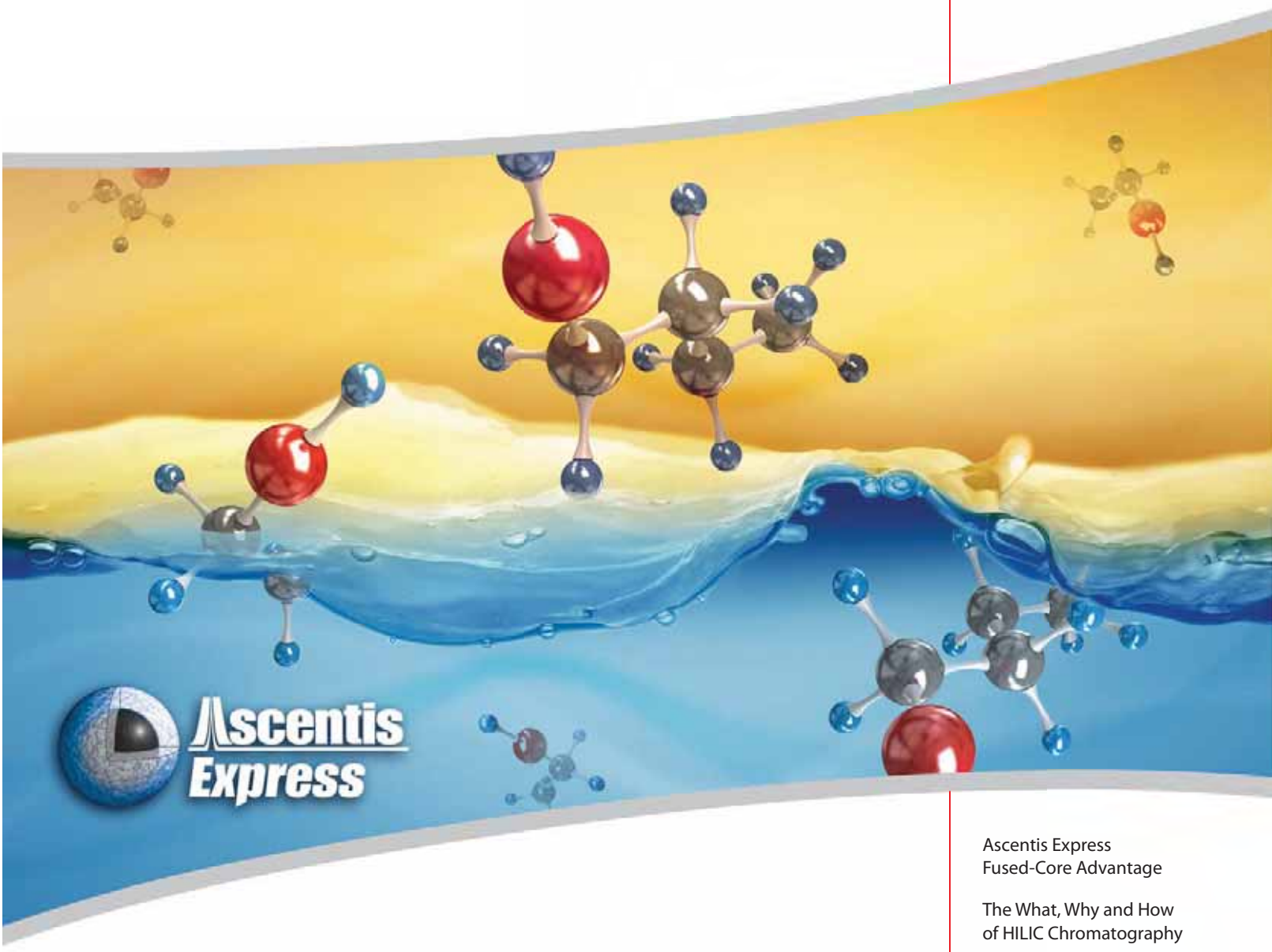
Ascentis® Express HILIC Guide

Faster Analysis of Polar Compounds

SUPELCO® Analytical



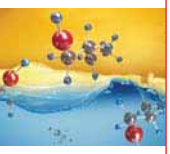
Ascentis Express
Fused-Core Advantage

The What, Why and How
of HILIC Chromatography

Choosing the Right
HILIC Phase

Developing HILIC Methods

Ascentis Express
HILIC Phases



Ascentis Express HPLC Columns for HILIC

Fused-Core Technology Columns Allow for Faster HPLC

Ascentis Express Fused-Core® columns are high-speed, high-performance liquid chromatography columns based on a new particle design. The Fused-Core columns particle provides a thin porous shell of high-purity silica surrounding a solid silica core. This particle design exhibits very high column efficiency due to the shallow diffusion paths in the 0.5 micron thick porous shell and the small overall particle size of 2.7 microns. The stationary phases of Ascentis Express HILIC (Hydrophilic Interaction Liquid Chromatography) columns can be used for separation of basic, acidic, or neutral polar compounds.

HILIC Phases

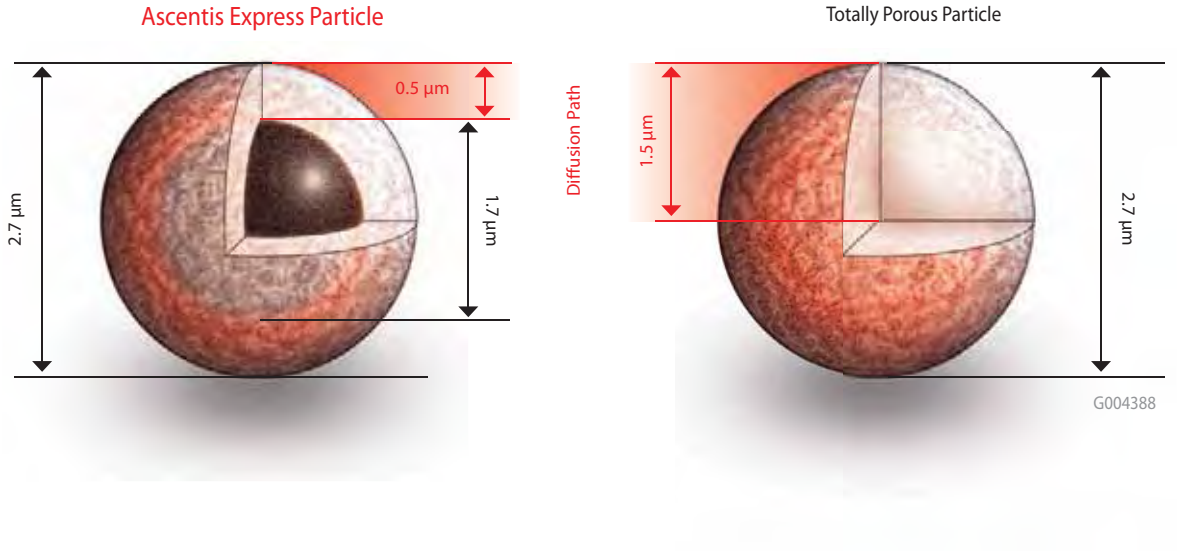
In this report, we present applications of four HILIC phases and their relative advantages as HILIC phases on our Ascentis Express Fused-Core particles.

