

Developing Hydrophilic Interaction Liquid Chromatography (HILIC) Separation Methods with HALO® HILIC and HALO® Penta-HILIC Columns

Introduction

Method developers rely on reversed-phase (RP) conditions for most of their separations. However, what happens when there are polar analytes that are not well retained under reversed-phase conditions? One option is to use Hydrophilic Interaction Liquid Chromatography (HILIC). Currently, there are two HILIC phases available from Advanced Materials Technology: HALO® HILIC and HALO® Penta-HILIC. This white paper will provide anyone unfamiliar with HILIC with some preliminary helpful knowledge and references on how to pick the right phase to use and some method development capability.

What is HILIC?

The term Hydrophilic Interaction Liquid Chromatography (HILIC) was first coined by Andrew Alpert (1). This mode of chromatography enables retention of polar analytes and ionizable compounds that are typically not well retained by reversed-phase. The hydrophobicity of a compound is given by its octanol-water partition coefficient (K_{ow} or $\log P_{ow}$). If the compound is more soluble in water, then it will have a value of $\log P_{ow} \leq 0$. For ionizable analytes, instead of $\log P_{ow}$, $\log D$ is reported, which is measured at different pHs. Those compounds with a $\log D \leq 0$ are candidates for HILIC. Values of $\log P_{ow}$ and $\log D$ are available from PubChem (2). HILIC is a mode of chromatography that uses a hydrophilic stationary phase and an organic solvent heavy mobile phase, typically acetonitrile, with water. HILIC stationary phases are classified as being neutral, acidic, basic, or other/novel. In HILIC the hydrophilic stationary phase support will uptake water forming a labile layer of surface adsorbed water. Polar neutral analyte retention happens by partitioning into and from the adsorbed water layer where hydrogen bonding, dipole-dipole interactions, and surface adsorption can take place. Additionally, ionizable analytes can retain and elute via electrostatic interactions with the stationary phase (3). Inverse to reversed-phase chromatography, water is the strong solvent. Thus, increasing the water content will shorten elution times. An example demonstrating this effect will be presented in Figure 8.

HALO® HILIC Products

Advanced Materials Technology offers two main stationary phases for HILIC: HALO® HILIC and HALO® Penta-HILIC. Figure 1 shows the structures of these two products. HALO® HILIC (acidic category) is a bare silica phase while HALO® Penta-HILIC (neutral category) is a bonded HILIC phase that consists of a ligand with 5 hydroxy groups bonded to the silica by a proprietary linker. Both of these phases are available in 2, 2.7, and 5 μm particle

sizes with 90 Å pore size. All HALO® particles are superficially porous particles comprised of solid silica cores and porous outer silica layers. For maximum efficiency 2 μm should be selected. For systems that are pressure limited 5 μm should be used and for a compromise between efficiency and back pressure, 2.7 μm should be selected. HALO® Penta-HILIC stationary phase was developed for its reduced ionic interactions compared to bare silica, which leads to improved peak shape and retention of acids and zwitterionic compounds in HILIC mode. See Figure 2 for the effect of buffer concentration on several types of compounds run on either HALO® HILIC or HALO® Penta-HILIC.

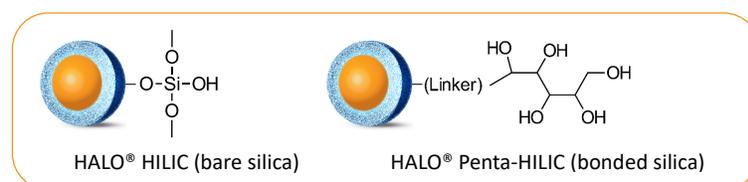


Figure 1. HALO® stationary phases for HILIC.

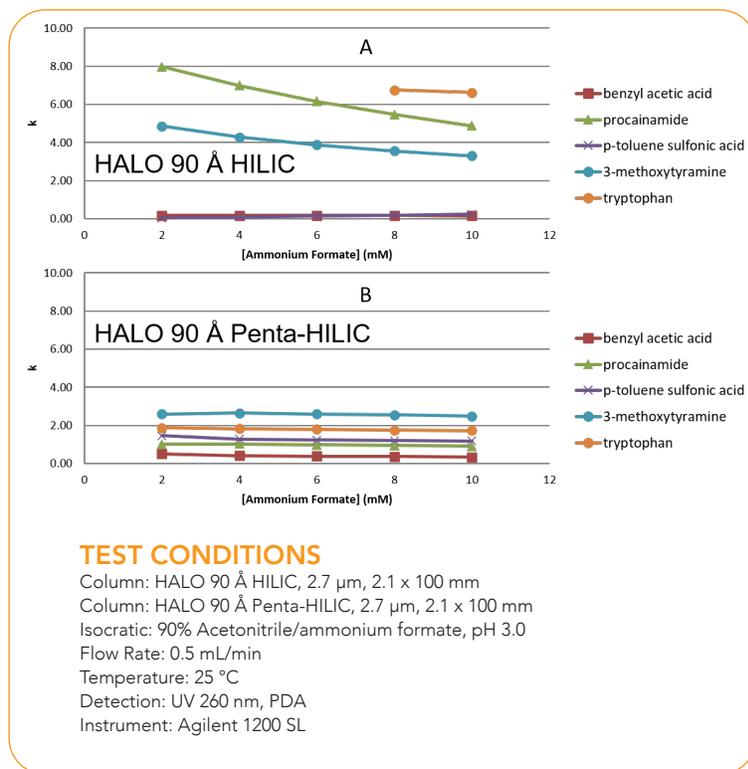


Figure 2A. Effect of buffer concentration on HALO® HILIC using benzyl acetic acid, procainamide, p-toluenesulfonic acid, 3-methoxytyramine, and tryptophan. 2B. Effect of buffer concentration on HALO® Penta-HILIC using the same compounds as in 2A.

With HALO® HILIC, as the buffer concentration is increased, the retention of basic compounds (procainamide and 3-methoxytyramine) decreases. There is little retention for the acidic compounds (benzyl acetic acid and p-toluenesulfonic acid) so there is no impact of buffer concentration on retention. The retention of the zwitterionic compound (tryptophan) is also not impacted much by increased buffer concentration. In contrast, with HALO® Penta-HILIC, there is no significant impact on the retention of the basic compounds with an increase in buffer concentration. The retention of the acidic compounds has increased compared to the retention with the HALO® HILIC column. The elution order of the compounds is different with the HALO® Penta-HILIC compared to the HALO® HILIC with the acidic compounds being more retained and the basic and zwitterionic compounds being less retained.

An example of the resolution and retention differences between HALO® Penta-HILIC compared to HALO® HILIC is demonstrated in Figure 3.

