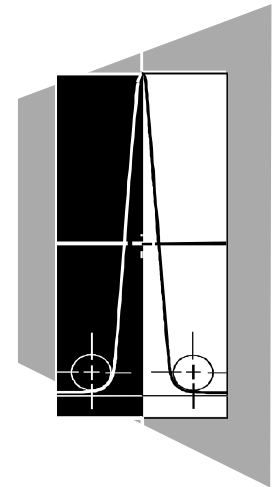


Gradient Operation in HPLC

Dr. Shulamit Levin
Medtechnica
levins@medtechnica.co.il

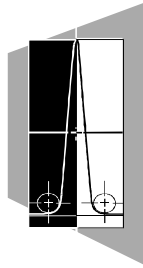
<http://www.forumsci.co.il/HPLC>
<http://shulalevin.tripod.com>



Gradient Operation in HPLC

Dr. Shulamit Levin
Medtechnica
levins@medtechnica.co.il

<http://www.forumsci.co.il/HPLC>
<http://shulalevin.tripod.com>

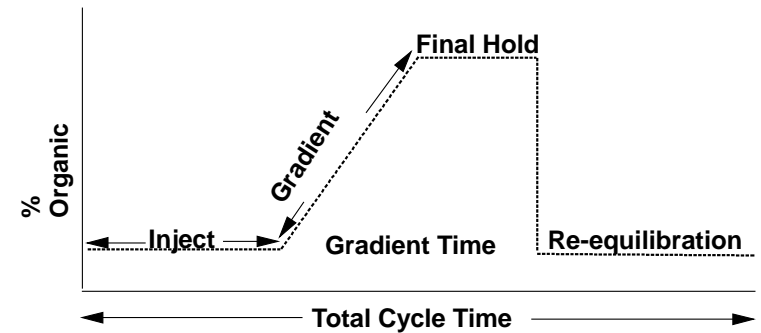


Outline

- Introduction
- Strategies for Higher Throughput Gradient Separations to Achieve Maximum Throughput and Optimal Resolution
 - ▶ system solutions
 - ▶ method solutions

Introduction - Optimizing Gradient Separations

- The following diagram illustrates the cycle time parameters that are used in a typical gradient



Typical Problems Encountered in Gradient Chromatography

- Non-reproducible retention times
- Difficulties to transfer from analytical to narrowbore columns
- Long reequilibration times
- Long cycle times (injection to injection)

More efficient analyses desired

Introduction - Options to Improve Sample Throughput

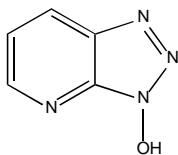
System Solutions:

- ▶ Reduce Gradient Delay Volume
- ▶ Decrease Re-equilibration time
- ▶ Reduce Injection Cycle time
- ▶ Modify Instrument
- ▶ Use Multiple Parallel Columns
- ▶ Adjust Detector Sampling Rate

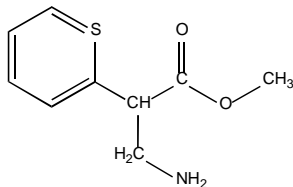
Method Solutions:

- ▶ Use Shorter Gradients
- ▶ Use Higher Flow Rates
- ▶ Use Shorter Columns
- ▶ Use a Smaller Particle Size
- ▶ Decrease Re-equilibration Time
- ▶ Increase Temperature

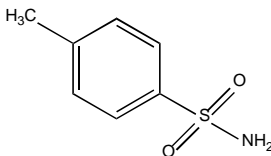
Test Probe Structures



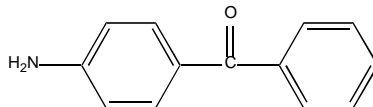
1-hydroxy-7-aza-benzotriazole



methyl 3-amino-2-thiophenecarboxylate



4-methylbenzene sulfonamide



4-aminobenzophenone

Outline

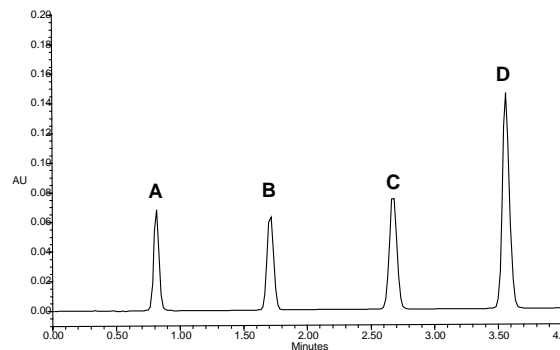
Introduction

Strategies for Higher Throughput Gradient Separations to Achieve Maximum Throughput and Optimal Resolution

- ▶ system solutions
 - reduce gradient delay volume
 - decrease re-equilibration time
 - reduce injection cycle time

- ▶ method solutions

Initial Separation and Conditions



Conditions:

Column: Symmetry® C₁₈, 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

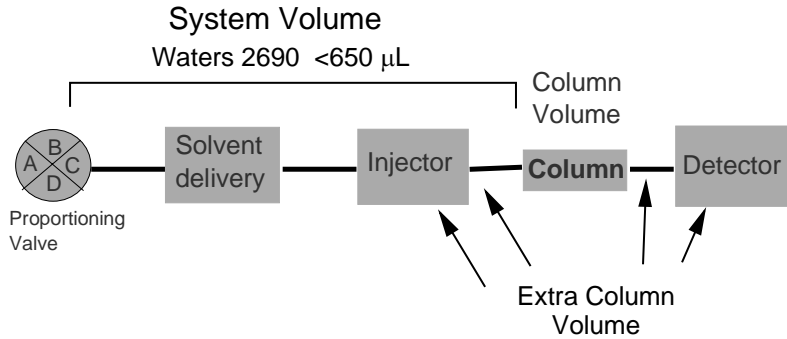
Flow rate: 1 mL/min.

