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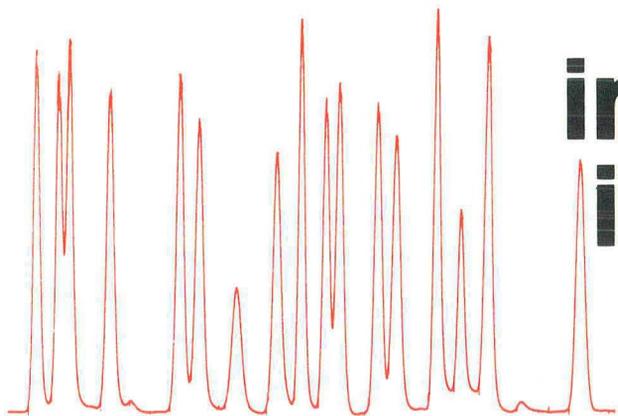
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QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIPS FOR HERBICIDES

REVERSED-PHASE LIQUID CHROMATOGRAPHIC RETENTION PARAMETER, log k_w, *versus* LIQUID–LIQUID PARTITION COEFFICIENT AS A MODEL OF THE HYDROPHOBICITY OF PHENYLUREAS, *s*-TRIAZINES AND PHENOXYCARBONIC ACID DERIVATIVES

THOMAS BRAUMANN*, GERT WEBER and L. HORST GRIMME

Universität Bremen, Fachbereich Biologie/Chemie, Postfach 330440, D-2800 Bremen 33 (G.F.R.)

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SUMMARY

The retention behaviour of phenylureas, *s*-triazines and phenoxycarbonic acid derivatives in a reversed-phase high-performance liquid chromatographie (RPLC) system has been examined. Using methanol–water or acetonitrile–water as the mobile phase, a linear relationship between the volume fraction of the organic modifier, φ, and the logarithm of the capacity factor, log k', is established for each solute. The different correlation curves for each compound indicate selective effects upon retention due to solute–solvent and solute–stationary phase interactions. It is shown that log k_w, a theoretical capacity factor obtained by extrapolation of retention data in binary solvent systems to pure aqueous eluent, is suitable for eliminating the selective effects and thereby for quantitatively describing the hydrophobic nature of solutes in a way which is strongly related to the partition coefficient, log P, of the standard *n*-octanol–water partitioning system. The dependence of log k_w on the nature of the organic modifier and an analysis of functional group behaviour in different eluents reveal that log P and log k_w are not completely interchangeable, because certain substituents, *i.e.*, methylthio and trifluoromethyl groups, behave differently in RPLC and a true liquid–liquid partitioning system. The consequences of this non-polar group selectivity in RPLC on the quality of quantitative structure–activity relationships for electron transport-inhibiting herbicides are demonstrated. The results suggest that log k_w might be a better model for the assessment of the hydrophobicity of drugs in biological systems.

INTRODUCTION

The aim of studies on quantitative structure–activity relationships (QSARs), introduced by Hansch and co-workers[1,2], is the correlation of biological activity with chemical structure for a congeneric series of compounds. Most of these studies, in-

cluding those with herbicides[3-5], have shown that the inhibitory action of drugs is predominantly a function of their hydrophobic nature. The use of partition coefficients, P, obtained from an n-octanol–water partitioning system has become a standard method[2] for modelling biological membranes and thereby quantifying the hydrophobicity of a given compound as: $\log P = \log C_{\mathrm{oct}} - \log C_{\mathrm{water}}$

Log P is either determined experimentally or calculated[2,6]. The conventional shaking flask method is laborious and time.consuming, often complicated by instability in aqueous media, impurities and the tendency for a compound to dissociate. Furthermore, this procedure only has a limited application range up to $\log P = 4$[6]. Calculation of log P using known Hansch π values is often only successful when the molecular framework of a given series of compounds exhibits roughly the same physico-chemical properties as those from which the π values were derived. Thus, there are innumerable compounds for which log P values have to be determined.

Alternatively, chromatographic techniques can be used for the determination of the hydrophobic nature of drugs, especially thin-layer chromatography (TLC)[7] and reversed-phase high-performance liquid chromatography (RPLC) (see refs. 8–10 for extensive references). The latter technique has been shown to produce very efficiently high-precision data with respect to retention which are believed to be a measure of the partition behaviour between the non-polar bonded stationary phase and the more polar eluent. The capacity factor, k', is given by

$$k' = (t_R - t_0)/t_0 \tag{1}$$

where t_R and t_0 are the retention times of a retained and an unretained solute, respectively. k' is directly related to the Gibbs free energy attributed to the retention process, ΔG_0, according to

$$\log k' = \log \varphi - \Delta G_0/2.3\ RT \tag{2}$$

where R, T and φ are the gas constant, temperature and phase ratio of mobile and stationary phases, respectively. Log k' is therefore equivalent to log P and can also be used to obtain extrathermodynamic substituent constants[8,11].

It has been shown[10] that within a group of compounds of comparable size, shape and polarity, good correlations between log k' and log P are observed. However, with octadecyl-silica as the stationary phase and water–organic mixtures as eluents, polar group selectivity[12] and non-polar group selectivity[10,13] appear which depend both on solute structure and mobile phase composition. To eliminate these selective effects, we have introduced log k_{w} as a measure of hydrophobicity in RPLC and as a better analogue to log P[10]. This approach is equivalent to the R_{m}^0 values, introduced for TLC[14], and has recently found further application in studies of the hydrophobic nature of phenols[15], the prediction of solubility in water[16] and in ion-pair RPLC[17].

In this study, we investigate in detail the relation between the reversed-phase retention behaviour and bulk liquid–liquid partition coefficients of several economically important herbicidal groups. The results indicate that log k_{w} can be used instead of log P as a hydrophobicity parameter for phenylureas and phenoxycarbonic acid derivatives. However, the two parameters are not completely interchangeable because

specific substituents behave differently in RPLC and a true liquid–liquid partitioning system. In the case of *s*-triazines, only RPLC provides hydrophobicity parameters which are strongly related to the inhibitory action of these compounds on photosynthetic electron transport.

EXPERIMENTAL

Materials

     All herbicides obtained from Riedel-de Haen (Hannover, G.F.R.) were of the highest purity available. In Table I are collected the common and chemical names of the compounds. Distilled water was prepared with an all-glass double distillation unit (Heraeus-Schott, Mainz, G.F.R.). All other reagents were of analytical reagent grade and used without further treatment. The column (25 cm × 4.6 mm I.D.) (E. Merck, Darmstadt, G.F.R.) was self-packed by the slurry technique using tetrachloroethylene as the suspending medium. The suspension was introduced into the column

TABLE I

COMMON NAMES AND CHEMICAL NAMES OF THE HERBICIDAL COMPOUNDS

Common name	Chemical name
Phenylureas	
Chloroxuron	3-[4-(4-Chlorophenoxy)phenyl]-1,1-dimethylurea
Chlortoluron	3-(3-Chloro-*p*-tolyl)-1,1-dimethylurea
Diuron	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
Fenuron	1,1-Dimethyl-3-phenylurea
Linuron	3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea
Metobromuron	3-(4-Bromophenyl)-1-methoxy-1-methylurea
Metoxuron	3-(3-Chloro-4-methoxyphenyl)-1,1-dimethylurea
Monolinuron	3-(4-Chlorophenyl)-1-methoxy-1-methylurea
Monuron	3-(4-Chlorophenyl)-1,1-dimethylurea
Neburon	1-Butyl-3-(3,4-dichlorophenyl)-1-methylurea
s-Triazines	
Atrazine	2-Chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine
Propazine	2-Chloro-4,6-di(isopropylamino)-1,3,5-triazine
Simazine	2-Chloro-4,6-di(ethylamino)-1,3,5-triazine
Prometryn	2,4-Di(isopropylamino)-6-methylthio-1,3,5-triazine
Desmetryn	2-Isopropylamino-4-methylamino-6-methylthio-1,3,5-triazine
Terbutryn	2-*tert.*-Butylamino-4-ethylamino-6-methylthio-1,3,5-triazine
Phenoxycarbonic acids	
2,4-D	2,4-Dichlorophenoxyacetic acid
Dichlorprop	2-(2,4-Dichlorophenoxy)propionic acid
Fenoprop	2-(2,4,5-Trichlorophenoxy)propionic acid
MCPA	4-Chloro-2-methylphenoxyacetic acid
MCPB	4-(4-Chloro-2-methylphenoxy)butyric acid
Mecoprop	2-(4-Chloro-2-methylphenoxy)propionic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
Phenoxycarbonic acid methyl esters	
All methyl esters of phenoxycarbonic acids listed above	

with the aid of a Haskel pump at 500 bar. The stationary phase was 10-μm Li-Chrosorb RP-18 (Merck, batch No. VV 1106). The column was used without further treatment in all experiments.

Chromatography

The liquid chromatograph consisted of a Series 2/2 reciprocating pump (Perkin-Elmer, Norwalk, CT, U.S.A.), a Model LC-55 variable wavelength UV–visible detector (Perkin-Elmer) and a Servogor Model S pen recorder (Metrawatt, Nürnberg, G.F.R.). The detector was set at 230 nm (*s*-triazines), 250 nm (phenylureas) and 280 nm (phenoxycarbonic acid derivatives).

A volume of 5 μl of a $2.5 \cdot 10^{-4}$ M sample solution was injected by means of a 10-μl precision syringe via a Rheodyne Model 7105 sample valve (Rheodyne, Berkeley, CA, U.S.A.), and the retention times were measured with a stop-watch. The reproducibility of the retention times was better than 1 %, so that in all experiments two independent runs were carried out.

The mobile phase consisted of different volume fractions of methanol and acetonitrile in water, respectively, prepared with the gradient former of the chromatograph. Instead of water, the phenoxycarbonic acids were analysed in a 0.5 M acetate buffer (pH 2.9) as the aqueous phase. The flow-rate was 1.7 ml/min at room temperature. The column dead time was determined by the injection of acetone dissolved in methanol or acetonitrile with 100 % methanol or acetonitrile, respectively, as the mobile phase. We have checked the reliability of t_0 obtained by this procedure by comparison with values derived from the linearization of the net retention time for homologous alkylbenzenes[18]. The t_0 values were identical within the experimental error. The standard error of log k' determinations was less than ± 0.005.

Measurement of the Hill reaction

Broken spinach chloroplasts were prepared according to a standard procedure[19]. The reaction medium contained 7 mM phosphate buffer (pH 6.4), 0.25 M sucrose, 0.1 mM dichlorophenolindophenol and 50 μg chlorophyll per ml and was illuminated for 45 sec with 50,000 lux by means of a projector lamp. The decrease in absorption was measured at 590 nm. At least four different herbicide concentrations were used for the determination of pI_{50}, the logarithm of the concentration of a herbicide which causes 50 % inhibition of the Hill reaction.

RESULTS AND DISCUSSION

When the reversed-phase packing of octadecyl groups attached to a silica surface does not contain unreacted silanol groups which contribute to the retention of a solute, ligand–solute interaction will be weak and non-selective[20]. Genieser *et al.*[21] estimated the ligand content of the stationary phase used in this study and found a very high surface coverage of about 22 %. Furthermore, determination of the dead volume with acetone gave the same values as derived from the linearization of the net retention time for homologous alkylbenzenes, which is very unlikely in the case of free silanol groups. It is therefore reasonable to assume that there are no accessible hydroxyl groups to contribute to retention. According to the solvophobic theory[20,22], retention will then be a function of solution behaviour in the mobile phase.

Variation of the capacity factor with mobile phase composition

The analytical tool to assess selective effects of specific molecular structures is the variation of the k' of the sample with the organic modifier content of the mobile phase[23]:

$$\log k' = \log k_w + S\varphi \tag{3}$$

where φ is the volume fraction of organic solvent in the water–organic solvent mixture, k_w represents the capacity factor of a solute with pure water as mobile phase (usually obtained by extrapolation to the intercept of the ordinate) and S, the slope of the regression curve, should be related to the solvent strength of the pure organic solvent[23]. Table II shows the retention data of herbicides at different volume fractions of methanol, φ_M, in water. Table III collects the data from regression analysis of the relation between $\log k'$ and φ_M, and includes the partition coefficients, $\log P$.

From Table III, it is seen that eqn. 3 describes the variation of sample k' with φ_M. The proposal of Snyder *et al.*[23] that S depends only on the solvent strength of the pure organic modifier and should therefore be constant for different kinds of solutes is obviously not correct. Values of S can vary over the range -3.0 to -6.0, which indicates that a given increment in organic modifier concentration causes large differences in retention. The same behaviour has been found for benzene derivatives[16] and for ion-pair RPLC of benzenoic acids[17]. Within the different groups of herbicides, functional group selectivity clearly exists, as indicated by a large variation of the free energy change of the sorption process with a given change in φ_M of the mobile phase. The capacity factor, k', is therefore not a good parameter to describe the hydrophobic nature of a solute since compounds with the same k' at a given φ_M do not necessarily exhibit the same retention mechanism due to different values of S. We have therefore suggested[10] that $\log k_w$ is a better measure of the hydrophobicity of a solute because selective effects are eliminated owing to the extrapolation to $\varphi_M = 0$. That S as well as $\log k_w$ is largely dependent on the hydrophobic surface area of the solute is demonstrated by the close correlation of these variables:

$$S = -0.719 \log k_w - 1.980 \ (n = 30, r = 0.952) \tag{4}$$

The fact that the herbicides belong to quite different chemical classes essentially does not influence the relationship between S and $\log k_w$. However, the different herbicidal groups show a small but significant deviation from the overall relation of eqn. 8 (Table IV).

If S and $\log k_w$ depend on the same properties of the solute, the slope of the regression curve will be -1.0. The smallest deviation from -1.0 is found for the *s*-triazines which possess only hydrophobic substituents symmetrically arranged around the heterocyclus. It is therefore reasonable to assume that the solvophobic effect is the dominant factor controlling retention. The phenoxycarbonic acids on the other hand are partly ionized at the pH of the mobile phase so that also electronic contributions play a considerable rôle with a concomitant by low slope of -0.458. The other herbicidal groups contain both hydrophilic and hydrophobic substituents and show a behaviour between the two extremes.

TABLE II

ISOCRATIC $\log k'$ VALUES OF HERBICIDES FOR DIFFERENT VOLUME FRACTIONS OF METHANOL, φ_M, IN WATER

M = Methyl ester.

Common name	Volume fraction, φ_M								
	0.90	0.85	0.80	0.75	0.70	0.65	0.60	0.55	0.50
Fenuron					-0.699	-0.538	-0.347	-0.184	0.036
Metoxuron				-0.734	-0.459	-0.292	-0.131	0.064	0.238
Monuron			-0.620	-0.420	-0.244	-0.060	0.100	0.279	0.459
Monolinuron			-0.456	-0.292	-0.102	0.076	0.253	0.446	0.637
Chlortoluron			-0.398	-0.299	-0.027	0.158	0.344	0.543	0.743
Metobromuron			-0.377	-0.215	-0.022	0.158	0.344	0.544	0.743
Diuron			-0.292	-0.102	0.090	0.276	0.476	0.686	
Linuron			-0.187	0.017	0.207	0.410	0.618	0.840	
Chloroxuron			-0.097	0.117	0.352	0.583	0.829		
Neburon			0.021	0.262	0.496	0.725	1.010		
Simazine	-0.796	-0.638	-0.469	-0.284					
Atrazine	-0.699	-0.509	-0.310	-0.125					
Propazine	-0.585	-0.377	-0.180	0.053					
Prometryn	-0.252	0.009	0.283	0.581					
Desmetryn	-0.208	0.061	0.336	0.619					
Terbutryn	-0.131	0.140	0.430	0.758					
2,4-D					-0.509	-0.337	-0.180	-0.018	0.127
MCPA					-0.420	-0.252	-0.092	0.083	0.230
2,4,5-T					-0.276	-0.097	0.072	0.233	0.407
Dichlorprop					-0.260	-0.092	0.076	0.248	0.394
Mecoprop					-0.194	-0.018	0.155	0.322	0.483
Fenoprop					-0.066	0.117	0.301	0.483	0.650
MCPB					0.127	0.292	0.484	0.642	0.816
2,4-D-M	-0.523	-0.301	-0.102	0.086	0.312				
MCPA-M	-0.481	-0.268	-0.060	0.137	0.360				
Dichlorprop-M	-0.377	-0.161	0.053	0.265	0.502				
Mecoprop-M	-0.357	-0.143	0.072	0.286	0.529				
2,4,5-T-M	-0.310	-0.092	0.117	0.330	0.569				
MCPB-M	-0.180	0.041	0.260	0.502	0.753				
Fenoprop-M	-0.187	0.053	0.274	0.509	0.770				

TABLE III

REGRESSION ANALYSIS OF THE RELATIONSHIP BETWEEN THE VOLUME FRACTION OF METHANOL, φ_M, AND log k': log $k' = \log k_w - S\varphi_M$

Partition coefficients, log P, were calculated with π values given by Norrington et al.[24]. Diuron (log P = 2.68) was the reference for the dimethylureas, linuron (log P = 2.76) for the methylmethoxyureas. Log P values of s-triazines were calculated with simazine (log P = 1.51) as the standard compound. The log P values of the reference compounds were taken from ref. 25. Log P values of the phenoxycarbonic acids and their methyl esters were calculated with acetic acid (log P = −0.24), propionic acid (log P = 0.29) and butyric acid (log P = 0.70) as the parent compound, respectively. M = Methyl ester.

Common name	log k_w	− S	r	log P
Fenuron	1.838	3.642	0.9984	1.18
Metoxuron	2.185	3.859	0.9977	1.98
Monuron	2.239	3.556	0.9997	1.91
Monolinuron	2.453	3.650	0.9998	1.99
Chlortoluron	2.640	3.813	0.9998	2.55
Metobromuron	2.603	3.746	0.9996	2.37
Diuron	2.816	3.891	0.9998	2.68
Linuron	3.072	4.081	0.9997	2.76
Chloroxuron	3.602	4.636	0.9997	3.65
Neburon	3.920	4.882	0.9992	4.31
Simazine	2.267	3.410	0.9994	1.51
Atrazine	2.759	3.842	0.9999	2.05
Propazine	3.211	4.222	0.9994	2.59
Prometryn	4.731	5.546	0.9995	1.91
Desmetryn	4.749	5.512	0.9999	2.46
Terbutryn	5.178	5.914	0.9991	2.56
2,4-D	1.726	3.182	0.9996	2.22
MCPA	1.872	3.270	0.9997	2.30
2,4,5-T	2.103	3.392	0.9998	2.99
Dichlorprop	2.051	3.296	0.9996	2.75
Mecoprop	2.182	3.388	0.9998	2.83
Fenoprop	2.498	3.662	0.9999	3.52
MCPB	2.546	3.456	0.9996	3.53
2,4-D-M	3.186	4.114	0.9995	2.64
MCPA-M	3.277	4.174	0.9998	2.72
Dichlorprop-M	3.551	4.368	0.9998	3.17
Mecoprop-M	3.599	4.402	0.9997	3.25
2,4,5-T-M	3.611	4.360	0.9997	3.41
MCPB-M	3.998	4.654	0.9995	3.95
Fenoprop-M	4.076	4.740	0.9996	3.94

Influence of the nature of the organic modifier on log k_w

Log k_w was defined before as the capacity factor with pure water as the mobile phase and should therefore be independent of the nature of the organic modifier. Additionally, log k_w should be an intrinsic property of the solute, indicating the non-polar surface area[17,26]. In order to verify these assumptions, we have estimated the k' values of the phenylurea herbicides with acetonitrile–water mixtures as the mobile phase. Table V collects the resulting values for S and log k_w and compares them to those obtained from the methanol–water system.

TABLE IV

REGRESSION ANALYSIS OF THE RELATIONSHIP $S = a \log k_w + b$

Herbicides	$-a$	$-b$	n	r
s-Triazines	0.857	1.471	6	0.999
Phenylureas	0.643	2.219	10	0.932
Phenoxyacetic acid methyl esters	0.688	1.916	7	0.996
Phenoxyacetic acids	0.458	2.398	7	0.893
Pyridazinones*	0.792	2.096	8	0.969

* Data taken from refs. 10.

Again, we find a linear relationship between the capacity factor and the volume fraction of acetonitrile, φ_A, in water. However, $\log k_{w(A)}$ and $S_{(A)}$ are quite different from $\log k_{w(M)}$ and $S_{(M)}$. A regression analysis yields:

$$\log k_{w(M)} = 1.435 \log k_{w(A)} + 0.279 \ (n = 10, r = 0.939) \tag{5}$$

The moderate correlation coefficient indicates that $\log k_w$ indeed reflects basically the same molecular properties of the solute in both solvents, but these properties contribute differently to retention. This is also seen in the poor correlation of $S_{(A)}$ to $\log k_{w(A)}$ in contrast to the good correlation of $S_{(M)}$ to $\log k_{w(M)}$:

$$S_{(A)} = -0.203 \log k_{w(A)} - 2.749 \ (n = 10, r = 0.410) \tag{6}$$

Most probably, these differences arise from the chemical nature of the two organic modifiers. Methanol exhibits both hydrogen donor and acceptor abilities and will therefore easily be incorporated into the network of water molecules, whereas aceto-

TABLE V

REGRESSION ANALYSIS OF THE RELATIONSHIP $\log k' = \log k_{w(A)} - S_{(A)}\varphi_A$

The corresponding values of $S_{(M)}$ and $\log k_{w(M)}$ are included.

Common name	Acetonitrile				Methanol	
	$\log k_{w(A)}$	$-S_{(A)}$	n	r	$\log k_{w(M)}$	$-S_{(M)}$
Fenuron	0.910	2.902	7	0.9995	1.838	3.642
Metoxuron	1.493	3.518	7	0.9975	2.185	3.859
Monuron	1.428	3.086	7	0.9984	2.239	3.556
Monolinuron	1.732	3.035	7	0.9989	2.453	3.650
Chlortoluron	1.524	2.837	7	0.9981	2.640	3.813
Metobromuron	1.789	3.024	7	0.9997	2.603	3.746
Diuron	1.673	2.929	7	0.9979	2.816	3.891
Linuron	2.025	3.064	7	0.9985	3.072	4.081
Chloroxuron	2.132	3.217	7	0.9985	3.602	4.636
Neburon	2.427	3.302	7	0.9979	3.920	4.882

TABLE VI

REGRESSION ANALYSIS OF THE RELATIONSHIP BETWEEN THE VOLUME FRACTION OF DIF-
FERENT ORGANIC MODIFIERS AND log k'

Retention data were taken from the compilation of ref. 27. Included are measured log P values, mostly published by
the Hansch group[1,2].

Compound	Methanol		Acetonitrile		Tetrahydrofuran		log P
	$-S_{(M)}$	log $k_{w(M)}$	$-S_{(A)}$	log $k_{w(A)}$	$-S_{(T)}$	log $k_{w(T)}$	
Acetophenone	2.73	1.92	2.28	1.42	2.71	1.21	1.66
Aniline	2.06	1.21	–	–	2.62	1.27	1.10
Anisole	2.66	2.15	2.62	1.86	3.21	1.81	2.11
Benzaldehyde	2.65	1.80	2.22	1.36	2.62	1.20	1.48
Benzene	2.56	2.16	2.57	1.86	3.08	1.85	2.13
Benzonitrile	2.63	1.77	2.44	1.54	3.01	1.45	1.56
Benzophenone	3.72	3.15	2.99	2.34	3.96	2.25	3.18
Benzyl alcohol	2.52	1.47	1.86	0.80	2.65	0.93	1.10
Biphenyl	4.23	3.89	3.25	2.88	3.99	2.54	4.02
n-Butylbenzene	4.54	4.32	3.34	3.03	–	–	4.26
Chlorobenzene	3.27	2.80	3.01	2.33	3.81	2.33	2.81
p-Chlorophenol	3.00	2.15	2.79	1.67	–	–	2.39
p-Chlorotoluene	3.76	3.37	3.01	2.50	–	–	3.33
o-Cresol	2.65	1.81	2.51	1.46	3.61	1.88	1.96
o-Dichlorobenzene	3.62	3.26	2.93	2.44	–	–	3.38
Diethyl phthalate	3.70	2.90	3.22	2.30	3.52	1.80	3.15
2,4-Dimethylphenol	3.09	2.31	2.74	1.77	3.94	2.15	2.30
Dimethyl phthalate	3.22	2.21	2.61	1.63	3.01	1.23	2.11
m-Dinitrobenzene	2.62	1.91	2.87	1.86	3.68	2.06	1.49
o-Dinitrobenzene	2.90	2.02	3.07	1.97	–	–	1.58
p-Dinitrobenzene	2.62	1.81	2.91	1.89	–	–	1.46
2,4-Dinitrotoluene	3.00	2.37	3.21	2.21	–	–	1.98
Diphenyl ether	4.34	3.91	3.52	2.91	4.00	2.54	4.20
Ethylbenzene	3.52	3.18	3.37	2.64	3.57	2.33	3.15
m-Fluoronitrobenzene	2.73	2.13	2.80	1.93	–	–	1.99
p-Fluoronitrobenzene	2.73	2.01	2.78	1.87	–	–	1.99
p-Fluorophenol	2.70	1.62	2.50	1.28	–	–	1.77
p-Hydroxybenzaldehyde	2.59	1.38	2.25	0.84	–	–	1.35
p-Methoxybenzaldehyde	2.79	1.97	2.35	1.42	–	–	1.68
p-Methylbenzaldehyde	2.92	2.15	2.54	1.73	–	–	2.04
Methyl benzoate	2.87	2.28	2.61	1.82	–	–	2.12
Naphthalene	3.58	3.22	3.01	2.46	4.13	2.54	3.37
p-Nitroacetophenone	2.77	1.95	2.65	1.68	3.28	1.68	1.53
p-Nitrobenzaldehyde	2.69	1.72	2.53	1.54	–	–	1.20
Nitrobenzene	2.70	2.03	2.66	1.80	3.36	1.80	1.85
m-Nitrophenol	2.72	1.80	2.68	1.46	4.10	2.10	2.00
o-Nitrophenol	2.54	1.90	2.50	1.61	–	–	1.79
p-Nitrophenol	2.79	1.77	2.81	1.49	–	–	1.91
Phenol	2.35	1.34	2.19	1.06	3.19	1.50	1.46
2-Phenylethanol	2.81	1.80	2.08	1.04	3.04	1.17	1.36
p-Phenylphenol	3.67	2.96	3.52	2.40	–	–	3.20
3-Phenylpropanol	3.06	2.19	2.43	1.41	–	–	1.88
n-Propylbenzene	4.15	3.82	3.29	2.83	–	–	3.68
Toluene	3.15	2.72	2.94	2.28	3.63	2.28	2.69
2,4,5-Trichlorotoluene	4.19	3.84	3.41	2.92	–	–	2.92

nitrile can serve only as an hydrogen acceptor and will change the structure of the mobile phase more drastically. The free energy change for the retention process will thus be dependent on the different molecular properties of the solute. In order to gain more insight into functional group selectivity in different organic modifiers, we have analysed the retention data of Schoenmakers et al.[27] with respect to S and $\log k_w$ (Table VI) and included measured $\log P$ values reported in the literature.

The contribution of a substituent to retention can be defined[8,11] as

$$\tau = \log \left(k_j'/k_i' \right) \tag{7}$$

where k' is the capacity factor of solutes j and i which differ by a substituent. When transformed to $\log k_w$, eqn. 7 becomes:

$$\tau_w = \log k_{w(j)} - \log k_{w(i)} \tag{8}$$

We have examined τ_w for the different monosubstituted benzenes of Table VI and with three different organic modifiers (Table VII).

In general, the methanol–water system is the most discriminating eluent with respect to substituent effects on retention. These differences are most pronounced for completely non-polar groups (Table VII). If we examine polar substituents, significant differences in polar group selectivity are found within the different mobile phases. Short alcoholic groups (as in 2-phenylethanol) produce polar contributions to

TABLE VII

FUNCTIONAL GROUP VALUES, τ_w, DERIVED FROM MONOSUBSTITUTED BENZENES OF TABLE VI

Log k_w for benzene is taken as reference. π values are taken from ref. 2.

Substituent	Methanol–water τ_M	Acetonitrile–water τ_A	Tetrahydrofuran–water τ_T	π
CH_3	+0.56	+0.42	+0.41	+0.56
CH_3CH_2	+1.02	+0.78	+0.48	+1.02
n-Propyl	+1.66	+0.97	–	+1.55
n-Butyl	+2.16	+1.17	–	+2.13
Phenyl	+1.73	+1.02	+0.73	+1.96
OH	−0.82	−0.80	−0.35	−0.67
CH_2OH	−0.69	−1.06	−0.92	−1.03
CH_2CH_2OH	−0.36	−0.82	−0.68	−0.77
$CH_2CH_2CH_2OH$	+0.03	−0.45	–	−0.25
Cl	+0.64	+0.47	+0.48	+0.71
NO_2	−0.13	−0.06	−0.05	−0.28
NH_2	−0.95	–	−0.58	−1.23
CN	−0.39	−0.32	−0.40	−0.57
CHO	−0.36	−0.50	−0.65	−0.65
$COCH_3$	−0.24	−0.44	−0.64	−0.55
OCH_3	−0.01	0.00	−0.04	−0.02

the benzene ring in the acetonitrile (AN) and tetrahydrofuran (THF) systems which are almost twice those in the methanol system. This selectivity difference has also been observed by Tanaka *et al.*[12] for different *n*-alcohols. These authors also showed that phenol is specifically retarded in the THF system, in accordance with our results (Table VII). Acetophenone and benzaldehyde also possess a greater polarity in THF and AN, but the functional group values in this case follow the order $\tau_M < \tau_A < \tau_T$. On the other hand, nitro and amino groups attached to the benzene ring result in a stronger retention in AN and THF (*i.e.*, τ_A and τ_T are smaller than τ_M). The dependence of τ for non-electrolytes on the nature of the organic modifier may be the result of electronic interactions between the solute and solvent, especially in the cases of polar group selectivity observed in the different phase systems.

Relationship between log k_w *and the partition coefficient,* log P, *of the n-octanol–water system*

The relation between log k_w and log P can be considered as a special case of the Collander equation[28] which relates the partition coefficients of different solvent systems:

$$\log P_2 = a \log P_1 + b \tag{9}$$

Using the values of log k_w and log P from Table VI, we obtain the following regression curves for the methanol system

$$\log k_{w(M)} = 0.870 \log P + 0.401 \ (n = 45, r = 0.958) \tag{10}$$

for the acetonitrile system

$$\log k_{w(A)} = 0.601 \log P + 0.525 \ (n = 44, r = 0.894) \tag{11}$$

and for the THF system:

$$\log k_{w(T)} = 0.460 \log P + 0.799 \ (n = 24, r = 0.827) \tag{12}$$

If a solute is equally distributed between octanol and water, *i.e.*, log $P = 0$, the intercept of the Collander equation gives a measure of the hydrophobicity of the non-aqueous phase in relation to the hydrophobicity of *n*-octanol; a positive value indicates that the solvent is less hydrophobic than octanol. The positive intercept in our case can be explained by recent findings[13,29] that the organic modifier is extracted into the stationary phase to solvate the octadecyl ligands and thereby decreases the hydrophobicity of the "effective" stationary phase. Furthermore, the mobile phase consists of water–organic mixtures which leads to a decrease in the free energy change of expulsion from the eluent relative to pure water. The overall effect is as if the non-polar phase is more hydrophilic than octanol.

The slope of the regression equation is a measure of the solvent system's sensitivity to changes in the hydrophobicity of solutes. Methanol again proves to be the most discriminating modifier and is in this respect quite similar to the octanol–water system. For THF, changes in solute structure will usually result in only half the

differences when compared to methanol or the octanol–water system. The magnitude of the correlation coefficient observed is only acceptable for the linear relation between $\log k_{w(M)}$ and $\log P$. We can therefore conclude from the different correlation coefficients of eqns. 9–11 that only methanol–water as the mobile phase produces retention data which are strongly related to $\log P$.

We will now examine the validity of these results for the more complicated structures of the herbicides. Since these compounds contain mostly hydrophobic substituents, it is expected that at least $\log k_{w(M)}$ will be closely related to $\log P$. Fig. 1 depicts this relationship for phenylureas and phenoxycarbonic acid derivatives. Indeed, the two hydrophobicity parameters may be regarded as equivalent in this instance. The poor correlation of $\log k_w$ to $\log P$ ($r = 0.923$, $n = 10$), when determined in acetonitrile–water, also fits into the framework established by the analysis of the simple benzene derivatives.

