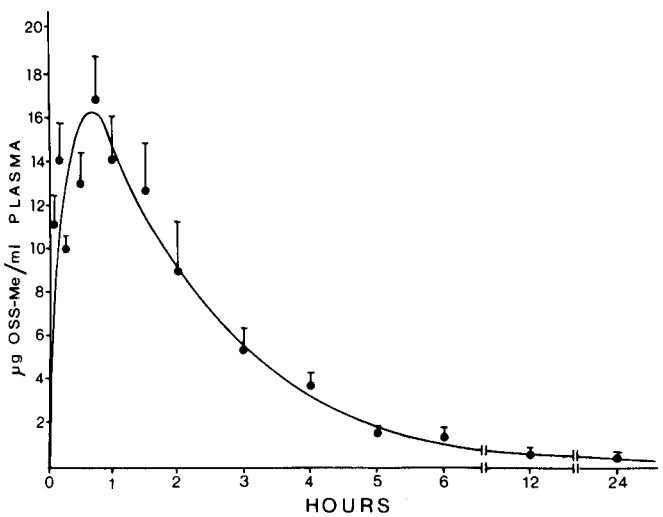

Fig. 3. Plasma concentration–time profiles in the rat after oral dosage of 25 mg/kg. Levels are presented as mean values ± standard error of the mean ($n = 4$).

curate and reliable for quantifying OSS-Me in small sample volumes of rat plasma and tissue. The employment of a high-resolution capillary column enhances both the sensitivity and specificity of the method. Since the assay involves only a single solvent extraction and a short analysis time on the column, it is simple and rapid.

Using the appropriate internal standard this assay procedure can be used to determine and study the kinetics of other organophosphorus triesters, including O,S,S-trimethylphosphorothioate and O,O,S-trimethylphosphorodithioate, which also occur as toxic impurities in technical malathion.

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GAS CHROMATOGRAPHIC DETERMINATION OF NOXYPTYLINE IN SUBSTANCE, TABLETS AND IN BIOLOGICAL MATERIAL

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SUMMARY

Noxyptyline, *i.e.* 5-(2-dimethylaminoethyloxyimine)-5*H*-dibenzo[*a*,*b*]cyclohepta-1,4-diene hydrochloride, is an antidepressant. A new, direct method for its determination in substance and in tablets by means of gas chromatography has been developed. The results were compared with those of the spectrophotometric method, and the systematic errors (coefficient of variation) were 2.19 % and 2.49 %, respectively.

Apropriate conditions were developed for the extraction of noxyptyline from plasma and urine, and the gas chromatographic method was applied for its determination. Within the concentration range 0.5–10 $\mu g/cm^3$, the systematic error after extraction from plasma was 4.61 %, and after extraction from urine it was 2.08 %. The recovery from plasma was 71.12 ± 8 %, and from urine it was 90.94 ± 1.5 %.

INTRODUCTION

Noxyptyline is administered[1] only in tablet form: the initial doses of 25–50 mg daily may be increased gradually to 100 and even 300 mg daily. Like other dibenzocycloheptadiene derivatives, it has been determined by titration and spectrophotometric methods described in pharmacopoeiae. Literature reports describe colorimetric methods for the determination of noxyptyline, involving the formation of complexes with bromothymol[2] and bromophenol[3], as well as two titration methods: one following precipitation with potassium titration methods: one following precipitation with potassium hexathiocyanogen chromate and ammonium tetrathiocyanogen chromate[4], the other applied after hydrolysis to dibenzocycloheptenone[2].

All the above methods for the determination of noxyptyline are not sensitive enough to be applied for determination of the same compound in body fluids (plasma, urine). It seems worth emphasising that no method for determination of noxyptyline in biological material has been quoted in literature.

Therefore, we have outlined the following aims of the present work:

(1) to work out conditions of determination of noxyptyline without synthesising its derivatives;

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(2) to develop conditions for the extraction of noxyptyline from human plasma and urine;

(3) to determine noxyptyline by gas chromatography of the extracts obtained.

These three problems are closely related to pharmacokinetic investigations of noxyptyline.

EXPERIMENTAL

Materials and reagents

The following chemicals were used: noxyptyline of 99.22% purity (Rzeszów Pharmaceutical Works, "POLFA"); noxyptyline of 99.42% purity (Bayer); noxyptyline tablets, 25 mg content of the active ingredient declared (Rzeszów); Oxazepam of 99.73% purity (Tarchomin Pharmaceutical Works, "POLFA"); 95° ethanol, hexane, silicone OV-17, and Gas-Chrom Q.

Apparatus

The following apparatus was used: Perkin-Elmer gas chromatograph, Model 3920 B; Mechanika Precyzyjna centrifuge, type 317a; Premed versatile shaker, type WV-3.

Qualitative determinations of noxyptyline

Noxyptyline used for the determinations melted in the range 188–189°C. The results of ultraviolet and infrared absorptiometric tests complied with the requirements of pertinent standards.

The substance was tested for purity with a thin-layer chromatography method in the following system: F_{254} Merck silica gel; chloroform–methanol (7:3). It exhibited one spot of R_F value 0.5, which complied with the standard. The active ingredient content determined with the titration method amounted to 99.22%.

Gas chromatographic determination of noxyptyline

The determination of noxypryline was conducted on a gas chromatograph equipped with a nitrogen detector. A glass column (1.8 m × 0.3 cm I.D.) filled with 1.5% OV-17 for Gas-Chrom Q qas used. The column was conditioned for 48 h at 280°C. The working temperatures were: column, 230°C; evaporator, 250°C; detector, 280°C. Nitrogen was used as a carrier gas at a flow-rate of 46 cm^3/min. The hydrogen flow-rate was 9.2 cm^3/min and the air flow-rate 150 cm^3/min.

The sensitivity of the measurement was 10 × 16. The internal standard was oxazepam, *i.e.* 7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one.

Ethanolic solutions of the tested samples and of the standard, both containing 5 mg% of oxazepam, were prepared within the noxyptyline concentration range: 0.5–2 mg%. Portions (1 μl) of the standard and tested solutions were injected on to the column. The chromatogram obtained is shown in Fig. 1, and the results are listed in Table I. The retention time for noxyptyline was 1 min 50 sec.

The relation between the concentration and the peak height ratio of noxyptyline and the internal standard was linear within the range 0.5–5 $\mu g/cm^3$.

The same method was used for determination of noxyptyline in the model

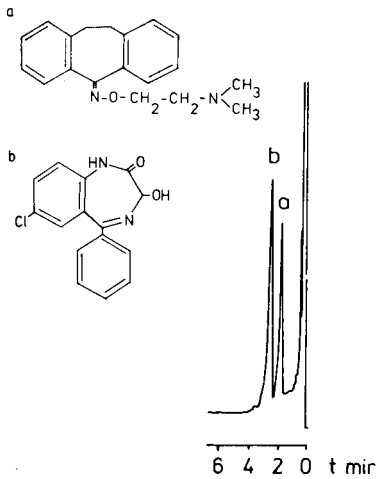

Fig. 1. Gas chromatograms of noxyptyline (a) and internal standard, oxazepam (b).

mixture containing talc, lactose, starch, magnesium stearate and polyvinylpyrrolidone in the amounts declared for the finished product (*i.e.*, for the tablets) by the producer.

A weighed portion of the model mixture, equivalent to one tablet, was placed in a 10-cm^3 measuring flask. Then 8 cm^3 of 95% ethanol was added. The flask was shaked on a shaker for 15 min then its contents were diluted with 95% methanol to 10 cm^3 and filtered. The filtrate was used for the preparation of a noxyptyline solution of 1 mg% concentration, to which 5 mg% of internal standard were added. Portions (0.001 cm^3) of the tested solution and the standard one of the same concentration were injected on to the column.

Noxyptyline was also determined in the tablets of declared 25 mg active substance content. The extraction of noxyptyline from the crushed tablets was conducted

TABLE I

PERCENTAGE OF NOXYPTYLINE IN SUBSTANCE, MODEL MIXTURE AND TABLETS

Materials	*Method*				
	Gas chromatography				*UV*
	$\bar{x}$	*S.D.* ($n = 5$)	*Confidence interval for* $p = 0.95$	*C.V.*	$\bar{x}$ ($n = 5$)
Noxyptyline in substance	99.09	2.17	99.09 ± 2.68	2.19	–
The model mixture (25 mg of noxyptyline and auxiliary substances)	97.19	2.42	97.19 ± 2.99	2.49	95.00
Noxyptyline in tablets of the declared 25 mg active substance content	99.64	1.74	99.64 ± 2.16	1.75	103.92

similarly to the process described above. Simultanously, noxyptyline extracted from the model mixture and from the tablets was determined with spectrophotometric methods. The results are presented in Table I.

In vitro determination of noxyptyline in human plasma

The determination of the tested compound in human plasma was conducted under the conditions worked out for the substance.

Two stock solutions were made up as follows: (1) 10 mg% noxyptyline ethanolic solution; (2) 2.5 mg% oxazepam ethanolic solution. From the stock solutions five standard solutions were prepared, containing 0.5, 1, 2, 3.5 and 5 μg of noxyptyline and 25 μg of oxazepam in 1 cm^3.

Preparation of calibration curves

From the five standard solutions the calibration curve I was constructed. After an extraction from plasma to which respective amounts of noxyptyline were added, the calibration curve II was prepared. In each of five centrifugal tubes 1 cm^3 of plasma was placed, then the following volumes of noxyptyline stock solution were added: 0.005, 0.01, 0.02, 0.35 and 0.05 cm^3. The contents of each tube were stirred and extracted three times with 4 cm^3 of hexane while being shaken for 15 min on a shaker. After 5 min centrifugation (4000 *g*) the upper layer was pipetted out into a conical flask and dried with anhydrous sodium sulphate. The mixture was filtered, and hexane was evaporated under vacuum below 30°C. The residue was dissolved in 0.5 cm^3 of the internal standard solution and 0.001 cm^3 of thus prepared solution was injected on to the column. The chromatogram obtained is shown in Fig. 2.

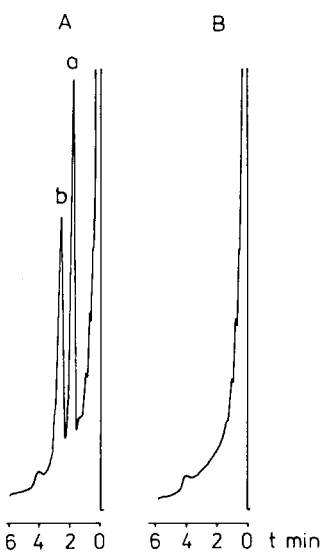

Fig. 2. Gas chromatograms of (A) extract from plasma containing noxyptyline (a) and internal standard, oxazepam (b); (B) extract from blank plasma.

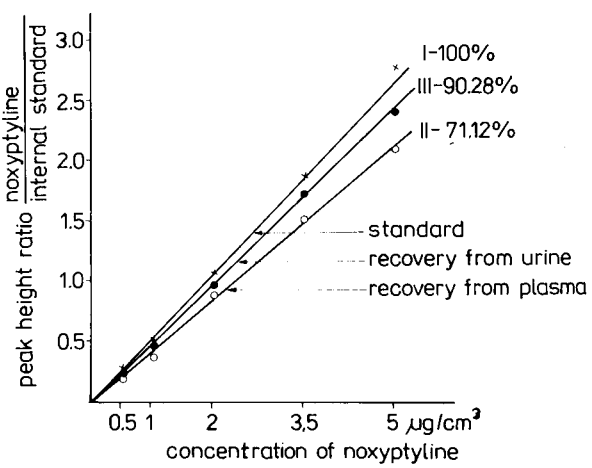

Fig. 3. Calibration curves of noxyptyline. I, Standard; II, after extraction from plasma; III, after extraction from urine.

The calibration curve I was used for determination of detector linearity. The calibration curves I and II show the relationship between the noxyptyline concentration and the peak height ratio of noxyptyline and the internal standard (Fig. 3).

Based on the curves obtained, the recovery of noxyptyline from human plasma was determined. The content of noxyptyline may be determined in 1–4 cm^3 of plasma under extraction conditions similar to those described for the construction of the calibration curve II. The concentration of noxyptyline was calculated from the calibration curve II prepared separately for each series of determinations.

Determination of noxyptyline in human urine

The human urine used for the determination was adjusted to pH 7 with borate buffer of pH 9.

Into five centrifugal tubes, each containing 1 cm^3 of human urine of pH 7, the following volumes of 10 mg % noxyptyline ethanolic solution were added: 0.005, 0.01, 0.02, 0.035 and 0.05 cm^3. The extraction process and the determination itself were conducted precisely as described for the determination of noxyptyline in human plasma. The chromatogram of the extract from urine is shown in Fig. 4.

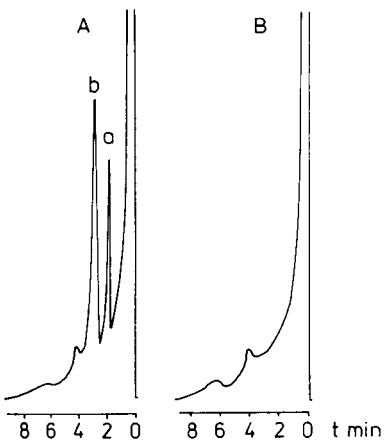

Fig. 4. Gas chromatograms of (A) extract from urine containing noxyptyline (a) and internal standard, oxazepam (b); (B) extract from blank urine.

Based on the results obtained the calibration curve I was constructed, and the calibration curve III was prepared after the extraction from urine (Fig. 3).

For determination of unknown amounts of noxyptyline 1–4 cm^3 of urine may be used, and the extraction should be conducted according to the instructions given for the calibration curve III preparation.

The noxyptyline content was read out from the calibration curve III prepared separately for each series of determinations.

RESULTS AND DISCUSSION

The gas chromatographic determination of noxyptyline was conducted with use of a nitrogen detector, which proved to be more sensitive to the tested compound than the flame ionization detector.

The results listed in the Table I show that the gas chromatographic method described can be used to determine noxyptyline in substance and in a model mixture with an error lower than that of the pharmacopoeial spectrophotometric method.

The use of hexane for extraction of noxyptyline from human plasma and urine was very convenient because the components of plasma and urine were only slightly soluble in the organic layer.

The extraction method worked out for isolation of noxyptyline from urine gave optimal results at pH 7 and therefore required adjustment of urine to neutral with borate buffer of pH 9. The retention times for the impurities from plasma and urine are different from those for noxyptyline and oxazepam.

The determination of noxyptyline in plasma and urine is possible in the concentration range 0.5–10 $\mu g/cm^3$. The recovery of noxyptyline from plasma amounted to 71.12 % ± 8 %. Statistical evaluation: $\bar{x}$ = 72.9 %; S.D. = 3.36; confidence interval 72.9 % ± 6.68 for p = 0.95 and n = 8; coefficient of variation (C.V.) = 4.6 %.

The recovery of noxyptyline from urine amounted to 90.28 % ± 7 %. Statistical evaluation: $\bar{x}$ = 90.94 %; S.D. = 1.82; confidence interval 90.94 % ± 1.51 for p = 0.95 and n = 8; C.V. = 2.08 %.

CONCLUSIONS

The gas chromatographic method described permits the identification and determination of noxyptyline in substance and tablets, with an error (coefficient of variation) of 2.19 % and 2.49 %, respectively.

The determination of noxyptyline in plasma and urine is possible in the concentration range 0.5–10 $\mu g/cm^3$. The sensitivity of the method is 0.1 $\mu g/cm^3$; the systematic error (coefficient of variation) is 4.61 % for extraction from plasma and 2.08 % for extraction from urine.

The method developed for extraction of noxyptyline from plasma and urine, and the method of its direct determination by gas chromatography, may be applied in bioavailability and pharmacokinetic investigations.

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Note

Studies on the gas chromatographic behaviour of organophosphorus esters

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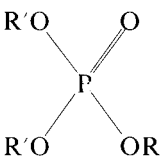
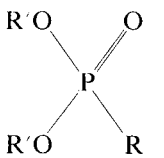
Although extensive gas–liquid chromatographic (GLC) data have been collected on commercially important organophosphorus (OP) esters, their retention behaviour has not been subjected to any systematic study and analysis. We report here the retention indices of diethyl alkyl phosphates (I), diethyl alkylphosphonates and di-*n*-propyl alkylphosphonates (II) on OV-17 stationary phase with *n*-alkanes as references. The calculated δI value for methylene addition at different side chains showed the possibility of predicting the retention index of a higher member with the help of the index of the parent compound.



Phosphates (I)



Phosphonates (II)

R′ = $-C_2H_5$ or $-C_3H_7$
R = $-CH_3$, $-C_2H_5$, $-C_3H_7$, $-C_4H_9$, $-C_5H_{11}$, $-C_6H_{13}$

EXPERIMENTAL

All the solvents and reagents used were of AnalaR grade (BDH, Poole, Great Britain) except *n*-alkanes which were obtained from Fluka (Buchs, Switzerland).

Synthesis of phosphates and phosphonates

Diethyl alkyl phosphates were synthesized[1] by reaction of diethyl chlorophosphate with sodium alkoxide in an inert solvent and purified by distillation under reduced pressure.

Diethyl and dipropyl alkylphosphonates were prepared from alkyl iodides and sodium diethyl phosphites under Michaelis–Becker reaction[2] conditions and purified by distillation.

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Gas chromatography

Three stainless-steel columns (6 ft. × 2 mm I.D.) packed with Gas-Chrom Q (80–100 mesh) loaded with 1.5%, 10% and 20% OV-17, respectively, were prepared and conditioned at 250°C for more than 8 h before use and checked for reproducibility.

The gas chromatograph used was a Perkin-Elmer Model 3920 B, with flame ionization detector. The temperatures of the injection port, column oven and detector were 200, 160 and 200°C respectively. The dead volume of the column was measured by injecting methane. Mixtures of the phosphates or phosphonates together with *n*-alkane reference were prepared in benzene. A 2-μl volume of the mixture was injected on the column and the retention times for each component were measured with the help of stop-watch. Each mixture was injected three times to check the reproducibility of retention times. The chromatograms were recorded on a Perkin-Elmer Model 23 recorder with sensitivity 1 mV for full scale deflection.

The raw retention data were corrected for dead volume and then used to calculate Kováts' retention indices[3].

RESULTS AND DISCUSSION

Kováts' indices are sensitive to column temperature[4], stationary phase loading and sample size[5,6]. The sensitivity to column loading in case of OP compounds had not previously been studied. Our results given in Table I showed that with 20% loading the indices are very consistent compared to those with low loadings.

TABLE I

EFFECT OF PERCENTAGE LIQUID LOADING ON KOVÁTS' RETENTION INDICES (*I*) OF TRIALKYL PHOSPHATES AND PHOSPHONATES AT 160°C

Compound	*I*		
	1.5% OV-17	*10% OV-17*	*20% OV-17*
Diethyl ethyl phosphate	1302	1298	1296
Diethyl butyl phosphate	1488	1483	1484
Diethyl hexyl phosphate	1682	1676	1674
Diethyl ethylphosphonate	1257	1250	1246
Dipropyl propylphosphonate	1520	1514	1510
Dipropyl pentylphosphonate	1702	1700	1700

The retention indices in Table II calculated by Kováts' method clearly show the dependence on chain length in phosphates and phosphonates. The average δI_a value for the addition of a methylene group was 93 ± 4 instead of 100. Such a deviation has been reported previously for esters of dibasic acids[7].

