APPLICATIONS

Investigation on the Impact of Using Different Mobile Phase Ratios of Acetonitrile to Methanol on Reversed Phase Phenyl Selectivity

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Introduction

Aliphatic hydrocarbon bonded phase columns such as C18 and C8 continue to be the most popular reversed phase HPLC columns on the market today. These phases are primarily designed for and excel at hydrophobic based LC selectivity and retention. However, for complex mixtures, additional selectivities may be needed to achieve the desired chromatographic separation. Phenyl phases are often the first choice in orthogonal selectivity for aromatic and polar compounds due to the π-π interaction offered by the double bonds within the phenyl ligand. The increase in selectivity correlates to the amount of π electrons in the analyte and can also be affected by the choice of organic modifier used in the mobile phase. For highest π-π interaction, methanol is the preferred solvent over acetonitrile as the π electrons associated with the nitrile (C≡N) bond in acetonitrile compete for the π-π interaction between the phenyl phase and the analyte. However, because of the higher viscosity of methanol/water mobile phases, the addition of acetonitrile with methanol when using phenyl phases is often recommended to reduce the mobile phase viscosity and thus reduce the backpressure associated with methanol/water mixtures. In this study, we show that the addition of acetonitrile to methanol does not disrupt the π-π interaction of a phenyl phase, but rather suppresses the interaction as a function of the relative amount versus methanol. Depending on the analyte characteristics, overall selectivity may also be improved.

Materials and Methods

Analyses were performed using an Agilent® 1200 (Agilent Technologies, Inc.) equipped with an API 4000™ MS/MS from SCIEX®. Reference standards for morphine, hydromorphone, codeine, hydrocodone, and diazepam were obtained from Cerilliant (Round Rock, TX) and prepared by diluting to 100 ng/mL. Reagents were obtained from JT Baker (Phillipsburg, NJ).

To investigate the impact of acetonitrile and methanol on phenyl phase selectivity, we used a reversed phase gradient for all runs comprised of: All columns used (C18, Phenyl-Hexyl, Biphenyl, F5) were from the same Kinetex® 2.6 µm Core-Shell family, all with dimensions of 50 x 4.6 mm. Since these phases all share the same material characteristics, such as pore volume and surface area, the only difference is in the bonded phase.

HPLC Conditions

Mobile Phase:	A: Water with 0.1 % Formic acid B: 0.1 % Formic acid in a solution of Methanol with Acetonitrile in ratios of 20 % increments: 100 % Methanol 80:20 Methanol/Acetonitrile 60:40 Methanol/Acetonitrile 40:60 Methanol/Acetonitrile 20:80 Methanol/Acetonitrile 100 % Acetonitrile
Gradient:	15 % B to 95 % B over 4 minutes
Flow Rate:	800 µL/min
Temperature:	Ambient

Discussion

Morphine, hydromorphone, codeine, hydrocodone, and diazepam were chosen as aromatic, polar, and basic standards to demonstrate phenyl selectivity. Chemical structures and properties are shown in **Table 1**.

Three separate phenyl phases (Kinetex Phenyl-Hexyl, Kinetex Biphenyl, and Kinetex F5) were used to evaluate the effect of various methanol ratios on retention and selectivity based on differing levels of π-π, hydrophobic, hydrogen bonding, and dipole interactions offered by each column. A Kinetex C18 was used as a comparative control for hydrophobic interaction selectivity. While increasing percentages of acetonitrile also reduced retention times on the Kinetex C18 due to acetonitrile being a stronger hydrophobic eluent (**Figures 1** and **12**), the reduced retention is comparatively higher on the Kinetex Biphenyl where π-π interaction is also increasingly suppressed (**Figure 2**). Diazepam, being the most hydrophobic of the tested compounds (**Table 1**), shows the largest decrease in hydrophobic retention with increasing acetonitrile on the C18 (**Figure 1**). Morphine, hydromorphone, codeine, and hydrocodone which are all more polar compounds, show a comparatively small difference in hydrophobic retention with increasing acetonitrile on the C18.

Figure 1.