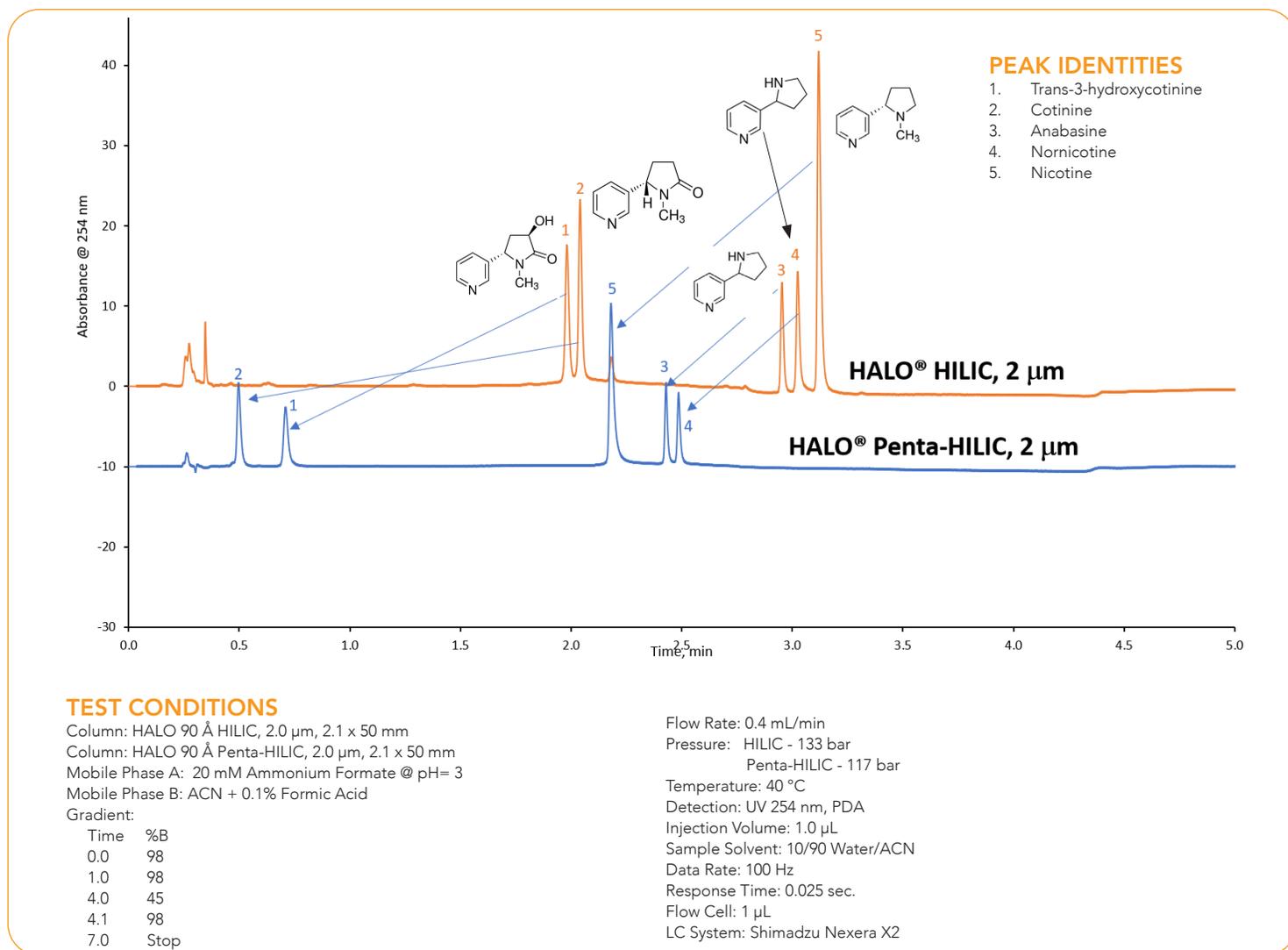


Figure 3. Differences between HALO® HILIC and HALO® Penta-HILIC in terms of retention and resolution.

With this separation of nicotine and its metabolites, there are examples of elution order changes when moving from a HALO® HILIC column to a HALO® Penta-HILIC column. All of the compounds are retained more on the HALO® HILIC column. This aligns with the graphs in Figure 2 since all of the compounds are basic. However, the resolution between trans-3-hydroxycotinine and cotinine (peaks 1 and 2) is improved on the HALO® Penta-HILIC column. Additionally, the HALO® Penta-HILIC is better able to resolve nicotine (peak 5) from the anabasine and nornicotine (peaks 3 and 4).

In terms of efficiency and peak shape, the HALO® Penta-HILIC outperforms the HALO® HILIC. See Figure 4 for the comparative chromatograms.

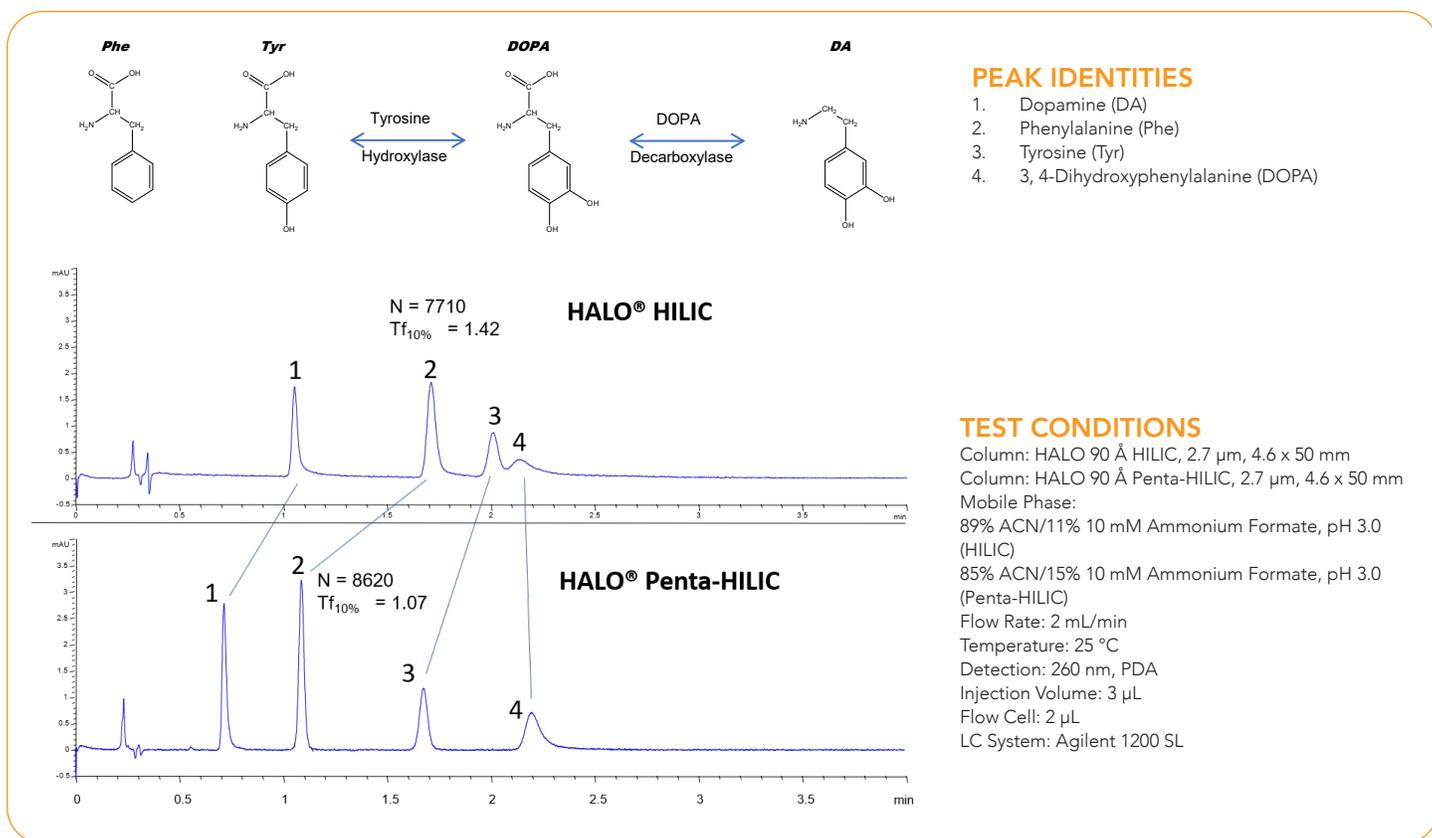


Figure 4. Differences between HALO® HILIC and HALO® Penta-HILIC in terms of efficiency and peak shape for a mix of catecholamines and amino acids.

