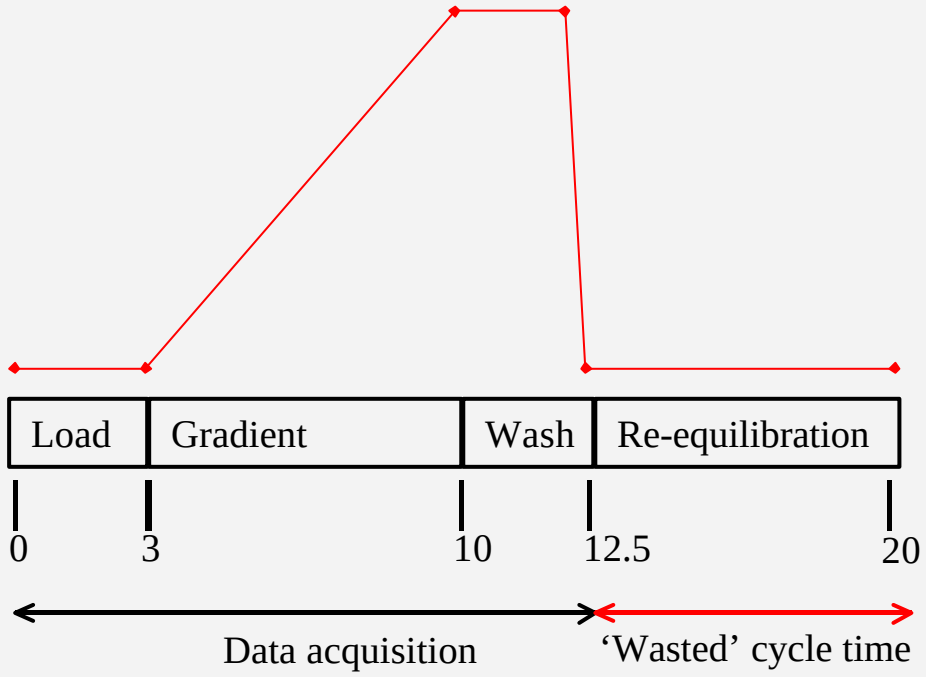
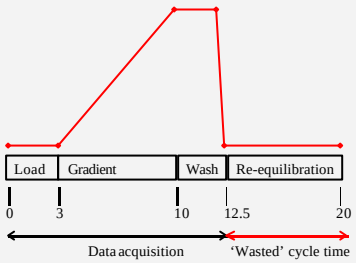
Theory of Gradient Separations: Example: Peptides

- Why do we use gradients?



Theory of Gradient Separations: Example: Peptides

- Why do we use gradients?



Why do we use gradients?...

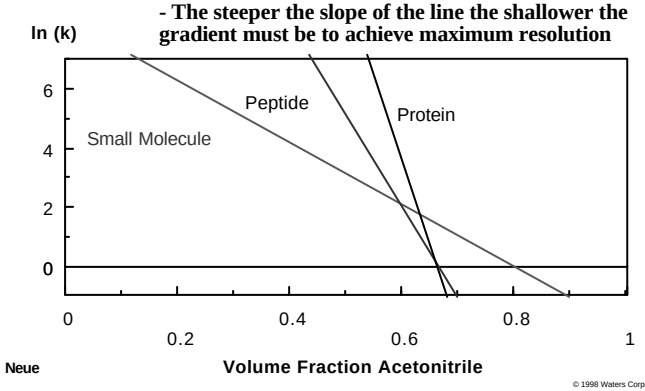
...because...

