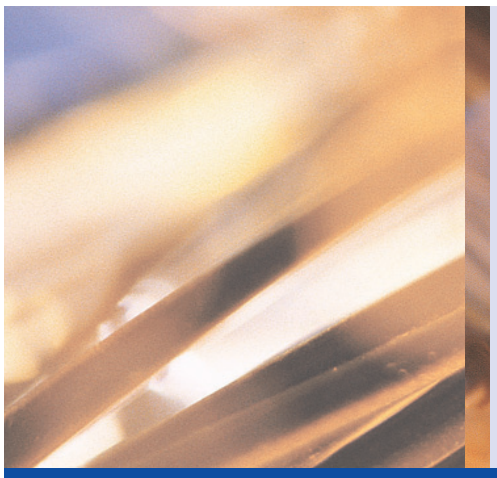

Chromatography Focus

HILIC - STRAIGHTFORWARD CHROMATOGRAPHY OF POLAR COMPOUNDS

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FOR SUCCESSFUL USE OF HILIC ONE MAY NEED TO THINK DIFFERENT, BUT THEN IT BECOMES A STRAIGHTFORWARD AND POWERFUL TOOL FOR THE SEPARATION AND ANALYSIS OF POLAR AND HYDROPHILIC COMPOUNDS

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STRAIGHTFORWARD CHROMATOGRAPHY

Techniques that directly and in a smooth way solve analytical or preparative problems is what chemists search for in a never ending struggle to earn more, but spend less. Chemists are not only easy-going they also tend to be smart, but it is sometimes an overwhelming job to keep updated in the information flow provided by suppliers and the scientific community. Therefore this paper should give you a convenient introduction to hydrophilic interaction (liquid) chromatography (HILIC), a straightforward separation technique for polar and hydrophilic compounds.

THE SELECTIVITY TOOLBOX

During the evolution of high performance liquid chromatography the mainstream trend has been towards reversed phase separations and that for good and well known reasons, numerous of separation problems are solved. The more problems solved, the more types of reversed phase columns in the toolbox. However, problems arises when the compounds are not adapted for this mode on conventional stationary phases. Small polar and hydrophilic compounds often lack retention on reversed-phase columns. This has lead to the introduction of so called polar embedded or polar end-capped groups columns. These columns often require highly aqueous eluents that generally are less suitable for LC-MS and may cause solubility problems. Similar difficulties are encountered when combining normal phase chromatography and LC-MS. Therefore a HILIC column is a must in a well equipped toolbox.

THE PRINCIPLE OF HILIC

HILIC conditions are accomplished when two criterions are fullfilled:

- A hydrophilic stationary phase
- The mobile phase has a high content of water miscible organic solvent

Using these conditions the retention mechanism is based on partitioning of polar compounds between the less polar mobile phase and an aqueous layer in the stationary phase [1,2], see *Figure 1*. The ability of the column to create and maintain a stable enriched water layer in the hydrophilic stationary phase is therefore crucial for successful and reproducible separations.

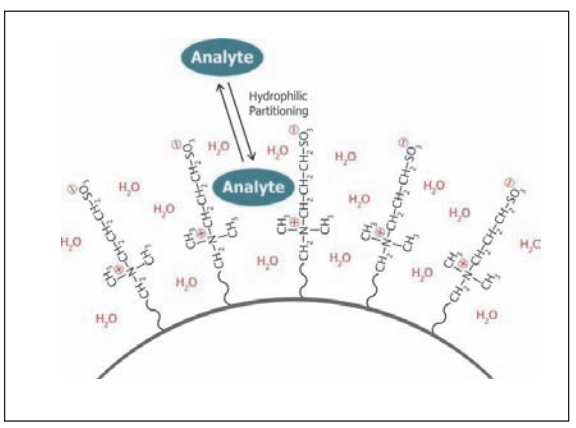

Figure 1. Hydrophilic partitioning to the water enriched stationary phase

In a typical application the mobile phase consists of 60-95% acetonitrile in aqueous ammonium acetate or formiate buffer or just simply water as in *Figure 2*. Water is the strong solvent and that is easy to say, but sometimes hard to remember in practical work.

This means that it may also be beneficial to adjust sample pre-treatment when running HILIC separations. A polar analyte can for instance be enriched on a reversed phase solid phase extraction cartridge and eluted in a small volume of acetonitrile.