Interestingly, s-triazines show a completely different behaviour in the two systems:

$$\log P = 0.196 \log k_{w(M)} + 1.432 \ (n = 6, r = 0.555) \tag{13}$$

The lack of correlation between $\log k_w$ and $\log P$ is not the result of polar group selectivity as observed for the benzene derivatives, but arises from the unusual behaviour of the non-polar methylthio substituent in RPLC. When s-triazines contain this functional group instead of a chloro-substituent, a small decrease in hydrophobicity should occur according to the π values of Cl ($+0.71$) and SCH_3 ($+0.67$)[2]. What is actually observed is a large increment in retention with a corresponding $\Delta \log k_M$ of 1.5, which is equivalent to an additional propyl group attached to the heterocy-
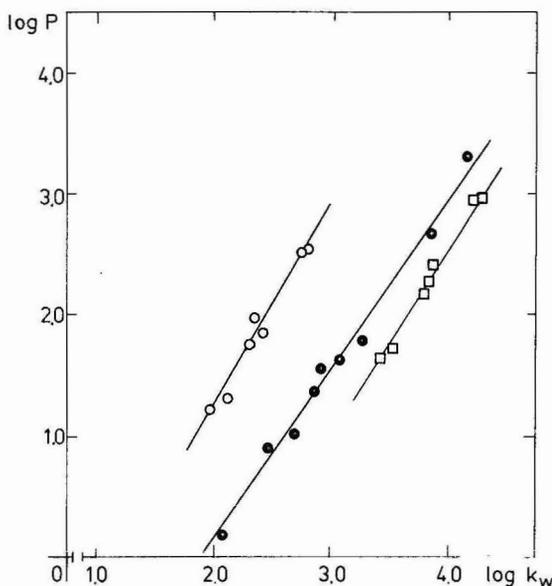
Fig. 1. Relationship between $\log P$ and $\log k_{w(M)}$ for phenylureas ($\bullet$), phenoxycarbonic acids ($\bigcirc$) and phenoxycarbonic acid methyl esters ($\square$).

clus. We have shown[10] that a trifluoromethyl group also gives an unusually large increase in hydrophobicity when compared to the π value ($\Delta\log k_w$ of about 1.5). Both results demonstrate that specific non-polar groups exert selective effects which are not found in liquid–liquid partitioning systems. As a consequence, we have measured the inhibitory activity of s-triazines to examine which hydrophobicity parameter is better suited for modelling the hydrophobicity of these herbicides.

Structure–activity relationships

Phenylureas and s-triazines inhibit the photosynthetic electron transport of chloroplasts. Since both herbicidal groups are known to bind to a specific protein near photosystem II[30], the ability to inhibit electron flow upon illumination is strongly dependent on the hydrophobic nature of these herbicides[5]. Therefore, QSAR with $\log k_w$ and $\log P$ is a good test of whether polar and non-polar group selectivity influences the quality of the relation between the biological response and the hydrophobicity of these compounds.

Using the I_{50} values for the inhibition of the Hill reaction from Table VIII, for phenylureas:

$$pI_{50} = 0.945 \log k_{w(M)} + 3.583 \ (n = 10, r = 0.841) \tag{14}$$

This equation is equivalent to those reported previously[4,5] and is also valid if $\log P$

TABLE VIII

pI_{50} VALUES FOR THE INHIBITION OF THE HILL REACTION BY PHENYLUREAS AND s-TRIAZINES

The reaction medium contained 7 mM phosphate buffer (pH 6.4), 0.25 M sucrose, 0.1 mM dichlorophenol-indophenol and 50 μg chlorophyll per ml and was illuminated for 45 sec with 50,000 lux. The decrease in absorption was measured at 590 nm.

Compound	pI_{50}	
	Measured	Reported[5]
Fenuron	4.9	4.6–5.5
Metoxuron	6.0	6.6
Monuron	5.5	5.6–6.8
Monolinuron	5.8	5.6–6.1
Chlortoluron	6.4	7.0
Metobromuron	5.6	6.0
Diuron	7.0	6.7–7.5
Linuron	6.7	6.7–7.0
Chloroxuron	6.7	6.8–7.3
Neburon	7.1	6.7–6.9
Simazine	5.7	5.4–6.4
Atrazine	6.0	6.1–6.6
Propazine	5.7	5.4–6.3
Prometryn	6.8	7.0
Desmetryn	6.4	–
Terbutryn	7.1	–
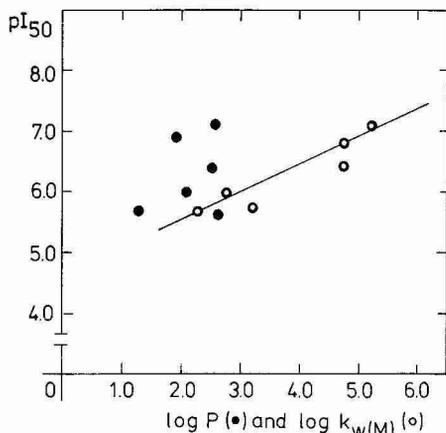
Fig. 2. Relationship between the inhibition of the photosynthetic electron transport by *s*-triazines and their log $k_{w(M)}$ and log P values, respectively. The inhibitory activity is given as pI_{50}, the logarithm of the concentration which causes 50% inhibition of the Hill reaction with dichlorophenolindophenol as electron acceptor.

values are used instead of log k_w. Obviously, log k_w can fully replace log P as a hydrophobicity parameter. This is also true for the phenoxycarbonic acids and their methyl esters and for a variety of compounds, *i.e.*, pyridazinones[10], phenols[15] and alkylbenzamides[31].

The *s*-triazines with SCH_3 substituents show non-polar group selectivity in RPLC so that log k_w and log P are only poorly correlated (eqn. 13). Interestingly, log k_w can be used to model the behaviour of *s*-triazines in thylakoid membranes whereas log P cannot (Fig. 2). The parallelism between the increase in activity and the increase in retention upon methylthio substitution cannot be interpreted at the moment. For that purpose, more QSAR studies with congeneric series would have to be performed where τ_w or log k_w of those substituents which show polar and non-polar group selectivity in RPLC should be related to their biological activity. Preliminary results with trifluoromethyl-substituted pyridazinones are promising in this respect.

CONCLUSIONS

Extrapolated log k_w values of different herbicides and of benzene derivatives can provide hydrophobicity parameters which are strongly related to log P in the standard octanol–water system. However, polar group selectivity and non-polar group selectivity is observed in RPLC for certain substituents, among them OH, SCH_3 and CF_3, which indicates that log k_w is basically an expression of the solvophobic effect but also includes additional information about the physico-chemical properties of a compound. The few QSAR studies which have employed retention parameters all show that log k' or log k_w can fully replace log P as a measure of the hydrophobicity of drugs. We have shown here that in the specific case of *s*-triazines with methylthio substituents only log k_w can be used for this purpose.

Biomembranes are highly compartmentalized structures with respect to their lipid and protein moieties, and are thus not homogeneous within their non-polar nor

polar regions. Thus, membranes should be better able to discriminate between minor structural differences than is indicated by the gross hydrophobic behaviour in liquid–liquid systems. This may be especially true for the protein-rich functional membranes of chloroplasts and mitochondria which are the preferred targets for herbicides. Furthermore, the lipids and proteins of the biomembranes are asymmetrically arranged and cannot move freely, laterally or transversely, so that steric and electronic requirements of the molecular structure come into play which do not operate in bulk liquids. Probably, the dynamic chromatographic process —the exclusion of a solute from the mobile phase into a more or less ordered stationary phase— is a better model of the behaviour of drugs during their passage through biomembranes.

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ANALYSIS OF SOME COMMERCIAL PREPARATIONS FOR MIGRAINE TREATMENT USING ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ADDITION OF SALTS TO THE MOBILE PHASE

GARY K. C. LOW and P. R. HADDAD*

Department of Analytical Chemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)

and

A. M. DUFFIELD

Biomedical Mass Spectrometry Unit, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033 (Australia)

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SUMMARY

When appropriate salts are added to the mobile phase in ion-pair high-performance liquid chromatography (HPLC) it is possible to arrive at an isocratic solvent with which complex mixtures of nitrogenous compounds of different pK_a values and lipophilic characteristics may be separated. Selectivity in the manipulation of solute retention depends on the salt type, its concentration, the percentage of organic modifier in the mobile solvent and the solute itself. In addition to a dramatic reduction in analysis time, the use of salts can also improve the resolution of closely eluted peaks. With judicious control of the pH of the mobile solvent, the addition of salts to the mobile phase can cause the retention of compounds of different pK_a values to alter in a contrasting manner. Under typical ion-pair HPLC conditions, an increase in salt concentration in the mobile solvent enhances the retention of neutral compounds and reduces the retention of ionized compounds. An inverse log–log relationship between the capacity factor of a solute and the salt concentration in the mobile phase was found. Examples are given of the use of salts in mobile solvents for ion-pair HPLC of a number of pharmaceutical preparations employed for the treatment of migraine.

INTRODUCTION

The analysis of compounds which contain amino groups is very important in biological and pharmaceutical applications. The conditions for separation of basic nitrogenous compounds by high-performance liquid chromatography (HPLC) have always been dependent on the chemistry of these bases[1]. Two important parameters regulating the elution profiles of these compounds from reversed-phase columns are their pK_a values and lipophilic characteristics. In the analysis of mixtures of bases which differ widely in these parameters, the most popular method for separation has

been gradient elution ion-pair HPLC[2]. Whilst this is adequate for qualitative analysis, the lack of precision has limited its application. It has recently been demonstrated[3] that chromatographic separation with gradient elution can only be reproducible when isocapacitive mobile solvents are used. These solvents are defined as mobile phases in which the pairing ion concentration is similar to that in the stationary phase.

The addition of salts to mobile phases is a standard practice in ion-exchange chromatography of ionized solutes, where the ions from the salt compete for the charged sites on the ion-exchange resin. In salting-out chromatography, salts have been used for the elution of neutral organic compounds from ion-exchange columns. In an attempt to relate the retention of a solute to the concentration of the salt in the mobile solvent, the following empirical formula was proposed[4,5]

$$\log k' = \log k_0' + K_s M$$

where k' and k_0' are the capacity factors of a solute eluted using mobile solvents with and without salt, respectively, M is the concentration of the salt in the mobile solvent and K_s is the salting-out constant. This expression is of the same form as the well known Setchenow equation[6] which describes the effects of salts on the solubility of non-electrolytes in aqueous solution. The salt constants in both equations depend on the nature of the salts used.

The use of salts in mobile solvents has been successfully applied to the separation of small neutral or ionized compounds on reversed-phase columns[7–10]. The enhancement of retention of neutral compounds with increasing salt concentration has been explained by the solvophobic theory[7] in terms of the combined effects of a reduction of electrostatic repulsion between solute molecules, an increase in surface tension of the eluent and the concomitant increase in the energy required for cavity formation to accommodate the solute molecule in the solvent. For ionized solutes, a more complicated relationship is involved: at low salt concentrations, the retention decreases with increasing salt concentration, whereas at sufficiently high salt concentration, an increase of salt in the mobile solvent increases retention[8]. Jandera et al.[9] have demonstrated that the solvophobic theory cannot be strictly applied to these retention phenomena.

Until recently, the addition of salts in ion-pair chromatography had been employed only to assist in the elucidation of the retention mechanism of ion-pair chromatography. A number of authors[10–12] have suggested that an inverse dependence of the capacity factor on the salt concentration in the mobile solvent can be used to support the dynamic ion-exchange hypothesis, however the same relationship is essentially consistent with a variety of alternative retention mechanisms[13–15]. In these studies, the solutes used have generally been small molecules such as biogenic amines and amino acids, and no allowances have been made for selectivity differences arising from the use of different salts.

In this report we discuss a number of attractive features of the use of salts in ion-pair HPLC of nitrogenous basic drugs, in particular with regard to selectivity in retention reduction and improvement in the resolution of closely eluting peaks. With judicious control of the pH of an isocratic mobile solvent containing a salt, it is possible to analyse a number of pharmaceutical preparations employed for migraine.

These preparations generally contain nitrogenous bases of varying pK_a and lipophilic properties, and the addition of salts to mobile solvents not only reduces analysis time but can also exert opposite effects on the retention of neutral and ionized molecules. The relationship of the capacity factors of a number of these nitrogenous drugs to the salt concentration in the mobile phase of ion-pair HPLC is also investigated.

EXPERIMENTAL

Standards and reagents

The standard drugs were obtained from various sources: ($\pm$)-ephedrine hydro-chloride, caffeine and theophylline from Sigma (U.S.A.); ergotamine tartrate from Fluka (Switzerland) and cyclizine hydrochloride, meclozine hydrochloride and diphen-hydramine hydrochloride from the National Biological Standards Laboratory (Can-berra, Australia). These materials were shown to be free from contaminants by gas chromatographic–mass spectrometric (GC–MS) analysis and test solutions were made up in methanol–water (50:50) at a concentration of 1.0 mg/ml.

Analytical grade ammonium sulphate, lithium sulphate, magnesium sulphate, sodium sulphate and sodium chloride were purchased from BDH (Australia). The salts were washed with anhydrous methanol and dried in a desiccator under vacuum before use. *n*-Heptanesulphonic acid (sodium salt) and acetic acid were obtained from Ajax Chemicals (Australia) and used without further purification.

Analysis of pharmaceutical formulations for migraine treatment

Four different formulations were purchased over the counter in Sydney, Aus-tralia. The ingredients and their concentrations in each preparation are shown in Table I. Twenty randomly selected tablets from each of the four formulations were crushed and an accurately weighed portion of powder equivalent to the average weight of a single tablet was dissolved in an appropriate volume of methanol–water

TABLE I

QUANTITATION OF SOME COMMERCIAL PHARMACEUTICAL PREPARATIONS FOR MI-GRAINE TREATMENT

Preparation	Manufacturer	Stated concentration of active ingredients (mg)	%* of stated content
Cafergot	Sandoz (Switzerland)	Ergotamine tartrate, 1.0	99 ± 1.2
		Caffeine, 100	101 ± 0.5
Ergodryl	Park Davis (Australia)	Ergotamine tartrate, 1.0	99.0 ± 1.0
		Caffeine citrate, 100	99.6 ± 1.2
		Diphenhydramine hydrochloride, 25	95.0 ± 1.3
Ergalan	Allen and Hanburys (Australia)	Ergotamine tartrate, 1.0	94.5 ± 2.0
		Caffeine, 100	99.0 ± 0.8
		Meclozine hydrochloride, 10	100.0 ± 1.0
Migral	Burroughs Wellcome (Australia)	Ergotamine tartrate, 2.0	98.0 ± 2.0
		Caffeine hydrate, 100	101.0 ± 1.3
		Cyclizine hydrochloride, 50	100.0 ± 1.2

* Results are expressed as the percentage of the declared content of the active ingredient ± stan-dard deviation. The standard deviation is estimated from the range of three individual determinations.

(50:50) in a volumetric flask, with the aid of a ultrasonic bath. The solution was then filtered and diluted, if necessary, to an appropriate concentration for injection onto the HPLC column.

Quantitations were made by using the chromatographic peak heights, and the concentrations of active ingredients in each tablet formulation were calculated with respect to their stated contents. All assays were performed in triplicate.

Instrumentation and chromatographic procedures

The liquid chromatograph system consisted of a Waters Model M6000A solvent pump, Model U6K injector, Model M450 variable wavelength detector (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe Model B5217-1 recorder. The detector was generally operated at 254 nm with a sensitivity setting of 0.1 a.u.f.s., however for separations of drugs in pharmaceutical preparations the wavelength was 245 nm. Because of the large differences in concentrations of active ingredients in these formulations, the setting of the detector at this wavelength tended to bias the sensitivity of detection towards the drugs of low concentration. All separations were carried out at 20°C and the flow-rate was set at 2.0 ml/min unless otherwise indicated.

Separation by HPLC was accomplished using a μBondapak C_{18} column (30 cm × 4.7 mm I.D., Waters Assoc.). The column was equilibrated with each mobile phase before use; a constant retention time was usually obtained after pumping 20–30 column volumes. When changing from one mobile phase to another, the column was washed with 10 ml of 0.01 N H_3PO_4 (pH 3.0) and 15 ml of methanol–water (50:50 v/v) before equilibrating with the new solvent. When mobile solvents containing sodium chloride were used, the HPLC system was washed with water immediately after use. A number of similar C_{18} columns were used in this study, however the column was not changed until a particular aspect of the study was completed.

Preparation of mobile phases

Analytical grade methanol was triply distilled from all-glass apparatus and water was distilled using a Millipore Milli-Q water purification system. The mobile phases were usually prepared immediately before use by dissolving 5.0 mmole of heptanesulphonic acid (sodium salt) and an appropriate concentration of salt in methanol–water mixtures containing 1.0 % (v/v) glacial acetic acid. All mobile solvents were prepared in single large batches of sufficient quantity for experiments on a particular aspect of the study, so that intrastudy variations were eliminated. The exact ingredients of the mobile phase used are given in the figure captions.

Mobile phases were aspirated through 0.7-μm glass microfibre paper filters (GF/F, Whatman), degassed in an ultrasonic bath and allowed to equilibrate to ambient temperature before use.

RESULTS AND DISCUSSION

Selection of solutes

The compounds selected for this study are listed in Table II together with their pK_a values and connectivity indices, χ[16]. The connectivity index is a useful indication of the lipophilic character of a molecule and is directly related to the cavity surface area, polarizability, solubility and partition coefficient of that molecule[17]. The calcu-

TABLE II

CONNECTIVITY INDICES AND pK_a VALUES FOR COMPOUNDS STUDIED

Name	Connectivity index, χ	pK_a*
Caffeine	5.37	< 1.0
Cyclizine	8.34	8.16
Diphenhydramine	8.27	9.12
Ephedrine	5.25	10.8
Ergotamine	12.12	6.4
Meclozine	9.41	–
Theophylline	5.13	< 1.0

* From ref. 25.

lated values of χ reported here are approximate, in that no corrections were made for the contributions of different functional groups, such as carbonyl and hydroxyl, in the molecule.

The compounds shown in Table II are widely distributed in their pK_a and connectivity index values. Under the mobile phase pH conditions used in this study (pH 3.5), theophylline and caffeine remain essentially neutral, whereas all of the other species are in their protonated forms. In view of the large differences in χ values, the separation of a mixture of these compounds, e.g., caffeine, ephedrine and ergotamine, would be difficult under isocratic conditions. Ion suppression methods[18] cannot be used here since the pH required would be in excess of that permissible with silica columns.
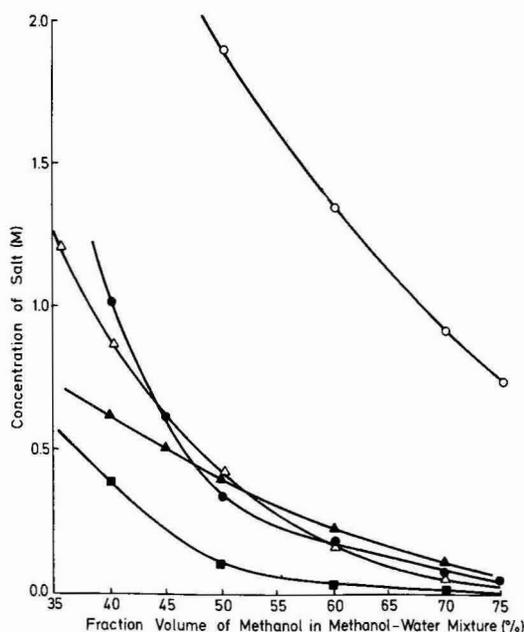
Fig. 1. Variation of the solubility of salts with changes in the fraction of methanol in methanol–water mobile solvents. ○ = NaCl; ● = $MgSO_4$, △ = $(NH_4)_2SO_4$; ▲ = Li_2SO_4; ■ = Na_2SO_4.

Salt effects

The solubilities of the various salts studied in methanol–water mixtures were determined experimentally by dissolving a known excess of salt in the solvent and accurately weighing the dried residue. Fig. 1 shows the solubilities of these salts at 20°C.

The effects of addition of the salts NaCl, $MgSO_4$, $(NH_4)_2SO_4$, Li_2SO_4 and Na_2SO_4 to the mobile phase in ion-pair HPLC on the retention behaviour of the solutes ergotamine, cyclizine, diphenhydramine, ephedrine and caffeine are shown in Fig. 2. For the first four compounds, inclusion of a salt in the mobile phase caused a reduction in retention, whereas for caffeine the retention was either unaffected or slightly increased. The different behaviour of caffeine is clearly due to the fact that it is not ionized under the pH conditions used. Similar results were obtained with theophylline, which is also not ionized. The general pattern observed for the protonated solutes was that a sharp decrease in retention occurred upon the initial addition of salt, after which only slight changes occurred with further addition of salt.

The results given in Fig. 2 indicate that the observed change in retention is strongly dependent on the type of salt added to the mobile phase. The general order of efficiency with which the salts reduced the retention of the solutes tested was $Li_2SO_4 > (NH_4)_2SO_4 > NaCl > Na_2SO_4 > MgSO_4$, although the order did vary slightly depending on the nature of the solute. At higher concentrations of salt than those shown in Fig. 2, a slight increase in retention was observed (Fig. 3) and this has been rationalized[13] in terms of the gradual dominance of the surface tension terms in the retention equation developed from the solvophobic theory. The same phenomenon has also been attributed to salting-out effects caused by the increased concentration of salt in the mobile phase[18].

The order of efficiency of retention reduction exhibited by the various salts is different for ephedrine than for the other protonated species. This difference must be
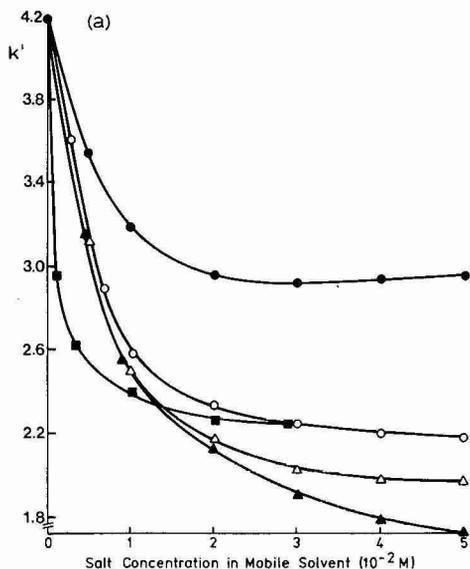
Fig. 2.
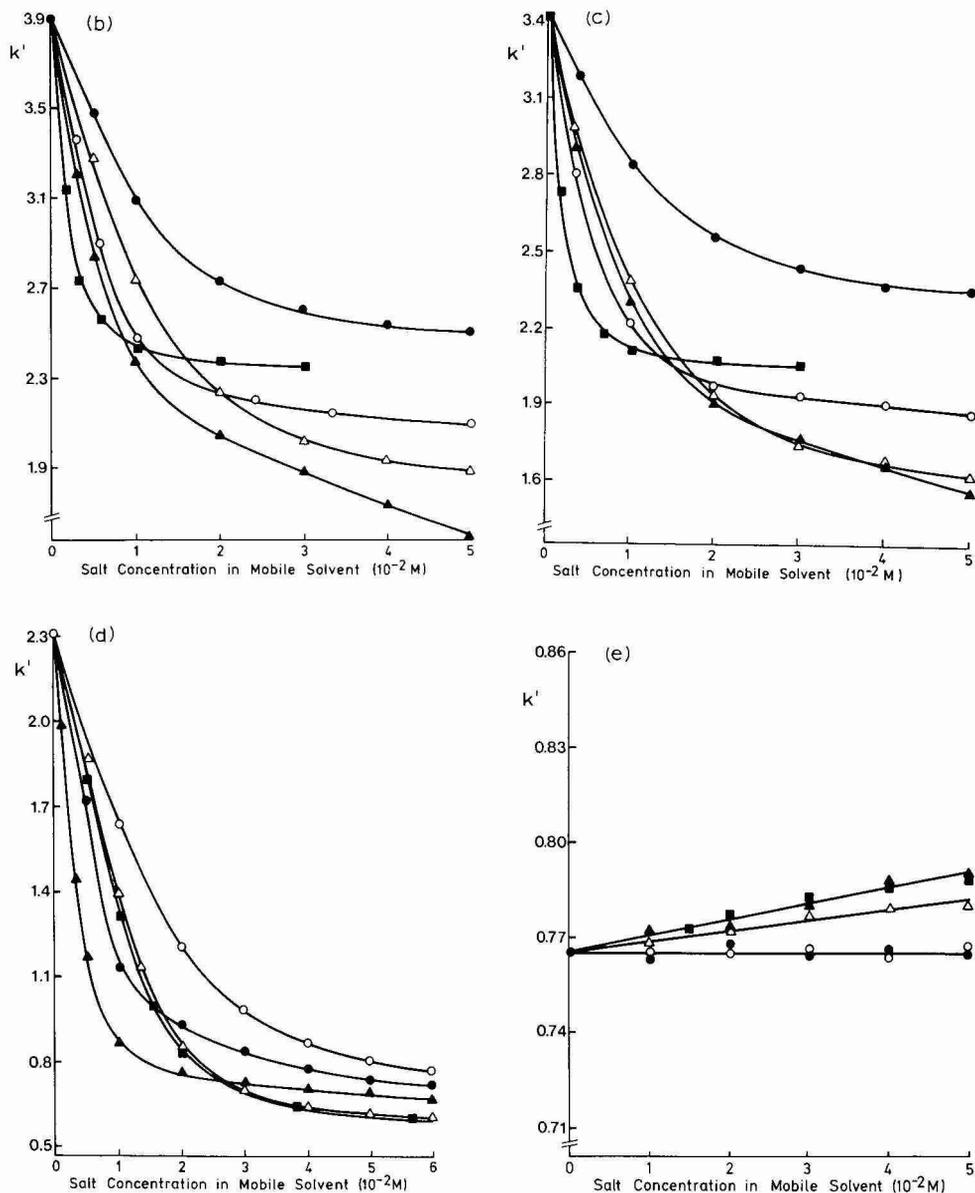
Fig. 2. Change of the capacity factor, k', with salt concentration in the mobile solvents. Salts are denoted as in Fig. 1. Solutes: a, ergotamine; b, cyclizine; c, diphenhydramine; d, ephedrine; e, caffeine. Mobile solvents: a–c, methanol–water (60:40) containing 5.0 mM sodium heptanesulphonate and 1.0% acetic acid; d, e, methanol–water (43:57) containing 5.0 mM sodium heptanesulphonate and 1% acetic acid.

partially attributed to the less lipophilic nature of ephedrine compared with the other compounds. A lower fraction of organic solvent was employed in the mobile phase used for ephedrine in order to obtain a workable k' value which would illustrate salt effects ($k' > 1.5$ at zero salt concentration).
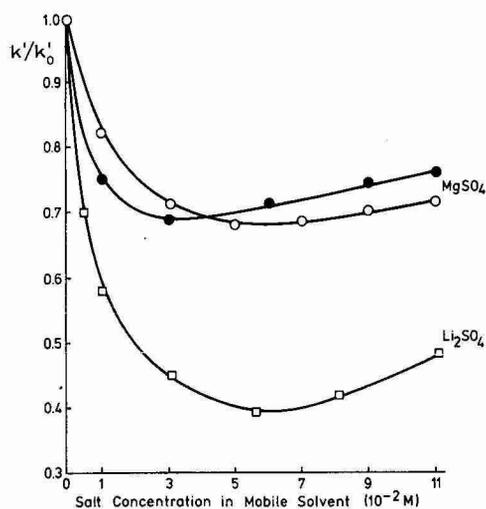
Fig. 3. Examples of the increase in retention at higher salt concentration in the mobile solvent. Mobile solvent as for Fig. 2a–c. $\square$, $\bigcirc$ = Diphenhydramine; $\bullet$ = ergotamine. k_0' is the capacity factor obtained without addition of salt to the mobile phase.

The effect of added salt was somewhat dependent on the proportion of methanol present in the mobile phase. In mobile phases containing low amounts of methanol the k' value reached a lower plateau level than that observed for higher amounts of methanol. In addition, the initial rapid decrease in k' occurring upon the first addition of salt was more dramatic in mobile phases containing higher proportions of methanol. This trend is expected since the concentration of pairing ion adsorbed onto the stationary phases decreases as the fraction of organic modifier is increased. Competition by added salt ions can therefore more readily reduce the retention of an ionized solute.
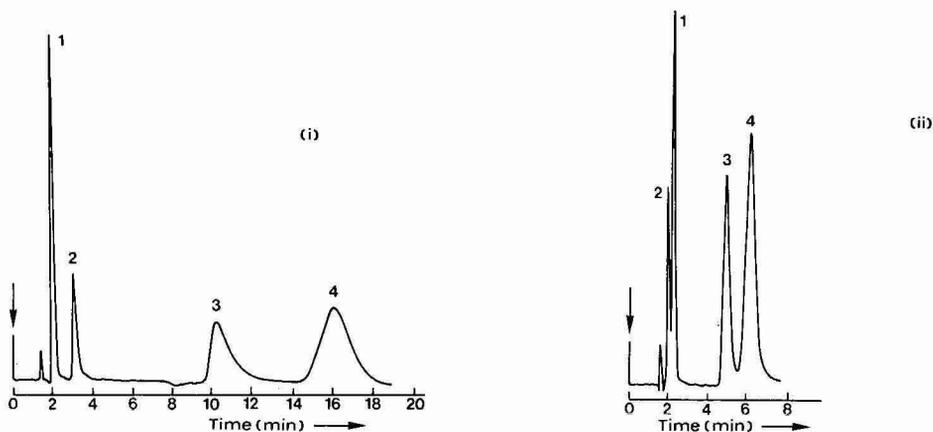
Fig. 4. Comparison of isocratic separations of synthetic mixtures of caffeine (1), ephedrine (2), diphenhydramine (3) and ergotamine (4) with two different mobile solvents. Mobile solvents: i, methanol–water (50:50) containing 5.0 mM sodium heptanesulphonate and 1.0% acetic acid; ii, as for i except with $7.0 \cdot 10^{-2}$ M Na$_2$SO$_4$ added.

Selectivity and resolution

The possibility of using the addition of salt to selectively manipulate retention is evident from the data in Fig. 2. It is clear that the retention of a solute in ion-pair HPLC is a function of the combined effects of the added salt type, salt concentration, the fraction of organic modifier in the mobile phase and the nature of the solute. An example of selective retention manipulation is given in Fig. 4 which depicts the isocratic separation of a mixture of caffeine, ephedrine, diphenhydramine and egotamine using mobile phases both with and without the presence of added salt. Addition of Na_2SO_4 resulted in a decreased analysis time, a reversal of the elution order of caffeine and ephedrine and an improvement in peak shape for all solutes.