- 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

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



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Outline

- Introduction
- Theory of Gradient Separations of Peptides
- Optimization of Separations to...
  - achieve maximum resolution
  - maximize throughput
  - maximize reproducibility
- Assay Reproducibility
- Conclusion

Calculation of Isocratic Mobile Phase

k-Dependence on Mobile Phase Composition in Reversed-Phase HPLC:

$$\ln(k) = \ln(k_0) - S \cdot F$$

Gradient Retention Times:

$$t_G = t_0 + t_d + \frac{1}{b \times S} \times \ln\left(b \times S \times t_0 \times k(F_0) + 1\right)$$

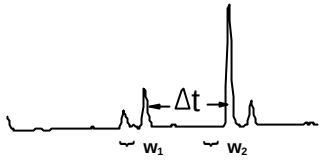
Column dead time; System delay time; Gradient slope; Slope of lnk vs. organic; k at gradient starting composition

Basic Resolution Equation

-Factors Influencing Resolution for an Isocratic Separation

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

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



Resolution Equations

-Factors Influencing Resolution for an Isocratic Separation

$$R_s = \frac{\Delta t}{w} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \times \underbrace{\frac{a - 1}{a}}_{\text{Selectivity}} \times \underbrace{\frac{k}{k+1}}_{\text{Retention}}$$

R_s = Resolution
t = Retention time
w = Peak width
N = Plate Count
a = Selectivity Factor
k = Retention Factor

-Factors Influencing Resolution for a Gradient Separation

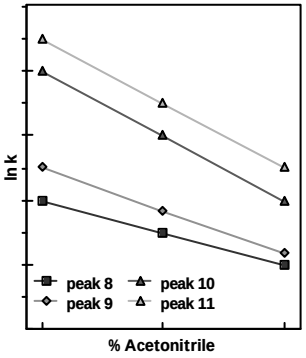
$$R_s = \frac{\Delta t}{w} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \times \underbrace{\ln a}_{\text{Selectivity}} \times \underbrace{\frac{1}{Bct_0 + 1}}_{\text{Retention}}$$

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 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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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**

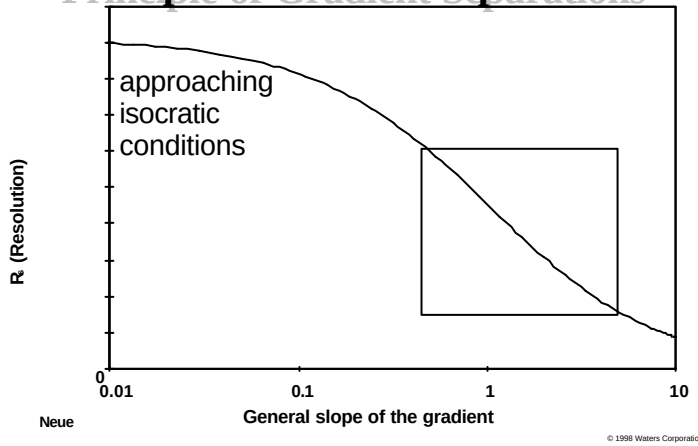
$$R_s = \frac{\Delta t}{w} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \times \underbrace{\ln \alpha}_{\text{Selectivity}} \times \underbrace{\frac{1}{Bct_0 + 1}}_{\text{Retention}}$$

What Factors Influence Gradient Slope?

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

- **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 (t_g) while keeping the Δ% organic of the mobile phase constant.**

Principle of Gradient Separations



What Factors Influence Gradient RP-HPLC Separations...

- Part I - Factors influencing efficiency and retention
  - Gradient Slope; c (%B/min.) - varied by changing the % organic across a specified gradient run time. All other variables are kept constant.

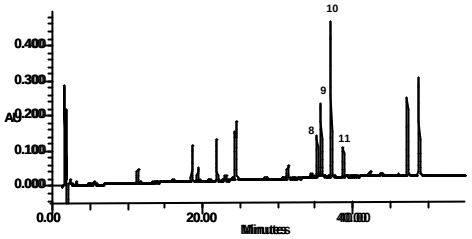
$$R_s = \frac{\Delta t}{w} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \times \underbrace{\ln\alpha}_{\text{Selectivity}} \times \underbrace{\frac{1}{Bct_0 + 1}}_{\text{Retention}}$$

t_g = gradient run time

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

Resolution as a Function of Gradient Slope

-Slope of the gradient = 0.66%/min



Conditions

-Column: Symmetry300™, C_{18}, 5 µm, 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

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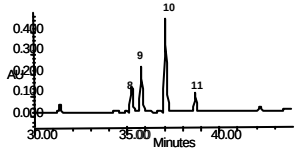
Resolution as a Function of Gradient Slope

