# Hydrophilic Interaction Liquid Chromatography: An Investigation into the Experimental Factors that Affect Selectivity

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## Overview

**Purpose:** The aim of this work was to characterize stationary phases typically used for HILIC applications and to develop areas of critical understanding by testing a range of HILIC experimental parameters.

**Methods:** A column characterization investigation was performed on ten different HILIC columns. In addition the following experimental factors were studied: effect of solvent content, effect of buffer concentration and effect of buffer pH on retention behaviour of polar analytes.

**Results:** From this study an understanding of the relationship between the chemical properties of the stationary phases and a selected series of test solutes and their interplay with the mobile phase was gained. This approach represents a good practical guide to adjustments of conditions that effect the selectivity of a separation.

## Introduction

HILIC is increasingly being employed in metabonomics, glycomics, food safety, proteomics, etc. The market potential for HILIC is vast and the need to provide selective columns and optimal separating conditions is reflected in that.

This study characterized five main classes of HILIC columns:

1. Bare silica
2. Neutral bonded ligands (e.g. amide and diol)
3. Charged ligands (e.g. positively charged amino phases)
4. Zwitterionic phases (e.g. sulfobetaine)
5. Mixed Mode phases (both bimodal – eg alkyl chain/diol – and trimodal – e.g. RP/WAX/SCX and HILIC/SAX/WCX).

In order to highlight and compare the fundamental differences in the column chemistries and their main interaction mechanisms, a column characterization investigation was carried out. The method chosen to investigate retention selectivity and interaction modes was based on two seminal characterization studies [1, 2] and consisted of identifying selectivity factors for pairs of similar chemical substances – one with properties promoting the particular interaction mode being probed and the other lacking such properties.

In order to identify the optimal conditions at which to operate each column, the following experimental factors were studied:

1. Effect of acetonitrile content on retention behaviour of neutral and charged analytes
2. Effect of buffer concentration on retention behaviour of charged analytes
3. Effect of buffer pH on retention behaviour of charged analytes

The understanding and characterization of primary and secondary retention mechanisms coupled with a better understanding of how common experimental parameters affect the separation mechanism will aid in the selection of the appropriate HILIC conditions when developing separations.

## Methods

**Instrument Set Up**

- Thermo Scientific™ Dionex™ UltiMate™ 3000 UHPLC system
- Column compartment temperature: 30 °C.
- Autosampler tray temperature: 15 °C.
- UV detection: 210 and 254 nm.
- Thermo Scientific™ Dionex™ Chromeleon™ 7.1.1.1127 software.

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### Experimental conditions

A list of columns used and experimental conditions are given in Table 1 and relevant legends. The following test mixtures were used:

test mixture 1a: t0 (toluene), uridine (U), 2'-deoxyuridine (2dU), to test hydrophilic selectivity – $\alpha_{(OH)dU}$;
test mixture 1b: t0, uracil (Ur), cytosine (CYS), to test hydrophilic selectivity – $\alpha_{(OH)CYS}$;
test mixture 2a: t0, uridine (U), 5-methyluridine (5MU), to test hydrophobic selectivity – $\alpha_{(CH2)MU}$;
test mixture 2b: t0, ethylimidazole (E-IMI), methylimidazole (M-IMI), to test hydrophobic selectivity – $\alpha_{(CH2)IMI}$;
test mixture 3a: uracil (Ur) and phenanthrene (Phe), to compare the degree of Hydrophilicity – $k_{Uracil}$;
test mixture 3a: uracil (Ur) and phenanthrene (Phe), to compare the degree of Hydrophilicity – $k_{Uracil}$;
test mixture 4a: t0, uracil (Ur), trimethylphenylammoniumchloride (TMPAC), to test cation exchange – $\alpha_{CXT}$;
test mixture 4b: t0, uridine (U), Nortriptylene, to test cation exchange – $\alpha_{CXN}$;
test mixture 5a: t0, uracil (Ur), sodium p-toluenesulfonate (SPTS), to test anion exchange – $\alpha_{AXS}$;
test mixture 6a: t0, vidarabine (V), adenosine (A), to test configurational selectivity – $\alpha_{V/A}$;
test mixture 7a: t0, adenosine (A), adenine (ADI), to test hydrogen bonding – $\alpha_{(ribose)}$;
test mixture 8a: t0, theophylline (Tp), theobromine (Tb), to test stationary phase surface pH – $\alpha_{Tb/Tp}$;
test mixture 9a: t0 (toluene), 1-vinylimidazole (V-IMI), 1-ethylimidazole (E-IMI), to test π-π interactions – $\alpha_{\pi-\pi}$.

