Hydrophilic interaction chromatography

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In the early 1990s, hydrophilic interaction (liquid) chromatography (HILIC) emerged as a new separation method. HILIC is becoming increasingly popular for the separation of polar compounds in aqueous-organic mobile phases rich in organic solvents (usually acetonitrile). It has become important for the separation of pharmaceuticals, neurotransmitters, nucleosides, nucleotides and other compounds.

This review presents an overview of HILIC separation systems, comparing it to several other recently described modes. © 2012 Elsevier Ltd. All rights reserved.

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1. Introduction

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

The vast applicability of RP-HPLC is due to its versatility and to the constant development of new stationary phases and instrumentation. However, the usual RP-HPLC separations still present some limitations. Analysis of highly polar and basic compounds has been problematic since the beginning of HPLC. Some polar compounds are so difficult to analyze that they require high concentrations of aqueous buffer, even on the most inert RP columns, resulting in imperfect peak shapes.

Another separation mode, normal phase (NP)-HPLC, which involves a polar stationary phase and organic eluents, presents low-efficiency separations with asymmetric chromatographic peaks when analyzing polar compounds.

Hydrophilic interaction (liquid) chromatography (HILIC) is an interesting alternative for the analysis of polar substances. HILIC can be defined as a separation mode that combines stationary phases usually used in the NP mode and mobile phases used in RP separations.

Thus, typical applications of HILIC involve highly hydrophilic stationary phases, (e.g., bare or chemically modified silica, or polar polymers). In most cases, the mobile phase is a polar organic solvent [most frequently acetonitrile (ACN), sometimes methanol] containing up to 30% water. These applications include the separation of substances [e.g., biomarkers [1], nucleosides [2], human and vegetable metabolites [2], pharmaceuticals [3], and proteins [4]], and many others that contribute to the development of medicinal chemistry, molecular biochemistry, metabolomics and other areas. The increase in HILIC applications is shown by the increase in publications on this theme, illustrating that this separation mode is increasingly being adopted by researchers, especially towards the end of the 2000s (Fig. 1).

This review explores several aspects of HILIC, as the use of this separation mode is becoming more relevant in HPLC.

1.1. History

In 1990, Alpert [5] suggested the term “HILIC” to describe a separation mode in which polar analytes interact with a
moderately polar stationary phase and are eluted with a relatively hydrophobic mobile phase. However, in 1975, Linden and Lawhead [6] described a very successful separation of saccharides using silica chemically modified with amine groups as stationary phase and a mixture of ACN and water as mobile phase. In the same year, Palmer [7] executed a similar study, in which saccharides were analyzed on amine-based columns, using ACN and water as eluent. Thus, other authors had already shown the application of HILIC as a separation mode before Alpert's insight in 1990.

Although the first analyses used carbohydrates as polar analytes and amine-modified or bare silica columns, Alpert's work [5] described a silica column coated with an organic cation-exchange polymer and a neutral polymeric coating for the separation of amino acids and peptides. This study confirmed that stationary phases other than bare silica could be used in the HILIC mode, but that all of the stationary phases useful for HILIC should have polar characteristics. Alpert was the first to demonstrate, experimentally, that separations involving different column types and a different class of analytes could be accounted for by a common mechanism.

After this start, various studies about the mechanisms involved in HILIC separations were initiated, to clarify the mechanisms of this separation mode [8–10].

2. Fundamentals

2.1. Separation mechanisms

The first relevant study about the separation mechanism in HILIC involved carbohydrates, because the authors believed that this class of compounds comprised ideal model analytes [6,7]. These studies were performed when the term “HILIC” had not yet been adopted, showing that the search for answers to the mechanisms involved in this separation mode were initiated with the first applications.

Some authors explained the separation of polar compounds in terms of the interaction of hydroxyl groups of the analytes with the polar groups on the stationary phase surfaces [11,12]. As in the NP mode, the proposed separation mechanism is governed by adsorption. However, this idea was limited to analytes that possessed hydroxyl groups in their structures, which reduced the scope of this hypothesis. Soon the appearance of new, better ideas led to replacement of the direct adsorption mechanism.

Several other authors concluded that the most relevant mechanism in the HILIC mode was partition, after the observation of carbohydrate separations using bare and amine-modified silica columns [13–15].

