CONSIDERATIONS IN SCALING UP A CHROMATOGRAPHIC RUN

Dr. Shulamit Levin

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Dr. Shulamit Levin

Tel: 03-9254040; Fax: 03-9249977
Email: levins@.net.il
home page: www.geocities.com/shulamit_1999
www.forumsci.co.il/HPLC

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

Problem Definition: Analysis

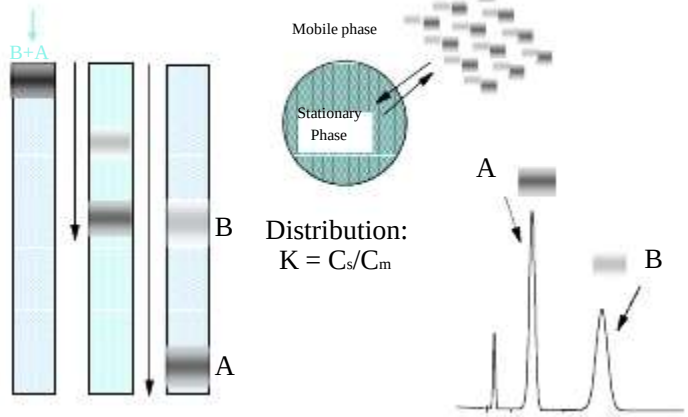
- What sensitivity is required?
- How complex is the sample?
- How many analyses will be performed?
- What degree of accuracy, precision, etc. is required?
- How easy (routine) does the assay need to be?

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

Preparative Chromatography Terminology

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

Chromatographic Process



$k' = \frac{tR - t0}{t0}$ $k' = \frac{Cs}{Cm}$

RETENTION FACTOR $k' = \frac{tR - t0}{t0}$

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

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

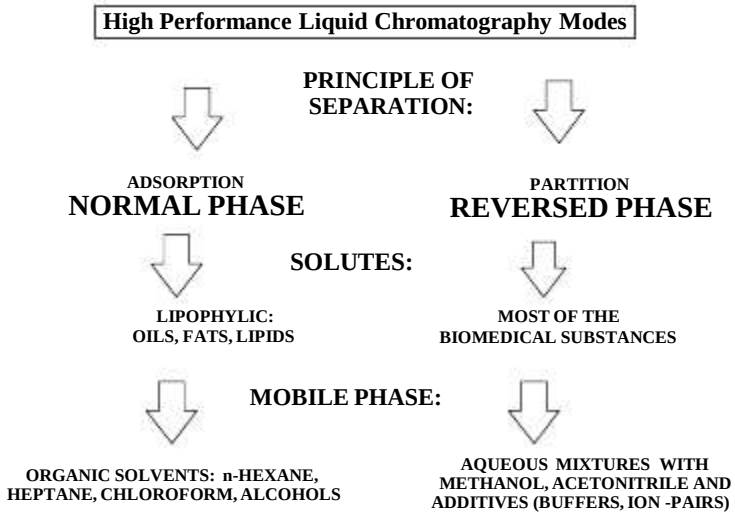
Seven Basic Considerations in Choosing HPLC Operating Parameters

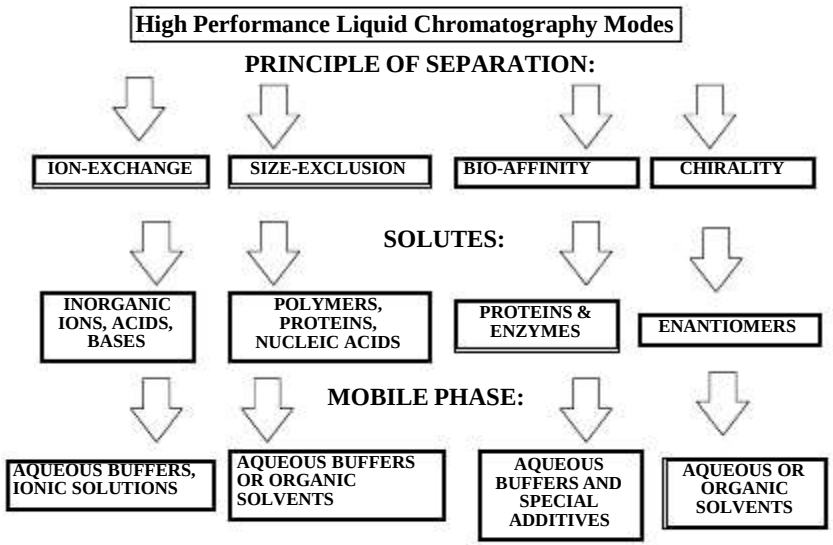
1) **Solubility** - Hexane, Chloroform, Methanol, Water (buffer pH), other?
2) **Molecular Weight** - Would GPC be useful in either the analysis or sample prep?
3) **Functional Groups** - Any ionizable groups? Acidic, Basic, or Neutral?
4) **Sample Matrix** - What amounts are expected in matrix for either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

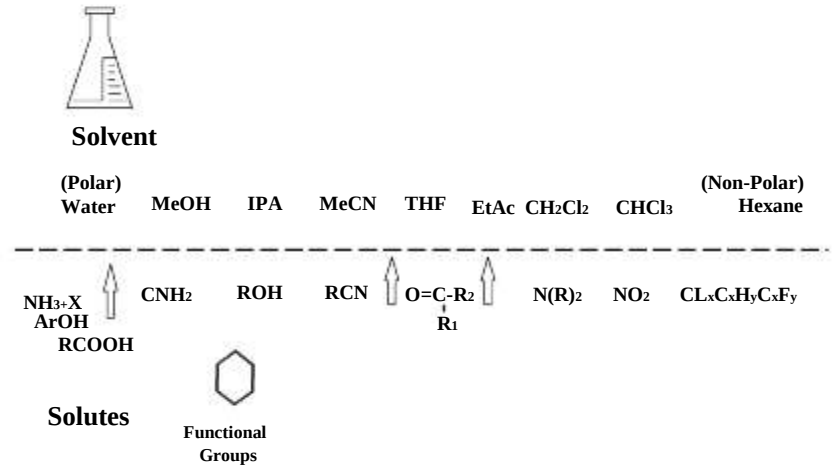
Selection of the appropriate mode of chromatography

