
# Method Development and Column Selection: How the FluoroPhenyl Phase Provides the Power of HILIC and Reversed-Phase Modes in One Column

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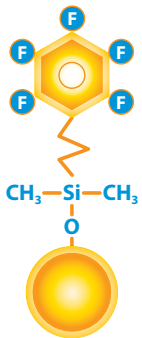
## Abstract

*Column selection is an important part of method development as the quality of critical separations is determined in large part by the retention and selectivity of the analytical column. Although C18 columns are commonly employed, FluoroPhenyl columns provide an orthogonal alternative for method development because they incorporate multiple retention mechanisms and can be used in both reversed-phase (RP) and hydrophilic interaction liquid chromatography (HILIC) modes. This paper explores how FluoroPhenyl columns can be used to improve separations, especially for compounds that are difficult to analyze on a C18, such as basic analytes or small polar compounds.*


## Introduction

While the C18 phase has historically been the workhorse of LC columns in many labs, it is not always the best choice for hydrophilic compounds due to limitations in retention and selectivity. C18 columns contain alkyl stationary phases that separate compounds primarily through dispersive interactions, resulting in hydrophobic retention. This single retention mechanism is the foundation for many reversed-phase (RP) applications, but it ultimately limits the utility of C18 columns to nonpolar analytes that can be separated based on their hydrophobicity. In contrast, Restek's FluoroPhenyl stationary phase operates through multiple retention mechanisms, including dispersion, shape selectivity, cation exchange, dipole, and pi-pi (π-π) interactions. The presence of multiple retention mechanisms gives FluoroPhenyl columns alternative, or orthogonal, selectivity to a C18 and makes them a much more powerful tool for difficult separations, including those for small polar compounds and basic analytes. The FluoroPhenyl phase achieves its unique selectivity by combining strongly electronegative fluorine atoms with a phenyl ring (Figure 1). This moiety results in a mixed-mode column that can be used in both hydrophilic interaction liquid chromatography (HILIC) and RP modes, giving method developers the flexibility to choose the mode that works best for their target analytes. In this article, we will examine method development and column selection and demonstrate the broader range of separations that can be accomplished using a FluoroPhenyl column when compared to a C18. We will also discuss how separations can be further controlled and optimized by modifying mobile phase parameters, including composition, acid strength, and acid concentration.

**Figure 1:** Raptor FluoroPhenyl LC Column Properties.

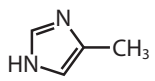



| | |
|---|---|
| **Stationary Phase Category:** | Pentafluorophenyl propyl (L43) |
| **Ligand Type** | FluoroPhenyl |
| **Particle:** | 2.7 µm or 5 µm superficially porous silica (SPP or "core-shell") |
| **Pore Size:** | 90 Å |
| **Surface Area** | 150 $m^2$/g (2.7 µm)<br>100 $m^2$ (5 µm) |
| **Recommended Usage:** | pH Range: 2.0–8.0<br>Max. Temperature; 80 °C<br>Max. Pressure: 600 bar (2.7 µm); 400 bar (5 µm) |

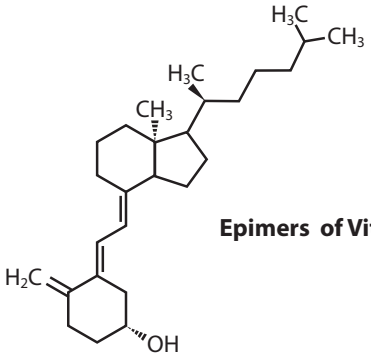
## Multiple Retention Mechanisms Give FluoroPhenyl Columns Alternate Selectivity to a C18

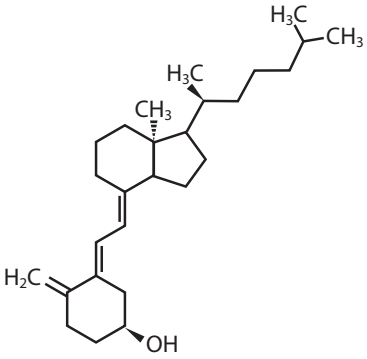
Efficient, effective method development depends on column selection. Early identification of the best column for the target analyte(s) can save time when developing a new method. The alternate selectivity provided by FluoroPhenyl columns, when compared to a C18, can aid method developers by allowing them to utilize multiple retention mechanisms in a single column. We chose several challenging target analytes (Figure 2) to illustrate the unique retention characteristics of the FluoroPhenyl phase and to examine its performance in both HILIC and RP modes. All of these analytes were selected because they exhibit poor retention, poor resolution—or both characteristics—on a traditional C18 stationary phase.