The difference in retention indices, δI_b, between phosphates and phosphonates having similar alkyl groups is 50 ± 2 for the various pairs studied (see Table III). This

TABLE II

KOVÁTS' RETENTION INDICES FOR AN HOMOLOGOUS SERIES OF PHOSPHATES AND PHOSPHONATES ON 20% OV-17 AT 160°C

Compound No.	*R'*	*R*	*I*	δI_a
Diethyl alkyl phosphates (A)				
1	$-C_2H_5$	$-CH_3$	1205	–
2	$-C_2H_5$	$-C_2H_5$	1296	91
3	$-C_2H_5$	$-C_3H_7$	1390	94
4	$-C_2H_5$	$-C_4H_9$	1482	92
5	$-C_2H_5$	$-C_5H_{11}$	1579	97
6	$-C_2H_5$	$-C_6H_{13}$	1674	95
Diethyl alkylphosphonates (B)				
7	$-C_2H_5$	$-C_2H_5$	1246	–
8	$-C_2H_5$	$-C_3H_7$	1340	94
9	$-C_2H_5$	$-C_4H_9$	1430	90
10	$-C_2H_5$	$-C_5H_{11}$	1525	95
Di-n-propyl alkylphosphonates (C)				
11	$-C_3H_7$	$-C_2H_5$	1420	–
12	$-C_3H_7$	$-C_3H_7$	1510	90
13	$-C_3H_7$	$-C_4H_9$	1604	94
14	$-C_3H_7$	$-C_5H_{11}$	1700	96

Average = 93 ± 4

difference appears to be due to the difference in polarities of phosphate and phosphonate esters.

Table IV shows the difference between the retention indices, δI_c, of phosphonates having the same R but different R' groups. This averages to 87 ± 3 and is significantly less than δI_a.

TABLE III

DIFFERENCE IN RETENTION INDICES BETWEEN PHOSPHATES AND PHOSPHONATES, δI_b, HAVING THE SAME ALKYL GROUPS ON 20% OV-17 AT 160°C

δI_b = *I* (phosphate) − *I* (phosphonate); *I* values from Table II.

R'	*R*	δI_b
$-C_2H_5$	$-C_2H_5$	50
$-C_2H_5$	$-C_3H_7$	50
$-C_2H_5$	$-C_4H_9$	52
$-C_2H_5$	$-C_5H_{11}$	49

Average = 50 ± 2

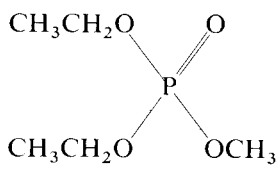
TABLE IV

EFFECT OF ADDITION OF A METHYLENE GROUP IN R′ FOR PHOSPHONATES ON 20% OV-17 AT 160°C

Values of indices taken from Table II.

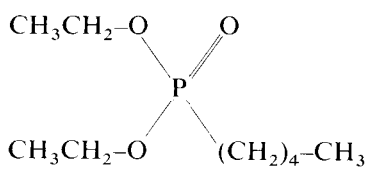
Phosphonate series B		*Phosphonate series C*		*Difference in methylene group at R′*	*Difference in index, δI_c, per methylene group*
R′	*R*	*R′*	*R*		
$-C_2H_5$	$-C_2H_5$	$-C_3H_7$	$-C_2H_5$	1 × 2	174/2 = 87
$-C_2H_5$	$-C_3H_7$	$-C_3H_7$	$-C_3H_7$	1 × 2	170/2 = 85
$-C_2H_5$	$-C_4H_9$	$-C_3H_7$	$-C_4H_9$	1 × 2	174/2 = 87
$-C_2H_5$	$-C_5H_{11}$	$-C_3H_7$	$-C_5H_{11}$	1 × 2	175/2 = 88
					Average = 87 ± 2

Amongst the compounds reported, it was possible to predict the retention indices of higher members by making use of the δI_a, δI_b and δI_c values. Thus taking diethyl methyl phosphate (a)



(a)

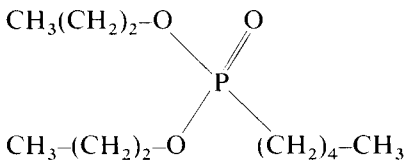
as the parent compound for the series, the retention index of diethyl pentylphosphonate (b) can easily be calculated.



(b)

The parent compound has a retention index of 1205, the contribution due to δI_a for the above compound is 4 × 93 = 372 and δI_b is −50, giving the retention index as

1527 which agrees well with the observed value of 1525 (Table II). Again, for dipropyl pentylphosphonate (c)



(c)

δI_a is $4 \times 93 = 372$, $\delta I_b = -50$ and $\delta I_c = 2 \times 87 = 174$. Adding this to 1205, the value obtained is 1691 compared with the observed value of 1700 (Table II).

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Note

Contributions to the chemistry of silicon–sulphur compounds

XXVI*. Relationships between chromatographic behaviour and structure of triorganosilanes, triorganosilanethiols, hexaorganodisilthianes and triphenylthioalkylsilanes

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There have been few reports[1–5] of the use of thin-layer chromatography (TLC) for separation of organosilicon compounds. The results indicate some relations between chromatographic behaviour and structure of the investigated compounds.

Uhle[1,2] reported that for triorganosilanes, silanols and siloxanes there are interactions between silica gel and both the aryl and silanol groups. Adsorption increases with decreasing electron withdrawing character of phenyl-ring substituents. Franc and Šenkýřová[3] pointed out the significant influence of the phenyl groups of a series of phenylmethylsiloxanes on their adsorption on silica gel. If these molecules also contain organoxy groups, the adsorption considerably increases. Wojnowska[4], who chromatographed ethylphenoxy- and phenylphenoxysilanes, has demonstrated, as have Franc and Šenkýřová[3], the rôle of hydrogen bridges between the oxygen of the organoxy group and OH group from the silica gel.

Although an increasing number of studies have been made on the chemistry of silicon–sulphur compounds[6], only one paper gives information about the TLC of these compounds. Wojnowski[5] observed that the adsorption of trialkoxysilanethiols decreases with increasing number of carbon atoms in the alkyl group and with its branching; adsorption decreases with the decreasing acidity of silanethiols.

The aim of our work was to obtain data for a few more classes of organic silicon–sulphur compounds and find a relationship between the chromatographic behaviour and structure.

EXPERIMENTAL

Samples

Triorganosilanes were prepared from trichlorosilane and appropriate Grignard reagents[7]. Triorganosilanethiols and hexaorganodisilthianes were prepared by insertion of sulphur into triorganosilanes[8]. Hexamethyldisilthiane was obtained ac-

* Part XXV, J. Pikies and W. Wojnowski, *Z. Anorg. Allg. Chem.*, submitted for publication.

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cording to Cumper *et al.*[9]. Triphenylthioalkylsilanes, triphenylsilanol and hexaphenyldisiloxane were prepared as described in ref. 10. Hexamethyldisiloxane was NMR grade from Petrarch Systems, U.S.A.

Stationary and mobile phases

Home-made plates were used: 0.25-mm layers were prepared from a suspension of 30 g of Kieselgel G (E. Merck) in 60 cm^3 water. After drying in the laboratory atmosphere, the plates were activated at 110°C for 1 h and stored without further treatment.

Seven mobile phases were used: A, hexane; B, cyclohexane; C, tetrachloromethane; D, trichloromethane–hexane (1:1); E, benzene; F, tetrachloromethane–hexane (1:1); G, hexane–ethyl acetate (40:1). All solvents were dried by the usual methods and distilled before use.

Chromatographic procedure

Solutions were prepared in benzene as solvent. Amounts of 1 μl were spotted on the chromatoplate. The sample concentrations, developing distances and spot visualizations were as follows:

(1) R_3SiH: 1% solution; 13 cm; water–alcohol solution of silver nitrate, black spots

(2) R_3SiSH and $(R_3Si)_2S$: 20% solution; 6 cm; water–alcohol solution of silver nitrate, yellow-brown spots, or iodine vapour, brown spots

(3) R_3SiSR: 1.5% solution; 13 cm; iodine vapour, brown spots

All experiments were carried out at room temperature.

RESULTS AND DISCUSSION

Tables I–III summarize the R_F values obtained.

TABLE I

R_F VALUES OF TRIORGANOSILANES

Et = Ethyl; Me = methyl; Ph = phenyl; Pr = propyl.

Compound	*Mobile phase*				
	A	*B*	*C*	*D*	*E*
$PhMe_2SiH$	0.76	0.57	0.80	0.91	0.93
$PhEt_2SiH$	0.78	0.60	0.83	0.91	0.93
$PhPr_2SiH$	0.79	0.63	0.86	0.91	0.94
Ph_2MeSiH	0.53	0.32	0.70	0.90	0.92
Ph_2EtSiH	0.55	0.33	0.73	0.90	0.92
Ph_2PrSiH	0.57	0.34	0.76	0.90	0.92
Ph_3SiH	0.30	0.18	0.63	0.89	0.92
$(p\text{-}CH_3C_6H_4)_3SiH$	0.25	0.15	0.60	0.89	0.92
$(m\text{-}CH_3C_6H_4)_3SiH$	0.28	0.14	0.60	0.89	0.91
$(o\text{-}CH_3C_6H_4)_3SiH$	0.37	0.15	0.64	0.89	0.91
$(p\text{-}ClC_6H_4)_3SiH$	0.55	0.38	0.82	0.90	0.92
$(p\text{-}BrC_6H_4)_3SiH$	0.51	0.37	0.82	0.90	0.92
$(p\text{-}MeOC_6H_4)_3SiH$	0.00	0.01	0.04	0.48	0.45
$(PhCH_2)_3SiH$	0.13	0.06	0.42	0.84	0.90

In the triorganosilane series (Table I) the most significant factor is the interaction of the phenyl groups attached to silicon with the silica gel. Adsorption decreases in the order: $Ph_3SiH > Ph_2AlkSiH > PhAlk_2SiH$, where Alk = alkyl. The R_F values can be correlated with the withdrawing effect of the phenyl-ring substituents and are in agreement with the data reported by Uhle[1,2]: $(p\text{-}CH_3C_6H_4)_3SiH > (m\text{-}CH_3C_6H_4)_3SiH > Ph_3SiH > (p\text{-}BrC_6H_4)_3SiH > (p\text{-}ClC_6H_4)_3SiH$.

Within the series of phenyldialkylsilanes and diphenylalkylsilanes there are small differences in R_F values (Table I), and adsorption decreases with increasing chain length. This could be connected with the affinity of the compound to the mobile phase. The extremely low R_F value of tribenzylsilane is due to the isolation of the phenyl groups by methylene bridges.

Table II shows that the triorganosilanethiols are less strongly adsorbed than the related disilthianes. It is also noted that the adsorption increases with the increasing number of aryl groups. Surprisingly, compounds with the same number of phenyl groups have similar R_F values, *e.g.*, $Ph_2PrSiSH$ and $(PhPr_2Si)_2S$. The oxygen analogues, *e.g.*, Ph_3SiOH, $(Ph_3Si)_2O$ and $(Me_3Si)_2O$ are more strongly adsorbed, $R_F = 0$.

TABLE II

R_F VALUES OF TRIORGANOSILANETHIOLS AND HEXAORGANODISILTHIANES

$R_F = 0$ for Ph_3SiOH, $(Ph_3Si)_2O$ and $(Me_3Si)_2O$ in both mobile phases. In trichloromethane or benzene the investigated compounds were eluted with the solvent front. Tailing was observed when the developing distance was longer than 6 cm probably caused by hydrolysis of the Si–S bond.

R_3Si–	*Mobile phase*			
	A		*F*	
	R_3SiSH	$(R_3Si)_2S$	R_3SiSH	$(R_3Si)_2S$
Me_3Si		0.40		0.80
$PhMe_2Si$	0.68	0.21		
$PhEt_2Si$	0.71	0.28	0.80	0.55
$PhPr_2Si$	0.71	0.32		
Ph_2MeSi	0.29	0.06		
Ph_2EtSi	0.31	0.10	0.63	0.33
Ph_2PrSi	0.32	0.18		
Ph_3Si	0.08		0.33	
$(p\text{-}CH_3C_6H_4)_3Si$	0.13			
$(m\text{-}CH_3C_6H_4)_3Si$	0.10			
$(o\text{-}CH_3C_6H_4)_3Si$	0.08			
$(p\text{-}ClC_6H_4)_3Si$	0.05			
$(PhCH_2)_3Si$	0.03			

The adsorption increases with the acidity of the silanethiol, $\Delta\bar{\nu}$(SH-THF)[11], according to the relationship:

$$R_F = -0.026\ \Delta\bar{\nu}(\text{SH-THF}) + 2.426 \qquad (1)$$
$$r = 0.882 \text{ for } n = 11$$

This very significant correlation (for a confidence level of 99% and ten pairs of variables, a critical value for r is 0.765) allows to conclude that the thiol group acts as an adsorption centre, particularly by hydrogen bond formation. This means that the interaction between the phenyl ring and the OH groups of the silica gel, which was the determining factor in the case of silanes containing a phenyl ring (Table I), plays a minor rôle in arylsilanethiols. $(PhCH_2)_3SiSH$ does not fit the correlation 1 because both adsorption centres (phenyl ring and SH group) are essential for strong adsorption.

According to the results in Table III the substitution of hydrogen in Ph_3SiH by an SR group results in a second adsorption centre (R_F of Ph_3SiH 0.77, R_F of Ph_3SiSMe 0.61) of minor but distinct influence. The substitution of hydrogen by an OR group introduces an even stronger adsorption centre.

TABLE III

R_F VALUES OF TRIPHENYLTHIOALKYLSILANES AND TRIPHENYLALKOXYSILANES

Mobile phase G. Values for Ph_3SiOR are taken from ref. 12. For Ph_3SiH, R_F = 0.77. Bu = Butyl.

R	*Ph_3SiSR*	*Ph_3SiOR*
H	0.48	0.04
Me	0.61	0.44
Et	0.64	0.51
Pr	0.66	0.59
Bu	0.67	0.62

Finally, the adsorption of derivatives with the same triorganosilyl group decreases in series: $Ph_3SiOH > Ph_3SiSH \gtrsim Ph_3SiOR > Ph_3SiSR > Ph_3SiH$.

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Note

Gas–liquid chromatographic analyses

III. Glass capillary gas chromatography of chloromethyl monochloro esters of aliphatic C_3–C_{12} *n*-carboxylic acids

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Recently, gas chromatographic (GC) separations of mixtures with a wide range of chain lengths of methyl monochloro[1] and methyl, methyl 2-chloro and chloromethyl esters[2] of aliphatic *n*-carboxylic acids have been reported on Carbowax 20M glass capillary columns.

This paper describes a GC study of chloromethyl monochloro esters of aliphatic C_3–C_{12} *n*-carboxylic acids. The separations of combined mixtures of even- and odd-carbon-number esters were studied, and elution times compared with those of the corresponding methyl esters by separating the mixtures with the same chain lengths under the same operating conditions.

EXPERIMENTAL

GLC analysis

A Varian Model 2400 gas chromatograph, equipped with a flame-ionization detector and 3% Carbowax 20M glass capillary column (50 m × 0.3 mm I.D.), was used for GC analyses. The column temperature was programmed from 50 to 190°C at 4°C/min and held at 190°C until the elution of peaks ceased. Nitrogen was used as the carrier gas at a flow-rate of 1 ml/min. The splitting ratio was 1:20, and the temperatures of injector and detector were 220 and 240°C, respectively.

Samples

Chloromethyl monochloro esters of aliphatic C_3–C_{12} *n*-carboxylic acids were prepared by chlorination[3] of the corresponding chloromethyl esters in the presence of benzene. Methyl monochloro esters were synthesized as described earlier[4–6]. The crude chlorination mixtures were used for GC analyses. To avoid the overlapping of peaks on GC of crude mixtures with a wide range of chain lengths, it is necessary to prevent the formation of higher chlorinated products by using a diluent and much less than an equimolar amount of chlorine.

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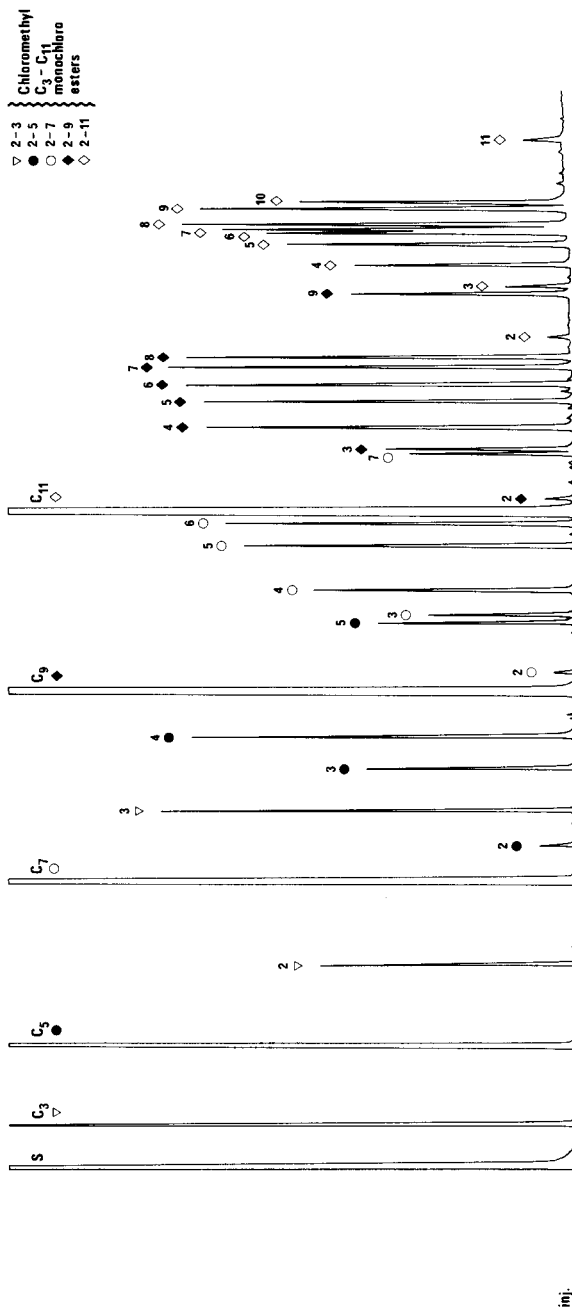

Fig. 1. Chromatogram of the mixture of chloromethyl monochloro esters of aliphatic odd-carbon-number C_3–C_{11} *n*-carboxylic acids. S = Solvent; peak number = position of Cl-substituent.

