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ORIGINAL

A Comparison of Silica C and Silica Gel in HILIC Mode: The Effect of Stationary Phase Surface Area

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Abstract In order to assess the effect of silica gel structure on retention in hydrophilic interaction chromatography, a test system was developed which used quaternary ammonium ions as probes with tetramethylammonium acetate (TMAA) as the counter-ion competing against the interaction of the test probes with ionised silanols in the stationary phase. Four silica gel columns and a silica hydride column were examined. Retention times were obtained for the test probes at 20, 40, 60, 80 and 90 % acetonitrile (ACN) with all the mobile phase mixtures containing 10-mM TMAA buffer at pH 6.0. All phases gave "U"-shaped plots for log *k* against percentage of ACN with the steepest rise in retention occurring between 80 and 90 % ACN. Benzyltrimethylammonium, the smallest quaternary ammonium ion, was the most strongly retained probe at 90 % ACN and was most retained on a high surface area 60 Å Kromasil column and least retained on a 300 Å ACE silica gel column. The ionic strength of the mobile phase was varied at 80 and 90 % ACN and plots of log *k* against the inverse of buffer strength followed by fitting of second-order polynomial curves allowed an assessment of the contribution from HILIC to the mixed HILIC/ion-exchange retention mechanism. Toluene and pentylbenzene were used to assess the decrease in accessible pore volume due to water absorption in HILIC mode.

Keywords HILIC · Silica gel · Type C silica · Pore size · Retention mechanism

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Introduction

The term hydrophilic interaction chromatography (HILIC) was coined by Alpert in 1990 to describe chromatographic retention as a consequence of the partitioning of substances into an adsorbed water layer on a polar stationary phase [1, 2]. Although the term HILIC suggests interaction with a water layer on the stationary phase, in fact, the interactions involved are much more complicated than this and remain to be completely elucidated [3, 4]. The applications of HILIC are expanding rapidly [4], particularly in metabolomic profiling where many biomolecules are polar and do not retain well on reversed phases [5]. In HILIC, the stationary phase is either polar or charged and phases include unmodified silica gel or most commonly silica gel modified with an increasing variety of polar ligands [3]. The mobile phase used is highly organic (>70 % solvent, typically acetonitrile) containing a small percentage of aqueous solvent/buffer. The majority of HILIC stationary phases are based on silica gel and even when utilising the best bonding technology (primary bonding plus endcapping) between 30 and 50 % of the silanols on the silica gel surface are not bonded with either the primary or secondary moieties [6]. In fact, although it is often proprietary information, manufacturers of HILIC columns usually leave silanol groups uncapped so that they can contribute to the HILIC process. In previous work, we found that even some conventional alkyl

columns exhibited HILIC-like interaction [7] with basic compounds with high levels of acetonitrile (ACN) in the mobile phase. The mechanism of HILIC even on the simplest phase silica gel remains poorly understood [3, 4]. Thus even in the case of bare silica gel, without the additional complication of surface modification, there is still work to be done with regard to gaining a better understanding of the retention mechanism in HILIC mode so that it can be more fully exploited. McCalley and Neue measured the thickness of the water layer on silica gel in HILIC mode using the hydrophobic probes benzene and toluene, which were excluded from the water-filled pores of the two stationary phases studied. Using this method plus pycnometry they concluded that the percentage of the pore volume of a particular packing occupied by water under HILIC conditions varied between 13 %, with acetonitrile/water (70:30) in the mobile phase, to 4 % with acetonitrile/water (95:5) in the mobile phase [8]. Although they did not state it, from the results reported in their paper it would appear that the greater the surface area of a silica gel the larger would be the volume of the water pseudo-stationary phase. Their measurements were carried out with no pH modifier in the mobile phase, and it is likely that with pH control the volume of adsorbed water might change with the degree of ionisation and consequent solvation of silanol groups. Gritti et al. [9] used pyridine as a test probe in combination with frontal analysis and concluded that the maximum absorption excess of water in ACN water mixtures occurred with 80 % ACN in the mobile phase. No buffer modifier was used in that study. Overall, the HILIC mechanism even on a simple phase like silica gel is complex and contributors to the effect include: ionic attraction and repulsion, the effect of percentage organic solvent on the p*K*a values of weak acids and weak bases and on the ionisation of buffer salts [10] and the effect of the concentration of buffer salts in the aqueous layer associated with the silica gel surface. Thus, it would appear there is still much to explore with regard to the HILIC mechanism. A recent study used computer-based simulation to try to understand the interaction between the mobile phase and the silica gel surface [11]. From the simulation, it was observed that the layer water within ca 0.5 nm of the silica gel surface was not greatly affected by the composition of the bulk mobile phase, whereas if an absorption isotherm was plotted for water enrichment within 2 nm of the surface the maximum absorption excess for water occurred at around 5:95 water/acetonitrile. Thus, apart from pure water, there may be water-enriched layers which may contribute to retention in HILIC mode. In the water-enriched layers, there is some acetonitrile content and this would be likely to affect the degree of ionisation of acidic and basic test probes and of buffer salts.