**Figure 2:** Test Probes Used to Demonstrate the Multiple Retention Modes of Raptor FluoroPhenyl Columns




**4-methylimidazole (4-MEI)**




**Epimers of Vitamin D3**

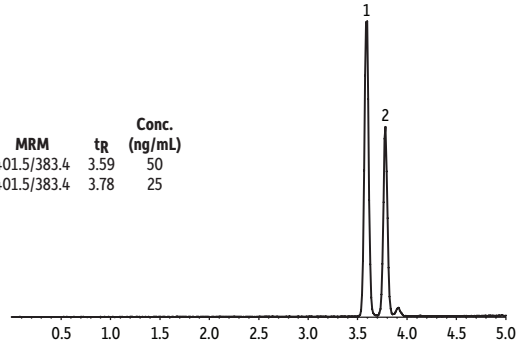
*Shape-Selective Retention of Vitamin D Isobars in Reversed-Phase Mode*

In our first example, we evaluated the analysis of 25-hydroxyvitamin D3 and its C3 epimeric form, a separation of increasing relevance in clinical labs around the world. 25-Hydroxyvitamin D3 is an important biomarker used in the determination of faulty vitamin D metabolism. The C3 epimeric form (3-epi-25-hydroxyvitamin D3) has much lower bioactivity and can cause biomarker values to be overestimated if not reported individually. Because these compounds are isobaric, chromatographic separation is essential for the accurate determination of biological levels. As shown in Figure 3, the use of a FluoroPhenyl column in standard RP mode allows complete separation of 25-hydroxyvitamin D3 and its C3 epimer in just five minutes. In contrast, on a C18 column the elution time was three times longer and adequate separation was never achieved. In this example, column selection clearly plays a key role in method development: the rigid structure of the FluoroPhenyl phase provides additional shape selectivity and substantially more resolving power than can be achieved using the hydrophobic retention of a C18 column.

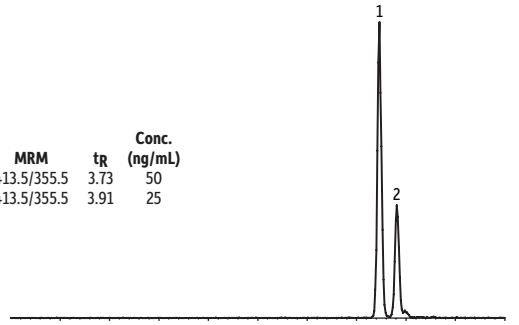
**Figure 3:** Vitamin D analysis is more accurate on a FluoroPhenyl column because its shape-selective retention separates the C3 epimer from the bioactive form.

## Raptor FluoroPhenyl

| Peaks | MRM | $t_R$ | Conc. (ng/mL) |
|---|---|---|---|
| 1. 25-Hydroxyvitamin D3 | 401.5/383.4 | 3.59 | 50 |
| 2. 3-Epi-25-hydroxyvitamin D3 | 401.5/383.4 | 3.78 | 25 |




| Peaks | MRM | $t_R$ | Conc. (ng/mL) |
|---|---|---|---|
| 1. 25-Hydroxyvitamin D2 | 413.5/355.5 | 3.73 | 50 |
| 2. 3-Epi-25-hydroxyvitamin D2 | 413.5/355.5 | 3.91 | 25 |




LC_CF0644

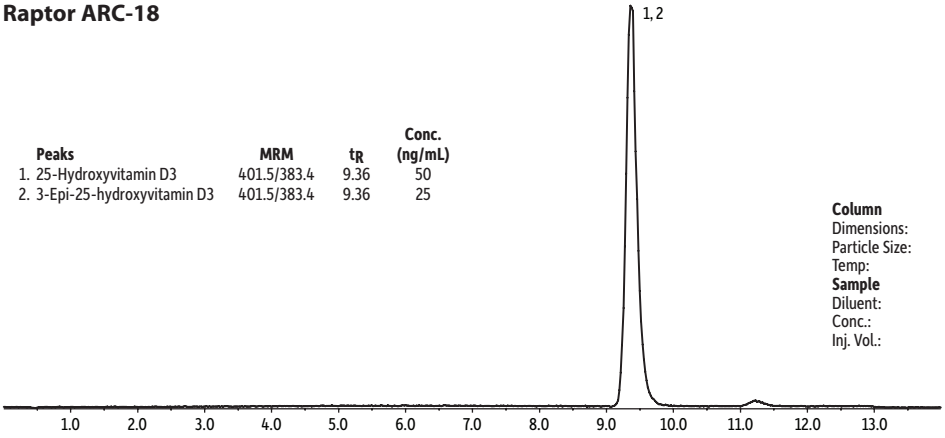
| | |
|---|---|
| **Column** | Raptor FluoroPhenyl (cat.# 9319A1E) |
| Dimensions: | 100 mm x 3 mm ID |
| Particle Size: | 2.7 µm |
| Temp.: | 30 °C |
| **Sample** | |
| Diluent: | Water:methanol (50:50) |
| Conc.: | 25-50 ng/mL |
| Inj. Vol.: | 5 µL |
| **Mobile Phase** | |
| A: | 0.1% Formic acid in water |
| B: | Methanol |

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.6 | 25 | 75 |
| 4.00 | 0.6 | 15 | 85 |
| 4.10 | 0.6 | 0 | 100 |
| 5.00 | 0.6 | 0 | 100 |
| 5.01 | 0.6 | 25 | 75 |
| 7.00 | 0.6 | 25 | 75 |

| | |
|---|---|
| **Detector** | MS/MS |
| Ion Mode: | ESI+ |
| Mode: | MRM |
| **Instrument** | HPLC |