The resolution of closely eluted peaks is also governed by the concentration of salt in the mobile phase. We have found that an increase in salt concentration leads to an increase in resolution, followed by a decrease at higher salt concentrations. This effect was particularly pronounced with columns of low efficiency and can be explained in terms of the binding of exposed silanol sites by the salt in the mobile phase. The results are illustrated in Fig. 5 which shows that the resolution of ergotamine and
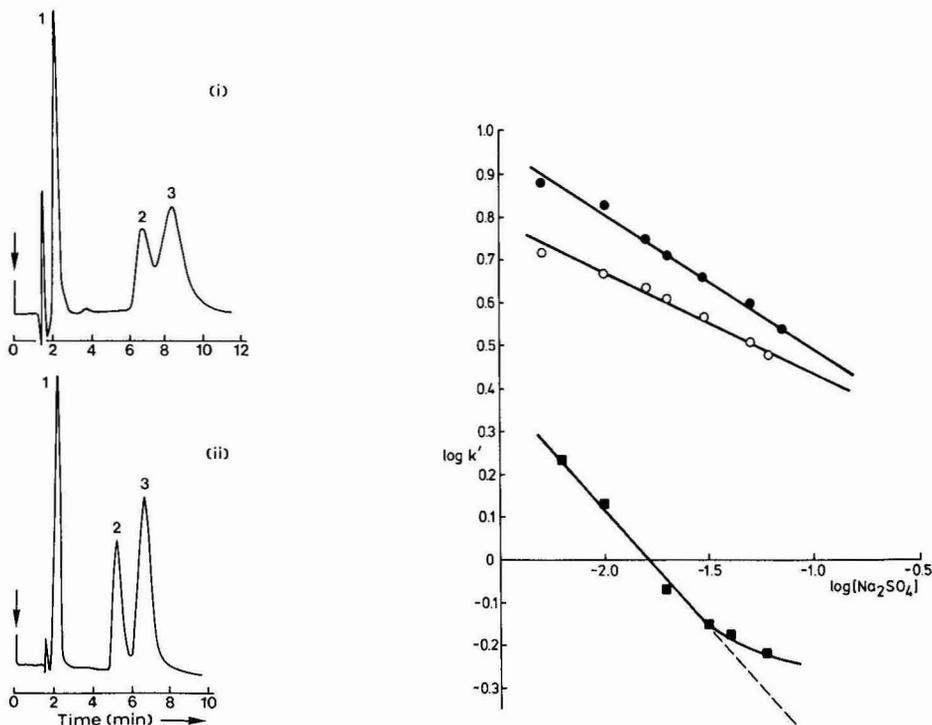
Fig. 5. An example of the improvement in the resolution of two closely eluted peaks produced by changing the salt concentration in the mobile solvent. Salt concentrations: i, $0.71 \cdot 10^{-2}$ M; ii, $2.63 \cdot 10^{-2}$ M in methanol–water (50:50) containing 5.0 mM sodium heptanesulphonate and 1.0% acetic acid. Peaks: 1 = caffeine; 2 = diphenhydramine; 3 = ergotamine.

Fig. 6. Observed log–log relationship between k' and Na_2SO_4 concentration in the mobile solvent. Mobile phase: methanol–water (50:50) containing 5.0 mM heptanesulphonate, 1% acetic acid and the indicated concentration of Na_2SO_4. ● = Ergotamine; ○ = diphenhydramine; ■ = ephedrine.
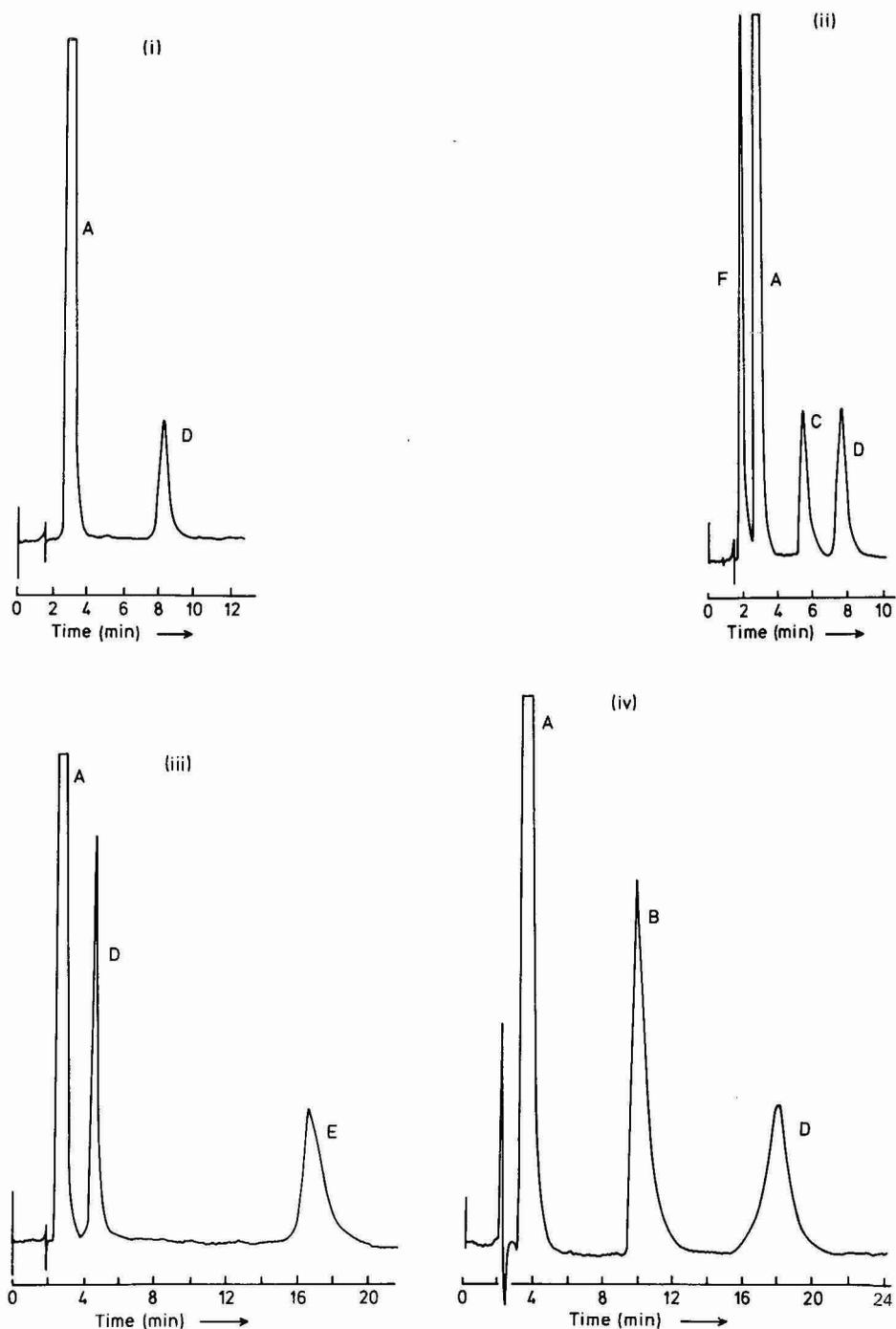
Fig. 7. Separations of pharmaceutical preparations for migraine treatment using methanol–water mobile solvents containing 5.0 mM sodium heptanesulphonate and 1.0% (v/v) acetic acid. i, Cafergot tablets, methanol–water (50:50) and $7.0 \cdot 10^{-2}$ M Na$_2$SO$_4$; ii, Ergodryl tablets, methanol–water (50:50) and $9.0 \cdot 10^{-2}$ M Na$_2$SO$_4$; iii, Ergalan tablets, methanol–water (51:43) and $4.0 \cdot 10^{-2}$ M Na$_2$SO$_4$; iv, Migral tablets, methanol–water (43:57) and $10 \cdot 10^{-2}$ M Na$_2$SO$_4$. Peaks: A = caffeine; B = cyclizine; C = diphenhydramine; D = ergotamine; E = meclozine; F = citric acid.

diphenhydramine is dramatically improved by an increase in the salt concentration, despite the fact that the retention of each species has been reduced. Addition of salt improves the resolution by narrowing the peaks, since the separation factors calculated from each of the chromatograms in Fig. 5 are almost identical (1.31 and 1.37 for Fig. 5i and ii, respectively). Improvements in resolution resulting from addition of salt to the mobile phase in ion-pair HPLC have been reported previously[20].

Relationship between k′ and [salt]

A number of explanations have been advanced for the mechanism by which addition of a salt to the mobile phase produces a reduction in retention in ion-pair HPLC. The uncertainty surrounding this mechanism is a direct result of the fact that considerable ambiguity still exists regarding the mechanism of ion-pair chromatography itself[14,15,20-24]. In many of the published studies, an inverse relationship has been reported between $k′$ and the concentration of salt in the mobile phase[11,12,14,15]. We have experimentally determined the relationship between $k′$ and [salt] for the solutes ephedrine, ergotamine and diphenhydramine; the pairing-ion concentration was maintained at 5 mM. The results of this study showed that, for the particular solutes and salt concentrations examined, the capacity factor was not inversely proportional to [salt]; rather an inverse log–log relationship was observed, as indicated by Fig. 6. The reasons for the disparity between these results and those previously reported could only be determined by further study, however we have used much larger solute molecules and a wider range of salt concentrations than in previous studies. These factors are likely to have a considerable effect.

Analysis of pharmaceutical preparations

To illustrate the utility of isocratic ion-pair HPLC with addition of salt to the mobile phase, several preparations employed for migraine treatment containing different mixtures of the test solutes were analysed. The salt concentration and the percentage of organic modifier in the mobile phase were adjusted to optimize the separation in the minimum analysis time. As far as possible, the salt concentration was maintained in the plateau region (see Fig. 2) to minimize variations in retention caused by slight changes in salt concentration. The chromatograms obtained are given in Fig. 7 and the results are summarized in Table I. These results clearly show that the proposed method of analysis has direct application to the analysis of such pharmaceutical formulations.

CONCLUSIONS

This study has demonstrated that the addition of salts to the mobile phase used in ion-pair HPLC can lead to selective changes in retention of solutes. The type of salt, the degree of ionization of the solute, the percentage of organic modifier, the salt concentration and the nature of the solute itself all play a part in determining solute retention. Adjustment of the salt concentration in the mobile phase can permit isocratic separation of a series of compounds previously separable only with gradient elution ion-pair HPLC.

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β-CYCLODEXTRIN AS A SELECTIVE AGENT FOR THE SEPARATION OF *o*-, *m*- AND *p*-XYLENE AND ETHYLBENZENE MIXTURES IN GAS–LIQUID CHROMATOGRAPHY

DANUTA SYBILSKA* and TOMASZ KOŚCIELSKI

Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44, Warsaw 01-224 (Poland)

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SUMMARY

The dependence of the elution patterns of *o*-, *m*- and *p*-xylenes and ethylbenzene on the β-cyclodextrin concentration in the formamide stationary phase deposited on Celite has been studied in the temperature range 50–70°C. It has been found that stereoselective molecular inclusion processes take place in the formamide medium, thus, modifying its chromatographic properties. Such systems result in a gain in stereoselectivity while at the same time retaining all the advantages of partition gas chromatography. The conditions for complete separation of the investigated compounds have been elaborated.

INTRODUCTION

The most remarkable property of cyclodextrins (CDs) is their great ability to form inclusion compounds with various molecules and ions. Attempts to make use of this phenomenon for the separation of various mixtures have mainly been by use of liquid chromatography[1,2]. There is little information concerning the behaviour of CDs in gas chromatography (GC). The retention data of various compounds were studied using stationary phases containing crystalline α- and β-CDs[3] and polyurethane incorporating CDs[4]. Under these conditions, molecular inclusion plays a significant rôle in the sorption process. The Japanese authors[4] proposed that CD-polyurethane resins be applied in analytical practice for the concentration of some aromatic compounds.

The aim of this work was to answer the question: can CDs be used to modify the resolution properties of liquid stationary phases in gas chromatography? In other words: can CDs be applied to the analytical separation of various compounds by partition GC?

The studies were performed using β-CD solutions in formamide; *o*-, *m*- and *p*-xylenes and ethylbenzene were chosen as the model compounds.

EXPERIMENTAL

Reagents

β-CD was supplied by Chinoin (Budapest, Hungary). Celite (80–120 mesh) for gas chromatography was from BDH (Poole, Great Britain). Pure formamide was supplied by POCh (Gliwice, Poland). All materials were of analytical or reagent grade and were used without further purification.

Apparatus and procedures

Chromatographic studies were performed using a Hewlett-Packard 7620 A gas chromatograph equipped with dual flame ionization detectors. Glass columns (2 m × 4 mm I.D.) were used. In the experiments special care was taken to maintain constant the inlet pressure (2.75 atm) and helium flow velocity (50 ml/min).

The compounds were injected (0.2 μl) separately or as mixtures using Hamilton microsyringes. The retention time of each compound was determined as the mean value (relative error <0.5%) from each of series of six injections.

The stationary phase was prepared as follows. An aqueous solution containing β-CD and formamide was deposited on Celite. The resulting slurry was shaken for about 10 min and then the excess of water was slowly evaporated at low pressure (20 mmHg) and at a temperature of 50°C. In all the experiments the quantities of formamide (4.54 g) and Celite (20 g) were constant. The amount of β-CD was varied: 0.3, 0.6, 0.9 or 1.2 g. The columns were weighed before and after packing to give the mass of coated support in the column; the mean value for all the columns was 12 ± 0.5 g.

In each case two columns were prepared: one with a given solution of β-CD in formamide and the second (reference) containing formamide alone. These two columns when placed in the chromatograph oven enabled performance comparisons under the same conditions, thus excluding many sources of error. The two flame ionization detectors were connected to two electrometers and could be operated with two columns at the same time. Each injection on the β-CD containing column was followed almost at once by the injection on the reference column.

The studies were carried out in the temperature range 50–70°C.

RESULTS AND DISCUSSION

The chromatograms presented in Fig. 1 show the dependence of the separation of a mixture of *o*-, *m*- and *p*-xylenes and ethylbenzene on the concentration of β-CD in the formamide solution used to coat the Celite column. Fig. 2 demonstrates the influence of the temperature on the separation process for the same mixtures. In these experiments the stationary phase comprised a 1.18 mol % solution of β-CD in formamide.

A comparison of the elution patterns in Fig. 1 leads to the conclusion that inclusion of the solutes in the β-CD cavity takes place in the formamide medium. For each species investigated, the adjusted retention times followed the relationship:

$$t'_R \ (\beta\text{-CD, formamide}) > t'_R \ (\text{formamide})$$
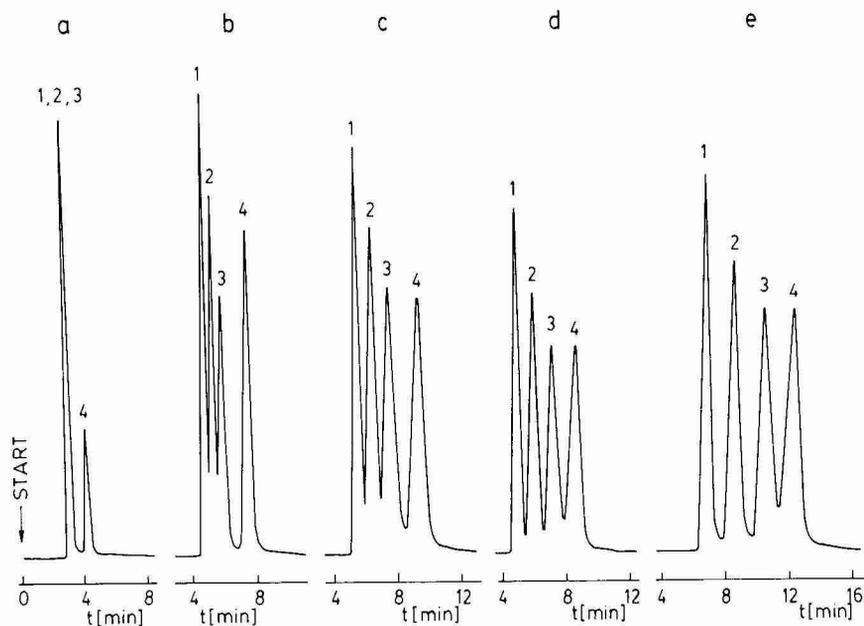
Fig. 1. Chromatograms of a mixture of *o*-(4), *m*-(1) and *p*-(2)xylenes and ethylbenzene (3) at 60°C on columns (2 m × 4 mm I.D.) packed with 0.0 (a), 0.296 (b), 0.59 (c), 0.88 (d) and 1.18 mol % (e) of β-CD in formamide solution, deposited on Celite (4.54 g per 20 g). Sample size: 0.3 μl; composition; *o*-xylene (0.7974 g), *m*-xylene (0.7546 g), *p*-xylene (0.7262 g) and ethylbenzene (0.7080 g).

Fig. 2. Chromatograms of the same mixture as in Fig. 1e at 50°C, 60°C and 70°C. Sample size: 0.3 μl. Helium flow: 50 ml/min.

TABLE I

SEPARATION FACTORS AT 70°C FOR COLUMNS WITH DIFFERENT CLNCENTRATIONS OF β-CD

o-, m-, p-X = o-, m-, p-Xylenes; etb = ethylbenzene.

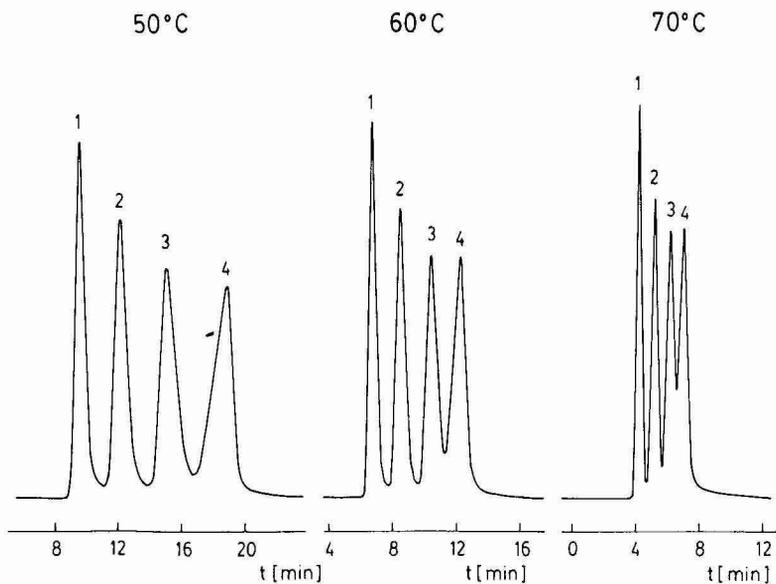| Concentration of β-CD in formamide (mol %)	$\alpha_{p\text{-}X/m\text{-}X}$	$\alpha_{etb/p\text{-}X}$	$\alpha_{o\text{-}X/etb}$	$\alpha_{etb/m\text{-}X}$	$\alpha_{o\text{-}X/m\text{-}X}$
0	0.965	0.974	1.485	0.940	1.396
0.30	1.114	1.107	1.277	1.233	1.574
0.59	1.145	1.116	1.222	1.278	1.562
0.88	1.221	1.135	1.244	1.386	1.724
1.18	1.223	1.364	1.057	1.668	1.763

A characteristic feature of this inclusion process is its stereoselectivity. The separation factors, α, of pairs of the compounds investigated, calculated as the ratios of their adjusted retention times, are given in Table I.

With the column containing formamide alone, only o-xylene forms a separate peak while all the other C_8H_{10} aromatics are eluted together; thus is due to the relatively great difference (about 4°C) between boiling point of o-xylene and those of the other compounds. However, in the presence of β-CD in formamide solution, the shape of the molecules is distinguished and each compound is eluted separately. The polarizability, which changes only slightly in the case of xylenes, plays a minor rôle.

The processes involved in the formation of β-CD molecular inclusion complexes which take place in formamide medium, except stereoselectivity and suitable capacity, meet the other requirements of the dynamic chromatographic method, primarily those of reversibility and fast equilibration. The elution curves are symmetrical and the retention of each compound is independent of its concentration and of the presence of other substances in the injected sample. Table II gives the number of theoretical plates, N, of the columns and Table III lists the resolution, R_s, established graphically for neighbouring peaks. It is evident that the loss of efficiency caused by complexation processes is relatively small, not exceeding 12–24 % of the reference value for the column containing formamide alone. Because of the gain in

TABLE II

NUMBER OF THEORETICAL PLATES, N, OF THE COLUMNS AT 60°C FOR DIFFERENT CONCENTRATIONS OF β-CD IN FORMAMIDE SOLUTION

Concentration of β-CD in formamide (mol %)	$N^\star$
0	1250
0.59	950
0.88	1050
1.18	1100

$\star$ Calculated on the basis of the peak of o-xylene.

TABLE III

RESOLUTION, R_s, FOR o-, m-, p-XYLENES AND ETHYLBENZENE AT DIFFERENT CONCENTRATIONS OF β-CD AND AT DIFFERENT TEMPERATURES

Concentration of β-CD in formamide (mol %)	R_s	Temperature		
		$50°C$	$60°C$	$70°C$
0.0	$\dfrac{R_{etb,\,p\text{-}X}}{R_{p\text{-}X,\,m\text{-}X}}$		<0.40	
	$R_{m\text{-}X,\,o\text{-}X}$	1.81	1.92	1.69
0.30	$R_{m\text{-}X,\,p\text{-}X}$	1.25	1.17	0.80
	$R_{p\text{-}X,\,etb}$	1.10	1.14	0.67
	$R_{etb,\,o\text{-}X}$	2.22	2.00	1.86
0.59	$R_{m\text{-}X,\,p\text{-}X}$	1.26	1.20	1.20
	$R_{p\text{-}X,\,etb}$	1.13	1.16	1.09
	$R_{etb,\,o\text{-}X}$	1.53	1.42	1.00
0.88	$R_{m\text{-}X,\,p\text{-}X}$	1.56	1.33	1.40
	$R_{p\text{-}X,\,etb}$	1.25	1.25	1.45
	$R_{etb,\,o\text{-}X}$	1.29	1.29	1.00
1.18	$R_{m\text{-}X,\,p\text{-}X}$	1.88	1.52	1.38
	$R_{p\text{-}X,\,etb}$	1.60	1.47	1.44
	$R_{etb,\,o\text{-}X}$	1.57	1.33	1.05

selectivity with molecular inclusion, these systems exhibit better resolution towards the compounds investigated.

Taking into account the time of separation and the values of R_s as a function of β-CD concentration and of temperature, it may be concluded that the following conditions are suitable for the analysis of o-, m- and p-xylene and ethylbenzene mixtures: β-CD concentration, 1.18 ml %; temperature, 60°C. Under these conditions the chromatographic columns are stable and may be used for several weeks.

Therefore we have obtained an affirmative answer for the question posed at the outset of this work. β-CD can modify the resolution properties of partition GC systems under suitably chosen conditions. The observed stereoselectivity seems to be a very promising feature for various chromatographic separations.

The opinion generally accepted until quite recently, that CDs form inclusion complexes exclusively in aqueous solutions, does not therefore seem to be correct. Formamide itself constitutes a suitable liquid matrix for β-CD inclusion processes.

In the course of this work special attention was paid to maintaining constant values of the inlet pressure and helium carrier gas flow velocity. Under these conditions it was possible to compare the relative stabilities of β-CD complexes while having no means of establishing their exact values[5-8]. This evaluation was performed on the assumption that only complexes of 1:1 stoichiometry are formed and hence

$$t'_{\beta\text{-CD}} = t'_0 \left(1 + K\,[\beta\text{-CD}]\right)$$

where $t'_{\beta\text{-CD}}$ and t'_0 are respectively the adjusted retention times of a solute on the column containing β-CD in formamide solution and on the reference column contain-

ing pure formamide; K is the stability constant of a 1:1 β-CD complex, *e.g.*, for *o*-xylene;

$$K_{ortho} = \frac{[o\text{-}X \cdot \beta\text{-}CD]}{[o\text{-}X] [\beta\text{-}CD]}$$

For dilute β-CD solutions ([β-CD] < 0.296 mol %) the following sequence has been etablished at 60°C (etb = ethylbenzene):

$$K_{ortho} < K_{meta} < K_{para} < K_{etb}$$

This sequence changes in more concentrated β-CD solutions, where the above straight line depencence (for 1:1 stoichiometry) is no longer valid, and may suggest that complexes of other than 1:1 stoichiometry are formed. Further studies on the application of CDs in partition GC are in progress.

ACKNOWLEDGEMENTS

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ION-EXCHANGE DERIVATIVES OF SPHERON

IV★. PHOSPHATE DERIVATIVES

O. MIKEŠ★, P. ŠTROP and Z. HOSOMSKÁ

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6 (Czechoslovakia)

M. SMRŽ

Research Institute of Pure Chemicals, Lachema 621 33, Brno (Czechoslovakia)

J. ČOUPEK and A. FRYDRYCHOVÁ

Laboratory Instruments Works, 162 03 Prague 6 (Czechoslovakia)

and

M. BAREŠ

Institute of Chemical Technology, 166 28 Prague 6 (Czechoslovakia)

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SUMMARY

Medium acidic phosphate derivatives of the hydroxyethyl methacrylate macro-porous gels Spheron 300 and 1000 were prepared by several procedures. The resulting cation exchangers had nominal capacities of 0.20–4.08 mequiv./g and were characterized by their capacity for small ions, static and dynamic capacities for proteins, elemental analysis, operating volume and inner surface area. Some samples were titrated potentiometrically to the first and second dissociation degrees and the results discussed in terms of the phosphorus content. The relationships between the nominal capacity and phosphorus content, between nominal capacity and static capacity for proteins and between the static and the dynamic capacity for proteins were investigated and discussed. Chromatographic experiments on a mixture of serum albumin, chymotrypsinogen and lysozyme showed a dependence of retention volumes on the nominal capacity. Applications to the separations of egg-white proteins and of cellulolytic enzymes from a cultivation liquid of *Trichoderma viride-reesei* are described. Experiments with the cation exchanger Spheron 300 phosphate, used as an "immobilized acid" for catalysis (esterification of alcohol and inversion of saccharose), are also reported.

INTRODUCTION

New types of ion exchangers based on the macroreticular poly(2-

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★ For Part I see ref. 2; for Part II, ref. 3 and for Part III, ref. 4.

hydroxyethyl)methacrylate, trade-name Spheron (Lachema, Brno, Czechoslovakia) or Separon HEMA (Laboratory Instruments Works, Prague, Czechoslovakia), have been developed since 1975 for the rapid chromatography of biopolymers and their fragments. In our introductory work[1], these ion exchangers were described and examples presented of the chromatography of proteins, polypeptides, amino acids, nucleic acids, oligonucleotides and nucleotides. The hydrodynamic properties of these new packings for high-performance liquid chromatography (HPLC) of biopolymers were also discussed. We subsequently reported[2] the characterization of the polymer matrix, in particular its macrostructure and porosity, and described in detail[3] the medium-basic diethylaminoethyl (DEAE) derivatives of Spheron, including examples of their application and a method of determination of the static capacity for proteins. The most recent paper[4] in this series dealt with the weakly acidic carboxylic derivatives of Spheron and described a determination of the dynamic capacity for proteins.

These ion-exchange derivatives of Spheron and other derivatives not yet described in detail have found broad application in biochemistry for the separation of technical enzymes[5-7] and other proteins and their fragments[8], in the chromatography of mono- and oligosaccharides[9,10] including cellodextrins[11], mono- and oligonucleotides[12,13] and organic acids[14]. The rapid chromatographic analysis of enzymes and examples of the application of Spheron ion exchangers have been reviewed[15-17].

This paper describes medium-acidic phosphate Spheron cation exchangers and their application in the rapid chromatography of proteins and in catalysis (*cf.*, ref. 18). A part of the results have been presented in a preliminary form[19].

EXPERIMENTAL

Materials

Spheron 300 (beads 10–20 and 20–40 μm) and 1000 (beads 20–40 and 40–60 μm) were products of Lachema. Spheron 300 was extracted before ionogenic modifications, as described[2]. Phosphorylated derivatives of hydroxyethyl methacrylate gels of capacity 2.20 or 2.28 mequiv./g, applied in catalysis, were prepared according to ref. 20 from non-extracted Spheron 300 (particle size 300–400 μm, specific inner surface area 103 m^2/g).

Proteins employed for testing chromatographic properties were those used earlier[1-4]. Phosphoryl chloride, amines and solvents required for ionogenic modifications, *n*-butanol, acetic acid and saccharose used for testing the catalytic efficiency of phosphorylated derivatives and all inorganic compounds and chemicals for determination of cellulolytic activity were supplied by Lachema. Whatman No. 1 chromatographic paper was supplied by Whatman Labsales (Maidstone, Great Britain). The cultivation liquid *Trichoderma viride-reesei* was kindly provided by the Research Institute of Food Industry, Prague.

Preparation of ion exchangers for chromatographic application

Spheron phosphates were prepared by reaction of phosphoryl chloride with Spheron as shown in Scheme I in Results and Discussion. One of the laboratory procedures is given in the legend of Fig. 1, which shows the effect of reaction time on the course of ionogenic substitution. Further modifications are described in Table I,
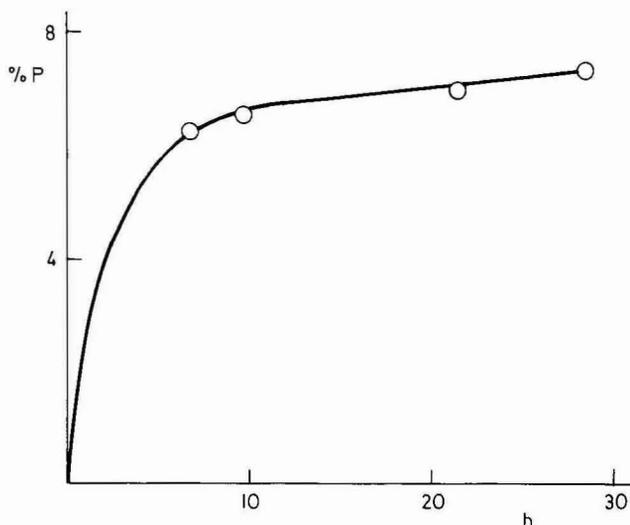
Fig. 1. Effect of reaction time on the ionogenic modification of Spheron. 2.5 g dry Spheron 300 (fraction of particle size 25 μm) dispersed in 25 ml ethyl acetate were phosphorylated, with stirring at $-25°$C, with 5 ml POCl$_3$ in the presence of excess of trimethylamine. The samples for analysis were washed with water and acetone, allowed to stand in 2 M NaOH for 2 h at laboratory temperature and further washed with water, ethanol, acetone and diethyl ether and dried.

which presents the effect of temperature, and in Table II, which gives characteristics of a series of ion exchangers with increasing nominal capacities.

In the preparation according to Scheme II (Results and Discussion), 10 g of Spheron were first transformed into a glycidyl derivative by alkaline swelling overnight in 50 ml of 50 % potassium hydroxide at 10°C, followed by rapid filtration by suction and dispersion in 40 ml of epichlorohydrin in dioxan. After boiling under

TABLE I

INFLUENCE OF REACTION CONDITIONS ON CHLORINE AND PHOSPHORUS CONTENTS IN THE PREPARATION OF SPHERON PHOSPHATES

A 10-g amount of dried Spheron 300 (particle size 25 μm) was dispersed in 100 ml dry ethyl acetate at $-30°$C and, in the presence of 20 ml dry triethylamine, substituted stepwise by addition of 20 ml phosphoryl chloride dissolved in ethyl acetate for the given time. In the case of sample c, the temperature was increased to $+25°$C. The samples for analysis were treated as described in Fig. 1; they are not further characterized in Table III.

Sample	Reaction time (h)		Elemental analysis (%)	
	At -25 to $-30°$C	At $+25°$C	Cl	P
a	1	—	0	2.68
b	3	—	0	3.76
c	3	15	3.20	5.27
d	6	—	0.12	7.25
e	7★	—	0.19	6.95

★ Pyridine was used instead of triethylamine and activated carbon was added after addition of POCl$_3$.

TABLE II

PREPARATION OF SPHERON PHOSPHATES OF INCREASING NOMINAL CAPACITY

Spheron 1000 (particle size 20–40 μm) was dispersed in chloroform, mixed with phosphoryl chloride at 0–5°C and pyridine dissolved in chloroform was added dropwise. After complete addition of pyridine, the mixture was stirred for another 2 h at the given temperature, filtered, washed and then subjected to hydrolysis in sodium bicarbonate solution. The resulting ion exchanger was cycled.

Sample No.[*]	Phosphoryl chloride (mmol/g of gel)	Pyridine (mmol/g of gel)	Nominal capacity[**] (mequiv./g)
7	1.09	1.2	0.29
8	2.18	2.4	0.45
9	3.27	3.6	0.73
10	4.36	4.8	1.24
11	6.53	7.2	2.34
12	7.85	8.6	3.14
13	12.00	13.2	4.18

[*] Numbering as in Table III.
[**] In contrast to the data in Table III, the values were read from titration curves.

reflux for 3 h the required derivative was collected on fritted glass filter and washed three times with acetone, once with water, three times with acetone and three times with diethyl ether. This glycidyl-Spheron 300 (0.3 g) was mixed with 2 g sodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) and 3 ml water and heated in a sealed tube to 98°C for 90 h. The product was refined and cycled by stepwise washing with water, 2 M hydrochloric acid, water, ethanol, acetone and diethyl ether and dried under vacuum.

We also tried to prepare Spheron phosphate according to Scheme III (Results and Discussion): 0.5 g Spheron 100 and 2 g sodium hydrogen phosphate were heated with 1.5 ml water in a narrowed-neck tube which was placed in a heating block at 175°C as long as water distilled off. After cooling a second portion (1.5 ml) of water was added and the process was repeated 10–15 times over 7 h. The product was washed with water, 0.5 M hydrochloric acid, water, methanol and acetone and dried.