0.66%/min



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

Conditions

-Column: Symmetry300™, C_{18}, 5 µm, 3.9 x 150 mm
- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20 µL
- Mobile Phase:
  Solvent A: 0.1% TFA in water
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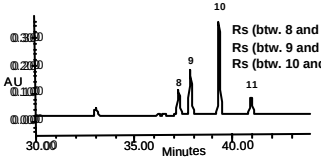
- Detection: 214 nm
- Flow rate: 0.75 mL/min.
- Temperature: 35 °C

0.62%/min (0-28%)



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

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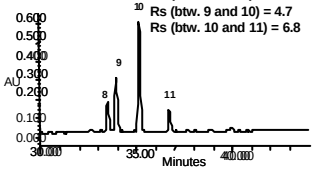
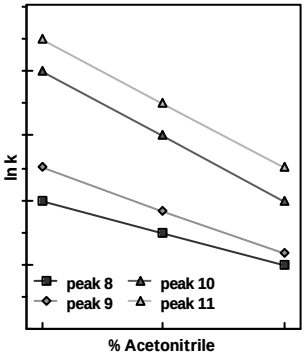
0.71%/min (0-35%)



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

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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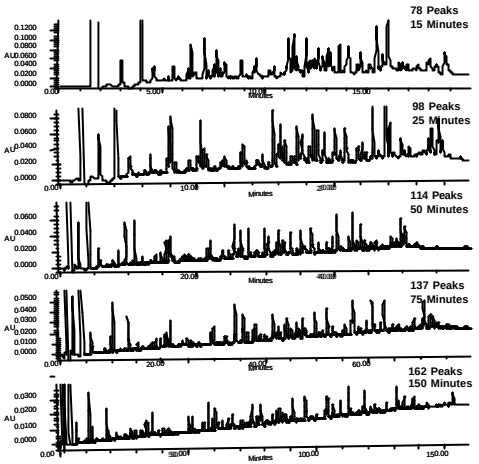
What Factors Influence Gradient RP-HPLC Separations...

- 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.

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

Resolution as a Function of Gradient Duration



Conditions

-Column: Symmetry300™, C_{18} 5 µm, 4.6 x 150 mm
- Sample: Tryptic digests of bovine serum albumin
- Injection: 20 µL
- 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 R_s

-$R_s \sim 1/c$ where c = gradient slope. All other variables are kept constant

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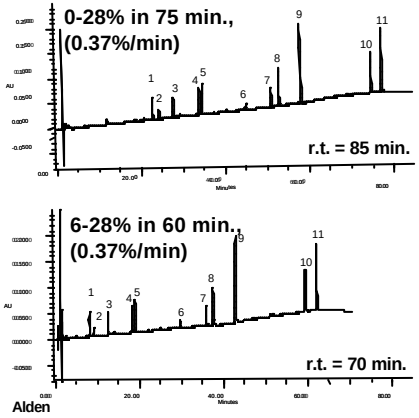
Gradient Modifications:
Initial ACN% in Peptide Separations



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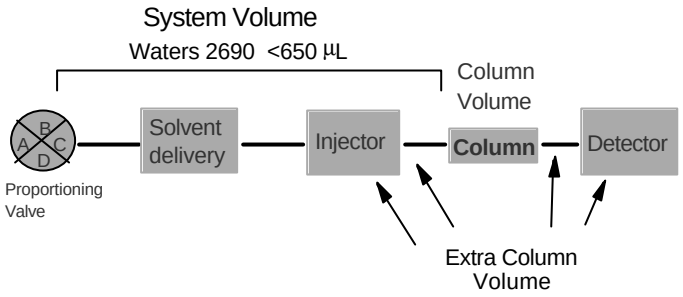
Gradient SLOPE is more important if peaks elute during gradient (not in initial condition)

-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.