Detector: 254 nm

Injection volume: 1 μL

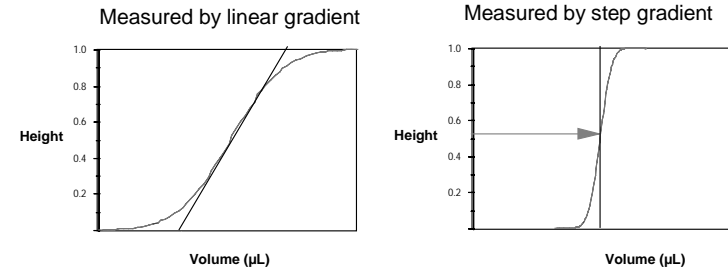
- A - 1-hydroxy-7-aza-benzotriazole
- B - 4-methylbenzene sulfonamide
- C - methyl 3-amino-2-thiophenecarboxylate
- D - 4-aminobenzophenone

Volumes in an HPLC System



Determination of System Precolumn Volume

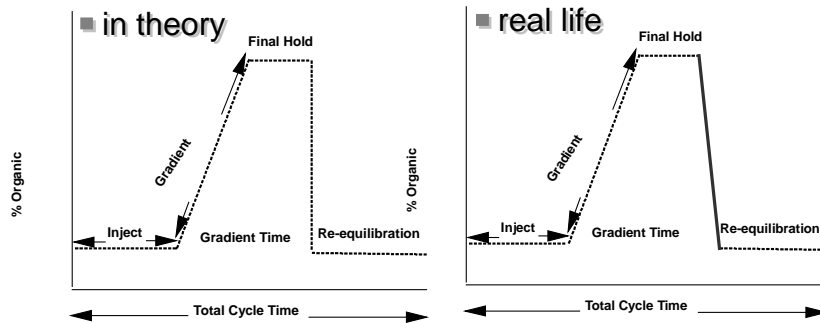
- **Definition:** Delay volume is the volume of plumbing between the point the gradient is formed and the inlet of the column.



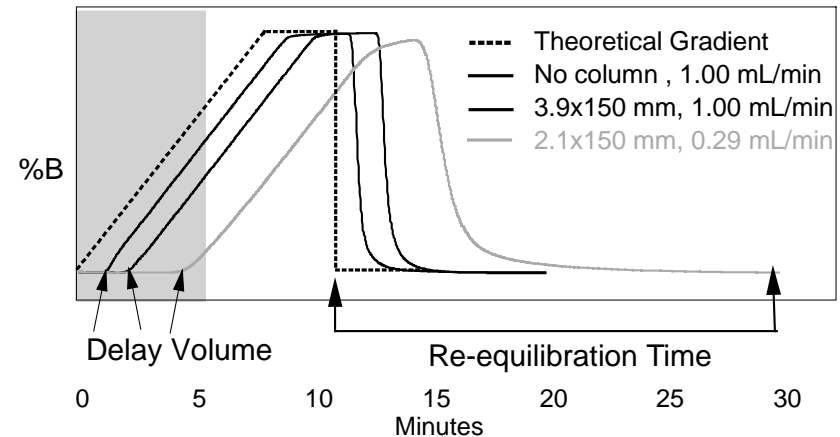
- **System components affecting dwell volume:**
 - Pump
 - Gradient Mixers
 - Injector

Effect of Precolumn Volume

- Reducing Delay Volume



Gradient Shape and Precolumn Volume



Outline

- Introduction
- Strategies for Higher Throughput Gradient Separations to Achieve Maximum Throughput and Optimal Resolution

- ▶ system solutions
 - reduce gradient delay volume
 - decrease re-equilibration time
 - reduce injection cycle time

- ▶ method solutions

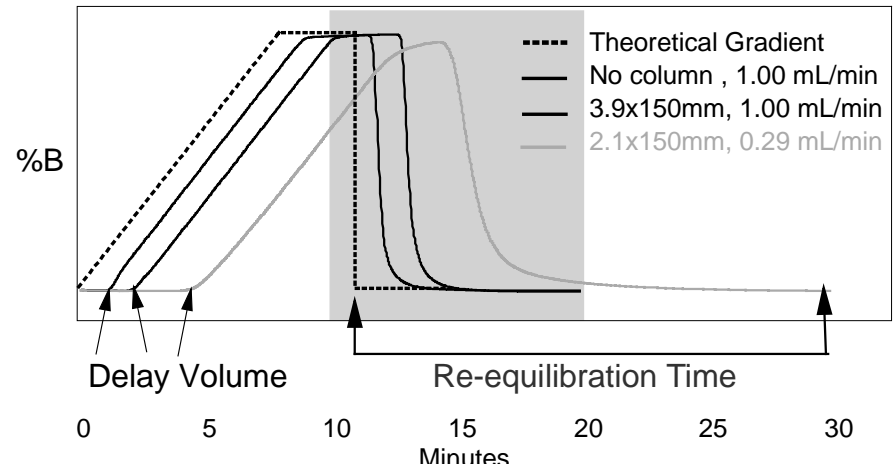
Calculation of Gradient Equilibration Volume

