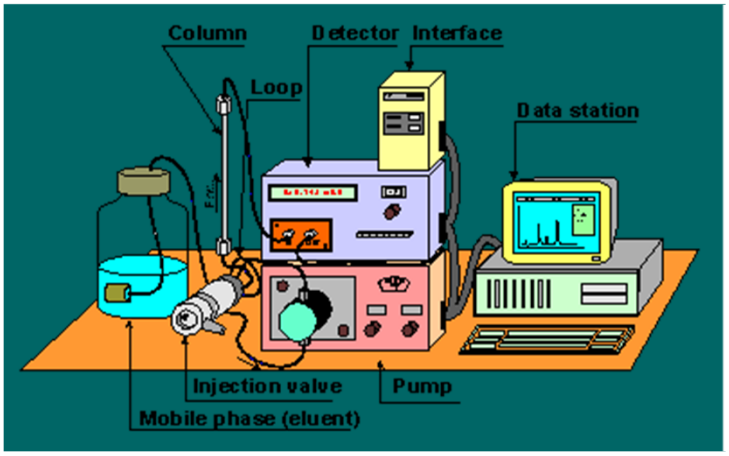
Updated: 3 November 2014

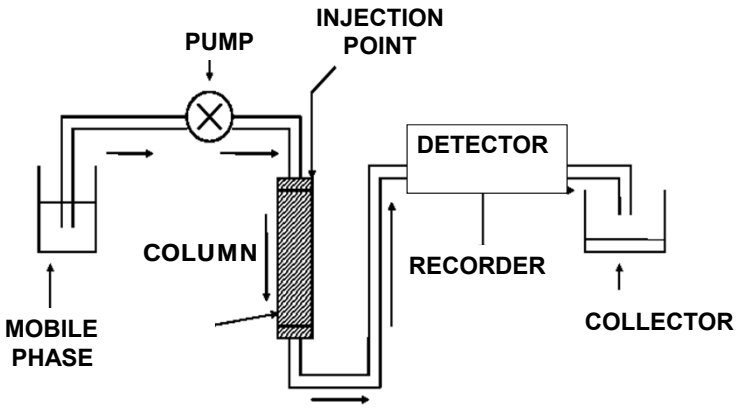
Print version

High Performance Liquid Chromatography

HPLC System



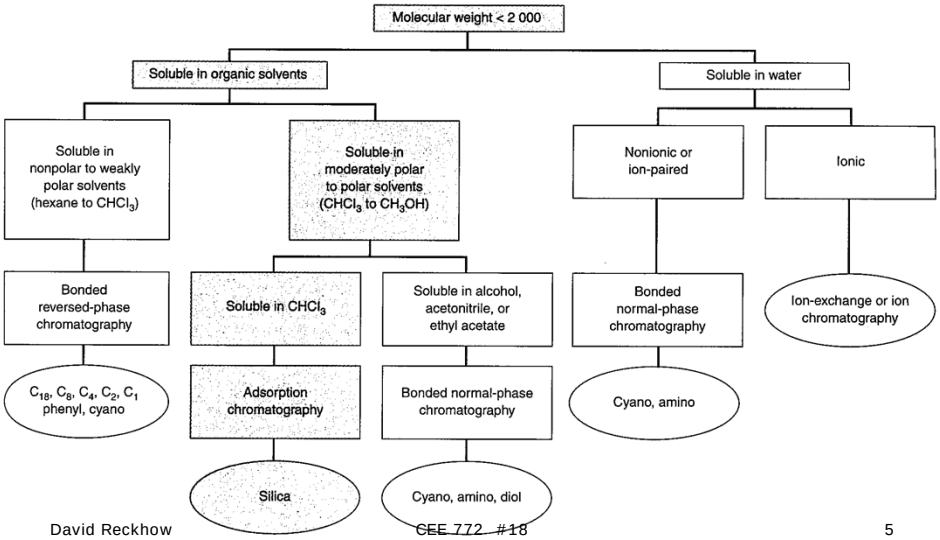
Instrument Basics



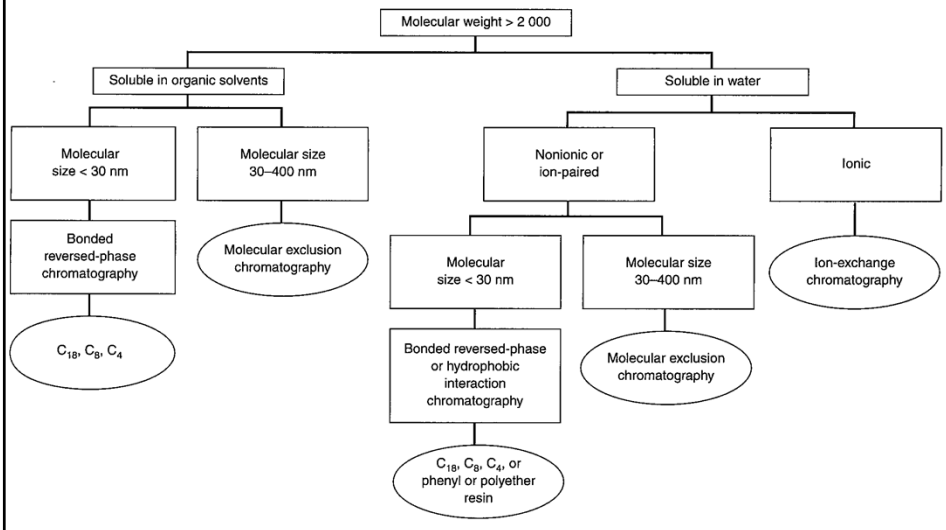
Types of HPLC

- Adsorption
  - Normal Phase – polar bed, non polar mobile phase (n-hexane, tetrahydrofuran)
  - Reverse Phase – non-polar bed w/ polar mobile phase (methanol, water, acetonitrile mixture)
  - * most common
- Ion Exchange
  - Stationary bed ionically charged surface, opposite to sample ions
  - Use with ionic or ionizable samples
  - Stronger charge = longer elution time
  - Mobile Phase – aqueous buffer
- Size Exclusion
  - Column material precise pore sizes
  - Large molecules first, then small

Separation mode selection



Separation mode selection



