Gradient Operation in HPLC

Dr. Shulamit Levin

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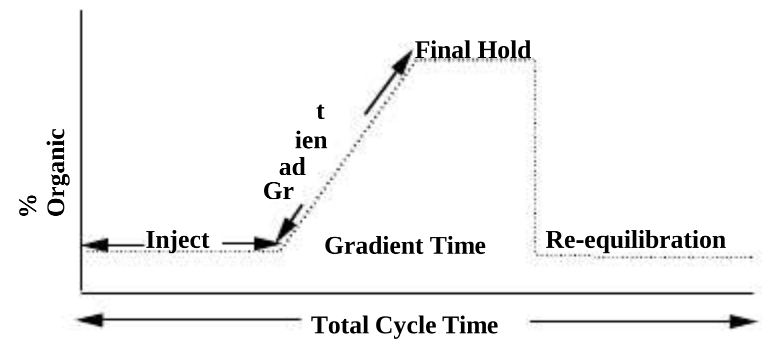
Introduction - Optimizing Gradient Separations

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



Outline

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

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

Outline

- Introduction
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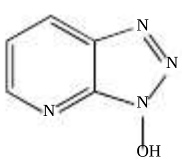
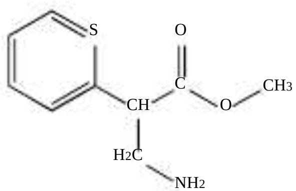
  - systemsolutions
    - reduce gradient delay volume
    - decreasere-equilibration time
    - reduce injection cycle time
  - method solutions

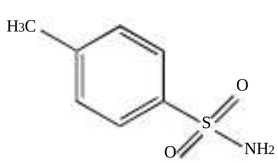
Test Probe Structures



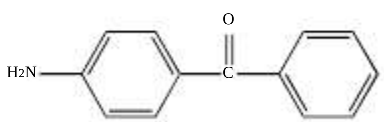
1-hydroxy-7-aza-benzotriazole



methyl 3-amino-2-thiophenecarboxylate



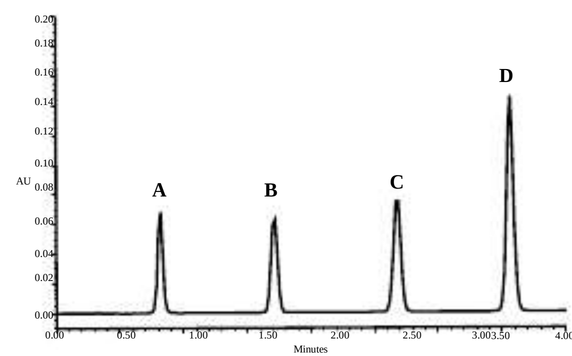
4-methylbenzene sulfonamide



4-aminobenzophenone

H. Weller, Bristol-Myers Squibb Pharmaceutical Research Institute

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

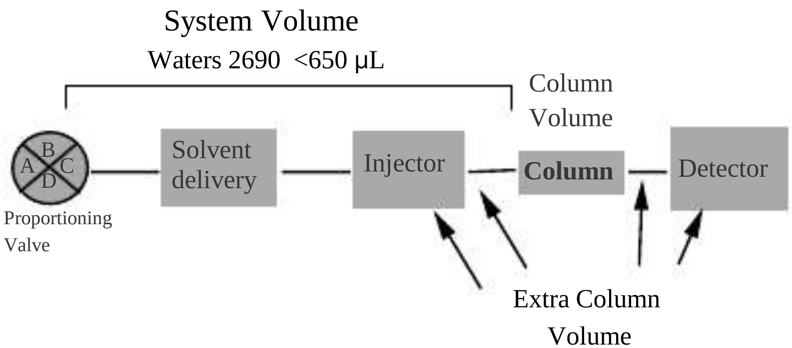
Column temperature: 30.0 ° C

Flow rate: 1 mL/min.

