

Hydrophilic Interaction Liquid Chromatography: Some Aspects of Solvent and Column Selectivity

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Key Words

Hydrophilic, HILIC, Porous Graphitic Carbon (PGC), Selectivity, Zwitterionic Phase

Abstract

The retention mechanisms of five stationary phases were investigated when using hydrophilic interaction liquid chromatography (HILIC) for the separation of small polar compounds. The effect on retention of changing acetonitrile content, column temperature and buffer concentration in the mobile phase was investigated. This study highlighted differences in retentivity and selectivity, which could assist during HILIC method development.

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, 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 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.

HILIC is a feasible alternative for the analysis of polar compounds. HILIC can be described as a variation of normal phase chromatography performed using a polar stationary phase (for example, unmodified silica, amino, cyano or diol bonded phases). The mobile phase employed is highly organic in nature (>70% solvent, typically

acetonitrile) containing also a small percentage of aqueous solvent/buffer or other polar solvent. The water/polar solvent forms an aqueous-rich sub-layer adsorbed to the polar surface of the stationary phase (as shown in Figure 1) into which analytes partition.

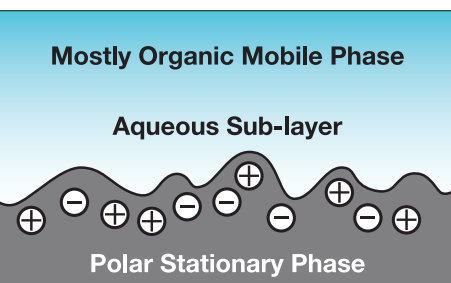


Figure 1: Schematic representation of hydrophilic partitioning



The retention mechanisms in HILIC are complex but are believed to be a combination of hydrophilic partitioning interaction, and secondary electrostatic and hydrogen bonding mechanisms. These mechanisms result in a retention order which is roughly the opposite of the order analytes elute from a reversed phase column.¹ Separation of analytes occurs based on analyte polarity and degree of solvation.

The five materials chosen for investigation were: Thermo Scientific™ Accucore™ HILIC (a solid core material), Hypersil GOLD™ HILIC, Synchronis™ HILIC, Hypersil GOLD Silica and Hypercarb™ (Porous Graphitic Carbon).

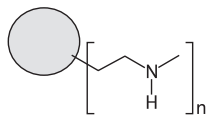


Figure 2: Functional group of Hypersil GOLD HILIC

Accucore HILIC and Hypersil GOLD Silica are unmodified silica materials; Hypersil GOLD HILIC is a weak anion exchanger, based on a polymeric amine ligand: polyethyleneimine (Figure 2). The main benefit of using a charged stationary phase lies in the extra selectivity brought about by the possible electrostatic interactions with the analyte. The strength of these interactions depends on the ionization of the solute and the stationary phase. The charge density is therefore pH-dependent and high buffer concentrations may be necessary in order to disrupt these interactions and allow the analyte to elute.

Synchronis HILIC is a zwitterionic phase able to provide weak electrostatic interactions, whose charge density is pH-independent. The electrostatic forces of each charge are weak because they are counterbalanced by the proximity of an ion of opposite charge.

Porous Graphitic Carbon (PGC) provides unique separation mechanisms, based on a combination of dispersive interactions between the analyte-mobile phase and analyte-graphitic surface together with charge induced interactions of polar analytes with the polarizable surface of the graphite (as schematically shown in Figure 3). As a consequence of these combined interactions, PGC material can retain polar analytes, both in typical reversed-phase and HILIC type mobile phase conditions. As illustrated in Figure 4, at acetonitrile concentrations between 90–60%, analyte retention increases as percentage of acetonitrile increases (HILIC retention behavior); between 10–60% acetonitrile, analyte retention decreases as the concentration of acetonitrile becomes greater (a reversed phase retention behavior).

