Journal of Chromatography, 142 (1977) 213–232

CHROM. 10,350

EXAMINATION OF FIVE COMMERCIALLY AVAILABLE LIQUID CHROMATOGRAPHIC REVERSED PHASES (INCLUDING THE NATURE OF THE SOLUTE–SOLVENT–STATIONARY PHASE INTERACTIONS ASSOCIATED WITH THEM)

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SUMMARY

Five commercially available reversed phases are examined, and the effects of chain length, carbon content and extent of derivatization on wettability, retention and selectivity are investigated. The nature of the stationary phase surface, when in contact with the mobile phase under conditions of wetting, is examined and the mechanism of solute–stationary phase interaction identified. The location of the ion-pair reagent, when the reversed phase is used for ion-pair chromatography, is identified for both lyophilic and lyophobic conditions of chromatographic development.

INTRODUCTION

The bonded phases were originally introduced for use in liquid chromatography to obviate the limitations of silica gel when used to separate mixtures of highly polar and ionic substances. Silica gel separates substances largely on a basis of polarity difference and is highly satisfactory for solutes having low or intermediate polarities. Relatively poor selectivity is frequently realized, however, when the polarities of the solutes approach that of the silica gel itself, and the poor selectivity is often accompanied by poor mass transfer characteristics, producing low efficiencies and peak tailing. Experience with the use of bonded phases, however, has demonstrated that, besides providing a substitute for silica gel for the separation of highly polar solutes, they exhibit unique selectivity that can be exploited in many other separation problems. Bonded phases can be prepared by reacting silica gel with an appropriate organic mono-, di- or trichlorosilane, producing a surface coating of organic material that replaces the surface hydroxyl groups as the interacting moieties of the stationary phase. The interacting organic groups can be simple hydrocarbon chains as in the case of a reversed-phase material, a hydrocarbon chain with a terminating polar functional group as in the case of a polar bonded phase, or an ion-exchange moiety as in the case of an ion-exchange bonded phase. Each class of bonded phase contains a number of different types, and in this paper several types of reversed phases are examined. The work is in two parts, the first examines the chromatographic properties of the reversed phases and relates them to the physical and

chemical properties of each respective phase; the second part examines the surface of the reversed phase, determines its nature and its mode of interaction with solutes during chromatographic development. Further, the mechanism of ion-pair chromatography carried out with reversed phases is examined and the part played by the ion-pair reagent with respect to the reversed phase identified.

THE CHROMATOGRAPHIC CHARACTERISTICS OF FIVE DIFFERENT REVERSED PHASES

The bonded phases examined were RP2, RP8 and RP18 supplied by E.M. Labs. (Elmsford, N.Y., U.S.A.) and ODS and ODS2 supplied by Whatman (Clifton, N.J., U.S.A.). All bonded phases were supplied as packing material, but Whatman also provided columns packed with ODS and ODS2 and E.M. Labs. also provided a prepacked column containing RP8. The columns containing RP2 and RP18 were packed in the laboratory, employing the slurry packing technique, using a 25% (v/v) solution of glycerol in methanol as the dispersing solvent. After packing, the columns were equilibrated directly with water–acetonitrile (35:65, v/v).

The apparatus used for testing the columns consisted of a Waters 6000M pump, a Valco high-pressure valve (Model ACV-6-UHPa-N60) and either an LDC UV monitor or an LDC refractometer, depending on the solutes to be examined. The output from the detector was fed directly to a 10-mV potentiometric recorder and was also processed by a Hewlett-Packard HPMX2108 computer with a 32K memory and a HP7900A disc. Data were acquired at a rate of 7.5 data points per second, at a threshold value of 10 μV and an averaging time of 0.5 sec. The mobile phase employed was water–acetonitrile (35:65, v/v) at a flow-rate of 1 ml/min. The solutes used were phenol, benzene, anthraquinone, 2-methylanthraquinone and 2-ethylanthraquinone in conjunction with the UV detector operated at 254 nm. In order to determine the dead volume of each column, 2 μl of water were injected on to the column and monitored by the refractometer detector.

The carbon content of each stationary phase was determined in duplicate by microanalysis, and the average of the duplicates was taken; no pair of duplicates differed by more than 0.3%. The surface area data for the parent silica gels were supplied by the manufacturers, as was the percentage derivatization. The wettability of each bonded phase was taken as the maximum water content that could be incorporated into the particular solvent without losing the ability to completely wet the bonded phase. Three different solvents were employed for each stationary phase, and the maximum water concentration that would maintain wetting was determined as follows. One hundred milligrams of stationary phase were placed in the bottom of a 10-ml graduated cylinder and 2 ml of the solvent added. Water was then added drop by drop with shaking until a light film of the bonded phase was seen to persist on the surface of the liquid. The volume of liquid and the volume of water added were then noted, the percentage (v/v) of water calculated and this value taken as the maximum water concentration to permit wetting. The wetting characteristics of the five bonded phases are given in Table I and the physical, chemical and chromatographic properties are summarized in Tables II and III.

It is seen from Table I that ODS is completely wetted by water, and this illustrates the effect of incomplete coverage of the bonded phase by the hydrocarbon

TABLE I

THE WETTING CHARACTERISTICS OF FIVE REVERSED PHASES

Reversed phase	*Carbon content (%, w/w)*	*Chain lenth of bonded material*	*Maximum water content to permit complete wetting (%, v/v)*		
			Solvent		
			Methanol	*Acetonitrile*	*Isopropanol*
ODS	5.0	18	100	100	100
ODS2	16.9	18	32	64	74
RP2	5.0	2	50	76	84
RP8	12.2	8	50	68	84
RP18	19.8	18	55	58	82

TABLE II

THE CHROMATOGRAPHIC PROPERTIES OF FIVE REVERSED-PHASE COLUMNS

N = number of theoretical plates.

Column	*Supplier*	*Column length (cm)*	*Column I.D. (mm)*	*Surface area of parent silica gel (m^2/g)*	*Carbon chain length*	*Derivatization (%)*	*Dead volume (ml)*	*N*
ODS	Whatman	25	4.6	400	18	—	2.51	2300 ($k' = 1.9$)*
ODS2	Whatman	25	4.6	400	18	—	2.04	5900 ($k' = 3.9$)*
RP18	E.M. Labs.	25	4.6	150	18	67.5	1.92	4363 ($k' = 10.9$)**
RP8	E.M. Labs.	25	4.6	250	8	75.0	2.61	4591 ($k' = 2.9$)*
RP2	E.M. Labs.	25	4.6	350	2	67.5	2.79	4472 ($k' = 1.3$)**

* Packed by supplier.
** Packed in the laboratory.

TABLE III

RETENTION DATA FOR A NUMBER OF SOLUTES SEPARATED ON DIFFERENT REVERSED-PHASE COLUMNS EMPLOYING ACETONITRILE–WATER (65:35, v/v) AS THE MOBILE PHASE

V' = Corrected retention volume; V_0 = dead volume.

