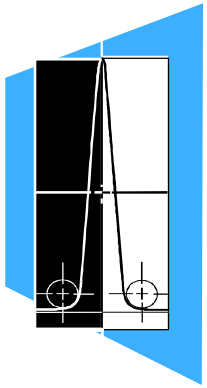
Poster presented at Drug Discovery '98 in Boston at the Hynes Convention Center, August 10-13th



Strategies for High Throughput HPLC Analysis for Combinatorial Chemistry

J. Carmody*, U. Neue, R. Crowley and C. Andrews, Waters Corporation, Milford, MA 01757

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Abstract

Traditionally, the search for biologically active molecules has involved the systhesis of one-molecule-at-a-time discovery strategies. This has proven to be a very time consuming and labor intensive process for the discovery of new drugs, catalysts and materials. New combinatorial chemistry techniques have reduced syntesis times by allowing simultaneous generation of a large number of chemical variants, several of which may be active leads. Once this lead generation process is complete the resultant combinatorial library is subjected to high throughput screening techniques, once of which is HPLC. Due to the plethora of compounds generated by the combinatorial chemistry technique, minimizing analysis time is paramount to meeting the major challenge of isolating the desired compound from other indigenous material as quickly as possible. Optimization of the HPLC screening process to achieve shorter analysis times is not always intrinsically straightforward. In order to achieve compressed analysis times there is a need to more completely understand the effect that the column characteristics (i.e. flow rate and gradient time) have on the selecitivity and resolution of the separation. The resolution and the selectivity being the two characteristics of the separation most affectd by changes in the column and operational parameters.

In this paper we will show straightforward HPLC strategies to developing fast gradient methods to quickly resolve target compounds from other inactive combinations.

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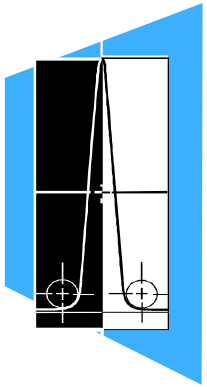

Outline

- Introduction
- Theory of Gradient Separations
- Optimization of Gradient Separations to...
  - achieve maximum throughput
  - maximize resolution
- Conclusion

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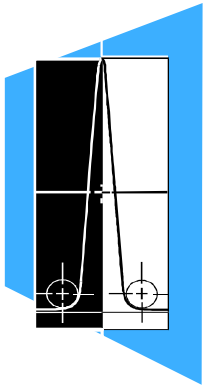

Introduction

- Goal...
  - Give you a strategy for rapid gradient methods development by showing you how to use operational parameters (such as gradient run time, flow rate and column length) to maximize the desirable aspects of a combinatorial separation:
    - Throughput
    - Resolution
    - Robustness

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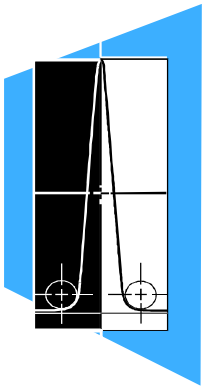

Factors Influencing Resolution in Gradient RP-HPLC Separations

- Resolution, R_s, between two closely resolved analytes in gradient RP-HPLC is a function of mean column efficiency N, mean selectivity α, and the effective retention factor:

$$R_s = \frac{\Delta t}{\overline{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \cdot \underbrace{\ln \alpha}_{\text{Selectivity}} \cdot \underbrace{\frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot t_0 + 1}}_{\text{Retention}}$$

$$\frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot \varepsilon_t \cdot \pi r^2 \cdot L/F + 1}$$

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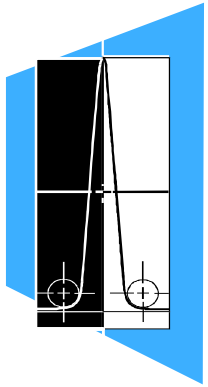

HPLC System

- Waters 2690XE Separations Module
- Waters Millennium^{32} Data System
- Waters 486 UV detector and 996 Photodiode Array detector