HALO® Penta-HILIC was more retentive compared to HALO® HILIC so a higher concentration ACN mobile phase was used for HALO® HILIC. The peak shapes of basic compounds are more symmetrical/less tailed on the HALO® Penta-HILIC column compared to the HALO® HILIC column. Specifically, the tailing factor for phenylalanine is 1.42 on the HALO® HILIC column and 1.07 on the HALO® Penta-HILIC column. Additionally, the efficiency is 10.5% higher on the HALO® Penta-HILIC column compared to the HALO® HILIC column. HALO® HILIC and HALO® Penta-HILIC are different and are suitable for different separation challenges. Since the two columns have different selectivity, it may be useful to screen both HALO® HILIC and HALO® Penta-HILIC to be more certain that all sample components are being resolved. When a suitable separation can be accomplished on both columns, HALO® Penta-HILIC is usually preferred because it has higher efficiency and is less sensitive to changes in pH and ionic strength; however, HALO® HILIC can often perform unique separations. HALO® HILIC has been used for the analysis of glucosylsphingosine and galactosylsphingosine in cerebrospinal fluid, plasma, and brain samples (4).

Modified Ikegami Characterization of HILIC Phases

Similar to the Tanaka characterization that has been developed for reversed-phase chromatography stationary phases, there is a set of test probes and conditions that has been developed to specifically characterize HILIC stationary phases (5). This method was further adapted by Corman et al. (6) in order to better distinguish different HILIC stationary phases from one another. In particular, the selectivity between 4-nitrophenyl α-D-glucopyranoside and 4-nitrophenyl β-D-glucopyranoside was removed and the retention factors of the anionic and cationic probes (sodium p-toluenesulfonate (SPTS) and (N,N,N-trimethylphenylammonium chloride (TMPAC)), respectively were plotted in separate bar graphs instead of being included in the radar plot. Table 1 lists the factors that are included in the radar plots.

Results on HALO® HILIC and HALO® Penta-HILIC

Figure 5 shows a radar plot for the 6 parameters listed in Table 1. The main differences between the HALO® HILIC and the HALO® Penta-HILIC are the retention factor of uridine $k(U)$ and the selectivity of hydroxyl addition $\alpha(OH)$. Increased retention of uridine is observed with HALO® Penta-HILIC. The $\alpha(OH)$ factor, determined by the selectivity between uridine and 2-deoxyuridine, has been shown to be a convenient marker of how hydrophilic the stationary phase surface is. It was demonstrated by Ikegami et al. to have a linear relationship to the thickness of surface adsorbed water (7). Hence, this is a simple assessment of the hydrophilic partitioning capabilities of the stationary phase materials wherein the HALO® HILIC is a weak partitioning support, and the HALO® Penta-HILIC is a strong partitioning support.

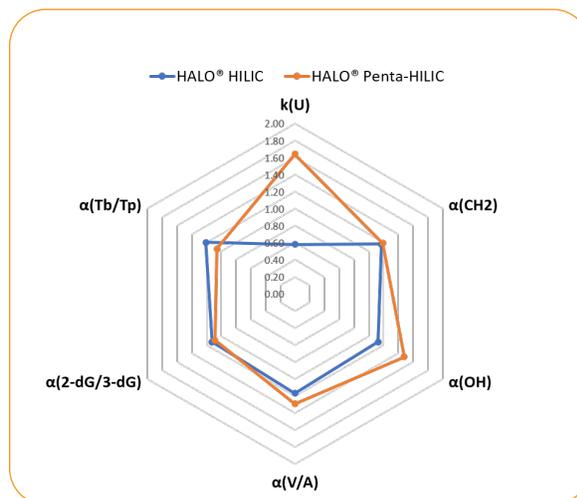
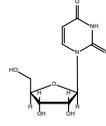
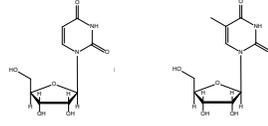
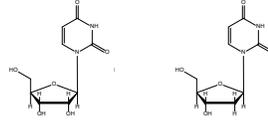
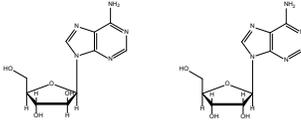
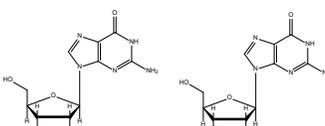
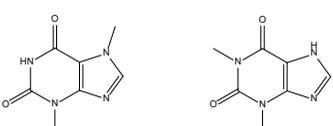


Figure 5. Radar plot comparing the Ikegami parameters for HALO® HILIC and HALO® Penta-HILIC.

Table 1 Parameters measured for HILIC Stationary Phase Characterization

PARAMETER	ABBREVIATION	WHAT IS MEASURED	STRUCTURE
Retention factor of uridine	$k(U)$	Absolute Retention	
Selectivity of methyl addition using uridine and 5-methyluridine	$\alpha(CH_2)$	Hydrophobicity	
Selectivity of hydroxyl addition using uridine and 2'-deoxyuridine	$\alpha(OH)$	Hydrophilicity	
Selectivity between Vidarabine & Adenosine	$\alpha(V/A)$	Degree of separation of configurational isomers	
Selectivity between 2-deoxyguanosine & 3-deoxyguanosine	$\alpha(2d/3d)$	Degree of separation of positional isomers	
Selectivity between Theobromine & Theophylline	$\alpha(Tb/Tp)$	Acidity/basicity of the stationary phase	

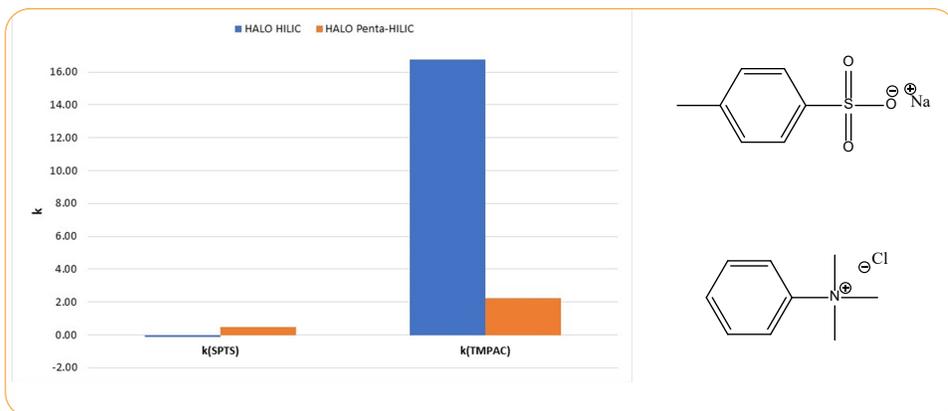


Figure 6. Bar graph indicating the ion exchange contributions for HALO® HILIC compared to HALO® Penta-HILIC.

As shown in Figure 6, for the ion exchange contributions to retention, it is clear there is little retention of the acidic compound SPTS on the HALO® HILIC phase compared to HALO® Penta-HILIC. The HALO® HILIC phase has many more surface silanols which repel acidic compounds unlike the HALO® Penta-HILIC. In contrast,

the HALO® HILIC is considerably more retentive towards basic compounds compared to the HALO® Penta-HILIC phase as indicated by the retention of TMPAC. Strong basic retention may require appreciable amounts of buffer salts to reduce retention and improve peak shape of, which is not ideal in some cases, especially when MS detection is required. The reduced electrostatic interactions towards basic compounds on the HALO® Penta-HILIC leads to better peak shapes overall.

