

CONSIDERATIONS IN SCALING UP A CHROMATOGRAPHIC RUN

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Problem Definition: Analysis

- What sensitivity is required?
- How complex is the sample?
- How many analyses will be performed?
- What degree of accuracy, precision, etc. is required?
- How easy (routine) does the assay need to be?

If the first step is gathering information on your sample;
the second is defining the analysis by the above criteria.

Problem Definition: Isolation

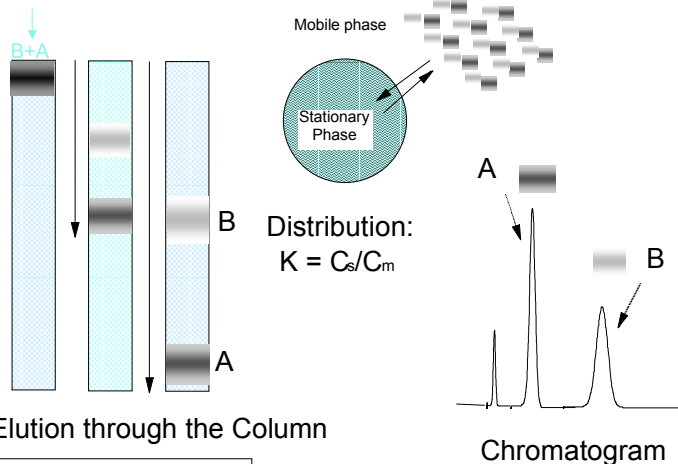
- What quantity of material needs to be isolated?
- Is the material a major or minor component?
- Do you need to maintain biological activity?
- What degree of purity (or specific activity) is required?
- How will purity or activity be verified?

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

Preparative Chromatography Terminology

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

Chromatographic Process



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

RETENTION FACTOR

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

CAPACITY RATIO

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

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

Scaling up

Seven Basic Considerations in Choosing HPLC Operating Parameters

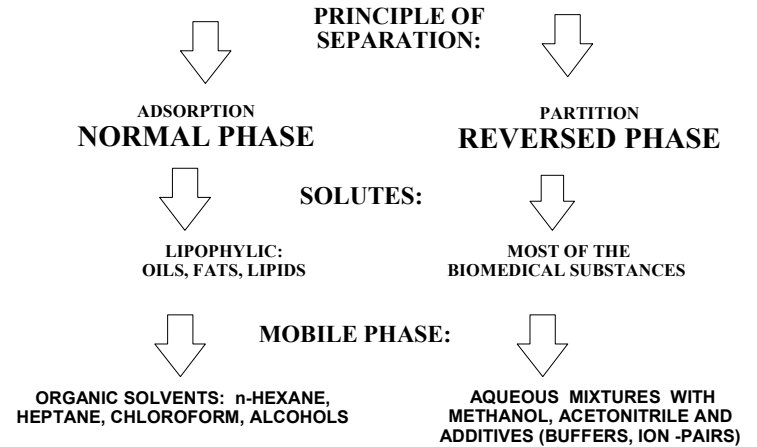
- 1) **Solubility** - Hexane, Chloroform, Methanol, Water (buffer pH), other?
- 2) **Molecular Weight** - Would GPC be useful in either the analysis or sample prep?
- 3) **Functional Groups** - Any ionizable groups? Acidic, Basic, or Neutral?
- 4) **Sample Matrix** - What amounts are expected in matrix 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 in the separation, especially if compounds are similar in their structure.

Selection of the appropriate mode of chromatography

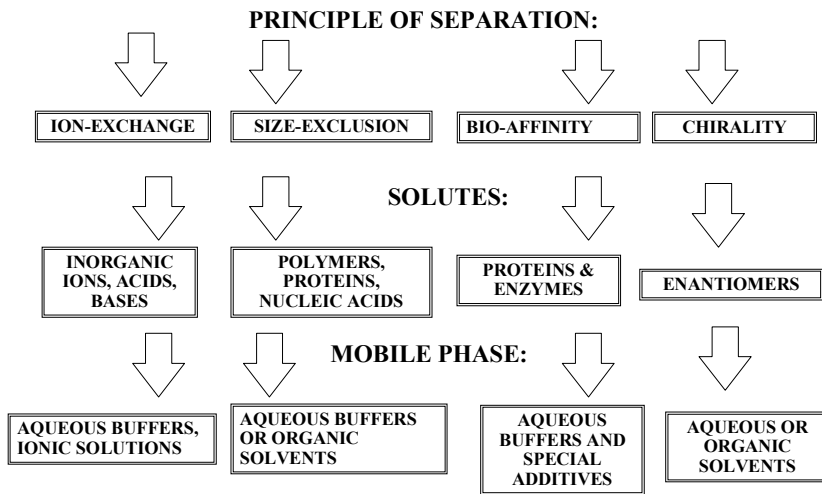
- Normal Phase
- Reverse Phase
- Ion Exchange
- Chiral
- Specialty

These are the most common modes of HPLC. They will be discussed throughout the course.

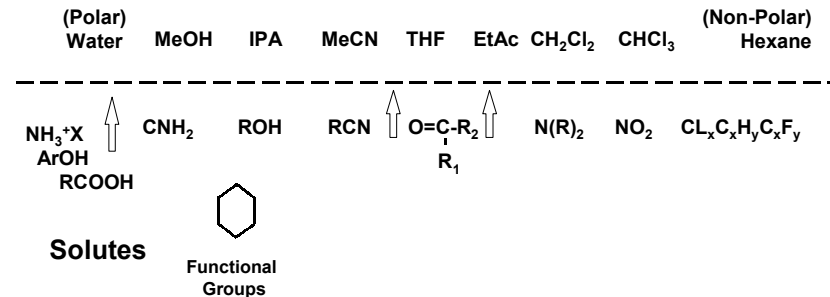
High Performance Liquid Chromatography Modes



High Performance Liquid Chromatography Modes



Solvent



The relationship between the polarity of sample functional groups and solvent polarity which is used to predict sample and solvent compatibility.

Strategy for Preparative Separation



Selection of the appropriate mode of chromatography



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

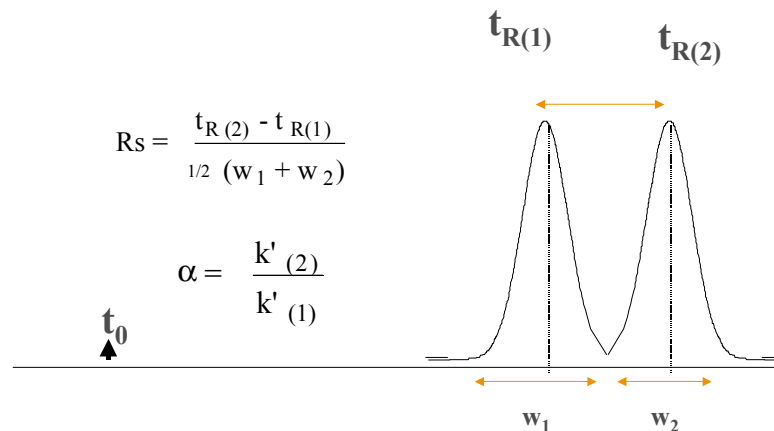


Optimization of the throughput
(Sample amount: Column Overloading)
Adsorption Isotherm & Competition



