Hydrophilic Interaction Liquid Chromatography: an Investigation into the Solvent and Column Selectivity

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This article summarises the results of an investigation into hydrophilic interaction liquid chromatography (HILIC) focusing on the retention mechanisms of ten stationary phases. Additionally to a column characterisation study, the effect on retention of changing acetonitrile content, column temperature, mobile phase pH and buffer concentration was investigated. The understanding and characterisation of HILIC retention mechanisms coupled with a better understanding of how common experimental parameters affect the separation mechanism will allow not only the judicious selection of the column, but also the appropriate HILIC conditions when developing separations.

Introduction

The ability to retain and separate polar and hydrophilic molecules can be very challenging during method development. If using conventional reverse phase liquid chromatography (RPLC), ion pair reagents, mobile phase pH modification, concentrated buffers or highly aqueous mobile phases have to be employed. Such options have potential detrimental effect upon column stability, mass spectrometric detection and sample solubility, and often still offer poor retention. If using normal phase liquid chromatography, poor reproducibility and difficulty in interfacing with mass spectrometry can be expected.

Hydrophilic interaction liquid chromatography (HILIC) is a viable alternative for the analysis of polar compounds. HILIC has been described as 'reversed reversed phase liquid chromatography' [1], where the stationary phase is either polar or charged (for example, unmodified silica, amino, cyano, amide, diol or ion exchange bonded phases). The mobile phase is highly organic (>70% solvent, typically acetonitrile) containing also a small percentage of aqueous solvent/buffer. The water/buffer solvent forms an aqueous-rich sub-layer adsorbed to the polar surface of the stationary phase into which polar analytes preferentially partition. The resulting retention order is approximately the opposite of the order analytes elute from a reversed phase chromatographic system [2].

McCalley and Neue demonstrated the existence of the water-rich layer on the silica surface under the typical HILIC conditions [3];

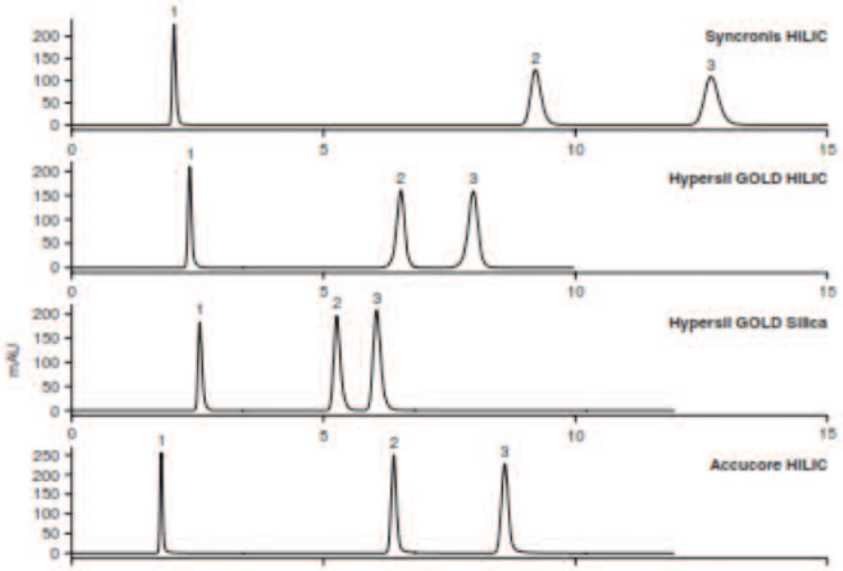


Figure 1: Chromatograms for α (CH2) test. Analyte: 1) toluene; 2) 5-methyluridine; 3) uridine.

Column Name	Phase type	Column dimension (mm)	Surface area (m^2/g)	Pore size(Å)
Syncronis HILIC (5 µm)*	Zwitterion	100 x 4.6	320	100
Hypersil GOLD HILIC (5 µm)*	Polyethyleneimine	100 x 4.6	220	175
Hypersil GOLD Silica (5 µm)*	Unbonded Silica	100 x 4.6	220	175
Accucore HILIC (2.6 µm)*	Unbonded Silica	100 x 4.6	130	80
Acclaim HILIC-10 (3 µm)	Proprietary^	150 x 4.6	300	120
Acclaim Mixed Mode HILIC-1 (5 µm)	Mixed Mode Diol	150 x 4.6	300	120
Hypersil GOLD Silica (1.9 µm)	Unbonded Silica	100 x 2.1	320	100
Syncronis Silica (5 µm)	Unbonded Silica	100 x 4.6	220	90
Experimental HILIC (3 µm)	Polyacrylamide	150 x 3.0	100	90
Acclaim Trinity P1 (3 µm)	NSH**	150 x 3.0	100	300

*Table 1: Specifications of the HILIC columns used. ** NSH: Nanopolymer Silica Hybrid. Columns marked with the asterisk were used in the chromatographic parameters investigation*

they were also able to observe that the water-rich layer increased in thickness as the aqueous content in the mobile phase increased up to 30%. The authors provided indirect evidence that hydrophilic partitioning is the main mechanism experienced at higher water content but other interactions (such as hydrogen bonding) might become more relevant as the water content decreases.

Subsequently, McCalley demonstrated the existence of a very complex mechanism, consisting of a combination of hydrophilic partitioning, adsorption, ionic interactions and even hydrophobic interactions [4]. Other authors also support this view, Liang et al. proposed a HILIC retention model, where the predominant mechanism depends on the analyte characteristics, the mobile phase composition and the nature of the stationary phase [5].

In HILIC an increase in the percentage of organic solvent leads to an increase in the retention times for polar analytes [1]; this phenomenon was investigated in our laboratory, particularly in order to assess whether partitioning is the main retention mechanism. Electrostatic interactions are secondary forces which can have important contributions to the retention in HILIC, since some polar compounds can be charged at the mobile phase pH conditions typically used [6]. We therefore investigated the electrostatic interaction contribution, by assessing the effect of mobile phase pH and salt concentration/salt type on the retention of polar acids and polar bases.