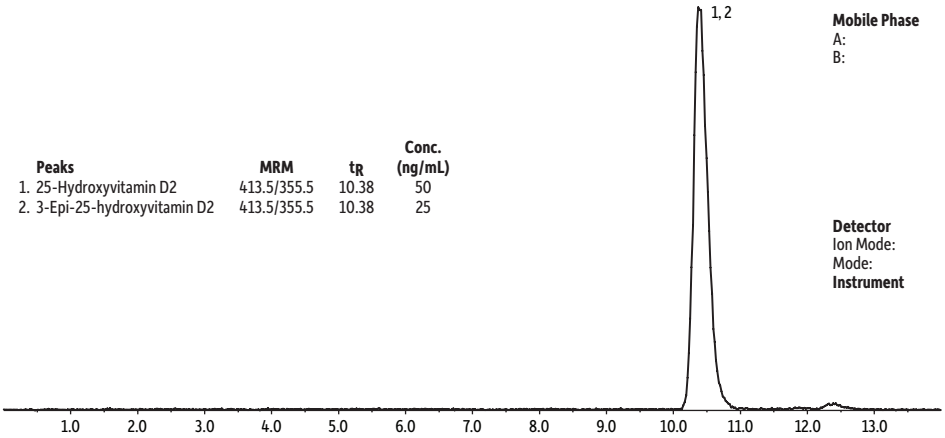
## Raptor ARC-18

| Peaks | MRM | $t_R$ | Conc. (ng/mL) |
|---|---|---|---|
| 1. 25-Hydroxyvitamin D3 | 401.5/383.4 | 9.36 | 50 |
| 2. 3-Epi-25-hydroxyvitamin D3 | 401.5/383.4 | 9.36 | 25 |




| | |
|---|---|
| **Column** | Raptor ARC-18 (cat.# 9314A12) |
| Dimensions: | 100 mm x 2.1 mm ID |
| Particle Size: | 2.7 µm |
| Temp: | 30 °C |
| **Sample** | |
| Diluent: | Water:methanol (50:50) |
| Conc.: | 25-50 ng/mL |
| Inj. Vol.: | 5 µL |

| Peaks | MRM | $t_R$ | Conc. (ng/mL) |
|---|---|---|---|
| 1. 25-Hydroxyvitamin D2 | 413.5/355.5 | 10.38 | 50 |
| 2. 3-Epi-25-hydroxyvitamin D2 | 413.5/355.5 | 10.38 | 25 |




LC_CF0649

| | |
|---|---|
| **Mobile Phase** | |
| A: | 0.1% Formic acid in water |
| B: | Methanol |

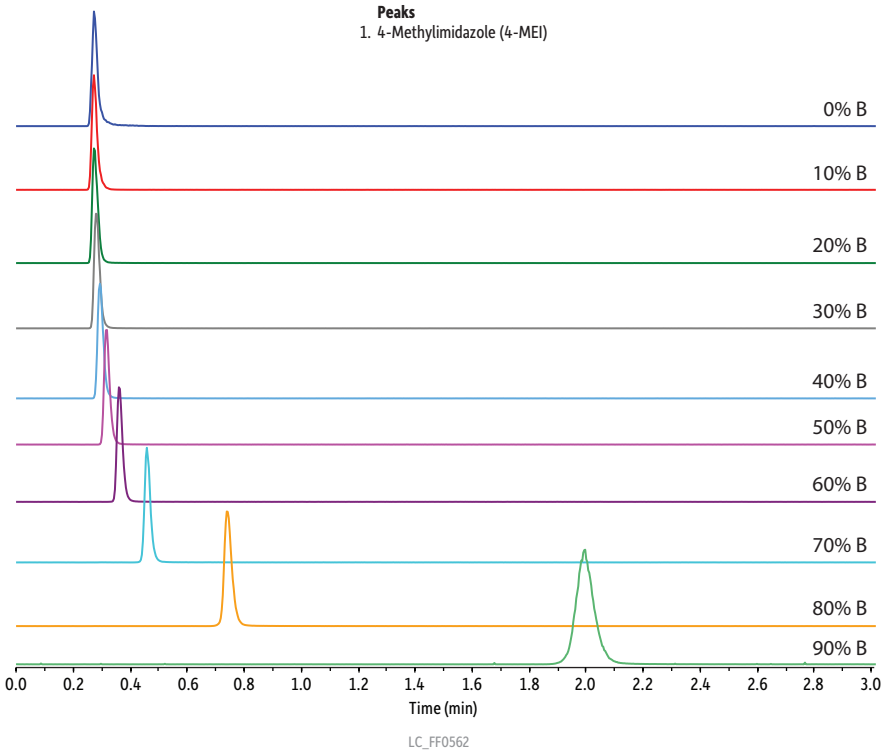
| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.5 | 25 | 75 |
| 4.00 | 0.5 | 20 | 80 |
| 12.00 | 0.5 | 20 | 80 |
| 12.10 | 0.5 | 25 | 75 |
| 14.00 | 0.5 | 25 | 75 |

| | |
|---|---|
| **Detector** | MS/MS |
| Ion Mode: | ESI+ |
| Mode: | MRM |
| **Instrument** | HPLC |

