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Chromatography

HILIC After the Hype: A Separation Technology Here to Stay

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Today Hydrophilic Interaction Liquid Chromatography (HILIC) is by far the fastest growing HPLC separation mode, currently being used by almost 20% of the HPLC instrument owners [1], although the relative number of applications still is considerably fewer. Does this mean HILIC is yet another technology-hype on the separation science scene, and that the interest will soon vanish? We believe not. This paper reviews the reasons behind the remarkable rise of HILIC and based on that, tries to predict future development within this separation technology. For an introduction to the basic separation requirements and mechanisms, we refer to previous texts [2] on the subject.

HILIC History

The story about modern HILIC started with a publication [3] more than 20 years ago: *'Hydrophilic-Interaction Chromatography for the Separation of Peptides, Nucleic Acids and other Polar Compounds'*.

The HILIC separation technique had actually by then been used for about 15 years for sugar analysis on aminopropyl silica columns, but it was with his landmark paper in 1990 that Andrew Alpert showed HILIC to be a universal separation mode, potentially applicable to separation of all types of polar compounds. The publication went by relatively unnoticed by the HPLC community, and for the next 12 years the number of people using HILIC were rather limited [2], many of whom did not even use the term HILIC.

By 2002 the number of publications in the scientific literature using HILIC was still only a handful, but the dormant phase of HILIC was now passed and within two years the scene changed dramatically. The small company SeQuant, a spin-off from Umeå University in northern Sweden, launched a new zwitterionic HILIC phase (the ZIC®-HILIC), Waters launched their plain silica Atlantis® HILIC column and Tosoh re-braded their TSKgel® Amide-80 column as a HILIC column. The number of HILIC publications (and with some delay also column sales) have since then been increasing by 30-40% each year and today scientific papers on new HILIC stationary phases are published in unmatched numbers and every HPLC column manufacturer have at least one HILIC stationary phase in their program.

Success of Selectivity

What have been the key ingredients in this tremendous success of HILIC? Naturally its perfect fit to mass spectrometry (MS) and the lack of need for investments in any new HPLC equipment have played a part by providing the soil for this growth, but what really has been the key driving force is the fact that HILIC focuses on selectivity.

With HILIC it is now possible to separate compounds previously impossible to retain on HPLC columns. The direction towards selectivity is in sharp contrast to the development in reversed phase (RP) over the last decades, where UHPLC, with sub-2 µm, core-shell particles or monolithic approaches, all have been attempts to optimise the separation physics. At the same time, selectivity was minimised by ever better synthesis schemes and end capping protocols, trying to make all C-18 columns similar. What HILIC thus has brought is an alternative view of what is important in HPLC separations, away from physics and plate counts, towards chemistry and selectivity. A quick glance at the equation for chromatographic resolution [4] shows that this is a wise path; increasing selectivity is the most effective way to improve a separation (*Figure 1*).

With its alternative selectivity, HILIC addresses a range of compounds that have been problematic to separate for many years; polar and hydrophilic compounds. Such molecules are found in many different application areas. Consequently, a continuous expansion of the application base has followed which will keep on increasing as the HILIC separation technology reaches new audiences.

Metabolomics and Clinical Analysis

HILIC columns can easily be interfaced with single and tandem MS, electrochemical detectors (ECD), inductive coupled plasma (ICP), and nuclear magnetic resonance spectroscopy (NMR) for high sensitivity and specificity. This makes HILIC very well suited for the detection and quantification of low-level biomarkers targeted in metabolomics [5] and clinical analysis [6] for diagnosis and disease monitoring. Many of these new clinical assays require only protein precipitation as sample preparation, use of isotopically labelled internal standards, and an isocratic chromatographic method, as exemplified by the 3-min HILIC-MS assay (see *Figure 2*) [7] for physiological levels of methyl malonic acid (MMA), which is an important biomarker for vitamin B_{12} deficiency.