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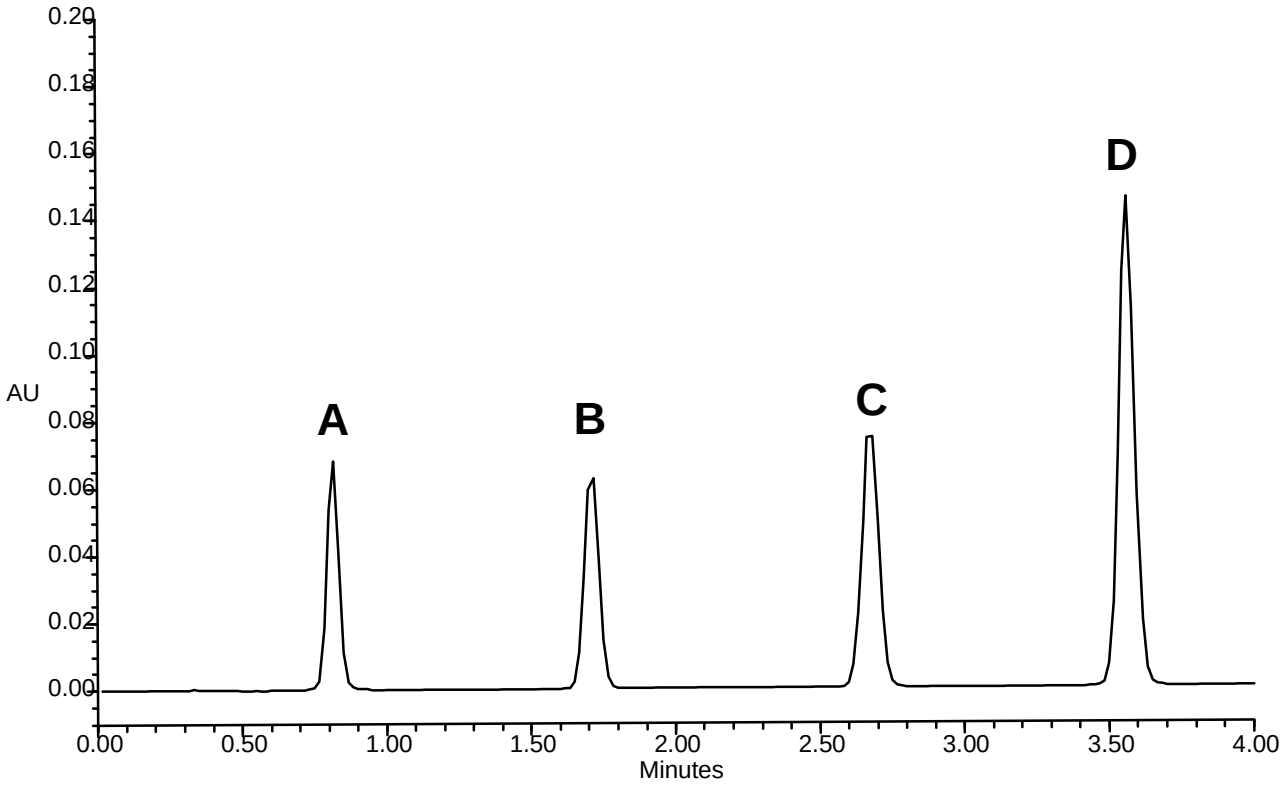
Initial Separation and Conditions



A - 1-hydroxy-7-aza-benzotriazole
B - 4-methylbenzene sulfonamide
C - methyl 3-amino-2-thiophenecarboxylate
D - 4-aminobenzophenone

Conditions:

Column: Symmetry® C_{18}, 5 µm, 4.6 X 50 mm

Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile

Gradient: 0-60% B in 8 minutes

Column temperature: 30.0 °C

Flow rate: 1 mL/min.

Detector: 254 nm

Injection volume: 1 µL

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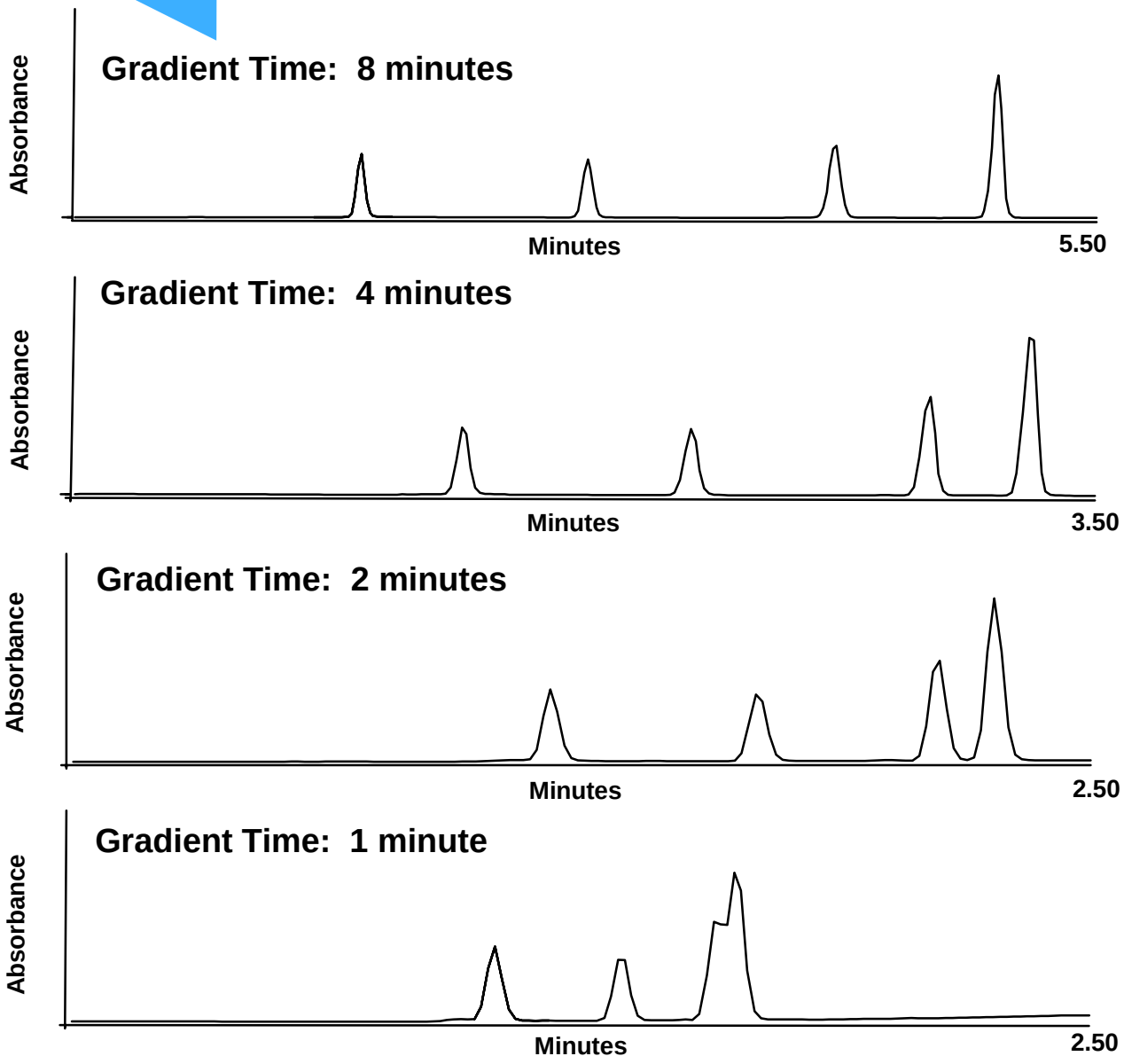
Impact of Reducing Gradient Time (t_g) on Resolution