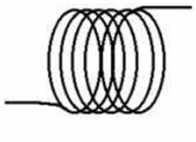
Pumps

- **Pumps solvent through stationary phase bed**
  - **Smaller packing requires higher pressure by pump**
  - **Larger packing and lower pump pressure is usable for most procedures, except SEC**
- **Stable flow rate - (not affected by pump)**
  - **0.01-10 mL/min**
  - **Normal flow rate stability < 1 %**
  - **Max psi 5000**
- **Pump should be inert to solvents, buffer salts and solutes**
  - **Stainless steel; titanium; resistant minerals (sapphires and ruby); PTFE (Teflon)**

Pump Types

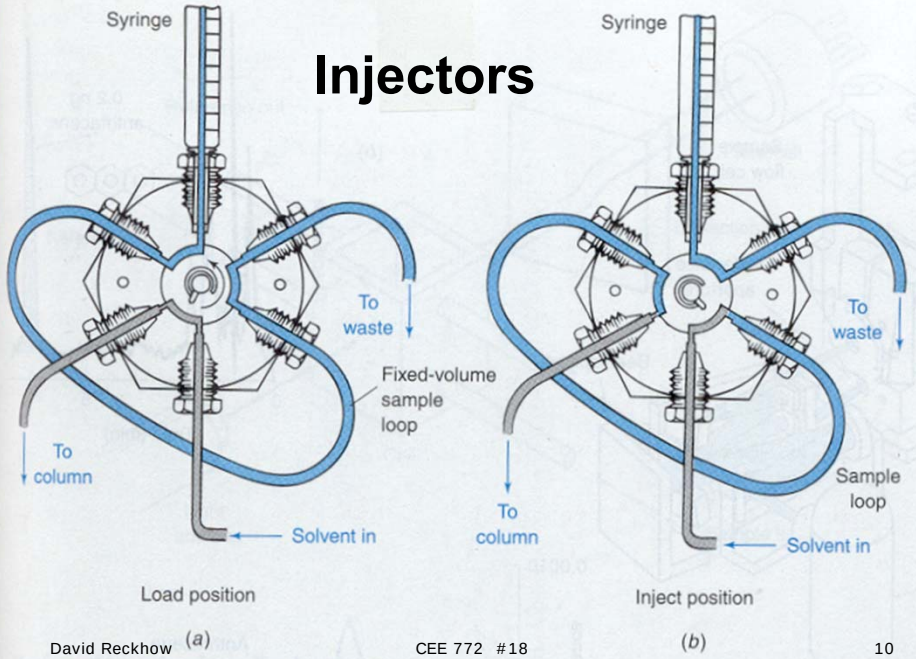
- I. Constant Pressure
  - **a) Pressurized coil**
  - **b) Pressure intensifier**
- II. Constant Flow Pump
  - **a) Piston *** most widely used**
  - **b) Syringe**
- Modern pumps are highly efficient and can be programmed to vary eluent ratios