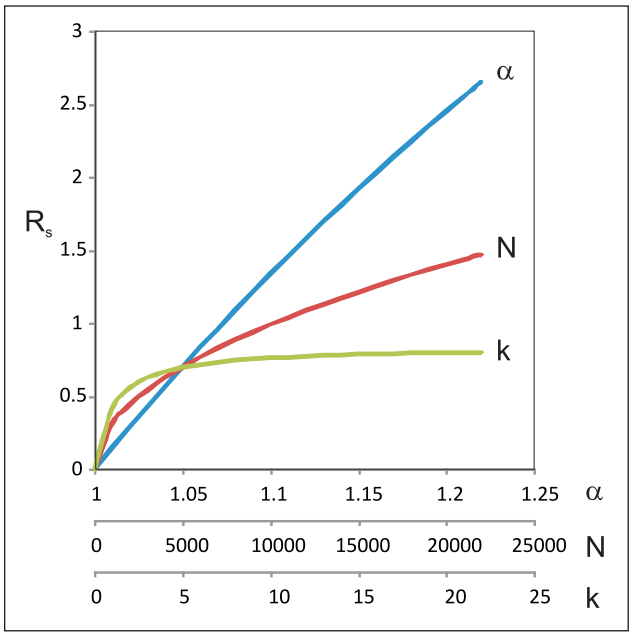

Figure 1. Relative impact of selectivity (α), efficiency (N) and retention (k) on chromatographic resolution (R_s). Graphs were obtained by plotting the resolution equation [4] against one parameter while keeping the others fixed at the intersection point values (α=1.05, N=5000, k=5).

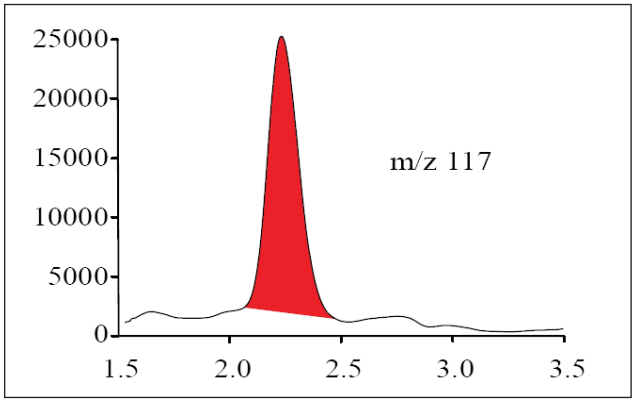

Figure 2. Separation of methylmalonic acid (MMA, m/z 117) from a precipitation-treated sample of patient plasma containing 0.137 mol/L MMA, using a HILIC column and an isocratic eluent [7].

Other biomarker analysis where HILIC already have been put to use are; amino acids (S-adenosyl-methionine [8], ornithine [9], 4-amino-3-hydroxyphenylalanine and 3-amino-4-hydroxyphenylalanine [10]; plasma-free metanephrines [11] ; morphine and its metabolites [12]; cocaine and its metabolites [13]; sulphated and glucurunated steroids [14]; quaternary ammonium compounds like acetylcholine, choline, and butyrobetaine [15]; acetylcholine precursor betaine and phospholipid precursor dimethylglycine [16]; nitrosamine metabolites [17], lung disease biomarkers hydroxyproline, nitrotyrosine, proline and tyrosine [18]; cardiovascular disease biomarker asymmetric dimethylarginine (ADMA) [19,20].

Evidently metabolomics and clinical analysis are major growth fields in HILIC separations. The reason for this is fundamentally simple; most metabolites and biomarkers found in body fluids are per se of polar and hydrophilic nature.