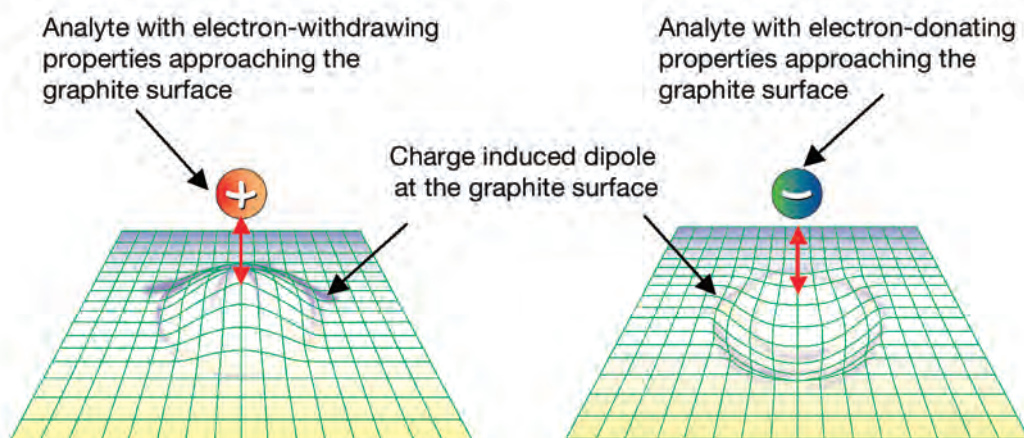


Figure 3: Schematic representation of charge induced interaction on the PGC surface

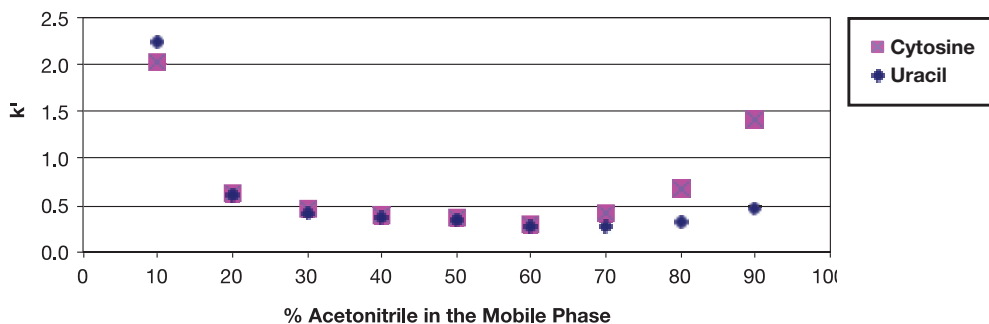


Figure 4: Analyte retention factor, k' as a function of acetonitrile percentage in mobile phase
 PGC column: 3 μm , 100 \times 2.1 mm
 Mobile phase: acetonitrile/water, containing 10 mM ammonium acetate, pH 4.7
 Flow rate: 0.2 mL/min
 Column temperature: 40 $^{\circ}\text{C}$
 Detection: ESI/MS

The objectives of this study were:

- Investigate the factors that influence retention in HILIC mode for five stationary phases
- Gain an understanding of the chromatographic processes that rule HILIC
- Provide a tool to facilitate method development and method optimization in HILIC and ultimately HILIC column selection

Experimental

Instrumentation:	HPLC system equipped with a quaternary pump, a DAD detector, a degasser, a column heater and an autosampler		
Columns:	As given in Table 1		
Basic test mixture:	1. uracil, 2. adenosine, 3. uridine, 4. cytosine, 5. cytidine		
Acid test mixture:	1. salicylamide, 2. salicylic acid, 3. aspirin, 4. 3,4-dihydroxyphenylacetic acid (dhpa)		
Mobile phases:	Various mobile phases were prepared by mixing the desired volumes of acetonitrile and stock buffer solutions. The pH of the salt solutions was not adjusted before mixing with acetonitrile. The salt concentrations reported in the individual results sections refer to the final concentrations of the salt on the column		
Instrumentation set-up:	Flow rate:	1.0 mL/min	
	UV:	228 nm for the acid mixture and 248 nm for the basic mixture	
	Injection volume:	5 μ L	
	Column temperature:	30 $^{\circ}$ C	