Detector: 254 nm

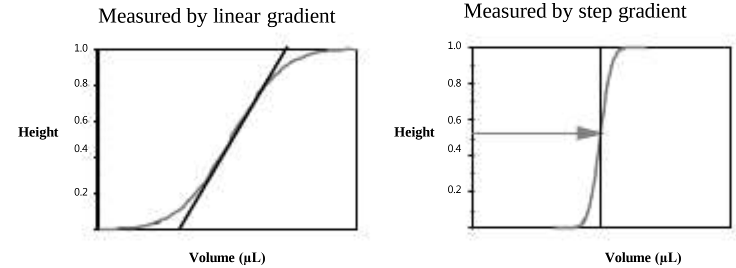
Injection volume: 1 µL

Volumes in an HPLC System



Determination of System Precolumn Volume

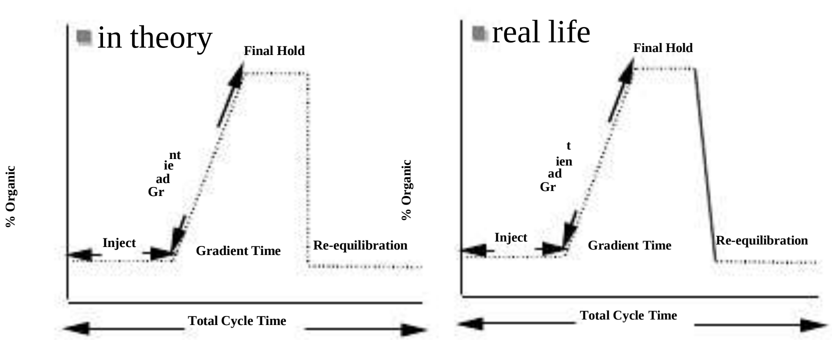
- **Definition: Delay volume is the volume of plumbing between the point the gradient is formed and the inlet of the column.**



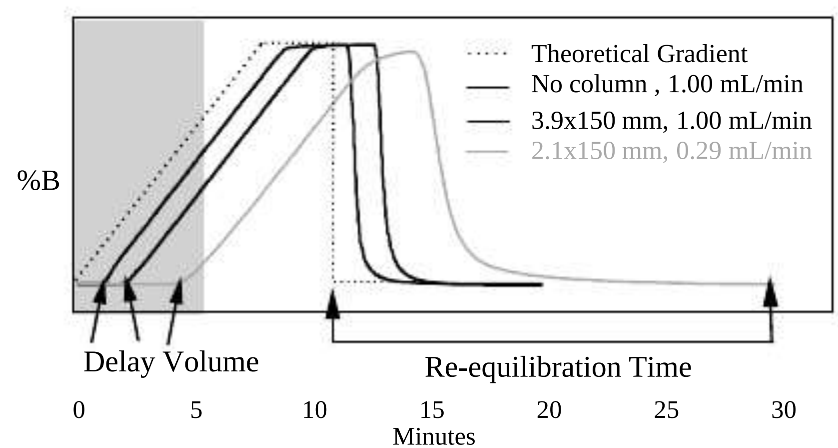
- **System components affecting dwell volume:**
  - **–Pump**
  - **–Gradient Mixers**
  - **–Injector**

