Stability and Performance of Cyano Bonded Phase HPLC Columns for Reversed-Phase, Normal-Phase and HILIC Applications

Fundamentals of Ascentis® ES Cyano
Column Selectivity Dominates Resolution Equation*

\[ R_s = \frac{\sqrt{N}}{4} \cdot \frac{k}{k+1} \cdot \frac{\alpha - 1}{\alpha} \]

\[ N = 16 \left( \frac{t_R}{w} \right)^2 \] or \[ N = 5.5 \left( \frac{t_R}{w} \right)^2 \]

\[ k = \frac{(t_R - t_0)}{t_0} \]

\[ \alpha = \frac{k_2}{k_1} \]

Selectivity term is powerful being nearly linear at low \( \alpha \) values

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Important HPLC Stationary Phases on Silica

Choice of column is a critically important variable in determining system selectivity, but chemical interaction at solid-liquid interfaces inside pores is complex and still not well understood.

**Reversed Phase**
- C18
- C8
- Embedded Polar Group (amide)
- Phenyl
- Cyano
- Fluorinated phenyl (PFP or F5)

**Normal Phase**
- Bare silica (no phase)
- Cyano
- F5 (PFP)
- Amino
- Diol
Selectivity Tools in Reversed-Phase HPLC*

Continuous variables (mobile phase):
- Solvent type (methanol, acetonitrile, etc.)
- pH (ionizable solutes most influenced)
- Additives (type and concentration)
- Solvent strength (% ACN or MeOH)
- Temperature (only tool available in GC)

Discontinuous variable (stationary phase):
- Column type (stationary phase and particle substrate)

* Used in part with permission by John Dolan

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## Classification by Chemical Interaction Types

<table>
<thead>
<tr>
<th>Bonded Phase</th>
<th>Dispersive (Hydrophobic)</th>
<th>H-Bonding</th>
<th>Dipolar</th>
<th>π-π</th>
<th>Shape</th>
<th>Ionic</th>
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<td>No</td>
<td>No</td>
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<td>Weak</td>
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<td>Strong</td>
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<tr>
<td>F5/PFP</td>
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<td>Moderate Acceptor</td>
<td>Strong</td>
<td>Strong Acceptor</td>
<td>Strong (planar)</td>
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</table>

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<table>
<thead>
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<th>Polarity</th>
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<th>Shape</th>
<th>Ionic</th>
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<tr>
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<td>Strong Acceptor</td>
<td>Strong (planar)</td>
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*a. Using Euerby variation of Snyder-Dolan-Carr Hydrophobic Subtraction Model*. 

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Alkyl Bonded Phase (C18 and C8)

- C18 reagents are bulky and can leave some silanols unreacted (ca. 50%).
- C8 reagents are smaller and provide better silanol coverage.
- At pH >4, silanols can ionize and add cation-exchange character.
Amide (EPG) Bonded Phase

- Possible Solute Shielding (basic solutes)- alkyl EPG phases with embedded polar groups have been reported to correlate much better with logP data than C18 columns due their higher base deactivation\(^1\).

- Possible H-bonding with solutes that are good H-bond donors (acids, etc.)
Phenyl Bonded Phase

- Phenyl is a Lewis base or electron donor; $\pi-\pi$ interaction can occur with solutes that are deficient in electrons (Lewis acids).

- Due to the rigid nature of the aromatic ring, solute shape can dictate selectivity (how closely solutes can approach the ring).
Pentafluoro-Phenyl (F5) Bonded Phase

- PFP is a Lewis acid or electron acceptor; π-π interaction can occur with solutes that are rich in electrons (Lewis bases).
- Due to the rigid aromatic ring, solute shape can also dictate selectivity.
- Dipolar and H-bonding interactions can also occur.

Free silanol

"Endcapping" reagent

Primary phase reagent

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Cyano Bonded Phase

- Cyano is a strong dipole that can interact with other dipoles on solutes.
- Cyano phases also have moderate hydrophobic character from alkyl ligands.
- Stability of newer Cyano phases is now comparable to all other HPLC phases.

Newest Porous Phase

Free silanol

Primary phase reagent

“Endcapping” reagent

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Ascentis ES-Cyano Features and Specifications

**Features:** Strong dipole attracts and separates polar molecules; works very well in both reversed-phase and normal-phase modes; extremely useful in HILIC mode; designed for high RP retention and long lifetime; very compatible with LC/MS.

