

Scaling up to Preparative Chromatography

Scaling Up to Preparative Chromatography

Dr. Shulamit Levin

home page: www.forumsci.co.il/HPLC

Strategy for Preparative Separation



Selection of the appropriate mode of chromatography



Optimization of the Separation
(Stationary phase, Mobile Phase, Temperature, Additives)

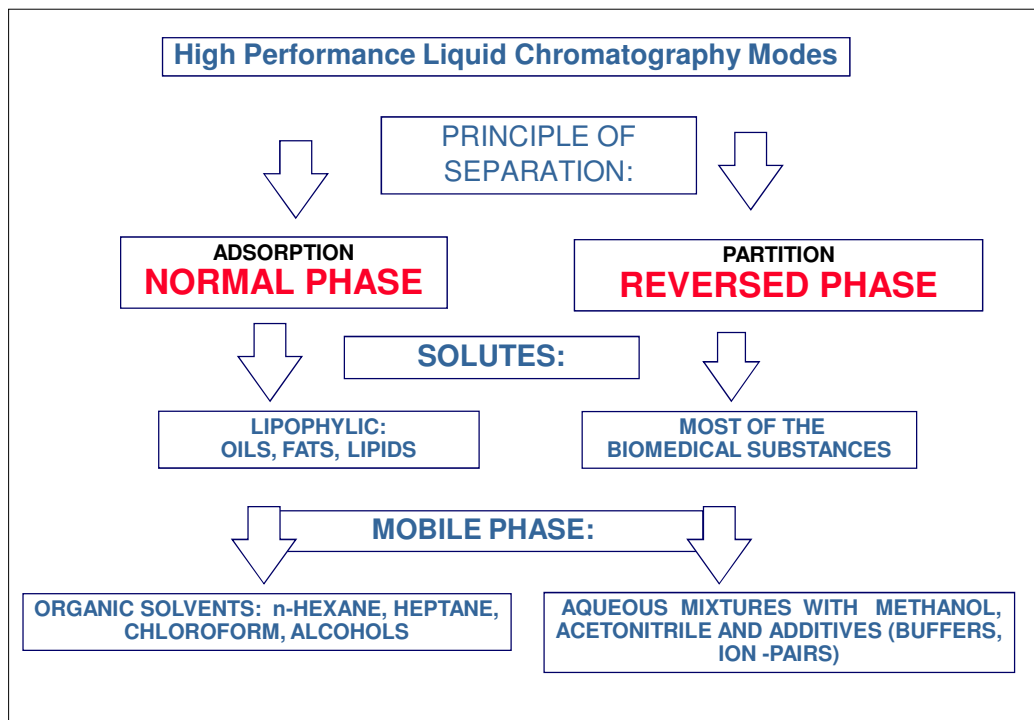


Optimization of the throughput
(Sample amount: Column Overloading)
Adsorption Isotherm & Competition

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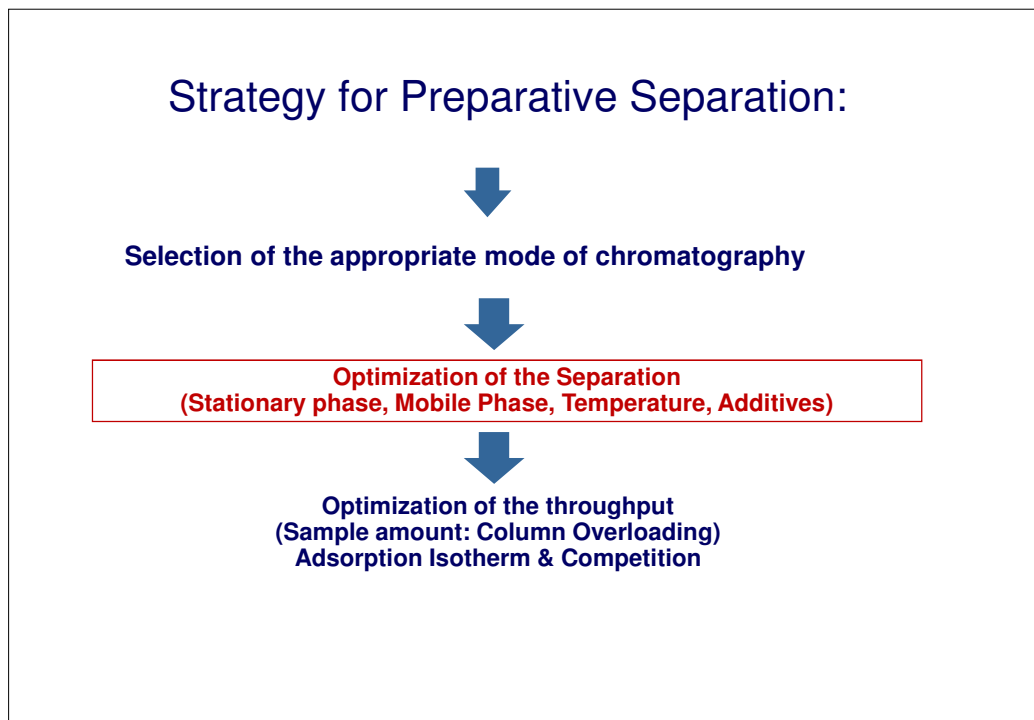
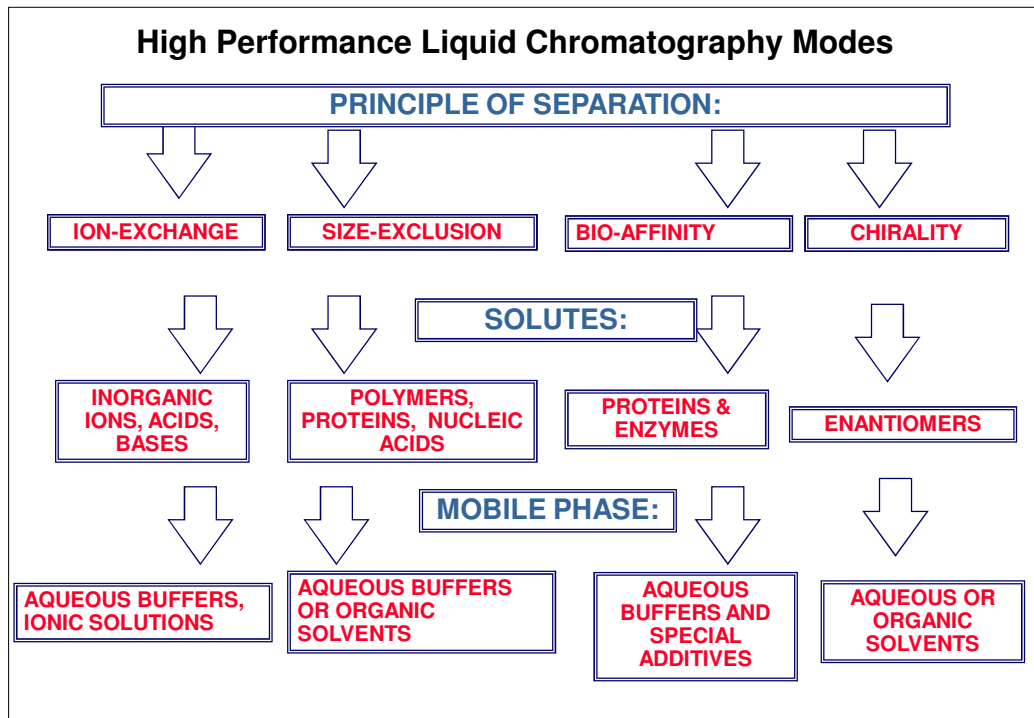
Seven Basic Considerations in Choosing HPLC Operating Parameters

- 1) **Solubility** - Hexane, Chloroform, Methanol, Water (buffer pH), other?
- 2) **Molecular Weight** - Would GPC be useful in either the analysis or sample prep?
- 3) **Functional Groups** - Any ionizable groups? Acidic, Basic, or Neutral?
- 4) **Sample Matrix** - What amounts are expected in matrix for either analytical or preparative isolation?
- 5) **Levels in Matrix** - What amounts are expected in matrix for either analytical or preparative isolation?
- 6) **Detectability** - Any chromophores or fluorophores?
Consider Redox or derivatization.
Together with point #5, an appropriate detector is chosen.
- 7) **How Do Species Differ** - An important clue to manipulate selectivity the separation, especially if compounds are similar in their in structure.



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PERFORMANCE CRITERIA BY ONE PEAK

Application

RETENTION FACTOR or CAPACITY RATIO

$$k' = \frac{t_R - t_0}{t_0} \quad k' = \phi \frac{C_s}{C_m}$$

ASYMMETRY FACTOR

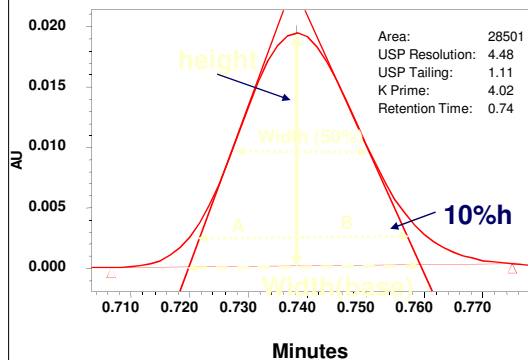
$$A_f = \frac{B_{(10\%h)}}{A_{(10\%h)}}$$