- Re-equilibration is a necessary part of gradient chromatography. Both the HPLC system and the column must be at initial conditions at the beginning of each run to ensure reproducible chromatographic separations.
- The re-equilibration volume can be divided into two parts, the system washout and the column re-equilibration.
- For good system/column equilibration

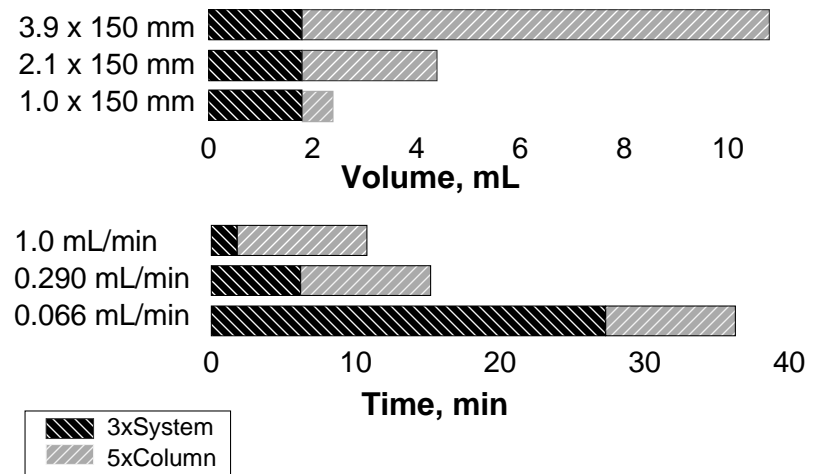
$$t_r = (3V_T + 5V_c)/F$$

where: t_r is the re-equilibration time in minutes,
 V_T is the total system volume,
 V_c is the column volume in mL
 F is the flowrate in mL/min.
 column volume = $0.7(\pi r^2 L/2)$
 system volume = 650-3000 μ L

Gradient Shape and Re-equilibration



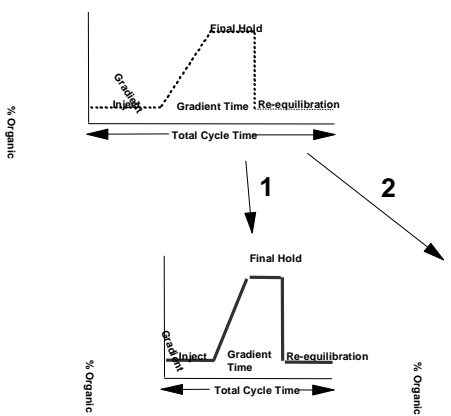
Column Re-equilibration



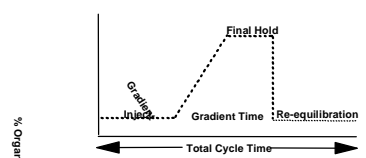
Reduction of Re-equilibration Time

Reduce Re-equilibration time, two Approaches:

1. increase flow rate
2. reduce column volume



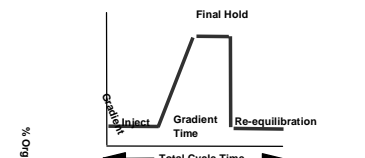
Reduction of Re-equilibration Time (Approach 1 - Increase Flow Rate)



Column: 3.9 X 50 mm

Column volume (c.v.) = 0.60 mL
 5 minute gradient @ 1 mL/min
 instrument delay volume (d.v.) = 650 µL
 gradient volume = $t_g \times c.v. = 3$

Total re-equilibration time, t_r
 $= \{3(0.65) + 5(0.7)(0.6)\}/1$
 $= 4.0/1$
 $= 4.0 \text{ min.}$



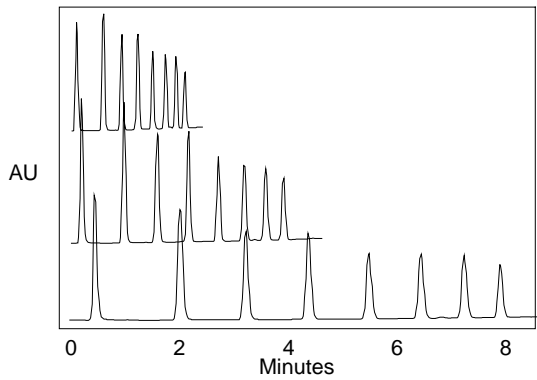
Column: 3.9 X 50 mm

Column volume (c.v.) = 0.60 mL
 5 minute gradient @ 2 mL/min
 instrument delay volume (d.v.) = 650 µL
 gradient volume = $t_g \times c.v. = 3$

Total re-equilibration time, t_r
 $= \{3(0.65) + 5(0.7)(0.6)\}/2$
 $= 4.0/2$
 $= 2.0 \text{ min.}$

re-equilibration time is reduced by 50%

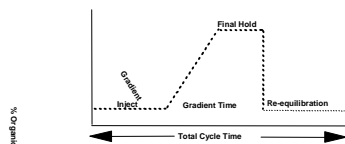
Reduction of Re-equilibration Time (Approach 1 - Increase flow rate)



Symmetry C18
 Alkylphenones
 3.9 x 50 mm
 40-100%B

3.0 min, 3 mL/min
 4.5 min, 2 mL/min
 9.0 min, 1 mL/min

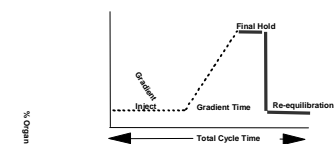
Reduction of Re-equilibration Time (Approach 2 - Reduce Column Volume)