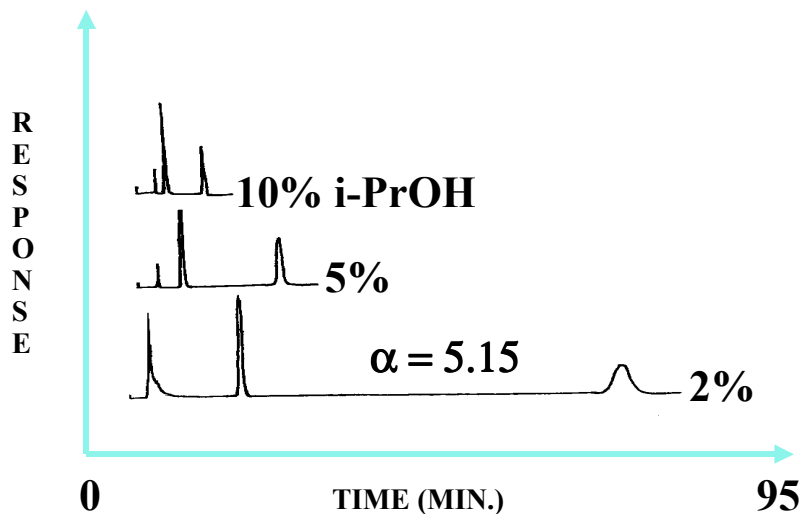
Scaling up

Optimization: Selectivity and Resolution



OPTIMIZATION

OF THE CHIRAL SEPARATION OF BENZOFURAN HU-249+250



Why Optimize the Small Scale Separation?

Maximizing selectivity factor will significantly impact:

- Throughput
- Size of packing material needed.
- Size of column needed to obtain desired throughput.
- Solvent Used
- Instrument Capability

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

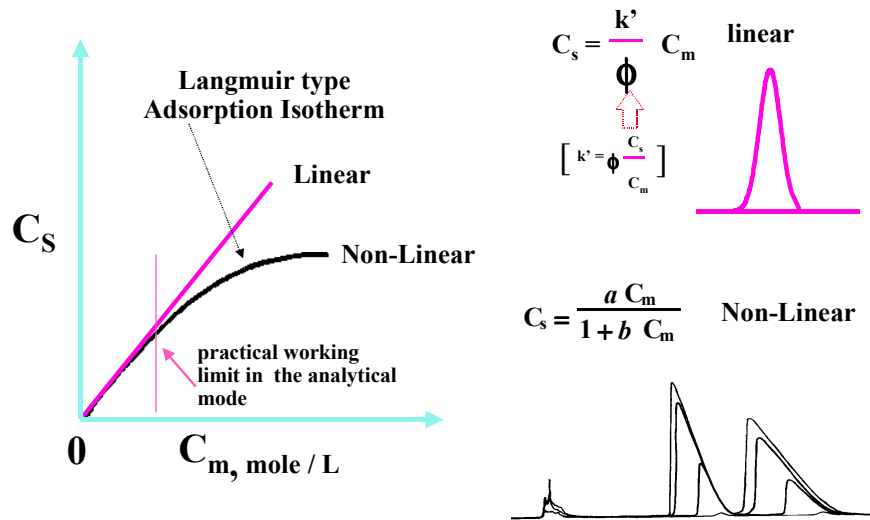


Scaling up

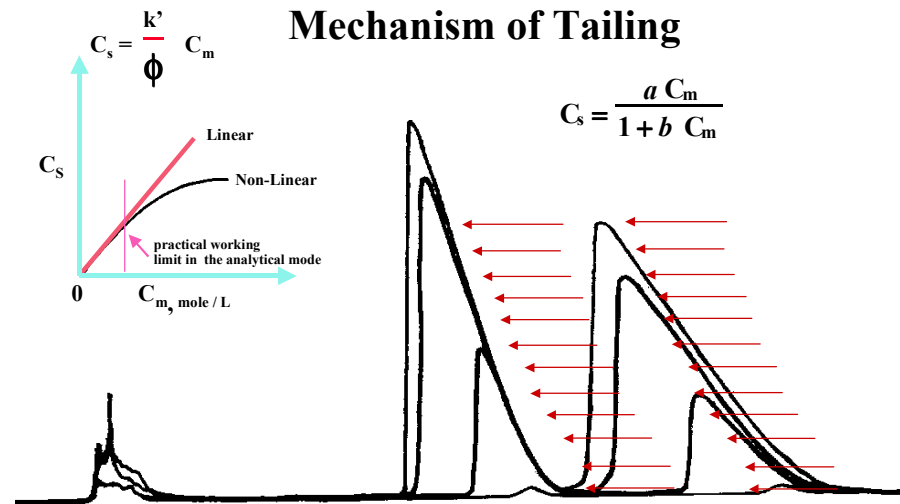
FIRST CHOICE: USING OVERLOADING CONCENTRATIONS

- 1- Availability of the analytical equipment
- 2- Good stability of the column packing
- 3- Low cost
- 4- Less solvent use, less environmental pollution

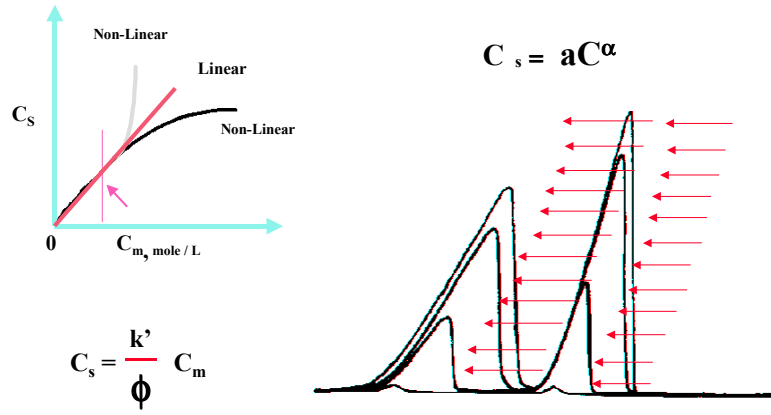
ADSORPTION ISOTHERMS: THE KEY TO RATIONAL SCALE-UP



