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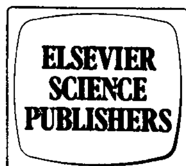

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edited by **P.L. DUBIN**, *Indiana-Purdue University*

(Journal of Chromatography Library, 40)

The rapid development of new packings for aqueous size-exclusion chromatography has revolutionized this field. High resolution non-adsorptive columns now make possible the efficient separation of proteins and the rapid and precise determination of the molecular weight distribution of synthetic polymers. This technology is also being applied to the separation of small ions, the characterization of associating systems, and the measurement of branching. At the same time, fundamental studies are elucidating the mechanisms of the various chromatographic processes.

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THE RÔLE OF THE STATIONARY PHASE IN MICELLAR LIQUID CHROMATOGRAPHY

ADSORPTION AND EFFICIENCY

ALAIN BERTHOD* and AGNES ROUSSEL

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(First received April 1st, 1988; revised manuscript received May 26th, 1988)

SUMMARY

In micellar liquid chromatography a surfactant solution containing micelles is used as a mobile phase. Ionic surfactants strongly adsorb on chromatographic stationary phases. This adsorption can be modified by adding various organic solvents in the micellar mobile phase: the organic modifier, *e.g.*, *n*-pentanol can also adsorb on the stationary phase and replace a part of the adsorbed surfactant. Efficiency in micellar liquid chromatography seems to be linked with the rigidity of the organic layer (bonded moiety + adsorbed molecules) coating the silica surface. Organic modifiers can decrease this rigidity and increase the solute diffusion coefficients in the stationary phase layer. This increase of mass-transfer rate improved the efficiency. *n*-Propanol and tetrahydrofuran were the most effective organic solvents to improve micellar chromatographic efficiencies. Micellar efficiency was shown to be solute-dependent. Quaternary ammonium salts exhibited low efficiency, even with *n*-propanol in the micellar phase, when other solutes (caffeine, toluene) were separated in the same experiment with good efficiency.

INTRODUCTION

The use of a micellar solution as a mobile phase in reversed-phase liquid chromatography (RP-HPLC) was first performed by Armstrong and Terrill[1]. The usefulness of these non-flammable, non-toxic and inexpensive mobile phases was demonstrated[2,3], and an increasing number of applications have been reported[4–9]. Nevertheless, in spite of the advantages of micellar liquid chromatography (MLC), it has not yet achieved widespread usage among practising chromatographers[10]. The main problem is a serious loss of efficiency when compared to traditional hydro-organic mobile phases. Dorsey *et al.*[11] explained that the efficiency problem was due to a poor mass transfer on bonded stationary phases due to the high water content of the micellar phases. They showed that the addition of 3% (v/v) *n*-propanol in a micellar mobile phase and the use of a column temperature of 40°C greatly improved the

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efficiency for non-ionic solutes with micellar mobile phases containing an anionic, a cationic or a non-ionic surfactant[12].

Different authors presented worthwhile improvements of the efficiency in MLC[12,13], but no fundamental study fully explained why the efficiency in MLC is so poor. The aim of the present work was to point out the importance of the stationary phase in MLC. Surfactants adsorb on stationary phases, so surfactant adsorption isotherms were extensively studied[14–17]. The change in surfactant adsorption induced by the addition of methanol, *n*-propanol, *n*-pentanol and tetrahydrofuran (THF) were investigated in this work.

The retention time of a solute allows one to obtain the dimensionless partition coefficients, K_{MW} and K_{SW}, between micelles (M) and bulk water (W) and between the stationary phase (S) and bulk water, respectively. The Armstrong–Nome equation was[18]

$$\frac{1}{k'} = \frac{1}{\varphi}\left[\frac{V\ (K_{MW} - 1)}{K_{SW}} \cdot C_m + \frac{1}{K_{SW}}\right] \quad (1)$$

in which k' is the capacity factor of the solute, φ is the phase volume ratio V_s/V_0 (where V_s is the stationary phase volume and V_0 the dead volume), V is the molar volume of the surfactant and C_m is the concentration of the surfactant in the micellar form, *i.e.*, the total surfactant concentration minus the critical micelle concentration (CMC).

The K_{MW} values measure the affinity of a solute for a micelle; K_{MW} should be dependent only on the mobile phase and be independent of the nature of the stationary phase. The K_{SW} values give information about the affinity of the solute for the surfactant-covered stationary phase. It may be fruitful to associate these partition coefficients with the observed efficiency for various solutes. Additives to the micellar mobile phase affect the two partition coefficients and the peak efficiency. The effects of some additives added to ionic micellar mobile phases is presented and discussed.

EXPERIMENTAL

Materials

Mobile phases were prepared with deionized and distilled water. Sodium dodecyl sulphate (SDS) and cetyltrimethylammonium bromide (CTAB) were obtained from Merck (Darmstadt, F.R.G.); SDS was biochemistry grade and CTAB was analytical grade. Micellar mobile phases were prepared by dissolution of the appropriate amount of surfactant in pure water, in alcoholic or in electrolytic solutions. Then, the solution was aspirated through 0.5-μm cellulose acetate filters (Millipore, Bedford, MA, U.S.A.) and degassed in an ultrasonic bath.

ODS Hypersil (Shandon, Runcorn, U.K.) was mainly used as the stationary phase. This bonded silica was made up of spherical 5-μm particles with a monolayer coverage of octadecyl groups (ODS)[17]. The physico-chemical characteristics of the ODS Hypersil silica were 104 m^2/g, 8.5% (w/w) and 2.5 $\mu mol/m^2$ for the silica surface, the carbon percentage and the bonding coverage, respectively. Columns (100 mm × 4.6 mm I.D.) were slurry packed in the laboratory using about 1 g of stationary phase. The chromatographic apparatus consisted of two Altex Model 110A pumps, a Model 70-10 six-port Rheodyne injection valve and a Altex Model 153 analytical UV detector.

Solutes of various polarities were tested. Toluene (Prolabo, Rhone-Poulenc, France) was chosen as an apolar solute. Caffeine (Serva, Hedelberg, F.R.G.) was chosen as a polar but non-ionic solute. Benzoic acid (Prolabo) ($pK_a = 4.2$) was an anionic solute at mobile phase pH values between 5.5 and 6.5. Benzyltrimethylammonium bromide (BTAB) was a cationic quaternary ammonium solute. Sodium *p*-octylbenzenesulphonate (SOBS) ($pK_a = 0.8$) was prepared by sulphonation of *p*-octylbenzene (Fluka, Italy) and neutralization by sodium carbonate. Cetylpyridinium chloride (CPC) was obtained from Merck. SOBS and CPC were chosen as ionic solutes having surfactant properties. They have an hydrophobic tail of the same length as that of SDS and CTAB, respectively. In the case of SOBS, the benzene ring of the hydrophobic tail is classed as a four-methylene sequence, as is usually done by industrial surfactant chemists. All experiments were performed at 25°C with a flow-rate of 1 ml/min, unless otherwise indicated.

Methods

The adsorption isotherms for SDS and CTAB were determined at 30°C by pumping the appropriate concentration of surfactant in the mobile phase through the column until constancy of the detector baseline with time. Methanol was used fully to desorb the surfactant, which was determined by selective titration. The procedure was described previously[14].

The column efficiency is commonly calculated by assuming a Gaussian model for peak shape. The general equation to obtain the number, N, of plates per column is

$$N = (t_R/\sigma)^2 \tag{2}$$

where t_R is the retention time for the peak and σ is the standard deviation or σ^2 is the variance measured in time units and related to various peak width measurements. In the case of a perfect Gaussian peak profile, the peak width at 60% of peak height, $W_{0.6}$, is two times the standard deviation. So, the formula most commonly used to calculate the column efficiency is:

$$N = 4(t_R/W_{0.6})^2 \tag{3}$$

The problem in MLC was that the peak shape of many solutes deviated from the ideal Gaussian shape by the appearance of a tail. To obtain the exact efficiency, it was necessary to use statistical methods: whatever the peak shape, (i) the retention time t_R is expressed as the first statistical moment of the elution curve and it occurs at the centre of mass of the peak, and (ii) the peak variance, σ^2, is the second central moment[19]. The moment method requires the use of computers and data acquisition techniques, and full calculations are not possible in case of slightly fused peaks. Foley and Dorsey have recently derived a simple and accurate manual method for the calculation of plate counts[20]. Their equation, which corrects for the asymmetry of skewed peaks, is

$$N = 41.7(t_R/W_{0.1})^2/(B/A + 1.25) \tag{4}$$

in which $W_{0.1}$ is the peak width measured at 10% of the peak height, and B/A measures the peak asymmetry, B and A are measured by using the peak maximum and

$A + B = W_{0.1}$. This equation was used in all the micellar efficiency calculations in this work.

It should be noted that the observed variance, σ^2, is the sum of independent variances arising from the column effects, but also from injector, connecting tubing or dispersion in the detector[21]:

$$\sigma^2 = \sigma^2_{col} + \sigma^2_{inj} + \sigma^2_{tube} + \sigma^2_{detec} + \sigma^2_{other} \quad (5)$$

The time constants of the electronic amplifiers of the detector and the recorder are represented by σ^2_{other}. The use of a modern injection valve, a low-volume detector cell and narrow bore (0.1 mm I.D.) connecting tubes reduced the extra-column band broadening but did not fully eliminate it. The major source of the extra-column variance was likely the detector time constant (about 1 s). We made the assumption of similar extra-column band broadenings when a classical hydroalcoholic mobile phase was used or when a micellar phase was used with exactly the same hardware (pumps, injector, column and detector). We compared the plate count, N, or the height equivalent to a theoretical plate, H (= L/N where L is the column length) obtained with a micellar phase to that obtained with an hydroorganic phase.

RESULTS AND DISCUSSION

Adsorption of ionic surfactant on various stationary phases

All the adsorption isotherms of ionic surfactants studied, except for the one of SDS on non-bonded silica, had the same shape. The amount of surfactant adsorbed increases rapidly and reaches a plateau for any surfactant concentration higher than the CMC (Fig. 1). We showed that (i) the quantity of surfactant adsorbed reached similar values (4–5 μmol/m^2) on different alkyl (C_1, C_8 or C_{18}) monolayer bonded phases, and that (ii) the plateau was not strictly horizontal in many cases[14]. The last

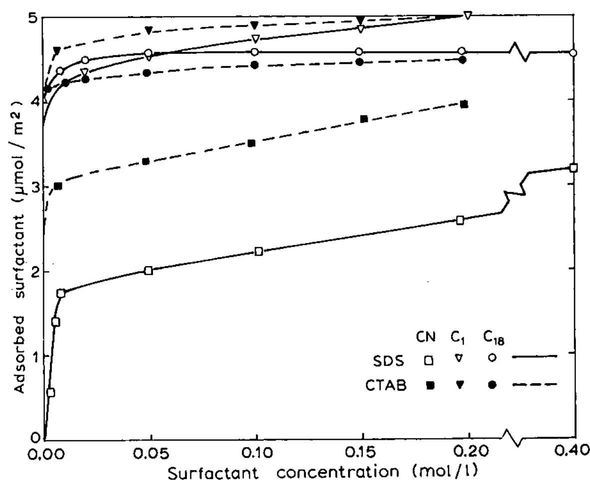

Fig. 1. Surfactant adsorption on three stationary phases: C_1 = SAS Hypersil; C_{18} =ODS Hypersil; CN = CPS Hypersil. Data from ref. 14.

point means that an additional adsorption occurred above the CMC. This further increase of adsorbed surfactant may be as high as 20% of the CMC value in the case of SDS on cyanopropyl bonded silica. A similar comportment was noted by Borgerding and Hinze[7] using the non-ionic surfactant Brij 35 and a Waters Radial-Pak C_{18} bonded stationary phase.

In other work[17] we studied the effect of the addition of 5% (v/v) methanol or 0.1 *M* sodium chloride on the SDS and CTAB adsorption isotherms on two monolayer alkyl (C_1 and C_{18}) bonded silica. 5% Methanol decreased the quantity of surfactant adsorbed by about 15%. The global effect of sodium chloride was an increase or a decrease in the amount adsorbed depending on the surfactant and the stationary phase. Other organic modifiers (*n*-propanol, *n*-pentanol and THF) added to the micellar solution produced a decrease in the plateau concentration of the adsorption isotherms. This decrease was proportional to the mole fraction of the organic modifier added, as shown by Fig. 2.

The adsorption of both SDS and CTAB did not seem to be directly related to the micelle state. The CMC of each surfactant was modified by the addition of organic solvent (Fig. 3). However, the variation of the free surfactant concentration, *i.e.*, the CMC, did not correspond to the isotherm plateau-concentration variations. The organic solvent seemed to compete with the surfactant for adsorption on the stationary phase. Scott and Simpson[22] showed that the longer the alkyl chain of an alcohol, the stronger is the adsorption on C_{18} phases. According to these results, *n*-pentanol was adsorbed on the C_{18} stationary phase more strongly than was *n*-propanol. Thus, it desorbed more surfactant molecules than did propanol. The order of surfactant desorption strength was the same as the order of stationary phase affinity: pentanol > propanol > methanol. The effect of THF, up to 5% (v/v) or a molar fraction, $X = 0.0116$, was similar to the effect of propanol (Fig. 2). The two solvents were given comparable properties when used as chromatographic effluents[21].

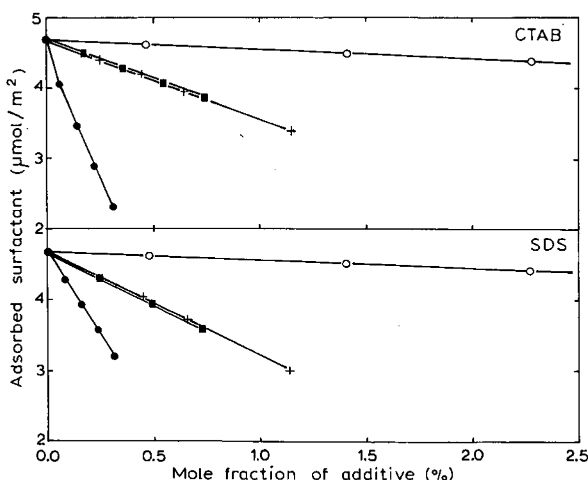

Fig. 2. Additive effects on surfactant adsorption. SDS concentration: 0.05 *M*; CTAB concentration: 0.02 *M*. $X = 2.28\%$ corresponds to 5% (v/v) methanol, $X = 1.15\%$ is 5% (v/v) THF, $X = 0.739\%$ is 3% (v/v) propanol, $X = 0.338\%$ is 2% (v/v) pentanol. Additives: ○ = methanol; + = THF; ■ = propanol; ● = pentanol.

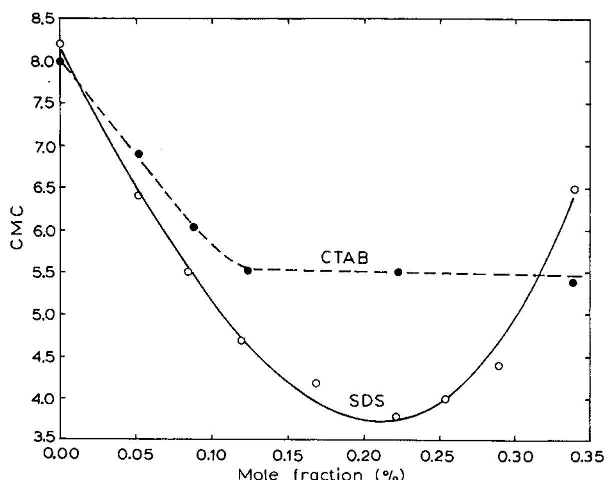

Fig. 3. Pentanol influence on CMC measured by conductimetry. ○, full line: SDS scale in 10^{-3} M. ●, dashed line: CTAB scale in 10^{-4} M. X =0.338% corresponds to 2% (v/v) pentanol.

Efficiency obtained with solutes of various polarities

As stated above, one of the early problems with MLC was rather poor chromatographic efficiency. This low efficiency was shown to be caused by slow mass transfer due principally to poor wetting of the stationary phase[11]. Fig. 4A shows a classical chromatogram obtained with a hydroalcoholic mobile phase. The peak of benzophenone, with a retention time of 3.3 min (k' = 2.15), allowed calculation of the

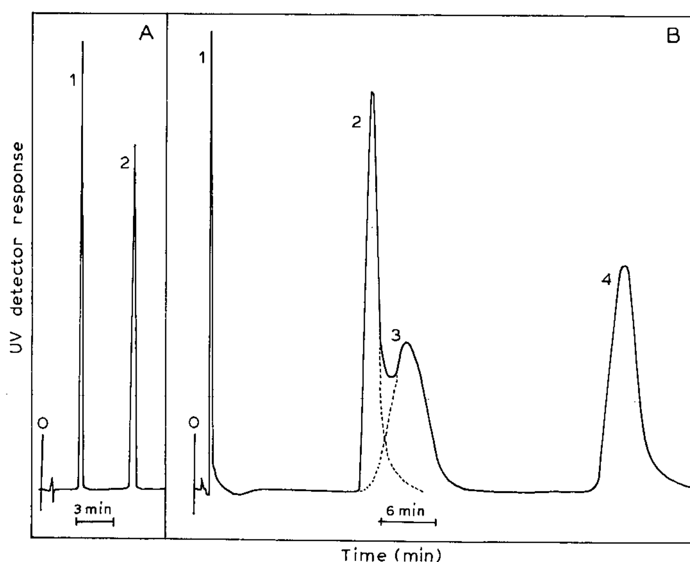

Fig. 4. Chromatograms on a 10-cm ODS Hypersil 5 μm column. A, Mobile phase methanol–water (75:25, v/v). Peaks: 1 = benzophenone; 2 = biphenyl (≈ 1 nmol). B, mobile phase 0.03 M CTAB in water. Peaks: 1 = caffeine (≈ 5 nmol); 2 = benzoic acid (≈ 30 nmol); 3 = CPC (≈ 30 nmol); 4 = toluene (≈ 60 nmol injected).

value of the efficiency, N, to be 3100 plates (H = 32 μm). The second peak (biphenyl), with a retention time of 7.8 min (k' = 6.43), was associated with an efficiency, N, of 4600 plates (H = 22 μm). Eqn. 2 showed that N is related to σ^2 (eqn. 5). Whereas the standard deviation σ_{col}, due to the column itself, was dependent on the elution time, the extra-column standard deviations were not time-dependent. Thus, the extra-column band broadening became less important for the most strongly retained solutes, and the efficiency seemed to increase with increasing retention times. Assuming that the standard deviations, σ_{col}, for benzophenone and biphenyl were strictly proportional to the respective retention times, and using eqns. 2 and 5, it was possible roughly to estimate the extra-column variance to be 1400 μl^2 (corresponding to a global extra-column dead volume of about 37 μl). This value was about 40% of the total variance for benzophenone which was 3500 μl^2 (eqn. 2), but only 10% of the total variance for biphenyl (13 300 μl^2). It was clear that extra-column band broadening had a less significant effect on highly retained solutes. This means that, given the characteristic of our system and with the ODS (C_{18}) column, only k' values greater than 7 were really significant for efficiency study. Fortunately, with high-water-content micellar mobile phases and ODS-bonded stationary phases, capacity factors, k', were higher than 7 for most of the solutes studied.

Fig. 4B shows a chromatogram obtained with the same hardware as for Fig. 4A, but with a micellar mobile phase comprising of 0.03 *M* CTAB. Table I lists the retention times, capacity factors and efficiencies for the solutes separated with CTAB (Fig. 4B) and SDS micellar mobile phases.

As stated in the literature[3–5,7,10–13], the efficiency was much lower with both anionic (SDS) and cationic (CTAB) micellar mobile phases, than with hydroalcoholic mobile phases. However, Table I and Fig. 4 show that the efficiency was strongly solute dependent. So, it seemed to be of interest to try to determine which parameters were involved in micellar efficiency.

TABLE I

CHROMATOGRAPHIC DATA WITH AQUEOUS MICELLAR PHASES

Column: 10 cm × 4.6 mm I.D., ODS Hypersil 5 μm, V_0 = 0.9 ml. Flow-rate: 1 ml/min. Average of three measurements, reproducibility 20%.

Mobile pase	*Solute*	t_R *(min)*	k'	N	H *(μm)*	B/A
0.05 *M* SDS	Caffeine	2.8	2.1	250	400	4.2
	Toluene	39.6	43.0	1350	74	3.4
	BTAB	46.7	50.9	160	625	2.1
	SOBS	6.7	6.4	1650	61	1.5
0.03 *M* CTAB	Caffeine	1.8	1.0	200	500	1.3
	Toluene	46.5	50.7	800	125	1.7
	Benzoic acid	19.5	20.6	490	200	2.2
	CPC	22.5	24.0	180	560	1.2

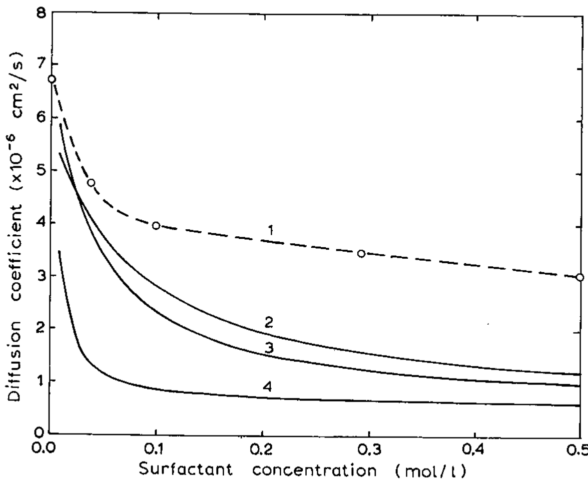

Fig. 5. Effect of micelles on solute diffusion coefficients. From data (compound 1, dashed line) and equations (compounds 2, 3 and 4) of ref. 24. 1 = Sodium 2,6-naphthalenedisulphonate; 2 = *p*-nitrophenol; 3 = *p*-nitroaniline; 4 = naphthol.

According to Snyder and Kirkland[21], the contributions to band broadening in a column can be represented by

$$H = C_e d_p + \frac{C_m d_p^2 u}{D_m} + \frac{C_d D_m}{u} + \frac{C_{sm} d_p^2 u}{D_m} + \frac{C_s d_f^2 u}{D_s} \tag{6}$$

in which the C values are constant plate height coefficients related to eddy diffusion (e), mobile phase mass transfer (m), longitudinal diffusion (d), stagnant mobile phase mass transfer (sm) and stationary phase mass transfer (s), d_p is the diameter of the packing particles, d_f the thickness of the stationary phase layer, D_m the solute diffusion coefficient in the mobile phase layer, D_s is the solute diffusion coefficient in the stationary phase layer and u is the mobile phase velocity.

The first solute-dependent parameter is the diffusion coefficient, D_m, in the mobile phase (terms 2, 3 and 4 in eqn. 6). In recent work[23] we showed that D_m was strongly dependent on the K_{MW} values. Fig. 5 shows the D_m evolution *versus* the surfactant concentration for three binding solutes of similar polarities, and a non-binding solute. Table II lists the efficiencies obtained with these solutes.

There were no significant differences in efficiency between the slow-diffusing solutes and the rapidly diffusing one. Furthermore, there were no significant efficiency differences at different surfactant concentrations[23]. These results indicated that the mobile phase mass-transfer effects were not mainly responsible for the poor efficiency obtained with micellar mobile phases.

The last term of eqn. 6 involves stationary phase mass transfer. It seems to be the most important term, overshadowing the other factors in the case of micellar mobile phases. The thickness, d_f, of the stationary phase layer was significantly increased by surfactant adsorption[7], and the structure of the layer was modified by the insertion of surfactant molecules. This may increases the viscosity of the stationary phase layer and

TABLE II

EFFICIENCY AND DIFFUSION COEFFICIENTS

Column: 30 cm × 4.6 mm I.D., polynitrile 10 μm (Varian). Flow-rate 1 ml/min; 20°C. A 3000-plate efficiency was obtained with a hydroalcoholic mobile phase and the same hardware, from ref. 23.

Solute	K_{MW}	K_{SW}	*0.005 M SDS*		*0.5 M SDS*	
			D_m *(10^{-6} cm²/s)*	*N (plates)*	D_m *(10^{-6} cm²/s)*	*N (plates)*
Naphthol	370	30	1.1	530	0.62	540
p-Nitroaniline	87	4.6	3.3	760	0.97	660
p-Nitrophenol	48	4	3.6	460	1.18	510
Sodium 2,6-naphthalene-disulphonate	*	*	4.5	560	3.06	560

* Non-micelle binding solute.

decrease the diffusion coefficient, D_s, of the solute in the liquid crystal-like layer[23].

The surfactant adsorption was not very different on four monolayer bonded stationary phases[14]. Table III lists the efficiency obtained with the solutes studied compared with the respective K_{SW} values which measure the solute affinity for the

TABLE III

EFFECT OF THE STATIONARY PHASE ON EFFICIENCY

Mobile phase: 0.03 *M* CTAB. Columns: 10 cm × 4.6 mm I.D., packed with Hypersil monolayer bonded stationary phases 5 μm; 25°C. Average of three measurements, reproducibility 20%.

Stationary phase		*Caffeine**	*Toluene*	*Benzoic acid*	*CPC*	*Biphenyl***
CPS cyanopropyl	k'	1.8	27	33	27	3.6
	K_{SW}	2.0	63	490	910	–
	N	430	1210	330	100	4000
	H (μm)	230	83	300	1000	25
SAS C_1	k'	2.4	18	37	26	3.9
	K_{SW}	3.6	55	870	850	–
	N	300	630	200	15	3400
	H (μm)	330	160	500	6700	29
MOS C_8	k'	1.3	39	23	21	5.2
	K_{SW}	1.8	190	760	1000	–
	N	160	600	150	70	3300
	H (μm)	620	170	670	1430	30
ODS C_{18}	k'	1	50.7	20.6	24	6.4
	K_{SW}	2.3	190	550	610	–
	N	200	800	490	180	4600
	H (μm)	500	130	200	560	22

* Efficiency values of caffeine were not very significant given its low retention time (high extra-column effects).

** Biphenyl was used to check the column efficiency with methanol–water (75:25, v/v) on ODS and MOS Hypersil and methanol–water (70:30, v/v) on SAS and CPS Hypersil.

TABLE IV

EFFECT OF SOME ADDITIVES ON EFFICIENCY IN MLC

Column: 10 cm × 4.6 mm I.D., ODS Hypersil 5 μm; 25°C. Average of three measurements, reproducibility 20%, 4600 plates were obtained with biphenyl and a methanol–water (75:25, v/v) mobile phase. Results obtained with caffeine were not very significant because the k' values were lower than 1.5.

Mobile phase	*Solute*	*Additive*					
		None	*0.1 M NaCl*	*5% Methanol*	*3% Propanol*	*0.5% Pentanol*	*3% (v/v) THF*
0.05 *M* SDS	Caffeine	250	230	260	800	450	1100
	Toluene	1350	1000	1000	4400	2300	1400
	BTAB	160	110	120	400	150	160
	SOBS	1650	1000	1300	1900	1300	1500
0.02 *M* CTAB	Caffeine	180	190	210	2000	140	2800
	Toluene	750	1100	800	3900	1100	4000
	Benzoic acid	380	420	600	2200	600	2000
	CPC	200	220	300	370	300	380

surfactant-covered stationary phase. It is apparent that, whatever the stationary phase, the lowest efficiency occurred with CPC and the highest with toluene. At this point, it seems that the higher the K_{SW} value, the lower is the efficiency. The surfactant adsorption on bonded stationary phases was significantly affected by additives in the micellar mobile phase[16,17]. The efficiencies obtained with each additive are listed in Table IV for SDS and CTAB micellar mobile phases.

As stated by Dorsey[10–12], the best improvement in micellar efficiency was obtained with the addition of 3% *n*-propanol in the micellar mobile phase. Although, the efficiency obtained for BTAB and CPC, with SDS and CTAB as micellar mobile phases, respectively, remained low, even when propanol was present (Table IV). These two compounds are quaternary ammonium salts. Amine and ammonium salts have an high affinity for residual surface silanols, which was the reason for the high K_{SW} values (Table III)[15,16]. The first reason for the significantly low efficiency noted with these solutes is the heteroenergetic retention process from the mixed partition–adsorption

TABLE V

EFFICIENCY EVOLUTION OF A COLUMN EXPOSED TO SURFACTANTS

A, New column; B, after 2 days of work with CTAB mobile phases and about 3 h of rinse with methanol (200 ml); C, after 2 days of work with SDS mobile phases and about 3 h of rinse with methanol (200 ml); D, after a couple of working days with CTAB + pentanol mobile phases and rinse with methanol; E, after 2 days of work with CTAB + propanol and rinse with methanol. Column: 10 cm × 4.6 mm I.D., ODS Hypersil 5 μm. Mobile phase: methanol–water (75:25, v/v), 25°C.

Solute	*Test*				
	A	*B*	*C*	*D*	*E*
Benzophenone	3100	2900	2700	3300	3200
Biphenyl	4600	3900	3600	4500	4200

interactions. The second reason is the fact that these silanols lie on the silica surface, which means that quaternary ammonium salts have to pass through the surfactant adsorbed layer and through the bonded layer (Fig. 6) to reach the silanols. This may result in a large d_f value and partly explain the poor efficiency for these solutes (term 5, eqn. 6).

Pentanol was adsorbed more strongly than any other additive studied (Fig. 2 and ref. 22), however, its effect on efficiency was not as good as that of propanol (Table IV). It seemed that an important physico-chemical parameter was the "rigidity" of the organic stationary phase layer. The term "rigidity" was used by De Gennes and Taupin[24] to explain the rôle of medium-chain-length *n*-alcohols on microemulsion formation. They showed that the major effect of those alcohols was to increase the flexibility of the layers separating the aqueous phase from the oil phase. Although the oil phase of a microemulsion is different from the stationary phase in MLC, the aqueous phases are quite similar. Then, in a very crude fashion, we propose to compare the interphases, *i.e.*, the surface of the surfactant-covered bonded phase may resemble the alcohol–surfactant layer separating the oil phase from the aqueous phase in an oil-in-water microemulsion.

With a pure aqueous mobile phase, the bonded layer was in a very rigid "collapsed state"[25] (Fig. 6). Surfactant may be adsorbed on this layer (Figs. 1 and 6, ref. 14) wich still remained rigid. Organic additives may modify and perhaps destroy the crystal-like "collapsed state"[26] (Fig. 6). Propanol seems to be the additive producing the less rigid organic layer which induced high diffusion coefficients, D_s, and higher efficiency. The partial displacement/replacement of adsorbed surfactant molecules by pentanol molecules seems to make up a composite layer less rigid than the pure adsorbed surfactant layer, but more rigid than the propanol–surfactant layer. THF, whose effect on adsorption was similar to that of propanol (Fig. 2), improved the efficiency almost as well as did propanol (Table IV).

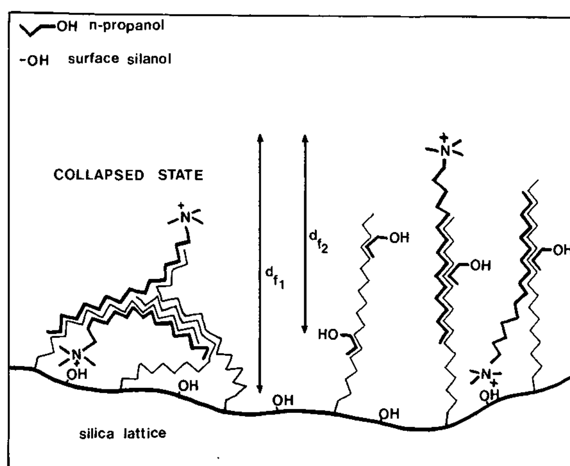

Fig. 6. Oversimplified model of the surfactant-covered stationary phase (CTAB and ODS Hypersil monolayer type). d_{f_1} = Thickness of the layer in the case of quaternary ammonium compounds; d_{f_2} = thickness of the layer in the case of non-polar compounds. Left side: the "collapsed state". Right side: a brush-type layer obtained with 3% (v/v) propanol in the micellar mobile phase.

It was pointed out[7] that the surfactant adsorbed layer was very stable, *i.e.*, it was impossible to desorb the surfactant even after a 24-h dynamic extraction with acetonitrile–water (30:70, v/v). However, pure methanol fully desorbed the entire adsorbed surfactant[14,16]. To confirm this, we determined the efficiency of a column exposed to both anionic and cationic surfactants with intermediate methanol rinsing. The pressure drop remained constant. Table V lists the efficiency evolution of the column. The constancy of the hydroalcoholic pressure drop and efficiency was good evidence of total surfactant desorption on monolayer bonded stationary phases. Indeed, if a part of SDS was irreversibly adsorbed onto the stationary phase, the negative charges would retain the CTA^+ ion during the CTAB exposure, producing a build-up of a thick adsorbed layer that could obstruct the column and/or produce a permanent low efficiency.

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METHODS FOR CHARACTERIZATION OF SELECTIVITY IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

IV. RETENTION BEHAVIOUR OF OLIGOMERIC SERIES

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SUMMARY

The retention behaviour of oligomeric series in reversed-phase systems with respect to the number of repeat structural units and the mobile phase composition can be described by the same set of equations as for homologous series, but the constants of these equations are strongly dependent on the structure of both the repeat structural unit and the structural residue in the molecule. The separation selectivity for the individual oligomers is determined mainly by the size (molar volume) and polarity (interaction index) of the repeat units and of the structural residue, but it also depends on the type and concentration of the organic solvent in the mobile phase. If the repeat structural unit increases both the size and polarity of the oligomer molecules, the selectivity may vary as a function of the structural parameters of the structural residue and of the mobile phase composition, and the retention may even decrease with increasing number of repeat structural units in a given series. This behaviour can be explained, characterized and even predicted (at least qualitatively) from the structural constants V_x, I_x on the basis of the interaction indices model. This retention behaviour was investigated and verified for oligostyrenes, oligoethylene glycols and oligoethylene glycol nonylphenyl ethers on various C_{18} and C_8 columns in mobile phases containing methanol, 1,4-dioxane or 2-propanol in water.

The effectiveness of gradient elution for the separation of oligomeric series depends strongly on their structure. The presence of cetyltrimethylammonium bromide (CTAB) in the mobile phase has only a minor influence on the separation of non-sulphated oligoethylene glycol nonylphenyl ethers, but it leads to an increase in the retention of sulphated ethers, with a separation selectivity similar to that of non-sulphated ethers. The individual oligomers in mixtures containing sulphated and non-sulphated surfactants of this type can be separated in a single run using CTAB-containing mobile phases.