Column temperature is an important parameter that can also affect retention of polar analytes in HILIC [6]. The equation that is often used is derived from chemical thermodynamics, where the equilibrium point is related to the temperature, and is referred to as the van't Hoff equation. In a chromatography sense the relationship between column temperature and retention factor is often described by the following:

$\ln k' = -\Delta H°/RT + \Delta S°/R + \ln \Phi$

where:

- $\Delta H°$ = enthalpy of interaction between stationary/mobile phase and analyte
- $\Delta S°$ = entropy of interaction between stationary/mobile phase and analyte
- R= universal gas constant
- T= column temperature in Kelvin
- Φ= phase ratio

The van't Hoff equation should also apply to

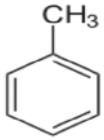
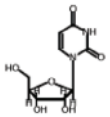
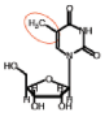
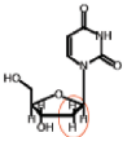
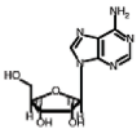
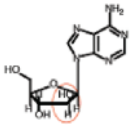
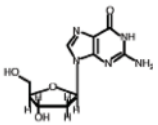
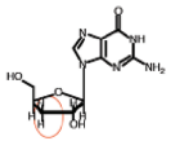
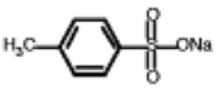
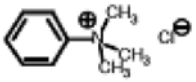
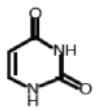
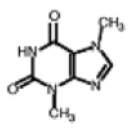
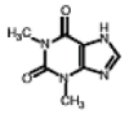
Chromatographic probes	Molecular Structure	Variable	pKa	LogD	Test Mixture
Toluene		t_0 marker	41	2.72	all
Uridine		Hydrophilic/ hydrophilic interaction	12.6	-1.58	1+2
5-Methyluridine		Hydrophobic interaction	12.0	-1.02	1
2'-Deoxyuridine		Hydrophilic interaction	13.9	-1.26	2
Adenosine		Configurational isomers selectivity	13.9	-1.03	3
Vidarabine		Configurational isomers selectivity	13.9	-1.02	3
2' -Deoxyguanosine		Positional isomers selectivity	13.5	-1.14	4
3'-Deoxyguanosine		Positional isomers selectivity	13.5	-1.14	4
Sodium p-toluenesulfonate		Anion exchange selectivity	-2.8	0.88	5
N,N,N-trimethylphenylammonium chloride		Cation exchange selectivity		-2.31	6
Uracil		Hydrophilic interaction	13.8	-1.08	5,6
Theobromine		Acidic-basic nature of stationary phase	10	-1.06	7
Theophylline		Acidic-basic nature of stationary phase	8.6	-0.5	7

Table 2: Structures of characterisation test solutes and their physiochemical properties (pKa and Log D values obtained from www.chemspider.com)

HILIC if the retention mechanism is consistent, in which case the relationship between ln k' and 1/T is linear [6].

Although it has been demonstrated that the organic modifier/aqueous ratio is the predominant factor in providing the necessary separation selectivity in HILIC [6], the choice of stationary phase is also very important. Chirita et al. suggested a column selection scheme and applied it to neurotransmitters analysis [7]. They advocated choosing HILIC columns according to the nature of the interactions between analyte and stationary phase. Fine-tuning the separation by optimising the organic solvent content, the buffer concentration and the mobile phase pH should follow in the decision tree. This approach to HILIC method development highlights the importance of column selection. Given the fact that the stationary phases used in HILIC are quite diverse (Hemström and Irgum described more than forty separation materials used for HILIC applications [1]), choosing the optimal column can be very challenging.

Systematic studies of HILIC materials chemistries and the roles of their functional groups have been limited. A combination of these factors has lead to confusion and difficulties during HILIC column selection for method development. Pontén recently commented on the fact that users are under the impression that 'HILIC columns' are interchangeable, despite the difference in the chemical structure of the various HILIC stationary phases [8]. This misconception has probably been encouraged by the increasing interest and demand for HILIC methodologies which in turn resulted in an increase in the 'HILIC' branded products. HILIC column comparison studies have been undertaken in the last few years; however, these studies concerned specific classes of compounds and probed specific interaction modes, but without discussing partial structure selectivity [4, 9-12].

More recently, both Ikegami's [13] and Irgum's [14] groups, independently suggested two comprehensive and seminal characterisation studies to classify HILIC columns and investigate HILIC retention mechanisms, focusing on specific interactions. Irgum and co-workers designed a method based on selectivity factors for pairs of similar chemical compounds, one with properties promoting the particular interaction being assessed and the second one lacking such properties. The HILIC interactions characterised by Irgum and his group were: hydrophilic, hydrophobic, electrostatic, hydrogen bonding, dipole-dipole, π-π interaction and shape selectivity [14].

Ikegami's group followed a similar approach but using different test compounds in their HILIC characterisation work [13]. The method they suggested could probe specific secondary interactions, namely: degree of hydrophilicity, selectivity for hydrophilic-hydrophobic groups, selectivity for positional and configurational isomers, evaluation of electrostatic interactions and evaluation of the acidic-basic nature of the stationary phases. The data from this study showed structure-selectivity relationship for the various HILIC phases and represent a good approach to HILIC column selection for when targeting separations whose analytes possess some of the same structural characteristics.

This testing scheme was applied in our laboratory, for examining columns with the following chemistries: bare silica, zwitterionic-, amino-, amide-, mixed-mode diol-, mixed-mode RP/anion-exchange/cation exchange (** in Table 1, Nanopolymer Silica Hybrid, NSH)- phases and a silica phase covalently modified with an hydrophilic group and an anion-exchanger (^ in Table 1).

The work presented in this paper can be divided into two main categories: the stationary phase characterisation and the investigation into the chromatographic parameters that have a major role in the HILIC selectivity for acidic and basic compounds.

Materials and Methods

Chemicals and reagents

HPLC grade acetonitrile, water and toluene, analytical grade ammonium acetate, ammonium formate and Optima grade acetic acid were obtained from Fisher Scientific (Loughborough, UK). Uridine, 5-methyluridine, 2'-deoxyuridine, adenosine, vidarabine, 2'-deoxyguanosine, 3'-deoxyguanosine, uracil, sodium p-toluenesulfonate, N,N,N-trimethylphenylammonium chloride, theobromine, theophylline, cytosine, cytidine, salicylamide, salicylic acid, aspirin and 3,4-dyhydroxyphenylacetic acid were purchased from Sigma-Aldrich (Poole, UK).

Chromatographic tests

The chromatographic conditions were kept unaltered throughout the column characterisation study; the mobile phase consisted of 90:10 (v/v) acetonitrile:ammonium acetate (20mM on the column, pH 4.7). The flow rate was fixed at 0.5mL/min. UV detection was carried out at 254nm. The injection volume was 5µL. All runs were done with active thermostatting of the columns at 30°C. The columns assessed in this study are reported in Table 1. They cover a range of surface chemistry and physical properties (with regards to particle size and pore size). All the columns were from Thermo Scientific (Runcorn, UK).

Retention factors were determined as the average of six injections and toluene was used as an unretained marker (t_0).

The chromatographic conditions used to investigate the effect of other experimental factors on retention had various mobile phase compositions, which were prepared by mixing the desired volumes of acetonitrile and stock buffer solutions. The pH of the salt solutions was adjusted before mixing with acetonitrile, but for the pH study only. The salt concentrations reported in the individual results sections refer to the final concentrations of the salt on the column. The flow rate was fixed at 1.0mL/min. UV detection was carried out at 228nm for the acid mixture and 248nm for the basic mixture. The injection volume was 5µL. The column temperature was maintained at 30°C. The columns assessed in this study are also reported in Table 1.

Instrument

Chromatographic experiments were carried out on an Accela UHPLC system (Thermo Scientific, San Jose, USA). ChromQuest 5.0 (Thermo Scientific, San Jose, USA) was used to control the UHPLC system, and to process the chromatographic data.