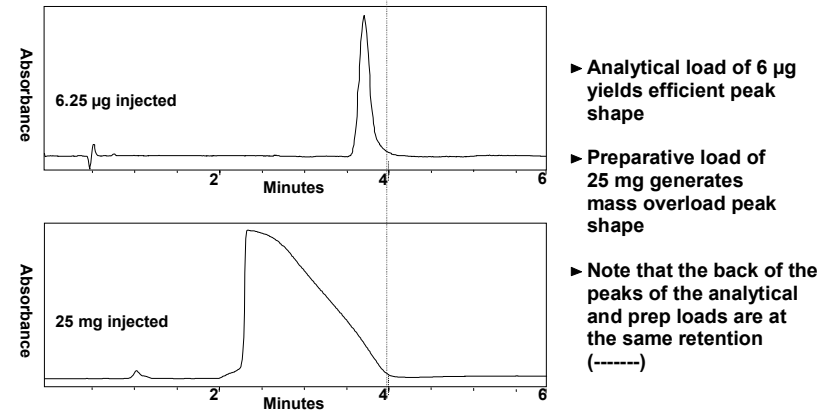
Mechanism of Tailing



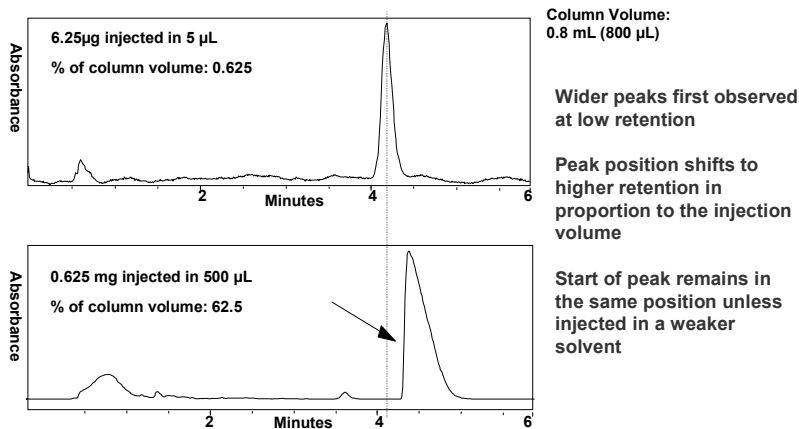
Mechanism of Diffuse Front



Mass Overload



Volume Overload

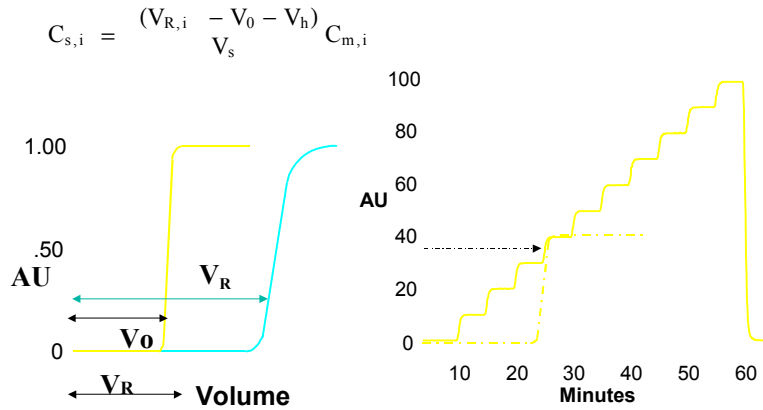


Experimental Methods Used to Measure Adsorption Isotherm

- 1- Frontal Analysis (FA)
- 2- Frontal Analysis by Characteristic point (FACP)
- 3- Elution by a Characteristic point (ECP)
- 4- Elution on Plateau (EP)
- 5- System Peaks Analysis (SPA) *

* S. Levin and S. Abu-Lafi, J. Chromatogr., 556, 277-285, 1991.

FRONTAL ANALYSIS



Frontal Analysis (FA)

$$C_{s,i} = \frac{(V_{R,i} - V_0 - V_h)}{V_s} C_{m,i}$$



STEPWISE:

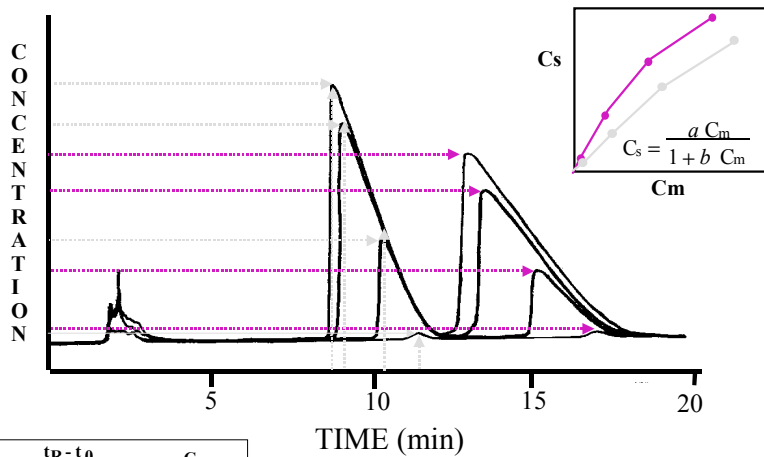
$$C_{s,i} = \int_0^{C_{m,i}} \frac{(V_{R,i} - V_0 - V_h)}{V_s} dC_{m,i}$$

V_0 = column void volume

V_s = stationary phase volume

V_h = hold-up volume (from the pump to the detector)

ELUTION BY A CHARACTERISTICS POINT (ECP)



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

ELUTION BY A CHARACTERISTICS POINT (ECP)

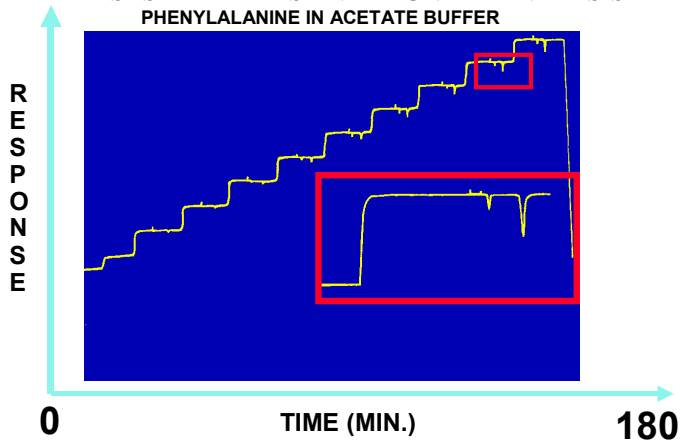
$$C_{s,i} = \frac{(V_{R,i} - V_0 - V_h)}{V_s} C_{m,i}$$

V_0 = column void volume

V_s = stationary phase volume

V_h = extra-column void volume (from the injector to the detector)

**Measurements of Adsorption Isotherm Using both Methods:
SYSTEM PEAKS AND FRONTAL ANALYSIS *
PHENYLALANINE IN ACETATE BUFFER**



*S. Levin and S. Abu-Lafi, J. Chromatogr., 556, 277-285, 1991.

Calculation of $C_{s,i}$:

System Peaks Analysis (SPA)

$$C_{s,i} = \frac{1}{\phi} \sum_0^{C_{m,i}} k'_i \Delta C_{m,i}$$

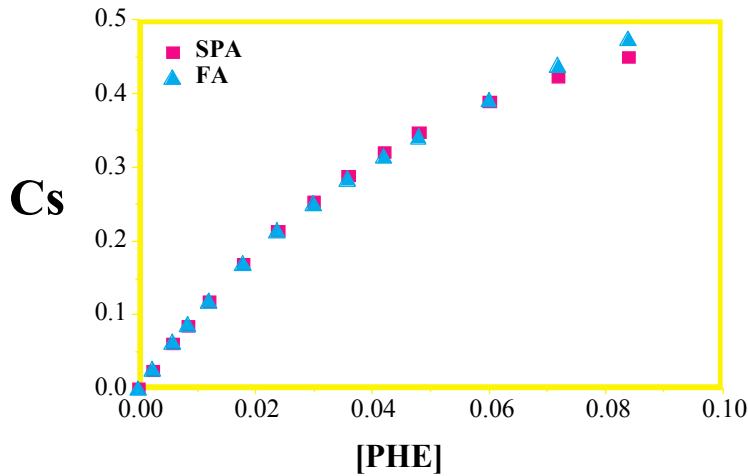
$C_{s,i}$ = concentration in the stationary phase