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

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







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

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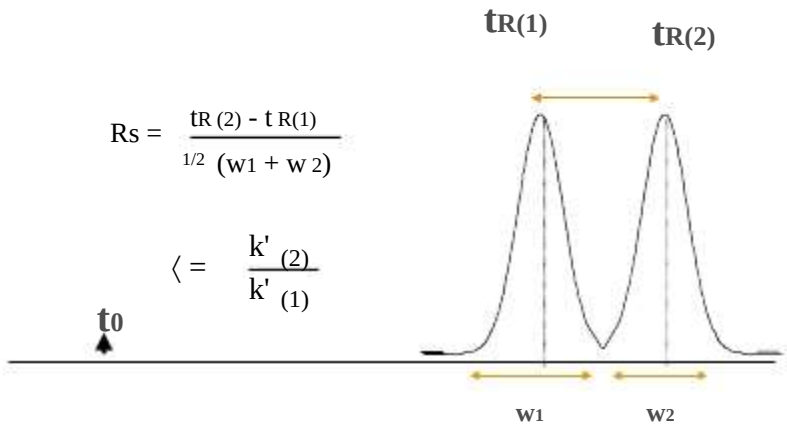
(Stationary phase, Mobile Phase, Temperature, Additives)

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

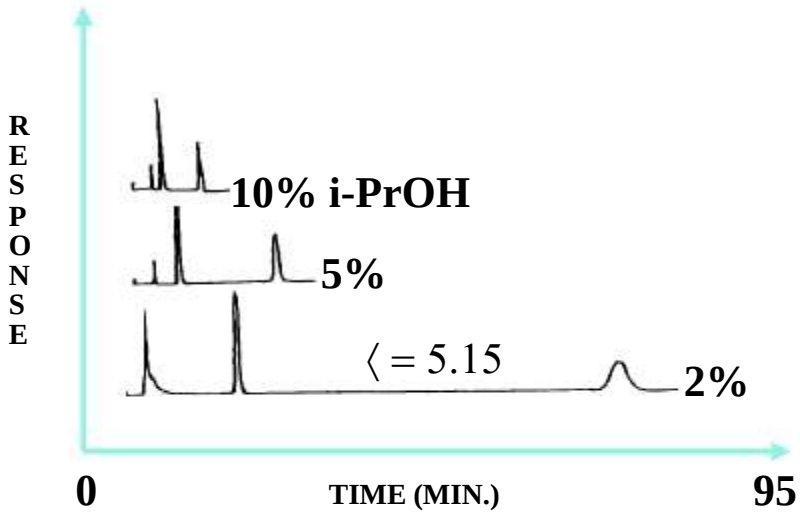
Scaling up

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)

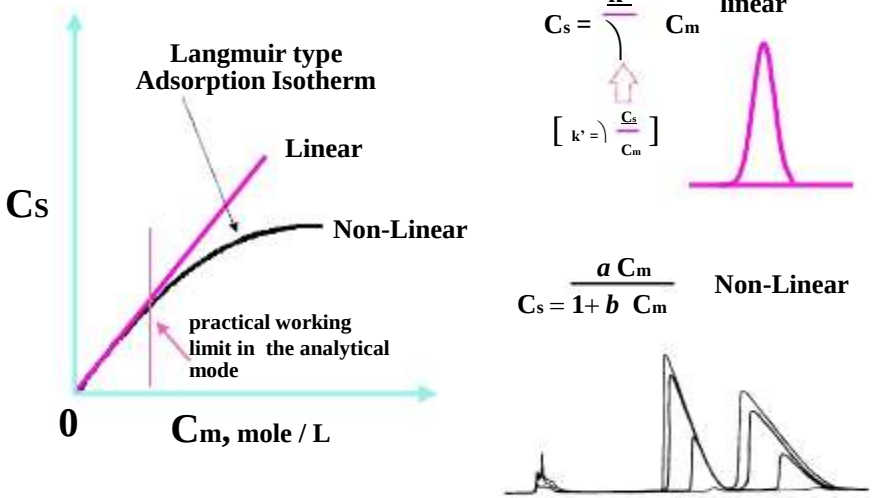
Optimization of the throughput
(Sample amount: Column Overloading)
Adsorption Isotherm & Competition

Scaling up

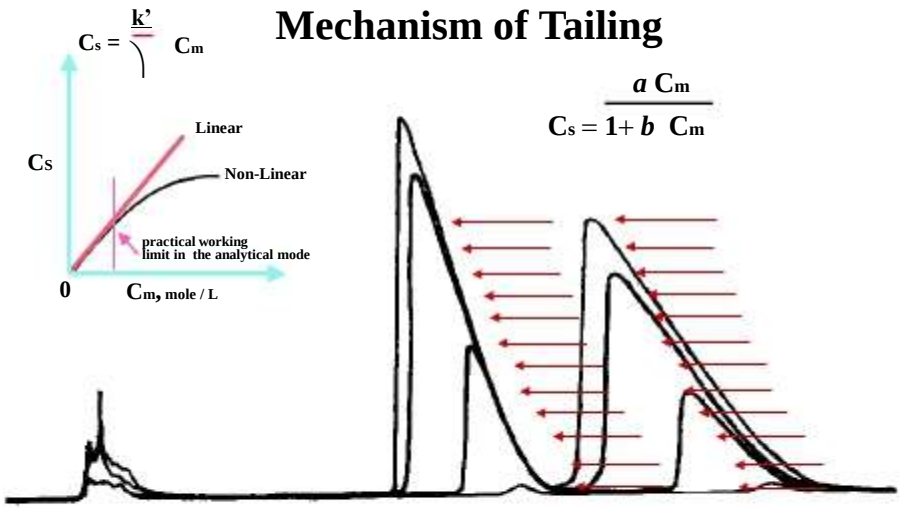
FIRST CHOICE:
USING OVERLOADING CONCENTRATIONS

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

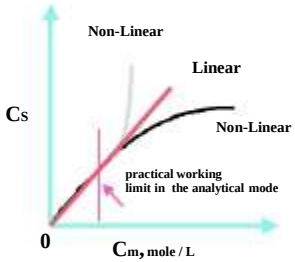
ADSORPTION ISOTHERMS: THE KEY TO RATIONAL SCALE-UP



