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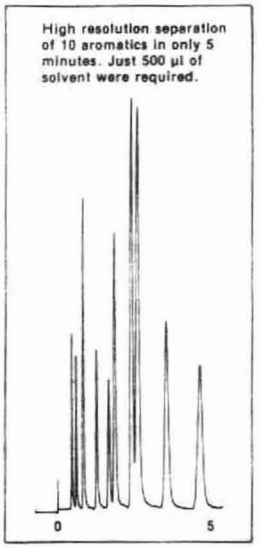



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TRIANGULAR CHARACTERIZATION OF GAS CHROMATOGRAPHIC STATIONARY PHASES

T. J. BETTS
School of Pharmacy, Western Australian Institute of Technology, Bentley, W.A. 6102 (Australia)
(Received September 25th, 1985)

SUMMARY

Three solute probes used by McReynolds, butanol, pyridine and octyne, were selected to check retention index (I_R) values on various packed columns. Their ratios proved more consistent than their absolute values. A triangular plot of relative I_R suggested three groupings of stationary phases: polar, non-polar and intermediate. Three selected polysiloxanes should suffice to represent these groups for most work.

INTRODUCTION

Shah *et al.*[1] recently published a Snyder Triangle method for characterization of gas chromatographic (GC) stationary phases. Snyder[2] constructed his triangular plot for evaluating solvents, not stationary phases, using 1973 data recorded by Rohrschneider[3]. Snyder used, in effect, the expression

$$x_e + x_d + x_n = 1 \quad (1)$$

where x_e is the fraction of the polarity index of the solvent contributed by its interaction with the test solute ethanol; and similarly for dioxane (d) and nitromethane (n). These x values were plotted triangularly in an eccentric manner, and allowed Snyder to circle eight groups of solvents inside his triangle. A resulting problem was that water and chloroform were grouped together! It is notable that the above three test solutes had been used by Rohrschneider[4] or McReynolds[5] as probes in their evaluations of GC stationary phases.

Klee *et al.*[6] in 1983 applied such triangular plots in an attempt to formulate a systematic approach to the selection of GC stationary phases. They used ΔI for x in formula 1, where "corrected indices for the solutes on a deactivated squalane column were subtracted" from the retention indices of the probe solutes on the test stationary phase. In their triangular plots they shift the spatial positions of Snyder's solvent group circles, including the reversal of numbering for the first two. The Klee *et al.*[6] plots in fact do not correspond to Snyder's[2], for an inspection of the latter's work shows two axes are for 0.23–0.73, whilst the x_d axis is for 0.04–0.54. The Klee *et al.* triangle has two axes of 0.2–0.7, with an x_d axis of 0.1–0.6, which shifts its

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position significantly. Their triangle is cut from a complete triangular plot near to the ethanol axis, and hence it is unrealistic for them to remark on the need for "representative phases ... lying in the corners of the selectivity triangle"[6]. They could have produced them by cutting a different triangle!

Shah *et al.*[1] studied the use of two more McReynolds solute probes, but never three together! It seems sensible to make use of the extensive literature on such probe ΔI values. Shah *et al.* concluded, not surprisingly, that "the classification of GC phases is solute dependent ... the selection of the solute probe can greatly affect the location of the stationary phase in the selectivity triangle". Equally so by changing the axes of the triangle!

In some much earlier work (1963) on stationary phases by Brown[7] retention volumes were used for values of x. Two of his solute probes were later chosen by Rohrschneider[4] or McReynolds[5]. Brown noted that "the non-polar phases are close the the *n*-decane apex (of the triangle) while the (electron) donor-type phases are towards the trichloroethane apex", an example being polyethyleneglycol.

The present work was undertaken initially to use and check published McReynolds values for x, to see if they could characterize GC stationary phases in a triangular plot. Three groups of GC stationary phases were determined as a consequence.

EXPERIMENTAL

Instrumentation

A Pye-Unicam GCD gas chromatograph was used, fitted with a flame ionisation detector. The recorder was a Hewlett-Packard 3390A integrator. The column oven temperature was always 120°C, the injection port temperature 225°C and the detector temperature 300°C. The mobile phase was high purity nitrogen at 40 ml min^{-1} for 4-mm I.D. columns (20 ml min^{-1} for the 2 mm I.D. columns, see Table I). Hydrogen was supplied to the detector at about 30 ml min^{-1}.

Columns

Packed columns from our laboratory "library" were used, some purchased over ten years ago, some newly prepared for this work. The GC stationary phase loadings ranged from 1.5 to 15% (when prepared) on 80–100 mesh supports, some silanized. Further details in Table I.

Solutes for injection

Volumes of 0.1–0.2 μl of commercial grade solutes were injected together with items from a "McReynolds' Kit" (PolyScience Corp.) using a Hamilton microsyringe. Inspection of published McReynolds' values[5] and some trials suggested the selection of the following three solute probes: (i) 2-octyne as an aliphatic, non-polar hydrocarbon showing more discrimination than a fully saturated substance (McReynolds' k), (ii) *n*-butanol as an aliphatic, oxygen-containing, electrophilic compound (McReynolds' y'), and (iii) pyridine as an aromatic, nitrogen-containing nucleophilic substance (McReynolds' s'). These three have fairly short retention times on various GC stationary phases at 120°C, and their elution sequence alters according to the type of stationary phase.

TABLE I

OBSERVED AND CALCULATED I_R AND x VALUES FOR SOME PACKED GAS CHROMATOGRAPHIC COLUMNS

x = (I_R of solute)/Σ(I_R three solutes). Values in italics calculated from literature[5,8]. (ΔI for phase + I_R squalane). UV absorbance studies[11] suggest the phenyl content of OV-17 is just over 40%. SP-2330 contains 2% carboxamide groups[12].

Stationary phase	*Column details*	*n-Butanol*		*Pyridine*		*2-Octyne*	
		I_R	x	I_R	x	I_R	x
Squalane	10% in 1.0 m × 4 mm I.D. steel	610 *590*	0.282 *0.277*	705 *699*	0.325 *0.328*	852 *841*	0.393 *0.395*
OV-1 (methyl polysiloxane)	2% in 1.5 mm × 4 mm I.D. glass	663 *645*	0.294 *0.287*	737 *741*	0.326 *0.329*	859 *864*	0.380 *0.384*
SE-30 (methyl polysiloxane)	2% in 1.0 m × 4 mm I.D. steel	645 *643*	0.287 *0.286*	743 *740*	0.330 *0.330*	860 *863*	0.383 *0.384*
SP-2100 (methyl polysiloxane)	2% in 1.0 m × 4 mm I.D. glass	651 *647*	0.288 *0.287*	746 *742*	0.331 *0.329*	860 *866**	0.381 *0.384*
OV-17 (phenyl-methyl polysiloxane)	1.5% in 1.5 m × 4 mm I.D. glass	747 *748*	0.293 *0.288*	871 *901*	0.342 *0.347*	930 *946*	0.365 *0.365*
SP-2250 (phenyl-methyl polysiloxane	3% in 2.0 m × 4 mm I.D. steel	738 *748*	0.288 *0.288*	887 *901*	0.346 *0.347*	936 *946**	0.366 *0.365*
OV-210 (trifluoropropyl-methyl polysiloxane)	15% in 1.5 m × 2 mm I.D. glass New column	815 *828*	0.304 *0.303*	977 *1009*	0.364 *0.369*	891 *897*	0.332 *0.328*
OV-225 (cyanopropyl-phenyl-dimethyl polysiloxane)	3% in 1.0 m × 4 mm I.D. glass New column	939 *959*	0.317 *0.316*	1046 *1085*	0.353 *0.357*	980 *991*	0.330 *0.327*
SP-2330 (nonacyanopropyl-methyl polysiloxane)	10% in 1.0 m × 4 mm I.D. steel New column	1263 *1315*	0.336 *0.333*	1397 *1477*	0.372 *0.375*	1097 *1153**	0.292 *0.292*
PEG 1000 (polyethylene glycol mol.wt. ≈ 1000)	10% in 1.5 m × 2 mm I.D. glass	1159 *1197*	0.337 *0.336*	1228 *1288*	0.357 *0.361*	1052 *1081*	0.306 *0.303*
PEG 20M (polyethylene glycol mol.wt. ≈ 15000)	10% in 1.0 m × 4 mm I.D. steel	1104 *1126*	0.332 *0.331*	1180 *1209*	0.355 *0.356*	1041 *1062*	0.313 *0.313*
DEGS (diethylene glycol-succinate polymer)	15% in 1.0 m × 4 mm I.D. steel	1229 *1323*	0.331 *0.333*	1363 *1490*	0.368 *0.375*	1115 *1162*	0.301 *0.292*

* Estimated values, none being found in the literature.

RESULTS AND DISCUSSION

Whilst the use of retention index (I_R) values should be more reliable than retention volumes, there seems to be no point in subtracting from them a constant set of I_R values for reference squalane to obtain ΔI. Thus the probe I_R values were calculated from the literature[5,8] and also determined experimentally. Discrepancies were found, in some cases, which do not seem to be due to the differing ages and histories of the columns used. Newly prepared columns are indicated in Table I, where average results are recorded. x values from this table are plotted in Fig. 1, where the axes have been selected to best display the results. The experimentally obtained x values are in good agreement with those derived from the literature, even though I_R values are not always close. Results for SE-30, in an old steel column, are in closest agreement. As the solute probe I_R values increase with the study of more polar phases, so do the discrepancies. These are negative for the polar stationary

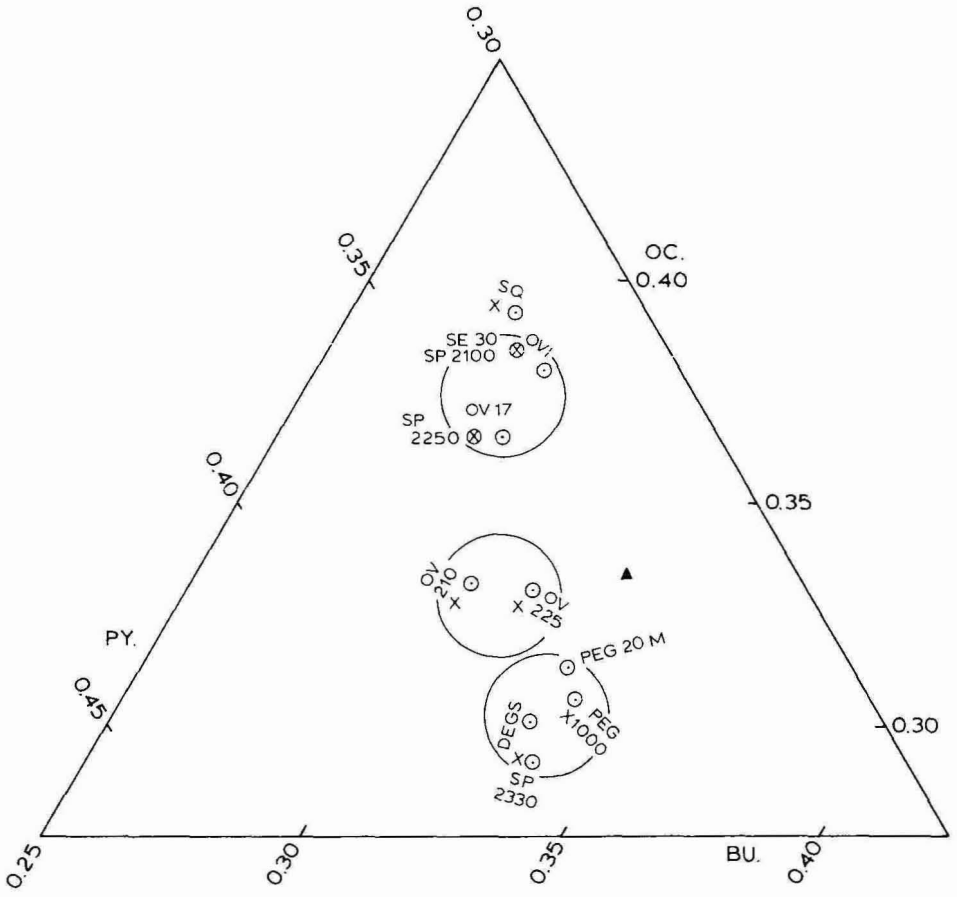

Fig. 1. Plot of relative I_R values of gas chromatographic stationary phases using solute probes of 2-octyne (OC.), *n*-butanol (BU.) and pyridine (PY.). (▲) Centre of full triangular plot; (⊙) experimental values; × values calculated from the literature, where different to experimental values. Given for squalane (SQ), OV-1, OV-17, OV-210, OV-225, PEG 1000, DEGS and SP-2330. See Table I for explanation of phases.

phases, but positive for the non-polar squalane. Shah *et al.*[1] reported great difficulty in obtaining I_R on squalane corresponding to "corrected" values of Klee *et al.*[6] and blamed this on free silanol support groups, and the need to deactivate the column wall. In theory, this should not matter, unless the probe solutes are retained differently to the reference alkanes used for I_R determinations. Also, theoretically, the column loading of stationary phase is irrelevant, for McReynolds[5] did not indicate what he used; nor if each column was freshly prepared for his studies, of the same dimensions, with the same mobile phase gas flow-rate. The only experimental detail in his paper is "all data were obtained at 120°C", and there is no indication of the reliability of his values, which have been quoted repeatedly in the literature without question. From the present work, it appears that ratios of I_R for several solutes may be more consistent than absolute I_R values. Second columns of SP-2100 and of PEG 20M gave very similar results to those quoted in Table I.

A GC stationary phase showing exactly equal affinity for each of the three solute probes will have the same x value for each, and will plot at 0.333 in the centre of the full triangle. As one x value increases, the other(s) must decrease, as the three total unity. The stationary phases coming nearest to 0.333 are polyethylene glycol 20M, and the cyanopropyl-, phenyl-, dimethylpolysiloxane OV-225, which are both favoured by the *British Pharmacopoeia*[9] in its monographs. Furthest removed from 0.333 are the fully methyl-polysiloxanes and, in a different direction within the triangle, the nonacyanopropyl-, methylpolysiloxane SP-2330. The lattermost forms a group of relatively polar GC stationary phases together with polyethylene glycols and the polyester phase which is enclosed on the triangular plot Fig. 1 with a circle of diameter 0.025 centred at about (0.365 pyridine, 0.333 butanol). The more stable polysiloxane phase could probably replace the others for general use as they all show the same sequence of solute probe retention times, octyne, butanol, pyridine (slowest), indicating their lack of affinity for the non-polar octyne.

The non-polar group of GC stationary phases is also enclosed by the same size circle in Fig. 1 centred at (0.375 octyne, 0.337 pyridine), consisting of the fully methyl and methyl-, phenylpolysiloxanes. Like squalane, their affinity for octyne is shown by the common solute sequence butanol, pyridine, octyne (slowest). This leaves an intermediate polarity group of phases characterised by the solute probe sequence butanol, octyne, pyridine. It includes the trifluoropropyl-, methylpolysiloxane OV-210, and OV-225 which are enclosed by a circle centred at (0.360 pyridine, 0.330 octyne). A conclusion that can be made is that GC laboratories, and *British Pharmacopoeia* monographs, could limit themselves to a "library" of columns of three types of polysiloxanes alone: the highly polar type with a high content of cyanopropyl side-chains; the low polarity type which are fully methyl; and an intermediate type with half the side chains trifluoropropyl groups. The only problem with the lattermost type of GC stationary phases is that a column loading of 15% or more is needed to avoid excessively brief retention times, and to show its "unique selectivity"[10].

Yancey[10] in a recent review of polysiloxane GC stationary phases notes the "selective retention of benzene" by cyanoalkyls, which is confirmed in this work by pyridine showing the highest I_R and x value of any phase examined. These stationary phases also show "a shorter retention for an acetylinic compound" confirmed by the SP-2330 x value for octyne.

The present author was dismayed to see that Klee *et al.*[6] concluded by antici-

pating a "computerised optimization" of mixed GC stationary phases. There are already far too many; let us not mix them! McReynolds in his paper, which consists mainly of a table of results, concluded that it would "show the similarity of many liquid phases now in use. It is hoped that this data will help to reduce the number of liquid phases being used"[5]. The publisher of the journal, Preston[13], supported this and suggested "selecting 10 or 20 of the most frequently used liquid phases and designating these as preferred". He listed those most used from recent (1969) publications, and they included the polysiloxanes SE-30 (fully methyl), QF-1 (partly trifluoropropyl), and XE-60 (partly cyanoethyl). These correspond to the three types recommended in this paper, fifteen years later!

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CHARACTERIZATION OF FUSED-SILICA CAPILLARY TUBING BY CONTACT ANGLE MEASUREMENTS

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SUMMARY

The capillary rise method has been used to obtain contact angle measurements on untreated fused silica and fused silica treated with a variety of deactivating reagents. The contact angle data were used in the construction of Zisman plots which allowed characterization of the wettability of the surfaces by their critical surface energies. The wettability of raw fused silica was found to be widely variable which adversely affects attempts to fully deactivate the surface. Hydrothermal treatment of the fused silica with nitric acid was found to be adequate for cleaning and hydroxylating the surface so as to allow complete deactivation. Simple silylating reagents, cyclic siloxanes, and polysiloxanes covering a wide range of polarity were used and evaluated as deactivating reagents.

INTRODUCTION

The production of high-efficiency capillary columns requires that the stationary phase be deposited as a thin, homogeneous film on the surface of the capillary tubing. To ensure a reasonable column lifetime, the stationary phase film must retain its uniformity through repeated temperature programming and large volume injections of a wide range of solvents. The ability of the column to resist performance degradation due to high-temperature rearrangement or washout of the phase can be dramatically improved by cross-linking or immobilizing the stationary phase inside the column. However, the initial attainment of a smooth, homogeneous film is dependent upon the ability of the stationary liquid to completely wet the surface of the tube wall. The importance of this wettability was first suggested in 1962 by Farre-Rius *et al.*[1] who used the concept of a Zisman plot[2] to determine the critical surface energies of various tubing materials. Since that time, a number of workers have used this method to determine the degree of wetting of glass surfaces by stationary phases[3–8].

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The technique of capillary rise has been used for the characterization of actual capillaries made of glass[4,9–11] and fused silica[11,12].

Because of the relatively high surface energy of fused silica[11], most stationary phases will wet the surface. However, columns coated without deactivation of the surface most often show undesirable activity toward sensitive analytes. Efforts to deactivate the surface while maintaining or enhancing wettability have been at the forefront of capillary column research for many years now.

Wettability of capillary columns by stationary phases can generally be enhanced in one of two ways: roughening the interior surface of the tubing or chemical modification of the surface. Increased roughness has been related to increased wettability by a roughness factor introduced by Wenzel[13] and further discussed by Cassie[14]. Roughening can be accomplished either by etching the inside surface or by deposition of a microcrystalline structure on the surface. These methods are summarized by Lee *et al.*[15] but are generally not applicable to fused-silica tubing material.

Chemical modification of fused silica is at present the most satisfactory technique for both the deactivation and the enhancement of wettability of the surface. Replacement of surface hydroxyl groups with silyl ether groups (silylation) containing functional groups similar or identical to those in the stationary phase is the most successful. Simple silylating reagents such as disilazanes, disiloxanes, and chlorosilanes have been widely used[16–19]. Other useful deactivating procedures include the thermal degradation of polysiloxane stationary phases[20] and the thermally induced ring opening of cyclic siloxanes[21–23].

In this paper we report the use of the capillary rise method to obtain data for the construction of Zisman plots to characterize both raw fused silica and fused silica subjected to various hydrothermal and deactivation procedures. Consistent with the high degree of surface coverage demonstrated, highly efficient and inert capillary gas chromatographic (GC) columns are produced which show chromatograms free from both reversible and irreversible adsorption of nanogram levels of alcohols, amines and acids.

THEORY

The theory of wetting at the solid–liquid interface is discussed in detail by Shaw[24] and Adamson[25] and summarized[11] elsewhere. For the sake of brevity, we present only the salient features here.

In the wetting of a solid by a liquid, the liquid spreads so as to increase the solid–liquid and liquid–gas interfacial areas while decreasing the solid–gas interfacial area. The surface tension of a liquid, γ_l, is the force per unit length on the liquid surface that opposes the expansion of the liquid surface area.

At a liquid–solid–gas interface there is a characteristic contact angle θ. The contact angle is defined as the angle formed between the tangent to the liquid drop and the solid surface, as drawn through the liquid. For a liquid that completely wets the surface the contact angle is zero. The tendency for the liquid not to wet the surface increases with the contact angle. This situation arises when the cohesive forces of the liquid are greater than the adhesive forces between the liquid and solid. Therefore, the contact angle is an inverse measure of the wettability of the solid by the liquid.

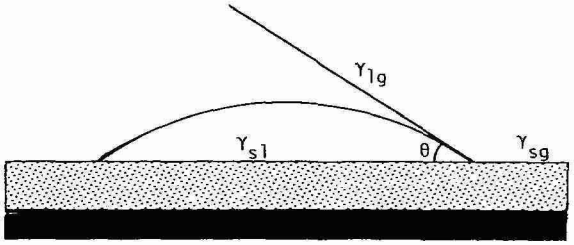

Fig. 1. Drop of liquid in contact with a solid surface showing surface tension of the liquid in equilibrium with its vapor (γ_{lg}), surface tension of the solid in equilibrium with vapor (γ_{sg}), surface tension of the solid in equilibrium with the liquid (γ_{sl}), and the contact angle (θ).

Fig. 1 shows a liquid drop in equilibrium with a solid surface and gas. At equilibrium the three interfacial tensions must balance along the line of contact (Fig. 2) so that:

$$\gamma_{sg} = \gamma_{sl} + \gamma_{lg}\cos\theta \tag{1}$$

where γ_{sg} is the surface tension of the solid in equilibrium with gas (vapor of the wetting liquid); γ_{lg} is the surface tension of the wetting liquid in equilibrium with its vapor; and γ_{sl} is the surface tension of the solid in equilibrium with the liquid.

Rearranging eqn. 1 gives the so-called Young's equation:

$$\cos\theta = \frac{\gamma_{sg} - \gamma_{sl}}{\gamma_{lg}} \tag{2}$$

Young's equation holds only if the solid is partially wet by the liquid; it fails if the solid is completely wet ($\cos\theta = 1$) or if the solid is not wet at all ($\cos\theta < 0$). The cosine of the contact angle can thus be used as a direct measure of the surface wettability.

The determination of θ (or $\cos\theta$) for liquids in contact with the inner surface of fused-silica capillary tubing can be made accurately by the technique of capillary rise since fused silica is inherently straight.

A capillary tube of radius r is shown immersed in a liquid of density ρ in Fig. 3. If the liquid at least partially wets the tube, the liquid rises. It continues to rise until the force due to the surface tension which is pulling the liquid upward is coun-

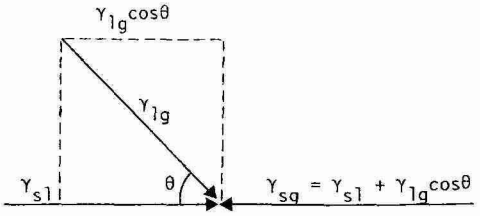

Fig. 2. Vector diagram illustrating the balance of the solid–liquid–gas interfacial tensions at equilibrium.

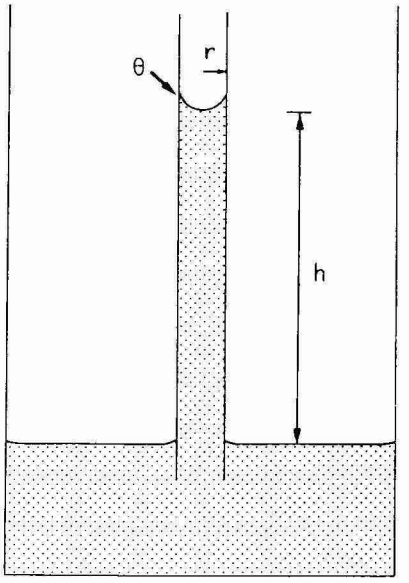

Fig. 3. Diagram of capillary rise experiment showing fused-silica capillary immersed in a reservoir of liquid (methanol–water mixtures).

terbalanced by the downward pull of the force of gravity. The height of rise of liquid in the tube is represented by h and is measured from the surface of the liquid to the lowest point of the meniscus. Only the vertical component of the upward force is effective in causing the liquid to rise up the capillary so that the upward force is $2\pi r\gamma_l \cos\theta$. The downward force is given by $\pi r^2 h\rho g$ where g is the acceleration of gravity. As before, at equilibrium these forces must balance so that:

$$2\pi r\gamma_l \cos\theta = \pi r^2 h\rho g \tag{3}$$

or

$$\cos\theta = \frac{h\rho g r}{2\gamma_l} \tag{4}$$

The exact solution for capillary rise[25] includes a correction for the deviation of the meniscus from sphericity and would use the density difference ($\Delta\rho$) between the liquid and the surrounding vapor instead of simply the liquid density. Good discussions of experimental aspects of the capillary rise method have appeared in the literature[26,27].

A useful parameter for characterizing the wettability of solid surfaces is that of the critical surface tension (or energy), γ_c, as proposed by Zisman and co-workers[2,28]. Critical surface energies are obtained from plots (Zisman plots) of $\cos\theta$ *vs.* γ_l for various liquids. In most cases these plots are linear and can be extrapolated to zero θ ($\cos\theta = 1$). The γ_l value corresponding to $\cos\theta = 1$ was proposed by Zisman to be characteristic of the particular solid and was designated the critical surface tension. The significance of the critical surface energy is that, in general, liquids with

$\gamma_l \leqslant \gamma_c$ will completely wet the surface while liquids with $\gamma_l > \gamma_c$ will wet the surface incompletely.

If the surface under investigation is heterogeneous, *i.e.*, composed of two types of material, then the observed contact angle for the composite surface is given by:

$$\cos \theta_{obs} = \sigma_1 \cos \theta_1 + \sigma_2 \cos \theta_2 \tag{5}$$

assuming the heterogeneities are effectively averaged and σ_1 and σ_2 are the fractional surface areas of each type of material with their respective contact angles (θ_1 and θ_2). The slopes of the Zisman plots can be used to determine the relative coverage of the two surface types if $\theta = 0$ for one of the surface structures and γ_c is the same for both the heterogeneous surface under study and a surface comprised of entirely type 1 structures, as has been described by Bartle *et al.*[11]. For this limiting case, if $\theta_2 = 0$ for the bare silica surface, then the magnitude of $\cos \theta_{obs}$ will decrease as σ_1 increases. As a result, the relative degree of surface coverage of the type 1 groups on two surfaces can be assessed from the ratio of the slopes of the two Zisman plots by the relationship:

$$\sigma_1 = \frac{\cos \theta_{obs} - 1}{\cos \theta_1 - 1} = \frac{\text{slope of Zisman plot for heterogeneous surface}}{\text{slope of Zisman plot for type 1 surface}} \tag{6}$$

This type of approach is used to assess the relative surface coverage of fused silica by different classes of silylating reagents carrying the same functional groups; the more negative the slope, the higher the relative surface coverage.

EXPERIMENTAL

Fused-silica capillary tubing (0.25 mm I.D.) was obtained from Polymicro Technologies (Phoenix, AZ, U.S.A.). Deactivating reagents used were: trimethylchlorosilane, hexamethyldisiloxane, octamethylcyclotetrasiloxane (D_4), octamethylcyclotetrasilazane, tetravinyltetramethylcyclotetrasiloxane, (3,3,3-trifluoropropyl) methylsiloxanes (mixed cyclics, F_3/F_4), octaphenylcyclotetrasiloxane (Ph_4), and tetrakis (β-cyanoethyl) tetramethylcyclotetrasiloxane from Petrarch Systems (Bristol, PA, U.S.A.); hexamethyldisilazane (HMDS) and hexamethylcyclotrisiloxane (D_3) from Aldrich (Milwaukee, WI, U.S.A.); SF-96 (dimethylpolysiloxane) and Carbowax 20M from Foxboro/Analabs (North Haven, CT, U.S.A.); Superox 20M and Superox 4 from Alltech/Applied Science (Deerfield, IL, U.S.A.); and diphenyltetramethyldisilazane (DPTMDS) from Fluka (Hauppauge, NY, U.S.A.).

Fused-silica treatment

Some tubing was rinsed with water followed by methanol while other tubing was subjected to intense hydrothermal treatment at 200°C with 20% nitric acid before deactivating as described elsewhere[29]. In all cases the tubing was dehydrated at 225°C for 90 min under nitrogen flow before proceeding.

To accomplish deactivation, a plug of the deactivating reagent was pushed

through the column with nitrogen. Reagents which are liquids at room temperature were used neat with the column ends flame-sealed immediately after expelling the reagents. Solid reagents were dissolved in either methylene chloride or pentane and the ends were sealed after 30 min of drying under nitrogen flow at 25°C for pentane and 40°C for dichloromethane solutions. Tubing to be heated above 350°C was wrapped in a protective jacket of aluminum foil and purged with nitrogen while

TABLE I

SUMMARY OF CRITICAL SURFACE ENERGY (C.S.E.) DETERMINATIONS FOR VARIOUS DEACTIVATION REAGENTS AND CONDITIONS

Surface	*Deactivant*	*Fused silica pretreatment*	*Temp. (°C)/Time (h)*	*C.S.E. (dyn/cm)*	*Slope (× 10^{-3} cm/dyn)*
1	None	None		28–48	
2	None	a[§]		44	
3	None	b[§]		46	
4	D_4*	a	400/1.5	23	−35
5	D_4*	b	400/1.5	21	−37
6	D_4	b	400/1.5	21	−68
7	D_3*	a	410/2	23	−49
8	D_3	b	400/1.5	21	−9
9	Hexamethyldisiloxane	b	400/1.5	21	−73
10	TMCS	b	400/1.5	21	−53
11	TMCS	a	150/0.5	25	−38
12	SF-96	b	400/1.5	20	−73
13	HMDS	b	400/1.5	20	−77
14	Octamethylcyclotetrasilazane (2% in pentane)	b	400/1.5	20	−80
15	Tetravinyltetramethylcyclotetrasiloxane (10% in pentane)	a	390/1.5	22	−54
16	F_3/F_4	b	400/1.5	22	−54
17	DPTMDS	a	400/10	25	−40
18	DPTMDS	b	400/10	26	−58
19	Ph_4 (10% in dichloromethane)	a	400/1.5	29	−49
20	Tetrakis (β-cyanoethyl) tetramethylcyclotetrasiloxane (2% in dichloromethane)	b	280/16	32	−24
21	Cyclic mixture**	b	400/1.5	28	−46
22	Polysiloxane***	b	400/1.5	21	−65
23	Carbowax 20M (2% in dichloromethane)	b	280/16	43	−8
24	Superox 20M (2% in dichloromethane)	b	280/16	44	−16
25	Superox 4 (0.25% in dichloromethane)	b	280/16	34	−14

* Measured with homologous series of alkanes.

** 7% Tetrakis (β-cyanoethyl)tetramethylcyclotetrasiloxane, 7% Ph_4, 2% tetravinyltetramethylcyclotetrasiloxane, and 84% D_4–2% in dichloromethane.

*** 7% Cyanoethyl, 7% phenyl, 1% vinyl, 85% methyl polysiloxane–2% in pentane.

§ Treatment a: water followed by methanol rinse. Treatment b: HNO_3 at 200°C for 10 h.

TABLE II

SURFACE TENSIONS (γ_l) AND DENSITIES (ρ) OF THE METHANOL–WATER MIXTURES USED IN THE CAPILLARY RISE EXPERIMENTS[30]

Composition methanol–water	γ_l *(dyn/cm)*	ρ *(g/ml)*
100:0	22.1	0.792
90:10	24.9	0.820
75:25	28.3	0.859
70:30	29.7	0.872
60:40	32.6	0.894
50:50	34.9	0.916
25:75	45.8	0.962
20:80	51.6	0.967
10:90	58.5	0.982
0:100	72.0	1.000

others were placed in an oven directly and heated at 10°C/min to various final temperatures and held for various lengths of time. After cooling to room temperature, the columns were rinsed with dichloromethane and briefly dried at 100°C under nitrogen flow. The individual conditions are noted in Table I.

The capillary rise method was used to measure the advancing contact angle of methanol–water mixtures on the various surfaces at a temperature of 25° ± 1°C. Surface tensions and densities of the mixtures are listed in Table II. The height of rise of the meniscus from the surface of the liquid was measured to ±0.01 cm with a cathetometer. Approximately 15 ml of solution was placed in a 100-ml color comparison tube and supported on a ring stand. A thermometer was placed through one hole of a three-hole No. 6 rubber stopper, a right angle 1/4-in. open glass tube placed through another hole and a 1/8-in. Swagelok union was threaded through the third hole. Sections of fused silica were held in place in the 1/8-in. union with a 1/8 in. × 0.4 mm graphite reducing ferrule. Frontal illumination against a white background allowed easy location of the meniscus in the capillaries.

RESULTS AND DISCUSSION

In utilizing the contact angle approach for characterizing fused-silica surfaces, there are several characteristics that must be emphasized. First of all, γ_c need not be independent of the probe liquids used and data obtained from Zisman plots using different liquids should not be quantitatively compared. This is apparent from a comparison of surfaces 5 and 6. Secondly, any data point for which $\cos\theta = 1$ cannot be used since γ_c must be obtained from extrapolation and finally, the extrapolation to γ_c should be kept short and in the linear region. There are some wettability studies which have appeared in which one or more of these guidelines apparently have been ignored.

The linearity of all the Zisman plots reported in this study was quite good. Correlation coefficients were 0.990 or better for all plots except for surface 7 which had a correlation of 0.98.

As previously mentioned, the exact solution for capillary rise takes into ac-

count a correction for the deviation of the meniscus from sphericity. For capillaries of 0.25 mm I.D., this correction would have added only 0.004 cm to the actual height measured and in all measurements here it was neglected. This factor, along with a small error encountered by not being able to exactly locate the level of the surface of the reservoir when sighting through the glass reservoir, can be corrected for by applying calibration measurements on capillaries of identical diameters with liquids which completely wet the surface[9–12]. Values obtained from eqn. 4 differed by less than 0.01 in cos θ from those obtained using calibration and thus calibration was deemed not necessary.

Critical surface energies and the slopes of the Zisman plots are given in Table I. As seen from the first entry, the critical surface energy of the raw fused silica is widely variable with values ranging from 28 to 48 dyn/cm. The majority of test pieces were determined to have energies in the low to mid 40's. These values are lower than those found by Lee and co-workers[11] who measured surface energies for some tubing to be greater than 72 dyn/cm. It is known that clean glass is a high energy surface[31], but as a result of this high energy the glass surface can easily be converted to a lower energy surface through adsorption and hydration[32,33]. Such processes can certainly occur during the handling and storage of the fused silica and are the probable cause for the discrepancies in wettability noted between different manufacturers of fused silica and also within material from the same manufacturer. This variability is also suspected to be the major factor in irreproducible deactivations and coatings noted by us and others[29,34].

Rinsing the fused silica was effective in restoring a moderately wettable surface (No. 2) but still showed insufficient deactivation (compare surface 4 with 5, 7 with 8, and 10 with 11). Methods to activate the raw fused-silica were investigated[29] and a 20% nitric acid hydrothermal treatment at 200°C for 10 h was found to yield a reproducible high energy surface (No. 3) that could be completely deactivated.

Eqns. 5 and 6 can be used to compare the relative surface coverages for surfaces that have the same γ_c. Comparing the extent of methylation for various deactivating reagents by this approach shows that for surfaces with γ_c = 21 dyn/cm (surfaces 6, 8, 9 and 10) the degree of coverage appears to decrease in the order hexamethyldisiloxane > D_4 > TMCS > > D_3. For surfaces with γ_c = 20 dyn/cm (surfaces 12–14) the degree of coverage appears to decrease in the order octamethylcyclotetrasilazane > hexamethyldisilazane > SF-96. It should be noted that the differences among any of these are not large and all are adequate for a high degree of surface coverage. The only treatment which failed to give a high degree of coverage was the D_3 (surface 8).

The high degree of deactivation of fused silica with D_4 is evident from a comparison of surfaces 5 and 6. The equivalence of γ_c for D_4 treated surfaces measured with alkanes (No. 5) and methanol–water mixtures (No. 6) is indicative of the film building tendency of cyclic siloxane deactivants as noted by Blomberg[35] and Lee *et al.*[36]. As discussed by Kováts and co-workers[9,10], the shielding of a siliceous surface by bonded methyl silyl groups is insufficient to prevent the interaction of polarizable and polar wetting agents (such as the methanol–water mixtures) with the bare silica surface. As a result, as the underlying dipole layer becomes more and more shielded, the difference between γ_c measured with alkanes and γ_c measured with more polar liquids will decrease.

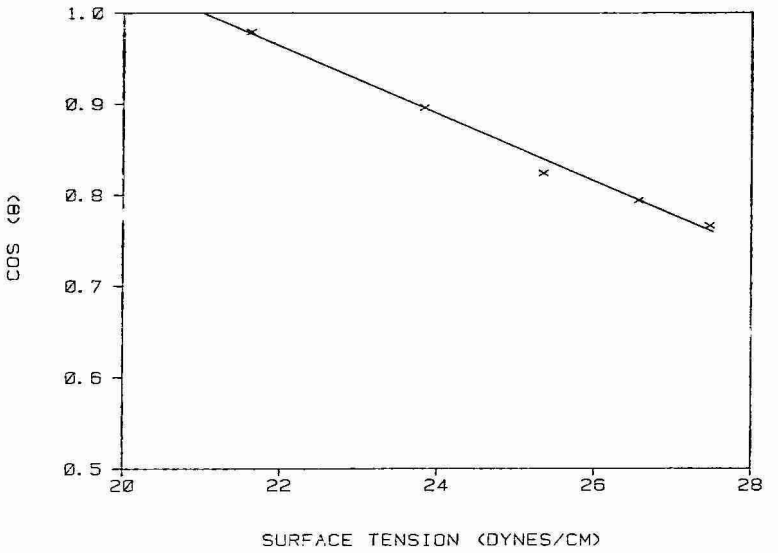

Fig. 4. Zisman plot obtained from fused silica hydrothermally treated with nitric acid and deactivated with D_4 (surface 6).

The Zisman plot for D_4 on an acid treated fused-silica surface (6) is shown in Fig. 4. Fig. 5 shows a chromatogram of the Grob comprehensive test mix II[37] on this same surface coated with OV-73 and cross-linked with azo-*tert.*-butane (ATB). The excellent inertness of the column is evident from the perfect elution (height and

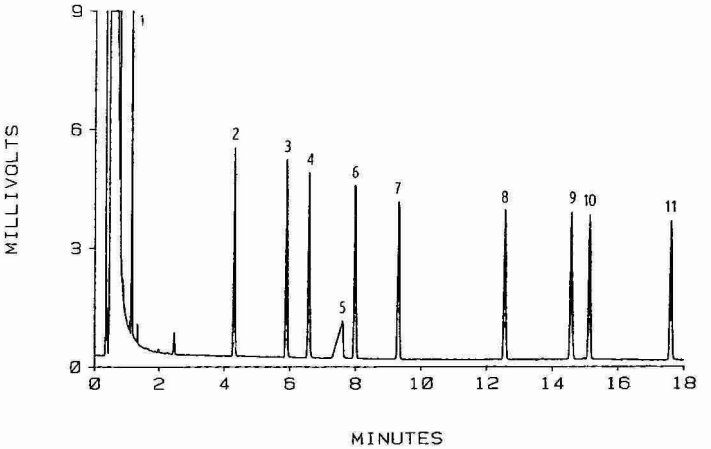

Fig. 5. Chromatogram of Grob Test Mix II. Column: 10 m × 0.25 mm I.D. fused silica, hydrothermally treated with 20% nitric acid at 200°C for 10 h, D_4 deactivated, coated with OV-73 (0.25 μm) and cross-linked with azo-*tert.*-butane. Temperature programmed from 40° to 130°C at 5°/min with H_2 carrier gas at 50 cm/s (measured at 40°C); 1 μl injection split 20:1; flame ionization detection. Peak identifications: 1 = 2,3 butanediol (2.7 ng), 2 = *n*-decane (1.4 ng), 3 = 1-octanol (1.8 ng), 4 = 2,6 dimethylphenol (1.6 ng), 5 = 2-ethylcaproic acid (1.9 ng), 6 = 2,6 dimethylaniline (1.6 ng), 7 = *n*-dodecane (1.5 ng), 8 = methyl decanoate (2.1 ng), 9 = dicyclohexylamine (1.6 ng), 10 = methyl undecanoate (2.1 ng), 11 = methyl dodecanoate (2.1 ng).

symmetry) of all the components of the mixture except for the free acid which is obviously overloaded. Examination of the area counts for the 2-ethylcaproic acid peak shows no evidence of irreversible adsorption.

Surfaces 4, 5; 7, 8; and 10, 11 cannot be compared by using the slopes of the Zisman plots since the critical surface energies are not the same. At first glance this might appear to be a failure of eqns. 5 and 6. However, this is not the case since in obtaining 6 from 5 it was assumed that the surface contained only two types of surface structures; one that was completely wet ($\theta_2 = 0$) and one that was incompletely wet ($\theta_1 > 0$). The values of γ_c obtained for surfaces 4, 7, and 11 are indicative of more than two types of surface structures present on the fused silica. Therefore, eqn. 5 must be expanded to include at least one additional term:

$$\cos \theta_{obs} = \sigma_1 \cos \theta_1 + \sigma_2 \cos \theta_2 + \sigma_3 \cos \theta_3 \quad (7)$$

where σ_3 is now the fractional area occupied by the type 3 surface and θ_3 is the contact angle characteristic of this surface. To probe the methylated surfaces, methanol–water mixtures were used which covered the range of 22–30 dyn/cm. If we arbitrarily assign σ_1 to be the fractional area occupied by methyl groups ($\gamma_c = 21$ dyn/cm) we see that each of the probe liquids used will incompletely wet σ_1. If σ_2 is the fractional area occupied by the bare silica surface (which is composite but is treated as a single structure, $\gamma_c \approx 45$ dyn/cm) each of the probe liquids used completely wets σ_2. If σ_3 were completely wet by the range of liquids used then eqn. 6 would still hold. Therefore, the surface energy of the structure giving rise to σ_3 must lie within the range of the surface tensions of the probe liquids used. In cases such as this the success of deactivation must be judged by the value of γ_c and not the slope of the Zisman plot. Whatever the surface structure is that gives rise to this phenomenon, the water and methanol rinses were not sufficient to remove it whereas the 20% nitric acid treatment was. This type of hydrothermal treatment then appears to be a necessary precaution to not only fully hydroxylate the surface[29] but also to clean it.

The surface energy of fused silica can be increased from 21 dyn/cm by the use of deactivants with functional groups more polar than methyl groups. Surface 16 was deactivated with (3,3,3-trifluoropropyl) methyl cyclic siloxanes which yields a surface compatible with the trifluoropropyl containing phases such as OV-210 and OV-215[22,38].

Higher surface energies are obtainable by utilizing deactivants with phenyl substituents. The entirely phenyl substituted cyclic tetramer (Ph_4) gave a critical surface energy of 29 dyn/cm. Diphenyltetramethyldisilazane (surfaces 17 and 18) is an excellent silylating reagent for introducing some phenyl substitution onto the surface. The conditions used here for the fused silica are analogous to those developed by Grob and Grob[17] for glass. As expected, the surface which was acid treated prior to deactivation is superior to the rinsed surface. We have used this deactivation procedure with only a 90 min hold at 400°C successfully with OV-73 as the liquid phase.

Recently there has arisen some controversy over the use of DPTMDS in improving wettability of glass and fused silica surfaces. Rutten *et al.*[39] have claimed that silylation with DPTMDS at temperatures of 400°C yields a surface identical to that formed when HMDS is used due to the loss of phenyl groups by DPTMDS. Our

results clearly demonstrate the increased wettability resulting from silylation with DPTMDS (surface 18 over surface 13) at least with fused silica as the substrate. These quantitative results are in agreement with the recent qualitative studies of Grob and co-workers[40,41].

The most polar cyclic siloxane we have used for deactivation is the tetrakis (β-cyanoethyl)tetramethylcyclotetrasiloxane (surface 20). A reaction temperature of 280°C for 16 h gives a good deactivation with a surface energy of 32 dyn/cm.

The Carbowax pyrolysis procedure of Cronin[42] was studied and compared with identical treatments for Superox 20M and Superox 4 (surfaces 23–25). The Carbowax 20M and Superox 20M both give very high surface energies which would be wettable by most stationary phases commonly used in fused-silica capillary columns. However, the temperature limit of a Carbowax 20M or Superox 20M pretreated column would now be that of the deactivation layer (about 250°C) and not that of the stationary phase. Also, the polarity of columns coated with the more non-polar phases is affected by the polyethylene glycol (PEG) pretreatment. For these reasons the PEG type deactivating procedures are not commonly used. There is a current interest in our laboratory in the use of these materials for the deactivation of fused-silica columns for supercritical fluid chromatography where the increased strength of the mobile phase as compared to GC minimizes the observance of column polarity changes and where column temperatures rarely exceed 150°C. Preliminary results with Carbowax 20M deactivation coupled with a cross-linked film of OV-1701 are quite encouraging[43].

Because of its higher molecular weight (4×10^6), Superox 4 has been used for production of glass and fused-silica columns of moderate to high polarity[44–46]. In our experience Superox 4 does not yield surfaces with as high a surface energy as Carbowax 20M or Superox 20M. Solutions of Superox 4 used for dynamically coating the capillaries for deactivation were more dilute than the Carbowax 20M and Superox 20M solutions due to the very high viscosities of the Superox 4 solutions.

The final comparison to be made is between the use of an intermediate polarity phase for polysiloxane degradation type deactivation *versus* a mixture of cyclic siloxanes. The stationary phase was synthesized utilizing cyclic siloxanes as the starting materials by a procedure described elsewhere[47]. The phase was cyanoethyl–phenyl–methyl–vinyl polysiloxane (cyanoethyl analog of OV-1701) (7:7:85:1). The mixture of cyclics used for deactivation was made to give equivalent percentages of each functional group from tetrakis (β-cyanoethyl)tetramethylcyclotetrasiloxane, tetravinyltetramethylcyclotetrasiloxane, Ph_4, and D_4. As seen from a comparison of surfaces 21 and 22, the use of the cyclic mixture yielded a fused-silica surface of moderately high energy which produced a stable and efficient column when coated with the phase. The polysiloxane degradation of the phase yielded a surface with very low energy (equivalent to methylation with a high surface coverage, see surface 6, etc.) which could not be coated efficiently with the phase. A similar phenomenon was noted for OV-17 on fused silica by Verzele *et al.*[48]. Presently we are unable to explain this observation.

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CHROM. 18 289

INFLUENCE OF ADDITIVES TO THE ELUENT ON HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF SIMPLE COMPOUNDS

III. THERMODYNAMICS OF INTERACTION OF *n*-ALCOHOLS WITH OCTYL-AGAROSE IN MIXTURES OF WATER WITH METHANOL, UREA OR ETHYLENE GLYCOL

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SUMMARY

Standard thermodynamic quantities for the interaction of *n*-alcohols with octyl-agarose in aqueous mixtures of methanol, ethylene glycol and urea are calculated from retention data at different temperatures. The use of Van 't Hoff plots is discussed. Results are compared with literature data and are interpreted with a simple model of hydrophobic hydration.

INTRODUCTION

This paper is the third in a series on the influence of organic additives in hydrophobic interaction chromatography with octyl-agarose. So far, we have studied the properties of the layer of octylglycidyl groups (OG) at 25°C. It was concluded that the retention can be described by a liquid–liquid partition model. The layer of OG has liquid properties closely resembling those of *n*-octanol. The variation of the partition constant with the composition of the eluent is caused solely by the variation of the activity coefficient of the solute in the eluent[1], even if the co-solvent is strongly sorbed by the stationary phase[2].

The present paper describes the influence of the temperature on the chromatography of *n*-alcohols in aqueous mixtures of methanol, urea, ethylene glycol and, for the sake of comparison with (scarce) literature values[3], in a single water–dimethylformamide (DMF) mixture. The first three are of interest in chromatography, as they have been used to elute sorbed protein[4–6], and DMF has been proposed as a reference co-solvent in calorimetric determinations of enthalpies of solution[7]. Thermodynamic standard quantities can be calculated from the temperature dependence of the measured elution volumes. The standard states of the solutes in the layer of OG are not subject to variations of sorbed amounts of additive with temperature, or with eluent composition, because of the low affinity of these co-solvents for the layer of OG. The

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decrease of the retention with increasing amounts of co-solvent can then be interpreted by a simple model, and the results can be compared with literature data on the vaporization of the *n*-alcohols from the partly aqueous solvents.

The thermodynamics of the interaction in an aqueous 25 m*M* phosphate buffer[8] has been described before. It was demonstrated that the values of ΔH°, ΔS° and ΔC_p° show all the characteristics of the transfer of an apolar solute from water to an organic medium, closely resembling *n*-octanol.

THEORETICAL

It is our aim to calculate values of ΔG°, ΔH°, ΔS° and ΔC_p° (henceforth denoted as ΔX°) from the variation of the retention volumes with temperature and to interpret these data as a function of the amount of co-solvent in the eluent. First, a discussion about the difficulties and the reliability of the method is given.

It is obvious that the standard state of a solute in the stationary phase must preferably not depend on the possible variation with temperature of the sorbed amounts of co-solvent, $W_{s,A}^\circ$ (grams of A per gram of OG groups). Such a variation would have a temperature-dependent impact on the retention volumes that would lead to corresponding trends in values of $\Delta G^\circ/T$ as a function of $1/T$ and, hence, to values of ΔX° that are difficult to interpret. It should be noted that such difficulties occur particularly with batch partition experiments in, *e.g.*, the octanol–water system. For in those systems, contrary to the chromatographic system with its bonded phase, the organic phase also may dissolve in the aqueous phase. Further, the experimental problems associated with batch partition experiments are much larger.

Fortunately, there exists a class of co-solvents that do not sorb on the layer of OG, *i.e.* electrolytes and highly polar organic substances such as sucrose and urea. In that case, the properties of the adsorbent resemble those of pure *n*-octanol at the same temperature, irrespective of the amount of co-solvent in the eluents, thus the same standard state in the stationary phase holds at all eluent compositions and temperatures. With methanol or ethylene glycol, however, where $W_{s,A}^\circ$ is not exactly zero, the possible influence of a small amount of these co-solvents in the layer of OG needs careful attention before the results are interpreted (see Results and discussion).

The density, d_{OG}, of the stationary phase is another topic that deserves attention. In previous papers[1,2], the mole fraction partition constant $K_{X,B}$ has been proved suitable for the description of the chromatographic system at a constant temperature. The value of d_{OG} is eliminated in that quantity. However, the temperature dependence of $-RT \ln K_{X,B}$ cannot be used for the calculation of readily interpretable values of ΔX°: the variations of the densities of the eluents with temperature and composition differ considerably from one eluent to another. This leads to artificial trends in values of ΔX°. Rather, it has been pointed out[9] that the molarity distribution constant, K_B, should be used. This implies that we have to face the problem of the unknown value of d_{OG}. Fortunately, it will be seen that various realistic assumptions about the value of d_{OG} lead to very small differences in the calculated values of ΔX_{OG}°. In the following paragraph, the standard free energy of transfer of 1 mole of B from an infinitely dilute solution in the eluent to the stationary phase, ΔG_{OG}°, is calculated, taking a small amount of sorbed co-solvent into account.

Calculation of ΔG°_{OG}

We assume that the chromatographic conditions do not differ significantly from the standard state of infinite dilution. The distribution constant K_B is given by

$$K_B = \frac{c_{B,og}}{c_{B,aq}} = \frac{n_{B,og}/V_{og}}{n_{B,aq}/V_{aq}} \tag{1}$$

Here, c_B denotes the concentration of a solute B (mol l^{-1}), V_{og} and V_{aq} are the volumes of the stationary and the mobile phase in the column, respectively, and n_B denotes the number of moles of B. V_{og} can be approximated as the sum of the volumes of the pure constituents: octylglycidyl (V_{OG}) and sorbed additive ($V_{A,or}$). If we substitute in eqn. 1 the expression for the capacity ratio, k_B

$$k_B = \frac{n_{B,og}}{n_{B,aq}} = \frac{V_e - V^{\circ}}{V^{\circ}} \tag{2}$$

we have

$$K_B = \frac{V_e - V^{\circ}}{V_{OG} + V_{A,or}} \tag{3}$$

Here, V° denotes the elution volume of a supposedly unretarded compound (hence $V^{\circ} = V_{aq}$, see ref. 1) and V_e that of B. Substitution of the expression for the specific retention volume[1], $V_g = (V_e - V^{\circ})/w_{OG}$ (w_{OG} denotes the weight of OG groups in the column) and transformation of volumes into amounts yield an expression for K_B, from which ΔG°_{OG} can be calculated as

$$\Delta G^{\circ}_{OG} = -RT \ln V_g + RT \ln [d_{OG}^{-1} + W^{\circ}_{s,A} d_A^{-1}] \tag{4}$$

Here, d_{OG} denotes the density of the layer of OG and d_A that of the pure additive (g ml^{-1}). Thus ΔG°_{OG} can be calculated from specific retention volumes at different temperatures, provided that values of $W^{\circ}_{s,A}$, d_A and d_{OG} are known at those temperatures. It follows from eqn. 4 that the methylene increments of ΔG°_{OG} can be obtained from specific retention volumes only:

$$\Delta G^{\circ}_{OG,CH_2} = -RT \ln V_{g,n}/V_{g,n-1} \tag{5}$$

where n denotes the number of carbon atoms. (Of course, this elimination of d_{OG} and $W_{s,A}$ does not lead to the nullification of the influence of sorbed additive on the methylene increments.

A particularly simple situation exists if the sorbed amounts are too small to have any influence on the values of ΔG°_{OG}. In that case, ambiguity about d_{OG} can be avoided by calculating the change of ΔG°_{OG} with changing eluent composition, from retention volumes only. From this change, and literature data on the free energy of hydration, the free energy of solvation in the partly aqueous eluent can then be calculated. The calculations proceed as follows. It holds that

$$\Delta G^{\circ}_{OG} = \Delta G^{\circ}_{s} - \Delta G^{\circ}_{h} \tag{6}$$

Here, ΔG_s^o denotes the free energy of solvation of 1 mole of solute in the layer of OG (transfer vacuum → layer of OG) and ΔG_h^o that in the partly aqueous eluent. Note that ΔG_s^o in eqn. 6 is independent of the eluent composition in the considered case. From eqn. 6

$$\Delta G_h^o = \Delta G_h^{o*} - \Delta G_{OG}^o + \Delta G_{OG}^{o*} \quad (7)$$

where the asterisks refer to the values in a pure aqueous eluent, and substitution of eqn. 4 transforms this into

$$\Delta G_h^o = \Delta G_h^{o*} + RT \ln (V_g/V_g^*) \quad (8)$$

EXPERIMENTAL

The experimental procedure has been described before[1,8]. Special care was given to preventing vaporization of the eluent constituents.

The elution peaks were symmetrical at temperatures above 0°C. Below this temperature, in aqueous methanol, $C_A = 0.098$ (grams of A per gram of eluent) peak shape changed drastically and serious tailing was observed. Here, the layer of OG might lose its liquid properties. Such phase transitions in bonded layers have been observed before[10,11] in reversed-phase high-performance liquid chromatography.

With two eluents, *i.e.* the highest concentration of urea and ethylene glycol at $t > 25$°C, the baseline stability was problematic. We were forced to increase the sample dose to 1 mg. This hardly affected the accuracy. However, measurements with higher homologues than *n*-octanol were not possible owing to long-term drifts.

RESULTS AND DISCUSSION

Table I lists values of V_g (millilitres per gram of OG groups) of *n*-alcohols on octyl-Sepharose CL-4B at various temperatures. The precision of these values is estimated to be *ca.* 0.4 ml g^{-1} or 0.8%, for small and large values of V_g, respectively. Control experiments with unsubstituted Sepharose showed no significant contribution of agarose to the retention. Before applying eqns. 4–8, we shall first discuss the possible influence of small amounts of additive in the layer of OG.

Influence of sorption

As we stated before, with some organic co-solvents $W_{s,A}^o$ is not exactly zero. Of the four additives, methanol probably has the largest affinity for the layer of OG. This can be inferred from the values of the octanol–water distribution constants: methanol, 0.15[12]; DMF, 0.10[13]; urea, 0.08[14]; and ethylene glycol, 0.012[15]. Therefore, the following discussion about methanol *a fortiori* holds for the other co-solvents.

The influence of sorption may become manifest in two different ways: (i) in the change of the amount of stationary phase with temperature (this effect is accounted for in eqn. 4); (ii) in the change of the nature of the layer of OG.

(i) From the data in Table I, for the model substance *n*-octanol in methanol–water eluents, we used eqn. 4 to calculate different estimates of $\Delta G_{OG}^o/T$ at each

TABLE I

SPECIFIC RETENTION VOLUMES OF *n*-ALCOHOLS ON OCTYL-SEPHAROSE CL-4B

Additive (A)	C_A	*t* (°C)	*Number of carbon atoms in model substance*					
			5	*6*	*7*	*8*	*9*	*10*
Methanol	0.000	4.2	12.8	46.3	172	641		
		10.4	13.9	50.9	189	700		
		18.0	15.9	57.9	211	775		
		25.7	17.5	62.6	225	814		
		32.9	18.4	66.1	233	827		
		40.3	18.9	68.5	239	832		
Methanol	0.098	5.6	11.7	40.7	148	532	1869	
		9.8	13.6	43.3	152	543	1897	
		18.2	13.1	44.6	156	548	1897	
		26.7	13.4	46.4	158	534	1793	
		34.4	14.3	47.2	157	514	1683	
		42.8	13.3	46.1	148	480	1544	
Methanol	0.199	3.4	9.2	36.9	126	426	1407	
		10.4	10.7	38.3	120	397	1255	
		18.0	12.8	37.7	117	367	1124	
		25.7	11.8	36.8	112	339	996	
		33.1	11.0	33.7	103	303	879	
		40.4	11.6	32.8	95	272	783	
Methanol	0.303	4.9	9.7	26.9	75.9	219	614	1697
		9.2	9.8	26.2	73.3	205	567	1560
		18.0	10.5	23.9	66.9	179	470	1247
		26.7	6.8	21.7	58.3	148	374	940
		34.6	8.3	20.7	51.4	126	314	738
		43.2	7.3	19.7	46.2	107	252	605
Methanol	0.414	1.9		15.1	35.1	84.2	198	457
		9.2		13.7	32.3	73.8	170	378
		18.0		14.4	29.9	67.5	146	312
		25.4		12.1	26.4	57.5	123	259
		33.9		13.2	22.8	48.1	102	213
		41.7		10.6	20.8	42.7	84	171
Urea	0.059	5.5	13.3	49.2	174	674		
		11.5	14.6	52.0	187	729		
		16.3	15.9	55.1	204	753		
		22.6	17.3	58.3	210	784		
		29.8	17.9	62.4	219	798		
		39.4	19.7	66.0	221	798		
Urea	0.172	3.6	12.0	40.9	157	583		
		12.4	13.1	45.3	168	603		
		18.8	14.0	49.5	178	613		
		26.3	16.5	51.5	182	634		
		33.4	15.5	53.4	183	632		
		44.3	14.9	51.7	177	610		

(Continued on p. 24)

TABLE I *(continued)*

SPECIFIC RETENTION VOLUMES OF *n*-ALCOHOLS ON OCTYL-SEPHAROSE CL-4B

Additive (A)	C_A	*t* (°C)	*Number of carbon atoms in model substance*					
			5	*6*	*7*	*8*	*9*	*10*
Urea	0.279	3.2	12.0	37.3	145	515		
		10.4	12.3	42.0	154	524		
		18.0	13.5	44.5	156	526		
		25.0	13.8	45.2	156	535		
		33.3	13.7	46.3	155	511		
		40.7	13.7	45.9	154	503		
Urea	0.380	4.9	–	34.1	118	387		
		12.1	12.3	36.8	123	392		
		18.4	13.8	38.7	127	399		
		25.2	13.4	40.8	130	387		
		33.7	14.7	44.4	128	–		
		41.1	–	40.9	126	369		
Ethylene glycol	0.153	3.6	11.1	47.3	175	641		
		11.3	11.9	49.9	185	674		
		18.1	13.6	52.6	195	679		
		25.3	15.3	55.7	192	658		
		34.1	15.5	56.2	190	645		
		42.6	17.5	55.8	187	619		
Ethylene glycol	0.299	0.0	12.9	47.5	173	621		
		6.3	13.2	47.9	167	583		
		13.5	13.5	47.3	161	558		
		19.6	13.9	47.1	158	514		
		26.8	14.1	46.2	150	475		
		33.9	13.2	44.2	139	–		
		40.6	13.2	41.3	127	390		
Ethylene glycol	0.440	0.8	13.7	44.2	140	464		
		6.8	12.8	41.8	131	416		
		12.7	12.6	40.0	122	382		
		20.7	11.6	37.4	111	336		
		29.8	11.4	35.1	100	282		
		39.4	11.6	31.5	84.0	230		
DMF	0.331	4.0		18.9	62.0	175		
		11.2		17.5	55.2	160		
		19.8		17.5	51.8	139		
		26.2		16.9	46.3	122		
		33.4		16.0	41.5	109		
		41.2		13.6	35.9	92.8		

experimental temperature by making various estimates of the values of d_{OG}, d_A and $W^{\circ}_{s,A}$ as a function of T. From the estimates of $\Delta G^{\circ}_{OG}/T$ we calculated ΔH°_{OG}, $T\Delta S^{\circ}_{OG}$ and $\Delta C^{\circ}_{p,OG}$ as before[8]. The results, presented in Fig. 1, show the small influence of d_{OG}, d_A and $W^{\circ}_{s,A}$. Clearly, the variation of $-R \ln V_g$ with T and with

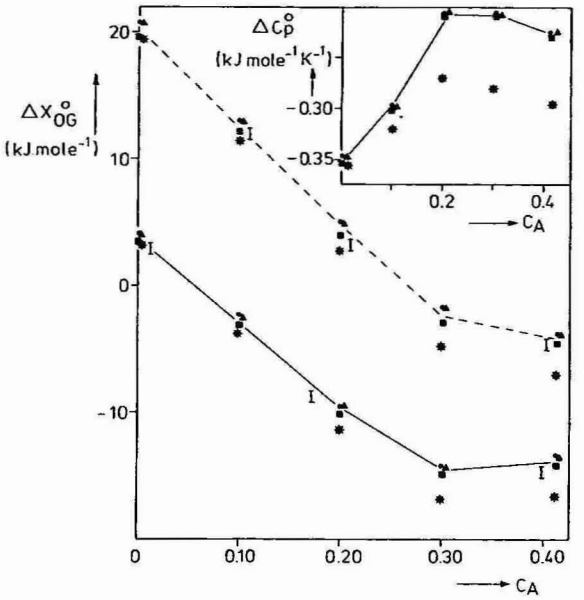


Fig. 1. Influence of model parameters on calculated thermodynamic standard quantities of *n*-octanol in methanol–water mixtures.

(a)	d_{OG}	$\frac{\partial d_{OG}}{\partial T}$	$W^{o}_{s,A}$	$\frac{\partial W^{o}_{s,A}}{\partial T}$	d_A	$\frac{\partial d_A}{\partial T}$
■	0.89[b]	(c)	Refs. 1 and 2 (20°C)	0[d]	0.8	Ref. 19
▲	1	0	Refs. 1 and 2 (20°C)	0	1	0
●	1	0	0	0	1	0
✳	0.89	(c)	Refs. 1 and 2	(e)	0.8	Ref. 19

(a) Full lines = ΔH^{o}_{OG}; broken lines = $T\Delta S^{o}_{OG}$.
(b) Density of octylglycidyl ether at 25°C[16].
(c) Values for octanol, ref. 19.
(d) The value of $W^{o}_{s,A}$ is probably independent of T. This follows from the constant value of the activity coefficient of methanol in all eluents between 40°C and 100°C (calculated from P–X diagrams[17,18]) and from the value of the activity coefficient of methanol in the layer of OG (*i.e.*, near unity[1]). Both are not expected to change much from 40°C to 5°C, and hence $W^{o}_{s,A}$ can be expected to be constant.
(e) An unrealistic, very large fluctuation of $W^{o}_{s,A}$ with T is assumed: $W^{o}_{s,A}$ (T°C) = $W^{o}_{s,A}$ (20°C) × $2^{\frac{T-20}{20}}$, *i.e.* a doubling of $W^{o}_{s,A}$ per 20°C temperature increase.

C_A outweighs that of d_{OG}, d_A and $W^{o}_{s,A}$. Only an unrealistic change of $W^{o}_{s,A}$ with T leads to different values, but even they show the same trend with eluent composition. These results lead us to neglect the sorbed amounts of co-solvent and to take d_{OG} = 1, which implies dropping the term $RT \ln [d_{OG}^{-1} + W^{o}_{s,A}\, d_A^{-1}]$ from eqn. 4.

(ii) The possibility that changing amounts of sorbed co-solvent as a function of the eluent composition, and especially of the temperature, contribute to the change of retention volumes must be considered in spite of the small values of $W^{o}_{s,A}$. Whether

this occurs can be investigated with eqn. 6, provided that values of ΔG°_{OG} and ΔG°_{h} are known, as a function of the eluent composition. In that case, ΔG°_{s} can be calculated and tested for being constant. Values of ΔG°_{OG} have been dealt with in (i). They are known but for a small constant term arising from d_{OG}. Values of ΔG°_{h} can be calculated as follows. Assuming that the vapour of the solute behaves ideally and that its activity coefficient in the eluent equals that of a saturated solution in the eluent[1], ΔG°_{h} follows from the ratio of the number density of the solute in the vapour phase and in the saturated eluent. It holds than that

$$\Delta G^{\circ}_{s} = \Delta G^{\circ}_{OG} - RT \ln \frac{RTS}{10^{-3}\, a\, P^{\circ}\, M_B} \tag{9}$$

where M_B denotes the molecular weight of the solute, a is its activity when saturated with the eluent and S is the solubility (g l^{-1}) of the solute in the eluent, and P° (Pa) is the vapour pressure of the pure solute.

We performed this calculation for *n*-octanol in methanol–water eluents at 25°C using the data from a previous paper[1]. Results, listed in Table II, show that ΔG°_{s} is constant within *ca.* 0.3 kJ mol^{-1}.

TABLE II

FREE ENERGIES OF TRANSFER AND OF SOLVATION FOR *n*-OCTANOL IN METHANOL–WATER MIXTURES

C_A (g g^{-1})	ΔG°_{OG}* (kJ mol^{-1})	S** (g l^{-1})	a**	ΔG°_{s} *** (kJ mol^{-1})
0.000	−16.60	0.572	0.79	−34.3
0.098	−15.59	0.730	0.72	−34.2
0.199	−14.44	1.16	0.64	−34.4
0.303	−12.48	2.57	0.57	−34.7
0.413	−9.61	6.52	0.48	−34.6

* Eqn. 4 with V_g from Table I, d_{OG} = 1 and $W^{\circ}_{s,A}$ = 0.
** See comments in ref. 1.
*** Eqn. 9, P° from ref. 20.

With enthalpy values another test can be performed, also for *n*-octanol. The value of ΔH°_{s} (C_A = 0), *i.e.* −70 kJ mol^{-1}, was obtained with the enthalpy equivalent of eqn. 6 from the value of ΔH°_{OG} (Fig. 1) and the literature value of ΔH°_{h}, both in water[20]. The value of ΔH° for solvation of *n*-octanol in pure methanol is −69 kJ mol^{-1} (found by extrapolation of literature values of lower alcohols with constant methylene increments[21]). As these values are nearly identical, the presence of a small amount of methanol in the layer of OG at higher values of C_A is not expected to influence ΔH°_{s} much.

We conclude that ΔX°_{s} does not depend on the eluent composition. This means that the variation of ΔX°_{OG}, and that of ΔX°_{h}, with eluent composition, are identical. Our chromatographic data on ΔX°_{OG} can thus be compared with literature data on ΔX°_{h} obtained by classical methods. The methods are complementary, as the chromatographic method works best with higher homologues that are well retained on

the octyl-agarose column, whereas classical methods work best with lower homologues that are well soluble in partly aqueous solvents and have a high volatility.

Influence of the temperature on the retention volumes

Fig. 2 presents a Van't Hoff plot ($\Delta G^{\circ}_{OG}/T = -R \ln V_g$ *vs.* T^{-1}) for *n*-octanol. The remarkable behaviour of pure water, especially at low temperatures, is clearly illustrated by this Figure. It is seen that the increase of the retention with temperature in pure water changes rapidly to the opposite behaviour if co-solvent (except urea) is added.

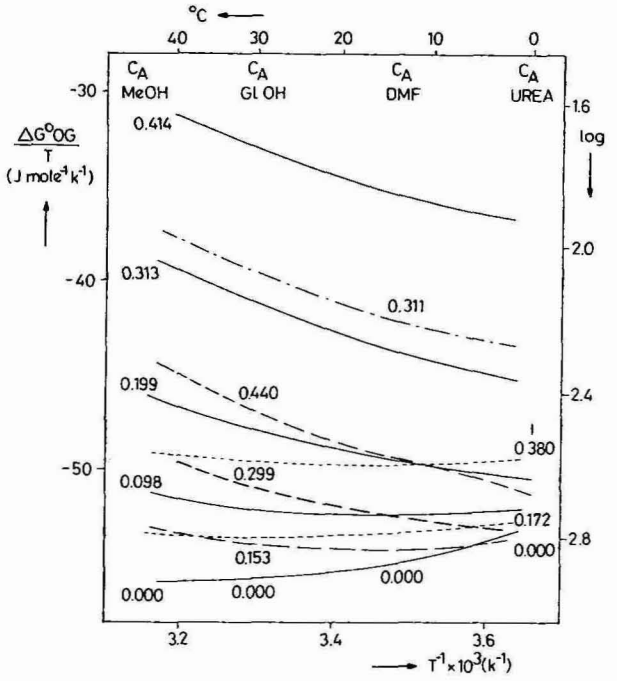


Fig. 2. Van't Hoff plot for *n*-octanol. Eluents: methanol (MeOH)–water = ———; ethylene glycol (GlOH) = – – –; urea = ...; DMF = –.–.–. Values of C_A (weight fraction of additive in the eluent) are indicated in the figure.

Thermodynamics of the retention process

Values of ΔX°_{OG} at 25°C were calculated as before[8]. They are listed in Table III. From the precision of the experimental data, an error of ± 0.07 kJ mol^{-1}K^{-1} can be predicted in $\Delta G^{\circ}_{OG}/T$ for values of $V_g > 100$ ml g^{-1}. Table III shows that S_y values resulting from the statistical treatment are indeed of this order of magnitude.

The methylene increments of values of ΔX°_{OG} in Table III are independent of the chain length of the alcohols, within experimental error. More accurate values of $\Delta X^{\circ}_{OG,CH_2}$ were established as follows. Values of $\Delta G^{\circ}_{OG,CH_2}$ were calculated from the ratio of $V_{g,n}$ and $V_{g,n-2}$ with $n = 8$ or 9, if measured (eqn. 5). Values of $\Delta X^{\circ}_{OG,CH_2}$ were calculated from a Van't Hoff plot. They are listed in Table IV.

TABLE III

VALUES OF ΔX°_{OG} AT 25°C FOR INTERACTION OF *n*-ALCOHOLS WITH OCTYL-AGAROSE IN VARIOUS ELUENTS

A (C_A)	*n*	$\Delta C^{\circ}_{p,OG}$	ΔH°_{OG}	$T\Delta S^{\circ}_{OG}$	ΔG°_{OG}	s^{*}	s_y^{**}
Methanol	5	0***	8.2	15.2	−7.0	0.8	0.28
(0.000)	6	−0.27	7.0	17.3	−10.24	0.2	0.06
	7	−0.30	5.5	19.0	−13.41	0.1	0.05
	8	−0.35	4.1	20.7	−16.60	0.2	0.06
Methanol	5	0***	2.1	8.5	−6.4	1.3	0.49
(0.098)	6	−0.24	2.0	11.5	−9.51	0.3	0.10
	7	−0.28	−0.3	12.3	−12.55	0.2	0.06
	8	−0.29	−2.5	13.1	−15.59	0.1	0.02
	9	−0.35	−4.5	14.1	−18.61	0.2	0.06
Methanol	5	0***	3.1	9.1	−6.0	2.3	0.86
(0.199)	6	−0.31	−4.0	5.0	−8.93	0.4	0.15
	7	−0.22	−6.1	5.6	−11.68	0.3	0.10
	8	−0.21	−9.5	5.0	−14.44	0.2	0.05
	9	−0.10	−11.8	5.4	−17.13	0.1	0.03
Methanol	5	0***	−6.3	−1.1	−5.3	2.9	1.10
(0.303)	6	0.07	−6.1	1.6	−7.70	0.2	0.09
	7	−0.18	−10.2	0.0	−10.19	0.3	0.12
	8	−0.21	−14.3	−1.8	−12.48	0.3	0.10
	9	−0.22	−17.5	−2.7	−14.82	0.2	0.08
	10	−0.19	−20.7	−3.6	−17.12	0.6	0.21
Methanol	6	0***	−5.2	1.1	−6.29	1.7	0.65
(0.414)	7	−0.22	−10.7	−2.6	−8.14	0.3	0.12
	8	−0.23	−13.4	−3.8	−9.61	0.5	0.14
	9	−0.28	−16.7	−4.8	−11.95	0.3	0.10
	10	−0.20	−18.5	−4.7	−13.81	0.3	0.12
Urea	5	−0.23	7.5	14.6	−7.10	0.4	0.12
(0.059)	6	−0.08	6.2	16.3	−10.13	0.2	0.05
	7	−0.35	4.1	17.4	−13.31	0.3	0.10
	8	−0.30	2.6	19.1	−16.55	0.1	0.03
Urea	5	0***	4.6	11.2	−6.62	1.8	0.65
(0.172)	6	−0.34	3.8	13.6	−9.72	0.3	0.11
	7	−0.28	1.7	14.6	−12.89	0.2	0.07
	8	−0.17	0.7	16.7	−15.97	0.2	0.09
Urea	5	0***	3.0	9.4	−6.45	1.5	0.54
(0.279)	6	−0.34	2.4	11.9	−9.49	0.3	0.10
	7	−0.19	0.2	12.7	−12.53	0.3	0.09
	8	−0.16	−1.1	14.4	−15.53	0.2	0.09
Urea	5	0***	5.2	11.7	−6.51	1.9	0.35
(0.380)	6	−0.30	3.6	12.8	−9.26	0.8	0.27
	7	−0.24	0.7	12.8	−12.05	0.1	0.05
	8	−0.18	−1.5	13.3	−14.79	0.2	0.08

TABLE III *(continued)*

VALUES OF ΔX°_{OG} AT 25°C FOR INTERACTION OF *n*-ALCOHOLS WITH OCTYL-AGAROSE IN VARIOUS ELUENTS

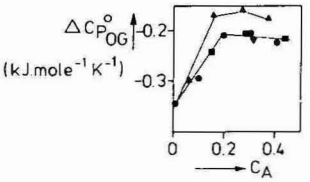
A (C_A)	*n*	$\Delta C^{\circ}_{p,OG}$	ΔH°_{OG}	$T\Delta S^{\circ}_{OG}$	ΔG°_{OG}	$s^{\star}$	$s_y^{\star\star}$
Ethylene glycol	5	0***	8.5	15.2	−6.63	0.8	0.30
(0.153)	6	−0.20	2.8	12.7	−9.92	0.3	0.10
	7	−0.26	0.4	13.4	−13.05	0.3	0.10
	8	−0.24	−1.6	14.6	−16.13	0.3	0.10
Ethylene glycol	5	−0.23	−0.8	5.7	−6.50	0.4	0.15
(0.299)	6	−0.22	−3.4	6.1	−9.50	0.2	0.07
	7	−0.23	−6.4	6.1	−12.43	0.2	0.10
	8	−0.21	−9.2	6.1	−15.32	0.2	0.07
Ethylene glycol	5	−0.23	−2.0	4.1	−6.08	0.4	0.13
(0.440)	6	−0.08	−6.5	2.4	−8.89	0.2	0.07
	7	−0.20	−10.2	1.3	−11.53	0.2	0.09
	8	−0.22	−14.0	0.3	−14.20	0.2	0.08
DMF	6	−0.35	−6.5	0.5	−7.01	0.8	0.28
	7	−0.24	−11.0	−1.5	−9.55	0.4	0.15
	8	−0.22	−13.1	−1.1	−12.00	0.2	0.08

* s denotes the error in values of ΔH°_{OG} and $T\Delta S^{\circ}_{OG}$ (kJ mol^{-1}).
** s_y denotes the error in a single value of $\Delta G^{\circ}/T$.
*** ΔC°_p was not evaluated from the data, but arbitrarily put equal to zero.

TABLE IV

VALUES OF METHYLENE INCREMENTS OF ΔX°_{OG} AT 25°C

Eluent	c_A	$\Delta C^{\circ}_{p,CH_2}$	$\Delta H^{\circ}_{CH_2}$	$T\Delta S^{\circ}_{CH_2}$	ΔG_{CH_2}	s	s_y
Methanol	0.000	−0.04	−1.48	1.70	−3.18	0.06	0.02
	0.098	−0.04	−2.09	0.94	−3.03	0.16	0.06
	0.199	+0.06	−2.83	0.10	−2.93	0.10	0.04
	0.303	−0.02	−3.70	−1.36	−2.34	0.13	0.05
	0.414	−0.01	−2.84	−0.92	−1.92	0.17	0.06
Urea	0.059	−0.12	−1.82	1.39	−3.20	0.14	0.04
	0.172	+0.09	−1.55	1.55	−3.10	0.14	0.05
	0.279	+0.09	−1.77	1.26	−3.03	0.21	0.08
	0.380	+0.06	−2.21	0.61	−2.82	0.20	0.06
Ethylene glycol	0.153	−0.02	−2.17	0.93	−3.10	0.25	0.09
	0.299	+0.01	−2.84	0.08	−2.92	0.12	0.05
	0.440	−0.03	−3.56	−0.92	−2.64	0.08	0.03
DMF	0.331	+0.05	−3.33	−0.82	−2.50	0.44	0.16



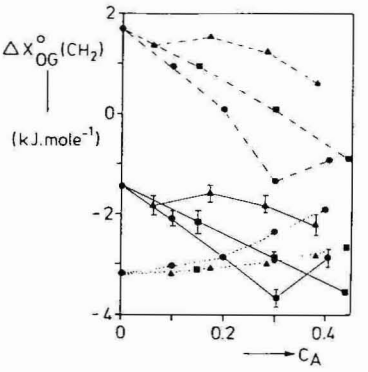


Fig. 3. Change of heat capacity values with eluent composition. Symbols: ● = methanol; ■ = ethylene glycol; ▲ = urea; ▼ = DMF.

Fig. 4. Thermodynamics of the interaction of a methylene group with the octylglycidyl layer. Symbols as in Fig. 3, plus free energy = ·····; enthalpy = ———; entropy = – – – –.

Fig. 3 shows values of $\Delta C^{\circ}_{p,OG}$ for *n*-octanol as a function of C_A, and the influence of co-solvents on the methylene increments of free energy, enthalpy and entropy is given in Fig. 4.

A comparison with recalculated literature data is shown in Fig. 5. Here, we plotted our values of ΔH°_{h} (open symbols) together with recalculated data from Rouw and Somsen[3] on the heats of solution of some lower alcohols in mixtures of DMF and water. It can be seen that the agreement between the two sets of data is good.

A graphical representation of eqn. 6 for methanol–water eluents is given in Fig. 6, and Fig. 7 presents the results for eluents with ethylene glycol and urea. In the latter figure, values of ΔH°_{h} for *n*-butanol, recalculated from data of Nwankwo and Wadsö[22], agree very well with our results for *n*-hexanol and *n*-octanol.

In the next few paragraphs, the values of the free energy, heat capacity, en-

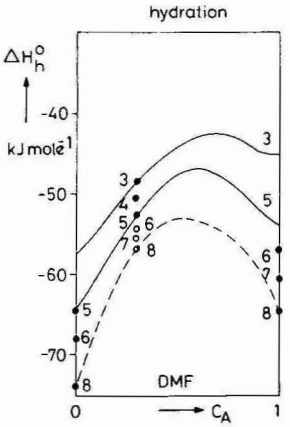


Fig. 5. Standard enthalpies of hydration of *n*-alcohols in DMF–water mixtures. Numbers in the figure denote the value of *n*. Open symbols are our data and closed symbols and full lines are recalculated data from Rouw and Somsen[3], on enthalpies of solution, ΔH°_{sol}. Note that $\Delta H^{\circ}_{h} = \Delta H^{\circ}_{sol} - \Delta H^{\circ}_{vap}$, where ΔH°_{vap} denotes the standard enthalpy of vaporization (from ref. 20).

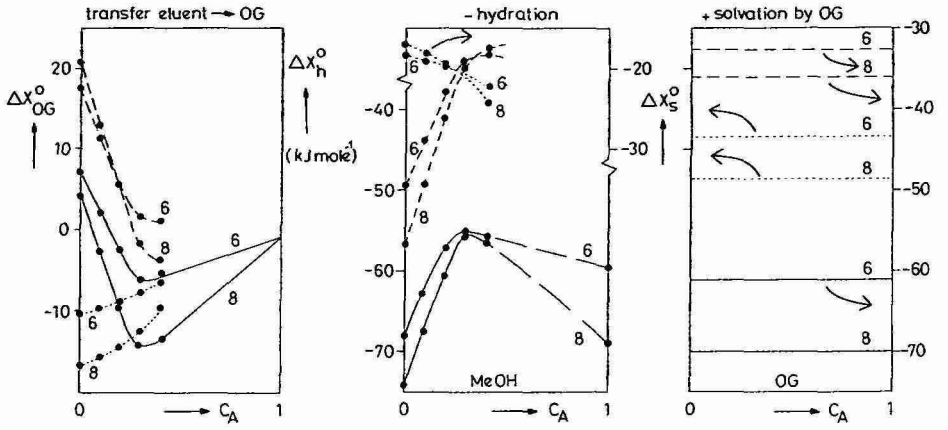


Fig. 6. Thermodynamics of transfer, solvation and hydration in methanol (MeOH)–water mixtures. Symbols as in Fig. 4. Solutes are *n*-hexanol and *n*-octanol (denoted with the value of *n* in the figure). Values were calculated with the ΔX equivalent of eqn. 8. ΔG_h^{o*} and ΔH_{hn}^{o*} values at $C_A = 0$ are from refs. 20 and 23; values at $C_A = 1$ are estimated by extrapolation with constant methylene increments from data in ref. 21.

thalpy and entropy will be discussed and interpreted according to current theories on the subject.

Free energy values

It can be seen in Figs. 6 and 7 that values of ΔG_{OG}^{o} become less negative with increasing amounts of co-solvent, in this case just as with the values of $-\log K_{X,B}$ discussed before[1]. The ratio of ΔG_{OG}^{o} and $\Delta G_{OG,CH_2}^{o}$ (Fig. 4) appears to have a nearly constant value of $(n - 2.8)$ in all eluents except for methanol ($C_A = 0.414$). Assuming constant methylene increments, this can be interpreted as a contribution of the polar head to ΔG_{OG}^{o} opposite to that of 2.8 methylene groups. This contribution thus decreases from 9 kJ mol^{-1} in water to *ca.* 6 in methanol ($C_A = 0.303$). The value for

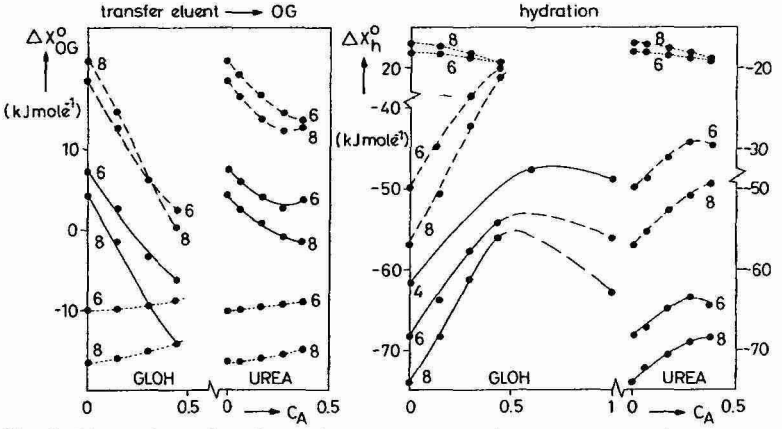


Fig. 7. Thermodynamics of transfer and hydration in eluents with ethylene glycol (GlOH) and urea. Symbols as in Fig. 4. With ethylene glycol, values for *n*-butanol were obtained from ref. 22. Values at $C_A = 1$ are estimated by extrapolation of values for lower homologues from ref. 24.

the free energy of dehydration of the polar head in water is much larger; *i.e. ca.* 24 kJ mol^{-1}[25]. Appreciable hydrogen-bond formation of the polar head in the layer of OG must therefore still persist, presumably with the spacer arm[8,26].

The methylene increments of ΔG_s° and ΔG_h° are equal to half the difference between the curves of *n*-hexanol and *n*-octanol for solvation and hydration, respectively, in Figs. 6 and 7. The methylene increments of ΔG_s° are large and negative, *i.e.* -2.7 kJ mol^{-1}. This is also observed with solvation in organic liquids[27,28]. In water, the methylene increments of ΔG_h° for $n = 6$–9 are small and positive, *i.e. ca.* $+0.5$ kJ mol^{-1}, thus contributing negatively to $\Delta G^\circ_{OG,CH_2}$. The large negative value of $\Delta G^\circ_{OG,CH_2}$ apparently results from a lack of interaction between water and methylene groups. By addition of co-solvents, $\Delta G^\circ_{h,CH_2}$ may change sign, as can be seen in Fig. 6. This reflects the increasing capability of the mixture to dissolve hydrophobic moieties. Water is not unique in showing positive values of $\Delta G^\circ_{h,CH_2}$. It can be inferred that solvation in hydrazine is also accompanied with small (positive) methylene increments[29]. The cause of this phenomenon lies probably in the large forces of cohesion in polar liquids as a result of extensive hydrogen-bond networks[30].

Heat capacity values

The heat capacity values of *n*-octanol (Fig. 3) change by *ca.* -0.35 kJ mol^{-1} K^{-1} upon transfer from water to the layer of OG. The large negative value of $\Delta C^\circ_{p,OG}$ water is in good agreement with the concept of a thermally labile hydrophobic hydration structure, that is thought to surround alkyl groups in water. It is seen that $\Delta C^\circ_{p,OG}$ becomes less negative by the addition of co-solvent.

Fig. 3 offers the same picture as can be obtained from literature data for the dissolution of *n*-butanol in aqueous mixtures of DMF[7] or ethylene glycol[22].

Enthalpy and entropy: general survey

The influence of co-solvents on these quantities is much more complicated than on free energies or heat capacities. Fig. 4, 6 and 7 yield the following four points of information.

(1) All co-solvents have the same effect on ΔH°_{OG} and $T\Delta S^\circ_{OG}$, both for alcohols and methylene groups: their values are lowered. The entropy decreases more rapidly than the enthalpy. A change to negative values may even occur. Note that $\Delta H^\circ_{OG,CH_2}$ in water is strongly negative. So this type of hydrophobic interaction is not solely entropy-driven, and it becomes less important with increasing temperature.

(2) The co-solvents are not equally effective in lowering values of ΔX°_{OG}. Of course, this depends also on the concentration unit used.

(3) Changes in ΔG°_{OG} can be accompanied by tenfold changes in $T\Delta S^\circ_{OG}$. Apparently, changes in entropy and enthalpy largely compensate each other.

(4) A considerable contribution of the polar head to values of ΔH°_{OG} and $T\Delta S^\circ_{OG}$ must exist. This follows from the positive values of $\Delta H^{\circ*}_{OG}$ and the negative value of $\Delta H^{\circ*}_{OG,CH_2}$. The possibility of non-linear methylene increments over the range $n = 0$–9 hampers the precise evaluation of the contribution of the polar head to $\Delta H^{\circ*}$ and to $T\Delta S^{\circ*}_{OG}$. However, a tentative extrapolation to $n = 0$ yields an enthalpic contribution of $+16$ kJ mol^{-1}, and an entropic contribution of $+7$ kJ mol^{-1} to the values of $\Delta H^{\circ*}_{OG}$ and $T\Delta S^{\circ*}_{OG}$, respectively. It follows that the partial dehydration of the polar head is the cause of the increase of retention with increasing temperature

in water (see Fig. 2), and not the hydrophobic interaction of the alkyl chain with the layer of OG.

We shall now turn to the interpretation of the values of ΔH° and $T\Delta S^\circ$ separately.

Enthalpy values

It can be seen in Fig. 6 that the values of $\Delta H_h^{\circ *}$ and ΔH_s° are nearly equal, in spite of $\Delta G_h^{\circ *}$ being much less negative (*ca.* 15 kJ mol^{-1} for *n*-octanol). This unexpected feature (for it costs a lot of heat energy to form a cavity in the hydrogen-bonded liquid) has been interpreted as the result of an extensive formation of hydrogen bonds around alkyl chains in aqueous solution. The nature of this labile, non-rigid "hydrophobic hydration" and its existence in (partly) aqueous media has been studied by various methods (see *e.g.* refs. 3 and 7, and refs. therein). Water is unique in this respect, for even hydrazine, about as lipophobic as water and also strongly hydrogen-bonded, does not show such abnormal enthalpies[29].

It has been found that hydrophobic hydration is strongly co-operative in nature. This explains the strikingly non-linear graphs of ΔH_h° and ΔH_{OG}° as a function of C_A in the Figs. 6 and 7. Apparently, relatively small amounts of co-solvent are capable of destroying a large portion of the hydrogen bonds around alkyl chains, so that for C_A *ca.* 0.3 the hydrophobic hydration of octanol is completely nullified. This is also reflected in Fig. 3 for the values of $\Delta C_{p,OG}^\circ$.

Following Rouw and Somsen, we adopted a simple model, developed by Mastroianni *et al.*[31] and based on the assumption that hydrophobic hydration causes a contribution ΔH_{hh}° in pure water, superimposed on the value obtained from a linear relation in the co-solvent-rich region between ΔH° and the mole fraction of water, X_{H_2O}. This value (in our case $\Delta H_h^{\circ *} - \Delta H_{hh}^\circ$) is obtained by extrapolation to $X_{H_2O} = 1$ of the supposedly linear part of the ΔH° *vs.* X_{H_2O} relationship at $X_{H_2O} <$ *ca.* 0.6. It is then assumed that the probability of finding N water molecules forming a "cage" around *e.g.* an alkyl group is equal to $X_{H_2O}^N$, and that even the exclusion of only one water molecule from the cage by co-solvent molecules leads to the nullification of the contribution of that cage to the value of ΔH° (in our case, ΔH_h°). For solvation in a partly aqueous eluent it can be derived that

$$\Delta H_h^\circ = X_{H_2O}(\Delta H_h^{\circ *} - \Delta H_{hh}^\circ) + (1 - X_{H_2O})\Delta H_{h,C_A=1}^\circ + X_{H_2O}^N \Delta H_{hh}^\circ \qquad (10)$$

where $\Delta H_{h,C_A=1}^\circ$ denotes the standard solvation enthalpy in the pure co-solvent A.

We applied this expression to our data on ΔH_h° by adopting successively several values of ΔH_{hh}° for the *n*-alcohols and then calculating the best value of N by the least-squares method. The curves were forced through the ΔH_h° values at $X_{H_2O} = 1$ and 0. It appears that good fits can be obtained only with different values of N for the various co-solvents (except ethylene glycol and methanol). This is shown in Fig. 8 for *n*-octanol, taking $\Delta H_{hh}^\circ = -24$ kJ mol^{-1}.

Fig. 9 is a plot of the calculated best values of N as a function of the adopted values of ΔH_{hh}° for the *n*-alcohols with $n = 6$, 7 and 8. The numbers in the graph denote the standard errors of the estimate of ΔH_h°, S_e (kJ mol^{-1}) data for $X_{H_2O} =$ 0 and 1 excluded. It can be seen in Fig. 9 that our data can be fitted with eqn. 10 with S_e values of *ca.* 1 kJ mol^{-1} over a range of ΔH_{hh}° values, which hampers the

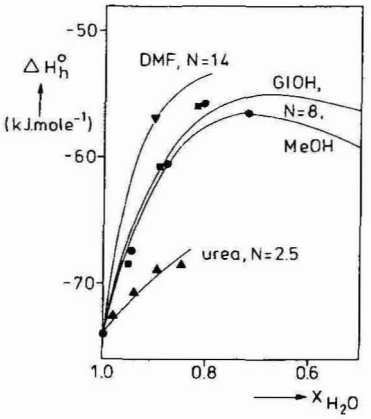


Fig. 8. Enthalpies of hydration of *n*-octanol and the cage model. Symbols as in Fig. 4. Lines are calculated values of ΔH_h°, with a ΔH_{hh}° value of -24 kJ mol^{-1}. The best values of N are indicated. With urea, the value of $C_A = 1$ (not shown) is assumed to be equal to that of ethylene glycol. However, even a change of 10 kJ mol^{-1} in this value does not lead to different conclusions.

extraction of both ΔH_{hh}° and N values. With DMF, the values of N are plotted that reproduce the single experimental ΔH_h° value for *n*-octanol. For the sake of comparison, values of N and of ΔH_{hh}° for lower homologue (symbol: ●, *n*-values in parentheses) obtained by Rouw and Somsen[3] with DMF–water mixtures also are shown. Extrapolation of these data to the curve for *n*-octanol in DMF gives the combination $\Delta H_{hh}^\circ = -24$ kJ mol^{-1}, $N = 14$. With this value of ΔH_{hh}° for *n*-octanol, a lower value for N is found in methanol–water and ethylene glycol–water mixtures, *viz.*, $N = 8$. With *n*-hexanol and *n*-heptanol, N values of 6 and 7 are found, respectively, if ΔH_{hh}° values are adopted in between those for $n = 5$ and 8. On doing so, N is found to increase with n, as could be expected.

However, quite independent of the ΔH_{hh}° values are the values of $N = 2$–3 found with urea (Fig. 9). They are very much lower than those for the other co-solvents. The low N values are related to the low value of the slope of the curve for urea in Fig. 8. Apparently, urea does not destroy the cage-like hydration structures

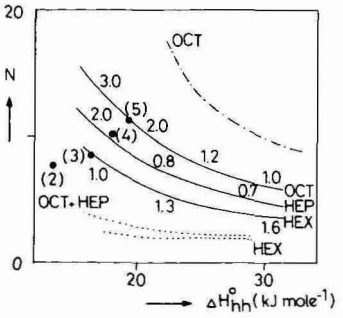


Fig. 9. Best values of N as a function of adopted values of ΔH_{hh}°. Numbers in the graph denote the standard deviation of estimates of ΔH_h°. The symbol ● (n) denotes a value from Rouw and Somsen[3]; *n* is the number of carbon atoms of the *n*-alcohol.

as effectively as DMF, for example. Rather, our results point to the existence of mixed solvation structures around alkyl groups in concentrated solutions of urea, as proposed by Nozaki and Tanford[32]. Recently, Kuharski and Rossky[33,34], in a molecular dynamics study, simulated the incorporation of urea in the hydration layer of an apolar solute. These authors showed that interactions between water–water and water–urea molecules in contact with the solute are indeed somewhat stronger than the same interactions in the bulk solution, although this enhancement was found to be smaller than in pure water. These findings explain our results: urea may be built-in into the hydrophobic hydration structure. However, it should be noted that the values of $\Delta C^{\circ}_{p,OG}$ in urea–water mixtures indicate even less structure around the alkyl chains than in mixtures of the other co-solvents with water.

Literature values of enthalpies of solution may also be used to calculate N values. We arrived at $N = 6$ for n-butanol in ethylene glycol–water mixtures[22]. This is lower than its value in DMF–water mixtures[3] ($N = 10$), just as we have found for $n = 6,7$ and 8. Such dependence of N values on the size and/or the polarity of the co-solvent molecules is also indirectly apparent in results of Kimura *et al.*[34] with alcohols as co-solvents. Here, the slopes of the enthalpy graph at $X_{H_2O} = 1$ are different, just as in our Fig. 8. This may indicate that the polar hydroxy group of the co-solvent can also be (partially) built-in into the hydration structure without destroying it, just as the hydroxy group of the solute probably participates in the cage structure[7,35]; it also may explain the lower N values we found for methanol and ethylene glycol as co-solvents.

Entropy values

Figs. 4, 6 and 7 show the same trends (in a somewhat more pronounced way) as enthalpy values. The large, negative values of $T\Delta S^{\circ *}_{h}$ are thought to arise from the increase of structure, the slowing down of translation and rotation, and the restriction of the configurational freedom of water in the hydration shell associated with hydrophobic hydration[36]. Enthalpy and entropy values compensate at least partially.

CONCLUSIONS

(1) Values of ΔG°, ΔH°, ΔS° and ΔC°_{p} for the interaction of n-alcohols with octyl-Sepharose can be interpreted with a simple partition model. Their variation with eluent composition is solely caused by the change of the standard state of the solute in the aqueous phase.

(2) The increase of the retention of the n-alcohols with temperature, with water as the eluent, is not caused by hydrophobic interaction but by dehydration of the polar head.

(3) By adopting the cage model of Mastroianni *et al.*[31], reasonable values of the number of water molecules N in the hydration cage of n-alcohols can be obtained from the enthalpy of hydration. The (single) value for the enthalpy of hydration of n-octanol in DMF–water can be described with $N = 14$. This figure corresponds with the value extrapolated from data given by Rouw and Somsen for lower homologues. With aqueous mixtures of methanol or ethylene glycol, lower N values are found, *viz.* 8, 7 and 6 for n-octanol, n-heptanol and n-hexanol, respectively.

(4) With urea, much lower N values indicate that co-solvent molecules are

built-in into the cage. This is not reflected in the values of ΔC_p^o in urea–water mixtures. These indicate even less structures around the alkyl chain than in mixtures of the other co-solvents with water.

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THERMODYNAMIC MODEL FOR REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH MULTISOLVENT MOBILE PHASES

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SUMMARY

A basic reversed-phase liquid chromatographic model is presented. It is based on the thermodynamic treatment of adsorption, dissolution and mixing of binary solvents. It has been applied to the prediction of the capacity ratio of five antiepileptic drugs in a multisolvent mobile phase. Correlation coefficients between the observed and the calculated values of the logarithm of the capacity ratio were *ca.* 0.95–0.98.

INTRODUCTION

Various attempts have been made to predict retention characteristics of multisolvent high-performance liquid chromatography (HPLC), both reversed phase (RP) and normal phase (NP), especially for the optimization of sample separation. It is possible to treat retention in RPLC in a very similar way to retention in NPLC[1]. Snyder[2] introduced the well-known empirical parameter, solvent strength, to predict the capacity ratio in the mobile phase of an organic–water mixture for NPLC. Glajch *et al.*[3] extended his work to predict the optimum mobile phase statistically by using the solvent selectivity triangle for NPLC and RPLC. Soczewinski[4] proposed an empirical equation for the prediction of capacity ratio for NPLC, and Hara *et al.*[5] systematically investigated its validity. Jandera and Churáček[6] and Tijssen *et al.*[7] predicted the capacity ratio on the basis of solubility parameters. Recently, Colin *et al.*[8] proposed an improved equation for RPLC by considering the polar interactions between two solvent molecules and between the sample solutes and the solvent molecules, and found a good agreement. However, they did not include the entropic contribution to the retention characteristics. Almost all these authors have based their reports on the linear relationship between the logarithm of the capacity ratio and the mole fraction of the organic solvent, and have added some terms or modified the shape.

This paper presents a basic RPLC model, based on the full thermodynamic treatment of adsorption, dissolution and mixing of solvents. It is applied to the prediction of the capacity ratio in a multisolvent mobile phase, and to obtain the resolution functions for the separation of five antiepileptic drugs.

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THEORETICAL

Sample molecules that are in equilibrium between the two-component mobile phase and the adsorbent are described in Fig. 1A. Here, S and B denote the sample molecule and the adsorbent, respectively. Both α and β denote each group of solvent molecules which solvate to the same area of adsorption site surface of sample molecule and adsorbent, respectively (Surface Site Model; SSM). Solvent molecules, which usually solvate on the sample molecule or on the adsorbent bed, regardless of the sample adsorption or desorption from the adsorbent, can be neglected for simplicity as illustrated in Fig. 1B. We need to deal only with species that definitely adsorb or desorb in the equilibrium process for a rough evaluation of the change in Gibbs free energy ΔG. So the equilibrium process is described as the following equation:

$$SB + \alpha\beta = S\alpha + B\beta + \Delta G \tag{1}$$

This equation is the sum of the following four equations:

$$SB = S + B - \Delta G_0 \tag{2}$$
$$S + \alpha = S\alpha + \Delta G_\alpha \tag{3}$$
$$B + \beta = B\beta + \Delta G_\beta \tag{4}$$
$$\alpha\beta = \alpha + \beta - \Delta G_\phi \tag{5}$$

Here, the values which are symbolized with Δ are corresponding Gibbs free energy changes. In case of RPLC, solvent molecules are far more polar than the adsorbent. The ratio of values of small non-polar affinity of the two solvent molecules to the adsorbent bed might be large, whereas there will not be such a difference between these two absolute values compared with the RT value. Therefore, as a first approximation, we assume that the ratio of binary solvent molecules on the adsorption site surface is equal to that in the mobile phase.

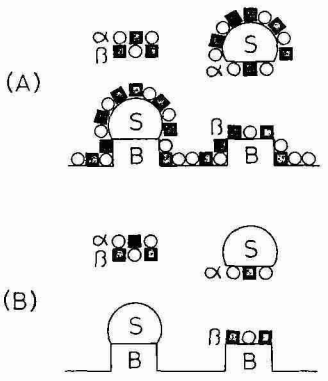

Fig. 1. Visualization of the dissolution–adsorption equilibrium in binary-solvent liquid chromatography. (A) Whole illustration as it is; S = sample molecule, B = adsorbent; open circles and filled squares show two kinds of solvent molecule; (B) unchanged solvent molecules can be neglected; see text for further discussion.

The ΔG_0 is described as the following equation:

$$\Delta G_0 = \Delta H_{50} - RT\, X_S \ln(X_S) \tag{6}$$

Here, ΔH_{50} is the corresponding enthalpy change of eqn. 2, and the second term corresponds to the entropy change of the same equation; X_S denotes the mole fraction of the sample. ΔG_α is defined by the following equation:

$$\begin{aligned}\Delta G_\alpha = {} & W_{HS}N_{HS} + W_{IS}N_{IS} + W_{SHH}M_{SHH} + W_{SII}M_{SII} + W_{SHI}M_{SHI} \\ & + RT\,[X_H \ln(X_H) + X_I \ln(X_I) - X_H(1 - X_S y) \ln\{X_H(1 - X_S y)\} - \\ & X_I(1 - X_S y) \ln\{X_I(1 - X_S y)\}]\end{aligned} \tag{7}$$

Here, H and I denote each solvent molecule in the binary solvent mobile phase; S denotes the sample molecule; W_{HS} and W_{IS} denote the adsorption energies of solvent molecules, H and I, to the adsorption site surface of the sample molecule, respectively; N_{HS} and N_{IS} denote the number of solvent H molecules and solvent I molecules that solvate to the adsorption site surface of the sample molecule; W_{SHH}, W_{SII} and W_{SHI} denote the interaction energy between each pair of two molecules HH, II and HI on the adsorption site, respectively, M_{SHH}, M_{SII} and M_{SHI} denote the number of corresponding pair interactions of the nearest neighbours on the adsorption site surface; X_H and X_I denote the mole fraction of each solvent; the factor y denotes the area of the adsorption site surface in units of solvent molecule cross-section; the final term corresponds to the entropy change of eqn. 3.

ΔG_β differs from ΔG_α only in that S is replaced by B; X_B is equal to X_S because the number of sample molecules equals the number of adsorption sites that will be adsorbed perfectly.

ΔG_ϕ is defined by the following equation:

$$\Delta G_\phi = W_{HH}(y n_S N)X_H^2 + W_{HI}(y n_S N)2X_H X_I + W_{II}(y n_S N)X_I^2 - RT\, X_S \ln(X_S) \tag{8}$$

Here, W_{HH}, W_{HI} and W_{II} denote the interaction energies of each pair of solvent molecules; N and n_S denote the Avogadro constant and the mole number of the sample in the system. The value in parentheses describes the number of all solvent molecules that are assigned to group α or β.

Therefore, ΔG can be written as the following equation:

$$\begin{aligned}\Delta G = {} & -\Delta H_0 + W_\alpha + W_\beta - (W_{HH}X_H^2 + 2W_{HI}X_H X_I + W_{II}X_I^2) y n_S N \\ & + 2\,RT\,X_S \ln(X_S) + 2\,RT\,\{X_H(1 - X_S y) \ln(1 - X_S y) + \\ & + X_I(1 - X_S y) \ln(1 - X_S y) - X_S y X_H \ln(X_H) - X_S y X_I \ln(X_I)\}\end{aligned} \tag{9}$$

where

$$W_\alpha = W_{HS}N_{HS} + W_{IS}N_{IS} + W_{SHH}M_{SHH} + W_{SII}M_{SII} + W_{SHI}M_{SHI} \tag{10}$$

$$W_\beta = W_{HB}N_{HB} + W_{IB}N_{IB} + W_{BHH}M_{BHH} + W_{BII}M_{BII} + W_{BHI}M_{BHI} \tag{11}$$

$$N_{HS} = N_{HB} = (y n_S N)X_H \tag{12}$$

$$N_{IS} = N_{IB} = (yn_S N)X_I \quad (13)$$

$$M_{SHH} = M_{BHH} = (yn_S N)X_H^2 \quad (14)$$

$$M_{SII} = M_{BII} = (yn_S N)X_I^2 \quad (15)$$

$$M_{SHI} = M_{BHI} = (yn_S N)2X_H X_I \quad (16)$$

Fig. 2 shows the various interactions between solvent molecules, sample molecules and adsorbent. Rearrangement of eqn. 9 gives the following equation:

$$\Delta G - X_H \Delta G_H - X_I \Delta G_I = -yn_S[2\,RT\,\{X_H \ln(X_H) + X_I \ln(X_I)\} + N X_H X_I(\Delta g - \Delta g_S - \Delta g_B)] \quad (17)$$

where

$$\Delta g = W_{HH} + W_{II} - 2W_{HI} \quad (18)$$

$$\Delta g_S = W_{SHH} + W_{SII} - 2W_{SHI} \quad (19)$$

$$\Delta g_B = W_{BHH} + W_{WII} - 2W_{BHI} \quad (20)$$

Here, ΔG_H and ΔG_I are the free energy changes of the adsorption processes in the case of pure solvent mobile phases. The Gibbs free energy change is related to the capacity ratio k' by the equation:-

$$\log k' = \log \varphi - \Delta G/(2.303RT) \quad (21)$$

where φ is the phase ratio of the HPLC column. Substitution of eqn. 21 into eqn. 17 gives the following equation:

$$\log k' = X_H \log k'_H + X_I \log k'_I + y\{2(X_H \log X_H + X_I \log X_I) + N X_H X_I(\Delta g - \Delta g_S - \Delta g_B)/(2.303RT)\} \quad (22)$$

Approximating Δg_S and Δg_B as equal to Δg, we can obtain the following equation:

$$\log k' = X_H \log k'_H + X_I \log k'_I + y\{2(X_H \log X_H + X_I \log X_I) - N X_H X_I \Delta g/(2.303RT)\} \quad (23)$$

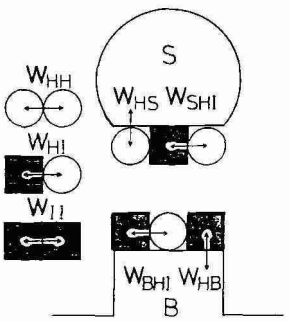

Fig. 2. Visualization of the solvation interaction.

which can also be expressed as:

$$\log k' = a(y)\, X_H^2 + b(y)\, X_H + c(y) + 2y\{X_H \log X_H + (1 - X_H)\log(1 - X_H)\} \quad (24)$$

Here, $a(y)$, $b(y)$ and $c(y)$ denote the coefficients as a function of y. The fourth term on the right-hand side is an additional term of the well-known quadratic equation.

Next, consider the separation factor or selectivity factor, α, defined as

$$\alpha = k'_1/k'_2 \quad (25)$$

Here, the subscripts 1 and 2 denote the different sample peaks. The logarithm of α is defined as follows:

$$\log \alpha = X_H(\log k'_{H1} - \log k'_{H2}) + X_I(\log k'_{I1} - \log k'_{I2}) + (y_1 - y_2)2(X_H \log X_H + X_I \log X_I) - N\Delta g(y_1 - y_2)X_H X_I/(2.303RT) \quad (26)$$

Here, the subscript of y and the second subscript of k' denote the peak number, whereas Δg has no subscript because it is independent of the sample molecules. The overall shape of eqn. 26 is about the same as that of eqn. 23.

Next, consider the resolution function R_S, defined as follows[9]:

$$R_S = \frac{1}{4}(\alpha - 1)\sqrt{N_{TP}}\frac{k'_1}{k'_1 + 1} = \frac{\sqrt{N_{TP}}}{2}\frac{k'_2 - k'_1}{k'_1 + k'_2 + 2} \quad (27)$$

where N_{TP} is the number of theoretical plates of the column. Substituting eqns. 23 and 26 into eqn. 27, we can obtain R_S values for any desired pair of peaks in the various mobile phase compositions.

Further, the SSM can be extended to multisolvent mobile phase systems in order to optimize the composition. Eqn. 23 can be extended to n solvents, as follows:

$$\log k'_1 = \sum_{i=1}^{n} X_i \log k'_{i1} + y\left\{2\sum_{i=1}^{n} X_i \log X_i - \frac{N}{2.3RT}\sum_{i}^{n}\sum_{\neq j} X_i X_j \Delta g_{ij}\right\} \quad (28)$$

where $\sum_{i=1}^{n} X_i = 1$ and Δg_{ij} denote the exchange energy of solvent molecules i and j. If it is desired to estimate the overall value of k' or R_S from experiments in the n-solvents system, it is necessary to evaluate only following number, Q, of unknown parameters in eqn. 28 from at least the same number of experiments:

$$Q = n + 1 + \frac{n(n-1)}{2} = \frac{n^2 + n + 2}{2} \quad (29)$$

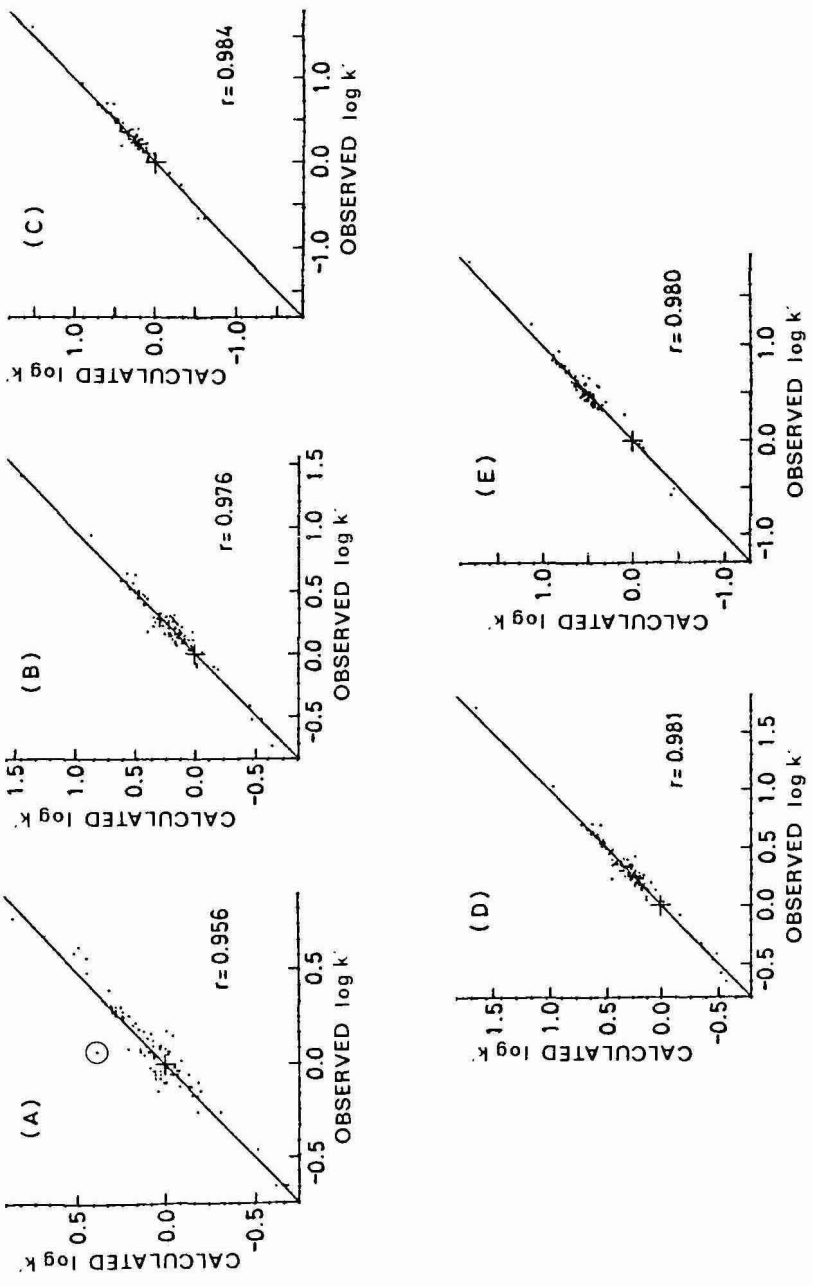

Fig. 3. Correlations between calculated and observed values of the logarithm of the capacity ratio for each drug by fitting eleven parameters. (A) Phenobarbital; (B) phenytoin; (C) nitrazepam; (D) clonazepam; (E) diazepam; the crosses indicate where $k' = 1$; r = correlation coefficient.

EXPERIMENTAL

Samples and columns

Antiepileptic drugs, phenobarbital, phenytoin, nitrazepam, clonazepam, and diazepam, were purchased from Fujinaga Pharmaceutical (Tokyo, Japan), Dainippon Pharmaceutical (Osaka, Japan), Sankyo (Tokyo, Japan), Sumitomo Chemical (Osaka, Japan), and Yamanouchi Pharmaceutical (Tokyo, Japan), respectively. ODS columns, μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.) were used.

Chromatography

A constametric metering pump Model 3 (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) and a Model KLS-3T injector (Kyowa Seimitsu, Tokyo, Japan) were linked to a Model UVIDEC 100 UV detector (Nihon Bunko, Tokyo, Japan). HPLC-grade solvents, acetonitrile, tetrahydrofuran, and methanol, were purchased from Wako (Osaka, Japan), Kanto Chemical (Tokyo, Japan), and Tokyo Kasei (Tokyo Japan), respectively. The flow-rate was 1.0 ml/min. Each sample was dissolved in methanol or ethanol. A volume of 10 μl of sample solution was injected into the column. The hold-up volume was measured by using water as a sample. The results were obtained at ambient temperature (22 ± 4°C).

RESULTS AND DISCUSSION

Five antiepileptic drugs were eluted by 114 arbitrary compositions of mobile phases, and the corresponding capacity ratios for each drug were evaluated. The ranges of the eluting solvents were 0–100% for tetrahydrofuran, 0–100% for acetonitrile, 0–100% for methanol and 0–80% for water. Eqn. 28 was fitted to the experimental points. Fig. 3 shows the correlations between the observed values and fitted (calculated) values of the logarithm of the capacity ratio. A good correlation was obtained with eleven parameters for quaternary solvent mobile phase HPLC. The circled point in Fig. 3A corresponds to an elution with 39% tetrahydrofuran and 61% water. Correlation coefficients between observed and calculated values of the logarithm of the capacity ratio were *ca.* 0.95–0.98. Better correlations can of course be obtained with few and completely empirical parameters.

Our attention is now focused on the values of these parameters rather than the correlation itself. All the parameters obtained are shown in Table I. It can be seen that the number of solvent molecules that adsorb on the sample is less than 1. Parameters that indicate the exchange energy of specific pairs of solvent molecules have the same sign and show similar tendencies. Next, we tried to predict the capacity ratios by using these exchange energy parameters, Δg_{ij}. These six parameters obtained from the fitting procedures for all drugs other than phenobarbital were averaged at each parameter and were used to predict the capacity ratios of phenobarbital. These Δg_{ij} values were substituted into eqn. 28, and the resulting equation was fitted to the experimental points to obtain the $\log k'_{i1}$ and y values specific to phenobarbital.

Fig. 4 shows the correlations between observed and calculated values of the logarithm of the capacity ratio for each drug from the fitted equation. Correlation coefficients between observed values and calculated values were *ca.* 0.94 – 0.97. This means that if we obtain Δg_{ij} values we can accurately predict the capacity ratio of

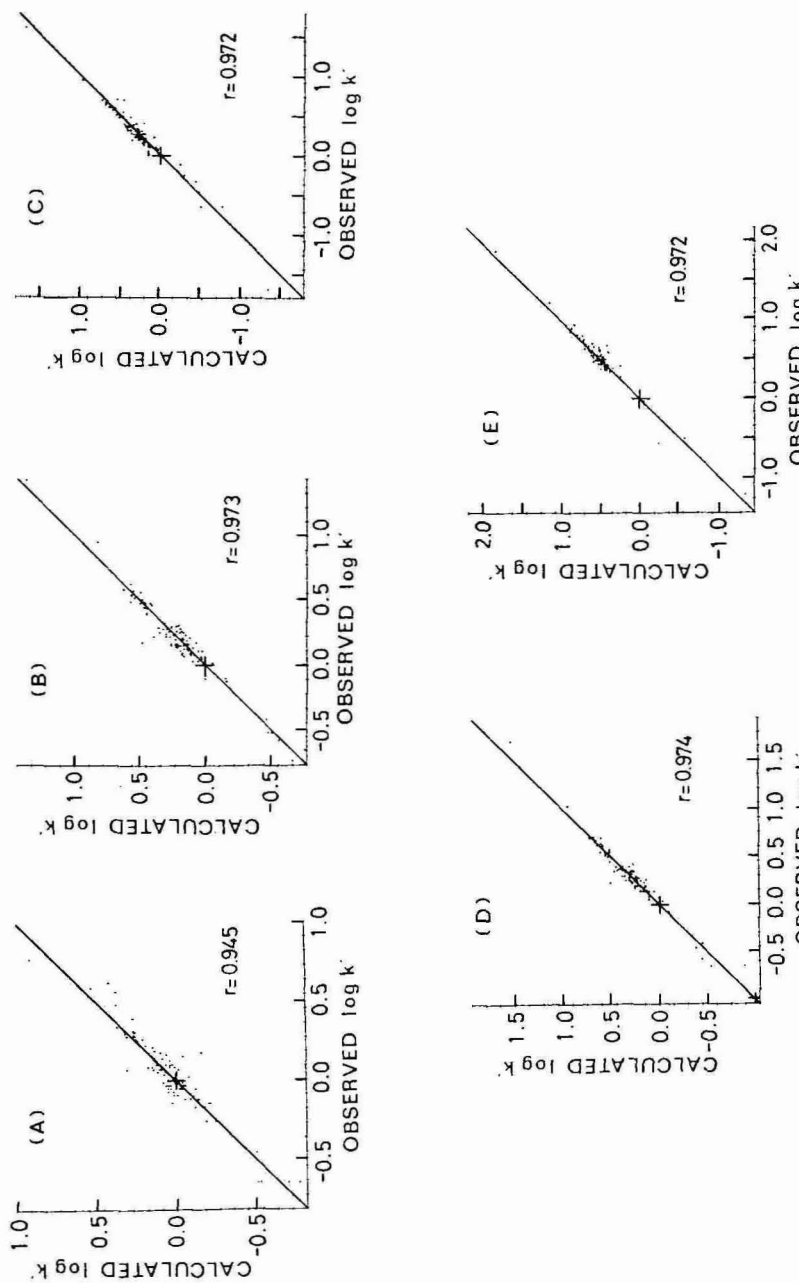

Fig. 4. Correlations between calculated and observed values of the logarithm of the capacity ratio for each drug by fitting five parameters. (A) Phenobarbital; (B) phenytoin; (C) nitrazepam; (D) clonazepam; (E) diazepam; the crosses indicate where $k' = 1$; r = correlation coefficient.

TABLE I

PARAMETERS OBTAINED FROM k' FITTING ON THE EXPERIMENTAL POINTS

α = Tetrahydrofuran; β = acetonitrile; γ = methanol; δ = water.

Parameter	*Antiepileptic drugs*				
	Phenobarbital	*Phenytoin*	*Nitrazepam*	*Clonazepam*	*Diazepam*
$\log k'_\alpha$	−0.66	−0.66	−1.69	−0.65	−1.21
$\log k'_\beta$	−0.67	−0.54	−0.35	−0.45	−0.11
$\log k'_\gamma$	−0.61	−0.63	−0.53	−0.57	−0.43
$\log k'_\delta$	1.83	2.85	2.95	3.08	3.16
y	0.42	0.38	0.79	0.55	0.68
$\frac{N}{2.3RT}\Delta g_{\alpha\beta}$	−2.94	− 3.84	− 4.50	− 2.61	−3.59
$\frac{N}{2.3RT}\Delta g_{\alpha\gamma}$	−12.44	−14.39	−10.19	−11.06	−8.66
$\frac{N}{2.3RT}\Delta g_{\alpha\delta}$	2.34	9.49	1.39	6.52	3.63
$\frac{N}{2.3RT}\Delta g_{\beta\gamma}$	− 7.35	− 9.05	− 6.97	− 8.04	−7.77
$\frac{N}{2.3RT}\Delta g_{\beta\delta}$	4.40	9.17	3.42	5.68	3.57
$\frac{N}{2.3RT}\Delta g_{\gamma\delta}$	3.09	5.73	1.19	3.11	0.64

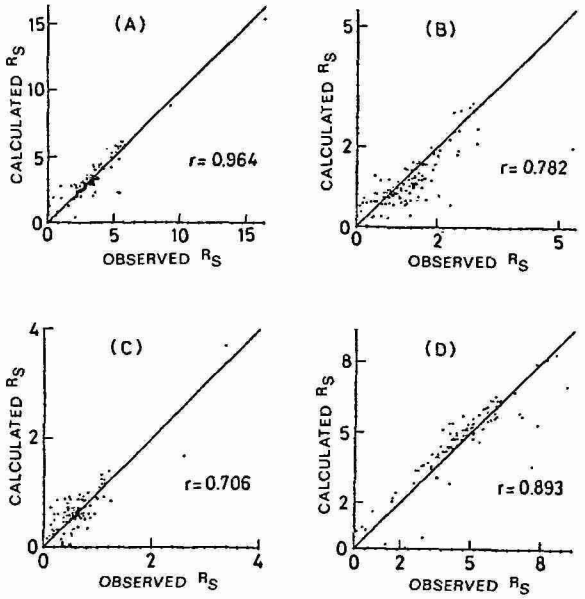

Fig. 5. Correlations between calculated and observed values of R_S for the nearest pairs of the drugs. (A) Phenobarbital–phenytoin; (B) phenytoin–nitrazepam; (C) nitrazepam–clonazepam; (D) clonazepam–diazepam; r = correlation coefficient.

each drug by using eqn. 28 and at least five capacity ratio data (the number specific values, log k'_{i1} and y, is five). Reliability of the prediction, of course, depends on the number of the capacity ratio data used.

Next, the R_S values were evaluated from the parameters obtained. Fig. 5 shows the correlation of observed and calculated R_S values: correlation coefficients were *ca.* 0.70–0.96, relatively low compared with those of the capacity ratios. This shows that the sensitive R_S parameter cannot be easily reproduced by the k' fitting equation, whereas the overall feature of k' itself can be reproduced well.

We may state that capacity ratios can be reproduced well by our thermodynamic model with several parameters. They seem to depend mainly on the exchange energies of the solvent molecules. It remains to be seen whether all these energy values can be related to the chemical thermodynamic values of binary liquids that have not yet been determined. It is still difficult to evaluate R_S values because of their sensitivity caused by the difference between two independent substances.

ACKNOWLEDGEMENTS

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REPETITIVE STRIPPING AND TRAPPING APPLIED TO THE DETERMINATION OF TRACE HYDROCARBONS IN AQUEOUS SAMPLES*

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SUMMARY

The method of repetitive stripping and trapping of analytes was investigated to determine the reliability of the quantitative results. Different experimental variants of the method, *viz.*, a closed circuit and an open arrangement, were tested by analysing water–air model systems with low μg/l levels of benzene, toluene, *n*-decane, *n*-undecane, *n*-dodecane as analytes in the aqueous phase. Whereas in a closed circuit the stripping/trapping process can be conducted either in a conservation or in an equilibration regime, in an open arrangement the conservation or pseudoequilibration (non-stationary conditions) mode of trapping is possible. All these variants yielded good quantitative results. The results of the determination of *n*-decane, *n*-undecane and *n*-dodecane consistently suffered from systematic negative errors of 20–40%, which was attributed to non-constant (concentration-dependent) matrix effects associated with the adsorption of the analytes at the water–air interface. The ability of the repetitive stripping/trapping method to eliminate such effects was tested by comparing the results for the determination of low μg/l levels of benzene, toluene and *n*-decane in a water–air and in a water–Carbowax 400 (9:1)–air system. Whereas the method of external calibration by means of a reference water–air system gave erroneous results for the system with Carbowax 400, the results obtained by the repetitive stripping/trapping method were correct.

INTRODUCTION

Dynamic headspace analysis in combination with sample-enrichment procedures affords a high sensitivity for the determination of volatile compounds in condensed matrices. The process which takes place during the quasi-equilibrium passage of a gas through a condensed material was described more than 20 years ago[1,2]. This process is utilized analytically in combination with a sample-enrichment unit (charcoal filters, short sorbent-packed columns) to which the analyte is gradually transferred from the condensed phase by the stripping process, either in an open arrange-

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ment[3,4] or in a closed circuit[5]. In order to obtain quantitative data, it is necessary to know the relationships between the initial amounts of the analytes in the material (system) analyzed and the amounts recovered from the trap. The amount of the analytes obtained from the trap is often called the recovery of the entire procedure. This recovery depends, among other factors, on the partition coefficients of the analytes in the given system and, in principle, cannot be assumed to reach 100% in any finite time, as has been documented by several authors[6,7]. Quantification based on a comparison with the analysis of a reference sample containing known amounts of the analytes is sometimes complicated by the so-called matrix effects, *i.e.*, by possible differences in the compositions of the matrices of the actual and reference samples and, consequently, the different partition coefficients of a given analyte[8]. One of the methods by which these matrix effects can be eliminated is repetitive gas-phase sampling. The potential of this method has been demonstrated by the determination of hydrocarbons in water[9] or solvent residues in polymers[10] in a static arrangement, and by the determination of alcoholic compounds in water[11] in a dynamic arrangement.

In this paper, the method is discussed for cases in which the stripping process is combined with sample enrichment in either a closed circuit or in an open arrangement. The potential of this method is demonstrated by the determination of traces of hydrocarbons in water at concentrations of a few μg/l.

PRINCIPLES OF QUANTIFICATION

If a stream of inert gas passes at a volumetric flow-rate, F, through the liquid and gaseous phases of volumes V_L and V_G in a gas–liquid system with initial mass m_{io} of analyte i, the change in the mass of the analyte with time, t, is[2,12]

$$\frac{dm_i}{dt} = -\frac{m_i F t}{V_G + K_i V_L} \tag{1}$$

where m_i is the instantaneous mass of the analyte in the system and K_i is the partition coefficient of the analyte, defined as the ratio of the equilibrium concentrations of analyte in the liquid and gaseous phases, *i.e.*, $K_i = c_{iL}/c_{iG}$. Integration and eqn. 1 for the initial conditions $t = 0$, $m_i = m_{io}$ gives:

$$\frac{m_i}{m_{io}} = \exp\left(-\frac{Ft}{V_G + k_i V_L}\right) \tag{2}$$

Eqns. 1 and 2 are valid provided the liquid matrix is virtually non-volatile (V_L = constant), and the analyte concentrations in the liquid phase and stripping gas bubbles leaving the liquid phase are in equilibrium. The stripping gas containing the equilibrium concentration of the analyte passes through a trap and, provided the analyte is captured quantitatively in the trap, the mass of the analyte entrapped in time t, m_{iS}, is:

$$m_{iS} = m_{io} - m_i \tag{3}$$

Combination of eqns. 3 and 2 gives[12]:

$$\frac{m_{iS}}{m_{io}} = 1 - \exp\left(-\frac{Ft}{V_G + K_i V_L}\right) \tag{4}$$

In dynamic headspace analysis, when the stripping process is combined with a sample-enrichment step, there are three variants of the method of repetitive gas-phase sampling, namely: (i) conservation trapping either in a closed circuit or in an open arrangement; (ii) equilibration trapping in a closed circuit and (iii) pseudoequilibration trapping, *i.e.*, trapping under conditions at which the frontal zone of the analyte has broken through the sorbent bed in the trap, in an open arrangement. The analytical procedure is the same with all three variants in that a liquid sample is stripped at least twice and the initial mass of the analyte in the sample is calculated from the analyte contents determined in the concentrates recovered from the trap in the two analytical steps. However, the processes of stripping/trapping differ in each variant and, consequently, the derivations of the equations relating the initial analyte mass to the experimental parameters are also different.

Variant (i)

If the trapping of the analyte from the stripping gas proceeds in a conservation regime, *i.e.*, if the analyte is totally captured in the sorbent of the trap, the amount of analyte entrapped in time t is given by eqn. 4. Hence, in the first step, after the stripping/trapping time $t = t_1$, the mass of analyte entrapped in the sorbent, m_{iS1}, is

$$\frac{m_{iS1}}{m_{io}} = 1 - \exp\left(-\frac{Ft_1}{V_G + K_i V_L}\right) \tag{5}$$

and the mass of analyte that remains in the system is $m_o - m_{iS1}$. In the second step, after the stripping/trapping time $t = t_2$ with a new trap, the analyte mass entrapped in the sorbent is m_{iS2}:

$$\frac{m_{iS2}}{m_{io} - m_{iS1}} = 1 - \exp\left(-\frac{Ft_2}{v_G + K_i V_L}\right) \tag{6}$$

Provided $t_1 = t_2$ and V_G, V_L and K_i are constant, the right-hand sides of eqns. 5 and 6 are identical (a system factor), and it is possible to write

$$m_{io} = \frac{m_{iS1}}{1 - \frac{m_{iS2}}{m_{iS1}}} = \frac{m_{iS1}}{1 - \frac{A_2}{A_1}} \tag{7}$$

where A_1 and A_2 are the areas of the analyte peaks in the chromatograms of the concentrates recovered from the trap in the first and the second step, respectively. The same relationship was derived earlier from the solute mass balance[13].

Variant (ii)

If the stripping/trapping process takes place in a closed circuit and the analyte is not retained too strongly in the sorbent of the trap, the frontal zone of the analyte breaks through the trap and after some time the entire gas–liquid–sorbent system reaches equilibrium. The mass of analyte contained in the trap at equilibrium conditions is given by[12]

$$\frac{m_{iS}}{m_{io}} = \left[\left(\frac{K_{iS}V_S}{V_{Gt} + K_{iS}V_S}\right)\left(\frac{V_G}{K_{iS}V_S} + \frac{K_iV_L}{K_{iS}V_S} + 1\right)\right]^{-1} \tag{8}$$

where K_{iS}, V_S and V_{Gt} are the partition coefficient of the analyte in the trapping sorbent–gas system, the volume of the sorbent in the trap and the void volume of the trap, respectively. Eqn. 8 differs from eqns. 5 and 6 in having a different system factor (the right-hand side of the equation). Provided all the quantities occurring in the system factor are kept constant during the entire analytical procedure, the same treatment as that applied with variant (i) leads again to relationship 7. Hence, we can conclude that with the closed-loop arrangement the method of repetitive stripping/trapping can be used for quantitative analysis no matter whether the process of trapping proceeds in a conservation or in an equilibration regime.

Variant (iii)

In the context of quantitative analysis, this variant is apparently the least favourable. For exact quantification the exponential decay of the analyte concentration with time would have to be taken into account. However, it can be supposed that in each successive stripping/trapping step accomplished under these non-stationary conditions the analyte mass retained in the trap is proportional to the mass that would be entrapped under the conditions of equilibration stripping and trapping. Thus, for steps 1 and 2, respectively

$$m^*_{iS1} = k_1 m_{iS1} \tag{9}$$

$$m^*_{iS2} = k_2 m_{iS2} \tag{10}$$

where m^*_{iS} is the mass of analyte entrapped under the non-stationary (pseudoequilibration) conditions and k is a proportionality constant. Provided $k_1 = k_2$, the substitution of eqns. 9 and 10 into eqns. 5 and 6 results in relation 7.

From the practical standpoint, there is a substantial difference between the methods of equilibration in a closed circuit and pseudoequilibration in an open arrangement. Namely, in the first case a constant amount of the analyte is retained in the trap while the analyte leaving the trap is returned back to the system analyzed after a state of equilibrium has been reached. Hence, the amounts of the analyte in both the trap and the system do not change with time at equilibrium, and it is then immaterial how long the stripping/trapping process is continued. However, in the second case the amounts of the analyte both in the trap and in the system decrease continuously with time during the stripping/trapping process, and it is mandatory to keep the stripping/trapping times in the consecutive analytical steps strictly equal in

order for $k_1 = k_2$. Clearly, the same qualification applies also to variant (i) if the system factor (right-hand sides of eqns. 5 and 6) is to be kept constant.

EXPERIMENTAL

Chemicals

Hydrocarbons of analytical grade purity (Fluka, Buchs, Switzerland) were used as model analytes. Standard solutions of the hydrocarbons were prepared using analytical grade acetone (Lachema, Brno, Czechoslovakia) as a solvent. The liquid matrices of the systems studied consisted of distilled water boiled for some time before use. To study the matrix effects, Carbowax 400 GC stationary phase (Carlo Erba, Milan, Italy) was used as an additive to the liquid phases of the systems. The Carbowax was purified by purging it with a 100 ml/min stream of pure nitrogen for 30 min at 80°C. Blank determinations of the hydrocarbon contents were negative.

Instrumentation

The stripping/trapping experiments in a closed circuit were carried out with a laboratory-made set-up[14] as illustrated in Fig. 1. The main component is a pump, consisting of a stainless-steel bellows unit (1) and a dual ball valve (3). The bellows unit is periodically depressed by a cam driven by a motor (2). The stripping gas (headspace air) pumped by the bellows passes via the valves and enters a glass vessel (4) containing the liquid to be analyzed. Near the bottom of the vessel there is a sintered-glass frit, above which a septum (5) is attached to a short thick-walled glass capillary to enable the introduction of samples and/or standards. In the outlet of the vessel a quartz-wool plug is inserted in order to prevent droplets of the liquid from being entrained further into the tubing. The volume of the vessel was about 80 ml. The compounds stripped from the liquid are captured in trap 6, consisting of a 6 cm × 3 mm I.D. glass tube packed with Tenax GC (30–60 mesh). The individual units of the set-up are connected with a 1 mm I.D. stainless-steel capillary, so that the gas

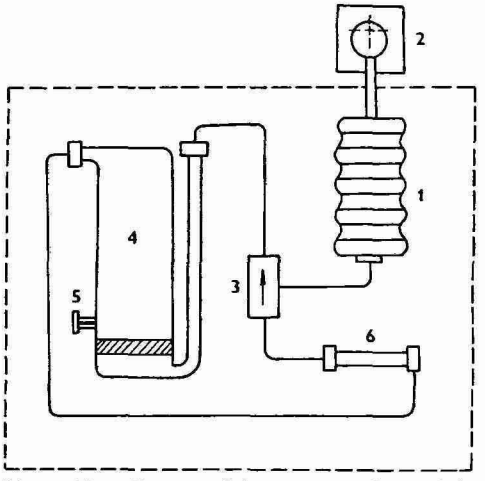

Fig. 1. Flow diagram of the apparatus for work in a closed circuit. 1 = Bellows; 2 = motor; 3 = ball valve; 4 = sample vessel; 5 = septum; 6 = trap.

circulating within the set-up is in contact only with glass and stainless steel. The whole arrangement is fixed to a console whereby it may be immersed in a thermostatted water-bath (shown by a dashed line in Fig. 1).

A schematic representation of the set-up for stripping/trapping in an open arrangement is shown in Fig. 2. The flow-rate of nitrogen (stripping gas) is controlled by needle valve 1 and led by the four-port stopcock 2 either via vessel 3 to trap 4 or to flow meter 5. Septum 6 serves the same function as in the set-up for work in a closed circuit. The thermostatting is again accomplished by use of a water-bath.

The analytes entrapped in the sorbent are thermally desorbed, and the concentrate is entrained by the carrier gas into the analytical column of the gas chromatograph. This is accomplished with the aid of the arrangement shown schematically in Fig. 3. The carrier gas is directed by four-port stopcock 1 into the sample-introduction port of the gas chromatograph, either directly or via trap 2, the latter being heated in oven 3.

The gas chromatographic (GC) analyses were carried out on a GC4A Shimadzu gas chromatograph (Shimadzu Seisakusho, Kyoto, Japan) with a flame ionization detector and a 1.5 m × 3 mm I.D. stainless-steel column containing 15% (w/w) Carbowax 20M on Chromosorb G (100–120 mesh) (Carlo Erba). The temperature of the GC column was 65°C and the sample inlet port and detector were kept at 130°C. The trap was heated to 180°C. The carrier gas (nitrogen) flow-rate was 35 ml/min. The peak areas were measured by a CI-100 integrator (Laboratory Instruments, Prague, Czechoslovakia).

Analytical procedure

The model samples were prepared directly in the vessel of the set-up for stripping/trapping by injecting 1 μl of a standard solution of the model hydrocarbons in acetone into the liquid phase [distilled water alone and/or distilled water and Carbowax 400 (9:1, w/w)]. The concentrations of the analytes in the standard solutions

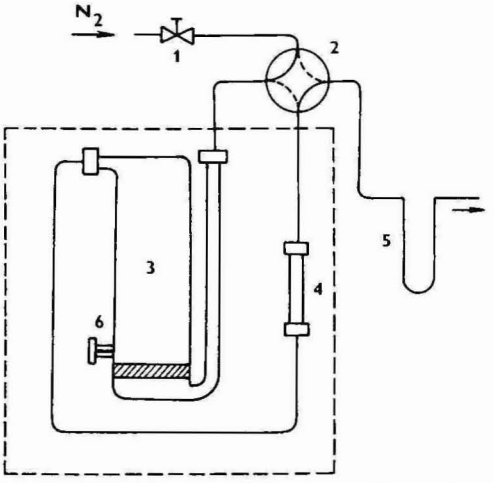

Fig. 2. Flow diagram of the apparatus for work in an open arrangement. 1 = Needle valve; 2 = four-port stopcock; 3 = sample vessel; 4 = trap; 5 = flow meter; 6 = septum.

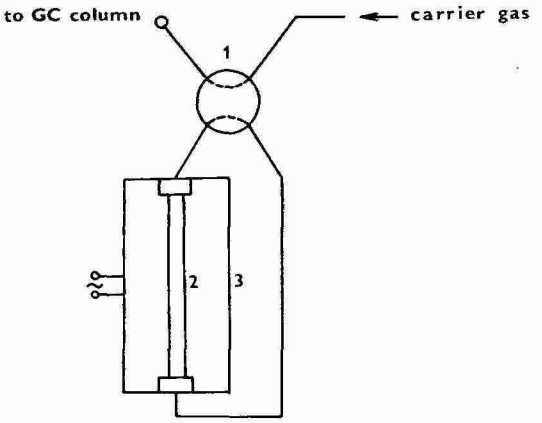

Fig. 3. Flow diagram of the arrangement for the thermal desorption of the concentrate and its introduction into the gas chromatograph. 1 = Four-port stopcock; 2 = trap; 3 = oven.

were chosen so as to obtain samples containing a few μg/l (ppb) of these analytes. The stripping/trapping process was commenced after the vessel had been shaken and heated to 40 ± 0.1°C (410 Ultrathermostat; VEB Prüfgeräte, Medingen, G.D.R.). After each analysis the vessel was washed with distilled water and the entire set-up was purged with a stream of nitrogen. The trap containing the analytes was heated for 3 min at 180°C, whereupon the concentrate was transferred to the gas chromatograph as described above (Fig. 3).

The results obtained by repetitive stripping/trapping were compared with those obtained by injecting samples of the standard solutions directly into the gas chromatograph. By considering the former data as "found" and the latter as "given", the accuracy of the results could be evaluated.

RESULTS AND DISCUSSION

In a previous paper[14], preliminary results obtained by the method of repetitive stripping/trapping in a closed circuit indicated some discrepancies that were attributed to mutual interferences of the analytes. These results have been thoroughly checked in this study, and the revised data are summarized in Table I. The model analytes chosen cover a wide range of boiling points and represent both volatile aromatics and high-boiling paraffins, which both show a tendency to adsorb at the water–air interface[15]. In order to elucidate the mutual interferences of the analytes and the effect of these on the results obtained by the repetitive stripping/trapping method, model samples containing single solutes, several solutes and a relative excess of *n*-dodecane (38 μg/l) were studied separately; the solubility of *n*-dodecane in water was estimated to be about 18 μg/l. For aromatic hydrocarbons the method gives reliable results, although with benzene in the presence of higher *n*-alkanes (especially when in excess) there is a higher probability of a systematic error. In spite of this, however, the relative bias and standard deviation scarcely exceed 10%.

With benzene and toluene the trapping in the Tenax column took place in an equilibration and a conservation regime under the experimental conditions employed

TABLE I

RESULTS OF THE DETERMINATION OF HYDROCARBONS IN WATER BY THE METHOD OF REPETITIVE STRIPPING AND TRAPPING IN A CLOSED CIRCUIT

Conditions: A, single analyte present in the system; B, the analyte in the presence of all the other ones at the concentrations quoted; C, the analyte in the presence of an excess (about 38 μg/l) of *n*-dodecane; D, B + C. The stripping/trapping time was 10 min at 80 ml/min for each step.

Analyte	*Concn. (μg/l)*	*Rel. bias (%)*	*S.D. (%)*	*Conditions*	*No. of measurements*
Benzene	1.8	−10.3	7.0	A	5
		−11.3	3.4	B	6
		−14.1	4.8	C	5
		−12.1	15.7	D	7
Toluene	1.8	−6.2	5.0	B	5
		−5.1	6.3	D	8
n-Decane	1.5	−22.6	3.7	A	7
		−20.8	6.4	B	5
		−35.8	6.1	C	5
		−34.1	5.7	D	8
n-Undecane	3.0	−27.0	6.0	B	5
		−40.0	3.6	D	7
n-Dodecane	7.5	−32.1	7.9	B	5

(with 16 mg of Tenax the retention volumes of benzene and toluene are about 200 and 1000 ml at 40°C, respectively), but the mode of trapping had no effect on the reliability of the results.

As for the systems with aliphatic hydrocarbons, with *n*-decane alone in water the determination suffers from a negative bias of about 20%, and in the presence of an excess of *n*-dodecane the bias of the *n*-decane determination is even larger. As the precision of determination in these cases is the same as that in the analyses of the aromatic hydrocarbons, systematic errors have to be envisaged in the analyses of systems containing higher alkanes. The adsorption of these alkanes at the water–air interface remains a possible cause of the errors. This explanation is corroborated by the significant effect of the presence of *n*-dodecane on the error. Being present in about a two-fold concentration over its solubility limit, *n*-dodecane probably forms a microphase at the water surface, which might enhance the tendency of other hydrocarbons to adsorb. However, this could be established only by exact measurements of the partition coefficients of the analytes in the systems studied. Though the gaseous phase contacts only glass and stainless steel, the surfaces of these materials are most likely covered with a layer of adsorbed water and thus act as water–air interfaces with respect to the analytes. The systematic negative error indicates that the overall matrix effects are not constant during the stripping/trapping process. Namely, as the capacity of the water surface and/or *n*-dodecane microlayer in the systems studied is small, even very low surface concentrations of the analytes adsorbed may exceed those corresponding to the Henry-law region. If the effective partition coefficient (bulk dissolution plus adsorption) of an analyte in the water–air

TABLE II

RESULTS OF THE DETERMINATION OF HYDROCARBONS IN WATER BY THE METHOD OF STRIPPING AND TRAPPING IN AN OPEN ARRANGEMENT

Conditions: E, trap containing 16 mg of Tenax, single solute; F, trap containing 98 mg of Tenax, single solute; G, trap containing 98 mg of Tenax, solute in the presence of an excess (about 38 μg/l) of *n*-dodecane. The stripping/trapping time was 5 min at 80 ml/min (nitrogen) in each step.

Analyte	*Concn. (μg/l)*	*Rel. bias (%)*	*S.D. (%)*	*Conditions*	*No. of measurements*
Benzene	1.8	−52.2	2.9	E	6
		−0.8	4.5	F	6
		−4.2	3.6	G	6
Toluene	1.8	−8.6	5.6	E	6
		−3.6	7.5	F	8
		−3.5	5.6	G	8
n-Decane	1.5	−12.1	4.1	E	5
		−18.5	4.2	F	7
		−26.8	5.9	G	9
n-Undecane	3.0	−18.7	10.1	E	5
		−18.7	8.9	F	10
		−8.8	31.8	G	8
n-Dodecane	7.5	−21.4	11.6	E	5
		−6.2	18.1	F	6

system decreases during the stripping/trapping process, the ratio A_2/A_1 in eqn. 7 becomes larger than that corresponding to a constant partition coefficient, thus resulting in a negative error.

The results obtained by repetitive stripping/trapping in an open arrangement suffer from practically the same errors as those obtained in a closed circuit (see Table II). With the aliphatic hydrocarbons the systematic errors are somewhat smaller as compared to the corresponding results obtained in a closed circuit, however, the large random error of the determination of *n*-undecane in the presence of *n*-dodecane suggests that the small bias in this case is most likely incidental.

TABLE III

RESULTS OF THE DETERMINATION OF HYDROCARBONS IN WATER–CARBOWAX 400 (9:1) BY THE METHODS OF EXTERNAL CALIBRATION (REFERENCE SYSTEM: PURE WATER AS A LIQUID MATRIX) AND REPETITIVE STRIPPING

Conditions: open arrangement, trap containing 98 mg of Tenax, stripping/trapping time 5 min at 80 ml/min (nitrogen) in each step. Six measurements in each case.

Analyte	*Concn. (μg/l)*	*External standard*		*Repetitive stripping*	
		Rel. bias (%)	*S.D. (%)*	*Rel. bias (%)*	*S.D. (%)*
Benzene	1.8	−13.0	5.7	−5.9	6.7
n-Decane	1.5	−60.8	3.4	−15.4	5.1
Toluene	3.6	−11.9	8.2	−4.6	4.6

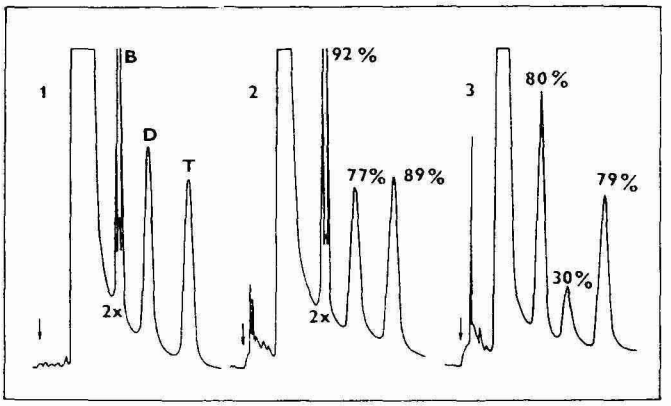

Fig. 4. Efficiency of the stripping of benzene (B), *n*-decane (D) and toluene (T) from liquid matrices of different compositions. (1) Chromatogram of 1 μl of a standard solution of B, D and T (each about 3 μg/l) in acetone; (2) and (3) chromatograms of the concentrates obtained by the 5-min stripping/trapping of B, D and T from systems with pure water and water–Carbowax 400 (9:1), each system having been doped with 1 μl of the standard solution.

In order to demonstrate the ability of the repetitive stripping/trapping method to eliminate the matrix effects, systems in which the liquid phase comprised water–Carbowax 400 (9:1, w/w) were analyzed by this method (open arrangement), and the results were compared with those obtained by external calibration using reference systems with pure water as the liquid matrix. Benzene, toluene and *n*-decane at concentrations of a few μg/l in the liquid phase were used as model analytes. The results of this comparison are summarized in Table III. The effect of the composition of the liquid matrix on the efficiency of stripping is shown in Fig. 4. The first chromatogram was obtained by injecting 1 μl of the standard solution of the model analytes in acetone directly into the gas chromatograph. The second and third chromatograms represent the analyses of the concentrates recovered from the Tenax trap after 5-min stripping/trapping of liquid–air systems with pure water and the water–Carbowax 400 mixture, respectively, each containing 1 μl of the above standard solution. The effect of the presence of Carbowax is manifested with all the analytes, most markedly with *n*-decane. If the peak areas of chromatogram 2 are used as reference data to quantify the analysis of the "actual sample" represented by chromatogram 3, the systematic errors are as quoted in Table III, whereas the repetitive stripping/trapping method yields fairly reliable results, independent of the presence of Carbowax.

CONCLUSIONS

The method of stripping and trapping of analytes both in a closed circuit and in an open arrangement gives good quantitative results. Whereas in a closed circuit the stripping/trapping process can be accomplished either in a conservation or in an equilibration regime, conservation or pseudoequilibration modes of trapping are possible when working in an open arrangement. All the above variants can be used for quantitative analysis, however, when working in an open arrangement better results are obtained by conservation trapping.

Larger negative errors in the determination of higher aliphatic hydrocarbons in water indicate that the matrix effects stemming from the adsorption of the analytes at the water–air interface are not quite constant during the stripping/trapping process. In spite of this, however, results obtained by the method of repetitive stripping and trapping are fairly insensitive to changes in the composition of the liquid matrix of the system studied.

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INTERFERENCE EFFECTS IN TRAPPING TRACE COMPONENTS FROM GASES ON TENAX-GC

SORPTION OF BENZENE IN THE PRESENCE OF *n*-BUTANOL, *o*-XYLENE AND *n*-PENTANE

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SUMMARY

Sorption equilibria were measured in systems of Tenax-GC and benzene–*n*-butanol–nitrogen, benzene–*o*-xylene–nitrogen and benzene–*n*-pentane–nitrogen gaseous mixtures at 19.9, 27.1 and 35°C. With all the systems the benzene (a model analyte) concentration was kept at 0.5 ppm and the concentrations of *n*-butanol, *o*-xylene and *n*-pentane (interferents) were varied over a range of four orders of magnitude. The dependences of the partition coefficient of benzene on the concentrations of the interferents are discussed in terms of the conservation and equilibration modes of concentrating trace substances in sorbent-packed trapping columns.

INTRODUCTION

The concentration of trace components of gaseous mixtures on solid sorbents is mostly carried out by employing the method of conservation trapping[1]. The method of equilibration trapping was introduced[2] as an alternative to conservation trapping and applied with the use of both liquid[3] and solid[4–7] sorbents as trapping column packings. Both modes of trapping have been discussed in detail in earlier papers[8,9]. The choice of the method of trapping depends above all on the properties of the material to be analysed, namely, the nature and concentration range of the components. These criteria are also most relevant for the choice of suitable sorbent packings for the trapping column.

In order to assess the breakthrough volume with the conservation method, it is necessary to know the sorption isotherm of the most weakly sorbed (most mobile) analyte in the trapping column and the plate number of the latter. The changes in the retention of the most mobile analyte in its zone migrating down the trapping column, due to displacement by the succeeding frontal zones of more strongly (less mobile) analytes, are usually negligible[10].

Two of the reasons why the conservation method has found such wide application are its simplicity and the possibility of easily estimating experimentally the

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safe sampling volume. Usually a control trapping column is connected to the outlet of the working column, and different sample volumes are drawn through the series of the columns while checking the control column for the content of the substances being trapped. The use of the method of equilibration trapping is limited to cases in which it is possible to determine reliably the partition coefficients of the analytes in the trapping sorbent–analysed mixture system under the actual conditions of analysis.

In this paper, the effect of *n*-butanol, *o*-xylene and *n*-pentane as interfering components on the partition coefficient of benzene on Tenax-GC in model systems of known compositions is demonstrated. The findings are interpreted and discussed in terms of the concentration of trace substances in sorbent-packed columns by the above two methods of trapping.

EXPERIMENTAL

Systems with three gaseous model mixtures were investigated: (i) benzene–*o*-xylene–nitrogen, (ii) benzene–*n*-butanol–nitrogen and (iii) benzene–*n*-pentane–nitrogen. With all the three systems the concentration of benzene was kept at 0.5 ppm while the concentrations of the other (interfering) components were varied over a range of about four orders of magnitude. The method of preparation of the model mixture and the measurement and calculation of the partition coefficients have been described earlier[8,11]. For each system, the partition coefficients of benzene and of the interfering component, as a function of the interferent concentration, were determined at three temperatures: 19.9, 27.1 and 35°C. The data obtained are presented graphically in Figs. 1–3.

RESULTS AND DISCUSSION

The model systems (i)–(iii) were chosen with a view to demonstrating three possible cases of the mutual relationship between the partition coefficients of benzene (K_1) and of the interferent (K_2), namely: (i) $K_2 > K_1$, (ii) $K_2 = K_1$ and (iii) $K_2 < K_1$, respectively. The partition coefficient is defined as the ratio of the sorbate concentrations in the sorbent (c_s, mol/g) and in the gaseous phase (c_g, mol/ml), and hence the dimensions of $K = c_s/c_g$ are ml/g.

With all the model systems, the partition coefficient of benzene decreases with increasing concentration of the interferent in the gaseous phase. In view of the conservation mode of trapping, the increase in the gas-phase concentration of benzene within its migrating frontal zone, caused by partial displacement of benzene from the sorbent by the more slowly advancing interferent frontal zone, is important, as it is this concentration that determines the migration velocity of the zone of benzene.

The ratio of the gas-phase concentration of an analyte within its migrating frontal zone to that in the mixture fed into the trapping column, F, has been defined[10] as

$$F = (K_2 - K_1^2)/(K_2 - K_1) \quad (1)$$

where K_2 and K_1 are the actual partition coefficients (at the actual concentrations of the respective compounds) of the interferent and analyte alone within their frontal

TABLE I

VALUES OF THE FACTOR F FOR BENZENE (ANALYTE) AND *o*-XYLENE, *n*-BUTANOL AND *n*-PENTANE (INTERFERENTS) AT THE BENZENE GAS-PHASE CONCENTRATION OF 0.5 ppm AND DIFFERENT GAS-PHASE CONCENTRATIONS (c_{g1}) OF THE INTERFERENTS

Interferent	*Parameter*	*Value*				
o-Xylene	c_{g1} (ppm)	0.08	0.25	0.8	2.5	7.9
	F	1.00	1.01	1.05	1.24	2.0
n-Butanol	c_{g1} (ppm)	0.8	2.5	8.0	25.0	79.0
	F	1.86	2.60	10.7	37.0	–

zones, and K_1^2 is the actual partition coefficient of the analyte within the mixed analyte–interferent zone. The values of the factor F for the benzene gas-phase concentration of 0.5 ppm and several gas-phase concentrations of *o*-xylene and *n*-butanol at 19.9°C are presented in Table I.

As quoted earlier[10], the interrelation between K_2 and K_1 renders it necessary to employ an iterative procedure when calculating the factor F using eqn. 1, in which the Henry-law K_1 value is used as the first approximation. However, as this K_1 value is larger than K_2 with benzene–*n*-butanol systems, eqn. 1 cannot be used to calculate the factor F in this case. Within the mixed benzene–*n*-butanol zone at *n*-butanol concentrations lower than 50 ppm, the benzene partition coefficient, K_1^2, is smaller than the partition coefficient of *n*-butanol, K_2, *i.e.* $K_2 > K_1^2$. Within this range of *n*-butanol concentrations, the displacing action of *n*-butanol induces a gradual increase in the benzene gas-phase concentration ahead of the mixed frontal zone, thus decreasing the partition coefficient of benzene within this zone and accelerating the latter.

The decrease in the partition coefficient of *n*-butanol on increasing its concentration is steeper than that with benzene. Hence, at certain concentrations of these two compounds their partition coefficients have the same values, *i.e.*, $K_2 = K_1$, and the value of F is infinity. Considering the mixed benzene–*n*-butanol frontal zone at *n*-butanol concentrations below 50 ppm from this point of view, we can imagine the following situation: as benzene is gradually being displaced by *n*-butanol from the migrating mixed zone, a leading zone of benzene alone is formed in wich the benzene gas-phase concentration gradually rises and the respective partition coefficient decreases until K_1 in this zone becomes equal to K_2 in the rear mixed zone. From that moment, a stationary state is established in which the benzene partition coefficient in the leading zone is only slightly smaller than that of *n*-butanol in the rear zone, and both zones migrate at virtually the same velocities. Clearly, this is a typical case of classical displacement chromatography[12].

The behaviour of *o*-xylene as an interfering component differs from that of *n*-butanol. Whereas with benzene–*n*-butanol mixtures the displaced benzene zone ahead of the mixed one develops gradually in the course of mixed zone migration, *i.e.*, during frontal chromatography of the two components, with benzene–*o*-xylene mixtures this benzene zone is developed immediately after the mixture enters the column. Hence, pure displacement chromatography takes place from the very beginning of the trapping process with benzene–*o*-xylene mixtures on Tenax-GC. Clearly,

this is so because *o*-xylene is a more strongly sorbed compound and, consequently, a more efficient displacer than *n*-butanol. In the case of the interference by *n*-butanol, the gas-phase concentration of benzene ahead of the mixed benzene–*n*-butanol zone increases proportionately to the volume of the gas mixture having been drawn through the trapping column until the benzene gas-phase concentration in the leading benzene zone reaches a value at which the partition coefficient of benzene within this zone becomes equal to that of *n*-butanol. The values of the factor F in Table I were calculated under the assumption that $K_1 = K_2$, using the experimental data published earlier[8]. At an *n*-butanol gas-phase concentration of 79 ppm the *n*-butanol frontal zone moves faster than that of benzene, and the interfering effect of *n*-butanol becomes identical with that of *n*-pentane.

As it is actually the sorbent-phase concentration of the interferent that controls the extent of displacement, the mere gas-phase concentrations of the interferents are not unequivocal criteria of the interfering effects. There is the same decrease in the benzene partition coefficient, relative to that in the absence of any interferent, at substantially different gas-phase concentrations of the three interferents studied. On the other hand, the sorbent-phase concentrations of these interferents that bring about the same displacement of benzene do not differ much from each other. Thus, at 19.9°C the sorbent-phase concentrations of *o*-xylene, *n*-butanol and *n*-pentane at which the benzene partition coefficient amounts to half of that in the absence of

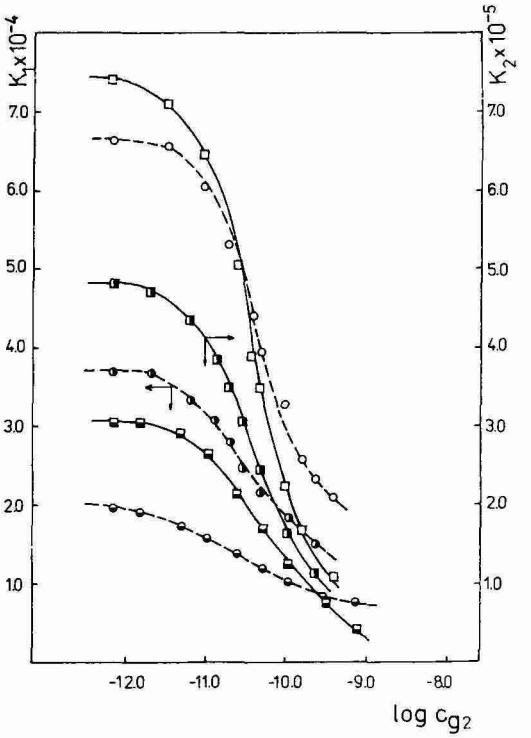
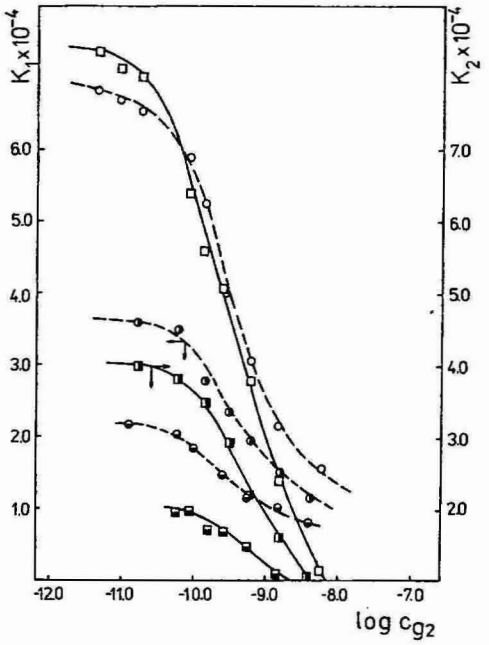


Fig. 1. Dependences of the partition coefficients of benzene (○, K_1, c_{g1} = 0.5 ppm) and *o*-xylene (□, K_2) on the gas-phase concentration (c_{g2}) of *o*-xylene. □, ○, 19.9°C; ◧, ◑, 27.1°C; ⬓, ◒, 35°C.



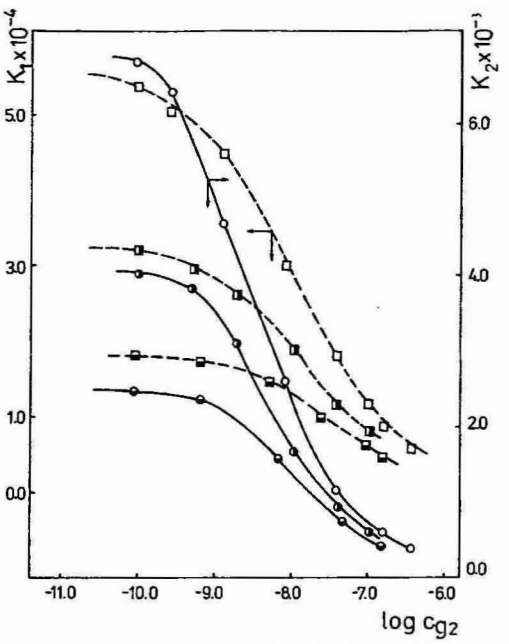


Fig. 2. Dependences of the partition coefficients of benzene (○, K_1, c_{g1} = 0.5 ppm) and *n*-butanol (□, K_2) on the gas-phase concentration (c_{g2}) of *n*-butanol. □, ○, 19.9°C; ◨, ◑, 27.1°C; ◒, ◒, 35°C.

Fig. 3. Dependences of the partition coefficients of benzene (○, K_1, c_{g1} = 0.5 ppm) and *n*-pentane (□, K_2) on the gas-phase concentration (c_{g2}) of *n*-pentane. □, ○, 19.9°C; ◨, ◑, 27.1°C; ◒, ◒, 35°C.

interferents are $2.05 \cdot 10^{-5}$, $1.96 \cdot 10^{-6}$ and $2.36 \cdot 10^{-5}$ mol/g, respectively. With benzene alone, the decrease in its partition coefficient to half of that at infinite dilution is achieved at $1.85 \cdot 10^{-5}$ mol/g of benzene in the sorbent.

The finding that the same displacement effect is achieved with all the sorbates investigated at about the same molar concentrations in the sorbent indicates the non-selective sorption of these compounds on Tenax-GC. Under these circumstances, it is possible to assess the change in the analyte partition coefficient from the known overall concentration of the interferents in the sorbent, which is very important in view of the equilibration mode of trapping. As can be seen in Figs. 1–3, the same relative changes in the sorbent-phase concentrations result in about the same relative changes in the partition coefficients with benzene and the interferents.

At as gas-phase concentration of 0.5 ppm, the effect of benzene on the partition coefficients of the interferents is negligible. Hence the data obtained can be used to estimate the breakthrough volumes of *o*-xylene, *n*-butanol and *n*-pentane on Tenax-GC. With all the model systems investigated, an increase in temperature results in widening of the concentration range over which Henry's law is valid for the sorbates involved. This behaviour is apparently due to the decrease in the partition coefficients and, consequently, the sorbent-phase concentrations of the sorbates.

CONCLUSION

When trapping trace substances from complex gaseous mixtures in columns packed with solid sorbents, significant interferences among the sorbates may occur. These interferences not only result in decreases in the partition coefficients of the analytes on the given sorbent, but they can even induce a change in the mechanism of the process taking place in the trapping column. In the absence of interferences, and/or if they are not very strong, the compounds being trapped are subjected to frontal chromatography in the trapping column and, after a state of equilibration has been attained with all the components of the gas sample mixture throughout the trapping column, the latter contains all these components in concentrations given by the products of their gas-phase concentrations and the respective actual partition coefficients on the given sorbent (provided that the contents of the components in the gas mixture occurring in the interstitial space of the trapping column are negligible). However, if there are great differences in the sorbabilities of the sample components, and some of the components are sorbed so strongly as to displace completely less strongly sorbed components, the latter will be subjected to displacement rather than frontal chromatography. In this instance, the components that form displacement zones during the sampling process are gradually pushed out of the trapping column, and only very small amounts (those present in the sampled gas mixture that occurs in the interstitial space within the trapping column) of them will be found in the trapping column in the state of final equilibration. Hence, with the conservation mode of trapping these strong displacement effects need not necessarily influence the situation drastically, but with equilibration trapping the displaced components become virtually lost for analysis.

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GAS CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF LINEAR AND BRANCHED-CHAIN ALKYL CHLORIDES

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SUMMARY

Polar and non-polar liquid stationary phases (Apiezon L, tricresyl phosphate and Carbowax 20M) were used for the analysis of C_5–C_8 linear and branched-chain alkyl chlorides. Specific retention volumes, retention index and relative molar heats of solution of alkyl chlorides with respect to the homomorphous alkanes were measured and correlated with molecular structure, in order to permit the identification of isomeric compounds in a mixture.

The behaviour of alkyl chlorides was compared with that of the corresponding bromo- and iodoalkanes, on the basis of previously published data.

INTRODUCTION

Increasing attention has been devoted in recent years to the analysis of halogenated compounds in the environment, owing to their possible carcinogenic effects. Trihalomethanes (THM) formed in the disinfection of water with chlorine, other volatile halocarbons resulting from water pollution by industrial and domestic solvents, pesticides and fumigation agents have all been detected and measured in various amounts[1–10]. Analyses of different environmental samples showed the presence of some halogenated compounds, not identified as commonly used solvents, that can be formed by thermal cracking or by biological degradation of polymeric substances, paints, complex halogenated molecules of different kinds, etc.

The identification of these unknown compound should be possible by gas chromatography–mass spectrometry (GC–MS), but this expensive technique is not available in many laboratories that routinely perform environmental analyses on restricted areas, river basins, water supplies, etc. Some attempts were therefore made to achieve such identification on the basis of GC only, by investigating the behaviour of many halogenated compounds on stationary phases of different polarities. In a first series of studies dealing with haloalkanes results were published on the analysis of alkyl bromides[11], and other experiments are now in progress in order to establish the GC behaviour and the electron-capture detection (ECD) sensitivity of compounds containing different halogen atoms in the same molecule.

This paper describes experiments performed with various stationary phases in

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order to obtain the complete separation of C_5–C_8 isomeric chloroalkanes and to identify them on the basis of GC results.

EXPERIMENTAL

The analyses were carried out on a Varian 3760 gas chromatograph with a thermal conductivity detector. The columns (3 m × 1/8 in. I.D.) were made of stainless steel or glass and filled with known amounts of 15% Apiezon L (APL), tricresyl phosphate (TCP) or Carbowax 20M (CW 20M) on Chromosorb W AW DMCS (80–100 mesh).

Pure helium was used as the carrier gas, and the inlet pressure was adjusted to give a constant flow-rate of 30 ± 0.5 ml/min at any temperature (75, 100 and 125°C). The flow-rate was measured after each analysis and a correction factor was applied in order to convert the retention values to the nominal flow-rate of 30 cm^3/min.

Samples of about 0.2 μl, pure or dissolved in *n*-hexane, were injected directly into the column inlet. No appreciable difference in the retention times was observed between glass and stainless-steel columns. A Varian Vista 402 data system was used to measure the retention times, with an accuracy of ±0.01 min. The adjusted retention times, t'_R, were calculated with respect to the retention time of air.

Specific retention volumes, V_g, were calculated[12] by using the equation

$$V_g = \frac{F(P_0 - P_w)}{P_0} \cdot \frac{1.5\,(P_i/P_0)^2 - 1}{(P_i/P_0)^3 - 1} \cdot \frac{273}{T\,W} \cdot t'_R \qquad (1)$$

where

F = flow-rate (ml/min) measured by a soap-bubble flow meter;
P_0 = pressure at the column outlet (Torr);
P_w = vapour pressure of water (Torr) at temperature T;
P_i = column inlet pressure (Torr);
T = temperature (K) at which the gas flow was measured;
W = weight (g) of liquid stationary phase in the column.

Relative molar heats of solution, ΔH_s^0, *i.e.*, the difference between molar heats of solution for the chlorinated compound and the homomorphous alkane, were calculated from the equation

$$\Delta H_s^0 = \frac{1.98\,(\ln r_1 - \ln r_2)}{1/T_1 - 1/T_2}, \qquad (2)$$

where r is the relative retention with respect to the homomorphous alkane, T is the absolute temperature of the column and subscripts 1 and 2 refer to the different temperatures[13]. The average values of ΔH_s^0 calculated between 75 and 100°C and between 100 and 125°C were used.

