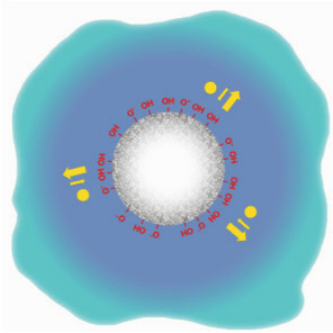
Understanding HILIC separation and improving your results!

Hydrophilic interaction liquid chromatography (HILIC) is especially suited for the analysis of ionised or polar solutes and provides a valuable technique which complements reversed phase (RP) chromatography. Because HILIC provides different separation mechanisms compared to RP it offers the possibility for 2-D orthogonal chromatography if solutes are easily retained in both modes. In addition, the use of a high organic content in the mobile phase leads to the advantages of reduced column back pressure and an increase in sensitivity when working with evaporative detectors such as evaporative light scattering (ELSD), charged aerosol detector (CAD) or electrospray ionisation mass spectrometry (ESI-MS). Here we provide a review highlighting influences on the separation mechanisms in HILIC to provide a helpful tool for method development and troubleshooting.

What can influence a HILIC separation?



In HILIC the three main mechanisms involved in analyte retention are:

1. the **adsorption of an analyte on polar groups** of the stationary phase as a result of hydrogen-bonding, electrostatic (coulombic) and dipole interactions,
2. the **dispersion of analytes between mobile phase and the surface water layer on the stationary phase** and
3. **ionic exchange interactions**.[1]

Consequently, various parameters have an effect on the retention of different compounds in a HILIC analysis and a number of studies have investigated the effects of different stationary phases, buffer pH and concentration, column temperature and organic solvent concentration on neutral, acidic and basic analytes.[2] The influence of each of these factors can be determined and ranked in the following order with the most influential on the left:

Stationary phase > mobile phase pH > organic solvent concentration > buffer concentration > column temperature

Stationary phase considerations

A large number of new supports have been proposed and tested as materials suitable for HILIC columns, but by far the most commonly used are silica-based materials, because the properties of silica and silica-hybrids are well understood and provide high separation efficiency and symmetric peak shapes. So it is no surprise that a wide variety of ligands have been bonded to silica and the properties of resulting stationary phases have been extensively studied and reviewed.[3-5]

Generally, HILIC stationary phases can be divided intuitively into groups based on their chemical properties and structure.[4] Some examples are:

Neutral charged ligands:	**diol, cyano, PFP, amide (e.g. YMC-Triart Diol-HILIC, YMC-Triart PFP)**
Positively charged ligands:	**amino, polyamine, imidazole (e.g. YMC-Pack NH2, YMC-Pack Polyamine II)**
Negatively charged ligands:	**bare silica, polyaspartic acid (e.g. YMC-Pack SIL)**
Zwitterionic ligands:	**peptides, sulfobetaine**

