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A Systematic Approach Towards UPLC Methods Development

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APPLICATION BENEFITS

- Demonstrates a systematic approach to method development
- Selectivity is manipulated using pH, column chemistry, and organic modifier
- UPLC provides a 6-fold improvement in throughput, reducing time and cost per sample in the analysis

WATERS SOLUTIONS

ACQUITY UPLC® System

ACQUITY UPLC BEH, BEH Shield, BEH Phenyl, and HSS T3 Columns

KEY WORDS

Method development, paroxetine hydrochloride and related compounds, pH, column chemistry, organic modifier, optimization, gradient slope, temperature

Reversed-phase HPLC methods development can take anywhere from weeks to months, incurring large operational cost. By utilizing UltraPerformance LC® (UPLC®) Technology for methods development, a 6-fold improvement in throughput can be realized. This, in turn, reduces cost per sample and time of analysis considerably while maintaining or improving separation integrity. By developing rapid, high resolution analytical methods, products can be brought to market faster, therefore, improving the overall profitability of the assay.

A new method can be developed efficiently if experimental design is well thought out. Common methods development approaches include: conducting a literature search, trial and error, a step-wise iterative approach or a systematic screening protocol. A systematic screening protocol that explores selectivity factors such as pH, organic modifier and column chemistry will be the premise of this strategy. This approach allows chromatographers to quickly determine which experimental parameters are most effective in manipulating the selectivity of a separation. By employing this strategy, the total number of steps necessary to develop a method are reduced, therefore, providing an efficient and cost effective approach.

In this application note, combinations of selectivity factors (pH, column chemistry, and organic modifier) in UPLC separations were examined to develop high resolution chromatographic methods. Once the best combination of factors was selected, gradient slope and temperature were optimized. This methods development approach is demonstrated by developing a separation for paroxetine hydrochloride and its related compounds.

EXPERIMENTAL

LC Conditions

System:	Waters ACQUITY UPLC
Columns:	ACQUITY UPLC® BEH C_{18} 1.7 µm, p/n 186002350 ACQUITY UPLC BEH Shield RP18 1.7 µm p/n 186002853 ACQUITY UPLC BEH Phenyl 1.7 µm, p/n 186002884 ACQUITY UPLC HSS T3 1.8 µm, p/n 186003538
Dimensions:	2.1 x 50 mm
Mobile phase:	
A1	20 mM Ammonium Formate, pH 3.0
A2	20 mM Ammonium Bicarbonate, pH 10.0
B1	Acetonitrile
B2	Methanol
Flow rate:	0.5 mL/min

Gradient:

Time (min)	Profile %A	%B
0.0	95	5
5.0	10	90
5.01	95	5
5.5	95	5

Injection vol.:	4.0 µL
Temperature:	30 °C
Detection:	UV Scan 200-350 nm
Sampling rate:	20 pts/sec
Time constant:	0.1
Instrument:	Waters ACQUITY UPLC with ACQUITY UPLC Column Manager and ACQUITY UPLC PDA Detector

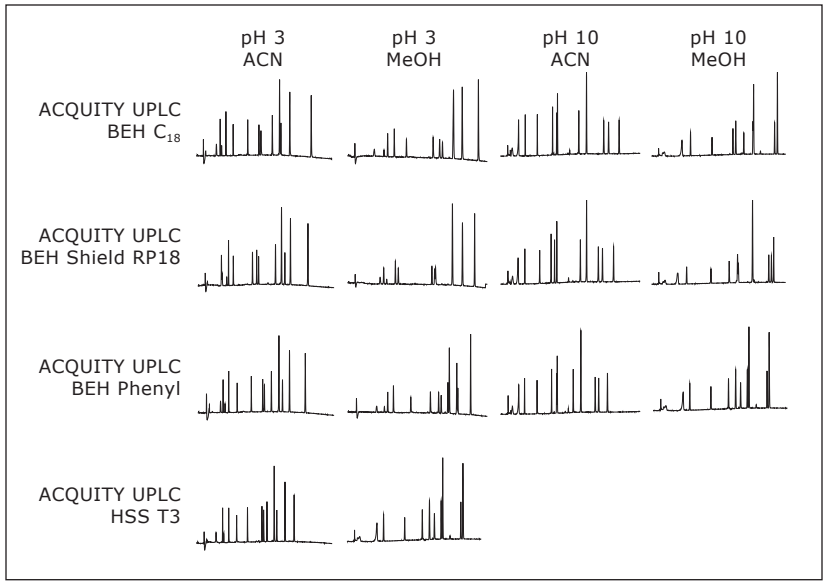

Figure 1. UPLC Methods Development Experimental Matrix.