Test Mixtures

Characterisation tests

All the stock solutions for the individual test probes were prepared in mobile phase at 1mg/mL. The test mixtures comprised selected pairs of compounds that were expected to vary in their interactions with the stationary phases, plus the t_0 marker. A total of seven test mixtures were prepared and they were:

- test mixture 1: t_0, uridine (U), 5-methyluridine (5MU)
- test mixture 2: t_0, uridine, 2'-deoxyuridine (2dU)
- test mixture 3: t_0, adenosine (A), vidarabine (V)

- test mixture 4: t_0, 2′-deoxyguanosine (2dG), 3′- deoxyguanosine (3dG)
- test mixture 5: t_0, uracil (Ur), sodium p-toluenesulfonate (SPTS)
- test mixture 6: t_0, uracil, N,N,N-trimethylphenylammonium chloride (TMPAC)
- test mixture 7: t_0, theobromine (Tb), theophylline (Tp)

The chemical structures of the test compounds used in this study, together with their physiochemical properties are given in Table 2.

Chromatographic parameters investigation

All the standard stock solutions for the individual test probes were prepared in 80:20 acetonitrile: water at 1mg/mL. Two test mixtures were then prepared: a basic test mixture, comprising uracil, adenosine, uridine, cytosine and cytidine; an acid test mixture, comprising salicylamide, salicylic acid, aspirin and 3,4-dihydroxyphenylacetic acid (dhpa).

Results

Characterisation tests

Hydrophobic Selectivity

The degree of hydrophobic interaction between the stationary phase and the test compounds is a useful characteristic in both RPLC [13] and HILIC. It can be measured from the selectivity for a methylene group, α (CH_2). Ikegami et al. obtained α (CH_2) from a comparison of k uridine (retention factor for uridine) and k 5-methyluridine (retention factor for 5-methyluridine), which differs by a CH_2. Uridine and 5-methyluridine were chosen as the α (CH_2) probe pair since they are polar enough to afford retention in HILIC [13]. Figure 1 shows chromatograms for four representative columns.

From Figure 1 it can be seen that uridine is more retained than 5-methyluridine, which reflects the fact that uridine is more hydrophilic than 5MU.

Hydrophilic Separation

The degree of hydrophilic interaction between the stationary phase and the test compounds was assessed using the selectivity for an hydroxy group, α (OH). In this study α (OH) was obtained from a comparison of k uridine and k 2′-deoxyuridine. Figure 2 shows chromatograms for four representative columns, where it can be seen that uridine is

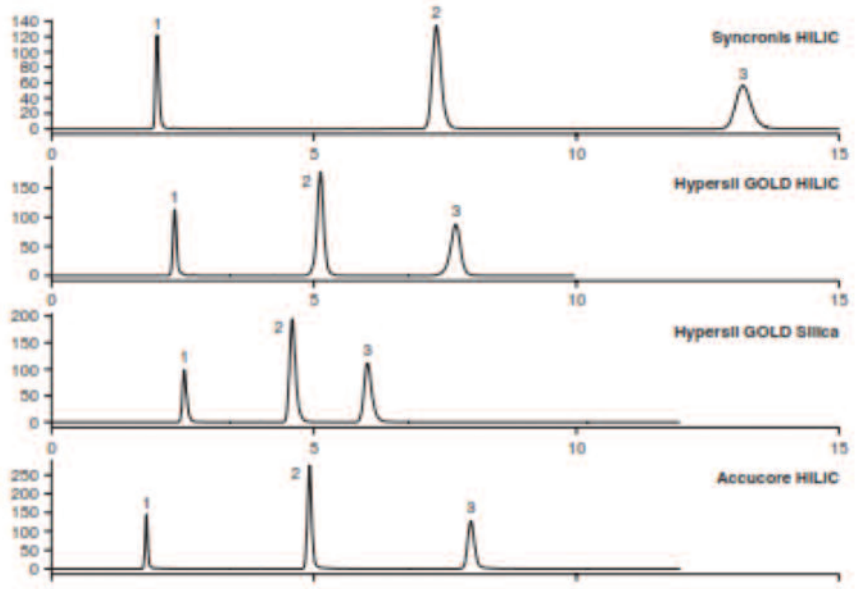


Figure 2: Chromatograms for α (OH) test. Analyte: 1) toluene; 2) 2′-deoxyuridine; 3) uridine.

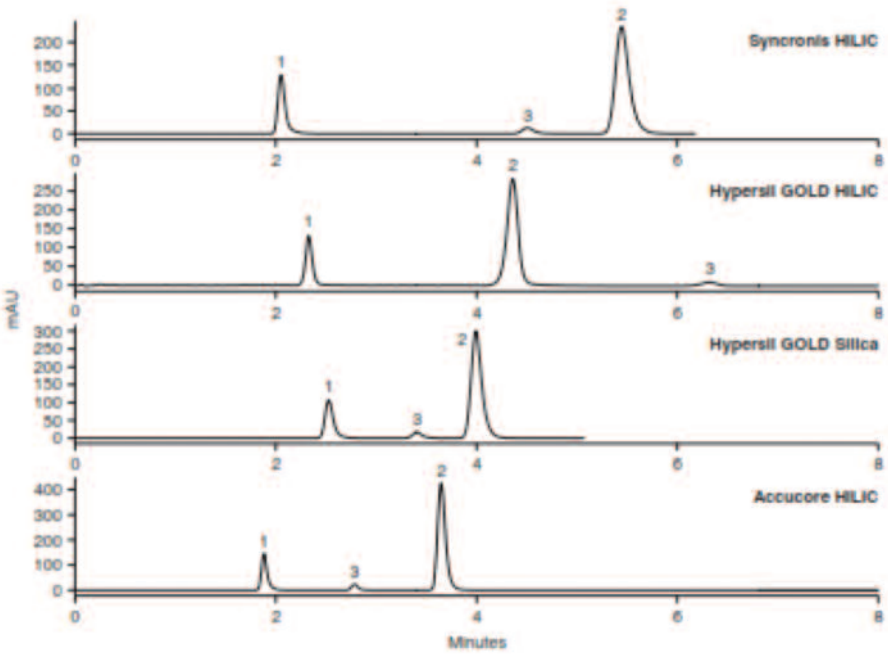


Figure 3: Chromatograms for α (AX) test. Analyte: 1) toluene; 2) uracil; 3) sodium p-toleuenesulfonate, SPTS.

Column Name	α (CH2)	α (OH)	k uridine
Syncronis HILIC (5 µm)	1.477	2.090	5.053
Hypersil GOLD HILIC (5 µm)	1.330	1.931	2.278
Hypersil GOLD Silica (5 µm)	1.291	1.697	1.377
Hypersil GOLD Silica (1.9 µm)	1.253	1.579	1.340
Syncronis Silica (5 µm)	1.302	1.518	3.152
Accucore HILIC (2.6 µm)	1.473	1.942	3.753
Acclaim Mixed Mode HILIC-1 (5 µm)	1.000	1.000	0.112
Acclaim HILIC-10 (3 µm)	1.117	1.521	1.836
Acclaim Trinity P1 (3 µm)	1.226	1.828	0.869
Experimental HILIC (3 µm)	1.530	2.182	3.513

Table 3: Separation factors for methylene α (CH2) and hydroxy α (OH) groups and retention factor for uridine.