Effect of Precolumn Volume

- Reducing Delay Volume



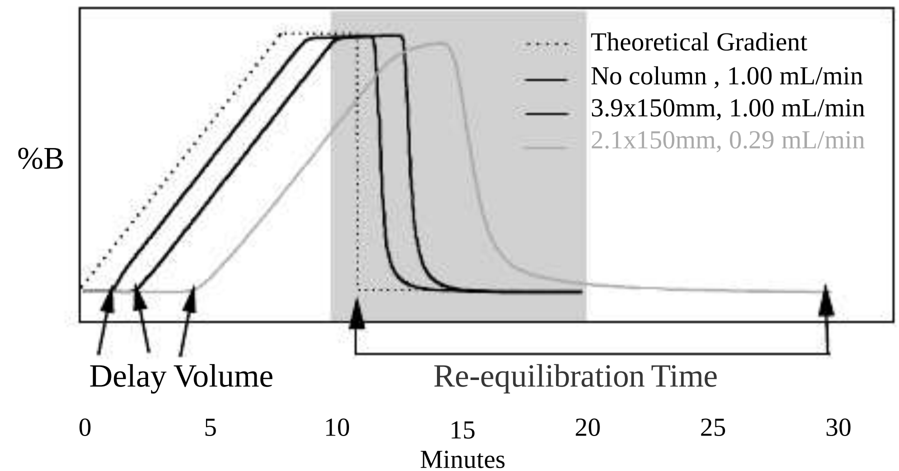
Gradient Shape and Precolumn Volume



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

Gradient Shape and Re-equilibration



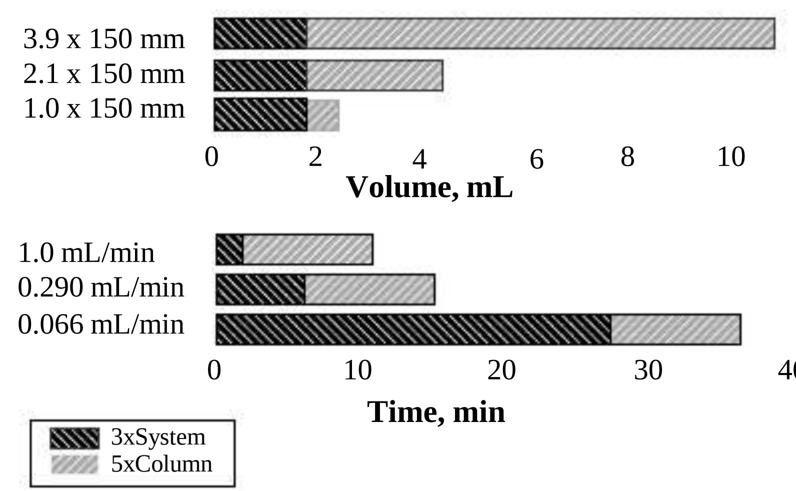
Calculation of Gradient Equilibration Volume

- 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

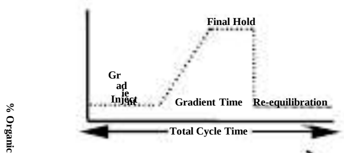
$$t_{rr} = (3V_T + 5V_c)/F$$

where: t_{rr} **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 µL

Column Re-equilibration



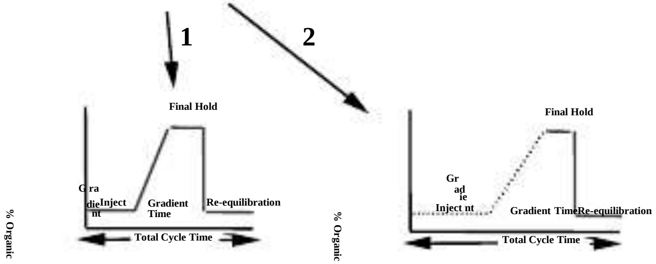
Reduction of Re-equilibration Time



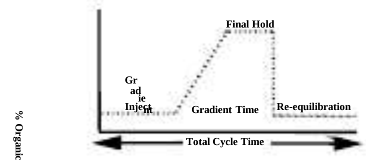
Reduce Re-equilibration time, two Approaches:

1. increase flow rate

2. reduce column volume



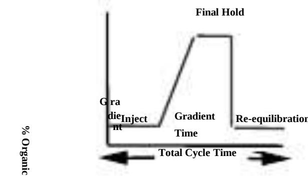
Reduction of Re-equilibration Time
(Approach 1 - Increase Flow Rate)



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 x c.v. =3

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



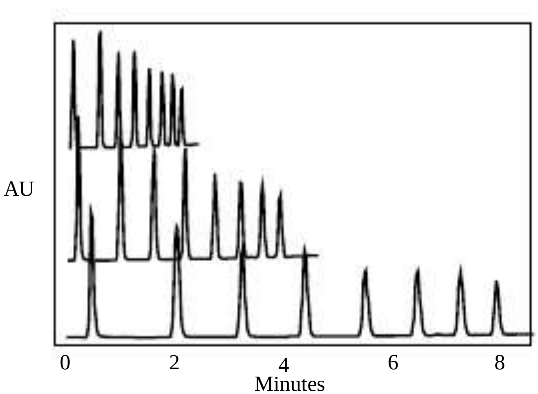
Column: 3.9 X 50 mm

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

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

re-equilibration time is reduced by 50%

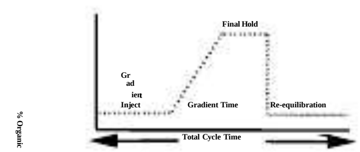
Reduction of Re-equilibration Time
(Approach 1 - Increase flow rate)