RESULTS AND DISCUSSION

As depicted in Figure 1, a result matrix of 14 chromatograms is generated by evaluating three Bridged Ethylene Hybrid (BEH) columns at low and high pH and a silica (HSS) column at low pH, with two different organic modifiers. Each experimental result was evaluated for retentivity, peak shape, and resolution.

Step 1: Select the pH

By first evaluating the data acquired at low and high pH, the retention characteristics, loadability, and overall resolution of the mixture of analytes can quickly be determined. Paroxetine is an alkaline species with a pKa of 9.8. It is, therefore, in its neutral charge state when the mobile phase is increased to pH 10. As seen in Figure 2, acidic mobile phase pH results in poor resolution of paroxetine and related compounds. Alkaline pH provides better retention and resolution of all components due to the neutral charged states of the analytes.

Step 2: Select column chemistry

Once pH is selected, a comparison of different stationary phases is made. As shown in Figure 3, all three BEH columns show potential for resolving all components. The ACQUITY UPLC BEH C_{18} was selected to carry out the separation.

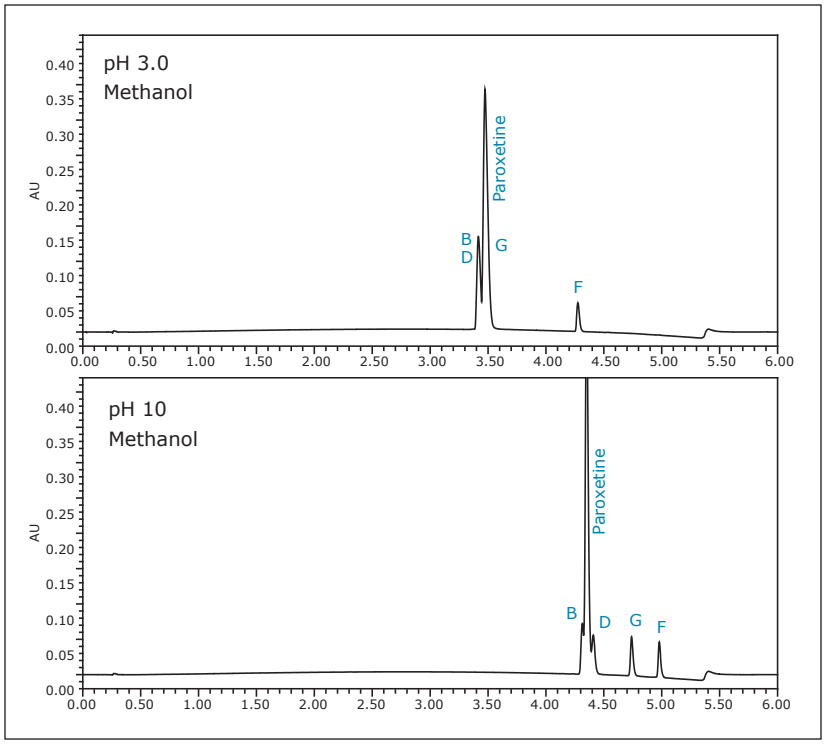

Figure 2. Evaluation of pH selectivity on ACQUITY UPLC BEH C_{18}.

