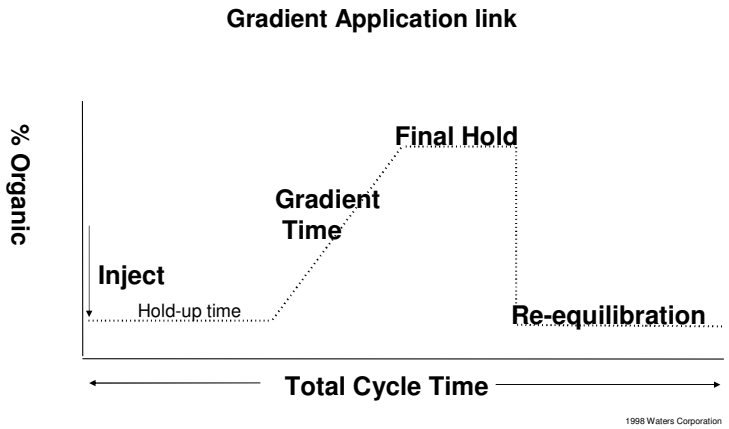
Gradient Considerations in LC

Operation in Gradients of Solvent Strength in LC



Layout

- Introduction
- LC System Considerations
- Gradient Parameters Affecting Resolution

Gradient Separations: Example: Peptides

Why do we use gradients?

Why do we use gradients?...

...because...

Properties of analytes

Retention (*k*) of the solutes has a steep dependence on the % organic in the mobile phase

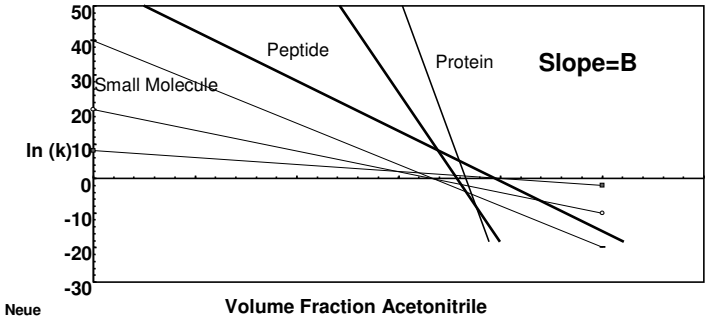
Wide range of differing hydrophobicities of the analytes

Gradient Considerations in LC

Why do we use gradients?

The retention (*k*) of peptides have a steep dependence on the % organic in the mobile phase

- The steeper the slope of the line the shallower the gradient must be to achieve maximum resolution

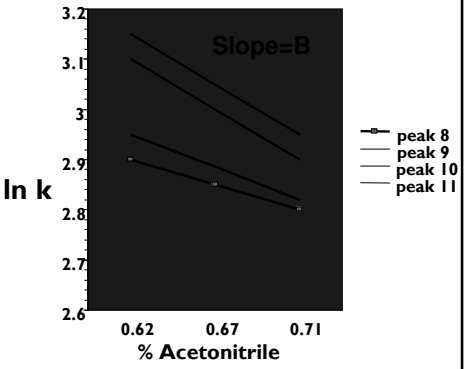


Neue

Analyte Retention as a Function of Gradient Slope

k (retention) for each analyte changes independently as the gradient slope changes.

Thus, the resolution between peaks changes.

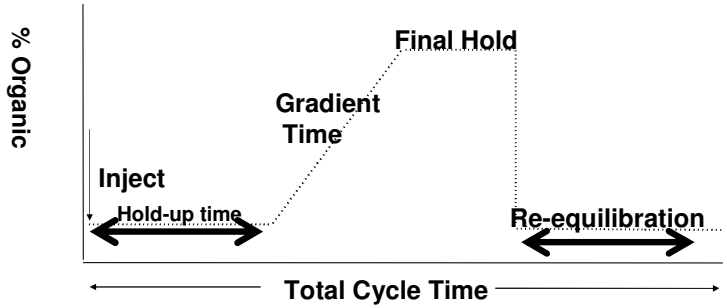


Carmody

Layout

- Introduction
- LC System Considerations
- Gradient Parameters Affecting Resolution

System Considerations When Working with Gradients



Gradient Considerations in LC

Different Configurations Create Different Time Delays

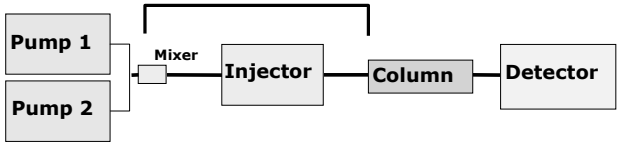
Mixing Valve Low – More Pipe Volume – More Time Delay