A Comparison of HALO® Penta-HILIC to Amide HILIC

When HALO® Penta-HILIC is compared to another commercial phase, Amide HILIC, some noticeable differences emerge. See Figure 7. Figure 7 shows slightly more retention for the acidic compound (Salicylic Acid – peak 3) compared to the 1.7 µm Amide HILIC. Both Amide HILIC columns show increased retention for the basic compound (thiamine - peak 4) and increased peak tailing/lower efficiency.

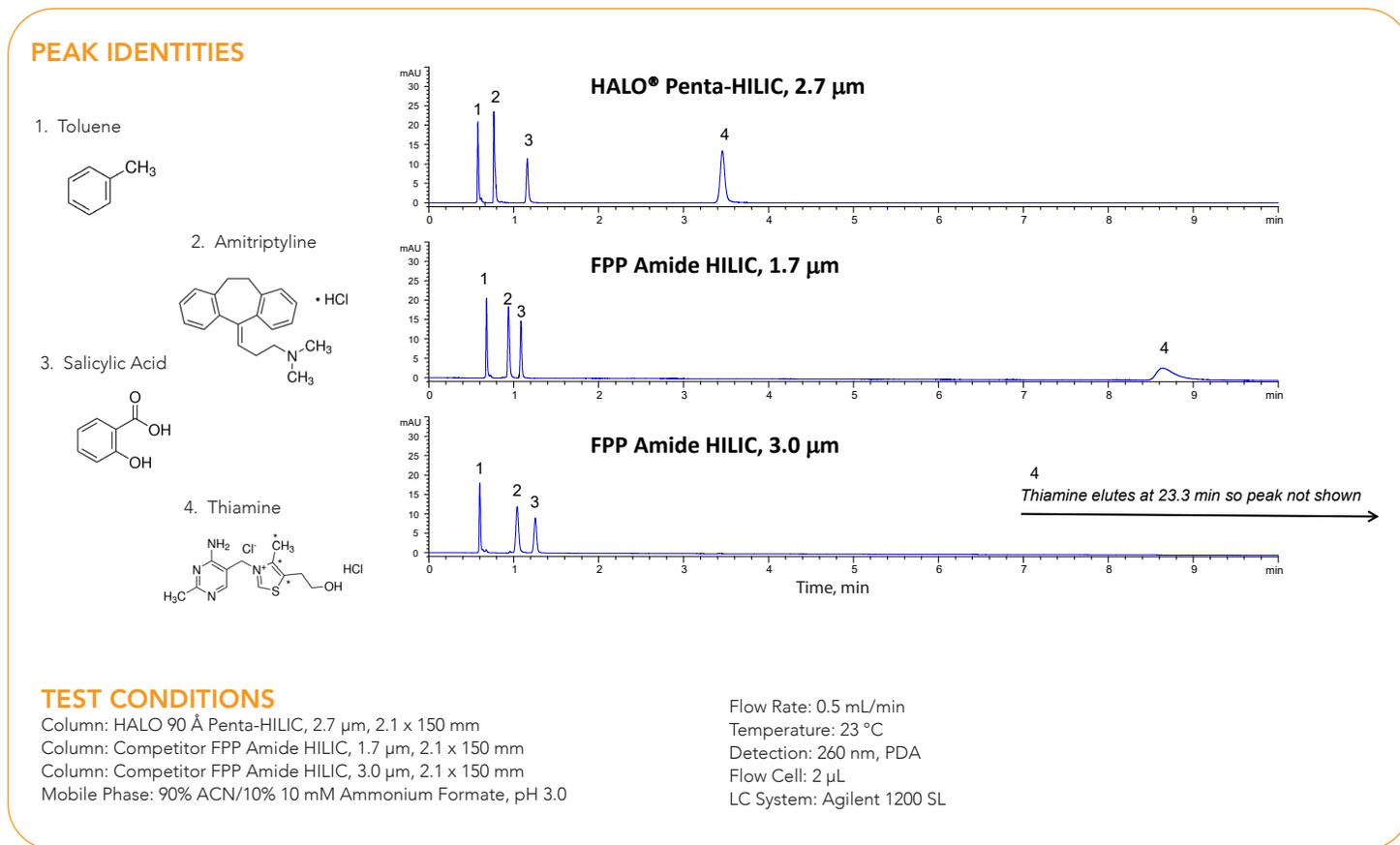


Figure 7. HALO® Penta-HILIC compared to fully porous Amide HILIC stationary phases. Elution order: toluene, amitriptyline, salicylic acid, thiamine.

Keys to Successful HILIC Method Development

In order to be successful with HILIC methods, a few key items should be kept in mind. First, remember that water is the strong solvent, which means more water in the mobile phase will give less retention.

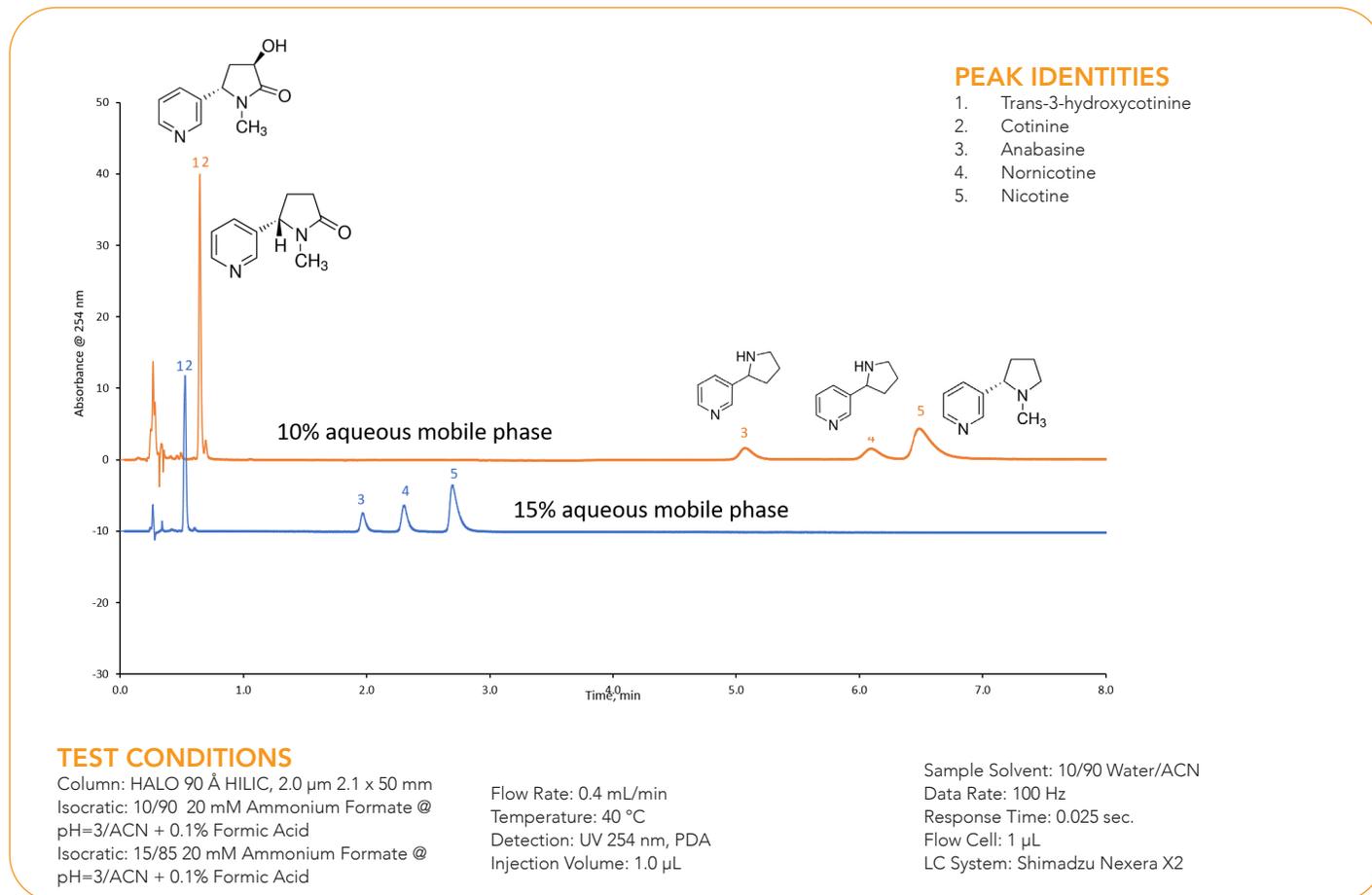


Figure 8. Impact of water in the mobile phase for HILIC. 10/90 aqueous/ACN is more retentive than 15/85 aqueous/ACN.