Over the last decade, a new type of silica-based stationary phase has been developed that is based on chemically modified silica containing very few silanols on the surface. This material is referred to as silica hydride or type C silica [12, 13]. In recent work, we compared a 100 Å silica gel column and type C silica columns using a range of acidic basic and neutral test compounds [14] and found that the unmodified type C silica appeared to be more retentive than the silica gel column, particularly for basic test compounds. We were unable to completely explain our observations and it was apparent that neither the Si–H surface nor indeed the Si–OH surface was fully understood and there has not been a comprehensive exploration of the difference between the silanol-bearing surface of silica gel and the type C silica surface. Thus, the primary reason for the current investigation was to study the main factors governing retention on a silica C column in comparison with bare silica gel. In order to the remove the effect of organic solvent content on ionisation of the test probes [10] and on the buffer ions used in the mobile phase three quaternary ammonium test probes (Fig. 1) with different alkyl substitution were studied, and the modifier used in the mobile phase was tetramethylammonium acetate (TMAA) at pH 6.0. We also used the

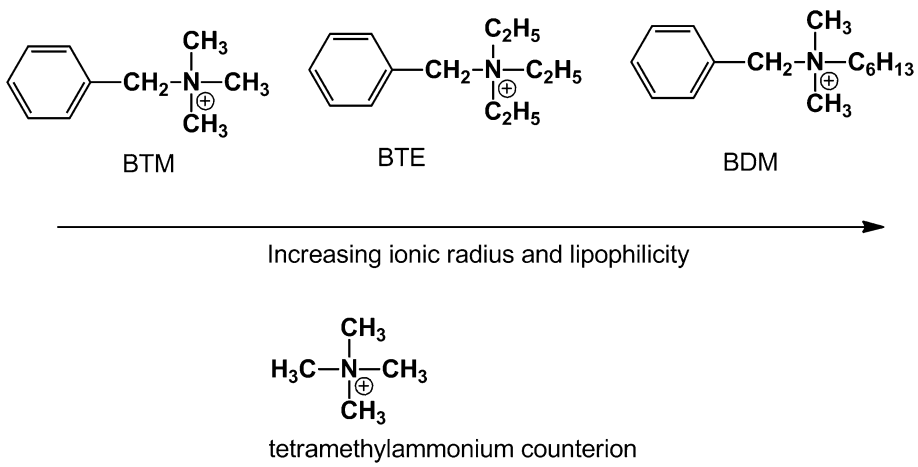

Fig. 1 Quaternary ammonium test probes and tetramethylammonium (TMAA competing counter ion)

Table 1 Manufacturers data for the columns tested (www.hichrom.co.uk) and http://www.kromasil.com/ plus V_o values determined using toluene as the test probe and anhydrous acetonitrile as the mobile phase

Column	Particle size (μm)	Pore size (Å)	Pore volume (mL g^{-1})	Packed density of silica gel (g mL^{-1})	Surface area (m^2/g)	V_o (mL)
ACE 100	5	100	–	–	300	2.1
ACE 300	5	300	–	–	100	2.1
Kromasil 60	5	60	1.2	0.45	540	2.3
Cogent Silica C	4	100	–	–	350	1.8
Kromasil 100	5	100	0.9	0.5	320	2.1

method of McCalley and Neue to estimate the thickness of the water layer on the different stationary phases at 80 and 90 % ACN and went on to examine the behaviour of the columns with acidic and neutral probes.

Experimental

Chemicals

HPLC grade acetic acid and acetonitrile were obtained from Fisher Scientific, Loughborough, UK and anhydrous ACN (<0.001 % water) and molecular sieve were obtained from Sigma Aldrich, Dorset, UK. Tetramethylammonium hydroxide, benzyltrimethylammonium chloride (BTM), benzyltriethylammonium chloride (BTE) and benzyldimethylhexyl ammonium chloride (BDM), *p*-hydroxybenzoic acid, uridine, pentylbenzene and toluene were obtained from Sigma Aldrich, Dorset, UK.

Buffer Stock Preparation

For mobile phase, preparation, stock solutions of 100 and 200 mM of tetramethylammonium acetate buffer were prepared by dissolving, respectively, 3.00 and 6.00 g of acetic acid in 300 mL of water and then adjusting to pH 6 with tetramethylammonium hydroxide. The stock solutions were then made up to 500 mL with water.

