

Hydrophilic Interaction Liquid Chromatography (HILIC)—A Powerful Separation Technique

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ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) is a variant of normal phase liquid chromatography provides that partly overlaps with other chromatographic applications such as ion chromatography and reverse phase liquid chromatography a way to separate small polar compounds on polar stationary phases effectively. The purpose of this work was to review the characterization of HILIC stationary phases and their applications and its column chemistry and also method development as HILIC increases the scope of possible applications of liquid chromatography.

Keywords: Hydrophilic interaction liquid chromatography; Stationary phase; Applications; Buffers

INTRODUCTION

Hydrophilic interaction liquid chromatography (HILIC) is an alternative high-performance liquid chromatography (HPLC) mode for separating polar compounds. For historical reasons, it has been reported that HILIC is a variant of normal phase liquid chromatography, but the separation mechanism used in HILIC is more complicated than that in NP-LC. While the acronym HILIC was first suggested by Alpert in 1990 [1]. Expensive ion pair reagents are not required in HILIC, and it can be conveniently coupled to mass spectrometry (MS), especially in the electrospray ionization (ESI) mode. In contrast to RP-LC, gradient elution HILIC begins with a low-polarity organic solvent and elutes polar Analytes by increasing the polar aqueous content [2]. It has been successfully applied to the analysis of carbohydrates [3,4] peptides [5-7] and polar pharmaceuticals [8,9] etc. HILIC also allows the analysis of charged substances, as in ion chromatography (IC).

Since the 1970s, the introduction of high performance liquid chromatography (HPLC) as a analytical separation technique has promoted significant progress in the pharmaceutical sciences. Reversed-phase (RP) separations technique using hydrophobic stationary phases with polar mobile phases have greatly increased the application of the chromatographic technique. Today, about 70% of HPLC separations are performed in the Reversed-phase mode [10]

The vast applicability of RP-HPLC is due to its versatility and constant development of new stationary phases and instruments.

However RP HPLC separations still presents some limitations such as analysing highly polar and basic compounds has been problematic. Even some polar compounds are very difficult to analyze because they require high concentrations of aqueous buffer, even on the most inert RP columns, resulting in imperfect peak shapes.

Another separation technique, normal phase (NP)-HPLC, which involves a polar stationary phase and organic eluents, presents low-efficiency separations with asymmetric chromatographic peaks while analysing polar compounds [10]

Hydrophilic interaction liquid chromatography (HILIC) is an alternative high-performance liquid chromatography (HPLC) mode proven to be a versatile analytical tool for the separation of polar and ionisable compounds. Over the past two decades HILIC has gained popularity in separation and analysis of many compounds in foods, organic nutrients (carbohydrates, phospholipids, amino acids, peptides, and proteins), contaminants, and toxic compounds. The term HILIC was first introduced by Andrew Alpert in the year 1990 and refers to combination of a polar stationary phase (SP) and a mobile phase (MP) containing minimum 2-3% of water and a substantial proportion of water-miscible organic solvent (>60%). Number of publications on HILIC has increased substantially since 2003, as outlined in the well-constructed review by Hemström and Irgum [11].

Typical applications of HILIC involve highly hydrophilic stationary phases such as silica, amino or cyano [12-15]. In most cases, the

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mobile phase used is similar to those employed in the RP-LC mode. Most frequently acetonitrile (ACN), sometimes methanol] containing up to 30% water. Applications of HILIC include the separation of substances [e.g., biomarkers [16], nucleosides [17], human and vegetable metabolites [17], pharmaceuticals [18], and proteins [19], and many others that contribute to the development of medicinal chemistry, molecular biochemistry, metabolomics and other areas. The increase in HILIC applications are shown by the increase in publications on this theme, illustrating that this separation mode is increasingly being adopted by researchers.

This paper covers fundamental developments in hydrophilic interaction liquid chromatography. The objective of the present work is to review options for the characterization of HILIC stationary phases and their applications to separations of polar compounds in complex matrices. Gaining a thorough understanding of retention behaviour in HILIC enhances the scope of applications of liquid chromatography. The separation mechanism can depend on many factors, such as the physicochemical properties of the stationary phase and hydro organic mobile phase, and the structures of the samples investigated. Precisely defining which mechanism prevails is currently a difficult and complicated task. This phenomenon is still waiting for theoretical elucidation.

Stationary Phases

First generation of stationary phases used in HILIC separation was started in the year of 1975 by using amino silica phase, linden et al., using a mixture of acetonitrile and water (75:25 v/v) they separated carbohydrates [20]. Diol and amide are second generation as Stationary phases were came into existence [21]. Out of all, DIOL-silica column has more advantages for the separation of proteins [22,23].