Reversed phase	V_0	V' *(ml)*					α $\left(\frac{\textit{2-Ethylantraquinone}}{\textit{Anthraquinone}}\right)$
		Phenol	*Benzene*	*Anthraquinone*	*2-Methylanthraquinone*	*2-Ethylanthraquinone*	
ODS	2.51	0.86	—	3.05	3.82	4.45	1.46
ODS2	2.04	1.37	8.25	10.03	15.39	20.72	2.07
RP2	2.79	1.91	—	3.18	3.50	3.89	1.22
RP8	2.61	1.36	3.88	4.92	6.57	8.75	1.78
RP18	1.92	1.25	5.10	6.82	10.56	14.83	2.17

moiety. The three bonded phases RP2, RP8 and RP18 that have a high percentage of derivatization have similar wetting properties in methanol and isopropanol but significantly different in acetonitrile. It is also interesting to note that the less polar the solvent, the more water can be tolerated to permit complete wetting. The wetting characteristics are not simply related to the carbon content of the bonded phase as RP18 with a carbon content of 19.8% (w/v) can tolerate more water under wetting conditions than the bonded phase ODS2, which has only 16.9% (w/w) carbon. This indicates that the carbon in ODS2 is in a significantly different form than that in RP18 although they both have organic moieties with a chain length of 18 carbon atoms. The wetting characteristics can be extremely important in the practical use of reversed phases. If the water content is increased, to increase selectivity, and the water present exceeds the wetting limit, a significant interfacial resistance to mass transfer effect could be produced, which would severely impair column efficiency. On the other hand, however, employing water concentrations in excess of the wettability has important implications when using a bonded phase for ion-pair chromatography.

The data in Table II permit the physical and chemical properties of the bonded phases to be compared with their respective chromatographic properties. It is seen that the phases RP2, RP8 and RP18 are made from parent silica gels having different surface areas. RP18 with the longest chain length being made from the silica with the smallest surface area and consequently the largest pore size. The selection was an obvious attempt to prevent the bonded material from blocking the pores of the silica, but as RP18 has the smallest dead volume, this approach appears to have been only partly successful. In fact, the dead volume of the ODS2 column is slightly greater than that of the RP18 column, and ODS2 was made from a silica having considerably smaller pore size; however, it is likely that the carbon chains on the ODS2 bonded phase are quite different from that on the RP18. The relationship between carbon content, carbon chain length and retention properties is not simple and has been studied by a number of workers[1–5]. In general the results shown in Table III are in agreement with the data of these workers, in that, the retention of a given solute under constant mobile phase conditions, increases both with carbon content of the reversed phase and chain length. In Fig. 1 chromatograms are shown, run under identical conditions and with the same solutes, on the bonded phases RP2, RP8 and RP18. It is clearly seen that solute retention increases with chain length. In Fig. 2 the retention volume of 2-ethylanthraquinone is plotted against carbon content for all five bonded phases, and it is seen that retention volumes obtained for RP2, RP8 and RP18 all fall on the same straight line. This linear relationship could be fortuitous with only three points involved, but in any event, it indicates that the relationship between retention volume and carbon content for the RP series is different from that for the ODS phases.

In Fig. 3 is shown the separation of the same solute mixture carried out under the same conditions on the three C_{18} columns ODS, ODS2 and RP18. It is seen that there is no correlation between retention and either carbon content or chain length for these three phases. The significant fact shown in Fig. 3 is the exceptionally high retention exhibited by ODS2 which has the same chain length as RP18 and has a significantly lower carbon content. This suggests that ODS bonded phases are of a distinctly different type from that of the RP phases. The RP bonded phases appear

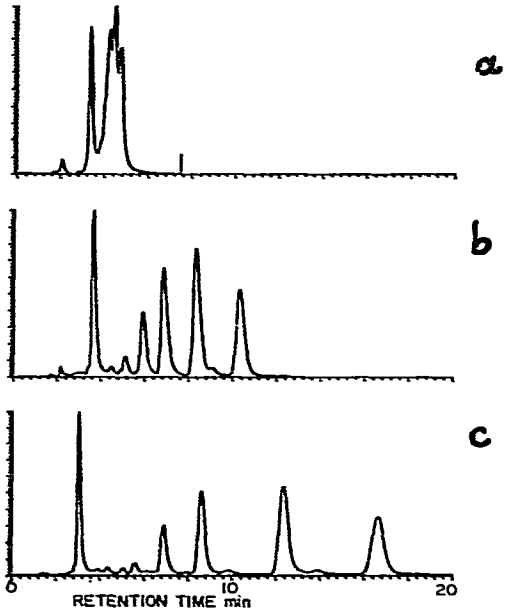

Fig. 1. Chromatograms demonstrating the effect of hydrocarbon chain length on solute retention. Mobile phase: water–acetonitrile (35:65, v/v); solutes: phenol, benzene, anthraquinone, 2-methylanthraquinone and 2-ethylanthraquinone. Carbon chain lengths: a, 2; b, 8; c, 18; carbon content: a, 5.0; b, 12.2; c, 19.18%; OH-groups reacted: 68 (a), 75 (b), 68 (c) %.

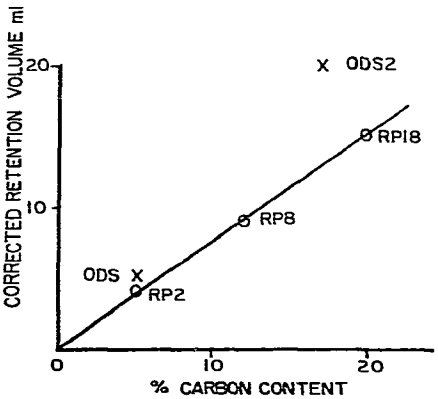

Fig. 2. Graph of corrected retention volume for 2-ethylanthraquinone against carbon content of reversed phase.

to be in the form of single carbon moieties bonded to the surface in "brush" form, as described by Halasz and Sebastian[6]. Such materials are usually prepared from silanes having monofunctional groups such as the monochlorosilanes. The ODS phase, on the other hand, appears to be in polymeric or "bulk-modified" form, as described by Unger *et al.*[7] and often results from the use of the trifunctional silanes, such as trichlorosilanes, in the preparation of the bonded phase. It is also seen from

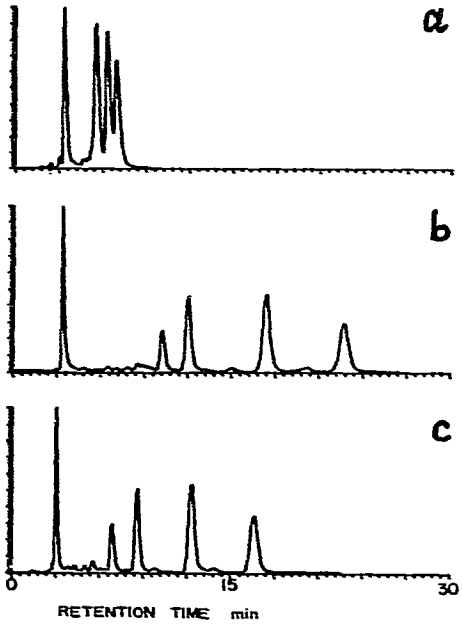

Fig. 3. Chromatograms demonstrating the effect of the carbon content of C_{18} bonded phases on solvent retention. Mobile phase: water–acetonitrile (35:65, v/v); solutes: phenol, benzene, anthraquinone, 2-methylanthraquinone and 2-ethylanthraquinone. (a) ODS, carbon content 5.0%, (b) ODS2, carbon contents 16.9%, (c) RP18, carbon contents 19.8%.