Mobile Phase Preparation

The mobile phases for studying the effect of percentage of ACN on retention of the test probes were prepared by mixing a fixed volume (50 mL) of 100-mM tetramethylammonium acetate buffer with varying volumes of water and ACN to give the required percentage of modifiers in a 500-mL final volume.

Different mobile phase concentrations were used to study the effect of ionic strength at 80 and 90 % ACN. At 80 % ACN, the mobile phases were prepared by diluting the 100- or 200-mM tetramethylammonium acetate buffer (pH 6) to 5-, 10, 15 and 20-mM in a 500-mL volumetric flask and adding a fixed proportion of 400 or 450 mL of ACN and varying amounts of buffer and water to give the required final ionic strength.

HPLC Columns

Cogent Silica C (4 μm, 100 Å-150 mm × 4.6 mm i.d.), Kromasil 60 Å and Kromasil 100 Å 5 μm silica gel (150 mm × 4.6 mm i.d.), ACE 5 100 and 300 Å, 5 μm silica gel (150 mm × 4.6 mm i.d.) were purchased from HiChrom Ltd., Reading, UK. In addition, silica hydride was prepared from 100 Å ACE silica gel removed from a commercially packed column using a previously described method [15] and was slurry packed into a column 50 mm × 2.1 mm i.d. The void volume (V_o) values for the columns were estimated by the injection of toluene after the columns had been flushed with anhydrous ACN, which was stored over molecular sieve, for 100 min at 1 mL min^{-1}. The manufacturers' data for the columns are shown in Table 1.

HPLC Instrumentation

HPLC analysis was carried out on a ThermoFinnigan HPLC system consisting of a P4000 pump, P6000 diode array detector and an AS4000 autosampler (ThermoFisher, Hemel Hempstead, UK) and Kontron 480 column oven (Kontron Instruments, Munich, Germany). The mobile phase compositions were mixed off-line in the proportions required as described above, and the HPLC system was used in isocratic mode. The injection volume was 5 μL, the flow rate was 1 mL min^{-1}, and the column was kept in column oven at 22 °C. The diode array detector was set to monitor specifically 263 nm, and the full PDA range was 200–350 nm.

Sample Preparation

Samples were prepared as stock solutions at 0.1 % w/v in methanol and diluted to a concentration of 100 μg mL^{-1} with the appropriate mobile phase starting composition.

Results and Discussion

Since high levels of organic solvent affect the pK_a values of weak acids and bases, increasing them and lowering them, respectively [10, 16], quaternary ammonium test probes with a positive charge which is unaffected by pH were chosen. Also the permanently charged competing counter-ion in the mobile phase, tetramethylammonium, was used to avoid any effect on its percentage ionisation due to the organic solvent content in the mobile phase. Thus, the only charge which could be affected by the percentage organic solvent was that on the silanol groups within the silica gel stationary phases. It is uncertain exactly what effect percentage ACN has on the ionisation of silanol groups, there might be little effect if, as suggested by Melnikov et al. [11], there is a 0.5-nm water layer next to the surface of the silica gel which is independent of the mobile phase composition. This layer is sufficiently thick for the silanol group p*K*a values, and hence pH dependent ionisation, to remain unaffected by the content of ACN in the mobile phase. The order of charge density on the test probes is BTM > BTE > BDM, and their lipophilicity is the reverse of this order (log *p* values calculated by ChemDraw: −2.44, −1.05 and 1.07, respectively).

Figure 2 shows the plots of log *k* for the test probes against percentage ACN for 60 and 100 Å Kromasil silica gel columns, a 300 Å ACE silica gel column and a Cogent Silica C column. In all cases, the most retained test probe at 20 % v/v ACN is BTE. If the mode of retention at 20 % v/v ACN was predominantly ion exchange, then it would have been expected that the more charge-dense BTM probe would have been more strongly retained. It has been known for many years that the siloxane bonds in bare silica gel are lipophilic, as a result of their inability to hydrogen bond which results from strong interaction between the lone pairs on the oxygen and the unoccupied d-orbitals of the flanking silicon atoms [6]. Thus, it is possible to propose that a major component in the retention of the test probes at 20 % ACN must be lipophilic interaction with the siloxane bonds of the silica gel. The least charge dense of the test probes BDM is almost as strongly retained as BTE, and this would suggest that the overall retention of these test probes at 20 % ACN is due to a combination of lipophilicity and ion exchange with the BDM exhibiting weaker ion exchange than BTE due to its larger size but having strong enough lipophilic interaction to retain longer than the more charge-dense BTM. Since the ion-exchange interactions of BTM should be greater than those of BTE, the additional retention of BTE over BTM must be indicative of lipophilic interaction. Thus, if the difference in the log *k* values for BTE and BTM at 20 % organic modifier is taken as being approximately indicative of lipophilic interaction it can be seen from Fig. 2 that the lipophilic interaction is greatest for the high surface area Kromasil 60 silica gel and for the Cogent Silica C column. As the percentage of ACN in the mobile phase increases, the retention of the test probes initially falls as lipophilic interaction is reduced. Thus, the minima in the plots of log *k* against percentage ACN at around 60 % ACN indicate the point where ion exchange is mainly responsible for the retention of the test probes. Above 60 % ACN, hydrophilic interaction begins to exert an effect and the most hydrophilic and most densely charged probe, BTM, is the most sensitive to the establishment of the HILIC layer which, as proposed by Melnikov