Column: 2.1 X 50 mm

Column volume (c.v.) = 0.170 mL
 5 minute gradient @ 1 mL/min
 instrument delay volume (d.v.) = 650 µL
 gradient volume = $t_g \times c.v. = 0.85$

Total re-equilibration time, t_r
 $= \{3(0.65) + 5(0.7)(0.17)\}/1$
 $= 2.5 \text{ min.}$



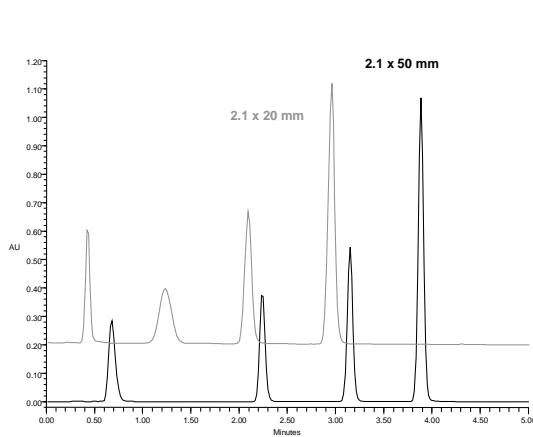
Column: 2.1 X 20 mm

Column volume (c.v.) = 0.069 mL
 5 minute gradient @ 1 mL/min
 instrument delay volume (d.v.) = 650 µL
 gradient volume = $t_g \times c.v. = 0.35$

Total re-equilibration time, t_r
 $= \{3(0.65) + 5(0.7)(0.069)\}/1$
 $= 2.0 \text{ min.}$

re-equilibration time is reduced by 20%

Reduction of Re-equilibration Time (Approach 2 - Reduce Column Volume)



Conditions:
 Symmetry® C₁₈, 5 μm
 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.

-Increase throughput by approximately 25% by reducing column volume from 0.170 (50 mm length) to 0.069 (20 mm length).

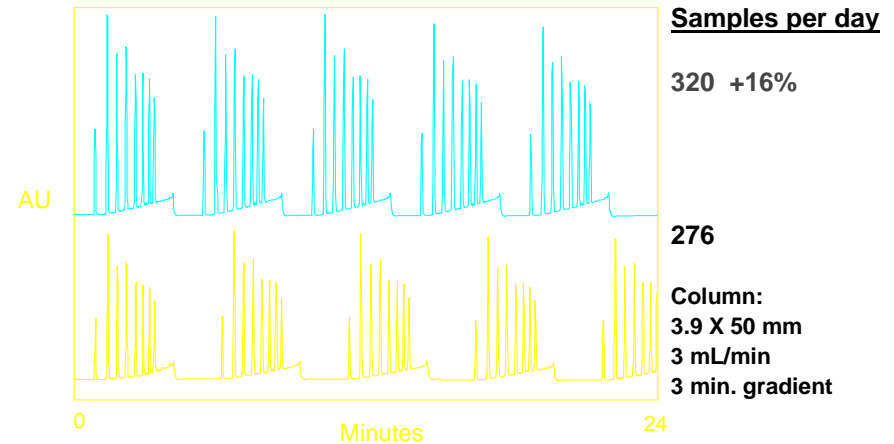
Outline

- Introduction
- Strategies for Higher Throughput Gradient Separations to Achieve Maximum Throughput and Optimal Resolution
 - ▶ system solutions
 - reduce gradient delay volume
 - decrease re-equilibration time
 - reduce injection cycle time
 - ▶ method solutions

Shorter Cycle Time

Reducing Total Cycle Time

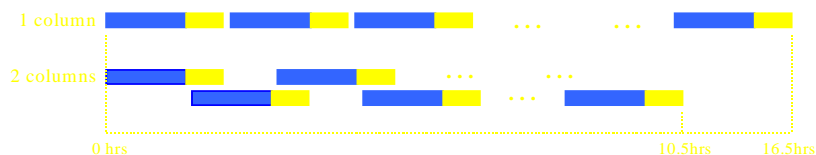
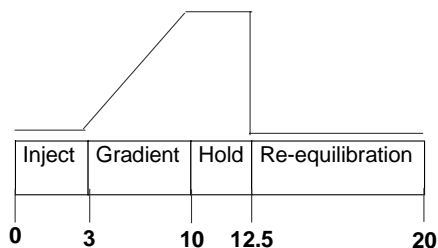
- Reduce Cycle Times by:
 - ▶ Programming a system purge in the method which occurs during the injection of the sample or...
 - ▶ Employing two columns and performing column switching.



Higher Throughput Through Column Switching

Column: Symmetry®, C₁₈, 5 µL, 19 X 50 mm
 Flow Rate: 20 mL/min.
 Re-equilibration requires 5 column volumes = 150 mL = 7.5 min.
 Re-equilibration period = unused time

Column switching can reduce runtimes by approx. 30%



Note: a second pump must be employed

Summary - System Solutions

- ▶ Reducing Gradient Delay Volume
 - Use 0.12 mm (0.005") i.d. tubing instead of 0.25 mm (0.009") to reduce system volume;
 - Shorten all tubing lengths;
 - Reduce the extra-column volume in the auto-injector by employing a smaller loop
 - Remove gradient mixers

Summary - System Solutions (cont'd)