This is demonstrated in Figure 8 in which a 10% aqueous mobile phase is compared to a 15% aqueous mobile phase. Note that the separation contains the same analytes as in Figure 3 and was not optimized for resolution. Whereas an aqueous rich mobile phase would mean more retention under reversed-phase conditions, the opposite is true when running in HILIC mode. Keeping this in mind, while in HILIC mode, columns are cleaned by going to higher aqueous content in the mobile phase.

Some other things to remember regarding the mobile phase is to avoid using mobile phase additives that are at or near the limit of solubility in the low water mobile phase. In terms of additives, buffers are better at controlling pH than acid additives alone. It is important to remember to pre-mix and degas the mobile phase. Specifically, use 5% ACN in water and 5% water in ACN. Most instruments have online degassers, but best practice is to degas after premixing the mobile phase.

Additionally, a different marker must be used for t_0 than what is typically used with reversed-phase conditions. Uracil would be retained under HILIC conditions so toluene is used as the t_0 instead. See Figure 7. Acenaphthene can also be used as a void volume marker. See Figure 9.

The next item to keep in mind is the impact of sample solvent on peak shape. Several investigations have been conducted on peak shape in HILIC mode (8-9). It is important to have a sample solvent that is weaker than the initial mobile phase composition. If too much water is present in the sample solvent, then peaks may show splitting or may front and/or show decreased retention. Figure 9 has a comparison of the correct sample solvent compared to a highly aqueous sample solvent.

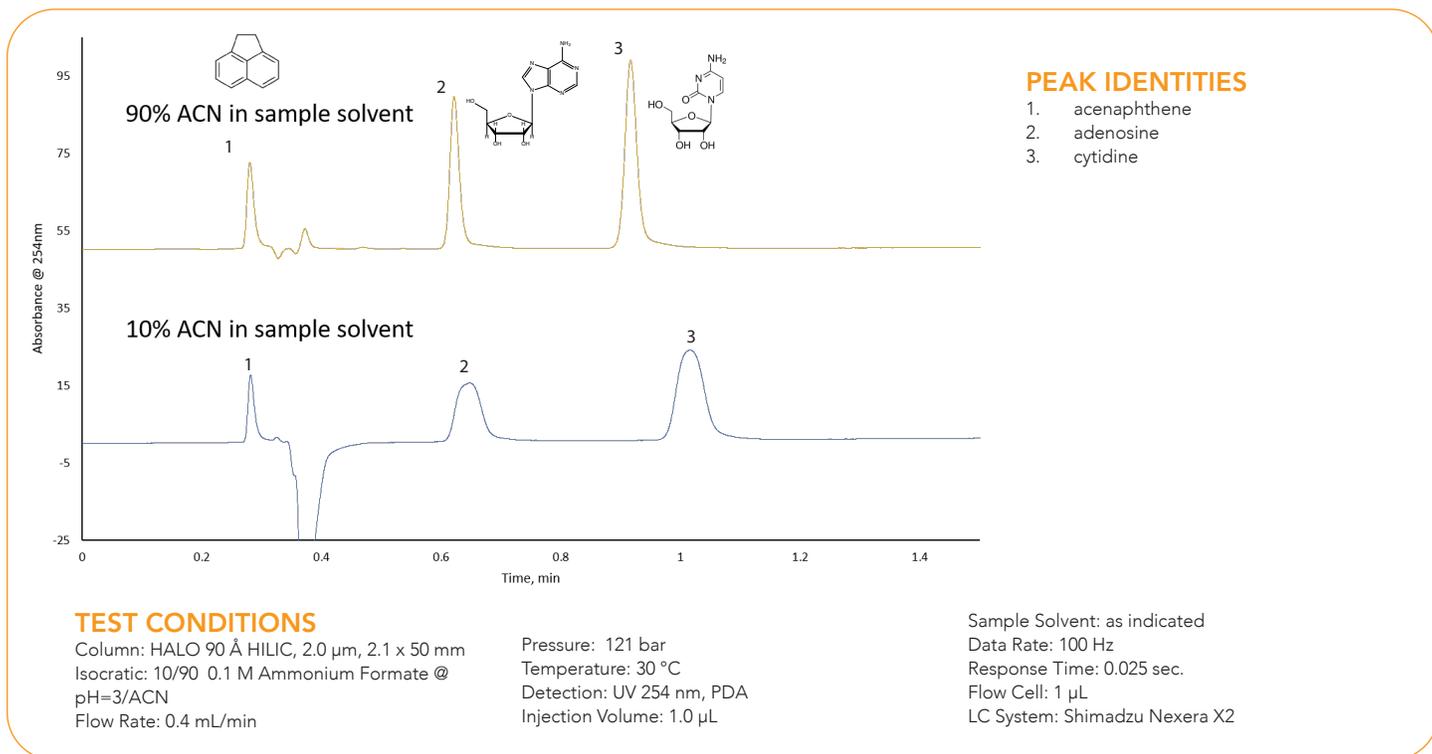


Figure 9. Impact of sample solvent composition on peak shape and retention in HILIC mode – 1 µL injection volume.

The HILIC method in Figure 9 uses 90% acetonitrile which means that the sample solvent should be near 90% acetonitrile to avoid peak splitting or peak broadening especially of the early eluting compounds. When only 10% ACN is present in the sample solvent, the peak shape suffers. Seeing a result such as this could make an analyst believe that the column has experienced a catastrophic failure. To demonstrate how devastating this type of sample solvent mismatch can be, the injection volume was increased to 2.5 µL – see Figure 10.