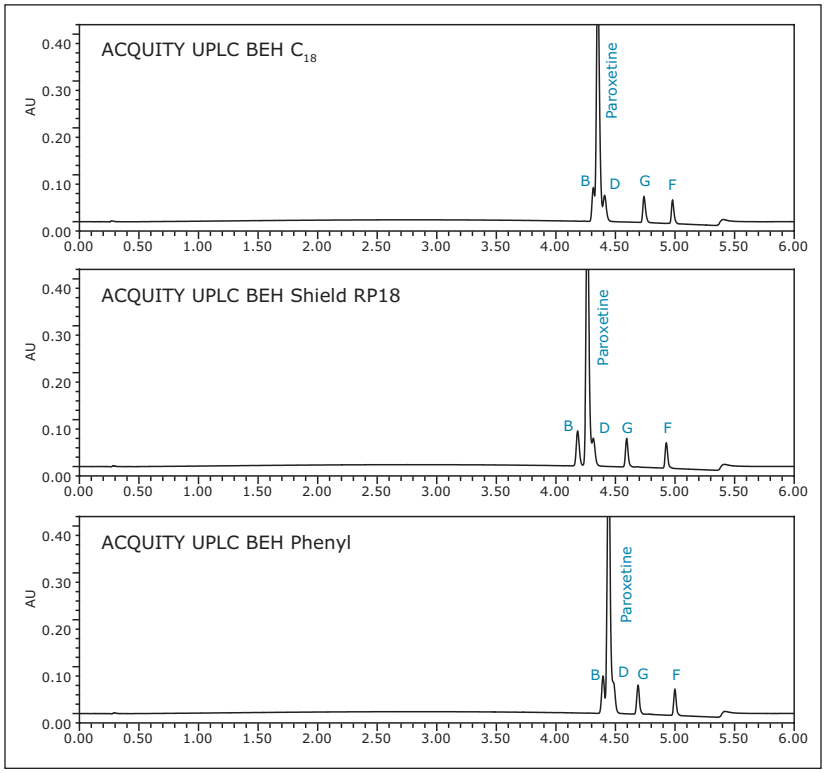

Figure 3. Comparison of column selectivity in methanol at alkaline pH.

Step 3: Select organic modifier

Lastly, the organic modifier is selected. Methanol offers a different selectivity than acetonitrile, and is a weaker elution solvent at equivalent concentration. This results in greater retention of the analytes. For this set of components, acetonitrile offers a better separation, as depicted in Figure 4.

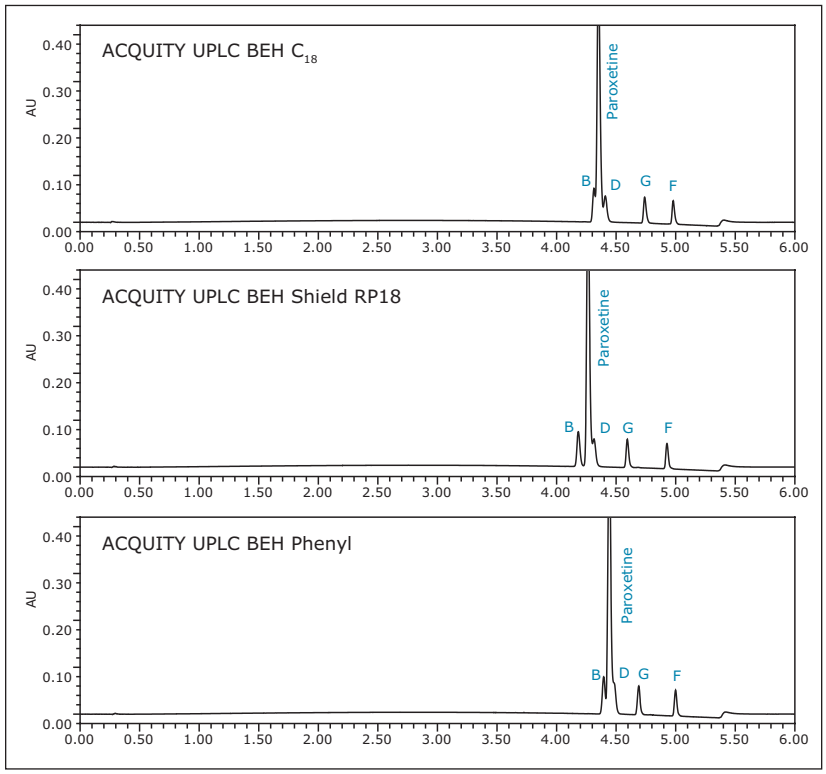

Figure 4. Evaluation of solvent selectivity on ACQUITY UPLC BEH C_{18}

Optimization

During our initial method screening, the related compounds were spiked into the solution at a 10% concentration level relative to paroxetine for ease of identification. For method optimization, the concentration of the related compounds was reduced from 10% of paroxetine to the target concentration of 0.1%, as shown in Figure 5. However, at the 0.1% concentration level, inadequate resolution among paroxetine and related compounds B and D resulted due to disparate levels of concentration making for a more challenging separation.

In efforts to improve the separation, gradient slope and temperature were manipulated.

