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THE ROLE OF ORGANIC MODIFIERS ON POLAR GROUP SELECTIVITY IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

The influence of the organic modifiers methanol (MeOH), acetonitrile (AN) and tetrahydrofuran (THF) on polar group selectivity in reversed-phase liquid chromatography has been studied. In order to elucidate polar group effects, we have first explored the influence of the surface coverage of bonded phase on selectivity. Using a series of synthesized *n*-octyl bonded phases, we have been able to observe significant differences in group contribution with bonded phase coverage, the largest differences arising from MeOH–H_2O as mobile phase and the least from THF–H_2O as mobile phase. The importance of using phases that minimize accessible silanol groups in order to study the influence of the mobile phase has been emphasized. We have selected a high coverage *n*-octyl phase that is silanized for these studies.

In order to examine polar group effects, we have normalized the methylene group increment in the MeOH–H_2O, AN–H_2O and THF–H_2O binary phases. As the hydrophobic selectivity is thus roughly normalized, meaningful relative polar group contributions are observed. Plots of log k' (THF–H_2O) *vs.* log k' (MeOH–H_2O) reveal particularly striking polar group differences. The practical usefulness of the plots is shown in the peak reversal of solute mixtures with the two mobile phases. Further studies reveal that polar group selectivity can be powerfully controlled using ternary phases of MeOH–THF–H_2O. Thus, the choice of mobile phase can greatly influence separation in reversed-phase liquid chromatography.

INTRODUCTION

At present reversed-phase liquid chromatography (RPLC) using *n*-alkyl bonded phases is the most frequently selected separation mode in high-performance liquid chromatography (HPLC). It is well-known that RPLC is an excellent method to separate substances based on size or alkyl group structure, as a consequence of hydrophobic or solvophobic interactions[1–5]. What may not be sufficiently realized is that RPLC can also be a highly selective method for separation based on polar group differences.

It is known that hydrophobic selectivity in RPLC is a sensitive function of

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the mobile phase, more specifically the type and amount of organic modifier mixed with water[1,2,5]. We should also note that the role of the mobile phase has been studied in the past using open-bed techniques[6,7].

The composition of the mobile phase can play a significant role on (1) retention, (2) hydrophobic group selectivity and (3) polar group selectivity. That mobile phase control can be a potentially powerful tool for the optimization of separation has been demonstrated in a recent paper, dealing with binary and ternary phases[8]. However, it is clear that an understanding of the role of mobile phase composition on retention and selectivity is difficult to achieve because of several factors. First, as correctly pointed out by Karch *et al.*, a well-defined and reproducible stationary phase is required before one can begin to understand mobile phase or bonded phase effects[9]. Second, without appropriate normalization, both hydrophobic and polar group selectivity can simultaneously change with organic modifier type and composition. Third, there is a lack of a full understanding of the mechanism of retention with bonded phases[1,10,11].

We have undertaken an examination of the role of organic modifier(s) on selectivity in RPLC. As we have already explored to some extent hydrophobic selectivity[2], our main emphasis will be on polar group selectivity. In this paper we wish to report our initial efforts in this direction. We first examine in detail the role of the quality of the bonded phase on retention and group selectivity of non-ionic polar substances, as well as to a limited extent ionic substances. We next turn to a comparison of binary solvents in terms of polar group selectivity. For this study, we have normalized the hydrophobic selectivity in order to examine more meaningfully polar group selectivity. Large variations in polar group selectivity are observed when various organic modifiers are used. Indeed, complete reversals in elution order for selected substances from one mobile phase to another can be found. Polar group selectivity under normalized hydrophobic conditions is also examined in ternary phases, and significant changes with composition are again seen. It is clear that mobile phase composition can significantly control separation in RPLC.

EXPERIMENTAL

Equipment

An HPLC instrument was set-up from Waters Assoc. (Milford, Mass., U.S.A.) components, consisting of a M6000A solvent delivery system, U6K injector, R401 refractive index detector and M440 absorbance monitor operated at 254 nm. The columns were maintained at 30 $\pm$ 0.1° by submerging them in a water bath.

Columns

The packing consisted of 5-μm Hypersil (Shandon Southern, Sewickley, Pa., U.S.A.). Chemical bonding was performed with octyldimethylchlorosilane (Silar Labs., Scotia, N.Y., U.S.A.), using conditions similar to those of Hemetsberger *et al.*[12]. Silanization after bonding was done in a similar fashion using hexamethyldisilazine and trimethylchlorosilane (Silar Labs.). The stationary phase was packed into columns of 15 cm $\times$ 4.6 mm I.D. tubing (Analabs, North Haven, Conn., U.S.A.) using conventional high-pressure slurry techniques.

Mobile phases and samples

The mobile phases were made up by volume from LC grade solvents (Burdick & Jackson, Muskegon, Mich., U.S.A.) and distilled, deionized water. Acetic acid (0.1%) was added, where appropriate, to control the pH of the mobile phase.

Sample solutes were from commercial sources and used without further purification. Most samples were made up with *ca.* 30% methanol (MeOH). Injection volumes were roughly 1 μl, consisting of less than 1 μg for aromatic compounds and 1–3 μg for aliphatic compounds. Separate measurements revealed no influence of retention on sample size at these levels, except in the case of ionic substances with packings containing significant numbers of unreacted silanol groups.