TAILING FACTOR

$$T_f = \frac{A + B}{2A} \quad (5\% h)$$

NUMBER OF THEORETICAL PLATES

$$N = 16 \left(\frac{t_R}{W} \right)^2$$



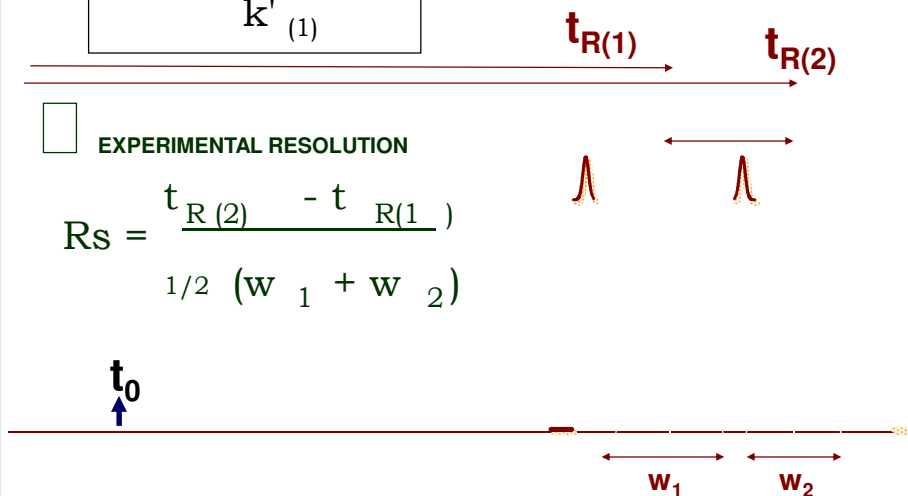
PERFORMANCE BY TWO PEAKS

SELECTIVITY FACTOR

$$\alpha = \frac{k'_{(2)}}{k'_{(1)}}$$

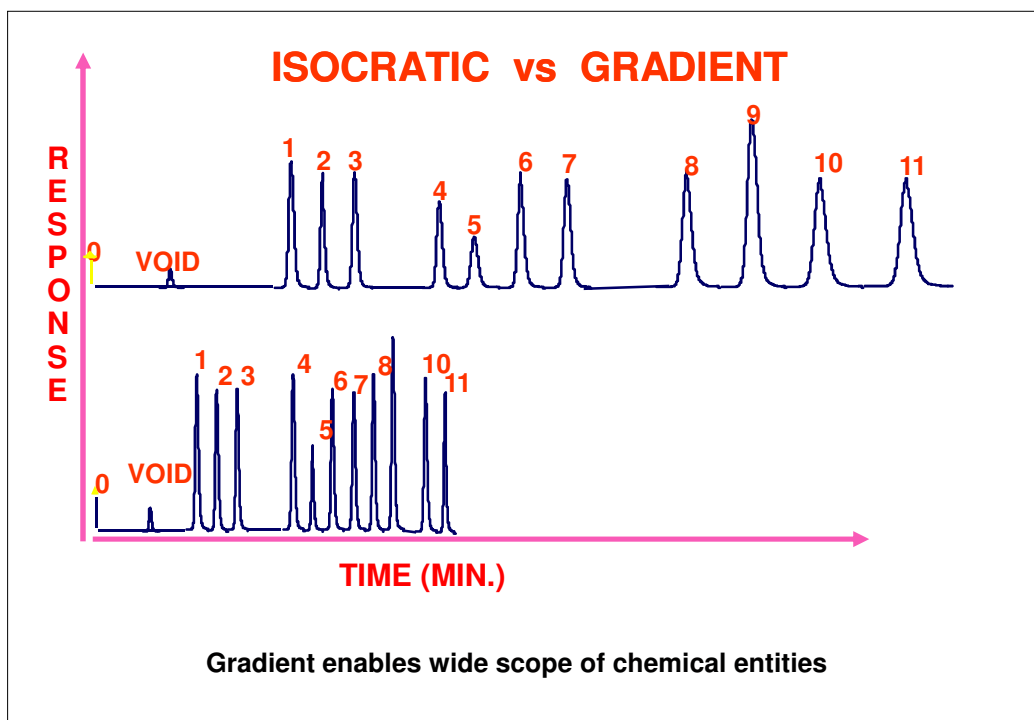
EXPERIMENTAL RESOLUTION

$$R_s = \frac{t_{R(2)} - t_{R(1)}}{1/2 (w_1 + w_2)}$$



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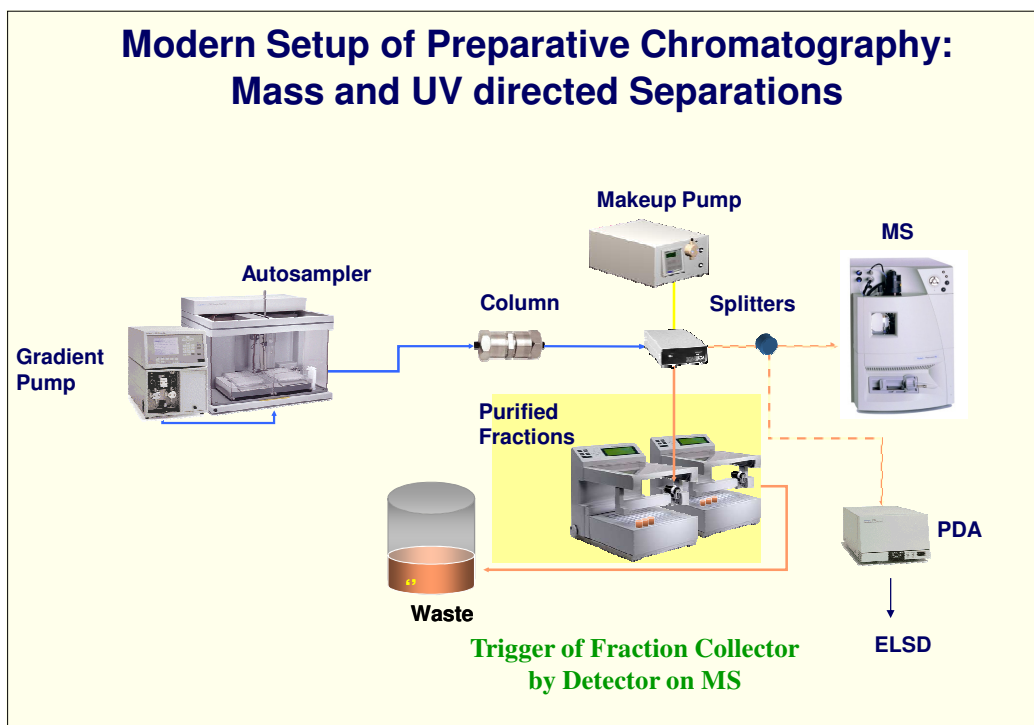
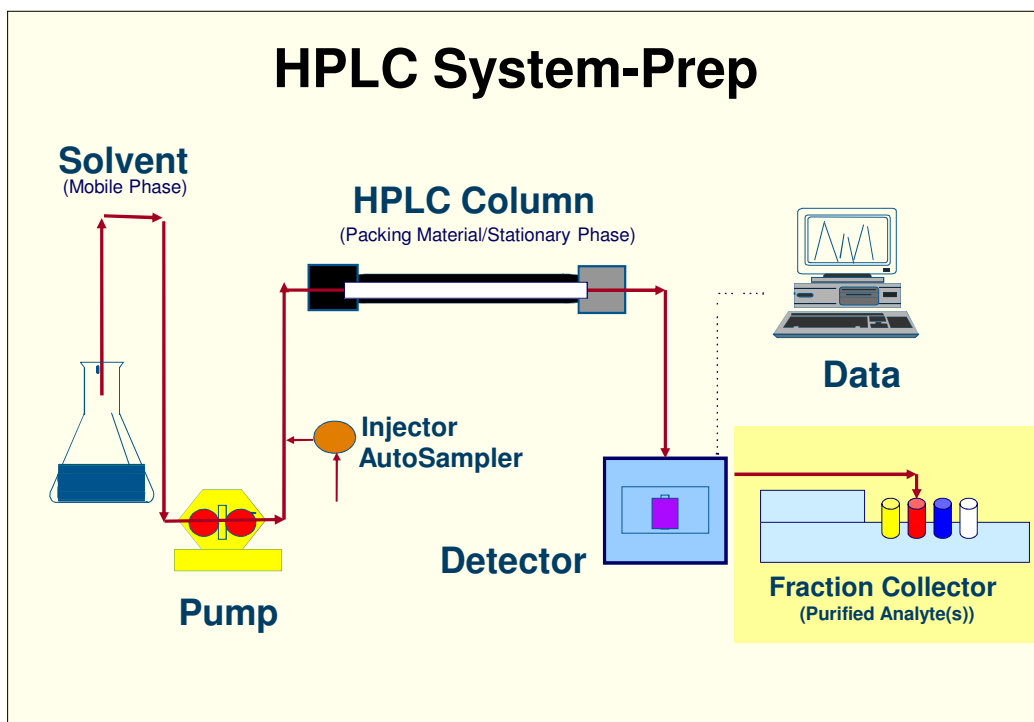
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General Introduction to Preparative Chromatography

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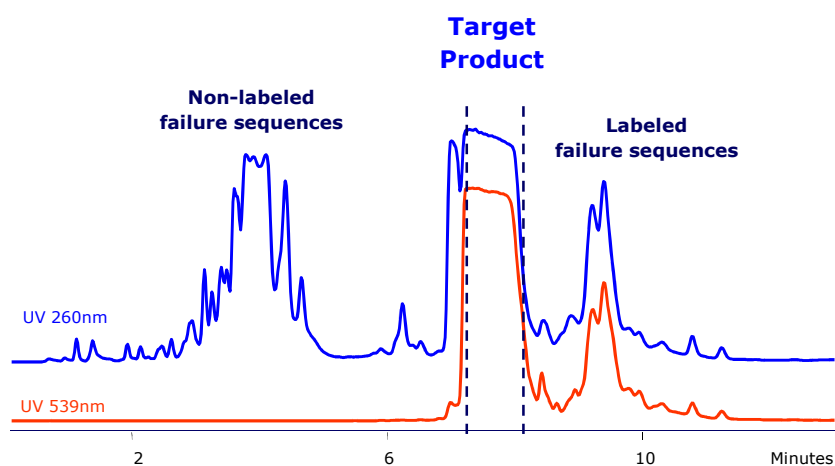
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Purification of 0.1 μ mole of 5'HEX labeled 25mer



Fountain et al., J. Chromatogr. B, 2003, vol. 783, 61-72

Various Dimensions of Preparative Columns



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Problem Definition: Purifications

- What quantity of material needs to be isolated?
- Is the material a major or minor component?
- Do you need to maintain biological activity?
- What degree of purity (or specific activity) is required?
- How will purity or activity be verified?

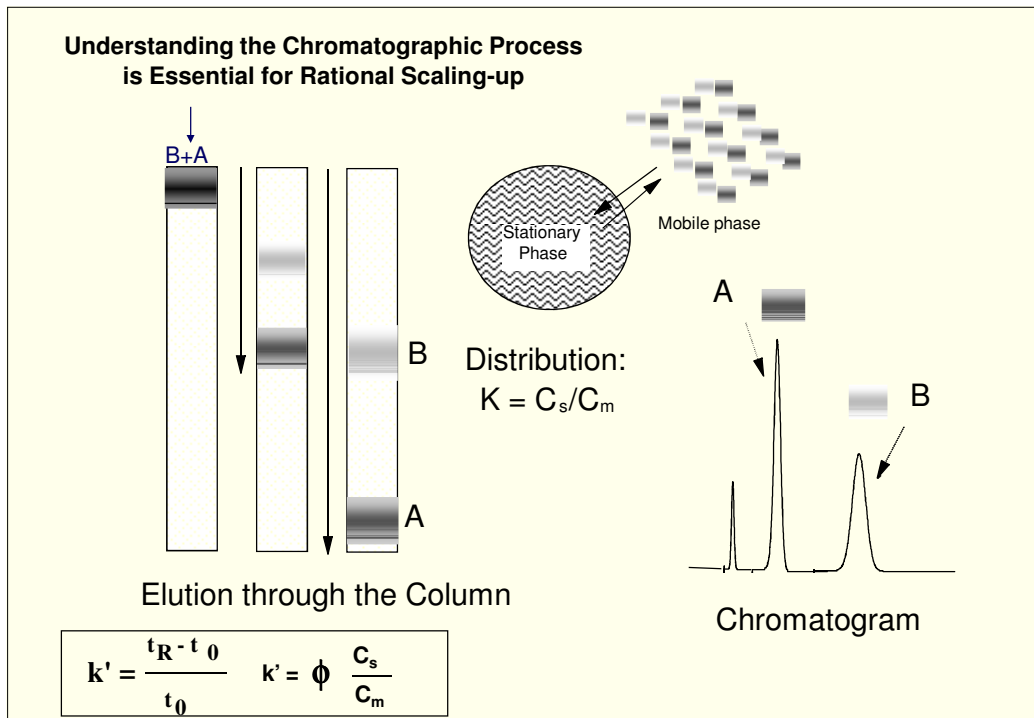
If analyzed sample is to be collected; the above questions must be answered

Preparative Chromatography Terminology: Different from Analytical Chromatography

- **Sample Solubility**
- **Load - Overload**
- **Throughput**
- **Purity**
- **Recovery/Yield from Column**
- **Recovery from Fractions**
- **Cost of Purification**

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RETENTION FACTOR = Capacity Factor

We know the measurement of k' from the analytical work:
 Retention Factor:

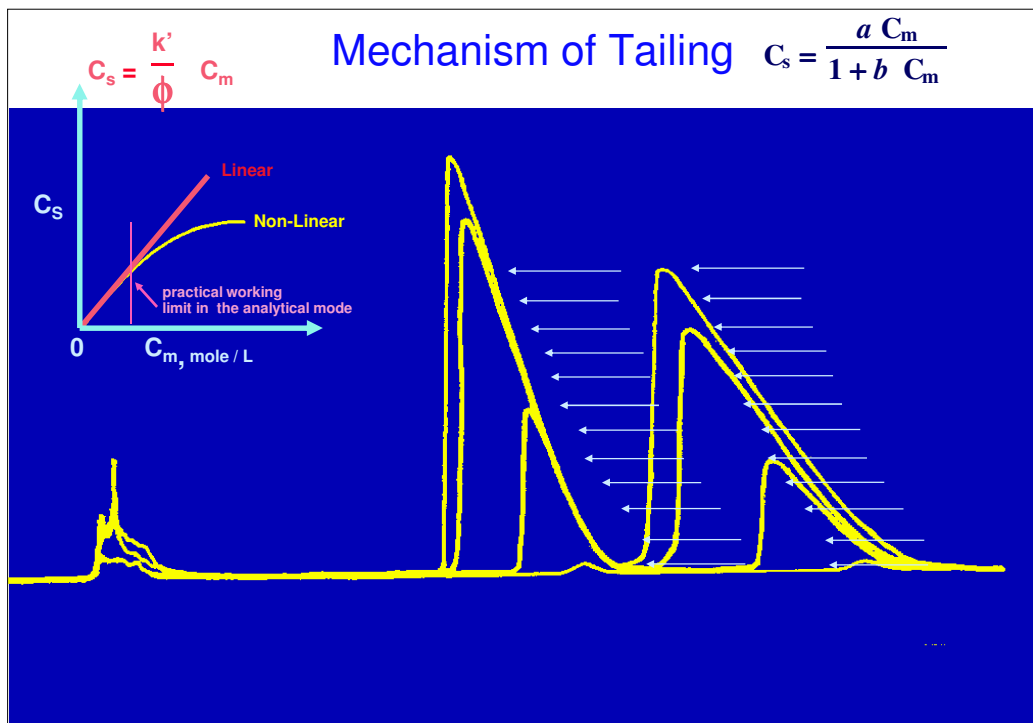
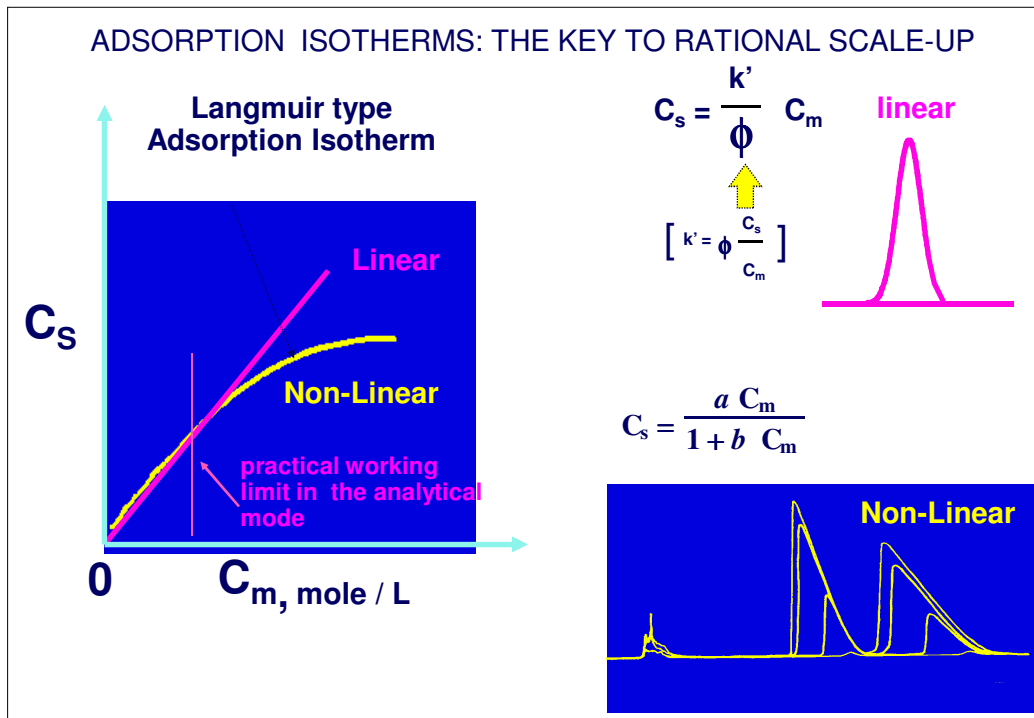
$$k' = \frac{t_R - t_0}{t_0}$$