Mixing Valve High – Less Pipe Volume – Less Time Delay

Volumes of Pumping Systems

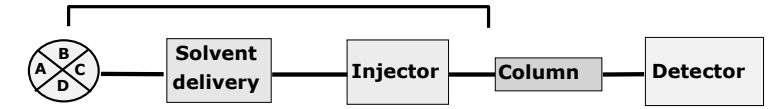
High Pressure Mixer: Multi-Pump

Smaller System Volume = Dwell volume



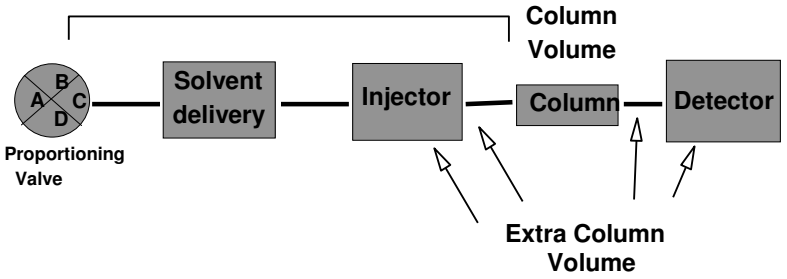
Low Pressure Gradient: Single Pump

Larger System Volume = Dwell volume

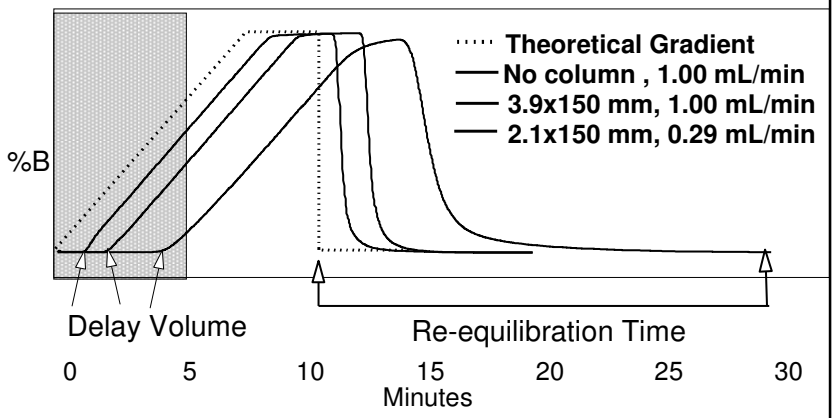


Delay Volume and Extra-Column Band Broadening Volume

System Volume



Delay Volume is Significant in Narrow Bore-Low Flow Applications

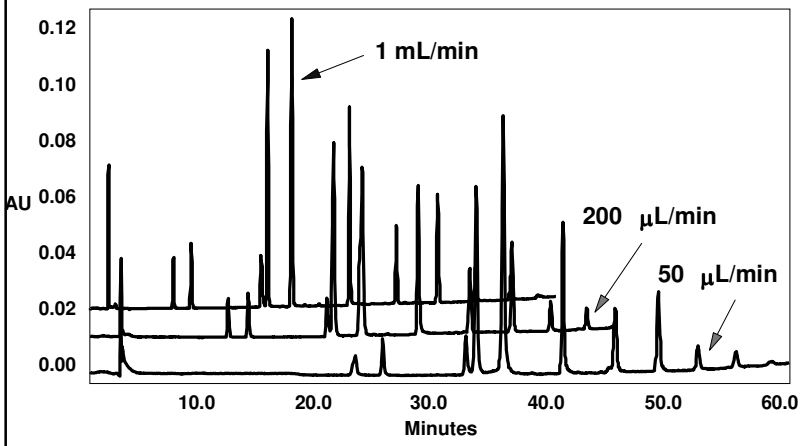


Gradient Considerations in LC

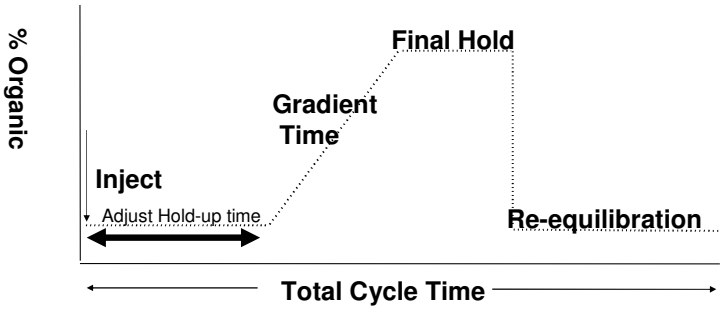
Linear Gradient Separation

Application

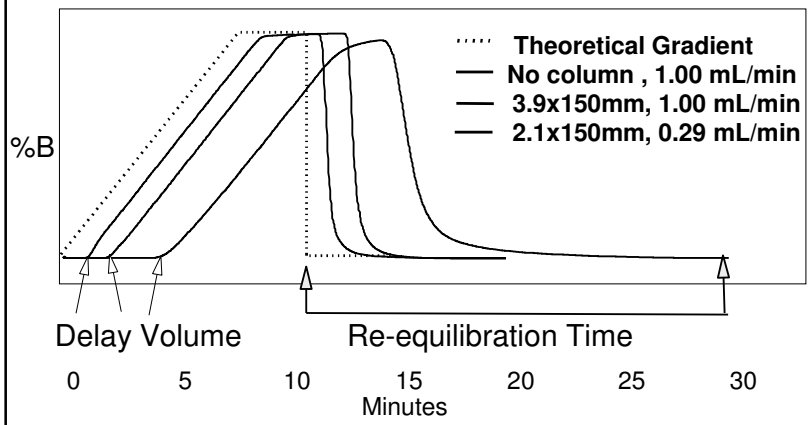
1 mL/min, 200 and 50 µL/min



System Considerations When Working with Gradients



Delay Volume is Significant in Narrow Bore-Low Flow Applications



Re-Equilibration Time

For good system/column equilibration

$$t_r = (3VT + 5Vc)/F$$

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

For example, Alliance system's volume = 650 µL

Gradient Considerations in LC

Layout

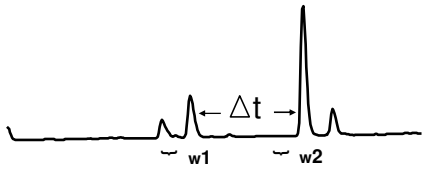
- Introduction
- LC System Considerations
- Gradient Parameters Affecting Resolution

Basic Resolution Equation

-Factors Influencing Resolution for an Isocratic Separation

$$Rs = \frac{\Delta t}{\overline{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \underbrace{\frac{\alpha - 1}{\alpha}}_{\text{Selectivity}} \underbrace{\frac{k}{k+1}}_{\text{Retention}}$$

Rs = Resolution
N = Plate Count
t = Retention Time
w = Average peak width
α = Selectivity Factor
k = Retention Factor



Resolution Equations

-Factors Influencing Resolution for an <u>Isocratic</u> Separation

$$Rs = \frac{\Delta t}{\overline{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \underbrace{\frac{\alpha - 1}{\alpha}}_{\text{Selectivity}} \underbrace{\frac{k}{k+1}}_{\text{Retention}}$$

B = Slope of ln (k) with solvent composition (an analyte dependent property)
c = Gradient Slope
t_0 = Time of elution for an unretained peak

-Factors Influencing Resolution for a <u>Gradient</u> Separation

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

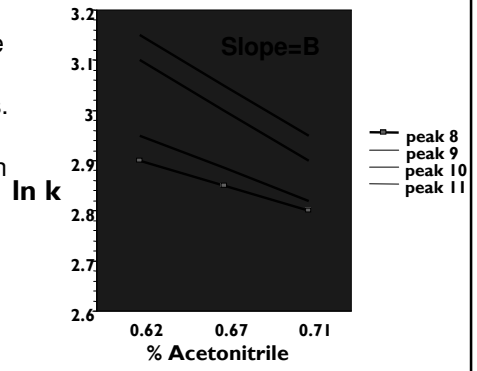
Rs = Resolution
t = Retention time
w = Peak width
N = Plate Count
α = Selectivity Factor
k = Retention Factor

-Factors are similar, however...