Table II that both RP18 and ODS2 give good selectivity and satisfactory efficiencies and so there seems little to choose between them from a chromatographic point of view.

In Fig. 4 the separation ratios of 2-ethylanthraquinone to anthraquinone are plotted against carbon content for each of the five bonded phases. It is seen that, whereas the corrected retention volumes for all phases did not correlate with carbon

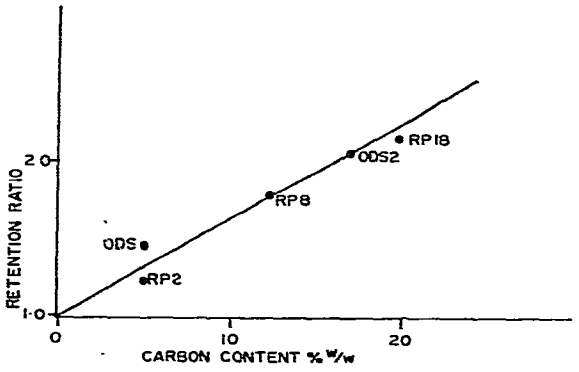

Fig. 4. Graph of retention ratio of 2-ethylanthraquinone to anthraquinone against carbon content of reversed phase.

content, the separation ratios do and this correlation is independent of whether the carbon moiety is monomeric in nature or polymeric. The significance of these two apparently conflicting effects is not clear. It would appear that the character of the interaction was related to carbon content but the extent of interaction was not. Obviously a wider range of reversed phases needs to be examined, having different types of monomeric and polymeric bonded material, before the exact relationship between carbon content, chain length and carbon chain type on retention and selectivity, respectively, can be determined.

The percent derivatization, that is the percentage of the available hydroxyl group on the parent silica gel that have reacted with the silane, was given by the manufacturer for RP2, RP8 and RP18 but not for ODS and ODS2. Free, unreacted hydroxyl groups on the bonded phase give it polar characteristics, and thus a measurement of the polarity of the bonded phase would be directly related to the extent of derivatization. The polarity of a bonded phase can readily be determined by using it in the forward-phase mode in a similar way to silica gel. The three C_{18} columns, RP18, ODS and ODS2 were equilibrated with *n*-heptane by passing six dead volumes of tetrahydrofuran, ethyl acetate, dichloroethane and *n*-heptane sequentially through each column. A mixture of benzene and nitrobenzene was injected into each column at a flow-rate of 1 ml/min and the chromatograms obtained are shown in Fig. 5. It is seen that nitrobenzene is retained strongly on ODS and retained to a lesser extent on ODS2. Significant retention is also shown by the column packed with RP18,

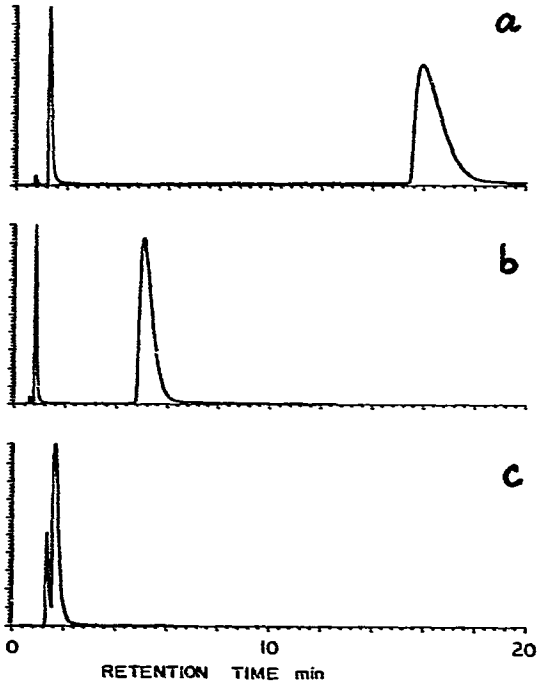

Fig. 5. Forward phase chromatograms from incompletely derivatized reversed-phase columns. Solutes: benzene, nitrobenzene; mobile phase: heptane. (a) ODS, C_{18}, 5.0% carbon, (b) ODS2, C_{18}, 16.9% carbon, (c) RP18, C_{18}, 19.8% carbon.

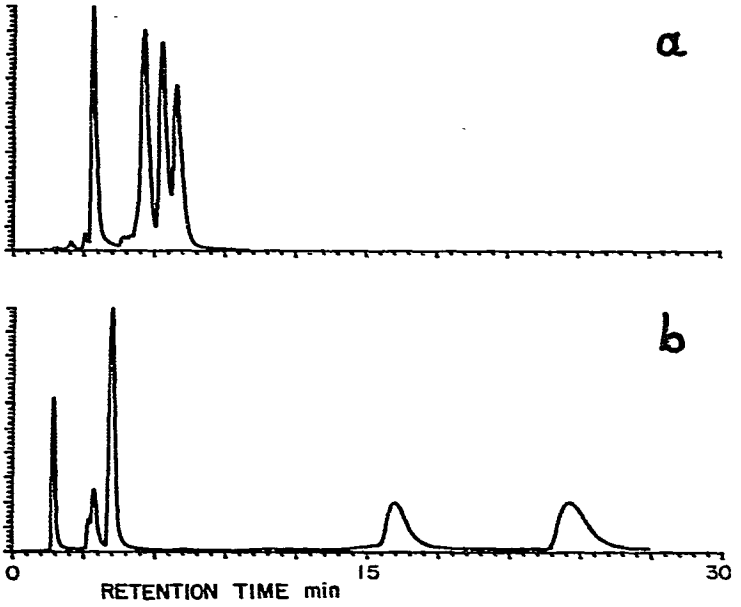

Fig. 6. Forward- and reversed-phase chromatograms from an incompletely derivatized octadecyl bonded phase. (a) Mobile phase, water–acetonitrile (35:65 v/v), solutes: benzene, anthraquinone, 2-methylantraquinone, and 2-ethylanthraquinone, (b) Mobile phase ethyl acetate–heptane (7:93 w/v), solutes benzene, acetophenone, α-phenyl alcohol and benzylalcohol.