Conditions:

Column: Symmetry® C_{18}, 5 µm, 4.6 X 50 mm
Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Gradient: 0-60% B in noted gradient time
Column temperature: 30.0 °C
Flow rate: 1 mL/min.
Detector: 254 nm
Injection volume: 1 µL

-Longest gradient time provides best resolution

-Shortest gradient time maximizes throughput

-Reducing just gradient time sacrifices resolution

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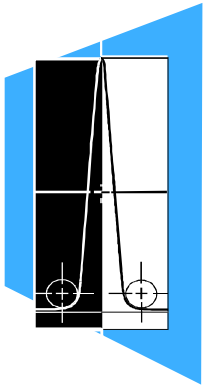

Summary - Impact of Gradient Run Time on Resolution

- Resolution increases as gradient run time increases
- Throughput decreases as gradient run time increases

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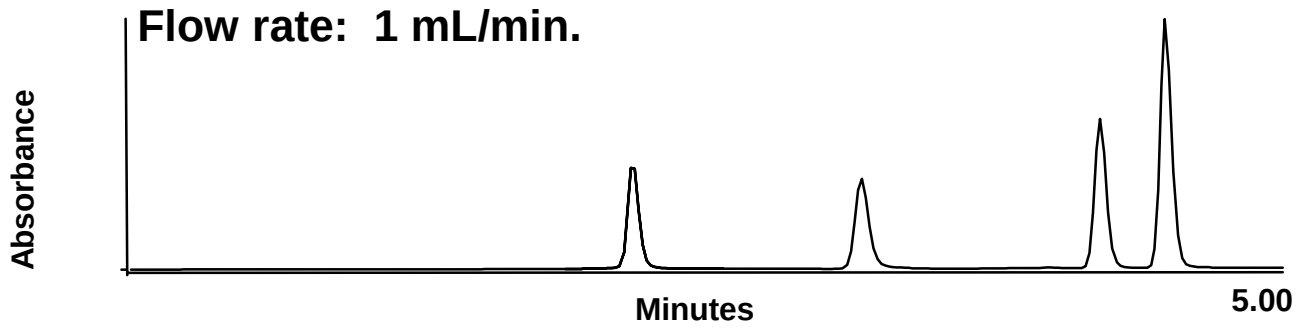
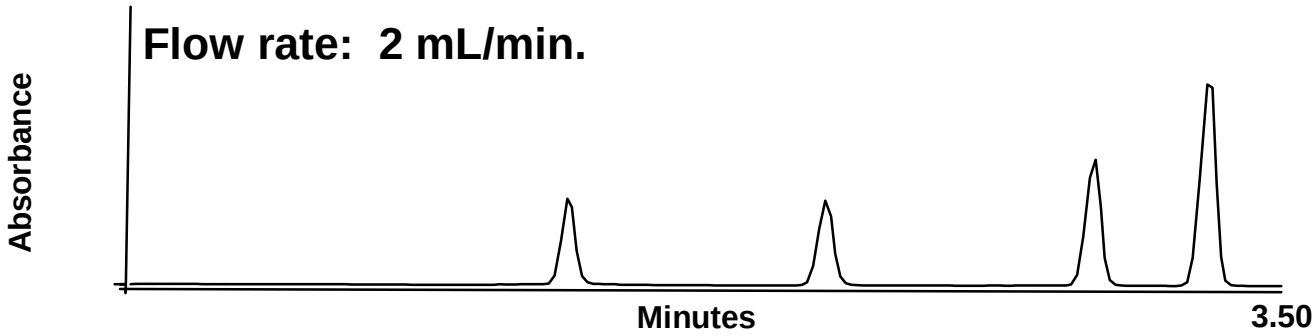
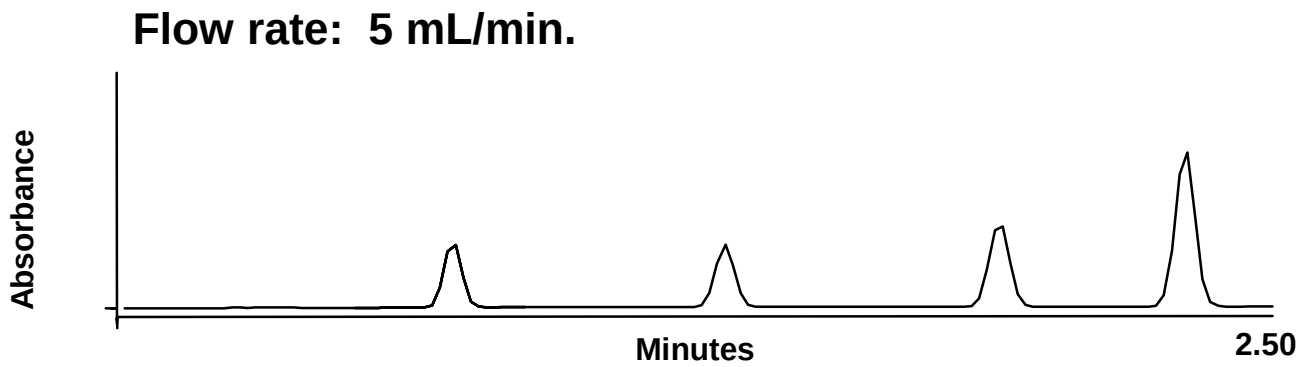
Impact of Flow Rate (F) on Resolution







