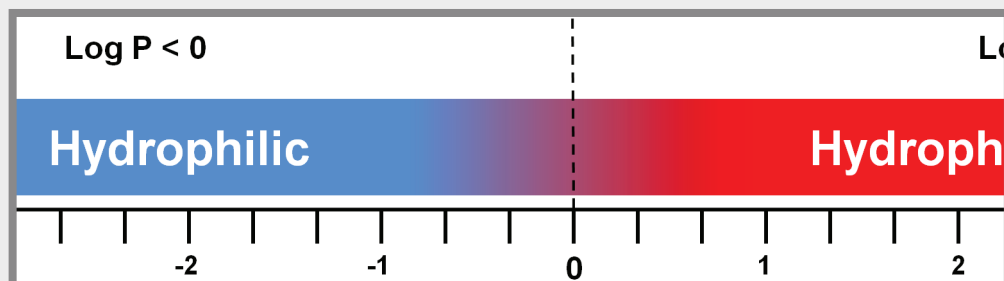


Separation Science is proud to launch 'HILIC Solutions' — a series that will provide you with useful tips and hints for developing robust and handy HILIC methods. We'll cover the theory, basics and practical handling of HILIC, explore stationary phase options and discuss how to select the best chromatographic parameters to enhance performance.

Tech Tip

Do you HILIC?



Are your analytes eluting close to the void volume of a reversed-phase (RP) column? Do you have problems retaining organic molecules under conventional reversed-phase liquid chromatography (RPLC) conditions? Have you increased the initial percentage of water in the mobile phase or tried a different mobile phase (and pH) or changed to a polar-embedded RP column without success? What do you do? Have you tried HILIC?

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Featured Applications

Agilent 1290 Infinity LC System – Applications requiring the Agilent Ultra-Low Dispersion Kit

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Reversed-Phase Analysis of a Monoclonal Antibody on an Accucore C4-150 HPLC Column

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Giorgia Greco



Thomas Letzel

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Hydrophilic interaction liquid chromatography, referred to as HILIC, has been known for several years as a 'non-robust', 'difficult' separation technique with long equilibration times. This statement has often been announced by analysts originally experienced in RPLC. However, this is not true if you are careful about several key aspects.

We will give you several important hints via this tip series, thus providing you with the basis of developing 'robust' and 'easy' HILIC methods.

Indeed HILIC is a very powerful new tool for the separation of polar compounds poorly retained under RPLC conditions and dates back to the 1970s. It is also known as 'reversed-RPLC' because the mobile phase is a mixture of water/ water-miscible organic solvent as with RPLC but solvent elution strengths are opposite. In gradient mode, HILIC starting conditions require a high percentage of organic solvent, typically 95%, and the elution is promoted by increasing the water content in the mobile phase up to 40%. The HILIC stationary phases are very polar materials such as silica or polar-bonded silica-based phases, whose main characteristic is to strongly adsorb water on their surface.

Hydrophilic compounds are highly retained in HILIC due to a favoured solubilization into the adsorbed water layer, whereas

Figure 1

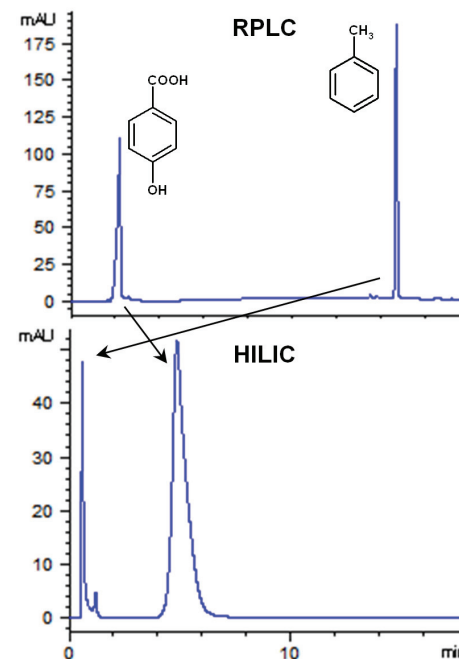


Figure 1. Separation of 4-hydroxybenzoic acid and toluene on RPLC and HILIC. Chromatographic conditions: RPLC: column, Zorbax ECLIPSE XDB-C18 (150 x 4.6 mm, 5 μ m; mobile phase, water/ ACN, 0–15 min, from 20 to 90% ACN; flow-rate: 0.6 mL/min; UV detection: 250 nm. HILIC: column, YMC-Diol (150 x 2.1 mm, 5 μ m); mobile phase, ACN/water (95/5, v/v), 10 mM ammonium acetate; flow-rate: 0.6 mL/min; UV detection: 250 nm.