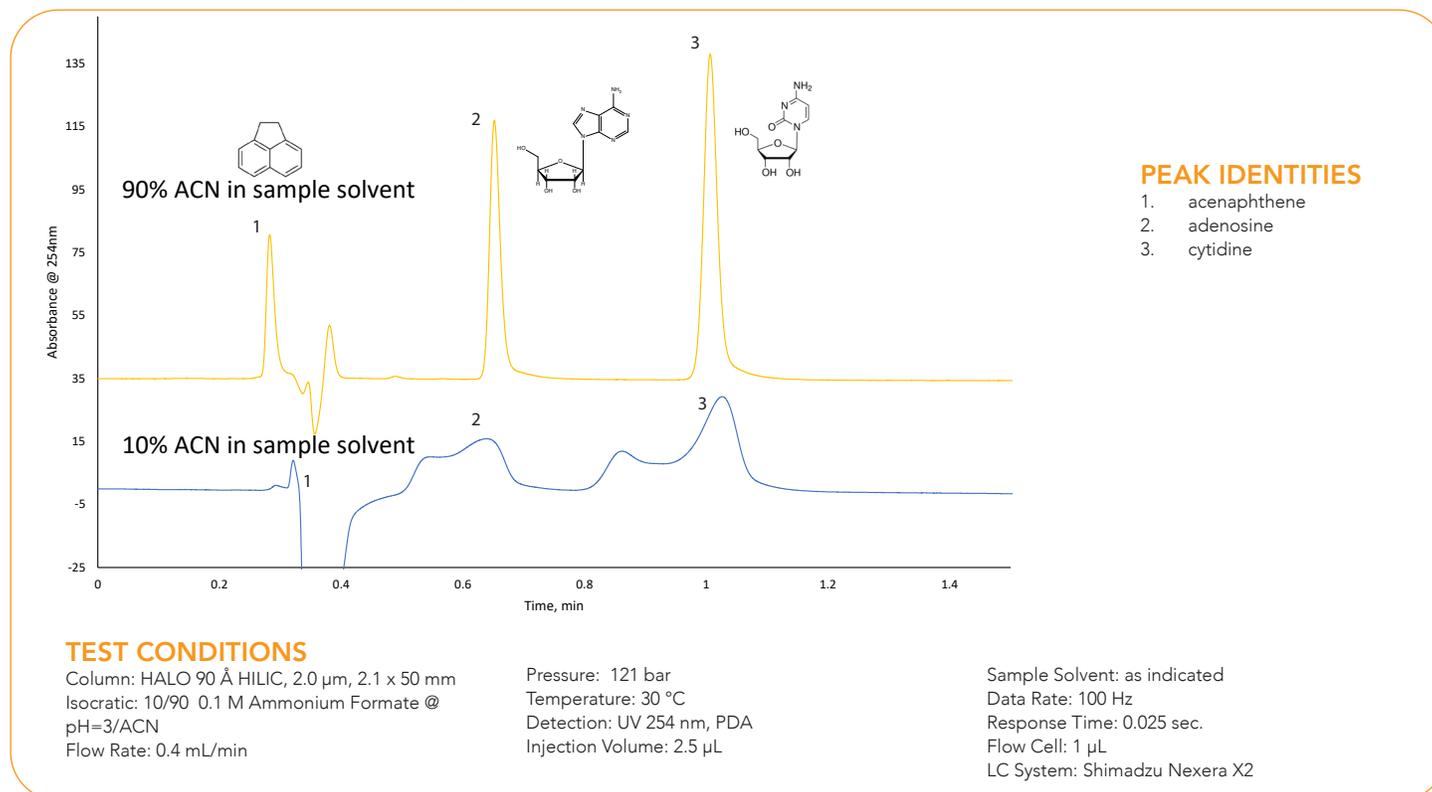


Figure 10. Impact of sample solvent composition on peak shape and retention in HILIC mode – 2.5 µL injection volume.

With the 2.5 μL injection volume, the peaks show splitting and even broader peak shape.

Naturally, the solubility of very polar analytes in organic solvents can be a concern. To overcome this, stock solutions of the polar analyte can be made using 50:50 water:organic and then the stock solutions can be diluted to higher organic concentration. Another method is to use 50:50 acetonitrile:isopropanol for working solutions. For peptides, 100% isopropanol or ethanol is an option. When analyzing potential drug candidates, dimethylsulfoxide (DMSO) in 80% acetonitrile or higher may be used.

HILIC methods can be equilibrated just as fast as reversed-phase and several published papers have delved into this topic (10-12). Superficially porous particle columns allow faster flow rates to be used which can lead to a faster equilibration time for the method. Simply increase the flow rate during the equilibration step in the gradient.

Finally, HILIC can be as reproducible as reversed-phase provided the following is true:

- Mobile phases are prepped consistently
- Buffers are used instead of acid additives alone
- Sample solvent is optimized
- Instrument rinse solvent is not composed of too much water (strong solvent)

HILIC Applications

Amino Acids

One application area that benefits from HILIC is metabolomics. Figure 11 demonstrates four amino acids from a yeast extract sample. HILIC mode allows for much higher retention of these amino acids when compared to reversed phase separations (13). A higher increase in signal is also observed when compared to RP due to the higher organic mobile phases being used.

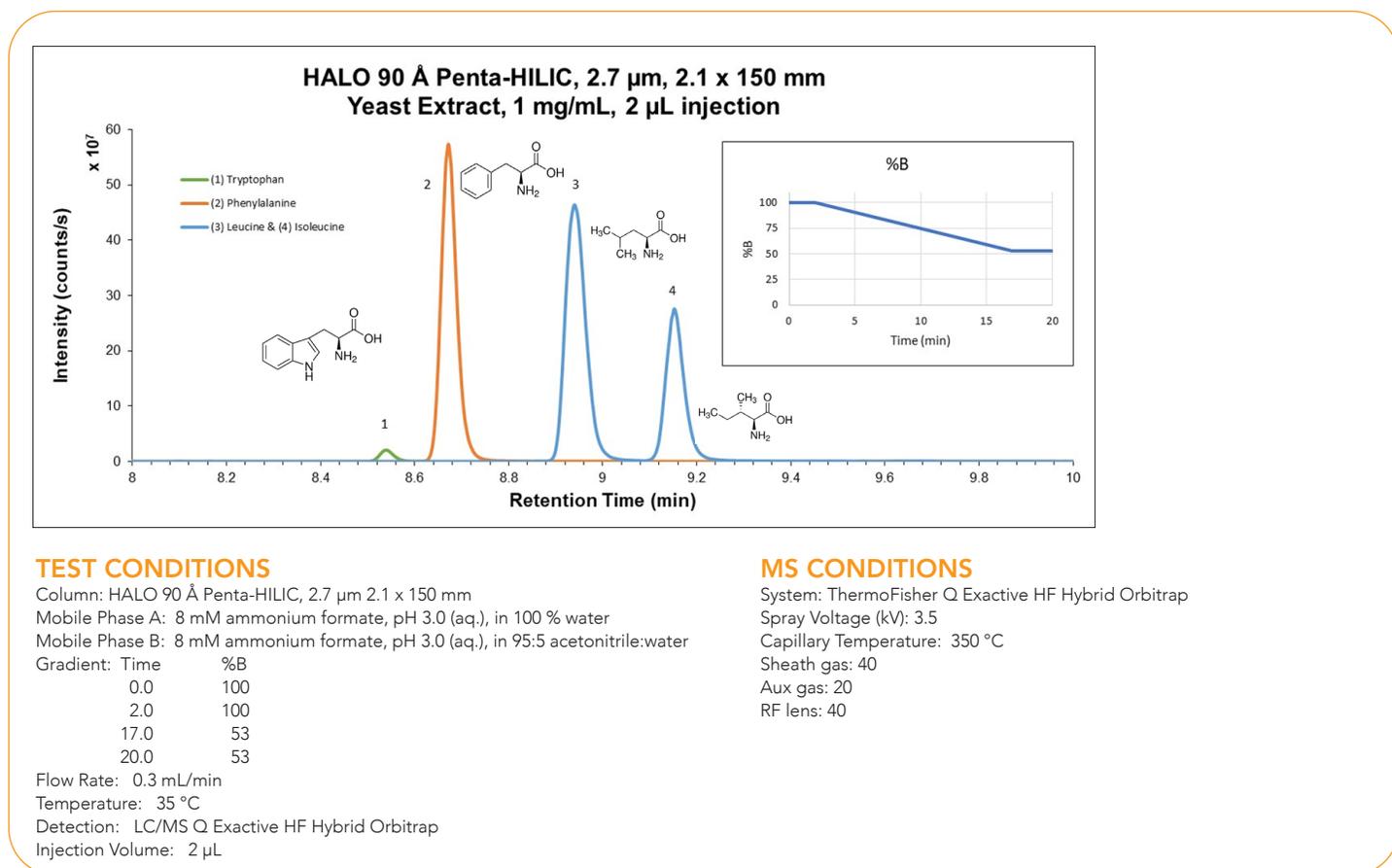


Figure 11. Separation of amino acids from yeast extract using a HALO® Penta-HILIC column.

