HILIC Method Development in a Few Simple Steps

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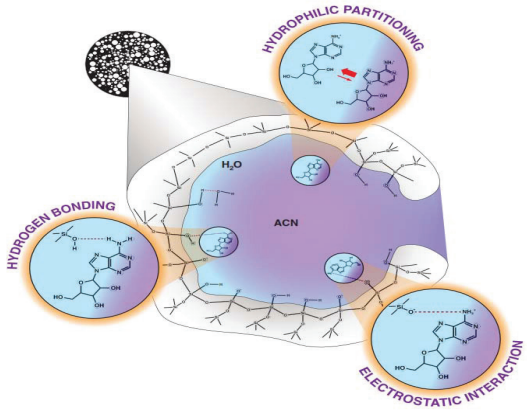
Overview

This poster presents a systematic approach to method development in HILIC.
Guidelines are provided for:
- column selection based on analyte(s) properties
- mobile phase selection, including composition, use of buffers and pH
- separation optimization

Introduction

Hydrophilic interaction liquid chromatography (HILIC) is arguably the most successful approach for the retention and separation of polar compounds. This technique can be described as a variation of reversed phase chromatography performed using a polar stationary phase. The mobile phase employed in HILIC is highly organic in nature (60-70% solvent, typically acetonitrile) containing a small percentage of aqueous solvent/buffer or other polar solvent. The aqueous portion of the mobile phase acts as the stronger solvent; it forms an aqueous-rich layer adsorbed to the polar surface of the stationary phase (as illustrated in Figure 1).

FIGURE 1. Schematic representation of the water-rich liquid layer within the stationary phase In HILIC



Polar analytes preferentially partition into this aqueous rich layer and evidence [1] suggests that they are retained through a complex, combination of:

- hydrophilic partitioning of the analyte between the aqueous-rich layer and the bulk of the mobile phase
- hydrogen bonding between polar functional groups and the stationary phase
- electrostatic interactions of ionized functional groups
- van der Waals interactions between the hydrophobic portions of the bonded ligands of the stationary phase and the non-polar part of the analytes.

In addition to the HILIC mechanism inherent complexity, there is variety of misinformation regarding the use of this technique.

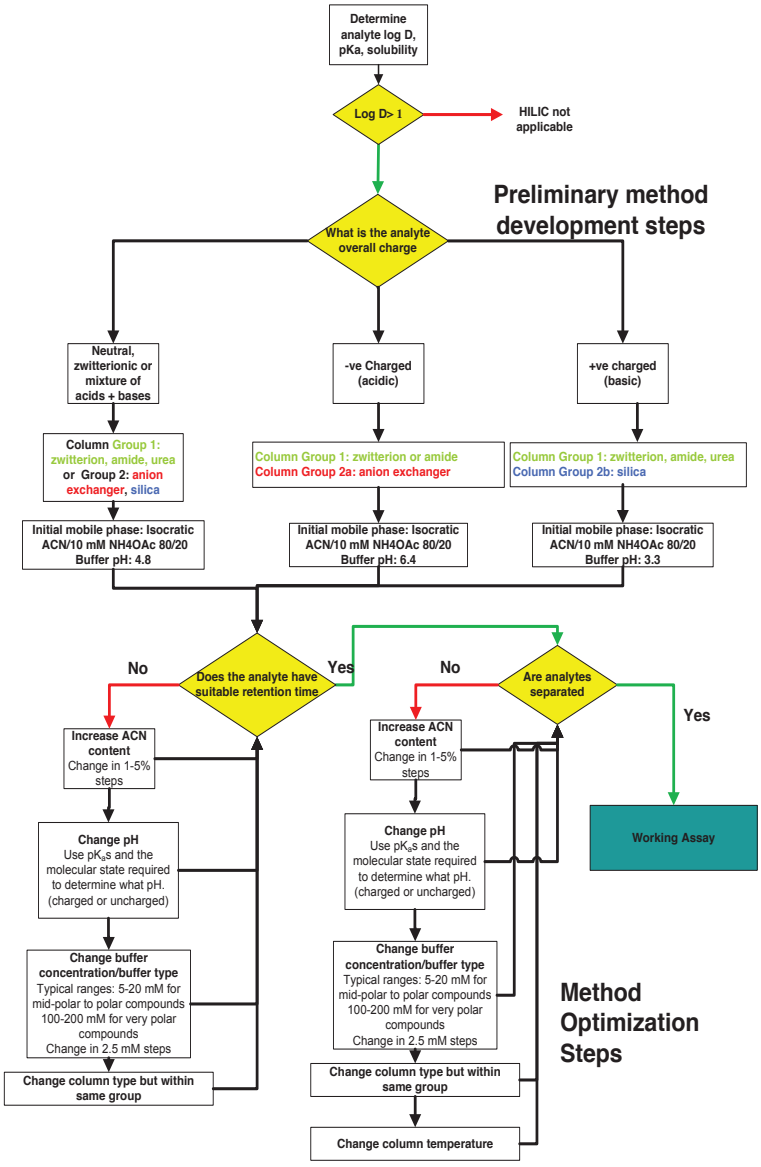
1. What column should be used?
2. What are the best mobile phase starting conditions?
3. What are the common issues in HILIC method development that need to be addressed?