Volumes in an HPLC System



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**

$$R_s = \frac{\Delta t}{w} \sim \underbrace{\frac{\sqrt{N}}{4}}_{\text{Efficiency}} \times \underbrace{\ln\alpha}_{\text{Selectivity}} \times \frac{1}{B \cdot \underbrace{\frac{\Delta\%}{t_g} \cdot t_0}_{\text{Retention}} + 1} \cdot \frac{1}{B \cdot \frac{\Delta\%}{t_g} \cdot e_t \cdot pr^2 \cdot L/F + 1}$$

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)

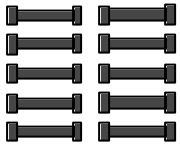
-Gradient volume scaled to column volume

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.

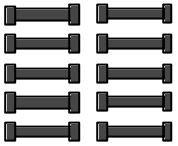


250 mm column

Column volume = 2.5 mL

25 minute gradient @ 1 mL/min

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



What Factors Influence Gradient RP-HPLC Separations...

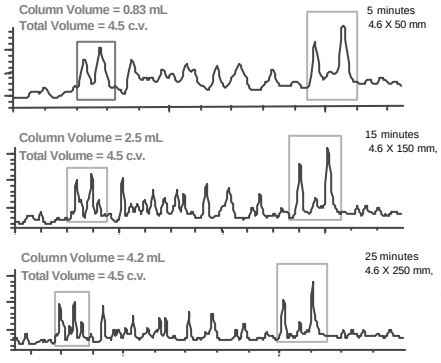
- Part I - Factors influencing efficiency and retention
  - Gradient Slope; c
  - Column Length; L and N

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

- 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™, C_{18}, 5 µm
- Sample: Tryptic digests of bovine serum albumin
- Injection: 20 µL
- 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.

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Column Volume to Gradient Volume Relationship (Approach 2)

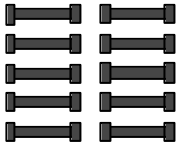
-Gradient volume **not** scaled to column volume

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.



250 mm column

Column volume = 2.5 mL

5 minute gradient @ 1 mL/min

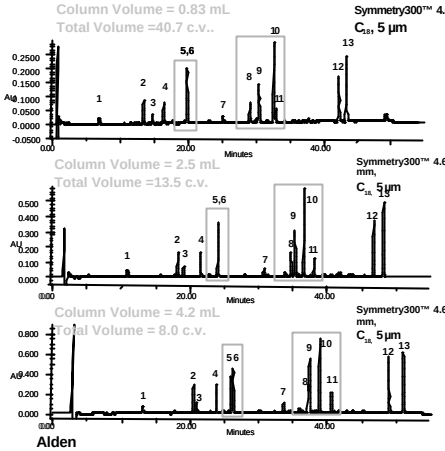
gradient volume = t_g x f.r. = 5
Total volume = g.v./c.v. = 2 column vols.

What Factors Influence Gradient RP-HPLC Separations...

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

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

Column Length Effects on Resolution at a Constant Gradient Duration



Conditions

- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20 µL
- 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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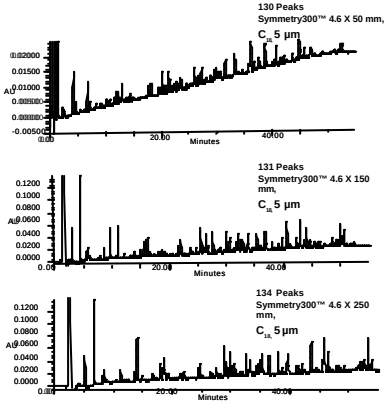
Column Length Effects on Resolution at a Constant Gradient Duration (cont'd)



Conditions

- Sample: Tryptic digests of bovine serum albumin
- Injection: 20 µL (7 µL 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.

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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 <u>not</u> 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

What Factors Influence Gradient RP-HPLC Separations...