k'_i = capacity factor

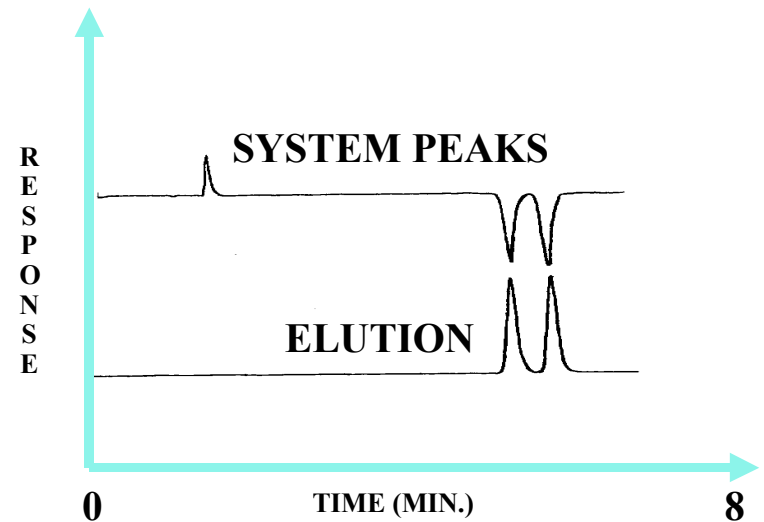
ϕ = phase ratio

$\Delta C_{m,i}$ = difference in concentration between every
.....two steps.

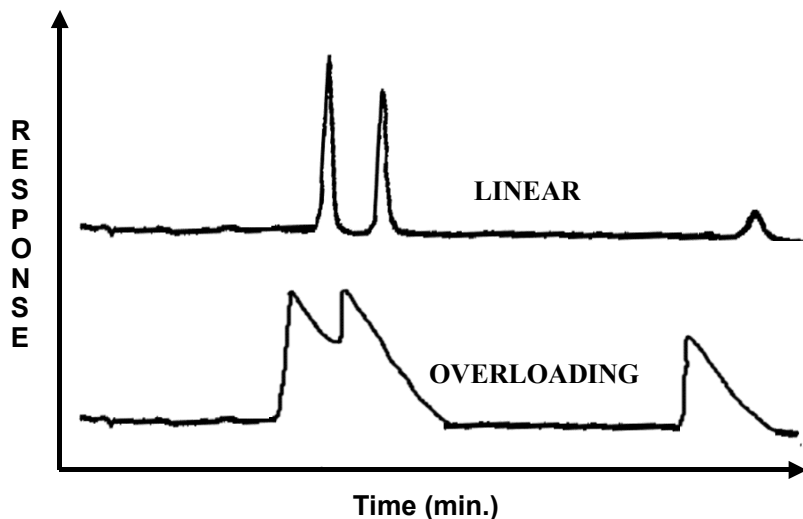
**ADSORPTION ISOTHERM OF PHENYLALANINE IN
0.1 M ACETATE BUFFER BY FA and SPA**



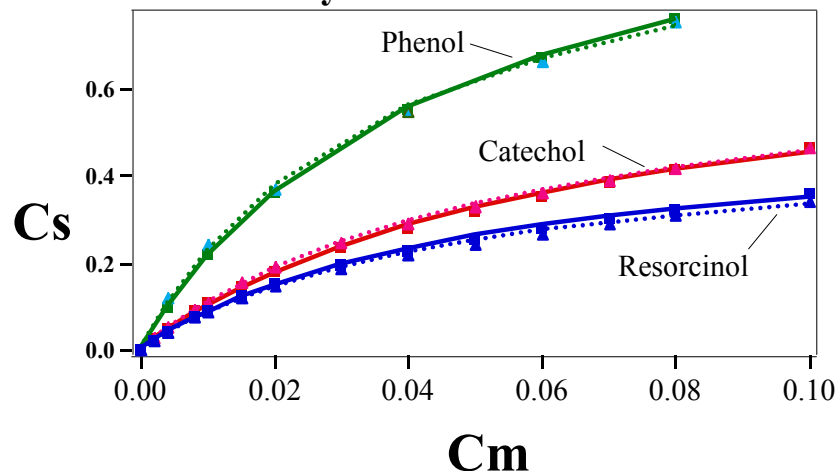
**MIXTURE OF (1:1) 1,8- AND 1,5-DCAQ
IN THE LINEAR RANGE**



CHROMATOGRAM OF RESORCINOL CATECHOL & PHENOL

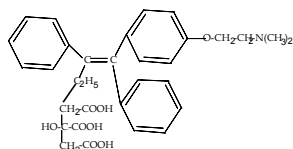
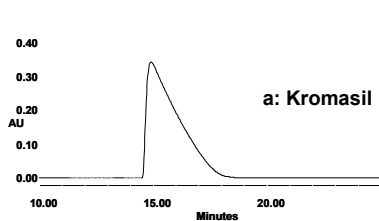


ADSORPTION ISOTHERMS by SPA AND FA*



S. Levin, S. Abu-Lafi, S. Golshan-Shirazi and G. Guiochon, *J. Chromatogr.*, 679, 213-229, 1994.y

Tamoxifen: Comparison of Loading Capacities of SymmetryPrep™ and Kromasil® Columns



Column:

a: Kromasil C18 7 μ m (4.6 x 150) mm

b: Symmetry Prep C18 7 μ m (4.6 x 150) m m

Mobile Phase:

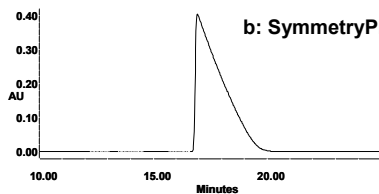
a: 44% acetonitrile / 46% 50mM potassium phosphate buffer, pH 3.0

b: 40% acetonitrile / 60% 50mM potassium phosphate buffer, pH 3.0

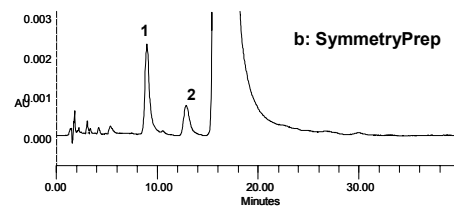
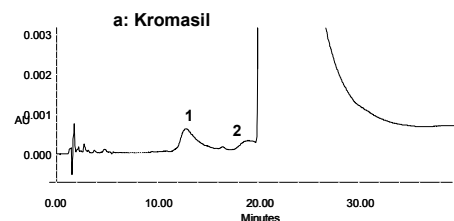
Flow Rate: 1.0 mL/min