Column Name	Column Dimension (mm)	Surface Area (m ² /g)	Pore Size (Å)
Synchronis HILIC (5 μm)	100 \times 4.6	320	100
Hypersil GOLD HILIC (5 μm)	100 \times 4.6	220	175
Hypersil GOLD Silica (5 μm)	100 \times 4.6	220	175
Accucore HILIC (2.6 μm)	100 \times 4.6	130	80
Hypercarb (5 μm)	100 \times 4.6	120	250

Table 1: HILIC stationary phases



Results and Discussion

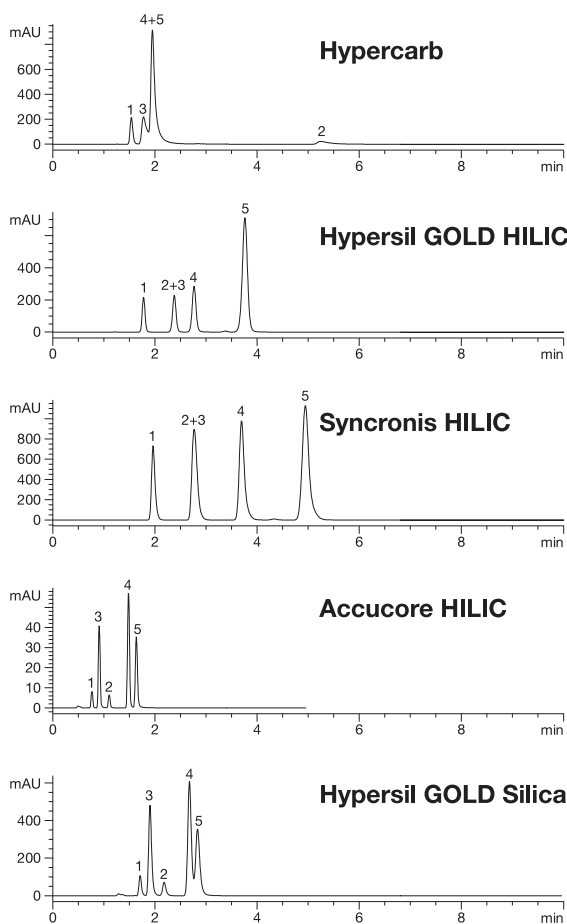
Column Selectivity

Separation of the two test mixtures was carried out on the five stationary phases, selected because they have very different surface chemistries. The resulting chromatograms are compared and reported in Figure 5.

Figure 5 shows that the elution orders for both acid and basic compounds are different for the five different stationary phases, with exception for the elution of polar basic compounds on Hypersil GOLD HILIC and Synchronis HILIC (Figure 5a). The five materials demonstrate different selectivity. The higher retentivity displayed by Synchronis HILIC is likely to be due to the higher surface

area of this material. There is a clear lack of retention on Hypercarb, demonstrating the different selectivity offered by Hypercarb under the HILIC mobile phase conditions (as illustrated in Figure 4). Salicylic acid (peak 2 on Figure 5b) is more strongly retained on the Hypersil GOLD HILIC column than on any other column, and ion exchange is likely to be responsible for this behavior. The resolution of aspirin is much improved on Synchronis HILIC (peak 3 on Figure 5b); this could be the result of electrostatic interactions between aspirin and the negatively charged groups of the stationary phase.