Pulse Dampener



- In-line metal coil system
  - **Reduces pulse to +/- 3 %**
  - **Low cost, possible contamination**
  - **Limited range +/- 50-100 psi**
- T-type
  - **flow does not pass through coil**
  - **< 0.1 % pulse reduction**
  - **Same limitations as above**
- Bellows, Spring Loaded
  - **best but most expensive**

Injectors



Detectors

- **UV Detector**
  - Substances that absorb light from 180 to 350 nm
  - 254 nm common
  - General detector, most organic compounds
  - Good for non UV absorbing solvents
- **Fluorescence**
  - very sensitive to a few analytes which do fluoresce (phenanthrene)
  - Derivative methods to attach 'fluorophores' to analytes
  - Excitation at 280-305 nm and emission at 340-500 nm
- **Refractive Index**
- **Electrochemical**
- **Conductivity**

Comparison between different detectors

	Fixed Wavelength UV	Variable Wavelength UV	RI	Fluorescence Detectors	Wire-FID
Range of Application	Selective	Selective	Universal	Very selective	Universal
Destructive or nondestructive	Nondestructive	Nondestructive	Nondestructive	Nondestructive	Nondestructive
Gradient compatible	Yes	Yes	No	Yes	Yes
Detectability					
Min. Physical Change	5×10^{-5} abs.	2×10^{-4} abs.	1×10^{-7} RI units	~0.2 mV	2×10^{-13} A
Minimum Concentration	1×10^{-9} g/ml	4×10^{-9} g/ml	7×10^{-7} g/ml	10^{-12} g/ml	3×10^{-6} g/ml
Minimum Det. Sample	$\sim 1 \times 10^{-11}$ g/sec	2×10^{-11} g/sec	$\sim 3 \times 10^{-9}$ g/sec		3×10^{-6} g/ml
Minimum Det. Quantity	< 1 ng	~1 ng	~1 ug	1 pg	1 µg
Linear Range	$\sim 10^4$	$\sim 10^4$	3×10^3	$10^4 - 10^6$	N.A.
Short Term Reproducibility	Better than 0.5%	Better than 1%	Less than 1%	Better than 1%	Better than 1%
Peak Shape	Positive only	Positive only	Positive or Negative	Positive	Positive only

Mobile Phase / Eluent

- **Purity**
- **Detector compatibility**
- **Solubility of sample**
- **Low viscosity**
- **Chemical inertness**
- **Price**

- All solvents “HPLC grade”
  - **Filtered using 0.2 µm filter**
  - **Extends pump life**
  - **Protects column from clogs**
- Solvent Degassing / Purging
  - **Displacement w/ less soluble gas**
  - **Vacuum application**
  - **Heat solvent**