Later, Alpert [5] proposed a complex mechanistic separation in the HILIC mode, still accepted today. In it, the polar groups attached to the different types of stationary phase attract water molecules, forming an aqueous layer over the surface. Thus, a polar analyte that is dissolved in the mobile phase undergoes partitioning between two liquid phases: the semi-immobilized aqueous layer and the mobile phase, also having some aqueous content. Polar solutes have higher affinities for the semi-immobilized aqueous layer than for the mainly organic mobile phase, since they are better solvated in the former. This preference leads to increased interaction of the solute with the aqueous layer, increasing retention.

Figure 1. Number of publications indexed on Web of Science with terms “HILIC” and “hydrophilic-interaction chromatography” 2000–11.
Orth and Engelhardt [14] showed that an increase of water content in the mobile phase could also increase the amount of superficially retained water in the stationary phase, producing a stationary liquid. The thickness of the water increases until a saturation limit, which depends on the number and the nature of the chemically-bonded polar groups on the stationary phase.

Reanalyzing these results, Hemström and Irgum [16] concluded that solute retention decreased with increased water content in the mobile phase, since, in separations governed by partition, the equilibrium partitioning of a solute between two phases depends on relative solubility, and, as the water content of the mobile phase increases, it becomes more similar to the retained aqueous layer. This leads to increased residence time of solute in the mobile phase and the consequent reduction in its retention. Thus, retention is directly proportional to the polarity of the solute and inversely proportional to the polarity of mobile phase and, in the HILIC separation mode, mobile phases with lower water contents are less polar (Fig. 2).

Currently, the separation mechanisms of HILIC are still under discussion. HILIC separations have been observed when both adsorption and partition processes are present, due to intermolecular forces (e.g., electrostatic interactions, hydrogen bonding, dipole-dipole interactions and weak hydrophobic interactions) [17–19]. This mechanism is multimodal, because it includes both hydrophilic and hydrophobic interactions, and is relevant for analytes with more than one functional group in their chemical structures.

Lü et al. [19] observed that the interaction of different analytes with a carboxymethyl-chitosan-coated silica column involves electrostatic interactions between ionized analytes and the chemically-bonded stationary phase and dipole-dipole interactions between the same groups and non-ionized polar analytes. These interactions suggest that processes of partition and adsorption are involved in the separation of analytes.

Jandera et al. [20] have proposed the simultaneous involvement of several possible mechanisms with polar stationary phases used for the separation of some flavonoids by RP and HILIC modes on the same column, just by varying the fraction of ACN in the mobile phase. This proposal is based on the observation of both hydrophobic type separations and hydrophilic ones with the same stationary phase, originally intended for the HILIC mode. These results also suggest that not all polar phases are candidates for multimodal mechanisms of separation, as the multimodal effect was observed with only the Luna HILIC column. This commercial column contains both oxyethylene and diol groups, with a tendency for separation by RP on the first and by HILIC on the second.
Recently, studies were carried out to confirm which interactions are involved in HILIC separations [21,22]. Dinh et al. [23] evaluated several commercial columns with different chemical properties (cationic, anionic, neutral and zwitterionic as well as those containing polar bonded groups) used in HILIC. Each group presented specific interactions with the test compounds, based predominantly on adsorption, which indicates that both adsorption and partition play important roles for retention in the HILIC mode.

The retention mechanism in HILIC depends not only on the stationary-phase type, but also on the chemical nature of the analyte and on the mobile-phase composition [17,24,25]. Karatapanis et al. [26] analyzed some water-soluble vitamins and proved that there is a transition from a partitioning mechanism into an adsorption mechanism when the percentage of organic solvent in the mobile-phase composition is augmented.

It is important to note that, although the mechanism of multimodal separation in HILIC is well accepted, many authors stress the importance of the partitioning process in this mode of separation, as initially proposed [27,28].

2.2. Stationary and mobile phases

2.2.1. Stationary phases. As in all modes of chromatography, the stationary phase is very important in HILIC separations, as the choice of phase influences the retention of the analyte and the separation of a mixture.

The first generation of stationary phases for HILIC included bare silica and silica chemically modified with aminopropyl groups [6]. The second generation contained diol and amide groups on silica, available since the 1980s and still applied for HILIC separations [2].