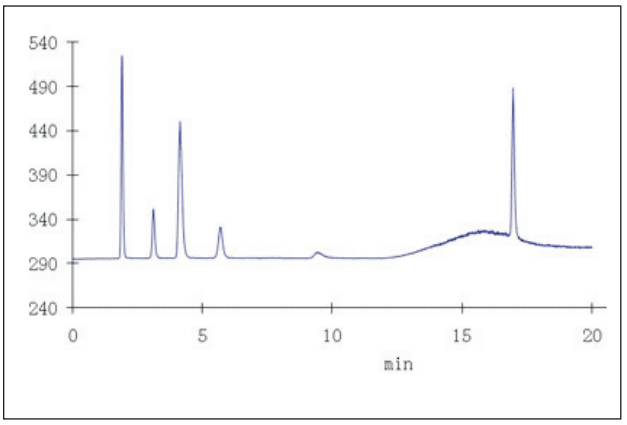

Figure 3. HILIC-ELSD separation of milk hydrolysate for detection of L-hydroxyproline (peak no. 4). Injection of 2 µL on ZIC-HILIC 150x2.1, 5 µm, 200 Å column operated at 0.8 mL/min using isocratic elution during 10 minutes with 85% acetonitrile in 50 mM ammonium acetate (pH 5.56) followed by acetonitrile gradients 10-15min 85%-65% and 15-20min 65%. Courtesy of Jeffery Lei, Merck China.

HILIC is thus an ideal technique for separating compounds from biological samples such as serum, plasma, cerebrospinal fluid, urine, dialysis fluid, hair, sweat, saliva, tissue, and breath condensates. Using a separation technique fit-for-the-purpose enables finding information that never previously was revealed, or at least was very difficult to retrieve with sufficient certainty at reasonable costs using traditional chromatographic methods. HILIC will certainly continue to expand strongly in this field and it is even likely that a majority of the newly developed methods for clinical routine analysis will be based on HILIC in the future.

Food Safety

In September 2008 the world witnessed how thousands of children were poisoned by Chinese milk products that had been manipulated by addition of melamine to increase the nitrogen content and mislead the Kjeldahl nitrogen analysers into reporting high protein content. Within a few weeks the Food and Drug Administration in USA had published recommended methods [21] based on HILIC separations and MS/MS detection for analysing melamine and its decomposition product cyanuric acid which together form an insoluble complex precipitating in the kidney.

Recently it was discovered that hydrolysed protein extracts from old leather scraps have been added to milk for the same nitrogen-rising purpose. HILIC separation approaches with detection by ELSD or MS/MS have been developed to screen for and determine L-hydroxyproline, which is an amino acid not naturally occurring in milk. (*Figure 3*).

Also this time the scene for the tampering is China, but it would be naïve to think that this problem is only limited to dairy products in a specific country. Potentially any compound rich in nitrogen can be used to fool Kjeldahl nitrogen analysers in any food product where protein content is important for price and sales. Analysis of native amino acids [22] or other small molecular indicators for changed composition of food products are thus emergent areas where HILIC will continue to make a difference for ensuring food safety.

Diethylene glycol is another contaminant that keeps coming back in different consumer products causing hundreds of human deaths with a frightening regularity.

It is typically used in place of glycerin or propylene glycol and there have been several epidemics due to the substitution by diethylene glycol into products like medicine and toothpaste in the past fifteen years. Development of rapid protocols based on HILIC-MS [23] have been vital to find and stop these poisoned products.

Aminoglycoside antibiotics have until recently been very difficult to analyse but there have been a large need due to the extensive use in veterinary medicine and animal husbandry. Utilising simultaneous HILIC and ion exchange separation mechanisms on a zwitterionic column, these compounds can now readily be separated and detected by MS [24].

All in all we can conclude that with the ever increasing food prices and amounts of industrially processed food the incentives and possibilities for food tampering increase. Many of the potential compounds in question here are polar and hydrophilic, wherefore HILIC would be the most obvious separation technique to choose when solving these analysis problems.

Simultaneous Anion and Cation Analysis

The possibility to simultaneously quantify inorganic anions and cations in one chromatographic run is an attractive feature of HILIC. It is straightforward when used with a zwitterionic column in combination with detection techniques like evaporative light scattering (ELSD) [25], charged aerosol (CAD) [26], or potentially also condensation nucleation light scattering (CNLSD). The pharmaceutical industry quickly realised the benefits of having one generic HILIC-based analysis method for determination of several counter ions in a range of different pharmaceutical preparations. The organic-rich eluents used in HILIC separations also proved to be beneficial for solubility of the often poorly water-soluble drugs.