Chromatographic methods and evaluation of fractions

The procedures were described earlier[1-4] (determination of static capacity, see Fig. 1[3]; determination of dynamic capacity, see Fig. 4[4]), as were the chromatographic instrumentation, packing of columns, regeneration (2 M sodium chloride), cycling (2 M sodium hydroxide, 2 M hydrochloric acid) and equilibration of Spheron ion exchangers. The effluent from chromatographic columns was continuously evaluated by measuring its absorbance at 285 and 254 nm; the conductivity and pH of collected fractions were also determined. The activity of cellulolytic enzymes (so called filter-paper activity) was ascertained for 100-μl aliquots of collected fractions according to Mandels and co-workers[21,22] using squares (1 × 1 cm) of Whatman No. 1 paper and incubation in 1 ml 0.05 M citrate buffer (pH 4.7) for 3–14 h at 40°C; 200 μl of the supernatant were then used for the determination of reducing sugars according to Somogyi[23] and Nelson[24]. For details see the disertation of Hostomská[25].

Measurement of the catalytic activity of Spheron phosphates

n-Butanol was continuously esterified in a vertical glass cylinder (100×3 cm I.D.) furnished with a thermostating jacket and heated by circulation of heating oil. A thermometer probe (thermocouple) was placed in the column axis. The column consisted of three sections: the upper inlet section contained a 19-cm layer or a preheating packing of porous ceramics followed by a 2-cm layer of glass wool; the central section contained a 56-cm layer of ion-exchange catalyst (Spheron phosphate, 2.28 mequiv./g) and a 2-cm layer of glass wool; the lower discharge section consisted of 19 cm of ceramic packing resting on a 2-cm layer of glass wool. The column was connected through a pump and a flow-controlling valve to a reservoir of processed liquid. The outlet of the column led to a separation vessel.

Continuous inversion of saccharose was carried out in a similar column (110×1.5 cm I.D.); the height of the catalyst section (Spheron phosphate, 2.20 mequiv./g) was 51 cm and the volume of this section was 90 ml.

RESULTS AND DISCUSSION

The phosphorylated derivatives of Spheron

Medium-acidic cation exchangers of the Spheron phosphate type are best prepared by the reaction of phosphoryl chloride with Spheron[1,2] or Separon HEMA[26] (Sph–OH), dispersed in an organic solvent in the presence of a base, followed by hydrolysis:

$$\text{Sph–OH} + \text{ClPOCl}_2 \xrightarrow{-\text{HCl}} \text{Sph–O–POCl}_2 \xrightarrow{+\text{H}_2\text{O}} \text{Sph–OPO(OH)}_2 \qquad \text{(I)}$$

Analytical characteristics of the derivatives prepared by this method are listed in Table III.

Other methods of preparation were also studied, *e.g.* via the glycidyl derivative (prepared by use of epichlorohydrin, Scheme IIa) and the reaction with sodium phosphate (Scheme IIb):

$$\text{Sph–OH} + \text{ClCH}_2\text{–CH–CH}_2 \xrightarrow{\text{KOH}} \text{Sph–O–CH}_2\text{–CH–CH}_2 \qquad \text{(IIa)}$$

$$\text{Sph–O–CH}_2\text{–CH–CH}_2 + \text{Na}_2\text{HPO}_4 \rightarrow \text{Sph–O–CH}_2\text{–CH–CH}_2\text{–O–PO(ONa)}_2 \qquad \text{(IIb)}$$
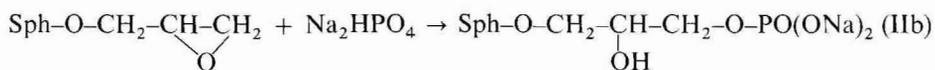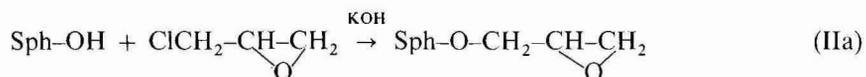
The attempted preparation by heating Spheron with sodium dihydrogen phosphate is illustrated by Scheme III:

$$\text{Sph–OH} + \text{HOPO(ONa)}_2 \xrightarrow{-\text{H}_2\text{O}} \text{Sph–O–PO(ONa)}_2 \qquad \text{(III)}$$
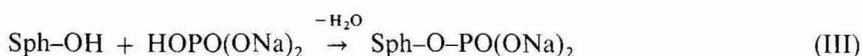
Other procedures, not described, were also tried. However, the route in Scheme I proved most suitable. It is of great importance in this case that the reaction product is the pure ion exchanger with a nominal capacity as high as possible, and it should not

TABLE III

ANALYTICAL CHARACTERISTICS OF THE PREPARED SPHERON PHOSPHATES

Data for the initial non-substituted Spherons are given for comparison, *cf.*, also refs. 2–4. Properties of the initial Spherons 300 and 1000 are given in ref. 2. Both types differ in pore size and in exclusion limit; the latter for Spheron 300 (after extraction) is 300,000–500,000 Daltons and for Spheron 1000 is 10^6 Daltons.

Property	Unit	Non-substituted Spheron		Cation exchangers	
		1	*2*	*3*	*4*
Starting type		300	1000	300*	300
Particle size	μm	20–40	20–40		40–60
Nominal capacity					
calculated (bifunctional groups)	mequiv./g	–	–	4.21	2.25
determined**	mequiv./g	0.03	0.04	3.00	1.50
Elemental analysis					
C	%	55.68	55.47	46.25	51.33
H	%	7.56	7.57	6.26	6.47
P	%	0	0	6.52	3.49
Cl	%	0	0	1.42	0
Ashes	%	0	0		0
Dry substances	%	100	96.69		97.66
Working volume**					
in 2 *M* NaOH	ml/g	3.95***	3.80	2.7	4.15
in 2 *M* HCl	ml/g		3.75	2.5	4.10
Specific inner surface area					
by Klyaschko Gurwich method**	m^2/g	42–62	139–155	55	102
Capacity for serum albumin (desorption)**					
by static method	mg/g	2–3	12		
by dynamic method	mg/g		5.5		

   * Spheron was subsequently twice phosphorylated and the preparation contained 0.16 % N.

  ** The methods are described in refs. 2–4.

  *** Valid for distilled water.

contain any chlorine which may result in undesirable reactions. As is seen from the analyses in Table III, our procedures for ion exchanger preparation meet these requirements quite well.

One problem in the preparation of medium-acidic Spheron phosphate cation exchangers was that most samples exhibited a higher content of phosphorus than that corresponding to the capacity for small ions[2] as determined by titration of the presumed bifunctional group (*cf.*, Tables III and IV). This can be explained by a partial reaction of phosphoryl chloride with two adjacent hydroxyl groups on the surface of Spheron according to Scheme IV:

$$\text{Sph}\overset{\text{OH}}{\underset{\text{OH}}{\diagdown}} \; + \; \overset{\text{Cl}}{\underset{\text{Cl}}{\diagup}}\text{POCl} \rightarrow \text{Sph}\overset{\text{O}}{\underset{\text{O}}{\diagdown}}\text{PO–OH} \tag{IV}$$

5	6	7	8	9	10	11	12	13
300	300	1000	1000	1000	1000	1000	1000	1000
40–60	40–60	20–40	20–40	20–40	20–40	20–40	20–40	20–40
3.54	4.22	0.17	0.30	0.70	1.50	2.94	3.38	5.04
2.60	3.40	0.20	0.37	0.65	1.21	2.21	3.04	4.08
47.62	46.66	55.04	55.06	53.35	50.80	48.31	46.06	42.00
6.64	7.02	7.37	7.57	7.46	7.13	6.93	6.93	6.90
5.48	6.54	0.18	0.47	1.08	2.32	4.56	5.24	7.81
0.34	0.36	0	0	0	0	0	0	0
0.37	0.28	0	0	0	0	0.38	0.19	0.31
97.38	97.41	96.43	96.57	97.53	97.05	96.45	95.73	95.60
4.0	3.8	3.75	3.68	3.50	3.50	3.40	3.25	3.10
3.65	3.65	3.70	3.65	3.45	3.45	3.30	2.90	2.80
100	100	73	88	96	92	99	94	92
		108	164	178	216	220	224	196
			14.5		21.8	23.7		27.5

The partial formation of polyphosphate chains in the anhydrous medium can also be considered, as in Scheme V:

$$\text{Sph–OH} + 2\ \text{ClPOCl}_2 \rightarrow \underset{\underset{\text{OH}}{|}}{\text{Sph–O–PO–O–PO(OH)}_2} \tag{V}$$
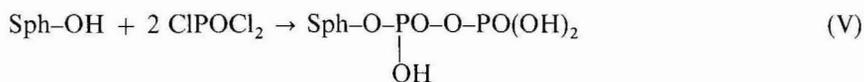
As follows from Schemes IV and V, the exclusive occurrence of the first reaction (IV) would cause a more significant lowering of the nominal capacity, *i.e.*, from a ratio $2H^+$:P for Scheme I to H^+:P for Scheme IV. On the other hand, the exclusive reaction of type V would decrease the capacity only to the ratio $3H^+$:2P.

The potentiometric titration of samples of Spheron phosphates carried out to the first degree (Table IV) always agreed with the content of phosphorus found by elemental analysis. On the contrary, potentiometric titration to the second degree (Table IV) showed a clearly lower value than that corresponding to the content of

TABLE IV

PHOSPHORUS CONTENTS AND CALCULATED AND MEASURED CATION-EXCHANGE CAPACITIES OF SPHERON PHOSPHATES FOR BOTH DISSOCIATION DEGREES

Numbers of samples 4–6 as in Table III. Samples f and g were prepared in small amounts and are not characterized in Table III.

Sample No.	Content of P		Nominal capacity (mequiv./g)	Experimentally determined		
	%	*mmol/g*	*Calculated from P content for the bifunctional group*	*Potentiometrically, read from titration curves to the degree*		*In effluent after passing titrated hydroxide through the column (H^+) and reverse titration using indicator according to ref. 2*
				I	*II*	
f	0.90	0.29	0.58	0.30	0.48	
4	3.49	1.13	2.25	1.02	1.62	
5	5.48	1.77	3.54	1.75	2.86	1.50
6	6.54	2.11	4.22	2.18	3.61	2.60
g	7.10	2.29	4.58	2.29	3.66	3.40

phosphorus. The nominal capacities, determined by filtration of an excess of titrated hydroxide through the ion-exchanger column according to ref. 2, exhibited even lower values (Table IV). This problem has not yet been solved fully, but it can be supposed that some rôle is played by those dissociable protons which would be potentiometrically titrated in the second degree, but which are eliminated by the formation of phosphoric diesters (IV) or polyphosphates (V).

Static and dynamic capacity of Spheron phosphates for sorption of serum albumin

The static sorption capacity of Spheron cation exchangers for proteins, as determined in slowly rotating sealed test-tubes[3], was first measured in several buffer solutions (pH 3–6) to find the optimal pH (see Fig. 2). The strongly acidic buffers prevented accurate measurements due to the formation of a turbidity in the solutions, in particular during desorption; on the other hand, the sorption decreased in the alkaline pH region. However, a shoulder was found in the dependence of static sorption on pH, for this ion exchanger, and the optimal pH, which enabled measurement of sorption and desorption, was at pH 5, *i.e.*, near the isoelectric point of serum albumin (pH 4.7). This is in contradiction to the usual belief that proteins are best sorbed on ion exchangers at a pH which differs by at least one pH unit from the isoelectric point (*cf.*, ref. 27).
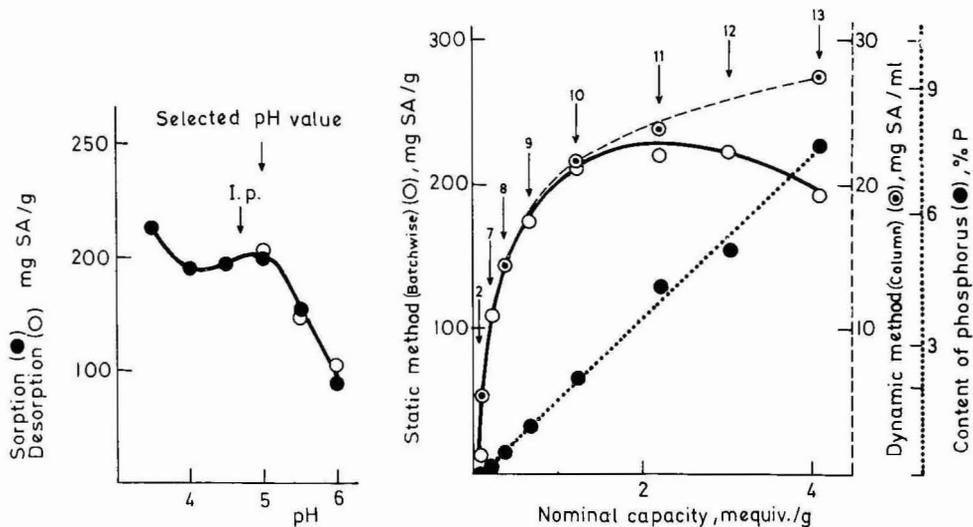
Fig. 2. Search for the optimal pH for the sorption of serum albumin on Spheron phosphate 300 of nominal capacity 3.4 mequiv./g (sample 6 in Table III), equilibrated with 0.1 *M* sodium acetate at various pH values. The sorption (and desorption in the same buffers adjusted to 1 *M* with NaCl) was studied under conditions described for the determination of static capacity by the batch method[3]. I.p. = Isoelectric point of serum albumin.

Fig. 3. Relationship between the nominal capacity of Spheron phosphates and the phosphorus content and between the static and dynamic capacities for sorption of serum albumin and the nominal capacity for small ions (Na+). The static capacity was determined according to ref. 3 by a batch method in the presence of excess of protein in a sorption buffer (0.1 *M* sodium acetate, pH 5) as long as the equilibrium was established, followed by desorption with the same buffer enriched with 1 *M*.NaCl. The dynamic capacity was determined at flow through a column according to ref. 4, under conditions given in Fig. 4. The sample numbers are as in Table III.

We have found (cf., Fig. 4 in ref. 3), that the static sorption on DEAE-Spheron anion exchanger follows a steep convex curve depending on its nitrogen content, whereas the capacity for small ions, e.g., Cl^-, has a linear dependence. We have also described the similar steeply convex dependence of the static capacity for serum albumin on the nominal capacity of carboxylic cation exchangers (CM-Spheron and Spheron C; cf., Fig. 7 in ref. 4), and explained this feature on the basis of the multiple sorption of proteins on these ion exchangers under the conditions used to determine the static capacity[3,4]. In the present study of medium-acidic cation exchangers of the Spheron phosphate type, we have confirmed the earlier findings, as follows from Fig. 3. Thus the nominal capacity for small ions (Na^+) is roughly linearly dependent on the phosphorus content. The static capacity for serum albumin again steeply increases at low nominal capacities for small ions up to values of 2–3 mequiv./g. From a comparison of the results obtained with the three mentioned types of ion exchangers, it is obvious that the relationships between the capacity for small ions and for proteins follow the same general rule, thus confirming and generalizing the above hypothesis[3,4]. However, at even higher values of nominal capactiy a distinct drop in the static capacity occurs. A weak indication of this phenomenon was also found in Fig. 7 of ref. 4 where the static capacity of sample 4 is somewhat lower than that of sample 3. At that time[4], we ascribed this to possible scatter due to experimental errors. The phenomenon is much more pronounced in the present Fig. 3, because the phosphate ion exchangers have higher nominal capacities. Further experiments will be required to elucidate completely the reasons for this behaviour.

The measurement of the dynamic capacity for serum albumin (SA) is illustrated in Fig. 4 and the values found are given in Table III. The relationship between the static and dynamic capacities (Fig. 3) is surprisingly different from the expected nearly linear relationship between dynamic capacity and nominal capacity, found earlier for proteins on the carboxylic derivatives of Spheron (Fig. 7 in ref. 4). The dynamic capacity first follows a convex dependence similar to the static capacity, but with much lower values (these are 3–4 times higher after recalculation of volume data in mg SA/ml to weight data in mg SA/g), and increases almost linearly only above the nominal capacity of about 1.2 mequiv./g. It may be concluded that the sorption is controlled by at least two different mechanisms, depending on the nominal capacity. The bifunctional phosphate groups obviously adsorb proteins at low capacities relatively more intensively than do monofunctional groups, so that the course of the dynamic sorption at low nominal capacities may be similar to that of the static sorption.

Chromatographic experiments

Spheron phosphates are well suited as medium-acidic ion exchangers for the rapid chromatography of proteins. They have relatively high nominal capacities and suitable flow-through properties. The examples given below show that for chromatographic purposes it is not necessary further to increase their capacity. Examples of application of Spheron phosphates to the chromatography of proteins have been presented in previous papers[1,6–8,15]. For this reason, we now describe only an example illustrating the effect of nominal capacity on the separation of a synthetic mixture of proteins and two examples of the separation of protein mixtures.

Separation of a synthetic mixture of human serum albumin (S), bovine chymo-
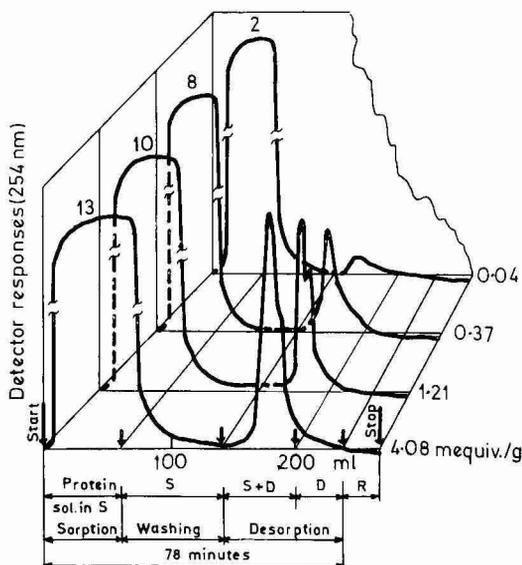
Fig. 4. Determination of the dynamic capacity of Spheron phosphates for serum albumin by the method of ref. 4 in a chromatographic column of 0.8 cm I.D. containing 10 ml of ion exchanger (height of packing about 20 cm). The numbers of the individual chromatographic profiles correspond to the ion-exchanger samples in Table III; their nominal capacities are given on the right. A 5 % solution of serum albumin in the sorption buffer (S) was pumped through the column which had previously been equilibrated with the buffer S. After saturation with protein, the column was washed with buffer S to remove serum albumin which was not ionically bonded, and then the desorption was carried out. The flow-rate was 3 ml/min during the whole experiment; 20°C; pressure drop 0.4–0.5 MPa. S = Sorption buffer, 0.1 M sodium acetate, pH 5; D = desorption buffer of the same composition, enriched with 2 M NaCl; S + D = region of linear gradient; R = regeneration with non-buffered solution of 2 M NaCl. The desorption effluent was collected, its volume measured and analyzed spectrophotometrically for the content of eluted serum albumin, which gave the dynamic capacity (mg SA/ml) of the ion-exchanger bed.

trypsinogen (C) and chicken lysozyme (L). Elution was first attempted on a column (0.8 × 20 cm) containing Spheron phosphate of high capacity (4.08 mequiv./g) equilibrated with the first buffer, using a series of linear-gradients of buffers with simultaneously increasing pH and ionic strength in the region from 0.05 M sodium formate (pH 3.5) to 1 M sodium acetate (pH 8); in the last gradient 1 M sodium acetate (pH 8) was used with the same buffer enriched to 1 M sodium chloride. Because serum albumin tended to be eluted together with chymotrypsinogen under these con-- ditions, a series of experiments were carried out with a gradient of ionic strength only, at pH 4, 5, 5.5, 6, 6.5 and 7.5 respectively. The results obtained resembled closely those from similar experiments carried out previously with the carboxylic cation exchanger (Fig. 10 of ref. 4); only the pH value at which S and C start to separate was different. Only two peaks (S and C were combined) were observed in both series of experiments at low pH, whereas the proteins S, C and L were separated at higher pH, but S and C were separated discontinuously (*cf.*, the discussion in ref. 4 relating to Fig. 10). The experiment with CM-Spheron[4] mentioned above showed a threshold pH value of 6.25, whereas for Spheron phosphate this appears at pH 5.5. Because this interesting chromatographic behaviour occurred with two different Spheron ion exchangers, we presume a similar interaction of both proteins. The value of pH 6.5 was chosen as optimum for further separations.

The following experiments were carried out to investigate the effect of the degree of ionogenic substitution on the separation of the mixture of proteins S, C and L under optimal conditions, starting with the unsubstituted Spheron (low nominal cation-exchanging capacity of 0.04 mequiv./g due to a trace of irremoved carboxyls and proceeding to the strongly modified Spheron phosphate (4.08 mequiv./g). In contrast to the earlier experiments with DEAE-Spheron[3] and CM-Spheron[4] where the ion exchanger with highest capacity proved most efficient, the present experiments revealed that the mixture of proteins is well separated on the ion exchanger with lower nominal capacities (*cf.*, Fig. 5). Of interest was the shifts in position of the peaks of the basic lysozyme L with decreasing capacity; these first appeared at higher retention volumes and then rapidly dropped to the hold-up volume, in contrast to an expected continuous decrease with decreasing capacity. This behaviour can be explained on the basis of an antagonistic effect of the spontaneously decreasing pH with the increasing nominal capacity upon the retention of absorbed proteins. The same tendency, although not so pronounced, can be seen with the other proteins C and S. It is obvious that Spheron phosphate with bifunctional groups differs from DEAE-Spheron and CM-Spheron not only in the dependences of the static and dynamic capacities for proteins
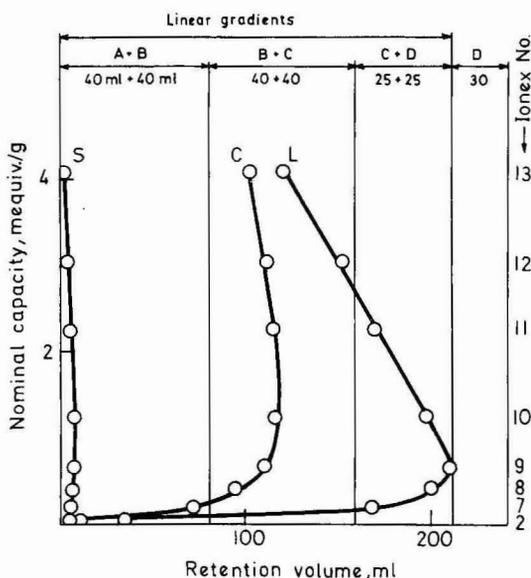
Fig. 5. Dependence of separation of 20.5-mg mixture of serum albumin S (10 mg), chymotrypsinogen C (7 mg) and lysozyme L (3.5 mg) on the nominal capacity of Spheron phosphate of particle size 20–40 μm. The column dimensions were in all cases 20 × 0.8 cm I.D. and the mixture of proteins was applied in 0.3 ml of the first buffer, which was also used for equilibration of the ion exchangers. The numbers of the ion exchangers are as in Table III. The points indicate the positions of chromatographic peak maxima. All buffers were prepared from ammonium hydroxide of the following concentrations adjusted by acetic acid to pH 6.5: A, 0.05 *M*; B, 0.3 *M*; C, 1 *M*; D, non-buffered 2 *M* NaCl. The flow-rate was 3 ml/min; pressure drop 0.5–0.9 MPa; ambient temperature. The pH during elution with the linear gradients was constant at 6.5 for unsubstituted Spheron (No. 2, Table III) but decreased for cation exchangers in the region between the first and second gradient, becoming more pronounced with increasing nominal capacity (from pH 6.4 to 6.2, whereas a slow but incomplete return to the original pH value took place with further elution at the end of gradients).
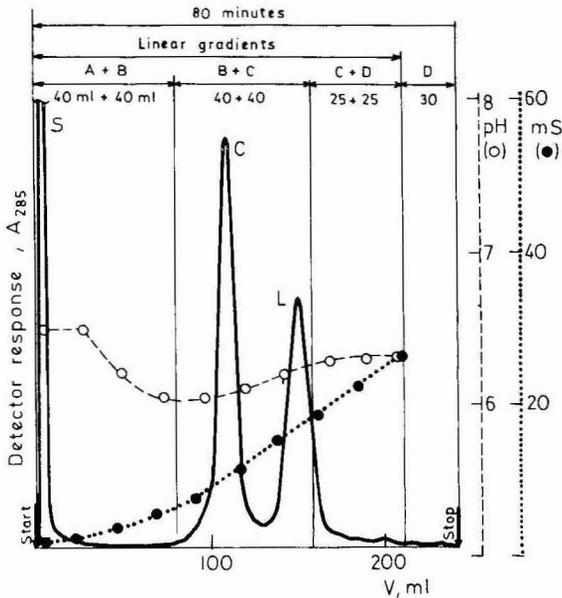
Fig. 6. Separation of serum albumin (S), chymotrypsinogen (C) and lysozyme (L) on Spheron phosphate 1000 of capacity 3.04 mequiv./g (preparation 12, Table III). For chromatographic conditions see Fig. 5. The volume of effluent, V, is shown on the horizontal axis.

on the nominal capacity for small ions, but also in the chromatographic retention. An example of the chromatographic profile of the separation of three proteins is shown in Fig. 6.

The egg-white proteins were chosen as an example of a natural mixture of proteins, as previously for CM-Spheron (Figs. 8 and 9 in ref. 4). Experiments only with a gradient of ionic strength in ammonium acetate buffers at pH 4, 7 and 9 failed. However, simultaneously increasing ionic strength and pH from 4.5–5.5 to 8–9 proved suitable for the separation of these proteins. In contrast to the behaviour shown in Fig. 5, the ion exchangers with the highest nominal capacities gave the best separation in these experiments, as with other types of Spheron ion exchangers. Fig. 7 shows the chromatogram obtained using Spheron phosphate of high capacity (4.08 mequiv./g). A reasonable separation was even obtained with ion exchangers of lower capacity down to 2.27 mequiv./g. Still lower capacities led to an unsatisfactory separation; the peaks broadened and the unsubstituted starting Spheron 1000 with gradients of ionic strength and pH did not separate the egg proteins.

An example of enzyme separation on Spheron phosphate is shown in Fig. 8 for the chromatography of cellulolytic enzymes from a cultivation liquid *Trichoderma viride-reesei*. The so-called "filter-paper activity" was only tested in this experiment, and was found to be fairly retained, although the ion exchanger (No. 3, Table III) contained small amounts of bonded chlorine. Other types of enzymes from the cellulolytic system of this organism retained full enzyme activity during their separation on Spheron phosphates. The separation of cellulolytic enzymes on Spheron ion exchangers proved very suitable[25] and will be reported in a separate paper[28].
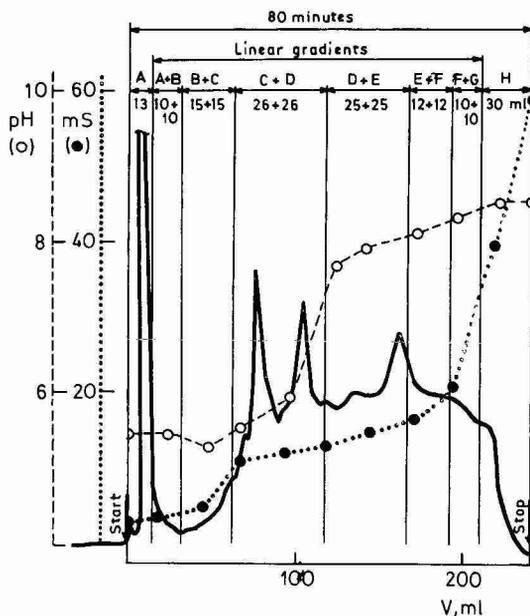
Fig. 7. Chromatography of 20-mg egg-white protein preparation on a column (20 × 0.8 cm I.D.) packed with Spheron phosphate 1000 of capacity 4.08 mequiv./g and particle size 20-40 μm (preparation 13, Table III). Buffers A and B were prepared from 0.01 M and 0.2 M NaOH adjusted to pH 5.5 and 6.5, respectively, with acetic acid. Buffers C (0.4 M), D (0.6 M), E (0.6 M), F (0.6 M) and G (0.6 M) were prepared from Tris adjusted with acetic acid to pH 7.5, 8.0, 8.5, 9.0 and 9.0 and enriched with 0.1 M, 0.2 M, 0.4 M, 1.0 M and 2.0 M NaCl. The non-buffered regeneration solution (H) was 2 M NaCl. Flow-rate 3 ml/min, 25°C, pressure drop 0.6 MPa. The fractions were collected every 90 sec.

Catalytic properties of Spheron phosphates

Cation exchangers in the hydrogen form are "immobilized acids" and may serve as catalysts in reactions which are otherwise catalyzed by hydrogen ions. We chose two types of such reactions to study the catalytic properties of Spheron phosphates: (a) continuous esterification of acetic acid with *n*-butanol and (b) the continuous inversion of saccharose.

The catalyst of initial nominal capacity 2.28 mequiv./g was used in the experiments on esterification of acetic acid with *n*-butanol. Best results (90 % conversion of acid) were achieved at the acid:alcohol molar ratio of 1:1.2, 116°C and flow-rate 20 ml/h. When the highest conversion degree was attained, the product formed on cooling was a two-phase system with the mixture of ester and alcohol in the upper layer. At lower degrees of conversion the product formed one phase. The degree of conversion of acid (but not the relative content of ester in the resulting mixture) rose with increasing proportion of alcohol (from 1:1 to 1:2), but decreased with decreasing temperature (from 110 to 60°C) or with increasing flow through. At flows higher by one order of magnitude (about 200 ml/h) the degree of conversion is very low. Traces of phosphorus were observed in the reaction products (*e.g.*, 2.5 × 10^{-3} %), slightly increased in amount with temperature at 60–70°C and rapidly increased above 85–90°C. A conversion degree of 74 % was obtained at 79°C, the acid:alcohol molar ratio
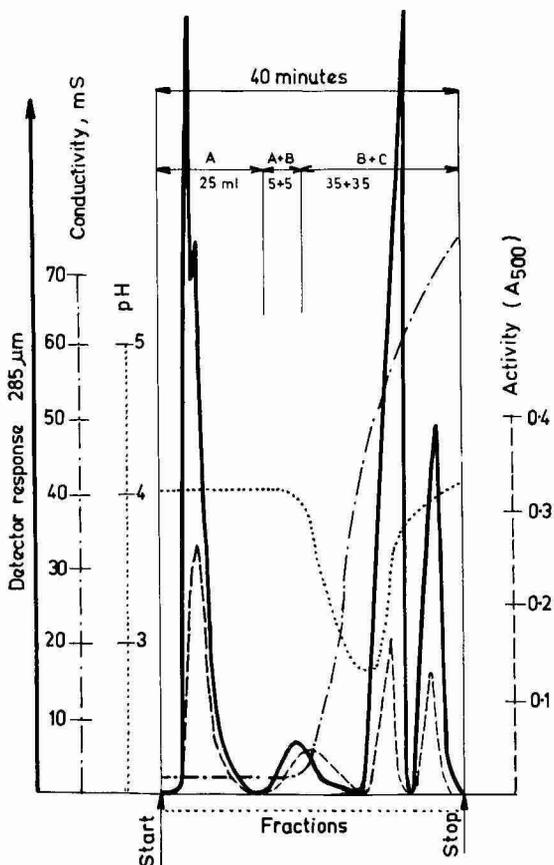
Fig. 8. Medium-pressure chromatography of a cellulolytic preparation from the cultivation liquid *Tricho-derma viride-reesei* on a column (20 × 0.8 cm I.D.) with Spheron phosphate 300 of capacity 3.0 mequiv./g (sample 3, Table III). Buffers: A, 0.05 M NaOH adjusted with acetic acid to pH 4; B, the same buffer enriched to 1 M NaCl; C, the same buffer enriched to 3 M NaCl. Flow-rate 2.4 ml/min, 20°C. Fractions of volume 4.8 ml were collected in 2-min intervals: pressure drop 0.5 MPa. A method for measurement of cellulolytic activity by the analysis of reducing sugars is described in the text.

being 1:2 and the flow through 67 ml/h. The long-term behaviour of the column was tested, following the decrease in content of phosphate groups by measuring the nominal capacity of the ion exchanger. After 12 h of operation the capacity had decreased from the original value of 2.28 to 2.12 mequiv./g. The esterification column still worked with a high efficiency after 25 l of the reaction mixture had been passed through it. After the end of the whole experiment (total operation period 275 h), the mean capacity of the bed was 1.7 mequiv./g, but this value was not evenly distributed over the column. The upper part was most (1.58 mequiv./g), the middle part less (1.82 mequiv./g) and the lower discharge part least exhausted (1.94 mequiv./g).