We use its thermodynamic expression to rationally scale up the separation:

CAPACITY RATIO $k' = \frac{C'_s}{C'_m} = \frac{C_s V_s}{C_m V_m} = \phi K$

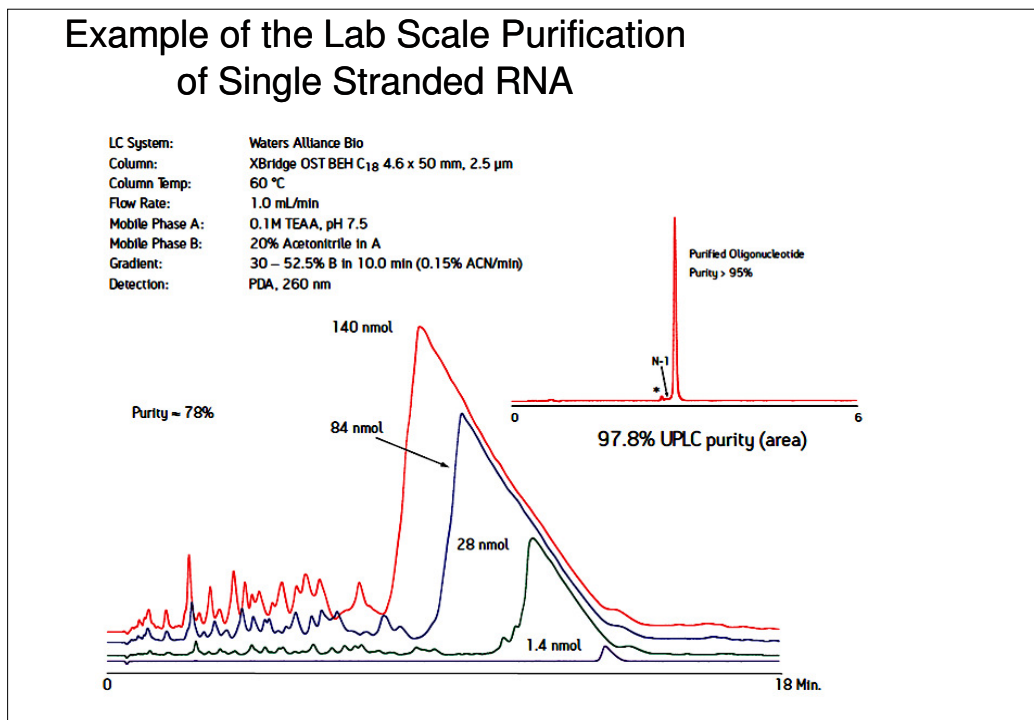
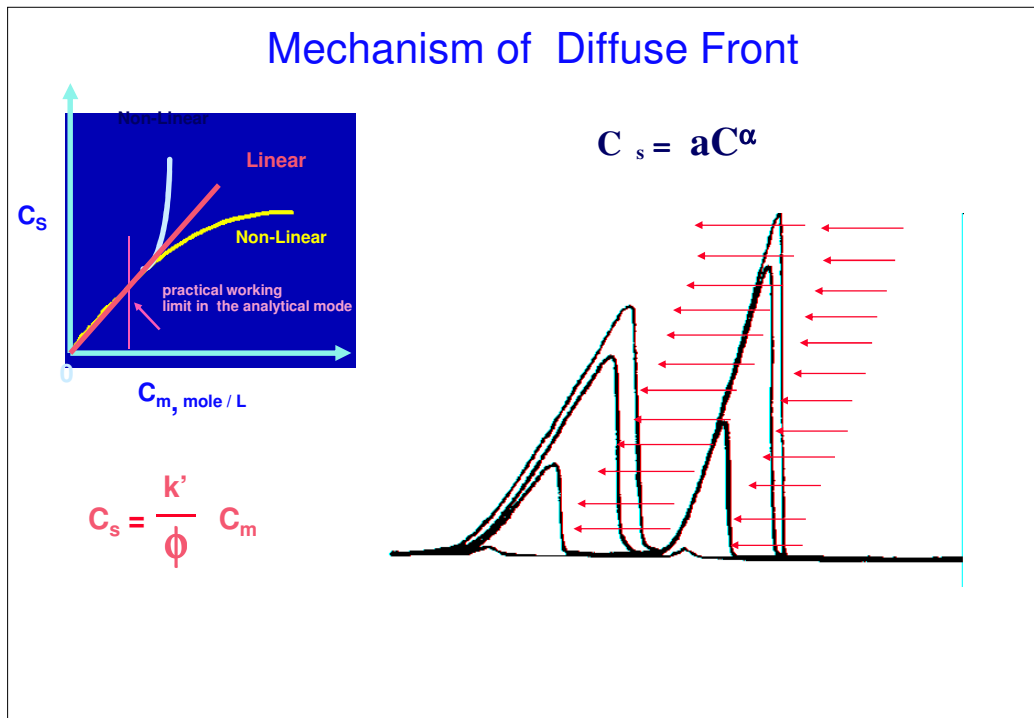
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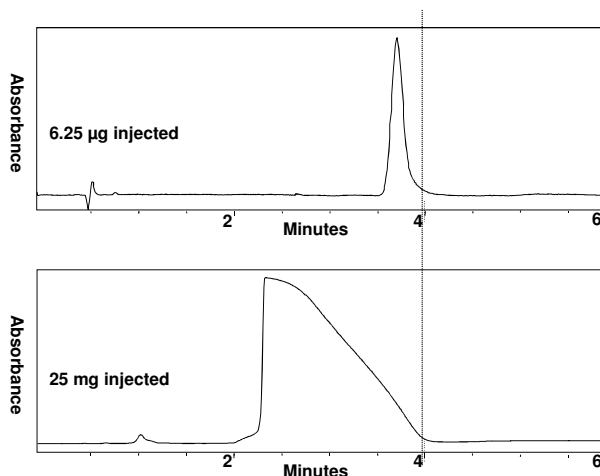
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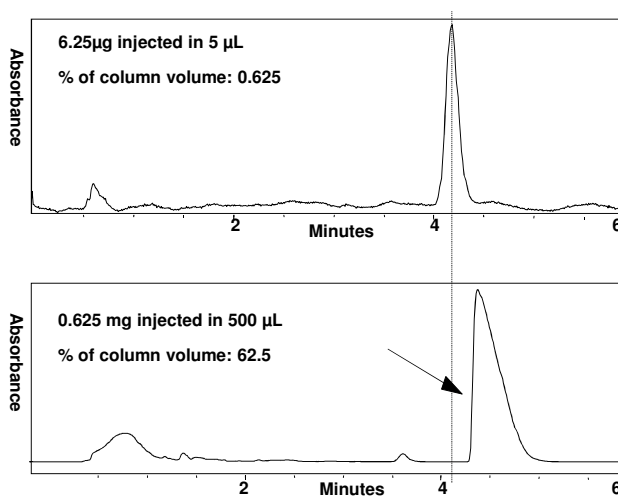
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Mass Overload



- Analytical load of 6 µg yields efficient peak shape
- Preparative load of 25 mg generates mass overload peak shape
- Note that the back of the peaks of the analytical and prep loads are at the same retention (-----)

Volume Overload



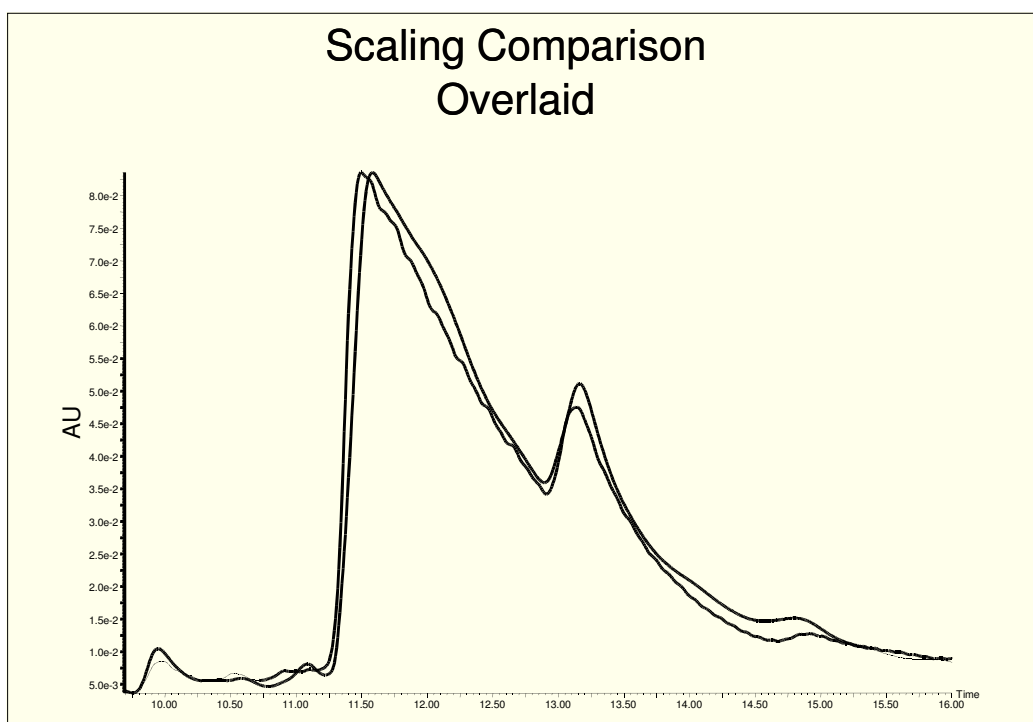
Column Volume:
0.8 mL (800 µL)

Wider peaks first observed
at low retention

Peak position shifts to
higher retention in
proportion to the injection
volume

Start of peak remains in the
same position unless
injected in a weaker solvent

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Strategy for Preparative Separation

↓

Selection of the appropriate mode of chromatography

↓

Optimization of the Separation
(Stationary phase, Mobile Phase, Temperature, Additives)

↓

Optimization of the throughput
(Sample amount: Column Overloading)
Adsorption Isotherm & Competition

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From Small Scale To Purification



Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
 - ◆ Dimensions
 - ◆ Particle Size
 - ◆ Chemistry
- Chemistry of the Mobile phase

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Scaling Mass Load and Injection Volume