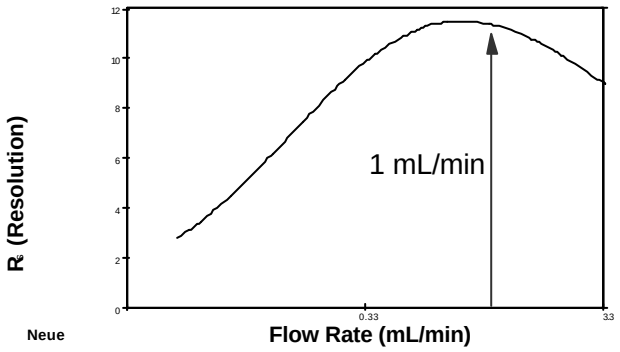
- Part I - Factors influencing efficiency and retention
  - Gradient Slope; c
  - Column Length; L and N
  - **Flow Rate; F**

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

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

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

(50 mm Column)



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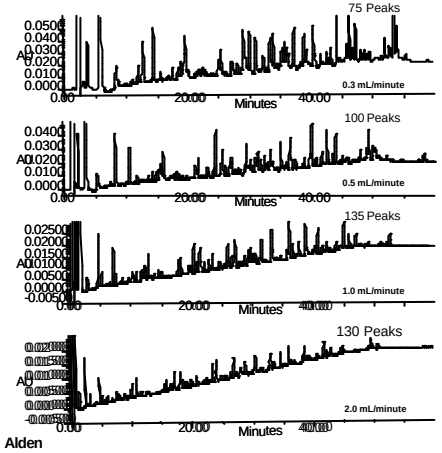
Flow Rate Effects on Resolution at a Constant Gradient Duration



Conditions

- Column: Symmetry300™, C_{18} 5 µm, 4.6x50mm
- Sample: Tryptic digests of bovine serum albumin
- Injection: 20 µL
- 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.

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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.

What Factors Influence Gradient RP-HPLC Separations...

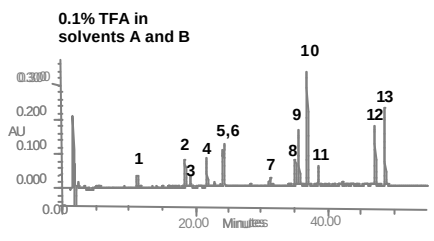
- Part I - Factors influencing efficiency and retention
  - Gradient Slope; c
  - Column Length; L and N
  - Flow Rate; F
- Part II - Factors influencing selectivity
  - **Concentration and Type of Modifier**
  - Temperature
  - Chemistry and Pore Size of the Packing Material
- Part III - Factors influencing 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™, C_{18}, 5 µm, 3.9x150mm,
- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20 µL
- 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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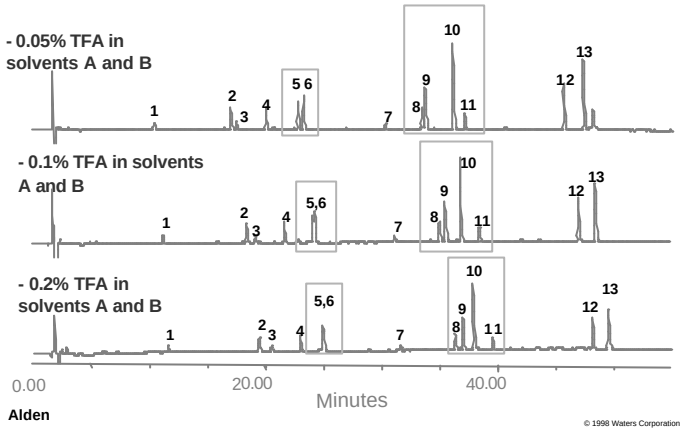
The Power of Different TFA Concentrations in Your Mobile Phase



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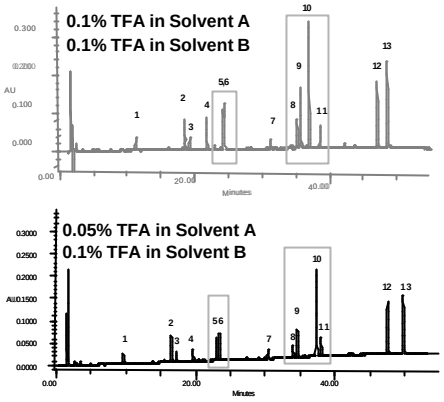
The Power of Different TFA Concentrations in Your Mobile Phase