    A blank experiment, to determine the contribution of autocatalysis to the esterification, was carried out in the same column but packed with inert glass balls of diameter 0.8 mm. The highest degree of autocatalysis was found at 80°C, flow through 19 ml/h and acid:alcohol ratio of 1:1:2; the degree of conversion of acid was

11.8 %. The kinetics of the esterification reaction catalyzed by the ion exchanger were also followed and compared with those in the presence of the equivalent amount of free phosphoric acid. Both reactions were found to be of the second order (with respect to the concentrations of acetic acid and n-butane), that catalyzed by the ion exchanger proceeding more than two times faster (rate constant $k_2 = 5.49 \times 10^{-4}$ l/mol · min) than the reaction catalyzed by the acid ($k_2 = 2.06 \times 10^{-4}$ l/mol · min). A more detailed analysis of these results can be found in ref. 18.

The cation exchanger of nominal capacity 2.20 mequiv./g was used for a continuous inversion of saccharose, $i.e.$, for the hydrolysis of this disaccharide to glucose and fructose. A 24 % solution of saccharose in distilled water was pumped through the column at rates ranging from 20 to 200 ml/h and temperature was maintained at 50, 60, 70°C or higher. The degree of conversion of saccharose was 100 % at flow-rates up to 70 ml/h at 70°C and the resulting solution remained colourless. The conversion was almost complete also at a flow-rate of 100 ml/h and 70°C. At higher temperature, $e.g.$, 90°C, the processed solution was coloured brown. A part of the functional groups of the catalyst was also lost during the preparation of invert sugar, the content of phosphorus in the products being in the range $2 \times 10^{-3} - 5 \times 10^{-3}$ %. Upon conclusion of the experiments, $i.e.$, after 5 days, when 13–14 l of the reaction mixture had continuously passed through the column, the nominal capacity decreased in the upper part of catalyst from the initial 2.20 to 0.85 mequiv./g at a depth of 4 cm from the surface to 1.10 mequiv./g and at a depth of 20 cm only to 1.84 mequiv./g. The lower layers of the catalyst bed were practically unaffected.

The volume velocity of catalysis, $i.e.$, the ratio of the flow-rate (per hour) to the volume of catalyst (in the same units), was 0.8–1.0. This value is interesting from the point of view of possible practical applications. However, the chemical instability at the higher temperatures required for continuous catalysis represents a problem.

CONCLUSIONS

The phosphate derivatives of Spheron may best be prepared by phosphorylation of Spheron with phosphoryl chloride in the presence of a soluble base. These medium-acidic ion exchangers absorb proteins under static conditions, even at lower nominal capacities, and can be prepared with relatively high nominal capacities of 3.5–4 mequiv./g. They proved suitable for medium-pressure chromatography of proteins at ambient or lower temperature. Under such conditions, they may be repeatedly regenerated and reused. Enzymes chromatographed on these ion exchangers retain full activity. Spheron phosphates were also used as immobilized acids in the catalysis of alcohol esterification with organic acid and for the inversion of saccharose to glucose and fructose. At higher reaction temperatures, small amounts of phosphorus are lost, particularly in the upper parts of the column.

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ARTIFICIAL PEAKS IN GEL PERMEATION CHROMATOGRAPHY DUE TO COMBINING COLUMN SUPPORTS WITH DIFFERENT PORE SIZES

JACQUES G. BINDELS* and HERMAN J. HOENDERS*

Department of Biochemistry, University of Nijmegen, Kapittelweg 46, 6525 EP Nijmegen (The Netherlands)

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SUMMARY

Contrary to the elution pattern of bovine eye lens proteins chromatographed on a TSK GEL G5000 PW type column, an additional peak, representing $4 \cdot 10^6$ dalton aggregates, was observed on a combined G5000 PW–G4000 SW type system. This extra peak, found between the G5000 PW total exclusion fraction and α-crystallin ($M_r = 1.1 \cdot 10^6$), appeared to correspond to the G4000 SW void volume. Simulation calculations substantiated that this peak is the result of coupling the two columns and does not originate from a distinct sub-population of molecules. Such artificial peaks may be expected whenever a broad molecular weight distribution extends beyond the fractionation limits of at least one of the constituents of a combined gel permeation system. It is inferred that the hypothetical exclusion limit for compact, globular, symmetrical proteins on TSK GEL G5000 PW is near 10^8 dalton.

INTRODUCTION

Semi- and non-rigid column packings allowing high-pressure and high-speed gel permeation chromatography (GPC) which have been used in non-aqueous applications for more then 10 years, have recently become popular in aqueous (bio)polymer characterization[1–12]. Two types of rigid hydrophilic column packings are available: chemically modified macroporous silica supports and microspheres consisting exclusively of hydrophilic polymer. The first type is preferred when proteins are investigated and the latter gives higher resolution for synthetic polymers[5]. The application of silica-based TSK GEL SW type high-pressure GPC in combination with a molecular weight-dependent detection system, using a low-angle laser light scattering photometer, greatly improved the size characterization of biological macromolecules[8]. In a previous paper we described[13] the chromatography of eye lens proteins, crystallins, on TSK GEL G3000 SW and G4000 SW columns. Since the fractionation range of the largest pore size of SW type columns is limited to molecules having radii below 20 nm (inferred from the exclusion limits and molecular dimensions for dextrans[4,7,14]), these supports are less appropriate than the conventional

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* Present address: Milupa A.G. Research Department, 6382 Friedrichsdorf/Ts, G.F.R.

agarose matrices for the separation of high-molecular-weight (HM)-crystallin with dimensions up to 500 nm[15]. Compared with the SW type columns, the polymer-based TSK GEL G5000 PW and G6000 PW type columns have larger pores[5,7]. Himmel and Squire[9] used a TSK GEL G5000 PW type column for size exclusion of. large proteins and viruses and Ozaki *et al.*[6] were able to fractionate human serum lipoproteins on G5000 PW or G6000 PW columns. Moreover, to obtain an optimal one-step fractionation of the lipoproteins, the latter authors used several combinations of PW and SW type columns.

The present communication deals with the results of fractionating lens proteins on a TSK GEL G5000 PW column and a combination of a G5000 PW and a G4000 SW type column. In the latter case an additional peak was found between the void volume and the α-crystallin peak, suggesting a discrete sub-population of inter-mediately sized HM-crystallin. However, because no indication whatever of such peak could be obtained using only the G5000 PW type column, simulation calculations were done and showed that the extra peak is artificial.

EXPERIMENTAL

High-pressure GPC was carried out at room temperature in prepacked columns containing TSK GEL SW and PW type columns which were, when combined, connected in sequence of descending pore size: GPWP (precolumn, 10 × 0.75 cm), G5000 PW and G4000 SW (60 × 0.75 cm each) (Toyo Soda). The carrier buffer was composed of 0.10 M sodium sulphate and 0.02 M sodium phosphate pH 6.9 and was Millipore-filtered and degassed under vacuum before use. Elution was performed at constant flow-rate of 0.4 ml/min for the PW type alone and 0.8 ml/min for the combined system (solvent delivery: Beckman/Altex Model 100A). Detection was accomplished with an ultraviolet absorbance detector at 212 nm (Hitachi/Altex). Samples of 100 μl, obtained from nuclei of calf lenses as described earlier[13,16] and diluted in elution buffer to a concentration of 2 mg/ml, were applied using a Valco loop injector.

Molecular weight determination was done with a flow-through low-angle laser light scattering photometer (Chromatix: KMX-6) and a differential refractive index detector (Melz: LCD 201) according to the relative method[8] and using a Hewlett-Packard 3353 data system as described previously[13].

RESULTS

GPC of high-molecular-weight lens proteins
Fig. 1 shows the result of applying a diluted extract from the innermost part (nucleus) of a calf lens to a TSK GEL G5000 PW type column (upper trace) and a G5000 PW–G4000 SW type combination (lower trace). The upper elution pattern shows that a good separation of the crystallins is obtained, especially between the largest HM-crystallin aggregates eluting in the void volume and the α-crystallin peak. Due to the pore characteristics of the G5000 PW support, the resolution of the other crystallins is limited. The peaks were identified by comparison of the profiles with the patterns obtained from SW type columns and by molecular weight determinations using the molecular weight-dependent low-angle laser light scattering device; the data
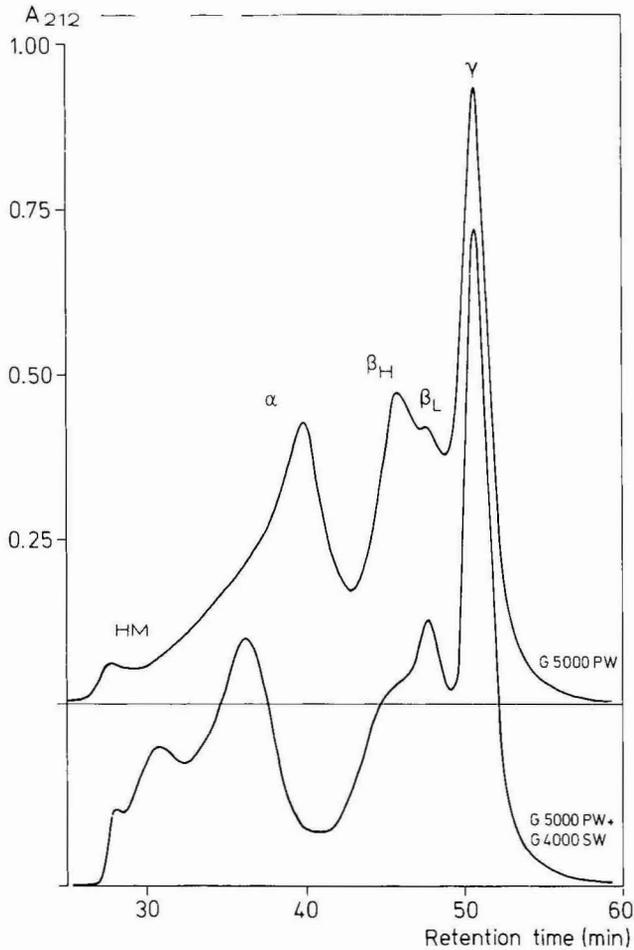
Fig. 1. High-performance GPC of calf nuclear water-soluble lens proteins. The upper elution profile was obtained using a TSK GEL G5000 PW type column (flow-rate 0.4 ml/min) and the lower one represents the pattern obtained from elution on a TSK GEL G5000 PW–G4000 SW column combination (flow-rate 0.8 ml/min).

were in accordance with those presented earlier[13] and are included in Table I.

To obtain a better resolution in the β- and γ-crystallin range we coupled a TSK GEL G4000 SW type column to the PW type column and a typical elution pattern is shown in the lower trace of Fig. 1. When both drawings are compared, the decreasing overlaps between α- and β- and β- and γ-crystallin indicate the improvement in resolution. The ratio of peak heights for $\beta_{H(igh)}$- and $\beta_{L(ow)}$-crystallin changes markedly; however, on comparing the areas under the curves for both chromatograms it was found that the proportions of β_H- and β_L-crystallin are similar in both analyses. Because of its heterogeneity[13], the β_H-crystallin group is eluted as a broader peak in the better resolving system, with a lower peak height.

The presence of the additional peak between the void volume and the α-crystal-

TABLE I

MOLECULAR WEIGHTS AND GPC ELUTION PARAMETERS FOR TSK GEL G5000 PW

t_e = Retention time of a particular fraction; t_t = retention time for totally permeated molecules; t_0 = retention time at which the void volume elutes; $K_d = (t_e - t_0)/(t_t - t_0)$; $F_{(v)} = (\sqrt[3]{t_e/t_t} - \sqrt[3]{t_0/t_t})/(1 - \sqrt[3]{t_0/t_t})$.

Compound	M_r	K_d	t_e/t_t	$F_{(v)}$	$\sqrt[3]{M_r \bar{v}}$
Tobacco mosaic virus	$39.4 \cdot 10^6$*	0	0.495*	0	306.4*
HM-crystallin	$> 20 \cdot 10^6$**	0	0.495**	0	> 245.5**
Sea worm chlorocruorin	$2.9 \cdot 10^6$*	0.343	0.668*	0.398	130.2*
α-Crystallin	$1.1 \cdot 10^6$**	0.442	0.718**	0.500	93.37**
Thyroglobulin	670,000*	0.477	0.736*	0.535	78.43*
Dimeric serum albumin	136,000*	0.636	0.816*	0.686	46.00*
β_H^5-crystallin	130,000**	0.640	0.818**	0.690	45.40**
Monomeric serum albumin	68,000*	0.699	0.848*	0.744	36.51*
β_L^2-crystallin	50,000**	0.711	0.854**	0.755	33.02**
γ_L-crystallin	20,000**	0.824	0.911**	0.854	24.33**
Cytochrome c	12,500*	0.871	0.935*	0.894	20.79*

  * Molecular weight and elution data were taken from ref. 9.
  ** Elution data correspond to Fig. 1; molecular weights were determined by low-angle laser light scattering and agree with those reported previously[13]. For α- and HM-crystallin we used a partial specific volume, $\bar{v}$, of 0.74 ml/g based on experimental data[20,21]; for β- and γ-crystallin we used a $\bar{v}$ value of 0.72 ml/g based on estimated values from amino acid compositions[22,23].

lin fraction was more puzzling. At first we thought of superior resolution of a discrete sub-population of intermediately sized HM-crystallin aggregates. Such a population has never been found as a peak fraction in a one-step GPC procedure[15-17]. Although van Kleef and Hoenders[18] isolated these aggregates ($s_{20,w}$ near 40 S) as a peak fraction on a Bio-Gel A-50m column, they obtained this by rechromatography of collected Bio-Gel A-5m void volume fractions.

We found molecular weight values near $4 \cdot 10^6$ by the low-angle laser light scattering device for the material eluting in the extra peak seen in the lower trace of Fig. 1. It thus seems possible that the aggregates eluting in this peak represent the earlier characterized HM2[18], high α-[15] and oligomeric α-crystallin[16]. However, the suggestion that this fraction represents a discrete sub-population of aggregates is uncertain. Since this material could never be isolated as a peak fraction on one-step agarose GPC, no indication of a corresponding peak in the elution pattern from the G5000 PW type column could be found and its molecular weight value coincides possibly with the G4000 SW exclusion limit for these proteins, we looked for an artificial origin of this peak. The results of simulation calculations, presented below, confirm the artificial character of this peak caused by coupling the two different columns.

The G5000 PW exclusion limit for compact globular protein

Although the literature concerning TSK GEL SW fractionation of proteins is rapidly expanding, few papers have described the elution of proteins on TSK GEL PW type supports[2,5,6,9]. To our knowledge Himmel and Squire[9] are the only authors to mention the fractionation range for native high-molecular-weight proteins chroma-
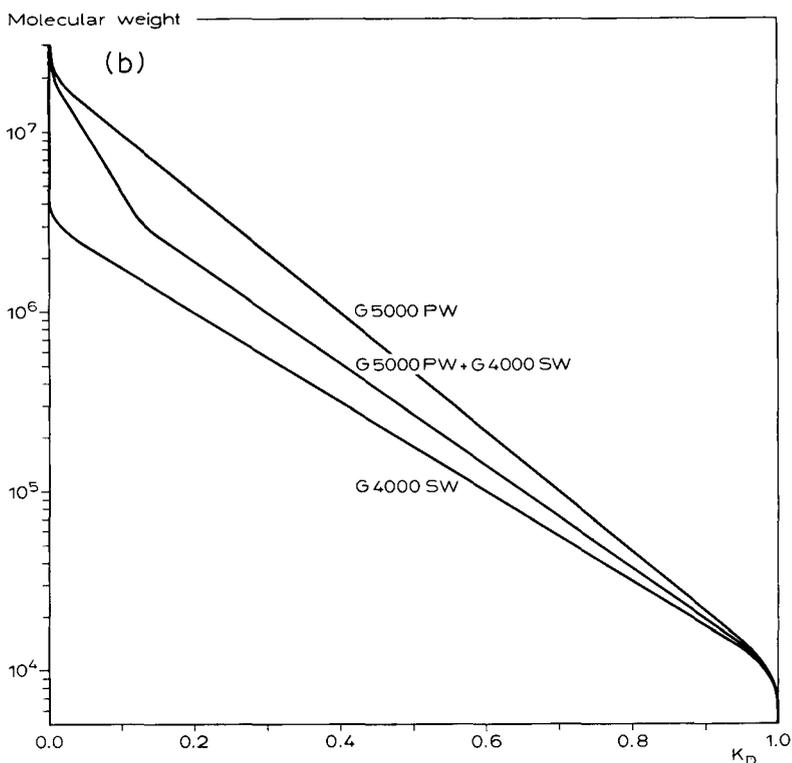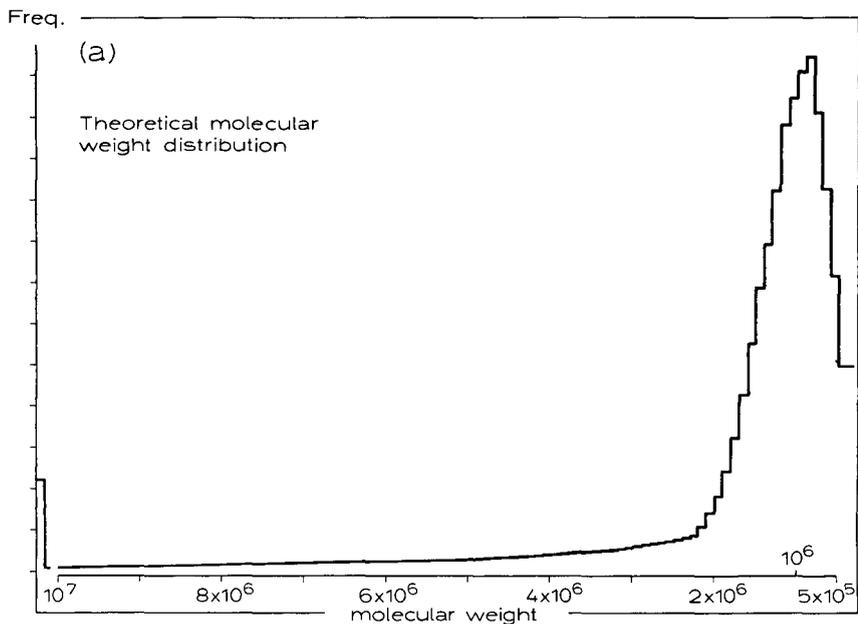
Fig. 2. Theoretical molecular weight distribution for calf lens nuclear HM- and α-crystallin (a) and calibration plots for the G5000 PW and G4000 SW type columns alone and in combination (b).

tographed on a TSK GEL G5000 PW column. Since the reported exclusion limit of $1.4 \cdot 10^6$ dalton is much lower than our estimate, we compared our results with a selection of elution data given by the previous authors. Table 1 lists several parameters of the elution shown in the upper part of Fig. 1 and those reported in ref. 9: M_r; distribution coefficient, K_d; the t_e/t_t value; the elution volume parameter, $F_{(v)}$, and the molecular weight function $\sqrt[3]{M_r \bar{v}}$. It can be seen that there is a good agreement between the elution parameters K_d and t_e/t_t for crystallins and other proteins of comparable size analyzed by Himmel and Squire[9].

It appears that the inconsistency between our exclusion limit estimate and that of Himmel and Squire[9] can be attributed to the assignment of the void volume peak. The latter authors considered most viruses and the sea worm chlorocruorin to be eluted in the void volume ($t_e/t_t = 0.642$–0.668) and tobacco mosaic virus to behave anomalously ($t_e/t_t = 0.495$). Moreover, in their elution pattern for the $5 \cdot 10^5$ dalton dextran sample a small void volume peak can also be found with a t_e/t_t value of 0.495. Whatever the structure of tobacco mosaic virus or the high-molecular-weight dextran component may be, it is theoretically impossible for them to be eluted earlier than the void volume. The reason why the three other viruses and the sea worm proteins elute at practically the same time remains to be established. We are convinced that the largest HM-crystallin aggregates elute in the totally excluded volume. Since the tobacco mosaic virus peak elutes at exactly the same position, our further calculations are based on the t_e/t_t value of 0.495 representing the void volume.

Fitting all data from Table I with the least-squares method revealed an excellent linear correlation between the distribution coefficient and the logarithm of the molecular weight (correlation coefficient 0.999). The exclusion limit, obtained by extrapolation of K_d to zero, amounts to $100 \cdot 10^6$ dalton. This value is higher than our estimate of $20 \cdot 10^6$ for HM-crystallin obtained using high-pressure GPC/low-angle laser light scattering and trypsin-treated HM-crystallin[19]; we found a weight average molecular weight of $30 \cdot 10^6$ for the smallest aggregates still eluting in the void volume and $15 \cdot 10^6$ for the aggregates which eluted immediately after the void volume peak. The discrepancy between both estimates may be explained by the structure of HM-crystallins which feature chain-like polymers mainly composed of α-crystallin entities[16].

Extrapolation using the method of Himmel and Squire[9,10], based on a linear relationship between the elution volume function, $F_{(v)}$, and $\sqrt[3]{M_r \bar{v}}$, yields a considerably lower value for the exclusion limit: $13 \cdot 10^6$ dalton (correlation coefficient 0.97).

Simulation of elution patterns

To investigate whether an extra peak may be generated when two columns with different pore sizes are coupled, simulation calculations were performed. Fig. 2a shows a theoretical molecular weight distribution for HM- and α-crystallin. Although precise data are still lacking, we think that this pattern represents a reasonable approximation of the actual distribution. The distribution profile is sliced into fractions of width 10^5 dalton. In Fig. 2b, theoretical calibration curves are presented for a TSK GEL G4000 SW column, a G5000 PW column and a combination of both. The curves for the G5000 PW and G4000 SW columns were constructed considering
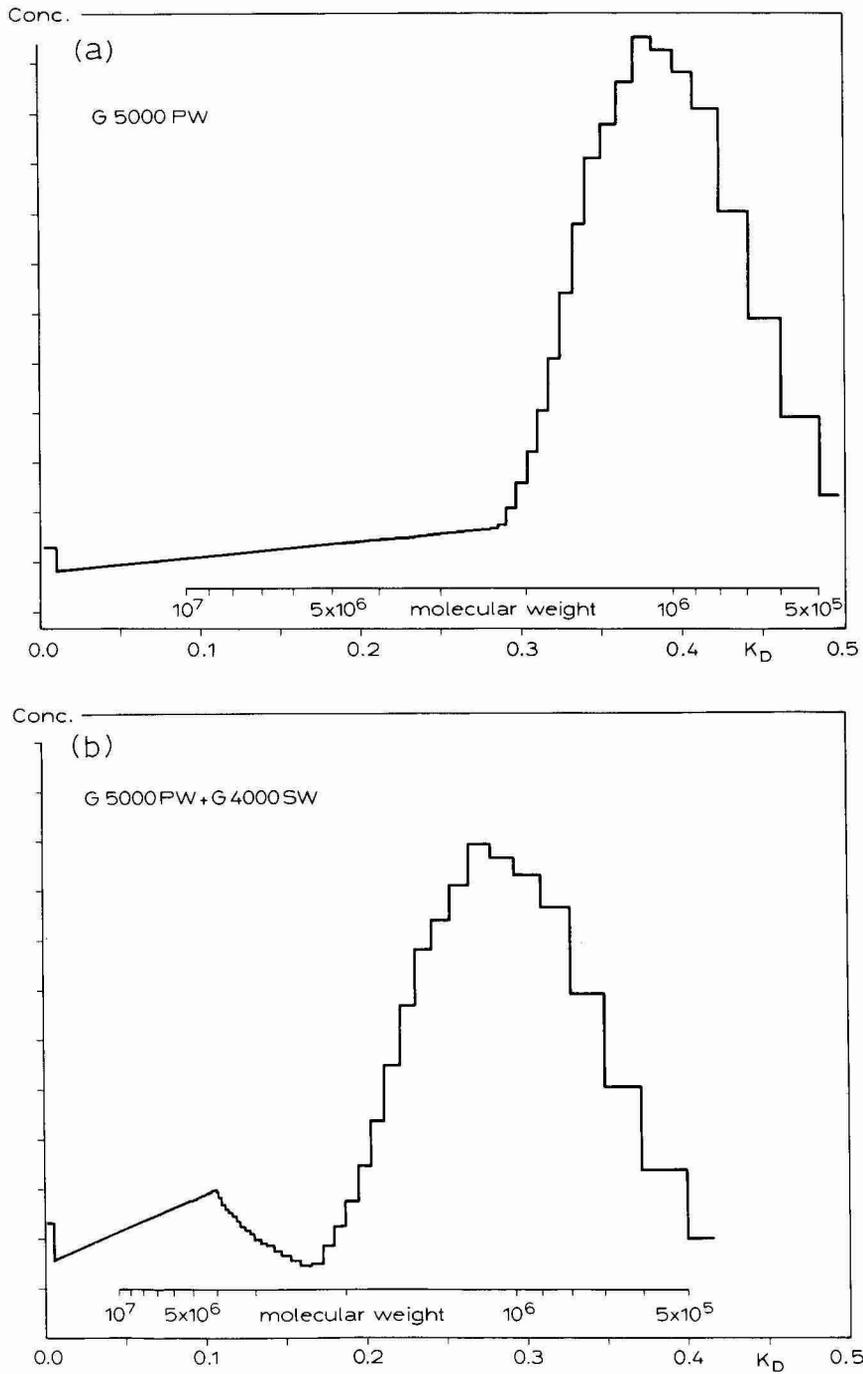
Fig. 3. Simulated chromatograms calculated from the molecular weight distribution and calibration plots given in Fig. 2. The pattern for a G5000 PW type column alone (a) and the profile corresponding to the coupled G5000 PW–G4000 SW type columns (b) are given.

exclusion limits of $4 \cdot 10^6$ and $20 \cdot 10^6$, respectively, and a total permeation limit of 10^4 for both supports. We think that a reasonable description is provided for the actual situation in the case of crystallins. The curve for the combined columns was obtained by plotting for each molecular weight the average distribution coefficient for the separate columns. This is permitted since both columns are of exactly the same size and appear to have similar pore volumes. The inflection point appearing at the upper exclusion limit of the G4000 SW type column is marked.

In fig. 3 simulated chromatograms are shown which were calculated from the theoretical molecular weight distribution and calibration curves. For each slice in Fig. 2a, representing molecules within a range of 10^5 dalton, the distribution coefficients at the molecular weight limits were determined from Fig. 2b. The simulated chromatograms were constructed using the minimal and maximal distribution coefficients for each slice (representing the slice width), and considering the areas of the different slices to be proportional to those in the molecular weight distribution plot (Fig. 2a). So, the slice heights were obtained from the ratio of the slice areas and the slice widths. The extra peak in the simulated elution pattern of the combined columns arising at the upper exclusion limit of the G4000 SW column is obvious.

It has to be stressed that the shape of the chromatograms is rather arbitrary; in fact we calculated Fig. 2a from Fig. 3a based on a linear increase of the chromatogram for eluting molecules between $20 \cdot 10^6$ and $2.1 \cdot 10^6$ dalton. If only straight lines were used in the theoretical calibration plot a saw-tooth shape for the artificial peak was obtained. For the sake of simplicity we neglected diffusion and/or band spreading effects in the simulation.

DISCUSSION

Whenever a broad continuous molecular weight distribution extends beyond the fractionation limits of the constituents of a combined GPC column system, additional peaks may be observed caused by the chromatographic system. This effect is observed with high-molecular-weight eye lens proteins on a combined TSK GEL G5000 PW and G4000 SW column system. While investigating the suitability of the semi-rigid TSK GEL Toyopearl packings for GPC analysis of dextrans, Barker et al.[12] also found an additional peak for which they had no explanation. Since it occurred only when columns were packed with certain mixtures of HW55S and HW65S and appeared to be related to the exclusion limit of the HW65S packing, we are convinced that they observed the same effect.

Using different column combinations, Mori[24] compared the average molecular weight distribution curves for polystyrene NBS 706, NBS 705 and a commercial polystyrene with a broad MW distribution. Although the average molecular weights were not affected by the column combinations, he found that the integral molecular weight distribution curves showed definite differences among several column sets. For a broad-range calibration column systems, Yau et al.[25] suggested the combination of two GPC columns having about one decade difference in pore size and approximately equal pore volumes for the two pore sizes. The desired situation is one in which the linear portions of the individual column molecular weight calibration graphs are substantially non-overlapping and the pore volume of each packing is such that the linear parts of the calibration graphs are essentially parallel. Unfortunately, such

supports are not yet available for aqueous high-pressure GPC; it appears that the TSK GEL SW and PW type columns principally differ in their upper exclusion limits and to a lesser extent in the lower ones, thus resulting in non-parallel calibration curves.

The accuracy of molecular weight determinations using empirically derived calibration plots is influenced by many factors. Besides the development of high-performance instrumentation, attention has been focused on obtaining linear calibration curves, thus reducing the number of necessary calibration points. However, it is obvious that for the determination of molecular weights on combined column systems, many calibration samples are needed properly to construct the calibration curves. An alternative would be the use of a molecular weight-sensitive detection system.

It should be clear that it is not strictly relevant to determine the exact value for the exclusion limit of TSK GEL G5000 SW type supports for globular and compact high-molecular-weight proteins since there are very few proteins which meet these demands; both HM-crystallin and tobacco mosaic virus are asymmetrical. However, the difference between the value of $1.4 \cdot 10^6$ of Himmel and Squire[9] and our estimate of $100 \cdot 10^6$ is too large. An intermediate value of $13 \cdot 10^6$ may be found if one recalculates the data of the previous authors using the method based on the $F_{(v)}$ vs. $\sqrt[3]{M_r \bar{v}}$ relation[9,10] and a different void-volume fraction. Support for the $100 \cdot 10^6$ value can be obtained if we consider the GPC elution behaviour of dextrans on TSK GEL G4000 SW and G5000 PW; the exclusion limits were experimentally found to be $0.5 \cdot 10^6$ (ref. 4) and $7 \cdot 10^6$ (ref. 3), respectively. Using the data compiled by Kuga[7], these values correspond with equivalent sphere radii of 17 and 50 nm, respectively; according to Senti et al.[14] ($R = 0.066 M_r^{0.43}$), radii of 19 and 58 nm, respectively, can be found. Since the approximately three-fold increase in radius corresponds with a 27-fold increase in volume and considering the exclusion limit of compact globular proteins on TSK GEL G4000 SW, $7 \cdot 10^6$ (ref. 4), the estimate of $100 \cdot 10^6$ for such proteins on G5000 PW type columns seems more reasonable than the lower values. For the chain-like HM-crystallin protein clusters a value of $20 \cdot 10^6$ is postulated for the molecular weight exclusion limit.

In conclusion, we would like to emphasize that checking of the elution behaviour on separate columns is needed to decide whether additional peaks or shoulders, observed in a high-resolution system consisting of supports with different pore sizes, represent discrete sub-populations of molecules or column artifacts. Although the method of Himmel and Squire[9] seems to have advantages over the logarithmic treatment for molecular weight estimations by interpolation of the data obtained from standard proteins, extrapolation of these data to obtain the exclusion limit should preferably be performed according to the logarithmic method.

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CLEAN-UP PROCEDURES FOR THE EXAMINATION OF SWABS FOR EXPLOSIVES TRACES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION AT A PENDENT MERCURY DROP ELECTRODE

J. B. F. LLOYD

Home Office Forensic Science Laboratory, Priory House, Gooch Street North, Birmingham B5 6QQ (Great Britain)

SUMMARY

A microfilter extraction assembly containing a mixture of alumina and octadecylsilylsilica is used in the preparation of small volumes of cleaned-up extracts from handswabs for their screening for explosives components by high-performance liquid chromatography at a pendent mercury drop electrode. Apart from the removal of lipids and lipophilic materials likely to degrade chromatographic performance, the adsorbent removes highly polar electroactive compounds, and traps out volatile explosives components when the swabbing solvent is removed. From used handswabs, including grossly soiled swabs, the recovery values of fourteen compounds in the range 1–50 ng per swab are given. Also are presented the results of a survey of 98 handswabs, the majority of which were collected from people occupied in heavy manual work. From these results, the sensitivity limits are on the order of 1–10 ng per used swab, which is subject to variation by the origin of the swab and by the identity of the compound of interest. Examples are given of the application of the technique to people who had handled explosives and a firearm, and to the use of the extraction assembly in a headspace sampling of bomb debris.