- **Mass Load**
 - ◆ Proportional to column volume
 - ◆ Limited by solubility in mobile phase
- **Injection volume**
 - ◆ Approximately proportional to column volume
 - ◆ Approximately proportional to both length and cross-sectional area
 - ◆ Most strongly dependent on sample solvent

Scale Up Equations - Mass

$$M_{prep} = M_{anal} \times \frac{V_{prep}}{V_{anal}}$$

M_{prep} = mass injected on prep column

M_{anal} = mass injected on analytical column

V_{prep} = volume of prep column

V_{anal} = volume of analytical column

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Scaling Equations Mass

$$M_2 = M_1 \times \frac{L_2}{L_1} \times \frac{d_2^2}{d_1^2} \quad \text{where :}$$

M_1 is Mass on Column 1 (Analytical)

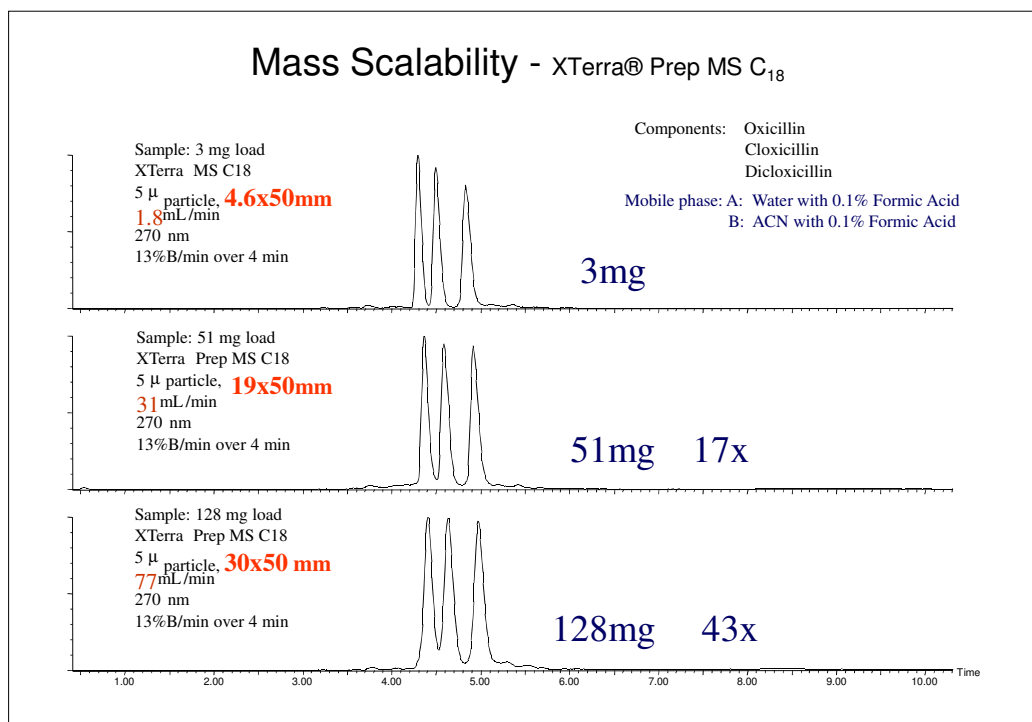
M_2 is Mass on Column 2 (Prep)

L_1 is Length of Column 1

L_2 is Length of Column 2

d_1 is Diameter of Column 1

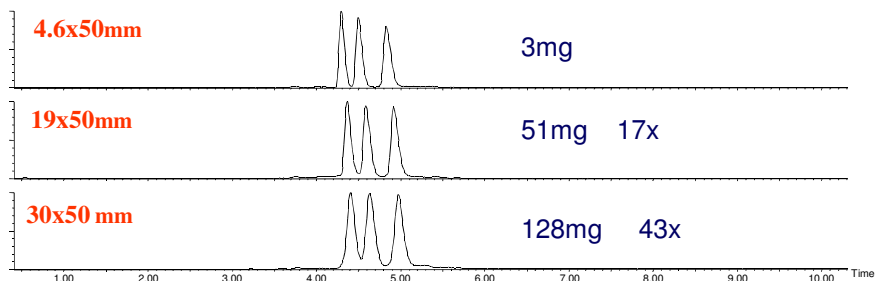
d_2 is Diameter of Column 2



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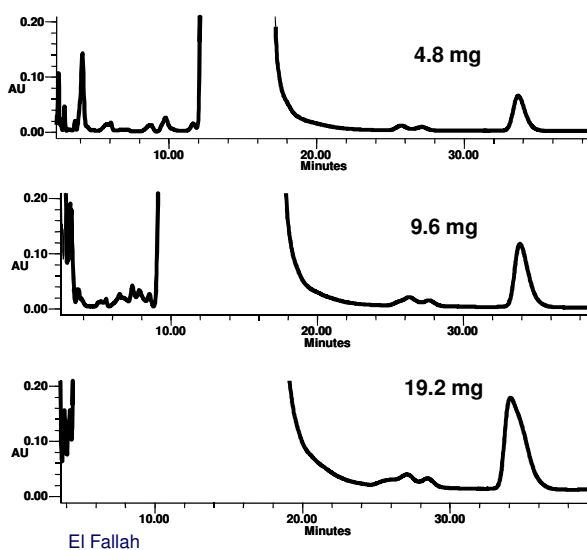
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Mass Scalability - XTerra® Prep MS C₁₈

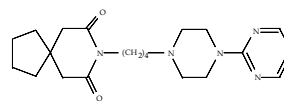


R (cm)	R ²	L (cm)	V (mL)	Scaling Factor	Flow	Mass Load
0.230	0.053	5	0.83		1.8	3
0.950	0.903	5	14.18	17	30.7	51
1.5	2.250	5	35.34	43	76.6	128

Buspirone Impurities II



Structure of Buspirone



Column: SymmetryPrep C18,
7 μ m (3.9 x 150) mm
Mobile Phase:
28% acetonitrile / 72%
0.18% TETA-MeCOOH pH 7.0
Flow Rate: 1.0 mL/min
Detection: UV at 360 nm
Sample: 12 mg/mL of Buspirone
Injection: from 0.4 to 1.6 mL

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Approximate Mass Loading Capacity

Many factors affect the mass capacity of preparative columns.
The listed capacities represent an “average” estimate

Mass Capacity (mg)

Length (mm)	Diameter (mm)											
	3.9	4.6	7.8	8	10	19	20	25	30	40	47	50
50	2	3	8		15	45	50		110			310
100	4	5	15	15	25	90	100	155	225	400		620
150	6	8	25		40	135	150		335			930
200				30				310		795		
250	10	13	40		60	225	250		560			1550
300	12	16	45	50	75	270	300	470	670	1195	1650	1860
Reasonable Flow Rate(ml/min)	1.0	1.4	4.0	4.2	6.6	24	27	42	60	105	145	164
Reasonable Injection Volume(µ l)	15	20	60	65	100	350	390	610	880	1565	2160	2450

Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
 - ◆ Dimensions
 - ◆ Particle Size
 - ◆ Chemistry
- Chemistry of the Mobile phase

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Scale Up of Flow Rate

- * Same Linear Velocity to maintain
N and Rs and Retention Time
- Van Deemter

Scaling Equations Flow Rate

$$F_2 = F_1 \times \frac{d_2^2}{d_1^2} \text{ where :}$$

F_1 is Flow Rate for Column 1 (Analytical)

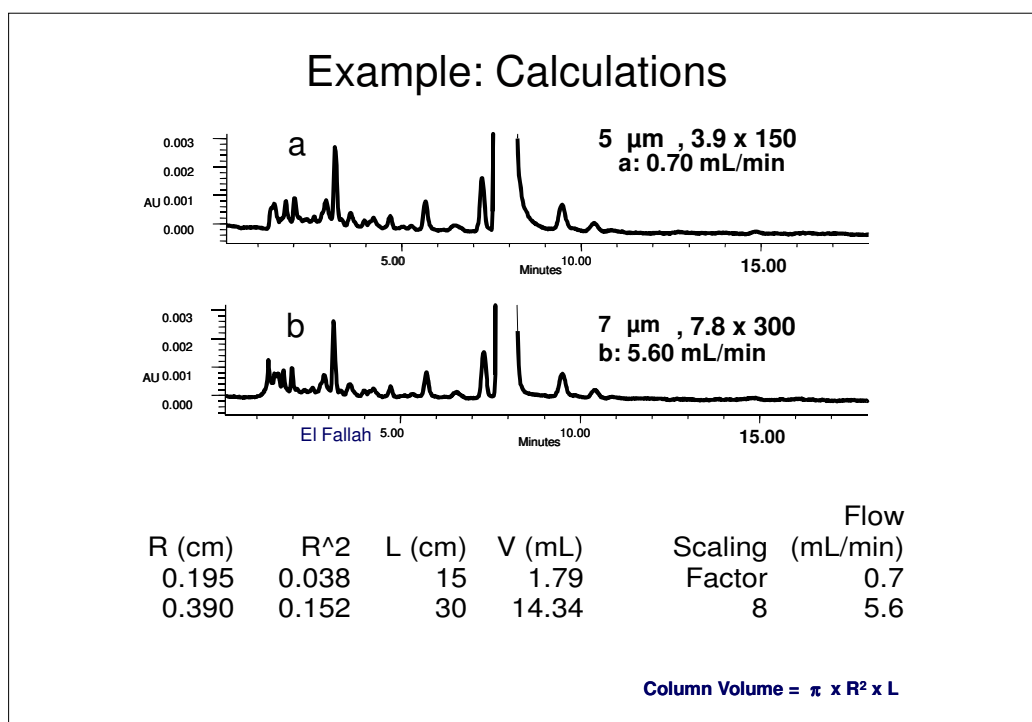
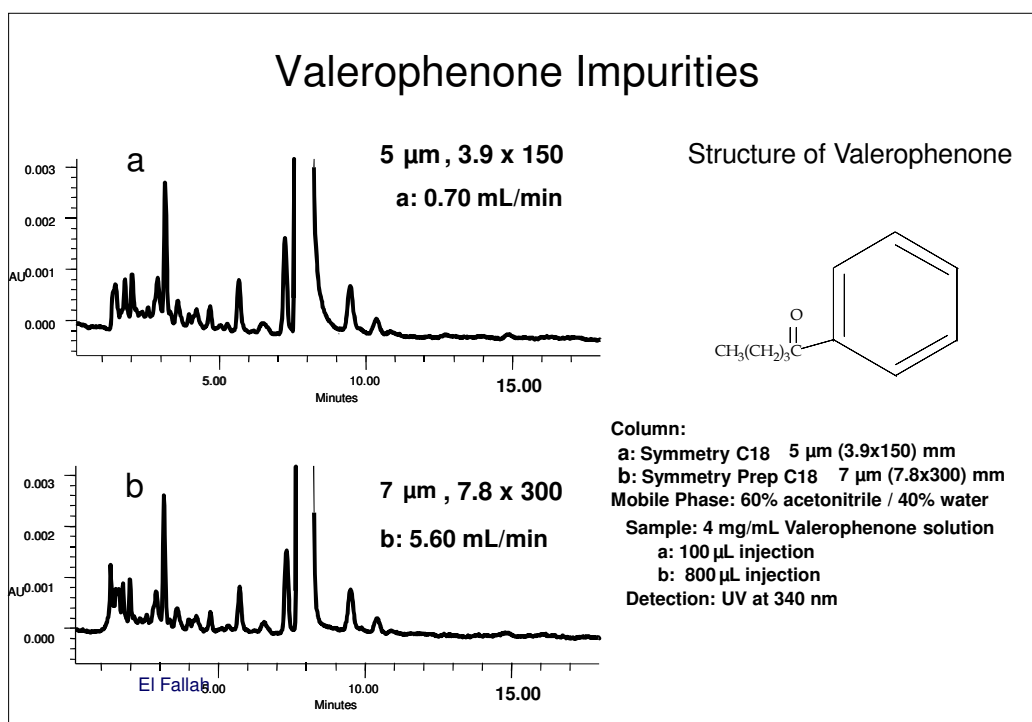
F_2 is Flow Rate Column 2 (Prep)