Analyte Retention as a Function of Gradient Slope

k (retention) for each analyte changes independently as the gradient slope changes.

Thus, the resolution between peaks changes.



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Gradient Considerations in LC

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention

- **Gradient Slope; c**
- Column Length; L and N
- Flow Rate; F

Part II - Factors influencing selectivity

- Concentration and Type of Modifier
- Temperature
- Chemistry and Pore Size of the Packing Material

Part III - Factors influencing reproducibility

- Column
- HPLC system

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

Part I - Factors influencing efficiency and retention

Gradient Slope; C (%B/min.) - increase in organic concentration per unit time

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

What Factors Influence Gradient Slope?

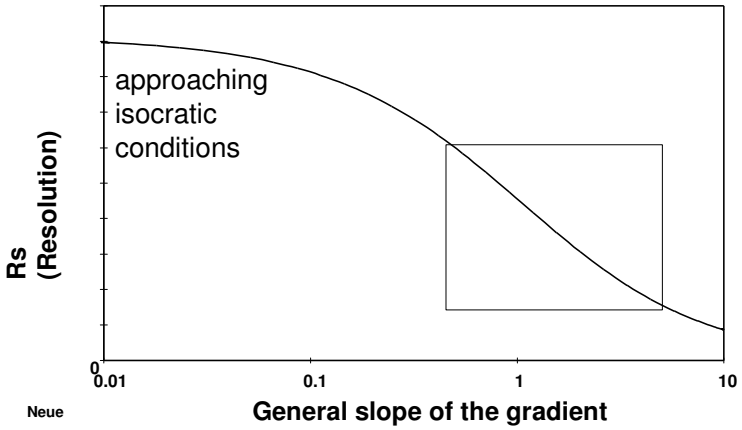
$$c = \%B/\text{minute} = \frac{\Delta\%}{tg}$$

Two ways to change the slope

change the percent organic (Δ %) of the mobile phase across a specified gradient run time.

change the gradient run time (tg) while keeping the Δ% organic of the mobile phase constant.

Principle of Gradient Separations



Gradient Considerations in LC

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention

Gradient Slope; c (%B/min.) - varied by changing the <u>% organic across a specified gradient run time</u>. All other variables are kept constant.

$$Rs = \frac{\Delta t}{\overline{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \underbrace{\ln a}_{\text{Selectivity}} \underbrace{\frac{1}{Bct_0 + 1}}_{\text{Retention}}$$

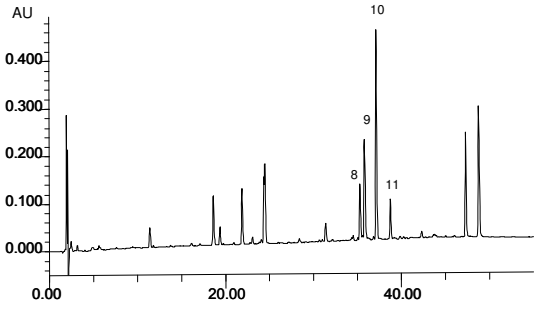
$$\downarrow$$

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

tg = gradient run time

Resolution as a Function of Gradient Slope

-Slope of the gradient = 0.66%/min



Alden

<u>Conditions</u>

-Column: Symmetry300™, C18, 5 m, 3.9 x 150 mm

- Sample: Tryptic digests of bovine cytochrome *c*

- Injection: 20µL

- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile

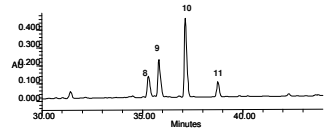
- Gradient: 0-45 min., 0-30%B

- Flow rate: 0.75 mL/min.

- Temperature: 35 C
- Detection: 214 nm

Resolution as a Function of Gradient Slope

0.66%/min

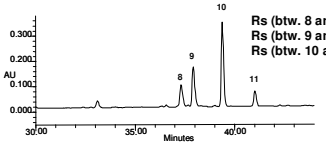


Rs (btw. 8 and 9) = 2.0
Rs (btw. 9 and 10) = 5.4
Rs (btw. 10 and 11) = 6.9

<u>Conditions</u>

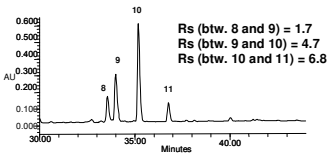
-Column: Symmetry300™, C18, 5 m, 3.9 x 150 mm
- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20 L
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Detection: 214 nm
- Flow rate: 0.75 mL/min.
- Temperature: 35 C

0.62%/min



Rs (btw. 8 and 9) = 2.3
Rs (btw. 9 and 10) = 6.0
Rs (btw. 10 and 11) = 6.8

0.71%/min



Rs (btw. 8 and 9) = 1.7
Rs (btw. 9 and 10) = 4.7
Rs (btw. 10 and 11) = 6.8

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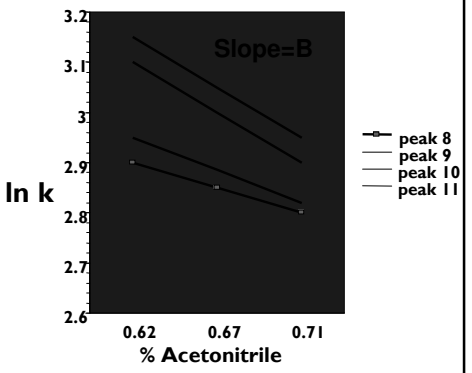
Analyte Retention as a Function of Gradient Slope

k (retention) for each analyte changes independently as the gradient slope changes.