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**

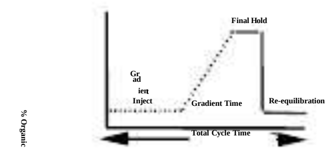
Reduction of Re-equilibration Time
(Approach 2 - Reduce Column Volume)



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 x c.v. = 0.85

Total re-equilibration time, t_r
= {3(0.65) + 5(0.7)(0.17)}/1
= 2.5 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 x c.v. = 0.35

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

re-equilibration time is reduced by 20%

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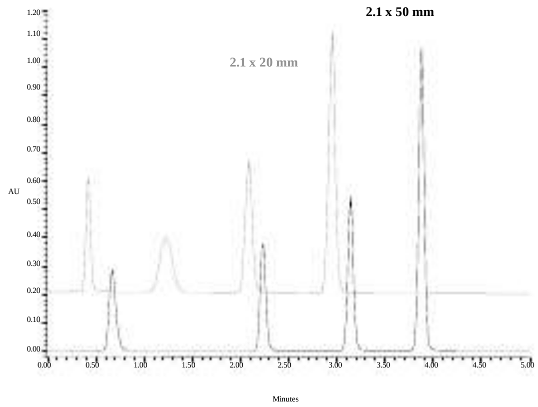
Reduction of Re-equilibration Time (Approach 2 - Reduce Column Volume)



Conditions:

Symmetry® C_{18}, 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).

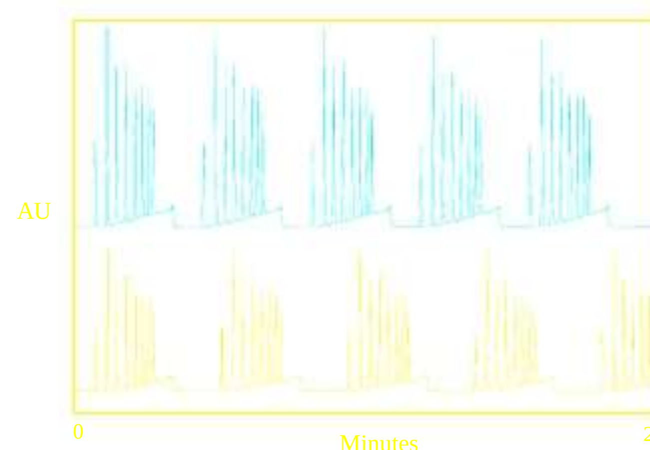
Outline

- 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

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.

Faster Chromatography - Shorter Cycle Time



Samples per day

320 +16%

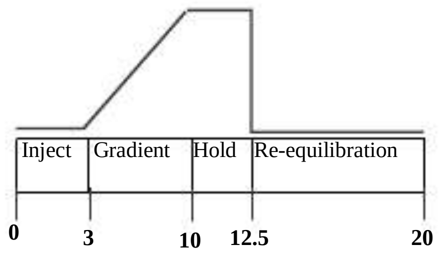
276

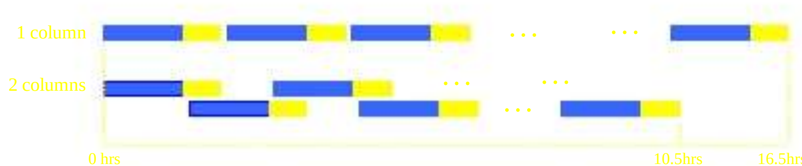
Column:
3.9 X 50 mm
3 mL/min
3 min. gradient

Higher Throughput Through Column Switching

Column: Symmetry®, C_{18}, 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**