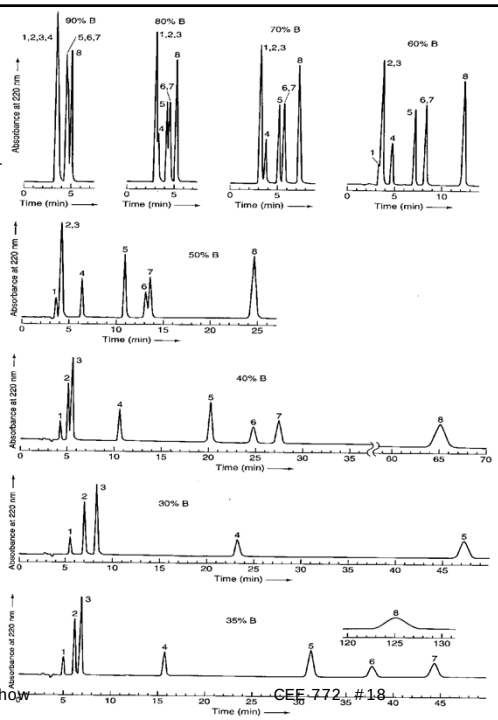


Figure 25-10 Isocratic HPLC separation of a mixture of aromatic compounds at 1.0 mL/min on a 0.46 × 25 cm Hypersil ODS column (C_{18} on 5-µm silica) at ambient temperature (~22° C): (1) benzyl alcohol; (2) phenol; (3) 3′,4′-dimethoxyacetophenone; (4) benzoin; (5) ethyl benzoate; (6) toluene; (7) 2,6-dimethoxytoluene; (8) *o*-methoxybiphenyl. Eluent consisted of aqueous buffer (designated A) and acetonitrile (designated B). The notation "90% B" in the first chromatogram means 10 vol % A and 90 vol % B. The buffer contained 25 mM KH_2PO_4 plus 0.1 g/L sodium azide adjusted to pH 3.5 with HCl.

Mobile Phase / Eluent

- **Isocratic elution**

--- Eluent composition remains constant

--- Single solvent or single solvent mixture

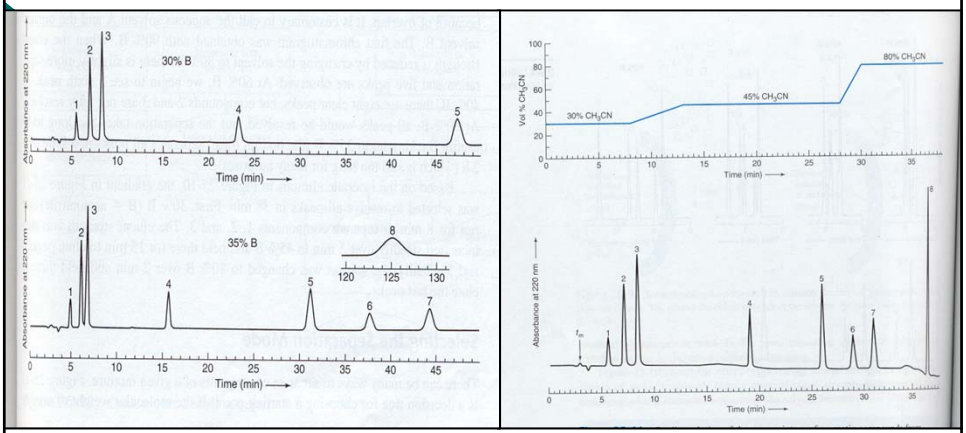
- **Gradient elution:**

--- Eluent composition (and strength) changed

--- Increases separation efficiency

--- Decreases retention time

--- Peak shape is improved (Less tailing)



Isocratic Separation (B : Acetonitrile) Gradient Separation

Conditions:

- Column : 0.46 * 25cm Hypersil ODS
- Flowrate : 1.0 mL/min
- Eluent : Aqueous Buffer (pH 3.5) and Acetonitrile

(1) benzyl alcohol, (2) Phenol
(3) 3', 4'- dimethoxy-toluene (4) benzoin (5) ethyl benzoate, (6) toluene
(7) 2, 6 -dimethoxytoluene, (8) o- methoxybiphenyl

HPLC Columns

- Stainless steel
- Common sizes:
  - 10,12.5, 15, 25 cm long
  - 4.6 mm i.d.
- Length for optimum separation dictated by theoretical plates needed for good resolution
- Filled with stationary phase material

Support Materials (Adsorption)