Conditions

- Column: Symmetry300™, C_{18}, 5 µm, 3.9x150mm
- Sample: Tryptic Digests of Bovine Cytochrome *c*
- Injection: 20 µL
- 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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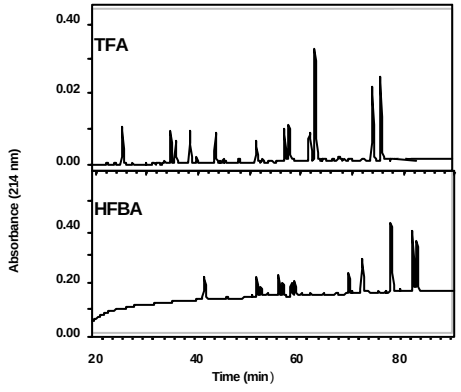
Alternate Ion Pairing Reagents TFA and HFBA (Heptafluorobutyric Acid)



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

Column: Delta-Pak™ C_{18} 5µm, 300Å, 2.0 x 150 mm

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

Gradient: 0-60 % B 120 min

Flow: 0.18 mL/min

Temp: 35 ºC

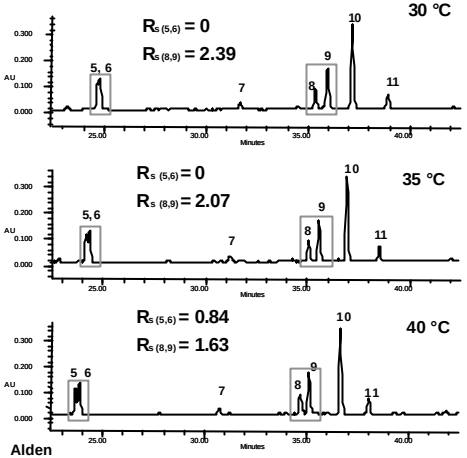
What Factors Influence Gradient RP-HPLC Separations...

- 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.

Temperature Effects on Resolution



Conditions

- Column: Symmetry300™, C_{18}, 5 µm, 3.9x150mm
- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20 µL
- 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

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What Factors Influence Gradient RP-HPLC Separations...

- Part I - Factors influencing efficiency and retention
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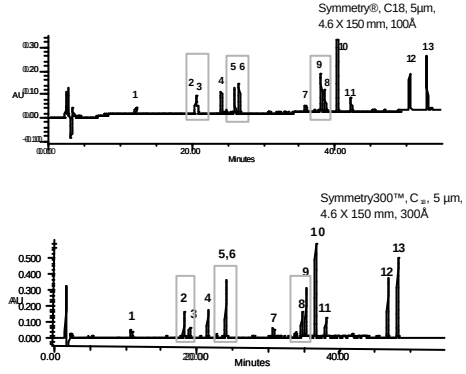
  - Flow Rate; F
- Part II - Factors influencing selectivity
  - Concentration and Type of Modifier
  - Temperature
  - **Pore Size and Chemistry of the Packing Material**
- Part III - Factors influencing reproducibility
  - Column
  - HPLC system

Pore Size Effects on Resolution



Conditions

- Sample: Tryptic digests of cytochrome *c* (bovine)
- Injection: 20 µL
- 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.

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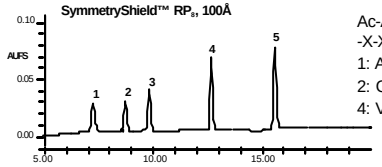
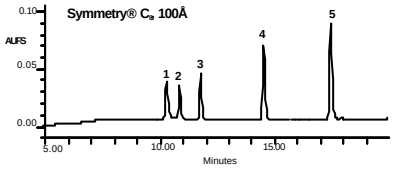
Selectivity Differences Between Packings

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
2: Gly-Gly 3: Ala-Gly
4: Val-Gly 5: Val-Val



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 µL Alberta Peptides, mix with 5 decapeptides**
Detector:	**214 nm**
Temperature:	**35°C**

What Factors Influence Gradient RP-HPLC Separations...