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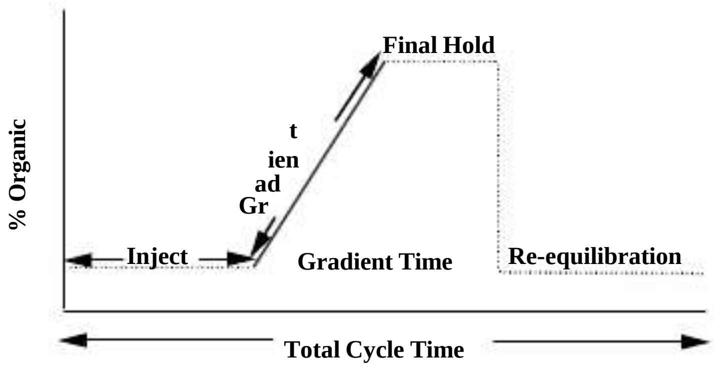
Optimizing Separations

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



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

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

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

c = %B/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....**

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, $\pi r^2 L/2$.

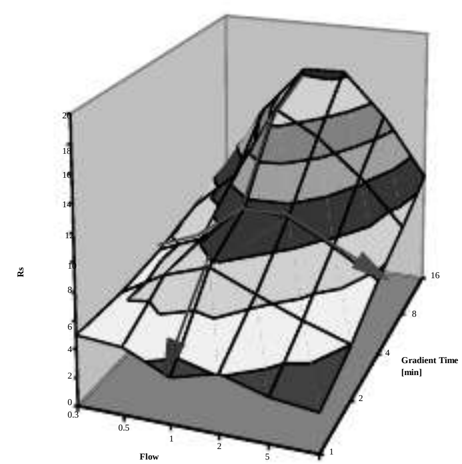
$$R_s = \frac{\Delta t}{\bar{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} * \underbrace{\ln\alpha}_{\text{Selectivity}} * \underbrace{\frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot t_0 + 1}}_{\text{Retention}}$$

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

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

Resolution Dependance on Gradient Time and Flow Rate for a Gradient Method

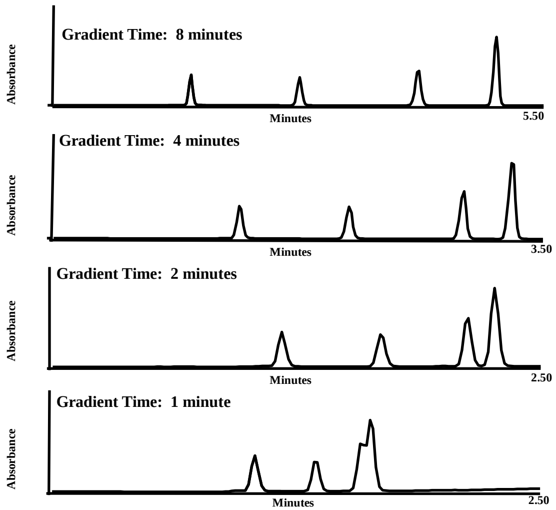
(Symmetry® C_{18}, 4.6 X 50 mm, 5 µm)



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

1. Effect of changing gradient run time, t_g
2. Effect of changing flow rate, F

Impact of Reducing Gradient Time (t_g) on Resolution



Conditions:

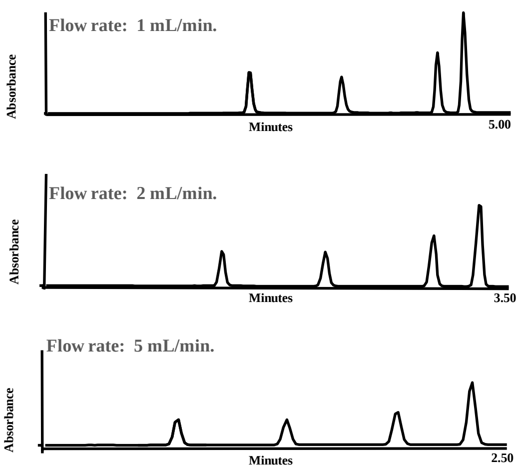
Column: Symmetry® C_{18}, 5 µm, 4.6 X 50 mm
Mobile phase: A=0.1% TFA in water, B=0.1% TFA in acetonitrile
Gradient: 0-60% B in noted gradient time
Column temperature: 30.0 ° C
Flow rate: 1 mL/min.
Detector: 254 nm
Injection volume: 1 µL