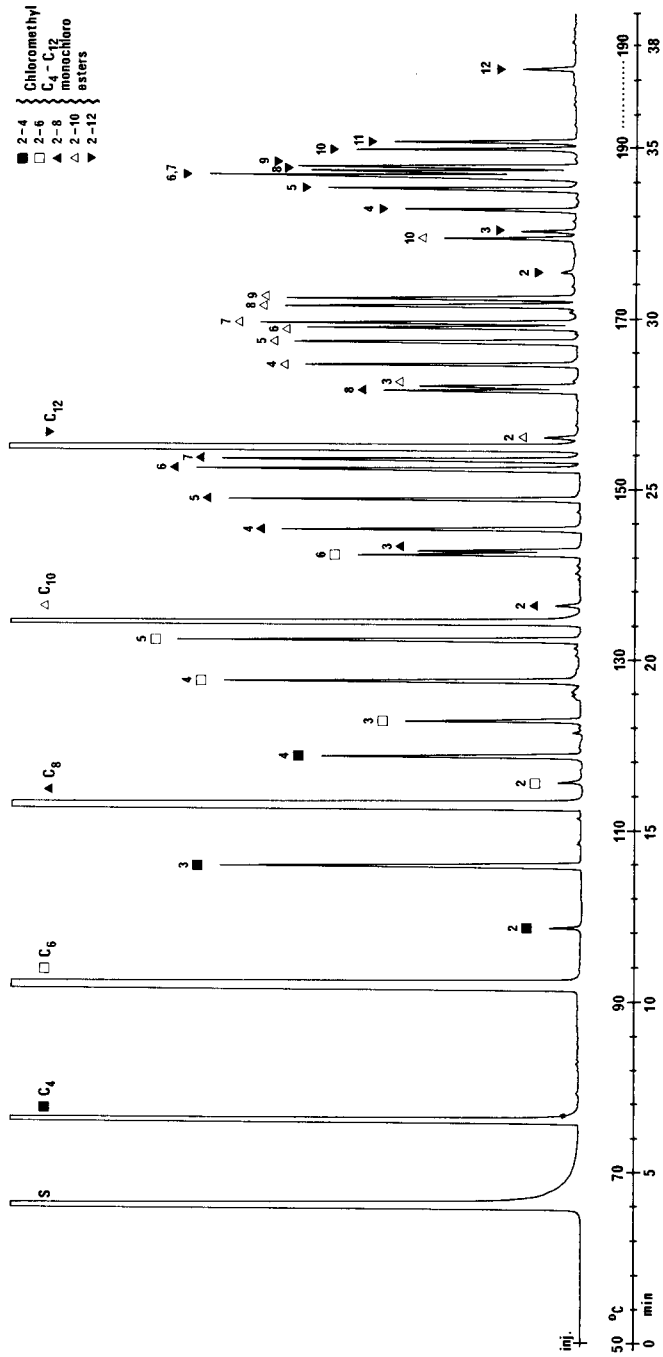

Fig. 2. Chromatogram of the mixture of chloromethyl monochloro esters of aliphatic even-carbon-number C_4–C_{12} *n*-carboxylic acids. S = Solvent; peak number = position of Cl-substituent.

TABLE I

ABSOLUTE AND RELATIVE RETENTION TIMES FOR CHLOROMETHYL ESTERS OF ALIPHATIC C_3–C_{12} *n*-CARBOXYLIC ACIDS

Chain length	*Absolute* and relative** retention times*										
	Chloromethyl ester	*Isomeric chloromethyl monochloro esters*									
		2-Cl	*3-Cl*	*4-Cl*	*5-Cl*	*6-Cl*	*7-Cl*	*8-Cl*	*9-Cl*	*10-Cl*	*11-Cl*	*12-Cl*
C_3	5.19	9.88	14.40								
	1.00	1.90	2.77								
C_4	6.50	12.14	13.91	17.15							
	1.00	1.87	2.14	2.64							
C_5	7.50	13.40	15.67	16.59	19.95						
	1.00	1.79	2.09	2.21	2.66						
C_6	10.45	16.41	18.19	19.35	20.55	23.07					
	1.00	1.57	1.74	1.85	1.97	2.21					
C_7	12.35	18.52	20.22	20.95	22.24	22.90	24.98				
	1.00	1.50	1.64	1.70	1.80	1.85	2.02				
C_8	15.77	21.60	23.18	23.80	24.70	25.57	25.81	27.88			
	1.00	1.37	1.47	1.51	1.57	1.62	1.64	1.77			
C_9	17.97	23.65	25.11	25.71	26.48	26.95	27.48	27.78	29.66		
	1.00	1.32	1.40	1.43	1.47	1.50	1.53	1.55	1.65		
C_{10}	21.12	26.53	28.00	28.62	29.29	29.69	29.85	30.34	30.57	32.33	
	1.00	1.26	1.33	1.36	1.39	1.40	1.41	1.44	1.45	1.53	
C_{11}	23.25	28.44	29.90	30.53	31.13	31.46	31.58	31.70	32.17	32.39	34.22	
	1.00	1.22	1.29	1.31	1.34	1.35	1.36	1.36	1.38	1.39	1.47	
C_{12}	26.21	31.32	32.54	33.20	33.80	34.16	34.18	34.30	34.43	34.91	35.16	37.28
	1.00	1.19	1.24	1.27	1.29	1.30	1.30	1.31	1.31	1.33	1.34	1.42

* Absolute retention times (min) measured from Figs. 1 and 2.

** Relative retention times for unchlorinated chloromethyl esters taken as 1.00.

TABLE II

RELATIVE RETENTION TIMES FOR CHLOROMETHYL ESTERS OF ALIPHATIC C_3–C_{12} *n*-CARBOXYLIC ACIDS

Chain length	*Relative retention time**											
	Chloromethyl ester	*Isomeric chloromethyl monochloro esters*										
		2-Cl	*3-Cl*	*4-Cl*	*5-Cl*	*6-Cl*	*(ω-5)-Cl*	*(ω-4)-Cl*	*(ω-3)-Cl*	*(ω-2)-Cl*	*(ω-1)-Cl*	*ω-Cl*
C_3	0.25	0.37	0.51								0.32	0.45
C_4	0.31	0.46	0.50	0.60						0.40	0.46	0.53
C_5	0.36	0.51	0.56	0.58	0.68				0.45	0.52	0.54	0.62
C_6	0.49	0.62	0.65	0.68	0.70	0.78		0.55	0.61	0.64	0.67	0.71
C_7	0.58	0.70	0.72	0.73	0.76	0.77	0.63	0.68	0.70	0.73	0.75	0.77
C_8	0.75	0.81	0.83	0.83	0.84	0.86	0.79	0.80	0.83	0.84	0.84	0.86
C_9	0.85	0.89	0.90	0.90	0.90	0.91	0.88	0.89	0.90	0.91	0.91	0.92
C_{10}	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C_{11}	1.10	1.07	1.07	1.07	1.06	1.06	1.07	1.06	1.06	1.06	1.06	1.06
C_{12}	1.24	1.18	1.16	1.16	1.15	1.15	1.17	1.16	1.15	1.15	1.15	1.15

* Relative retention times for C_{10} derivatives taken as 1.00.

RESULTS AND DISCUSSION

As expected, the isomeric monochloro chloromethyl esters are eluted in direct order from 2-chloro to ω-chloro compound, as are the corresponding methyl derivatives[1]. The gas chromatograms of the combined mixtures of odd-carbon-number C_3–C_{11} and even-carbon-number C_4–C_{12} chloromethyl monochloro esters are illustrated in Figs. 1 and 2. The absolute and relative retention times are presented in Tables I and II. All retention times were measured from sample injection and are tabulated relative to unchlorinated chloromethyl esters = 1.00 (Table I) and C_{10} derivatives = 1.00 (Table II).

The results show that all compounds are resolved except for chloromethyl 6-chloro- and 7-chlorododecanoates (Fig. 2). Bearing in mind the GC separations of methyl monochloro esters[1,5–7], it is evident that the chloromethoxy group makes the polarities of the mid-chain isomers similar, leading to overlapping peaks. On the other hand, owing to their higher boiling points, the elution times of the chloromethyl isomers increase as compared with methyl esters (Fig. 3 and Table III), causing poorer separation of the long-chain isomers.

To compare the elution times of chloromethyl and methyl esters, GC separations of the mixtures with the same chain lengths were performed under the same operating conditions; the results are presented in Table III. It can be seen that the relative retention times of chloromethyl 2-chloro isomers are smaller than those of the 3-chloro derivatives. The values of the latter, on the other hand, are always the greatest ones. This can clearly be seen from Fig. 3; the time between the elution of chloromethyl 2-chloro- and 3-chlorodecanoates is twice as long as that between the

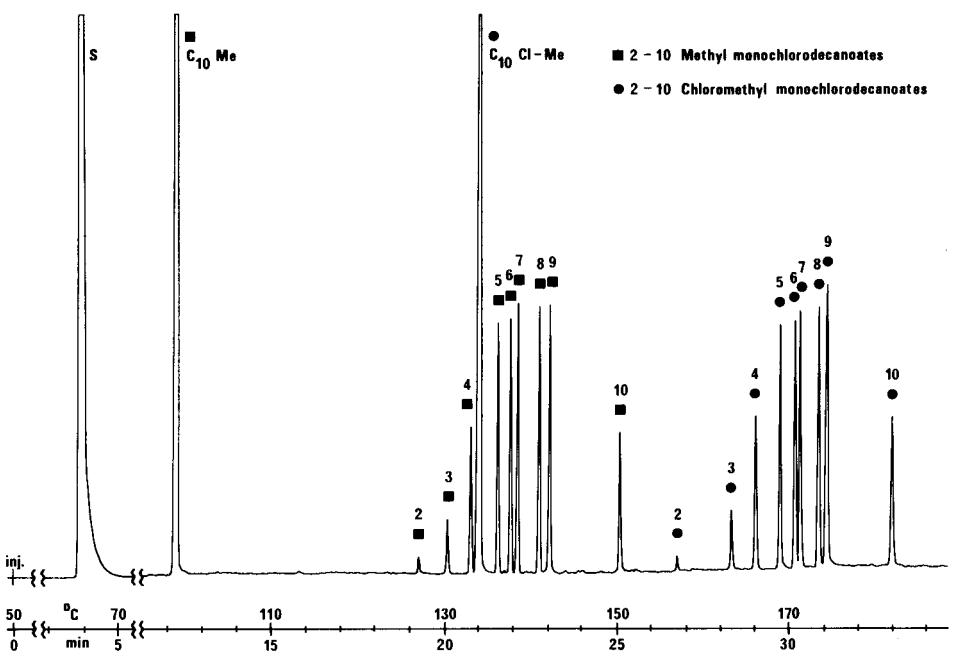

Fig. 3. Chromatogram of the mixture of methyl and chloromethyl decanoates. S = Solvent; peak number = position of Cl-substituent.

TABLE III

RELATIVE RETENTION TIMES FOR CHLOROMETHYL ESTERS OF ALIPHATIC C_3–C_{12} *n*-CARBOXYLIC ACIDS

Chain length	*Relative retention time**										
	Chloro-methyl ester	*Isomeric chloromethyl monochloro esters*									
		2-Cl	*3-Cl*	*4-Cl*	*5-Cl*	*6-Cl*	*7-Cl*	*8-Cl*	*9-Cl*	*10-Cl*	*11-Cl*	*12-Cl*
C_3	1.31	1.87	2.10									
C_4	1.41	1.92	2.04	2.00								
C_5	1.49	1.92	2.06	2.05	1.89							
C_6	1.74	1.87	1.93	1.88	1.81	1.68						
C_7	1.93	1.66	1.68	1.64	1.59	1.57	1.49					
C_8	1.87	1.53	1.54	1.52	1.50	1.48	1.47	1.41				
C_9	1.83	1.47	1.48	1.46	1.44	1.43	1.41	1.40	1.36			
C_{10}	1.66	1.37	1.39	1.37	1.36	1.36	1.35	1.34	1.34	1.31		
C_{11}	1.52	1.31	1.33	1.32	1.31	1.31	1.31	1.31	1.30	1.30	1.29	
C_{12}	1.40	1.26	1.30	1.29	1.29	1.30	1.29	1.29	1.29	1.29	1.30	1.31

* Relative retention times for the corresponding methyl esters taken as 1.00, determined from the mixtures of methyl and chloromethyl esters with the same chain lengths.

elution of the corresponding methyl isomers. The chloromethoxy group has the greater effect on the 2-position making 2-chloro isomers less polar and leading to the relatively short elution times on a polar Carbowax 20M column. On a non-polar SE-52 column, however, owing to the better solubilities of less polar 2-chloro isomers, shorter times between the elution of isomers are observed.

The relative retention times of ω-chloro compounds are in general noticeably short, but with increasing chain length the differences from the other isomers decrease. Owing to the isothermal running conditions after reaching the final temperature, however, the greatest value is obtained for ω-chlorododecanoate (Table III).

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Note

Electron-capture gas chromatographic determination of sulphide as a new pentafluorobenzyl derivative

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Sulphide is a significant environmental pollutant derived partly from industrial effluents. It contaminates water supplies and results in distressing odours.

Several methods, including titrimetric[1–3], colorimetric[4], fluorimetric[5,6], ultraviolet spectrophotometric[7,8], coulometric[9] and electrode[10,11] techniques, have been used for the determination of sulphide in water. In addition, direct gas chromatography (GC) with β-ionization detection[12] has been used to analyze hydrogen sulphide in air at ppm levels. However, there is still a need for a specific and more sensitive method for quantitation of sulphide in complicated matrices.

In this paper, a new approach based on detector-oriented derivatization of sulphide, as bis(pentafluorobenzyl) sulphide, is described for quantitative analysis of inorganic sulphide (S^{2-}) down to ppb* levels. The method has been applied to the analysis of S^{2-} in spring-water. The results indicate that the method is specific and highly sensitive.

EXPERIMENTAL

GC conditions

A Yanaco (Kyoto, Japan) Model G2800EN gas chromatograph equipped with an electron-capture detector (ECD) of non-radioactive type (Kyoto, Japan) was used. The column was a coiled glass tube (3.0 m × 3 mm I.D.) packed with 3% silicone gum SE-30 on Chromosorb W AW DMCS (60–80 mesh). The injection port and detector temperatures were kept at 250°C and that of the column was isothermally set at 180°C. Helium was used as the carrier gas at a flow-rate of 30 ml/min. A Yanaco VR-101 recorder with a chart speed of 5 mm/min was used. The peak area ratios were computed by a Shimadzu (Kyoto, Japan) Chromatopac E1A digital integrator.

* Throughout this article, the American billion (10^9) is meant.

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Materials

Pentafluorobenzyl phenyl thioether, pentafluorobenzyl *n*-butyl thioether and bis(pentafluorobenzyl) sulphide (BPFBS) were synthesized at this laboratory and their structures were confirmed by mass spectrometry (MS). Pentafluorobenzyl bromide (PFBBr or α-bromo-2,3,4,5,6-pentafluorotoluene) (Aldrich, Milwaukee, WI, U.S.A.) and 3% silicone gum SE-30 on Chromosorb W AW DMCS (60–80 mesh) (Nishio, Tokyo, Japan) were used without further treatment. Sodium sulphide nonahydrate ($Na_2S \cdot 9H_2O$), toluene and other reagents were of analytical grade. Deionized and distilled water was used to prepare aqueous solutions.

Solutions of the internal standard (I.S.), 0.5 μM, and of the reference standard were separately prepared by dissolving pentafluorobenzyl phenyl thioether in toluene and a suitable amount of $Na_2S \cdot 9H_2O$ in 0.01 *N* sodium hydroxide (NaOH). The sample solution was prepared by dilution of an equal volume spring-water in 0.02 *N* NaOH solution, and further dilution in 0.01 *N* NaOH if necessary. The PFBBr solution, 0.4 μM, was prepared by dissolving PFBBr in acetone.

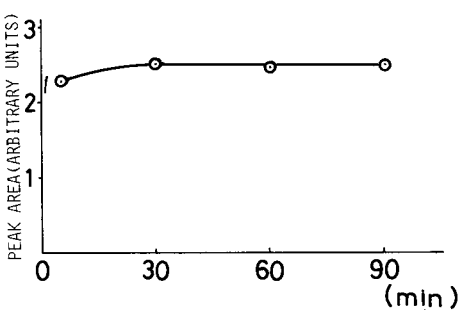
Procedure

A 0.5-ml volume of PFBBr solution was added to a 15-ml glass-stoppered flask containing 0.1 ml of the reference standard or sample solution. The reaction mixture was magnetically stirred for 1 h at room temperature and then evaporated under a nitrogen stream for 2 min at room temperature. To the concentrated solution, 1.0 ml of I.S. solution was added and mixed well, followed by a small pinch of anhydrous sodium sulphate. The GC determination was performed by injecting about 0.4 μl of the clear toluene layer.

RESULTS AND DISCUSSION

Derivatization

Alkylation of S^{2-} with PFBBr in an alkaline medium proceeds quickly at room temperature and the reaction equilibrium can be attained in 30 min as shown in Fig. 1. GC analysis could be run after this time, but the analytical data presented here were obtained after 1 h unless otherwise indicated. Derivatization of S^{2-} in neutral water instead of 0.01 *N* NaOH solution resulted in slower formation of the derivative, and in stronger alkali (0.06 *N* NaOH) a complicated reagent background is formed.



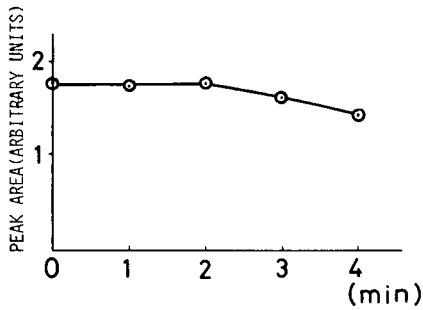


Fig. 1. Effect of reaction time on the formation of the sulphide derivative.

Fig. 2. Effect of evaporation time on loss of the sulphide derivative.

This background interferes with the analysis of the S^{2-} derivative and stems probably from the products of hydrolysis of PFBBr. Concentration of the reaction solution by evaporation under a nitrogen stream can lessen the reagent tailing in the chromatogram, but a concentration period longer than 2 min as shown in Fig. 2 results in lower analyses for the S^{2-} derivative. The same phenomenon is found in the case of evaporation of the reaction solution with a rotary evaporator under reduced pressure, and is due probably to loss of the volatile S^{2-} derivative. The I.S. is also volatile, so it was added to the reaction solution after the concentration step.

Interference

Several anions were investigated for interference with 0.3 ppm of S^{2-}, as shown in Table I. The method does not seem to suffer interference from acetate, sulphite, sulphate, chloride, carbonate, thiosulphate, cyanide, nitrate and nitrite at the levels found in environmental samples. Thus the method is quite specific for S^{2-} analysis in the presence of these anions.

Aliphatic and aromatic mercaptans, such as *n*-butanethiol and thiophenol, could also be converted into their pentafluorobenzyl derivatives[13], but the S^{2-} derivative can be well separated from these derivatives, the elution order under the present GC conditions being as follows: pentafluorobenzyl *n*-butyl thioether; pentafluorobenzyl phenyl thioether; the S^{2-} derivative. Pentafluorobenzyl phenyl thioether, which is structurally similar to BPFBS, is used as I.S. in this work. If a water sample contains thiophenol, the S^{2-} value measured will be lower than the real value. The presence of thiophenol in a sample can be corrected for by running a chromatogram without addition of I.S.

Analytical calibration

Seven samples containing the reference standard at about 0.8–65 ng of S^{2-} were analyzed to construct a calibration graph of the amount of S^{2-} against peak-area ratios of the S^{2-} derivative to I.S. A linear regression equation ($y = 0.05871\ x +$

TABLE I

INTERFERENCE STUDY

Concentration of sulphide investigated: 0.3 ppm.

Anion spiked	*Concentration spiked (ppm)*	*Recovery (%)**
None	–	100.0 ± 2.3
CH_3COO^-	100	99.1 ± 2.7
SO_3^{2-}	100	101.9 ± 2.5
SO_4^{2-}	100	101.6 ± 1.2
Cl^-	100	99.7 ± 3.4
CO_3^{2-}	100	98.0 ± 1.0
$S_2O_3^{2-}$	50	102.2 ± 2.2
CN^-	50	102.8 ± 0.6
NO_3^-	20	102.0 ± 2.3
NO_2^-	20	100.8 ± 2.4

* Mean of triplicate analyses.

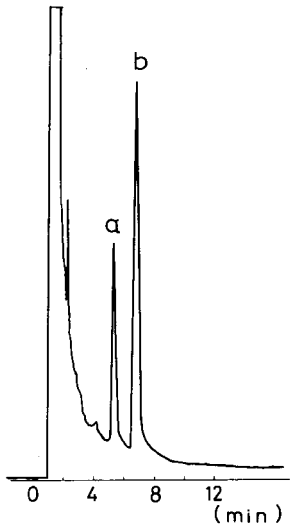


Fig. 3. Typical gas chromatogram of pentafluorobenzyl phenyl thioether (a, I.S.) and bis(pentafluorobenzyl) sulphide (b).