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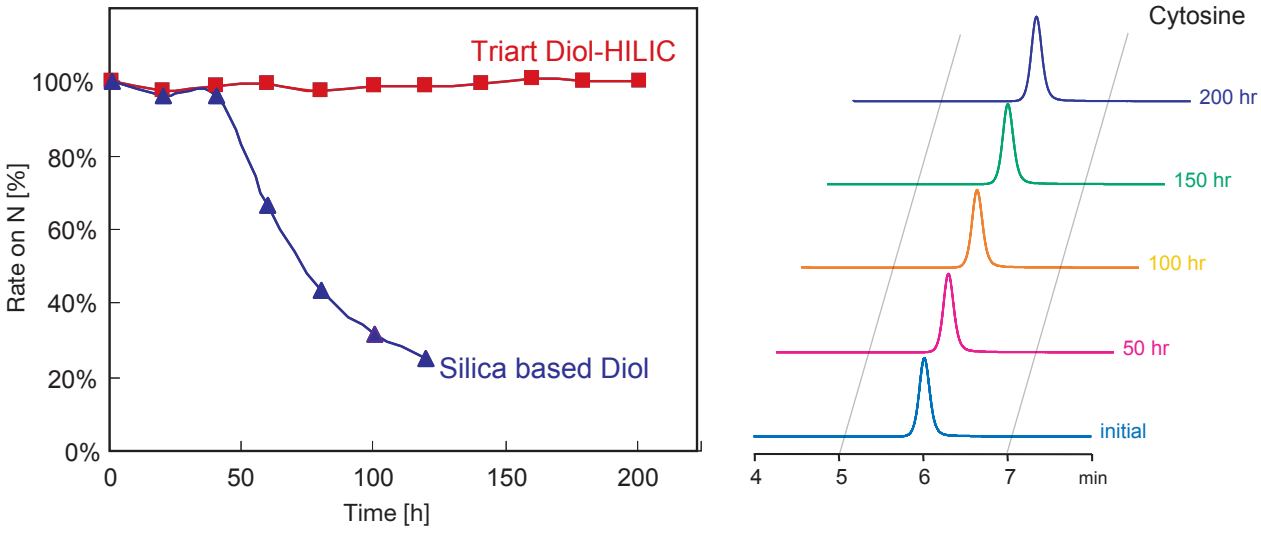
Since stationary phase selection has the greatest effect on selectivity it is important for the development or optimisation of a method to have a “tool kit” of different materials. For example, a **bare silica** phase shows poor correlation of retention factors for most other phases, due to the high electrostatic repulsion and reduced retention of acidic analytes and increased retention for bases. Therefore, it is a good first candidate for the chromatographers’ tool kit, since its selectivity is very different from the other materials.[2,6]

Due to their neutral ligands **diol** and **amide** phases show good correlation, but different selectivities and high retention for neutral polar compounds. They also show reduced secondary interactions for ionised analytes, which makes them a suitable choice for applications where symmetrical peak shapes and high sensitivity is of benefit and should also be included in the tool kit. Besides the good hydrophilic selectivity, differences are mostly due to ionic interaction effects of **amide** phases, which can lead to peak tailing in some cases.

Acidic analytes are well retained on **amino** phases due to the strong effect of electrostatic and ion exchange interactions, while bases are mostly repelled from these positively charged phases. It is well known that in many applications **amino** phases suffer from a reduced life time. This is probably due to the surface pH of the material being higher than the pH of the mobile phase. This effect has been counteracted by novel materials using mixed amines to reduce surface pH and by using polymeric coating or silica-hybrid technology.

When considering the stationary phase for your analysis, the choice of pH in the mobile phase plays another complex role. Applications at extreme pH values put chemical stress on the stationary phase which can lead to faster degradation and loss of column performance. At pH values over 6 dissolution of silica can occur, which for **bare silica** material is even more significant because of the missing bonded groups protecting its surface.[2] Modified **silica-hybrid materials** have a wider pH stability and are not easily hydrolysed at high or low pH. They provide an improvement in column live times and show less residual silanol activity, which all silica-based materials display whilst superimposing a cation exchange mechanism onto the separation. Modifiers such as TFA and HFBA can reduce these effects further, but are not fully compatible with some detection methods and most buffer systems.

Stability at high pH (pH 11, 50 °C)*



* pH ≤ 10 is recommended for regular use

Column: 5 µm, 150 x 4.6 mm ID
Part No.: TDH12S05-1546PTH
Eluent: acetonitrile / water / NH_3 (90/10/0.1) pH 11.3
Flow rate: 1.0 mL/min
Temperature: 50 °C
Sample: Cytosine