Kinetex® C18 Solvent Effect

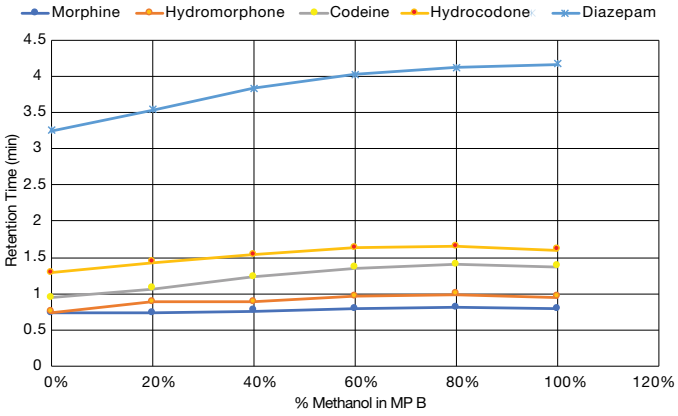
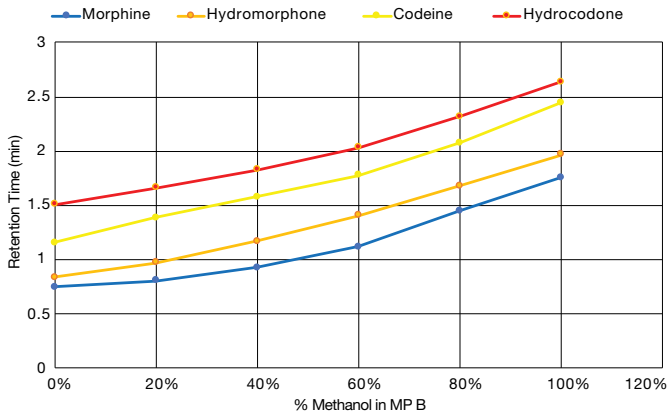


Figure 2.

Kinetex Biphenyl Solvent Effect



Kinetex Biphenyl, being the most aromatic phase of the group, demonstrated the highest level of π-π interaction. This is exhibited by the largest increase in retention for the four columns as the percentage of methanol increases. While each analyte (**Figure 2**) shares a general trend of increasing retention time, the relative increase is higher for the more polar morphine, hydromorphone, and codeine than the less polar hydrocodone, demonstrating the increased polar interaction and retention offered by the Biphenyl phase. Codeine responds somewhat linearly to increasing percentages of methanol, while morphine exhibits a stronger response when methanol comprises at least 60 % of mobile phase B.

It is also important to note that increased retention does not necessarily correlate with highest selectivity. The isomer pairs morphine/hydromorphone and codeine/hydrocodone exhibit highest retention in 100 % methanol, but selectivity is actually highest at 80 % and 100 % acetonitrile, respectively (α = 1.76 and 1.63), during which π-π interactions are suppressed and hydrophobic interactions are allowed to dominate. The lowest selectivity for both pairs is actually exhibited at 100 % methanol with α = 1.18 and 1.10, respectively (**Figure 3**, **9**).

When comparing the methanol effect on the Kinetex Phenyl-Hexyl versus the Kinetex Biphenyl, we see a similar but overall lower increase in retention with increasing percentage of methanol (**Figure 4**). As the Phenyl-Hexyl has only a single aromatic group, the potential for π-π interaction is expected to be lower than what is observed for the Biphenyl phase. However, the Phenyl-Hexyl offers the strongest hydrophobic interaction of each phenyl phase due to its longer six carbon hexyl linker.

A slightly different selectivity trend is shown on Phenyl-Hexyl, with highest selectivity for morphine and hydromorphone at 40 % methanol (α = 1.76) and lowest selectivity and complete coelution at 0 % methanol (α = 1). Comparatively, codeine and hydrocodone exhibited highest selectivity at 0 % methanol (α = 1.97) and lowest at 100 % methanol (α = 1.19) (**Figures 5** and **10**).

The Kinetex F5 (pentafluorophenyl) phase demonstrates a similar trend to the Phenyl-Hexyl, both of which feature comparable levels of π-π interaction (**Figures 6** and **11**). The F5 differs from the Phenyl-Hexyl with respect to a having shorter three carbon propyl linker, lower hydrophobicity, and the presence of the highly electronegative fluorine moieties that offer unique dipole-dipole, induced dipole, and hydrogen bonding interactions for polar compounds.

Figure 3.