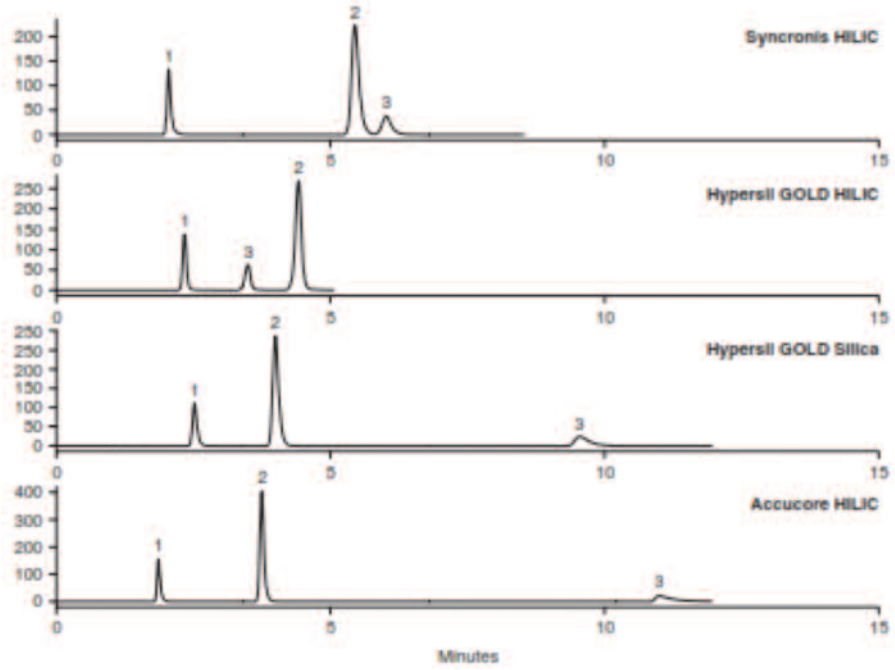


Figure 4: Chromatograms for α (CX) test. Analyte: 1); 2) uracil; 3) N,N,N-trimethylphenylammoniumchloride, TMPAC.

Column Name	α (V/A)	α (2dG/3dG)
Syncronis HILIC (5 µm)	1.403	1.129
Hypersil GOLD HILIC (5 µm)	1.444	1.082
Hypersil GOLD Silica (5 µm)	1.255	1.092
Hypersil GOLD Silica (1.9 µm)	1.214	1.092
Syncronis Silica (5 µm)	1.270	1.100
Accucore HILIC (2.6 µm)	1.327	1.114
Acclaim Mixed Mode HILIC-1 (5 µm)	1.000	1.102
Acclaim HILIC-10 (3 µm)	1.222	0.963
Acclaim Trinity P1 (3 µm)	1.409	1.023
Experimental HILIC (3 µm)	1.336	1.111

Table 4. Separation factors for configurational isomers α (V/A) and positional isomers α (2dG/3dG)

more retained than 2'-deoxyuridine; this reflects the fact that uridine is more hydrophilic than 2dU. The average α (CH_2), α (OH) and kuridine values are summarised in Table 3 and reported in Figure 5.

Structural and Configurational Isomers

An important property that HILIC can offer is the ability to separate structural isomers and configurational isomers, typically found in saccharides and peptides. The selectivity of configurational isomers, α (V/A) - obtained from a comparison of k vidarabine and k adenosine, α (V/A) - and positional (regio) isomers, α (2dG/3dG) - calculated from the k 2dG/k 3dG ratio - were investigated. These selectivity values also reflect shape selectivity [13]. Table 4 and Figure 5 summarise the mean α (V/A) and α (2dG/3dG) values for the stationary phases tested.

Ion Exchange Interactions

Ion-exchange interactions can be influential in HILIC, leading to drastic changes in selectivity, particularly when separating ionic species [15]. To evaluate the degree of ion exchange nature of the stationary phases a relatively hydrophobic organic anion, sodium p-toluenesulfonate (SPTS) and a relatively hydrophobic organic cation, N,N,N-trimethylphenylammoniumchloride (TMPAC) were chosen to see the effect of anion and cation interactions [13]. It is reasonable to postulate that these compounds would also be retained by hydrophilic interactions [13], so the retention factors k SPTS and k TMPAC were divided by k Uracil to account for the hydrophilic interaction contribution. The chromatography for both the anion and