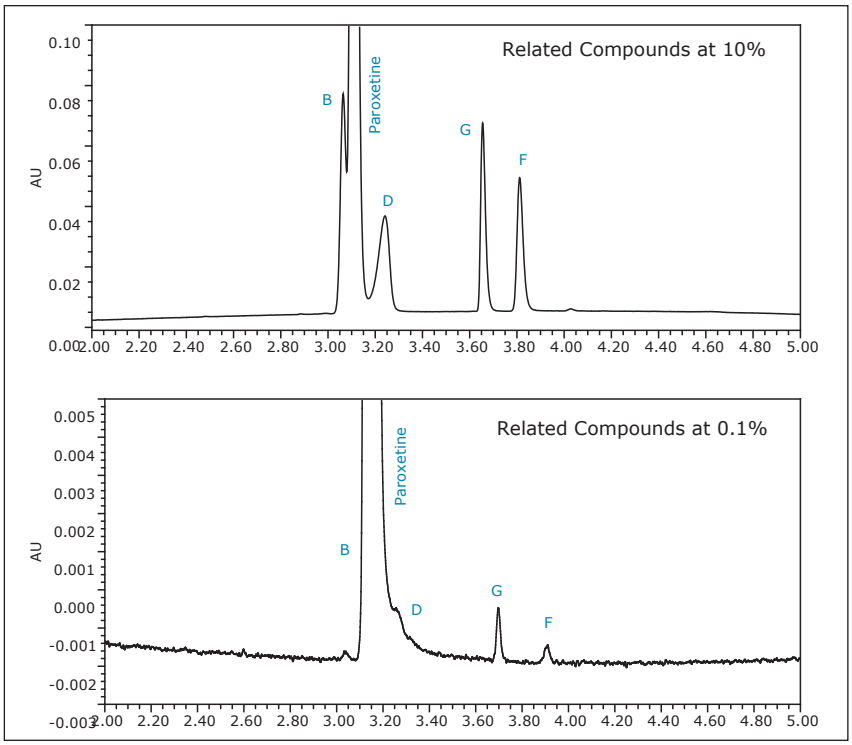

Figure 5. Related compounds at 10% vs. 0.1% of paroxetine.

Optimization: Gradient slope

Changing gradient slope is often a balance between resolution and sensitivity. Although selectivity change can occur, most often a steeper gradient slope will result in a reduction in resolution and an increase in sensitivity, while a shallower gradient slope will result in an increase in resolution and a decrease in sensitivity.

In efforts to improve resolution, the gradient slope was flattened by changing the % organic at the start and then endpoint of the gradient. In this case, marginal improvement was made by altering the gradient slope as depicted in Figure 6. Using the 20 – 65% acetonitrile gradient, the influence of column temperature was then explored.