**Specifications:**
- USP Code- L10 (Cyano)
- Phase- Diisopropylcyanopropyl main phase; silanols endcapped
- Phase load- 10% carbon by wt
- Particle substrate- Spherical silica, type B (<5ppm metals)
- Surface area- 450 m²/g
- Particle Pore size- 100Å
- Particle size- 3µm and 5µm
- pH range for longest life- 1-8
Toluene Van Deemter Plot
Efficiency vs Flow Velocity
Reduced Plate Height
Ascentis ES Cyano 100x4.6mm

Optimum Flow Rates:
- 5µm – 1.5mL/min
- 3µm – 2.25mL/min

Velocity too low
B-term effect

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Batch Test 1 – Ascentis ES Cyano, 5µm

50% Acetonitrile 50% Water
1.5mL/min, 35ºC, 254nm, 10µL
150x4.6mm 5µm BL: 7122

Elution Order:
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene

Comparison to Express Fused-Core®
Plates: 30-35,000 /15cm
Batch Test 1 – Ascentis ES Cyano, 3µm

50% Acetonitrile 50% Water
35°C, 254nm, 10µL
100x4.6mm 3µm, BL 7141

Elution Order:
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene

Comparison to Express Fused-Core®
Plates: 20-25,000 /10cm
Chromatography Mode Switching*

50% Acetonitrile 50% Water
1.5mL/min, 35°C, 254nm, 10µL
150x4.6mm 5µm BL: 7122

Procedure:
1. Reverse phase
2. Normal phase
3. Reverse phase
4. 100% aqueous
5. Normal phase
6. Reverse phase

Elution Order:
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene

N: 15,191
As: 1.01
k: 2.43

N: 14,610
As: 1.05
k: 2.51

*Column dedication is highly recommended
Mass Spectral Bleed TIC – Same Scales

Ascentis C18 Control

Ascentis ES Cyano

Cyano D

Cyano Z (popular competitor)

Cyano S (early cyano)
Over many column volumes of mobile phase at elevated temperature, Ascentis ES Cyano proved more stable than the leading CN phase.

Mobile Phase: 35% Acetonitrile 65% Water 0.1% TFA
Flow Rate: 0.8mL/min
Temperature: 50°C
Detection: UV-254nm
Injection: 2µL; one injection every 60 minutes
Sample: Uracil (5µg/mL), Nitrobenzene (130µg/mL), Butyl paraben (10µg/mL)
Dimension: 150x2.1mm, 5µm
Ascentis ES Cyano Stability- RP Mode 0.1% TFA

Elution Order:
1. Uracil
2. Nitrobenzene
3. Butylparaben

Elution order:
1. Uracil
2. Nitrobenzene
3. Butylparaben

30,000 Voids
- N: 9,500
- As: 1.07
- K': 3.52

15,000 Voids
- N: 9,500
- As: 1.08
- K': 3.54

0 Voids
- N: 9,600
- As: 1.09
- K': 3.54

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## Quantitative Column Classification Data

<table>
<thead>
<tr>
<th>Column Type</th>
<th>$k_{pb}$</th>
<th>$\alpha_{CH2}$</th>
<th>$\alpha_{T/O}$</th>
<th>$\alpha_{C/P}$</th>
<th>$\alpha_{BA/P}$ (7.6)</th>
<th>$\alpha_{BA/P}$ (2.7)</th>
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<tr>
<td>Ascentis C18</td>
<td>7.35</td>
<td>1.50</td>
<td>1.59</td>
<td>0.37</td>
<td>0.31</td>
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<td>Ascentis RP-Amide</td>
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<td>1.44</td>
<td>1.68</td>
<td>0.22</td>
<td>0.19</td>
<td>0.03</td>
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<tr>
<td>Ascentis C8</td>
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<td>0.93</td>
<td>0.31</td>
<td>0.25</td>
<td>0.07</td>
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<tr>
<td>Ascentis Phenyl</td>
<td>2.54</td>
<td>1.37</td>
<td>0.97</td>
<td>0.95</td>
<td>0.38</td>
<td>0.11</td>
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<tr>
<td>Ascentis ES-Cyano</td>
<td>0.91</td>
<td>1.21</td>
<td>2.57</td>
<td>0.64</td>
<td>0.88</td>
<td>0.18</td>
</tr>
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</table>

Interaction strength-
hydrophobic solute

2. Euerby (Tanaka)
Good Correlation between Same Phases on Different Silica: **Ascentis C18 vs Express C18**

Fitted Line Plot

$$C18\_1 = 0.1089 + 1.158 \times C18\_\text{Express} \_1$$

Porous vs Fused-Core® silica

- 13mM ammonium acetate, 30:70 H2O:ACN, pH 6.91, 50 solutes (acid, base, neutral)

If two columns having the same phase are very different (nonideal), either main phase, capping or substrate properties may be different.