INTRODUCTION

A recently described high-performance liquid chromatography (HPLC) technique, with detection at a pendent mercury drop electrode (PMDE) gave detection limits in the range 7–49 pg for fourteen representative organic explosives components in 20-μl samples[1]. The technique is amongst the most sensitive now available, and offers particular advantages in its ease of use and its freedom from problems due to the thermal lability of many explosives. To date, the development of the technique has been especially directed to the general screening of samples for a variety of common explosives components. This application to handswabs, which are frequently submitted to forensic science laboratories for an examination for explosives traces, is the main topic of the present paper.

In a related paper, Bratin *et al.*[2] have described the use of their thin-layer electrode–HPLC technique in the examination, without prior clean-up, of some handswab extracts for firearms discharge traces. Douse[3] has developed a clean-up procedure based on Amberlite XAD-7 resin, which is appropriate to his silica capillary column gas chromatography (GC) electron-capture detector (ECD) technique[4]; Twibell *et al.*[5] for their GC–ECD technique used a thin-layer chromatography clean-up on silica gel; and for their HPLC–ECD work on explosion debris Krull *et al.*[6] also used silica gel. Earlier references are given by these authors. Although no clean-up procedure may be necessary when relatively high amounts of explosives are present, as in the application of Bratin *et al.*[2], for the multi-sample screening of swabs for low nanogram amounts some degree of clean-up is required, not only to remove compounds interfering in the detection of those of interest, but simply as a protection of both the separation and the detection systems against fouling by any materials strongly retained on them. Earlier results in the present research[1] indicated that this is just as true for the thin-layer electrode technique as for the GC techniques referred to; and although the PMDE is relatively unaffected by fouling, because it is instantly renewable, for its effective routine use at high sensitivities sample clean-up is obligatory.

EXPERIMENTAL

Chromatography

The details have been given before[1], they are summarized here. The separations are made on 150 × 4.5 mm columns of 3-μm ODS-Hypersil, in methanol–aqueous potassium phosphate (100:86, v/v) with a flow-rate of 1.0 ml min^{-1}. The aqueous component is 0.025 M orthophosphoric acid adjusted to pH 3.0 with potassium carbonate. To eliminate oxygen, the contents of the eluent reservoir are maintained under reflux. When heavily soiled swabs are being processed regularly, the column is purged, *e.g.*, weekly, with methanol followed by ethyl acetate to preserve resolution. Samples are deoxygenated, prior to injection, in a modified syringe[7]. Usually, the injection volumes are 20 μl, but slightly higher resolution is obtained with 10-μl volumes. Detection is at a PMDE normally operated at −1.0 V *vs.* Ag/AgCl.

Explosives components

With the addition of 2,6-dinitrotoluene in some experiments, these are the same as before[1]. The abbreviations used are given in the caption to Fig. 1, which is of a chromatogram of a standard mixture.

Processing of handswabs

Materials. Methanol is HPLC grade (Rathburn); the other chemicals are AristaR grade (BDH). BP-quality cotton wool is Soxhlet-extracted in methanol for 8 h. Aqueous potassium phosphate, 0.061 M, is prepared by the addition of potassium carbonate to 3.5 g of orthophosphoric acid (86 %) dissolved in 500 ml of water to give a pH of 3.0. Sulphamic acid solution (5 %, w/v) is prepared (when required for the removal of nitrite) from 0.5 g of the acid dissolved in 5 ml of water, and made up to 10 ml with methanol. This solution is filtered through a 0.2-μm polytetrafluoroethylene membrane. The adsorbent suspension is a mixture of 2.5 g of Spherisorb ODS-silica,
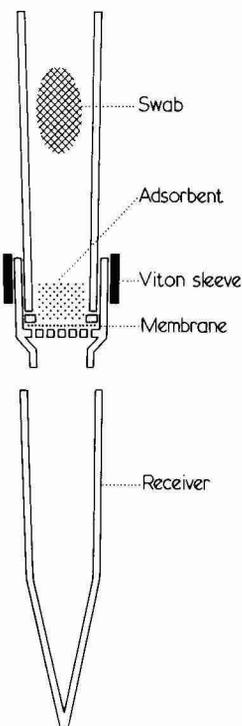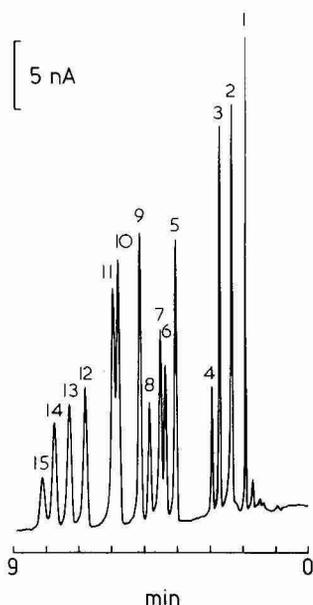
Fig. 1. Chromatogram of standard compounds (0.5 ng each in 10 μl) run on 150 $\times$ 4.5 mm ODS-Hypersil, 3 μm, in deoxygenated methanol–aqueous potassium phosphate (0.025 M, pH 3.0) (100:86, v/v) at 1 ml min^{-1}, with detection at a PMDE (-1.0 V $vs.$ Ag/AgCl). The compounds are: 1 = octogen (HMX); 2 = picric acid (PA); 3 = hexogen (RDX); 4 = ethyleneglycol dinitrate (EGDN); 5 = 1,3-dinitrobenzene (DNB); 6 = tetryl (TET); 7 = nitrobenzene (NB); 8 = nitroglycerine (NG); 9 = 2,4,6-trinitrotoluene (TNT); 10 = 2,6-dinitrotoluene (26DNT); 11 = 2,4-dinitrotoluene (24DNT); 12 = 2-nitrotoluene (2NT); 13 = 4-nitrotoluene (4NT); 14 = 3-nitrotoluene; (3NT); 15 = pentaerythritol tetranitrate (PETN).

Fig. 2. Cross-sectional diagram of a centrifugal microfilter (Bioanalytical Systems) prepared for use as a clean-up extraction assembly.

10 μm, and 2.5 g of Spherisorb alumina, 10 μm (Phase Separations), in 50 ml of ethanol. A glass-sleeved magnetic stirrer bar is kept in the mixture's storage vessel.

Extraction assemblies. These are made from centrifugal microfilters (Bioanalytical Systems). Prior to its use, each dismantled filter is soaked overnight in methanol containing 2% (w/v) sodium hydroxide, 0.2% ethylenediaminetetraacetic acid and 0.2% thiourea. Explosives components in particular are rapidly degraded by the mixture (the thiourea acts as a source of sulphide); even so, microfilters that have come into contact with explosives are not used subsequently for trace analysis. After they have been rinsed in water, methanol and dried (50°C), each microfilter is fitted with a 0.2-μm cellulose membrane, and assembled with a piece of Viton sleeving (Soxhlet-extracted in methanol) pushed over the membrane holder (see Fig. 2) to enable the holder itself later to be sleeved by an inlet of a vacuum manifold.

To each assembled microfilter is added 1 ml of the adsorbent suspension, transferred by means of a wide-bore dropping pipette from the vigorously stirred

mixture. The adsorbent is then centrifuged down at 1850 g onto the filter membrane. The assemblies are stored in a sealed container, in the absence of ethanol, until they are required for use, when they are washed through at the centrifuge with 1 ml of ethanol. Fig. 2 shows the completed assembly.

Clean-up procedure. The following is used for swabs consisting of 90–110 mg of cotton wool and *ca.* 200 μl of ethanol. This volume of solvent is left after a swab holding 500 μl has been rubbed thoroughly over a hand.

A swab is inserted loosely into the top of a prepared microfilter assembly, from which the receiving tube has been removed. The assembly is attached to a vacuum manifold so that a stream of air can be sucked through the swab and then through the adsorbent. A silica desiccant trap (made from a 5-ml glass hypodermic syringe, the cone of which is pushed through a hole made in a microfilter cap) is attached to the entrance of the filter. With an air flow of 75 ml min^{-1}, once the bulk of the ethanol has evaporated from the adsorbent, the solvent is removed completely (mass loss less than 1 mg min^{-1}) within 30 min at an ambient temperature of about 20°C.

The outlet tip of the microfilter is washed with acetone, shaken dry and fitted with a tared receiver tube. The swab is packed down tightly onto the adsorbent bed, treated with 250 μl of methanol–water (100:35, v/v) and centrifuged for 5 min at 460 g and then at 1850 g for 10 min. The volume collected, *ca.* 160 μl, is calculated from the mass collected on the assumption that the eluate's density is 0.86 g ml^{-1}. For chromatography, a 0.37-volume ratio of the aqueous 0.061 M phosphate is added either to the whole of the eluate, or to an aliquot when the presence of phosphate could interfere in other work on the eluate. If nitrite is to be removed (see below), 1 μl of the sulphamic acid solution is added per 20 μl of the diluted eluate.

For swabs of the stated composition, from which the results described here were obtained, the drying time and the volume of the collected eluate are sufficiently reproducible that it is unnecessary to weigh the microfilter and the receiver tube. This is necessary, however, in the examination of swabs collected under unstandardized conditions. In some cases solvent exudes when an over-wet swab is inserted in the microfilter. This surplus may be removed and put aside, as sufficient remains on the swab for analysis. Alternatively, the surplus is returned to the swab after the initial drying. When over-wet swabs are being dealt with, the extraction assembly is inverted during the evacuation in order to avoid any drainage of the solvent onto the adsorbent. As the dried adsorbent is loose, the assembly is set upright before the vacuum is turned off. Up to 250 mg cotton wool swab may be handled by the technique, with a proportionate increase in the volume of eluent. Aliquots are taken of larger swabs.

Recovery experiments

These are made with DNB as an internal standard. Known amounts of explosives components in ethanol or aqueous methanol are distributed with a syringe throughout the swab, and the swab is then processed. To the extract is added DNB in an amount comparable to the explosives components, and the recoveries are calculated from peak heights with reference to standards in the usual way.

RESULTS AND DISCUSSION

Factors relevant to the swabbing solvent

For much of the present work a relatively large injection volume, for 3-μm column packings, of 20 μl was adopted as a compromise between the requirements of resolution and sensitivity. (With reference to a 10-μl injection, as in Fig. 1, the increases in peak width and the inverse variation in peak height of a constant amount of HMX in 20- and 50-μl injections are 1.14 and 1.47, respectively.) Because of this it is important that the injection solvent must closely match the chromatography eluent in composition, otherwise disastrously distorted chromatograms are obtained. If the composition of the injection solvent is to be readily and accurately controlled, a clean-up technique must include a step in which the swabbing solvent is removed. The chromatography eluent or one of its components cannot be used as a swabbing solvent unless it is then removed, because its composition is changed uncontrollably by the effects of evaporation and by the accumulation of moisture from sweat. Neither are dry swabs practicable. Although they efficiently remove explosives from skin surfaces[8], presumably by attrition of lipid material in which the explosives may dissolve[2], the presence of a solvent is necessary to inhibit the loss of volatile compounds from the swab between the times of its use and extraction[9].

At present, ethanol is a frequently used swabbing solvent, mainly because the amounts of compounds extracted that interfere in the commonly used GC–ECD techniques are relatively low[9]. Although it does not follow that this is the case for the HPLC–PMDE technique, the present work has been conducted with ethanol-containing swabs because in the short term, at least, it is likely that they will remain in common use. However, the clean-up technique has been designed to deal with any solvent that is appreciably volatile at ambient temperatures.

Clean-up adsorbent

There are three important functions performed by the mixed adsorbent (alumina plus ODS-silica) in the extraction assemblies. The first is the removal of lipids and strongly lipophilic materials, such as mineral oil, that otherwise either are thrown out of solution when the aqueous phosphate is added to the microfilter eluate, or are strongly retained on the chromatography column and degrade its performance. These materials are retained on the ODS-silica.

The second function is the removal of the large amounts of the highly polar reducible species present in some handswabs, which give rise to an intense broad peak that obscures the early part of the chromatogram, and leaves behind a disrupted baseline. An illustration is given in Fig. 3, which compares chromatograms from a pair of left and right handswabs that had been intermixed and divided. One part (A) was dried, then extracted directly with the chromatography eluant. The other (B) was cleaned-up as described. The effective adsorbent here is the alumina. Less satisfactory adsorbents examined were a cation-chelating resin. Florisil and silica.

Thirdly, the adsorbent traps out any explosives components that volatilize during the solvent removal stage, particularly if the time-to-dryness is exceeded. This is shown in Table I, where the quantities recovered separately from the adsorbent and from two unused swabs are compared after 30 ng of each compound had been added to the swabs before the solvent was removed. At 29 min the swabs had almost reached
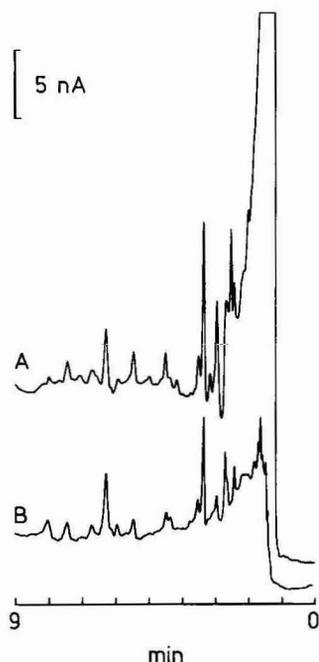
Fig. 3. Example of the effect of the clean-up procedure on handswab extracts. Sample A is from half of a bulked pair of handswabs which was extracted directly; B is an extract from the other half made by the described clean-up procedure. The chromatography conditions are as in Fig. 1.

dryness, but some transfer to the adsorbent was already occurring. On further pumping for a similar period, up to 5 ng amounts of the more volatile components were transferred, which otherwise would have been lost. Other experiments indicated that on a proportionate basis this potential loss increases as the amounts of the compounds decrease. With the prolonged pumping time in the given example, evidently some break-through occurred, e.g., 7 ng of NB were lost. The retentivity of the adsorbent mixture can be increased by the use of a more heavily silylated silica, but the advantage gained in this respect is offset by the increased difficulty with which PETN can be subsequently eluted.

The possible use of proprietary clean-up cartridges was examined; but because of the amounts of adsorbent they contain, and its large particle size, they are unsuitable for the recovery of samples into small volumes of solvent unless an evaporative concentration step is introduced, with consequent loss of volatile compounds: e.g., when the volume of a methanol–water (100:20, v/v) solution from a cartridge was reduced to 1/3rd in a stream of nitrogen at ambient temperature, the losses of NB and EGDN were 38 % and 20 % respectively. A 1/5th reduction gave the respective losses of 50 % and 86 %. No losses due to sorption of the compounds in the plastics composing the microfilters have been encountered, in contrast to the losses reported for some organomercury compounds[10].

The microfilter clean-up technique is readily adapted for other adsorbents that are more selective for individual classes of compound, although such adsorbents are unsuitable for the comprehensive detection of all of the compounds of interest. Ex-

TABLE I

RECOVERY OF EXPLOSIVES COMPONENTS FROM UNUSED SWABS AND THE ADSOR-
BENT AFTER SOLVENT REMOVAL IN AN EXTRACTION ASSEMBLY

The swabs contained 250 μl of ethanol and 30 ng of each compound initially.

Compound*	Recoveries (ng)			
	Drying time 29 min		Drying time 59 min	
	Swab	Adsorbent	Swab	Adsorbent
HMX	31	nd**	29	nd
PA	25	nd	24	nd
RDX	30	nd	29	nd
EGDN	28	nd	22	4.2
TET	28	nd	26	nd
NB	28	0.63	18	4.8
NG	29	nd	27	1.3
TNT	29	nd	27	0.15
26DNT	29	nd	25	2.2
24DNT	28	nd	26	1.4
2NT	27	0.33	20	5.4
4NT	26	0.48	22	4.2
3NT	31	0.24	21	5.1
PETN	28	nd	29	nd

* Full names are given in the caption to Fig. 1.
** nd = None detected.

periments were made with Amberlite XAD-7 in a modified form of Douse's tech-
nique[3], with results in general agreement with his except that NB was recovered,
probably because of the improved measures that can be taken to avoid the loss of
volatile compounds. Selective and generally higher recoveries were obtained with
LiChrosorb-NH$_2$. However, both techniques involved extra steps, because the ad-
sorbent was additionally extracted with isopentane, and gave poor recoveries for
some compounds. Where specific compounds are sought though, such adaptations
could be important, and applied to the products of an initial non-selective clean-up.

Interferences not removed in the extraction assembly

Substantial amounts of nitrite were found in swabs taken from two lathe oper-
ators. Presumably, the nitrite was from an anticorrosion additive in cutting fluid. As
Fig. 4 shows, an intense, distinctively shaped peak is produced, which is readily
removed by the treatment with sulphamic acid. Because nitrite could be of signifi-
cance in an explosives investigation, and because of its infrequent occurrence in
amounts likely to prove a nuisance, the extracts are not routinely treated with sul-
phamic acid. It should be noted that apart from its reaction with nitrite, the sul-
phamic acid is added in amount sufficient to reduce the pH of the extract to the point
where the reaction rapidly occurs. Chromatographic resolution and recoveries are
unaffected.

A high level of calcium was present on two occasions in handswabs from
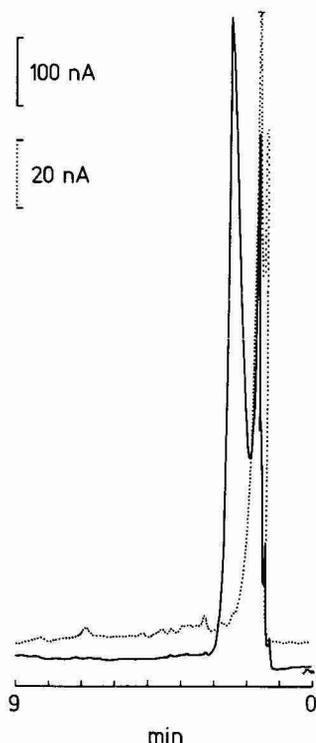
Fig. 4. Chromatograms of a handswab sample from a lathe operator, before (full line) and after (dotted line) the extract had been treated with sulphamic acid.

bricklayers, and precipitated as phosphate when the extracts were diluted. This was readily removed at the centrifuge. The effect was obvious and caused no particular difficulty, but as a precaution against column blockage a filter was subsequently installed between the injector and the column.

Quantitative recovery experiments

The variation of recovery, with a constant volume of varied eluent, of 10-ng amounts of explosives components from typical used swabs processed as described under Experimental is shown in Table II. For most of the compounds there is slight variation in recovery as the water–methanol ratio is increased to 35:100 (v/v). Beyond this, recoveries decrease considerably for the compounds more strongly adsorbed on ODS-silica. This could be countered by an increase in volume of the eluent, but with poorer detection limits because of the resulting dilution of the eluate. From heavily soiled handswabs, lipid-type materials are eluted when the solvent ratio is reduced below 35:100, hence this composition is adopted for general use. In the present instance the average recovery is 90 %.

The presence of large amounts of lipid and mineral oil on a swab do to some extent retard the elution, but the effect is fairly small. Recovery data from some exceptionally soiled handswabs are given in Table III. These, which were collected from five garage mechanics, contained large quantities of mineral oil and other

TABLE II

COMPOSITION OF CLEAN-UP ELUENT: RECOVERIES OF EXPLOSIVES COMPONENTS FROM USED HANDSWABS

The amount of each compound was 10 ng; the volume of eluent, 250 μl.

Compound	Recoveries (%)			
	Volumes of water per 100 volumes of methanol			
	25	35	45	55
HMX	85	88	81	82
PA	75	88	70	80
RDX	94	96	86	82
EGDN	94	103	90	86
TET	74	78	64	55
NB	92	95	79	68
NG	99	97	87	73
TNT	83	86	68	54
24DNT	83	88	65	43
2NT	90	93	72	51
4NT	84	90	64	41
3NT	85	89	65	37
PETN	90	84	70	47
Mean	86.8	90.4	73.9	61.5

TABLE III

RECOVERIES OF 50-ng AMOUNTS OF EXPLOSIVES COMPOUNDS FROM FIVE HEAVILY SOILED GARAGE MECHANICS HANDSWABS: EFFECT OF ELUTION VOLUME DURING CLEAN-UP

Compound	Recoveries (%)			
	Elution volume, 250 μl		Elution volume, 450 μl	
	Mean	Range	Mean	Range
HMX	64	41–80	85	68–99
PA	66	38–88	93	88–96
RDX	76	49–95	92	85–98
EGDN	91	84–102	98	87–105
TET	73	45–89	84	75–91
NB	80	71–86	88	86–90
NG	79	56–94	93	89–98
TNT	68	44–87	84	73–91
24DNT	62	37–86	80	58–93
2NT	75	65–90	85	73–96
4NT	67	53–84	80	66–87
3NT	72	63–86	86	78–90
PETN	72	45–91	87	79–96

TABLE IV

RECOVERIES OF 10-ng AMOUNTS OF EXPLOSIVES COMPOUNDS FROM FIVE HEAVILY
SOILED GARAGE MECHANICS HANDSWABS

The extraction assembly was eluted with 250 μl in each case.

Compound	Recovery (%)	
	Mean	Range
HMX	73	62–78
PA	80	68–88
RDX	90	82–94
EGDN	86	76–97
TET	68	44–82
NB	65	44–84
NG	80★	78–89
TNT	70	39–82
24DNT	64	34–80
2NT	71	62–80
4NT	77★	72–82
3NT	79	51–93
PETN	72	60–81

★ Only four results, the peak in the other samples was obscured.

debris, and were far more soiled than any that have been encountered in case work in
this laboratory. The swabs were spiked with 50-ng amounts of the compounds, and
yielded the results shown for 250-μl and 450-μl elution volumes. On average the
increase in recovery due to the larger volume of eluent is from 73 % to 87 %. Even for
the poorest single recovery in the smaller elution volume (24DNT, 37 %), which is
remedied in the larger one (81 % in this particular sample), the increase in concentra-
tion of the compound in the approximately doubled volume of eluate is negligible.
However, if a high recovery must be assured the larger elution volume is used with
this type of sample. Alternatively, a second elution of the extraction assembly is made
if a positive result is given by a first 250-μl elution.

Further recovery data from five other garage mechanics' handswabs spiked
with 10-ng quantities are given in Table IV, and data from a set of swabs taken to
represent a more usual level of soiling and spiked with 1-ng quantities are given in
Table V. The latter data are in the region of the sensitivity limits, given swabs of this
type, as discussed later. Above this level, the average recovery under the standard
conditions from Tables II–IV is 75.8 %. The associated variation, which also is a
function of the soiling present on the swab, on average is ± 15 %. This, from the
results in Table III, is reduced to ± 8.5 % by an increased elution volume.

Detection limits and interferences on handswabs

As the sensitivity of detection is increased, explosives-free handswabs increas-
ingly yield a number of chromatographic peaks that sometimes appear at positions
corresponding to explosives components, or, if they are not coincident, overlap these
positions. Consequently, minor amounts of explosives components may be obscured.
An extensive quantitative variation in such peaks occurs between handswabs, al-

TABLE V

RECOVERIES OF 1-ng AMOUNTS OF EXPLOSIVES COMPOUNDS FROM FIVE MODER-
ATELY-SOILED HANDSWABS

The extraction assembly was eluted with 250 μl in each case.

Compound	Recovery (%)		No. of results*
	Mean	Range	
HMX			0
PA	79	74–84	2
RDX	84	62–98	4
EGDN	80	71–90	2
TET	38		1
NB	69	54–79	3
NG	78	73–84	3
TNT	70	51–89	5
26DNT	71	70–73	2
24DNT	52	48–57	2
2NT	76	57–95	2
4NT			0
3NT			0
PETN			0

* Successful analyses could be made only in a restricted number of cases. In others the peaks were
obscured.

though the qualitative variation is inconsiderable: some peaks are common to many
samples but vary widely in amplitude. For example, the strong peaks shown in Fig. 3
at 3.4 and 6.3 min are particularly common and normally the most intense apart from
the unretained peak. They are not, however, coincident with explosives components,
but usefully indicate the level of interference to be expected in other parts of the
chromatograms. The amounts of explosives that can be seen against this type of
background is indicated by the results in Tables IV and V. With the heavily soiled
garage mechanics' handswabs (Table IV), 10-ng additions of NG and 4NT to the
whole swabs were obscured in one out of the five swabs used. In the other set of less-
soiled swabs (Table V) 1-ng additions could not be determined in many instances.

Further examples are given in Figs. 5 and 6. Fig. 5 shows chromatograms from
explosives-free handswabs exhibiting the most intense peaks encountered in this
work. Superimposed (dotted line) is a chromatogram representing 1-ng amounts of
the standard compounds, which is equivalent to the contribution expected from the
complete recovery from the swabs of 11 ng of each compound. Fig. 6 is an example
from a more characteristically soiled swab with a superimposed contribution ex-
pected from 1 ng of each explosives compound per swab.

These examples are taken from the examination of a collection of 98 different
swabs. Of these, 25 were from members of the general public; the remainder were
from groups of people occupied largely in manual work, the majority of whose swabs
were extensively soiled. Of the latter, 12 were collected from garage mechanics, 8 at a
steel fabrication plant, 14 at a light engineering works, 22 at a building site, 15 at a rail
terminal and 2 from laboratory staff. One swab from each person, generally from the
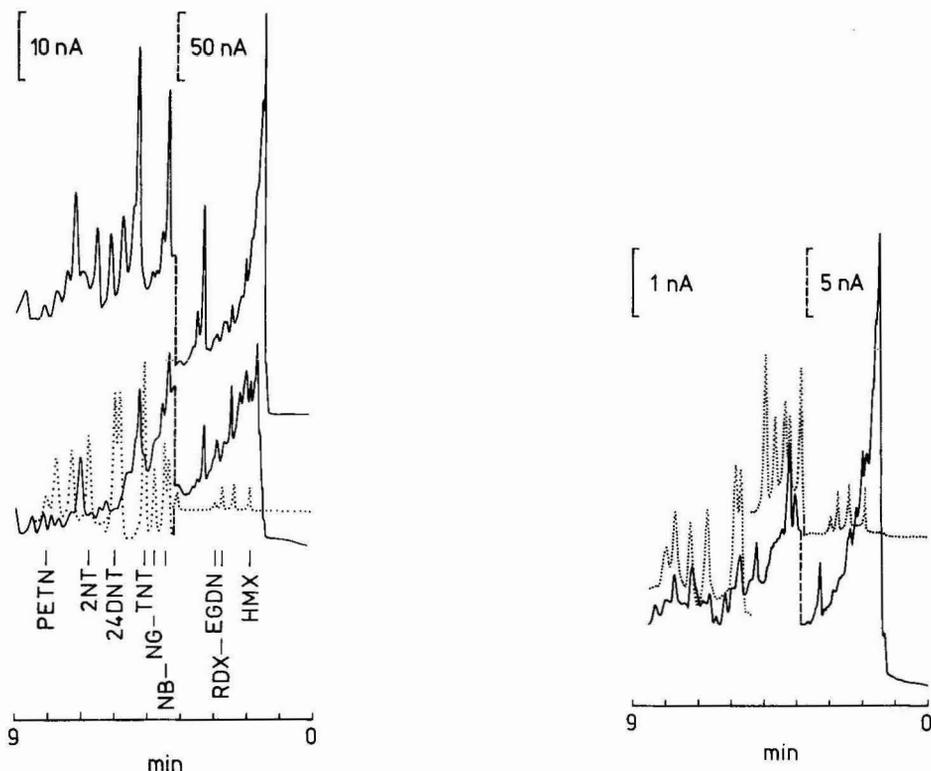
Fig. 5. Chromatograms from two heavily soiled, explosives-free handswabs (full lines), and 1 ng of each of the standard compounds (dotted line), of which some of the more important are labelled. The abbreviations are identified in the caption to Fig. 1. The chromatography conditions are as in Fig. 1, except the injection volume is 20 μl.

Fig. 6. Chromatogram from a typically soiled, explosives-free handswab (full line) with a superimposed chromatogram of 91-pg amounts of the standards, equivalent to 1-ng amounts in the swab. The chromatography conditions are as before, with an injection volume of 20 μl.

right hand, was examined for the presence of chromatographic peaks coincident with the explosives components run consecutively to each swab sample. Coincidence was taken to be an agreement in retention times of within 1 % (the maximum variation in retention time detectable between any pair of compounds in ten consecutive standard chromatograms was 0.6 %). The results expressed in terms of the number of swabs giving a response within different concentration ranges (on the assumption that losses during recovery were insignificant in the context of the ranges used) are summarized in Table VI. From these results it is apparent that for most of the compounds there is usually little interference at levels above 1 ng per swab. Major exceptions commonly seen in the 1–10 ng interference range are at the 3NT position, which is unimportant because this compound only occurs as an explosives trace as a minor component of the nitrotoluenes mixture, and at the TET position, which compound is of rare occurrence. Lesser exceptions are RDX and PETN. The RDX coincidence typically occurs in heavily soiled swabs, the other is more generally distributed but readily

TABLE VI

INTERFERENCE PEAKS IN A COLLECTION OF 98 USED HANDSWABS

Any interfering peak is allotted to the concentration range of the corresponding explosives component.

Compound	Number of swabs in the indicated range		
	Less than 1 ng per swab	Between 1 and 10 ng per swab	Greater than 10 ng per swab
HMX	93	5	0
PA	95	2	1
RDX	86	12	0
EGDN	95	2	1
DNB	98	0	0
TET	70	20	8
NB	95	3	0
NG	95	3	0
TNT	97	1	0
26DNT	97	1	0
24DNT	94	3	1
2NT	95	3	0
4NT	92	6	0
3NT	41	53	4
PETN	88	8	2

distinguished from PETN voltammetrically (see below). In general, from the data given in Tables II–VI, the sensitivity limits of the present technique applied to used handswabs are on the order of 1–10 ng per 100-mg swab.

Voltammetric selectivity

The chemical identities of the interference peaks are at present unknown, but in
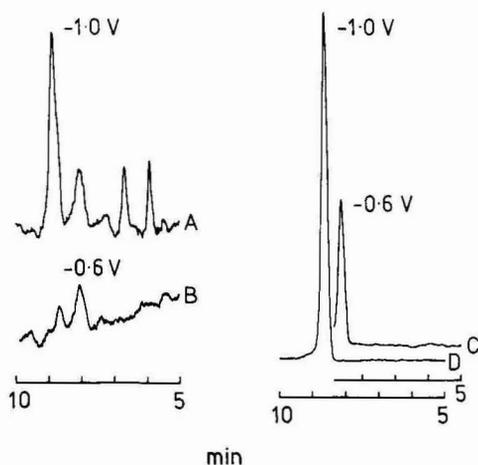
Fig. 7. Sections of the PETN region of chromatograms of a handswab extract (A and B) and a PETN standard (C and D) with the PMDE potential set at -1.0 V (A and D) and -0.6 V (B and C) *vs.* Ag/AgCl. The same sensitivity setting was used for both chromatograms of each sample. Other chromatography conditions are as in Fig. 1.
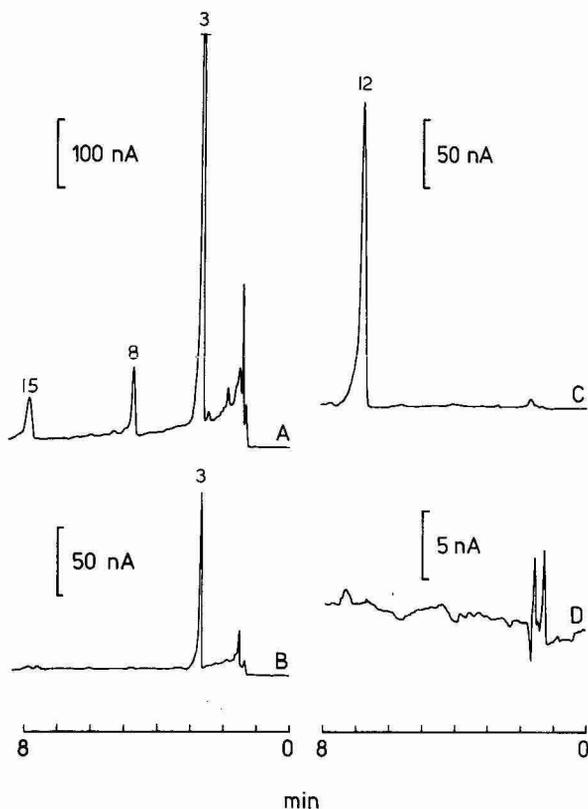
Fig. 8. Chromatograms of various samples: (A) handswab collected after a brief handling of explosives 3 h before sampling, and an unknown number of hand washes in the interim; (B) a swab collected from a beer glass used by a person who had handled an RDX-based explosive 2.5 h before sampling, and had subsequently washed his hands; (C) an extract from an extraction assembly used to vapour-sample the debris remaining after the explosion, 1 day previously, of a device containing 2NT; (D) a blank handswab. The peaks are labelled as in Fig. 1. The chromatography conditions are as in Fig. 1, with 20-μl injection volumes.

some cases, at least, it is possible to distinguish the peaks from explosives components voltammetrically, if not by modified chromatography conditions. Thus, PETN is readily distinguished when the potential of the PMDE is raised to −0.6 V. An example is shown in Fig. 7. For the handswab material (chromatograms A and B) the reduction in the response of the peak at the PETN position is by a factor of 0.12 when the potential is changed from −1.0 to −0.6 V, whereas for PETN (C and D) the factor is 0.41. As the coefficients of variation of the peaks are in the region of 3 %[1], PETN and the handswab compound are completely differentiated.