Kinetex Biphenyl Solvent Effect on Selectivity

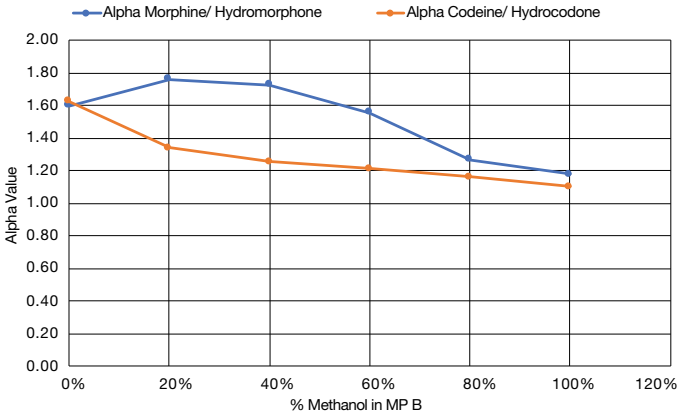


Figure 4.

Kinetex Phenyl-Hexyl Solvent Effect

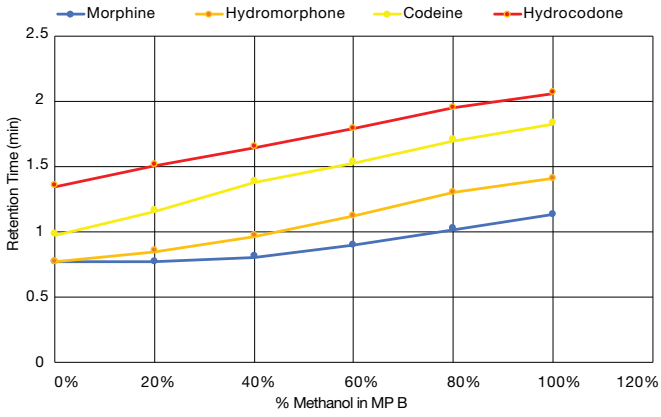


Figure 5.

Kinetex® Phenyl-Hexyl Solvent Effect on Selectivity

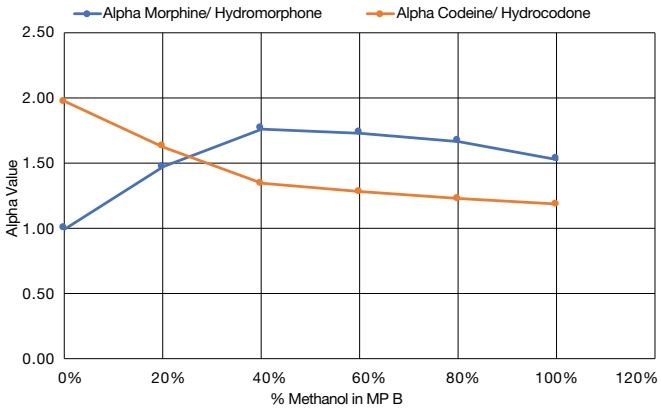


Figure 6.

Kinetex F5 Solvent Effect

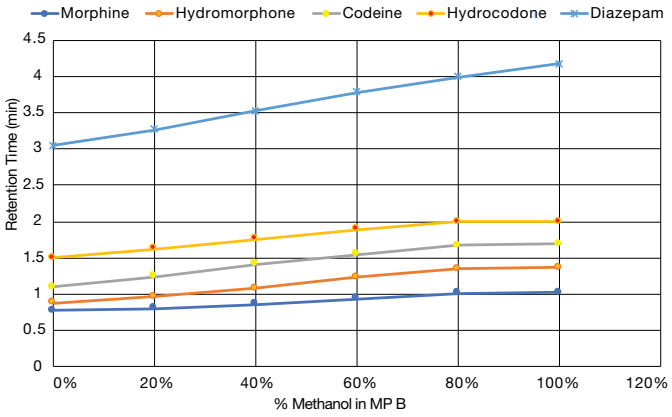


Figure 7.