Mechanism of Tailing

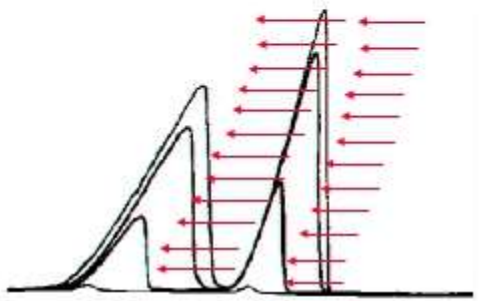


Mechanism of Diffuse Front

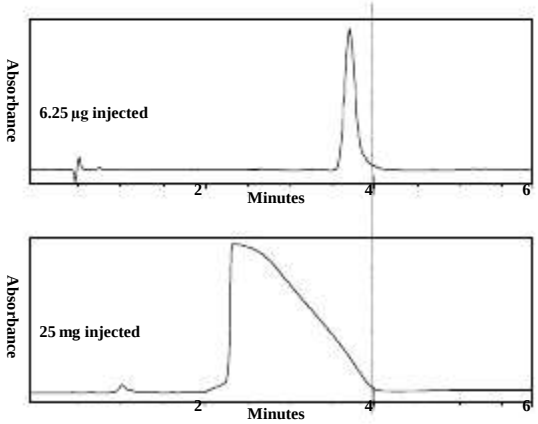


$C_s = aC$

$C_s = \frac{k'}{\alpha} C_m$

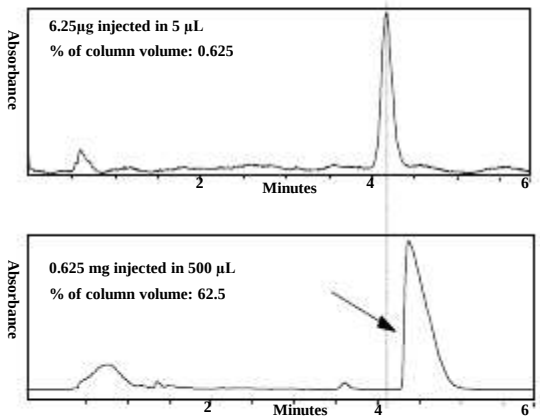


Mass Overload



- 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

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)**

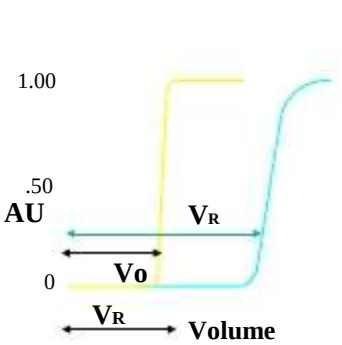
4- **Elution on Plateau (EP)**

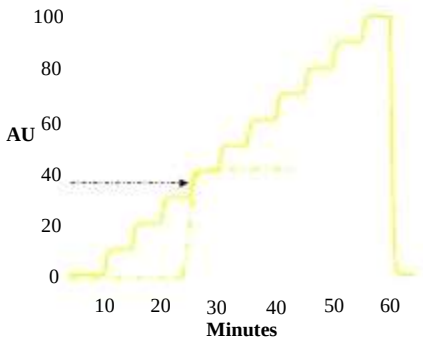
5- **System Peaks Analysis (SPA) ***

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

FRONTAL ANALYSIS

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





Frontal Analysis (FA)

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

STEPWISE:

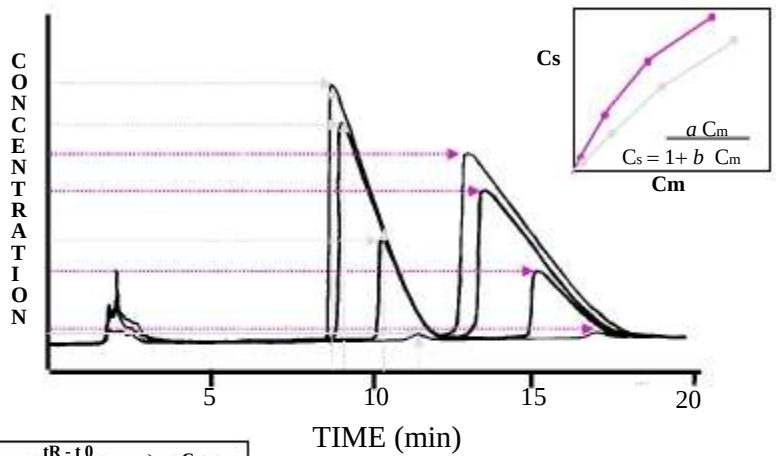
$$C_{s,i} = \int_0^{C_{m,i}} \frac{(V_{R,i} - V_0 - V_h)}{V_s} dC_{m,i}$$

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)



$$k' = \frac{tR - t0}{t0} \qquad k' = \left(\frac{Cs}{Cm}\right)$$

ELUTION BY A CHARACTERISTICS POINT (ECP)

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

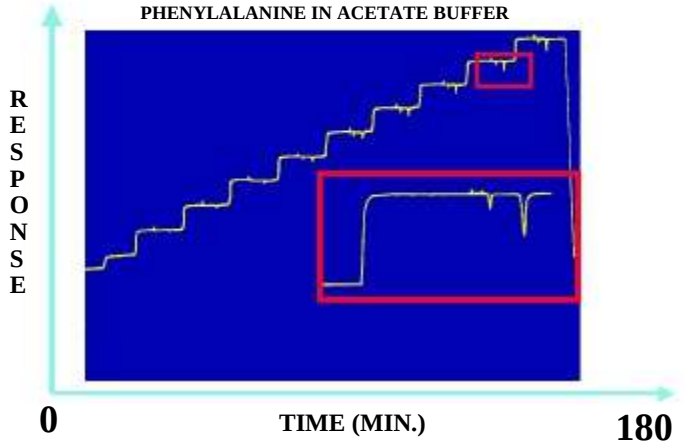
V_0 = column void volume

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. ýChromatogr., 556, 277-285, 1991.ý*