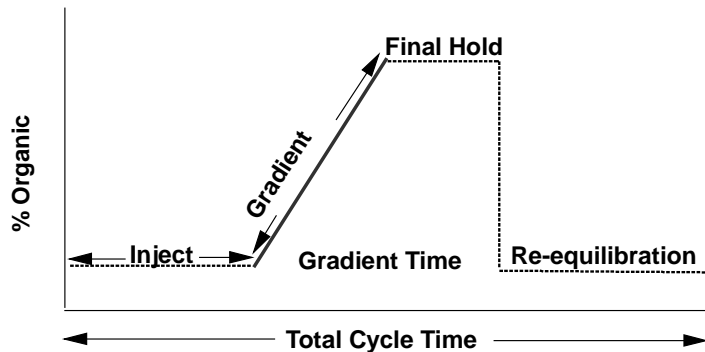
- Achieve Faster Gradient Chromatography By...
 - ▶ Reducing Re-equilibration Time
 - Reduce column volume
 - Increase flow rate
 - ▶ Reducing Cycle Time
 - Program injection to occur during re-equilibration
 - Implement column switching

Outline

- Introduction
- Strategies for Higher Throughput Gradient Separations to achieve maximum throughput and maximize resolution
 - ▶ system solutions
 - ▶ method solutions
 - use shorter gradients
 - use higher flow rates
 - use shorter columns
 - use smaller particle sizes
 - increase temperature

Optimizing Separations

- The following diagram illustrates the cycle time parameters that were optimized to achieve high throughput goals.



What Factors Influence Gradient RP-HPLC Separations...

- ...further derivatization of this term shows the relationship between resolution and flow rate, F , and column length, L , or column volume, $\pi r_2^2 L/2$.

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

$\frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot \varepsilon_t \cdot \pi r_2^2 \cdot L/F + 1}$

- It is the effect of these variables, F , t_g and L , that we will investigate.

Factors Influencing Resolution in Gradient RP-HPLC Separations...

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

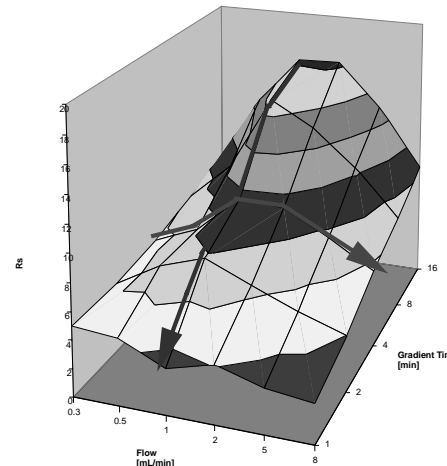
$$R_s = \frac{\Delta t}{W} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} * \underbrace{\ln \alpha}_{\text{Selectivity}} * \underbrace{\frac{1}{Bct_0 + 1}}_{\text{Retention}}$$

$c = \%B/\text{minute} = \frac{\Delta\%}{t_g}$

$$\frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot t_0 + 1}$$

- Upon substitution of the actual variables ($\Delta\%/t_g$ (gradient time)) for c , gradient slope, one can see the relationship between gradient time and resolution, and...

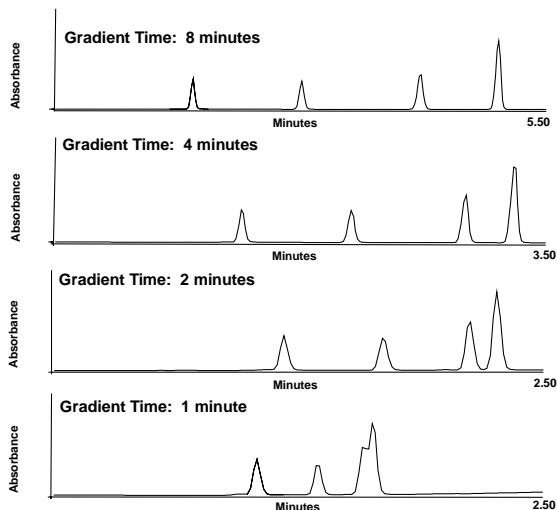
Resolution Dependence on Gradient Time and Flow Rate for a Gradient Method (Symmetry® C₁₈, 4.6 X 50 mm, 5 μm)



$$R_s = \frac{\Delta t}{W} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} * \underbrace{\ln \alpha}_{\text{Selectivity}} * \underbrace{\frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot \varepsilon_t \cdot \pi r_2^2 \cdot L/F + 1}}_{\text{Retention}}$$

- Effect of changing gradient run time, t_g
- Effect of changing flow rate, F

Impact of Reducing Gradient Time (t_g) on Resolution



Conditions:

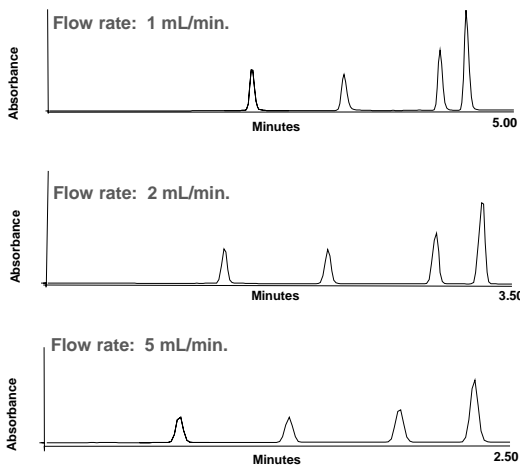
Column: Symmetry® C₁₈, 5 μm, 4.6 X 50 mm
 Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
 Gradient: 0-60% B in noted gradient time
 Column temperature: 30.0 °C
 Flow rate: 1 mL/min.
 Detector: 254 nm
 Injection volume: 1 μL