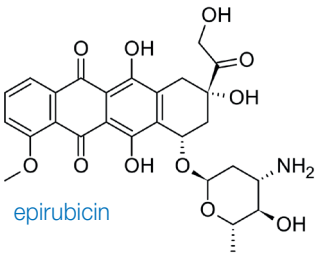
Mobile phase considerations

In HILIC the mobile phase contains high concentrations of organic solvent (60–9%) and low concentrations of water (3–40%). This provides the advantage of a lower viscosity and reduced back pressures, even with relatively long columns. The high volatility of the eluent leads to increases in sensitivity of evaporative detectors.[7] However the different overlapping interaction mechanisms in HILIC and especially the formation of a stable water layer on the stationary phase surface also leads to longer equilibration times, which is even more important when using gradient methods.
Acetonitrile (AcN) is the most widely used organic solvent in analytical HILIC, due to its good UV transparency and low viscosity. When increasing the AcN content in the mobile phase and reducing the water concentration, retention significantly increases, but the increase in retention factor doesn't follow a linear trend.[2]

This is due to the overlap of adsorption and partition mechanisms in HILIC separations and the change in the thermodynamic pH, which can occur when the concentration of an organic compound in the mobile phase changes. Full or partial substitution of AcN with alcohols such as methanol (MeOH), ethanol (EtOH) or isopropanol (IPA) will reduce retention by weakening hydrogen-bonding and disrupting the formation of the stationary water layer, which usually has a negative effect on reproducibility. [8-12]

A ranking of solvents in order of relative solvent strength is not easily estimated in HILIC because of the different overlapping separation mechanisms and strongly depends on the choice of stationary phase. For instance, separation of polar analogues of the inhibitory drug epirubicin on bare silica gives a ranking in the order of:

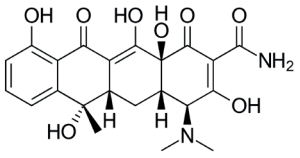
methanol <isopropanol <tetrahydrofuran <ACN



epirubicin

whereas polar tetracycline antibiotics on an amino-bonded phase show the ranking in order of:

tetrahydrofuran <methanol <isopropanol <ACN



tetracycline

Notably AcN is the weakest solvent regardless of testing conditions.[13]

Retention of weaker acids and bases is greatly affected by mobile phase pH, while strong acids and bases are affected to a lesser extent. The main retention mechanism for these compounds are coulombic interactions between the stationary phase charged groups and the charged analytes. The influence of pH on the charged state of the stationary phase is also a big factor for these compounds.

Small acidic mobile phase modifiers such as **formic acid, acetic acid** or phosphoric acid can act as reasonable "buffers" or at least keep a constant pH, but generally do not have adequate ionic strength in high AcN concentrations to reduce silanol activity and produce symmetric peak shapes.[14-15] Because silanols on the stationary phase surface will be fully protonated at pH 2–3, TFA or HFBA is often used to reduce the net charge of residual silanols to zero and to suppress the cationic exchange mechanism with basic analytes.[16] However this can produce an effect of increased repulsion and even exclusion of acidic analytes from the stationary phase especially on **bare silica** and **amide** phases. It is believed this may be due to the enrichment of hydronium ions on the water/eluent interface and/or charged AcN artifacts due to hydrolysis but these effects still require further investigation.[17-19]

The use of **salts derived from formic acid and phosphoric acid, as well as oxalic acid and citric acid as buffer ions** is a well-established tool to alter retention times and improve peak shapes, but this shows little effect on the selectivity of the separation itself.[15,20] This means that higher resolution and sensitivity can be achieved by screening a method for the appropriate buffer concentration and its components. The most widely used buffer systems in HILIC are **ammonium formate and -acetate** because of their solubility in high AcN concentrations and also their volatility, which makes them a suited buffer system for use in ESI-MS.

The use of buffer systems of pH 9 or higher can significantly change selectivity, especially as quaternary ammonium compounds show high retention under these conditions due to ionic interactions with deprotonated silanols. Since most silica-based materials will rapidly dissolve under these conditions, the use of **hybrid-silica** phases is a novel tool to overcome this obstacle and make it possible to screen a high pH values in method development.