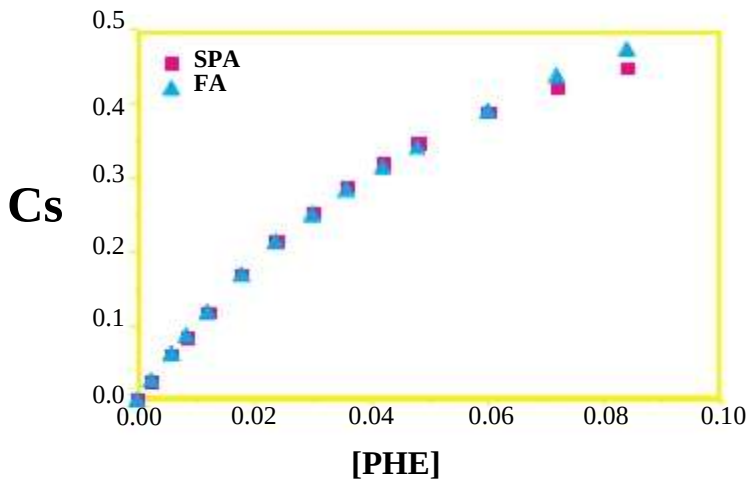
Calculation of Cs,i:

System Peaks Analysis (SPA)

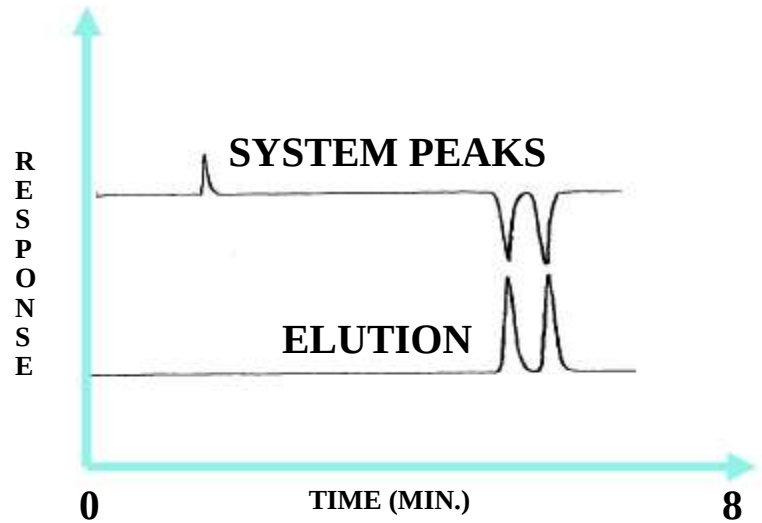
$$C_{s,i} = \frac{1}{\varphi} \int_{0}^{C_{m,i}} k'_i \, dC_{m,i}$$

Cs,i = concentration in the stationary phase
k'i = capacity factor
φ = phase ratio
dCm,i= difference in concentration between every .....two steps.

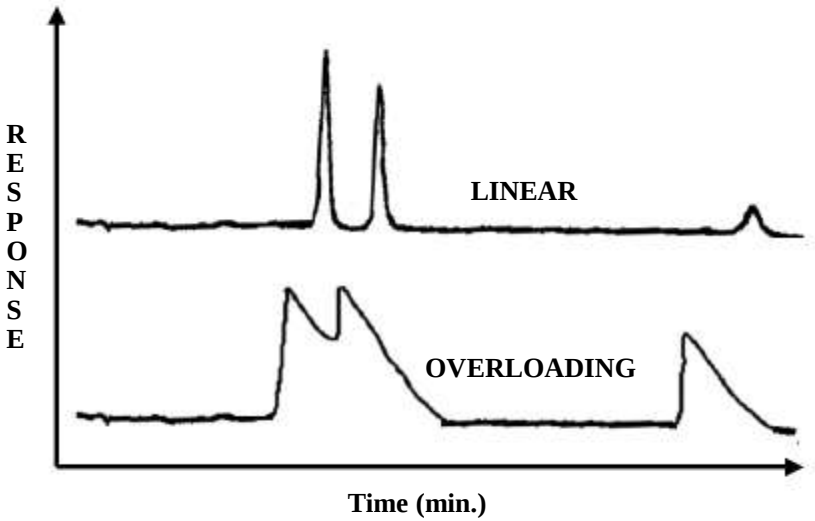
ADSORPTION ISOTHERM OF PHENYLALANINE IN 0.1 M ACETATE BUFFER BY FA and SPA



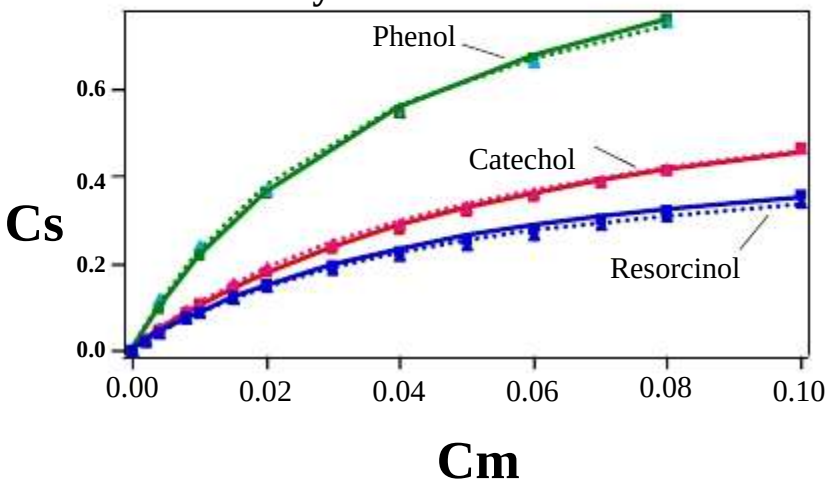
MIXTURE OF (1:1) 1,8- AND 1,5-DCAQ IN THE LINEAR RANGE



CHROMATOGRAM OF RESORCINOL CATECHOL & PHENOL

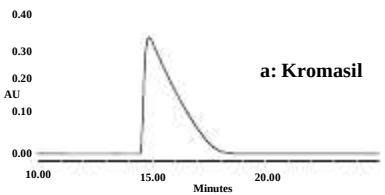


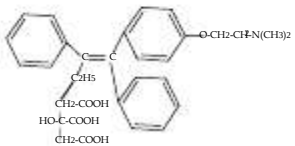
ADSORPTION ISOTHERMS by SPA AND FA*

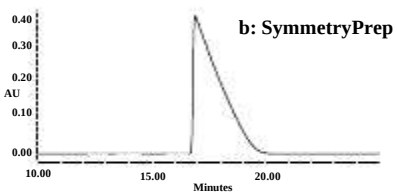


S. Levin, S. Abu-Lafi, S. Golshan-Shirazi and G. Guiochon, J. Chromatogr., 679, 213-229, 1994.ý

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







Column:

a: Kromasil C18 7 µm (4.6 x 150) mm
b: Symmetry Prep C18 7 µm (4.6 x 150) m m

Mobile Phase:

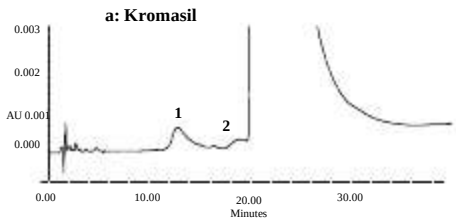
a: 44% acetonitrile / 46% 50mM potassium phosphate buffer, pH 3.0
b: 40% acetonitrile / 60% 50mM potassium phosphate buffer, pH 3.0

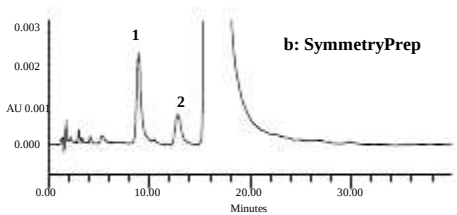
Flow Rate: 1.0 mL/min

Sample: 14 µL of 5 mg/mL Tamoxifen solution

Detection: UV at 254 nm

Prochlorperazine: Effect of Loading Capacity on the Separation of Impurities





Column:

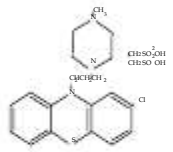
a: SymmetryPrep C18 7µm (4.6x150) mm
b: Kromasil C18 7µm (4.6x150) mm

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

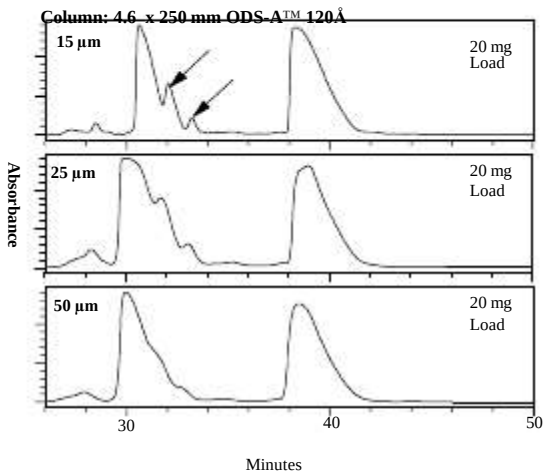
Flow Rate: 1 mL/min

Detection: UV at 254 nm

Sample: 10 µL of 0.97 mg/mL solution

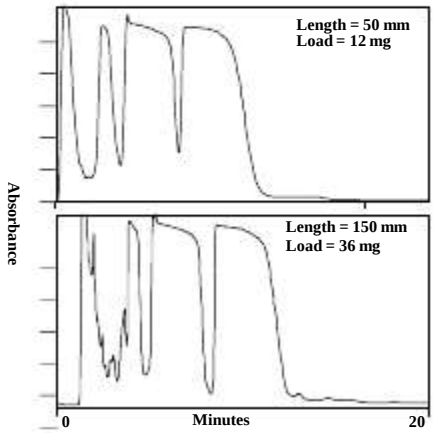


Effect of Particle Size on Capacity



- As particle size increases, resolution of the minor impurities decreases (→)
- Well resolved components not affected by change in particle size

Effect of Column Length on Capacity

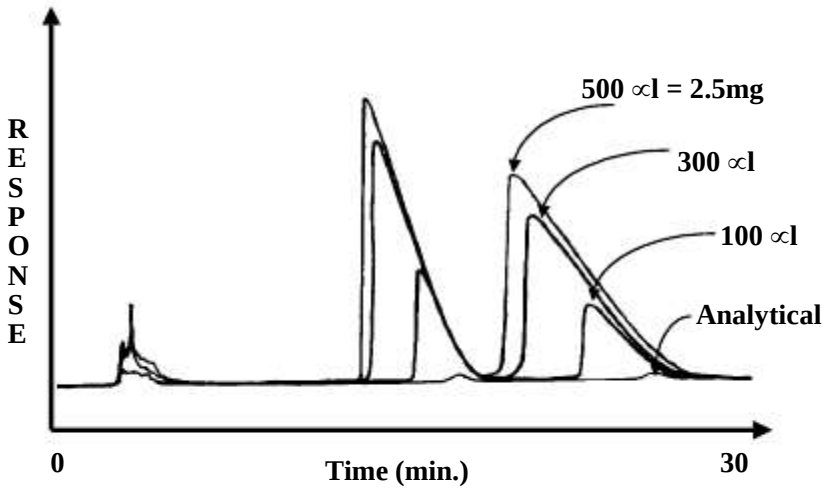


- As can be seen in this example, there is a linear relationship between column length and loading capacity - 3X increase in column length generates a >3X increase in loading capacity