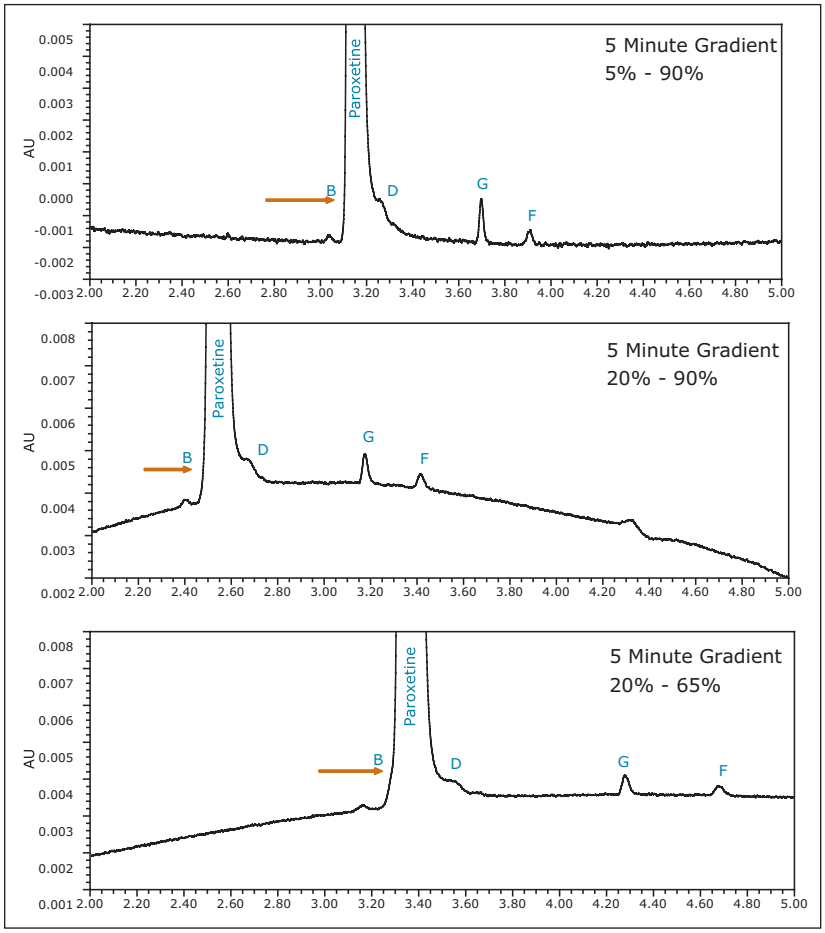

Figure 6. Monitoring influence of gradient slope reduction.

Optimization: Temperature

Temperature affects every chemical process that occurs. Analyte diffusivity, sample loadability and peak shape dramatically improved with increasing temperature. At 60 °C, adequate separation of related compounds from paroxetine was achieved; therefore, no further optimization was necessary.

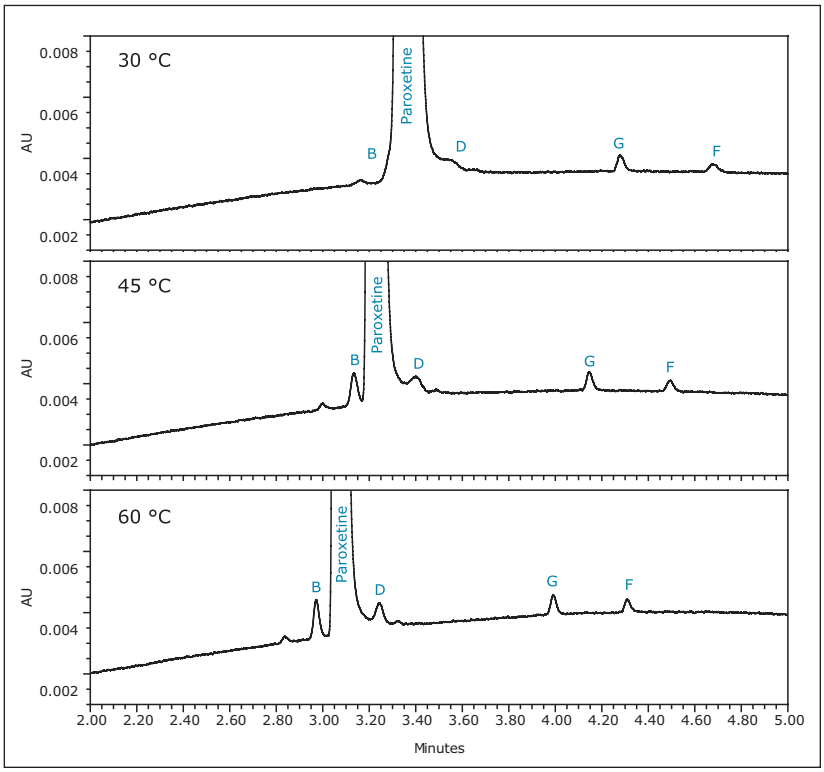

Figure 7. Influence of temperature on separation.

Final conditions

Separation was performed on a ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7-µm column at 60 °C. Mobile Phase A contained 20.0 mM ammonium bicarbonate with 1.2% ammonium hydroxide. Mobile Phase B was acetonitrile. A 5 minute gradient from 20 to 65% acetonitrile was performed. Flow rate was 0.5 mL/min.