which according to the manufacturers was derivatized to the extent of 67.5% and thus still exhibited some polar characteristics, indicating that some of the unreacted hydroxyl groups were still sterically available to the solute. Owing to the highly polar character of ODS it would act as an effective silica gel column, as well as a reversed-phase column, and in Fig. 6 this dual capability is clearly demonstrated. In the upper chromatogram the separation is shown for the solutes benzene, anthraquinone, 2-methylanthraquinone and 2-ethylanthraquinone. The column was operated in the reversed-phase mode, employing water–acetonitrile (35:65, v/v) as the mobile phase. In the lower chromatogram the same column was operated in its polar mode, using a mobile phase consisting of ethyl acetate–*n*-heptane (7:93, w/v). The solutes separated were benzene, acetophenone, α-phenethyl alcohol and benzyl alcohol, and it is seen that useful separations can be achieved in both modes of operation although the peaks for α-phenethyl alcohol and benzyl alcohol exhibit serious tailing. In Table IV

TABLE IV

COMPARATIVE RETENTION DATA FOR ODS REVERSED PHASE AND PARTISIL 10 SILICA GEL

Solute	*ODS*		*Partisil 10*	
	k'	α	*k'*	α
α-Phenethyl alcohol	5.44		6.85	
		1.54		1.45
Benzyl alcohol	8.37		9.91	

the k' values and separation ratio for α-phenethyl alcohol and benzyl alcohol are given for the ODS column and a Partisil 10 column, the latter data taken from the literature[8]. It is seen that both the respective k' values and the separation ratio (α) for the solutes on the two phases are very similar, which indicates that the ODS column operated in the polar mode is, in effect, acting as a silica gel column. It would appear that under polar conditions of development the hydrocarbon moieties on the bonded phase were completely deactivated by the non-polar solvent heptane and under reversed-phase conditions of development the free silanol groups were completely deactivated by the water present in the mobile phase. Thus the reversed-phase containing a significant proportion of silanol group can operate under conditions where stationary phase interactions were, on the one hand, largely confined to the silanol hydroxyl group and, on the other, were associated mostly with the hydrocarbon chains of the bonded phase.

The above results suggest the interesting concept of a column that is specifically designed to operate in both polar and non-polar modes. Such a column would be extremely useful for the preliminary assessment of the best conditions to separate a hitherto unknown sample. The sample could be chromatographed over a range of polarities from heptane to alcohol, employing the material as a polar stationary phase and then, by adding water, using it in the reversed mode to obtain the optimum conditions for separation. Such a bonded phase would have to be carefully tailored to provide adequate efficiencies and peak symmetry and retention characteristics when operating in both the polar and non-polar modes. It may be that a column packed with an equal mixture of silica gel and reverse phase having the same mean particle diameter might be a simpler and more effective approach to providing such a dual-purpose column.

INTERACTION ON THE SURFACE OF REVERSED BONDED PHASES

A satisfactory chromatographic separation is realized if the individual solutes in a mixture are retained to different extents on the column and are eluted discretely. Solutes will be retained owing to their interaction with the stationary phase, which in the case of a bonded phase will occur at the surface. It is therefore important to understand both the nature of the surface and the nature of the interactions, if the mechanism of the interaction between solute and stationary phase is to be understood. There are a number of questions that need to be asked and answers found to them. (1) What is the nature of the interacting surface under chromatographic conditions? Is the carbon chain of the bonded phase directly available to the solute or is it coated with one component of the mobile phase? (2) How does the solute interact with the stationary phase? Does it interact directly with the carbon moiety, or if the carbon chains are covered with an adsorbed film of the mobile phase, does it interact with the coating or does it displace the coating and interact with the carbon chain? (3) When a reversed phase is used in conjunction with an ion-pair reagent does the reagent exist solely in the mobile phase or does some reside on the stationary phase? If the ion-pair reagent associates with the stationary phase, does it reside on the surface of a solvent coating or does it displace the solvent coating and interact directly with the carbon chain of the bonded phase? (4) In ion-pair chromatography on reversed-phases, is the ion pair, formed by the solute and reagent, produced in

TABLE V

EQUILIBRIUM DATA FOR THREE DIFFERENT SOLVENTS AND FIVE DIFFERENT STATIONARY PHASES

Solvent	*Chain length of bonded phase*	*Mass of solvent added to system (g)*	*Mass of solvent in mobile phase (g)*	*Mass of solvent on stationary phase (g)*	*Mean mass of solvent on stationary phase (g)*	*Mass of bonded phase in system (g)*	*Concentration of solvent in mobile phase (%, w/v)*	*Index of determination*	*Slope*	*Mass of solvent on stationary phase (mg/g)*	*Surface area of solvent molecule (Å²)*	*Calculated surface coverage by solvent (m²/g)*
Isopropanol	C_8 (RP8)	7.869	7.386	0.483			18.92					
		15.681	15.218	0.463	0.472	7.218	31.78	1.0000	1.001	65.4	28.1	184
		23.455	22.986	0.469			40.33					
Acetonitrile	C_8 (RP8)	10.382	9.854	0.528			25.19					
		18.353	17.931	0.422	0.453	7.378	36.13	1.0000	1.008	61.4	21.1	190
		26.288	25.880	0.408			44.23					
Methanol	C_8 (RP8)	14.368	14.136	0.232			35.94					
		22.913	22.580	0.333	0.246	7.143	45.43	0.9999	1.003	34.4	22.3	144
		30.643	30.470	0.173			51.45					
Isopropanol	C_{18} (RP18)	6.343	6.010	0.333			15.40					
		14.110	13.756	0.354	0.332	5.024	28.65	1.0000	1.000	66.1	28.1	186
		21.837	21.529	0.308			37.34					
Isopropanol	C_2 (RP2)	6.840	6.377	0.463			16.31					
		14.604	14.203	0.401	0.392	5.364	29.57	1.0000	1.010	73.1	28.1	206
		22.328	22.016	0.312			42.47					
Isopropanol	C_{18} (ODS)	6.833	6.447	0.386			16.49					
		14.596	14.094	0.502	0.434	5.261	29.36	0.9999	0.998	82.5	28.1	232
		22.320	21.906	0.414			38.19					
Isopropanol	C_{18} (ODS2)	6.818	6.348	0.470			16.23					
		14.582	14.125	0.457	0.448	5.031	24.43	1.0000	1.003	89.0	28.1	251
		22.307	21.889	0.418			38.16					

the mobile phase or on the surface of the stationary phase? When the ion pair interacts with the stationary phase, does it interact with a surface coating of solvent or displace a solvent layer and interact directly with carbon chain of the bonded phase?

Experiments were designed to provide answers to some of these questions which are described sequentially in the following section together with the results and conclusions.