- **OH5**: Pentahydroxy phase
- **F5**: Pentafluorophenylpropyl stationary phase
- **ES-Cyano**: Cyano linked by a propyl chain
- **HILIC (Si)**: Bare silica

Highlights

- HILIC chromatography offers orthogonal selectivity to reversed-phase chromatography
- Superior resolution and sensitivity with Fused-Core 2.7 µm particle size
- Double the efficiency of 3 µm columns
- Same efficiency of sub-2 µm columns at half the backpressure
- Rugged design capable of ultra high pressure operation

Comparison of Architecture of Fused-Core Particles vs. Porous 2.7 µm Particles

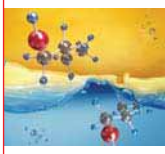


Testimonial

"The key advantages of the Fused-Core particle columns for pharmaceutically relevant analyses is their substantially lower back pressures which allows them to be used at much higher flow rates than porous sub-2 µm particle phases for fast LC applications, or the column length to be increased to improve separation efficiency without exceeding the capabilities of conventional HPLC equipment."

Abrahim et. al./Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 131-137

What is HILIC and Why You Should Select It

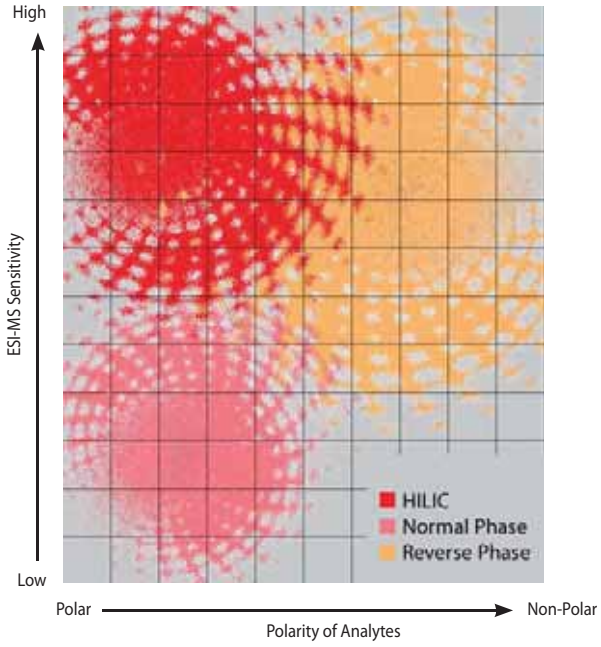


HILIC Introduction

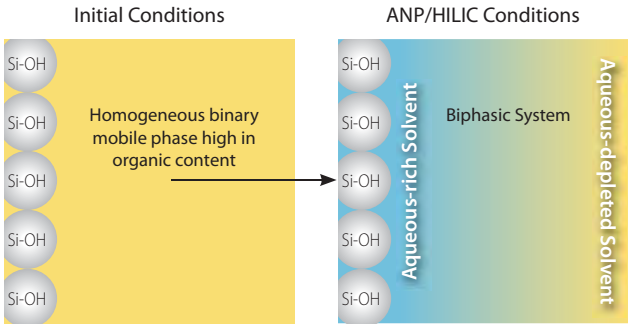
The separation of polar analytes continues to be an exceptional challenge to scientists. Reversed phase (RP) chromatography, though most commonly adopted, is not well-suited for analytes that are hydrophilic, due to poor retention. HILIC is a technique that has been adopted for analysis of hydrophilic analytes by researchers in recent years, owing to its complimentary nature to reversed phase (RP) and normal phase (NP) chromatography. HILIC often provides retention and selectivity that RP and NP techniques lack (1).

The HILIC technique can be successfully used to improve separation and resolution of very polar analytes by improving their retention (2). HILIC can also provide a mode of separation for mixtures of polar and ionizable compounds. In addition, HILIC may also provide increased LC-MS response (3). These benefits have made HILIC a potential solution for separation of polar analytes and an alternate technique to RP for challenging separations.

Complimentary Nature of HILIC to RP Liquid Chromatography Allows Better Separation of Polar Compounds and Enhanced ESI-MS Response



HILIC Partitioning



HILIC Retention Mechanism

Understanding HILIC retention mechanisms is critical before selecting the right column and phase for a HILIC application. HILIC retention mechanisms consist of a complex combination of liquid-liquid partitioning, ion exchange retention and dipole interaction.

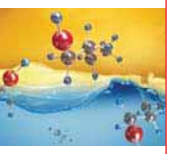
The retention mechanisms in HILIC is complex, consisting of:

- Partitioning between a layer of water held on the surface and the bulk organic enriched mobile phase
- Specific adsorption of polar functional groups on HILIC phase
- Ionic retention on ionized groups or on ionized silanols of the base silica
- Reversed-phase retention on the hydrophobic portions of bonded ligands (4).

These complex mechanisms lead to different retention patterns on different HILIC stationary phases. A significant discrimination between HILIC columns is whether they rely mainly on adsorption and hydrogen bonding, or hydrophilic partitioning and multipoint interactions. All plain silica columns exhibit adsorption selectivity, whereas zwitterionic columns generally exhibit a selectivity pattern that could be attributed to partitioning (5).

References

1. Hydrophilic Interaction Liquid Chromatography (HILIC) and Advanced Applications, Wang Perry G., He Weixuan, CRC Press, Taylor & Francis Group.
2. Alpert, A. J. , *J. Chromatogr., A.* **1990**, 499, 177-196.
3. Needham, S.R., Bell, D., *J. Chromatogr., A.* **2000**, 869, 159-170.
4. McCalley, D. V., *J. Chromatogr., A.* **2010**, 1217, 3408-3417.
5. Dinh, N. P., Jonsson T., Irgum K., *J. Chromatogr., A.* **2010**, 1217, 3408-3417.