RESULTS AND DISCUSSION

Tables I and II give the specific retention volumes, V_g, and retention indices, I, of the alkyl chlorides on APL, TCP and CW 20M columns.

The logarithms of the V_g values of straight-chain 1-, 2- and 3-chloroalkanes followed a linear relationship (correlation coefficient $r > 0.99$) with the number of carbon atoms, n, at all temperatures on all of the stationary phases tested. Table III shows the coefficients of the equation $\ln V_g = b_0 + b_1 n$ for straight-chain alkyl chlorides. The Arrhenius plots ($\ln t'_R = m + n/T$, where T is the absolute temperature of analysis) were also linear ($r > 0.97$) in the investigated temperature range for both straight- and branched-chain compounds.

The behaviour of the V_g values of 1-chloroalkanes is compared with those of linear alkanes and of other 1-haloalkanes[11,14] in Fig. 1. From Fig. 1 and the data in Tables I and II, one can see that the increase in the retention indices of halogenated compounds with increasing polarity of the column is due to the greater decrease in the retention of alkanes with respect of that of the alkyl halides. This can be expressed in terms of "equivalent methylenes" (EM), which should be added to the homomorphous alkane in order to obtain a straight-chain alkane with the same retention as the halogenated compounds.

The values in Table IV were calculated by averaging the ΔI values (I of a haloalkane minus I of the alkane with the same number of carbon atoms) obtained at 75, 100 and 125°C and therefore do not take into account the effect of the column temperature. A fine structure of the EM values as a function of temperature was observed, mainly for 1-halo compounds and when the EM values are high enough to largely overcome the experimental fluctuations.

As an example, on the upper graph in Fig. 2 (average EM values for 1-iodoalkanes) are superimposed the EM values for the same compounds measured at 75, 100 and 125°C, showing that the average values are very close to the EM measured at the intermediate temperature of 100°C. The same behaviour was observed for other homologous series of 1-, 2- and 3-haloalkanes but, when the EM values decreased, the effect of temperature was of the same order of magnitude as the standard deviation of the EM averaged over compounds with a different number of carbon atoms. Fig. 2 also shows that the polarity of the TCP column is nearly intermediate between those of the APL and CW 20M columns for the separation of haloalkanes. Table IV shows that the replacement of a single hydrogen atom with a halogen drastically increases the retention time of the compounds, mainly when the polarity of the column is greater and when the substitution takes place at the end of the straight alkane chain. In contrast, small differences were observed between 2-, 3- and (where available) 4-halo compounds. The difference in the EM values between haloalkanes with the substituent atom at the end of the chain or in an internal position was about 0.4–0.5 on the APL, 0.6 on the TCP and 0.7–0.8 on the CW 20M column (see Table IV).

Branched-chain compounds showed similar behaviour (see Tables I and II): the increase in retention was greater when the halogen atom was in the 1-position, mainly when the side methyl group was in the 2-position. In contrast, when the halogen atom was on the same carbon atom of the side methyl group, the increase in the retention had the smallest value.

TABLE I

SPECIFIC RETENTION VOLUMES, V_g, OF ALKYL CHLORIDES ON DIFFERENT COLUMNS AT VARIOUS TEMPERATURES

Carrier flow-rate: 30 cm³/min. Amount of stationary phase: 15% (w/w).

Compound	*Apiezon L*			*Tricresyl phosphate*			*Carbowax 20M*		
	75°C	*100°C*	*125°C*	*75°C*	*100°C*	*125°C*	*75°C*	*100°C*	*125°C*
1-Chloropentane	8.17	3.28	1.72	7.27	3.66	1.92	4.76	2.10	1.16
2-Chloropentane	5.55	2.51	1.33	4.88	2.46	1.33	2.99	1.40	0.83
3-Chloropentane	5.88	2.51	1.33	5.28	2.51	1.46	3.07	1.50	0.92
1-Chlorohexane	16.46	6.61	3.20	16.73	7.27	3.51	9.18	3.68	1.91
2-Chlorohexane	11.68	4.92	2.44	10.65	4.79	2.44	5.58	2.38	1.31
3-Chlorohexane	11.38	4.95	2.45	10.92	4.84	2.50	5.54	2.49	1.40
1-Chloroheptane	36.32	13.40	5.98	37.51	14.54	6.39	18.20	6.59	3.35
2-Chloroheptane	30.70	9.70	4.40	22.68	9.45	4.41	10.76	4.18	2.16
3-Chloroheptane	24.76	9.02	4.44	22.88	9.39	4.40	10.29	4.21	2.26
4-Chloroheptane	23.95	9.41	4.40	20.29	9.19	4.30	10.18	3.90	2.08
1-Chlorooctane	75.54	26.88	11.13	87.97	29.61	11.83	35.56	12.60	5.09
1-Chloro-2-methylbutane	7.26	2.81	1.49	5.84	2.95	1.51	3.38	1.66	0.92
1-Chloro-3-methylbutane	6.34	2.61	1.40	6.72	2.67	1.46	3.25	1.50	0.92
2-Chloro-2-methylbutane	4.27	1.84	1.06	3.28	1.74	1.11	2.34	1.08	0.70
1-Chloro-2-methylpentane	12.93	5.45	2.70	12.29	5.36	2.75	6.06	2.81	1.54
2-Chloro-2-methylpentane	8.19	3.45	1.85	7.93	4.05	1.69	3.56	1.66	1.00
3-Chloro-2-methylpentane	10.79	4.67	2.41	10.82	4.43	2.23	4.96	2.28	1.22
3-Chloro-3-methylpentane	10.35	4.36	2.11	8.78	4.33	2.17	4.65	2.17	1.22
1-Chloro-3,3-dimethylbutane	9.65	4.12	2.11	8.56	4.00	2.07	4.55	2.07	1.16
2-Chloro-2,3-dimethylbutane	9.61	4.07	2.08	7.39	3.66	1.85	4.07	1.96	1.00

TABLE II

RETENTION INDICES, *I*, OF ALKYL CHLORIDES ON DIFFERENT COLUMNS AT VARIOUS TEMPERATURES

Carrier flow rate: 30 cm^3/min. Amount of stationary phase: 15% (w/w).

Compound	*Apiezon L*			*Tricresyl phosphate*			*Carbowax 20M*		
	75°C	*100°C*	*125°C*	*75°C*	*100°C*	*125°C*	*75°C*	*100°C*	*125°C*
1-Chloropentane	810	800	808	858	877	879	943	938	945
2-Chloropentane	763	762	768	806	818	818	874	869	880
3-Chloropentane	770	762	768	816	821	834	878	880	900
1-Chlorohexane	897	897	908	968	979	981	1041	1034	1041
2-Chlorohexane	854	857	865	908	917	920	967	959	969
3-Chlorohexane	856	858	865	913	918	923	965	967	982
1-Chloroheptane	991	999	1008	1073	1083	1083	1142	1135	1150
2-Chloroheptane	971	953	959	1008	1018	1020	1064	1055	1065
3-Chloroheptane	945	943	960	1009	1017	1019	1057	1057	1074
4-Chloroheptane	941	949	959	993	1014	1016	1052	1044	1058
1-Chlorooctane	1085	1098	1109	1185	1090	1186	1242	1247	1232
1-Chloro-2-methylbutane	796	778	786	829	845	840	891	898	900
1-Chloro-3-methylbutane	779	768	776	848	830	834	886	880	900
2-Chloro-2-methylbutane	731	719	732	755	768	789	837	826	849
1-Chloro-2-methylpentane	867	871	880	927	934	940	979	988	1000
2-Chloro-2-methylpentane	811	807	820	870	892	858	899	898	916
3-Chloro-2-methylpentane	845	850	862	911	905	905	944	952	956
3-Chloro-3-methylpentane	839	840	841	883	902	900	939	944	956
1-Chloro-3,3-dimethylbutane	831	832	841	880	890	892	936	936	945
2-Chloro-2,3-dimethylbutane	830	831	836	860	877	873	919	927	916

TABLE III

COEFFICIENTS OF THE EQUATION $\ln V_g + b_0 + b_1 n$ FOR STRAIGHT-CHAIN CHLOROALKANES ON DIFFERENT STATIONARY PHASES

Homologous series	*Temperature (°C)*	*Apiezon L*		*Tricresyl phosphate*		*Carbowax 20M*	
		b_0	b_1	b_0	b_1	b_0	b_1
1-Chloroalkanes	75	−1.65	0.75	−2.16	0.83	−1.80	0.67
	100	−2.37	0.71	−2.20	0.70	−2.11	0.57
	125	−2.57	0.66	−2.38	0.60	−2.34	0.50
2-Chloroalkanes	75	−2.60	0.85	−2.25	0.77	−2.11	0.64
	100	−2.46	0.68	−2.47	0.67	−1.91	0.45
	125	−2.70	0.60	−2.71	0.60	−2.70	0.50
3-Chloroalkanes	75	−1.83	0.72	−2.00	0.73	−1.91	0.60
	100	−2.26	0.64	−2.38	0.66	−2.18	0.52
	125	−2.73	0.60	−2.38	0.55	−2.34	0.45

The increase in the retention when hydrogen atom is replaced with an halogen is not linearly correlated with the increase in the boiling point, as shown previously for bromoalkanes[11], or with the increase in molecular weight. The EM values of chloroalkanes on the non-polar stationary phase APL are similar to the ration between the weight of chlorine and that of a methylene group (2.54), but this is not true for bromo (Br/CH_2 ratio $\approx$ 5.7) and iodo compounds (I/CH_2 ratio $\approx$ 9.06) on

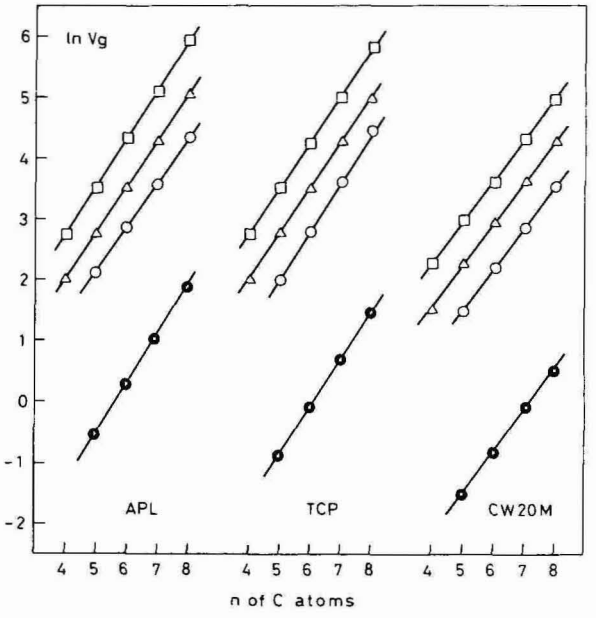

Fig. 1. Effect of the substituent halogen atom in the 1-position on the retention volume as a function of the chain length. ●, Alkanes; ○, 1-chloroalkanes; △, 1-bromoalkanes; □, 1-iodoalkanes. Temperature, 75°C. Carrier gas flow-rate, 30 cm^3/min.

TABLE IV

AVERAGE VALUES OF THE "EQUIVALENT METHYLENES", EM, THAT SHOULD BE ADDED TO STRAIGHT-CHAIN ALKANES TO OBTAIN COMPOUNDS WITH THE SAME RETENTION TIMES AS THE VARIOUS HALOALKANES ON DIFFERENT STATIONARY PHASES

Halogen	*Position in the chain*	*Carbon chain length*	*Stationary phase*		
			APL	*TCP*	*CW 20M*
Cl	1	5–8	2.96	3.78	4.41
	2	5–7	2.61	3.15	3.67
	3	5–7	2.59	3.19	3.73
Br	1	4–8	3.92	4.70	5.59
	2	4–7	3.46	4.14	4.85
	3	4–7	3.45	4.15	4.77
I	1	4–8	4.97	5.75	6.63
	2	4–7	4.49	5.23	5.94
	3	4–7	4.43	5.18	5.88

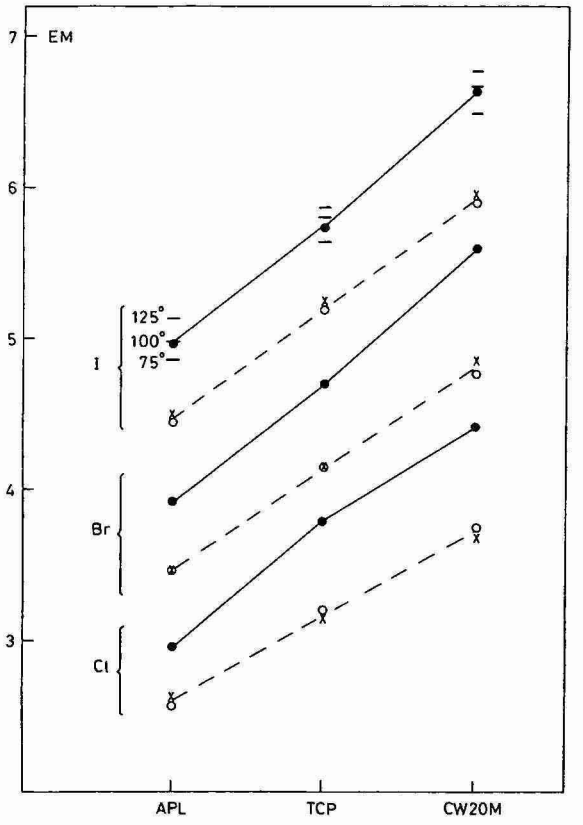

Fig. 2. Average values of equivalent methylenes (EM) for straight-chain chloro-, bromo- and iodoalkanes on different stationary phases. ●, 1-Halo; ×, 2-halo; ○, 3-halo. For 1-iodoalkanes the effect of column temperature is also shown (see text).

TABLE V

VALUES OF ΔH_s^0 OF CHLOROALKANES WITH RESPECT TO THE HOMOMORPHOUS ALKANES ON DIFFERENT STATIONARY PHASES

Compound	ΔH_s^0 *(kcal/mol)*		
	APL	*TCP*	*CW 20M*
1-Chloropentane	3.18	2.56	3.56
2-Chloropentane	2.49	2.39	2.85
3-Chloropentane	2.80	2.28	2.43
1-Chlorohexane	2.60	2.89	3.45
2-Chlorohexane	2.21	2.40	2.79
3-Chlorohexane	2.26	2.40	2.40
1-Chloroheptane	2.54	3.21	3.42
2-Chloroheptane	2.27	2.49	2.98
3-Chloroheptane	2.05	2.54	2.46
4-Chloroheptane	1.94	2.03	1.85
1-Chlorooctane	2.10	3.54	4.15
1-Chloro-2-methylbutane	3.70	3.10	3.28
1-Chloro-3-methylbutane	3.30	2.98	3.03
2-Chloro-2-methylbutane	2.66	2.29	2.80
1-Chloro-2-methylpentane	3.24	3.95	2.89
2-Chloro-2-methylpentane	2.79	3.29	2.32
3-Chloro-2-methylpentane	2.87	3.39	3.08
3-Chloro-3-methylpentane	2.73	2.46	2.76

either non-polar, medium-polar or polar stationary phases. The increase on changing from Cl to Br to I is close to one EM on all of the stationary phases tested.

The values of ΔH_s^0 calculated using eqn. 2 are shown in Table V. These data refer to the difference in intermolecular forces for the halogen-substituted and unsubstituted alkane molecules. The values show appreciable fluctuations owing to the small temperature range available for calculation on the data. Notwithstanding this, as previously seen for bromoalkanes[11], independent of the polarity of the stationary phase for straight-chain chlorohexanes and chloroheptanes, the ΔH_s^0 values increase when the halogen atom is bonded near to the extremity of the chain and show minimum values when it is connected to the central carbon atoms.

The ΔH_s^0 values for branched compounds also increase on all the stationary phases when the halogen atom moves from the inner part of the carbon structures to their extremities. Molecules with the chlorine atom closely surrounded by several methyl groups and therefore with a compact and rounded shape shown the lowest ΔH_s^0 values, as previously observed for bromoalkanes. This effect increases with increasing weight and shape of the substituent halogen atom; as an example, the ratios between the ΔH_s^0 values of 1-halo-2-methylpentanes and those of 3-halo-3-methylpentanes are (for Cl, Br and I, respectively) 1.19, 1.74, 2.07 on APL and 1.04, 1.27, 2.63 on CW 20M columns.

The resolution of the columns used (about 1000 theoretical plates per metre for APL and 1500 for TCP and CW 20M) did not permit the complete separation of all the isomers.

The chromatograms obtained with mixed samples showed that CW 20M elutes all the compounds having a retention time smaller than that of 4-chloroheptane as

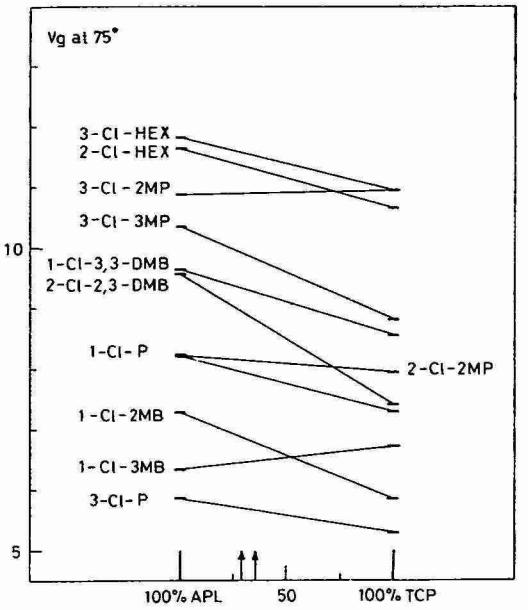

Fig. 3. Separation of closely eluting chloroalkane isomers on similar columns of different polarity and graphical choice of a suitable mixed column. Arrows show the phase composition range for the best separation.

four groups of closely grouped peaks. A longer packed or a capillary Carbowax column is therefore necessary for complete resolution. In contrast, the length of both the APL and TCP columns used permits a satisfactory resolution of most of the compounds. Interference of 1-chloropentane with 2-chloro-2-methylpentane and of 2-chloro-2,3-dimethylbutane with 1-chloro-3,3-dimethylbutane was observed at 75°C on the APL column, whereas on the TCP column coincident or closely eluting peaks were given, at the same temperature, by 1-chloropentane and 2-chloro-2,3-dimethylbutane, by 1-chloro-3,3-dimethylhexane and 3-chloro-3-methylpentane and by 3-chloro-2-methylpentane and 3-chlorohexane. Fig. 3 shows that a mixed column containing about 30% of TCP and 70% of APL, or an equivalent series arrangement of the two columns, should permit the best possible resolution of the grouped peaks. Further enhancement of the separation was obtained by temperature programming, but the observed changes in relative retention were too complex to be predicted on the basis of the available data measured on single columns at constant temperatures.

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SOLUBILITY PARAMETERS OF POLY(α-METHYLSTYRENE–CO-ACRYLONITRILE) FROM GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Solubilities of fourteen polar and non-polar solvents in poly(α-methylstyrene–co-acrylonitrile) have been measured by the gas chromatographic method over the temperature range 165–195°C. The Flory–Huggins χ parameters, partial molar heats of mixing ($\Delta\bar{H}_1^\infty$) and heats of solution (ΔH_s) were calculated and the infinite-dilution solubility parameter (δ_2^∞) for the polymer was estimated following the method of Di Paola-Baranyi and Guillet. At 180°C (midpoint of the temperature range investigated), δ_2^∞ was found to have a value of 8.0 $(cal/ml)^{1/2}$. By linear extrapolation, the χ parameter at 25°C was also estimated, which yielded a value of 9.9 for δ_2^∞ at 25°C.

INTRODUCTION

Since the pioneering work of Smidsrød and Guillet[1], inverse gas chromatography has become a method of much use in polymer studies. It has been used to evaluate various properties of polymers, such as the melting and glass transition points, crystallinity, diffusion coefficient, and surface as well as thermodynamic properties. It has been generally established that thermodynamic data obtained from this method can be as reliable and accurate as those determined by the static methods. The practical requirements and proper procedure required to derive reliable thermodynamic data from such a method have been given by Young[2]. A great advantage of the method is its speed, which makes it preferable to the time-consuming conventional methods.

In this paper we report the determination of solubility parameters of poly(α-methylstyrene–co-acrylonitrile), MSAN, by inverse gas chromatography. Studies on this polymer have been scarce and there is little information relating to its thermodynamic properties. This work is part of our continuing project to study the miscibility of MSAN with solvents of small molecules and with other polymers[3].

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EXPERIMENTAL

The MSAN polymer used was Luran KR 2556U, manufactured by BASF. It contains 30% (w/w) acrylonitrile and has a weight-average molecular weight $\bar{M}_w$ of 160 000. All solutes (the compounds to be injected into the column) were obtained from standard suppliers and were used without further purification.

The columns were prepared in the usual manner. The polymer was dissolved in 2-butanone and then deposited on Chromosorb G AW DMCS (60–80 mesh) by evaporation of the solvent. After vacuum drying for about 72 h with slight heating, the coated support was resieved and packed into 1/4-in. (O.D.) copper tubing. The exact loading of polymer on the support was determined by calcination using a suitable blank correction. To assure reproducibility, two columns were prepared with loadings of 8.01 and 8.78% (w/w), the weights of polymer in the columns being 1.493 and 1.277 g, respectively.

Chromatographic measurements were carried out on a Hewlett-Packard 5790 gas chromatograph equipped with a dual flame ionization detector. The apparatus, with a slight modification to facilitate measurement of column inlet pressure, can be operated with conditions meeting the requirements of Young. The carrier gas used in all measurements was nitrogen. Flow-rates chosen at *ca.* 20 ml/min were measured at the end of the column with a soap-bubble flow-meter. The column inlet pressure was measured with a mercury manometer, while the outlet pressure was always atmospheric. The oven temperature was measured to 0.01°C. Solute samples, mixed with a small amount of methane marker, were injected manually with a 10-μl Hamilton syringe. To keep the sample size as small as possible, the solutes were usually injected in vapoour form. In general, the solutes were characterized by symmetrical elution peaks, exhibiting sample size-independent retention volumes.

Data reduction followed standard procedures[4,5]. The parameters of the solutes required were obtained as follows. Vapour pressures were calculated from the Antoine equation using the constants taken from standard sources[6,7]. Second virial coefficients, B_{11}, were computed using the method of O'Connell and co-workers[8,9], except those for the hydrocarbons, which were calculated from the corresponding state equations of McGlashan and Potter[10]. Molar volumes, except those of *n*-dodecane and *n*-tetradecane[11], were calculated from the modified Rackett equations[9,12]. The specific volumes of MSAN were estimated from the Simha–Boyer relation[13].

RESULTS AND DISCUSSION

Fourteen solutes of differing molecular structures and polarities have been used in this study. Retention volumes were measured at 13 temperatures in the range 165–195°C. The temperatures are more than 50° above the T_g (115°C) of MSAN, a recommended condition for the thermodynamic study of polymers by the chromatographic method[14].

Specific retention volumes were corrected to 0°C. The weight fraction activity coefficients and the χ parameters, derived from the specific retention volume results, are listed in Table I at selected temperatures. Both these quantities show, in all cases except dichloromethane, a decrease in their values with increasing temperature. This

TABLE I

THERMODYNAMIC INTERACTION PARAMETERS FOR VARIOUS SOLUTES IN MSAN

Solute	*165°C*		*180°C*		*195°C*	
	$(a_1/w_1)^\infty$	χ	$(a_1/w_1)^\infty$	χ	$(a_1/w_1)^\infty$	χ
n-Dodecane	79.20	2.910	54.56	2.526	45.64	2.335
n-Tetradecane	82.39	2.978	60.73	2.664	50.81	2.476
Benzene	7.37	0.650	6.61	0.537	6.16	0.467
Toluene	8.09	0.745	7.27	0.624	7.16	0.602
n-Butylbenzene	11.08	1.066	9.70	0.923	9.13	0.851
Dichloromethane	4.54	0.577	4.52	0.577	4.64	0.608
Chloroform	4.41	0.671	4.01	0.576	3.90	0.551
Chlorobenzene	4.83	0.506	4.49	0.421	4.46	0.401
n-Butanol	10.71	0.932	9.13	0.768	8.29	0.673
Cyclohexanol	8.57	0.909	6.98	0.689	6.38	0.584
n-Butyl acetate	11.56	1.109	9.87	0.942	9.27	0.877
Dioxane	5.94	0.607	5.50	0.519	5.52	0.517
Tetrahydrofuran	8.04	0.752	6.65	0.562	6.59	0.556
2-Butanone	8.64	0.712	7.88	0.620	7.57	0.583

suggests that the solubilities of the solutes in MSAN increase with temperature. The largest activity coefficients and χ parameters are observed for the two *n*-alkanes, which are typical non-solvents for the polymer.

The partial molar heats of mixing ($\Delta\bar{H}_1^\infty$) and heats of solution (ΔH_s) for the solutes in MSAN are shown in Table II. Partial molar heats of mixing are given by

$$\Delta\bar{H}_1^\infty = R\partial\ln(a_1/w_1)^\infty/\partial(1/T) \tag{1}$$

TABLE II

PARTIAL MOLAR HEATS OF MIXING AND HEATS OF SOLUTION OF VARIOUS SOLUTES IN MSAN

Temperature range is 165–195°C.

Solute	$\Delta\bar{H}_1^\infty$ *(kcal/mol)*	$-\Delta H_s$ *(kcal/mol)*
n-Dodecane	7.49 ± 0.37	3.98 ± 0.35
n-Tetradecane	6.23 ± 0.26	7.16 ± 0.24
Benzene	2.02 ± 0.14	4.41 ± 0.14
Toluene	1.63 ± 0.20	5.63 ± 0.18
n-Butylbenzene	2.53 ± 0.15	7.31 ± 0.14
Dichloromethane	−0.60 ± 0.20	5.95 ± 0.20
Chloroform	1.77 ± 0.16	4.08 ± 0.15
Chlorobenzene	1.03 ± 0.12	6.96 ± 0.11
n-Butanol	3.84 ± 0.15	5.76 ± 0.13
Cyclohexanol	3.77 ± 0.24	6.16 ± 0.21
n-Butyl acetate	2.71 ± 0.22	5.24 ± 0.20
Dioxane	1.07 ± 0.20	6.18 ± 0.19
Tetrahydrofuran	2.79 ± 0.32	3.15 ± 0.31
2-Butanone	1.66 ± 0.12	4.80 ± 0.11

Heats of solution can be obtained in a similar manner from the temperature dependence of the specific retention volume

$$\Delta H_s = -R\partial(\ln V_g)/\partial(1/T) \tag{2}$$

The uncertainty limits in the table refer to the standard errors in the slope, as given by a linear least-squares analysis of the data. The values of $\Delta\bar{H}_1^\infty$ vary, in general, with the solutes in a manner consistent with that observed from the interaction parameters.

The polymer solubility parameter δ_2 was estimated in the manner described by Di Paola-Baranyi and Guillet[15], from the following equation

$$\chi = (V_1/RT)(\delta_1 - \delta_2)^2 \tag{3}$$

Eqn. 3 can be rewritten

$$\frac{\delta_1^2}{RT} - \frac{\chi}{V_1} = \left(\frac{2\delta_2}{RT}\right)\delta_1 - \frac{\delta_2^2}{RT} \tag{4}$$

and δ_2 can be obtained from plotting $(\delta_1^2/RT - \chi/V_1)$ against δ_1. The required solubility parameters of the solutes (δ_1) can be evaluated from the definition

$$\delta_1 = [(\Delta H_v - RT)/V_1]^{1/2} \tag{5}$$

while ΔH_v, the heat of vaporization of solute, can be obtained directly from chromatographic data from the relation[15]

$$\Delta H_v = \Delta\bar{H}_1^\infty - \Delta H_s \tag{6}$$

Values of δ_1 and ΔH_v so obtained at 180°C (midpoint of the temperature range investigated), as well as V_1, the molar volumes of the solutes, are summarized in Table III. It has been found that the present ΔH_v values agree very well with the literature values available[6]. A plot of $(\delta_1^2/RT - \chi/V_1)$ against δ_1 at 180°C is shown in Fig. 1a. A straight line is obtained, as would be expected from eqn. 4, with a slope of $(2\delta_2/RT)$ and an intercept of $(-\delta_2^2/RT)$. A linear least-squares analysis gives 8.0 ± 0.2 and 8.2 ± 0.2 $(cal/ml)^{1/2}$ from the slope and the intercept, respectively.

The variation of χ with temperature has been found to fit the following equation

$$\chi = \alpha + \beta/T \tag{7}$$

The constants α and β, determined from a linear least-squares analysis, are shown in Table IV. The linear correlation in most cases, except dichloromethane, is good. if such a relationship is assumed to extend to lower temperatures, values of χ at 25°C can be obtained by extrapolation. A value of δ_2 at 25°C can then be estimated as above (see Fig. 1b). The values of δ_2 so obtained are 9.9 ± 0.4 and 10 ± 0.4 $(cal/ml)^{1/2}$ from the slope and the intercept, respectively. There are no literature values of δ_2 for MSAN for comparison. However, a value of 10.4 at 25°C has been estimated by

TABLE III

V_1, δ_1 AND ΔH_v VALUES FOR THE SOLUTES AT 180°C

Solute	V_1 *(ml/mol)*	ΔH_v *kcal/mol*	δ_1 *(cal/ml)*$^{1/2}$
n-Dodecane	272.2	11.47 ± 0.72	6.23
n-Tetradecane	307.4	13.39 ± 0.50	6.37
Benzene	110.3	6.43 ± 0.28	7.08
Toluene	131.3	7.26 ± 0.38	6.96
n-Butylbenzene	189.4	9.84 ± 0.29	6.87
Dichloromethane	78.8	5.35 ± 0.40	7.51
Chloroform	98.4	5.85 ± 0.31	7.10
Chlorobenzene	121.3	7.99 ± 0.23	7.64
n-Butanol	114.8	9.24 ± 0.28	8.52
Cyclohexanol	128.4	9.93 ± 0.45	8.36
n-Butyl acetate	163.6	7.95 ± 0.42	6.57
Dioxane	105.6	7.25 ± 0.39	7.75
Tetrahydrofuran	100.0	5.94 ± 0.63	7.10
2-Butanone	111.8	6.46 ± 0.23	7.05

means of the Small method[16], which should be able to provide some indication as to the validity of the present determination.

As has been noted in many earlier studies[17,18], the δ_2 values obtained from the intercept are slightly higher than those derived from the slope. Moreover, the χ parameters recalculated from eqn. 3, using the values of δ_2 obtained from the slope, are always lower than those observed. Table V shows the differences (γ) between such calculated and observed χ, and it is noted that the two *n*-alkanes appear to be rather odd, with γ values much larger than those for the others, which are more or less constant around an average of 0.54.

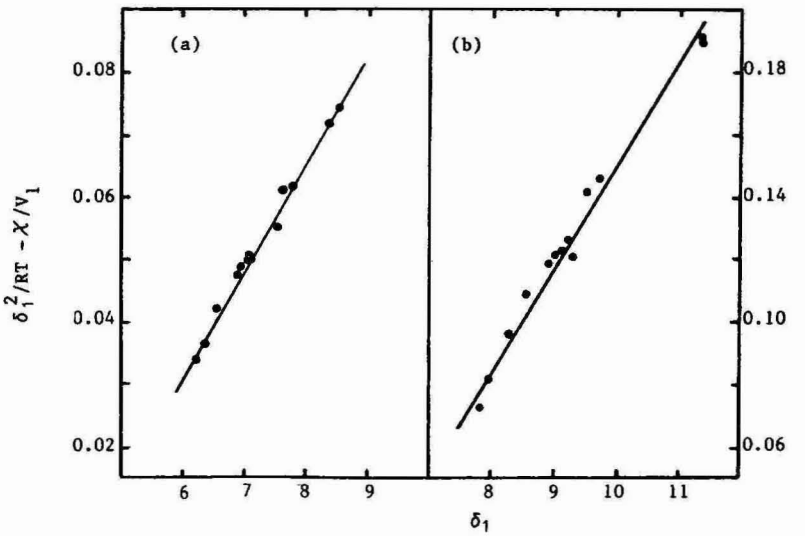

Fig. 1. Plot of $(\delta_1^2/RT - \chi/V_1)$ against δ_1 at (a) 180°C and (b) 25°C.

TABLE IV

TEMPERATURE DEPENDENCE OF THE χ PARAMETER OF VARIOUS SOLUTES IN MSAN

Temperature range is 165–195°C. The data were fitted to an equation of the form $\chi = \alpha + \frac{\beta}{T}$.

Solute	α	β	*Correlation coefficient*
n-Dodecane	−6.076	3915.5	0.9859
n-Tetradecane	−4.509	3260.0	0.9900
Benzene	−1.739	1038.1	0.9694
Toluene	−1.483	964.2	0.9296
n-Butylbenzene	−2.199	1418.5	0.9820
Dichloromethane	1.340	−346.2	0.6806
Chloroform	−1.194	807.8	0.9240
Chlorobenzene	−1.087	690.5	0.9549
n-Butanol	−3.163	1784.1	0.9859
Cyclohexanol	−3.921	2098.1	0.9796
n-Butyl acetate	−2.233	1449.3	0.9607
Dioxane	−0.923	656.8	0.8553
Tetrahydrofuran	−2.539	1426.8	0.9205
2-Butanone	−1.159	811.4	0.9631

Eqn. 3 is the original solubility parameter equation based on the Hildebrand–Scatchard and the Flory–Huggins theory. Huggins[19] and also Scott and Magat[20,21] deduced later that χ should be more properly expressed by

$$\chi = (V_1/RT)(\delta_1 - \delta_2)^2 + \gamma \tag{8}$$

TABLE V

COMPARISON OF OBSERVED AND CALCULATED χ PARAMETERS FOR VARIOUS SOLUTES IN MSAN

Solute	χ_{obs}	χ_{cal}	γ $(\chi_{obs} - \chi_{cal})$	γ/V_1
n-Dodecane	2.526	0.95	1.58	0.0058
n-Tetradecane	2.664	0.91	1.75	0.0057
Benzene	0.537	0.10	0.44	0.0040
Toluene	0.624	0.16	0.46	0.0035
n-Butylbenzene	0.923	0.27	0.65	0.0034
Dichloromethane	0.577	0.02	0.56	0.0071
Chloroform	0.576	0.09	0.49	0.0050
Chlorobenzene	0.421	0.02	0.40	0.0033
n-Butanol	0.768	0.03	0.74	0.0064
Cyclohexanol	0.689	0.02	0.67	0.0052
n-Butyl acetate	0.942	0.37	0.57	0.0035
Dioxane	0.519	0.01	0.51	0.0048
Tetrahydrofuran	0.562	0.09	0.47	0.0047
2-Butanone	0.620	0.11	0.51	0.0046

* Calculated from $\chi = (V_1/RT)(\delta_1 - \delta_2)^2$, with $\delta_2 = 8.0$.

where γ is essentially an empirical parameter and could be considered as an entropy correction term. If this equation is used in place of eqn. 3, it will give, on rearrangement

$$\frac{\delta_1^2}{RT} - \frac{\chi}{V_1} = \left(\frac{2\delta_2}{RT}\right)\delta_1 - \left(\frac{\delta_2^2}{RT} + \frac{\gamma}{V_1}\right) \tag{9}$$

This shows that a plot of $(\delta_1^2/RT - \chi/V_1)$ against δ_1 will now give $(2\delta_2/RT)$ as the slope, but $-(\delta_2^2/RT + \gamma/V_1)$ instead of $-(\delta_2^2/RT)$ as the intercept. It follows then that the δ_2 values obtained from the slope of a plot of eqn. 4 should be the more accurate ones.

Table V also gives the γ/V_1 values for all the MSAN-solute systems. They are all about constant now, including those for the two *n*-alkane systems, with an average of 0.0048. Using this average γ/V_1 value and the value of δ_2 obtained from the slope of the plot of eqn. 4, the values of χ can be recalculated from

$$\chi = \frac{V_1}{RT}(\delta_1 - \delta_2)^2 + \left(\frac{\gamma}{V_1}\right)V_1 \tag{10}$$

which are in good agreement with the observed χ values.

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GAS CHROMATOGRAPHIC RETENTION INDICES FOR ALL C_1- AND C_2-ALKYLATED BENZOTHIOPHENES AND THEIR DIOXIDES ON THREE DIFFERENT STATIONARY PHASES

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SUMMARY

The retention indices for all C_1- and C_2-alkylated benzothiophenes and their dioxides were determined on three stationary phases of different polarity: methylphenylsiloxane, polyethylene glycol and cyanopropylsiloxane. In many instances isomeric dioxides were better separated than the corresponding unoxidized benzothiophenes. The influence of the substitution pattern on the retention indices is discussed.

INTRODUCTION

Sulphur-containing compounds constitute an important part of petroleum products of various kinds. Interest in such compounds has increased markedly in the last few years following the realization of the deleterious effects that sulphur dioxide, formed by burning of, *e.g.*, organically bound sulphur, can have in the form of acid rain. A multitude of sulphur-containing compounds are known to occur in fossil fuels[1], one of the major classes of which are the polycyclic aromatic sulphur heterocycles (PASH).

The simplest representative of the PASHs is benzo[*b*]thiophene, corresponding to naphthalene among the polycyclic aromatic hydrocarbons (PAHs). Benzo[*c*]thiophene, possessing a quinoid structure and consequently being fairly reactive, is known only from synthesis. While alkylated benzothiophenes are minor components in some types of petroleum and petroleum-derived products, such as coal tar and carbon black, where mainly non-alkylated PASHs with larger ring systems are found[2,3], they abound in others, *e.g.*, in shale oils[2,4] and crude oils[5] where they, together with alkylated three-ring PASHs, make up the bulk of the PASHs.

The interest in determining individual alkylated isomers in complex mixtures has been spurred by the realization that in the same way that aromatic compounds, whose basic ring structures are isomeric, can display widely different degrees of carcinogenicity, so the alkylation of aromatic compounds can have an equally profound influence on their carcinogenicity. As little is known about this property of alkylated PASHs, a PAH example may serve well. The ability of monomethyl derivatives of benzo[*a*]pyrene (BaP) to initiate mouseskin tumours has been studied[6]; 1-, 3- and

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TABLE I

COMPARISON OF THE NUMBER OF ALKYLATED DERIVATIVES OF NAPHTHALENE AND BENZOTHIOPHENE AS A FUNCTION OF THE TOTAL NUMBER OF CARBON ATOMS (x) IN THE SIDE-CHAINS

x	*Naphthalenes*	*Benzothiophenes*
1	2	6
2	12	21
3	30	62

11-methyl-BaP show higher activity than BaP itself, 4- and 12-methyl-BaP are about as effective as BaP and 2-, 5- and 6-methyl-BaP display a reduced ability in this respect; 7-, 8-, 9- and 10-methyl-BaP are inactive. The determination of individual alkyl isomers present in a complex mixture can thus be an important task.

Because of the asymmetry introduced into the molecule by the sulphur atom, there are many more isomers of the alkylated benzothiophenes possible than of the alkylated naphthalenes (the same is, of course, true for aromatic compounds containing other heteroatoms). Thus there are six methylbenzothiophenes but only two methylnaphthalenes. This is further illustrated by the data in Table I, which gives the number of C_1-, C_2- and C_3-alkylated benzothiophenes and naphthalenes theoretically possible (C_x denotes a total of x carbon atoms in all side-chains added together).

A fair amount of work has been carried out on the analysis of the dimethylnaphthalenes in crude oils[7], often for geochemical reasons, but no corresponding work is known for the more complex case of the benzothiophenes. The analytical challenge will be greater because of the larger number of isomers that need to be separated and because in most practical samples the benzothiophenes occur as minor constituents, obscured by the more abundant naphthalenes of the same degree of alkylation.

Traditionally, PASHs are determined by capillary gas chromatography (GC) on non-polar stationary phases such as SE-52 and DB-5[8], although more polar stationary phases have been shown to give enhanced selectivity for three-ring PASH isomers[9]. Superox 20M (a polyethylene glycol phase), alone or mixed with SE-52, was found to be superior to SE-52 because of increased interaction between the PASHs and the Superox phase[9]. For the considerably more polar benzothiophene dioxides, which contain the highly polarized SO_2 group, even more polar stationary phases might yield improved selectivity. Dioxides of PASHs do not seem to have been investigated by GC up to now.

In this work the retention data for all C_1- and C_2-benzothiophenes (with the exception of the 4-ethyl isomer) and their dioxides on three different stationary phases of various polarity were determined as a basis for the analysis of alkylated benzothiophenes in environmental and other samples.

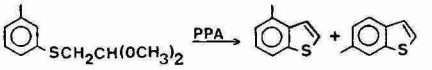
EXPERIMENTAL

A Pye Unicam 4500 gas chromatograph equipped with a flame photometric detector was used throughout. The following columns were employed: 30 m × 0.25

mm I.D. DB-5 (J & W Scientific), film thickness 0.1 μm; 26 m × 0.20 mm I.D. Carbowax 20M (Hewlett-Packard); and 50 m × 0.33 mm I.D. CP Sil 88 (Chrompack), film thickness 0.22 μm. Hydrogen was employed as the carrier gas at a linear flow-rate of *ca.* 80 cm/s (measured at 60°C with a bubble flow meter at the detector). A Shimadzu C-R34 integrator or a Spectra-Physics Model SP 4100 computing integrator was used for retention time determinations. The temperature programme included an initial isothermal period of 2 min at 60°C, then programming at 4°C/min to a point just beyond the last peak. For the dioxides on CP Sil 88, the programme was 160°C for 2 min followed by a programming rate of 4°C/min.

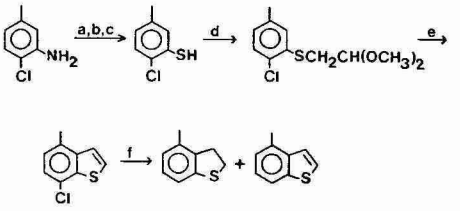
The benzothiophenes were dissolved in hexane and the dioxides in ethyl acetate at a concentration of *ca.* 50 μg/ml. Between five and eight compounds were usually determined at a time. The total volume injected never exceeded 2 μl. Each compound was determined four to eight times; 96% of the compounds showed a standard deviation (σ) below 0.15 retention index units. In general, σ for the dioxides was higher than that for the unoxidized compounds.

The benzothiophenes were synthesized according to standard procedures through ring closure of an appropriately substituted α-(phenylthio)ketone or α-(phenylthio)acetal[10] with polyphosphoric acid (PPA). 2-Alkylated compounds were prepared through reaction of the anion of a suitable benzothiophene (generated with butyllithium) and an alkylating agent (dimethyl sulphate, ethyl *p*-methylbenzene sulphonate, *n*-propyl bromide). The crude products were purified by distillation and/or column chromatography on alumina with hexane as eluent.



(1)

For *meta*-substituted (phenylthio)ketones, two isomeric benzothiophenes can be formed, a 4-alkyl and a 6-alkyl derivative (eqn. 1). In order to correlate the GC peaks of the products with the structures, an unambiguous synthesis of 4-methylbenzothiophene was devised, outlined in eqn. 2. In the last step, lithium aluminium



(2)

(a) $NaNO_2$, HCl

(b) $KSC(=S)OC_2H_5$

(c) NaOH

(d) $ClCH_2CH(OCH_3)_2$, KOH

(e) Polyphosphoric acid, toluene

(f) Na, tert-BuOH/THF

hydride failed to give any products, so sodium in *tert.*-butanol[11] had to be used. The main product surprisingly turned out to be 4-methyl-2,3-dihydrobenzothiophene

[identified by mass spectrometry ($M^+ = 150$) and NMR (multiplet at δ 3.15–3.30 ppm in $CDCl_3$)]. The products were separated by column chromatography on alumina. The minor, later eluting peak in the gas chromatogram of the products in eqn. 1 showed the same retention as 4-methylbenzothiophene from the reactions in eqn. 2. In other instances where the analogous isomerism may occur, the smaller peak was assigned to the 4-alkyl derivative, in agreement with this result and with steric considerations, which should favour the less crowded 6-substitution in the ring closure. This situation pertains to the following five pairs of compounds: 4-methyl- and 6-methyl-, 4-ethyl and 6-ethyl-, 2,4-dimethyl- and 2,6-dimethyl-, 3,4- and 3,6-dimethyl-, and 4,5- and 5,6-dimethylbenzothiophene.

No entries are given for 4-ethylbenzothiophene, because in all chromatograms of the product from the attempted synthesis of this compound (as a mixture with the 6-ethyl derivative), only one peak was found; also, in the oxidized fraction only one peak appeared. As the steric hindrance in the ring-closure reaction should favour 6-ethylbenzothiophene and as the GC data for the peak fit the expected data for the latter compound much better than for the former, probably no (or very little) of the 4-ethyl isomer is formed in the reaction.

The dioxides were synthesized by reaction at room temperature with *m*-chloroperbenzoic acid in chloroform for 30 min, followed by washing with solutions of sodium thiosulphate and sodium carbonate. They were purified by recrystallization from dilute ethanol and/or column chromatography on silica gel with benzene to elute non-polar substances followed by benzene–methanol (1:1) to elute the dioxides.

RESULTS AND DISCUSSION

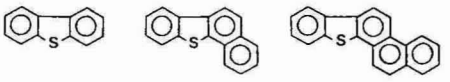
In the GC analysis of real samples, there are in principle two different ways to circumvent the obscuring effect of the much more abundant PAHs on the PASHs: either to use a procedure that contains a step to separate the two types of compounds, or to employ a sulphur-selective detection, commonly flame photometric detection (FPD). Several methods have been proposed for the separation of PASHs from other aromatics[12–14], but none could be made to work satisfactorily when tested for this work. Therefore, FPD remained the choice for detecting sulphur-containing samples and was used exclusively here.

Retention data are preferentially expressed in the form of retention indices (RI). The RI system is based on comparison of the retention times of the compounds of interest with those of calibration standards which, by definition of the original Kováts retention index scale, are the homologous *n*-alkanes. The choice of FPD means that a new series of calibration standards containing a sulphur atom had to be found. The problem is similar to that caused with electron-capture detection, which is widely used for the detection of compounds containing electronegative substituents, typically halogen atoms. In that case standards containing halogen atoms had to be used; the homologous series of the *n*-alkyl trichloroacetates was adopted and has been widely accepted[15].

In the analysis of PAHs, problems were encountered when RI were calculated on the basis of *n*-alkanes[16,17], which led Lee and co-workers to substitute a series of aromatic compounds (benzene, naphthalene, phenanthrene, chrysene and picene) for the *n*-alkanes. This reduced the dependence of RI on factors such as film thickness,

temperature fluctuation and wear of the column, leading to much more reproducible values. The wear of the column with time can have a profound influence on the RI if aromatic compounds are analysed against *n*-alkane standards. A case involving PCB analyses illustrates this[18]; the problem was solved by using PCBs as (aromatic) standards rather than *n*-alkanes, as the retention volumes of PCBs relative to each other remained constant.

In agreement with this approach, the following PASHs were selected as RI standards for use with the flame photometric detector: thiophene, benzothiophene, dibenzothiophene (I), benzonaphtho[2,1-*d*]thiophene (II) and benzophenanthro-[2,1-*d*]thiophene (III). These compounds were (arbitrarily) assigned the exact RI values of 100, 200, 300, 400 and 500, respectively. However, thiophene and III were not needed in this work. The width of the intervals between consecutive standards will be considerably larger with a limited number of aromatic standards rather than with the more numerous *n*-alkanes (*ca.* 5.5 times larger on DB-5), but experimentally this does not seem to lead to poorer precision or accuracy[17]. Under the present conditions, one unit corresponds to between 9 and 10 s, depending on the column and interval.



(I) (II) (III)

The RI values were calculated according to the equation commonly used for temperature-programmed analyses[16,19]:

$$RI = 100 \cdot \frac{t_{R_x} - t_{R_z}}{t_{R_{z+1}} - t_{R_z}} + 100z$$

where t_R is retention time, x is the compound of interest and z and $z + 1$ are the number of aromatic rings in the standard thiophenes eluting immediately prior to and after the compound, respectively. All compounds eluted during the programming.

Lee and co-workers[16,17] published the RI values of a large number of polycyclic aromatic compounds including some PASHs and nitrogen-containing aromatic compounds. In order to increase the usefulness of the body of data and to facilitate comparisons, the experimental conditions in this work were kept as close as possible to those in their revised work[17]. This includes a 2 min initial isothermal period followed by programming at 4°C/min. Instrumental limitations determined the present starting temperature of 60°C rather than 40°C as in the published work. Further, the choice of our starting temperature was also dictated by the recommended minimum working temperatures of 60 and 55°C, respectively, for two of our stationary phases, Carbowax and CP Sil 88. The higher starting temperature will have some influence on the RI of the faster eluting of the two-ring compounds but hardly any on those of the three-ring and higher PASH.

In Table II the RI values determined on the three stationary phases for all C_1- and C_2- and 2-propylbenzothiophene (except for 4-ethylbenzothiophene, see Exper-

TABLE II

RETENTION INDICES OF ALKYLATED BENZOTHIOPHENES AND BENZOTHIOPHENE DIOXIDES ON DB-5, CARBOWAX 20M AND CP SIL 88

Compound	*Benzothiophenes*			*Dioxides*		
	DB-5	*Carbowax 20M*	*CP Sil 88*	*DB-5*	*Carbowax 20M*	*CP Sil 88*
Benzothiophene	200.00	200.00	200.00	276.77	339.06	364.64
2-Methyl	217.97	207.82	208.30	284.44	325.91	346.10
3-Methyl	220.59	213.01	214.30	303.22	355.84	389.16
4-Methyl	220.53	214.15	214.90	302.26*	354.72	384.34
5-Methyl	219.49	212.88	212.85	299.82*	353.24	379.25
6-Methyl	219.29	212.54	212.86	298.65*	350.41	375.38
7-Methyl	216.49	209.04	207.06	285.57	333.79	351.41
2-Ethyl	236.86	219.61	218.78	300.61*	332.76	351.24
3-Ethyl	238.71	224.01	223.91	320.21	361.80	397.50
5-Ethyl	238.23	224.72	223.75	317.20	362.49	387.79
6-Ethyl	238.25	224.32	223.75	315.04	357.26	381.78
7-Ethyl	233.50	219.48	216.05	298.68*	339.01	352.49
2,3-Dimethyl	242.19	226.24	225.49	312.80	347.81	373.94
2,4-Dimethyl	239.40	223.97	223.08	308.27	343.18	365.91
2,5-Dimethyl	238.73	222.20	220.16	306.79	341.94	360.58
2,6-Dimethyl	238.11	221.71	220.11	304.68	338.10	357.02
2,7-Dimethyl	235.74	218.79	215.17	293.37	321.48	334.29
3,4-Dimethyl	249.64	238.56	238.84	336.62	382.55	–**
3,5-Dimethyl	241.58	227.66	227.13	326.63	371.27	404.79***
3,6-Dimethyl	241.61	227.36	226.16	324.70	366.97	398.68
3,7-Dimethyl	238.60	223.50	220.64	312.02	350.89	374.53
4,5-Dimethyl	247.01	236.47	235.74	332.75	379.00	412.29***
4,6-Dimethyl	240.91	228.85	226.60	322.69	366.26	394.13
4,7-Dimethyl	238.53	224.25	220.96	311.09	350.69	370.27
5,6-Dimethyl	245.60	234.79	234.10	326.90	372.48	401.15§
5,7-Dimethyl	236.81	223.16	219.06	307.54	348.99	365.42
6,7-Dimethyl	242.88	230.18	227.99	313.33	354.42	373.92
2-Propyl	254.05	230.17	227.74	316.02	340.40	357.39

* Retention index determined with benzothiophene dioxide and 2,5-dimethylbenzothiophene dioxide as references (see text) and recalculated to the normal system.

** This compound does not elute from the column below the upper temperature limit.

*** Retention index based on dibenzothiophene and naphthobenzothiophene (see text).

§ Retention index determined with dibenzothiophene and 4,5-dimethylbenzothiophene dioxide as references (see text) and recalculated to the normal system.

imental) and their dioxides are given. The stationary phases are, in order of increasing polarity, DB-5, Carbowax 20M and CP Sil 88. DB-5 is a methylphenylsilicone phase containing 5% of phenyl groups and is considered to be non-polar. Carbowax 20M is a polyethylene glycol whose ether oxygen atoms make it a polar material. CP Sil 88 is 100% cyanopropylsiloxane and is highly polar. The sums of the five McReynolds constants for the three phases are *ca.* 337, 2300 and 3700, respectively.

Benzothiophenes on DB-5

From the data in Table II and Fig. 1A it is obvious that on DB-5 a mixture

of the six methylbenzothiophenes will give rise to four signals only; 3- and 4-methylbenzothiophene coelute, as do the 5- and 6-methyl isomers. This was also reported[17] for determinations on SE-54 (which is generally considered to be of the same polarity as DB-5) when the starting temperature was 40°C. Table III compares the RI of the eight compounds which were determined both in this work and on SE-54 by Vassilaros *et al.*[17]; the RI based on the thioaromatic scale (this work) have been converted into values based on the PAH scale, using the RI values 201.43 and 295.39 from ref. 17 for benzothiophene and dibenzothiophene, respectively. The following equation was used for the conversion:

$$RI_{PAH} = 0.9396(RI_{PASH} - 200) + 201.43$$

where 0.9396 is the scale factor for the RI units in going from the PASH RI scale to the PAH RI scale [0.9396 = (295.39 − 201.43)/100].

The difference between the two sets of determinations for the monomethyl compounds is *ca.* 2.4 RI units in all instances. This general agreement between the two sets of data, determined on different stationary phases under different conditions, is highly satisfactory.

For the dimethylbenzothiophenes some generalizations can be made. An isomer with a methyl group in the 2- or 7-position has a lower RI than the other isomers. If the two methyl groups are adjacent to each other, a considerably longer retention time is observed than would otherwise be expected. This is seen for all substitution patterns. This *ortho*-effect is known from methylbenzenes[20] and methylnaphthalenes[21] and also from chloroaromatics[22] and is said to be a function of the lower vapour pressure normally exhibited by *ortho*-isomers[20]; on non-polar columns there is a linear relationship between retention and the logarithm of the vapour pressure. This effect, which also operates for *peri*-substituted compounds (*e.g.*, 3,4-dimethylbenzothiophene), is actually seen on all three columns.

The effect increases with increasing polarity of the stationary phase, which means that the elution range of compounds of type *a*,*x*-dimethylbenzothiophene, where *a* is fixed and *x* varies, is largest on CP Sil 88. The difference between the first and the last eluting *x*,6-dimethylbenzothiophene is 14 units on the most polar stationary phase but only 7.5 units on DB-5. This effect, which is displayed by both the non-polar benzothiophenes and the polar dioxides, is summarized in Table IV for the three columns.

Benzothiophenes on Carbowax 20M

Since some previous work[9] yielded indications that Carbowax or similar stationary phases might show increased selectivity toward PASHs, its inclusion in this study on alkyl derivatives of a simple PASH was justified. However, for the monosubstituted benzothiophenes there is no improvement in selectivity compared with DB-5; this time 3-, 5- and 6-methylbenzothiophene coelute (Fig. 1B). As on the other stationary phases, 2- and 7-methylbenzothiophene emerge from the column well separated ahead of the other isomers. Similar considerations apply to the ethyl derivatives; here the 3- and 6-ethyl peaks coalesce.

The *ortho*-effect is even more pronounced than on DB-5, as is the lowering of the RI values for 2- and 7-substituted compounds. 2-Propylbenzothiophene elutes

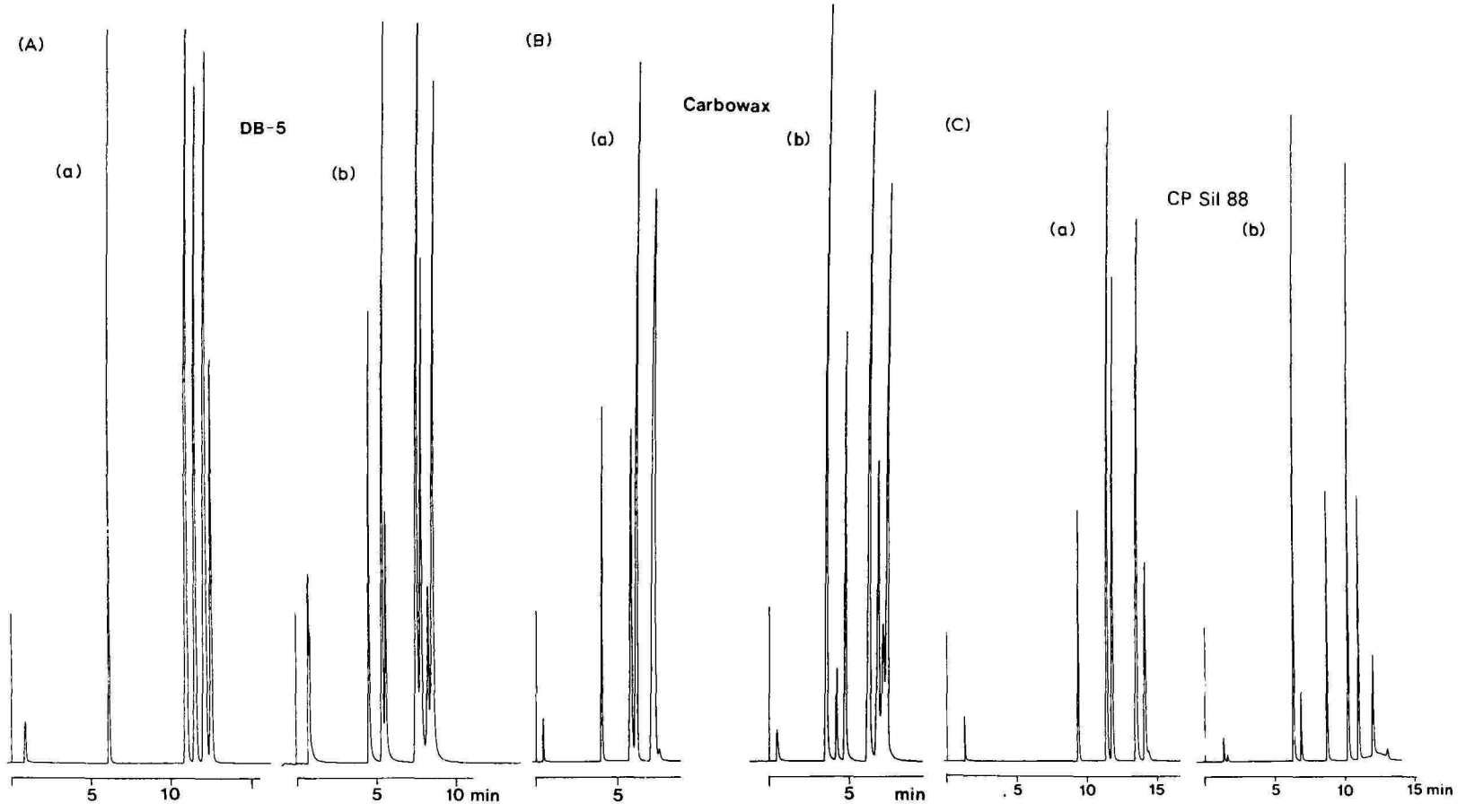


Fig. 1. Gas chromatograms of (a) benzothiophene and the six monomethylbenzothiophenes and (b) the corresponding dioxides. Except for the oven temperature, the conditions are as reported under Experimental. The peaks are identified here by the numbers in parentheses, which refer to the position of the methyl group. 0 = benzothiophene. A + sign indicates coelution. (A) DB-5: (a) 75°C (0, 7, 2, 5 + 6, 3 + 4); (b) 135°C (0, 2, 7, 6, 5, 4, 3). (B) Carbowax 20M: (a) 75°C (0, 2, 7, 3 + 5 + 6, 4); (b) 175°C (0, 2, 7, 6, 5, 4, 3). (C) CP Sil 88: (a) 105°C (0, 7, 2, 5 + 6, 3, 4); (b) 230°C (2, 7, 0, 6, 5, 4, 3).

ahead of four of the C_2 derivatives, as opposed to DB-5, where the elution order strictly follows the number of carbon atoms in the side-chain. Obviously vapour pressure is no longer the sole determining factor for the retention but polar interactions become significant.

Benzothiophenes on CP Sil 88

Also on the very polar stationary phase CP Sil 88 the six monomethyl derivatives appear as four peaks only. The 3- and 4-methyl isomers coelute, as do the 5- and 6-methyl isomers (Fig. 1).

The *ortho*-effect is very prominent, as is the low RI of 2- and 7-substituted compounds. 2-Propylbenzothiophene elutes ahead of four C_2-benzothiophenes. Although the C_2 compounds are spread out over a larger interval on the more polar columns (*cf.*, Table IV) (16.14 units between the first and the last eluting compound on DB-5, 19.81 units on Carbowax 20M and 26.06 units on CP Sil 88), the number of overlapping peaks is only slightly less than on DB-5. Twelve to fifteen of the twenty C_2-isomers coelute with one or more other isomers on the different columns. Just as none of the stationary phases will separate all the monomethylbenzothiophenes, less surprisingly, none will separate all the C_2 compounds either.

Benzothiophene dioxides

The three types of stationary phases investigated here cover the whole range of polarities available. The very similar results obtained make it unlikely that other stationary phases would provide much enhanced selectivities unless they exhibit very special properties. For the analysis of complex samples, the selectivity of the common stationary phases is simply insufficient.

However, at least with the lower PASHs, there might be a simple way to improve this situation, namely to use a rapid and quantitative derivatization reaction. Oxidation to the dioxides with *m*-chloroperbenzoic acid in, *e.g.*, chloroform is complete in a few minutes and side-reactions appear not to play a role. This oxidant is superior to hydrogen peroxide, which requires higher reaction temperatures and fre-

TABLE III

COMPARISON OF RETENTION INDICES* OF EIGHT ALKYLATED BENZOTHIOPHENES DETERMINED ON DB-5 (STARTING TEMPERATURE 60°C, THIS WORK) WITH THOSE DETERMINED ON SE-54 (STARTING TEMPERATURE 40°C, REF. 17)

Compound	*SE-54***	*DB-5*	*Difference*
2-Methyl	220.76	218.31	2.45
3-Methyl	223.08	220.78	2.30
4-Methyl	223.15	220.72	2.43
5-Methyl	222.09	219.74	2.35
6-Methyl	222.11	219.55	2.56
7-Methyl	219.16	216.92	2.24
5-Ethyl	236.14	237.35	−1.21
3,5-Dimethyl	243.56	240.50	3.06

* Based on naphthalene/phenanthrene as standards (see text).

** From ref. 17.

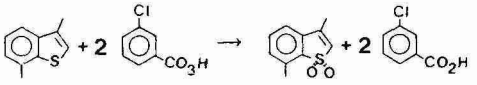
TABLE IV

SIZE OF THE ELUTION RANGE FOR *a,x*-DIMETHYLBENZOTHIOPHENES AND THEIR DIOXIDES (IN RETENTION INDEX UNITS)

Compounds	*a*	*DB-5*	*Carbowax 20M*	*CP Sil 88*
a,x-Dimethylbenzothiophenes	2	6.45	7.45	10.32
	3	11.04	15.06	18.20
	4	11.11	14.59	17.88
	5	10.20	14.27	16.68
	6	7.49	13.08	13.99
	7	7.14	11.39	12.82
a,x-Dimethylbenzothiophene dioxides	2	19.43	26.33	39.65
	3	24.60	34.74	>30.85*
	4	28.35	39.37	>46.38*
	5	25.96	37.06	51.71
	6	22.22	34.38	44.13
	7	19.96	32.94	40.24

* 3,4-Dimethylbenzothiophene dioxide could not be determined on CP Sil 88 (see text).

quently gives rise to side-products. The reaction is exemplified by the oxidation of 3,7-dimethylbenzothiophene (eqn. 3).



(3)

The dioxides have a vapour pressure high enough to allow their analysis by GC. They have been shown to be stable under normal GC conditions[23]. In order to explore their behaviour on the three columns used here, all 28 dioxides of the C_0-, C_1-, C_2- and 2-propylbenzothiophenes were synthesized and analysed in the same way as the parent compounds.

Benzothiophene dioxides on DB-5

Owing to their polar nature, the dioxides have a lower vapour pressure than the parent compounds and consequently elute considerably later. On DB-5 the monomethyl dioxides have retention times comparable to that of dibenzothiophene. The most striking difference from the methylbenzothiophenes, however, is the complete resolution of the six isomers. In Fig. 1 the chromatograms obtained for benzothiophene and its six monomethyl derivatives and also for the corresponding dioxides are shown for all three stationary phases. The excellent improvement on going from the benzothiophenes to the dioxides is obvious, regardless of the stationary phase.

The influence of the substituent position is very striking. While the 3-, 4-, 5- and 6-methyl dioxides elute fairly close together, the 2- and 7-methyl dioxides display a much shorter retention time. While this trend is also true for the unoxidized parents, the effect is considerably more pronounced for the dioxides (and is further enhanced on the more polar stationary phases, see below). This strong dependence on the substitution pattern has the undesirable consequence of moving some C_2-oxides into the region of the C_1-oxides. There are three of the former (2-ethyl, 7-ethyl and 2,7-

dimethyl) eluting before the last eluting C_1-oxide. It is thus clear that isomers with substituents flanking the functional group elute ahead of the others.

This effect is numerically expressed by the difference in RI between a dioxide and its parent compound. This difference is in a sense the RI increment of the dioxide group. As can be seen in Table V, in which the increment is listed for the monosubstituted compounds, this increment can be sensitive to changes in substitution pattern, generally being lower for ethyl than for methyl derivatives and much lower for 2- and 7-substituted dioxides, causing them to elute ahead of the isomeric compounds. Again, the extremes are displayed by the most polar stationary phase where this increment can vary between 178 units (for 3,5-dimethyl-) and 119 units (for 2,7-dimethylbenzothiophene dioxide).

The chain length of the substituents still plays a role, of course. The difference in RI units between the dioxides of 2-propyl-, 2-ethyl- and 2-methylbenzothiophene dioxide is *ca.* 16 units per methylene group (less than the value for the unoxidized parent compounds), but this is overshadowed by the effect of the substituent position. For the dimethyl dioxides, the difference in RI units between the last and the first eluting isomer is no less than 43 units (compared with 11.6 units for the parent compounds), meaning that the exact structure of the dioxides plays a much larger role for the retention than with the parent compounds. That the effect of the chain length on the retention is subordinate to the effect of the substitution pattern is further demonstrated by 2-propylbenzothiophene dioxide, which elutes ahead of eight (of the twenty) C_2-dioxides.

Several dioxides nearly coalesced with dibenzothiophene. Their RI values were determined (under the usual chromatographic conditions) with 2,5-dimethyl- and benzothiophene dioxides as reference compounds and the values thus obtained were recalculated to the normal scale used.

Dioxides on Carbowax 20M

The general trend for the monomethyl derivatives is the same as on DB-5, but

TABLE V

DIFFERENCE IN RETENTION INDEX BETWEEN MONOSUBSTITUTED BENZOTHIOPHENE DIOXIDES AND THE CORRESPONDING BENZOTHIOPHENES (INCREMENTS FOR THE DIOXIDE FUNCTIONAL GROUP)

Substituent	*DB-5*	*Carbowax 20M*	*CP Sil 88*
None	76.77	139.06	164.64
2-Methyl	66.47	118.09	137.80
3-Methyl	82.63	142.83	174.80
4-Methyl	81.70	140.57	169.44
5-Methyl	80.33	140.36	166.40
6-Methyl	79.36	137.87	162.52
7-Methyl	69.08	124.75	144.35
2-Ethyl	63.75	113.15	132.46
3-Ethyl	81.50	137.79	173.59
5-Ethyl	78.97	137.77	164.04
6-Ethyl	76.79	132.94	158.03
7-Ethyl	65.18	119.53	136.44
2-Propyl	61.97	110.23	129.65

the more polar stationary phase will retard the (polar) dioxides more than the (non-polar) reference compounds and thus the RI values will tend to be higher on this stationary phase. On Carbowax 20M benzothiophene dioxide itself elutes later than all those monosubstituted dioxides which carry a methyl or ethyl group in the 2- or 7-position. Possibly the flanking alkyl group exerts some steric hindrance to efficient interactions with the stationary phase. This is evident for 2-propylbenzothiophene dioxide also, which elutes ahead of all but four of the C_2- and all but two of the C_1-dioxides.

Dioxides on CP Sil 88

The results on this very polar stationary phase are similar to those on Carbowax 20M, but more pronounced. Several C_1- and C_2-dioxides, substituted in the 2- and/or 7-positions, including the 2-propyl derivative, elute before benzothiophene dioxide itself, underlining the hindering effect of the flanking alkyl groups on the interactions of the SO_2 group with the stationary phase. Also in this instance, four of the six monomethyl dioxides are more strongly retained than the 2-propyl derivative.

On this column some dioxides emerge after benzo[2,1-*d*]naphthothiophene (II), the standard used to define the RI value 400. As the next standard, benzophenanthro[2,1-*d*]thiophene, did not elute below the upper temperature limit of the stationary phase, the RI values for those compounds have been calculated on the basis of dibenzothiophene/benzonaphthothiophene. 5,6-Dimethylbenzothiophene dioxide nearly coincided with the latter standard and its RI value had to be determined indirectly using dibenzothiophene and 4,5-dimethylbenzothiophene dioxide as secondary reference compounds. 3,4-Dimethylbenzothiophene dioxide did not elute at all at temperatures below 260°C, above the upper temperature limit for the stationary phase; consequently, no RI could be determined for this compound on CP Sil 88.

Some of the effects discussed above are further demonstrated by the data in Table VI, which shows the RI increments for a methylene unit in the side-chain of the monosubstituted benzothiophenes and their dioxides. For the parent compounds, the RI increment is larger for the first methylene unit (going from a methyl to an ethyl derivative) than for the second one (from an ethyl to a propyl derivative); this is true for all the stationary phases. The increment varies slightly with the position of the substituent, mainly in that the 7-substituted compounds generally show a smaller increment than other isomers.

TABLE VI

RETENTION INDEX INCREMENTS FOR A METHYLENE UNIT IN SOME ALKYLATED BENZOTHIOPHENES (BT) AND THEIR DIOXIDES (BTO_2)

Substituents compared	*DB-5*		*Carbowax 20M*		*CP Sil 88*	
	BT	*BTO_2*	*BT*	*BTO_2*	*BT*	*BTO_2*
2-Pr–2-Et	17.19	15.41	10.56	7.64	8.96	6.15
2-Et–2-Me	18.89	16.17	11.79	6.85	10.48	5.14
5-Et–5-Me	18.74	17.38	11.84	9.25	10.90	8.54
7-Et–7-Me	17.01	13.11	10.04	5.22	8.99	1.08

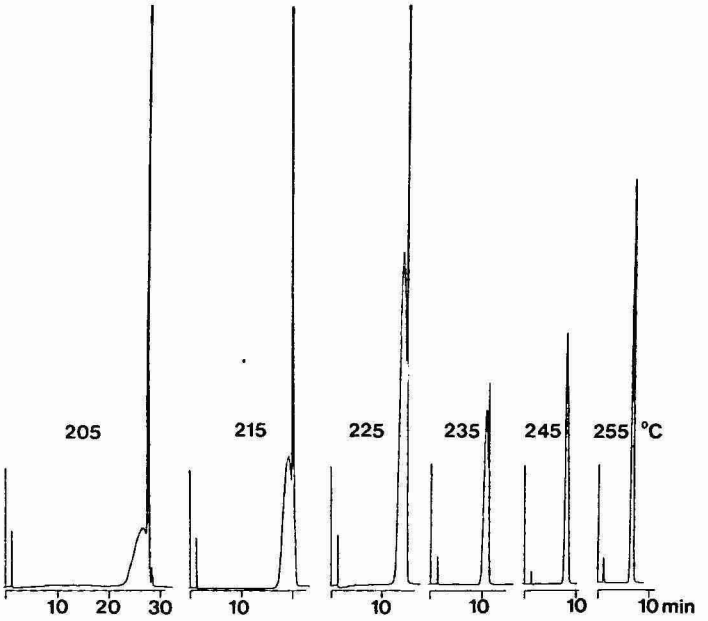


Fig. 2. 3-Methylbenzothiophene dioxide chromatographed at different temperatures on CP Sil 88. Injector and detector temperature, 250°C.

Elongation of the alkyl chain by a methylene unit has a smaller effect on the RI the more polar the stationary phase and the more polar the molecule is, reflecting how polar interactions become more important for the retention. The extreme case is the 7-alkylated dioxides on CP Sil 88, where the RI difference between the ethyl and the methyl derivatives is only 1 unit.

Finally, the curious behavior displayed by 3-methylbenzothiophene dioxide on CP Sil 88 should be mentioned. On this stationary phase only, this dioxide, but no other isomer, appeared as a broad peak followed by the normal narrow peak [compare the peaks in Fig. 1C (b)]. The retention time of the latter peak was used and fits well into the general picture. Probably a decomposition occurs on the column. The temperature dependance is illustrated in Fig. 2. At lower temperatures (205°C) relatively little of the broad peak is visible and the normal sharp peak dominates. However, at 255°C only the "broad" peak appears, which at this temperature is narrow and more "normal" looking.

The initial assumption that it is a temperature effect and for that reason only seen on CP Sil 88, where the elution temperature under normal conditions is considerably higher than for the other phases (*ca.* 152, 183 and 245°C for DB-5, Carbowax 20M and CP Sil 88, respectively) was tested in a separate experiment. The compound was sealed in a melting point capillary and heated at 250°C for 5 min. However, the product of this treatment gave a gas chromatogram which was indistinguishable from that of the starting material. When another column with a cyanopropylsiloxane stationary phase (SP 2340) was used, no sign of decomposition in the temperature range 185–225°C was observed and a chromatogram of all the monomethylbenzothiophene dioxides contained a peak of the correct size for the 3-methyl

derivative. Hence it is probably a specific effect of the CP Sil 88 column, the origin of which is unknown.

Many of the features discussed above can be brought out more clearly graphically, and for that reason some of the data in Table II are plotted in Fig. 3. In Fig. 3a the good correlation between the RI on CP Sil 88 and those on DB-5 is displayed. Different straight lines are obtained depending on the number of carbon atoms in the substituents. Compounds with a substituent in the 2-position (filled circles in Fig. 3a) and, to a lesser extent, those with a 7-substituted pattern generally lie below the other points because they are less retained than other isomers on CP Sil 88.

A similar good correlation is obtained for the dioxides on CP Sil 88 and DB-5 (Fig. 3b), with a correlation coefficient for the C_2 compounds of 0.991. The slope of

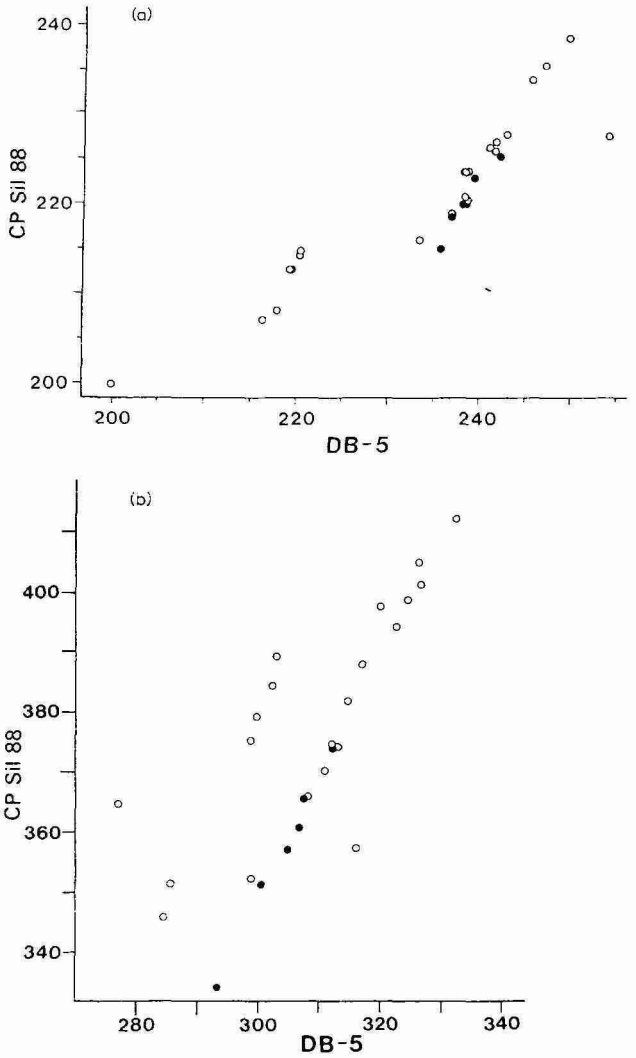


Fig. 3.

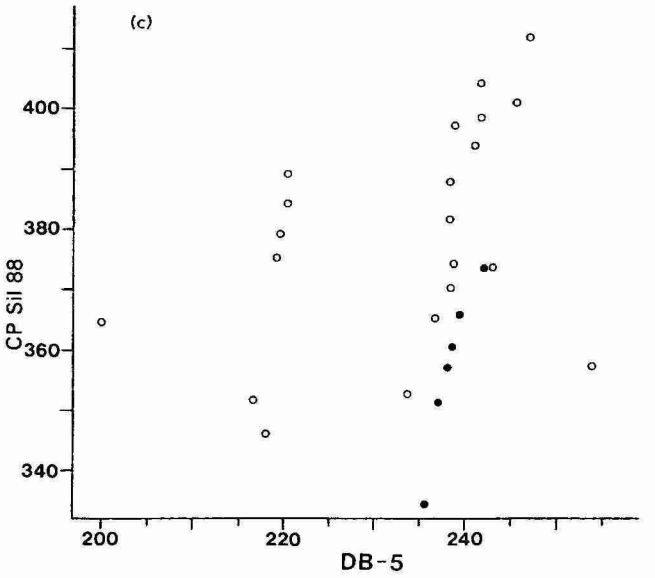


Fig. 3. Plots of retention indices for (a) alkylated benzothiophenes on DB-5 and CP Sil 88, (b) alkylated benzothiophene dioxides on DB-5 and CP Sil 88 and (c) alkylated benzothiophene dioxides on CP Sil 88 *vs.* alkylated benzothiophenes on DB-5. Filled circles represent 2-substituted compounds.

1.99 shows how CP Sil 88 spreads the compounds over a larger elution interval. There are fewer overlapping peaks with the polar stationary phase, but this may simply be due to the fact that the compounds elute over a larger interval (slope > 1) and less to some unique selectivity of this phase which should have manifested itself in a low correlation coefficient. In this plot the 2-substituted C_2 isomers lie on the same straight line as the other isomers. Fig. 3b also makes it visually clear how the dioxides of benzothiophene and the C_1-benzothiophenes on this polar stationary phase fall in the same interval as those of the C_2-benzothiophenes. Similar correlations are obtained if the data from Carbowax 20M are plotted against those from the other stationary phases.

The high degree of correlation makes it unlikely that any combination of these three stationary phases would lead to much additional information compared with the use of only one column. The high correlation also shows that the choice of stationary phase appears not to be very critical, unless specific separation problems must be solved.

The last case, a plot of the RI values of dioxides against those of the parent compounds, is presented in Fig. 3c. The unoxidized benzothiophenes were measured on DB-5 and their dioxides on CP Sil 88. In this instance the points are much less correlated with each other; this is true also if the same stationary phase is used for both classes of compounds, but the effect is not as pronounced as in Fig. 3c.

The procedure of oxidizing a sample and chromatographing both the unoxidized and oxidized samples for the identification of individual alkylated benzothiophenes is currently under investigation.

CONCLUSIONS

Of the three stationary phases studied in this work, the most polar, consisting of 100% cyanopropylsiloxane, proved more capable of resolving a large number of alkylated benzothiophenes than the less polar Carbowax and DB-5. On oxidation with *m*-chloroperbenzoic acid, benzothiophenes yield the corresponding dioxides, which can be chromatographed with ease.

The substitution pattern of both parent and oxidized benzothiophene has a great influence on their retention:

(a) 2- and 7-substituted compounds always elute faster than other isomers; this effect is particularly striking for the dioxides;

(b) *ortho*-substitution contributes to a longer retention time;

(c) on polar stationary phases, the contribution of the dioxide group to the retention often alters the normal elution order of C_1-substituted derivatives eluting before C_2-substituted derivatives and so on;

(d) the substitution pattern influences the retention of the dioxides more than that of the unoxidized benzothiophenes, causing them to elute over a larger interval;

(e) to obtain the largest number of resolved peaks, benzothiophenes should be chromatographed as dioxides.

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ION-PAIR REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY OF ORGANIC ACIDS

RE-INVESTIGATION OF THE EFFECTS OF SOLVENT pH ON R_F VALUES

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SUMMARY

The effect of solvent pH on the ion-pair reversed-phase thin-layer chromatography of four organic acids (salicylic acid, gentisic acid, 2,5- and 2,6-dihydroxybenzoic acid) has been re-investigated. These acids were chromatographed on silica gel, paraffin-impregnated silica gel and on C_{18}-bonded silica gel with a total of four ion-pair reagents.

The R_F values of these compounds were unaffected by solvent pH when tetra-*n*-butylammonium iodide and tetraheptylammonium bromide were used as ion-pair reagents. However, when cetrimide and cetyltrimethylammonium bromide were used a significant dependence of R_F on the solvent pH was observed.

Some possible explanations for the observed differences between these ion-pair reagents are discussed, together with the potential usefulness of this phenomenon.

INTRODUCTION

Ion-pair (IP) chromatography has been extensively employed in the field of high-performance liquid chromatography (HPLC) in order to obtain the retention of highly polar ionic substances. However, despite the advantages of IP techniques for such compounds, there have been relatively few applications of ion pairing in thin-layer chromatography (TLC). Some examples which show the usefulness of IP-TLC are described in refs. 1–4. In a previous paper we reported the results of studies in which the reversed-phase (RP) IP-TLC of a range of organic acids was investigated using a number of IP reagents[4]. This work was performed with the aim of determining the importance of factors such as pH, solvent composition etc., on chromatography. Our results demonstrated that, using tetra-*n*-butyl ammonium iodide (TBA) as a model IP reagent, varying the pH of the solvent between 2 and 10 had no effect on the R_F values of the test acids.

We were therefore intrigued by the report of Szepesi *et al.*[3], who found in their studies that "the effect of the pH of the eluent on the selectivity and efficiency of the

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separation on KC-18 (a C_{18}-bonded plate manufactured by Whatman) was similar to that in HPLC using a chemically bonded phase". These workers found that a pH of 8 was optimal for bonded phases, whilst a pH of 5 gave the best results on silica gel.

A number of differences between the studies of Szepesi *et al.*[3] and our own investigations[4] are apparent. In particular, they used an over-pressurised TLC system, and cetrimide as the IP reagent. However, their results appeared so at variance with our experience that they prompted us to re-investigate the role of solvent pH in IP-RP-TLC using a wider range of IP reagents, including both TBA and cetrimide.

EXPERIMENTAL

Chemicals

The ion-pair reagents used in this study were purchased from either Fisons [cetrimide (bromide salt), cetyltrimethylammonium bromide (CTA), tetra-*n*-butylammonium iodide (TBA)] or Eastman-Kodak [tetraheptylammonium bromide (THA)]. Solvents were of analytical grade.

The test acids were obtained from the Aldrich (Gillingham, U.K.) and were used as received. All the TLC plates used in this study incorporated a fluorescent indicator (254 nm).

Preparation of TLC plates

Silica gel TLC plates (20 × 20 cm, plastic backed, Macherey-Nagel, Camlab, Cambridge, U.K.) were coated with the appropriate ion-pair by dipping them in a 0.07 *M* solution of reagent dissolved in a volatile solvent. In the case of TBA and THA the dipping solution was dichloromethane and ethanol (4:1, v/v). For cetrimide and CTA the dipping solution was dichloromethane.

Paraffin-coated silica gel TLC plates, were prepared as described in ref. 4, by dipping silica gel TLC plates (Macherey-Nagel, 20 × 20 cm, plastic backed) in a 0.07 *M* solution of the IP reagent containing $7\frac{1}{2}$% (v/v) of heavy refined paraffin ("Nujol"). Apart from the inclusion of paraffin, the composition of the dipping solutions were the same as those used for coating the silica gel TLC plates with IP reagent alone.

C_{18}-bonded silica gel HPTLC plates

The C_{18}-bonded plates (manufactured by E. Merck, supplied by BDH, Poole, U.K.) were also coated with IP reagent by dipping. The coating solutions were the same as those employed for coating the silica gel TLC plates.

Chromatography

Ascending chromatography was performed in glass TLC tanks 20 × 20 × 5 cm using solvent systems composed of methanol and 0.01 *M* potassium phosphate buffer at pH 2, 4, 7, 9 and 11.

All three types of IP-impregnated TLC plates (silica gel, paraffin-coated silica gel and C_{18}-bonded silica gel) were developed simultaneously in the same tank to minimise experimental variations.

RESULTS

The effect of solvent pH on the R_F of four polar organic acids (gentisic acid, salicylic acid, 2,5-dihydroxybenzoic and 2,6-dihydroxybenzoic acid) was investigated over the pH range 2–11. TBA, THA, cetrimide and CTA were used as IP reagents in these studies, coated onto a variety of TLC adsorbents. The adsorbents used were silica gel, paraffin-coated silica gel, and C_{18}-bonded silica gel. In the case of the paraffin-coated plates the IP reagent was applied to the plate in the same solution as the paraffin, as described elsewhere[4]. Unless the reagents were coated onto the adsorbents IP formation was either inefficient or did not occur at all[3,4]. The results obtained for each of the IP reagents are described below.

TBA

In the absence of TBA all four acids had R_F values of approximately 0.9, irrespective of adsorbent, eluotropic strength of the solvent or pH. A similar result was obtained when TBA was coated onto silica gel. However, when coated onto either C_{18}-bonded or paraffin-coated plates TBA gave excellent results, with any desired R_F between 0 and 0.9 possible using a solvent of appropriate eluotropic strength. For the same solvent composition the paraffin-coated plates gave a higher R_F than the C_{18}-bonded ones. These results compare well with those obtained in the previous study[4]. When the effect of solvent pH was re-examined using both C_{18}-bonded and paraffin-coated TLC plates the R_F value of the test compounds showed

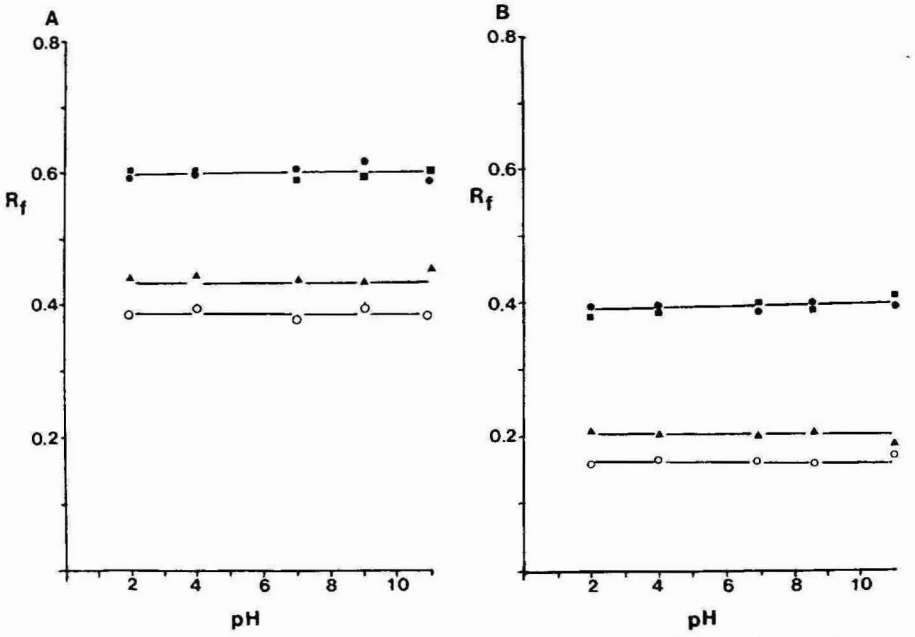

Fig. 1. Plots of R_F *versus* pH of the solvent [methanol–water (1:1)] showing the absence of any variation in the R_F values of the test compounds using TBA as IP reagent on paraffin-coated silica gel (A) and C_{18}-bonded RP-TLC plates (B). Key: (■) gentisic acid; (●) 2,5-dihydroxybenzoic acid; (○) 2,6-dihydroxybenzoic acid, (▲) salicylic acid.

no significant variation over the whole range, as illustrated in Fig. 1. These results confirmed our original observation and reinforced our view that solvent pH was not an important factor in the control of R_F in IP-RP-TLC using TBA. This result did not however, preclude the possibility that TBA might be an atypical IP reagent. For this reason we continued our investigations with THA, cetrimide and CTA.

THA

Chromatography on THA-coated silica gel was more succesful than on TBA-coated plates. Thus, not only did the test compounds not chromatograph at the solvent front, but it was also possible to vary their R_F by changing the solvent composition. However, the chromatographic characteristics of THA-coated silica gel were not particularly good, with irregular spot shape and excessive tailing. Despite these limitations the results obtained when the solvent pH was varied showed that the R_F value did not change appreciably between pH 2 and 11. Similarly the R_F values of the test compounds was unaffected by solvent pH when the same experiments were performed using C_{18}-bonded and paraffin-coated RP-TLC plates. As was observed with TBA, for the same solvent composition paraffin-coated RP-TLC plates gave a higher R_F value than their C_{18}-bonded equivalents. The TBA-coated silica gel gave the highest R_F values of all.

Cetrimide

In marked contrast to TBA and THA cetrimide-coated silica gel gave excellent

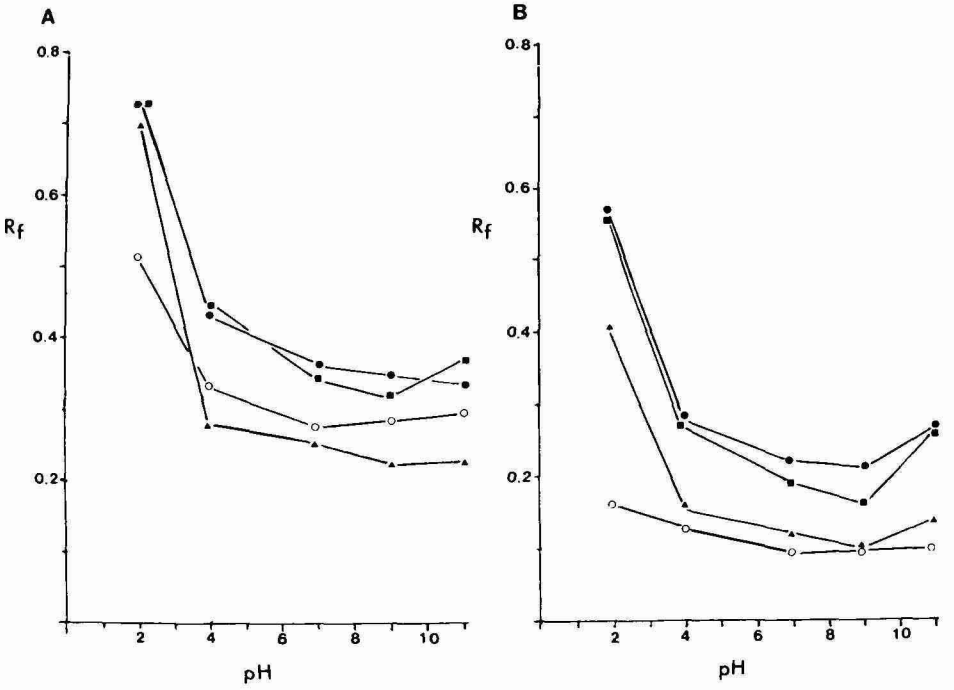

Fig. 2. Plots of R_F values of the test acids *versus* solvent pH on silica gel coated with cetrimide (A) or paraffin-coated silica gel also coated with cetrimide (B). Symbols as for Fig. 1. The solvent was methanol–water (1:1).

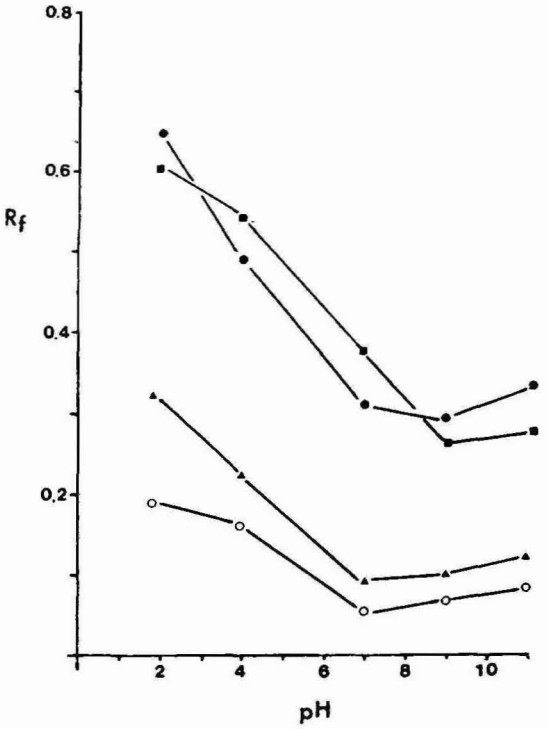

Fig. 3. Plot of R_F *versus* pH on C_{18}-bonded RP-TLC plates with cetrimide as IP reagent. The solvent was methanol–water (6:4). Symbols as for Fig. 1.

results when the chromatography of the test compounds was examined. Similarly paraffin-impregnated and C_{18}-bonded RP-TLC plates coated with cetrimide also gave good results for the four acids tested. For the same solvent composition the highest R_F values were observed on silica gel, with paraffin-coated plates giving higher R_F values than C_{18}-bonded phases. This result is therefore similar to that obtained with THA. However, when the effects of solvent pH on R_F were examined we were surprised to note a marked pH dependence in R_F for a constant solvent composition. So, for example, at pH 2 compounds such as gentisic acid, and 2,5-dihydroxybenzoic acid had R_F values (on silica gel coated with cetrimide) of approximately 0.76. These R_F values decreased dramatically at pH 4 to approximately 0.45, with a further, less marked, decrease when the pH was increased towards pH 11 (Fig. 2A). At pH 11 a slight increase in R_F was observed compared to the value obtained at pH 9. Essentially similar results were seen using paraffin-coated RP-TLC plates (Fig. 2B).

When the same experiments were performed on C_{18}-bonded RP-TLC plates a decrease in R_F with increasing solvent pH was also observed. However, unlike the silica gel and paraffin-coated plates there was no sudden reduction in R_F between pH 2 and 4, but instead a more regular decrease was obtained (Fig. 3).

CTA

In many respects the chromatographic properties of CTA-coated plates were similar to those impregnated with cetrimide. Thus excellent results were obtained

when this reagent was coated onto either silica gel, paraffin-coated silica gel, or C_{18}-bonded RP-TLC plates. Like cetrimide this IP reagent showed a clear dependence of R_F of the test compounds on pH, with the highest values obtained for acidic solvent systems. On silica gel and paraffin-coated silica gel RP-TLC plates the large decrease in R_F between pH 2 and 4, originally observed with cetrimide, was repeated with CTA. On the C_{18}-bonded plates a more gradual decrease in R_F was noted.

DISCUSSION

The mechanism(s) of IP chromatography are still a matter for considerable debate[5]. For IP-HPLC three models have been proposed, the IP model, the dynamic ion-exchange model, and the ion-interaction model. In the IP model reagent and solute combine in the solvent to form a neutral species which is then able to partition into the stationary phase. The ion-exchange model assumes that the solute interacts with IP reagent adsorbed on the stationary phase. In the case of the ion-interaction model a somewhat more complex situation is proposed requiring the formation of an electrical double layer at the surface of the stationary phase. The primary layer is composed of IP reagent whilst an oppositely charged secondary layer of ions forms above this. The sample solute competes for access to the secondary layer, from which it is electrostatically attracted into the primary layer, where it may also be subject to lipophilic attraction to the non-polar stationary phase. Absorbtion of a solute ion into the primary layer results in an extra charge being present and a further molecule of IP reagent then enters to neutralise it. Thus a pair of ions is now present in the primary layer, although not necessarily as as ion pair. This partition, or adsorption, of ions onto the stationary phase is a dynamic process, and chromatographic separation is therefore possible.

Whether the models proposed for IP-HPLC apply equally well to IP-TLC is not yet clear. However, whatever mechanisms are responsible for IP-RP-TLC the IP reagents which we have examined fall into two distinct classes, those which show pH-dependent effects on R_F and those which do not. Clearly the explanation for this must reside in some physicochemical difference between the two classes of IP reagent. If only TBA and cetrimide are considered, the former showing no pH dependency whilst the latter giving a marked dependence of R_F on solvent pH, then the difference cannot simply be molecular weight as they differ by only a single methylene group. In shape however TBA and cetrimide are very different (see Fig. 4). Thus TBA is a symmetrical and (by comparison with cetrimide) compact molecule. Cetrimide in contrast is unsymetrical, and has it's quaternary nitrogen placed at one end of a long, lipophilic, alkyl chain. It should also be noted that as a consequence of these differences in lipophilicity the water solubility of TBA is much greater than that of cetrimide. It does not therefore seem unreasonable to suppose that such different IP reagents will give rise to quite different layers when coated onto TLC plates.

The type of layer formed seems also to depend in part on the adsorbent. Thus whilst silica gel and paraffin-coated silica gel gave rather similar results for cetrimide the C_{18}-bonded plates showed a different type of pH-dependent behaviour. This may be explicable in terms of the acidic silanol groups present on the silica gel. In the case of silica gel and the paraffin-coated silica gel all of the silanol groups are present and available to interact with the basic quanternary ammonium IP reagent. It can be

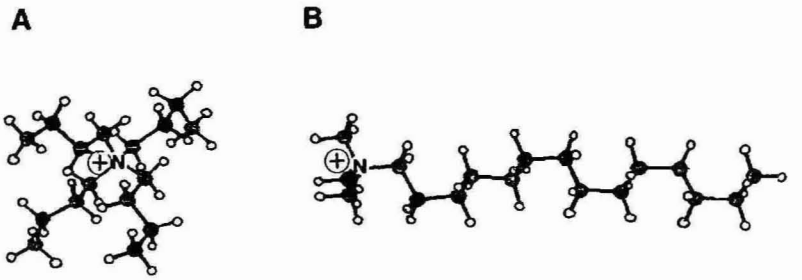

Fig. 4. Structures of TBA (A) and cetrimide (B) produced by a Chemical Graphics program (Chemical Design, Oxford, U.K.) on a Vax 11/750 computer.

postulated that, for an IP reagent like cetrimide on silica gel, this would lead to the production of a lipophilic layer on the surface of the silica gel in which the "tails" of further cetrimide molecules might sit, with their hydrophilic ion-pairing ends protruding into the aqueous mobile phase. This would enable cetrimide to act with an ion-exchange (or perhaps ion-interaction) type of mechanism when acidic compounds, such as the test acids, were chromatographed upon such plates. This type of layer is illustrated in Fig. 5A. As the pH of the mobile phase was lowered the ionisation of the silanols would be suppressed abolishing (or greatly reducing) the extent of the interaction between them and the IP reagent. This might lead to the situation shown in Fig. 5B, where a simple IP mechanism might apply.

In the case of C_{18}-bonded plates many of the silanol groups are removed when the silica gel is reacted with the silylating reagents used to prepare the bonded phase. Thus, the number available for interaction with cetrimide is limited.

As was the case with silica gel and paraffin-coated silica gel when the pH is lowered that portion of the cetrimide interacting with the silanols is reduced. However, whereas with the silica gel and paraffin-coated plates the reduced interaction of the IP reagent with the silanol groups might lead to the breakdown of the postulated layer (Fig. 5B), the C_{18} layer would still provide a lipophilic zone in which the lipophilic portion of cetrimide might sit. This possibly explains in part why the dra-

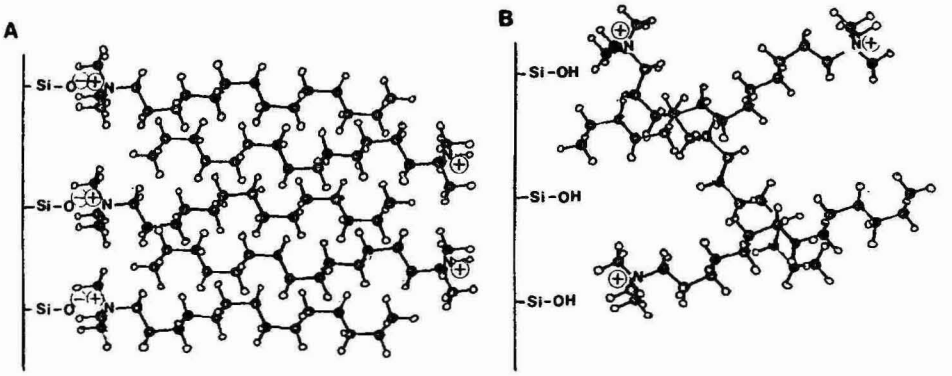

Fig. 5 (A) Hypothetical bilayer resulting from the interaction of cetrimide with ionised silanol groups on silica gel. (B) The breakdown of the bilayer illustrated in A because of the suppression of ionisation of the silanol groups at a solvent pH of 2.

matic increase in R_F noted with cetrimide on silica gel and paraffin-coated plates on going from pH 4 to 2 is not seen on C_{18}-bonded plates. An ion-interaction or ion-exchange mechanism rather than ion-pairing seems likely on these C_{18}-bonded plates.

With IP reagents such as TBA the construction of the hypothetical layer postulated for cetrimide (and similar compounds) looks less likely. For, although a proportion of the available TBA may indeed interact strongly with surface silanols on the silica gel they will not produce a non-polar layer as, unlike cetrimide, the quaternary ammonium ion is in the centre of the molecule and not at one end. Furthermore, TBA (and THA) are hydrophilic, water soluble, compounds. Thus, it is very likely that even were a layer formed it would rapidly breakdown as the reagent dissolved in the advancing solvent. Layers of cetrimide (and CTA) on the other hand, being much less water soluble, would probably be more stable. Thus this difference in water solubility may provide a partial explanation for the lack of ion-pairing effect with TBA coated onto silica gel compared with cetrimide.

It must be emphasised that, in the absence of further experimental evidence, much of the above discussion is highly speculative.

CONCLUSIONS

Whatever their cause the differences observed between the two types of IP reagent in this study probably explains why some workers have encountered pH-dependent effects in IP-RP-TLC whilst we did not. These results are both interesting and useful, for, whilst the underlying reasons for the different types of behaviour requires further study, there are immediate practical benefits to the chromatographer. Thus systems may be chosen where pH is an important factor in the separation or alternatively pH effects may be excluded completely. This, therefore provides another way in which the selectivity of an IP-RP-TLC system might be manipulated. For example, it should be possible using a pH "sensitive" IP reagent to change the R_F values of acidic compounds whilst leaving non-acids unaffected, thus enabling a separation to be optimised.

Further work is clearly required in this area in order to provide explanations for the behaviour of the different IP reagents, and to explain differences between the bonded and paraffin-coated TLC plates. Studies designed to provide further insights into these problems are in progress.

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CHROMATOGRAPHIC BEHAVIOR OF DIASTEREOISOMERS

VIII. SOLVENT SELECTIVITY EFFECTS IN SEPARATIONS ON SILICA

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SUMMARY

Solvent strength values and solvent selectivity effects have been studied for twenty diastereoisomeric compounds (ten *threo* and *erythro* pairs) as a function of mobile phase composition, in thin-layer chromatography on silica. Forty-six different mobile phases were studied, composed of twelve different solvents, and comprising almost 500 k' values. These data are compared with predictions from a previous model and found to give generally good agreement between experiment and theory. The general importance of solvent–solute localization in determining changes in band-spacing (α-values) was confirmed.

INTRODUCTION

Previous papers in this series[1–8] have dealt with various aspects of the separation of diastereoisomers on silica. It has been shown for over 100 diastereoisomeric pairs that steric hindrance to adsorption controls which isomer (*threo* or *erythro*) will be preferentially retained. The choice of mobile phase can have a profound effect on the relative separation of a given diastereoisomeric pair[6], and our paper[7] has provided a theoretical analysis of these mobile-phase selectivity effects. It appears that *solvent–solute localization* as described in refs. 9–11 accounts for change in separation factor α as the composition of the mobile phase in varied, allowing quantitative prediction of these solvent effects for given diastereoisomers.

In the present study we have broadened our data base for the separation of diastereoisomers by different mobile phases, in an effort to confirm further the basic model of separation, and to provide more information on the specific selectivity effects of a broad range of solvent types. Our findings are expected to be applicable beyond the special case of diastereoisomers as solutes; *i.e.*, these results should be

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generally useful for all samples in the optimization of band-spacing in separations on silica. In this paper we will look mainly at solvent effects *per se*. In the following paper we will examine the implications of these data concerning the *a priori* prediction of retention order in this series of diastereoisomers.

THEORY

Previous experimental work with columns of silica and alumina[9,12] has established the importance of solvent–solute localization: a major contributor to solvent selectivity effects in separations by liquid–solid chromatography (LSC). These localization effects are particularly important in LSC systems that involve solute band-pairs of similar molecular size and mobile phases where strong hydrogen-bonding between sample and solvent molecules does not occur. The diastereoisomeric solutes described here, and most of the mobile phases used by us, fit these latter requirements closely. Therefore the present LSC systems are well suited as probes for the effects of solvent–solute localization.

Localization in LSC systems is based on the tendency of polar molecules of sample or of mobile phase to attach to specific adsorption sites on the adsorbent surface: surface silanols in the case of silica[13]. For sufficiently polar molecules (more specifically, molecules with very polar *groups* or substituents), this attachment of adsorbed molecules to the surface will be strong enough to result in *localization* of the molecule over an adsorption site (silanol). Localized molecules are restricted in their movement across the surface (slower diffusion; see ref. 14), and more importantly have a higher energy of adsorption than for the non-localized state. The consequences of localized adsorption as regards solvent selectivity effects is then as follows. Assume first a non-localizing mobile phase B and two solutes of equal retention (k') in that mobile phase: a localizing solute X and a non-localizing solute Y. Now replace mobile phase B with a localizing mobile phase C of similar strength. Because localizing molecules cover only a fraction of the total adsorbent surface (*ca.* 75% maximum), non-localizing solute molecules Y can continue to adsorb without competing with localizing mobile phase molecules C. However, localizing solute molecules X must now compete with localizing solvent molecules C for a place on the adsorbent surface. Because the adsorption energy of localized molecules is larger, and because LSC retention involves a competition between adsorbing molecules of sample and of solvent[10,13], this means that the net adsorption energy of solute X will be smaller in mobile phase C *vs.* B. Therefore while X and Y have similar k' values in mobile phase B, k' for X will be much smaller in mobile phase C. This leads then to a change in band-position or band-spacing of the compounds X and Y; *i.e.*, a change in their separation factor α.

Previous work[9,10,12] has developed a quantitative model for solvent-solute localization and its effect on solute α-values. The model predicts that as the mobile phase composition is changed by substituting different solvents or by changing their concentrations, the separation of two compounds will vary as

$$\log \alpha = A + Bm \tag{1}$$

Here α is the separation factor of the two compounds in question, m is the localization

parameter of the mobile phase (increasing with increased localization of the mobile phase), and A and B are constants for a given combination of sample and specific localizing solvents. To a first approximation, A and B will be constant for a given pair of solutes and for all mobile phases.

The mobile phase localization parameter m can in turn be calculated from a knowledge of the composition of the mobile phase[9,10], where for a binary mobile phase A/C

$$m = m^0 \, f(\theta_C) \tag{2}$$

Here m^0 is the m value of pure solvent C as mobile phase, $f(\theta_C)$ is some function of the surface coverage θ of localizing solvent C, and solvent A is assumed not to localize. For the case of more than one localizing solvent in the mobile phase, a slightly more complicated version of eqn. 2 results[9,10], but we can ignore this here. The function $f(\theta_C)$ varies from 0 to 1 as θ_C varies from 0 (no C in the mobile phase) to 1 (pure solvent C). The surface coverage θ_C can in turn be calculated from a knowledge of certain other properties of the LSC system:

$$\theta_C = K_{ca}N_C/(N_A + K_{ca}N_C) \tag{3}$$

Here N_A and N_C are mole fractions of A and C in the mobile phase, and K_{ca} is the equilibrium constant for the competitive adsorption of A and C onto the surface of the adsorbent:

$$K_{ca} = 10^{\alpha' n_b(\varepsilon_C - \varepsilon_A)} \tag{4}$$

Here α' is the adsorbent activity parameter (equal to 0.57 for silica), n_b is the relative molecular area of solvent C, and ε_A and ε_C are the solvent strength parameters ε^0 for pure solvents A and C. Values of these latter solvent parameters are given in ref. 10 for numerous common solvents. Values of m^0 are available for a few solvents and silica[10], and approximate values can be estimated for other solvents from similar data for alumina as adsorbent. However, one of the objectives of the present study is to obtain additional m^0 values for various solvents and silica as adsorbent.

Data were also obtained from the present study on the solvent strength values ε^0 for various mobile phases. It was of interest to compare these experimental values of ε^0 with values that can be calculated from the model described in refs. 10 and 13. Experimental R_F values can be converted to values of the capacity factor k':

$$R_M = \log[(1 - R_F)/R_F] = \log k' \tag{5}$$

and the relative solvent strengths ε_1 and ε_2 of two mobile phases 1 and 2 can be obtained from

$$\log(k_2/k_1) = \alpha' A_s(\varepsilon_1 - \varepsilon_2) \tag{6}$$

Here, for a given solute, k_1 and k_2 are k' values obtained for mobile phases 1 and 2, respectively, α' is the adsorbent activity factor cited above, A_s is the relative molecular area of the solute. Non-polar solvents such as hexane are assigned an ε^0 value

of zero, which permits measurement of ε^0 for other mobile phases from eqn. 6. We can also calculate values of ε^0 for various mobile phase mixtures from

$$\varepsilon^0 = \varepsilon_A + \log(N_A/\theta_A) \quad (7)$$

Here θ_A is the surface mole-fraction of adsorbed solvent A, equal to $(1 - \theta_C)$ for binary-solvent mobile phases A/C. Values of θ_A are obtained from eqn. 3. It should be further noted that the value of ε_C (localizing solvents) is not constant, but varies both with the values of θ_C (and therefore N_C) and the nature of solvent A (its solvent strength). These details are described in ref. 10 and will not be repeated here.

EXPERIMENTAL

Silica gel (Riedel de Haen, Hannover, F.R.G.) was used as previously[1–6]. A slurry of 30 g of adsorbent and 80 ml of distilled water was spread on four plates (20 × 20 cm) by means of an apparatus according to Stahl; the coating thickness was *ca.* 0.5 mm. The coated plates were air-dried (see p. 374 of ref. 15). The samples were applied at a distance of 2 cm from the edge of the plate, with a separation of 1.5 cm between samples. The length of run was 18 cm. Chromatograms were developed in a glass chamber (20 × 10 × 20 cm) using 30 ml of mobile phase. Separation at ambient temperature without presaturation of the chamber was employed; however, equivalent results were obtained with and without presaturation. Developed plates were sprayed with iodine–ether solutions to effect detection of separated compounds. The reproducibility of measured R_F values was ±0.02 units. The R_F values of Tables II and III are arithmetic means of two to six measurements.

The solvents used were p.a. grade or were treated with molecular sieve 4A and distilled in glass. Methyl *tert.*-butyl ether was prepared by modification of the procedure of ref. 16.

The amidoesters and diamides of the diastereoisomeric 2,3-diphenylglutaric acids (compounds 1–20 of Tables II and III) were prepared by Michael addition reaction and their relative configurations have been assessed by NMR spectroscopy[17–19].

RESULTS AND DISCUSSION

Ten pairs (twenty solutes) of diastereomeric compounds were investigated, as summarized in Table I. These compounds were run as groups of ten in 46 different mobile phases. Compounds 1–10 of Table I are less strongly retained, and were run in mobile phases having $0.31 \leqslant \varepsilon^0 \leqslant 0.38$. Compounds 11–20 required mobile phases that were stronger: $0.42 \leqslant \varepsilon^0 \leqslant 0.49$. Retention data for these various LSC systems are summarized in Tables II and III. In this study we focused on the separation of individual diastereoisomeric pairs, although the separation of different compounds (non-isomer pairs) could also have been examined as a function of mobile phase composition. A cursory examination of the R_F values and separation factors α in Tables II and III demonstrates large changes in separation as a function of mobile phase composition. For example, log α for diastereomers 11 and 12 of Table I varies from 0.08 to 0.90 — a variation in α of almost seven-fold.

TABLE I

DIASTEREOISOMERIC SOLUTES USED IN THE PRESENT STUDY

C_6H_5-CH(X)-CH(Y)-C_6H_5

X	Y	Configuration	Compound No.	A_s*
CH_2COOCH_3	$CON(CH_3)_2$	*threo*	1	37.5
		erythro	2	
CH_2COO-*tert.*-butyl	$CON(CH_3)_2$	*threo*	3	38.6
		erythro	4	
CH_2COOCH_3	$CON(C_2H_5)_2$	*threo*	5	38.3
		erythro	6	
CH_2COOCH_3	CON(isopropyl)$_2$	*threo*	7	38.9
		erythro	8	
CH_2COOCH_3	CON(cyclohexyl)$_2$	*threo*	9	46.3
		erythro	10	
$CH_2CON(CH_3)_2$	$CON(CH_3)_2$	*threo*	11	39.3
		erythro	12	
$CH_2CON(CH_3)_2$	$CON(C_2H_5)_2$	*threo*	13	40.1
		erythro	14	
$CH_2CON(CH_3)_2$	CON(*n*-propyl)$_2$	*threo*	15	40.7
		erythro	16	
$CH_2CON(CH_3)_2$	CON(isopropyl)$_2$	*threo*	17	40.7
		erythro	18	
$CH_2CON(CH_3)_2$	CON(cyclohexyl)$_2$	*threo*	19	48.1
		erythro	20	

* Values of A_s are calculated as in ref. 13.

Solvent strength values

The present study offers additional opportunity to test the accuracy of eqn. 7 for predictions of solvent strength as a function of mobile phase composition. For 46 mobile phases, which include nine localizing solvents and three non-localizing solvents, experimental ε^0-values (eqns. 5 and 6) agree with calculated values (eqn. 7) within ± 0.04 units in ε^0. This is slightly poorer than in previous studies[20], where almost 200 different mobile phases gave agreement between experiment and theory of ± 0.02 units. Estimates of ε^0 for the pure solvents diisopropyl ether, tetrahydrofuran and ethanol reported in ref. 10 were replaced here with more accurate (measured) values:

Solvent	ε'	ε''
Diisopropyl ether	0.45	0.35
Tetrahydrofuran	0.73	0.48
Ethanol	2.0	0.65

TABLE II

EXPERIMENTAL VALUES OF R_F AND DERIVED VALUES OF log α FOR DIASTEREOISOMERIC COMPOUNDS 1–10 OF TABLE I, IN DIFFERENT MOBILE PHASES

*Solute**	*R_F for indicated mobile phase***									
	PE	*10% EE/B*	*14% EE/B*	*16% EE/B*	*18% EE/B*	*50% EE/B*	*60% EE/B*	*33% EE/H*	*20% EE/CS*	*50% PE/H*
1	0.10	0.09	0.13	0.18	0.18	0.44	0.54	0.04	0.09	0.02
2	0.24	0.15	0.21	0.28	0.29	0.61	0.68	0.08	0.21	0.06
3	0.25	0.14	0.20	0.24	0.27	0.60	0.68	0.08	0.16	0.06
4	0.37	0.21	0.27	0.33	0.36	0.69	0.74	0.13	0.25	0.11
5	0.34	0.19	0.24	0.30	0.33	0.67	0.74	0.13	0.21	0.09
6	0.52	0.23	0.31	0.37	0.40	0.73	0.78	0.18	0.29	0.19
7	0.74	0.34	0.45	0.52	0.54	0.86	0.89	0.33	0.39	0.47
8	0.73	0.34	0.45	0.52	0.54	0.86	0.89	0.33	0.39	0.47
9	0.83	0.53	0.63	0.67	0.69	0.94	0.94	0.44	0.51	0.68
10	0.81	0.48	0.57	0.62	0.64	0.94	0.94	0.39	0.47	0.58
ε^0_{expt}***	0.348	0.328	0.336	0.341	0.343	0.373	0.378	0.320	0.331	0.321
ε^0_{calc}§	0.340	0.305	0.322	0.330	0.337	0.397	0.403	0.313	0.301	0.261
	log α for indicated mobile phase									
1/2§§	0.45	0.25	0.25	0.25	0.27	0.29	0.26	0.32	0.42	0.50
3/4	0.25	0.21	0.17	0.19	0.18	0.17	0.12	0.23	0.24	0.28
5/6	0.32	0.10	0.15	0.14	0.13	0.12	0.10	0.17	0.19	0.37
7/8	−0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
9/10	−0.06	−0.08	−0.11	−0.10	−0.10	0.00	0.00	−0.09	−0.07	−0.19

	*R_F for indicated mobile phase***								
	25% PE/CS	*33% PE/CS*	*30% MBE/H*	*20% EA/H*	*30% EA/H*	*40% EA/H*	*4% AN/B*	*10% AN/B*	*18% AN/B*
1	0.06	0.08	0.14	0.06	0.16	0.31	0.06	0.20	0.45
2	0.14	0.20	0.21	0.13	0.28	0.47	0.07	0.27	0.51
3	0.11	0.15	0.23	0.14	0.33	0.55	0.08	0.31	0.53
4	0.23	0.31	0.27	0.21	0.43	0.64	0.10	0.36	0.56
5	0.14	0.22	0.27	0.18	0.40	0.60	0.10	0.37	0.54
6	0.29	0.42	0.32	0.26	0.49	0.68	0.10	0.37	0.54
7	0.52	0.67	0.49	0.44	0.65	0.83	0.18	0.47	0.69
8	0.56	0.72	0.49	0.44	0.65	0.83	0.17	0.46	0.69
9	0.77	0.91	0.63	0.56	0.75	0.90	0.32	−0.61	0.82
10	0.73	0.89	0.58	0.53	0.71	0.90	0.28	0.56	0.82
ε^0_{expt}***	0.333	0.345	0.337	0.329	0.348	0.366	0.313	0.341	0.360
ε^0_{calc}§	0.178	0.196	0.339	0.316	0.348	0.379	0.296	0.348	0.395
	log α for indicated mobile phase								
1/2§§	0.40	0.46	0.21	0.36	0.31	0.30	0.07	0.17	0.11
3/4	0.38	0.40	0.10	0.21	0.19	0.16	0.11	0.10	0.05
5/6	0.40	0.41	0.10	0.21	0.16	0.15	0.00	0.00	0.00
7/8	0.07	0.10	0.00	0.00	0.00	0.00	−0.03	−0.02	0.00
9/10	−0.10	−0.09	−0.09	−0.05	−0.09	0.00	−0.08	−0.09	0.00

TABLE II *(continued)*

*Solute**	*R_F for indicated mobile phase***							
	4% AC/B	*8% AC/B*	*17% AC/B*	*25% THF/H*	*30% THF/H*	*40% THF/H*	*6% PA/H*	*3% EAL/B*
1	0.13	0.28	0.50	0.21	0.32	0.51	0.20	0.18
2	0.20	0.36	0.58	0.28	0.37	0.58	0.27	0.22
3	0.18	0.37	0.59	0.33	0.45	0.65	0.28	0.21
4	0.22	0.43	0.64	0.36	0.48	0.65	0.31	0.25
5	0.20	0.40	0.62	0.40	0.53	0.68	0.32	0.23
6	0.24	0.44	0.65	0.40	0.53	0.68	0.34	0.26
7	0.32	0.56	0.80	0.58	0.73	0.84	0.39	0.32
8	0.32	0.56	0.80	0.54	0.69	0.84	0.39	0.32
9	0.42	0.72	0.91	0.68	0.80	0.88	0.42	0.43
10	0.38	0.66	0.91	0.62	0.76	0.88	0.39	0.39
ε^0_{expt}***	0.329	0.348	0.368	0.345	0.355	0.372	0.336	0.331
ε^0_{calc}§	0.318	0.363	0.417	0.368	0.403	0.438	0.364	0.464
	log α for indicated mobile phase							
1/2§§	0.23	0.16	0.14	0.17	0.10	0.12	0.17	0.11
3/4	0.11	0.11	0.09	0.06	0.06	0.00	0.06	0.10
5/6	0.10	0.08	0.06	0.00	0.00	0.00	0.04	0.08
7/8	0.00	0.00	0.00	−0.07	−0.08	0.00	0.00	0.00
9/10	−0.07	−0.12	0.00	−0.12	−0.10	0.00	−0.05	−0.07

* Compound numbers as in Table I.

** Solvent abbreviations (% indicates vol. %): PE, diisopropyl ether; EE, diethyl ether; B, benzene; H, hexane; CS, carbon disulphide; MBE, methyl-*tert.*-butyl ether; EA, ethyl acetate; AN, acetonitrile; AC, acetone; THF, tetrahydrofuran; PA, isopropyl alcohol; EAL, ethyl alcohol.

*** Measured from experimental R_F values according to eqn. 5.

§ Calculated as described in ref. 10 from parameters given in Table I of that reference.

§§ Diastereoisomer pairs for corresponding α-values.

These latter values (see Table I of ref. 10) agree within a few hundredths of a unit with ε' and ε'' values reported earlier. So our confidence in other estimated values of these solvent parameters (in ref. 10) remains high.

Solvent selectivity effects

We initially attempted to calculate values of the localization parameter m for the various mobile phases of Tables II and III, using eqns. 2–4 and estimated values of m^0 from ref. 10. Eqn. 1 was then tested for its ability to predict values of log α for the various diastereoisomeric solute pairs of Table I. The resulting plots showed more scatter than we had observed for similar studies earlier[7,9,12], and so we examined the data more closely. One difference in the mobile phases reported here *vs.* those studied earlier in terms of solvent–solute localization and eqn. 1 is the use of more polar non-localizing solvents such as benzene and carbon disulphide, in place of the usual non-polar mobile-phase carriers such as pentane and hexane. Whereas moderate concentrations of polar solvents such as acetonitrile ($N_C > 0.05$) in mobile phases composed mainly of non-polar solvents lead to large θ_C values with $m \approx m^0_C$ for the mobile phase, the model of ref. 10 does not predict this to be true for mobile phases

TABLE III

EXPERIMENTAL VALUES OF R_F AND DERIVED VALUES OF log α FOR DIASTEREOISOMERIC COMPOUNDS 11–20 OF TABLE I, IN DIFFERENT MOBILE PHASES

*Solute**	*R_F for indicated mobile phase***										
	PE	*60% EE/B*	*65% EE/B*	*75% EE/B*	*EE*	*90% MBE/H*	*80% EA/H*	*90% EA/H*	*50% AN/B*	*60% AN/B*	*70% AN/B*
11	0.00	0.02	0.02	0.02	0.03	0.15	0.03	0.08	0.19	0.22	0.59
12	0.00	0.10	0.12	0.13	0.18	0.42	0.19	0.41	0.33	0.39	0.71
13	0.00	0.07	0.07	0.08	0.10	0.48	0.11	0.30	0.41	0.49	0.81
14	0.02	0.16	0.18	0.21	0.31	0.64	0.30	0.60	0.41	0.49	0.81
15	0.01	0.18	0.18	0.22	0.36	0.77	0.27	0.64	0.56	0.65	0.91
16	0.03	0.25	0.27	0.31	0.48	0.82	0.43	0.72	0.51	0.61	0.91
17	0.05	0.30	0.31	0.35	0.57	0.90	0.43	0.74	0.62	0.71	0.92
18	0.05	0.30	0.31	0.35	0.51	0.82	0.43	0.74	0.59	0.63	0.92
19	0.11	0.45	0.47	0.51	0.76	0.96	0.68	0.88	0.76	0.84	0.96
20	0.06	0.34	0.36	0.41	0.61	0.90	0.57	0.82	0.66	0.74	0.96
ε^0_{expt} ***		0.425	0.426	0.429	0.441	0.471	0.437	0.460	0.453	0.458	0.489
ε^0_{calc} §		0.403	0.425	0.407	0.430	0.470	0.461	0.472	0.467	0.478	0.487
	log α for indicated mobile phase										
11/12§§	–	0.74	0.82	0.86	0.85	0.61	0.88	0.90	0.32	0.36	0.23
13/14	–	0.40	0.46	0.48	0.60	0.28	0.54	0.55	0.00	0.00	0.00
15/16	0.49	0.18	0.23	0.20	0.22	0.13	0.31	0.16	−0.08	−0.08	0.00
17/18	0.00	0.00	0.00	0.00	−0.10	−0.29	0.00	0.00	−0.05	−0.16	0.00
19/20	−0.28	−0.20	−0.20	−0.18	−0.31	−0.43	−0.21	−0.21	−0.21	−0.27	0.00

*Solute**	*R_F for indicated mobile phase***								
	36% AC/B	*50% AC/B*	*15% AC/EE*	*50% THF/H*	*60% THF/H*	*70% THF/H*	*28% PA/H*	*7% EAL/B*	*10% EAL/B*
11	0.31	0.62	0.11	0.14	0.26	0.45	0.19	0.19	0.28
12	0.50	0.70	0.51	0.28	0.43	0.70	0.48	0.23	0.30
13	0.51	0.74	0.56	0.40	0.53	0.79	0.52	0.24	0.32
14	0.58	0.77	0.68	0.47	0.58	0.83	0.70	0.27	0.34
15	0.62	0.83	0.79	0.61	0.75	0.89	0.77	0.28	0.36
16	0.62	0.83	0.70	0.61	0.75	0.89	0.77	0.28	0.36
17	0.66	0.86	0.84	0.68	0.83	0.91	0.82	0.30	0.41
18	0.62	0.86	0.78	0.60	0.77	0.91	0.74	0.27	0.38
19	0.71	0.94	0.88	0.79	0.89	0.93	0.89	0.35	0.45
20	0.66	0.82	0.76	0.67	0.84	0.93	0.83	0.30	0.42
ε^0_{expt} ***	0.459	0.479	0.467	0.454	0.467	0.484	0.467	0.435	0.443
ε^0_{calc} §	0.459	0.482	0.455	0.465	0.485	0.500	0.460	0.483	0.496
	log α for indicated mobile phases								
11/12§§	0.35	0.16	0.93	0.38	0.33	0.28	0.60	0.10	0.04
13/14	0.12	0.08	0.23	0.13	0.09	0.11	0.34	0.07	0.04
15/16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
17/18	−0.08	0.00	−0.17	−0.15	−0.16	0.00	−0.21	−0.06	−0.05
19/20	−0.10	−0.53	−0.37	−0.27	−0.19	0.00	−0.22	−0.10	−0.05

* Compound numbers as in Table I.

** Solvent abbreviations as in Table II.

*** Measured from experimental R_F values according to eqn. 6.

§ Calculated as described in ref. 10 from parameters given in Table I of that reference.

§§ Diastereoisomer pairs for corresponding α-values.

TABLE IV

COMPARISON OF SELECTED α-VALUES FOR SOLUTES 1 AND 2 OF TABLE I AND DIFFERENT MOBILE PHASES (DATA OF TABLE II)

Mobile phase	N_C	*Calc. m*	*log α*
Diethyl ether–benzene (10:90)	0.09	0.11	0.25
Diethyl ether–benzene (16:84)	0.14	0.18	0.25
Diethyl ether–benzene (50:50)	0.46	0.39	0.29
Diethyl ether–benzene (60:40)	0.56	0.40	0.26
Acetonitrile–benzene (4:96)	0.07	0.16	0.07
Acetonitrile–benzene (10:90)	0.16	0.43	0.17
Acetonitrile–benzene (18:82)	0.27	0.81	0.11

TABLE V

FIT OF DATA OF TABLES II AND III TO EQN. 1

Assuming $m = m^0$ for the various mobile phases; values of m^0 derived from these data.

*Solute pair**	*A***	*B***	*Standard deviation*
1/2	0.51	−0.36	±0.05
3/4	0.36	−0.28	0.04
5/6	0.40	−0.38	0.04
7/8	0.04	−0.06	0.03
9/10	−0.10	0.04	0.05
11/12	1.8	−1.5	0.16
13/14	1.3	−1.2	0.05
15/16	0.57	−0.58	0.04
17/18	0.02	−0.12	0.09
19/20	−0.32	0.12	0.13
	overall agreement		±0.07

Localizing solvent	m^0 *(expt)****	m^0 *(10)*§
Diisopropyl ether	0.10	
Ethyl acetate	0.60	0.60
Diethyl ether	0.66	0.43
Methyl-*tert.*-butyl ether	0.82	0.82
Isopropyl alcohol	(0.85)§§	
Acetone	0.95	0.87
Tetrahydrofuran	1.00	0.65
Acetonitrile	1.05	1.19
Ethyl alcohol	1.05	

* From Table I.

** Best fit to eqn. 1; data of Tables II and III.

*** Best fit to eqn. 1; assumes $m = m^0$.

§ Predicted values from ref. 10.

§§ Value derived on the basis of two mobile phases only.

composed mainly of solvents such as benzene. One reason, despite the large difference in ε^0-values for solvents such as acetonitrile and benzene, is that so-called *site-competition delocalization* predicts that the ε^0-value for acetonitrile will be reduced by the co-adsorption of molecules of benzene on the surface. This in turn reduces K_{ca} and θ_C, and consequently also reduces m (eqn. 2).

The present study suggests that this reduction in m predicted above does not occur for the case of mobile phases such as acetonitrile–benzene when N_C (acetonitrile) > 0.05. This is illustrated in Table IV, using data from Table II for solutes 1 and 2 (Table I) and several mobile phases. At first glance, there appears to be no correlation between log α and m, as predicted by eqn. 1. However, if $\theta_C \approx 1$ and $m = m^0$, as would be the case in the absence of site-competition delocalization, then the data make reasonable sense. The m^0 values of pure diethyl ether and acetonitrile are 0.66 and 1.05, respectively (see below); *i.e.*, quite different. The corresponding log α-values for mixtures of diethyl ether and acetonitrile (Table IV) are respectively 0.26 ± 0.02 and 0.12 ± 0.05. That is, the data agree within ±0.02–0.05 units with a

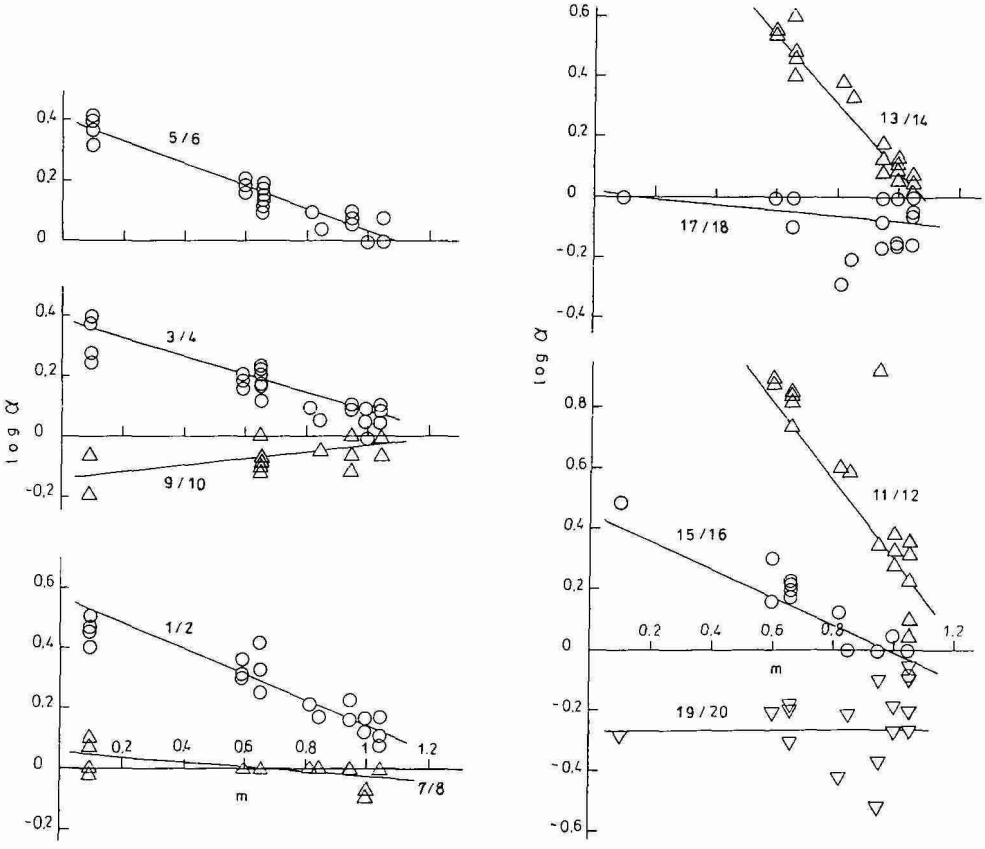

Fig. 1. Comparison of experimental data of Table II in terms of eqn. 1. Parameter values of Table V assumed, with $m = m^0$.

Fig. 2. Comparison of experimental data of Table III in terms of eqn. 1. Parameter values of Table V used, with $m = m^0$.

model that assumes $m \approx m^0$ for these mobile phase systems. Carrying this hypothesis further, we have fit the data of Tables II and III to eqn. 1 with the assumption that in each case $m \approx m^0$. Resulting values of A and B from eqn. 1 are listed in Table V, along with the deviation of experimental points from eqn. 1. The data of Tables II and III are also plotted in terms of eqn. 1 in Figs. 1 and 2.

The resulting plots show somewhat more scatter than for similar plots reported in ref. 7 for other diastereoisomers, but the agreement of the data with eqn. 1 is adequately precise to clearly see the importance of solvent–solute localization in these LSC systems (overall S.D. = ± 0.07 units). The greater scatter here *vs.* ref. 7 can be attributed to the stronger solvent systems used (where other effects become more important —see discussion of ref. 10). We also see general verification of the rule that maximum separation will occur either for mobile phases of large m or small m— but not for solvents of intermediate m value. Other correlations parallel to those drawn in ref. 7 can be made, but we will defer discussion of these to a later time.

Values of m^0 derived from these studies are presented in Table V. These can be compared with values from ref. 10, and it is seen that agreement is generally good; *i.e.*, we were successful in ref. 10 in predicting which solvents will exhibit large solvent–solute localization effects.

CONCLUSIONS

Almost 500 retention measurements were made for twenty diastereoisomeric solutes, using 46 different mobile phases composed of twelve different solvents. These data were used to test a previous theory of solvent selectivity effects in LSC. Agreement of experimental data with the theory was found to be acceptable, but the data suggest that the combination of polar non-localizing and localizing solvents (*e.g.*, benzene, non-localizing; acetonitrile, localizing) is generally dominated by the localizing solvent. Specifically, when the mole-fraction of the localizing solvent is greater than 0.05, solvent selectivity effects are constant and characteristic of the pure localizing solvent. This result was not predicted by our previous model, and constitutes an effect that should be studied further.

The previous model also predicts solvent strength as a function of composition. This was tested here for these 46 different mobile phases, and the model was found to predict ε^0-values within ± 0.04 units (1 S.D.). Thus our model has now been tested for almost 300 different mobile phases (binary, ternary, quaternary; silica and alumina) and found to give generally good agreement with experimental data.

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DETERMINATION OF INORGANIC ANIONS BY ION-PAIR CHROMATOGRAPHY

HETAERON ADSORPTION CHARACTERISTICS ON SOME ALKYL-BONDED SILICA STATIONARY PHASES*

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SUMMARY

A non-buffered ion-pair chromatographic system with alkyl-bonded silica was used to determine inorganic anions. Some octyl-bonded stationary phases showed unexpected behaviour such as a dependence on pre-wetting and on the flow-rate during the dynamic equilibration. On the other hand, octadecyl-bonded stationary phases were not influenced by these effects. The influence of an organic co-solvent and the efficiency with 3-μm particles in ion-pair chromatography were also studied.

INTRODUCTION

In a previous paper[1] we described the separation of inorganic anions by ion-pair reversed-phase liquid chromatography monitored by indirect photometry. A lipophilic ionic hetaeron ($C_8H_{17}NH_3^+$) is adsorbed on octyl-bonded silica and the ionic solute competes with the UV-absorbing counter ion. A non-buffered aqueous mobile phase is used in order to decrease the conductivity of the eluent and permit double detection by conductimetry and indirect photometry[2]. We have previously shown[3] that pre-wetting of the octyl-bonded silica influences the amount of the surfactant ($C_8H_{17}NH_3^+CH_3C_6H_4SO_3^-$) adsorbed, although the concentration of the surfactant in the aqueous mobile phase is kept constant, and that an increase in the amount of surfactant adsorbed causes an increase in the capacity factors of inorganic

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TABLE I

AMOUNT (A) OF ADSORBED SURFACTANT AND CAPACITY FACTORS (k') WITH AQUEOUS MOBILE PHASE, OA OR TBA HETAERON AND (PTS) COUNTER ION ON OCTYL- AND OCTADECYL-BONDED SILICA

Columns: Zorbax C_8, 240 × 4 mm I.D.; Zorbax ODS, 90 × 4 mm I.D. Mobile phase: 5 mM, surfactant, pH 4.5. Flow-rate: 2 ml min^{-1}.

Zorbax C_8							*Zorbax ODS*			
Pre-wetting: methanol (60 ml) then water (ml)	*OA-PTS*			*TBA-PTS*			*Pre-wetting: methanol (60 ml) then water (ml)*	*OA-PTS*		
	A (μmole)	*k' (I^-)*	*k' (SO_4^{2-})*	*A (μmole)*	*k' (I^-)*	*k' (SO_4^{2-})*		*A (μmole)*	*k' (I^-)*	*k' (SO_4^{2-})*
0	800	4.36	18.6	370	10.3	1.17	0	390	1.6	3
5	515	2.9	12.3	170	4.3	0.6	20	390	1.6	3
20	55	0.4	1.2	80	1.6	0.16	40	390	1.6	3
60	52	0.4	1.2	30	0.3	0.01	60	390	1.6	3

solutes. Other workers[4–7] have found that the amount of surfactant adsorbed depends on the concentration of either the pairing ion or the ionic and/or organic modifier in the eluent.

In this work, we studied the effects of pre-wetting of two types of alkyl-bonded silica with two types of alkylammonium surfactants. The influence of the flow-rate of the mobile phase during the equilibration was investigated. With octyl-bonded silica, an irreversible modification was observed after several adsorption and desorption cycles and we propose a simple test to demonstrate this modification. Further studies indicated that the efficiency of a column in ion-pair chromatography is lower than that in reversed-phase chromatography, and that this efficiency is drastically reduced when 3-μm microparticles are used.

EXPERIMENTAL

The chromatographic equipment, eluent preparation, column loading and column desorption were as described elsewhere[1–3].

The columns were slurry packed in the laboratory with 7-μm Zorbax C_8 and ODS (DuPont, Wilmington, DE, U.S.A.), 5-μm LiChrosorb RP-8 and RP-18 (Merck, Darmstadt, F.R.G.) and 5-μm Spherisorb C_8 (Phase Separations, Queensferry, U.K.). For the study of the influence of flow-rate, an Ultrasphere C_8 column (Beckman, Fullerton, CA, U.S.A.) was used. Situated before the Rheodyne sample valve, a pre-column packed with octyl-bonded silica guarded the analytical column. In all experiments the following order of operations was adopted.

For the pre-wetting of the apolar phase, different finite volumes of liquid were pumped through the pre-column–analytical column system, first 60 ml of methanol and then from 0 to 60 ml of water. The analytical column was then disconnected and the pre-column was equilibrated separately with the eluent containing the surfactant. After equilibrium of the pre-column, the analytical column was switched into the same eluent flow line. The amount of surfactant adsorbed of the analytical column was calculated by the breakthrough method[3] (Table I).

Aqueous eluents were prepared with octylammonium (OA) or tetrabutylammonium (TBA) hetaeron and *p*-toluenesulphonate (PTS), phthalate (P), chloride (Cl) or bromide (Br) counter ions. A comparative study of adsorption was carried out with hydro-organic eluents.

RESULTS AND DISCUSSION

Influence of pre-wetting of alkyl-bonded silica

Before pumping the surfactant (hetaeron–counter ion) dissolved in water through the column, the apolar stationary phase (Zorbax C_8 or ODS) was pre-wetted with 60 ml of methanol followed by water. The various volumes of water desorb a portion of the organic solvent adsorbed on the apolar moiety of the stationary phase and the lipophilic interactions between the surfactant and the stationary phase are decreased.

We have noted[1] that on Zorbax octyl-bonded silica the amount of OA-PTS (or TBA-PTS) surfactant adsorbed decreases with increase in the volume of water used in the pre-wetting stage. This decrease causes a decrease in the capacity factors of the solutes (Table I).

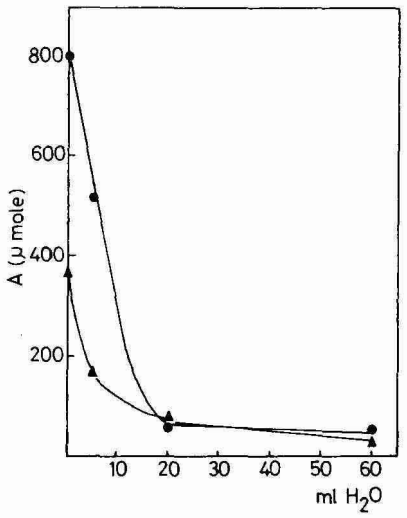

Fig. 1. Plots of amount of surfactant adsorbed as a function of the volume of water in the pre-wetting step following the methanol flushing. ●, 5 m*M* OA-PTS; ▲, 5 m*M* TBA-PTS.

Table I and Fig. 1 show a smaller amount of hetaeron adsorbed (A) for TBA than for OA, a greater decrease in A for OA than for TBA and a difference in the capacity factors of solutes for the two different adsorbed hetaerons (OA and TBA).

Two points can be made about the TBA-PTS system: first, the k' value of the sulphate dianion is lower than that of the iodide monoanion, and second, the k' value of the iodide monoanion is high.

Regarding the first point, similar dianion elution has been observed previously in ion-exchange chromatography with a high concentration of salt in the eluent[8,9], with ion-pair systems[10–12] and with a particular surfactant[2,13] (TBA-nitrobenzene-sulphonate). In this instance sulphate is eluted as the chloride or bromide faster than as the nitrate or iodide.

The second point can be explained by the different mechanisms involved in TBA hetaeron ion pairing in the mobile phase and in the stationary phase and in OA hetaeron ion pairing in the stationary phase, and by the diffuse charge of the iodide anion, *i.e.*, strong hydrophobic adsorption of the solute on the alkyl-bonded surface is possible when a small amount of TBA surfactant is adsorbed.

Hence the pre-wetting of Zorbax C_8 has a marked effect on the retention of the solutes. The reproducibility and the prediction of retention are difficult without a detailed knowledge of the pre-wetting stage, which may be one explanation for the numerous problems encountered in many laboratories when using such techniques.

OA-PTS was adsorbed on octadecyl-bonded silica (Zorbax ODS) following the same pre-wetting procedure as for the octyl-bonded stationary phase. Table I shows that the amount of OA-PTS adsorbed and the capacity factors of mono- and dianions do not depend on the pre-wetting.

We can propose an explanation for the different behaviours of these apolar bonded silicas. Before flushing with water, the methanol molecules are intercalated between the apolar chains and can behave like a surfactant. When water (60 ml) is

TABLE II

CAPACITY FACTORS OF SUGARS USING AN OCTYL-BONDED SILICA COLUMN

Column: Zorbax C_8, 240 × 4 mm I.D. Mobile phase: water. Flow-rate: 0.8 ml min^{-1}.

Packing	*k'*		
	Glucose	*Sucrose*	*Raffinose*
Unmodified	0.14	0.14	0.17
Irreversibly modified	0.73	1.29	1.68

flushed through the column, methanol is easily removed from the short-chain octyl-bonded layer, which leaves a chaotic structure that collapses owing to increased hydrophobic association[14]. With water, which is a very bad wetting agent for these apolar chains, a disordered folded state is favoured like a dry packing[15], and the surfactant is less adsorbed.

On the other hand, with ODS columns after the same flushing with water, some methanol molecules remain between the longer octadecyl chains, favouring an ordered "bristle" or chaotic state but non-collapsed. The residual methanol can be desorbed with a larger amount of water[14] or by heating[14,16]. With an octadecyl layer wetted by pure methanol or by methanol–water the same amount of surfactant can be adsorbed. Recently, spectral studies of ion-pair interactions have shown that a surfactant in water unfolds the octadecyl chain more than the octyl chain, as the shorter chains have less freedom to rearrange[17].

To confirm this difference in structure of alkyl chains after flushing with water, we can compare the separation of hydrophilic compounds such as glucose, sucrose and raffinose on C_8 and C_{18} unmodified columns with water as the eluent. On C_8 bonded silica (Table II) no selectivity is obtained and in this collapsed structure the carbohydrates have a retention volume close to the void volume. In contrast, on C_{18} bonded silica a good separation of carbohydrates is obtained with a low-loaded octadecyl-bonded packing having residual silanol groups[18] and also with a high-loaded Zorbax ODS packing[19]. For this last stationary phase residual methanol from the conditioning of the column may permit a chaotic structure to be preserved and the separation of carbohydrates by a sieve phenomenon.

Influence of organic co-solvent

With the same concentration of surfactant in the mobile phase, various amounts can be adsorbed and various capacity factors can be obtained by adding an organic solvent to the mobile phase.

We have shown[2,3] that after methanol and water pre-wetting, an aqueous–organic eluent increases the amount adsorbed and the capacity factors, but the breakthrough curves show a diffuse shape. After pre-wetting with methanol without water, an increase in the percentage of organic solvent in the eluent (to more than 5–10%) decreases the amount adsorbed because the hydrophobic effect is decreased[6]. However, as can be seen in Fig. 2a, a small amount of organic co-solvent (0–2%) allows more surfactant to be adsorbed than the neat aqueous mobile phase. This small

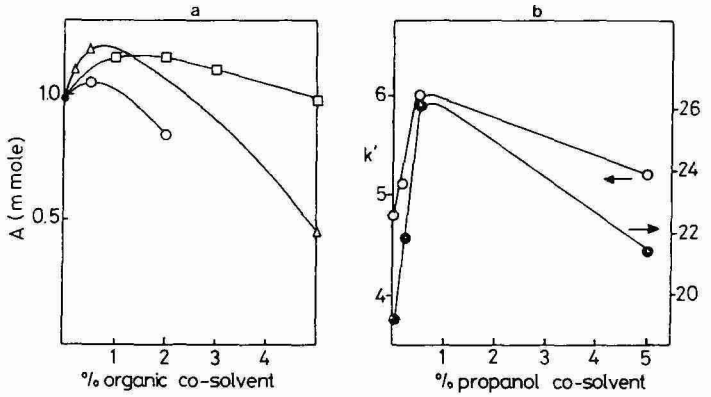

Fig. 2. (a) Amount of surfactant adsorbed as a function of the percentage of organic solvent in the mobile phase (5 m*M* OA-PTS): ○, Tetrahydrofuran; △, propanol; □, acetonitrile. (b) Capacity factors of solutes as a function of the percentage of propanol in the mobile phase (5 m*M* OA-PTS). ●, SO_4^{2-}; ○, I^-. Column: Zorbax C_8, 250 × 4.6 mm I.D. Mobile phase: 5 m*M* OA-PTS (pH 4.5) + organic co-solvent. Flow-rate: 2 ml min^{-1}. Pre-wetting: 60 ml of methanol.

modification to the mobile phase considerably increases the capacity factors (Fig. 2b).

In contrast a concentration of organic solvent higher than 2% decreases the amount adsorbed and also the capacity factors. In this instance the greater the affinity of the organic modifier for the apolar chain, the more it desorbs the lipophilic surfactant. This affinity can be characterized by the dispersion component, δ_d, of the Hildebrand parameter[20]. Tetrahydrofuran (δ_d = 7.6) is the most lipophilic (propanol, δ_d = 7.2; acetonitrile, δ_d = 6.5).

Fig. 2 shows that the same retention of a solute can be obtained with different amounts of surfactant using different percentages of propanol co-solvent (0.5 or 5%).

Finally, to increase the capacity factors it is possible to use a small amount of organic co-solvent, but the injection of aqueous solutions disturbs such systems[2].

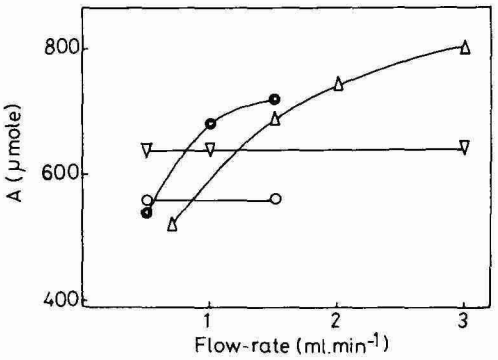

Fig. 3. Plots of amount of surfactant adsorbed [5 m*M* OA-P (pH 6.38)] as a function of the flow-rate of the equilibration. ●, Ultrasphere C_8; △, Zorbax C_8; ○, Spherisorb C_8; ▽, LiChrosorb RP-8.

Influence of flow-rate on equilibration

In their application notes, some manufacturers (*e.g.* Shodex) recommend changing the flow-rate of the mobile phase during the equilibration process, but the reason for this variation is not obvious. This fact, and the presence of some unexplained irregularities in the breakthrough curves[3], prompted us to study the influence of flow-rate during the equilibration.

After pre-wetting with methanol, the stationary phase was equilibrated using various flow-rates (3, 2, 1.5, 1 and 0.5 ml min^{-1})compatible with the maximum pressure of the columns. For each value of these flow-rates the amount of surfactant adsorbed was measured.

This was tried with various packing materials (LiChrosorb RP-8 and RP-18, Ultrasphere C_8, Spherisorb C_8 and Zorbax C_8 and ODS) using OA as the hetaeron and P as the counter ion. The results presented in Fig. 3 for C_8 columns show a difference between two columns for which the amount of surfactant adsorbed is independent of the flow-rate (Spherisorb and LiChrosorb) and the other two columns (Zorbax C_8 and Ultrasphere C_8) for which there is a clear dependence on these parameters. An increase in the flow-rate induces an increase in the amount of surfactant adsorbed. The similarity between these two columns confirms the observations of Majors[21] and Goldberg[22], who found a similarity in the capacity factors of either apolar or acidic or basic polar compounds on these two stationary phases. Similar studies with the C_{18} phases indicated complete independence of these parameters.

In order to verify the influence of the nature of the hetaeron and counter ion on this effect, the same test was carried out using TBA and OA as the hetaeron and PTS, P, Cl and Br as counter ions. Table III gives the results with the organic counter ions. A similar variation with flow-rate was obtained in all four instances. Replacing the organic with inorganic counter ions[3] gave the same results.

It was found that this effect is not constant during the life of the column, being noticeable only when the column is either new or has been never used in ion-pair chromatography. After a few adsorption and desorption cycles it becomes saturated and eventually the amount of surfactant adsorbed becomes independent of the

TABLE III

INFLUENCE OF EQUILIBRIUM FLOW-RATE ON THE AMOUNT OF SURFACTANT ADSORBED AND ON THE CAPACITY FACTORS

Column: Zorbax C_8, 7 μm, 240 × 4 mm I.D. Mobile phase: 1 m*M* surfactant. Pre-wetting: methanol.

Surfactant	*Flow-rate (ml min^{-1})*	*A (mmole)*	*k' (NO_3^-)*	*k' (I^-)*	*k' (SO_4^{2-})*
OA-PTS	3	0.57	5.1	6.2	28.5
OA-PTS	0.5	0.18	1.5	1.8	6.4
OA-P	3	0.45	3.6	5.3	10.8
OA-P	0.5	0.28	2.2	2.9	6.6
TBA-PTS	2.5	0.40	7.2	20.9	11.3
TBA-PTS	0.5	0.27	4.7	13	7.8
TBA-P	2.5	0.2	12.9	44	20.7
TBA-P	0.5	0.14	8.7	29	14.4

flow-rate used in equilibration (the same value as at a high flow-rate). Therefore, after utilization of these columns, the stationary phase becomes modified. We have devised a simple test to demonstrate the state of these columns. As can be seen from Table II, there is a great difference between the separation of a mixture of three specific sugars (glucose, sucrose and raffinose) on a new and on a modified column, the separation being impossible on a new column and very easy when it is modified. Hence the simple injection of a mixture of these sugars immediately indicates whether the modification to the column has occurred.

We think that a small amount of surfactant remains adsorbed after the desorption process with methanol–water (1:1). We have tried to desorb it by cleaning the column with solvents other than this methanol–water mixture, *viz.*, ethanol–propanol (1:1) at 50°C, methanol–chloroform (1:2) and 1 m*M* perchloric acid at 50°C, but the test with sugars showed that the column was still modified. A small amount of surfactant may be irreversibly adsorbed. In fact, Bij *et al.*[23] have shown that amines with a bulky alkyl chain such as octylamine are associated with residual silanol groups of bonded silica. These silanophilic interactions are stabilized by hydrophobic interactions between the alkyl chain of the amine and the alkyl moiety of the stationary phase.

These hypotheses do not provide any explanation for the separation of carbohydrates on the modified columns. One can only postulate that the phenomenon is similar to that observed with C_{18} bonded silica for the separation of the same compounds.

Efficiency of the ion-pair chromatographic system

The efficiency of apolar columns (C_8 and C_{18}) is usually assessed by chromatographing aromatic solutes with methanol–water as the mobile phase[24]. However, we have found that this evaluation of the performance of an apolar packing material has no meaning when it is used in an ion-pair chromatographic rather than in a reverse-phase system.

Table IV illustrates the results obtained with 7- and 3-μm Zorbax octadecyl-

TABLE IV

COMPARISON OF EFFICIENCY OF ZORBAX ODS PACKINGS IN REVERSED-PHASE AND ION-PAIR CHROMATOGRAPHIC SYSTEMS

System	*Mobile phase*	*Solute ion*	*Column*			
			Zorbax ODS 7 μm (150 × 4.6 mm I.D.)		*Zorbax ODS, Golden 3 μm (80 × 6.2 mm I.D.)*	
			Plates/m (N)	*k'*	*Plates/m (N)*	*k'*
Reversed-phase	Methanol–water (70:30)	Nitronaphthalene	40 400	6.20	65 950	4.75
		Naphthalene	55 440	9.74	95 920	8.11
Ion-pair	1 m*M* OA-PTS	NO_3^-	17 430	1.73	14 420	2.49
		ClO_3^-	18 680	2.54	16 310	3.39
		ClO_4^-	22 320	3.78	17 330	4.58
		SO_4^{2-}	27 150	9.51	22 100	10.58

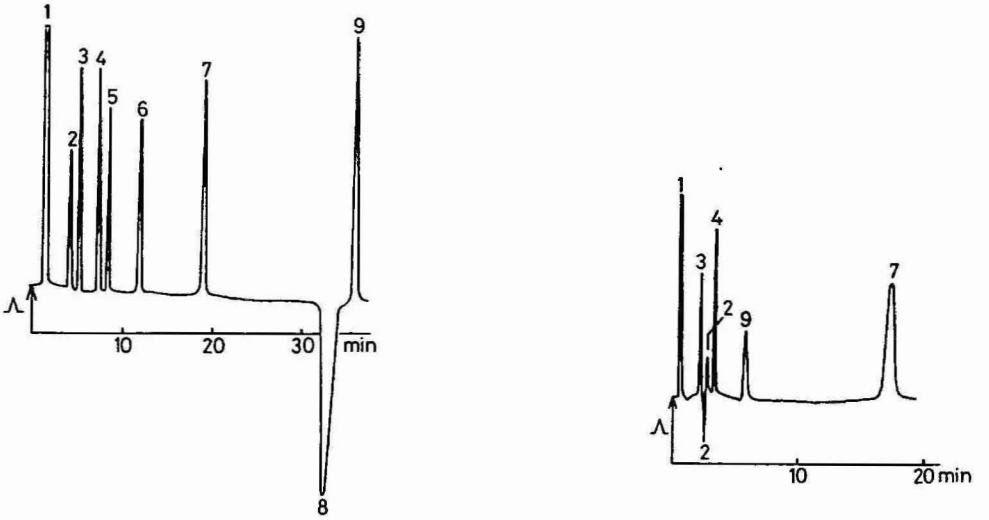

Fig. 4. Chromatogram of a test mixture of inorganic anions. Sample loop: 20 μl. 1 = Cations; 2 = carbonate, 0.6 μg; 3 = chloride, 0.36 μg; 4 = nitrite, 0.92 μg; 5 = bromide, 1.58 μg; 6 = nitrate, 1.24 μg; 7 = sulphate, 0.96 μg; 8 = induced peak; 9 = iodide, 2.52 μg. Column: Zorbax C_8, 240 × 4 mm I.D. Eluent: 1 m*M* tetrabutylammonium phthalate, pH = 6.2, flow-rate = 1.5 ml min^{-1}. Conductivity detection.

Fig. 5. Chromatogram of a test mixture of inorganic anions. Solutes as in Fig. 4. Multiple peaks for carbonate. Column: Zorbax ODS, 90 × 4 mm I.D. Eluent: 0.2 m*M* Tetrabutylammonium phthalate, pH = 6, flow-rate = 1.5 ml min^{-1}. Conductivity detection.

bonded stationary phases. Two observations can be made: first, the opposite variation of efficiency with granulometry in the reversed-phase (RP) and the ion-pair (IP) systems (N = 55 440 at a 7 μm particle size and 95 920 at 3 μm in the RP system; N = 27 150 at 7 μm and 22 100 at 3 μm in the IP system); and second, the decrease in efficiency in the ion-pair system (for the same k' value, N = 55 440 in the RP system and 27 150 in the IP system at 7 μm; N = 95 920 in the RP system and 22 100 in the IP system at 3 μm). This decrease in efficiency can be explained by the numerous equilibria involved in the retention process and by the small velocity of mass transfer in ion-pair chromatography.

In conclusion, a realistic test for an ion-pair chromatographic system needs to be devised. Moreover, one must remember that the arguments applied by chromatographists to reversed-phased systems are not always transposable to ion-pair chromatography.

CONCLUSION

Non-buffered ion-pair reverse-phase liquid chromatography was found to be a successful system for the determination of inorganic anions. The equilibration process is longer with octadecyl- than with octyl-bonded stationary phases, and requires particular attention with octyl-bonded stationary phases. The selectivity depends on the alkyl-bonded stationary phase. For example, Figs. 4 and 5 show that for the determination of carbonate, an octyl-bonded stationary phase must be selected.

Finally, we cannot choose between the C_8 and C_{18} systems, and we consider that the efficiency of an ion-pair system must be measured by tests different from those used with classical reversed-phase systems.

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EFFECT OF HYDROTHERMAL TREATMENT IN THE PRESENCE OF SALTS ON THE CHROMATOGRAPHIC PROPERTIES OF SILICA GEL

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SUMMARY

The effects of hydrothermal treatment of silica gel in the presence of some inorganic salts on the chromatographic properties of the product have been studied. In addition to the specific surface area and the mean pore diameter, the selectivity and retention properties of the treated silica gel as well as some hydrodynamic properties are also changed. The extent of these changes depended both on the duration of the hydrothermal treatment and on the nature of the salt added. The treatment with phosphate ion solution produced a decrease in the specific pore volume and in the apparent specific weight of the silica gel skeleton, along with significant changes in its selectivity.

INTRODUCTION

The hydrothermal treatment of silica gel[1,2] usually results in the modification of its specific surface area and/or the mean pore diameter. It has been stated in various contexts that the chromatographic properties of silica gel[3–10] and/or other kinds of gel behaviour[11] depend on the so-called history of the particular gel, *e.g.*, the method of its production[10] and the influence of substances with which it comes into contact during routine application. It must therefore be assumed that the influence of inorganic salts, which can alter the rate of hydrothermal treatment, will also be manifested in the properties of the resulting product[12].

Sodium and potassium salts of hydrofluoric, carbonic, boric and orthophosphoric acids were chosen as the inorganic salts. After the hydrothermal treatment, sodium and/or potassium cations, which react with sufficiently acidic surface silanols by ion exchange[13], were washed out with dilute hydrochloric acid. The decrease in the specific surface area of the silica gel was regarded as a measure of the hydrothermal treatment of the starting irregular high-surface-area silica gel. The effects of the change in specific surface area or of the anion used for the hydrothermal treat-

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ment on the following characteristics were measured: specific pore volume, mean pore diameter, apparent specific weight of the silica gel skeleton, retention and selectivity properties of the silica gel treated and the hydrodynamic characteristics of the bed. The validity of the selected measurements and tests was verified on several randomly chosen samples of commercial silica gels.

EXPERIMENTAL

Apparatus and materials

The starting material for hydrothermal treatment was a coarser fraction (d_p = 15 μm) of the commercial irregular silica gel Silasorb (Lachema, Brno, Czechoslovakia). A fraction with mean particle diameter 7.5 μm, selected from the same batch, was used as reference sample.

The silica gel was treated with a solution of fluoride, borate, carbonate or orthophosphate under atmospheric pressure at 373 K. The duration of hydrothermal treatment ranged from 20 to 60 min depending on the salt used and on the decrease in specific surface desired. The silica gel was then washed with water, until free from the anion of the salt used and then with diluted hydrochloric acid. Finally it was washed with distilled water until free from chloride.

The system of measurements and tests used for evaluation of the silica gel treated hydrothermally was verified on the initial Silasorb sample and on the following commercial silica gels selected at random: Nucleosil 100-10 (Macherey-Nagel, F.R:G.), Hypersil (Shandon Southern Products, U.K.), Separon SI-VSK (Laboratory Instruments, Prague, Czechoslovakia), LiChrospher Si 100 (E. Merck, F.R.G.) and LiChrosorb Si 60 (E. Merck).

The chromatographic apparatus was described previously[14] and the set-up used for the packing of unpolished stainless-steel columns, 200 × 6 mm, is shown in Fig. 1. The packing procedure, uniform for all silica gels listed in Tables I and V, was a variant of the high-pressure viscosity-filtration technique[15]. The suspension was prepared by stirring of silica gel (7 ml) ultrasonically in 1.5 volumes (10.5 ml) of cyclohexanol–methanol (95:5). After the apparatus had been assembled (Fig. 1), the column outlet (Fig. 2a) was plugged and both the column (9) and the connecting piece (8) were filled with chloroform. The freshly prepared suspension was introduced into the reservoir (7). Then the chloroform was let out of the column, the suspension was overlaid with 1–2 ml of suspending liquid and both the reservoir (7) and the reservoir head (6) were filled with the pressure liquid, a technical mixture of hydrocarbons free from polar impurities. The pressure reservoir (3) was pressurized to the packing pressure of 25 MPa and this pressure was then suddenly applied to the suspension reservoir and during packing was maintained within ±0.3 MPa. The bed was stabilized by washing with the pressure liquid at the maximum output of the pump (about 1.5 l/h) for 1 h. After washing the bed with 250 ml of methanol–denatured ethanol, the column inlet was adjusted for sampling by use of microsyringes[16] (Fig. 2a).

The basic component of the mobile phases was *n*-hexane (Soyuzkhimexport, U.S.S.R.), to which ethanol (UV-grade) was added at concentrations of 0.7 or 0.05% (v/v). For measurement of the streaming current responses from the sorbent beds[17], butanol p.a. was employed instead of ethanol (both from Lachema) at a concentra-

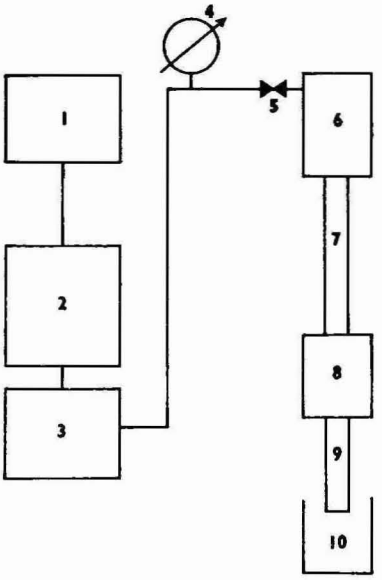

Fig. 1. Diagram of the packing apparatus. 1 = Low-pressure reservoir of pressure liquids; 2 = dual-head membrane pump, Orlita type; 3 = high-pressure reservoir of pressure liquid (volume 2 l); 4 = measurement of pressure; 5 = high-pressure valve; 6 = head of suspension reservoir; 7 = suspension reservoir (500 × 6 mm); 8 = connecting piece; 9 = packed column; 10 = waste.

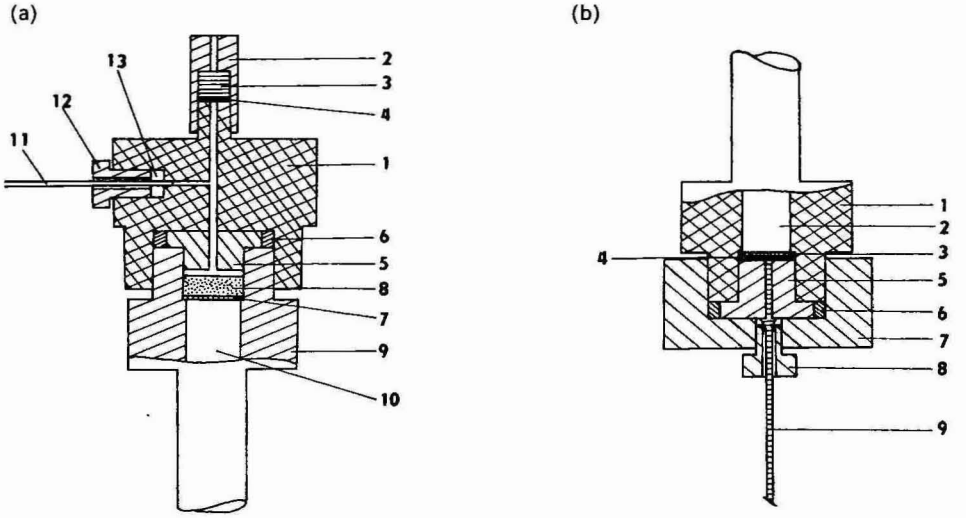

Fig. 2. Detail of the inlet (a) and outlet (b) of the column. (a) 1 = Injector body; 2 = needle guide; 3 = soft silicone rubber; 4 = hard hydrocarbon-resistant rubber; 5 = PTFE inlet seal; 6 = supporting ring; 7 = filter-paper; 8 = glass beds; 9 = column; 10 = silica gel bed; 11 = inlet capillary; 12 = screw; 13 = PTFE seal. (b) 1 = Column; 2 = silica gel bed; 3 = filter-paper; 4 = stainless-steel cloth; 5 = PTFE outlet seal; 6 = supporting ring; 7 = nut; 8 = screw; 9 = outlet capillary.

tion of 5% (v/v). Before use, the hexane was cleaned and dried by passing through an alumina bed. The alumina was activated for 8 h at 623 K. The alcohols were added to the mobile phase without drying. The dead volumes were measured using *n*-octane p.a. (Loba-Chemie, Austria) as a solute. The streaming current responses were generated by butanol injections. Nitrobenzene (NB), *m*-dinitrobenzene (DNB) and 2,4,6-trinitrotoluene (TNT) dissolved in the mobile phase with the addition of benzene (B) were used for the evaluation of column efficiency. Two other model mixtures were employed for the retention and selectivity evaluation. The mixture of oxygen-containing compounds consisted of anisole (AN), acetophenone (AP), *o*-hydroxyacetophenone (HAP), dimethyl phthalate (DMP) and 3-nitroacetophenone (NAP). The mixture of nitrogen-containing compounds was made up of diphenylamine (DPA), 2-bromopyridine (BP) and carbazole (CB). These model mixtures were dissolved in the mobile phase with the addition of ethanol. The samples were injected into the mobile phase stream with syringes.

The specific surface areas of the adsorbents were measured by the dynamic method of nitrogen heat desorption[18] using alumina as the standard (S = 203 m^2/g). The specific pore volumes, mean pore diameters, apparent specific weights of the silica gel skeleton, exclusion bed volumes and mobile phase volumes inside the sorbent particles were calculated from the retention volumes of the streaming current responses generated by butanol injections[14].

Measurement procedure and evaluation of results

Each column was weighed prior to packing. After packing, the column was equilibrated with a mobile phase containing 0.7% of ethanol. Its dead volume, the dependence of its efficiency on the mobile phase flow-rate and the retention data for all model solutes were then measured. Finally the column was washed with 250 ml of dried, freshly distilled acetone and equilibrated with a mobile phase containing 0.05% of ethanol. The course of equilibration was always monitored by injecting the nitro derivatives used for measuring the column efficiency. The column dead volume and the retention characteristics were again measured. After washing the column with hexane–butanol (95:5), the exclusion column volume and the elution volume of the butanol vacant response[14] were measured using the streaming current detection. The column was then dried in a nitrogen stream at 160–190°C and weighed.

The reduced height equivalent to a theoretical plate, h, and the reduced linear velocity of the mobile phase, v, were calculated in the usual way. The retention time of the non-retained mobile phase component[19], measured by injection of *n*-octane[14], was considered as the dead retention time. The diffusion coefficients of solutes in the mobile phase, D_m, were determined from the Wilke–Chang equation[20]. The capacity factors were calculated from the retention times at the peaks and then normalized to 1000 m^2 of the sorbing surface. During normalization, the measured capacity factors were multiplied by the correction factor $10^3/S_t$, where S_t is the total surface area of the silica gel in the column. The values of S_t are given in Tables IV and VIII.