Thus, the resolution between peaks changes.



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Gradient Considerations in LC

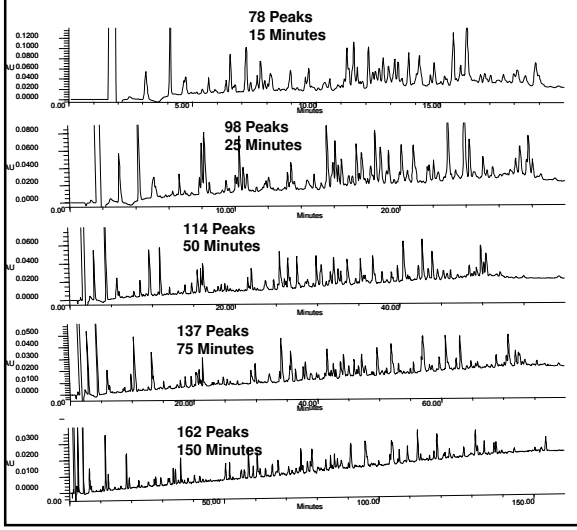
What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention

Gradient Slope; c (%B/min.) - varied by changing the gradient run time. All other variables are kept constant.

$$Rs = \frac{\Delta t}{\bar{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \quad \underbrace{\ln \alpha}_{\text{Selectivity}} \quad \underbrace{\frac{1}{B \cdot \frac{\Delta\%}{tg} \cdot t0 + 1}}_{\text{Retention}}$$

Resolution as a Function of Gradient Duration



Conditions

-Column: Symmetry300™, C18, 5 m, 4.6 x 150 mm
- Sample: Tryptic digests of bovine serum albumin
- Injection: 20 L
- Mobile Phase:
Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
- Detection: 214 nm
- Flow rate: 0.75 mL/min.
- Temperature: 35 C

-Longer RT; Shallower the slope; Increases Rs

-Rs ~ 1/c where c = gradient slope. All other variables are kept constant

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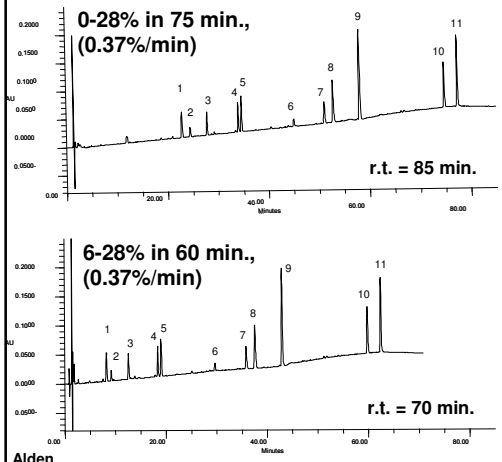
Gradient Modifications: Initial ACN% in Peptide Separations



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Gradient SLOPE is more important if peaks elute during gradient (not in initial condition)

-By changing the initial mobile phase conditions, but keeping the gradient slope the same, the run time can effectively be shortened without a loss in resolution.

Summary of Part I - Gradient Slope

- Gradient Slope is one of the most powerful operational parameter you have at your disposal
  - Resolution increases as gradient slope decreases.
  - Change in the initial percent organic can decrease the run time, maintain the resolution of your separation and preserve your elution pattern.

Gradient Considerations in LC

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention
- Gradient Slope; c
- **Column Length; L and N**
- Flow Rate; F

Part II - Factors influencing selectivity
- Concentration and Type of Modifier
- Temperature
- Chemistry and Pore Size of the Packing Material

Part III - Factors influencing reproducibility
- Column
- HPLC system

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention ▪
Gradient Slope; c–
Column Length; L and N↗

$$Rs = \frac{\Delta t}{w} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \underbrace{\ln \alpha}_{\text{Selectivity}} \underbrace{\frac{1}{B \cdot \frac{\Delta\%}{tg} \cdot t_0 + 1}}_{\text{Retention}} \frac{1}{B \cdot \frac{\Delta\%}{tg} \cdot \varepsilon_t \cdot \pi r^2 \cdot L/F + 1}$$

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).

Column Volume to Gradient Volume Relationship (Approach 1)

-Gradient volume scaled to column volume

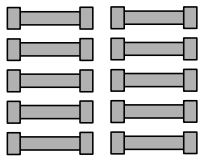
5 minute gradient = 1 c.v./min
Total volume = 10 column volumes

50 mm column

Column volume = 0.5 mL

5 minute gradient @ 1 mL/min

gradient volume = tg x f.r. = 5
Total volume = g.v./c.v. = 10 column vols.

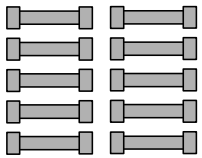


250 mm column

Column volume = 2.5 mL

25 minute gradient @ 1 mL/min

gradient volume = tg x f.r. = 25
Total volume = g.v./c.v. = 10 column vols.



Gradient Considerations in LC

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention

Gradient Slope; c

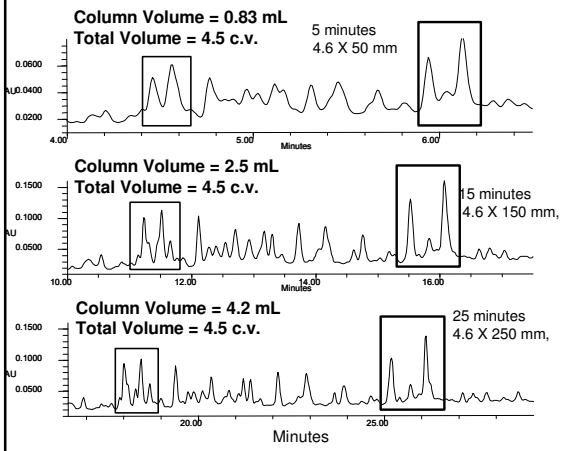
Column Length; L and N

$$Rs = \frac{\Delta t}{\overline{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \underbrace{\ln \alpha}_{\text{Selectivity}} \underbrace{\frac{1}{B \cdot \frac{\Delta\%}{tg} \cdot \varepsilon_t \cdot \pi r_2 \cdot L/F + 1}}_{\text{Retention}}$$