0.0610) was obtained with a correlation coefficient of 0.9997. This indicates high linearity over the range examined. The equation was then used to calculate the S^{2-} content in samples.

A typical chromatogram presented in Fig. 3 demonstrates the good resolution and high symmetry of the peak of the S^{2-} derivative. The retention time of peak b is identical to that of BPFBS, synthesized by scaling up the amounts of S^{2-} in 0.01 *N* NaOH and PFBBr in acetone and allowing them to react at room temperature for 1 h. The crystals obtained were examined by MS using a Hitachi RMU-6E mass spectrometer with an ionization source temperature of 200°C, an electron energy of 70 eV and an acceleration energy of 1.8 kV. The mass spectrum obtained exhibits a parent ion at m/e = 394, representing the formation of BPFBS, and some other peaks are tentatively assigned as in Fig. 4.

Further comparison of the retention time of peak b in Fig. 3 and that of BPFBS was carried out using a Yanaco G180 flame-ionization gas chromatograph

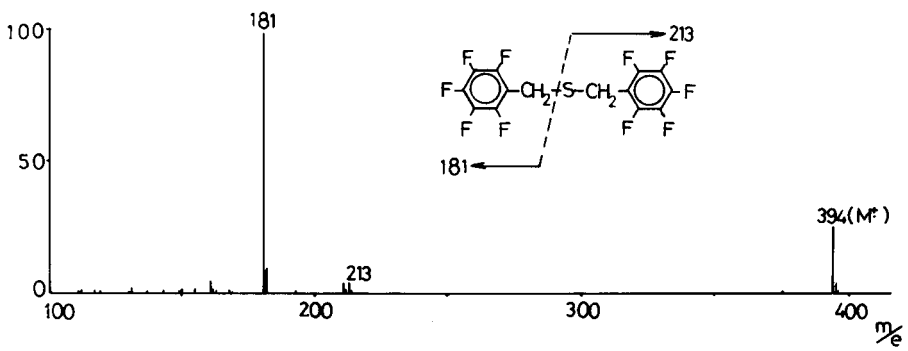


Fig. 4. Mass spectrum of bis(pentafluorobenzyl) sulphide.

TABLE II

COMPARISON OF THE ANALYSIS OF SULPHIDE CONTENT IN SPRING-WATER BY THE TWO METHODS

Sample	*GC–ECD**	*Colorimetry (ppm)*
A	2.55 ± 0.02 ppm	2.52
B	1.71 ± 0.09 ppm	1.76
C	1.04 ± 0.02 ppm	1.05
D	5.50 ± 0.17 ppb	**
E	**	**

* Average of triplicate analyses.
** Below detection limit.

with: (1) a coiled glass column (2 m × 3 mm I.D.) of 10% silicone OV-17 on Shimalite W AW DMCS (60–80 mesh), carrier gas (nitrogen) flow-rate 55 ml/min; and (2) a stainless-steel column (2 m × 3 mm I.D.) of 5% PEG-HT on Uniport HT (60–80 mesh), carrier gas (nitrogen) flow-rate 31 ml/min. In both cases the injection temperature was 275°C and the column temperature was 170°C. The results of these chromatographic separations based on stationary phases of weak and strong polarity gave the same retention times for peak b and BPFBS. Therefore, peak b in Fig. 3 can be assigned as BPFBS.

The method was applied to the analysis of S^{2-} in spring-water and the results compared to those obtained by a colorimetric method[4] based on methylene blue formation. The results in Table II reveal good agreement between the two methods, and the reliability of the proposed method. The high sensitivity of our method is reflected in the analysis of sample D. The S^{2-} content in sample E is below the detection limit of both methods, and is estimated to be less than 0.5 ppb based on the detection limit of the proposed GC–ECD method.

Possible applications of this method to other anions, such as nitrite and cyanide, using pentafluorophenyl-type reagents are being investigated.

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Note

High-performance liquid chromatographic determination of pergolide and its metabolite, pergolide sulfoxide, in microbial extracts

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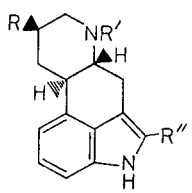
The pharmacological effects of ergot alkaloids are well documented, and the motive for recent research with ergoline derivatives has been to design drugs for the treatment of prolactin-dependent disorders, such as galactorrhea and amenorrhea, prolactin-dependent breast tumors, and Parkinson's disease[1–3]. During a study undertaken to define the structural requirements necessary for prolactin inhibition[4], the drug pergolide (I) (see Fig. 1) was synthesized and was later found to be one of the most potent *in vitro* and *in vivo* dopamine agonists and inhibitors of prolactin secretion[4–6]. Clinical studies indicated that pergolide may be efficacious in the treatment of prolactin-dependent disorders[7].



I. R = CH_2SCH_3, R′ = $CH_2CH_2CH_3$, R″ = H

II. R = $CH_2S\overset{\overset{O}{\uparrow}}{C}H_3$, R′ = $CH_2CH_2CH_3$, R″ = H

III. R = $CH_2C{\equiv}N$, R′ = CH_3, R″ = Cl

Fig. 1. Structures of pergolide (I), pergolide sulfoxide (II) and lergotrile (III).

We are currently examining the use of microorganisms to prepare metabolites of pergolide for biological evaluation and for comparison to metabolites found in mammalian species. Studies involving the microbial transformation of pergolide sulfoxide (II) by a *Helminthosporium* species (NRRL 4671) has necessitated the development of a new analytical procedure for pergolide and its metabolite; thus, high-performance liquid chromatography (HPLC) was chosen for its demonstrated utility in the rapid analysis of microbial extracts[8]. The developed method may be useful in other pharmaceutical, pharmacokinetic, and/or clinical studies with ergoline derivatives.

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EXPERIMENTAL

Materials

Pergolide (I), pergolide sulfoxide (II) and lergotrile (III) were provided by Eli Lilly, Indianapolis, IN, U.S.A. Ergoline spectra (mass spectra, proton magnetic resonance spectra) were consistent with anticipated results, as were physical data (m.p.). The compounds were found to be homogeneous by thin-layer chromatography and HPLC.

Water for use in HPLC was deionized and double-distilled in glass; acetonitrile was HPLC-grade (OmniSolv; MCB Reagents, Cincinnati, OH, U.S.A.). The mobile phase was prepared by the filtration of individual solvents through glass fiber filter pads (GF/F grade; Whatman, Clifton, NJ, U.S.A.), mixing and degassing prior to use. All other solvents and reagents were analytical reagent quality.

All glassware used in extractions was silylated using 2% trimethylsilylchloride (TMSCl) (Aldrich, Milwaukee, WI, U.S.A.) in toluene, rinsed thoroughly and dried prior to use.

Chromatographic procedure

A Beckman Model 110A pump, an Altex Model 210 injector with a 50-μl sample loop (Beckman, Silver Springs, MD, U.S.A.) and a Tracor Model 970 variable-wavelength detector (Tracor, Austin, TX, U.S.A.) were employed for all HPLC analyses. An Altex Model C-R1A integrator (Beckman) at an input sensitivity of 1 mV/min was used for peak area measurements and chromatographic recording. A μBondapak C_{18} column, 300 × 3.9 mm I.D. (Waters Assoc., Milford, MA, U.S.A.), was used. The mobile phase consisted of acetonitrile–0.01 *M* ammonium carbonate buffer, pH 8.4 (3.75:2). The flow-rate was 2 ml/min (1000 p.s.i.). Under these conditions, retention times were as follows: pergolide, 6.74 min; pergolide sulfoxide, 3.22 min; and lergotrile, 2.42 min.

Helminthosporium cultivation

The *Helminthosporium* species (NRRL 4671) was maintained on mycophyll agar slants (BBL, Cockeysville, MD, U.S.A.) which were refrigerated at 4°C. Incubations were performed in a two-stage fermentation procedure as described earlier[9]. Cultures were harvested, combined and fully homogenized (Polytron; Brinkmann, Westbury, NY, U.S.A.) before HPLC analysis.

Extraction and analysis

Spiked samples were prepared using a single extraction step. Stock solutions at levels of 1 mg/ml in methanol were prepared for pergolide, pergolide sulfoxide and lergotrile. Portions of 500, 250, 150, 100, 50 and 20 μl of pergolide and pergolide sulfoxide solutions and 100 μl of the internal standard solution of lergotrile, were placed in 125 × 16 mm silylated glass extraction tubes and the solvent was removed under a nitrogen stream. A total of 2 ml of the *Helminthosporium* culture homogenate was added to the tube and the mixture was alkalinized with 2 ml of 0.1 *M* sodium carbonate–sodium bicarbonate buffer (pH 8.5) and extracted with 4 ml of isoamyl alcohol. Samples were agitated for 30 min (30 oscillations per min) on a Lab-Tek aliquot mixer, and centrifuged (1230 *g*) for 10 min before 2 ml of the isoamyl alcohol

layer were removed and dried under a nitrogen stream; samples containing 250 μg/ml of I and II were reconstituted in 500 μl of mobile phase; the remainder of all samples was dissolved in 250-μl portions of mobile phase, filtered and subjected to HPLC analysis.

Samples at levels of 250, 125, 75, 50, 25 and 10 μg/ml of pergolide and pergolide sulfoxide were prepared without extraction for comparison of peak areas with extracted samples for the calculation of absolute recovery values. The absolute recovery of lergotrile was determined at a level of 100 μg/ml only. Resultant peak areas were used to prepare standard curves where peak area ratios (standard/lergotrile) were plotted *vs.* μg of standard compound per ml of culture.

RESULTS AND DISCUSSION

Fig. 2 illustrates the HPLC separation system developed for pergolide (I), pergolide sulfoxide (II) and the internal standard, lergotrile (III). A series of indole compounds (*e.g.*, tryptamine) were examined as possible internal standards, and lergotrile was chosen based on its structural similarity, resolution from all peaks in the chromatogram and absolute recovery from isoamyl alcohol (91.1 ± 2.9%, $n = 6$). The wavelength chosen for detection (290 nm) was optimal for all three compounds.

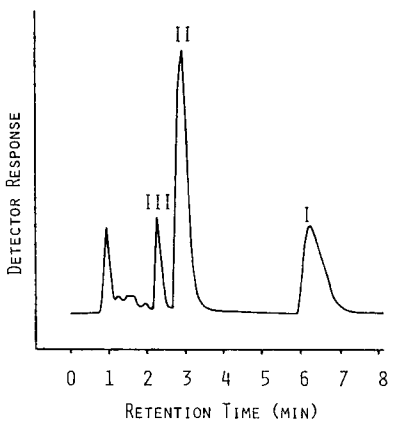

Fig. 2. Chromatographic separation of pergolide (I), pergolide sulfoxide (II) and lergotrile (III) from a culture extract of *Helminthosporium*. Chromatographic conditions are described in the Experimental section.

The final composition of the developed mobile phase resulted from systematic variation of the components in the mixture until adequate resolution was obtained while retaining a short analysis time (8 min). Alkaline conditions (pH 8.4) were chosen to allow for development of totally unionized species and did not appear to effect the integrity of the HPLC column over a period of nine months.

A simple extraction procedure was developed which allows for the HPLC analysis of a *Helminthosporium* culture after a single extraction step. Culture homogenates were spiked with levels of standards of pergolide and pergolide sulfoxide based on maximum substrate levels and theoretically maximal product formation (250 μg/ml). Isoamyl alcohol was chosen for extraction based on absolute recovery experi-

ments with an array of organic solvents (chloroform, dichloromethane, diethyl ether, ethyl acetate) and a lack of interference from co-extracted media components. Good absolute recoveries of pergolide (71.2 $\pm$ 7.6%, $n = 6$) and pergolide sulfoxide (91.5 $\pm$ 5.2%, $n = 6$) were obtained with this solvent. Resulting standard curves for pergolide and pergolide sulfoxide produced satisfactory results; a typical pergolide standard curve yielded a slope of 0.0128 (y-intercept = 0.0685, $r = 0.9954$) and the pergolide sulfoxide standard curve typically yielded a slope of 0.0244 (y-intercept = -0.0096, $r = 0.9997$). This demonstrates the utility of this analysis over a concentration range anticipated for both compounds in microbial transformation experiments.

In summary, a method has been developed for the simple extraction and rapid analysis of pergolide and its metabolite, pergolide sulfoxide, in *Helminthosporium* cultures. This method has been applied to growing cultures of a *Helminthosporium* species to determine the amount of enzymatic *vs.* spontaneous air-oxidation of pergolide (in preparation). This method may have application in the analytical determination of additional ergolines and their metabolites, and the preparative-scale separation of these compounds.

ACKNOWLEDGEMENT

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Note

High-performance liquid chromatographic determination of N,N-dimethylcolchiceinamide and its metabolites, N-methylcolchiceinamide and colchiceinamide, in microbial culture

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(Received June 30th, 1981)

Although colchicine (I) has been used for centuries in the treatment of gout, recent interest in colchicine derivatives stems from their potential use as antineoplastic agents[1]. There is ample evidence to indicate that certain colchicine analogues, particularly colchiceinamide derivatives such as II–IV (see Fig. 1), exhibit higher therapeutic indicies, and hold more promise for clinical application[2,3].

We are currently examining the use of micro-organisms to prepare colchicine derivatives metabolically and to study their metabolic alteration[4]. Studies involving the microbial transformations of N-methylcolchiceinamide (II) and N,N-dimethylcolchiceinamide (III) have necessitated the development of new analytical procedures for these substrates and their metabolites. We chose high-performance liquid chromatography (HPLC) for its demonstrated utility in the rapid analysis of microbial extracts and delineation of metabolic pathways[5]. The devised method may also be useful in other pharmaceutical, pharmacokinetic, and clinical studies of colchiceinamide derivatives used as antineoplastic agents.

EXPERIMENTAL

Reagents

All solvents and reagents were analytical grade or better. Solvents for chromatography were chromatographic grade (LiChrosorb; MCB, Cincinatti, OH, U.S.A.). Water was deionized and double distilled in glass. Mobile phases were prepared by the filtration of individual solvents through glass fiber pads (GF/F grade; Whatman, Clifton, NJ, U.S.A.), mixing, and degassing prior to use.

Standard compounds

Colchicine (I) and N-methylcolchiceinamide (II) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Colchiceinamide (IV) was obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Compounds I, II, and IV gave spectral and physical data consistent with literature reports[4]. N,N-Dimethylcolchiceinamide (III) was synthesized from colchicine according to the method of Hartwell *et al.*[6], and recrystallized from light petroleum (b.p. 60–68°C). This compound gave the follow-

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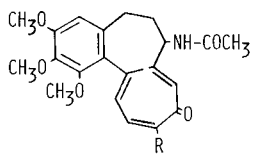

I, R = OCH_3

II, R = $NH\text{-}CH_3$

III, R = $N(CH_3)_2$

IV, R = NH_2

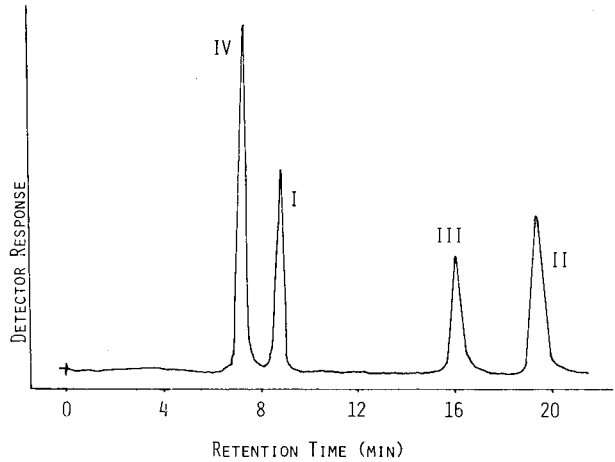

Fig. 1. Structures of colchiceine (I) and the colchiceinamide derivatives N-methylcolchiceinamide (II), N,N-dimethylcolchiceinamide (III) and colchiceinamide (IV).

Fig. 2. HPLC separation of colchicine (I) and the colchiceinamide derivatives colchiceinamide (IV), N-methylcolchiceinamide (II), and N,N-dimethylcolchiceinamide (III). Chromatographic conditions are described in the Experimental section.

ing analytical data: m.p. 138–143°C, re-solidifies, and re-melts at 173–174°C [rept.[6] 145°C (foams), 203–205°C (melts); and[7] 174–176°C]; proton magnetic resonance spectrum ($CDCl_3$) ppm (multiplicity, assignment); 1.97 (s, 3H, $COCH_3$), 2.0–2.6 (m, 5H, C-5, C-6, C-7), 3.15 (s, 6H, $N(CH_3)_2$, 3.60, 3.85 and 3.93 (3s, 9H, C-1–, C-2– and C-3–OCH_3), 6.45 and 6.58 (m, 2H, C-4 and C-11), 7.20 and 7.33 (m, 2H, C-2 and C-8), identical with reported spectrum[8]; mass spectrum, *m/e* (% relative abundance), 412 (100), 398 (39), 384 (12), 369 (11), 357 (16), 341 (12), 325 (18), 310 (15), 298 (10), consistent with analogous spectra[9]. All of the standard compounds were homogeneous as determined by thin-layer chromatography and HPLC.

Chromatographic system

A Tracor Model 950 pump and 970A variable-wavelength detector (Tracor Industries, Austin, TX, U.S.A.) with a 20-μl loop injector (Rheodyne, Berkeley, CA, U.S.A.) were employed for all HPLC analyses. Detection was at 370 nm, which represents a maximal compromise for all four compounds analyzed. An HP Model 3380A reporting integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.) at an input sensitivity of 0.1 v/a.u. and a slope sensitivity of 1 mV/min was used for peak area measurements and chromatographic recording. The column used was a 5-μm Ultrasphere-ODS (Beckman), 25 × 0.46 cm I.D. The mobile phase consisted of 0.1 *M* triethylamine hydrochloride in phosphate buffer (0.02 *M*, final pH adjusted to 2.2)–acetonitrile–methanol (715:200:85).

Extraction and analysis

Spiked samples were analyzed using a single extraction step. A stock solution was prepared consisting of 1 mg each of colchiceinamide (IV), N-methylcolchiceinamide (II), and N,N-dimethylcolchiceinamide (III), in 25 ml of chloroform.

Portions of 0.625, 1.25, 2.50, 3.75, and 5.00 ml were pipeted into duplicate 125 × 16 mm I.D. silylated glass extraction tubes, and the solvent was removed under a gentle nitrogen stream. The residue was taken up in 1 ml of *Streptomyces griseus* culture (NRRL B-599) grown according to a two-stage fermentation procedure[4]. Samples were alkalinized with 1.0 ml of saturated aqueous sodium bicarbonate solution, and extracted with 2.0 ml of chloroform containing 100 μg of colchicine (I) as the internal standard. The mixture was agitated for 20 min at 18 rpm on an Alignot mixer Model 4651 (Ames Co., Elkhart, IN, U.S.A.) and centrifuged. A 1-ml portion of the chloroform layer was taken to dryness under a nitrogen stream, reconstituted in 1.0 ml of acetonitrile–methanol (1:1), filtered, and subjected to HPLC analysis.

The resultant peak areas were used to plot standard curves for II–IV as peak area ratio (standard compound/colchicine) *vs.* μg of standard compound per ml of culture. Typical standard curves were as follows: colchiceinamide, slope = 0.018, *y*-intercept = 0.03, $r = 0.999$; N-methylcolchiceinamide, slope = 0.13, *y*-intercept = 0.02, $r = 0.998$; N,N-dimethylcolchiceinamide, slope = 0.090, *y*-intercept = 0.01, $r = 0.999$.