Zwitterionic stationary phases were introduced in the late 1980s [29,30]. Initial applications included ion chromatography [31,32]. Zwitterionic phases have short carbon chains with groups that are positively or negatively charged, linked to silica or to a polymeric support. This type of phase has great acceptance for many different applications, including HILIC separations, even today [33–36].

Silica is the most frequently employed chromatographic support for HILIC [36]. A wide range of functional groups are chemically bonded to the silica surface in order to prepare the modified stationary phases for HILIC analysis. These include aminopropyl, amide and cyano groups and polyols bonded to silica. Polar polymers, highly cross-linked carbohydrates or other solids capable of attracting water molecules to form the aqueous layer are also used in this mode of separation, often coated onto a silica support. The number and the variety of different hydrophilic groups have increased in recent years to overcome some drawbacks in the HILIC mode. Most of these stationary phases are commercially available.

As is well known, the chemical characteristics of different stationary phases influence the separation of any group of compounds. This also occurs in HILIC because the formation of an aqueous layer over the stationary phase depends on the number and the nature of the chemically-bonded groups, which determine the partitioning of the solute [37]. In addition, the chemical nature of the polar bonded group also influences the adsorption phenomena, resulting in surface interactions between analyte and stationary phase (e.g., hydrogen bonding, dipole interactions and ion exchange) [23].

Generally, polar interactions increase in the following sequence: cyanopropyl < diol < aminopropyl < silica (i.e. for the same analyte, bare silica columns usually have significantly higher retention than cyanopropyl-type columns) [38]. However, the strong electrostatic attraction presented by bare silica can cause residual interactions and peak asymmetry in the HILIC mode, reducing the separation efficiency for some classes of analytes [39]. The poor reproducibility on bare silica is reduced when using polar bonded stationary phases or hydride silica.

According to Kawachi et al. [21], to select the proper stationary phases for a separation target, one has to know the retention, the selectivity and the separation efficiency of HILIC columns for that specific application. They suggested an inclusive test scheme for HILIC stationary phases using nucleosides, saccharides, xanthines, sodium p-toluenesulfonate and trimethylphénylammonium chloride to describe some parameters (e.g., degree of hydrophilicity, selectivity for hydrophobic and hydrophilic groups, positional selectivity, configuration of hydrophilic groups, anion-exchange and cation-exchange properties, local pH conditions on the stationary phases, and shape selectivity). They observed that strongly hydrophilic phases included amide-bonded phases and zwitterionic phases. Certain phases (e.g., cyclodextrin-, diol-, triazole-, amine-bonded and bare silica) could be categorized as weakly hydrophilic.

McCalley [18] compared the retention of various types of ionizable polar compounds using different stationary phases in the HILIC mode – bare silica, amide, diol, zwitterionic and mixed-mode (long carbon chains with polar groups) – using the same mixture of water and ACN as eluent. This author concluded that, for the same compound, chromatographic parameters (e.g., retention factor and plate height) change according to the stationary phase used. As previously mentioned, the chromatographic behavior depends on certain factors (e.g., the stationary phase and the formation of the aqueous layer), which can change the interactions established by the analyte during the separation. Another important observation was the high retention factor of analytes on silica, whose retention times were much longer than with the other stationary phases (Fig. 3).
Polymer-based stationary phases are little used for separations in HILIC [40–42]. Ikegami et al. [2] showed that the separation efficiency with these columns is less than with silica-based columns, as is also seen in RPLC. However, polymeric columns can be used with strongly acidic or strongly basic conditions, which are not
indicated for silica-based columns in general, although bare silica can be used at low pH, as can silica with coatings not based on silanes [43].

2.2.2. Mobile phases. The content of water in HILIC separations is very important. The percentage of water in the mobile phase can vary (5–40%) [39]. A minimum of 2% is essential for formation of the aqueous layer involved in the separation and provides for the complete solubilization of the hydrophilic analytes, especially for preparative chromatography. However, a high aqueous content in the mobile phase can dramatically reduce hydrophilic analyte retention and lead to elution in the column-void volume [5].

Many polar organic solvents can be used, but the favorable characteristics of ACN (e.g., low viscosity and efficient separations at low pressures) make it the most used solvent in HILIC. ACN has a lower elution strength $e_0 = 0.65$, compared with methanol $e_0 = 0.95$, ethanol $e_0 = 0.89$ or isopropanol $e_0 = 0.82$, and the use of stronger eluting components can lead to elution of some analytes in the column void volume [44]. Although the strength of elution of acetone $e_0 = 0.58$ is lower than that of ACN, its high absorbance cut-off value $\lambda = 330 \text{ nm}$ makes it impractical for applications where detection is in the UV region, although it can be used with mass spectrometric (MS) detection.