The nature of the interacting surface

In order to determine whether a coating of solvent existed on the surface of the bonded phase, the loss of solvent to the stationary phase was determined by a mass-balance procedure. A known mass of bonded phase was equilibrated with a known volume of water–solvent mixture of known composition. In these experiments solvent mixtures were employed that permitted complete wetting of the stationary phase as these are the normal conditions used in simple reversed-phase separations. After equilibrium had been established duplicate samples of the mobile phase were analyzed by gas chromatography (GC) and the difference between the total mass of solvent added and the total mass determined in the mobile phase was taken as the mass adhering to the stationary phase. The initial concentration of solvent used was about 2% (w/v) above the wettability limit. Two subsequent masses of solvent were then added, equilibrium established, and the mobile phase was analyzed again. Careful corrections were made to take into account the change in volume and mass of solvent resulting from the samples withdrawn for analysis and the volume change on mixing. The sample of bonded phase and solvent were equilibrated in a 70-ml bottle closed by a PTFE-lined serum cap to prevent loss of solvent vapor. The bottle was shaken for 30 min in a thermostated bath maintained at 25 $\pm$ 0.1° and then allowed to stand for 20 min in the bath to allow the bonded phase to settle before samples were taken for analysis. The solvent samples were analyzed on a Hewlett-Packard 5710A gas chromatograph fitted with an automatic sample injector and computer data processing facilities; using a 9-ft. column packed with 10% PEG 20M on Chromosorb W. The chromatograph was operated at a temperature of 70 or 80°, depending on the nature of the solvent; quantitative analysis was obtained against an added internal standard of either 1-butanol or dioxane using peak area measurements. The bonded phase RP8 was examined using the three solvents isopropanol, acetonitrile and methanol whereas the bonded phases RP2, RP18, ODS and ODS2 were examined using one solvent only, isopropanol. Isopropanol was chosen because it gave very symmetrical peaks on the gas chromatograph and thus provided more precise data. The results obtained are summarized in Table V.

It is seen from Table V that all bonded phases adsorb a layer of solvent on the surface and that this applies to all three solvents, methanol, acetonitrile and isopropanol. The quantity of solvent adsorbed does not vary with the concentration of solvent and this is confirmed by the linear correlation coefficient of unity for the curve relating mass of solvent added to the system to the mass of solvent in the mobile phase. Further confirmation of this constant mass of solvent adsorbed for any given bonded phase is provided by the fact that the slope of this linear curve is also close to unity. The area of each solvent molecule was determined approximately from accurate molecular models having a scale of $1.25{:}10^{-8}$. Models of each solvent were made and their perimeter contours obtained by placing them on photographic paper

under a strong light. The images of the molecules were cut out and the paper images weighed together with a standard square of the same paper representing 100 Å^2. The values for the area of each solvent molecule obtained in this way are also shown in Table V. From the mass of solvent adsorbed per gram of stationary phase, the molecular weight of the solvent and molecular area, the effective area of the bonded phase could be calculated. The effective area available to each solvent on the respective bonded phase is shown in the last column of Table V. It is seen that the available area for solvent interaction of each bonded phase is in line with the surface areas of the parent silica gels given in Table II, albeit somewhat smaller. The slight reduction in surface area relative to the parent silica gel probably results from partial blocking of the silica pores by the bonded material. Owing to the limited precision of the GC analysis, the absolute values for surface areas given in Table V will only by approximate, particularly for the solvent methanol. In the GC analysis of methanol, the peak for this solvent gave a significant tail, which rendered the accurate measurement of peak area extremely difficult. The values for the surface areas of the bonded phases that are covered with solvent molecules, calculated in this way, however, gave a strong indication that the solvent layer is only one molecule thick. A bimolecular layer, if present, would be expected to give surface area values of twice the magnitude given in Table V. It can be concluded that under chromatographic conditions, where the stationary phase is wetted by the solvent, a reversed-phase adsorbent is covered by a layer of the solvent that is present in the aqueous mobile phase mixture and this layer is probably one molecule thick.

Solute interaction with the surface of the bonded phase

In order to investigate the interaction of the solute with the surface of the reversed phase, it is necessary to design experiments to determine whether the solute associates with the solvent layer on the surface, or whether it displaces the solvent layer and interacts directly with the carbon chain. Three different methods were employed, the first two employing a mass-balance approach and the third using a chromatographic approach. A known mass of RP8 reverse phase (5.11 g) was placed in a 70-ml flask, 50 ml of acetonitrile–water (*ca.* 42:58, w/v) mixture were added and the bottle was sealed with a PTFE-lined serum cap. The mixture was equilibrated for 30 min at 25 $\pm$ 0.1° in a thermostated bath with continuous shaking and then allowed to stand for 20 min to permit the bonded phase to settle. Samples were withdrawn and subjected to GC analysis using the same conditions as described previously, except that the column was programmed up to 200° to elute the solute acetophenone when present; 25 ml of the equilibrated mobile phase were also removed and 1.04 g of acetophenone dissolved in it. Four milliliters of this solution were then added to the bottle and the contents equilibrated. Samples were taken for GC analysis and another 4 ml of the acetophenone solution added and the process repeated for a total of six additions. The mass of solute on the stationary phase was calculated as the difference between the total mass added and the mass of solute in the mobile phase, taking into account the appropriate corrections for the mass and volume of the samples withdrawn for analysis. The results obtained are shown in Fig. 7 as graphs relating the concentration of acetonitrile in the mobile phase and, the concentration of acetophenone on the stationary phase to concentration of acetophenone in the mobile phase. Acetophenone is eluted from a column run under the same mobile