- **Silica gel :**
  - **polymer composed of tetrahedral silicon atoms connected through oxygen atoms (siloxane, Si-O-Si) with silanol (S-OH) groups present at the surface**
  - **Spherical (superior, more expensive)**
    or non-spherical forms
  - **Particle size and shape, surface area, and pore size help to get good separation**
  - **Also, pH of gel surface, # active silanol groups, presence of metal ions**

Effect of chain length on performance

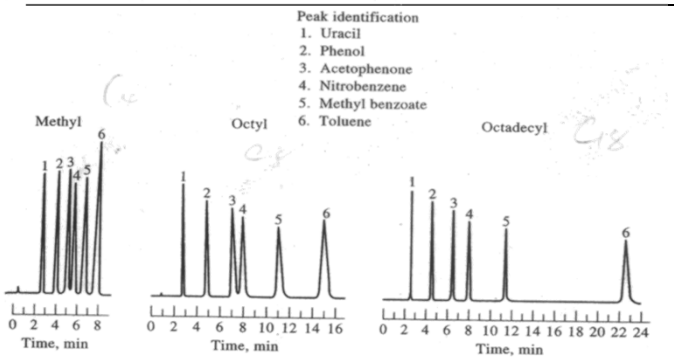


Figure 28-15 Effect of chain length on performance of reversed-phase siloxane columns packed with 5-μm particles. Mobile phase: 50/50 methanol/water. Flow rate: 1.0 mL/min.

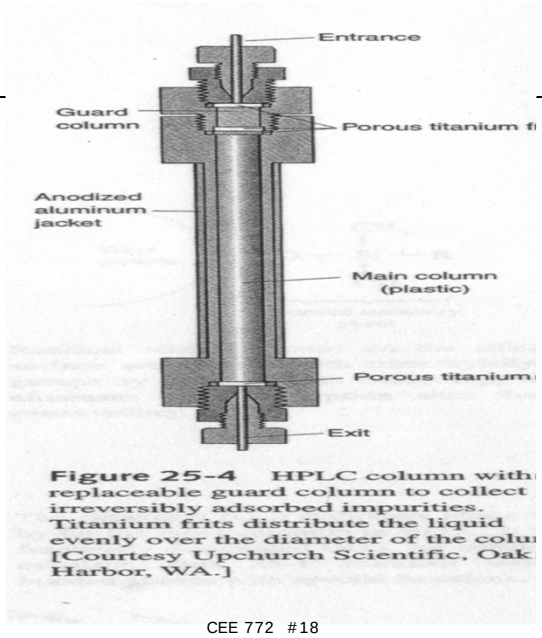


Figure 25-4 HPLC column with replaceable guard column to collect irreversibly adsorbed impurities. Titanium frits distribute the liquid evenly over the diameter of the colu [Courtesy Upchurch Scientific, Oak Harbor, WA.]

Normal phase column

-- stationary phase: high polar rigid silica, or silica-based compositions

-- mobile phases: relatively nonpolar solvent, hexane, methylene chloride, or mixtures of these

-- more polar solvent has higher eluent strength

-- the least polar component is eluted first

- **Elution**

  is described as a displacement of solute from the stationary phase by solvent.

- **Eluent strength**

  is a measure of solvent adsorption energy. The greater the eluent strength, the more rapidly will solutes be eluted from the column.

Reverse phase column

-- stationary phases: nonpolar hydrocarbons, waxy liquids, or bonded hydrocarbons (such as C18, C8, etc.)

-- mobile phase: polar solvents or mixtures such as methanol-water or acetonitrile-water

-- the most polar component is eluted first

-- less polar solvent has higher eluent strength

-- less sensitive to polar impurities

∇ Avoid to measure a sample that pH value is greater than 7.5 in a reversed –phase column, because of hydrolysis of the siloxane.