Examples of various applications

In Fig. 8, chromatogram A shows the result from a handswab of a person who had briefly handled wrapped sticks of two explosives and some Cordtex detonating fuse 3 h before the sample was taken, during which time the hands had been washed at least once. The numbered peaks correspond to RDX (289 ng per swab), NG (108
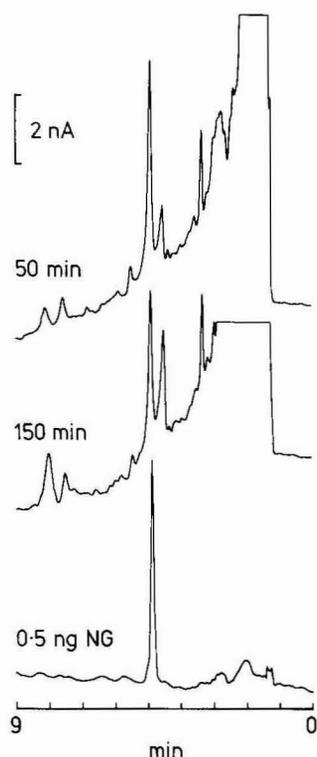
Fig. 9. Handswab chromatograms from two experiments in each of which two shots were fired from a Smith and Wesson Model 10, .38 Special revolver before the indicated sampling times. From each extract 10 μl were injected representing *ca.* 5 % of the material on the swabs. The chromatography conditions are as in Fig. 1.

ng per swab) and PETN (141 ng per swab), which are the major components of each of the explosives. The NG-containing explosive also contained EGDN, which is probably responsible for the weak shoulder present in the tail of the intense RDX peak. Chromatogram B is from a swabbed drinking glass used by a person who had handled an RDX-based explosive 2.5 h previously, and subsequently washed his hands: the peak corresponds to 28 ng per swab of RDX. Chromatogram C is of the eluate from an extraction assembly through which the headspace over the debris from a device containing 2NT, exploded 24 h earlier, had been sampled. This was done at ambient temperature for 20 min with the outlet of the extraction assembly attached to a filter pump whilst the inlet collected the headspace within the Polythene bag containing the debris. A twenty-fold dilution of the eluate was necessary to obtain the 2NT peak in the chromatogram. A direct solvent extraction of the debris gave a similar peak, but with much higher levels of background. A result from a blank swab is shown at D.

Fig. 9 shows the chromatograms from handswabs taken 50 and 150 min after two rounds on each occasion had been discharged in a Smith and Wesson Model 10, .38 Special revolver. The NG peaks seen, from injections representing 5 % of the recovered material, correspond to 9.1 and 5.8 ng per swab, respectively. Two other

experiments yielded *ca.* 1 ng per swab of NG. This amount of potentially available explosive is well within the sensitivity range of the chromatography system, but a satisfactory characterization requires clean-up techniques more specific than the relatively non-selective one presented here.

CONCLUSIONS

Other recently reported results for the detection of explosives traces in hand-swabs include GC–ECD techniques giving limits of 0.5 ng per swab[5] and 10 ng per swab[3] for NG, and 10–50 ng per swab for six other compounds[3]. GC mass spectrometry, for NG, gives 2 ng per swab[5]. The earlier introduced thin-layer chromatography for NG[11] gives, with sample clean-up, 5 ng per swab[5] and 20 ng per swab[3]. Because of the variations in the samples used, in the specificity of the techniques, and in the criteria applied in the assessment of these results, close comparison cannot be made between them and the limits in the region of 1–10 ng per swab obtained with the present technique. Even so, the conclusion seems to be justified that for all of the compounds examined the HPLC–PMDE technique is amongst the most sensitive that have been developed to date. The technique is not subject to operating difficulties analogous to those characteristic of GC–ECD techniques, *e.g.*, detector fouling cannot occur because the detector electrode is replaceable for or during each chromatogram; the only routine maintenance work required is the periodic solvent-purge of the column; and the technique is robust to the multiple analysis of contaminated samples.

Although the clean-up technique has been designed as a general-purpose screening technique for use with HPLC–PMDE, it could be readily modified to suit other detection systems, or to vary its selectivity, by means of other adsorbents and solvents. Probably, slight modification will be needed for its use in the recovery of inorganic explosives components and of nitrocellulose.

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OFF-LINE LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDIES OF o-PHTHALALDEHYDE–PRIMARY AMINE DERIVATIVES

RICHARD C. SIMPSON*, JIMMY E. SPRIGGLE and HANS VEENING*

Department of Chemistry, Bucknell University, Lewisburg, PA 17837 (U.S.A.)

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SUMMARY

The structures of the products formed between several *n*-aliphatic amines and *o*-phthalaldehyde (OPT) during routine post-column derivatization after high-performance liquid chromatographic (HPLC) elution, were determined by gas chromatography–mass spectrometry. OPT derivatization studies were carried out separately in the presence of mercaptoethanol (MCE) and in the presence of ethanethiol (ETH). Comparison of the mass spectra of the silylated OPT–MCE derivatized amines with those of the non-silylated OPT–ETH derivatives indicated a consistent fundamental structure with similar fragmentation patterns. It was shown that the structure of the fluorescent product obtained under analytical post-column HPLC derivatization conditions is a 1-alkylthio-2-alkyl-substituted isoindole. This finding is in agreement with results reported earlier for preparative-scale reactions.

INTRODUCTION

High-performance liquid chromatographic (HPLC) separations of simple aliphatic amines pose a special problem involving detection. The amines do not absorb in the UV region of the spectrum nor do they possess native fluorescence. Therefore, it is necessary to derivatize these compounds to a more easily detected form. Derivatization usually involves chromophoric or fluorophoric labeling of the molecule prior to detection. The advantage of using the latter method lies in the superior specificity and sensitivity of fluorescence detection over ultraviolet (UV) or visible (VIS) absorption[1].

Various reagents are currently available for fluorescent labeling of primary amines. These include fluorescamine[2,3], dansyl chloride[4,5], and the increasingly popular reagent, *o*-phthalaldehyde (OPT). Since OPT was first introduced by Roth[6], it has been used in a wide variety of applications[7–9]. In 1976, Simons and Johnson[10] presented the first study elucidating the structure of the fluorescent product formed in the

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* Present address: Department of Chemistry, University of Rhode Island, Kingston, RI 02881, U.S.A.

preparative reaction of a primary amine with OPT and 2-mercaptoethanol (MCE). Based upon data collected from 100-MHz proton NMR spectra of derivatized *n*-butylamine, they concluded that the structure of the product was a 1-alkylthio-2-alkyl-substituted isoindole[10,11], as shown in Fig. 1. In a later publication, they demonstrated that ethanethiol (ETH) could replace MCE to yield a more stable product of the same general structure[12].

X = OH for 2-mercaptoethanol
X = H for ethanethiol

Fig. 1. Structure of the derivative as proposed by Simons and Johnson[10,11].

In a later investigation by Simons and Johnson[13] they reported results based on the UV spectra of the derivatization reaction mixture of various primary amines to confirm the presence of the isoindole ring system. They also performed mass spectral (MS) analysis of various amines derivatized on a preparative scale in order to establish further the structure of the fluorescent product. However, due to the difficulty in isolating isoindoles[13], several measures, including long reaction times (15 min), the use of bulky alkyl thiols and low temperatures of crystallization (0°C), were taken to permit precipitation and isolation of the fluorescent adduct. Although these conditions did achieve preparative isolation of the product, they are not typical of those normally used in OPT-derivatization of primary amines in HPLC applications. Normally, on-line HPLC procedures are carried out over a much shorter period of time (less than 1 min) in alkaline aqueous buffers, which are at or above room temperature. Thus, it is not necessarily valid to assume that the fluorescent products, obtained through the use of each of these vastly different conditions, are the same.

The purpose of our study was to determine the structure of the major product obtained for amines actually eluted from HPLC columns and subjected to post-column derivatization. This was achieved by MS analysis of extracts of the derivatization reaction mixtures. Various aliphatic primary amines were derivatized with OPT and MCE and again with OPT and ETH. Thus, the structure of the product was observed as a function of amine structure and as a function of using either ETH or MCE. The results obtained were then cross-checked against each other to establish a consistent structure of the fluorescent adduct.

We have presented detailed MS fragmentation patterns for several of the primary amine–OPT derivatives in this paper. The availability of such data for these important HPLC derivatives should be useful as the trend toward combined HPLC–MS continues to grow.

EXPERIMENTAL

Apparatus

The HPLC system was a Spectra-Physics SP-8700 solvent delivery system (Spectra-Physics, Santa Clara, CA, U.S.A.) equipped with a Model 7120 injection

valve (Rheodyne, Cotati, CA, U.S.A.), fitted with a 100-μl sample loop. The column used was 30 cm × 3.9 mm I.D., packed with Zorbax C_8 bonded phase material (DuPont, Wilmington, DE, U.S.A.). The post-column OPT derivatizing reagent was pumped into the column effluent by a Model 39-650 high pressure pump (Rainin Instrument, Woburn, MA, U.S.A.) through a Swagelok "T" connector (Allentown Valve and Fitting, Emmaus, PA, U.S.A.). On-line post-column derivatization occurred in a reaction coil which was 305 cm × 0.023 mm I.D. coiled stainless steel tubing (Supelco, Bellefonte, PA, U.S.A.). The fluorophores were detected by a Schoeffel FS-970 spectrofluoromonitor (Kratos, Westwood, NJ, U.S.A.) with the excitation monochromator set at 340 nm and equipped with a Type 440 emission filter (Kratos).

The gas chromatograph–mass spectrometer was a Finnigan Model 4021 (Finnigan, Sunnyvale, CA, U.S.A.). The gas chromatograph was equipped with a 25 m × 0.25 mm I.D. SE-54 (film thickness 0.25 μm) fused silica column (J & W Scientific, Rancho Cordova, CA, U.S.A.).

Reagents

Aliphatic amines (methylamine, 40 % aqueous; ethylamine, 70 % aqueous; *n*-propylamine, 98 %; *n*-butylamine, 96 %; *n*-amylamine, 99 %), ETH and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). OPT was purchased from Sigma (St. Louis, MO, U.S.A.). Reagent grade boric acid and anhydrous sodium sulfate were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Matheson, Coleman & Bell (Norwood, OH, U.S.A.) was the source of reagent grade MCE. HPLC grade methylene chloride and methanol were purchased from Burdick & Jackson Lab. (Muskegon, MI, U.S.A.). Ultrapure water was produced in-house by a Milli-Q reagent grade water system (Millipore, Bedford, MA, U.S.A.). All other incidental chemicals were of reagent grade purity.

The first derivatizing reagent, OPT–MCE, was prepared by dissolving 800 mg of OPT and 600 μl of MCE in 10 ml of methanol; this mixture was then added to approximately 950 ml of a 0.50 M potassium borate buffer at pH 9.0. The total volume was then brought to 1.00 l by addition of more borate buffer. The second derivatizing reagent, OPT–ETH, was prepared identically. Multicomponent amine mixtures were prepared by mixing 1-ml samples of each amine and shaking thoroughly.

Procedure

A 100-μl sample of the five component amine mixture was injected into the HPLC system and eluted through the column with 0.20 M sodium acetate buffer (pH 4.50) at a flow-rate of 2.0 ml/min. The OPT–MCE (or OPT–ETH) reagent was added to the column effluent at a rate of 0.7 ml/min. The detector effluent was collected for the duration of full-scale detector response. The collected fraction was then extracted with two 2-ml portions of methylene chloride. The methylene chloride layer was removed and rendered anhydrous by being passed through a short Pasteur pipette containing a plug of glass wool and anhydrous sodium sulfate. The resulting methylene chloride fraction was collected and evaporated under a stream of dry nitrogen, to a volume of approximately 100 μl. The OPT–MCE derivatized amines were then further derivatized for GC–MS analysis by adding 100 μl of BSTFA to the methylene

chloride concentrate, mixing thoroughly and warming gently for 1 min to insure complete silylation. This part of the procedure was omitted for the OPT–ETH derivatized amines. A 0.1-μl aliquot of either the silylated methylene chloride concentrate, or the untreated OPT–ETH sample was injected into the GC–MS system at an injector temperature of 310°C, using a 15:1 split ratio and a helium carrier flow-rate of 1 ml/min. The column temperature was initially held at 100°C for 2 min, then increased to 300°C at a rate of 15°C/min for OPT–MCE derivatives and 12°C/min for OPT–ETH derivatives. The mass spectrometer was used in the electron-impact (EI) mode, with the electron energy set at 70 eV. The scan time was 1 sec over a mass range of m/z 33 to m/z 500.

RESULTS AND DISCUSSION

The purpose of the current HPLC–MS study was to expose aliphatic amines to conditions actually encountered in a typical HPLC separation and on-line post-column derivatization, even though minimal separation of these compounds was achieved by reversed-phase HPLC. Each of the amines was derivatized with OPT–MCE and OPT–ETH in separate experiments. This permitted observation of the derivative structures for each amine as a function of either MCE or ETH addition to the OPT derivatizing reagent. Due to the presence of a hydroxyl group in the derivative formed with OPT–MCE (see Fig. 1), it was necessary to silylate the anhydrous extract concentrate of the on-line HPLC derivatization reaction in order to obtain a product which was sufficiently volatile to survive gas chromatographic (GC) separation.

The reconstructed ion current gas chromatogram (RIC) of the silylated extract of the OPT–MCE derivatization is shown in Fig. 2. The peaks at scan numbers 722,
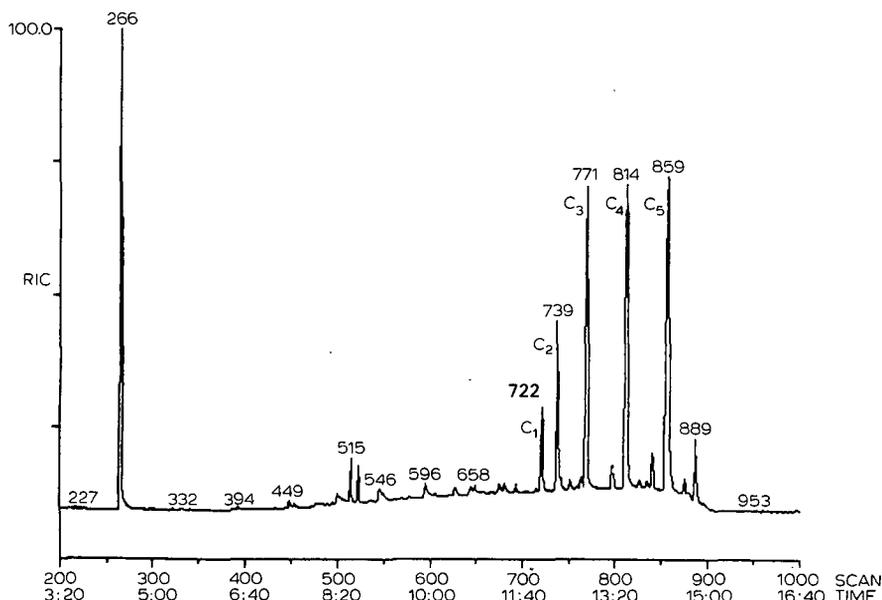
Fig. 2. RIC of silylated OPT–MCE amine derivatives.
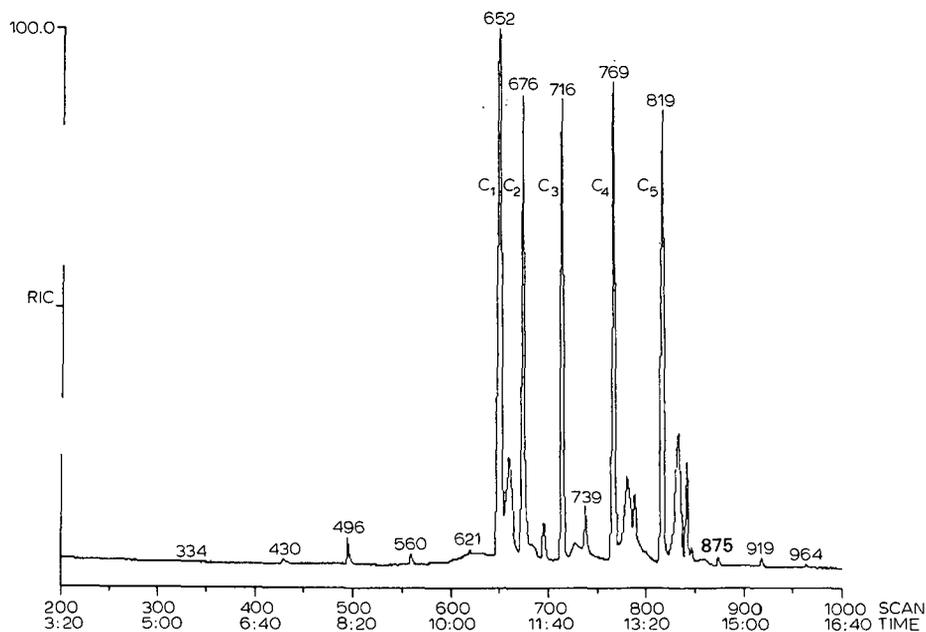
Fig. 3. RIC of OPT–ETH amine derivatives.
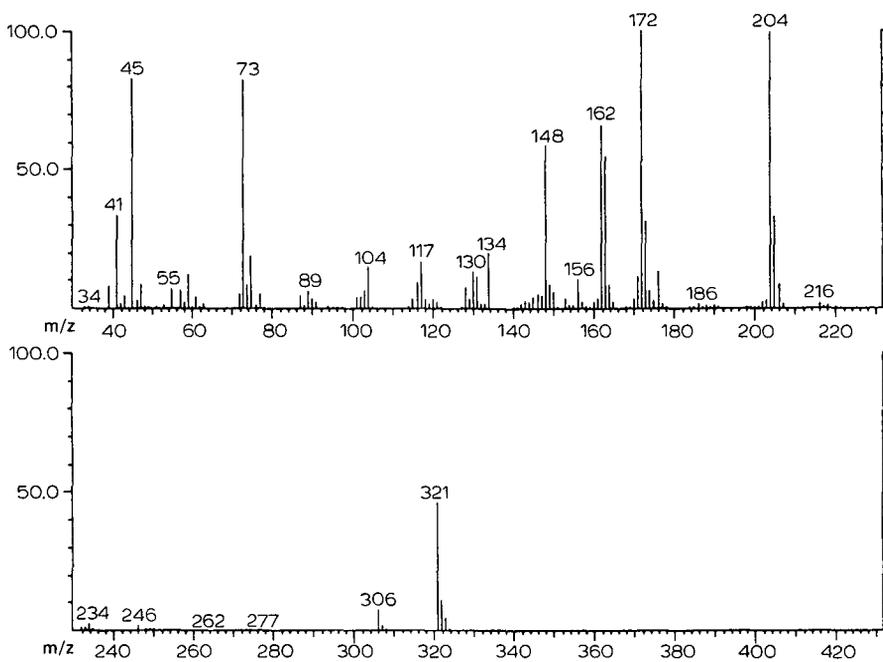
Fig. 4. EI mass spectrum of silylated OPT–MCE *n*-butylamine derivative.

TABLE I

EI MASS SPECTRAL FRAGMENTATION OF n-BUTYLAMINE DERIVATIVES

OPT–MCE n-butylamine derivative (silylated)			OPT–ETH n-butylamine derivative (unsilylated)		
m/z	Rel. int. (%)	Assigned fragment	m/z	Rel. int. (%)	Assigned fragment
321 (M⁺)	46	(isoindole with SCH₂CH₂OSi(CH₃)₃ and N–(CH₂)₃CH₃ substituents)	233 (M⁺)	68	(isoindole with SCH₂CH₃ and N–(CH₂)₃CH₃ substituents)
204	99	(isoindole, S⁺, N–(CH₂)₃CH₃)	204 (base)	100	(isoindole, S⁺, N–(CH₂)₃CH₃)
172 (base)	100	(isoindole, S⁺, N–(CH₂)₃CH₃)	162	82	(isoindole, S⁺, NCH₃)
162	66	(isoindole, S⁺, NCH₃)	148	86	(isoindole, S⁺, NH)
148	59	(isoindole, S⁺, NH)			
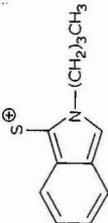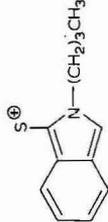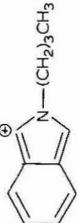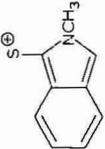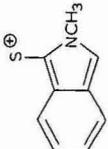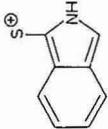
739, 771, 814 and 859 represent the silylated forms of derivatized methyl-, ethyl-, *n*-propyl-, *n*-butyl- and *n*-amylamine, respectively. Fig. 3 is the RIC of the products resulting from on-line HPLC derivatization with OPT–ETH. The peaks at 652, 676, 716, 769 and 819 represent the derivatives obtained for methyl-, ethyl-, *n*-propyl-, *n*-butyl- and *n*-amylamine, respectively. Other peaks in both chromatograms (Figs. 2 and 3) are due to derivatized isomeric amines, incompletely derivatized amines, impurities and decomposition products.

In this discussion, only the structural analysis of derivatized *n*-butylamine will be presented; although, the structures for the other four derivatized amines were found to be analogous. The EI mass spectra of the silylated OPT–MCE and the OPT–ETH derivatives of *n*-butylamine are shown in Figs. 4 and 5, respectively. In Fig. 4, the molecular ion (M^+) and the base peak were observed at m/z 321 and m/z 172, respectively. Fig. 5 showed M^+ at m/z 233 and the base peak at m/z 204. Table I lists the major m/z values, their relative MS peak intensities and the assigned molecular fragments. The other analogous silylated OPT–MCE and unsilylated OPT–ETH amine derivatives exhibited similar patterns consistent with their respective molecular weights. Table II summarizes the m/z values of the respective M^+ ions and base peaks from the mass spectrum for each amine derivatized by both methods.
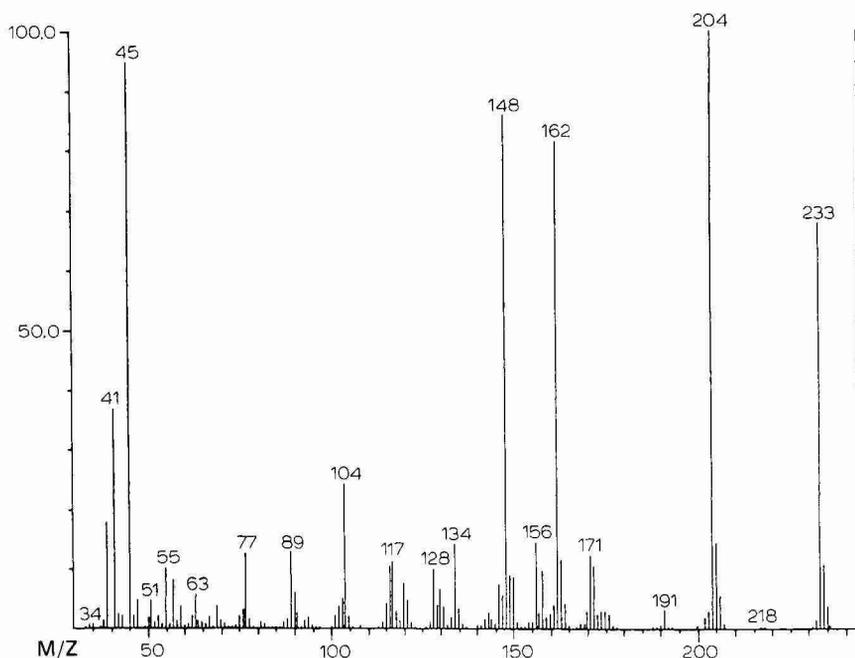
Fig. 5. EI mass spectrum of OPT–ETH *n*-butylamine derivative.

Examination of the data in Table I suggests a consistent basic structure of the derivative, regardless of which reagent was used to produce it. The difference in the M^+ m/z values is due to the presence of a trimethylsilylated hydroxyl group in the OPT–MCE derivative which is absent in the OPT–ETH derivative, thus resulting in a consistent difference of 88 m/z units for the two respective molecular ions for each

TABLE II

SUMMARY OF MS DATA FOR C_1-C_5 DERIVATIZED ALIPHATIC AMINES

Parent amine	OPT–MCE derivative (silylated)		OPT–ETH derivative (unsilylated)	
	M^+ (m/z)	Base peak (m/z)	M^+ (m/z)	Base peak (m/z)
Methylamine	279	162	191	162
Ethylamine	293	176	205	146
n-Propylamine	307	190	219	190
n-Butylamine	321	172	233	204
n-Amylamine	335	186	247	162

amine. The data presented in Table II, along with more complete fragmentation patterns not presented in this paper, show that the fundamental structure of OPT–MCE or OPT–ETH amine derivatives was independent of the amine used.

This study has demonstrated that under conditions normally used for HPLC on-line post-column derivatization of amines: (a) no preparative isolation of the product is necessary for structural analysis; (b) the fundamental structure of the fluorophore was consistently present in all derivatives; (c) substitution of ETH for MCE in the OPT reagent gives an analogous non-hydroxylated derivative (as expected) and (d) the structure of the fluorescent product obtained under analytical post-column derivatization HPLC conditions is a 1-alkylthio-2-alkyl-substituted isoindole, in agreement with that reported in the literature for preparative-scale reactions.

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Note

Determination of some polyaromatic compounds by reversed-phase liquid chromatography with electrochemical detection

W. LOWRY CAUDILL, MILOS V. NOVOTNÝ and R. MARK WIGHTMAN*

Department of Chemistry, Indiana University, Bloomington, IN 47405 (U.S.A.)

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Synthetic fuels derived from sources such as coal and oil shale are being increasingly analyzed for components that may present certain health hazards. While the multicomponent separation and identification methods combined with extensive fractionation schemes[1-6] are important to fully characterize these materials, simple measurements are also needed for the selected compounds that possess mutagenicity and carcinogenicity. Polycyclic aromatic primary amines (APAHs) were found among the potent mutagens contained in petroleum substitutes[4-8].

A key feature of liquid chromatography (LC) with electrochemical detection (ElCD) is the selectivity that can be afforded by the judicious choice of the applied potential. In this communication, we show that amperometric detection can be employed to selectively determine APAHs in the presence of other polycyclic aromatic compounds (PACs). This selectivity may, therefore, be of great utility for routine determinations. The electrochemical oxidation of PACs in solvents such as acetonitrile has been investigated[9,10]. In general, APAHs are oxidized at less positive potentials than polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic nitrogen heterocycles (PANHs) are more difficult to oxidize than either of the above classes.

APAHs have been previously determined by strong cation-exchange LC–ElCD[11]. However, recent advances in column technology and cell design provide considerable improvement in this analysis scheme. The use of glassy carbon electrodes and inert materials throughout the amperometric cell permit a wide range of solvents to be employed. While separation of PACs can be achieved by reversed-phase LC with acetonitrile as the mobile phase, electrochemical detection is still possible. Since non-aqueous LC–ElCD is an unexplored area, we have characterized the performance of the detector in this application.

EXPERIMENTAL

Reagents

Standard solutions of anthracene (Gold Label; Aldrich, Milwaukee, WI, U.S.A.) and 2-aminoanthracene (Aldrich) were prepared immediately before chromatographic analysis in acetonitrile (HPLC Grade, Fisher, Cincinnati, OH, U.S.A.) – water (doubly distilled) (90:10) which contained sodium perchlorate (0.1 M or 0.01 M). The 2-aminoanthracene was sublimed (200°C) prior to use. Standard solutions of

anthracene, 2-aminoanthracene and acridine $(1 \cdot 10^{-3}$ M, Sigma, St. Louis, MO, U.S.A.) were prepared immediately before cyclic voltammetric characterization in acetonitrile which contained sodium perchlorate (0.1 M).

Apparatus

LC system consisted of a constant-flow reciprocating pump (Model 396 mini pump; Milton Roy, Riviera Beach, FL, U.S.A.), a loop injector (50 µl, Model 7010; Rheodyne, Berkeley, CA, U.S.A.) and a reversed-phase column (Chromanetics Spherisorb, ODS-2, 25 cm × 5 mm I.D., 5 µm particle size; PJ Cobert Assoc., St. Louis, MO, U.S.A.). A stainless-steel tube (0.1 m × 5.0 mm I.D.) between the pump and the loop injector served as a pulse dampener. The mobile phase, which contained sodium perchlorate (0.1 M or 0.01 M) in acetonitrile–water (90:10) was recycled by pumping the effluent into the buffer reservoir. The mobile phase reservoir was sealed to prevent solvent evaporation. The flow-rate was 1.2 ml/min.

Amperometric detection was accomplished with a working electrode of glassy carbon (Tokai GC-20; Atomergic, New York, NY, U.S.A.). The glassy carbon was polished to a mirror finish with the use of 600 grit sandpaper followed by polishing successively on a felt cloth with 5 µm, 0.3 µm, and 0.05 µm alumina (Fisher, Cincinnati, OH, U.S.A.). The auxiliary electrode, which is also the upper portion of the cell, was made of stainless steel as described previously[12]. A pre-cut spacer (51 µm thick, Tefzel; DuPont, Wilmington, DE, U.S.A.) was laminated (20 min at 300°C) onto the auxiliary electrode. The lamination of the Tefzel spacer was necessary to prevent the leakage that occured between the auxiliary electrode and spacer when PTFE or polyethylene spacers were employed. The entire assembly was then clamped together with 4 bolts. The working electrode area was 0.46 cm^2 and the cell volume was 2.3 µl. The reference electrode in these studies was a silver/silver perchlorate (saturated) electrode in acetonitrile–water (90:10). The reference electrode potential was checked daily *versus* a saturated calomel electrode and was found to be 0.40 V more positive. Voltammetric studies employed a PARC Model 174A (Princeton, NJ, U.S.A.) and a disk-shaped glassy carbon electrode (area 0.058 cm^2).

RESULTS AND DISCUSSION

The cyclic voltammograms of 2-aminoanthracene, anthracene and acridine in acetonitrile with sodium perchlorate (0.1 M) as supporting electrolyte at a glassy carbon electrode gave oxidation peak potentials +0.16 V, +0.82 V and +1.17 V, respectively. These results are similar to those obtained at a rotating platinum electrode in acetonitrile[9,10]. Analysis of the relative peak potential differences indicates that LC–ElCD should be able to determine 2-aminoanthracene in the presence of anthracene and acridine. However, as indicated in the introduction, a detector which is physically stable (*i.e.* will not degrade, dissolve, etc.) in acetonitrile, was needed. To achieve this requirement an amperometric detector was constructed in which the mobile phase contacts only stainless steel, Tefzel, glassy carbon, and the reference electrode.