Chemically bonded stationary phases with specific structural properties have been prepared by Buszewski et al. [24-27]. In this method the Unmodified bare silica gel was found to be advantageous when compared to chemically bonded stationary phases.

Chemically bonded stationary phases contains amino-propyl ligands bonded to silica (SG- NH₂); and an alkyl amide packing phase (SG-AP) and a mixed phase (SG-MIX) containing different types of ligands (-NH₂, -CN, -Ph, -C₈, -C₁₈) bonded to support. Over the last 20 years, It was progressed into 2nd & 3rd generation implementations, which involved mixed or multiple-interaction solid phases. Some novel separation materials for HILIC have attracted increasing attention in recent years [28-33].

Type A silica gels, prepared by precipitation from the solutions of

silicates, Type B silica gels are formed by the aggregation of silica sols in air, which contains very low amount of metals, and also more stable at intermediate even in higher pH values (up to at least pH 9) than xerogel-type materials. Type B especially for basic samples provides better separations, because of their high purity and less acidic “sol-gel” spherical silica particles [34].

Silanol groups are ionized at higher pH Suppressing silanol ionization through the addition of TFA may promote the ion-pairing mechanism. Silica gel type C with a hydrosilated surface populated with nonpolar silicon hydride Si-H groups instead of silanol groups [35].

Similar effects have also been observed in HILIC on monolithic silica gel columns, which offer higher permeability when compared to that of particle-packed HILIC columns [36].

It can be used to separate acids or bases in the HILIC mode in buffered mobile phases containing more than 50-70% organic solvent (acetonitrile).

So, the structural variations of HILIC stationary phases are wider than those found in RP- systems. HILIC phases can be grouped into ionic surfaces or neutral polar which show good reproducibility and also selectivity for the separation of compounds which are polar. However, In HILIC different types of separation materials will have different separation selectivities and retention characteristics. Cyclodextrin-silica stationary phases that possess several linked glucopyranoside units and have chiral recognition properties which are useful for HILIC chiral separations [37]

Zwitterionic stationary phases have also been introduced for HILIC separations. Zwitterionic columns are commercially available under the trade names ZIC-HILIC (on a silica gel support) and ZICpHILIC (on a polymer support) [38].

The separation of neutral compounds on Ion exchangers under typical HILIC conditions has been known about for a very long time. Only the retentions of some polar compounds (e.g., carbohydrates and related substances) increase with increasing ethanol concentration in the mobile phase. For other compounds, the opposite effects have been observed [39-41].

A mixed-mode HILIC/ion-exchange mechanism controls the retention, which may cause specific selectivity, due to the presence of ion-exchange groups.

For the analysis and purification of compounds from natural products, the mixed anion-exchange/cation-exchange/HILIC mechanism that occurs on silica-based, small-pore, weak ion exchange resins was found to be useful (Figure 1).

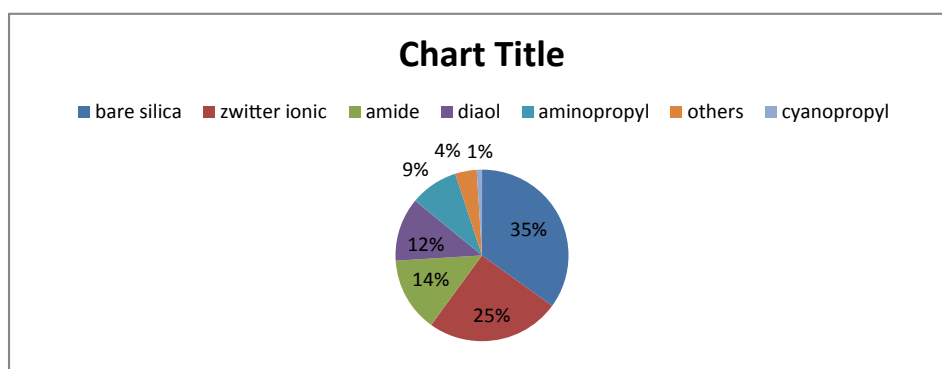


Figure 1: Typical HILIC Stationary phases and its Applications. (Bare silica, zwitter ionic, amide, diaol, amino propyl, cyan propyl, others).