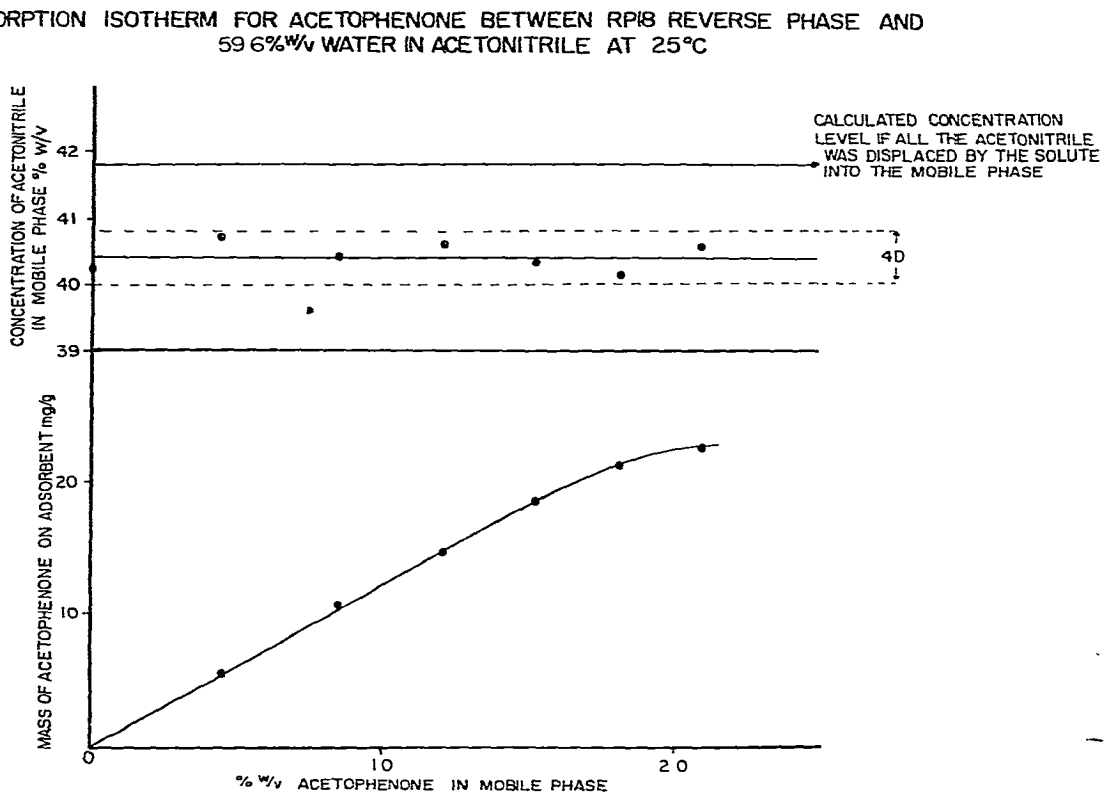

Fig. 7. Adsorption isotherm for acetophenone between RP18 reversed phase and water–acetonitrile (59.6:40.4, w/v) at 25°.

phase conditions at a k' value of about 1.6. It is seen that as the concentration of acetophenone on the stationary phase rises linearly to about 22 mg/g, the concentration of acetonitrile in the mobile phase remains sensibly constant. There is no observed change in acetonitrile content, which would be expected to rise from 40.4% (w/v) to a maximum of 41.8% (w/v), if all the solvent was displaced by the solute from the stationary phase into the mobile phase. Unfortunately, although no change in acetonitrile concentration is observed, owing to the scatter of the points, the confidence limit of this conclusion is only about 80%.

A similar experiment was carried out using the bonded phase RP8, a mobile phase of about 46.3% (w/v) water in acetonitrile and the solute, 2-ethylanthraquinone. If chromatographed under these conditions, 2-ethylanthraquinone would be eluted at a k' value of about 3.9. A similar experiment to that described previously was carried out, but in this case, six replicate samples of the equilibrated mobile phase were analyzed for acetonitrile and after one addition of the solute a further six replicate samples were analyzed. The concentration of 2-ethylanthraquinone was determined gravimetrically. This procedure was adopted in an attempt to improve the precision of the GC analysis and thus improve the confidence level of the conclusion. The results obtained are summarized inTable VI. It is seen that the mean concentrations of acetonitrile before and after the addition of 2-ethylanthraquinone were 54.71% (w/v) and 54.68% (w/v), respectively, and thus no increase in aceto-

TABLE VI

THE CONCENTRATION OF ACETONITRILE IN THE MOBILE PHASE BEFORE AND AFTER THE ADDITION OF 2-ETHYLANTHRAQUINONE AS SOLUTE

Mobile phase			*Mass of 2-ethylanthraquinone on stationary phase*
Concentration of acetonitrile in the absence of 2-ethyl-antraquinone (%, w/v)	*Concentration of acetonitrile in the presence of 2-ethyl-anthraquinone (%, w/v)*	*Concentration of 2-ethylanthraquinone (g/ml)*	
54.15	54.42		
54.68	54.76		
54.58	54.59		
54.80	54.22	0.226	0.066 g = 12.8 mg/g
54.89	54.58		
55.14	55.00		
Mean			
54.71	54.68		
S.D.			
0.334	0.282		

nitrile content could be observed. However, the precision of the analysis was still not sufficient to conclude with a confidence level of more than 80% that no acetonitrile was desorbed from the stationary phase.

A chromatographic procedure was then adopted as an alternative, confirmatory method to determine whether the solute displaced acetonitrile from the mobile phase. If a retained solute did, indeed, displace the acetonitrile layer from the adsorbent, then, if the solute was fed continuously onto the column as in frontal analysis, the desorbed solvent would be eluted at the front of the frontal analysis step and would be clearly identified. In this experiment the RP8 column was again employed and the solute 2-ethylanthraquinone was eluted at a k' value of 3.9 with a mobile phase having the composition the same as used in the previous experiment, namely 70.0% (w/v) acetonitrile. A sample of 16 ml of equilibrated mobile phase containing 64 mg of 2-ethylanthraquinone was fed onto the column at a flow-rate of 1 ml/min and the elution curve monitored by a refractive index detector. The results obtained are shown in Fig. 8. The upper chromatogram shows the peak for 8 mg of pure acetonitrile eluted at the dead volume, detected at one third of the sensitivity employed in the frontal analysis curve, and demonstrates the sensitivity of the system to acetonitrile. It should be noted that the peak for acetonitrile is positive. The center chromatogram was obtained using the UV detector for a 1-mg sample of the solute and identified its retention time. The lower chromatogram shows the negative deflection of the frontal analysis step for 2-ethylanthraquinone. It should be noted that there is no sign of a positive peak or step for acetonitrile, prior to the breakthrough of the solute step, and this was confirmed by expanding the baseline section of the chromatogram, prior to the frontal analysis step, to the point where the detector noise was clearly seen. As the mass of acetonitrile that would be released from the adsorbent, if it had been displaced, would be commensurate with the mass of solute injected, *viz.* 64 mg, it follows that, if acetonitrile had been displaced, it would have been clearly seen.

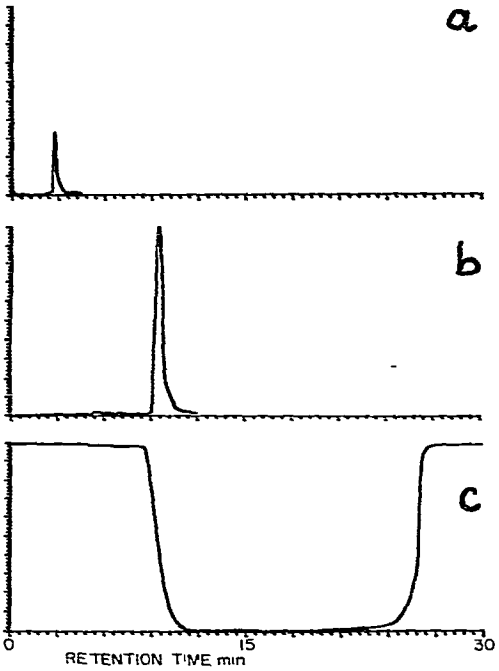

Fig. 8. Chromatograms demonstrating the persistence of the adsorbed solvent layer on the bonded phase during chromatographic development. Stationary phase: RP8; mobile phase: water–acetonitrile (3:7, v/v). (a) Refractometer detector, 8 mg of acetonitrile, + signal sensitivity, ×3; (b) UV detector, 0.6 mg of 2-ethylanthraquinone, sensitivity, ×32; (c) refractometer detector sensitivity × 1, 68 mg of 2-ethylantraquinone, — signal, frontal analysis.