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INTRODUCTION

The retention mechanism in reversed-phase liquid chromatography still remains controversial. The solvophobic theory of Horváth *et al.*[1,2], the molecular statistical theory of Martire and Boehm[3] and the models using molecular connectivity[4], Hildebrand solubility parameters[5–7] and interaction indices[8,9] have been proposed to describe and characterize retention in reversed-phase systems. Various scales of retention indices based on alkyl aryl ketones[10,11], alkan-2-ones[12,13] or polyaromatic hydrocarbons[14] have been suggested for calibrating retention in reversed-phase systems. The scale of interaction indices based on various simple aromatic compounds was intended for the same purpose[8,9,15]. A disadvantage of these methods is that they do not take full account of the selectivity changes with changing mobile phase composition.

A calibration method using alkylbenzene calibration series and two indices to characterize the retention of each solute over a wide range of mobile phase compositions has been introduced recently[16]. One index, n_{ce}, accounts for the hydrophobicity of a solute and the other, q_i, for the polarity of its functional group(s) interacting with mobile phase components. This approach makes it possible to predict the selectivity changes induced by changes in the mobile phase composition and may be used to predict relative and absolute retentions under isocratic[17,18] or gradient elution[19] conditions in binary and ternary mobile phases. The lipophilic and polar indices may be calculated from the additive contributions of structural elements[17].

As with homologous series[20], a regular increase in the logarithms of the capacity factors with increasing number of repeat structural units was observed for some oligomeric series studied systematically in reversed-phase systems, such as for styrene oligomers[21] and for oligoethylene glycol phenyl ethers[22], in agreement with the Martin rule.

Lower styrene oligomers have been separated on octadecylsilica columns by elution wih pure acetonitrile[23], or using gradient elution with increasing concentration of tetrahydrofuran in water[24–26], tetrahydrofuran in methanol[24,27] or dichloromethane in methanol[28,29]. Isocratic separations of styrene oligomers on phenyl-bonded phases using elution with tetrahydrofuran–water, acetonitrile–water or tetrahydrofuran–*n*-hexane mobile phases were also successful[21]. Separations on nitrile-bonded phases[30] and on silica gel[31–34] have also been reported.

Reversed-phase chromatography has only exceptionally been employed to separate the individual oligomers in oligoethylene glycol series[35] and in samples of their adducts with phenol[22]. Rather, normal-phase chromatography on silica[36,37], nitrile-bonded[37], amino-bonded[38] or diol-bonded[39] phases is used for the separation of the oligomers in various commercial oligoethylene glycol adducts.

It was the purpose of this work to investigate the possibilities of the characterization and prediction of selectivity and retention in oligomeric series, using the approach employed earlier for homologous series[20] and the calibration method based on the lipophilic and polar indices[17]. To verify the theoretical approach, various series were selected, including oligostyrenes, oligoethylene glycols and sulphated and non-sulphated oligoethylene glycol nonylphenyl ethers. A further aim was to investigate and to attempt to explain possible differences between the chromatographic behaviour of homologous series and that of oligomeric series and to attempt to

formulate some general rules, which could make it possible to predict (at least qualitatively or semi-quantitatively) the selectivity in oligomeric series on the basis of some structural indices in a given oligomeric series.

THEORETICAL

Retention equations for oligomeric series

The description of retention and selectivity of the members of an oligomeric series can be derived using the same approach as that reported earlier for homologous series[20], which is based on the model of interaction indices[8,9]. In both types of series, the molar volumes V_x and the interaction indices I_x, which are a measure of the solute polarities, increase regularly with increasing number of repeat structural units, *i.e.*, the degree of polymerisation, n:

$$V_x = V_{0x} + \Delta V_x n \quad (1)$$
$$I_x = I_{0x} + \Delta I_x n \quad (2)$$

The starting point of the derivation is the basic equation of the interaction indices model for reversed-phase systems. For chromatography in binary mobile phases composed of water (interaction index I_{H_2O}) and an organic solvent (interaction index I_{org}) it reads as follows[8]:

$$\log k' = \log \Phi + \frac{V_x c_M I_{H_2O}}{2.3\,RT}(c_M I_{H_2O} - c_x I_x) - \frac{V_x c_M}{2.3\,RT}(2c_M I_{H_2O} - c_x I_x) \times (I_{H_2O} - I_{org})\varphi + \frac{V_x c_M^2}{2.3\,RT}(I_{H_2O} - I_{org})^2\varphi^2 \quad (3)$$

where k' is the capacity factor of a sample solute with molar volume V_x and interaction index I_x, Φ is the phase ratio, V_S/V_M (V_S and V_M are the volumes of the stationary and mobile phases, respectively, in the column used), R is the gas constant, T is temperature (K), c_M and c_x are proportionality constants relating to the mobile phase and to the solute, respectively, and φ is the concentration of the organic solvent in the mobile phase [% (v/v) $\cdot$ 10^{-2}].

The combination of the eqns. 1, 2 and 3 yields the following expressions for k' of the members of a homologous or oligomeric series[20]:

$$\log k' = \log \beta + (\log \alpha)n + (\log \gamma)n^2 \quad (4)$$

and

$$\log k' = a - m\varphi + d\varphi^2 \quad (5)$$

where n is the number of the repeat structure units in a given member of a homologous or oligomeric series. Eqn. 4 predicts a quadratic dependence of log k' on the number of oligomeric or homologous units, in contrast to the Martin rule, but as long as the

product of the increments $\Delta V_x \Delta I_x$ is low enough, the quadratic term ($\log \gamma$) can be neglected and the retention is described by the well known expression[20]

$$\log k' = \log \beta + (\log \alpha)n \tag{4a}$$

Eqn. 5 for the dependence of $\log k'$ on φ is generally quadratic, in agreement with both the interaction index[8] and solubility parameter[6,7] theories, but often simple linear expression may be used for description of reversed-phase systems:

$$\log k' = a - m\varphi \tag{5a}$$

where

$$a = a_0 + a_1 n \tag{6}$$
$$m = m_0 + m_1 n \tag{7}$$
$$\log \beta = \beta_0 - \beta_1 \varphi \tag{8}$$
$$\log \alpha = \alpha_0 - \alpha_1 \varphi \tag{9}$$

Using eqn. 3, it can be shown that the constants of eqns. 4a and 5a depend on the interaction indices of water (I_{H_2O}) and of the organic solvent used in the mobile phase (I_{org}), on the contribution of a structural repeat unit to the interaction index of a given oligomer (ΔI_x), on an analogous contribution to the molar volume of the oligomer (ΔV_x), and on the contribution of the structural residue to the interaction index (I_{0x}) and to the molar volume (V_{0x}) of the oligomer:

$$a_0 = \beta_0 = \log \Phi + c_1 V_{0x} I_{H_2O}(c_M I_{H_2O} - c_x I_{0x}) \tag{10}$$
$$m_0 = \beta_1 = c_1 V_{0x}(2c_M I_{H_2O} - c_x I_{0x})(I_{H_2O} - I_{org}) \tag{11}$$
$$a_1 = \alpha_0 = c_1 I_{H_2O}[(c_M I_{H_2O} - c_x I_{0x})\Delta V_x - c_x \Delta I_x V_{0x}] \tag{12}$$
$$m_1 = \alpha_1 = c_1(I_{H_2O} - I_{org})[(2c_M I_{H_2O} - c_x I_{0x})\Delta V_x - c_x \Delta I_x V_{0x}] \tag{13}$$

where c_1 is a constant depending on temperature and on the nature of the components of the mobile phase:

$$c_1 = \frac{c_M}{2.3RT} \tag{14}$$

Oligomeric series differ from homologous series in the character of the increments ΔI_x and ΔV_x, which are identical for various homologous series in which a methylene group is the repeat structural unit, but may differ considerably between the individual oligomeric series. Thus, the retention behaviour in homologous series represents a special case of the behaviour in oligomeric series. As in homologous series, the following equations apply also for oligomeric series, provided that the linear eqns. 4a and 5a are valid:

$$m = q + pa \tag{15}$$
$$\log k' = (a_0 + a_1 n)(1 - p\varphi) - q\varphi \tag{16}$$

The constants p and q can be defined as the correlation parameters between the slope and the intercept of log k' *versus* φ dependences for the individual members of a given homologous or oligomeric series. These constants depend on the organic solvent used in the mobile phase and on the polarities and sizes of the structural increment and of the structural residue in a given series[20].

The structural dependence of the correlation parameter p can be described by the following equation, obtained after combination of eqns. 5a–7, 12, 13 and 15:

$$p = \left(1 - \frac{I_{org}}{I_{H_2O}}\right) \Bigg/ \left(1 + \frac{1}{1 - \frac{c_x}{c_M I_{H_2O}} \cdot Q}\right) \tag{17}$$

where Q is a combined structural parameter including the size (ΔV_x, V_{0x}) and polarity (ΔI_x, I_{0x}) parameters of both the repeat structural unit and the structural residue in a given oligomeric series:

$$Q = \frac{V_{0x}\Delta I_x + I_{0x}\Delta V_x}{\Delta V_x} \tag{18}$$

The numerator in eqn. 18 represents a combination term, which can be distinguished in eqns. 12 and 13 for the constants $\alpha_0 = a_1$ and $\alpha_1 = m_1$ and which characterizes the magnitude of the interaction between the sizes and polarities of the repeat structural unit and of the structural residue in a given series. Hence the combined structural parameter Q has the physical meaning of the relative magnitude of the intramolecular repeat unit–structural residue interaction with respect to the size increment of the repeat unit.

Separation selectivity in oligomeric series

The selectivity of separation in a given oligomeric series is understood here as the relative retention of the neighbouring oligomers. If the quadratic term log γ in eqn. 4 can be neglected, the selectivity is constant in a given series and chromatographic system (under isocratic conditions) and is given by the term log α. The combination of eqns. 9, 12, 13 and 18 yields the following expression for the selectivity:

$$\log \alpha = c_1 c_x \Delta V_x \left\{\left(2 \cdot \frac{c_M}{c_x} \cdot I_{H_2O} - Q\right) [I_{H_2O} - (I_{H_2O} - I_{org})\varphi] - \frac{c_M}{c_x} \cdot I^2_{H_2O}\right\} \tag{19}$$

eqns. 12 and 13 can be further rearranged:

$$\alpha_0 = c_1 c_x I_{H_2O} \Delta V_x \left(\frac{c_M}{c_x} \cdot I_{H_2O} - Q\right) = K_1(K_3 - Q)\Delta V_x \tag{20}$$

$$\alpha_1 = c_1 c_x (I_{H_2O} - I_{org}) \Delta V_x \left(2 \cdot \frac{c_M}{c_x} \cdot I_{H_2O} - Q\right)$$

$$= K_2(2K_3 - Q)\Delta V_x \tag{21}$$

According to eqn. 19, the separation selectivity in a given oligomeric series depends on the molar volume of the structural increment, ΔV_x, on the combined structural parameter Q, on the polarity (I_{org}) and concentration (φ) of the organic solvent in the mobile phase and on temperature. The possible influence of the stationary phase on selectivity, which is of relatively minor importance, is not considered in eqn. 19 but, if present, it could modify the values of the constants c_1 and c_x to some extent.

From eqn. 19, it follows that the selectivity, $\log \alpha$, in an oligomeric series may be either positive or negative, which means that the retention may either increase or decrease with increasing number of the repeat structural units, n. The former case occurs more often and it can be derived from eqn. 19 that $\log \alpha > 0$ if the following condition applies:

$$Q < \frac{c_M}{c_x} \cdot I_{H_2O} \left[2 - \frac{1}{1 - \left(1 - \frac{I_{org}}{I_{H_2O}}\right)\varphi} \right] \tag{22}$$

On the other hand, the retention would decrease with increasing n ($\log \alpha < 0$) if

$$Q > \frac{c_M}{c_x} \cdot I_{H_2O} \left[2 - \frac{1}{1 - \left(1 - \frac{I_{org}}{I_{H_2O}}\right)\varphi} \right] \tag{23}$$

The physical meaning of eqns. 22 and 23 can be interpreted as follows. The combined structural parameter Q is low if ΔI_x is low or negative, *i.e.*, if the repeat structural unit is relatively non-polar (such as methylene groups in homologous series, where $\Delta I_x < 0$). In such a case, eqn. 22 applies and the retention increases with increasing n. If $\Delta I_x < 0$, the actual values of the structural parameters V_{0x} and I_{0x} in eqn. 18 should have only a minor effect on selectivity. Hence ΔV_x and ΔI_x of the repeat structural unit are almost independent of the type of homologous series and the constants V_{0x}, I_{0x} of the structural residue often tend to compensate the influence of each other. This means that if one homologous series has a bulkier structural residue than the other series, this residue is often less polar. Consequently, the selectivity, $\log \alpha$, is nearly constant in various homologous series, as has been found experimentally[20].

The structural combined parameter Q in oligomeric series becomes larger with increasing positive ΔI_x and decreasing ΔV_x, *i.e.*, with increasing polarity and decreasing size of the repeat structural unit the probability increases that the eqn. 23 applies and elution occurs in order of decreasing number of oligomeric units. This is more likely in mobile phases containing high concentrations of relatively low-polarity organic solvents, as the right-hand sides of eqns. 22 and 23 decrease with decreasing I_{org} and increasing φ. This also means that for some oligomeric series a concentration φ_0 could be found for which Q equals the right-hand sides of eqns. 22 and 23 and $\log \alpha = 0$, *i.e.*, all the members of this series would be eluted in a single peak. In mobile phases where $\varphi > \varphi_0$, the elution order of the oligomers would be the reverse of that when $\varphi < \varphi_0$.

Eqn. 19 predicts that the selectivity ($\log \alpha$) should depend also on the character of the structural residue in the oligomeric series if $\Delta I_x > 0$. The combined structural

parameter Q and the probability of elution in order of decreasing number of repeat structural units, n, increase with increasing size (molar volume, V_{0x}) and polarity (interaction index, I_{0x}) of the structural residue (eqn. 23). Consequently, if two oligomeric series contain the same repeat structural unit, but the structural residue in one oligomeric series is significantly bulkier or more polar than the residue in the other series, important differences between the selectivities in the two series may be expected according to eqn. 19 as the combined structural parameters Q of the two oligomeric series differ significantly in this instance. According to eqn. 18, these differences in selectivities should become more significant for oligomeric series in which the repeat structural unit is relatively bulky and polar, as Q increases with increasing ΔI_x and ΔV_x and the possible influence of the structural residue on selectivity increases with increasing Q (eqn. 19). Otherwise, Q is small and the separation selectivity should be almost independent of the structural residue.

The dependence of the selectivity on the character of the structural residue may appear strange at first glance, but it can be understood as follows. The mobile phase, which forms a part of the environment of repeat structural units, has an important influence on the separation selectivity in homologous and oligomeric series. The structural residue is also part of the environment of the repeat structural units. Its influence on the separation selectivity, although less apparent, manifests itself as the result of the interaction between the contributions of the polarities and of the sizes of both the repeat structural units and of the structural residue. This conclusion follows from the assumption that both the molar volume and the polarity of an oligomer change regularly with increasing number of the repeat structural units, n, and is expressed mathematically by eqns. 12 and 13.

The above-derived equations apply if the quadratic terms in $\log k'$ *versus* φ and in $\log k'$ *versus* n relationships can be neglected. Otherwise, additional terms are required for more accurate predictive calculations.

Calibration of retention in oligomeric series

Using the method for calibration of retention introduced recently[16–19], k' of each oligomer can be calculated from two characteristic indices:

$$\log k' = (a_{0B} + a_{1B}n_{ce})(1 - p_B\varphi) - q_i\varphi \qquad (24)$$

where n_{ce} is the lipophilic index, *i.e.*, the number of methylene groups in a (hypothetical) member of a calibration homologous series (*n*-alkylbenzenes) with an equal retention to the sample solute in the absence of an organic solvent in the mobile phase, q_i is the polar index, which characterizes the magnitude of the polar interactions of the solute with the mobile phase containing a given organic solvent, φ is the concentration of the organic solvent in the mobile phase and a_{0B}, a_{1B} and p_B are the characteristic constants a_0, a_1 and p in eqn. 16 for the reference calibration homologous series (*n*-alkylbenzenes) on a given column, which are determined by regression analysis of the retention–composition plots for several members of the calibration homologous series. The constants n_{ce} and q_i can be calculated from the constants a and m in eqn. 5a for each solute, using eqn. 24 and the known constants a_{0B}, a_{1B} and p_B[16].

After combination of eqns. 16, 26, 6 and 7, we find that

$$n_{ce} = \frac{a_0 - a_{0B}}{a_{1B}} + \frac{a_1}{a_{1B}} \cdot n = n_{ce0} + \Delta n_{ce} n \tag{25}$$

$$q_i = q + (a_0 + a_1 n)(p - p_B) = q_{0i} + \Delta q_i n \tag{26}$$

This means that the indices n_{ce} and q_i should regularly increase (or decrease) with increasing number of oligomeric units in a given series; n_{ce0} and q_{0i} are the n_{ce} and q_i indices of the structural residue in this series, and Δn_{ce} and Δq_i are the contributions of the repeat structural unit to the n_{ce} and q_i indices of the individual oligomers. It should be kept in mind that these indices have been defined as relative values with respect to the calibration homologous series (*n*-alkylbenzenes). In various homologous series, the contribution of the methylene group to retention and selectivity is approximately constant (approximately constant ΔI_x and ΔV_x and small values of the combined structural constant Q). Consequently, $a_1 \approx a_{1B}$ and $p \approx p_B$, which means that the increments $\Delta n_{ce} \approx 1$ and $\Delta q_i \approx 0$ in various homologous series. This need not necessarily apply for various oligomeric series with different repeat structural units (and possible significant influence of the constant Q on selectivity) and a regular change of n_{ce} and q_i according to the eqns. 25 and 26 may be generally expected.

EXPERIMENTAL

Some experiments were performed using an M 6000A pump (or two M 6000A pumps and an M 660 gradient controller), an U6K injector and an M 440 UV detector operated at 254 nm (all from Waters Assoc., Milford, MA, U.S.A.) and a TZ 4221 line recorder (Laboratory Instrument Works, Prague, Czechoslovakia). An HP 1090M liquid chromatograph equipped with a UV–VIS diode-array detector, automatic sample injector, 3DR solvent delivery system, thermostated column compartment, a Series 79994A workstation, an HP 2225 Think-Jet printer and a 7475A plotter (Hewlett-Packard, Avondale, PA, U.S.A.) was used for other experiments. For chromatography of oligoethylene glycols, Waters Assoc. instrumentation was employed with an R 401 refractometric detector instead of the M 440 UV detector.

Stainless-steel columns were packed in the laboratory with spherical octadecylsilica gel (Silasorb C_{18} SPH, 10 and 7.5 μm) and with spherical octylsilica gel (Silasorb C_8 SPH, 10 and 7.5 μm) (both from Lachema, Brno, Czechoslovakia) using a high-pressure slurry packing technique. A stainless-steel column packed with spherical silica gel (Separon SIX C_{18}, 5 μm) was purchased from Laboratory Instrument Works.

Column dead volumes, V_M, were determined using methanol and ^2H_2O as dead volume markers and refractometric detection with an R 401 differential refractometer.

The compounds used as sample solutes included *n*-alkanes and *n*-alkylbenzenes (various sources), oligoethylene glycols (Carbowax 200, Michrome, Gurr, London, U.K.), oligostyrenes (a polystyrene standard of a nominal molecular weight 2350, Waters Assoc.), oligomeric ethoxylated nonylphenols (Serdox NNP 4, Servo, Delden, The Netherlands) and oligomeric anionic surfactants prepared by sulphation of

ethoxylated nonylphenols[40]. The solutes were dissolved in mobile phases at appropriate concentrations to yield a good UV or RI detector response.

The mobile phases were prepared by mixing water (doubly distilled in glass with addition of potassium permanganate) with the organic solvents [and with cetyltrimethylammonium bromide (CTAB)] in the required volume ratios. Methanol, 2-propanol and 1,4-dioxane were of spectroscopic or analytical-reagent grade (Lachema). CTAB was purchased from Serva (Heidelberg, F.R.G.). All the solvents were filtrated using a Millipore 0.45-μm filter and the mobile phases were de-gassed by ultrasonication before the use, or were prepared directly in the HP 1090M instrument from the pure solvents continuously stripped by a stream of helium.

Evaluation of the data

The retention volumes, V_R, of the members of the homologous and oligomeric series tested were measured at different mobile phase compositions. The capacity factors, k', were calculated from the mean V_R value of three repeated experiments under given conditions ($k' = V_R/V_M - 1$). The order of elution was checked and the individual oligomers were identified by comparison of the chromatograms of the monomers and of the samples of different nominal degrees of polymerization, *i.e.*, of different average molecular weights (oligoethylene glycols 200 and 400, oligostyrenes 2350 and 3500 and oligomeric ethoxylated nonylphenols NNP4, NNP8 and NNP12) under the conditions where the low oligomers were best separated. This identification of the peaks was compared with previous experimental peak identification in normal-phase systems[34] and, with the oligomeric ethoxylated nonylphenols, it was further checked using mass spectrometry (further details are given under Results and Discussion).

Linear regression analysis was used to calculate the constants log α and log β in eqn. 4a from the experimental plots of log k' *versus* n at different mobile phase compositions and the constants a and m in eqn. 5a from the experimental plots of log k' *versus* φ for the individual homologues or oligomers. Some log k' *versus* φ plots in propanol–water and in dioxane–water mobile phases were slightly curved at the lower concentrations of the organic solvent in the mobile phase; the parts of the plots that deviated more than 5% from linearity were not considered in the regression analysis. The plots of the constants a and m in eqn. 5a *versus* n for the individual oligomers and the plots of the constants log α and log β in eqn. 4a *versus* φ in the mobile phase were subjected to linear regression analysis to determine the constants a_0, a_1, m_0, m_1, α_0, α_1, β_0, β_1, q and p (eqns. 6–9 and 15). The values of these constants for the series and chromatographic systems studied are given in Tables I–IV, VI and VII.

RESULTS AND DISCUSSION

Comparison of the chromatographic behaviour of the members of homologous and oligomeric series

The validity of the present retention model in reversed-phase systems was tested on the chromatographic behaviour of *n*-alkanes, *n*-alkylbenzenes, oligostyrenes, oligoethylene glycols and ethoxylated nonylphenols on several C_{18} and C_8 columns in various mobile phases, containing water and methanol, acetonitrile, 1,4-dioxane or 2-propanol as the organic solvents. For each series, the validity of eqns. 4a, 5a, 6–9, 15,

25 and 26 was tested. The values of the constants a, m, log α, log β, $a_0 = \beta_0$, $m_0 = \beta_1$. $a_1 = \alpha_0$, $m_1 = \alpha_1$, p, q, n_{ce}, q_i, n_{ce0}, Δn_{ce}, q_{0i} and Δq_i of these equations and the corresponding correlation coefficients, which indicate the validity of the above equations, are given in Tables I–IV. The constants a_{0B}, a_{1B}, m_{0B}, m_{1B}, q_B and p_B of the calibration homologous n-alkylbenzenes series, necessary for calculations of the indices n_{ce} and q_i (eqns. 24–26), were determined in an analogous way from the experimental k' values of C_2–C_5 alkylbenzenes measured at four or five different mobile phase compositions and are also given in Tables I–IV. The indices n_{ce} and q_i were calculated from the following equations:

$$n_{ce} = \frac{a - a_{0B}}{a_{1B}} \tag{27}$$

$$q_i = m - p_B a \tag{28}$$

and the constants $n_{ce}{}^0$, Δn_{ce}, q_{0i} and Δq_i in eqns. 25 and 26 were calculated using linear regression of the $n_{ce}{}^0$ *versus* n and q_i *versus* n plots. The calculated values of these constants (Tables I–IV) were in all the instances virtually identical with the values determined directly from the constants a_0, a_1 and p in eqn. 16 applying for a given

TABLE I

EXPERIMENTAL VALUES OF THE PARAMETERS α_0, α_1, β_0, β_1, a_0, a_1, m_0, m_1, q, p, n_{ce0}, Δn_{ce}, q_{0i} AND Δq_i IN EQNS. 6–9, 15, 25 AND 26 FOR n-ALKANES ON A C_8 COLUMN IN METHANOL–WATER AND ACETONITRILE–WATER MOBILE PHASES, CALCULATED USING LINEAR REGRESSION OF THE EXPERIMENTAL DATA ACCORDING TO THE EQUATIONS

R = correlation coefficient. Column: Silasorb C_8, 7.5 μm (300 × 3.8 mm I.D.).

Mobile phase: methanol–water; n = 5–10:				*Mobile phase: acetonitrile–water; n = 5–10:*			
Methanol (%, v/v)	*Log β*	*Log α*	*R*	*Acetonitrile (%, v/v)*	*Log β*	*Log α*	*R*
60	−0.506	0.236	0,9999	50	−0.332	0.192	0.9999
70	−0.617	0.179	0.9999	55	−0.328	0.169	0.9998
80	−0.763	0.131	0.9990	60	−0.405	0.154	0.9999
90	−0.979	0.091	0.9995	70	−0.530	0.131	0.9998
				80	−0.714	0.114	0.9997
Log α = 0.521 − 0.483φ;			R = 0.9974	Log α = 0.311 − 0.252φ;			R = 0.9818
Log β = 0.456 − 1.563φ;			R = 0.9886	Log β = 0.376 − 1.329φ;			R = 0.9801
a = 0.618 + 0.494n;			R = 0.9996	a = 0.518 + 0.287n;			R = 0.9979
m = 1.757 + 0.450n;			R = 0.9991	m = 1.527 + 0.219n;			R = 0.9939
m = 1.193 + 0.912a;			R = 0.9999	m = 1.249 + 0.766a;			R = 0.9985
n_{ce} = −1.85 + 0.99n;			R = 0.9996	n_{ce} = −2.04 + 0.98n;			R = 0.9980
q_i = 1.18 − 0.01n;			R = 0.9000	q_i = 1.08 − 0.03n;			R = 0.9454
Calibration series of n-alkylbenzenes:				*Calibration series of n-alkylbenzenes:*			
a_{0B} = 1.542;	a_{1B} = 0.501;		R = 0.9982	a_{0B} = 1.114;	a_{1B} = 0.293;		R = 0.9994
m_{0B} = 2.635;	m_{1B} = 0.466;		R = 0.9966	m_{0B} = 2.082;	m_{1B} = 0.254;		R = 0.9990
q_B = 1.195;	p_B = 0.932;		R = 0.9997	q_B = 1.118;	p_B = 0.864;		R = 0.9998

TABLE II

EXPERIMENTAL VALUES OF THE PARAMETERS α_0, α_1, β_0, β_1, a_0, a_1, m_0, m_1, q, p, n_{ce0}, Δn_{ce}, q_{0i} AND Δq_i IN EQNS. 6–9, 15, 25 AND 26 FOR OLIGOSTYRENES ON A C_{18} COLUMN IN 1,4-DIOXANE–WATER MOBILE PHASES, CALCULATED USING LINEAR REGRESSION OF THE EXPERIMENTAL DATA ACCORDING TO THE EQUATIONS

R = correlation coefficient. Column: Silasorb C_{18} SPH, 7.5 μm (300 × 4.1 mm I.D.).

Mobile phase: 1,4-dioxane–water; n = 3–11:

Dioxane (%, v/v)	*Log β*	*Log α*	*R*
75	0.149	0.148	0.9980
77	0.099	0.134	0.9992
80	−0.012	0.102	0.9988
83	−0.116	0.082	0.9984
85	−0.155	0.062	0.9984
86	−0.176	0.058	0.9988

$\log \alpha = 0.768 - 0.826\varphi$;		$R = 0.9992$
$\log \beta = 2.487 - 3.116\varphi$;		$R = 0.9932$
$a = 2.487 + 0.767n$;		$R = 0.9980$
$m = 3.116 + 0.826n$;		$R = 0.9979$
$m = 0.437 + 1.077a$;		$R = 0.9999$
$n_{ce} = 2.00 + 2.30n$;		$R = 0.9980$
$q_i = 0.82 + 0.12n$;		$R = 0.9964$
Calibration series of n-alkylbenzenes:		
$a_{0B} = 1.820$;	$a_{1B} = 0.334$;	$R = 0.9980$
$m_{0B} = 2.724$;	$m_{1B} = 0.308$;	$R = 0.9966$
$q_B = 1.041$;	$p_B = 0.923$;	$R = 0.9998$

oligomeric series, using eqns. 25 and 26. The two calculation methods are identical as far as the log k' *versus* φ, log k' *versus* n, n_{ce} *versus* n and q_i *versus* n plots are linear and the agreement between the values calculated using the two methods can be considered as evidence for the applicability of the linear forms of the above relationships in the systems studied.

Homologous n-alkylbenzenes and n-alkanes

Both log k' *versus* φ and log k' *versus* n plots for these two series are linear in methanol–water mobile phases and only slightly curved outside the concentration range 50–80% (v/v) of acetonitrile in acetonitrile–water mobile phases. The selectivity constants $\alpha_0(a_1)$ and $\alpha_1(m_1)$ are almost the same for the two series in mobile phases containing a given organic solvent and are higher in methanol–water than in acetonitrile–water mobile phases (Table I). $\Delta n_{ce} \approx 1$ and $\Delta q_i \approx 0$, which is in agreement with general theoretical predictions for homologous series; q_i and p are also almost the same for the two series in the mobile phases studied. From the comparison of n_{ce0} values it follows that the contribution of the benzene ring to lipophilic selectivity in the n-alkylbenzene series corresponds to *ca.* two methylene groups.

TABLE III

EXPERIMENTAL VALUES OF THE PARAMETERS α_0, α_1, β_0, β_1, a_0, a_1, m_0, m_1, q, p, n_{ce0}, Δn_{ce}, q_{0i} AND Δq_i IN EQNS. 6–9, 15, 25 AND 26 FOR OLIGOETHYLENE GLYCOLS ON A C_{18} COLUMN IN METHANOL–WATER MOBILE PHASES, CALCULATED USING LINEAR REGRESSION OF THE EXPERIMENTAL DATA ACCORDING TO THE EQUATIONS

R = correlation coefficient. Column: Silasorb C_{18}, 10 μm (300 × 4.2 mm I.D.).

Mobile phase: methanol–water; n = 3–9:

Methanol (%, v/v)	*Log β*	*Log α*	*R*
10	−1.233	0.330	0.9994
15	−1.278	0.308	0.9999
20	−1.310	0.258	0.9975
30	−1.338	0.188	0.9977

$\log \alpha = 0.409 - 0.734\varphi$; $R = 0.9944$
$\log \beta = -1.195 - 0.507\varphi$; $R = 0.9601$
$a = -1.061 + 0.363n$; $R = 0.9938$
$m = 0.614 + 0.598n$; $R = 0.9863$
$m = 2.358 + 1.655a$; $R = 0.9978$
$n_{ce} = -5.31 + 0.66n$; $R = 0.9494$
$q_i = 1.56 + 0.28n$; $R = 0.9678$

Calibration series of n-alkylbenzenes:*
$a_{0B} = 1.915$; $a_{1B} = 0.536$; $R = 0.9985$
$m_{0B} = 2.628$; $m_{1B} = 0.466$; $R = 0.9971$
$q_B = 0.960$; $p_B = 0.871$; $R = 0.9999$

* Measured in 70–90% (v/v) methanol–water.

Oligostyrenes

Because oligostyrenes are not soluble in methanol, it was not possible to investigate their behaviour in methanol–water mobile phases. Fig. 1 shows the plots of log k' *versus* n for oligostyrenes on a C_{18} column in 1,4-dioxane–water mobile phases. The log k' *versus* φ plots are linear in the range 75–87% dioxane; outside this range curvature is observed and the absolute retention increases rapidly in mobile phases containing less than 75% dioxane. The plots of increasing log k' with degree of polymerization n are linear and only the k' for the first members of the series show slight negative deviations. An example of the isocratic separation of oligostyrenes in 83% dioxane is shown in Fig. 2. The separation achieved is approximately comparable to that in chromatography on a silica gel column[34].

The repeat structural unit [$-CH_2$(phenyl)$-CH_2-$] shows a contribution to lipophilic selectivity approximately equivalent to 2.3 methylene groups. Both lipophilic, Δn_{ce}, and polar, Δq_i, indices for the repeat unit are positive, in contrast to the behaviour in homologous series (Table II); $n_{ce0} = 2$, which corresponds to the contribution to lipophilic selectivity of the structural residue equivalent to four methylene groups, in agreement with the structure of the butyl end-group for an anionically polymerized polystyrene sample. The constant p in the oligostyrene series is only slightly (*ca.* 10%) higher than the corresponding values for homologous n-alkylbenzenes and n-alkanes in methanol–water mobile phases.

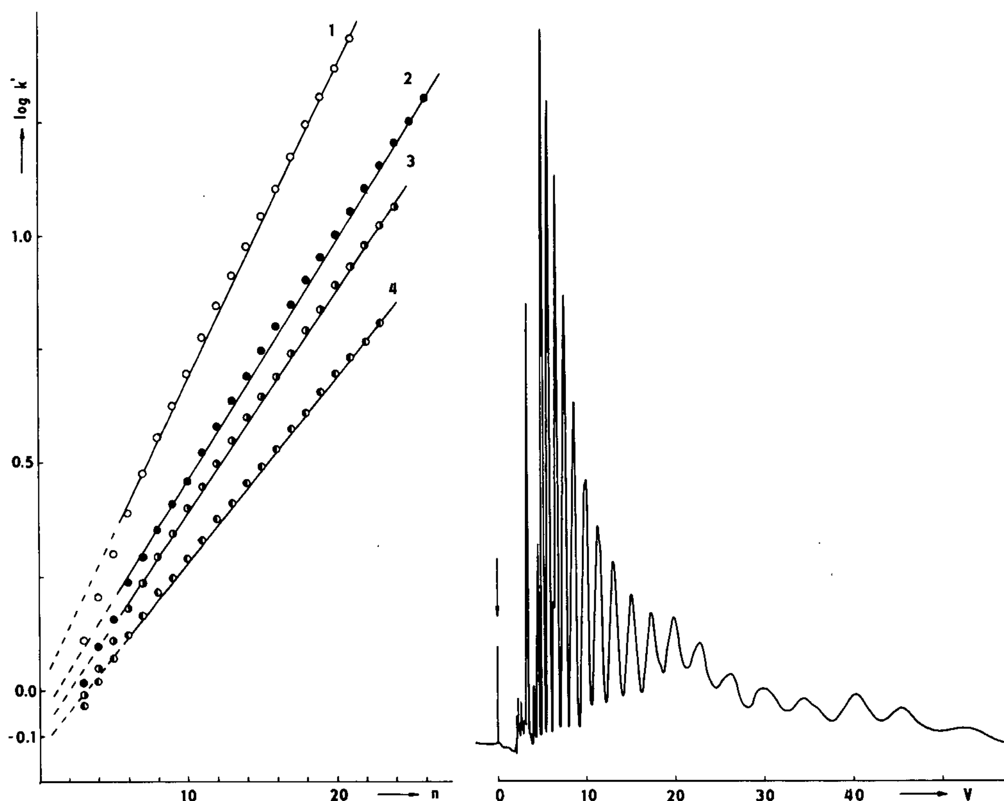

Fig. 1. Dependence of retention (k') of oligostyrenes on the number of structural repeat units, n, in mobile phase composed of 1,4-dioxane and water; (1) 83:17; (2) 85.3:14.7; (3) 86:14; (4) 87:13. Column: Silasorb C_{18} SPH, 7.5 μm (300 × 4.1 mm I.D.).