-Longest gradient time provides best resolution

-Shortest gradient time maximizes throughput

-Reducing just gradient time sacrifices resolution

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

Summary - Impact of Gradient Time on Resolution

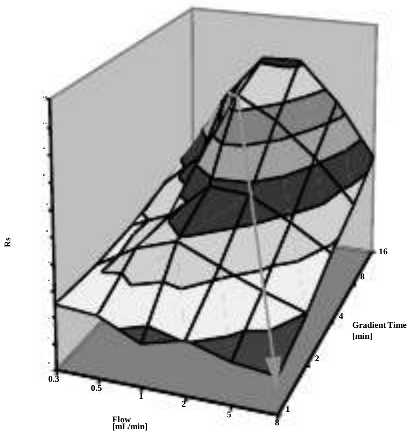
- Resolution increases as gradient time increases
- Throughput decreases as gradient time increases

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 Dependance on both Flow Rate and Gradient Time for a Gradient Method

(Symmetry® C_{18}, 4.6 X 50mm, 5 µm)

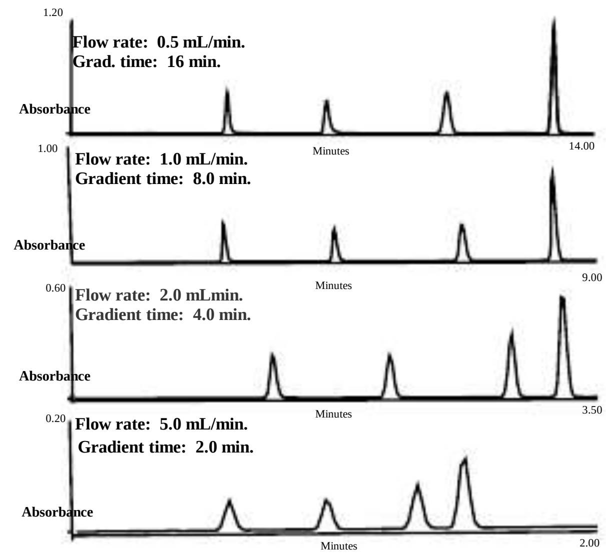


$$R_s = \frac{\Delta t}{w} \sim \frac{\sqrt{N}}{4} \cdot \ln\alpha \cdot \frac{1}{\frac{\Delta\%}{t_g} \cdot B \cdot \varepsilon_t \cdot o r_2 \cdot L/F + 1}$$

Efficiency Selectivity Retention

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

Reduction of Cycle Time



Conditions:

Column: Symmetry® 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.**

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.

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

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.

The Number of Column Volumes per Minute Impacts Resolution

- 2 Approaches:
  - Approach 1: Gradient volume in 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 proporionally 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 x f.r. = 5

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

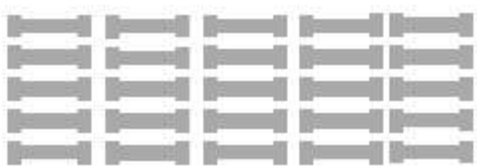
20 mm column

Column volume = 0.2 mL

5 minute gradient @ 1 mL/min

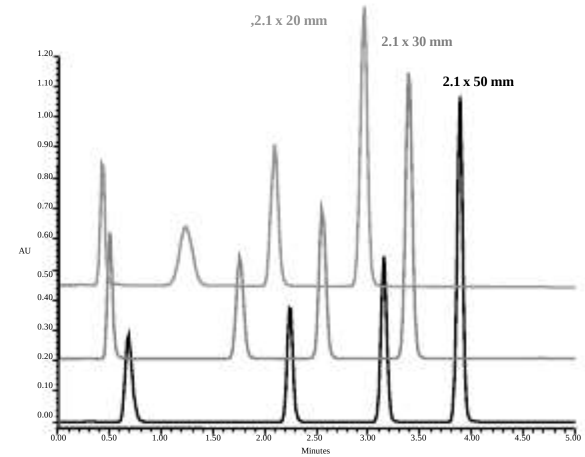
gradient volume = t_gx f.r. = 5

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



Impact of Column Length on Resolution (Approach 1)

-Gradient volume not scaled to column volume



Conditions:
Symmetry® C_{18}, 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.