RESULTS AND DISCUSSION

Stationary phase characteristics

A number of studies have been concerned with the synthesis and characterization of chemically bonded phases (*e.g.* refs. 9 and 11–19). A particularly detailed examination of the synthesis of such phases can be found in the work of Kovats *et al.*[20]. It is well known that unreacted and accessible silanol groups can lead to undesirable effects with respect to retention and band asymmetry, particularly with basic substances[14–19,21]. Therefore, workers attempt to maximize bonded phase coverage in order to minimize silanol groups from partaking in the retention process.

It is important to know the role of unreacted silanol groups of a particular bonded phase on polar group selectivity of solute molecules (non-ionic and ionic). This information is necessary if we wish to explore the influence of type and composition of organic modifier on selectivity. Obviously, the conclusions reached in any study are only valid if the effect of unreacted silanol groups is minimized.

In this work we have made a series of *n*-octyl bonded phases using several different batches of commercial 5-μm Hypersil. We used *n*-octyldimethylchlorosilane, rather than the corresponding di- or trichlorosilanes in order to eliminate the possibility of polymerization and of the reformation of silanol groups upon the hydrolysis of unreacted Si–Cl bonds[19]. In several cases we have followed the bonding step with a silanization reaction to reduce the number of remaining and accessible unreacted silanols. In one example we have purposely stopped the bonding reaction at approximately 70% of full coverage, in order to elucidate more fully the role of unreacted silanols.

There are a variety of ways of characterizing bonded phases; however, no method to date is entirely satisfactory. Table I shows 5 of the phases that we have made, along with several of the more popular methods of characterization. We should note that 3 different batches of Hypersil have been used, in order to obtain some appreciation of the variation of silica from a commercial source. The specific surface areas, as shown in Table I, were approximately the same, as reported by the manufacturer.

The first method of characterization is based on the surface concentration of bonded phase, as deduced from the elemental analysis[17]. As can be seen, values of coverages of 3.4 μmoles/m^2 were obtained, in agreement with the values of high coverages from other workers[11,12,14,17]. Column IV was purposely not bonded at full coverage, as we have already noted.

TABLE I

CHARACTERISTICS OF STATIONARY PHASES

Phase number: I and II, same batch (192 m^2/g); III, 187 m^2/g; IV and V, same batch (195 m^2/g). TMS = trimethylsilyl.

Phase number	*Bonded phase*	*Surface coverage (μmole/m^2)*	*k' with n-heptane mobile phase*		
			Anisole	*Methylbenzoate*	*Acetophenone*
I	C_8+TMS	3.4	<0.1	0.20	0.55
II	C_8	3.4	0.2	1.6	5.3
III	C_8	3.3	0.5	3.3	—
IV	C_8	2.4	1.4	≈9	—
V	C_8+TMS	3.4	0.1	0.45	1.15

As will be seen, significant differences exist in the chromatographic behavior of columns I, II, III and V, even though they possess the same coverages. Errors may arise in the determinations of the elemental composition and of the surface area, leading to imprecision in the surface coverage. In addition, the pore structure of different silicas may differ from one another, particularly in the extent of micropores, and this can lead to unreacted and accessible silanols. Thus, the surface coverage measurement can only be used as a first order estimation of the quality of phase. Moreover, because of the imprecision of the measurement and the high carbon content, it is invalid to conclude that silanization after bonding has no influence on the quality of the phase, if the surface coverage does not change. Other methods of evaluating bonded phases must be used.

A popular method of characterization for accessible unreacted silanol groups is to measure the retention of small polar molecules in dry heptane[11,12,16,19]. In order to obtain a valid measure, great care must be exercised. One must remove all traces of polar eluents from the bonded phase in order to be able to measure the true characteristic of the stationary phase. In our work, we have washed a reversed-phase column with at least 100 ml of MeOH, 150 ml of THF and 200 ml of *n*-heptane prior to chromatographic determinations. At this point polar compounds in as small a quantity as possible (<0.1 μg) were injected after every 100 ml of *n*-heptane until no increase in retention was observed. We found that unless proper washing precautions were taken, much lower k' values than the actual values were obtained.

We used small polar aromatic solutes, such as anisole, methyl benzoate and acetophenone, to measure the extent of coverage, as low sample amounts could then be employed. Non-ultraviolet active species, such as diethyl ether and MeOH, require significantly larger quantities of sample as the less sensitive refractive index detector must be used. Chromatographic peaks for such aliphatic substances are distorted due to overloading and incorrect retention times may be observed. In Table I we show k' values for the aromatic species.

Consider first phases I and II, which arise from the same batch of silica gel. In phase II, the high coverage of *n*-octyl groups still leads to a significant retention of methyl benzoate and acetophenone. It should be noted that the reproducibility in k' for any aromatic solute with heptane as mobile phase was 20% between two bonded phases from the same silica gel batch. Silanization reduced the retention of the solutes to a significant extent (phase I). Knox and Jurand[21], among others, have also noted the beneficial effects of silanization of the bonded phase.

It is interesting to compare next phases II and III in which the same reaction conditions were employed; however, two different batches of silica gel were used. Very significant differences in retention are observed, in spite of the same surface coverage and the roughly similar surface areas reported from the manufacturer. These differences are real, as the reproducibility in k' was again 20% with the same batch of silica gel. A comparison of phases I and V shows further the influence of different batches of silica. These results emphasize the need for good quality control (and perhaps the absence of micropores) of the base silica gel upon which bonding is to occur. As silanols may influence retention of polar species in RPLC, it is necessary to have reproducible silica gel in order to achieve good reproducibility in retention from column to column.