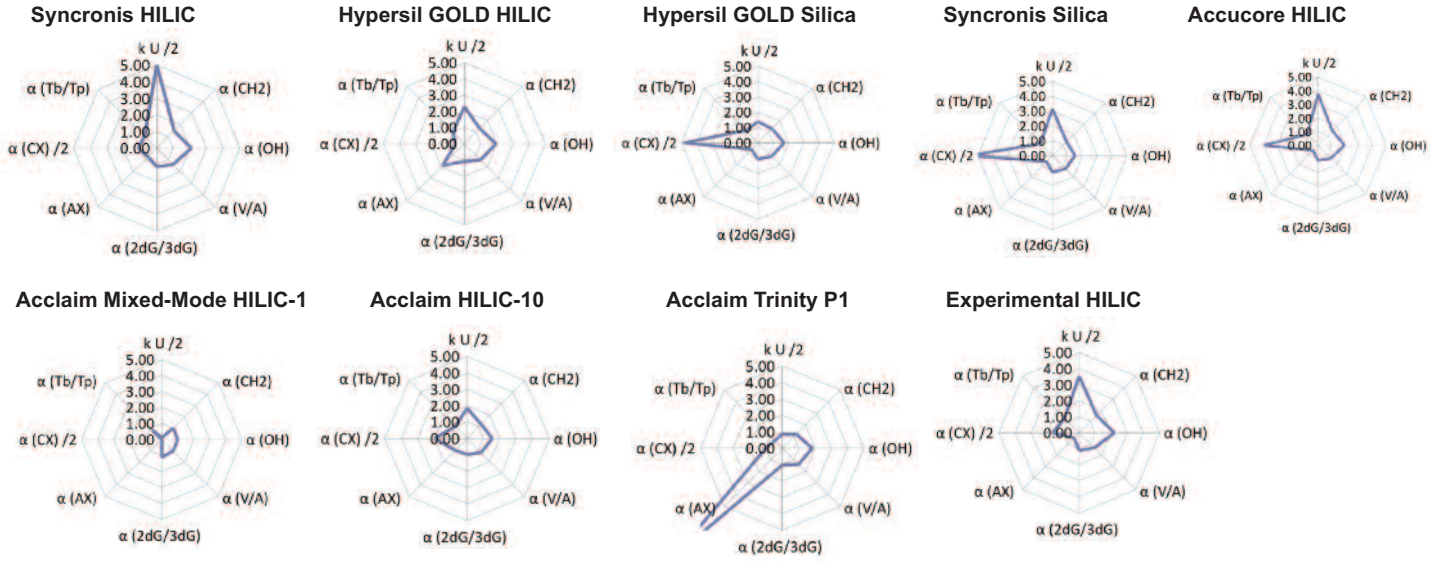


Figure 5: Radar plots for HILIC stationary phases

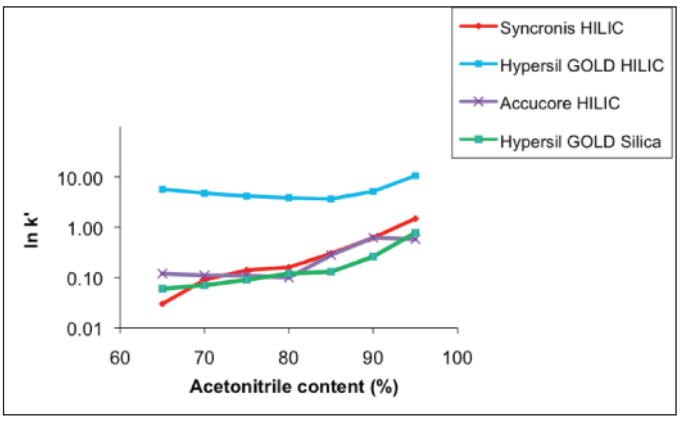


Figure 6a

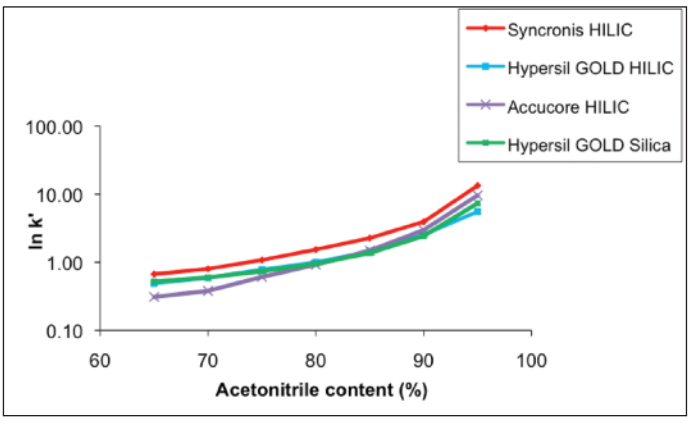


Figure 6b

Figure 6a and 6b. Effect of acetonitrile content on the retention of salicylic acid (a) and cytosine (b)
Column temperature: 30°C. Flow rate: 1mL/min. UV detection at 228nm for salicylic acid and 248nm for cytosine.

cation exchange interactions on the selected columns is shown in Figure 3 and Figure 4, respectively. The resulting mean separation factors, α (AX) and α (CX) for the stationary phases tested are reported in Table 5; the values are also plotted in Figure 5.

Figure 3 and Table 5 highlight the variability in elution orders obtained for the different columns. The majority of columns show that the elution order is toluene (t_0 marker), uracil, and SPTS, although Hypersil GOLD HILIC and Acclaim Trinity P1 have a reversal of the two test analytes. Anion exchange selectivity also varied for Acclaim Mixed Mode HILIC-1, where SPTS was not retained and it eluted before toluene and for Acclaim Mixed Mode HILIC-10, where SPTS co-eluted with uracil.

From Figure 4 and Table 5 a similar set of results demonstrating a variability in elution order can be seen when performing the cation test. Thus, it can be observed that for some materials TMPAC elutes after uracil, apart from Hypersil GOLD HILIC, where it elutes before uracil. Different cation exchange selelctivity was also exhibited by Acclaim Mixed Mode HILIC-1 and Acclaim Trinity P1, with TMPAC co-eluting with uracil.

Column Name	α (AX)	α (CX)
Syncronis HILIC (5 µm)	0.723	1.115
Hypersil GOLD HILIC (5 µm)	1.878	0.554
Hypersil GOLD Silica (5 µm)	0.609	4.832
Hypersil GOLD Silica (1.9 µm)	0.549	5.951
Syncronis Silica (5 µm)	0.581	5.614
Accucore HILIC (2.6 µm)	0.521	3.992
Acclaim Mixed Mode HILIC-1 (5 µm)	-	0.000
Acclaim HILIC-10 (3 µm)	1.000	1.919
Acclaim Trinity P1 (3 µm)	9.241	1.000
Experimental HILIC (3 µm)	0.454	1.660

Table 5: Separation factors for α (AX) and α (CX)

Column Name	α (Tb/Tp)	pH conditions of stationary phase
Syncronis HILIC (5 µm)	1.000	Neutral
Hypersil GOLD HILIC (5 µm)	1.000	
Acclaim HILIC-10 (3 µm)	1.000	
Acclaim Mixed Mode HILIC-1 (5 µm)	0.860	Basic
Acclaim Trinity P1 (3 µm)	0.671	
Syncronis Silica (5 µm)	1.151	Acidic
Hypersil GOLD Silica (1.9 µm)	1.102	
Hypersil GOLD Silica (5 µm)	1.091	
Accucore HILIC (2.6 µm)	1.189	
Experimental HILIC (3 µm)	1.269	

Table 6: Separation factors for α (Tb/Tp)

Acidic-Basic nature of the stationary phase surface

Since many compounds analysed in HILIC have ionisable functional groups, knowing the acid-base properties of the stationary phase is important for controlling the separation [13]. Xanthine derivatives have been used as test samples in HILIC; the pKa values for theophylline and theobromine have been reported as pKa= 8.6 and pKa= 10 respectively, so theobromine is more basic than theophylline. If an HILIC mode separation applies, an elution order of theophylline < theobromine would be expected, based on their log D values.

It follows that the selectivity values, k Tb/k Tp should be > 1 [10]. From the selectivity data obtained during our investigation (reported in Table 6 and Figure 5) it can be seen that this is the case for the acidic bare silica materials. This elution order is the opposite