The above experiment was repeated using the ODS2 column and a mobile phase composition of water–acetonitrile (35:65, v/v) under which circumstances the 2-ethylanthraquinone was eluted at a k' value of about 10. A 16-ml sample containing 64 mg of anthraquinone was again used and the results obtained are shown in Fig. 9. All chromatograms were obtained using the refractive index detector and the top chromatogram again demonstrates the sensitivity of the detector to 8 mg of acetonitrile at one third sensitivity of that used for the frontal analysis curve. The center chromatogram is for a 2-mg sample of 2-ethylanthraquinone and identifies the solute retention time. The lower chromatogram is the frontal analysis curve for anthraquinone, this time eluted at a k' value of 10. No trace of displaced acetonitrile is observed and again, this was confirmed by expanding the sensitivity along the baseline prior to the solute step, so that detector noise was clearly visible. No positive peak or step was observed.

The results clearly and unambiguously indicate that for substances eluted up to a k' value of 10 under conditions where the stationary phase is wetted by the solvent, the monomolecular layer of solvent adsorbed on the surface of the bonded phase is not displaced by a solute. Thus in the chromatographic development, the solute interacts with the solvent layer and not directly with the carbon moiety.

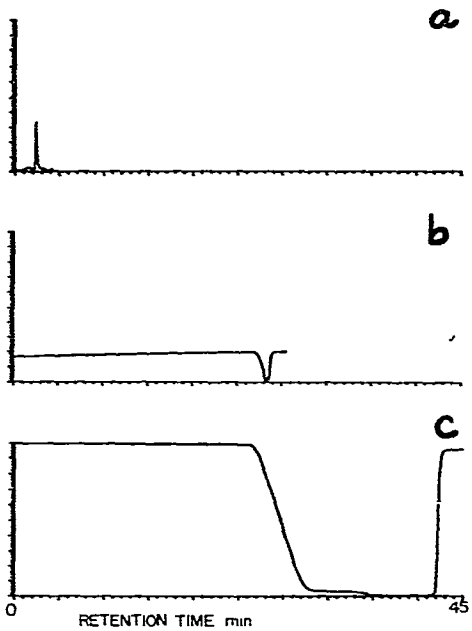

Fig. 9. Chromatograms demonstrating the persistence of the adsorbed solvent layer on the bonded phase during chromatographic development. Stationary phase: ODS2; mobile phase: water–acetonitrile (35:65, v/v); refractometer detector. (a) 8 mg of acetonitrile, + signal sensitivity ×3; (b) 1 mg 2-ethylantraquinone, — signal sensitivity × 1; (c) 68 mg 2-ethylanthraquinone, — signal sensitivity × 1.

The interaction of ion-pair reagents with reversed phases

Ion-pair reagents are used both with silica gel and reversed phases to modify the retention and selectivity exhibited by the chromatographic system. In practice, the ion-pair reagent, which can be an alkyl sulfonic acid or salt in the case of acidic reagents or a tertiary alkyl ammonium salt in the case of basic reagents, is dissolved at a level of between 0.1 and 1.0% (w/v) in the mobile phase. The reagent can modify selectivity, either by direct interaction with the solute in the mobile phase, or be adsorbed on the surface of the packing and interact with the solute on the stationary phase.

The distributions of the ion-pair reagents, *p*-toluenesulfonic acid and the sodium salt of octanesulfonic acid, were determined by a mass-balance method and by a chromatographic procedure, under conditions where the stationary phase is wetted by the solvent. A known mass of reversed phase was equilibrated with a mobile phase containing 30% (v/v) water in acetonitrile in a sealed bottle situated in a thermostat bath and 8 ml of the equilibrated solvent was withdrawn; about 0.75 g of the ion-pair reagent was dissolved in it and 2-ml portions of the mixture were then added to the equilibrated mixture. The mixture was shaken for 30 min, allowed to stand for 20 min, and samples were withdrawn and the quantity of reagent per milliliter of mobile phase was determined. From the results, the mass of reagent in the mobile phase was calculated (taking into account volume and mass changes resulting from the removal of samples for analysis). The mass adsorbed by the

TABLE VII

MASS OF ION-PAIR REAGENT ADSORBED BY THE STATIONARY PHASE

For sodium octanesulfonate: mass of stationary phase (ODS), 5.2 g; mobile phase water–acetonitrile (3:7, v/v). For *p*-toluenesulfonic acid: total mass of stationary phase (RP18), 5.02 g; mobile phase, water–acetonitrile (3:7, v/v).

Sodium octane sulfonate (gravimetric method)				*p-Toluenesulfonic acid (volumetric method)*			
Concentration of reagent in mobile phase (%, w/v)	*Mass of reagent added (mg)*	*Mass of reagent in mobile phase (mg)*	*Mass of reagent on stationary phase (mg/g)*	*Concentration of reagent in mobile phase (%, w/v)*	*Mass of reagent added (mg)*	*Mass of reagent in mobile phase (mg)*	*Mass of reagent on stationary phase (mg/g)*
0.46	200.4	213.9	−2.6	0.30	140	135.3	+1.0
0.86	391.4	399.9	−1.6	0.57	280	255.1	+5.0
1.28	574.8	595.2	−3.9	1.06	420	465.6	−9.1
1.61	749.6	748.7	+0.2	6.60	643	660.6	−3.5

stationary phase was taken as the difference between the mass added and the mass contained in the mobile phase. Two reversed phases and two reagents were employed in two different experiments using two different analytical procedures. In the first experiment ODS2 and sodium octanesulfonate were used and analyzed quantitatively by evaporating the sample down to dryness in a preweighed weighing bottle. In the second experiment RP18 and *p*-toluenesulfonic acid were employed and the quantity of the reagent in the solvent sample determined by direct titration with 0.01 *N* sodium hydroxide solution using phenolphthalein as an indicator.

The results obtained are shown in Table VII. It is seen that neither reagent is adsorbed onto the stationary phase and the sulfonates appear to reside entirely in the mobile phase. If, indeed, the ion-pair reagents exist solely in the mobile phase, then they would also be eluted in the dead volume if injected as a sample directly on the column with the same mobile phase. The dead volumes of the ODS, ODS2 and RP18 columns were determined together with the retention volumes of sodium octanesulfonate and *p*-toluenesulfonic acid and the results are shown in Table VIII. The results given in Table VIII confirm that the two reagents are indeed eluted at the dead volume of the column.