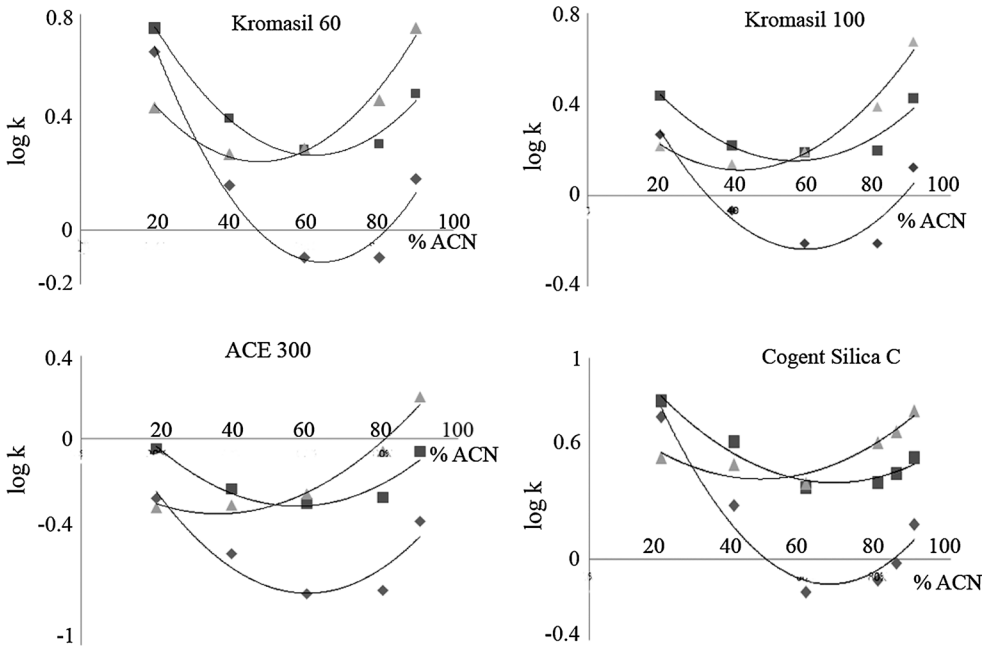

Fig. 2 Plots showing the approximate effect of percentage acetonitrile on log *k* for the test probes (BTM *black up-pointing triangle*, BTE *black square*, BDM *black diamond suit*) run on the following columns: Kromasil 60, Kromasil 100, ACE 300 and Cogent Silica C (all 4.6 × 150 mm) with 10 mM TMAA in each mobile phase composition