The volume of liquid phase in the interparticle space of the silica gel bed (exclusion volume) is equal to the elution volume of the exclusion streaming current response, V_E[14,21]. The volume of the liquid in the pores of the silica gel bed, V_l, is equal to the difference between the elution volumes of the butanol vacant response[14],

V_V, and of the exclusion streaming current response[14]:

$$V_I = V_V - V_E \tag{1}$$

For the specific pore volume, V_p, the following relationship holds:

$$V_p = \frac{1}{G}(V_V - V_E) \tag{2}$$

The weight of the silica gel in the column, G, was determined from the weight of the empty column and the weight of the packed column after evaporation of the hexane–butanol mobile phase. The mean pore diameter, D, was calculated from[22]

$$D = \frac{4\,V_p}{S} \cdot 10^{-3} \tag{3}$$

using the measured specific surface areas, S. After substituting V_p (cm^3/g) and S (m^2/g) in eqn. 3, the mean pore diameter is obtained in nm[23]. The apparent specific weight of the silica gel skeleton, ρ, was calculated from the relationship[14]

$$\rho = \frac{G}{V_0 - V_V} \tag{4}$$

where V_0 is the volume of the empty column. Columns with $V_0 = 5.65$ ml were used.

Choice of tests

For the measurements, columns of 200 mm in length and of 6 mm in diameter were chosen. Larger columns are convenient for measuring the elution volumes of streaming current responses, from which some characteristics of hydrothermally treated silica gels were calculated. Furthermore, when measuring the efficiency of such columns, the apparatus band broadening is less important. The contribution to band broadening caused by dispersions in the connections and the detector cell of the Variscan spectrophotometer (Fig. 3) was measured by the method described[24]. Its extrapolated value, used for the correction of variances read from the chromatograms, was $140 \cdot 10^{-6}$ cm^6. The correction was negligible for packings with $d_p = 10$ μm. In the case of finer grain sizes it was significant only at mobile phase velocities close to the optimum.

The test mixtures were chosen so as to obtain symmetrical peaks in the measurements involving the mobile phase containing 0.7% of ethanol. Solutes with small molecules were preferred in order to avoid any distortion of the retention measurement by steric exclusion effects[14,25].

A mixture of nitroderivatives was chosen for measuring the efficiency. All its components have groups of equal polarity which interact with the stationary phase primarily by dipole interaction[26,27]. Their peaks can therefore be expected to be symmetrical on all samples of silica gels, even with a lower content of ethanol in the mobile phase. The oxygen-containing solutes were used to compare the differences in selective interaction attributed to surface silanols. Therefore, they were chosen so

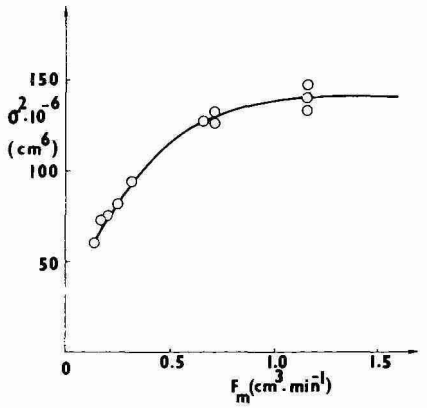

Fig. 3. Flow dependence of the band broadening caused by the Variscan spectrophotometer.

as to contain various oxyen functional groups and their combinations. The nitrogen-containing compounds were used chiefly for evaluation of the differences in the acidity of the surfaces[28,29].

The loss in column efficiency at linear mobile phase velocities exceeding the optimum value is reflected in the magnitude of the C term in the Van Deemter equation. In view of the considerable internal diameter of the columns used (6 mm), however, it was not possible to measure the flow dependence of the column efficiency over a sufficiently wide range of linear mobile phase velocities, necessary for an accurate determination of this constant. Therefore another criterion was chosen to express the loss in column efficiency: the increase in the reduced height equivalent to a theoretical plate, Δh, induced by an increase in the reduced mobile phase velocity, v, to three times that of the reduced optimum velocity of the mobile phase, v_{opt}. This criterion is sufficient for a mutual comparison of the packings, and, moreover, clearly illustrates the changes in column efficiency in the range of flow-rates of most practical interest.

The specific surface areas of the silica gels, and in the case of commercial preparations also their weights in the bed, exhibited significant differences (Tables I, IV and V). A direct comparison of the measured capacity factors would give a distorted picture of the sorption properties of the surfaces of the silica gels studied. Therefore, the measured capacity factors were normalized to an equal area of the sorbent surface, 1000 m^2.

The purpose of all the tests chosen was to express the differences in retention properties, selectivity properties and the geometrical characteristics of silica gels, as well as in the efficiency of their beds, caused by treatment of the silica gels with solutions of different salts, or with a single salt for various durations. It was therefore necessary to verify the effectiveness of the whole series of tests. Silica gels from different manufacturers, differing in shape and declared geometrical characteristics (specific surface area, specific pore volume, mean pore diameter), were chosen. Obviously, at least some of them must have been produced by different technological procedures.

The results which characterize the selected commercial silica gels and their beds are presented in Tables I–IV. As is seen, differences in the measured characteristics,

TABLE I

CHARACTERISTICS OF COMMERCIAL PACKINGS

sph = Spherical, irr = irregular.

Sorbent	*Particle shape*	d_p* *(μm)*	*S (m^2/g)*		*V_p (cm^3/g)*		*D (nm)*		*ρ (g/cm^3)***
			*cert.***	*exptl.*	*cert.***	*exptl.*	*cert.***	*exptl.*	
Nucleosil	sph	10	300	380	1.0	0.7	10	7.4	2.3
Hypersil	sph	5	200	200	–	0.7	10	14	2.4
Separon	sph	10	450	470	1.5	1.5	–	13	2.2
LiChrospher	sph	10	250	540	1.2	1.2	10	10	1.8
LiChrosorb	irr	10	550	590	–	0.7	6	4.8	1.8
			400		0.8		6		
Silasorb	irr	7.5	500	520	0.7–0.8	0.6	–	4.3	1.8

* Declared mean particle diameter.

** Declared values for Nucleosil, Hypersil and LiChrospher from ref. 31; for LiChrosorb the first values are from ref. 30, the second from ref. 10. Declared data for Separon and Silasorb are those given by the suppliers.

*** Apparent specific weight of the silica gel skeleton.

expected at least in some of the preparations, were found to occur between all the samples. The suitability of the technique used for measuring the geometrical characteristics of the silica gels had been demonstrated previously[14].

RESULTS

Properties of the silica gels

The influence of the duration of the hydrothermal treatment of the silica gel by an aqueous solution containing only one anion on the chromatographically important properties of the adsorbents and their beds was studied with a series of samples designated as Q15. The effect of the nature of the anion used in the hydro-

TABLE II

RELATIVE RETENTIONS ON COMMERCIAL SILICA GELS ELUTED BY HEXANE MODERATED WITH 0.7% ETHANOL

Figures in parentheses indicate capacity factors normalized to 1000 m^2 of the sorbent surface used as reference for the given group of solutes.

Sorbent	*Nitro derivatives*			*Nitrogen-containing compounds*			*Oxygen-containing compounds*				
	NB	*TNT*	*DNB*	*DPA*	*BP*	*CB*	*AN*	*HAP*	*AP*	*DMP*	*NAP*
Nucleosil	(0.27)	2.7	3.9	(0.32)	2.3	5.5	0.14	0.56	(0.44)	2.6	3.0
Hypersil	(0.45)	2.5	3.7	(0.48)	2.7	5.9	0.17	0.60	(0.75)	2.5	3.9
Separon	(0.40)	2.5	3.6	(0.44)	2.6	5.0	0.11	0.55	(0.62)	2.6	3.0
LiChrospher	(0.30)	2.7	4.4	(0.41)	2.4	5.8	0.12	0.54	(0.54)	2.7	3.2
LiChrosorb	(0.27)	2.6	4.8	(0.41)	2.8	5.9	0.10	0.53	(0.54)	2.6	3.8
Silasorb	(0.28)	2.8	4.8	(0.35)	2.7	4.1	0.098	0.54	(0.52)	2.7	3.0

TABLE III

RELATIVE RETENTIONS ON COMMERCIAL SILICA GELS ELUTED BY HEXANE MODERATED WITH 0.05% ETHANOL

Figures in parentheses indicate capacity factors normalized to 1000 m^2 of the sorbent surface used as reference for the given group of solutes.

Sorbent	*Nitro derivatives*			*Nitrogen-containing compounds*			*Oxygen-containing compounds*				
	NB	*TNT*	*DNB*	*DPA*	*BP*	*CB*	*AN*	*HAP*	*AP*	*DMP*	*NAP*
Nucleosil	(1.1)	8.8	7.8	(1.5)	5.7	3.7	0.082	0.43	(6.7)	3.5	>3.8
Hypersil	(1.9)	8.3	7.3	(2.2)	7.6	3.8	0.078	0.41	(8.6)	4.5	6.6
Separon	(1.7)	9.1	7.5	(2.2)	5.8	4.1	0.075	0.48	(9.6)	3.9	6.3
LiChrospher	(1.7)	13.0	9.7	(2.9)	4.5	2.5	0.085	0.40	(6.8)	2.6	6.5
LiChrosorb	(1.0)	8.6	8.2	(1.4)	5.6	4.4	0.074	0.39	(4.8)	4.2	>6.5
Silasorb	(1.0)	8.2	7.7	(1.4)	5.3	4.5	0.075	0.39	(4.5)	4.1	>7.9

TABLE IV

CHARACTERISTICS OF COMMERCIAL SILICA GEL BEDS

G = Weight of sorbent in the 200 × 6 mm bed; S_t = total sorbent surface in bed, $S_t = SG$; V_E = exclusion volume of bed; V_I = volume of mobile phase inside the particles of the packing; h_{opt} = reduced height equivalent to a theoretical plate measured at the optimum reduced linear velocity of the mobile phase, v_{opt}; Δh = increase in h caused by increasing v from v_{opt} to $3v_{opt}$.

Sorbent	G (g)	S_t (m^2)	V_E (ml)	V_I (ml)	*Benzene*			*m-Dinitrobenzene*		
					v_{opt}	h_{opt}	Δh	v_{opt}	h_{opt}	Δh
Nucleosil	3.16	1191	2.11	2.15	5.5	2.0	3.1	4.7	4.1	1.5
Hypersil	3.22	631	2.19	2.11	2.3	2.1	2.2	4.5	3.8	3.2
Separon	1.97	916	2.07	2.88	5.0	2.0	3.5	5.0	2.4	2.6
LiChrospher	2.03	1089	2.13	2.38	4.5	1.8	4.7	5.0	2.2	2.4
LiChrosorb	2.74	1622	2.33	1.88	5.2	2.5	1.4	4.3	3.1	1.8
Silasorb	3.02	1570	2.26	1.70	4.0	2.0	1.3	7.5	3.3	1.8

TABLE V

CHARACTERISTICS OF HYDROTHERMALLY TREATED SILICA GELS

S = Specific surface area; V_p = specific pore volume; D = mean pore diameter; ρ = apparent specific weight of the silica gel skeleton.

Sorbent	*Modifying ion*	S (m^2/g)	V_p (cm^3/g)	D (nm)	ρ (g/cm^3)
Silasorb	–	520	0.6	4.3	1.8
Q15-2AW	F^-	450	0.6	5.4	2.0
Q15-6AW	F^-	290	0.6	8.3	1.9
Q15-10AW	F^-	190	0.6	13	2.0
P129-3AW	F^-	290	0.6	8.3	2.0
P129-5AW	$H_2BO_3^-$	290	0.6	8.3	2.0
P129-7AW	CO_3^{2-}	290	0.6	8.3	1.9
P129-8AW	PO_4^{3-}	290	0.5	8.3	1.6

thermal treatment was investigated in the sample series P129. When treating the silica gels with different anions, the duration of treatment was chosen so as to attain approximately the same value of the specific surface area.

It follows from Table V that during the hydrothermal modification of the specific surface area of irregular silica gel in aqueous solutions of inorganic salts, the specific pore volume varied only when phosphate was used for the modification. The increase in the apparent specific weight of the silica gel skeleton, ρ, following the hydrothermal treatment with fluoride, carbonate and borate solutions can most readily be explained by the extinction of micropores inaccessible to the molecules of the mobile phase[31]. When measuring the quantity ρ using the method described[14], their volume is included in the volume of the bed occupied by the silica gel skeleton and the calculated apparent specific weight, ρ, must therefore, be lower than the real one. The cause of the decrease ρ in the case of the silica gel treated with the aqueous solution of phosphate buffer, pH $\approx$ 7, cannot be stated unambiguously on the basis of the tests performed. However, in view of the differences in retention properties of the silica gel washed with hydrochloric acid and of the same sample washed additionally with phosphate buffer, as described[11], and on the basis of some other yet unpublished findings, we assume that a chemical reaction takes place between the phosphate ions and the surface of the silica gel. This is in accord with the formation of a highly stable complex of phosphoric acid with surface silanol groups[32]. When other strong inorganic acids are added, the complex is produced even at room temperature[32]. At higher temperatures the silica gel dissolves in excess of orthophosphoric acid, even without adding another acid[33]. Depending on the reaction conditions, silicon phosphates or pyrophosphates of different compositions are produced.

The differences found in both the absolute and relative retentions are distinctly smaller in the mobile phase containing 0.7% ethanol than in the mobile phase containing lower alcohol concentrations (Tables VI, VII). Similar behaviour was found on the commercial silica gels (Tables II, III). If the alcohol content in the mobile phase is reduced from 0.7 to 0.05%, but the silica gel bed is not dried with acetone, then the absolute as well as the relative retentions of the solutes remain almost un-

TABLE VI

RELATIVE RETENTIONS ON HYDROTHERMALLY TREATED SILASORBS ELUTED BY HEXANE MODERATED WITH 0.7% ETHANOL

Figures in parentheses indicate capacity factors normalized to 1000 m^2 of the sorbent surface used as reference for the given group of solutes.

Sorbent	*Nitro derivatives*			*Nitrogen-containing compounds*			*Oxygen-containing compounds*				
	NB	*TNT*	*DNB*	*DPA*	*BP*	*CB*	*AN*	*HAP*	*AP*	*DMP*	*NAP*
Silasorb	(0.28)	2.8	4.8	(0.35)	2.7	4.1	0.098	0.54	(0.52)	2.7	3.0
Q15-2AW	(0.37)	2.4	3.4	(0.40)	2.9	4.2	0.14	0.57	(0.56)	2.6	3.0
Q15-6AW	(0.43)	2.4	3.3	(0.50)	2.4	4.0	0.12	0.56	(0.62)	2.6	3.0
Q15-10AW	(0.42)	2.7	5.0	(0.56)	2.5	5.9	0.20	0.59	(0.82)	2.4	3.2
P129-3AW	(0.39)	2.7	5.7	(0.50)	2.8	6.1	0.12	0.54	(0.68)	2.6	3.8
P129-5AW	(0.40)	2.7	5.5	(0.53)	3.2	6.0	0.13	0.51	(0.70)	2.5	3.8
P129-7AW	(0.40)	2.7	5.4	(0.54)	2.7	6.0	0.11	0.54	(0.70)	2.6	3.7
P129-8AW	(0.43)	2.6	5.4	(0.60)	2.8	6.2	0.15	0.57	(0.79)	2.5	3.8

TABLE VII

RELATIVE RETENTIONS ON HYDROTHERMALLY TREATED SILASORBS ELUTED BY HEXANE MODERATED WITH 0.05% ETHANOL

Figures in parentheses indicate capacity factors normalized to 1000 m^2 of the sorbent surface used as reference for the given group of solutes.

Sorbent	*Nitro derivatives*			*Nitrogen-containing compounds*			*Oxygen-containing compounds*				
	NB	*TNT*	*DNB*	*DPA*	*BP*	*CB*	*AN*	*HAP*	*AP*	*DMP*	*NAP*
Silasorb	(1.0)	8.2	7.7	(1.4)	5.3	4.5	0.075	0.39	(4.5)	4.1	7.9
Q15-2AW	(1.1)	8.6	7.9	(0.88)	5.4	4.4	0.076	0.39	(5.7)	3.9	6.3
Q15-6AW	(1.3)	9.2	8.3	(1.9)	5.1	4.3	0.079	0.38	(5.6)	4.0	6.5
Q15-10AW	(1.2)	11.0	9.9	(2.2)	5.0	4.5	0.084	0.40	(6.8)	4.0	6.2
P129-3AW	(1.3)	8.2	8.2	(2.0)	5.3	4.7	0.093	0.40	(5.8)	4.0	6.2
P129-5AW	(1.3)	8.2	7.8	(2.5)	3.1	3.1	0.094	0.45	(7.0)	3.5	6.0
P129-7AW	(1.3)	8.5	7.8	(2.1)	4.2	5.4	0.062	0.38	(5.9)	4.0	4.7
P129-8AW	(1.2)	7.3	7.2	(1.6)	5.0	4.2	0.096	0.41	(4.1)	1.9	2.7

changed. This is accounted for by the deactivation of the silica gel surface by water adsorbed from the mobile phase containing 0.7% ethanol. Polar acetone, which has an unlimited ability to dissolve water, causes a very rapid drying of the column. In the mobile phase containing 0.05% ethanol, high retentions are established after washing the column with about fifty times the dead volume and remain constant during the whole measurement.

Properties of the beds

For the silica gel with a declared mean particle size of 15 μm, the hydrothermal treatment under different conditions did not produce any measurable differences in the volume of mobile phase in the interparticle space. Likewise, there was hardly any change in the volume of liquid phase inside the sorbent particles. Only on application of the neutral phosphate buffer was it reduced by 0.4 ml compared with the other beds, although the amount of sorbent in the column was diminished by only 0.1–0.2 g. This decrease in V_i was manifested also in a smaller specific pore volume of the resulting product (Table V). The surface reaction of phosphate ions with the silica gel, considered earlier in connection with the decrease in the apparent specific weight of the skeleton of this sample, might again be responsible for the above behaviour.

The small differences in the volume of liquid phase in the interparticle space of the beds of hydrothermally treated samples, as well as the high reproducibility of the amounts of differently treated silica gels in the bed with dimensions of 200 × 6 mm are, among other things, determined by an effectiveness and reproducibility of the column-packing technique. Nevertheless, the h values at the minimum of Van Deemter's curve are surprisingly low. The mean particle diameter was not determined after the hydrothermal treatment. Therefore, we cannot say whether the observed high bed efficiencies are produced by a relatively narrow distribution of the particles of the coarser silica gel, or by a change in the mean particle diameter due to the hydrothermal treatment. In the treated samples, $h_{opt} \approx 2$ for $d_p = 12.5$ μm. Even in that case, however, the Δh values measured would remain lower than for the starting silica gel (Table VIII).

TABLE VIII

CHARACTERISTICS OF BEDS OF THE HYDROTHERMALLY TREATED SILASORBS

Details as in Table IV.

Sorbent	*G* (g)	S_t (m^2)	V_E (ml)	V_t (ml)	*Benzene*			*m-Dinitrobenzene*		
					v_{opt}	h_{opt}	Δh	v_{opt}	h_{opt}	Δh
Silasorb	3.02	1570	2.26	1.70	4.0	2.0	1.3	7.5	3.3	1.8
Q15-2AW	2.79	1250	2.73	1.50	7.0	1.7	0.9	6.5	2.0	1.2
Q15-6AW	2.72	792	2.68	1.57	4.7	1.4	0.8	4.5	1.6	1.2
Q15-10AW	2.70	510	2.67	1.63	6.3	1.7	0.9	3.5	2.0	0.8
P129-3AW	2.69	769	2.65	1.67	4.8	1.6	0.4	6.0	2.4	1.2
P129-5AW	2.70	791	2.79	1.50	5.0	1.6	0.8	6.2	2.4	1.6
P129-7AW	2.68	766	2.67	1.59	5.5	1.8	0.5	7.0	2.7	1.6
P129-8AW	2.59	761	2.72	1.26	5.5	1.4	1.2	5.1	2.0	1.6

The measurements of efficiency indicate that the hydrodynamic properties of the column as characterized by h_{opt} and Δh (and/or their sum) were improved upon hydrothermal treatment of irregular silica gel. The lower the specific surface area of the sorbent after the treatment with fluoride solution, the better were the properties of its bed in terms of the magnitude of Δh. When the specific surface area was normalized, fluoride ions were the most effective in decreasing Δh. The rather poor quality of the initial preparation cannot be regarded as causing a smaller decrease in the efficiency of the hydrothermally treated silica gel beds. As regards the maximum possible bed efficiency, h_{opt}, and the loss in bed efficiency, Δh, the Silasorb used was equivalent to the LiChrosorb sample, and both these irregular silica gels were at least comparable with the spherical silica gels (Fig. 4, Table IV).

Lower efficiency losses were also found for two laboratory samples of spherical silica gel, examined under other circumstances (Fig. 5). Noteworthy is the fact that

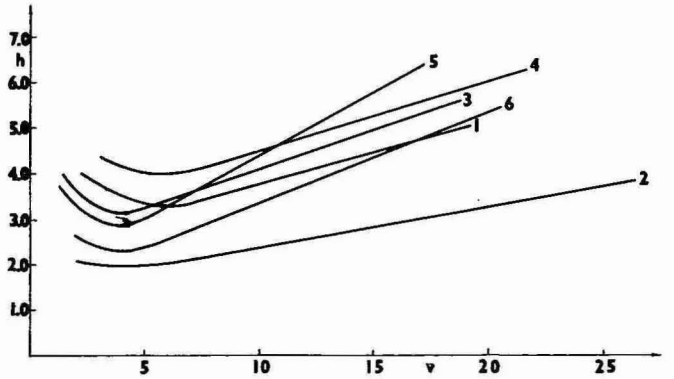

Fig. 4. Flow dependences of the bed efficiency of some commercial silica gels and Silasorb hydrothermally treated with fluoride solution. Solute = *m*-dinitrobenzene. Curves: 1, Silasorb before hydrothermal treatment; 2, Silasorb after hydrothermal treatment; 3, LiChrosorb; 4, Nucleosil; 5, Hypersil; 6, LiChrospher.

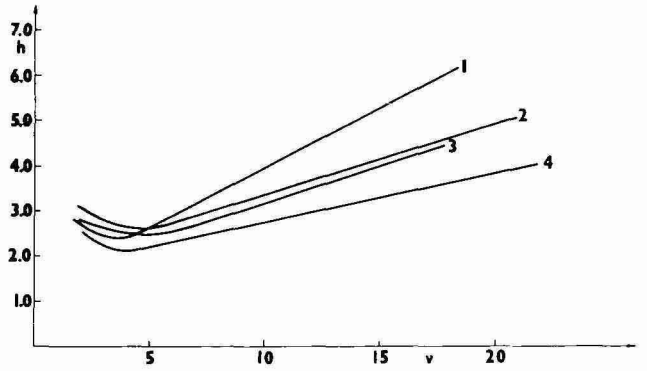

Fig. 5. Effect of hydrothermal treatment of laboratory samples of spherical gels on the bed efficiency. Solute = *m*-dinitrobenzene. Curves: 1, sample A before hydrothermal treatment (specific surface area, S = 510 m^2/g; specific pore volume, V_P = 1.0 cm^3/g; mean pore diameter, D = 8.1 nm); 2, sample A after hydrothermal treatment (S = 280 m^2/g, V_P = 1.2 cm^3/g, D = 17 nm); 3, sample B before hydrothermal treatment (S = 300 m^2/g, V_P = 1.8 cm^3/g, D = 24 nm); 4, sample B after hydrothermal treatment (S = 250 m^2/g, V_P = 2.0 cm^3/g, D = 32 nm).

after the treatment with the fluoride ion solution, Δh decreased even for a sample with an initial specific surface area of 300 m^2/g and a pore volume of 1.8 cm^3/g. According to the commonly used relationship 3, the mean pore diameter corresponding to these values is 24 nm.

DISCUSSION

At the moment no integrated set of generally recognized and accepted quantitative criteria is available for a comprehensive evaluation of silica gel as a chromatographic material. The limiting bed efficiency for a non-retained solute under ideal conditions, $h_{opt} \approx 2$ for v lying in the range 3–6, is the only quantitatively determined and generally accepted criterion. There are recommendations concerning the loss in bed efficiency with increasing linear velocity of the mobile phase, expressed by the magnitude of the C term in Van Deemter's equation (C of the order of 10^{-3})[34], of the mean pore diameter ($D \geqslant 6$ nm)[10] or of the specific pore volume (V_P not exceeding 1 cm^3/g for conventional silica gels)[35]. However, no criteria are available for the evaluation of the retention and selectivity properties of silica gels.

The set of measurements used in the present study was chosen so as to provide information, with minimum expenditure of time and effort, about those characteristics of silica gels or their beds which can be regarded, on the basis of theoretical studies, practical experience or traditional views, as chromatographically important. Chromatographic measurements were preferred. The numerical data summarized in this study are only one means of evaluating the occurrence and extent of changes caused by the hydrothermal treatment of silica gels with aqueous solutions of different salts. They are not meant to be instrumental in drawing conclusions concerning the suitability of the silica gels for practical uses or their quality.

The method of measuring efficiency applied is based on that proposed by Bristow and Knox[34]. The results (Table VIII) show that the hydrothermal treatment of

Silasorb with salt solutions always improved the attainable bed efficiency for the sorbed solutes. The extent of improvement depended on the particular salt anion. Silica gel can thus be treated even after sieving. Of note are the decrease in Δh caused by the hydrothermal treatment and the dependence of the magnitude of this decrease on the particular salt anion. Although the decrease in Δh was found also with two laboratory samples of spherical silica gel (Fig. 5), a closer examination of the effect of the hydrothermal treatment on the magnitude of the C term in Van Deemter's equation is necessary before the hydrothermal treatment in the presence of salts may be declared as a process suited to improve the migration of the solutes inside the sorbent particles.

The data in Tables V and VIII seemingly imply that the decrease in Δh occurring with the beds of silica gels whose specific surface area was diminished by treatment in the fluoride solution is caused by an increase in the mean pore diameter. However, it is evident from the data summarized in Tables I and IV, as well as from the decrease in Δh for the spherical laboratory preparation initially having $D = 24$ nm (Fig. 5), that there is no straightforward relationship between D and Δh. Also, a greater mean pore diameter by itself is no guarantee of better hydrodynamic properties of the bed at higher linear velocities of the mobile phase. The measurements obtained show that it is possible, at the same reduced mobile phase velocities, that two silica gels having the same particle shape and the same mean pore diameter differ in the magnitude of Δh, or that the bed of material having smaller D is distinctly more effective. Mere differences in the magnitudes of the mean pore diameter cannot explain why both the irregular silica gels, LiChrosorb and Silasorb, exhibited a lower Δh than did some spherical silica gels. At the same time, the mean pore diameter found for the two irregular types of silica gel was below the 6-nm limit considered to be essential for high bed efficiency[10]. None of these facts can be satisfactorily explained only on the basis of the measurements taken and existing knowledge. It is obvious, however, that a modification of the surface, associated with the hydrothermal treatment in aqueous solutions of the salts, improves the conditions for solute migration inside the particles of the sorbent. The effects of the different anions might be connected with the mechanism of this modification, or with irreversible interactions of the anions present in the solution with the surface of the silica gel. The differences in selectivity properties of the silica gels treated with solutions of salts containing diverse anions seem to be in accord with the latter possibility.

As is seen, qualitative judgements concerning the conditions of solute transport in the particles of different silica gels, in the range of D values usually exhibited by chromatographic silica gels, also cannot be made from the magnitudes of the mean pore diameter (at least from the values obtained by the procedure[22,23] used in the present work).

It is commonly accepted that the structure of the silica gel surface changes during modification. Hence, upon hydrothermal treatment involving a certain salt solution, the relative retentions of the solutes would also be expected to vary to some extent with the decreasing specific surface area. However, there are surprising differences in retentions, and especially in relative retentions, the decrease in the specific surface being the same upon treatment with solutions of different salts (series P129). The relatively small differences between the samples treated with fluoride, carbonate or borate solutions might perhaps be explained as being due to differences in the

rates of decrease of the silica gel specific surface area in the solutions of these salts. However, the substantially more marked differences found with the sample treated with the phosphate medium cannot be so explained. As with specific pore volume and specific skeleton weight, the retention also suggests that, during the hydrothermal treatment, phosphate ions react with silica gel. This is in agreement with some previous measurements[11].

The duration of hydrothermal treatment of the tested samples varied in the order of tens of minutes. The kinetics of the reaction of phosphate ion with the silica gel is unknown. However, the reaction of silica gel with phosphate ions may take place also at room temperature, and is expected[32] to increase in rate with increasing mobile phase acidity. Taking into consideration the proposed mechanism of degradation of silica gel in a phosphoric acid medium[32], it is probable that the phosphoric acid buffers not only alter, through their chemical action, the selectivity of the silica gel, but also unfavourable affect the lifetime of the chemically bound stationary phases.

As a whole, the measurements demonstrate that the hydrothermal treatment with salt solutions exerts a considerable influence on all the chromatographically important properties of silica gel. The extent of this influence depends not only on the decrease in the specific surface area, but also on the anion of the salt added.

The hydrothermal treatment invariably has a positive influence on the transport of solutes in the sorbent particles. As for the mobile phase linear velocities, of practical importance, the dependence of the bed efficiency on the linear velocity decreases. In this respect, fluoride appeared to be the most effective among the anions tested. The effect on the bed efficiency at the optimum mobile phase velocity probably depends on the mean pore diameter of the silica gel before the treatment. With the irregular Silasorb which possesses a small mean pore diameter (D = 4.3 nm), the bed efficiency was improved in all cases tested, but remained unchanged in the case of spherical gels having the initial D values greater than 10 nm.

The practical impact and importance of the changes in selectivity properties induced by the hydrothermal treatment cannot generally be evaluated on the basis of the present measurements. It is evident, however, that the interaction of silica gel with salt solutes during its production, especially at elevated temperatures, is one of the factors responsible for the familiar differences in selectivity properties of commercial silica gels. Further changes in the retention and selectivity properties may take place if aqueous or aqueous–organic solutions of salts are used as mobile phases.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ALKYLBIPHENYLS ON SILICA

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SUMMARY

The influence of the molecular structure of 58 alkylbiphenyls and related compounds on their retention behaviour on silica has been studied. The moisture control system was incorporated into a closed circuit to maintain the long-term equilibration of the water content between the mobile and the stationary phase. The retention data have been expressed in the form of logarithms of capacity factor k'. The retention of the measured compounds is influenced by the number of alkyl groups, their length, shape and arrangement.

Retention increases if two or more substituents are in *ortho*-positions on one of aromatic rings. On the other hand, decreased retention is observed if there is substitution in the 2,6,2′,6′ positions, *i.e.* in *ortho*-positions with respect to the link between two aromatic rings. If these positions are more highly substituted the angle between two aromatic rings is increased and the approach of biphenyl molecule to the adsorption surface is impeded.

INTRODUCTION

The relation between the molecular structure of some mono- and diaromatic hydrocarbons and their adsorptivity on silica and alumina has been studied by Popl *et al.*[1–3]. High-performance liquid chromatography (HPLC) enables the more precise study of the relationship between the structure and the retention behaviour. In this relation, the retention data of alkylbenzenes[4] and alkylnaphthalenes[5] were measured, with silica as the stationary phase and *n*-pentane as the mobile phase. The next stage in this project is to measure retention data for 58 alkylbiphenyls under the same conditions.

EXPERIMENTAL

Apparatus

A Varian 8500 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) with a syringe pump was used. Sample injection was performed by the stop-flow technique

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with a 10-μl syringe (Hamilton, Bonaduz, Switzerland) directly into the column, using a septumless injector. A UVM-4 multi-wavelength UV detector (Vývojové dílny ČSAV, Prague, Czechoslovakia) was operated at 254 nm. Chromatograms were recorded and retention times were measured with a 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.). A stainless-steel column (250 × 8 mm I.D.) with a tapered inlet (Varian), was filled using the slurry-packing technique with 7.5 μm irregularly shaped silica gel (Silasorb; Lachema, Brno, Czechoslovakia). The column and the lower part of injector were inserted in a water-jacket, kept at a constant temperature. The water content in the recycling mobile phase was maintained by a moisture control system (MCS) incorporated in closed circuit. The MCS was filled with 175 g of silica containing 0, 0.5 or 1% of water. The silica had been activated by heating for 4 h at 200°C. Deactivation was performed by addition of 0.5 or 1% of water, respectively.

Both the column and the MCS were kept at 25 ± 0.1°C. The MCS apparatus was described in detail previously[5]. Retention data were calculated on a HP-85 personal computer (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a 82905 B impact printer, a 82901 M dual disc drive and a 9872 A plotter. Visicalec software was used to prepare, compute, and print the table.

Chemicals

Of the series of 58 alkylbiphenyls used, some were synthesized[6–8] and the other 30 higher-molecular-range alkylbiphenyls were kindly given by Dr. E. A. Johnson of the National Institute for Biological Standards and Control (London, U.K.).

Mobile phase

n-Pentane (analytical reagent grade) was used as the mobile phase. Before use, it was distilled in glass and dried by percolating it over a silica gel column (Mikrosil, 100–150 μm; Lachema) that had been dried for 4 h at 200°C. The flow-rate of the mobile phase was 100 ml/h.

Procedure

Three or four injections were carried out for each sample. The samples were 10% solutions in isooctane. Before measurements, the column was stabilized by washing with fresh recycling mobile phase. The column activity was checked before the beginning of the measurement, then several times during the analysis and after the completion of the measurements, by injection of a test mixture of benzene, naphthalene and phenanthrene in isooctane. The dead volume of the column was determined by measuring the retention time of *n*-hexane.

RESULTS AND DISCUSSION

The values of retention times (t_x) and capacity factors (k') are given in Table I. Each value represents an average of three or four measurements of individual alkylbiphenyls. As in the case of alkylbenzenes[4], the range of retention data broadens with increasing number of carbon atoms. Polymethylbiphenyls with methyl groups in 4-, 3,4-, 3,4,3′,4′- and 3,4,5,3′,4′,5′-positions, respectively, have the largest retention times in the range of corresponding compounds with the same number of carbon

TABLE I

RETENTION DATA FOR ALKYLBIPHENYLS

t_R = Retention time (min); k' = capacity factor; $t_X = t_R - t_0$ = adjusted retention time (min).

Compound	*Abbreviation*	*Deactivation of silica (concentration of water in moisture control system)*					
		0%		*0.5%*		*1%*	
		t_X	k'	t_X	k'	t_X	k'
Biphenyl	BIP	29.97	5.59	27.12	4.96	26.11	4.74
2-Methylbiphenyl	2-M-B	32.77	6.20	29.03	5.38	28.14	5.18
3-Methylbiphenyl	3-M-B	33.42	6.35	29.62	5.51	27.51	5.05
4-Methylbiphenyl	4-M-B	34.03	6.48	30.84	5.78	28.04	5.16
2,3-Dimethylbiphenyl	2,3-DM-B	40.31	7.86	35.75	6.86	32.07	6.05
2,4-Dimethylbiphenyl	2,4-DM-B	37.85	7.32	33.35	6.33	30.56	5.72
2,5-Dimethylbiphenyl	2,5-DM-B	36.60	7.04	32.11	6.06	29.27	5.43
3,4-Dimethylbiphenyl	3,4-DM-B	43.68	8.60	38.19	7.39	33.55	6.37
3,5-Dimethylbiphenyl	3,5-DM-B	38.29	7.42	34.22	6.52	31.71	5.97
3,4′-Dimethylbiphenyl	3,4′-DM-B	41.19	8.05	35.49	6.80	31.14	5.84
2,4′-Dimethylbiphenyl	2,4′-DM-B	39.95	7.78	34.00	6.47	31.02	5.82
3,3′-Dimethylbiphenyl	3,3′-DM-B	33.61	6.39	30.54	5.71	29.00	5.37
4,4′-Dimethylbiphenyl	4,4′-DM-B	39.00	7.57	35.29	6.76	32.19	6.07
2,2′-Dimethylbiphenyl	2,2′-DM-B	34.13	6.50	31.00	5.81	28.46	5.25
2,6-Dimethylbiphenyl	2,6-DM-B	35.02	6.70	30.93	5.80	27.94	5.14
2,3′-Dimethylbiphenyl	2,3′-DM-B	35.73	6.85	31.25	5.87	28.19	5.20
2,6,2′-Trimethylbiphenyl	2,6,2′-TM-B	32.84	6.22	29.37	5.45	27.01	4.94
3,5,4′-Trimethylbiphenyl	3,5,4′-TM-B	38.03	7.36	36.43	7.01	34.20	6.52
2,3,2′,3′-Tetramethylbiphenyl	2,3,2′,3′-TM-B	42.92	8.43	40.38	7.87	38.38	7.44
2,4,2′,4′-Tetramethylbiphenyl	2,4,2′,4′-TM-B	40.46	7.89	37.02	7.14	36.68	7.06
2,5,2′,5′-Tetramethylbiphenyl	2,5,2′,5′-TM-B	37.28	7.19	34.68	6.62	32.62	6.17
2,6,2′,6′-Tetramethylbiphenyl	2,6,2′,6′-TM-B	32.14	6.06	27.43	5.03	24.59	4.40
3,4,3′,4′-Tetramethylbiphenyl	3,4,3′,4′-TM-B	58.74	11.91	54.17	10.91	50.65	10.13
3,5,3′,5′-Tetramethylbiphenyl	3,5,3′,5′-TM-B	44.99	8.89	41.87	8.20	39.35	7.65
2,4,6,2′,4′,6′-Hexamethylbiphenyl	2,4,6,2′,4′,6′-HM-B	40.58	7.92	35.19	6.73	31.40	5.90
3,4,5,3′,4′,5′-Hexamethylbiphenyl	3,4,5,3′,4′,5′-HM-B	75.16	15.52	71.73	14.76	68.73	14.11
2-Ethylbiphenyl	2-E-B	32.00	6.03	28.72	5.31	25.97	4.71
3-Ethylbiphenyl	3-E-B	32.06	6.05	28.76	5.32	26.73	4.87
4-Ethylbiphenyl	4-E-B	32.84	6.22	29.51	5.49	27.21	4.98
Biduryl	BIDU	37.60	7.26	32.39	6.12	30.34	5.67
Biisoduryl	BI-i-DU	59.10	11.99	54.35	10.95	52.12	10.45
o-Terphenyl	O-TERPH	93.12	19.47	87.27	18.18	76.99	15.92
m-Terphenyl	M-TERPH	86.12	17.93	77.42	16.02	69.65	14.31
p-Terphenyl	P-TERPH	88.08	18.36	80.07	16.60	73.41	15.13
2-*n*-Propylbiphenyl	2-nP-B	29.44	5.47	26.57	4.84	24.67	4.42
4-*n*-Propylbiphenyl	4-nP-B	27.51	5.05	24.54	4.39	22.89	4.03
2-Isopropylbiphenyl	2-iP-B	30.00	5.59	27.31	5.00	25.92	4.70

(Continued on p. 148)

TABLE I *(continued)*

Compound	*Abbreviation*	*Deactivation of silica (concentration of water in moisture control system)*					
		0%		*0.5%*		*1%*	
		t_X	k'	t_X	k'	t_X	k'
3-Isopropylbiphenyl	3-iP-B	30.82	5.77	27.88	5.13	25.33	4.57
4-Isopropylbiphenyl	4-iP-B	28.34	5.23	27.58	5.06	25.02	4.50
2-*n*-Butylbiphenyl	2-nB-B	27.21	4.98	24.36	4.35	22.69	3.99
4-*n*-Butylbiphenyl	4-nB-B	26.84	4.90	24.19	4.32	20.45	3.49
2-*tert.*-Butylbiphenyl	2-tB-B	27.95	5.14	24.58	4.40	22.56	3.96
3-*tert.*-Butylbiphenyl	3-tB-B	27.12	4.96	25.72	4.65	25.00	4.49
4-*tert.*-Butylbiphenyl	4-tB-B	27.53	5.05	25.54	4.61	24.82	4.45
3,4-Diisopropylbiphenyl	3,4-D-iP-B	29.66	5.52	25.67	4.64	23.00	4.05
3,4′-Diisopropylbiphenyl	3,4′-D-iP-B	29.19	5.42	25.33	4.57	23.44	4.15
3,5-Diisopropylbiphenyl	3,5-D-iP-B	27.01	4.94	22.57	3.96	19.17	3.21
3,3′-Diisopropylbiphenyl	3,3′-D-iP-B	27.50	5.04	25.01	4.50	23.30	4.12
2,2′-Di-*tert.*-butylbiphenyl	2,2′-D-tB-B	23.22	4.10	21.44	3.71	20.19	3.44
4,4′-Di-*tert.*-butylbiphenyl	4,4′-D-tB-B	24.13	4.30	22.62	3.97	21.47	3.72
Biphrehnithyl	BIPR	79.98	16.58	76.71	15.86	72.21	14.87
2-Isopropyl-5-methylbiphenyl	2-iP-5M-B	32.17	6.07	28.02	5.16	25.46	4.60
1,1-Diphenylethane	1,1-DPh-Et	30.22	5.64	27.73	5.09	25.78	4.67
1,2-Diphenylethane	1,2-DPh-Et	36.56	7.04	32.88	6.23	30.06	5.61
2-Methyldiphenylmethane	2-MDM	53.24	10.70	47.75	9.49	42.41	8.32
3-Methyldiphenylmethane	3-MDM	38.06	7.36	35.02	6.70	34.21	6.52
4-Methyldiphenylmethane	4-MDM	54.96	11.08	48.93	9.75	45.00	8.89
Cyclopropylmethane	CyP-Ph-M	16.18	2.56	14.27	2.14	12.98	1.85

atoms (see Fig. 1). The minimum retention times of the compounds with the same number of carbon atoms correspond to alkylbiphenyls where the *n*-alkyl chain is in position 2.

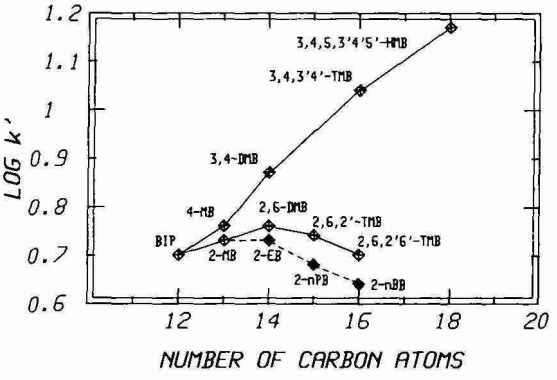

Fig. 1. Dependence of log k' of alkylbiphenyls on the number of carbon atoms. The dashed line represents data for alkylbiphenyls with an *n*-alkyl chain in 0.5% water in the MCS.

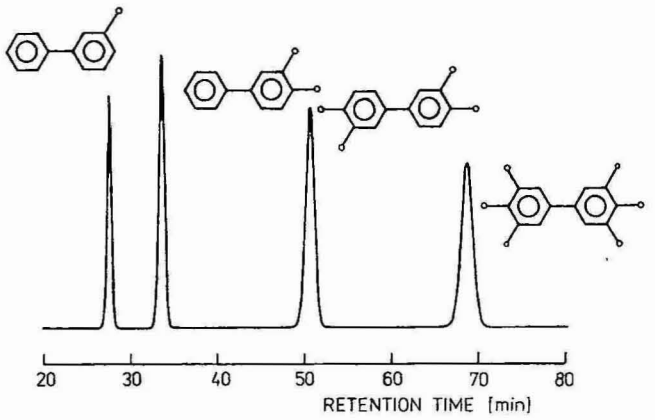

Fig. 2. Chromatogram of some methylbiphenyls with different numbers of methyl groups. Column, 250 × 8 mm I.D. packed with 7.5 μm silica gel; mobile phase, moisture-controlled *n*-pentane; 0.5% water in the MCS; flow-rate, 100 ml/h.

The main factors affecting the retention of alkylbiphenyls are: (i) the number of alkyl groups; (ii) the length of the alkyl groups; (iii) the arrangement of the alkyl groups (*ortho*-effect); (iv) the orientation of the aromatic rings; (v) the shape of the alkyl groups.

Number of alkyl groups

As the number of carbon atoms increases so the range of retention times broadens. With an increasing number of methyl groups in positions 3 and (especially) 4, the retention times increase (Fig. 2). On the other hand, introduction of further isopropyl groups to 3-isopropylbiphenyl results in lower retention times; thus 3,5-, 3,3′-, 3,4′-, and 3,4-diisopropylbiphenyl have lower retention times than both 3-iP-B and biphenyl itself. Also, introduction of an alkyl group to a sterically sensitive position (2), *i.e.*, the *ortho*-position with respect to the link between two aromatic rings, results in lowered retention times (Fig. 1).

Length of alkyl groups

As in the case of alkylbenzenes[4], the retention time decreases with increasing alkyl chain length. The largest effect of this type is observed for the 4-*n*-alkylbiphenyls measured, *viz.* 4-methyl-, 4-ethyl-, 4-*n*-propyl- and 4-*n*-butylbiphenyl. The dependence of log k' on the number of carbon atoms for 2-*n*-alkylbiphenyls is presented in Fig. 1. Fig. 3 shows a chromatogram of 2-methyl-, 2-ethyl-, 2-*n*-propyl-, and 2-*n*-butylbiphenyl. As for alkylbenzenes, it was expected that no substantial decrease in retention times would be observed for a further increase in the length of the *n*-alkyl chain.

Alkyl arrangement

For methylbiphenyls and ethylbiphenyls, there is a general increase in retention time in the order of 2-methyl, 3-methyl, 4-methyl, and similarly 2-ethyl, 3-ethyl, 4-ethyl. In case of larger alkyl groups, the situation is complicated by the length, shape, and position of the alkyl groups and subsequent effects on the orientation of the two

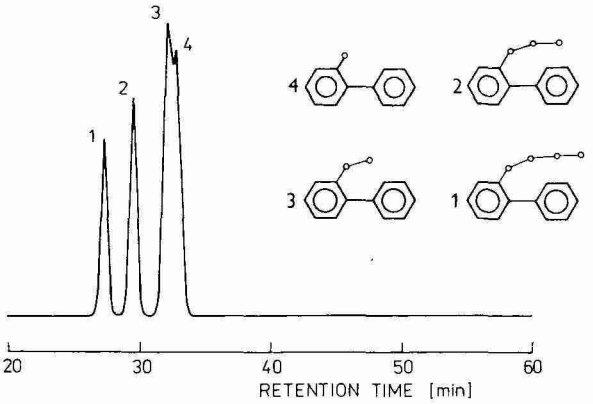

Fig. 3. Chromatogram of some *n*-alkylbiphenyls. Column, 250 × 8 mm I.D. packed with 7.5 μm silica gel; mobile phase, moisture-controlled *n*-pentane; 0.5% water in the MCS; flow-rate, 100 ml/h.

aromatic rings. In the case of methylbenzenes, *ortho*-substituted compounds are characterized by a markedly higher retention time than *meta*- and *para*-derivatives (the elution times of the latter two groups are almost identical). In the case of alkylbiphenyls, substitution in positions 2,6,2′,6′ results in an increase in the angle between the ring planes, which complicates the adsorption mechanism.

For methylbiphenyls, the retention time increases as the methyl groups are positioned nearer to each other. The highest retention is achieved when the methyl groups are mutually *ortho*. This trend is observed also in case of more substituted methylbiphenyls (see *e.g.* an apparent increase in retention for 3,5,4′ trimethylbiphenyl with respect to 2,6,2′-trimethylbiphenyl, or even more substantial retention increase for 3,4,5,3′,4′,5′-hexamethylbiphenyl with respect to 2,4,6,2′,4′,6′-hexamethylbiphenyl.

Fig. 4 describes the dependence of log k' on the number of substituents for methylbiphenyls substituted in positions 3 and 4. An approximately linear depen-

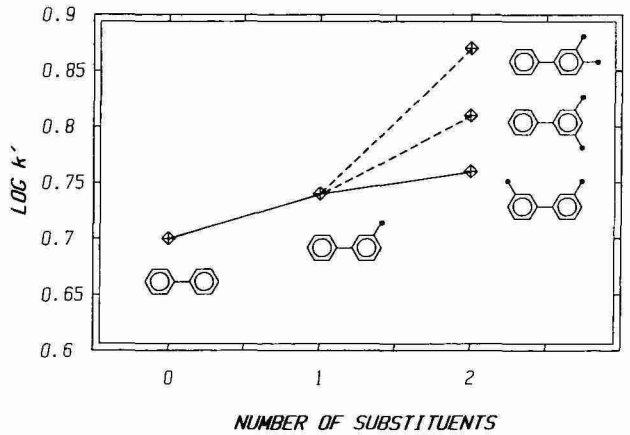

Fig. 4. Dependence of log k' on the number of carbon atoms for biphenyl, 3-methylbiphenyl and some dimethylbiphenyls substituted in positions 3 and 4; 0.5% water in the MCS.

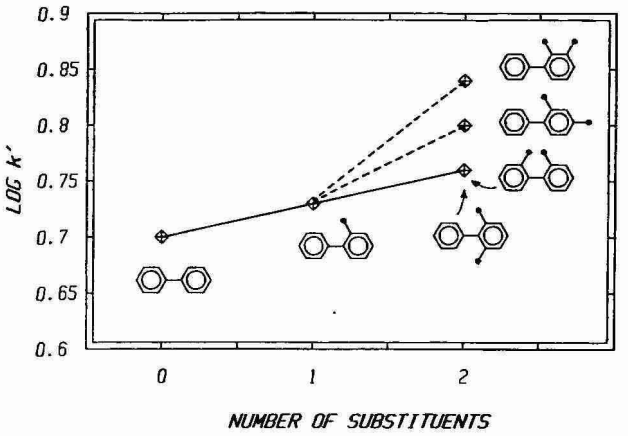

Fig. 5. Dependence of log k' on the number of carbon atoms for biphenyl, 2-methylbiphenyl and some dimethylbiphenyls substituted in position 2; 0.5% water in the MCS.

dence is observed in the series of biphenyl, 3-methylbiphenyl, 3,3′-dimethylbiphenyl. A longer retention time is observed for 3,5-dimethylbiphenyl, where two substituents are located on a single ring. The longest retention time observed was that of 3,4-dimethylbiphenyl, which has mutually *ortho* methyl groups.

Similarly, the retention also increases for biphenyls with one or more substituents in positions 2 or 6. Fig. 5 shows the dependence of log k' on the number of substituents for the mentioned compounds. A linear dependence is again observed between log k' and the number of methyls in the order biphenyl, 2-methylbiphenyl 2,2′-dimethylbiphenyl. 2,6-Dimethylbiphenyl has the same retention time as 2,2′-dimethylbiphenyl. Retention times of the other dimethylbiphenyls increase in the following order: 2,6- < 2,5- < 2,4- < 2,3-dimethylbiphenyl. The highest value was observed for 2,3-dimethylbiphenyl, *i.e.* two methyl groups in mutually *ortho* positions.

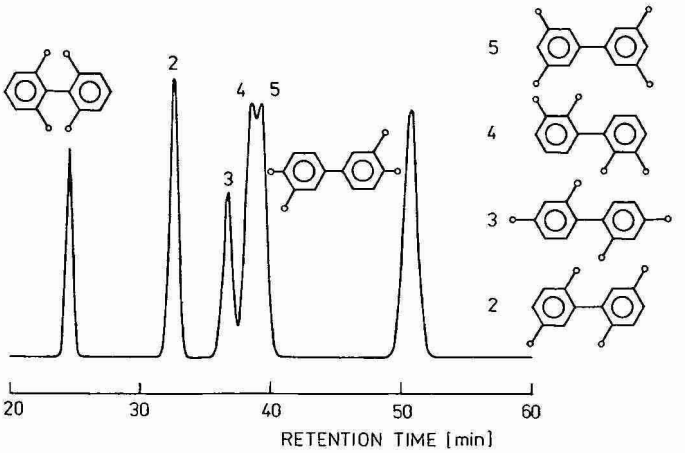

Fig. 6. Chromatogram of some tetramethylbiphenyls. Column, 250 × 8 mm I.D. packed with 7.5 μm silica gel; mobile phase, moisture-controlled *n*-pentane; 0.5% water in the MCS; flow-rate, 100 ml/h.

Orientation of the rings

Most of the biphenyl derivatives do not have planar molecules when in solution. The interplanar angle of biphenyl itself has been estimated at 23–26° (ref. 10). The introduction of substituents into the sterically sensitive 2,6,2′ and 6′ positions causes the angle between the ring planes to increase. This angle is 70° for 2,6-dimethyl-, 73° for 2,2′-dimethyl-, and 80° for 2,6,2′,6′-tetramethylbiphenyl, respectively (ref. 10). On the other hand, substitution in the position 4 results in a decrease of the interplanar angle: 4-methylbiphenyl and 4,4′-dimethylbiphenyl have planar molecules[11,12].

Changes in the retention behaviour of biphenyl derivatives due to rotation of benzene ring planes are of substantial importance for their adsorption on alumina[13,14]. The chromatographic behaviour of biphenyl compounds on silica gel is also affected, although this change has less marked effects because of the different adsorption mechanism on silica gel. Fig. 6 shows a chromatogram for tetramethylbiphenyls: the 2,6,2′,6′ derivative is eluted first, with a retention time similar-to-those of the methylbiphenyls; next, tetramethylbiphenyls substituted in positions 2 and 2′ are eluted, *viz.* the 2,5,2′,5′, 2,4,2′,4′ and 2,3,2′,3′ derivatives, the retention is gradually increasing with the decreasing distance between methyl groups on a single ring; the final two tetramethylbiphenyls are not substituted in positions 2 and 2′, and the longest retention time is that of 3,4,3′,4′-tetramethylbiphenyl, in which the *ortho* effect is at its strongest.

The three octamethylbiphenyls may serve as another example. The shortest retention time is observed for biduryl (2,3,5,6,2′,3′,5′,6′-octamethylbiphenyl), which is substituted in all sterically sensitive positions (*viz.* 2,6,2′ and 6′). Its retention time is in the range of those of the dimethylbiphenyls. It is followed by biisoduryl (2,3,4,6,2′,3′,4′,6′-octamethylbiphenyl), whose longer retention time may be ascribed to *ortho*-substitution in positions 3,4,3′ and 4′. The longest elution time is that of biprehnithyl (2,3,4,5,2′,3′,4′,5′-octamethylbiphenyl), which has a smaller interplanar angle than either of the other two octamethyl compounds; moreover, a substantial increase in retention is due to substitution in positions 3,4,5,3′,4′ and 5′.

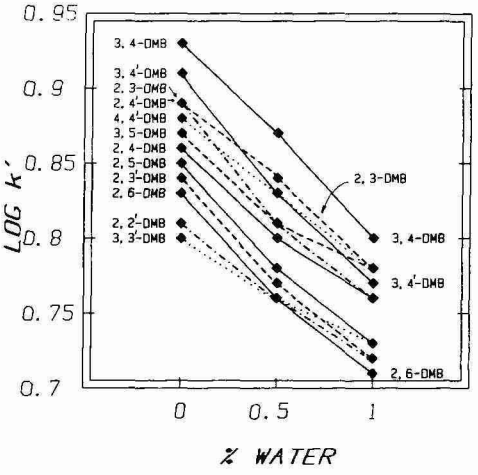

Fig. 7. Variation of log k' for a series of dimethylbiphenyls with the content of water in the MCS.

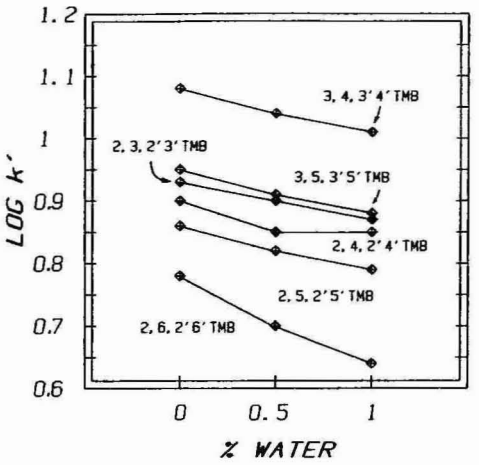

Fig. 8. Variation of log k' for a series of tetramethylbiphenyls with the content of water in the MCS.

Shape of the alkyl group

Alkylbiphenyls with short alkyl groups (especially methyl) have longer retention times than those with long substituents in the same positions. As in the case of alkylbenzenes, compounds with the alkyl chain branched at the α-carbon atom have longer retention times than the corresponding compounds with one alkyl group and the same number of carbon atoms: *e.g.*, the retention times of 2- and 4-isopropylbiphenyls are longer than those of 2- and 4-*n*-propylbiphenyls, and 2- and 4-*tert.*-butylbiphenyls have also higher retention times than the corresponding 2- and 4-*n*-butylbiphenyls.

Influence of water content in the mobile phase

The elution times of the alkylbiphenyls investigated decrease with increasing water content in the mobile phase. Fig. 7 shows the dependence of log k' on the water content as determined by the MCS for a series of dimethylbiphenyls, and Fig. 8 shows the same dependence for tetramethylbiphenyls. It is evident from Fig. 7 that selectivity is strongly influenced by changes in the water content. The separation of dimethylbiphenyls is best when the mobile phase contains the minimum of water, namely dry *n*-pentane. On the other hand, the application of the MCS leads to a good reproducibility of the retention data. The reproducibility at water content concentrations of 0.5 and 1.0% is better when the MCS is applied, therefore the recommended minimum water content is 0.2%[15].

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SOLID-PHASE DERIVATIZATION OF THIOLS

APPLICATION TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The use of functionalized silica materials packed in a short column has been investigated for the determination of thiol compounds in solution and in chromatographic eluates. The influences of the silica treatment, grafted disulphide, pH and solvent effects are described. These silica-based postcolumn reagents exhibit high capacities, result in low peak broadening and are compatible with organic solvents. They permit the detection of 0.05 nmol of thiol.

INTRODUCTION

Thiol compounds are of great importance in biochemical processes and in chemical systems. Thus, substantial efforts have been made to design highly specific reagents for their determination[1–12]. Some methods involve the release of highly coloured[5–9] or fluorescent products[10–12]. However, in many cases, the thiols must be separated before derivatization. High-performance liquid chromatography (HPLC) with precolumn[13–17] or postcolumn derivatization[18–20] has been reported as an useful means for the analysis of thiol mixtures; the former technique is often time-consuming and the latter can introduce peak broadening which can affect the resolution, peak shape and height and minimum detection limits.

Within the past few years, novel approaches for performing pre- or postcolumn derivatization without excessive dilution have been described[21,22].

In the case of thiols and of disulphides, thiol reagents immobilized on soft Sepharose gels have been introduced by Studebaker[23,24]. In order further to minimize peak broadening in this technique, the thiol polymers can be replaced by thiol-functionalized silica materials[25,26]; this may allow operations at high flow-rates, and the use of microparticulate silica may improve the derivatization kinetics. We have studied two solid-phase reagents (SPRs) obtained from 10-μm diameter silica particles. After activation with a disulfide R'S–SR', they allow the determination of thiol compounds in solutions or in chromatographic eluates with high sensitivity.

The elution of a small amount of a thiol RSH on a disulphide silica column

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may proceed by two reactions

$$\text{Silica}\sim\text{S-SR}' + \text{RSH} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{Silica}\sim\text{S-SR} + \text{R}'\text{SH} \quad (1)$$

$$\text{Silica}\sim\text{S-SR}' + \text{RSH} \overset{K_2}{\rightleftharpoons} \text{Silica}\sim\text{SH} + \text{RS-SR}' \quad (2)$$

When R′ is aromatic, the $R'S^-$ anion is stabilized by resonance in a thione form, and reaction 1 becomes predominant; moreover, the thiol–disulphide interchange can readily be monitored by electronic absorption spectroscopy[5,27]. Thus, we have used aromatic disulphides.

In order to improve the sensitivity of the method, we have investigated the influence of different parameters such as eluent pH, solvent and flow-rate. The nature of the silica surface and that of the radical R′ are of great importance; free silanol groups on the silica, may result in additional phenomena such as retention of the released thiol or thione and tailing effects. These drawbacks can be avoided by grafting diol functions onto the silica surface[28]. Thus, by studying two thiol silica supports whose silanol groups are masked or not, and by using two disulphides, 2,2′-dipyridyl disulphide (2 PDS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), whose corresponding thiols have very different solubilities in water, we have been able to prepare silica-based materials and use them as postcolumn solid-phase reagents.

EXPERIMENTAL

Materials

LiChrospher Si 100, 2-thiopyridone, 2-mercaptoethanol and cysteamine hydrochloride were obtained from Merck. (3-Mercaptopropyl)trimethoxysilane, 2 PDS, DTNB and dithioerythritol (DTE) were obtained from Aldrich. L-Cysteine hydrochloride, acetonitrile, sodium dihydrogenphosphate, disodium hydrogenphosphate, toluene and EDTA were purchased from Prolabo. Glycidoxypropyltrimethoxysilane was obtained from Polysciences.

Preparation of mercaptopropyl-bonded silica (support I)

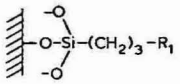
A suspension of LiChrospher Si 100 (1 g) in toluene (9.5 ml) was placed in an ultrasonic bath at 80°C. After addition of 0.5 ml of freshly distilled mercaptopropyltrimethoxysilane, the mixture was kept for 1 h under these conditions. After several washings with toluene and ethanol, the thiol silica was dried at 40°C and used immediately. The thiol content was determined with a 2 PDS solution[29], and the sulphur content by elemental analysis.

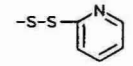
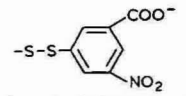
Preparation of mercaptopropyl- and diol-bonded silica (support II)

The introduction of diol functions onto silica was achieved by treating silica with glycidoxypropyltrimethoxysilane in an aqueous medium as described previously[30]. Then, 1 g of diol silica (9% carbon) was suspended in 8 ml of toluene, and placed in an ultrasonic bath at 80°C. After the addition of 2 ml of mercaptopropyltrimethoxysilane, the mixture was kept for 2 h under these conditions. Washing, drying and determination of the thiol and sulphur contents were performed as for product I (Table I).

TABLE I

STRUCTURE AND CHARACTERISTICS OF THE SILICA-BASED SUPPORTS



Support No.	R_1	*S content* (mmol/g)*	*SSR' content** (mmol/g)*
I	–SH	0.36–0.5	–
II	–O–CH_2–CH(OH)–CH_2–OH and –SH	0.03–0.05	–
III		–	0.35–0.5
IV	–O–CH_2–CH(OH)–CH_2–OH and 	–	0.025–0.05
V		–	0.35–0.5

* Determined by elemental analysis.
** Determined with a 2 PDS solution.

Instrumentation

For all experiments, the eluent was delivered by an Altex pump (Model 110); thiol samples were injected within an U6K injector (Waters). The released thione was monitored by a 450 (Waters) variable-wavelength detector, and recorded on an Omniscribe recorder (Houston Instrument). The thiol silica was slurry packed in a 5 cm × 4 mm I.D. stainless-steel column[31], and then activated by pumping a $5 \cdot 10^{-3}$ *M* disulphide solution in 0.1 *M* phosphate buffer–ethanol (1:1), at 0.2 ml/min, for 16 h. Three kinds of supports were investigated (see Table I): mercaptopropyl-bonded silica activated by 2 PDS (support III), mercaptopropyl- and diol-bonded silica activated by 2 PDS (support IV) and mercaptopropyl-bonded silica activated by DTNB (support V).

Standard procedure

Thiol solutions were prepared in buffers containing 1 m*M* EDTA, to prevent oxidation. 50-μl Aliquots were injected on the disulphide column. The response was evaluated by measuring the height, H, and/or the surface area, S_1. of the released thione peak at 350 nm for supports III and IV, at 412 nm for support V.

The amount, ρ (%), of the thiol–disulphide interchange was calculated by comparing S_1 with the surface area, S_0, of the peak obtained by injection of the theoretical amount of thione onto a 5-cm length of an inert column (LiChrosorb Diol, Merck):

$$\rho = S_1/S_0 \tag{3}$$

RESULTS AND DISCUSSION

Detection of thiol compounds on a 2 PDS-activated mercaptopropyl silica (support III)

Calibration curve. The injection of a thiol such as DTE on support III yielded a peak at 350 nm corresponding to the released thione (see reaction 1 and Fig. 1a). When increasing concentrations of DTE were injected, the calibration curve derived from the peak surface areas was linear from $5 \cdot 10^{-4}$ to 10^{-5} *M* (lowest detectable concentration), contrary to that obtained from the peak heights which showed a noticeable curvature. This discrepancy is caused by peak asymmetry which is dependent on the injected thiol concentration: when concentrated thiol solutions are injected on the solid-phase reagent (SPR) the thiol–disulphide interchange is more rapid and the released thione is eluted with a sharper peak than with diluted solutions.

Influence of acetonitrile. Different thiols were detected on this support (see

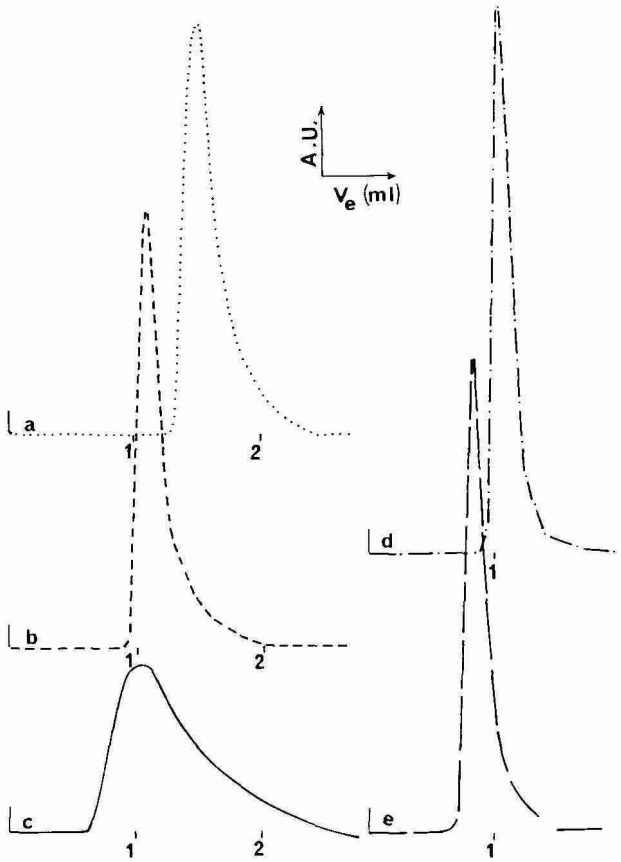


Fig. 1. Response of various solid-phase reactors to 10^{-4} *M* DTE injections. Experimental conditions: columns, 5 × 0.4 cm I.D.; flow-rate, 0.5 ml/min. (a) Support III; 0.15 *M* phosphate buffer, pH 6.0; λ = 350 nm at 0.2 a.u.f.s. (b) Support III; 0.15 *M* phosphate buffer, pH 6.0–acetonitrile (80:20); λ = 350 nm at 0.2 a.u.f.s. (c) 2 PDS-activated thiopropyl Sepharose; 0.15 *M* phosphate buffer, pH 8.0, 10^{-3} *M* EDTA; λ = 350 nm at 0.1 a.u.f.s.(d) Support IV; 0.15 *M* phosphate buffer, pH 8.0; λ = 350 nm at 0.2 a.u.f.s. (e) Support V; 0.15 *M* phosphate buffer, pH 7.95; λ = 412 nm at 0.4 a.u.f.s.

TABLE II

DETECTION OF THIOL COMPOUNDS ON SUPPORTS III–V

Conditions: thiol concentration, 10^{-4} *M*; flow-rate 0.5 ml/min; injection volume, 50 µl; eluent, 0.15 *M* phosphate buffer, 10^{-3} *M* EDTA. V_e = Elution volume. S_1 = Detector response (0.2 a.u.f.s.; chart speed 2.5 cm/min).

Injected thiol	*Support*	*pH*	*Acetonitrile (%)*	V_e *(ml)*	S_1	$\rho = \frac{S_1}{S_0} \cdot 100$
DTE	III	6.0	0	1.46	262	130
			10	1.05	203.5	101
			20	1.09	173.3	86
	IV	6.0	0	1.09	82.6	41
		7.0	0	1.04	193.5	96
		8.0	0	1.1	197.6	98
	V	7.0	0	0.91	270	81
		7.9	0	0.84	324	97
Cysteine	III	6.0	0	1.45	162	79
			10	1.0	159.2	79
			20	1.0	163.2	81
	IV	6.0	0	1.05	90.1	45
		7.0	0	1.0	153.2	76
		8.0	0	1.03	181.4	90
	V	7.0	0	0.9	236	70
		7.9	0	0.83	290	86
Cysteamine	III	6.0	0	1.49	117	58
			10	1.01	102.8	51
			20	1.1	112.9	56
	IV	6.0	0	1.09	65.3	32
		7.0	0	1.05	110.9	55
		8.0	0	1.1	121.0	60
	V	7.0	0	0.91	194	58
		7.9	0	0.8	212	63

Table II). The elution volumes of the released thiones are constant, V_e from 1.45 to 1.49 ml. However, when injected on a 5-cm long LiChrosorb Diol reference column (Merck), 2-thiopyridone is eluted at a lower volume, V_e = 1 ml, and as a sharper peak (see Fig. 2a). This difference results from the reaction kinetics and/or from an affinity of the injected or of the released compounds for the support.

By adding to the eluent a component such as acetonitrile, the retention volume of the released thione decreases, V_e = 1 ml, and tailing effects are reduced (see Fig. 1b). Under these conditions, interactions between the thione and this support are minimized. Moreover, in the presence of acetonitrile the amount of thiol–disulphide interchange, ρ, becomes less than 100% for DTE, but is unaffected for cysteine and cysteamine (see Table II).

One can conclude that, in purely aqueous media, DTE has an affinity toward the modified silica. Due to the presence of two SH groups in the DTE molecule, several reactions with the SPR can be proposed. In the absence of acetonitrile, after reaction 4, DTE is strongly retained by the SPR, and there is a possibility of reaction 5:

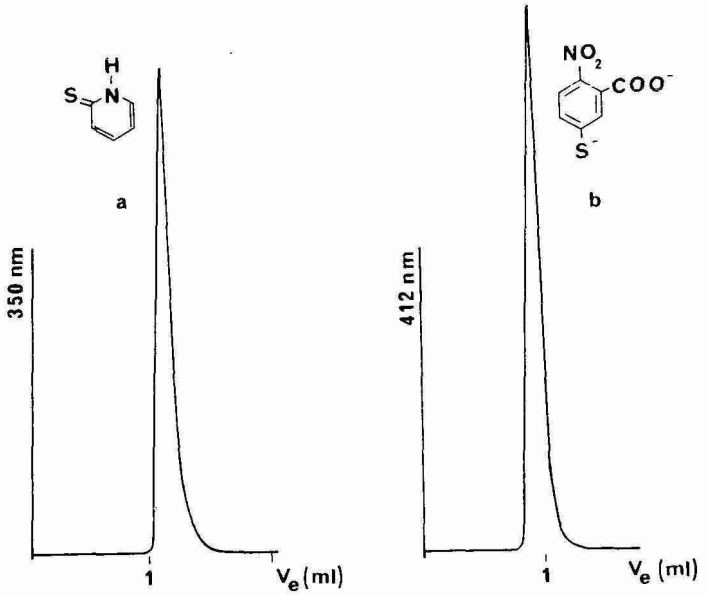
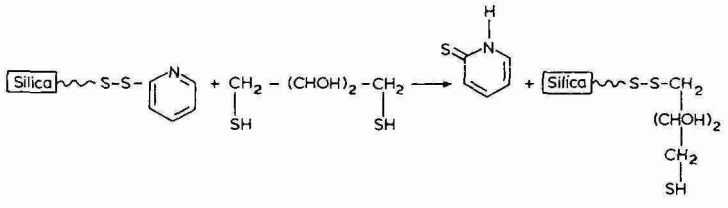
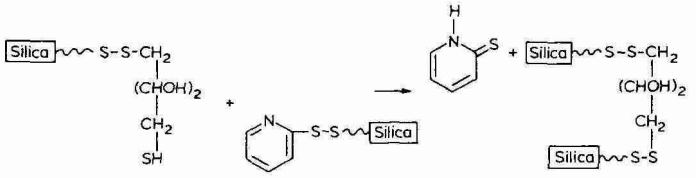


Fig. 2. Injection of 2-thiopyridone (a) and of 5-thio-2-nitrobenzoate (b) anion into a reference column (5 × 0.4 cm I.D.; LiChrosorb Diol, Merck; particle diameter, d_p = 10 μm). Flow-rate: 0.5 ml/min. Eluent: phosphate buffer, pH 8.0.

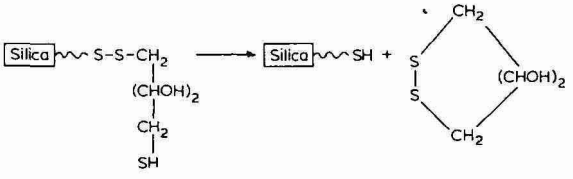


(4)



(5)

Two moles of thione are released per DTE molecule, which can explain ρ values higher than 100%. In the presence of acetonitrile, after reaction 4, the usual cyclization of the oxidized DTE[32] can take place (reaction 6)



(6)

and leads to the release of one thione per DTE molecule, lowering the ρ value, as observed experimentally.

Influence of flow-rate and pH. The influence of pH has been investigated in order to improve the sensitivity of the detection. The reaction yield is practically constant for pH values between 4.5 and 6.5 and decreases at higher pH. Similarly, by lowering the flow-rate from 1 to 0.2 ml/min, which involves a greater residence time of the thiol compound in the column, there is only a slight increase in the thiol–disulphide interchange.

Thus, under these conditions (0.01 a.u.f.s., maximum sensitivity of the UV detector used), this support permits a detection limit of $5 \cdot 10^{-10}$ mol of thiol.

Detection of thiol compounds on a 2 PDS-activated, mercaptopropyl and diol silica (support IV)

In order to avoid the addition of acetonitrile to the eluent, we have studied support IV whose silanol groups are masked. When thiol solutions are injected on a column filled with this material, the released thione peak is eluted at low retention volumes and presents no tailing effects (see Fig. 1d), demonstrating that adsorption phenomena are minimized with this support.

Influence of flow-rate on calibration curve. With a flow-rate of 1 ml/min, the response of support IV to cysteine injections is linear from $5 \cdot 10^{-4}$ to $5 \cdot 10^{-6}$ M (minimum detectable concentration), and the amount, ρ, of the thiol–disulphide interchange is practically constant, 35–40%; the correlation coefficient for 15 injections was found to be $\sigma = 0.999$. At lower flow-rates, ρ decreases when the thiol concentration becomes smaller than 10^{-4} M (see Fig. 3). This can be explained by the onset of the reverse reaction of the thiol–disulphide interchange (reaction 1) which prevents a higher extent of reaction under these conditions.

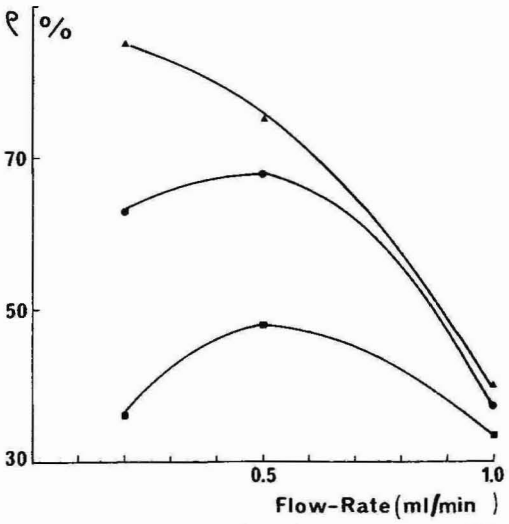
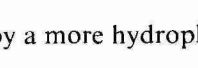


Fig. 3. Thiol–disulphide interchange on support IV as a function of the flow-rate at different cysteine concentrations: ▲, $5 \cdot 10^{-4}$, $2 \cdot 10^{-4}$ and 10^{-4}; ●, $5 \cdot 10^{-5}$; ■, 10^{-5} M. Experimental conditions: injection volume, 50 μl; eluent, 0.15 M phosphate buffer, 10^{-3} M EDTA, pH 7.0.

Influence of pH and of acetonitrile. The detector response at pH 6.0 is lower than with support III, but at higher pH values (up to 8) the released thione peaks are sharper and the amount of the diol–disulphide interchange increases (see Table II). Owing to the presence of the thiol function, the addition of acetonitrile (10–20%) to the eluent has little influence on the retention volumes and on ρ, but may be useful for the detection of poorly water-soluble thiol compounds. Under optimum conditions (pH 8.0, flow-rate = 1 ml/min), $2.5 \cdot 10^{-11}$ mol of thiol can be detected on this support.

For comparison, another thiol silica, whose silanol groups are masked by diol functions, was prepared in the same way as support IV, from LiChrosorb Diol (Merck); this support has a high thiol content ($1.2 \cdot 10^{-3}$ SH groups per g) and has been tested after activation by 2 PDS. The study of the released thione peaks revealed strong retention, even in the presence of acetonitrile in the eluent. This affinity may be due to the presence of a high concentration of $-(CH_2)_3$–SSPy groups (Py = pyridyl) on the solid phase. Due to this defect, this support was not studied further.

Detection of thiol compounds on a DTNB-activated mercaptopropyl silica (support V)

By replacing the –SPy moiety by a more hydrophilic one, , support V

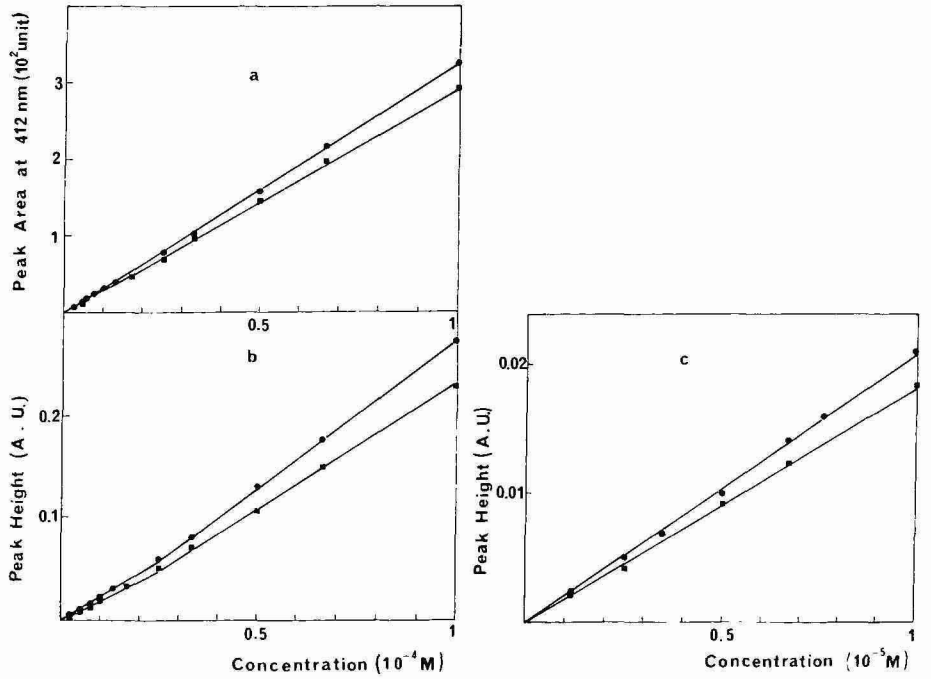


Fig. 4. Response of support V to cysteine (□) and DTE (○) injections. Experimental conditions: eluent, 0.15 *M* phosphate buffer, pH 7.95, 10^{-3} *M* EDTA; flow-rate, 0.5 ml/min; injection volume, 50 μl. (a) Peak area at 412 nm. (b) Peak height at 412 nm (absorbance units). (c) Peak height at 412 nm for thiol concentrations less than 10^{-5} *M* ($7 \cdot 10^2$ units corresponds to 10^4 mm²).

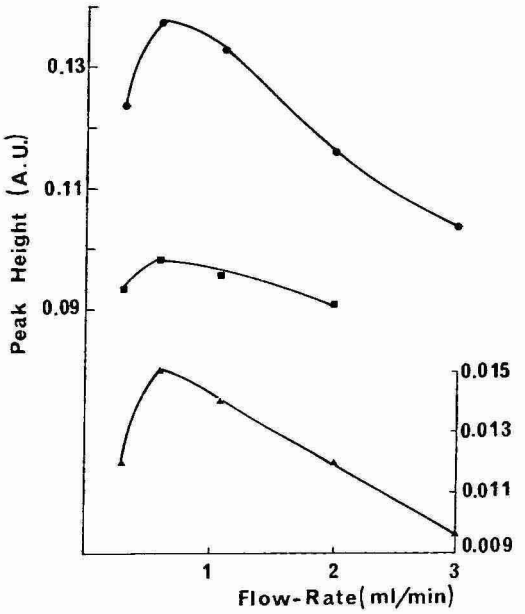


Fig. 5. Response of support V as a function of the flow-rate to different cysteine concentrations: ●, $7 \cdot 10^{-5}$; ■, $4 \cdot 10^{-5}$; ▲, $8 \cdot 10^{-6}$ *M*. Experimental conditions: injection volume, 50 µl; eluent, 0.15 *M* phosphate buffer, pH 7.95, 10^{-3} *M* EDTA.

has been obtained as described under Experimental. When thiol compounds are injected, the released thione, which has a 2-charge at pH values higher than 6.0 (thiol $pK_a = 4.53$[6]), is eluted as a sharp peak (see Fig. 1e) at smaller retention volumes than on other supports (see Table II).

Calibration curve. As shown in Fig. 4a, the response of this SPR to DTE and cysteine is linear from $2 \cdot 10^{-4}$ to 10^{-6} *M* (lowest detectable concentration), when the peak surface area, S_1, is plotted against thiol concentration ($\sigma = 0.999$ for 21 cysteine injections; $\sigma = 0.999$ for 27 DTE injections). The calibration curve obtained with peak heights (Fig. 4b) exhibits a slight change in slope, but can be used for thiol concentrations between $2 \cdot 10^{-5}$ and 10^{-6} *M* (see Fig. 4c).

Influence of pH, flow-rate and acetonitrile. The amount of the thiol–disulphide interchange increases when the pH rises from 6.0 to 8.0 (see Table II). However, at pH higher than 7.5, a spontaneous thione release sometimes took place as revealed by a strong increase in the baseline absorbance.

We have also investigated the influence of the flow-rate. From Fig. 5, it is seen that for any thiol concentration there is an optimum flow-rate between 0.5 and 1 ml/min; at lower flow-rates, a contribution of the reverse reaction (reaction 1) can be invoked.

Between 0.02 and 0.3 *M*, the ionic strength of the eluent has a very small influence on the detector response. It enables detection of thiol compounds whose elution from chromatographic columns requires concentration gradients. For example, thiol separations on a silica-based anion exchanger, prepared in our laboratory[33], are shown in Fig. 6.

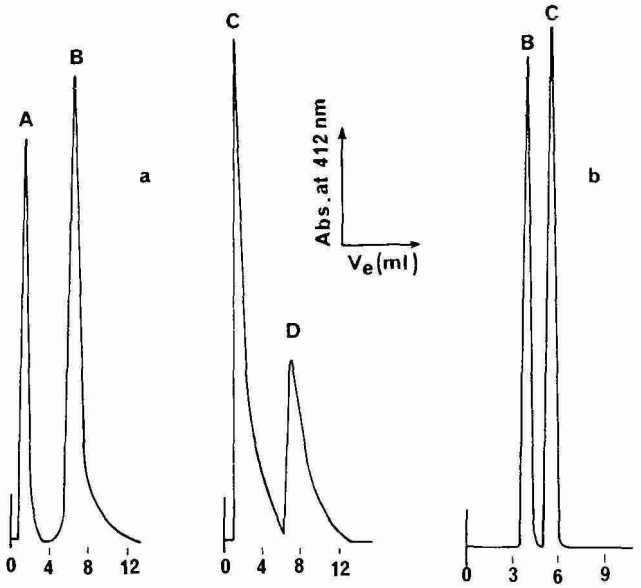


Fig. 6. Detection with support V of thiol mixtures after separation on an analytical column. Experimental conditions: (a) analytical column, anion-exchange silica, 7.5 × 0.4 cm I.D.; eluent, linear gradient from 5 to 95% B in 5 min, A = 0.02 *M* Tris–acetate, pH 8.0, B = 0.02 *M* Tris-acetate, 0.3 *M* sodium chloride, pH 7.7; flow-rate, 0.8 ml/min. (b) Analytical column, C_{18} μBondapak (25 × 0.4 cm I.D.); eluent, acetonitrile–0.15 *M* Tris-HCl, pH 7.9 (20:80); flow-rate, 1 ml/min. Peaks: A = DTE; B = reduced glutathione; C = 2-mercaptoethanol; D = cysteine.

Fig. 7 shows the influence of the addition of organic solvents such as acetonitrile or methanol to the eluent. An organic modifier in amounts as high as 50% (v/v) can be used, without drastic decrease of the detector response. This enables the detection of poorly water-soluble thiol compounds and of thiol mixtures separated on reversed-phase columns. Fig. 6b shows an example of a separation of thiol compounds on a C_{18} μBondapak column, detected after derivatization with support V.

Thus, under optimum conditions (pH 7.9, flow-rate = 0.5 ml/min), $5 \cdot 10^{-11}$ mol of thiol can be detected on support V with the detector presently used.

Peak broadening

As shown in Fig. 1, the silica-based materials result in less peak broadening than thiol Sepharose material tested under the same conditions (Fig. 1c).

In order to evaluate the peak broadening due to the chemical interchange reaction, we have compared the released thione peaks eluted from these SPRs (length 5 cm, d_p = 10 μm) and those obtained by injecting the same amount of the corresponding thione on a 5 cm long LiChrosorb Diol reference column (d_p = 10 μm). From the results summarized in the Table III, it appears that solid-phase reactions induce an increase in the thione peak width of 30% in the best cases (supports IV and V) and of 60% in the worst. This peak broadening could probably be reduced by using smaller silica particles.

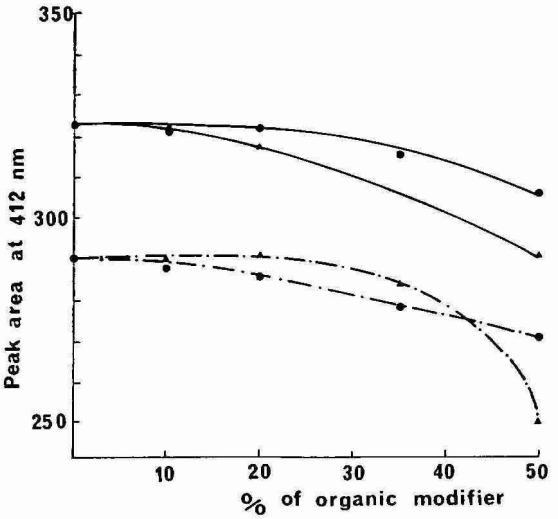


Fig. 7. Response of support V to DTE (——) and cysteine (— · — ·) injections as a function of the organic modifier (●, methanol; ▲, acetonitrile). Experimental conditions: thiol concentration, 10^{-4} *M*; flow-rate, 0.5 ml/min; eluent, 0.15 *M* Tris–HCl, pH 8.0 and an organic modifier; injection volume, 50 µl.

Column lifetime

About 200 10^{-4} *M* thiol solutions have been injected on support IV, corresponding to 1/20 of the SSR′ content of the solid-phase reactor, without noticeable decrease in the response. With supports III and V of greater SSR′ content, the number of injections would be ten-fold higher. We have not yet achieved this number. In spite of this, we have examined the possibility of regenerating support IV: the column was washed with a mercaptoethanol solution (10^{-2} *M*; pH 8.0) until the thione was completely released, and then reactivated by a disulphide. After these operations, the performance of the SPR was found to be reduced: for example, ρ which was close to 95% before regeneration, dropped to 60% afterwards.

It seems that the major limitation in the durability of these reactors is the slow dissolution of the silica in aqueous media, which leads to the occurrence of a small void in the column after use for about 10 weeks.

TABLE III

PEAK WIDTH AT HALF HEIGHT, δ, AS A FUNCTION OF THE NATURE OF THE SUPPORT

Conditions: column, 5 cm × 4 mm I.D.; d_p = 10 µm; thiol concentration, 10^{-4} *M*; flow-rate, 0.5 ml/min.

Support	*Injected compound*	δ *(ml)*
LiChrosorb Diol*	2-Thiopyridone	0.125 ± 0.006
	5-Thio-2-nitrobenzoate	0.138 ± 0.006
Support III**	DTE	0.200 ± 0.006
Support IV*	DTE	0.163 ± 0.006
Support V*	DTE	0.187 ± 0.006

* 0.15 *M* phosphate buffer, pH 8.0.

** 0.15 *M* phosphate buffer, pH 6.0–acetonitrile (80:20).

CONCLUSIONS

Solid-phase reagents for thiol detection based on silica materials can easily be obtained; columns (5 cm × 4 mm I.D.) containing 10–20 μmol SSR (support IV) and 140–200 μmol SSR (supports III and V) have been prepared. These allow numerous detections of thiol compounds without changing or regenerating the support.

Moreover, by using such silica-based SPRs, peak broadening is considerably reduced (see Fig. 1), compared to analogous soft gels. Compatibility with organic solvents such as acetonitrile or methanol is good, which allows detection of poorly water-soluble thiol compounds, and thiol separations on reversed-phase columns.

When using supports IV and V, no interaction of the released thione with the silica support was observed. They are operated at pH values from 7.0 to 8.0. Peaks obtained with the former are somewhat sharper, but the SSR′ content of the columns is lower and linearity of the response is not as good as on support V. Support III gives satisfactory results at low pH values (4.5–6.5), but the addition of 20% acetonitrile to the eluent is recommended.

Despite the slight contribution of the chemical interchange reaction to peak broadening, these solid-phase reactors make it possible to determine thiol compounds with high accuracy, since the minimum detectable amount has been evaluated as $5 \cdot 10^{-11}$ mol with the UV detector presently used.

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PRACTICAL NARROW-BORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM USING COMMERCIAL EQUIPMENT AND 1-mm BORE COLUMNS PACKED IN THE LABORATORY

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SUMMARY

The construction of a robust narrow-bore high-performance liquid chromatograph is described which involves a commercial pump, injector and detector and columns (300 × 1 mm I.D.) prepared in the laboratory. The columns were constructed from glass-lined stainless-steel tubing with unique zero-dead volume end assemblies. Full details of the column packing procedure are given which uses a conventional column packing pump at pressures less than 10 000 p.s.i. Results are presented from a study to establish the most appropriate slurry liquids and slurry concentrations. Both silica and ODS-silica columns have been prepared and their performance is discussed with reference to their suitability for routine analysis.

INTRODUCTION

Most high-performance liquid chromatography (HPLC) is currently conducted with columns of 4–5 mm internal diameter but the last five years has seen considerable interest in the utilisation of columns with reduced internal diameters. This is demonstrated by a large number of research papers (and several extensive reviews[1–6]) concerning this topic. Three different types of column have emerged, (*viz.* narrow-bore packed, packed microcapillaries and open-tubular) but are often referred to collectively as "microbore" columns which can be very confusing. At the present time only narrow-bore packed columns appear to be suitable for routine work and these are essentially conventional HPLC columns with reduced internal diameters containing packing materials of 3–10 μm.

Despite the vast literature concerning the development of narrow-bore columns few analytical laboratories are currently using them for routine applications despite numerous claims of their advantages over conventional columns. This can be attributed to several factors. Firstly, until relatively recently little commercial equipment specifically designed for narrow-bore work was available and few application laboratories have been prepared to make the extensive modifications to existing hardware as demonstrated in the literature. Secondly, commercial narrow-bore columns have been relatively expensive while few published papers give sufficient experimental

details to facilitate the packing of such columns in the laboratory. Finally, some confusion remains concerning the merits of narrow-bore columns in routine analysis and many laboratories await further clarification before making the considerable investment necessary to convert existing systems. With these considerations in mind the practicality of using narrow-bore HPLC columns for routine work has been examined and the present paper describes the construction of 1 mm bore columns, gives details of our packing procedure and demonstrates the performance of the columns using readily available commercial equipment requiring minimal modification. A second paper[7] will examine the potential advantages of these columns over conventional HPLC columns with particular reference to the types of problem encountered in forensic drug analysis.

It is now well established that the packing of small particles (3–10 μm) into conventional HPLC columns requires pumping a slurry of the material into the empty column at a relatively high pressure. Nevertheless numerous variations on this basic theme have been published as has been indicated in recent reviews[8,9]. The many variables include the nature of the slurry liquid and the pressurising solvent, the concentration of the slurry, the method of dispersal (*e.g.* ultrasonic vibration), the use of constant pressure or constant flow pumps, the packing pressure, the shape and size of the slurry reservoir, and the use of procedures to prevent sedimentation (*e.g.* upward packing, slurry stirring). In addition, several criteria are used to evaluate the success of a column packing method and these include the column plate count, the permeability and stability of the packed bed, the production of symmetrical peaks and the speed and reproducibility of the procedure. Practical experience has demonstrated that good columns can be produced using a wide range of different procedures but attempts to optimise the many variables and identify the best methods have met with limited success. The results of such studies have often been contradictory and few general guidelines have emerged. After many such experiments Verzele *et al.*[10] have even concluded that every packing material, including different commercial brands of the same type, requires an individualised packing procedure to produce columns of optimum performance.

It is generally agreed that narrow-bore HPLC columns are more difficult to pack than conventional columns. Scott and Kucera[1,11,12] have advocated the use of very high packing pressures (25 000 p.s.i.) and have recommended low viscosity balanced density slurries for silica packings and acetonitrile for octadecyl-silica (ODS-silica). Balanced density slurries have also been used by Hermansson[13], Bowermaster and McNair[14], Apffel *et al.*[15] and Menet *et al.*[16] for a wide range of different packing materials. The last publication also recommends the use of very high packing pressures with dilute slurries and stresses the importance of a cone shaped connector between the slurry reservoir and the empty column. Vadukul and Loscombe[17,18] have packed narrow-bore columns using low viscosity slurry solvents and a stirred reservoir. Meyer and Hartwick[19] have also used a low viscosity slurry solvent, isopropanol, for packing 1 mm bore columns and describe systematic studies to optimise the procedure. The above publications all involved the packing of stainless-steel (SS) columns of around 1 mm I.D. but recent work by Takeuchi and Ishii[20], Yang[21], Hirata and Jinno[22] and Novotny *et al.*[23] has involved the use of glass or fused-silica tubing (0.1–0.5 mm I.D.) for column fabrication. These columns have been packed at lower pressures (typically $< 10\,000$ p.s.i.) with low viscosity slurry solvents.

The operation of narrow-bore HPLC columns requires the use of miniaturized injectors and detector flow-cells in order to avoid extra-column band broadening and thus realise the true performance of the columns. The emphasis of the present paper is on the feasibility of setting up reliable narrow-bore HPLC systems for routine analysis and hence the selection of the hardware (pump, injector, UV detector) has been based on its commercial availability, deliberately avoiding the need for extensive modifications in the laboratory. The valve injector selected had loop sizes ranging from 0.5 to 5 μl while the UV detector had a flow-cell of 0.3 μl being the smallest available in any UV detector on the British market at the time of commencing this work[24]. The use of 1 mm I.D. columns in this study was influenced by the dimensions of these components since an excessive loss in performance might be expected with columns of smaller internal diameters[25]. In addition, such columns are usually operated at flow-rates between 10 and 100 μl/min and a dual-piston analytical pump was modified by the manufacturer to deliver these flow-rates. Adhering to the general philosophy of using equipment which is readily available all column packing has been conducted with a commercial packing pump capable of pressures up to about 10 000 p.s.i.

EXPERIMENTAL

Materials

Methanol and hexane were HPLC grade obtained from Rathburn Chemicals (Walkerburn, U.K.). Acetone, ethanediol, nitrobenzene, pentane, phenol and toluene were analytical grades from BDH (Poole, U.K.). Anisole, *p*-cresol, 2,5-dimethylphenol, 3,5-dinitrobenzonitrile, 3,5-dinitrobenzyl chloride and 2,6-dinitrotoluene were obtained from Aldrich (Gillingham, U.K.). All other chemicals were general purpose grades from BDH. Water was distilled in glass in the laboratory.

Three HPLC packing materials were obtained from Shandon Southern Products (Runcorn, U.K.), *viz.* Hypersil-silica with mean particle diameters of 4.13 and 5.73 μm respectively and Hypersil-ODS with a mean particle diameter of 5.49 μm.

Column construction

Columns were constructed from glass-lined SS tubing (30 cm × 1/8 in. O.D. × 1 mm I.D.) obtained from Scientific Glass Engineering (Milton Keynes, U.K., Part No. 2737). Each end of the column was terminated in the same way with a 1/4-in. O.D. tube brazed onto the glass-lined tubing and a conventional zero-dead volume end-fitting (Fig. 1).

A length of SS rod (1/4 in. O.D.) was cut on a lathe (3.5 cm) and drilled along its length (No. 30 British Standard bit) such that the glass-lined tubing gave a close fit when inserted into the hole. The metal surfaces to be brazed were first cleaned with fine emery-paper and then immersed in 50% sulphuric acid for 15 min. After rinsing with distilled water, the glass-lined tube was inserted into the outer tube taking care to align the ends of the tubes. The assembly was clamped in a vertical position with the ends pointing down. A short length of a silver brazing alloy (melting point 620–630°C) was wrapped around the glass-lined tubing and allowed to rest on the top of the outer tube. The region of the joint was then covered with a thick paste of Tenacity Flux No. 5 in water. Heat was carefully applied to the outer tube using

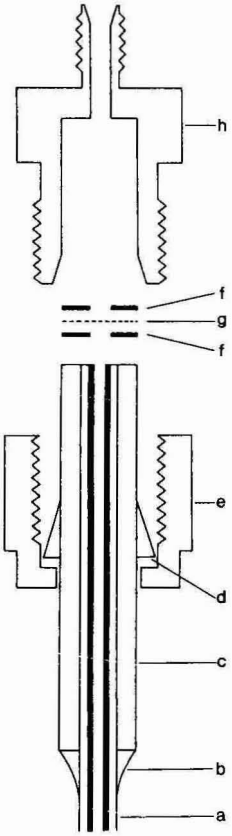

Fig. 1. Construction of narrow-bore column end assemblies. a = Glass-lined SS tubing (30 cm × 1/8 in. O.D. × 1 mm I.D.); b = silver brazing; c = SS rod (3.5 cm × 1/4 in. O.D.) drilled along its length to accommodate the glass-lined tubing; d = SS ferrule (1/4 in.); e = SS nut (1/4 in.); f = PTFE washer (0.005 in. thick); g = SS mesh (1/4 in.); h = drilled out zero-dead volume end-fitting (1/4 in.).

a fine gas-air torch until the brazing alloy was seen to melt when the source of heat was quickly removed to prevent any damage to the glass-lining of the inner tube. After allowing the joint to cool slowly to room temperature the excess flux was removed.

The end-fitting used was a Parker-Hanifin drilled out SS 1/4–1/16 in. reducing union (HETP, Macclesfield, U.K., Part No. 200) and was held in position by a 1/4-in. SS compression ferrule. The packing material was held in position by a 1/4-in. SS mesh disc (HETP, Part No. 206) which was sandwiched between two 1/4-in. washers made from PTFE sheet (0.005 in.) having 1-mm diameter holes cut in the centres.

Column packing

The columns were packed with conventional HPLC column packing equipment (Shandon Southern Products) based on a pneumatic amplifier pump and capable of pressures up to about 10 000 p.s.i. The apparatus included a 3-way switching

valve such that the liquid being pumped could be changed without interrupting the flow. The outlet from the pump was connected to a coil of SS tubing (1/16 in. O.D. × 1.09 mm I.D.) from Phase Separations (Queensferry, U.K., Part No. T1009) which acted as a slurry reservoir (Fig. 2). Three such reservoirs were constructed with tubing lengths of 150, 375 and 1000 cm having volumes of *ca.* 1.4, 3.5 and 9.4 ml respectively. The connection between the pump and the slurry reservoir involved a drilled out 1/16-in. union with a side arm (HETP, Part No. 387) and a straight through 2-way stop valve (Scientific Glass Engineering, Part No. SSI-15MW-A200). The outlet side of this valve was terminated with a luer syringe connector (HETP, Part No. 437). This arrangement allowed the slurry reservoir to be loaded rapidly from a luer syringe without the need to disconnect any fittings.

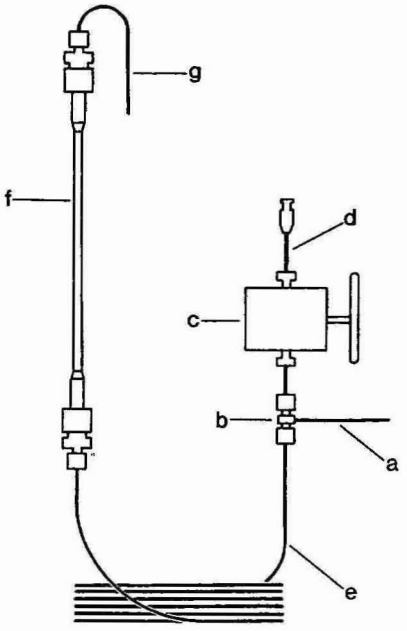

Fig. 2. Narrow-bore HPLC column packing apparatus. a = SS inlet tube (1/16 in.) from the HPLC packing pump; b = drilled out union (1/16 in.) with a side-arm brazed into position; c = straight through stop valve; d = luer syringe connector; e = slurry reservoir constructed from SS tubing (1/16 in. O.D. × 1.09 mm I.D.); f = empty narrow-bore column; g = PTFE outlet tube (1/16 in. O.D. × 0.8 mm I.D.).

The outlet end of the slurry reservoir was connected to the empty column using a 1/16-in. SS nut and ferrule. The outlet end of the column was assembled as shown in Fig. 1 while the mesh and PTFE washers were removed from the inlet end. Columns were packed in an upward direction with a PTFE tube (1/16 in. O.D. × 0.8 mm I.D.) connected to the column outlet such that the waste solvent could be safely collected. This was particularly important when handling the toxic halogenated slurry solvents.

Various slurry liquids were tested during the course of this study including methanol, methanol–ethanediol (1:1, v/v), aqueous ammonium hydroxide (0.001 *M*), iodomethane–methanol (9:1, w/w) and tetrabromoethane–methanol (4:1, w/w). The

last two mixtures, containing halogenated solvents, tended to discolour on storage; this was effectively removed by passing the liquids through disposable cartridges (*ca.* 23 × 9 mm I.D.) packed with silica (Sep-Pak silica) obtained from Waters Assoc. (Northwich, U.K.) immediately before use.

The packing procedure now used routinely for both silica and ODS-silica is described below. The equipment was set up as shown in Fig. 2 with the 1.4-ml slurry reservoir in position and the liquid reservoirs of the packing pump containing hexane, methanol and 50% aqueous methanol. After priming the pump with hexane and setting the pressure to 8500–9000 p.s.i. against a stop valve the pump was switched off and the pressure allowed to subside. The slurry reservoir filling valve was opened and a compressed air line applied to the luer fitting such that solvent was pushed out of the waste line leaving the slurry reservoir and column completely empty. The glass barrel of a gas-tight syringe (V. A. Howe, London, U.K., Part No. 1001TLL, 1 ml) was then attached to the luer fitting.

Packing material (190 mg) was weighed into a graduated glass test tube (5 ml) with a stopper and the slurry liquid [tetrabromoethane–methanol, 4:1 (w/w), 1 ml] was added. The tube was shaken vigorously to disperse the packing material and then placed in an ultrasonic bath for 15 min. The slurry was decanted into the syringe barrel using a pasteur pipette and pushed into the slurry reservoir using the PTFE tipped plunger although this was not always necessary as the slurry often moved into the coil without assistance. The slurry reservoir filling valve was then closed and the packing pump switched on. Hexane was pumped for about 15 min and then methanol for a further 15 min when the packing was considered to be complete. With ODS-silica packing materials the column was further conditioned with 50% aqueous methanol. Towards the end of the packing procedure the column was inverted by gently bending the tubing of the slurry reservoir and then the pump was switched off. When the pressure had subsided the column was disconnected and the SS mesh with PTFE washers placed in position.

Column testing

Chromatography was performed with a Waters M6000A pump modified by the manufacturer to slow down the speed of the electric motor, with an internal switch used to select a reduction in flow-rate of × 10 or × 100. Samples were introduced with a Rheodyne 7410 microbore injection valve fitted with a loop filler port (Model 70-11). A range of internal sample loops (0.5, 1, 2 and 5 μl) were used with this valve. The eluate was monitored with a Jasco Uvidec-100-III UV spectrophotometer (Lea Scientific, Milton Keynes, U.K.). Chromatograms were recorded with a Linear Model 500 chart recorder having a pen response of <0.5 s full scale.

The UV detector was fitted with a micro-cell cassette available from the manufacturer which contained a small flow-cell (0.3 μl) consisting of a length of quartz tubing (0.6 mm O.D., 0.3 mm I.D.). The construction is shown in Fig. 3 along with the details of the connections from the end of the narrow-bore column used to minimise the dead-volume. A further reduction in dead-volume was achieved by each column having its own unique inlet connection consisting of a short length of SS tubing (5 cm × 1/16 in. O.D. × 0.006 in. I.D.) held into the end-fitting by a SS ferrule such that the tube outlet touched the mesh disc. This approach eliminated problems arising from variations in the drilling of each end-fitting.

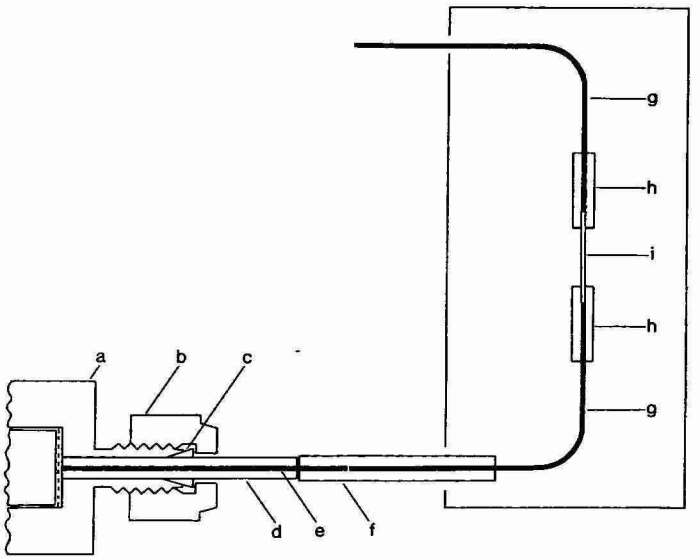

Fig. 3. Connections between the column outlet and the UV detector flow-cell. a = Narrow-bore end-fitting; b = SS nut (1/16 in.); c = PTFE ferrule (1/16 in.); d = PTFE tubing (3.5 cm × 1/16 in. O.D. × 0.023 in. I.D.); e = SS tubing (4 cm × 0.7 mm O.D. × 0.1 mm I.D.); f = PTFE tubing (3 cm × 2 mm O.D. × 0.5 mm I.D.); g = SS tubing (6 cm × 0.6 mm O.D. × 0.3 mm I.D. with two 0.15-mm wires inserted into the bore to reduce dead volume); h = PTFE tubing (1 cm × 1/16 in. O.D. × 0.5 mm I.D.); i = quartz tubing (1.25 cm × 0.6 mm O.D. × 0.3 mm I.D.).

The column testing procedures were essentially those recommended by Bristow and Knox[26]. An eluent of hexane–methanol (99.5:0.5, v/v) was used for silica columns and methanol–water (60:40, v/v) for ODS-silica columns. Detection wavelengths of 235 and 270 nm were used for the normal-phase and reversed-phase systems respectively. The injection valve was fitted with a 0.5-μl loop for column testing while the performance of the pump was checked at regular intervals by connecting an appropriate graduated syringe with no plunger to the outlet from the detector and timing the progress of the liquid along the scale.

The test mixture for silica columns was prepared by dissolving acetophenone (30 mg), 1,3-dinitrobenzene (15 mg), 3,5-dinitrobenzonitrile (20 mg), 3,5-dinitrobenzyl chloride (20 mg), 2,6-dinitrotoluene (20 mg) and nitrobenzene (40 mg) in methanol (1 ml), adding pentane (50 ml) and then making up to a volume of 200 ml with hexane. Toluene (1.5 mg per ml) was also added to this solution for some test procedures.

The test solution for ODS-silica columns was prepared by dissolving acetone (716 mg), phenol (15 mg), *p*-cresol (29 mg), 2,5-dimethylphenol (29 mg), anisole (24 mg) and phenetole (35 mg) in methanol–water (80:20, v/v) (50 ml).

The measurements taken from the test chromatograms were used to calculate the conventional parameters used to assess the performance of packed HPLC columns. These included plate number (N), height equivalent of a theoretical plate (HETP), reduced plate height (h), separation impedance (E), flow resistance parameter (φ) and total column porosity (ε_{tot}). Calculations followed published proce-

dures[26] using peak widths at half height to estimate column efficiencies. They were carried out using an interactive computer program written in BASIC for the Prime 550 computer by the author.

RESULTS AND DISCUSSION

The selection of glass-lined SS tubing for the column construction was based on published data which suggests that columns with smooth interior walls give higher plate counts when packed under identical conditions[20,27,28]. The performance of narrow-bore columns fabricated from glass or fused-silica tubing (0.1–0.5 mm)[20–23] has most closely approached the theoretical limits which can be achieved with conventional columns. However, the flexibility of such tubing is a practical disadvantage for routine handling which is overcome by the use of rigid glass-lined tubing.

The end-fittings of conventional SS HPLC columns are usually held in position by compression ferrules on the column tubing. Initial experiments with the glass-lined tubing (1 mm I.D.) using SS one-piece ferrules to attach 1/8-in. zero-dead volume end-fittings cracked the glass-lining. Similar results were obtained using two-piece SS or brass ferrules. The method which was finally adopted is shown in Fig. 1 and involved silver brazing a short length of 1/4-in. diameter SS tubing onto the end of the glass-lined tubing and then using a 1/4-in. zero-dead volume end-fitting with a SS ferrule. The performance of columns constructed in this way was greatly improved by the inclusion of PTFE washers each side of the mesh disc to minimise any dead volume associated with the drilling of the end-fitting leaving a slightly conical surface at the bottom of the 1/4-in. hole.

Preliminary experiments using a conventional slurry reservoir (*ca.* 30 ml, 8 mm I.D.) to pack silica (5.73 μm) into the 1-mm bore columns (300 mm) gave poor efficiencies while the packing procedure took several hours. The adoption of a slurry reservoir of comparable internal diameter to the column (Fig. 2) gave columns of satisfactory performance with the total procedure taking less than one hour. Using this apparatus a series of experiments was conducted to examine the effect of different slurry liquids on the column packing procedure and the results are presented in Table I. All packing procedures were identical except for the selection of the appropriate slurry liquid and the solvent used to push the slurry into the column. Two columns were used in this study and each packing procedure was tested on both columns. The slurry liquids were selected to be representative of the different approaches to slurry packing which have been reported in the literature, *viz.* low viscosity[29–33], high viscosity[27,34], aqueous ammonia[35] and balanced density[36–39] slurries. The plate counts for six compounds in the silica test mixture measured at a flow-rate of 80 μl/min indicated that the balanced density slurries of iodomethane–methanol and tetrabromoethane–methanol gave the best results. The results did not demonstrate any clear advantages between the two balanced density slurry liquids.

The influence of slurry concentration was investigated by packing a single column with the same weight of silica dispersed in different volumes of tetrabromoethane–methanol. In each experiment the slurry reservoir was selected to have a volume just large enough to accommodate the slurry such that the column started to pack as soon as the packing pump was switched on with no time delay. The results are shown in Table II and clearly indicate that concentrated slurries are

TABLE I

EFFECT OF DIFFERENT SLURRY LIQUIDS ON COLUMN PACKING

Columns: A and B both 300 × 1 mm I.D. Slurry preparation: Hypersil-silica (5.73 μm), 175 mg dispersed in 3 ml of the slurry liquid. Packing pressure: 8500 p.s.i. Column testing procedure involved an eluent of hexane–methanol (99.5:0.5, v/v) at a flow-rate of 80 μl/min. Compounds 1–6 are nitrobenzene, acetophenone, 2,6-dinitrotoluene, 1,3-dinitrobenzene, 2,6-dinitrobenzylchloride and 2,6-dinitrobenzonitrile respectively with capacity factors (k') ranging from 0.44 to 5.92.

Packing procedure		*Column*	*Plate counts**					
Slurry liquid	*Pressurising solvent*		*1*	*2*	*3*	*4*	*5*	*6*
Methanol	Methanol	A	1932	1844	1799	1824	1683	1634
		B	2435	2446	2585	2620	2422	2560
Methanol–ethanediol (1:1, v/v)	Methanol	A	2043	1940	1929	2079	1755	1632
		B	2214	2262	2212	2436	2287	1206
Ammonium hydroxide (0.001 *M*)	Methanol	A	2527	2670	2550	2788	2649	2867
		B	1583	1504	1470	1590	1480	1472
Iodomethane–methanol (9:1, w/w)	Hexane	A	9239	8716	10 743	11 745	11 732	11 849
		B	8293	8453	10 084	11 841	11 811	12 890
Tetrabromoethane–methanol (4:1, w/w)	Hexane	A	9204	10 334	10 633	12 411	12 140	12 823
		B	8197	9239	9204	10 991	10 870	10 642

* Mean of 3 determinations.

preferred. This has the additional advantage of speeding up the packing procedure since less time is required to pump the relatively viscous slurry liquid through the column. Balanced density slurry packing procedures for conventional HPLC columns have become rather unpopular in recent years and this can be attributed to the toxicity and high cost of the halogenated solvents used in the preparation of the slurry liquids. The small slurry volumes used for packing narrow-bore columns in this study overcome these objections both from an economic viewpoint and by the ease with which such small quantities can be safely contained thus reducing exposure risks.

TABLE II

EFFECT OF SLURRY CONCENTRATION ON COLUMN PACKING

Column: 300 × 1 mm I.D. Slurry liquid: tetrabromoethane–methanol (4:1, w/w). Packing material: Hypersil-silica (5.73 μm). Slurry concentration: 170 mg of packing material dispersed in the appropriate volume of slurry liquid. Packing pressure: 8500 p.s.i. Column testing procedure involved an eluent of hexane–methanol (99.5:0.5, v/v) at a flow-rate of 80 μl/min.

Slurry volume (ml)	*Slurry reservoir volume (ml)*	*Plate count for 2,6-dinitrotoluene*
1	1.4	14 294
3	3.5	12 502
9	9.4	12 036

The present slurry packing procedures have now been used for over 18 months and have been applied to a wide range of different packing materials. The procedures have proved to be very reproducible and a series of identical columns packed with the same batch of Hypersil-silica gave plate counts with a coefficient of variation of 6.7% (n = 7).

The procedure now adopted routinely for packing narrow-bore columns involves a slurry liquid of tetrabromoethane–methanol (4:1, w/w) with a slurry volume of 1 ml. This procedure has been applied to materials of different particle sizes and Fig. 4 shows the results of experiments to examine the relationship between the column efficiency and the eluent flow-rate for a test compound (2,6-dinitrotoluene, k' = 1.22) on typical columns packed with 4.13 and 5.73 μm silica. Fig. 4A gives a plot of plate count (N) against flow-rate for the two materials with both columns giving 14 500–15 000 plates at optimum velocity. Thus, no improvement in plate count was achieved by using the material of smaller particle size. Fig. 4B shows the same data plotted as reduced plate height (h) against reduced velocity (v). These are dimensionless parameters which allow comparisons to be made with the theoretical expectations for packed columns[26]. The 4.13-μm material gave minimum reduced

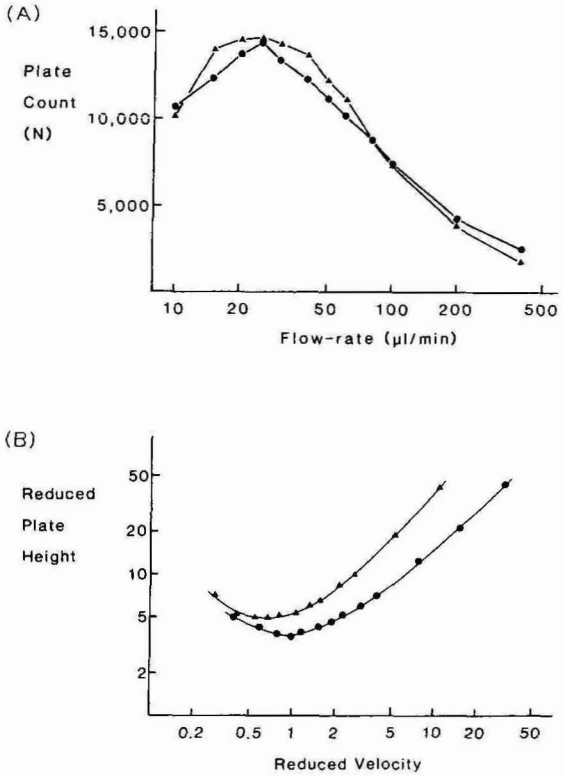

Fig. 4. The performance of silica narrow-bore HPLC columns. (A) Plots of plate count against flow-rate; (B) plots of reduced plate height against reduced velocity. Eluent: hexane–methanol (99.5:0.5, v/v). Detection: 235 nm. Test compound: 2,6-dinitrotoluene (k' = 1.22). Injection volume: 0.5 μl. Packing materials: Hypersil-silica (mean particle size: ● = 5.73 μm, ▲ = 4.13 μm).

plate heights of about 5 while the 5.73-μm material gave values of about 3.5. It is clear that the smaller particle size material is less successfully packed using the present packing procedure.

Although the optimum performance of the narrow-bore columns packed with 5.73-μm silica (h = *ca.* 3.5) falls short of that which can be achieved with well packed conventional columns (h = 2–3) the results compare favourably with previous publications involving the packing of 1 mm bore columns[12–19] where the procedures used have often been less convenient involving high pressures (>20 000 p.s.i.) or long packing times. The packing procedure has also been applied to ODS-silica (5.49 μm) using the same slurry liquid and Fig. 5 shows a plot of reduced plate height against reduced velocity for *p*-cresol (k' = 1.03) on a typical column. As for silica columns typical reduced plate heights at optimum velocity were *ca.* 3.5. It is interesting to note that Scott and Kucera[11] found that different slurry liquids were required for packing silica and ODS-silica into 1 mm bore columns.

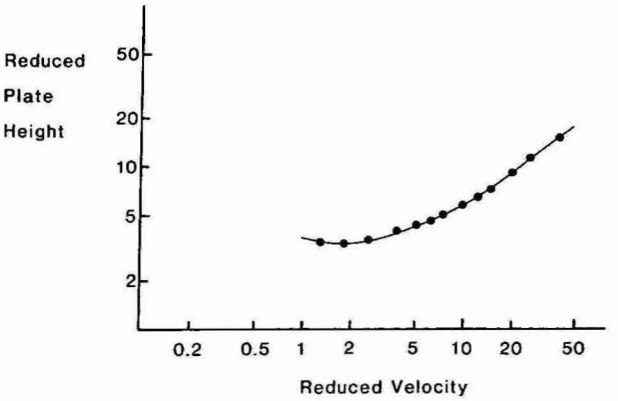

Fig. 5. The performance of an ODS-silica narrow-bore HPLC column. Eluent: methanol–water (60:40, v/v). Detection: 270 nm. Test compound: *p*-cresol (k' = 1.03). Injection volume: 0.5 μl. Column: Hypersil-ODS (5.49 μm).

The column testing procedures adopted were essentially those recommended by Bristow and Knox[26]. The test mixture for ODS-silica columns was identical to that described, *viz.* acetone, phenol, *p*-cresol, 2,5-dimethylphenol, anisole and phenetole. However, the mixture used for testing silica columns was modified from that recommended, replacing 1,3,5-trinitrobenzene which is difficult to obtain, with three new compounds (1,3-dinitrobenzene, 3,5-dinitrobenzylchloride and 3,5-dinitrobenzonitrile) which extend the retention range to k' = 5.9. Fig. 6 shows chromatograms obtained for the test mixture on a 5.73-μm silica column at two different flow-rates. The slower flow-rate of 25 μl/min is close to the minimum in the reduced plate height against reduced velocity curve (Fig. 4B) and although demonstrating optimum efficiencies the analysis times are rather long. The second chromatogram at a flow-rate of 80 μl/min, which is equivalent to a flow-rate of 2 ml/min with a conventional (5 mm) column, indicates that a satisfactory performance can be achieved on these columns with reasonable analysis times. Fig. 7 shows a chromatogram obtained with

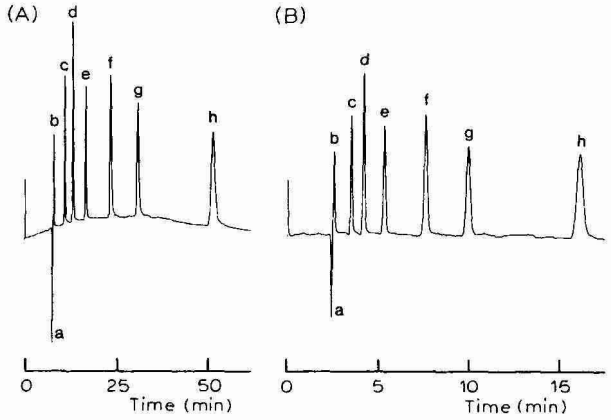

Fig. 6. Chromatograms of a test mixture on a silica narrow-bore column. Flow-rate: (A) 25 μl/min; (B) 80 μl/min (with operating pressures of 120 and 390 p.s.i. respectively). Column: Hypersil-silica, 5.73 μm (300 × 1 mm I.D.). Eluent: hexane–methanol (99.5:0.5, v/v). Detection: 235 nm. Injection volume: 0.5 μl. Peaks: a = pentane; b = toluene; c = nitrobenzene; d = acetophenone; e = 2,6-dinitrotoluene; f = 1,3-dinitrobenzene; g = 2,6-dinitrobenzylchloride; h = 2,6-dinitrobenzonitrile. The test compounds (a–h) have capacity factors (k') of 0, 0.05, 0.44, 0.72, 1.22, 2.13, 3.13 and 5.92 respectively.

the test mixture on an ODS-silica column. The peaks in all chromatograms show good peak shapes and it is clear that the column efficiencies are very satisfactory for routine analysis. Furthermore the operating pressures at typical working flow-rates were comparable to those which would be expected with conventional columns at identical linear flow-rates.

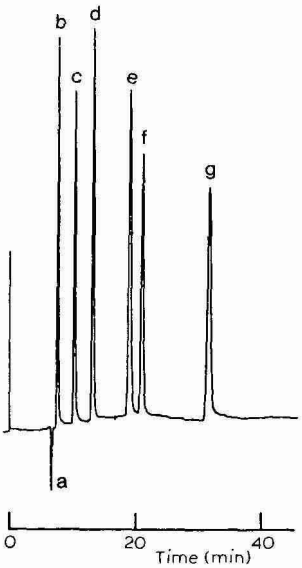

Fig. 7. Chromatogram of a test mixture on an ODS-silica narrow-bore column. Column: Hypersil-ODS, 5.49 μm (300 × 1 mm I.D.). Eluent: methanol–water (60:40, v/v). Flow-rate: 25 μl/min. Pressure: 850 p.s.i. Detection: 270 nm. Injection volume: 0.5 μl. Peaks: a = methanol–water disturbance; b = acetone; c = phenol; d = *p*-cresol; e = 2,5-dimethylphenol; f = [illegible]ole; g = phenetole. The test compounds (a–g) have capacity factors (k') of 0, 0.17, 0.60, 1.03, 1.90, 2.20 and 3.82 respectively.

Further comparison of the narrow-bore columns with conventional columns has been made by the calculation of flow resistance parameters (φ). This is a dimensionless parameter which measures the resistance to eluent flow allowing for the column length, the eluent viscosity and the particle size of the packing material. A value of about 500 is typical for conventional slurry packed HPLC columns while the present narrow-bore columns (both silica and ODS-silica) have all shown similar values (500 $\pm$ 100). This must indicate that the density of the packed bed in the narrow-bore columns is similar to that in the larger bore columns and this was confirmed by measurements of the total porosity (ε_{tot}). This parameter represents the fraction of the column bed volume which is occupied by the mobile phase and shows typical values of 0.7–0.8 for conventional columns. The narrow-bore columns showed ε_{tot} values of *ca.* 0.72 for ODS-silica (5.49 μm) and *ca.* 0.83 for silica (5.73 μm).

The separation impedance (E) is generally regarded as one of the best parameters to describe the overall performance of packed HPLC columns[26]. It is dimensionless and measures performance in terms of a high plate count, low elution time and the economical use of pressure; a decrease in E represents an improved performance. It is simply related to the flow resistance parameter and the reduced plate height by $E = h^2\varphi$ and thus an optimum value of *ca.* 2000 is expected for conventional columns with $h = 2$ and $\varphi = 500$. The present narrow-bore columns packed with 5.73-μm silica and 5.49-μm ODS-silica gave E values of 5500–6000 at optimum flow-rates. As the flow resistance parameter for the narrow-bore columns was measured to be similar to that in conventional columns it is clearly the non-optimal efficiency (high h value) which gives the high E value.

The application of the present narrow-bore columns to practical analytical problems requires some guidelines concerning the influence of sample injection on column performance and experiments have been conducted to examine both injection volume and mass loading. A reversed-phase HPLC system was chosen for these studies involving a Hypersil-ODS column (300 $\times$ 1 mm I.D.) and a buffered aqueous methanolic eluent [methanol–0.1 M sodium dihydrogenphosphate (50:50, v/v)] with injections of the drug phenacetin dissolved in the eluent. Phenacetin has a capacity factor of *ca.* 1.6 on this HPLC system. In the first series of experiments the same weight of drug (2 μg) was injected onto the column using the four different loops available for the injection valve. In each case the sample loop was completely filled with a solution of phenacetin at the appropriate concentration. An eluent flow-rate of 50 μl/min was used with detection at 220 nm. The results clearly demonstrated that there was little difference in column efficiency between the 0.5-, 1- and 2-μl loops (N = 6305, 6378 and 6347 respectively) while the 5-μl loop led to a fall in the observed column performance (N = 5637). Although a limiting injection volume of 2 μl is very small compared to conventional HPLC columns and may require samples to be further concentrated before analysis, the selection of an injection solvent with a lower elution strength should enable the injection volume to be further increased while maintaining the column performance[40].

The critical mass loading of a column has been suggested as the mass of sample which causes a 30% deterioration in the column efficiency observed with small loads[41]. Fig. 8 shows a plot of plate count against the weight of phenacetin injected in a 2-μl sample loop and using the above definition it can be seen that the maximum loading is around 10 μg for the present reversed-phase HPLC system.

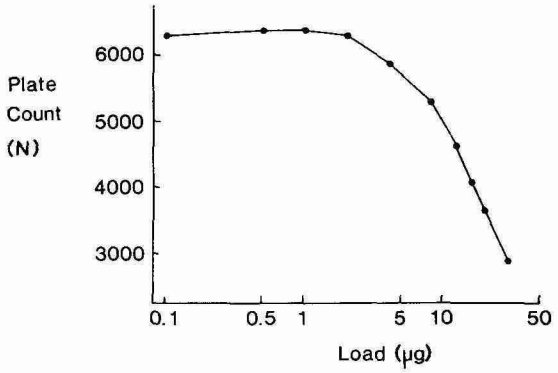

Fig. 8. The influence of mass loading on the observed column performance. Column: Hypersil-ODS, 5.49 μm (300 × 1 mm I.D.). Eluent: methanol–aqueous sodium dihydrogen phosphate (0.1 *M*) (50:50, v/v). Flow-rate: 50 μl/min. Compound injected: phenacetin dissolved in the eluent. Injection volume: 2 μl.

CONCLUSION

The present work has demonstrated a practical narrow-bore HPLC system which uses commercial equipment and columns (1-mm bore) packed in the laboratory. The columns are constructed from glass-lined SS tubing being terminated by unique end assemblies. Full details of a packing procedure are described which has proved to be both easy and reliable for both silica and ODS-silica. The packing procedure has been used in the authors laboratory for over 18 months and has consistently given columns with satisfactory performance with a very low failure rate. Furthermore the operation of these columns with the commercial hardware described has proved to be as straightforward as using conventional HPLC systems.

The performance of the columns is similar to that which has already been achieved by other workers with 1-mm bore columns but the simplicity of the packing procedure using a conventional HPLC column packing pump at < 10 000 p.s.i. is a considerable advantage. The failure to achieve reduced plate heights with the present 1-mm bore columns as good as those obtained with conventional columns may have several causes, but it is most probably the simple consequence of the narrow-bore columns being less well packed. Nevertheless, such factors as the influence of the column wall on the chromatography, extra column band broadening from the valve injector, detector flow-cell and column inlet/outlet fittings, and slow response times from the detector and chart recorder may all contribute to the overall band broadening observed in the chromatogram during a performance test. Undoubtedly, improvements could be attempted in many of these areas. There is clearly much scope for further optimisation of the packing procedure and the use of special home-made equipment may help to overcome some of the other problems as has already been demonstrated in the literature. However, it was not the intention of the present paper to strive for the maximum column performance but to describe a practical narrow-bore system with a performance satisfactory for routine analysis which can easily be set up in any laboratory.

It is hoped that the demonstration of such a practical narrow-bore system will

encourage analysts to consider the use of these columns for applications in their own fields. Data from routine analysis are urgently required by which the various claims and counter claims concerning the advantages of narrow-bore columns can be tested and the analyst can be guided in the purchase of new HPLC instrumentation with regard to the necessity for "microbore" capability. With this intention, a second paper[7] will consider the potential advantages of 1-mm bore HPLC columns with reference to routine applications in the field of forensic drug analysis.

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APPRAISAL OF NARROW-BORE (1 mm I.D.) HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COLUMNS WITH VIEW TO THE REQUIREMENTS OF ROUTINE DRUG ANALYSIS

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SUMMARY

The potential advantages of using 1-mm bore high-performance liquid chromatography columns to replace conventional 4–5-mm bore columns for routine drug analysis have been considered in the light of various claims and counter-claims in the scientific literature. Experimental work has been conducted to measure the changes in mass sensitivity when using both UV and electrochemical detection. Further work has investigated the coupling of these narrow-bore columns in order to achieve high plate counts with particular reference to the influence of peak asymmetry. Finally the linking of the narrow-bore columns to a mass spectrometer with a moving-belt interface has been examined. All results are discussed with reference to the needs of the routine analyst.

INTRODUCTION

High-performance liquid chromatography (HPLC) has found widespread application in the field of drug analysis over the last decade where most routine work is now conducted with columns of 4–5-mm bore. Recently there has been much general interest in the use of columns with reduced internal diameters and we are currently considering the potential advantages of using such columns in forensic toxicology. In a previous paper[1] one of us has described a practical narrow-bore HPLC system using 300 × 1 mm I.D. columns constructed from glass-lined stainless-steel tubing with unique zero dead-volume end assemblies. The columns, both silica and octadecyl-silica (ODS-silica), were packed in the laboratory at less than 10 000 p.s.i. and operated with a commercial pump, injector and ultraviolet (UV) detector. The study clearly demonstrated that the performance of 1-mm bore columns is very satisfactory for routine work, the packing of the columns is relatively straightforward and reproducible, and the equipment is as robust and reliable as that used with conventional columns. Nevertheless, the question remains as to whether such narrow-bore columns offer any significant advantages.

Before considering the potential advantages of narrow-bore columns over con-

ventional HPLC columns it is important to acknowledge that there are also disadvantages. Conventional HPLC equipment is generally unsuitable for operating narrow-bore columns and often requires extensive modifications by the analyst. Many instrument manufacturers are now starting to offer commercial equipment suitable for such columns but the replacement of existing hardware clearly represents a large capital investment. Flow-rates are reduced in proportion to the column cross-sectional area and hence the pumps used with 1-mm bore columns must be capable of flow-rates ranging from about 10 to 200 μl/min. Similarly peak volumes are considerably reduced and hence injectors and detector flow-cells must be of low volume to minimise extra column band broadening. The small injection volumes may also be a disadvantage from the viewpoint of sample preparation as samples may require extra concentration before analysis. Another significant disadvantage is the fact that narrow-bore columns are more difficult to pack than conventional columns. Our own technique of packing 1-mm bore columns can achieve reduced plate heights of about 3.5 which compares with typical values of 2–3 for well packed conventional columns[1].

There have been many claims published in the literature concerning the advantages of narrow-bore over conventional HPLC columns, but several questions remain relating to their application in routine analysis. Reductions in the quantities of HPLC packing materials and the volumes of eluent used have obvious economic advantages (*e.g.* a change from 5 mm to 1 mm I.D. leads to 96% savings). It is also claimed that the reduction in the peak volumes eluting from narrow-bore columns, resulting in less dilution of the injected sample, facilitates lower detection limits[2,3]. This is sometimes referred to as an increase in mass sensitivity. Some authors have highlighted the ease with which narrow-bore columns can be coupled together in series to achieve high plate counts in order to solve difficult separation problems[4,5] while others have suggested that narrow-bore columns provide an effective method of achieving high-speed HPLC separations[6–8]. Also the reduced internal diameters facilitate more effective temperature control within the HPLC column making the use of temperature programming feasible[9]. One further advantage claimed for narrow-bore HPLC is the possibility of using new detection principles arising from the reduced eluent flow-rates[10,11].

A close examination of the advantages claimed for narrow-bore columns indicates three features which would be expected to enhance the capabilities of any laboratory engaged in the routine analysis of drugs. These are the increase in mass sensitivity, the decrease in analysis time and the coupling of columns to achieve high resolution. In view of these potential advantages it is perhaps surprising that few laboratories are presently using narrow-bore HPLC columns for routine analytical work. Thus, the present paper considers these claims with particular reference to the types of problem encountered in forensic toxicology. In addition the coupling of the 1-mm bore columns with a mass spectrometer has been examined using a moving-belt interface. Although much research is underway on the development of new types of detector compatible with the low eluent flow-rates of narrow-bore columns, the mass spectrometer is probably the only new "detector" which is currently commercially available and which is likely to find application in drug analysis in the near future.

The concept of using narrow-bore columns to achieve high-speed separations in HPLC has recently been reviewed by Hartwick and Dezaro[12]. They clearly dem-

onstrate that narrow-bore packed columns offer no theoretical advantages, as the equations derived for optimising separation speeds do not include any terms relating to the column internal diameter. High-speed separations in HPLC are achieved through the use of short columns, small particles, low eluent viscosities, high pressures and high linear flow-rates. However, the authors do point out that narrow-bore columns do perhaps offer practical advantages in this field. Firstly, the fact that narrow-bore columns can be operated at high linear velocities with relatively low volume flow-rates is an important economic factor. Secondly, they point out that the optimisation of column length is an important approach to developing high-speed separations and narrow-bore columns are more easily cut and coupled to achieve this. In view of this thorough examination of narrow-bore columns for achieving high-speed separations no experimental work on this topic has been conducted in the present study. It is clear that narrow-bore columns do not offer a unique method of achieving rapid HPLC separations in drug analysis.

THEORETICAL ASPECTS OF MASS SENSITIVITY

The term "mass sensitivity" is widely used in discussions concerning narrow-bore columns and it is used in the present paper. In the current context it is really synonymus with the limit of detection (LOD) concept in chromatography. The LOD for an HPLC system is defined as that weight of a given analyte, injected on to the column, which gives a peak detectable with reasonable certainty above the baseline noise. It is normal practice to set a limiting peak height of 2 or 3 times the noise level. It is important to appreciate that the LOD is a function of the complete analytical system taking into account the dilution of the sample in the HPLC column before reaching the detector. Furthermore, the term must not be confused with the concept of minimum detectability (MD) which is a property of the detector alone and is defined for a concentration sensitive device, as the minimum concentration of analyte to give a signal discernible above the noise, generally twice the peak to peak noise. The MD has been called the minimum detectable concentration of the detector by some authors. The close similarity between the definitions for LOD and MD has led to much confusion in the chromatographic literature and the clarification of these concepts has been discussed recently[13].

It can be shown that the maximum concentration (C_{max}) of a solute at the apex of a peak eluting from an HPLC column is given by the following equation[14]:

$$C_{max} = m(N/2\pi)^{\frac{1}{2}}/V_R \tag{1}$$

where m is the mass of solute injected, N is the column plate count and V_R is the retention volume of the solute. This equation assumes a gaussian peak profile. It is possible to express the retention volume in terms of the column dimensions

$$V_R = \varepsilon_{tot} D^2 L\, \pi(1 + k')/4 \tag{2}$$

where ε_{tot} is the total porosity of the column, D is the column internal diameter,

L is the column length and k' is the solute capacity factor. Combination of eqns. 1 and 2 results in

$$C_{max} = m(8N/\pi^3)^{\frac{1}{2}}/D^2\varepsilon_{tot}L(1 + k') \quad (3)$$

Examination of eqn. 3 reveals that in an ideal situation, when all other parameters remain constant, changing the column diameter from 5 to 1 mm gives a 25-fold increase in the peak concentration of the solute reaching the detector. Assuming that the MD of the detector remains constant this represents the increase in mass sensitivity claimed for narrow-bore systems. However, it is necessary to consider whether this potential increase can be realised in practice with particular reference to the assumption concerning the MD of the detector.

The practical problems of achieving the theoretical increases in mass sensitivity are well illustrated with UV detection where most modern detectors are equipped with a range of exchangeable flow-cells having different volumes for different applications. The confusion over this topic in the published literature has mainly arisen because authors have not always differentiated between the various types of experiment which can be conducted to compare mass sensitivity. The present section attempts to clarify this situation. Fig. 1 illustrates the types of experiment which can be conducted. As before, the columns being compared are considered to be identical in every respect (L, ε_{tot} and N) except for the different internal diameters and each is operated at the same linear flow-rate. Thus the injection of a given weight of solute will give a 25-fold difference in concentration at the detector (eqn. 3).

In the first experiment (Fig. 1a) both columns are connected in turn to the same flow-cell (X) in the detector. The selection of a microbore flow-cell (typically < 1 μl) allows both columns to operate without any loss in column efficiency arising

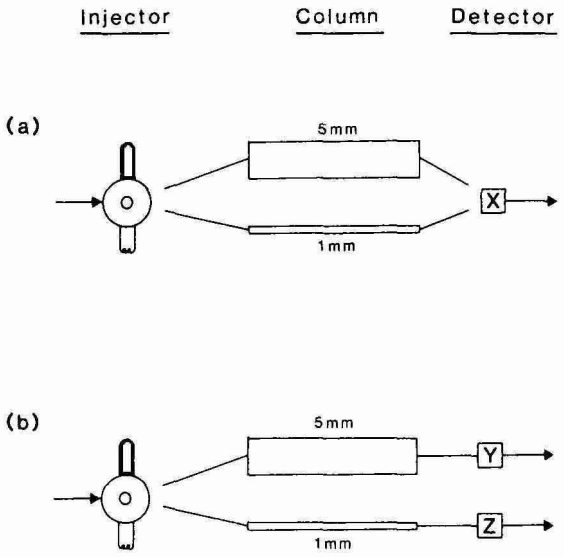

Fig. 1. Schematic diagram showing different types of experiment to compare mass sensitivities of 5- and 1-mm bore columns using a UV detector with exchangeable flow-cells. (a) Comparison using the same flow-cell (X); (b) comparison using different flow-cells (Y and Z).

from extra-column band broadening. Such experiments involve no changes to the detector and unless the difference in volume flow-rate alters the noise level, the MD of the detector will remain the same. Under such conditions the 25-fold increase in mass sensitivity should be observable. However, this experiment uses a flow-cell which is unnecessarily small for the 5 mm bore column placing it at a severe disadvantage in the mass sensitivity comparison. Fig. 1b represents an alternative experiment where each column is used with its own flow-cell of appropriate volume. For example, flow-cells Y and Z may have volumes of 8 and 0.5 μl respectively such that both columns can still be operated without any loss in performance from extra-column band broadening. Under these circumstances it is unlikely that the MD of the detector will remain unchanged since the narrow-bore flow-cell (Z) will inevitably have a shorter optical pathlength giving a reduction in detector response. Changes to the detector noise level may also occur. Thus if flow-cells Y and Z have optical pathlengths of 10 and 1 mm respectively, the potential increase in mass sensitivity is reduced from 25 $\times$ to 2.5 $\times$ based on the expected detector response. Scott and Kucera[2] have argued that such reductions are compensated by a comparable decrease in the noise level of the detector.

A third type of experiment can be considered in which a single flow-cell is used to compare the mass sensitivities but is selected to have a volume more suitable for 5-mm bore columns (*e.g.* Fig. 1a where X has a volume of 8 μl). In this case the MD of the detector is unlikely to change and any departure from the 25-fold potential increase in mass sensitivity is the result of the peaks from the narrow-bore column experiencing band broadening in the large flow-cell.

EXPERIMENTAL

Materials

Hexane, methanol and acetonitrile were HPLC grades obtained from Rathburn Chemicals (Walkerburn, U.K.). Ammonium perchlorate, 2,6-dinitrotoluene, 3,5-dinitrobenzonitrile and 3,5-dinitrobenzylchloride were obtained from Aldrich (Gillingham, U.K.). All other chemicals were obtained from BDH (Poole, U.K.). Water was distilled in glass in the laboratory. All drugs came from the collection of the Central Research Establishment, Home Office Forensic Science Service.

Columns

Narrow-bore HPLC columns (300 $\times$ 1 mm I.D.) were constructed from 1/8 in. O.D. glass-lined stainless-steel tubing as in the previous study[1] although 1/4-in. inverted end-fittings (Part No. 204, HETP, Macclesfield, U.K.) were used on some columns instead of the traditional type. The packing procedure for both silica and ODS-silica involving balanced density slurries in tetrabromoethane–methanol (4:1, w/w) has been described previously[1].

Conventional HPLC columns were 160 $\times$ 5 mm I.D. (Shandon Southern Products, Runcorn, U.K.) packed using familiar slurry procedures. Silica was packed by dispersal of the packing material in methanol and pressurising with methanol, while ODS-silica was slurried in isopropanol and pressurised with hexane.

Chromatography

Five different HPLC systems were used during the present study all using 5-μm packing materials. An eluent of hexane–methanol (99.5:0.5, v/v) on Hypersil-silica (Shandon Southern Products) was used for the separation of a test mixture of pentane, toluene, nitrobenzene, acetophenone, 2,6-dinitrotoluene, 1,3-dinitrobenzene, 2,6-dinitrobenzyl chloride and 2,6-dinitrobenzonitrile as previously described[1]. Hypersil-ODS (Shandon Southern Products) has been used with an eluent of methanol–water (60:40, v/v) for a test mixture of phenol, *p*-cresol, 2,5-dimethylphenol, anisole and phenetole[1], and with an eluent of aqueous sulphuric acid (0.02 *N*)–methanol–acetonitrile (5:8:9, v/v/v) for the characterisation of cannabis resin. This latter eluent represents a slight modification of a literature procedure by Baker *et al.*[15]. The same reversed-phase packing material has also been used for the chromatography of codeine using an eluent of methanol–aqueous sodium dihydrogen phosphate (0.1 *M*) (30:70, v/v). Several basic drugs have been chromatographed on Spherisorb-silica S5W (Phase Separations, Queensferry, U.K.) using an eluent prepared by dissolving ammonium perchlorate in methanol (0.01 *M*) and then adding a 0.1 *M* solution of sodium hydroxide in methanol (1 ml per l) giving a pH of 6.7[16].

Equipment

Chromatography was performed with a Waters M6000A pump modified by the manufacturers to give a flow-rate range from 0.001 to 9.9 ml/min. Injections were made with a Rheodyne 7410 microbore injection valve fitted with a loop filler port (Model 70-11) and equipped with a range of internal sample loops (0.5–5 μl). Chromatograms were recorded with a Linear Model 500 chart recorder having a pen response of < 0.5 s full scale.

Three variable wavelength UV detectors have been used for the studies on mass sensitivity each being equipped with exchangeable flow-cells supplied by the manufacturers. These were the Jasco Uvidec-100-III (Lea Scientific, Milton Keynes, U.K.) with 0.3- and 8-μl flow-cells, the Spectroflow 773 (Kratos Analytical Instruments, Urmston, U.K.) with 0.5-, 8- and 12-μl flow-cells and the LKB2151 (LKB Instruments, Croydon, U.K.) with 0.8- and 10-μl flow-cells. The Jasco detector with the 0.3-μl flow-cell was used for all other experiments with the 1 mm bore columns.

Electrochemical detection was performed with a wall-jet amperometric flow-cell constructed in the laboratory[17]. The flow-cell had an active cell volume of about 0.3 μl with a glassy carbon working electrode (3.1-mm diameter), a stainless-steel auxiliary electrode and a silver–silver chloride reference electrode. The inlet connections of the flow-cell were specially designed to minimise band broadening for narrow-bore HPLC. The flow-cell was operated using a LC4 electronic control unit (Bioanalytical Systems, West Lafayette, U.S.A.). A working potential of +1.2 V *vs.* silver–silver chloride reference was used for the detection of diphenoxylate.

The mass spectrometer used for interfacing experiments was a VG MM 16F magnetic sector instrument operated with a VG2250 Data System (VG Analytical, Altrincham, U.K.). A VG moving belt interface was used having an endless polyimide belt which passes through a narrow channel into the source housing of the mass spectrometer where a small nose heater vapourises the sample. All work reported in this paper has involved electron impact (EI) spectra with an accelerating voltage of 4 kV, 70 eV electron energy, 100 μA trap current and source temperatures from 190

to 230°C. The effluent from the narrow-bore HPLC columns was passed through a short length of 1/16 in. PTFE tubing (5 cm × 0.006 in. I.D.) which was connected by a 1/16-in. zero-dead volume union to a piece of stainless-steel capillary tubing (3.9 cm × 0.006 in. I.D.). The outlet end of this tube had been filed to give a sharp point and was positioned just above the surface of the moving belt.

RESULTS AND DISCUSSION

Mass sensitivity

Theoretical considerations concerning the design of experiments to compare the mass sensitivities of narrow-bore and conventional bore HPLC systems have been presented above. Although a change from 5- to 1-mm bore columns has the potential of achieving a 25-fold increase in mass sensitivity, severe practical problems are predicted and the present section describes experimental work to explore these limitations. The theoretical discussions have considered two columns which are identical in every respect except for having different internal diameters. Our experience with packing 1-mm bore columns[1] has indicated that they are more difficult to pack than conventional columns confirming other observations in the literature. The plate counts achieved with these columns are typically 50–60% of what might be expected for 5-mm bore columns of identical length while the total porosity values are similar to those observed for conventional columns. It is apparent that practical experiments to compare mass sensitivities can only be set up with two columns which have other differences besides their internal diameters. In the present work a narrow-bore column (300 × 1 mm I.D.) and a conventional bore column (160 × 5 mm I.D.) have been compared, the difference in length being selected to give similar plate counts. The columns were packed with 5-μm silica and operated with an eluent of methanol containing ammonium perchlorate (0.01 *M*) at pH 6.7[16]. Diphenoxylate and protriptyline were selected as the test compounds having capacity factors of 0.2 and 1.7 respectively on this system.

Table I shows the results obtained from experiments comparing the mass sensitivities of 1- and 5-mm bore columns using UV detection. In all experiments volume flow-rates of 24 and 600 μl/min were used for the narrow-bore and conventional columns respectively, representing identical linear flow-rates. The work has involved three commercial UV detectors each having a range of exchangeable flow-cells. No experiments have been conducted in which the two columns were compared using a "microbore" flow-cell (Fig. 1a, X having a volume of < 1 μl) since it has already been pointed out that such comparisons are predicted to show a large increase in mass sensitivity for the narrow-bore column but that this is a gross misrepresentation of the true situation as a conventional column would not normally be operated with such a small flow-cell.

The first 3 experiments shown in Table I correspond to the illustration in Fig. 1b where different flow-cells (Y and Z) are selected for each column to be as large as possible without causing extra-column band broadening. Each of these experiments was conducted with a different detector and in each case the smallest flow-cell available was selected for the 1-mm bore column (ranging from 0.3 to 0.8 μl) while flow-cells ranging from 8 to 12 μl were selected for the 5-mm bore column. It can be seen that the optical pathlengths of these flow-cells varied considerably. Operating

TABLE I

COMPARISON OF UV DETECTOR NOISE AND RESPONSE FOR INJECTIONS OF DIPHENOXYLATE AND PROTRIPTYLINE ON 1- AND 5-mm BORE HPLC COLUMNS

Each experiment involved a comparison of two columns (300 × 1 mm I.D. and 160 × 5 mm I.D.) packed with 5-μm silica (Spherisorb S5W) operated at the same linear flow-rates (*i.e.* 24 and 600 μl/min respectively). Eluent: methanol containing ammonium perchlorate (0.01 *M*) pH 6.7. Injection: diphenoxylate (1 ng) and protriptyline (0.2 ng) in methanol (2 μl).

Expt. No.	*Detector**	*Flow cell (1 mm column)*		*Flow-cell (5 mm column)*		*Detector noise (A.U. × 10^5)*		*Relative peak height response (1 mm/5 mm)*	
		Volume (μl)	*Optical pathlength (mm)*	*Volume (μl)*	*Optical pathlength (mm)*	*1 mm column*	*4 mm column*	*Diphenoxylate***	*Protriptyline***
1	A	0.8	3	10	10	3.2	2.4	2.28	2.41
2	B	0.5	1	12	8	1.2	1.2	0.99	★★★
3	C	0.3	0.3	8	10	1.3	1.6	0.13	0.09
4	B	8	10	8	10	★★★	★★★	5.07	7.47

* A = LKB2151, B = Spectroflow 773, C = Uvidec-100-III.

** Diphenoxylate and protriptyline had capacity factors (k') of 0.2 and 1.7 respectively.

*** Data not available.

the detectors at high sensitivity ranges with an appropriate eluent flow-rate enabled the short term noise levels to be measured. The results are included in Table I where it can be seen that the change of flow-cell had little effect on the noise level observed with any of the three detectors. These results differ from those reported by Scott and Kucera[2], using home-made flow-cells in a commercial detector, where the noise level was found to decrease in proportion to the optical pathlength. For example, with Detector C where the optical pathlengths of the flow-cells differ by a factor of 33 the measured noise levels were 1.3×10^5 and 1.6×10^5 AU for the 1 and 5 mm columns respectively.

The relative peak height responses (1 mm/5 mm) for injections of the drugs on to the two columns with detectors A, B and C are shown in the right hand columns of Table I (expts. 1–3). As the noise levels were relatively constant these figures are good approximations for the relative mass sensitivities. It can be seen that the narrow-bore column only showed an increase in mass sensitivity in expt. 1. The columns showed similar mass sensitivities in expt. 2 while the 5-mm bore column showed the advantage in expt. 3 by a factor of about 10. These results reflect the increasing difference between the optical pathlengths of the two flow-cells selected for each experiment with detectors A–C.

The average increase in mass sensitivity observed in the best case (expt. 1) of about 2.35 is a long way from the potential 25-fold advantage in changing from 5- to 1-mm bore columns. The use of a 1-mm bore column with approximately double the length of the 5-mm bore column in order to produce similar plate counts is predicted to reduce this factor to about 12.5 (see eqn. 3). Furthermore, the optical pathlengths of the flow-cells in expt. 1 (3 and 10 mm) differ by a factor of 3.3 which further reduces the factor to about 3.7. Consequently, the experimental result is not unreasonable in view of the approximate nature of the calculations and the unknown influence of extra-column band broadening.

The final results in Table I (expt. 4) involve a different type of experiment where both columns were tested using a single 8-μl flow-cell in detector B. In this case the narrow-bore column showed increases in mass sensitivity of 5.07 and 7.47 for diphenoxylate and protriptyline respectively. As before it can be predicted that the drug concentrations at the column exits should differ by a factor of about 12.5 but the peaks from the narrow-bore column will experience band broadening in the relatively large flow-cell such that the observed increase in mass sensitivity will be less than this figure. Once again the experimental results are reasonable and the different values for the two drugs can be explained since diphenoxylate ($k' = 0.2$) had a shorter elution time than protriptyline ($k' = 1.7$) and hence, having a smaller peak volume, experienced greater band broadening.

The operation of narrow-bore columns with conventional flow-cells provides the most effective method of using these columns to achieve significant increases in mass sensitivity but does so at the cost of sacrificing column efficiency. The practical value of such approaches depends on the type of analytical problems to be tackled. A method involving the separation of the analyte from a complex mixture of matrix peaks may not be viable with any significant loss in plate count while other methods involving a small number of well resolved components may tolerate considerable losses in the chromatographic resolution.

The practical problems of achieving large increases in mass sensitivity with

TABLE II

ELECTROCHEMICAL DETECTOR NOISE AND RESPONSE FOR THE INJECTION OF DIPHENOXYLATE ON 1- AND 5-mm BORE HPLC COLUMNS

Wall-jet electrochemical flow-cell (0.3 μl) with a glassy-carbon working electrode (3.1 mm) operated at +1.2 V *vs.* silver–silver chloride reference electrode. Packing material: Spherisorb S5W, 5 μm. Eluent: ammonium perchlorate (0.01 *M*) pH 6.7. Injection of diphenoxylate: 2 μl containing 100 ng. The drug had a k' value of 0.2 on the HPLC system.

Column	*Flow-rate (μl/min)*	*Detector* noise (nA)*	*Relative peak height response (1 mm/5 mm)*
300 × 1 mm I.D.	24	0.25	2.5–3.1
160 × 5 mm I.D.	600	0.5	

* Noise measurements were made with the 5-mm bore column operated at two flow-rates (24 and 600 μl/min) to avoid disturbing the electrochemical flow-cell between measurements.

narrow-bore columns using UV detection arise from the reductions in optical path-lengths which occur in "microbore" flow-cells designed to avoid extra-column band broadening. The miniaturisation of an electrochemical detector does not suffer from this type of problem and flow-cells with nano-litre volumes have been described (*e.g.* ref. 18). It might be expected that such detectors would be ideal for achieving the potential increases in mass sensitivity of narrow-bore columns. Table II shows the results of experiments to compare the 1- and 5-mm bore columns already considered with UV detection. A wall-jet flow-cell was used having a volume of about 0.3 μl[17] and this was expected to give little band broadening with either column. It can be seen that the eluent flow-rate had a significant effect on detector noise with the narrow-bore flow-rate (24 μl/min) giving half the noise level seen with the 5 mm bore flow-rate (600 μl/min). Experiments to determine the relative peak height response (1 mm/5 mm) for 100-ng injections of diphenoxylate gave values between 2.5 and 3.1 which translates to an overall increase in mass sensitivity for the 1-mm bore column of 5–6 when the change in noise level is also considered.

Once again the observed increase in mass sensitivity of narrow-bore columns with electrochemical detection is relatively small. In this case the problem arises from the volume flow-rates used with the two columns. Theoretical work[19] indicates that the signal from a wall-jet amperometric flow-cell is directly proportional to $V^{3/4}$, where V is the volume flow-rate. The situation is little better with a thin-layer amperometric flow-cell where the signal is directly proportional to $U^{1/2}$, where U is the linear fluid velocity within the flow-cell. In both cases the signal is also directly proportional to the concentration of the electroactive solute. The term relating to flow-rate markedly reduces the expected response from the narrow-bore column and accounts for the relatively low increase in mass sensitivity.

It is clear that the practical increases in mass sensitivity which can be achieved with 1-mm bore columns using both UV and electrochemical detection are not as large as might be expected from some claims in the literature. Whether the relatively small increases demonstrated in the present work are sufficient incentive to convert existing HPLC methods over to narrow-bore columns will depend on the nature of the analytical problem. Moreover it is important to identify those areas in routine

drug analysis where an increase in mass sensitivity in HPLC could provide significant advantages. Where samples are large and/or re-sampling is easy an increase in mass sensitivity is of little consequence. Furthermore, the use of conventional bore columns rather than narrow-bore columns should be encouraged since the handling of large sample volumes should improve the precision of a quantitative analysis. Some aspects of the work performed in forensic toxicology fall into this category *e.g.* the examination of body fluids and tissues following an autopsy. In contrast, where samples are small and re-sampling is difficult or impossible, an increase in mass sensitivity would allow smaller aliquots to be taken for each analysis. A good example of this type of problem in forensic toxicology is the blood samples presented for examination following road traffic offences where the volumes are often less than 5-ml and multiple analyses are required to screen for unknown drugs. In such cases the relatively small increases in mass sensitivity may still prove to be valuable.

Column coupling

The production of high plate counts in HPLC through the use of long columns of conventional bore (> 30 cm, 4–5 mm I.D.) is not practical as such columns are very difficult to pack. Many workers have coupled conventional columns in an attempt to increase plate counts but this has often not been completely successful with the overall plate count being less than the sum of the plate counts of the individual columns (*e.g.* ref. 4). Nevertheless the literature does contain examples where such column concatenation has achieved additive plate counts[20–22]. At the present time it is not clear why different authors have experienced different results but discussions have centred on the method of coupling (butting columns end to end or using narrow capillary tubing) as well as on the intrinsic properties of the columns to be coupled. It has been argued that additive plate counts can only be achieved with columns showing good peak symmetries[23] and more recently that the columns require special packing techniques[24]. Despite the difficulties experienced with conventional columns, the coupling of small bore columns (≤2 mm I.D.) appears to be much more successful and additive plate counts have been reported by several workers[4,5,25–27].

Experiments have been conducted with the present 1 mm bore columns coupled together with short lengths (5 cm) of narrow capillary tubing (0.006 in. I.D.). It has been found that the coupling of two or three columns has consistently given additive plate counts when making measurements using the peak width at half-height method. Furthermore, the influence of peak asymmetry on the additivity of plate counts[23] has also been tested. Three narrow-bore silica columns were selected with greater than average asymmetry factors ranging from 1.33 to 1.43 when operated with the standard test mixture. Nevertheless, on coupling these three columns, the overall plate count was found to be additive (Table III). A further experiment was conducted in which a large peak asymmetry was deliberately induced by the use of codeine as the test compound and the selection of a methanol–phosphate buffer eluent with an ODS-silica column. It is well known that many basic drugs show poor peak shapes on ODS-silica unless amine additives are present in the mobile phase (*e.g.* ref. 28). In this second experiment (Table IV) codeine showed peak asymmetries of 3.42 and 2.85 on the two separate ODS-silica columns yet additive plate counts were still observed when the columns were coupled. It is interesting that the coupled columns showed a further deterioration of the codeine peak shape in this case.

TABLE III

PLATE COUNTS AND ASYMMETRY FACTORS FOR THE COUPLING OF THREE SILICA COLUMNS

Columns (A, B and C): each 300 × 1 mm I.D. packed with Hypersil-silica (5 μm). Eluent: hexane–methanol (99.5:0.5, v/v). Flow-rate: 25 μl/min. Detection: 235 nm. Solute: 2,6-Dinitrotoluene (k' = 1.23). Injection: 0.5 μl of eluent containing 100 ng of solute.

Column(s)	*Plate count (N)*	*Asymmetry factor (A_s)*
A	13 140	1.33
B	13 156	1.43
C	13 413	1.34
A + B + C	40 459	1.34

TABLE IV

PLATE COUNTS AND ASYMMETRY FACTORS FOR THE COUPLING OF TWO ODS-SILICA COLUMNS

Columns (X and Y): each 300 × 1 mm I.D. packed with ODS-Hypersil (5 μm). Eluent: methanol–aqueous sodium dihydrogen phosphate (0.1 *M*) (30:70, v/v). Flow-rate: 50 μl/min. Detection: 284 nm. Solute: codeine (k' = 0.58). Injection 0.5 μl of eluent containing 2.2 μg of solute.

Column(s)	*Plate count (N)*	*Asymmetry factor (A_s)*
X	3209	3.42
Y	2766	2.85
X + Y	5982	4.31

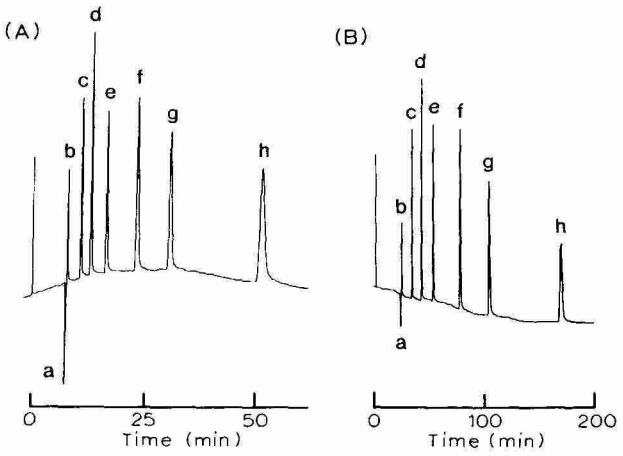

Fig. 2. Chromatograms of a test mixture on silica narrow-bore columns (300 × 1 mm I.D.). (A) Single column; (B) three columns coupled in series using capillary tubing (5 cm × 0.006 in. I.D.). Packing material: Hypersil-silica, 5 μm. Eluent: hexane–methanol (99.5:0.5, v/v). Flow-rate: 25 μl/min. Detection: 235 nm. Injection volume: 0.5 μl. Peaks: a = pentane; b = toluene; c = nitrobenzene; d = acetophenone; e = 2,6-dinitrotoluene; f = 1,3-dinitrobenzene; g = 2,6-dinitrobenzyl chloride; h = 2,6-dinitrobenzonitrile.

In our experience the coupling of 1-mm bore columns is a practical and reliable method of achieving high plate counts and hence improving the resolution between compounds in HPLC. This is demonstrated in Fig. 2 for the separation of the standard test mixture on three silica columns. Nevertheless it must always be borne in mind that the increase in column efficiency is achieved at the expense of longer analysis times (*cf.* Fig. 2A and B). In the context of routine drug analysis it is debatable under what circumstances such extended analysis times might be tolerated to achieve the advantages of better resolution.

For the analysis of drugs in complex matrices, often of biological origin, it is common to encounter a situation where the compound of interest is not resolved from the other components in the sample. Although it might be possible to achieve base-line resolution by modification to the eluent or to the sample preparation pro-

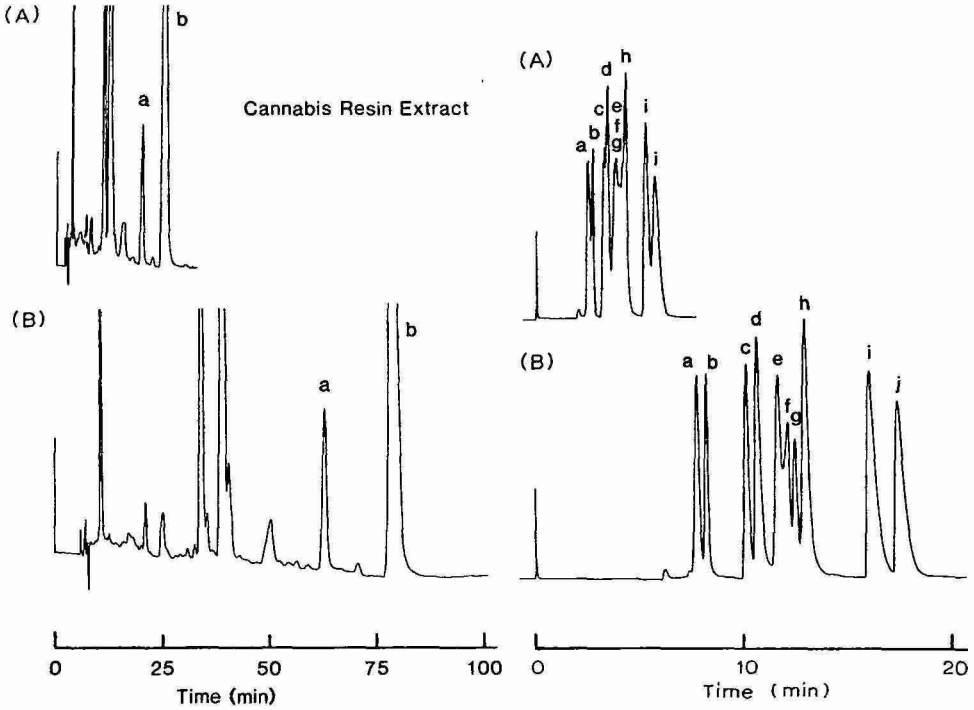

Fig. 3. Chromatograms of a cannabis resin extract on ODS-silica narrow-bore columns (300 × 1 mm I.D.). (A) Single column; (B) three columns coupled in series. Packing material: ODS-Hypersil, 5 μm. Eluent: sulphuric acid (0.02 *N*)–methanol–acetonitrile (5:8:9, v/v/v). Flow-rate: 70-μl/min. Operating pressures: 1250 and 4900 p.s.i. for one and three columns respectively. Detection: 220 nm. Resin extracted with methanol–chloroform (4:1, v/v) with injections of 2 μl. Peaks: a = cannabinol (CBN); b = delta-9-tetrahydrocannabinol (THC).

Fig. 4. Separation of ten basic drugs on silica narrow-bore columns (300 × 1 mm I.D.). (A) Single column; (B) three columns coupled in series. Packing material: Spherisorb S5W, 5 μm. Eluent: ammonium perchlorate (0.01 *M*) in methanol adjusted to pH 6.7 with sodium hydroxide. Flow-rate: 80 μl/min. Operating pressures: 800 and 2750 p.s.i. for one and three columns respectively. Detection: 254 nm. Drugs dissolved in methanol, 2 μl injection. Peaks: a = diphenoxylate; b = caffeine; c = methysergide; d = lysergamide; e = anileridine; f = amphetamine; g = tyramine; h = practolol; i = protriptyline; j = desipramine.

cedure, or even by the selection of a new column packing material or the use of a more specific detector, in some circumstances it may be more convenient to use the same HPLC system with coupled columns of higher efficiency. In a similar way column coupling may prove to be useful for the comparison of illicit drugs of biological origin (*e.g.* cannabis, opium) where the chromatogram shows a complex pattern of largely uncharacterised components. Such patterns can be very useful for intelligence purposes[15]. Fig. 3 shows how three coupled ODS-silica columns can provide a more detailed "fingerprint" of a cannabis resin extract by the separation of many more components.

At the present time one of the important roles of HPLC in forensic toxicology is to provide a tentative identification of unknown drugs from retention measurements. In this respect many HPLC systems have been developed for the separation of drugs within a given class (*e.g.* barbiturates[29], amphetamines and narcotic analgesics[30], local anaesthetics[31]). Such systems depend on the number of compounds which can be resolved across the chromatographic range and this can be considerably increased by the use of columns with higher efficiency. It is in this area that coupled narrow-bore columns might find further applications. Fig. 4 shows the separation of 10 basic drugs on Spherisorb S5W using an eluent system recommended for basic drug screening[16]. It is clear that it is possible to differentiate between many more compounds using the coupled columns. Furthermore it is particularly worth noting that all 10 drugs elute with k' values of less than 2, a region in an HPLC chromatogram where resolution is generally poor.

The mass spectrometer as a detector

Several interfaces are now commercially available for the on-line combination of HPLC with mass spectrometry (MS) including moving-belt, direct liquid inlet (DLI) and thermospray devices. Recent reviews have described the current progress in this area[32,33]. In the present study only a moving-belt interface on a magnetic sector instrument operated in the electron-impact (EI) mode has been examined. This interface involves the deposition of the effluent from the HPLC column on to a moving polyimide belt which passes through a narrow channel into the source region of the mass spectrometer where it is heated to effect volatilisation.

As a detector for HPLC the mass spectrometer has enormous potential for drug analysis. It can be operated as a universal detector by continuous scanning over a wide mass range or as a very selective detector operated in the multiple ion detection (MID) mode. In theory the continuous scanning mode should offer the greatest flexibility since through computer reconstruction methods it should be possible to obtain chromatograms using the total ion current (TIC) or at any specific mass ion as well as obtaining a complete mass spectrum for any separated component. However, experience with the belt interface has shown that there are numerous background ions originating from the belt itself which limit the value of the scanning mode when analytes have significant ions at low masses[34]. Useful mass spectra with ions below m/z 150 are often difficult to obtain, even with the application of computer background subtraction methods, except when drug concentrations are very high. Furthermore, the TIC detection mode often gives noisy chromatograms and hence is rather insensitive for drug detection. In contrast, the detection limits for many drugs using the MID mode are in the low ng range[34].

The HPLC–MS belt interface relies upon the efficient evaporation of solvents from the eluent before the analyte on the belt reaches the source region of the mass spectrometer. Initial experiments using conventional HPLC columns encountered considerable problems associated with the use of flow-rates $\geqslant 1$ ml/min and the use of eluents with a high water content. Satisfactory results could only be obtained when a powerful infra-red heater was positioned over the belt in the inlet chamber immediately after the effluent was deposited on the belt. Furthermore the inlet chamber had to be maintained under vacuum. The intense source of heat was responsible for the thermal decomposition of several sensitive drugs and for a considerable reduction in the useful life of the moving belt[34].

The low flow-rates used with narrow-bore HPLC columns have obvious advantages for use with belt interfaces in HPLC–MS and several workers have now explored this approach[35–39]. Previous HPLC–MS studies in our laboratory have used 0.5 mm I.D. columns constructed from thick-walled PTFE tubing, but these columns showed very poor plate counts. Their replacement with the present 1 mm bore columns has greatly improved the chromatographic performance and reliability of the HPLC–MS equipment. With the use of flow-rates $\leqslant 100$ μl/min it has been possible to operate the interface with the infrared heater switched off and with the inlet chamber at atmospheric pressure while the use of eluents containing a high proportion of water is now feasible.

Experiments have been conducted to examine the chromatographic performance of the 1 mm bore HPLC columns using the mass spectrometer as a detector. The plumbing was arranged such that the column effluent passed through *ca.* 9 cm of capillary tubing (0.006 in. I.D.) before deposition on the belt. An ODS-silica column was used with an eluent of 60% methanol and injecting a test mixture containing phenol, *p*-cresol, 2,5-dimethylphenol, anisole and phenetole. As the molecular weights of these compounds are relatively small, ranging from 94 to 122, the MID detection mode was necessary to facilitate sensitive detection. Fig. 5 shows the chromatograms obtained for the 5 compounds at three detection ions (*m/z* 94, 108 and 122) and clearly demonstrates the facility of the mass spectrometer to act as a very selective detector for HPLC. By the superposition of the appropriate peaks from Fig. 5 on to a single time axis (Fig. 6) it is possible to appreciate the excellent chromatographic resolution which can be achieved with the present equipment. Furthermore, comparison of Fig. 6 with the test chromatograms using UV detection shown in the previous paper[1] indicates that the loss in chromatographic performance with the mass spectrometer as a detector is very small. This agrees with the work of Games *et al.*[40] who have concluded that the moving belt interface on a mass spectrometer has a low time constant and a low effective dead volume.