Sample: 14 μ L of 5 mg/mL Tamoxifen solution

Detection: UV at 254 nm



Prochlorperazine: Effect of Loading Capacity on the Separation of Impurities



Column:

a: SymmetryPrep C18 7 μ m (4.6x150) mm

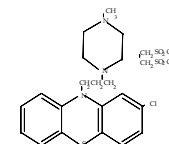
b: Kromasil C18 7 μ m (4.6x150) mm

Mobile Phase: 75% methanol / 25% 20 mM phosphate buffer pH 7.0

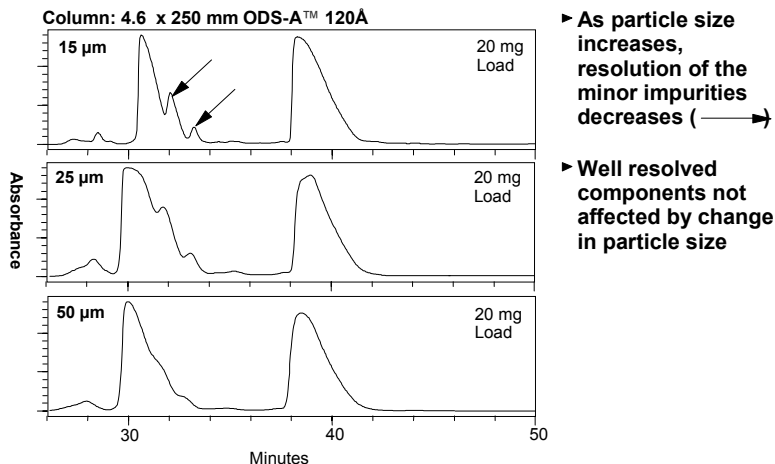
Flow Rate: 1 mL/min

Detection: UV at 254 nm

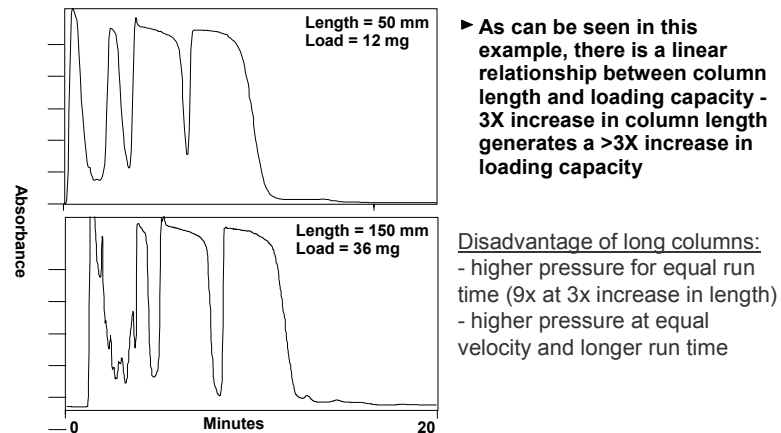
Sample: 10 μ L of 0.97 mg/mL solution



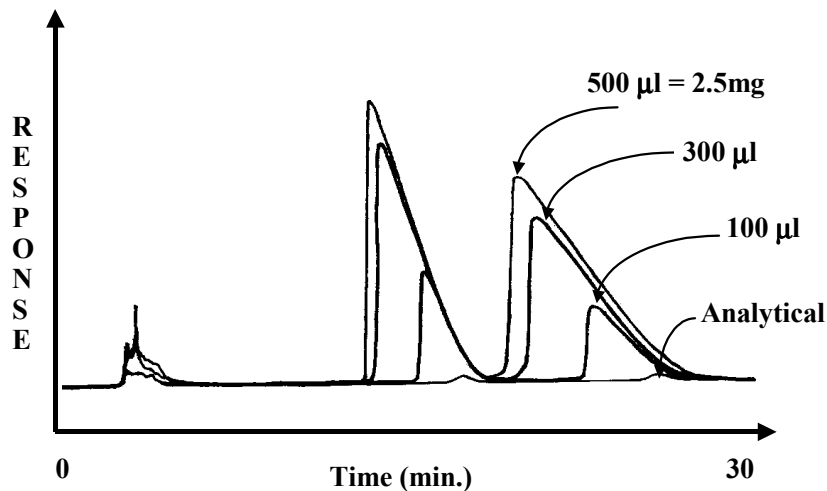
Effect of Particle Size on Capacity



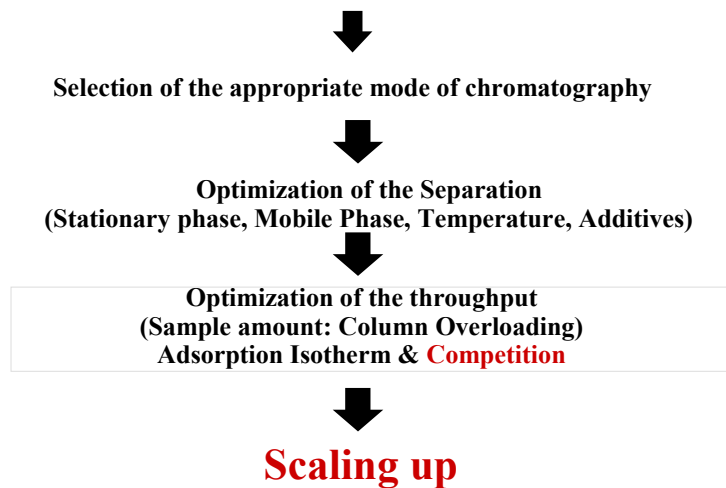
Effect of Column Length on Capacity



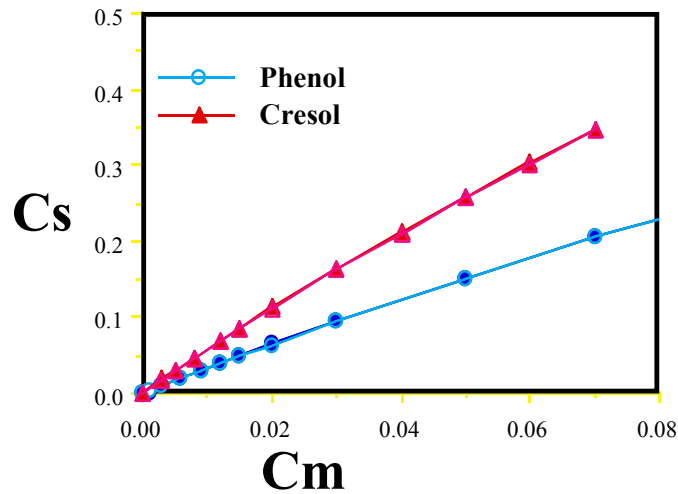
Optimization of the throughput



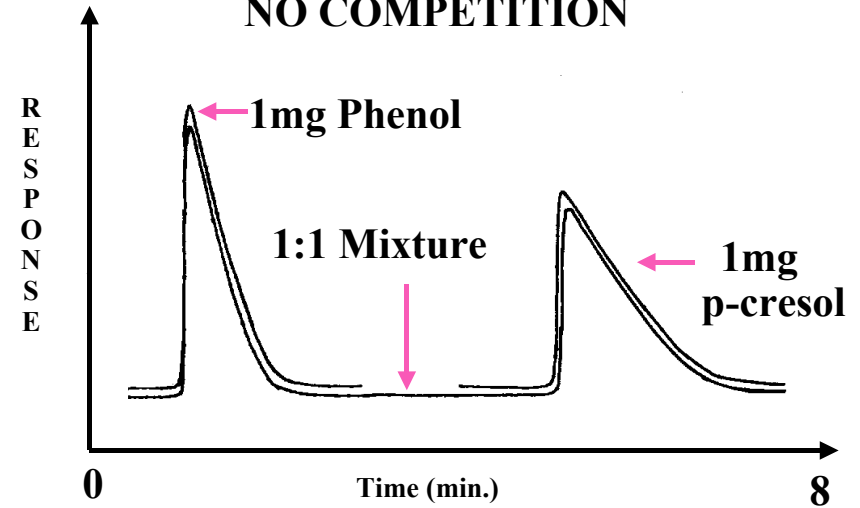
Strategy for Preparative Separation



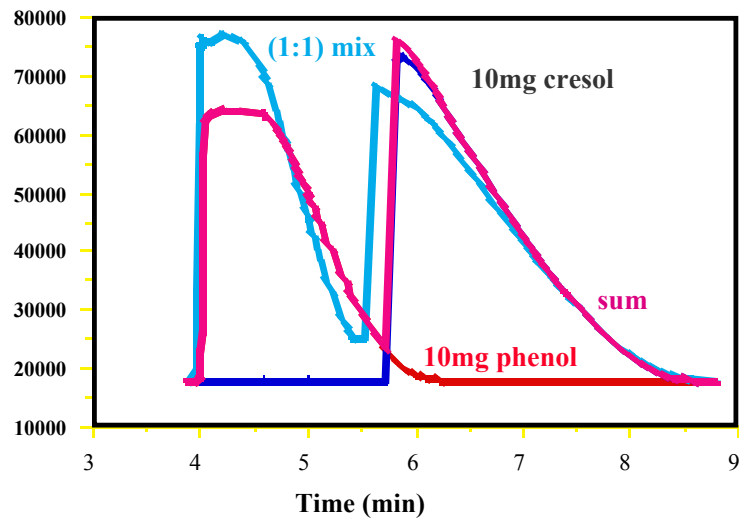
ADSORPTION ISOTHERMS: HIGH CAPACITY - NO COMPETITION



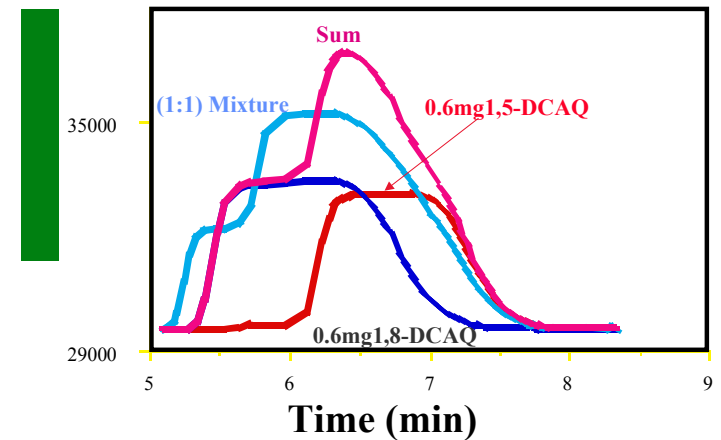
PEAK SHAPE AT OVERLOADING CONCENTRATIONS: NO COMPETITION