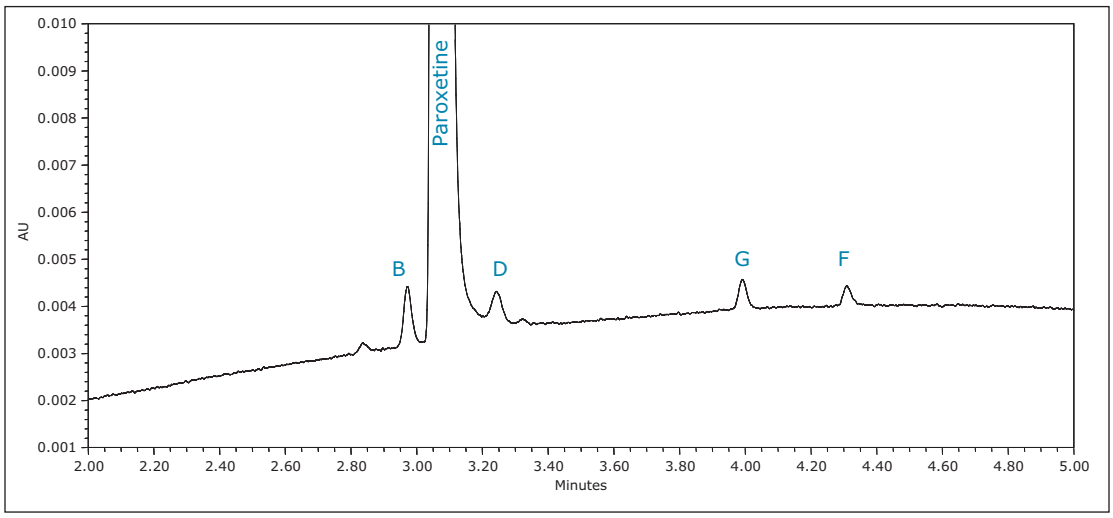

Figure 8: Final separation of Paroxetine and related compounds B, D, G, and F at the 0.1% level.

BUSINESS IMPACT

Productivity improvements associated with employing UPLC technology for methods development are depicted below in Table 1. By comparing the UPLC methods development strategy outlined previously to one directly scaled to conventional HPLC, a 6-fold improvement in time is observed. This significantly reduces the overall instrument time required to develop chromatographic methods to one work day opposed to one work week with conventional HPLC.

Methods Development Time					
UPLC Technology 2.1 x 50 mm, 1.7 µm, 0.5 mL/min				**Conventional HPLC** 4.6 x 150 mm, 5 µm, 1.0 mL/min	
pH 3	**acetonitrile**	**Time**		**pH 3**	**acetonitrile**	**Time**
Flow Ramp		5 min		Flow Ramp		5 min
Column Conditioning (2 blanks)		11 min		Column Conditioning (2 blanks)		79.2 min
Sample Injection (2 replicates)		11 min		Sample Injection (2 replicates)		79.2 min
pH 3	**methanol**			**pH 3**	**methanol**	
Flow Ramp		5 min		Flow Ramp		5 min
Column Conditioning (2 blanks)		11 min		Column Conditioning (2 blanks)		79.2 min
Sample Injection (2 replicates)		11 min		Sample Injection (2 replicates)		79.2 min
Column Purge		6 min		Column Purge		43.2 min
pH 10	**acetonitrile**			**pH 10**	**acetonitrile**	
Flow Ramp		5 min		Flow Ramp		5 min
Column Conditioning (2 blanks)		11 min		Column Conditioning (2 blanks)		79.2 min
Sample Injection (2 replicates)		11 min		Sample Injection (2 replicates)		79.2 min
pH 10	**methanol**			**pH 10**	**methanol**	
Flow Ramp		5 min		Flow Ramp		5 min
Column Conditioning (2 blanks)		11 min		Column Conditioning (2 blanks)		79.2 min
Sample Injection (2 replicates)		11 min		Sample Injection (2 replicates)		79.2 min
Column Purge		6 min		Column Purge		43.2 min
Time		120 min		Time		740 min

Screening Time			
3 Hybrid (BEH) Columns	6 Hours	**3** Hybrid (BEH) Columns	36.9 Hours
1 Silica (HSS) Column	1 Hour	**1** Silica Column	6.1 Hours
Total Screening Time	**7 Hours**	**Total Screening Time**	**43 Hours**

Table 1. Comparison of productivity between UPLC Technology and HPLC for methods development.

CONCLUSION

A systematic approach towards chromatographic methods development that monitors selectivity change in a separation by manipulating pH, column chemistry and organic modifier was described. By utilizing UPLC Technology for methods development, a 6-fold improvement in throughput can be realized. This, in turn, reduces cost per sample and time of analysis considerably while maintaining or improving separation integrity. By developing rapid, high resolution analytical methods, products can be brought to market faster, therefore, improving the overall profitability of the assay.

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