Drugs of Abuse

Drugs of abuse and their metabolites can be analyzed in HILIC mode as shown in Figure 12. Resolution of the codeine/hydrocodone isomers is enabled by the HALO® Penta-HILIC phase. This rapid separation is completed in less than 3 minutes using isocratic conditions and mass spectrometry detection. HILIC mode is ideal for LC-MS because of highly volatile conditions that facilitate ionization and often improve signal response and sensitivity.

TEST CONDITIONS

Column: HALO 90 Å Penta-HILIC, 2.7 µm, 2.1 x 100 mm
Isocratic: 95/5 ACN/water with 5 mM Ammonium Formate, pH 3
Flow Rate: 0.5 mL/min
Temperature: 30 °C
Injection Volume: 1 µL
Flow Cell: 1 µL
LC System: Shimadzu Nexera

MS CONDITIONS

System: LCMS 2020
ESI: +2kV
Heat Block: 400 °C
Capillary: 225 °C

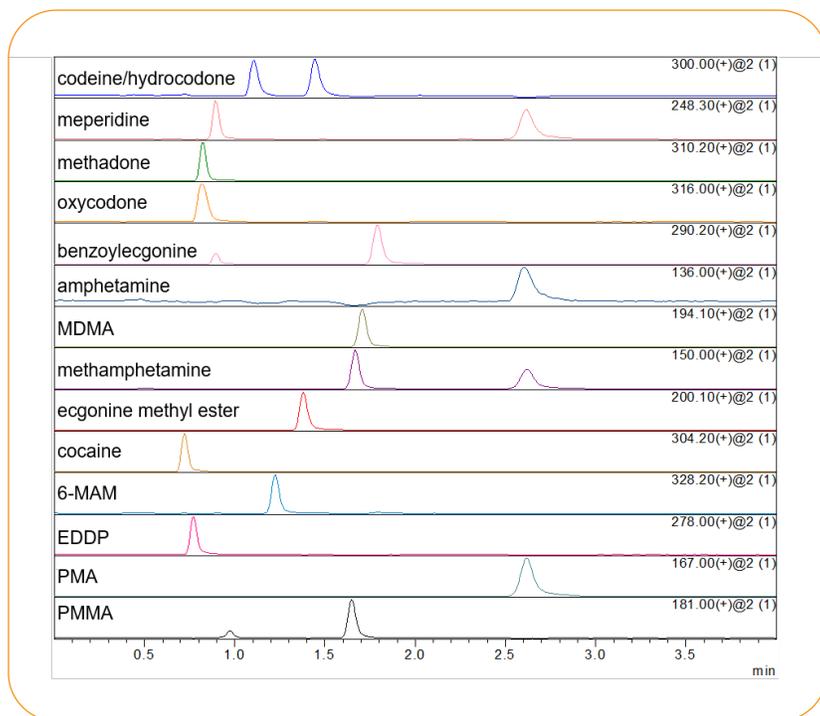


Figure 12. Separation of 15 Drugs and Metabolites Using HALO® Penta-HILIC

Sugar Analysis

HILIC analysis of simple sugars as shown in Figure 13 enables honey to be tested for the presence of adulterants. Honey contains fructose and glucose and should not contain any other sugars. If other sugars are found, then the honey has been adulterated. For this analysis, ELSD was used since the sugars have no UV chromophores. Furthermore, the column is run at 65 °C to prevent peak splitting of reducing sugars (all except sucrose which lacks a free aldehyde or ketone group).

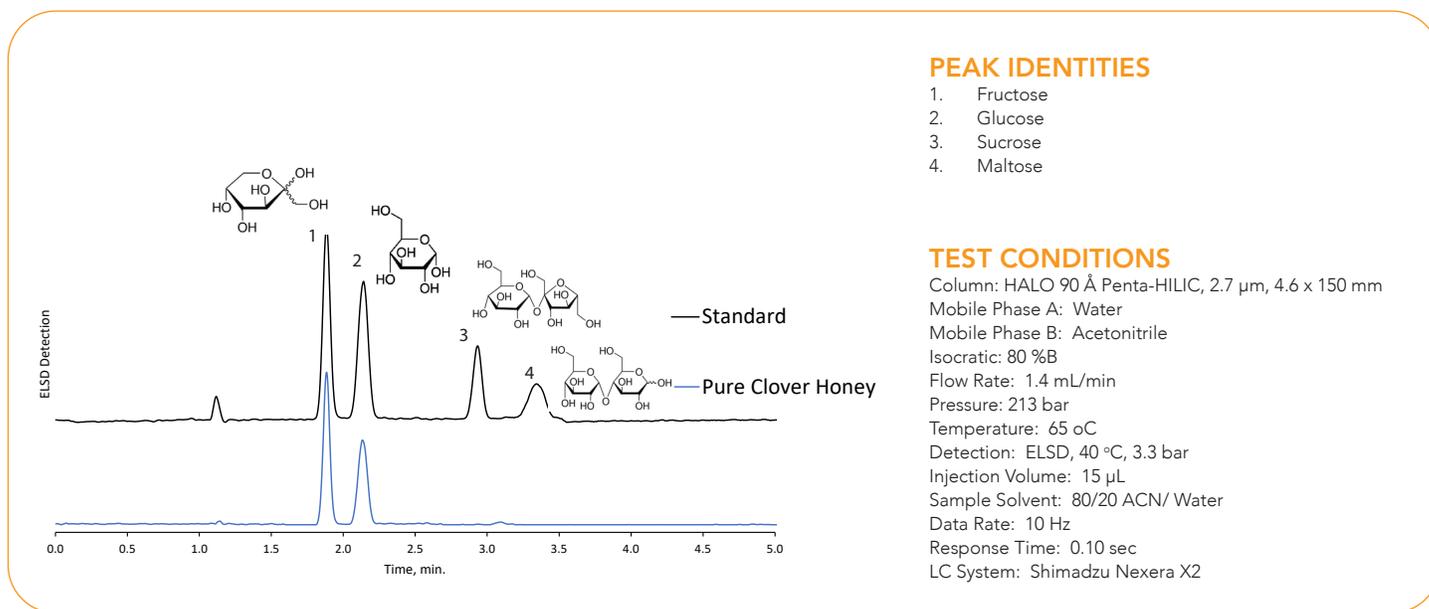


Figure 13. Separation of sugars in pure clover honey using HALO® Penta-HILIC (blue trace). Black trace shows standard sugars.

A HALO® Penta-HILIC column was used to monitor beer fermentation as shown in Figure 14. Before yeast addition, there are high levels of glucose, sucrose, maltose, and maltotriose (blue trace). As the fermentation occurs, the glucose and sucrose are consumed after day 1 (red trace). By day 7, not much sugar remains in the beer (pink trace).