Finally, phase IV shows a very great retention of methyl benzoate even though 2.4 μmoles/m^2 of *n*-octyl groups are bonded to the silica gel. This phase will be used as a reference for studies in RPLC.

From the results in Table I we see that elution in *n*-heptane is a much more sensitive probe of unreacted silanols than the measure of surface coverage. It can be argued, however, that even this retention measure is not fully correct, since the wetting properties of *n*-heptane would be expected to be very different from those of the solvents normally used in RPLC. We have therefore explored these bonded phases in RPLC and sought their relationship to the results in Table I.

Stationary phase effects in RPLC

Bonded phases, including some of the phases in Table I, have been first examined by RPLC using acetonitrile–water (AN-H_2O, 33:67) at pH 3.3 with components such as benzene, toluene and acetophenone. In addition, as protonated amines are known to be sensitive to unreacted silanols (see *e.g.* ref. 21), we have included two basic drugs: procainamide and N-acetylprocainamide. Fig. 1 shows

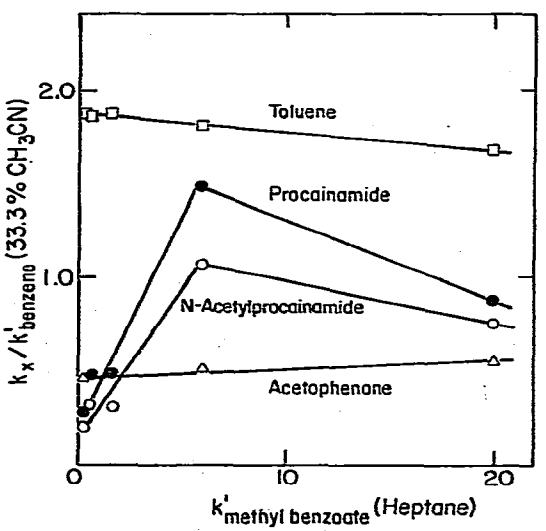

Fig. 1. Plot of retention ratio $k'_x/k'_{benzene}$ in AN–H_2O (33:67; pH 3.30) against k' of methyl benzoate in heptane for stationary phases of different coverage. The values for acetophenone are from AN–H_2O (30:70).

the results of retention of these substances as a function of k' of methyl benzoate in heptane. Note that the retention axis is normalized to the k' value of benzene.

We first note that the relative retention of toluene to benzene decreases with more exposure of silanol groups. Acetophenone, relative to benzene, appears to increase slightly with an increased number of exposed silanol groups. Thus, the contribution of hydrophobic selectivity appears to decrease with the decrease in bonded phase coverage, while the contribution of polar group selectivity increases with the decrease in surface coverage.

The behavior of the ionic species is of the greatest interest. Procainamide, which is less hydrophobic than N-acetylprocainamide, elutes later than its metabolite. The peaks for both substances are somewhat tailed in acetonitrile, but in MeOH the peak shape is much worse. We note an increase of the relative retention of each ionic species to benzene up to a certain coverage ($k' \approx 6$ for methyl benzoate on heptane). The interaction of surface silanols with ammonium ion appears to be predominant in the retention of these species.

With material of the lowest surface coverage retention was observed to decrease and peak shape was found to improve dramatically. It is at present believed that a change in retention mechanism may occur with the low coverage bonded phase material. It may well be that the relatively large number of silanols are accessible to the aqueous mobile phase, resulting in liquid–liquid partition between an imbibed aqueous phase and the mobile phase, in a manner considered by Hill[22]. While this retention behavior for basic species with low coverage bonded phase is interesting, it is obviously more desirable to maximize bonded phase coverage in order to enhance the column lifetime[17].

We next examined the influence of bonded phase coverage on the retention of non-ionic polar and non-polar aromatic compounds as a function of organic modifier type and composition in the mobile phase. For this work we used phases I (best coverage with silanization), III (high coverage but no silanization) and IV (70% of maximum coverage with no silanization). For mobile phases we employed MeOH–H_2O (50:50), AN–H_2O (30:70) and tetrahydrofuran(THF)–H_2O (25:75). As will be discussed later, these compositions have been selected to normalize the methylene group increment on phase I. The results of this study in terms of k' and group increments $\alpha^x = k'_{\varphi-x}/k'_{\varphi}$ for a series of monofunctional aromatic substances are shown in Tables II–IV.

Consider first the retention of benzene. For MeOH–H_2O, retention is found to decrease by *ca.* 35% from phase I to IV, for AN–H_2O by *ca.* 28% from phase I to IV and for THF–H_2O by only *ca.* 2%. (Note that the specific surface areas as reported by the manufacturer are approximately the same for all three phases, see Table I). We should point out that our results with MeOH–H_2O agree with Roumeliotis and Unger[19] who found significant retention differences for anthracene on bonded phases with different concentrations of unreacted silanol groups. The change of retention of hydrocarbons with coverage has been reported as due simply to the amount of stationary phase present[11]. However, the effect of solvent on this change is rather unexpected.

Consider next hydrophobic selectivity as measured by α^x for the aromatic hydrocarbons. For all three solvent systems, we observe a small but discernible trend to lower α^x values as the coverage is decreased from phase I to IV. This small loss

TABLE II

INFLUENCE OF SURFACE COVERAGE OF BONDED PHASE ON RETENTION AND GROUP CONTRIBUTION

 —R. Columns I, III and IV, see legend to Table I. $\alpha^x = k'_{\varphi-x}/k'_{\varphi}$. Mobile phase: MeOH–$H_2O$ (50:50); pH, 4.3; temperature, 30°.