RESULTS AND DISCUSSION

Fig. 2 illustrates the chromatographic separation of colchiceinamide (IV), the internal standard, colchicine (I), N-methylcolchiceinamide (II), and N,N-dimethylcolchiceinamide (III). Several modifications of the previously reported methods for the separation of colchicine derivatives[10] were necessitated by the observation that the N-methyl derivative, II, had the same retention time as the N,N-dimethyl derivative, III. In addition, colchiceinamide (IV) exhibited no separation from the desired internal standard, colchicine (I).

The first modification involved the use of a 5-μm, instead of a 10-μm column, thus increasing the resolution of II and III. Second, the addition of triethylamine hydrochloride to the mobile phase[11] was found to improve the peak shape. We found a linear increase in the number of theoretical plates with reference to the peaks for II and III as the concentration of triethylamine was increased from zero to 0.8%, due entirely to improved peak shape with no alteration of retention time. Our results indicate that compounds II–IV are not protonated at the pH of the mobile phase (pH 2.2) (see below). It appears that triethylamine, in its protonated form at this pH, functions to improve peak shape by a "competing base" mechanism[11].

The use of an acidic pH (2.2) allowed for the resolution of colchiceinamide (IV) and colchicine (I). Colchicine is the only readily available compound of similar chemical structure to II–IV which would not be a potential metabolite; hence, it was chosen as an appropriate internal standard. Colchicine and colchiceinamide exhibit the same retention time in a chromatographic system reported earlier[10] for analyzing colchicine metabolites and related derivatives. However, Fig. 3 illustrates that a drastic decrease in k' values occurs only for the colchiceinamide derivatives (II–IV) as the pH is decreased below 3.0, presumably due to the protonation of the relatively non-basic vinylagous amide of the tropolone ring. Colchicine, on the other hand, exhibits little pH effect, as would be expected. Thus, baseline resolution of I and IV was accomplished at pH 2.2, as indicated in Fig. 2.

A method has been developed for the simple extraction and rapid analysis of

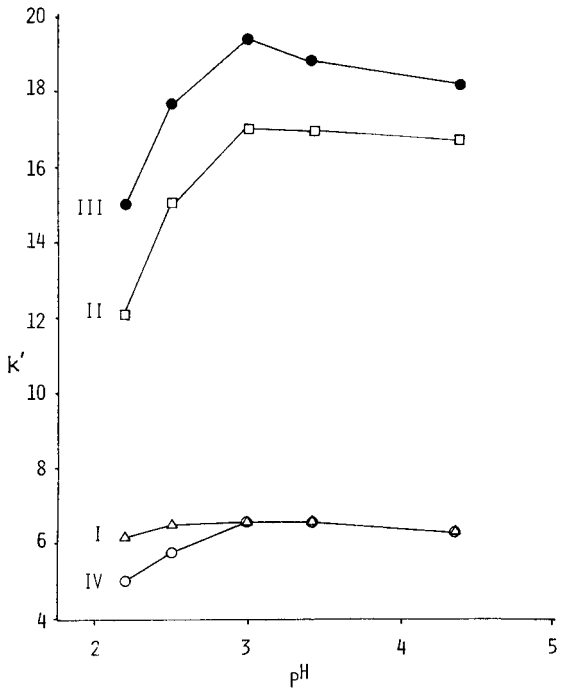

Fig. 3. Variation in capacity factor (k') as a function of pH for colchiceinamide (IV) (○), colchicine (I) (△), N-methylcolchiceinamide (II) (□), and N,N-dimethylcolchiceinamide (III) (●). Chromatographic conditions are described in the Experimental section.

colchiceinamide derivatives in microbial cultures. The procedure has been applied to growing cultures of *Streptomyces griseus* to follow the sequential N-dealkylation of III to II to IV (results to be published elsewhere). The method may also have application in pharmaceutical and clinical analyses of these important antineoplastic agents.

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Note

Separation of the N-trifluoroacetyl dimethyl esters of leukotriene D and E isomers by semi-preparative high-performance liquid chromatography

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Leukotrienes D (LTD) and E (LTE) are members of a series of 7,9,11,14-eicosatetraenoic acids[1]. Recent evidence has shown that LTD is one of the biologically active components of the human slow reacting substance of anaphylaxis (SRS-A)[2]. SRS-A is one of the chemical mediators, the presence of which gives rise to the clinical symptoms of asthma[3]. To investigate this situation further, a series of pure LTD and the related LTE isomers were required for comparative pharmacological studies.

LTD and LTE can be readily prepared from leukotriene A methyl ester (1b). In the convergent synthesis of 1b three additional major geometric isomers are formed which are isomeric about the 9,10- and 11,12-double bonds[4–7]. The separation of these isomers is described elsewhere[8]. Each geometric isomer of leukotriene A methyl ester exists as a racemic mixture. Individual reaction of 1b with the N-trifluoroacetyl methyl ester of either optically pure L-cysteinylglycine or L-cysteine gives protected LTD[9] (2bii) and LTE[10] (3bii), respectively, and their corresponding isomer pair (2bi and 3bi). A further six isomers of protected LTD and LTE were similarly formed by reaction of 1a, 1c and 1d (Table I). These N-trifluoroacetyl dimethyl ester derivatives were synthesised for ease of chromatographic separation. After chromatographic purification all the derivatised molecules were converted to the corresponding unprotected molecules for comparative pharmacological studies.

The use of semi-preparative high-performance liquid chromatography (HPLC) to separate all of these isomeric molecules is described. Mass spectrometry and ultraviolet (UV) spectroscopy were utilised to characterise the chromatographic peaks.

EXPERIMENTAL

Reagents

The Spherisorb S5W, S5NH, S5CN and S5ODS column packing materials

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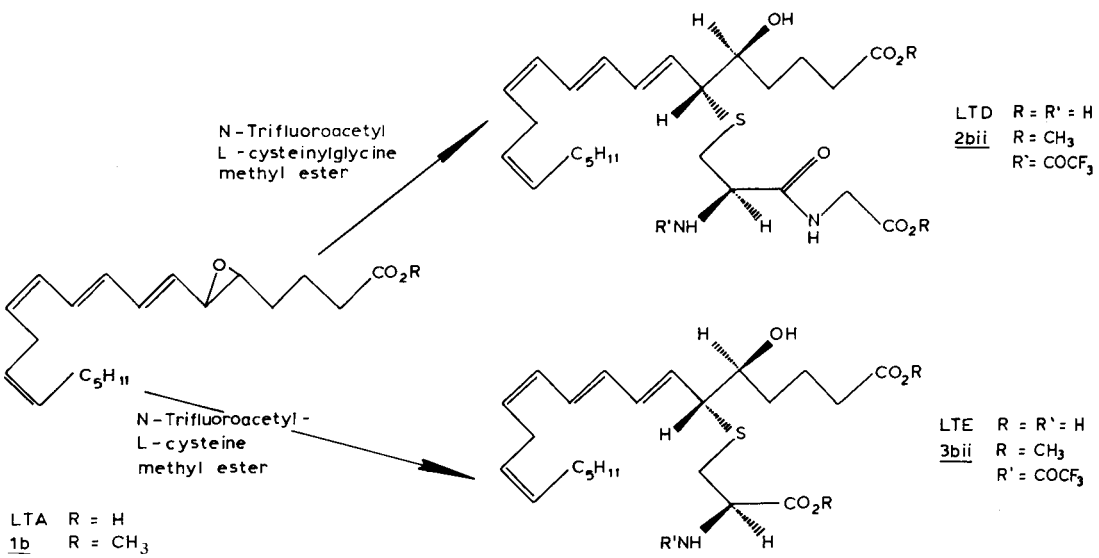
TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF LTD AND LTE ISOMER DERIVATIVES

*Isomer**	*Isomer No.*	*Capacity factors, k'*			λ_{max} *(nm)*		
		S5NH	*S5CN*	*S5W*			
5*R*, 6*S*,9-*cis*, 11-*trans*-LTD	2ai	2.5			272.0(sh)	279.5	288.0(sh)
5*S*, 6*R*, 9-*cis*, 11-*trans*-LTD	2aii	3.4			270.5(sh)	278.5	286.5(sh)
5*R*, 6*S*-LTD	2bi	2.8	1.47	10.5	274.0(sh)	282.5	290.5(sh)
LTD (natural 5*S*, 6*R*)	2bii	3.4	1.57	10.5	274.0(sh)	281.5	289.5(sh)
5*R*, 6*S*, 9-*cis*-LTD	2ci	2.6			277.0(sh)	282.0	290.5(sh)
5*S*, 6*R*, 9-*cis*-LTD	2cii	3.4			275.0(sh)	281.5	291.5(sh)
5*R*, 6*S*, 11-*trans*-LTD	2di	2.6			272.0(sh)	279.0	286.5(sh)
5*S*, 6*R*, 11-*trans*-LTD	2dii	3.0			272.0(sh)	278.5	286.5(sh)
5*R*, 6*S*, 9-*cis*,11-*trans*-LTE	3ai	3.8			273.0(sh)	278.5	286.5(sh)
5*S*, 6*R*, 9-*cis*,11-*trans*-LTE	3aii	4.3			272.0(sh)	279.0	286.5(sh)
5*R*, 6*S*-LTE	3bi	3.8	2.8	3.1	277.0(sh)	282.5	292.5(sh)
LTE (natural 5*S*,6*R*)	3bii	4.1	2.9	3.1	276.0(sh)	282.0	291.5(sh)
5*R*, 6*S*, 9-*cis*-LTE	3ci	3.8			277.0(sh)	282.5	290.5(sh)
5*S*, 6*R*, 9-*cis*-LTE	3cii	4.2			277.0(sh)	282.0	289.5(sh)
5*R*, 6*S*, 11-*trans*-LTE	3di	3.4			272.0(sh)	278.5	287.5(sh)
5*S*, 6*R*, 11-*trans*-LTE	3dii	3.6			272.0(sh)	278.0	287.5(sh)

* Order of elution of isomeric pairs assumed to be analogous to that of LTD and 5*R*,6*S* LTD. The stereochemistry of the 7–8 (*trans*) and 14–15 (*cis*) double bonds remains fixed.

were supplied by Phase Separations (Queensferry, Great Britain). HPLC grade hexane, dichloromethane and methanol were purchased from Fisons (Loughborough, Great Britain) and analytical-reagent grade acetic acid from May & Baker (Dagenham, Great Britain).



Instrumentation

A constant-flow Milton Roy Constametric 11G pump coupled to a Cecil Model 212 variable-wavelength UV monitor was utilised for the chromatographic separation. The UV monitor was set at 280 nm or 305 nm for the analytical and semi-preparative work, respectively. The analytical 10-mm path length cell was replaced with one of 1 mm for semi-preparative chromatography. Samples were injected using a Rheodyne variable-volume valve injector (20-μl volume loop for analytical and a 2.0-ml volume loop for semi-preparative separations).

Chromatography

All stainless-steel columns were packed in a vertically upwards mode using a methanol or isopropanol (S5ODS only) slurry of the packing material. Analytical chromatography was performed on 12.5 cm × 5 mm I.D. columns packed with Spherisorb S5W, S5NH, S5CN or S5ODS. For the Spherisorb S5W, S5NH and S5CN columns dichloromethane–methanol (200:1) was used to elute LTD-type molecules and hexane–dichloromethane–methanol (75:25:1) the LTE-type molecules at flow-rates of 1 ml/min. Identical eluting solvents (5 ml/min) were used for the 50 cm × 8 mm I.D. Spherisorb S5NH semi-preparative columns. For the Spherisorb S5ODS column, optimum separations were achieved with either a methanol–water–acetic acid (75:25:0.1) eluent for protected LTDs (2bi and 2bii) or methanol–water–triethylamine (80:20:1) for protected LTEs (3bi and 3bii).

Mass spectrometry

The required HPLC eluate was manually collected and reduced in volume before transference to a sample tube where it was blown to dryness. All spectra were obtained using an LKB 9000S mass spectrometer. Samples (3 μg) were analysed by direct insertion probe at 90°C using an ion accelerating voltage of 3.5 kV, an electron voltage of 20 eV and a source temperature of 270°C.

Ultraviolet spectroscopy

The UV absorption spectra were recorded in cyclohexane on a Pye-Unicam SP8-100 spectrophotometer, calibrated at 279.4 nm with a Holmium filter.

RESULTS AND DISCUSSION

Both μBondapak C_{18} (refs. 6, 10, 11) and Nucleosil C_{18} (ref. 12) analytical HPLC columns have been used to purify microgramme quantities of LTD and to check the co-elution of samples from biological and synthetic sources. Compound 2bii has also been chromatographed[6] on a μBondapak C_{18}column. Minimal attention has yet been paid to the HPLC of LTE and 3bii although both compounds have been chromatographed[13] using reversed-phase HPLC. The performance of these analytical columns or their suitability for scaling up to semi-preparative requirements was not discussed.

It was anticipated that for both the comparative pharmacological studies and complete spectral characterisation of all isomers, milligramme rather than microgramme quantities of material would be required. Consequently, semi-preparative rather than analytical columns were utilised for the chromatographic separation.

Preparative HPLC usually involves a compromise between sample capacity, column resolution and separation time[14]. As the chromatographic separation between isomeric peaks was expected to be small, it was considered important to carry out the analytical work on high efficiency columns, which can be scaled up for semi-preparative operations without loss in column performance. In practice it would not be possible to sacrifice the separation of the diastereoisomers for increased sample throughput. Consequently, to compare their separation characteristics, four analytical columns, 12.5 cm in length, were packed with 5-μm spherical packing material.

Analytical chromatography

It was anticipated that the presence of the carboxylic acid and amino-group functionalities in the parent eicosatetraenoic acids would diminish differential chromatographic characteristics observed between the optical-isomer pairs. Hence separations were developed on the fully derivatised (2ai–2dii and 3ai–3dii) rather than underivatised molecules. The initial HPLC separation was developed for isomers 2bi and the natural isomer 2bii. This latter isomer was the most important and the most readily available. It was assumed that conditions selected for one optical-isomer pair could be utilised for the separation of the remaining isomeric pairs. A similar approach was used for isomeric pairs 3ai–3dii.

The separations achieved for the four pairs of optical isomers of each type are shown in Table I. For 2bi and 2bii optimum separations were obtained on a Spherisorb S5NH column. When using a dichloromethane–methanol eluent an α value of 1.2 was obtained. No separation was observed on a Spherisorb S5W column using the same solvent system even though surprisingly long retention times were observed. Partial separations were obtained on the less retentive Spherisorb S5CN (α = 1.07) and S5ODS phases (α = 1.1). As similar separations were achieved for the optical isomers of 2a, 2c or 2d, the Spherisorb S5NH column was used for scaling up to semi-preparative separations. Although the optical-isomer pairs were well resolved, the geometric isomers (2ai–2di) are not completely separated, confirming the need to chromatograph 1a–1d at the previous synthetic stage. Similar observations can be made for the alternative geometric isomers (2aii–2dii).

Optical isomers 3bi and 3bii were more difficult to separate than those in the corresponding LTD-type series. Using a less polar eluent the optimum separation was again achieved using a Spherisorb S5NH column but with a reduction in α value to only 1.1. The Spherisorb S5ODS column gave a separation of 3bi and 3bii showing an α value of 1.09. The reduction in polarity and increase in size of the 6-substituent would therefore seems to be advantageous in separating the LTD-type isomers compared with those of LTE type.

Reaction following of LTE formation

It was observed during the development of the separation for 3bi and 3bii that samples were contaminated with the corresponding 11-*trans* LTE isomers (3di and 3dii). As 3di and 3dii are only slightly less retained than 3bi, its purification was made more difficult by the presence of these *trans*-isomers. Monitoring the formation of 3bi and 3bii from the appropriate isomer of LTA methyl ester indicated that the reaction was essentially complete in 20 min. Isomers 3di and 3dii were only formed when the reaction products were allowed to stand in methanol–triethylamine. Hence, by ensur-

ing the chromatographic separation was carried out soon after completion of the reaction, minimal formation of interfering 3di and 3dii was observed.

Semi-preparative chromatography

Using a 50 cm × 8 mm I.D. Spherisorb S5NH column with over 40,000 theoretical plates, up to 3 mg of a mixture of 2bi and 2bii dissolved in 1 ml of eluent were separated per injection. The attempted separation of larger amounts of material caused loss in column resolution to an unacceptable level. The relatively low sample capacity of this column (0.2 mg/g packing material) reflects[9] the difficulty of the separation. Surprisingly, volume overload effects were also observed when the isomers were dissolved in greater than 1 ml of eluent. This rate of separation allowed the collection of several milligrams of 2bi and 2bii, although less of the other three isomer pairs was collected. A chromatogram showing the elution positions of 2bi and 2bii and a spiked mixture of all eight optical isomers of protected LTD is shown in Fig. 1a and b, respectively.

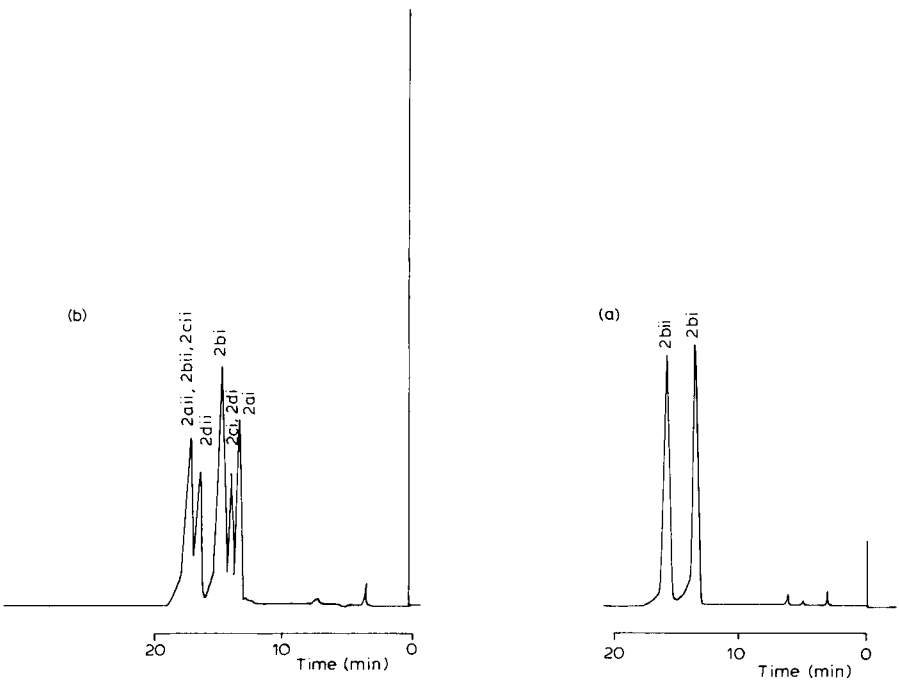


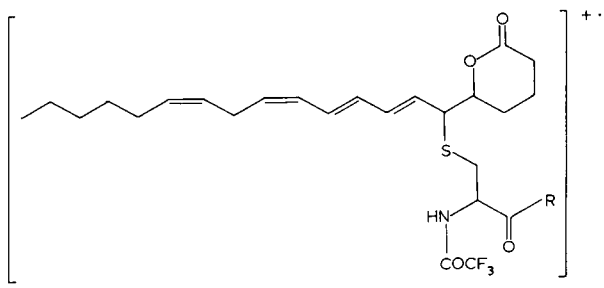
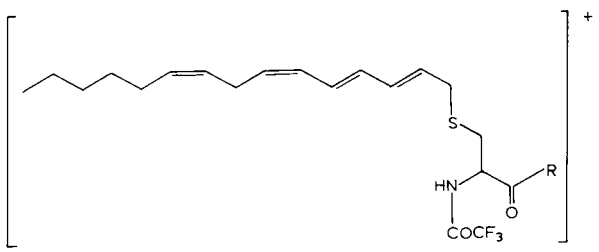
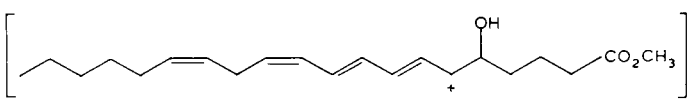
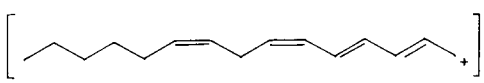
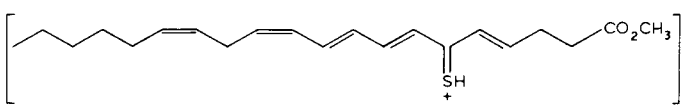
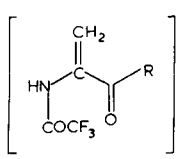
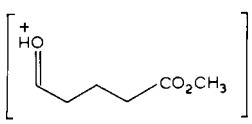
Fig. 1. (a) Chromatogram of derivatised leukotriene D isomers 2bi and 2bii. (b) Chromatogram of a mixture of all eight isomers of derivatised leukotriene D.