(a) Basic Test Mixture



(b) Acid Test Mixture

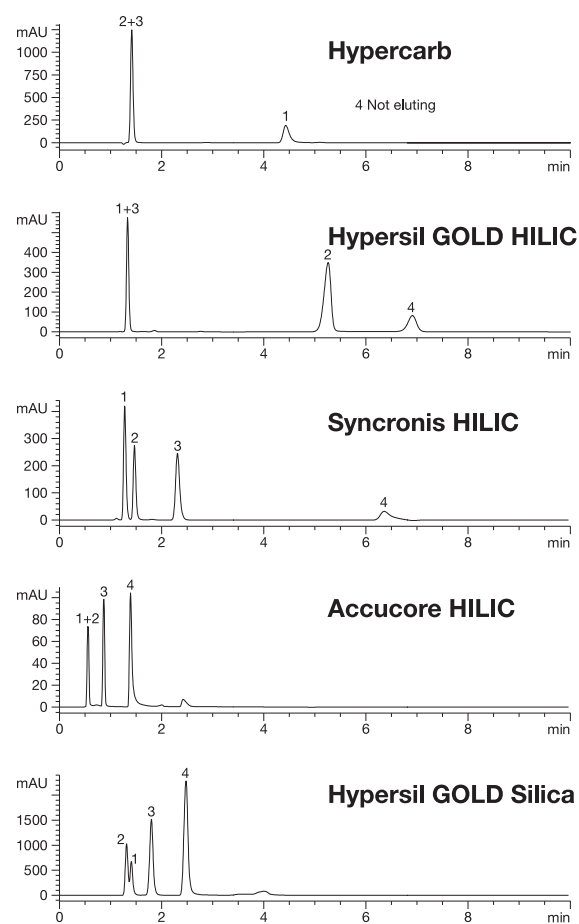


Figure 5: Column selectivity for bases and acids

Mobile phase: acetonitrile/water (85/15, v/v), containing 10 mM ammonium acetate

The Effect of Acetonitrile Content on Retention

In HILIC, the level of organic solvent in the mobile phase has a large influence on retention. Separation of the two test mixtures was carried out on the five stationary phases. The percentage of acetonitrile in the mobile phase was varied whilst keeping ammonium acetate concentration constant at 10 mM. 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 typical HILIC behavior of decreasing retention with decreasing acetonitrile content on Synchronis HILIC and on Hypersil GOLD Silica. On Hypercarb its retention levels off when the acetonitrile content is 80% and below. 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 behavior is likely to be due to ion-exchange interactions. Ion-exchange interactions between salicylic acid and the amine ligand of Hypersil GOLD HILIC would also explain the higher retentivity observed for this analyte.

Cytosine shows HILIC behavior of decreasing retention with decreasing acetonitrile content on the five stationary phases. This is also true for the other components of the basic test mixture (data not shown). This behavior indicates that partitioning is the main retention mechanism in these separations, regardless of the radically different chemistries.