| Column Name | Phase Type | Column dimension (mm) | Particle size (µm) | Surface area ($m^2$/g) | Pore size(Å) | Mobile Phase Flow Rate (mL/min) | Injection Volume (µL) |
|---|---|---|---|---|---|---|---|
| Syncronis™ HILIC | Zwitterion | 100 x 4.6 | 5 | 320 | 100 | 0.7 | 2 |
| Hypersil™ GOLD HILIC | Polyethyleneimine | 100 x 4.6 | 5 | 220 | 175 | 0.7 | 2 |
| Hypersil GOLD Silica | Unbonded Silica | 100 x 4.6 | 5 | 220 | 175 | 1.0 | 2 |
| Accucore™ HILIC | Unbonded Silica | 100 x 2.1 | 2.6 | 130 | 80 | 0.5 | 1 |
| Betasil™ Diol | Diol | 250 x 4.6 | 5 | 100 | 310 | 1.0 | 5 |
| Accucore Urea HILIC | Urea | 100 x 2.1 | 2.6 | 130 | 80 | 0.4 | 1 |
| Acclaim Mixed Mode HILIC-1 | Alkyl/Diol | 150 x 4.6 | 5 | 300 | 120 | 0.7 | 5 |
| Accucore-150-Amide-HILIC | Polyamide | 100 x 2.1 | 2.6 | 80 | 150 | 0.4 | 1 |
| Acclaim Trinity P1 | RP/WAX/SCX | 100 x 3.0 | 3 | 300 | 100 | 0.5 | 2 |
| Acclaim Trinity P2 | HILIC/WCX/SAX | 150 x 3.0 | 3 | 300 | 100 | 0.5 | 2 |

**TABLE 1. Specifications of the Thermo Scientific™ columns and experimental conditions used. Mobile phase flow rates were varied according to individual column geometry [3], in order to maximize peak efficiency.**

## Results

The results generated from the characterization tests are summarized as radar plots in Figure 1.

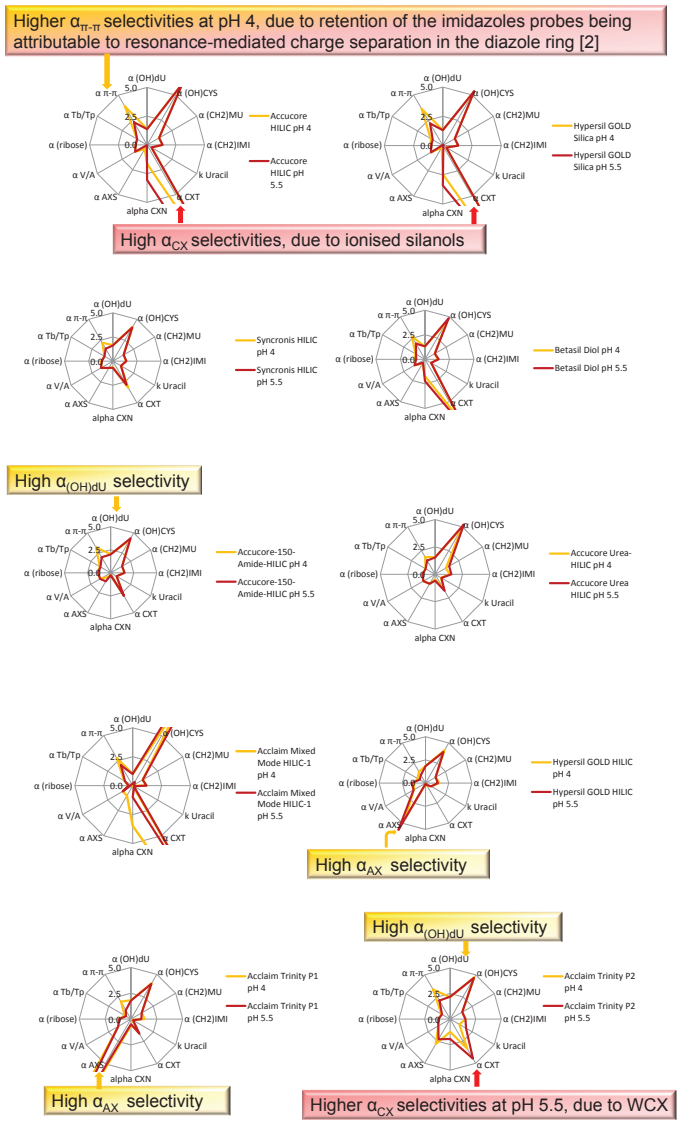


**FIGURE 1. Summary of results obtained from the characterization study of the Thermo Scientific HILIC and mixed mode columns using acetonitrile/10 mM ammonium acetate, pH 4.0 and pH 5.5 (90/10, v/v) mobile phase.**

In order to adequately adjust HILIC separation conditions, **the effect of the changes in organic content on retention were characterized.** The selectivity variation thus afforded are reported in Figure 2, where representative radar plots from the organic content 90–50% range are shown. From these plots it is clear to see that decreasing the concentration of acetonitrile from 90 to 50% leads to overall average decreases in retention, attributable to an increase partitioning for the hydrophilic test analytes into the mobile phase.