For the routine drug analyst the prospect of an HPLC detector with the versatility of a mass spectrometer is very exciting. In particular, the high selectivity of MID promises less arduous sample preparation procedures for the quantification of drugs in body fluids, while continuous scanning offers information on the identity of unknown components. Although continuous scanning is at present rather insensitive there are some problem areas where samples are plentiful and the technique has proved to be particularly useful, *e.g.* the identification of acid/neutral impurities in illicit heroin samples[41]. This last example clearly demonstrates the need for on-line HPLC–MS as the chromatograms are extremely complex such that off-line fraction collection and transfer to a mass spectrometer would be virtually impossible.

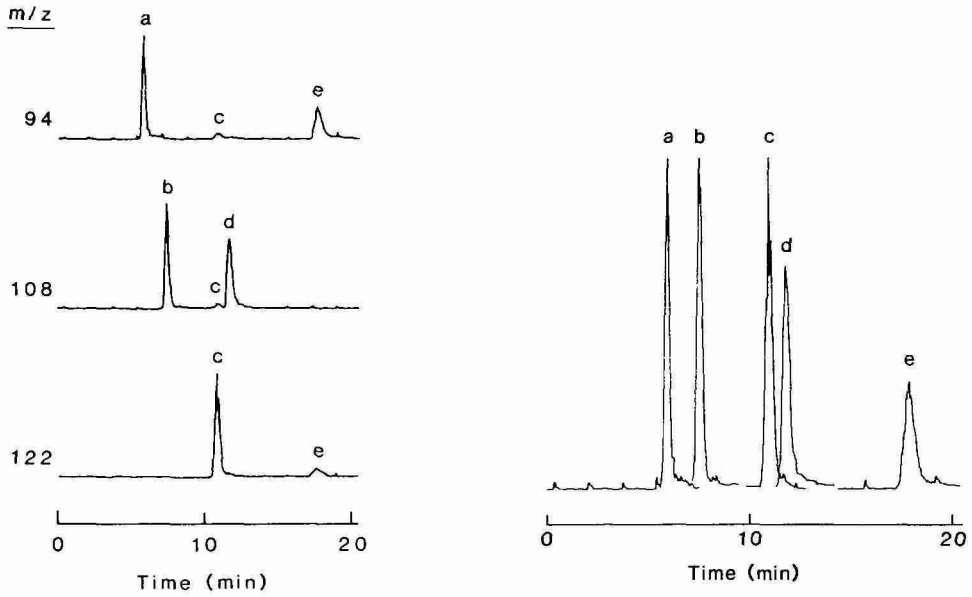

Fig. 5. HPLC–MS of a standard test mixture on an ODS-silica narrow-bore column using multiple ion detection (MID) at *m/z* 94, 108 and 122. Column: Hypersil-ODS, 5 μm (300 × 1 mm I.D.). Eluent: methanol–water (60:40, v/v). Flow-rate: 80 μl/min. Injection volume: 2 μl (test compounds in eluent). Peaks: a = phenol; b = *p*-cresol; c = 2,5-dimethylphenol; d = anisole; e = phenetole.

Fig. 6. Chromatogram of a standard test mixture on an ODS-silica narrow-bore column produced by overlaying the MID traces shown in Fig. 5. Peaks: a = phenol (*m/z* 94); b = *p*-cresol (*m/z* 108); c = 2,5-dimethylphenol (*m/z* 122); d = anisole (*m/z* 108); e = phenetole (*m/z* 94).

CONCLUSION

The present paper has addressed the question as to whether narrow-bore HPLC columns (1 mm I.D.) offer any important advantages over conventional columns for routine drug analysis. Large savings (>95%) on consumables (packing materials and solvents) are certainly significant when considering the running costs of narrow-bore systems. Nevertheless it is questionable whether this alone would be enough to justify the necessary capital investment in replacement pumps, injectors and detectors to accommodate such columns in the short term. Such economic factors might only prove to be important when expensive solvents or packing materials (*e.g.* chiral stationary phases for the analysis of enantiomeric drugs) are used.

The present experimental work has demonstrated some increases in mass sensitivity for the narrow-bore columns over conventional columns using both UV and electrochemical detection. However, it is clear that the increases in mass sensitivity which might be predicted from simple considerations of decreasing column diameter are difficult to achieve in practice. The problems encountered with UV detection are particularly significant as the vast majority of routine analyses use these detectors. Such relatively small increases (5–7.5 in the most favourable circumstances) would probably only prove to be of value in circumstances where the sample size is severely restrictive.

On a more encouraging note the coupling of 1 mm bore columns has proved to be an extremely effective method of producing columns with very high plate counts. Such coupled columns have been shown to be capable of separating complex drug mixtures. Nevertheless it is questionable how many routine drug analyses require more than the 10 000–15 000 plates currently achievable with a single column of conventional bore.

Finally, our experience has confirmed that narrow-bore (1 mm) HPLC columns solve many of the chromatographic problems associated with the use of the moving-belt HPLC–MS interface. In a similar way narrow-bore columns are being used with other types of HPLC–MS interface[32,33]. Research and development into interface design continues at a rapid pace but HPLC–MS is still in its infancy and no single device is yet able to handle all types of compound successfully. Consequently HPLC–MS can not be described as a routine tool at this stage. As narrow-bore columns have been shown to be advantageous with several types of interface they would appear to have a secure future in this area. It is worthy of note that the latest commercial interface, the thermospray[42], can operate with virtually any solvent including water with flow-rates up to at least 2 ml/min and hence is well suited to conventional columns.

Thus 1 mm bore HPLC columns do offer some advantages over the use of conventional columns, but these advantages are perhaps not as great as might be suspected from the vast literature which has been published over the last five years. Furthermore, for the routine drug analyst it may seem difficult to justify the necessary investment to change over to such columns at the present time. Nevertheless practical 1 mm bore systems are easy to set up and reliable in operation, and will always find applications where difficult analytical problems dictate that the properties of such narrow-bore columns are truely significant.

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CHROM. 18 228

SIMPLE METHOD TO PREPARE NON-CHARGED, AMPHIPHILIC AGAROSE DERIVATIVES, FOR INSTANCE FOR HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

γ-Glycidoxypropyltrimethoxysilane is coupled in an aqueous medium to agarose via the trimethoxy groups. The epoxide ring can then be employed for attachment of, for instance, alcohols in solutions of dioxane or acetone and in the presence of boron trifluoride diethyl etherate as a catalyst. In the range pH 4–10 the stability of the linkage between the ligand and agarose is similar to that of ligands coupled to agarose via an ether bond. A method is also described where the epoxide reaction with alcohols is catalyzed by stannic chloride. Both catalysts can degrade cross-linked agarose. However, by using appropriate experimental conditions for the stannic chloride-catalyzed reaction the degradation is negligible, which is of importance when high flow-rates are desired as in high-performance liquid chromatography.

INTRODUCTION

We have previously described a method for the preparation of agarose derivatives by which glycidyl ethers of the components to be attached are allowed to react with the hydroxy groups in the agarose beads[1]. This technique was used particularly for the preparation of amphiphilic, neutral agarose beads for hydrophobic interaction chromatography. The first step in this coupling method involves the synthesis of the glycidyl ether from an alcohol and epichlorohydrin in the presence of boron trifluoride diethyl etherate as a catalyst[2]. We will now describe a coupling method in which this relatively time-consuming synthesis step is avoided. This simple immobilization method, which we have used for several years with good results, involves as a first step the preparation of an epoxide-containing agarose derivative. Compounds con-

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taining hydroxy groups, for instance alcohols, are then coupled to the agarose via the epoxide group with either boron trifluoride or stannic chloride as a catalyst.

MATERIALS

γ-Glycidoxypropyltrimethoxysilane (Silane Z-6040) was a gift from Dow Corning (Midland, MI, U.S.A.). Boron trifluoride diethyl etherate was obtained from Merck-Schuchardt (Darmstadt, F.R.G.) and stannic chloride from Mallinckrodt (St. Louis, MI, U.S.A.). Sepharose® 4B, Sepharose® CL-4B and Octyl-Sepharose® CL-4B were gifts from Pharmacia Fine Chemicals. Agarose (EEO, −0.17) was obtained from Réactifs IBF (Villeneuve, La Garenne, France).

Phycoerythrin and phycocyanin from *Ceramium rubrum* were prepared as described[3].

EXPERIMENTAL AND RESULTS

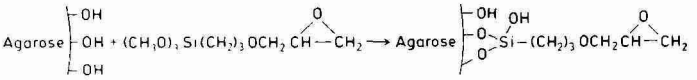
Synthesis of γ-glycidoxypropylsilylagarose

The reaction scheme is outlined in Fig. 1a. About 35 g of sedimented Sepharose 4B (agarose concentration: about 4%) was washed three or four times with 100-ml portions of water, and resuspended in 100 ml of water; 10 ml of γ-glycidoxypropyltrimethoxysilane were added dropwise from a pipette. The suspension was stirred at room temperature for 30 min. The pH was around 6; it was not adjusted to any particular value, since the reaction is not pH sensitive (see below). The gel beads were washed several times with dioxane (or acetone) and then suspended in 100 ml of dioxane (or acetone).

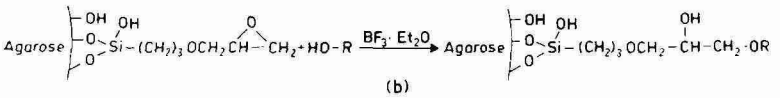
Coupling of alcohols to γ-glycidoxypropylsilylagarose with boron trifluoride as a catalyst (method A)

The principle of the reaction is shown in Fig. 1b. The coupling procedure will be described for octanol, although the same technique can be used for other alcohols (although the reaction times may be different).

A 5-ml volume of boron trifluoride diethyl etherate ($BF_3 \cdot Et_2O$) was added with stirring to the above suspension of γ-glycidoxypropylsilylagarose in dioxane (or



(a)



(b)

Fig. 1. (a) The reaction between agarose and γ-glycidoxypropyltrimethoxysilane. The number of bonds to the agarose (here assumed to be two) depends, among other things, on the number of sterically available hydroxy groups in the agarose. (b) The reaction between alcohols and γ-glycidoxypropylsilylagarose.

acetone) followed by 5 ml of *n*-octanol. After stirring for 45 min the gel was washed on a büchner funnel or by centrifugation at 1000 *g* according to the following procedure: dioxane, 100 ml; dioxane–acetone (1:1), 100 ml; acetone, 100 ml; five 100-ml portions of water.

Test for covalent attachment of octanol to agarose

Part of the Sepharose 4B gel, presumed to contain octyl groups, was packed into a Pasteur pipette and equilibrated with 0.01 *M* sodium phosphate (pH 6.8) containing 4 *M* sodium chloride. The red protein phycoerythrin was adsorbed strongly to the column in the latter buffer, but was eluted easily upon omission of the sodium chloride. In a control experiment, phycoerythrin was not adsorbed to γ-glycidoxypropylsilylagarose treated with boron trifluoride as described above but without addition of octanol. The adsorption of the protein was accordingly due to hydrophobic interaction with the octyl groups[4,5]. An indication that these groups were covalently coupled to agarose was the fact that the adsorption of phycoerythrin was not affected by extensive washings of the gel with various solvents: acetone–water (1:2, 2:1); acetone; ethylene chloride–acetone (2:1, 1:2); ethylene chloride; tripropylene glycol methyl ether–water (1:2, 2:1); tripropylene glycol methyl ether; dioxane–water (1:2, 2:1); dioxane; hexane–dioxane (1:2, 2:1); hexane; decanol–dioxane (1:2, 2:1); decanol. Additional evidence for covalent binding of the ligand was obtained as follows. The gel was first liquified by boiling. When the temperature decreased to room temperature a gel was formed again. The gel block was granulated by pressing it through a net[6]. The gel granules still retained the capacity to adsorb phycoerythrin at high (but not low) ionic strength.

The long-term pH stability of the amphiphilic agarose derivatives

It is of some importance to know the pH-stability of the bond between the ligand and agarose. To measure this we first tried to determine the amount of silicon in the gels, but could not obtain reproducible analyses. Therefore, we decided to measure the protein capacity of the gels, *i.e.*, the amount of phycoerythrin adsorbed to octylagarose treated with buffers of various pH; a decrease in adsorption indicates a breakage of the ligand bond. The experiment was performed as follows.

Portions (2 ml) of a suspension of a uniform slurry containing 1 g of sedimented octylagarose, prepared as described above, were pipetted into each of 55 Pasteur pipettes equipped at the bottom with a plug of glass wool. These columns were divided into eleven groups (1–11), each group containing five columns (I–V). The columns I–V in each group were equilibrated with 0.1 *M* sodium citrate–phosphate buffers (the molarity refers to the phosphate ions) of pH 4.0, 5.6, 7.0 and 0.1 *M* glycine–sodium hydroxide buffers of pH 8.6 and 9.8, respectively; 5.0 ml of each of these five buffers were used for equilibration, which proceeded for about 2 h. The columns in group 1 were then equilibrated with 5.0 ml of 0.01 *M* sodium phosphate buffer, pH 7, containing 4 *M* sodium chloride. A 2-ml volume of phycoerythrin in this buffer ($A_{500}^{1\,cm} \approx 0.35$) was applied to each of these five columns. After washing with 1 ml of the same buffer, the absorbance, A_e, at 500 nm of the 3 ml of eluate was measured. The absorption, A_s, of 2 ml of the sample solution of phycoerythrin diluted with 1 ml of the buffer was also determined. The amount of phycoerythrin adsorbed is proportional to $A_s - A_e$.

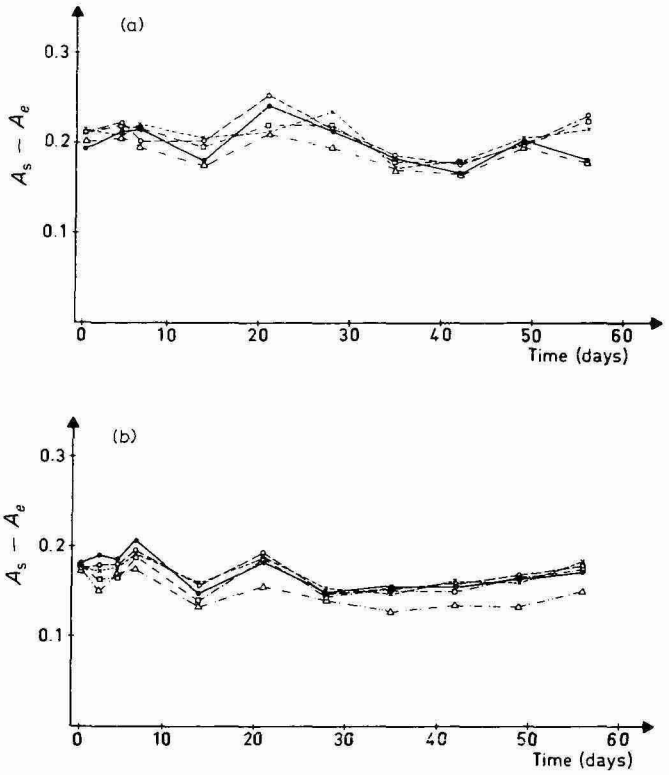


Fig. 2. A test of the pH-stability of octylagarose prepared by method A (a) and of commercial octyl-Sepharose CL-4B (b). $A_s - A_e$ is a measure of the protein capacity of the column. The capacity (which is related to the ligand density) was measured after exposure of the gels to different pH values during 8 weeks. As seen, the stability is good in the range pH 4–10. Octyl-Sepharose CL-4B is cross-linked, whereas octylagarose, prepared as described above, is not. pH values: 4.0 (△— · · —△); 5.6 (●—●); 7.0 (○---○); 8.6 (×---×); 9.8 (□— · —□).

The columns in group 2 were left to stand at the five different pH values for 3 days and were then equilibrated with the sodium chloride-containing phosphate buffer of pH 7. Phycoerythrin solution was added and the amount adsorbed was calculated from absorption measurements as above. The same procedure was then repeated for the columns in groups 3–11 after 5, 7, 14, 21, 28, 35, 42, 49 and 56 days, respectively; to increase the accuracy the experiments were done in duplicate, 110 columns being used in all. The results are shown in Fig. 2a.

For comparison the same experiments were performed on columns packed with the commercial Octyl-Sepharose CL-4B (Fig. 2b). Sepharose CL-4B (without octyl ligands) showed no adsorption of phycoerythrin in the 0.01 *M* phosphate buffer containing 4 *M* sodium chloride. Fig. 2a and b shows that the non-cross-linked octylagarose prepared by the present method has about the same stability in the range pH 4–10 as the commercial cross-linked product prepared by reaction with octyl glycidyl ether[1], which gives a stable ether bond.

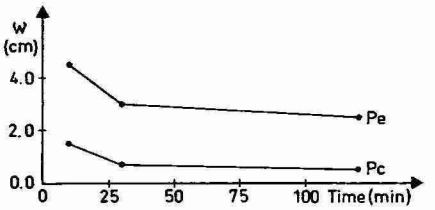


Fig. 3. Effect of the reaction time on the synthesis of γ-glycidoxypropylsilylagarose. w = Width of the phycoerythrin (Pe) and the phycocyanin (Pc) zones.

Effects of reaction time and pH on the synthesis of γ-glycidoxypropylsilylagarose

One batch of γ-glycidoxypropylsilylagarose prepared exactly as described above was compared with two others prepared in the same way except that the reaction times were 10 and 120 min, respectively. After coupling of octanol by the procedure described (using $BF_3 \cdot Et_2O$ as a catalyst) the three batches were packed into Pasteur pipettes and equilibrated with 0.01 *M* sodium phosphate buffer, pH 7.0, containing 4 *M* sodium chloride. A sample of 50 μl of the coloured proteins phycoerythrin and phycocyanin was applied to each column followed by 5 ml of the buffer. The widths of the two protein zones were measured. These are related to the protein capacity, *i.e.*, the number of available octyl groups of the columns: the smaller the width the larger is the capacity. The results are presented in Fig. 3, which shows that the reaction between γ-glycidoxypropyltrimethoxysilane and agarose is complete within 30 min.

The effect of pH on this reaction was studied by a similar technique: four batches of γ-glycidoxypropylsilylagarose were prepared, at initial pH values of 4.0, 6.5, 9.0 and 11, respectively (adjusted by addition of hydrochloric acid or potassium hydroxide). After 5 min the pH values were 4.0, 6.2, 7.7 and 8.5 and after 30 min they were 3.9, 6.0, 7.0 and 8.1 respectively. After attachment of octanol the relative capacities of these batches for phycoerythrin was estimated by packing them into Pasteur pipettes and measuring the width of the protein zone as described. These experiments indicated that the capacity, and therefore the synthesis of γ-glycidoxypropylsilylagarose, is independent of the pH at which the synthesis is performed (at least in the interval pH 4–11).

Coupling of alcohols to γ-glycidoxypropylsilylagarose with stannic chloride as a catalyst (method B)

The use of this catalyst will also be described in detail because it has some advantages over boron trifluoride (see Discussion). The dioxane (or acetone) in the suspension of γ-glycidoxypropylsilylagarose was exchanged for the alcohol to be coupled by the following washing steps: dioxane–water (1:1), 100 ml; dioxane, 100 ml; dioxane–the alcohol to be coupled (1:1), 100 ml; the alcohol, 100 ml.

The beads (cross-linked by a method to be described elsewhere) were then suspended in 100 ml of the alcohol and the catalyst stannic chloride ($SnCl_4 \cdot 5H_2O$) was added to a concentration of 0.4% (w/v) with stirring. The coupling was allowed to proceed with stirring for 1 h at room temperature. The beads were washed and transferred to water as described above for procedure A. The stannic chloride-cata-

TABLE I

CAPACITY FACTOR, k', FOR TRANSFERRIN AND THE RIGIDITY OF THE AGAROSE BEADS (EXPRESSED AS MAXIMUM FLOW-RATE) FOR VARIOUS CATALYST CONCENTRATIONS AND REACTION TIMES

$SnCl_4 \cdot 5H_2O$ (%, w/v)	Reaction time (h)	k'	V_{max} (relative values)
0.1	0.5	1.3	–
0.1	1	1.8	4
0.1	2	3.0	3
0.1	3	3.5	3
0.1	4	∞	1
0.2	1	∞	5
0.4	1	∞	5

lyzed reaction has been used for coupling of pentanol and octanol. Other alcohols may require other reaction times and catalyst concentrations.

The reaction time (1 h) and the amount of stannic chloride (0.4%) in the above protocol were selected from the data in Table I, which shows the capacity factor, k' (which is related to the ligand density), and the relative rigidity of the beads (expressed as the maximum flow-rate obtained for a bed packed with the beads) for different reaction times and catalyst concentrations. The experiments were performed in 0.05 *M* sodium phosphate (pH 7) containing 1.3 *M* ammonium sulphate. Table I shows that when the reaction time is 1 h the catalyst concentration should be 0.2–0.4% for strong adsorption of transferrin and for a high flow-rate. The low flow-rates obtained at long reaction times indicate that some bonds in the cross-linked agarose are broken by stannic chloride, as is the case with boron trifluoride (see Discussion).

The coupling conditions for methods A and B above refer to 4 and 12% agarose beads respectively. Some changes may be required when agarose beads of other concentrations are used.

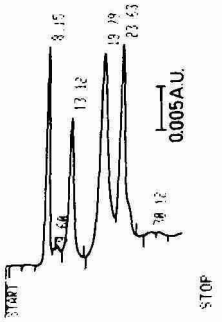


Fig. 4. High-performance hydrophobic interaction chromatography on octylagarose. The octyl groups were attached to 12% agarose by method B. Bead size: 5–7 μm. The sample consisted of 20 μl of a mixture of cytochrome *c* (retention time, t_R = 8.15), ribonuclease (t_R = 13.12), transferrin (t_R = 19.79) and lysozyme (t_R = 23.63). The elution was performed at a flow-rate of 0.2 ml/min on a 63 × 6 mm I.D. column. Desorption was achieved by isocratic elution during 5 min in 0.05 *M* sodium phosphate (pH 7.0) containing 1.0 *M* ammonium sulphate and then during 25 min by a negative linear salt gradient formed from this solution and 0.05 *M* sodium phosphate (pH 7.0).

Applications

We have recently shown that agarose beads prepared under special conditions can be used with advantage for high-performance liquid chromatography (HPLC) of biopolymers[7–16]. We have attached octyl groups to these beads by the techniques described in this paper. Fig. 7 in ref. 10 shows an example of an experiment done on an octylagarose column 1 year after the preparation of the beads, indicating that the columns can be employed over a long period of time (the coupling was by method A). During the intervening year the column was kept at pH 7 and was used occasionally. The use of method B for the preparation of amphiphilic agarose columns for high-performance hydrophobic interaction chromatography is illustrated in Fig. 4 and in ref. 17. The agarose beads were prepared as described in ref. 18 (the agarose concentration was 12%).

DISCUSSION

The method described herein for the coupling of alcohols to agarose with boron trifluoride as a catalyst is similar to that used by Chang *et al.*[19] for attachment of various ligands to silica beads used in HPLC. However, in sharp contrast to the amphiphilic derivatives of silica, those of agarose are stable even above pH 8 (Fig. 2). This is of importance for optimum utilization of a chromatographic bed material. In hydrophobic interaction chromatography, for instance, the relative retention times for the proteins to be separated, and thereby the resolution, change with pH, particularly above pH 8[17] where the silica columns unfortunately are not stable.

The γ-glycidoxypropylsilylagarose is prepared from a trimethoxysilane and can therefore probably be considered as a "bulk-modified material" with cross-linkings between the silane molecules, which decreases the risk of ligand leakage[20,21]. This risk is further reduced if the silicon atom can have two (as outlined in Fig. 1b) or three bonds to the agarose matrix. Three bonds can occur only (and two bonds perhaps more readily) if hydroxy groups from more than one agarose chain are involved. The stability of γ-glycidoxypropylsilylagarose is evident not only from Fig. 2a, but also from the finding that octylagarose prepared as described herein retained its amphiphilic character upon storage for 1 year[10].

The catalyst stannic chloride may not have been employed previously for the derivatization of chromatographic bed materials, although it was mentioned as early as 1956 as a very efficient catalyst for the reaction between epoxides and alcohols[22]. We noticed that boron trifluoride catalyzes cleavage of ether bonds when applying the above coupling method A on beads of agarose cross-linked by epichlorohydrin, since the flow-rate, and thereby the rigidity of the beads, decreased after the coupling procedure. (In order to avoid the cleavage of ether bonds, a smaller amount of boron trifluoride was used for attachment of ligand; however, the ligand density also decreased since proteins were only slightly adsorbed in a buffer containing 1.3 *M* ammonium sulphate.) Since stannic chloride at the concentration and with the reaction time employed in method B does not catalyze observable cleavage of any bonds (to judge from the finding that the flow-rate did not decrease upon coupling) we use this method for coupling ligands to agarose matrices designed for HPLC experiments where high flow-rates are essential. However, an increase in reaction time from 1 to 4 h (at constant catalyst concentration) increases the capacity factor, *i.e.*, the ligand

density (Table I). At the same time, the flow-rate decreases (Table I), indicating that stannic chloride can also catalyze degradation of cross-linked agarose. In this respect it resembles boron trifluoride (see above) and tribromide[23].

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CHROM. 18 265

LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF POROUS VINYLPYRIDINE POLYMER AS A WEAK ANION-EXCHANGE COLUMN PACKING

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SUMMARY

The retention behaviour of aromatic acids on a pyridine polymer column in ion-exchange high-performance liquid chromatography was investigated, and compared with that on a pyridinium polymer column. The effect of the eluent pH on the capacity factor, k', of the acids on these polymer columns may be explained by the extent of dissociation of both the solute and the polymer. The relationship between the concentration of methanol in the eluent and k' showed that both polymer columns exhibit similar hydrophobic interactions with the solutes. On the pyridine polymer column, good resolution of structural isomers of aminobenzoic and hydroxybenzoic acids was obtained. It is suggested that undissociated pyridyl groups contribute to the solute retention.

INTRODUCTION

In the analysis of aromatic acids by high-performance liquid chromatography (HPLC), a variety of separation modes such as ion-exchange, ion-exclusion, reversed-phase, normal-phase and ion-pair chromatographies are available[1]. We recently reported an evaluation of a porous vinylpyridine polymer cross-linked with divinylbenzene (pyridine polymer) for use as a column packing in HPLC[2]. So far, there have been no reports of the application of polymers containing pyridyl groups as column packings in ion-exchange HPLC, except for an investigation of a picolyl Kel-F column[3].

This paper describes the separation of aromatic acids by ion-exchange chromatography on a pyridine polymer column. In general, the capacity of a weak anion-exchange resin is dependent on the pH of the eluent, and the retention of acidic compounds based on the ion-exchange mechanism is easily controlled by changing the eluent pH. Moreover, the interaction between the solute and the undissociated pyridine group is expected to influence significantly on the retention of the solute. The retention behaviours of the acids on the pyridine polymer have also been compared with those on a pyridinium polymer prepared by quaternization of the pyridine polymer with methyl bromide.

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EXPERIMENTAL

Apparatus and materials

An EYELA PLC-10 high-performance liquid chromatograph (Tokyo Rikakikai Co.) equipped with a UV spectrophotometric detector and a sample injector with a 10-μl loop was used.

The chemicals used as chromatographic solutes and buffers were reagent grade from a variety of sources. Methanol and water used as the mobile phase were distilled from glass apparatus before use.

Preparation of column packings

Macroporous pyridine polymer (20% cross-linked with divinylbenzene, particle size 10–15 μm) was prepared by the method described previously[2]. The pyridinium polymer was prepared as follows. A 5-g amount of the pyridine polymer (nitrogen content: 7.96%) suspended in 20 ml of methanol was placed in a 50-ml pressure bottle and cooled with a mixture of ice and salt. Methyl bromide (25 ml) was added to the cooled mixture and the mixture was heated at 60°C for 15 h. The product was filtered off and washed with methanol. The nitrogen and bromine contents of the quaternized polymer were 4.76% and 28.04%, respectively.

Chromatography

The columns (25 × 0.26 cm I.D.) were made from stainless-steel tubing and packed with a methanol slurry of the packing materials. Unless stated otherwise, methanol–water (1:1) containing 0.05 *M* phosphate buffer and 0.05 *M* sodium nitrate was used as the eluent at a flow-rate of 0.5 ml/min. The eluent pH was measured after mixing the buffer and organic modifier. The column was maintained at 55°C by external circulation. The sample solutions (1 mg/ml) were prepared in methanol. The dead volume of the column was determined by using pyridine unretained solute.

RESULTS AND DISCUSSION

In ion-exchange chromatography, the retention of solutes is affected by the nature and concentration of the salt added in the eluent owing to the competition between the solute and other ions. A comparison of the effects of monovalent anions in the eluent on the retention of nitrobenzoic acids is presented in Table I. The order of the capacity factor, k', of the acids on the pyridine polymer was $H_2PO_4^-$ > Cl^- > NO_3^- > ClO_4^-, and a similar behaviour was observed on the pyridinium polymer except that the values for $H_2PO_4^-$ and Cl^- were nearly equal.

The apparent pK_a value of the pyridine polymer determined in 50% aqueous methanol is nearly equal to the value (4.2) determined in water[4], and aromatic carboxyl groups have pK_a values in the region of 2–5. Therefore, the k' values of the acids vary with the pH of the eluent by which dissociation of the functional groups on the polymer and acidic solute is affected. The relationships between eluent pH and k' for hydroxybenzoic and nitrobenzoic acids on pyridine and pyridinium polymers are shown in Fig. 1. Over the pH range examined, the degree of dissociation of 2-hydroxybenzoic acid and nitrobenzoic acids is approximately constant. Therefore, the decrease in k' values of these acids with increasing eluent pH is due to the decrease

TABLE I

EFFECT OF VARIOUS IONS ON THE CAPACITY FACTOR, k', OF NITROBENZOIC ACIDS ON PYRIDINE POLYMER (I) AND PYRIDINIUM POLYMER (II) COLUMNS

Eluent: methanol–water (1:1) containing 0.05 *M* phosphate buffer and 0.025 *M* of the above ions (pH 5.52).

Compound	*k' on I*				*k' on II*			
	$H_2PO_4^-$	Cl^-	NO_3^-	ClO_4^-	$H_2PO_4^-$	Cl^-	NO_3^-	ClO_4^-
2-Nitrobenzoic acid	2.67	1.08	0.67	0.38	8.39	9.00	6.47	3.86
3-Nitrobenzoic acid	11.79	4.69	2.97	2.08	13.73	13.26	6.77	3.66
4-Nitrobenzoic acid	10.17	4.28	2.69	1.81	10.36	10.81	6.23	3.74

in dissociation of the pyridine polymer. As the pH decreased, the pH profiles of k' for 3- and 4-hydroxybenzoic acids tended to flatten out, due to a contribution from the undissociated acids. In the case of the pyridinium polymer which is completely dissociated in the pH range examined, the k' values for all the acids tested increase with eluent pH up to their maximum dissociation. The results shown in Fig. 1 may be accounted for in terms of the extent of dissociation of both the solute and the polymer.

Organic solvents in the eluent also affect not only the dissociation of functional groups but also the hydrophobic interactions between the solute and polymer. In the retention of organic compounds on a C_{18}-modified silica gel column[5], porous polymer columns[6] and ion-exchange columns[7–10], linear relationships between the logarithm of k' or the distribution coefficient and the concentration of organic modifier in binary water–organic eluents have been observed. Similar relationships were found for the pyridine and pyridinium polymer columns. The slopes of the lines obtained at 30–60% aqueous methanol are listed in Table II. The values for the acids having nearly equal pK_a values tend to increase with increasing hydrophobic nature of the

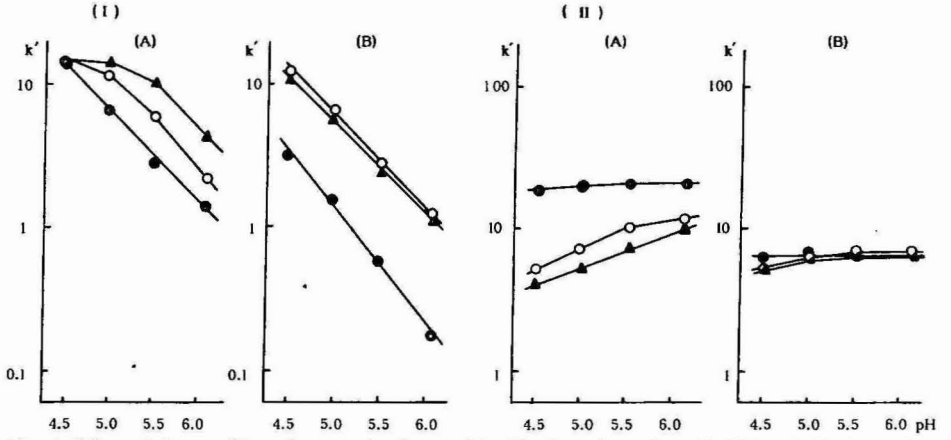

Fig. 1. Effect of eluent pH on the capacity factor, k', of hydroxybenzoic acids (A) and nitrobenzoic acids (B). Column: I = pyridine polymer; II = pyridinium polymer. ● = 2-, ○ = 3- and ▲ = 4-substituted benzoic acid. Eluent: methanol–water (1:1) containing 0.05 *M* phosphate buffer and 0.05 *M* sodium nitrate.

TABLE II

SLOPE, a, AND CORRELATION FACTOR, r, FOR THE RELATIONSHIP BETWEEN THE CONTENT OF METHANOL IN THE ELUENT, c, AND log k' ON PYRIDINE POLYMER (I) AND PYRIDINIUM POLYMER (II) COLUMNS

log $k' = ac + b$, where c = percentage of methanol in water.

Compound	pK_a	*log p**	*I*		*II*	
			a	*r*	*a*	*r*
3-Hydroxybenzoic acid	4.08	1.50	−0.009	−0.998	−0.007	−0.995
4-Chlorobenzoic acid	3.99	2.75	−0.016	−0.998	−0.017	−0.998

* Log p are hydrophobicity values[15].

compounds. A similar behaviour was previously found by Sherma and Rieman[9] for the retention of ketones on a Dowex 50-X8 column with aqueous solutions of various organic solvents as eluents. The slope obtained on the pyridine polymer is similar to that on the pyridinium polymer. Although the polymers have different functional

TABLE III

CAPACITY FACTOR, k', OF AROMATIC ACIDS ON PYRIDINE POLYMER (I) AND PYRIDINIUM POLYMER (II) COLUMNS

Eluent: methanol–water (1:1) containing 0.05 M phosphate buffer and 0.05 M sodium nitrate (pH 5.52).

Compound	pK_a	k'	
		I	*II*
2-Aminobenzoic acid	2.05, 4.95	8.08	6.55
3-Aminobenzoic acid	3.07, 4.74	2.06	4.64
4-Aminobenzoic acid	2.38, 4.89	4.78	4.06
2-Hydroxybenzoic acid	3.00, 13.40	2.75	20.27
3-Hydroxybenzoic acid	4.08, 9.93	5.94	9.89
4-Hydroxybenzoic acid	4.58, 9.23	10.11	7.19
2-Nitrobenzoic acid	2.17	0.56	6.36
3-Nitrobenzoic acid	3.49	2.67	6.66
4-Nitrobenzoic acid	3.44	2.33	6.23
2-Chlorobenzoic acid	2.94	1.11	4.05
3-Chlorobenzoic acid	3.82	4.69	5.96
4-Chlorobenzoic acid	3.99	5.25	5.13
1,2-Benzenedicarboxylic acid	2.95, 5.41	2.52	19.47
1,3-Benzenedicarboxylic acid	3.62, 4.60	2.25	35.23
1,4-Benzenedicarboxylic acid	3.54, 4.46	1.97	29.03
2-Methylbenzoic acid	3.91	3.84	2.09
3-Methylbenzoic acid	4.27	5.28	2.71
4-Methylbenzoic acid	4.37	5.31	2.55
Benzoic acid	4.21	3.31	3.17
Nicotinic acid	4.82, 12.00	0.25	1.30
Isonicotinic acid	4.84, 12.33	0.17	1.34
4-Methylbenzenesulphonic acid	1.70	0.39	10.07

groups, they have the same polymer backbone, it is this which mainly contributes to the hydrophobic interaction.

Under the chromatographic conditions in which the pyridyl groups of the polymer are slightly dissociated, the retention behaviour of various acids was investigated and the results are summarized in Table III together with those on the pyridinium polymer. Benzenedicarboxylic acids and some aromatic acids having low pK_a values such as 2-hydroxybenzoic, 2-nitrobenzoic and 4-methylbenzenesulphonic acids were strongly retained on the pyridinium polymer, but only weakly retained on the pyridine polymer. From these results, it is presumed that the contribution of ion exchange to the retention on the pyridine polymer is small. Among the structural isomers tested, the acids having low pK_a values were eluted more rapidly on the pyridine polymer. However, this was not the case on the pyridinium polymer.

It is considered that non-ionic interactions between the undissociated acids and the pyridyl groups on the polymer contribute to the retention of the acids. Accordingly, it is necessary to take into account that both ionic and non-ionic interactions are involved in retention of the acids on the polymers. The interactions were therefore analyzed by a dual binding model, which has been employed previously to explain the retention of compounds in ion-pair chromatography[11,12] and to interpret the silanophilic and hydrophobic interactions of silica-bonded hydrocarbonaceous stationary phases[6,13,14]. Accordingly to Bij *et al.*[13], this model can be expressed by the equations

$$\frac{A}{k'_0 - k'} = \frac{1}{k_{20}K_A} + \frac{A}{k_{20}} \tag{1}$$

$$k' = k_1 + k_2 \tag{2}$$

$$k'_0 = k_1 + k_{20} \tag{3}$$

where k'_0 and k' are the measured capacity factors in the absence and presence of a competing agent (CA) for ionic interaction, k_1 and k_2 are non-ionic and ionic ca-

TABLE IV

CONTRIBUTION OF NON-IONIC k_1, AND IONIC k_2, CAPACITY FACTORS TO THE RETENTION OF COMPOUNDS ON PYRIDINE POLYMER (I) AND PYRIDINIUM POLYMER (II) COLUMNS

Eluent: methanol–water (1:1) containing 0.05 *M* phosphate buffer and 0.05 *M* or without sodium nitrate (pH 5.52).

Compound	*I*			*II*		
	k_1	k_2		k_1	k_2	
		Without	*0.05 M*		*Without*	*0.05 M*
4-Nitrobenzoic acid	2.47	9.20	−0.14	4.99	9.75	1.24
4-Hydroxybenzoic acid	10.07	2.82	0.04	3.19	39.95	4.00
4-Aminobenzoic acid	4.73	0.52	0.05	3.33	4.56	0.73

pacity factors, k_{20} is the ionic capacity factor in the absence of CA, K_A is the binding constant of CA and A is the concentration of CA. Under constant chromatographic conditions, except for the concentration of CA, k_1 is constant independently of the concentration of CA.

Sodium nitrate was used as CA and its concentration was varied from 0 to 0.005, 0.025 and 0.05 M. The concentration of the phosphate buffer used to adjust the eluent pH was constant. The value of k'_0 was the capacity factor measured in the absence of sodium nitrate. The k' values measured at various concentrations of sodium nitrate were employed in eqn. 1. An excellent linear relationship was obtained between $A/(k'_0 - k')$ and A. The reciprocal of the slope of this line represents the value of k_{20}. The value of k_1 was obtained by substitution of k_{20} into eqn. 3. The value of k_2 in the presence of various concentrations of sodium nitrate was calculated from eqn. 2. The data obtained are listed in Table IV. On the pyridine polymer column, the k_1 values of 4-hydroxybenzoic and 4-aminobenzoic acids were greater than k_2 in the absence of CA, but in the case of 4-nitrobenzoic acid the oposite was found. When CA is present, k_2 decreases, therefore it is clear that these compounds (shown in Table IV) are mainly retained by non-ionic interaction. Despite the presence of CA, strong ionic interaction contribute to the solute retention on the pyridinium polymer. These results suggest that the solute retention on the pyridine polymer is due predominantly to non-ionic interactions and in very small part only to ionic interactions under the chromatographic conditions shown in Table III. However, it is almost certain that ionic interactions do exist in this chromatographic system, for the dissociation of the pyridyl groups affected the retention of the acids. Considerable ionic interaction must be involved in solute retention on the pyridinium polymer under the same conditions.

The separation of structural isomers of aminobenzoic and hydroxybenzoic acids is shown in Figs. 2 and 3, respectively. The good resolution of each isomer of the aminobenzoic acids on the pyridine polymer is due to the non-ionic interactions. The elution order of the hydroxybenzoic acids on this polymer differs from that on the pyridinium polymer. 2-Hydroxybenzoic acid was strongly retained on the latter,

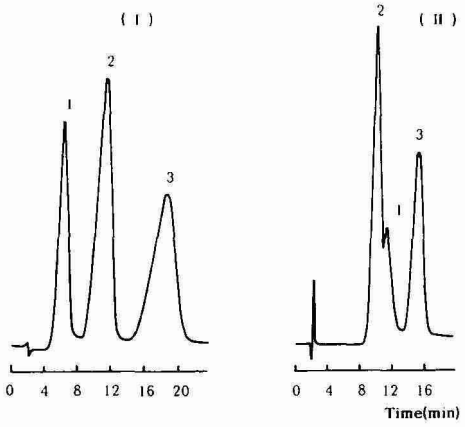

Fig. 2. Separation of aminobenzoic acids on pyridine polymer (I) and pyridinium polymer (II) columns. Peaks: 1 = 3-aminobenzoic acid; 2 = 4-aminobenzoic acid; 3 = 2-aminobenzoic acid. Other conditions as in Table III.

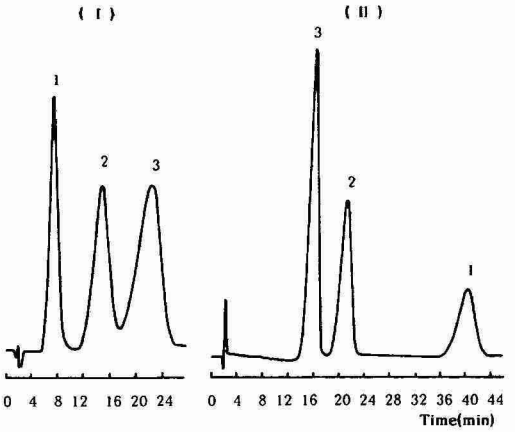

Fig. 3. Separation of hydroxybenzoic acids on the pyridine polymer (I) and the pyridinium polymer (II). Peaks: 1 = 2-hydroxybenzoic acid; 2 = 3-hydroxybenzoic acid; 3 = 4-hydroxybenzoic acid. Other conditions as in Table III.

but weakly on the pyridine polymer. An excellent separation of aromatic acids is obtained on the pyridine polymer column under conditions in which the pyridyl groups are slightly dissociated. The weak ionic interactions of the pyridine polymer result in faster elution of acids having low pK_a values, and non-ionic interactions contribute to the separation of the structural isomers.

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PREPARATION OF NOVEL, CURABLE CAPILLARY GAS CHROMATOGRAPHIC SYSTEMS AND THEIR APPLICATION TO THE ANALYSIS OF UNDERIVATISED BARBITURATES AND OTHER CONTROLLED DRUGS OF FORENSIC INTEREST

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SUMMARY

Three novel stationary phases have been prepared for the analysis of underivatised barbiturates by incorporating different monomers, N,N′-di(but-3-enyl)amylobarbital, N,N′-di(pent-4-enyl)amylobarbital and N,N′-di(hex-5-enyl)amylobarbital into a standard SE-54 matrix. The chromatographic behaviour of these columns is compared with a standard immobilised SE-54 column. They are shown to be less polar than the previously reported N,N′-diallylamylobarbital incorporated column. Of the three new phases, the one incorporating the monomer N,N′-di(but-3-enyl)-amylobarbital shows the best performance for the separation of a mixture containing 22 barbiturates but all are selective, very efficient, inert and can be used up to at least 300°C. The versatility of these new phases is demonstrated further by the analysis of other drugs of abuse. They offer unique selectivities not previously available.

INTRODUCTION

Recent developments of capillary column technology for gas chromatography (GC) have been reviewed by several research workers[1–3]. Polar, immobilised phases such as SE-54 into which have been incorporated N,N′-diallylamylobarbital (IV) have recently been prepared in our laboratory[4]. These phases are efficient, selective, thermally stable and exhibit very good tailing behaviour. We now report the preparation of three new, crosslinkable selective stationary phases by incorporating different monomers, N,N′-di(but-3-enyl)amylobarbital (I), N,N′-di(pent-4-enyl)amylobarbital (II) or N,N′-di(hex-5-enyl)amylobarbital (III), into an SE-54 matrix. Their chromatographic performance was evaluated by the use of Grob test mixtures, while the selectivity towards the separation of underivatised barbiturates was tested with a standard barbiturate mixture. The versatility of these columns can be demonstrated further by the analysis of other controlled drugs, especially the benzodiazepines.

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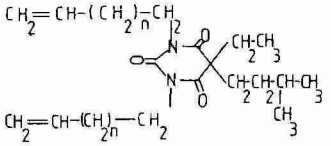

I, n = 1; IIm, n = 2; III, n = 3; IV, n = 0.

EXPERIMENTAL

Characterisation of barbiturate monomers

Unless otherwise stated, NMR spectra were recorded in deuterated chloroform solution using a Perkin-Elmer R32 NMR spectrometer, and are given in parts-per-million (δ) downfield from an internal tetramethylsilane (TMS) standard. The abbreviations s, d, t, q and m refer to singlet, doublet, triplet, quarted and multiplet peaks, respectively.

Infrared (IR) spectra were recorded on a Perkin Elmer 157 prism spectrophotometer. The abbreviations s, m and w refer to strong, medium and weakly absorbing peaks, respectively.

Synthesis of N,N'-di(but-3-enyl)amylobarbital (I)

To a stirred solution of 2 g of amylobarbital (May and Baker, Dagenham, U.K.) in 10 ml of acetone (distilled) in a round-bottomed flask were added 3 ml of (*ca.* 2.5 equiv.) triethylamine (freshly distilled). To this were added dropwise 4 g, (*ca.* 3 equiv.) of 4-bromo-1-butene (Aldrich). The reaction mixture was stirred overnight at room temperature. The white precipitate formed was dissolved in 50 ml of water and the reaction mixture extracted into diethyl ether (3 times 50 ml), washed with 50 ml of 1 *M* sodium hydroxyde, 50 ml of water, dried (anhydrous sodium sulphate) and filtered. The solvent was removed under vacuum to obtain a pale yellow oil. After purification by column chromatography [silica gel 60; acetone–chloroform (3:7, v/v)], a colourless oil [1.6 g, 56%, R_F 0.56; acetone–chloroform (3:7, v/v)] was obtained.

IR (film): 3050 cm^{-1} (m, =C–H stretch), 1700 cm^{-1} (s, C=O), 1000 and 840 cm^{-1} (s, =C–H bend).

NMR (C^2HCl_3) δ 1.80–2.20 (m, 4H, CH_2–C=C), δ 3.80–4.20 (t, 4H, CH_2–N), δ 4.80–5.30 (m, 4H, CH_2=C), δ 5.40–6.10 (m, 2H, CH=C).

$C_{19}H_{30}N_2O_3$ requires: C = 68.26, H = 8.98, N = 8.38; found: C = 67.90, H = 9.15, N = 8.65.

Synthesis of 5-bromo-1-pentene

To a stirred solution of 10 g of 4-penten-1-ol (Aldrich) at −10°C in a three-necked round-bottomed flask was added 4.5 g (0.33 equiv.) of phosphorus tribromide (Aldrich) dropwise over a period of 5 min, moisture being kept out by means of a Drierite-filled tube. The reaction mixture was stirred overnight at room temperature. After pouring into 50 ml of ice-water, the reaction mixture was extracted into diethyl ehter (3 times 50 ml), washed with 50 ml of water, 10% (w/v) sodium carbonate solution (2 times 100 ml), 50 ml of water, dried (anhydrous sodium sulphate) and filtered. The solvent was removed under vacuum and the residue was distilled to give

5-bromo-1-pentene as a colourless liquid [7.2 g; 42%; R_F 0.30; diethyl ether–chloroform (2:8, v/v)].

IR (film): 3050 cm^{-1} (m, =C–H), 1640 cm^{-1} (m, C=C), 1000 and 920 cm^{-1} (s, =C–H bend).

NMR (C^2HCl_3): δ 1.00–2.40 (m, 4H, CH_2–CH_2), δ 3.20–3.50 (t, 2H, CH_2–Br), δ 4.80–5.20 (m, 2H, CH_2=C), δ 5.50–6.10 (m, 1 H, CH=C).

C_5H_9Br requires: C = 40.29, H = 6.04, Br = 53.65; found: C = 40.04, H = 6.30, Br = 53.35.

Synthesis of N,N'-di(pent-4-enyl)amylobarbital (II)

A solution of 2 g of amylobarbital in 10 ml of acetone, 3 ml of triethylamine and 4 g of 5-bromo-1-pentene was treated under the same conditions as described for the synthesis of I to give II as a colourless oil [1.6 g; 52%, R_F 0.56; diethyl ether–chloroform (3:7, v/v)].

IR (film): 3050 cm^{-1} (m, =C–H stretch), 1700 cm^{-1} (s, C=O), 1000 and 840 cm^{-1} (s, =C–H bend).

NMR (C^2HCl_3):δ 1.80–2.20 (m, 4H, CH_2–C=C), δ 3.80–4.20 (t, 4H, CH_2–N), δ 4.80–5.20 (m, 4H, CH_2=C), δ 5.50–6.20 (m, 2H, CH=C).

$C_{21}H_{34}N_2O_3$ requires: C = 69.61, H = 9.39, N = 7.73; found: C = 69.38, H = 9.62, N = 7.95.

Synthesis of 6-bromo-1-hexene

Amounts of 10 g of 5-hexen-1-ol (Aldrich) and 8.98 g (0.33 equiv.) of phosphorus tribromide (Aldrich) were treated under the same conditions as described for the synthesis of 5-bromo-1-pentene to give 6-bromo-1-hexene as a colourless liquid [6.3 g; 39%, R_F 0.33 diethyl ether–chloroform 2:8, v/v)].

IR (film): 3050 cm^{-1} (m, =C–H), 1640 cm^{-1} (m, C=C), 1000 and 920 cm^{-1} (s, =C–H bend).

NMR (C^2HCl_3): δ 1.40–2.20 (m, 6H, CH_2–CH_2–CH_2), δ 3.30–3.50 (t, 2H, CH_2–Br), δ 4.80–5.20 (m, 2H, CH_2=C), δ 5.50–6.10 (m, 1H, CH=C).

$C_6H_{11}Br$ requires: C = 44.20, H = 6.75, Br = 49.05, found: C = 43.95, H = 7.04, Br = 48.90.

Synthesis of N,N'-di(hex-5-enyl)amylobarbital (III)

A solution of 2 g of amylobarbital in 10 ml of acetone, 3 ml of triethylamine and 4 g of 6-bromo-1-hexene was treated under the same conditions as described for the synthesis of I to give III as a colourless oil [1.8 g; 54%, R_F 0.44; diethyl ether–chloroform (2:8, v/v)].

IR (film): 3050 cm^{-1} (m, =C–H stretch), 1700 cm^{-1} (s, C=O), 1000 and 940 cm^{-1} (s, =C–H bend).

NMR (C^2HCl_3): δ 1.80–2.20 (m, 4H, CH_2–C=C), δ 3.80–4.20 (t, 4H, CH_2–N), δ 4.80–5.20 (m, 4H, CH_2=C), δ 5.50–6.20 (m, 2H, CH=C).

$C_{23}H_{38}N_2O_3$ requires: C = 70.76, H = 9.74, N = 7.18; found: C = 70.55, H = 9.96, N = 7.42.

Synthesis of N,N'-diallylamylobarbitaol (IV)

The monomer IV was synthesised by diallylation of 5-ethyl-5-isoamyl barbituric acid (May and Baker) with allyl bromide according to a procedure described[5].

Column preparation

Glass capillaries (0.23 mm I.D. and 0.80 mm O.D.) were drawn from Pyrex glass tubes (3.5 mm I.D. and 6.0 mm O.D.) on a Carlo Erba GCDM 60 glass-drawing machine. Cleaning of glass tubing, leaching followed by rinsing and dehydration were carried out as described earlier[5]. Persilylation was performed with divinyltetramethyldisilazane (DVTMDS)[4].

A solution for static coating was prepared by first dissolving SE-54 (0.29%, Phasesep) in pentane (AnalaR grade; BDH) and then adding 30% (w/w) of the synthesised unsaturated barbiturate monomer (I, II, III or IV) [2% (w/w) in methylene chloride]. Finally, 2.5% (w/w) dicumyl peroxide (DCUP) (2% in toluene) was added, with the exception of the column incorporating IV in which 1.5% (w/w) DCUP was used. Columns were statically coated as described earlier[5] and 2 m at both ends were kept free of coating. After static coating, the columns were flushed with dry nitrogen for 1 h and cured according to the method of Blomberg *et al.*[6] in which the capillary was sealed under vacuum and then placed in an oven at 140°C for 30 min. The oven was cooled rapidly and each column flushed with dry nitrogen for 3 h. The ends of the columns were straightened using a Carlo Erba GESM 102-20 automatic electrical end-straightening machine under a flow of dry nitrogen. The columns were conditioned at 300°C overnight and test run. Finally, each column was washed with 5 ml methylene chloride (AnalaR grade; BDH), dried in a stream of nitrogen and reconditioned at 300°C before re-testing.

Preparation of the human urine standard spiked with ten drugs

A urine sample was spiked with amphetamine, ephedrine, phendimetrazine, benzocaine, procaine, methaqualone, cocaine, codeine, ethyl morphine and morphine. The urine standard was prepared by a simple, one-step extraction with methylene chloride from the sample which was made alkaline with ammonium hydroxide, the organic layer dried, evaporated and reconstituted with ethyl acetate. A portion of the reconstituted material was injected onto the column.

Gas chromatography

Capillary GC was performed with a standard Hewlett Packard Model 5710A gas chromatograph modified for capillary GC analysis and equipped with a flame ionisation detector (air, 240 ml/min; hydrogen 30 ml/min). The carrier gas was nitrogen with injector and detector temperatures of 300°C. A split ratio of 50:1 was used. Nitrogen was used as the make-up gas (20 ml/min).

RESULTS AND DISCUSSION

Chromatograms of Grob test mixtures for SE-54 into which has been incorporated N,N′-di(but-3-enyl)amylobarbital (I), N,N′-di(pent-4-enyl)amylobarbital (II) and N,N′-di(hex-5-enyl)amylobarbital (III) show that the column incorporating monomer (I) is more polar than the column incorporating monomer II which in turn is more polar than the column incorporating III. This means that increasing the carbon chain length of the unsaturated substituents linked to the nitrogen atom of the incorporated monomer leads to a decrease in the polarity of the resultant phase. Columns incorporating monomers I and II are slightly basic in character whereas the

TABLE I

CHARACTERISTICS OF THE NEW STATIONARY PHASES PREPARED

*Stationary phase**	*TZ*	*k' for hexadecane at 140°C*	*HETP*** *(mm) for hexadecane at 140°C*	*CE*	*Decrease in k' of hexadecane at 140°C after rinsing with solvent (%)*
SE-54 + 30% I	27.3	5.75	0.23	85.2	3.5
SE-54 + 30% II	27.4	4.89	0.24	80.4	3.9
SE-54 + 30% III	27.4	5.65	0.22	89.2	4.0

* All columns are of the same dimensions: 15 m × 0.23 mm I.D.
** Height equivalent to a theoretical plate.

column incorporating monomer III is slightly acidic. All three phases show very good tailing behaviour and are very efficient as evidenced by their high Trennzahl (TZ) values (Table I). They also show very high coating efficiencies (CE) and can routinely be used up to at least 300°C. All phases were immobilised and extraction with methylene chloride led to a decrease in capacity factor (k') values of only *ca.* 3.5–4.0%. This reflected the observed minor differences in chromatographic performance before and after the dichloromethane washings of the prepared columns.

The separation of a mixture of 22 underivatised barbiturates commonly encountered in toxicological cases on a standard SE-54 column and the four columns incorporating the different monomers are shown in Fig. 1. It is apparent that the

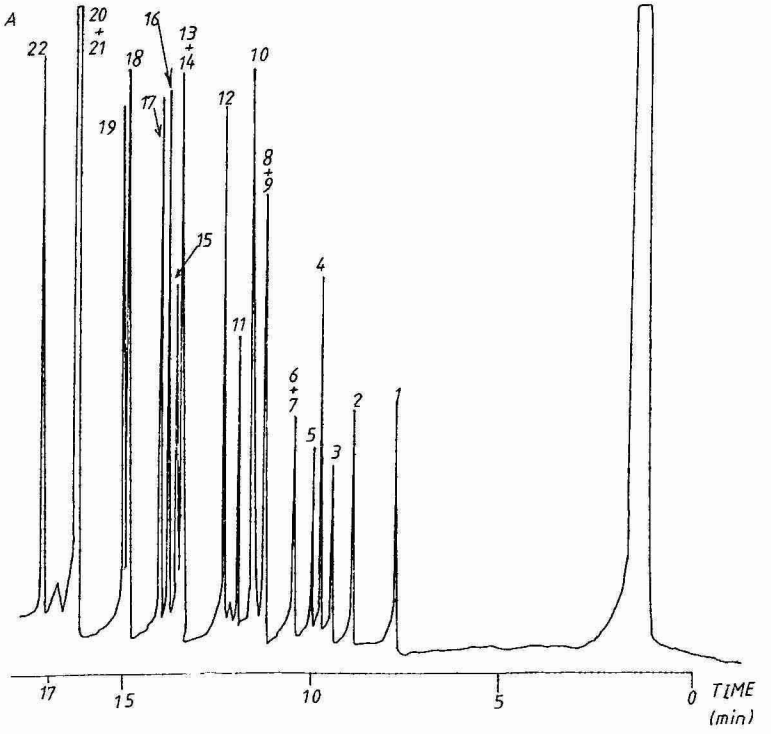

Fig. 1.

(Continued on p. 224)

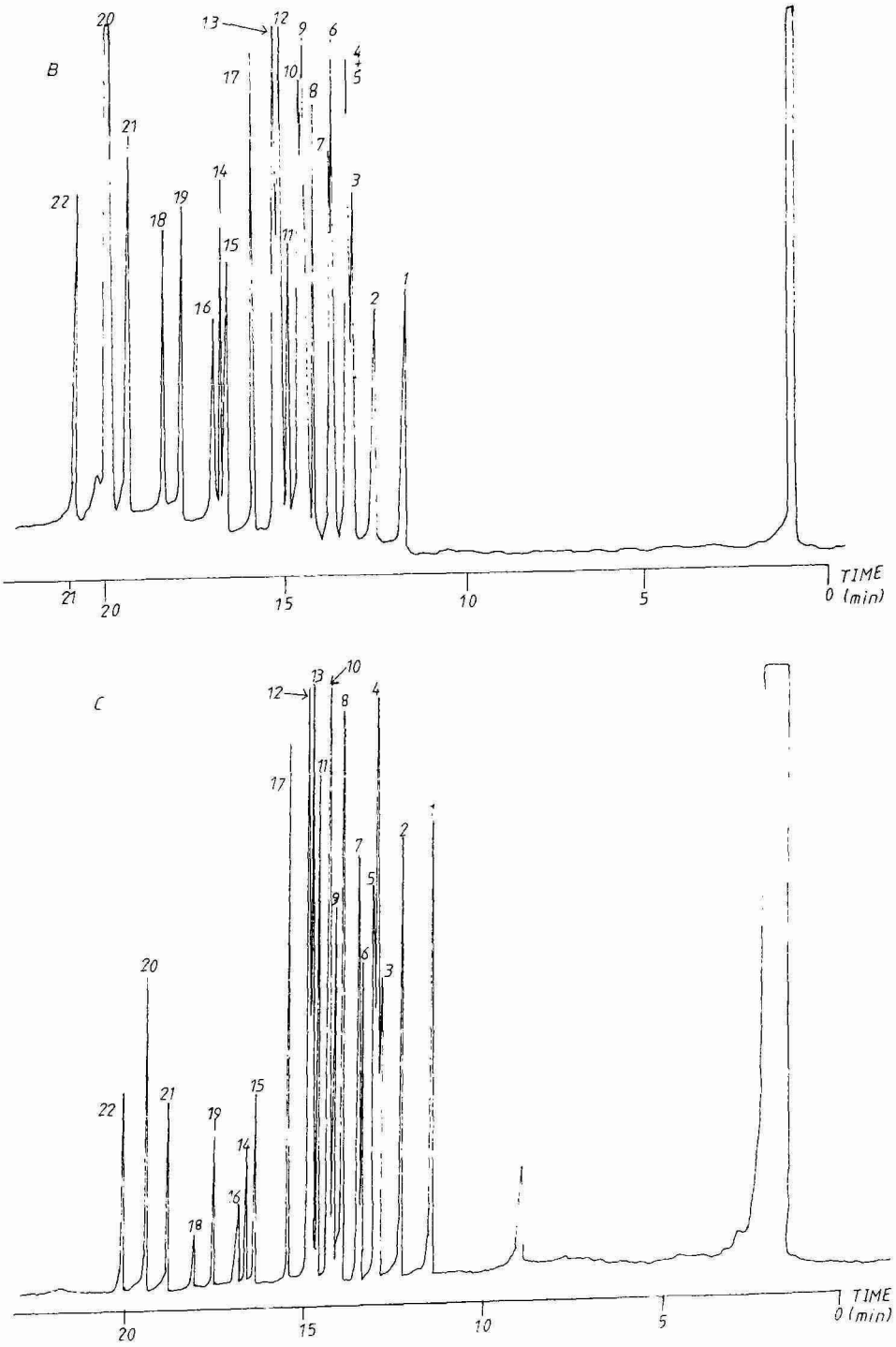
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Fig. 1.

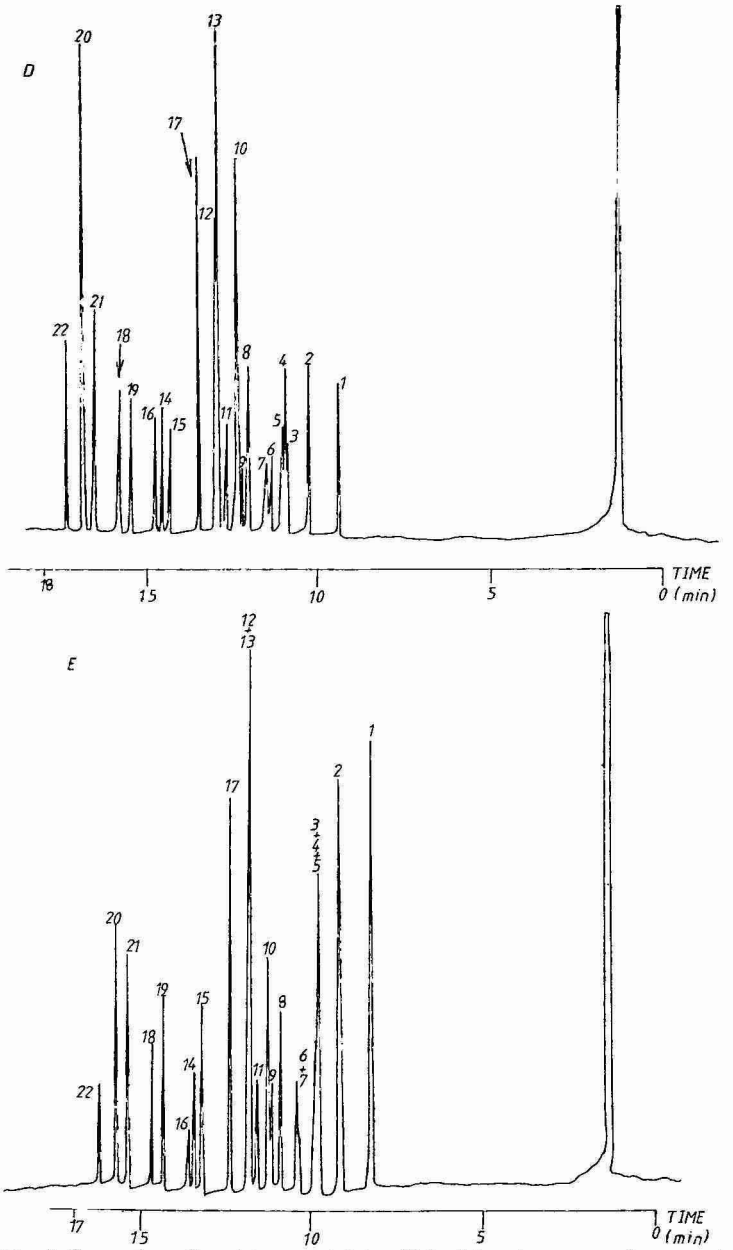

Fig. 1. Separation of a mixture containing 22 barbiturates commonly encountered in toxicological cases on five different columns. Chromatographed at 120°C for 2 min, programmed at 8°C/min to 240°C and held at the upper temperature for 5 min. Chart speed: 600 mm/h. (A) SE-54 column; (B) SE-54 column with 30% IV incorporated; (C) SE-54 column with 30% I incorporated; (D) SE-54 column with 30% II incorporated; (E) SE-54 column with 30% III incorporated. Peaks: 1 = barbital; 2 = probarbital; 3 = allobarbital; 4 = aprobarbital; 5 = ethylcrotylbarbital; 6 = butobarbital; 7 = butalbital; 8 = amylobarbital; 9 = nealbarbital; 10 = pentobarbital; 11 = vinbarbital; 12 = quinalbarbital; 13 = hexobarbital; 14 = brallobarbital; 15 = cyclopentobarbital; 16 = ibomal; 17 = methylphenylbarbital; 18 = phenobarbital; 19 = cyclobarbital; 20 = 5-ethyl-5-tolylbarbital; 21 = heptabarbital; 22 = reposal.

different phases show differences in selectivities towards the barbiturates. The SE-54 column is capable of resolving only 18 out of the 22 barbiturates and the column incorporating monomer III was also unable to resolve all the barbiturates. The column incorporating monomer IV resolves 21 out of 22 but the best resolution was provided by the one incorporating monomer I in which some resolution of all 22 barbiturates was demonstrated over 20 min.

The versatility of the new phases is further demonstrated by the analysis of some controlled drugs which are frequently abused. The best separation of six benzodiazepines is illustrated in Fig. 2 for the new phase incorporating monomer III and is compared with the result obtained using a standard SE-54 column. Different selectivities were shown for the separation of the benzodiazepines using columns incorporating the different monomers as illustrated by the different order of elution.

The separation of some common central nervous system stimulant drugs on the column incorporating monomer I is shown in Fig. 3, while several important drugs of abuse extracted from a human urine, as would be the case for a drug screen, is illustrated in Fig. 4. All components were resolved using either the columns incorporating monomers I or II although the separations of methaqualone and cocaine (peaks 6 and 7, respectively) and of codeine and ethyl morphine (peaks 8 and 9, respectively) were slightly better in the latter column.

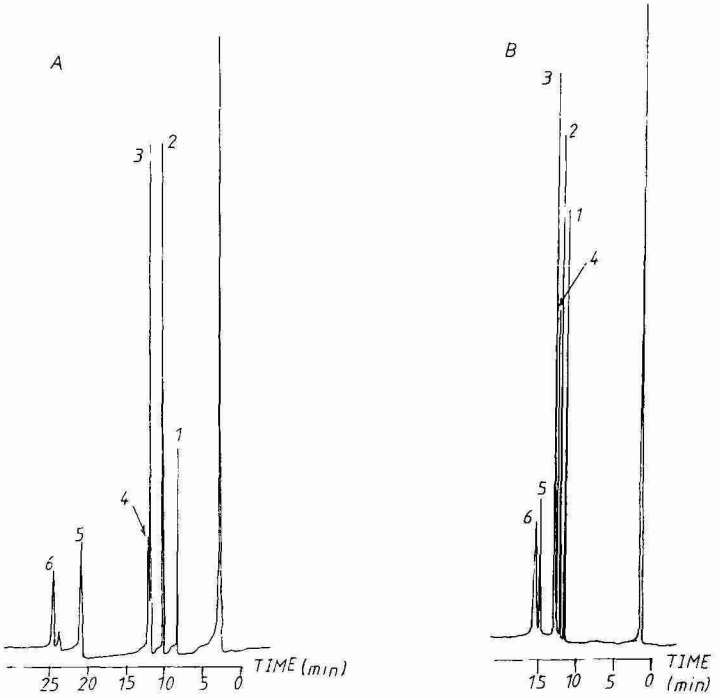

Fig. 2. Separation of six commonly abused benzodiazepines (chart speed: 120 mm/h). (A) SE-54 column, chromatographed at 250°C for 16 min, programmed at 1°C/min to 260°C. (B) SE-54 column incorporating monomer III, chromatographed at 130°C for 2 min, programmed at 16°C/min to 300°C and held at the upper temperature for 5 min. Peaks: 1 = oxazepam; 2 = diazepam; 3 = desmethyl diazepam; 4 = librium; 5 = nitrazepam; 6 = clonazepam.

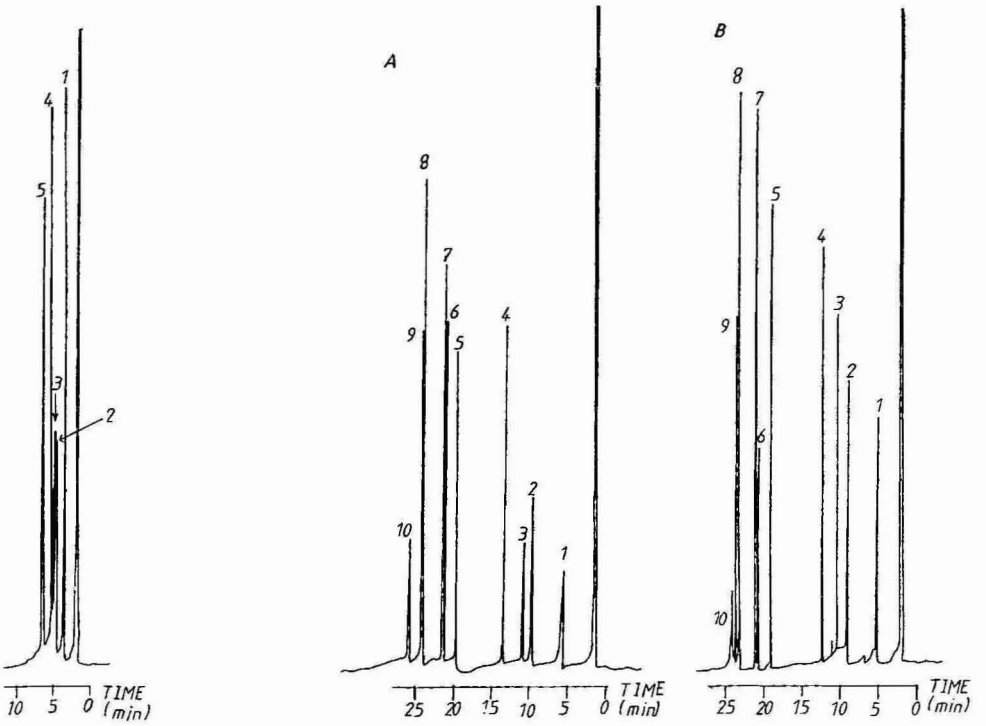

Fig. 3. Separation of some common central nervous system stimulants on the column incorporating monomer I. Oven temperature: isothermal at 120°C. Chart speed: 120 mm/h. Peaks: 1 = cyclopentamine; 2 = norfenfluramine; 3 = phentermine; 4 = methylamphetamine; 5 = ethylamphetamine.

Fig. 4. Separation of several important drugs of abuse in a human urine drug screen. (A) Column incorporating monomer I; (B) column incorporating monomer II. Chromatographed at 100°C for 2 min, programmed at 8°C/min to 280°C and held at the upper temperature for 5 min. Chart speed: 120 mm/h. Peaks: 1 = amphetamine; 2 = ephedrine; 3 = phendimetrazine; 4 = benzocaine; 5 = procaine; 6 = methaqualone; 7 = cocaine; 8 = codeine; 9 = ethylmorphine; 10 = morphine.

Narcotic analgesics, without derivatisation, can be resolved on a column incorporating monomer I (Fig. 5) and underivatised tricyclic antidepressants can also be resolved on this same column.

The usefulness of these columns is indicated by Fig. 6 which shows the forensic analysis of a "street" heroin sample. Separation of heroin from the impurities, without derivatisation, was achieved using the column incorporating monomer I. Information derived from such impurities can be used to help trace the heroin source[7].

The new phases are immobilised which means that they are also suitable for samples presented in aqueous solution since the immobilised phase cannot be displaced from the capillary surface by water. Any non-volatile material deposited in the column inlet system can also be removed by solvent rinsing, provided that any contamination is soluble. It must be borne in mind that injection of acids and bases together with water can lead to hydrolysis of the stationary phase, a problem also known to occur with HPLC silanized silica. The chromatogram of an aqueous solution of LSD 25 (0.1 mg LSD 25 tartrate in 1 ml water) is demonstrated in Fig. 7

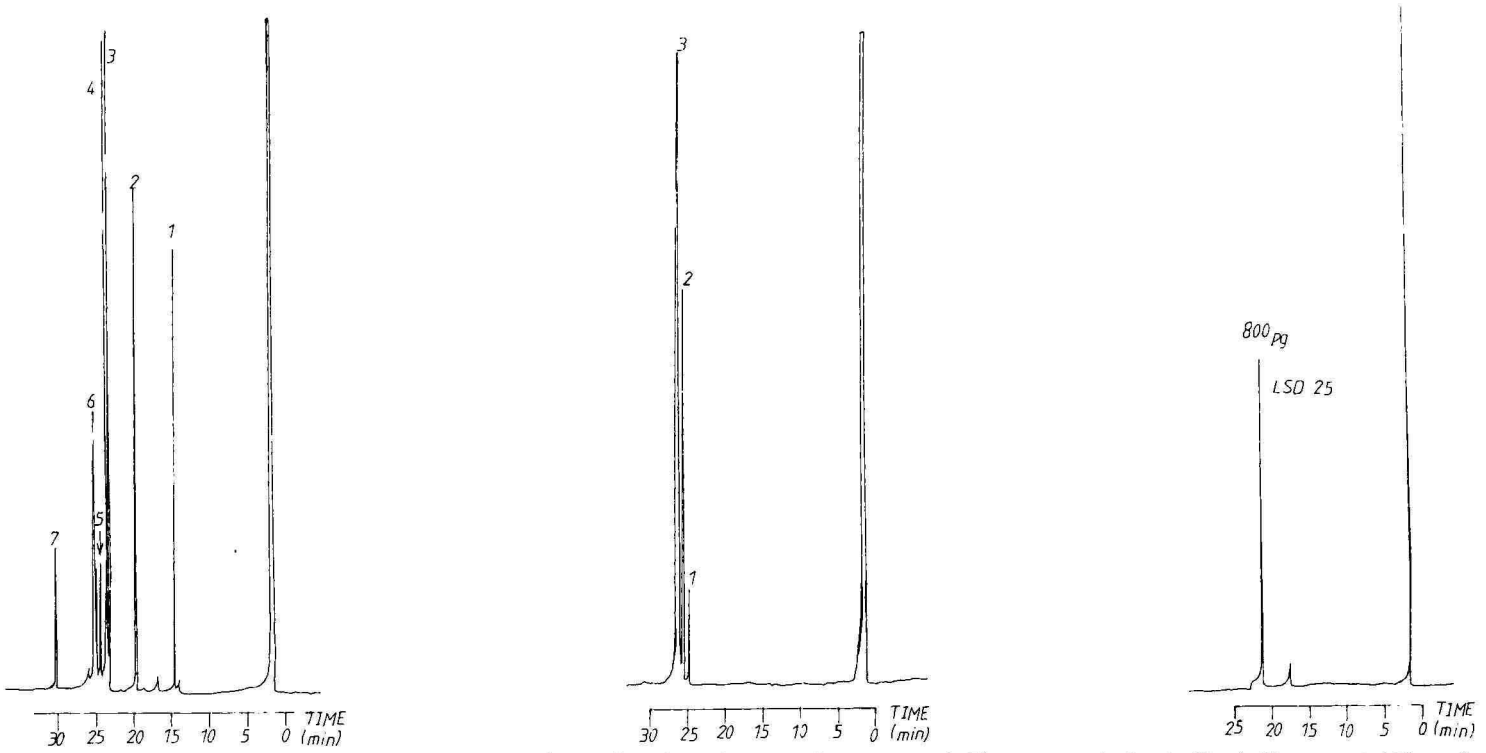

Fig. 5. Total separation of some common narcotic analgesics on the column incorporating monomer I. Chromatographed as in Fig. 4. Chart speed: 120 mm/h. Peaks: 1 = pethidine; 2 = methadone; 3 = dipipanone; 4 = codeine; 5 = morphine; 6 = heroin; 7 = dextromoramide.

Fig. 6. Forensic analysis of a "street" heroin sample in methanol on the column incorporating monomer I. Chromatographed as in Fig. 4. Chart speed: 120 mm/h. Peaks: 1, 2 = impurities; 3 = heroin.

Fig. 7. Chromatogram of an aqueous solution of LSD 25 (0.1 mg LSD 25 tartrate in 1 ml water) on the column incorporating monomer I. Chromatographed as in Fig. 4. Chart speed 120 mm/h. Attenuation: 1 × 256. Injection volume: 0.4 μl.

TABLE II

DRUG LIBRARY FOR THE COLUMN INCORPORATING MONOMER I

GG conditions: chromatographed at 100°C for 2 min, programmed at 8°C/min to 280°C and held at the upper temperature for 5 min. Chart speed: 120 mm/h.

No.	*Drug*	t_R*	*No.*	*Drug*	t_R*
1	Amphetamine	11.0	11	LSD 25	41.8
2	Ephedrine	19.0	12	Methaqualone	42.0
3	Phendimetrazine	21.5	13	Cocaine	42.8
4	Benzocaine	26.5	14	Desipramine	43.0
5	Pethidine	28.5	15	Dipipanone	46.0
6	Methadone	39.0	16	Codeine	47.5
7	Procaine	39.5	17	Ethylmorphine	48.5
8	Imipramine	41.0	18	Morphine	49.5
9	Trimipramine	41.0	19	Heroin	50.5
10	Nortriptyline	41.5	20	Dextromoramide	60.0

* Absolute retention time (mm).

on the column incorporating monomer I. The injection volume was 0.4 μl which enables *ca.* 800 pg of the hallucinogen to enter the column. The drug shows good peak shape and indicates the potential of such a column for forensic analysis.

Finally, it should be mentioned that the same temperature programme rate was employed for the separation of the human urine drug screen, the tricyclic antidepressants, the narcotic analgesics, the "street" heroin sample and the aqueous sample of LSD 25 on the column incorporating monomer I, hence a drug library, if desired, can easily be generated for identification purposes (Table II).

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MODIFICATION OF CATION-EXCHANGE PROPERTIES OF ACTIVATED CARBON BY TREATMENT WITH NITRIC ACID*

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SUMMARY

The uptake of inorganic cations by high-surface-area activated carbon can be increased by an order of magnitude by controlled exposure to high concentrations of nitric acid at elevated temperatures. Distribution coefficients of cations are also increased. Acid strength of the functional groups from the nitric acid treatment is greater than those of the starting material. Surface area measurements and small-angle neutron scattering indicate that the increase in effective ion-exchange capacity is not accompanied by gross changes in the structure of the material. ^{13}C NMR on solid samples suggests that the concentration of carboxyl and phenolic functional groups in the carbon is increased by the treatment.

INTRODUCTION

Adsorption by activated carbon of inorganic ions has received less attention than that of other substances, although there is a considerable literature on the subject. Various aspects have been covered in reviews (see, *e.g.*, refs. 1–3) or in book chapters (*e.g.*, ref. 4). A book[5], referenced by Smith[3], deals with adsorptive properties in terms of ion exchange. Smith also cites a number of Russian papers. Nelson *et al.*[6] demonstrated the distribution of Fe(III) between activated carbon and concentrated solutions of hydrochloric acid and lithium chloride paralleled in a remarkable way the distribution observed with organic ion exchangers and in solvent extraction.

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Kraus and Nelson[7] also showed that the distribution of acids from concentrated electrolyte solutions was analogous for activated carbon and ion exchangers. Activated carbon can have either positive or negative surface charge[2,8].

The ion-exchange capacity of conventional activated carbons is usually not high except in contact with solutions at high pH. It has been realized for some time that cation-exchange capacity can be sharply increased by exposure to oxidizing environments. Weller and Young[9] reported the effect on acid and base uptake of activated carbon exposed to oxygen at 400°C. There was a decrease in hydrochloric acid uptake, and an increase in sodium hydroxide, up to *ca.* 2 mequiv./g when the final sodium hydroxide concentration was above 0.1 *M*. Puri *et al.*[10] treated charcoal with nitric acid at elevated temperature and attained neutralizations of barium hydroxide of up to 12 mequiv./g. Ivanova *et al.*[11] reported properties of charcoal oxidized by atmospheric oxygen, hydrogen peroxide and nitric acid.

The literature survey here is by no means complete; there are many other citations in the references listed. Here we report effects on ion-exchange properties of nitric acid treatment as a function of time, temperature, and nitric acid concentration. We also report observations on other properties of the materials that we believe shed some light on the changes in the solids accompanying the changes in ion-exchange properties.

EXPERIMENTAL

Activated carbons

Four samples were used in oxidation studies. Three were of high surface area (*ca.* 1000 m^2/g) coconut charcoals. One of these was a Nucon material (referred to as N). Two from Barnebey Cheney (referred to as BC-1 and BC-2) differed in some details of preparation not known to us. The other was a low-surface-area coconut charcoal, also from Barnebey Cheney (BC-3). It was stated to have a surface area of about 50 m^2/g. Two mesh sizes were investigated, -100, $+200$ and -6, $+14$.

A limited evaluation of commercial oxidized carbons from Nuclear Consultants, is also reported.

Oxidation procedures

The charcoal samples were dried at 110°C for at least 2 days. In early experiments, they were outgassed under vacuum at 500°C for 24 h, in conformance with the practice of Puri *et al.*[10]. Although the outgassing procedure, as we shall describe, had effects on properties of the as-received material, differences between as-received and outgassed appeared to be insignificant after nitric acid treatment, and the outgassing step was omitted in later stages.

Most oxidations were carried out under reflux on samples of from five to ten grams. Ten ml of nitric acid was added per gram of charcoal. Time, temperature, and nitric acid concentrations were varied. After the desired time, samples were cooled; excess acid was poured off; the samples were neutralized with 0.1 *M* sodium hydroxide, and rinsed with distilled water, with the final wash adjusted to pH 6 by acid or base addition; and samples were dried at 110°C overnight. In presenting results, concentrations in the solid phase are in terms of amounts per kg of dry charcoal. Except for outgassed samples, "dry charcoal" is defined as material dried at 110°C.

Batch equilibrations

With a few exceptions, measurements of uptake and distribution coefficients were carried out by equilibrating a known amount of solid with a solution of known composition tagged with a radioactive tracer. The solution was counted after equilibration, and the amount on the solid determined by difference from the original amount in the solution.

The details of procedures were varied as appropriate, and aspects thought significant will be discussed in connection with presentation of results. Carbon samples were usually pre-equilibrated with the composition desired several times before addition of the tracer. In most cases, tests were carried out in centrifuge tubes, were shaken at least overnight, and were centrifuged at 2000 *g* for 20 min to remove solid. The samples were further equilibrated in cases where there was doubt concerning attainment of equilibrium. In some instances, pH changed during equilibration, owing to adsorption of atmospheric carbon dioxide or other causes; when the shift had significant consequences, the acidity was readjusted to the desired value and the sample re-equilibrated. Tracers used were ^{22}Na, ^{85}Sr, ^{64}Cu, and ^{57}Co.

Activated carbon in the pH range of these measurements behaves as a weak-acid ion exchanger. Consequently, its apparent capacity, if evaluated, *e.g.*, as moles of replaceable sodium ion/kg solid, will vary with pH. We consequently prefer to discuss "uptake", measured under specified conditions, rather than "capacity", which might be defined as the maximum uptake, at pH values high enough for all protons to be replaced from the solid. Uptake was obtained from batch equilibrations by isotope dilution: w kg of charcoal is pre-equilibrated with a desired concentration (c, moles or equivalents per liter) of electrolyte without tracer, and then with V liters of the same concentration plus a known number of counts of tracer of the cation per unit volume per unit time (n_0, initial; n at equilibrium). Uptake in moles or equivalents/kg charcoal is

$$\text{Uptake} = \frac{(n_0 - n)cV}{nw}$$

Distribution coefficients (D in l/kg) are measured by the same procedure, the value sought being counts of tracer of the ion whose D is being measured per kg of solid after equilibration/counts per liter of equilibrated solution.

$$D = \frac{(n_0 - n)V}{nw}$$

In carrying out measurements as a function of pH, it is frequently desirable to stabilize systems by adding a low concentration of a buffer. Acetate is convenient to maintain pH values around 5; bicarbonate, 8; and carbonate or ammonia in the range 10–11.

Preloaded column[12]

Another technique used in some cases for measurements of distribution coefficients, including one set reported here, utilizes a column of carbon which has been precontacted in a batch equilibration with a solution containing the ion of interest, traced with a radioisotope. This approach is particularly convenient for measurement

of high values of D at a fixed solid loading of the ion of interest, over a range of concentrations of a salt of a displacing ion. If distribution coefficients of the ion of interest are high, relatively little of it is removed from the solid in establishing equilibrium with the eluting solution passed through the column. Consequently, the loading changes only at the top of the column, and the bottom stays at the initial loading, which is known from analysis after the initial equilibration. Alternatively, the initial loading may be accomplished by passing a solution of known concentration and known specific activity through a column until the effluent concentration equals the incoming. The loading may be computed from the amount of the ion taken up from the solution put through in the process. In either case, one knows the amount of the ion both in moles and counts per kg of solid and by counting the solution effluent from the column can determine the concentration in equilibrium, and thereby can compute D.

Small-angle neutron scattering

Small-angle neutron scattering from several samples of carbon was measured on the 30-m (source-to-detector) instrument of the National Center for Small-Angle Scattering Research, located at the High Flux Isotope Reactor, Oak Ridge National Laboratory. The maximum range of κ [$\kappa = (4\pi/\lambda)\sin\theta$, where λ is the wavelength of the neutrons, here 4.75 Å, and 2θ is the scattering angle] was about 0.003–0.3 $Å^{-1}$, attained by using several different sample-to-detector distances from 1.4 to 16 m. Data are collected with a two-dimensional position-sensitive detector, and combined as a radial average corrected for detector background, empty-beam, and detector sensitivity by programs provided by the Center. Intensities were converted to an absolute scale (cm^{-1}) with factors provided by the Center, based on scattering by water, vanadium or other substances of known cross-sections. Runs at different distances may be combined by an available program.

Cross polarization/magic angle spinning–^{13}C NMR

Solid state nuclear magnetic resonance measurements were made on a modified Varian-XL-100 spectrometer equipped with an external ^{19}F field frequency lock, Nicolet NMR-80 data system, and a home-built, double-tuned single coil probe operating at 25.16 and 100.07 MHz for ^{13}C and ^{1}H, respectively. A single contact cross polarization experiment with radio-frequency field strengths ${}_{\gamma C}H_{1C} = {}_{\gamma H}H_{1H} = 30$ kHz was used. The Hartmann-Hahn match was adjusted with 1 dB precision. Dipolar decoupling during acquisition was accomplished with a *ca.* 40 kHz proton radio frequency field. Spectra were recorded with a 1.0 ms contact time and 2.0 s repetition rate. Spin temperature alternation was employed for the cancellation of artifacts. Powder samples were packed in a hollow screw cap Kel-F rotor (*ca.* 90 μl volume) of the Andrew type and spun at the magic angle ($\pm 0.3°$) at 2.5 ± 0.1 kHz. No attempt has been made to correct the center band areas for the overlap of first order sideband contributions to the spectra. The distortion of the aromatic centerband from the first lower aliphatic resonance sideband, similar for both samples, does not overlie the region of carboxylic acid resonances, and is estimated to introduce less than 2% error in the aromatic resonance areas.

RESULTS

Uptake. Effect of nitric acid treatment

High-surface-area charcoals

In comparisons, we frequently used an arbitrary combination of conditions: 90% (fuming) nitric acid at 70°C for 35 minutes. In Table I, we compare the effect of exposure of the three high-surface-area materials on uptake of sodium from 0.01 *M* sodium carbonate, the measured pH values scattering about 10.5 in most cases.

The uptakes of the as-received carbons varied substantially, over a range of about a factor of five. After oxidation, the values are much more similar, the maximum differences being less than 50%. They are also much greater after oxidation, the uptakes increasing from a few tenths of a mole/kg to about 1.5. Outgassing decreased uptakes in comparison to the values as received, but there is little, if any, difference outside scatter in uptakes of outgassed and as-received after nitric acid treatment. Likewise differences between coarse and fine particle sizes are probably not significant. Apparently the kinetics of the reactions with nitric acid is such that diffusion into the particles is not limiting. The times of contact in batch equilibrations also seem long enough for essential equilibrium in distribution of sodium tracer to be attained for both particle sizes. Reproducibility of uptakes of the same starting material in duplicate oxidations also seems to be good.

Effect of pH. The increase in sodium uptake after nitric acid treatment is found over the range of pH investigated, from slightly below neutral to 10–11 (Figs. 1 and 2). The conditions of the adsorption measurements were not exactly the same in all cases; Na(I) concentration was varied sometimes to obtain maximum precision (50% of initial tracer removed at equilibrium), for example. However, differences in conditions are not enough to obscure the general picture. Fig. 1 shows the lack of effect

TABLE I

SODIUM UPTAKES OF CHARCOALS BEFORE AND AFTER NITRIC ACID TREATMENT

90% HNO_3 at 70% for 35 min, measured from 0.01 *M* Na_2CO_3.

Charcoal	*Mesh size*	*As received*		*Oxidized*		*Outgassed*		*Outgassed and oxidized*	
		pH	*Na(I) equiv./kg*	*pH*	*Na(I) equiv./kg*	*pH*	*Na(I) equiv./kg*	*pH*	*Na(I) equiv./kg*
BC-1	−100, +200	10.9	0.047					10.5	1.70
	−6, +14	10.7	0.054	10.4	1.56			10.4	1.72
BC-2	−100, +200	11.1	0.30	10.4	1.81	10.7	0.10	10.5	1.72*
								10.6	1.68*
	−6, +14	10.4	0.35			10.8	0.21	10.5	1.55
Nucon	−100, +200	10.7	0.15			10.4	0.08	10.5	1.47
	−6, +14	10.4	0.15	10.5	1.26	10.4	0.16	10.4	1.40
		11.4	0.30						

* These values are for samples prepared in separate oxidations.

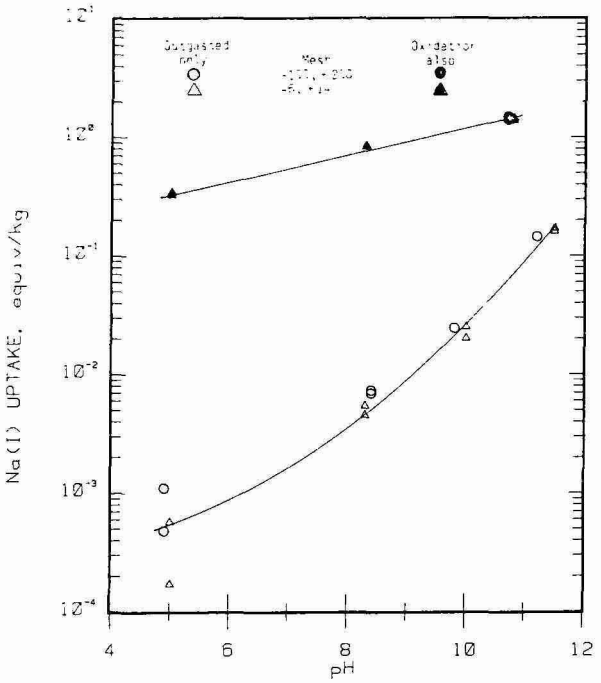

Fig. 1. Effect of particle size on sodium ion uptake of Nucon. Oxidation conditions: 70°C, 90% HNO_3, 35 min.

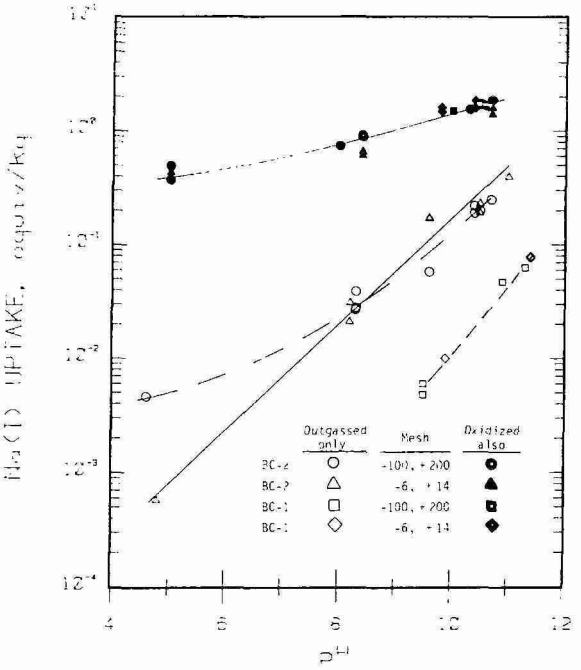

Fig. 2. Comparison of monovalent ion uptake by activated carbons. Oxidation conditions: 70°C, 90% HNO_3, 35 min.

of particle size. Fig. 2 shows that differences between samples disappear after HNO_3 treatment.

A general characteristic of these figures is an increase in sodium ion uptake with pH, the percentage increase being much greater for carbons which had not been subjected to nitric acid treatment. These substances are weak-acid exchangers, and the average pK of the protonated groups is lower (*i.e.*, the groups are stronger acids) after nitric acid treatment.

Effect of salt concentration. If the increase in uptake with pH arises from neutralization of weak-acid groups, one would expect higher sodium uptakes with higher sodium chloride concentration, owing to Na^+–H^+ exchange. Fig. 3 shows that this does occur. Fig. 4 indicates that the effects of salt concentration are relatively somewhat greater for materials not treated with nitric acid than for those that were; the values for oxidized materials fall within a narrow band on a logarithmic ordinate.

Divalent ions. Use of sodium ions to compare capacities avoids ambiguities from possible precipitation or valence state change. However, it is, of course, important to validate that the values obtained are at least relatively applicable to other ions of interest. Cu(II) is convenient to measure at pH 5, buffered with acetate, and at pH above 10, from an ammonia solution. Co(II) poses no problems from acetate buffered solutions at pH 5. Ion exchange of Co(II) from ammonium chloride–ammonia solutions (*ca.* pH 10) were found to be reversible and to follow fairly well predictions of simple theory in short-term experiments. However, on long exposure to activated carbons, distribution coefficients continued to increase and adsorption became irreversible. The reasons are not altogether clear. One possible explanation

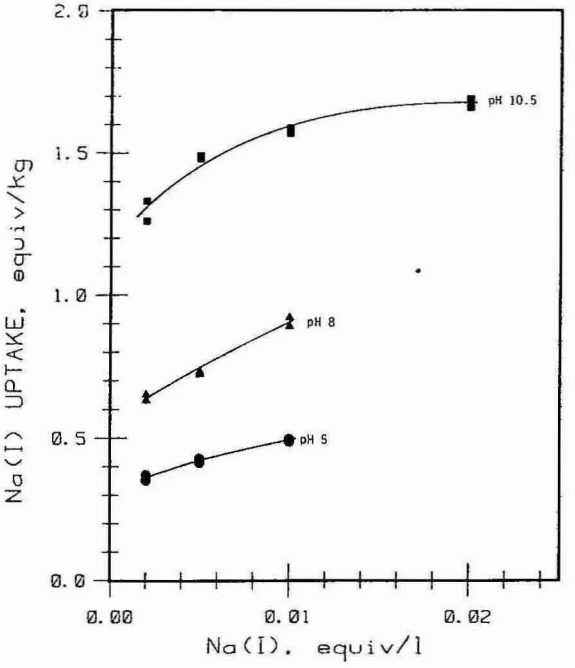

Fig. 3. Effect of salt concentration on uptake of monovalent ion on oxidized BC-2. −100, +200 mesh; oxidation conditions: 70°C, 90% HNO_3, 35 min.

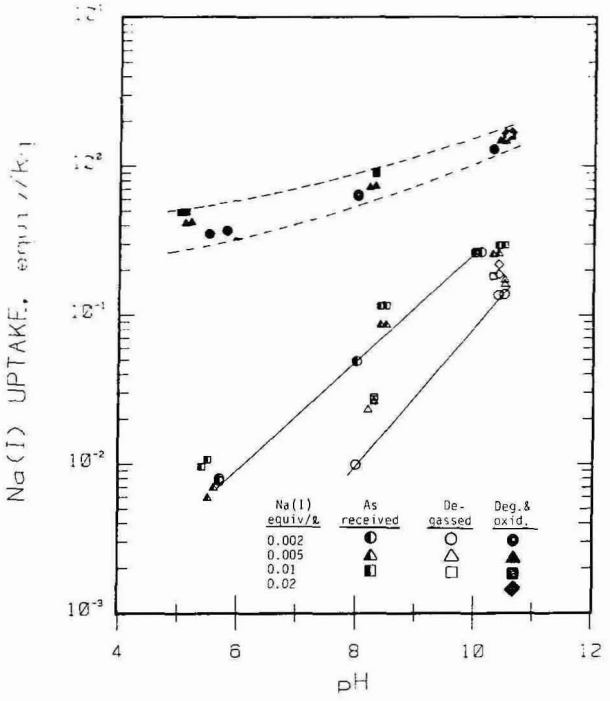

Fig. 4. Salinity and pH effects on monovalent ion uptake on carbons. BC-2; −100, +200 mesh; oxidation conditions: 70°C, 90% HNO_3, 35 min.

is oxidation to Co(III). Arguing against this is the fact that adsorption could not be reversed by reducing agents. In addition, in other studies we have found even oxidized carbon to be itself highly reducing; it will, for example, reduce pertechnetate [Tc(VII)] to lower valence states in solutions of neutral or mildly alkaline pH. The fact that 1 *M* hydrochloric acid rapidly removes irreversibly adsorbed cobalt suggests that precipitation of hydrolyzed species (in spite of the stabilization which might be expected from formation of ammine complexes) is the cause of observed irreversibility.

Fig. 5 compares uptakes of Na(I), Cu(II), and Co(II) as a function of pH for oxidized and outgassed-only Nucon. In these measurements, acetic acid was added to adjust pH to *ca.* 5, and the solutions at pH *ca.* 10 were 0.5 *M* in ammonia. For oxidized carbon, equilibrium concentrations were 0.005 *M* copper chloride or 0.005 *M* cobalt chloride at both pH values. The solutions equilibrated with non-oxidized charcoal were 0.005 *M* at pH 10 and 0.002 *M* Cu(II) or Co(II) at pH 5. Na(I) results reported in other figures are included for comparison. It can be seen that at pH 5, uptake of Cu(II) by unoxidized material is much higher than of Na(I), and Co(II) is intermediate. Relative differences appear to be less at higher pH. For the oxidized material, differences between uptakes of the various ions are not great.

Low-surface-area charcoal

The nominal 50 m^2/g BC-3 responded to nitric oxide treatment drastically differently. In contrast to other materials, it appeared to undergo considerable de-

composition, evidenced by blackening of the liquid during oxidation. Subsequently, we were unable to wash from the solid materials which blackened contacting solutions. Solutions of hydrochloric acid, of sodium hydroxide, and of sodium carbonate, as well as acetone, were tried. We carried out no further evaluation of this material, but the results with it show that nitric acid treatment does not replace the standard activation process, even though it does wash out previous differences in ion-exchange properties of various activated carbons.

Selectivity

Cation exchange between solution and solid phases is conventionally represented by an equilibrium of the type

$$M^{n+} + n[Na^+]_{solid} \rightleftarrows [M^{n+}]_{solid} + n[Na^+] \tag{1}$$

with an equilibrium constant

$$K = \frac{[M^{n+}]_{solid}\,[Na^+]^n\,(\gamma_{\pm MCl_n})^{n+1}_{solid}\,(\gamma_{\pm NaCl})^{2n}}{[M^{n+}]\,[Na^+]^n_{solid}\,(\gamma_{\pm MCl_n})^{n+1}\,(\gamma_{\pm NaCl})^{2n}_{solid}},$$

$$K = \frac{D_M[Na^+]^n\Gamma}{[Na^+]^n_{solid}} \tag{2}$$

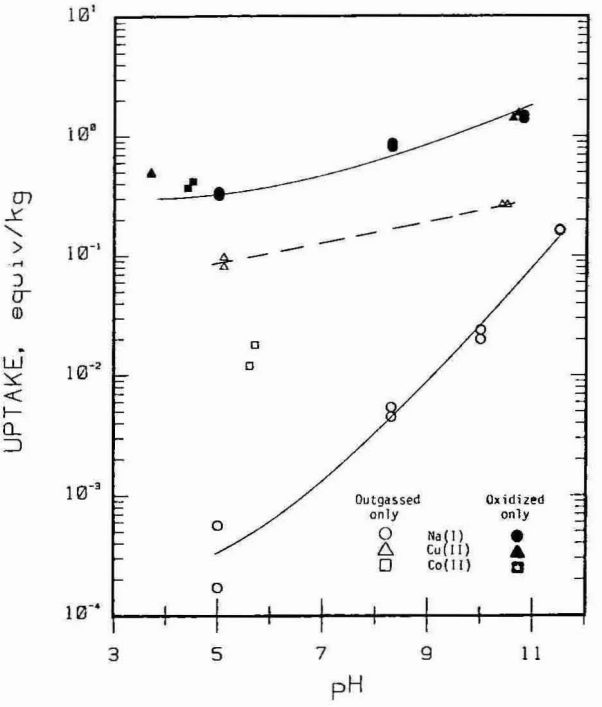

Fig. 5. Uptake of monovalent and divalent ions. Nucon, −6, +14 mesh; oxidation conditions: 70°C, 90% HNO_3, 35 min.

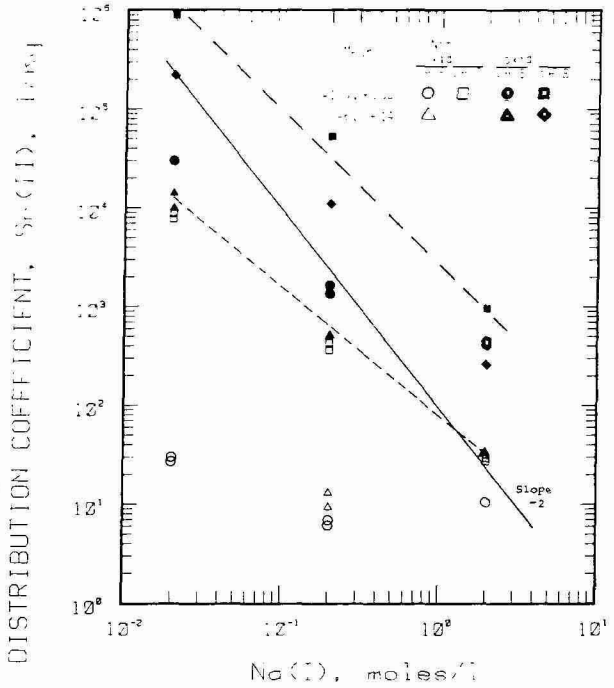

Fig. 6. Distribution of divalent ion between solution and BC-2 as a function of monovalent ion concentration. Sodium chloride + 0.01 *M* acetate, pH 5; +0.01 *M* bicarbonate, pH 8; oxidation conditions: 70°C, 90% HNO_3, 35 min.

Γ summarizing the activity coefficient ratio. If units of concentration are moles/kg solid and moles/l solution (no subscript), the units of the distribution coefficient D will be l/kg. If variations in Γ can be neglected and if coion (chloride in this example) invasion is low enough to be ignored, and if the concentration of M^{n+} is small, so that $[Na^+]_{solid}$ is essentially equal to C, the cation-exchange capacity, and therefore constant, a plot of log D_M *vs.* log $[Na^+]$ should be linear with a slope of $-n$. (This will also be true at appreciable loading of M^{n+} provided loading is constant, *i.e.*, $[M^{n+}]_{solid}/[Na^+]_{solid}$ does not vary.) Similar expressions can be obtained when coion invasion does occur, but we have made no measurements of coion invasion on activated carbons. It is probably small in most cases here, and eqns. 1 and 2 will be adequate for discussion.

Even if Γ is constant, coion invasion small, and the solution concentration of sodium ions constant, eqn. 2 requires that D_M decrease with loading of M on the solid when it becomes appreciable in comparison with the capacity, *i.e.*, $[Na^+]_{solid}$ begins to vary. Another cause of variation of D_M is of significance here, the change in apparent capacity from Na^+–H^+ exchange on these weak-acid exchangers.

Low loading of charcoals relative to capacity

Fig. 6 compares distribution coefficients of Sr(II) on BC-2 as a function of sodium ion concentration. The strontium tracer was not carrier-free, and loading of the solid (perhaps up to a tenth of Na^+ uptake) has a small effect at the highest D

value. At pH 8, the slope for the nitric acid-treated charcoal is not far from the ideal ion-exchange value of -2, at least for the small mesh size. At pH 5, the slope is less for oxidized carbon and D is almost invariant for as-received. Presumably this non-ideality reflects change of effective capacity because of Na^+–H^+ exchange. Distribution coefficients are higher for nitric-acid treated solid, other conditions being equal. They are also higher at higher pH. Substantial differences between different particle sizes may imply that equilibrium was not attained in all cases.

High loading

Distribution coefficients of another divalent ion, the Cu(II)–ammonia complex, were measured as a function of monovalent ion solution concentration at appreciable, but constant, Cu(II) loading of the carbons by the preloaded column technique. The carbons were first loaded by passing through columns of them a solution of 0.002 M in Cu(II) and 1 M in ammonium chloride and in ammonia until the effluent reached the influent concentration. The amount of Cu(II) on the carbon was computed from the amount taken up in the process, analyses being by flame atomatic absorption. Uptakes of Cu(II) are listed in Fig. 7. The concentration of ammonium chloride and ammonia (always the same) was then varied and the effluents analyzed. The results (Fig. 7) plotted as log D_{Cu} *vs.* log[NH_4^+] fall roughly about the ideal ion-exchange slope, -2.

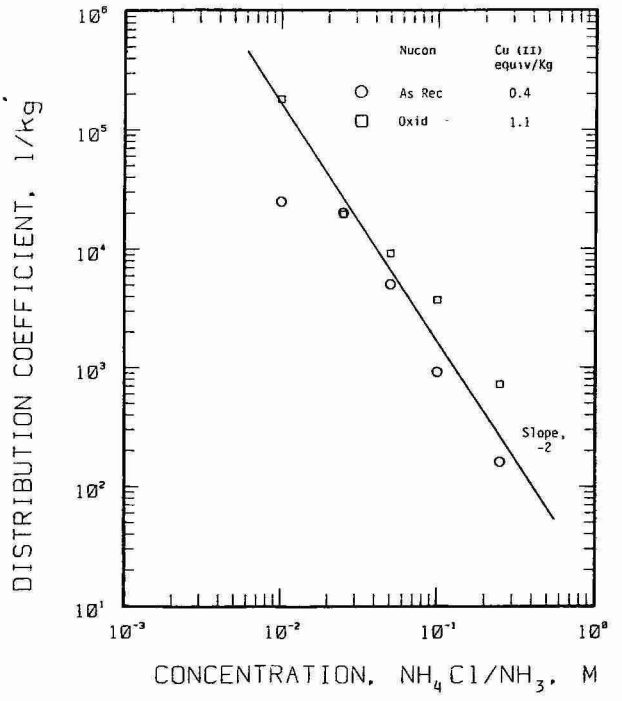

Fig. 7. Distribution of Cu(II) between activated carbon and NH_4Cl solutions at high Cu(II) loading. NH_3 concentration equal to NH_4Cl, pH ≈9.4; oxidation conditions: 70°C, 90% HNO_3, 35 min; −100, +200 mesh.

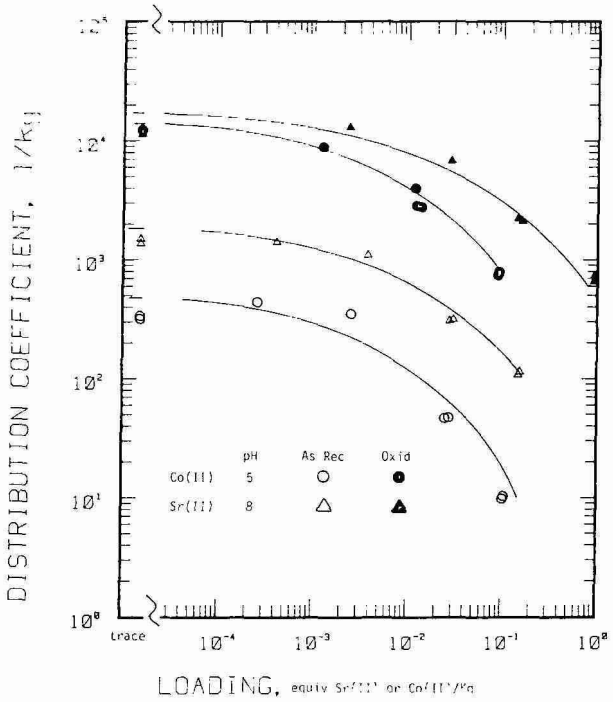

Fig. 8. Effect of loading on distribution of divalent ions. BC-2, −100, +200 mesh; oxidation conditions: 70°C, 90% HNO_3, 35 min; 0.2 *M* sodium chloride + 0.01 *M* acetate or bicarbonate.

Loading effects

Distribution coefficients of one ion (here, a divalent ion) in exchange with a constant solution concentration of another (here, monovalent) are expected to decrease as the concentration of the first on the solid increases (eqn. 2, as $[Na^+]_{solid}$ decreases). Fig. 8 shows that this is indeed the case for Co(II) and Sr(II) adsorbed from 0.2 *M* sodium chloride on both oxidized and as-received charcoal. The pH values were selected to avoid possible difficulties with hydrolysis. We do not have measurements of sodium ion uptake on these materials from 0.2 *M* sodium chloride, but we estimate from other results that they would be about 0.01 equiv./kg at pH 5 and 0.1 at pH 8 for as-received and about 0.5 equiv./kg at pH 5 and 1 at pH 8 for oxidized. If these numbers are correct, significant declines in *D* are seen sooner (*i.e.*, at lower ratios of loading of divalent ion to sodium uptake) than ideal ion-exchange behavior (eqn. 2, constant Γ, constant equivalent Na^+ plus divalent on solid) would predict, at least for the oxidized material. The estimates of Na(I) uptake are too uncertain for a detailed analysis to be worthwhile.

Effect of conditions of nitric acid treatment

Our initial attempt at nitric acid treatment was adapted from Puri *et al.*[10]: charcoal was degassed for 28 h at 500°C in a vacuum, 5 g were treated with 150 ml of fuming nitric acid added in increments, with increasing temperature to a final value of about 110°C. This procedure converted the charcoal we used almost com-

pletely to a colloidal material, unusable as a solid adsorbent. However, milder treatment such as that used to obtain the results described so far (exposure to 90% nitric acid at 70°C) gave solids on which batch equilibrations could be carried out and from which columns could be prepared. The next three figures show the effect of variation of these treatment parameters.

Fig. 9 gives the dependence on nitric acid concentration of sodium ion uptake on Nucon exposed for 100 min at 70°C. Uptake rises with nitric acid concentration as expected.

Fig. 10 gives the variation of uptake with temperature of oxidation treatment. As one would expect, uptake increased, although the dependence (a large increase over untreated, but about the same at 60 and 70°C, and a sharp rise thereafter) seems odd.

Fig. 11 shows increase of uptake with increasing oxidation time for several combinations of temperature and nitric acid concentration.

Characterization of charcoals

We have attempted in several ways to obtain information contributing to an understanding of the effects of nitric acid treatment on charcoals.

Surface area

The increase in ion-exchange uptakes could plausibly result either from an

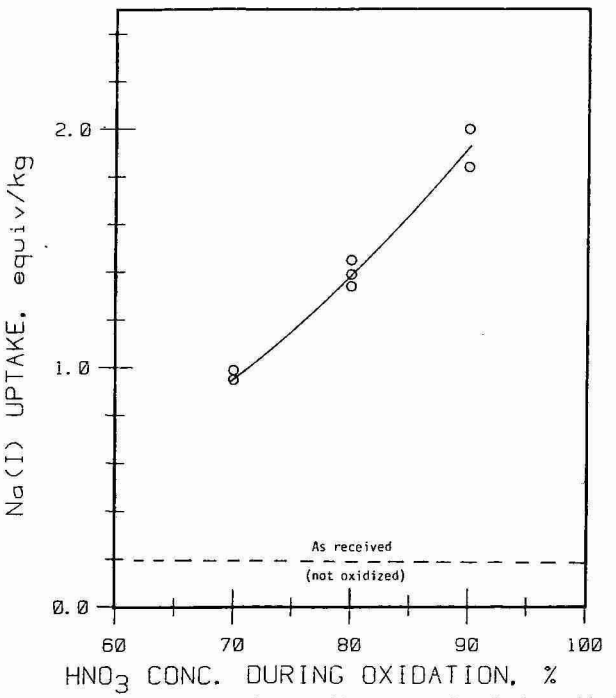

Fig. 9. Dependence of uptake on acid concentration during oxidation. Nucon, −6, +14 mesh; oxidation conditions: 70°C, 100 min; uptake from 0.01 *M* Na_2CO_3, pH 10.7.

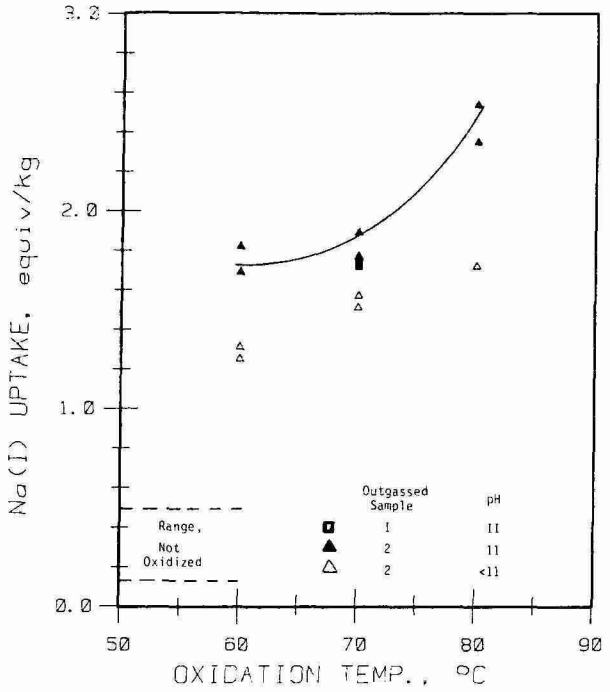

Fig. 10. Effect of oxidation temperature. BC-2; oxidation conditions: 90% HNO_3, 35 min.

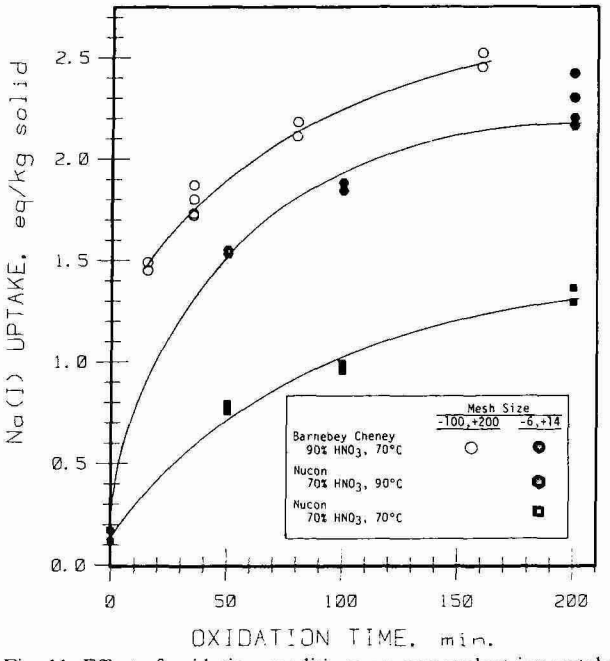

Fig. 11. Effect of oxidation conditions on monovalent ion uptake by charcoals. Measured from 0.01 *M* Na_2CO_3, pH 10.5.

TABLE II

SURFACE AREAS AND HELIUM DENSITIES OF NUCON CHARCOAL SAMPLES*

	Sample wt. (g)	*Helium density (g/cm^3)*	*Surface area (m^2/g)*
As received	0.0507	2.013	1020
After HNO_3 treatment	0.0509	2.062	970

* Carried out by J. D. Kintigh of the ORNL Metals and Ceramics Division.

increase in surface area or from an increase in density of ionizable groups per unit area. BET nominal surface area measurements were carried out on as-received, −100, +200 mesh, Nucon and on the same material after treatment with 90% nitric acid at 70°C for 35 min. The results are given in Table II. The sorbing gas was nitrogen. There is no significant increase in gas adsorption or exposed nominal surface area, from oxidation. Differences between samples in both area and in helium apparent density are considered insignificant.

NMR characterization

We carried out ^{13}C NMR scans on solid samples of hydrogen-form, −100, +200 mesh, BC-2 as-received and after nitric acid treatment (90% acid at 70°C for 35 min). Conversion to the hydrogen form in this case was by two contacts with 10 ml of 1 *M* hydrochloric acid solution. The results are shown in Fig. 12. The spectra resemble those of bituminous coals with exaggerated low field "aromatic" resonances which represent phenolic (145–165 ppm) and carboxylic acid (170–185 ppm) moieties. The integrated intensity of the spectra in Fig. 12 is only about 10% of that obtained for coals measured under similar conditions. The bulk of this hydrogen-poor material apparently does not yield a cross polarization signal. However, relative peak areas of the detected signal in these two samples show significant differences. The carboxylic acid group resonance is substantially higher in the oxidized sample. There also appears to be a less well-defined area increase in the phenolic region of the spectrum.

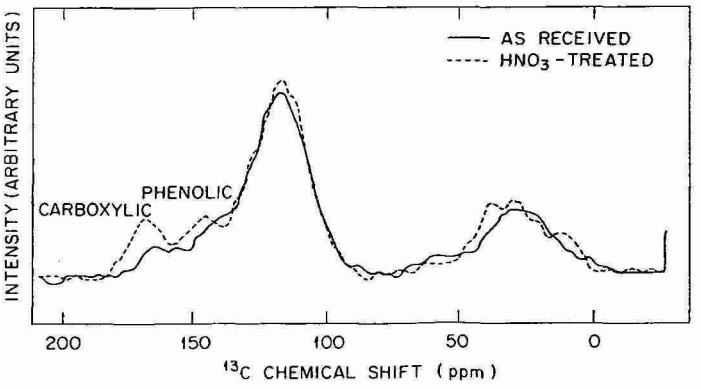

Fig. 12. NMR scans of −100, +200 mesh BC-2 activated carbon, before and after nitric acid treatment. 90% HNO_3 at 70°C for 35 min.

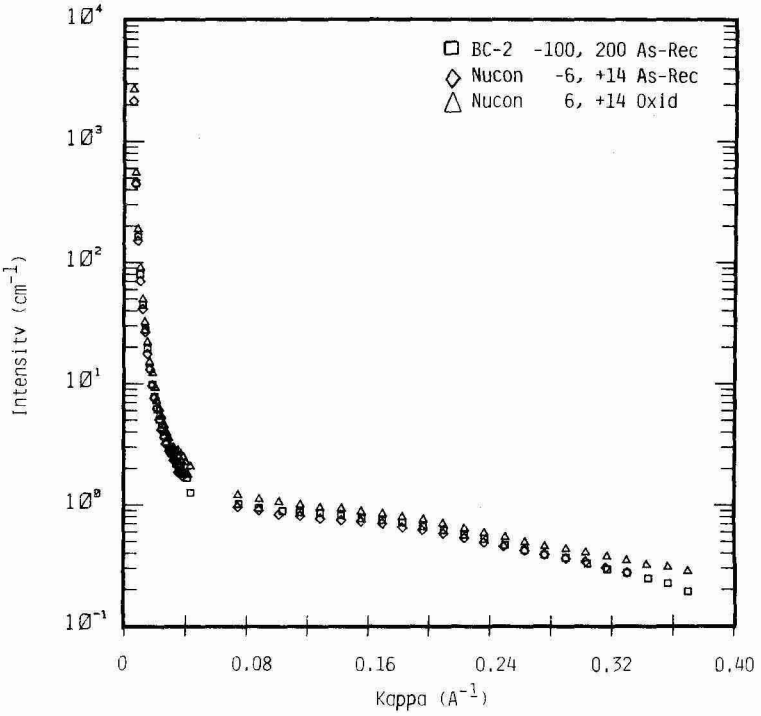

Fig. 13. Small-angle neutron scattering from as-received and HNO_3-treated carbons. Oxidation conditions: 70°C, 90% HNO_3, 35 min.

Taken together with ion-exchange behavior, these observations suggest strongly that increased sodium uptake with nitric acid treatment arises from an increase in the density of carboxylic and phenolic groups on the charcoal.

Small-angle neutron scattering

Neutrons are scattered at small angles from these charcoals because of inhomogeneities arising from the existence of pores. Patterns can therefore be used to detect changes in pores arising from treatment with nitric acid. Recently Hall and Williams[13] reported scattering from activated carbon. We obtained a pattern for as-received Nucon extending from $\kappa \approx 0.003$ to 0.3. We do not reproduce it here because our inferences are essentially the same as theirs: that there is a distribution of void sizes, difficult to analyze in detail; and that from the results at the high end of the κ range, where the effect of interpore interferences is diminished, a Guinier dimension[14] of about 6 Å can be obtained.

More pertinent to present concerns is a comparison of patterns taken at two distances from an as-received charcoal and from the same material exposed to 90% nitric acid for 35 min at 70°C (Fig. 13). Differences are slight, in agreement with the comparison of surface areas. A pattern from BC-2 included in the figure is similar to those from Nucon.

DISCUSSION

The results presented here, in agreement with the literature, show that oxidative treatment can increase the effective cation exchange capacity of active carbon at a given pH by an order of magnitude. The functional groups contributing to uptake are more strongly acidic than those on the untreated charcoal. Distribution coefficients are also enhanced by oxidative treatment. There is some sacrifice in particle strength, but probably not to a prohibitive extent for many column applications.

In contrast to the observations of Coughlin and Ezra[15], the increases in uptake with oxidation were not accompanied by change in surface area. They found increases in uptake (as measured by base consumption from aqueous solutions of bases of different strength) similar to ours, but the nominal surface area of the active carbon they investigated (Union Carbide Columbia carbon LC325) was less than half the original 1200 m^2/g) after oxidation. They also found that the increase in uptake of ions was accompanied by a decrease in adsorption of phenol and nitrobenzene both per unit weight and per unit surface area. Because small-angle neutron scattering also indicated that no great changes in the structure of our material resulted from nitric acid treatment, we conclude that there are basic differences between their activated carbon and ours.

In much of the literature, increase in ion exchange capacity with oxidation has been attributed to "chemisorption" of carbon dioxide, which then forms salts when contacted with alkaline solutions. In at least some authors' minds, formation of carboxylate groups attached to hydrocarbon-type structures would appear to be included under this term. Puri *et al.*[10] for one has a more specific model, however, based on such evidence as equivalence between adsorption of Ba^{2+} and carbon dioxide removed by outgassing. Such a picture would imply here that there is carbon dioxide held entrapped by the carbon surface, not rapidly exchangeable under the conditions of our equilibrations with carbonate in solution phases. The ready exchange of other inorganic ions raises questions concerning this mechanism in our case.

Puri's conditions in nitric acid treatment and in other experiments were considerably more severe than ours. To us, the simplest inference is that the increase in uptake results from increase in carboxylate and phenolate groups on the carbon. The NMR results support this explanation of capacity increase. In this connection, if ortho diphenols were formed, their acid strength would be greater than monophenols. We had analyses carried out on as-received and nitric acid-treated samples by a commercial laboratory. There were increases in oxygen content with oxidation, but we are not sure how much adsorbed gases may have contributed to the results. One inference can be safely drawn from the analyses. Nitrogen was low with samples before and after oxidation and increased only slightly from the treatment. Nitration of the carbon thus appears to have made only a small contribution.

We measured as a function of pH uptakes of two commercial samples of oxidized activated carbon, Spherical Oxycarbon G-30-16 × 35 and Oxycarbon G-80-16 (Nuclear Consulting Services, Columbus, OH, U.S.A.). Uptakes were comparable to nitric acid samples we prepared, with somewhat greater dependence on sodium ion concentration.

Particle attrition is frequently a serious problem in use of adsorbents in col-

umns, and the effect of oxidation procedures on particle strength is of concern. Evaluation by crushing tests has been carried out, and is the subject of another planned communication[16]. Here we comment only that there is an appreciable loss of strength accompanying the increased ion-exchange capacity, but that it does not appear prohibitive for many applications. Our hope that this proves correct is increased by the fact that the increased capacity may not only be helpful in separations using the materials discussed here, but it also increases the amounts of other adsorbents with which the pores of the carbons can be loaded[17]. Adsorbents of desirable selectivity but difficult to make in forms of size, homogeneity, and stability suitable for chromatography can frequently be used when held in carbon.

ACKNOWLEDGEMENTS

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ON-LINE ELECTROCHEMICAL REAGENT PRODUCTION FOR FLUORESCENCE DETECTION OF PHENOTHIAZINES IN LIQUID CHROMATOGRAPHY

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(Received October 16th, 1985)

SUMMARY

Thioridazine was detected by fluorimetry after separation by reversed-phase liquid chromatography, using a post-column derivatization system in which electrochemically generated bromine is applied as an oxidant. Analysis of plasma samples did not involve an extraction step. The detection limit was 0.5 ng or 5 ng/ml in plasma. The peak-area reproducibility was ±4.6% for plasma samples (n = 10). This inexpensive derivatization system compares well with alternative techniques. It can also be applied to the detection of other phenothiazines.

INTRODUCTION

As fluorescence measurement is generally the most sensitive and selective detection method for high-performance liquid chromatography (HPLC), much effort has been put into the development of derivatization techniques for compounds that lack a strong native fluorescence. Of these, pre-column methods have the advantage that the fluorescence labelling can be combined with an improvement in the separation properties of the compounds of interest and with sample clean-up. On the other hand, post-column techniques are also often applied as artefacts are less easily generated and automation is simpler. Several post-column derivatization techniques can be applied, such as the addition of reagent solutions, liquid–liquid phase reactions, solid phase reactions, including enzyme reactions, light irradiation and electrochemical conversion. Recently an extensive review on fluorescence derivatization methods for HPLC has been published[1].

In previous work[2,3] we have presented an electrochemical cell for the on-line production of reagents in the column effluent. It was used in conjunction with an amperometric detector, which monitored the excess of reagent. However, electrochemical reagent generation can be applied just as well with other detection princi-

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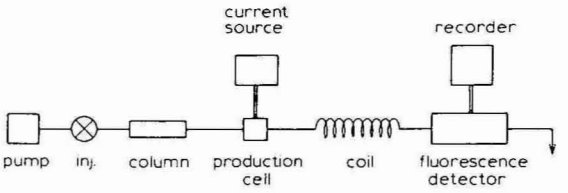
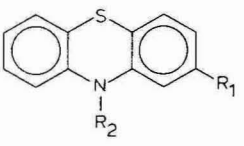


Fig. 1. Set-up for fluorescence detection in HPLC with on-line generated reagents.

ples, such as fluorimetry. Fig. 1 shows a scheme of this method. It has several advantages compared with other post-column techniques: (i) as opposed to the addition of reagent solutions, there is no extra peak dilution, no mixing problems, less pump noise and no need for an extra pump; (ii) in comparison with solid-phase reactors, the system is more versatile and the reactor does not wear out; (iii) with photochemical reactors the reaction time is usually long and the reaction often unspecific; as will be shown, this is not necessarily the case with electrochemically generated reagents; (iv) direct electrochemical conversion of a compound into a fluorescent product is also a promising technique, as has been shown[4] with vitamin K_1. When a coulometric cell is used, the compound of interest can be fully converted, and the same sensitivity may be expected as with the indirect method of electrochemical reagent generation, if the heterogeneous and the homogeneous reactions give the same products. An advantage of the indirect method presented in this paper is that it is less susceptible to electrode deactivation.

As a model compound we have chosen thioridazine (I), which is widely used as a psychotherapeutic drug. Its determination in plasma is difficult because of the low therapeutic concentrations (10–200 ng/ml). Normal- and reversed-phase HPLC separations of thioridazine and its metabolites have been used with UV[5,6], electrochemical[7,8] and fluorescence[9–11] detection. Thioridazine exhibits a weak native fluorescence, but it has been recognized in the past that oxidation of phenothiazines enhances the fluorescence intensity[12–14]. Determination by HPLC with fluorescence detection is therefore based on post-column oxidation. Muusze and Huber[9] oxidized thioridazine and its metabolites after normal-phase HPLC separation by addition of a permanganate solution and subsequent removal of the excess of permanganate with hydrogen peroxide. Wells *et al.*[10] modified this method. Scholten *et al.*[11] applied a post-column photochemically induced oxidation with fluorescence detection.

An attempt to effect the direct electrochemical conversion of thioridazine was not successful. Therefore, we studied a post-column system in which electrochemically generated bromine is the oxidant, and compared it with the above-mentioned methods for the determination of thioridazine in plasma. The applicability of the system to other phenothiazines has also been investigated.



I $R_1 = -S-CH_3$

II $R_1 = -SO-CH_3$

III $R_1 = -SO_2-CH_3$

$R_2 = -CH_2CH_2-$ (N-CH_3)

EXPERIMENTAL

Apparatus

Excitation and emission spectra were registered with a Perkin-Elmer 3000 scanning spectrofluorimeter with 1 × 1 cm quartz cuvettes. The chromatographic equipment consisted of a Perkin-Elmer 601 pump, a Rheodyne injection valve with 20- and 200-μl loops, a 250 × 4.6 mm I.D. column packed with LiChrosorb RP-8 (5 μm), a Kontron SFM 23 spectrofluorimeter, a laboratory-built integrator and a Kipp BD-41 recorder. In optimization studies a Metrohm 1096/2 amperometric detector was used with a 3-mm glassy carbon working electrode and a silver–silver chloride–1 *M* lithium chloride reference electrode. For on-line electrochemical reagent production a KOBRA-cell, manufactured in the Free University Chemistry Department workshop, and described in the literature[2], was used with a home-made constant-current source. Electrochemical batch experiments were performed with a rotating platinum electrode, silver–silver chloride–1 *M* lithium chloride reference electrode and a platinum auxiliary electrode in a three compartment vessel.

Reagents and solutions

Thioridazine(I), mesoridazine besylate(II) and sulforidazine(III) were obtained from Sandoz. Other phenothiazines were obtained from various sources. The other chemicals used were of analytical-reagent grade. Phenothiazine stock solutions were prepared in methanol and stored at 4°C in the dark. The mobile phase was 70% (v/v) methanol–water containing 0.1 *M* lithium nitrate, 0.05 *M* tetraethylammonium perchlorate, 1 m*M* potassium bromide and 0.1 m*M* EDTA. The EDTA was added shortly before use.

Plasma sample preparation

Human pooled plasma was stored at −18°C. After thawing it was spiked with the desired amount of thioridazine and incubated for 30 min. For deproteination 1 ml of methanol containing 0.1 *M* sulphuric acid was added to 1 ml of plasma. The mixture was shaken vigorously and centrifuged for 10 min in a table-top centrifuge. Of the supernatant 200 μl were injected.

RESULTS AND DISCUSSION

Electrochemical and chemical oxidation

In first instance we investigated the direct electrochemical oxidation of thioridazine. With a rotating platinum electrode in a 0.1 *M* acetic acid buffer a well defined, diffusion-limited wave was obtained with a half-wave potential of +0.53 V *vs.* silver–silver chloride. Under the assumption that the electrode reaction is reversible, from the slope of the wave a value of $n = 1$ for the number of electrons transferred was calculated. On prolonged electrolysis at +0.8 V, a compound was produced with a dark blue colour. This compound was unstable, especially when exposed to light. It is likely that under the conditions employed direct electrochemical oxidation yields initially the radical cation of thioridazine[15]. Probe measurements showed that the 320/465 nm fluorescence of the unoxidized thioridazine decreased during electrolysis, and a new, stable fluorescent compound with maxima at 320/390 nm was found.

However, the intensity was not higher than that of the original thioridazine. Fluorescence signal enhancement was thus not possible by direct electrochemical conversion, and we directed our study to the on-line generation of bromine as homogeneous oxidant.

We studied the bromine oxidation of thioridazine and two of its metabolites, mesoridazine(II) and sulforidazine(III). When an excess of bromine was added to 10^{-4} *M* solutions of these compounds in an acetic acid buffer, the fluorescence intensity increased drastically. As can be seen in Fig. 2, the excitation spectra of the oxidized compounds show the typical crescendo-like wave paterns with three maxima such as have been reported for oxidation with potassium permanganate[13]. The spectra of the oxidation products of thioridazine and mesoridazine are identical and that of sulforidazine deviates slightly. Maximum fluorescence intensity with sulforidazine was obtained with a stoichiometric amount of bromine and for the other compounds an excess of bromine had to be added. Although the absolute fluorescence intensity for thioridazine is lower than that of the other compounds, the relative gain was largest for thioridazine, as the fluorescence of the unoxidized form is weak.

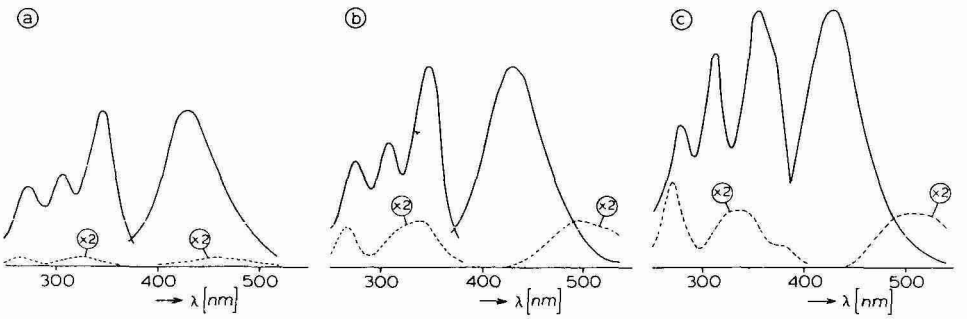


Fig. 2. Fluorescence excitation and emission spectra of thioridazines, before (---------) and after (———) oxidation with bromine. (a) Thioridazine; (b) mesoridazine; (c) sulforidazine. For the underivatized thioridazines the signal is amplified twice.

Optimization of the post-column system

The optimization of the post-column system was carried out with flow-injection experiments. In the early stages of our experimental work we used 0.1 *M* potassium bromide in methanol–water mixtures with acetate or formate buffers as the solvent in which bromine was generated electrochemically. In contrast to the batch experiments, the gain in fluorescence intensity was disappointingly low. High generating currents (1 mA, corresponding to a bromine concentration of 0.31 m*M* at a flow-rate of 1 ml/min) and long reaction capillaries (1 min) were needed to oxidize thioridazine to a sufficient extent. Only under these conditions was the fluorescence enhanced. By thermostating the reaction capillary at 60°C the reaction was accelerated, and the capillary could be shortened to reduce band broadening. A considerably faster reaction was observed with the unbuffered methanol–water mixtures containing only 1 m*M* KBr, which we used in subsequent studies. The decrease in bromide concentration leads to less formation of tribromide ion, which is less reactive than

bromine. Apart from this, the buffers appeared to have an inhibitory effect on the oxidation reaction.

The relationship between bromine consumption and fluorescence intensity for the three thioridazine derivatives was investigated by varying the reaction time and generating current. The bromine consumption was calculated from peak areas obtained with an amperometric detector instead of the spectrofluorimeter. This amperometric detector monitored the excess of bromine after the reaction coil. The reaction time multiplied by the concentration of bromine generated was taken as an indication of the "oxidation strength" of the derivatization system. As can be seen in Fig. 3, mesoridazine and sulforidazine react with at most one stoichiometric amount of bromine, mesoridazine with the higher reaction rate. The fluorescence intensity, measured at 345/425 nm, is proportional to the progress of the reaction. Thioridazine reacts with more than one equimolar amount of bromine at higher oxidation strengths. The largest gain in fluorescence intensity is in the first stage of the reaction.

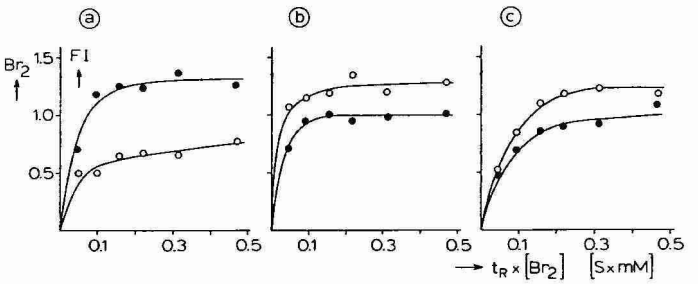


Fig. 3. Dependence of the amount of bromine reacted (●) and fluorescence intensity of thioridazines (○) on the reaction time and generating current. (a) Thioridazine; (b) mesoridazine; (c) sulforidazine. The bromine uptake is in moles of Br_2 per mole injected. For details see text.

Although there is still some ambiguity between the results of the batch and flow-injection experiments, some general conclusions on the mechanisms involved may be drawn. (i) Oxidation of the ring sulphur to a sulphoxide, the first step in the reaction of the thioridazines, is essential to obtain a high fluorescence. (ii) Oxidation of the side-chain sulphur enhances the fluorescence further. (iii) With oxidized side-chain sulphurs, as in sulforidazine, the rate of oxidation of the ring sulphur is decreased.

We proceeded with a reaction coil with a 12 s hold-up time and a generating current of 100 μA, corresponding to a bromine concentration of 3.1×10^{-5} *M* at a flow-rate of 1 ml/min. Under these conditions the fluorescence intensities of the three compounds virtually reach a plateau value.

Determination of thioridazine in plasma

Separation of thioridazine from interfering compounds was achieved with an RP-8 column and an aqueous mobile phase containing 70% (v/v) methanol, 0.1 *M* lithium nitrate, 0.05 *M* tetrathylammonium perchlorate, 1 m*M* potassium bromide and 0.1 m*M* EDTA. The addition of the quaternary ammonium salt to the mobile phase was necessary to reduce peak tailing. A guard column in front of the injection

valve improved the stability of the analytical column, probably by preventing the accumulation of metal salts from the pump on the top of the analytical column. Sulforidazine, mesoridazine and thioridazine eluted with k' values of 0.35, 0.6 and 2.3, respectively. The signal obtained with standard thioridazine injections was linear with the amount injected up to 40 ng. Above this amount signals were lower than expected. This may be caused by an internal fluorescence quenching effect, or by a depletion of the available reagent. The improvement of the sensitivity by the post-column system was determined by comparing signal-to-noise ratios obtained with underivatized and oxidized thioridazine at the appropriate wavelengths. As can be seen from Table I, the bromine generation system improves the detection limit by a factor of 16. A signal-to-noise ratio of 2 will be obtained with 0.5 ng injected.

TABLE I

SENSITIVITY OF FLUORESCENCE DETECTION OF THIORIDAZINE WITH AND WITHOUT POST-COLUMN OXIDATION WITH BROMINE

i_G (μA)	*Detection wavelengths*		*Signal*,***	*Noise**	*Signal-to-noise ratio*
	λ_{exc} (nm)	λ_{em} (nm)			
0	325	465	3.5	0.8	4.4
0	345	425	<2	1.1	<2
100	325	465	19	0.8	24
100	345	425	76	1.1	69

* Fluorescence intensity in arbitrary units.
** 0.1 nmole of thioridazine injected.

The clean-up procedure for plasma samples was a simple deproteination technique. An equal volume of acidified methanol was added to the plasma and the mixture was centrifuged. Of the supernatant 200 μl were injected. Fig. 4 shows the chromatograms of a standard solution, a blank plasma and a plasma sample spiked with 20 ng/ml of thioridazine.

The recovery of thioridazine from spiked plasma samples was determined by comparing peak areas with those of standard solutions (Table II). The recoveries are close to 100%. No interfering compounds were found in plasma samples, and the detection limit is the same as with standard solutions, *viz.*, 0.5 ng, corresponding to a plasma concentration of 5 ng/ml. The peak-area variance was $\pm 3.8\%$ ($n = 4$) for a standard solution and $\pm 4.6\%$ ($n = 10$) for a plasma supernatant, both containing 100 ng/ml of thioridazine.

The above investigations were performed with a pulseless pump and a custom-made current source. We also tested a reciprocating pump and replaced the constant-current source by a simple 12 V d.c. supply, with a 100 kΔ resistance in series with the cell. As the voltage drop over the production cell was approximately 2 V, the current was close to 100 μA. The signal-to-noise ratios were unchanged. Therefore, apart from the reagent production cell, the extra investment for the post-column derivatization system can be minimal.

TABLE II

RECOVERY OF THIORIDAZINE FROM PLASMA AFTER DEPROTEINATION WITH AN EQUAL VOLUME OF ACIDIFIED METHANOL

Thioridazine concentration added to plasma (ng/ml)	*Recovery (%) (mean ± S.D., n = 3)*
200	95 ± 2
100	101 ± 6
20	93 ± 9

Other phenothiazines

Preliminary experiments were performed to establish the applicability of the bromine derivatization system to the determination of other phenothiazines. Excitation and emission spectra of these compounds were measured before and after addition of an excess of bromine. Chromatograms were recorded with the fluorescence detector at the optimal wavelength for the underivatized and oxidized compounds, with the generating current on and off, respectively. No effects were made to optimize the separation or the derivatization; the same conditions as for thioridazine were applied. Table III summarizes the results. Changes in the noise level of the detector are not accounted for in this table. Although the increase in the fluorescence intensity is not as large as with thioridazine, the sensitivity for the phenothiazines can be improved 2–14 times with the post-column system. Further improvements may be possible by optimization of the reaction time or generating current for the individual compounds.

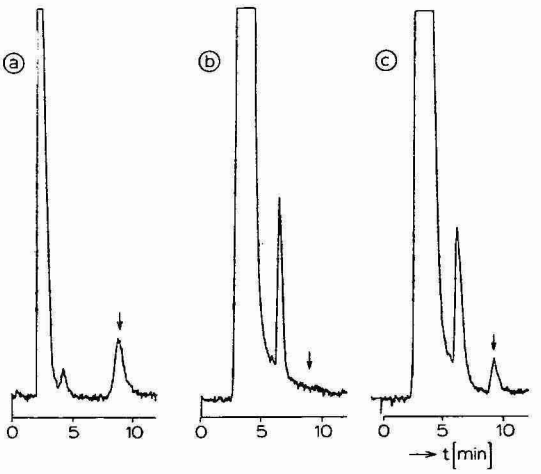
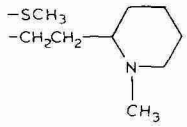
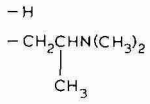
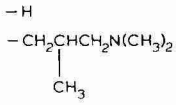
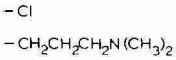
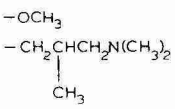
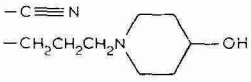


Fig. 4. Chromatograms of (a) standard solution of thioridazine (20 ng/ml); (b) blank plasma, deproteinated; (c) plasma, spiked with 209 ng/ml thioridazine and deproteinated with an equal volume of acidified methanol. For details see text.

TABLE III

FLUORESCENCE INTENSITY OF PHENOTHIAZINES WITH AND WITHOUT POST-COLUMN OXIDATION WITH BROMINE

Phenothiazine	R_1, R_2	*Unoxidized*			*Oxidized*			*Gain*
		λ_{ex}	λ_{em}	*F.I.**	λ_{ex}	λ_{em}	*F.I.**	
Thioridazine		325	465	1.00	345	425	19	19
Promethazine		315	440	0.8	335	375	3.2	4
Alimemazine		315	445	1.4	340	375	16	12
Chlorpromazine		315	440	0.45	340	375	6.2	14
Levomepromazine		315	445	1.8	340	375	3.5	1.9
Propericiazine		340	510	8.6	375	435	16	1.8

* F.I. = fluorescence intensity relative to unoxidized thioridazine.

TABLE IV

COMPARISON OF HPLC DETECTION METHODS FOR THIORIDAZINE

*Separation**	*Detector*	*Post-column oxidation*	*LOD*** (ng)*	*Plasma extraction****	*Lowest plasma concn. (ng/ml)*	*Ref.*
NP	UV, 254 nm	–	2	5	10	5
RP	UV, 254 nm	–	1	2	1	6
RP	LC–ED, +0.9 V	–	1	3	–	7
RP	LC–ED, +0.9 V	–	0.1	4	1	8
NP	Fluorescence, filters	Permanganate/H_2O_2	0.8	2	2	10
RP	Fluorescence, 340/378 nm	Photochemical	0.5	–	50	11
RP	Fluorescence, 345/425 nm	Bromine	0.5	–	5	This work

* NP = normal phase; RP = reversed phase.

** LOD = limit of detection, signal-to-noise ratio = 2.

*** Number of extraction steps in clean-up procedure.

CONCLUSION

In Table IV our detection system is compared with other techniques reported in the literature. Fluorescence detection after post-column oxidation is not superior to other detection modes in respect of absolute detection limits, but the selectivity is such that elaborate sample extraction procedures are not necessary. Other workers determined lower thioridazine concentrations in plasma than we can by concentrating the sample as part of the clean-up procedure. Although it was not attempted, the results indicate that our system is also compatible with sample concentration methods, *e.g.*, pre-column techniques, as the instrumental noise overrules any chemical noise from trace sample compounds. In this way lower plasma concentrations can be determined if necessary. Table IV shows that our inexpensive derivatization method compares well with alternative techniques. Studies on the electrochemical generation of other reagents for optical signal enhancement are under way.

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PENTAFLUOROBENZYL *p*-TOLUENESULPHONATE AS A NEW DERIVATIZING REAGENT FOR ELECTRON-CAPTURE GAS CHROMATOGRAPHIC DETERMINATION OF TRACE INORGANIC ANIONS

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SUMMARY

Inorganic anions in water were reacted with pentafluorobenzyl *p*-toluenesulphonate in dichloromethane, using tetra-*n*-amylammonium chloride as a phase-transfer catalyst. Bromide, iodide, thiocyanate and nitrite at relatively low concentrations can be pentafluorobenzylated, and the resulting derivatives can be detected by electron-capture gas chromatography. The interferences of some anions were examined. The recovery test of bromide, iodide or thiocyanate spikes and an intercomparison study for nitrite were performed. This gas chromatographic method has been successfully applied to the determination of bromide and iodide in kelp extract, and thiocyanate and/or nitrite in human urine or saliva.

INTRODUCTION

To date, gas chromatography (GC) has been widely used for separation and analyses of volatile compounds. Furthermore, by using derivatization, non-volatile compounds can be determined by GC. Derivatization has been employed not only to volatilize the analyte but also to introduce a detector-oriented tag into the molecule. Electron-capture detection (ECD) has been studied in conjunction with derivatization to introduce a detector-oriented tag, due to its high sensitivity, and several derivatizing reagents have been developed for GC–ECD[1]. The determination of inorganic anions by GC with derivatization has not been extensively studied[2], compared to that for inorganic cations. This is due to the failure to develop a suitable method applicable to the derivatization of various anions.

MacGee and Allen[3] reported a method for determining halide ions, which were converted into their tetraalkylammonium salts on an ion-exchange resin and then into the corresponding alkyl halides by thermal decomposition in the injection port

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of the gas chromatograph. Moore[4] has modified the method by using *n*-butyl *p*-toluenesulphonate as the alkylating reagent. Faigle and Klockow[5] have also developed the method using *n*-decyl methanesulphonate: in this case, cyanide, thiocyanate and nitrate can be determined, as well as halides. These methods are complicated and time-consuming, because the inorganic anions must first be isolated or dried, and then derivatized in the water-free organic solvent. We have improved Moore's method: aqueous inorganic anions were extracted into the organic layer as their tetraalkylammonium salts and then converted into their *n*-butyl derivatives, using *n*-butyl *p*-toluenesulphonate as the derivatizing reagent[6]. The method is not very sensitive because the use of flame ionization detection (FID) could not be avoided, owing to the introduction of the hydrocarbon moiety from the derivatizing reagent. Therefore, in order to use ECD, which is much more sensitive than FID, we have attempted pentafluorobenzylation of inorganic anions instead of *n*-butylation as in the above method. A new derivatizating reagent, pentafluorobenzyl *p*-toluenesulphonate (TsO-PFB) has been synthesized, and its applicability to the simultaneous derivatization of inorganic anions has been studied by GC–FID[7]. As a result, bromide, iodide, cyanide, thiocyanate, nitrite, nitrate and sulphide can be derivatized and detected simultaneously. However, this technique is an ECD-oriented derivatization designed to enhance sensitivity. In this work, we have investigated the determination of trace amounts of inorganic anions by pentafluorobenzylation with TsO-PFB, using GC–ECD.

EXPERIMENTAL

Apparatus

A Shimadzu GC-4BM gas chromatograph equipped with a ^{63}Ni ECD unit (Kyoto, Japan) was used. A stainless-steel column (4 m × 3 mm I.D.) was packed with 5% OV-225 (2.5 m) and 5% OV-210 (1.5 m), both of which were coated on 60-80 mesh Uniport HP, obtained from Gasukuro Kogyo (Tokyo, Japan). Nitrogen was used as the carrier gas at a constant flow-rate of 30 ml/min. The detector and injection port temperatures were maintained at 200°C, and the column temperature was kept at 130°C for the simultaneous determination of bromide and iodide, and at 190°C for that of thiocyanate and nitrite. A Shimadzu Chromatopac C-R3A data processor was used as the recorder and integrator.

Reagents

Commercial grade tetra-*n*-amylammonium chloride (TAAC) was obtained from Wako (Osaka, Japan). The derivatizing reagent, TsO-PFB, was prepared from *p*-toluenesulphonyl chloride and pentafluorobenzyl alcohol by a modification of the literature method[8], and recrystallized from ethanol. It was identified by mass spectrometry and infrared spectroscopy. The inorganic anions were used as their sodium or potassium salts, of analytical-reagent grade. Dichloromethane and deionized water were distilled before use for analysis. All other chemicals were also of analytical-reagent grade.

Procedure

The procedure for the determination of bromide, iodide, thiocyanate and ni-

trite was as follows. A brown-coloured test tube with a screw cap (*ca.* 10 ml) was used as the reaction vessel, in order to protect the contents from the ligth. To 1.0 ml of sample solution were added 0.10 ml of an aqueous solution of TAAC ($2.0 \cdot 10^{-2}$ *M*), 1.0 ml of a dichloromethane solution of TsO-PFB ($2.5 \cdot 10^{-3}$ *M*), and 0.10 ml of a buffer solution (pH 7) containing disodium hydrogenphosphate (0.1674 *M*) and citric acid (0.01765 *M*). Then, the vessel was sealed tightly with the screw cap and shaken mechanically for 30 min at room temperature. After shaking, 0.10 ml of the dichloromethane layer was separated from the aqueous layer. In the analyses of human urine or saliva, the organic and aqueous layers often became muddy and inseparable, so the organic layer was separated from the aqueous layer after ultrasonic irradiation. The dichloromethane solution was evaporated carefully under a nitrogen stream for 2 min at room temperature, and then 1.0 ml of *tert.*-butyl methyl ether solution was added to the residue. The *tert.*-butyl methyl ether solution contained 1,2,4-trichlorobenzene ($2.0 \cdot 10^{-4}$ *M*) or 1,2,3,4-tetrachlorobenzene ($1.0 \cdot 10^{-4}$ *M*) as an internal standard for the determination of bromide and iodide or thiocyanate and nitrite, respectively. An aliquot (0.10 μl) of the *tert.*-butyl methyl ether solution was injected into the gas chromatograph.

Samples

Kelp. "Wakame" seaweed grown in Japanese waters were washed with water and then ashed in a crucible. About 0.3 g of the resulting ash (*i.e.* kelp) was weighed accurately, and bromide and iodide in the kelp were extracted with water (8 ml) by shaking for 2 h at 60°C. Then, the solution was used for analysis after filtration and 50-fold dilution with water for bromide determination or 10-fold dilution for iodide determination.

Human urine and saliva. The samples were offered from the young investigators in our laboratory. Immediately after collection, urine and saliva were diluted 2- and 10-fold, respectively, with water and used for analysis. In the determination of nitrite in saliva, saturated aqueous silver sulphate solution was added to the sample in order to prevent the interference of chloride.

RESULTS AND DISCUSSION

Pentafluorobenzylation of inorganic anions with TsO-PFB

As previously reported[7], relatively high concentrations (> *ca.* 50 μg/ml) of bromide, iodide, cyanide, thiocyanate, nitrite, nitrate and sulphide can be pentafluorobenzylated with TSO-PFB, and their PFB derivatives determined by GC–FID. Therefore, pentafluorobenzylation of the above anions was studied at lower concentrations (< *ca.* 5 μg/ml), and their PFB derivatives were detected by GC–ECD. At lower concentrations, only four anions (bromide, iodide, thiocyanate and nitrite) gave peaks for their PFB derivatives (*i.e.* pentafluorobenzyl bromide, pentafluorobenzyl iodide, pentafluorobenzyl thiocyanate and α-nitro-2,3,4,5,6-pentafluorotoluene, respectively). The others did not give GC peaks corresponding to their PFB derivatives, even when derivatization was performed under varying reaction conditions. Therefore, further work was concentrated on the above four anions.

Dichloromethane was the most suitable organic solvent for pentafluorobenzylation. As this solvent is apparently unfavourable for ECD, various organic sol-

vents immiscible with water were tried in order to find a suitable solvent for pentafluorobenzylation followed by ECD. Among the solvents *tert.*-butyl methyl ether, methyl acetate, ethyl acetate, benzene, toluene, ethylbenzene, 1-heptanol, 1-octanol, 1-decanol, methyl isopropyl ketone, methyl isobutyl ketone and methyl isoamyl ketone, *tert.*-butyl methyl ether gave the highest derivatization yield for each inorganic anion: only *ca.* 10% of the yield with dichloromethane. In this work, therefore, dichloromethane was used for the PFB derivatization reaction and then *tert.*-butyl methyl ether was used for ECD, as described in the procedure in the Experimental section.

Determination of bromide and iodide

As the gas chromatograph used was equipped with a single ECD unit, GC determination with column temperature-programming is very difficult. Therefore, the determination of bromide and iodide was performed at a different column temperature from that for the determination of thiocyanate and nitrite.

Ten standard reference samples containing bromide and iodide were determined over the concentration ranges 0.16–1.6 and 0.38–3.8 μg/ml, respectively, to evaluate the quantitative application of the method for the determination of both anions. Each calibration curve was constructed by plotting the concentration of analyte (x) *vs.* the peak-area ratio of its PFB derivative to internal standard (y). Linear regression equations, $y = 0.3113x + 0.0086$ for bromide and $y = 0.1091x + 0.0022$ for iodide, were obtained with correlation coefficients of 0.9967 and 0.9971, respec-

TABLE I

INTERFERENCE STUDY FOR THE DETERMINATION OF Br^- AND I^-

Concentrations of Br^- and I^- are 1.0 and 3.0 μg/ml, respectively.

Anion	*Added as*	*Concentration (μg/ml)*	*Relative peak area**	
			Br^-	I^-
Standard			100.0 ± 1.1	100.0 ± 4.7
F^-	KF	100	98.5 ± 2.8	100.5 ± 2.7
Cl^-	KCl	100	101.6 ± 3.7	103.7 ± 0.7
Br^-	KBr	100	–	105.2 ± 0.9
I^-	KI	10	97.6 ± 2.0	–
CN^-	KCN	100	95.8 ± 1.9	100.7 ± 3.5
SCN^-	KSCN	50	100.9 ± 1.5	100.6 ± 2.6
S^{2-}	Na_2S	10	99.7 ± 4.8	–
		50	**	101.5 ± 3.2
NO_2^-	$NaNO_2$	10	97.9 ± 3.0	104.2 ± 2.6
NO_3^-	$NaNO_3$	10	96.3 ± 3.3	–
		50	**	101.5 ± 3.2
CO_3^{2-}	Na_2CO_3	50	100.6 ± 2.9	–
		100	**	97.8 ± 2.6
SO_3^{2-}	Na_2SO_3	100	99.1 ± 1.6	100.3 ± 2.6
SO_4^{2-}	Na_2SO_4	50	101.3 ± 3.6	103.6 ± 2.1
$H_2PO_4^-$	NaH_2PO_4	100	97.5 ± 1.9	104.0 ± 2.7
HPO_4^{2-}	Na_2HPO_4	100	101.5 ± 3.1	101.5 ± 2.0

* Mean ± S.D. of five replicate analyses.

** Interference was observed.

TABLE II

RESULTS OF ANALYSES OF KELP EXTRACT WITH Br^- AND I^- RECOVERY TESTS

Amount added (μg/ml)		*Amount found (μg/ml)*		*Recovery (%)*	
Br^-	I^-	Br^-	I^-	Br^-	I^-
0.00	0.00	(0.30 ± 0.012)*	(1.17 ± 0.026)*	–	–
0.30	0.50	0.61	1.65	103.3	96.0
0.60	1.00	0.93	2.10	105.0	93.0
0.90	1.50	1.18	2.64	97.8	98.0
1.20	2.00	1.48	3.20	98.3	101.5

* Mean ± S.D. of five replicate analyses.

tively. This indicates good applicability of the method to quantitation of bromide and iodide. The interferences of some anions with the determination of bromide and iodide are shown in Table I. The anions selected are those usually found in environmental and biological samples. These anions do not appear to interfere with this determination, except for sulphide (50 μg/ml), nitrate (50 μg/ml) and carbonate (100 μg/ml). However, the interferences were not observed at 10 μg/ml sulphide and nitrate and at 50 μg/ml carbonate.

In order to investigate the applicability of the method to the analyses of real samples, bromide and iodide in kelp extract were determined by the present method. Recovery tests for bromide and iodide added to the samples were performed. The results are given in Table II, and Fig. 1 shows the gas chromatograms obtained on analysis of the kelp extract. For both anions, the recoveries are all >90% and the relative standard deviations of the method are <5%.

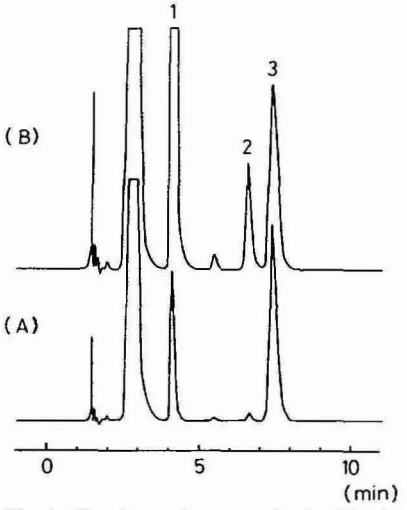

Fig. 1. Gas chromatograms obtained in the determination of bromide (A) and iodide (B) in kelp extract. Peaks: 1 = pentafluorobenzyl bromide; 2 = pentafluorobenzyl iodide; 3 = 1,2,4-trichlorobenzene (internal standard).

Determination of thiocyanate and nitrite

The calibration curves for thiocyanate and nitrite were constructed in the same manner as those for bromide and iodide. Good linear relationships were obtained; linear regression equations are $y = 0.296x - 0.0185$ and $y = 0.0839x - 0.00453$, and correlation coefficients are 0.9988 and 0.9995 for thiocyanate (0.5–5.0 μg/ml) and nitrite (1.0–10.0 μg/ml), respectively. The interferences of some anions with the determination of thiocyanate and nitrite were also examined, and the results are given in Table III. None of the anions at the concentrations examined interfere with the present method. The method was applied to the determination of thiocyanate in human urine samples, and recovery tests were also performed, as in Table IV. The recoveries are all >90%. Thiocyanate and nitrite in human saliva were also determined by the present method. When nitrite co-exists with >10 μg/ml thiocyanate, the analytical value for nitrite is lower than its actual value. This is presumably due to the preferential reaction of thiocyanate with TsO-PFB. Unfortunately, human saliva often contains >10 μg/ml thiocyanate. Therefore, in the determination of nitrite, silver sulphate was added to each saliva sample solution in order to precipitate thiocyanate and to stop it from interfering. In this case, nitrite could not be determined simultaneously with thiocyanate. Fig. 2 shows the gas chromatogram of the analysis, and the results are given in Table V, together with the values for nitrite concentration determined by the widely used colorimetric method[9]. The nitrite concentrations for each sample obtained by the two methods are in good agreement.

TABLE III

INTERFERENCE STUDY FOR THE DETERMINATION OF SCN^- AND NO_2^-

Concentrations of SCN^- and NO_2^- are 5.0 μg/ml.

Anion	*Added as*	*Amount added (μg/ml)*	*Relative peak area**	
			SCN^-	NO_2^-
Standard			100.0 ± 3.0	100.0 ± 5.0
F^-	KF	100	102.5 ± 4.2	97.4 ± 2.0
Cl^-	KCl	1000	97.9 ± 2.9	95.6 ± 4.3
Br^-	KBr	10	100.9 ± 4.1	103.0 ± 1.4
I^-	KI	1	104.2 ± 4.9	97.2 ± 2.8
CN^-	KCN	10	104.6 ± 5.8	98.5 ± 1.8
SCN^-	KSCN	10	–	97.8 ± 2.7
S^{2-}	Na_2S	10	101.1 ± 1.5	97.5 ± 1.8
NO_2^-	$NaNO_2$	100	99.6 ± 2.0	–
NO_3^-	$NaNO_3$	50	98.3 ± 2.5	97.5 ± 3.2
CO_3^{2-}	Na_2CO_3	100	100.9 ± 3.2	102.5 ± 5.9
SO_3^{2-}	Na_2SO_3	100	103.8 ± 1.0	97.4 ± 2.0
SO_4^{2-}	Na_2SO_4	100	103.9 ± 5.0	96.3 ± 2.1
$H_2PO_4^-$	NaH_2PO_4	100	102.1 ± 3.3	102.5 ± 5.8
HPO_4^{2-}	Na_2HPO_4	100	103.7 ± 5.4	97.8 ± 2.4

* Mean ± S.D. of five and three replicate analyses for SCN^- and NO_2^-, respectively.

TABLE IV

RESULTS OF ANALYSES OF HUMAN URINE WITH SCN^- RECOVERY TESTS

Urine No.	*Amount added (μg/ml)*	*Amount found (μg/ml)*	*Recovery (%)*
1	0.0	(1.5 ± 0.03)*	–
	2.0	3.3	93.1
	4.0	5.6	103.2
	6.0	7.5	99.7
	10	12.2	106.9
2	0.0	(0.9 ± 0.03)*	–
	2.0	3.0	102.0
	4.0	4.9	98.6
	6.0	7.1	102.0
	10.0	10.7	97.7
3	0.0	(1.4 ± 0.03)*	–
	2.0	3.4	99.0
	4.0	5.6	103.8
	6.0	7.5	101.7
	10.0	10.9	94.9

* Mean ± S.D. of five replicate analyses.

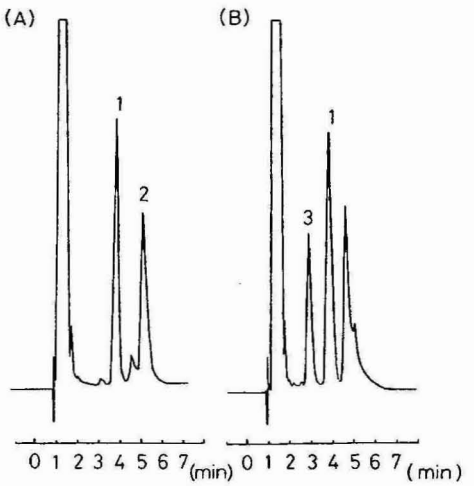

Fig. 2. Gas chromatograms obtained in the determination of thiocyanate (A) and nitrite (B) in human saliva. Peaks: 1 = 1,2,3,4-tetrachlorobenzene (internal standard); 2 = pentafluorobenzyl thiocyanate; 3 = α-nitro-2,3,4,5,6-pentafluorotoluene.

CONCLUSIONS

From the results mentioned above, the present method is applicable to the simultaneous determination of bromide and iodide or to that of thiocyanate and nitrite at low concentrations in real samples. As bromide, iodide, thiocyanate and nitrite at low concentrations are pentafluorobenzylated simultaneously, their simul-

TABLE V

RESULTS OF ANALYSES OF HUMAN SALIVA WITH INTERCOMPARISON STUDY FOR NO_2^- DETERMINATION

Saliva No.	*Concentration of* SCN^-* *(μg/ml)*	*Concentration of* NO_2^- *(μg/ml)*	
		*GC**	*Colorimetry***
1	6.71 ± 0.01	5.64 ± 0.03	5.64
2	7.41 ± 0.06	1.93 ± 0.01	1.98
3	30.43 ± 0.26	5.60 ± 0.14	5.59
4	12.77 ± 0.25	7.33 ± 0.10	7.22
5	7.80 ± 0.10	4.43 ± 0.23	4.61
6	14.95 ± 0.21	1.87 ± 0.32	1.81

* Mean ± S.D. of four replicate analyses.
** Colorimetric method using *n*-(1-naphthyl)-ethylenediamine and sulphanilic acid.

taneous determination may be performed by using the column temperature-programming technique.

Besides the above four anions, cyanide, nitrate and sulphide can also be pentafluorobenzylated at relatively high concentrations, as reported previously[7]. Cyanide, nitrate and sulphide at relatively low concentrations are not converted into their PFB derivatives because: none of these three anions at low concentrations are effectively extracted into the organic layer containing TsO-PFB; in the previous method using FID, 1.0 ml of a 0.1 *M* solution of TsO-PFB was used for the derivatization reaction, whereas in the present method 1.0 ml of a $2.5 \cdot 10^{-3}$ *M* was used. This means that there is a shortage of the reagent and the three anions cannot therefore be derivatized. The injection of larger amounts of TsO-PFB than that used in this work decreased the initial standing current of an ECD cell and the sensitivity of the detector. Moreover, it is difficult and time-consuming to remove TsO-PFB from the organic layer injected into the gas chromatograph. An attempt is now in progress to increase the concentration of TsO-PFB in the derivatization reaction without decreasing the detector sensitivity. It is of interest to try to derivatize anions with TsO-PFB in a homogeneous mixture using a water-miscible organic solvent, instead of the biphasic reaction system described here.

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IMMUNO-AFFINITY PARTITION OF CELLS IN AQUEOUS POLYMER TWO-PHASE SYSTEMS

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SUMMARY

Poly(ethylene glycol) (PEG) was covalently coupled to IgG antibody preparations directed against human red blood cells. This modification reduces the tendency of the antibody to agglutinate cells and increases its affinity for the upper phase in dextran–PEG aqueous two-phase systems. These effects are related to the molecular weight of the PEG used for modification and to the number of PEG molecules attached to the antibody.

Exposure of human red blood cells to PEG-modified antibody causes a substantial and specific increase in cell partition into the PEG-rich phase in a number of PEG–dextran aqueous two-phase systems. Pertinent phase-system parameters were examined. Following a single incubation with PEG-derivatized antibody, a mixture of sheep and human red blood cells was completely separated in 100 min by a 30-transfer countercurrent extraction using a two phase system which normally offers little resolution.

INTRODUCTION

There is considerable need both in biomedical research and in diagnostic medicine for reliable techniques for specifically separating a discrete population of cells from a mixture. The ideal technique should provide a good yield of cells of high purity while cell function is maintained. The present work indicates that immuno-affinity phase partition has the potential for becoming such a technique.

Aqueous solutions of low concentrations of the polymers dextran and poly(ethylene glycol) (PEG) form two-phase systems consisting of a less dense, PEG-rich phase floating on a dextran-rich phase. When buffered and made isotonic by the addition of salts or other low molecular weight solutes, these aqueous polymer two-phase systems can be used for analytical and preparative separation of a wide range of biological substances including cells, organelles, nucleic acids, and proteins[1–4]. Separation results from differential partitioning of substances between the two phases

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or, in the case of particles, between the liquid–liquid interface and either of the phases. Resolution can be enhanced by the repeated partitioning provided by countercurrent distribution and countercurrent chromatography[1–7].

Factors which influence the partition of macromolecules and particles include size, surface charge, and surface hydrophobicity[1–3]. System characteristics such as interfacial tension and salt composition also influence partition and can be altered by changing the type and concentration of polymers and salts[1–3].

Another method for altering partition is through the use of affinity ligands[7–15]. A ligand that selectively binds to a particle or macromolecule of interest is covalently coupled to one of the polymers (typically PEG). When mixed in a two-phase system, and allowed to settle, the PEG-bound ligand partitions predominately into the phase rich in that polymer, pulling the target material with it[8–15]. These ligands may be specific, such as enzyme inhibitors for enzyme purification[8], or nonspecific, like dyes for enzyme or nucleic acid purification[9–12] or fatty acid esters for cell purification[15].