The preparative separation of the LTE-type isomers was, as expected from the analytical work, more difficult than that for the corresponding LTD-type isomers. Although "heart cutting" of peaks was employed it was necessary to re-inject a sample up to three times to obtain the required purity. Over a milligramme of 3bi and 3bii was collected but less of the other six isomers.

TABLE II

CHARACTERISTIC IONS OBSERVED IN THE MASS SPECTRUM OF LTD- AND LTE-TYPE DERIVATIVES

LTD type: R = $NHCO_2CH_3$; LTE type: R = OCH_3.

m/e values		*Ion structure*
LTD-type derivative	*LTE-type derivative*	
620	563	$[M]^{+\cdot}$
588	531	
490	433	
333	333	
203	203	
347	347	
254	197	
131	131	

Purity and chromatographic yield

As the presence of small amounts of one isomer in another could markedly affect the biological activity of the major component, strict control of isomer purity was essential. The purity of the eight protected LTD isomers as measured by analytical HPLC and assuming equi-molar UV response was >99.5% and that for the LTE series >99%. The recovery of a sample of protected LTD following re-injection of 2bii on the semi-preparative Spherisorb S5NH column was almost quantitative. No stability problems were thus met during the time elapse of the chromatographic separation although a considerable amount of material was lost when samples were stored at ambient temperatures or taken to dryness. The isomer appears to adhere to the glassware. Hence, once isolated, it was important to store the isomers in solution at –60°C to minimise loss.

Mass spectrometry

The spectra of each isomer of protected LTD and LTE showed a characteristic range of ions although significant differences were observed in the relative abundance of these ions. It is anticipated one will be able to correlate these variations in ion abundance with stereochemical assignments within the molecules. A weak molecular ion and the loss of water and methanol is observed for all isomers. Fragment ions characteristic of the eicosatetraenoate and peptide portions of the isomers are observed (see Table II).

Ultraviolet spectroscopy

The UV absorption maxima in cyclohexane of eight isomers of both derivatised LTD and LTE are listed in Table I. The geometric-isomer pairs showed characteristic absorption maxima although, as expected, the optical isomers with the same double-bond geometry were very similar.

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Note

High-performance liquid chromatographic analysis of carvacrol and thymol in the essential oil of *Thymus capitatus*

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Phenolic compounds are widely distributed in plants, but their functions are not yet known[1–3]. Most of them are formed via the shikimic acid pathway, which leads also to amino acids (phenylalanine, tryptophan), growth substances (indole acetic acid) and secondary products such as lignins, coumarins, flavones and anthocyanins. The metabolism of phenolic substances is related to phenylalanine ammonialyase (PAL), the activity of which is affected by external and internal factors. In particular, the different amounts of thymol in the essential oil of *Thymus serrulatus* grown in different environments has been related to water supply[4].

In *Thymus capitatus* the phenolic fraction, which is the main component of the essential oil (60–90%), comprises only two isomeric phenolics, carvacrol (2-*p*-cymenol) and thymol (3-*p*-cymenol), whose synthesis can be related to external factors[5]. Several researchers have attempted to separate and quantitate these two isomers by using gas-chromatographic (GC) or chemical methods in which phenolics were first separated from non-phenolics by alkaline extraction[6–10]. The results obtained were not satisfactory because the chromatographic peaks were not completely resolved and/or the alkaline extraction was not quantitative. Recently, the use of glass capillary columns in GC has allowed a very efficient separation of these two phenols[11].

For the same purpose, we have developed a fast and accurate method involving reversed-phase high-performance liquid chromatography (HPLC), which has already proved successful in the separation of different phenolics[12–16].

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph consisted of: two Waters Assoc. Model 6000 pumps, a Model U6K universal injector, and a Model 660 solvent programmer; two columns, (1) 300 × 3.9 mm I.D., packed with μBondapak C_{18} (Waters Assoc.), (2) 250 × 4.6 mm I.D., RP-18 (Brownlee Labs.) packed with 10-μm LiChrosorb (E. Merck); a variable-wavelength detector, Perkin-Elmer LC-55 UV–visible spectrophotometer with a 8-μl sample cell; a Leeds & Northrup Speedomax XL 68 Mark II recorder.

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Reagents

A carvacrol and thymol standard was obtained from Fluka. The solvents were acetonitrile RS and methanol RS for HPLC (Carlo Erba, Milan, Italy). Twice distilled water was filtered through a 0.2-μm Millipore system. The phenolic standard solutions were 200 ppm carvacrol and thymol in methanol. Acetonitrile–water (40:60) was employed as mobile phase.

Extraction of essential oil

The essential oil of *T. capitatus* was extracted by steam distillation at atmospheric pressure and then separated from the aqueous phase with petroleum ether. The ethereal phase was washed with water, dried over anhydrous Na_2SO_4 and the solvent removed in a rotary evaporator at low pressure and 30°C.

Separation of "phenolic fraction"

The phenolic fraction was separated by the alkaline method[9,10] shown schematically in Fig. 1. The yields of phenolic and non-phenolic fractions from 2 g of essential oil were 1.55 g and 0.45 g, respectively.

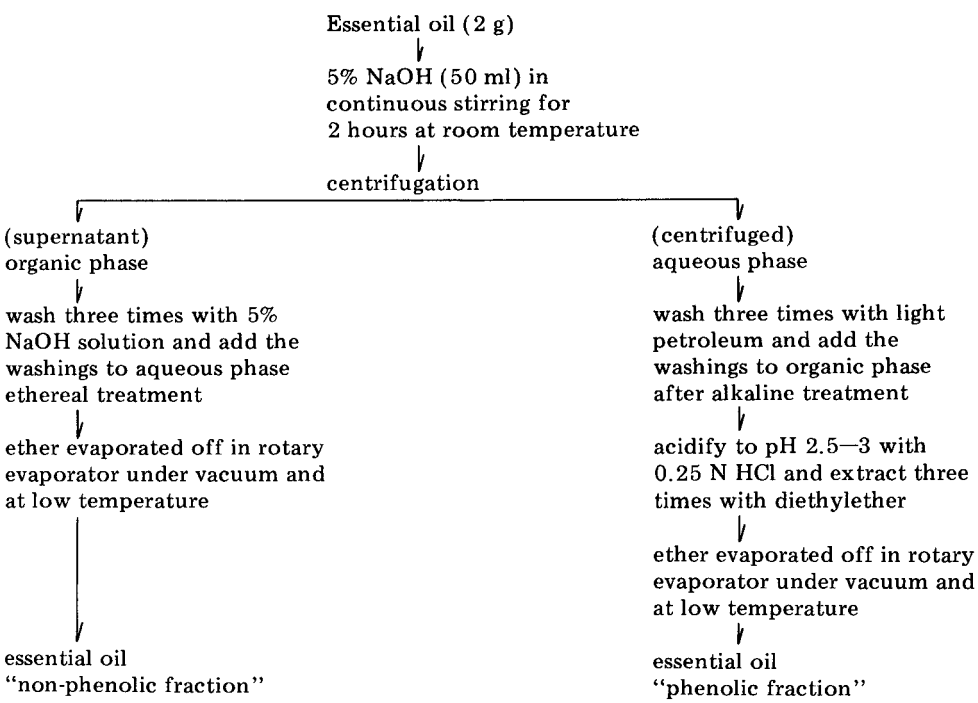

Fig. 1. Flow diagram illustrating the alkaline separation of the "phenolic fraction" from the "non-phenolic fraction" of the essential oil.

Determination

In order to find the best chromatographic conditions for separation of the two isomers, the retention time, t_R, capacity factor, k', relative retention, α, and resolution, R_s, were calculated for the two columns tested (Table I).

TABLE I

RETENTION TIMES, t_R, CAPACITY FACTORS, k', RELATIVE RETENTIONS, α, AND RESOLUTIONS, R_s, OF CARVACROL AND THYMOL ON μBONDAPAK C_{18} AND RP-18 USING WATER–ACETONITRILE AS ELUENT

Compound	*μBondapak* C_{18}				*RP-18*			
	t_R *(min:sec)*	k'	α	R_s	t_R *(min:sec)*	k'	α	R_s
Carvacrol	17:10	7.55	1.14	1.45	17:50	7.75	1.12	1.39
Thymol	19:30	8.65			19:40	8.70		

Calibration curves were constructed by using standard solutions containing different ratios of the two phenols and plotting the amount of pure component against the peak area (Fig. 2). Each point of the curve was calculated as the average value from three injections. Good results were also obtained by plotting the amount of each compound against the heights or weights of the peaks, as demonstrated by the correlation coefficients calculated by using a simple regression equation.

For the quantitation of the two phenolics, solutions in methanol at different concentrations (200 and 2000 ppm) of the essential oil and of the two fractions obtained by alkaline extraction were subjected to HPLC.

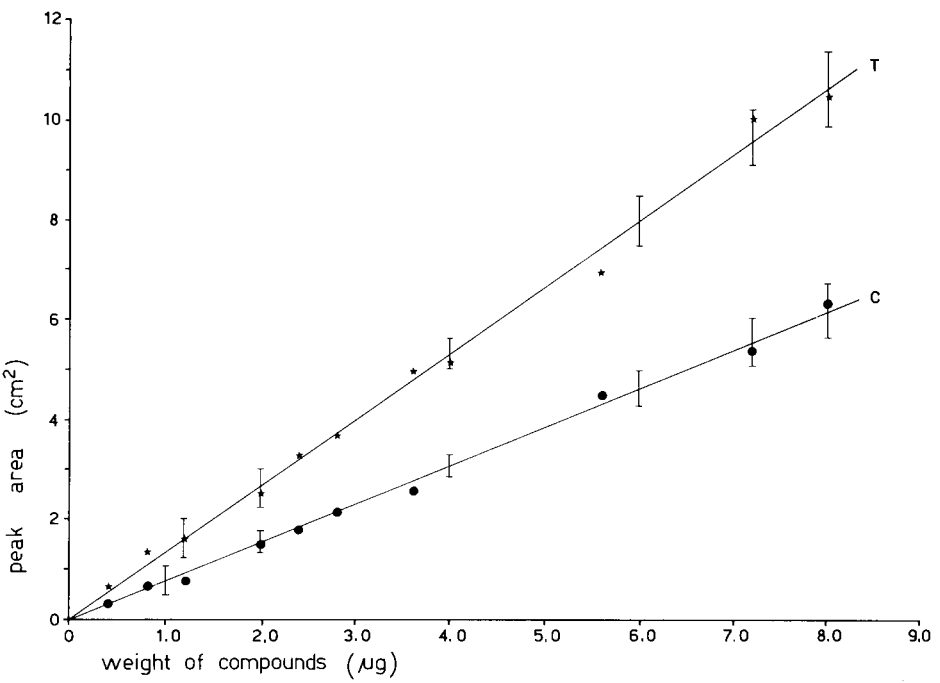

Fig. 2. Calibration graphs for carvacrol (C) and thymol (T). Each point shown is the average from three determinations. The lines indicate the 95% confidence limits for some points on the calibration curves.

RESULTS AND DISCUSSION

The HPLC separation of the two isomers carvacrol and thymol was attempted in reversed-phase mode because of the low polarity and solubility of both chemicals in water. Excellent results were obtained when a μBondapak C_{18} column, an acetonitrile–water (40:60) mobile phase and a flow-rate of 1.5 ml/min were used. No improvements were achieved by varying either the flow-rate or the mobile phase composition. The analysis time and the resolution of two bands decreased as the acetonitrile content increased. The analysis time was unnecessarily long and resolution did not improve by increasing the water content.

No appreciable variations were obtained under the same chromatographic conditions but with the RP-18 column, the packing of which is somewhat more retentive than that of μBondapak C_{18}. The two columns had approximately equal resolution powers for the two isomeric phenolics as demonstrated by the α parameter, 1.12 on RP-18 and 1.14 on μBondapak C_{18} (Table I).

Thus, all results reported are those obtained with μBondapak C_{18}.

The correlation coefficients between the amount of compound and the area, height or weight of the peaks (Fig. 2) indicate linearity over the investigated concentration ranges (0–8 μg).

Some chromatograms of mixtures of carvacrol and thymol at different ratios are reported in Fig. 3. The two isomers are well separated as demonstrated by the R_s values (about 1.4), which indicate less than 1 % overlap of bands. From the same tests it is possible to calculate the detection limits under these chromatographic conditions as the amount of sample which gives a response equal to the noise of the detector[17]: these are 0.02 μg for carvacrol and 0.15 μg for thymol.

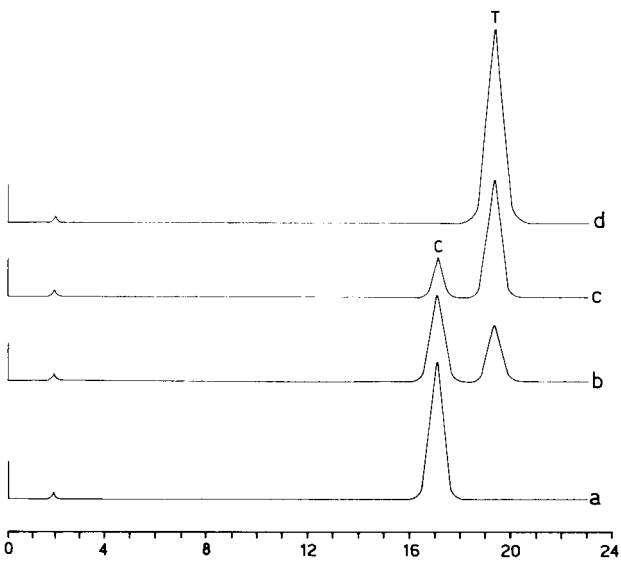

Fig. 3. HPLC chromatograms of 8-μg mixtures of carvacrol (C) and thymol (T) in different ratios: a, 100% C; b, 70% C + 30% T; c, 30% C + 70% T; d, 100% T. Conditions: mobile phase, water–acetonitrile (60:40); column, μBondapak C_{18}, 300 × 3.9 mm I.D.; flow-rate, 1.5 ml/min; pressure, 700–1000 p.s.i.; detector, UV at 283 nm.

In order to check the effectiveness of the alkaline extraction, equal amounts of the "non-phenolic fraction" and of the "phenolic fraction", obtained according to Fig. 1, were injected. The respective chromatograms (Fig. 4) show that the two fractions have about the same percentage of phenolics. This demonstrates that the alkaline extraction is not quantitative, as already pointed out[10], and that the phenols present in the essential oil of *T. capitatus* are partitioned between the aqueous and organic phases, with a preference for the former. Consequently, in this case, the alkaline extraction should be avoided if the exact content of phenolics is to be determined.

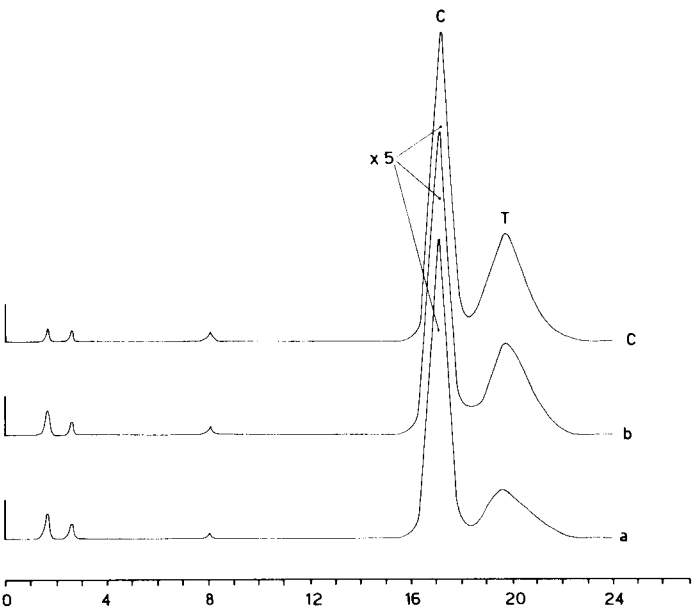

Fig. 4. HPLC chromatograms of 80 μg of the "non-phenolic fraction" (a), "phenolic fraction" (b) and "essential oil" (c) of *Thymus capitatus*. Peaks: C = carvacrol; T = thymol. Conditions as in Fig. 3.

A chromatogram of the essential oil which was not subjected to alkaline extraction is also shown in Fig. 4. A large amount of oil (80 μg) was injected, in order to reveal the thymol, which is present in very small amount in comparison with carvacrol. The separations were not as good as those obtained in calibration tests, as demonstrated by the lower R_s value (about 1.0). Nevertheless, this value indicates that the separation is still efficient and that reasonable estimations of the contents can be made.

On the other hand, the retention times did not change in the concentration range examined.

Only the thymol was quantitated from the chromatogram in Fig. 4c, whereas carvacrol was determined from a chromatogram (not shown) obtained by injecting 8 μg of the essential oil.

By means of the calibration graphs, it has been calculated that 94% of the essential oil of *T. capitatus* is constituted by the "phenolic fraction", of which 91% is carvacrol and 9% is thymol.

In conclusion, the present study has demonstrated that:

(1) the sample of the essential oil must be injected without alkaline separation,

(2) the elimination of preliminary purification and separation steps notably shortens the analysis time and reduces the risk of sample loss,

(3) HPLC allows the separation and quantitation of phenolic compounds, even of isomers, in some essential oils.

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Note

Séparation des acides ascorbique et isoascorbique par chromatographie de paires d'ions sur phase inverse

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(Reçu le 26 juin 1981)

L'acide ascorbique et l'acide isoascorbique sont épimères au niveau de leur carbone 5. Ils ont des propriétés chimiques très comparables, notamment le groupe ènediol-1,2 s'oxyde aisément en dione-1,2, conférant à ces acides des propriétés antioxydantes. Pour cette raison, ils sont largement utilisés dans les aliments. L'acide ascorbique qui se trouve dans de nombreux végétaux, possède une activité vitaminique antiscorbutique (vitamine C) 20 fois supérieure à celle de l'acide isoascorbique. Dans certains pays, en France notamment, seul l'acide ascorbique est autorisé comme additif alimentaire malgré son coût plus élevé et il est donc important de pouvoir le distinguer de l'acide isoascorbique.

Les méthodes usuelles de dosage utilisent les propriétés d'oxydo-réduction du groupement ènediol-1,2 (volumétrie[1], spectrophotométrie[2], polarographie[3], réactions enzymatiques[4]) mais ne permettent pas de différencier ces deux acides. La polarographie a courant alternatif[5], l'isotachophorèse[6] et surtout les méthodes chromatographiques permettent de les séparer et de les doser. Ces acides sont séparés par chromatographie sur papier ou sur couche mince[7] mais les résultats les plus intéressants sont obtenus par chromatographie liquide à haute performance. Deux publications rétentes très semblables, celle de Bui-Nygen[8] et de Geigert *et al.*[9] décrivent leur séparation sur colonne échangeuse d'anions faibles (silice greffée NH_2, éluant: mélange eau–acetonitrile faiblement tamponné).