Morphine Solvent Effect

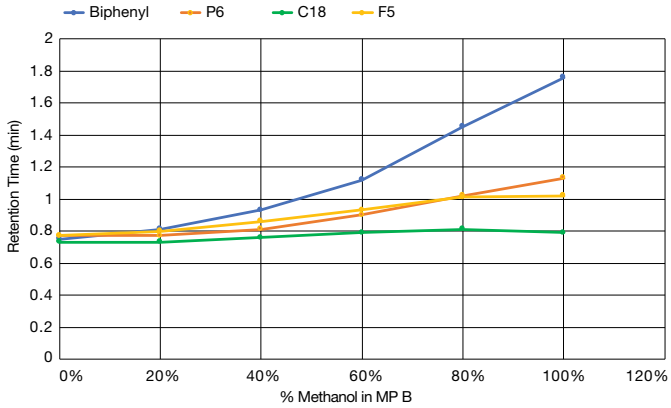
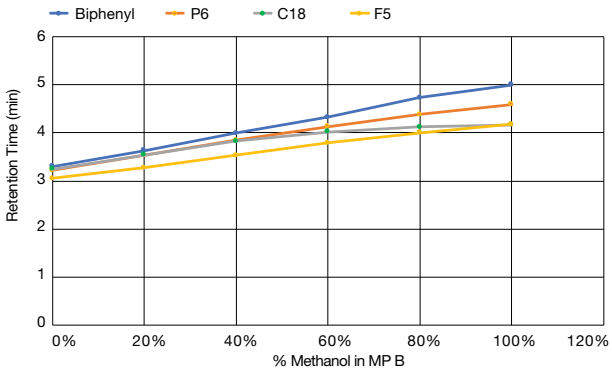


Figure 8.

Diazepam Solvent Effect



Looking at the results for morphine (**Figure 7**), we see that Kinetex Biphenyl exhibits by far the highest retention for the polar basic drug. It is possible the high π electron density created by the dual ring structure of the biphenyl phase gives behavior similar to a weak cation exchanger, leading to enhanced retention for positively charged basic analytes in addition to the increased aromatic retention in high methanol conditions. When we compare morphine to the nonpolar, neutral drug diazepam, we see the two phenyl phases trending closer to each other in retention times with π-π and hydrophobic interactions dominating (**Figure 8**).

Figures 9-12 show the effect of % Methanol on the overall separation for these compounds. Note that space limitations only allow inclusion of the chromatograms for 0, 20, 60, and 100 % Methanol. The chromatograms for 40 and 80 % Methanol can be found on the Phenomenex website.

Conclusion

When compared to acetonitrile, methanol promotes an increase in π-π interaction with phenyl phases, resulting in increased retention for aromatic compounds. While acetonitrile suppresses π-π interaction with the phenyl phases to some degree as a function of its relative amount to methanol, the interaction is never completely inhibited while methanol is present. Not only can the addition of acetonitrile result in lower backpressure, the suppression of π-π selectivity may result in increased hydrophobic selectivity that may improve resolution among certain analytes. When developing a method, it can be beneficial to explore different acetonitrile/methanol mixtures to achieve the desired degree of both π-π and hydrophobic selectivity. In addition, Biphenyl, Phenyl-Hexyl, and F5 all offer a unique combination of polar and aromatic selectivities when hydrophobic interaction alone on a C18 is insufficient for the job.

Table 1. Structures and Chemical Properties

Structure	Morphine	Hydromorphone	Codeine	Hydrocodone	Diazepam
LogP	0.90	1.47	1.34	1.96	3.08
LogD (pH2.7)	-2.30	-1.69	-2.16	-1.54	2.65