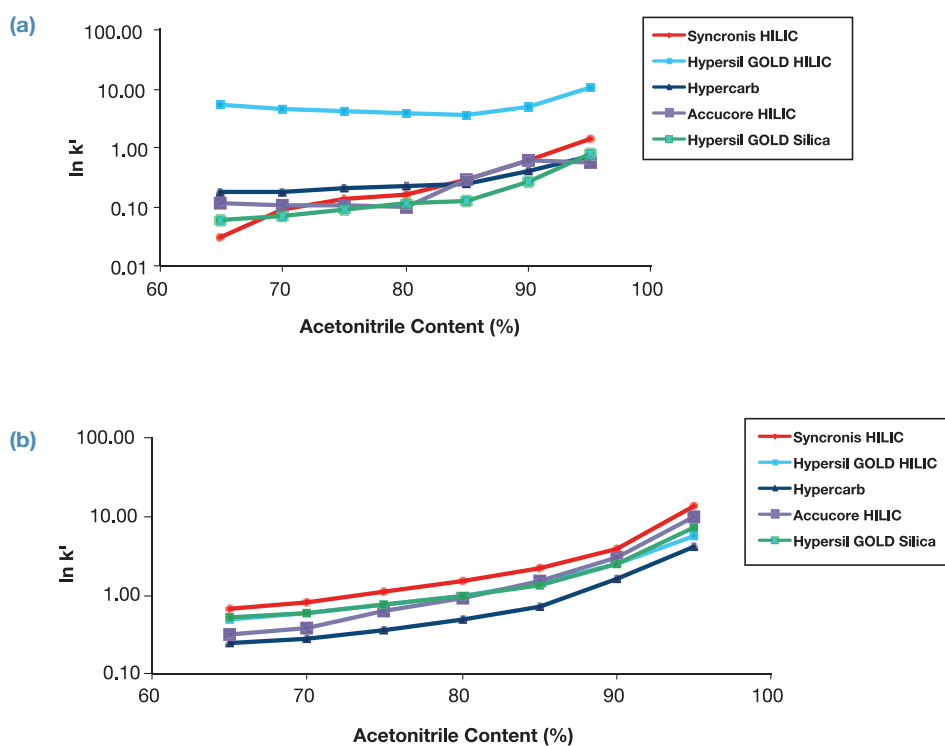


Figure 6: Effect of acetonitrile content on the retention of salicylic acid (a) and cytosine (b)

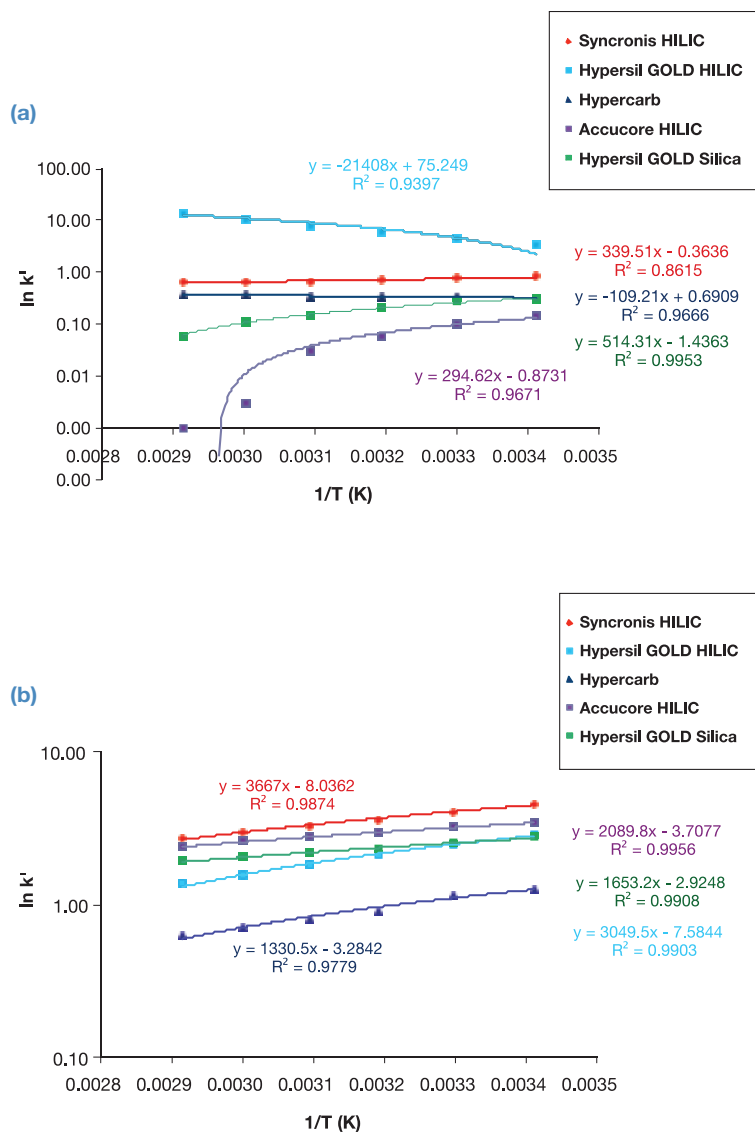


Figure 7: Effect of column temperature on retention of salicylic acid (a) and cytosine (b)
Mobile phase: acetonitrile/water (90/10, v/v), containing 10 mM ammonium acetate

The Effect of Column Temperature on Retention

Column temperature is an important parameter that can affect retention of polar analytes in HILIC. In reverse-phase LC, the relationship between column temperature and retention factor is often described by the van't Hoff equation:

$$\ln k' = -\Delta H^\circ/RT + \Delta S^\circ/R + \ln \phi$$

Where:

ΔH° – retention enthalpy

ΔS° – retention entropy

R – gas constant

T – column temperature

ϕ – phase ratio

The van't Hoff equation should also apply to HILIC; if retention is through partitioning between the mobile phase and the immobilized layer of water on the stationary phase, then the relationship between $\ln k'$ and $1/T$ is linear.