These are the types of question that HILIC users face and will be addressed within this poster.

HILIC Method Development

We recommend the following sequential method development steps:

FIGURE 1. HILIC method development flow chart



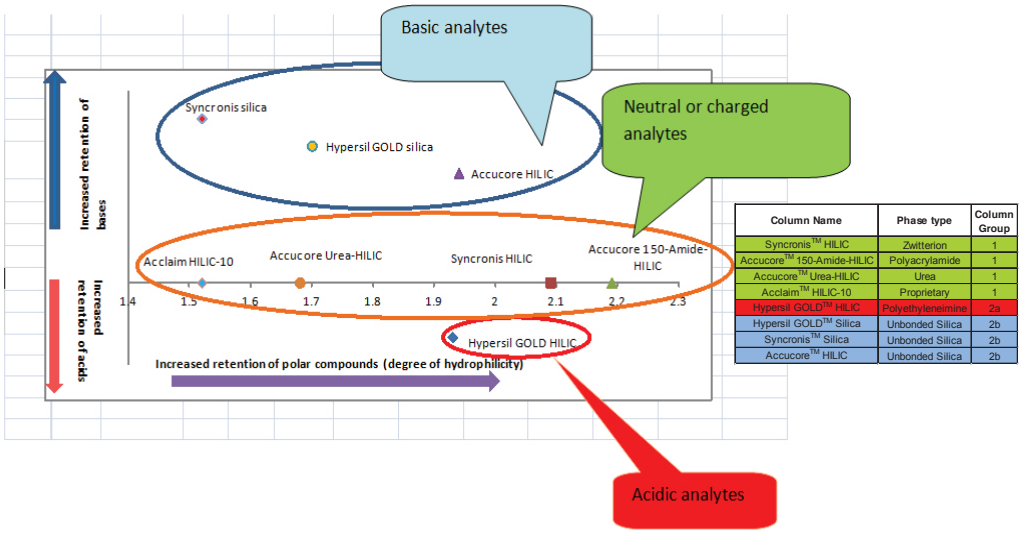
Further information on method parameters is given in next section.

Method Parameters Considerations

Column Selection

It is suggested to match the analyte log P or log D values to the degree of polarity of the HILIC phases. In general terms, the more negative the log P or log D value for an analyte, the greater the degree of stationary phase polarity required to retain it. The following chart, which illustrates the relative hydrophilicity and ion-exchange properties for Thermo Scientific™ HILIC columns, can be used as a guide in stationary phase selection at this stage:

FIGURE 3. Relative polarity and ion-exchange characteristics for various HILIC phases



Column Name	Phase type	Column Group
Syncronis™ HILIC	Zwitterion	1
Accucore™ 150-Amide-HILIC	Polyacrylamide	1
Accucore™ Urea-HILIC	Urea	1
Acclaim™ HILIC-10	Proprietary	1
Hypersil GOLD™ HILIC	Polyethyleneimine	2a
Hypersil GOLD™ Silica	Unbonded Silica	2b
Syncronis™ Silica	Unbonded Silica	2b
Accucore™ HILIC	Unbonded Silica	2b

Mobile Phase – Organic Content

In HILIC the mobile phase is highly organic (generally 60-70%; at least 3% water is required). It has been demonstrated that besides the selection of a suitable column chemistry, the organic modifier/aqueous ratio is a major factor controlling the separation selectivity. An increase in the percentage of organic solvent leads to an increase in retention.

Although acetonitrile is the most popular solvent used in HILIC, several other polar, water-miscible organic modifiers can be used. The elutropic strength is generally the inverse to what observed in RPLC.

Mobile Phase – Do I need a Buffer?

As a general guideline buffers are added to the mobile phase to reduce peak tailing and/or retention of charged analytes.

Due to their good solubility in organic solvents, the recommended buffers for HILIC are ammonium salts of acetic and formic acids. These buffers also have the advantage of being volatile for use with mass spectrometry and charged aerosol detection.

Generally, stationary phases with a net positive or negative charge require higher concentrations of buffers than neutral or zwitterionic phases.

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. The presence of buffers in the mobile phase can reduce electrostatic interactions (both attractive and repulsive) between charged analytes and the stationary phase.