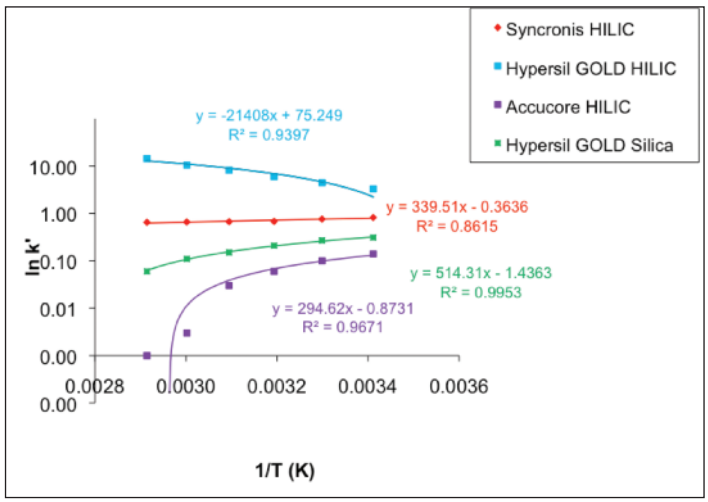


Figure 7a

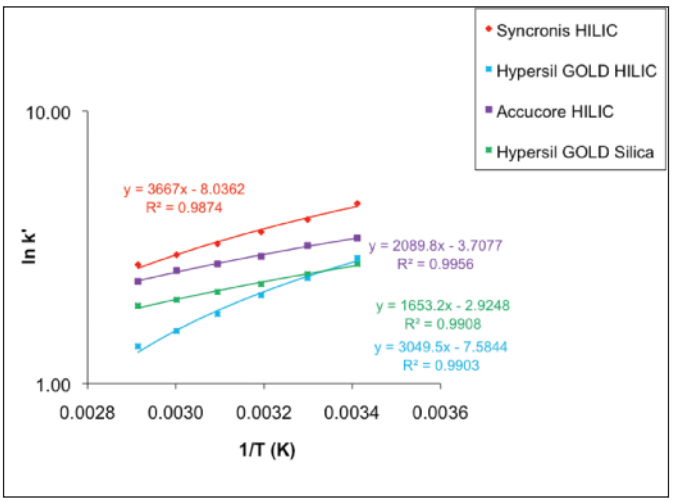


Figure 7b

Figure 7a and 7b. Effect of column temperature on retention of salicylic acid (a) and cytosine (b). Mobile phase: acetonitrile/water (90/10, v/v), containing 10mM ammonium acetate.

on the basic Acclaim Mixed Mode HILIC-1, which produced a negative selectivity value; the stronger retention of theophylline on this material would indicate an additional ionic interaction between the more acidic theophylline and the column surface as compared to theobromine. On the remaining columns the two xanthines co-elute, resulting in selectivity values = 1.

Chromatographic parameters investigation: their effect on HILIC retention

The effect of acetonitrile content on retention

In HILIC, the level of organic solvent in the mobile phase can have a large influence on retention. Separation of the two test mixtures was carried out on the four stationary phases (marked with an asterisk in Table 1). The percentage of acetonitrile in the mobile phase was varied whilst keeping ammonium acetate concentration constant at 10mM. The logarithmic retention factors (ln k) for two model compounds (salicylic acid and cytosine) were plotted against the acetonitrile content (Figure 6a and 6b respectively).

Salicylic acid shows HILIC behaviour of decreasing retention with decreasing acetonitrile content on Syncronis HILIC and on Hypersil GOLD Silica. On Hypersil GOLD HILIC and on Accucore HILIC the retention of salicylic acid decreases initially as the acetonitrile content decreases, but as this reaches 80% the retention gradually increases. This behaviour could be due to ion-exchange interactions. Cytosine shows HILIC behavior of decreasing retention with decreasing acetonitrile content on the four stationary phases. This is also true for the other components of the basic test mixture.

The effect of column temperature on retention

In this study the temperature effect on the retention of salicylic acid and cytosine was investigated for the four stationary phases; the column temperature was varied from 20 to 70°C. The logarithmic retention factors for the two model compounds were used to generate van't Hoff diagrams. Figure 7a shows van't Hoff plots for salicylic acid and Figure 7b for cytosine, on the four selected columns. From Figure 7a it can be seen that a decrease in retention, indicating a negative heat of enthalpy and hence an exothermic reaction, is observed as the column temperature is increased on Accucore HILIC, Hypersil GOLD Silica and to a lesser extent on Syncronis HILIC. The retention increases with the temperature on Hypersil GOLD HILIC. Positive enthalpy was obtained for Hypersil GOLD HILIC, indicating an endothermic process of transferring salicylic acid from the mobile phase to the stationary

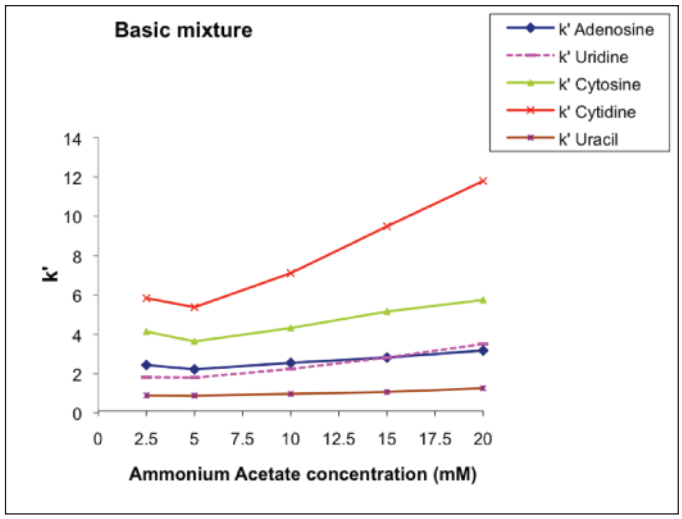


Figure 8a

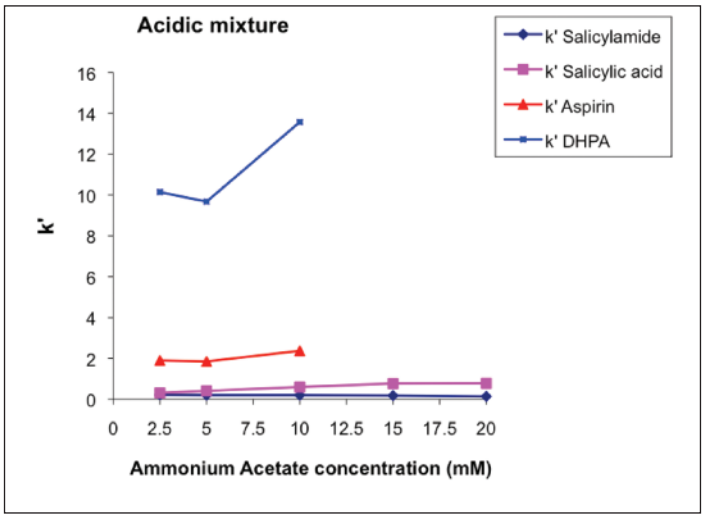


Figure 8b

Figure 8a and 8b. Effect of buffer concentration on retention of basic and acidic mixtures, for Syncronis HILIC.

phase. For Hypersil GOLD HILIC, the positive enthalpy of salicylic acid could also be the evidence of both ion exchange and partitioning processes taking place.

Figure 7b shows a decrease in retention on the four stationary phases as the column temperature is increased. Linear van't Hoff plots resulted from this study. Negative retention enthalpy values were obtained, indicating an exothermic process. The enthalpy values derived were very different, which could indicate the existence of strong specific (secondary) interactions between cytosine and the functional groups of the four stationary phases [6].