d_1 is Diameter of Column 1

d_2 is Diameter of Column 2

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Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
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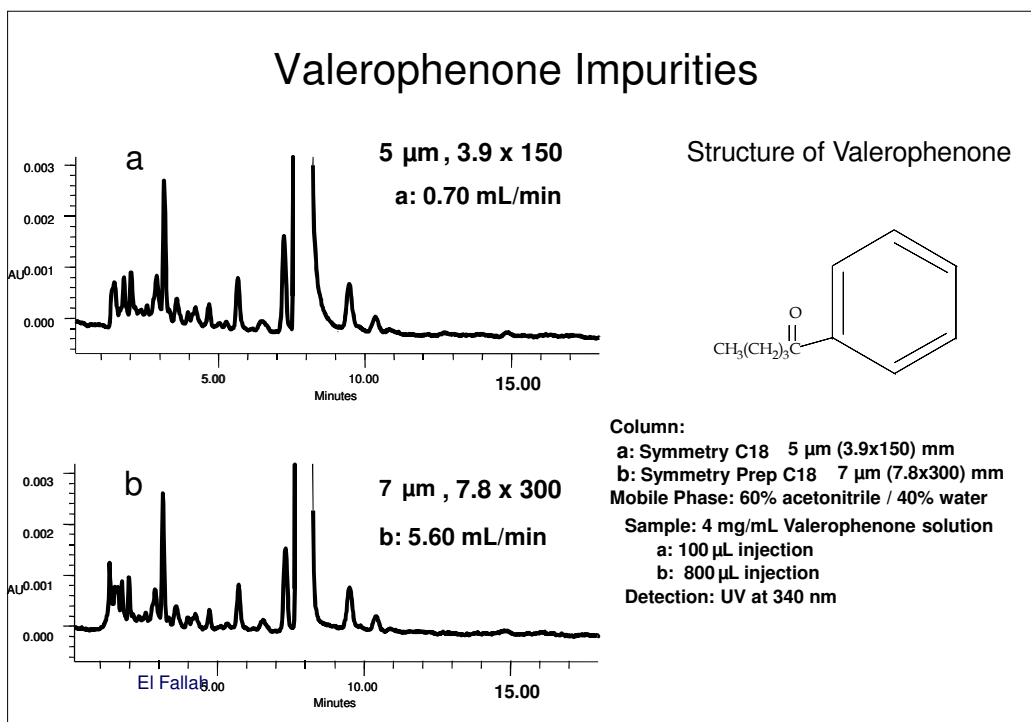
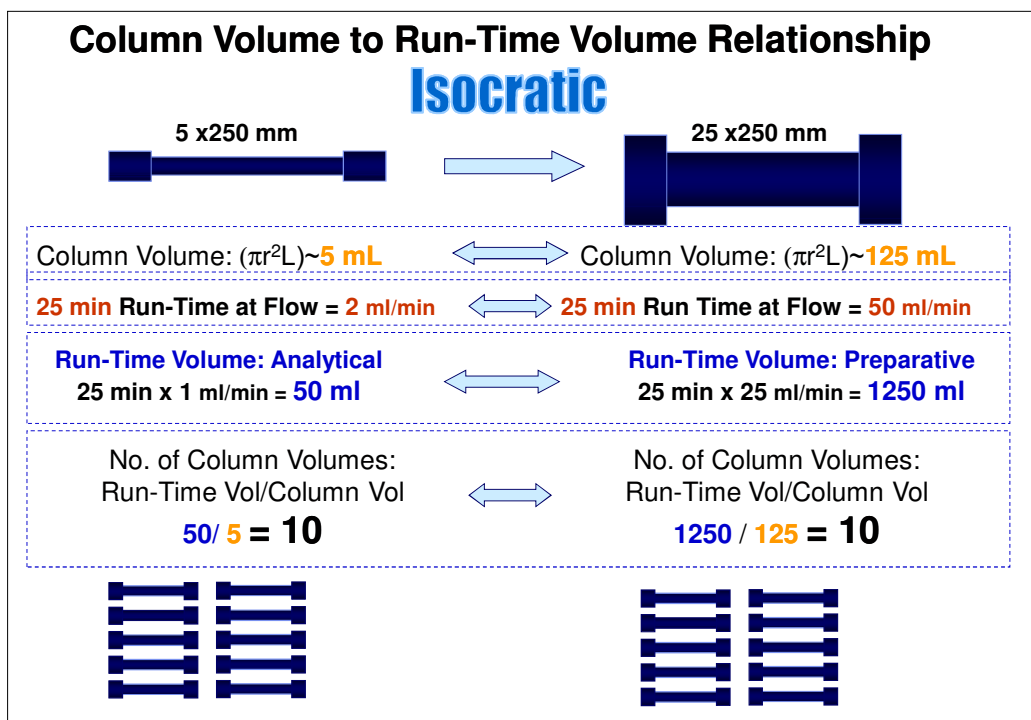


Understanding the Concept of **Column Volumes**

$$\text{Column Volume} = \pi \times R^2 \times L$$

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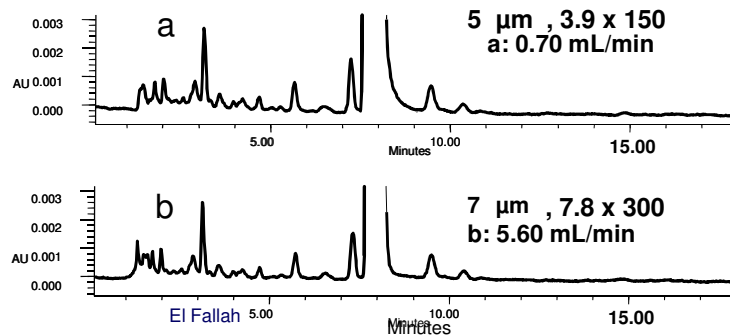
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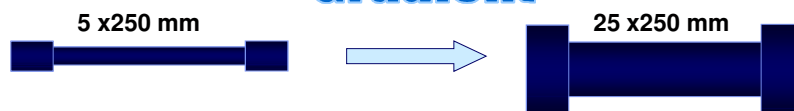
Example: Calculations



R (cm)	R ²	L (cm)	V (mL)	Scaling Factor	Flow	Run-Time (Min)	Column Volumes
0.195	0.038	15	1.79		0.7	15	5.9
0.390	0.152	30	14.34	8	5.6	15	5.9

Column Volume to Gradient Volume Relationship

Gradient



Column Volume: $(\pi r^2 L) \sim$ **5 mL** \longleftrightarrow Column Volume: $(\pi r^2 L) \sim$ **125 mL**

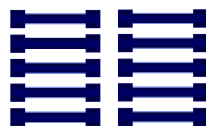
25 min Gradient at Flow = **2 mL/min** \longleftrightarrow **25 min** Gradient at Flow = **50 mL/min**

Gradient Volume: Analytical
25 min x 2 mL/min = **50 mL**

Gradient Volume: Preparative
25 min x 50 mL/min = **1250 mL**

No. of Column Volumes:
Gradient Vol/Column Vol
50 / 5 = 10

No. of Column Volumes:
Gradient Vol/Column Vol
1250 / 125 = 10



Scaling up to Preparative Chromatography

Scale Up Equations - Gradient

$$t_{prep} = t_{anal} \times \frac{V_{prep}}{V_{anal}} \times \frac{F_{anal}}{F_{prep}}$$

t_{prep} = run time with prep column

t_{anal} = run time with analytical column

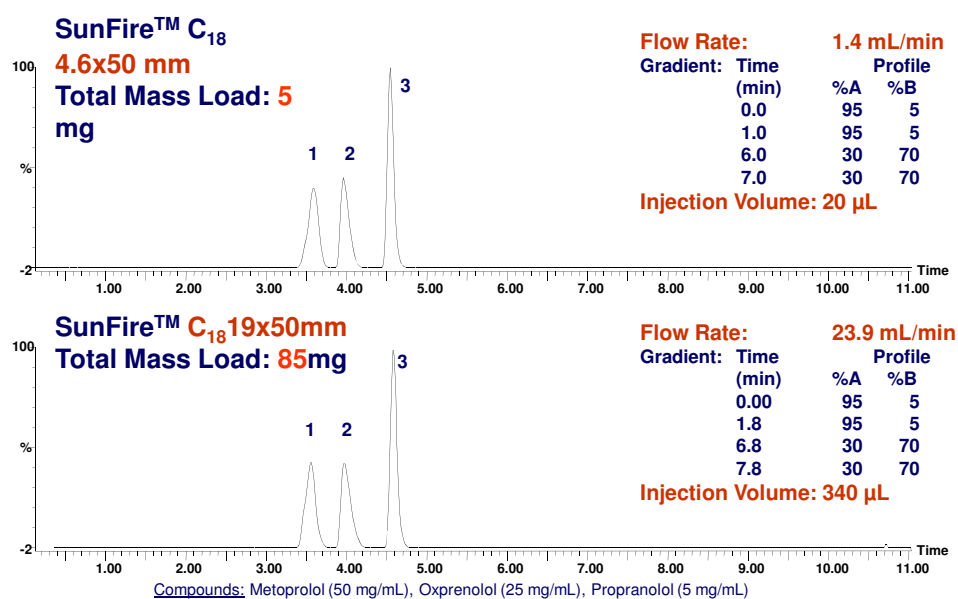
V_{prep} = volume of prep column

V_{anal} = volume of analytical column

F_{prep} = prep flow rate

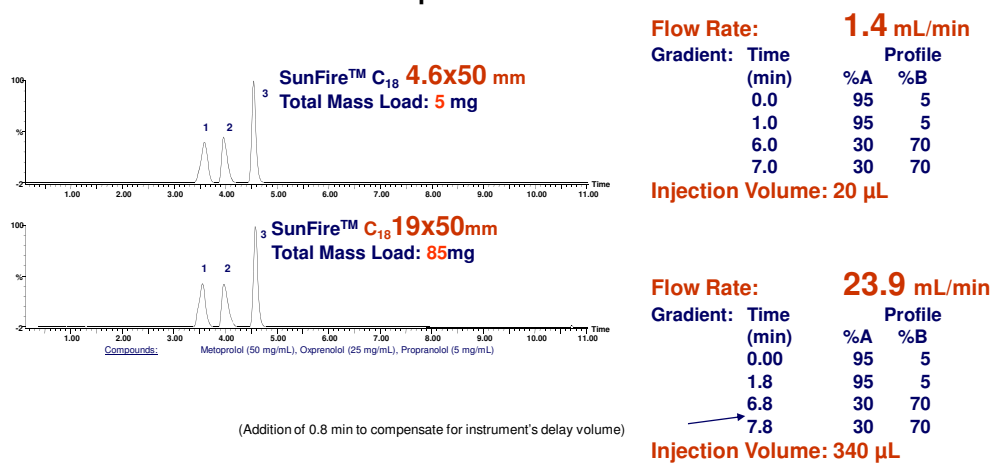
F_{anal} = analytical flow rate

Example: Scale-up Gradient



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Scale-up Gradient



R (cm)	R ²	L (cm)	V (mL)	Scaling Factor	Flow
0.230	0.053	5	0.83		1.4
0.950	0.903	5	14.18	17	23.9

Prep Calculator

Basic Gradient Scalar Calculation

This calculation requires input for dimensions of the two columns as well as a gradient table used for column 1. All 10 lines in the gradient table should be completed to avoid any errors. An example is shown on the Help Page.

Note 1: You may observe differences in retention times due to the dwell volume of the systems. If you know

Column Dimensions

Column	I.D. (mm)	Length (mm)	Column Volume (mL)
Column 1	4.6	100	1.395
Column 2	3.0	150	0.890

Number of runs on column 2:

Calculate

Print

Reset

Help

Exit

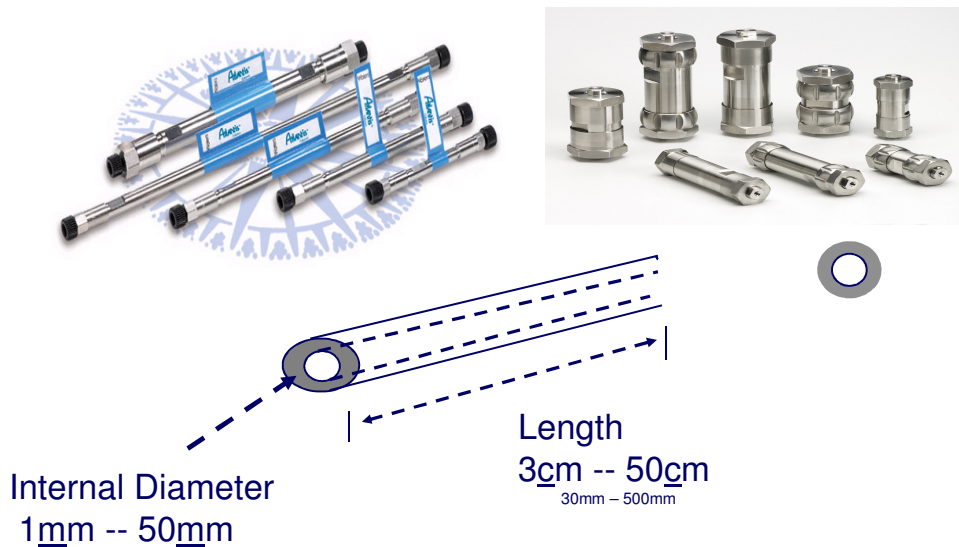
Step	Time	Flow	%A	%B	%C	%D	Curve
Init Cond.	0	1	50	40	5	5	-
Init Hold	2	1	50	40	5	5	-
3	12	1	40	50	5	5	6
4	37	1	20	70	5	5	6
5	41.9	1	20	70	5	5	6
6	42	1	50	40	5	5	6
7	48	1	50	40	5	5	6
8	48	1	50	40	5	5	6
9	48	1	50	40	5	5	6
10	48	1	50	40	5	5	6