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.

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_gx 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_gx f.r. = 2

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

What Factors Influence Gradient RP-HPLC Separations...

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

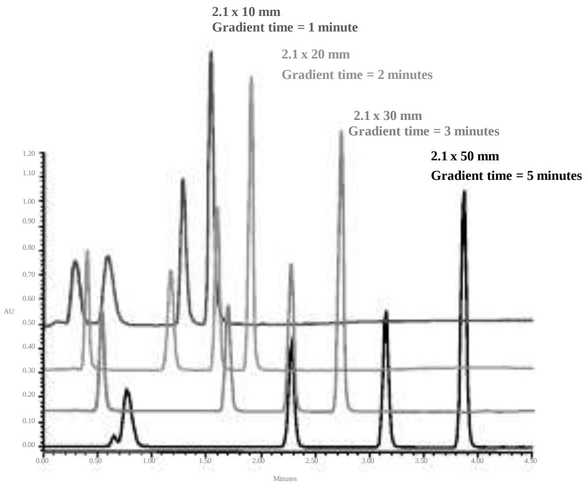
$$R_s = \frac{\Delta t}{w} \sim \frac{\sqrt{N}}{4} * \ln\alpha * \frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot \varepsilon_t \cdot \pi r^2 \cdot L/F + 1}$$

Efficiency Selectivity Retention

Terms are constant

Impact of Column Length on Resolution (Approach 2)

-Gradient volume scaled to column volume



Conditions:
Symmetry® C_{18}, 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.

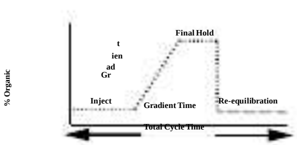
-Reduce analysis time by >50%.

-Trade-off: reduction in resolution

Summary - Impact of Column Length on Resolution

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

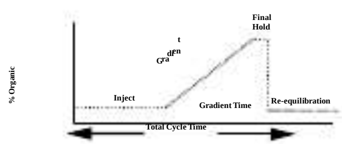
Gradient Delay Time



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_gx 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.



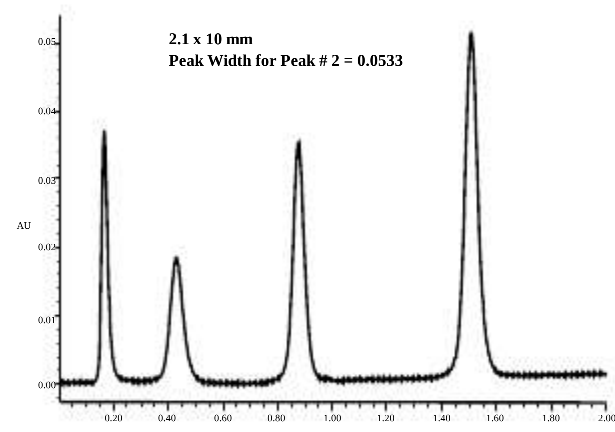
Column: 2.1 X 50 mm

Column volume (c.v.) = 0.17 mL
5 minute gradient @ 0.2 mL/min
instrument delay volume (d.v.) = 650 µL
gradient volume = t_gx 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