R² = 0.99
Columns with Similar Phases Should Also Show High Correlation: Ascentis C18 vs C8

\[ \log k_{C8} = -0.06344 + 0.9851 \log k_{C18} \]

\[ R^2 = 0.97 \]

13mM ammonium acetate, 30:70 H2O:ACN, pH 6.91, 50 solutes (acid, base, neutral)
Columns with Different Phases Should Be Orthogonal (Different): **Ascentis C18 vs Amide**

**Fitted Line Plot**

\[
\log k_{\text{RPA}} = -0.08420 + 1.014 \log k_{\text{C18}}
\]

- \( R^2 = 0.70 \)

13mM ammonium acetate, 30:70 H2O:ACN, pH 6.91, 50 solutes (acid, base, neutral)
Ascentis® Cyano vs Discovery® Cyano*, RP Mode

13mM ammonium acetate (70:30 water:acetonitrile) pH 7.0

Fitted Line Plot
log k Ascentis Cyano = 0.4228 + 1.658 log k Discovery Cyano

R² = 0.91

Retention due to combined polar and nonpolar interactions

* Discovery silica is 200Å and lower surface area
Ascentis Cyano vs Discovery Cyano, HILIC Mode

2mM ammonium formate (5:95 water:acetonitrile) pH 7.2

Fitted Line Plot

\[ \log k_{\text{Ascentis Cyano}} = -0.1195 + 1.124 \log k_{\text{Discovery Cyano}} \]

\[ R^2 = 0.92 \]

Retention due to mainly to polar interactions
Ascentis Cyano vs Ascentis Si, HILIC Mode

2mM ammonium formate (5:95 water:acetonitrile) pH 7.2

Fitted Line Plot
\[ \log k \text{ Ascentis Cyano} = -0.1874 + 0.5236 \log k \text{ Ascentis Si} \]

\[ R^2 = 0.33 \]

Silica surface is very polar and more retentive in HILIC than any bonded phase.
Applications of Ascentis ES Cyano
Antimalarial Compounds

Mefloquine

Quinine

Chloroquine

Primaquine
Anti-Malarial Drugs - RP Mode

Ascentis ES Cyano

Elution Order:
1. Chloroquine
2. Quinine
3. Primaquine
4. Mefloquine

Competitor CN

Elution Order (Note elution order change):
1. Chloroquine
2. Primaquine
3. Quinine
4. Mefloquine
Anti-Malarial Drugs - HILIC Mode

Ascentis ES Cyano

Competitor CN

column: Ascentis Cyano or Competitor CN 5 cm x 4.6 mm I.D.
Both 5 µm particles
mobile phase A: Water/Acetonitrile (5:95 v/v); 2 mM ammonium formate
mobile phase B: Water/Acetonitrile (10:90 v/v); 10 mM ammonium formate

flow rate: 1.0 mL/min.
temp.: 35 °C
det.: MS in ESI(+), SIR mode
injection: 5 µL
sample: 1 µg/mL each in mobile phase A
gradient:

<table>
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<tr>
<th>min</th>
<th>% A</th>
<th>% B</th>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Elution Order (reversed vs RP):
1. mefloquine
2. quinine
3. primaquine
4. chloroquine

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Hydrophobic Bases on ES-Cyano

Quinidine

Reversed Phase Mode

Naphthalene
Neutral Hydrophobic

Diphenhydramine

Fluoxetine
2° Amine and Fluorines (Polar)
<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa</th>
<th>Log P</th>
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<tr>
<td>Diphenhydramine</td>
<td>8.76</td>
<td>3</td>
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<tr>
<td>Fluoxetine</td>
<td>10.05</td>
<td>3.93</td>
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<tr>
<td>Quinidine</td>
<td>12.8</td>
<td>2.82</td>
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<tr>
<td>Quinidine</td>
<td>9.28</td>
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<tr>
<td>Hydroquinidine</td>
<td>4.77</td>
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<td>Dihydroquinidine</td>
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<td>4.77</td>
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DFQ – pH Change (Ascentis C18)

Elution Order:
1. Uracil
2. Naphthalene
3. Quinidine
4. Diphenhydramine
5. Hydroquinidine
6. Fluoxetine

65% Methanol 35% 10mM Potassium Phosphate
1.0mL/min, 35ºC, 220nm, 10µL(DFQN)
150x4.6mm, 5µm

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DFQ – pH Change (Ascentis ES Cyano)

65% Methanol 35% 10mM Potassium Phosphate
1.0mL/min, 35ºC, 220nm, 10µL (DFQN)
150x4.6mm, 5µm

Elution Order:
1. Uracil
2. Naphthalene
3. Quinidine
4. Diphenhydramine
5. Hydroquinidine
6. Fluoxetine
### DFQ – Ionic Strength pH 5.5 (C18)

65% Methanol 35% Potassium Phosphate pH 5.5
1.0mL/min, 35°C, 220nm, 10µL(DFQN)