Fig. 2. Separation of a polystyrene standard, nominal molecular weight 2350, on a Silasorb C_{18} SPH column in 1,4-dioxane–water (83:17). Flow-rate, 0.5 ml/min; detection, UV (254 nm); sample volume, 10 μl. V = volume of eluate (ml).

Oligoethylene glycols

Oligoethylene glycols are readily soluble in methanol and a linear decrease in log k' with increasing concentration of methanol in the mobile phase is observed up to *ca.* 40% (v/v). At higher methanol concentrations, the retention of all the oligomers is low and these are eluted as a single peak with a retention volume close to the column dead volume. The log k' values increase linearly with increasing number of oligomeric units, n (Table III).

The increase in retention with increasing n at lower methanol concentrations was confirmed experimentally by comparison of the elution pattern of two oligoethylene glycol samples with different average molecular weights. The elution pattern of the sample with higher molecular weight was shifted towards larger elution volumes than that of the lower molecular weight sample.

The repeat structural unit $-CH_2CH_2O-$ contributes to the lipophilic selectivity (Δn_{ce}) as the equivalent of 0.7 methylene units, whereas its contribution Δq_i to polar

TABLE IV

EXPERIMENTAL VALUES OF THE PARAMETERS α_0, α_1, β_0, β_1, a_0, a_1, m_0, m_1, q, p, n_{ce0}, Δn_{ce}, q_{0i} AND Δq_i IN EQNS. 6–9, 15, 25 AND 26 FOR ETHOXYLATED NONYLPHENOLS ON C_{18} AND C_8 COLUMNS IN 2-PROPANOL–WATER MOBILE PHASES, CALCULATED USING LINEAR REGRESSION OF THE EXPERIMENTAL DATA ACCORDING TO THE EQUATIONS

R = correlation coefficient.

Column: Separon SIX C_{18}, 5 μm (250 × 4 mm I.D.): Mobile phase: 2-propanol–water; n = 1–5:

2-Propanol (%, v/v)	*Log β*	*Log α*	*R*
45	0.946	−0.040	0.9973
50	0.734	−0.041	0.9991
55	0.529	−0.044	0.9995
60	0.354	−0.047	0.9998

Equation	R
$\text{Log}\ \alpha = -0.018 - 0.048\varphi$;	$R = 0.9798$
$\text{Log}\ \beta = 2.721 - 3.962\varphi$;	$R = 0.9991$
$a = 2.694 - 0.033n$;	$R = 0.9951$
$m = 3.890 - 0.023n$;	$R = 0.9639$
$m = 5.694 - 0.669a$;	$R = 0.9330$
$n_{ce} = 4.27 - 0.12n$;	$R = 0.9952$
$q_i = 1.47 + 0.05n$;	$R = 0.9978$

Calibration of n-alkylbenzenes:

$a_{0B} = 1.486$;	$a_{1B} = 0.283$;	$R = 0.9999$
$m_{0B} = 2.257$;	$m_{1B} = 0.279$;	$R = 0.9999$
$q_B = 0.963$;	$p_B = 0.897$;	$R = 0.9948$

Column: Silasorb C_{18} SPH, 7.5 μm (300 × 3.6 mm I.D.): Mobile phase: 2-propanol–water; n = 1–7:

2-Propanol (%, v/v)	*Log β*	*Log α*	*R*
45	0.762	−0.037	0.9995
50	0.525	−0.037	0.9992
55	0.335	−0.039	0.9999
60	0.164	−0.039	0.9994

Equation	R
$\text{Log}\ \alpha = -0.030 - 0.016\varphi$;	$R = 0.8944$
$\text{Log}\ \beta = 2.530 - 3.968\varphi$;	$R = 0.9972$
$a = 2.535 - 0.033n$;	$R = 0.9965$
$m = 3.973 + 0.011n$;	$R = 0.8972$
$m = 4.82 - 0.335a$;	$R = 0.8577$
$n_{ce} = 3.43 - 0.10n$;	$R = 0.9889$
$q_i = 1.00 + 0.05n$;	$R = 0.9934$

Calibration series of n-alkylbenzenes:

$a_{0B} = 1.423$;	$a_{1B} = 0.327$;	$R = 0.9999$
$m_{0B} = 2.583$;	$m_{1B} = 0.385$;	$R = 0.9996$
$q_B = 0.905$;	$p_B = 1.179$;	$R = 0.9998$

Column: Silasorb C_8 SPH, 10 μm (300 × 4.2 mm I.D.): Mobile phase: 2-propanol–water; n = 2–5:

2-Propanol (%, v/v)	*Log β*	*Log α*	*R*
35	1.103	−0.028	0.9996
40	0.756	−0.029	0.9988
45	0.488	−0.030	0.9994
50	0.269	−0.032	0.9999

Equation	R
$\text{Log}\ \alpha = -0.019 - 0.026\varphi$;	$R = 0.9827$
$\text{Log}\ \beta = 3.009 - 5.540\varphi$;	$R = 0.9946$
$a = 3.024 - 0.024n$;	$R = 0.9999$
$m = 5.569 + 0.016n$;	$R = 0.9974$
$m = 7.567 - 0.661a$;	$R = 0.9967$
$n_{ce} = 5.05 - 0.07n$;	$R = 0.9999$
$q_i = 1.24 + 0.05n$;	$R = 0.9999$

Calibration series of n-alkylbenzenes:

$a_{0B} = 1.306$;	$a_{1B} = 0.340$;	$R = 0.9997$
$m_{0B} = 2.830$;	$m_{1B} = 0.487$;	$R = 0.9996$
$q_B = 0.947$;	$p_B = 1.431$;	$R = 0.9999$

selectivity is about twice that for a repeat unit in the oligostyrene series. n_{ce0} is much lower and q_{0i} is larger than the corresponding values for *n*-alkanes, *n*-alkylbenzenes and oligostyrenes. The values of these constants in Table III may be subject to some error, as it was necessary to use the calibration constants for *n*-alkylbenzenes determined in the region with much larger concentrations of methanol (in 10–30% methanol mobile phases, alkylbenzenes are too strongly retained). The constant p is almost twice that for homologous *n*-alkanes and *n*-alkylbenzenes. Consequently, an equivalent change in methanol concentration has a larger effect on the selectivity of oligoethylene glycols than on that of *n*-alkanes or *n*-alkylbenzenes.

Ethoxylated nonylphenols

Log k' of the individual oligomers decrease linearly with increasing concentration of 2-propanol in the mobile phase in the concentration range 40–60% (v/v) of 2-propanol for C_{18} columns and in the range 30–50% of 2-propanol for C_8 columns. Outside these ranges the plots are slightly curved. In the mobile phases studied, the retention decreases with increasing number of repeat oligoethylene units. This behaviour, which could appear strange at first glance, was confirmed in two independent ways: (i) fractions containing the individual oligomers were collected and subjected to gas chromatography–mass spectrometry, which confirmed that the molecular weights of the oligomers decrease with increasing elution volumes of the fractions, and (ii) several samples with different average molecular weights were subjected to high-performance liquid chromatography under identical conditions and it was found that the maxima of the elution patterns for higher molecular weight samples are shifted towards lower elution volumes. The same behaviour was observed

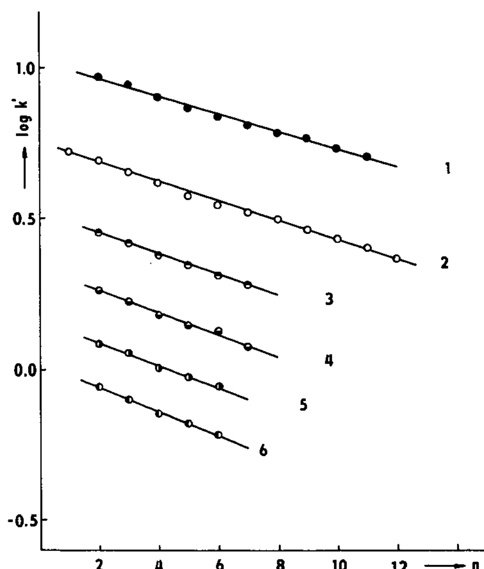

Fig. 3. Dependence of retention (k') of oligoethylene glycol nonylphenyl ethers on the number of structural repeat units, n, in mobile phases composed of 2-propanol and water: (1) 40:60; (2) 45:55; (3) 50:50; (4) 55:45; (5) 60:40; (6) 65:35. Column: Silasorb C_{18} SPH, 7.5 μm (300 × 3.6 mm I.D.).

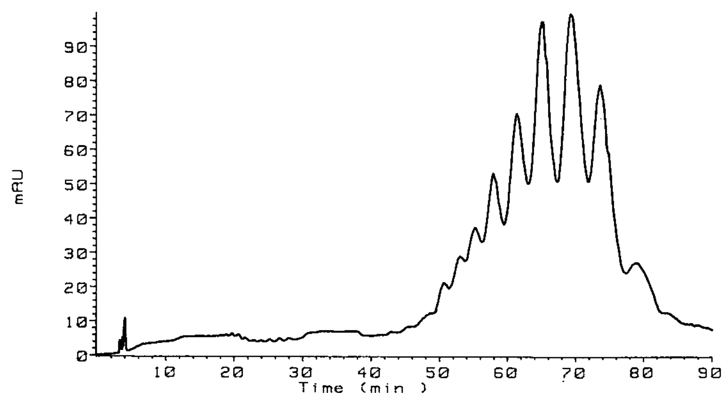

Fig. 4. Separation of a commercial sample of Serdox NNP 4, containing oligoethylene glycol nonylphenyl ethers on a Silasorb C_{18} SPH column in 2-propanol–water (35:65). Flow-rate, 0.5 ml/min; detection, UV (230 nm); instrument, HP 1090M; sample volume, 5 μl.

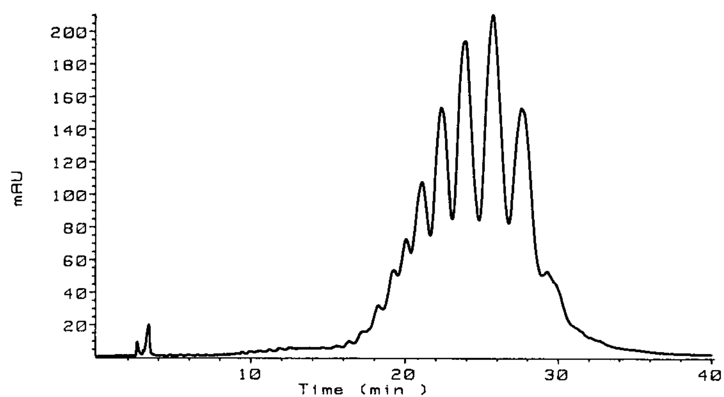

Fig. 5. Separation of Serdox NNP 4 in 2-propanol–water (45:55). Other conditions as in Fig. 4.

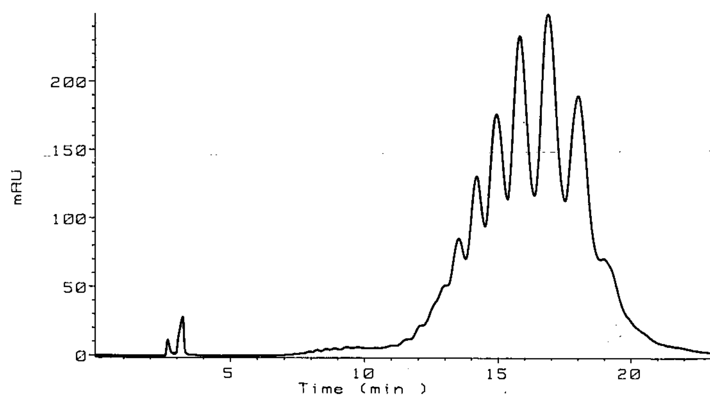

Fig. 6. Separation of Serdox NNP 4 in 2-propanol–water (50:50). Other conditions as in Fig. 4.

on different C_{18} and C_8 columns (Table IV) and log k' decreased linearly with increasing number of repeat structural units (Fig. 3). A chromatogram of a commercial sample of oligethylene glycol nonylphenyl ethers is shown in Fig. 4.

The experimental selectivity constants α_0 are negative, whereas α_1 are positive; the constants p are negative. This means that the absolute value of the selectivity for neighbouring oligomers increases with increasing concentration of the organic solvent in the mobile phase, in contrast to the behaviour of all the other series tested. The experimental values of $\alpha_0(\alpha_1)$ and $\alpha_1(m_1)$ are very close to zero, which means that the selectivity is low. The slight increase in selectivity with increasing concentration of propanol in the mobile phase is compensated for by a decrease in the contribution of the capacity term to resolution. The separation of the lower oligomers (the later eluted ones) is approximately independent of the mobile phase composition, whereas the separation of the earlier eluted higher oligomers is slightly impaired as the concentration of propanol in the mobile phase increases, as is demonstrated in Figs. 4–6, showing the separation achieved with 35, 45 and 50% (v/v) propanol in water, respectively, as the mobile phases.

The repeat structural unit in the ethoxylated nonylphenol series is the same as that in oligoethylene glycols but, in contrast to the latter series, the lipophilic index n_{ce} decreases with the number of repeat structural units ($\Delta n_{ce} < 0$) and the polar index q_i is approximately constant, *i.e.*, Δq_i is relatively very small. The contribution of the structural residue to q_i, q_{0i}, is similar to that in the oligoethylene glycol series, but the contribution to the lipophilic index, n_{ce0}, is much higher, equivalent to approximately ten methylene units.

As the absolute retention of oligoethylene glycol nonylphenyl ethers decreases with increasing number of repeat structural units, the separation selectivity in this homologous series is expected to decrease with increasing polarity of the organic solvent in the mobile phase (see Theoretical) and the experimental behaviour agrees with this prediction. The separation selectivity is much lower in methanol–water than in propanol–water mobile phases and all the individual oligomers are eluted in a single common peak (Fig. 7). The absolute retention increases as the concentration of methanol decreases, but the resolution does not improve.

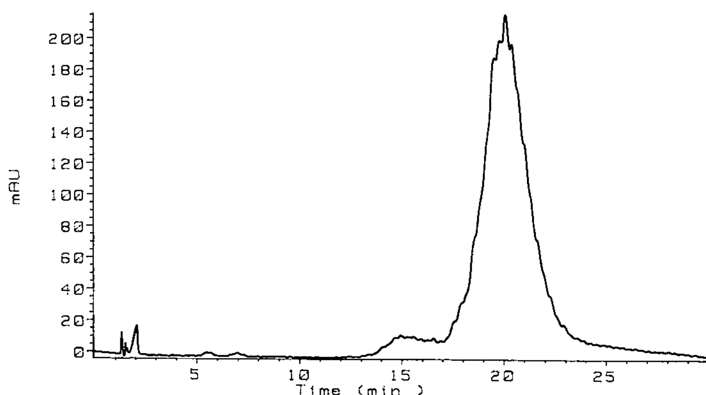

Fig. 7. Separation of Serdox NNP 4 in methanol–water (75:25). Other conditions as in Fig. 4.

Structural dependence of separation selectivity

The experimental values of the selectivity constants $\alpha_0(e)$ and $\alpha_1(e)$ (eqns. 12 and 13) for the homologous and oligomeric series tested are summarized in Table V. These values vary significantly for different series, which is understandable, as the structures of the repeat oligomeric units and also the solvents used in the mobile phases differ for the individual series studied. The differences in selectivity and in the constants α_0 and α_1 between the individual oligomeric series could be explained, at least qualitatively, on the basis of eqns. 20 and 21.

TABLE V

EXPERIMENTAL AND ESTIMATED STRUCTURAL INDICES V_{0x}, ΔV_x, I_{0x}, ΔI_x (EQNS. 1 AND 2), COMBINATION TERMS ($V_{0x}\Delta I_x + I_{0x}\Delta V_x$), COMBINED STRUCTURAL PARAMETERS Q (EQN. 18) AND EXPERIMENTAL [$\alpha_0(e)$ AND $\alpha_1(e)$] AND CALCULATED [$\alpha_0(c)$ AND $\alpha_1(c)$ (CALCULATED FROM THE EXPERIMENTAL OR ESTIMATED STRUCTURAL INDICES AND Q USING EQNS. 20 AND 21)] SELECTIVITY CONSTANTS α_0 AND α_1 (EQNS. 12 AND 13)

The experimental data relate to a Silasorb SPH C_{18} column, except for *n*-alkanes (Silasorb C_8 column). The constants $K_1 = 0.315, K_2 = 0.166, K_3 = 10.72$ in eqns. 20 and 21 were calculated using the following data from ref. 8 (LiChrosorb RP-18 column, methanol–water mobile phases): $I_{H_2O} = 47$, $I_{CH_3OH} = 22.2$, $c_M = 0.291$, $c_x = 1.274$. The calculated selectivity constants $\alpha_0(c)$ and $\alpha_1(c)$ apply for methanol–water mobile phases.

The constants V_{0x}, ΔV_x, I_{0x} and ΔI_x for alkylbenzenes and *n*-alkanes were calculated directly from the linear regression analysis of V_x and I_x as a function of n (eqns. 1 and 2). For other series, they were roughly estimated as follows:

(1) For oligostyrenes: V_{0x}, I_{0x} for *n*-butyl group (anionically polymerized polystyrene sample): $I_{0x} \approx 1.40 - 4 \cdot 0.32 = 0.12$; $V_{0x} \approx 0.35 + 4 \cdot 0.16 = 0.99$. $\Delta V_x \approx V_x$ (ethylbenzene) $= 0.90 + 2 \cdot 0.16 = 1.22$; $\Delta I_x \approx I_x$ (ethylbenzene) $= 2.94 - 2 \cdot 0.25 = 2.44$.

(2) For oligoethylene glycols: $V_{0x} \approx V_x$ (water) $= 0.18$; $I_{0x} \approx 2I_x$ (OH group) $- I_x$ (–O– group) $\approx 2[I_x$ (benzyl alcohol) $- I_x$ (toluene)] $-[I_x$(di-*n*-butyl ether) $- 2I_x$(*n*-butane)] $= 2 \cdot (7.44 - 2.69) - (2.35 - 2 \cdot 1.40 + 8 \cdot 0.32) = 7.39$. $\Delta V_x \approx V_x$ (di-*n*-butyl ether) $- V_x$ (*n*-hexane) $= 1.70 - 0.35 - 6 \cdot 0.16 = 0.39$; $\Delta I_x \approx I_x$ (di-*n*-butyl ether) $- I_x$ (*n*-butane) $- I_x$ (ethane) $= 2.35 - 0.12 - 0.76 = 1.47$.

(3) For oligoethylene glycol nonylphenyl ethers: $V_{0x} \approx V_x$ (phenol) $+ 9\Delta V_x$ (CH_2) $= 0.88 + 9 \cdot 0.16 = 2.32$; $I_{0x} \approx I_x$ (*p*-cresol) $+ 8\Delta I_x$ (CH_2) $= 6.60 - 8 \cdot 0.25 = 4.60$. ΔV_x and ΔI_x as for oligoethylene glycols.

Parameter	*Series*				
	Alkylbenzenes	*n-Alkanes*	*Oligostyrenes*	*Oligoethylene glycols*	*Ethoxylated nonylphenols*
Structural repeat unit	$-CH_2-$	$-CH_2-$	$-CH_2C(C_6H_5)H-$	$-CH_2CH_2O-$	$-CH_2CH_2O-$
Structural residue	C_6H_5, H	H, H	C_4H_9	OH, OH − (–O–)	$C_9H_{19}C_6H_4$, OH
V_{0x}	0.90	0.35	0.99	0.18	2.32
ΔV_x	0.16	0.16	1.22	0.39	0.39
I_{0x}	2.94	1.40	0.12	7.39	4.60
ΔI_x	−0.25	−0.32	2.44	1.47	1.47
$(V_{0x}\Delta I_x + I_{0x}\Delta V_x)$	0.24	0.11	2.56	3.15	5.20
Q	1.53	0.70	2.10	8.07	13.34
$\alpha_0(c)$	0.46	0.51	3.31	0.33	−0.32
$\alpha_1(c)$	0.53	0.55	3.92	0.87	0.52
$\alpha_0(e)$	0.54	0.52	0.77	0.41	−0.015
$\alpha_1(e)$	0.47	0.48	0.83	0.78	0.052
Mobile phase for experimental data	Methanol–water	Methanol–water	1,4-Dioxane–water	Methanol–water	2-Propanol–water

Each of the constants α_0(e) and α_1(e) has almost the same value for different homologous series (*n*-alkylbenzenes and *n*-alkanes) in methanol–water mobile phases. The structural incremental indices ΔV_x and ΔI_x for the methylene group (eqns. 1 and 2) are approximately equal in different homologous series and the polarity increments are negative (Table V). Consequently, the combination term $\Delta V_x I_{0x} + \Delta I_x V_{0x}$ (the numerator in eqn. 18) is close to zero and the combined structural parameter Q is relatively small (0.7 for *n*-alkanes and 1.53 for *n*-alkylbenzenes), so that they have no significant effect on the separation selectivity, *i.e.*, on the values of the selectivity constants α_0 and α_1. This leads to approximately equal separation selectivities and α_0 and α_1 values for various homologous series. The selectivity in homologous series is controlled primarily by the size (molar volume, ΔV_x) of the methylene group and by the polarity (I_{org}) and concentration (φ) of the organic solvent in the mobile phase.

Similar conclusions should obviously apply also for the oligomeric series with relatively non-polar repeat structural units. The oligomeric unit in the oligostyrene series has a relatively high positive polarity contribution index ΔI_x and an almost eight times larger volume contribution index ΔV_x than the methylene group (Table V). Consequently, the combination term ($\Delta V_x I_{0x} + \Delta I_x V_{0x}$) and the combined structural parameter Q (eqn. 18) are greater than in homologous series, but the influence of the size, ΔV_x, of the repeat oligomeric unit on the separation selectivity obviously prevails over the influence of the combination term. This leads to a greater retention and selectivity [and constants α_0(e) and α_1(e)] than in homologous series (Table V). Therefore, only a limited concentration range of the mobile phase is available for the practical separation of oligostyrenes, the separation selectivity changes more significantly with changing mobile phase composition and the correct choice of the mobile phase composition is more critical than for the separation of the other series studied.

In the oligoethylene glycol series and in the series of ethoxylated nonylphenols, the repeat structural unit $-CH_2CH_2O-$ contributes significantly to the polarity (ΔI_x), but its contribution to the molar volume (ΔV_x) is about three times lower than ΔV_x in the oligostyrene series and about three times higher than ΔV_x of the methylene group (Table V). The combination term ($\Delta V_x I_{0x} + \Delta I_x V_{0x}$) and the structural parameter Q (eqn. 18) are relatively large, which means that their influence on the separation selectivity may become important.

As the repeat structural unit $-CH_2CH_2O-$ is the same in these two oligomeric series, the selectivity log α should also be the same in a mobile phase of given composition, provided that the structural residue has no influence on the separation selectivity. The retentions of the two types of oligomers are very different, so that it was not possible to compare directly the separation selectivities for the two series in the same mobile phase. Table V shows the experimental selectivity constants α_0(e) and α_1(e) for oligoethylene glycols in water–methanol mobile phases. The constant α_0 is lower and the constant α_1 is higher than the corresponding values for the homologous series tested. The separation selectivity for ethoxylated nonylphenols is very low in water–methanol mobile phases and the individual oligomers are eluted in a single common peak with very bad resolution (Fig. 7). A change in methanol concentration does not influence the bad quality of the separation and only causes a shift of the elution volume of the common peak. Hence it was not possible to evaluate quantitatively the selectivity and the constants α_0 and α_1 (eqns. 12 and 13) in

water–methanol mobile phases, but it can be concluded that they are close to zero; otherwise, the separation could have been improved by a change in methanol concentration. This means that the α_0 and α_1 values for ethoxylated nonylphenols are different to those for oligoethylene glycols, which suggests an important influence of the structural residue on the separation selectivity for the oligomeric series with $-CH_2CH_2O-$ repeat units.

In dioxane–water mobile phases, it was also impossible to resolve the individual oligomeric ethoxylated nonylphenols. These compounds could be separated in propanol–water mobile phases. The selectivity constants α_0(e) and α_1(e) applying for ethoxylated nonylphenols in these mobile phases are very low and a negative value of α_0 means that the oligomers are eluted in order of decreasing number of oligomeric units, n (Table V). The selectivity constant α_0 is (theoretically) independent of the organic solvent used in the mobile phase and the constant α_1 is expected to be larger in mobile phases containing less polar organic solvents (eqn. 21). This further confirms the essential differences between the selectivity constants α_0 and α_1 of ethoxylated nonylphenols and the corresponding values for oligoethylene glycols, which are eluted in order of increasing number of the oligomeric units in propanol–water mobile phases.

This behaviour may be explained by the influence of the combination term $(\Delta I_x V_{0x} + \Delta V_x I_{0x})$ and of the combined structural parameter Q (eqn. 18) on the separation selectivity. These terms have higher values for the series with $-CH_2CH_2O-$ repeat units than for the other series tested and are higher for ethoxylated nonylphenols (Q = 13.34) than for oligoethylene glycols (Q = 8.07). If the combination term is large, the structural parameter Q may be even greater than the term involving the interaction index of water in eqns. 20 and 23 applies, which means that α_0 is negative, whereas α_1 may still be positive, as has been observed experimentally for ethoxylated nonylphenols.

Eqns. 20 and 21 make it possible to calculate the selectivity log α and the selectivity constants α_0 and α_1 from the structural indices I_{0x}, ΔI_x, V_{0x} and ΔV_x, provided that the constants K_1, K_2 and K_3 in these equations are known. To calculate these selectivity constants, K_1–K_3 were taken from earlier work[8] and the indices I_{0x}, ΔI_x, V_{0x} and ΔV_x necessary for the calculations were either measured or estimated from the structural indices of other compounds and are given in Table V together with the calculated parameters Q and selectivity constants α_0(c) and α_1(c). The agreement between the predicted and experimental α_0 and α_1 values is relatively good for the homologous series of n-alkylbenzenes and n-alkanes and for oligoethylene glycols, the retention data of which were measured in methanol–water mobile phases. For the oligostyrene series, where the retention data were measured in dioxane–water mobile phases, and for the ethoxylated nonylphenols, where propanol–water mobile phases were employed, large differences between the numerical values of α_0(c) and α_0(e) and of α_1(c) and α_1(e) were found (Table V), which can be attributed to the following possible sources of errors: (a) the experimental data are compared with the data calculated using the constants K_1–K_3 in eqns. 20 and 21, which were determined in water–methanol mobile phases; (b) slight curvatures of the log k'–φ plots in the range of lower φ in propanol–water and dioxane–water mobile phases were neglected to a first approximation; and (c) the structural indices for the oligomeric series studied were calculated from the values measured for other compounds, assuming additivities for various structural elements, which possibly is not strictly obeyed.

In spite of these significant differences in the numerical values of the experimental and calculated selectivity constants α_0 and α_1, their comparison may be interesting, as the following qualitative agreement between the calculated and experimental values is observed: (1) both the experimental and the calculated values of α_0 for ethoxylated nonylphenols are negative, whereas all the other experimental and calculated α_0 and α_1 have positive values; and (2) both the calculated and experimental α_0 and α_1 values for oligostyrenes are higher than the corresponding values for the other series.

Gradient elution chromatography

Gradient elution reversed-phase liquid chromatography is well suited to improving the separation of homologous series, where approximately equal distances between the neighbouring peaks can be easily achieved. The situation is not so simple for oligomeric series, where the dependence of retention and selectivity on mobile phase composition is very important.

In the oligostyrene series (Table II), the absolute retention, selectivity and their concentration dependences are greater than in homologous series. Generally, the retention volumes in gradient elution chromatography decrease with increasing initial concentration A of the organic solvent in the mobile phase at the start of the gradient and with increasing product mB [B is the slope of the gradient expressed as the change in φ, in % (v/v) $\cdot$ 10^{-2}, per unit volume of the eluate][41]. This implies that low values of B are required to compensate for large m and a relatively high A should be used in gradient elution separations of oligostyrenes in order to keep the separation time within reasonable limits. Fig. 8 shows the reversed-phase separation of a sample of

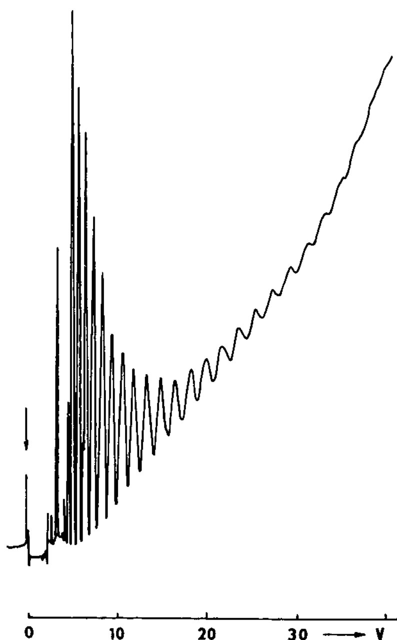

Fig. 8. Separation of a polystyrene standard (MW 2350) using gradient elution with linear gradient from 83 to 90% of 1,4-dioxane in water in 2 h. Other conditions as in Fig. 2.

oligostyrenes using such a shallow gradient. The improvement in the separation in comparison with isocratic conditions (Fig. 2) is not as significant as is usual in reversed-phase gradient elution separations of homologous series, for two reasons: (1) shallow gradients cause less band compression than steeper gradients and (2) restricted diffusion within the pores of the column packing and possible effects of molecular conformation contribute to additional band broadening of the large molecules of higher oligomers[42].

Gradient elution chromatography is suitable for improving the separation of oligoethylene glycols, but it is incompatible with refractometric detection and UV detection at 185 nm[35] requires the use of very pure solvents as mobile phase components.

The retention of ethoxylated nonylphenols is considerably higher than that of oligoethylene glycols under identical conditions and, as expected, it decreases with increasing concentration of the organic solvent (propanol) in the mobile phase. However, as α_0 is negative and α_1 is positive (Table V), the selectivity decreases slightly with decreasing concentration of the organic solvent in the mobile phase, in contrast to the other series studied (see Fig. 3). A decrease in selectivity with decreasing elution strength of the mobile phase has an important consequence for gradient elution separations of ethoxylated nonylphenols. Generally, a decrease in the gradient slope or initial concentration of the stronger eluting component in the mobile phase leads to increased selectivity. However, a decreased gradient slope and initial concentration of propanol cause an increase in the elution volumes of the ethoxylated nonylphenols, but also a slightly impaired separation selectivity and resolution of the individual oligomers (Fig. 9B in comparison with Fig. 9A).

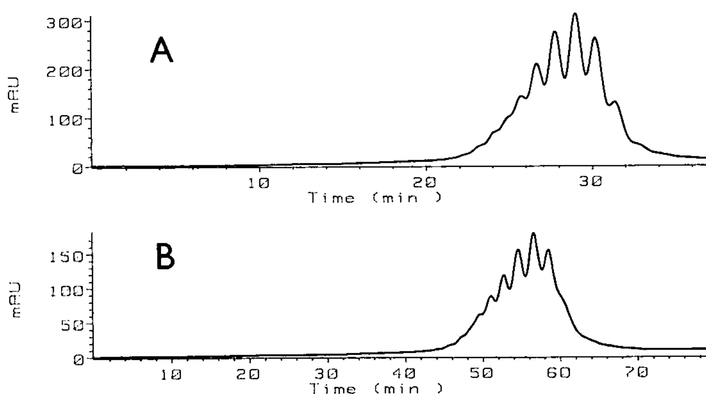

Fig. 9. Gradient elution separation of Serdox NNP 4 using linear gradients (A) from 35 to 65% 2-propanol in water in 1 h and (B) from 30 to 60% 2-propanol in water in 2 h. Other conditions as in Fig. 4.

Chromatographic behaviour of non-sulphated and sulphated oligoethylene glycol nonylphenyl ethers in mobile phases containing CTAB

Sulphated oligoethylene glycol nonylphenyl ethers are commonly used anionic surfactants prepared by sulphation of oligoethylene glycols. As the sulphated oligomers contain strongly acidic groups, they are eluted in a single non-resolved peak

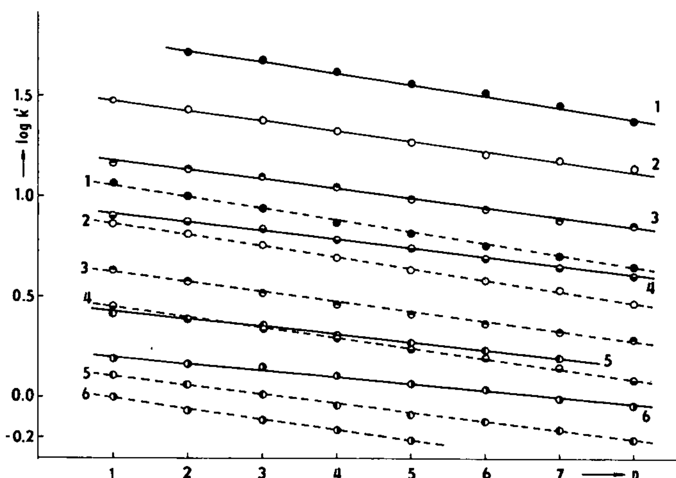

Fig. 10. Dependence of retention (k') of non-sulphated (broken lines) and sulphated (full lines) oligoethylene glycol nonylphenyl ethers on the number of structural repeat units, n, in mobile phases containing 0.04 M CTAB with different ratios of 2-propanol to water: (1) 35:65; (2) 40:60; (3) 45:55; (4) 50:50; (5) 60:40; (6) 65:35. Column: Silasorb C_{18} SPH, 7.5 μm (300 × 3.6 mm I.D.).

near the column dead volume in reversed-phase systems using mobile phases composed of an organic solvent and water. Addition of an ion-pairing reagent such as CTAB to the mobile phase increases the retention and the individual oligomers can be separated. In propanol–water mobile phases containing CTAB, the retention of both sulphated and non-sulphated ethers decreases linearly with increasing concentration of propanol in the mobile phase and with increasing number of repeat structural units, as in the chromatography of oligoethylene glycol nonylphenyl ethers in mobile phases without CTAB (Fig. 10). The retention of a sulphated ether is always considerably higher than that of the corresponding non-sulphated ether, which can be utilized for the separation of the two groups of compounds (substrates and reaction products) in a single run[40] (Fig. 11).