Compound (R)	*I*		*III*		*IV*	
	k'	α^x	k'	α^x	k'	α^x
H	3.19	1.00	2.82	1.00	2.09	1.00
CH_3	6.38	2.00	5.51	1.95	3.81	1.83
CH_2CH_3	12.35	3.87	10.3	3.64	6.73	3.23
$CONH_2$	0.35	0.11	0.42	0.15	0.45	0.21
CH_2OH	0.96	0.30	0.88	0.31	0.79	0.38
OH	0.96	0.30	0.88	0.31	0.75	0.36
CHO	1.28	0.40	1.42	0.50	1.20	0.57
CN	1.47	0.46	1.50	0.54	1.36	0.65
CH_2CH_2OH	1.47	0.46	1.42	0.50	1.20	0.57
$COCH_3$	1.60	0.50	1.73	0.61	1.51	0.72
NO_2	2.04	0.64	2.18	0.77	1.83	0.88
OCH_3	3.00	0.94	2.86	1.01	2.19	1.05
CO_2CH_3	3.29	1.03	3.43	1.22	2.76	1.32
Cl	6.54	2.05	5.82	2.06	4.10	1.96
$CO_2CH_2CH_3$	6.48	2.03	6.36	2.26	4.82	2.31
$CO_2CH(CH_3)_2$	11.93	3.74	11.3	4.02	8.08	3.87

TABLE III

INFLUENCE OF SURFACE COVERAGE OF BONDED PHASE ON RETENTION AND GROUP CONTRIBUTION

 —R. Columns I, III and IV: see legends to Table I. $\alpha^x = k'_{\varphi-x}/k'_{\varphi}$. Mobile phase: AN–$H_2O$ (30:70); pH, 3.5; temperature, 30°.

Compound (R)	*I*		*III*		*IV*	
	k'	α^x	k'	α^x	k'	α^x
H	6.78	1.00	6.21	1.00	4.88	1.00
CH_3	13.62	2.00	12.0	1.93	9.00	1.84
CH_2CH_3	27.20	4.00	23.2	3.74	16.6	3.40
$CONH_2$	0.46	0.07	0.50	0.08	0.57	0.12
CH_2OH	1.06	0.16	1.06	0.17	1.06	0.22
OH	1.59	0.23	1.53	0.25	1.43	0.29
CHO	2.77	0.41	2.81	0.45	2.50	0.51
CN	3.62	0.53	3.68	0.59	3.29	0.68
CH_2CH_2OH	1.58	0.23	1.68	0.27	1.62	0.33
$COCH_3$	3.11	0.46	3.17	0.51	2.87	0.59
NO_2	5.42	0.80	5.36	0.86	4.60	0.94
OCH_3	6.67	0.98	6.22	1.00	5.08	1.04
CO_2CH_3	6.19	0.91	5.95	0.96	5.08	1.04
Cl	14.44	2.13	12.8	2.06	9.84	2.02
$CO_2CH_2CH_3$	12.47	1.84	11.6	1.87	9.31	1.91
$CO_2CH(CH_3)_2$	24.67	3.64	22.0	3.54	16.6	3.40

TABLE IV

INFLUENCE OF SURFACE COVERAGE OF BONDED PHASE ON RETENTION AND GROUP CONTRIBUTION

 —R. Columns I, III and IV: see legend to Table I. $\alpha^x = k'_{\phi-x}/k'_{\phi}$. Mobile phase: THF–$H_2O$ (25:75); pH 3.6; temperature, 30°.

Compound (R)	*I*		*III*		*IV*	
	k'	α^x	k'	α^x	k'	α^x
H	8.28	1.00	9.01	1.00	8.16	1.00
CH_3	16.12	1.95	17.3	1.92	15.7	1.92
CH_2CH_3	30.20	3.65	—	—	29.9	3.66
CO_2NH_2	0.50	0.06	0.50	0.06	0.63	0.08
CH_2OH	1.43	0.17	1.54	0.17	1.63	0.20
OH	3.70	0.45	3.95	0.44	3.91	0.48
CHO	2.53	0.31	2.68	0.30	2.81	0.34
CN	3.48	0.42	3.86	0.43	3.91	0.48
CH_2CH_2OH	2.10	0.25	2.26	0.25	2.37	0.29
$COCH_3$	2.58	0.31	2.85	0.32	2.88	0.35
NO_2	6.61	0.80	7.28	0.81	7.39	0.91
OCH_3	7.26	0.88	8.01	0.89	7.60	0.93
CO_2CH_3	5.32	0.64	6.01	0.67	6.04	0.74
Cl	18.64	2.25	20.2	2.24	18.7	2.30
$CO_2CH_2CH_3$	10.15	1.23	11.1	1.23	11.0	1.35
$CO_2CH(CH_3)_2$	18.31	2.21	20.0	2.22	19.4	2.38

in hydrophobic selectivity with percent carbon content has also been observed by Scott and Kucera[11]. As in the case of benzene above, the percent change in α^x from phase I to IV is largest in MeOH–H_2O and smallest in THF–H_2O. As further evidence of this trend, for *n*-alcohols (not shown in Table II) α^{CH_2} decreased by 6% from phase I to IV in MeOH–H_2O and only 2.5% in THF–H_2O. Thus, it may be concluded that under the conditions of Table II, hydrophobic retention and selectivity is most affected by MeOH and least by THF, as the surface coverage (and accessible silanol groups) is varied. From this point of view, relative to MeOH, AN and THF may lead to more reproducible results with phases which contain accessible silanols.