The effect of buffer concentration on retention

In this study the effect of salt concentration on the retention was investigated. Separation of the two test mixtures was carried out on Syncronis HILIC and Hypersil GOLD HILIC. The content of acetonitrile in the mobile phase was kept constant, whilst varying the ammonium acetate concentration from 2.5 to 20 mM. The capacity factors for the two model compound mixtures were plotted against the buffer molar concentrations for the two columns. Figure 8a and 8b show the data relative to the basic and acid test mixtures respectively obtained on Syncronis HILIC. The five bases are fully resolved on Syncronis HILIC when low levels of ammonium acetate are used (2.5, 5 and 10 mM). Further increase in the salt content leads to an increase in retention, with co-elution of adenosine and uridine. Apart from uracil, the other 4 bases show an increase in retention as the ammonium acetate concentration is

Column	pH	k salicylic acid	k aspirin	k cytosine	k cytidine
Accucore HILIC					
	3.3	0.21	0.43	3.07	3.49
	4.0	0.25	1.26	3.22	3.88
	4.8	0.24	1.61	3.12	3.80
	6.4	0.26	1.79	3.23	3.98
Hypersil GOLD HILIC	3.3	3.73	0.50	3.34	6.50
	4.0	3.89	0.54	3.42	6.56
	4.8	3.98	5.70	3.22	6.02
	6.4	4.18	5.90	3.26	6.11
Syncronis HILIC	3.3	1.11	0.97	4.88	8.40
	4.0	1.17	2.15	4.90	8.82
	4.8	1.06	2.60	4.62	8.13
	6.4	1.06	2.60	4.68	8.23
Hypersil GOLD Silica	3.3	0.40	0.61	2.41	2.55
	4.0	0.43	1.20	2.49	2.76
	4.8	0.42	1.43	2.41	2.66
	6.4	0.40	1.50	2.37	2.64

Table 7. The effect of buffer pH on retention of model compounds

increased above 5mM, the increase being more pronounced for cytidine. Interestingly, the retention decreases when the buffer concentration is increased from 2.5 to 5 mM. The general trend of increased retention as the buffer concentration increases has been related to a hydrophilic partitioning process [6]. The partitioning model for HILIC assumes the presence of a water-rich liquid layer on the stationary phase. Salt is more soluble in the aqueous rich sub-layer, so increasing the organic will effectively increase the concentration in this layer. The four acids are not fully resolved on Syncronis HILIC, as shown in Figure 8b, with salicylic acid co-eluting with salicylamide when low levels of ammonium acetate are used (2.5, 5 and 10 mM). Further increase in the salt concentration to 15mM leads to an increase in retention for salicylic acid and salicylamide. It is possible that electrostatic repulsions from the negatively charged sulphonate groups prevented the acid molecules from reaching the quaternary amine groups (located closer to the silica surface), resulting in lower retentions. Higher salt concentration could have weakened these repulsions, leading to salicylic acid and salicylamide being more retained. Aspirin elutes as a split peak and dhpa is very broad when the salt content was increased to 15mM and above.

Figure 9a and 9b show the data relative to the basic and acid test mixtures respectively, obtained on Hypersil GOLD HILIC.

To elucidate the different behaviours exhibited by the two columns, a comparison,

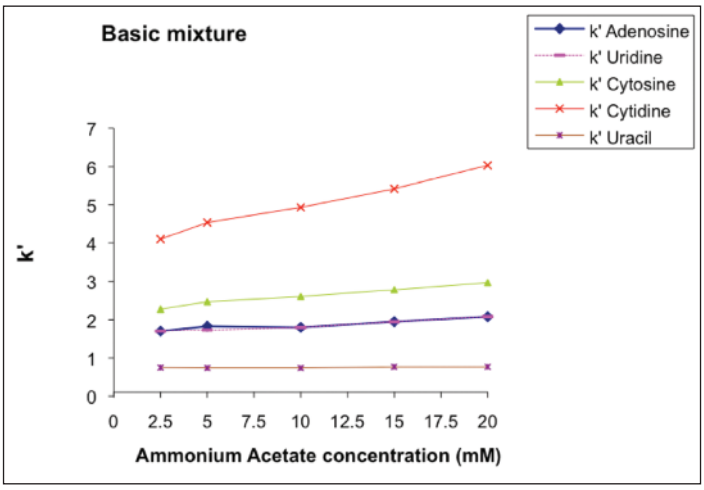


Figure 9a

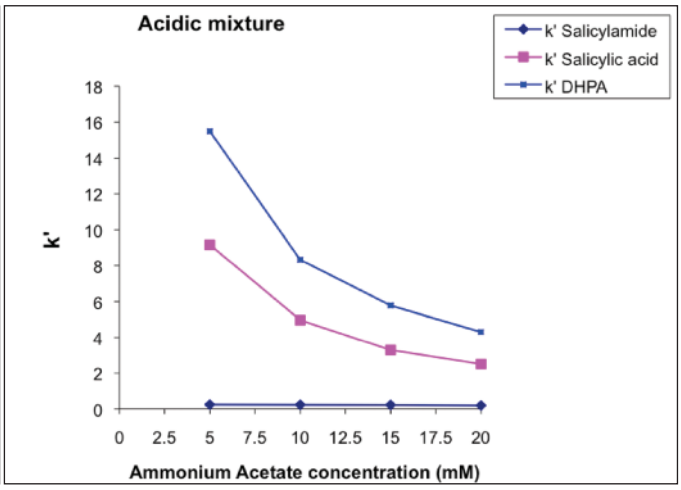


Figure 9b

Figure 9a and 9b. Effect of buffer concentration on retention of basic and acidic mixtures, for Hypersil GOLD HILIC.

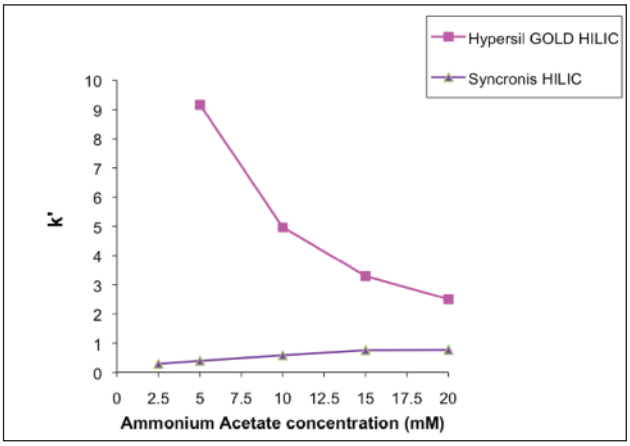


Figure 10. Effect of buffer concentration on retention of salicylic acid.

in terms of salicylic acid retention, is given in Figure 10. On Hypersil GOLD HILIC salicylic acid exhibits an opposite behaviour to what observed on Syncronis HILIC, with a dramatic decrease in retention, as the buffer concentration increases. The ion exchange interaction on this phase could have a major effect on the retention of acids. Salicylic acid is charged (mobile phase buffer pH= 6.7, pKa for salicylic acid= 2.9) and its retention drops as the buffer concentration increases (pH of mobile phase increases), which is typical of an ion exchange mechanism.