Quite a substantial part of the analysis traditionally performed with ion chromatography can thus be transferred to HILIC. Applications where low detection limits and very complex samples make analysis difficult, will however not likely be performed with HILIC in the foreseeable future. It is thus not superior performance that will make ion analysis by HILIC a success, but ease of use and the possibility to run both anions and cations in one run.

Environmental Analysis

Environmental analysis literally exploded with the realisation that a compound that was incredibly useful in industry or households was a potential disaster agent when finally ending up in nature. Hydrophobic compounds like DDT, PBC and other chlorinated organic compounds were quickly banned since they could be detected after enrichment and biomagnification in the food chain.

Today environmental analysis faces other, but potentially as dangerous compounds, many of which are polar and hydrophilic [27]. Among these we find algal and cyanobacterial toxins, disinfection by-products, hormones and other endocrine disrupting compounds, drugs of abuse and their metabolites, organometallics, organophosphate flame retardants and plasticisers, pharmaceuticals and personal care products, polar pesticides together with their degradation and transformation products, and surfactants and their metabolites. It would also make sense to add artificial sweeteners to the list.

The overall ongoing trend in environmental analytical chemistry is that contaminants, and in particular their degradation products and metabolites, are more hydrophilic. Consequently they are more easily transported by water from the point of emission into streams and lakes diluting, but not necessarily degrading them. Finding ways of concentrating and analysing such samples will be an immense task where HILIC absolutely will have a role to play.

Biomolecules: Peptides and Oligonucleotides

Separation of peptides has since Alpert´s pioneering paper [3] been an important application area for HILIC separations. In particular has HILIC enabled separation and extraction of post translational modified peptides, subjected to glycosylation [28, 29] and phosphorylation [30]. More recently the complementarily of HILIC and RP have been used to accomplish orthogonal 2D mapping separations of protein digests and thereby demonstrating supreme protein identification capabilities [31, 32].

Today the available methods for separating oligonucleotides are either ion-pairing RP or ion chromatography [33]. With both these techniques the selectivity is mainly derived from the number of negatively charged phosphate groups, such as, from the length of the oligonucleotide. The difficulty of achieving good separation between different oligonucleotides of the same length has resulted in numerous attempts to use mass spectrometry for resolution, and although this has had some success, both chromatography and detection is severely influenced by the ion-pairing agent concentration.

Scientists are now turning to HILIC to separate oligonucleotides and the advantages are obvious; no need for ion-pairing reagents and the happy marriage to MS. Because the partitioning process causing retention in HILIC is governed by the overall hydrophilicity of the oligonucleotide, the nucleotide bases will also affect the selectivity and make it possible to separate oligonucleotides of the same size but with different composition or base sequence, see *Figure 4* [34].

It is not hard to predict that HILIC will continue to be a very strong contributor to analysis of small biomolecules such as peptides and oligonucleotides. The needs for analysis in these fields will continue to increase, especially for oligonucleotide separations which often is mentioned as the next big challenge [33] due to the growing importance of these molecules and the drawbacks of present separation approaches.

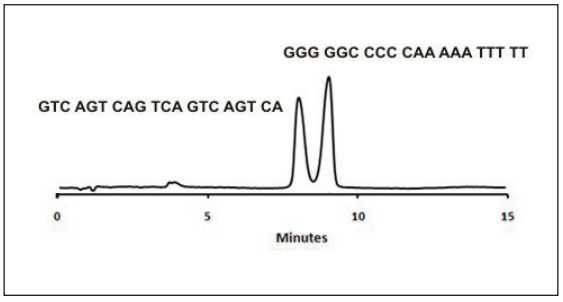

Figure 4. HILIC-MS separation of two 20 unit heterogeneous oligonucleotides of same composition but different sequence. Separation on ZIC-HILIC 100x2.1, 3.5 µm, 200 Å HPLC column at 50°C, operated at 0.4 mL/min using linear gradient elution from 65% to 55% acetonitrile in water during 7.5 minutes with constant 10 mM ammonium acetate (pH 5.8). Courtesy of Lingzhi Gong, University of Oxford, UK [34].