*Cation-Exchange Retention of Small Polar Compounds in HILIC Mode*

Our next example demonstrates the cation-exchange mechanism of the FluoroPhenyl phase and its effectiveness in retaining small polar compounds. 4-Methylimidazole (4-MEI) is an additive that is used as a caramel coloring in food and beverages. Like other small polar compounds, it is difficult to analyze using standard RP chromatography and a C18 column because it has limited retention on a nonpolar stationary phase. However, in an acidic solution, 4-MEI has a positive charge, which makes it very amenable to cation exchange and easy to retain on a FluoroPhenyl column under HILIC conditions. This occurs because the electron withdrawing nature of the five fluorine atoms imparts a slight negative charge to the stationary phase. As shown in Figure 4, when a high proportion of acetonitrile is used in the mobile phase, cation-exchange interactions dominate retention and 4-MEI is retained well. Under reversed-phase conditions, retention of the FluoroPhenyl column is dominated by dispersive interactions and 4-MEI is poorly retained. The FluoroPhenyl phase gives analysts the flexibility to evaluate performance in both RP and HILIC modes in order to base method development decisions on which mode is most effective for their compounds of interest.

**Figure 4:** Small polar compounds, such as 4-MEI, are difficult to retain under RP conditions, but adequate retention is easily obtained under HILIC conditions on a FluoroPhenyl column.

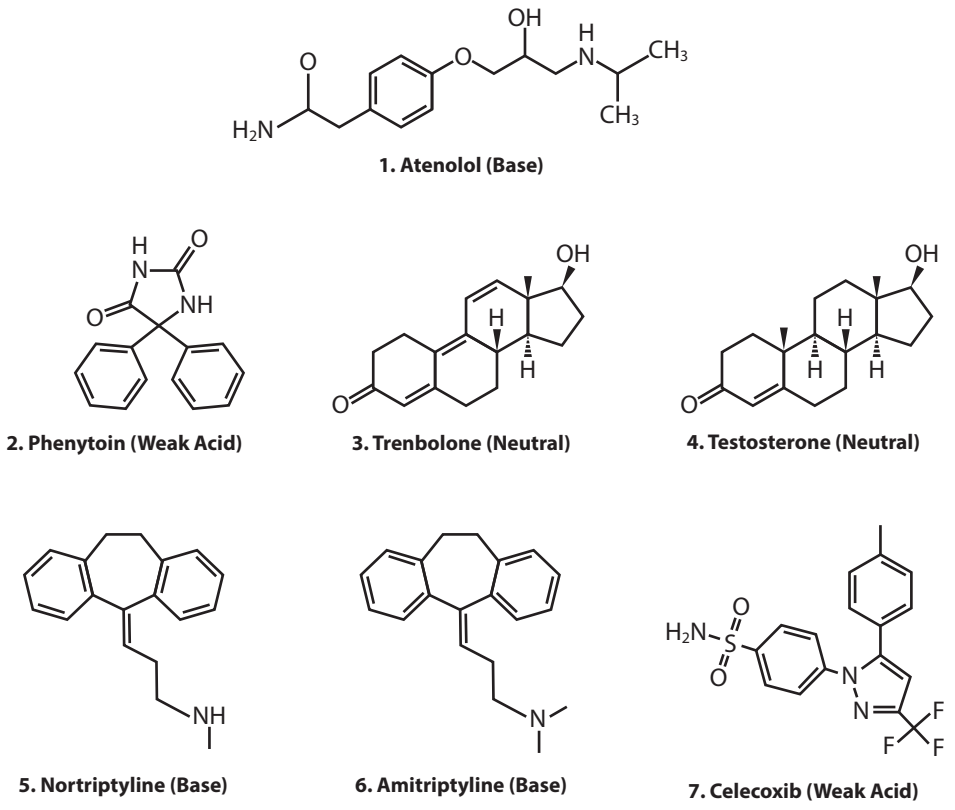



| | |
|---|---|
| **Column** | Raptor FluoroPhenyl (cat.# 9319A52) |
| Dimensions: | 50 mm x 2.1 mm ID |
| Particle Size: | 2.7 µm |
| Temp.: | 35 °C |
| **Sample** | |
| Diluent: | Acetonitrile |
| Conc.: | 100 ng/mL |
| Inj. Vol.: | 5 µL |
| **Mobile Phase** | 0.1% Formic acid in water:0.1% formic acid in acetonitrile |
| Flow: | 0.6 mL/min |
| **Detector** | MS/MS |
| Ion Mode: | ESI+ |
| Mode: | MRM |
| **Instrument** | UPLC |

### Fine-Tune Retention and Selectivity with Modified Mobile Phase Parameters

As previously established, effective method development depends on careful column selection, and the multiple retention modes of the FluoroPhenyl phase provide more flexibility than is possible with single retention mode C18 columns. In this section, we will explore ways to optimize separations by modifying mobile phase composition, acid strength, and acid concentration. For these experiments, several common drug compounds were selected to demonstrate the varied retention behaviors of the FluoroPhenyl stationary phase (Figure 5). These chemical probes represent bases, weak acids, and neutral compounds and highlight differences in the selectivity and the affinity of the FluoroPhenyl phase for hydrophilic and basic compounds.