HILIC Method Development

Knowing Your Analytes for HILIC Method Development

An important guideline to starting an HPLC method development on an unknown analyte or mixture is to gather information about its chemical and physical properties. Knowing key analyte characteristics Partition Coefficient (Log P), Distribution Coefficient (Log D), and pKa, can lead to successful HPLC method development. An easy estimation of these coefficients is offered by modern chemistry software.

Is HILIC Right for My Analyte?

For a preliminary analysis, a good practical judgment of analyte polarity or hydrophilicity can be made from an RP-Amide, C8 or C18 run in RP mode (1). Analytes eluting early are generally more polar than ones eluting later. Retention times on a C8 or C18 run can therefore serve as a good estimation of analyte log P and log D values.

$$log\ P_{oct/wat} = log\left(\frac{[Analyte]_{octanol}}{[Analyte]_{un\text{-}ionized\ water}}\right)$$

Log P is the ratio of concentrations of an un-ionized compound in the two phases of a mixture of immiscible solvents at equilibrium.

The resulting log P value indicates the extent to which the measured compound is hydrophobic or hydrophilic. A log P > 0 indicates a more hydrophobic analyte, while a log P < 0 signifies a more hydrophilic analyte.

The distribution coefficient is the ratio of the sum of the concentrations of all forms of the compound (ionized plus un-ionized) in each of the two phases.

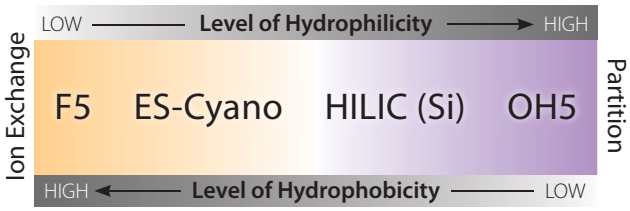
$$log\ D_{oct/wat} = log\left(\frac{[Analyte]_{octanol}}{[Analyte]_{ionized\ water} + [Analyte]_{neutral\ water}}\right)$$

Using log P and log D as a Guide to Phase Selection

Log P is a measure of compound polarity, and log D is an indicator of compound ionization state in solution at a particular pH. A combination of these characteristics can be used to choose the right HILIC phase for separation of a mixture of polar compounds. Modern chemistry software allows users to calculate theoretical log D and log P values for a given structure of compound.

The use of these physiochemical properties, log P and log D can offer reduction in the method development time without compromising quality.

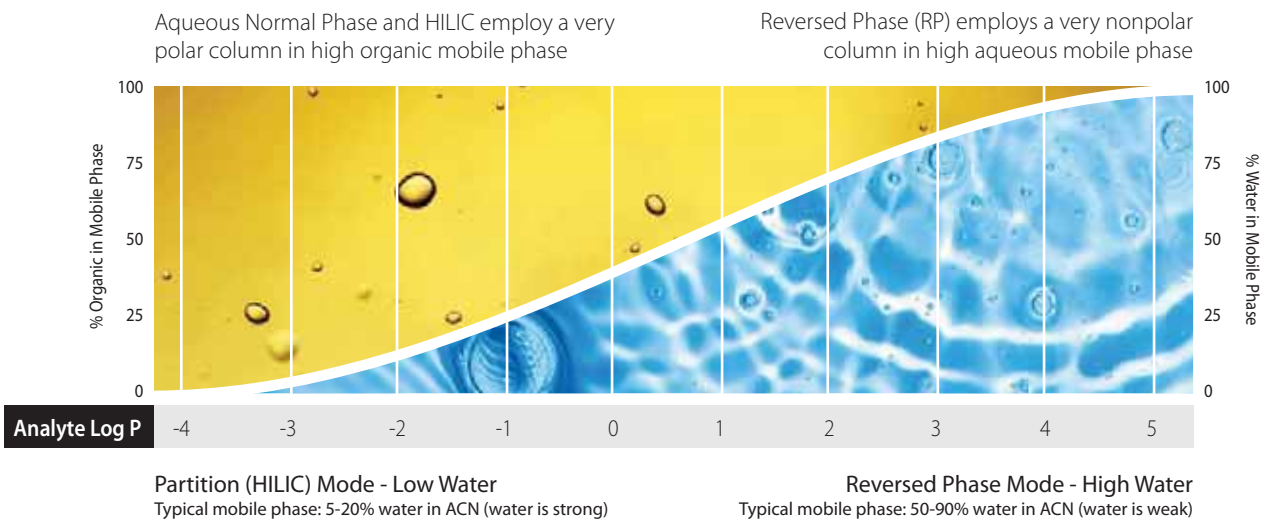
Relative Hydrophilicity of various HILIC Phases



Reference

1. Grushka, E., Behhaim, D., *J. Chromatogr., A.* **2010**, 1217, 65-74.

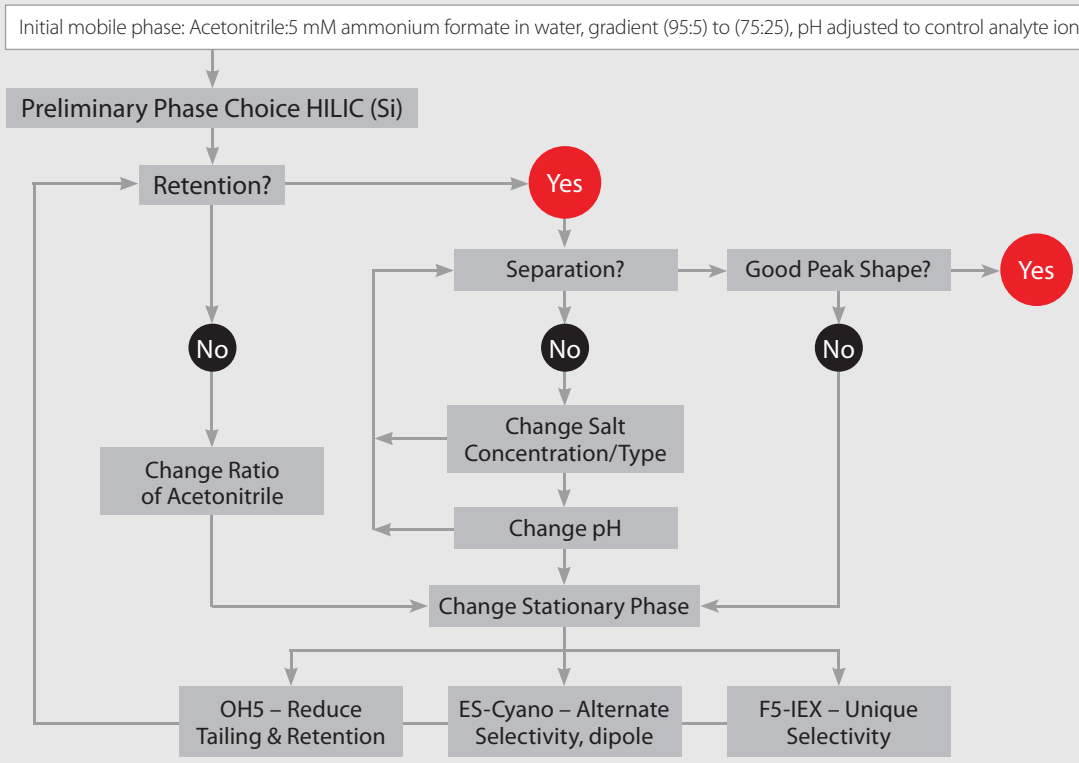
Using Log P Values as a Guide to Mode Selection