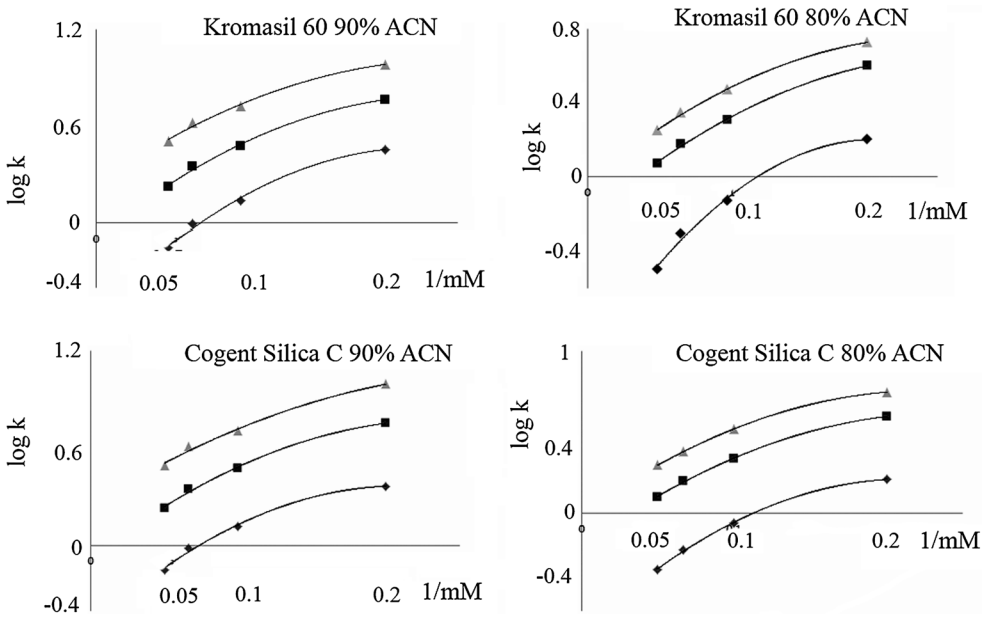

Fig. 3 Plots of log *k* against the inverse of the buffer strength for the test probes (BTM *black up-pointing triangle*, BTE *black square*, BDM *black diamond suit*) on Kromasil 60 and Cogent Silica C columns with 80 % acetonitrile and 90 % acetonitrile as the mobile phase modifier

et al., is composed of regions with varying degrees of water enrichment as well as a fixed layer of water close to the surface of the silica gel. The retention of the other two probes also increases above 60 % ACN although the retention of the BDM is generally lower at 90 % ACN than at 20 % ACN again indicating that it is exhibiting relatively weak ion exchange and, at 90 % ACN, very little hydrophilic interaction. Taking the retention of BTM as being most indicative of the development of a HILIC layer, the 100 and 300 Å ACE silica gel columns show the most marked increase in log *k* up to 90 % ACN although overall the 60 Å Kromasil column gives the strongest retention of BTM at 90 % ACN.

In order to distinguish the relative contribution of HILIC and ion exchange to the retention on the different columns, the molarity of the TMAA buffer was varied within the mobile phases containing 80 % v/v ACN and 90 % v/v ACN. The retention times of the test probes varied by $< \pm 2$ % between runs ($n = 3$). Figure 3 shows plots of log *k* against 1/mM for the 60 Å Kromasil column and the Cogent Silica C column at 80 % ACN and 90 % ACN. Such plots would be expected to be linear if the retention mechanism was due to a single parameter, as is accepted in models proposed for reversed-phase partition chromatography [17]; but the plots were curved and it was possible to fit second-order polynomial curves through the points indicating that a mixed mode retention mechanism was in operation. The r^2 values for the curves fitted according to second-order polynomials were >0.99 apart from 5 out of the 36 curves, where r^2 was between 0.969 and 0.989. According to the hypothesis proposed by McCalley [18], the intercepts for these retention plots with the *y*-axis would occur with infinite ionic strength in the mobile phase where there is no possibility of ion-exchange interactions between the stationary phase and the test probes. Thus, because of the absence of ion-exchange interactions at infinite ionic strength the intercepts indicate the contribution of HILIC to the overall retention [18, 19]. The curves remain hypothetical and are only intended as a guide to what may happen. Table 2 shows the intercepts obtained from the polynomial plots for each column [19].

As judged from the intercepts, the greatest HILIC interactions occurred with BTM on all columns. In Table 3, data for the retention factors obtained for the probes and the intercepts obtained from the polynomial plots are combined and the percentage contributions of HILIC to retention factor are shown at each buffer strength. From this data, it can be seen that increasing ionic strength increases the percentage contribution of HILIC to the overall retention and this varies between about 15 % at 5 mM buffer strength to 50 % or over at 20 mM buffer strength. In addition, it is apparent that the contribution of HILIC to retention at the different buffer strengths, in most cases, does not vary as much as might be expected. This is clearest at 20 mM, where the RSD overall percentage contributions from HILIC for all the probes run on all the columns is ± 16 %. Furthermore, the data suggest that hydrophilic partitioning increases in proportion to the ion-exchange capacity of a stationary phase. If the surface area per gram for the different stationary phases is plotted against the *k* values of the probes at 90 % ACN, Fig. 4 is obtained which shows a degree of correlation between *k* values and surface area. Figure 5 shows chromatograms of BTM on the Kromasil 60 and 100 columns and on the Cogent Silica C column. The peak shapes obtained for the quaternary ammonium probes were generally good. The Cogent Silica C column, which is based

Table 2 Intercepts for the polynomial curves fitted through the plots of log *k* against 1/ionic strength (mM) and the slopes obtained for plots of log *k* against log ionic strength (mM)