-Longest gradient time provides best resolution

-Shortest gradient time maximizes throughput

-Reducing just gradient time sacrifices resolution

Impact of Flow Rate (F) on Resolution



Conditions:

Column: Symmetry® C₁₈, 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

Summary - Impact of Gradient Time on Resolution

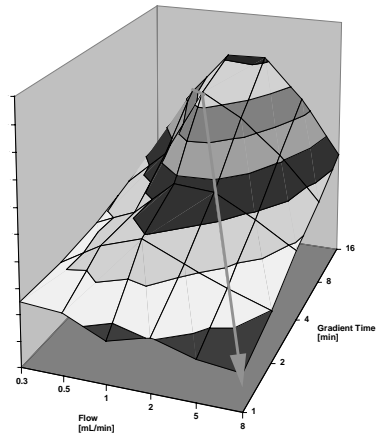
- Resolution increases as gradient time increases
- Throughput decreases as gradient time increases

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 is approximately 1 to 2 mL/min for most practical separation problems

Resolution Dependence on both Flow Rate and Gradient Time for a Gradient Method

(Symmetry® C₁₈, 4.6 X 50 mm, 5 μm)

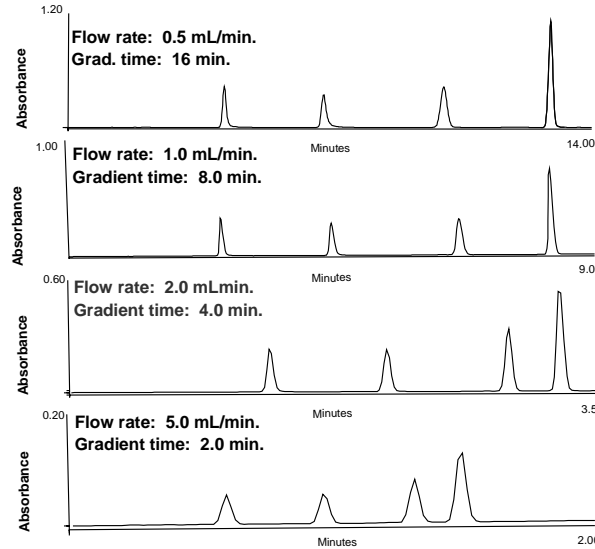


$$R_s = \frac{\Delta f}{W} \sim \frac{\sqrt{N}}{4} \Delta \ln \alpha \cdot \frac{1}{B \cdot \frac{t_g}{t_r} \cdot \epsilon \cdot O_r \cdot L/F + 1}$$

Efficiency Selectivity Retention

3. Effect of changing gradient run time, t_g , and flow rate, F

Reduction of Cycle Time



Conditions:

Column: Symmetry® C₁₈, 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

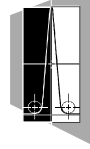
- ▶ Flow rate increased proportional to gradient time decrease.
- ▶ Elution pattern is maintained as cycle time is decreased resulting in an increase in throughput.

Summary - Reduction of Cycle Time

- To obtain the maximum sample throughput the gradient time must be adjusted inversely 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.

Outline

- Introduction
- Strategies for Higher Throughput Gradient Separations to achieve maximum throughput and maximize resolution
 - ▶ system solutions
 - ▶ method solutions
 - use shorter gradients
 - use higher flow rates
 - use shorter columns
 - use smaller particle sizes
 - increase temperature



The Number of Column Volumes per Minute Impacts Resolution

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.


■ 2 Approaches:

- Approach 1: Gradient volume is not proportion to the column volume (gradient run time constant while changing the column length).
- Approach 2: Scale gradient volume in proportion to the column volume (change the gradient run time proportionally with the column length).

Column Volume to Gradient Volume Relationship (Approach 1)

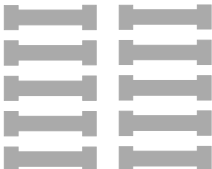
-Gradient volume not scaled to column volume

50 mm column




Column volume = 0.5 mL
5 minute gradient @ 1 mL/min

gradient volume = $t_g \times f.r. = 5$
Total volume = $g.v./c.v. = 10$ column vols.

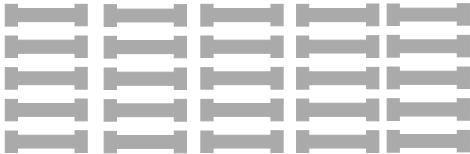


20 mm column



Column volume = 0.2 mL
5 minute gradient @ 1 mL/min


gradient volume = $t_g \times f.r. = 5$
Total volume = $g.v./c.v. = 2$ column vols.



Column Volume to Gradient Volume Relationship (Approach 2)

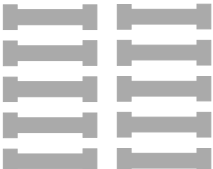
-Gradient volume scaled to column volume

50 mm column




Column volume = 0.5 mL
5 minute gradient @ 1 mL/min

gradient volume = $t_g \times f.r. = 5$
Total volume = $g.v./c.v. = 10$ column vols.