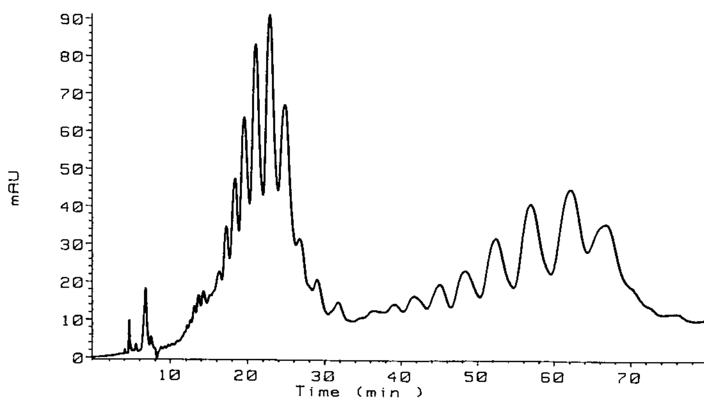

Fig. 11. Separation of partially sulphated Serdox NNP 4 in 2-propanol–water (45:55) + 0.04 M CTAB. Other conditions as in Fig. 4. The first group of peaks are non-sulphated oligoethylene glycol nonylphenyl ethers and the second group of peaks are oligomers in the sulphated product.

Even though the retention behaviour and separation selectivity of the non-sulphated ethers on C_{18} and C_8 columns in mobile phases containing 0.04 *M* CTAB were similar to those in mobile phases without CTAB, some differences are apparent (Table VI): the constants α_1 (m_1) are negative in CTAB-containing mobile phases and positive in mobile phases without CTAB, whereas the opposite holds true for the constants p. The decrease in n_{cc} with increasing number of repeat structural units, n, is more significant and q_i values are higher in CTAB-containing mobile phases. This means that the separation selectivity improves slightly with decreasing concentration of propanol in the mobile phase, in contrast to mobile phases without CTAB, but this difference is not very significant in practice. The influence of 0.04 *M* CTAB in the mobile phase on the retention and selectivity of the separation of the non-sulphated ethers is of only minor importance, but it might be useful to investigate the behaviour of non-ionic surfactants in CTAB-containing mobile phases in more detail.

The sulphated oligoethylene glycol nonylphenyl ethers are retained much more strongly and their separation selectivity is similar to, but slightly lower than, that of the non-sulphated ethers (Table VII). The contributions of the structural residue to both the hydrophobic (n_{cc0}) and polar (q_{0i}) selectivities are higher with the sulphated ethers whereas the decrease in n_{cc} with the number of repeat structural units, n, (Δn_{ce}) is similar and q_i is almost independent of n for both series. The larger q_{0i} for sulphated

TABLE VI

EXPERIMENTAL VALUES OF THE PARAMETERS $\alpha_0, \alpha_1, \beta_0, \beta_1, a_0, a_1, m_0, m_1, q, p, n_{cc0}, \Delta n_{cc}, q_{0i}$ AND Δq_i IN EQNS. 6–9, 15, 25 AND 26 FOR ETHOXYLATED NONYLPHENOLS ON C_{18} AND C_8 COLUMNS IN 2-PROPANOL–WATER MOBILE PHASES CONTAINING CTAB, CALCULATED USING LINEAR REGRESSION OF THE EXPERIMENTAL DATA ACCORDING TO THE EQUATIONS

R = correlation coefficient.

Column: Silasorb C_{18} SPH, 7.5 μm (300 × 3.6 mm I.D.): Mobile phase: 2-propanol–water + 0.04 M CTAB; n = 1–7

2-Propanol (%, v/v)	Log β	Log α	R
35	1.090	−0.055	0.9962
40	0.894	−0.050	0.9942
45	0.670	−0.049	0.9969
50	0.489	−0.046	0.9968

Equation	R
$\log \alpha = -0.074 + 0.056\varphi$;	$R = 0.9661$
$\log \beta = 2.514 - 4.066\varphi$;	$R = 0.9993$
$a = 2.258 - 0.076n$;	$R = 0.9983$
$m = 3.523 - 0.054n$;	$R = 0.9911$
$m = 1.908 + 0.716a$;	$R = 0.9953$
$n_{cc} = 3.93 - 0.31n$;	$R = 0.9983$
$q_i = 1.48 + 0.02n$;	$R = 0.9503$

Calibration series of n-alkylbenzenes:

		R
$a_{0B} = 1.313$;	$a_{1B} = 0.238$;	$R = 0.9999$
$m_{0B} = 2.319$;	$m_{1B} = 0.213$;	$R = 0.9999$
$q_B = 1.147$;	$p_B = 0.893$;	$R = 0.9998$

Column: Silasorb C_8 SPH, 10 μm (300 × 4.2 mm I.D.): Mobile phase: 2-propanol–water + 0.04 M CTAB; n = 2–7:

2-Propanol (%, v/v)	Log β	Log α	R
30	1.100	−0.051	0.9958
35	0.851	−0.047	0.9979
40	0.636	−0.042	0.9986
45	0.436	−0.041	0.9987

Equation	R
$\log \alpha = -0.071 + 0.070\varphi$;	$R = 0.9726$
$\log \beta = 2.411 - 4.414\varphi$;	$R = 0.9987$
$a = 2.335 - 0.070n$;	$R = 0.9999$
$m = 4.247 - 0.066n$;	$R = 0.9970$
$m = 2.046 + 0.942a$;	$R = 0.9970$
$n_{cc} = 4.54 - 0.33n$;	$R = 0.9995$
$q_i = 1.71 + 0.01n$;	$R = 0.9160$

Calibration series of n-alkylbenzenes:

		R
$a_{0B} = 1.356$;	$a_{1B} = 0.217$;	$R = 0.9987$
$m_{0B} = 2.927$;	$m_{1B} = 0.236$;	$R = 0.9974$
$q_B = 1.454$;	$p_B = 1.086$;	$R = 0.9997$

TABLE VII

EXPERIMENTAL VALUES OF THE PARAMETERS $\alpha_0, \alpha_1, \beta_0, \beta_1, a_0, a_1, m_0, m_1, q, p, n_{ce0}, \Delta n_{ce}, q_{0i}$ AND Δq_i IN EQNS. 6–9, 15, 25 AND 26 FOR SULPHATED ETHOXYLATED NONYLPHENOLS ON C_{18} AND C_8 COLUMNS IN 2-PROPANOL–WATER MOBILE PHASES CONTAINING CTAB, CALCULATED USING LINEAR REGRESSION OF THE EXPERIMENTAL DATA ACCORDING TO THE EQUATIONS

R = correlation coefficient.

Column: Silasorb C_{18} SPH, 7.5 μm (300 × 3.6 mm I.D.): Mobile phase: 2-propanol–water + 0.04 M CTAB; n = 1–7:

2-Propanol (%, v/v)	*Log β*	*Log α*	*R*
45	1.218	−0.047	0.9975
50	0.951	−0.043	0.9971
60	0.461	−0.038	0.9971

Equation	R
$\log \alpha = -0.073 + 0.059\varphi$;	$R = 0.9921$
$\log \beta = 3.471 - 5.022\varphi$;	$R = 0.9998$
$a = 3.471 - 0.073n$;	$R = 0.9972$
$m = 5.022 - 0.059n$;	$R = 0.9948$
$m = 2.233 + 0.803a$;	$R = 0.9986$
$n_{cc} = 9.07 - 0.31n$;	$R = 0.9972$
$q_i = 1.92 + 0.01n$;	$R = 0.9089$

Calibration series of n-alkylbenzenes

		R
$a_{0B} = 1.313$;	$a_{1B} = 0.238$;	$R = 0.9999$
$m_{0B} = 2.319$;	$m_{1B} = 0.213$;	$R = 0.9999$
$q_B = 1.147$;	$p_B = 0.893$;	$R = 0.9998$

Column: Silasorb C_8 SPH, 10 μm (300 × 4.2 mm I.D.): Mobile phase: 2-propanol–water + 0.04 M CTAB; n = 2–7:

2-Propanol (%, v/v)	*Log β*	*Log α*	*R*
35	1.414	−0.042	0.9996
40	1.142	−0.042	0.9999
55	0.338	−0.033	0.9996

Equation	R
$\log \alpha = -0.058 + 0.045\varphi$;	$R = 0.9656$
$\log \beta = 3.295 - 5.377\varphi$;	$R = 0.9999$
$a = 3.421 - 0.060n$;	$R = 0.9990$
$m = 5.712 - 0.050n$;	$R = 0.9913$
$m = 2.881 + 0.828a$;	$R = 0.9960$
$n_{cc} = 9.19 - 0.22n$;	$R = 0.9634$
$q_i = 1.91 + 0.03n$;	$R = 0.8720$

Calibration series of n-alkylbenzenes:

		R
$a_{0B} = 1.356$;	$a_{1B} = 0.217$;	$R = 0.9987$
$m_{0B} = 2.927$;	$m_{1B} = 0.236$;	$R = 0.9974$
$q_B = 1.454$;	$p_B = 1.086$;	$R = 0.9997$

ethers may be attributed to the presence of the sulphuric acid ester end-group and larger n_{ce0} values (equivalent to about five methylene units) to the formation of ion pairs between the sulphated ethers and CTAB. Hence the mobile phase composition and structural effects on the retention of compounds such as the sulphated oligoethylene glycol nonylphenyl ethers in mobile phases containing CTAB can be explained in a closely analogous manner to the reversed-phase mechanism of the retention of the non-sulphated oligoethylene glycol nonylphenyl ethers.

CONCLUSIONS

In reversed-phase chromatography, the retention of oligomeric series as a function of the number of repeat structural units and of the mobile phase composition can be described formally by the same set of equations as the retention in homologous series. The constants of these equations have different values, depending on the structures of the oligomeric repeat unit and of the structural residue in the molecule. The retention decreases with increasing concentration of the organic solvent in the mobile phase, φ, and the $\log k'$–φ plots are linear at least over a limited range of mobile phase compositions. The dependences of $\log k'$ on the number of repeat structural units, n, at different φ are also linear for a more or less limited number of oligomers.

The selectivity of separation for a pair of neighbouring oligomers is constant in an oligomeric series under given separation conditions and depends on the contributions of both the repeat structural units and the structural residue to the lipophilic and polar selectivity, characterized by the indices n_{ce0}, Δn_{ci}, q_{0i} and Δq_i. If a repeat structural unit is non-polar, the retention increases with n, but if it contributes to both the size and polarity of oligomer molecules, the retention may, under certain circumstances, even decrease with n, such as in the oligoethylene glycol nonylphenyl ether series. This behaviour depends also on the concentration and type of the organic solvent in the mobile phase and can be explained and predicted, at least qualitatively, from the molar volumes and interaction indices of repeat units and the structural residue, using the theoretical model of interaction indices.

Gradient elution reversed-phase chromatography significantly improves the separation of homologous series and it usually also improves the separation of oligomeric series with relatively non-polar oligomeric units; the individual oligomers of such series are relatively strongly retained and have large values of m in eqn. 5a. Relatively shallow gradients are usually required for separation of these oligomeric series, such as the lower oligostyrenes. However, the application of gradient elution may have little advantage in the separation of oligomeric series, where the structural repeat unit is relatively polar and the structural residue is relatively large and non-polar. In such series, the separation selectivity may not change significantly or may even decrease with decreasing elution strength of the mobile phase, such as in the reversed-phase chromatography of ethoxylated nonylphenols, where the application of gradient elution is not successful.

In mobile phases containing CTAB, the retention behaviour of non-sulphated ethoxylated nonylphenols is similar to that in mobile phases without CTAB, but the presence of CTAB may influence the selectivity and its dependence on concentration to a certain extent. Anionic oligomers, such as sulphated ethoxylated nonylphenols, form ion pairs with CTAB, which leads to a very significant increase in retention. The separation selectivity for the neighbouring sulphated oligomers and the dependence of selectivity on mobile phase composition and on the number of repeat structural units are similar to those for the non-sulphated oligomers. In propanol–water mobile phases containing CTAB, the separation of the individual oligomers in both non-sulphated and sulphated ethoxylated nonylphenols may be achieved in a single chromatographic run.

SYMBOLS

a	extrapolated log k' in pure water as the mobile phase, *i.e.*, the intercept in eqns. 5 and 5a;
a_0	a of the structural residue in a homologous or oligomeric series, *i.e.*, the intercept in eqn. 6;
a_{0B}	a_0 for the calibration n-alkylbenzene homologous series;
a_1	slope of the a *versus* n relationship (eqn. 6);
a_{1B}	a_1 for the calibration n-alkylbenzene homologous series;
c_1	temperature- and organic solvent-dependent constant in eqns. 10–13 (eqn. 14);

c_M temperature- and organic solvent-dependent constant in eqns. 3 and 10–13;
c_x temperature- and solute-dependent constant in eqns. 3 and 10–13;
d second-power term constant in eqn. 5;
k' capacity factor of solute;
m first-power term constant in eqns. 5 and 5a, *i.e.*, the slope of the log k' *versus* φ relationship, equivalent to the term S introduced by Snyder[43];
m_0 m of the structural residue in a homologous or oligomeric series (intercept in eqn. 7);
m_1 slope of the m *versus* n relationship, *i.e.*, contribution of one repeat structural (oligomeric or homologous) unit to m (eqn. 7);
n number of repeat structural units in a given homologue or oligomer;
n_{ce} lipophilic structural index, *i.e.*, the number of methylene groups in a hypothetical member of the calibration homologous *n*-alkylbenzene series with the same retention as the solute in water (eqn. 24);
n_{ce0} n_{ce} of the structural residue in an oligomeric series (eqn. 25);
Δn_{ce} contribution of one oligomeric unit to n_{ce} in a given series (eqn. 25);
p slope of the linear dependence of m on a, applying for a given homologous or oligomeric series and organic solvent (eqn. 15);
p_B p applying for *n*-alkylbenzene homologous calibration series;
q intercept of the linear dependence of m on a, applying for a given homologous or oligomeric series and organic solvent in the mobile phase (eqn. 15);
q_i polar structural index, characterizing the magnitude of the polar interactions of the solute with the mobile phase (eqn. 24);
q_{0i} q_i of the structural residue in an oligomeric series (eqn. 26);
Δq_i contribution of one oligomeric unit to n_{qi} in a given series (eqn. 26);
A concentration of the stronger eluting solvent in a binary mobile phase at the start of gradient elution;
B gradient slope, *i.e.*, the change in φ [% (v/v) $\cdot 10^{-2}$] per unit volume (1 cm^3) of the eluate during gradient elution;
I interaction index as a quantitative measure of polarity[8];
I_{H_2O} I of water;
I_{org} I of the organic solvent in a binary mobile phase;
I_x I of solute;
I_{0x} I_x of the structural residue in a given homologous or oligomeric series (eqn. 1);
ΔI_x contribution of one repeat structural (homologous or oligomeric) unit to I_x (eqn. 1);
K_1, K_2, K_3 temperature- and organic solvent-dependent constants in eqns. 20 and 21;
Q combined structural parameter in a given homologous or oligomeric series (eqn. 18);
R gas constant;
T absolute temperature;
V_M volume of the mobile phase in the column;
V_S volume of the stationary phase in the column;
V_x molar volume of solute (cm^3 mol^{-1} $\cdot 10^{-2}$);

V_{0x}	V_x of the structural residue in a given homologous or oligomeric series (eqn. 2);
ΔV_x	contribution of one repeat structural (homologous or oligomeric) unit to V_x (eqn. 2);
α	relative retention, *i.e.*, selectivity in a given homologous or oligomeric series, the ratio of k' of the neighbouring members (eqn. 4);
α_0	selectivity constant, *i.e.*, intercept of log α *versus* φ relationship (eqns. 9 and 12);
α_1	selectivity constant, *i.e.*, slope of log α *versus* φ relationship (eqns. 9 and 13);
β	log k' of the zeroth member of a given homologous or oligomeric series (eqn. 4);
β_0	intercept of log β *versus* φ relationship (eqns. 8 and 10);
β_1	slope of log β *versus* φ relationship (eqns. 8 and 11);
γ	second-power term constant in eqn. 4;
φ	concentration of the organic solvent in the mobile phase $[\%(v/v) \cdot 10^{-2}]$;
φ_0	φ at which the selectivity $\alpha = 0$;
Φ	phase ratio in column, $\Phi = V_S/V_M$.

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ENTHALPY–ENTROPY COMPENSATION IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SERIES OF ARYLOXOALKANOIC AND ARYLHYDROXYALKANOIC ACIDS

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SUMMARY

The capacity factors of various arylacetic (I), aryloxoalkanoic (II), arylhydroxyalkanoic (III) and substituted benzyloxyphenylacetic (IV) acids have been determined at various temperatures on a μBondapak C_{18} reversed-phase system with buffered aqueous methanol, acetonitrile or tetrahydrofuran (pH 3.0) as the mobile phases. Three groups of acids were evaluated, the lipophilicity and retention behaviour of which were affected by intramolecular interactions. A series of acids I, used for comparison, showed a linear correlation between the logarithms of capacity factors, log k', and the corresponding changes in enthalpy, $-\Delta H^0$. Interactions of two hydrophilic groups (so-called H/H interactions) affect the lipophilicity and retention properties of the acids II, III in a similar way. Thus it is possible to calculate log P values from the linear dependence log P *vs.* log k', regardless of the modifier used. These interactions did not show in the enthalpy–entropy compensation of the systems containing methanol or tetrahydrofuran as a modifier. Thus, the linear relationships between log k' and ΔH^0 are valid for the whole group of acids I–III. An effect of intramolecular hydrophobic interactions upon the retention properties of acids IV in 50% methanol was accompanied by a deviation from the isokinetic relationship derived for the acids I–III. This type of interaction did not occur in 40% tetrahydrofuran and the entropy–enthalpy compensation operated over the whole group of acids I–IV. The enthalpy changes were minimal in the system with 40% acetonitrile as the mobile phase and thus the isokinetic relationship was affected by a large error.

INTRODUCTION

Partition chromatography is widely used for the evaluation of the lipophilicity of bioactive compounds[1–6]. Statistically highly significant regression equations were obtained[7,8] for series of arylalkanoic acids, in which logarithms of the partition coefficients were correlated with the chromatographic parameters R_M or log k'. Silica gel impregnated with a silicone oil or silanized silica gel were used as the stationary phases in thin-layer chromatography (TLC). High-performance liquid chromato-

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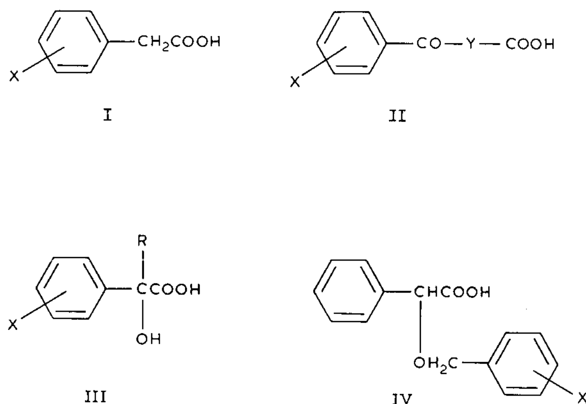
graphy (HPLC) using various C_{18} silanized packings[7] was also employed without any change in statistical significance of the regression equations. The best results were obtained with buffered water (pH 3.5) as the mobile phase in a mixture with a suitable organic solvent; acetone for TLC or methanol for HPLC gave[8] a linear dependence of log P and log k' for groups of acids containing arylacetic acids (I), aryloxoalkanoic acids (II) and arylhydroxyalkanoic acids (III). Introduction of an hydrophilic group in the vicinity of the carboxyl in acids II and III results[9] in so-called H/H interactions[10] which influence the lipophilicity.



For the groups of acids I–IV, the dependence of the retention characteristics on temperature was studied as well as the enthalpy–entropy compensation in systems with different modifiers in the mobile phase, *i.e.*, methanol, acetonitrile, tetrahydrofuran. The equation derived by Melander *et al.*[11]

$$\log k'_T = -\frac{\Delta H^0}{2.3R}\left(\frac{1}{T} - \frac{1}{\beta}\right) - \frac{\Delta G^0_\beta}{2.3R\beta} + \log \varphi \tag{1}$$

was used where k'_T is the capacity factor of a solute at temperature T, β is the compensation temperature, ΔH^0 is the change in standard enthalpy, ΔG^0_β is the Gibbs energy of a process at temperature β and φ is the phase ratio of the column. The value of ΔH^0 for the transfer of the solute from the mobile phase to the stationary phase can be calculated from the slope of the linear relationship between log k' and $1/T$

$$\log k' = -\frac{\Delta H^0}{2.3R} \cdot \frac{1}{T} + \frac{\Delta S^0}{2.3R} + \log \varphi \tag{2}$$

where ΔS^0 is the corresponding change in standard entropy. This method of verification of the isokinetic relationship was used for a series of arylacetic acids[12] including derivatives the lipophilicity of which was affected by the intramolecular hydrophobic interaction of the aromatic nuclei and by steric hindrance of solvation. A difference in the slope of the linear equation 1 was found in both cases in comparison with the standard series of arylacetic acids.

EXPERIMENTAL

HPLC

Experiments were carried out using a liquid chromatograph assembled from a Model 6000 A pump, an U6K injector, a 440 fixed-wavelength detector and a M 730 data module (Waters Assoc., Milford, MA, U.S.A.). To maintain the temperature ($\pm$ 0.1°C), a water-jacketed column connected to a water-circulating bath was used. A μBondapak C_{18} column (300 mm $\times$ 3.9 mm) was obtained from Waters Assoc. 0.0025 *M* Aqueous phosphate buffer (pH 3.0) modified with 50% (v/v) methanol, 40% (v/v) acetonitrile and 40% (v/v) tetrahydrofuran, respectively, was used as a mobile phase. Doubly distilled water, filtered through 0.45-μm Millipore filters, was used throughout. The eluent flow-rate was 1 ml/min. Detection was performed by UV absorption at 200 μm, range 0–0.01 a.u. The retention time of sodium nitrate (0.2% solution) was taken as t_0 and the capacity factor, k', was evaluated from the retention time, t_R, of the solute using the relationship $k' = (t_R - t_0)/t_0$.

Determination of partition coefficients

Partition coefficients, P_{exp}, of acids Ia, IIa, f, IIIc, d, IVa, b, c, were determined by the shake-flask method[13] in an octanol–water system at 20°C, with both phases presaturated with the other. To eliminate the effect of dissociation of the acids, the aqueous phase employed was an acetate buffer (pH 3.4). The concentrations of the acids in the two phases were determined spectrophotometrically and the partition coefficients, P, were calculated as the ratio of concentrations in the octanol and aqueous phases, $P_{exp} = c_0/c_w$.

Sample preparation

The arylacetic acids (I) were prepared[14] by the Wilgerodt reaction or by hydrolysis of the corresponding arylacetonitriles. To prepare aryloxoalkanoic acids (II) we used the Friedel–Crafts reaction of anhydrides of dicarboxylic acids with appropriate aromatic acids[15]. The acids III were obtained[16] from the esters of aryloxocarboxylic acids by reactions with methylmagnesium iodide and subsequent hydrolysis. The acids IV were obtained[16] by reaction of phenylhydroxyacetate with the corresponding benzyl chlorides in the presence of sodium hydride, followed by hydrolysis.

Calculations

The log P values of the remaining acids I were calculated using the parameters π derived[17] for the arylacetic acids. For the acids II the parameters π were derived[17] for the substituted benzoic acids, and for the acids III and IV the parameters π were taken from those derived for the substituted benzyl alcohols. The sums of the parameters π for the 3-chloro-4-alkoxy derivatives were reduced by 0.23, in accordance with the results[14,18] of partition chromatography of those derivatives of arylaliphatic acids. The log P value of the unsubstituted acid IVa was calculated by the fragmental method according to the equation:

$$\begin{aligned}\log P\,(\text{IVa}) &= 2f(C_6H_5) + f(CH_2) + f(CH) + f^{1R}(COOH) + f^{1R}(\text{–O–}) + \\ &\quad + 4F_b + F_{gBr} - 0.42\,[f^{1R}(COOH) + f^{1R}(\text{–O–})] \\ &= 3.80 + 0.66 + 0.43 - 1.03 - 1.52 - 0.48 - 0.22 + 1.07 \\ &= 2.71\end{aligned}$$

where fragmental constants f and fragmental factors F were taken from refs. 10 and 19. The coefficients in the regression equations were calculated from the experimental results by multiple regression analysis. The statistical significances of the regression equations were tested by the standard deviation, s, the coefficient of multiple correlation, r, and the Fischer–Snedecor criterion, F.

RESULTS AND DISCUSSION

The experimental values of the capacity factors, k', of the series of acids I–IV determined in the temperature range of 22–57°C are given in Tables I–III. The corresponding regression relationships between log P and log k' are summarized in Table IV; the log P values introduced in Table I were used in the calculations. Eqns. 3–7 hold for the system, where methanol was used as a modifier of the mobile phase. Eqns. 8–12 are valid for acetonitrile and eqns. 13–17 for tetrahydrofuran. The statistical significance of the linear relationships log P *vs.* log k' for the groups of acids I–III is not influenced by a change of the modifier or by the temperature. The slope,

TABLE I

LOGARITHMS OF CAPACITY FACTORS (log k') OF ACIDS I–IV AT VARIOUS TEMPERATURES

50% Methanol was used as the mobile phase.

Compound			*log P***	*Temperature (°C)*				
No.	*X*	*Y (R)**		*22*	*30*	*40*	*50*	*58*
				1/T ($K^{-1} \cdot 10^3$)				
				3.390	*3.300*	*3.195*	*3.096*	*3.030*
Ia	H		1.45***	0.226	0.154	0.060	0.028	−0.034
Ib	4-Cl		2.15	0.591	0.516	0.397	0.325	0.290
Ic	4-C_2H_5		2.43	0.819	0.751	0.631	0.565	0.510
Id	4-*iso*-C_3H_7		2.85	1.086	0.994	0.877	0.796	0.724
Ie	4-*tert.*-C_4H_9		3.13	1.297	1.198	1.076	0.983	0.908
If	4-*n*-$C_5H_{11}O$		3.46	1.541	1.435	1.288	1.184	1.108
IIa	H	CH_2CH_2	1.23***	0.201	0.139	0.021	−0.004	−0.040
IIb	3-Cl-4-CH_3O	CH_2CH_2	1.91	0.610	0.516	0.394	0.321	0.247
IIc	4-*iso*-C_3H_7O	CH_2CH_2	2.11	0.815	0.727	0.614	0.543	0.435
IId	4-Br	CH_2CH_2	2.21	0.675	0.589	0.482	0.413	0.344
IIe	4-*iso*-C_3H_7	CH_2CH_2	2.63	1.039	0.952	0.827	0.729	0.641
IIf	H	$CH_2CH(CH_3)$	1.62***	0.437	0.373	0.260	0.221	0.180
IIh	3-Cl-4-*iso*-C_3H_7O	$CH_2CH(CH_3)CH_2$	3.40	1.532	1.416	1.272	1.158	1.075
IIIa	H	CH_3	0.80	−0.040	−0.104	−0.206	−0.232	−0.300
IIIb	4-CH_3O	CH_3	0.81	−0.030	−0.095	−0.241	−0.249	−0.289
IIIc	4-*iso*-C_4H_9	CH_3	2.75***	1.152	1.052	0.923	0.829	0.751
IIId	4-*iso*-C_4H_9	C_2H_5	3.37***	1.432	1.327	1.184	1.083	1.007
IVa	H		2.00***	0.875	0.772	0.641	0.543	0.471
IVb	3-Cl-4-CH_3O		2.45***	1.158	1.038	0.876	0.753	0.687
IVc	4-Cl		2.60***	1.264	1.149	0.991	0.880	0.801

* Y applies to the acids II and R to the acids III.
** Calculated, *cf.*, Experimental.
*** Determined in octanol–buffer (pH 3.5) by the shake-flask method.

TABLE II

LOGARITHMS OF CAPACITY FACTORS (log k') OF ACIDS I–IV AT VARIOUS TEMPERATURES

40% Acetonitrile was used as the mobile phase.

No.	*Temperature (°C)*				
	22	*30*	*40*	*50*	*55*
	1/T (K⁻¹ · 10³)				
	3.390	*3.300*	*3.195*	*3.096*	*3.049*
Ia	0.102	0.066	0.013	−0.030	−0.071
Ib	0.348	0.304	0.248	0.193	0.165
Ic	0.483	0.441	0.391	0.339	0.312
Id	0.656	0.616	0.563	0.509	0.475
Ie	0.795	0.754	0.701	0.845	0.609
If	0.975	0.932	0.875	0.812	0.778
IIa	0.089	0.053	0	−0.045	−0.070
IIb	0.325	0.283	0.228	0.178	0.139
IIc	0.463	0.427	0.382	0.345	0.304
IId	0.408	0.364	0.308	0.256	0.219
IIe	0.630	0.591	0.541	0.487	0.460
IIf	0.248	0.210	0.159	0.112	0.089
IIg*	0.422	0.382	0.325	0.272	0.248
IIh	0.954	0.915	0.863	0.806	0.770
IIIa	−0.185	−0.206	−0.241	−0.268	−0.308
IIIb	−0.172	−0.195	−0.229	−0.268	−0.294
IIIc	0.589	0.563	0.523	0.475	0.443
IIId	0.828	0.794	0.749	0.695	0.667
IVa	0.541	0.487	0.422	0.345	0.304
IVb	0.737	0.676	0.602	0.525	0.487
IVc	0.788	0.728	0.654	0.577	0.539

* IIg belongs to 5-(3′-chloro-4′-methoxyphenyl)-5-oxopentanoic acid (X = 3-Cl-4-CH_3O, Y = $CH_2CH_2CH_2$) with log P (measured experimentally) 2.17.

which is a measure of the solvent system selectivity to changes in solute lipophilicity, rises with temperature. These linear relationships can be used for calculation of log P for the acids I–III from the corresponding experimental values of log k' (Table V). However, this is not true in the case of acids IV (Table VI), the lipophilicity of which is influenced by intramolecular hydrophobic interaction of both aromatic nuclei. The lipophilicity, expressed by the log P_{exp} values, is lower in comparison with the log P_{calc} values calculated by the fragmental method. The effect of this hydrophobic interaction on the retention behaviour in HPLC is exhibited to a lesser extent when methanol or acetonitrile is used as a modifier. The corresponding values, log P_{chrom}, lie between the values of log P_{exp} and log P_{calc}. Such an interaction is almost absent in the case of tetrahydrofuran.

From the experimental log k' values summarized in Tables I–III, the dependencies of log k' on temperature were calculated (*cf.*, Table VII). They show a regular course over the whole range of temperature used, provided methanol or acetonitrile is

TABLE III

LOGARITHMS OF CAPACITY FACTORS (log k') OF ACIDS I–IV AT VARIOUS TEMPERATURES

40% Tetrahydrofuran was used as the mobile phase.

No.	*Temperature (°C)*				
	20	*30*	*40*	*50*	*55*
	1/T ($K^{-1} \cdot 10^3$)				
	3.413	*3.300*	*3.195*	*3.096*	*3.049*
Ia	0.380	0.338	0.314	0.305	0.371
Ib	0.675	0.612	0.556	0.524	0.571
Ic	0.731	0.671	0.617	0.587	0.634
Id	0.897	0.818	0.752	0.711	0.749
Ie	1.030	0.947	0.851	0.809	0.841
If	1.169	1.069	0.978	0.915	0.939
IIa	0.290	0.259	0.230	0.225	0.305
IIb	0.498	0.433	0.383	0.362	0.421
IIc	0.590	0.537	0.497	0.477	0.533
IId	0.686	0.615	0.556	0.522	0.573
IIe	0.792	0.725	0.663	0.626	0.668
IIf	0.450	0.416	0.386	0.376	0.440
IIg	0.590	0.523	0.466	0.436	0.490
IIh	1.030	0.947	0.861	0.824	0.858
IIIa	0.243	0.211	0.188	0.189	0.262
IIIb	0.174	0.151	0.131	0.130	0.211
IIIc	0.955	0.868	0.793	0.745	0.780
IIId	1.208	1.112	1.022	0.960	0.983
IVa	0.981	0.897	0.826	0.780	0.785
IVb	1.083	0.990	0.907	0.852	0.881
IVc	1.268	1.157	1.055	0.983	1.001

TABLE IV

REGRESSION EQUATIONS: $\log P = a \log k' + b$

The log P values summarized in Table I were used for calculation of regression eqns. 3–17.

Eqn.	*Temperature (°C)*	*a*	*b*	*n*	*r*	*s*	*F*
3	22	1.657	0.949	17	0.992	0.114	903.9
4	30	1.708	1.054	17	0.992	0.111	961.5
5	40	1.738	1.245	17	0.993	0.105	1072.8
6	50	1.852	1.307	17	0.993	0.108	1011.3
7	57	1.892	1.418	17	0.992	0.111	962.5
8	22	2.431	1.175	18	0.991	0.114	911.5
9	30	2.459	1.253	18	0.992	0.111	966.5
10	40	2.481	1.367	18	0.992	0.108	1005.0
11	50	2.531	1.474	18	0.991	0.113	925.1
12	55	2.533	1.555	18	0.992	0.113	931.7
13	20	2.602	0.478	18	0.982	0.162	435.9
14	30	2.796	0.521	18	0.981	0.166	413.8
15	40	3.032	0.541	18	0.980	0.172	383.0
16	50	3.261	0.507	18	0.980	0.173	380.6
17	55	3.506	0.198	18	0.979	0.174	373.7

TABLE V

EXPERIMENTAL AND CALCULATED VALUES OF log P FOR ACIDS I–III

Compound			*log P_{exp}**	*log k′***			*log P_{chrom}****		
No.	*X*	*Y*		*A*	*B*	*C*	*A*	*B*	*C*
Ig	3-Cl-4-$CH_2=CHCH_2O$		2.75	1.082	0.632	0.865	2.74	2.71	2.73
IIi	4-CH_3O	CH_2CH_2	1.38	0.195	0.098	0.360	1.27	1.41	1.42
IIk	3-Cl-4-CH_3O	$CH_2CH_2CH_2$	2.17	0.803	0.438	0.623	2.28	2.24	2.10
IIl	3-Cl-4-CH_3O	$CH_2CH(CH_3)CH_2$	2.60	1.052	0.585	0.812	2.69	2.60	2.59
IIIe	4-C_6H_5	CH_3	2.71	1.187	0.698	0.945	2.91	2.87	2.94

* Values were determined in octanol–buffer (pH 3.5) by the shake-flask method.