The present article describes how we have refined the technique of phase partitioning to include specific binding of cell surface antigens by antibody ligands attached to PEG. Several steps are required to demonstrate the efficacy of immuno-affinity cell partition: (1) PEG must be attached to the antibody; (2) PEG-derivatized antibody (PEG-Ab) must partition into the PEG-rich phase and it must remain active; (3) the PEG-Ab must pull the cells of interest into the top phase; and (4) this cell partitioning must be selective for the targeted cells.

We have chosen to examine the partitioning of human and sheep red blood cells (RBCs) as a simple model system. RBCs have been extensively studied in phase partitioning[1–3] and have the additional advantage of being easily quantified; in addition, native antibodies are commercially available for immuno-affinity partitioning of these RBCs. Also, one aspect of antibody activity can easily be measured by microtiter hemagglutination assay; but it should be noted that PEG-modified antibody may be rendered effectively monovalent, and thus may still be active and capable of binding RBCs when it can no longer agglutinate RBCs. We chose a polymer phase system in which both human and sheep RBCs partitioned in favor of the interface and then used PEG-bound antibodies to the cell membrane of the human RBC to specifically increase the partition of human RBCs into the top, PEG-rich phase. The effects of phase-system parameters and the influence of PEG molecular weight and degree of derivatization were also investigated. In addition, we have examined the usefulness of PEG-antibodies in countercurrent distribution of cells.

MATERIALS AND METHODS

Unless specified otherwise all reagents were ACS grade, or better, quality from commercial sources. Distilled, filtered, 12 MΩ/cm water was used throughout.

Antibody preparations

Rabbit IgG directed against stroma from human RBCs (native antibody) was obtained from Cooper Biomedical, Malvern, PA, U.S.A. (lot 22196). Fab fragments derived from this same lot were prepared by controlled papain digestion by Jackson Immunoresearch, Avondale, PA, U.S.A. An enriched IgG fraction from sheep, also directed against human RBCs, was kindly provided by Dr. D. E. Brooks, University

of British Columbia, Vancouver, Canada. This fraction, which had been derived from whole serum by ammonium sulfate precipitation, was then further purified by ion exchange chromatography using a DEAE-silica gel column[16]. Both IgG samples agglutinated human RBCs at concentrations less than 0.2 μg/ml but did not agglutinate sheep RBCs.

Modification of antibodies with PEG

In a typical preparation, 12 mg of protein in 0.5 ml 0.05 *M* $Na_2B_4O_7$, pH 9.2, was added to 1.5 ml 0.1 *M* borate buffer. A volume of 1 ml of cyanuric chloride-activated[17,18] PEG 5000 or PEG 1900 monomethyl ether in the borate buffer was then added at 4°C, and the mixture was stirred for 1 h. Unattached PEG was removed by diafiltration (50-ml Amicon cell, PM-30 membrane, 30 000 molecular weight cut-off) with 10 volumes of 0.05 *M* borate buffer and 0.025 *M* sodium azide to a final volume of 2 ml. Typically 27 mg of activated PEG 5000, approximately equimolar relative to IgG lysine amino groups (90 per molecule)[19] resulted in about 50% modification of the lysine amino groups; only 70 of the 90 lysine groups were sensitive to our method of analysis (below). About half this amount of activated PEG (14 mg) gave 30% modification.

Analysis was performed by the Biuret and Habeeb methods as described previously[17,18]. For the latter method (based on reaction of trinitrobenzenesulfonic acid with free lysine groups), we found it necessary to remove unattached PEG and to keep protein concentrations at approximately 0.6 mg/ml in order to obtain accurate and reproducible results. The 0.1 *M* borate buffer was used rather than 4% $NaHCO_3$ called for in the original procedure.

RBC preparation

Blood samples were obtained by venipuncture from healthy individuals and treated with 10 m*M* EDTA as an anticoagulant. Samples were used fresh following brief storage at 4°C. Shortly before use, 1-ml aliquots were washed four times by suspension in twenty volumes of isotonic buffer (Isoton II, Coulter Electronics, Hialeah, FL, U.S.A.) and centrifugation for 10 min at 1000 *g*, followed by removal of the supernatant and the buffy coat.

RBC quantification

Quantification of mixtures of sheep and human RBCs was based on size differences as disclosed by an impedance cell counter (Coulter Electronics) equipped with a 100 channel size analyzer interfaced with an Apple II+ computer. In some experiments agglutination hindered use of this method, and visible spectroscopy was used to measure hemoglobin concentrations and thus cell numbers. For this procedure, partitioning samples were centrifuged at 1000 *g* for 10 min, the supernatant removed, and 0.4 ml cyanomethemoglobin reagent (Hycel, Houston, TX, U.S.A.) was added to lyse the cells. After centrifugation to remove cell stroma (8800 *g* for 10 min), absorbance measurements were taken at 540 nm and compared to the absorbance of a sample, treated identically, containing a known number of cells.

Direct hemagglutination assay

The method for passive hemagglutination described by Amman and Pelger[20]

was modified to directly assay the agglutinating ability of both PEG-Ab and native antibody fractions. Human and sheep RBCs (between 1 and 7 days old) were collected and washed as described above. Packed, washed cells (0.05 ml) were resuspended in 3.0 ml of solution I [100 ml 0.01 *M* phosphate buffered saline, pH 7.2, 1.6 ml 30% bovine serum albumin (BSA), and 100 mg dextrose]. This suspension could be kept for up to 5 days at 4°C. Before testing, a further 1:7 dilution in solution I was made.

In V-bottom microtiter plates (Cooke Enginnering, Alexandria, VA, U.S.A.) serial two-fold dilutions (0.05 ml) of each sample to be tested were made using solution II (100 ml 0.01 *M* phosphate buffered saline, pH 7.2, 0.05 ml Tween 80, 1.0 mg polyvinylpyrrolidone, and 1.5 ml 30% BSA). Human or sheep RBCs (0.05 ml) were added to each well.

Microtiter plates were placed on an automatic rotator shelf for mixing (10 min) and then incubated on a flat surface overnight at 25°C. Prior to screening, plates were elevated at a 60° angle for 15 min. Positive agglutination resulted in no "run-down" pattern of RBCs. Results were expressed as the minimum concentration of antibody which caused appreciable agglutination.

Preparation of two-phase systems

Polymer phase systems were prepared as described previously[2,3,5,6] by mixing appropriate weights of the following aqueous stock solutions (all %, w/w): 20% dextran T500 (Pharmacia, Piscataway, NJ, U.S.A., lot IE32126 or lot HD26066), 30% PEG 8000 (Union Carbide, lot B529-9104 or lot B-739), 20% dextran T40 (Pharmacia, lot FL18974), 0.6 *M* NaCl, and 0.22 *M* Na_2HPO_4, 0.07 *M* NaH_2PO_4, pH 7.2 buffer. Sodium azide was added to a final system concentration of 0.02% to retard bacterial contamination. Concentrations of dextran solutions were determined by polarimetry[1-3]. All other concentrations were determined gravimetrically. After preparation, phase systems were sterilized by filtration through a 0.2-μm filter and allowed to settle at room temperature overnight. The phases were then separated and stored at 4°C but returned to room temperature before use.

Seven well characterized two-phase polymer systems were used in this work[2,5,21]: (a) (5, 4) I, 5% dextran T500, 4% PEG 8000, 109 m*M* Na_2HPO_4, 35 m*M* NaH_2PO_4, pH 7.2 (buffer I); (b) (5, 4) II, 5% dextran T500, 4% PEG 8000, 50 m*M* NaCl, 73 m*M* Na_2HPO_4, 23 m*M* NaH_2PO_4, pH 7.2 (buffer II); (c) (5, 3.5) V, 5% dextran T500, 3.5% PEG 8000, 150 m*M* NaCl, 7.3 m*M* Na_2HPO_4, 2.3 m*M* NaH_2PO_4, pH 7.2 (buffer V); (d) (4.6, 3.9) V, 4.6% dextran T500, 3.9% PEG 8000, buffer V; (e) (5, 4) V, 5% dextran T500, 4% PEG 8000, buffer V; (f) (7, 4) V, 7% dextran T500, 4% PEG 8000, buffer V; (g) (7, 5) V, 7% dextran T40, 5% PEG 8000, buffer V.

These systems were chosen to represent a range of interfacial tensions, electrostatic bulk phase potentials, and dextran molecular weight[1-5,21].

Single tube partition

The ability of various concentrations of PEG-Ab to increase the partition coefficient *K* (the ratio of cells in the top phase to the remainder of cells) of human RBCs was studied in single-tube experiments. In 75 × 12 mm tubes, 0.5 ml upper phase containing $2 \cdot 10^7$ human RBCs was incubated with 0.2 ml PEG-Ab solution for 15

min at 37°C. The cells were pelleted by centrifugation at 1000 *g* for 10 min and the supernatant was removed. After being washed once in fresh upper phase, cells were resuspended in 1.0 ml upper and 1.0 ml lower phase. The suspension was mixed by inversion twenty times and allowed to settle 15 min for equilibration. Mixing was repeated, the system allowed to settle for 15 min and 0.7 ml of top phase was then promptly removed. A portion of this sample (0.2 ml) was used for impedance counting and the remaining 0.5 ml was centrifuged and quantified by spectrophotometric analysis, as described above.

Inhibition studies

Prior to single tube partitioning, 0.5 ml human RBCs at a concentration of $4 \cdot 10^7$/ml in upper phase was first incubated at 37°C for 15 min with 0.2 ml of unmodified Fab. Cells were then washed once in fresh upper phase and resuspended in 0.5 ml upper phase for incubation with PEG-Ab.

Antibody partition

Both PEG-Ab and native antibody preparations (0.2 ml) at a concentration of 2 mg/ml were added to two ml of a (5, 4.25) V system to produce a (4.9, 3.6) V system. Each tube was mixed twenty times by inversion and allowed to equilibrate for 15 min. After remixing, the systems were centrifuged at 1000 *g* for 5 min. An aliquot of the resulting upper phase was mixed with an equal volume of buffer and its absorbance measured at 280 nm. This value was compared to that of the 100%-control tube in which 0.2 ml of the original sample was added to 0.9 ml of upper phase and 1.1 ml buffer.

BSA partition

Comparison of the partition of unmodified bovine serum albumin (BSA) and PEG-modified BSA was carried out in a (5, 4) V system as follows: preparations (0.1 ml) at a concentration of 4 mg/ml were added to 2 ml of phase system. Tubes were mixed by vortexing twice at 10-min intervals, and were then centrifuged 5 min at 200 *g*. Aliquots from each phase (0.7 ml) were diluted to 3 ml with phosphate-buffered saline (PBS) and fluorescence excitement and monitoring were done at 280 nm and 380 nm, respectively, in a Turner spectrofluorometer with the appropriate phase acting as a zero control.

Automated countercurrent distribution experiments

Countercurrent distribution (CCD) experiments were performed as previously described[1–3,5,6], using a 60-chamber Biosheff MK II apparatus (Biochemistry Department, University of Sheffield, Sheffield, U.K.). The lower cavity volume for this machine is 850 μl. Since all runs described in this work consisted of 30 transfers, either duplicate or two different experiments could be run simultaneously, one on either side of the rotor.

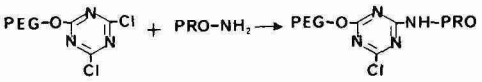
Antibody treatment of RBCs consisted of incubating cells or mixtures of cells with PEG-Ab (1.3 mg/ml) at 37°C for 15 min in the same proportions as for the single tube partitioning experiments. After incubation, cells were washed once with fresh upper phase and resuspended to a load mix concentration of $2 \cdot 10^7$–$4 \cdot 10^7$ cells per ml of upper phase. For partitioning of cells, 800 μl of lower phase was added

to each of the 60 cavities. A volume of 900 μl of sample load mix was added to cavities 0, 1, 30 and 31. A volume of 900 μl of upper phase alone was added to the remaining cavities. In every run, a mixing time of 12 s and a settling time of 3 min was used. Phases were broken by adding 1.0 ml of buffer to each cavity. Cells were quantified as described above.

RESULTS AND DISCUSSION

Antibody modification

Of the several methods available for protein-PEG coupling[22], we chose the method of Abuchowski and Davis[17] in which the PEG is first activated with cyanuric chloride and then coupled to lysine groups on the protein, eqn. 1[18,23].



(1)

Using this reaction we could vary the number of lysines substituted with PEG (as shown by reduction in the number of primary lysine amino groups).

The effect of PEG substitution on protein partitioning in a polymer two-phase system was first tested with BSA, Table I. As can be seen from the shifts in BSA partitioning, attaching PEG to a protein can have a dramatic effect on protein partitioning. Similarly, antibody partitioning can also be shifted to favor the top phase, although partitioning shifts are not as dramatic as with BSA, Table I. Nonetheless, modified antibody was found to strongly favor the top phase, indicating that the proposed affinity partitioning was feasible and that the partition behavior of high MW molecules could be altered by PEG coupling.

Earlier works[14,23,24], especially those of Abuchowski and Davis[17] have amply demonstrated that PEG-substituted enzymes remain active although the loss of activity resulting from substitution can be severe with some enzymes and with some

TABLE I

EFFECTS OF PEG SUBSTITUTION ON BSA AND NATIVE ANTIBODY PARTITIONING IN DEXTRAN–PEG TWO-PHASE SYSTEMS

Protein modified	*Lysines modified (%)*	*Partition (%)****
BSA*	0	31 ± 3
BSA	10	89 ± 3
BSA	39	96 ± 1
BSA	47	96 ± 1
BSA	76	98
Native-Ab**	0	50 ± 3
Native-Ab	47	87 ± 3

* Bovine serum albumin in a (5, 4) V system, dextran T500, PEG 8000; see Materials and methods.

** Sheep IgG anti-human RBCs in a (4.6, 3.9) V system; dextran T500, PEG 8000; see Materials and methods.

*** Percentage of total protein partitioning into the upper phase; BSA ($\bar{x}$ ± SD, n = 2); native-Ab ($\bar{x}$ ± SD, n = 4).

TABLE II

EFFECTS OF PEG SUBSTITUTION ON ABILITY OF ANTIBODY TO AGGLUTINATE RBCs

*PEG $\bar{M}_w$**	*Lysines modified (%)*	*Minimal hemagglutination concentration (μg/ml)***
Control	0	<0.2
(A) 5000	27	23 ± 8
(B) 5000	40	19 ± 8
(C) 5000	51	5 ± 2
(D) 1900	45	1 ± 0
(E) 1900	70	3 ± 1

* Molecular weight of PEG used for substitution of sheep IgG anti-human RBCs. Letter designation refers to Fig. 1.

** Lowest antibody concentration exhibiting appreciable hemagglutination in microtiter assay ($\bar{x}$ ± SD, n = 3).

coupling methods. The present study is the first to our knowledge utilizing PEG-substituted antibody[25]. As shown in Table II, PEG-Ab loses some of its ability to agglutinate human RBCs. This effect shows an apparent dependence on PEG mol.wt., the higher mol.wt. causing more deactivation. The effect of percentage substitution on antibody activity is not a simple relationship since the PEG 5000 is more active with a higher percentage substitution while the PEG 1900 is less active, Table II. However, measurements of agglutination and percentage substitution are sufficiently approximate to make this latter conclusion uncertain. We are presently using more precise techniques to examine the effects of PEG substitution on enzyme activ-

TABLE III

PARTITIONING OF HUMAN RBCs AS A FUNCTION OF PEG-Ab CONCENTRATION AND DEGREE OF ANTIBODY MODIFICATION

Modification (%, MW, origin)*	*Partition (%)***					
	[PEG-Ab] (μg/ml)					
	5.35	*10.7*	*21.4*	*42.8*	*85.7*	*171*
27% 5000 SHE (A)	47	46	49	53	79	92
40% 5000 SHE (B)	33	39	48	56	77	84
50% 5000 SHE (C)	36	38	38	45	54	80
45% 1900 SHE (D)	41	34	39	44	55	57
70% 1900 SHE (E)	40	45	46	47	54	61
47% 5000 RAB***	3.0	3.0	4.0	12	35	80

* Given as lysines modified (%) by PEG; MW of PEG used; and origin (sheep or rabbit) of antibody; A–E designation refers to Fig. 1.

** Duplicate determinations with average standard deviation of 2.4%. Partitioning was done in separate batches of (4.6, 3.9) V phase system (dextran T500, PEG 8000; see Materials and methods). In absence of PEG-Ab 2% partition of human RBCs was observed in the rabbit experiments and 35% in the sheep experiments (see Discussion).

*** Additional values of [PEG-Ab]/% partition: 322/93; 643/90; 1286/84; and 2200/83.

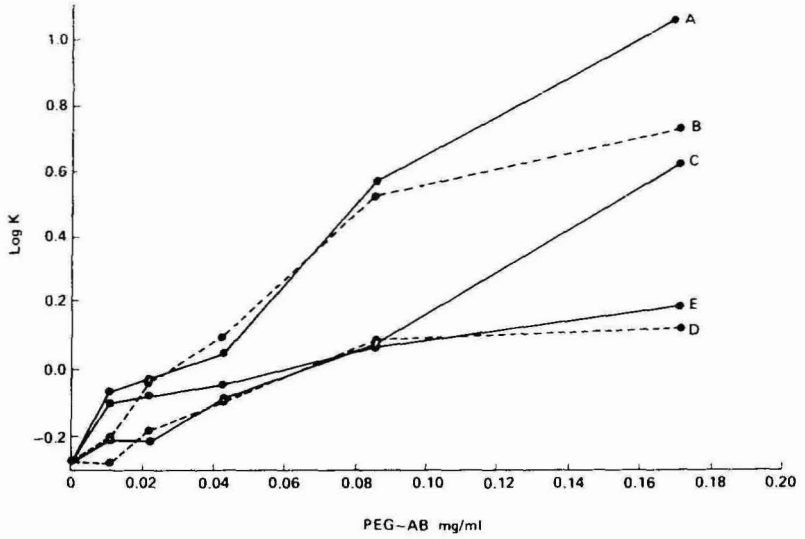


Fig. 1. Effects of PEG molecular weight, extent of modification, and incubation concentration of PEG-Ab (derived from sheep) on the upper-phase affinity (log K) of human RBC. K is defined as the ratio of the number of cells in the top phase to the remainder. (A) PEG 5000, 27% modification; (B) PEG 5000, 40% modification; (C) PEG 5000, 51% modification; (D) PEG 1900, 45% modification; (E) PEG 1900, 70% modification.

ity. For the present study, however, these effects are not of critical importance, since all that is needed is PEG-substituted antibody which is active and partitions to the top phase. In a control experiment PEG-Ab (antibody from sheep) was shown to have no agglutinating activity toward sheep RBCs.

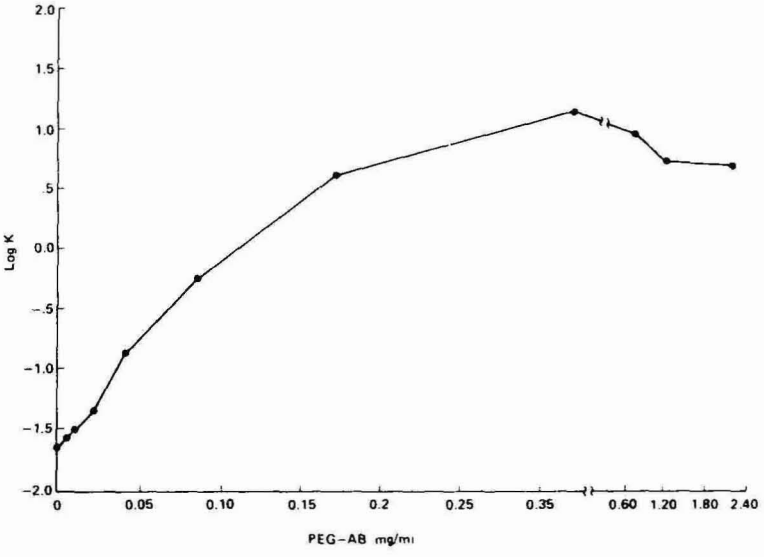


Fig. 2. Effect of incubation concentration of PEG-Ab (derived from rabbit) on the upper-phase affinity (log K) of human RBC.

Single tube partition

Next the ability of the PEG-Ab to alter the partitioning of human RBCs was examined. These results are shown in Table III and Figs. 1 and 2. As we had hoped, the PEG-modified antibody is quite effective at pulling human RBCs into the top phase; the increase in partition varies directly with the amount of PEG-Ab added. Note also that the partitioning shift is observed with both sheep- and rabbit-derived antibody. Unmodified antibody did not alter RBC partition, either because of extensive agglutination and sedimentation or because PEG is necessary to pull the cells into the PEG-rich phase.

Antibody modified with PEG 5000 gives more dramatic effects, especially evident at the higher concentrations of modified antibody; for example, at 171 μg/ml the PEG-5000 conjugate gave an average partition of 85% while the PEG 1900 conjugate gave an average partition of 59%. Interestingly, the least agglutinating PEG-Ab gave the highest percentage partition; with the more active antibody, agglutination and sedimentation of cell clumps was a problem. Thus it appears that the reduction in antibody agglutinating ability which results upon PEG coupling is advantageous from the standpoint of affinity partitioning.

Finally, it remains to demonstrate that the PEG-Ab is selective in pulling only specific cells into the top phase. Single-tube control experiments clearly showed that partitioning of sheep RBCs was unaffected by PEG-modified antibody. Thus all the components of immuno-affinity phase partitioning of cells are in hand: PEG-modified antibodies will dramatically and selectively shift the partitioning of the targeted cells into the top PEG-rich phase.

In a related study, Sharp *et al.*[25] have shown that human RBCs bind approximately 15 000 PEG-Ab molecules of rabbit-derived anti-human antibody. This study indicates that the present technique may be applicable to cells having this approximate receptor density.

As an additional control experiment, we also examined the ability of monovalent Fab fragments prepared from the same lot of rabbit IgG anti-human RBC (unmodified by PEG attachment) to inhibit the shift in human RBC partitioning that results in the presence of PEG-modified antibody. For this experiment the following concentrations were used: Fab, 686 μg/ml; 47% modified PEG 5000 IgG, 600 μg/ml; and $2 \cdot 10^7$ human RBCs in 2 ml of a (4.6/3.9) phase system. In the absence of Fab 89% of the RBCs partitioned to the top phase. Incubation of the cells with Fab prior to their incubation with PEG-Ab gave a reduction in partitioning to 69% (expected from 150 μg of PEG-Ab). Unfortunately, this experiment was confused somewhat by unexpected cell aggregation by the Fab (which surprisingly, occurred in phase system but not in buffer). Nonetheless, Fab did compete with the PEG-modified IgG in binding with the RBCs and thus gave a reduction in the affinity partitioning effect.

System variation

After these initial experiments, several other phase systems were examined to determine the effects of various phase system parameters on affinity partitioning, Table IV. In brief, we observed that PEG-Ab addition produced the most dramatic shifts in cell partition in systems having a low interfacial tension and a low electrostatic bulk phase potential. As would be expected, increasing the interfacial tension pulled the cells to the interface, making it more difficult to pull the cells into either phase [*e.g.*, compare the (5, 3.5) V, (5, 4) V, and (7, 4) V systems].

TABLE IV

EFFECT OF PHASE SYSTEM COMPOSITION ON ABILITY OF PEG-Ab TO INDUCE ALTERATION OF RBC PARTITION

*Two-phase system**	*Interfacial tension (μN/m)***	*Electrostatic bulk phase potential (mV)***	*Cell location****	*Partition (%)*§*	
				No PEG-Ab	*PEG-Ab*
(5, 4) I	11.0	2.2	Upper	14 ± 1	58 ± 4
(5, 4) II	8	1.0	Upper	6 ± 1	64 ± 1
(5, 3.5) V	0.7	0.2	Upper	2 ± 1	77 ± 1
(4.6, 3.9) V	3	0.2	Upper	2 ± 1	69 ± 3
(5, 4) V	4.9	0.3	Upper	0 ± 1	30 ± 1
(7, 4) V	17.2	0.2	Upper	0 ± 1	4 ± 2
(7, 5) V*	5.0	0.2	Upper	0 ± 1	1 ± 1
			Interface	45 ± 3	97 ± 3
			Lower	55 ± 3	3 ± 1

* All systems compounded with PEG 8000 and dextran T500 of the same lots except (7, 5) V which was prepared with dextran T40; see Materials and methods.

** Taken from ref. 21.

*** Referring to the upper or lower phase or to the interface between them.

§ Mean ± S.D. of two independent determinations using 643 μg/ml of PEG-Ab (from rabbit).

The effects of electrostatic bulk phase potential are difficult to determine since increasing potential (by salt variation) is accompanied by an increase in interfacial tension (*e.g.*, compare the I, II, and V versions of the 5, 4 system). Generally, however, it appears that increasing the potential is counterproductive for affinity partitioning since it increases the partition coefficient K (ratio of cells in top to the remainder of cells) in the absence of affinity ligand. Also, the high interfacial tension for high potential systems [*e.g.*, (5, 4) I] makes it difficult for the affinity ligand to pull cells away from the interface; note the decrease in partition coefficient in the presence of ligand upon moving from V to II to I versions of the (5, 4) system. The net result is that the largest shifts in partitioning from ligand addition are found with the low tension systems such as the (5, 3.5) V system.

The final entry in Table IV is for a (7, 5) V system made from dextran of molecular weight 40 000. The lower dextran molecular weight, as expected[1,2], gives a lower partition coefficient, with a significant percentage of the cells moving to the

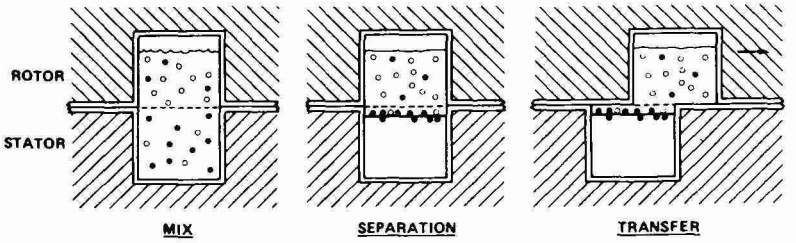


Fig. 3. Thin-layer countercurrent distribution (CCD) in aqueous polymer two-phase systems. The phases are mixed, allowed to separate, and material distributed between cavities is separated by transferring the upper cavity onto a fresh lower cavity. This process can be repeated (from refs. 5 and 6).

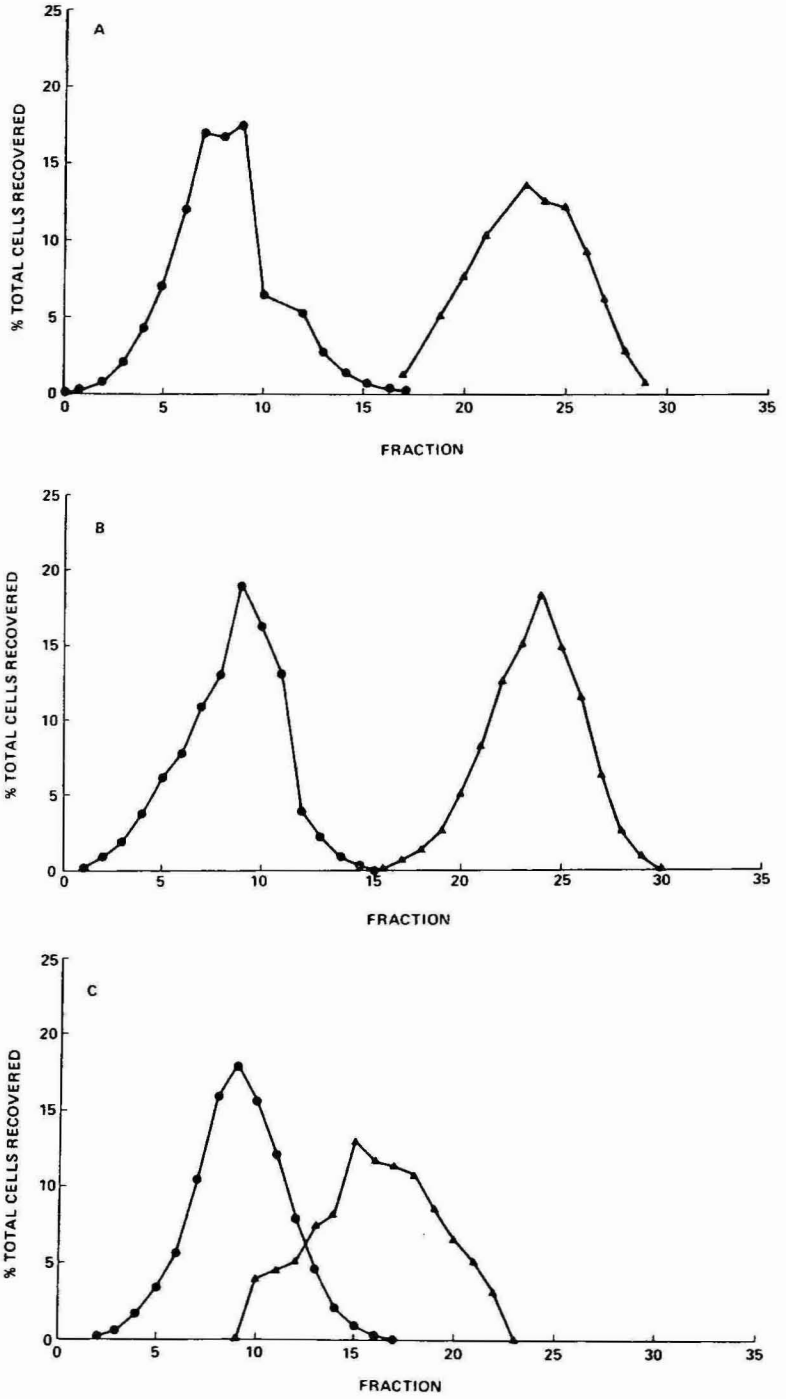


Fig. 4. Superimposed 30-transfer CCD runs of human RBC in a (4.6, 3.9) V two phase system. (A) RBC incubated with 1.3 mg/ml PEG-Ab (30% modification) and washed prior to CCD (▲); control RBC incubated in buffer only and washed prior to CCD (●). (B) Identical to A except a different preparation of PEG-Ab used for incubation (47% modification). (C) Identical to B except that an incubation concentration of 0.325 mg/ml was used.

lower, dextran-rich phase. This system appears to be of little use for affinity partitioning since the ligand acts to move cells from the bottom phase to the interface, which already contains a large number of the cells. However, it is noteworthy that the PEG-Ab binding is powerful enough to pull the cells from the bottom phase.

Finally, it should be noted that the (4.6, 3.9) V entry (without ligand) for Table IV is quite different from the same entry for Table III. These two results differ because different lots of PEG 8000 were used. It is not uncommon in partitioning to observe this sort of unexplained dependence on polymer lots[21].

Countercurrent distribution

The shifts in partitioning produced by adding PEG-Ab are not large enough to produce total separation in one step. This is a familiar situation in phase partitioning with aqueous polymer two-phase systems, and the usual approach is to utilize an automated device which provides repeated partitioning[1–7]. In the present case we have performed 30-transfer CCD experiments with a Biosheff MK2 apparatus. In a CCD device the cells are mixed with the two-phase system in one cavity, then the top phase is shifted to the adjacent bottom phase and the bottom phase is shifted to the adjacent top phase, Fig. 3. These affinity CCD experiments could be performed in two ways. Either PEG-Ab could be added to every cavity, in which case a large quantity of PEG-Ab would be required, or the cells could be incubated once with the PEG-Ab prior to CCD; in this latter case little PEG-ligand would be required.

In Fig. 4 we have presented the results of three CCD experiments. In the first, Fig. 4a, untreated human RBCs (circles) and human RBCs previously incubated with PEG-Ab for 15 min at 37°C (triangles) were subjected to separate 30-transfer CCD runs. As would be expected on the basis of the higher partition coefficient for human RBCs in the presence of PEG-Ab (from single tube experiments), the human RBCs incubated with PEG-Ab gave a peak well separated from that of the untreated human RBCs. Fortunately, the incubation technique provided sufficient exposure of the RBCs to antibody, thus dramatically reducing the amount of PEG-Ab required to perform CCD experiments.

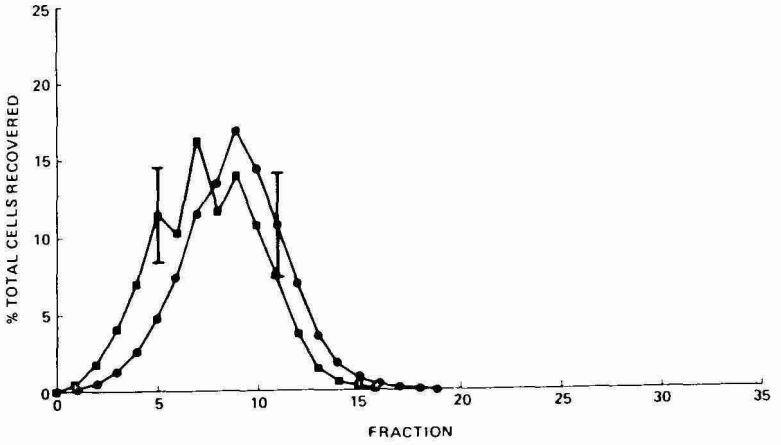


Fig. 5. Superimposed 30-transfer CCD runs of human (●) and sheep (□) RBCs in a (4.6, 3.9) V two-phase system. Results are averages of 4 runs for human and 7 runs for sheep.

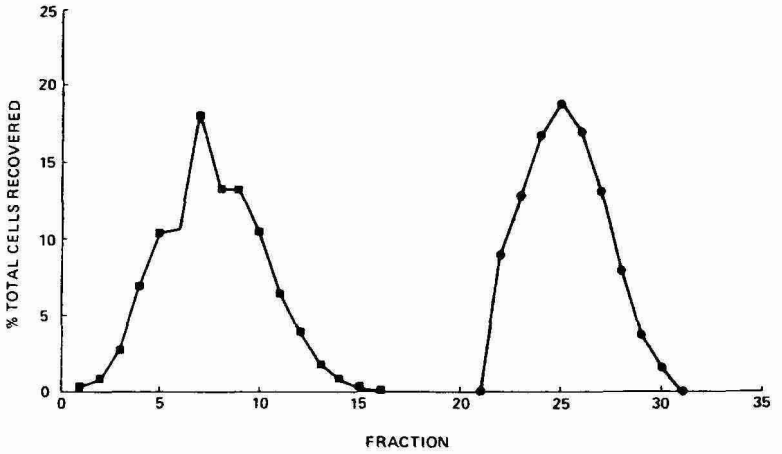


Fig. 6. 30-transfer CCD of a mixture of $7 \cdot 10^7$ human (●) and sheep (■) RBCs in a (4.6, 3.9) V two-phase system following incubation with 1.3 mg/ml PEB-Ab (from rabbit) and washing with phase system to remove unadsorbed antibody.

The experiment of Fig. 4a was performed with rabbit-derived PEG-Ab with 30% of the lysines substituted by PEG. This particular PEG-Ab sample gave extensive cell agglutination. To avoid cell sedimentation in this case we used a large excess of PEG-Ab in our incubation procedure to lessen the likelihood of a particular antibody encountering more than one cell. Interestingly, not all PEG-Ab preparations gave this same behavior. For example in Fig. 4c we show a CCD curve for untreated and treated human RBCs in which incubation was done with a relatively small amount of PEG-Ab in which the IgG was rabbit-derived and had 47% of its lysines substituted with PEG. In this case an antibody excess was not required because cell sedimentation was minimal. The poor separation of Fig. 4c results apparently because insufficient PEG-Ab is bound to the human cells. In Fig. 4b we show CCD curves for the weakly agglutinating antibody of Fig. 4c but at the same concentration as in Fig. 4a; nearly identical results are obtained.

These results emphasize that appreciable variation in PEG-Ab preparations was found. On the negative side, we must say that the origin of this variation is unclear, while on the positive side, we can note that all the variants were effective in immuno-affinity cell partitioning.

Prior to attempts to separate mixtures of human and sheep RBCs, control experiments were done, Fig. 5. In these experiments, sheep and human RBCs which were not incubated with PEG-Ab revealed identical CCD behavior, with both RBCs peaking in early fractions.

In our final experiment, shown in Fig. 6, sheep and human RBCs were mixed, incubated with PEG-Ab, then subjected to a 30-transfer CCD run. Again, the human and sheep RBCs were cleanly separated, with the human RBC's moving far down the CCD train.

CONCLUSION

In summary, we have demonstrated that immuno-affinity phase partitioning

with PEG-modified antibodies as affinity ligands is an effective technique for cell purification in an erythrocyte model system. This advance might reasonably be expected to be applicable to cell separations of significant biomedical interest. Work is in progress in our laboratories to explore this prospect.

ACKNOWLEDGEMENTS

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CHROMATOGRAPHIC ANALYSIS OF FATTY ACID DIMERS

COMPARISON OF GAS–LIQUID CHROMATOGRAPHY, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY WITH FLAME IONIZATION DETECTION

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SUMMARY

For monitoring fatty acid polymerization and isolation processes, a sufficiently rapid and reproducible analytical procedure is still lacking. This paper compares the results of the analysis of mixtures of monomeric and oligomeric fatty acids by means of gas–liquid chromatography, high-performance liquid chromatography, and thin-layer chromatography with flame ionization detection. For serial analyses, the last mentioned method appeared to be the most suitable.

INTRODUCTION

The composition and chemical structure of dimeric fatty acids prepared by thermal polymerization of unsaturated fatty acids has been investigated by various methods. The presence of components such as unreacted monoenoic and dienoic acids and their stereoisomers, linear dimeric, monocyclic and dicyclic dimeric and higher oligomeric fatty acids is well known[1]. In the dimerization process and for the quality control of end-products, analytical determination of the main component classes, *i.e.* monomers, dimers and trimers, is sufficient.

At first, molecular-still separations[2,3] and column liquid chromatography[4–6] were used and, in some cases, thin-layer chromatography (TLC) was used[6,7]. During the early and prolific days of instrumental chromatographic development, attempts were made to use gel permeation chromatography (GPC)[8–14], from which all three main component classes could be determined. Nevertheless, the extended time-scale for GPC separations on different gels is a disadvantage[7,11,12,14]. Efforts to apply gas–liquid chromatography (GLC) were hindered at first by the high retention of the

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trimeric component, so that its content was evaluated from the mass differences[11,13,15,16]. In spite of the possibility of obtaining complete separation by GLC with the aid of temperature programming[17,18], the main disadvantages of GLC are (i) the need to sample derivatize in order to produce the corresponding methyl esters, and (ii) the necessity to operate at temperatures of 350–400°C.

Surprisingly, papers reporting the use of high-performance liquid chromatography (HPLC) and thin-layer chromatography with flame ionization detection (TLC–FID)[19,20] for oligomeric fatty acid separations could not be found in the literature until recently. In this paper, we discuss the suitability of the application of these chromatographic methods in the control of the production of fatty acid dimers. For comparison, GLC separations were also used to test both of the above-mentioned techniques on selected samples.

EXPERIMENTAL

Reagents and chemicals

Samples of fatty acid dimers were prepared in our Institute during the study of unsaturated fatty acid dimerization[21] according to methods previously published[22]. Fatty acids from rape oil with low erucic acid content were used, having a high enough content of C_{18} unsaturated fatty acids. Dimerization was performed at 260°C under nitrogen for 2 h, applying bentonite as catalyst. From the reaction mixtures, fractions of unreacted monomers and enriched dimers were isolated by thin-film molecular distillation for further technological experiments, application testing and analysis. The remaining distillation residue was purified, bleached and also tested and analysed. Some of the isolated fractions of monomers and dimers, showing purities of 93–96%, were used as standards for identification in analysis or were used for the preparation of model mixtures for analytical method testing for reliability and accuracy.

The samples of commercial fatty acid dimers were obtained from Koospol (Foreign Trade Agency, Prague, Czechoslovakia); a list is included in Table I, along with a comparison of the compositions calculated by the manufacturers with those found by us using GLC.

TABLE I
CALCULATED COMPOSITIONS OF COMMERCIAL FATTY ACID DIMERS AND THEIR MONOMER (M), DIMER (D) AND TRIMER (T) CONTENT (IN MASS %) FOUND BY GLC

Product	*Calculated composition*			*Composition found by GLC*		
	M	*D*	*T*	*M*	*D*	*T*
Empol 1014	1	95	4	0.7	95.3	4.0
Empol 1018	1	81–83	17–19	2.6	81.8	15.6
Empol 1022	3	75–80	20–22	2.6	76.2	21.2
Hystrene 3695	1	95	4	3.6	87.6	8.8
Dimak S	5–7	67–69	24–28	5.1	68.0	26.9
Dimak DS	7–10	72–76	15–18	8.7	71.7	19.6
Emery 3020	–	–	–	5.2	72.2	22.6
Pripol 1022	–	80	20	3.5	73.8	22.7

All chemical used were of reagent grade; all solvents were dried and distilled in glass before use.

For GLC separations, all samples were converted to methyl esters using the current esterification procedure of reacting 0.2 g of fatty acid dimer with 50 ml of methanol plus three drops of concentrated sulphuric acid and heating for 3 h under reflux on a water-bath; the resulting methyl esters were isolated after addition of 100 ml of water by extraction into 3 × 50 ml of diethyl ether. For samples with a substantial concentration of higher oligomers present, the sample to be esterified can be promoted by the addition of 1–2 ml of chloroform.

For analyses by HPLC and TLC–FID, original free fatty acid samples were applied without any modification.

Instruments and operating conditions for GLC, HPLC and TLC–FID

Corresponding data are summarized in Table II.

RESULTS AND DISCUSSION

GLC separation

Using GLC, various commercial samples and fractions from fatty acid dimer preparation were analysed. Because of the lack of standards, the separated peaks were assigned to monomers, dimers and trimers according to their retention order given by the relative volatility. This identification is in agreement with the literature[17,18]. The evaluation of GLC results was made from peak-area measurements by applying empirical corretion factors taken from the calculated and found compositions of commercial products. By choosing the dimer as a relative standard (K_D = 1.0), the factors for monomer and trimer peak-area corrections were K_M = 0.9 and K_T = 2.0, respectively. From the very high K_T value caused by the very low FID response, it may be assumed that some of the less volatile trimer or higher oligomers are retained on the column.

An example of GLC separation is shown in Fig. 1. The retention temperatures under our operating conditions were 140–160°C for unreacted fatty acids (*i.e.* isomeric monomers in one main peak and some trace peaks of different shape, their asymmetry being determined by the isomer distribution), *ca.* 260°C for dimers (where a slight asymmetric peak includes all linear and cyclic isomers), and *ca.* 330°C for trimers. Some results of GLC separations are summarized in Table I. The compositions of commercial products, as determined by our procedures, were comparable with those stated in product specifications confirmed by GLC.

HPLC separation

At first, attempts were made to separate fatty acid dimer samples by reversed-phase liquid chromatography (RPLC) on C_2, C_8 and C_{18} type packings using a methanol–water mobile phase. In comparison with GLC separations, more peaks appeared, especially on the C_{18} bonded phase. However, assignment of the individual oligomers was not as simple as in GLC. In addition, the retention time of the trimer fractions was very long, indicating the possible presence of higher oligomers, which could be completely retained on the column. Therefore, application of the back-flushing technique was unavoidable.

TABLE II

INSTRUMENTS AND OPERATING CONDITIONS

	GLC	*HPLC*	*TLC–FID*
Instruments	Fractovap GV-200	LC chromatograph with 52.00 pump and Rheodyne 7120 sampling valve	Iatroscan TH-10 (two instruments: Mark II and Mark III)
	C. Erba, Milan, Italy	H. Knauer, Bad Homburg, F.R.G.	Iatron Labs., Japan, distributed by Newman-Howells, Winchester, U.K.
Integrator	–	–	For Mark II: Spectra-Physics System I Computing Integrator; for Mark III: analogue built-in integrator
Detector	FID, operated at 350°C	Differential refractometer 61.00 (Knauer)	FID, scanning speed of 0.42 cm/s, H_2 flow of 180 ml/min, air flow of 2100 ml/min
Column	Dual, stainless steel, 60 cm × 4 mm I.D.	stainless steel, 25 cm × 4.6 mm I.D.	–
Separation media	Column packing of 3% JXR on Chromaton N HMDS 100–120 mesh	Column packing of LiChrosorb NH_2 (Merck), 10 μm	Silica gel of 5-μm grain size on Chromarod S II
Operating temperature	Programmed from 120 to 350° at a rate of 8°C/min	Ambient under detector thermostating	Ambient
Mobile phase	N_2 carrier gas at rate of 60 ml/min	Methanol + 0.05% acetic acid at a flow-rate of 1.0 ml/min corresponding to 5 MPa inlet pressure approx.	(A) *n*-Pentane–diethyl ether–acetic acid (85:15:1) (B) *n*-hexane–diethyl ether–formic acid (94:3:3) (C) Methylene chloride–methanol–acetic acid (98:0.5:1.5)
Sample application	Injected volume: 0.2–1 μl of sample diluted by *n*-hexane	Injected volume: 20 μl of 5% solution in the mobile phase	Spotted volume: 1 μl of the sample solution (25–300 mg in 1 ml of chloroform)
Remark	Methyl esters were prepared using diazomethane or methanol/sulphuric acid reagent	Back-flushing system was used applying six-way two-position Rheodyne 7010 valve coupled with column inlet and outlet	One-step elution was performed in saturated developing chamber, migration distance of the front from the start was 10 cm

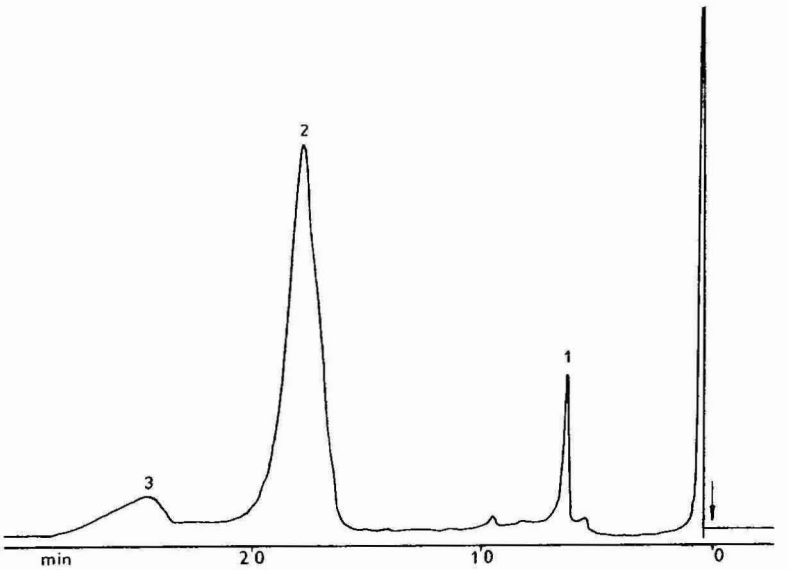

Fig. 1. GLC separation of a commercial fatty acid dimer mixture after the conversion to methyl esters. Peaks: 1, monomers; 2, dimers; 3, trimers.

Much more reliable results have been achieved on NH_2-bonded phases, where separation proceeds according to carboxyl group number, evidently due to the expected ion-exchange mechanism of the separation in methanol to which acetic acid has been added (0.01–0.1%, v/v). The retention is strongly influenced by the acetic acid concentration: while at concentrations above 0.1% all components were poorly separated and eluted in a short time, at 0.05% a sharp separation appeared in approximately 20 min.

Examples of HPLC separations are shown in Fig. 2. In the chromatograms, there are five characteristic peaks. Peak 1 is a trace component (<1%) of apolar nature, presumably representing products of decarboxylation. Peak 2 is a system peak generated by acetic acid freed from the column-packing by the separated carboxylates. Monocarboxylic fatty acid components are included in peak 3; its shape is mostly asymmetrical due to the presence of various isomers. Peak 4 contains all of the dicarboxylic components. After elution, tricarboxylic components together with other higher oligomers are displaced from the column by reversed elution, applying the back-flushing valve: thus, peak 5 contains all the remaining components.

For the quantitative evaluation of peak areas, correction by using relative detector responses should have been considered, but the non-availability of pure standards and the absence of appropriate n_D data in the literature[1] made such corrections impossible. Nevertheless, even without correction, the results coincided with the GLC analyses of commercial products: from this, one can deduce that the difference in n_D data between oligomers is small. On the other hand, the peak areas from independent and parallel UV detection, applied to several samples, showed a strongly increased response to dimer and trimer fractions, proving the presence of cyclic structures[1].

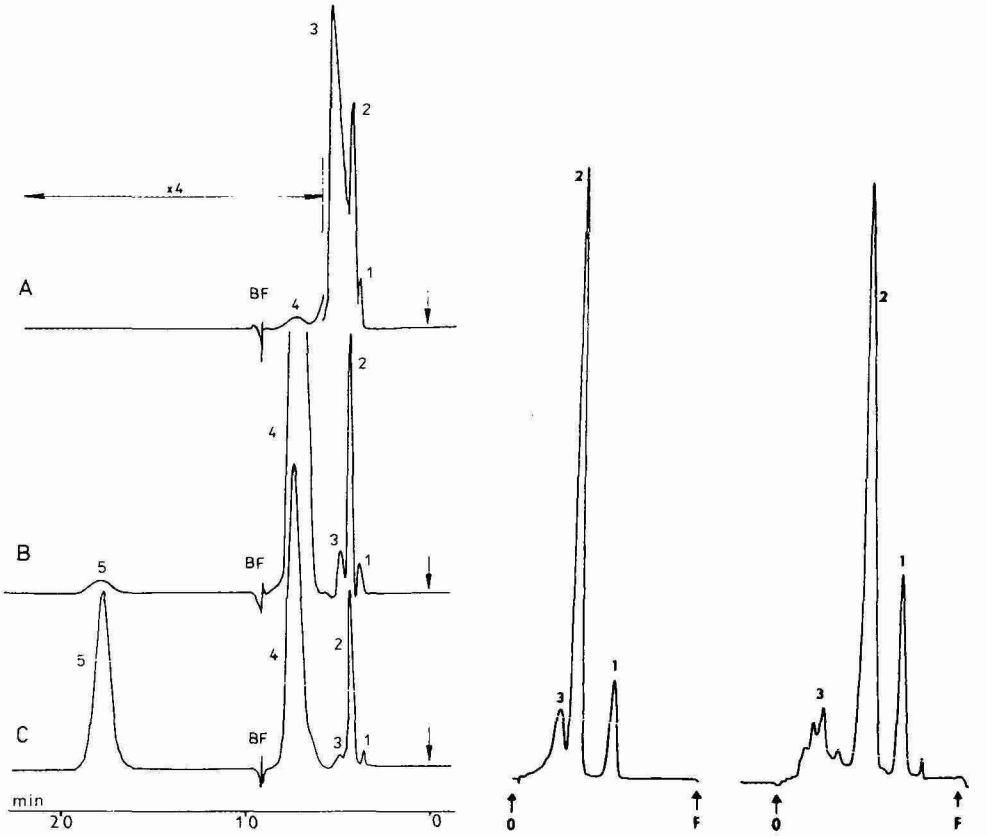

Fig. 2. HPLC separation of fractions isolated from the reaction mixture in fatty acid dimerization experiment. A, monomer fraction as first fraction from molecular distillation; B, dimer fraction as main product from molecular distillation; C, trimer fraction in distillation residue. Peaks: 1, decarboxylation products; 2, system peak; 3, monomers; 4, dimers; 5, trimers and other higher oligomers. BF = back-flush; $\times 4$ = amplification degree of detection. HPLC analyses were performed applying original samples, *i.e.* in the form of free acids only.

Fig. 3. TLC–FID chromatogram of fatty acid dimer mixture on Chromarod S II using mobile phase B. O = Origin (starting point), F = elution front. Peaks: 1, monomers; 2, dimers; 3, trimers and higher oligomers.

Fig. 4. TLC–FID analysis of the same sample as in Fig. 3. Chromarod S II, first elution in mobile phase B (10 cm), second elution in mobile phase C (2.5 cm above the starting point); for description, see Fig. 3.

TLC separation with FID quantification

In optimizing the separating conditions, several mobile phase systems were tried. They were combined from various organic solvents (alkanes, benzene, chloroform, dichloromethane, diethyl ether, ethyl acetate and acetone) and formic or acetic acids. After numerous preliminary experiments in two independent laboratories, each laboratory decided to use its own mobile phase (A and B in Table II).

The main factor that complicated the resolution of the individual oligomer

classes is the presence of one or more free carboxylic groups affecting the shape of the particular adsorption isotherm. This is markedly evident in neutral solvent systems, where the separated zones are often broad, deformed and exhibit tailing toward the origin. In non-polar solvents, such as *n*-hexane, all components remain unresolved at the origin. Diethyl ether, ethyl acetate or acetone increase the R_F value of all oligomers, which migrate in one broad and deformed peak. Chromatography in benzene, dichloromethane or chloroform gives a group of fused peaks in the first third of the migration path along the Chromarod.

After the addition of organic acids to such systems, separation improves dramatically; the peaks are narrower, better resolved and their shape is almost Gaussian, all of which provides a better basis for quantification. Satisfactory separations can be achieved in mixtures of *n*-hexane (or *n*-pentane), diethyl ether and formic (or acetic) acid, as well as in systems based on chloroalkanes and acetic acid that contain small amounts of methanol. For example, chromatography in dichloromethane–methanol–acetic acid (mobile phase C in Table II) gave a similar separation to the more widely used *n*-hexane–diethyl ether–formic acid mixtures (mobile phase B in Table II). However, the latter systems are less hazardous, have higher flow-rates and seem to be preferable due to the lower noise during scanning of Chromarods in FID. Optimum elution strength of these types of solvent systems can be achieved by adjusting the ratio of diethyl ether and organic acid. For example, mobile phase A, containing 15% (v/v) diethyl ether and 1% (v/v) acetic acid, has a very similar resolving power to mobile phase B containing only 3% (v/v) diethyl ether and the same amount of formic acid. An elution mixture with a lower diethyl ether content and a higher acid content was found to be more sensitive to changes in composition, as a result of the loss of diethyl ether due to its high volatility and its preferential sorption in the lower region of the thin layer.

The trimer fraction, remaining close to the origin (Fig. 3), can be moved by the second elution in mobile phase C over a short migration distance (*ca.* 2 cm above the starting point, see Fig. 4). As can be seen, the zone of higher oligomers is split into approximately four fused peaks.

For quantitative evaluation, no correction factors were applied, presuming the relative responses of all components in FID to be equal due to the similar structure and combustible effective carbon atom number in the molecule. This approximation, although not quite correct[19,20], proved to be admissible, as demonstrated from the analysis of samples prepared by mixing commercial products of known composition (Table III).

The reproducibility of the TLC–FID analyses is markedly dependent on the amount of sample analysed (Table IV). Poor reproducibility in the range below 1 μg of substance spotted is a well-known problem in TLC–FID quantification[23,24], therefore, it is advantageous to operate with higher loads (50 μg or more) to obtain sufficient reproducibility for minor components.

Comparison of GLC, HPLC and TLC–FID analyses

The results of the collaborative testing of three mixed model samples are shown in Table V. The individual samples were prepared by mixing pure monomer fraction A and pure dimer fraction B, the composition of which was determined by means of all three methods independently. From these data, the compositions of the tested

TABLE III

TLC–FID ANALYSES OF SAMPLES OF KNOWN COMPOSITION (MODEL MIXTURES) ON CHROMAROD S II USING MOBILE PHASE B, SPOTTED AMOUNT 100 μg

Components for model mixtures	*M (%)*	*D (%)*	*T (%)*
a Distilled monomer fraction	93.0	2.0	5.0
b Distilled dimer fraction	3.9	93.4	2.7
c Purified trimer fraction from distillation residue	0.8	19.8	79.4

Sample	*Mass proportions of components in model mixtures*			*M (%)*		*D (%)*		*T (%)*	
	a	*b*	*c*	*Calc.*	*Found*	*Calc.*	*Found*	*Calc.*	*Found*
1	1	9	0	12.8	11.1	84.3	86.6	2.9	2.4
2	5	5	0	48.5	51.6	47.7	44.4	3.9	4.0
3	9	1	0	84.1	87.9	11.1	7.9	4.8	4.2
4	1	0	9	10.0	7.0	18.1	17.8	71.9	75.2
5	5	0	5	46.9	46.9	10.9	10.2	42.2	42.9
6	9	0	1	83.8	87.7	3.7	2.8	12.5	9.5
7	0	1	9	1.1	0.9	27.2	26.1	71.7	73.0
8	0	5	5	2.4	1.9	56.6	58.0	41.0	40.0
9	0	9	1	3.6	3.0	86.0	90.7	10.4	6.3
10	3	2	4	32.2	30.6	30.2	30.7	37.6	38.7

mixtures C, D and E were calculated separately for GLC, HPLC and TLC–FID in two laboratories.

It is apparent from the results given above that the best correlation between

TABLE IV

THE DEPENDENCE OF THE REPRODUCIBILITY OF TLC–FID ANALYSIS ON THE SPOTTED AMOUNT OF SAMPLE CHROMAROD S II, TWO-STEP ELUTION IN MOBILE PHASE D (10 cm) AND MOBILE PHASE C (2.5 cm)

Spotted amount (μm)	*M*		*D*		*T*	
	Mass (%)	*C.V. (%)*	*Mass (%)*	*C.V. (%)*	*Mass (%)*	*C.V. (%)*
25	49.4	4.8	50.6	5.3	–*	–
50	50.8	3.3	47.8	3.7	1.4	35.6
100	50.2	3.5	47.1	2.6	2.7	11.6
200	47.5	2.1	50.5	2.3	2.0	8.4
300	48.7	2.2	48.9	1.2	2.4	6.5
Calculated composition	49.4		48.5		2.1	

* Very low and broad peak that cannot be integrated.

TABLE V

COMPARISON OF THE GLC, HPLC AND TLC–FID ANALYSES OF FATTY ACID MONOMERS AND DIMER FRACTIONS AND THEIR MIXTURES

TLC–FID I: one-step elution in mobile phase A on Chromarod S II, Iatroscan TH-10 Mark III; TLC–FID II: one-step elution in mobile phase B on Chromarod S II, Iatroscan TH-10 Mark II. Average values are presented: for GLC from two determinations, for HPLC from 2–3 determinations and for TLC–FID from five determinations.

Sample		*Method*	*Found*					
			M (%)		*D (%)*		*T (%)*	
A	Pure unreacted monomer fraction isolated by distillation	GLC	90.9		9.1		–*	
		HPLC	95.6		3.8		0.6	
		TLC–FID I	95.5		0.6		3.9	
		TLC–FID II	96.0		1.5		2.5	
B	Pure dimer fraction isolated by distillation	GLC	7.4		92.6		–*	
		HPLC	3.5		95.6		0.9	
		TLC–FID I	3.1		93.1		3.8	
		TLC–FID II	2.8		95.6		1.6	
			M (%)		*D (%)*		*T (%)*	
			Calc.	*Found*	*Calc.*	*Found*	*Calc.*	*Found*
C	Mixture A:B (8:2)	GLC	74.2	76.8	25.8	23.2	–	–
		HPLC	77.2	77.9	22.2	21.8	0.6	0.2
		TLC–FID I	76.5	80.2	19.3	15.8	4.2	4.0
		TLC–FID II	77.4	79.1	20.3	19.2	2.3	2.3
D	Mixture A:B (5:5)	GLC	49.2	52.3	50.8	47.7	–	–
		HPLC	49.6	49.9	49.7	49.6	0.7	0.5
		TLC–FID I	48.9	51.5	47.0	45.4	4.1	3.2
		TLC–FID II	49.4	47.5	48.5	50.5	2.1	2.0
E	Mixture A:B (2:8)	GLC	24.1	21.0	75.9	79.0	–	–
		HPLC	21.9	22.0	77.2	77.4	0.6	0.8
		TLC–FID I	21.3	21.8	74.8	74.9	3.9	3.3
		TLC–FID II	21.4	20.3	76.8	77.4	1.8	2.3

* In GLC, trace trimers concentration cannot be determined.

calculated and experimental data was found using HPLC. There is sufficient agreement of results for monomers and dimers from all methods. For trimers of trace level, the GLC response was not sufficient to be exactly evaluated; a possible explanation may be the irreversible sorption or thermal decomposition of higher oligomers during analysis at high temperatures.

Higher results of TLC–FID for trimers, compared with HPLC can be explained by the influence of different retention principles in both procedures. In the given HPLC system, strongly specific interactions with carboxyl groups is decisive, whereas in the given TLC–FID system, adsorption is predominant without specific interactions of functional groups. Because of the existence of various structures in dimers and trimers[1,22] and the possible presence of trimers having two carboxyl groups only, the response for trimer components cannot be identical in both HPLC

and TLC–FID. Moreover, the trend for more efficient separation of dimer and trimer components in TLC–FID compared with simple HPLC separation according to carboxyl group number is evident also from Figs. 2 and 4.

It is interesting to note that the TLC–FID results are obtained in two independent laboratories. In spite of different elution systems, the differences in experimental results are relatively small. The higher content of trimers in elution system A suggests that the resolution between trimers and dimers is not identical in both systems.

Conclusions

Fatty acid dimers, containing a complicated mixture of isomeric oligomers, can be analysed by GLC, HPLC and TLC–FID. As a result, the contents of monomers, dimers and trimers can be evaluated. The best correlation between calculated and found composition was achieved by means of HPLC; however, for the monitoring of dimerization process control, TLC–FID appears to be most convenient method.

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CHEMICAL CHANGES OF ORGANIC COMPOUNDS IN CHLORINATED WATER

X*. FORMATION OF POLYCHLORINATED METHYLPHENOXYMETHYLPHENOLS (PREDIOXINS) DURING CHLORINATION OF METHYLPHENOLS IN DILUTE AQUEOUS SOLUTION

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SUMMARY

Aqueous methylphenol (*o*-, *m*- and *p*-isomers) solutions were treated with hypochlorite at 20°C under various experimental conditions. Changes in the compositions of the chlorination products in water were determined by using gas chromatographic (GC) and GC–mass spectrometric analyses of diethyl ether extracts. The chlorination of methylphenols in dilute aqueous solutions produced a series of highly chlorinated compounds, including chloromethylphenols, chloromethylbenzoquinones and chlorinated carboxylic acids. Tentative identifications were also made for other compounds with following molecular formulae: $C_{14}H_5Cl_2O_2$, $C_{14}H_4Cl_3O_2$ and $C_{14}H_3Cl_4O_2$. On the basis of the thin-layer chromatographic and GC behaviour and mass fragmentation patterns, these compounds are considered to be chlorinated methylphenoxymethylphenols. The production of some of these compounds is dependent on the number of equivalents of chlorine per mol of compound and the reaction pH. These results show that treatment with chlorine of water contaminated with phenolic compounds leads to the production of chlorinated phenoxyphenols (predioxins), which are precursors of the highly toxic chlorinated dibenzo-*p*-dioxins.

INTRODUCTION

Polychlorinated phenoxyphenols (PCPPs) occur as the main contaminant of technical chlorophenol formulations[1,2]. Attention has been drawn to such compounds because their 2-hydroxy isomers (predioxins) have been shown to undergo both thermal and photochemical ring closure to form polychlorinated dibenzo-*p*-dioxins (PCDDs)[3–5]. In addition, the chlorophenols themselves may react photo-

* For Part IX, see ref. 13.

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chemically to give PCDDs[6–8]. Although the discussion of the origin of PCDDs and PCPPs has been focused on the above two potential sources, recently Lamparski *et al.*[9] and Weerasinghe *et al.*[10] have demonstrated the presence of PCDDs in municipal sewage sludges.

Chlorination is used extensively in waste-water treatment in order to disinfect and deodorize effluents prior to discharge, particularly where the water may subsequently be used for recreational purposes or as a source of potable water. It seems, therefore, that PCPPs and PCDDs are formed mainly by condensation reactions after chlorination of phenolic substances in the water supplies and wastes[9,10]. Direct support for this hypothesis has been provided by our recent findings which demonstrate that phenolic compounds react with chlorine to form the condensation products of their chlorinated compounds[11–13]. We now present more detailed findings on the characteristics of the condensation products formed upon reaction of chlorine with methylphenols in dilute aqueous solution.

EXPERIMENTAL

Materials

Methylphenols (*o*-, *m*- and *p*-isomers) were obtained from Tokyo Chemicals (Tokyo, Japan). Several chlorinated methylphenols, benzoquinones and carboxylic acids, which may be expected to be formed during the reaction of each methylphenol with hypochlorite in dilute aqueous solution, were commercially available reagents. Standard solutions of these compounds both alone and as mixtures were prepared by dissolving the compounds in methanol, with subsequent dilutions. Hypochlorite solutions were prepared by diluting sodium hypochlorite solution (*ca.* 10% available chlorine; Nakarai Chemicals, Kyoto, Japan) in distilled water and were adjusted to the required pH by addition of 0.1 *M* disodium hydrogenphosphate–potassium dihydrogenphosphate buffer solution. The hypochlorite concentrations were determined by iodometric titration.

Treatment of aqueous methylphenol solutions with hypochlorite and extraction of reaction mixture

Aqueous methylphenol solutions (50 μmol/l) were treated with hypochlorite at 20°C for 1 h with stirring at various pH values and with various equivalents of chlorine per mol of compound. After the desired reaction time, the unreacted chlorine was removed by addition of sodium thiosulphate solution. The reaction mixture was then acidified to pH 2.0 with 0.1 *M* hydrochloric acid before extracting with three 200-ml volumes of diethyl ether. The solvents were dried over anhydrous sodium sulphate and concentrated under vacuum at 40°C to suitable volumes for gas chromatographic (GC), GC–mass spectrometric (GC–MS) and thin-layer chromatographic (TLC)–MS analyses. More detailed results of TLC–MS studies of diethyl ether extracts from a chlorine-treated methylphenol solution will be presented subsequently.

Product resolution and characterization

A Shimazu GC-6A gas chromatograph equipped with a flame ionization detector and 2 m × 3 mm I.D. glass column packed with 10% Apiezon L on Chro-

mosorb W AW DMCS (60–80 mesh) was programmed from 180 to 280°C at 5°C/min. The nitrogen carrier gas flow-rate was 50 ml/min. A Shimazu Model Chromatopac-1A data system was used to determine the retention times and peak areas on the chromatograms.

An Hitachi M-80 mass spectrometer–gas chromatograph equipped with an Hitachi M-003 data processing system was used for the qualitative analyses of samples under the following conditions. The ion source was operated at 250°C with a trap current of 70 μA and an electron energy of 70 eV. A glass column (2 m × 3 mm I.D.) packed with 5% SE-30 on Chromosorb W AW DMCS (60–80 mesh) was used for the GC separation of the diethyl ether extracts. The oven temperature of the gas chromatograph was programmed from 220 to 300°C at 5°C/min. The products were identified by comparison of their retention times and mass spectra with those of authentic compounds.

RESULTS AND DISCUSSION

Production of chlorinated methylphenol dimers

Typical GC (with flame ionization detection) and GC–MS (total ion) traces of diethyl ether extracts of methylphenol solutions after treatment with hypochlorite at 5 equiv. chlorine per mol of compound are shown in Figs. 1 and 2. Some of the peaks were identified on the basis of the retention times of known compounds.

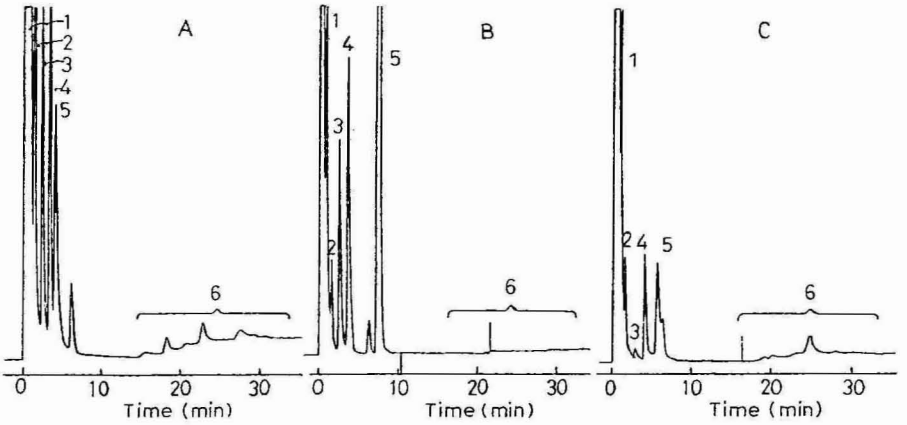


Fig. 1. Typical gas chromatograms (with flame ionization detection) of diethyl ether extracts of 2- (A), 3- (B) and 4-methylphenol (C) solutions (50 μmol/l) after treatment with hypochlorite (5 equiv. of chlorine per mol of compound) at 20°C for 1 h. For GC conditions, see Experimental. Peaks: (A) 1 = 2-methylphenol, 2 = 6-chloro, 3 = 4-chloro, 4 = 4,6-dichloro, 5 = quinone body, 6 = 2-methylphenol dimers; (B) 1 = 3-methylphenol, 2 = 6-chloro, 3 = 4-chloro, 4 = 4,6-dichloro, 5 = 2,4,6-trichloro, 6 = 3-methylphenol dimers; (C) 1 = 4-methylphenol, 2 = 2-chloro, 3 = quinone body, 4 = 2,6-dichloro, 5 = quinone body, 6 = 4-methylphenol dimers.

Compounds corresponding to other peaks were determined from the mass spectrum of each peak (Figs. 3 and 4). In addition, in order to investigate the detailed mass fragment patterns for these chlorination products, the extract was further separated on Polyamid 11 F 254 pre-coated TLC plates (E. Merck, Darmstadt, F.R.G.)

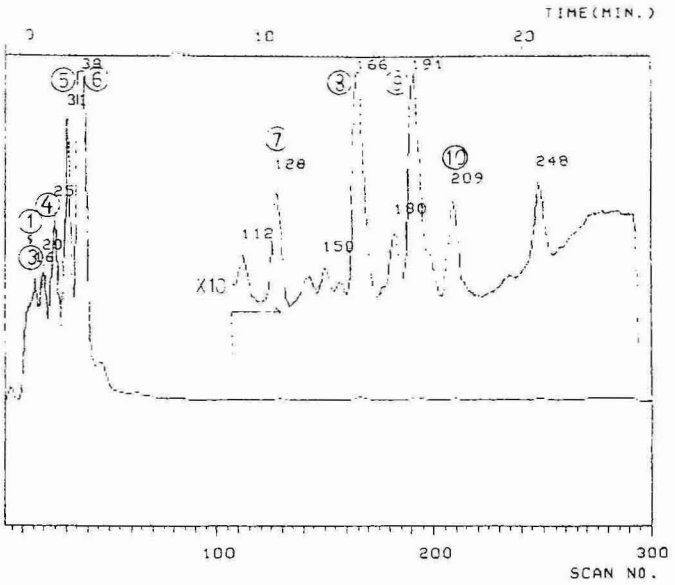


Fig. 2. Mass chromatogram (total ion current) of a diethyl ether extract of a 2-methylphenol solution (50 μmol/l) after treatment with hypochlorite (5 equiv. of chlorine per mol of compound) at 20°C for 1 h. Compounds as in Table I. For GC–MS conditions, see Experimental.

using chloroform as the developing solvent (Fig. 5), and the separated components were then analyzed by mass spectrometry.

Fig. 3A shows the mass spectrum of the dichlorinated compound, peak 7 in Fig. 2, occurring in the diethyl ether extract from a chlorine-treated 2-methylphenol solution. The molecular ion (M^+) is at *m/e* 282 and the most abundant fragment ion is at *m/e* 212, which arises by the loss of two chlorine atoms from the molecular ion. Other fragment ions at *m/e* 247 ($M^+ - Cl$), 142 ($M^+ - 2Cl - C_4H_6O$) and 77 ($M^+ - 2Cl - C_8H_7O_2$) are observed. The same molecular ion with two chlorine atoms was also observed for peak 9 (Fig. 4A), but the fragmentation pattern of this compound is different from that of peak 7. This seems to indicate that two isomers of a methylphenol dimer with two chlorine atoms are present in the diethyl ether extract from chlorine-treated 2-methylphenol solution.

The mass spectrum of peak 8, which was the most abundant among the chlorinated 2-methylphenol dimers (Fig. 2), was interpreted as being that of a 2-methylphenol dimer with three chlorine atoms (Fig. 3B). The molecular ion at *m/e* 316 and three fragment ions at *m/e* 246 ($M^+ - 2$ Cl), 142 ($M^+ - 3Cl - C_4H_5O$) and 77 ($M^+ - 3Cl - C_8H_6O_2$) were observed. Fig. 4B shows that peak 10 is a 2-methylphenol dimer with four chlorine atoms. The molecular ion is at *m/e* 350 and the most abundant fragment ion at *m/e* 280, which arises by the loss of two chlorine atoms from the molecular ion. Other fragment ions at *m/e* 142 ($M^+ - 4Cl - C_4H_4O$) and 77 ($M^+ - 4Cl - C_8H_5O_2$) were observed.

The GC–MS trace (not shown) of the diethyl ether extract of a chlorine-treated 4-methylphenol solution showed the occurrence of 4-methylphenol dimers with two, three and four chlorine atoms.

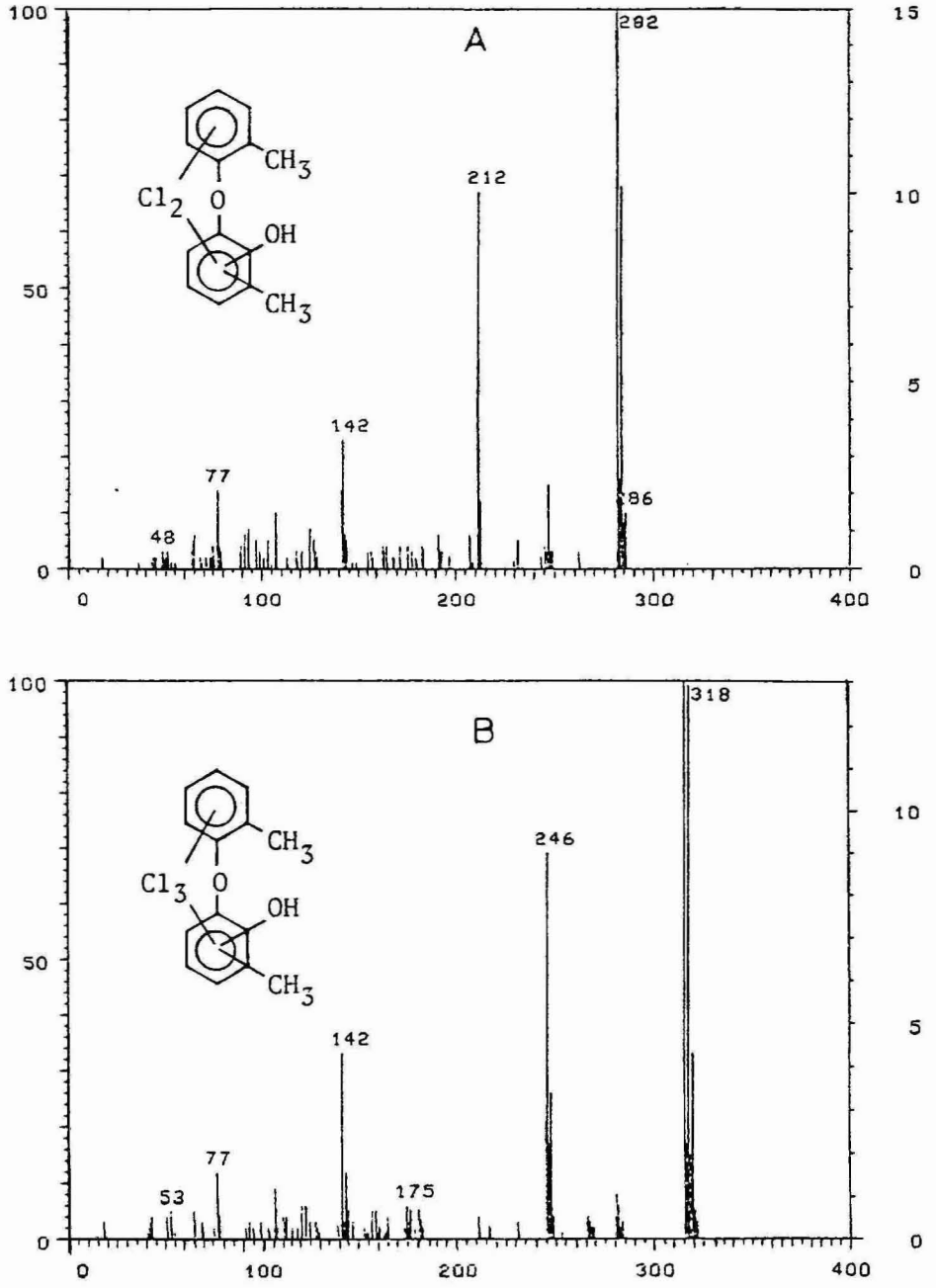


Fig. 3. Mass spectra of dichlorinated (A) and trichlorinated 2-methylphenol (B) dimers obtained from peaks 7 and 8, respectively (*cf.*, Fig. 2).

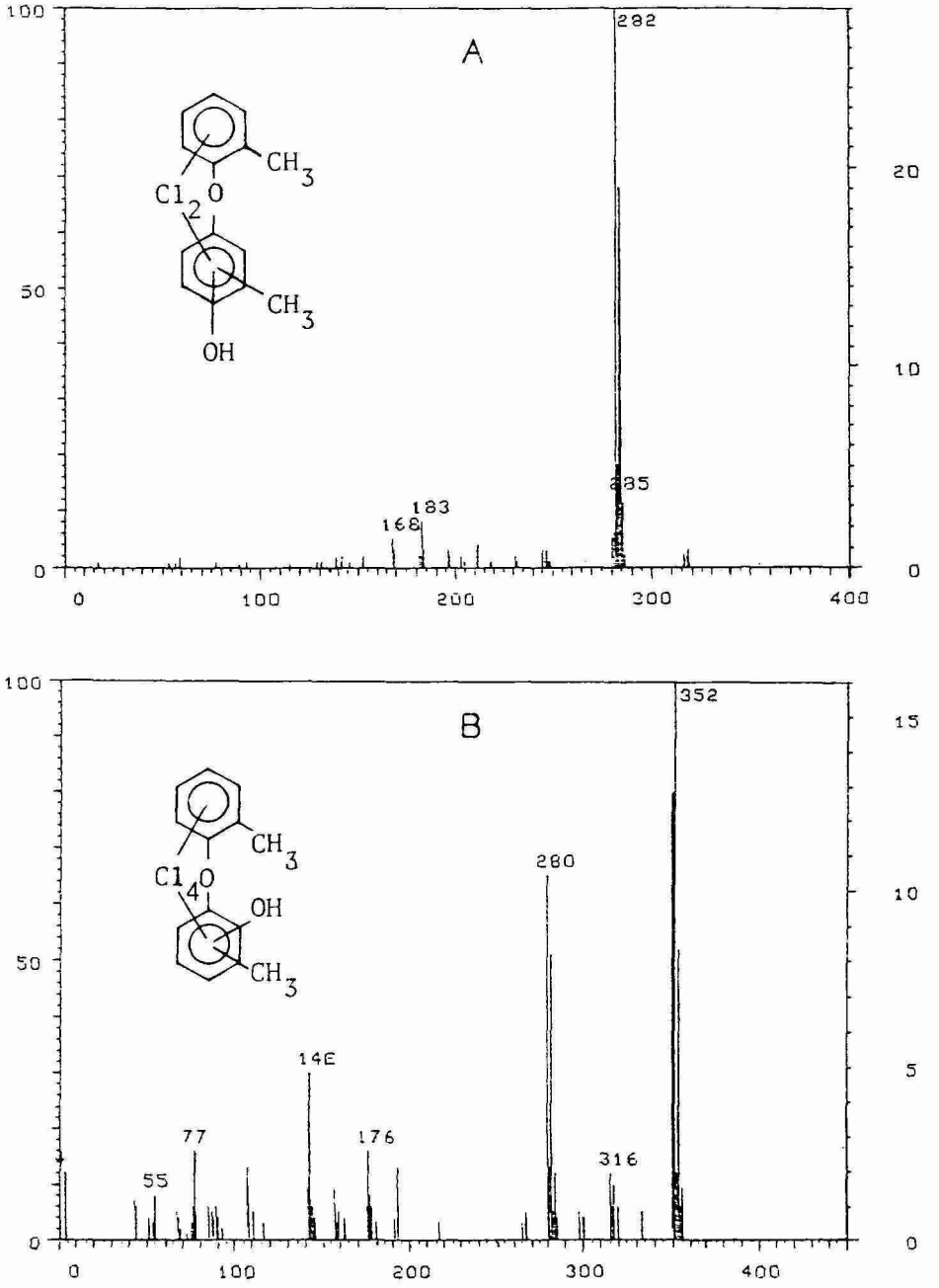


Fig. 4. Mass spectra of dichlorinated (A) and tetrachlorinated 2-methylphenol (B) dimers obtained from peaks 9 and 10, respectively (*cf.*, Fig. 2).

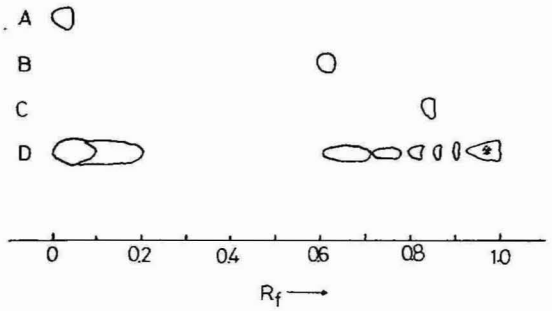


Fig. 5. Typical thin-layer chromatogram of a diethyl ether extract of the products from a chlorine-treated 4-methylphenol solution, on a Polyamid plate with chloroform as the developing solvent. A, Trichloroacetic acid B, 4-methylphenol; C, 2,6-dichloro-4-methylphenol; D, diethyl ether extract. The asterisk indicates chlorinated 4-methylphenol dimers. Based on the R_F values, each separated component was scraped from the plate with diethyl ether and then analyzed by GC–MS.

Several compounds corresponding to the polychlorinated methylphenol dimers were observed on the gas and mass chromatograms of diethyl ether extracts of mixtures obtained upon reaction of methylphenols with hypochlorite in water (Figs. 1 and 2). However, earlier workers[14,15] demonstrated the presence of the corresponding chlorinated phenol dimers (ethers and dioxins) in the mass spectra of polychlorinated phenols and concluded that these dimers are formed by pyrolysis of the chlorinated compounds. In this work, the corresponding dimers could not be detected in the mass spectra of the chlorinated methylphenols. Therefore, it is concluded that the production of several chlorinated methylphenol dimers occurs during the reactions of methylphenols with hypochlorite in dilute aqueous solution.

A summary of these chlorination products identified or determined from their GC retention times and mass spectrometric interpretation is presented in Tables I and II. On the basis of TLC, GC and GC–MS (Figs. 1, 2 and 5), and mass spectrometric characterization (Figs. 3 and 4), these methylphenol dimers containing different numbers of chlorine atoms are considered to be chlorinated methylphenoxymethylphenols, but their exact structures are not clear.

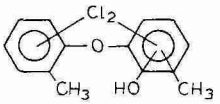
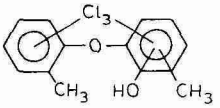
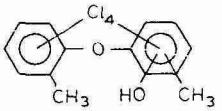
GC determinations of residual amounts of chlorinated methylphenol dimers in water

GC analysis of the diethyl ether extracts indicated that a marked reduction in the original amount of methylphenols in water occurs with an increase in the molar ratio of hypochlorous acid (Fig. 6). The chlorinated methylphenols were found to be present at high concentrations in water at low molar ratios of hypochlorous acid. At moderate molar ratios, chlorinated methylbenzoquinones and polychlorinated methylphenol dimers (*m/e* 282, 316 and 350 in Tables I and II) were detected as the intermediates of reaction of methylphenol solutions with hypochlorite in water. At molar ratios of hypochlorous acid to the compound of >20 only a small amount of these chlorinated intermediates was found (Fig. 6).

Polychlorinated dibenzo-*p*-dioxins were recently found in dried municipal sludge from Milwaukee (Milorganite)[9] sampled in 1933, 1981 and 1982 and it was speculated that their presence may have resulted from condensation reactions after

TABLE I

REACTION PRODUCTS OF 2-METHYLPHENOL WITH HYPOCHLORITE IN DILUTE AQUEOUS SOLUTION AT 20°C

No.	*Compound*	*Scan No.*	*m/e (order of ion intensity)*
1	6-Chloro-2-methylphenol*	–	142 (M^+), 107 ($M^+ - Cl$)
2	4-Chloro-2-methylphenol*	–	142 (M^+), 107 ($M^+ - Cl$)
3	Chloromethyl-*p*-benzoquinone*	–	158 (M^+), 123 ($M^+ - Cl$)
4	4,6-Dichloro-2-methylphenol*	13	141 ($M^+ - Cl$), 176 (M^+)
5	Dichloromethyl-*p*-benzoquinone*	30	192 (M^+), 157 ($M^+ - Cl$)
6	Unknown	38	165, 163, 43, 145, 89, 101
7	$C_{14}H_{12}Cl_2O_2$** 	128	282 (M^+), 212 ($M^+ - 2Cl$), 142 ($M^+ - 2Cl - C_4H_6O$), 247 ($M^+ - Cl$), 77 ($M^+ - 2Cl - C_8H_7O_2$)
8	$C_{14}H_{11}Cl_3O_2$** 	166	316 (M^+), 246 ($M^+ - 2\ Cl$), 142 ($M^+ - 3Cl - C_4H_5O$), 77 ($M^+ - 3Cl - C_8H_6O_2$)
9	Isomer of compound 7**	191	282 (M^+)
10	$C_{14}H_{10}Cl_4O_2$** 	209	350 (M^+), 280 ($M^+ - 2Cl$), 142 ($M^+ - 4Cl - C_4H_4O$), 77 ($M^+ - 4Cl - C_8H_5O_2$), 315 ($M^+ - Cl$)

* Complete identification based on mass spectral interpretation and confirmed by comparison with a reference spectrum and the retention time of the substance.

** Tentative structure; identification based on mass spectral interpretation.

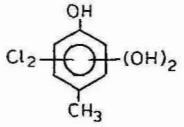
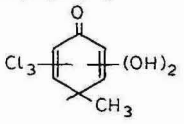
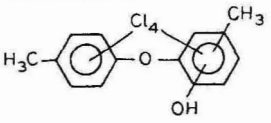
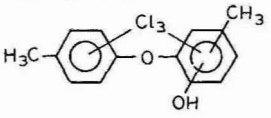
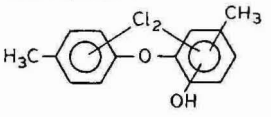
chlorination of phenolic substances in the water supply and wastes. Although a reduction in the amounts of chlorinated methylphenol dimers in water was observed with increasing molar ratio of hypochlorous acid to the compound (Fig. 6), the formation of polychlorinated methyldibenzo-*p*-dioxins in chlorine-treated water was not tested in the present work. Further research would therefore be required to determine how the chlorinated methylphenol dimers are converted into other chlorinated substances.

Fig. 7 shows the results of GC determinations of diethyl ether extracts from methylphenol solutions after treatment with an excess of hypochlorite at various pH values for 1 h. Several chlorinated methylphenols, further oxychlorinated products of chlorinated methylphenols and the chlorinated methylphenol dimers were detected at high concentrations in acidic solutions, although small amounts of these chlorinated compounds were obtained under neutral and alkaline conditions. These results were also in agreement with the values of the chlorine demands of methylphenols obtained at various pH values (data not shown).

Under acidic and neutral conditions, chlorinated products of 4-methylphenol formed upon treatment with hypochlorite in water were detected at higher concen-

TABLE II

REACTION PRODUCTS OF 4-METHYLPHENOL WITH HYPOCHLORITE IN DILUTE AQUEOUS SOLUTION AT 20°C

No.	*Compound*	*Scan No.*	*m/e (order of ion intensity)*
1	2-Chloro-4-methylphenol*	–	142 (M^+)
2	$C_7H_6Cl_2O_3$** 	19	89 ($M^+ - 2Cl - 3CO$), 53 ($M^+ - 2Cl - 3CO - C_3H$), 123 ($M^+ - 2CO - CHO$), 208 (M^+)
3	2,6-Dichloro-4-methylphenol*	12	141 ($M^+ - Cl$), 176 (M^+), 77 ($M^+ - 2Cl - CHO$)
4	$C_7H_5Cl_3O_3$** 	25	123 ($M^+ - COCl - 2CO$), 87 ($M^+ - COCl - CHO - CO - Cl$), 53 ($M^+ - COCl - 2CO - 2Cl$), 179 ($M^+ - COCl$), 242 ($M^+$)
5	Unknown	30	123, 53, 87, 179
6	$C_{14}H_{12}Cl_2O_2$** 	144	146 ($M^+ - 2Cl - C_4H_2O$), 282 (M^+), 247 ($M^+ - Cl$), 212 ($M^+ - 2Cl$), 219 ($M^+ - 2Cl - CO$), 267 ($M^+ - CH_3$)
7	$C_{14}H_{11}Cl_3O_2$** 	172	316 (M^+), 246 ($M^+ - 2Cl$), 301 ($M^+ - CH_3$), 281 ($M^+ - Cl$), 267 ($M^+ - Cl - CH_3$), 146 ($M^+ - 3Cl - C_4HO$)
8	Isomer of compound 7**	187	180 ($M^+ - 3Cl - CH_3O$), 316 (M^+), 281 ($M^+ - Cl$), 246 ($M^+ - 2Cl$), 253 ($M^+ - COCl$)
9	$C_{14}H_{10}Cl_4O_2$** 	214	350 (M^+), 280 ($M^+ - 2Cl$), 315 ($M^+ - Cl$), 335 ($M^+ - CH_3$)

* Complete identification based on mass spectral interpretation and confirmed by comparison with a reference spectrum and the retention time of the substance.

** Tentative structure; identification based on mass spectral interpretation.

trations than those observed for 2- and 3-methylphenol solutions (Fig. 7). This could be explained by steric shielding of the *para*-position in the phenol by a methyl group substituent in the aromatic ring, which hinders the oxidative degradation of chlorinated methylphenols with hypochlorous acid in acidic aqueous solutions.

Aqueous chlorine is used to disinfect sewage water and raw water destined for

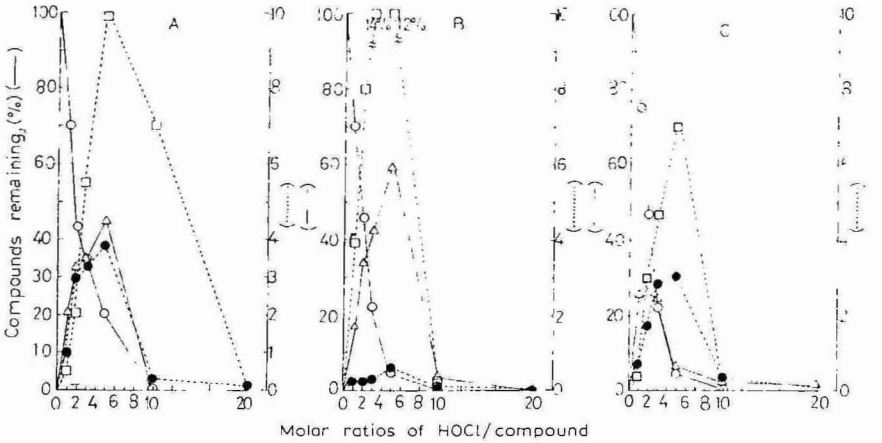


Fig. 6. Residual amounts of reaction products in aqueous 2- (A), 3- (B) and 4-methylphenol (C) solutions (50 μmol/l) after treatment with hypochlorite (various equivalents of chlorine per mol of compound) at 20°C for 1 h. The yields are derived from GC peak areas, relative to the peak area of each starting material. ○—○, Starting material; △—△, chlorinated methylphenols; □—□, polychloropolyhydroxylated methylphenols; ●—●, chlorinated methylphenol dimers.

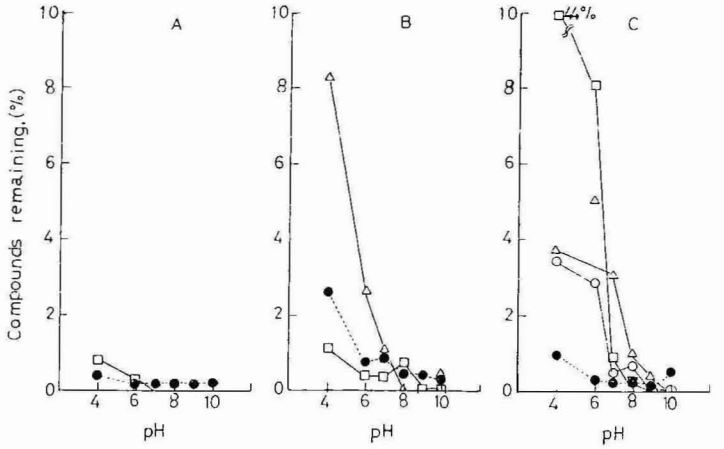


Fig. 7. Residual amounts of reaction products in aqueous 2- (A), 3- (B) and 4-methylphenol (C) solutions (50 μmol/l) after treatment with an excess of hypochlorite at various pH and 20°C for 1 h. Other details as in Fig. 6.

human consumption. Methylphenols have been shown to produce many chlorinated aromatic compounds upon reaction with hypochlorite in water (Tables I and II). The production of these compounds is greatly dependent on the molar ratio of hypochlorous acid and the reaction pH, with higher concentrations formed at moderate chlorine doses (Fig. 6) and under acidic conditions (Fig. 7). Acidic chlorinated waters are generally adjusted to neutral conditions or diluted in water at the water-treatment station. Therefore, most of the chlorinated aromatic compounds are destroyed by this process and the hazard to public health may be substantially decreased. However,

the chlorinated methylphenol dimers are particularly interesting, as they are present in chlorinated-treated methylphenol solutions and are also precursors of the highly toxic chlorinated dioxins.

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COMPARISON AND CRITICAL EVALUATION OF SIX PUBLISHED EXTRACTION AND CLEAN-UP PROCEDURES FOR AFLATOXIN M_1 IN LIQUID MILK

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SUMMARY

A practical evaluation has been carried out of six previously published extraction and clean-up methods for aflatoxin M_1 in liquid milk. The procedures evaluated incorporated the most widely used stages of clean-up including solvent extraction and silica gel chromatographic clean-up, selective solvent extraction of the extracted residue, the use of deproteination prior to hydrophilic column liquid–liquid partition or solvent extraction and the use of pre-packed reversed-phase cartridges for the direct extraction of aflatoxin M_1 from the milk. Analysis times for each method, recoveries and relative costs are reported together with fluorescence high-performance liquid chromatography chromatograms, obtained under identical conditions to compare the relative cleanliness of the final extracts produced by each method. A pre-packed reversed phase cartridge method was shown to be the most satisfactory in terms of speed, cost and cleanliness of the final residue.

INTRODUCTION

There continues to be considerable interest in aflatoxin M_1 contamination of dairy products as evidenced by the number of papers reporting survey results[1–3] and further stimulated by the imposition of various national statutory limits[4,5]. There have been a number of recent papers[6–14] proposing new analytical methods for aflatoxin M_1 and the analyst involved in routine monitoring needs continually to reassess his choice of a particular procedure in the light of possible improved techniques. For arbitration of any possible dispute over aflatoxin M_1 levels in a particular commodity, or for critical samples one is necessarily obliged to employ methods like that of the AOAC[15] which have been thoroughly collaboratively tested. However for routine surveillance and for quality control the most rapid methods available need to be employed, provided adequate specificity can be achieved through efficacious extraction and clean-up stages. In this laboratory over the course of several years, we have routinely employed a modified Stubblefield extraction and clean-up method[16], for the analysis of several hundred samples of milk for aflatoxin M_1, using

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both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) for the final determination[1]. This method has proved to be reliable and reasonably rapid and has given good results when employed in the U.K., the European Economic Community (Community Bureau of Reference), and internationally organised collaborative trials.

Recent publications[9–14] do however suggest that alternative procedures might offer the advantages of more rapid analyses, reduced consumption of expensive solvents, and cleaner final extracts. A comparison of these methods and assessment of the various claims is not always easy without carrying out experimental work as the final determination step may be different in each case and criteria like ease and speed of analysis can to some extent be subjective. Therefore, in order to evaluate alternative methodologies for aflatoxin M_1 in liquid milk we have undertaken a critical practical appraisal of six published procedures. The results of this assessment are reported in this paper.

Although there have been a large number of methods published for aflatoxin M_1, the differences between procedures are often only slight and the six methods evaluated in this paper were chosen as being representative of a number of different principles which have been employed in the extraction and clean-up stages. These methods are summarised in Table I. The Stubblefield method[16] is based on chloroform extraction of milk and silica gel column clean-up, whilst the Chang and DeVries method[9] employs a similar extraction of the milk but selective solvent extraction of the residue replaces the chromatographic stage. The Gauch *et al.* method[10] was chosen to exemplify the use of hydrophilic liquid–liquid partition columns (Extrelut), whilst Chambon *et al.*[11] have employed a deproteination step prior to solvent extraction. The Ferguson-Foos and Warren procedure[12] uses a pre-packed Sep-Pak C_{18} cartridge for the extraction from the milk followed by a silica gel column clean-up, whilst the Takeda method[13] is similar in its use of a reversed-phase Sep-Pak for the extraction but the choice of different conditions avoids the need for any further clean-up.

TABLE I

SUMMARY OF AFLATOXIN M_1 METHODS UNDER EVALUATION

Method	*Volume of milk (ml)*	*Preliminary treatment*
Stubblefield[16]	50	None
Chang and DeVries[9]	50	None
Gauch *et al.*[10]	50	Hydrochloric acid (0.4 ml), methanol (15 ml)
Chambon *et al.*[11]	20	Zinc sulphate–sodium hydroxide deproteination
Ferguson-Foos and Warren[12]	20	Warm water (20 ml)
Takeda[13]	10	Water (10 ml)

These methods have been variously developed for both TLC and HPLC determinations, but as HPLC is generally less demanding of the cleanliness of the final extract because of its increased resolution over TLC, this has been employed under identical conditions for the analysis of all the final extracts. Thus after gaining experience with each method, and modifying (but only where absolutely necessary) to facilitate operation in our hands, the speed of analysis, unit cost, recovery, and cleanliness of the final extract have been assessed under identical conditions and are reported in this paper together with a commentary on the methods.

EXPERIMENTAL

Materials

Pasteurized bulked commercial whole milk which contained less than 0.005 μg/kg of the toxin was stored frozen and a thawed aliquot spiked with a solution of aflatoxin M_1 in acetonitrile to give the desired concentrations. The same supply of milk was used throughout to avoid any differences in potential interferences, although spiking in each case was carried out immediately before analysis. Aflatoxin M_1 standard was obtained from Sigma (Poole, U.K.), and quantitative solutions were standardised using the recommended procedure[15]. Sep-Pak C_{18} and Extrelut pre-packed columns were obtained from Waters Chromatography Division of Millipore (London, U.K.) and BDH (Poole, U.K.), respectively.

Methods

Full detailed descriptions of each method are given in the original publications, but for comparative purposes the principle of each is outlined here together with an indication of any modifications that were found necessary to operate the methods successfully in our hands. Any such modifications were kept to the absolute minimum.

Stubblefield method[16]. This involves chloroform extraction of milk by shaking

Extraction	*Clean-up*	*Published recovery (%)*	*Published determination*
Chloroform (120 ml)	Silica-gel column	80	TLC (one dimension)
Chloroform (120 ml)	Acetonitrile–light petroleum extract	100 ± 13	HPLC (TFA derivative)
Extrelut column	Eluate (dichloromethane–toluene) washed with sodium hydroxide and hydrochloric acid	78 ± 4	TLC (two developments)
Chloroform extract of filtrate	None	83–89	HPLC
Sep-Pak C_{18}	Silica gel column	99–103	HPLC (silica gel packed flow cell)
Sep-Pak C_{18}	None	72–95	HPLC (TFA derivative)

together with saturated sodium chloride solution, evaporating the solvent to dryness and clean-up on a silica gel column with three solvent washings prior to elution with acetone–chloroform. Modifications were as described elsewhere[1], involving a reduction in the volume of sodium chloride solution added to the milk to 5 ml while the chloroform extractant was not heated to 35°C. The hexane wash was increased to 15 ml and elution of M_1 was with acetone–chloroform (2:3) rather than (1:4).

Chang and DeVries method[9]. This entails extraction of aflatoxin M_1 into chloroform by gentle rolling of the milk with the solvent, evaporation of the chloroform to dryness and then extraction of the residue into acetonitrile, washing with light petroleum and evaporation to dryness, redissolving in chloroform prior to analysis. The only modifications that were introduced were extraction for 10 min rather than the 3 min proposed in the method and the replacement of light petroleum with hexane.

Gauch et al. method[10]. This stipulates milk protein precipitation with concentrated hydrochloric acid, heating to assist coagulation and then extraction of the filtrate on an Extrelut column. The eluate in dichloromethane–toluene is washed with sodium hydroxide and hydrochloric acid prior to analysis. No modifications were introduced.

Chambon et al. method[11]. This requires deproteination of the milk by the addition of zinc sulphate and sodium hydroxide followed by filtration. The filtrate is washed with hexane (this step has been mistakenly left out of the written procedure given in ref. 11 although it may be inferred from the text) and then extracted into chloroform, evaporating to dryness. The method was followed exactly but it was found necessary to record the volume of chloroform recovered (usually 60–80%) and to use this to correct the final recovery.

Ferguson-Foos and Warren method[12]. This is based on extraction of toxin by passage of the warmed milk, diluted with water, through a reversed-phase Sep-Pak previously primed with methanol, washing and then eluting with diethyl ether. This is followed by a clean-up on a silica gel column using vacuum elution of the aflatoxin in methylene chloride–ethanol solvent.

Slight modifications were made in that only 10 ml of milk were extracted (as opposed to the recommended 20 ml) as this was found simpler to pass through the Sep-Pak, and also loading onto the cartridge was under syringe pressure rather than the proposed vacuum system. A number of the silica gel columns were extracted simultaneously using a vacuum manifold system which speeded up this stage of the clean-up.

Takeda method[13]. This relies upon extraction of the aflatoxin M_1 by passing the diluted milk through a reversed-phase Sep-Pak C_{18}, washing with firstly 10% basic acetonitrile, then 10% acidic acetonitrile and finally eluting with 30% acidic acetonitrile. No changes were introduced into this method.

HPLC analysis

All results were obtained under optimum conditions for aflatoxin separations[17], using a Varian 5500 ternary chromatograph and a 250 × 4.9 mm I.D. 5-μm Spherisorb ODS column, maintained at 35°C and eluted with water–acetonitrile–methanol (60:30:10) at a flow-rate of 0.75 ml/min. The solvents were purged with helium and a back-pressure regulator adjusted to 2 bar was attached to

the outlet of the fluorimeter. A Waters in-line filter with a 2-μm frit was placed between the injector (Rheodyne 7125 with a 20-μl loop) and the column. Fluorescence detection was employed using a Perkin Elmer Model LS-4 spectrofluorimeter with the excitation and emission monochromators set at 355 and 433 nm respectively, as indicated by stopped-flow scanning of an M_1 standard.

Batches of six samples were analysed in all cases, consisting of duplicates each of a blank, a 0.016-μg/kg spike and a 0.16-μg/kg spike. All sample residues were made up immediately prior to analysis in a volume of water–acetonitrile (80:20) such that 100 μl represented 25 ml of milk. Aliquots (10 μl) of these solutions were injected onto the HPLC system using the partial loop-fill method in order to conserve sample. Results were displayed on a chart recorder at a speed of 5 mm/min under identical conditions for each method of analysis. Calculations were made on a peak height basis.

RESULTS AND DISCUSSION

One of the main difficulties of evaluating methods is that familiarity with a given procedure tends to be prejudicial, and for this reason each new method was carried out many times during the course of several weeks, and repeated until we were satisfied that it was performing reproducibly and with good recovery according to the claims of the originators of the method. Assessment to some extent has been relative to the Stubblefield method, as this procedure had been employed in these laboratories for several years for aflatoxin M_1 analyses and has given clean extracts for both TLC and HPLC determinations. The only problems with the Stubblefield method has been that in inexperienced hands the initial extraction can give rise to emulsions, and in addition we have not found it to be a particularly rapid procedure, a batch of six milk samples usually taking a complete day for analysis including the determination step and allowing time for preparation of columns and reagents.

In Table II the analysis time per sample is given for the six methods together with recoveries, detection limits and the relative costs of the different procedures. The two most rapid procedures were those based on reversed-phase Sep-Pak extraction of aflatoxin from the milk (Ferguson-Foos and Warren, and Takeda methods) and these took less than half the time of the Stubblefield method to carry out. Generally the more rapid methods were those which were also less expensive on solvent and other material requirements, although in assessing overall relative costs of different methods the labour element is often far more important. With high assigned labour costs, the order of total costs was identical to the order in which methods are placed in terms of analysis times.

The recoveries (Table II) were generally acceptable, ranging from 60 to 84%, based on milk spiked at 0.16 μg/kg. Similar values were found at the lower concentration, 0.016 μg/kg, for all methods except for that of Gauch *et al.*, where the recovery fell to 42%. All results quoted are averages from four samples (the duplicate milks spiked at 0.16 μg/kg in both of the last two batches analysed using each method). The higher detection limit of the Gauch *et al.* method was a direct consequence of its lower recovery. This was attributed to losses during the alkaline wash stage in the procedure which although carried out rapidly as stipulated in the method might still give rise to degradative losses of aflatoxin M_1. It should however be noted

TABLE II

CRITICAL COMPARISON OF AFLATOXIN M_1 METHODS

Method	*Analysis time per sample (h)*	*Average recovery (%)*	*Limit of detection (μg/kg)*	*Relative costs (solvents/materials)**	*Total relative costs (including labour)**
Stubblefield[16]	4	77	0.005	1.3	2.2
Chang and DeVries[9]	2.5–3.0	80	0.005	1.7	1.7
Gauch *et al.*[10]	5.5–6.0	60	0.01	4.0	3.5
Chambon *et al.*[11]	3.5–4.0	74	0.005	1.0	2.1
Ferguson-Foos and Warren[12]	1.5	84	0.005	1.8	1.0
Takeda[13]	1.5–2.0	70	0.005	1.2	1.2

* Costs are calculated relative to the lowest cost per method. The cost of labour is estimated at 3.7 times greater per hour than unit cost of solvent/materials.

that this method is reported to give satisfactory results with a wide range of dairy products, including powdered milks, baby food and cheeses. Low recoveries of chloroform were encountered during the extraction stage of the Chambon *et al.* method and recovered volumes were recorded in order to correct for losses at this stage.

Manipulative difficulties were experienced with the large volumes of solvent applied to the Extrelut columns in the Gauch *et al.* method and the columns themselves seemed to be prone to leaking around the tips, requiring constant attention by the operator, although this might be attributable to our lack of experience with this technique. A general disadvantage in methods like that of Takeda and that of Gauch

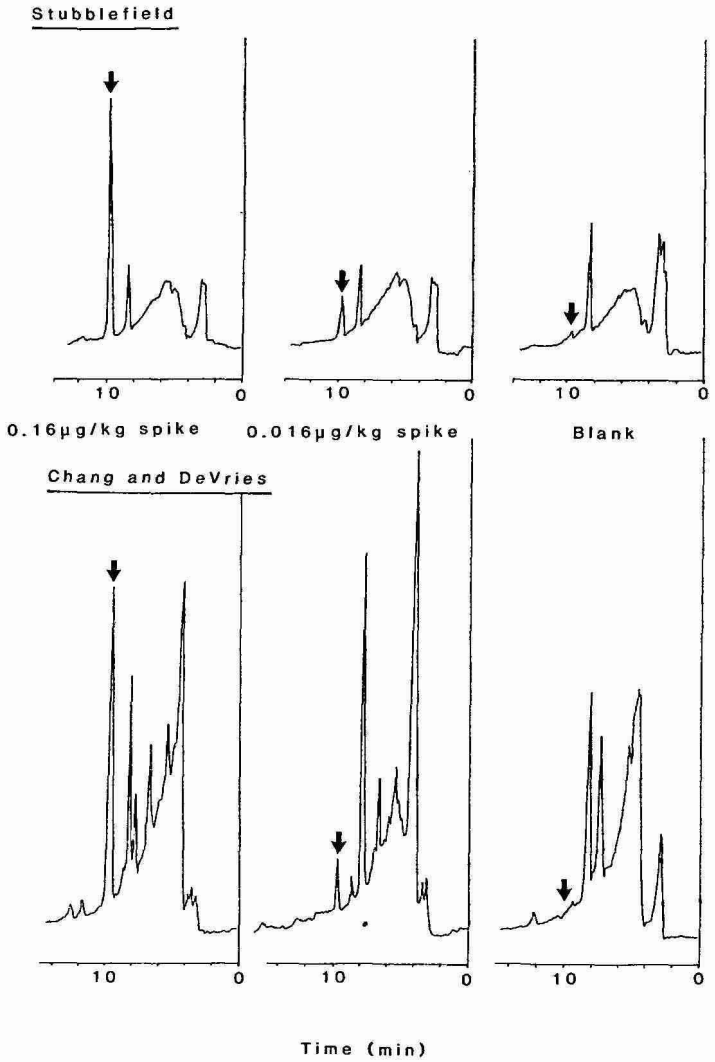

Fig. 1. HPLC fluorescence chromatograms illustrating the clean-up for aflatoxin M_1 (arrows) by the Stubblefield[16] and the Chang and DeVries[9] methods. HPLC conditions as described in the Experimental section.

et al. was that final solvents were water or other high boiling point solvents such as toluene, when the final evaporation to dryness may be the most time-consuming step in the procedure (depending upon the efficiency of the vacuum pump used) and also potentially cause losses of aflatoxin M_1. This difficulty is avoided in the method of Ferguson-Foos and Warren where the additional chromatographic stage allows transfer of the extract into a more suitable final solvent for evaporation. An additional partition stage could be added to the Takeda method to transfer the toxin into chloroform[18] but it is doubtful whether this would be cost effective. Four of the methods eliminate direct solvent extraction of the milk, a procedure which can be a source of emulsion difficulties and low recoveries, but the deproteination stages in-

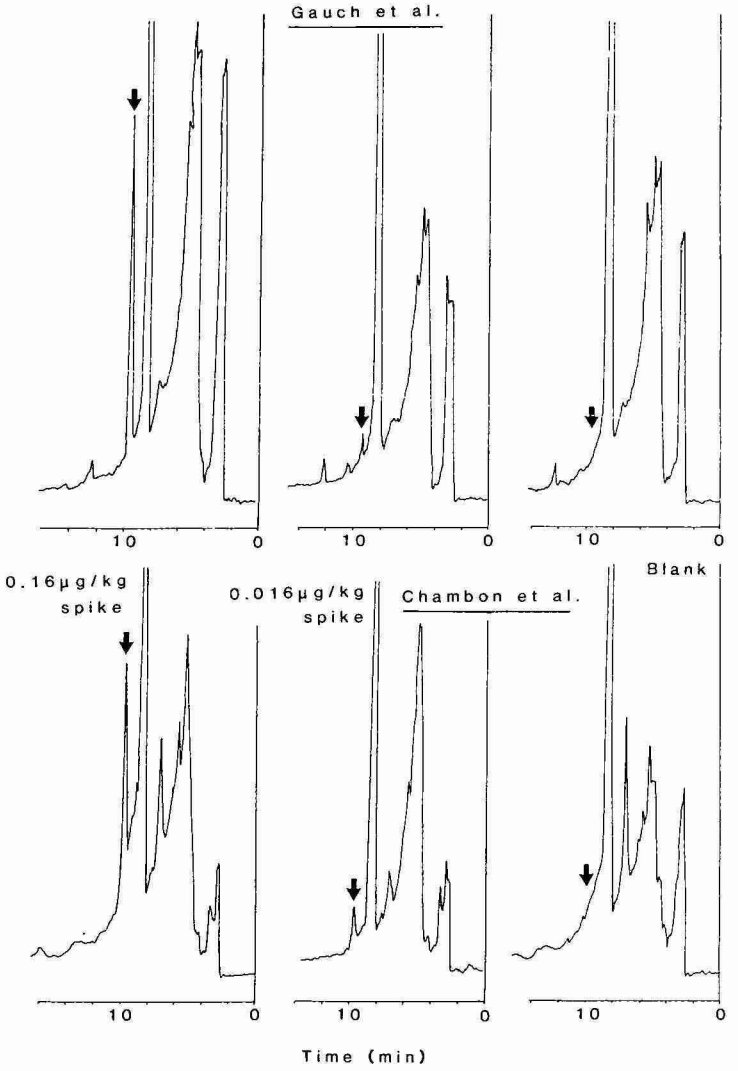

Fig. 2. HPLC fluorescence chromatograms illustrating the clean-up for aflatoxin M_1 (arrows) by the Gauch *et al.*[10] and Chambon *et al.*[11] methods. HPLC conditions as described in the Experimental section.

volved in the Gauch *et al.* and the Chambon *et al.* methods are themselves critical and therefore seem to offer little advantage. In contrast using a prepacked cartridge for the extraction stage results in a much simpler operation, allowing significant time savings over the solvent extraction, and this is a considerably attractive aspect of both the Takeda and the Ferguson-Foos and Warren procedures.

One major constraint upon sensitivity is the quality of fluorescence detector employed. For the LS-4 with a new lamp, 2 pg of M_1 injected on column could be detected at a signal-to-noise ratio of 3:1. However, the critical feature in the assess-

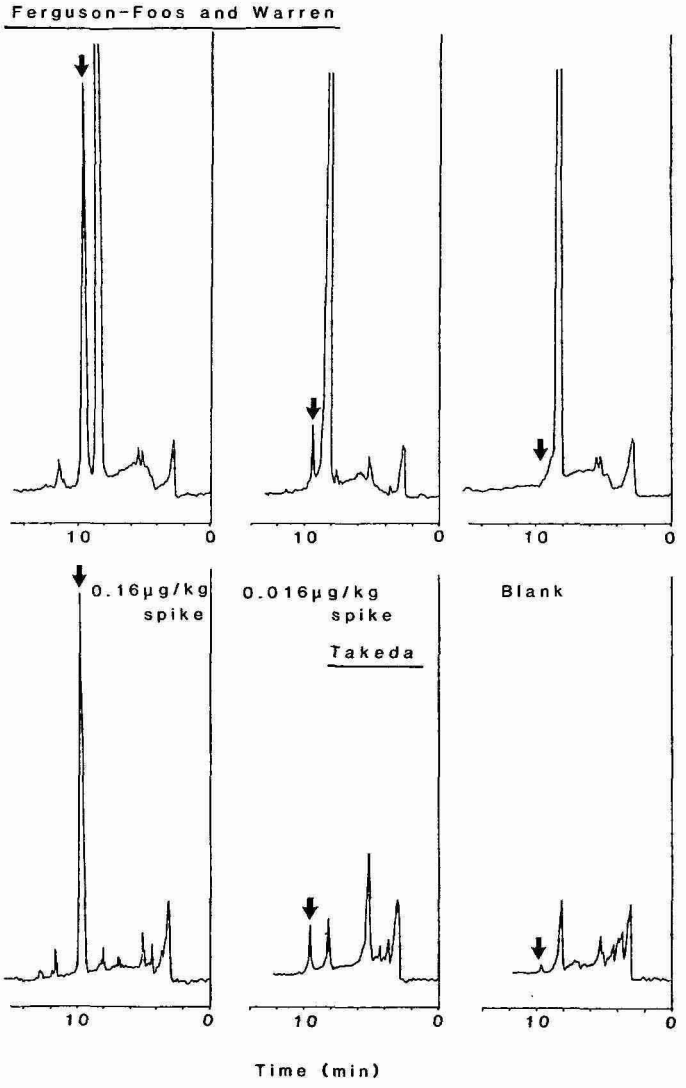

Fig. 3. HPLC fluorescence chromatograms illustrating the clean-up for aflatoxin M_1 (arrows) by the Ferguson-Foos and Warren[12] and the Takeda[13] methods. HPLC conditions as described in the Experimental section.

ment of any method of analysis for aflatoxin M_1 is the cleanliness of the final extract, in terms of background noise components affecting the limit of detection and the likely extent of interferences giving rise to false positives. The detection limits of the six methods (Table II) were broadly similar although, as discussed above, the Gauch *et al.* method gave a slightly higher value due to the lower recovery obtained. The HPLC chromatograms for milk blanks, and for spikes into milk at 0.016 μg/kg and 0.16 μg/kg are shown for each of the six methods in Figs. 1–3. The Chang and DeVries, Gauch *et al.*, and Chambon *et al.* methods in our view give unacceptable background components in the extracts which although not directly interfering with the aflatoxin M_1, do mean that the peak is eluting on the tail of a comparatively large extraneous component. The Ferguson-Foos and Warren, and the Takeda methods both give chromatograms that are cleaner than the Stubblefield method in the initial region of the trace, but the Ferguson-Foos and Warren method does give a rather large additional component eluting immediately before aflatoxin M_1, which might be a source of some difficulty if operating under less than optimum HPLC conditions. Overall the Takeda method gave the cleanest chromatogram, with the blank showing possibly 0.002 μg/l of M_1, a level also observable in the Stubblefield trace, although not confirmed. It is of interest that only the later two methods produced chromatograms where such a low level of contamination would be observable.

We did not employ trifluoroacetic acid (TFA) derivative formation prior to HPLC (unlike Takeda) because the fluorescence detector employed here gives high sensitivity without the factor of three enhancement found for the TFA reaction product. A further consideration is that the derivative elutes much earlier than M_1 from the HPLC column, in an area of the chromatogram where potential interferences are often found. Confirmation of positive results is however best achieved by formation and chromatography of the aflatoxin M_1–TFA product, and it is therefore encouraging that the Takeda method gives good results for both the toxin and its derivatives.

CONCLUSIONS

The evaluation exercise discussed in this paper has clearly demonstrated the benefits of a Sep-Pak procedure for the extraction and clean-up of aflatoxin M_1 from milk, both in terms of time savings and also improved cleanliness of the final extract. In our view the Takeda method was clearly shown to be the preferable method, bearing in mind the constraints of our assessment procedure, primarily that only one particular milk has been examined. In addition, this method should be more amenable to automation than other procedures. It should be noted that the AOAC is currently collaboratively testing the Ferguson-Foos and Warren method[19]. We intend in the future to adopt the Takeda method for the routine analysis of aflatoxin M_1 in milk and milk related products; preliminary results indicate that this method is also suitable for the analysis of milk powders. However, the ultimate test of any method is continued use over a period of time, and only after routine application can our initial assessment finally be confirmed.

ACKNOWLEDGEMENT

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SEPARATION OF AROMATIC HEPTAENE ANTIBIOTICS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Thin-layer chromatography and high-performance liquid chromatography have been used to separate and characterize representatives of the aromatic heptaene group of antifungal antibiotics. Most antibiotics were complex mixtures that could be placed in well defined groups. The identity of candicidin and levorin was confirmed in one group. Aureofungin, DJ400 and hamycin shared many components in common in the second group. The proportion of the individual components of lucknomycin and trichomycin was markedly different from each other and from the antibiotics in the other two groups. Lucknomycin stood out as the most homogeneous of the antibiotics examined.

INTRODUCTION

The aromatic heptaene antibiotics are a group of antifungal antibiotics possessing a large lactone ring containing a hydroxylated portion and a characteristic system of seven conjugated double bonds, an amino sugar (mycosamine or perosamine) and an aromatic entity (*p*-aminoacetophenone or N-methyl-*p*-aminoacetophenone). Most of these antibiotics are available only as complex mixtures[1]. Only two[2,3] of the previous high-performance liquid chromatographic (HPLC) studies[2–5] have attempted a comprehensive comparison of the heptaenes available.

In order to determine the identity of the recently isolated and purified heptaene antibiotic lucknomycin[6], it has been compared with other antibiotics in this group by HPLC and thin-layer chromatography (TLC).

EXPERIMENTAL

Materials

The aromatic heptaene antibiotics examined are listed with their suppliers in Table I. Pre-coated chromatoplates silica gel 60 HPTLC, 100 × 200 mm (Art 5641, E. Merck), acetonitrile (HPLC grade, Rathburn), ammonium pentaborate (Sigma) and analytical-grade solvents and reagents (BDH) were used.

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TABLE I

THE AROMATIC HEPATAENE ANTIBIOTICS EXAMINED

Antibiotic	*Source*
Aureofungin	Hindustan Antibiotics, Pimpri, India
Candicidin	Dumex, Copenhagen, Denmark
Candicidin	S. B. Penick Corp., New Jersey, U.S.A.
DJ400	Schering, Berlin, F.R.G.
Hamycin	Hindustan Antibiotics Ltd., Pimpri, India
Levorin	U.S.S.R. Research Technological Institute for Antibiotics and Enzymes for Medical Use, Leningrad, U.S.S.R.
Lucknomycin	UCB, Brussels, Belgium
Trichomycin	National Institute of Health, Tokyo, Japan

TLC

Plates were sprayed with 0.1 *M* ammonium pentaborate then dried at 140°C for 60 min. The mobile phase consisted of the lower phase of a mixture of chloroform–methanol–dioxan–glacial acetic acid–0.1 *M* ammonium pentaborate (33:38:9:1:19).

The filter-paper-lined chromatography tank was equilibrated with the mobile phase for 24 h at room temperature, then 3 h at 37°C. The antibiotics were dissolved in the lower phase of a mixture of chloroform–methanol–borate buffer (pH 8.3) (4:4:2), 3 mg ml^{-1} except lucknomycin 1.5 mg ml^{-1}, and 8-μl aliquots were applied to the surface of the plate with a micro-syringe. The plates were developed over a distance of 15 cm in a fully saturated tank at 37°C, then they were air-dried, sprayed with anisaldehyde–sulphuric acid reagent (5%, v/v, anisaldehyde and 5%, v/v, sulphuric acid, sp.gr. 1.84, in methanol) and heated 100°C for 5 min.

For biological detection, air-dried plates were covered with a 4 mm thick layer of candicidin assay agar[7] inoculated with a suspension of *Saccharomyces cerevisiae* NCYC 10716 and incubated at 32°C for 18 h. Antifungal activity was revealed as a clear zone of inhibition of growth.

Individual components were located on the plate under ultraviolet light, and the appropriate area of silica gel was scrapped off and extracted with methanol. The resulting suspension was passed through a low-volume filter unit, 0.45 μm APD (Millex HV_4, Millipore) then examined by HPLC.

HPLC

The apparatus consisted of two reciprocating pumps, a gradient controller (Constametric I, Constametric II G, and a gradient master, Model 1601, Laboratory Data Control) and a variable-wavelength spectrophotometer (Model CE272, Cecil Instruments) fitted with a 75-μl flow-through cell. A presaturation column, Spherisorb S10, ODS1 (100 × 4.6 mm I.D.) was placed between the solvent mixing chamber and the injector; a Spherisorb S5, C_8 column (200 × 4.6 mm I.D.) was used for the analytical separation. The mobile phase was filtered through a glass microfibre filter and degassed prior to use. It consisted of acetonitrile, 0.1 *M* sodium dodecyl sulphate and 0.005 *M* ammonium acetate–succinic acid buffer (pH 4.6). The selected gradient elution profile gave an exponential increase (exponent = 2) in acetonitrile concen-

tration from 40 to 46% v/v in 30 min. The flow-rate was 0.5 ml min^{-1}. The antibiotics were dissolved in dimethyl sulphoxide 2.8 mg ml^{-1}, except lucknomycin 1.4 mg ml^{-1}, diluted 1:10 with methanol and injected through a Rheodyne injector (Model 7125) with a 20-μl fixed loop. The eluent was monitored at 380 nm, sensitivity 0.2 a.u.f.s. The relative composition of the samples was determined by normalisation of peak areas measured using a computing integrator (Model SP 4270, Spectra Physics); it was assumed that the heptaene components have the same absorbance at 380 nm.

RESULTS AND DISCUSSION

TLC

TLC revealed the complex nature of the aromatic heptaene antibiotics examined, lucknomycin appeared by far the most homogeneous. The hR_{S_t} values of the major components in the antibiotics are given in Table II, and a typical chromato-

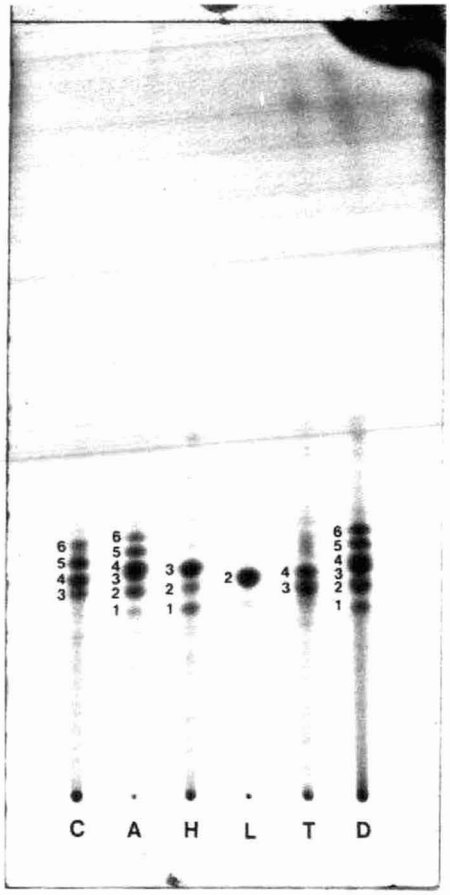

Fig. 1. Thin-layer chromatogram of aromatic heptaene antibiotics detected with anisaldehyde–sulphuric acid reagent. Silica gel 60 HPTLC; lower phase of mixture chloroform–methanol–dioxan–glacial acetic acid–0.1 *M* ammonium pentaborate (33:38:9:1:19). C = Candicidin; A = aureofungin; H = hamycin; L = lucknomycin; T = trichomycin; D = DJ400.

TABLE II

THE hR_{St} VALUES OF THE COMPONENTS OF THE AROMATIC HEPTAENE ANTIBIOTICS

hR_{St} values calculated from thin-layer chromatograms of aromatic heptaene antibiotics. Silica gel 60 HPTLC, lower phase of mixture chloroform–methanol dioxan–glacial acetic acid–0.1 *M* ammonium pentaborate (33:38:9:1:19).

$$hR_{St} = \frac{\text{distance of sample spot from origin}}{\text{distance of reference spot (L2) from origin}} \times 100.$$

Candicidin		*Aureofungin*		*Hamycin*		*Lucknomycin*		*Trichomycin*		*DJ400*	
Ident. No.	hR_{St}	*Ident. No.*	hR_{St}	*Ident. No.*	hR_{St}	*Ident. No.*	hR_{St}	*Ident. No.*	hR_{St}	*Ident. No.*	hR_{St}
										D11	177
										D10	170.5
								T10	167.5		
		A9	165	H6	164.5					D9	163.5
						L4	157	T9	157.5	D8	156
		A8	137								
		A7	129	H5	129					D7	128.5
C7	126							T8	125.5		
		A6	122							D6	121
C6	119.3							T7	118		
		A5	115	H4	117			T6	114.5	D5	115
C5	111					L3	111.8	T5	110.5		
		A4	108							D4	108
C4	103.5	A3	105	H3	106			T4	102.5	D3	103.8
						L2	100				
C3	97.5	A2	97.1	H2	97.5			T3	94.6	D2	95.8
C2	90.4	A1	88	H1	87.6	L1	87.2	T2	88	D1	85.6
								T1	78.4		
C1	74.9										

gram is shown in Fig. 1. The antibiotics reacted to give distinctive colours with the anisaldehyde spray as follows: candicidin and levorin, dark pink; lucknomycin, mauve; aureofungin, DJ400 and hamycin, grey-green; and trichomycin, dark green. Individual components in aureofungin, DJ400 and hamycin were different colours: A1, D1, H1, green; A2, C3, D2, H2, pink; A3, D3, H3, green; A4, D4, pink; A5, D5, mauve; A6, D6, grey. Anisaldehyde–sulphuric acid spray is used to identify carbohydrates separated by TLC[8]. Different colour reactions of the components of aureofungin, DJ400 and hamycin suggest that these components differ by the presence of different sugars attached to the aromatic heptaene moiety, although to date only two different sugars, mycosamine and its isomer perosamine, have been found in the aromatic heptaene antibiotics.

Bioautography showed that all the major components had antifungal activity. Individual components were collected from the silica plate to be extracted and examined by HPLC. Attempts to compare the major components as revealed by TLC using HPLC were abandoned when it was found that some of the components degraded during the extraction process and that the extracted material was extremely unstable. HPLC showed lucknomycin to consist of 70% (peak 13) and 22% (peak 17), examination of the freshly extracted major component (hR_{St} 100) showed 35% (peak 13) and 61% (peak 17), 60 min later the composition of this extract had changed to 23% (peak 13) and 71% (peak 17). The instability of candicidin[3], lucknomycin[6] and DJ400[9] has been reported previously.

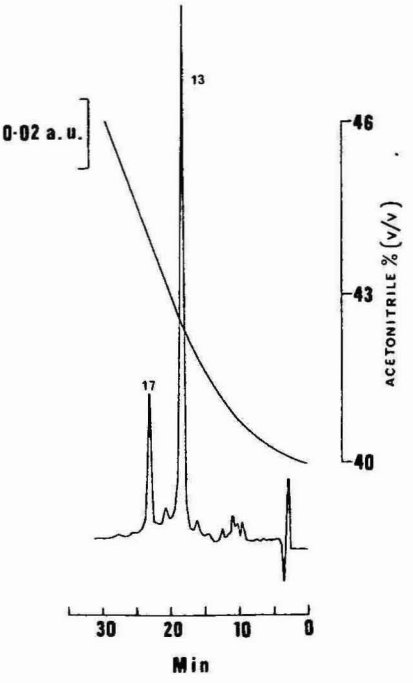

Fig. 2. Chromatogram of lucknomycin, showing gradient profile of increase of acetonitrile concentration in eluent. Peak identification and chromatographic conditions as in Table III.

TABLE III

COMPOSITION OF THE AROMATIC HEPTAENE ANTIBIOTICS

Composition (%) of the aromatic heptaene antibiotics based on the measurement of peak areas of the components separated by HPLC. Major components (> 10%) boxed. Spherisorb C_8 column (200 × 4.6 mm I.D.); eluent, 0.1 *M* sodium dodecyl sulphate, 0.005 *M* ammonium acetate–succinic acid buffer (pH 4.6), acetonitrile (40% v/v increasing exponentially, exponent = 2 to 46% v/v in 30 min); flow-rate, 0.5 ml min^{-1}; measured at 380 nm.

	Peak No.											
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Capacity factor (*k'*)	0.85	1.01	1.31	1.70	1.96	2.08	2.44	2.57	2.82	3.22	3.36	3.73
Candicidin 1	–	0.49	–	–	–	–	–	–	–	–	–	1.00
2*	–	2.01	0.29	–	0.68	–	0.68	–	–	–	0.42	0.92
Levorin	–	1.26	–	–	–	–	–	–	–	–	0.80	–
Aureofungin 1*	0.14	1.00	0.25	–	*14.19*	6.82	*10.58*	–	3.68	–	2.23	9.97
2	1.32		0.77	–	*10.17*	4.21	*12.21*	–	2.44	–	1.40	7.07
DJ 400	–	1.53	0.49	1.53	*21.53*	*11.27*	*16.09*	–	2.01	–	0.33	6.87
Hamycin 1*	0.16	1.08	0.24	3.24	*23.62*	*12.00*	*11.69*	–	*26.96*	–	6.93	2.88
2*	1.77	1.30	0.29	0.64	*43.84*	*19.23*	*14.10*	–	*16.65*	–	2.19	–
Lucknomycin*	–	–	–	–	1.75	2.82	1.45	–	0.13	–	1.19	–
Trichomycin*	0.13	5.61	5.55	4.23	2.19	3.59	–	*30.61*	7.23	*19.58*	9.42	–

* Chromatogram shown.

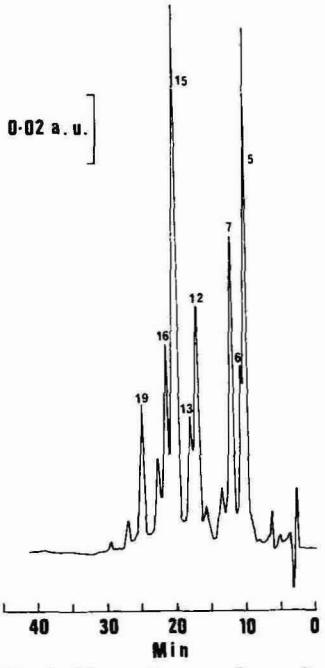

Fig. 3. Chromatogram of aureofungin, sample 1. Peak identification and chromatographic conditions as in Table III.

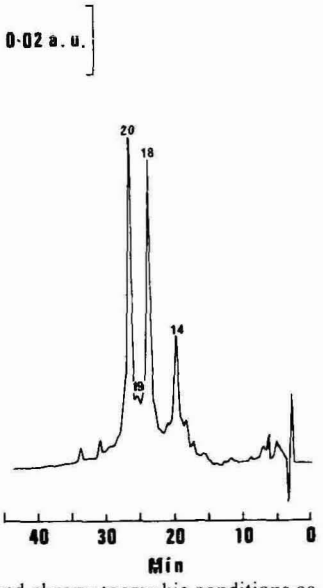

Fig. 4. Chromatogram of candicidin, sample 2. Peak identification and chromatographic conditions as in Table III.

13	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*
3.92	4.33	4.50	4.87	5.06	5.42	5.71	6.05	6.16	6.89	7.14	7.76	9.25	10.36
–	*13.06*	4.08	–	–	*31.89*	7.59	*30.39*	–	1.13	1.58	7.82	–	–
1.43	*13.49*	2.47		–	*34.52*	5.59	*35.82*	–	–	–	–	–	–
–	*23.29*	1.80		–	*41.63*	3.92	*24.28*	.0.14	–	2.23	0.65	–	–
6.38	–	*24.59*	7.55	4.21	–	6.28	–	–	0.88	0.24	–	–	–
5.45	–	*21.33*	*13.15*	5.60	–	*10.59*	–	–	1.78	0.60	–	–	–
4.83	–	*12.54*	*11.25*	4.12	–	5.01	–	–	0.58	–	–	–	–
2.69	–	3.42	1.09	2.19	–	1.70	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
69.88	–	1.12	–	*21.66*	–	–	–	–	–	–	–	–	–
1.99	2.23	2.22	–	2.48	–	–	–	–	–	–	–	1.81	0.83

HPLC

The method developed confirmed the heterogeneous nature of the aromatic heptaene antibiotics and revealed up to 26 different components in the antibiotics examined (Table III). The best separation obtained utilised a gradient elution system as shown in Fig. 2. The peaks have been numbered and the major components marked in the chromatograms to assist in recognising those components that occur in more than one antibiotic. Chromatograms of separations of lucknomycin, aureofungin, candicidin, hamycin and trichomycin are shown in Figs. 2–7. The two samples of candicidin from different sources were very similar to each other and to levorin, confirming earlier reports that candicidin and levorin are identical, differing only in the relative composition[1–3]. Two different samples of the same antibiotic hamycin showed a marked difference in their relative composition: sample 1 contained 23.62% peak 5 and sample 2 contained 43.84% (Figs. 5 and 6).

Aureofungin, DJ400, hamycin, lucknomycin and trichomycin shared a number of minor components. Aureofungin, DJ400 and hamycin also shared a number of major components. Similarity between the HPLC profiles of aureofungin, DJ400, hamycin and trichomycin have been noted before[2]. Detailed examination of the relative composition can differentiate aureofungin and hamycin, DJ400 appeared to be a mixture of the two antibiotic types. The proposition of the individual components of lucknomycin and trichomycin was markedly different from each other and from the other aromatic heptaene antibiotics examined.

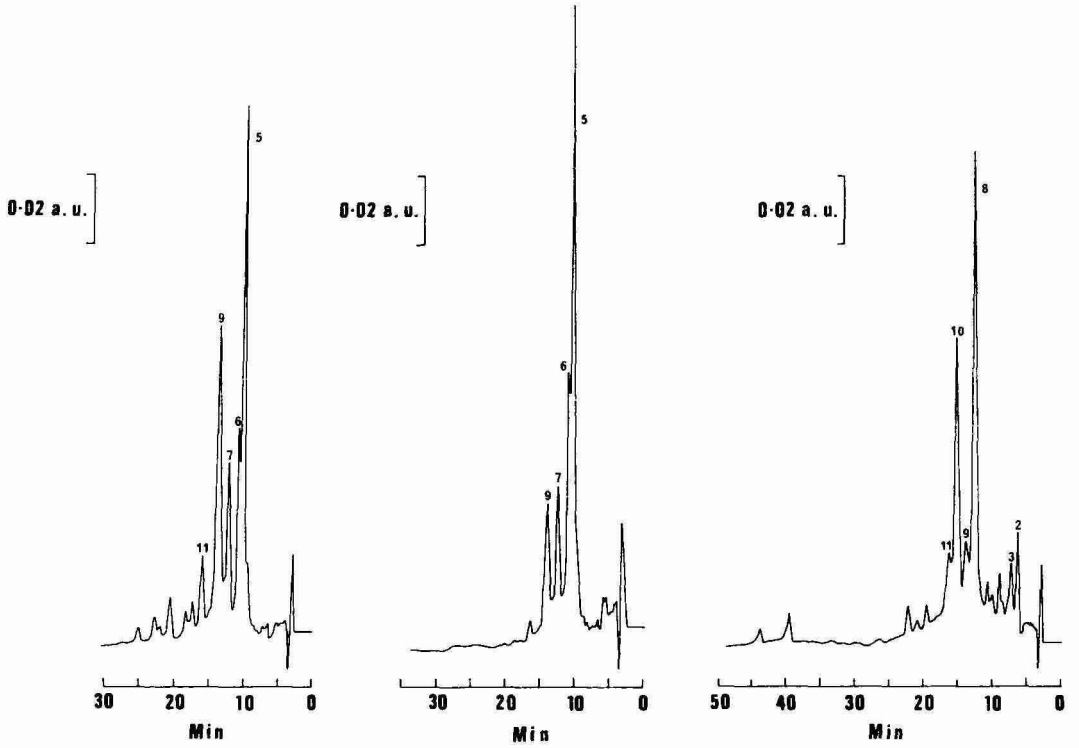

Fig. 5. Chromatogram of hamycin, sample 1. Peak identification and chromatographic conditions as in Table III.

Fig. 6. Chromatogram of hamycin, sample 2. Peak identification and chromatographic conditions as in Table III.

Fig. 7. Chromatogram of trichomycin. Peak identification and chromatographic conditions as in Table III.

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CHROM. 18 255

RESOLUTION OF ALIPHATIC 2,4-DINITROPHENYL-DL-AMINO ACIDS ON A NATIVE CELLULOSE COLUMN*

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SUMMARY

Aliphatic DL-amino acids derivatized with fluorodinitrobenzene (2,4-dinitrophenyl-DL-amino acids) were resolved on a native cellulose (B-27) column. High resolution factors (α) and resolution rates (R_s) were obtained. It is suggested that an increase in the molecular size and an amplification of distortion resulting from the modification of the amino group may play an important role on their complete resolutions.

INTRODUCTION

Our primary concern in this work is to examine the critical mechanism for the resolution of DL-amino acids in a chiral environment. For the purpose of this study, a simplified system without any complicated modification can be recommended. We have already succeeded in resolving non-derivatized DL-amino acids by using native cellulose thin-layer and column chromatography[1–5]. Those previous reports gave evidence that the resolving power depends on the bulkiness of DL-amino acids[4] and the hydrophobicity of the environment[5]. It was also indicated that all the tested aliphatic DL-amino acids were not satisfactorily resolved because of their small side-groups[4].

We have therefore modified the amino group attached to the α-carbon atom with fluorodinitrobenzene to yield more bulky 2,4-dinitrophenyl (DNP)-amino acids. Such a modification should increase the asymmetry of the α-carbon atom, so that the modified racemic amino acids should be recognized more readily by cellulose molecules than the unmodified.

To date, there have been no reports published of the resolution of DNP-DL-amino acids. We describe here the resolution of aliphatic DNP-DL-amino acids by cellulose column chromatography.

* LEB/OU contribution No. 81, Japanese patent pending.

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MATERIALS AND METHODS

Cellulose column

Specially manufactured native cellulose (tentative name: B-27, Japanese patent pending) was kindly provided by Daicel, Osaka, Japan. Another native cellulose (Lot No. 2331, Merck, F.R.G.) was also used in order to compare the resolution capability.

The cellulose was suspended in 1 *N* hydrochloric acid to eliminate some contaminants, and then washed with glass distilled water to remove the acid. The cellulose was then suspended in *n*-butanol containing ethanol (4:1, v/v) and packed into a glass column (60 × 0.85 cm I.D.; bed height, 50 cm). A shorter column (15 × 0.85 cm I.D.; bed height, 10 cm) was also used. The packed cellulose was washed with the same mixture of alcohols (4:1, v/v) in order to eliminate all traces of water, since the absolute amount of water in a column is the limiting factor for resolution.

Reactivation of cellulose column

Repeated use of the cellulose column leads to a decrease in the resolution capability, but it can be reactivated by the following elution procedure: (1) 5 ml of *n*-butanol; (2) 2 ml of 1 *N* hydrochloric acid; (3) 20 ml of water (approximately equivalent to a bed volume); (4) 10 ml of 0.1 *N* sodium hydroxide to neutralize residual hydrochloric acid; (5) 20 ml of water; (6) 5 ml of *n*-butanol; (7) 20 ml of *n*-butanol–ethanol (4:1, v/v) and (8) equilibration with 100 ml of the elution mixture, *n*-butanol–ethanol–water (4:1:0.1, v/v/v). When the column has been reactivated, perfectly resolved patterns are observed on chromatograms, so once the column has been packed it can be used repeatedly.

DNP-amino acids

Amino acids were purchased from Sigma (U.S.A.) and Wako (Osaka, Japan). A 6-mg amount of each DL-amino acid was dissolved in 1 ml of pure water, and 1 ml of 4 mg/ml of sodium bicarbonate was added. To this solution, 1 ml of 1-fluoro-2,4-dinitrobenzene ($5 \cdot 10^{-2}$ *M*) and 3 ml of ethanol were added. After mixing, the solution was warmed in a water-bath (80°C). In order to eliminate by-products that interfere with the chromatograms 35 min after the start, an aliquot (1 ml) of the reaction mixture was taken into a test-tube and completely dried at 60°C under nitrogen. Then 1 ml of 0.5 *N* hydrochloric acid was added to the tube and DNP-DL-amino acid was extracted with 4 ml of ether. The top quarter of ether phase was taken out and dried under nitrogen. This procedure was repeated twice. The dried sample was dissolved into 0.1 ml of 4 mg/ml of sodium bicarbonate, to which 5 ml of *n*-butanol was added. The sample solution thus prepared was used for the present experiment. However, the samples, *e.g.* alanine and α-aminobutyric acid, were not treated in this way.

Column chromatography

The cellulose column prepared by the above method was used for the present experiment. Prior to loading the sample solution, 5 ml of *n*-butanol were passed through the column to eliminate traces of water. Then, 0.05–0.1 ml of a sample solution containing 25–50 μg of DNP-DL-amino acids was eluted with the elution

mixture, *n*-butanol–ethanol–water (4:1:0.1, v/v/v). The eluent (flow-rate, 90 ml/h) was continuously monitored at 360 nm for all chromatograms.

Assignment of resolution of DNP-DL-amino acids

The resolved enantiomers were assigned by co-chromatography of different ratios of DNP-D- and -L-amino acids. For instance, 10 μg of DNP-L- and 30 μg of DNP-D-amino acids were coeluted. For the DNP-DL-amino acids tested, the L-enantiomers were eluted faster than the D-enantiomers. Circular dichroism (CD) measurements of the eluents can also be used for assignment provided a sufficient amount of the resolved enantiomers (more than 50 μg/ml) in available. The CD absorbance (θ value) was no greater than we expected, so we did not use CD measurements in the present study.

RESULTS AND DISCUSSION

Resolution of aliphatic DNP-DL-amino acids

Fig. 1 shows chromatograms of aliphatic DNP-DL-amino acids. The fact that the L-enantiomers were eluted faster than the D-enantiomers in co-chromatography

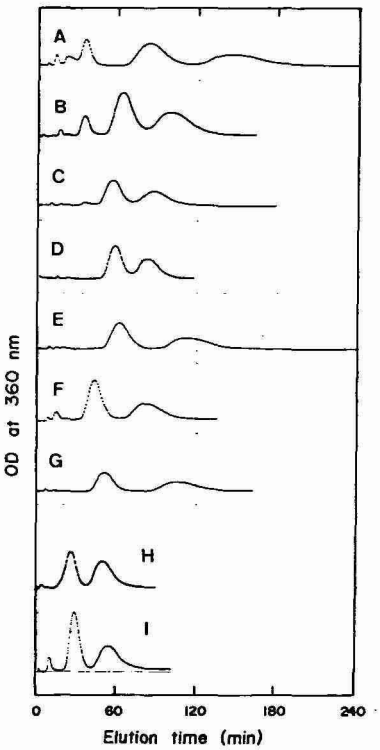

Fig. 1. Chromatograms of aliphatic DNP-DL-amino acids. (A) Ala (alanine); (B) AABA (α-amino-*n*-butyric acid); (C)nVal (normal valine); (D) nLeu (normal leucine); (E) Val (valine); (F) Leu (leucine); (G) Ile (isoleucine); (H) Ile; (I) Leu. Column, 50 × 0.85 cm I.D. (A–H) and 10 × 0.85 cm I.D. (I); applied sample, 25–50 μg; eluent, *n*-butanol–ethanol–water (4:1:0.1, v/v/v); flow-rate, 90 ml/h (A–G), 120 ml/h (H), and 30 ml/h (I). monitored at 360 nm (Hitachi Effluent Monitor, Tokyo, Japan); chart speed, 3 cm/h.

(data not shown) indicates that the latter interact more strongly with the cellulose. DNP-amino acids were not extracted with ether from the acidified sample solution (*cf.* Methods) peak(s) (30–40 min) apparently containing dinitrophenol and dinitroaniline was observed (A and B), and incomplete extraction also results in a small peak (C). However, no peaks of byproduct(s) appear when ether extraction is carried out (D–G).

By using different conditions from that for A–G, we obtained complete resolutions (H and I). Chromatogram I in particular has an extremely high theoretical plate number, because a shorter column was used.

Table I shows the resolution factors (α) and the resolution rates (R_s). Although non-proteinic amino acids gave relatively low values ($\alpha = 1.50$, $R_s = 0.97$), others were satisfactory. The reason why we did not obtain high R_s values seems to be that the column was incompletely filled with cellulose; it is also possible that improved elutions conditions may lead to much higher values.

Content of water in the column and choice of elution mixture

In order to resolve DNP-DL-amino acids on the cellulose column, we had to pay particular attention to the content of water in both the column and the elution mixture, as the limiting factor. First of all, the washed cellulose powder should be dried overnight in an oven at 50°C to eliminate traces of water trapped in the cellulose. When a column is reactivated, extra care is necessary in washing out water from the cellulose. If the cellulose contains excess water, no resolution is observed. It should be mentioned that some non-aliphatic DNP-DL-amino acids (DNP-DL-Trp and -Phe, data not shown) can be resolved even with relatively high water content in the elution mixture, *n*-butanol–ethanol–water (4:1:0.25).

We used *n*-butanol–ethanol as the elution mixture, because water and/or etha-

TABLE I

RESOLUTION OF DNP-DL-AMINO ACIDS

$\alpha = (t_2 - t_0)/(t_1 - t_0)$, where $t_0 = 7$ min (void volume), and t_1 is for the peak of L-enantiomer and t_2 is for that of D-enantiomer. $R_s = 2\Delta t/(w_1 + w_2)$, where w_1 is for the theoretical base of the eluted L-enantiomer and w_2 is for that of D-enantiomer, and $\Delta t = t_2 - t_1$.

DNP-DL-amino acids	*Elution time (min)*		α	R_s
	t_1	t_2		
Ala	83	143	1.77	1.15
AABA	64	100	1.63	0.97
nVal	57	87.5	1.61	1.03
nLeu	58.5	83	1.50	1.11
Val	62.5	112	1.89	1.30
Leu	43	82	2.08	1.25
Ile	51	105	2.23	1.50
Ile*	26	50	2.26	1.16
Leu**	29	54	2.14	1.11

* Fig. 1H; $t_0 = 6$ min.
** Fig. 1I; $t_0 = 7$ min.

nol carries the sample to the void volume, but the sample cannot be eluted only with *n*-butanol. The elution mixture described here provides a relatively hydrophobic environment for cellulose, so that the samples were eluted sufficiently slowly.

Comparison of cellulose B-27 with another cellulose

We investigated another cellulose (Merck), but all the samples were eluted in the void volume and no resolved chromatograms were obtained. This is probably because we may not have completely eliminated the water from the cellulose. Cellulose B-27, on the other hand, seems to be easily dried by the present procedure. It can thus be concluded that the difference between the celluloses might be due to different water-retaining properties.

Evaluation

We have already resolved non-derivatized amino acids on native cellulose[4]. However, those resolutions took a long time (Leu and Ile, 10 h; nVal, nLeu and Val, 12 h; AABA, 15 h; Ala, 22 h) and required a long column (250 × 0.85 cm I.D.). Furthermore the resolutions were unsatisfactory, especially for alipohatic amino acids. We seem to have overcome these problems in the present studies. The results are quite reasonable, since amino acids become much more bulky and distorted after modification. The samples were quickly eluted, and the α-values and R_s values stand comparison with those obtained by high-performance liquid chromatography. Judging by its resolution capability and the modification process, the method reported here should be applicable to every laboratory study.

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CHROM. 18 279

SELECTIVE GAS CHROMATOGRAPHIC ANALYSIS OF CHLORINATED PHENOLIC COMPOUNDS USING THE NITROGEN–PHOSPHORUS DETECTOR

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SUMMARY

A new gas chromatographic method, using a nitrogen–phosphorus detector to selectively analyse chlorinated phenolic compounds, is described and its usefulness evaluated. In this scheme of analysis the chlorinated phenolic substances are converted into their cyanoethyldimethylsilyl ether derivatives by a one-step reaction with 2-cyanoethyldimethyl(diethyl)aminosilane prior to analysis. Results indicate that the ethers have good gas chromatographic properties and can be detected with high sensitivity by the nitrogen–phosphorus detector. The response is linear over a wide range of concentrations and the limits of detection are $(5 \pm 2) \cdot 10^{-13}$ g N. Furthermore, the relative response factors calculated for chlorophenols and chlorinated hydroxybiphenyls, when using this detector, are close to unity. The mass spectrometric behavior of cyanoethyldimethylsilyl ethers is also discussed with reference to structure specificity and detection at low levels by selected-ion monitoring gas chromatography–mass spectrometry.

INTRODUCTION

The determination of chlorinated phenolic compounds at trace levels is of analytical interest because these substances are found in the environment. Chlorophenols are used mostly as pesticides and fungicides and are considered as toxic contaminants[1–4]. Chlorinated hydroxybiphenyls, which are substituted chlorophenols, are of biological interest because they are involved in the metabolism of polychlorinated biphenyls[5]. In recent years, methods of analysis of these compounds have been developed, many of them using gas chromatography (GC)[6–14] and high-performance liquid chromatography (HPLC)[15–18].

In GC, the selective detection of chlorophenols has been done using flame photometric detection (FPD)[13] and electron-capture detection (ECD)[6,7,11,14]. ECD has the advantage of a high sensitivity and selectivity for the chlorinated phenolic substances but problems can be encountered due to contamination and mostly because this detector has a wide range of response factors for different isomers[6–9,11,12,19,20]. The latter situation is inherent to ECD and can create difficulties

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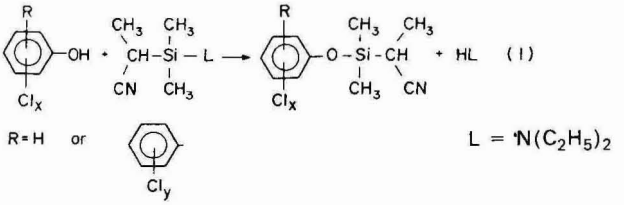
if several isomers of different compounds, present in a wide range of concentrations, are to be analyzed in the same sample.

In order to develop a sensitive method of analysis in which the relative response factors for the chlorinated phenolic substances would be less affected by either the position or the number of chlorine radicals, we have considered the utilisation of another selective detector. The nitrogen–phosphorus detector of the Kolb type[21] offers analytical potential since it has high sensitivity to nitrogen (10^{-13} g N/s), high selectivity (10^4), good stability, a wide dynamic range (10^5) and is fairly resistant to contamination[20]. Chlorinated phenolic compounds are not detected directly by this detector and they must be chemically tagged through derivatization with the proper reagent. Derivatization of the phenolic hydroxy group can be accomplished using a one-step silylating reaction and this approach is depicted in reaction 1.



The formation of cyanoethyldimethylsilyl ethers of the compounds of interest should yield derivatives amenable to selective detection by the nitrogen–phosphorus detector with high sensitivity because the cyano radical is believed to be involved in the mechanism of detection of organic compounds by this detector[24]. Moreover, these ethers should have similar relative response factors when analyzed by this detector since they all contain the same "detection site". To evaluate the usefulness of this new approach, chlorinated phenols and hydroxybiphenyls have been studied and the results are presented.

EXPERIMENTAL

Derivatization

All solvents and chemicals used in this study were purified by standard procedures. Chlorophenols were obtained from Aldrich, except for 2,3,4,6-tetrachlorophenol (Pfaltz & Bauer) and they were purified by sublimation prior to use. Chlorinated hydroxybiphenyls were synthesized in this laboratory[22], except for 3-chloro-2-hydroxy- and 3-chloro-4-hydroxybiphenyls, which were obtained from Aldrich. The silylating reagent, 2-cyanoethyldimethyl(diethyl)aminosilane (CEDMSDEA) was prepared in this laboratory according to a procedure published elsewhere[23], and the silylation reactions were done in ethyl acetate under an inert atmosphere of nitrogen. The Reacti-Vials® where pretreated with dimethyldichlorosilane prior to utilization because it was noted that some chlorophenols interact strongly with glass giving irreproductible results. Unless otherwise specified, the reaction time was 15 min and the reagent 80 times more concentrated than the chlorophenols (*ca.* 1 m*M*). In all cases, an aliquot (1 μl) of the reaction mixture was directly injected into the gas chromatograph without prior separation of reactants from the products.

Instrumentation

GC analyses were made on a Perkin-Elmer (Norwalk, CT, U.S.A.) Sigma 2B gas chromatograph equipped with dual detectors; flame ionization and nitrogen–phosphorus. Unless otherwise specified the column was 15 m × 0.23 mm I.D., and the stationary phase was polydimethylsiloxane (DB-1). The temperature was programmed from 120 to 280°C at a rate of 12°C per min. The injector and the detector were maintained at 300°C and the split ratio on the injector was 32:1. In experiments where the nitrogen–phosphorus detector was used, helium was the make-up gas with a flow-rate of 30 ml/min, and the flow-rates of air and hydrogen at the detector were 100 and 2.6 ml/min, respectively. The nitrogen–phosphorus detector was optimized for maximum selectivity[26], and the bead current was kept below maximum sensitivity in order to increase the lifetime of the bead. The carrier gas was helium with an initial flow-rate of 0.7 ml/min.

GC–mass spectrometry (MS) experiments were conducted on a Kratos MS-50TA mass spectrometer. In all cases the electron energy was 70 eV, the source temperature was 220°C and the transfer lines were kept at 260°C. The GC parameters were similar to those described above, scanning speeds varied depending on the separation and helium was the carrier gas.

RESULTS AND DISCUSSION

The kinetic parameters for the silylation reaction of chlorophenols and chlorinated hydroxybiphenyls by CEDMSDEA, were studied at three temperatures in order to optimize the analytical procedure. In these experiments the nineteen chlorophenol isomers and some chlorinated hydroxybiphenyls (see Table I) were treated with CEDMSDEA in ethyl acetate at temperatures of 25, 55 and 75°C. It was found that the compounds reacted readily with an excess of the reagent (80:1) and that varying the temperature had no significant effect on the overall reaction yield. The yield was calculated by measuring the disappearance of the reactant and for all the above mentioned compounds it was found to be >97.5% within experimental error. Fig. 1 shows the variation of the reaction yield with time at different temperatures for 3,4-dichloro- and 2,4,6-trichlorophenols. It can be seen that the reaction is completed within minutes and that the overall yield does not vary when the temperature is increased from 25°C to 75°C. Because of this, a reaction time of 15 min is considered sufficient to derivatize quantitatively these phenolic compounds and was used in other experiments. The results obtained indicate that CEDMSDEA is a good analytical silylating reagent for these phenolic compounds since the reaction is rapid, quantitative and can be performed under very mild conditions.

The GC behavior of the formed ethers of chlorophenols and chlorinated hydroxybiphenyls was examined and found to be similar to that of the corresponding trimethylsilyl ethers. Fig. 2 shows the chromatogram obtained by analyzing a mixture containing the nineteen isomers of chlorophenol with the nitrogen–phosphorus detector. The peaks corresponding to the compounds of interest (numbered 1–19) have a symmetrical shape and no tailing can be observed. Comparison of the retention times obtained for these ethers with those of the corresponding trimethylsilyl homologues analyzed (with the flame ionization detector) on the same stationary phase, indicates that cyanoethyldimethylsilyl derivatives have retention which are 1.6–2.5

TABLE I

MEAN VALUES FOR RELATIVE RETENTION TIMES AND RELATIVE RESPONSE FACTORS OF THE NITROGEN–PHOSPHORUS DETECTOR FOR CHLORINATED PHENOLIC COMPOUNDS

No.	*Compound*	*Relative retention time*	*Relative response factor*
1	2-Chlorophenol	0.585	0.60
2	3-Chlorophenol	0.605	0.77
3	4-Chlorophenol	0.629	0.86
4	2,3-Dichlorophenol	0.788	0.84
5	2,4-Dichlorophenol	0.764	0.82
6	2,5-Dichlorophenol	0.747	0.88
7	2,6-Dichlorophenol	0.754	0.85
8	3,4-Dichlorophenol	0.812	0.81
9	3,5-Dichlorophenol	0.754	0.98
10	2,3,4-Trichlorophenol	0.980	0.96
11	2,3,5-Trichlorophenol	0.916	0.95
12	2,3,6-Trichlorophenol	0.936	0.78
13	2,4,5-Trichlorophenol	0.930	1.03
14	2,4,6-Trichlorophenol	0.905	0.94
15	3,4,5-Trichlorophenol	0.974	1.00
16	2,3,4,5-Tetrachlorophenol	1.122	1.11
17	2,3,4,6-Tetrachlorophenol	1.100	1.07
18	2,3,5,6-Tetrachlorophenol	1.087	1.04
19	2,3,4,5,6-Pentachlorophenol	1.277	1.17
20	Triphenylamine	1.000	—
21	3-Chloro-2-hydroxybiphenyl	1.153	0.78
22	3-Chloro-4-hydroxybiphenyl	1.335	0.65
23	5,4′,5′,6′-Tetrachloro-4-hydroxybiphenyl	—	0.52

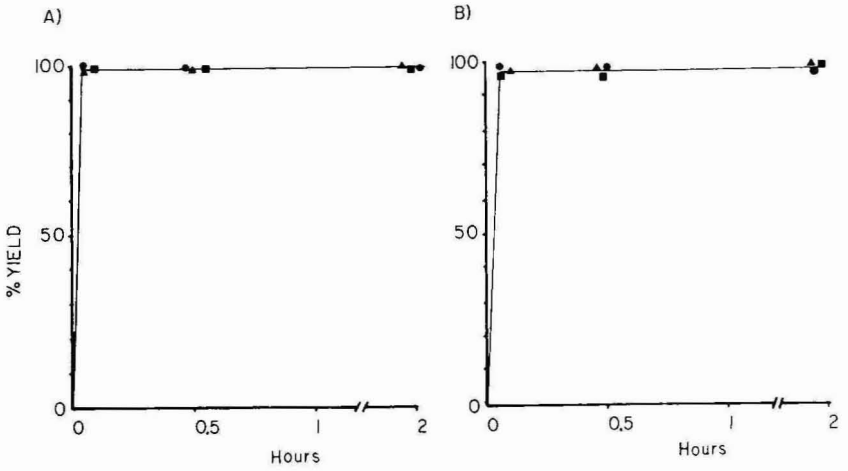


Fig. 1. Kinetic data for the reaction of chlorophenols with 2-cyanoethyldimethyl(diethyl)aminosilane; (A) 3,4-dichlorophenol; (B) 2,4,6-trichlorophenol. Temperatures: ■ = 25°C; ● = 55°C; ▲ = 75°C.

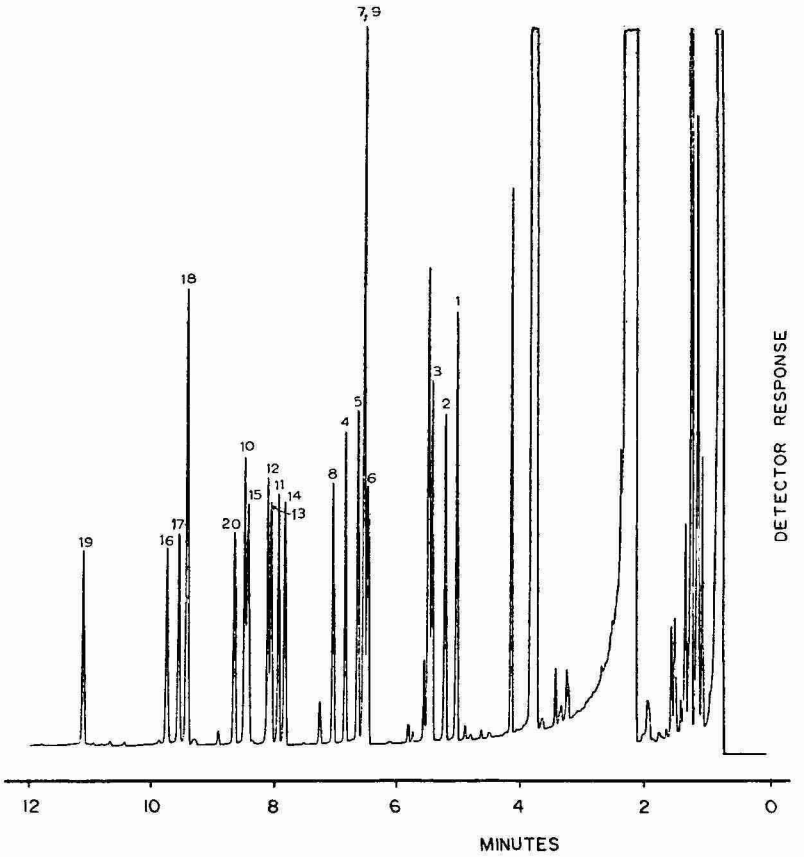


Fig. 2. Chromatogram of a mixture containing the nineteen chlorophenols. Detector; nitrogen–phosphorous column, 15 m DB-1; temperatures, 120°C to 280°C at 12°C min^{-1}. Numbers 1–20 refer to Table I.

times longer. This is to be expected since the cyanoethyl group in the ethers is bulkier than the methyl group in trimethylsilyl and the derivatives are less volatile. One can use this to advantage since it permits the separations of analytes from volatile impurities in the mixture. The chromatographic conditions used to obtain the data shown on Fig. 2 were not optimized for resolution but represent a compromise between resolution and time of analysis. In this example temperature programming at 12°C/min was used and it can be seen that the baseline of the nitrogen–phosphorus detector is not affected thus permitting rapid analysis.

Since one of the objectives of this project was to study the response of the detector to cyanoethyldimethylsilyl derivatives, pertinent analytical parameters involved in the detection of the studied compounds were measured. Parameters that can be used to evaluate the usefulness of the approach are sensitivity, linearity of response over a wide range of concentrations, values of the relative response factors for the isomers and low limits of detection. To calculate relative response factors and verify the linearity of response with concentration, standard calibration curves were plotted for all compounds mentioned in this study. Fig. 3 shows typical response

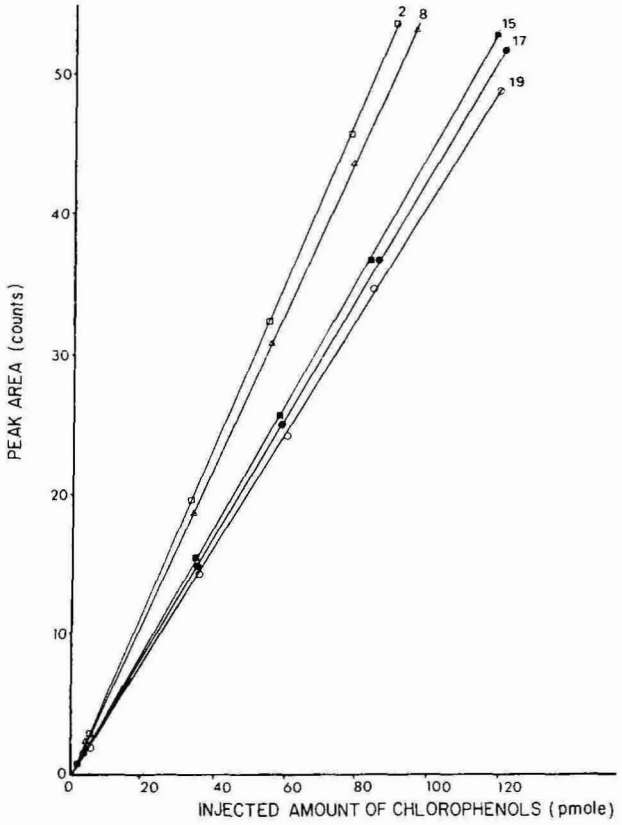


Fig. 3. Concentration response plots for 2-chloro-(2) 3,4-dichloro-(8) 3,4,5-trichloro-(15) 2,3,4,6-tetrachloro-(17) and pentachloro-(19) phenols.

plots obtained for mono-, di-, tri-, tetra- and pentachlorophenols. The relative response factors calculated from the slope (ratios) are listed in Table I. The response of the nitrogen–phosphorus detector to all phenolic compounds studied was found to be linear from 500 ng (upper amount injected) to the limit of detection which was established at $(5 \pm 2) \cdot 10^{-13}$ g N (signal-to-noise ratio = 5) under our experimental conditions. Lower limits can be obtained by increasing the bead current but the detector becomes unstable and unreliable. It is also possible to tune the detector to achieve a better sensitivity but selectivity, which is an important feature in the experimental signal-to-noise ratio, is lowered.

The relative response factors of the chlorophenols are listed in Table I with those of a few hydroxybiphenyls. It can be seen that the values obtained for the various compounds studied are close to unity. Examination of the relative response factors for chlorophenols and chlorinated hydroxybiphenyls reveals that both the position and the number of chlorine atoms on the molecules have little effect on the response of the detector towards the derivatives. The mean value of the relative response factors is 0.88 with minimum and maximum values of 0.60 and 1.17 for chlorophenols. This is to be expected if the formation of the cyano anion is mostly

responsible for the detection of these substances[24], since the nearby environment of the cyano radical is essentially the same in all compounds and it is isolated from the chlorinated organic moiety. The finding that the detection limits is approximately the same in g N for all substances also supports this rationalization. These results demonstrate that the use of the nitrogen–phosphorus detector permits the quantitative analysis of chlorinated phenols in mixtures without the disadvantages caused by the disparities in response factors observed with the electron-capture detector, which is more sensitive to the number and position of the halogens. A comparison between the detection parameters obtained with the nitrogen–phosphorus and flame ionization detectors was also made and is shown on Fig. 4. The sensitivity of the cyanoethyldimethylsilyl ethers measured with the former is 10–12 times larger than the sensitivity of the cyanoethyldimethylsilyl and the trimethylsilyl ethers to the latter, neglecting the selectivity of the nitrogen–phosphorus detector, which has an important influence on the signal-to-noise ratio and on the limits of detection. Under our experimental conditions the selectivity of the detector was *ca.* 10^4 as measured from benzonitrile and decane, but this value can vary according to the matrix from which the analytes are obtained.

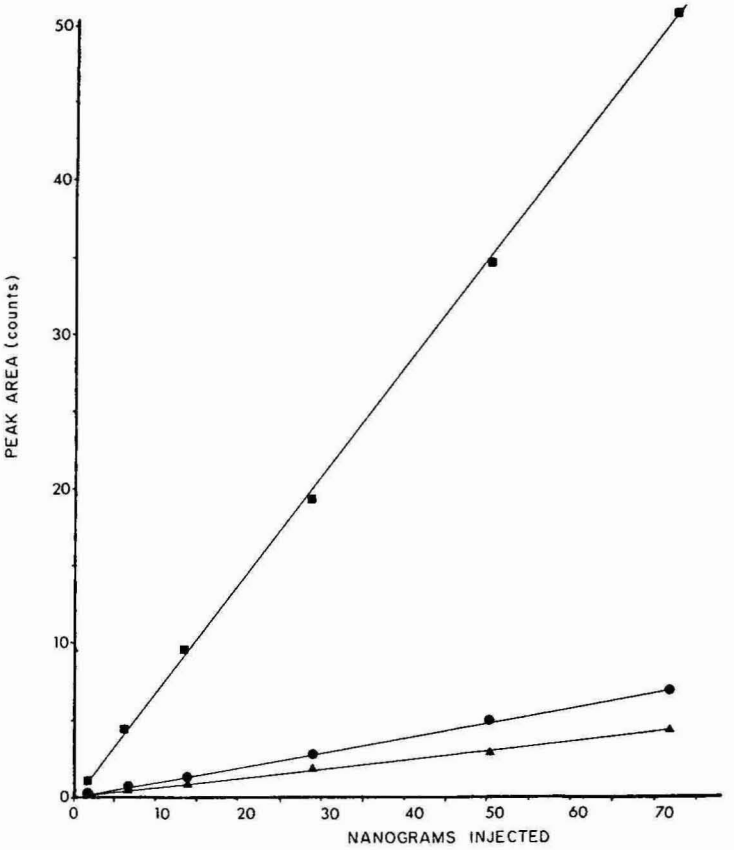


Fig. 4. Concentration response plot for 3,4,5-trichlorophenol derivatives: ■ = cyanoethyldimethylsilyl (NPD); ▲ = cyanoethyldimethylsilyl (FID); ● = trimethylsilyl (FID).