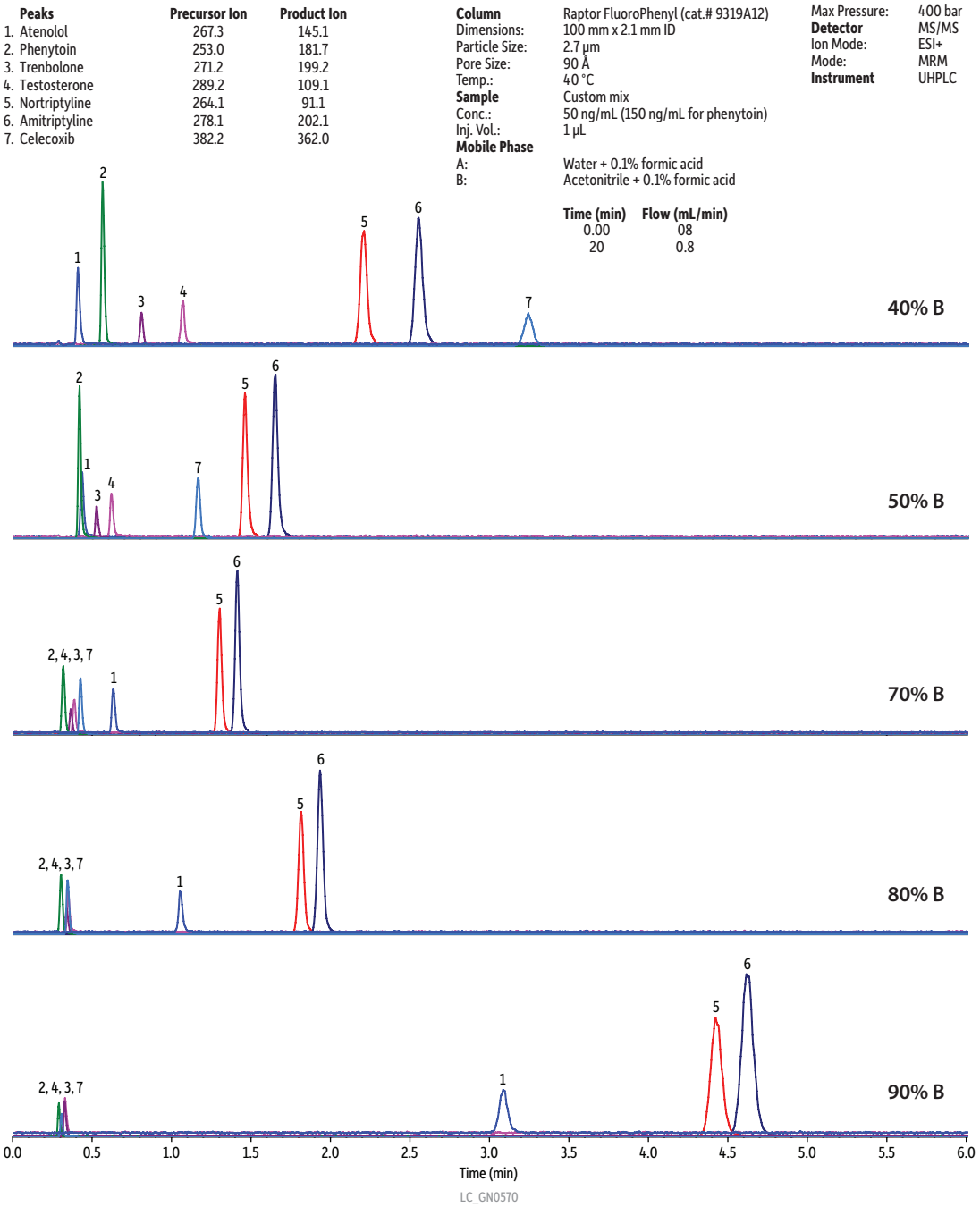
**Figure 5:** Test Probes Used to Demonstrate the Multiple Retention Modes of Raptor FluoroPhenyl Columns



*Effects of Mobile Phase Composition*

One of the primary method development advantages of selecting a FluoroPhenyl column is that it can be used in both HILIC mode and RP mode. By modifying the mobile phase composition, changes in retention and selectivity occur. Under RP conditions, analytes are retained on the FluoroPhenyl column by dispersive and polar interactions. However, as the mobile phase composition becomes increasingly organic, cation exchange dominates retention and the FluoroPhenyl stationary phase becomes preferentially selective for positively charged bases, with neutrals and acids eluting near the column void (Figure 6). The ability of the Fluoro-Phenyl column to operate using multiple retention mechanisms is clearly demonstrated by the "U-shaped" retention profile that is observed for some of the basic compounds as the mobile phase composition moves from high aqueous (RP) concentrations to high organic (HILIC) concentrations.

**Figure 6:** Changing the mobile phase composition from RP (40% B) to HILIC (90% B) conditions results in a U-shaped retention profile that demonstrates the changes in retention and selectivity that occur due to the multiple retention mechanisms that are present the stationary phase.



In addition to affecting retention and selectivity, changing to a highly organic HILIC mobile phase when using electrospray ionization mass spectrometry (ESI-MS) can significantly affect sensitivity by improving desolvation efficiency. Table I compares intensity values for the basic compounds analyzed in Figure 6 and demonstrates that increased sensitivity is observed for both nortriptyline and amitriptyline. (Sensitivity for atenolol is unchanged as it switches from an unretained compound in RP mode to a retained compound in HILIC mode.)