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

Terms are constant

Variation in Column Lengths at Equal Ratio of Gradient Volumes to Column Volumes



Conditions

- **Column: Symmetry300™, C18, 5 m**
- **Sample: Tryptic digests of bovine serum albumin**
- **Injection: 20 L**
- **Mobile Phase:**

Solvent A: 0.1% TFA in water
Solvent B: 0.1% TFA in acetonitrile

- **0 - 30 %B in the time shown.**
- **Flow rate: 0.75 mL/min.**
- **Detection: 214 nm**
- **Temperature: 35 C**

- **Elution pattern stays the same.**

- **Resolution inc. as the # of plates inc.**

- **Run time inc. as column length inc.**

Alden

Column Volume to Gradient Volume Relationship (Approach 2)

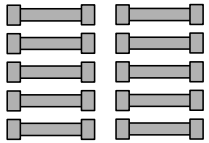
-Gradient volume not scaled to column volume

5 minute gradient = 1 c.v./min
Total volume = 10 column volumes

50 mm column

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

gradient volume = tg x f.r. = 5
Total volume = g.v./c.v. = 10 column vols.



250 mm column

Column volume = 2.5 mL
5 minute gradient @ 1 mL/min

gradient volume = tg x f.r. = 5
Total volume = g.v./c.v. = 2 column vols.

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention

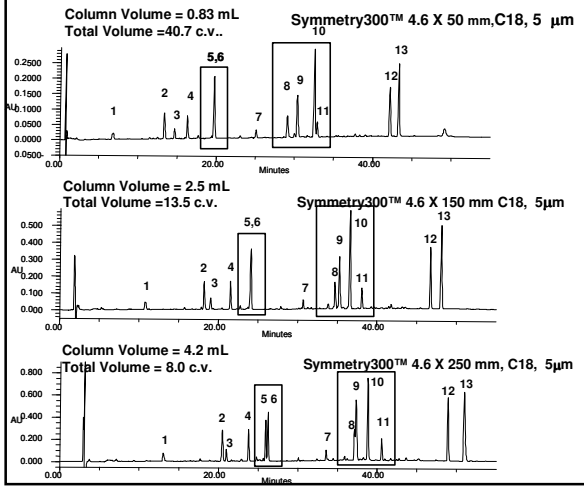
Gradient Slope; c

Column Length; L and N

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

Gradient Considerations in LC

Column Length Effects on Resolution at a Constant Gradient Duration



Conditions

- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20 L
- Mobile Phase:
  Solvent A: 0.1% TFA in water
  Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
- Flow rate: 0.75 mL/min.
- Detection: 214 nm
- Temperature: 35 C

-Will observe elution pattern changes.

-Resolution changes

-Run time remains the same.

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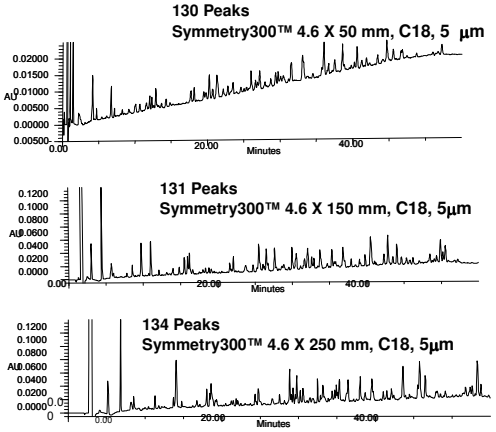
Column Length Effects on Resolution at a Constant Gradient Duration (cont'd)



Conditions

- Sample: Tryptic digests of bovine serum albumin
- Injection: 20 L (7 L for 4.6 X 50 mm)
- Mobile Phase:
  Solvent A: 0.1% TFA in water
  Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
- Temperature: 35 C
- Flow rate: 0.75 mL/min.
- Detection: 214 nm

-50 mm column has a similar resolving power as 250 mm column if the gradient duration remains the same.

Alden

Summary Part I - Column Length

If the gradient volume is scaled proportionally to the column volume

Elution pattern does not change
Resolution increases with column length.

If the gradient volume is **not** scaled in proportion to the column volume

Elution pattern and resolution changes
50 mm column exhibits similar resolving power to a 250 mm column.

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention
- Gradient Slope; c
- Column Length; L and N
- **Flow Rate; F**

Part II - Factors influencing selectivity
- Concentration and Type of Modifier
- Temperature
- Chemistry and Pore Size of the Packing Material

Part III - Factors influencing reproducibility
- Column
- HPLC system

Gradient Considerations in LC

What Factors Influence Gradient RP-HPLC Separations...

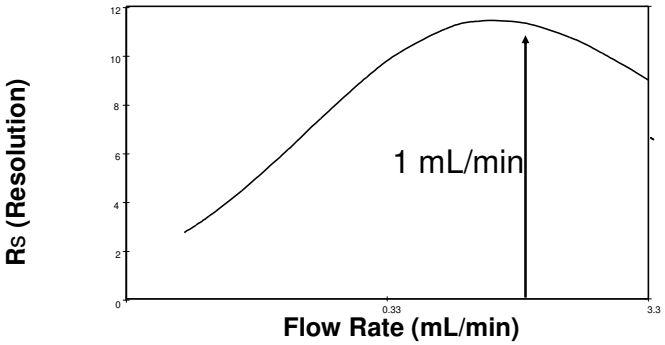
Part I - Factors influencing efficiency and retention