Because MS is often used with GC to confirm the identity or to analyze these compounds at low levels, the behaviour of cyanoethyldimethylsilyl ethers under electron impact ionization was also studied. The mass spectrum of the 3-chloro-4-hydroxybiphenyl derivative is shown in Fig. 5 and typical ions in the mass spectra of the derivatives of phenolic compounds are listed in Table II. The mass spectra of cyanoethyldimethylsilyl ethers are very similar to the trimethylsilyl and *tert.*-butyldimethylsilyl ethers and the differences can be attributed to the presence of the cyanoethyl radical. The molecular ions are always present with variable relative intensities (10–45%) and are seen at odd masses since they contain an odd number of nitrogen. The elimination of the cyanoethyl radical, which is preferred over the methyl, produces important ions observed at (M − 54). These ions, although similar to the (M − 57) encountered in the mass spectra of *tert.*-butyldimethylsilyl ethers, are more specific since a loss of 54 a.m.u. is quite uncommon in the spectra of organic compounds. Whereas the presence of chlorine in the *ortho* position yields an intense rearrangement ion at *m/z* 93 as can be seen on Table II, the (M − 54) ion is the base peak if there is no chlorine in the *ortho* position. This feature is not specific to cyanoethyldimethylsilyl ethers and has previously been reported for trimethylsilyl ethers[25]. The electron impact fragmentation is structure sensitive and the presence of the molecular ion with the intense ion at (M − 54), combined to the ion at *m/z* 93 permits the specific detection of these substances using selected-ion monitoring (SIM). The *m/z* 93 ion can be used as a probe to detect the presence of chlorinated phenolic compounds and the M^+ to confirm the identity of the compound.

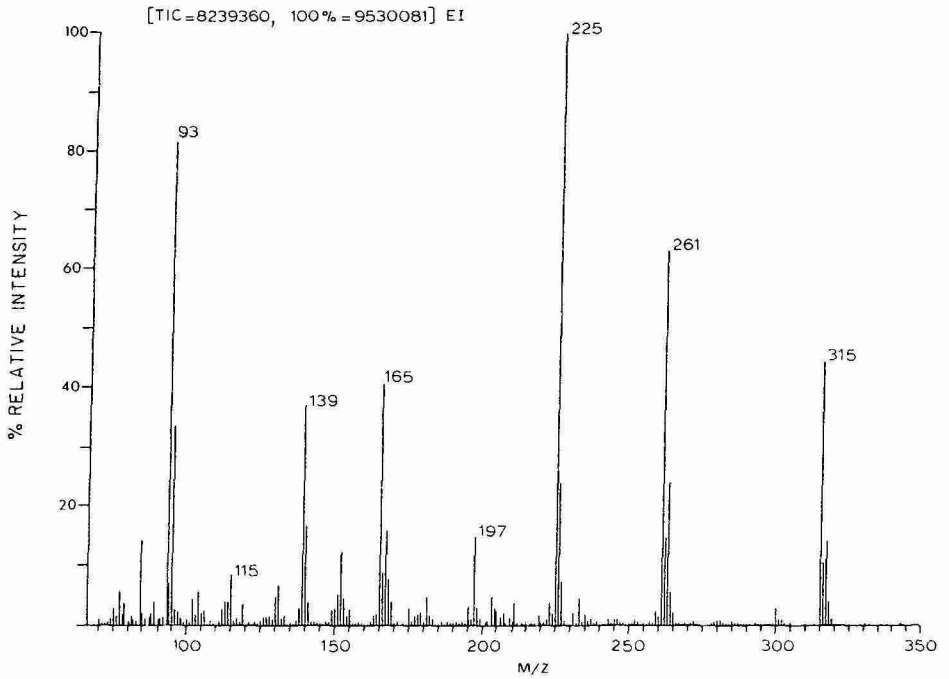


Fig. 5. Mass spectrum of 3-chloro-4-hydroxybiphenyl cyanoethyldimethylsilyl ether.

TABLE II

SPECIFIC IONS IN THE MASS SPECTRA OF CYANOETHYLDIMETHYLSILYL ETHERS OF CHLORINATED PHENOLIC COMPOUNDS

Compound	*Mol. wt.*	*Ion intensity as % of base peak*			
		M^+	$(M - 15)^+$	$(M - 54)^+$	93^+
2-Chlorophenol	239	10	3	90	100
3-Chlorophenol	239	25	2	100	22
4-Chlorophenol	239	20	1	100	18
2,3-Dichlorophenol	273	6	3	77	100
2,4-Dichlorophenol	273	13	2	70	100
2,5-Dichlorophenol	273	9	2	86	100
2,6-Dichlorophenol	273	8	4	100	65
3,4-Dichlorophenol	273	33	2	100	27
3,5-Dichlorophenol	273	23	1	100	20
2,3,4-Trichlorophenol	307	6	2	50	100
2,3,5-Trichlorophenol	307	7	2	45	100
2,3,6-Trichlorophenol	307	7	3	85	100
2,4,5-Trichlorophenol	307	6	2	44	100
2,4,6-Trichlorophenol	307	20	4	83	100
3,4,5-Trichlorophenol	307	20	2	100	19
2,3,4,5-Tetrachlorophenol	341	9	2	40	100
2,3,4,6-Tetrachlorophenol	341	15	2	60	100
2,3,5,6-Tetrachlorophenol	341	7	3	56	100
Pentachlorophenol	375	7	4	100*	92
3-Chloro-2-hydroxybiphenyl	315	10	3	100	93
3-Chloro-4-hydroxybiphenyl	315	44	3	64	84

* For this compound the (M + 2) isotope is the base peak.

CONCLUSION

The results presented in this study indicate that the combination of cyanoethyldimethylsilyl ethers with the nitrogen–phosphorous detector can be useful in the quantitative analysis of chlorinated substances. Use of CEDMSDEA offers a rapid one-step derivatization reaction for these substances under mild conditions and the detector provides a selective and sensitive detection system with a good dynamic range for which the response factors of all phenolic compounds are close to unity. It also offers an alternative to ECD and its operation is not severely affected by contamination from the matrix or from the presence of halogens in the analytes. The cyanoethyldimethyl ethers also provide adequate features for analysis or confirmation by GC–MS since they can be detected by SIM using the M^+, the $(M - 54)^+$ and the m/z 93 ions which are abundant and highly specific to the structure of the chlorinated phenolic compounds.

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CHROM. 18 344

CARACTÉRISATION PAR CHROMATOGRAPHIE SUR COUCHE MINCE DES COMPOSÉS BENZOTHIAZOLIQUES ET DU β-SITOSTÉROL CÉDÉS PAR LES SERINGUES NON RÉUTILISABLES

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(Reçu le 16 juillet 1985; manuscrit modifié reçu le 16 novembre 1985)

SUMMARY

Thin-layer chromatographic characterization of benzothiazole derivatives and β-sitosterol leached by disposable syringes

A thin-layer chromatographic study of 2-mercaptobenzothiazole, 2-(methylthio)benzothiazole, 2-(2-hydroxy-ethylthio)benzothiazole, 2-hydroxybenzothiazole, zinc 2-mercaptobenzothiazolate, mercaptobenzothiazole disulfide, and β-sitosterol with three developing solvents and eleven spray reagents is reported. A simple method is described which highlights the leaching of 2-mercaptobenzothiazole and 2-(2-hydroxy-ethylthio)benzothiazole from the rubber plunger-seals of disposable syringes: contact of rubber with bidistilled water, extraction of this liquid with chloroform, chromatography on silica gel with the solvents previously studied and spraying with N-chloro-2,6-dichloro-*p*-benzoquinone monoimine. The method may also be used to discriminate those syringes sterilized with ethylene oxide, the only ones able to leach 2-(2-hydroxy-ethylthio)benzothiazole, from those sterilized by irradiation. Owing to the β-sitosterol traces leached, it may also be used to investigate whether the plunger-seals are made of natural rubber. The toxic effects of the compounds leached are briefly reported.

INTRODUCTION

Les médicaments injectables sont administrés presqu'exclusivement à l'heure actuelle avec des seringues non réutilisables en matière plastique[1]. Ce matériel d'autre

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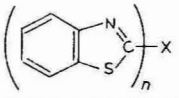
part n'est pas d'un emploi étroitement spécialisé, mais au contraire tout individu a reçu, ou recevra un jour, au moins quelques injections avec de telles seringues. Ces seules considérations suffiraient à expliquer l'intérêt des Pharmacopées et des organismes de normalisation pour le contrôle de la qualité de ces matériels et à justifier les travaux entrepris en ce sens.

La Pharmacopée française[2], notamment, prescrit que "les préparations injectables administrées à l'aide de seringues en matière plastique ne doivent subir au contact de celles-ci aucune modification de leurs propriétés physico-chimiques et toxicologiques". Cependant certains d'entre nous ont montré antérieurement[3] que les têtes de piston en élastomère de seringues de bonne qualité[4] cédaient très rapidement cinq composés de structure benzothiazolique (Tableau I). Le mercapto-2 benzothiazole (I) est un accélérateur de vulcanisation couramment utilisé et il a été établi que les composés III, IV et V prenaient naissance au sein de l'élastomère lors de la stérilisation par l'oxyde d'éthylène[3]. Cette réaction ne se produit cependant qu'à partir du mercapto-2 benzothiazolate de zinc (VI), également très employé, et non pas à partir du mercapto-2 benzothiazole. Mais en fait l'intérêt de cette distinction est surtout théorique, car en raison des réactions qui se produisent au cours de la vulcanisation, l'élastomère renferme finalement de l'oxyde de zinc, du mercapto-2 benzothiazole et du mercapto-2 benzothiazolate de zinc, quel que soit celui de ces deux composés qui a été utilisé[5]. Le mercapto-2 benzothiazole peut aussi se former à partir d'autres accélérateurs tels que le disulfure de benzothiazyle (VII), ou diverses benzothiazylsulfénamides qui libèrent en outre leurs amines respectives.

TABLEAU I

COMPOSÉS CÉDÉS PAR LES SERINGUES (I–V) ET PRODUITS DE RÉFÉRENCE (I–IV et VI–VIII)



No.	*Composé*	*n*	*X*
I	Mercapto-2 benzothiazole	1	–SH
II	Méthylthio-2 benzothiazole	1	$-SCH_3$
III	(Hydroxy-2 éthylthio)-2 benzothiazole	1	$-SCH_2CH_2OH$
IV	Hydroxy-2 benzothiazole	1	–OH
V	(Hydroxy-2 éthoxy)-2 benzothiazole	1	$-OCH_2CH_2OH$
VI	Mercapto-2 benzothiazolate de zinc	2	–SZnS–
VII	Disulfure de benzothiazyle	2	–S–S–
VIII	β-Sitostérol		

Nous n'avions pu identifier les cinq produits cités qu'en recourant à la spectrométrie de masse. Il a paru utile d'élaborer en vue de contrôles de routine une méthode plus simple telle que la chromatographie sur couche mince (CCM). Ultérieurement, il a également paru intéressant de vérifier en même temps si l'élastomère des têtes de piston était à base de caoutchouc naturel, en mettant en évidence le β-sitostérol (VIII), qui n'est présent que dans le polymère d'origine végétale et n'est pas retrouvé dans les élastomères de synthèse[6].

PARTIE EXPÉRIMENTALE

Les essais ont été préparés selon deux méthodes, à partir de seringues stérilisées par l'oxyde d'éthylène (sept marques) ou par les rayonnements ionisants (une marque):

Essai A: une seringue de 5 ml a été maintenue remplie d'eau bidistillée pendant 24 h à la température ambiante, puis le liquide a été extrait trois fois par 0,5 ml de chloroforme, les extraits réunis constituant l'essai A.

Essai B: cent têtes de piston de seringues de 5 ml, séparées des corps de seringues et des tiges des pistons, ont été immergées pendant 24 h à la température ambiante dans 100 ml d'eau bidistillée, le liquide étant ensuite soumis à la même extraction que ci-dessus pour donner l'essai B.

Les solutions A et B ont été déposées sur des couches minces de silice ordinaires (Merck, réf. 5721) et avec zone de concentration (Merck, réf. 11845), éventuellement fluorescentes (Merck, réf. 5729 et 11798). Les dépôts étaient de 2 μl pour les couches ordinaires et de 20 μl ou 100 μl pour celles à zone de concentration.

Les produits de référence suivants ont été retenus: mercapto-2 benzothiazole (I, Tableau I), qualité "pour analyses" (Prolabo, réf. 25.342.181); méthylthio-2 benzothiazole (II), qualité "reagent" (Eastman Kodak, réf. 4008); hydroxy-2 éthylthio)-2 benzothiazole (III), obtenu par synthèse à partir de I et du chloro-2 éthanol[7]. Son identité et/ou sa pureté ont été contrôlées par détermination du point de fusion[7], par spectrométries UV[3], de masse[3] et de résonance magnétique nucléaire du proton; hydroxy-2 benzothiazole (IV), qualité "reagent" (Eastman Kodak, réf. 3993); mercapto-2 benzothiazolate de zinc (VI), qualité industrielle (Vanderbilt, réf. Zetax, agent de vente en France: Polyplastic); disulfure de benzothiazyle (VII), qualité industrielle (Société Landaise de Produits Chimiques, réf. MBTS); β-sitostérol (VIII), qualité "pour laboratoire" (Merck, réf. 3741).

Pour la partie qualitative, les produits de référence étaient généralement en solution à 0,5% (m/v) dans l'éthanol absolu, ou en solution saturée en cas de solubilité insuffisante (composés VI et VII). Les limites de détection ont été déterminées par dilutions progressives. Pour la partie quantitative, le composé III était en solution dans l'éthanol aux concentrations de 2,5, 5, 7,5 et 10 mg pour 100 ml.

Les chromatographies ont été développées sur 15 cm dans les mélanges: (a) benzène–acétate d'éthyle–acétone (100:5:1); (b) benzène–acétate d'éthyle–acétone (100:20:5); (c) dichlorométhane–cyclohexane–éthanol (50:30:20). Plusieurs réactifs ont été utilisés pour la révélation (les concentrations indiquées sont données en m/v):

R_1: Vaporiser une solution aqueuse de tétraborate de sodium à 2,34% et d'hydroxyde de sodium à 0,33%, puis une solution méthanolique de N-chloro dichloro-2,6 *p*-benzoquinone monoimine à 0,1%.

R_2: Vaporiser une solution chloroformique à 0,035% de N-bromosuccinimide et, après séchage, une solution obtenue par mélange de 3 ml de solution de fluorescéine à 0,33% dans la soude 0,1 *N* avec 97 ml d'éthanol.

R_3: Vaporiser un mélange de 3 ml de solution aqueuse d'acide hexachloroplatinique (IV) à 15,3%, 97 ml d'eau et 100 ml de solution aqueuse d'iodure de potassium à 6%.

R_4: Dissoudre 1,5 g de pentacyanonitrosylferrate de sodium dans 5 ml d'acide chlorhydrique 2 *N*, 95 ml de méthanol et 10 ml de solution d'ammoniaque à 25%.

Observer les taches apparues avec ce réactif, puis vaporiser une solution de cyanure de potassium à 2% dans un mélange eau distillée–méthanol (5:95). Porter les plaques à l'étuve à 100°C.

R_5: Vaporiser une solution de nitrate d'ammonium–cérium(IV) à 10% dans l'acide nitrique 0,125 *N*.

R_6: Vaporiser une solution de N-éthylmaléimide à 0,625% dans l'isopropanol absolu, puis après séchage à l'air une solution aqueuse d'hydroxyde de potassium à 1,40%.

R_7: Vaporiser une solution éthanolique de rhodamine B à 0,5%, puis après séchage une solution aqueuse à 10% de carbonate de sodium anhydre. Observer sur plaques fluorescentes à la lumière du jour et sous rayonnement de longueur d'onde 365 nm.

R_8: Vaporiser une solution extemporanée de nitrite de sodium à 0,5% dans l'acide chlorhydrique 1 *N* et porter les plaques à l'étuve à 100°C.

R_9: Vaporiser une solution acétonique de dithizone à 0,025%.

R_{10}: Dissoudre dans 400 ml d'eau 17 g d'acétate de sodium trihydraté, 6 g de tétraborate de sodium décahydraté et à chaud 2 g de thiosemicarbazide. Filtrer après refroidissement. Ajouter 50 ml de solution méthanolique de (thiazolyl-2 azo)-4 résorcinol à 0,08%. Ajuster le pH à 7,5 et diluer à 500 ml (délai de conservation: 4 jours).

R_{11}: Vaporiser une solution d'acétate de cuivre(II) à 3% dans une solution aqueuse d'acide phosphorique à 8% et porter les plaques à 100°C pendant 15 min.

RÉSULTATS ET DISCUSSION

Les Tableaux II et III présentent les résultats obtenus pour les produits de référence et les essais, respectivement. Les R_F sur plaques ordinaires et avec zone de concentration ne sont pas significativement différents. Le signe "–" signifie que le réactif considéré ne révèle pas la tache correspondante. Les changements de couleurs, spontanés ou provoqués par la chaleur, sont indiqués par le signe "→". Le signe "↓" signale les taches qui disparaissent à chaud, le signe "↑" celles qui n'apparaissent qu'à chaud ou les changements de couleurs observés après chauffage. L'adjectif "blanc" indique que les taches ne sont visibles que grâce à la différence de mouillabilité de la silice à leur niveau, par rapport au fond de la plaque.

Aucun solvant de développement, même non rapporté ici, n'a permis de distinguer le mercapto-2 benzothiazole (I) et le mercapto-2 benzothiazolate de zinc (VI). Les séparations parfois décrites avec des solvants très proches de certains de ceux utilisés[8], ou très différents[9], n'ont pas été retrouvées: il paraît probable que dans certains cas au moins ces comportements apparemment différents sont dus au fait que les produits n'ont pas été chromatographiés sur la même plaque. Les autres composés sont convenablement séparés par les solvants proposés, à l'exception cependant des produits IV et VIII dans le solvant (b) et des produits VII et VIII dans le solvant (c).

La N-chloro dichloro-2,6 *p*-benzoquinone monoimine (R_1), proposée pour la révélation du mercapto-2 benzothiazole[10], met en évidence tous les dérivés benzothiazoliques considérés et le β-sitostérol, avec des différences de coloration permettant dans une certaine mesure de les distinguer (Fig. 1). Aucune modification des couleurs

n'a été notée en l'absence de prévaporisation de la solution tampon, préconisée dans le cas des phénols pour élargir l'éventail des couleurs[11,12]. Le réactif R_2 a été décrit pour la révélation de certains insecticides organo-phosphorés soufrés[13,14] et du mercapto-2 benzothiazole[15]. Il met en évidence tous les produits analysés. Réactif des alcaloïdes[16] employé plus récemment pour la localisation de plusieurs adjuvants pour élastomères[8], l'iodoplatinate de potassium (R_3) révèle également tous les produits analysés. Lui seul donne une coloration spécifique avec l'hydroxy-2 benzothiazole (IV), identique à celle que prend la zone de concentration lorsque de telles plaques sont utilisées. Son inconvénient pour des analyses répétées est son coût. La pentacyanonitrosylferrate de sodium (R_4) met en évidence les thiols[17–19]. Le cyanure de potassium coupe les disulfures organiques et les fait réagir à leur tour, ce qui permet de distinguer les composés I et VII. Néanmoins, et bien qu'il révèle tous les produits considérés, ce système de révélation ne présente que peu d'intérêt, car les colorations sont pâles et peu caractéristiques. Egalement décrit pour les thiols[20], le réactif R_5 révèle tous les produits analysés, avec une modeste possibilité de différenciation sur la base des colorations obtenues. La N-éthylmaléimide (R_6) permet en principe de distinguer les thiols des disulfures, qui ne réagissent pas[19]. Néanmoins, le produit VII a été révélé. Mais seule la différence de mouillabilité au niveau des taches permet de localiser les composés II et III, substitués sur le soufre, et le composé IV, non soufré en position 2. Ce réactif est par ailleurs lacrymogène. Employée comme réactif de certains lipides[19,21,22], la rhodamine B (R_7) a été retenue pour la mise en évidence de composés hétérocycliques sur plaques fluorescentes[23]. Elle révèle de façon identique tous les composés analysés. Le nitrite de sodium (R_8), proposé pour les dérivés indoliques[24] et thiazoliques[25] ne présente pas d'autre intérêt que de localiser les taches par leur différence de mouillabilité. La tache du composé I ne reste jaune qu'une ou deux secondes et le composé VII n'est pas mis en évidence. D'abord retenue à cause de la présence de zinc dans le produit VI[26], la dithizone (R_9) permet en fait de révéler tous les produits considérés. La tache rose de R_F 0 sur plaques ordinaires, identique à celle observée avec un sel minéral de zinc, reste sur la ligne de dépôt dans le cas des plaques à zone de concentration, sans aucune migration vers la zone de chromatographie. Elle est cependant sans intérêt pour différencier les composés I et VI dans les essais en raison des réactions qui se produisent au cours de la vulcanisation. Le réactif R_{10}, proposé pour le dosage du zinc[27], n'a été essayé que pour tenter de distinguer ces deux composés, mais la même conclusion qu'avec le réactif précédent s'impose. L'acétate de cuivre en solution phosphorique (R_{11}), utilisé pour la révélation du β-sitostérol dans les extraits organiques de caoutchouc naturel[6], donne aussi des colorations avec les composés I, VI et VII et inversement la plupart des autres réactifs testés réagissent avec le β-sitostérol. Enfin, avec des dépôts de 2 μl sur couches ordinaires fluorescentes, tous les composés benzothiazoliques présentent au moins une tache sombre sous rayonnement de 254 nm. Toutefois, dans le cas du composé VII, c'est la tache du composé I, présent en tant qu'impureté, qui apparaît ainsi. Des taches secondaires sont également visibles dans ces conditions pour le β-sitostérol, mais non la tache principale correspondant aux R_F indiqués. Avec des dépôts de 20 μl sur plaques fluorescentes à zone de concentration, apparaissent en outre à 254 nm la deuxième tache du composé I et la troisième du composé VII, et à 365 nm les premières taches des composés I et VII et celle du composé VI. Ces constatations sont en accord avec les caractéristiques d'absorption des divers produits que nous avons établies ailleurs[3].

TABLEAU II

PRODUITS DE RÉFÉRENCE

Plaques ordinaires, fluorescentes pour l'observation sous UV, dépôts 2 μl.

Composé	$R_F \times 100$ *dans les solvants*			*Couleurs obtenues avec les différents réactifs (R)*				
	a	*b*	*c*	R_1	R_2	R_3	R_4	R_5
I	26	45	67	Orangé	Vert → jaune	Jaune	Bleu → mauve ↑	Blanc → mauve → jaune
	50	60	78	Jaune → brun ↑	–	Jaune	mauve ↑	–
II	51	57	71	Jaune pâle ↓	Rose	Jaune pâle	Jaune sale	Blanc → jaune
III	11	24	62	Jaune pâle	Rose	Jaune pâle	Jaune pâle	Mauve → jaune halo mauve
IV	15	29	63	Beige	Vert → jaune	Bleu argenté ↑	Beige	Brun
VI	0	0	0	–	–	–	–	–
	26	45	67	Orangé	Vert → jaune	Jaune pâle	Bleu violacé → mauve ↑	Blanc
VII	26	45	67	Orangé	Vert → jaune	Jaune	Bleu → mauve ↑	Blanc → mauve
	32		72	Jaune → gris	Vert → jaune	Jaune*	–	Blanc → mauve
	50	60	78	Jaune	Rose	Jaune	Mauve ↑	Blanc → mauve
VIII	12	29	67	Bleu → blanc	Vert → jaune	Jaune	Blanc → gris vert	Jaune → mauve
Fond des plaques				Blanc	Rose pâle	Brique	Gris-vert	Jaune

* N'apparaît pas dans le solvant c.

Quoiqu'il s'agisse d'un produit de qualité "pour analyses", le mercapto-2 benzothiazole (I) utilisé contient du disulfure de benzothiazyle (VII), mais en proportion trop faible pour que certains des réactifs le mettent en évidence au sein du produit I (R_2 par exemple). Inversement, le disulfure de benzothiazyle est souillé de mercapto-2 benzothiazole, qui lui, est décelé par la plupart des réactifs. La nature d'une troisième tache de R_F intermédiaire n'a pas été élucidée, mais la présence d'impuretés est ici compréhensible, le produit n'étant destiné qu'à l'industrie. Enfin, tous les composés de référence montrent avec la dithizone (R_9) une légère tache rose, mais uniquement avec des dépôts de 20 μl ou 100 μl sur plaques à zone de concentration: elle traduit la présence de faibles quantités de zinc.

						UV	
R_6	R_7	R_8	R_9	R_{10}	R_{11}	*254 nm*	*365 nm*
Mauve	Rose violacé foncé	Jaune → blanc	Turquoise → violet	Crème	Vert → jaune ↑	+	–
–	–	–	jaune	–	–	–	–
Blanc	Rose violacé foncé	Blanc	Turquoise → violet		Blanc	+	–
Blanc	Rose violacé foncé	Blanc	Turquoise → jaune		Blanc	+	–
Blanc	Rose violacé foncé	Blanc	Turquoise → violet		Blanc	+	–
–	–	–	Rose	Rose	–	+	+
Mauve pâle	Rose violacé foncé	Blanc	Turquoise halo jaune → brun	Crème ↓	Jaune	+	–
Violet	Rose violacé	–	Turquoise		Vert → jaune ↑	+	–
Violet*	Rose violacé*	–	Blanc		–	–	–
Jaune pâle	Rose violacé	–	Jaune → blanc		–	–	–
Blanc	Rose violacé foncé	Blanc	Turquoise → jaune		Brun ↑	–	–
Blanc	Rose Violacé pâle	Blanc	Vert → gris violacé	Blanc	Blanc		

Les composés II, IV, VI et VII n'ont été mis en évidence dans les essais dans aucune des conditions opératoires retenues, sur aucune marque de seringues et quel qu'ait été le mode de stérilisation. Les dérivés I, III et VIII ont par contre été retrouvés dans plusieurs cas (Tableau III). Ainsi, pour toutes les marques de seringues stérilisées par l'oxyde d'éthylène, le composé III a été caractérisé dans les extraits préparés à partir d'une seule unité (essai A), mais uniquement avec un dépôt de 20 μl sur plaque à zone de concentration. Dans les extraits obtenus à partir de 100 têtes de piston (essai B), le composé III a été décelé même avec un dépôt de 2 μl sur plaque ordinaire. Sur couche à zone de concentration, un dépôt de 20 μl a en outre mis en évidence le composé I, mais la tache, petite, n'était révélée que par le réactif R_1 et disparaissait

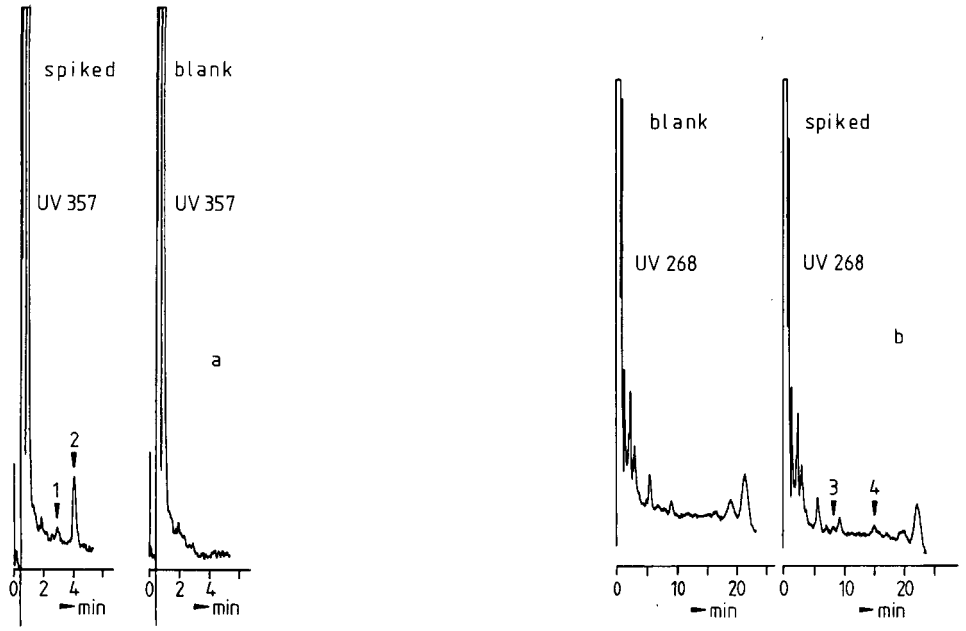


Fig. 2. Chromatograms of a deproteinized plasma sample, spiked with tetracycline, and its degradation products. Conditions: column PRP-1 (10 μm); eluent: (a) acetonitrile–dichloromethane–0.2 *M* acetate buffer (pH 3.6) + 0.025 *M* EDTA (6:1:94, v/v); (b) acetonitrile–dichloromethane–0.2 *M* acetate buffer (pH 3.6) + 0.025 *M* EDTA (10:1:90, v/v). Peaks: 1 = ETC; 2 = TC; 3 = EATC; 4 = ATC. TC peak corresponds to 172 ng/ml, EATC peak to 97 ng/ml and ATC peak to 363 ng/ml.

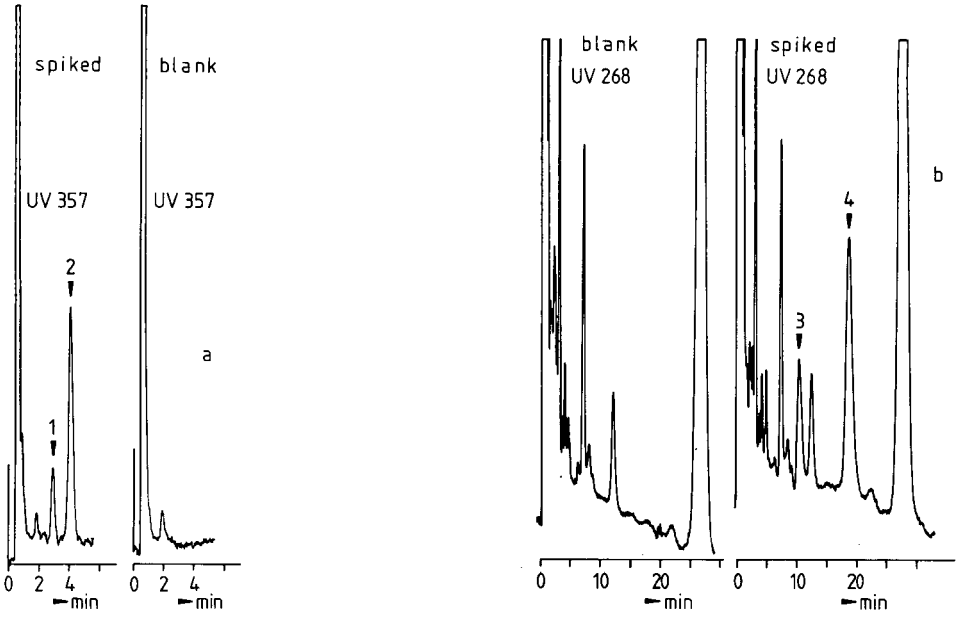


Fig. 3. Chromatogram of an extract of a plasma sample, spiked with tetracycline, and its degradation products. For conditions see Fig. 2.

			UV		*Conclusion*
			254 nm	*365 nm*	
R_7	R_9	R_{11}			
Rose violacé	Turquoise	–	+	–	Composé III
Rose violacé	Turquoise	–	+	–	Composé III
Rose violacé	Turquoise	Blanc	+	–	Composé III
–	–	–	+	+	Composé I
–	–	Brun ↑	–	–	β-Sitostérol
Rose violacé	–	Vert → jaune ↑	+	+	Composé I
–	–	Brun ↑	–	–	β-Sitostérol

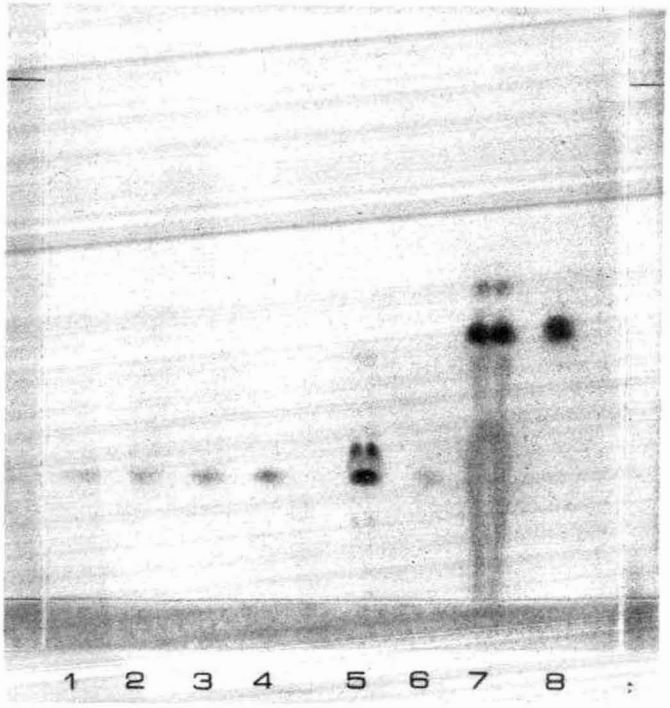


Fig. 2. Chromatographie des solutions essais et estimation de la migration du composé III. Plaque à zone de concentration; dépôts: 100 µl (sauf composé I: 2 µl); solvant (b); révélation par le réactif R_1. 1, 2, 3 et 4 = composé III, en solution à 2,5, 5, 7,5 et 10 mg pour 100 ml, respectivement; 5 et 6 = seringues stérilisées par l'oxyde d'éthylène, essais B et A, respectivement; 7 = seringues stérilisées par irradiation, essai B; 8 = composé I en solution à 0,5% (m/v).

rapidement. Un dépôt de 100 μl a été nécessaire pour observer le composé VIII. Dans le cas des seringues stérilisées par irradiation, l'emploi d'une seule unité n'a mis en évidence aucune migration, malgré le recours à des plaques à zone de concentration et l'augmentation des volumes déposés. Pour les extraits préparés avec 100 têtes en revanche, un dépôt de 20 μl sur ce même type de plaques a montré la présence du composé I: la tache obtenue avec le réactif R_1 était ici plus grande et pouvait être révélée par d'autres réactifs sans intérêt dans le cas précédent. A nouveau, un dépôt de 100 μl a seul permis de caractériser le composé VIII.

Pour un essai donné (A ou B) et des conditions chromatographiques fixées, les différents procédés de révélation n'ont pas tous mis en évidence les mêmes produits: ce sont le réactif R_1 et l'observation à 254 nm qui ont permis le plus grand nombre de caractérisations. Les limites de détection avec le réactif R_1 ont été trouvées égales à 0,1 μg pour le composé I, 5 μg pour les composés III et IV, et 10 μg pour le composé II. En rapprochant ces valeurs des résultats du Tableau III, on voit que les seringues stérilisées par l'oxyde d'éthylène cèdent essentiellement le composé III et à un bien moindre degré seulement le composé I, puisque celui-ci n'a pu être caractérisé qu'avec un dépôt plus important alors qu'il est plus facilement décelable. Cette conclusion, logique puisqu'une grande part du composé I s'est transformée en composé III, est d'autre part en accord avec les résultats obtenus en spectrométrie UV[3], les spectres observés étant superposables à celui du composé III (maximum à 280 nm) et très différents de celui du composé I (maximum à 320 nm). Au contraire, les seringues stérilisées par irradiation ne peuvent céder que le seul composé I puisqu'il n'a pas été transformé et celui-ci, présent en plus grande quantité que dans le cas précédent, migre de façon plus importante. Il est certain que les solubilités respectives des deux produits dans l'eau interviennent aussi, en plus de leur taux dans l'élastomère, dans le niveau de concentration atteint.

Ces conclusions sont illustrées par la Fig. 2, dans laquelle tous les dépôts ont été de 100 μl pour une meilleure visibilité, sauf celui du composé I, plus facile à déceler. Dans le cas de seringues stérilisées par l'oxyde d'éthylène, la tache du composé III dans l'essai A a une intensité comprise entre celles des solutions à 2,5 et 5 mg pour 100 ml, et très proche de cette dernière. En tenant compte de la concentration due à l'extraction chloroformique, cela signifie qu'une seringue de 5 ml cède à son contenu, en 24 h et à la température ambiante, près de 75 μg du composé III et que la concentration atteinte est donc voisine de 1,5 mg pour 100 ml. Ce résultat est très proche de celui déterminé antérieurement par spectrophotométrie, qui était de 1,34 mg pour 100 ml[28]. La migration du composé I à partir des seringues stérilisées par l'oxyde d'éthylène ou par irradiation n'a pas été estimée, car elle n'est visible que dans l'essai B, dont les modalités de préparation sont éloignées des conditions habituelles d'emploi des seringues.

Les résultats présentés permettent de proposer pour le contrôle rapide du relargage des seringues une méthode simple. L'essai sera préparé selon les modalités A dans le cas (le plus fréquent) de la stérilisation par l'oxyde d'éthylène, ou selon les modalités B en cas d'irradiation, et 20 μl de la solution essai seront déposés sur une plaque à zone de concentration. On emploiera les solvants (a) et (c), ou (b) et (c), et on localisera les taches par le réactif R_1 ou par observation à 254 nm si la plaque est fluorescente. Le composé III devant être synthétisé, il est à la rigueur possible pour des contrôles de routine de ne déposer que le composé I, et d'attribuer au composé

III la tache éventuellement observée qui aurait un R_F voisin de celui indiqué pour ce produit dans les mêmes conditions opératoires (Tableau II): la comparaison des rapports des R_F du composé I et de la tache considérée avec ceux observés ici permettrait tout de même d'affirmer la présence de l'(hydroxy-2 éthylthio)-2 benzothiazole. Si l'on souhaite vérifier l'origine de l'élastomère, on déposera sur une plaque à zone de concentration 100 μl d'essai B et de solution de β-sitostérol et on utilisera le réactif R_{11}. Cela ne nécessite pas de chromatographie supplémentaire, car il est possible de vaporiser le réactif R_{11} sur la plaque déjà révélée par le réactif R_1. La méthode peut être éventuellement utilisée pour déterminer le mode de stérilisation s'il est ignoré. Dans le cas d'un dépôt de 20 μl de l'essai B sur plaque à zone de concentration, la mise en évidence du composé I permet d'affirmer l'emploi d'un système de vulcanisation mercaptobenzothiazolique et la migration du mercapto-2 benzothiazole au moins. Selon qu'on observe ou non une seconde tache correspondant au composé III, les seringues ont été stérilisées par l'oxyde d'éthylène ou par irradiation.

Le protocole proposé peut paraître critiquable en ce qui concerne le contact prolongé de l'eau avec les têtes de piston et la concentration que réalise l'extraction par un faible volume de chloroforme: on peut en effet penser que ces modalités ne reflètent pas les conditions habituelles d'utilisation des seringues. Mais le contrôle d'un objet ou d'un matériau se fait toujours dans des conditions plus draconiennes que celles de son emploi, de façon à préserver une marge de sécurité, et d'ailleurs dans la réalité, l'emploi de telles seringues est loin d'être extemporané. En milieu hospitalier, les injections sont trop souvent préparées à l'avance[29]. Les diabétiques qui se soignent eux-mêmes à domicile placent fréquemment dès le matin au réfrigérateur toutes les seringues pleines d'insuline nécessaires pour la journée: une récente étude qui a montré l'absence de contamination bactérienne des seringues réutilisées pendant 7 jours n'a pu que les conforter dans cette habitude[30]. Enfin dans les pousse-seringues et pompes portables actuellement de plus en plus utilisés, les temps de contact sont de plusieurs heures ou de plusieurs jours. Or, nous avons montré que la migration de l'(hydroxy-2 éthylthio)-2 benzothiazole commence dès les premières secondes et ne fait que croître ensuite[28].

Comme déjà dit, aucune migration dans les médicaments n'est admise actuellement. Cette prescription cependant pourra éventuellement apparaître trop stricte et il n'est pas absolument exclu que l'on en vienne à tolérer une migration "acceptable" de produits bien identifiés et reconnus atoxiques. En ajustant les conditions opératoires (nombre de têtes de piston, temps et température de contact, volumes d'eau et de solvant extracteur, volume déposé, nature des plaques) de façon à obtenir des résultats négatifs pour les relargages inférieurs à ceux qui seraient admis, la méthode constituerait un essai limite analogue à ceux fréquemment prescrits par les Pharmacopées. Avec le réactif R_1, elle permettrait d'évaluer l'importance de la migration des composés benzothiazoliques. Avec d'autres réactifs ou des procédés de révélation non spécifiques (observation sous UV, vapeurs d'iode), elle pourrait être étendue à d'autres constituants des élastomères susceptibles de migrer.

Si la migration de traces de β-sitostérol reste probablement sans incidence toxicologique, celle des dérivés benzothiazoliques est plus préoccupante. En effet, bien que le mercapto-2 benzothiazole (I) soit autorisé, parfois d'ailleurs avec des restrictions, par la Food and Drug Administration et certains autres organismes dans les élastomères venant au contact des aliments[31], il faut se souvenir que les effets

d'un produit peuvent être très différents suivant la voie d'administration. Le mercapto-2 benzothiazole a ainsi été rendu responsable de réactions dermatologiques[32–34] et on a pu lui attribuer avec assez de vraisemblance des effets embryotoxiques et mutagènes chez l'animal[35,36]. Des effets du même ordre et des modifications de l'activité des transférases et des oxydases ont aussi été rapportés à propos du dimère qu'est le disulfure de benzothiazyle (VII) en cas d'inhalation[37]. L'(hydroxy-2 éthylthio)-2 benzothiazole (III) n'est pas utilisé dans l'industrie et la littérature toxicologique à son sujet est donc à peu près inexistante. Cependant, une étude chez la souris[38] a conclu que sa toxicité lors d'une seule administration était plus importante que celle du mercapto-2 benzothiazole, celui-ci étant au contraire plus néfaste en cas d'administrations répétées. Les effets les plus nets dans le cas d'une dose importante des deux composés étaient une stimulation du système nerveux central, une vasodilatation périphérique et une sialorrhée. En cas d'intoxication subaiguë, le mercapto-2 benzothiazole avait des effets hépatiques marqués, qu'on pouvait également observer à un moindre degré avec l'(hydroxy-2 éthylthio)-2 benzothiazole. Dans les études de toxicité aiguë, les deux composés se montraient plus toxiques par voie parentérale que par voie orale. Enfin, peut-être faut-il attribuer à l'(hydroxy-2 éthylthio)-2 benzothiazole, étant donné le nombre des marques dans lesquelles nous l'avons trouvé, les inhibitions de cultures de liquide amniotique constatées lorsque les amniocentèses étaient faites au moyen de seringues à usage unique[39,40].

CONCLUSION

La mise au point de la méthode proposée nous a amenés à étudier le comportement chromatographique de différents composés benzothiazoliques dans diverses conditions. En dépit de sa simplicité, le protocole présenté permet en une seule opération de contrôler la qualité des seringues du point de vue de la migration de leurs composants, de connaître leur mode de stérilisation et de préciser l'origine de leur élastomère. Il est utilisable aussi bien par le pharmacien hospitalier pour le choix d'une marque que par le fabricant de seringues pour le contrôle des têtes de piston qui lui sont livrées par l'industrie du caoutchouc. Il pourrait être utilisé comme méthode d'essai limite dans l'hypothèse où un taux acceptable de migration serait toléré. D'ores et déjà, il a permis de montrer la présence de mercapto-2 benzothiazole dans des médicaments présentés en seringues préremplies. Les données toxicologiques brièvement rapportées permettent de considérer que les résultats obtenus ne sont pas sans intérêt pour la sauvegarde de la santé publique.

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RÉSUMÉ

Le mercapto-2 benzothiazole, le méthylthio-2 benzothiazole, l'(hydroxy-2 éthylthio)-2 benzothiazole, l'hydroxy-2 benzothiazole, le mercapto-2 benzothiazolate de zinc, le disulfure de benzothiazyle et le β-sitostérol ont été étudiés en CCM avec

trois solvants de développement et onze réactifs de révélation. Une méthode simple est proposée pour la mise en évidence de la migration du mercapto-2 benzothiazole et de l'(hydroxy-2 éthylthio)-2 benzothiazole à partir des têtes de piston en élastomère des seringues à usage unique: contact de l'élastomère avec de l'eau bidistillée, extraction du liquide par le chloroforme, chromatographie sur gel de silice avec les solvants précédemment étudiés et révélation par la N-chloro dichloro-2,6 *p*-benzoquinone monoimine. La méthode permet aussi de distinguer les seringues stérilisées par l'oxyde d'éthylène, qui seules cèdent de l'(hydroxy-2 éthylthio)-2 benzothiazole, et celles stérilisées par irradiation. Elle est également utilisable pour préciser si les têtes de piston sont ou non en caoutchouc naturel, grâce aux traces de β-sitostérol cédées. Les effets toxiques des composés relargués sont brièvement rapportés.

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SIMULTANEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STABILITY-INDICATING ANALYSIS OF ACETAMINOPHEN, CODEINE PHOSPHATE, AND SODIUM BENZOATE IN ELIXIRS

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SUMMARY

A stability-indicating high-performance liquid chromatographic method has been developed for the simultaneous determination of acetaminophen, codeine phosphate and sodium benzoate in an elixir formulation. The reversed-phase paired-ion method utilizes UV detection at 214 nm, a C_{18} column at 50°C and requires *ca.* 10 min per analysis. The method has been validated for use with elixirs containing 120 mg of acetaminophen, 12 mg of codeine phosphate and 7.5 mg sodium benzoate preservative per 5 ml. The known potential degradation products *p*-aminophenol, codeine N-oxide and codeinone are separated for quantitation simultaneous with the parent compounds. The method has been shown to be linear, reproducible, specific, sensitive and rugged.

INTRODUCTION

The analysis of liquid dosage forms of pharmaceutical preparations is a difficult task, since many of these preparations contain more than one active constituent, along with various dyes, flavors, preservatives and sweeteners. The use of high-performance liquid chromatography (HPLC) has become a powerful technique in the successful analysis of these dosage forms. The HPLC techniques employed include normal-phase[1], reversed-phase[2] and paired-ion chromatography[3–5].

Simultaneous quantitative determinations of all active components, especially in multicomponent preparations have been difficult. One procedure[6] required separate spectrophotometric methods to determine two antihistamines which would not separate by HPLC. Other procedures[1,7] have used HPLC for the analysis of only one of the active constituents.

Further, differences in amounts of active constituents or their absorptivities or their position in the chromatogram have necessitated either shifts in detector sensitivity or wavelength during the analysis to accurately and precisely quantitate the constituents[2,8,9].

Pharmaceutically active moieties which have, nonetheless, been analyzed in

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elixirs by HPLC, include acetaminophen, codeine phosphate, pheniramine maleate, pyrilamine maleate, phenylpropanolamine, guaifenesin, and dextromethorphan hydrobromide. Preservatives such as sodium benzoate and the parabens have also been quantified using HPLC.

The simultaneous quantitation of more than one active constituent along with potential degradation products in liquid dosage forms has not been previously reported. This report documents the methodology used for the simultaneous analysis of acetaminophen, codeine phosphate, and their potential degradation products[10] as well as the preservative sodium benzoate. This method has been successfully employed in stability studies and product release.

EXPERIMENTAL

Materials, equipment and liquid chromatographic conditions

A μBondapak™ C_{18} column 30 cm × 3.9 mm I.D. (Waters Assoc.) was used with a mobile phase of 80:20 pH 4.8 buffer–methanol at a flow-rate of 2.0 ml/min. The mobile phase reservoir was maintained at ambient temperature, while the column was heated to 50°C. The chromatographic hardware consisted of a DuPont model 850 high-pressure liquid chromatograph equipped with a DuPont automatic sampler with a 20-μl loop, a DuPont column oven, a Waters Assoc. Model 440 absorbance detector equipped with a 214-nm wavelength extension kit, and a DuPont 4100 integrator. The integrator sensitivity was adjusted to 0.08 a.u.f.s. A Hewlett-Packard Model 1040A diode-array detector was used for specificity studies of stressed and unstressed samples.

Reagents and solutions

The methanol, water, potassium phosphate monobasic (KH_2PO_4), phosphoric acid, and triethylamine were all HPLC grade or equivalent. The 1-butane sulfonic acid sodium salt was reagent grade. The buffer was made by adding 2.4 g of 1-butane sulfonic acid sodium salt (0.015 *M*) and 2.04 g of potassium phosphate monobasic (0.015 *M*) and 2 ml triethylamine per liter of water. The pH of this solution was adjusted to 4.8 ± 0.1 with dilute phosphoric acid. The solution was filtered before use with a 0.45-μm pore diameter Millipore® filter (Catalogue No. HAWP04700). The pH of the filtered buffer was rechecked and readjusted if necessary. The mobile phase was made by adding 200 ml of methanol to 800 ml of buffer and mixing thoroughly.

The standard solvent was prepared by adjusting distilled water to pH 3.7 ± 0.1 with dilute phosphoric acid.

Standards and sample preparation

The standards were prepared in duplicate in the following manner: accurately weigh *ca.* 30 mg of codeine phosphate standard into a 100-ml volumetric flask; accurately weigh *ca.* 30 mg of acetaminophen standard into a 50-ml volumetric flask; accurately weigh *ca.* 46.8 mg of sodium benzoate standard into a 25-ml volumetric flask; dissolve the sodium benzoate standards in distilled water and dilute to volume; transfer by pipet a 10.0-ml aliquot of a sodium benzoate standard to the 100-ml volumetric flask containing the codeine phosphate standard and dilute to volume

with distilled water; transfer by pipet 10.0 ml of the codeine phosphate–sodium benzoate solution to the 50-ml volumetric flask containing the acetaminophen standard and dilute to volume with standard solvent. The pH of the final solution was checked to ensure it was close (±0.3) to the pH of the sample solutions.

The samples were prepared using a "To Contain" pipet. A 5.0-ml aliquot of elixir was accurately transferred to a 200-ml volumetric flask and diluted to volume with distilled water.

Assay procedure

The instrument was assembled as previously indicated and the column equilibrated for at least 20 min with the mobile phase flowing. The system suitability was determined by doubling the integrator chart speed and injecting 20 μl of the standard solution. The resolution between sodium benzoate and the codeine peaks should be at least 1.25 as determined using the resolution equation[11].

The precision of the chromatographic system was determined using the relative standard deviation (R.S.D.) of the response factors (area/μg) for the acetaminophen peak in the injections of the standard solutions. Typically, the R.S.D. was less than 2.0%.

Samples were analyzed with standard solutions chromatographed before, after and interspersed with the samples if a large number of analyses were to be performed. The typical retention times for acetaminophen, sodium benzoate and codeine phosphate were 2.6, 3.8 and 4.7 min, respectively.

Calculations

It is important to correct the weight of codeine phosphate standard for the degree of hydration so that results are reported on an exact hemihydrate basis. A response factor (R) for each weighing of a standard is determined using the equation $R = A/W$ where A is the average acetaminophen, sodium benzoate or codeine phosphate peak area for each individual weighing, and W (mg) is the weight of each individual standard compound. The percent label acetaminophen, sodium benzoate, or codeine phosphate or weight percent impurity is determined using the equation:

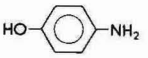
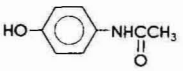
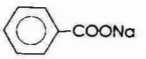
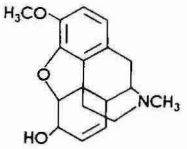
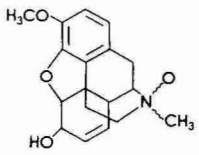
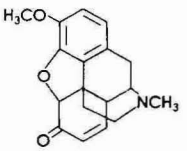
$$\% \text{ label} = \frac{A_u}{R_s} \cdot \frac{100}{W_{ts} \cdot F}$$

where A_u is the average acetaminophen, sodium benzoate, codeine phosphate or impurity peak area for each pipetting of the sample; R_s is the average acetaminophen, sodium benzoate, or codeine phosphate response factor for all weighings of the standard bracketing the sample; W_{ts} is the theoretical amount of acetaminophen, sodium benzoate, or codeine phosphate standard to be initially weighed for the preparation of a standard; and F is the sensitivity factor for an individual compound (see Table I for specific F values).

The sensitivity factor for an individual impurity accounts for the difference in sensitivity between either acetaminophen or codeine phosphate (from whichever the impurity is generated) and the individual compound under these analytical conditions. Unidentified peaks are quantified using an F value of 1 and the acetaminophen response factor. The sensitivity factor is calculated by dividing the response factor

TABLE I

STRUCTURES, SENSITIVITY FACTORS, RETENTION TIMES AND DETECTION LIMITS FOR THE INVESTIGATED COMPOUNDS

Structure	*Name*	*Retention time (min)*	*Sensitivity factor (F)**	*Detection limit (wt. %)**
	*p*-Aminophenol	1.8	7.23	0.02
	Acetaminophen	2.8	1.00	NA**
	Sodium benzoate	3.9	NA	NA**
	Codeine phosphate	4.9	1.00	NA**
	Codeine N-oxide	6.4	1.12	0.25
	Codeinone	8.8	1.15	0.25

* With respect to parent compound.
** Not applicable.

(area/μg) of acetaminophen or codeine phosphate into the response factor of the individual impurity.

Accelerated thermal decomposition

Acetaminophen with codeine phosphate elixir was placed in a clear flint-glass bottle, capped and placed in a 60°C oven for two weeks. The elixir darkened and would have failed visual assessment of acceptability. Samples of this material were treated as directed in this procedure and the acetaminophen, sodium benzoate, and codeine phosphate peaks were analyzed on a diode-array detector. These scans were compared with those obtained from a similar preparation of an unstressed sample.

Precision and recovery analysis

Precision was evaluated using a stock placebo containing sodium benzoate spiked with *ca.* 1% *p*-aminophenol, 1% codeinone, 1.4% codeine N-oxide, 100% acetaminophen and 100% codeine phosphate. The impurity compound amounts represent weight percents compared with the parent compound. Aliquots were drawn and samples prepared from this stock solution by three analysts, with each analyst making six preparations. The recovery of acetaminophen and codeine phosphate was further evaluated by adding 80, 100 and 120% of the labeled amounts of these constituents to a placebo, while sodium benzoate recovery was also evaluated by adding 10 and 20% excess to the same spiked placebo.

Linearity

The linearity of each component was determined individually by making serial dilutions of stock solutions and chromatographing each solution as described in the *Materials, equipment and liquid chromatographic conditions* section.

Ruggedness

The ruggedness of the chromatographic system was determined by evaluating alterations of several key parameters. The results of these experiments were used to establish variation limits for each of the parameters investigated. The parameters evaluated included temperature, pH, paired-ion concentration, potassium phosphate monobasic salt concentration, and organic concentration of the mobile phase.

RESULTS AND DISCUSSION

The analytical procedure presented represents a specific, precise, accurate, linear and stability-indicating method for the simultaneous quantitation of acetaminophen, sodium benzoate and codeine phosphate, as well as potential degradation products *p*-aminophenol, codeine N-oxide and codeinone. A typical chromatogram of a spiked standard solution containing each of the components given above in a placebo matrix is shown in Fig. 1. A standard solution and a thermally stressed elixir sample were chromatographed and detected using a diode-array detector as seen in Fig. 2. Excipients in both stressed and non-stressed samples exhibited no interferences with the quantitation of any of the compounds investigated. Diode-array scans of the acetaminophen, codeine phosphate and sodium benzoate peaks in the degraded sample were superimposable with those obtained from the standard preparation as seen in Figs. 3–5. This demonstrates the quantitation of the major investigated components in the elixir dosage form to be free of interferences, thereby establishing specificity.

The precision of the method was evaluated for each of the components mentioned. The relative standard deviations or the standard deviations obtained from individual sample preparations and their chromatographic evaluation are presented in Tables II and III. These data demonstrate this method to be sufficiently precise to quantitate each of these components.

The recovery of each component investigated was determined. The recovery of potential impurities was evaluated at *ca.* 1% and 3% by weight, while the recovery of the main components was determined at 80–120% label for acetaminophen and

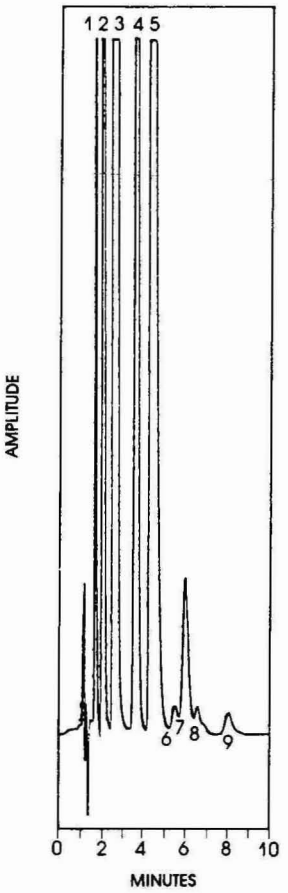


Fig. 1. Typical chromatogram of a spiked standard solution. Peaks: 1 = *p*-aminophenol; 2 = saccharin; 3 = acetaminophen; 4 = sodium benzoate; 5 = codeine phosphate; 6 = codeine N-oxide; 7 = excipient; 8 = excipient; 9 = codeinone.

codeine phosphate and at 100–120% label for sodium benzoate. These data are presented in Table IV.

The area *versus* concentration plots for each component were evaluated and found to be linear over the range of interest. These plots are shown in Figs. 6 and 7. The concentrations used in the assay for each of the major components are well within their investigated linear range.

The use of 214 nm for detection represents a wavelength where the ratio of the absorbance of codeine phosphate to acetaminophen is near a maximum, thereby increasing the sensitivity for codeine while extending the linear response range for acetaminophen. This, as a result, necessitates no adjustments in either wavelength or detector sensitivity in order to accurately and precisely quantitate any of the investigated components. This approach has been previously used and reported[12].

The ruggedness of the method was evaluated by varying certain chromatographic parameters, evaluating their qualitative effects on the analysis and extrapo-

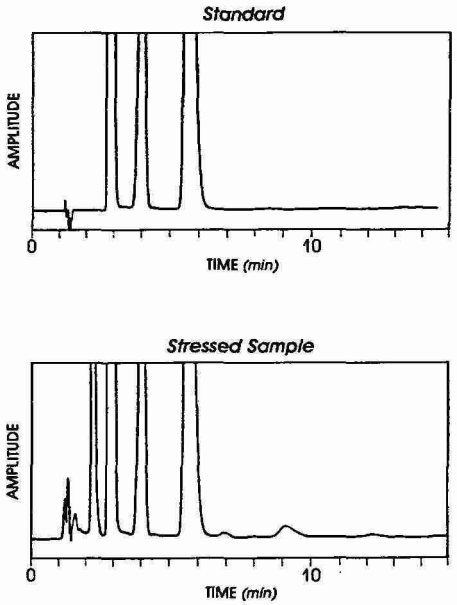


Fig. 2. High-performance liquid chromatograms of acetaminophen with codeine elixir standard and stressed samples.

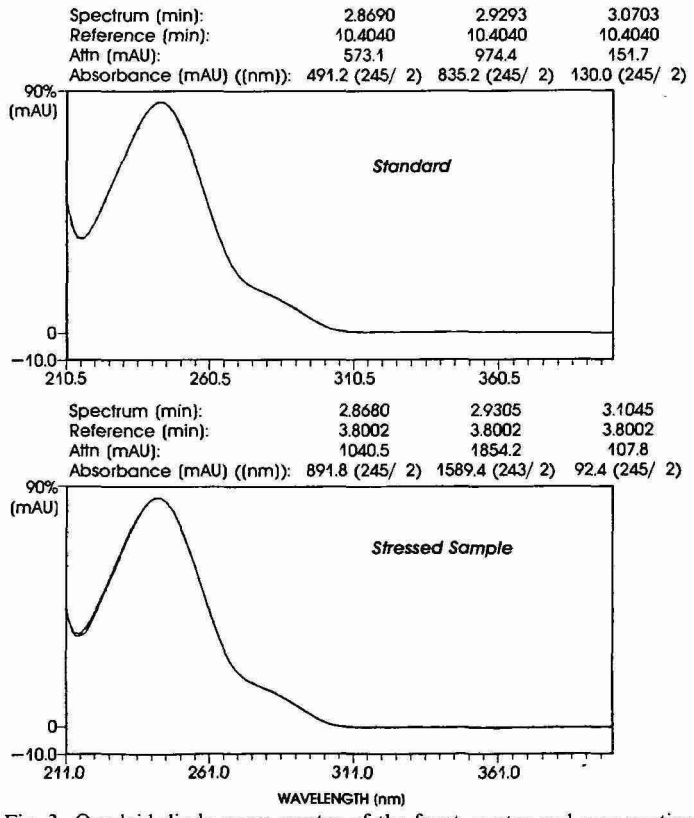
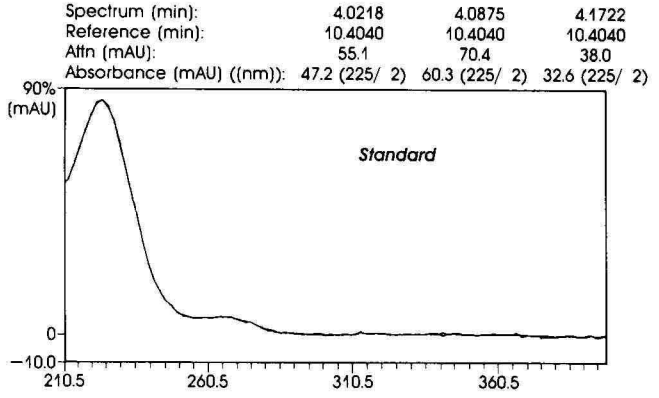
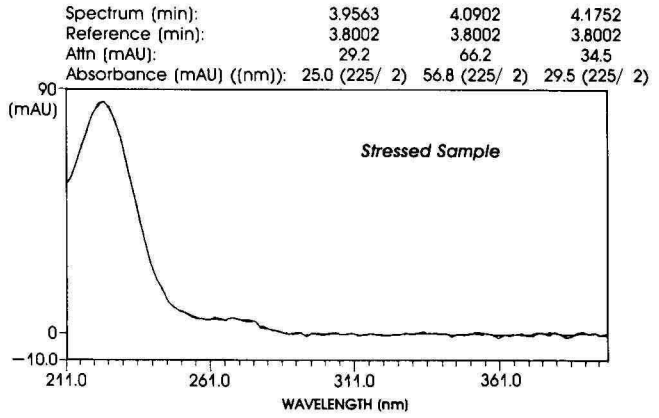
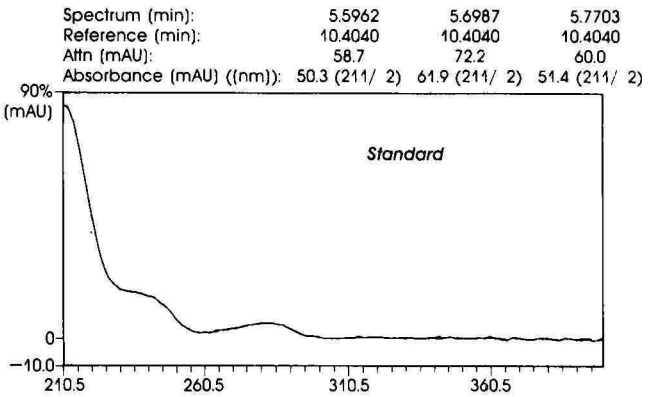


Fig. 3. Overlaid diode-array spectra of the front, center and rear portions of the acetaminophen component of acetaminophen with codeine elixir standard and stressed sample.







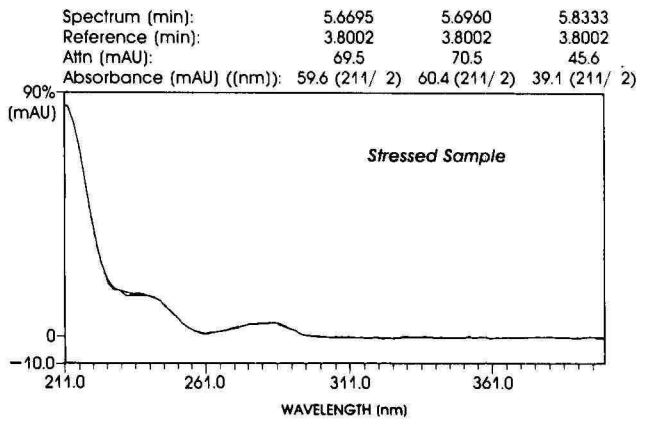


Fig. 4. Overlaid diode-array spectra of the front, center and rear portions of the sodium benzoate component of acetaminophen with codeine elixir standard and stressed sample.

Fig. 5. Overlaid diode-array spectra of the front, center and rear portions of the codeine phosphate component of acetaminophen with codeine elixir standard and stressed sample.

TABLE II

PRECISION DATA: POTENTIAL DEGRADATION PRODUCTS

Values are weight percent of the label.

Sample	*p-Aminophenol*		*Codeine N-oxide*		*Codeinone*
	Inst 1	*Inst 2*	*Inst 1*	*Inst 2*	*Inst 1*
1	0.92	0.94	1.28	1.82	1.14
2	0.94	0.95	1.77	1.87	1.10
3	0.94	0.95	1.54	1.82	1.12
4	0.94	0.95	1.55	1.55	1.19
5	0.94	0.94	1.72	1.84	1.05
6	*	0.95	*	1.75	*
7	0.92	0.92	1.32	1.60	1.10
8	0.93	0.94	1.45	1.29	1.06
9	0.92	0.92	1.60	1.69	1.21
10	0.93	0.91	1.46	1.79	1.10
11	0.93	0.93	1.42	1.05	1.15
12	0.94	0.91	1.42	1.70	1.01
13	0.92	0.92	1.03	1.45	1.22
14	0.92	0.93	1.23	1.62	1.07
15	0.93	0.94	1.17	1.44	1.18
16	0.92	0.93	1.25	1.50	1.02
17	0.92	0.91	1.08	1.71	1.21
18	0.91	0.90	1.27	1.54	1.12
$\bar{x}$	0.93	0.93	1.38	1.61	1.12
S.D.	0.0097	0.00161	0.210	0.214	0.066
Overall S.D.	0.013		0.212		0.067

* Sample was not injected by autosampler.

lating these to quantitative implications. The extent of each individual variation was chosen to represent extreme experimental deviations from the prescribed conditions. The criteria used in the qualitative evaluation consisted of ascertaining the resolution between peak pairs and the positioning in the chromatogram of the peaks of interest. The quantitative extrapolation of these data was based on the acceptability of the chromatography to assay adequately the samples for acetaminophen, sodium benzoate, codeine phosphate, and any potential decomposition products.

The pH of the aqueous buffer was the most critical parameter to control for acceptable resolution of the major compounds of interest. The pH should be controlled between 4.7 and 4.9, for outside this range the codeine phosphate and sodium benzoate coelute. The potential decomposition products are also acceptably resolved within the 4.7–4.9 range. Below pH 4.7 a flavor peak merges with codeine N-oxide, while above 4.9 a dye begins to coelute with codeine N-oxide.

The temperature of the column was varied over the 45–55°C range, with the retention times moving predictably, *i.e.*, a decrease in retention time with an increase in temperature. Within this range, all majors were resolved from each other, excipients and potential decomposition products. Above 50°C the dye and codeine N-

TABLE III

PRECISION DATA: MAJOR COMPONENTS

Values are weight percent of the label.

Sample	*Acetaminophen Inst 1*	*Sodium benzoate*		*Codeine phosphate*	
		Inst 1	*Inst 2*	*Inst 1*	*Inst 2*
1	95.1	100.0	101.1	109.9	110.4
2	95.3	100.1	101.0	110.2	110.7
3	95.2	100.2	101.4	109.7	110.6
4	95.2	100.5	100.7	110.1	110.2
5	95.6	100.3	101.3	110.4	110.1
6	*	*	101.2	*	110.3
7	93.7	98.6	99.4	108.1	108.3
8	93.6	98.5	99.5	107.9	108.4
9	93.4	98.0	98.4	107.7	108.0
10	93.8	98.4	98.4	108.0	108.0
11	94.8	99.6	99.1	109.4	108.2
12	95.0	99.8	99.9	109.3	109.9
13	94.4	99.8	99.4	109.3	108.6
14	94.2	99.6	100.1	109.0	109.0
15	94.1	100.1	99.4	109.5	106.6
16	93.8	99.3	99.6	109.2	108.5
17	94.1	99.7	99.5	109.0	108.2
18	94.0	99.3	98.6	109.4	108.0
$\bar{x}$	94.4	99.5	99.9	109.2	109.0
S.D.	0.696	0.737	1.006	0.822	1.185
R.S.D. (%)	0.74	0.74	1.01	0.75	1.09
Overall R.S.D. (%)	0.74	0.90		0.93	

* Sample was not injected by autosampler.

TABLE IV

RECOVERY DATA

Sample	*Recovery (%)*					
	Acetamin-ophen	*Sodium benzoate*	*Codeine phosphate*	*p-Amino-phenol*	*Codeine N-oxide*	*Codeinone*
Placebo	–	100.0	–	–	–	–
80% Spike	101.7	100.6	101.0	–	–	–
100% Spike	100.8	100.2	100.6	–	–	–
125% Spike	100.4	100.6	101.4	–	–	–
1% Impurity Spike	100.8	99.6	100.2	96.0	93.6	115.1
3% Impurity Spike	100.7	99.6	99.8	96.2	96.0	113.9
Average recovery	100.9	100.1	100.6	96.1	94.8	114.5

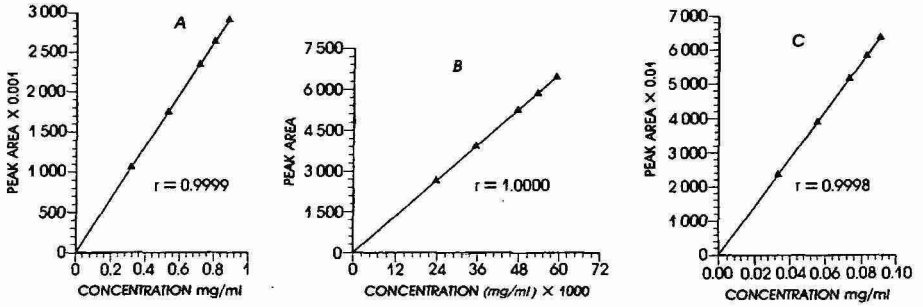


Fig. 6. Linearity plots of major components. (A) Linearity of acetaminophen at 214 nm; (B) linearity of sodium benzoate at 214 nm; (C) linearity of codeine phosphate at 214 nm.

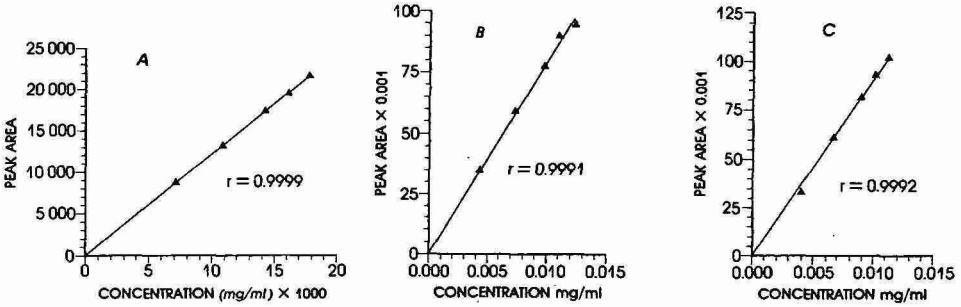


Fig. 7. Linearity plots of potential degradation products. (A) Linearity of *p*-aminophenol at 214 nm; (B) linearity of codeine N-oxide at 214 nm; (C) linearity of codeinone at 214 nm.

oxide would begin to lose resolution, and at *ca.* 55°C would reverse their elution order. The potential decomposition product *p*-aminophenol may have a problem being properly quantified above 50°C, owing to its rapid elution. Likewise, codeine N-oxide, if present, may not be adequately assayed above 50°C. However, at 45°C the resolution is more than adequate.

The use of a stable elevated temperature enhanced the chromatography and aided in the precision of the analysis and the stability of the capacity factors for all compounds investigated. A constant thermal environment is particularly useful when automated analyses are conducted over long periods of time. The effects of temperature and temperature control in liquid chromatography have been documented[13,14].

The concentration of the 1-butane sulfonic acid sodium salt was varied ±0.1 g/l. All major components were adequately resolved over this range, with no problem observed in the assay due to inadequate resolution. However, codeine N-oxide and codeinone, if present, would not be adequately resolved from the dye at either extreme.

The proportion of methanol in the mobile phase was varied from 15 to 24%, with the major components always being resolved from each other, the excipients, and the potential decomposition products. At 25% methanol the sodium benzoate and codeine peaks were not completely resolved: The codeine N-oxide and dye are not completely baseline resolved at 20% methanol; the resolution of the codeine

N-oxide and codeinone from the dye and a flavor excipient is very sensitive. However, no corresponding sensitivity was noted for the resolution of quantitation of the major components.

The concentration of potassium phosphate monobasic was varied by ±0.1 g/l. At each extreme all major components were baseline resolved with only a slight change in retention times. No apparent quantitation problems should be encountered for the major components.

CONCLUSION

This method represents a rugged stability-indicating analytical procedure for the simultaneous quantitation of acetaminophen, codeine phosphate, their known potential decomposition products, and sodium benzoate. The sample preparation is simple, the analysis time is short and the elution is isocratic. The method is amenable to the analysis of large numbers of samples with precision and accuracy comparable with individual component analyses. Overall, this analytical procedure is extremely time- and cost-effective, resulting in significant increases in productivity.

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CHROM. 18 283

SIMULTANEOUS QUANTITATIVE DETERMINATION OF BUTIZIDE, POTASSIUM CANRENOATE AND CANRENONE IN TABLETS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A procedure for the simultaneous quantitative determination and selective identification of potassium canrenoate and butizide is proposed. The amount of canrenone, the degradation product of potassium canrenoate, is also determined. Elution of these compounds is investigated on LiChrosorb RP-18 and RP-8 columns. The analysis time was shorter on a RP-8 column while the specific identification was still possible. Therefore, the LiChrosorb RP-8 column was chosen. The eluent consisted of an acetonitrile–0.05 *M* phosphate buffer, pH 4 (45:55) mixture. Quantitative determination was performed at the absorption maximum of each compound: 286 nm for potassium canrenoate and for canrenone, 271 nm for butizide. This change in wavelength is performed automatically by the computer that controls the spectrophotometric diode array detector.

INTRODUCTION

Potassium canrenoate (KCAN; a potassium-sparing diuretic) and butizide (BUT; a thiazide diuretic) are often administered together for the treatment of hypertension. Canrenone (CAN), the degradation compound of KCAN, still preserves some diuretic activity. Therefore, the amount of CAN is also analysed.

Until now, there has been no method available for the simultaneous quantitative determination of those compounds. BUT has been analysed by a spectrophotometric method[1]. The determination of KCAN is not described in the literature. CAN has been determined by several methods[2–5]. This paper describes the development of the simultaneous determination of KCAN, BUT and CAN. The accent is put on analysis time and selectivity. In a previous paper, the elution of thiazide, potassium-sparing and loop diuretics was investigated on a LiChrosorb RP-18 column[6]. Starting from these data, the separation of these compounds was optimized with regard to efficiency, speed and selectivity.

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EXPERIMENTAL

Apparatus

An SP 8770 isocratic pump (Spectra Physics, Darmstadt, F.R.G.) was used, equipped with a HP 1040A UV spectrophotometric detector (Hewlett-Packard, Palo Alto, CA, U.S.A.), a HP 85 computer, a HP 82901M flexible disc drive and a HP 3390A integrator. The HP 1040A UV detector contains a photodiode array. This detector can follow up to eight different wavelengths at the same time. The wavelength selection can be changed automatically by the program of the HP 85 computer. This is used for changing the wavelength from 286 to 271 nm for the detection of KCAN and BUT, respectively. CAN, if present in the sample, was measured at 286 nm.

The eluent was filtered through a 5-μm filter and degassed with helium. A Valco six-port injection valve with a 10-μl sample loop was used. The temperature of the column was thermostatted with a water bath.

Chromatographic procedure

LiChrosorb RP-18 and RP-8 columns (Chrompack, Middelburg, The Netherlands) of the same dimensions were used. The chromatographic conditions are listed in Table I. Unless otherwise specified, these conditions were used. The eluents were prepared by mixing the stated volume percentages.

Chemicals and reagents

All reagents were of analytical grade. Acetonitrile was HPLC grade. The diuretics were obtained from different pharmaceutical firms: KCAN (Searle), CAN (Searle), BUT (Boehringer Pharma), hydroflumethiazide (HFM) (Squibb), ethacrynic acid (ETA) (Merck, Sharp & Dohme) and polythiazide (PT) (Pfizer). KCAN and BUT were analysed in Canrenide® (Prospa) at a label claim of 50 and 5 mg per tablet, respectively.

Determination of potassium canrenoate, butizide and canrenone

Internal standard solutions. Two methanolic standard solutions containing 15 mg/ml HFM and 1.2 mg/ml PT were prepared.

Standard solutions. Three different methanolic standard solutions were prepared containing 2.5 mg/ml KCAN, 1 mg/ml BUT and 0.5 mg/ml CAN. A concentrated working standard solution was prepared by diluting 20 ml of the KCAN solution, 5 ml of the BUT solution, 3 ml of the CAN solution, 5 ml of the HFM

TABLE I

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CONDITIONS

Column	LiChrosorb RP-8, 5 μm, 150 × 4.6 mm I.D.
Eluent	Acetonitrile–0.05 *M* phosphate buffer, pH 4 (45:55)
Flow-rate	1 ml/min
Temperature	25°C
Detector	UV detector, wavelength 286 nm for KCAN and for CAN, 271 nm for BUT
Recorder	Chart speed 0.5 cm/min
Sample loop	10 μl

internal standard solution and 5 ml of the PT internal standard solution to 50 ml with methanol. The working standard solution was prepared by diluting this concentrated solution ten-fold with an acetonitrile–water (1:1) mixture.

Sample preparation. An amount of ground tablet powder corresponding to 50 mg of KCAN and 5 mg of BUT was weighed into a 50-ml volumetric flask. A 5-ml volume of each internal standard solution was added and the mixture was diluted to 50 ml with methanol. This solution was stirred for 10 min and filtered. The filtrate was diluted ten-fold with an acetonitrile–water (1:1) mixture, and refiltered.

RESULTS AND DISCUSSION

Optimization of the chromatographic parameters

The purpose of this paper was to develop a specific, fast and reproducible determination of BUT, KCAN and CAN. The chromatographic behaviour of these diuretics, together with some other thiazide, potassium-sparing and loop diuretics was investigated on a LiChrosorb RP-18 stationary phase[6]. In an acetonitrile–water (40:60) mixture, BUT eluted within a reasonable analysis time ($k' = 2.6$). KCAN eluted close to the column dead-volume. The retention of KCAN can be altered by changing the pH of the eluent, while the retention of BUT remains constant. Therefore the pH of the phosphate buffer was acidified to pH 3. This resulted in an increased retention of KCAN. However, CAN, the degradation product of KCAN, eluted rather late from the column ($k' = 10.5$). 4-Amino-6-chlorobenzene-1,3-disulphonamide, the hydrolysis product of BUT, eluted close to the column dead-volume. As we also wanted a quick determination of the amount of CAN present in the tablets, we needed a system where CAN eluted faster from the column. On a LiChrosorb RP-8 column (Table II), CAN eluted somewhat faster compared to the LiChrosorb RP-18 column. Therefore, we decided to use a LiChrosorb RP-8 column.

A further increase of the acetonitrile content of the eluent (up to 45%) and the phosphate buffer at pH 4 resulted in a good compromise between selectivity and speed (Table II and Fig. 1). Indeed, BUT is separated from other thiazide diuretics

TABLE II

k' VALUES OF KCAN, BUT AND THEIR DEGRADATION COMPOUNDS

Eluent A: acetonitrile–0.05 *M* phosphate buffer, pH 3 (40:60); eluent B: acetonitrile–0.05 *M* phosphate buffer, pH 4 (45:55).

Compound	*Column*		
	LiChrosorb RP-18	*LiChrosorb RP-8*	
	Eluent A	*Eluent A*	*Eluent B*
4-Amino-6-chlorobenzene-1,3-disulphonamide	0.42	0.58	0.38
KCAN	2.1	2.4	1.3
BUT	2.7	3.0	1.9
CAN	10.5	9.3	5.0

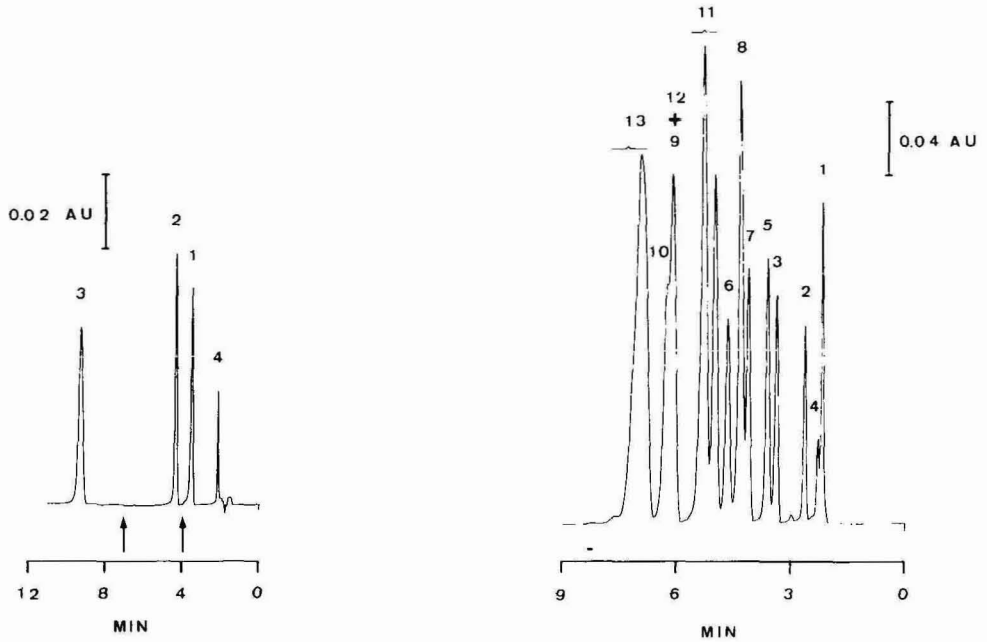

Fig. 1. Separation of KCAN (1) and BUT (2) from their respective degradation compounds: CAN (3) and 4-amino-6-chlorobenzene-1,3-disulphonamide (4) on a LiChrosorb RP-8 column. Mobile phase: acetonitrile–0.05 *M* phosphate buffer, pH 4 (45:55). The arrows indicate the time when the wavelength was changed (Table I).

Fig. 2. Selective identification of BUT. BUT (8) is separated from other thiazide diuretics: hydrochlorothiazide (1), hydroflumethiazide (2), trichlormethiazide (3), chlorthalidone (4), methylclothiazide (5), epithiazide (6), althiazide (7), polythiazide (9), bendroflumethiazide (10), cyclothiazide (11), cyclopenthiazide (12) and mebutizide (13). Chromatographic conditions: see Table I. Detection wavelength: 275 nm.

(Fig. 2). With higher amounts of acetonitrile (50%) in the eluent, a decrease in selectivity occured: co-elution of BUT and epithiazide. At pH 3, KCAN cannot be differentiated from furosemide, a loop diuretic, on the RP-8 column. The influence of eluent pH was investigated on a LiChrosorb RP-18 column[6]. On increasing the pH from 3 to 4, the k' value of furosemide decreased to a larger extent than the k' value of KCAN. This resulted in a greater selectivity between the two compounds. As we expected a similar behaviour on the LiChrosorb RP-8 column, we increased the pH to 4. Indeed, at this pH, KCAN can be separated from some other currently used potassium-sparing and loop diuretics (Fig. 3).

Quantitative determination

KCAN and BUT have different UV absorption maxima: 286 nm and 271 nm, respectively. Measurements carried out at the absorption maximum are more selective and more reproducible. We therefore decided to change the wavelength from 286 to 271 nm after KCAN eluted from the column. This change was performed automatically by the program of the HP 85 computer that controls the spectrophotometric diode array detector.

As the concentration of KCAN and BUT in the tablets differed by a factor of

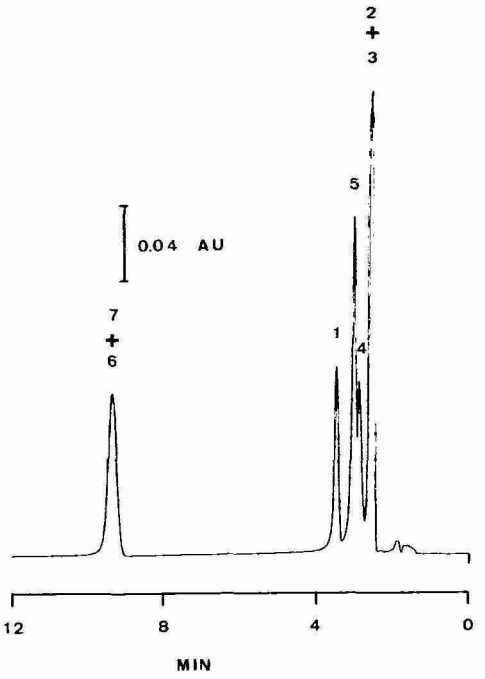

Fig. 3. Selective identification of KCAN. KCAN (1) is separated from: amiloride hydrochloride (2), furosemide (3), triamterene (4), ethacrynic acid (5), spironolactone (6) and CAN (7). Chromatographic conditions: see Table I. Detection wavelength: 275 nm.

10, we selected two internal standards: one for each compound. We selected ETA and PT, respectively, according to their structure relationship with KCAN and BUT and according to their elution behaviour[6]. Indeed, ETA contains a carboxylic acid function, as does KCAN. However, aqueous solutions containing both compounds were not stable: ETA, an acidic compound, catalysed the degradation of KCAN to CAN[7]. Thus, acidic compounds could not be used as internal standard. We therefore selected HFM as internal standard for KCAN. These solutions were stable.

The proposed method was linear for KCAN (r = 1.0000) and for BUT (r = 1.0000) over a concentration range of 50–150% of the amount of drugs normally present in the tablets.

TABLE III

DETECTION LIMIT (SIGNAL-TO-NOISE RATIO = 4)

Compound	*Detection limit (ng injected)*	*Wavelength (nm)*
KCAN	2	286
BUT	2	271
CAN	3	286
4-Amino-6-chlorobenzene-1,3-disulphonamide	6	286

TABLE IV

RECOVERY OF KCAN AND BUT FROM SYNTHETIC TABLET MIXTURES

Recovery (% of amount added)	
KCAN	*BUT*
99.8	99.1
99.9	100.3
100.0	100.0
100.0	99.7
99.2	100.1
99.8*	99.8*
0.34**	0.47**

* Average (%).
** Relative standard deviation (%).

TABLE V

RESULTS OF THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF KCAN AND BUT IN TABLETS

Compound	*% Found of the label chain*	*R.S.D. (%)*	*n*
KCAN	101.3	1.1	8
BUT	96.9	1.2	8

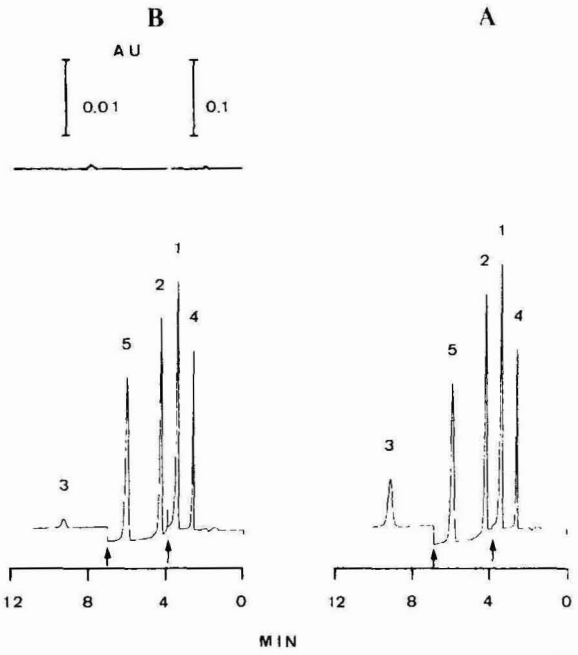

Fig. 4. Chromatogram of the simultaneous determination of KCAN (1), BUT (2) and CAN (3). HFM (4) and PT (5) are the internal standards. Chromatographic conditions: see Table I. A = Standard sample; B = canrenide sample.

The sensitivity of the proposed method is listed in Table III. Small amounts of CAN and 4-amino-6-chlorobenzene-1,3-disulphonamide can be determined.

The drugs were extracted from the pharmaceutical preparation with methanol. Table IV lists the results of the standard-addition recovery experiments on artificial mixtures at various drug levels. A recovery of 99.8% (relative standard deviation = 0.34%) for KCAN and 99.8% (relative standard deviation = 0.47%) for BUT was obtained.

The reproducibility of the method was investigated by performing several analyses on a commercial formulation. These results are shown in Table V. These data indicate that the proposed method is reliable and reproducible. Fig. 4 shows a chromatogram of the determination of KCAN, BUT and CAN: baseline separations were obtained.

CAN, the degradation product of KCAN, may be present in the tablet up to 3% of the amount of KCAN in the tablet. This corresponds to 1.5 mg of CAN per tablet. This maximum concentration is shown in the standard mixture in Fig. 4. The concentration of CAN in this tablet formulation was far below the maximum concentration: 0.25 mg of CAN per tablet.

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CHROM. 18 278

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DIMETHYLDITHIOCARBAMATE RESIDUES IN SOME AGRICULTURAL PRODUCTS

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SUMMARY

Dimethyldithiocarbamates are widely used in agriculture as active fungicides. The degradation of dimethyldithiocarbamates (ferbam, thiram) confirmed the fact that they are not stable and decompose very rapidly. The aim of this work was to apply the results obtained in high-performance liquid chromatographic quantitative analysis of residues of dithiocarbamate fungicides in some agricultural products (strawberries, maize, tobacco).

The developed method enables very simple control analysis of low concentrations of dimethyldithiocarbamate residues in very short time. All limits of detection correspond with the criteria of FAO (*Codex Alimentarius*).

INTRODUCTION

We have previously studied the degradation of dimethyldithiocarbamates (DTCs) and found that metal dimethyldithiocarbamates are not stable and decompose very quickly. Three degradation products were identified and the optimal conditions for the simultaneous high-performance liquid chromatographic (HPLC) separation of ferbam–iron(III) dimethyldithiocarbamate, thiram–tetramethyl thiuram disulphide and their possible degradation products in the minimum time and with maximum resolution were established. Adsorption and reversed-phase chromatography have been investigated[1,2].

Ferbam and thiram are widely used in agriculture as active fungicides[3–5] and the HPLC analysis of DTC fungicides using UV detection has been reported[6,7]. Extraction recoveries have been published for some agricultural products[8]. Maximum allowed residue concentrations of pesticides and fungicides for various kinds of vegetables and fruit have been given[9]. Other papers have described the determination of DTCs by HPLC using transition metal satls as 'ion-pair' reagents[10,11] and the HPLC of sodium and ammonium salts of DTCs using a chemically bonded stationary phase[12].

The aim of this work was to apply the results obtained using HPLC to the determination of residues of DTC complexes in some agricultural products.

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TABLE I

EXTRACTION RECOVERIES OF THIRAM FROM STAWBERRIES AND MAIZE

Column: Separon SIX, 5 μm (150 × 3.2 mm I.D.). Mobile phase: 45% chloroform in cyclohexane.

Sample	*Strawberries*				*Maize*			
	Chloroform extract (%)	*Methanol extract (%)*	*Extraction recovery (%)*	*Standard deviation (%)*	*Chloroform extract (%)*	*Methanol extract (%)*	*Extraction recovery (%)*	*Standard deviation (%)*
1	–	–	–	–	–	–	–	–
2	50.3	38.4	88.7	2.9	56.6	36.3	92.9	1.2
3	53.0	33.0	86.0	3.7	56.7	34.6	91.3	1.4

EXPERIMENTAL

Apparatus

All experiments were carried out on a Packard Model 8200 HPLC system with a UV detector (254 nm). For HPLC separations three glass columns (150 × 3.2 nm I.D., particle diameter 5 μm) were used, packed with Separon SIX, Separaton C_{18} or Separon NH_2 (Laboratorní přístroje, Prague, Czechoslovakia). Chloroform–cyclohexane and acetonitrile–methanol–water mixtures were used as the mobile phases.

Chemicals

All standards were synthetized by literature procedures[13–15] and their purities were verified by elemental analysis using a Model 1102 elemental analyser (Carlo Erba, Milan, Italy) and mass spectrometry using a 902S mass spectrometer (AEI, Manchester, U.K.).

Procedures

For the extraction of real samples (maize, strawberries and tobacco) the solvents applied were chloroform and methanol. A 100-g amount of the sample was extracted with three 100-cm³ volumes of chloroform and methanol. Each extraction was performed for about 10 min. Natural samples were extracted in large series and all data given in Tables I and II are average values from five extractions.

RESULTS AND DISCUSSION

Determination of thiram in strawberries

Thiram (TMTD) is widely used as an active fungicide in strawberry growing (Hermal L-50, Wolfen, Thiuram 85, Benlate T 20). The thiram concentrations applied here conformed with the amounts allowed in agricultural applications.

For HPLC separations of thiram and its two possible degradation products (TMTM = tetramethylthiuram monosulphide and TMTU = tetramethylthiourea), a mixture of 45% chloroform in cyclohexane and a silica gel column (Separon SIX)

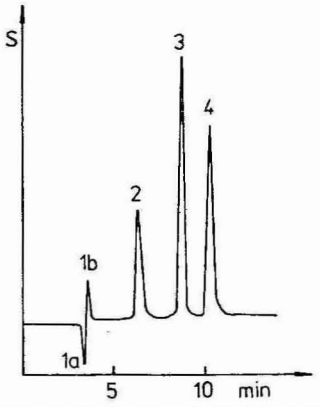

Fig. 1. Separation of TMTD, TMTM and TMTU (model mixture) on a silica gel column. Column: Separon SIX, 5 μm (150 × 3.2 mm I.D.). Mobile phase: 45% chloroform in cyclohexane. Flow-rate: 0.30 cm³/min. Pressure: 5 MPa. Peaks: 1 = inert; 2 = TMTU; 3 = TMTM; 4 = TMTD.

were used. The optimal flow-rate of the mobile phase was 0.3 cm^3/min and the pressure was 5 MPa.

The $R_{j/j}$ values for the optimal separation conditions were $R_{TMTD/TMTM} = 2.25$ and $R_{TMTM/TMTU} = 1.52$. The optimal linear velocity was determined according to the H–u curve (0.71 mm/s).

The HPLC separation of TMTD, TMTM and TMTU on the Separon SIX is demonstrated in Fig. 1.

Three samples of strawberries were analysed. Thiram was added to strawberries at the concentration recommended for agricultural applications: sample 1, no thiram; sample 2, 0.03 g/kg; and sample 3, 0.01 g/kg. The samples were extracted and, after evaporation of the extracts and dissolution of the residues in the mobile phase, the solutions were injected into the chromatograph. The determination of thiram was effected using optimal separation conditions.

Calibration graphs were statistically evaluated by linear regression ($n = 5$). For injected amounts of 5 mm^3 the equation was $y = 0.9x + 0.07$, correlation coefficient $r_{xy} = 0.9995$ and $S_{xy} = 2.3\%$. The limit of detection (the amount that gives a peak equivalent to three times the baseline noise) of thiram in strawberries was 0.08 mg/kg. The extraction recoveries are given in Table I.

Determination of thiram in maize

Thiram has also been used for the protection of maize before seeding. Samples of maize were mixed with dry thiram and extracted as described above. Separon SIX and Separon NH_2 columns were used to establish the optimal separation system. Suitable separation conditions for the Separon SIX column were described above (Fig. 1). For the Separon NH_2 column, the following conditions were established: mobile phase, 20% chloroform in cyclohexane, $R_{12} = 1.35$, $R_{23} = 1.03$; linear velocity of the mobile phase, 1.27 mm/s; flow-rate, 0.54 cm^3/min; and pressure, 7 MPa.

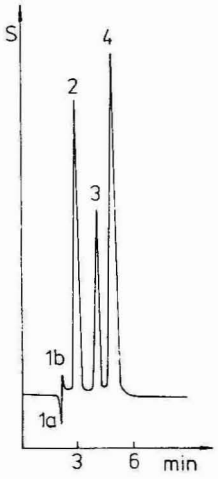

Fig. 2. Separation of TMTD, TMTM and TMTU (model mixture) on a column with chemically bonded stationary phase. Column: Separon NH_2, 5 μm (150 × 3.2 mm I.D.). Mobile phase: 20% chloroform in cyclohexane. Flow-rate: 0.54 cm^3/min. Pressure: 7 MPa. Peaks: 1 = inert; 2 = TMTU; 3 = TMTM; 4 = TMTD.

The separation of TMTD, TMTM and TMTU on a Separon NH_2 column is demonstrated in Fig. 2. Three maize samples were analysed: sample 1, no thiram; sample 2, 0.3 g/kg; and sample 3, 0.2 g/kg. The extraction recoveries are given in Table II.

Various mobile phases were investigated and the optimal separation conditions were chosen for the two columns used (silica gel and NH_2 phase). The numbers of theoretical plates (N_R), minimal separation times necessary for the separation of the last component (t_R), detection limits and optimal flow-rates of the mobile phases were as follows: silica gel column, $N_{R(TMTU)} = 5720$, $t_{R(TMTU)} = 10$ min, detection limit of thiram, 0.012 mg/kg maize and flow-rate, 0.30 cm^3/min; NH_2 phase column, $N_{R(TMTU)} = 2434$, $t_{R(TMTU)} = 6$ min, detection limit of thiram, 0.089 mg/kg maize and flow-rate, 0.54 cm^3/min.

It is obvious that the N_R values for adsorption chromatography were higher and the detection limit of thiram was considerably lower, so we adopted the silica gel column for the analysis of maize extracts. However, both columns were convenient for the successful separation and determination of DTCs and their degradation products in an acceptable time.

Determination of ferbam in tobacco

Ferbam is used as an active fungicide in tobacco growing. It was added to tobacco leaf samples as follows: sample 1, no ferbam; sample 2, 0.03 g/kg; and sample 3, 0.02 g/kg. The conditions used were as follows: column, Separon C_{18}; mobile phase, acetonitrile–methanol–water (40:35:25); flow-rate, 0.4 cm^3/min; pressure; 9.5 MPa. The limit of detection of ferbam in tobacco was 0.014 mg/kg.

The determination of ferbam was more complicated owing to its instability[1,2,16]. It was found that two of its degradation products (TMTD and TMTM) were present in all tobacco extracts. For these reasons, the calibration graphs were measured for three standards (ferbam, TMTD and TMTM). TMTU has not been identified in tobacco extracts.

The equations of the straight lines were as follows:

for ferbam:

$$y = 2.7x - 0.12$$
$$r_{x,y} = 0.9996$$

TABLE II

EXTRACTION RECOVERIES OF FERBAM, TMTD AND TMTM FROM TOBACCO

Column: Separon C_{18}, 5 μm (150 × 3.2 mm I.D.). Mobile phase: acetonitrile–methanol–water (40:35:25).

Sample	*Chloroform extract (%)*			*Methanol extract (%)*			*Extraction recovery (%)*	*Standard deviation (%)*
	Ferbam	*TMTD*	*TMTM*	*Ferbam*	*TMTD*	*TMTM*		
1	–	–	–	–	–	–	–	–
2	57.7	15.0	7.3	8.0	1.3	0.4	89.7	2.3
3	50.4	16.4	9.1	9.1	1.5	1.0	87.5	2.8

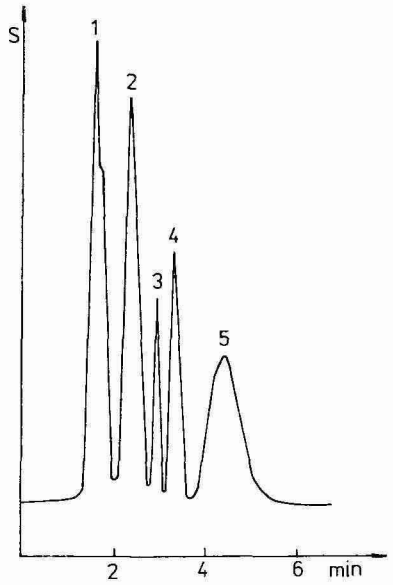

Fig. 3. Chromatogram of tobacco extract in chloforom. Column: Separon C_{18} 5 μm (150 × 3.2 mm; I.D.). Mobile phase: acetonitrile–methanol–water (40:35:25). Flow-rate: 0.42 cm^3/min. Pressure: 9.5 MPa. Peaks 1 and 2 = unidentified; 3 = TMTM; 4 = TMTD; 5 = ferbam.

for TMTD:

$$y = 2.4x - 0.23$$
$$r_{x,y} = 0.9994$$

for TMTM:

$$y = 1.9x - 0.35$$
$$r_{x,y} = 0.9996$$

Extraction recoveries for ferbam and its degradation products in tobacco are given in Table III and the chromatogram of a tobacco extract in chloroform is shown in Fig. 3. The detection limit of ferbam in tobacco was 0.032 mg/kg.

CONCLUSION

The method developed for the determination of dimethyldithiocarbamate fungicides in natural products permits very simple control analyses of low concentrations of DTC residues and their degradation products simultaneously with short separation times (6–10 min). All components were soluble in mobile phases containing chloroform. This fact reduced the detection limits of DTC fungicides published previously. All limits of detection correspond to the criteria of the FAO (*Codex Alimentarius*) concerning methods for the determination of DTC residues in food. The method has

already been accepted for the control analysis of DTC fungicides applied to some agriculatural products in Czechoslovakia.

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RESOLUTION OF MOUSE HEPATIC CYTOCHROME P-450 ISOZYMES BY CHROMATOFOCUSING

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SUMMARY

Constituent cytochrome P-450 isozymes from mouse hepatic microsomes were fractionated by chromatofocusing on Polybuffer Exchanger 94 over the pH range 5.3–8.3 and characterized by polyacrylamide gel electrophoresis. Eight isozymes were detected in fractions from unpretreated mice and seven from phenobarbitone-pretreated animals. Isozyme-containing fractions were reconstituted and shown to produce specific monohydroxy metabolites from both testosterone and Δ^9-tetrahydrocannabinol.

INTRODUCTION

Cytochrome P-450, the major enzyme system mediating biotransformation of lipophilic xenobiotics and certain endogenous compounds, exists as a group of selectively inducible isozymes having different, although somewhat overlapping, substrate specificities[1]. The isolation and purification of these isozymes has posed some difficult biochemical problems, recently reviewed by Guengerich[2]. A large number of chromatographic techniques have been used including such methods as high-performance liquid chromatography (HPLC)[3], *n*-octylamino-Sepharose chromatography[4], DEAE-cellulose chromatography[5] and affinity chromatography[6]. In our work on toxic metabolites formed from drugs and other xenobiotics we required a method for separation of cytochrome P-450 isozymes in an active form for reconstitution and metabolism studies with a variety of substrates. Chromatofocusing appeared to offer the potential for resolution of isozymes as it has greater resolving power than straightforward ion-exchange chromatography but, until recently[7], the technique does not appear to have been exploited for cytochrome P-450 separation. In this paper we describe a chromatofocusing system capable of resolving several cytochrome P-450 isozymes from phenobarbitone-treated and untreated mice and the reconstitution of the enzymes for the study of their catalytic activity.

EXPERIMENTAL

Pretreatment of mice

Mice (Charles River, male, 20–24 g) were treated with phenobarbitone (pH

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adjusted to 7.4 with 1 *M* hydrochloric acid) by including it in their drinking water (0.1%, w/v) for six days. The phenobarbitone was withdrawn 24 h before the animals were killed by cervical dislocation. A second group of mice was supplied with water without phenobarbitone.

Preparation of cytochrome P-450 fractions for chromatofocusing

This was based on the method described by Van der Hoeven and Coon[8]. The livers were removed from the treated and non-treated mice after removal of the gall bladder and placed in ice-cold buffer [0.1 *M* Tris–acetate (pH 7.4), containing 0.1 *M* potassium chloride, 0.1 m*M* EDTA and $2.3 \cdot 10^{-5}$ *M* butylated hydroxytoluene (BHT)]. All subsequent procedures were carried out at 0–4°C. The tissue was cut into approximately 5-mm cubes and homogenized in a Waring blender in four volumes of buffer. The homogenate was centrifuged at 10 000 *g* for 30 min, the supernatant was filtered through cheese cloth and centrifuged further at 105 000 *g* for 90 min. The resulting pellet was suspended in 0.1 *M* potassium pyrophosphate buffer (pH 7.4), containing 0.1 m*M* EDTA and $2.3 \cdot 10^{-5}$ *M* BHT to the same volume as the original homogenate and centrifuged at 105 000 *g* for 60 min. The microsomes were suspended in 10 m*M* Tris–acetate buffer (pH 7.4), containing 20% (v/v) glycerol and 0.1 m*M* EDTA (buffer A) to a protein concentration of about 24 mg/ml for storage at −20°C. The microsomes were then diluted with three volumes of 0.1 *M* Tris–acetate buffer (pH 7.4), containing 0.1 *M* potassium chloride, 20% (v/v) glycerol, 1.0 m*M* EDTA, 1.0 m*M* dithiothreitol and $2.3 \cdot 10^{-5}$ *M* BHT recently flushed with nitrogen. This suspension was solubilized with 10% (w/v) sodium cholate which was added dropwise with stirring to give a cholate-to-protein ratio of 3:1 and was stirred for a further 30 min. Fractional precipitation from this solution was achieved by adding 50% (w/v) polyethylene glycol 8000 solution to give 4%, 6%, 8%, 10% and 13% fractions. The precipitate was obtained by centrifugation at 10 000 *g* for 10 min between each addition of polyethylene glycol. The 8–10% and the 10–13% precipitates, which contained the cytochrome P-450, were suspended in buffer A and dialysed overnight against 50 volumes of buffer A. After dialysis, the fractions were combined and stored at −20°C until required. Before chromatofocusing, the samples were dialysed against 25 m*M* Tris–acetate buffer, (pH 7.4) (three times 1-l), containing 1.0 m*M* dithiothreitol and 0.5% (v/v) Tergitol NP-10. Protein was measured by the method of Lowry *et al.*[9] and cytochrome P-450 was measured by the method of Omura and Sato[10].

Chromatofocusing of cytochrome P-450

This was based on the technique described by Ono *et al.*[11] for squalene epoxidase. A 10-mg/ml sample of 500 mg total protein of the dialysed fraction from the unpretreated mice was applied to a 200-ml column of Polybuffer Exchanger 94 (PBE 94, Pharmacia, Uppsala, Sweden) equilibrated in 25 m*M* Tris–acetate buffer, pH 8.3. For the fraction from the phenobarbitone-pretreated mice, a 5.2-mg/ml sample of 250 mg total protein was applied to a 100-ml column of PBE 94 equilibrated at the same pH. The concentration of material applied to each column was equivalent to 2.5 mg of protein to each ml of column material. Elution of the two columns was performed with 2.5 l and 1.2 l respectively of a mixture of Polybuffer 96 (Pharmacia) (30%) and Polybuffer 74 (70%) adjusted to pH 5.0 with acetic acid at flow-rates of

30 and 20 ml/h respectively. The pH range was chosen following isoelectric focusing of a small sample of the dialysed material on flat bed polyacrylamide gels using the method described by the manufacturer (LKB, Cambridge, U.K.). The gels were stained for protein using Coomassie blue. Fractions of 5.7 and 6.6 ml were collected from the two chromatofocusing columns respectively, and further material was eluted using a salt gradient. Elution profiles were determined either by measuring the absorbance at 405 nm using a flow cell and chart recorder or by measuring at both 270 nm and 405 nm in each fraction. The pH of the fractions was determined within 24 h of elution using a pH meter and electrode calibrated to the pH of the starting buffer. The fractions were combined into samples containing individual cytochrome P-450s as far as could be judged from the elution profiles and frozen at −20°C until required. Under these conditions the cytochrome P-450 was stable for at least 36 weeks. The cytochrome P-450 content was determined as above.

Separation of cytochrome P-450 from polybuffer and detergent

Calcium phosphate gel[12] (approximately 0.5 ml for each 10 ml of cytochrome P-450-containing solution) was added to the fractions and mixed. The gel was centrifuged at 2000 *g* for 15 min and the absorbance of the supernatant was measured at 405 nm to confirm the complete precipitation of the cytochrome P-450. The gel was washed with buffer A until the absorbance at 278 nm was less than 0.1 unit, indicating that the Tergitol had been removed. The cytochrome P-450 was extracted from the gel with 3 ml of 0.3 *M* phosphate buffer containing 20% (v/v) glycerol and 1.0 m*M* EDTA and was centrifuged at 10 000 *g* for 10 min to recover the gel. This procedure was repeated at least three times. The efficiency of the calcium phosphate gel extraction in removal of the Tergitol NP-10 and polybuffers was 97.5% and 100%, respectively, as determined by the absorbances at 273 and 253 nm. The extracts were stored at −20°C until required.

Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with slab gels using the system of Fairbanks *et al.*[13]. Gels were stained for protein with Coomassie brilliant blue R (Sigma, Poole, U.K.), silver stain[14] or, for cytochrome P-450 activity, by peroxidase activity[15]. Molecular weight markers (Sigma) consisting of six proteins with molecular weights between 14 000 and 66 000 daltons were applied to the plates in two positions for development concurrently with the cytochrome P-450. Calibrations were obtained from a regression analysis of the R_F values. Concentration of the samples prior to electrophoresis, if required, was performed by containing the sample in a dialysis bag surrounded by dry Sephadex G 25 to act as a drying agent. This was allowed to stand at 4°C until the required concentration was achieved. Densitometer tracings of the gels stained with Coomassie blue were recorded with a Cecil CE 599 spectrophotometer fitted with a Cecil CE 570 gel scanner. The recording was made at 590 nm at a scan-rate of 5 s/mm.

Reconstitution of cytochrome P-450 for metabolism studies

Reconstitution experiments were performed in 1-ml cuvettes with a light path of 10 mm using a method based on that of Coon[16]. The sample of cytochrome P-450 (0.1 n*M*), cytochrome P-450 reductase (500 units, see below) and dilauroyl-

L-α-phosphatidylcholine (20 μl of a sonicated 0.1% aqueous suspension) were mixed and allowed to stand for 1 min at room temperature. The following components were then added: 0.5 *M* sodium 4-(2-hydroxyethyl)-1-piperazineethanesulphonate (HEPES) buffer at pH 7.4 (100 μl), 0.1 *M* magnesium chloride (50 μl), 0.5% (w/v) sodium deoxycholate (20 μl), 10 m*M* reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (20 μl) and distilled water to 1 ml. The mixture was incubated for 3 min at 37°C before the addition of substrate which was either Δ^9-tetrahydrocannabinol (Δ^9-THC, 40 μg) in ethanol (2 μl) or testosterone (40 μg) in ethanol (4 ml). After further incubation at 37°C for 1 h, the metabolites were extracted with ethyl acetate (three times 1 ml), the combined ethyl acetate fractions were concentrated, transferred to 0.3-ml conical micro-vials, blown to dryness and dissolved in N,O-bis-(trimethylsilyl)trifluoroacetamide (20 μl). This was heated at 60°C for 10 min and aliquots were examined by gas chromatography–mass spectrometry (GC–MS).

GC–MS

GC–MS was performed with a VG Micromass 12B mass spectrometer interfaced to a VG 2050 data system and, via a glass jet separator, to a Varian 2440 gas chromatograph which was fitted with a 2 m × 2 mm glass column packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q. Helium at 30 ml/min was used as the carrier gas and the column was temperature programmed from 170 to 300°C at 2°C/min. The injector and separator temperatures were both 300°C. The mass spectrometer was operated under the following conditions: electron energy, 25 eV; accelerating voltage, 2.4 kV; trap current, 100 μA; ion-source temperature, 260°C; scan speed, 3 s/decade.

Preparation of cytochrome P-450 reductase

This was obtained from the 13% polyethylene glycol supernatant of microsomes from the cytochrome P-450 purification (above) using a method based on that described by Shepard *et al.*[17].

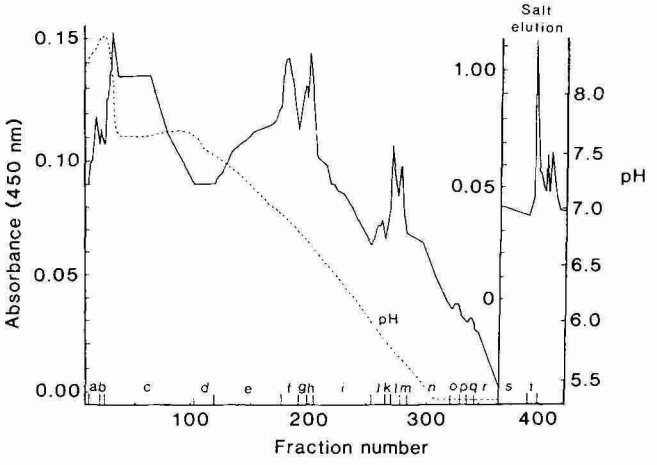


Fig. 1. Elution profile of mouse hepatic cytochrome P-450 (——) and pH (– – –) obtained by chromatofocusing.

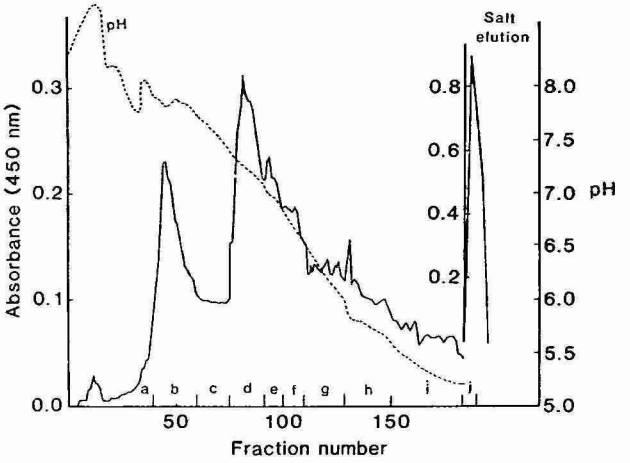


Fig. 2. Elution profile of phenobarbitone-pretreated mouse hepatic cytochrome P-450 (——) and pH (---) obtained by chromatofocusing.

RESULTS

The elution profiles and pH from the two chromatofocusing columns are shown in Figs. 1 and 2 for the untreated and phenobarbitone-treated mice, respectively. The profile from the unpretreated animals was obtained by monitoring the column eluent in a flow cell whereas that from the other column was constructed using the absorbance of each fraction determined in a scanning spectrophotometer. The slope of the absorption towards lower values during the elution is thought to be due to changes in the absorbance of the buffer.

Difference spectroscopy[18] revealed that both cytochrome P-450 and P-420 were eluted from the column but varied between the fractions. For example, fraction 42 from the phenobarbitone-induced P-450 gave a reduced carbon monoxide spectrum with maxima at 449 nm and 550 nm whilst fraction 82 from the same column gave substantial maxima at 420 and 450 nm and no detectable maximum at higher wavelength. Removal of the tergitol NP-10 and Polybuffers altered the spectral characteristics of several fractions to show increased absorbance at 450 nm and reduction of the peak at 420 nm. The reverse situation was not observed. This was most apparent in fraction 82 from the phenobarbitone-pretreated mice where, after removal of the detergent and Polybuffers, the difference spectrum maximized almost entirely at 450 nm with a broad absorbance band at 550 nm. Fractions and their properties are shown in Tables I and II for the unpretreated and phenobarbitone treated mice, respectively.

The quantity of recovered cytochrome P-450 was measured by the method described by Omura and Sato[10]. The total recovery of cytochrome P-450 with chromatofocusing was about 40% from both columns. Values for the individual fractions are listed in Tables I and II.

Individual fractions were examined by SDS-PAGE and the gel scans of the fractions, recorded at 550 nm, are shown in Figs. 3 and 4. Molecular weights and other properties are listed in Tables I and II.

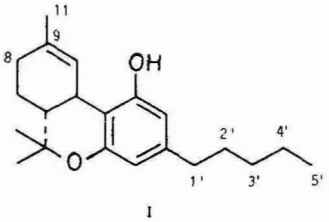
TABLE I

PROPERTIES OF THE FRACTIONS OBTAINED FROM CHROMATOFOCUSING OF MOUSE HEPATIC CYTOCHROME P-450

Fraction	*Elution pH*	*Absorption maximum (nm)*	*Molecular weight (× 1000)*	*Specific activity (nM/g/min)*	*Total P-450 recovery (%)*
b	8.3	449.0	45	–	
c	7.8	451.0	49	5.1	69.3
f	7.0	451.0	51, 56	3.3	5.8
g	6.7	449.0	46, 51, 53	1.7	1.7
h	6.6	451.5	46, 51	1.3	1.2
i	6.0–6.6	451.0	50, 55	1.6	7.8
j	6.0	451.0	50, 55	0.8	11.6
k	5.8	450.0	50, 55, 61	1.1	1.0
l	5.7	448.0	–	0.8	0.3
m	5.6	449.0	55, 57	1.3	1.5

Compounds eluted with salt at the end of the chromatofocusing phase were mainly cytochrome b_5 and cytochrome P-450 reductase.

Reconstitution experiments were successful for every cytochrome P-450-containing fraction and produced metabolites from both Δ^9-THC (I) and testosterone. Fractions not containing P-450 were inactive. All fractions from both untreated and phenobarbitone-pretreated mice produced the two major monohydroxy allylic



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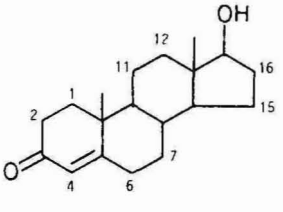
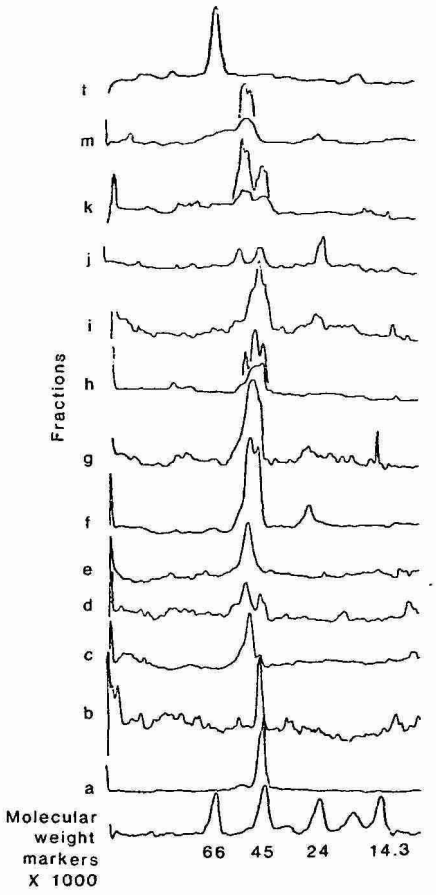


II

TABLE II

PROPERTIES OF THE FRACTIONS OBTAINED FROM CHROMATOFOCUSING OF PHENOBARBITONE-PRETREATED MOUSE HEPATIC CYTOCHROME P-450

Fraction	*Elution pH*	*Absorption maximum (nm)*	*Molecular weight (× 1000)*	*Specific activity (nM/g/min)*	*Total P-450 recovery (%)*
a	8.3	449.5	45, 54	0.3	1.2
b	8.0	451.5	47, 51	1.7	17.3
c	7.3–8.0	451.0	49, 51	2.9	29.6
d	7.3	451.5	49	1.7	21.8
e	7.0	451.0	51	1.0	8.5
f	6.6	451.5	51	1.4	12.5
g	6.2	450.0	51, 59	0.8	3.7
h	5.7	449.0	60	0.4	2.2
i	5.4	449.0	50, 59	0.3	3.3



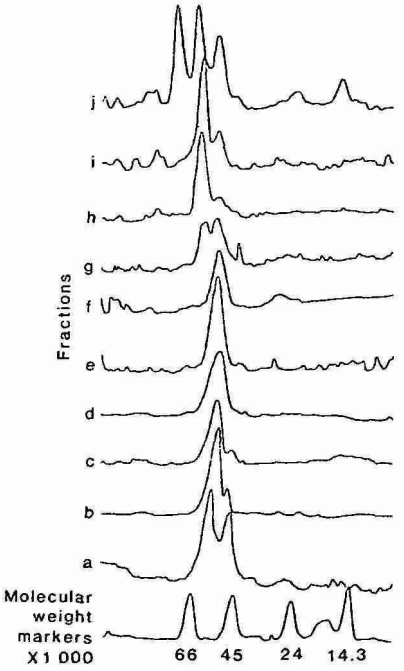


Fig. 3. Scans of SDS-PAGE of fractions from chromatofocusing of mouse hepatic cytochrome P-450.

Fig. 4. Scans of SDS-PAGE of fractions from chromatofocusing of phenobarbitone-pretreated mouse hepatic cytochrome P-450.

metabolites of Δ^9-THC, namely 8α- and 11-hydroxy-Δ^9-THC, although in different proportions. Fractions k–m from the untreated mice produced the side-chain monohydroxy metabolites, 3′- and 4′-hydroxy-Δ^9-THC with 3′-hydroxy-Δ^9-THC being the major metabolite in fraction m. This fraction also produced large quantities of a fourth but, as yet, unidentified metabolite. The profiles from the phenobarbitone-pretreated mice were similar in that all fractions, except fraction f, produced more 8α- than 11-hydroxy-Δ^9-THC and, in addition, this fraction produced substantial quantities of another unidentified metabolite. This metabolite was detected in the profile from fraction f from the unpretreated mice but not in any of the other fractions. Identification of the known metabolites was based on a comparison of their GC–MS properties with those of authentic standards and with published spectra[19]. Full details of the metabolites of Δ^9-THC identified in these fractions will be published later.

Monohydroxy metabolites of testosterone (II) were identified by a comparison

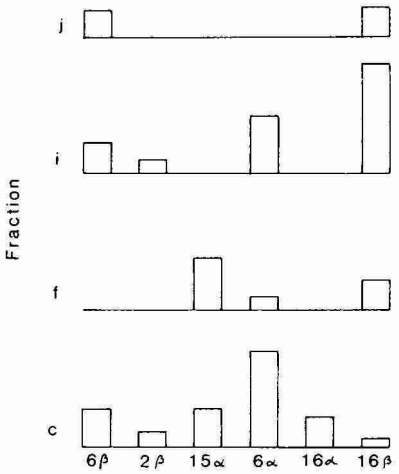


Fig. 5. Testosterone metabolites identified by GC–MS from reconstituted fractions following chromatofocusing of mouse hepatic cytochrome P-450. Compounds are listed in order of elution from the GLC column.

of their GC–MS properties with those of published spectra as follows; 2-β-, 16-α- and 16-β-[20], 6-α-[21], 6-β-[22] and 15-α-hydroxy-testosterone[23]. Their relative proportions produced in four fractions from the unpreteated mice are shown in Fig. 5.

Table III lists the properties of the cytochrome P-450 reductase present in microsomes and recovered from the affinity column.

DISCUSSION

With this study we have demonstrated the use of chromatofocusing as a technique for preliminary fractionation of cytochrome P-450 isozymes. Most fractions, designated by letters in the figures, obtained by combination of the individual fractions from the column, contained mixtures of enzymes, but this can partly be ascribed to the way in which the individual fractions from the chromatofocusing column were combined. The highest purity from the experiments described, was obtained with

TABLE III

PROPERTIES OF THE CYTOCHROME P-450 REDUCTASE ISOLATED FROM MOUSE LIVER MICROSOMES

Fraction	*Total protein (mg)*	*Total* activity (μM/min)*	*Specific activity (μM min^{-1} mg^{-1})*	*Degree of purification*	*Yield (%)*
Microsomes	585.0	98	0.167	1	100
Solubilized microsomes	516.0	145	0.281	1.68	149
Affinity column	2.1	55	26.2	157	56

* Activity is expressed as *μM* of cytochrome *c* reduced per minute at 30°C.

fraction 42 from the phenobarbitone-pretreated mice. A gel scan of the SDS-PAGE experiment indicated that 97% of the material in the P-450 was concentrated in a single band (mol. wt. 47 000). Better control over the combination of the individual column fractions would undoubtedly allow more of the isozymes to be obtained in a reasonably pure state. This technique, however, appears to be a useful method for preliminary fractionation of the isozymes and provides a number of physical characteristics as listed in Tables I and II. Recovery was generally good and twice that obtained by Huang *et al.*[24] who used DEAE-cellulose fractionation for mouse cytochrome P-450.

The only other report of cytochrome P-450 separation by chromatofocussing appears to be that of Pasanen and Pelkonen[7] who used the technique to separate twenty-one pools of the enzyme over the pH range 5–7.5. Their technique differs from ours in that preliminary purification was accomplished by chromatography on both phenyl-Sepharose and DEAE-cellulose and thus has the capability of achieving a more complete separation. Our method, however, has been demonstrated to work on a less pure preparation of the original enzyme mixture and to achieve substantial separation of the isozymes.

As it has been suggested that the elution pH of proteins from chromatofocusing columns can be considered as approximations of their isoelectric points[25], the elution pH values determined in the present experiments can be compared with the published pI values of cytochrome P-450. These are, for example, in the same range (pH 5.0–8.0) as those observed for cytochrome P-450 from rat liver[26,27]. Results from separation of cytochrome P-450 from the mouse[28] on DEAE-cellulose suggest the presence of two isozymes with p*I* values above pH 7.8 and three below. The elution of phenobarbitone-induced mouse liver isozymes[24] from DEAE-cellulose suggests two isozymes with p*I* values above pH 7.7 and two below. Chromatofocusing has given more accurate estimates of these values and has also suggested the presence of more isozymes; eight from the untreated mice and seven from the phenobarbitone-induced animals. The p*I* values of cytochrome P-450 reductase and cytochrome b_5 from the rat[27] of 5.4 and 4.9, respectively, confirm that these enzymes should be retained on the chromatofocusing column under the conditions used.

Experiments on the suitability of isoelectric focusing to separate microsomal proteins[29] demonstrate that these techniques can produce artefactual heterogeneity. It was suggested that this could be caused by the binding of carrier ampholites and strong protein-protein interactions. In these chromatofocusing experiments, these problems may have been overcome by the use of Polybuffers which were subsequently removed by calcium phosphate gel extraction and by using a sample loading of only 2.5 mg protein per ml of chromatofocusing gel.

Reconstitution experiments demonstrated that the fractions isolated from the chromatofocusing column were active and that they metabolized the two substrates tested with a certain degree of specificity. As most of the fractions contained a mixture of isozymes it is not possible from these experiments to determine the individual catalytic profile of each isozyme but the results are generally in agreement with work of others that some of the isozymes catalyse the production of individual metabolites. It is interesting that for Δ^9-THC, the major 8α- and 11-hydroxy-metabolites appear to be produced by most of the isolated isozymes whereas the production of the minor metabolite in the mouse[30], 3′-hydroxy-Δ^9-THC is much more specific. This undoubt-

edly explains why side-chain hydroxylated metabolites are produced in higher concentration by the lung[31] where the profile of cytochrome P-450 isozymes would be expected to be different. Work is underway to purify these enzymes further and to characterize fully their catalytic activity.

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CHROM. 18 271

TRACE ANALYSIS OF EXPLOSIVES BY THERMOSPRAY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A series of explosives were analyzed by thermospray high-performance liquid chromatography–mass spectrometry (TSP-HPLC–MS). Both positive and negative ion spectra were acquired in thermospray and chemical ionization (filament-on) modes. The explosives proved most sensitive in the negative ion mode under chemical ionization conditions. The spectra for the explosives analyzed provided molecular weight information with few fragment ions. Detection limits from 200 pg for trinitrotoluene to 5 ng for ammonium picrate were obtained under full scan mass spectrometer conditions. TSP-HPLC–MS proved useful in separating and identifying components in commercial explosive mixtures without interferences from plasticizers as well as in detecting explosives from hand swabs with excellent sensitivity and specificity. The plasticizers primarily were detected in the positive ion mode while the explosives were most sensitive with negative ion detection.

INTRODUCTION

The analysis of explosives has become of increasing importance in both forensic and environmental applications[1]. The analysis of post-explosion residues in debris material is important in the criminalistic investigation of a bombing because it can help find the link between the explosive used and the suspect. The resulting analysis of the explosive could later on be used as evidence in judicial proceedings. The identification of the type of explosive is often complicated by the small quantities of material available after separation from the debris on the explosion site.

During the handling of explosives, particles of explosives may adhere to the person's hands. Removal of explosive residues from the hands of suspects and their identification is therefore important. The suspect's hands are swabbed with cotton swabs soaked in a solvent. The extracts are then analyzed for traces of explosives. Only trace amounts of explosives, mixed with a variety of other organic compounds, are expected in such cases[2].

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Environmental contamination by explosives is becoming an important issue in the manufacturing and disposal of explosives. Environmental trace analysis of water and soil contaminated by explosives has become necessary due to the discharge of wastewaters produced in ammunition manufacturing and burial of obsolete ammunition and explosives in the soil.

The requirements of an analytical method for these applications should include good separation, high specificity and sensitive detection. The liquid chromatography–mass spectrometry (LC–MS) system seems to be the method of choice for these applications as it is also suitable for thermally sensitive and involatile compounds.

Several LC–MS systems have been used for the analysis of explosives[3–6]. These systems used the split-type direct liquid introduction (DLI) high-performance liquid chromatography (HPLC)–MS interface with either positive or negative chemical ionization or HPLC with off-line chemical ionization MS detection. The sensitivity of the DLI system is limited because only 1–2% of the effluent enters the mass spectrometer. On the other hand, off-line techniques require a much greater effort to collect and concentrate the fractions and can suffer a loss of the HPLC resolution.

In an effort to find a more sensitive LC–MS method for these applications, a thermospray (TSP)-LC–MS system has been used for the separation and identification of a series of standard and technical explosive mixtures, using positive and negative ion detection, with and without filament chemical ionization. The TSP interface appears to offer the greatest capability for analyzing explosives separated by HPLC with high sensitivity. The interface allows high flow-rates (near 2.0 ml/min) of aqueous solvents and volatile buffers into the system and provides the gentle ionization necessary to obtain molecular ion[7–10].

The pure compounds studied included 2,4,6-trinitrotoluene (TNT), 2,4,6-N-tetranitro-N-methylaniline (tetryl), 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX), 1,3,5,7-tetranitro-1,3,5,7-tetrazacyclooctane (HMX), pentaerythritol tetranitrate (PETN) and ammonium nitrate. Two technical mixtures were examined, one an explosive used in letter bombs containing RDX and PETN and another explosive containing TNT, nitroglycerin (NG), 2,4,6-trinitrophenol (picric acid), ammonium nitrate and 2,4-dinitrotoluene (DNT).

EXPERIMENTAL

Samples

Pure and technical explosives were obtained from the Israeli Police Analytical Laboratory. The standards were dissolved in acetone or acetonitrile (HPLC-grade) to known concentrations. Solvent used in this study were HPLC-grade and were filtered through 0.45-μm filters before use. Hand swabs on commercial explosive mixtures were performed by holding about 1 g of explosive in one of the author's hands for 1 min followed by wiping the hands with a paper towel. After 2 min a cotton swab soaked in acetone was wiped across the hand. This swab was washed in 4 ml acetone to remove the residual explosives from the cotton and then the acetone was evaporated with nitrogen to about 0.2 ml. About 50% of the extract was injected for HPLC–MS analysis.

HPLC–MS

Many details of the HPLC–MS system and operation have been given earlier[11]. The HPLC separations were performed on a Zorbax C_8, 25 cm × 4.6 mm I.D. column using a gradient of 25% methanol to 75% methanol in water containing 0.1 *M* ammonium acetate at 1.4 ml/min in 15 min. The final conditions were held for 10 min before cycling back to the initial conditions for the next analysis.

The Vestec thermospray interface (Vestec, Houston, TX, U.S.A.) was used for the HPLC–MS analysis of explosives. The vaporizer was operated at 170–180°C which resulted in the most intense and stable ion current for TNT. Source block temperatures from 120 to 300°C (by 50°C) were evaluated for both positive and negative ion detection of explosives. Low temperatures were preferred if molecular weight information was to be obtained from these thermally unstable compounds. However, temperature played an important role in negative ion detection. Electron-capture ionization of explosives showed the best sensitivity at the lowest operating temperatures (120°C) while providing abundant molecular weight information. Positive ion detection was less temperature-dependent and the source could be operated at 120–180°C without significant changes in the spectra or sensitivity. When the TSP interface was operated in the chemical ionization mode, a 1000 eV filament was turned on and the emission set to 0.15 mA. The mass spectrometer was operated under full scan acquisition (*m/z* 150–650) with a 2-s scan. All detection limits were obtained using full scan acquisition.

RESULTS AND DISCUSSION

The positive ion and mass chromatograms (TSP ionization) of a standard mixture are shown in Fig. 1. Only three components were observed: RDX, HMX and tetryl. The others (PETN, ammonium picrate and TNT) were not amenable to TSP ionization in the positive ion mode. The mass spectrum of RDX contained one major ion at *m/z* 240 which is due to $[M+NH_4]^+$. These types of ions are typical to TSP-LC–MS where ammonium acetate is used as a buffer[9,11]. Similarily, the mass spectrum of HMX contained one major ion at *m/z* 314, again due to $[M+NH_4]^+$.

The mass spectrum of tetryl (Fig. 2) had its base peak at *m/z* 290 due to $[M+NH_4-CH_3]^+$, a common ion in TSP-LC–MS. Other major ions were $[M+NH_4]^+$ at *m/z* 305 and the ion at *m/z* 242 which is a typical ion in both the electron ionization and chemical ionization mass spectra of tetryl[12,13]. This ion is due to loss of CH_3NO from the molecular ion.

Switching on the filament in positive ion detection mode did not produce any different mass spectra in this mixture other than some additional low abundance fragment ions.

The negative ion and mass chromatogram (TSP ionization) of the same standard mixture (Fig. 3) detected all six components. The mass spectrum of TNT contained one major ion, $[M-H]^-$, at *m/z* 226. The mass spectrum of tetryl (Fig. 4) had its base peak at *m/z* 241 due to the $[M-NO_2]^-$ ion. It contained another major ion at *m/z* 257, due to $[M-NO]^-$. Other characteristic ions, but of low abundance, were $[M+CH_3O]^-$ at *m/z* 318 and $[M+H]^-$ at *m/z* 288.

The mass spectrum of RDX contained mainly one ion, $[M+CH_3COO]^-$, at *m/z* 281 (typical to TSP-LC–MS) and a low abundance ion (<2%)

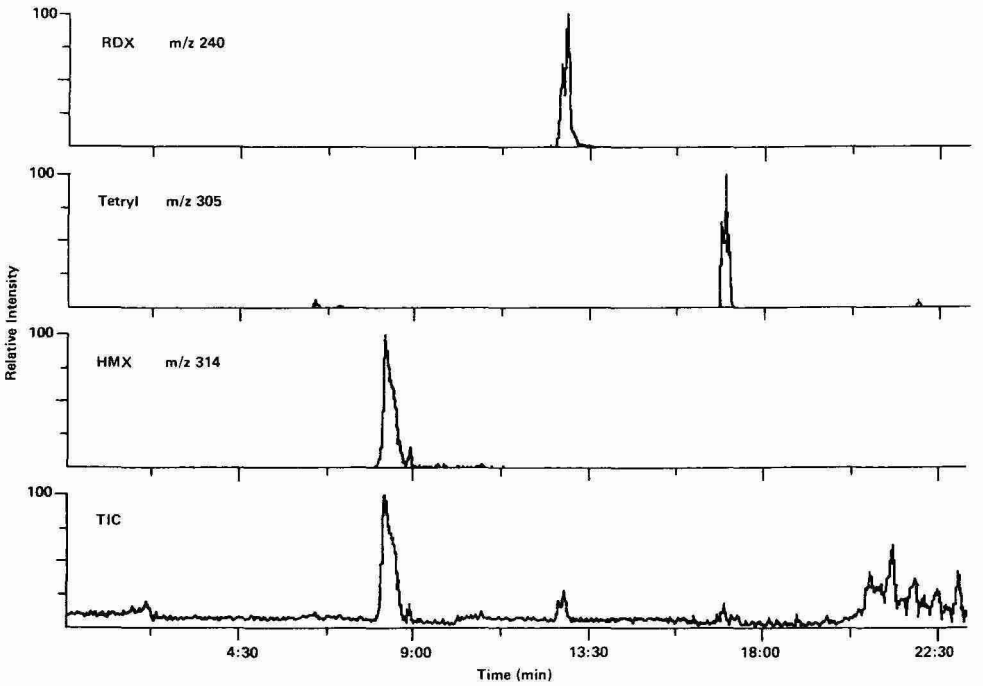

Fig. 1. Ion chromatogram and total ion current (TIC) for positive ion TSP ionization HPLC–MS analysis of the standard explosive mixture (5 μg injected). Note only RDX, HMX and tetryl were detected in the mixture. TNT, ammonium picrate, and PETN were not detected in the positive ion mode.

$[2M + CH_3COO]^-$ at *m/z* 503. The mass spectrum of HMX contained one major ion, $[M + CH_3COO]^-$, at *m/z* 355. Also, an ion at *m/z* 651, due to $[2M + CH_3COO]^-$, having an abundance of about 1% was observed. The mass spectrum of PETN (Fig. 5A) had a base peak at *m/z* 375, due to $[M + CH_3COO]^-$ and major ions at *m/z* 378, due to $[M + ONO_2]^-$, and at *m/z* 315, due to $[M - H]^-$ (ref. 14). An additional series of ions were observed at *m/z* 330, 285, 240 and 195. These ions were formed by

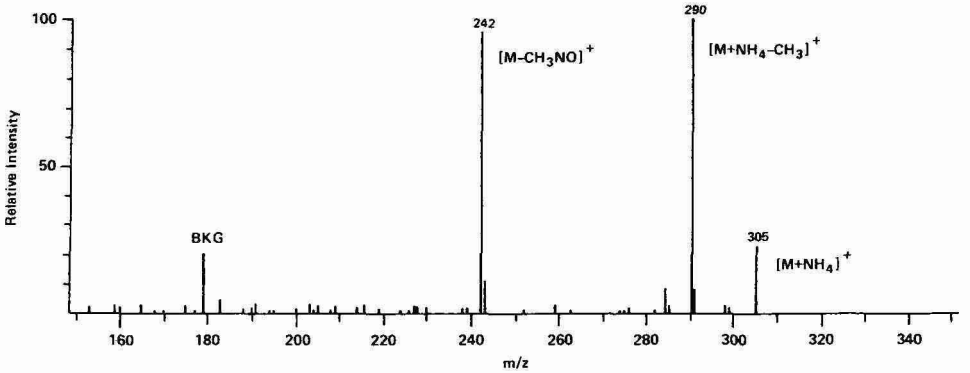

Fig. 2. TSP ionization positive ion spectrum of tetryl.

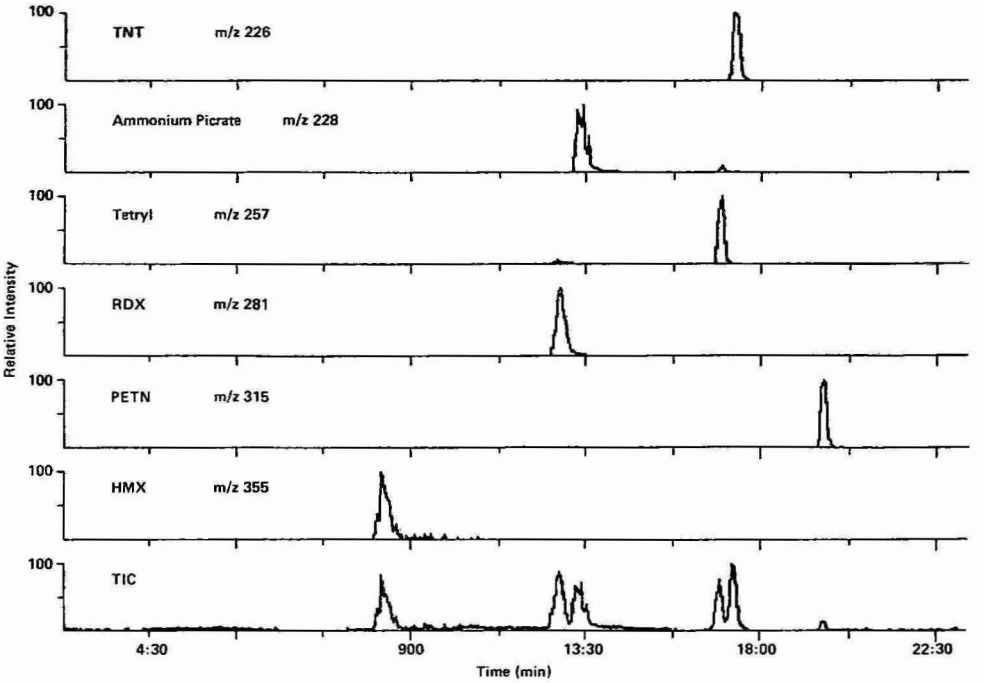

Fig. 3. Ion chromatograms and TIC chromatogram for negative ion TSP ionization of standard explosive mixture (5 μg injected).

consecutive losses of 45 mass units from the $[M+CH_3COO]^-$ ion at m/z 375. In order to explain the mechanistic formation of these ions we looked at the negative ion mass chromatogram of pure PETN (Fig. 5B). The chromatographic mass peak due to $[M+CH_3COO]^-$ was broader than the $[M-H]^-$ peak. Each consecutive loss of 45 mass units produced an even broader peak: the peak at m/z 330 was broader than the one at m/z 375, and the peak at m/z 285 was broader than the one at m/z 330. This consecutive loss of 45 mass units could be due to losses of COOH from

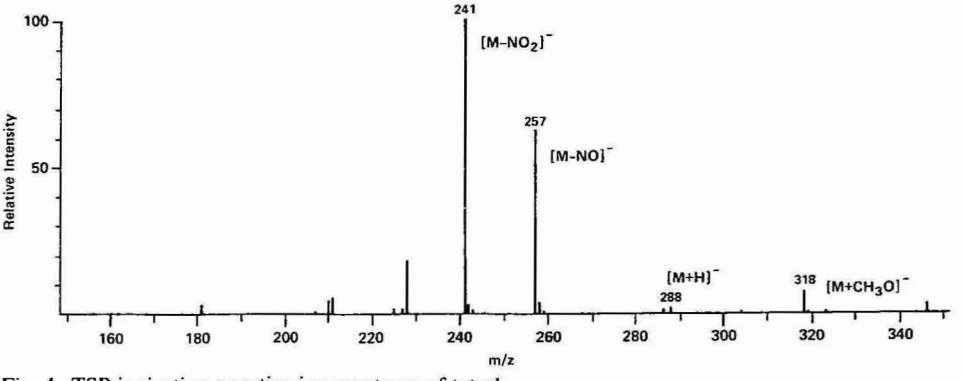

Fig. 4. TSP ionization negative ion spectrum of tetryl.

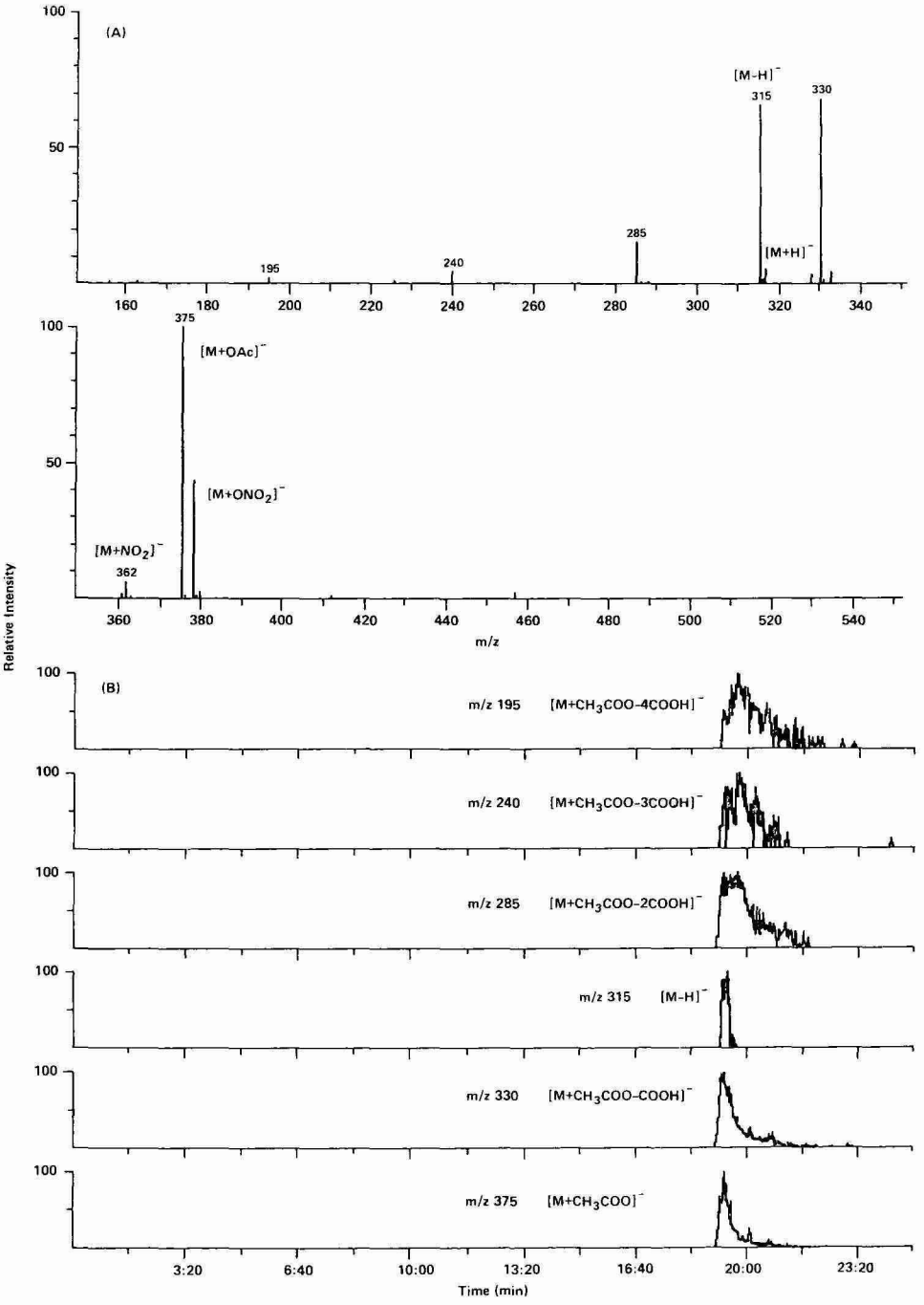

Fig. 5. (A) TSP ionization negative ion spectrum of PETN. (B) Ion chromatograms for PETN showing $[M+CH_3COO]^-$ anion with consecutive losses of 45 mass units (*m/z* 330, 285, 240, 195). These decomposition ions exhibit an increasing peak width with decreasing mass compared to the $[M-H]^-$ ion at *m/z* 315.

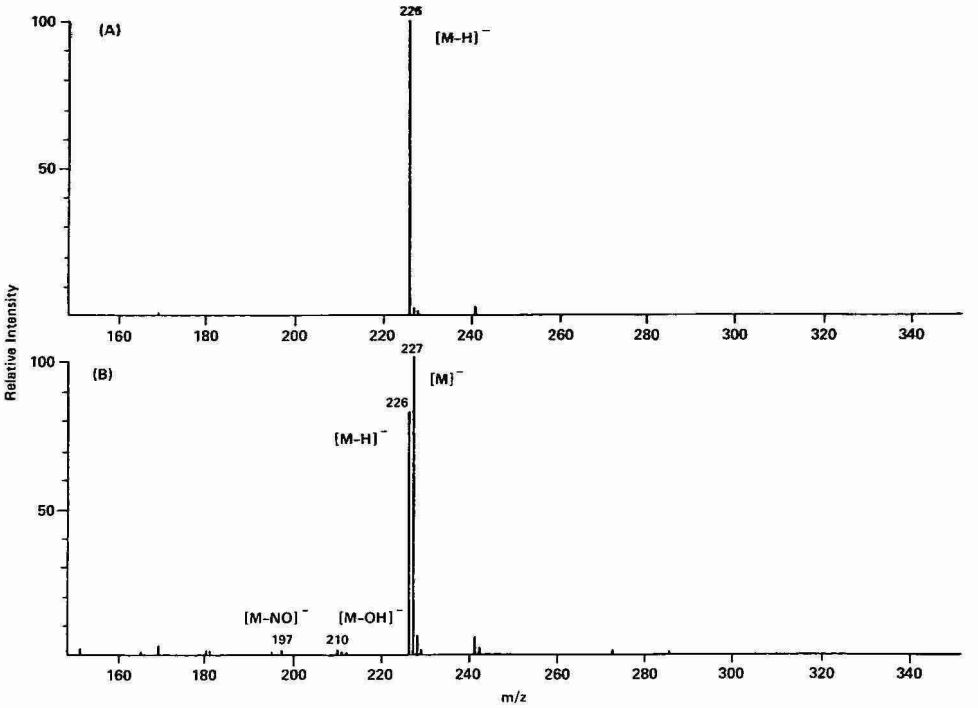

Fig. 6. HPLC–MS spectra of TNT. (A) Negative ion detection TSP ionization. (B) Negative ion detection filament ion chemical ionization.

$[C(CH_2ONO_2)_4 + CH_3COO]^-$ ion followed by rearrangements. Also, this could have occurred through thermal decomposition of $[PETN + CH_3COO]^-$ on the hot source block surface. The peak broadening and tailing behavior is probably explained by the condensation of PETN aerosol on the source block wall with subsequent thermal decomposition and possible reaction with the solvent system[15]. More experiments with isotopically-labelled PETN need to be performed to verify these assumptions.

The mass spectrum of ammonium picrate had one major ion, $[M-NH_4]^-$, at m/z 228. The loss of NH_4 from ammonium picrate had been observed in its positive ion mass spectra in both electron ionization[12] and chemical ionization[16].

When switching on the filament, the only difference was in the mass spectrum of TNT. Fig. 6A and B shows the mass spectra of TNT without and with filament-generated reagent ions respectively. In addition to the $[M-H]^-$ ion, an $[M]^-$ ion at m/z 227 was produced, forming the base peak. Low abundance ions[17,18] were observed at m/z 210, $[M-OH]^-$, and at m/z 197, $[M-NO]^-$.

Sensitivity

Better sensitivity was obtained in both positive and negative ion mode with filament-on. In the positive ion mode, when a sample of the standard mixture was injected, only RDX, HMX and tetryl were observed, as stated earlier. Less than 5 ng of RDX and HMX could be detected. However, the minimal amount of tetryl which

could be detected was more then 50 ng. In the negative ion mode, 200 pg of TNT, 1 ng of RDX, HMX, and PETN (Fig. 7) could be detected. The detection limits for tetryl were determined to be 5 ng and ammonium picrate to be 50 ng.

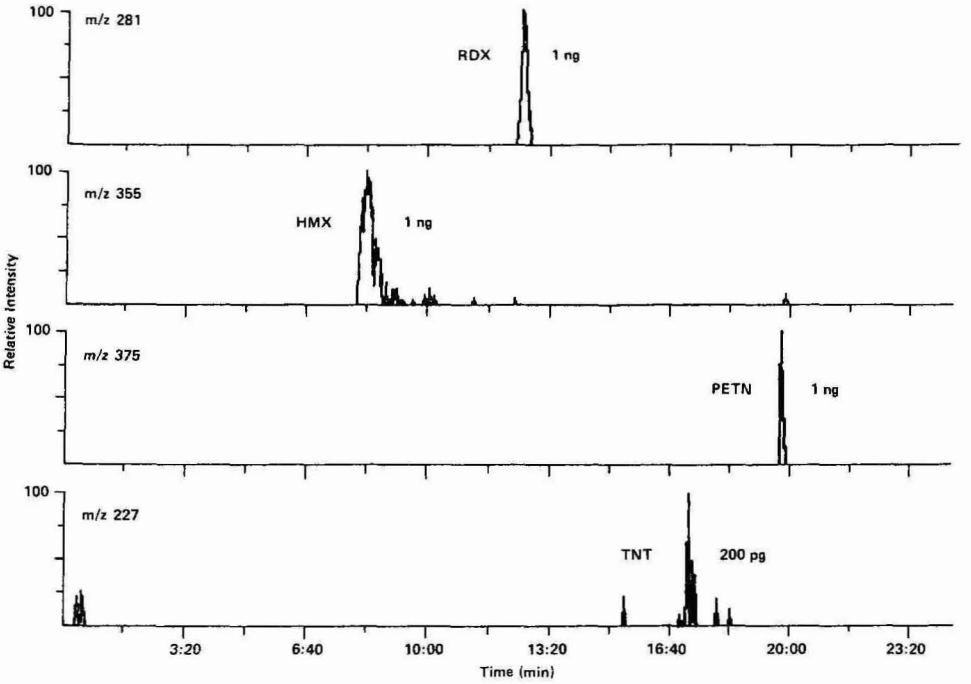

Fig. 7. HPLC–MS ion chromatograms for 200 pg of TNT and 1 ng of each of RDX, HMX and PETN using negative ion detection with filament chemical ionization.

Analysis of commercial explosive mixtures

The analysis of a letter bomb explosive using both positive and negative filament chemical ionization is shown in Fig. 8. It is interesting to note that positive ion detection was specific for the plasticizers (early eluting peaks in the total ion current) while negative ion detection was specific for the explosives. The letter bomb explosive was made from a mixture of RDX and PETN (RDX is also detectable in positive ion detection, but with less sensitivity).

Another explosive mixture examined by TSP-HPLC–MS is shown in Fig. 9. This technical explosive mixture contained DNT, TNT, NG, picric acid and ammonium nitrate along with various isomers of the above ingredients. At least three isomers of TNT were observed at m/z 227. The isomer having the highest concentration was the 2,4,6-isomer. This was proven by comparing the retention time of this isomer with the retention time of a standard of 2,4,6-TNT. Similarily, the three isomers of DNT, observed at m/z 182, contain the 2,4-DNT at highest concentration. The mass spectrum of picric acid contained one major ion, $[M]^-$ at m/z 229. The

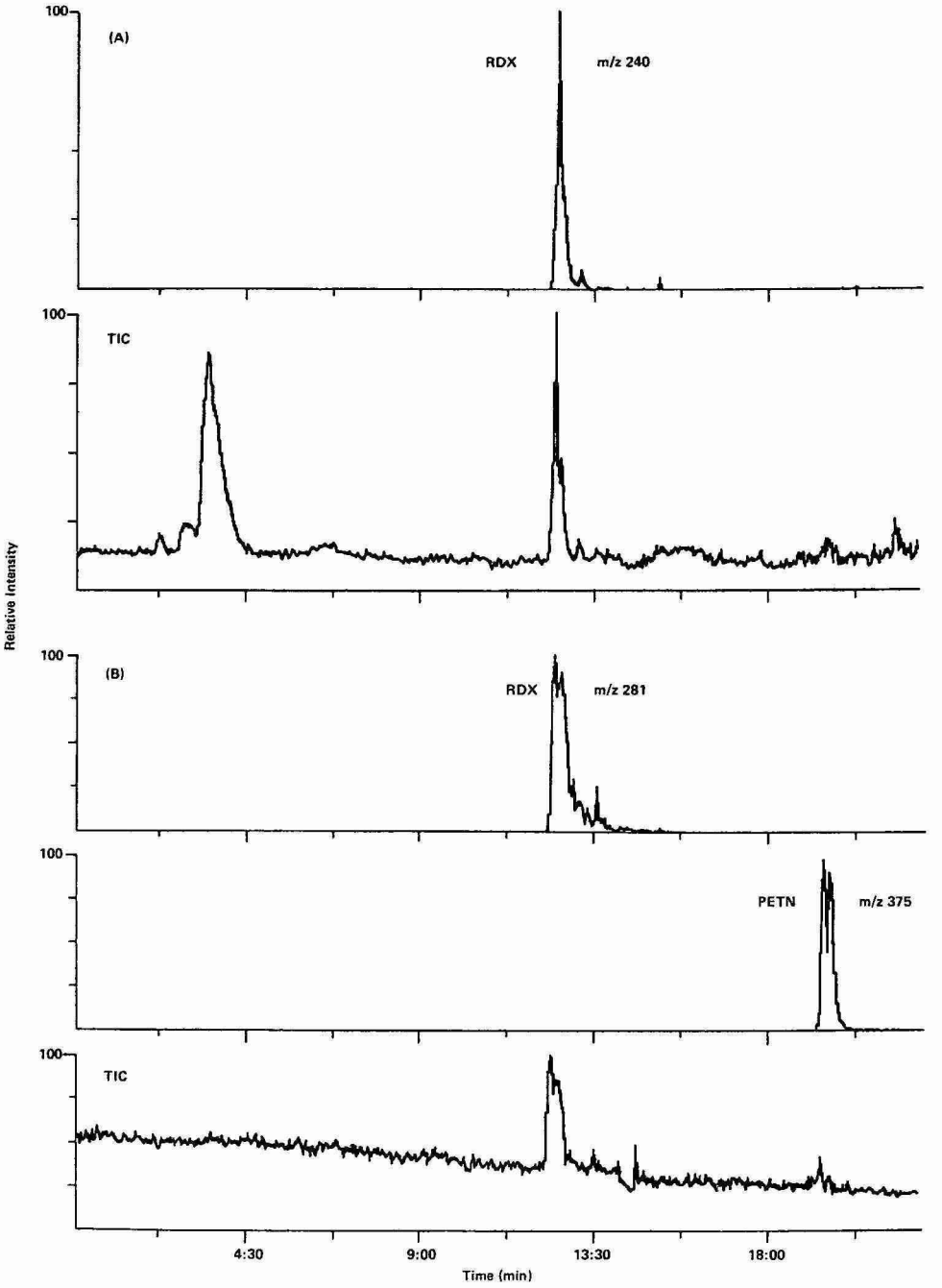

Fig. 8. HPLC–MS ion and TIC chromatograms for the analysis of a commercial letter bomb explosive using filament chemical ionization. (A) Positive ion detection. (B) Negative ion detection.

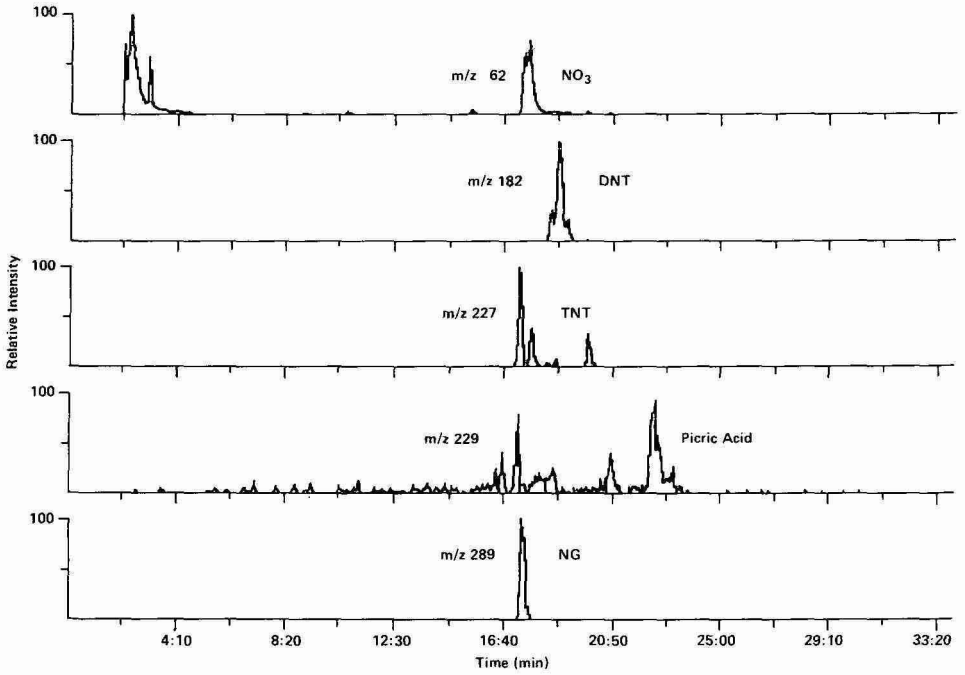

Fig. 9. HPLC–MS ion chromatograms for ammonium nitrate, DNT, TNT, picric acid and NG found in a technical explosive using filament negative ion chemical ionization.

other peaks in the ion chromatogram were due to isotope ions from TNT and isomers of picric acid. Ammonium nitrate produced one major ion, $[NO_3]^-$, at *m/z* 62. The mass spectrum of the last component, NG, (Fig. 10) had a base peak at *m/z* 289, formed by $[M+ONO_2]^-$ (ref. 14). Additional major ions in the spectrum were $[M+CH_3COO]^-$ at *m/z* 286, $[M]^-$ at *m/z* 227, and $[M-H]^-$ at 226. As in PETN, two ions were also formed by consecutive losses of COOH from the $[M+CH_3COO]^-$

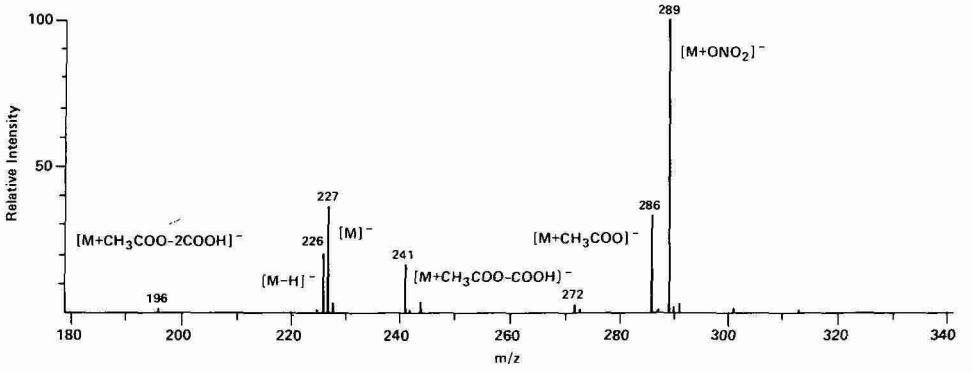

Fig. 10. Negative ion HPLC–MS spectrum of NG from a technical explosive mixture (filament chemical ionization).

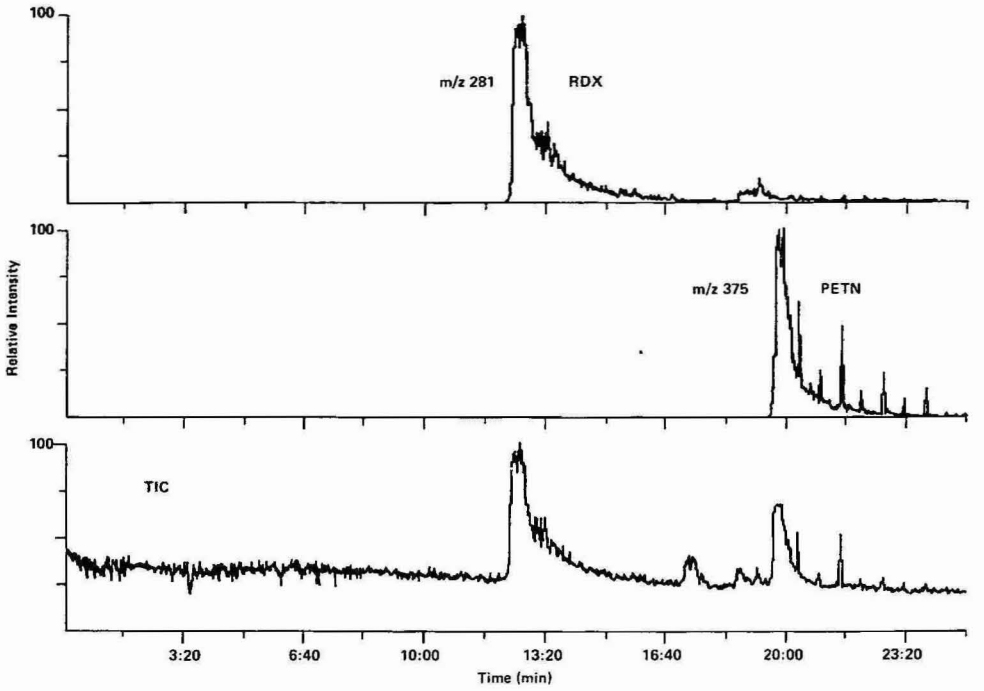

Fig. 11. Hand swab analysis of a letter bomb explosive in negative filament chemical ionization mode. The HPLC–MS ion chromatograms show the presence of RDX and PETN with few interferences.

ion: at *m/z* 241 and *m/z* 196. Also, NG exhibited an ion at *m/z* 62 $[NO_3]^-$ as seen in Fig. 9.

The positive ion chromatogram (filament chemical ionization) of this mixture does not show any of the explosive components but reveals three peaks which represent the additives (including the plasticizer) of this explosive mixture. The retention times of these three peaks were similar to the retention times of the additive peaks in the ion chromatogram of the letter bomb mixture (Fig. 8A), In order to demonstrate the sensitivity of the system for the detection and identification of traces of explosives, each one of the explosive mixtures was held (in two separate experiments) by one of the authors. The acetone extracts produced the mass chromatogram presented in Fig. 11 (letter bomb explosive) and Fig. 12 (technical explosive). The main explosive ingredients in both experiments can be clearly characterized and match the previous recorded spectra. The high ion count recorded points out that, even in cases were the time of contact with the explosive was shorter, or a longer time has passed between the handling of the explosive and the hand swab, it would be possible to detect the explosives in the extracts. However, this should be the subject of a separate study.

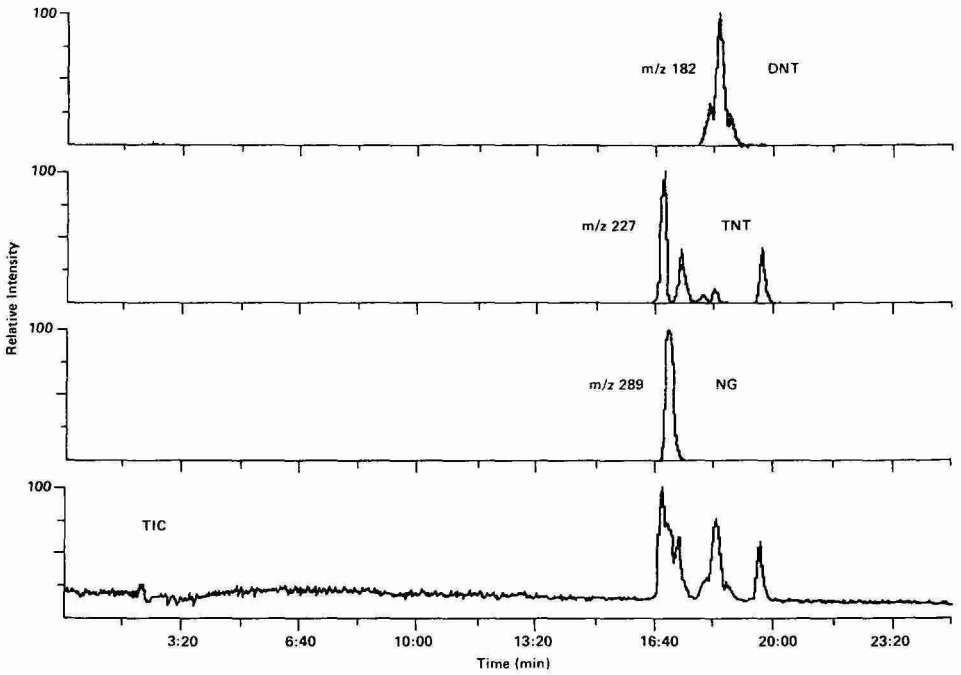

Fig. 12. Hand swab analysis of a technical explosive in negative filament chemical ionization mode. The HPLC–MS ion chromatograms clearly show the presence of DNT, TNT and NG with no interferences.

CONCLUSIONS

TSP-HPLC–MS proved very sensitive and specific for analysis of explosives. Sensitivities of a number of explosives are comparable to other analytical techniques for detection of explosive residues[2]. The real advantage of TSP-HPLC–MS is in the area of specificity. No interference with the target explosive was detected in the commercial mixture or hand swab extracts. Even when components do co-elute or elute very closely (such as in the case of the technical explosive containing DNT, TNT and NG which have very close retention times) the individual components can be resolved by mass.