In this study the temperature effect on the retention of salicylic acid and cytosine was investigated for the five 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 five columns.

From Figure 7a it can be seen that a decrease in retention is observed as the column temperature is increased on Accucore HILIC, Hypersil GOLD Silica and to a less extent on Synchronis HILIC. The retention increases with the temperature on both Hypersil GOLD HILIC and Hypercarb. Negative retention enthalpy values were obtained for Synchronis HILIC, Hypersil GOLD Silica and Accucore HILIC, indicating an exothermic process of transferring salicylic acid from the mobile phase to the stationary phase. Positive retention enthalpy values were derived for Hypercarb and Hypersil GOLD HILIC, suggesting an endothermic process of transferring salicylic acid from the mobile phase to the stationary 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. The large enthalpy variation of salicylic acid on this material could indicate the change in the degree of relative contribution to the overall retention from the two types of interaction.

Figure 7b shows a decrease in retention on the five 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 five stationary phases.²

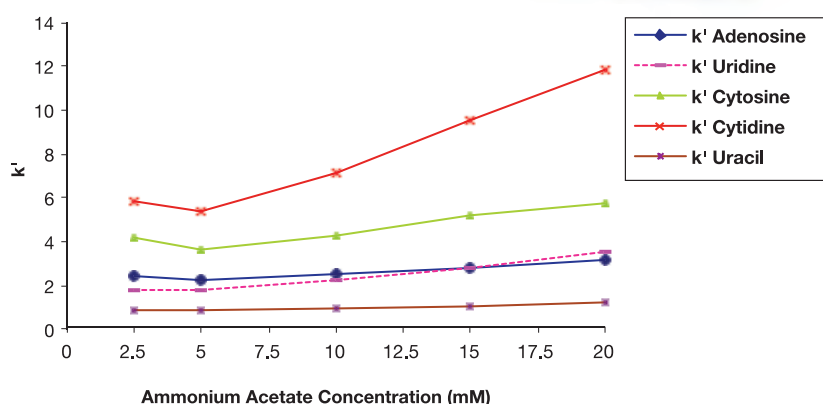
The Effect of Buffer Concentration on Retention

In this study the effect of salt concentration on the retention was investigated. Separation of the two test mixtures was carried out on Hypercarb, Synchronis 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 three columns. Figure 8a and 8b show the data relative to the basic and acid test mixtures respectively obtained on Synchronis HILIC. The five bases are fully resolved on Synchronis 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. This retention increase could be related to a partitioning process. Apart from uracil, the other 4 bases show an increase in retention as the ammonium acetate concentration is increased above 5 mM, 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.² The partitioning model for HILIC assumes the presence of a water-rich liquid layer on the stationary phase. High levels of organic in the mobile phase could make the salt prefer to be in the water-rich liquid layer. This would result in increased volume or hydrophilicity of the liquid layer, thus leading to stronger retentions.

The four acids are not fully resolved on Synchronis HILIC, as shown in Figure 8b, with salicylic acid coeluting with salicylamide when low levels of ammonium acetate are used (2.5, 5 and 10 mM). Further increase in the salt content to 15 mM leads to an increase in retention for salicylic acid and salicylamide. It is possible that electrostatic repulsions from the negatively charged sulfonate 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 15 mM and above.