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

Optimization of the throughput



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(Sample amount: Column Overloading)
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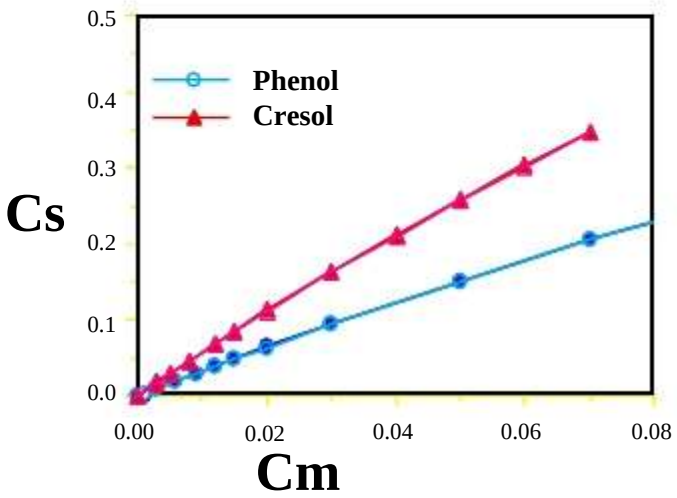
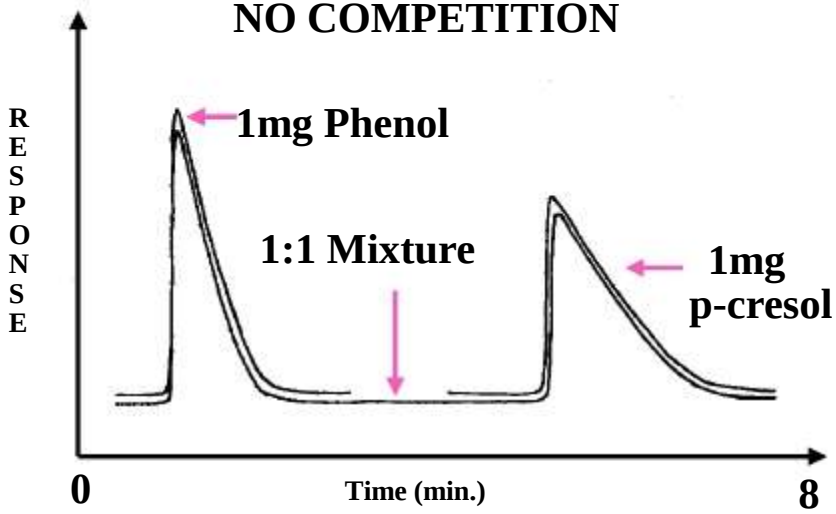
Scaling up

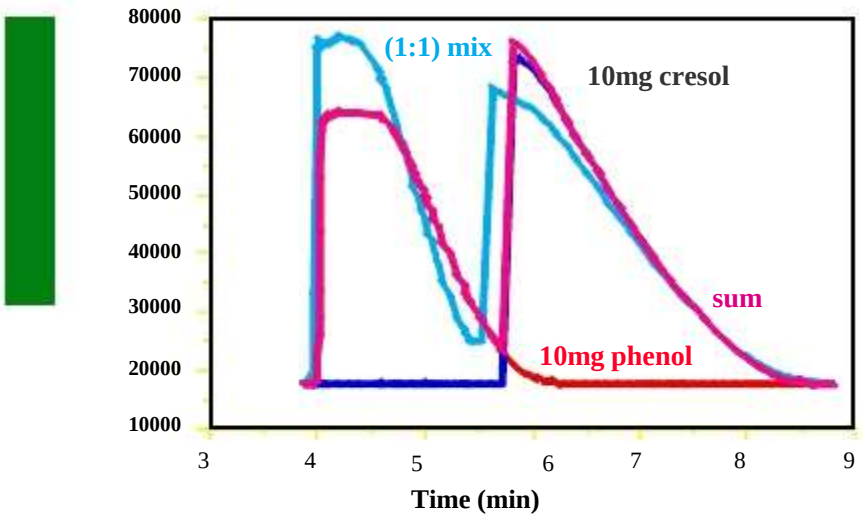
ADSORPTION ISOTHERMS: HIGH CAPACITY - NO COMPETITION



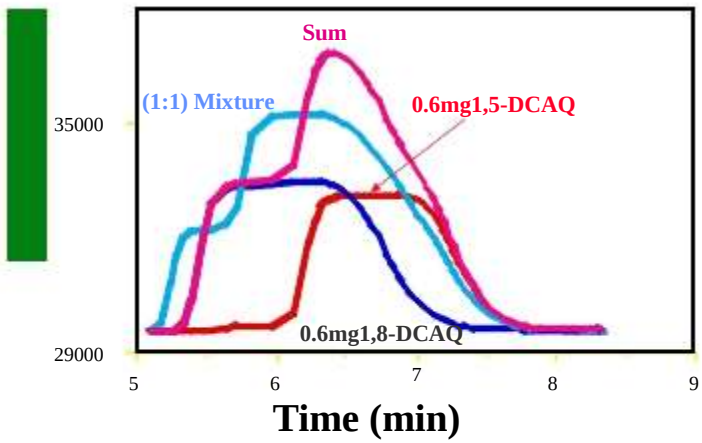
PEAK SHAPE AT OVERLOADING CONCENTRATIONS: NO COMPETITION



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

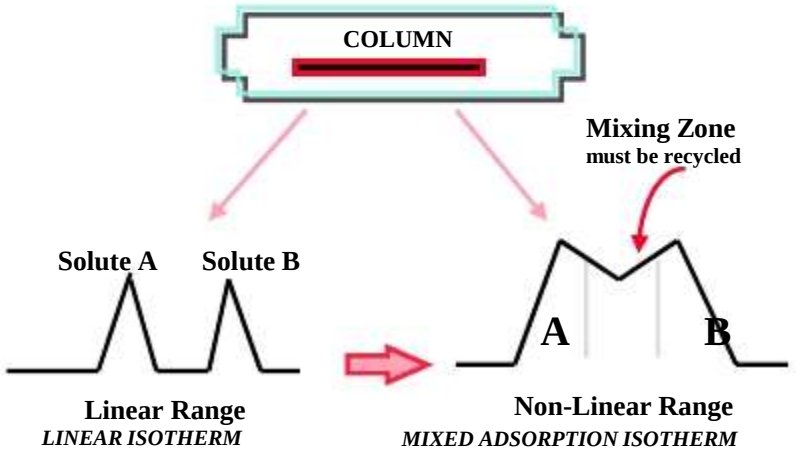
$$C_{s,1} = \frac{a_1 C_{m,1}}{1 + b_1 C_{m,1} + b_2 C_{m,2}}$$