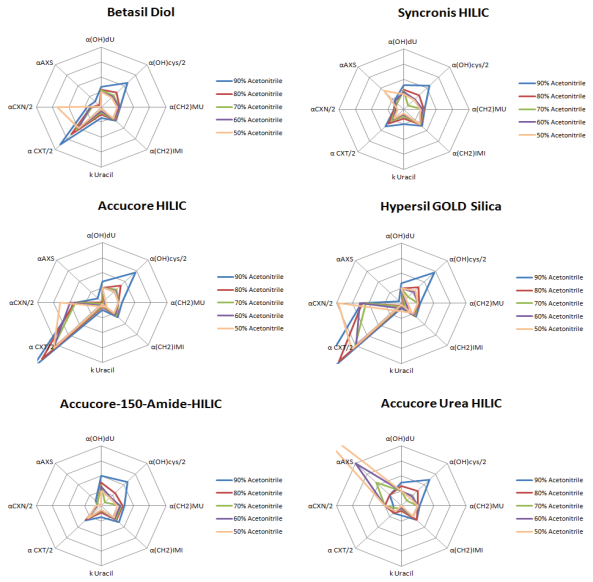


**FIGURE 2. Effect of acetonitrile content in 10 mM ammonium acetate buffer, pH 4.0 on hydrophilic, hydrophobic and ion exchange selectivities.**

Overall, an **increased electrolyte concentration** in the mobile phase leads to a reduction in the differences between stationary phases as a plateau is observed for most columns. Representative columns are discussed below.

Hypersil GOLD HILIC and Acclaim Trinity P2 displayed good anionic retention (SPTS), with a decreasing trend as the buffer concentration increases. This phenomenon is illustrated in Figure 3. The ligands of these materials offer higher retentions at lower ionic strength. The acetate ions in the buffer (ammonium acetate) compete with the anionic probe for the cationic site of the stationary phase; the higher the buffer ionic strength, the lower the retention for SPTS.

A general trend of decreased retention for positively charged analytes as the buffer concentration increases – as reported in Figure 4 – indicates that ion exchange controls the retention mechanism. It is very important to fine tune the mobile phase buffer pH, when using phases with high IEX selectivities

Ion exchange capacity is higher at pH 4, due to the higher ionization of their WAX moieties

Ionization of silanols of underlying silica is responsible for the increase in cationic retention in the weaker buffer. This phenomenon is clearly more pronounced at pH 5.5 – where the proportion of deprotonated silanols increases – whereas arguably little variation in retention is shown at pH 4.0.

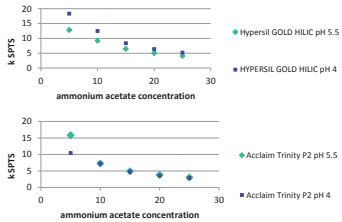


**FIGURE 3. Effect of ammonium acetate buffer concentration on SPTS (anion) retention, under 50/50 acetonitrile/aqueous conditions.**

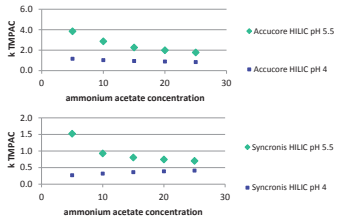


**FIGURE 4. Effect of ammonium acetate buffer concentration on TMPAC (cation) retention, under 50/50 acetonitrile/aqueous conditions.**

## Conclusion

- The characterization study highlighted that, beside partitioning the main contributing interactions are: π-π, cationic and anionic interactions - predominant on stationary phases with either a distinct ion exchange functionality or silanols activity. Low specific interactions were observed on neutral materials.
- An increase in organic solvent lead to an increase in retention.
- A general trend of decreased retention for charged analytes as the buffer concentration increases was demonstrated.
- The charge state of the stationary phase can affect HILIC retention of ionizable compounds, depending on the mobile phase pH. It is very important to fine tune the mobile phase buffer pH, when using phases with high IEX selectivities, in order to avoid poor/long retention times.

### References

[1] Kawachi Y., Ikegami T., Takubo H., Ikegami Y., Miyamoto M. and Tanaka N. 2011, J.Chromatogr. A, 1218, 5903.
[2] Dinh N.P., Jonsson T. and Irgum K., 2011, J. Chromatogr. A, 1218, 5880.
[3] HILIC Technical Guide (http://apps.thermoscientific.com/media/cmd/flipbooks/TG-21003-HILIC-TG21003-EN_flipbook/index.html).

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