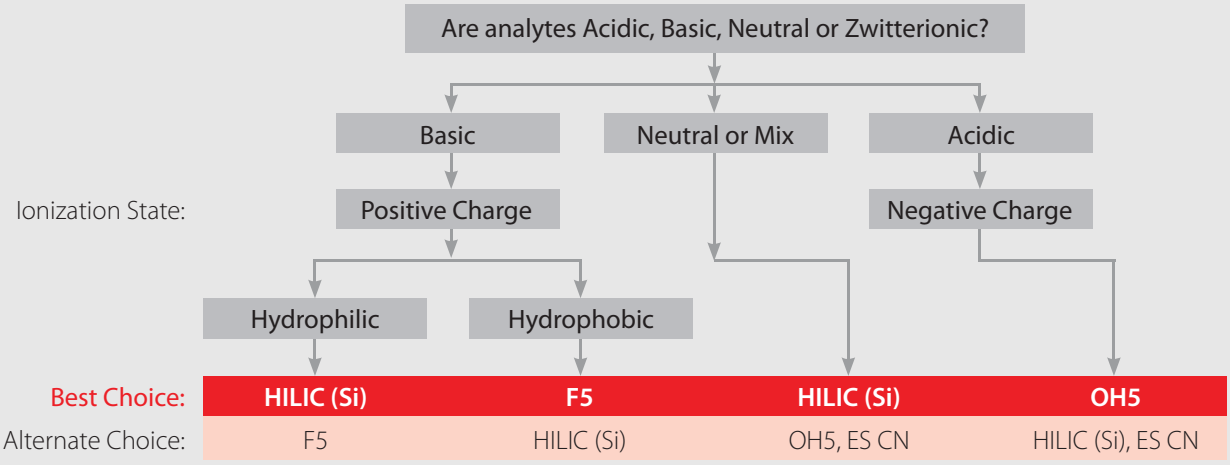
Partition (HILIC) Mode - Low Water
Typical mobile phase: 5-20% water in ACN (water is strong)

Reversed Phase Mode - High Water
Typical mobile phase: 50-90% water in ACN (water is weak)

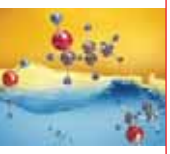
HILIC Method Development and Optimization Chart



HILIC Phase Selection Guide



For fast HPLC application support, go to *sigma-aldrich.com/hplc-support*



Ascentis Express OH5 HPLC Columns

Ascentis Express OH5 phase is a high speed, high-performance liquid chromatography column based on a new stationary phase. The HILIC OH5 phase is a highly polar ligand that possesses 5 hydroxyl groups tethered to the silica via novel proprietary linkage phase chemistry. The phase exhibits enhanced retention and performance by the unique phase chemistry coupled with Fused-Core technology. The phase is designed to provide enhanced HILIC partitioning and limited ion exchange retention.

Highlights

- Exhibits HILIC IEX retention, limited silanol anionic character, and is relatively insensitive to ionic strength
- High column stability
- Column efficiency is as good, and sometimes better than, sub-2 µm totally porous materials

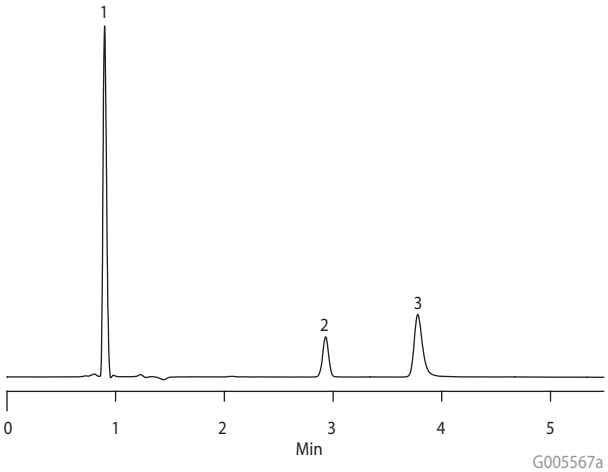
The impact of partitioning and ion exchange in HILIC separations, is demonstrated by a mixture of acidic, neutral, and basic polar compounds (**Figure 1**).

All three analytes have similar log D values, both acid and base are ionized at mobile phase pH.

Figure 1. Mixed Polar Compounds on Ascentis Express OH5

column: Ascentis Express OH5, 10 cm x 3.0 mm I.D., 2.7 µm particles (53769-U)
mobile phase: 5 mM ammonium formate (95:5 acetonitrile:water) pH 6.8
flow rate: 0.6 mL/min
pressure: 965 psi
temp.: 30 °C
det.: 254 nm
injection: 1 µL
sample: 100 µg/mL in 25:75, water:methanol

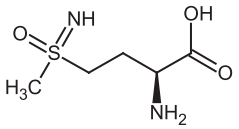
1. Caffeine (neutral, pKa = 0.52, log D = -0.63)
2. Fenoprop (acid, pKa = 2.93, log D = -0.05)
3. Codeine (base pKa = 8.29, log D = -0.02)



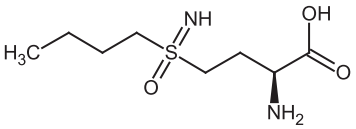
L-methionine sulfoximine (MSX) and Related Compounds using Ascentis Express HILIC and Ascentis Express OH5 Columns

L-methionine sulfoximine and L-buthionine sulfoximine (BSX) are used to prevent additional enzyme activity in Chinese hampster ovary (CHO) cell lines with supplemental Glutamine Synthase (GS). It is of interest to be able to monitor both purity of such molecules in order to control addition to cell media as well as for the assay of parent molecules during use. The analytes are highly polar and thus should be amenable to HILIC-LC-MS analysis. This application investigates the capability of Ascentis Express HILIC and Ascentis Express OH5 stationary phases for the separation of the methionine analog and related compounds as well as methionine and buthionine separations.