The question can be raised as to why the three solvents have different behavior with phases I, III and IV. In order to explore this question, we have briefly examined isopropanol(i-PrOH)–H_2O (25:75) on phases I and IV. As with MeOH, we observed a 30% decrease in k' from phase I to IV for benzene ($k' = 7.2$ and 5.1, respectively) and toluene ($k' = 16.4$ and 11.0, respectively). The dependence of retention on surface coverage for nonpolar species thus appears to be more related to the solvent class (*i.e.* functionality) of the organic modifier than to its hydrophobicity (*e.g.* i-PrOH is more hydrophobic than MeOH). It is known that the organic modifier is preferentially extracted into the hydrophobic stationary phase[10,23]. In the case of THF or AN, the polarity of the stationary phase (due to the silanols) may be more compensated than in the case of alcohols. More studies will be necessary in order to elucidate the causes of this effect.

Let us next turn to the retention of the polar monofunctional aromatic

solutes on the three bonded phase systems as a function of the three mobile phases. If we first examine MeOH–H_2O, we note that retention does not vary greatly from phase I to IV for highly polar molecules (*e.g.* benzonitrile, benzamide and benzaldehyde). As the group attached to the aromatic ring becomes more hydrophobic, a decrease in retention is, however, observed. Thus, for example, the k' value for chlorobenzene is reduced by *ca.* 35% from phase I to IV, in agreement with the trend for benzene.

The consequence of the varying trends in k' value with surface coverage and silanization for the MeOH–H_2O system is that the group contributions $\alpha^x(=k'_{\varphi-x}/k'_{\varphi})$ are a strong function of accessible silanol groups. For example, k' values of acetyl (–$COCH_3$) and cyano (–CN) groups vary by 40% from phase I to IV. Other polar group contributions vary by *ca.* 10–30% between these phases. As the group becomes more hydrophobic (*e.g.* chloro) the change in α from phase I to IV is small, since k' for benzene and the monofunctional aromatic substance change roughly to the same extent.

It is interesting to point out that the reproducibility of α^x in MeOH–H_2O (50:50) was better than $\pm 1.5\%$ on both unsilanized and silanized stationary phases from a single batch of silica gel, whereas four different batches of silica gel gave reproducibility of $\pm 3\%$ for silanized stationary phases and $\pm 6\%$ for unsilanized stationary phases. This indicates that the silanization leads to the better reproducibility of polar group selectivity. Moreover, if less than maximum coverage of bonded phase were to occur, and silanization were not performed, an even poorer reproducibility would result. Variable numbers of accessible silanol groups are undoubtedly a significant cause of poor reproducibility with particular bonded reversed-phase materials.

If we next examine AN–H_2O and THF–H_2O, we find much smaller variations in α^x values from phase I to IV. In the case of AN–H_2O the variation is *ca.* 10–30%, whereas with THF–H_2O the variation is generally less than 15%. The results in Tables II to IV indicate that surface silanols interact strongly with groups containing electron rich atoms (e.g. $>C=O$, $-C\equiv N$, $-NO_2$, $-CH_2OH$), and that organic solvents can compete with solutes for silanol sites, as shown by the smaller effect of silanols in THF-H_2O than in MeOH-H_2O.

The trends in Tables II–IV lead to some important conclusions. First, studies of polar group selectivity as a function of mobile phase composition must be performed on bonded phases with minimum accessible silanol groups. For the studies to be discussed in the next section, we have used only phase I (high coverage *n*-octyl + TMS). We observed that the differences between phase I and V (see Table I) are negligible in terms of α^x (see Fig. 1).

Second, as group contributions are most sensitive to accessible silanols using MeOH–H_2O, this mobile phase ought to be selected for determining column to column retention reproducibility. Moreover, non-polar and polar aromatic substances, e.g. acetophenone, methyl benzoate, benzene and toluene, should be simultaneously chromatographed. Finally, for the severest test of column to column reproducibility, protonated amines should be selected (see Fig. 1).

Solvent selectivity in RPLC

As was mentioned earlier, the type and composition of organic modifier can

influence both hydrophobic and polar group selectivity. As a consequence there is a complex relationship between various organic modifiers with respect to selectivity. In order to quantify effects with the ultimate hope of prediction of selectivity, we must simplify the problem. In this work, our goal has been to examine polar group selectivity of different organic modifiers relative to each other. We have therefore used mobile phase combinations in which the hydrophobic selectivity has been approximately normalized.

The hydrophobic selectivity may be assumed to be roughly constant when α^{CH_2} is the same with different H_2O–organic modifier phases. In this work, we found MeOH–H_2O (50:50), AN–H_2O (30:70) and THF–H_2O (25:75) resulted in equal methylene group increments of *ca.* 2.0.