Scaling up to Preparative Chromatography

Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
 - ◆ Dimensions
 - ◆ Particle Size
 - ◆ Chemistry
- Chemistry of the Mobile phase

Chromatography



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Scale Up – Column's Dimensions

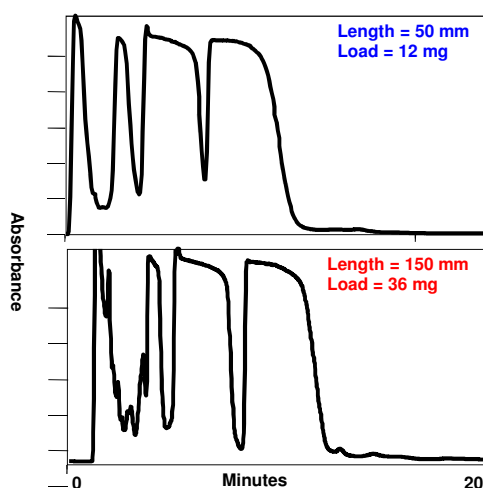
- Dimensions
 - ◆ Mass capacity is proportional to column volume
 - ◆ Elution volume is proportional to column volume
 - ◆ Shorter columns preferred for speed and pressure
 - ◆ Longer columns preferred for resolution

Effect of Column Length

- Efficiency increases with length
- Backpressure increases with length
- Run time increases with length
- Cost increases with length

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Effect of Column Length on Capacity



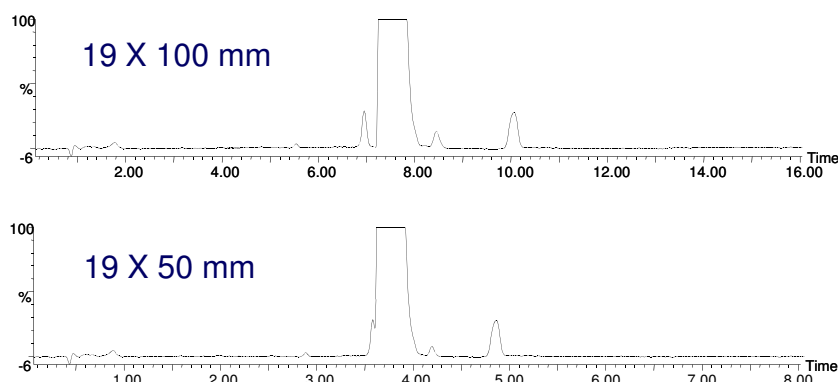
► As can be seen in this example, there is a linear relationship between column length and loading capacity - **3X increase in column length generates a >3X increase in loading capacity**

Disadvantage of long columns:

- higher pressure for equal run time (9x at 3x increase in length)
- higher pressure at equal velocity and longer run time

Effect of Column Length

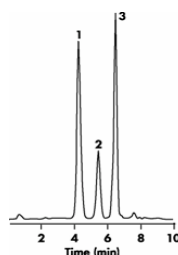
XTerra® MS C₁₈, OBD™, 5 µm



As column length decreases, resolution between the minor impurity and main peak decreases. Loading capacity will also decrease.

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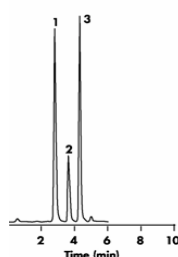
XTerra® Prep Columns for Speed – 40% Reduction in Retention and Peak Volume



Column: XTerra® Prep MS C₁₈
19 x 50 mm, 5 µm
Flow Rate: 20 mL/min
Mobile Phase: A: 10mM NH₄HCO₃, pH 10
B: ACN
Gradient: 5-40% B in 1.1 min
40-90% B from 1.1 min to 7.5 min
Injection Vol: 500 µL

Analytes: Conc. (mg/mL DMSO)
1: diphenhydramine 20
2: oxybutynin 20
3: terfenadine 20

- Peak # 1 13 ml
- Peak # 2 9 ml
- Peak # 3 13 ml



Column: XTerra® Prep MS C₁₈
19 x 30 mm, 5 µm
Flow Rate: 20 mL/min
Mobile Phase: A: 10mM NH₄HCO₃, pH 10
B: ACN
Gradient: 5-40% B in 0.6 min
40-90% B from 0.6 min to 4.5 min
Injection Vol: 500 µL

Analytes: Conc. (mg/mL DMSO)
1: diphenhydramine 20
2: oxybutynin 20
3: terfenadine 20

- Peak # 1 8 ml
- Peak # 2 6 ml
- Peak # 3 7 ml

Scaling to Smaller Columns Allows:

- Faster chromatography
- Peak volume reduction
- Less expensive column
- Depending on the application, how far is it possible to downsize?
- Plate-to-plate mapping, injecting from a 96 well plate and collecting fractions in another 96 well plate, analytical size columns are suitable for this work

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Load Considerations

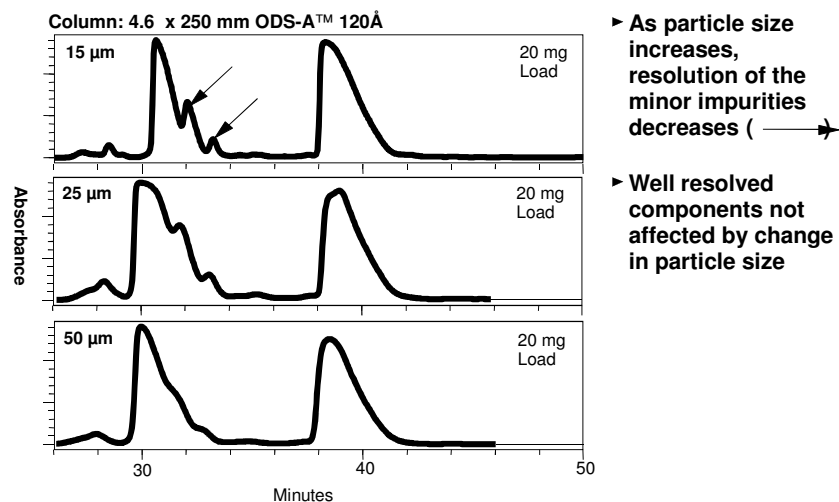
- Mass Capacity
 - ◆ Proportional to column volume
- Injection Volume
 - ◆ Scale in proportion to the column volume
 - ◆ Usually larger for gradient separations
 - ◆ Cannot be considered separately from sample solvent
- Sample Solvent
 - ◆ Good solvent needed to load high sample concentrations
 - ◆ Good sample solvents degrade chromatography
 - ◆ Good chromatographic diluents give low sample concentrations

Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
 - ◆ Dimensions
 - ◆ Particle Size
 - ◆ Chemistry
- Chemistry of the Mobile phase

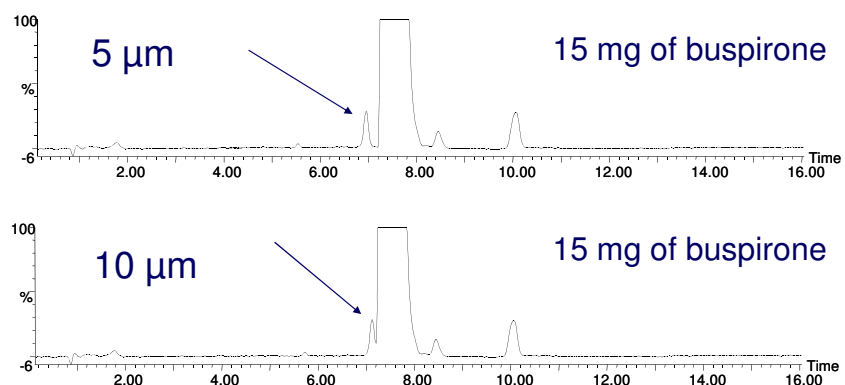
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Effect of Particle Size on Capacity



Effect of Particle Size

XTerra® MS C₁₈, OBD™, 19 X 100 mm



As particle size increases, resolution between the minor impurity and the main peak decreases.

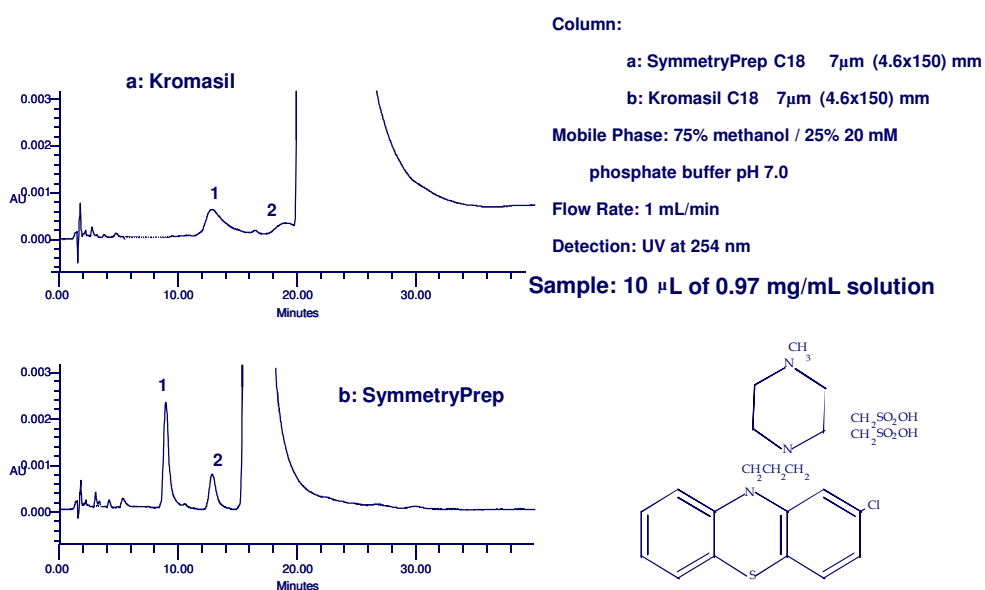
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- Mass Load
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- Chemistry of the Mobile phase

Prochlorperazine: Effect of Loading Capacity on the Separation of Impurities



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Impact of Column Chemistry

Conditions:

Columns: 4.6 x 150 mm, 5 μ m

Mobile Phase A: 0.1% HCOOH in H₂O

Mobile Phase B: 0.1% HCOOH in ACN

Flow Rate: 1.4 mL/min

Gradient:	Time (min)	%A	%B
	0.0	70	30
	10.0	10	90
	15.0	10	90

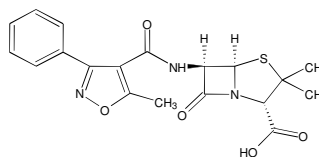
Injection Volume: 10.0 μ L

Sample Diluent: DMSO

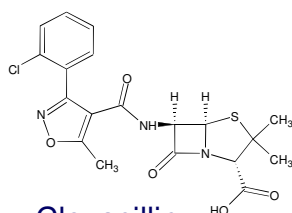
Temperature: 30 $^{\circ}$ C

Detection: UV @ 254 nm

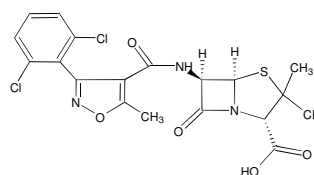
Instrument: Alliance[®] 2695 with 2996 PDA



Oxacillin



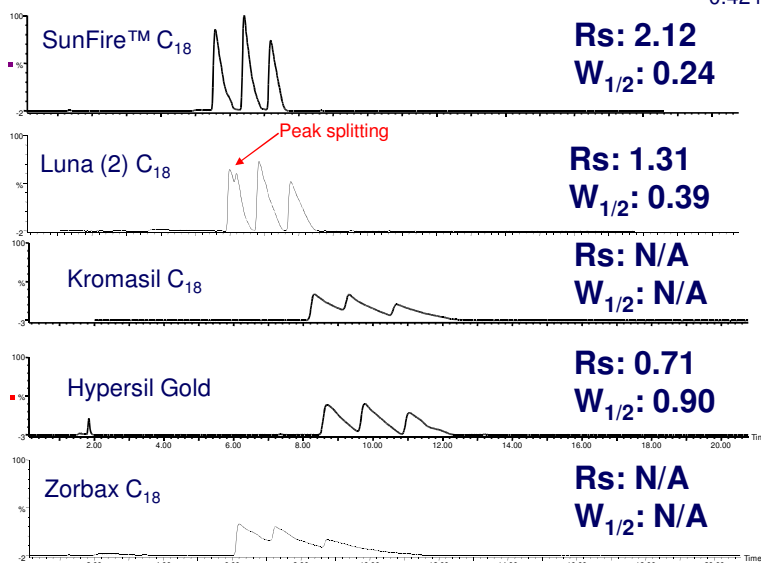
Cloxacillin



Dicloxacillin

All C₁₈ Columns are NOT the Same

0.42 mg Total Load



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Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
 - ◆ Dimensions
 - ◆ Particle Size
 - ◆ Chemistry
- Chemistry of the Mobile phase