Mobile Phase Buffer Type

Ammonium formate and ammonium acetate do not provide significant differences in retention times of acid and basic model compounds on neutral and zwitterionic phases.

However, the acetate ion has a greater neutralising effect of the electrostatic attractions between the surface of the charged stationary phase and the oppositely charged analyte, providing shorter retention times than ammonium formate [2].

Mobile Phase Buffer Concentration

When electrostatic attractions are prevalent, an increase in the salt concentration leads to a decrease in retention of charged solutes on the stationary phases of opposite charge. This phenomenon is illustrated in Figure 4, which show the separation of an acidic mixture on an anion exchanger, with the retention of the anionic analytes decreasing as the concentration of ammonium acetate increases.

Increased salt concentrations result in increased retention of positively charged solutes on stationary phases with same charge, as demonstrated in Figure 5, where the retention of cytosine and cytidine on an anion exchanger increases with the salt concentration. Enhanced hydrogen-bonding interactions (between the analyte and the stationary phase) are responsible for this behavior. The hydrogen-bonding interactions are facilitated by the increased population of solvated salt ions in the mobile phase (salting-out effect).

FIGURE 4. The effect of ammonium acetate concentration on the separation of a mixture of acids on Thermo Scientific™ Hypersil GOLD™ HILIC (anion exchanger). Mobile phase: 90/10 acetonitrile/ammonium acetate. Analytes: 1. Salicylamide; 2. Salicylic acid; 3. Aspirin

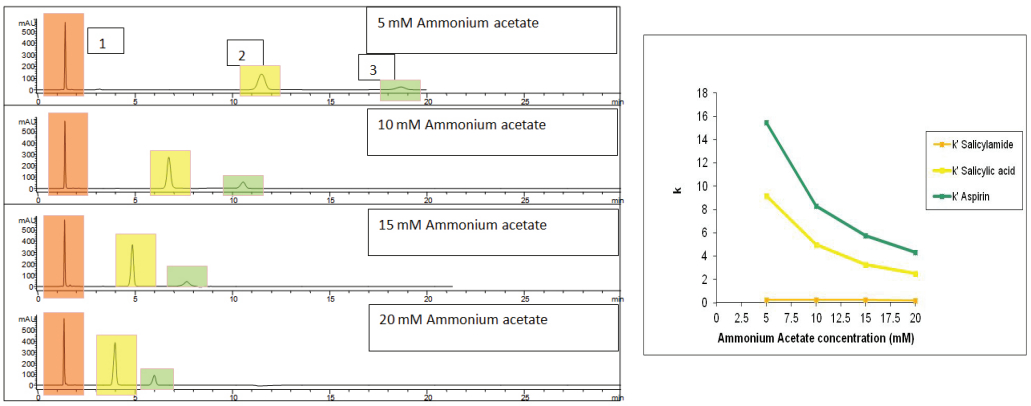
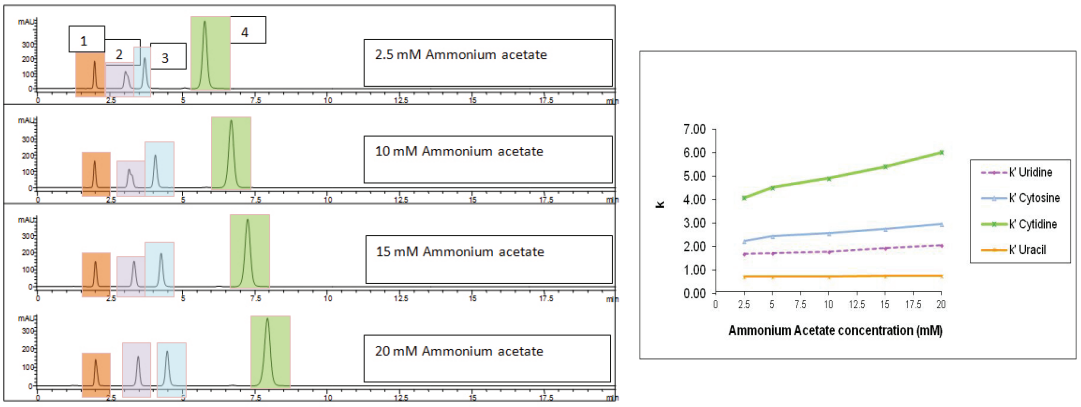