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

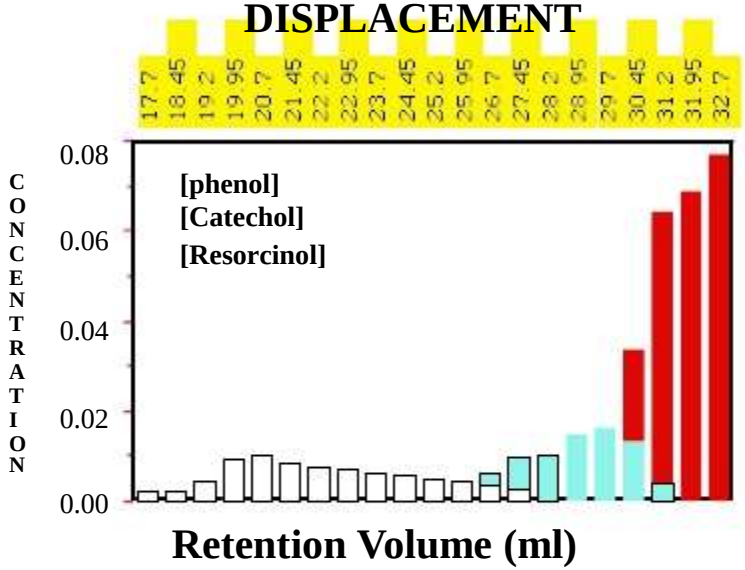
MULTI-COMPONENT

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

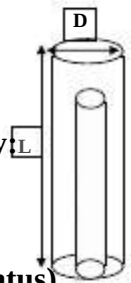
COMPETITION IN PREPARATIVE



HISTOGRAM FROM DISPLACEMENT



Scale-up of Flow



Scale-up flow rate to maintain constant linear velocity:

Flow rate = Flow rate $_{small\ scale}$ **x** $[(D_{Prep})^2 / (D_{small\ scale})^2]$

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

Load = Load $_{small\ scale}$ **x** $[(D_{Prep})^2 / (D_{small\ scale})^2 \times (L_{Prep} / L_{small\ scale})]$

D = Diameter of Columns
L = Length of Columns

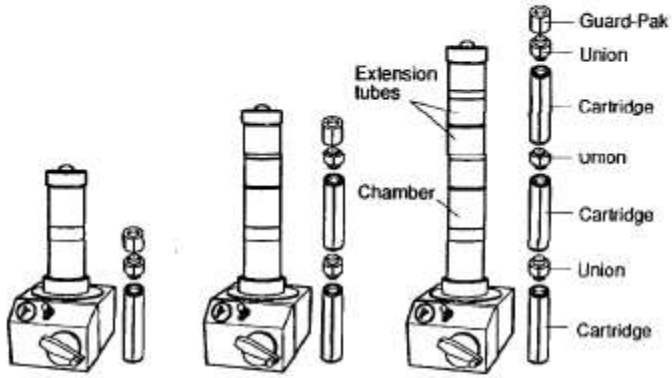
Approximate Mass Loading Capacity

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

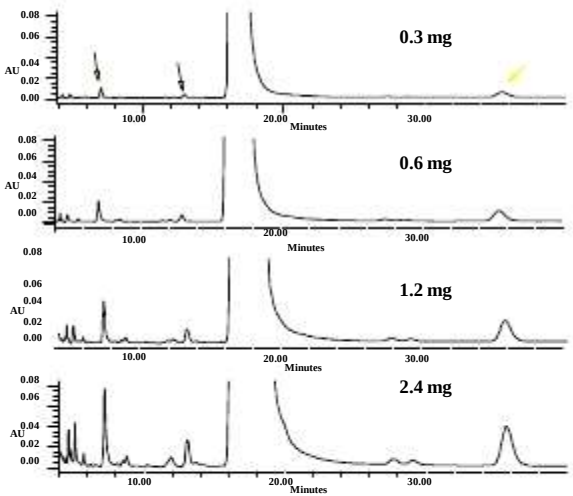
Mass Capacity (mg)

Diameter (mm)												
Length (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**

PrepPak Base 40mm ID Options



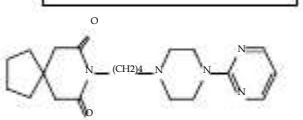
Buspirone: Effect of Increasing Load on the Separation of Impurities



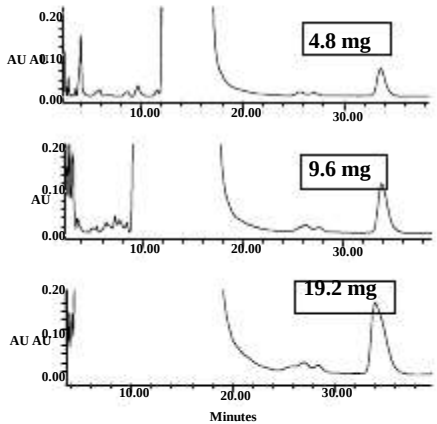
Column: SymmetryPrep C18, 7µm (3.9 x 150) mm
Mobile Phase:
28% acetonitrile / 72%
0.18% TETA-MeCOOH pH 7.0
Flow Rate: 1.0 mL/min
Detection: UV at 360 nm
Sample: 1.2 mg/mL of Buspirone

Injection: from 0.25 to 2.0 mL

Structure of Buspirone

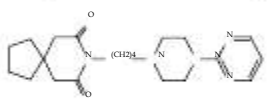


Buspirone: Effect of Increasing Load on the Separation of Impurities

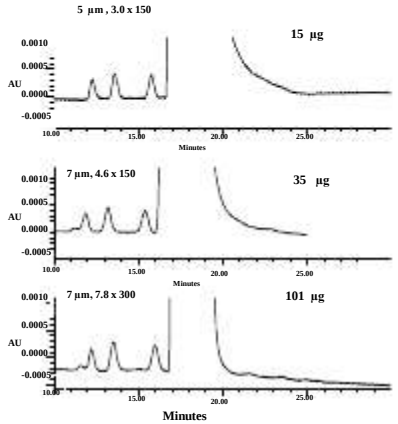


Column: SymmetryPrep C18, 7µm (3.9 x 150) mm
Mobile Phase:
28% acetonitrile / 72%
0.18% TETA-MeCOOH pH 7.0
Flow Rate: 1.0 mL/min
Detection: UV at 360 nm
Sample: 12 mg/mL of Buspirone
Injection: from 0.4 to 1.6 mL