Column	Analyte	*y* intercepts for binomial curves at 90 % ACN	*y* intercepts for binomial curves at 80 % ACN	Slope of log *k* plotted against log mM at 90 % ACN	Slope of log *k* plotted against log mM at 80 % ACN
ACE 100	BTM	−0.0974	−0.312	−0.724	−0.854
	BTE	−0.434	−0.511	−0.856	−0.920
	BDM	−0.876	−1.15	−0.984	−1.11
ACE 300	BTM	−0.346	−0.693	−0.822	−0.826
	BTE	−0.756	−0.959	−0.944	−0.992
	BDM	−1.33	−2.14	−1.18	−1.68
Kromasil 60	BTM	0.241	−0.0263	−0.782	−0.793
	BTE	−0.0918	−0.213	−0.889	−0.874
	BDM	−0.550	−0.987	−1.01	−1.14
Kromasil 100	BTM	0.166	−0.1902	−0.788	−0.812
	BTE	−0.173	−0.371	−0.871	−0.885
	BDM	−0.563	−1.10	−0.968	−1.15
Cogent Silica C	BTM	0.250	−0.00050	−0.817	−0.756
	BTE	−0.0766	−0.202	−0.862	−0.827
	BDM	−0.499	−0.732	−0.843	−0.923
Silica hydride based on 100 Å ACE	BTM	−0.166	−0.636	−0.692	−0.933
	BTE	−0.504	−0.975	−0.7685	−1.15
	BDM	−0.916	−4.01	−1.02	−1.17

The retention times of the test probes varied in all cases by < ±2 % (usually < ±0.1 %) between runs ($n = 3$)

on a 100 Å silica gel with a high surface area (Table 1), behaves just like a silica gel column where the retention of the test probes depends on ionic strength despite the fact that it is claimed that there are very few free silanol groups in this stationary phase. The silica hydride column, which was prepared in house exhibited slightly stronger HILIC interaction than the 100 Å ACE silica from which it was prepared, but exhibited much less HILIC interaction than the Cogent Silica C column.

In order to further differentiate ion exchange and HILIC contributions to the retention mechanism, a neutral and an acidic test probe were run on the Kromasil 60 Å, the Kromasil 100 Å and the Cogent Silica C column. Figure 6a shows the plots of the log *k* values for the two test probes against percentage ACN for the Kromasil 60 Å column. The plots obtained reflect the low lipophilicity of the probes, which are not strongly retained with 20 % v/v ACN in the mobile phase. Above 60 % v/v, there is a marked increase in retention as the HILIC effect increases. For the acidic and neutral probes, there can be no additional ion-exchange contribution to retention. With a mobile phase containing acetonitrile/buffer (80:20) there was no marked effect evident from plotting 1/ionic strength against the retention factors of uridine, but the retention time of hydroxybenzoic acid increased with increasing ionic strength suggesting that ionic repulsion effects were being overcome as the concentration of the TMAC counter-ion increased (Fig. 6b). In a mobile phase containing acetonitrile/buffer (90:10), ionic strength had no marked effect on the retention of uridine or on the retention of hydroxybenzoic acid (Fig. 6c). The lack of effect on hydroxybenzoic acid might be explained by the fact that the concentration of the TMAC counter-ion is likely to be higher in the HILIC layer with 90 % ACN in the mobile phase, since its partition coefficient will be altered in favour of the aqueous phase.

Following the idea of McCalley and Neue [8], to get some estimation of the thickness of the absorbed water layer on the silica gel columns toluene and pentylbenzene were used as probes. Since it was proposed that some of the HILIC activity resides in water-rich regions rather than pure water [11], pentylbenzene should be sensitive to this because it would be less likely to partition into regions of the HILIC layer which are enriched with water. Table 4 shows the V_o values of the probes under different conditions; the experiments were carried out at a flow rate of 1 mL min^{-1}, thus retention volumes and retention times are equivalent. As might be expected, the 60 Å Kromasil column had the largest *Vo* with an apparent volume of 2.3 mL which was determined in 100 % anhydrous ACN with no modifier being added. From the data in table 1, it would be expected from that a 15 × 0.46 mm Kromasil 60 column

Table 3 The variation in the percentage contribution of HILIC to the overall capacity factor for the different columns estimated from the intercepts of the polynomial plots of log *k* against 1/ionic strength (mM)