Columns Used In HILIC

HILIC method uses the columns that contains hydrophilic stationary phase, may be charged sometimes. The choice for the selection of an appropriate column is mainly depends upon the separation of solutes from the sample. The two factors that mainly plays important role while choosing a column are both the selectivity and capacity factor. The column capacity factor mainly determines the retention time and it also tells that, the analyte gives the relative speed of the solute through the column and is calculated by $k = (t_R - t_0) / t_0$ where, t_R is the retention time of the solute to is the required time for the void volume [42].

Column Care in HILIC

As with any other column, to maximize HILIC column life ensure that samples and mobile phases are particle-free. We also recommend the use of guard columns or inline filters between the switching valve and the column (Table 1) [43,44].

Mobile phase

It is commonly believed that in HILIC, the mobile phase forms a water-rich layer on the surface of the polar stationary phase vs. the water-deficient mobile phase, creating a liquid/liquid extraction system. The analyte is distributed between these two layers.

Typical eluents for HILIC consist of Acetonitrile in water or a volatile buffer (40-97%). water should be added at least of 3% in the mobile phase to get reproducible results, This addition of water is in order to ensure sufficient hydration of the stationary phase particles. In addition to it, high concentration of organic solvent in the mobile phase will increase the retention. Several polar, water-miscible organic solvents can be used in HILIC, although Acetonitrile is by far the most popular and the relative solvent strength can be outlined as:

Acetone < Acetonitrile < iso-propanol < ethanol < methanol < water.

Any aprotic solvent which is miscible with water (e.g., tetrahydrofuran, THF, and/or dioxane) can also be used also Alcohols can be used, though higher concentration is needed to achieve the same degree of retention of the analyte relative to an aprotic solvent-water combination. An allotropic row is useful for selection of a suitable organic modifier for the mobile phase.

HILIC separations are performed either in isocratic mode or in gradient mode. In isocratic mode with a high percentage of organic solvent. In gradient mode starting with a high percentage of organic solvent and ending with a high proportion of aqueous solvent. Sometimes if needed, a solvent system is used where there is no water at all, e.g. with methanol as the stronger solvent, but this does not show any added selectivity, because of the pH-dependency

of the charge of the stationary phase it is especially important to fix the pH of the mobile phase with a buffer. The components of such buffers need to be soluble in all compositions of the mobile phase, and commonly consist of ammonium acetate or formate salts. The choice of buffering salt may be important for the selectivity of the separation.

Buffer Recommendations and pH

Additives: Suitable buffers for HILIC are ammonium salts of acetate and formate, but also formic and acetic acids are recommended, which are added usually to control the mobile phase pH and its ion strength. Low solubility buffers are avoided to prevent precipitation if needed then they should be used with caution. Either ammonium carbonate or Ammonium hydroxide are suitable alternatives when a high pH is desired. In this they contribute to the polarity of the analyte, resulting in differential changes in retention. For extremely polar Analytes (e.g. amino glycosides: gentamicin or Adenosine triphosphate), higher concentrations of buffer (ca. 100mM) required to assure that the analyte will be in a single ionic form, Otherwise asymmetric peak shape, chromatographic tailing, and poor recovery from the stationary phase will be seen. For the separation of neutral polar Analytes Buffers are not necessary.(e.g. carbohydrates).

To increase the mobile phase polarity and to effect the elution usage of other salts such as 100-300mM sodium perchlorate which are soluble in high organic solvent mixtures,(ca. 70%-90% Acetonitrile), can be used, which are not volatile as well. An occasional "wash" with water is required as all the ions partitions into the stationary phase to some extent, to ensure a reproducible stationary phase [45].

Choice of pH: The choice of the pH can affect the Ionic nature of the column chemistry and also affects the polarity of the solutes. The pH was adjusted so that to reduce the selectivity towards functional groups with the same charge as the column, or it can also be increased for oppositely charged functional groups. Incase of the column surface chemistries that are strongly ionic, and are resistant to pH values in the range of the pH scale (pH 3.5-8.5), these separations will be reflective of the polarity of the analytes alone, and hence it will be easier to understand while doing method development.

Working of HILIC

HILIC works on inverse of Reverse Phase Chromatography. It is generally believed in HILIC that the mobile phase forms a water-rich layer over the surface of the polar stationary phase which creates a liquid/liquid extraction system [46,47]. It separates the compounds by passing a hydrophobic or mostly organic mobile phase across a neutral hydrophilic stationary phase, causing solutes to elute in order of increasing hydrophilicity. One may use 15mM

Table 1: Shows the column care parameters like pH stability followed by column washing and storing conditions.

p ^H stability	Thermo Scientific HILIC columns are silica based. A suitable pH range is 2–8.
Washing conditions	Prior to storage, buffered solutions used during HILIC analysis should be washed out of the column (and the HPLC system). A suitable wash solution is 60% organic solvent in water.
Storing conditions	HILIC columns should be stored under HILIC conditions. A mixture of organic solvent and water in a 90:10 ratio is recommended for storage. The Product Manual will give specific advice. Ensure the column end-fittings are sealed with end-plugs to prevent the stationary phase from drying.

ammonium formate with neutral peptides on reverse organic conditions. The analyte is normally distributed over these two layers. Highly charged molecules require low amounts (e.g., 10 mM) of salt for ion suppression, and a slight per chlorate or sulfate gradient to effect desorption.