The **concentration of the buffer** used has a large effect on retention of analytes using electrostatic interactions as the main separation mechanism. Variation in the range of 5–20 mM buffer concentration can lead to a drastic change of selectivity.[2] Combined with the nature of (high organic) liquids to evaporate over time this is a common source for non-reproducible results when using eluents which have not been freshly prepared. On the other hand this "shielding" effect of silanols due to counter ions in the mobile phase can lead to useful selectivity effects if buffer concentration can be maintained reproducibly. Furthermore increased salt concentration also increases the volume of the adsorbed water layer which successively increases the retention of neutral solutes.[21-22]

Column temperature

An increase in column temperature is always accompanied by a reduction in backpressure due to the decrease in viscosity of the mobile phase. But in HILIC mode temperature has a complex effect on the ionisation of stationary phase bonded groups, silanols, buffer components and analytes. Variation in the temperature range of 30-50°C generally shows a decrease in analyte retention of 3-30% with increasing temperature.[2, 12] However some basic analytes show increased retention with increasing temperature, which is an effect that currently cannot be explained.
Due to the relatively small effect of temperature changes on selectivity, it is often an overlooked parameter in method development. To ensure reproducible results column temperature should at least be set to a value just over room temperature to compensate for possible temperature fluctuations in the environment.

Sample injection

The influence of the injection solvent on HILIC separations and the resulting peak shapes is well studied on small molecules as well as peptides.[23] Generally samples should be injected in **pure AcN** to give best peak shapes and resolution. **Higher water concentrations in the injection solvent will proportionally lead to loss of efficiency** due to a plug of strong solvent and analytes being eluted through the column.[20]
In cases where it is not possible to inject in AcN because of the solubility of sample constituents or aggregation of peptides, it has been shown that reduction in injection volume can minimise this effect. As an example protein and antibody biopharmaceutical analysis in HILIC gave satisfactory results at concentrations of 65–80 % AcN when injecting in pure water, but decreasing injection volume to 0.1% of the column volume.[24] This strategy might also be applicable to environmental samples from pure water, but the generally low analyte concentrations in these applications may require very sensitive detection methods suited for HILIC or an enrichment of analyte via sample preparation techniques. For small molecules which are insoluble in AcN, it may be possible to inject in IPA or IPA/AcN mixtures providing solubility can be assured. For peptide analysis a switch to pure organic solvents such as EtOH or IPA can limit denaturation from occurring.[23]

Detection procedures

Because high concentrations of volatile solvents are used in HILIC, the response of different detection methods in which the mobile phase is evaporated is greatly enhanced.[25] Furthermore the formation of charged droplets in CAD or ESI-MS detection is facilitated by the low surface tension of HILIC mobile phases. The design of the ESI source has a huge influence on the relative sensitivity, but for most analytes an increase of signal-to-noise ratio compared to RP can be observed in the range of one order of magnitude and can increase by up to a factor of 800.[26]
It is very important particularly for methods using these detectors to maintain a constant pH and buffer concentration. When working with ESI-MS screening at different buffer concentrations can help to evaluate the ion suppression effects of buffer salts which can counteract some of the sensitivity gained.

Conclusions and summary

As an orthogonal separation mode HILIC offers unique advantages over reversed phase chromatography due to the high organic eluent contents and different interaction mechanisms. As a result of the water layer on the stationary phase, mass transfer and analyte diffusivity play a greater role in HILIC kinetics. Consequently HILIC is less well understood than other LC modes.
The greatest changes in selectivity are achieved by using different stationary phase chemistries, while changing organic solvent concentration and running gradients usually has a bigger influence on retention than selectivity itself. pH and the use of different buffer salts and concentrations have a considerable effect on analyte charge, stationary phase charge, water layer volume and selectivity of the stationary phase due to silanol activity quenching. Temperature has little effect on selectivity, but it is advised to keep it constant to maintain reproducibility.
In summary, we can give the following general tips for reproducible HILIC method development and troubleshooting:

- Stationary phase selectivities are very different in HILIC analysis.
  Screening different phases may give you a more appropriate phase for your analytes.
- The mobile phase should contain at least 3% and a maximum of 40% water
  to produce a stable water layer on the stationary phase.
- Depending on the application we suggest buffer concentrations up
  to 10 mM and to buffer both mobile phases for better miscibility and reproducibility
- Recommended buffers are ammonium salts of acetic or formic acid, bicarbonate salts or triethylamine phosphate for high solubility in organic solvents and suppression of secondary interactions. Acids such as formic acid or TFA as modifier may give poorer results than the use of genuine buffers.
- Use aprotic solvents such as acetonitrile, THF or acetone as weak eluent.
  Use of protic solvents including alcohols generally decreases retention and disturbs the stationary phase water layer.
- Keep in mind the pKa of your analytes and their charged state at a given pH. It is good practice to work 1–2 pH units above or below the pKa of your analytes and screen using at least 3 different pH values in your method development.
- Dissolve your sample in high organic concentration (pure AcN or other aprotic or protic solvents) or up to the starting composition of your eluent to suppress dilution and negative partition effects. Never inject samples in higher content of water or if this is not possible reduce the injection volume significantly.
- Give your HILIC phase sufficient time for equilibration. We recommend at least 20 column volumes prior to analysing samples and/or after gradient elution. If running a gradient keep the gradient slope relatively low or run small plateaux.

[1] D.V. McCalley, Understanding and manipulating the separation in hydrophilic interaction liquid chromatography, J.Chromatgr. A 1523 (2017) 49-71

[2] A. Kumar, J.C. Heaton, D.V. McCalley, Practical investigation of the factors that affect the selectivity in hydrophilic interaction chromatography, J.Chromatogr. A 1276 (2013) 33–46.

[3] P. Jandera, Stationary and mobile phases in hydrophilic interaction chromatography: a review, Anal. Chim. Acta 692 (2011) 1–25.

[4] Y. Guo, S. Gaiki, Retention and selectivity of stationary phases for hydrophilic interaction chromatography, J. Chromatogr. A 1218 (2011) 5920–5938.

[5] L.Z. Qiao, X.Z. Shi, G.W. Xu, Recent advances in development and characterization of stationary phases for hydrophilic interaction chromatography, Trac-Trend Anal. Chem. 81 (2016) 23–33.

[6] N.P. Dinh, T. Jonsson, K. Irgum, Probing the interaction mode in hydrophilic interaction chromatography, J. Chromatogr. A 1218 (2011) 5880–5891.

[7] D.V. McCalley, Evaluation of the properties of a superficially porous silica stationary phase in hydrophilic interaction chromatography, J. Chromatogr.A 1193 (2008) 85–91.

[8] S.M. Melnikov, A. Holtzel, A. Seidel-Morgenstern, U. Tallarek, A molecular dynamics view on hydrophilic interaction chromatography with polar-bonded phases: properties of the water-Rich layer at a silica surface modified with diol-functionalized alkyl chains, J. Phys. Chem. C 120 (2016)13126–13138

[9] S.M. Melnikov, A. Holtzel, A. Seidel-Morgenstern, U. Tallarek, Evaluation of aqueous and non-aqueous binary solvent mixtures as mobile phase alternatives to water-acetonitrile mixtures for hydrophilic interaction liquid chromatography by molecular dynamics simulations, J. Phys. Chem. C119 (2015) 512–523

[10] J.Y. Wu, W.G. Bicker, W.G. Lindner, Separation properties of novel and commercial polar stationary phases in hydrophilic interaction and reversed-phase liquid chromatography mode, J. Sep. Sci. 31 (2008)1492–1503

[11] Z.G. Hao, C.Y. Lu, B.M. Xiao, N.D. Weng, B. Parker, M. Knapp, C.T. Ho, Separation of amino acids, peptides and corresponding Amadori compounds on a silica column at elevated temperature, J. Chromatogr. A 1147 (2007)165–171.