That the constancy of the methylene group increment normalizes the hydrophobic selectivity may be seen in the following manner. Consider a simplified form of the equation of Horvath for retention in RPLC of a non-polar solute[1]:

$$\ln k' = \varphi + \frac{N\Delta A\gamma}{RT} - C + B + E \tag{1}$$

where φ is the phase ratio, ΔA the contact area decrease upon solute binding with the stationary bonded phase, N Avogadro's number, γ the surface tension, R the gas constant, T the absolute temperature, C the free energy contribution for dispersion force interaction in the association of the solute with the bonded phase, B the solvent-dependent terms and E the free energy contribution for dispersion force interaction of solute with the mobile phase. A methylene group increment then consists of the relative retention:

$$\ln k_2' - \ln k_1' = \ln \alpha^{CH_2} \approx \frac{N\Delta A^{CH_2}\gamma}{RT} \tag{2}$$

where φ and B cancel directly and C and E are assumed to roughly cancel. Thus, to a first approximation, the hydrophobic selectivity follows the surface tension of the mixed solvent. For the three mobile phase compositions cited above, we find that the surface tensions agree within *ca.* 5% of each other, *e.g.* 34.5 dynes/cm for MeOH–H_2O (50:50), 35.3 dynes/cm for AN–H_2O (30:70) and 33.8 dynes/cm for THF–H_2O (25:75) (Refs. 24 and 25). (Note that this is not a sensitive test *per se*, since the surface tension does not vary greatly with composition for all three solvent mixtures in this composition region.) It may be further pointed out that in our previous paper[2], we showed plots of log α^{CH_2} *vs.* percent organic modifier. These plots also appear to follow the variation of surface tension with composition.

We should note that because of the solvent-dependent term B in eqn. 1, the retention of a non-polar species (*e.g.* benzene) is not the same in the three mobile phase compositions. For example, in Tables II and IV k' for benzene varies from 3.19 to 8.28 from MeOH–H_2O (50:50) to THF–H_2O (25:75). An obvious second approach for comparison of solvent selectivity is to normalize the retention of a standard substance, such as benzene. This is the approach taken by Bakalyar *et al.*[8] and ourselves in our previous paper[2]. While this approach has practical value, it obviously leads to mixed group contributions (hydrophobic and polar) that are more difficult to interpret. By normalizing the hydrophobic selectivity we can study the relative polar group selectivity directly.

The retention of the monofunctional aromatic substances in Tables II and IV along with some difunctional substances and naphthalene were measured in the three solvent systems using phase I, the bonded phase with the least accessible silanol groups. In order to gain some initial insight into the differences of the three phases we have plotted in Fig. 2 the log k' values for the three solvent systems *vs.* log P (partition between octanol and water), a measure of hydrophobic behavior[26,27].

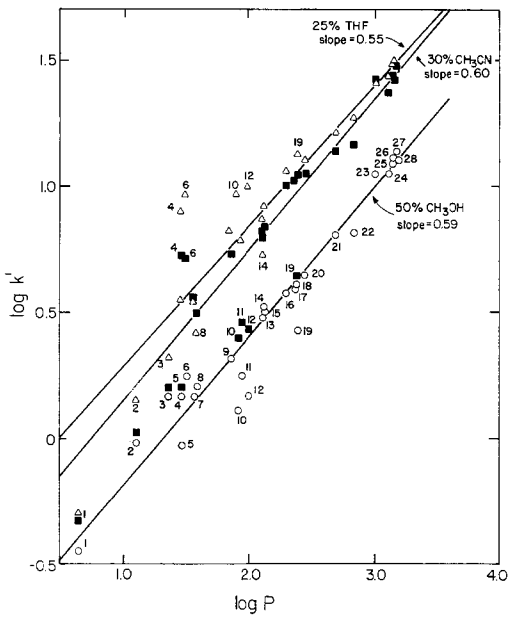

Fig. 2. Plot of log k' against log P for substituted benzenes in three solvents with stationary phase I (see Table I). Solutes are, in the order of increasing log P, (1) benzamide, (2) benzyl alcohol, (3) 2-phenylethyl alcohol, (4) *p*-dinitrobenzene, (5) phenol, (6) *m*-dinitrobenzene, (7) benzonitrile, (8) acetophenone, (9) nitrobenzene, (10) *p*-nitrophenol, (11) *p*-cresol ,(12) *m*-nitrophenol, (13) anisole, (14) methyl benzoate, (15) benzene, (16) *o*-nitrotoluene, (17) *p*-nitrotoluene, (18) *p*-nitrochlorobenzene, (19) *p*-chlorophenol, (20) *m*-nitrotoluene, (21) toluene, (22) chlorobenzene, (23) naphthalene, (24) *o*-xylene, (25) ethylbenzene, (26) *p*-xylene, (27) *m*-chlorotoluene, and (28) *m*-xylene. Slopes were calculated from solutes 15, 21, 23–26 and 28. For AN–H_2O (30:70) and THF–H_2O (25:75), numbers are listed only for the points that deviate drom the lines. ○, MeOH–H_2O (50:50); ■, AN–H_2O (30:70); △, THF–H_2O (25:75).

We have examined first only the aromatic hydrocarbons. Linear behavior is found for these substances between log k' and log P and the lines through these points are shown in Fig. 2. It can be seen that the slopes for the three mobile phases are approximately the same, indicative of the normalization of the hydrophobic selectivity.