Figure 9. Kinetex® Biphenyl

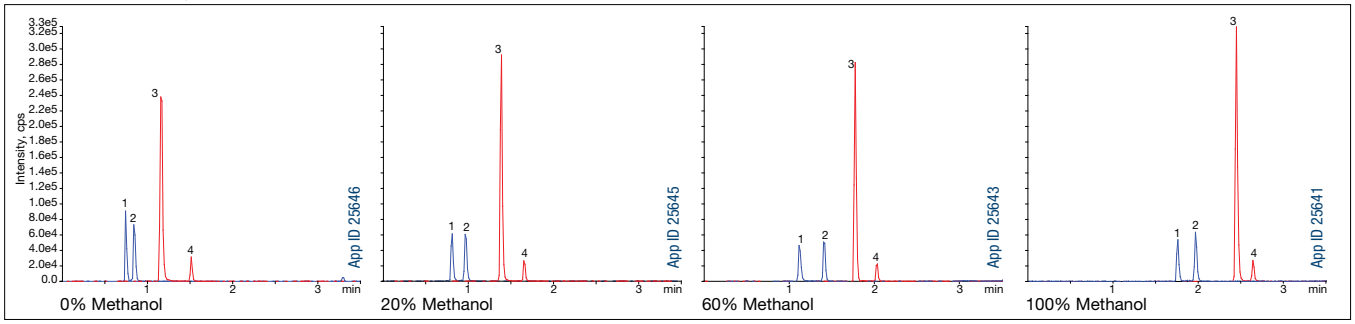


Figure 10. Kinetex Phenyl-Hexyl

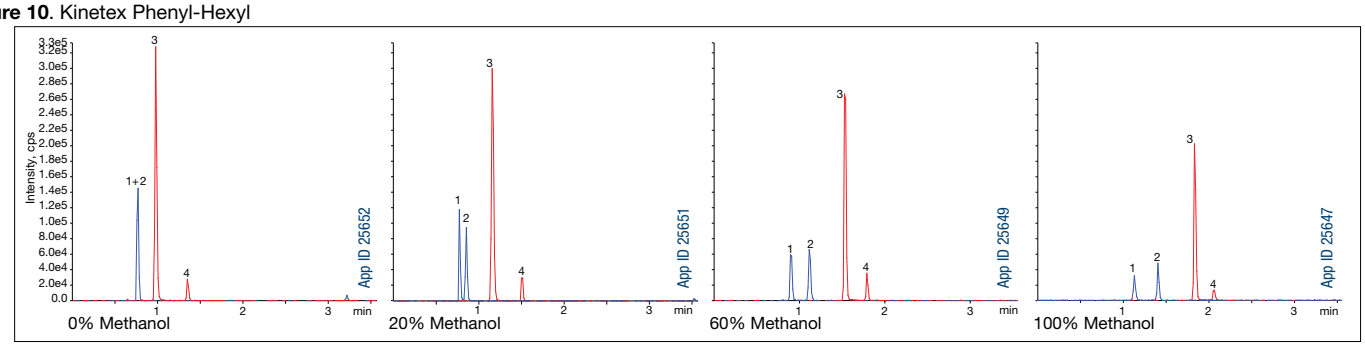


Figure 11. Kinetex F5

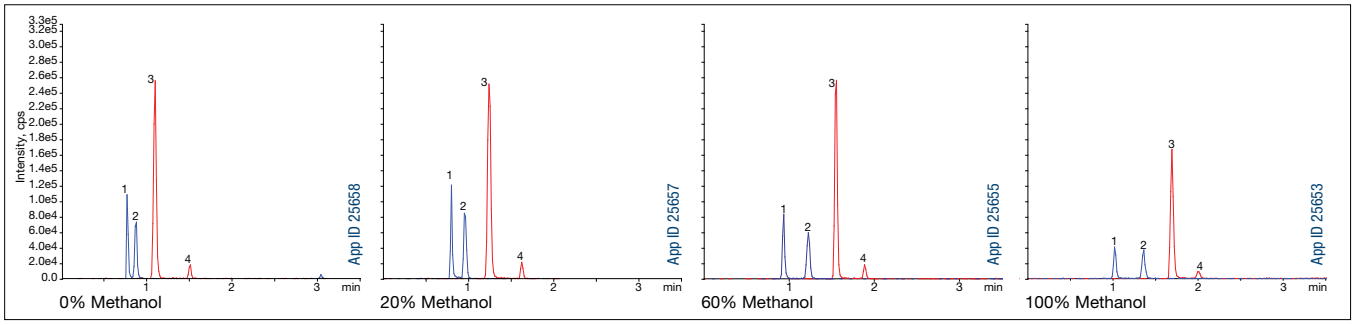
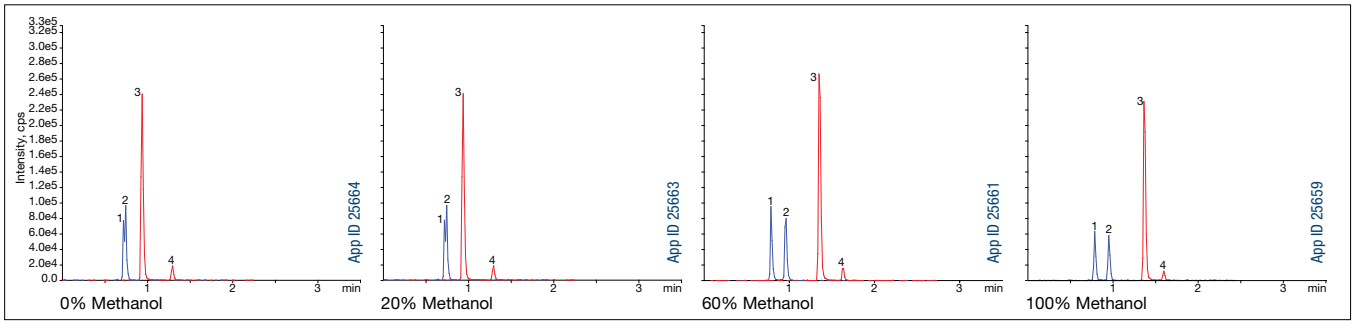