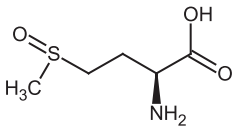
Figure 2. Structures of MSX and Related Compounds



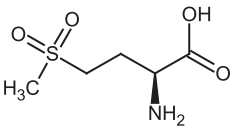
L-methionine sulfoximine (MSX)
Monoisotopic Mass = 180 Da



L-Buthionine (S,R)-sulfoximine (BSX)
Monoisotopic Mass = 222 Da



L-methionine sulfoxide (Sulfoxide)
Monoisotopic Mass = 165 Da



L-methionine sulfone (Sulfone)
Monoisotopic Mass = 181 Da

Figures 3 and 4 show extracted ion currents for the three methionine sulfoximine related compounds on Ascentis Exress HILIC (Si) and Ascentis Express OH5, respectively. The HILIC (Si) phase provides selectivity for all three compounds, whereas the OH5 does not discriminate between the sulfone and the MSX analytes. The OH5, however, does provide improved peak shape for all of the analytes as compared to HILIC (Si). Separation of the sulfone (most likely impurity) and MSX on the OH5 would be favored.

Figure 3. Extracted Ion Chromatogram of MSX, Sulfone and Sulfoxide on Ascentis Express HILIC

column: Ascentis Express HILIC (Si), 10 cm x 3.0 mm I.D., 2.7 µm particles (56370-U)
mobile phase: 0.1% formic acid, pH to 3.5 w/ammonium hydroxide: acetonitrile (LC-MS grade), 25:75, v/v
flow rate: 0.4 mL/min
pressure: 1350 psi

temp: ambient
det: ESI (+), scan m/z 150-300
injection: 5 µL
sample: 10 µg/mL in 90% methanol (LC-MS grade)

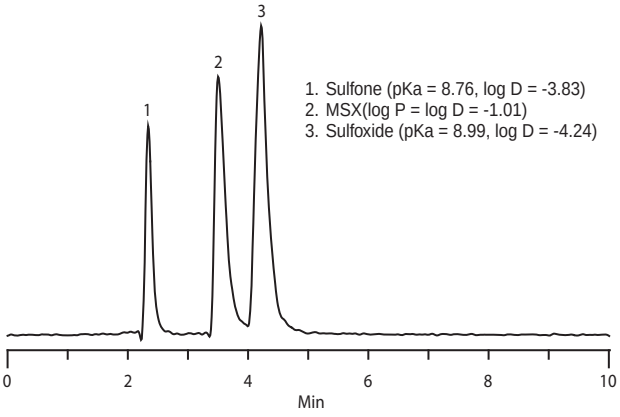
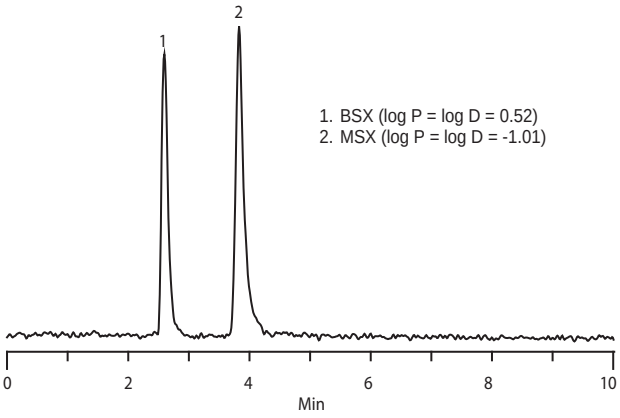


Figure 4. Extracted Ion Chromatogram of MSX, Sulfone and Sulfoxide on Ascentis Express OH5

column: Ascentis Express OH5, 10 cm x 3.0 mm I.D., 2.7 µm particles (53769-U)
mobile phase: 0.1% formic acid, pH to 3.5 w/ammonium formate: acetonitrile (LC-MS grade), 25:75, v/v flowrate: 0.4 mL/min
pressure: 1350 psi
temp: ambient
det: ESI (+), scan m/z 150-300
injection: 5 µL
sample: 10 µg/mL in 90% methanol (LC-MS grade)



Figures 5 and 6 show the separation of methionine sulfoximine and buthionine sulfoximine on the Ascentis Express HILIC and OH5 phases, respectively. Although both provide adequate separation and peak shape, the OH5 exhibits improved selectivity as well as peak efficiency for the pair of analytes.

Figure 5. Extracted Ion Chromatogram of Methionine Sulfoximine (MSX) and Buthionine Sulfoximine (BSX) on Ascentis Express HILIC

same conditions as Figure 3

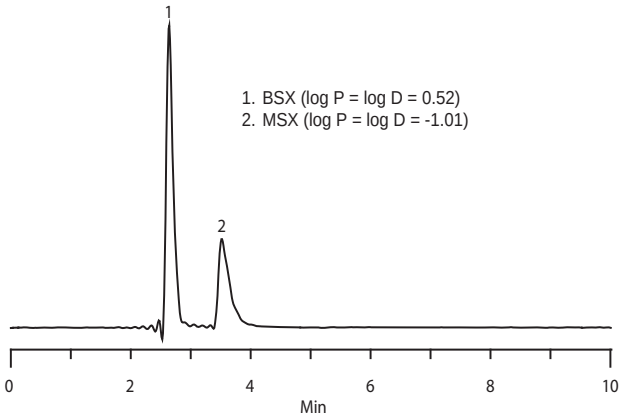
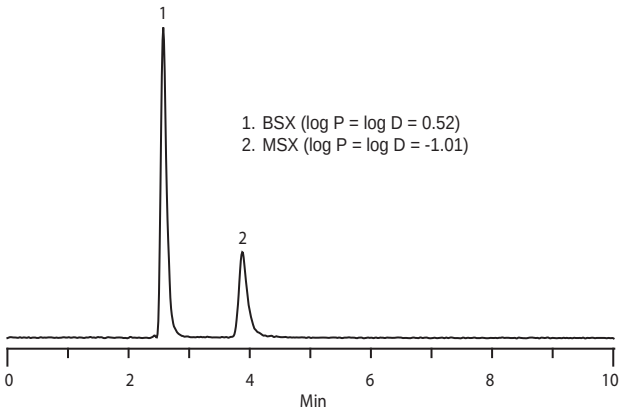
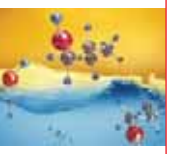