** For mobile phases with 50% methanol (A), 40% acetonitrile (B) and 40% tetrahydrofuran (C).

*** Calculated from eqn. 3 (A), eqn. 8 (B) and eqn. 13 (C).

TABLE VI

EXPERIMENTAL AND CALCULATED VALUES OF log P FOR ACIDS IV

No.	log P_{exp}*	log P_{calc}**	log P_{chrom}***		
			A	B	C
IVa	2.00	2.71	2.40	2.49	3.03
IVb	2.45	3.32	2.87	2.97	3.30
IVc	2.60	3.57	3.04	3.09	3.78

* Values were determined in octanol–buffer (pH 3.5) by the shake-flask method.
** See Experimental.
*** Calculated from eqn. 3 (A), eqn. 8 (B) and eqn. 13 (C).

used in the mobile phase. Anomalous behaviour was observed for tetrahydrofuran. The dependence log k' *vs.* $1/T$ shows curvature at 50–55°C. In this case, ΔH^0 values for the distribution process were calculated from log k' values at 20, 30, 40 and 50°C. The departure from linearity is the more striking the lower is the log k' of the compound. For example, the log k' value of compounds IIa, IIIa, IIIb at 55°C is even higher than that at 20°C. The slope of the dependence log k' *vs.* $1/T$ is positive at temperatures lower than 50°C and the corresponding ΔH^0 is negative, while it becomes positive at temperatures over 50°C. An influence of the anomalous temperature dependencies is evident also in the relationships between log P and log k'. The lines corresponding to eqns. 13–17 intersect.

The values of ΔH^0 were calculated from the temperature dependencies according to eqn. 2 and are summarized in Table VII. Relationships between log k' and ΔH^0 are expressed by eqns. 18–20 for the system in which 50% methanol is used as the mobile phase:

	n	r	s	F	
$\log k'_{313} = 0.128\,(-\Delta H^0) - 1.627$	6	0.983	0.092	116.8	(18)
$\log k'_{313} = 0.128\,(-\Delta H^0) - 1.842$	11	0.969	0.136	138.3	(19)
$\log k'_{313} = 0.128\,(-\Delta H^0) - 1.765$	17	0.949	0.141	136.4	(20)

The values of k'_{313} were measured at 313 K, close to the harmonic mean temperature. Eqn. 18 is valid for the arylacetic acids I, eqn. 19 for the acids II and III, the lipophilicity of which is influenced by an interaction of the hydrophilic fragments. The isokinetic relationship in both groups of compounds is characterized by the same value of the slope and by very similar values of the intercept. The combination of all three series of acids I–III gives rise to eqn. 20. The acids IV, where the lipophilicity and retention behaviour are affected by the intramolecular hydrophobic interaction of the aromatic nuclei, cannot be included in eqn. 20. The deviation of these acids from eqn. 20 is apparent from Fig. 1. For clarity, the line corresponding to eqn. 21, fitted only through three points, is also shown:

	n	r	s	F	
$\log k'_{313} = 0.094\,(-\Delta H^0) - 1.394$	3	0.968	0.063	15.1	(21)

TABLE VII

THERMODYNAMIC QUANTITIES OF RETENTION FOR ACIDS I–IV

Values correspond to the general linear relationship $\log k' = a(1/T) + b$; values of the correlation coefficient for all equations are higher than 0.990.

No.	50% Methanol			40% Acetonitrile			40% Tetrahydrofuran		
	Slope, a	Intercept, b	$-\Delta H^0$ ($kJ\ mol^{-1}$)	Slope, a	Intercept, b	$-\Delta H^0$ ($kJ\ mol^{-1}$)	Slope, a	Intercept, b	$-\Delta H^0$ ($kJ\ mol^{-1}$)
Ia	699.0	−2.152	13.46	493.6	−1.567	9.51	303.4	−0.658	5.84
Ib	864.6	−2.345	16.65	523.3	−1.427	10.08	545.8	−1.188	10.51
Ic	873.2	−2.141	16.81	489.0	−1.175	9.42	522.9	−1.054	10.07
Id	998.5	−2.302	19.23	526.0	−1.123	10.13	665.3	−1.375	12.81
Ie	1075.5	−2.352	20.71	539.0	−1.027	10.38	774.5	−1.612	14.91
If	1211.8	−2.569	23.34	561.8	−0.928	10.82	876.0	−1.821	16.87
IIa	682.2	−2.121	13.14	456.6	−1.459	8.79	275.1	−0.649	5.30
IIb	997.6	−2.779	19.21	534.1	−1.482	10.28	455.2	−1.306	8.77
IIc	1016.2	−2.627	19.57	445.6	−1.044	8.58	427.0	−0.869	8.22
IId	907.1	−2.404	17.47	545.0	−1.436	10.49	596.5	−1.351	11.49
IIe	1101.5	−2.690	21.21	486.3	−1.018	9.36	591.6	−1.227	11.39
IIf	725.4	−2.029	15.86	457.0	−1.303	8.80	293.6	−0.552	5.65
IIg				503.4	−1.285	9.69	568.9	−1.353	10.95
IIh	1270.2	−2.777	24.46	534.2	−0.851	10.29	774.5	−1.612	14.91
IIIa	729.0	−2.502	14.04	344.5	−1.337	6.63	197.3	−0.500	3.80
IIIb	700.7	−2.420	13.49	340.4	−1.333	6.55	252.6	−0.620	4.86
IIIe	1110.3	−2.614	21.38	424.1	−0.841	8.17	743.2	−1.583	14.31
IIId	1186.6	−2.593	22.85	459.6	−0.728	8.85	852.9	−1.703	16.42
IVa	1124.4	−2.940	21.65	691.3	−1.797	13.31	711.1	−1.447	13.69
IVb	1234.0	−3.370	24.69	714.0	−1.685	13.75	807.3	−1.673	15.54
IVc	1296.9	−3.136	24.97	711.7	−1.626	13.71	976.8	−2.066	18.81

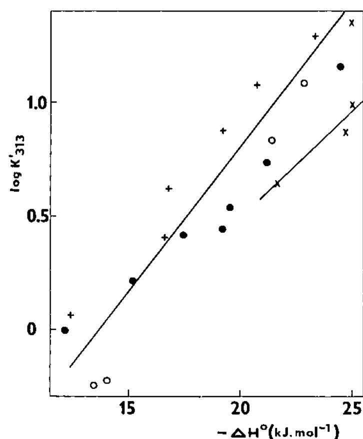


Fig. 1. Compensation plot, $\log k'_{313}$ *vs.* $-\Delta H^0$ for the acids I–IV. The $\log k'_{313}$ and $-\Delta H^0$ were taken from Tables I and VII, respectively. Mobile phase: 50% methanol. Solutes: +, arylacetic acids (I); ○, aryloxoalkanoic acids (II); ●, arylhydroxyalkanoic acids (III); ×, substituted benzyloxyphenylacetic acids (IV).

Deviations in the slope and intercept in comparison with eqn. 20 are similar to those ascertained for the arylalkoxyarylacetic acids[7,9], where the retention behaviour and lipophilicity are influenced by the same intramolecular hydrophobic interactions.

The enthalpic changes, ΔH^0, connected with the distribution process where acetonitrile was used as a modifier altered only over a narrow range. Consequently, the isokinetic relationship is subject to large errors and is statistically not significant. The following equations were derived for the system with 40% tetrahydrofuran as a mobile phase, eqn. 22 for the acids I, eqn. 23 for the acids II and III and eqn. 24 for the acids I–III:

	n	r	s	F	
$\log k'_{313} = 0.060\,(-\Delta H^0) - 0.031$	6	0.993*	0.032	271.2	(22)
$\log k'_{313} = 0.063\,(-\Delta H^0) - 0.094$	12	0.960	0.081	119.0	(23)
$\log k'_{313} = 0.063\,(-\Delta H^0) - 0.085$	18	0.970	0.068	252.0	(24)

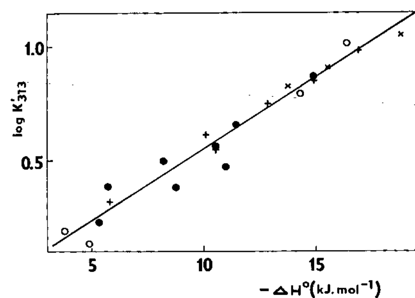


Fig. 2. Compensation plot, $\log k'_{313}$ *vs.* $-\Delta H^0$ for the acids I–IV. The $\log k'_{313}$ and $-\Delta H^0$ were taken from Tables III and VII, respectively. Mobile phase: 40% tetrahydrofuran. Symbols for solutes as in Fig. 1.

The values of ΔH^0 were calculated from the temperature dependencies in the range 20–50°C and so the isokinetic relationships are valid only in this range. The benzyloxy derivatives IV can also be introduced into the eqn. 24; no change in the slope and intercept resulted (*cf.*, eqn. 25):

	n	r	s	F	
$\log k'_{313} = 0.063\,(-\Delta H^0) - 0.080$	21	0.975	0.064	368.3	(25)

The validity of the isokinetic relationship for the acids I–IV is evident also from the plot in Fig. 2. It can be assumed that such an uniformity of the isokinetic relationship is due to the absence of intramolecular hydrophobic interactions in the distribution of these acids.

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NEW STRATEGIES FOR THE SCREENING OF A LARGE NUMBER OF IMMOBILIZED DYES FOR THE PURIFICATION OF ENZYMES

APPLICATION TO THE PURIFICATION OF ENZYMES FROM HUMAN HAEMOLYSATE

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SUMMARY

A method is presented for screening immobilized dyes applicable to the purification of enzymes from haemolysate (haemolysate can be considered as a nearly pure solution of haemoglobin containing only marginal amounts of enzymes). Haemolysate is loaded on immobilized dye mini-columns until haemoglobin and the studied enzymes are found in the column eluate at the same concentrations as those present in the haemolysate. Such a frontal mode of screening allows those dyes to be selected which, displaying a higher affinity for the enzyme of interest than for haemoglobin, can be used to displace the unwanted protein (haemoglobin) from the column by the enzyme of interest (present at a much lower concentration).

INTRODUCTION

Pioneering work in the late 1960s[1] showed that enzymes could interact strongly with textile dyes. The potential for using this interaction for protein purification was rapidly appreciated[2,3] and has since shown great development[4]. A landmark came when Qadri and Dean[5] in the early 1980s introduced a concept that for purifying a given protein from a crude extract it could be extremely rewarding to search in a systematic way for a dye displaying selective affinity for the protein of interest. Subsequently Hey and Dean[6] stressed that it could be useful to use in a first purification step a negative column, that is a column that binds as many as possible of the unwanted proteins in the crude extract but not the protein of interest, and in a second and potentially final step an immobilized dye able to retain totally the protein of interest and the minimal number of unwanted proteins.

Scopes[7–9] later studied the influence of several factors that can play a role in protein retention by immobilized dye columns and gave precise guidelines intended to

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make the systematic screening of immobilized dyes a rapid, problem-solving procedure for the purification of proteins.

Protocols for screening indicate that one has to deposit on to the columns of immobilized dyes to be tested a finite volume of starting material and determine the amount of retained proteins as a whole, together with the retained amount of the protein to be purified. In this paper we present arguments which indicate that it can be fruitful to switch from such a zonal mode of screening to a frontal mode, particularly when the starting material in the purification procedure is haemolysate, which can be regarded as a nearly pure solution of a single protein (haemoglobin) containing only trace amounts of the proteins of interest. A study was undertaken with the initial aim of devising methods for the purification of two enzymes, namely 6-phosphogluconate dehydrogenase (6PGD) and glucose phosphate isomerase (GPI).

EXPERIMENTAL

Materials

Dyes were gifts from ICI France (Clamart, France) and from Hoechst France (Nanterre, France). Brand names of the different dyes used are given in Table I together with their abbreviations. Precise chemical structures of most of these dyes are unknown but some insight can be obtained from published information[4].

Agarose beads (Ultrogel A4) were obtained from IBF (Villeneuve la Garenne, France). Chemicals were purchased from Merck (Darmstadt, F.R.G.) and Carlo Erba (Milan, Italy). Bio-Rad Labs. (Richmond, CA, U.S.A.) polypropylene Econo columns were used for the screening procedure. Their funnels were cut 4 cm from the top. The columns were closed at the top with a simple piston-like Delrin part (machined by SAE, Amboise, France) allowing the dead space above the gel surface to be kept to a minimum.

Immobilization of dyes

The immobilization protocol is similar to those in common use[4]. To obviate the tedious manual procedure of rinsing the gels after dye immobilization, a simple machine was built from commercial components to permit rinsing of four different supports automatically. The precise protocol for dye coupling is as follows.

Wash 20 ml of Ultrogel A4 in a Büchner funnel with about 200 ml of deionized water followed by 100 ml of 0.2 *M* sodium hydroxide solution containing 2% (w/v) of sodium chloride.

Weigh in a screw-capped vessel 400 mg of the dye, and transfer the gel into the vessel together with 20 ml of 0.2 *M* sodium hydroxide solution containing 2% (w/v) of sodium chloride.

Tumble the vessel with the gel suspension for 1 h at 60°C.

Centrifuge the gel suspension for 2 min at low speed and discard the supernatant, which often contains a noticeable amount of undissolved dye.

Pour the gel into one of the four chromatographic columns of the rinsing machine, rinse automatically with 10 m*M* sodium hydroxide solution (200 ml), water (300 ml), 6 *M* urea (300 ml) and finally water containing 0.02% (w/v) of sodium azide. Store the gel in the latter solution.

When gels of higher dye substitution were required, after centrifugation the gels

TABLE I

BRAND NAMES OF THE DYES USED IN THE AUTOMATED SCREENING PROCEDURE AND THEIR ABBREVIATIONS

Procion is a Trade Mark of ICI and Remazol a Trade Mark of Hoechst.

Abbreviation	*Name*	*Abbreviation*	*Name*
A1	Procion Black P-2R	R1	Procion Red HE-3B
A2	Procion Black HE-XL	R2	Procion Red MX-8B
A3	Procion Black P-N	R3	Procion Red HE-7B
A4	Remazol Black B	R4	Procion Red H-EXL
A5	Diazol Black RN Quad	R5	Procion Red P-8B
		R6	Procion Red MX-7B
B1	Procion Navy P-2R	R7	Procion Red P-4BN
B2	Procion Navy MX-4RD	R8	Procion Red MX-G
B3	Procion Havy MX-RB	R9	Procion Red MX-5B
B4	Procion Havy HE-R150	R10	Procion Red H-3B
B5	Remazol Brilliant Blue R	R11	Procion Scarlet P-2R
B6	Procion Blue SP-3R	R12	Procion Rubine MX-B
B7	Procion Blue MX-G	R13	Remazol Brilliant Red 6B
B8	Procion Blue MX-R	R14	Procion Scarlet HE-3G
B9	Procion Blue MX-2GN	R15	Remazol Brilliant Red BB
B10	Procion Blue HE-RD	R16	Remazol Brilliant Red F3B
B11	Procion Blue MX-7 RX	R17	Remazol Brilliant Red RB
B12	Procion Blue MX-2R		
B13	Procion Blue P-4R	T1	Procion Turguoise H-A
B14	Procion Blue P-5R	T2	Procion Turquoise SP-2G
B15	Procion Blue MX-4GD	T3	Procion Turquoise MX-G
B16	Procion Blue HE-GN	T4	Procion Turquoise P-GX
B17	Procion Blue P-7RX		
B18	Procion Navy HE-RN	V1	Procion Violet P-3R
C1	Procion Brown MX-GRN	Y1	Procion Yellow P-3R
C2	Procion Brown P-4RD	Y2	Procion Yellow MX-4R
C3	Procion Brown H-3R	Y3	Procion Yellow P-4G
C4	Procion Brown P-GR	Y4	Procion Yellow MX-4G
C5	Procion Brown HE-XL	Y5	Procion Yellow Brown HE-XL
C6	Procion Brown P-2R	Y6	Procion Yellow MX-8G
C7	Procion Brown MX-5BR	Y7	Procion Yellow HE-6R
		Y8	Procion Yellow HE-4R
G1	Procion Green HE-4BD	Y9	Procion Yellow HE-6G
G2	Procion Olive P-7G	Y10	Procion Yellow MX-3R
G3	Remazol Brilliant Green 6B	Y11	Procion Yellow SP-8G
G4	Procion Green P-4BD	Y12	Procion Yellow HE-3G
		Y13	Procion Yellow MX-GR
H1	Diazol Light Grey 6-BLN	Y14	Remazol Yellow GR
		Y15	Remazol Yellow GL
O1	Procion Orange MX-2R	Y16	Remazol Yellow 4GL
O2	Procion Orange P-2R	Y17	Remazol Yellow RNL
O3	Procion Orange HE-R		
O4	Procion Orange MX-G		
O5	Remazol Brilliant Orange 3R		

were resuspended in a freshly prepared solution of dye. This procedure could be repeated several times (gels identified later in the text with the symbol III following the abbreviation were incubated three times with fresh dye solution).

Quantitative appreciation of dye fixation on agarose

To evaluate the incorporation of dye in agarose, we used an acid hydrolysis method[5]: one volume of gel was hydrolysed with ten volumes of 50% acetic acid at 110°C for 15 min. The amount of dye liberated was evaluated by absorbance measurements at a wavelength found appropriate on the basis of spectral recordings of free dyes.

Assays of proteins

The classical Drabkin method[10] was used to assay haemoglobin by automated flow injection analysis[11]. Enzymatic activities were measured with Zeiss PMQ II or Beckman DU 8 instruments essentially according to methods given by Beutler[12].

Chromatographic procedures

An automated system was devised to apply simultaneously to eight mini-columns (containing 1.6 ml of gel) the samples, buffers and washing solutions and to collect appropriately the eight eluates. Full details concerning the construction of the automatic set-up can be obtained from the authors on request.

Zonal procedure. Starting material was prepared as follows. Human erythrocytes were first washed in saline, then the cells were lysed by dilution (three times) in distilled water and by freezing and thawing. The resulting haemolysate was diluted 11-fold with initial buffer (see below) and centrifuged.

The buffers used for screening were those described by Scopes[9]. Buffer A contained 10 m*M* potassium hydroxide, 2 m*M* magnesium chloride and 30 m*M* sodium chloride and was adjusted to pH 6.5 with morpholinoethanesulphonate. Buffer B had the same composition as A except that it contained 2 *M* sodium chloride and no magnesium chloride.

The flow-rate was 3 ml/h. Before deposition of starting material, the columns were rinsed for 2 h with 6 *M* urea and then for 2 h with buffer B. They were then equilibrated with buffer A.

The zonal procedure itself was essentially that described by Scopes, *i.e.*, 3 ml of starting material were deposited on to the columns (1.6-ml bed volume), which were subsequently rinsed with 6 ml of buffer A, then they were automatically developed with 6 ml of, successively, buffer B, 6 *M* urea and water containing 0.02% (w/v) of sodium azide. Columns filled with immobilized dyes and fitted with their piston-like upper part could be stored as such and were ready for subsequent use.

The percentages of total protein and of enzymatic activity retained by the dyes were evaluated by assays of two fractions of 3 ml each, collected from the beginning of sample deposition.

Frontal procedure. The same instrument, buffers and flow-rates used in the zonal method were also employed for the frontal procedure. However, a large volume of haemolysate was deposited on to the columns, and the haemoglobin concentration and enzymatic activities were measured in 0.5-ml fractions.

Determination of bleed volumes. The bleed volume was considered to correspond to an eluent volume for which 50% of the concentration of haemoglobin or enzyme present in the haemolysate was found in the column effluent.

RESULTS AND DISCUSSION

Zonal procedure

The results are presented in Fig. 1. Such results can be appreciated in the light of Scopes' guidelines: "The object is to find two columns, one which binds as much as protein as possible without binding the enzyme, the other which just holds on to the enzyme, and as little protein as possible, so that it may be eluted with only a slight change to the buffer conditions"[9]. Dye Y14 can be seen as a likely candidate for a negative column. It has to be stressed that stripping the starting material of haemoglobin with this dye would need a column with a large volume.

The choice of a positive column is not as easy as it might seem. For instance, it could be possible to choose A1 or O2, which appeared to bind all of the 6PGD activity and nearly the same amount of haemoglobin. Nevertheless, before making a definite choice, it would be interesting to know which of the two immobilized dye columns has a greater capacity for the enzyme. Moreover, C6 and B9 seemed to behave in the same fashion with regard to 6PGD and haemoglobin (enzyme and haemoglobin are both totally retained). However, this experiment did not give any information on the relative affinities of the dyes for 6PGD and for haemoglobin. An immobililized dye with a greater affinity for 6PGD than for haemoglobin could be used in the displacement mode; we could plan to load the column with starting material until the

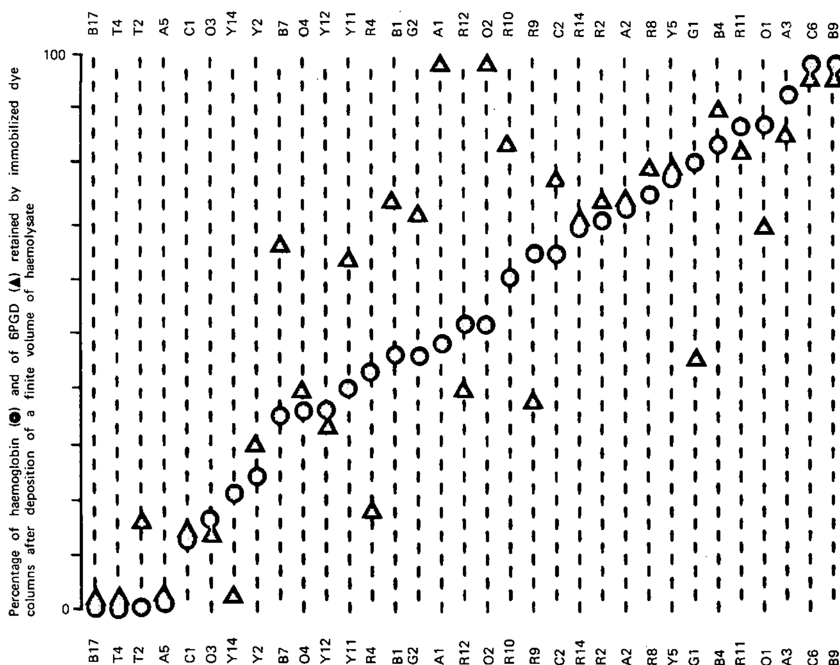

Fig. 1. Experimental results obtained with the automatic screening machine using the zonal method: small finite amounts of starting material were deposited on the columns (see Experimental). Abscissa: abbreviations as in Table I. Ordinate: percentages of whole protein (practically haemoglobin) retained from the starting material (O) and of enzymatic activity (6PGD; ▲).

enzyme begins to displace haemoglobin from the column. In this instance it would also be interesting to evaluate the absolute capacity of the dye columns for the enzyme because a greater capacity would mean a column of smaller volume. Displacement chromatography with a dye that has a lower affinity for 6PGD than for haemoglonin could also be used for 6PGD purification. However, a high concentration of haemoglobin in the starting material would obviously require a large column in order to treat a reasonable volume of haemolysate.

Hence, after zonal screening of haemolysate, for a few immobilized dyes little may be known about the relative affinities of the dyes for the enzyme and for haemoglobin or about the capacity of the columns for the enzyme, although such information would be useful to direct the sound choice of a dye for enzyme purification. This information can easily be obtained by frontal analysis[13].

Frontal procedure

Results for haemoglobin concentration and of enzymatic activity assays for two selected dyes are shown in Fig. 2. The data obtained with B18 show that haemoglobin is able to displace the studied enzymes from the column; the enzymatic activities in the

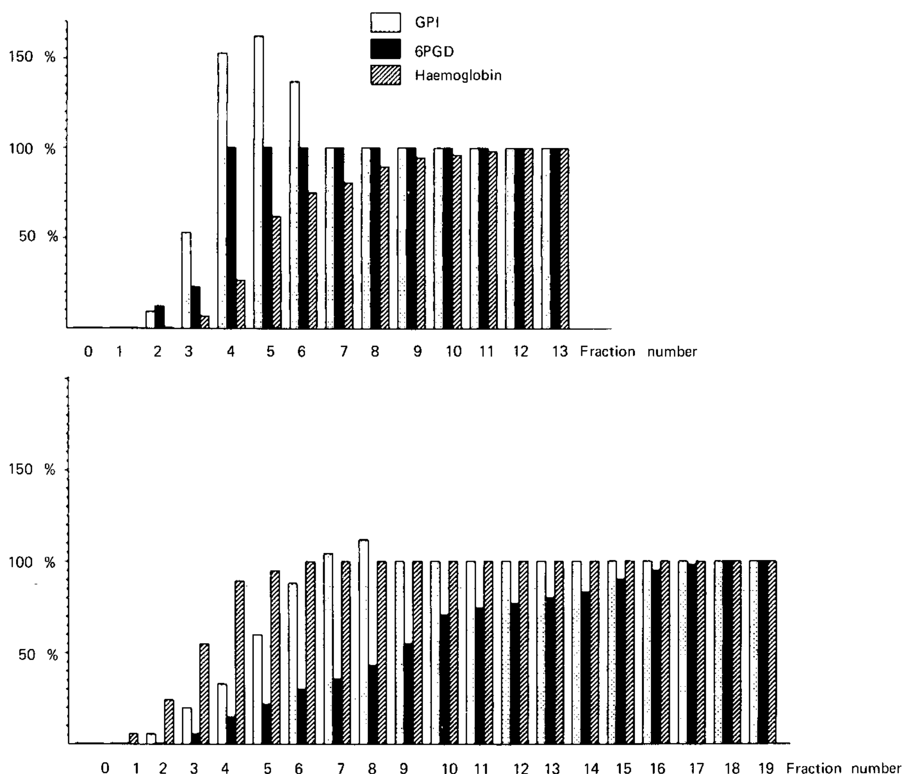

Fig. 2. Haemoglobin concentrations and enzymatic activities measured in 0.5-ml collected fractions with two different immobilized dyes. The value of 100% on the ordinate corresponds to the value measured in the original starting material. Top, results obtained with B18; bottom results obtained with A3.

collected fractions preceding haemoglobin breakthrough rise to higher levels than in haemolysate. With dye A3, 6PGD leaves the column later than haemoglobin, hence the enzyme probably displaces haemoglobin from the column. The low concentration of 6PGD compared with that of haemoglobin explains why the haemoglobin level does not rise to an appreciably higher level before breakthrough of the enzyme.

The results of the measurements of the bleed volumes of haemoglobin and of the two studied enzymes with 79 different dyes are shown in Fig. 3.

Comparison of the results obtained with dyes O2 and A1 demonstrate the interest in using frontal analysis for studies of dye–protein interactions in haemolysate; they could have been considered as equivalent on the basis of the results of zonal analysis because both dyes retained all 6PGD activity present in the 3-ml sample of haemolysate and nearly the same amount of haemoglobin (Fig. 1). The results of frontal analysis (Fig. 3) show, however, that the O2 column has a much greater capacity than the A1 column.

We should point out that the criteria for the choice of a positive column and the strategy for its use that we propose are not identical with those defined by Scopes. Thus, we no longer search for a dye that "just holds on to the enzyme" and use it for elution chromatography[9]. Instead, we look for an immobilized dye with a higher affinity for the protein of interest than for the unwanted protein (haemoglobin) and deposit the starting material in such a way that permits the displacement of the unwanted protein as much as possible by the protein of interest. Obviously bleed volume measurements are not rigorous measurements of the affinity of the proteins studied, but nevertheless it can be inferred from theoretical studies on frontal affinity chromatography[13] that among a mixture of proteins it is the one with the greatest affinity for the immobilized ligand that will have the greatest bleed volume.

The precise ranking of different dyes on the abscissa of Fig. 3 is probably strongly dependent on the actual amount of each dye immobilized on agarose. Immobilization of B16, O1, B4 and C3 at different concentrations showed that an increasing dye loading did not change the order of elution of the proteins, although the protein capacities increased.

It is worth noting that some immobilized dyes columns exhibited a very high capacity for proteins; for instance, 105 mg of haemoglobin were retained by a 1.6-ml column of dye B4 III before that any haemoglobin had left the column, and O1 III retained 57 mg of haemoglobin (the two dyes have the same bleed volume but the breakthrough curve of O1 III is less steep than that of B4 III).

Inclusion of ligands in the haemolysate buffer was used to check rapidly if affinity elution would be effective in desorbing the enzymes from the dye columns (as shown in Table II, the bleed volumes were in fact lower in every instance although the effect was more dramatic with dyes with which a large bleed volume had been found).

NADP was also seen in some instances to be able to lower significantly the bleed volume for haemoglobin, even though haemoglobin it is not known as an NADP-binding protein.

The results of frontal screening did not allow us to devise a purification procedure for GPI using a dye column in the first step (the bleed volumes for GPI and haemoglobin, at least in the buffer system used, were far too close). However, a simple purification procedure for 6PGD derived from the results described in this paper is presented in the following paper[14].

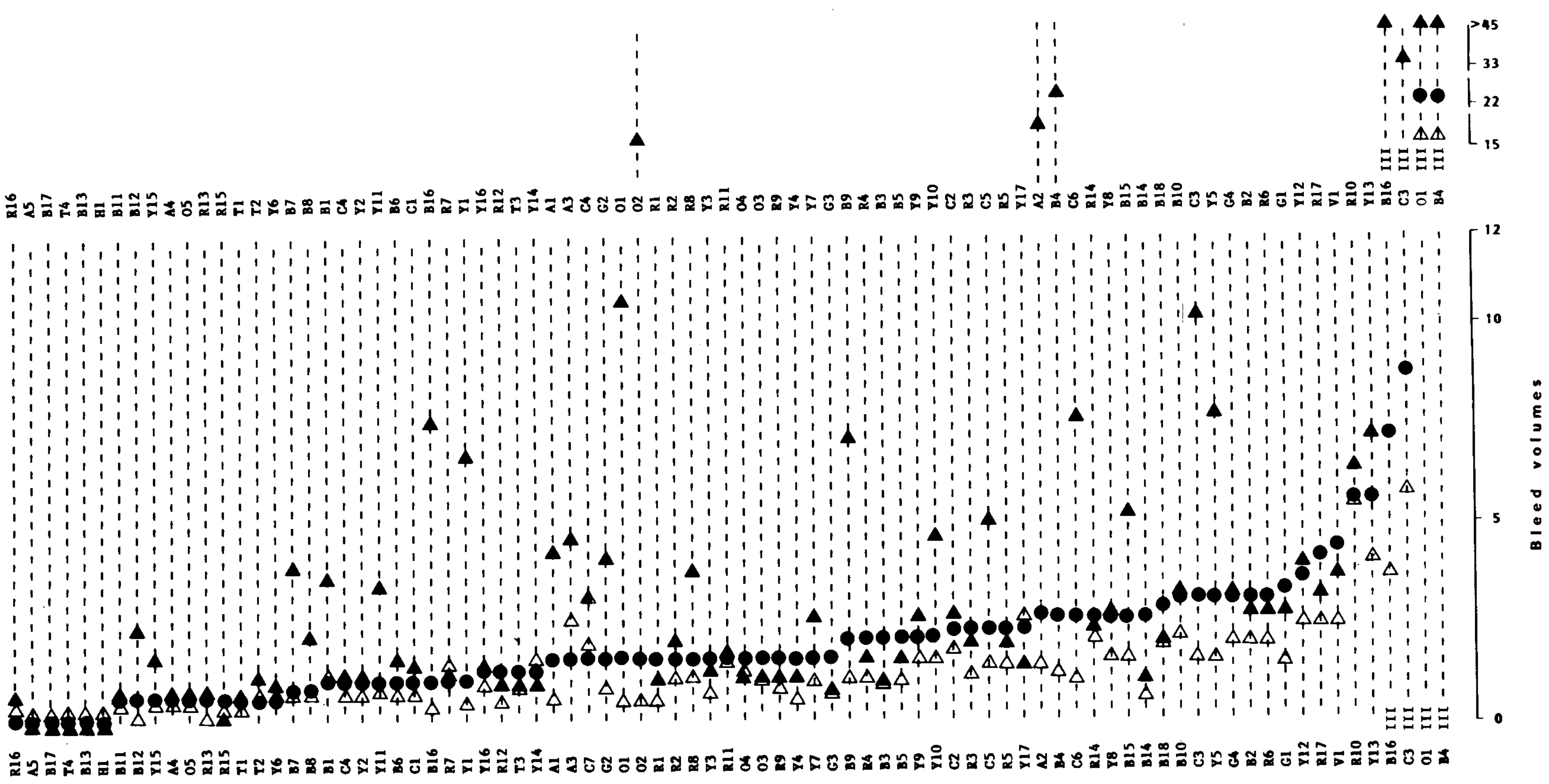

Fig. 3. Bleed volumes obtained with 79 immobilized dyes for haemoglobin (●), 6PGD (▲) and GPI (△). Bleed volumes were measured with 1.6-ml columns. Volumes (expressed in ml) are on the ordinate: for graphical representation, experimental values were reduced by the bleed volume of an unsubstituted agarose column. Abscissa: abbreviations of dyes as in Table I. The different dyes are arbitrarily ranked in order of their increasing affinity for haemoglobin. The dyes B16, C3, O1 and B4 were immobilized on agarose at two different levels of substitution (dye incorporations were respectively 1.5, 1.3, 1.0 and 0.8 mg per ml of gel for the lower level of substitution and 6, 2.6, 7.5 and 3.6 mg per ml of gel for the higher level of substitution; these dyes are followed by the symbol III).

TABLE II

BLEED VOLUMES MEASURED WITH A SMALL NUMBER OF DYES IN THE ABSENCE AND PRESENCE OF NADP

Dye	*6PGD*		*Haemoglobin*	
	Without NADP	*With NADP*	*Without NADP*	*With NADP*
A2	18	0.5	2.5	2.5
B4	27	0.5	2.5	1.5
Y8	2.5	1	2.5	2.5
B14	1	0.5	2.5	2.5
C3	10	1	3	2.5
R10	6.5	2	5.5	3.5
Y13	7	1.5	5.5	3.5
B16 III	>45	1	7	2.5

In conclusion, we should stress that the modifications that we have described to the screening procedures proposed previously by Scopes[9] and Hey and Dean[6] are of the greatest use in instances where haemolysate (*i.e.*, a quasi-pure solution of haemoglobin) is the starting material of the purification procedure. Choosing an immobilized dye with a greater capacity for the enzyme means that column volume needed to treat a given volume of haemolysate will be minimized. However, there are also some drawbacks to such a choice, as has been stressed by Scopes[15], *i.e.*, one could experience some difficulties in trying to elute a protein from a too tight binding immobilized dye.