- Gradient Slope; c
- Column Length; L and N
- **Flow Rate; F**

F (flow rate) is varied.
All other variables are kept constant

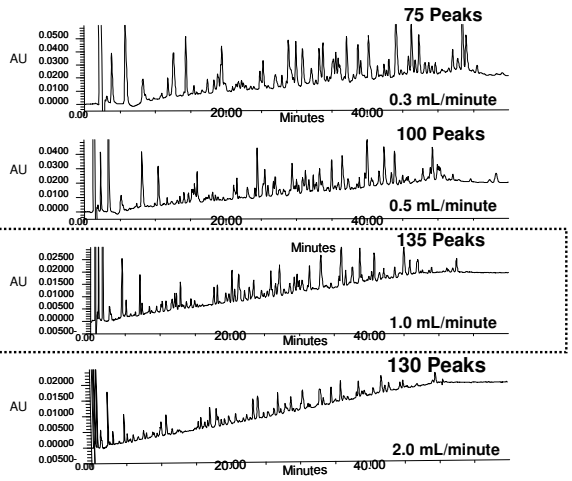
$$Rs = \frac{\Delta t}{\overline{w}} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \underbrace{\ln \alpha}_{\text{Selectivity}} \underbrace{\frac{1}{B \cdot \frac{\Delta\%}{tg} \cdot \varepsilon_t \cdot \pi r_2 \cdot L/F + 1}}_{\text{Retention}}$$

Resolution as a Function of Flow Rate at a Constant Gradient Duration



Neue

Flow Rate Effects on Resolution at a Constant Gradient Duration



Conditions

- **Column: Symmetry300™, C18, 5µm, 4.6x50mm**
- **Sample: Tryptic digests of bovine serum albumin**
- **Injection: 20µL**
- **Mobile Phase:**
  Solvent A: 0.1% TFA in water
  Solvent B: 0.1% TFA in acetonitrile
- **Gradient: 0-45 min., 0-30%B**
- **Temperature: 35 C**
- **Detection: 214 nm**

-Best resolution occurred at a flow rate of 1.0 mL/min. for this peptide under these conditions.

-Elution pattern changes.

Alden

Summary of Part I - Flow Rate

Maximum resolution is achieved at an optimal flow rate:

As flow rate changes *N* changes

As flow rate changes the elution pattern changes.

Gradient Considerations in LC

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention
- Gradient Slope; c
- Column Length; L and N
- Flow Rate; F

Part II - Factors influencing selectivity
- **Concentration and Type of Modifier**
- Temperature
- Chemistry and Pore Size of the Packing Material

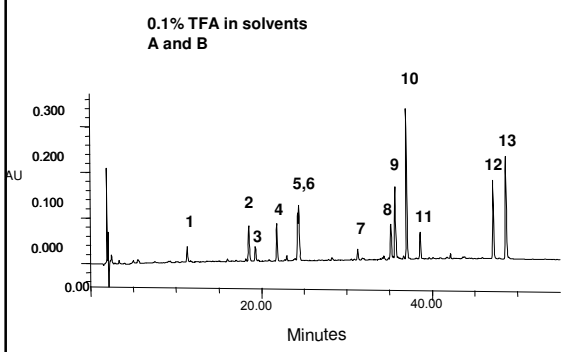
Part III - Factors influencing reproducibility
- Column
- HPLC system

Type of Modifiers

- Solvation
- Ionization
- Ion-pairing
- Volatility (Collection)

Effects of TFA Concentration on Resolution

- Typical gradient conditions Conditions



- Column: Symmetry300™, C18, 5 µm, 3.9x150mm,
- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20µL
- Mobile Phase:
  Solvent A: water
  Solvent B: acetonitrile
- **Gradient: 0-45 min., 0-30%B**
- Flow rate: 0.75 mL/min.
- Temperature: 35 C
- Detection: 214 nm

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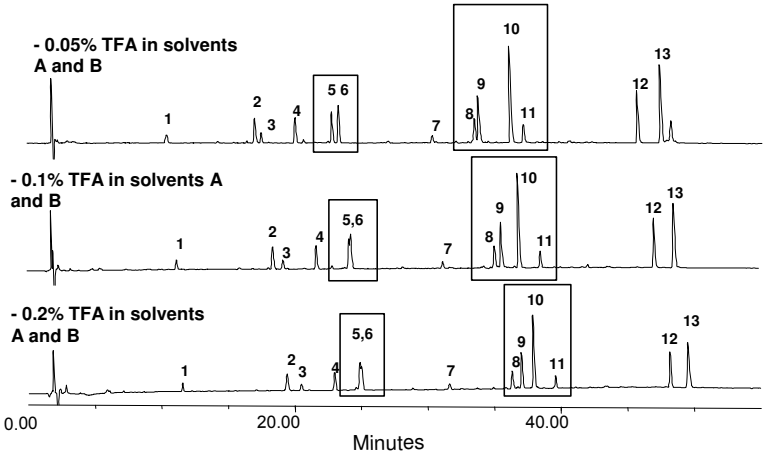
The Power of Different TFA Concentrations in Your Mobile Phase



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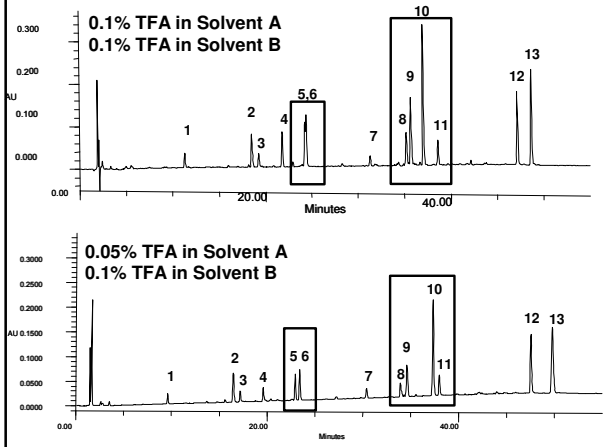
Gradient Considerations in LC

The Power of Different TFA Concentrations in Your Mobile Phase



Conditions

- Column: Symmetry300™, C18, 5µm, 3.9x150mm
- Sample: Tryptic Digests of Bovine Cytochrome *c*
- Injection: 20µL
- Mobile Phase:
  Solvent A: water
  Solvent B: acetonitrile
- Gradient: 0-45 min., 0-30%B
- Flow rate: 0.75 mL/min.
- Temperature: 35 C
- Detection: 214 nm