Figure 6. Extracted Ion Chromatogram of Methionine Sulfoximine and Buthionine Sulfoximine on Ascentis Express OH5

same conditions as Figure 4



The objective was to obtain good chromatographic separation of MSX and related compounds. Both the Ascentis Express HILIC (Si) and OH5 are shown to be good candidates for MSX analysis.



Ascentis Express F5 HPLC Columns

The pentafluorophenylpropyl stationary phase of Ascentis Express F5 provides a stable, reversed-phase packing with electron-deficient phenyl rings due to the presence of electronegative fluorines. In addition to forming π-π and mildly steric interactions, F5 phases also retain compounds by polar interactions. As a result of having both polar and non-polar character, F5 phases can show dual-mode retention behavior, sometimes producing a "U-shaped" retention as a function of acetonitrile content of the mobile phase, with retention increasing at both low and high concentrations of acetonitrile (reversed-phase and HILIC retention modes).

Fluorinated phases have been shown to exhibit greater ion-exchange character and thus often provide excellent chromatographic results when analytes to be separated differ in their ionization constants or where some ion exchange is necessary for the retention of polar metabolites or degradation products. A second important attribute of the fluorinated phases lies in their apparent increased shape selectivity relative to common stationary phase chemistries. Ascentis Express F5 can be used for basic, acidic, or neutral compounds.

Highlights

- Alternate selectivity where ion-exchange is a desired HILIC retention mechanism
- Retains bases more and hydrophobes less than C18
- Stable, low bleed for LC-MS

The multi-modal retention mechanisms in HILIC, which offers orthogonal selectivity to reversed-phase is evident in the following separation of selegiline and amphetamines on Ascentis Express F5 (**Figure 8**). Selegiline under HILIC condition elutes last where as the same under reversed phase condition elutes first. Selegiline, therefore is retaining primarily based in RP partitioning, whereas the amphetamines are retaining primarily by IEX.

Figure 7. Related Structures

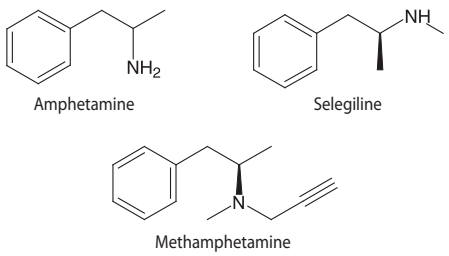
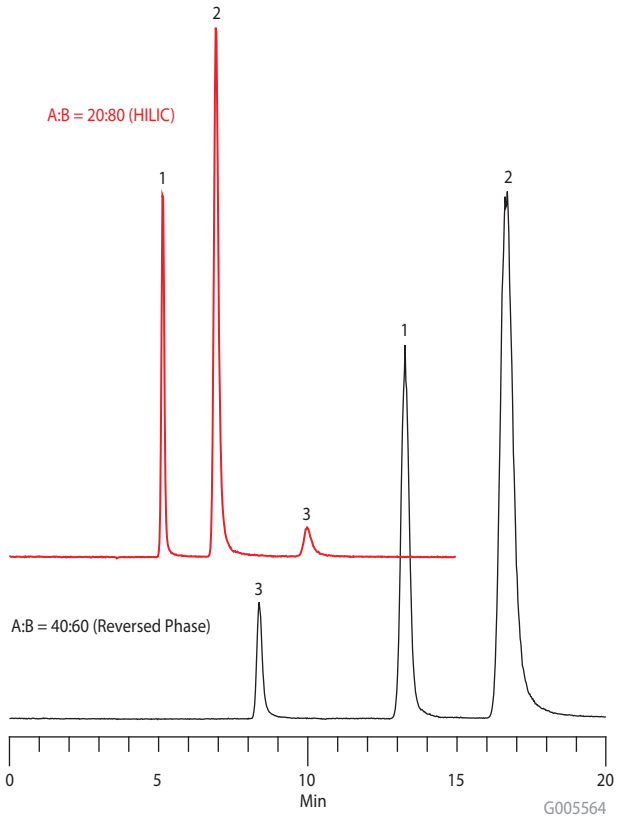


Figure 8. Separation of Selegiline and Amphetamines on Ascentis Express F5 in Reversed Phase and HILIC modes

column: Ascentis Express F5, 10 cm x 4.6 mm I.D., 2.7 µm particles (53590-U)
mobile phase: (A) 10 mM ammonium acetate, pH 4.0 with acetic acid; (B) acetonitrile; (20:80, A:B - HILIC), (40:60, A:B - reversed phase)
flow rate: 0.6 mL/min
pressure: 260 bar (2300 psi)
temp.: 35 °C
det.: MS ESI (+), SIR m/z 136, 150, 188
injection: 2 µL
sample: 10 µg/mL in methanol

1. Amphetamine
2. Methamphetamine
3. Selegiline



Note: The differences in retention time are due to the change in organic solvent and the buffer concentration.

Ascentis Express ES-Cyano HPLC Columns

Ascentis Express ES-Cyano brings together the highly efficient, robust 2.7 µm Fused-Core particle technology with a cyano phase for the successful separation of polar and non-polar organic compounds. Ascentis Express ES-Cyano is moderately polar in nature and highly suited for the separation of acids, bases, and neutrals. Ascentis Express ES-Cyano columns utilize a steric-protected cyano bonded-phase with extremely high resistance to acid-catalyzed hydrolysis of the siloxane bond that attaches the cyanopropyl chain to the surface. Thus, the combination of low pH and elevated temperature operation of the column is well tolerated. Ascentis Express ES-Cyano offers the following key advantages in the HILIC mode.