A careful examination of MeOH–H_2O system (50:50; circles in Fig. 2) will reveal that most points are close to the straight line. Only phenols are found to deviate significantly from this line. Davis[28] has already noted that phenols can hydrogen bond with *n*-octanol, leading to a deviation from strictly hydrophobic behavior. In the case of AN–H_2O (30:70) and most particularly THF–H_2O (25:75) we observed significant

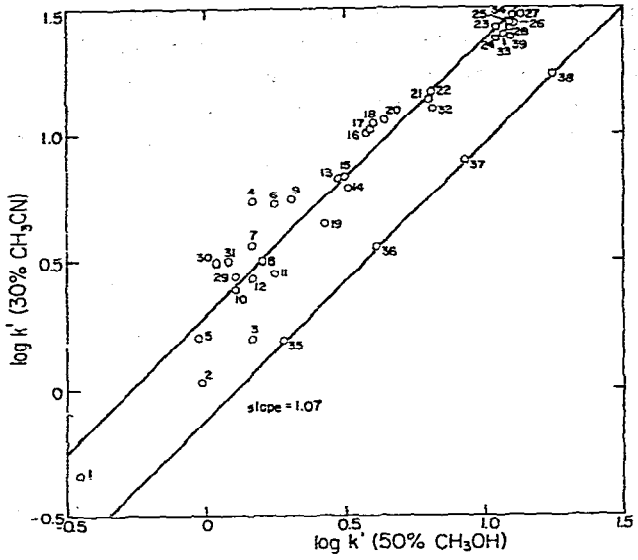

Fig. 3. Plot of log k' in AN–H_2O (30:70) against log k' in MeOH–H_2O (50:50) with stationary phase I. Solutes are the same as Fig. 2 for 1–28 and (29) benzaldehyde, (30) *m*-nitrobenzaldehyde, (31) *p*-nitrobenzaldehyde, (32) ethyl benzoate, (33) isopropyl benzoate, (34) *p*-dichlorobenzene, (35) 1-pentanol, (36) 1-hexanol, (37) 1-heptanol, (38) 1-octanol, and (39) cyclopentane.

deviations from the straight line for many substances. This means that these solvents offer specific polar group selectivities relative to MeOH–H_2O (50:50).

In order to elucidate more clearly these differences, Fig. 3 shows a plot of log k' in AN–H_2O (30:70) *vs.* log k' in MeOH–H_2O (50:50), while Fig. 4 shows a

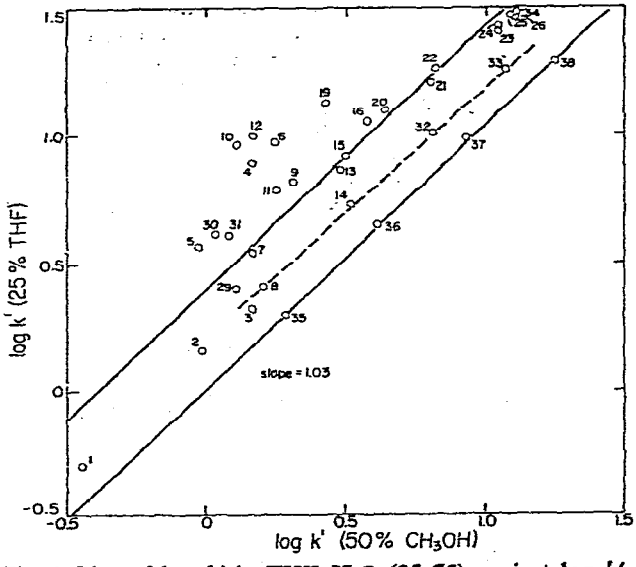

Fig. 4. Plot of log k' in THF–H_2O (25:75) against log k' in MeOH–H_2O (50:50) with stationary phase I. See Figs. 2 and 3 for solute classification.

similar plot of $\log k'$ in THF–H_2O (25:75) *vs.* $\log k'$ in MeOH–H_2O (50:50). We note that a linear relation is observed for a homologous series of *n*-alcohols with a slope close to unity, as required by the normalization of the hydrophobic selectivity. (The slope is clearly a function of the differences in methylene group increment in the two phases). A line parallel to the *n*-alcohol line has then been drawn through the point for benzene in Figs. 3 and 4. Deviations from this line give directly the relative polar group selectivity in the two phase systems. The plots thus constitute useful visual aids in observing the differences in polar group selectivity of the two phases.

We first observe that hydrophobic substances such as chlorobenzene, toluene, naphthalene and cyclopentane are close to the calibration line drawn through benzene in both figures. This result is expected from the normalization of the hydrophobic selectivity.

We next note that in general deviations from the calibration line are greater for the THF–MeOH system (Fig. 4) than the AN–MeOH system (Fig. 3). This result means that polar selectivity tends to be greater in the THF–MeOH system and that THF and MeOH would constitute an interesting pair to be used with water in a ternary mixture mobile phase for control of separation of substances with different functional groups (see later).

If we examine Figs. 3 and 4 in more detail, we observe that phenols (*e.g.* Nos. 5, 10, 12, 19) are significantly retarded in THF–H_2O (25:75), relative to MeOH–H_2O (50:50), whereas in AN–H_2O (30:70) no special selectivity for phenols is observed, relative to MeOH–H_2O. That these polar group increments are large in the case of the THF–MeOH system can be seen from the example that *m*-nitrophenol is retarded 2.8 times longer in THF–H_2O than benzonitrile, whereas both compounds showed similar retention in MeOH–H_2O. Other examples of specific retardation in THF–H_2O include dinitrobenzenes and *m*- and *p*-nitrobenzaldehyde. These species are also retarded in the AN–H_2O phase, relative to MeOH–H_2O (see Fig. 3).

Some substances are also seen to be retarded in the MeOH–H_2O phase relative to THF–H_2O. Thus, alkyl benzoates (Nos. 14, 32 and 33) are significantly retained in MeOH–H_2O. Note the linear behavior (dotted line, Fig. 4) for alkyl substitution, with a slope roughly parallel to the benzene calibration line. No special selectivity difference is seen for benzoates in the AN–H_2O *vs.* MeOH–H_2O phase systems. We also observe large retardations of *n*-alcohols (Nos. 35, 36, 37, 38) in the MeOH–H_2O phase, relative to THF–H_2O and AN–H_2O. Moreover, benzyl alcohol and benzamide are also relatively more retarded in MeOH–H_2O.