Conditions:

Column: Symmetry® C_{18}, 5 µm, 4.6 X 50 mm
Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Gradient: 0-60% B in 4 minutes
Column temperature: 30.0 °C
Detector: 254 nm
Injection volume: 1 µL

- Resolution goes through an optimum due to the combination of gradient expansion and decrease in plate count

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Summary - Impact of Flow Rate on Resolution

- Resolution goes through an optimum due to the combination of gradient expansion and decrease in plate count
- Optimum resolution around 1 to 2 mL/min for most practical separation problems

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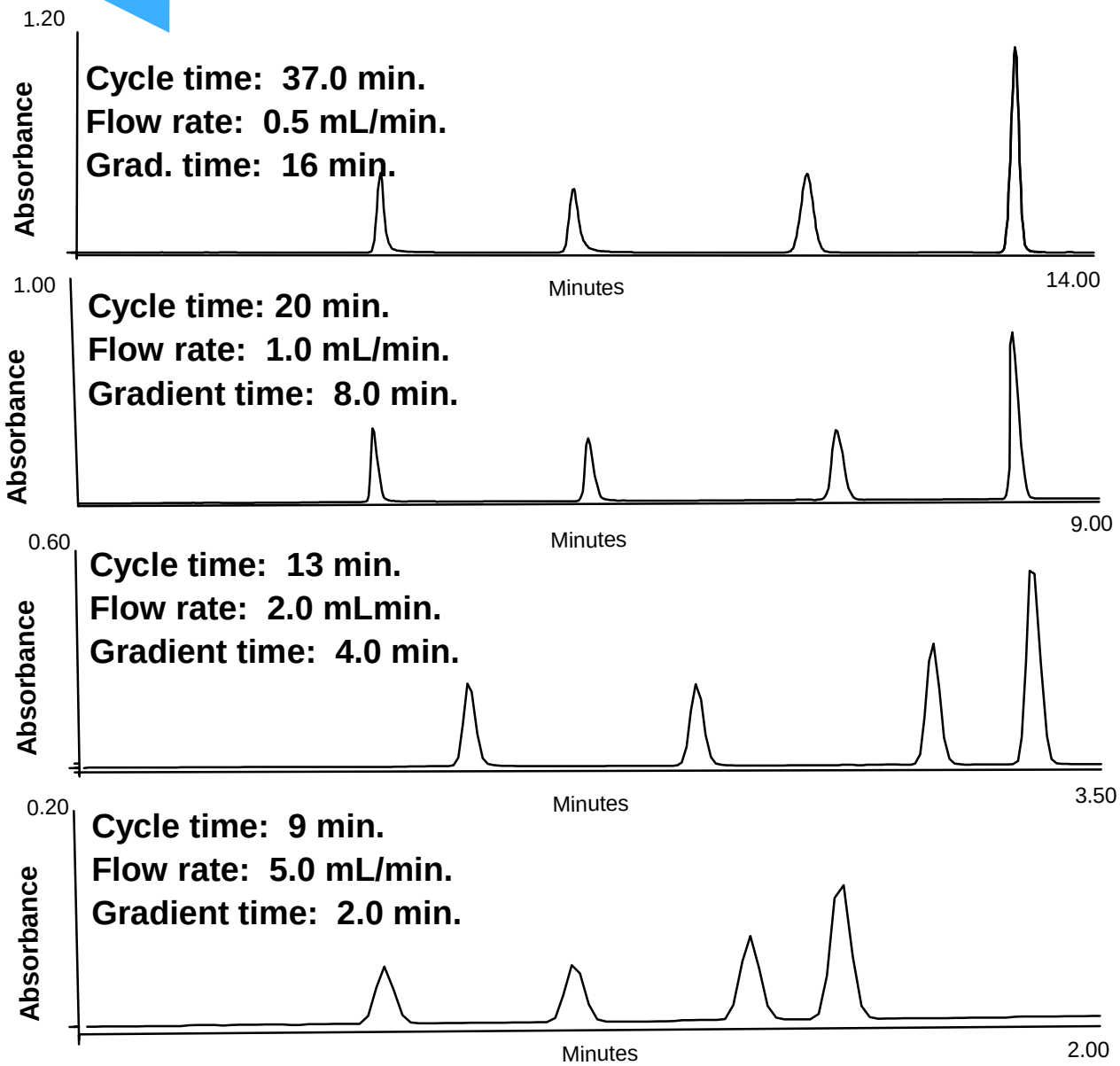
Optimization of Cycle Time Without Change in Elution Pattern



Conditions:

Column: Symmetry® C_{18}, 5 µm, 4.6 X 50 mm
Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Column temperature: 30.0 °C
Detector: 254 nm
Injection volume: 1 µL

-Flow rate increased proportional to gradient time decrease.