Future of HILIC columns

When so many application areas are relying on HILIC, combined with that the basic requirement on a HILIC stationary phase only is to hold the water layer in place for hydrophilic partitioning, it is not surprising that more than 40 different stationary phases have been tested for HILIC-type separations [2]. There is certainly a need for several different selectivities in HILIC, especially considering that one great advantage with HILIC is that changing stationary phase actually does change selectivity, but such immense diversity will most likely only be temporary. Still it would be surprising if a harmonization of HILIC phases to the same extreme as seen in RP (all manufacturers make very similar C-18 phases) will occur. But naturally, the HPLC society will need tools to better judge what stationary phases are similar and which to select for what type of separation.

Today four major groups of HILIC column chemistries exist on the market; plain silica, and bonded phases with functional groups of zwitterions, amide and diol. A survey of scientific literature from 2009 [35] confirms the picture but also show that mixed-mode columns of various types are being tested for HILIC despite their inherently lower hydrophilicity and consequently limited retention window. Although development of new HILIC stationary phases have been the topic for several scientific papers, the range of HILIC stationary phases in actual use is not likely to change dramatically within the next few years, with the exception of a continued decline of plain silica phases.

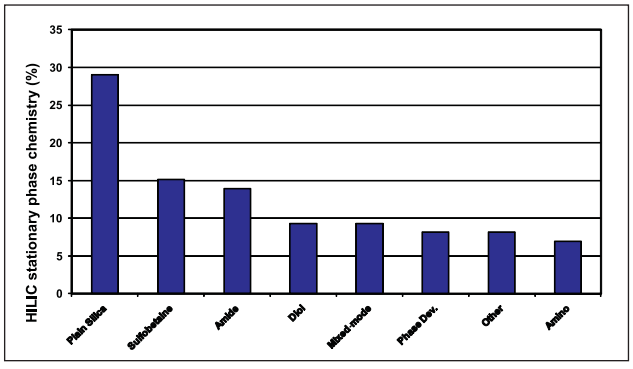

Figure 5. Relative number of published scientific papers using different stationary phase chemistries for HILIC separations. Data from literature survey performed in 2009 [35].

Plain silica does have a number of inherent drawbacks such as pH-dependent surface charge and thereby hydrophilicity, and strong irreversible adsorption or even reactivity towards certain analytes [36, 37]. For these reasons it has been concluded that plain silica is not suitable for biological analysis [36]. Although numerous HILIC applications still are performed with plain silica, the reasons for this are mainly a) availability in desired separation format, or b) lower cost. It is thus expected that as the knowledge and experience of HILIC increase among users and manufacturers, and column prices are reduced, bonded phases will come to dominate in HILIC.

The lower apparent hydrophilicity [38] of diol columns will likely limit their usefulness in HILIC since they will require higher content of organic solvent to generate retention. On the other hand there are indications [38] the retention mechanism on diol chemistries are influenced by direct hydrogen donor bonding between the stationary phase and the analyte, a feature which could give these columns a different selectivity compared to columns more relying on partitioning and ionic interactions for retention.

Columns based on amide and zwitterionic functionality seems to be the most promising bonded phase chemistries for HILIC although they do differ in several aspects. Amide columns are supposedly neutral with very limited electrostatic interactions [39] whereas zwitterionic columns also rely on weak ionic attraction and repulsion interactions [40] Recent data [41] also show that zwitterionic sulfobetaine type phases are remarkably good in establishing the water-enriched layer necessary for HILIC partitioning.

There have been some initial attempts to classify columns and to make generalisations regarding differences in column retention and suitability for various applications. Chirita et.al. [42] tested 12 different columns and came to the conclusion:

i) For anionic compounds, a cationic or zwitterionic phase will provide good retention

ii) For cationic compounds, a neutral, anionic or zwitterionic phase will provide good retention

iii) For neutral and zwitterionic compounds, any HILIC phase is likely to provide good retention

However, since selectivity is the main contributor to separation, what really is needed to leave the trial and error approach to column selection are studies of selectivity differences between phases. Some attempts are in preparation [43], meaning that within a few years scientific evidence will allow a systematic approach to column selection in HILIC.