90 % acetonitrile					80 % acetonitrile			
Kromasil 60								
mM	5	10	15	20	5	10	15	20
BDM	9.8	20.2	28.1	40.9	6.3	13.4	20.4	31.4
BTE	13.9	26.9	36.2	48.4	15.5	30.5	40.7	52.4
BTM	18.0	32.6	41.7	54.2	17.5	31.7	42.3	52.7
Kromasil 100								
BDM	10.9	21.1	32.4	41.0	5.9	12.1	19.6	29.4
BTE	13.9	25.5	37.2	45.9	13.6	25.8	36.4	46.6
BTM	17.4	30.7	43.2	51.3	14.4	24.1	35.4	43.8
ACE 100								
BDM	8.1	14.9	21.5	33.3	6.7	13.6	21.9	31.5
BTE	12.1	20.9	29.0	40.9	13.3	26.3	36.8	47.6
BTM	16.3	25.6	33.4	45.1	14.2	25.6	36.8	46.0
ACE 300								
BDM	6.1	13.7	20.5	32.9	1.6	5.1	7.9	19.1
BTE	11.9	23.6	32.9	45.2	10.8	22.4	31.6	43.6
BTM	17.8	33.1	43.3	56.5	14.3	25.1	35.6	45.2
Cogent Silica C								
BDM	13.6	23.9	32.6	44.6	11.4	21.2	30.8	41.3
BTE	14.7	27.8	37.5	49.1	15.8	28.8	39.7	49.7
BTM	17.9	34.8	43.6	56.9	17.8	30.0	41.6	50.3

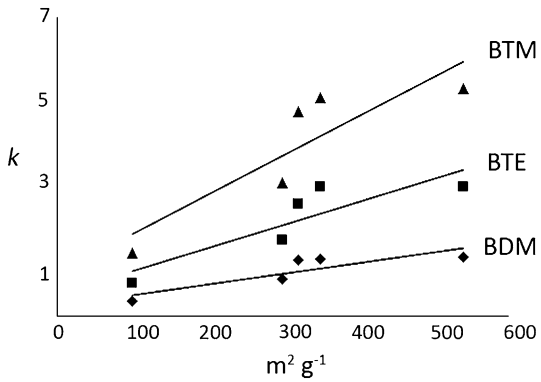

Fig. 4 Plot of retention times for the test probes (BTM *black up-pointing triangle*, BTE *black square*, BDM *black diamond suit*) on five columns, with acetonitrile/10 mM TMAA buffer (90:10) as the mobile phase, against silica gel surface area

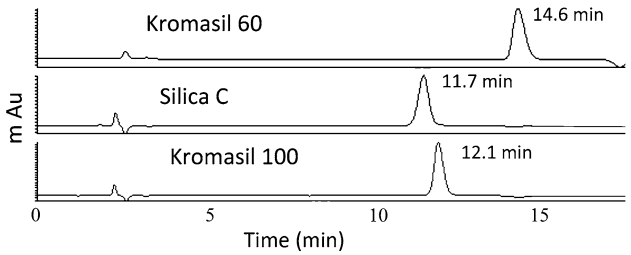

Fig. 5 HPLC chromatograms for the test probe BTM run on three columns with acetonitrile/10 mM TMAA buffer (90:10) as the mobile phase. UV detection at 263 nm

(2.5 cm^2 total volume) that it would have an internal pore volume of 2.5 × 0.45 × 1.2 = 1.35 mL. The interstitial pore volume produces most of the additional contribution to the total V_o value. In the mobile phase containing acetonitrile/buffer (90:10), there was a marked decrease in the residence time of toluene from 2.3 to 1.9 min indicating a decrease in available internal pore volume of 0.4 mL. This equates to about 29.6 % of the internal pore volume of the column being occupied by water. This is higher than the volume estimated by McCalley and Neue, which was around 6.9 % occupation of the internal pore volume at 90 % ACN; however, their estimates were made without buffer in the mobile phase where there would be much less ionisation of the silanol groups of the silica gel. McCalley and Neue observed a large change in the thickness of the water layer on the silica gel moving between 10 and 20 % water in the mobile phase with the percentage of the internal pore volume occupied by water being around 11.1 % at 20 % water. In the current case, there was a negligible change in the residence time of toluene upon moving from 10 to 20 % buffer. This observation is more consistent with