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>Time (min)</th>
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<tr>
<td>30mM</td>
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<tr>
<td>20mM</td>
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</tr>
<tr>
<td>10mM</td>
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</tr>
<tr>
<td>3.5mM</td>
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**Elution Order:**
1. Uracil
2. Naphthalene
3. Quinidine
4. Diphenhydramine
5. Hydroquinidine
6. Fluoxetine

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Ascentis C18 (pH 5.5)

- Naphthalene
- Quinidine
- Diphenhydramine
- Hydroquinidine
- Fluoxetine

Molarity (mM)

K'

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DFQ – Ionic Strength pH 5.5 (Cyano)

65% Methanol 35% Potassium Phosphate pH 5.5
1.0mL/min, 35°C, 220nm, 10µL(DFQN)

Elution Order:
1. Uracil
2. Naphthalene
3. Quinidine
4. Diphenhydramine
5. Hydroquinidine
6. Fluoxetine
Ascentis ES Cyano (pH 5.5)

- Naphthalene
- Quinidine
- Diphenhydramine
- Hydroquinidine
- Fluoxetine
Comparison of Tricyclic Antidepressants - RP Mode

85% RP Correlation

Ascentis ES Cyano

Competitor CN

column: as indicated; 15 cm x 4.6 mm, 5 µm particle
mobile phase A: 20 mM potassium phosphate, dibasic (pH 7.0 with phosphoric acid)
mobile phase B: acetonitrile
mobile phase C: methanol
mixing proportions: A:B:C, 25:60:15
flow rate: 2.0 mL/min
temp.: 25 °C
det.: 215 nm
injection: 5 µL
sample: 100 µg /mL in 70:30, water: methanol

1. Trimipramine
2. Doxepin
3. Amitriptyline
4. Imipramine
5. Nortriptyline
6. Nortriptyline
7. Desipramine
8. Protriptyline

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Hydrocortisone and Hydrocortisone Acetate- Normal Phase Mode

**Column**: Ascentis Cyano 15 cm x 4.6 mm, 5 µm (577306-U)

**Mobile phase**: 90:10, heptane:ethanol

**Flow rate**: 1.0 mL/min

**Temperature**: 25°C

**Detection**: UV-254 nm

**Injection**: 5 µL

**Sample**: 50 µg/mL in 92.5:2.5:5, (heptane:2-propanol:ethanol)

**Elution Order**: 1. Hydrocortisone acetate 2. Hydrocortisone

Ascentis ES Cyano
Isocyanate Derivatives - Reversed Phase Mode (30% ACN)

Column: as indicated; 15 cm x 4.6 mm, 5 µm particle
Mobile phase A: water with 0.1% ammonium acetate
Mobile phase B: acetonitrile with 0.1% ammonium acetate
Mixing proportions: A:B, 70:30
Flow rate: 1.0 mL/min
Temperature: 35ºC
Detection: UV-254 nm
Injection: 5 µL
Sample: 20 µg/mL in 80:12:8, water:acetonitrile:dimethylsulfoxide

Elution Order:
1. 2,6-TDI
2. 1,6-HDI
3. 2,4-TDI
4. 4,4'-MDI

Time (min)
Water Soluble Compounds- Reversed Phase Mode (5% MeOH)

Elution Order:
1. Norepinephrine
2. Normetanephrine
3. 3,4-dihydroxymandelic acid
4. 3,4-dihydroxyphenyl glycol
5. 4-hydroxy-3-methoxymandelic acid
6. 4-hydroxy-3-methoxyphenylglycol

Column: 15 cm x 4.6 mm, 5 µm particle
Mobile phase A: 95% Water 5% Methanol w/ 0.1% Formic acid
Flow rate: 1.0 mL/min
Temperature: 35ºC
Detection: UV-220 nm
Injection: 10 µL
• The cyano group has a strong dipole that can interact with polar moieties found in most analytes.
• Ascentis porous silica particles are available in range of particle sizes and columns- fast LC to prep LC.
• The stable Ascentis ES Cyano phase may the most versatile of all HPLC phases:
  • Reversed-phase mode (100% aqueous to 100% ACN or MeOH).
    – Ideal for fast LC-MS (balance of retention and selectivity).
  • Aqueous Normal Phase (HILIC) mode (> 70% ACN or MeOH).
  • Normal Phase mode (completely non-aqueous); Cyano may be to normal phase what C18 is to reversed-phase.
Acknowledgements and Trademarks

• The assistance of Craig Aurand, Hillel Brandes, Hugh Cramer and David Bell and others at Supelco Division of Sigma-Aldrich is greatly appreciated.

• Ascentis and Discovery are registered trademarks of Sigma-Aldrich Corporation.

• Fused-Core is a registered trademark of Advanced Materials Technology, Wilmington, DE.
Selectivity References