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CHROM. 18 270

DEVELOPMENT OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODS FOR TRACE METAL ANALYSIS

DETERMINATION OF TRACE COBALT IN SIMULATED PRESSURIZED WATER REACTOR PRIMARY COOLANT

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(First received September 19th, 1985; revised manuscript received October 7th, 1985)

SUMMARY

A high-performance liquid chromatographic system was developed to determine trace metals in simulated Pressurized Water Reactor primary coolant. Cobalt is the main species of interest in this report and an on-line preconcentration column was used to increase sensitivity. Separation was achieved using an Aminex A9 analytical column and a mobile phase containing tartaric acid. Detection was by post-column reaction with eriochrome black T and up to ten metal species could be separated using this system. Good linear calibrations were obtained from 2.5–100 ng of cobalt absolute weight, using volumes up to 200 ml. The detection limit for a 200-ml sample was 0.01 ng ml^{-1}.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) for rapid, multi-element trace analysis, has increased steadily in the past ten years, with a number of separation and detection methods having been developed[1–4]. One of the major advantages of HPLC is its potential for on-line and automated analysis of streams and circuits in a variety of industrial processes. It was considered that an HPLC system could be ideal for the monitoring of trace metal species in the primary coolant of a pressurized water reactor (PWR). Such an HPLC system would not only allow continuous on-line monitoring of chemical and physical transients, but also reduce the problem of adventitious sample contamination associated with discrete sampling methods. A further advantage is the multi-element capability which would help to keep the number of analytical techniques required to a minimum.

The ratio of metal alloys in contact with the primary coolant, in terms of surface area, is *ca.* 75% Inconel, 20% Zircalloy and 5% stainless steel. The influence of PWR circuit chemistry conditions on the release and deposition of corrosion products species such as manganese, iron, cobalt and nickel, has to be studied and optimized to minimize out-of-core radiation fields. There is particular interest in ^{59}Co

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since it is the precursor of ^{60}Co, the predominant activation product found in circuit deposits. Reducing out-of-core activity is important as it minimizes maintenance costs and therefore assists in the economic viability of the plant.

A number of difficulties have been experienced in the analysis of the coolant by currently available techniques. These arise from a combination of very low elemental concentrations [0.01–1.0 ng ml^{-1} (ppb)] and interferences caused by lithium and borate ions present in the coolant. At present the determination of manganese, iron, cobalt and nickel is carried out by either the lengthy preconcentration onto ion-exchange filters followed by X-ray fluorescence and/or electrothermal atomic absorption spectroscopy. Recently differential pulse polarography (DPP)[5] and differential pulse stripping voltammetry[6] have been proposed as alternative methods of analysis, with detection limits in the order of 0.01–1.0 ng ml^{-1}, for nickel and cobalt; however, such methods cannot be readily adapted to on-line automation.

Electrochemical[4] and photometric[1] measurement of metal complexes are the two main detection systems currently used in the inorganic application of HPLC, with reported detection limits in the range 0.1–1.0 ng ml^{-1}. In order to achieve limits in the pg ml^{-1} range, a number of approaches have been adopted, namely: the use of large injection volumes[7], new detector designs[8] and the use of fluorescent[9] and radiochemical[10] detection systems. Sample preconcentration, either directly onto the chromatography column[11] or onto a precolumn[12], offers the most versatile approach to ultra-trace analysis.

This paper describes the development of an HPLC method for the analysis of trace metal species in both simulated PWR primary coolant and distilled deionised water samples, with particular reference to the analysis of cobalt at the pg ml^{-1} level.

EXPERIMENTAL

Apparatus

The liquid chromatographic system (Fig. 1) consisted of a high-pressure stainless-steel eluent pump (Constametric III; Laboratory Data Control (LDC), Riviera Beach, FL, U.S.A.) and a high-pressure stainless-steel reagent pump (Model AA dual-piston Eldex pump; Eldex Labs., Mento Park, CA, U.S.A.). Sample enrichment was carried out with a high-pressure PTFE-lined sample plump (Model AA dual-piston Eldex pump), connected to a Model 7010 Rheodyne injector valve (Rheodyne, Cotati, CA, U.S.A.) fitted with a preconcentration column. The detector was a double beam UV–visible spectrophotometer (SF770; Shoeffel, Westwood, NJ, U.S.A.), and the 1 cm length flow-cell of 10 μl capacity was monitored at 610 nm. The LDC pump, the data from the spectrophotometer and the printer (Houston Model, LDC) were controlled by a central microprocessor control unit (Control Command Module, LDC).

The analytical column was maintained at 60°C by immersion in a Grant-type JB1 water-bath (Grant Instrumentation, Cambridge, U.K.). The conditions used throughout were an eluent flow-rate of 1.5 ml min^{-1}, a post-column reagent flow-rate of 1.0 ml min^{-1}, and a PTFE post-column reaction coil of 0.6 ml.

Materials and reagents

Distilled deionised water (DDW) was used throughout, with all samples, stan-

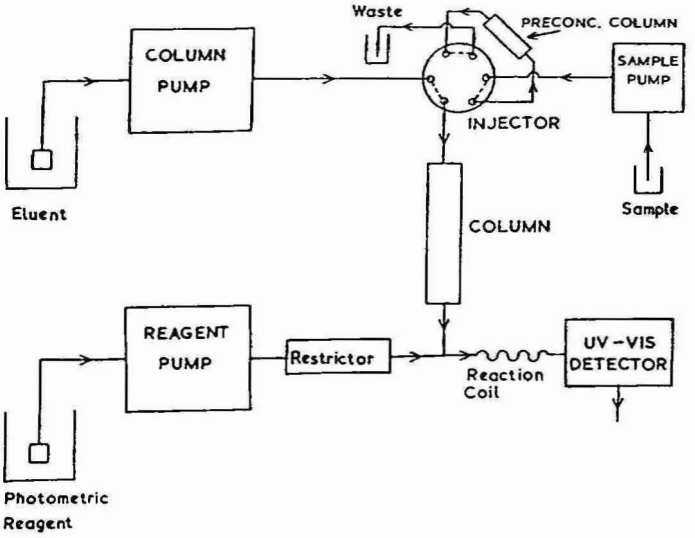

Fig. 1. Schematic diagram of apparatus.

dards and reagents being stored in polyethylene bottles, which had previously been contacted with IM Aristar-grade nitric acid for 16 h, followed by five separate washings with DDW. Whenever possible Aristar grade chemicals (BDH, Poole, U.K.) were used.

Standards were prepared by sequential dilution from 1000 ppm stock solutions. A stock solution of eriochrome black T (EBT) reagent (4 g l^{-1}), in 2 *M* ammonia was prepared, from which the following daily working solution was prepared: EBT–DDW–2 *M* ammonia (4:296:200). The lithium borate solution (LBS), contained 1200 mg l^{-1} of borate (typical of a PWR primary coolant), adjusted to pH 6.5 with 1.8 mg l^{-1} lithium hydroxide.

The cation-exchange resin (Aminex A9, 11.0 ± 0.5 μm, Bio-Rad) was slurry-packed at 3500 p.s.i. into both the 100 mm × 4.6 mm I.D. stainless-steel analytical column and the 50 mm × 4.6 mm I.D. titanium preconcentration column.

Preliminary considerations

Selection of the column material was based upon the requirement to operate over a long period of time. Therefore the cation-exchange resin (Aminex A9) was chosen because of its known long-term stability[3,12]. This necessitated the selection of a more strongly chelating polycarboxylic acid, and tartaric acid was used as eluent[3,12]. The detection system was the EBT inverse photometric method described in detail elsewhere[13], having an absolute detection limit for 100-μl injection of *ca.* 1 ng for Ni^{2+}, Co^{2+}, Mn^{2+} and Fe^{2+}. Consequently to achieve the desired detection limit of 10 pg ml^{-1}, sample preconcentration was required. Techniques such as solvent extraction and sample evaporation are not particularly suitable for this application because they are generally time-consuming and cannot readily be automated for on-line analysis. Direct preconcentration onto an analytical column requires a longer analysis time per sample, is less flexible in operation and does not offer the

facility for guarding the analytical column from contamination. Preconcentration of the sample was achieved by collecting a known volume of sample onto a 50 mm × 4.6 mm I.D. titanium precolumn, packed with Aminex A9. The preconcentrated sample was backflushed off the precolumn using 0.2 *M* tartaric acid onto an Aminex A9 stainless-steel analytical column (100 mm × 4.6 mm I.D.). After 90 s the Rheodyne injector was switched back into the load position, enabling another sample to be concentrated, while the first was being analysed. To illustrate the capability of the separation system for the determination of the metals of interest, a typical chromatogram is shown in Fig. 2.

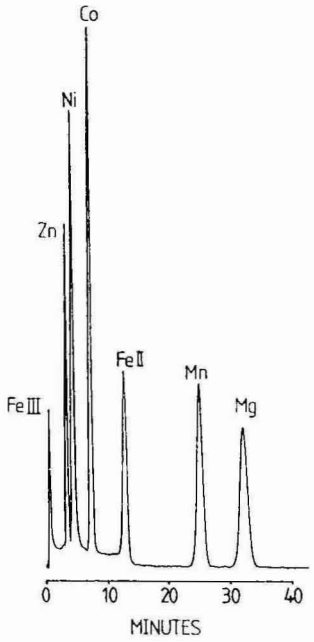

Fig. 2. Separation of mixed metal standard (100-μl injection). Metal ion concentrations: 5 ppm Fe^{3+}, Zn^{2+}, Ni^{2+}, and Mn^{2+}; 10 ppm Mg^{25}; and 25 ppm Fe^{2+}. Chromatographic conditions: 100 × 4.6 mm I.D. Aminex A9 column; eluent, 0.2 *M* tartaric acid (pH 4.3) at 1.5 ml min^{-1}; detection, post-column reaction with $7 \cdot 10^{-5}$ *M* EBT solution pumped at 1.0 ml min^{-1}; wavelength 610 nm.

RESULTS AND DISCUSSION

Prior to the investigation into the effectiveness of the preconcentration system, it was necessary to examine the stability of extremely low concentration of metal ions in different solutions. This was assessed by measuring the peak height response for 10 ml of a 10 ppb mixed metal solution. Four different aqueous systems were investigated and preconcentration volumes were chosen to give a calibration graph for each metal over a 20–100 ng weight range.

The four aqueous systems were: (i) DDW; (ii) 0.0014 *M* nitric acid; (iii) 0.001 *M* citrate buffer; and (iv) simulated primary coolant (LBS).

The peak height response for cobalt was similar for all four aqueous systems; however, on standing 24 h, a repeat analysis showed a decrease in the peak height response for standards made up in DDW. Cassidy and Elchuk[12] also studied the stability of standards at trace levels and reported a similar effect for nitric acid solutions after 100 h. The work reported here was only carried out over a 24-h period, and showed no decrease for standards prepared in nitric acid. However, higher blanks for nickel and iron were obtained, presumably due to the greater ability of the nitric acid to leach metals from wetted components. Consequently control standards were prepared and stabilized in 0.001 *M* citrate buffer and compared with spiked solutions of simulated primary coolant water (LBS) in subsequent work.

Investigation of preconcentration system

The effectiveness of the preconcentration system for the determination of cobalt at the required concentration was investigated by considering the following criteria: (a) system contamination; (b) reproducibility of analysis; (c) quantitative performance of the preconcentration column; (d) stability of very low concentrations (ng ml^{-1}) of metal ions; (e) detection limit for large preconcentration volumes.

(a) Contamination is one of the major problems of ultra-trace analysis. When considering the effects of contamination on the system described here it is best treated as two separate units, the preconcentration section and the separation section. The separation section (the pumps connecting tubing and analytical column) is composed entirely of stainless-steel components. The tartrate eluent flows continuously and any metal leached from the stainless-steel surfaces will reach a steady state producing a constant background. However, leaching is best kept to a minimum otherwise too much of the EBT reagent will be consumed. Thus the metal frits in the analytical column, which have a very high surface area, were replaced with PTFE frits. The situation regarding the preconcentration section is very different. Any metals leached from the wetted components will build up on the preconcentration column. Since preconcentration volumes can be 200 ml of more, leaching of metals could result in significant contamination. To reduce this contamination. PTFE tubing was used for all connections, the Eldex sample pump was PTFE lined, and the preconcentration column was made of titanium with PTFE frits as bed supports. The only remaining stainless steel in the preconcentration system was the small amount in the inlet and outlet ports of the Rheodyne valve. Some contamination will still be present mainly from the reagents and deionized water. This will build up on the preconcentration column when a sample is preconcentrated affecting blank levels and thus the limit of detection. This will be discussed in section (e).

((b) The reproducibility of analysis, at both the 1 ng ml^{-1} and the 10 ng ml^{-1} levels, was determined for freshly prepared standards throughout a working day. In each case a total weight of 50 ng was preconcentrated for each determination. Table I shows the statistical interpretation of these results. The results show good reproducibility throughout a working day, and are an indication that there were no adsorption losses, even for a 1 ng ml^{-1} LBS standard.

(c) In order to investigate the quantitative response of the preconcentration column, different concentrations of cobalt in differing volumes of both LBS and citrate stabilised standard solutions were analysed. The results are shown in Table II. The absolute amounts of cobalt preconcentrated vary from 2.5 to 100 ng. The

TABLE I

REPRODUCIBILITY OF PRECONCENTRATED SAMPLES

	Citrate solutions		*LBS solutions*	
	10 ng ml^{-1}	*1 ng ml^{-1}*	*10 ng ml^{-1}*	*1 ng ml^{-1}*
Number of replicate analyses	6	8	6	8
Standard deviation	2.52	1.17	2.62	6.25
Coefficient of variation (%)	3.8	2.2	4.9	6.4

TABLE II

QUANTITATIVE RESPONSE OF PRECONCENTRATION COLUMN

	Volume concentrated (ml)	*Concentration (ng ml^{-1})*	*Weight concentrated (ng)*	*Corrected peak height*	*Normalized* peak height*
Lithium borate solutions	100	1	100	182	182
	54	1	54	86	159
	25	1	25	39	156
	190	0.1	19	34	179
	100	0.1	10	17	170
	50	0.01	5	8.5	170
	25	0.1	2.5	3.75	150
Citrate stabilized solutions	10	10	100	146	146
	5	10	50	71	142
	25	1	25	39	156
	10	1	10	14	140
	200	0.1	20	28	140
	100	0.1	10	14	140
	25	0.1	2.5	4	160

* Peak height has been normalized to give value for 100 ng of cobalt preconcentrated.

normalized figures show the response is very consistent, indicating good linearity of preconcentration over a wide range of volumes and concentrations (absolute weight range 2.5–100 ng). The minimum levels of cobalt in a PWR primary coolant may be very low (0.01 ng ml^{-1}), thus preconcentrated samples will have small absolute weights concentrated. Therefore a further set of calibration graphs for both LBS and citrate stabilized solutions were produced to assess the linearity of preconcentration of different volumes of 0.1 ng ml^{-1} solutions over an absolute weight range of 2.5–20 ng (Fig. 3). Both graphs show a good linear response for the preconcentration of large volumes of dilute concentrations of cobalt in both LBS and citrate stabilised solution.

(d) Following the initial investigation of the stability of these very low concentrations of metal ions in solution, a long term stability trial was set up, to measure the stability of solutions at both the 1 ng ml^{-1} and the 10 ng ml^{-1} concentrations.

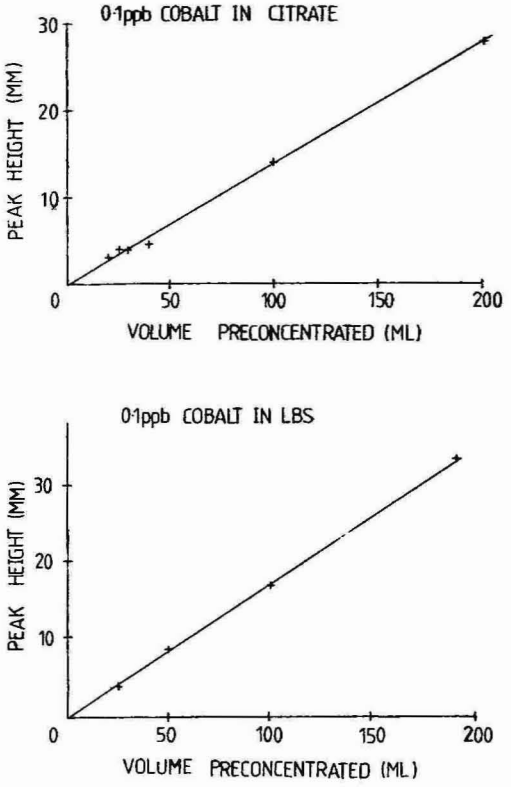

Fig. 3. Calibration graphs for blank corrected peak height response against volume preconcentrated.

In each trial 50 ng of cobalt were preconcentrated and the cobalt peak height of the aged sample was divided by the cobalt peak height of a freshly prepared standard and plotted against time, thus allowing for any variation in peak height response arising from daily preparation of eluent and post column reagent. For the 10 ng ml^{-1} and the 1 ng ml^{-1} concentrations the ratios were measured over nine days and eight days respectively. Statistical variation of these ratios against time is shown in Table III. The results demonstrate good stability of metal ion solutions at the 10 ng ml^{-1} level over a nine-day period, in citrate-stabilized solutions as well as in LBS.

TABLE III

STABILITY TRIAL OF COBALT STANDARD SOLUTIONS

	Citrate solutions		*LBS solutions*	
	10 ng ml^{-1}	*1 ng ml^{-1}*	*10 ng ml^{-1}*	*1 ng ml^{-1}*
Mean ratio	1.024	0.973	1.001	0.823
Standard deviation	0.069	0.039	0.085	0.174
Coefficient of variation (%)	6.8	4.0	8.5	21.1

However at the 1 ng ml^{-1} level over an eight-day period, the LBS was less stable, giving a coefficient of variation of 21.1%. The citrate-stabilized solution shows better long-term stability at very low concentrations and is therefore considered more suitable for making up reference standards.

(e) To assess the detection limit, large volumes of spiked samples and blanks were preconcentrated and analysed. Results are shown in Figs. 4 and 5. From Fig. 4, the detection limit for cobalt (twice the peak-to-peak baseline noise) in a 190-ml preconcentrated sample of simulated PWR primary coolant, is *ca.* 10 pg ml^{-1}. This clearly shows the ability of the system to achieve the desired limit of detection in the solution matrix of a PWR primary coolant. Contamination from boric acid was particularly noticeable for cobalt and magnesium and to a lesser extent zinc, since a 200-ml citrate reagent blank (Fig. 5) gave much lower peak heights for these metals, the cobalt peak height being reduced by a factor of six. Iron levels were lower in LBS than citrate probably owing to the greater ability of citrate solution to leach metals from the small amount of 316 stainless-steel wetted components. For nickel and iron the levels of contamination are at present acceptable because the expected levels in a primary coolant are two to three orders of magnitude higher than those in the blank.

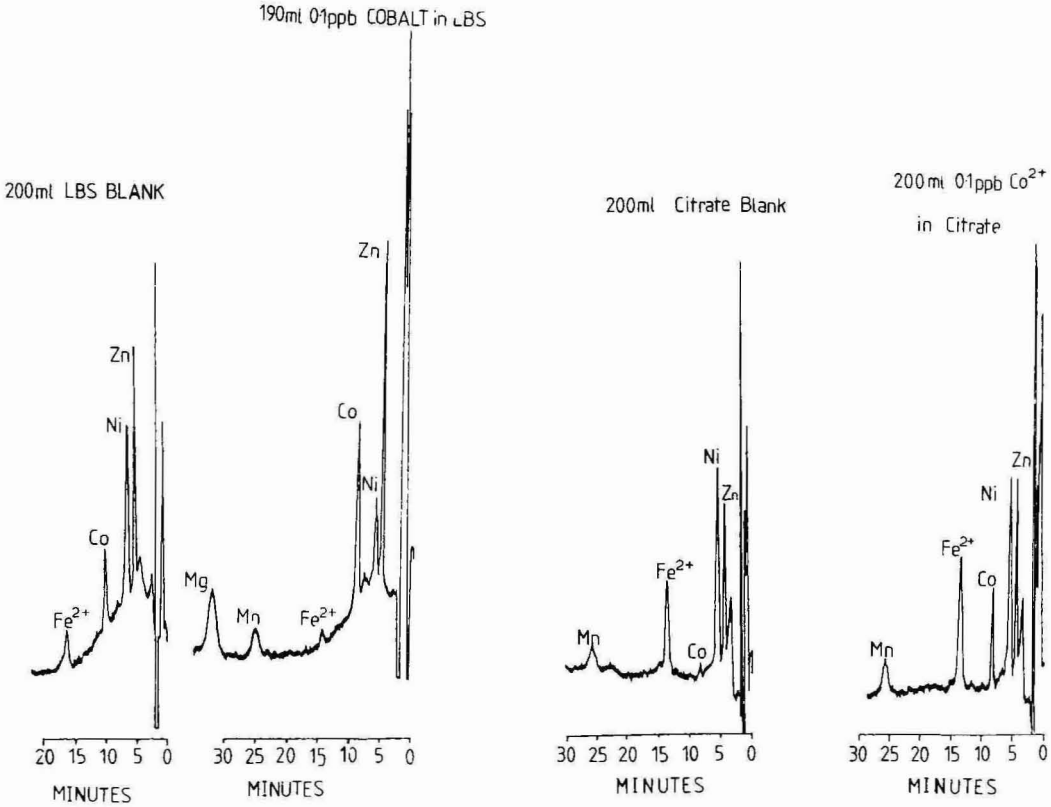

Fig. 4. Analysis of LBS, spiked with cobalt. Chromatographic conditions similar to Fig. 2.

Fig. 5. Analysis of citrate solution (0.001 *M*), spiked with cobalt. Chromatographic conditions similar to Fig. 2.

CONCLUSIONS

This work demonstrates the ability of HPLC systems to determine cobalt at pg ml^{-1} concentrations in the sample matrix of the primary coolant of a PWR. The performance of the preconcentration system gave quantitatively reproducible determinations with a good linear response over two orders of magnitude (2.5–100 ng). Blank levels could be reduced further and thus decrease the limit of detection, by additional purification of reagents using techniques such as recrystallization, manganese dioxide precipitation and constant electrolysis at a mercury pool electrode. The stability trials indicate the feasibility of the storage of samples for up to 7 days without adsorption losses when stabilized by a citrate buffer.

The system is easily automated and offers considerable flexibility with easily changed preconcentration and analytical columns, the precolumn also acting as a guard column. Sample volumes may be varied to suit requirements of either time or limit of detection, and the post-column reagents can be selected to vary both sensitivity and selectivity.

The system has been tried out on autoclaves at Winfrith Atomic Energy Establishment, and is presently being used for chemistry studies on a half Megawatt Loop, which simulates PWR operating conditions. Preliminary results have been very encouraging and have already shown the potential of the HPLC approach. The effect of varying PWR coolant chemistry on sub ppb cobalt levels could be monitored continuously where previously other methods had failed. Although not discussed here the detection of manganese, iron and nickel at the ppb level has also shown promising results and work is continuing on the multi-element performance of the developed HPLC system.

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SEPARATION AND QUANTIFICATION OF ADP-D-GLYCERO-D-MANNO-HEPTOSE AND ADP-L-GLYCERO-D-MANNOHEPTOSE BY BORATE LIQUID CHROMATOGRAPHY AND ITS USE IN THE ASSAY OF ADP-D-GLYCERO-D-MANNOHEPTOSE 6-EPIMERASE

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SUMMARY

A high-performance liquid chromatographic method using a borate–formate buffer system and an Aminex A-27 ion-exchange resin has been developed for the separation of heptose nucleotides from bacterial lipopolysaccharide biosynthesis. The main advantages of this system are isocratic elution with volatile buffer components, low working pressure, short analysis time, insensitivity to high salt contents in samples, stability of resin and adaptability to analytical and preparative separations.

INTRODUCTION

L-Glycero-D-mannoheptose (LD-heptose) is a typical component of the lipopolysaccharide core region of Gram-negative bacteria. Eidels and Osborn[1] have postulated a pathway for its biosynthesis. According to this scheme, starting from sedoheptulose-7-phosphate, a nucleotide-diphospho D-glycero-D-mannoheptose (DD-heptose) is synthesized which is then converted into a nucleotide-diphospho LD-heptose by the action of a 6-epimerase. We have recently isolated both types of nucleotide sugars from *Shigella sonnei* and *Salmonella typhimurium* R-mutants and the nucleotide moiety has been identified as ADP[2,3]. These results have been confirmed by Coleman[4], who found ADP-DD-heptose also in *Escherichia coli* K12.

The major problem in the determination of epimerase activity is the difficulty in separating the mixture of nucleotide sugars formed, because of the small differences between the structures of the substrate and products. Previously reported procedures for the separation of nucleotide sugar epimers such as paper[5] and thin-layer chromatography[6,7], paper electrophoresis[8] and lectin affinity chromatography[9,10] are cumbersome, time-consuming and hardly quantitative. In other cases, the nucleotide sugars are hydrolysed during the lengthy chromatographic procedure[11].

Weckbecker[12] and Keppler developed a method for the separation of UDP derivatives of galactose, glucose, N-acetylglycosamine, N-acetylgalactosamine, glucuronate and galacturonate as their borate complexes by high-performance liquid chromatography (HPLC) on a silica-based primary amine-containing anion exchanger.

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The main drawbacks of this system are the poor resistance of the silica matrix to even mildly alkaline solvents, and the use of glycerol in solvents which causes relatively high working pressures. Furthermore, the system is sensitive to the higher salt concentrations used in enzyme tests and needs a laborious desalting procedure. A gradient elution was applied and re-equilibration was necessary after each analysis.

To overcome all of these difficulties, we have developed an HPLC method for the separation of ADP-DD-heptose and ADP-LD-heptose which uses conventional anion-exchange resins with isocratic elution. It is less sensitive to high ion concentrations and buffer salts can easily be removed thus allowing application to preparative-scale separation of these nucleotide sugars. The method is able to separate NAD-, AMP-, ADP-, ATP- and ADP-heptoses in about 70 min and suitable for the quantitation of ADP-DD-heptose 6-epimerase.

MATERIALS AND METHODS

Chemicals and standard materials

Formic acid, boric acid and ammonium hydroxide (25% aqueous solution) were obtained in analytical grade from E. Merck (Darmstadt, F.R.G.). AMP, ADP, ATP and nicotiamide–adenine dinucleotide (oxidized, NAD) were obtained from Sigma (St. Louis, MO, U.S.A.). ADP-D-glycero-D-mannoheptose and ADP-L-glycero-D-mannoheptose were prepared in our laboratory as described elsewhere[2,3].

Preparation of buffer

Boric acid (18.55 g, 0.3 mol) was dissolved in 500 ml of 1 *M* formic acid and then concentrated ammonium hydroxide (about 43 ml) was added under constant stirring. The final pH was adjusted to 8.2 at room temperature to and the solution diluted to 1000 ml with distilled water. The buffer was filtered through a membrane filter (pore diameter: 0.45 μm) and deaerated before use.

Chromatographic procedures

Analytical columns (100 × 4.5 mm) stainless steel, fitted with 2-μm stainless-steel frits, were filled with Aminex A-27 anion-exchange resin (Bio-Rad Labs., Richmond, CA, U.S.A.) which is an 8% cross-linked styrene–divinylbenzene copolymer with quaternary ammonium functional groups. Before the packing procedure, the resin was slurried in two volumes of buffer and deaerated. The column was equilibrated with buffer (120 ml). Separations were performed at 70°C, with a flow-rate of 60 ml/h. Preparative separations were carried out at 60°C, with a flow-rate of 72 ml/h, on ANX4 anion-exchange resin (Hamilton, Bonaduz, Switzerland), in a jacketed glass column (250 × 9 mm; Glenco Scientific, Houston, TX, U.S.A.) heated with a water-circulation system. The sample volume was 1 ml.

For separations, a DuPont high-performance liquid chromatograph consisting of a Model 870 pump and thermostatic oven fitted with a Rheodyne Type 7125 injection valve (20-μl and 1000-μl sample loops) was used. The absorbance of the effluent was monitored at 254 nm with a DuPont Model 860 absorbance monitor (light paths 10 and 2 mm). The peak areas were determined with an electronic integrator built in our laboratory.

RESULTS AND DISCUSSION

Choice of buffer and pH

The only difference in the structure of ADP-DD- and ADP-LD-heptose is the steric configuration of the OH group on the C6 carbon atom of heptose. Therefore, the use of borate as the complexing buffer was crucial, because other systems available for nucleotide separation will not discriminate between structurally similar 6-epimeric ADP-sugars. Complete formation of the sugar borate complexes can be achieved with 0.3 *M* borate at pH 8.2. At this pH no serious decomposition of the pyrophosphate bond was observed, even at elevated temperature[13]. In this buffer, the nucleotides were strongly adsorbed onto the ion exchanger and it is necessary to advance the elution by addition of 0.5 *M* formate. A similar effect can be achieved with chloride salts as lithium, sodium or potassium chloride. The use of such salts, however, is not recommended because halogen ions are corrosive towards some types of stainless-steel components of HPLC pumps[14] and are non-volatile. Ammonium was chosen as the counter ion of the buffer system. Thus, the solvent contains only three ions which are easily removable in volatile form when necessary in preparative separations. Ammonium borate was used for the separation of ribo- and deoxyribonucleosides on Dowex 50 cation exchanger by Moran and Werkheiser[15]. These authors gave a detailed description of the method of removal of this buffer salt.

Effect of temperature

Separations at different temperatures in the range of 40–70°C were examined as shown in Fig. 1. With increasing temperature, a significant decrease in pressure and a considerable improvement in resolution were observed. From the resulting

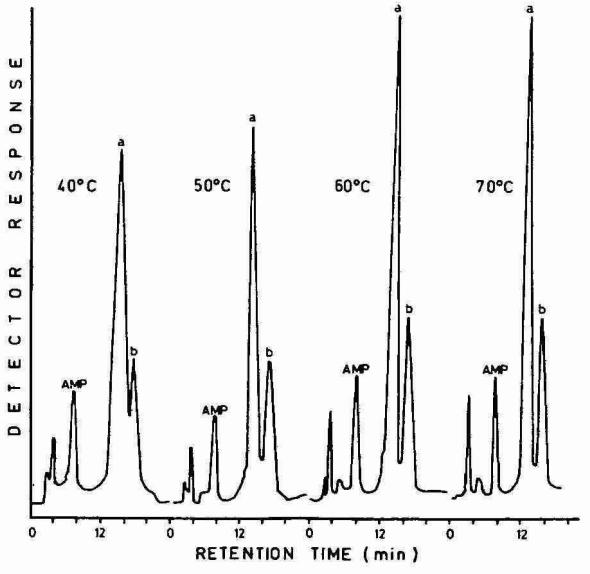


Fig. 1. Effect of temperature on the resolution and retention times of nucleotides. A standard mixture of nucleotides (20 µl) was assayed on the analytical column at the temperatures shown as described in Materials and methods. For other parameters see Table I. Peaks: a = ADP-LD-heptose; b = ADP-DD-heptose.

TABLE I

EFFECT OF TEMPERATURE ON THE CHROMATOGRAPHIC PARAMETERS OF HEPTOSE NUCLEOTIDES

For abbreviations see text.

	Working temperature (°C)			
	40	*50*	*60*	*70*
Pressure (MPa)	6.08	5.57	4.86	4.56
(p.s.i.)	882	808	705	661
Retention time (min)				
ADP-LD-heptose	15.03	14.24	13.68	12.95
ADP-DD-heptose	17.60	17.04	16.53	15.59
Δt (min)	2.57	2.84	2.85	2.64
$(W_{b1} + W_{b2})/2$ (min)	7.06	6.69	4.31	2.72
Resolution	0.36	0.42	0.66	0.97
Integrator counts				
AMP	90	69	71	55
ADP-LD-heptose	562	480	487	404
AMP/ADP-LD-heptose	0.16	0.14	0.15	0.14

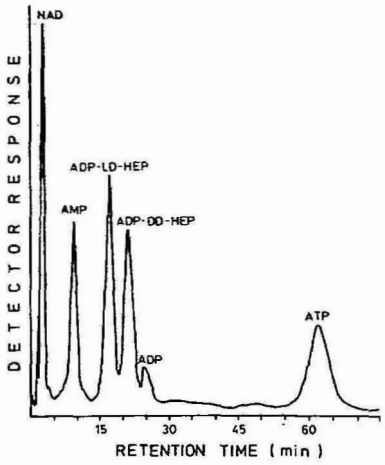
data (Table I), the optimum temperature for complete separation of ADP-sugars at pH 8.2 was established as 70°C. Retention times were not significantly influenced by the change in temperature. On the basis of the AMP to sugar nucleotide ratio, no deterioration was found even at 70°C. This is in accord with the observations of Khym[13], who separated nucleotides at the same pH and temperature as employed here. The resolution, R_s, of heptose nucleotides was nearly complete as established from the equation

$$R_s = \frac{\Delta t}{(W_{b1} + W_{b2})/2}$$

where Δt = the distance between the two peak maxima and W_{b1} and W_{b2} are the widths of the peaks at the base[16].

Analytical separation

To test the separation and quantitative recovery under the working conditions described in Materials and methods, standard mixtures of known concentrations of nucleotide components participating in heptose biosynthesis were examined. A typical chromatogram of NAD, AMP, ADP, ATP and of the corresponding sugar nucleotides is shown in Fig. 2. The separation of these components can be achieved in about 70 min and is sufficient for exact computing of the concentrations by the integrator. It is to be noted that normally ATP is not present in the reaction mixtures and quantitative analyses can be carried out within 30 min, in contrast to gas chromatographic determinations of heptose which need complicated derivatization procedures. The sensitivity of the method is in the range of 1–10 nmol of nucleotide sugar at 0.010 absorbance unit full scale. An examination of other nucleotide sugars, such as GDP-mannose, UDP-glucose and UDP-galactose on the same analytical



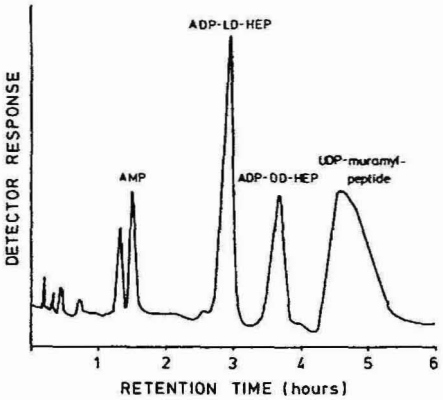


Fig. 2. Analytical separation of the mixture of nucleotides (20 μl) formed in heptose biosynthesis. For chromatographic conditions see Materials and methods.

Fig. 3. Preparative separation of heptose nucleotides. A 1-ml sample of the heptose nucleotide fraction extracted from 180 g (wet weight) of *Shigella sonnei* R-mutant bacteria and prepared by formate ion exchange[3] was loaded on the preparative column as described in Materials and methods.

column (not shown) indicated that, by alterations of the chromatographic parameters, *e.g.*, buffer concentration, use of gradient, etc., more complex mixtures of such components could similarly be resolved.

Preparative separation

Anion-exchange chromatography of a nucleotide extract from bacteria in the ammonium formate buffer system resulted in the separation of a peak containing both of the heptose nucleotides coeluted with an UDP-muramyl peptide[3]. To separate these components and to obtain in pure form, paper chromatography in which chromatograms were developed for 6 days or more and further rechromatography was needed. In such cases, the yield of the heptose nucleotides was low because of extensive hydrolytic degradation of pyrophosphate bonds.

In contrast, preparative separation in the borate–formate system described here yields high recovery and complete separation in 6–7 h, even in the presence of UDP-peptide contaminant (Fig. 3). About 1–1.5 μmol in 1 ml (calculated from the specific absorption of adenosine) can be loaded. In order to obtain high recovery and to avoid hydrolysis of nucleotide sugars, immediately after the appearance of the desired fractions in the effluent it is advisable to adjust the pH to 5.0 by the addition of 1 *M* formic acid and recover the nucleotides by adsorption on charcoal as described[2]. When solutions at pH 8.2 in borate buffer were left to stand even at -20°C, hydrolytic degradation and loss of heptose nucleotides resulted.

*Assay of ADP-*D*-glycero-*D*-mannoheptose 6-epimerase*

ADP-LD-heptose is formed from ADP-DD-heptose by the action of a 6-epimerase enzyme[3]. To test the activity of this enzyme during its preparation and to study the parameters influencing the enzyme kinetics, the analytical separation tech-

nique was adapted to replace the previously used gas chromatographic method, *i.e.*, the determination of heptose derivatives obtained from hydrolysates of nucleotide-containing reaction mixtures. The activity of the 6-epimerase was tested by incubation of a mixture of the enzyme preparation and ADP-DD-heptose as described previously[3,17]. 100-μl Aliquots were taken at given intervals and the enzyme was inactivated in a bath of boiling water. Precipitated protein was removed by centrifugation (15 000 *g*, for 5 min). The supernatant (20 μl) was immediately injected onto an analytical column and eluted as described in Materials and Methods.

Through the action of the 6-epimerase, the peak of ADP-DD-heptose was diminished, with a concomitant increase in the ADP-LD-heptose peak, as shown in Fig. 4. The relatively large peak of AMP was attributed to the activity of a phosphodiesterase coextracted in the enzyme preparation. In a control test mixture without enzyme preparation (see Fig. 4A), no deterioration of the heptose nucleotide was observed. Retention time and resolution were not influenced by buffer and other salts (Tris, magnesium chloride etc.). The ratio of the concentrations of both nucleotides when plotted against elapsed time is characteristic of the kinetics of enzyme action (see Fig. 4, inset). It is obvious that the effect of DD-heptose 6-epimerase reaches an equilibrium at 67% of ADP-LD-heptose. A shift of this reaction to the right takes place when LD-heptose nucleotide is consumed in lipopolysaccharide biosynthesis by transferases.

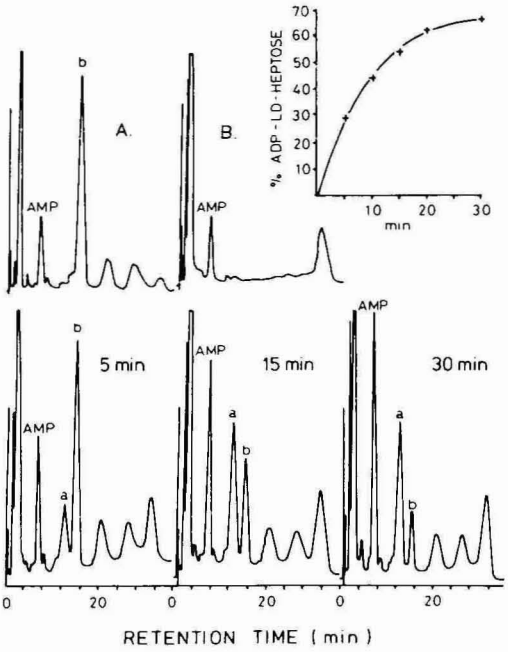


Fig. 4. Assay of ADP-DD-heptose 6-epimerase on the analytical column. After incubation with enzyme, samples were taken at given intervals and 20-μl inactivated aliquots were injected. (A) Reaction mixture without enzyme preparation; (B) reaction mixture without DD-heptose nucleotide substrate. Peaks: a = ADP-LD-heptose; b = ADP-DD-heptose. Inset: per cent of ADP-LD-heptose formed at different times during incubation of ADP-DD-heptose with 6-epimerase.

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TANDEM PURIFICATION OF IgM MONOCLONAL ANTIBODIES FROM MOUSE ASCITES FLUIDS BY ANION-EXCHANGE AND GEL FAST PROTEIN LIQUID CHROMATOGRAPHY

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SUMMARY

A tandem chromatographic procedure was used to isolate rapidly mouse IgM monoclonal antibodies. Mouse ascites fluids containing IgM monoclonal antibodies were first chromatographed on an anion-exchange Mono Q column connected to a fast protein liquid chromatography system. The IgM-rich fractions from the Mono Q column were then injected on a gel filtration Superose 6 column equilibrated with a low-ionic strength buffer and eluted with a high-ionic strength buffer. Assessment of the purity of isolated IgM monoclonal antibodies was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis together with a Coomassie Brillant Blue R 250 staining technique. Assessment of the immunoreactivity of isolated IgM monoclonal antibodies was performed by a enzyme linked immunosorbent assay using a solid phase adsorbed antigen against which IgM monoclonal antibodies were directed. The chromatographic procedure described provides a new method for the rapid purification of mouse IgM monoclonal antibodies to a high degree of purity and in a immunoreactive state.

INTRODUCTION

The purification of IgM monoclonal antibodies usually requires multistep procedures involving DEAE-cellulose anion-exchange chromatography[1,2], Ultrogel Ac A34 or G-200 Sephadex gel filtration chromatography[1–3] and protamine sulphate-Sepharose or concanavalin A-Sepharose affinity chromatography[4,5]. Most of these purification procedures are combined with a preliminary precipitation step[1,3–5] known

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to denature IgM antibodies[2,6]. In addition, these techniques rarely avoid contamination with alpha-2 macroglobulin[1,6]. Direct isolation of IgM monoclonal antibodies from human pathological sera by G-200 Sephadex gel filtration chromatography was made possible by increasing the retention time of IgM antibodies onto the column with a low-ionic strength buffer[6]. However, classical gel filtration chromatography remains of limited use due to the length of time involved for each separation[1]. Very little information is currently available on the rapid purification of IgM monoclonal antibodies from mouse ascites fluids under conditions where the purified antibody remains immunoreactive and is free of protein contaminants. Recent developments in high-performance liquid chromatography have opened new possibilities in the isolation of mouse monoclonal antibodies[7–10] and their antigen binding fragments (Fab)[8].

We describe in this study a tandem chromatographic procedure to isolate rapidly, from mouse ascites fluids, IgM monoclonal antibodies free from contaminating proteins and in a immunoreactive state. Mouse ascites fluids containing IgM monoclonal antibodies were first chromatographed on a Mono Q anion-exchange column connected to a fast protein liquid chromatography (FPLC) system. Eluted peaks containing IgM monoclonal antibodies were then chromatographed on a gel filtration Superose 6 column equilibrated with a low-ionic strength buffer. Assessment of the purity of isolated IgM antibodies was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) together with a Coomassie staining technique. Assessment of the immunoreactivity of isolated IgM antibodies was performed by enzyme linked immunosorbent assay (ELISA) using the antigen against which IgM antibodies were directed. The chromatographic procedure described provides a new method for the rapid isolation of pure IgM antibodies in a immunoreactive state.

EXPERIMENTAL

The FPLC separations were performed on a Mono Q HR 5/5 prepacked anion-exchange column (50 × 5 mm) and a Superose 6 HR 10/30 prepacked gel filtration column (300 × 10 mm) (Pharmacia, Uppsala, Sweden). Materials and chemicals, unless stated otherwise, were as previously indicated[8]. Rabbit anti-mouse IgM was from Miles Labs., U.S.A.) and goat anti-rabbit IgG conjugated with alkaline phosphatase was from Miles-Yeda (Israel).

Seven mouse ascites fluids containing IgM monoclonal antibodies were obtained from the following sources: P13, P14, P15, P16, P17 were IgM monoclonal antibodies directed against a human blood platelet alpha-granule glycoprotein called thrombospondin[11]; MAS 037 C, directed against human fibronectin, was obtained from Sera-Lab; 633 A, specific for furunculosis fish disease (*Aeromonas salmonicida*), was obtained from BioScot (Edinburgh, U.K.). Mouse ascites fluids were passed through 0.45-μm filters (Millipore) prior to chromatographic separations on a anion-exchange Mono Q column.

Anion-exchange chromatography

Mono Q HR 5/5 column. Mouse ascites fluids containing IgM monoclonal antibodies (1–2 ml) were equilibrated in the starting buffer (20 ml) and then injected

via a 50 ml Superloop (335 × 30 mm) (Pharmacia) onto a Mono Q anion-exchange column. The starting buffer was 20 m*M* L-histidine, pH 6.0, and the final buffer was 20 m*M* L-histidine, pH 6.0, containing 0.5 *M* sodium chloride. The gradient was generated over 25 min at a flow-rate of 1 ml/min.

Gel filtration chromatography

Superose 6 HR 10/30 column. The method of Bouvet *et al.*[6] involving G-200 gel filtration chromatography with a low-ionic strength buffer was used with minor modifications and was adapted, in this study, to the FPLC system. IgM-rich fractions from the Mono Q column were concentrated by ultrafiltration (Amicon) (0.5–0.7 ml) and injected at a flow-rate of 0.5 ml/min onto a gel filtration Superose 6 column previously equilibrated with a low-ionic strength buffer containing 5 m*M* L-histidine, pH 6.0. The eluting buffer was 50 m*M* L-histidine, pH 6.0, containing 1.7 *M* sodium chloride. In some experiments a buffer containing 0.5 *M* sodium chloride in 50 m*M* L-histidine, pH 6.0, was used to equilibrate the Superose 6 column and to elute the IgM monoclonal antibodies.

SDS-PAGE

Following the method of Mason and Williams[12], 100 μl of sample mixed with 100 μl of buffer containing 5% (w/v) SDS, 25% (w/v) glycerol and 2 m*M* N-ethylmaleimide were heated at 100°C for 5 min. Unreduced proteins were subjected to electrophoresis on a 3–10% gradient polyacrylamide gel in the presence of SDS according to Laemmli[13]. Gels were fixed in 40% (v/v) methanol–7% (v/v) acetic acid and then stained with Coomassie Brillant Blue R 250.

ELISA

Mouse IgM antibodies present in peaks eluted from the Mono Q column were detected by an ELISA similar to that described by Beards and Bryden[14] with minor modifications. Briefly, 100 μl of purified antigen (human platelet thrombospondin) (5 μg/ml) diluted in phosphate–buffered saline (pH 7.4) was added to poly(vinylchloride) microtitre plates (Nunc) and incubated overnight at 4°C. Wells coated with thrombospondin were then washed three times with 250 μl/well of phosphate-buffered saline containing 0.05% Tween 20 (washing buffer). The last wash was left for 30 min at room temperature. After removing the washing buffer, 100 μl of all the eluted peaks were added to the wells and left for 1 h at 37°C. A negative control was performed by the use of phosphate-buffered saline instead of eluted peaks. A positive control was performed by including the mouse ascites fluids containing IgM antibodies. After the incubation, the content of the wells was aspirated and rinsed 3 times with the washing buffer. One hundred microliters of rabbit anti-mouse IgM (diluted to 1/300) were added to each well for an additional 1 h incubation at 37°C. After aspirating and rinsing 3 times with the washing buffer, 100 μl of goat anti-rabbit IgM conjugated with alkaline phosphatase (diluted to 1/3000) were added to each well and incubated for 1 h at 37°C. The content of the wells was then aspirated and rinsed 3 times with the washing buffer. *p*-Nitrophenylphosphate (1 *M*) dissolved in diethanolamine buffer was then added to each well (100 μl). The enzyme reaction was allowed to proceed for 1 h at 37°C and then stopped with 10 μl/well of 0.5 *M* sodium hydroxide. The absorbance was read at 405 nm using a Titertek Multiskan MC photometer (Flow Laboratories).

RESULTS

Isolation of IgM monoclonal antibodies from mouse ascites fluids by FPLC anion-exchange chromatography

Mouse ascites fluids containing IgM monoclonal antibody P13 showed 5 major peaks when separated on a Mono Q column with a gradient of 0–0.5 *M* sodium

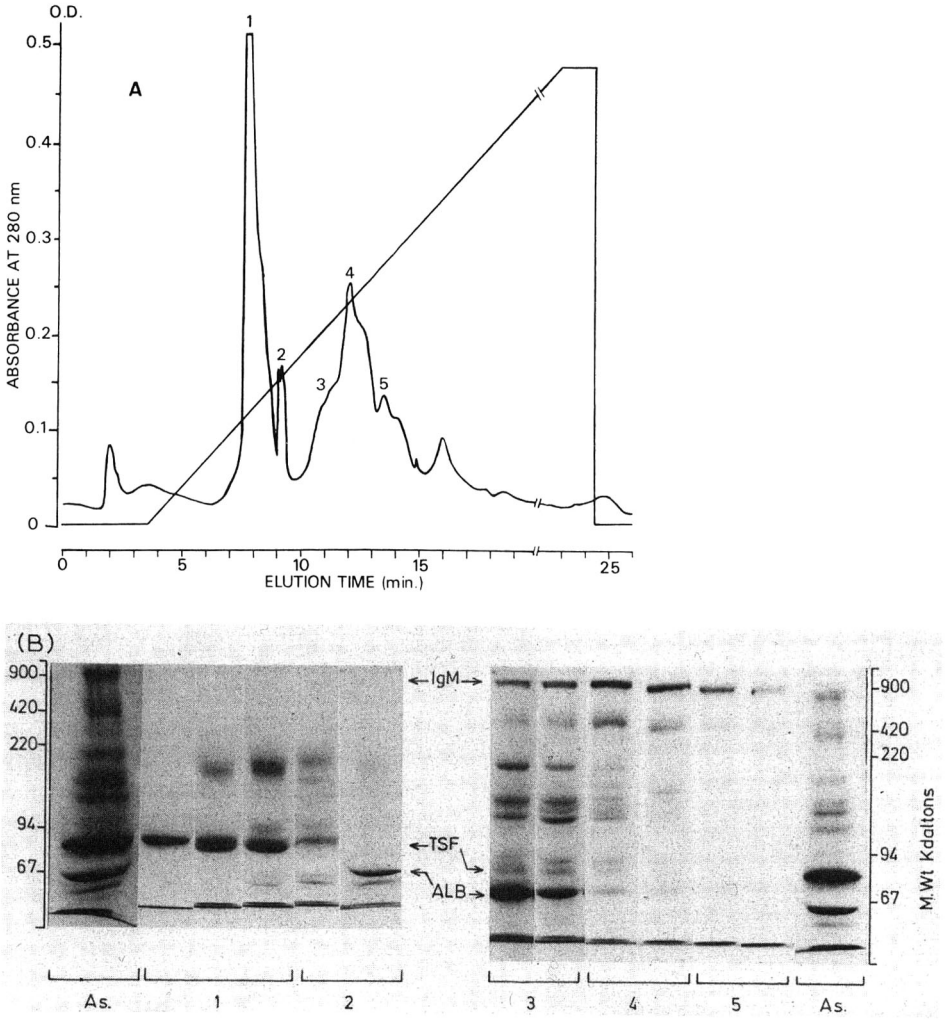

Fig. 1. (A) Chromatographic separation of mouse IgM monoclonal antibody P13 present in ascites fluid on a Mono Q column connected to a FPLC system. Ascites fluid (3 mg of protein per 15 ml) was injected into the column via a 50-ml Superloop. The gradient of 0–0.5 *M* NaCl in 20 m*M* L-histidine (pH 6.0) was generated over 25 min at a flow-rate of 1 ml/min. (B) Eluted peak fractions were treated with SDS and subjected to electrophoresis under non-reducing conditions on 3–10% gradient SDS-polyacrylamide gels. Gels were fixed and stained with Coomassie Brillant Blue R 250. Lanes As. contain mouse ascites fluid prior to the chromatography on a Mono Q column. Numbers below each lane correspond to peak numbers. Lanes 3 and 4 were loaded with 80–200 μg of eluted material whereas lane 5 was loaded with 30–60 μg. IgM = immunoglobulin M; TSF = transerrin; ALB = albumin.

chloride (Fig. 1A). Similar elution patterns were obtained with 6 different mouse ascites fluids containing IgM monoclonal antibodies (P14, P15, P16, P17, MAS 037 C and 633 A) (results not shown).

Eluted peak fractions were subjected to electrophoresis under non-reducing conditions on 3–10% gradient SDS-polyacrylamide gels and the separated proteins were identified by Coomassie staining (Fig. 1B). In typical electrophoretic separations of eluted peaks, peaks 1 and 2 which were eluted at 0.05 *M* and 0.08 *M* sodium chloride respectively, mainly contained transferrin and a 150 kDA protein band (Fig. 1B; lanes 1 and 2). In addition to protein bands between 80 and 420 kDa, albumin and a 900 kDa protein band corresponding to the apparent molecular weight of IgM antibodies[2] were present in peaks 3 and 4 which were eluted between 0.18 and 0.2 *M* sodium chloride (Fig. 1B; lanes 3 and 4). Only the IgM protein band and traces of a 420 kDa protein band corresponding to the molecular weight of α_2-macroglobulin[6] were present in peak 5 which was eluted at 0.25 *M* sodium chloride (Fig. 1B; lane 5). Using rocket imunoelectrophoresis, the recovery of IgM monoclonal antibodies from the Mono Q column was 80% of the amount of IgM antibodies originally present in the ascites fluid.

Assessment of the immunoreactivity of isolated IgM monoclonal antibodies by enzyme linked immunosorbent assay

In order to assess the immunoreactivity of IgM monoclonal antibody P13, eluted peaks from the Mono Q column were added to plastic wells coated with the

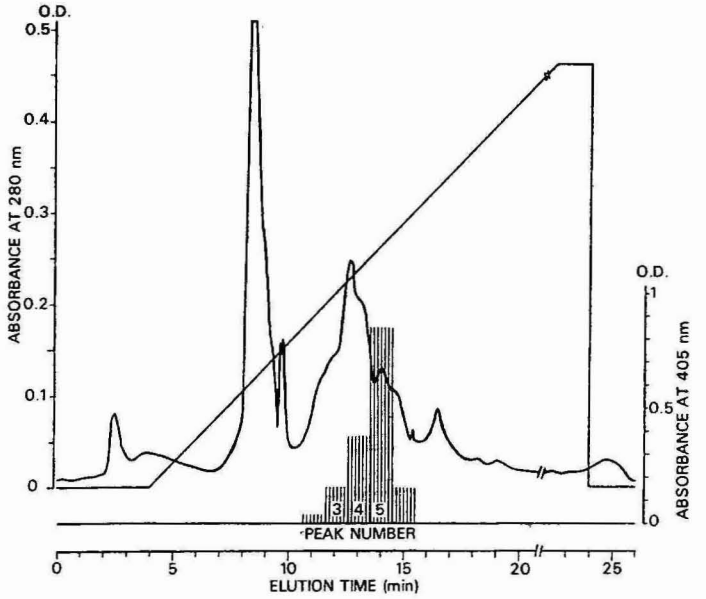

Fig. 2. Assessment of the immunoreactivity of isolated IgM monoclonal antibody P13 using an ELISA. Each eluted peak fractions of the Mono Q chromatographic profile were added to plastic wells coated with thrombospondin, the antigen against which was directed the IgM antibody P13. The upper part shows the chromatographic separation. The bottom part shows the binding of IgM monoclonal antibody P13 to adsorbed thrombospondin using rabbit anti-mouse IgM and goat anti-rabbit IgG conjugated with alkaline phosphate.

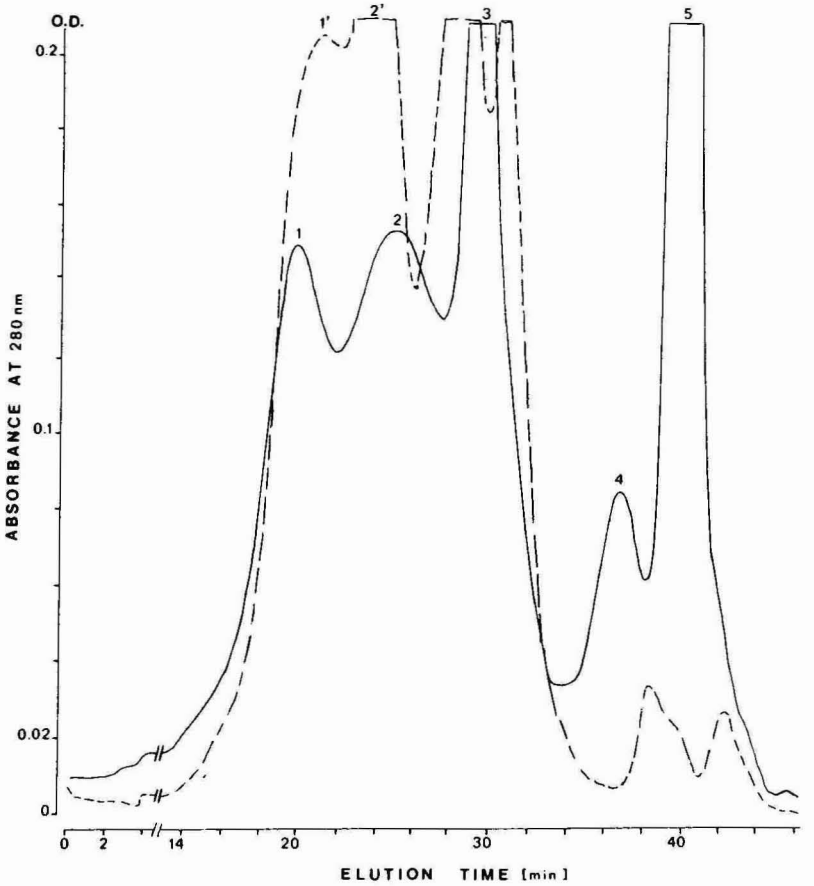

Fig. 3. Gel permeation chromatography on a Superose 6 column of mouse IgM monoclonal antibody P13 isolated from the Mono Q column. The IgM-rich fractions from the Mono Q column (peaks 4 and 5) were concentrated by ultrafiltration (0.5–0.7 ml) and injected into the column at a flow-rate of 0.5 ml/min. (———) Prior injection, the Superose 6 column was equilibrated in 5 m*M* L-histidine, pH 6.0. The IgM sample was then injected and eluted from the column with a buffer containing 1.7 *M* NaCl in 50 m*M* L-histidine, pH 6.0. (– – – –) Under normal ionic strength conditions, the IgM sample was injected into the Superose 6 column previously equilibrated with 50 m*M* L-histidine, pH 6.0, containing 0.5 *M* NaCl and eluted with the same buffer.

antigen (human platelet thrombospondin) against which monoclonal antibody P13 was directed. Results obtained confirmed the presence of IgM antibodies P13 in peaks 3, 4 and 5 and showed a strong anti-thrombospondin activity in peak 5 (Fig. 2). Similar results were obtained with four other mouse ascites fluids containing IgM antibodies (P14, P15, P16 and P17) directed against human platelet thrombospondin (results not shown). Recovery of the IgM antibodies in peak 5, based on recovery of immunological activity, was about 75% of the activity originally present in the ascites fluids.

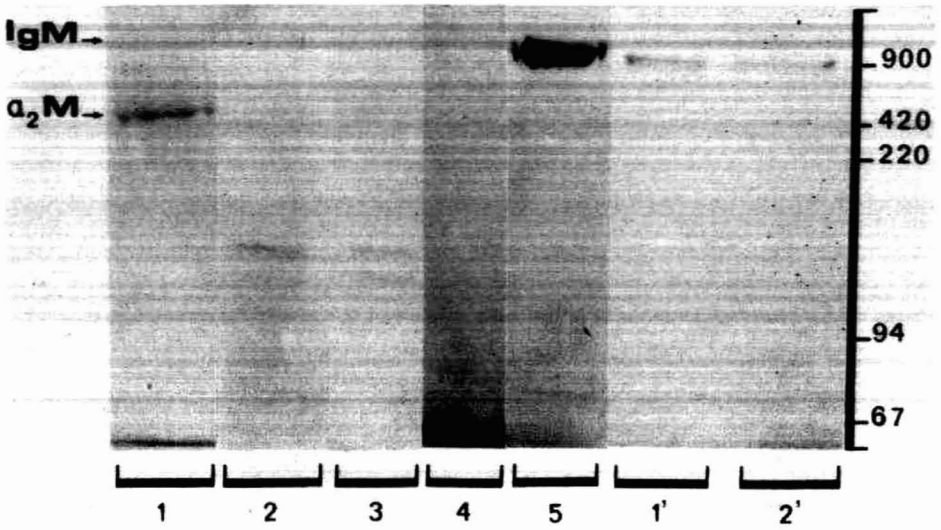

Fig. 4. Assessment of the purity of isolated IgM monoclonal antibodies by SDS-PAGE and Coomassie staining. The eluted peaks from the Superose 6 gel permeation column were treated with SDS and subjected to electrophoresis under non-reducing conditions on a 3–10% gradient SDS-polyacrylamide gel. The gel was fixed and stained with Coomassie Brillant Blue R 250. Numbers below each lane correspond to peak numbers. Lane 5 was loaded with about 100 μg of eluted material. IgM = immunoglobulin M; α_2M = α_2-macroglobulin.

Purification of isolated IgM monoclonal antibodies by FPLC gel permeation chromatography

The IgM-rich fractions from the Mono Q column shown to be strongly immunoreactive (peaks 4 and 5) were injected on a gel permeation Superose 6 column either equilibrated with a low-ionic strength buffer (Fig. 3A) or a regular buffer (Fig. 3B). The eluted peaks were subjected to electrophoresis under non-reducing conditions on a 3–10% gradient SDS-polyacrylamide gel. Coomassie brillant blue staining of electrophoresed proteins clearly showed that IgM monoclonal antibodies eluted in peak 5 (Fig. 3A) with a high degree of purity (Fig. 4; lane 5) when IgM-rich fractions were gel-filtered through a column equilibrated with a low-ionic strength buffer and eluted with a high-ionic strength buffer. On the other hand, IgM monoclonal antibodies eluted in peak 1′ (Fig. 3B) and tended to co-elute with α_2-macroglobulin as shown by SDS-PAGE (Fig. 4; lanes 1′ and 2′) when using normal ionic strength conditions of chromatography. The use of an enzyme linked immunosorbent assay with solid phase adsorbed antigen indicated that IgM monoclonal antibodies present in peaks 1′, 2′ and 5 were immunologically active (results not shown).

DISCUSSION

Results obtained in this study demonstrate that mouse IgM monoclonal antibodies are rapidly isolated from ascites fluids with a high degree of purity using anion-exchange followed by gel filtration chromatography on a FPLC system. Purification of mouse IgM monoclonal antibodies from ascites fluids using classical

methods usually requires a preliminary precipitation step[1,3–5] which has been shown to denature IgM monoclonal antibodies[2,6]. The results presented here indicate that pure IgM antibodies, isolated without prior precipitation, remain immunoreactive. While a tandem procedure has been employed in this study, it was observed during the anion-exchange step that most proteins from the mouse ascites fluids were found, like IgM antibodies, in more than one eluted peak. It is possible that interactions occur between IgM antibodies and other proteins, such as albumin and α_2-macroglobulin, to explain their elution at the same salt concentration. Similar observations were previously reported using the FPLC-Mono Q system[17,18]. Alternatively, the IgM antibodies in peak 3, eluting at a lower salt concentration than IgM antibodies contained in peaks 4 and 5, may represent inactive IgM monoclonal antibodies. The presence of inactive IgM monoclonal antibodies in peak 3 could explain the weak immunoreactivity obtained by ELISA. Stanker *et al.*[10] purifying mouse IgG_1 antibodies from ascites fluids by hydroxylapatite chromatography, also reported the elution of a inactive IgG_1 antibody at a slightly lower phosphate concentration than does the active IgG_1 antibody. Most of the classical IgM purification methods rarely avoid contamination with α_2-macroglobulin[1,6]. Using a modification of the gel filtration technique described by Bouvet *et al.*[6] as the second step of the tamdem purification of IgM antibodies, we shown in this study that purified mouse IgM monoclonal antibodies were free of contaminants including alpha α_2-macroglobulin. Furthermore, these mouse IgM antibodies were devoid of protease activity since long-term storage did not result in loss of antibody immunoreactivity. Recently, Stanker *et al.*[10] reported the purification of mouse IgG and IgM monoclonal antibodies from ascites fluids by hydroxyapatite chromatography with a 0.01–0.3 *M* phosphate gradient (pH 6.8). Under the chromatographic conditions used by the authors, both IgG and IgM antibodies eluted between 0.15 and 0.2 *M* sodium phosphate. Since ascites fluids are often contaminated with host mouse IgG antibodies[10,15], the isolation of mouse IgM monoclonal antibodies from ascites fluids by hydroxyapatite chromatography cannot avoid a contamination of IgM with non-specific mouse IgG antibodies. In this respect, the purification of mouse IgM antibodies from ascites fluids by hydroxyapatite chromatography remains possible only when Balb/c mice are immunosuppressed or irradiated to avoid the presence of host mouse immunoglobulins in ascites tumors. On the other hand, the isolation on a Mono Q column of mouse IgM monoclonal antibodies from ascites fluids allows their separation from non-specific mouse IgG antibodies since both immunoglobulin classes elute differently from the Mono Q anion-exchange column[16,19]. In this respect, the chromatographic procedure described in this study provides a new method for the rapid isolation (80 min) of mouse IgM monoclonal antibodies to a high degree of purity (greater than 90% pure) and in a functionally active state. Furthermore, this procedure can be adapted to the large scale purification of IgM monoclonal antibodies using preparative scale FPLC techniques.

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Note

Structural investigations on reversed-phase silicas

II. Evidence for endcapping trimethylsilyl groups

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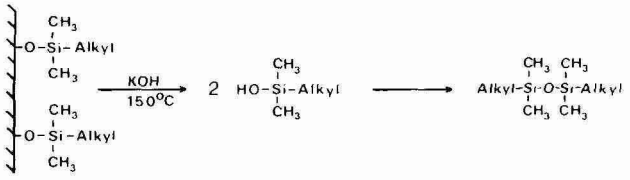
Reversed-phase (RP) silicas are extremely useful in solving many separation problems in modern liquid chromatography. Nevertheless there are still problems with the reproducibility of chromatographic results obtained with these materials. One important obstacle to the improvement of RP silicas, with respect to constant properties and quality, might be the lack of reliable analytical methods giving detailed information about the silica matrix itself and about the ligands attached to the surface.

In our studies we focus on the analysis of ligands. Items of interest are the structure of the ligand and its purity, its degree of substitution and its distribution on the surface as mono-, di- and trifunctional bonded alkyl groups, and mono- and polymeric substitution. Other important parameters are the proportion of unchanged silanol groups and the degree of endcapping.

For elemental analysis offers only poor information about these structural parameters, several authors have contributed to an improved characterization of RP silicas[1–16]. With special respect to endcapping trimethylsilyl (TMS) groups, methods using pyrolysis[4,9,12,14], thermogravimetry[2], ^{13}C and ^{29}Si NMR measurements[3,8,10] and hydrofluoric acid digestion[16] as well as chromatographic tests[5] have already been published.

We found the alkali fusion reaction to be a simple and valuable tool for the ligand analysis of silica-bonded stationary phases[7,11]. RP silicas with monofunctional ligands yielded alkyldimethylsilanol and its dimer 1,1,3,3-tetramethyl-1,3-dialkyldisiloxane during alkali fusion[15].



Monofunctional bonded ligand | Alkyldimethylsilanol | 1,1,3,3-Tetramethyl-1,3-dialkyldisiloxane

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For the TMS group, which is the most simple monofunctional ligand, we expected to find trimethylsilanol and hexamethyldisiloxane by gas chromatographic (GC) analysis of alkali fusion products from endcapped RP silicas.

EXPERIMENTAL

To 20 mg of the derivatized silica in a glass reaction tube (110 mm × 5 mm I.D.), 200 mg of dry powdered potassium hydroxide and 100 μl of triethylene glycol dimethyl ether (triglyme) were added. The tube was heated to 150°C for 30 min in an oil bath and two 100-μl lots of diethyl ether were added subsequently. Analysis of the ether extract was performed with a 437 S gas chromatograph (Packard Instruments, Delft) equipped with a non-polar 50-m OV-1 fused-silica capillary column (Orion, Espoo, Finland) and a flame ionization detector. The oven temperature was set to 33°C isothermally and the carrier gas flow-rate was 2 ml/min. After analysis, the oven temperature was set to 280°C to remove the reaction products of the main ligand from the column.

Structural characterization of organosilicon compounds was achieved by GC–mass spectrometry.

Unreliable results are possible when the glass surface of the reaction tube is silanized in order to promote antiwetting properties.

RESULTS

Laboratory-made TMS silica was fused with potassium hydroxide in triglyme suspension at 150°C for 30 min. The ether extract of the reaction products was analysed for trimethylsilanol and its condensation product hexamethyldisiloxane. Both components were found in the chromatogram (Fig. 1), but the silanol peak rapidly

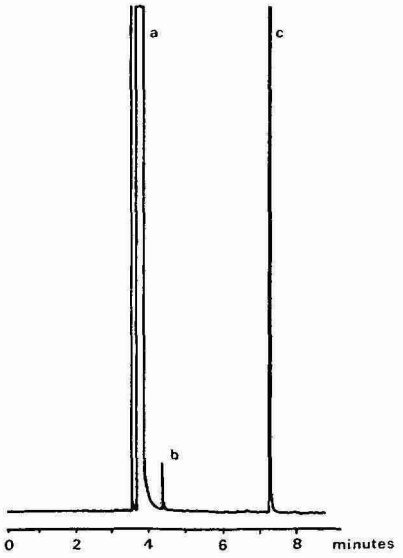

Fig. 1. Gas chromatogram of alkali fusion products from endcapped RP silicas (0.5 h, 150°C, triglyme suspension). Peaks: a = diethyl ether (solvent); b = trimethylsilanol; c = hexamethyldisiloxane.

TABLE I

ANALYSIS OF COMMERCIALLY AVAILABLE RP-8 SILICAS FOR ENDCAPPING TMS GROUPS BY ALKALI FUSION AND SUBSEQUENT GC PROOF OF HEXAMETHYLDISILOXANE

Sample	*Batch No.*	*Endcapping*
Kieselgel 60 HPLC C_8 (Riedel de Haen)	–	Yes
LiChroprep RP-8 (Merck)	8597034	No
LiChrosorb RP-8 (Merck)	VV 1137	No
LiChrosorb RP-8 (Merck)	VV 2159	No
MOS Hypersil C_8 (Shandon)	12772	No
Nucleosil 10 C_8 (Macherey & Nagel)	–	No
SIL-X5 C_8 (Perkin-Elmer)	829081	Yes
SIL-X10 C_8 (Perkin-Elmer)	820011	Yes

decreased during repeated injections. Methane, which is also evolved in small amounts, was not detected because of the experimental conditions. The presence of hexamethyldisiloxane caused us to check commercially available RP silicas with respect to TMS groups resulting from endcapping procedures with trimethylchlorosilane or other trimethylsilylating agents. Tables I and II show the results for several RP-8 and RP-18 materials based on GC analysis for hexamethyldisiloxane. During fusion the main ligand (RP-8/RP-18) is also cleaved, and products such as alkanes, alkanols and alkylsiloxanes as well as triglyme can be identified in temperature-programmed GC runs[11], so simultaneous determination of the main ligand structure is also possible.

TABLE II

ANALYSIS OF COMMERCIALLY AVAILABLE RP-18 SILICAS FOR ENDCAPPING TMS GROUPS BY ALKALI FUSION AND SUBSEQUENT GC PROOF OF HEXAMETHYLDISILOXANE

Sample	*Batch No.*	*Endcapping*
Kieselgel 60 HPLC C_{18} (Riedel de Haen)	CH-B2174	Yes
LiChroprep RP-18 (Merck)	8572024	No
LiChrosorb RP-18 (Merck)	VV 1106	No
Micro-Pak MCH-10 (Varian)	–	No
Nucleosil 7 C_{18} (Macherey & Nagel)	3022	Yes
Nucleosil 10 C_{18} (Macherey & Nagel)	–	Yes
ODS Hypersil (Shandon)	11/1067	Yes
ODS SIL-X5 (Perkin-Elmer)	–	Yes
ODS SIL-X10 (Perkin-Elmer)	–	Yes
Sep-Pak C_{18} (Waters)	–	Yes
Spherisorb S5 ODS (Separations Group)	18/157	Yes
Co:Pell ODS (Whatman)	100727	No

DISCUSSION

The condensation of trimethylsilanol can yield not only the symmetrical hexamethyldisiloxane but also mixed siloxanes with alkylsilanols derived from the main ligand. Normally the degree of substitution of the main ligand should be considerably higher than that of the TMS group, so that mixed siloxanes would be expected to be the main TMS-containing reaction products.

However, in the case of endcapped difunctional RP-8 silica we found only traces of 1,1,1,3,5,5,5-heptamethyl-3-octyltrisiloxane; the main TMS-containing product was hexamethyldisiloxane.

Alkali fusion of endcapped monofunctional RP-8 material yielded only traces of 1,1,1,3,3-pentamethyl-3-octyldisiloxane; the main TMS-containing product was again hexamethyldisiloxane.

These findings may be due to a possible concentration of TMS ligands in narrow pores of the silica matrix.

ACKNOWLEDGEMENT

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CHROM. 18 351

Note

Separation of orthophosphoric, phosphorous and hypophosphorous acids using single-column ion chromatography with conductivity detection

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The chromatographic separation of the lower oxo acids of phosphorus, *i.e.* hypophosphorous acid (H_3PO_2), phosphorous acid (H_3PO_3) and orthophosphoric acid (H_3PO_4), either as acids or as salts, and particularly in the presence of other organophosphorus compounds, has rarely been reported. The three acids have been eluted from a cation-exchange column[1] but no resolution was observed. The anions as orthophosphate, phosphonate and phosphinate were separated[2] on an anion-exchange column and detected using a flow injection system as a post-column detector employing oxidation to orthophosphate followed by chromogenesis with molybdenum reagent. Classical suppressed ion chromatography[3] was used to determine the three acids in nickel plating baths although limits of detection are not quoted. However, in this work the more flexible single-column system was chosen for further investigation.

Initially the "standard" potassium hydrogen phthalate eluent was used but it produced poor peak shapes and insufficient resolution. It has been shown[4] that weak organic acids are useful eluents for resin-based anion exchangers giving increased sensitivity due to low dissociation, while retaining sufficient eluting power for early eluting analytes.

In this work, therefore, succinic acid was investigated as the eluent, and after optimisation a quantitative method was developed which enabled the determination of all three oxo phosphorus anions at ppm levels and in the presence of polyorganophosphorus compounds.

EXPERIMENTAL

A Waters Assoc. Model 6000A solvent pump incorporating a Rheodyne 7125 injector with a 100-μl loop was used to deliver the eluent, an 0.02 *M* aqueous solution of succinic acid degassed with helium. The separation was carried out at a flow-rate of 4 ml/min with a 25 × 0.45 cm Vydac 302 silica-based anion-exchange column. The anions were detected using an LDC Conductomonitor III thermostatically controlled conductivity detector, and the chromatograms were recorded on a Spectra-Physics Model 4270 integrator.

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The phosphorus acids, their potassium salts and the succinic acid were obtained from BDH, Poole, U.K.

RESULTS AND DISCUSSION

When succinic acid was used at its natural pH of 3.0, the three oxo phosphorus anions were eluted in order of decreasing ionic size, *i.e.* $H_2PO_4^-$, $H_2PO_3^-$, and $H_2PO_2^-$. According to Ujimoto *et al.*[5] the average negative charge on each of these species within the pH range of 3–5 is close to one. The effect of varying ion concentration and pH was investigated. As may be seen from Fig. 1, decreasing succinic acid concentration in the eluent resulted in increased retention of $H_2PO_4^-$, although the effect was only marked at very low concentrations where $H_2PO_3^-$ and $H_2PO_2^-$ were excessively retained. On increasing the pH of the eluent by addition of sodium hydroxide, retention and resolution were reduced (Fig. 2). As the first pK_a value of succinic acid at pH 4.2 was approached with a consequent increase in dissociation, which led to a higher eluent conductance, the peak shape was adversely affected and the sensitivity dramatically reduced. The area response to $H_2PO_4^-$ on the conductivity detector was reduced by a factor of 25 on changing the pH from 3.0 to 3.8.

Maleic acid and phthalic acid proved unsuccessful as eluents for this system, showing poor sensitivity and peak shape. The separation was optimised at 0.02 *M*

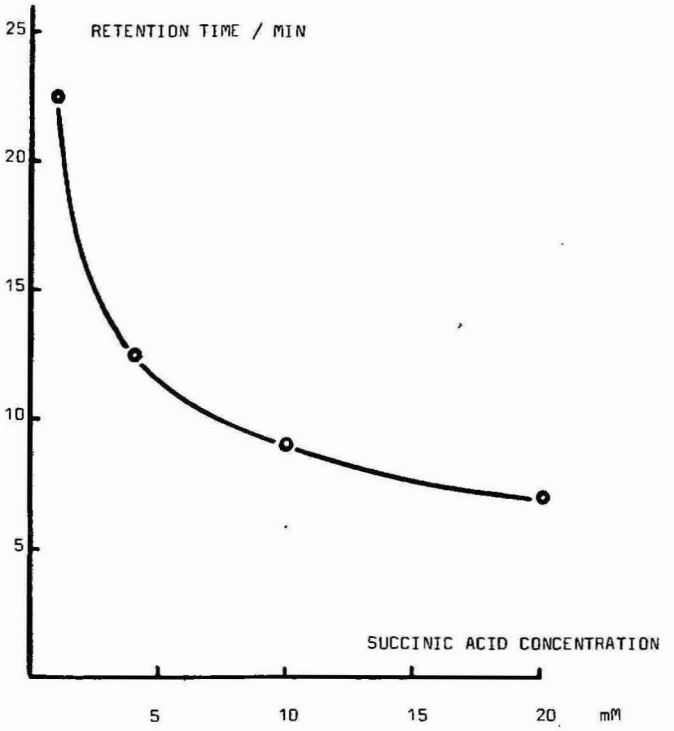

Fig. 1. Plot of variation in retention of $H_2PO_4^-$ with change in the succinic acid eluent concentration.

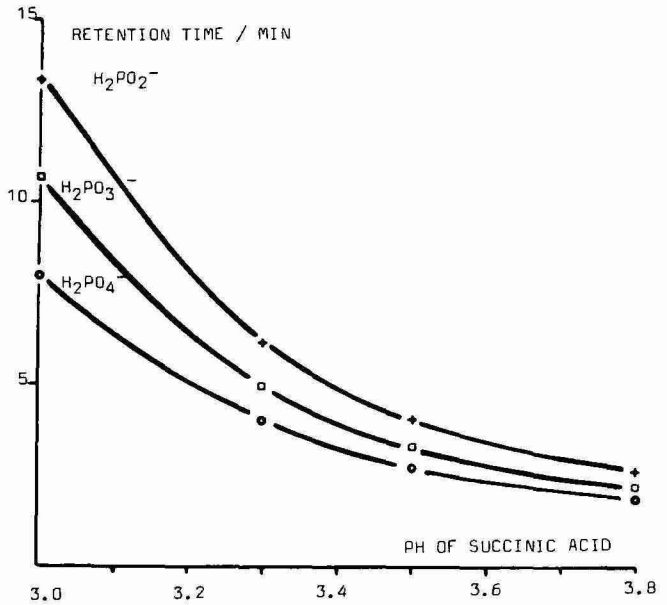

Fig. 2. Plot of variation in retention times of oxo phosphorus acids with change in pH of 0.02 *M* succinic acid eluent, adjusted with sodium hydroxide.

succinic acid without pH adjustment and with a flow-rate of 4 ml/min; this allowed separation to be completed within 20 min (Fig. 3). No system peak interference has been experienced. Despite the high flow-rate an efficiency of approximately 20 000 plates/m was obtained on the last eluted acid, $H_2PO_2^-$.

The limits of detection using these conditions were found to be 0.2 ppm for a 100-μl injection, with a response of three times the average noise level. The linearity for each acid was tested from 5 ppm to 50 ppm using peak area integration (Fig.4).

The method has been used for the determination of residual oxo phosphorus acids in a range of polycarboxyphosphinates and phosphonates over a period of six months. After more than 400 injections on to the same column the efficiency and resolution are still good even though the retention times have become progressively shorter. Duplicate columns of these same type give very similar separations and relative retention times but somewhat different absolute retention times.

CONCLUSION

Single-column ion chromatography using succinic acid as eluent and conductivity detection was found to provide a suitable and sensitive method for the separation of hypophosphorous, phosphorous and phosphoric acids down to the sub-ppm level. The method is robust and has been used for the quantitative determination of the acids in polycarboxyphosphorus compounds.

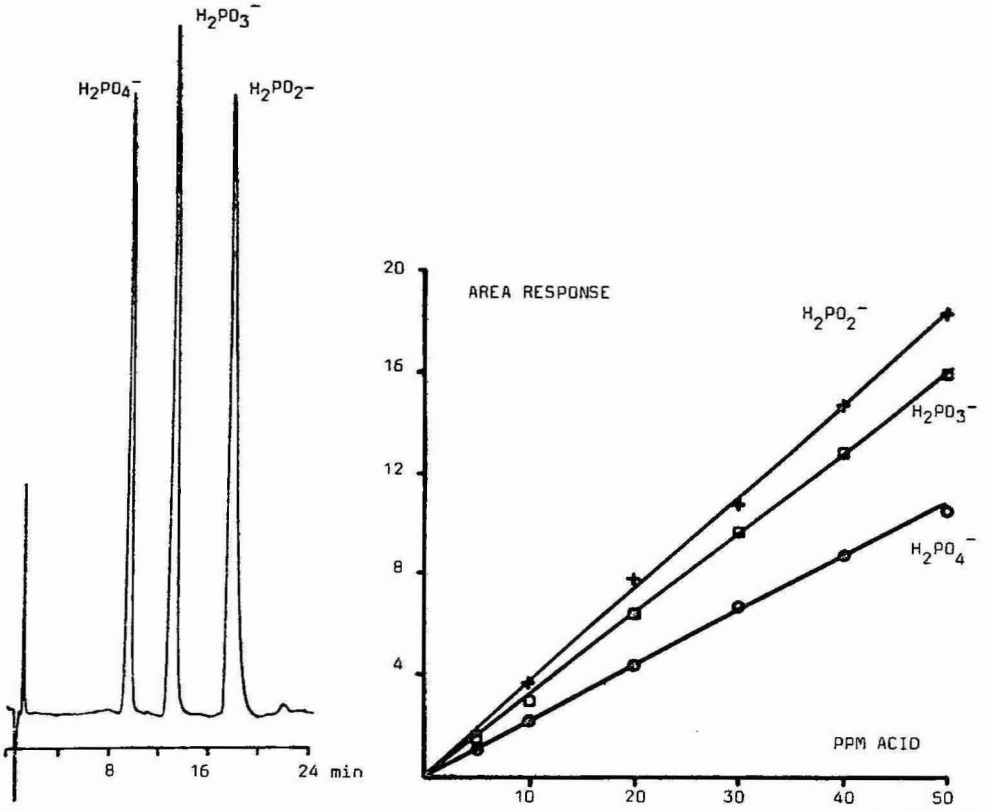

Fig. 3. Separation of oxo phosphorus acids on a Vydac 302, 25 × 0.45 cm I.D. column with 0.02 *M* succinic acid. LDC conductomonitor at 3μS., 100-μl injection containing 50 ppm of each acid.

Fig. 4. Calibration plots of oxo phosphorus acids using 100-μl injections.

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Note

Determination of polycyclic aromatic amines in skin by liquid chromatography with electrochemical detection

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(First received July 29th, 1985; revised manuscript received November 25th, 1985)

The development of synthetic fuels has raised concerns over the health hazards associated with coal derived liquids. Recent health effects studies have shown that some coal liquids contain quantities of genotoxic and carcinogenic polycyclic aromatic amines[1–3]. The potency of many of these aromatic amines has given impetus to studies aimed at understanding their mode of action. Of particular interest is the disposition of polycyclic aromatic amines following dermal exposure because that is considered to be the most likely route of exposure for synfuels workers. This report describes methodology developed in our laboratory for the analysis of several polycyclic aromatic amines in rat and mouse skin using liquid chromatography with electrochemical detection (LC–ED). Although the methodology was developed for measurements of skin absorption rates, it should be adaptable to the analysis of aromatic amines in other tissues.

There are a number of recent reports in the literature on the analysis of aromatic amines in coal liquids in which thin layer, column or liquid chromatography were used for sample clean-up in preparation for analysis by gas chromatography or gas chromatography–mass spectrometry[4–6]. Polyaromatic amines in synthetic fuel mixtures have also been analyzed directly by LC using peroxyoxalate chemiluminescence detection[7]. However, little attention has been given to the analysis of this compound class in biological matrices, although Shaikh *et al.*[8] have measured 2-aminoanthracene and its metabolites after *in vitro* incubations with rat liver S9 fractions by LC after conversion to methylurea derivatives.

The analysis of biological tissues and fluids generally requires methods that are highly selective as well as sensitive. Since the primary aromatic amines are readily oxidized at the carbon electrode, LC–ED offers an attractive approach to their analysis. The sensitivity and selectivity of the LC–ED technique is well documented and has resulted in analytical methods for a variety of other compound types in complex biological matrices. Several reports have illustrated the potential of LC–ED for the analysis of aromatic amines. The detection of picomole quantities of naphthylamines has been demonstrated by Mefford *et al.*[9]. Caudill *et al.*[10] reported on the ability of

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the electrochemical detector to discriminate between the aromatic amine, 2-aminoanthracene, and the corresponding aromatic hydrocarbon, anthracene. The response of the electrochemical detector to a number of one- and two-ring aromatic amines has been examined by Concialini *et al.*[11]. Our report illustrates the utility of the LC–ED technique for the analysis of polycyclic aromatic amines in rodent skin samples. The method presented here also provides a basis for development of procedures for analysis of polycyclic aromatic amines in body fluids and other tissue types.

EXPERIMENTAL

Materials

Aromatic amines, with the exception of 6-aminochrysene, were purchased from Ultra Scientific Corporation (Hope, RI, U.S.A.). 6-Aminochrysene was purchased from Aldrich (Milwaukee, WI, U.S.A.). All solvents were Burdick & Jackson (Muskegon, MI, U.S.A.) distilled-in-glass. Citric acid and trisodium citrate were Baker (Phillipsburg, NJ, U.S.A.) reagent grade and sodium perchlorate was obtained from MCB (Gibbstown, NJ, U.S.A.). All chemicals were used as received.

Apparatus

The liquid chromatograph consisted of a Waters 6000A pump, a Waters WISP 710B automatic sampler, a Kratos Spectroflow 773 variable-wavelength ultraviolet detector and a Bioanalytical Systems Model LC-4A electrochemical detector. Chromatographic peaks were integrated using a Hewlett-Packard 3390A integrator. The electrochemical detector was equipped with a glassy carbon thin-layer electrode referenced to a Ag/AgCl electrode.

High-performance liquid chromatography

Separations were performed on a DuPont Zorbax ODS 25 cm × 4.6 mm I.D. column operated at room temperature (23°C). DuPont C_8 and Waters CN columns were also evaluated during method development. The mobile phase was composed of acetonitrile–citric acid buffer. The buffer was prepared from equimolar quantities of citric acid and trisodium citrate (total citrate concentration from 2.5 m*M* to 5.0 m*M*) and sodium perchlorate (0.1 *M*) in water. For the analysis of skin samples, the acetonitrile–buffer ratio was 70:30. The retention volume for 6-aminochrysene was 12.20 ml at a solvent flow-rate of 1 ml/min. A 20–100-μl volume of tissue extract or of a standard prepared in acetonitrile was injected for each analysis.

Skin painting and extraction

Studies were conducted using Sprague-Dawley CD rats and CD-1 mice (Charles River, Portage, MI, U.S.A.). For extraction efficiency studies, the animals were shaved, killed with carbon dioxide, and skin samples excised and placed on ice immediately. Mouse skin samples were approximately 2 cm in diameter and rat skin approximately 4 cm. Aromatic amines were added to the skin samples by application of either 25 μl or 250 μl of an acetone solution containing approximately 40 μg/ml of each of the amines to be tested. The solution was applied evenly to the external surface of the skin sample using a disposable glass-tipped micropipet. Mouse skin

samples were extracted twice by sonicating for 10 min with 2-ml aliquots of acetonitrile, followed by a final rinse with 1 ml of acetonitrile. The acetonitrile portions were combined in a 5-ml volumetric flask, diluted to volume with acetonitrile, and analyzed without further treatment. Rat skin samples were handled in the same manner, except that two 10-ml aliquots of acetonitrile were used for the extraction and a 5-ml aliquot for the rinse, due to the larger size of the samples. Extracts from rat skin were diluted to a final volume of 25 ml and analyzed without further treatment.

The dermal absorption of 6-aminochrysene in mice was studied by applying 9.00 μg of 6-aminochrysene in acetone to the shaved backs of live aminals. The study in rats was performed in the same manner, except that 9.85 μg was used. Animals were sacrificed with carbon dioxide at selected intervals after dosing, the area of application identified under an ultraviolet lamp, and the skin excised immediately. Skin samples were taken 0, 1, 4 and 24 h after treatment for rats, and at 0, 0.25, 0.5, 1, 4, and 24 h for mice. Excised skin samples were placed in the first extraction volume of acetonitrile (2 ml for mice, 10 ml for rats) in a vial and stored refrigerated overnight before continuing with the extraction and analysis. The extraction and analysis were performed in the same manner as for the recovery studies.

RESULTS AND DISCUSSION

After examining a number of reversed-phase chromatographic systems (C_8, C_{18} and CN), we found that a C_8 or C_{18} column with a mobile phase composed of 40–70% acetonitrile in a aqueous citric acid buffer provides a flexible system for analyzing aromatic amines. The chromatography can be optimized for specific amines by adjusting the percentage of acetonitrile and the pH of the citrate buffer. The effect of acetonitrile content on the capacity factors of several aromatic amines is shown in Table I. Reducing the pH of the mobile phase by substituting citric acid for the citric acid–trisodium citrate buffer caused a dramatic reduction in the retention of the amines. Sodium perchlorate was incorporated in the solvent as a supporting electrolyte for the electrochemical detection. The separation of several aromatic amine standards is shown in Fig. 1.

The selectivity of the electrochemical detector for aryl amines relative to N-

TABLE I

CAPACITY FACTORS (k') OF SELECTED AROMATIC AMINES AS A FUNCTION OF THE ACETONITRILE–CITRATE BUFFER RATIO IN THE MOBILE PHASE

Column: DuPont Zorbax ODS 25 cm × 4.6 mm. Buffer: 5 m*M* citrate (2.5 m*M* citric acid, and 2.5 m*M* trisodium citrate), 0.1 *M* sodium perchlorate. $k' = (t_R - t_m)/t_m$ where t_R is the elution time for the analyte and t_m is the elution time for the solvent (or an unretained solute).

Compound	*Percentage acetonitrile*		
	45	*55*	*65*
2-Aminonaphthalene	5.7	3.6	2.2
4-Aminobiphenyl	9.1	5.1	2.8
2-Aminoanthracene	15.5	7.8	3.9
6-Aminochrysene	36.2	15.7	6.6

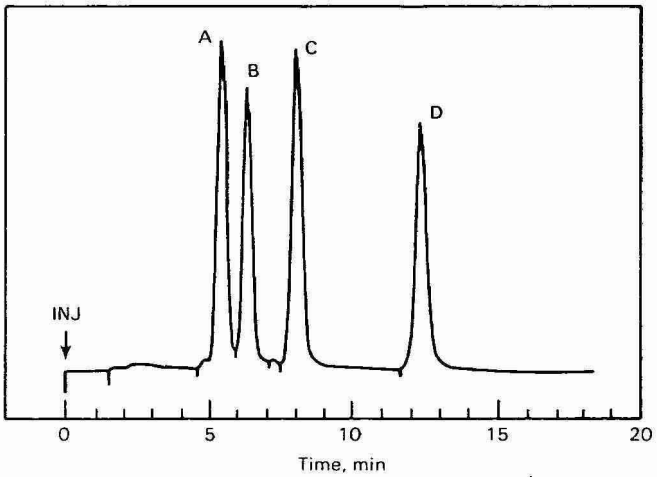

Fig. 1. Separation of aromatic amine standards with acetonitrille. 5 m*M* citrate (65:35). Peaks: A = 2-aminoaphthalene, 43 ng; B = 4-aminobiphenyl, 38 ng; C = 2-aminoanthracene, 41 ng; D = 6-amino-chrysene, 39 ng. Chromatographic conditions as described in the text.

heterocyclic compounds was tested by chromatographing several compound types and simultaneously monitoring the electrochemical signal and the ultraviolet absorption at 254 nm. Compounds chosen were representative of the types commonly encountered in synfuels. The results are summarized in Table II. As was anticipated, the aromatic heterocyclic nitrogen compounds were not readily oxidized at the glassy carbon electrode and were not electrochemically detected even at concentrations several orders of magnitude greater than the minimum detectable concentrations of the primary aromatic amines. Carbazole was the only heterocyclic nitrogen compound tested that gave a response. However, it differs from the other heterocycles in being a secondary amine with the nitrogen external to the aromatic system rather than a part of it.

Although carbazole was detected electrochemically, it is possible to detect the

TABLE II

ELECTROCHEMICAL DETECTOR RESPONSE TO AROMATIC NITROGEN COMPOUNDS

ND = None detected. Conditions: C_{18} column with 1 ml/min acetonitrile–5 m*M* citrate (45:55) and glassy carbon electrode at +0.9 V *versus* an Ag/AgCl reference electrode.

Compound	*Nanomoles injected*	*Area/mole*
Quinoline	3.11	ND
4-Azafluorene	1.26	ND
Benzo[*c*]cinnoline	1.27	ND
5,6-Benzoquinoline	1.18	ND
Carbazole	0.392	0.0745
4-Aminobiphenyl	0.0437	4.41
2-Aminonaphthalene	0.0552	6.20
2-Aminoanthracene	0.0823	4.01
6-Aminochrysene	0.168	3.28

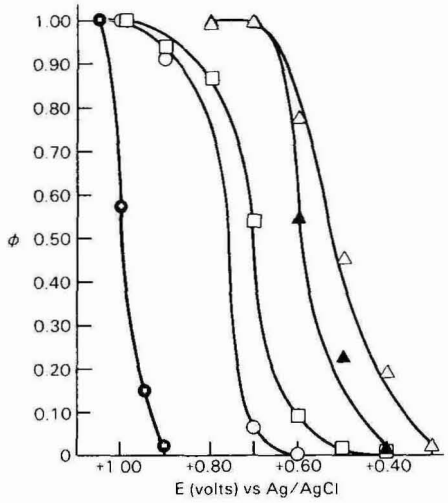

Fig. 2. Hydrodynamic voltammograms of several aromatic nitrogen compounds. ϕ (= the ratio of the peak current to the limiting current) for carbazole was calculated as the ratio of the peak current to the current at +1.05 V. Compounds: ● = carbazole; ○ = 4-aminobiphenyl; □ = 2-aminonaphthalene; △ = 2-aminoanthracene; ▲ = 6-aminochrysene.

aromatic amines in its presence without interference by appropriate selection of the working electrode potential. Some selectivity among the different aryl amines can also be achieved by careful selection of the working electrode potential. The relationship between detector response and voltage for several aryl amines is shown in the hydrodynamic voltammograms in Fig. 2. Adjustment of the electrode potential to +0.7 V, for example will allow detection of 6-aminochrysene without interference from carbazole, which is much more difficult to oxidize. Compounds that have lower oxidation potentials will be detected, while those that have much higher potentials will not.

The electrochemical detector response for three of the aromatic amines was examined in detail and found to be linear over the range of 10 pmoles to at least 500 pmoles injected. Linear regression parameters for several of the compounds are given in Table III. Deviations from detector linearity with injections of 1 nmole or more are typical of many compounds analyzed by LC–ED. If the analytical system were optimized for sensitivity, it would not be unrealistic to expect a minimum detection limit of approximately 0.1 picomole for the more easily oxidized amines. However,

TABLE III

LINEAR REGRESSION PARAMETERS

y = Area response; x = moles of amine.

Compound	*Regression line*	*Correlation coefficient*
2-Aminonaphthalene	$y = 2.22x \cdot 10^6 + 1.35 \cdot 10^4$	0.998
2-Aminoanthracene	$y = 3.30x \cdot 10^6 + 4.04 \cdot 10^3$	0.995
6-Aminochrysene	$y = 2.77x \cdot 10^6 + 5.88 \cdot 10^3$	0.999

TABLE IV

RECOVERY OF SELECTED AROMATIC AMINES FROM SPIKED SKIN

Compound	*Recovery (%)**		
	Mouse		*Rat*
	1 μg	*10 μg*	*10 μg*
2-Aminonaphthalene	98.5– 99.5	99.2– 99.6	101.2–102.0
2-Aminobiphenyl	104.1–107.1	98.9–101.7	103.0–105.1
2-Aminoanthracene	99.5–104.0	96.8– 98.7	97.9– 98.6

* The range of recoveries is given for 3 replicates.

a significant effort would be required to achieve such high sensitivity, and analytical precision would be reduced.

The LC–ED method has been used to monitor the disappearance of 6-ami-

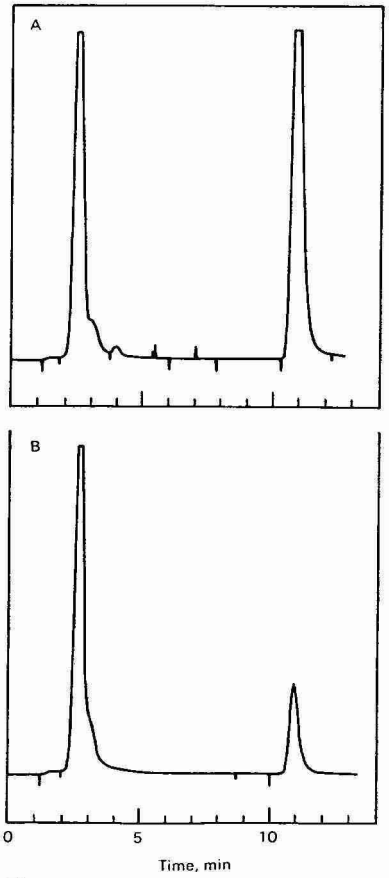

Fig. 3. Chromatograms of mouse skin tissue extracts after application of 9.0 μg 6-aminochrysene at (A) 0 h and (B) 4 h.

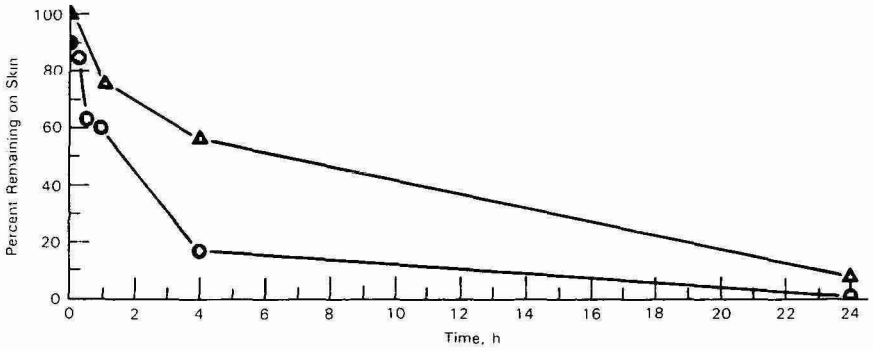

Fig. 4. Percentage of 6-aminochrysene remaining in skin *vs.* time after skin painting. ▲ = Rat, O = mouse.

nochrysene from skin following topical application to rats and mice. Extraction of the skin with acetonitrile gave nearly quantitative recoveries for all the primary aromatic amines studied, as shown in Table IV. The recovery of 6-aminochrysene from rat skin was 98.9% ± 3.6 (S.D.), $n = 6$ at the 1-μg level and 97.4% ± 3.7 (S.D.), $n = 6$ at the 10-μg level. Chromatograms of the tissue extracts were very clean, as shown in Fig. 3, and permitted the quantification of at least 40 pmoles of a primary aromatic applied to the skin. Increased sensitivity could be gained by concentration of the acetonitrile; however, sensitivity was not a limiting factor in our studies and the effect of sample concentration was not explored. Fig. 4 shows representative data obtained by LC–ED for the skin painting of 6-aminochrysene in both the rat and mouse and illustrates the species variation for skin clearance.

The LC–ED approach provides a rapid, sensitive and selective method for analysis of primary polycyclic aromatic amines in epidermal tissue. In addition, the methodology presented may serve as a basis for the analysis of aromatic amines in other tissues and fluids.

ACKNOWLEDGEMENT

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Note

High-performance liquid chromatography of tryptophan and its irradiation products using UV photometric and voltammetric detection

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Investigation of the radiolysis of amino acids is of great importance, as information is provided on the effect of high-energy radiation on proteins (and thus also on living organisms). Data on radiolysis in solution are necessary in the preparation, storage and purification of amino acids labelled with radionuclides.

Tryptophan belongs among the biologically most important amino acids and its radiolysis has recently been extensively studied (ref. 1 and references therein). Radiolysis of tryptophan in aqueous solution involves complex radiation–chemical reactions in which intermediates and final, stable products are formed due to the interaction of the active species produced by radiolysis of water, *e.g.* OH, H and e_{aq}^-, with tryptophan molecules[2]. Radiation transformation of the indole part of the tryptophan molecule is considered to predominate, but deamination, recombination and other processes also occur.

To study these radiation processes, reliable, selective and sensitive analytical methods are required. So far, tryptophan and its metabolites have been widely analysed in biological fluids and tissues using thin-layer chromatography[3], gas chromatography–mass spectrometry (GC–MS)[5] and especially high-performance liquid chromatography (HPLC) with UV photometric and fluorescence detection[6,7].

Most of the products of tryptophan metabolism or degradation are readily oxidizable (hydroxy derivatives are usually formed) and thus voltammetric HPLC detection of these substances is highly sensitive, with an additional advantage of the selectivity of voltammetry that is useful in analyses of complex mixtures. Hence, numerous papers have described these determinations in biological samples (for a survey see ref. 8, and more recently refs. 9 and 10). In the study of the radiolysis of tryptophan, HPLC with UV photometric detection was used[6,7,11,12].

In view of the high sensitivity and selectivity of voltammetric detection, we studied this method as a means of monitoring radiolysis of aqueous solutions of tryptophan under various experimental conditions. The present paper describes the properties of the analytical method, while the results thus obtained for the radiolysis process are given elsewhere[1].

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EXPERIMENTAL

Apparatus

The HPLC measurements were carried out on a Varian Aerograph 4100 instrument (Varian, U.S.A.) at laboratory temperature, in a reversed-phase system with glass columns (150 cm × 4 mm I.D.) packed with Separon SI C_{18} or Separon SI CN, 5 μm (Laboratorní Přístroje, Czechoslovakia). The separation was obtained using the following mobile phases: I, $1.2 \cdot 10^{-4}$ *M* EDTA (aq.)–20% methanol (pH 5.23); II, 0.0001 *M* KH_2PO_4 (aq.), (pH 5.47); III, 300 ml of 0.1 *M* citric acid + 160 ml of 0.1 *M* sodium acetate (pH 4.5) containing 7% methanol. The samples were injected through a stopped-flow valve using 10- and 25-μl syringes (Hamilton, U.S.A.). Two detectors were connected in series at the column outlet, a variable-wavelength Varian UV photometric detector followed by a carbon fibre voltammetric detector constructed by us and described elsewhere[13]. The electrode potentials are referred to a saturated silver chloride electrode.

Chemicals

All the chemicals employed were of p.a. purity (Lachema, Czechoslovakia) and were not further purified. The standard substances involved L-tryptophan (Koch-Light Labs., U.K.), 5-hydroxy-L-tryptophan, L-kynurenine, 3-indolepropionic acid (Fluka, Switzerland), 3-indoleacetic acid (Lachema, Czechoslovakia) and formyl-L-kynurenine (Calbiochem, U.S.A.). Methanol for UV spectroscopy was used.

Preparation of tryptophan radiolytic products

Tryptophan was dissolved in redistilled water to obtain a concentration of 10^{-2} mol/l and the solution was irradiated in 10-ml ground-glass-stoppered test tubes. The pH was adjusted with sulphuric acid or sodium hydroxide and some solutions were bubbled with nitrogen monoxide. A ^{60}Co gamma-source with an intensity of 2.7 kGy/h was used and the doses varied from 20 to 60 kGy. Samples of the solutions after the irradiation were directly injected into the column.

The substances were identified by comparing their retention data with those of the standard substances. For the determination, peak heights were measured, as they exhibited a better precision than the peak areas; and the calibration curve method was used.

RESULTS AND DISCUSSION

Because of great differences in the character of the tryptophan degradation products (acidic, neutral and weakly basic compounds are involved) it was impossible to carry out a satisfactory isocratic separation on a single column[6]. The problem can be solved either by a step change in the pH of the mobile phase during the separation[6] or by using two different columns. It was found that satisfactory separation of kynurenine, 5-hydroxytryptophan and tryptophan can be achieved using a C_{18} column with mobile phases I–III. 3-Indoleacetic and 3-indolepropionic acids are eluted with the solvent front when using mobile phases I and II. On the other hand, they are eluted after tryptophan with long retention times by mobile phase III. Good results are then obtained when mobile phase III is combined with a CN-stationary phase.

In selecting the mobile phase, the requirement of a sufficient electrical conductivity had to be satisfied if voltammetric detection was to be used.

The effect of the mobile phase flow-rate was followed from 0.1 to 1.0 ml/min, from the point of view of optimization of voltammetric detection. The signal of the detector was virtually independent of the flow-rate within this interval (*cf.* also ref. 13) and thus a value of 0.3 ml/min was selected, which was optimal from the point of view of the pressure in the system and the time of analysis.

To find the optimal detection conditions, the UV absorption curves and the voltammograms of the substances were obtained directly in the detector cell in the appropriate mobile phases. The UV absorption maxima lie at wavelengths of 220 and 250–290 nm. As the mobile phase itself absorbs strongly at 220 nm, a wavelength of 256 nm was selected for the determination. Typical hydrodynamic voltammograms are given in Fig. 1; on their basis, an electrode potential of +1.4 V was selected as the value at which the highest signal-to-noise ratio is obtained, in agreement with the literature[14].

The very high sensitivity of voltammetric detection, compared with UV photometric detection, and its suitability for monitoring tryptophan radiolysis products are demonstrated in Fig. 2a and b. The figure shows that the radiolysis products include kynurenine, 5-hydroxytryptophan, 3-indoleacetic and 3-indolepropionic acid. Formylkynurenine was not found among the products. The other substances that appeared in the chromatogram could not be identified because of a lack of the appropriate standards.

The calibration data for the UV photometric and voltammetric detection are given in Table I. The table shows that the voltammetric detector exhibits good lin-

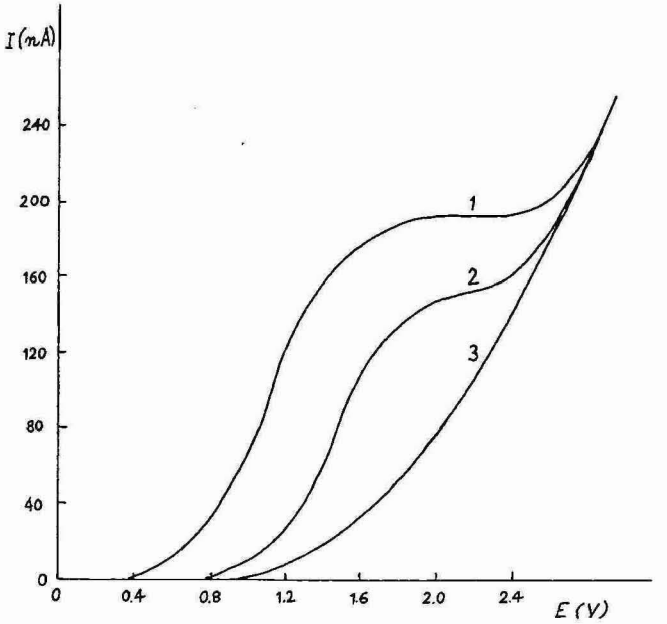

Fig. 1. Hydrodynamic voltammograms of 5-hydroxytryptophan (1), tryptophan (2) and the mobile phase (3). Mobile phase, 0.001 *M* KH_2PO_4; flow-rate, 0.3 ml/min.

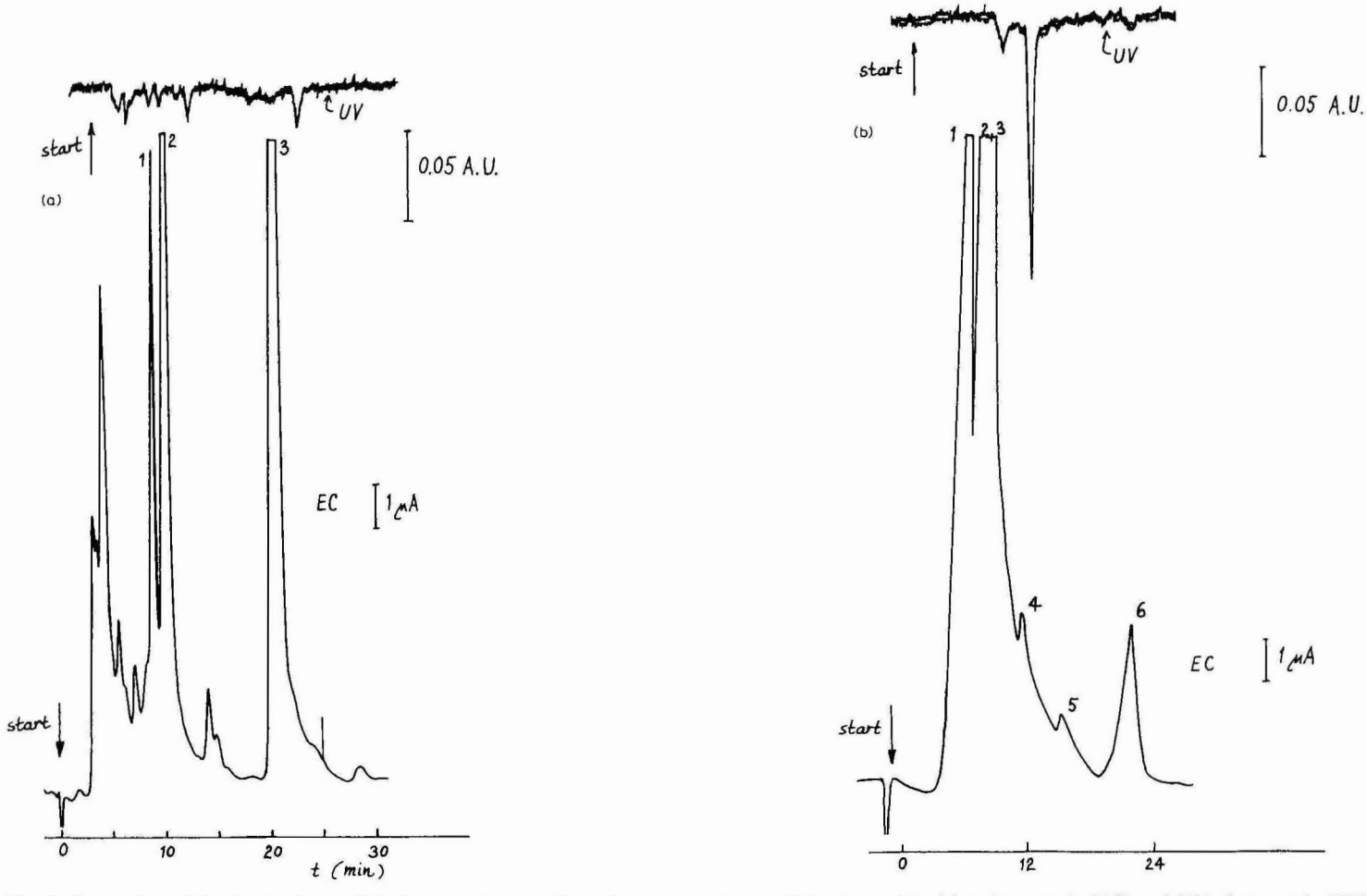

Fig. 2. Separation of the tryptophan radiolytic products on a C_{18} column (a) and on a CN column (b) with voltammetric (EC) and UV photometric (UV) detection. Mobile phase III, flow-rate 0.3 ml/min. (a) 1 = Kynurenine, 2 = 5-hydroxytryptophan, 3 = tryptophan. (b) 1 = Hydroxytryptophan, 2 = kynurenine, 3 = tryptophan, 4 = 3-indoleacetic acid, 5 = unidentified, 6 = 3-indolepropionic acid.

TABLE I

CALIBRATION DATA FOR THE UV PHOTOMETRIC AND VOLTAMMETRIC DETECTOR

For experimental conditions, see the text. The detection limit equals twice the peak-to-peak noise.

Substance	*UV photometric detector*			*Voltammetric detector*		
	Corr. coeff.	*Slope of regression dependence (A.U./ng)*	*Detection limit (ng)*	*Corr. coeff.*	*Slope of regression dependence (μA/ng)*	*Detection limit (ng)*
3-Indolepropionic acid	0.999	$7.59 \cdot 10^{-3}$	3.9	0.999	$3.75 \cdot 10^{-2}$	0.7
3-Indoleacetic acid	0.998	$6.63 \cdot 10^{-3}$	4.8	0.995	$1.77 \cdot 10^{-2}$	1.8
Kynurenine	0.997	$6.92 \cdot 10^{-3}$	4.3	0.999	$3.72 \cdot 10^{-2}$	0.9
5-Hydroxytryptophan	0.999	$5.58 \cdot 10^{-3}$	6.7	0.999	$6.30 \cdot 10^{-2}$	0.5
Tryptophan	0.993	$6.98 \cdot 10^{-3}$	4.1	0.998	$6.10 \cdot 10^{-2}$	0.8
Formylkynurenine	0.999	$9.38 \cdot 10^{-3}$	1.3	Electro-inactive		

earity, is more sensitive than the UV photometric detector and yields substantially lower detection limits for the test substances, with the exception of formylkynurenine which is electro-inactive. The connection of a UV photometric and a voltammetric detector in series is especially advantageous, as comparison of the detector responses facilitates identification of solutes in complex mixtures.

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Note

Reversed-phase high-performance liquid chromatographic separation of substituted phenolic compounds with a β-cyclodextrin bonded phase column

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The high-performance liquid chromatographic (HPLC) analysis of phenols has been an active field due to its practical applications in biochemical, clinical, forensic, and wood chemistry as well as in food inspection and environmental pollution control. For example, Åkerblom and Lindgren[1] have separated *o*- and *p*-chlorophenol isomers using an octadecylsilica (ODS) column with a methanol–acetic acid mobile phase system. The United States Environmental Protection Agency has listed eleven priority phenolic pollutants with a variety of substituents such as chloro, methyl, and nitro groups. An investigation of a group of multi-substituted phenols by Buckman *et al.*[2] indicates that the more number of chlorine atoms on phenol, the longer the retention time, using an ODS column with an acetonitrile–water–acetic acid mobile phase system. Separations of several hydroxyphenols using a silica column[3] and a diamine bonded phase column[4] have also been reported. Despite all of the activities in the separation of phenols (in most cases, with ODS columns), no detailed study has been reported using the newly commercialized β-cyclodextrin bonded phase column.

It is noted that the initial effective use of cyclodextrins (CD) in chromatography was as modifiers in thin-layer chromatography (TLC)[5,6]. Polymerized CD gels were also used as stationary phases in column chromatography with varying degrees of success[7]. Attempts to prepare stable, fully derivatized high-performance CD-silica packings via different amine or ethylenediamine linkages were later reported by two Japanese groups[8–10]. However, problems such as poor hydrolytical stability, low cyclodextrin loading, low efficiency, and tedious synthetic procedures and poor reproducibility were encountered[11].

The newly commercialized β-CD-bonded phase column by Advanced Separation Technology is packed with 5-μm silica material covalently bonded with β-CD molecules. The β-CD molecule, a non-ionic, toroidal shaped and cyclic carbohydrate, is considered to be the host molecule chemically bonded to silica gel via a stable spacer (non-nitrogen-containing) 6 to 10 atoms in length[12]. In the hollow truncated cone, there are seven primary hydroxyl groups on the side with the smaller circum-

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ference and fourteen secondary hydroxyl groups, seven in clockwise directions and seven counterclockwise, on the side with the larger circumference[13]. Inside the cavity there are no hydroxyl groups which provides a hydrophobic environment. Because of the rigid cavity size of β-CD, only those guest molecules of proper size such as naphthalene can form strong β-CD inclusion complexes[14]. Molecules that do not form strong inclusion complexes with β-CD can also be separated by either a normal phase or a reversed-phase mode[15]. However, due to the hydrolytic stability of this particular β-CD-bonded phase it is often used with water–methanol eluent systems. The unique selectivities exerted by the β-CD column toward a large number of enantiomers[16] and its use for routine separations have warranted large numbers of applications. In this paper, we report its use for the efficient separation of various phenolic compounds.

EXPERIMENTAL

Apparatus

A Beckman 332 liquid chromatograph with two Altex 110 pumps and a microprocessor control unit together with a Micromeritics 786 variable-wavelength (200–600 nm) detector with a deuterium lamp was used. A Micromeritics Model 7500 LC system was also used. This latter system was equipped with a Model 750 solvent delivery system, a Model 752 ternary solvent mixer, and a Model 731 column compartment with a universal injector and a variable-temperature controller from ambient temperature to 150°C.

Pressure-Lok series C-160, 10-μl and 25-μl syringes were used for sample injection. Chromatograms were recorded with a Linear Model 555 single-channel recorder.

Materials

Partisil PXS ODS column, 5 μm, 25 cm × 4.6 mm I.D., from Whatman was used for reversed-phase separation. β-CD column, 25 cm × 4.6 mm I.D., was obtained from Advanced Separations Technology.

HPLC-grade methanol, 2-propanol, and *n*-heptane were obtained from Fisher Scientific. HPLC water (18 MΩ) was obtained by passing boiled deionized water through a Milli-Q Type I reagent-grade water system. All other chemicals were reagent grade and obtained from various sources.

Chromatographic procedures

The general procedures to study the effects of solvent composition were described in a previous publication with minor modifications[17]. Mobile phase pre-equilibration was always achieved before any separation occurred. A flow-rate of 1 ml/min was used. Published methods were used to determine the t_0 value (retention time of an unretained compound) for the β-CD column[16].

RESULTS AND DISCUSSION

Table I lists the capacity factors of several substituted phenols using a β-CD column with various methanol–water mobile phase systems. In general, the elution

TABLE I

CAPACITY FACTORS (k') OF SEVERAL SUBSTITUTED PHENOL ISOMERS IN A β-CD-COLUMN USING A METHANOL–WATER MOBILE PHASE SYSTEM

		Percentage of methanol in water (v/v)							
		10	*20*	*30*	*40*	*50*	*65*	*80*	*100*
Aminophenol	*o-*	1.08	0.76	0.57	0.41	0.28	0.17	0.13	0.11
	m-	0.72	0.51	0.37	0.24	0.14	0.10	0.08	0.08
	p-	0.59	0.43	0.29	0.19	0.10	0.08	0.08	0.07
Cresol	*o-*	2.60	1.87	1.11	0.61	0.33	0.12	0.04	0.03
	m-	3.22	2.51	1.37	0.68	0.40	0.15	0.06	0.03
	p-	4.87	3.64	2.19	1.12	0.61	0.25	0.08	0.05
Nitrophenol	*o-*	4.09	2.80	1.92	1.19	0.70	0.29	0.15	0.13
	m-	4.22	2.86	2.02	1.21	0.70	0.24	0.08	0.06
	p-	8.30	6.33	4.22	2.53	1.53	0.61	0.27	0.24
Chlorophenol	*o-*	5.33	3.08	2.20	1.06	0.54	0.22	0.06	0.04
	m-	6.92	5.10	2.99	1.43	0.72	0.29	0.07	0.05
	p-	8.05	5.85	3.64	1.87	0.96	0.33	0.09	0.06

order for the positional isomers of cresol and chlorophenol is always *ortho* < *meta* < *para* regardless of mobile phase composition. However, the sequence is *para* < *meta* < *ortho* for aminophenol isomers. On the other hand, the elution order for nitrophenols is *ortho* < *meta* < *para* when methanol in mobile phase is 10% or 15%. This changes into *meta* < *ortho* < *para* when mobile phases of 65–100% (v/v) methanol in water are used. These results are not quite the same as those of unmodified and modified CD columns prepared by the Japanese groups, because most of their studies on retention dependence on eluent composition stopped at 40% methanol in water, and their studies were also complicated by the presence of amine groups in the bonded phases[8–10].

If all compounds are considered, it is observed that *p*-aminophenol and *m*-aminophenol are eluted as the first two compounds among twelve, and *p*-chlorophenol and *p*-nitrophenol the last two, using 10–65% methanol–water mobile phase system. At higher concentration of methanol in water, *i.e.* 80–100%, most of compounds coelute with *o*-cresol and *m*-cresol showing the shortest retention times and *o*-aminophenol, *o*-nitrophenol, and *p*-nitrophenol as the last three. The best condition for the separation of all twelve compounds is when a mobile phase of methanol–water (10:90) is used. (See chromatogram in Fig. 1). Although not all the peaks are well resolved, the separation is considered most favorable as compared to those done by other systems. In fact, our preliminary test for the separation of the twelve compounds using either a silica column under a normal phase mode or an ODS column under a reversed-phase mode does not give satisfactory results. For example, Fig. 2 shows the poor separation of all twelve substituted phenols using a Partisil PXS ODS column with 10% 2-propanol–water (10:90, v/v) mobile phase system under simple optimized, isocratic binary solvent conditions. It should be noted that although further optimization of the separation can be performed on all separations, including

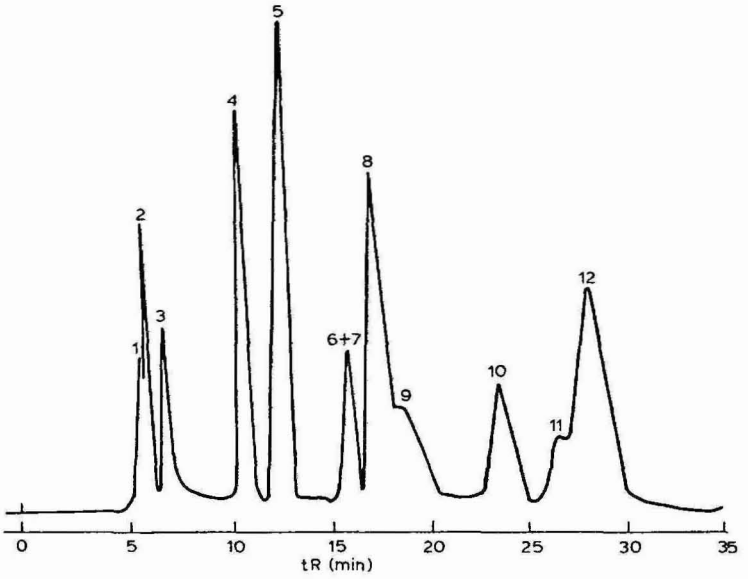

Fig. 1. Chromatogram for the separation of twelve substituted phenols, β-CD column, methanol–water (10:90), flow-rate = 1 ml/min. Peak identification: 1 = *p*-aminophenol, 2 = *m*-aminophenol, 3 = *o*-aminophenol, 4 = *o*-methylphenol, 5 = *m*-methylphenol, 6 = *o*-nitrophenol, 7 = *m*-nitrophenol, 8 = *p*-methylphenol, 9 = *o*-chlorophenol, 10 = *m*-chlorophenol, 11 = *p*-chlorophenol, 12 = *p*-nitrophenol.

the present separation of substituted phenols using a β-CD and an ODS columns, the intrinsic separation power of the β-CD column is clearly evident.

A specific feature involved in the separation using β-CD bonded phase is the possibility of inclusion process. A recent study by Chang *et al.*[18] proposed both vertical and flat inclusion configuration of several aromatic ligands and their organo-

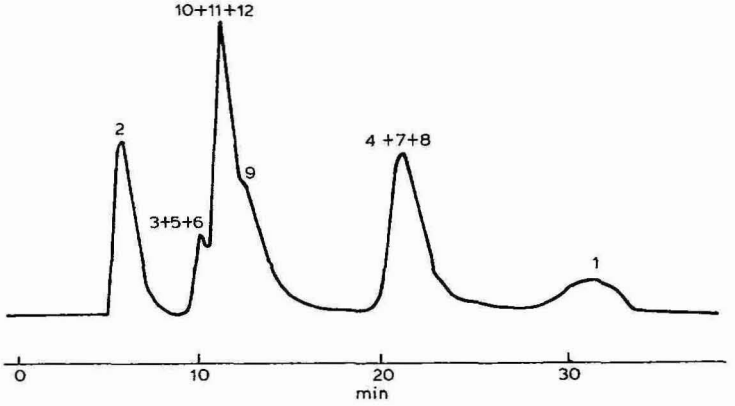

Fig. 2. Chromatogram for the separation of twelve substituted phenols, Partisil PXS ODS column, 2-propanol–water (10:90), flow-rate = 1 ml/min. Peak identification: 1 = *p*-aminophenol, 2 = *m*-aminophenol, 3 = *o*-aminophenol, 4 = *o*-methylphenol, 5 = *m*-methylphenol, 6 = *o*-methylphenol, 7 = *p*-chlorophenol, 8 = *m*-chlorophenol, 9 = *o*-chlorophenol, 10 = *p*-nitrophenol, 11 = *m*-nitrophenol, 12 = *o*-nitrophenol.

metallic compounds. In the present work, a reversed-phase behavior is observed for all phenolic compounds, *i.e.* retention time increases with increase of water content in the mobile phase. This is consistent with the inclusion phenomenon because the hydrophobic cavity of β-CD provides a reversed-phase environment. On the other hand, when 100% methanol is used as mobile phase, the inclusion process is probably not significant as evidenced by the fact that resolution of enantiomers cannot be achieved either[19]. In this case, normal phase behavior is probably dominant. Thus, mixed retention mechanisms are involved in the separation of phenols, similar to what has been found for the separation of several benzoic acids using a β-CD-bonded phase column[15]. This is advantageous when a complex separation is considered because one should be able to adjust the selectivity at a higher degree of freedom.

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Note

Comparison of refractive index, low-wavelength UV and UV visualisation detection methods for the high-performance liquid chromatographic determination of hexamethonium bromide in an injection formulation

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N,N-Hexamethylenebis(trimethylammonium)dibromide, (hexamethonium bromide) a quaternary ammonium ganglion-blocking agent with actions similar to those of pempidine tartrate, is used in the treatment of hypertension and is administered by either subcutaneous or intramuscular injection[1].

The British Pharmaceutical Codex[2] contains a monograph for hexamethonium bromide in which a non-specific titration method is described to determine the purity of the compound. No chromatographic assay procedure appears to have been published. A suitable method is required for the evaluation of hospital manufactured 2.5% (w/v) injection solutions of hexamethonium bromide and, as quaternary ammonium compounds have been assayed previously in this laboratory using ion-pair reversed-phase high-performance liquid chromatography (HPLC)[3,4], this method was chosen for the estimation of hexamethonium bromide. The compound does not contain any UV light absorbing groups and so the classical choice of detection would be differential refractive index (RI) detection. However, papers published in recent years suggest that low-wavelength UV[5] and UV visualisation detection[6] should be considered. In this communication HPLC assays for hexamethonium bromide are presented and the use of the three detection methods evaluated.

EXPERIMENTAL

Instrumentation and reagents

The HPLC system used consisted of an Applied Chromatography Systems 750/03 pump, a Rheodyne 7125 injector valve fitted with a 20-μl sample loop, a Pye-Unicam PU 4020 variable-wavelength absorbance detector operated at 262 and 210 nm, an Applied Chromatography Systems 750/13 differential refractometer, a Perkin Elmer Model 56 dual pen recorder (chart speed, 5 mm/min) with an operating voltage of 10 mV and a Perkin Elmer Sigma 15 integrator. The assays were carried out using a Waters 300 × 4.6 mm I.D. μBondapak C_{18} column. The mobile phases used were: solvent A, methanol–water (50:50) containing 0.005 *M* 1-heptanesulphonic acid sodium salt, pH adjusted to 3.5 with glacial acetic acid; solvent B,

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methanol–water (25:75) containing 0.005 *M* *p*-toluenesulphonic acid sodium salt, pH adjusted to 3.5 with glacial acetic acid. A flow-rate of 2.0 ml/min was found to give acceptable retention times for both solvents.

All solvents and reagents were chromatographic grade (Fisons, Loughborough, U.K.), except toluenesulphonic acid sodium salt (BDH, Eastleigh, U.K.). In all cases pure eluent was used to fill the reference cell of the RI detector.

UV scans of hexamethonium bromide and potassium bromide were carried out using a Perkin Elmer Model 200 UV spectrophotometer.

Methods

A 2.5% (w/v) solution of hexamethonium bromide (Sigma, U.K.) in water was autoclaved at 115°C for 30 min. Samples were then diluted to 0.5% (w/v) before introduction into the injector, using the loop filling technique, with a 100 μl syringe. Standard solutions for the calibration curves were prepared by dissolving a suitable amount of hexamethonium bromide in water.

The RI detector did not have a suitable output for use with the integrator so peak height measurement was used to quantify the results, but both peak height and area were used to calculate the results from the UV detector.

RESULTS AND DISCUSSION

Quaternary ammonium compounds have been assayed successfully in this laboratory by reversed-phase HPLC using an alkylsulphonic acid (usually heptanesulphonic acid) as an ion-pairing agent[3,4]. As a result, the initial assay development work on hexamethonium bromide was carried out using a RI detector and 0.005 *M* heptanesulphonic acid sodium salt in the solvent and varying the methanol–water content to obtain a suitable retention time and to try to detect breakdown products which may be present in the autoclaved injection solution. No breakdown products were detected and the most suitable solvent mixture was found to be solvent A, which gave a retention time for hexamethonium of 4.7 min (Fig. 1a). Linear response was found, using peak height measurement, over the range 80–110 μg injected on column. The minimum detectable quantity of hexamethonium bromide was found to be 2.5 μg.

The UV spectrum of a 0.5% (w/v) solution of hexamethonium bromide shows an E_{max} at around 210 nm. As a result low-wavelength HPLC detection was investigated using solvent A with the UV detector set at 210 nm and 0.04 A.U.F.S. The UV detector was operated in series with a RI detector. Fig. 1b shows that no peak was detected for hexamethonium at 210 nm and so this is not a suitable method of detection for this compound. The UV spectrum of potassium bromide indicates that the low-wavelength UV absorbance exhibited by hexamethonium bromide is due to the bromide in the compound.

UV visualisation detection of hexamethonium bromide was investigated using solvent B containing toluenesulphonic acid as the UV absorbing counter-ion. The UV detector was set at 262 nm, the E_{max} for toluene sulphonic acid, and 0.16 A.U.F.S. The UV detector was operated in series with the RI detector operated at range × 1. Retention times for hexamethonium bromide for RI and UV visualisation detection were 4.0 min (Fig. 2a) and 3.7 min (Fig. 2b) respectively. Linear calibration

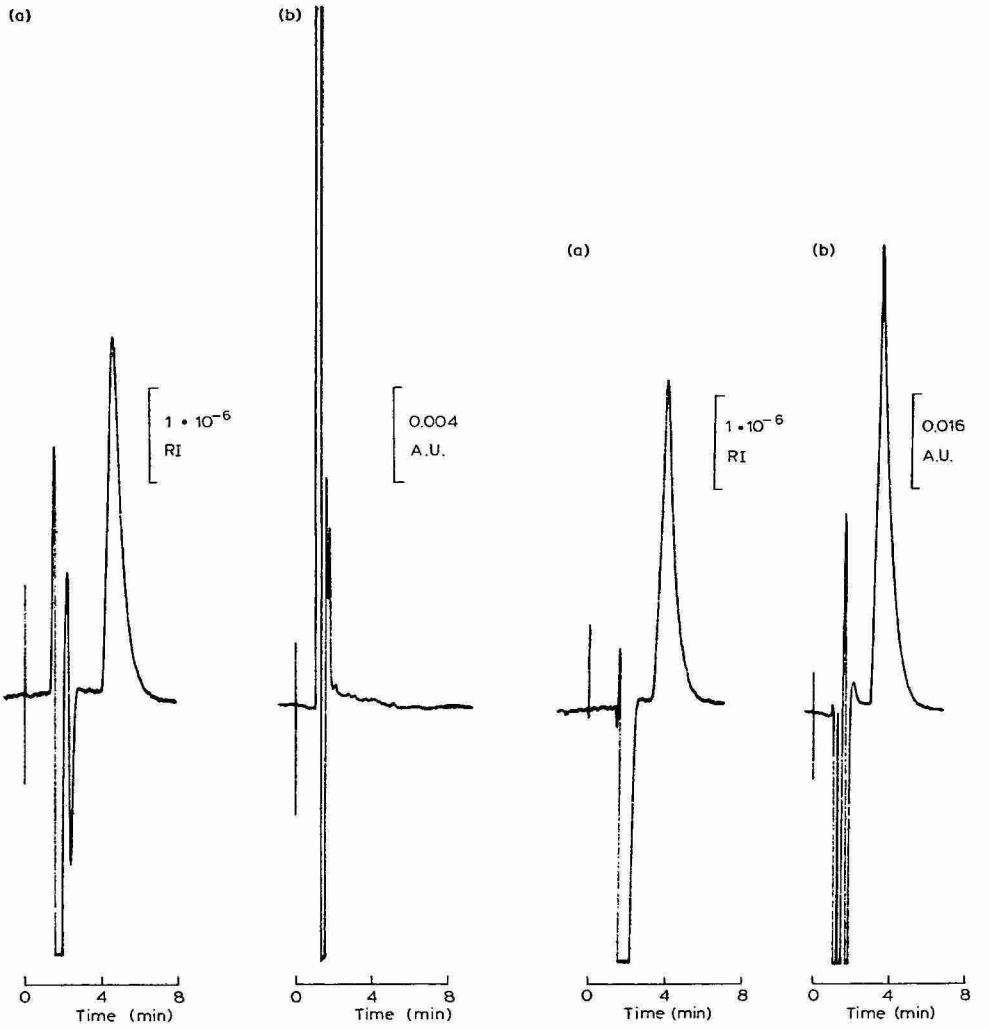

Fig. 1. Chromatograms of 0.5% (w/v) hexamethonium bromide using (a) RI detection (range × 1) and (b) UV detection 210 nm (0.04 A.U.F.S.). Column: μBondapak C_{18}. Eluent: methanol–water (50:50) containing 0.005 *M* heptanesulphonic acid sodium salt, pH 3.5. Flow-rate: 2 ml/min.

Fig. 2. Chromatograms of 0.5% (w/v) hexamethonium bromide using (a) RI detection (range × 1) and (b) UV visualisation detection 262 nm (0.16 A.U.F.S.). Column: μBondapak C_{18}. Eluent: methanol–water (25:75) containing 0.005 *M* toluenesulphonic acid sodium salt, pH 3.5. Flow-rate: 2 ml/min.

curves were obtained for the UV visualisation detection method, using both peak height and peak area measurements, and for the RI detection method using peak height measurement over the range 80–110 μg on column. The minimum detectable quantity of hexamethonium bromide using UV visualisation detection was 0.5 μg (0.04 A.U.F.S.) and for RI detection 2.5 μg (range × 1).

Using a 0.1% (w/v) solution of toluenesulphonic acid sodium salt the system peak for solvent B was found to elute at the solvent front.

TABLE I

ASSAY RESULTS FOR 2.5% (w/v) HEXAMETHONIUM BROMIDE INJECTION USING REFRACTIVE INDEX AND UV VISUALISATION DETECTION METHODS

Detection method	*Quantification method*	*Label claim found (%)*
Refractive index	Peak height	102.75
UV visualisation	Peak height	103.50
UV visualisation	Peak area	103.00

The loop injection valve gave good peak height and area reproducibility for both detection methods, with a relative standard deviation of ± 1.1%.

Stabilisation time for the RI and UV detectors using both solvents A and B was approximately 60 min.

Table I shows the assay results obtained for a production batch of 2.5% (w/v) hexamethonium bromide, 5 ml injection, using solvent B and both RI and UV visualisation detection. The results all fall within the limits: mean ± 0.5% showing that the three quantification methods give very comparable results. Assay limits for the injection were set at 95–105% label claim.

CONCLUSIONS

Of the three HPLC detection methods evaluated for the assay of hexamethonium bromide, RI and UV visualisation give acceptable results, but low-wavelength UV detection, at 210 nm, is unable to detect hexamethonium. The UV visualisation detection method is more sensitive than the use of a differential refractometer and eliminates the need for this latter type of detector for the assay of this non-UV light absorbing compound. From the results obtained it is concluded that the HPLC procedure described is a suitable method for determination of hexamethonium bromide in injection solutions.

ACKNOWLEDGEMENTS

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Note

High-sensitivity amino acid analysis of stained peptides and proteins from a sodium dodecyl sulphate-polyacrylamide slab gel

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The automated amino acid analyser based on ion-exchange chromatographic procedures was first devised by Moore *et al.*[1]. The separated amino acids were determined by colorimetry after post-column derivatization with ninhydrin[1,2]. This device was widely employed for quantitative amino acid analysis and the chemical characterization of peptides and proteins in the living system.

More recently, reversed-phase separation procedures and high-performance liquid chromatography (HPLC) have been developed. The classical method of amino acid analysis was first subjected to the HPLC system, and subsequently ninhydrin has often been replaced by fluorescamine[3,4] or *o*-phthalaldehyde[7–9] offers high sensitivity but suffers from disadvantages relating to the lack of the proline reaction, instability of the fluorescent products and difficulties in quantitation owing to the sensitivity to quenchers. Quantitation of proline requires special oxidative procedures[8] for opening the ring, thus greatly complicating the design of *o*-phthalaldehyde-based analysers. This method requires improvement for the determination of proline, which is one of the important amino acids constituting biologically active peptides.

Pre-column derivatization for amino acid analysis has been reported with dansyl[10,11] and phenylthiohydantoin (PTH)[12,13] derivatives of amino acids. The HPLC analysis of the pre-column derivatives (PTH- and dansyl-amino acids) is relatively rapid and exhibits good efficiency and sensitivity. However, the methods suffer from a lack of stability of the derivatives, low quantitative yields of PTH-amino acids and interference by peaks of byproducts originating from dansyl chloride.

More recently, high-sensitivity analysis of amino acids, including proline, derivatized quantitatively with phenyl isothiocyanate (PITC) has been described[14,15]. This technique, especially with reversed-phase columns, offers higher efficiency, greater ease of use and a higher speed of analysis than the conventional ion-exchange techniques. This method is not suitable, however, for samples that contain a large amount of ammonium chloride, a byproduct occurring during acid hydrolysis of peptides and proteins, because the separation of phenylthiocarbamoyl (PTC)-proline and a large amount of phenylthiourea derived from ammonium chloride is not achieved.

The purpose of this study was to establish a method for the separation of

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PTC-amino acids, especially PTC-proline and phenylthiourea, and to apply it to the amino acid analysis of acid hydrolysates of insulin B-chain, insulin, cytochrome *c* and other peptides and proteins extracted from a polyacrylamide slab gel after electrophoresis and staining with Coomassie brilliant blue. They were considered to produce a large amount of ammonium chloride on acid hydrolysis. We achieved their separation by the addition of 0.1% of sodium dodecyl sulphate (SDS) to the mobile phase. The results of amino acid analysis showed that the numbers of amino acids in these samples were each close to those calculated from their amino acid sequences, with a few exceptions. This method may be suitable for the routine microanalysis of the hydrolysates of peptides and proteins which are separated and stained on SDS-polyacrylamide slab gels.

EXPERIMENTAL

Materials

Reagents were obtained from the following sources: acetonitrile, triethylamine (TEA), amino acid standards and constant-boiling hydrochloric acid, Pierce (Rockville, IL, U.S.A.); sodium acetate, glacial acetic acid, PITC, acrylamide, Coomassie brilliant blue, and cytochrome *c* (Cyc, from horse heart), Wako (Osaka, Japan); oxytocin, bradykinin, angiotensin I and insulin B-chain, Protein Research Foundation (Osaka, Japan); SDS, BDH Chemicals (Poole, U.K.); and insulin (from bovine pancreas, crystalline), Sigma (St. Louis, MO, U.S.A.). High-purity water was prepared with a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.).

Apparatus and chromatography

The apparatus consisted of a Waters new sample preparation module (Pico-Tag Workstation) and a Waters liquid chromatograph (Pico-Tag amino acid analyser) (Waters Assoc., Milford, MA, U.S.A.). The former incorporated two separate functions in a compact design for the batchwise preparation of protein and peptide hydrolysates and for derivatization of the free amino acids. The latter was an HPLC system that consisted of two Model 6000A solvent delivery systems and a Model 440 fixed-wavelength detector (254 nm) controlled with a Model 680 controller. The temperature was controlled at 38 + 1°C with a column heater (Waters Assoc.). Sample were injected in volumes ranging from 1 to 40 μl using a Waters Model 710B autosample injector. The chromatographic separation was achieved in the presence of 0.1% of SDS on a Pico-Tag octadecylsilane column (particle size 5 μm, 150 × 3.9 mm I.D.) with a solvent system consisting of two eluents: eluent A, 131 m*M* sodium acetate buffer (pH 6.4) containing 6% of acetonitrile and 0.05% of TEA; eluent B, 60% acetonitrile in water. The gradient controller was programmed as follows: eluent B set to 0–46% in 10 min using a convex curve (No. 5), 46–100% in 0.5 min using a linear gradient (No. 6) and 100% for 2 min.

Hydrolysis (110°C, 24 h in 6 *M* HCl gas) of peptides and proteins and derivatization of the resultant amino acids with PITC were carried out according to the method of Bildingmeyer *et al.*[15].

Polyacrylamide gel electrophoresis

Polyacrylamide slab gels, 12 × 13 cm in size and 1–2 mm average thickness,

were prepared according to the method of Laemmli[16]. Prior to electrophoresis, the gels were subjected to an electric current (40 mA, 100 V, 3 h) in the absence of peptide and protein samples in order to remove unpolymerized materials.

Samples, each of 10 μg dissolved separately in 10 μl of deionized water, and 10 μl of deionized water alone for reference gels were boiled in the loading buffer[16] for 3 min and applied, after cooling, to the top of the same slab gel at 20-mm intervals in a volume not exceeding 50 μl. After electrophoresis (40 mA, 100 V, 2 h), the gel was stained with 0.2% Coomassie brilliant blue for 3 h. The stained gel was destained with 500 ml of methanol–acetic acid–water (5:7:88) and rinsed successively with 500 ml each of deionized water and high-purity water, five times in each instance. During the destaining procedures, the glycine in the gel from the running buffer (Tris–glycine) was also eliminated. The stained bands of peptides and proteins and the reference gels corresponding to the running positions of the stained bands were cut, each in a size of 2 × 8 mm, from the slab gel. The cut-off gels were kept in 200 μl of 2% TEA with gentle shaking at 37°C for 30 min in Pyrex glass tubes in order to extract the stained peptides and proteins. The supernatants, lightly coloured with Coomassie brilliant blue, were removed from the tubes with a microsyringe and used as samples for amino acid analysis. The drying and hydrolysis (110°C, 24 h, in 6 *M* HCL gas) of the samples and derivatization of the resultant amino acids with PITC were carried out in the Pico-Tag Workstation according to the method of Bidlingmeyer *et al.*[15]. Prior to the above experiment, all the glass tubes were heated in an electric oven at 550°C for 1 h.

RESULTS AND DISCUSSION

Separation of PTC-amino acids

Fig. 1 shows the separation of PTC-amino acids at the 250 pmol level. PTC-proline and phenylthiourea (shown as NH_3 in the figures) were chromatographed with the same retention time in the absence of 0.1% SDS at a flow-rate of 1 ml/min. PTC-β-alanine (shown as β-A in the figures) and PTC-histidine were also eluted at identical positions (Fig. 1A). The addition of 0.1% of SDS to the mobile phase and a simultaneous decrease in the flow-rate from 1.0 to 0.7 ml/min led to the clear separation of PTC-proline and phenylthiourea and also of PTC-β-alanine and PTC-histidine, as shown in Fig. 1B. PTC-arginine was eluted before PTC-threonine in the absence of SDS (Fig. 1A). In the presence of SDS, it was chromatographed with an increased retention time, appearing before and after PTC-alanine and PTC-proline, respectively (Fig. 1B). All the PTC-amino acids were thus separated completely by the addition of 0.1% of SDS.

Detection limits

Fig. 2 shows the analysis of standard amino acid mixtures at 25–250 pmol levels. In other experiments, 5 and 10 pmol of the standard amino acids could be detected on the chromatograms, but their peaks were all very small (not shown). Quantitation of less than 10 pmol is not practical because of background contributions from the solvents, chemicals, glasswares and pipette tips.

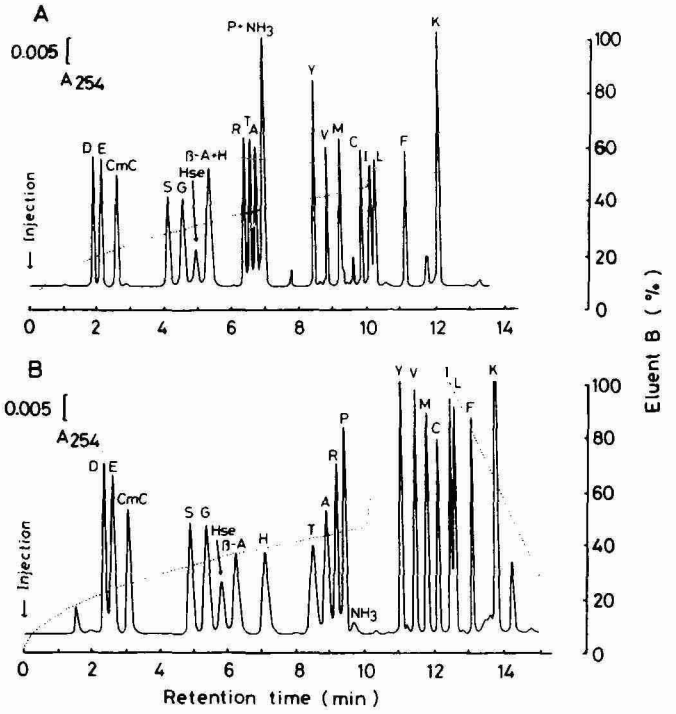

Fig. 1. HPLC of PTC-amino acids in the presence and absence of 0.1% of SDS. Standard amino acids (all 250 pmol) were derivatized with PITC according to the method of Bidlingmeyer *et al.*[15]. Eluent A: 131 m*M* sodium acetate (pH 6.4) containing 6% acetonitrile and 0.05% TEA. Eluent B: 60% acetonitrile in water. Gradient: 0–46% B in 10 min on a concave curve (No. 5), 46–100% in 0.5 min on a linear gradient (No. 6) and 100% B for 2 min. Column: Pico-Tag. Detector: 254 nm at 0.05 a.u.f.s. The one-letter symbols for amino acids are shown in Table I. (A) In the absence of SDS: flow-rate, 1.0 ml/min. (B) In the presence of 0.1% of SDS: flow-rate, 0.7 ml/min. CmC, carboxymethylcysteine; Hse, homoserine; β-A, β-alanine. -----, Gradient profile.

High-sensitivity analysis of peptides and proteins

The method was applied to the analysis of acid hydrolysates of some peptides and proteins at picomole levels, and its practicability was investigated. Aliquots (250 pmol) of the hydrolysed samples were injected into the amino acid analyser. The results are shown in Table I. For the low-molecular-weight peptides oxytocin, bradykinin, angiotensin I, and insulin B-chain, the numbers of residues per molecule determined by the amino acid analysis were each very close to those calculated from their amino acid sequences. With insulin and Cyc, however, the number of half-cystine residues observed was smaller than that in their sequences. Cysteine, generally determined as half-cystine or a cysteine derivative (cysteic acid or carboxymethylcysteine, is usually obtained with a value lower than that expected because of its instability to acid hydrolysis. In this experiment, cysteic acid was chromatographed before aspartic acid (data not shown).

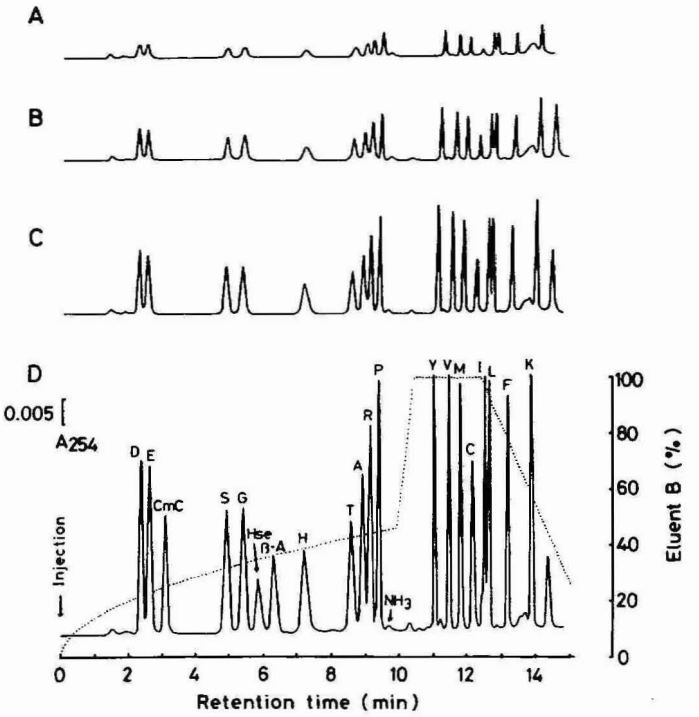

Fig. 2. High-sensitivity analysis of different amounts of standard amino acids. (A) 25; (B) 50; (C) 100; and (D) 250 pmol. Other conditions and abbreviations as in Fig. 1.

TABLE I

AMINO ACID ANALYSIS OF PEPTIDES AND PROTEINS

Amino acid	*Oxy-tocin*	*Brady-kinin*	*Angio-tensin I*	*Insulin B-chain*	*Insulin*	*Cyc*
Asp (D)	1.0(1)*		0.4(1)	0.6(1)	3.2(3)	8.1(8)
Glu (E)	1.0(1)			2.4(3)	6.8(6)	12.5(12)
Ser (S)		0.9(1)		0.9(1)	2.6(3)	
Gly (G)	1.0(1)	1.0(1)		3.2(3)	5.0(5)	11.3(12)
His (H)			2.1(2)	2.0(2)	1.9(2)	2.9(3)
Thr (T)				1.0(1)	0.9(1)	10.2(10)
Ala (A)				2.0(2)	3.0(3)	6.0(6)
Arg (R)		1.9(2)	1.0(1)	1.1(1)	1.0(1)	2.2(2)
Pro (P)	1.0(1)	3.0(3)	1.2(1)	1.0(1)	1.0(1)	4.2(4)
Tyr (Y)	0.9(1)		1.1(1)	2.2(2)	3.7(4)	4.2(4)
Val (V)			1.1(1)	4.0(3)	4.6(5)	3.7(3)
Met (M)						1.8(2)
½-Cys (C)	1.0(1)			0.7(2)**	4.7(6)	1.0(2)
Ile (I)	0.9(1)		1.1(1)		0.6(1)	5.8(6)
Leu (L)	1.1(1)		1.2(1)	4.4(4)	5.7(6)	5.8(6)
Phe (F)		1.9(2)	1.2(1)	3.4(3)	3.6(3)	3.9(4)
Lys (K)				1.0(1)	0.8(1)	19.2(19)

* Residues per molecule determined by amino acid analysis. Numbers in parentheses are those calculated from the sequences.

** Half-cystine calculated as cysteic acid.

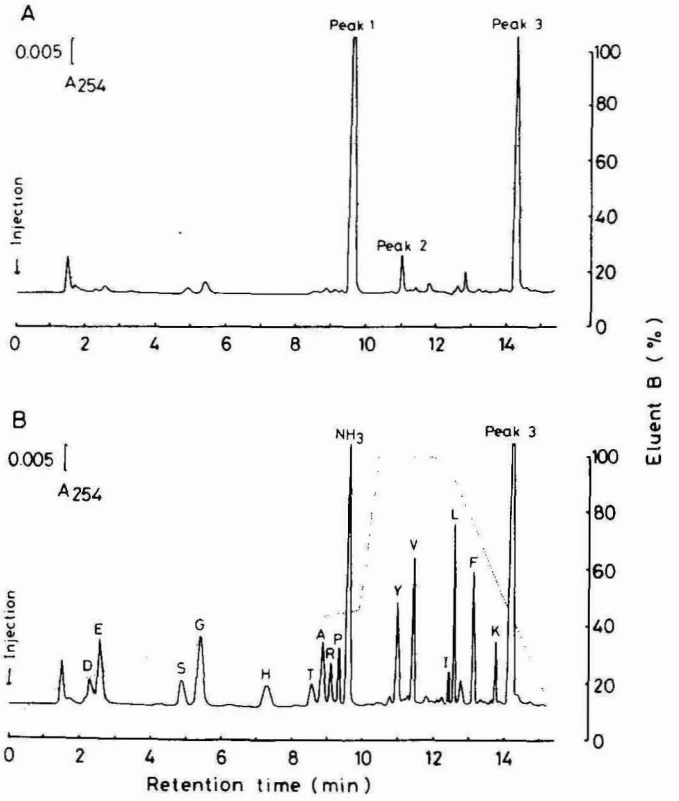

Fig. 3. High-sensitivity amino acid analysis of stained insulin from SDS-polyacrylamide slab gel. (A) Hydrolysate of the extract from reference gel; (B) hydrolysate of stained insulin extracted from SDS-polyacrylamide slab gel. -----, Gradient profile.

Amino acid analysis of stained peptides and proteins from SDS-polyacrylamide slab gel

Fig. 3 shows chromatograms obtained with insulin(B) and the corresponding reference gel(A). The PITC-derivatized hydrolysate of the extract from the reference gel gave two large peaks, 1 and 3, and several small peaks, including peak 2 (Fig. 3A). Peak 1 was identified as phenylthiourea, as both appeared at the same position. This phenylthiourea was probably formed by the reaction of PITC with ammonium chloride, which was produced from polyacrylamide gel on acid hydrolysis. Peak 3, not identified, was chromatographed after lysine. This peak also seems to have originated from polyacrylamide gel. Peak 2 eluted at the same position as PTC-tyrosine. The net amount of PTC-tyrosine in the peptide or protein sample was therefore obtained by subtracting the amount calculated from peak 2 from the amount of PTC-tyrosine observed in the sample. The first-appearing peaks having a retention time of 1.5 min in Fig. 3A and B can probaboly be ascribed to the same compound originally contained in the solvents or reagents used, as both peaks have similar heights. As Fig. 3B shows, PTC-proline and phenylthiourea (indicated by NH_3) were

TABLE II

AMINO ACID ANALYSIS OF STAINED PEPTIDES AND PROTEINS FROM SDS–POLYACRYLAMIDE SLAB GEL

Amino acid	*Insulin B-chain (7% recovery)*	*Insulin (14% recovery)*	*Cyc (5% recovery)*
Asp (D)	0.7(1)*	2.1(3)	7.3(8)
Glu (E)	2.0(3)	4.7(6)	11.8(12)
Ser (S)	0.7(1)	1.6(3)	0.6(0)
Gly (G)	3.1(3)	4.9(5)	16.4(12)
His (H)	1.7(2)	2.6(2)	2.5(3)
Thr (T)	0.8(1)	1.5(1)	9.0(10)
Ala (A)	2.0(2)	3.0(3)	6.0(6)
Arg (R)	0.9(1)	1.6(1)	2.0(2)
Pro (P)	1.3(1)	1.8(1)	3.8(4)
Tyr (Y)	4.4(2)	3.2(4)	2.3(4)
Val (V)	3.5(3)	4.8(5)	0.7(3)
Met (M)	0.5(0)	0.7(0)	0.7(2)
½-Cys (C)	N.D.**(2)***	0.7(6)	N.D.(2)
Ile (I)	0.4(0)	0.3(1)	4.4(6)
Leu (L)	4.0(4)	5.9(6)	5.3(6)
Phe (F)	3.2(3)	5.0(3)	3.0(4)
Lys (K)	1.0(1)	1.6(1)	13.3(19)

* Residues per molecule determined by amino acid analysis. Numbers in parentheses are those calculated from the sequences.

** N.D., not determined.

*** Half-cystine calculated as cysteic acid.

separated clearly by the addition of 0.1% of SDS to the moble phase. They could not be separated under the conditions examined unless SDS was added.

The amino acid compositions of stained insulin B-chain, insulin and Cyc are shown in Table II. Their recoveries from the SDS–polyacrylamide slab gel were in the range 5–14%, as calculated on the basis of amino acid analysis. The amino acid compositions of insulin B-chain, insulin and Cyc were close to the numbers calculated from their sequences, except for cystine and lysine. Quantitation of these two amino acids, which were considered to be sensitive to Coomassie brilliant blue, was very poor, probably because they were broken down during acid hydrolysis under the influence of the dye. This is similar to the poor lysine quantitation of fluorescamine-labelled peptides after acid hydrolysis, as observed in a protein kinase sequence study[17].

This highly sensitive method showed be widely applicable to the amino acid analysis of peptides and proteins separated by SDS–polyacrylamide gel electrophoresis.

ACKNOWLEDGEMENTS

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CHROM. 18 356

Note

Silica gel G interference in the cucumber hypocotyl bioassay

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Thin-layer chromatography was used in an attempt to isolate a plant growth synergist. The present report shows that components of the supposedly inert silica gel G interfere in the isolation of such a material not only by stimulating growth themselves but also by acting synergistically with growth regulators in the assay system.

MATERIALS AND METHODS

Thin-layer chromatographic methods were patterned after MacMillan and Suter[1]. Thin-layer plates of silica gel G (Merck, Darmstadt, F.R.G.) were prepared and activated at 110 to 120°C for 1–2 h. The blank plates were then developed and eluted. The origin was taken as a line 2 cm from one edge of the plate. The plates were removed from the solvent when the front was 3 cm from the opposite edge of the plate for a total migration distance of 15 cm. The plates were developed in one of three solvents: (a) diisopropyl ether–glacial acetic acid (95:5, v/v)[1]; (b) *n*-butanol–ethanol–water (4:1:5, v/v) organic phase[2]; (c) *n*-butanol–acetone–water (5:2:1, v/v)[2].

To elute material from the adsorbent the plate was divided into ten 1.5-cm zones with the first zone near the origin. The absorbent was scraped from each zone and transferred to a centrifuge tube, suspended in 3 ml 95% (v/v) ethanol and centrifuged for 10 min at 600 *g*. The elution was repeated three times after which the 9 ml eluate of each zone was allowed to dry. To test the residue on the bioassay system the residue of each eluted zone was dissolved in 1.0 ml 95% (v/v) ethanol.

The cucumber assay was patterned after the methods of Katsumi *et al.*[3]. Seeds (*Cucumis sativus Linneaus* "National pickling", Burpee Seed Co.) were soaked in water for 2 h and planted in vermiculite. Approximately five days after planting when the hypocotyl length was 2.5 cm an Indian ink mark was placed 2 cm below the cotyledonary node, and the apical bud was removed. The solution to be assayed was placed on the apex of each of seven seedlings chosen at random (0.01 ml per seedling). After three days the distance between the ink mark and the node was measured and recorded. The results from each set of seven seedlings was averaged and a standard error calculated. The growth response of the silica zones in ethanol was considered significant when the response and standard error exceeded the response and standard error of ethanol alone. The growth response of the silica zones in ethanol and growth hormone was considered significant and was described as synergistic when the re-

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sponse and standard error exceeded the response and standard error of the growth hormone in ethanol.

3-Indolylacetic acid (IAA) and gibberellic acid (GA3) was obtained from California Biochemical (Los Angeles, CA. U.S.A.). An amount of 10 μg of IAA or GA3 was dissolved in 0.01 ml ethanol and applied alone or in combination with extracts from the silica plates, and was inoculated at the site of the removed apical bud of the cucumber hypocotyl.

RESULTS AND DISCUSSION

Cucumber seedlings were innoculated with the eluates in ethanol of ten 1.5-cm zones from blank whole plates of silica gel G developed in solvent a (Fig. 1A and B). Plants treated with the eluates from zones 1–3 and 6–10 were significantly longer than those treated only with ethanol. When 10 μg gibberellic acid was added to each of the solutions, plants treated with eluates of zones 4, and 7–10 showed a synergistic growth response. The experiment was repeated with similar results.

Additional silica plates were developed in solvent b and combined so that each test solution contained the equivalent of two and a half plates (Fig. 1C and D). The ethanol solutions were inoculated alone and in combination with 10 μg IAA. Plants treated with silica plate extract in ethanol were not significantly longer than those

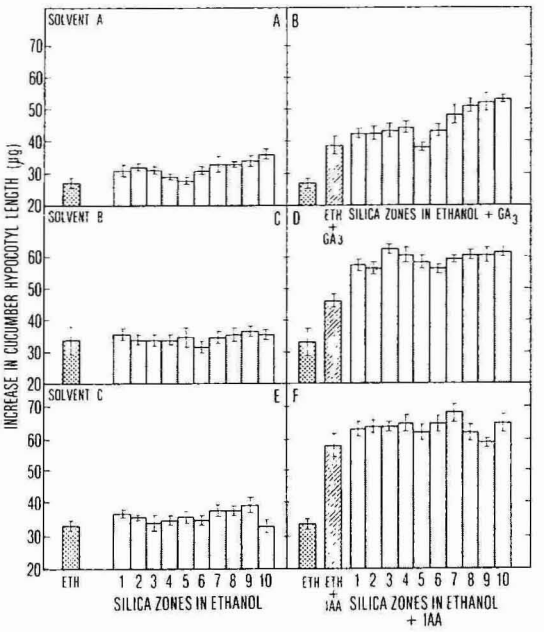

Fig. 1. Effect of silica zone extracts on cucumber hypocotyl growth, alone and in combination with plant growth hormones: (A and B) silica zones extracted with diisopropyl ether–glacial acetic acid (95:5, v/v), (A) silica zones in ethanol, (B) silica zones in ethanol plus GA3; (C and D) silica extracted with *n*-butanol–ethanol–water (4:1:5, v/v), (C) silica zones in ethanol (D) silica zones in ethanol plus IAA; (E and F) silica extracted with *n*-butanol–acetone–water (5:2:1, v/v), (E) silica zones in ethanol, (F) silica zones in ethanol plus IAA. Each column represents the mean value of seven seedlings. The vertical bars represent the standard error. ETH = Ethanol.

treated only with ethanol (Fig. 1C), but a definite synergistic response was obtained when plants were treated with silica extracts of all zones and IAA (Fig. 1D). In solvent c a significant growth increase was obtained with silica zones 7–9 in ethanol and a synergistic response was obtained when plants were treated with IAA and silica extracts of zones 4, 6, 7 and 10 (Fig. 1E and F).

The results indicate that some substance extracted from the silica plates stimulates the growth of the cucumber hypocotyl alone and when this substance is combined with GA3 or with IAA. The ionic mobility of the interfering ion varied according to the solvent system.

The silica gel had a manufacturer's analysis of 0.03% (w/w) maximum iron and 0.02% (w/w) maximum chloride. Although theoretically the silica gel is inert iron in the form of ferrous chloride is soluble in ethanol as is amorphous silica[4]. Dissolved calcium sulfate from silica gel G is reported to contribute to erroneously high values in lipid analysis[5]. Ferrous ions are reported to act as synergists of IAA-induced growth[6–9]. It is possible that the interference of silica gel G in the bioassay is due to the action of dissolved iron, calcium sulfate or even amorphous silica.

We hoped that the silica plates could be used to extract a growth factor from lichens but the growth stimulating property of the ingredients of the silica gel itself interfered in the assay.

While thin-layer chromatography is an excellent and frequently used tool it must be used cautiously when the separated compounds are to be assayed on biological systems.

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Note

Determination of DL-asparagine by gas chromatography

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A great deal of effort has been made over the last few years to analyze amino acids by gas chromatography (GC) or high-performance liquid chromatography (HPLC). Especially the resolution of DL-Asn has always been difficult. Using HPLC, separation factors (α) of 0.87 (ref. 1), 1.23 (ref. 2), 1.25 (ref. 3) and 1.5 (ref. 4) have been achieved. Using GC, the main problem has always been to avoid the degradation, due to acid hydrolysis, of the amides, Asn and Gln. Makita *et al.*[5] introduced a derivatization method in which the volatile derivatives N(O,S)-isobutoxycarbonyl(iso-Boc)-methyl esters of amino acids were prepared by two-step procedures involving iso-*n*-butoxycarbonylation with isobutyl chloroformate (iso-BCF), followed by esterification with diazomethane. The use of trimethylsilylation has been reported by Ruhlmann and Giesecke[6]. Hušek[8] reported another derivatization method, forming cyclic compounds using 1,3-dichlorotetrafluoroacetone in combination with reactive anhydrides such as heptafluorobutyric anhydride. But, due to the planar structure of oxazolidinones, no resolution of D- and L-enantiomers was obtained. In the other papers mentioned, no effort of enantiomer separation has been made; hence, it seemed necessary to find a quick and sensitive method with which even DL-Asn resolution is possible.

EXPERIMENTAL

Reagents and materials

DL- and D-asparagine monohydrate were purchased from Sigma (St. Louis, MO, U.S.A.) and DL-aspartic acid and L-asparagine were from Schwarz/Mann (Orangeburg, NY, U.S.A.). Acetylchloride was supplied by Mallinckrodt (KY, U.S.A.); 2-propanol, trifluoroacetic anhydride, acetone (Photorex) and dichloromethane were from J. T. Baker (Phillipsburg, NJ, U.S.A.).

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Sample preparation

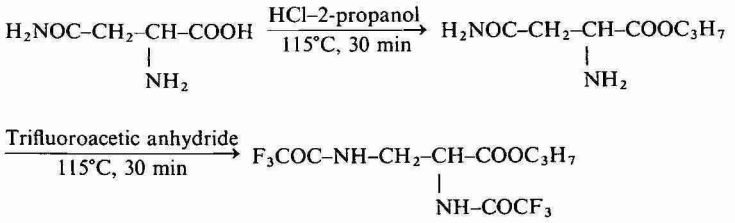
A 10-μl volume of aqueous amino acid solution (*ca.* 50 mg/ml) was evaporated either by gentle nitrogen stream or by vacuum freeze-drying. After addition of 250 μl 1.5 *N* hydrochloric acid–2-propanol, the mixture was heated at 115°C for 30 min, then cooled in an ice bath, and solvent evaporated off. A 1-ml volume of trifluoroacetic anhydride–methylene dichloride (50:50) was added and the solution heated again at 115°C for 10 min. After cooling in ice and evaporating off the excess reagents, the derivatized amino acid was dissolved in 500 μl methylene dichloride and 1-μl aliquots were injected into the gas chromatograph.

Gas chromatography

For GC analysis a Hewlett-Packard Model 5790 gas chromatograph equipped with a 60 m × 0.9 mm fused-silica Chirasil-Val column was used with helium as the carrier gas at an inlet pressure of 10 p.s.i. (0.7 bar). The nitrogen–phosphorus detector injector port was maintained at 250°C. The system was programmed as follows: injection at 40°C, after 30 sec a rapid temperature increase to 80°C, then to 180°C at 4°/min. The peaks were detected with a Hewlett-Packard nitrogen–phosphorus detector with an air flow-rate of 50 ml/min and a hydrogen flow-rate of 30 ml/min. The peaks were computed with a Hewlett-Packard 3390 A electronic integrator.

RESULTS AND DISCUSSION

Because of the mild hydrolysis conditions the asparagine is preserved and not, as is usually the case, converted into aspartic acid. It is assumed that only partial hydrolysis occurs which maintains the amide character of Asn. This leads to the di-N-trifluoroacetyl-Asn-isopropyl ester rather than to the N-trifluoroacetyl-diisopropyl ester of aspartic acid:



Their different retention times allow a clear distinction from each other as is seen in Fig. 1. By using the Chirasil-Val column and a temperature program of 1.5°C/min, baseline separation of the single enantiomers can be achieved. The Asn peaks are eluted later than the Asp peaks. The Asn:Asp ratio seems to be directly dependent on the hydrolysis conditions. Under the mildest conditions, essentially only Asn appears, whereas under the strongest conditions, only Asp is observed (Fig. 2). The best results for obtaining Asn have been achieved by treatment with 1.5 *N* hydrochloric acid and heating at 115°C for 30 min. Unfortunately, it has not been possible to obtain only Asn. Using strong hydrolysis conditions (4 *N* hydrochloric acid and heating at 115°C for 3 h) only Asp is obtained.

Since glutamine differs from asparagine only by one CH_2 group, it is expected



Fig. 1. Resolution of DL-asparagine and DL-aspartic acid. Aspartic acids are the by-products of asparagines; for hydrolysis, derivatization and separation conditions see text. The Asp and Asn peaks appear after ca. 30 min.

that preservation of the amide could also be possible. Similar treatment of Gln, as described for Asn, shows equivocal results (data not shown). Only once has a second peak pair been observed, but since it has not been reproducible we assume that an artifact was present. Even the mildest hydrolysis conditions seem to be too strong to preserve the amide in glutamine.

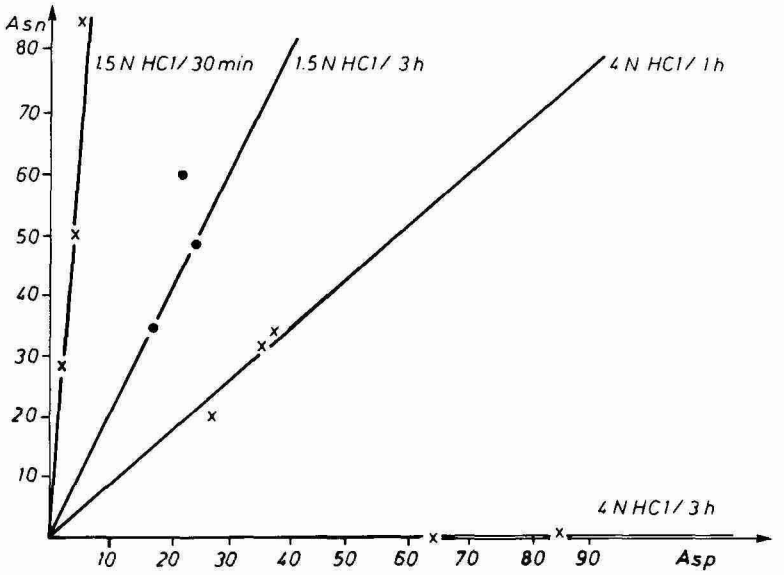


Fig. 2. Yields of asparagine *vs.* aspartic acid as a function of hydrochloric acid concentration and reaction time. The axis scales give the peak area in arbitrary units.