Figure 12. Kinetex C18



Kinetex® Ordering Information

5 µm Minibore Columns (mm)					SecurityGuard™ ULTRA Cartridges‡
Phases	**30 x 2.1**	**50 x 2.1**	**100 x 2.1**	**150 x 2.1**	**3/pk**
F5	—	00B-4724-AN	00D-4724-AN	00F-4724-AN	AJ0-9322
Biphenyl	00A-4627-AN	00B-4627-AN	00D-4627-AN	—	AJ0-9209
C18	00A-4601-AN	00B-4601-AN	00D-4601-AN	00F-4601-AN	AJ0-8782
Phenyl-Hexyl	—	00B-4603-AN	—	—	AJ0-8788

for 2.1 mm ID

5 µm MidBore™ Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	**50 x 3.0**	**100 x 3.0**	**150 x 3.0**	**3/pk**
F5	—	00D-4724-Y0	00F-4724-Y0	AJ0-9321
Biphenyl	00B-4627-Y0	00D-4627-Y0	00F-4627-Y0	AJ0-9208
C18	00B-4601-Y0	00D-4601-Y0	00F-4601-Y0	AJ0-8775
Phenyl-Hexyl	00B-4603-Y0	00D-4603-Y0	—	AJ0-8781

for 3.0 mm ID

5 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	**50 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**3/pk**
F5	00B-4724-E0	00D-4724-E0	00F-4724-E0	00G-4724-E0	AJ0-9320
Biphenyl	00B-4627-E0	00D-4627-E0	00F-4627-E0	00G-4627-E0	AJ0-9207
C18	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJ0-8768
Phenyl-Hexyl	00B-4603-E0	00D-4603-E0	00F-4603-E0	00G-4603-E0	AJ0-8774

for 4.6 mm ID

2.6 µm Minibore Columns (mm)						SecurityGuard ULTRA Cartridges‡
Phases	**30 x 2.1**	**50 x 2.1**	**75 x 2.1**	**100 x 2.1**	**150 x 2.1**	**3/pk**
Biphenyl	00A-4622-AN	00B-4622-AN	—	00D-4622-AN	00F-4622-AN	AJ0-9209
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
Phenyl-Hexyl	00A-4495-AN	00B-4495-AN	00C-4495-AN	00D-4495-AN	00F-4495-AN	AJ0-8788
F5	00A-4723-AN	00B-4723-AN	—	00D-4723-AN	00F-4723-AN	AJ0-9322

for 2.1 mm ID

2.6 µm MidBore™ Columns (mm)						SecurityGuard ULTRA Cartridges‡
Phases	**30 x 3.0**	**50 x 3.0**	**75 x 3.0**	**100 x 3.0**	**150 x 3.0**	**3/pk**
Biphenyl	—	00B-4622-Y0	—	00D-4622-Y0	00F-4622-Y0	AJ0-9208
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
Phenyl-Hexyl	—	00B-4495-Y0	—	00D-4495-Y0	00F-4495-Y0	AJ0-8781
F5	—	00B-4723-Y0	—	00D-4723-Y0	00F-4723-Y0	AJ0-9321

for 3.0 mm ID

2.6 µm Analytical Columns (mm)							SecurityGuard™ ULTRA Cartridges‡
Phases	**30 x 4.6**	**50 x 4.6**	**75 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**3/pk**
Biphenyl	—	00B-4622-E0	—	00D-4622-E0	00F-4622-E0	00G-4622-E0	AJ0-9207
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	00G-4462-E0	AJ0-8768
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	00G-4495-E0	AJ0-8774
F5	—	00B-4723-E0	—	00D-4723-E0	00F-4723-E0	00G-4723-E0	AJ0-9320

for 4.6 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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