**Table I:** Using a high-organic HILIC mobile phase in conjunction with ESI-MS can significantly increase sensitivity.

| Analyte | RP Intensity (40% B, Isocratic) | HILIC Intensity (90% B, Isocratic) | Increase in Response |
|---|---|---|---|
| Atenolol | 5.0e4 | 5.0e4 | – |
| Nortriptyline | 7.5e4 | 1.3e5 | 73% |
| Amitriptyline | 8.5e4 | 1.7e5 | 100% |

*Effects of Mobile Phase Acid Strength and Concentration*

As discussed above, modifying the aqueous:organic composition of the mobile phase can result in significant changes to FluoroPhenyl retention and selectivity, providing a valuable tool for method developers wishing to optimize specific separations. In addition, for acidified mobile phases, the strength of the acid as well as its concentration can affect the cation-exchange retention mechanism and influence the separation. As shown in Figure 7, the use of mobile phases containing a stronger acid, such as formic acid, will reduce the retention of basic compounds compared to identical analyses run using the same concentration of a weaker acid, such as acetic acid. However, in both cases there is minimal change in the retention of neutral compounds and weak acids. Similarly, the

**Figure 7:** Effects of Acid Strength on FluoroPhenyl Retention and Selectivity in RP Mode

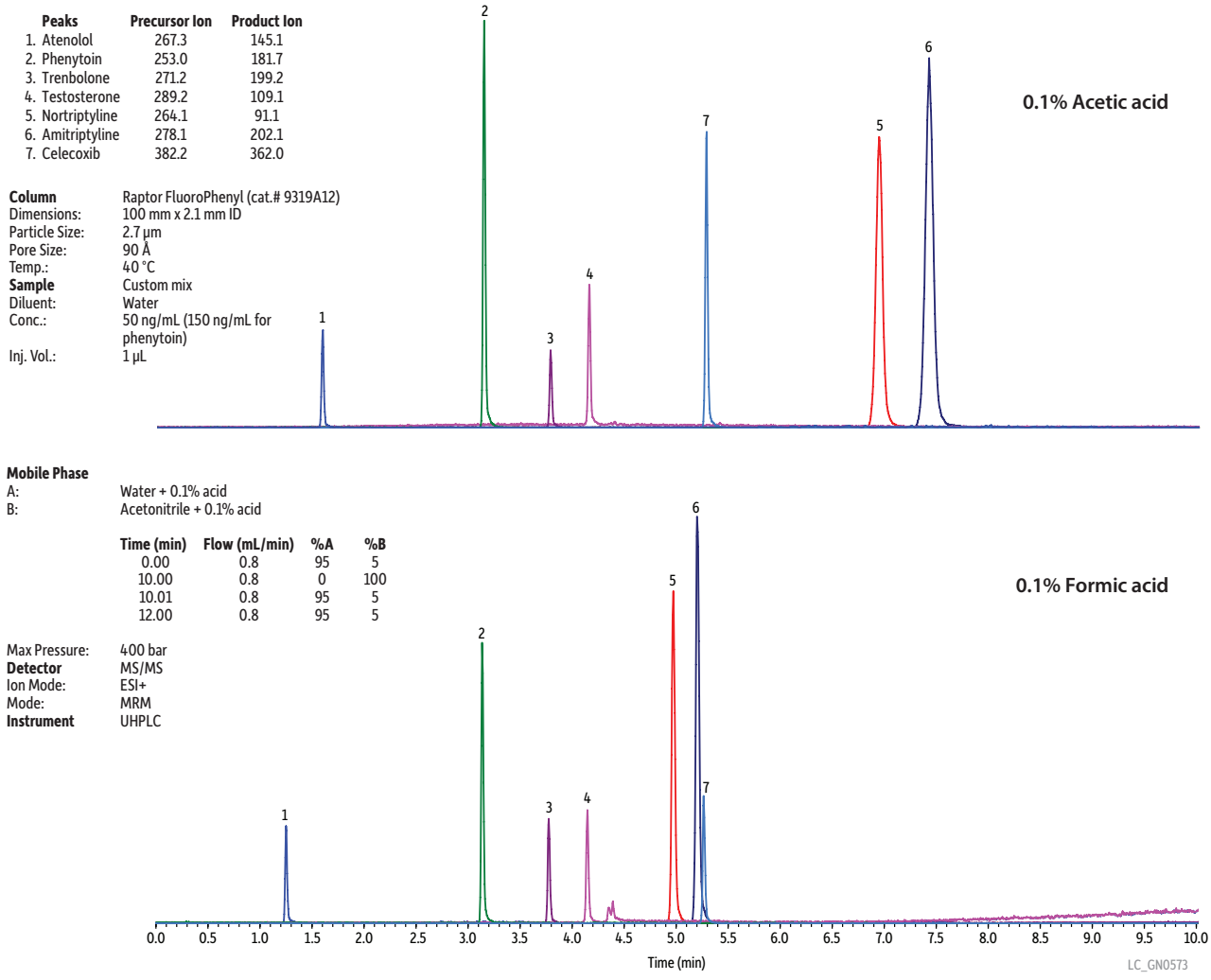
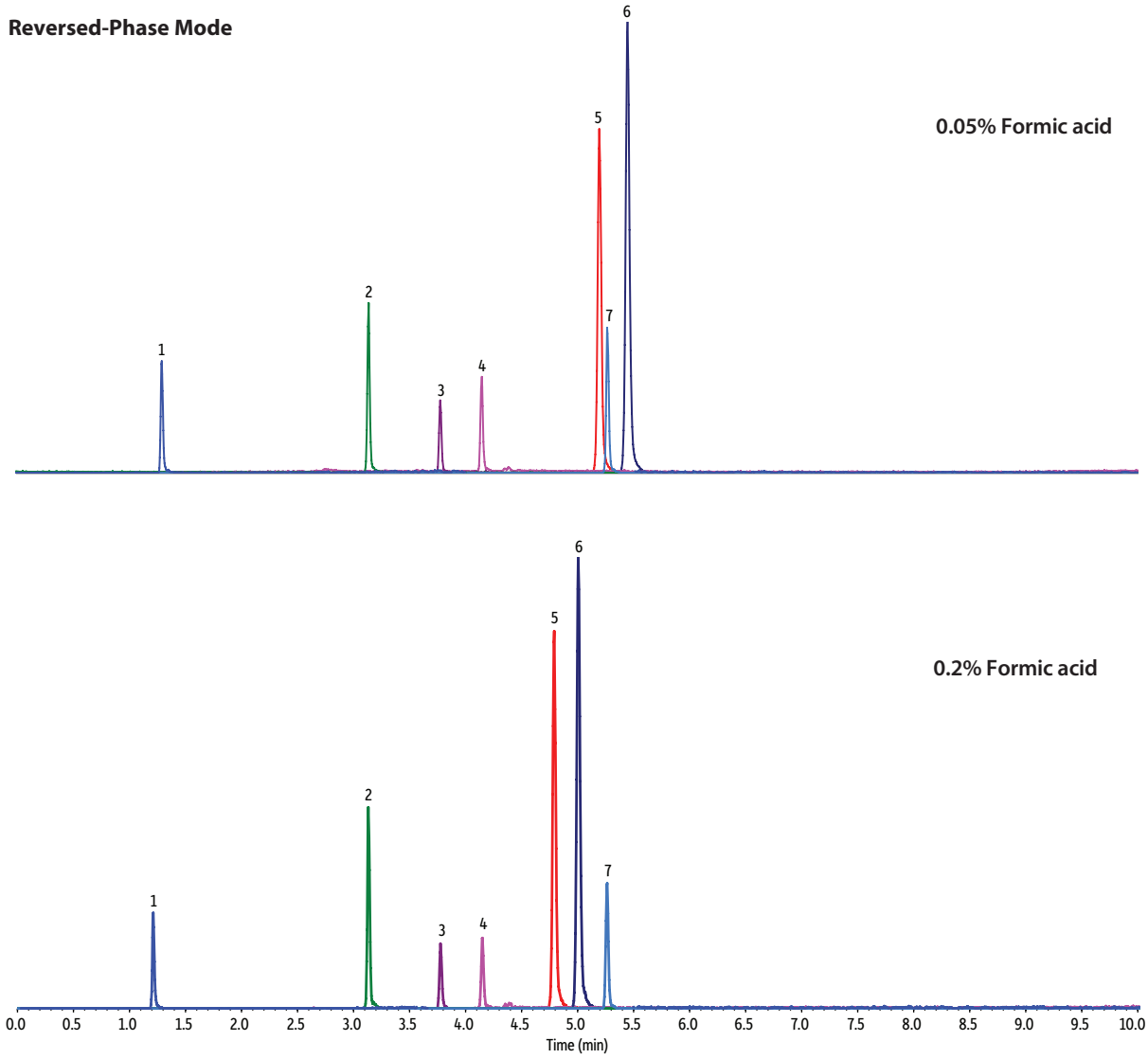


**Figure 8:** Effects of Acid Concentrations on FluoroPhenyl Retention and Selectivity




| Peaks | Precursor Ion | Product Ion |
|---|---|---|
| 1. Atenolol | 267.3 | 145.1 |
| 2. Phenytoin | 253.0 | 181.7 |
| 3. Trenbolone | 271.2 | 199.2 |
| 4. Testosterone | 289.2 | 109.1 |
| 5. Nortriptyline | 264.1 | 91.1 |
| 6. Amitriptyline | 278.1 | 202.1 |
| 7. Celecoxib | 382.2 | 362.0 |

**Column** Raptor FluoroPhenyl (cat.# 9319A12)
Dimensions: 100 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Temp.: 40 °C
**Sample** Custom mix
Diluent: Water
Conc.: 50 ng/mL (150 ng/mL for phenytoin)
Inj. Vol.: 1 µL
**Mobile Phase**
A: Water + x% formic acid
B: Acetonitrile + x% formic acid