Obviously these modifications could also be applied in more common situations such as the purification of proteins from bacterial extracts. However, in such instances, zonal screening has proved to be efficient for the selection of both a "negative" and a "positive" column to be used in tandem. Zonal screening is obviously much more easy to perform as a large number of enzymatic assays are needed in order to complete a frontal screening procedure, whereas zonal screening needs only one assay to evaluate the amount of retained enzyme. The choice of frontal analysis would therefore seem justified only if one would like to select a dye for a positive column with a high affinity for the protein of interest with the inherent drawback that possibly this stronger binding column would no longer allow the protein of interest to be "eluted with only a slight change to the buffer conditions"[9]. It is worth noting that in one procedure described for the purification of proteins of bacterial origin, displacement of one protein from a dye column by another of greater affinity was used advantageously[16].

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PURIFICATION OF HUMAN 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM HUMAN HAEMOLYSATE WITH CHROMATOGRAPHY ON AN IMMOBILIZED DYE AS THE ESSENTIAL STEP AND USE OF AUTOMATION

SIMULTANEOUS PURIFICATION OF LACTATE DEHYDROGENASE

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SUMMARY

The screening procedure described in the preceding paper allowed a practical purification procedure to be devised that was automated for human 6-phosphogluconate dehydrogenase. The purification needed only two chromatographic steps, first on immobilized Procion Blue HE-GN and then on Phenyl-Sepharose. This technique also gave purified lactate dehydrogenase. Both enzymes showed single bands in SDS polyacrylamide gel electrophoresis.

INTRODUCTION

The results of the screening procedure described in the preceding paper prompted us to devise a complete purification procedure for human 6-phosphogluconate dehydrogenase (6PGD) from human haemolysate using an immobilized dye, Procion Blue HE-GN. The first trial on a large scale showed that 6PGD could be obtained in a fairly purified state by NADP elution. Only one major contaminant could be seen by gel electrophoresis, and this 36 kDa contaminant was identified as lactate dehydrogenase (LDH). Separation of the two enzymes was easily achieved by differential elution procedures from the immobilized dye column. Diverse procedures for the specific elution of the enzymes from the dye columns were tried and the results are presented in this paper.

EXPERIMENTAL

The dye, chemicals and most of the methods employed were the same to those described in the preceeding paper. Nucleotides were obtained from Boehringer (Mannheim, F.R.G.) or Sigma (Saint Louis, MO, U.S.A.).

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The procedure used for dye immobilization was also essentially the same. However, the results described in this paper were obtained with dye immobilized on Sepharose CL 4B from Pharmacia (Uppsala, Sweden) and not on Ultrogel A4 (IBF, Villeneuve la Garenne, France). The dye incorporation was 6.2 mg/ml of wet Sepharose [the dye incorporated in Ultrogel A4–B16 III (see the preceding paper) was 6.0 mg/ml]. Bleed volume measurements (made with 1.6-ml columns, see the preceeding paper) gave values of 90 ml for Procion Blue HE-GN–Ultrogel and of 155 ml for Procion Blue HE-GN–Sepharose. The better performance of the latter gel is reminiscent of similar results obtained by Dean *et al.*[1], who studied the effect of the matrix used for immobilization on the performance of dye columns.

The buffers used for the purification procedure were as follows:

(A) 10 m*M* potassium hydroxide adjusted to pH 6.5 with solid morpholinoethanesulphonate containing 30 m*M* sodium chloride and 2 m*M* magnesium chloride[2]; (B) the same as buffer A but adjusted to pH 7.5; (C) the same as buffer A but the sodium chloride concentration was 2 *M* and magnesium chloride was absent; (D) 6 *M* urea; (E) the same as buffer A but containing ammonium sulphate at 20% saturation; (F) the same as buffer A but containing ammonium sulphate at 80% saturation.

The nucleotide solution for the elution of LDH from the Procion Blue HE-GN column was 5 m*M* AMP dissolved in buffer A, and that for the elution of 6PGD from the Procion Blue HE-GN column was NADP dissolved in digestion buffer hydrolysed completely to NMN and 2′5′-ADP by snake venom phosphodiesterase (see below for details).

Lactate dehydrogenase (LDH) was assayed according to Beutler[3].

The isozymic pattern of LDH was determined by electrophoresis (Helena Labs., Beaumont, TX, U.S.A.).

The protein (haemoglobin) content in the starting material was determined according to Drabkin[4]. Other protein assays were performed according to the Bradford procedure[5] using reagents from Bio-Rad Labs. (Richmond, CA, U.S.A.). However, the protein contents of totally purified 6PGD pools were evaluated from optical density measurement at 280 nm using an $A_{1\,\mathrm{cm}}^{1\%}$ value of 10 (ref. 6).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was conducted according to Laemmli[7]. Molecular weight markers were obtained from Pharmacia.

Hydrolysis of NADP by phosphodiesterase from *Crotalus durissus* (Boehringer) was carried out in a buffer of the same composition as buffer A but containing 20 m*M* Tris and adjusted to pH 8. Completion of digestion was checked by chromatography at 37°C on an octadecylsilica column (25 × 0.46 cm I.D.) (Merck, Darmstadt, F.R.G.). The mobile phase[8] was 75 m*M* dipotassium hydrogenphosphate buffer adjusted to pH 4.12 with phosphoric acid, containing 1.4 m*M* undecanoic acid (Aldrich, Milwaukee, WI, U.S.A.) and 12% (v/v) methanol. This procedure gives a clear separation of NADP, NMN and 2′5′-ADP.

Several experiments intended to study the conditions for elution by nucleotides were conducted on a small scale on columns of 1.5-cm I.D. filled to a height of 2.7 cm. The volume of deposited haemolysate (prepared as mentioned below) was 60 ml, the flow-rate was 12 ml/h and volume of collected fractions was 3 ml.

After deposition, the columns were rinsed for 2.5 h with buffer B, then for 2 h with buffer A. The columns were then developed with 15 ml of the various solutions

of nucleotides in buffer A (readjustment of pH was sometimes necessary) and further rinsed with buffer A.

RESULTS AND DISCUSSION

Elution of enzymes by nucleotides

A large number of enzymes were purified by taking advantage of the fact that inclusion of the enzyme cofactor elutes the protein from the immobilized dye column. Nevertheless, this fact obviously does not prove that the dye binding site is identical with the substrate binding site. In at least one instance (with Cibacron Blue and alcohol dehydrogenase[9]) it was definitely proven by careful crystallographic studies that the dye did overlap with the nucleotide binding site, but was linked to the protein by other residues not involved in nucleotide enzyme interactions. If the enzyme does bind to the dye by just one part of the nucleotide binding site, one can expect that a fragment of the nucleotide will be effective in promoting enzyme desorption. Moreover, one can speculate that a combination of fragments may be effective. The impetus to search for eluents other than the coenzyme itself can also be based on economic reasons: fragments of the nucleotide can be cheaper than the nucleotide itself.

TABLE I

RECOVERY OF 6PGD AND LDH FROM IMMOBILIZED PROCION BLUE HE-GN

Numerical values given are percentages of enzymatic activities deposited on the columns which were found in the eluate after switching to nucleotide containing developer.

Eluent	*Concentration (mM)*	*6PGD (%)*	*LDH (%)*
NADP	0.5	0	n.d.*
	1	23	5
	2	60	n.d.
	5	80	23
2′(3′),5′-ADP	0.5	0	0
	1	43	n.d.
	2	60	n.d.
	10	75	0
2′,5′-ADP	1	65	n.d.
3′,5′-ADP	1	0	n.d.
NAD	5	0	67
AMP	5	0	72
NMN	5	0	0
NMN + 2′(3′),5′-ADP	0.5 + 1	56.5	n.d.
	1 + 2	95	0
NMN + AMP	1 + 2	0	16
	2.5 + 2.5	n.d.	32
	5 + 5	n.d.	67
NADP + 2′(3′),5′-ADP	1 + 1	71	n.d.
	1 + 2	100	0.5
NAD + 2′(3′),5′-ADP	1 + 2	74	n.d.
NADP hydrolysed by phosphodiesterase I (snake venom)	2	96	0

* Not determined.

The aim of the studies described below was to find rapidly an efficient and cheap way of eluting the enzymes from the immobilized dye column. It is understood that results obtained by the empirical method used (see Experimental) are only qualitative and mostly interesting from a practical point of view. In no way do we claim that they could give a true quantitative description of the interactions of the protein with the dye in the presence of interacting substances.

The results of trials with several eluting agents for 6PGD and LDH are given in Table I.

NADP was observed to be able to elute 6PGD. The mixed isomer 2′(3′),5′-ADP was also able to elute the enzyme efficiently. The active species in the isomer mixture was shown to be the 2′,5′-ADP isomer. The phosphate grafted in the 2′-position of the ribose ring seems to be of the greatest importance for competition with the dye because neither NAD, AMP nor 3′,5′-ADP is able to elute 6PGD. Nicotinamide mononucleotide has no eluting power for 6PGD but a mixture of 1 m*M* NMN and 2 m*M* 2′(3′),5′-ADP is clearly more efficient than 1 m*M* NADP or 2 m*M* 2′(3′),5′-ADP used alone to elute the enzyme from the dye column. This suggests on the one hand that NMN at least interacts with the enzyme and on the other that it assists the other half of the cofactor molecule to elute the enzyme from the column. From these results one can guess that it would be interesting to check analogues of NADP possessing a longer or possibly more flexible link between 2′(3′),5′-ADP and NMN parts with regards to their ability to elute the enzyme from the dye column.

A mixture with low concentrations of 2′(3′),5′-ADP and NADP is extremely powerful for elution of the enzyme from the column. Yet more striking is the fact that a mixture of 2′(3′),5′-ADP and NAD is able to elute efficiently 6PGD from the column. Therefore, one can guess that in this instance NAD is an analogue of NMN and that the other part of the molecule (*i.e.*, the "AMP part") does not play any role in the elution of the enzyme from the immobilized dye.

The fact that a mixture of NMN and 2′(3′),5′-ADP was very efficient in eluting 6PGD from the column gave the opportunity to prepare easily a (relatively) inexpensive eluent for the enzyme from the immobilized dye column: NADP was dissolved to a final concentration of 4 m*M* in digestion buffer and submitted to the action of phosphodiesterase from snake venom (final concentration 0.26 μg/ml). Digestion was complete after incubation for 18 h at 37°C. The mixture was then diluted once with buffer A and adjusted to pH 6.5 and used directly to elute the enzyme. The results in Table I demonstrate that, as expected, hydrolysed NADP is more effective than unhydrolysed NADP at the same concentration.

The results obtained with LDH will be discussed in the same way as those described previously for 6PGD. However, first, one should note that a significant part of the LDH activity was not retained by the dye, thus explaining that yields evaluated as mentioned in Table I are never quantitative, even with a powerful eluent such as NAD or AMP. The isozymic patterns of LDH both retained and not retained by the column were checked by cellulose acetate electrophoresis. Haemolysate showed three bands corresponding to the A2B2, A1B3 and B4 isozymes; only the last two were seen in the breakthrough volume. LDH eluted from the column consisted of a small proportion of B4 but mostly A1B3 and A2B2, with the latter isozyme eluted last in the activity peak. This finding suggests that Procion Blue HE-GN does not have the same affinity for the two different constitutive subunits of LDH. A similar observation was

made with Blue dextran agarose[19] and also with Cibacron Blue linked to silica, which was used to separate rapidly B4 and A4 isoenzymes[11].

NAD and AMP can elute LDH retained by the dye; NMN is ineffective. NADP can elute a significant portion of retained LDH activity, showing that grafting a phosphate group on the 2′-position of the ribose ring does not impair totally the power of the other part of the molecule to compete with the dye [however, 2′(3′),5′-ADP is ineffective]. It is interesting that at "equivalent" concentrations, mixtures of AMP and NMN are less powerful than NAD in eluting the enzyme, which is contrary to the result obtained with 6PGD; 2 m*M* hydrolysed NADP could not elute any LDH activity.

AMP is preferred to NAD for elution of LDH because of cost. Hydrolysed NADP is the cheapest eluent for 6PGD.

Large-scale elution of enzymes by a salt gradient from the immobilized dye column

Chromatography was performed on a column of 5 cm I.D. filled with immobilized dye to a bed height of 5 cm; the flow-rate was 150 ml/h. The deposited haemolysate volume was 1400 ml. After sample deposition the column was rinsed with 1000 ml of buffer B and then developed with a linear gradient obtained by automated mixing (with a Mixograd apparatus; Gilson, Villiers Le Bel, France) of 450 ml of initial buffer (buffer A) and 450 ml of final buffer (buffer C).

This linear gradient could not separate LDH and 6PGD significantly. SDS-PAGE of aliquots of the collected fractions showed the presence of several protein bands. This result is in sharp contrast to the satisfactory results obtained with nucleotide elution in a separate pilot experiment on the same scale. We therefore used the latter procedure for the elution of enzymes from the immobilized dye column in the routine automated purification procedure described below.

Automated complete purification of human 6PGD and purification of LDH from the same starting material

The rationale for using automation for the relatively large-scale production of enzymes in the research laboratory has been described[6]. Briefly, with the equipment available in a standard research laboratory, it is easier to repeat purifications starting from a small amount of starting material (*i.e.*, around 1 l) than to purchase specially dedicated columns or centrifuges necessary for the processing of large volumes of starting material. The drawback of such an approach is obviously the time needed to repeat the purifications. The solution for us was, as before, to automate the purification procedure.

The instrument, which is operated in a cold room, is under the control of a simple programmer made by associating electromechanical timers, liquid and voltage level sensors and electronics incorporating a diode matrix. The working principle of the machine has already been described[12] and a detailed description is beyond the scope of this paper. However, Fig. 1, showing a flow chart of the machine, and Fig. 2, showing the time schedule of activation of its elements and a description of the purification procedure itself given below, will allow the reader to understand how the machine works. Full details of the construction of the automatic machine and its simple programmers can be obtained from the authors on request.

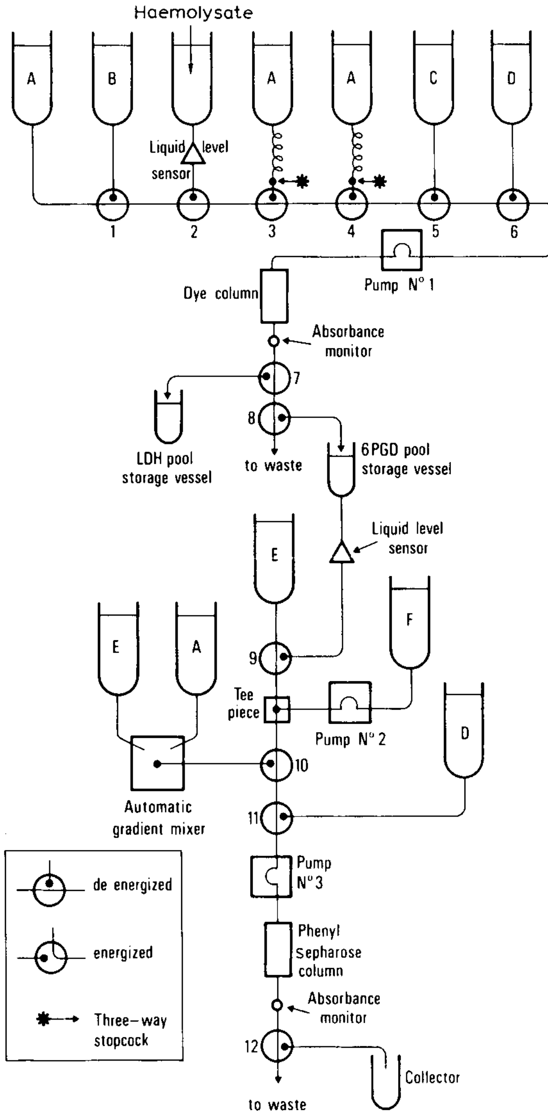

Fig. 1. Diagram of the automatic chromatographic system. The valves and pumps are numbered as in Fig. 2. A–F are buffers.

Overall procedure

Preparation of haemolysate. We use routinely as starting material either bags of outdated packed red cells or blood of patients undergoing therapeutic phlebotomy for primary polycythaemia (in this instance red cells are collected by centrifugation and washed once in phosphate-buffered saline). Red cells are lysed by 3-fold dilution with water and freezing and thawing. The lysate is then diluted twice with buffer A and cleared from membrane remnants by centrifugation at 10 000 *g* for 45 min.

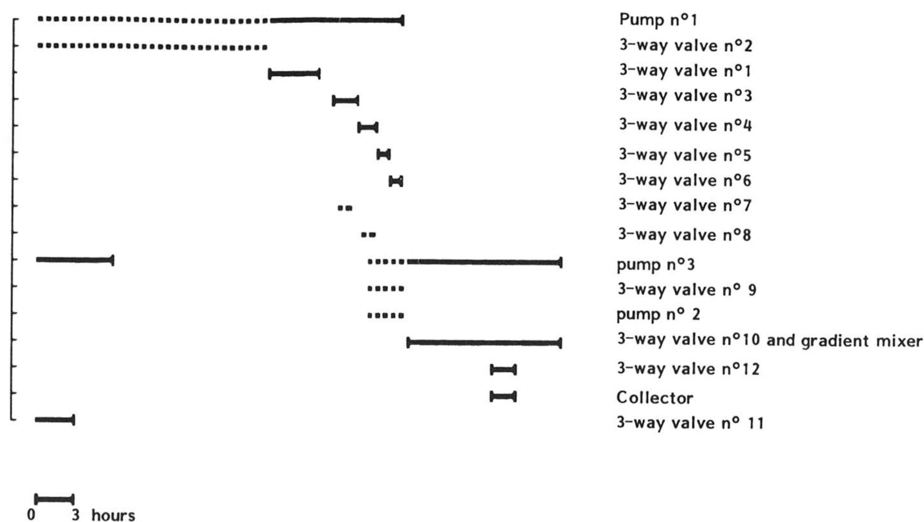

Fig. 2. Diagram of the programme routinely used for the automatic purification of 6PGD. The horizontal lines show when power is brought to the valves, pumps, gradient mixer and collector. Most of the programming relies on time counting (solid lines) but the end of starting material deposition and collection of LDH and 6PGD pools relies on sensing of liquid levels and voltage levels, respectively (dotted lines).

Chromatography on immobilized Procion Blue HE-GN. Centrifuged haemolysate (the volume is usually *ca.* 1400 ml) is poured into the appropriate reservoir of the machine as shown in Fig. 1 and the instrument is turned on. The haemolysate will therefore flow to the immobilized dye column (I.D. 5 cm, height 5 cm, flow-rate 150 ml/h).

When the haemolysate reservoir becomes empty the column is rinsed for 1 h with buffer B, then the flow-rate is automatically increased to 300 ml/h and the dye column is rinsed for a further 3 h with buffer B (the higher pH of buffer B allows some unwanted proteins to be eluted). The column is thereafter rinsed for 1 h with buffer A, then 300 ml of 5 m*M* AMP dissolved in buffer A (contained in a storage loop fixed under a buffer A reservoir, see Fig. 1) are pumped on to the column. When AMP begins to leave the column (LDH being eluted at the same time), the effluent from the column is adequately collected in the LDH pool storage vessel . The column is then developed with buffer A for 1 h. The column is thereafter developed with 300 ml of 2 m*M* hydrolysed NADP. The eluate is diverted to the 6PGD pool storage vessel. The immobilized dye column will then be rinsed with buffer C and D and subsequently re-equilibrated with buffer A in order to be ready for the next use.

Phenyl-Sepharose chromatography for 6PGD purification. The 6PGD-containing pool is automatically mixed with buffer F in such a way as to make it 20% saturated in ammonium sulphate. It is deposited on to the Phenyl-Sepharose column (I.D. 2.2 cm, height 26 cm) at 72 ml/h. The column is then rinsed for 3 h with buffer E and developed with a linear gradient between buffer E and buffer A for 6 h. The effluent from the Phenyl-Sepharose column is adequately collected in fractions. The Phenyl-Sepharose column will then be rinsed by buffer D and re-equilibrated with buffer

TABLE II

TYPICAL PURIFICATION OF HUMAN ERYTHROCYTIC 6PGD AND LDH FROM A GIVEN STARTING MATERIAL

On a routine basis specific activities are checked in the starting material and final products only.

Enzyme	*Sample or collected peak*	*Applied or collected volume (ml)*	*Activity (IU/ml)*	*Yield (%)*	*Protein concentration (mg/ml)*	*Specific activity (IU/mg)*	*Purification factor*
6PGD	Haemolysate	1400	0.28	100	63	0.0045	1
	Procion Blue HE-GN	235	1.57	94	0.080	19.6	4400
	Phenyl-Sepharose	115	2.31	68	0.092	25	5600
LDH	Haemolysate	1400	3.0	100	63	0.047	1
	Procion Blue HE-GN	400	7.25	69	0.036	201.4	4285
	Phenyl-Sepharose	220	10.6	55.5	0.026	407	8659

E during the early stages of the next automatic purification cycle so as to be ready for the next use.

Pure 6PGD is eluted from the Phenyl-Sepharose column as a symmetrical optical density peak after 3.5 h of the beginning of the linear gradient between buffers E and A.

Quantitative data on one typical purification procedure are given in Table II. The purity of the enzyme pool is routinely checked by SDS-PAGE. Fig. 3 shows the results of such an electrophoresis. This photograph and also Table II demonstrate the high efficiency of the immobilized dye chromatography step. The specific activity of 6PGD routinely obtained at the end of the purification procedure is 25 IU/mg, which corresponds to a 5600-fold purification. The overall yield of the purification procedure, which was repeated many times, usually lies between 60 and 70%. These

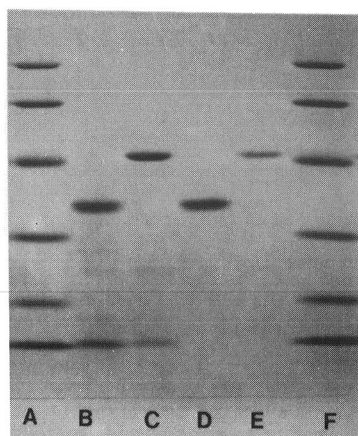

Fig. 3. Electrophoresis (SDS-PAGE) of the products. Lanes A and F, molecular weight markers (from top to bottom 94, 67, 43, 30, 20 and 14.4 kDa); lanes B and C, aliquots of the LDH and 6PGD pools, respectively, obtained after the immobilized Procion Blue. HE-GN chromatography step; lanes D and E, aliquots of purified LDH and 6PGD, respectively, obtained after Phenyl-Sepharose chromatography.

results seem as good as (with regard to specific activity) or better than (with regard to yield) than those produced by the technique formerly used[6], based on a much more expensive affinity chromatography support (2′,5′-ADP–agarose). If one is not interested in simultaneous LDH purification, it is possible to elute 6PGD directly from the Procion Blue HE-GN column directly with hydrolysed NADP, without prior use of AMP. It was checked also on a large scale that no LDH was eluted by hydrolysed NADP, the 6PGD obtained at the end of the purification procedure is of same purity as that produced by the complete procedure.

Phenyl-Sepharose chromatography for LDH purification. The dimensions of the Phenyl-Sepharose column used for LDH purification are 3.2 cm I.D. and height 23 cm. Phenyl-Sepharose chromatography is conducted manually. Solid ammonium sulphate is added up to 10% saturation to the LDH pool obtained in the Procion Blue HE-GN chromatographic step. The LDH pool is then deposited at a flow-rate of 110 ml/h on to the column equilibrated in buffer E containing ammonium sulphate at 10% saturation. After completion of sample deposition, the column is developed with a non-linear gradient obtained by adequately mixing in an automatic gradient former (Gilson) initial buffer and buffer E. LDH is obtained in pure form (see Fig. 3). Quantitative data on one typical purification procedure are given in Table II.

General comments

It has already been described by others that it was possible to elute enzymes from dye columns with substances not known to interact with them (for instance, 2,3-diphosphoglycerate can elute glucose phosphate isomerase from a dye column as shown by Prehu *et al.*[13], who also gave several other examples of the elution of enzymes with non-natural ligands). It is tempting to speculate that every substance which in some way mimics a dye (*i.e.*, which associates a high density of charged groups with hydrophobic parts) has the potential to elute a protein from a dye column. It has even been demonstrated that metallic complexes could elute with some specificity enzymes from dye columns[14]. Hence we can hypothesize that to find for a given protein a more or less specific eluent that is readily available at a low price will be mostly a matter of systematic trial.

It has to be stressed, however, that another approach does exist to reducing the costs of affinity elution. Scopes[15] has demonstrated the usefulness of associating non-specific elution modes with affinity elution: a specific eluent has to be applied to the column only when by non-specific means (*e.g.*, by increasing the ionic strength or pH and/or possibly by using a buffer with no divalent cation added) the protein of interest "is just beginning to move down the column"; in this way it is possible to use low concentrations of specific eluents[16,17]. Obviously it could be interesting to combine both approaches.

Our work on erythrocytic 6PGD and LDH purification is in accordance with the now generally accepted notion that immobilized dye columns can compete in terms of efficiency with more expensive affinity chromatography matrices. Immobilized dye chromatography media are often used in the later steps of purification protocols for obtaining enzymes from haemolysate[18–20]. Our results confirm work by others[21,22] demonstrating that immobilized dye chromatography can also be used as the first step in purification from crude haemolysate. Screening procedures such as those described in the preceding paper can be very useful in choosing a dye well adapted to a specific purification problem.

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THE PEPTIDE TOXIN OF THE CYANOBACTERIUM *MICROCYSTIS AERUGINOSA* PCC 7941

ISOLATION AND ANALYSIS BY NUCLEAR MAGNETIC RESONANCE AND FAST ATOM BOMBARDMENT MASS SPECTROSCOPY

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SUMMARY

Toxin was obtained from the cyanobacterium *Microcystis aeruginosa* PCC 7941 by extracting freeze-dried cells with water-saturated, acidified *n*-butanol, diethyl ether–water distribution, reversed-phase thin-layer chromatography and silica high-performance liquid chromatography (HPLC). Two toxic peptide fractions resulted from HPLC. One of these fractions was analyzed by UV and NMR spectroscopy, amino acid analysis and fast atom bombardment mass spectroscopy. The following amino acids were identified: β-methyl-Asp, Thr, Glu, Ala, Val, Leu, Phe, Arg, N-methyldehydro-Ala and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. Yet the mass spectroscopic data showed that the fraction was still composed of several, most likely cyclic peptides that did not stain with ninhydrin.

INTRODUCTION

Microcystis aeruginosa, an ubiquitous, waterbloom-forming cyanobacterium, is known to produce hepatotoxins. Death of livestock and game due to these toxins has been reported from Australia[1,2], South Africa[3–6], North America[7–12], Europe[13–18] and Asia[19,20]. The lethal dose of the toxins to mice is reportedly of the order of 70 μg/kg or less[1].

Pathological findings include swollen, blood-engorged liver with haemorrhargic necrosis, oedematous lung and loss of architecture of the hepathocystes. Except for lung congestion, other organs seem not to be significantly affected. Under lethal doses, mice usually die within 30 min to 4 h, and death is preceded by pallor and prostation with terminal episodes of unprovoked leaping and twitching. Sublethal doses cause

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respective chronic damage. Gastroenteritis and liver damage have been observed in humans as well[1,12,21–25].

Attempts to identify the structure of the toxins from *Microcystis aeruginosa* had been rather confusing[1,3,7,11,26–32] until 1984, when Botes *et al.*[27] published a cyclic heptapeptide structure for a toxin isolated from a laboratory clone of *Microcystis aeruginosa* that originated from a natural bloom in Witbank Dam, South Africa. This toxin consists of D-Ala–L-X–*erythro*-β-methyl-D-iso-Asp–L-Y–3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,-dienoic acid (Adda)–D-iso-Glu–N-methyldehydro-Ala (X and Y represent variable L-amino acids) giving a total molecular mass of 909. The authors assumed some variation with respect to the two L-amino acids in the toxins of other *Microcystis* sp. isolates.

This report describes the isolation and analysis of the toxin from *Microcystis aeruginosa* strain PCC 7941 (syn. NRC-1[11]). On comparison with the toxins described by Botes *et al.*[27,28], the structural similarity with only minor modification becomes obvious. Furthermore, the presence of non-toxic peptides, having properties very similar to those of the toxin, will be pointed out.

EXPERIMENTAL

Materials and chemicals

All solvents and chemicals were of analytical grade. Uvasol grade methanol and chloroform were used when necessary. Ninhydrin spray and thin-layer chromatography (TLC) plates (RP_{18} F 254 S and cellulose) were obtained from Merck (Darmstadt, F.R.G.). β-Methyl-Asp was from Sigma (München, F.R.G.) analytical (25 cm × 0.8 cm) and preparative (25 cm × 3 cm) high-performance liquid chromatographic (HPLC) columns (Partisil-5) from Knaur (Bad Homburg, F.R.G.) and Sephadex G-15 and G-25 from (Pharmacia, Freiburg, F.R.G.). Sep-Pack C_{18} cartridges were from Waters (Eschborn, F.R.G.). The unialgal strain of *Microcystis aeruginosa* PCC 7941 (syn. NRC-1), originating from Little Rideae Lake, Ontario[11], was from the strain collection of the Institut für Biologie II, Mikrobiologie, Universität Freiburg. The cultures were cultivated in BG 11 medium[33] at 25°C in 1-l erlenmeyer flasks or in a 10-l fermentor. Male mice (10–11 weeks old, mean weight 33.3 ± 3 g) were kindly supplied by H. Mönig, Institut für Biophysik und Strahlenbiologie, Universität Freiburg. The mice received a normal laboratory diet from Altromin, Lage/Lippe, and acidified tap-water (pH 3) *ad libitum.*

Buffers

The following buffers were used: (A) 0.1 *M* sodium phosphate pH 8.0; (B) 27 m*M* Tris, 142 m*M* glycine pH 8.2–8.5; (C) 150 m*M* Tris–HCl pH 8.8.

Toxin isolation

Microcystis was harvested from cultures at the end of their exponential growth phase. Lyophilized cells (10–30 g) were extracted for 20 min at room temperature with water-saturated *n*-butanol (300 ml) containing 1% acetic acid. After centrifugation (1830 *g*, 15 min), the pellet was reextracted and the pooled supernatants were dried under reduced pressure (35°C). The residue was redissolved in water, the pH adjusted to 8 and lipids were extracted into diethyl ether by gentle mixing and subsequent

centrifugation (1830 *g*, 4°C, 5 min). The aqueous phase was separated from the non-toxic interlayer and the ether phase. It was dried under reduced pressure (35°C) and the residue was applied to silica gel RP_{18} thin-layer plates in methanol. The plates were developed in methanol–water–acetic acid (60:38:2, v/v/v), and the fluorescence-quenching band at R_F 0.2 was extracted into propan-2-ol. The extract was dried, the residue was redissolved in chloroform–methanol (7:3, v/v) and the solution was filtered through an HV 0.45 filter (Millipore, Eschborn, F.R.G.) prior to HPLC separation on Partisil-5.

Native polyacrylamide gel electrophoresis (PAGE)

Gradient PAGE was performed on slabs (1 mm × 100 mm × 100 mm, 11.5–18% acrylamide) at 15 mA in buffers B (electrode) and C (separation). The developed gels were cut into 2-mm sections, and the sections were extracted overnight with buffer A. Protein in the extracts was determined spectrophotometrically (mg protein per ml = 1.55 E_{280} − 0.76 E_{260}), and toxin by its maximum at 240 nm. The toxicity of the extracts was tested by the mouse bioassay.

HPLC

Separation on Partisil-5 by HPLC (Beckman or Waters) was accomplished by a linear gradient made up from 30 to 80% methanol–acetic acid (99:1, v/v) in chloroform–acetic acid (99:1, v/v) at a flow-rate of 2 ml/min (analytical column) or 10 ml/min (preparative column). The elution was monitored at 254 nm.

Spectrometry

Ultraviolet spectra were recorded on a Kontron UVIKON 810 photometer. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker WM 300 spectrometer (Max-Planck-Institut für Immunobiologie, Freiburg, F.R.G.), using tetramethylsilane (TMS) as an internal reference. Fast atom bombardment mass spectroscopy (FAB-MS) was performed on a Kratos MS 50 RF spectrometer equipped with a Kratos source (Gesellschaft für Biotechnologische Forschung, Braunschweig, F.R.G.). Glycerol was used as a matrix.

Amino acid analysis

Toxin (*ca.* 50 μg), corresponding to the HPLC fraction with t_R 16.45 min, was hydrolyzed in 6 *M* hydrochloric acid (0.5 ml) for 24 h at 105°C in sealed tubes, and the samples were subsequently subjected to amino acid analysis in a Biotronik LC 6001 amino acid analyzer with an automatic sample injector BT 7040. Elution of the amino acid derivatives was monitored at 570 and 440 nm (proline).

RESULTS

Toxin purification

The toxin present in *Microcystis aeruginosa* PCC 7941, which caused rapid death of mice in the bioassay, was extracted completely from the lyophilized cells by one extraction with acidified, water-saturated *n*-butanol, as revealed by control extractions of the extracted cells with aqueous buffers or acidified *n*-butanol. Furthermore, the

butanol extracts contained comparatively little protein. Purification of the toxins was therefore started routinely from these extracts.

Lipids were subsequently removed from the *n*-butanol extracts by a gentle diethyl ether–water (1:1, v/v) distribution. A clear, slightly yellow aqueous phase and a clear, dark green ether phase, separated by a solid orange interphase, resulted. The toxin, recovered in the aqueous phase (approximately 90% of total), was subjected to gel chromatography on Sephadex G-15. The entire toxic activity was eluted with the void volume, indicating a molecular weight exceeding 1500 daltons. Upon re-chromatography on Sephadex G-25, the toxin was slightly retarded compared with the void volume, suggesting a molecular weight of roughly 4000 daltons.

The toxic material was subjected to TLC on silica RP_{18} and the toxin recovered from a fluorescence-quenching band at R_F 0.2 revealed by irradiation at 254 nm. This chromatography replaced the previously used filtration through Sep-Pak C_{18} cartridges and elution of the toxin with *n*-butanol. The compounds in the fluorescent band showed no colour reaction with ninhydrin, and subsequent HPLC on Partisil-5 revealed three major ultraviolet-absorbing fractions (Fig. 1). Fractions at t_R 9.99 and 16.45 min, respectively, contained the toxic activity. Rechromatography by HPLC under the same conditions of either of these toxic fractions again resulted in two toxic fractions. Various modifications of the elution conditions did not significantly change this result nor was the degree of purification improved.