However, HILIC not only works by just simple partitioning but also includes hydrogen donor interactions between neutral polar species as well as weak electrostatic mechanisms under the high organic solvent conditions which are used for retention, this mechanism of HILIC distinct it from the ion exchange chromatography. Highly polar compounds will have a stronger interaction with the stationary aqueous layer than that of less polar compounds. Thus, a separation takes place based on a compound's polarity as well as degree of solvation.

Separation Mechanisms

There are essentially three possible ways to show the separation mechanism. The first is partitioning of analyte between the stationary and mobile phases; The second is the analyte adsorption onto the surface of the adsorbent; The third assumes that there will be a preferential adsorption of the mobile phase which is organic modifier onto the surface of the adsorbent, further followed by the partitioning of this analyte into the adsorbed layer.

The retention phenomenon in HPLC is simultaneously depends on various types of intermolecular interactions between the solute and the stationary phase, the solute and the mobile phase, and the stationary and mobile phases [48-51].

HILIC Method Development

A systematic approach to method development should be taken when developing a method in HILIC [52].

Method parameters should be considered and adjusted sequentially, one at a time.

This section provides some guidelines for:

- which type of stationary phase is the most appropriate to use
- what effects the mobile phase composition and pH can have on the separation
- how the column temperature can influence HILIC analysis [53-55].

How to Avoid Common Problems with HILIC Methods

Column conditioning and re equilibration are essential for retention time reproducibility:

The first step to ensure success with HILIC method understands that the analytical column must be properly conditioned before use and also sufficiently equilibrated before injections. Failure of conditioning and equilibration may result in the water layer not being fully re established on the particle surface, which may lead to irreproducible retention times.

To condition the column: It must be flushed with mobile phase that will be used during analysis. For isocratic method 50 column volumes should be used and for gradient method at least 10 blank injections running the full time programme should be performed.

Condition also required when you change the mobile phase composition or concentration of any additives.

Re equilibration of column: In addition to conditioning equilibration of column also very important between the injections as the separation mechanism in HILIC involves adsorption of a water layer on the particle surface, it is very important to completely re-establish or re set this water layer in between injections to ensure retention time reproducibility.

Injection solvent: Injection solvent matching is critical for good peak shape.

Mobile phase pH: Mobile phase pH effects are analyte dependent.

Buffer choice: choice of buffer influences both chromatographic and instrument performance.

Trouble Shooting

This section is intended as an aid in troubleshooting specific chromatographic problems that could occur during the running of HILIC separations. It is not meant to be an exhaustive guide to HPLC column and instrument troubleshooting. Advice specific to your column will be found in the Product Manual [56].

The following topics will be covered:

- Solubility
- Allowable buffers
- Retention reproducibility (peak retention drift)
- pH extremes affecting column stability
- Injection solvent
- Injection volume
- Syringe wash

ROLE OF HILIC IN PHARMACEUTICAL ANALYSIS

HILIC is one of chromatographic mode of analysis that has been gaining importance for the last few years, particularly with the increasing need for analysis of a large variety of biologically active substances including pharmaceutical compounds, amino acids, peptides, neurotransmitters, oligosaccharides, carbohydrates, nucleotides and nucleosides [57].

Several pharmaceutical applications have been reported over the last 10 years with HILIC, including the analysis of drug compounds, their impurities and metabolites in either bulk material, pharmaceutical formulations or body fluids (blood, plasma, serum and urine) [58].

In a study, 45 drugs possessing log D_{pH3} between -3 and 4 were selected by Rota et al. [59]. Because most of the drugs were ionisable, more than 90% were sufficiently retained under HILIC conditions, where it found that the achieved selectivity was very different from the reversed-phase LC.

In another work, an extended set of 82 drugs with log P values between -2 and 9 was performed using numerous HILIC phases and mobile phase conditions. Again, the retention of drugs was satisfactory (around 90%) with various mobile and stationary phase conditions. Only the less polar acids were hardly retained because of electrostatic repulsions with the negatively charged surface of the HILIC phases [60].