The enriched analyte can then be directly injected on a HILIC column since acetonitrile is the weak solvent. The procedure can likewise be used in the opposite way [3] and this orthogonality between HILIC and reversed phase will surely be more utilised in sample work up and separation of complicated samples.

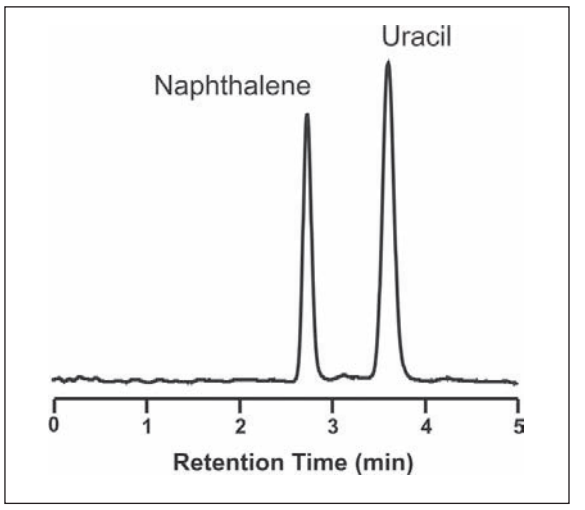

Figure 2. An example on a HILIC separation where the non-polar naphthalene elutes in the void volume while uracil has retention. Mobile phase: 70 % acetonitrile: 30% water; Column: ZIC®-HILIC 150 x 0.3 mm, 5µm.

STATIONARY PHASES

The characteristics of the hydrophilic stationary phase may affect and in some cases limit the freedom of choise when deciding on mobile phase composition, ion-strength, or buffer pH value, since other mechanisms than hydrophilic partitioning could take action. Only few stationary phases have specifically been designed for HILIC and some of these orginates from Alpert that also suggested the acronym "HILIC" in 1990 [1]. The ZIC®-HILIC column was introduced in 2002 and it has a zwitterionic stationary phase, see *Figure 3*.

Independent of pH it has a very high ability to bind water and provide a selectivity benefiting from both hydrophilic interactions and weak electrostatic interactions, while low eluent ionic strength may be used. In fact, since almost 50 years sugar separations have been performed on conventional ion exchangers using eluents [4] with water and an organic solvent. Still ion exchangers and even plain silica columns are used for HILIC despite the availability and often superior performance of tailor made phases [2].

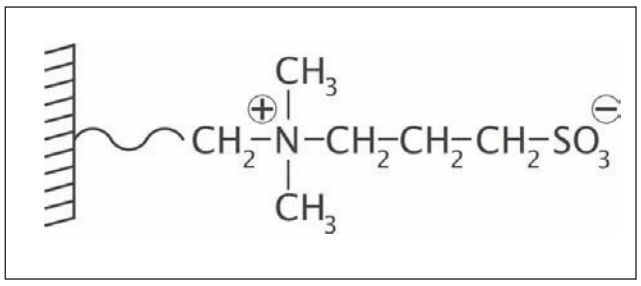

Figure 3. The zwitterionic stationary phase ZIC®-HILIC

HILIC AND MS – A HAPPY MARRIAGE

When struggling to retain very polar and hydrophilic compounds on a reversed phase column it is often neccessary to use a mobile phase containing 95%(v/v) or more of buffer/water. Such conditions are rarely optimal for electrospray ionisation (ESI) and often resulting in low sensitivity.

By instead utilising HILIC this category of compounds can be retained using a high percentage of organic modifier (read acetonitrile) and that offers an additional advantage, namely a comparatively lower surface tension of the mobile phase, which eases the drop formation during the electrospray ionisation process and significantly improves the formation of ions in the gaseous phase [5]. Hence, HILIC demonstrates large advantages compared to reversed phase chromatography in regard to the detection sensitivity of polar substances in the ESI-MS process, and by changing from reversed phase to HILIC a 10-1000 fold increase in sensitivity is often observed for hydrophilic analytes [6].

GRADIENT ELUTION

A rule of thumb says that if a compound doesn't have retention on a reversed phase column then it will be retained in HILIC. However, there are a considerable number of compounds that have retention in both reversed phase and HILIC. Therefore, gradient runs in HILIC may be just as powerful way to control retention of compounds with varying polarity and the elution order will be reversed compared to reversed phase, useful when evaluating peptide maps and enabling orthogonal and two-dimensional separations [7].