PEAKS SHAPE AT OVERLOADING CONCENTRATIONS



COMPETITION: THE MIXTURE IS NOT A SIMPLE SUM OF THE INDIVIDUAL COMPONENTS

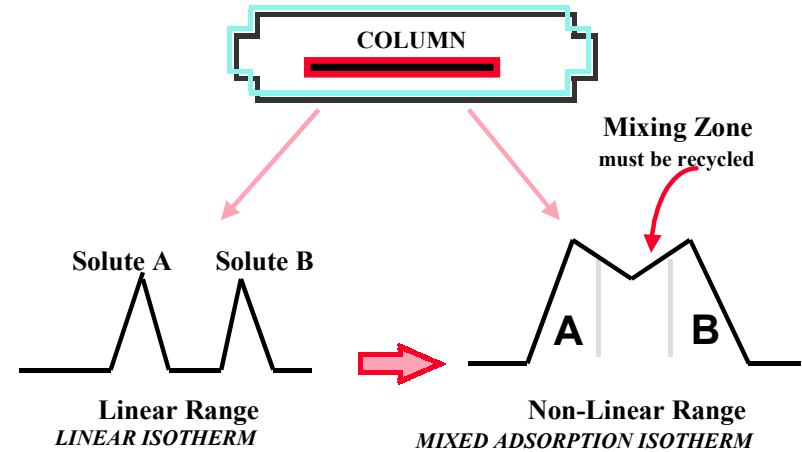


LANGMUIR EQUATIONS

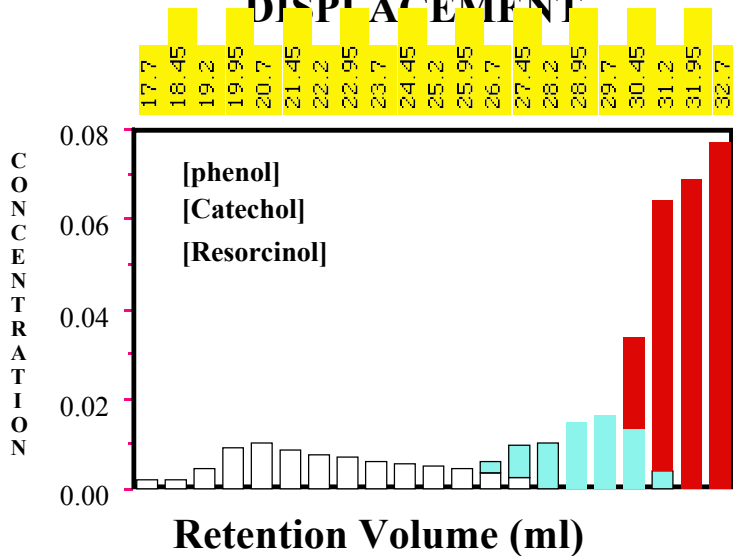
SINGLE COMPONENT $C_s = \frac{a C_m}{1 + b C_m}$

TWO COMPONENTS $C_{s,1} = \frac{a_1 C_{m,1}}{1 + b_1 C_{m,1} + b_2 C_{m,2}}$
 $C_{s,2} = \frac{a_2 C_{m,2}}{1 + b_1 C_{m,1} + b_2 C_{m,2}}$

MULTI-COMPONENT $C_{s,i} = \frac{a_i C_{m,i}}{1 + \sum_j b_j C_{m,j}}$



HISTOGRAM FROM DISPLACEMENT



Scale-up of Flow

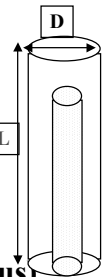
Scale-up flow rate to maintain constant linear velocity:

$$\text{Flow rate} = \text{Flow rate}_{\text{small scale}} \times \left[\frac{(D_{\text{Prep}})^2}{(D_{\text{small scale}})^2} \right]$$

Scale-up of sample load (Maintain the overloading status)

$$\text{Load} = \text{Load}_{\text{small scale}} \times \left[\frac{(D_{\text{Prep}})^2}{(D_{\text{small scale}})^2} \times \left(\frac{L_{\text{Prep}}}{L_{\text{small scale}}} \right) \right]$$