- Part I - Factors influencing efficiency and retention
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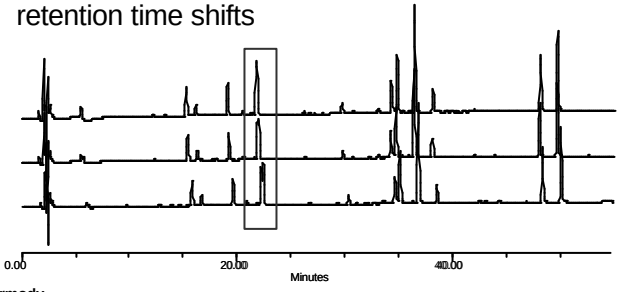
  - Concentration and Type of Modifier
  - Temperature
  - Chemistry and Pore Size of the Packing Material
- Part III - Factors influencing reproducibility
  - Column
  - HPLC system

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



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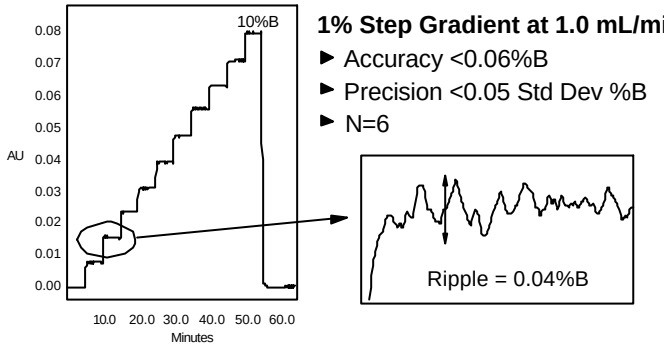
Waters 2690 Separations Module

Gradient Accuracy & Precision

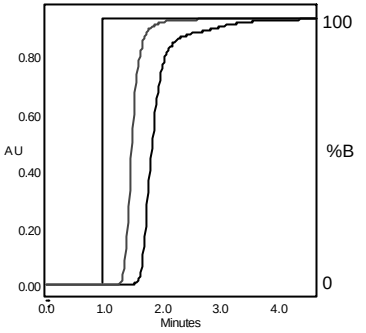


1% Step Gradient at 1.0 mL/min

- Accuracy <0.06%B
- Precision <0.05 Std Dev %B
- N=6

Waters 2690 Separations Module

System Volume



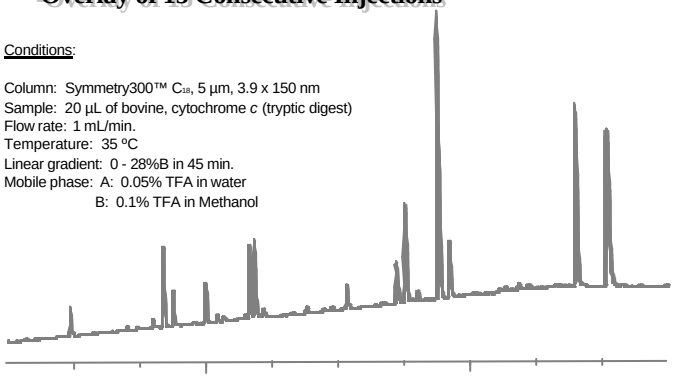
- Step 0-100%B
- Solvent delivery <250 µL
- System volume <650 µL
  typically <550 µL

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

Reproducible Gradient Delivery
-Waters Alliance™ HPLC System
-Overlay of 15 Consecutive Injections

Conditions:

Column: Symmetry300™ C_{18}, 5 µm, 3.9 x 150 nm
Sample: 20 µL of bovine, cytochrome *c* (tryptic digest)
Flow rate: 1 mL/min.
Temperature: 35 ºC
Linear gradient: 0 - 28%B in 45 min.
Mobile phase: A: 0.05% TFA in water
B: 0.1% TFA in Methanol



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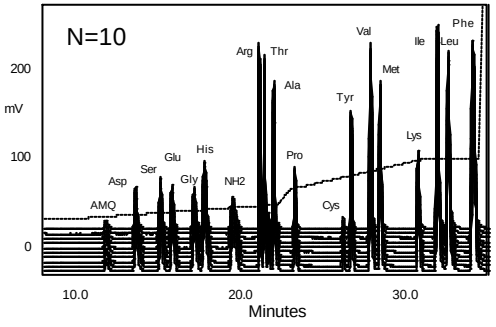
Reproducibility of Complex Gradients
AccQ·Tag? Amino Acid Analysis