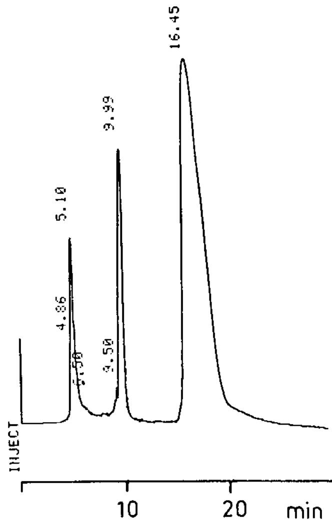

Fig. 1. UV absorbance profile (at 254 nm, in methanol) of the HPLC separation of the toxin of *Microcystis aeruginosa* PCC 7941. The fractions with t_R 9.99 and 16.45 min showed toxic activity.

Analysis of the toxic fractions

The UV absorbance spectra of either of the toxic fractions from HPLC showed maxima at 232 and 238 nm (Fig. 2). These maxima are similar to those described by Botes *et al.*[3] and Eloff[4,34] for the cyanoginosins from a South African *Microcystis aeruginosa* strain and might result from a conjugated diene.

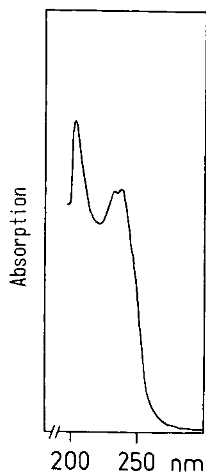

Fig. 2. UV absorbance spectrum (in methanol) of the toxin (fraction at t_R 16.45 min in Fig. 1). Maxima: 203, 232 and 238 nm.

Also the ^1H NMR spectra (Fig. 3), particularly the aromatic and olefinic resonance patterns between 5.1 to 7.3 ppm, were very similar to those reported previously by Botes *et al.*[3]. Resonance signals for the unusual amino acid Adda (H-4, quartet at 5.4 ppm; H-7, doublet at 5.50 ppm; H-5, doublet at 6.3 ppm; phenyl residue,

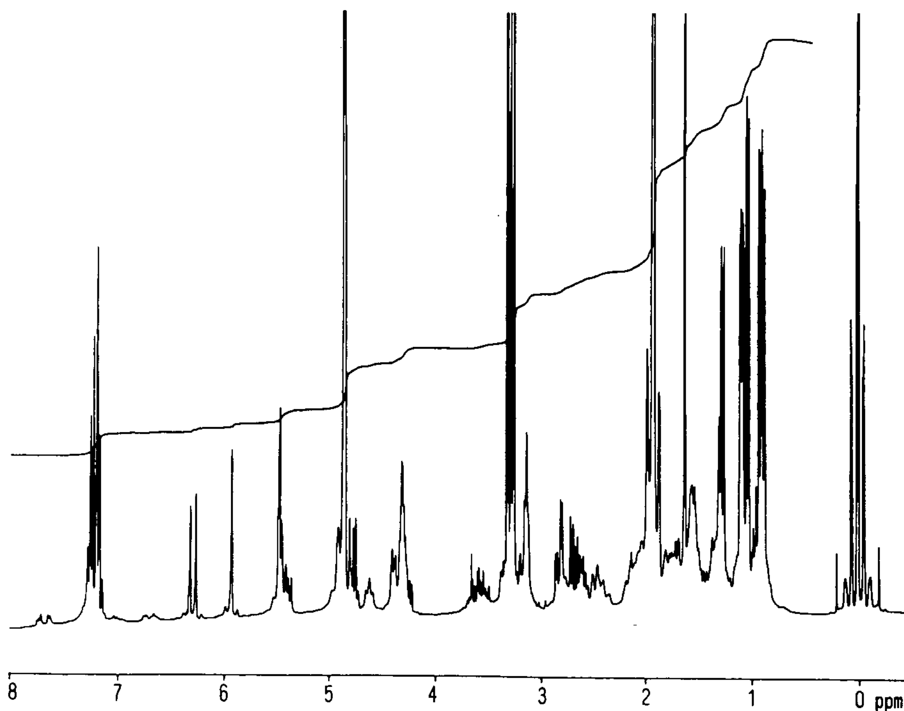

Fig. 3. ^1H NMR spectrum (in ^2H_2O; TMS, δ = 0) of the toxin (fraction at t_R 1.45 min in Fig. 1).

7.0–7.3 ppm; 6-O-methyl, singlet at 1.62 ppm) and N-methyldehydro-Ala (H-3, singlets at 5.50 and 5.94 ppm) were apparent in the spectra. Homonuclear proton–proton decoupling experiments revealed the coupling of H-4 (5.4 ppm) with H-5 (6.3 ppm) and of H-4 (5.4) with H-3 (4.6 ppm) in the Adda moiety. Furthermore, the methoxy group of Adda was identified by ^{1}H NMR (3.28 ppm) and ^{13}C-NMR spectroscopy in $^{2}H_2O$ (58.4 ppm), as well as in $C^2H_3O^2H$ (58.9 ppm) (^{13}C NMR data not shown). The N-methyl group of N-methyldehydro-Ala was apparent at 3.25 ppm in the ^{1}H NMR spectrum and at 38.5 ppm in the ^{13}C NMR spectrum in $C^2H_3O^2H$. A characteristic signal at 88.4 ppm in the ^{13}C NMR spectrum in $C^2H_3O^2H$ or at 88.0 ppm in $^{2}H_2O$ might be assigned to C-9 of Adda. The poor spectral resolution between 1.8 and 5.0 ppm in the ^{1}H NMR spectrum (Fig. 3) did not allow the assignment of individual signals. The presence of Ala was, however, apparent by coupling of the respective methyl resonance (1.25 ppm) with that of the α-proton, as well as by the methyl signal in the ^{13}C NMR spectrum (17.3 ppm) (coupling data not shown). The doublet at 0.9 ppm and the CH_3 resonances at 21.2 and 23.3 ppm in the ^{1}H NMR spectrum, as well as a signal at 40.5 ppm in the ^{13}C NMR spectrum in $C^2H_3O^2H$ (C-3) were assigned to Leu, which had been identified by the amino acid analyzer. The additional four methyl doublets in the NMR spectrum most likely originated from Adda (two doublets), β-methyl-Asp and Val. The identification of β-methyl-Asp was corroborated by signals at 16.9 (CH_3) and 36.9 ppm (C-3) in the ^{13}C NMR spectrum in $^{2}H_2O$. The small signal at 1.55 ppm perhaps originated from the methyl group of Thr. This resonance was coupled with a signal at 4.4 ppm, which most likely must be assigned to H-3 of Thr.

Analysis of the amino acids, after complete hydrolysis of the peptides, showed β-methyl-Asp (3–4), Thr (1), Glu (4), Ala (5), Val (3), Leu (6), Phe (2) and Arg (4), the values in parentheses representing molar ratios. The presence of β-methyl-Asp was

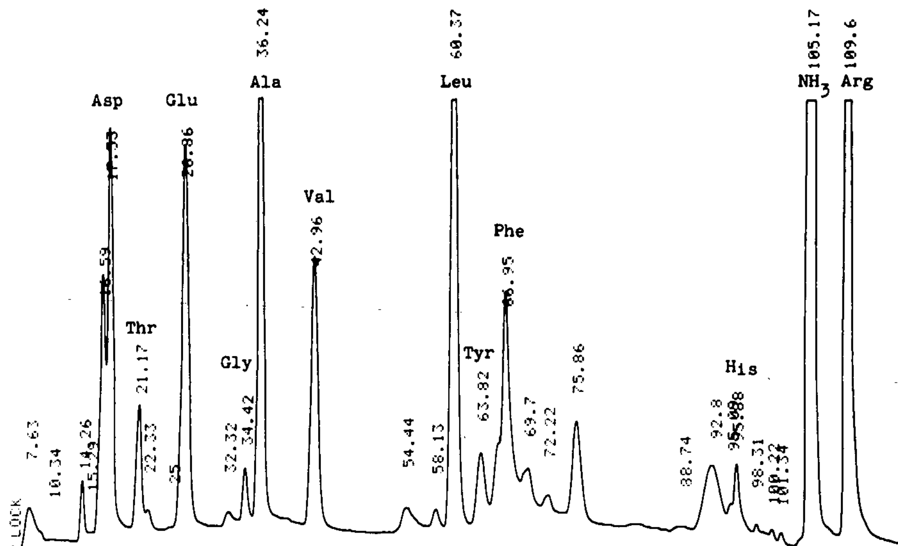

Fig. 4. Analysis by the automatic amino acid analyzer of the toxin (fraction at t_R 16.45 min in Fig. 1).

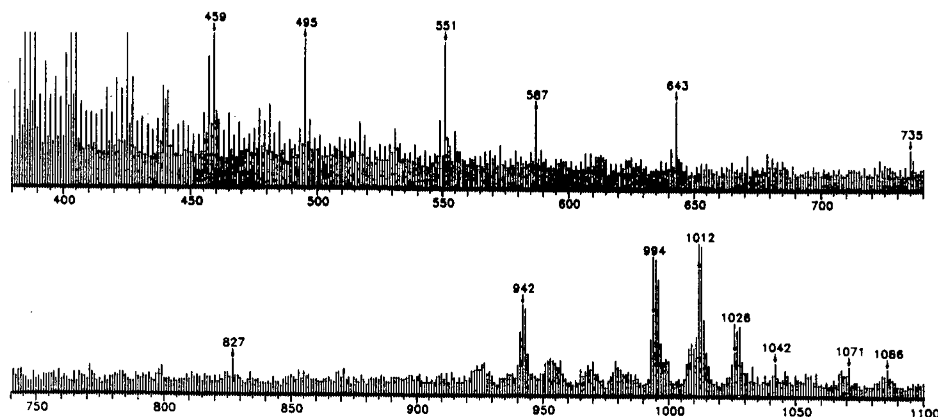

Fig. 5. Positive FAB mass spectrum of the toxin (fraction at t_R 16.45 min in Fig. 1).

derived from the double peak eluted at the Asp position in the amino acid analyzer (Fig. 4), which very likely arises from *threo*- and *erythro*-configurations from β-methyl-Asp during hydrolysis[26]. Since no reference samples of Adda and N-methyl-dehydro-Ala were available, these amino acids could not be identified in the hydrolysis mixture. Nevertheless, the large molecular weights, determined by FAB-MS, m/z 943 and higher, might accommodate also these unusual amino acids.

The molecular weights, determined by FAB-MS (Figs. 5 and 6), differ considerably from those extrapolated from the above mentioned gel filtrations. This might be due to either an unusual molecular conformation or to aggregation of the toxins in aqueous solution, a further aspect being here the lack of homogeneity of the HPLC-purified toxin fraction, as became obvious from the FAB-MS data. It should be noted that molecular weights ranging over 600 to 20 000 daltons had been reported for toxins from various *Microcystis sp.* strains[7,35]. Gel exclusion profiles in propanol-containing buffers may provide an answer to this point.

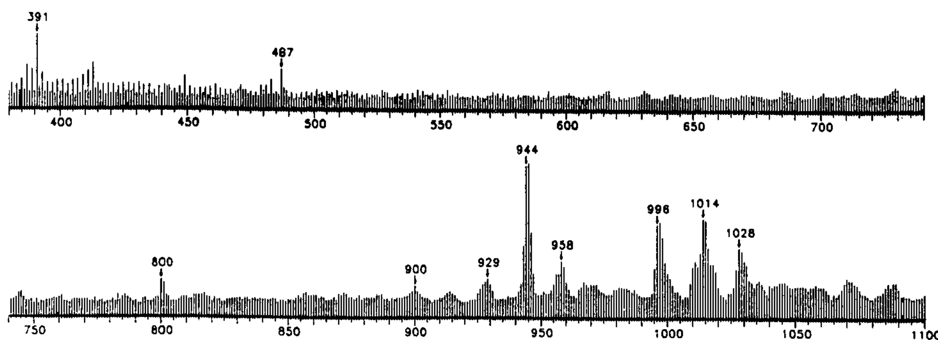

Fig. 6. Negative FAB mass spectrum of the toxin (fraction at t_R 16.45 min in Fig. 1). Glycerol signals at 459, 551, 643, 735 and 827 nm.

DISCUSSION

The solubility of the toxin from *Microcystis aeruginosa* PCC 7941 in alcohols and in slightly alkaline aqueous buffers, but not in diethyl ether or chloroform, indicated its amphilic character. This was also confirmed by the relative mobility (R_F 0.2) of the toxin observed in reversed-phase TLC. Gel electrophoresis at pH 8.2–8.5 revealed the negative charge of the toxin.

The unusual aromatic amino acid "Adda", which had been reported previously as part of the cyanoginosins[27], was also identified in the toxic fraction from *Microcystis aeruginosa* PCC 7941. The identification was based, in particular, on the resonance signals between 5.3 and 7.5 ppm in the ^1H NMR spectrum (Fig. 3) and on the signal in ^{13}C NMR spectrum assigned to carbon 9 (88.4 ppm in $C^2H_3O^2H$; 88.0 ppm in ^2H_2O), as well as on the ultraviolet absorbance with maxima at 232 and 238 nm[3,4]. Furthermore, the presence of N-methyldehydro-Ala and β-methyl-Asp, which also form part of the cyanoginosins[27], was detected by ^1H and ^{13}C NMR spectroscopy. It should be noted, however, that the material used for spectroscopy consisted of at least four peptides, which at present does not allow us to assign Adda, N-methyldehydro-Ala and β-methyl-Asp to one toxic peptide of *Microcystis aeruginosa* PCC 7941.

The peak eluted from an HPLC column (Fig. 1) indicates heterogeneity (for comparison see ref. 36) of the toxin fraction. Also, the ultraviolet absorbance spectrum of the sample does not prove its purity. Nevertheless, we demonstrated that the same amino acids that had been described as "invariant" in the cyanoginosins from a South African strain[27] are present in the toxin fraction from the North American strain of *Microcystis aeruginosa* used in this work. An homologous structural composition of the toxins from the two strains can, therefore, be anticipated. The failure to stain with ninhydrin as well as the lack of typical peptide fragmentation in FAB-MS point to cyclic structures for all peptides present in the toxic fraction. This is also supported by their solubility in organic solvents.

ACKNOWLEDGEMENTS

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Note

Quantitative determination of 2-amino-1,3-propanediol and its impurities by capillary gas chromatography

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2-Amino-1,3-propanediol (serinol) is an important intermediate in the synthesis of (*S*)-N,N′-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[(2-hydroxy-1-oxopropyl)-amino]-2,4,6-triiodo-1,3-benzenedicarboxamide (iopamidol)[1], a non-ionic water-soluble iodinated X-ray contrast medium[2]. The purity of serinol is an essential prerequisite for obtaining a contrast medium suitable for uro-angiographic and myelographic examinations, which require injection of highly concentrated solutions.

Serinol, prepared on an industrial scale according to reported methods[3–8], is often contamined by small amounts of amino alcohols as by-products that are difficult to separate from the main product prior to its quantitative determination.

The separation of amino alcohols by gas chromatography (GC) after derivatization with trifluoroacetic anhydride (TFAA) is a well know technique[9], but literature concerning serinol and related compounds is scarce. The most recent paper[10] describing a GC method using such derivatization and a packed column was found to lack the ability to separate the amino alcohol impurities from serinol.

This paper describes a GC method which, using a capillary column and stream splitting, allows the simultaneous quantitation of serinol and contaminants.

EXPERIMENTAL

Materials

All the reagents were analytical-reagent grade unless specified otherwise. Ethanolamine (ETH), 1-amino-2,3-propanediol (ISO) and 2-aminopropanol (AP) were commercially available and were used as standards without further purification. Ultrapure serinol (SER), used as a standard, was prepared and purified as described in previous work[10]. 2,3-Diaminopropanol (DAP) and 2-aminomethyl-1,3-propanediol (NME) were obtained by lithium aluminium hydride reduction[11] of the corresponding methyl esters of 2,3-diaminopropionic acid and 2-aminomethyl-3-hydroxypropionic acid, respectively.

1-Methoxy-2-aminopropanol (OME) was obtained by partial demethylation of the corresponding 1,3-dimethoxy-2-aminopropane by hydrogen bromide. A 1% (w/w) solution of methyl palmitate in chloroform was used as the internal standard in GC analyses.

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Instruments

GC analyses were performed using a Hewlett-Packard HP 5890 gas chromatograph equipped with an HP 7393A atuomatic sampler and an HP 3393A integrator. A DB 1701 fused-silica capillary column (30 m × 0.25 mm I.D.; film thickness 0.25 μm), supplied by J & W Scientific, was used under the following operation conditions: injector temperature, 200°C; flame ionization detector temperature, 220°C [hydrogen flow-rate, 33 ml/min; air flow-rate, 375 ml/min; helium flow-rate (auxiliary, 29 ml/min]; carrier gas, helium (column flow-rate, 1 ml/min; split flow-rate, 62 ml/min; purge flow-rate, 4 ml/min).

The following temperature programme was used: an initial hold of 145°C for 6.5 min followed by a ramp of 15°C/min to 10°C, held for 1 min and then a second ramp of 30°C/min to 205°C, held for 16 min.

Injections of 4-μl aliquots were performed automatically. Gas chromatography–mass spectrometry (GC–MS) was carried out on a Finnigan-MAT 8222 mass spectrometer equipped with an INCOS data system and coupled to a Varian 3400 gas chromatograph working as described above. The column was directly connected to the electron-impact source (EI) and the spectra were taken at 70 eV; filament emission, 0.5 mA; source temperature, 200°C; resolving power, 1250 (10% valley).

The molecular weight was confirmed by performing GC–MS under chemical ionization (CI) conditions using methane as reagent gas. Infrared (IR) spectra were recorded on a Perkin-Elmer Model 882 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC 200 FT spectometer equipped with an ASPECT 3000 computer. All the spectra were recorded in hexadeuterated dimethyl sulphoxide using tetramethylsilane as an internal standard; chemical shifts (δ) are expressed in ppm.

Analytical derivatization procedure

Solutions of serinol (200 mg) in water (240 mg) spiked with various concentrations of a mixture of known impurities, each covering the range from 0.2 to 1.5% (w/w), were prepared. Each solution was derivatized in a standard screw-capped vial by adding trifluoroacetic anhydride (10 ml) cooled in a bath of solid carbon dioxide–acetone. The mixture was cautiously warmed to 40°C, then held at this temperature for 30 min; subsequently, after cooling to room temperature, methyl palmitate internal standard (100 mg) was added. Standard solutions of serinol, spiked with 0.7% of each impurity individually, were similarly derivatized and used as calibrating solutions.

Preparative derivatization procedure

To a well stirred mixture of amino alcohol (0.1 mol) and trifluoroacetic acid (0.25 mol), trifluoroacetic anhydride (0.6 mol) was added under nitrogen at 0°C. The reaction mixture was cautiously warmed to room temperature and then at 40°C for 1 h. The trifluoroacetyl derivative was recovered from the reaction mixture after evaporation of the solvent and purification either by distillation under vacuum or by crystallization from anhydrous diethyl ether. The products obtained (90–95% yield) were characterized as shown in Table I.

TABLE I

AMINO ALCOHOL TRIFLUOROACETYL DERIVATIVES PREPARED

Amino alcohol	*B.p.** *(°C)*	*Molecular formula***	*IR* *(cm^{-1})*	d_{20} *($g\ ml^{-1}$)*	n_D^{20}	*^1H NMR*	*^{13}C NMR*
AP	108–110	$C_7H_7F_6NO_3$ 267.14	3308, 3101, 2992, 1788, 1707, 1553, 1461, 1405, 1386, 1354, 1164, 775, 734, 725 (neat)	1.4380	1.3640	9.4 (1H, bs, NH); 4.5 (1H, bm, CHNH); 4.3 (2H, bd, CH_2O); 1.25 (3H, bd, CH_3)	157.2 ($OCOCF_3$, J_{CCF} = 30 Hz); 156.3 ($NHCOCF_3$, J_{CCF} = 30 Hz); 115.9 ($NHCOCF_3$, J_{CF} = 289.4 Hz); 114.2 ($OCOCF_3$, J_{CF} = 289.4 Hz); 69.05 (CH_2OCO); 44.164 (CHNH); 15.13 (CH_3)
ETH	117–119	$C_6H_5F_6NO_3$ 253.12	3321, 3109, 2973, 1787, 1710, 1552, 1453, 1404, 1378, 1349, 1164, 1017, 775, 732 (neat)	1.4099	1.3730	9.65 (1H, bs, NH); 4.5 (2H, t, CH_2O); 3.5 (2H, dt, CH_2N, J = 4.2 Hz)	157.1 ($OCOCF_3$, J_{CCF} = 30 Hz); 156.8 ($NCOCF_3$, J_{CCF} = 30 Hz); 115.8 ($NCOCF_3$, J_{CF} = 289.4 Hz); 114.1 ($OCOCF_3$, J_{CF} = 289.4 Hz); 65.87 (CH_2OCO), 37.90 (CH_2NHCO)
OME	111–115	$C_8H_9F_6NO_4$ 297.16	3430, 3318, 3098, 2998, 2939, 2906, 2839, 1792, 1718, 1550, 1479, 1461, 1403, 1385, 1352, 1166, 894, 775, 735, 727 (neat)	1.3257	1.3730	9.5 (1H, bd, NHCO); 4.6 (1H, bm, CHNH); 4.5 (2H, bm, CH_2O); 3.6 (2H, bd, CH_2OCH_3); 3.3 (3H, s, CH_3O)	156.7 ($OCOCF_3$, J_{CCF} = 30 Hz); 155.4 ($NCOCF_3$, J_{CCF} = 30 Hz); 115.7 ($NHCOCF_3$, J_{CF} = 289.4 Hz); 114.1 ($OCOCF_3$, J_{CF} = 289.4 Hz); 69.69 (CH_2OCO); 66.28 (CH_2OCH_3); 58.11 (CH_3O); 48.12 (CHNH)
NME	140–141	$C_{10}H_8F_9NO_5$ 393.17	2974, 1794, 1691, 1464, 1420, 1402, 1352, 1158, 1092, 833, 774, 762, 733 (neat)	1.4465	1.3700	4.7 (5H, bm, CH_2CHCH_2); 3.1 (3H, s, CH_3N)	157.5 ($NCOCF_3$, J_{CCF} = 40 Hz); 156.0 ($OCOCF_3$, J_{CCF} = 40 Hz); 115.9 ($NCOCF_3$, J_{CF} = 289.4 Hz); 114.5 ($OCOCF_3$, J_{CF} = 289.4 Hz); 63.25 (CH_2O); 52.75 (CHN); 30.29 (NCH_3)

SER	143–146***	$C_9H_6F_9NO_5$ 379.14	3295, 3110, 2982, 2895, 1786, 1709, 1565, 1464, 1394, 1342, 1226, 1152, 989, 953, 919, 897, 882, 845, 800, 773 (neat)	1.6175***	1.3690***	9.7 (1H, bd, NH); 4.6 (5H, bm, CH_2CHCH_2)	156.86 ($NCOCF_3$, J_{CCF} = 37.4 Hz); 156.22 ($OCOCF_3$, J_{CCF} = 42.2 Hz); 115.5 ($NCOCF_3$, J_{CF} = 287.6 Hz); 114.2 ($OCOCF_3$, J_{CF} = 285.5 Hz); 65.29 (CH_2O); 47.17 (CHN)
ISO	149–150[§]	$C_9H_6F_9NO_5$ 379.14	3318, 3100, 2960, 1787, 1712, 1667, 1558, 1435, 1350, 1168, 799, 775, 731, 706 (neat)	–	–	9.75 (1H, bd, NH); 5.5 (1H, bs, CHO); 4.7 (2H, m, CH_2O); 3.7 (2H, bm, CH_2N)	157.35 ($OCOCF_3$, J_{CCF} = 40 Hz); 136.30 ($NHCOCF_3$, J_{CCF} = 40 Hz); 119.96 ($NCOCF_3$, J_{CF} = 289.4 Hz); 115.91 ($OCOCF_3$, J_{CF} = 289.4 Hz); 73.74 (CHO); 65.98 (CH_2O); 38.58 (CH_2NH)
DAP	142–143[§§] (m.p.)	$C_9H_7F_9N_2O_4$ 378.15	3310, 3115, 1786, 1696, 1563, 1447, 1407, 1388, 1356, 1316, 1254, 1212, 1170, 1049, 976, 930, 918, 886, 850, 827, 715, 737, (KBr)	–	–	9.5 (1H, bd, NH); 4.6 (1H, m, CHN); 4.4 (2H, bd, CH_2O); 3.5 (2H, bs, CH_2N)	156.9 ($NCOCF_3$, J_{CCF} = 30 Hz); 157.2 ($OCOCF_3$, J_{CCF} = 30 Hz); 115.96 ($NCOCF_3$, J_{CF} = 289.4 Hz); 114.37 ($OCOCF_3$, J_{CF} = 289.4 Hz); 66.61 (CH_2O); 48.12 (CHNH); 39.23 (CH_2NH)

* Determined at 15 mmHg and uncorrected.
** Satisfactory microanalyses obtained: C ± 0.23, H ± 0.27, N ± 0.21, F ± 0.18%.
*** Lit.[5]: b.p. = 125°C (3 mmHg), d_{20} = 1.6175, n_D^{20} = 1.3693.
§ After distillation the product solidified (m.p. 62–63°C).
§§ From diethyl ether.

TABLE II

GC–MS (EI) FRAGMENTATION OF AMINO ALCOHOL TRIFLUOROACETYL DERIVATIVES

Relative abundances (%).

Ion	m/z (%)						
	AP	*ETH*	*OME**	*NME*	*SER*	*ISO*	*DAP*
M	267(<1)	253(<1)	297(<1)	393(4)	379(<1)	379(<1)	378(<1)
M + 1**	268(<1)	254(1)	298(1)	394(<1)	380(1)	380(1)	379(1)
$M-CH_3$	253(<1)	–	–	–	–	–	–
M−F	–	–	278(<1)	374(5)	360(6)	380(2)	359<1)
$M-CF_3$	198(5)	184(20)	228(8)	324(9)	310(2)	310(1)	309(3)
$M-OCH_3$	–	–	266(1)	–	–	–	–
$M-CH_3OH$	–	–	265(<1)	–	–	–	–
$H-COCF_3$	–	156(1)	–	296(1)	282(<1)	282(<1)	281(<1)
$M-CO_2CF_3$	154(16)	140(50)	184(34)	280(32)	266(68)	266(24)	265(41)
$M-CO_2HCF_3$	153(5)	139(83)	183(5)	279(21)	265(36)	265(58)	264(33)
$M-CH_2OCOCF_3$	140(100)	126(100)	170(41)	266(99)	252(83)	252(2)	251(13)
$C_2H_4NHCOCF_3$	140(100)	–	140(7)	140(9)	140(8)	140(65)	140(50)
$CH_2NHCOCF_3$	126(7)	126(2)	126(3)	126(2)	126(30)	126(100)	126(59)
CO_2CF_3	113(8)	113(3)	113(2)	113(1)	113(18)	–	113(4)
$M-CO_2CF_3-CO_2HCF_3$	–	–	–	166(28)	152(79)	152(45)	151(17)
$M-CH_2OCOCF_3-CH_2HCF_3$	–	–	–	152(100)	138(100)	138(8)	137(100)
$M-CH_2OCOCF_3-CF_3$	–	–	–	210(3)	106(48)	196(9)	195(14)
$M-CO_2HCF_3-COCF_3$	–	–	–	182(1)	168(9)	168(9)	167(3)

* Base peak m/z 45.

** Confirmed by CI.

RESULTS

The separation achieved by GC analysis of serinol containing other amino alcohols after derivatization is shown in Fig. 1. Material corresponding to each peak, analysed in conjunction with the mass spectrometer, showed, in addition to a molecular ion, the typical fragmentation pattern of N,O-trifluoroacetyl derivatives[9]. The same fragmentations were observed by GC–MS (EI) analysis of the trifluoroacetyl derivatives obtained preparatively. Table II gives the results.

The quantitation of serinol and amino alcohol contaminants was subsequently examined. The precision of the method was characterized by the standard deviation (S.D.) obtained from five replicate analyses and the accuracy was characterized as the percentage error (Δ%) in the analyses of six samples (A–F) with different pre-determined compositions (Table III).

CONCLUSION

The procedure described represents a simple, accurate and precise method that allows the quantitation of serinol and amino alcohol contaminants. There is no *a priori* reason why the method should not also be valid outside the composition range described. Further, to our knowledge, except with serinol, none of the trifluoroacetyl derivatives, which have been fully characterized here, have been reported previously.

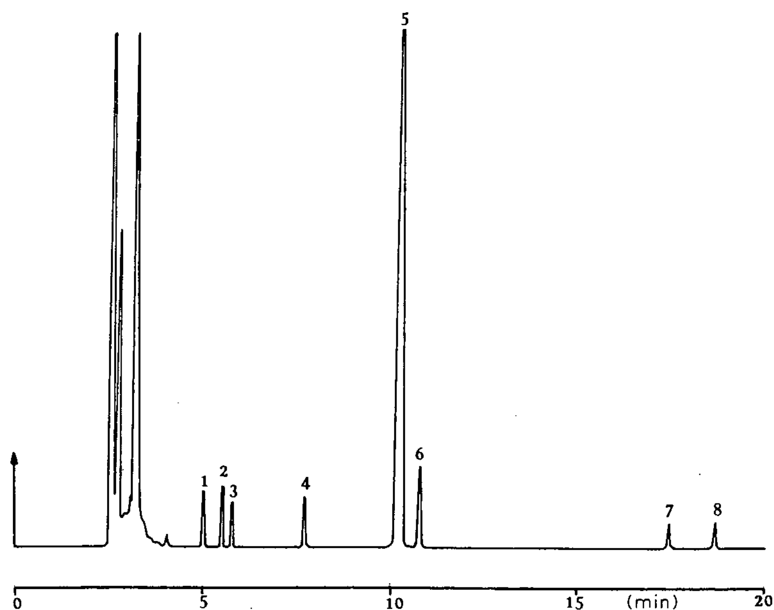

Fig. 1. Gas chromatogram of a sample of serinol and its impurities after derivatization. Peaks and retention times: 1 = 2-aminopropanol (5.003 min); 2 = 2-ethanolamine (5.535 min); 3 = 1-methoxy-2-aminopropanol (5.821 min); 4 = 2-aminomethyl-1,3-propanediol (7.776 min); 5 = serinol (10.404 min); 6 = 1-amino-2,3-propanediol (10.861 min); 7 = 2,3-diaminopropanol (17.486 min); 8 = methyl palmitate (18.789 min).

TABLE III

ACCURACY AND PRECISION OF ANALYTICAL PROCEDURE

Amino alcohol	*Parameter*	*Sample*					
		A	*B*	*C*	*D*	*E*	*F*
AP	Content (mg)	1.155	1.991	1.991	3.270	0.209	0.852
	Found ± S.D. (mg)	1.221 ± 0.004	1.465 ± 0.010	2.128 ± 0.005	3.438 ± 0.022	0.022 ± 0.002	0.912 ± 0.007
	Δ%	5.74	4.78	6.88	5.14	5.26	7.04
ETH	Content (mg)	2.207	3.071	0.249	0.687	1.216	1.565
	Found ± S.D. (mg)	2.301 ± 0.016	3.259 ± 0.026	0.271 ± 0.001	0.723 ± 0.010	1.314 ± 0.047	1.676 ± 0.012
	Δ%	4.26	6.12	8.84	5.24	8.06	7.00
OME	Content (mg)	1.577	2.031	3.003	0.282	0.615	1.017
	Found ± S.D. (mg)	1.668 ± 0.006	2.147 ± 0.016	3.158 ± 0.023	0.307 ± 0.004	0.672 ± 0.002	1.082 ± 0.014
	Δ%	5.77	5.71	5.16	8.87	9.27	6.29
NME	Content (mg)	3.263	0.255	0.641	1.115	1.531	2.233
	Found ± S.D. (mg)	3.488 ± 0.011	0.278 ± 0.003	0.695 ± 0.014	1.194 ± 0.008	1.641 ± 0.052	2.412 ± 0.0215
	Δ%	6.90	9.02	8.91	7.09	7.18	8.02
SER	Content (mg)	200.40	201.40	225.70	214.60	202.10	197.50
	Found ± S.D. (mg)	201.90 ± 0.004	203.73 ± 0.008	216.10 ± 0.010	216.10 ± 0.012	203.41 ± 0.002	198.64 ± 0.006
	Δ%	0.75	0.66	0.79	0.70	0.65	0.58
ISO	Content (mg)	0.255	0.711	1.134	1.670	2.342	3.454
	Found ± S.D. (mg)	0.268 ± 0.009	0.767 ± 0.003	1.189 ± 0.020	1.770 ± 0.022	2.484 ± 0.032	3.636 ± 0.018
	Δ%	5.10	5.06	4.89	5.99	6.06	5.27
DAP	Content (mg)	0.691	0.988	1.426	2.259	3.312	0.310
	Found ± S.D. (mg)	0.727 ± 0.008	1.047 ± 0.039	1.497 ± 0.022	2.365 ± 0.031	3.462 ± 0.013	0.327 ± 0.015
	Δ%	5.21	5.97	4.98	4.47	4.53	5.48

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Note

Determination of phenolic antioxidants in JP-5 jet fuels by gas chromatography–mass selective detection*

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Throughout the petroleum industry, the desirable light, sweet crudes are increasingly being replaced by heavy crudes containing undesirable amounts of metals, sulfur, nitrogen, and oxygen. This has led to a number of problems with the currently available jet fuels (such as the Navy's JP-5). These fuels can increase in peroxide number, corrosiveness, and viscosity during storage.

Commercial antioxidant mixtures containing hindered phenols are available to protect jet fuels against storage instability, and these contain either fully hindered or partially hindered phenolic types. Because of the current problems with the degradation of stored jet fuels, a project was initiated to study the depletion of antioxidants and changes in chemical composition occurring when stored at elevated temperatures. The project required development of analytical technology that would give accurate measurement of the specified antioxidants down to the 1-ppm level in JP-5 jet fuel. A desirable technique would either analyze the fuel directly or with a minimum of pretreatment.