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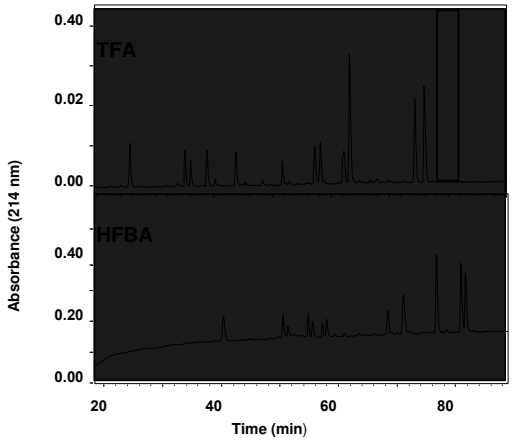
Alternate Ion Pairing Reagents TFA and HFBA (Heptafluorobutyric Acid)



Sample: Rabbit cytochrome *c* tryptic digest, 500 pmol

Column: Delta-Pak™ C18, 5 µm, 300 , 2.0 x 150 mm

Eluents: A=water/ 0.1% TFA or HFBA
B=acetonitrile/ 0.1% TFA or HFBA

Gradient: O-60 % B 120 min

Flow: 0.18 mL/min

Temp: 35 C

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention
- Gradient Slope; c
- Column Length; L and N
- Flow Rate; F

Part II - Factors influencing selectivity
- Concentration and Type of Modifier
- **Temperature**
- Chemistry and Pore Size of the Packing Material

Part III - Factors influencing reproducibility
- Column
- HPLC system

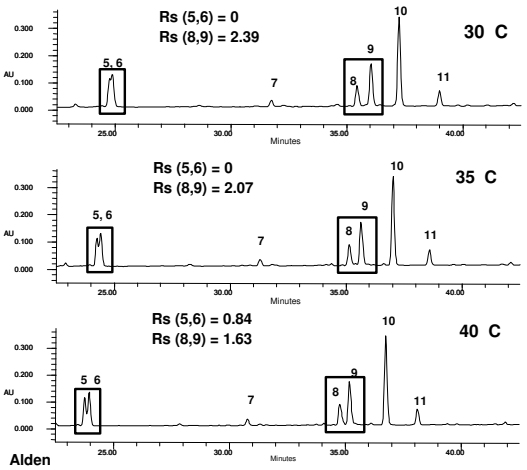
Temperature Effects on Resolution

Resolution is temperature dependent

Temperature is a critical parameter to control in order to achieve reproducible separations.

Gradient Considerations in LC

Temperature Effects on Resolution



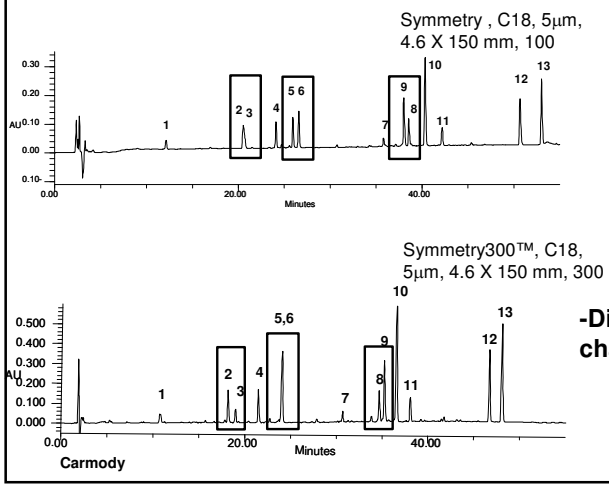
Conditions

- Column: Symmetry300™, C18, 5µm, 3.9x150mm
- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20µ L
- Mobile Phase:
  Solvent A: 0.1% TFA in water
  Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
- Flow rate: 0.75 mL/min.
- Detection: 214 nm

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention ▪
Gradient Slope; c–
Column Length; L and N–
Flow Rate; F–
Part II - Factors influencing selectivity ▪
Concentration and Type of Modifier–
Temperature–
Pore Size and Chemistry of the Packing Material↗
Part III - Factors influencing reproducibility ▪
Column–
HPLC system–

Pore Size Effects on Resolution



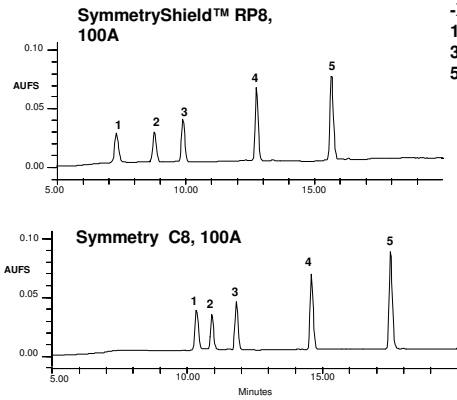
Conditions

- Sample: Tryptic digests of cytochrome *c* (bovine)
- Injection: 20µL
- Mobile Phase:
  Solvent A: 0.1% TFA in water
  Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-50 min., 0-30%B
- Temperature: 35 C
- Flow Rate: 0.75 mL/min.
- Detection: 214 nm

-Different pore sizes change selectivity.

Selectivity Differences Between Packings

Alberta Peptides on Symmetry Reversed-Phase Columns



Ac-Arg-Gly-X-X-Gly-Gly-Leu-Gly-LysAmide
-X-X-:
1: Ala-Gly with free alpha amino group
3: Ala-Gly 2: Gly-Gly
5: Val-Val 4: Val-Gly

Conditions:

Columns:	3.9 mm x 150 mm
Flow Rates:	0.8 mL/min
Mobile Phase:	A. 0.1% TFA aqueous; B. acetonitrile with 0.1% TFA
Gradient:	10% to 40% B in 30 minutes, step to 60% B for 5 min
Sample:	9 L Alberta Peptides, mix with 5 decapeptides
Detector:	214 nm
Temperature:	35 C

Gradient Considerations in LC

What Factors Influence Gradient RP-HPLC Separations...