The effect of mobile phase pH on retention

Mobile phase pH can have a significant contribution on the variation of retention and selectivity in HILIC separations [6]. In this study the pH of stock salt solutions was changed before mixing with acetonitrile. Ammonium formate was used (because of its relatively low buffering range, pH 2.8-4.8); the pH of the 100mM ammonium formate stock solution (pH~ 6.4) was adjusted with formic acid to pH 3.3, 4.0 and 4.8. Table 7 summarises the retention data of the model compounds on the four columns. The retention is affected when the analyte ionisation state changes in the pH range considered for the study. This is the case for aspirin, where its retention gradually decreases with the buffer pH on the two silica materials, since aspirin is protonated and less hydrophilic. A similar behaviour is demonstrated by salicylic acid on Hypersil GOLD HILIC.

Aspirin retention is instead unchanged in the buffer range 6.4-4.8 on the zwitterionic material, but then the retention time drops when the pH is lowered to 3.3, probably due to ion-exchange effect no longer taking place. Cytidine and cytosine retentions only fluctuate slightly on the four columns in the pH range investigated.

Discussion

Characterisation tests

The data sets for the separation factors generated in this study highlighted important retention characteristics and differences (Table 3-6). Stationary phase characteristics have been visually illustrated by radar graphs [13, 16], which allow to express multi-dimensional data in a two-dimensional format and ultimately allow to visually assess and compare columns. The separation factors obtained in the course of this assessment were therefore arranged in radar plots, which are shown in Figure 5. The following discussion will concern both tabulated data and their corresponding graphical representations.

From the radar plots illustrated in Figure 5 it is interesting to note that there is a positive correlation, for all of the materials, between α (CH_2) and α (OH)which matches the observations by Ikegami and his group [13]. A tentative interpretation for this observation is that the chemistry of the stationary phases does not have a substantial role on the selectivity of these two particular groups. Alternatively, the k uridine data demonstrate that the stationary phase chemistry has an effect on the absolute retention, probably due to the absolute volume of the water layer. It can be seen that the bare silica materials, the Trinity P1 and the mixed mode HILIC-1 exhibit lower values for k uridine. Syncronis HILIC demonstrated to be the most retentive material for uridine. The bare silica of Hypersil GOLD provided different kuridine, α (OH) and α (CH_2) values from the silica in Accucore HILIC and Syncronis Silica. These differences could be due to differences in pore volume, surface area and particle morphology for the three silica types. Syncronis Silica showed a higher retentivity than Hypersil GOLD Silica due to its higher nominal surface area. Accucore HILIC, in turn demonstrated higher k uridine, α (OH) and α (CH_2) values than the other bare silica columns.

From Table 4 it can be observed that Syncronis HILIC provided the best selectivity for α (V/A) and α (2dG/3dG). Similar data were reported by Ikegami's group for Nucleodur HILIC and ZIC-HILIC which have similar zwitterionic functionality [13]. Mixed Mode HILIC-1 cannot discriminate between the two configurational isomers, as demonstrated by the α (V/A) value of 1.0. This diol material showed a similar α (2dG/3dG) value to the 1.06 value reported by Ikegami et al. for Lichrosphere Diol [13].

The fact that α (2dG/3dG) values are about 1.1 for most materials (apart from HILIC-10) would indicate less specificity for positional isomers. From the radar plots it can be observed some correlation between α (V/A) and α (2dG/3dG) for most phases, although the small variations for α (2dG/3dG) data are not sufficiently significant. These small variations were also observed on the materials characterised by Ikegami and his group [13], suggesting that these probes are not selective enough. From Table 5 it can be observed that Hypersil GOLD HILIC and Acclaim Trinity P1 have the strongest anion interactions; these results are expected, considering that both materials possess amino groups, which work as AX functionalities at the pH experimental conditions of 4.7. The bare silica materials exhibited the highest α (CX) values; bare silica phases are known to possess cation exchange ability due to their acidic silanols (SiOH) functionality.

For the mixed mode HILIC-1 the value for α (AX) was not reported, and the value for α (CX) was zero, since SPTS eluted faster than t_0 and TMPAC co-eluted with t_0. It has been observed that some ligands exclude TMPAC and SPTS from the pore volume, resulting in these compounds not being retained [13]. Pore exclusion could be advocated for the early elution of SPTS and TMPAC experienced on the mixed mode HILIC-1.

From the AX and CX characterisation study it can be concluded that cation exchange interactions have important effects in HILIC on bare silica phases. Syncronis HILIC showed considerable CX character, due to the sulfo group in the phase; however, the α (CX) value for Syncronis HILIC was much lower than the values recorded by Ikegami's group for Nucleodur HILIC and ZIC-HILIC (3.46 and 4.41 respectively) [13]. Experimental HILIC also demonstrated some CX character. The degree of ion exchange interactions has a major impact on the shape of the radar plots, as illustrated in Figure 5, with a distinct dichotomy between (i) the bare silica materials, which have strong cation exchange ability, and (ii) Trinity P1 and GOLD HILIC, which exhibit strong anion exchange activity. Very little ion exchange interactions were demonstrated by HILIC-10 and mixed mode HILIC-1.

In the study by Lämmerhofer et al. [10] it was

shown that basic stationary phases give α (Tb/Tp) <1; neutral phases give α (Tb/Tp)= 1 and acidic phases give α (Tb/Tp)>1. Based on these observations, the materials under current investigation were classified accordingly, as reported in Table 6. The acidic phases comprise the silica and the amide materials. Amide materials are supposedly neutral in terms of the nature of their functionality [13], but Experimental HILIC demonstrated a high α (Tb/Tp) value and it could therefore be expected to show an acidic nature in terms of retentions. The zwitterionic material, Syncronis HILIC proved to be neutral. Interestingly, Ikegami and his group found that some zwitterionic phases (i.e. ZIC-HILIC) were acidic, whereas others (i.e. Nucleodur HILIC) were neutral [13]. Irgum et al. confirmed these findings and suggested that ligand loading could be responsible for this dual nature of zwitterionic materials, since ZIC-HILIC columns are polymerically functionalised, whereas Nucleodur HILIC columns are monomerically functionalised and therefore have a lower ligand loading [14]. Syncronis HILIC, being monomerically functionalised and neutral, confirms Irgum's suggestion.

Chromatographic parameters investigation

This study highlighted that in HILIC the level of organic solvent in the mobile phase has a large influence on retention. HILIC behaviour of decreasing retention with decreasing acetonitrile content was generally observed, indicating that partitioning is the main retention mechanism in these separations, regardless of the radically different chemistries.

The exception to the HILIC behaviour was demonstrated by Hypersil GOLD HILIC (weak anion exchanger), which exhibited secondary interactions when analysing acid compounds.

The presence of secondary interactions was also confirmed by the thermodynamic data, where the high differences in the enthalpy values would indicate the existence of strong specific interactions between the analytes and the functional groups of the stationary phases.

Salt content proved to be fundamental for the full resolution of both acids and bases. Its effect was particularly significant on Syncronis HILIC; the general increase in retention as the salt concentration was increased provided indirect evidence for the hydrophilic partitioning model.

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