Liu et al. [45] proposed the use of different alcohols as weak solvent alternatives to ACN in the separation of hydrazines in order to allow the use of an alternative universal detector (nitrogen chemiluminescence detector), which does not permit the presence of nitrogen in the mobile phase. They concluded that ACN may be replaced by ethanol, whereas methanol gave separations with low retention times and isopropanol presented both longer separation times and lower efficiencies.

By contrast, Fountain et al. [46] evaluated the use of methanol, ethanol and acetone as solvents to replace ACN for separation of different classes of polar compounds (pharmaceuticals, nucleotides and others) on bare silica columns. The results showed that the analytes were eluted in $t_m$ when methanol or ethanol was used as mobile phase components. The same elution behavior was observed when a mixture of isopropanol with tetrahydrofuran was used. Replacement of ACN by acetone caused a significant loss in detection using MS, which led the authors to conclude that acetone is not a good alternative to ACN for HILIC-MS.

Karatapanis et al. [26] evaluated the influence of organic solvent type on the separation of six hydrophilic vitamins, using diol columns. The separations using methanol and isopropanol were less selective, whereas separations with ACN as eluent were highly selective (Fig. 4).

Another advantage of using ACN over other organic solvents is its chemical structure, which does not favor the formation of hydrogen bonds. Thus, the use of ACN is very promising because it can avoid competition between solvent and water molecules for the stationary-phase interaction sites. Replacement of water molecules with an organic solvent would prejudice the formation of the aqueous semi-stationary layer, making it more hydrophobic and reducing the retention of polar analytes [47].

The presence of buffers or acids in the mobile phase can cause a major impact on separations in HILIC, interfering in the processes of partition and surface adsorption. Ionization of the analyte undergoes variations as a function of the pH of the chromatographic analysis. Moreover, the polar groups linked to the stationary phase can also be ionized, depending on the value of $pK_a$ of these groups. Thus, electrostatic interactions could be increased or decreased, and that may influence the adsorption process and the retention of some analytes.

The most important drawback of mobile phases in the HILIC mode is aqueous content. With high water content, hydrophobic interactions are non-negligible and can influence the hydrophilic interactions, reducing the retention of polar analytes [5].

3. Recent advances

3.1. HILIC-MS and HILIC-MS/MS

An important development in HILIC was its coupling to MS detection. Before MS, the most used detectors were based on absorbance and fluorescence, which depend on the nature of the analyte, sometimes requiring an additional step of derivatization. Separations in HILIC-MS and HILIC-MS/MS can overcome the limitations of other types of detection [48–50]. In recent years, the use of HILIC-MS/MS was popularized. A review of recent publications shows that coupling HILIC-MS was widely adopted by researchers (e.g., environmental analysis [51], food [52,53], natural products [54] and omics [55–57]).

The term “-omic” is currently used to describe studies with biological and biochemical systems containing a given group of similar compounds, leading toward an understanding of an even larger set [57]. Metabolomics, genomics and proteomics are the most representative “-omics” commonly encountered. The targets of such studies are often highly-polar or easily-ionized analytes, whose separations were previously limited to low-efficiency separations in RP. Developments in -omics have been driven by advances in separation techniques (e.g., HILIC) and the popularization of MS for detection [58].

In 2010, Eckert et al. [59] used HILIC-MS/MS for the determination of biochemical markers of exposure to several industrial carcinogens. Such monitoring is extremely important in occupational health. The results
showed that one important marker was present in the urine of both exposed and unexposed people, but to a lesser degree in the latter (reflecting endogenous production). The low detection limits of these analytes (3.0–7.0 ng/mL) permitted differentiation of the two health conditions.

Quintela et al. [60] proposed the use of HILIC-MS/MS for non-invasive evaluation of cocaine in hair samples. This complex matrix provides information on the intensity and the duration of consumption of this illicit material, while blood and urine do not provide such information. The HILIC method developed by the authors was shown to be suitable for application in forensic science, as its figures of merit (e.g., precision and accuracy) were similar to those of the reference method.