-Elution pattern is maintained as cycle time is decreased resulting in an increase in throughput.

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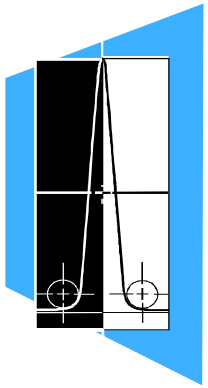

Summary - Optimization of Cycle Time Without Sacrificing Resolution

- To obtain the maximum sample throughput without sacrificing resolution the gradient time must be adjusted proportionally to the flow rate.

- As shown in the previous slide the sample throughput was increased by 800% upon increasing the flow rate to 5 mL/min. and reducing the gradient time to 2 minutes.

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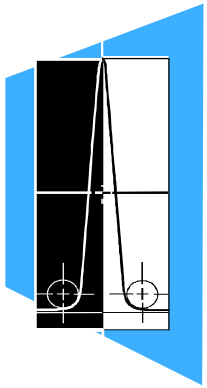

Impact of Column Length on Resolution

- **How Short is Too Short?**
  - It is not the column length which influences the separation in so much as the number of gradient volumes moving across the column.

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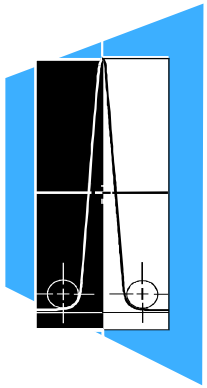

The Number of Column Volumes per Minute Impacts Resolution

- 2 Approaches:
  - Approach 1: scale gradient volume in proportion to the column volume (such as change the gradient run time with the column length).
  - Approach 2: do not scale the gradient volume in proportion to the column volume (such as keep the gradient run time constant while changing the column length).

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Column Volume to Gradient Volume Relationship (Approach 1)

-Gradient volume scaled to column volume

50 mm column

Column volume = 0.5 mL

5 minute gradient @ 1 mL/min

gradient volume = t_g x f.r. = 5

Total volume = g.v./c.v. = 10 column vols.

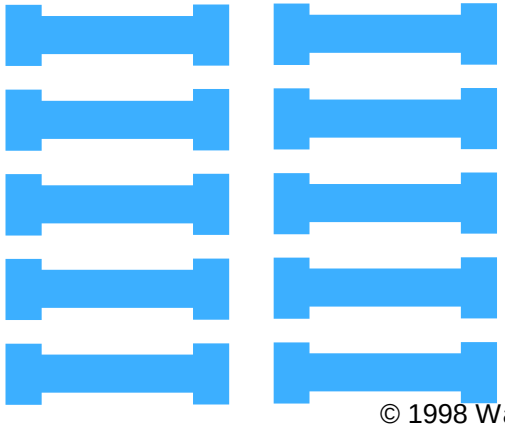
20 mm column

Column volume = 0.2 mL

2 minute gradient @ 1 mL/min

gradient volume = t_g x f.r. = 2

Total volume = g.v./c.v. = 10 column vols.



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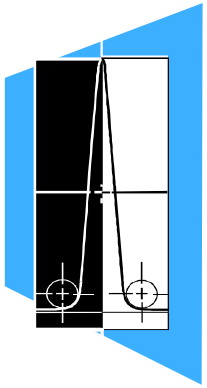

What Factors Influence Gradient RP-HPLC Separations...

- L (column length) is varied. Gradient volume is scaled in proportion to the column volume.

$$R_s = \frac{\Delta t}{\overline{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \cdot \boxed{\underbrace{\ln \alpha}_{\text{Selectivity}} \cdot \frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot \varepsilon_t \cdot \pi r^2 \cdot L/F + 1}}$$