These two studies confirm that the applicability of HILIC in

achieving orthogonal selectivity and retention is more than compared to that of any other chromatography particularly reversed-phase LC. Thus HILIC was considered to analyse various types of polar (APIs) in formulations. In addition to it HILIC was also found to be used in drug development for the separation of synthetic starting material, impurities, intermediates, excipients and APIs. Reversed-phase LC and HILIC columns were coupled in series to broaden the elution window for the simultaneous analysis of both polar and non polar drugs. This innovative method applied for the concurrent analysis of sugars and sulphonamide, Genotoxic impurities, including aryl amines and amino pyridines.

Advantages

- Hydrophilic interaction chromatography (HILIC) is fast becoming and also the preferred technique when encountered with polar and/or basic solutes.
- HILIC affords several advantages making the technique reliable over Normal and reversed phase HPLC.
- The main advantage is that it has ability to retain hydrophilic compounds without the need for solvents which are toxic and expensive such as those generally used in normal-phase liquid chromatography, and also without significant amount of salts or ion-pairing reagents, which hardly compatible with MS detection [61].
- Because of its the strong contribution of an ion exchange mechanism, the achieved selectivity is generally very different in HILIC as opposed to reversed-phase LC, for example, in the case of peptides separation [62].
- Major benefit of the HILIC mode is the enhanced signal in MS, explained by efficient desolvation of highly organic mobile phases. A sensitivity gain of up to 10 times can be achieved in hydrophilic interaction liquid chromatography-electro spray ionization-mass spectrometry (HILIC-ESI-MS) compared with reversed-phase LC-ESI-MS [63].
- A low viscosity of highly organic mobile phase (on average 2-3 times lower than reversed-phase LC), the generated backpressure is also limited, which allows higher flow rates with longer columns and smaller particle sizes to be used. This has been illustrated by McCauley [64], who reached efficiencies in excess of 100,000 plates within reasonable analysis times (less than 15 min) when using a 45-cm column.
- In general, it yields much higher solute diffusivity, increased sensitivity with ESI-MS and also peak shapes which are highly symmetrical.

Disadvantages

- The main disadvantage is that it depends heavily on aprotic solvent; Acetonitrile will be viewed as the major disadvantage of HILIC preponderantly from a sourcing perspective.
- It is perceived that HILIC is a less flexible technique over RP-HPLC methodology as most studies done on RP-HPLC

Applications [65-74]

- This method is applicable in the fields of pharmaceutical chemistry, proteomics, glycomics, metabolomics, medical

science, agricultural and food chemistry.

- HILIC mode is extensively used to separate some bio molecules through differences in polarity as well as both organic and inorganic molecules.
- HILIC Using Silica Columns for the Retention of Polar Analytes and Enhanced ESI-MS Sensitivity.
- It is used for the analysis of polar contaminants in food and environmental samples.
- HILIC offers a tenfold increase in sensitivity over RP-chromatography because the organic solvent is much more volatile.
- hydrophilic interaction chromatography method was Improved for both identification as well as quantification of glucosinolates.
- These type of separations were made easier when combined with several detection techniques, such as fluorescence (FL), ultraviolet light absorbance (UV), evaporative light scattering (ELSD), refractive index (RI), and mass spectrometry (MS), charged aerosol (CAD).
- It is well suited to the sensitive LC-MS analysis of water-soluble (polar) compounds, because of its high organic content in the mobile phase which leads to rapid evaporation of the solvent during ESI.
- Determination of the Stabilizer Sucrose in a Plasma-Derived Antithrombin Process Solution by HILIC with Evaporative Light-Scattering Detection (Table 2).

Table 2: Application of some Herbal plants.

S no	Name of herbal plant	Group detected	Reference
1	Quillasaponaria	Saponins	[72]
2	Sapindusarak	Oleanetriterpene, oligoglycosides, rarasaponins, rarasoside, other saponins and sesquiterpene glycosides	[73]
3	Arabidopsis thaliana	Phytochelatin	[74]
4	Mitragynaspeciosa	Mitragynine: the primary active alkaloid	[75]

CONCLUSION

As a result of recent advances in mass spectrometry and computation, developed for proteomics and metabolomics, it is now possible to characterize and identify thousands of compounds upon elution in chromatography. This makes it feasible to study the mechanisms in HILIC through an inductive approach, using the chromatographic behaviour of numerous analytes to identify trends in retention. The progress in proteomics made possible by HILIC may be reciprocal. To quote Mikhail Tswett: "Every scientific advance is an advance in method".

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