These two examples show that coupling HILIC to MS enables determination of analytes present in low concentrations in a sample, as is the case of specific metabolites (e.g., biomarkers). The specificity of MS/MS also allows analyses without complex components that may interfere with identification of the analytes.

### 3.2. Monolithic HILIC columns

Monolithic columns comprise a single rod having two general categories:

1. Silica based; and,
2. Rigid organic polymer-based.

The key advantages that they offer over packed columns include:

1. The absence of frits to retain the packed bed;
2. A stable chromatographic bed that cannot develop void space, or undergo changes in bed porosity or pore diameter; and,
3. A low column back pressure at higher mobile phase flow rates [61,62].

Despite these advantages, there have been only a few studies involving the use of bare silica monoliths in HILIC [63–65]. Developments aiming at applications in HILIC have been directed at preparing monolithic silica modified with polar groups or covered by polymers. The coating of the silica monolith can be done using various types of polymers. The derivatives of polyacrylamide and polyacrylate are the most used [66]. For this, the silica surface is prepared with specific alkoxysilane agents that serve as anchors. These agents allow the polymerization of several types of monomers to be carried out on the surface of the monoliths, bringing versatility to these stationary phases.

In general, modified monolithic silica columns are more efficient than particulate columns [66]. Horie et al. [67] compared the plate height in HILIC-mode separations with particulate and monolithic columns and concluded that the highest efficiencies were observed using monolithic columns. The better efficiencies of separation were shown by lower plate heights for the same linear velocity (μ) of mobile phase. Moreover, in monolithic columns, the highly macroporous structure allows higher velocities without significantly reducing efficiency, so decreasing analysis time.

As an alternative to silica monoliths, organic polymer monoliths were introduced in the late 1990s [68]. However, the higher pressures employed during chromatographic separations makes these phases less attractive than particulate ones, thereby limiting their use in HILIC [2].

### 3.3. Alternative stationary phases

Researchers are constantly looking for alternatives that might decrease the drawbacks imposed by the most commonly used stationary phases in the HILIC mode. The first was the development of new silica-bonded phases, more selective and useful than bare silica, which could expand the application possibilities of this separation mode. Several bonded phases (e.g., those with alkyl chains with embedded polar groups, sulfosalicylates, cyclodextrins, and polypeptides, as well as polar polymeric phases) have been reported [39]. Dai et al. [69] described the preparation of a tetrazole-functionalized silica for separation of nucleosides and nucleobases and concluded that this stationary phase could be an interesting alternative for the separation of these analytes.

Modifications of the silica surface were also proposed to replace bare silica. Hydride silica has gained attention as a stationary phase in HILIC separations. Also called silica type C, this phase has about 95% of Si-OH groups replaced by Si-H groups during the manufacturing process [70]. This less hydrophilic surface is less attractive for water molecules than bare silica and makes possible the separation of moderately-polar compounds using HILIC, although its principal applications have been with aqueous NP, a variation of HILIC [71].

Another option is the use of metal-oxide stationary phases in HILIC separations, most commonly titania or zirconia. The use of stationary phases based on titania was described some years ago. As to advantages over bare silica, titania is mechanically sturdier and is stable over a wider pH range. In addition, the surface of titania acts as an anion exchanger at low pH and as a cation exchanger at high pH, unlike silica, which offers only cation-exchange sites at high pH. This property of titania can be advantageous in the separation of ionized species.

In 2008, Randon et al. [72] reported the preparation of titania monoliths for application in capillary LC. Xanthine separations in the HILIC mode on this monolithic column showed good selectivity and resolution. However, although promising as a substitute for silica in HILIC, only a small number of papers reporting the use of titania were published by 2011 [73–76].

Rigney et al. [77] described some advantages of zirconia as a material for chromatography:

1. High chemical and thermal stabilities;
2. Presence of hydroxyl groups on the surface;
3.4. UHPLC in the HILIC mode

In the search for faster, more efficient separations, the introduction of ultra-high-performance LC (UHPLC) was a landmark. With this chromatographic technique, the use of stationary phase particles with diameters less than or equal to 2 µm, considerably smaller than those used in HPLC before then, permitted chromatographic analyses with greater efficiency and better resolutions in a shorter analysis time [80]. However, as UHPLC separations are carried out under high pressure (up to 1000 bar) due to greater resistance to the passage of the mobile phase through columns packed with these small particles, more mechanically resistant materials are required, although the types of stationary phase used in UHPLC are the same as those used in HPLC.