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

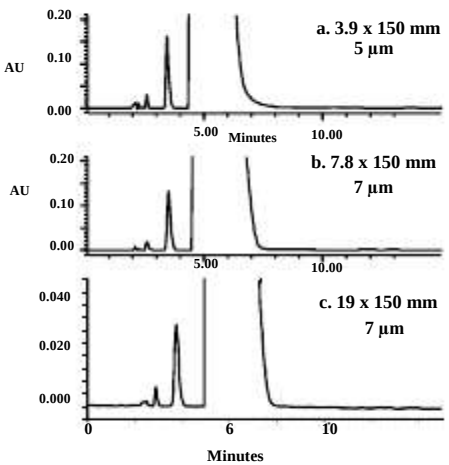
b: 0.97 mL/min

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 C 18 5 µm

b., c. SymmetryPrep C 18 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

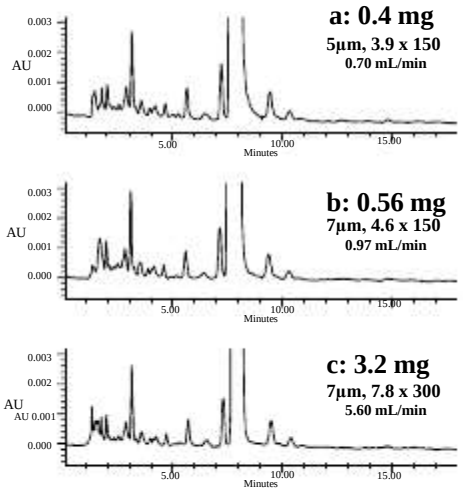
Sample: diltiazem

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

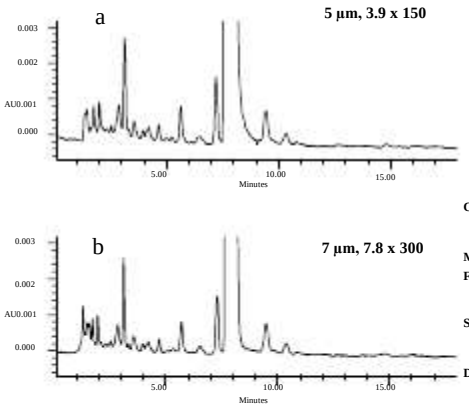
Sample: 4 mg/mL Valerophenone solution

a: 100 µL injection

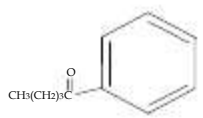
b: 140 µL injection

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 * r^2*L)

Keeping the separation at smaller flow rates:

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

? min = 50 min x $\frac{100}{1}$ x $\frac{1}{50}$ = 100 min

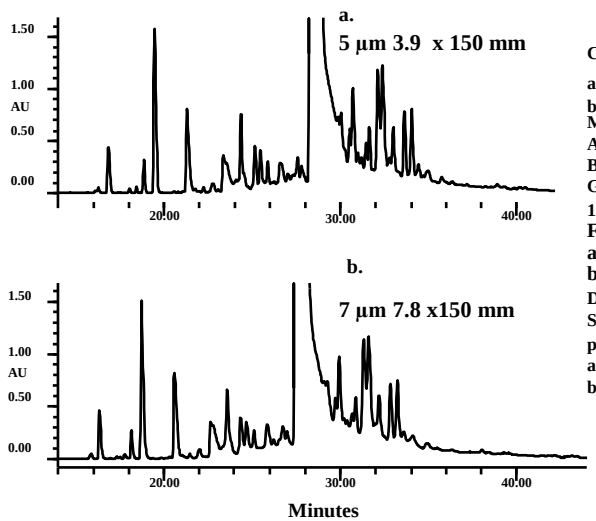
Scale-up a gradient run

	Column Diameter(mm)	Gradient Duration(min)	Flowrate (ml/min)	Voidvolume (ml)
Smallscale	4	30	1	3.14
Largescale	40	30	100	314.0
Semi-prep	10	30	6.25	19.6
Semi-prep	10	37.5	5	19.6

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 C 18

b. SymmetryPrep C 18

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

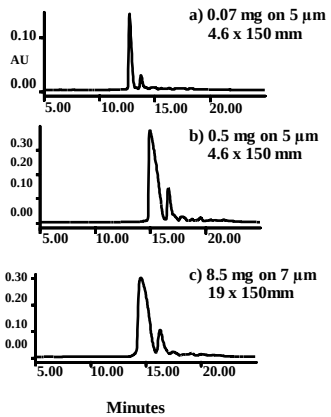
Sample:

prochlorperazine edisylate

a. 0.8 mg

b. 3.2 mg

Scale-up of Insulin Impurity Isolation to 19 mm SymmetryPrep C 18 Column



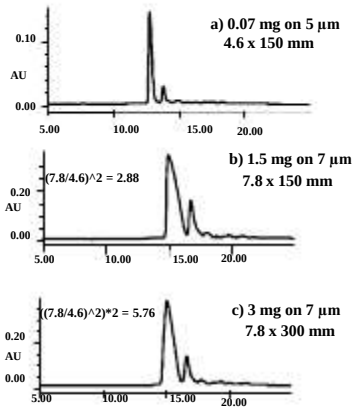
Mobile Phases: **A. 0.1% TFA aqueous**
B. 0.1% TFA/ acetonitrile
26% B to 33% B in 14 minutes

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 C $_{18}$ 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

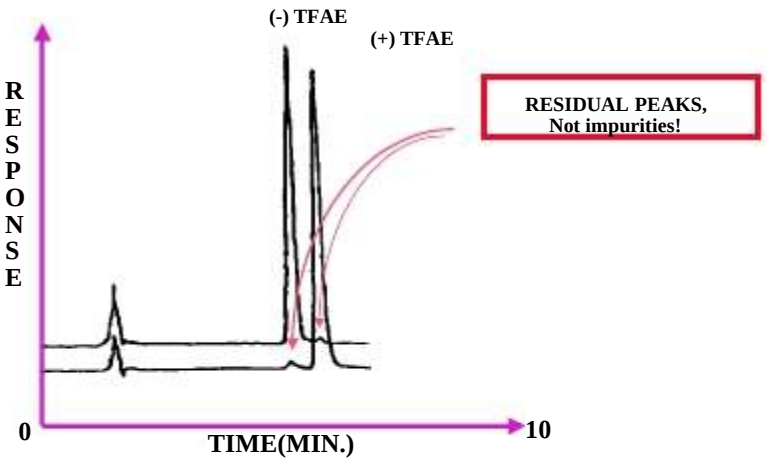
HPLC System:

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.ý

Scale-up Strategy - Summary

1. **Define the problem** → **Find the chromatographic mode.**
2. **Develop and optimize the separation** → **Increas 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.**