CONCLUSION

As the above results demonstrate, the best preservation of asparagine has been achieved by derivatizing according to our protocols with 1.5 *N* hydrochloric acid at 115°C for only 30 min. Since no reproducibility was obtained for glutamine, milder hydrolysis conditions have to be found. Because of its excellent resolving power and very high sensitivity, this method could be applied to amino acid analysis of proteins and peptides.

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CHROM. 18 303

Note

Determination of diethylene glycol in wine

Rapid purification with a Carbopack B cartridge and quantitation by gas chromatography

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Very recently, the detection of a hazardous additive in wine, diethylene glycol (DEG), prompted a search for a reliable, gas chromatographic (GC) procedure able to determine small amounts of DEG in wine.

The determination of glycols in aqueous solution by GC is difficult as almost all stationary phases are intolerant to water and solid supports are insufficiently inert to achieve elution of glycols as untailed peaks. Further, the determination of DEG in wine is made even more difficult by the presence of endogeneous, high-boiling compounds which, on accumulating in the initial part of the column packing, induce partial chemisorption of DEG, which can give rise to "ghosting" effects.

The aim of this work was to devise a reliable and sensitive GC procedure able to determine DEG in wine. Rapid sample purification was achieved passing 2 ml of a wine sample through a Carbopack B[1–4] cartridge. Subsequently, 2 μl of the last 500 μl of the eluate were injected into the GC column containing Carbopack C modified with 0.8% (w/w) of tetrahydroxyethylethylenediamine (THEED), which is able to elute glycols as untailed peaks[5].

EXPERIMENTAL

Purification apparatus

The Carbopack B trap is an experimental kit developed and kindly supplied by Supelco (Bellefonte, PA, U.S.A.). The cartridge consists of a cylindrical polypropylene tube which is one-sixth full of 250 mg of Carbopack B graphitized carbon black with a particle size less than 125 μm. Polyethylene frits are located above and below the Carbopack B bed to hold the minute particles in place and keep the chromatographic column intact. This cartridge fitted directly into the vacuum manifold. Vacuum was obtained with a water pump, taking no care to ensure low and constant flow-rates of the samples percolating through the cartridge.

Purification procedure

The Carbopack B cartridge was cleaned by passing 5 ml of water, then 1.5 ml of the sample, to which had been added 1 g/l of 1,4-butanediol as an internal stan-

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dard, was passed through the cartridge and the effluent was discarded. A further 0.5 ml of the wine sample was passed through and the effluent was collected, and 2 μl of this eluate were injected into the GC column.

GC instrumentation

GC analysis was performed on a DANI (Milan, Italy) Model 3400 gas chromatograph equipped with a flame ionization detector. The glass column (1.0 m × 2 mm I.D.) was packed with Carbopack C graphitized carbon black, modified with 0.8% of THEED (Supelco). The GC column was conditioned overnight under flow, by holding the injector, detector and oven temperatures at 120°C. The column was operated by maintaining the same temperature conditions as used for the column conditioning. The operating temperature was critical in order to ensure a long life of the column. When the injection temperature was set at 130°C, we observed a slow but continuous degradation of the column packing, resulting in changes in the retention time of DEG and the appearance of some glycol peak tailing. Helium was used as the carrier gas. The dead time was 5 s.

RESULTS AND DISCUSSION

The effect on the GC packing of repeated injections of unpurified wine was evaluated. Several runs, each consisting of 30 injections of 2 μl of a red wine sample spiked with 2 g/l of DEG, were made. After each run, 2 μl of the same wine sample, but not fortified with DEG, were injected into the GC column. In the latter instance the chromatogram showed one "ghost" peak having the same retention time as DEG and apparently corresponding to a DEG content in wine of about 0.15 g/l. Moreover, after four injection runs, some glycol peak tailing appeared. Probably progressive accumulation on the injection port of unknown, high-boiling compounds present in wine are responsible for these two effects.

The same experiment was repeated by injecting a wine sample that had been submitted to the purification step described under Experimental. After each injection run we observed a "ghost" peak corresponding to a constant DEG concentration not exceeding 0.013 g/l. Further, no ghost peak was observed after a second injection of wine that had not been fortified with DEG. The extent of DEG "ghosting" measured after wine purification is certainly very low and it may be considered negligible in comparison with the level of DEG usually detected in adulterated wines. Anyway, in order to eliminate completely the presence of a "ghost" peak for DEG, we found it useful, after GC analysis of a sample containing a high concentration of DEG and before chromatographing another wine sample, to inject 2 μl of water containing 1 g/l of ethylene glycol. In this way, even traces of DEG remaining chemisorbed on the initial part of the columns were completely displaced by ethylene glycol.

Another advantage of injecting purified wine samples over injecting unpurified samples is that the life of the column packing is substantially prolonged. Even after 180 injections of purified wine during 1 week of operation, the chromatographic characteristics of the column packing remained unaltered and the peak for DEG showed no tailing.

Glycerol, which is a natural product present in wine in relatively large amounts, co-eluted from the Carbopack B cartridge with DEG and 1,4-butanediol. Under our

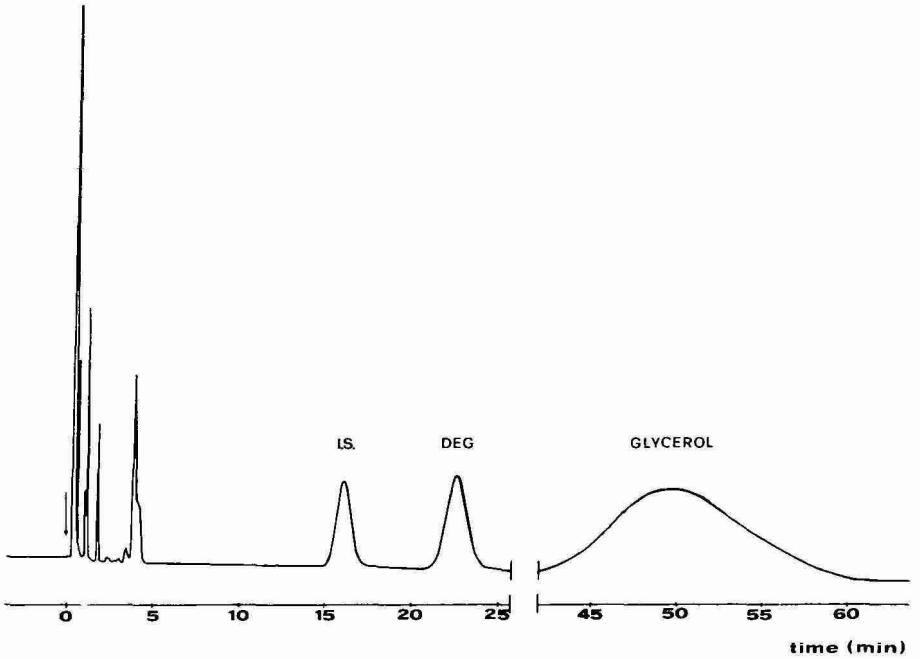

Fig. 1. Typical chromatogram of purified wine spiked with 2 g/l of DEG.

GC conditions, glycerol has a much higher retention time than DEG, and it is eluted as a very broad peak. This may cause interference during successive GC analyses. This problem was eliminated by injecting wine samples at constant intervals of 30 min. In this way, the peak for glycerol appeared in a zone of the chromatogram free from the two peaks for DEG and the internal standard.

Fig. 1 shows a typical GC profile for a purified wine sample spiked with DEG.

The recovery and precision of the method were assessed by analysing wine samples spiked with known amounts of DEG of 5, 2, 0.6 and 0.1 g/l. The recovery was calculated by measuring the peak heights of DEG relative to those of the internal standard and comparing them with those measured by direct injections of wine samples that had not been purified. Six replicate analyses at each DEG concentration gave an average recovery of 99.2% (range 96.9–102%). The coefficients of variation (C.V.) were 2.8 and 1.4% for the lowest and highest concentrations considered, respectively. The limit of sensitivity (signal-to-noise ratio = 3) at which DEG could be measured with a C.V. of 6.9% was 1 mg/l. At this concentration, a well defined chromatographic peak for DEG could still be obtained.

The specificity of the assay was evaluated by analysing 30 different DEG-free wine samples. No detectable peak having the retention time of DEG was observed. Moreover, adsorption studies were performed on the effectiveness of the Carbopack B surface to retain compounds dissolved in a wine-simulating aqueous solution, *viz.*, water–ethanol (88:12, v/v). The results showed that only very polar compounds, such as the lower members of mono- and polycarboxylic acids, alcohols, ketones, aldehydes and hydroxy and keto acids, passed almost unretained through the Carbopack

B cartridge and were co-eluted with DEG. However, these compounds are not potential sources of interference in the analysis as, when injected on to the above GC column packing, some of them were eluted with retention times much lower than that of DEG and the others were not detectable under the GC conditions chosen.

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CHROM. 18 248

Note

Gas chromatographic determination of cysteic acid

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The development of a satisfactory gas chromatographic (GC) procedure for the analysis of cysteic acid has been hampered by the difficulty of preparing a suitable volatile derivative of this compound. The N-heptafluorobutyryl isobutyl ester (HBB)[1,2] and the trimethylsilyl (TMS)[3,4] derivatives used in previous studies appear to be inadequate for reliable quantitative work in view of the fact that the HBB derivative is thermally unstable[1] and the TMS derivative is very sensitive to moisture[4].

Recently, we developed a specific and reliable method for the determination of taurine by GC in which taurine was analysed as its N-isobutoxycarbonyl (N-isoBOC) dibutylamide derivative[5], and we demonstrated that complex biological materials such as urine and blood could be analysed accurately and precisely by the method[5,6]. In the present paper, we report the logical extension of this work to the analysis of cysteic acid.

EXPERIMENTAL

Reagents

Cysteic acid and homocysteic acid as an internal standard (I.S.) were purchased from Nakarai (Kyoto, Japan), and each was dissolved in water to make a stock solution at a concentration of 1 m*M*. Isobutyl chloroformate (isoBCF) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Cetyldimethylbenzylammonium chloride (CDMBA-Cl)[7] obtained from Tokyo Kasei Kogyo was dissolved in methanol at the concentration of 10%. Hydrogen chloride in methanol (HCl–methanol) obtained from Tokyo Kasei Kogyo was diluted with methanol at the concentration of 0.5 *M*. Thionyl chloride and *n*-dibutylamine (DBA) were purchased from Nakarai and used after distillation. All other chemicals were of analytical grade.

Analytical derivatization procedure

An aliquot of the sample solution (containing 5–500 nmol of cysteic acid) was pipetted into a 10-ml pyrex glass tube with a PTFE-lined screw-cap. After addition of 0.25 ml of the I.S. solution (0.1 or 1.0 m*M*), 0.1 ml of 0.5 *M* sodium hydroxide was added and then the total reaction volume was made up to 1 ml with distilled water if necessary. Immediately after the addition of 0.1 ml of isoBCF, the mixture was shaken with a shaker set at 300 rpm (up and down) for 5 min at room temper-

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ature. The reaction mixture was washed twice with 3 ml of diethyl ether after adjustment to pH 1–2 with 0.5 *M* hydrochloric acid. Subsequently, 0.05 ml of 10% CDMBA-Cl was added to the aqueous layer, and the ion-pair compounds formed were extracted into 2 ml of methylene chloride by shaking for 3 min at room temperature. After centrifugation for 1 min the organic layer was transferred to another tube, and the solvent was evaporated to dryness under a stream of nitrogen. To the residue was added 0.2 ml of 0.5 *M* HCl–methanol solution, and the tube was tightly capped and heated at 80°C for 15 min. The excess HCl–methanol was removed at 80°C under a stream of nitrogen. To the residue was added 0.2 ml of thionyl chloride, and the tube was tightly capped and heated at 80°C for 15 min. The excess thionyl chloride was removed at 80°C under a stream of nitrogen. To the residue was added 0.2 ml of 2 *M* DBA in acetonitrile and the mixture was allowed to stand for 5 min at room temperature after tightly capping. The reaction mixture was acidified with 1 ml of 20% orthophosphoric acid and then extracted twice with 3 ml of *n*-pentane. After the solvent was evaporated to dryness at 60°C the residue was dissolved in 0.1 ml of ethyl acetate, and 2–4 μl of this solution were injected into the gas chromatograph.

Preparation of reference compound

A reference sample of methyl β-dibutylsulphamoyl-α-(isobutoxycarbonylamino)propionate, m.p. 55–57°C, was prepared from 50 mg of cysteic acid in essentially the same manner as the analytical derivatization procedure. The data for elemental analysis are as follows: calculated for $C_{17}H_{34}N_2O_6S$: C, 51.75; H, 8.69; N, 7.10. Found: C, 52.07; H, 8.80; N, 7.08.

Gas chromatography

GC analysis was carried out with on a Shimadzu 4CM gas chromatograph equipped with a hydrogen flame ionization detector. The column packing, 1.0% OV-17–0.2% FFAP on Uniport HP (100–120 mesh) was prepared using toluene as a coating solvent according to the solution coating technique[8], and was poured into a silanized glass column (1.0 m × 3 mm I.D.). The packed column was conditioned at 275°C for 24 h with nitrogen at a flow-rate of 30 ml/min. The operating conditions were as follows: nitrogen flow-rate, 45 ml/min; hydrogen flow-rate, 35 ml/min; air flow-rate, 0.75 l/min; injection and detector temperature, 280°C; oven temperature, 220°C; chart speed, 1.0 cm/min; sensitivity, 10^2 (× 10^6 Ω); range, 4–32 (× 0.01 V).

Gas chromatography–mass spectrometry (GC–MS)

A Shimadzu-LKB 9000 gas chromatograph–mass spectrometer with the same type of column as used for GC analysis was employed under the following conditions: trap current, 60 μA; ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion-source temperature, 270°C; separator temperature, 260°C; helium flow-rate, 40 ml/min.

RESULTS AND DISCUSSION

Cysteic acid (α-amino-β-sulphopropionic acid) could be successfully converted into its stable volatile derivative, methyl β-dibutylsulphamoyl-α-(isobutoxycarbonylamino)propionate by essentially the same procedure as that used in the derivatiza-

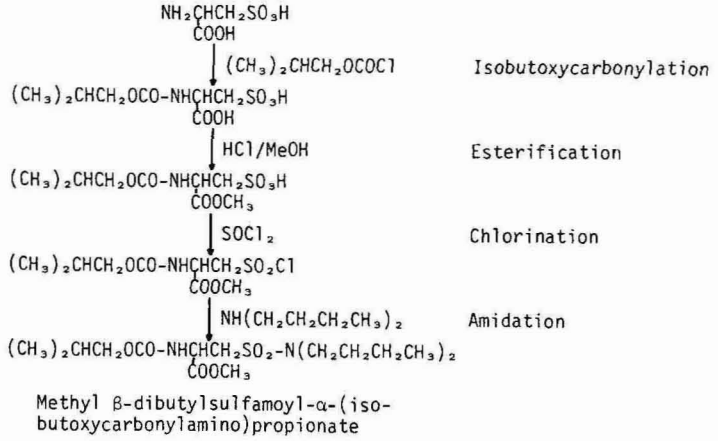

Fig. 1. Derivatization of cysteic acid.

tion of taurine[5] except for an additional step, methylation of carboxyl group, which was easily carried out with HCl–methanol before chlorination of the sulphonic acid group. The best reaction conditions for the methylation were determined to be at 80°C for 15 min (for discussion on the reaction conditions for other steps, see ref. 5). The derivatization process is outlined in Fig. 1. It should be noted that ion-pair extraction technique using CDMBA as a counter-ion results in rapid and quantitative extraction into organic layer of N-isoBOC cysteic acid dissolved in the aqueous medium. The mean derivatization yield throughout the procedure was determined to be 97% by comparison with the synthetic reference compound. The derivative preparation took *ca.* 60 min, but several samples could be treated simultaneously.

The structure of the derivative was confirmed both by GC–MS and by elemental analysis. Although the molecular ion peak (*m/e*, 394) was not observed, the prominent fragment ion peaks [$M^+ - CH_2CH(CH_3)_2$, $M^+ - N(C_4H_9)_2$, $M^+ - CH_2CH(CH_3)_2$ and $N(C_4H_9)_2$] were useful for structure elucidation. The values of

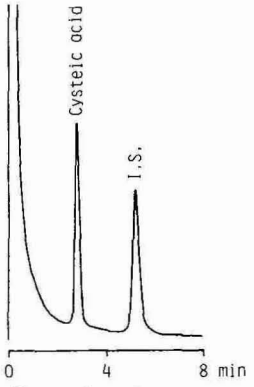

Fig. 2. Gas chromatogram obtained from a standard solution containing 250 nmol each of cysteic acid and I.S. (homocysteic acid). Column, 1.0% OV-17–0.2% FFAP on 100–120 mesh Uniport HP, 1.0 m × 3 mm I.D., glass; temperature, 220°C (isothermal). Each peak represents *ca.* 5 nmol.

elemental analysis agreed with the theoretical values calculated for the structure expected.

The derivative was very stable to moisture and, therefore, no precaution to exclude moisture was necessary in the handling and storage of it.

The derivative of cysteic acid gave a single clean peak on GC with a OV-17–FFAP column (Fig. 2); no extraneous peaks due to thermal decomposition were observed. A chromatographic run was completed within 8 min.

The calibration curve for cysteic acid prepared from peak height ratios relative to the I.S. was found to be linear in the range 5–500 nmol, corresponding to 0.1–10 nmol of cysteic acid in the portion injected, and the reproducibility of the calibration curve was found to be satisfactory.

These experiments have conclusively demonstrated that the proposed method is suitable for quantitative determination of cysteic acid. Further investigations on the application of this method to biological samples are in progress.

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CHROM. 18 354

Note

High-performance liquid chromatographic detection and quantitation of synthetic acid fast dyes with a diode array detector

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A previously published high-performance liquid chromatographic (HPLC) method for the analysis of synthetic dyes[1] has been re-examined. In this study, we have used tetrabutylammonium phosphate as an ion-pairing agent to produce a more acceptable separation, and a diode array detector to enhance detection sensitivity. For the nine dyes examined, detection limits ranging from 0.08 to 0.29 ppm have been realized representing a better than ten-times increase in sensitivity. Further, the method as presented more effectively resolves the dyes FDC Green 3 and FDC Blue 1 into their major constituents.

EXPERIMENTAL

Apparatus

The liquid chromatograph used was an Altex Model 322 with two, Model 110A pumps and a C_{18} column, 5 μm, 250 × 4.6 mm (Altex), fitted with a Rheodyne 20-μl syringe loading sample loop injector. Detection was by means of a Hewlett Packard 1040A diode array detector (6 mm path length, 4.5 μl volume), coupled with an HP85B computer, an HP2225 printer (for data output) and an HP7470 plotter (Hewlett Packard).

Reagents

Mobile phase. (A) Tetrabutylammonium dihydrogen phosphate (Aldrich) (1.70 g) was dissolved in 1 l HPLC-grade water (Caledon Labs., Georgetown, Canada), containing ACS-grade disodium hydrogen phosphate (1.42 g) and potassium dihydrogen phosphate (1.36 g). (B) A volume of 200 ml of mobile phase (A) was diluted to 1 l with HPLC-grade methanol (Caledon Labs.).

Standards. Acid fast dyes (see text) of known dye strength from McCormick-Stange Flavor Division (Chicago, IL, U.S.A.).

Sample preparation. Solutions of standards were prepared by dissolving 100 mg in 1 l water and further diluting 10 ml to 100 ml. Samples are prepared by methods appropriate to the matrix containing them (*e.g.* see ref. 1).

Chromatographic conditions

A solvent gradient of 45% B to 100% B in 35 min at a flow-rate of 1 ml/min was used.

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TABLE I

FDC DYES, WAVELENGTHS USED FOR QUANTITATION AND LIMITS OF DETECTION

Dye	*Wavelength (nm)*	*μg detectable per 20 μl injected*
FDC Blue 2	290	0.0035
FDC Yellow 5	428	0.0039
Amaranth	500	0.0057
FDC Yellow 6	500	0.0045
FDC Red 40	500	0.0038
FDC Red 4	500	0.0043
FDC Red 3	528	0.0016
FDC Green 3	595	0.0040
FDC Blue 1	595	0.0052

RESULTS AND DISCUSSION

The nine dyes were initially chromatographed individually using detection at 280 nm (which we had earlier used on a fixed-wavelength detector) and on-the-fly spectra were recorded. From the wavelength maxima found, five wavelengths were selected that were close to optimal for the nine dyes. This is given in Table I, together with the number of micrograms detectable (with a pre-set area reject of 10 milli-absorbance units on the detector).

The separation efficiency of the system is shown in Fig. 1. We found 280 nm to be the best single wavelength to use for mixtures of the dyes. Fig. 2 shows the effect on sensitivity with variation in detection wavelength. In Fig. 3, part of the chromatogram recorded at 595 nm is shown in expanded form, showing a reasonable

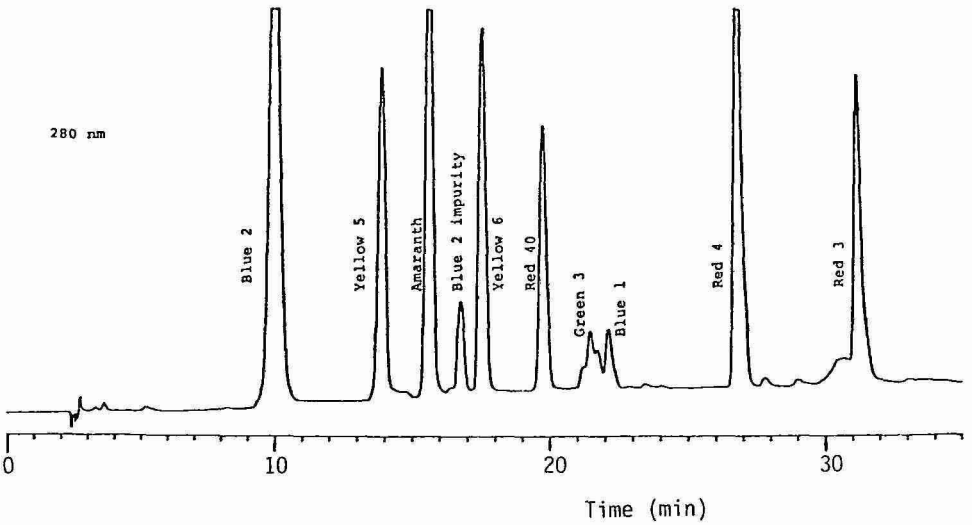

Fig. 1. Chromatogram illustrating the separation of nine acid fast dyes.

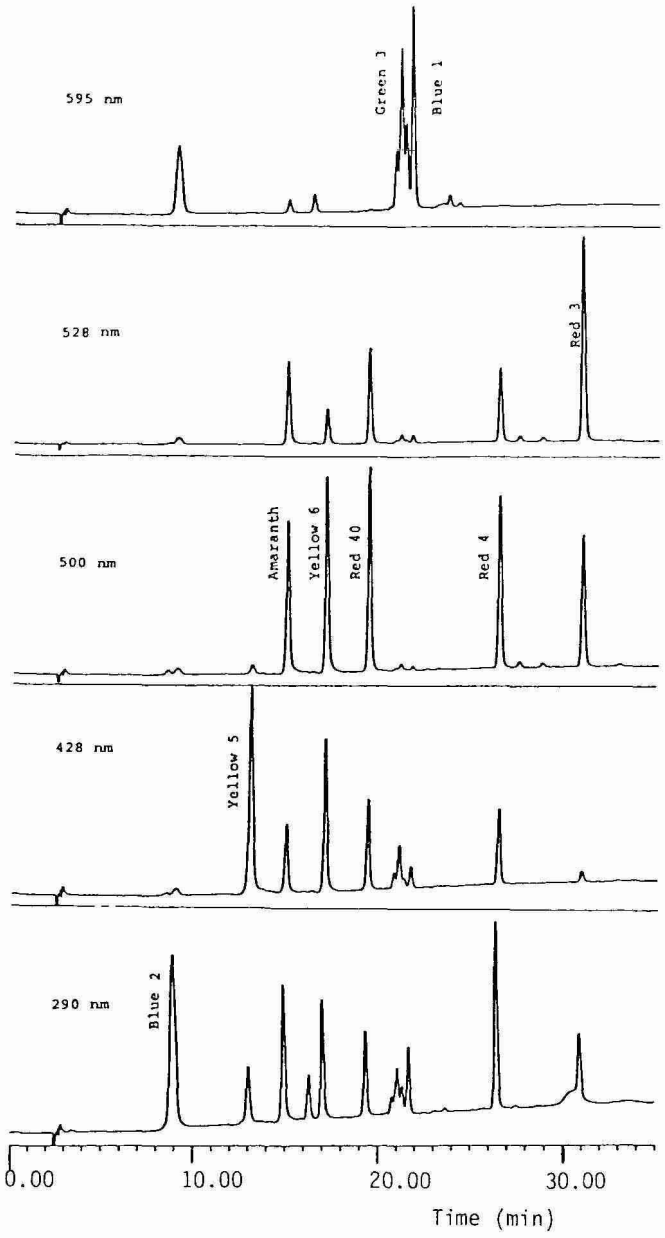

Fig. 2. Chromatograms showing effect of changes in detection wavelength on sensitivity.

separation between FDC Green 3 and FDC Blue 1, as well as between the minor components of each dye. On-the-fly spectral scan of the FDC Blue 1 peaks, and of the FDC Green 3 peaks, shows that for each dye, the UV–VIS spectra for the components are almost identical and that there is no wavelength that could be used to determine one of the components without interference from the other.

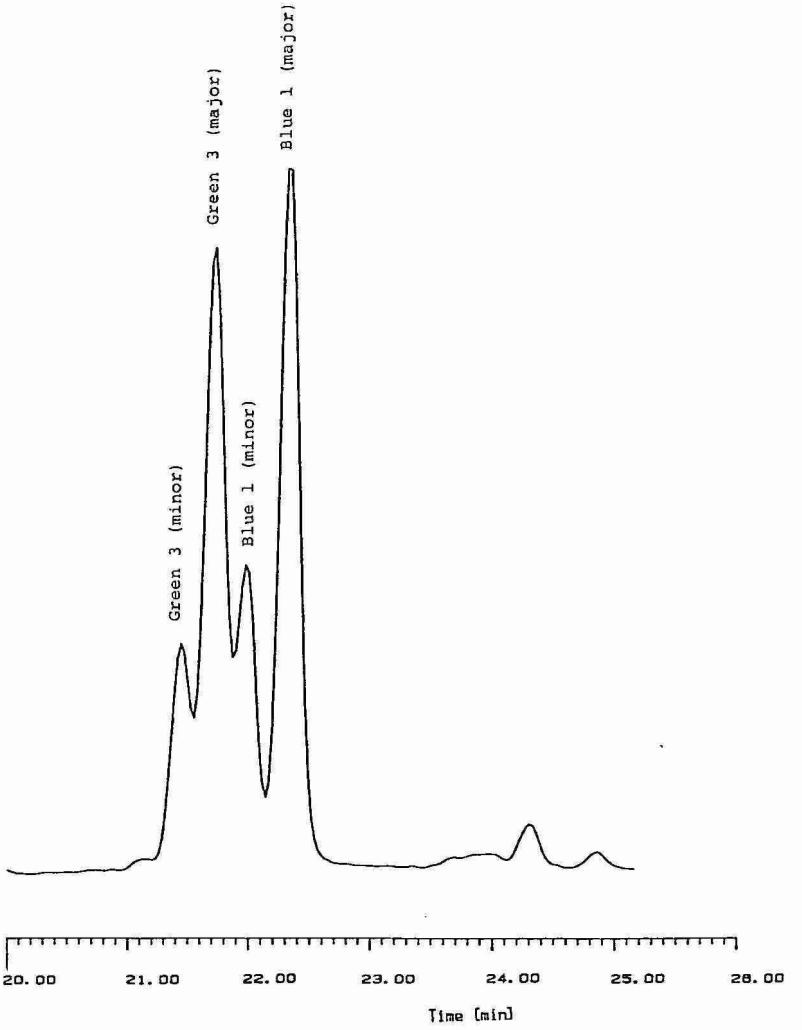

Fig. 3. Part chromatogram (595 nm) showing components of FDC Green 3 and FDC Blue 1.

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CHROM. 18 388

Note

High-performance liquid chromatographic method for the determination of nitrate and nitrite in cured meat

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Cured meat is normally meat which has been treated with nitrate and/or nitrite and immersed in brine. The nitrite (either added or produced from nitrate-reducing organisms) reacts with myoglobin to produce the red colour of pickled meats[1]. Nitrate can act as a reservoir for nitrite[2] but as a preservative it is similar in effectiveness to sodium chloride[1], the difference being that nitrate prevents the growth of *Clostridium botulinum*. Nitrate may react with amines to form carcinogenic nitrosamines and consequently it has become increasingly important to monitor nitrate and nitrite levels in meat products. New Zealand regulations[3] do not permit wet cured meats to contain more than 125 mg/kg of nitrite and nitrate (calculated as sodium nitrite) and do not allow the addition of nitrates. The usual methods for nitrate determination involve cadmium reduction to nitrite, followed by diazotisation, coupling and spectrophotometric estimation. These methods are time consuming and suffer from interferences which become critical at 10–20 mg/kg levels of nitrate and nitrite[4].

High-performance liquid chromatographic (HPLC) techniques have been examined as alternative procedures for the determination of nitrate and nitrite. Suppressed ion chromatography is perhaps an obvious alternative, but complications arise with the oxidation of nitrite and nitrate in the acidic conditions used on the suppressor column[5]. Jackson *et al.*[6] have assessed many of the HPLC methods and have proposed a method using a low-capacity silica-based anion-exchange column with 11 m*M* chloromethanesulphonic acid as eluent and UV detection. Excellent resolution of nitrate and nitrite was obtained and no interference from the high concentrations of chloride found in cured meats was observed. Two recent methods have made use of ion-interaction chromatography. Wootton *et al.*[7] used a silica-based reversed-phase column (Waters Radial Pak C_{18}) and direct UV detection and found that matrix interference was a problem with meat samples. De Kleijn and Hoven[8] found that a neutral styrene–divinylbenzene copolymer column (Hamilton PRP-1) and UV detection at 240 nm gave a clean chromatogram with low sensitivity. A fourth method for the determination of nitrate and nitrite in cured meats makes use of a high-capacity anion-exchange column, 0.04 *M* sodium perchlorate as eluent and UV detection. This method has a detection limit of about 17 mg/kg nitrate in ham. Improved resolution was obtained with 0.2 *M* methanesulphonate as eluent. At high mobile phase concentrations, however, the column was damaged[9].

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In a laboratory where many samples are analysed it is necessary to have quality assurance checks regularly and to be able to assess the precision of the method at frequent intervals. This can be accomplished easily using an automated HPLC method. We have used a high-capacity polymethacrylate gel anion-exchange (quaternary ammonium) column with dipotassium phosphate as eluent and detection by UV spectrophotometry at 215 nm. As nitrite is susceptible to oxidation at pH values less than 5[4,5] and will not be retained on an ion-exchange column near or below its pK_a of 3.37, dipotassium hydrogen phosphate (5 m*M*) was chosen as eluent for it has sufficient buffer capacity at pH 9 and is an effective anion at displacing nitrate and nitrite. The response of nitrate is considerably enhanced if it is detected at 190 nm[10]. We chose 215 nm as the detection wavelength as it is a suitable compromise between sensitivity and interference from other compounds.

EXPERIMENTAL

Instrumentation

An HPLC system (Waters Assoc., Milford, MA, U.S.A.), a Model 8700 solvent delivery system (Spectra-Physics, San Jose, CA, U.S.A.), a Model PU4020 variable-wavelength spectrophotometer (Pye Unicam, Cambridge, U.K.), and a Model SP4270 integrator (Spectra-Physics) were used. The column was an IC-Pak anion column (Waters Assoc.) with a capacity of 30 mequiv./ml and it was maintained at 40°C using a purpose-built column heater.

Mobile phases

Dipotassium hydrogen phosphate solution (5 m*M*) was prepared from analytical grade reagent (Ajax Chemicals) and sodium methanesulphonate (30 m*M*) was prepared from methanesulphonic acid (Riedel de Haën, Prosynth grade) titrated with sodium hydroxide (0.1 *M*) to pH 6.0. The flow-rate was 0.6 ml/min.

Sample preparation

Sample preparation follows the method of Fudge and Truman[11]. The samples of meat were purchased from a retail outlet. A 250-g sample was homogenised in a food processor (Toshiba, Model TFP-1200), and 10.00 g were slurried thoroughly with hot distilled water (100 ml) at 80°C, saturated borax solution (5 ml) and activated charcoal (0.5 g) and then placed on a boiling water bath for 15 min. After cooling to room temperature, Carrez Reagents I and II were added (2.0 ml of each), followed by saturated borax solution (5 ml) and the solution was made up to 250 ml in a volumetric flask. After standing for 30 min, the solution was filtered through Whatman No. 44 paper (previously washed with 50 ml of 0.1 *M* hydrochloric acid), the first portion of the filtrate being discarded, and then through a 0.45-μm membrane filter: aliquots of 20 μl were injected into the chromatograph.

RESULTS AND DISCUSSION

The chromatogram of a standard solution containing 5 ppm nitrite and nitrate (both as sodium nitrite) recorded at 215 nm using 5 m*M* dipotassium phosphate as eluent is shown in Fig. 1a. The relative standard deviation for replicate injections

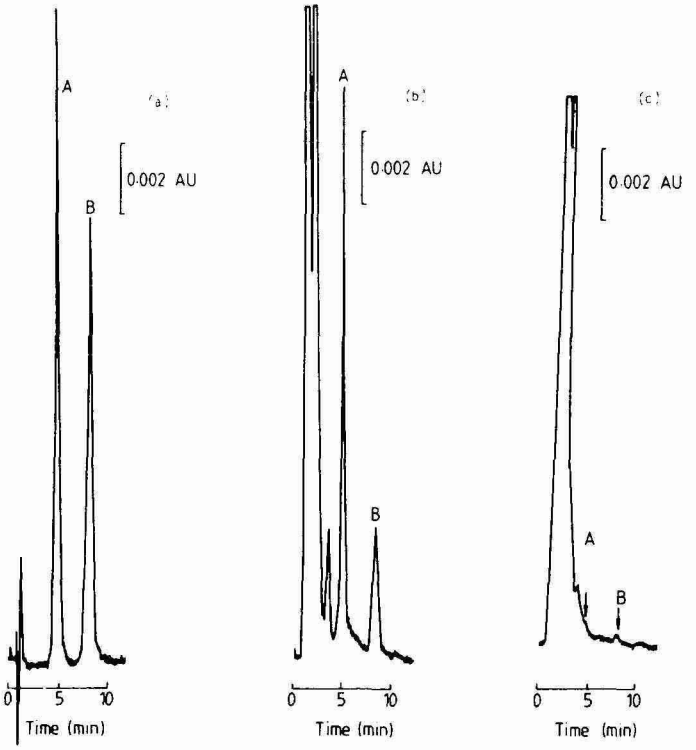

Fig. 1. Separation of nitrite (A) and nitrate (B). (a) 5 ppm of nitrite and nitrate. (b) Corned beef sample. The estimated concentration in the aqueous extract corresponds to 57 mg of nitrite and 32 mg of nitrate per kg corned beef. (c) Beef sample. Conditions: Waters IC-Pak A column with 5 m*M* dipotassium phosphate; flow-rate, 0.8 ml/min; injection volume, 20 μl, detection wavelength, 215 nm.

obtained under repeatability conditions[11] was dependent on the amount of solute injected and varied from 1 to 4%. The regression of peak area *versus* concentration over the range 0.2–100 ppm (as sodium nitrite or sodium nitrate) was linear for nitrite and nitrate. Fig. 1b shows a chromatogram of an aqueous extract of cured meat. The estimated concentration in the extract corresponds to 58 mg of nitrite and 25 mg of nitrate per kg of cured meat. Fig. 1c shows the chromatogram of a sample of similar beef which was not cured. The method has also been used on luncheon sausage, saveloys and frankfurters and shown to be free from interferences.

When 30 m*M* methanesulphonic acid (adjusted to pH 6 with sodium hydroxide) was used as eluent, retention times of nitrite (5.6 min) and nitrate (9.2 min) were similar to those obtained with the above eluent. First proposed by Ivey[13] as an eluent for the chromatography of inorganic anions using both UV absorption and conductivity detection, methanesulphonic acid offers no advantages in this application.

Prior to the development of this method, this laboratory determined nitrate in cured meat samples by reduction with cadmium followed by diazotisation of sulphanilamide and coupling with N-(1-naphthyl)ethylenediamine hydrochloride. The absorbance of the resulting solution was measured at 540 nm[11].

Samples of cured meat were extracted and analysed for nitrite and nitrate by both methods and the results are compared in Table I. The agreement for nitrite is excellent while for some samples the nitrate is quite variable. Usher and Telling[4] in their critical review on the analysis of nitrate and nitrite with special reference to meat products discuss in detail the methods for nitrate which use reduction by spongy cadmium. They find the disadvantages are the variable sample blanks which can range from 0 to 30 mg/kg and occasionally up to 80 mg/kg, and inconsistent reduction efficiencies, particularly if polyphosphate is present in the meat. They conclude by suggesting the method is not ideal and is suspect for low levels of nitrate (10 mg/kg) in food samples.

TABLE I

COMPARISON OF CHROMATOGRAPHIC AND FUDGE AND TRUMAN'S[11] METHODS FOR DETERMINATION OF NITRITE AND NITRATE IN CURED MEATS

Nitrite (mg/kg)		*Nitrate (mg/kg)*	
HPLC	*Fudge and Truman*[11]	*HPLC*	*Fudge and Truman*[11]
8.7	8.5	21.7	27.1
17.5	17.0	27.8	28.8
58.0	57.9	33.0	25.1
38.4	38.7	229	178
17.0	17.2	18.4	34.6
13.7	13.1	29.7	49.0
42.3	41.9	25.2	23.7
11.7	11.6	18.3	18.0
318	330	48.0	48.8
23.8	24.5	174	165
12.6	12.6	19.4	20.2

In contrast the HPLC method, particularly when automated, is considerably more convenient and rapid and the results for nitrate are more precise with a lower detection limit (approximately 1 mg/kg with a 100-μl injection volume of the extract).

We have found that peaks arising from malic and tartaric acids are not fully resolved from that of nitrate and care should be taken if these are suspected additives to cured meats, or if the method is used for the determination of nitrate in plant tissues.

The microbiological stability of aqueous solutions of nitrate is always open to question and the nitrate concentrations in unpreserved samples can be dramatically reduced in a short time. The meat extracts are preserved with borax and standards should also be preserved.

CONCLUSIONS

The use of a polymethacrylate gel ion-exchange column with dipotassium phosphate as eluent and detection by UV spectrophotometry permits the precise measurement of nitrite and nitrate at the lower levels required by present legislation.

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CHROM. 18 246

Note

Simple and direct determination of pyridinoline and 2'-deoxypyridinoline by automatic analysis

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Since discovery of pyridinoline[1] and 2'-deoxypyridinoline[2] crosslinks in collagen molecules, experimental evidence[3,4] suggests that they are age-dependent products in metabolic pathways which involve reducible crosslinks, and that the content varies broadly with the type of tissue. The sparse occurrence of the crosslinks in these tissues, however, makes it very difficult to determine their content in collagen unless time-consuming enrichment is carried out by Biogel P-2 or phosphocellulose column chromatography. This paper describes an elaboration of the elution system for a simple and effective method of determination of pyridinium crosslinks in collagen hydrolysate.

MATERIALS AND METHODS

A standard amino acid mixture and reagents were obtained from Wako. Bovine Achilles tendon was a product of Worthington Diagnostic System. Pyridinoline and 2'-deoxypyridinoline were prepared from bovine bone insoluble collagen[5] by the previous method[2]. Desmosine and isodesmosine were isolated from elastin of bovine *ligamentum nuchae* according to the method of Partridge *et al.*[6], after pre-concentration of desmosines on a cellulose column[7].

An analytical standard solution was made up to contain *ca.* 10–20 nmol of pyridinium crosslinks and 60 nmol of each amino acid in 1 ml of 0.01 *M* hydrochloric acid.

Insoluble collagen was hydrolysed in 6 *M* hydrochloric acid in a sealed tube under nitrogen for 24 h at 110°C. The hydrolysate was rotary-evaporated to dryness and redissolved in 0.01 *M* hydrochloric acid. Amino acid analysis was then carried out with a Hitachi Model 835 analyser fitted with a sampling valve with a 50-μl loop. Single-column analyses were normally performed at 53°C on a 150 × 4 mm I.D. column of sulphonated polystyrene resin (10% divinylbenzene, 7 μm), Hitachi No. 2619. The flow-rates were set to 0.225 ml/min for buffer and 0.3 ml/min for ninhydrin. The buffers and the ninhydrin reagent were prepared according to the Hitachi technical manual, except Buffer 5. All the sodium citrate buffers contained 0.4% (v/v) Brij-35 and 0.01% (v/v) octanoic acid. Buffers 1, 2, 3 and 5 contained 0.5% (v/v) thiodiglycol. Buffers 1 and 2 contained 13% and 2% (v/v) ethyl alcohol, respectively.

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TABLE I

ELUTION SYSTEM FOR THE SEPARATION OF PYRIDINIUM CROSSLINKS FROM AMINO ACID MIXTURE

Programme: 0–1 min, Buffer 1; 1–8 min, Buffer 2; 8–25 min, Buffer 3; 25–33 min, Buffer 5; 33–50 min, Buffer 4; 50–56 min, Buffer 6; 56–60 min, Buffer 2; 60–82 min, Buffer 1.

Buffer no.	*Composition*
1	0.2 *M* (Na^+) Citrate (pH 3.3), 13% ethyl alcohol
2	0.2 *M* (Na^+) Citrate (pH 3.3), 2% ethyl alcohol
3	0.2 *M* (Na^+) Citrate (pH 4.3)
4	1.2 *M* (Na^+) Citrate (pH 4.9), 0.5% benzyl alcohol
5	0.2 *M* (Na^+) Citrate (pH 5.28)
6	0.2 *M* Sodium hydroxide

Buffer 5 contained 0.5% (v/v) benzyl alcohol. Table I summarizes the operating conditions. To obtain optimum resolution of pyridinoline and 2′-deoxypyridinoline from the other amino acids in the hydrolysate, an elution system was adopted with the addition of a buffer (5) between Buffers 3 and 4.

RESULTS AND DISCUSSIONS

As shown in Fig. 1, the pyridinium crosslinks are clearly separated in distinct peaks with very fine resolution from those due to the other amino acids, and from desmosine and isodesmosine if there is contamination by elastin. In fact, even an excess sample charge (0.75 mg of hydrolysate in a loop, *ca.* 150 times as large as usual) allows a satisfactory determination of pyridinoline and 2′-deoxypyridinoline (Fig. 2). Thus, we can determine the amino acid and pyridinium crosslink composition with a single elution system by sequentially putting the usual and concentrated samples into an autosampler system if necessary. A linear response of pyridinolines with the ninhydrin reaction was obtained in the range 0.5–10 nmol (Leu equivalent). The detection limit is usually below 0.3 nmol per injection, and depends on the purity

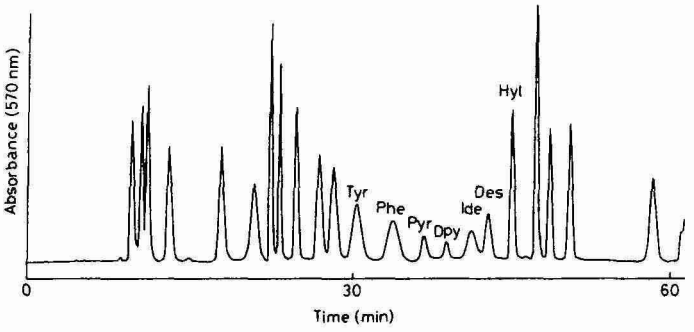

Fig. 1. An amino acid chromatogram for the analytical standard. Each amino acid (3.0 nmol per injection): Pyr = pyridinoline (0.62 nmol Leu equivalent); Dpy = 2′-deoxypyridinoline (0.39 nmol Leu equivalent); Ide = isodesmosine (0.91 nmol Leu equivalent); Des = desmosine (1.1 nmol Leu equivalent).

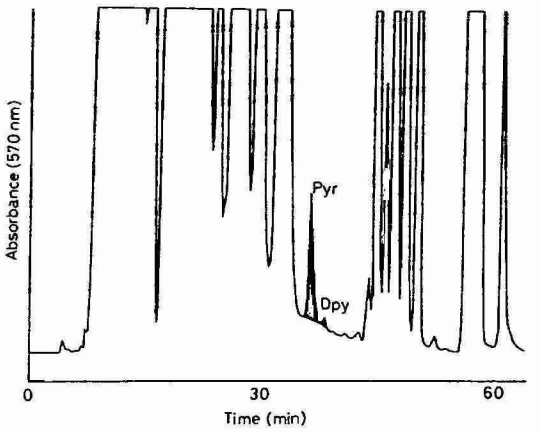

Fig. 2. An amino acid chromatogram for the concentrated hydrolysate of bovine Achilles tendon (*ca.* 0.75 mg per injection).

of collagen samples and the content of crosslinks in collagen. The method needs only 80 min for a run and precisely determines the pyridinium crosslinks in the collagen hydrolysate using a conventional analyser system with ninhydrin detection. It can be conveniently applied in connective tissue research, for example on age or pathological changes in bone, tendon, ligament, and aorta.

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CHROM. 18 294

Note

Determination of small amounts of 2-naphthalenesulphonic acid in 1-naphthalenesulphonic acid by high-performance liquid chromatography

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Naphthalenesulphonic acids (NSA) are important raw materials used in the manufacture of dyestuffs, pesticides, pharmaceuticals, wetting agents and dispersants. Depending on the sulphonating conditions of naphthalene, a mixture of 1-NSA, 2-NSA, di-, tri- and tetrasulphonic acids and sulphones is obtained[1,2]. High purity 1-NSA is required for the manufacture of pesticides *e.g.*, carbaryl, napropamide and 1-naphthoxyacetic acid, and of pharmaceuticals *e.g.* hepalande, propranolol and alphol. A simple analytical method is needed for the estimation of 1-NSA and 2-NSA in the mixture.

Qualitative separation of 1-NSA and 2-NSA by thin-layer chromatography[3] and paper chromatography[4] and their quantitative estimation by gas–liquid chromatography (GLC)[5] after derivatization and by spectroscopic techniques such as ultraviolet, infrared[6] and NMR[7] have been reported. Since sulphonic acids are freely soluble in aqueous solvents, reversed-phase high-performance liquid chromatography (HPLC) and ion-exchange chromatography[8] are potentially applicable to the analysis of these compounds. Ion-pair reagents, such as tetrabutyl ammonium phosphate and cetrimide, or strong electrolytes are added to the mobile phase during reversed-phase HPLC determination of NSA and related compounds[9–17]. Retention behaviour of various aromatic sulphonic acids in reversed-phase HPLC with ion-pair reagents or inorganic salts added to the mobile phases have been compiled[18,19]. Marmion[20] had separated trace amounts of 1-NSA present in large amounts of 2-NSA by HPLC at 40°C and recommended that improvements have to be carried out to develop a suitable method for the estimation of 2-NSA present in 1-NSA. Thus in this paper we describe a simple and improved isocratic HPLC method using a μBondapak CN column at ambient temperature for the estimation of 1–10% of 2-NSA in 1-NSA.

EXPERIMENTAL

Materials and reagents

All reagents used were of analytical grade unless otherwise stated: glass-distilled water; ammonium sulphate; 2-propanol; *m*-nitrobenzenesulphonic acid sodium salt.

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1-NSA and 2-NSA sodium salts were prepared in the laboratory by the sulphonation of naphthalene with chlorosulphonic acid, and purified by repeated complex formation with benzidine hydrochloride. The purity was confirmed by UV and NMR, spectroscopy.

Chromatographic system

A high-performance liquid chromatographic system Model ALC/GPC 244 with a Model M 6000 A high-pressure pump, U6K injector, μBondapak CN (30 cm × 3.9 mm I.D., 10 μm size) column, and a Model 440 absorbance detector with UV-254 nm fixed wavelength from Waters Assoc. (Milford, MA, U.S.A.) combined with a Chromatopak E1A integrator (Shimadzu) were used.

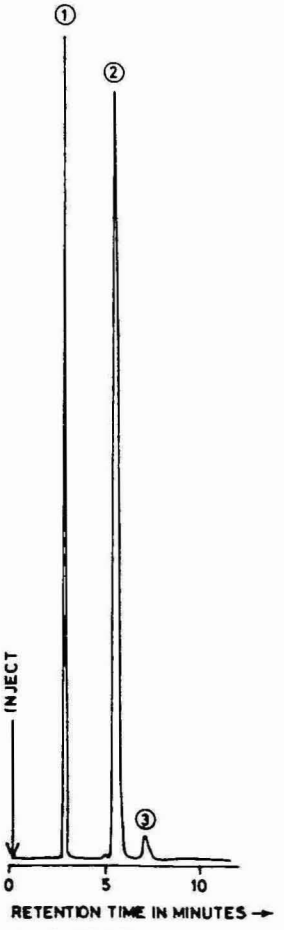

Fig. 1. HPLC trace of naphthalenesulphonic acid sodium salts on a μBondapak CN (30 cm × 3.9 mm I.D., 10 μm) column at a flow-rate of 2 ml/min with 0.15 *M* ammonium sulphate in water–2-propanol (97.5:2.5, v/v) as eluent and UV detection at 254 nm. Peaks: 1 = *m*-nitrobenzenesulphonic acid sodium salt (internal standard); 2 = 1-naphthalenesulphonic acid sodium salt; 3 = 2-naphthalenesulphonic acid sodium salt.

TABLE I

RETENTION DATA

Sample number	*Compound*	*Retention time (min)*	*Relative retention time*
1	*m*-Nitrobenzenesodium sulphonate	2.9	1.00
2	1-Naphthalenesodium sulphonate	5.7	1.97
3	2-Naphthalenesodium sulphonate	7.5	2.59

Sample preparation for HPLC

1-Naphthalenesulphonic acid sodium salt, 2-naphthalenesulphonic acid sodium salt and *m*-nitrobenzenesulphonic acid sodium salt [internal standard (I.S.)] were dissolved in the mobile phase.

Analytical procedure

A standard mixture of 1-NSA, 2-NSA and *m*-nitrobenzenesulphonic acid sodium salts was injected by means of a 10-μl Hamilton syringe, through a U6K injector. The mobile phase used was 97.5% of 0.15 *M* ammonium sulphate in water and 2.5% of 2-propanol. The analysis was carried out under isocratic conditions at a flow-rate of 2 ml/min and a chart speed of 0.5 cm/min at room temperature (28°C). The peaks were identified by injecting the individual compounds.

TABLE II

RESPONSE FACTOR DETERMINATION FOR 1-NAPHTHALENESULPHONIC ACID SODIUM SALT

Sample number	*Weight of 1-NSA · Na (mg)*	*Weight of I.S. (mg)*	*Area of 1-NSA · Na*	*Area of I.S.*	*Response factor**
1	27.1	4	129 903	78 275	4.0824
2	27.1	4	131 411	79 209	4.0837
3	28.0	4	145 752	84 293	4.0483
4	28.0	4	128 770	74 459	4.0476
5	29.0	4	143 230	81 781	4.1449
6	29.0	4	143 045	81 485	4.1299
7	29.0	4	144 039	81 590	4.1067
8	30.2	4	147 315	81 099	4.1564
9	30.2	4	152 439	83 918	4.1563
10	30.2	4	144 529	79 079	4.1309
Mean					4.10871
Standard deviation					0.04149
% Standard deviation					1.00980

* Response factor of 1-NSA · Na $= \frac{\text{area of I.S.}}{\text{wt. of I.S.}} \times \frac{\text{wt. of 1-NSA} \cdot \text{Na}}{\text{area of 1-NSA} \cdot \text{Na}}$

Standard mixtures containing 4 mg of internal standard, 27–30 mg of 1-NSA and 0.3–3 mg of 2-NSA sodium salts per 10 ml were prepared by dissolving the weighed amounts of the compounds in the mobile phase. Each standard mixture of 5 μl was injected and chromatographed under the above-mentioned conditions. From the areas of peaks the response factors of 1-NSA and 2-NSA sodium salts with respect to the internal standard were calculated.

Five synthetic mixtures were analysed under identical conditions: 75 mg of synthetic mixture and 10 mg of internal standard were dissolved in 25 ml of mobile phase and chromatographed. The percentages of 1-NSA and 2-NSA were calculated from the peak areas, and the response factors were determined as previously.

RESULTS AND DISCUSSION

HPLC separation of 1-NSA and 2-NSA sodium salts and *m*-nitrobenzenesulphonic acid sodium salt is shown in Fig. 1. From the chromatogram it is clear that 1-NSA and 2-NSA are resolved completely. The retention time data are given in Table I. Response factors determined for 1-NSA and 2-NSA (in the ranges 90–100% of 1-NSA and 10–0% of 2-NSA) are given in Tables II and III. The response factors for 1-NSA and 2-NSA remained constant throughout this range. The results obtained

TABLE III

RESPONSE FACTOR DETERMINATION FOR 2-NSA SODIUM SALT

Sample number	*Weight of 2-NSA · Na (mg)*	*Weight of I.S. (mg)*	*Area of 2-NSA · Na*	*Area of I.S.*	*Response factor**
1	0.3	4	2232	75 429	2.5346
2	0.3	4	2142	68 614	2.4024
3	0.6	4	4310	75 883	2.6405
4	0.6	4	4437	75 123	2.5396
5	0.9	4	6769	77 924	2.5105
6	0.9	4	6717	76 873	2.5750
7	1.2	4	8891	73 954	2.4953
8	1.2	4	9015	73 841	2.4572
9	1.5	4	10 782	73 895	2.5700
10	1.5	4	10 849	75 160	2.5979
11	1.8	4	13 062	74 608	2.5703
12	1.8	4	15 595	90 293	2.5466
13	2.1	4	15 515	74 556	2.5228
14	2.1	4	15 864	76 569	2.5339
15	2.4	4	17 499	74 844	2.5662
16	2.4	4	17 428	75 208	2.5892
17	3.0	4	21 704	75 399	2.6054
18	3.0	4	21 939	73 202	2.5024
Mean					2.5422
Standard deviation					0.0565
% Standard deviation					2.2220

* Response factor of 2-NSA · Na $= \frac{\text{area of I.S.}}{\text{wt. of I.S.}} \times \frac{\text{wt. of 2-NSA} \cdot \text{Na}}{\text{area of 2-NSA} \cdot \text{Na}}$

TABLE IV

ANALYTICAL DATA FOR THE SYNTHETIC MIXTURES

Sample number	*1-NSA · Na (%)*		*Error (%)*	*2-NSA · Na (%)*		*Error (%)*
	Taken	*Found**		*Taken*	*Found**	
1	98.72	101.54	+2.86	1.28	1.31	+2.34
2	98.02	96.72	−1.33	1.98	2.03	+2.53
3	97.44	97.32	−0.12	2.56	2.55	−0.39
4	91.96	92.66	+0.76	8.04	8.01	−0.37
5	90.35	91.93	+1.75	9.65	9.86	+2.18

* Average values obtained from duplicate determinations.

from the synthetic mixtures shown in Table IV are in good agreement with the actual values. It should be mentioned that this analytical procedure is suitable not only for determining trace amounts of 2-NSA in 1-NSA but also for the determination of 1-NSA in large amounts of 2-NSA. The method is simple, rapid and inexpensive compared with the methods reported earlier[9–19], which employ gradient elution and ion-pair reagents.

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CHROM. 18 290

Note

Clean-up and quantification of abscisic acid from citrus leaves by reversed-phase high-performance liquid chromatography

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(Received October 16th, 1985)

Abscisic acid (ABA) is an important plant hormone, which is known to control different phases of plant growth processes, and its physiological effects have recently been investigated[1].

The production of ABA by citrus tissues is of interest for its possible role in abscission, senescence and stomatal control[2].

Recently, various methods for the analytical quantification of ABA have been developed. These include gas chromatographic (GC) analysis of the methyl ester of ABA using electron-capture detection (ECD)[3] or flame ionization detection (FID)[4].

More recently, high-performance liquid chromatography (HPLC) has been used for the rapid separation and quantitation of ABA in a wide variety of plant tissue extracts[5–10]. Detection was based on the UV absorbance of underivatized ABA.

Certain plant tissues have been shown to contain, besides free ABA, a polar fraction referred to as "bound ABA" and identified as (+)-abscisyl-β-D-glucopyranoside[11]. This paper describes a simple, sensitive assay for free and bound ABA in citrus leaves by reversed-phase HPLC.

EXPERIMENTAL

Reagents and chemicals

All solvents used in these studies were ACS grade. Standards of cinnamic acid and abscisic acid were purchased from Fluka (Buchs, Switzerland).

Instrumentation

The liquid chromatograph consisted of a Kontron LCS 620 Gradient System (Kontron A.G., Zurich, Switzerland) equipped with a Model 7125 injector with a 20-μl injection loop (Rheodyne, Berkeley, CA, U.S.A.), a Kontron UV Uvikon 722 LC variable-wavelength spectrophotometric detector and a Kontron Anacomp 220 data processor. Chromatography was performed at ambient temperature on a stainless-steel column (25 × 0.4 cm I.D.) packed with Hypersil ODS 5 μm (Policonsult Scientifica, Rome, Italy).

Detection was by UV absorbance at 254 nm and 200 mV full scale.

The flow-rate and chart speed were set at 1 ml/min and 0.5 cm/min, respectively. The mobile phase was 0.2 *N* acetic acid–acetonitrile (6:4, v/v).

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Extraction and purification of free and bound ABA

Fresh tissue (5 g) was homogenized at 5°C in 50 ml of methanol–water (8:2, v/v) containing 200 mg/l of sodium diethyldithiocarbamate. The homogenized tissue was stirred overnight at 5°C and the mixture filtered. The filtrate was concentrated *in vacuo* and filtered, and the solution was divided into two halves.

Free ABA. The first fraction was treated with an equal volume of 0.1 *M* disodium hydrogen phosphate, the pH was adjusted to 10.0 with 0.1 *M* sodium hydroxide, and the solution was washed three times with diethyl ether. The aqueous phase was adjusted to pH 3.0 with 0.1 *M* hydrochloric acid and extracted three times with equal volumes of diethyl ether. The extract was dried over sodium sulphate and evaporated to dryness, the residue was dissolved in 1 ml of methanol–2 m*M* phosphoric acid (75:25, v/v) and introduced on a disposable Sep-Pak C_{18} cartridge (Waters Assoc.) previously washed with 2 ml of 2 m*M* phosphoric acid–methanol (90:10, v/v). Then 2 ml of methanol–2 m*M* phosphoric acid (75:25, v/v) eluted free ABA from the cartridge.

Total ABA. The second fraction was treated with an equal volume of 0.1 *M* sodium hydroxide at 50°C for 30 min; after washing of the solution with diethyl ether, the procedure described above was used.

Calibration curve

Standard solutions containing 0.6, 1, 4, 9, 18 and 25 μg/ml at ABA in HPLC eluent were prepared. The peak height of cinnamic acid (internal standard) is plotted against concentration.

RESULTS

The chromatograms obtained in this study are shown in Figs. 1 and 2. The retention times of ABA and internal standard are 3.7 and 5.0 min, respectively. The calibration curve shows a good correlation coefficient, $r = 0.9997$, between peak heights and ABA concentration in the range 0.6–25 μg/ml. The slope is 0.16 and intercept −0.047.

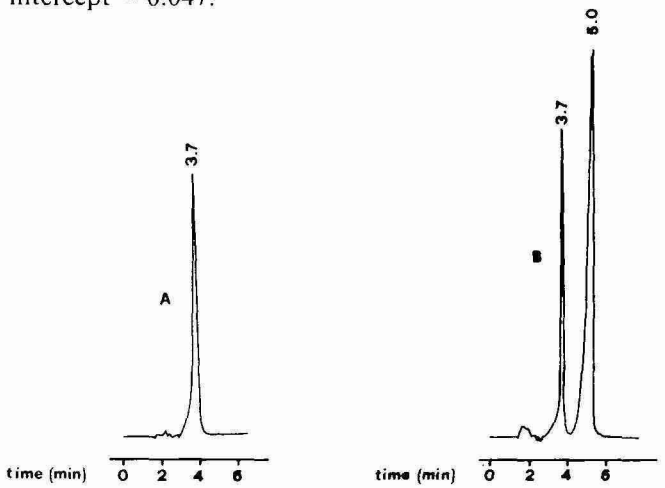

Fig. 1. Chromatography of standards: (A) ABA; (B) ABA and internal standard.

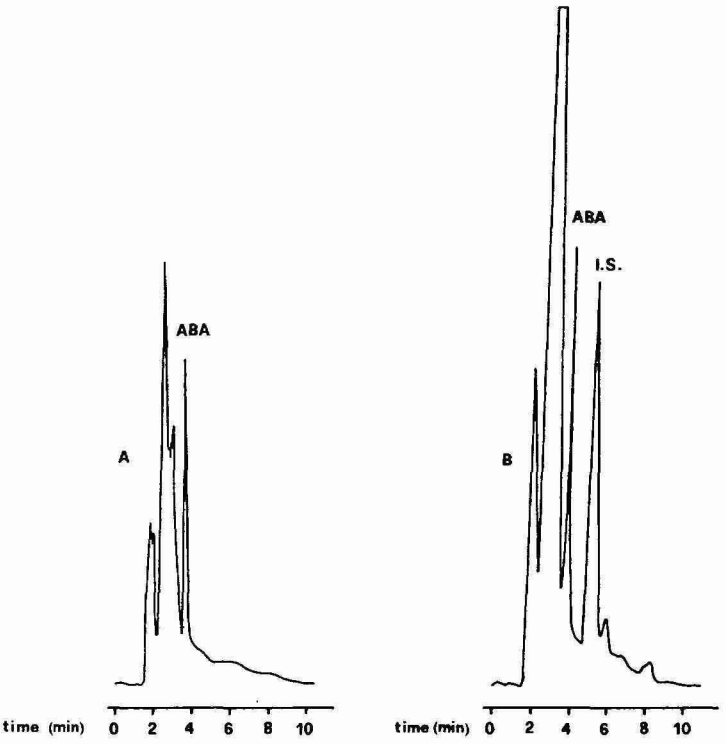

Fig. 2. Chromatography of citrus leaves extract: (A) total ABA (35 nmol/g fresh tissue); (B) total ABA (47 nmol/g fresh tissue) and cinnamic acid (I.S.).

Recovery and sensitivity

We have estimated the recovery by adding the solution of ABA standard in leaves extract containing the same amount of ABA. The extraction percentage is *ca.* 80%. Results are shown in Table I. The limit of sensitivity was 1–2 nmol/ml.

DISCUSSION

To demonstrate the biological applicability of the method, the values of free ABA were determined in twenty samples of citrus leaves, all from different trees. The

TABLE I

RECOVERY OF ABA FROM TISSUE

Number of samples is 5.

Amount added (μg/ml)	*Amount found (μg/ml)*	*Coefficient of variation (%)*	*Recovery (%)*
4	3.2 ± 0.21	6.5	80
9	7.24 ± 0.18	2.5	80.4
18	14.34 ± 0.30	2.1	79.6

TABLE II

LEVELS OF ABA FROM CITRUS LEAVES

Free ABA ± S.D. (μg/g wet wt. of tissue)	*Bound ABA ± S.D. (μg/g wet wt. of tissue)*
0.46 ± 0.31	15.14 ± 2.99

peaks of ABA were compared with the peak of the internal standard which had been added in the sample before extraction. The obtained values are shown in Table II.

The clean-up method based on using a C_{18} cartridge is simple and efficient; it allows rapid analysis of various biological samples containing ABA. The determination of ABA is made easier by the introduction of cinnamic acid as internal standard, which presents similar recovery of ABA.

The reversed-phase cartridge used in this HPLC method also facilitates the determination of ABA in complex biological matrices.

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Note

Preparative gel chromatography of acidic oligosaccharides using a volatile buffer

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Gel chromatography is a valuable technique for analytical and preparative separation of mixtures of neutral oligosaccharides[1,2]. It has, however, proved less satisfactory for acidic sugars. Uronic acid-containing oligosaccharides elute earlier than expected on the basis of molecular size and are poorly resolved[3,4]. Elution with buffers of low pH or high ionic strength has been proposed to correct this effect and achieve satisfactory size separation, but studies have been limited to chromatography on an analytical scale and at elevated temperatures[2,5]. Preparative separations, using buffers containing non-volatile salt and ethanol, have been disappointing[3,4].

We report here the preparative separation of acidic oligosaccharides by gel chromatography, using elution at room temperature with an volatile acidic buffer.

EXPERIMENTAL

Formic acid (90%) and trimethylamine (30%) were obtained from Merck-Schuchardt, Darmstadt, F.R.G. Uronic acid-containing oligosaccharides were obtained by graded acid hydrolysis of exopolysaccharides from *Rhizobium* strains[6].

To prepare a 0.2 *M* buffer, formic acid (10 ml) was mixed in water (800 ml), the pH adjusted to the desired value with trimethylamine and the volume made to 1 l.

Chromatography was carried out with a column (95 × 1.5 cm I.D.) packed with Biogel P-2 (fine), which had been slurry-packed at a flow-rate of 16 ml/h. Oligosaccharide mixtures (typically 80 mg of total sugar) were applied in 0.5 ml of buffer and eluted at 8 ml/h. Aliquots of the 1.0-ml fractions were monitored for sugar content by the phenol–sulphuric acid[7] and carbazole[8] methods.

RESULTS AND DISCUSSION

Gel chromatography of sugars is not a simple phenomenon: for both dextran[5] and polyacrylamide[1,2,9,10] matrixes, separation on a size basis is complicated by

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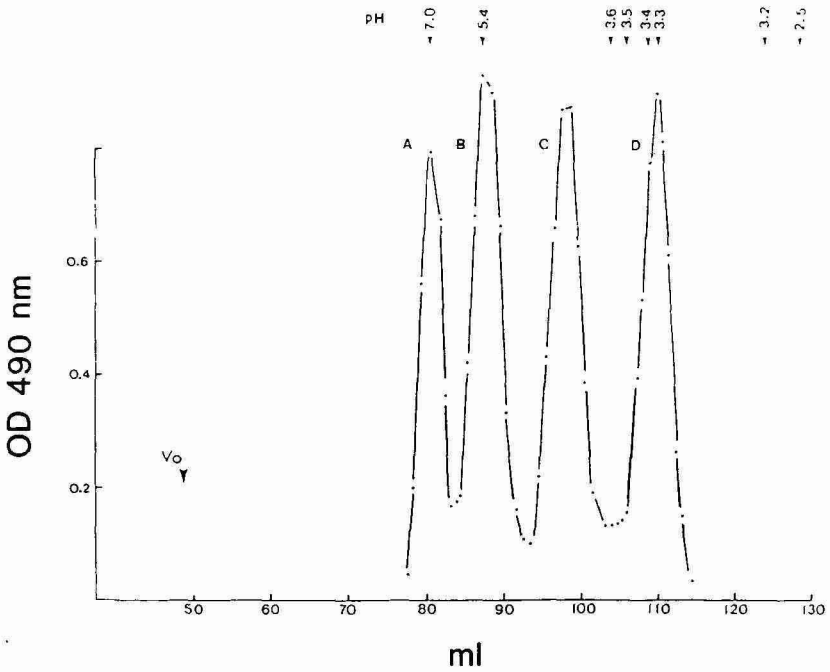

Fig. 1. Influence of pH on elution of glucuronic acid. Column, 95 × 1.5 cm I.D. Biogel P-2 (fine); buffer, 0.20 *M* trimethylammonium formate (pH 7.0); flow-rate, 8 ml/h; mixture, glucuronic acid, (15 mg), raffinose (10 mg) cellobiose (10 mg) and glucose (10 mg). Peaks: A = glucuronic acid; B = raffinose; C = cellobiose; D = glucose. The superimposed arrows show the elution positions of glucuronic acid at pH values.

associative interactions between the sugars and the matrix. Reduced[2], methylated[11] and deoxy sugars[10], which have decreased interactions, elute earlier than the parent sugars and have greater apparent molecular sizes.

The early elution of acidic sugars[3–5], on the other hand, indicates that their behaviour is dominated by ion exclusion from the negatively charged matrix[12]. This will be at a maximum when the sugar is fully ionized, *i.e.*, at pH values significantly above its pK_a value. This for a uronic acid, is *ca.* 3.4[13].

Accordingly, at pH 5.4 and above, glucuronic acid elutes earlier than a neutral trisaccharide (Fig. 1). At pH 2.5, where the ionization is significantly depressed, association with the gel matrix dominates and the sugar elutes later than glucose. Depending on the ionization equilibrium, the uronic acid can elute at any position between the two extremes (Fig. 1).

A pH of 3.3 gives "well-behaved" elution of glucuronic acid, *i.e.*, elution at the same position as glucose. At this pH, oligosaccharides containing two uronic acid residues have the same elution positions as neutral oligomers with the same number of sugar units (Fig. 2). Column capacity and chromatographic resolution are comparable with those for neutral sugars.

Furthermore, it is possible to exploit the earlier elution of acidic sugars that have ether[11] or deoxy[10] groups. This enables preparative separation of 4-O-methyl-D-glucuronic acid from glucuronic acid. Similarly, acidic oligosaccharides con-

taining the deoxysugar, L-rhamnose, elute from a Biogel P-2 column earlier than glucose-containing oligomers with the same number of residues (Fig. 2). It is possible to separate acidic tetrasaccharides that differ by only a single methyl ether substituent (Fig. 2).

Finally, the aberrant elution of acidic oligosaccharides can be exploited. For example, a mixture of hexoses and uronic acid can be separated more conveniently, and with better recovery, using Biogel P-2 with 1% formic acid as eluent, rather than

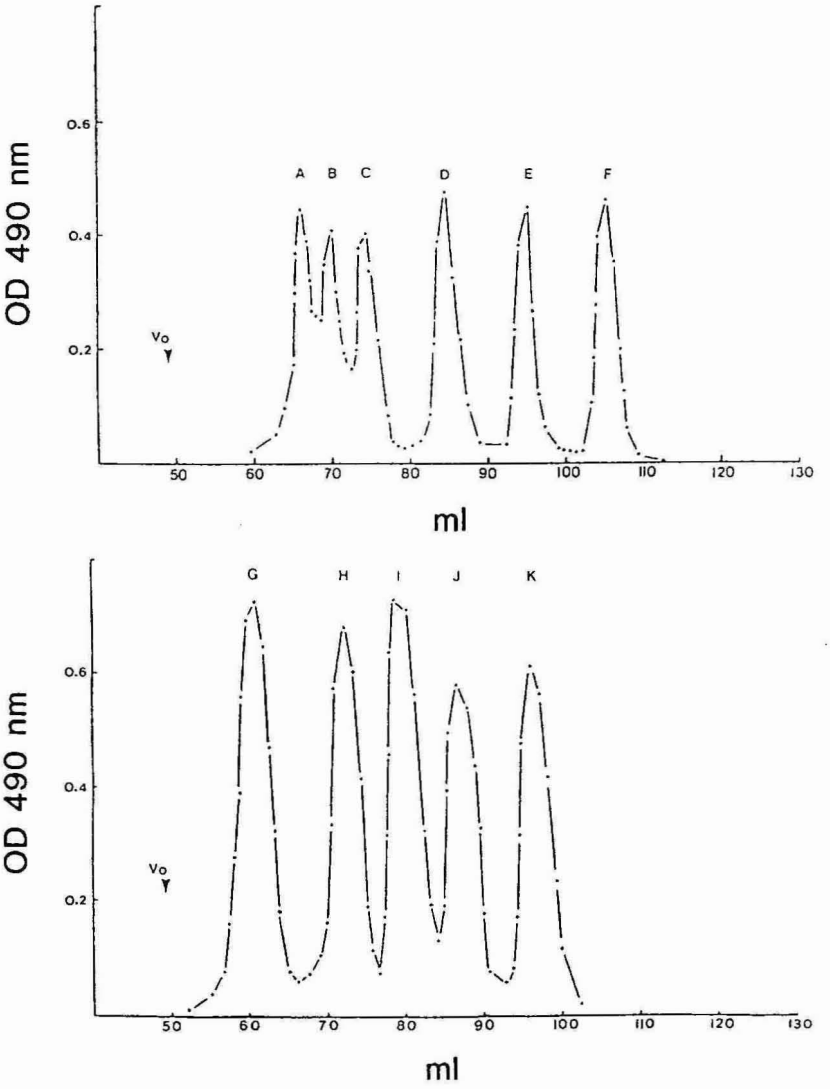

Fig. 2. Preparative separations of two oligosaccharide mixtures at pH 3.3. The column and operating conditions were as in Fig. 1. In each case, *ca.* 80 mg of mixture were applied. Peaks: A = $(Rha)_2$(2-O-methyl-Rha)$_1$(4-O-methyl-GlcA)$_1$; B = $(Rha)_3$(4-O-methyl-GlcA)$_1$; C = $(Rha)_2$(4-O-methyl-GlcA)$_1$; D = $(Rha)_1$(4-O-methyl-GlcA)$_1$ + $(Rha)_2$; E = 4-O-methyl-GlcA + 2-O-methyl-Rha; F = Rha; G = $(Glc)_4(GlcA)_2$; H = $(Glc)_3(GlcA)_2$; I = $(Glc)_2(GlcA)_2$; J = $(Glc)_1(GlcA)_2$; K = $(GlcA)_2$.

by ion-exchange chromatography. Conversely, elution with a volatile buffer at neutral pH permits very simple desalting of uronic acids, which are eluted well ahead of salts.

ACKNOWLEDGEMENTS

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Note

High-performance metal chelate affinity chromatography of proteins

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(Received October 21st, 1985)

Metal chelate affinity chromatography of proteins was introduced in 1975 by Porath *et al.*[1]. Since then, it has been applied successfully to many kinds of proteins[2] and is becoming a standard tool for the isolation of proteins. It has been especially useful for group separation between two classes of proteins with and without affinity for heavy metal ions. However, it seems difficult to achieve good separation among the proteins having affinity for heavy metal ions. Furthermore, most separations have been performed at low speed using agarose derivatives. If these aspects were to be improved, this technique would become much more useful.

Very recently, a new support for metal chelate affinity chromatography has become commercially available under the trade-name of TSKgel Chelate-5PW (Toyo Soda, Tokyo, Japan). According to the manufacturer, it is prepared by introducing iminodiacetic acid into TSKgel G5000PW[3], which is a hydrophilic resin-based material of large pore size (particle diameter 10 μm) employed for high-performance gel filtration. Therefore, the schematic structure is believed to be as shown in Fig. 1. The amount of immobilized iminodiacetic acid is about 20 μmol per ml swollen gel. We have evaluated this new support for protein separation and the results are now described.

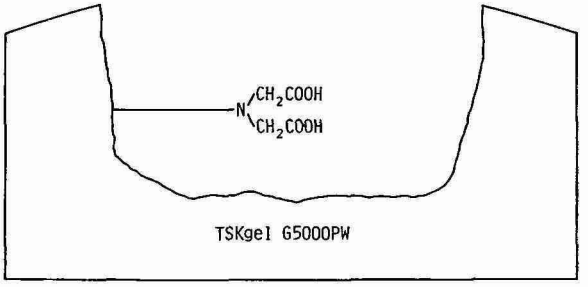

Fig. 1. Schematic structure of TSKgel Chelate-5PW.

EXPERIMENTAL

All chromatographic measurements were performed on 75 × 7.5 mm I.D. stainless-steel columns with a high-speed liquid chromatograph Model SP8700 (Spectra-Physics, San Jose, CA, U.S.A.) equipped with a variable wavelength UV detector

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Model UV-8 (Toyo Soda). Proteins were usually separated on a zinc-loaded column by a 60-min linear gradient of glycine from 0 to 0.2 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* sodium chloride and at a flow-rate of 0.5 ml/min at 25°C; detection was at 280 nm, unless stated otherwise. Before each separation, the column was washed with *ca.* 10 ml of 0.05 *M* ethylenediaminetetraacetic acid, tetrasodium salt containing 0.5 *M* sodium chloride. Then, the column was equilibrated with *ca.* 5 ml of the initial buffer and 2 ml of 0.2 *M* zinc chloride were applied to the column by using a sample injector. This amount of zinc chloride was enough to saturate the column with zinc ion. After the column had been re-equilibrated with *ca.* 15 ml of the initial buffer, the sample dissolved in the initial buffer was applied. The gradient of glycine was started at the same time as sample injection.

The recovery of proteins was determined from the areas of the eluted peaks. As controls, we used areas obtained when the column was replaced by empty 1 mm I.D. stainless-steel tubing of 1 ml total inner volume and the elution was performed isocratically at the eluent compoositions at which each protein was eluted in gradient elution. Amounts of 50 μg of each protein were injected. The peak areas were measured with a data processor Model CP-8000 (Toyo Soda).

Ribonuclease A (bovine pancreas), transferrin (human), carbonic anhydrase (bovine erythrocytes), myoglobin (horse skeletal muscle), lactate dehydrogenase (rabbit muscle), alcohol dehydrogenase (yeast), L-glutamic dehydrogenase (bovine liver) and carboxypeptidase A (bovine pancreas) were purchased from Sigma (St. Louis, MO, U.S.A.). Lipoxidase (soya bean) was obtained from P-L. Biochemicals (Milwaukee, WI, U.S.A.) and malate dehydrogenase (yeast) from oriental Yeast (Osaka, Japan).

RESULTS AND DISCUSSION

Fig. 2 shows the separation of an artificial mixture of rather pure proteins which have affinity for zinc ion. All the proteins were eluted as fairly sharp peaks. It may be concluded that metal chelate affinity chromatography on TSKgel Chelate-5PW has a resolution comparable to that of high-performance ion-exchange chromatography, reversed-phase chromatography or hydrophobic interaction chro-

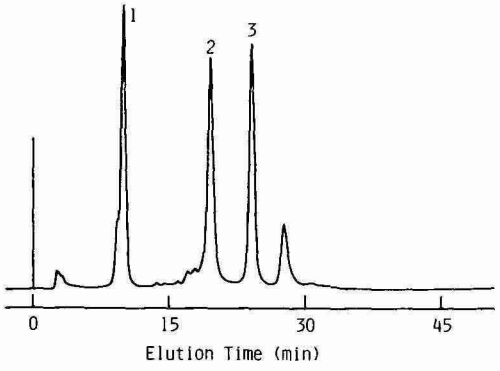

Fig. 2. Chromatogram of a mixture of ribonuclease A (1), transferrin (2) and carbonic anhydrase (3) obtained on Zn^{2+}-Chelate-5PW. The flow-rate was 1 ml/min.

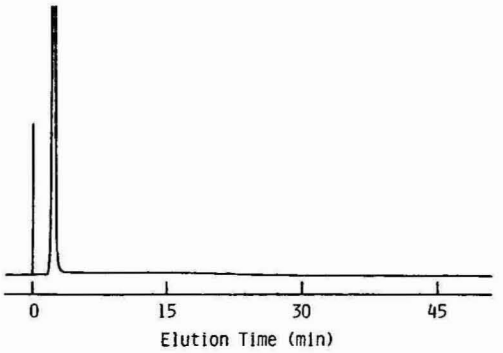

Fig. 3. Chromatogram of the same mixture as in Fig. 2 obtained without loading zinc chloride on the column. The flow-rate was 1 ml/min.

matography. The same protein mixture was chromatographed without loading zinc ion on the column (Fig. 3). All the proteins were eluted in the void volume of the column. Consequently, it seems that the proteins were retained by interaction with chelated zinc ion in the separation shown in Fig. 2. Other proteins (myoglobin, lactate dehydrogenase, alcohol dehydrogenase, L-glutamic dehydrogenase and carboxypeptidase A) which were retained on Zn^{2+}-Chelate-5PW were also eluted in the void volume of the column in the absence of zinc ion. Therefore, non-specific adsorption of proteins on TSKgel Chelate-5PW must be minimal and this support whould be very useful to separate selectively proteins which have affinity for heavy metal ions.

The recoveries of proteins from Zn^{2+}-Chelate-5PW are listed in Table I. All proteins tested were recovered in greater than 80% yield.

TABLE I

RECOVERY OF PROTEINS FROM Zn^{2+}-CHELATE-5PW COLUMN

Protein	*Recovery (%)*
Ribonuclease A	93
Myoglobin	93
Carbonic anhydrase	86
Transferrin	100
Lactate dehydrogenase	95

Fig. 4 shows an example of the application of metal chelate affinity chromatography on TSKgel Chelate-5PW to protein purification. Commercial crude lipoxidase was separated. Many components were retained on Zn^{2+}-Chelate-5PW in 0.02 *M* Tris–HCl buffer (pH 8.0) containing 0.5 *M* sodium chloride, and they were eluted separately by increasing the glycine concentration from 0 to 0.2 *M*. Because the peak eluted at *ca.* 30 min was confirmed to be lipoxidase by an enzymatic activity test, the column effluent was collected between the two vertical lines shown in Fig. 4 and examined for the recovery of enzymatic activity and the degree of purification. The

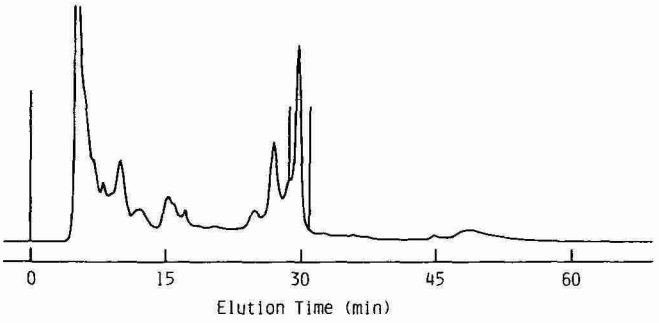

Fig. 4. Separation of commercial lipoxidase (0.5 mg) on Zn^{2+}-Chelate-5PW.

recovery of enzymatic activity was 90%, and the degree of purification based on the specific activity was 7.5-fold. The fraction was also subjected to high-performance ion-exchange chromatography and reversed-phase chromatography (Figs. 5 and 6, respectively). One main peak and a few very small peaks are seen in each chromatogram of the fraction. The main peak in the ion-exchange chromatogram exhibited enzymatic activity, suggesting that the main peaks in the chromatograms of the fraction correspond to lipoxidase. Consequently, it can be said that fairly pure lipoxidase was obtained from a commercial sample containing many impurities in a single step by metal chelate affinity chromatography on TSKgel Chelate-5PW.

Fig. 7 shows the separation of commercial malate dehydrogenase. The peak eluted at *ca.* 25 min was identified as malate dehydrogenase by an enzymatic activity test, and hence the column effluent between two vertical lines was collected. Of the applied enzymatic activity, 95% was found in the fraction. The degree of purification was 3.9-fold. Malate dehydrogenase recovered in the fraction was of high purity according to high-performance liquid chromatography.

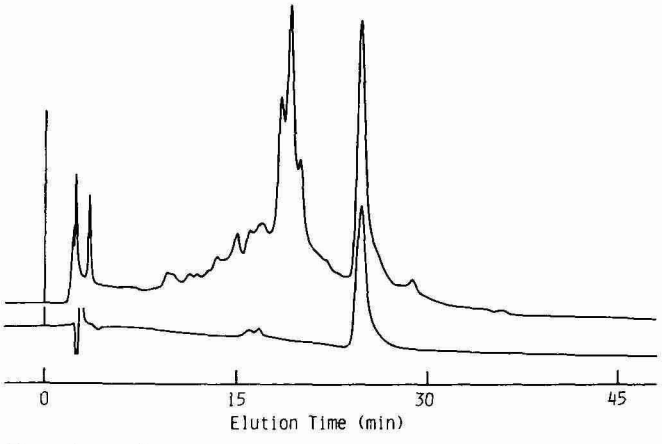

Fig. 5. Ion-exchange chromatograms of a crude lipoxidase sample (upper curve) and the fraction in Fig. 4 (lower curve). The samples were separated on a TSKgel DEAE-5PW column (75 × 7.5 mm I.D.) with a 60-min linear gradient of sodium chloride from 0 to 0.5 *M* in 0.02 *M* Tris–HCl buffer (pH 8.0) at a flow-rate of 1 ml/min at 25°C and detected at 280 nm.

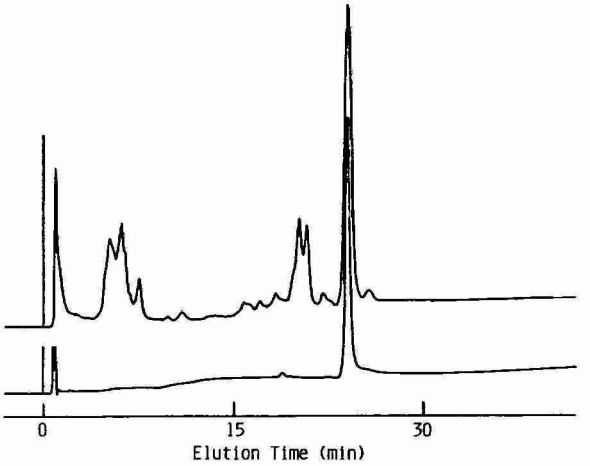

Fig. 6. Reversed-phase chromatograms of a crude lipoxidase sample (upper curve) and the fraction in Fig. 4 (lower curve). The samples were separated on a TSKgel Phenyl-5PW RP column (75 × 4.6 mm I.D.) with a 2-min linear gradient of acetonitrile from 5 to 20% followed by a 48-min linear gradient of acetonitrile from 20 to 80% in 0.05% trifluoroacetic acid at a flow-rate of 1 ml/min at 25°C and detected at 220 nm.

Fig. 8 shows the effect of the flow-rate and gradient time on resolution. Higher resolution was obtained at longer gradient times. However, this effect was diminished at gradient times longer than 120 min, except in the case of very low flow-rates. Because a longer gradient time results in a longer separation time, gradient times of 60–120 min, corresponding to 0.1–0.2 *M* glycine per h, seem to be a good compromise between resolution and separation time. Using these times, the highest resolution was obtained at flow-rates of *ca.* 0.5 ml/min. Although an increase in flow-rate results in a slight decrease in separation time, it also results in more dilution of the sample during separation. Therefore, the optimum flow-rate is around 0.5 ml/min.

The effect of eluent pH was investigated by separating a mixture of transferrin and carbonic anhydrase in 0.02 *M* Tris–HCl buffers of pH 7.0–9.0 (Fig. 9). Proteins were more strongly retained at lower pH, although the affinity of proteins and amino acids for heavy metal ions should be diminished[1,4,5]. This is probably because the affinity of glycine for zinc ion decreased more than that of proteins when the eluent pH was decreased. The peak width and peak interval of the two proteins were also dependent on the eluent pH. Although both characteristics became broader as the eluent pH decreased, the resolution was maximal at pH 7.5–8.0.

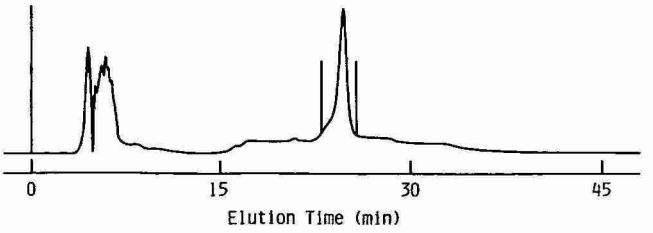

Fig. 7. Separation of commercial malate dehydrogenase (0.1 mg) on Zn^{2+}-Chelate-5PW.

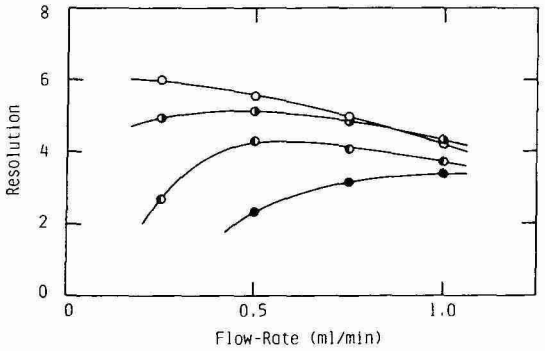

Fig. 8. The effect of the flow-rate and gradient time on resolution. A mixture of transferrin and carbonic anhydrase was separated on Zn^{2+}-Chelate-5PW at flow-rates of 0.25–1 ml/min and gradient times of 30–240 min. The resolution was calculated from the peak widths and elution volumes of the two proteins. Gradient times: 30 (●—●), 60 (◐—◐), 120 (◑—◑) and 240 min (○—○).

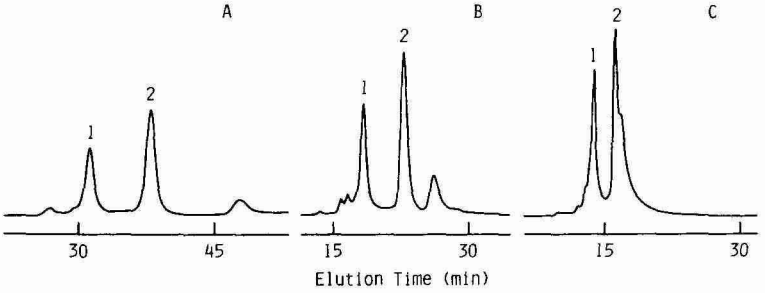

Fig. 9. Chromatograms of a mixture of transferrin (1) and carbonic anhydrase (2) obtained on Zn^{2+}-Chelate-5PW at pH 7.5 (A), 8.0 (B) and 8.5 (C). The flow-rate was 1 ml/min.

The support capacity was studied by separating various amounts of commercial crude lipoxidase. Almost identical separations were obtained with sample loadings up to 2 mg as shown in Fig. 10, but the peaks gradually became broader with higher sample loadings. Therefore, the maximum sample loading resulting in the highest resolution is about 2 mg for this sample, although it is believed to depend on

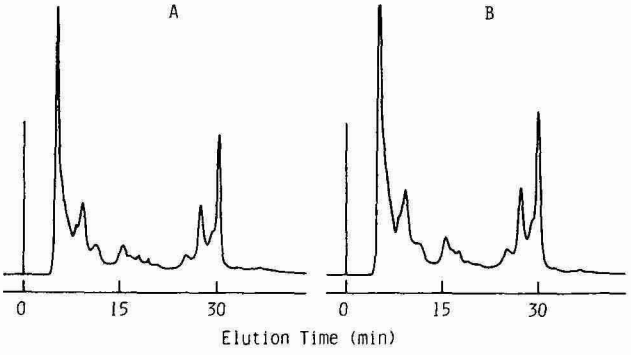

Fig. 10. Chromatograms of commercial crude lipoxidase obtained on Zn^{2+}-Chelate-5PW with sample loadings of 0.2 (A) and 2 mg (B).

the sample, as observed in other modes of high-performance liquid chromatography of proteins[6–8].

As demonstrated above, TSKgel Chelate-5PW is very useful for metal chelate affinity chromatography of proteins. Proteins which have affinity for zinc ion could be separated rapidly with high resolution and recovery. Moreover, it should be possible to apply TSKgel Chelate-5PW to very high-molecular-weight proteins because it has pores with a diameter of several thousand ångströms. In addition, it is chemically very stable. No change in the iminodiacetic acid content was observed when it was suspended in 0.5 *M* sodium hydroxide or 20% acetic acid for 10 days at 25°C. Therefore, metal chelate affinity chromatography on TSKgel Chelate-5PW should become an important technique for the separation of proteins.

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Note

Determination of sterigmatocystin in feed by high-performance liquid chromatography with column switching

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Sterigmatocystin (Fig. 1) is a toxic mould metabolite and recognized carcinogen[1–3] recently detected in feed (7.75 mg/kg) associated with acute clinical symptoms of bloody diarrhoea and death in dairy cattle[4]. It is produced by strains of *Aspergillus versicolor, Aspergillus nidulans, Bipolaris sorokiniana* and other species of the genus *Aspergillus*[5]. The main producer, *Aspergillus versicolor*, is of ubiquitous distribution and a common contaminant of a wide variety of foods and feeds[6]. Because it has been found to grow on roughages[7,8], it is possible that hay and straw intended for feeding purposes may contain sterigmatocystin. Numerous studies on the occurrence and analysis of sterigmatocystin in various foods and feeds using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) have been published[9–15]. The application of these methods to the analysis of hay and straw for sterigmatocystin yields unsatisfactory results, because some interfering compounds cannot be separated completely from sterigmatocystin by either TLC or HPLC. For this reason, we have developed an efficient and simple method for the determination of sterigmatocystin at low ppb levels in straw and hay by HPLC with column switching.

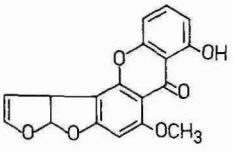

Fig. 1. Structure of sterigmatocystin.

EXPERIMENTAL

Chemicals

Sterigmatocystin was prepared in our laboratory[16] and its identity was confirmed by mass spectrometry, nuclear magnetic resonance spectroscopy and UV spectrometry. Its purity was determined by TLC and HPLC to be as high as 99%. A stock solution (5 μg/ml) was prepared in methanol. Other solutions were obtained by dilution. All solvents were distilled before use. Silica gel 60 for column chromatography (70–230 mesh) was activated for 2 h at 120°C.

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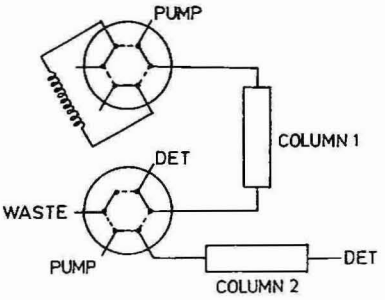


Fig. 2. Column switching system for the determination of sterigmatocystin in straw and hay (see text for details).

Apparatus

Fig. 2 is a schematic diagram of the column switching assembly employed. The chromatographic system consists of a double-headed Orlita high-pressure pump (Hewlett-Packard Model 1010 B liquid chromatograph) with two-solvent capability equipped with a sampling valve (OE-320; Labormim, Budapest, Hungary) and an additional six-port valve (Rheodyne Model 7010). A LiChrosorb RP-18 (particle size 7 μm) column (25 × 0.46 cm I.D.) for analytical work and a LiChrosorb RP-8 (particle size 10 μm) column (10 × 0.46 cm I.D.) were used. The injection and switching valve work manually, but the system can be automated easily for routine analysis. For detection a Hewlett-Packard Model 1030B variable-wavelength detector and an LCD 254 UV monitor (Laboratorní Přístroje, Prague, Czechoslovakia) were used.

Extraction and separation

The extraction and clean-up procedure is based on the AOAC method for the determination of sterigmatocystin in barley and wheat[17]. Extraction of 10 g of dried and finely ground straw or hay with acetonitrile–water containing 4% potassium chloride (9:1) was followed by partition against *n*-hexane and chloroform. After evaporation of the solvent, the residue was purified on 10 g of silica gel 60 (70–230 mesh), eluted with cyclohexane–ethyl acetate (4:1). The eluate was vacuum evaporated at 40–50°C. The residue was transferred quantitatively into a 10-ml flask with sequential portions of methanol (3 × 1 ml) and the solvent was evaporated to dryness under a gentle stream of nitrogen. The sample was redissolved in an appropriate volume (0.5–5.0 ml) of acetonitrile–3.3 · 10^{-3} *M* phosphoric acid (45:55) and after filtration it was injected into the HPLC system.

The switching time can be derived from the retention time of sterigmatocystin, which was determined daily by three injections of a standard solution onto column 1 (Fig. 2), followed by elution with acetonitrile–3.3 · 10^{-3} *M* phosphoric acid (45:55) at a flow-rate of 1 ml/min.

Feed samples were injected onto the pre-column. After washing with acetonitrile–3.3 · 10^{-3} *M* phosphoric acid (45:55) for about 5 min, the eluate from column 1 containing sterigmatocystin was switched to the analytical column. When the desired volume was diverted from column 1 to column 2, the valve was placed in its original position and chromatography proceeded on column 2 with acetonitrile–3.3 · 10^{-3} *M* phosphoric acid (60:40) as the mobile phase at a flow-rate

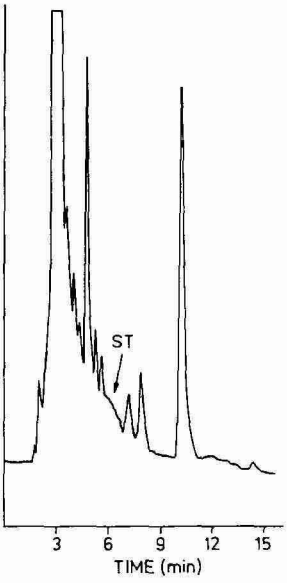
of 1.5 ml/min. Calibration graphs for sterigmatocystin in the concentration range 10–100 ng per injection were monitored in the same manner.

Detection of sterigmatocystin was performed by UV absorption measurement at 325 nm. The sensitivity was set to 0.02 a.u.f.s. The effluent from the pre-column was monitored by UV detection at 254 nm. The injection volume was 20 μl in all instances.

RESULTS AND DISCUSSION

Chromatograms of straw and hay samples extracted and purified by the AOAC method for the determination of sterigmatocystin in barley and wheat and analysed by HPLC are given in Figs. 3 and 4. The chromatograms illustrate that this procedure did not provide adequate clean-up of extracts. Compounds were detected in control samples that were not separated completely from sterigmatocystin. These peaks are variable amount and interfere with accurate quantitation. For this reason, additional purification steps are necessary, which are tedious to perform and often give rise to loss of toxin. These drawbacks can be circumvented by HPLC with column switching, reviewed by Freeman[18].

The application of two reversed-phase columns allows direct switching of an effluent segment of the pre-column to the analytical column without trapping loops or manual solvent evaporation steps. The column length, alkyl chain length of the chemically modified silica and particle size were selected in such a way that column 1 can be eluted with a markedly weaker solvent than column 2. This enables relatively



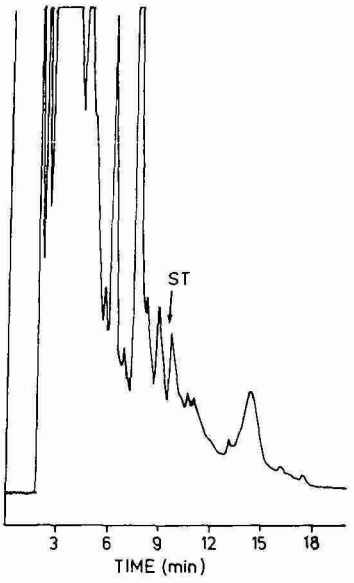
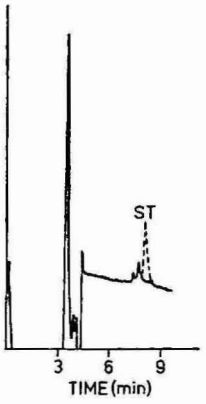


Fig. 3. HPLC separation of a control straw sample on a LiChrosorb RP-18 (particle size 7 μm) column at a flow-rate of 1.5 ml/min with acetonitrile–3.3 · 10^{-3} *M* phosphoric acid (60:40) as eluent and UV detection at 325 nm. ST = sterigmatocystin.

Fig. 4. HPLC separation of a control hay sample. Flow-rate, 1.0 ml/min; other conditions as in Fig. 3. ST = sterigmatocystin.



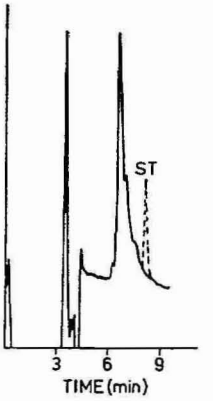


Fig. 5. Chromatogram of a straw sample spiked with 50 ppb of sterigmatocystin (ST) obtained by HPLC with column switching. The solid line refers to the response of a blank and the broken line to the spiked sample.

Fig. 6. Chromatogram of a hay sample spiked with 100 ppb of sterigmatocystin (ST) obtained by HPLC with column switching. The solid line refers to the response of a blank and the broken line to the spiked sample.

large volume segments of the pre-column to be switched directly to the analytical column without undesirable band spreading of the compound of interest.

The HPLC traces of unspiked and artificially contaminated straw and hay samples shown in Figs. 5 and 6 show the high purity of the sample extracts transferred to the analytical column by the column switching technique. No interfering peaks were detected at the retention time of sterigmatocystin and accurate quantitation was possible. The total HPLC analysis time is about 15 min. At the end of the analytical run the pre-column does not need flushing with stronger solvents in most instances. A time of 15 min is sufficient for elution of strongly retained compounds and reconditioning of the column. Another advantage of the method is the prolonged lifetime of the analytical column. It has been used in our laboratory for 3 months with daily injections without any decrease in column efficiency. Column 1 has to be repacked after about 200 injections, depending on the type of samples injected. The detection limit for sterigmatocystin in hay and straw was 25 ppb*.

TABLE I

REPRODUCIBILITY OF RETENTION TIME FOR STERIGMATOCYSTIN

Standard solutions and spiked straw samples were injected.

Parameter	*Value*
No. of injections	30
Retention time:	
Range (s)	457–481
Mean (s)	473
Standard deviation (s)	6.6
Coefficient of variation (%)	1.4

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

TABLE II

REPRODUCIBILITY OF PEAK HEIGHT FOR STERIGMATOCYSTIN

20-μl aliquots of an 11.6 ng/μl sterigmatocystin standard solution in methanol were injected: the detector sensitivity was set to 0.04 a.u.f.s.

Parameter	*Value*
No. of injections	10
Peak height:	
Range (mm)	132.5–136
Mean (mm)	134.8
Standard deviation (mm)	1.26
Coefficient of variation (%)	0.93

The retention times and peak heights ascertained for a sterigmatocystin standard solution and for contaminated straw samples provide evidence of the high reproducibility of the HPLC column switching technique employed (Tables I and II). The relationship between peak height and amount of sterigmatocystin injected was linear over the range 10–250 ng. Average recoveries of sterigmatocystin added to straw and hay in amounts from 50–200 ppb were 71.5 and 59.4%, respectively.

The column switching technique was successfully employed for the analysis of various naturally sterigmatocystin-contaminated hay samples (Fig. 7). Preliminary studies have shown that the procedure described may also be applied to the determination of sterigmatocystin in cereals, maize and silage.

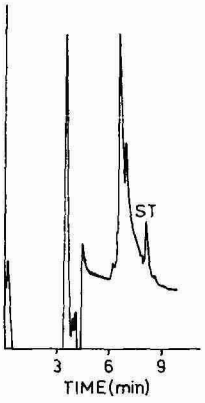


Fig. 7. Chromatogram of a hay sample naturally contaminated with 72 ppb of sterigmatocystin (ST) obtained by HPLC with column switching.

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CHROM. 18 350

Note

Continuous-flow monitoring of hexuronic acid by carbazole reaction during gel filtration of proteoglycans in urea solutions

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Gel filtration is one of the methods frequently employed for purification and fractionation of proteoglycans[1–3]. Since proteoglycans are known to form large aggregate structures under associative conditions[4,5], gel filtration can be used to study aggregation. *In vitro*, this association is reversible and is largely dictated by the solvent. Proteoglycans are usually isolated from many tissues by extraction with dissociative solvents such as 4 *M* guanidine hydrochloride or 6 *M* urea[5,6], followed by cesium chloride isopycnic centrifugation and/or gel filtration. Isolation of aggregates by gel filtration is achieved by using salt solutions of low concentration, *e.g.*, 0.4 *M* guanidine · HCl, while 4 *M* guanidine · HCl or 6 *M* urea is used for isolation of proteoglycan monomers. After gel filtration, fractions generally are dialyzed to remove salts and then monitored for hexuronic acid by carbazole reaction. We recently described a procedure for continuous-flow analysis of proteoglycans during gel filtration in guanidine · HCl by an orcinol–sulfuric acid reaction[7]. This is a general reaction for sugars and is not specific for uronic acids.

This report describes a procedure for continuous-flow monitoring of proteoglycans by a carbazole–sulfuric acid reactions using the Technicon sugar chromatography system. The proteoglycans were eluted from gel filtration by a dissociative solvent, 6 *M* urea.

EXPERIMENTAL

Materials

Separhose CL-6B was purchased from Pharmacia (Piscataway, NJ, U.S.A.). Reagent-grade (ACS grade) sulfuric acid was obtained from DuPont (Wilmington, DE, U.S.A.), potassium tetraborate and urea from J. T. Baker (Phillipsburg, NJ, U.S.A.), and carbazole from Eastman Organic Co. (Rochester, NY, U.S.A.). Glu-

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curonolactone was purchased from Corn Products Refineries (New York, NY, U.S.A.). Bovine aorta proteoglycan was available in the laboratory from other studies[8–10]. Glycosaminoglycan standards were donated by Drs. J. A. Cifonelli and M. B. Matthews, University of Chicago.

Sulfuric acid–borate reagent. Potassium tetraborate (19.1 g) was dissolved in 200 ml distilled water, and to this solution 2300 ml of concentrated sulfuric acid (97%) were carefully added in the cold.

Carbazole reagent. Carbazole (1.0 g) was dissolved in 1 l of 95% ethanol and stored at 4°C.

Glucuronolactone standard solution. Standard solutions of glucuronolactone were prepared either in distilled water or in different concentrations of urea in 0.5 *M* sodium acetate, pH 5.8.

Methods

Isolation of proteoglycans from bovine aorta. Proteoglycans were isolated from bovine aorta intima as previously described[9–11]. Proteoglycans were extracted with 4 *M* guanidine · HCl in the presence of protease inhibitors. The extract was clarified by centrifugation, and the density was adjusted to 1.33 g/ml by the addition of cesium chloride. The solution was centrifuged at 100 000 *g* for 19 h at 4°C. The bottom two-fifths of the gradient was adjusted to a density of 1.46 g/ml with cesium chloride recentrifuged as before. Fractions of the bottom two-fifths gradient were pooled, dialyzed and lyophilized.

Automated gel filtration procedure. The flow diagram of the gel filtration procedure is illustrated in Fig. 1. A chromatographic column (76 × 2 cm I.D., 3400 Series; Glenco Scientific, Houston, TX, U.S.A.) filled with Sepharose CL-6B was used. The flow-rate was kept constant at 0.5 ml/min by negative pressure, *i.e.*, by passing the column outlet tube through the proportioning pump manifold. Pump tubing sizes are shown in the figure. Colorimeter and recorder settings were the same as those described previously[12,13]. A 530-nm filter was used in the colorimeter.

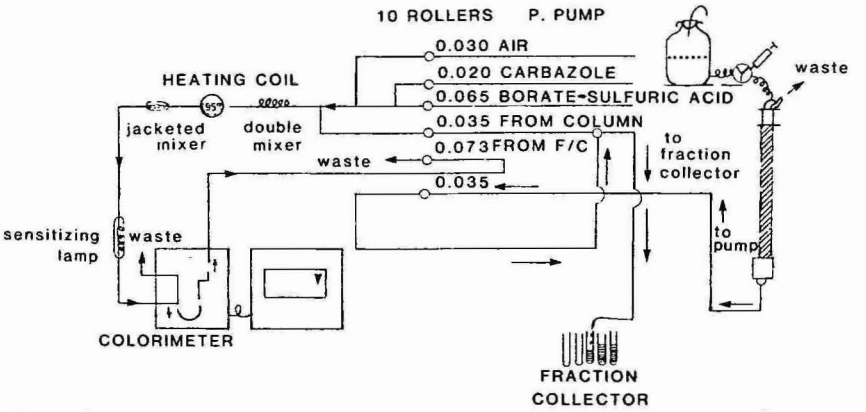

Fig. 1. Diagram of continuous-flow monitoring of hexuronic acid by the carbazole-sulfuric acid reaction in the Technicon sugar chromatography system. All connections are with 1.6 mm glass–0.090 in. Acidflex sleeve; tube size is given in inches. Colorimeter is equipped with a 15-mm tubular flow cell (F/C) and a 530-nm filter.

The analyzer was first primed with 95% ethanol before adding carbazole reagent. If carbazole reagent is used directly for priming, a possibility exists of precipitation of carbazole in the tubing and this would block the pumping of the reagent. Precaution also should be taken that the carbazole reagent should mix with sulfuric acid before it joins the inflowing sample solution in the manifold (*i.e.*, carbazole reagent should be introduced in the system when the other reagents are being pumped freely into the system).

Prior to gel filtration of proteoglycans, the Sepharose column was equilibrated with 6 *M* urea in 0.5 *M* sodium acetate, pH 5.8. Samples of proteoglycans or glycosamidoglycan were dissolved in this buffer and added to the top of the column. The column was then eluted with buffer and the effluent was monitored continuously in the sugar analyzer by carbazole–sulfuric acid.

Determination of glucuronic acid. Manual determination of glucuronic acid in distilled water or in urea solutions was performed by the procedure of Bitter and Muir[14]. Solutions of different concentrations of glucuronic acid in 0.5 *M* sodium acetate, pH 5.8, and in 6 *M* urea in 0.5 *M* sodium acetate, pH 5.8, were analyzed by the autoanalyzer. Glucuronic acid samples were introduced through the sample inlet of the manifold and analyzed by the carbazole reaction. The areas of the peaks on the recorder chart were calculated by triangulation.

RESULTS AND DISCUSSION

Earlier, Ford and Baker[15] reported an automated procedure for hexuronic acid determination by carbazole–sulfuric acid reaction using the Technicon sugar analyzer. In this method carbazole was dissolved in sulfuric acid–borate and pumped into the system. Unfortunately, this reagent is not stable even at 4°C and gradually turns yellow in 4–6 h. Since many gel filtration procedures require long durations, we found that the procedure is not suitable for continuous-flow monitoring during chromatography. In the present method we introduced carbazole reagent and sulfuric acid–borate reagent separately into the system and allows them to mix with each other before mixing with uronate samples. Both reagents are stable indefinitely when stored at 4°C. The manifold in the autoanalyzer was modified to accommodate the changes (Fig. 1).

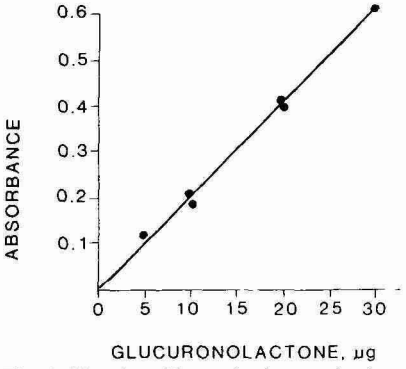

Fig. 2. Uronic acid standard curve in the presence or absence of 6 *M* urea. Both curves were identical. Absorbance was measured at 530 nm.

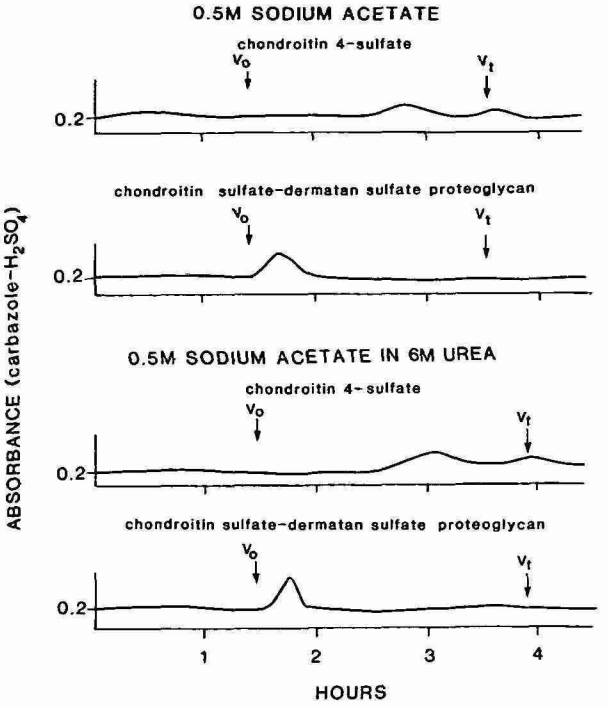

Fig. 3. A comparison of gel filtration profiles of chondroitin 4-sulfate and a chondroitin sulfate–dermatan sulfate proteoglycan from bovine aorta in the presence or absence of 6 *M* urea in sodium acetate monitored by the carbazole–sulfuric acid reaction. Absorbance was measured at 530 nm.

Fig. 2 illustrates the effect of urea on the color absorbance of carbazole reaction of glucuronic acid[14] in a test tube. Urea in 0.5 *M* sodium acetate, even at a concentration of 6 *M*, did not suppress the color. However, when protease inhibitors were included in the solvent along with 6 *M* urea, an approximately 7% decrease in absorbance occurred at a concentration of 60 μg/ml uronic acid.

Glucuronic acid solutions (either in sodium acetate or in 6 *M* urea in sodium acetate) gave peak areas of identical values when analyzed in the autoanalyzer by the procedure described. This suggests that urea does not interfere in the method. Analyses of ten identical samples of glucuronic acid in 6 *M* urea in sodium acetate gave a coefficient of variation of 3%. When known concentrations of glucuronic solution in 6 *M* urea and 0.5 *M* sodium acetate were analyzed in the autoanalyzer, the areas of the peaks on the recorder trace were proportional to the glucuronic acid concentration, suggesting the method is suitable for monitoring proteoglycans or glycosaminoglycans.

Fig. 3 illustrates the gel filtration profiles of chondroitin 4-sulfate and a chondroitin sulfate–dermatan sulfate proteoglycan from bovine aorta in sodium acetate and in 6 *M* urea in sodium acetate. The peaks in chromatograms developed with 6 *M* urea in sodium acetate are somewhat sharper than those developed with sodium acetate alone, but the areas of the peaks in both buffers were the same. Chondroitin sulfate and the chondroitin sulfate–dermatan sulfate proteoglycan peaks eluted from

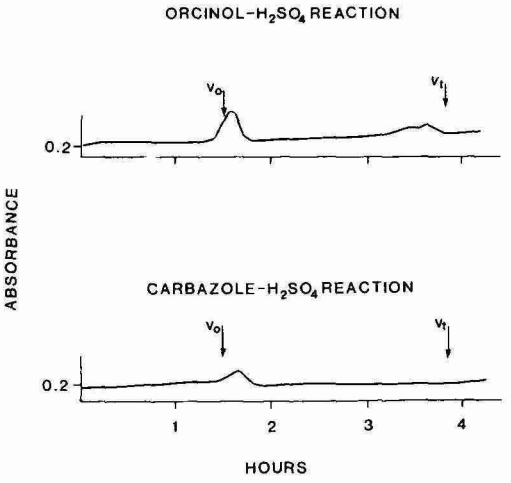

Fig. 4. Comparison of orcinol–sulfuric acid reaction (measured at 420 nm) and carbazole–sulfuric acid reaction (measured at 530 nm) for monitoring hexuronic acid during gel filtration of a chondroitin sulfate–dermatan sulfate proteoglycan from bovine aorta intima.

the column somewhat slower in urea. Our observations suggest that the procedure is adequately sensitive for monitoring hexuronic acid during chromatography of proteoglycans in 6 *M* urea.

Fig. 4 shows a comparison of gel filtration profiles of a partially purified sample of chondroitin sulfate–dermatan sulfate proteoglycan continuously monitored during gel filtration by carbazole–sulfuric acid reaction and by orcinol–sulfuric acid reaction. Both chromatograms show one major peak near the void volume. The peak in the chromatogram analyzed by carbazole–sulfuric acid had a smaller area than the same peak in the chromatogram analyzed by orcinol–sulfuric acid. In the orcinol–sulfuric acid analyzed chromatogram a second peak of a much lower molecular weight appeared, which was absent in the carbazole–sulfuric acid monitored run. This documents the increased specificity of the carbazole–sulfuric acid reaction over the orcinol–sulfuric acid reaction.

Carbazole–sulfuric acid is at least five times as sensitive to hexuronic acid than orcinol–sulfuric acid. The differences in the areas of peaks between the chromatograms are likely due to either short oligosaccharide chains present on the core protein of the proteoglycan or contaminant glycoproteins in the preparation. Although the carbazole–sulfuric acid reaction is specific for hexuronic acid, neutral sugars, particularly at high concentrations, will produce some color.

In conclusion, the method described affords a procedure for monitoring proteoglycans during gel fitlration by a color reaction specific to hexuronic acids and will significantly aid isolation of proteoglycans.

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Note

Isotachophoretic determination of hyaluronate oligosaccharide-degrading enzyme activities

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The unbranched glycosaminoglycan hyaluronic acid (HA) consists of the basic disaccharide unit β-D-glycanopyranuronosyl-1→3-O-(2-acetamido-2-deoxy-β-D-glycopyranosyl)-1→4 and is initially hydrolysed by endoglycanohydrolases such as bovine testicular hyaluronidase (E.C. 3.2.1.35) and leech hyaluronidase (E.C. 3.2.1.36). The even-numbered oligosaccharides obtained are further degraded by exoglycanohydrolases such as β-N-acetylglucosaminidase (E.C. 3.2.1.30) and β-glucuronidase (E.C. 3.2.1.31), leading to odd-numbered oligosaccharides with terminal glucuronic acid (GlcUA) or N-acetylglucosamine (GlcNAc).

These defined oligosaccharides are useful tool for the detection and characterization of these enzymes. This paper presents an isotachophoretic method for the quantification and identification of HA oligosaccharide hydrolysis products. This method opens up the possibility of characterizing hyaluronidases, β-N-acetylglucosaminidases and β-glucuronidases with defined biogenic substrates without any sample pre-treatment. The method provides an easy and accurate quantification and high resolving power.

EXPERIMENTAL

Preparation of oligosaccharides

Disaccharide (βGlcUAl–3GlcNAc), tetrasaccharide (βGlcUAl–3βGlcNAc) and trisaccharide (βGlcNAcl–43βGlcNAc) were produced by digesting HA from umbilical cords with bovine testicular hyaluronidase[1] and β-N-acetylglucosaminidase-free β-glucuronidase from *Patella barbara*[2].

Disaccharide* (βGlcNAcl–4GlcUA), tetrasaccharide* (βGlcNAcl–4βGlcUAl-3)$_2$ and trisaccharide* (βGlcUAl–3βGlcNAcl–4GlcUA) were produced by using leech hyaluronidase, bovine spleen β-N-acetylglucosaminidase and β-glucuronidase[2].

The hydrolysis products were separated by chromatography on DEAE-Sephacel (formate form) with a linear gradient of formic acid[3]. The purity of the oligosaccharides was checked by high-performance liquid chromatography (HPLC)[4] and isotachophoresis.

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Instrumental

Analyses were performed with an LKB 2127 Tachophor (LKB, Gräfelfing, F.R.G.) in a 230-mm PTFE capillary with 7 mmol/l hydrochloric acid glycine–glycine/0.3% hydroxymethylcellulose as the leading electrolyte; 0.01 mol/l caproic acid was used as the terminating electrolyte. Detection was carried out at 254 nm and by thermal measurement. Quantification was effected by measuring the widths of thermal and UV peaks and referring them to a calibration graph. The injection volume was 1.5 μl and runs were made within 12 min at 12°C with a detection current of 50 μA.

Enzyme assays

Purified β-glucuronidase from *Patella barbara*[2], β-N-acetylglucosaminidase from bovine spleen[5] and bovine testicular hyaluronidase[1] were incubated at 37°C with 0.01 mol/l substrate in 0.005 mol/l acetate buffer (pH 4.0), then 1.5-μl aliquots were analysed in a time-dependent manner until total hydrolysis of the substrate oligosaccharides used was achieved.

RESULTS AND DISCUSSION

Some biogenic HA oligosaccharides show clear thermal step-height differences in the described isotachophoretic system (Table I, Fig. 1D). A differentiation of even-numbered disaccharides and tetrasaccharides with terminal reducing GlcUA or GlcNAc is impossible by isotachophoresis because there is no difference in charge or molecular size and, as a consequence, they do not differ in their mobility. In contrast, odd-numbered oligosaccharides, like trisaccharides, differ in their GlcUA/GlcNAc ratio and therefore in their net charge and show distinct isotachophoretic behaviour (Table I). Trisaccharide*, for instance, with two GlcUA per molecule with a higher net charge than the other trisaccharide, shows a higher mobility and consequently a lower thermal step height than trisaccharide. Surprisingly, GlcUA and trisaccharide, with a lower molecular size and higher net charge than tetrasaccharide, are separated inadequately from it as shown by thermal detection (Fig. 1D).

For enzyme assay of β-glucuronidases, β-N-acetylglucosaminidases and hyaluronidases, biogenic HA oligosaccharides are useful substrates when the following requirements are fulfilled. First, the substrate must be hydrolysed by the investigated

TABLE I

RELATIVE THERMAL STEP HEIGHTS OF BIOGENIC HYALURONATE

Saccharides with terminal reducing glucuronic acid are labelled with an asterisk.

Saccharide	*Thermal step height (%)*
Trisaccharide*	26
Glucuronic acid	28
Tetrasaccharide	28
Tetrasaccharide*	28
Disaccharide	36
Disaccharide*	36
Trisaccharide	48

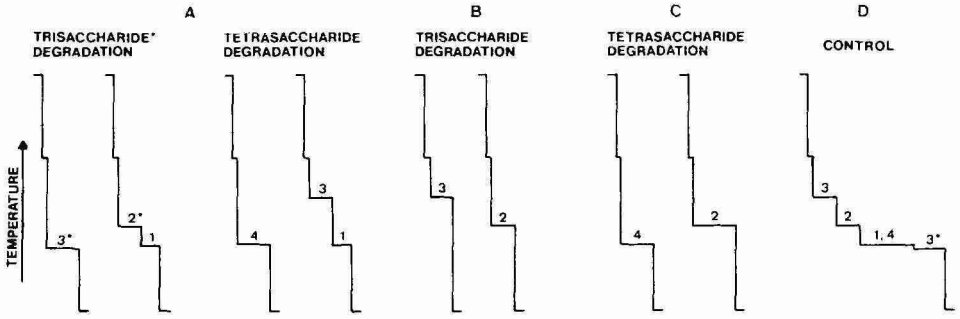

Fig. 1. Total degradation of hyaluronate oligosaccharides by (A) β-glucuronidase, (B) β-N-acetylglucosaminidase and (C) bovine testicular hyaluronidase monitored by isotachophoresis. Saccharides: 1 = glucuronic acid; 2 = disaccharide; 2* = disaccharide*; 3 = trisaccharide; 3* = trisaccharide*; 4 = tetrasaccharide.

enzyme and second, it must be degraded to hydrolysis products that are separable from the substrate and detectable by isotachophoresis. Useful substrate–enzyme combinations for enzyme characterization are as follows.

For β-glucuronidase determinations, trisaccharide* and tetrasaccharide are useful substrates: trisaccharide* → disaccharide* + GlcUA, tetrasaccharide → trisaccharide + GlcUA. In the trisaccharide* hydrolysis, disaccharide* and GlcUA are formed in equimolar concentrations. As can be seen in Fig. 1A, GlcUA is not well separated from trisaccharide* but the enzyme activity is determinable by the disaccharide* formed. In tetrasaccharide hydrolysis, tetrasaccharide and GlcUA are not separable and the enzyme activity is determined by the trisaccharide formed (Fig. 1A).

For β-N-acetylglucosaminidase activity detection, trisaccharide with terminal non-reducing GlcNAc is a useful substrate (Fig. 1B): trisaccharide → disaccharide + GlcNAc. In this case the disaccharide hydrolysis product and the substrate are detectable and both can be used for activity determination.

Hyaluronidase activity is measurable with tetrasaccharide as substrate: tetrasaccharide → 2 disaccharides. The substrate and the hydrolysis products are well separated by isotachophoresis (Fig. 1C). Further, the proof of hyaluronidases with HA as substrate is better by isotachophoresis than by HPLC. In HPLC problems can arise with the column system used for HA but not for HA oligosaccharides as substrates. The commonly used HPLC column systems for this separation are based on weak ion-exchange materials, which suffer from irreversible adsorption problems with the high-molecular-weight substrates. Further, the analysis takes longer, higher sample volumes are needed and the column lifetime is limited.

In contrast to chemical methods, isotachophoresis facilitates the simultaneous qualitative and quantitative detection of substrate and hydrolysis products. The small sample volumes, no sample pre-treatment and the short analysis time are advantages. Although this isotachophoretic system is not generally superior to HPLC systems, it is advantageous in the separation of disaccharide and trisaccharide, which is difficult by HPLC.

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Note

Assay of polyamines by thin-layer chromatography and a combination of double-isotope dilution and scintillation methods

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The sensitive and precise determination of polyamines is of importance in the investigation of their biochemistry[1–3]. The most widely used methods are based on quantitation by high-performance liquid chromatography of the native of dansylated samples[4,5]. These methods are excellent but rely on the complete recovery of the sample and require special instrumentation. Regier and Kafatos[6] and Airhart *et al.*[7] have used radiolabelled dansyl chloride to analyse the quantity and specific activity of amino acids by a combination of double-isotope dilution and the fluorometric methods. I have adapted this technique to the quantitation of polyamines in biological samples. The main advantage of this method is that it does not require complete sample recovery nor special instrumentation, and relies primarily on the availability of a liquid scintillation spectrometer.

EXPERIMENTAL

Chemicals

^{14}C-labeled polyamines and [^{3}H]dansyl chloride were purchased from New England Nuclear. Thin-layer chromatography plates (silica 60 F254) were obtained from E. Merck. All reagents used were analytical grade.

Materials

Rat liver homogenates, *Escherichia coli* sonicates and L1210 leukemia cell lysates in 50 m*M* Tris–HCl were extracted for 3 min at 0°C with an equal volume of 0.4 *M* perchloric acid, and centrifuged at 2000 *g* for 15 min at 4°C prior to use. Etiolated pea internodes (6 days old) were similarly extracted and centrifuged at 48 000 *g* for 20 min at 4°C.

Procedure

To the tissue extracts (100 μl) was added a mixture of known amounts of ^{14}C-labeled polyamines (1 nmol [^{14}C]putrescine, $1 \cdot 10^{-2}$ μCi/nmol; 15 nmol [^{14}C]spermidine or [^{14}C]spermine, $9 \cdot 10^{-4}$ μCi/nmol) followed by 100 μl [^{3}H]dansyl chloride (0.46 mg/100 μl acetone, $1 \cdot 10^{-3}$ μCi/nmol). A ten-fold excess (in terms of

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neutralizing equivalents) of saturated sodium carbonate was added and the mixture incubated in the dark at 37°C for 2 h. The unreacted dansyl chloride was decomposed with 0.4 mmol proline, and the reaction mixture extracted three times with benzene or diethyl ether saturated with a sodium carbonate (saturated)–acetone–water (1:1:1). The benzene or ether layers were pooled and evaporated to dryness under a stream of nitrogen. To the dry residue, benzene or ether was added, and a portion of the resulting solution was spotted on silica gel thin-layer plates. These were developed in the dark, preferably in two dimensions. The solvents for the first dimension were cyclohexane–ethyl acetate (4:5) and for the second dimension, chloroform–triethylamine (100:8). The fluorescent spots were scraped off the plate, and placed in 0.5 ml New England solubilizer (NES) plus 5.0 ml Econofluor and counted in a liquid scintillation spectrometer for ^{14}C and ^{3}H.

Controls

Standards were prepared by danyslating the original ^{14}C-labeled polyamines with [^{3}H]dansyl chloride. These were added to a separate sample of the tissue extract and isolated in parallel with the experimental samples.

Calculations

The amount of unlabeled polyamine in the samples was calculated by the equation

$$U + S = S(A/B) \tag{1}$$

where U = amount (mol) of the unknown polyamine in the sample, S = amount (mol) of the radioactive polyamine added, A = ratio of $^{14}C/^{3}H$ in the control dansylated polyamine sample and B = ratio of $^{14}C/^{3}H$ in the experimental dansylated polyamine sample.

RESULTS AND DISCUSSION

The two dimensional chromatographic solvent systems described enable an excellent separation of the dansylated derivatives of putrescine (put), spermidine (spd) and spermine (spm) (Fig. 1).

The reliability of the method is indicated by Table I, which shows the recovery of known amounts of polyamines added to *E. coli* extracts. The recovery was optimal when two-dimensional chromatography was used.

Eqn. 1 takes into account the modification of the concentration of the unknown polyamine, U, by that of the added polyamine, S. It is, therefore, applicable to values of S that are larger, within certain limits, than U. Experimentally, reliable results can be obtained for $S \leqslant U$.

Another limitation of the assay is imposed by the spillover of ^{14}C into the ^{3}H channel in the Beckman spectrometer. This spillover is 20% in Econofluor; consequently, approximate limits for accurate determination of B are $0.4 \leqslant B \leqslant 7$. These limits can be greatly extended, especially at the lower values, if in each experiment the value of A is determined experimentally by TLC rather than theoretically. In this way the various experimental fluctuations, including quenching, are taken into account.

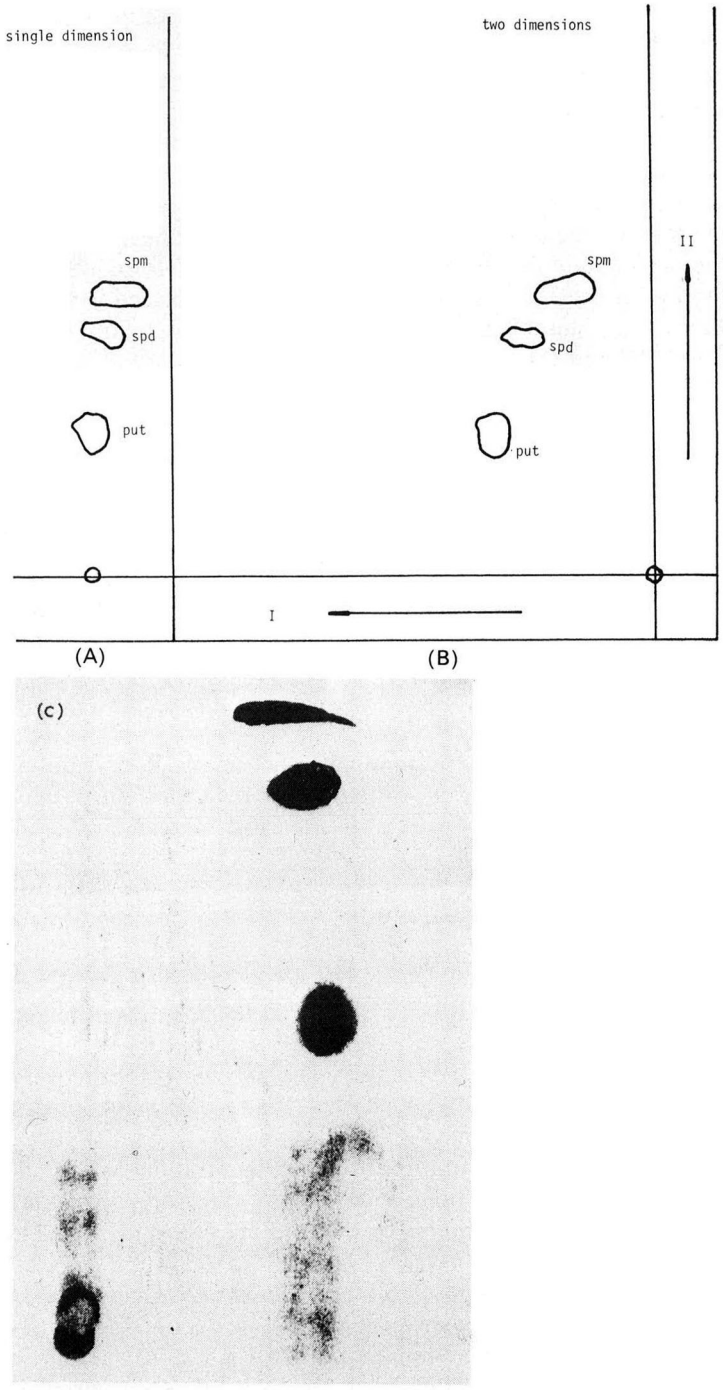

Fig. 1. Chromatography of dansylated derivatives of putrescine, spermidine and spermine. (A) One-dimensional TLC of dansylated polyamines. (B) Two-dimensional TLC of dansylated polyamines: I, cyclohexane–ethyl acetate (4:5); II, chloroform–triethylamine (100:8). (C) Autoradiography of dansylated [^{14}C]polyamines after two-dimensional TLC; the sample used in dansylation and chromatography was a mixture of [^{14}C]polyamines and *E. Coli* extract.

TABLE I

RECOVERIES OF KNOWN AMOUNTS OF POLYAMINES ADDED TO *E. COLI* EXTRACTS FROM SINGLE REPRESENTATIVE EXPERIMENTS

Polyamine	*Amount added (nmol)*	*Content in cell extract, A (nmol)*	*Cell extract + known amount of polyamines B (nmol)*	*Determined value (B−A) (nmol)*	*Recovery (%)*
(A) One-dimensional TLC, chloroform–triethylamine (100:8)					
Putrescine	2	1.16	3.03	1.87	93.5
Spermidine	20	0	16.92	16.92	84.6
Spermine	20	0.18	17.30	17.12	85.6
(B) Two-dimensional TLC					
Putrescine	2	1.84	3.82	2.02	101
Spermidine	20	0	18.39	18.39	91.95
Spermine	20	0	18.80	18.80	94.0

The stated specific activities of commercial samples of dansyl chaloride were found to be reliable. Should verification be required, because of radiation damage of the sample, this can be done by dansylating a commercial sample of a ^{14}C-labeled amino acid which is more stable than the commercially available polyamines and by determining the $^{14}C/^{3}H$ ratio. For most routine assays, the specific activity of commercial [^{3}H]dansyl chloride is too high, and should be diluted 20–100 fold. For many purposes, it may be considered adequate to add a known amount of radioactive

TABLE II

APPLICATION OF DOUBLE-ISOTOPE DILUTION METHOD TO THE DETERMINATION OF POLYAMINES IN DIFFERENT BIOLOGICAL MATERIALS

The values are the means ± S.D. for two or three separate experiments, except for the single determination in L1210 cells.

Material	*Polyamine content (nmol per 0.1 mg cell dry weight)*		
	put	*spd*	*spm*
E. Coli			
Sample 1	5.96 ± 0.21	7.03 ± 0.28	No
Sample 2	5.72 ± 0.015	6.22 ± 0.00	No
(nmol per 10 mg fresh weight)			
Etiolated pea internodes			
Sample 1	2.8 ± 0.1	4.4 ± 0.0	0.8 ± 0.01
Sample 2	5.3 ± 0.1	2.8 ± 0.3	0.9 ± 0.1
Sample 3	5.0 ± 0.1	4.4 ± 0.3	0.8 ± 0.0
(nmol per 10^5 cells)			
L1210 cells	0.18	0.72	0.29
(nmol per ml homogenate)			
Rat livers	1.8 ± 0.6	210 ± 0.00	224 ± 10.0

[^{3}H]dansyl chloride to a known quantity of non-radioactive dansyl chloride in order to establish a specific activity for the stock solution of dansyl chloride with an error of 1–5%.

In the present experiments, polyamines were determined in the nanomol and picomol range (Table II). Theoretically, the limits of detection are much lower and are determined by the specific activities of the undiluted radioactive reagents.

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Book Review

Gradient elution in column liquid chromatography, theory and practice (Journal of Chromatography Library, Vol. 31), by P. Jandera and J. Churáček, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1985, XX + 510 pp., price Dfl. 245.00. US$ 90.75, ISBN 0-444-42124-6.

The above book is devoted to an important field in liquid chromatography — gradient elution. This technique is somewhat analogous to temperature programming in gas chromatography and has evolved into a rigorous scientific discipline during the last ten years. However, relatively few books dealing entirely with gradient elution have been published. Thus, under the principle that "nature abhors the vacuum", this book fills a very important niche ignored by most other publications dealing with chromatography. It should be noted that only topics dealing with theory, practice, and application of the gradient elution technique are covered, and therefore some preliminary knowledge of chromatographic techniques, particularly liquid chromatography, is required.

The book is logically divided into four parts, with Part I devoted entirely to the fundamentals of isocratic development. The authors discuss the role of the mobile phase composition on solute retention and solute/solvent and solute/stationary phase interactions. Retention models of Snyder, Soczewinski, Scott, and Kucera are described here in great detail. This part is a well-written authoritative review of the literature covering the time period from 1962–1982. However, the problems of prediction of resolution and optimization of chromatographic separations are dealt with only marginally; this part could have been expanded to include recent optimization strategies.

Part II concentrates mainly on theoretical aspects of gradient elution chromatography. Various models of gradient elution, including different gradient profiles, calculation of retention parameters, and optimization of gradient systems, are discussed. This part, like the first, is put together with great knowledge of many theoretical aspects of the technique and is highly recommended to a serious student or research worker in the field of chromatography.

The final two parts cover some practical issues associated with the use of the technique. Part III discusses the instrumental aspects of gradient elution systems, such as various devices generating gradients, pumps, solenoid valves, columns, solvents, etc., and also deals with detection and quantitation issues.

Part IV presents examples of the application of gradient elution techniques to separation of different classes of compounds in various chromatographic systems. In a project of this nature, it is usually rather difficult to keep up-to-date on instrumentation and applications because of the rapid changes and developments in these areas. Thus, by necessity, some of the instruments described in the book have been discontinued or are no longer available, and new systems have appeared which are not covered in these discussions.

On the whole, the book is well-produced with many useful diagrams, graphs, tables, and chromatograms. The references cover the literature up to 1982. This book is highly recommended to graduate students, research workers, and anyone wishing to improve his or her skills in liquid chromatography.

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Book Review

Affinity chromatography: a practical approach, edited by P. D. G. Dean, W. S. Johnson and F. A. Middle, IRL Press, Oxford, Washington, DC, 1985, XVI + 215 pp., price £ 11.00, US$ 20.00, ISBN 0-904147-71-1.

This appears to be the first book in the field of chromatography to be written along the lines of "Organic Syntheses" or "Inorganic Syntheses", in that it gives complete methods of synthesis for affinity chromatography supports and is also written by a multitude of authors, twenty-five to be exact.

It is divided into eight main chapters. The first deals with matrix preparations and applications and makes interesting reading. Besides the usual matrices it also mentions Eupergit C and Kimal, both not familiar to the reviewer. Table 3 lists the commercial sources of supports. The coverage seems adequate, but unfortunately no addresses of the firms are given.

Chapter 2 describes activation procedures, including even one for cellulose paper.

In the next chapter on "Cross-linking agents for coupling matrices to spacers", a useful table lists the bifunctional compounds. The various methods also draw attention to the possible hazards in the preparation methods. A wise precaution, as many biochemists nowadays are not experienced in preparative organic chemistry.

Chapter 4 is entitled "Operational Methodologies". The exact meaning of this compound term still escapes me, but the subsections may enlighten the reader, namely: Determination of ligand concentration, Ligand leakage, Ligand concentration effects, Effect of column dimensions, pH and temperature effects, Ionic strength, Desorption methods, Use of cylindrical baffles in large scale affinity columns, Design and use of high performance affinity chromatography minicolumns.

Ligands for immobilisation is the topic of Chapter 5 and covers the topic adequately, but of course not completely in 48 pages and 74 references. Chapters 6 and 7 survey quantitative and analytical aspects.

The book concludes with a chapter on cell separation via cleavable mercury–sulphur bonds. There is a subject but no author index. In addition to providing methods the book is also a welcome, if incomplete, reference work. It should be on the shelf of every chromatographer.

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Errata

J. Chromatogr., 329 (1985) 428–433

Page 433, in Table I, last column, the fourth value from the bottom: "1.85" should read "5.12".

J. Chromatogr., 350 (1985) 237–244

Page 237, last line: "as opposed to the older techniques" should read "as opposed to the other techniques".

corrected
ch. S.
12 Apr. 86

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journal of chromatography news section

NEW BOOKS

Techniques for the optimization of HPLC separations, by J.C. Berridge, Wiley, Chichester, New York, 1985, XII + 203 pp., price £22.00, ISBN 0-471-90861-4.

Méthodes chromatographiques couplées à la spectrométrie de masse, by J. de Graeve, F. Berthou and M. Prost, Masson, Paris, New York, Barcelona, 1986, X + 381 pp., price FF 280.00, ISBN 2-225-80627-6.

The practice of quantitative gel electrophoresis, by A. Chrambach, Verlag Chemie, Weinheim, 1985, XVI + 265 pp., price DM 110.00, US$ 43.60, ISBN 3-537-26039-0.

Mass spectrometry in the health and life sciences (*Analytical Chemistry Symposia Series*, Vol. 24), edited by A.L. Burlingame and N. Castagnoli, Jr., Elsevier, Amsterdam, Oxford, New York, Tokyo, 1985, XX + 638 pp., price US$ 133.25 (U.S.A. and Canada), Dfl. 360.00 (rest of world), ISBN 0-444-42562-4.

Characterization of spilled oil samples. Purpose, sampling, analysis and interpretation, edited by J.A. Butt, D.F. Duckworth and S.G. Perry, Wiley, Chichester, New York, 1986, X + 95 pp., price £17.50, ISBN 0-471-90890-8.

Nitrated polycyclic aromatic hydrocarbons, edited by C.M. White, Hüthig, Heidelberg, Basel, New York, 1985, XII + 376 pp., price DM 128.00, US$ 64.00, ISBN 3-7785-1029-0.

OBITUARY

Professor Wiktor Kemula, the Honorary Chairman of the Scientific Council of the Institute, member of the Polish Academy of Sciences, doctor honoris causa of the Warsaw University, suddenly died on October 17th, 1985. He won the respect and admiration for his very outstanding, over 60 years scientific activity.

He served as President of the Polish Chemical Society and, recently, as the honorary President. He was also Honorary Member of several foreign scientific societies, incl. Deutsche Akademie der Naturforscher "Leopoldina", the New York Academy of Sciences, Societé de la Chimie Industrielle (Paris), Japánese Society of Analytical Chemistry, Czechoslovak Chemical Society, Royal Society of Chemistry (London). He served also as a National Representative to the Analytical Division of I.U.P.A.C. and the President of the Division in 1969–1972. The results of his scientific research, published in over 400 original papers, have been widely recognized throughout the scientific world.

Professor Kemula was a co-founder of the Institute of Physical Chemistry of the Polish Academy of Sciences. Until the last day he was actively engaged in organization and scientific research in the Institute.

ANNOUNCEMENTS OF MEETINGS

CHROMEXPO-1986, ROCKVILLE, MD, U.S.A., APRIL 10, 1986

CHROMEXPO-1986 will feature exhibits of the newest instrumentation, and poster sessions of the latest scientific developments in chromatographic separations. The exhibition will be held at the Crowne Plaza Hotel, 1750 Rockville Pike, Rockville, MD, U.S.A. It will be sponsored by the Washington Chromatography Discussion Group. Requests for booth space and Abstracts of posters should arrive before January 14th, 1986.

For further information contact: Barr Enterprises, CHROMEXPO-1986, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898 3772.

1st SYMPOSIUM ON PATTERN RECOGNITION METHODS IN ANALYTICAL SPECTROSCOPY, SALT LAKE CITY, UT, U.S.A., JUNE 16–18, 1986

The goal of the meeting is to provide a direct interface between the fields of analytical spectroscopy and chemometrics.

The following topics fall within the scope of the programme: Spectral Enhancement and Deconvolution, Data Reduction and Compression, Library Storage and Retrieval, Cluster and Classification Analysis, Factor and Discriminant Analysis, Multicomponent ("mixture") Analysis, Automated Spectral Interpretation, Multisource Data Base Integration, Modeling and Prediction. Keynote lectures will be presented by: J. Thomas Clerc, Peter R. Griffiths, Stephen R. Heller, Peter C. Jurs, Hal J.H. MacFie, Edmund R. Malinowski, Harald Martens, Abraham Savitzky, Charles L. Wilkins, Willem Windig, Carla Wong and Hugh B. Woodruff.

The three-day schedule is patterned after the well-known Gordon Research Conference format with morning and evening lecture sessions and afternoons available for social interactions or recreational activities. Each morning or evening session features only two keynote speakers with ample time for discussions. The official conference language is English.

All contributed papers will be presented in poster form. Posters will remain displayed during the entire conference.

Exhibition space will be available for companies interested in demonstrating relevant software or hardware products.

The symposium will be held at the Snowbird Resort at the foot of Hidden Peak Mountain (11 000 ft.) in beautiful Little Cottonwood Canyon, only 31 miles from Salt Lake City International Airport. Transportation to and from the airport will be arranged. A broad choice of individual afternoon activities include hiking, sightseeing, cable tram rides, swimming, exercising, tennis and volleyball.

Further information may be obtained from: Melinda Van, Conference Secretariat Biomaterials Profiling Center, University of Utah, Salt Lake City, UT, U.S.A. Tel.: (801) 581-8431, telex: GRAPHNET 3789459 UNIV UTAH SLC

INTERNATIONAL CONFERENCE ON DEVELOPMENTS IN ANALYTICAL METHODS AND PHARMACEUTICAL, BIOMEDICAL AND FORENSIC SCIENCES, VERONA, ITALY, JUNE 25–27, 1986

The University of Verona, together with the Italian Group for Mass Spectrometry in Biochemistry and Medicine, is organizing an international conference on Developments in Analytical Methods in Pharmaceutical, Biomedical and Forensic Sciences in Verona, Italy, Wednesday 25th–Friday 27th June, 1986. Lectures and communications will be held at the Medical Faculty of the University of Verona, in the Main Lecture Hall.

The conference will illustrate and discuss the latest aspects of chromatographic, spectroscopic, immunological methods applied to: drug research, clinical biochemistry, forensic medicine, pharmacology and toxicology, nutrition science, pollution, and improvements in the methodology. The conference will consist of lectures by invited speakers, oral communications, posters.

For those registering before April 30, 1986, the registration fee is US $130 (for Italian participants: Lit. 200.000). After this date, the registration fee will be: US $170 (for Italian participants: Lit. 250.000).

For further information please contact the secretariat of the conference: Dr. Alberto Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, via Eustachi 36, 20129 Milan, Italy.

2nd WORLD CONGRESS ON COMPOUNDS IN BIOLOGICAL AND CHEMICAL WARFARE: TOXICOLOGICAL EVALUATION – INDUSTRIAL CHEMICAL DISASTERS, GHENT, BELGIUM AUGUST 24–26, 1986

The above-mentioned symposium will be held at the State University of Ghent, Department of Toxicology, Ghent, Belgium, August 24–26, 1986. The Chairman will be Professor A. Heyndrickx.

The scientific programme will cover the following topics. Human and medical factors: new evidence; effects of chemicals, the methods of detection, mortality and morbidity rates, environmental problems, defensive needs, protection. Diagnosis, treatment, influence: methods of diagnosis, assignment to treatment modalities. Action programme: international legislation and rules, enforcement, sanctioning strategies, interaction between public information and enforcement; evidence of chemical warfare in SE Asia and Afghanistan. Industrial chemical disasters: flow sheet and toxicity of basic compounds, intermediates and toxic waste; organisation of warning systems; medical treatment and disaster plans; control systems in industry; ecotoxicology.

All information can be obtained from: Professor A. Heyndrickx, Head of the Department of Toxicology, State University of Ghent, Hospitaalstraat 13, B-9000 Ghent, Belgium. Tel.: (32) 91 25 10 21, telex: 11.558 A.Z. Gent – Toxicology.

5th INTERNATIONAL SYMPOSIUM ON ISOTACHOPHORESIS (ITP '86), MAASTRICHT, THE NETHERLANDS, SEPTEMBER 3–5, 1986

The 5th International Symposium on Isotachophoresis will be held September 3–5, 1986 in Maastricht, The Netherlands. The subtitle of the 1986 symposium is "New Applications in Biomedical, Pharmaceutical and Chemical Analysis". This title reflects the fields in which most progress has been made since the last symposium on isotachophoresis in 1984 in Hradec Králové, Czechoslovakia.

An analysis of literature reports over the last two years has shown that especially in biomedical research and in pharmaceutical and chemical analysis isotachophoresis is used more and more. All those who are interested in working with this electrophoretic technique, are most cordially invited to attend the 1986 symposium.

Who should attend ITP '86? analytical chemists; biochemists; biomedical researchers, especially in the field of body fluid analysis; clinical chemists; pharmacologists and pharmacists; specialists from food inspection departments; control authorities; industrial developers of equipment; toxicologists; environmental chemists; analytical biotechnologists.

The local organizing committee consists of Th. P.E.M. Verheggen, Prof. Dr. Ir. F.M. Everaerts and Prof. Dr. C.H.M.M. De Bruijn.

The organizers hope that many colleagues, active in the fields mentioned above, will come to Maastricht from 3 to 5 September 1986, and that they will present their recent work. In addition it is hoped that fruitful discussions will lead to new and stimulating contacts.

For further details please contact the Secretariat of ITP '86, c/o Th. Verheggen and F.M. Everaerts, Department of Chemical Engineering, Laboratory of Instrumental Analysis, Eindhoven University of Technology, 5600 MB Eindhoven, The Netherlands.

CHROMATOGRAPHY '86, INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY, HOUSTON, TX, U.S.A. SEPTEMBER 15–18, 1986

The 22nd International Symposium on Advances in Chromatography will be held September 15–18, 1986 at the Hotel Inter-Continental in Houston, TX, U.S.A. The scope of the meeting will cover papers and informal discussion groups by outstanding researchers from throughout the world in

all fields of chromatography. There will also be a commercial exhibition of the latest instrumentation and books. Participation in the symposium will be on the basis of invited papers as well as unsolicited contributions. Authors desiring to present papers must submit a 200-word abstract by March 15, 1986. Complete manuscripts of accepted authors will be due on September 15 at the meeting in Houston.

All correspondence pertaining to the symposium and exhibition space should be directed to: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, Tel. (713)-749-2623. The Gulf Coast Analytical Conference will be held at the same location later in the week.

FACSS XII, FEDERATION OF ANALYTICAL CHEMISTRY AND SPECTROSCOPY SOCIETIES 1986 MEETING, ST. LOUIS, MO, U.S.A., SEPTEMBER 29–OCTOBER 3, 1986

The Federation of Analytical Chemistry and Spectroscopy Societies, FACSS, will hold their 1986 meeting from September 29 until October 3 at the Cervantes Convention Center in historic and newly refurbished downtown St. Louis, MO, U.S.A. Researchers who would like to make oral or poster presentations of original work should send their title, author(s), address, and phone no. to the program manager, Dr. Sydney Fleming, by March 28, 1986. A 250-word abstract will be due by June 1, 1986.

Workshops and short courses will again be offered prior to, during, and after the conference. The FACSS Employment Bureau will again be available to conference attendees. Centrally located at the meeting will be an exhibition of scientific instrumentation, services, and publications. For information on exhibiting at the 1986 FACSS meeting contact the Exhibit Director, Dr. Edward G. Brame Jr.

The contact addresses are: Dr. Sydney Fleming, Program Manager, FACSS (Titles), 24 Crestfield Rd., Wilmington, DE 19810, U.S.A. Tel.: (302) 772-4572 and Dr. Edward G. Brame Jr., FACSS Exhibit Director, 13 North Cliffe Dr., Wilmington, DE 19089-1623, U.S.A. Tel.: (215) 798-5161.

3rd SYMPOSIUM ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY, PALMA DE MALLORCA, SPAIN, OCTOBER 8–10, 1986

The organisation of this third event – the first was held in Lausanne, Switzerland in November 1983, the second in Freiburg, F.R.G., in October 1985 – is in hands of the International Association of Environmental Analytical Chemistry and the University of Palma de Mallorca and sponsored by national bodies. A strong industrial participation is planned. It is the intention to bring together specialists in this field who can give a good account of the state-of-art in their respective specialty and to present first-hand experience in sample handling. Robotics, continuous flow extraction techniques, solid surface sample handling with pre-column technology (on-line and off-line) pre-chromatographic use of derivatization techniques, colum switching methodology for handling of complex samples are some of the topics that will be treated and extensively discussed. Special emphasis will be placed on techniques with automation potential and actually automated procedures suitable for routine handling of larger series of samples. Much of this methodology and "philosophy" can be applied to different types of matrices and problem solving but it is the intention to concentrate on applications to biological (urine, blood, tissue, plant material) and environmental samples (water, waste water, air) with drugs (pharmaceuticals) and priority pollutants as the analytes.

The coordinators will be: Prof. Dr. V. Cerdá, Department of Analytical Chemistry, University of Palma de Mallorca, Spain, and Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, Amsterdam, The Netherlands.

For further information and for submission of contributions contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5485379.

6th INTERNATIONAL SYMPOSIUM ON HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS, PEPTIDES AND POLYNUCLEOTIDES, BADEN-BADEN, F.R.G., OCTOBER 20–22, 1986

In the fast developing field of high-performance liquid chromatography and other high resolution techniques, the International Symposium on High-performance Liquid Chromatography of Proteins Peptides and Polynucleotides has become the established, major scientific meeting devoted to the

latest advances in experimental procedures and concepts in this dynamic discipline. The 6th scientific meeting will take place October 20–22, 1986 in Baden-Baden, F.R.G.

Baden-Baden, conveniently located in the heart of the Black Forest, has been chosen as the site for the 6th International Symposium. The relaxed atmosphere and pleasant surroundings of this famous Spa, together with excellent congress facilities, will guarantee a successful symposium.

Oral presentations and poster sessions will be organized into various topics, including: protein conformation and chromatographic behaviour; column techniques and support materials; peptide mapping and microsequencing; polynucleotides; membrane proteins; preparative chromatography; analytical applications; special topics, *e.g.* high resolution electrophoresis, clinical applications.

If you wish to present original research results in any of the above areas, you are kindly requested to submit an abstract by June 15, 1986. The Scientific Committee welcomes your suggestions for additional topics to be covered in the symposium.

Abstract forms, registration information and full details of the symposium can be obtained from: Secretariat, 6th ISPPP, P.O. Box 3980, D-6500 Mainz, F.R.G. Participants of the 5th International Symposium on high-performance liquid chromatography in Toronto will receive details automatically.

4th SYMPOSIUM ON LC–MS AND MS–MS, MONTREUX, SWITZERLAND, OCTOBER 22–24, 1986

This symposium will feature invited and contributed lectures and posters on the latest developments in this field. Fundamental, instrumental and application papers are considered. Panel discussions will deal with various segments of LC–MS, SFC–MS and MS–MS coupling.

An instrument exhibition is planned and proceedings of the workshop will be published as a special issue in the *Journal of Chromatography*. The symposium is preceeded by a 2-day short course (lecturers Dr. Dai Games and Dr. Jack Henion).

For further information and for submission of contributions contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5485379.

2nd INTERNATIONAL SYMPOSIUM ON QUANTITATIVE LUMINESCENCE SPECTROMETRY IN BIOMEDICAL SCIENCES, MAY 11–14, 1987, GHENT, BELGIUM

An international symposium on quantitative luminescence spectrometry in biomedical sciences, sponsored by the Faculty of Pharmaceutical Sciences of the State University of Ghent, the National Foundation of Scientific Research (N.F.W.O. - F.N.R.S.), and the Ministry of Education, will be held in Ghent, Belgium, from Monday, 11th May to Thursday, 14th May 1987, at the Farmaceutisch Instituut. The scope of the symposium will be similar to that of the first meeting held in Ghent, 1984, and will cover current research on all aspects of luminescence analysis.

The scientific programme will consist of plenary and invited lectures, submitted research papers and posters.

Five plenary lectures will be presented by outstanding specialists in the field of quantitative luminescence spectrometry: U.A. Brinkman (Free University of Amsterdam), luminescence detection in high-performance liquid chromatography; J.C. Hummelen (State University of Groningen), thermochemiluminescence techniques; L.J. Kricka (Queen Elizabeth Medical Centre, Birmingham), chemiluminescence immunoassays; S.G. Schulman (University of Florida), fluorescence techniques; O.S. Wolfbeis (Karl-Franzens University, Graz), fiber optic sensors.

Contributed papers (20 min lectures or poster communications) will cover the following topics: drug and bioanalysis via fluorescence and phosphorescence; fluorescence and chemiluminescence immunoassays; detection techniques in chromatography; solid surface luminescence methods; thermochemiluminescence techniques; chemical derivatization methods; use of fluorescence labels; luminescence applications and drug metabolism, clinical chemistry, biochemistry, pharmacokinetics, toxicology, ecology, protein tagging.

The conference language will be English. There will be an exhibition of commercial instruments for luminescence analysis.

As with the earlier symposium, the Proceedings will be published in a special issue of *Analytica Chimica Acta*. Information for authors will be provided in later announcements.

Authors who wish to present papers (oral or poster) should submit abstracts before December 31, 1986.

The symposium registration fee will be about 100 U.S.$ or 5500 BF. This fee includes a copy of the final programme, an abstracts volume; a copy of the Proceedings; part of the social events and refreshments during the breaks. Wagons-Lits Tours, Cantersteen 41, B-1000 Brussels has been appointed the Official Travel Agent.

All correspondence should be sent to: Dr. W. Baeyens, Symposium Chairman, Laboratory of Pharmaceutical Chemistry and Drug Analysis, State University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

AWARD

1986 DAL NOGARE AWARD

Professor Gerhard Schomburg will receive the 1986 Dal Nogare Award for his outstanding contributions in the fields of gas and liquid chromatography. The award is sponsored by the Chromatography Forum of the Delaware Valley and will be presented on 11 March, 1986 at the Pittsburgh Conference in Atlantic City, NJ, U.S.A.

Dr. Schomburg was born in Bochum, Nordrhein-Westfalen, F.R.G., in 1929, and received his doctorate degree from the Technical University of Aachen in 1965 where his thesis work was in IR spectroscopy. He remained at this institute to organize and develop a department for gas chromatography which later was expanded to include liquid chromatography. He also developed a laboratory at the Max-Planck-Institut für Strahlenchemie for radiation and photochemistry of biological and non-biological material.

He is the author or co-author of over 150 scientific publications, the author of a textbook of chromatography, and an editor of *Chromatographia*. He has been the recipient of several awards presented at international chromatography symposia.

Dr. Schomburg's work in gas chromatography has included the use of high-efficiency columns and cold injection sampling techniques, and automated multi-dimensional analysis. In gas chromatography–mass spectrometry he helped to develop the open-split interface technique. In liquid chromatography his work has dealt with metal complexation and synthesis of bonded high-performance liquid chromatographic phases.

At the present time he is teaching at the University of Wuppertal and the University of Bochum, and is Chairman of the Board of the Chromatography Group of the Gesellschaft Deutscher Chemiker.

CALENDAR OF FORTHCOMING MEETINGS

March 25–June 6, 1986 Uppsala, Sweden	**Biochemical Separation Methods, Uppsala Separation School** Contact: Ingela Strömberg, Institute of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden. (Further details published in Vol. 331, No. 2.)
April 10, 1986 Rockville, MD, U.S.A.	**CHROMEXPO-1986** Contact: Barr Enterprises, CHROMEXPO-1986, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898 3772.
April 13–18, 1986 New York, NY, U.S.A.	**Advances in Size Exclusion Chromatography** Contact: Theodore Provder, Ph. D., Principal Scientist, Polymer Research & Computer Science, Glidden Coatings and Resins, Division of SCM Corporation, 16651 Sprague Road, Strongsville, OH 44136, U.S.A. Tel.: (216) 826-5289, or Dr. C. Kuo, tel.: (216) 826-5346. (Further details published in Vol. 347, No. 3.)

April 14–16, 1986 Deerfield Beach, FL, U.S.A.	**The Liquid Chromatography–Biotechnology Interface** Contact: Kay Lioy, Division of Continuing Education, Florida Atlantic University, Boca Raton, FL 33431, U.S.A. Tel.: (305) 393-3099. (Further details published in Vol. 350, No. 2.)
April 22–24, 1986 Noordwijkerhout The Netherlands	**Anatech '86 – An International Symposium on Applications of Analytical Chemical Techniques to Industrial Process Control** Contact: Prof. W.E. van der Linden, Laboratory for Chemical Analysis, Department of Chemical Technology, Twente University of Technology, P.O. Box 217, 7500 AE Enschede, The Netherlands. (Further details published in Vol. 322, No. 3.)
April 23–25, 1986 Vienna, Austria	**Liquid Chromatography in Gene Technology** Contact: Mrs. Romana Zeilinger, Hewlett-Packard GmbH, Lieblgasse 1, Postfach 72, A-1222 Vienna, Austria. (Further details published in Vol. 330, No. 2.)
May 1–3, 1986 Brussels, Belgium	**XXXIVth Colloquium, Protides of the Biological Fluids** Contact: Colloquium "Protides of the Biological Fluids" Secretariate, Institute for Medical Biology, Alsembergsesteenweg 196, Chaussée d'Alsemberg, B-1180 Brussels, Belgium. Tel.: (02) 348 05 11, telex: CDHBRU 26501.
May 11–14, 1986 Nagara, Japan	**7th International Symposium on Capillary Chromatography** Contact: Dr. D. Ishii, Department of Applied Chemistry. Nagoya University, Nagoya 464, Japan. (Further details published in Vol. 331, No. 2.)
May 12–14, 1986 Minneapolis, MN, U.S.A.	**Minnesota Chromatography Forum, A Focus on Macromolecular Separation** Contact: Meeting Management, 1421 East Wayzata Boulevard, Suite 50, Wayzata, MN 55391, U.S.A. Tel.: (612) 473 0318.
May 12–14, 1986 Washington, DC, U.S.A.	**2nd Symposium on Preparative Liquid Chromatography** Contact: Barr Enterprises, P.O. Box 279, Walkersville, MD 21793, U.S.A. Tel.: (301) 898-3772. (Further details published in Vol. 330, No. 2.)
May 18–23, 1986 San Francisco, CA, U.S.A.	**HPLC '86. New Frontiers in HPLC. 10th International Symposium on Column Liquid Chromatography** Contact: Ms. Shirley Schlessinger, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A. (Further details published in Vol. 331, No. 2.)
May 26–29, 1986 Lerici, Italy	**III CAC – Meeting of the Chemometrics Society** Contact: Prof. M. Forina, Istituto di Analisi e Tecnologie Farmaceutiche ed Alimentari, Via Brigata Salerno (ponte), I-16147 Genova, Italy. Tel.: (010) 3993656. (Further details published in Vol. 330, No. 2.)
June 3–6, 1986 Munich, F.R.G.	**Analytica 86, 10th International Trade Exhibition and 10th International Conference 'Biochemical Analytics'** Contact: Dr. Rosemarie Vogel, Nymphenburgerstrasse 70, D-8000 München 2, F.R.G. (Further details published in Vol. 330, No. 2.)
June 23–27, 1986 Harrogate, U.K.	**1986 Hewlett-Packard Analytical Symposium** Contact: Tina Mears, Hewlett-Packard Ltd., Analytical Instrumentation Group, Miller House, The Ring, Berkshire, RG12 1XN, U.K. Tel.: Bracknell (0344) 424898, telex 848733. (Further details published in Vol. 347, No. 3.)

June 25–27, 1986 Verona, Italy	**International Conference on Developments in Analytical Methods in Pharmaceutical, Biomedical and Forensic Sciences** Contact: Dr. Alberto Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, via Eustachi 36, 20129 Milan, Italy.
July 20–26, 1986 Bristol, U.K.	**SAC 86 – International Conference and Exhibition on Analytical Chemistry** Contact: Miss P.E. Hutchinson, Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, U.K. Tel.: (01) 734-9971. (Further details published in Vol. 299, No. 1.)
Aug. 24–26, 1986 Ghent, Belgium	**2nd World Congress on Compounds in Biological and Chemical Warfare: Toxicological Evaluation – Industrial Chemical Disasters** Contact: Professor A. Heyndrickx, Head of the Department of Toxicology, State University of Ghent, Hospitaalstraat 13, B-9000 Ghent, Belgium. Tel.: (32) 91 25 10 21, telex: 11.558 A.Z. Ghent-Toxicology.
Aug. 25–29, 1986 Antwerp, Belgium	**10th International Symposium on Microchemical Techniques** Contact: Dr. R. Dewolfs, University of Antwerp, Department of Chemistry, Universiteitsplein 1, B-2610 Wilrijk, Belgium. Tel.: 03/828.25.28 (ext. 204), Telex: 33646.
Aug. 27–30, 1986 Brussels, Belgium	**12th International Congress of the European Association of Poison Control Centres (EAPCC)** Contact: Administrative Secretariat, Mrs. D. Shanni, SDR Associated, Rue Vilain XIIII, 17a, B-1050 Brussels, Belgium. Tel.: (02) 647 87 80. Telex: 61434 SDRBRU B.
Aug. 27–30, 1986 Brussels, Belgium	**3rd World Congress of the World Federation of Associations of Clinical Toxicology and Poison Control** Contact: Administrative Secretariat, Mrs. D Shanni, SDR Associated, Rue Vilain XIIII, 17a, B-1050 Brussels, Belgium. Tel.: (02) 647 87 80. Telex: 61434 SDRBRU B.
Aug. 31–Sept. 3, 1986 Ghent, Belgium	**6th International Symposium on Mass Spectrometry in Life Sciences** Contact: Professor Dr. A. De Leenheer, Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: (091) 218951, ext. 324. (Further details published in Vol. 350, No. 2.)
Sept. 4–8, 1986 Szeged, Hungary	**A Symposium on New Advances in Liquid Chromatography** Contact: Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, Budapest 1445, Hungary. (Further details published in Vol. 347, No. 3.)
Sept. 8–10, 1986 Freiburg, F.R.G.	**4th International Symposium on Bioluminescence and Chemiluminescence** Contact: Dr. J. Schölmerich, Medizinische Universitätsklinik, D-7800 Freiburg, F.R.G.
Sept. 9–12, 1986 London, U.K.	**5th Meeting of the International Electrophoresis Society, "Electrophoresis '86"** Contact: Dr. M.J. Dunn, Muscle Research Unit, Royal Postgraduate Medical School, DuCane Road, London W12 0HS, U.K. Tel.: 01-743-2030 ext. 338.
Sept. 15–18, 1986 Houston, TX, U.S.A.	**22nd International Symposium on Advances in Chromatography** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, 77004 TX, U.S.A. Tel.: (713) 749 2623.

Sept. 21–26, 1986 Paris, France	**16th International Symposium on Chromatography** Contact: GAMS, 88 bd Malesherbes, 75008 Paris, France. Tel.: (1) 563-9304. (Further details published in Vol. 331, No. 2).
Sept. 29–Oct. 3, 1986 St. Louis, MO, U.S.A.	**FACSS '86, Federation of Analytical Chemistry and Spectroscopy Societies 1986 Meeting** Contact: Dr. Marshall Fishman, U.S. Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, PA 19118, U.S.A. Tel.: (215) 233-6450.
Oct. 7–9, 1986 Tokyo, Japan	**23nd International Symposium Advances in Chromatography** Contact: Prof. N. Ikekawa, Department of Chemistry, Tokyo Institute of Technology, Ohokayama, Meguro-ku, Tokyo 152, Japan; or Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 347, No. 3.)
Oct. 8–10, 1986 Palma de Mallorca, Spain	**3rd Symposium on Handling of Environmental and Biological Samples in Chromatography** Contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5485379.
Oct. 12–16, 1986 Washington, DC, U.S.A.	**100th Annual AOAC International Meeting** Contact: Margaret Ridgell, AOAC, 1111 North 19th Street, Suite 210, Arlington, VA 22209, U.S.A. Tel.: (703) 522-3032.
Oct. 16–17, 1986 Stockholm, Sweden	**International Symposium on Progress in Capillary Chromatography** Contact: The Analytical Section, The Swedish Chemical Society, Upplandsgaten 6A, S-111 23 Stockholm, Sweden.
Oct. 20–22, 1986 Baden-Baden, F.R.G.	**6th International Symposium on High-Performance Liquid Chromatography of Proteins, Peptides and Polynucleotides** Contact: Secretariat 6th ISPPP, P.O. Box 3980, D-6500 Mainz, F.R.G.
Oct. 20–24, 1986 New York, NY, U.S.A.	**The Silver Jubilee Eastern Analytical Symposium** Contact: Dr. S. David Klein, EAS Publicity, 642 Cranbury Cross Road, North Brunswick, NJ 08902, U.S.A. Tel.: (201) 846-1582. (Further details published in Vol. 347, No. 3.)
Oct. 22–24, 1986 Montreux, Switzerland	**4th Symposium on Liquid Chromatography–Mass Spectrometry and Mass Spectrometry–Mass Spectrometry** Contact: Professor Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5485379.
April 28–May 1, 1987 Sydney, Australia	**9th Australian Symposium on Analytical Chemistry** Contact: The Secretary 9AC, Mr. John Eames, P.O. Box 137, North Ryde, N.S.W. 2133, Australia. Tel.: (02) 887-8688. (Further details published in Vol. 350, No. 2.)
May 11–14, 1987 Ghent, Belgium	**2nd International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences** Contact: Dr. W. Baeyens, State University of Ghent, Laboratory of Pharmaceutical Chemistry and Drug Quality Control, Harelbekestraat 72, B-9000 Ghent, Belgium.

PUBLICATION SCHEDULE FOR 1986

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

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 329, No. 3, pp. 449–452. A free reprint can be obtained by application to the publisher.)

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

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June 21–26, 1987 Toronto, Canada	**XXV Colloquium Spectroscopium Internationale** Contact: Mr. L. Forget, Executive Secretary CSI XXV, National Research Council of Canada, Ottawa, K1A 0R6 Canada. Tel.: (613) 993-9009, telex: 053-3145. (Further details published in Vol. 330, No. 2.)
June 28–July 4, 1987 Amsterdam, The Netherlands	**HPLC '87, 11th International Symposium on Column Liquid Chromatography** Contact: Organisatie Bureau Amsterdam bv, Europaplein, 1078 GZ Amsterdam, The Netherlands. Tel.: (31) 20-440807, telex: 13499 raico nl. (Further details published in Vol. 331, No. 2.)
August 17–21, 1987 Oberammergau, F.R.G.	**7th International Symposium on Affinity Chromatography and Interfacial Macromolecular Interactions** Contact: Prof. Dr. H.P. Jennissen, Institut für Physiologie, Physiologische Chemie und Ernährungsphysiologie, Universität München, Veterinärstr. 13, D-8000 München 22, F.R.G.