Retention

Terms are constant

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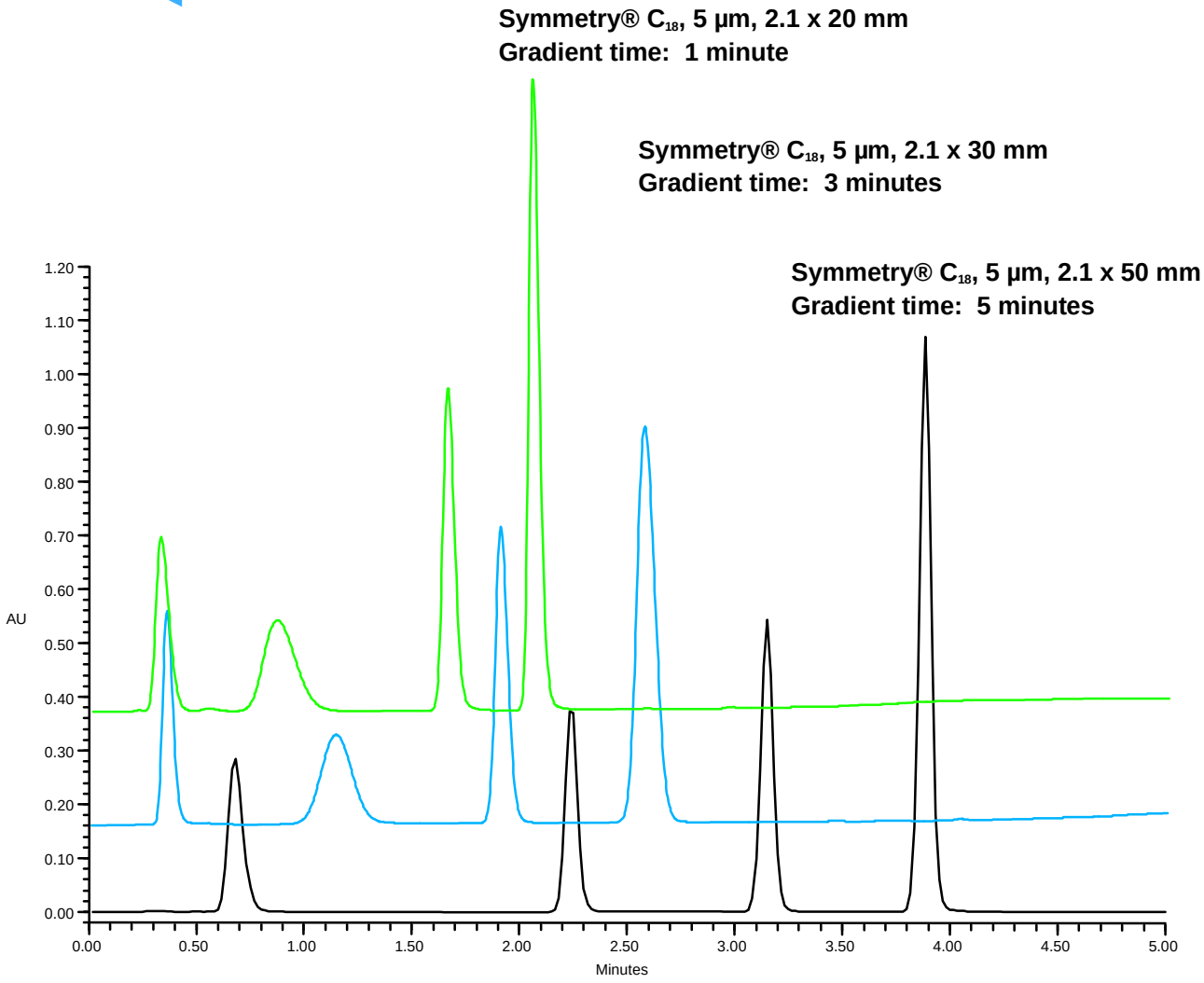
Impact of Column Length on Resolution (Approach 1)



Conditions:

Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Gradient: 0-60% B in noted time
Column temperature: 30.0 °C
Detector: 254 nm
Injection volume: 1 µL
Flow rate: 1 mL/min.

-Maintain resolution when scaling gradient volume proportionally to column volume.

-Reduce analysis time by >50%.

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Column Volume to Gradient Volume Relationship (Approach 2)

-Gradient volume not scaled to column volume

50 mm column

Column volume = 0.5 mL

5 minute gradient @ 1 mL/min

gradient volume = t_g x f.r. = 5

Total volume = g.v./c.v. = 10 column vols.

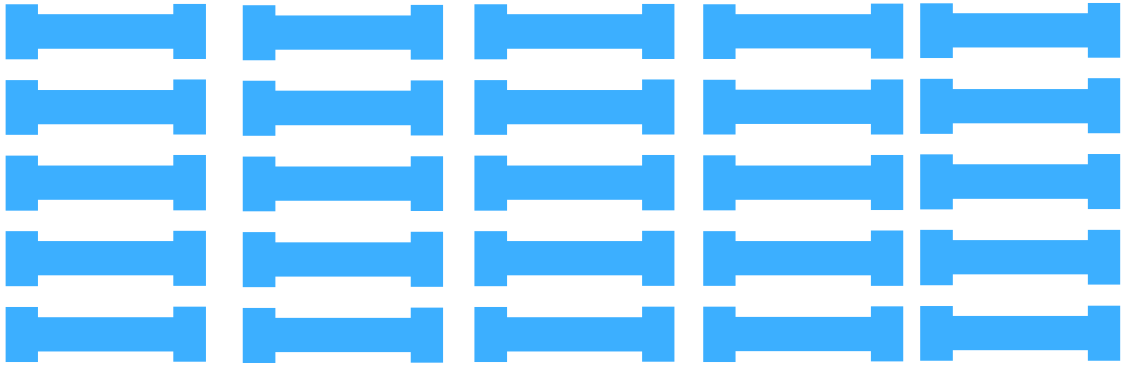
20 mm column

Column volume = 0.2 mL

5 minute gradient @ 1 mL/min

gradient volume = t_g x f.r. = 5

Total volume = g.v./c.v. = 2 column vols.