(a) Basic Mixture



(b) Acidic Mixture

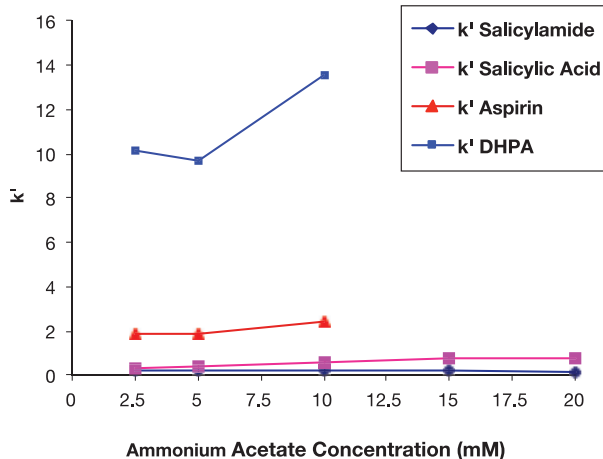


Figure 8: Effect of buffer concentration on retention of basic and acidic mixtures, for Synchronis HILIC
Mobile phase: acetonitrile/water (90/10, v/v)

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

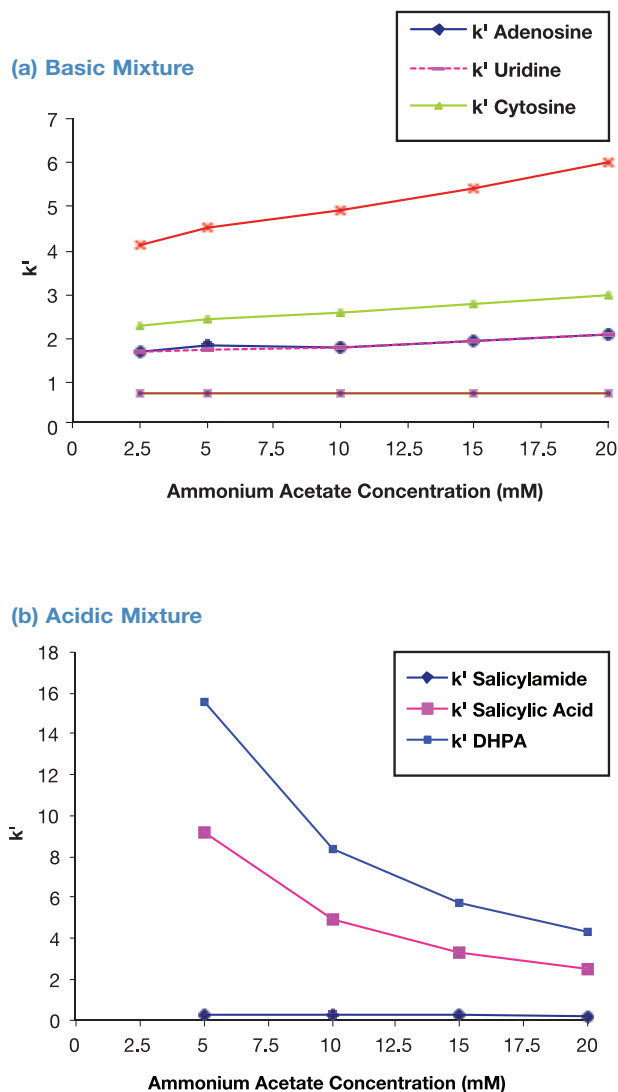


Figure 9: Effect of buffer concentration on retention of basic and acidic mixtures, for Hypersil GOLD HILIC
Mobile phase: acetonitrile/water (90/10, v/v)

Figure 10a and 10b show the data relative to the basic and acid test mixtures respectively, obtained on Hypercarb. On Hypercarb, under the HILIC conditions relative to this study uracil and uridine are not retained. Again, we must refer back to Figure 4 to remind ourselves of the dual behavior exhibited by Hypercarb and its capabilities to retain uracil.

The other three bases are resolved, but their retention is mostly unaffected by the variation of the buffer concentration. The components of the acidic mixture are not resolved on Hypercarb, apart from salicylamide; their retentions are not affected by the variation of the buffer concentration.