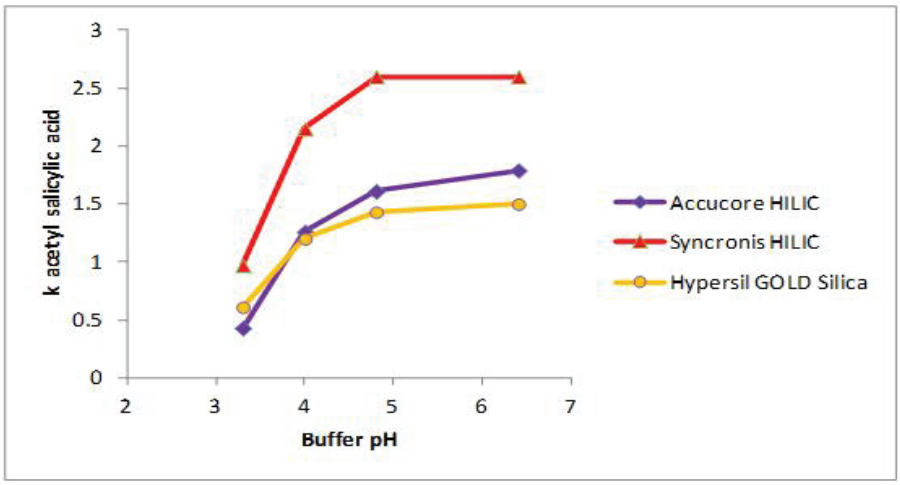
FIGURE 5. The effect of ammonium acetate concentration on the separation of a mixture of bases on Hypersil GOLD HILIC (anion exchanger). Mobile phase: 90/10 acetonitrile/ammonium acetate. Analytes: 1. Uracil; 2. Uridine; 3. Cytosine; 4. Cytidine



Mobile Phase Buffer pH

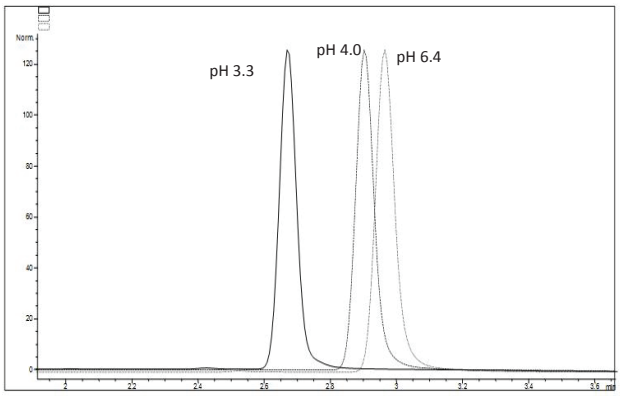
In general, charged compounds are more hydrophilic, and therefore are more retained in HILIC. The figure below shows the retention factor of acetylsalicylic acid increasing with the buffer pH, on bare silica and zwitterionic phases:

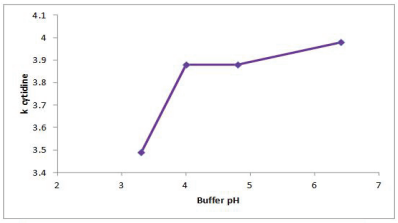
FIGURE 6. The effect of mobile phase buffer pH on the retention of acetylsalicylic acid. Mobile phase: 90/10 acetonitrile/100 mM ammonium formate. The mobile phase buffer pH was measured before the addition of acetonitrile



The mobile phase buffer pH can also affect the stationary phase charge state; this, for example is the case for bare silica phases, where the silanol ionisation varies with the mobile phase buffer pH. At pH>4-5, the silanols are deprotonated, making the silica surface negatively charged. This will have an effect on the retention of positively charged analytes. The increased retention for cytidine, illustrated in Figure 7 demonstrates this phenomenon.

FIGURE 7. The effect of mobile phase buffer pH on the retention of cytidine on a bare silica phase. Mobile phase: 90/10 acetonitrile/100 mM ammonium formate





It is very important to fine tune the mobile phase buffer pH, when using bare silica phases for the analysis of basic compounds, in order to avoid excessively long retention times and peak broadening

Conclusions

These are some key tips in method development and optimisation:

- Use acetonitrile or other polar, water-miscible organic modifiers. Remember that the elutropic strength is inverse to what observed in RPLC. Aprotic solvents give longer retention than protic solvents.
- Have a high organic content, between 60 to 97%; a minimum of 3% water is necessary to ensure sufficient hydration of the stationary phase.
- An increase in organic solvent will lead to an increase in retention.
- Use buffer salts such as ammonium acetate and ammonium formate to avoid peak tailing and to control retention times of charged analytes.
- Buffer salts concentrations are 2-20 mM, although 20 mM is recommended for organic content of up to 90%. Higher concentrations would not be soluble in high levels of organic and could impair MS or CAD signals.
- When using gradients, buffer both mobile phases, do not run buffer gradients.
- Do not run gradients from 100% organic to 100% aqueous. We suggest a 97-60% organic gradient.
- The charge state of the stationary phase can affect HILIC retention of ionisable compounds, depending on the mobile phase pH.

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