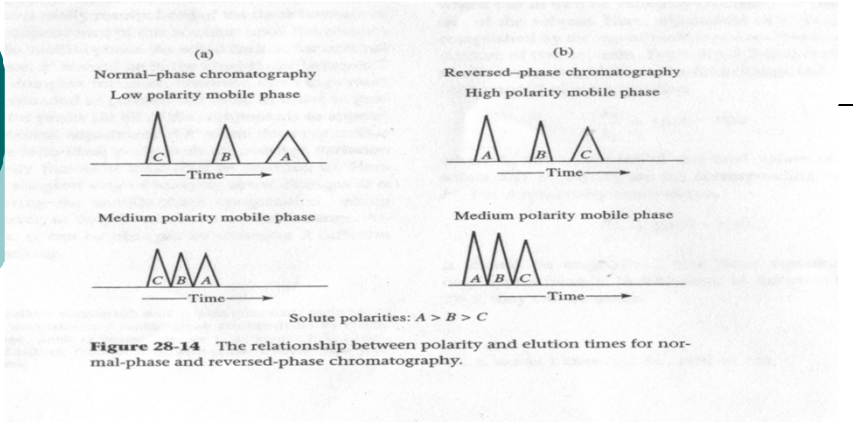


Figure 28-14 The relationship between polarity and elution times for normal-phase and reversed-phase chromatography.

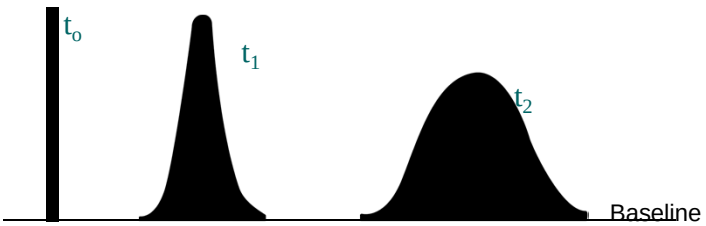
--- In a normal-phase column, decreasing the polarity of solvent will increase separation the components. In a reverse-phase column, the reverse is true

--- In normal-phase column, less polar solute is eluted first; in a reverse-phase column, the reverse is true

Guard Columns

--- Anterior to the separating
--- Filter or remove :
- ◊ particles
- ◊ compounds and ions
- ◊ compounds: precipitation upon contact with the stationary or mobile phase
- ◊ compounds: co-elute and cause extraneous peaks and interfere with detection and/or quantification.
- ◊ Prolongs the life of the analytical column

Column Efficiency

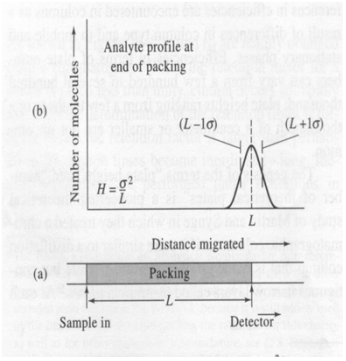


◊ each solute band spreads as it moves through the column
◊ the later eluting bands will spread more
◊ peak shape follow a Gaussian distribution

Column efficiency

◊ **Plate height, H=σ² /L**

The breadth of a Gaussian curve is directly related to the variance σ² or standard deviation σ



◊ **Plate count plates, N**

◊ **N=L/H**

Factors affecting Column efficiency

- Particle size of packings
- Column diameters
- Extra-column volume

  ---that volume in an HPLC system between and including the injector and the detector;
- Effect of mobile-phase flow rate

Particle size of packings:

-- the smaller size, the more plates and the higher efficiency

N=3500L(cm)/d_p(um)