Figure 2

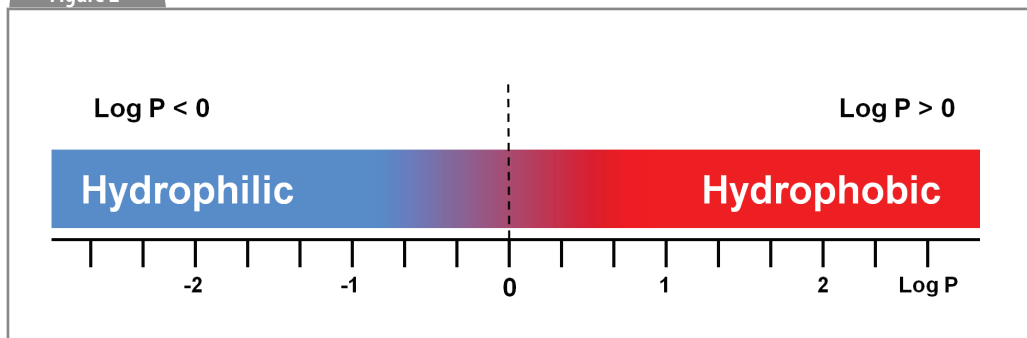


Figure 2. Graphical representation of log P values in relation to analyte polarity. Hydrophilicity increases with the decreasing of log P value.

hydrophobic compounds are eluted earlier.

Therefore, inversion of the elution order is quite a common experience when HILIC columns are compared with RPLC columns.

Let us consider two analytes with different polarity: toluene and 4-hydroxybenzoic acid. The first is very hydrophobic while the second is hydrophilic. As shown in Figure 1, toluene is strongly retained while 4-hydroxybenzoic acid is not retained by a conventional C18 RP column. Moreover, when the two analytes are injected on a HILIC diol phase, the elution order is reversed as 4-hydroxybenzoic is retained longer.

The separation of small polar compounds such as organic acids, basic amines and water soluble vitamins, that can be difficult on a C18 column, can easily be performed in HILIC.

But how can we decide between RPLC and HILIC?

Analyte polarity is the first parameter to be considered when we have to select the right HPLC method. Both RPLC and HILIC retention mechanisms are based on a partitioning process. Even if interactions of a different nature contribute to the separation, from a practical point of view analyte polarity can be well described by the logarithm of the partition coefficient, log P,

$$\log P_{\text{oct/wat}} = \log ([\text{analyte}]_{\text{oct}} / [\text{analyte}]_{\text{wat}})$$

where P is defined as the ratio of concentrations of an unionized analyte in *n*-octanol and water phases at equilibrium. Clearly, hydrophobic compounds are preferentially solubilized in the *n*-octanol phase and hydrophilic ones in water (Figure 2). In the case of ionizable analytes such as amines or organic acids, the polarity changes with the pH. As a consequence, in the case

Figure 3

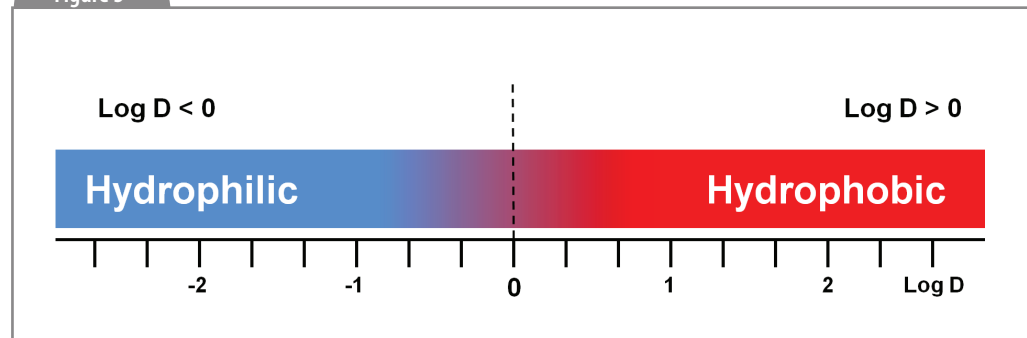


Figure 3. Graphical representation of log D values in relation to analyte polarity. Hydrophilicity increases with the decreasing of log D value.

of ionizable analytes such as amines or organic acids, the polarity changes with the pH. Charged analytes are more hydrophilic than their neutral forms and for them the logarithm of the distribution coefficient, log D, should be considered instead of log P,


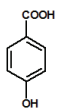
$$\log D_{\text{oct/wat}} = \log ([\text{analyte}]_{\text{oct}} / [\text{charged analyte} + \text{neutral analyte}]_{\text{wat}})$$

where D is the ratio between the concentration of the analyte in *n*-octanol and the sum of the concentrations of the charged and neutral form in water at a given pH (Figure 3).

In contrast to log P, log D is pH dependent and the pH at which it has been measured must be specified. Similarly to log P, log D can be measured experimentally. However, such a procedure is quite long and would not be compatible with routine analyses. A number of different software packages for a log P and a log D calculation can assist us for a rapid evaluation of analytes' polarities.

Among them, www.chemicalize.org and www.chemspider.com are free

Table 1

Compound	Structure	Marvin values		Retention
Toluene		log P = 2.5	> 0 hydrophobic	RPLC
4-hydroxybenzoic acid		log D (pH 7) = -1.4	< 0 hydrophilic	HILIC

databases that contain log P and log D values predicted with Marvin and ACD/PhysChem Suite software, respectively.