La chromatographie de paires d'ions a été utilisée pour doser l'acide ascorbique dans des mélanges multivitaminiques[10,11] et plus récemment Finlay et Duang[12] en séparant l'acide ascorbique de l'acide deshydroascorbique sur deux colonnes μBondapak en série, séparent partiellement l'acide isoascorbique. Ce dernier a un facteur de capacité compris entre celui de l'acide ascorbique ($k' \approx 0.50$) et celui de l'acide deshydroascorbique ($k' \approx 0.75$).

La présente communication décrit une méthode de séparation de l'acide ascorbique et l'acide isoascorbique par chromatographie de paires d'ions sur une seule colonne de silice greffée octyle ou octodécyle en utilisant un éluant eau–méthanol (9:1, v/v) tamponné et contenant le contre-ion cétyltriméthylammonium. La détection est effectuée à 264 nm.

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PARTIE EXPÉRIMENTALE

Matériel

Pompe Varian 5020 (Palo Alto, CA, U.S.A.), équipée d'un injecteur de 10 μl; spectrophotomètre ultra-violet Pye-Unicam opérant à 264 nm. Colonne en acier inoxydable de 150–200 mm × 4.6 mm I.D. remplie au laboratoire de LiChrosorb RP-8 ou RP-18, 5 μm ou 7 μm (Merck, Darmstadt, R.F.A.) par la méthode de la bouillie sous 400 bars de pression. Ensemble de filtration en inox de 13 mm I.D. adaptable sur seringue (1–10 ml), ainsi que des filtres en épaisseur et des membranes de 0.45 μm de porosité (Millipore, Bedford, MA, U.S.A.).

Réactifs

Bromure de cétyltriméthylammonium (Merck) méthanol, dihydrogenophosphate de potassium (Prolabo, Paris, France), acide ascorbique, acide isoascorbique, acide deshydroascorbique (Fluka, Buchs, Suisse), acide métaphosphorique (Carlo Erba, Milan, Italie).

Phases mobiles

Les solutions 0.1 *M* KH_2PO_4 ou (0.05 *M* KH_2PO_4 + 0.05 *M* K_2HPO_4) $5 \cdot 10^{-3}$ *M* cétyltriméthylammonium sont préparées dans un mélange eau–méthanol (9:1, v/v). Cette solution est filtrée avant usage sur membrane 0.45 μm de 47 mm de diamètre. Cette opération a le double avantage d'éliminer les particules microscopiques en suspension et les gaz dissouts, notamment l'oxygéne.

Conditions opératoires

La colonne est mise en équilibre avec la phase mobile pendant plusieurs heures, ou bien pendant la nuit à débit réduit. À la fin d'une série de mesures, la colonne est rincée à l'eau puis au méthanol et conservée dans ce même solvant.

Préparation des échantillons

Chaque échantillon est introduit dans la boucle d'injection au travers d'un filtre en épaisseur et d'une membrane de porosité 0.45 μm.

Jus de fruit. Le fruit est pressé et le jus est injecté, éventuellement dilué avec une solution aqueous d'acide métaphosphorique à 2 % (m/v).

Mélange d'additifs. L'échantillon est dissout dans de l'acide métaphosphorique à 2 % puis injecté après dilution convenable.

Charcuterie (jambon). L'échantillon ($\approx$ 30 g) est homogénéisé au mixer avec de l'acide métaphosphorique à 2 % (50–75 cm^3). Après filtration sur coton de verre, la solution est injectée.

RESULTATS ET DISCUSSION

La Fig. 1 montre la séparation de l'acide L-ascorbique et D-isoascorbique.

Le choix d'une phase mobile à force ionique et à pouvoir tampon élevés a pour but de limiter les variations des facteurs de capacité[13] en fonction des autres solutés coinjectés (sucres, sels) qui sont parfois en concentration très supérieures à celle de acide ascorbique ou isoascorbique.

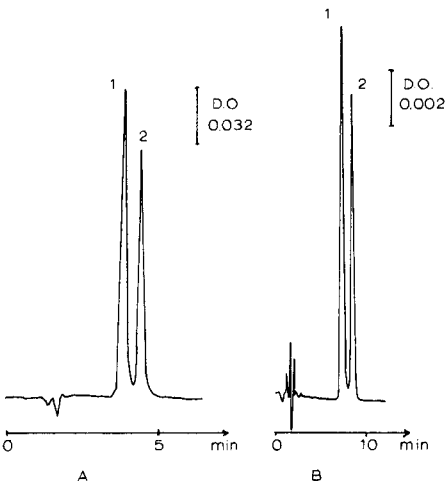

Fig. 1. (A) Colonne LiChrosorb RP-8, 7 μm (200 × 4.7 mm I.D.). Phase mobile: eau–méthanol (9:1, v/v), 0.05 *M* KH_2PO_4–0.05 *M* K_2HPO_4–$5 \cdot 10^{-3}$ *M* cétyltriméthylammonium. Débit: 1.5 cm^3 min^{-1}; U.V.: 264 nm. 1 = acide ascorbique, 2 μg injecté; 2 = acide isoascorbique, 2 μg injecté. (B) Colonne LiChrosorb RP-8, 5 μm (150 × 4.7 mm I.D.) Phase mobile: eau–méthanol (9:1, v/v)–0.1 *M* KH_2PO_4–$5 \cdot 10^{-3}$ *M* cétyltriméthylammonium. Débit: 1.5 cm^3 min^{-1}; U.V. : 264 nm. 1 = acide ascorbique, 0.1 μg injecté; 2 = acide isoascorbique, 0.1 μg injecté.

En l'absence d'ion cétyltriméthylammonium (TMCA) l'acide ascorbique et isoascorbique ont un facteur de capacité nul. La présence de TMCA est nécessaire pour accroître la rétention et la sélectivité permettant de séparer ces deux acides.

Par chromatographie sur échangeur d'anions faibles (silice griffée NH_2) l'acide

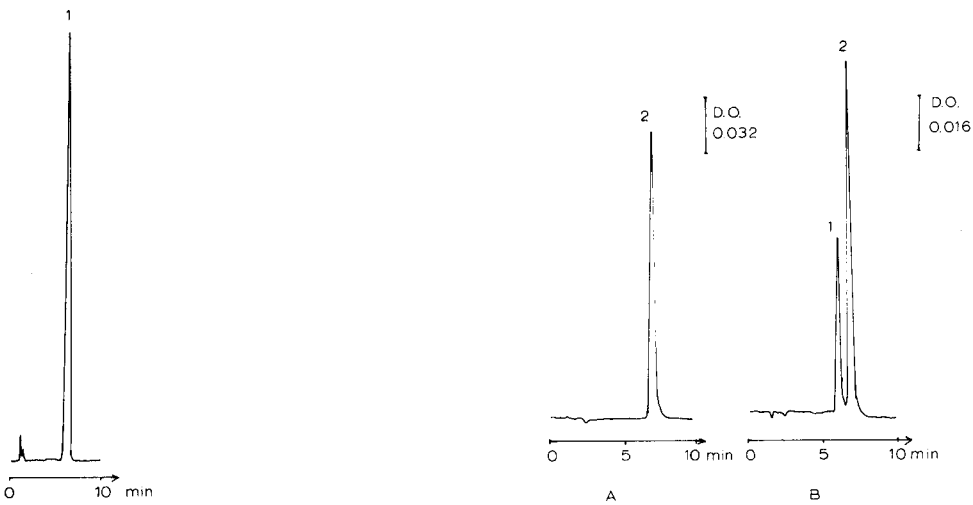

Fig. 2. Jus de citron contenant 286 mg l^{-1} d'acide ascorbique. Colonne LiChrosorb RP-18, 5 μm (150 × 4.7 mm I.D.). Phase mobile: eau–méthanol (9:1, v/v)–0.1 *M* KH_2PO_4–$3 \cdot 10^{-3}$ *M* cétyltriméthylammonium. Débit: 1.5 cm^3 min^{-1}; U.V. : 264 nm. 1 = acide ascorbique.

Fig. 3. Mélange d'additifs pour charcuterie contenant de l'acide isoascorbique. Mêmes conditions chromatographiques que sur la Fig. 2. 1 = acide ascorbique; 2 = acide isoascorbique. (A) Mélange d'additifs pour charcuterie; (B) Mélange d'additifs pour charcuterie additionnée d'acide ascorbique.

isoascorbique est élué en premier, par contre dans nos conditions chromatographiques, sur silice greffée octyle (ou octadécyle) cet ordre est inversé et l'acide ascorbique a un facteur de capacité inférieur à celui de l'acide isoascorbique, ceci est en accord avec les résultats de Finlay et Duang[12]. Cette propriété peut-être utilisée pour confirmer la présence de ces acides dans un mélange complexe.

La sensibilité de la méthode permet de déceler 0.01 μg d'acide ascorbique dissout dans de l'acide métaphosphorique à 1–2%, la réponse du détecteur reste linéaire pour des quantités injectées supérieurs à 2 μg. Le résultat est valable aussi bien pour l'acide ascorbique qu'isoascorbique car ils possèdent le même coefficient d'extinction moléculaire. Pour des chromatogrammes complexes, la sensibilité de la méthode est moindre, de plus l'acide deshydroascorbique n'est pas détecté à 264 nm et n'interfère donc pas.

Les Figs. 1–5 illustrent les séparations obtenues à partir de différents aliments par application de la méthode décrite ci-dessus.

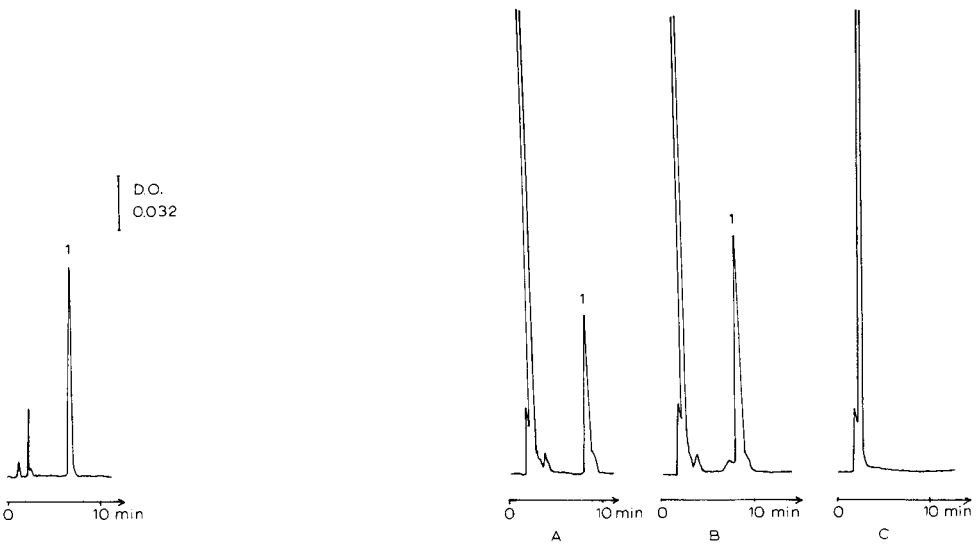

Fig. 4. Préparation multivitaminique en sirop. Colonne LiChrosorb RP-8. 5 μm (150 × 4.7 mm I.D.). Phase mobile: eau–méthanol (9:1, v/v)–0.1 *M* KH_2PO_4–$5 \cdot 10^{-3}$ *M* cétyltriméthylammonium. Débit: 1.5 cm^3 min^{-1}; U.V.: 264 nm. 1 = acide ascorbique annoncé 7.5 g l^{-1}, trouvé 7.7 g l^{-1}.

Fig. 5. Acide ascorbique dans un jambon. Colonne LiChrosorb RP-8, 5 μm (150 × 4.7 mm I.D.). Phase mobile: eau–méthanol (9:1, v/v)–0.1 *M* KH_2PO_4–$5 \cdot 10^{-3}$ *M*, cétyltriméthylammonium. Débit: 1 cm^3 min^{-1}; U.V.: 264 nm. 1 = acide ascorbique (40 mg kg^{-1} de jambon). A = Extrait de jambon; B = après ajout d'acide ascorbique; C = après addition de quelques gouttes d'une solution d'iode.

Cette méthode chromatographiques est utilisée dans notre laboratoire depuis plusieurs mois. La colonne subit un vieillissement qui se traduit par un accroissement de la perte de charge, un tassement de la phase stationnaire et une diminution des facteurs de capacité sans modification notable de la sélectivité. Ces phénomènes qui s'observent surtout avec les phases de 5 μm sont vraisemblablement dûs à une dissolution partielle de la phase stationnaire.

L'usage d'une précolonne garnie de silice, située entre la pompe et l'injecteur

(permettant de saturer l'éluant en silice[14]) ainsi que l'usage d'une phase stationnaire à granulométrie supérieure (7 μm), permettent d'allonger considérablement la durée de vie des colonnes.

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Note

Histamine analysis on a single column amino acid analyzer

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(Received June 23rd, 1981)

Histamine toxicity, also called scombroid food poisoning, is a well-recognized syndrome which appears after eating spoiled, histamine-containing tuna and related fish[1–5]. The associated symptoms include flushing of facial areas, dizziness, cramps and nausea. Histamine is formed from free histidine by action of histidine decarboxylase. Although histamine in very small amounts is a normal body constituent[6], ingestion of histamine-containing foods can be hazardous. For this reason, accurate, fast and inexpensive methods are needed for monitoring the histamine content of foods. Various procedures for measuring the histamine content of foods have been proposed[7–18]. These are summarized by Arnold and Brown[2]. They include methods based on fluorometry, colorimetry, gas, ion-exchange and thin-layer chromatography. A major disadvantage of most of these is that histamine has to be separated before analysis. Since single-column amino acid analyzers are now widely used, we examined the possibility of using standard amino acid analysis techniques to measure histamine along with the amino acids present in hydrolysates of casein with added histamine and in tuna. The results show possible advantages of this method.

EXPERIMENTAL*

Histamine · 2HCl was obtained from Sigma (St. Louis, MO, U.S.A.). Spoiled tuna was a gift from Professor L. F. Bjeldanes, Department of Nutritional Sciences, University of California at Berkeley. This sample has been implicated in an outbreak of scombroid poisoning[5].

Amino acid analyses

A weighed sample of Animal Nutrition Research Council (ANRC) casein (5 mg + 1.6 μmol/ml histamine) or tuna fish (50 mg) was hydrolyzed in 10 ml of 6 *N* HCl in a commercial hydrolysis tube. The tube was evacuated, placed in an acetone–dry ice bath, evacuated and flushed with nitrogen before being placed in an oven at 110°C for 24 h. The cooled hydrolysate was filtered through a disc funnel and evaporated to dryness at 40°C with the aid of an aspirator. The residue was suspended in

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water and evaporated to dryness. Amino acid analysis of an aliquot of soluble hydrolysate was carried out on a Durrum amino acid analyzer, Model D-500, under the following conditions: single-column ion exchange chromatography method; resin, Durrum DC-4A (bead diameter, 8 ± 1 μm) buffer pH, 3.25, 4.25 and 7.90; photometer, 440 and 590 nm; column, 40 cm × 1.75 mm I.D.; analysis time, 130 min. Norleucine was used as an internal standard[19].

The average histamine color factor per 10 nmol for five separate determinations with histamine concentrations ranging from 1.23–2.03 μmol/ml was 275,029 ± 1628, with a coefficient of variation of 0.592%. The color factor corresponds to a leucine equivalent (ratio) of 0.476.

Histamine in serum and urine

The procedures for sample preparation were adapted from Pickering[20]. To 1 ml of mouse serum in a centrifuge tube were added 50 mg of sulfosalicylic acid. The tube was shaken manually and centrifuged for 5 min. The supernate was filtered through a 0.45-μ Millipore filter membrane. Histamine was then added to the filtrate to a final concentration of 1.5 μmol/ml. The analysis on the amino acid analyzer was carried out with a 20-μl aliquot after a second filtration.

About 0.4 ml of mouse urine was acidified to pH 2 with 12 *N* HCl. The urine was shaken manually, centrifuged and filtered. Histamine was then added to the filtrate to a final concentration of 1.5 μmol/ml. The analysis on the amino acid analyzer was carried out with a 20-μl aliquot after a second filtration.

Fluorometric assay

The official Association of Official Analytical Chemists' (AOAC) method[21] was used: 10 g of spoiled tuna were extracted with 90 ml of methanol; the methanol extract was passed through an ion-exchange column to remove the free amino acids; the histamine in the eluate was derivatized to a fluorescent compound with *o*-phthalidicarboxaldehyde (OPT); and the derivative was assayed with a Perkin-Elmer Model 203 spectrophotometer.

RESULTS AND DISCUSSION

The available methods for determining histamine in foods usually require large samples. Special precautions are often needed to avoid interference. None of the available methods can be used to measure histamine along with the free and protein-derived amino acids in a single determination. An ideal method for histamine analysis with an amino acid analyzer should meet the following requirements: (a) the analysis should be carried out with the original sample without prior isolation of histamine by extraction or chromatographic separation; (b) histamine should be eluted in a convenient position as a separate peak; (c) the intensity of the peak should be linear with concentration; and (d) histamine should be stable to protein hydrolysis conditions.

Results in Figs. 1–3 show that assay for histamine along with amino acids can be carried out on single-column amino acid analyzer by extending the time from 95 to *ca.* 125 min. Histamine appears at *ca.* 120 min as a single, well-resolved peak, as determined with histamine added to a standard mixture of amino acids (Fig. 1), cohydrolyzed with casein (Fig. 2), and in a spoiled tuna hydrolysate (Fig. 3). The

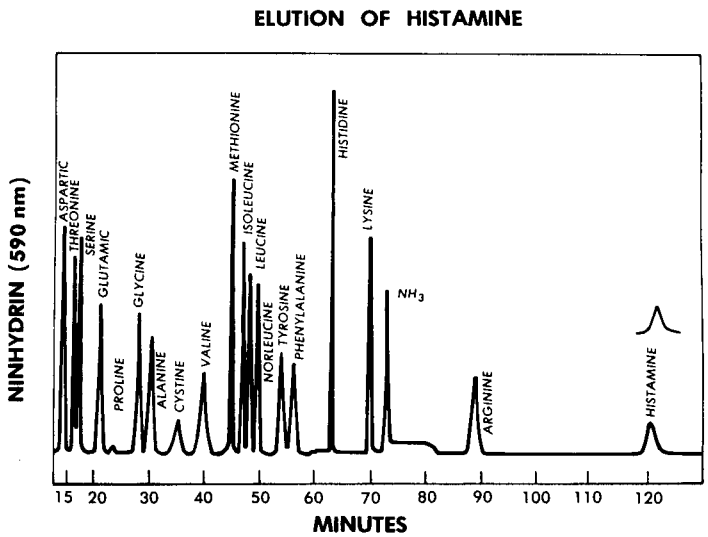

Fig. 1. Elution of histamine (0.86 μmol/ml) added to a standard mixture of amino acids. The 440 nm histamine peak is shown above the 590 nm peak.