The use of the HILIC mode in UHPLC was reported in the recent literature [81–83]. Bones et al. [81] used HILIC in UHPLC for mapping biochemical markers associated with cancer. The abnormal glycosylation of proteins present in plasma from patients, indicative of disease progression, was evaluated. The results showed that particles using saccharide-specific separations in HILIC-UHPLC had higher selectivity and quickly performed the desired separation.

Although HILIC-UHPLC looks promising, the number of studies shows that separations applying HILIC mode in UHPLC is still limited, possibly due to the requirement to use equipment appropriate to this technique and the lower efficiency seen with HILIC, compared to RPLC.

3.5. ERLIC

A few years ago, Alpert [43] suggested a variation of HILIC – ERLIC (electrostatic repulsion-hydrophilic interaction (liquid) chromatography) – which involves an ion-exchange stationary phase and a mobile phase similar to those used in HILIC. This separation mode is based on the electrostatic interactions between analytes and stationary phase, in addition to the hydrophilic interactions.

In ERLIC, ionized analytes interact with the aqueous layer on the ionic stationary phase, even though they may have the same charge as this phase. This is because hydrophilic interaction occurs simultaneously with electrostatic interaction (attractive or repulsive), and that separates ionized compounds by partitioning, with electrostatic interaction as an additional factor in the selectivity of the separation, primarily attributed to affinity for the aqueous layer.

Gan et al. [84] compared the isolation of phosphopeptides by cation-exchange chromatography and ERLIC, both with detection by MS. They concluded that, although both techniques can be exploited for this type of sample, ERLIC had higher efficiency and higher selectivity, permitting more analytes to be separated and detected.

Hao et al. [85] used ERLIC in mapping tissue-specific proteins from mice using a silica column coated with an anion-exchange polymer. ERLIC allowed fractionation of analytes that are difficult to separate by ion chromatography (e.g., those of similar mass and charge), using ion-exchange and hydrophilic interactions simultaneously, since the selectivity in ERLIC was higher than that observed in ion-exchange chromatography.

These results indicate that ERLIC could be applied in proteomics, where the matrices are usually complex and contain analytes with very similar chemical and physical characteristics, requiring chromatographic analyses that are more selective than those currently applied.

The stationary phases that can be used in ERLIC are not limited to cation-exchange or anion-exchange materials, as originally proposed by Alpert [43]. Zhou and Lucy [73] proposed the use of titania in the separation of carboxylic acids by ERLIC. The zwitterionic characteristic of titania allowed application of this phase without loss of selectivity.

3.6. Aqueous normal phase (ANP) versus HILIC

The ANP mode involves a dual mechanism, which combines interactions of RP-like and NP-like mechanisms. At high water concentrations, hydrophobic analytes are retained and hydrophilic ones are eluted in the column void volume. With high organic content percentage, the opposite is observed: hydrophobic analytes are rapidly eluted, while hydrophilic ones are retained [71].

This dual-retention capability allows an ANP column to operate over a broad range of mobile-phase compositions, changing only the water content of the mobile phase. Thus, a simple gradient elution can resolve a mixture of polar and non-polar compounds.
Special stationary phases need to be used in ANP separations because they must provide retention for both hydrophobic and hydrophilic species. Silica hydride is the stationary phase most used in the ANP mode because it has dual hydrophobic–hydrophilic retention properties. Recently, metal oxides were reported as stationary phases for ANP [86,87]. These materials have an amphoteric character and can work as anion exchange or cation exchange phases, depending on the mobile-phase pH.

Kučera et al. [87] compared three hydrophilic stationary phases (bare zirconia, zirconia modified with polybutadiene, and bare silica) using mobile phases with different ACN contents. Using the bare silica column, the polar analytes were strongly retained at high ACN percentage (over 80%), consistent with HILIC fundamentals. However, both zirconia columns showed a “U-shape” retention behavior for carboxylic acids (4-aminobenzoic, 4-hydroxybenzoic and 3,4-diaminobenzoic acids), where these compounds were strongly retained with both low and high ACN contents. In this case, the authors suggested that, at low aqueous concentration, the compounds entered the water-rich layer and showed HILIC behavior, while, at low ACN contents, the carboxylic moieties interacted via ligand-exchange with the Lewis sites on the zirconia surface, in accordance with ANP chromatography.