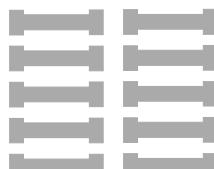


20 mm column



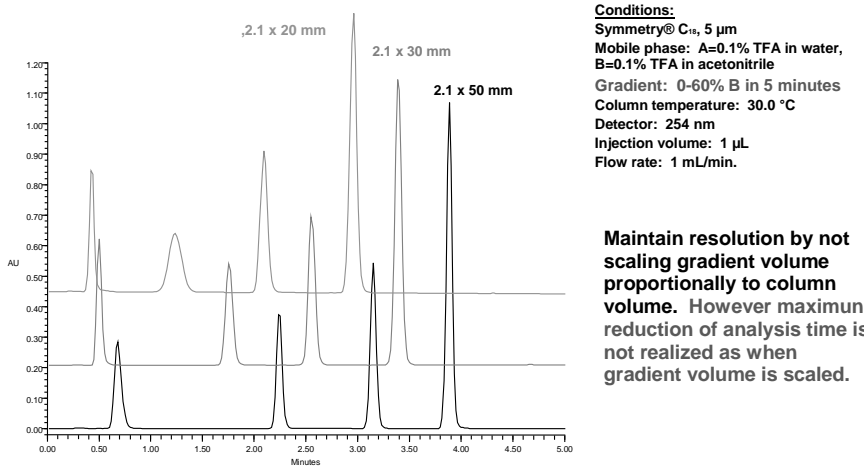
Column volume = 0.2 mL
2 minute gradient @ 1 mL/min

gradient volume = $t_g \times f.r. = 2$
Total volume = $g.v./c.v. = 10$ column vols.



Impact of Column Length on Resolution (Approach 1)

-Gradient volume not scaled to column volume



What Factors Influence Gradient RP-HPLC Separations...

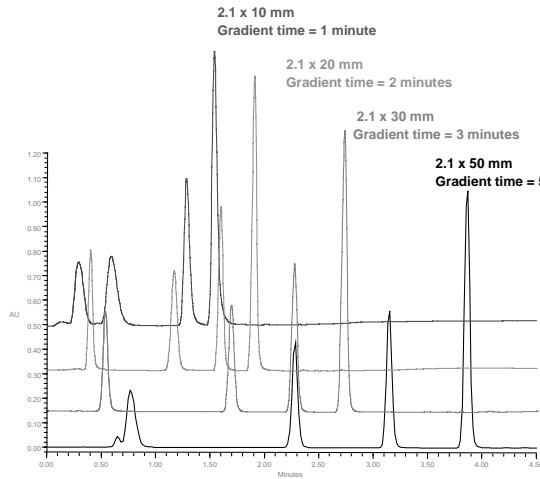
► L (column length) is varied. Gradient volume is scaled in proportion to the column volume.

$$R_s = \frac{\Delta t}{W} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} * \underbrace{\ln \alpha}_{\text{Selectivity}} * \underbrace{\frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot \epsilon_t \cdot \pi r_2 \cdot L/F + 1}}_{\text{Retention}}$$

Terms are constant

Impact of Column Length on Resolution (Approach 2)

-Gradient volume scaled to column volume

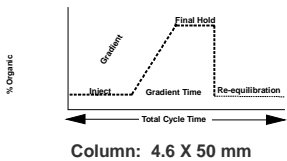


Conditions:
 Symmetry® C₁₈, 3.5 μm
 Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
 Gradient: 0-60% B in noted time
 Column temperature: 30.0 °C
 Detector: 254 nm
 Injection volume: 1 μL
 Flow rate: 1 mL/min.

-Reduce analysis time by >50%.

-Trade-off: reduction in resolution

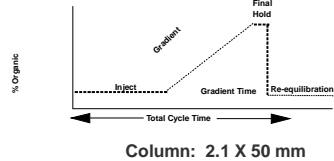
Gradient Delay Time



Column: 4.6 X 50 mm

Column volume (c.v.) = 0.83 mL
 5 minute gradient @ 1 mL/min
 instrument delay volume (d.v.) = 650 μL
 gradient volume = $t_g \times c.v. = 6$
 Total system volume = $0.7(c.v.) + d.v.$
 = 1.2 mL

Column re-equilibration time = total vol./f.r.
 = 1.15/1
 = 1.2 min.



Column: 2.1 X 50 mm

Column volume (c.v.) = 0.17 mL
 5 minute gradient @ 0.2 mL/min
 instrument delay volume (d.v.) = 650 μL
 gradient volume = $t_g \times c.v. = 6$
 Total system volume = $0.7(c.v.) + d.v.$
 = 0.72 mL

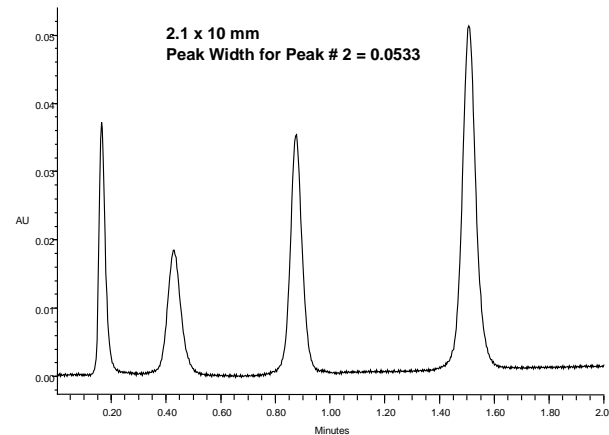
Column re-equilibration time = total vol./f.r.
 = 0.67/0.2
 = 3.6 min.

gradient is delayed by a factor of 3

Summary - Impact of Column Length on Resolution

- Maximum sample throughput is realized when the gradient volume is scaled proportionally to the column volume.