Trends in HILIC Selectivity Tuning

Changing the ionisation stage of a molecule has a significant impact on its chromatographic properties. That is the reason why pH always has been such an important tool for changing selectivity in HPLC. Ionised and charged solutes are less hydrophobic and have lower retention in RP separations and pH has thus been used to suppress ionisation. When ion suppression has not been possible, ion-pairing techniques have been the route to overcome the fundamental mismatch and tweak RP into separating charged species, but the technique has always struggled with drawbacks in terms of poor MS detection sensitivity and inherently reduced selectivity due to the complexes becoming more similar than the original ions.

With HILIC separations the opposite is true; charged compounds have more retention due to their increased hydrophilicity. Changing pH to deliberately induce ionisation is thus one of the most powerful ways of altering the selectivity in HILIC mode separations, and the incentive to go to pH-extremes will thus be greater in HILIC than in RP.

An additional feature of HIILIC is that the use of charged stationary phases easily adds another dimension of selectivity while the main HILIC separation mechanism still is present. Naturally the ionic interaction properties and the response to buffer salt type and concentrations differ whether the HILIC phases are anionic, cationic or zwitterionic, but the principle is similar. Opposite net charges on the material and the analyte will induce attraction and same net charges will cause repulsion.

By combining pH tuning and charged stationary phases it is possible to utilise controlled molecular orientation to enhance selectivity [44]. The concept is called ERLIC (or sometimes eHILIC) and is based on repulsion of commonly occurring charged functional groups of strongly retained species having the same charge as the stationary phase at the same time as the counteracting HILIC mechanism increase retention.

The increased benefit of going to extreme pH combined with the water layer wetting a HILIC phase increases its exposure to hydrolysis. These factors will together increase the need for pH-stable HILIC columns, for example with polymeric base particles. A pH stable column with a hydrophilicity and surface charge independent of pH makes it possible to run pH gradients with predictable results. The FDA method for simultaneous quantification of melamine and cyanuric acid [21] is a striking example of the usefulness of this approach.

Sample Preparation by HILIC

The need for sample preparation is increasing with the move towards smaller particle sizes (UPLC), and to match sample preparation throughput to these fast separations, online coupled techniques will be needed.

One aspect that largely has been overlooked when considering the applications of HILIC is the opposite elution strengths of solvents compared to RP. For medium polarity solutes that have retention in both HILIC and RP one can couple sample preparation by SPE in one dimension with chromatographic separation in the other while benefiting from automatic band sharpening. To reduce the variability in proteomic analysis of urine proteins Loftheim et al. [45] minimised the number of sample handling steps by applying protein precipitation by salting out, followed by centrifugation, tryptic cleavage, enrichment on a RP SPE cartridge, and direct injection of the eluate on a HILIC separation column. Conversely, removal of phospholipids was accomplished by Lindegardh et al. [46] by using protein precipitation in acetonitrile, followed by centrifugation, enrichment on HILIC 96-well plates, and direct injection of the eluate on a RP separation column.

In summary, combining RP and HILIC in sample preparation and HPLC separation either way can drastically simplify sample preparation schemes of biological samples where protein precipitation is needed before analysis of small molecules. The ease of automation of this combination is likely to attract a lot of interest in the near future.

Conclusions

There is no doubt HILIC has come to stay and that tweaks like ion-pairing RP to separate polar molecules will decline as a consequence. Our prediction is that HILIC very soon will be second to RP in terms of practitioners and eventually also in HPLC column sales. We believe that the initial excitement period over HILIC already have passed and now come the test how this separation mode will perform practically in competition with other techniques.

There are many separation technology trends that have risen rapidly in popularity among scientists only to fall short when it comes to practical use, followed by a more sober establishment after a few years. For HILIC we foresee a development without the decline phase for the following reasons; the hype is lower, the application pool is very large, and no instrument investments are needed. To summarise the present status of HILIC development we borrowed the words from a rather famous Britain [47]:

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

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