[12] Z.G. Hao, B.M. Xiao, N.D. Weng, Impact of column temperature and mobile phase components on selectivity of hydrophilic interaction chromatography(HILIC), J. Sep. Sci. 31 (2008) 1449–1464.

[13] R.P. Li, Y. Zhang, C.C. Lee, L.M. Liu, Y.P. Huang, Hydrophilic interaction chromatography separation mechanisms of tetracyclines on amino-bonded silica column, J. Sep. Sci. 34 (2011) 1508–1516.

[14] D.V. McCalley, Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed-phase liquid chromatography for the analysis of ionisable compounds? J. Chromatogr. A 1171 (2007) 46–55.

[15] J.C. Heaton, J.J. Russell, T. Underwood, R. Boughtflower, D.V. McCalley, Comparison of peak shape in hydrophilic interaction chromatography using acidic salt buffers and simple acid solutions, J. Chromatogr. A 1347 (2014)39–48.

[16] M. Kosmulski, The pH dependent surface charging and points of zero charge.VI. Update, J. Colloid Interf. Sci. 426 (2014) 209–212.

[17] D.V. McCalley, Study of retention and peak shape in hydrophilic interaction chromatography over a wide pH range, J. Chromatogr. A 1411 (2015) 41–49

[18] D.V. McCalley, Effect of mobile phase additives on solute retention at low aqueous pH in hydrophilic interaction liquid chromatography, J.Chromatogr. A 1483 (2017) 71–79.

[19] X.R. Lei, C. Gong, Y.L. Zhang, X. Xu, Influence of the acetamide from acetonitrile hydrolysis in acid-contained mobile phase on the ultra violet detection in high performance liquid chromatography, Chromatographia 79(2016) 1257–1262.

[20] J.C. Heaton, D.V. McCalley, Some factors that can lead to poor peak shape in hydrophilic interaction chromatography, and possibilities for their remediation, J. Chromatogr. A 2016 (1427) 37–44.

[21] D.V. McCalley, U.D. Neue, Estimation of the extent of the water-rich layer associated with the silica surface in hydrophilic interaction chromatography, J. Chromatogr. A 1192 (2008) 225–229.

[22] S.M. Melnikov, A. Holtzel, A. Seidel-Morgenstern, U. Tallarek, A molecular dynamics study on the partitioning mechanism in hydrophilic interaction chromatography, Angew. Chem. Int. Edit. 51 (2012) 6251–6254.

[23] J. Ruta, S. Rudaz, D.V. McCalley, J.L. Veuthey, D. Guillarme, A systematic investigation of the effect of sample diluent on peak shape in hydrophilic interaction liquid chromatography, J. Chromatogr. A 1217 (2010)8230–8240.

[24] A. Periat, S. Fekete, A. Cusumano, J.L. Veuthey, A. Beck, M. Lauber, D.Guillarme, Potential of hydrophilic interaction chromatography for the analytical characterization of protein biopharmaceuticals, J. Chromatogr. A1448 (2016) 81–92.

[25] C.R. Mitchell, Y. Bao, N.J. Benz, S.H. Zhang, Comparison of the sensitivity of evaporative universal detectors and LC/MS in the HILIC and the reversed-phase HPLC modes, J. Chromatogr. B 877 (2009) 4133–4139.

[26] A. Periat, J. Boccard, J.L. Veuthey, S. Rudaz, D. Guillarme, Systematic comparison of sensitivity between hydrophilic interaction liquid chromatography and reversed phase liquid chromatography coupled with mass spectrometry, J. Chromatogr. A 1312 (2013) 49–57.