D = Diameter of Columns
 L = Length of Columns

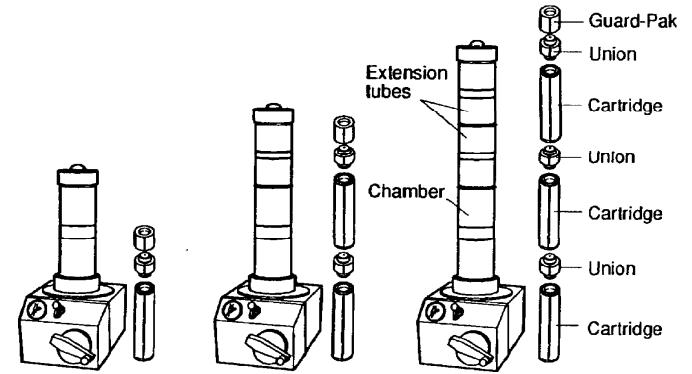


PrepPak Base 40mm ID Options

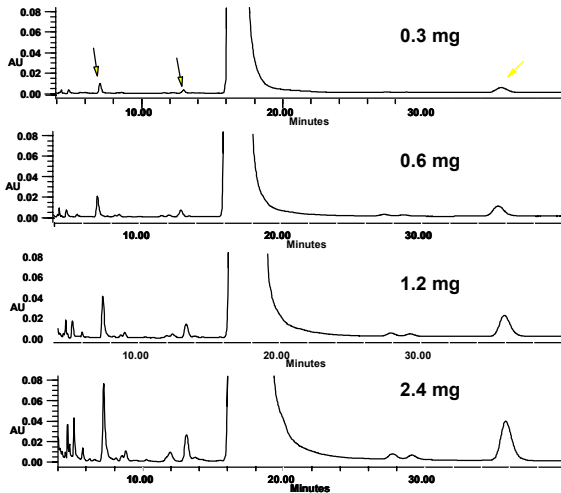
Approximate Mass Loading Capacity

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

| 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 (μl) | 15 | 20 | 60 | 65 | 100 | 350 | 390 | 610 | 880 | 1565 | 2160 | 2450 |

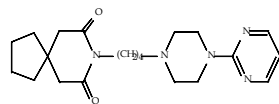


Buspirone: Effect of Increasing Load on the Separation of Impurities

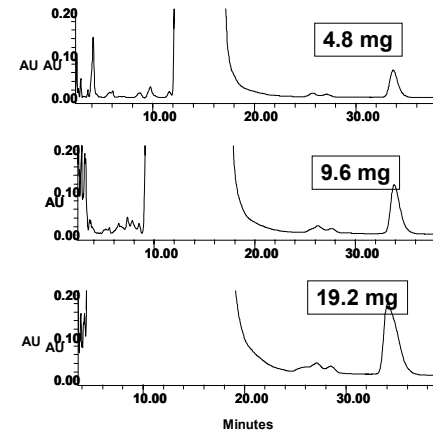


Column: SymmetryPrep C18, 7μm (3.9 x 150) mm
 Mobile Phase: 28% acetonitrile / 72% 0.18% TETA-MeCOOH pH 7.0
 Flow Rate: 1.0 mL/min
 Detection: UV at 360 nm
 Sample: 1.2 mg/mL of Buspirone
 Injection: from 0.25 to 2.0 mL

Structure of Buspirone

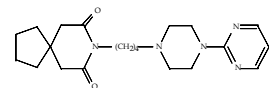


Buspirone: Effect of Increasing Load on the Separation of Impurities

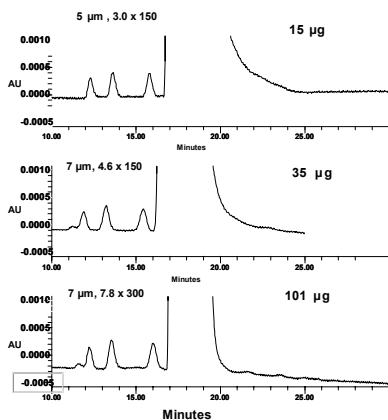


Column: SymmetryPrep C18, 7μm (3.9 x 150) mm
 Mobile Phase: 28% acetonitrile / 72% 0.18% TETA-MeCOOH pH 7.0
 Flow Rate: 1.0 mL/min
 Detection: UV at 360 nm
 Sample: 12 mg/mL of Buspirone
 Injection: from 0.4 to 1.6 mL

Structure of Buspirone



Tamoxifen Impurities: Scaling up from Symmetry C18, 5 µm to SymmetryPrep C18, 7 µm



Column:

- a: Symmetry C18 5µm (3.0x150) mm
- b: Symmetry Prep C18 7µm (4.6x150) mm
- c: Symmetry Prep C18 7µm (7.8x300) mm

Mobile Phase: 40% acetonitrile / 60% 50mM potassium phosphate buffer, pH 3.0

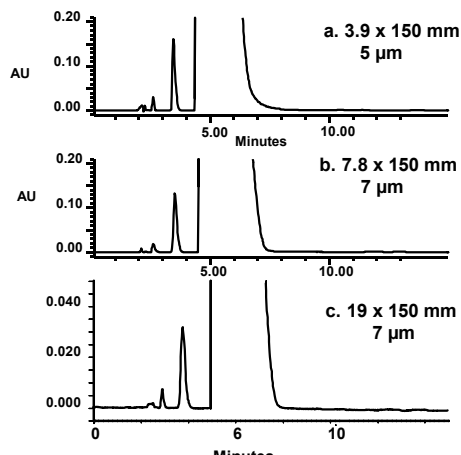
Flow Rate:

- a: 0.41mL/min
- b: 0.97 mL/min
- c: 5.60 mL/min

Sample: 5 mg/mL Tamoxifen solution

Detection: UV at 254 nm

Isolation of Diltiazem Impurities on SymmetryPrep 7 µm Columns



Columns:

- a. Symmetry C₁₈ 5 µm
- b., c. SymmetryPrep C₁₈ 7 µm

Mobile Phase:

30% acetonitrile/
70% 0.1%TFA aqueous

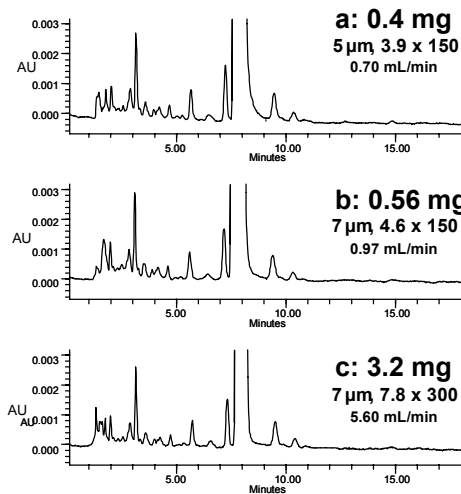
Flow Rates:

- a. 0.7 mL/min
- b. 2.8 mL/min
- c. 16.6 mL/min

Sample: diltiazem

- a. 0.5 mg
- b. 2.0 mg
- c. 11.9 mg

Valerophenone Impurities: Scaling up from Symmetry 5 µm to SymmetryPrep 7 µm



Mobile Phase:

60% acetonitrile / 40% water

Columns:

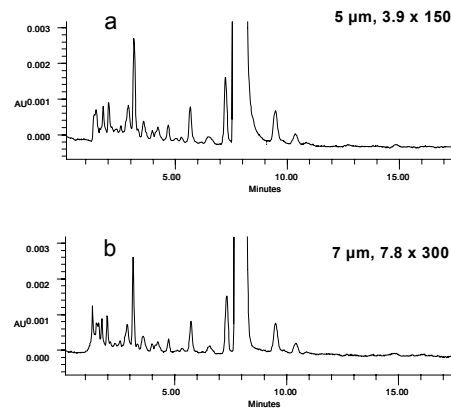
Symmetry C18

Detection: UV at 340 nm

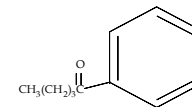
Sample: 4 mg/mL Valerophenone solution

- a: 100 µL injection
- b: 140 µL injection
- c: 800 µL injection

Valerophenone Impurities



Structure of Valerophenone



Column:

- a: Symmetry C18 5 µm (3.9x150) mm
- b: Symmetry Prep C18 7 µm (7.8x300) mm

Mobile Phase: 60% acetonitrile / 40% water

Flow Rate:

- a: 0.70 mL/min
- b: 5.60 mL/min

Sample: 4 mg/mL Valerophenone solution

- a: 100 µL injection
- b: 800 µL injection

Detection: UV at 340 nm

El Fallah

Scale-up a gradient run

Keeping the gradient duration the same:

$$\frac{\text{Gradient Duration}_{large}}{\text{Gradient Duration}_{small}} = \frac{(\text{Void Volume}_{large})}{(\text{Void Volume}_{small})} \times \frac{(\text{Flow Rate}_{small})}{(\text{Flow Rate}_{large})}$$

Gradient Occurs Over an Equivalent Number of Column Void Volumes (3.14 * r²*L)

Keeping the separation at smaller flow rates:

$$\text{Gradient Duration}_{large} = \text{Gradient Duration}_{small} \times \frac{(\text{Void Volume}_{large})}{(\text{Void Volume}_{small})} \times \frac{(\text{Flow Rate}_{small})}{(\text{Flow Rate}_{large})}$$

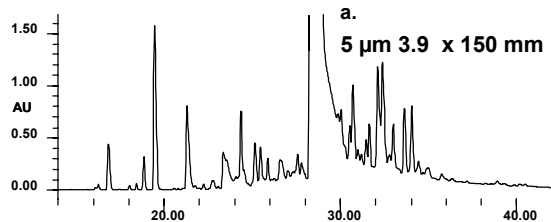
$$? \text{ min} = 50 \text{ min} \times \frac{100}{1} \times \frac{1}{50} = 100 \text{ min}$$

Scale-up a gradient run

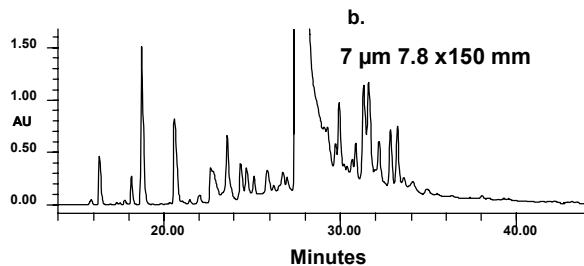
| | Column Diameter (mm) | Gradient Duration (min) | Flow rate (ml/min) | Void volume (ml) |
|-------------|----------------------|-------------------------|--------------------|------------------|
| Small scale | 4 | 30 | 1 | 3.14 |
| Large scale | 40 | 30 | 100 | 314.0 |
| Semi-prep | 10 | 30 | 6.25 | 19.6 |
| Semi-prep | 10 | 37.5 | 5 | 19.6 |

$$\text{Gradient Duration (semiprep)} = 30 * (19.6/3.14) * (1/5) = 37.5$$

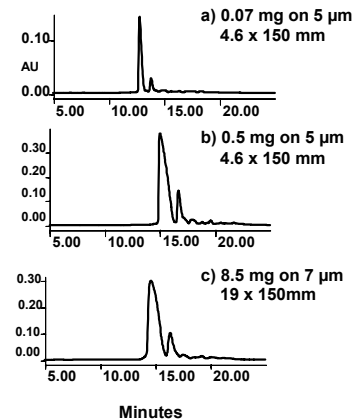
Degradation Products of Prochlorperazine Scale-up to 7 μm SymmetryPrep Column



Columns:
a. Symmetry C₁₈
b. SymmetryPrep C₁₈
Mobile Phase:
A. 0.1% TFA aqueous;
B. acetonitrile
Gradient:
10% to 60% B in 50 minutes
Flow Rates:
a. 0.7 mL/min
b. 2.8 mL/min
Detection: UV at 280 nm
Sample:
prochlorperazine edisylate
a. 0.8 mg
b. 3.2 mg



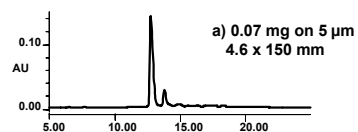
Scale-up of Insulin Impurity Isolation to 19 mm SymmetryPrep C₁₈ Column



Mobile Phases: A. 0.1% TFA aqueous
B. 0.1% TFA/ acetonitrile
26% B to 33% B in 14 minutes
Flow Rates:
a. and b. 1 mL/min;
c. 17 mL/min
Sample:
Bovine Pancrease Insulin, 10 mg/mL

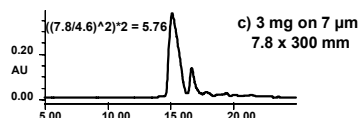
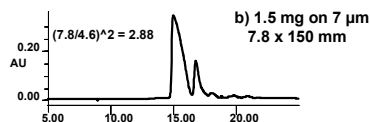
HPLC System: a. analytical system
b. and c. system modified for prep analysis, larger syringe and loop in injector and 0.04 in. i.d. tubing

Scale-up of Insulin Impurity Isolation to 7.8 mm SymmetryPrep C₁₈ Columns



Mobile Phases:
 A. 0.1% TFA aqueous
 B. 0.1% TFA in acetonitrile
 26% B to 33% B in 14 minutes

Sample:
 Bovine Pancrease Insulin,
 10 mg/mL in 0.01N HCl

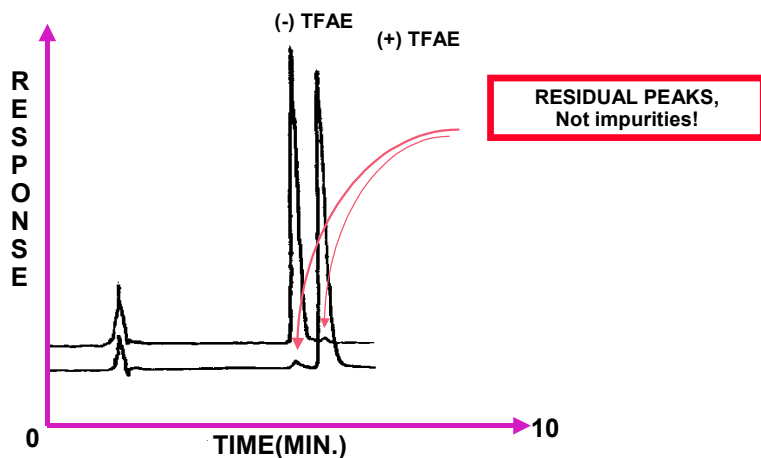


HPLC System:
 a. analytical system
 b. and c. system modified
 for prep analysis,
 larger syringe and loop
 in injector and 0.04 in. i.d.
 tubing

Preparative Chromatography Terminology

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

VISUALIZATION OF IRREVERSIBLE ADSORPTION VIA THE SYSTEM PEAKS OF THE RESIDUAL ENANTIOMERS*



S. Levin and S. Abu-Lafi, Chirality, 6, 148-155, 1994.

Scale-up Strategy - Summary

1. Define the problem ➡ Find the chromatographic mode.
2. Develop and optimize the separation ➡ Increases selectivity > 1.5
3. Maximize throughput ➡ Measure adsorption isotherm.
4. Increase sample mass and volume to the maximum while meeting purity objectives. ➡ Examine the competition
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.