As a consequence of the trends discussed in Figs. 3 and 4, we can conclude that significant polar group selectivity differences can be observed in the different phase systems. We shall next illustrate these differences in more detail.

Fig. 5 shows the separation of 4 substances with different functional groups (*p*-nitrophenol, *p*-dinitrobenzene, nitrobenzene and methyl benzoate) with MeOH–H_2O (50:50) and THF–H_2O (25:75). It can be seen that the elution order is exactly reversed with the two phases. This example, while trivial from a separation point of view, clearly illustrates the significant role that polar group selectivity can play as the organic modifier is changed. The longer elution time in THF–H_2O (25:75) could, of course, be reduced by a several percent increase in the THF content of the mobile phase.

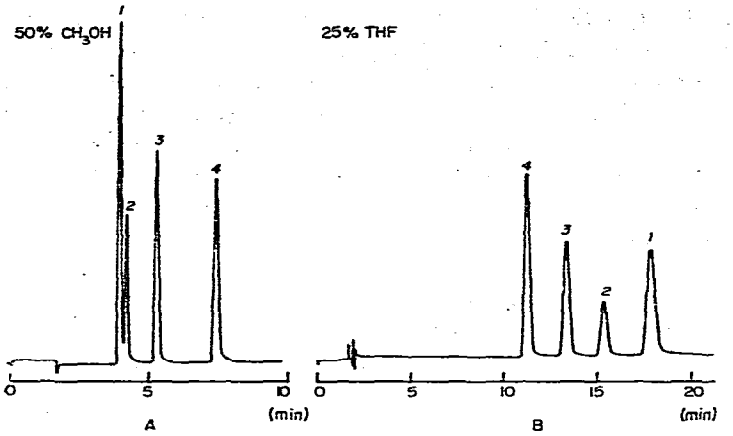

Fig. 5. Chromatograms illustrating difference in functional group selectivity caused by organic solvents with stationary phase I. A, MeOH–H_2O (50:50); B, THF–H_2O (25:75). Solutes are (1) *p*-nitrophenol, (2) *p*-dinitrobenzene, (3) nitrobenzene, and (4) methyl benzoate. Flow-rate, 1 ml/min.

Another example of polar group selectivity variations can be seen in the use of the ternary phase system THF–MeOH–H_2O. Fig. 6 shows the change in group selectivity α^x as the mobile phase is varied from MeOH–H_2O (50:50) to THF–H_2O (25:75) with intermediate ternary phase compositions of MeOH–THF–H_2O (30:10:60) and MeOH–THF–H_2O (17:17:66). The compositions were selected such that the methylene group increment was maintained constant. Also included in this figure are the absolute k' values for benzene at the different compositions.

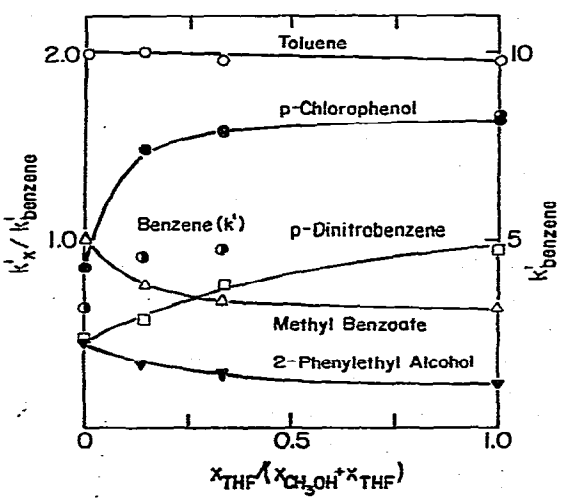

Fig. 6. Variation of $k_x/k_{benzene}$ with the change of mobile phase composition with stationary phase I. Abscissa indicates mole fraction of THF in organic solvents, while $k'_{toluene}/k_{benzene}$ was kept constant by adjusting the amount of water. Also shown are k' values of benzene at each solvent composition.

We first observe that α^x for toluene relative to benzene is fairly constant over the composition range, as expected by the normalization of the methylene group increment. However, significant variations in polar group selectivity are seen over the composition range with some groups increasing and others decreasing with addition of THF to the MeOH–H_2O phase. We further note that in general the major selectivity changes occur with the addition of small amounts of THF to MeOH–H_2O. Further addition of THF does not alter polar group selectivity greatly, but k' for benzene continues to increase. Thus, for this particular separation, small additions of THF to MeOH–H_2O should be used in order to influence selectivity without strongly affecting overall retention. The rapid change in selectivity with addition of THF to MeOH–H_2O is expected from the behavior in Fig. 4. From the results in Fig. 6, ternary phases are thus seen to be powerful tools for the control of polar group selectivity.

CONCLUSION

In this paper we have developed a procedure to examine polar group selectivity differences between several water–organic modifier mobile phases. These selectivity differences have been observed to be significant. A powerful control on polar group separation is thus possible by variation of the mobile phase composition and type. More data of this type will be necessary before one attempts to correlate these group contributions with semi-empirical classification schemes[29,30]. Ultimately, it may be possible to classify such mixed binary (and ternary) phases with respect to their hydrophobic and polar group selectivity, for estimation of mobile phase composition in order to achieve separation. We are continuing our studies in this area.

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