To elucidate the different behaviors exhibited by the three columns, a comparison, in terms of salicylic acid retention, is given in Figure 11. On Hypersil GOLD HILIC salicylic acid exhibits an opposite behavior to what observed on Synchronis 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, pK_a for salicylic acid = 2.9) and its retention drops as the buffer concentration increases, which is typical of an ion exchange mechanism. The electrostatic effect was not applicable for the bases since they are not charged in the mobile phase. The retention of salicylic acid on Hypercarb is not affected by variations of the salt concentration.

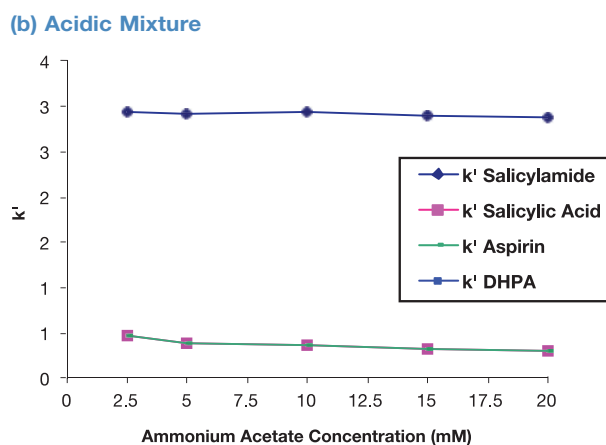
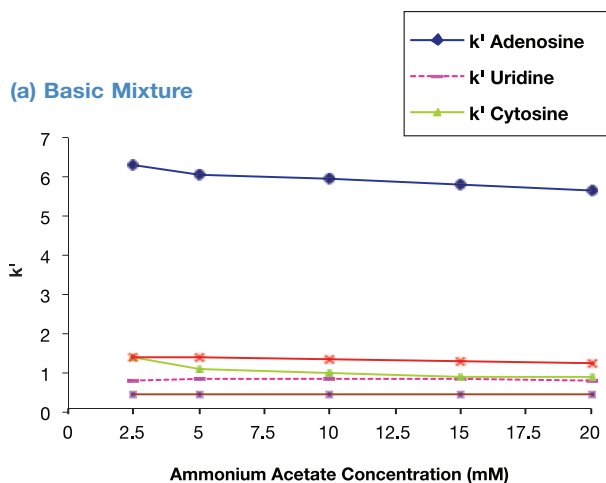


Figure 10: Effect of buffer concentration on retention of basic and acidic mixtures, for Hypercarb
Mobile phase: acetonitrile/water (90/10, v/v)

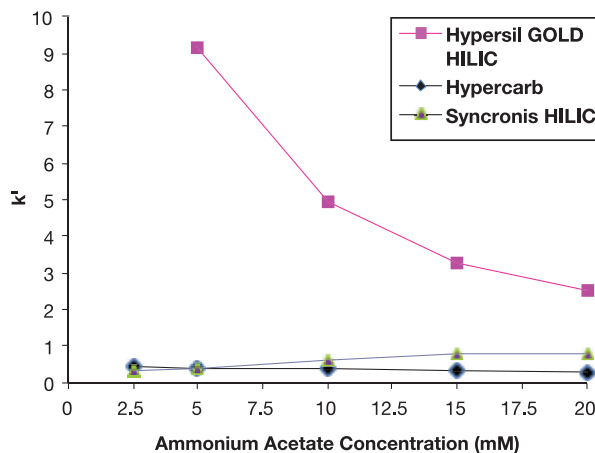


Figure 11: Effect of buffer concentration on retention of salicylic acid
Mobile phase: acetonitrile/water (90/10, v/v)

Conclusion

This study has demonstrated different selectivity when using the same mobile phase on five stationary phases, thus proving that partitioning is not the only separation process involved.³ Secondary interactions such as ion exchange (for Hypersil GOLD HILIC) and electrostatic interaction (for Hypercarb) could explain different elution patterns when analyzing acid compounds. This interpretation is consistent with 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 five stationary phases.

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

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