Let us consider again toluene and 4-hydroxybenzoic acid. The mobile phase pH is neutral in both conditions presented in Figure 1.

Keep in mind that in the case of unionizable compounds, such as toluene, $\log P = \log D$.

In order to select the most appropriate HPLC separation mode, you can follow this scheme:

$\log P$ or $\log D > 0 \rightarrow$ RPLC
 $\log P$ or $\log D < 0 \rightarrow$ HILIC

Now look at the polarity of your analytes. If they are hydrophilic, they are efficiently separated in HILIC.

In the upcoming instalments of this series, we will present the basics of HILIC, the main stationary phases commercially available and how to select the chromatographic parameters (i.e., organic solvent, salt content, mobile phase pH, and column temperature) for your best performance.

Until next time!

Giorgia Greco is currently a Post Doc researcher at the Analytical Research Group at the Technische Universität München, Germany. She received a PhD in Chemistry at the University of Naples, Italy. During her research, she specialized in the analysis and separation of metabolites from human and food matrices, as well as of organic contaminants in waste water samples, by hyphenated RPLC/MS and HILIC/MS techniques. She has also focused on the theoretical elucidation of the HILIC retention mechanism with the aim of providing scientific bases for the fast development of HILIC separations.

Thomas Letzel, Associate Professor, is head of the Analytical Research Group at the Technische Universität München, Germany. He received his PhD in Chemistry with Aerosol Analysis, worked as Post-Doc performing pharmaceutical analysis, built up his research group in bioanalysis with Habilitation in 2009 and extended his analytical experience from then in food and water analysis. In all areas he developed novel analytical platforms based on LC–MS for the characterization of organic molecules in complex matrices. Thereby techniques (such as HILIC or RP-UHPLC) are applied for new analytical solutions often in direct flow-coupling with (bio)functional assays. He is the author of more than 50 publications and two books.

Thomas Letzel wants to share his experience in liquid chromatography, especially in HILIC, with the community to accelerate the dissemination about HILIC theory and practical handling.

FEATURED APPLICATIONS



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Agilent 1290 Infinity LC System – Applications requiring the Agilent Ultra-Low Dispersion Kit

Company: Agilent Technologies

Improvements with respect to peak width, peak capacity, and plate number were evaluated using the Agilent Ultra-Low Dispersion Capillary Kit and the Agilent Ultra-Low Dispersion Flow Cell for the Agilent 1290 Infinity LC System. Based on the experimental results presented in this Technical Overview, we recommend using the Ultra-Low Dispersion (ULD) Kit for short runs with 50-mm columns, especially for columns with 2.1-mm and 3-mm id. For longer runs with 2.1-mm and 3-mm id columns, the improvements using the ULD kit were negligible. However, for 100-mm and 150-mm \times 4.6-mm columns, the increased pressure, for example, due to smaller capillary ids, exceeds the limit of 600 bar for sub-2 μ m 4.6-mm id columns when used with gradient runs. Therefore, the ULD kit is not compatible with these columns or for long gradient or low-organic isocratic runs.



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Extraction of Synthetic Cannabinoids (SPICE) and Metabolites from Urine, Plasma and Whole Blood using ISOLUTE SLE+ Prior to LC-MS/MS Analysis

Company: Biotage

This application note describes the extraction and quantitation of Cannabimimetic Naphthoylindoles (Synthetic Cannabinoids) and their metabolites (JWH Series) from various matrices using Supported Liquid Extraction (SLE). Synthetic Cannabinoids or SPICE as they are commonly known have become an increasing problem as one of the newest forms of illicit drugs being consumed today. These compounds bind to the cannabinoid receptors in mammals triggering similar euphoric symptoms as Tetrahydrocannabinoids (THC). Currently robust and fast analytical methods of analysis are required to aid in the screening and detection of this growing class of compounds. The recoveries obtained for the synthetic cannabinoids parent and metabolites ranged from 70-98 %.



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Reversed-Phase Analysis of a Monoclonal Antibody on an Accucore C4-150 HPLC Column

Company: Thermo Fisher Scientific

Accucore HPLC columns use Core Enhanced Technology to facilitate fast and highly efficient separations. The 2.6 μm diameter particles are not totally porous but have a solid core and a porous outer layer. The optimized phase bonding creates a series of high-coverage, robust phases. The tightly controlled 2.6 μm diameter of the Accucore particles results in much lower backpressures than typically seen with sub-2 μm materials. For the analysis of large biomolecules, the Accucore pore size has been further optimized, and a C4 phase with reduced hydrophobic retention has been prepared. This 150 Å pore size enables effective analysis of molecules that are unable to penetrate small pore sizes, and the lower hydrophobicity allows elution of hydrophobic proteins that are too strongly retained on C18.

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In the next *HILIC Solutions*: The Basics of HILIC

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