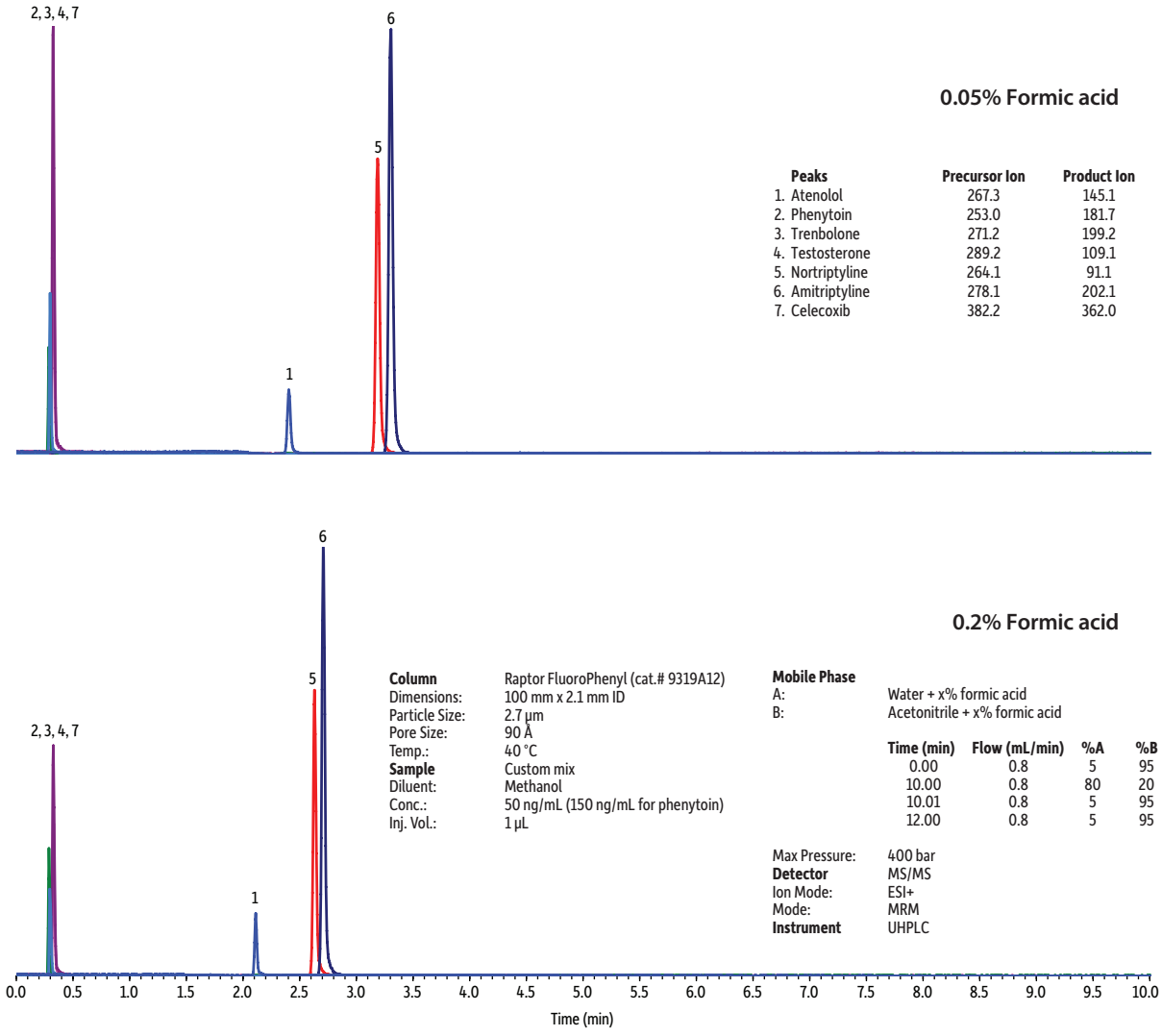
| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| 0.00 | 0.8 | 95 | 5 |
| 10.00 | 0.8 | 0 | 100 |
| 10.01 | 0.8 | 95 | 5 |
| 12.00 | 0.8 | 95 | 5 |

Max Pressure: 400 bar
**Detector** MS/MS
Ion Mode: ESI+
Mode: MRM
**Instrument** UHPLC

concentration of a given acid can also affect the cation-exchange retention mechanism of FluoroPhenyl columns. In this case, the use of mobile phases with higher acid concentrations serves to reduce the retention of the bases, with minimal change in retention for the neutrals and the weak acids, in both reversed-phase and HILIC modes (Figure 8).

**Figure 8:** Effects of Acid Concentrations on FluoroPhenyl Retention and Selectivity *(continued)*

**HILIC Mode**




## Conclusions

A large part of successful method development depends on column selection. While C18 columns are a popular choice, careful consideration should be given to the chemical moieties of the target analytes in conjunction with the capabilities of the stationary phase. Because the FluoroPhenyl stationary phase operates using multiple retention mechanisms (dispersion, shape selectivity, cation exchange, dipole, and pi-pi [π-π]), FluoroPhenyl columns offer orthogonal selectivity to method developers, compared to C18 columns, which only utilize dispersion. As demonstrated here, the multiple retention mechanisms of the FluoroPhenyl column allow it to be used in both RP and HILIC modes to achieve separations that cannot be accomplished on a C18. In addition, separations can be further refined by modifying the mobile phase composition, acid strength, and acid concentration, giving analysts more retention and selectivity options.

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