Highlights

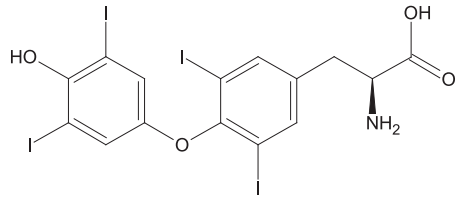
- Offers ion-exchange mechanism in a HILIC mode
- Stable at extremely low pH and high temperature
- Ideal for non-polar bases in HILIC mode

The ES-Cyano stationary phase of Ascentis Express provides enhanced HILIC separation by ion exchange mechanism. This phase, as a result of having both polar and non-polar character, can show dual mode retention. As a result these phases can be used for non-polar basic compounds in HILIC mode and will provide retention by ion exchange mechanism.

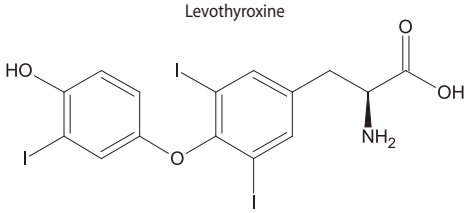
The ion exchange in HILIC separation is demonstrated by a mixture of acidic, neutral, and basic polar compounds on the ES-Cyano phase (**Figure 10**).

All components (**Figure 9**) have similar log D (distribution coefficient) values, both acid and base are ionized at mobile phase pH (**Figure 11**).

Figure 9. Structures of Levothyroxine and Liothyronine



Levothyroxine



Liothyronine

Figure 10. Mixed Polar Compounds on Ascentis Express ES-Cyano

column: Ascentis Express ES-Cyano, 10 cm x 3.0 mm I.D., 2.7 µm particles (53481-U)
mobile phase: 5 mM ammonium formate (95:5 acetonitrile:water) pH 6.8
flow rate: 0.6 mL/min
pressure: 1060 psi
temp.: 30 °C
det.: 254 nm
injection: 1 µL
sample: 100 µg/mL in 25:75, water:methanol

1. Caffeine (neutral, pKa = 0.52, log D = -0.63)
2. Fenoprop (acid, pKa = 2.93, log D = -0.05)
3. Codeine (base pKa = 8.29, log D = -0.02)

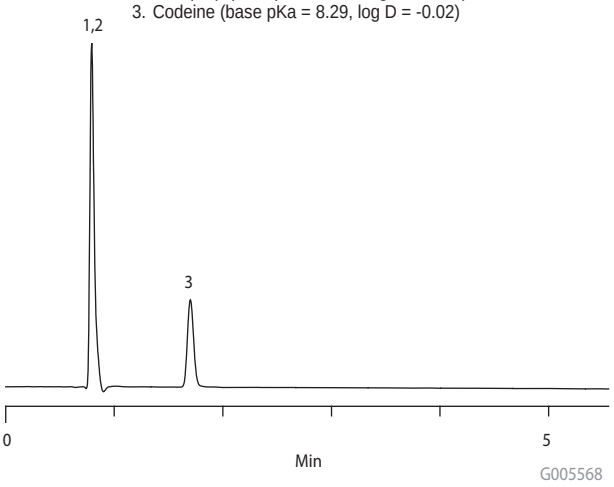
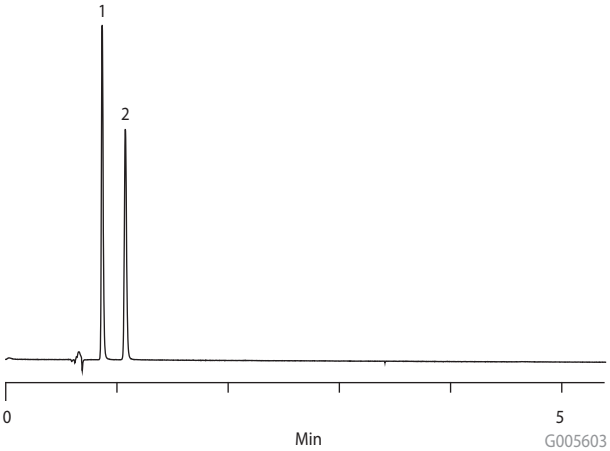
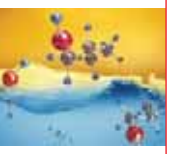


Figure 11. Levothyroxine and Liothyronine on Ascentis Express ES-Cyano

column: Ascentis Express ES-Cyano, 10 cm x 2.1 mm I.D., 2.7 µm particles (53491-U)
mobile phase: (A) water with 0.05% phosphoric acid; (B) acetonitrile with 0.05% phosphoric acid; (60:40, A:B)
flow rate: 1.5 mL/min
pressure: 3270 psi
temp.: 30 °C
detector: UV, 225 nm
injection: 5 µL
sample: 20 µg/mL in mobile phase

1. Liothyronine
2. Levothyroxine





Ascentis Express HILIC (Si) HPLC Columns

Ascentis Express HILIC (Si) offers mainly high surface area and high surface deactivation, which combine to give Ascentis Express Silica an exceptional performance as a HILIC phase. Besides being the underlying support for all Ascentis Express phases, Ascentis Express HILIC (Si) has applications in its own right. Silica is widely used to separate positional isomers and polar compounds in normal phase mode. Silica is also used in organic synthesis to purify reaction mixtures. In each case, a high purity, controlled and uniform surface is necessary to impart the desirable chromatographic performance.

Polar biomolecules, like amino acids, nucleotides and nucleosides, typically require derivatization for their analysis by reversed phase HPLC. The HILIC mode offered by Ascentis Express HILIC (Si) permits the retention and resolution of these compounds without derivatization, eliminating a time-consuming sample preparation step.