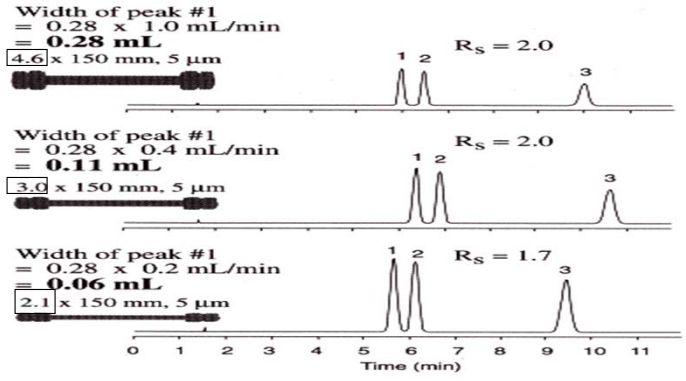
where d_p is the particle diameter

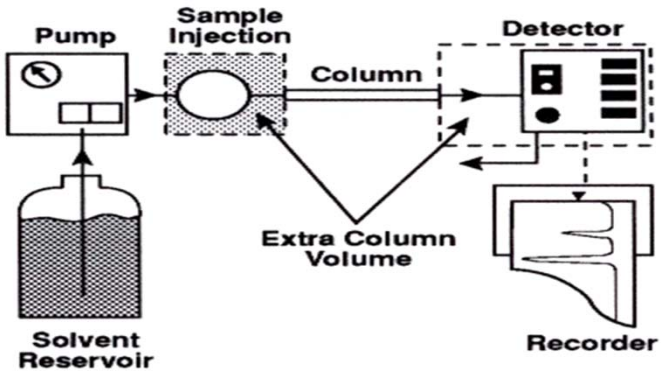
-- provide more uniform flow through the column, then reducing the multiple path term

-- the smaller the particles, the less distance solute must diffuse in the mobile phase

-- resistant to solvent flow. High pressure is required

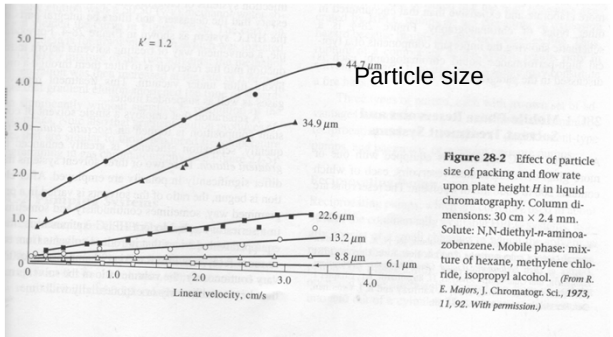
Column diameters





Extra-Column-Volume = sample volume + connecting tubing volume + fitting volume + detector cell volume

○ Effect of mobile-phase flow rate



A minimum in H (or a maximum in efficiency) at low flow

Effect of chain length on performance

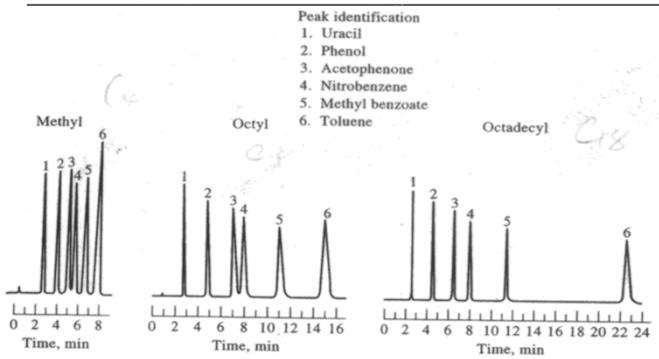
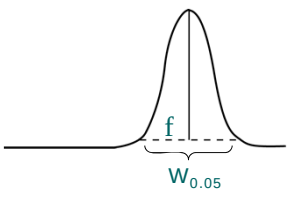


Figure 28-15 Effect of chain length on performance of reversed-phase siloxane columns packed with 5-μm particles. Mobile phase: 50/50 methanol/water. Flow rate: 1.0 mL/min.

Peak Tailing (A_s)

- A properly packed HPLC column will give symmetrical or Gaussian peak shapes.
- Changes in either the physical or chemical integrity of the column bed can lead to peak tailing.



Causes	Cure
Sample solvent stronger than the mobile phase	Dissolve sample in mobile phase or at least reduce the strength of the sample solvent as much as possible
Sample mass overload	Reduce the amount (mass) of sample injected.
Silanol interaction with amines (affects late eluting peaks most)	1. Reduce mobile phase pH to < 3.0 2. Increase mobile phase ionic strength. 25mM to 50mM recommended 3. Add a competing amine to the mobile phase. 10 mM TEA is usually sufficient. 4. Select a stationary phase with a lower silanol activity. See Figure 6 for a ranking of C18 phases by silanol activity
Adsorption of acids on silica	1. Increase salt concentration in the mobile phase 25 mM to 50 mM is usually sufficient 2. Reduce the pH of the mobile phase to < 3.0 3. Add a competing organic acid. 1% acetic acid or 0.1% TFA is usually sufficient
Column void (affects early eluting peaks most)	1. Replace the HPLC column. 2. Attempts to fill in the void are seldom worth the effort.