Part I - Factors influencing efficiency and retention
- Gradient Slope; c
- Column Length; L and N
- Flow Rate; F

Part II - Factors influencing selectivity
- Concentration and Type of Modifier
- Temperature
- Chemistry and Pore Size of the Packing Material

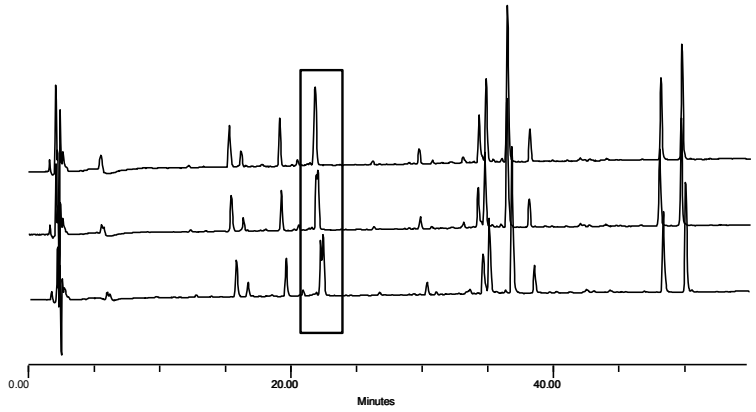
Part III - Factors influencing reproducibility
- **Column**
- **HPLC system**

Peptide Mapping Validation -Robustness Testing

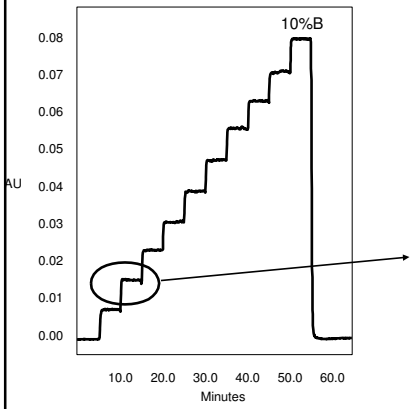
Choice and quality of enzyme
Digestion conditions
HPLC conditions
Equipment
- System
- Column

Effects of Irreproducible Gradient Delivery
-Traditional HPLC System

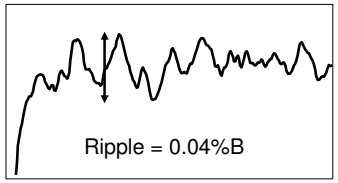
Experience resolution differences and retention time shifts



Separations Module
Gradient Accuracy & Precision



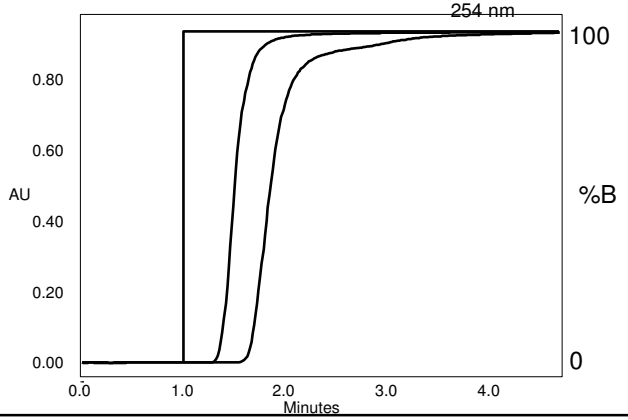
1% Step Gradient at 1.0 mL/min
Accuracy <0.06%B
Precision <0.05 Std Dev %B
N=6



Gradient Considerations in LC

Separations Module
System Volume

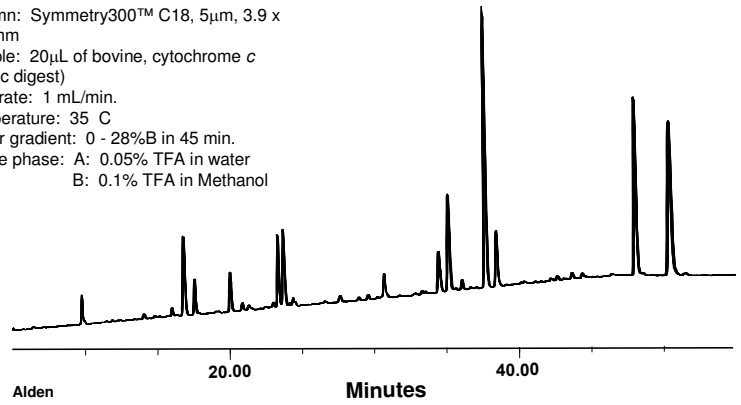
A = Methanol
B = Methanol+ Propylparaben
1 mL/min
254 nm



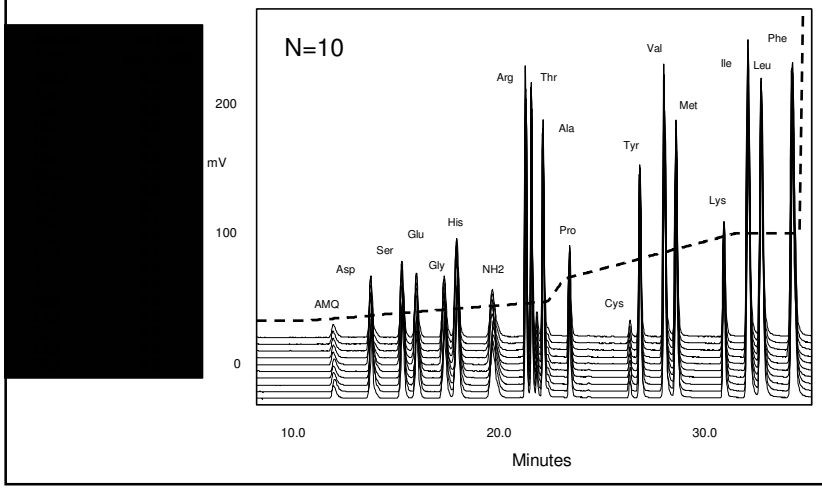
Reproducible Gradient Delivery
-HPLC System
-Overlay of 15 Consecutive Injections

Conditions:

Column: Symmetry300™ C18, 5μm, 3.9 x 150 mm
Sample: 20μL of bovine, cytochrome *c* (tryptic digest)
Flow rate: 1 mL/min.
Temperature: 35 C
Linear gradient: 0 - 28%B in 45 min.
Mobile phase: A: 0.05% TFA in water
B: 0.1% TFA in Methanol



Reproducibility of Complex Gradients
AccQ Tag Amino Acid Analysis



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...and the Symmetry Team, **WATERS**