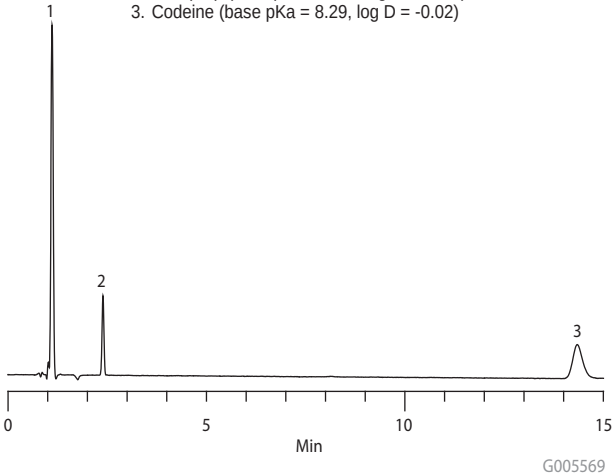
Highlights

- High-loading capacity
- Offers both ion-exchange and partition mechanisms of separation in a HILIC mode
- Ultra-pure silica
- Ideal for polar compounds

Figure 12. Mixed Polar Compounds on Ascentis Express HILIC (Si)

column: Ascentis Express HILIC (Si), 10 cm x 3.0 mm I.D., 2.7 µm particles (53769-U)
mobile phase: 5 mM ammonium formate (95:5 acetonitrile:water) pH 6.8
flow rate: 0.6 mL/min
pressure: 855 psi
temp.: 30 °C
det.: 254 nm
injection: 1 µL
sample: 100 µg/mL in 25:75, water:methanol

1. Caffeine (neutral, pKa = 0.52, log D = -0.63)
2. Fenoprop (acid, pKa = 2.93, log D = -0.05)
3. Codeine (base pKa = 8.29, log D = -0.02)

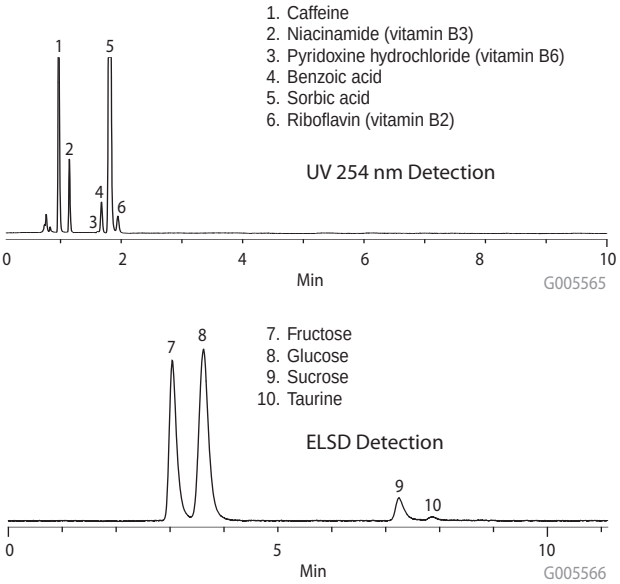


Caffeinated energy drinks contain a variety of ingredients that usually includes a sweetener (sugars, synthetic sugar substitutes, zero-calorie natural sweeteners), vitamin B supplements, and, of course, caffeine. They may also include amino acids, organic acids, and various plant extracts. The sample complexity makes it important to use highly-efficient, highly-selective phases and columns that are compatible with different detection systems to maximize the information from HPLC experiments. Ascentis Express Fused-Core columns meet these requirements.

The results on the Ascentis Express HILIC column are found in (**Figure 13**). Here, UV and ELSD detection was used to detect different types of compounds; ELSD allowed us to see the non-UV absorbing sugars. The HILIC conditions yielded extremely rapid analysis (under 2 minutes) and MS-friendly mobile phase. The low back-pressure of HILIC mobile phases also permits high flow rates for fast analysis.

Figure 13. Analysis of the Energy Drink Rock Star Using Ascentis HILIC (Si) with UV and ELSD Detection in Series

column: Ascentis Express HILIC (Si), 10 cm x 3.0 mm I.D., 2.7 µm particles (53970-U)
mobile phase: (A) 100 mM ammonium acetate, pH 5.0 with acetic acid; (B) water; (C) acetonitrile; (9:1:90, A:B:C)
mixing proportions: A:B:C = 9:1:90
flow rate: 0.6 mL/min
pressure: 815 psi
temp.: 35 °C
det.: UV at 254 nm; ELSD, 55 °C, 3.5 bar nitrogen
injection: 2 µL
sample: dilute 1:9 in acetonitrile



Selecting Your Ascentis Express HPLC Column

Which column ID is best for my needs?

If you are doing Mass Spec ..... **2.1 mm I.D.**
If you want solvent savings ..... **3.0 mm I.D.**
If you are doing standard HPLC ..... **4.6 mm I.D.**

Which column length is best for my needs?

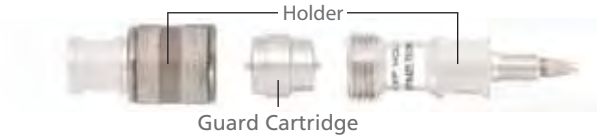
If you want to maximize the speed of your application ..... **2 to 7.5 cm**
If you want a balance of resolution and speed ..... **10 cm**
If you want the best resolution possible ..... **15 cm**

Ordering Information

ID (mm)	Length (cm)	OH5	F5	HILIC (Si)	ES-Cyano
Ascentis Express Columns					
2.1	2	53779-U	53592-U	—	53494-U
2.1	3	53748-U	53566-U	53933-U	53468-U
2.1	5	53749-U	53567-U	53934-U	53470-U
2.1	7.5	53755-U	53568-U	53938-U	53472-U
2.1	10	53757-U	53569-U	53939-U	53473-U
2.1	15	53764-U	53571-U	53946-U	53475-U
3.0	3	53766-U	53574-U	53964-U	53476-U
3.0	5	53767-U	53576-U	53967-U	53478-U
3.0	7.5	53768-U	53577-U	53969-U	53479-U
3.0	10	53769-U	53578-U	53970-U	53481-U
3.0	15	53771-U	53579-U	53972-U	53483-U
4.6	3	53772-U	53581-U	53974-U	53484-U
4.6	5	53774-U	53583-U	53975-U	53486-U
4.6	7.5	53775-U	53584-U	53977-U	53489-U
4.6	10	53776-U	53590-U	53979-U	53491-U
4.6	15	53778-U	53591-U	53981-U	53492-U
Ascentis Express Guard Cartridges, pk. of 3					
2.1	—	53780-U	53594-U	53520-U	53495-U
3.0	—	53781-U	53597-U	53521-U	53496-U
4.6	—	53782-U	53599-U	53523-U	53497-U

Ascentis Express Guard Columns

Description	Cat. No.
Universal Guard Holder	
Holder w/EXP Titanium Hybrid Ferrule (cartridge not included)	53500-U



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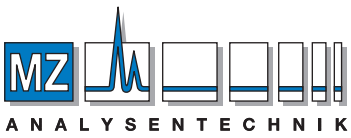

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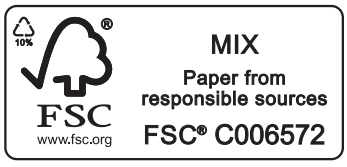

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