TABLE VIII

RETENTION DATA FOR ION-PAIR REAGENTS ON REVERSED PHASES (UNDER CONDITIONS WHERE THE STATIONARY PHASE WAS WETTED BY THE SOLVENT)

Reversed phase	V_R *(ml)*		
	Water (dead volume)	*p-Toluenesulfonic acid*	*Sodium 1-octanesulfonate*
ODS	2.51	2.44	2.51
ODS2	2.04	2.04	2.04
RP18	1.92	1.91	1.93

It must therefore be concluded that the ion-pair reagents, in the absence of a solute and when the stationary phase is wetted by the solvent, reside entirely in the mobile phase and are not adsorbed as a layer on the stationary phase in a similar manner to the solvent. Under the given conditions the conclusion is in direct conflict with the views of Kissinger[9], who assumes that the ion-pair reagent is adsorbed onto the reversed phase and modifies its interacting properties irrespective of the wetting conditions.

The effect of the ion-pair reagents on solute retention was then examined both under conditions where the solvent wetted the bonded phase and under conditions where it did not. Three solvent concentrations were employed, namely acetonitrile–water (65:35), which wetted the stationary phase, together with acetonitrile–water (25:75) and acetonitrile–water (20:80), which did not wet the stationary phase. The solutes 3-pyridylcarbinol and 6-amino-*m*-cresol were chromatographed on the ODS2 column using each of the three solvent mixtures and their retention volumes measured. The retention volumes of the ion-pair reagents, sodium octanesulfonate and *p*-toluenesulfonic acid were also determined under the same conditions. Sodium octanesulfonate was then added to each solvent mixture to provide a 0.005 *M* solution together with acetic acid to provide a 1% solution. The retention volumes of the same solutes were then determined in each of the three solvents, now containing the ion-pair reagent. The results are shown in Table IX. It is seen that for the solvent mixture that permitted wetting neither ion-pair reagent is retained and so no reagent is present on the surface of the bonded phase. When the solvent contains an ion-pair reagent, the retention volume of each solute decreases. This indicates that the ion-pair reagent, contained entirely in the mobile phase, merely introduces ionic interactions with the solute, which combines with the polar and dispersive interactions of the solute with the water and acetonitrile. Thus, as the total interactions of the solute in the mobile phase increase and the solute interactions with the stationary phase remain the same, the solutes are eluted more rapidly. The second mobile phase did not wet the station-

TABLE IX

RETENTION DATA FROM ODS2 WHEN USED WITH ION-PAIR REAGENTS

Solvent composition	*Solute*	V_R *(ml)*	
		In the absence of ion-pair reagent	*In the presence of 0.005 M sodium octanesulfonate and 1% acetic acid solution*
Acetonitrile–water (65:35)	3-Pyridylcarbinol	3.52	3.23
	6-Amino-*m*-cresol	4.56	3.63
	Sodium octanesulfonate	2.04	2.04
	p-Toluenesulfonic acid	2.04	2.04
Acetonitrile–water (25:75)	3-Pyridylcarbinol	4.12	4.78
	6-Amino-*m*-cresol	8.68	10.17
	Sodium octanesulfonate	4.06	
	p-Toluenesulfonic acid	2.51	
Acetonitrile–water (20:80)	3-Pyridylcarbinol	5.91	7.53
	6-Amino-*m*-cresol	15.27	18.30
	Sodium octanesulfonate	12.80	
	p-Toluenesulfonic acid	7.66	

ary phase, and it is seen that the retention volume of both solutes has increased and that the ion-pair reagents are now significantly retained, forming a layer on the surface. It is also seen from Table IX that the solutes, eluted by the same mobile phase but containing the ion-pair reagent, now show an increase in retention volume. This indicates that ionic interactions in the stationary phase are now becoming significant relative to the increased ionic interactions in the mobile phase. Increasing the water content of the solvent in the third mobile phase emphasizes this effect. The retention of 3-pyridylcarbinol and 6-amino-*m*-cresol is now significantly increased by the presence of the ion-pair reagent and the ion-pair reagent itself is more strongly held on the bonded phase. Thus under non-wetting conditions Kissinger's[9] explanation of ion-pair interaction could be correct.

It has been shown, therefore, that ion-pair reagents reduce retention by introducing ionic interactions in the mobile phase under conditions where the mobile phase can wet the reversed phase. Under conditions where the mobile phase does not wet the reversed phase and at sufficiently high water contents, the ion-pair reagent can be retained and form a surface coating. This coating permits ionic interactions with the stationary phase and thus increases the retention of solutes having complementary acid or basic characteristics with respect to the ion-pair reagent.

CONCLUSIONS

The commercially available reversed phases examined differed significantly in carbon content, chain length and degree of derivatization. The maximum concentration of water in the solvent mixture that would permit wetting varied widely between the reversed phases themselves and the solvent employed, the less polar the solvent the higher the concentration of water that would maintain wetting. The concentration of water in the solvent mixture at which the system changed from a lyophilic condition to a lyophobic condition is important to the practicing chromatographer as the separation process appears to change at that water concentration. The retentive capacity of the reversed phase generally increased with carbon content and chain length but depended also on the nature of the hydrocarbon moieties and in particular on whether it had been surface or bulk modified. The bulk-modified or polymeric bonded phase appears to provide greater retention than a surface-modified stationary phase of the same chain length and carbon content. Both surface- and bulk-modified material can provide columns having good chromatographic performance. The selectivity as determined by the retention ratio of 2-ethylanthraquinone to anthraquinone under the same solvent conditions increased regularly with the carbon content of the bonded phase, but this may not be a general relationship.

The presence of unreacted silanol groups on the surface of the bonded phase does not appear to affect seriously its selectivity or the column efficiencies obtained from it. A dual-purpose column that contains either a partially reacted bonded phase or a mixture of a fully reacted bonded phase and silica gel of the same particle size could be an interesting and useful column system. Such a column could be used as a silica gel column and a reversed-phase column by merely changing the solvent system and the data used to determine the best phase system to separate a hitherto unknown mixture.

Under conditions of wetting the reversed phase is covered by a layer of solvent

which is, on average, probably one molecule thick. Solutes retained up to a k' value of 10 interact with this layer of solvent and not with the hydrocarbon chain of the reversed phase. During interaction, the solute molecules associate with the solvent on the stationary phase but do not displace it. In ion-pair chromatography, where the reversed phase is wetted, the ion-pair reagent exists solely in the mobile phase. The normal polar and dispersive interactions between the solute and the mobile phase are thus supplemented by ionic interaction and the counter ionic solute species are eluted more rapidly and thus retention decreases. Under lyophobic conditions, however, and at higher concentrations of water in the mobile phase the ion-pair reagent is adsorbed as a film on the surface of the reversed phase. This coating of ion-pair reagent now permits ionic interaction between the solute and the stationary phase and thus the solute retention increases.

ACKNOWLEDGEMENTS

We would like to thank Dr. M. Gurkin of E.M. Laboratories Inc. for providing samples of their RP2, RP8 and RP18 reversed phases and Mr. D. Southern of Whatman, Inc., for providing samples of their ODS and ODS2 reversed phases.

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