Fig. 1A shows the chromatogram of 2-aminoanthracene (5 pmol) at +0.4 V in the presence of anthracene (5 pmol). The mobile phase was acetonitrile–water (90:10) with 0.1 M sodium perchlorate as the supporting electrolyte. The working electrode
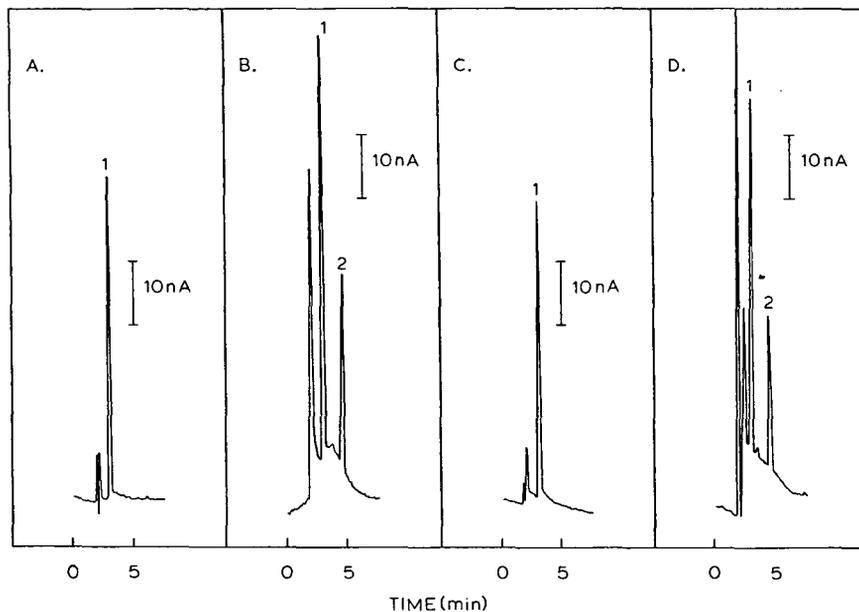
Fig. 1. Chromatograms of standard solutions of 2-aminoanthracene (5 pmol) and anthracene (5 pmol) with acetonitrile–water (90:10) mobile phase. (A) Working electrode potential, $E_{app} = +0.4$ V with 0.1 M sodium perchlorate as supporting electrolyte; (B) $E_{app} = +0.8$ V with 0.1 M sodium perchlorate as supporting electrolyte; (C) $E_{app} = +0.4$ V with 0.01 M sodium perchlorate as supporting electrolyte; (D) $E_{app} = +0.8$ V with 0.01 M sodium perchlorate as supporting electrolyte. Peaks: 1 = 2-aminoanthracene; 2 = anthracene.

potential was then altered to $+0.8$ V and the simultaneous detection of 2-aminoanthracene and anthracene was accomplished (Fig. 1B). The signal-to-noise ratios are reported in Table I and these compare favorably with those observed with this detector with aqueous LC–ElCD[13]. Thus, LC–ElCD with non-aqueous solvents does not compromise the high sensitivity associated with aqueous LC–ElCD. For 2-aminoanthracene, the calculated minimum detection limit (assuming a signal-to-noise ratio of 2) was less than 50 fmoles.

For the analysis of the non-polar PACs and for the longevity of the reversed-

TABLE I

SIGNAL-TO-NOISE RATIOS OF 2-AMINOANTHRACENE AND ANTHRACENE

E_{app}	Sodium per-chlorate concentration*	2-Aminoanthracene (5 pmol)	Anthracene (5 pmol)
$+0.4$ V**	0.1 M	226	
	0.01 M	419	
$+0.8$ V**	0.1 M	99.2	45.2
	0.01 M	68.2	30.6

* Supporting electrolyte concentration in acetonitrile-water (90:10).
** Versus Ag/AgClO$_4$ (saturated) reference electrode.

phase column, it may be advantageous to use relatively low concentrations of supporting electrolyte. As seen in Fig. 1C and D, chromatograms obtained with 0.01 M sodium perchlorate in the mobile phase are essentially identical to those with 0.1 M sodium perchlorate. The signal-to-noise ratios are also given in Table I for the low sodium perchlorate concentration and are virtually the same as those obtained with 0.1 M sodium perchlorate. The residual currents, however, increased from 57 nA to 81 nA at +0.4 V and from 560 nA to 700 nA at +0.8 V. An increase of this nature may arise because the lower supporting electrolyte concentration increases the solution resistance.

This work clearly indicates that non-aqueous LC–EICD at carbon electrodes may be a viable technique for the selective analysis of PACs. As shown, the method possesses the selectivity (detection of 2-aminoanthracene in the presence of anthracene), sensitivity (subpicomole) and low cost (recycling of buffer) that are associated with aqueous LC–EICD. For the analysis of very complex samples (coal tars and oil shale), the technique may provide valuable qualitative information in sample screening efforts (e.g., APAHs in the presence of PAHs). For fractionated samples, the technique may provide supportive information for identification purposes. If more component resolution becomes a necessity, a miniaturized version of this detector[14] could be used in conjunction with high-efficiency capillary LC[15]. Further, while gradient elution is problematic with electrochemical detection (due mainly to residual current shift), it has been shown that quantitative results on the picomole level can be obtained with small (<10%) gradients[16]. Larger gradients may also be possible; however, it is expected that there will be a decrease in detection limits.

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CHROM 15,723

Note

Rapid molecular weight determination for native glucocerebrosidase from human placenta using high-performance liquid chromatography

P. M. STRASBERG* and J. A. LOWDEN

Division of Neurosciences, Research Institute, The Hospital for Sick Children, and the Departments of Pediatrics and Clinical Biochemistry, The University of Toronto, Ontario (Canada)

Glucocerebrosidase (GCase) is a membrane-bound lysosomal hydrolase. Defects in the activity of this enzyme result in storage of glucocerebroside in reticuloendothelial tissues. The disorder is called Gaucher disease.

GCase is generally extracted from membranes with detergents. Estimations of "subunit" M_r on sodium dodecyl sulfate-polyacrylamide gel systems (SDS–PAGE)[1–3] yielded values ranging from 56,000 to 75,000. When whole tissue extracts were examined by gel exclusion chromatography (Sephadex G-200)[4] with a running buffer of Triton X-100 in 5 mM phosphate buffer, a M_r of 160,000–200,000 was observed for the fibroblast enzyme and 150,000 for the spleen in either 5 or 150 mM NaCl. Partially purified placental enzyme[5], examined on a similar column in 20 mM citrate–phosphate buffer containing cutscum and glycerol, was eluted at a volume corresponding to a M_r of 240,000, whereas a whole tissue placental extract revealed a 240,000 peak flanked by multiple activity peaks. GCase was thus thought to exist *in vivo* as a tetramer (in placenta) or perhaps a dimer (in spleen and fibroblasts). These studies suffered from a lack of sufficient and suitable protein standards, from working too close to the void volume of the Sephadex and from a failure to examine a completely purified protein, no longer in association with other proteins.

We have recently purified placental GCase to homogeneity by an affinity chromatography technique[1] and have examined the M_r of purified enzyme by the new and rapid procedure of high-performance liquid chromatography (HPLC). The technique is highly sensitive and obviates the necessity of making detectable derivatives, multiple activity assays and laborious elution volume measurements. Each run is complete in *ca.* 20 min. Only microgram quantities of protein are required[6,7].

EXPERIMENTAL

The HPLC system consisted of a Beckman 421 microprocessor, a single piston pump (Beckman Model 110A), variable wavelength detector (Beckman Model 155-40) set at 220 nm, a 250-μl sample injection loop and an Altex Model CRIA integrator-printer. The two columns used in series were Beckman size exclusion aqueous Spherogel, Micropak TSK Types SW 3000 and 4000, 7.5 mm × 30 cm, particle size and exclusion limits, M_r, being 10 μm and 3.0 · 10^5 and 13 μm and over 7.0 · 10^5 respectively.

An in-line silica precolumn was used. Such columns consist of a hydrophilic phase bonded to microparticulate silica so that the nature of this surface keeps protein–silanol interactions to a minimum for neutral or acidic proteins[8]. GCase has an isoelectric point close to 5[1]. Organic modifiers can also be used to reduce hydrophobic interactions[8]. The flow-rate was maintained at 0.9 ml/min, and the separations were performed at room temperature.

When the six molecular weight standards (Pharmacia) were separated on this system (Fig. 1) using a running buffer of 100 mM sodium phosphate and 50 mM NaCl at pH 6, a linear relationship was maintained between retention time (directly related to elution volume) and log M_r between M_r 669,000 and 13,700.
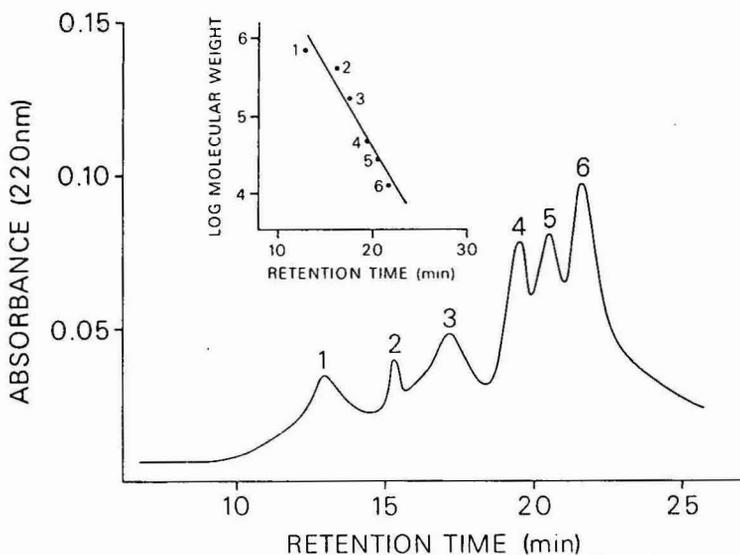
Fig. 1. HPLC separation of six molecular weight standards: 1 = thyroglobulin, M_r 669,000: 2 = ferritin, M_r 440,000; 3 = aldolase, M_r 158,000; 4 = ovalbumin, M_r 45,000; 5 = chymotrypsinogen A, M_r 25,000; 6 = ribonuclease, M_r 13,700. Conditions were as described in the text.

RESULTS AND DISCUSSION

GCase was injected immediately as it emerged from our affinity column. The enzyme was not concentrated or treated in any way before injection. In Fig. 2 the elution profile is compared to the separate elutions of lysosomal hexosaminidase (HEX) A and B from human placenta, purified in our laboratory[9]. The elution times of HEX A and B (17.74 and 18.45 min) correspond to $M\sigma$ of 120,000 and 87,000 respectively, in agreement with previously published values which ranged from 100,000 to 140,000; GCase, at 18.76 min, thus has a M_r of 74,000. These results did not change upon use of the following buffers: 1, 100 mM phosphate, 200 mM NaCl, pH 6; 2, 100 mM phosphate, 200 mM NaCl, 10 % ethylene glycol, pH 6; 3, 100 mM phosphate, 50 mM NaCl, 10 % ethylene glycol, pH 6; 4, 100 mM phosphate, 200 mM NaCl, 0.1 % sodium taurocholate, pH 5 (230 nm). However, as the ionic strength decreased (50 mM phosphate, 50 mM NaCl) the apparent M_r of GCase increased. Finally, when salt was omitted from the buffer and detergents were present (100 mM phos-
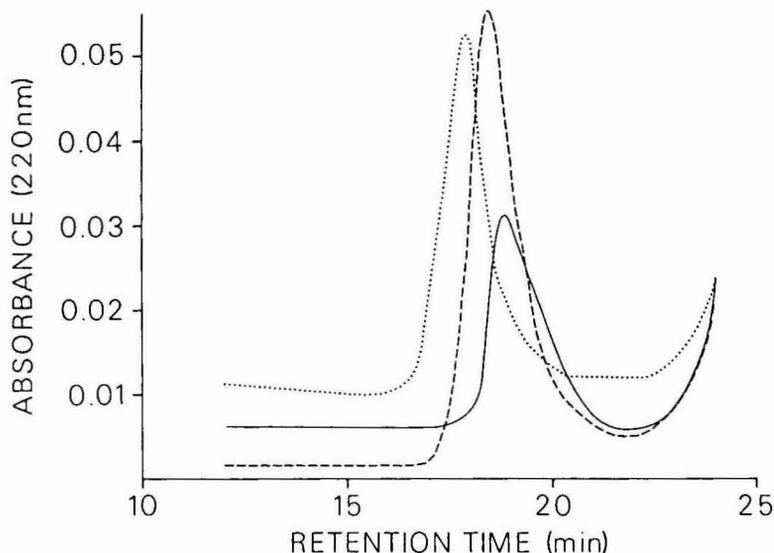
Fig. 2. HPLC analysis of GCase (——, 3 μg), HEX A (···, 4 μg) and HEX B (———, 4 μg). Conditions were as described in the text.

phate, 10 % ethylene glycol; 100 mM phosphate, 0.1–0.5 % sodium taurocholate; 50 mM phosphate, 0.1 % sodium taurocholate), the apparent M_r of both GCase and HEX B increased to 174,000. We believe this is due to an artefactual aggregation phenomenon because the change is also seen with HEX B, an enzyme which has been characterized as to M_r in many laboratories[9]. It is interesting that HEX B behaves
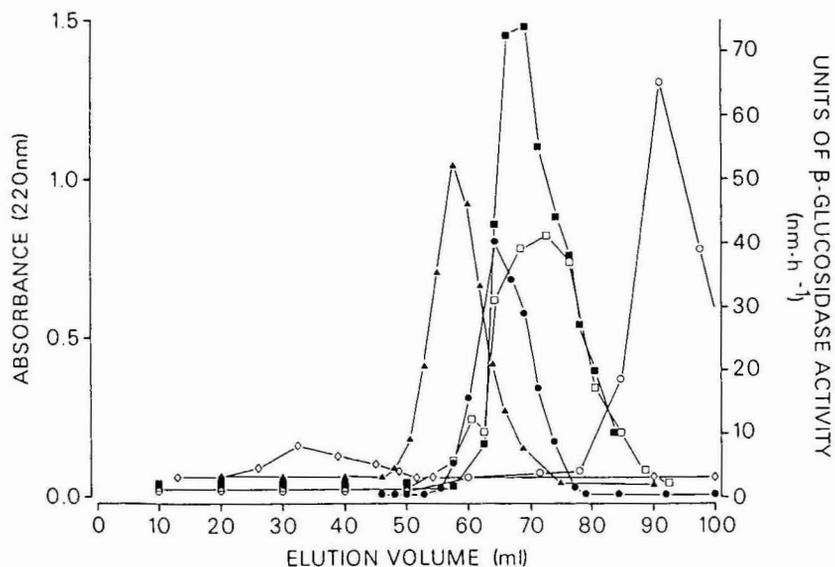
Fig. 3. Sepharose CL-6B analysis of GCase (□ or ■, two different preparations), HEX A (●), catalase (▲, 232,000), blue dextran (◇) and vitamin B_{12} (○). GCase was detected by activity[11], the others by absorbance at 220 nm.

in this fashion since this form of HEX is the more hydrophobic and tends to aggregate[10]. GCase, being a membrane protein, is also hydrophobic by nature.

GCase, HEX A, catalase, blue dextran and vitamin B_{12} were eluted separately from a 1.5×50 cm Sepharose CL-6B column, equilibrated with 100 mM phosphate, 50 mM NaCl, at pH 6 (Fig. 3). GCase emerges from the column later than, and therefore is smaller than, HEX A. This corroborates the HPLC results on the size of the enzyme.

After HPLC analysis of GCase, HEX A and HEX B, we analyzed the catalytic activity of the enzymes using 4-methylumbelliferyl substrates. The recovery of activity was highest for the least hydrophobic enzymes HEX A (90 %) and lower for the more hydrophobic HEX B (67 %) and GCase (40–50 %). The retention times for HEX A and B give apparent M_r values for the native enzymes which are compatible with those in the literature and thus we believe that for GCase in its native state M_r is 74,000. When GCase is reduced and alkylated[1] it migrates on HPLC in 6 M guanidine hydrochloride (our data, not presented) and on SDS-PAGE[1] as a single peak with apparent M_r of 70,000 and 75,000 respectively. Our results indicate GCase is a monomer in its native active state.

This HPLC technique will prove useful in analyzing GCase from Gaucher tissue.

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CHROM. 15,748

Note

Separation of glyceollin isomers I–III by thin-layer chromatography

TAMÁS KÖMIVES★

Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, CA 94720 (U.S.A.)

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Environmental stress of soybeans by chemical treatment (herbicide[1], heavy metal[2], ozone[3], etc.) or microbial infection[2,4–15] leads to accumulation of the anti-fungal metabolites glyceollin I, II and III (GI–III), the levels and relative ratios of which strongly depend on the plant tissue studied and the nature of the elicitor[1,6,11,12]. These isomeric phytoalexins can be analyzed by gas–liquid chromatography (GLC)[4,8] or high-performance liquid chromatography (HPLX)[1,6,11,12,15] but no separation is achieved with the usual thin-layer chromatographic (TLC) systems for phenolic compounds[1–5,7–15]. Accordingly, GI–III are usually quantitated after several TLC purification steps as "glyceollin", a mixture of the three compounds[1–5,7–15].

glyceollin I
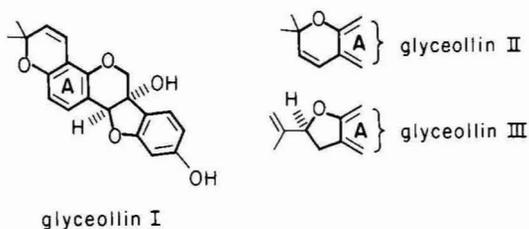
The present study evaluated a large number of cellulose, polyamide or silica gel TLC systems suggested for analysis of phenols[16,17] but without success for separation of GI–III (data now shown). However, these compounds are easily separated by using multiple developments of formamide-impregnated silica gel layers, a sorbent described for TLC of *Umbelliferae* drugs[18]. Quantitation of GI–III in plant extracts is then achieved by using this system in place of the last purification–quantitation steps[1–15], followed by normal UV spectrometry of the eluted spots. This method, when combined with radioautography, may also find application in radiotracer studies of the biosynthesis of GI–III.

★ Permanent address: Central Research Institute for Chemistry of the Hungarian Academy of Sciences, H-1025 Budapest, Hungary.

EXPERIMENTAL

Cut soybean seedlings (5 g) were treated with the diphenyl ether herbicide acifluorfen (sodium 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate; 5 ppm, stem uptake for 72 h). The GI–III mixture was extracted with 40% ethanol and purified by TLC according to Ingham et al.[11]. The purified GI–III mixture and standards of GI, II and III (0.2, 1 and 5 μg; both separately and together) were spotted onto formamide-impregnated silica gel TLC plates [prepared by developing silica gel 60 F254, 0.25 mm thick TLC plates (Merck) twice with 5% formamide in acetone and drying at room temperature] and developed four times with diethyl ether–hexane (3:1). UV-absorbing spots of GI, II and III were detected at R_F values of 0.50, 0.42 and 0.35, respectively, and the compounds were quantitated by UV spectrophotometry (using the following absorption maxima and molar absorption coefficients: GI, 285 nm, 8300 l mol^{-1} cm^{-1}; GII, 285 nm, 8700 l mol^{-1} cm^{-1}; GIII, 292 nm, 9600 l mol^{-1} cm^{-1})[4,6] after scraping off the spots and eluting with 2 × 1 ml ethanol. The levels of GI, II and III found in acifluorfen-treated soy bean seedlings were 7, 19 and 38 μg/g fresh weight, respectively (compared to 4, 28 and 44 μg/g fresh weight determined by HPLC in a separate experiment[1]). The GI–III content in the untreated leaves was <1 μg/g fresh weight.

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Errata

J. Chromatogr., 254 (1983) 91–107
    Page 96, formula in Fig. 2D, the methoxime and *tert.*-butyldimethylsilyl groups
    should be located on carbon atoms 11 and 12, respectively.

J. Chromatogr., 259 (1983) 511–514
    Page 511, first author's name should read "T. Nakatsuji".

corrected
A.| 6 11 03

NEW BOOKS

Essential oils analysis by capillary gas chromatography and carbon-13 NMR spectroscopy, by V. Formacek and K.-H. Kubeczka, Wiley, Chichester, New York, 1983, *ca.* 400 pp., price *ca.* US$ 138.00, £ 63.00, ISBN 0-471-26218-8.

L'Analyse biochimique quantitative par nanochromatographie en couche mince, by M. Bounias, Masson, Paris, 1983, VI + 198 pp., price FFr. 160.00, ISBN 2-225-78914-2.

Isoelectric focusing: theory, methodology and applications (Laboratory Techniques in Biochemistry and Molecular Biology, edited by T.S. Work and R.H. Burdon, Vol. 11), by P.G. Righetti, Elsevier Biomedical, Amsterdam, New York, 1983, XVI + 386 pp., price US$ 80.75 (U.S.A. and Canada), Dfl. 190.00 (rest of world), ISBN 0-444-80498-6 (hardback); US$ 25.50 (U.S.A. and Canada), Dfl. 60.00 (rest of world), ISBN 0-444-80467-6 (paperback).

Analytical aspects of environmental chemistry, edited by D.F.S. Natusch and P.K. Hopke, Wiley, Chichester, New York, 1983, *ca.* 224 pp., price *ca.* US$ 39.90, £ 24.90, ISBN 0-471-04324-9.

Manual of laboratory pharmacokinetics: Experiments in biopharmaceutics, biochemical pharmacology and pharmacokinetics, with a consideration of relevant instrumental and chromatographic techniques, by S.H. Curry and R. Whelpton, Wiley, Chichester, New York, 1983, *ca.* 208 pp., price *ca.* US$ 21.45, £ 9.75, ISBN 0-471-10247-4.

Wilson and Wilson's Comprehensive analytical chemistry, Vol. XVIII, edited by G. Svehla, Elsevier, Amsterdam, Oxford, New York, 1983, 446 pp., price US$ 110.00 (U.S.A. and Canada), Dfl. 275.00 (rest of world), subscription price US$ 106.50 (U.S.A. and Canada), Dfl. 250.00 (rest of world), ISBN 0-444-99685-0.

Supplement to the second edition of Rodd's Chemistry of carbon compounds, Vol. I, Aliphatic compounds, Part FG, edited by M.F. Ansell, Elsevier, Amsterdam, Oxford, New York, 1983, XX + 404 pp., price US$ 115.00 (U.S.A. and Canada), Dfl. 270.00 (rest of world), subscription price US$ 104.25 (U.S.A. and Canada), Dfl. 245.00 (rest of world), ISBN 0-444-42183-1.

Flow phenomena in porous media: fundamentals and applications in petroleum, water, and food production, by R.A. Greenkorn, Marcel Dekker, New York, Basel, 1983, XII + 550 pp., price SFr. 214.00, ISBN 0-8247-1861-5.

MEETINGS

GORDON RESEARCH CONFERENCE ON ION EXCHANGE

The Gordon Research Conference on Ion Exchange will be held on July 18–22, 1983, at Plymouth State College in Plymouth, New Hampshire, U.S.A. Howard S. Sherry and Irving M. Abrams will be Chairperson and Vice Chairperson, respectively.

Further details may be obtained from Alexander M. Cruickshank, Director, Gordon Research Conferences, Pastore Chemical Laboratory, University of Rhode Island, Kingston, RI 02881, U.S.A. Tel.: (401) 783-4011/3372.

A detailed program is listed below.

July 18: *Fundamentals of ion exchange* (Jacob A. Marinsky, session chairperson): George Boyd, "Interpretation of ion exchange properties using the manning condensation model"; Eric Hogfeldt, "Equilibria in non-ideal systems"; Russell Petterson, "Thermodynamics of ion exchange in microcrystals of hydrous oxides"; Kurt Bunzl, "Kinetics of ion exchange."

July 19: *Ion exchange properties of zeolites* (John D. Sherman, session chairperson): Richard M. Wallace, "Fundamental properties of zeolites that are important for radioactive waste treatment"; Rodney P. Townsend, "Thermodynamics of ternary ion exchange in zeolites"; Pochen Chu, "Ion exchange properties of ZSM-5"; Richard M. Barrer, "Ion exchange and isomorphous replacement in zeolites and feldspars."

July 20: *Ion exchange in analytical chemistry* (Gabriella Schmuckler, session chairperson): Michael J. Hudson, "Use of coordinating polymers in hydrometallurgy"; R.M. Cassidy, "Separation of lanthanides by HPLC." *Ion exchange for drinking water and water reuse* (Dennis Clifford, session chairperson): Wolfgang Hoell, "Partial demineralization of drinking water combined with the use of carbon dioxide as regenerant"; Dennis Clifford, "The structure of resins as it affects the uptake of polyprotic acids such as chromic, carbonic, arsenic, etc."

July 21: *Ion exchange properties of clay minerals* (Adrian Cremers, session chairperson): Garrison Sposito, "Ternary cation exchange in clay minerals"; Oscar Talibudeen, "Calorimetry of ion exchange reactions of the phyllosilicates in relation to their properties and composition"; Andre Maes, "How to make cesium high selectivity sites in montmorillonite." *Special lecture*: Calvin C. Calmon, "The history of ion exchange." *Elections. Poster session.*

July 22: *Industrial applications* (George Crits, session chairperson): Howard L. Simpson, "Applications of ion exchange resins in glucose/fructose separations"; E.C. Hitz. "Innovative ion exchange at Three Mile Island nuclear station."

CAPILLARY CHROMATOGRAPHY – 2nd INTERNATIONAL SYMPOSIUM

The 2nd International Symposium on Capillary Chromatography will be held on Oct. 10–12, 1983, at the Westchester Marriott Hotel in Tarrytown, NY, U.S.A. The symposium will consist of invited and submitted papers on all aspects of capillary chromatography, given by leading authorities from throughout the world. Informal discussions will permit the free exchange of ideas on various current questions related to these techniques and their applications. There will also be an exhibition of chromatography instrumentation.

Further information may be obtained from: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.

WORKSHOP ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY

This workshop will be held at the Lausanne Congress Centre in Lausanne, Switzerland, on Nov. 24–25, 1983, and is organized by the International Association of Environmental Analytical Chemistry and sponsored by national organizations. The Workshop is intended to bring together specialists in this field who can give a good account of the state-of-the-art in their particular specialty and to present first-hand experience in sample handling. Strong industrial participation and ample discussion time are planned. Poster contributions to the workshop can be submitted until October 15, 1983.

A short course on "sample handling in liquid chromatography" will be offered prior to the workshop on Nov. 22–23, 1983, in the same location.

For further information contact: Prof. R.W. Frei, The Free University of Amsterdam, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

EUROANALYSIS V, CRACOW, POLAND

The 5th European Conference on Analytical Chemistry will be held in Cracow, Poland, on Aug. 26–Sept. 1, 1984. On behalf of the Working Party on Analytical Chemistry of the Federation of European Chemical Societies, this conference will be organised by the Polish Chemical Society and the Committee for Analytical Chemistry of the Polish Academy of Sciences. The conference will aim, like earlier conferences, at the broadest possible coverage of analytical chemistry. The programme is being planned to appeal both to practising analytical chemists in industrial and control laboratories and to those teaching and doing research on analytical chemistry at universities and research institutes. Topics will encompass all classical and instrumental analytical techniques of determination and separation. Special sessions will be devoted to Computer-Based Analytical Chemistry (COBAC III) and to Speciation in Trace and Environmental Analysis. The official language of the conference will be English.

Further details may be obtained from Professor Zygmunt Kowalski, Secretary-General, Euroanalysis V, Academy of Mining and Metallurgy, Mickiewicza 30, 30-059 Kraków, Poland.

NEW JOURNAL

MATERIALS CHEMISTRY AND PHYSICS

Materials Chemistry, previously published by Cenfor (Genoa, Italy), will be published by Elsevier Sequoia S.A. in a new and enlarged format as *Materials Chemistry and Physics* (Editor: Professor Vincenzo Lorenzelli, Università di Genova, Genoa, Italy). *Materials Chemistry and Physics* is an international journal for publishing results of experimental and theoretical research on the physico-chemical properties and behaviour of different classes of materials, as related to their structure, environment and fabrication processes.

Materials Chemistry and Physics will be published in two volumes (12 issues in total) in 1983. Subscription price for 1983 (Volumes 8 and 9) SFrs. 420.00 (*ca.* US $227.00). A free sample copy can be obtained from the publishers, Elsevier Sequoia S.A., P.O. Box 851, CH-1001 Lausanne 1, Switzerland.

PROFESSOR EDGAR LEDERER CELEBRATES 75th BIRTHDAY

Professor Edgar Lederer, one of the founding fathers of chromatography, will be celebrating his 75th birthday on the 5th of June 1983.

The editor and the publishers of the *Journal of Chromatography* would like to take the opportunity to thank him for all that he has done for the journal through the years since its inception and to congratulate him on the achievements that he can look back on and the great influence that he has been on the scientific community.

CHROMATOGRAPHY

Fundamentals and Applications of Chromatographic and Electrophoretic Methods

Part A: Fundamentals and Techniques
Part B: Applications

ERICH HEFTMANN, U.S. Department of Agriculture, Berkeley, CA, U.S.A. *(editor)*

Journal of Chromatography Library Vol. 22 A + B

This two-part handbook is an up-to-date treatment of the entire field of chromatography and electrophoresis, written by scientists who are internationally recognized as the foremost authorities in their specific fields.

Chromatography and electrophoresis are probably the most widely used analytical techniques today. Since these techniques were last presented in a comprehensive treatise in 1975, significant changes have taken place, both in the techniques themselves and in their applications to analytical problems.

The work is in two parts:
Part A – Fundamentals and Techniques – presents an introduction to the field, including the development and theory of chromatography and electrophoresis, and then gives the theoretical and instrumental basis of each technique.
Part B – Applications – is a critical review of the isolation and quantification methods in current use for various classes of substances.

Both parts of this work belong in all libraries serving chromatographers at technical colleges, research institutes, universities, pharmaceutical companies and instrument manufacturers. Practising chromatographers and analysts will refer to it often when faced with new or unusual analytical problems and situations.

CONTENTS

ELSEVIER

Pyrolysis Mass Spectrometry of Recent and Fossil Biomaterials

Compendium and Atlas

by H.L.C. MEUZELAAR, *Salt Lake City, UT, U.S.A.*, J. HAVERKAMP, *Amsterdam, The Netherlands* and F.D. HILEMAN, *Dayton, OH, U.S.A.*
Techniques and Instrumentation in Analytical Chemistry, Vol. 3

Since the 3rd International Symposium on Analytical Pyrolysis in 1976, the importance of pyrolysis mass spectrometry (Py-MS) as an analytical method has increased considerably. Specially designed Py-MS systems using galvanically heated filament or direct probe pyrolysis have been quite successful in structural investigations and kinetic studies involving synthetic polymers and model compounds. The Curie-point Py-MS has, however, made a lasting impression on the field, being unique in those applications that require maximum reproducibility. This book presents an in-depth discussion of basic principles (including sample requirements, short- and long-term reproducibility problems and data processing approaches) of Py-MS techniques.

The Compendium has been put together by authors who have themselves been deeply involved in an extensively used fully-automated Py-MS system. It is thought that this system, particularly useful in characterising bacteria, could revolutionise clinical methods which have not changed since Pasteur.

The advent of commercially available Curie-point Py-MS systems has prompted the authors to include a small Atlas of over 150 reference spectra of carefully selected biomaterials which should help users of these systems to "tune" their instruments to the existing systems and to evaluate unknown spectra.

An asset to any chemistry library and particularly useful to those scientists considering the application of Py-MS techniques to their own specific problems in the analysis and data processing of biomaterials.

Contents: Part I. Compendium of Basic Principles and Applications.
1. Origins and Development of Pyrolysis Mass Spectrometry of Biomaterials.
2. From Fingerprinting to Structural Investigation. 3. Pyrolysis Mechanisms in Biomaterials. 4. The Technique of Curie-Point Py-MS. 5. Reproducibility in Curie-Point Py-MS. 6. Data Analysis Procedures. 7. Selected Applications to Biomaterials. **Part II. Atlas of Selected Pyrolysis Mass Spectra.**

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PUBLICATION SCHEDULE FOR 1983

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. 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 review articles, 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.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

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