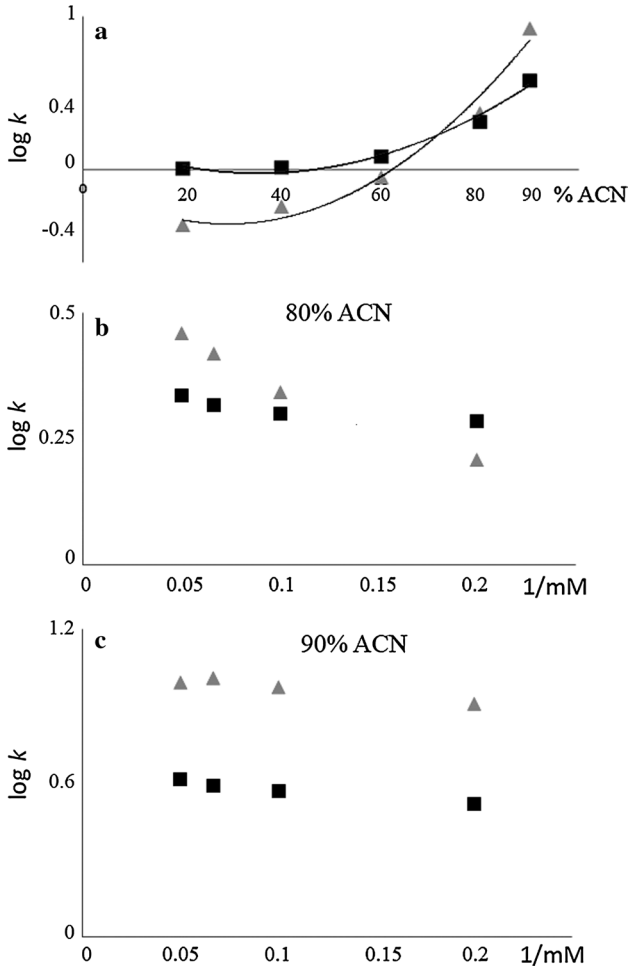

Fig. 6 **a** Plots of log *k* against % acetonitrile, **b** and **c** plots of the effect of 1/ionic strength (mM) on a neutral probe (uridine *black square*) and an acidic probe (*p*-hydroxybenzoic acid *black up-pointing triangle*) at 80 and 90 % acetonitrile in the mobile phase, respectively

the fact that the retention times of the quaternary ammonium test probes increased markedly between 20 and 10 % buffer driven by decreased affinity for the high organic solvent content in the mobile phase and a stable water layer close to the surface of the silica gel. The residence time of the pentylbenzene probe with 10 % buffer in the mobile phase was similar to that of toluene, but there was marked decrease in V_o of 0.2–1.7 mL with 20 % buffer in the mobile phase suggesting that there might be an increase in a water-enriched layer into which the very lipophilic probe does not partition. The 100 Å Kromasil column, as might be expected, had a lower V_o than the 60 Å column of 2.1 mL (from the data in Table 1 its internal pore volume would be expected to be 1.125 mL) and moving to 10 % buffer reduced the residence volume of toluene by 0.3 mL suggesting that about 26 % of the original internal pore volume is occupied by water which is in line with the lower HILIC contribution to retention on the 100 Å Kromasil column. The Cogent Silica C behaved differently from the silica gel columns apparently having a smaller total pore volume of 1.8 mL. The data to enable calculation of the internal pore volume of the Silica C column is not available, since at 90 % ACN there was only a 0.1 mL decrease in the pore volume occupied by water. Thus, although similar to silica gel the behaviour of Silica C is not identical. A recent study used microcalorimetry to measure water absorption on a silica hydride column in comparison with the base silica gel from which it was prepared [20]. The silica hydride surface adsorbed methanol more strongly than the base silica gel, and it was proposed that this was due to bonding with Si–H groups. In addition, it was also observed that the water excess adsorption on the silica hydride column was lower than that on silica gel, which is consistent with our current observations of a lower effect on the internal pore volume for this phase.

Conclusion

There is still a lack of knowledge of the processes which occur on the simplest HILIC phase, silica gel and this is a hindrance to fully exploiting the HILIC mechanism. While different silica gels have been explored previously with regard to their HILIC properties, there has been no systematic study of the influence of silica gel surface area on HILIC. McCalley proposed a model [18], which was later used by Bicker et al. [19] to dissect out the relative contributions of ion exchange and HILIC for different HILIC columns. In the current study, we clearly observed that an increase in silica gel surface area led to an increase in hydrophilic interaction. For bases, the contribution of HILIC to overall retention increases as the ionic strength of the competing counter-ion is increased, and if it is desired that the contribution of HILIC to retention should

Table 4 Void volumes for the alkyl benzene test probes on the different columns with 80 and 90 % acetonitrile in the mobile phase a 10 mM TMAA

Column	V_o toluene			V_o Pentyl benzene		
	100 % ACN	90 % ACN	80 % ACN	100 % ACN	90 % ACN	80 % ACN
Kromasil 60	2.3	1.9	1.9	2.2	1.8	1.6
Kromasil 100	2.1	1.8	1.7	2.1	1.8	1.6
Cogent Silica C	1.8	1.7	1.6	1.8	1.7	1.5

be promoted then it would be better to work at higher ionic strength. However, the binomial curve fitting used to assess the contribution of HILIC to overall retention neglects the effect of the ionic strength in the HILIC layer. Although we have yet to study this, the ionic strength of the mobile phase modifier is likely to be much higher in the HILIC layer than in the bulk, since ionic modifiers will partition strongly into the aqueous layer. The level of such modifier partitioning should also depend on the particular counter-ions used, both anions and cations which again provide a topic for future study.

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