3.7. *Per aqueous liquid chromatography (PALC) versus HILIC*

The high consumption of organic solvents and concern about reducing the environmental impact of this consumption has led to a search for alternatives to reduce both the initial cost of these solvents and the cost of their disposal. This is especially critical in HILIC and RPLC due to the use of mobile phases with high percentages of ACN.

Re-evaluating several chromatographic modes in the framework of green chromatography, Pereira et al. [88] proposed the use of a mobile phase having a low proportion of ACN, or even totally aqueous mobile phases, for the separation of polar compounds on HILIC columns. This separation mode is called *per aqueous LC (PALC)*. They evaluated the separation of seven amino acids by HILIC and PALC modes, both using MS detection. The results showed that the selectivity obtained in PALC was higher than that observed in HILIC, since the latter is based principally on hydrophilic interactions and PALC also involves hydrophobic interactions. For these analytes, the side chain influenced the elution order.
more polar analytes [e.g., glutamic acid and lysine (which contain a second carboxyl group or a second amine side chain, respectively)] showed less retention than analytes of lower polarity (e.g., leucine, isoleucine and proline). This result is in agreement with the proposition that the more polar analytes have lower retentions in the PALC mode (Fig. 5).

Gritti et al. [89] compared the separation of caffeine by PALC and HILIC. The results showed that a reduction in the fraction of ACN in the mobile phase (below 2.5%) caused a significant increase in the asymmetry of the chromatographic peak of the analyte. However, when the fraction of ACN in the mobile phase was close to 15%, the efficiency of the separation and the asymmetry of the caffeine peak in PALC were very similar to those obtained in HILIC, where 88% ACN was used.

PALC has been proposed as an alternative to HILIC for the separation of some polar compounds. PALC is very attractive because it involves both hydrophilic and hydrophobic interactions, and it has the advantage of using lower ACN concentrations. Thus, PALC should be the subject of intense research in the coming years in order to elucidate the mechanisms involved in these separations.

4. Advantages and disadvantages

Many advantages of HILIC make separations using this mode a good alternative to NPLC and RPLC separations. Moreover, its viability is supported from technical and economic points of view.

One of the main advantages of HILIC is the efficient separation of polar and ionized compounds, which is difficult to achieve using RPLC mode. In RPLC, some compounds have poor retention and many polar analytes elute in the column-void volume [4]. The order of elution of polar solutes in HILIC is usually the opposite of that found in RPLC separations, which suggests that the HILIC mode can be applied to aid confirmation of compound identity [27].

The separation mechanisms observed in HILIC decrease problems of irreversible retention and peak asymmetry, frequently seen in separations of polar analytes using the NPLC mode [90].

In HILIC, the use of mobile phases with predominantly low-viscosity organic solvents allows the separation to be carried out at lower pressures than in RPLC and the resulting higher flow rates allow separations with reduced analysis times [18]. HILIC-mode operation requires no adjustments to the equipment used in chromatographic separations in RPLC and NPLC and uses stationary and mobile phases common to both. Thus operating costs are very similar to those involved in RPLC and NPLC.

Although advantageous in some aspects, HILIC has some limitations in comparison to RPLC. The separation mechanism of HILIC is less well understood than that of RP, which makes it difficult to predict the effect of changing separation conditions [91]. Also, HILIC is unsuitable for hydrophobic compounds, because they often do not present sufficient interactions to promote as good a separation as that with RP.

Another limitation discussed in the literature is the difficulty of separating a mixture containing a large number of structurally-similar analytes due to the similarity of their hydrophilic interactions [92]. However, this statement has been contradicted by other experimental observations [79,93,94]. Hao et al. [85] proved that a mixture of peptides with differences of only one amino acid in the same position can be separated by ERLIC by a combination of electrostatic repulsion and hydrophilic interaction mechanisms. Other examples of successful HILIC separations for similar compounds include separations of carbohydrate isomers [95] and sulfur-containing amino acids [96].