Chemistry of the Sample Solution: Maximizing Sample Load

- Mobile phase solvent choice
- Buffer the mobile phase
 - ◆ Control of chromatographic pH conditions
 - ◆ Reduces breakthrough of sample
 - ◆ Use volatile buffers
 - Reduces sample handling and energy required for evaporation
 - Minimizes contamination of purified fraction
 - Minimizes risk of target decomposition
 - Mass spectrometry compatible
- Mobile phase pH impacts load
 - ◆ Low pH maximizes acid load
 - ◆ High pH maximizes base load

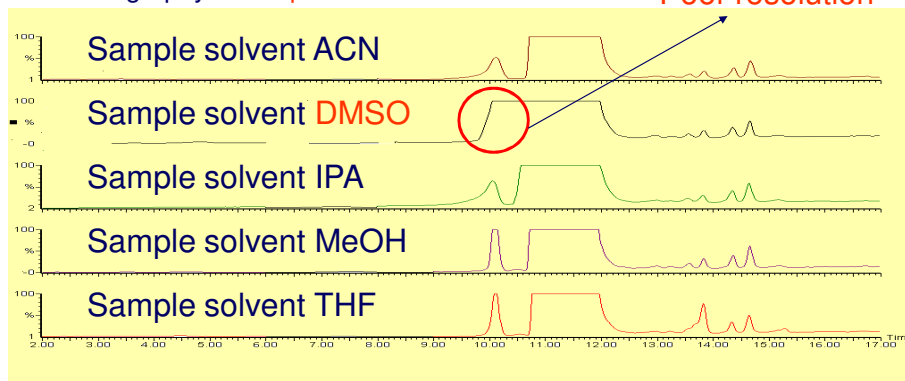
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Sample Solvent Affects Resolution and Peak Shape

Chromatography run at pH 3.8

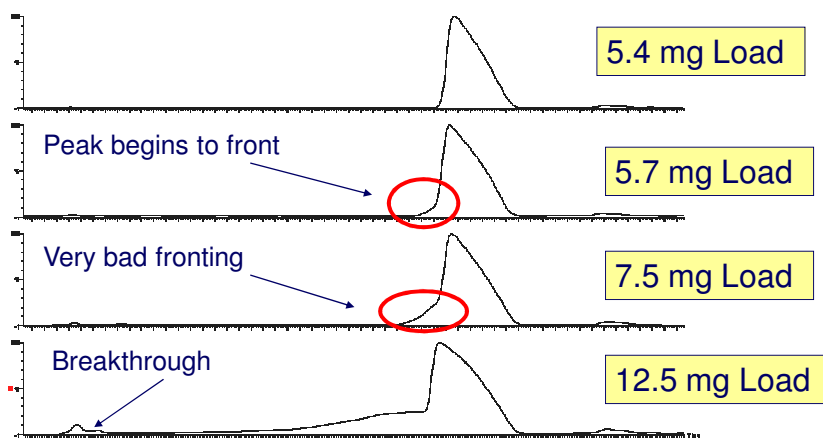
Poor resolution



DMSO in high aqueous mobile phases generates a high viscosity pressure “spike” (observed post injection) resulting in poor resolution of the chromatographic peaks. All weaknesses in HPLC system will become evident.

Increasing Load in DMSO: Mass Overload

Column: XTerra® MS C₁₈, 4.6 × 50 mm, 5 µm. Mobile phase A: water + 50 mM formic acid; mobile phase B: acetonitrile + 50 mM formic acid. Flow rate: 1.8 mL/min. Gradient: 35-65% B in 5 min. UV: 280 nm.

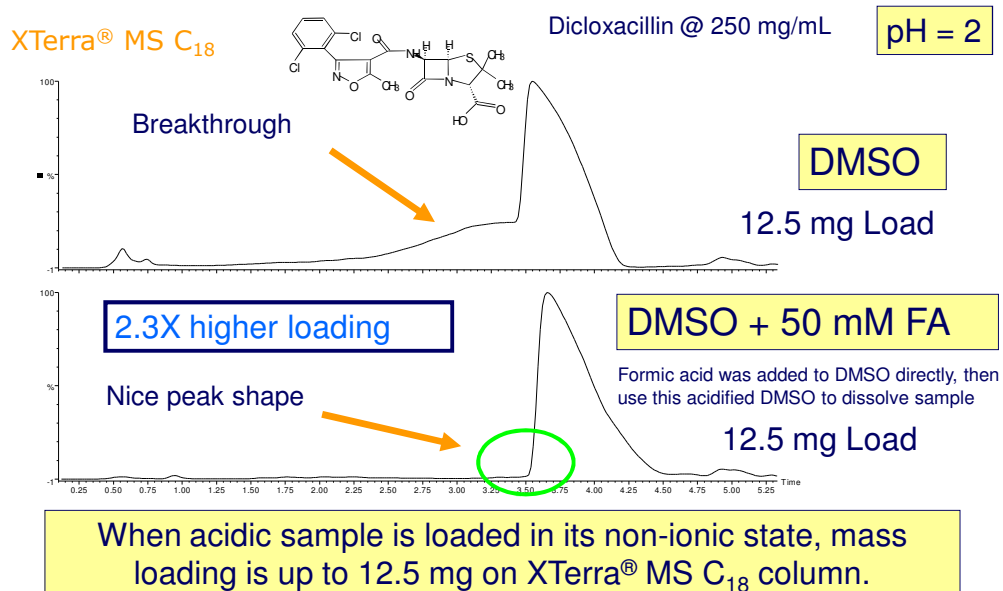


When DMSO is used as the diluent, mass loading is 5.4 mg.

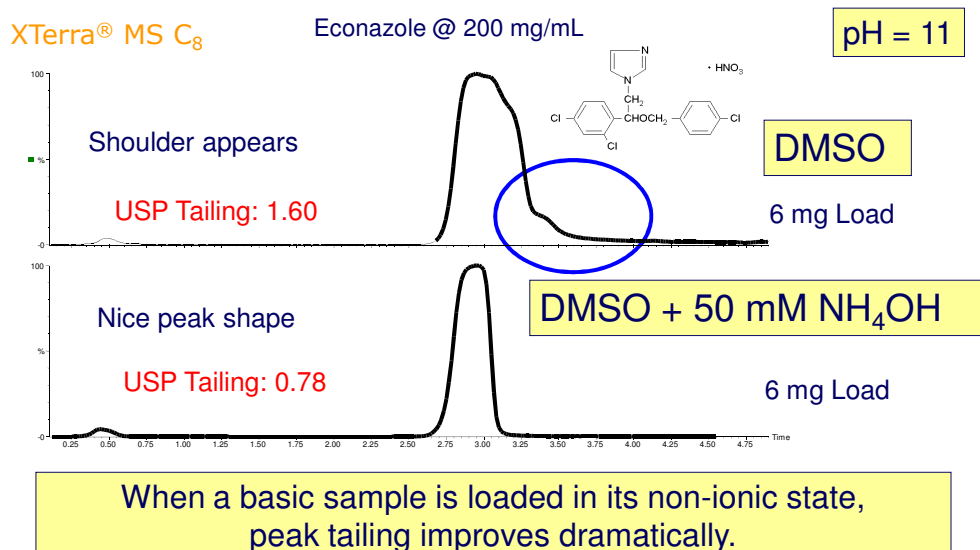
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Non-Ionic Loading: Acids



Non-Ionic Loading: Bases

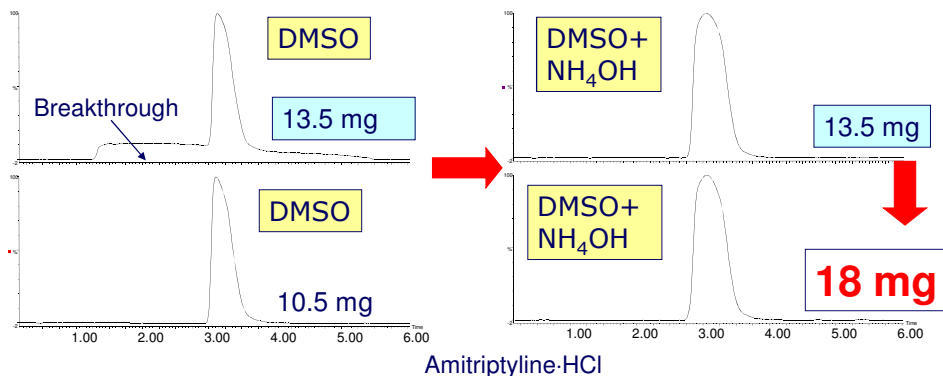


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Loading a Base under Acidic Mobile Phase Conditions

Column: XTerra® MS C₁₈, 4.6 × 50 mm, 5 µm. Mobile phase A: water + 50 mM formic acid; mobile phase B: acetonitrile + 50 mM formic acid. Flow rate: 1.8 mL/min. Gradient: 5-95% B in 5 min. UV: 280 nm.

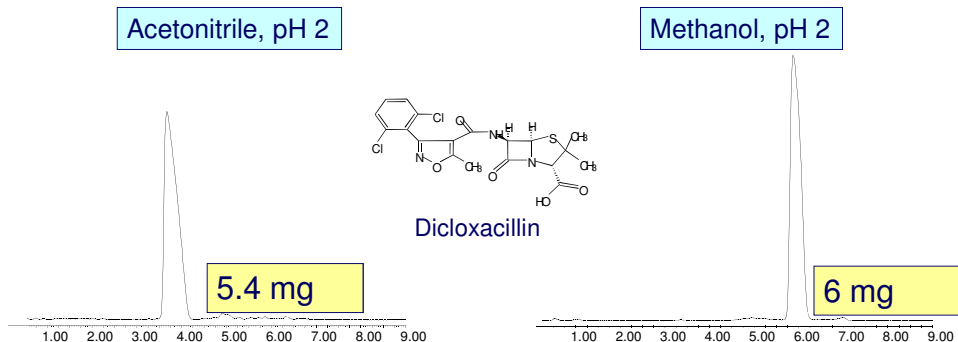


When NH₄OH is added to the DMSO, no breakthrough was observed.

1.8X high mass load by adding base to the diluent.

Choice of Strong Solvent (Acetonitrile vs. Methanol)

Column: XTerra® MS C₁₈, 4.6 × 50 mm, 5 µm. Mobile phase A: water + 50 mM formic acid; mobile phase B: acetonitrile/methanol + 50 mM formic acid. Flow rate: 1.8 mL/min. Gradient: 35-65% B in 5 min. UV: 254 nm.



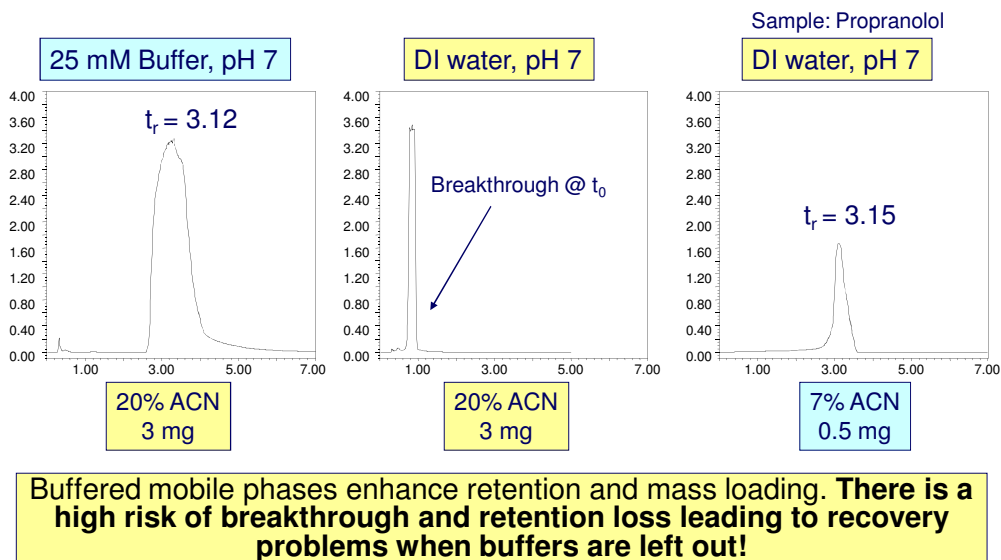
Applying the same gradient with both organic solvents, sample elutes later with methanol than with acetonitrile.