Successful binary gradient runs require that one keep some basics in mind:

(I) Start at a high ratio 80-95% of organic solvent

(II) Decrease organic solvent down to 40-50%, and not lower.

(III) Try to have the same buffer salt concentration in both eluents

(IV) Dissolve and dilute sample in the weak solvent, i.e., in organic solvent

Firstly, one should never run with 100% organic solvent since this will dehydrate the stationary phase ("dewetting", "phase collapse") and to avoid this at least 3% water is needed in the mobile phase. Likewise, it does not make sense to run the gradient lower than 40% solvent since below this point the HILIC mechanism will not be in action any longer, see *Figure 4*. Keeping this in mind, returning to starting conditions for re-equilibrating the stationary phase goes faster and sample throughput is increased. If ion exchangers are used and the charged analytes does not elute under HILIC conditions (i.e., >40% organic solvent), then ion exchange is the dominating or only retention mechanism and the solvent may not be needed at all. A standard salt gradient could be sufficient.

APPLICATION AREAS

In general the interest for HILIC arises when it becomes apparent that the retention of the analyte in reversed phase mode is low, and when the use an ion-pairing agent or pre-column derivatisation are not desirable. There are also applications were the HILIC separation can facilitate both sample pre-treatment and detection. Among numerous of new applications are here some examples.

DRUG DISCOVERY

Urine samples were analysed and metabolic fingerprints were obtained by analysing rat urine with both HILIC and reveresed phase using ESI-MS and multivariate data analysis [8]. By combining these separation techniques the number of biomarkers detected increased since the very polar compounds were not discarded.

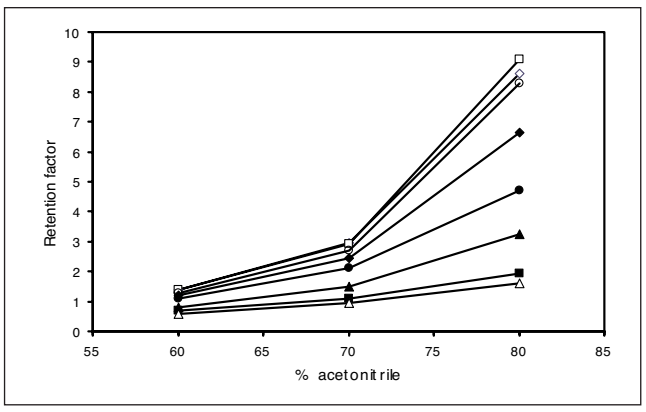

Figure 4. Retention of amino acids versus acetonitrile ratio in the mobile phase. Mobile phase: acetonitrile/20 mM ammonium acetate, pH 6.8; Column: ZIC®-HILIC 150 x 4.6 mm, 5 µm;
□ Asp; ◇ Gly; ○ Glu; ◆ Thr; ● Pro; ▲ Tyr; ■ Leu; △ Phe

PROTEOMICS

One of the first HILIC application was separation of peptides [1] and a lot of peptides have retention in both reversed phase and HILIC, while the retention order is opposite [7]. Even more hydrophilic compounds like the glucopeptides have in some cases only been detected when HILIC was used for microextraction [9] or separation of isomeric glucopeptides [10], while these are eluted in the void on a reversed phase column. Consequently, the concept of two dimensional separations based on the combination of reversed phase and HILIC can be expected to grow, also within the field of metabolomics.

ENVIRONMENTAL ANALYSIS

The awarness of pharmaceutical compounds becoming environmental pollutants when secreted is subject to more studies. Water soluble drugs and metabolites thereof are then particular difficult to assay and such compounds have been analysed using HILIC, for example cytotoxic drugs for cancer treatment like fluorouracil, cytarabine, and gemcitabine [11]. Among other applications HILIC has also been applied for herbicides like the quaternary amines chlormequat and mepiquat [12].

ION ANALYSIS

Traditionally small ions have been analysed by Ion Chromatography (IC). Most popular for inorganic ions, but also frequently used for small organic acids and amines. The number of papers describing HILIC separations of amino acids, carboxylic acids, quaternary and primary amines, acrylamides, nucleotides, and zwitterionic compounds are now rapidly increasing [13] and ESI-MS is mostly used as being more sensitive than the conductivity detector.

CONCLUSION

For successful use of HILIC one may need to think different, but then it becomes a straightforward and powerful tool for the separation and analysis of polar and hydrophilic compounds. The rapidly increasing interest is also reflected by the fact that most HILIC papers have been published during the last few years [13].

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