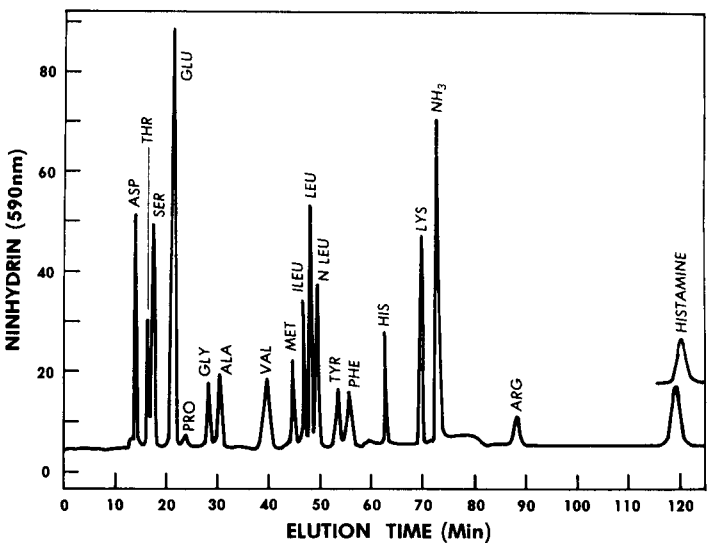

Fig. 2. Elution position of histamine cohydrolyzed with casein [5 mg casein + histamine (1.6 μmol/ml) hydrolyzed as described in the Experimental section].

calculated recovery of histamine from the cohydrolysate shown in Fig. 2 was 98.9%. This result shows that histamine is stable under acid conditions used for protein hydrolysis. The area of the histamine peak was proportional to concentration, as determined by separate analyses at five different concentrations (Fig. 4). The equation for the linear plot shown in Fig. 4 has a coefficient of determination (R^2) of 0.999 (n = 5). The lower limit of sensitivity of the analysis is estimated to be *ca.* 1 nmol of histamine on the column.

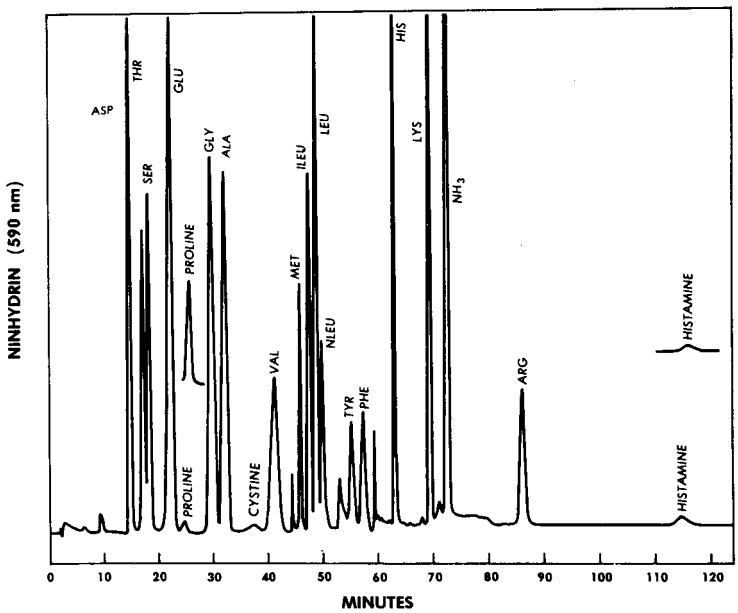

Fig. 3. Elution position of histamine in a standard amino acid hydrolysate of spoiled tuna.

Studies of the effects of varying buffer pH revealed that the elution time of histamine can be reduced to *ca.* 80 min by using a single, pH 7.9 citrate buffer as eluent instead of the three-buffer system described in the Experimental section (Fig. 5). This modification causes some of the amino acid peaks (but not histidine, which eluted at *ca.* 12 min) to coalesce on the chromatogram, permitting analysis of histamine but not most of the other amino acids.

The histamine value of spoiled tuna (154.0 mg/100 g) obtained by fluorescence analysis is somewhat lower than the value (165.7 mg/100 g) obtained by amino acid analysis of a tuna fish hydrolysate. The lower value could be due to an incomplete extraction of histamine in the AOAC method; inhomogeneity of histamine distribution in the tuna fish, especially in view of the fact that the AOAC method requires 10 g of tuna and the amino acid analysis procedure only 50 mg; or a combination of errors of both methods.

To assess the potential of the method for clinical and nutritional studies, the elution behaviour of histamine added to mouse serum and urine was also examined. Analyses of histamine added to mouse serum and urine show that components in the two physiological liquids do not affect the elution position of histamine on the short column.

The following biogenic amines (cadaverine, putrescine, tryptamine, tyramine, spermine and spermidine) do not seem to interfere with the histamine analysis since they were not found to elute between 0 and 160 min under the standard amino acid analysis conditions or with the pH 7.9 buffer.

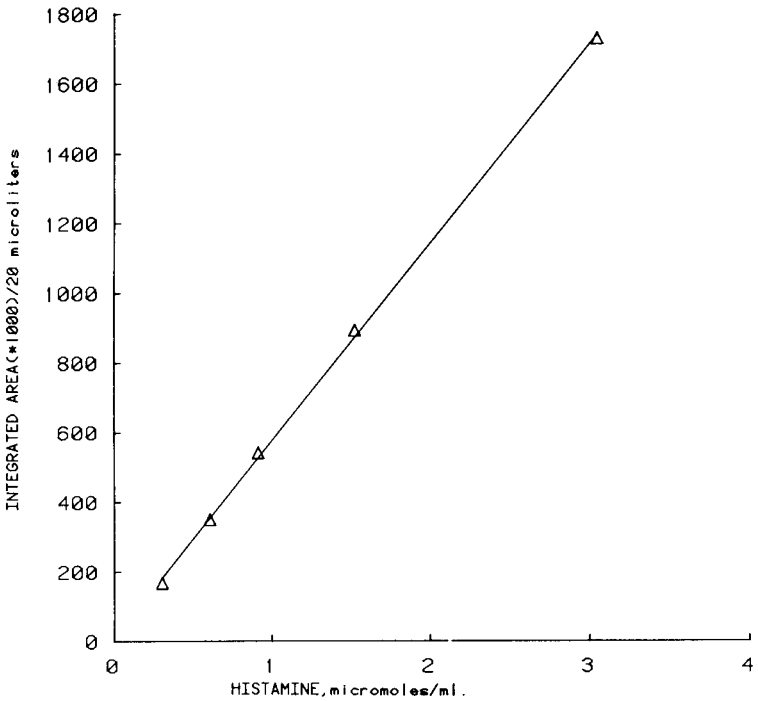

Fig. 4. Beer's law plot for histamine on an amino acid analyzer.

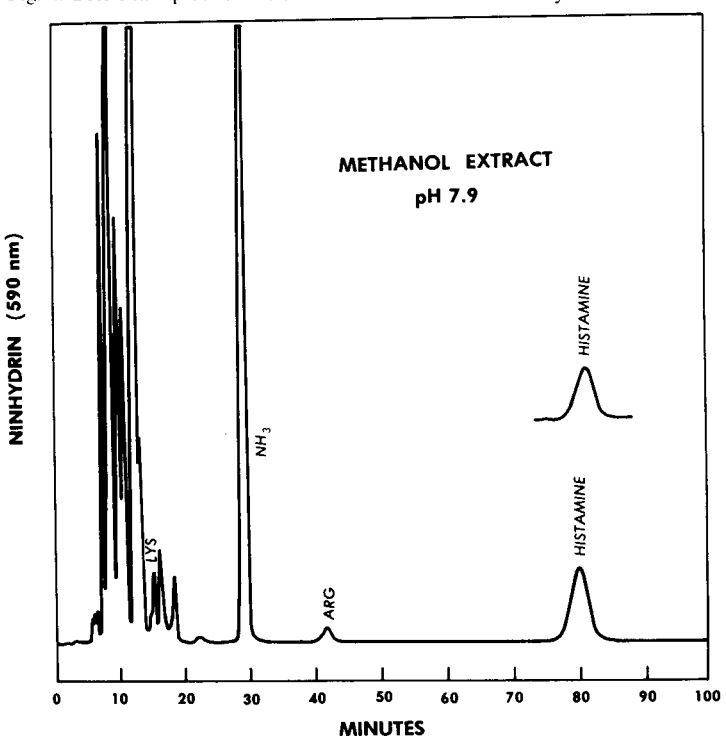

Fig. 5. Elution position of histamine at 80 min: 10 ml of the methanol extract were evaporated to dryness; the residue was dissolved in 1 ml of pH 2.2 buffer; 20 μl of this solution were applied to the column and eluted with pH 7.9 citrate buffer only.

CONCLUSIONS

The amino acid analysis method for histamine complements currently available procedures and may offer certain advantages. For instance, special extraction and purification techniques for histamine need not be applied since histamine can be measured in the presence of other amino acids. This may be important for comparing histamine to histidine (or to other amino acids) as a measure of food spoilage due to decarboxylation of histidine (or other amino acids). Application to nutritional and clinical studies designed to measure histamine along with free amino acids in body tissues and fluids should also be possible. Another advantage is that the analysis is automated, permitting after hydrolysis the sequential unattended assay of as many as 80 samples.

In summary, the described assay for histamine on a single-column amino acid analyzer complements available procedures and may have advantages in some applications. The method may be of special value for laboratories that do not have fluorometers.

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We thank Tina Porcuna of National Food Processors, Berkeley, CA, for assaying the histamine content of the spoiled tuna by the fluorometric method, and Dr. T. F. Schatzki for constructive contributions.

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Note

Thin-layer chromatographic analysis of aromatic hydrocarbons in crude oil and in petroleum products as their η^6-arene-η^5-cyclopentadienyliron hexafluorophosphates

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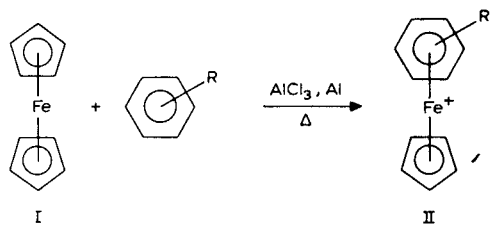
(Received July 6th, 1981)

The ligand-exchange reaction between benzene and substituted benzenes and ferrocene (I) generally leads to the formation of η^6-arene-η^5-cyclopentadienyliron cations (II)[1,2]:



I II

This reaction appears to be a general one for aromatics since many polycyclic aromatics[2] and polycyclic heteroaromatic systems[3–6] have been shown to undergo the ligand-exchange reaction. I have investigated the reaction as a possible novel method for the qualitative determination of aromatics, especially in petroleum[7]. This followed from a need to evaluate the types of aromatics present in a recent crude oil spillage in the delta areas of Nigeria. Subjecting petroleum samples to the ligand-exchange reaction led to a selective complexation of aromatics. Pyrolytic mass spectral analysis of the resulting complexes revealed a number of mono-, di- and tricyclic aromatic compounds. This analytical approach for aromatics in petroleum offers some advantages over conventional methods as it is insensitive to the presence of non-aromatic components and does not necessarily require prior fractionation of the sample.

I now report the results of an extension of this work involving the application of thin-layer chromatography (TLC) to the study of arene complexes from crude oil and some petroleum products.

EXPERIMENTAL

Ferrocene (Aldrich), aluminium chloride (BDH), aluminium powder (BDH), ammonium hexafluorophosphate (Alfa Products) and decahydronaphthalene (decalin) (Aldrich) were of reagent grade and were used as received. *n*-Hexane, cyclo-

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hexane, petroleum ether (b.p. 40–60°C), diethyl ether, dichloromethane and acetone were distilled before use. The petroleum samples and arenes were dried over a mixture of anhydrous calcium chloride and magnesium sulphate before each ligand exchange reaction. Authentic samples of η^6-arene-η^5-cyclopentadienyliron hexafluorophosphates for benzene[1], toluene, *ortho*-, *meta*- and *para*-xylenes[8], naphthalene[9], 1-methylnaphthalene (as for 2-methylnaphthalene)[10], phenanthrene[11], dibenzothiophene[3] and fluorene[12] were prepared as described in the literature. Thin-layer chromatography was performed on Selecta F1500 silica gel plates (Schleicher & Schüll).

Preparation of arene complexes from petroleum samples

Arene complexes were prepared from crude oil samples BSAP-W6, BKKRF-1, BKKRF-2, RKC-2, RBMF-2, ROBR-4T, ROBF-12T and IREGB-1 and from petroleum products, Super and 5-star petrol, diesel fuel and kerosene, following the procedure described previously[7].

Preparation of arene complexes of mixtures of arenes

Mixture A was prepared from ferrocene (9.3 g, 50 mmol), $AlCl_3$ (13.3 g, 100 mmol), Al (1.35 g, 50 mmol), *p*-xylene (5.3 g, 50 mmol), naphthalene (6.4 g, 50 mmol) and fluorene (8.3 g, 50 mmol) in decalin (80 ml) under reflux at 135°C for 5 h. The reaction mixture was worked up as previously described[7] to give yellow crystals (5 g).

Mixture B was prepared from ferrocene (6.5 g, 35 mmol), $AlCl_3$ (9.3 g, 70 mmol), Al powder (0.95 g, 35 mmol), benzene (2.7 g, 35 mmol), toluene (3.2 g, 35 mmol), *p*-xylene (3.7 g, 35 mmol), naphthalene (4.5 g, 35 mmol), 1-methylnaphthalene (4.9 g, 35 mmol), phenanthrene (6.2 g, 35 mmol), dibenzothiophene (6.4 g, 35 mmol) and fluorene (5.8 g, 35 mmol) in refluxing cyclohexane (80 ml) for 20 h. A brown crystalline solid (3 g) was obtained on working up the reaction mixture[7].

TLC of the arene complexes

The complexes dissolved in acetone were spotted on the plates and developed in acetone–dichloromethane (1:4, v/v). The spots were located with iodine vapour.

RESULTS AND DISCUSSION

Thin-layer chromatography of authentic samples of the individual arene complexes gave the following R_F values: benzene (0.29), *p*-toluene (0.43), *p*-xylene (0.56), naphthalene (0.60), dibenzothiophene (0.61), 1-methylnaphthalene (0.67), phenanthrene (0.68) and fluorene (0.73). Good separations were obtained for mixtures containing authentic arene complexes of benzene, toluene, *o*-, *m*- or *p*-xylene, naphthalene and any of dibenzothiophene, 1-methylnaphthalene, phenanthrene or fluorene; however, mixtures of the last four arene complexes could not be separated owing to extensive overlapping of the bands.

The scope of the applicability of this method to the analysis of mixtures of arenes was evaluated by subjecting a mixture of benzene, toluene, *p*-xylene, naphthalene and fluorene to the ligand exchange reaction. The resulting mixed complexes (mixture A) were analysed by TLC and the chromatogram found to be identical with that of a mixture of authentic samples of the respective arene complexes. When the complexes (mixture B) from a similar mixture containing benzene, toluene, *p*-xylene,

naphthalene, dibenzothiophene, 1-methylnaphthalene, phenanthrene and fluorene were analysed, a separation pattern identical to that observed for a mixture of the respective authentic arene complexes was obtained.

Thin-layer chromatograms of the crude oil samples and petroleum products indicated the presence of benzene (except in kerosene), toluene, xylene and naphthalene and some poorly resolved bands with R_F 0.90–0.93 in all the samples. These unresolved bands are strongly suspected to be due to polyaromatics, as judged from the TLC results which showed the R_F values for the complexes to be in order: fluorene > phenanthrene ⩾ 1-methylnaphthalene > dibenzothiophene ⩾ naphthalene > xylene > toluene > benzene. Moreover, a mass spectral analysis of the arene complexes from some crude oil samples[7] has identified some polyaromatics in addition to the aromatics identified in this study.

These results suggest that this analytical technique could be utilized for the analysis of monocyclic aromatics and naphthalenes and for the separation of monocyclic aromatics from polycyclic aromatic ring systems. It could also complement analytical procedures employing charge-transfer complex formation, for example, with 2,4,7-trinitrofluorenone[13], which are more sensitive to di-, tri- and other polyaromatics. The violet band obtained for the benzene complex after iodine treatment of the chromatoplate is characteristic, easily recognizable and well-separated from other bands. This method could therefore serve as a reliable means for the identification of benzene in mixtures of arenes.

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Book Review

Analytiker Taschenbuch, Band 2, edited by R. Bock, W. Fresenius, H. Günzler, W. Huber and G. Tölg, Springer, Berlin, Heidelberg, New York, 1981, VI + 351 pp., 50 figs., 85 tables, price DM 78.00, *ca.* US$ 37.20, ISBN 3-540-10338-4.

This handbook contains three excellent chapters on chromatographic techniques.

Dr. W. Brümmer has written a 33-page introduction to affinity chromatography which is readable and well balanced. It is a pity that he lists only general references and does not give the references to the work discussed by him.

There is an equally good chapter by Professor H. Engelhardt and G. M. Ahr explaining high-performance liquid chromatography (21 pp.). Although the latest papers and books quoted are from 1979, some information is already slightly outdated, for example, that relating to preparative separations or plate numbers obtainable. This is no fault of the authors, but due to the fact that the field is still progressing quickly.

Dr. G. Schwedt deals with gas chromatographic separations and determinations of traces of inorganic substances. This chapter consists mainly of tables and is more of the condensed information type that one would expect in an analysts' handbook. However, he covers these topics very ably and very thoroughly.

Lausanne (Switzerland) M. JANOVSKY

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Gas Chromatography

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21. PURINES, PYRIMIDINES, NUCLEIC ACIDS AND THEIR CONSTITUENTS

21a. Purines, pyrimidines, nucleosides, nucleotides

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See also 6547, 6556, 6600.

23. OTHER SUBSTANCES CONTAINING HETEROCYCLIC NITROGEN

23a. Porphyrins and other pyrroles

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19a. General techniques

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Thin-Layer Chromatography

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BIBLIOGRAPHY SECTION

SUPPLEMENT TO THE
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INDEXES

INTRODUCTION

As in previous years we present here the Subject Index and the Index of Types of Compounds Chromatographed. Because the methodological part differs substantially in individual techniques, we have retained the subdivision system, using the following abbreviations: G = gas chromatography, C = liquid column chromatography, E = electrophoresis, P = paper chromatography, and T = thin-layer chromatography. In the Index of Types of Compounds Chromatographed all types of methods are indicated in the individual entries by appropriate abbreviations. In entries that are heavily populated by chromatographic papers we made a further subdivision into Techniques and Applications. Reviews are always referred to separately. In the Subject Index, materials and procedures in common use are not quoted as special entries.

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Brno (Czechoslovakia) J. JANÁK

Subject Index

Please note that this index refers to the entry numbers in the Bibliography Section. The entries have been published according to the following schedule:

Entries	*Vol./No.*	*Entries*	*Vol./No.*
1-1808	207/2	3861-5769	214/2
1809-3860	211/1	5770-7217	219/2

Gas Chromatography

Liquid Column Chromatography

Paper and Thin-Layer Chromatography

Electrophoresis

Index of Types of Compounds Chromatographed

In this Index identical rules as in the Bibliography books (Supplementary volumes of the *Journal of Chromatography*) were used, e.g. references of general interest and techniques are listed first, followed by application and finally by papers limited to a certain area of applications only.

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PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	212/3 213/1 213/2	213/3 214/1 214/2	214/3 215 216	217 218 219/1	219/2 219/3
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Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2	224/3	225/1	225/2	226/1	226/2

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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Ionic Hydration in Chemistry and Biophysics

by B. E. CONWAY,
Department of Chemistry, University of Ottawa, Ontario, Canada.

STUDIES IN PHYSICAL AND THEORETICAL CHEMISTRY 12

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This monograph is a comprehensive coverage of all aspects of ionic hydration. Not only is the nature of the phenomenon described, but also its importance in electrochemistry, inorganic and physical chemistry, biophysics and biochemistry. The basic background of techniques and approaches (e.g. electrostatic theory) required for the study and interpretation of ionic hydration in chemistry is included.

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