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>Detection</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine and metabolites</td>
<td>Embedded polar group</td>
<td>ACN:formate buffer, pH 2.9 (gradient elution)</td>
<td>ESI-MS/MS</td>
<td>Lower resolution than RPLC for polar and aromatic analytes</td>
<td>[92]</td>
</tr>
<tr>
<td>Pterines (biomarkers)</td>
<td>Aminopropyl-silica</td>
<td>ACN:acetate buffer, pH 6.0 (isocratic elution)</td>
<td>Fluorescence spectroscopy (λ_em = 350/450 nm)</td>
<td>Lower retention when MeOH replaced ACN</td>
<td>[97]</td>
</tr>
<tr>
<td>Glycans from monoclonal antibodies</td>
<td>Zwitterionic</td>
<td>ACN:acetic acid, pH 3.0 (gradient elution)</td>
<td>ESI-MS</td>
<td>Alternative to aminopropyl stationary phase</td>
<td>[98]</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>β-cyclodextrin</td>
<td>ACN:formate buffer, pH 6.8 (gradient elution)</td>
<td>UV (λ = 254 nm)</td>
<td>Better separation than with RPLC</td>
<td>[99]</td>
</tr>
<tr>
<td>Antiviral drugs</td>
<td>Bare silica</td>
<td>ACN:formate buffer, (gradient elution)</td>
<td>ESI-MS/MS</td>
<td>Good performance for fresh samples</td>
<td>[100]</td>
</tr>
</tbody>
</table>
By using a larger volume of organic solvents than in RPLC, HILIC is environmentally more aggressive than RPLC. However, compared to NPLC, HILIC appears much more appropriate, avoiding the use of large volumes of highly toxic organic solvents.

5. Some recent applications

Table 1 summarizes information from selected papers that have used HILIC separations, indicating the versatile application of this way of separating biomolecules. Common among these studies is the complexity of the matrix from which they originate (e.g., blood, urine, environmental samples and foods). We describe two further examples below.

Li et al. [101] applied HILIC due to the limitations of other analytical methods used in the monitoring of aromatic amines in the environment. The authors proposed development and validation of a rapid method for the analysis of five aromatic amines. They also evaluated the effect of mobile-phase pH and the amount of polar organic solvent used in the separation. The separation was performed using a bare silica column (5 µm Kromasil 100-5Sil, 250 mm × 4.6 mm i.d.). The mobile phase comprised a mixture of 85% ACN and 15% 10 mmol/L phosphate buffer, at a flow rate of 1.0 mL/min.

The chromatographic parameters were determined after optimizing the analysis conditions, and that showed that the plate number was in the range 10,200–14,900, the asymmetry factor was 1.01–1.1, and resolution between two adjacent peaks was 1.6–9.9. After validation, the authors concluded that the HILIC method was adequate to replace those used previously (gas chromatography, capillary electrophoresis and RP-HPLC).

Martínez-Villalba et al. [53] proposed determination of the concentration of the antiparasitic ammonium (AMP) in food using the HILIC mode with detection by MS/MS. The presence of AMP was investigated in muscles of birds, eggs and animal feed. They used a fused-core bare silica column (2.7 μm, 100 mm × 2.1 mm i.d.) and isocratic elution (60:40 v/v ACN:50 mmol/L ammonium formate, pH 4.0). The analytical curve was linear over the range 75–5000 ng/L, with r² > 0.999. The evaluation of precision at two concentration levels (75 ng/L and 500 ng/L) showed repeatability (n = 6) and intermediate precision (n = 6, 3 days) with RSD less than 13%, consistent with the requirement of <20%.

6. Conclusions and perspectives

The mechanisms involved in separations using the HILIC mode are much more complex than those initially proposed by Alpert. The literature has shown that each compound has a predominant mechanism for its retention under different chromatographic conditions. Thus, before selecting the HILIC separation mode, attention should be paid to both chemical and physical characteristics of the analyte and the matrix, in order to select the best chromatographic conditions for separation.

Although HILIC has great potential applications for separations of polar compounds, it presents challenges to be overcome (e.g., replacement of the separation columns used in the RP mode, responsible for more than 70% of the applications of HPLC). However, HILIC offers an interesting alternative for separations that are problematic with NPs and RPs, or even with ion-pair or ion-exchange chromatography.

As a general trend in separation techniques, we expect that the development of HILIC will be focused on miniaturization to reduce costs and waste generation, and to make analyses faster. Microdevices are already being applied in RPLC and HILIC, and they should be included in research in this direction.

Studies of new stationary phases, mainly new bonded phases and monoliths, provide a short-term perspective, since, in other methods of separation, the use of monolithic phases has been gaining acceptance.

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