Sample Preparation

- Samples in solution
- Solutions must be filtered, centrifuged
- Some samples may need to be extracted using various solid phase extraction techniques
- pH is important for ionized species

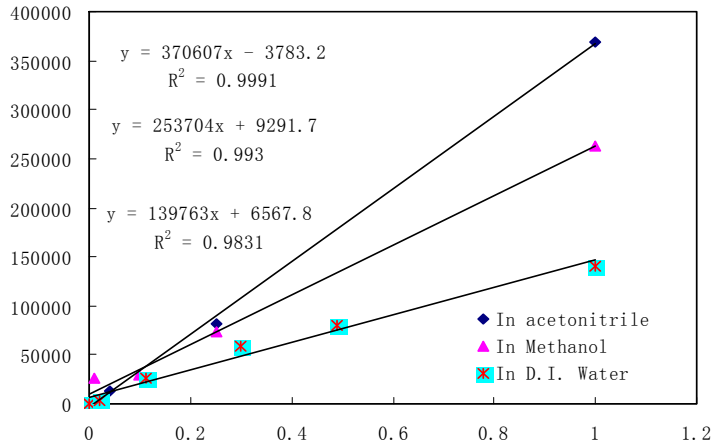
Research interest

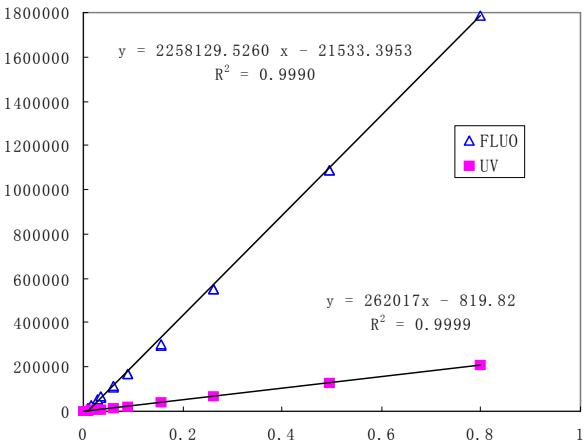
- Evaluate the maximum wood sorption capacity on PAHs.
- Competitive sorption with metal and between PAHs
- Sorption and desorption under different pH values or different temperature

PAHs

- Sources:
  - nature: forest fire
  - human behaviors: fuel burning, excess pesticide…
- Characteristic
  - low solubility --- accumulation
  - toxic, carcinogenic, mutagenic
- Research interest
- Analysis
  - GC/FID: sensitivity but background interferences
  - HPLC : necessary sensitivity and higher specificity

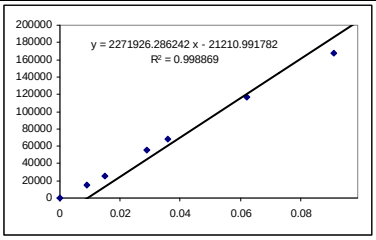
- **UV detector: MeOH : Water=90:10**

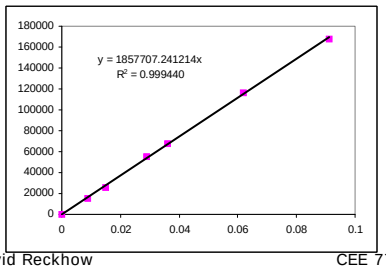


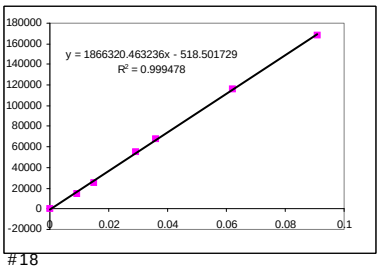


- **UV and Fluo detector : in solution with 0.01 M $CaCl_2$ and 200 mg/L NaN_3,**
- **MeOH : Water=90:10**
- **C18**

- **Fluorescence detector:**
  excitation—252 nm
  emission—370 nm







- To next lecture