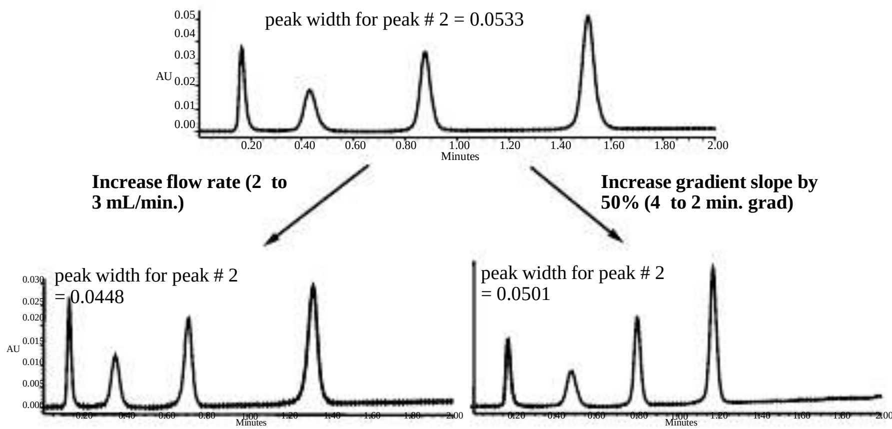
Impact of the Number of Column Volumes on Peak Shape



Conditions:
Symmetry® C_{18}, 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

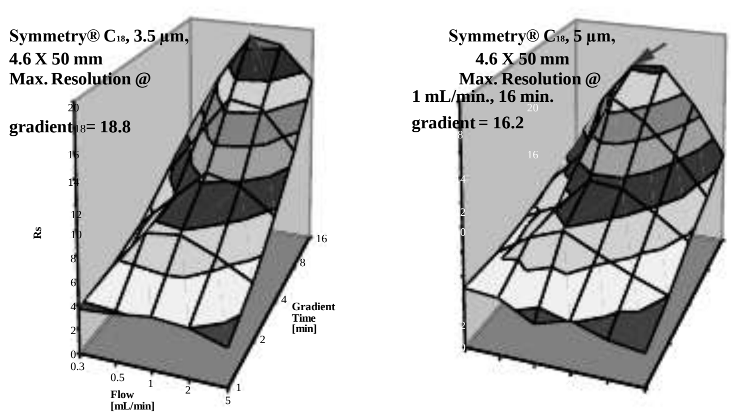
- Make gradient steeper by increasing the flow rate or decreasing the gradient time



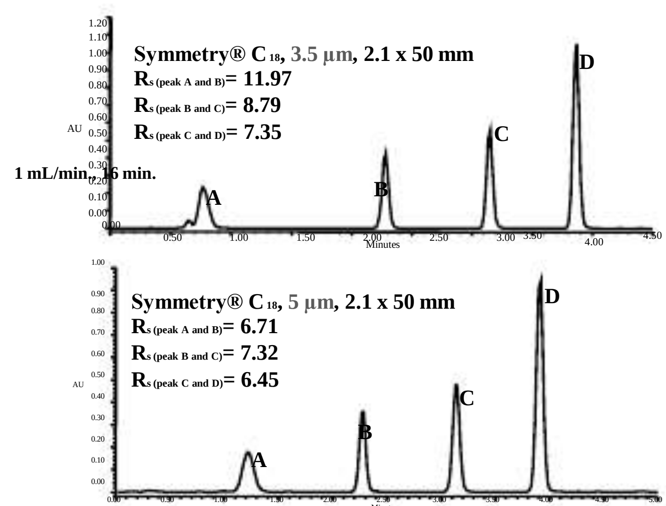
Outline

- 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

Comparison of Resolution Dependence on Particle Size



Impact of Particle Size (dp) on Resolution



Conditions:

Columns: Symmetry® C18 5 µm, 4.6 X 50 mm and Symmetry® C18 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
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.

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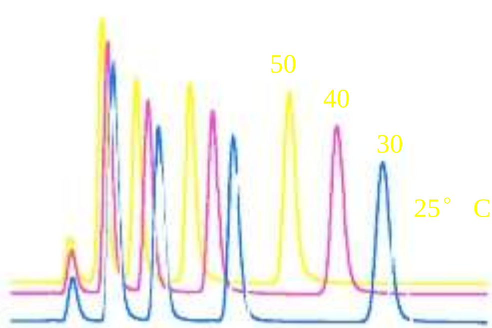
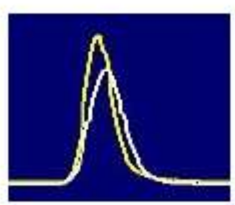
Impact of Temperature



- Faster Separtation
- Narrower Peaks
- Lower Backpressure
- Lower Viscosity
- Greater Sensitivity-



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