Changing the organic solvent may improve peak shape due to the additional interaction with the sample.

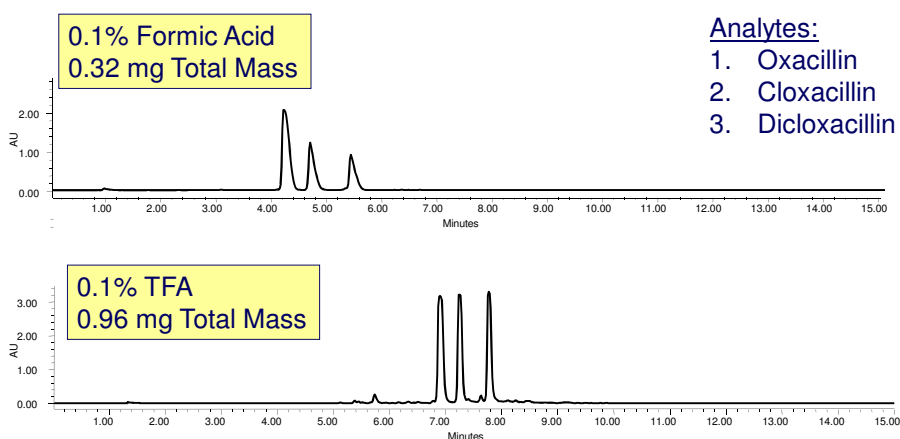
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Use of Buffers



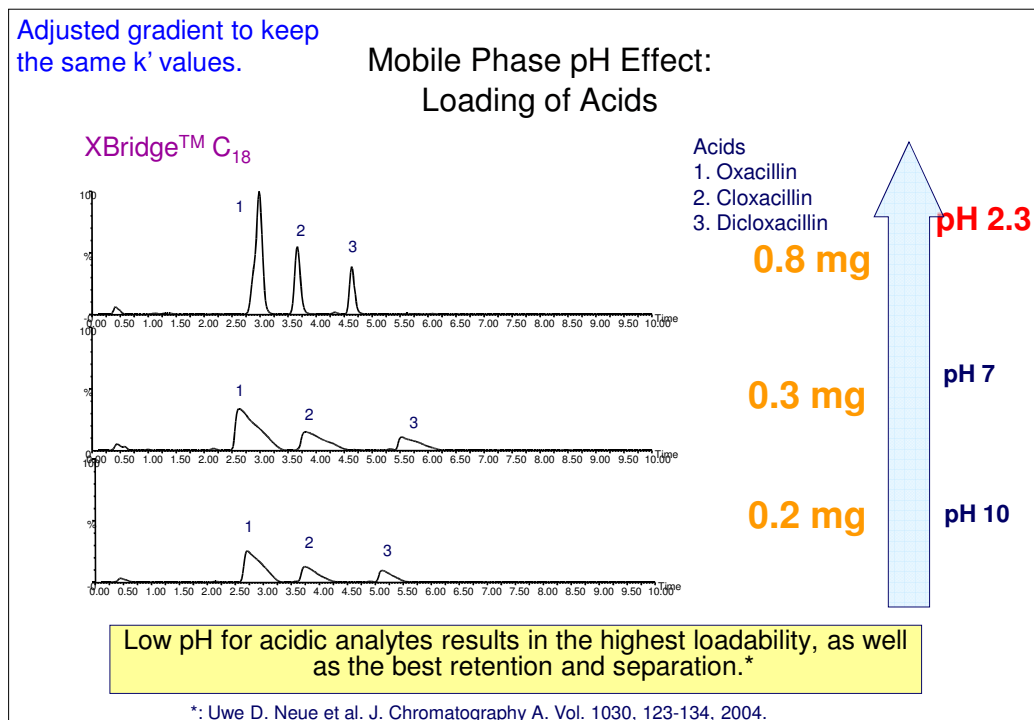
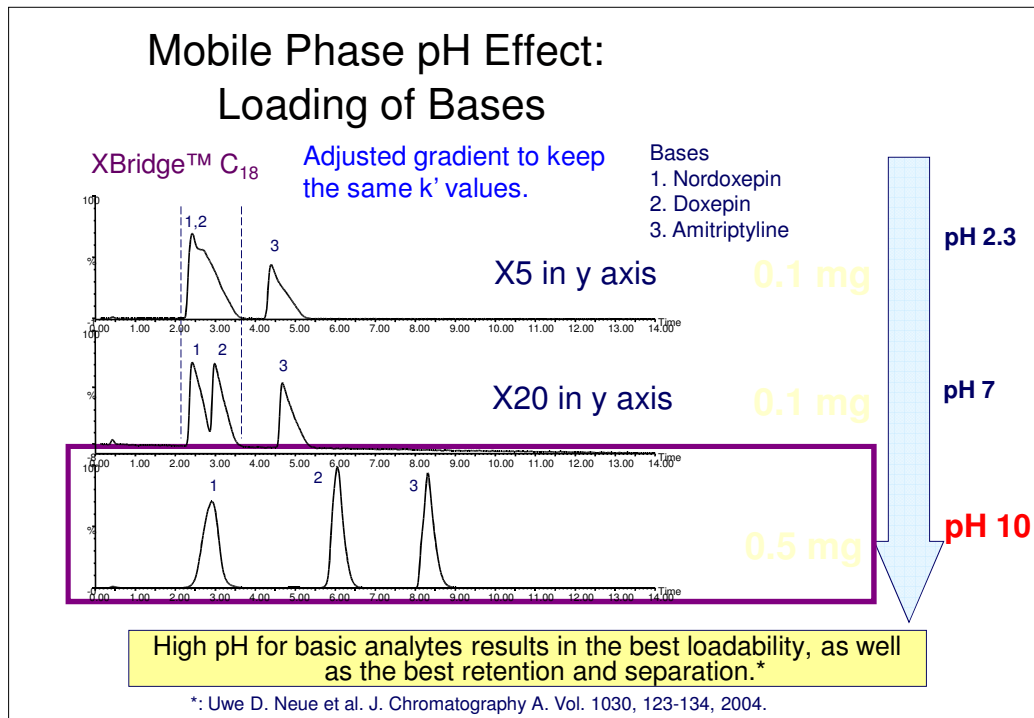
Modifier Impact on Load



As sample mass / volume loading increases, strong pH shifts can occur, affecting chromatographic peak shapes.

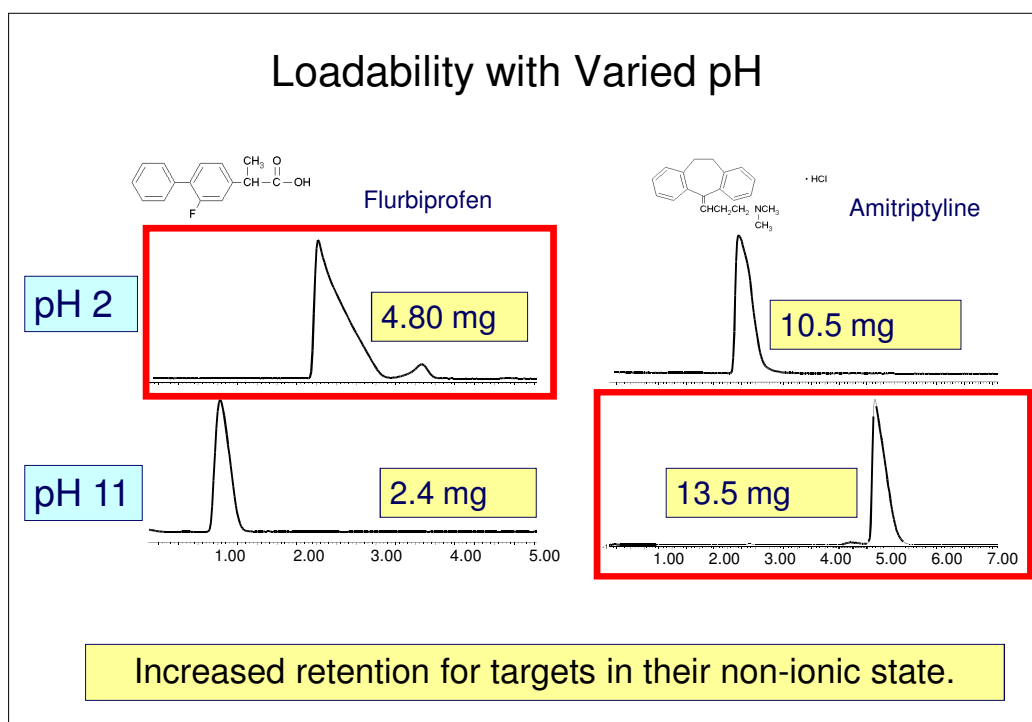
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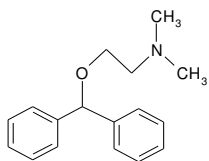
Utilizing pH to Manipulate Target Retention

- pH is the most powerful tool to manipulate retention of ionizable compounds
- Elution Order (Standard Gradient Mode)
 - ◆ early eluting peaks can be isolated more quickly
 - ◆ later eluting peaks can be purified at higher loadings and elute in higher concentration organic solvent
- Basic compounds chromatographed in their unionized state (2pH units above pKa) are retained longer and exhibit excellent peak shape
- A popular high pH volatile buffer is 0.1% NH₄OH

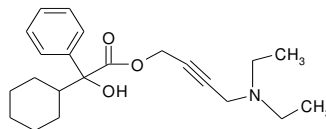
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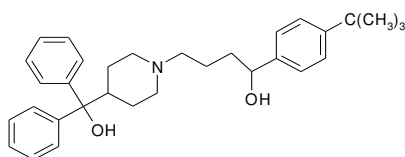
Example: Basic Test Compounds



Diphenhydramine

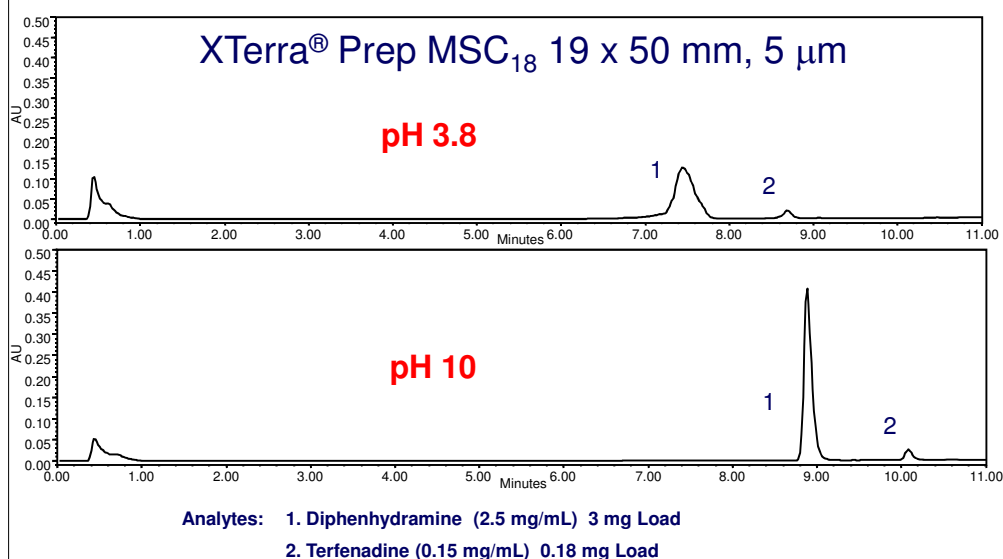


Oxybutynin



Terfenadine

Peak shape and retention comparison: Basic Compounds at Low and High pH



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Scale-up Strategy - Summary

1. Define the problem ➡ Find the chromatographic mode.
2. Develop and optimize the separation ➡ Increase selectivity > 1.5
3. Maximize throughput ➡ Measure adsorption isotherm.
4. Increase sample mass and volume to the maximum
 while meeting purity objectives. ➡ Examine the interferences
5. Determine recovery ➡ Examine residuals on the column
6. Scale up to desired column size to meet
 throughput/load objectives. ➡ Keep the flow rate and sample load ratio
7. Pool fractions of comparable purity and rerun if
 necessary.
8. Check fraction purity using analytical column.