Techniques for determining phenolic antioxidants in a variety of matrices have been described[1–12]. The majority of these techniques[1–9] was not applicable to the requirements of our particular project either because of a different matrix[1–7], or lack of a low ppm detection limit[8]. One technique[9] applied only to partially hindered phenols. Of the techniques which determined trace antioxidants in jet fuel[9–12], one was directly applicable but required fractionation and concentration of the fractions[10]. Two techniques that appeared to be directly applicable were selected for evaluation and adaptation to our specific uses. Hillman and Hayes[11] described a reversed-phase high-performance liquid chromatographic (HPLC) technique using electrochemical

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detection (ED) that appeared to have potential for detecting all fully and partially hindered phenolic antioxidants with sensitivity and selctivity. Masoud and Cha[7] reported a similar method for non-fuel matrices. Bartl and Schaaff[12], in a brief communication, outlined the use of gas chromatography–mass spectrometry (GC–MS) to identify and detect specific antioxidants in AVTUR aviation turbine fuel. No details concerning linearity or precision were given, and some of the operating parameters of the GC were not discussed.

This report describes work done to extend the use of GC–MS to the determination of antioxidants in a JP-5 jet fuel and to evaluate its usefulness in identifying and quantitating trace levels of specific antioxidants.

EXPERIMENTAL

Instrumentation

The analyses were carried out using a Hewlett-Packard (Palo Alto, CA, U.S.A.) 5890 gas chromatograph with a HP 5970A mass selective detector, and the data were processed with a HP 9825B calculator. The GC column was a Supelcowax 10 (30 m × 0.25 mm) fused-silica capillary column (Supelco, Bellefonte, PA, U.S.A.), and injections were in the splitless mode with a 0.5-min purge. The column enters the detector in the capillary direct mode.

Reagents

The HPLC grade *n*-heptane was obtained from J. T. Baker. Pure phenolic compounds were obtained from Aldrich. Commercial antioxidant A (DuPont AO-29) contained over 99% of 2,6-di-*tert.*-butyl-4-methylphenol (BHT). Commercial antioxidant B (DuPont AO-33) contained approximately 66% of 2,4-di-*tert.*-butylphenol plus 14% of 2,4,6-tri-*tert.*-butylphenol, and 12% of 4-*tert.*-butylphenol. The last two compounds were identified by GC and GC–MS data. Commercial antioxidant C (DuPont AO-30) was used as the internal standard in the determination of antioxidants A and B. It contained approximately 82% of 6-*tert.*-butyl-2,4-dimethylphenol. A fuel equivalent to a JP-5 fuel but without additives was used to prepare standards and samples for this work.

Standard blends. Blends of antioxidants A and B at the 0.2–0.3% (w/w) level in a JP-5 jet fuel were prepared. The jet fuel used was the same as that used for the preparation of samples for the storage and depletion tests. Dilute blends approximating 20 ppm were prepared from these standards as required.

Internal standard blend. A blend of antioxidant C at the 0.2–0.3% (w/w) level in *n*-heptane was prepared. Dilute blends approximating 20 ppm were prepared from this standard as required.

Calibration blend. Internal standard (2 ml) and standard blend (2 ml) were mixed. The detector was calibrated with these blends before unknown samples were analyzed.

Sample preparation. Sample (2 ml) and internal standard (2 ml) were mixed prior to analysis.

Operating conditions

Gas chromatography. The gas chromatograph was operated with the injection port at 280°C and the detector manifold at 220°C. The column was programmed from 50°C to 220°C at a rate of 5°C/min for antioxidant A and 8°C/min for antioxidant B. The flow through the column was 1 ml/min of helium with a column head pressure of 2 p.s.i.

Mass selective detector. The detector was operated using a dwell time of 200 ms and a mass window of 1.0 a.m.u. For antioxidant A, ions monitored were at m/z 205.1 and 135.1, and for B they were at m/z 191.1 and 135.1. Also for antioxidant B, the ion at m/z 247.2 was sometimes monitored to allow measurement of the 2,4,6-tri-*tert.*-butylphenol impurity.

RESULTS AND DISCUSSION

The majority of the analyses were carried out on samples from a stability storage test at 60°C. In each case, only the major component of each of the commercial antioxidants under study was monitored, and the analytical technology was developed to determine only this major component. Information about other phenols in each commercial mixture is included only as background information.

Choice of suitable ions

With the mass selective detector in the peak finder mode (scanning), blends of each of the three antioxidants in *n*-heptane were used to determine their most abundant ions. The most abundant ions and their relative abundances are shown in Table I. The molecular ion M^+, $[M - 15]^+$ and $[M - 43]^+$ fragments are common to hindered phenols (13) and other *tert.*-butyl substituted aromatics.

The usefulness of each particular ion was evaluated with blends prepared in jet fuel. The criterion was to be able to monitor the ion and detect and measure the antioxidants without interference from jet fuel components. It was found necessary to use a Carbowax-type (Supelcowax 10) fused-silica capillary column to separate the antioxidants from components of the jet fuel. A program rate of 8°C/min successfully separated the antioxidant B (2,4-di-*tert.*-butylphenol), which is partially hindered, from the jet fuel components, but a program rate of 5°C/min was required to separate

TABLE I

RELATIVE ABUNDANCE OF MOST ABUNDANT IONS

M is the molecular ion in each case.

Compound	M^+ (%)	m/z	$[M - 15]^+$ (%)	m/z	$[M - 43]^+$ (%)	m/z
2,6-Di-*tert.*-butyl-4-methylphenol (antioxidant A)	25	220	100	205	10	177
2,4-Di-*tert.*-butylphenol (major component of antioxidant B	16	206	100	191	16	163
6-*tert.*-Butyl-2,4-dimethylphenol (antioxidant C)	39	178	100	163	51	135

antioxidant A from the jet fuel components. Being completely hindered, antioxidant A (2,6-di-*tert*.-butyl-4-methylphenol) eluted much earlier than antioxidant B and required a slower program rate.

Choice of internal standard

Another hindered phenol, 6-*tert*.-butyl-2,4-dimethylphenol (antioxidant C), was chosen for the internal standard. This eluted between antioxidants A and B in the GC program. Two ions that were abundant showed jet fuel interference (see Table I), so the ion at m/z 135.1 was selected as it did not have interfering jet fuel components in the chosen jet fuel.

Analytical parameters

Typical chromatograms of antioxidants A and B in jet fuel are shown in Figs. 1 and 2. These show clearly the separation of the selected ion peaks from the interfering jet fuel components. The absence of interfering fuel components is further demonstrated in Fig. 3 which shows selected ion chromatograms of the jet fuel used in this study without added antioxidants.

Over the range of 0–40 ppm of antioxidant in the jet fuel, satisfactory linearity was demonstrated with a linear regression analysis that gave a correlation coefficient of 0.9998 for antioxidant A and 0.9996 for antioxidant B. The precision of the technique was determined by preparing ten aliquots of one sample with internal standard and analyzing each portion. Relative standard deviations of 2.3% for antioxidant A and 3.9% for antioxidant B were obtained. The accuracy of the

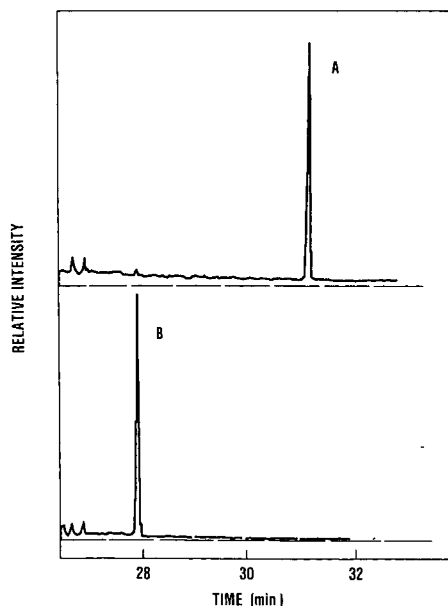

Fig. 1. GC–MSD of antioxidant A in jet fuel 1, monitored at (top) m/z 135.1, (bottom) m/z 205.2. Peaks: A = internal standard (antioxidant C, 25 ppm), B = antioxidant A (17 ppm). Conditions as described in Experimental.

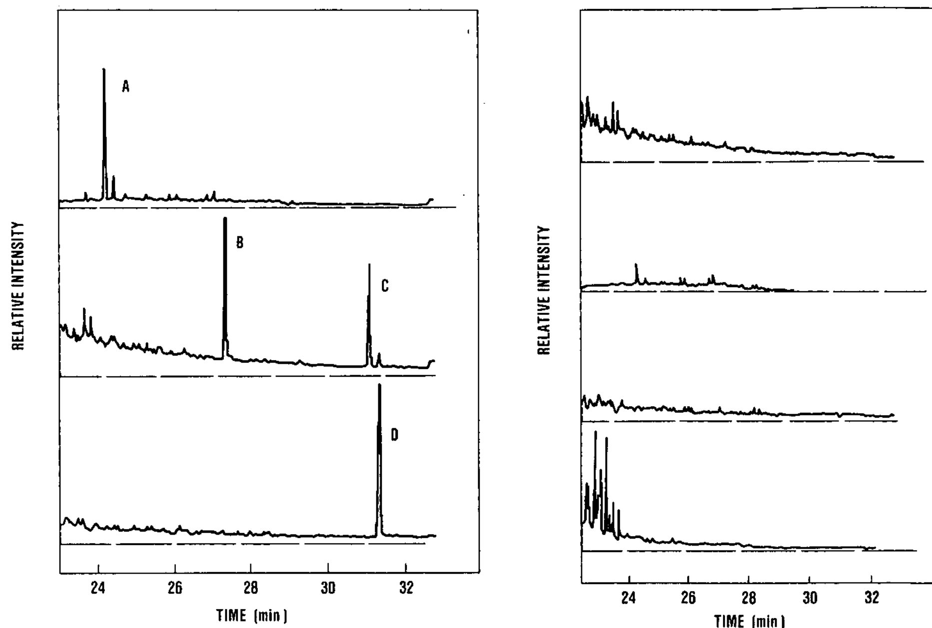

Fig. 2. GC–MSD of antioxidant B in jet fuel 1, monitored at (top) m/z 247.2, (middle) m/z 135.1, (bottom) m/z 191.1. Peaks: A = tri-*tert.*-butylphenol, B = internal standard (antioxidant, 19 ppm), C = impurity in antioxidant B (4-*tert.*-butylphenol), D = antioxidant B (20 ppm). Conditions as described in Experimental.

Fig. 3. GC–MSD of jet fuel 1 without additives, monitored at (from top to bottom) m/z 135.1, m/z 247.2, m/z 191.1, and m/z 205.1. Conditions as described in Experimental.

technique should be similar to the precision. The minimum detection level is 0.2 ppm with a 0.2-μl splitless injection, or approximately 0.2 ng of each antioxidant (assuming a signal-to-noise ratio of 3).

Analysis of samples from storage at 60°C

For the storage stability test, large quantities of blends of antioxidants A and B at 12 and 24 μg/g in a JP-5 jet fuel were prepared and stored at 60°C. Samples were withdrawn and the antioxidant level determined at two-week intervals. During the first twelve weeks, no reduction in antioxidant level was found within the limits of precision of the method. These data were averaged and are shown in Table II. It can be seen that the values agree well with the theoretical values.

Interference from oxidation products

A brief, two-week storage test of this jet fuel at 90°C rapidly caused antioxidant depletion. When these samples were analyzed, a large number of new components (see Fig. 4) appeared on the select ion at m/z 135.1 (used to monitor the internal standard). These oxidation products are quite polar and are probably hydroxy compounds. The internal standard peak was overlapped by interfering compounds in the analysis of antioxidant B. This problem was overcome by reducing the programming rate from 8°C/min to 6°C/min which eluted the components over a longer period and gave

TABLE II

COMPARISON OF MEASURED *VERSUS* THEORETICAL LEVELS OF ANTIOXIDANT IN JP-5 JET FUEL

Antioxidant A (μg/g)			Antioxidant B (μg/g)		
Theory	*Measured*	*n*	*Theory*	*Measured*	*n*
12	11.9	6	12	11.8	5
24	24.1	6	24	23.8	5

complete resolution of the internal standard peak. The other ions at m/z 191.1, 205.1, and 247.2 did not show any new components. If, in future work, interference with the internal standard ion becomes worse, it would be quite feasible to use antioxidant A as the internal standard for B, and *vice versa.*

Another phenomenon that emerged during the analysis of these (90°C storage) samples was the depletion of antioxidant A, apparently during vaporization in the injection port. This problem was uncovered when antioxidant A was used as an internal standard. This loss of antioxidant is thought to be due to reaction with peroxides (1170 ppm) and/or the newly formed oxygenated compounds that were in the jet fuel. At the injection port temperature of 280°C, reaction could occur rapidly. Antioxidant B, however, was not affected in this manner.

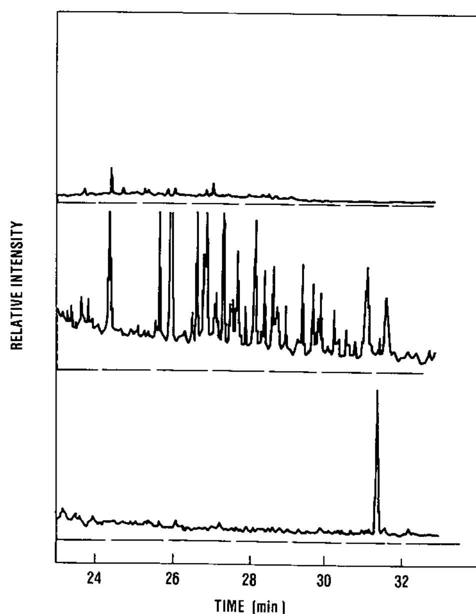

Fig. 4. GC–MSD of jet fuel 1 with antioxidant B after storage at 90°C, monitored at (top) m/z 247.2, (middle) m/z 135.1, (bottom) m/z 191.1. Many new components are present on the m/z 135.1 trace. Compare with Fig. 2. Conditions as described in Experimental.

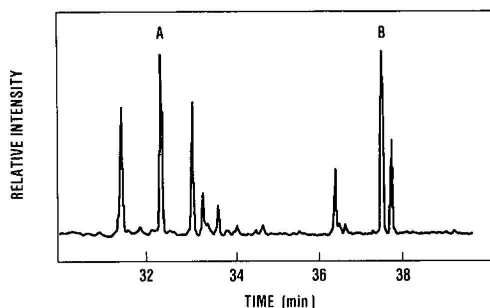

Fig. 5. GC–MSD of jet fuel 4, monitored at m/z 163.1. Peaks: A = internal standard (antioxidant C), B = major component of antioxidant D. Conditions as described in Experimental.

After the above occurrence, the injection technique was switched to a cool, on-column injection. This eliminates exposure of the sample to high temperatures. The work reported below used the cool, on-column technique.

Extension of technique to other fuels and another antioxidant

Three other JP-5 jet fuels that were on hand have also been examined by GC–mass selective detection (MSD) to establish if they contained interfering impurities. No impurities were found for ions at m/z 135, 191, and 205.

Other phenolic antioxidants can be determined by this technique. Three outside samples of JP-5 jet fuels were submitted for determination of their specified antioxidants. The first and second contained antioxidants B and C, respectively, and the third contained antioxidant D, which GC–MSD showed was predominantly *tert.*-butyldimethyl or *tert.*-butylethylphenols concentrated mainly in three major components. Although these components were isomeric with antioxidant C, they all eluted later than C (see Fig. 5) suggesting that they are less hindered than antioxidant

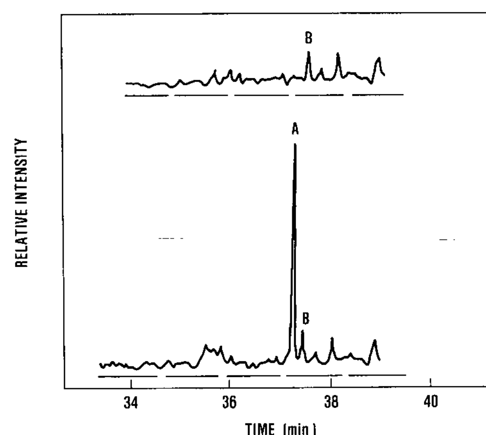

Fig. 6. GC–MSD of jet fuel 2, monitored at m/z 191.1. (Top) Neat fuel, (bottom) fuel containing 3.4 ppm of added antioxidant B. Peaks: A = antioxidant B, B = reference peak. Conditions as described in Experimental.

C (more polar). The latter was used as an internal standard, and measurement for both antioxidants (the largest component was used to quantitate D) were made using ion of *m/z* 163. The level of antioxidant D was found to be 18.5 ppm.

A different quantitation technique was developed for the other two samples of JP-5 jet fuels. A GC–MSD scan revealed that none of the specified antioxidants were present, but in each case a broad fuel component eluted at the same retention time as the antioxidant. Addition of ppm levels of antioxidant to the fuel resulted in a narrow peak riding on a broad peak (see Fig. 6). Calculation of detection limit was made by using an adjacent fuel component to correlate sample size and measuring peak heights with and without the added antioxidant. In both cases, the antioxidant level was found to be less than 1 ppm.

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Note

Improvement of chemical analysis of antibiotics

XIV*. Identification of the components of bacitracin using normal- and reversed-phase thin-layer chromatography

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Bacitracin (BC) is a basic and cyclic peptide antibiotic produced by *Bacillus subtilis* and *Bacillus licheniformis*[1]. Commercial BC consists of various components that have different antimicrobial activities, and their proportions are not always constant. The components have been classified into BC-A, -B, -C, -D, -E, -F and -G based on their UV spectra[2,3]. The major antimicrobial active components are BC-A and -B and major degradation product is BC-F, which is devoid of antimicrobial activity and also shows nephrotoxicity[1]. The structures of the components have not been clarified, except for BC-A and -F.

Although the analysis of BC has been often carried out by bioassays, we considered that a chemical method would be more suitable because it would be possible to analyse each component of BC, including deactivated ones such as BC-F. For chemical analysis high-performance liquid chromatographic (HPLC)[3–7], paper chromatographic (PC)[8] and thin-layer chromatographic (TLC)[8–16] methods have been reported. Most reversed-phase (RP) HPLC methods use solvent gradient systems with consequent difficulties in reproducibility and the ion-exchange HPLC method gave a poorer resolution of the components of BC than did RP-HPLC. PC is time consuming and gives poor resolution. In general, TLC is a simple and inexpensive technique, and a number of workers have tried to separate the components of BC using adsorbent layers of silica gel[8–12], cellulose[10,14], ion-exchange resin[15] and RP-type silica gel[16]. Although many components exists in BC, the previously reported TLC methods using silica gel, cellulose and ion-exchange showed only one or two spots on the TLC plates[8–15]. Aszalos and Aquilar[16] analysed only BC-A on an RP-type TLC plate but we obtained a poor resolution of the components of BC under their TLC conditions.

For the identification of the components of BC, we considered that the combined use of different modes such as normal-phase (NP) and RP- chromatography would be the most suitable approach, because BC contains various components as

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mentioned above. Therefore, we attempted to establish simple and reliable methods using NP- and RP-TLC for the components of BC.

EXPERIMENTAL

Materials

Chloroform, methanol, isopropanol, ethyl acetate, *n*-butanol, ethanol, acetic acid, trifluoroacetic acid, trichloroacetic acid, phosphoric acid, oxalic acid, sodium hydroxide, aqueous ammonia, triethylamine, zinc chloride, calcium chloride, potassium sulphate, sodium sulphate, magnesium chloride and ninhydrin were analytical-reagent grade chemicals. Bacitracin was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). TLC plates precoated with silica gel and C_8- and C_{18}-modified silica gel (E. Merck, HPTLC plates 15696, 13725 and 13724, respectively) were used.

Bacitracin solution

BC (100 mg) was weighed accurately into a 10-ml volumetric flask and diluted to volume with methanol.

Thin-layer chromatography

Solvent systems in the optimization step. Various solvent systems were prepared by mixing chloroform, methanol and aqueous acetic acid for NP-TLC and by mixing methanol, various salt solutions (potassium sulphate, sodium sulphate, calcium chloride, magnesium chloride, manganese chloride and zinc chloride) and triethylamine for RP-TLC.

Optimal solvent system in NP-TLC. Chloroform–methanol–0.75% aqueous acetic acid (30:20:4) was used.

Optimal solvent system in RP-TLC. Methanol–0.1 *M* aqueous potassium sulphate solution (pH 2.0) (7:3) containing 1.0% triethylamine was used. The solvent system was prepared by the following procedure; 1 g of triethylamine was dissolved in 30 ml of 0.1 *M* aqueous potassium sulphate solution and the pH value of the solution was adjusted to 2.0 with phosphoric acid and then 70 ml of methanol was added to the solution.

Development. After applying 1 μl of the BC solution to a TLC plate, chromatography was carried out by placing the plate in a 24 × 11 × 20 cm glass chamber at 25°C. The tank was saturated with the respective solvent system for 30 min before introducing the plate.

Detection of bacitracin. The TLC plate was sprayed evenly with 0.5% ninhydrin in *n*-butanol and then heated at 120°C to produce coloured spots.

RESULTS AND DISCUSSION

BC-A and -F are the major antimicrobial and nephtrotoxic components[1], respectively, and the structures of the other components have not yet been clarified. Therefore, the separation of BC-A and -F from the other components was investigated.

Normal-phase TLC

For NP-TLC, eight spots of the components of BC were observed on a silica gel HPTLC plate using chloroform–methanol–0.75% aqueous acetic acid (30:20:4) as the mobile phase. A typical separation obtained using the optimal conditions is illustrated in Fig. 1A. Various examinations were carried out to obtain these optimal conditions on the silica gel TLC plate, but we describe mainly the optimization of the concentration of acetic acid in the solvent system below.

In order to optimize the concentration of acetic acid in the aqueous solution, using chloroform–methanol–aqueous acetic acid (30:20:4) the influence of the acid concentration on the R_F values and the shape of the spots was examined. When 0.75% aqueous acetic acid was used, the most suitable R_F values and a good shape of the spots were obtained. Therefore, we chose chloroform–methanol–0.75% aqueous acetic acid (30:20:4) as the optimal solvent system with a silica gel TLC plate to separate the components.

RP-TLC

Using RP-TLC, as shown in Fig. 1B the components of BC were separated into ten spots on a C_8 TLC plate using methanol–0.1 *M* aqueous potassium sulphate (pH 2.0) (7:3) containing 1.0% of triethylamine as the solvent system.

Comparison of C_8 and C_{18} plates. The suitability of C_8 and C_{18} TLC plates for the separation of the components of BC was tested using methanol–0.1 *M* aqueous potassium sulphate solution (pH 2.0) (7:3) containing 1.0% of triethylamine as the solvent system. When a C_{18} TLC plate was used, two spots with $R_F \approx 0.6$ showed tailing and overlapping. With a C_8 TLC plate, the components that overlapped on the C_{18} TLC plate were separated into two spots, so that we could observe ten spots of the components. Accordingly, we chose the C_8 TLC plate for subsequent work.

Addition of triethylamine. In general, BC gives extremely tailing spots on RP-

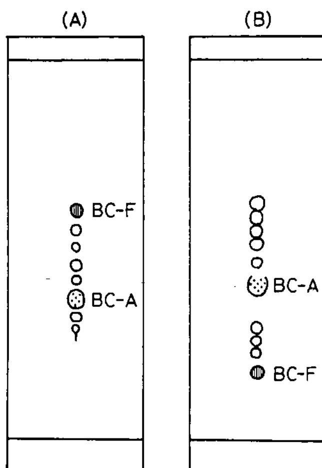

Fig. 1. Separation of the components of bacitracin. (A) Silica gel TLC plate; solvent system, chloroform–methanol–0.75% acetic acid (30:20:4); (B) C_8 TLC plate; solvent system, methanol–0.1 *M* potassium sulphate (pH 2.0) (7:3) containing 1.0% of triethylamine.

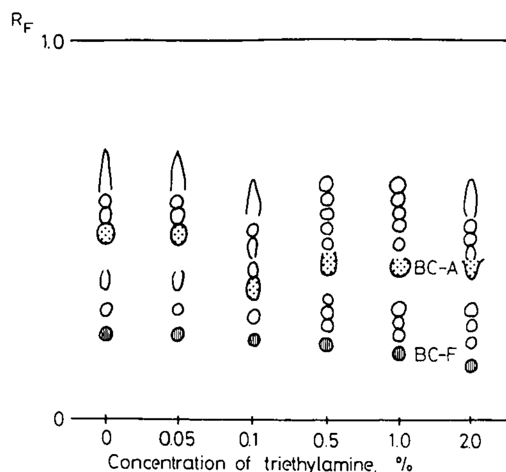

Fig. 2. Effect of triethylamine concentration in RP-TLC. C_8 TLC plate; solvent system, methanol–0.1 *M* potassium sulphate (pH 2.0) (7:3) containing 0–2% of triethylamine.

TLC plates. We tried using a solvent system containing triethylamine[17] to avoid this tailing. Using methanol–0.1 *M* aqueous potassium sulphate (pH 2.0) (7:3) containing triethylamine as the solvent system, the influence of the triethylamine concentration on the separation and the shape of spots was examined. As shown in Fig. 2, only seven spots with tailing appear on the chromatogram using a triethylamine-free solvent system, but the separation and the shape of the spots are improved with increasing concentration of triethylamine and satisfactory results were obtained between 0.5 and 1.5%. However, because the spots showed tailing when triethylamine concentrations above 1.5% were used, we chose to use 1.0% triethylamine in the solvent system in subsequent work.

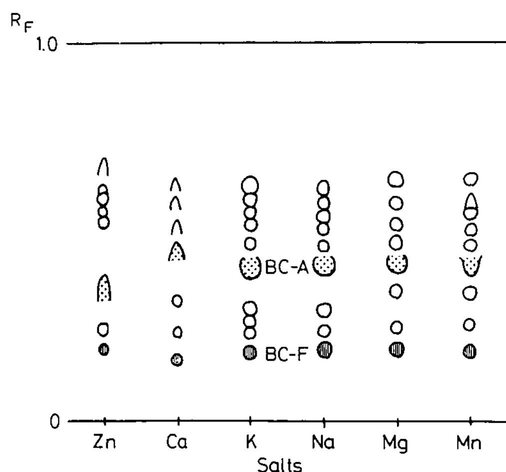

Fig. 3. Comparison of salts in the solvent system on the resolution of the spots. C_8 TLC plate; solvent system, methanol–0.1 *M* salt (pH 2.0) (7:3) containing 1.0% of triethylamine.

Addition of salt. Effect of the addition of zinc chloride, calcium chloride, potassium sulphate, sodium sulphate, magnesium chloride and manganese chloride on the separation and the shape of the spots was examined using methanol–0.1 *M* aqueous salt solution (pH 2.0) (7:3) containing 1.0% of triethylamine. As shown in Fig. 3, only when potassium sulphate was used we observe ten spots of the components on the plate. Next, the optimization of the concentration of aqueous potassium sulphate was investigated using methanol–aqueous potassium sulphate (pH 2.0) (7:3) containing 1.0% triethylamine. The best separation was obtained when a 0.1 *M* aqueous solution is used and we therefore chose 0.1 *M* aqueous potassium sulphate for subsequent work.

pH of aqueous solution. The influence of the pH of the aqueous solution on the separation and the shape of spots was investigated using methanol–0.1 *M* aqueous potassium sulphate (7:3) containing 1.0% triethylamine. The pH values were varied with 85% phosphoric acid as described under Experimental. The best separation of the components was given at pH 2.0 (Fig. 4).

Proportions of methanol and aqueous potassium sulphate solution. We investigated the influence of the proportions of methanol and aqueous potassium solution on the separation of the components using various mixtures of methanol–0.1 *M* aqueous potassium sulphate (pH 2.0) containing 1.0% of triethylamine as solvent systems. As shown in Fig. 5, the best separation was achieved when methanol and the aqueous solution were in the ratio 7:3. We therefore recommend methanol–0.1 *M* aqueous potassium sulphate (pH 2.0) (7:3) containing 1.0% of triethylamine as the solvent system for separating the components of BC on a C_8 HPTLC plate.

CONCLUSION

A technique for the identification of components of BC using NP- and RP-TLC plates has been established with the following characteristics. For NP-TLC, a solvent

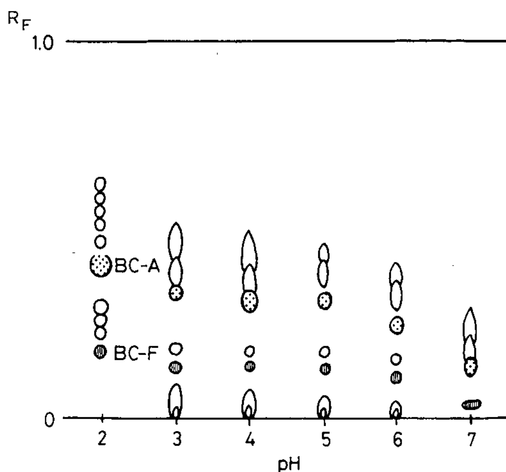

Fig. 4. Influence of pH of aqueous potassium sulphate solution. C_8 TLC plate; solvent system, methanol–0.1 *M* potassium sulphate (7:3) containing 1.0% of triethylamine.

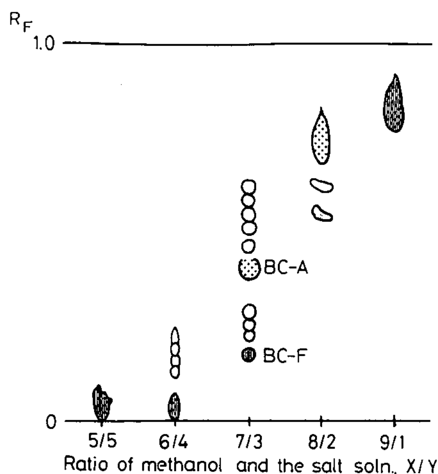

Fig. 5. Influence of ratio of methanol and 0.1 *M* aqueous potassium sulphate. C_8 TLC plate; solvent system, methanol–0.1 *M* potassium sulphate (pH 2.0) (*X*:*Y*) containing 1.0% of triethylamine.

system containing acetic acid made possible a reliable separation of the components of BC. The separation of the spots was dependent on the concentration of acetic acid and good separation was obtained at 0.75%. A combination of a silica gel TLC plate and chloroform–methanol–aqueous acetic acid (30:20:4) as the solvent system gave a satisfactory separation of the components. With respect to RP-TLC, a solvent system containing triethylamine and potassium sulphate gave a reliable separation of components. The separation and the shape of the spots were improved with increasing potassium sulphate and triethylamine concentrations and good results were obtained above 0.1 *M* and 1.0%, respectively. A satisfactory separation was achieved on a C_8 TLC plate using methanol–0.1 *M* aqueous potassium sulphate (pH 2.0) (7:3) containing 1.0% of triethylamine as the solvent system. Both TLC methods gave better separation of the components of BC than previously reported TLC methods. Because with this combination of NP- and RP-TLC techniques the identification of the components was able to be carried out simply, especially for BC-A and -F, we intend to apply it to the study of the structural characterization of the components of BC in the near future.

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Book Review

Flow perturbation gas chromatography (*Chromatographic Science Series*, Vol. 42), by N. A. Katsanos, Marcel Dekker, New York, Basle, 1988, XI + 304 pp., price US$ 99.75 (U.S.A. and Canada), US$ 119.50 (other countries), ISBN 0-8247-7833-2.

This book deals with the principles and applications of two techniques of physico-chemical measurement employing gas chromatographic instrumentation, *viz.*, stopped-flow and the reversed-flow gas chromatography. The latter technique was introduced in 1980 by the author of the present book. Both methods make use of perturbations imposed on the carrier gas flow.

The book opens with an introductory chapter on the theory of conventional gas chromatography. The second chapter presents a detailed theoretical analysis of ideal stopped-flow gas chromatography for different kinds of chemical reactions occurring in the stationary phase. An application of the stopped-flow gas chromatography to the measurement of diffusion coefficients is also discussed here.

The remainder of the book concerns the reversed-flow technique. In the third chapter, the general experimental setup is described and the general chromatographic sampling equation for the ideal reversed-flow gas chromatography is derived. In the remaining three chapters, the theory and applications of three different experimental arrangements of reversed-flow gas chromatography are described. References and symbols appear at the end of each chapter. Some repetition is therefore involved.

The central theme of the book lies in the theoretical treatments presented. For various experimental arrangements, the respective theoretical analyses lead to partial differential equations with constant coefficients. The equations are solved by successive Laplace transformations. Some of the inverse transformations are difficult but the remainder of the derivations are detailed enough to be followed even by readers who know only a few basic properties of the Laplace transformation.

The flow perturbation techniques were introduced primarily in order to study rate processes. In certain instances, however, they also lend themselves to the determination of some equilibrium quantities. This happens when the respective thermodynamic parameter enters the problem through the boundary conditions. In all instances, the theoretical analysis makes it possible to extract from experimental data an impressive amount of information on the process being studied.

Some minor comments about the book are as follows. The author's concept of ideal chromatography appears to differ from the common understanding of this term. Usually, the term implies the absence of *all* zone-broadening factors so that a chromatographic zone would move down the column without any changes in shape. In the author's classification, however, the term merely implies an instantaneous equilibration of the solute between the two phases, axial diffusion being allowed; therefore, the peaks would be Gaussian in linear ideal chromatography (*cf.*, p. 4).

The theoretical treatment of the measurement of diffusion coefficients by reversed-flow gas chromatography (Chapter 4, Section I.B) indicates that the method is

not sensitive to the particular mode of introduction of the solute into the diffusion column. This represents a significant advantage of the reversed-flow method over the Taylor dispersion technique.

Eqn. 1-52 is not a Gaussian function of t since the variance depends on t (*cf.*, eqn. 1-53).

It is difficult to agree with the claim (p. 202) that the gas-phase imperfection correction is not necessary in the determination of activity coefficients by reversed-flow gas chromatography. The partial molar excess enthalpies derived from the temperature dependence of activity coefficients are always of inferior accuracy compared with direct calorimetric results obtained with modern instrumentation. This statement is not limited to classical chromatographic techniques but applies regardless of the particular method of determination of the activity coefficients. Therefore, the gas-phase imperfection correction should be applied to activity coefficients whenever the partial molar excess functions are to be derived.

The heats of adsorption listed in Table 6.6 should carry a negative sign (*cf.*, p. 274 and eqn. 6-63).

The number of misprints is low given the large number of equations in the book.

To conclude, this beautiful book made a decisively positive impression despite the few minor criticisms voiced above. It presents an inspiring example of what human ingenuity can accomplish when combined with perseverance. Hence the author should be sincerely congratulated, and the book can be recommended to anyone interested in physico-chemical measurements by gas chromatography.

Brno (Czechoslovakia)

MICHAL ROTH
JAROSLAV JANÁK

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(Data Handling in Science and Technology, 2)

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