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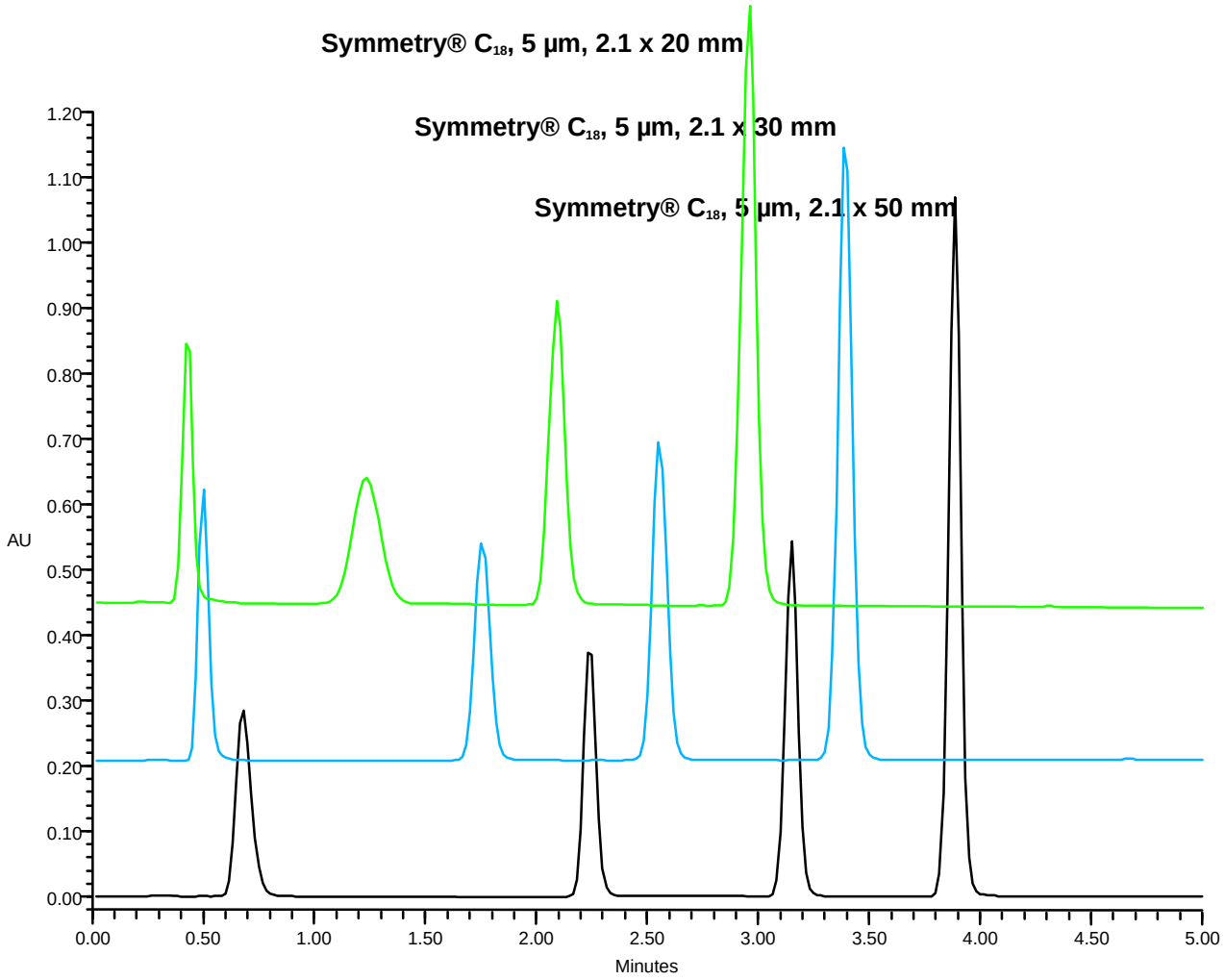
Impact of Column Length on Resolution (Approach 2)



Conditions:

Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Gradient: 0-60% B in 5 minutes
Column temperature: 30.0 °C
Detector: 254 nm
Injection volume: 1 µL
Flow rate: 1 mL/min.

-Maintain resolution by not scaling gradient volume proportionally to column volume. However maximun reduction of analysis time is not realized as when gradient volume is scaled.

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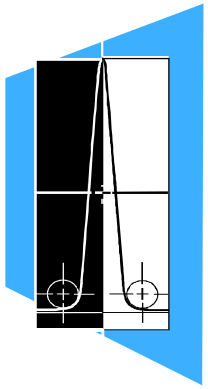

Summary - Impact of Column Length on Resolution

- Resolution is maintained when taking either approach (scaling or not scaling).
- However maximum sample throughput is realized when the gradient volume is scaled proportionally to the column volume.