Amino Acid	Std Dev (seconds)
AMQ	1.3
Asp	1.8
Ser	1.5
Glu	1.8
Gly	1.7
His	2.0
NH3	1.8
Arg	0.8
Thr	0.7
Ala	0.7
Pro	0.9
Cys	1.1
Tyr	1.0
Val	1.0
Met	1.0
Lys	0.8
Ile	0.8
Leu	0.9
Phe	1.3



Complex Gradient Conditions:

- Column: AccQ Tag™ Column, 3.9 x 150 mm
- Eluent A: AccQ Tag™ Eluent A
- Eluent B: Acetonitrile
- Eluent C: Water
- Flow Rate: 1.0 mL/min.
- Column Temp.: 37 °C
- Detection: Fluorescence, k_{ex} = 250 nm, k_{em} = 395 nm
- Sample: 50 pmol Hydrolysate Standard
- Sample Temp.: 5 °C
- Gradient:

Time	Flow	%A	%B	%C	%D	Curve
0.00	1.00	100.0	0.0	0.0	0.0	*
0.50	1.00	99.0	1.0	0.0	0.0	11
18.0	1.00	95.0	5.0	0.0	0.0	6
19.0	1.00	91.0	9.0	0.0	0.0	6
29.5	1.00	83.0	17.0	0.0	0.0	6
33.0	1.00	0.0	60.0	40.0	0.0	11
36.0	1.00	100.0	0.0	0.0	0.0	11
65.0	1.00	0.0	60.0	40.0	0.0	11
100.0	0.00	100.0	0.0	0.0	0.0	6

Why is Column Reproducibility Important?

- Validated RP-HPLC assays require HPLC columns to be reproducible from column-to-column and batch-to-batch.
- Columns which perform reproducibly in terms of selectivity and separation characteristics from batch-to-batch ensures reliable, reproducible and robust assays over the life of the product.

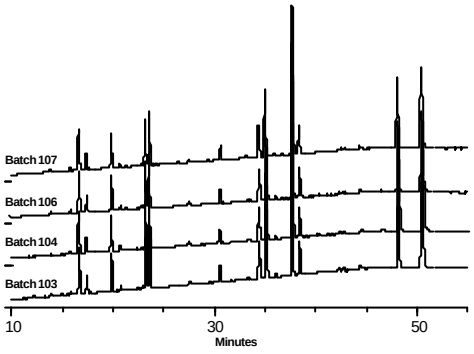
"Consideration must be given to column-to-column variation attributed to production differences from a recommended manufacturer. Column-to-column variability was recently bemoaned as the 'Achilles heel' in the HPLC of protein pharmaceuticals (20). Although this problem has been both ignored by many and overstated by others, peptide mapping does place very high demands on column performance. Thus, column-to-column variability is of special concern (21,22)."

Garnick et al. *Biologicals* (1996) 24, 255-275

Column-to-Column Reproducibility

- Employed 8 different Symmetry300™, C_{18}, 5 µm columns from the same batch (#107)
- Conducted evaluation over a 3-week time period
- Different mobile phase preparations for each run

Symmetry300™ Batch-to-Batch Reproducibility



Conditions

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- Sample: Tryptic digests of cytochrome *c* (bovine)
- Injection: 20 µL
- Mobile Phase:
  Solvent A: 0.05% TFA in water
  Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-28%B
- Flow rate: 1.0 mL/min.
- Temperature: 35 °C
- Detection: 220 nm

- Instrument: Waters Alliance™ HPLC system

Costello

Conclusion

- There are several factors to assess when developing/optimizing a gradient RP-HPLC method.
  - Primary tools for gradient optimization are gradient slope, column length and modifier type.
  - Secondary tools to be used for fine tuning your gradient are temperature, modifier concentration and flow rate.

Conclusions (continued)

- For Rugged/Robust gradient method development consider...
  - System performance
    - reproducibility of gradient delivery
    - system delay volume
  - Column performance
    - column-to-column reproducibility
    - batch-to-batch reproducibility

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...and Waters' Symmetry® Team