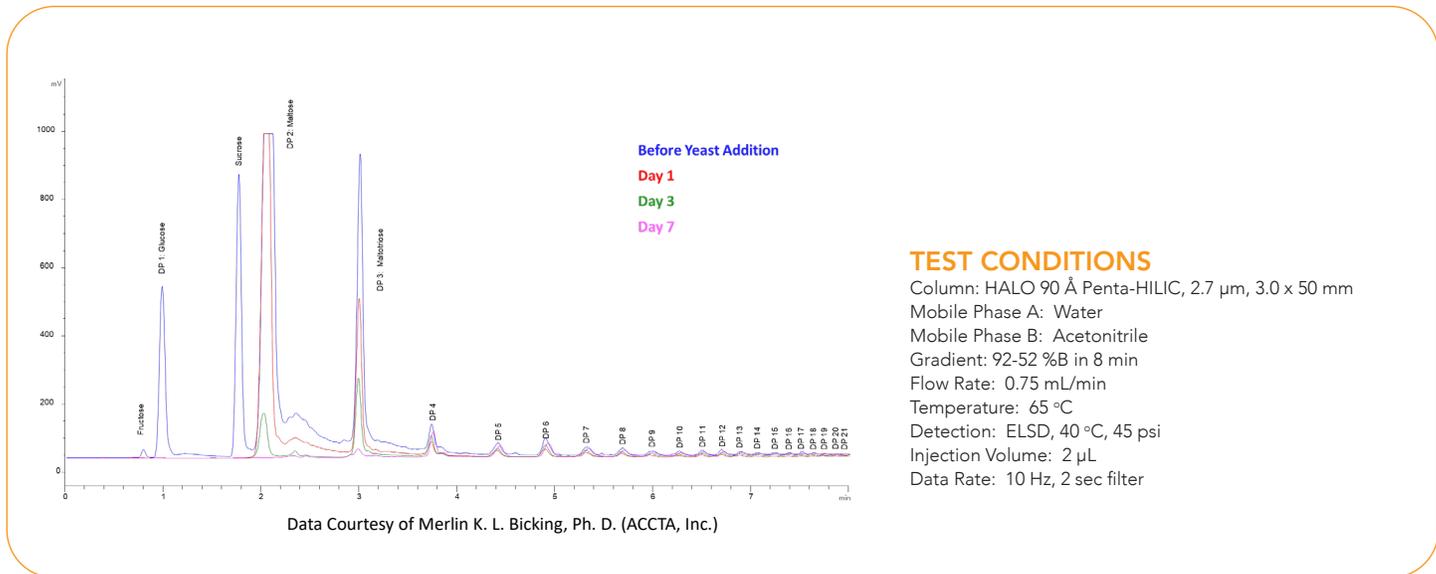


Figure 14. Beer Fermentation Analysis using HALO® Penta-HILIC.

Another type of sugar analysis is shown in Figure 15. Here a comparison of the reversed-phase analysis of glycopeptides in a bovine Ribonuclease B tryptic digest is shown versus the HILIC analysis. The increased resolution and retention in HILIC mode is advantageous for these types of samples and is not possible when reversed-phase conditions are used. The colored traces are the SIMs while the black traces are the TICs.

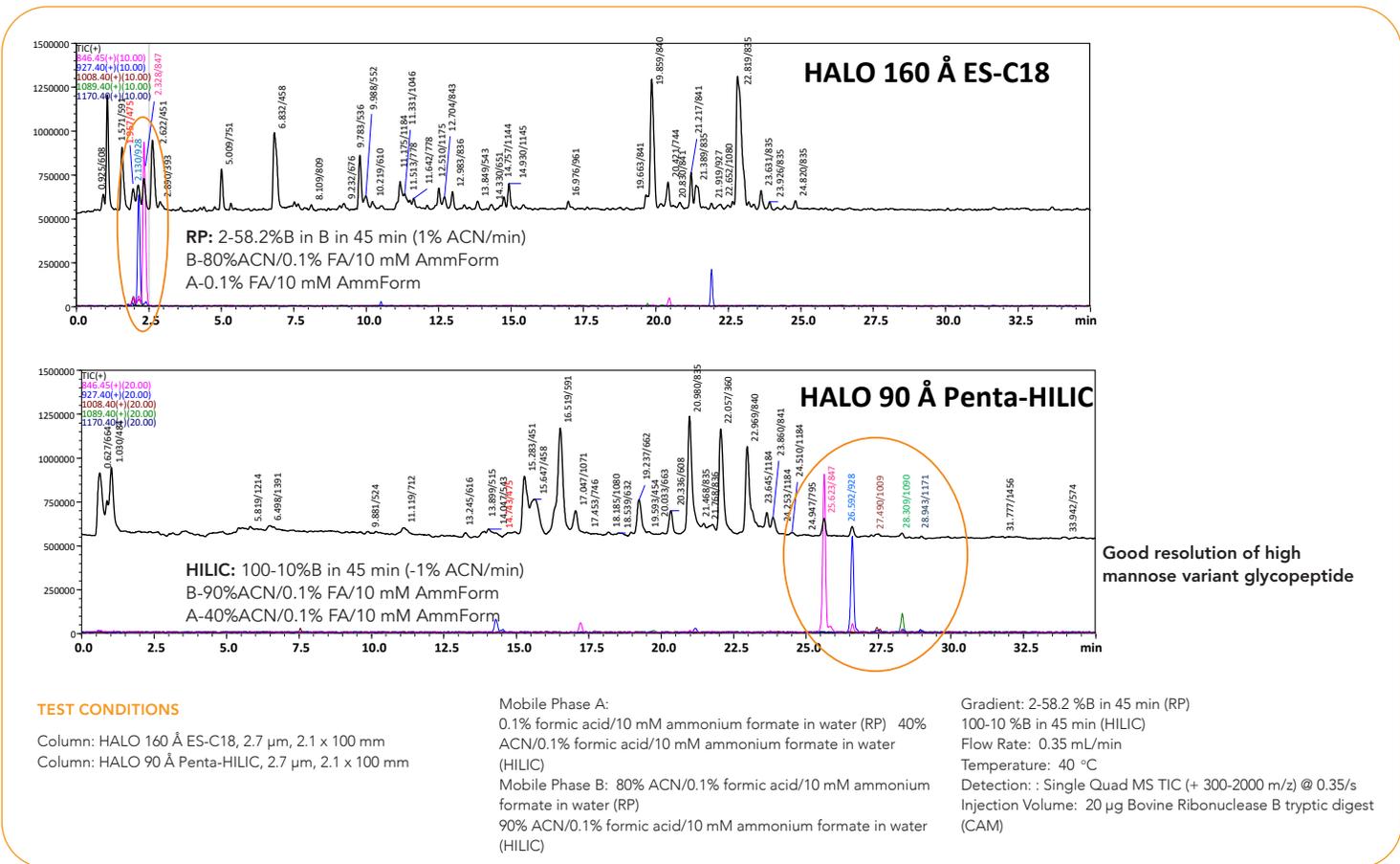


Figure 15. Advantage of HILIC mode for glycopeptide separations using HALO® Penta-HILIC.

Summary

HILIC continues to gain popularity as more highly polar and ionizable compounds are developed. No exotic solvents are required – mainly acetonitrile and water with buffer. HILIC is a useful option for retaining polar compounds and shows improvements compared to reversed-phase with MS detection. The HILIC phases available on HALO® Fused-Core® particles include HALO® HILIC and HALO® Penta-HILIC. Analysts may be reluctant to try HILIC, but the methods can be designed to be as robust and reproducible as reversed-phase methods. HILIC solves challenging separations and should be appreciated and utilized.

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Company Profile

Advanced Materials Technology, founded in 2005, has been focused on one mission – Improving the presentation of the sample to the detector. Using our novel Fused-Core® particle design, we have challenged conventional wisdom and engineered innovative solutions for the separations community.

All company operations and functions are proudly located in Wilmington, Delaware, USA with world-wide distribution and accessibility.

AMT invites a company culture of diversity, respect and pride in delivering quality products. We embrace ISO 9001 standards in our work systems and daily work. We pledge to have a dynamic leadership team which promotes our culture of excellence embedded in every employee.

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