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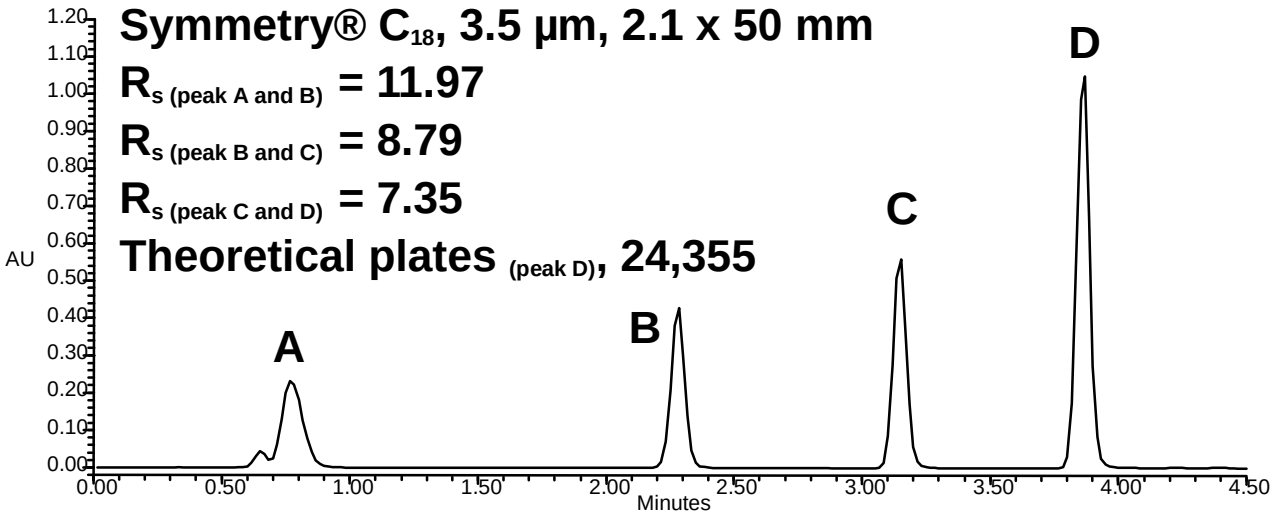
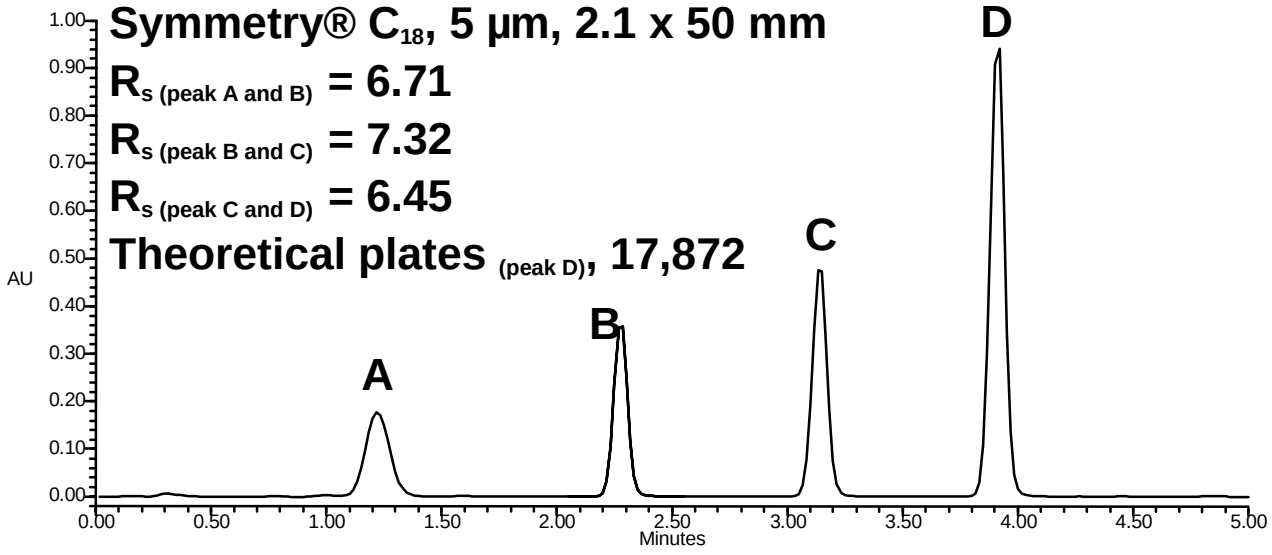
Impact of Particle Size (dp) on Resolution





Conditions:

Columns: Symmetry® C_{18}, 5 µm, 4.6 X 50 mm and Symmetry® C_{18}, 3.5 µm, 4.6 X 50 mm
Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Gradient: 0-60% B in 4 minutes
Column temperature: 30.0 °C
Detector: 254 nm
Injection volume: 1 µL

-Achieve increased resolution with the smaller particle size material in the same gradient time

-Increase throughput and resolution with smaller particle size if flow rate is increased

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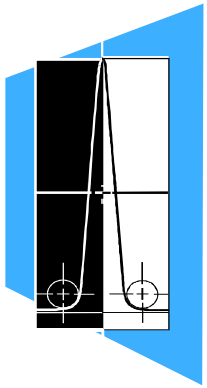

Summary - Impact of Particle Size on Resolution

- Resolution is increased as a result of using a smaller particle size. This is due to the increase in the number of theoretical plates.

- If the flow rate is increased as well as the particle size being decreased, an increase in sample throughput is realized with increasing resolution.

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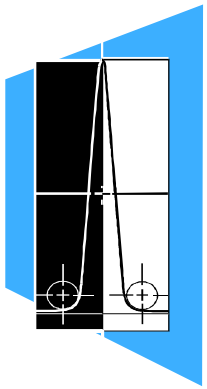

Conclusions

- To maximize resolution and sample throughput: use shorter columns with a smaller particle size (i.e. 2.1 X 20 or 10 mm, 3.5 µm) with faster flow rates and scale gradient volume in proportion to column volume.

- To maximize resolution with some increase in sample throughput: increase the flow rate or decrease the particle size.

- Areas of investigation: effects of DMSO and maximizing column lifetimes.

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