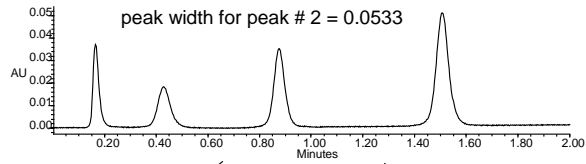
Impact of the Number of Column Volumes on Peak Shape



Conditions:
 Symmetry® C₁₈, 5 μm
 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
 Flow rate: 2 mL/min.

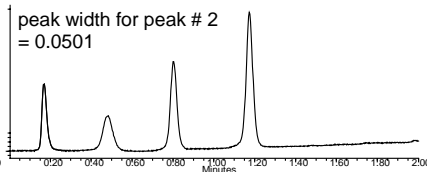
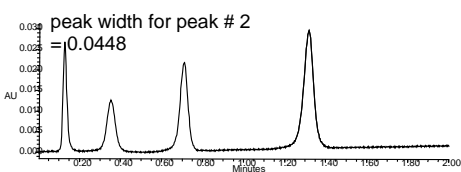
Reducing the Effect of Gradient Delay Volume

- Make gradient steeper by increasing the flow rate or decreasing the gradient time

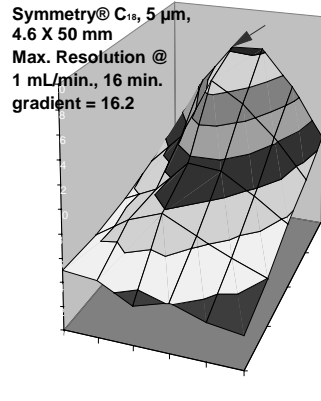
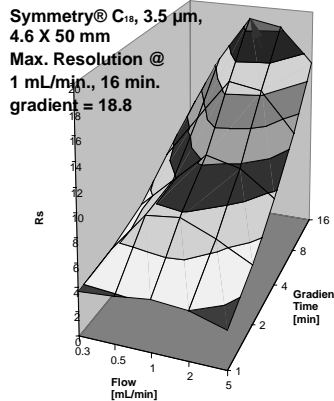


Increase flow rate (2 to 3 mL/min.)

Increase gradient slope by 50% (4 to 2 min. grad)



Comparison of Resolution Dependence on Particle Size



Outline

Introduction

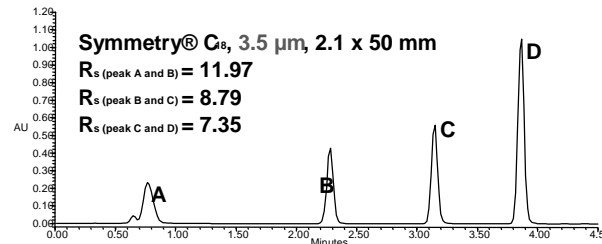
- Strategies for Higher Throughput Gradient Separations to achieve maximum throughput and maximize resolution

system solutions

method solutions

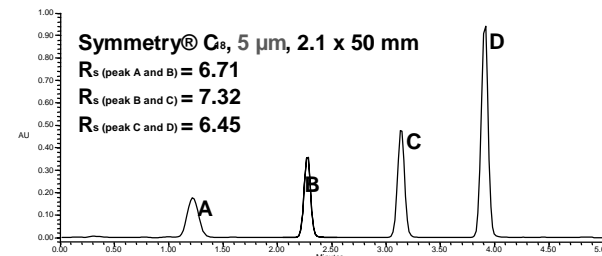
- use shorter gradients
- use higher flow rates
- use shorter columns
- use smaller particle sizes
- increase temperature

Impact of Particle Size (dp) on Resolution



Conditions:

Columns: Symmetry® C₈ 3.5 µm, 4.6 X 50 mm and Symmetry® C₈ 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
Flow rate: 1 mL/min.



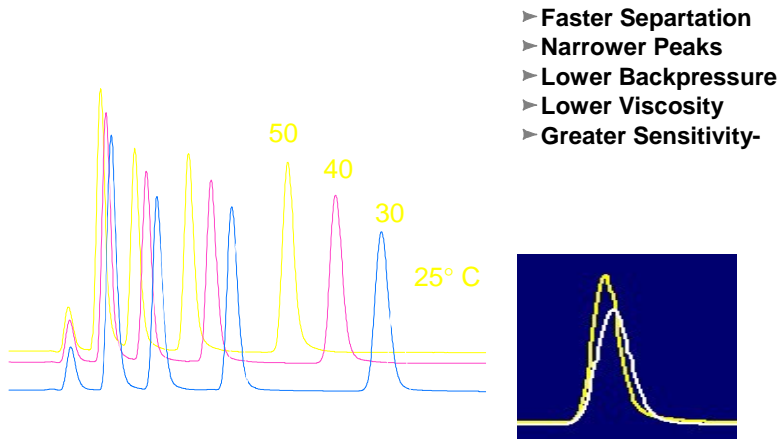
-Achieve increased resolution with the smaller particle size material in the same gradient time

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

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.

Impact of Temperature



Outline

- Introduction
- Strategies for Higher Throughput Gradient Separations to achieve maximum throughput and maximize resolution
 - ▶ system solutions
 - ▶ method solutions
 - use shorter gradients
 - use higher flow rates
 - use shorter columns
 - use smaller particle sizes
 - increase temperature

Summary - Method Solutions

- To obtain the fastest throughput:
 - ▶ increase flow rate
 - ▶ decrease column volume
 - ▶ decrease particle size
 - ▶ scale gradient volume with decrease in column volume
 - ▶ increase temperature to reduce viscosity of mobile phase allowing increases in flow rate