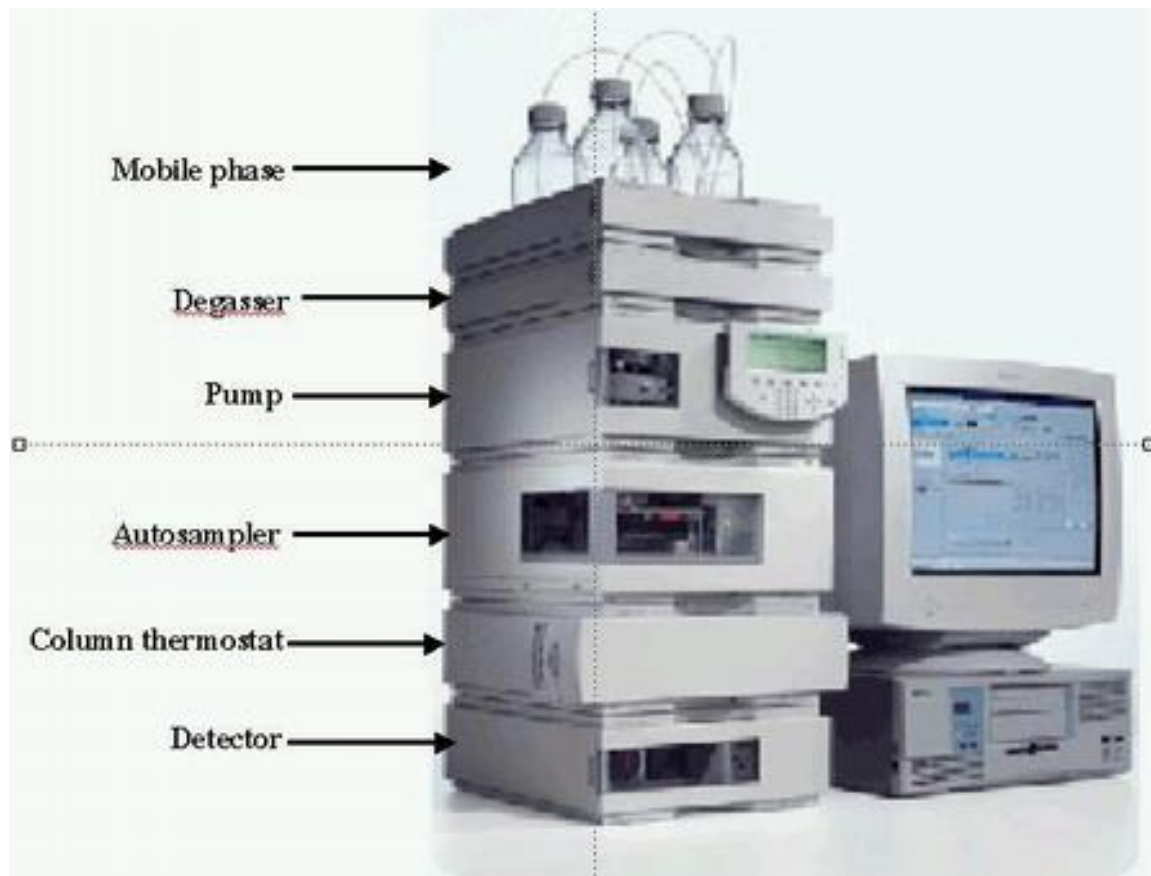


HPLC II

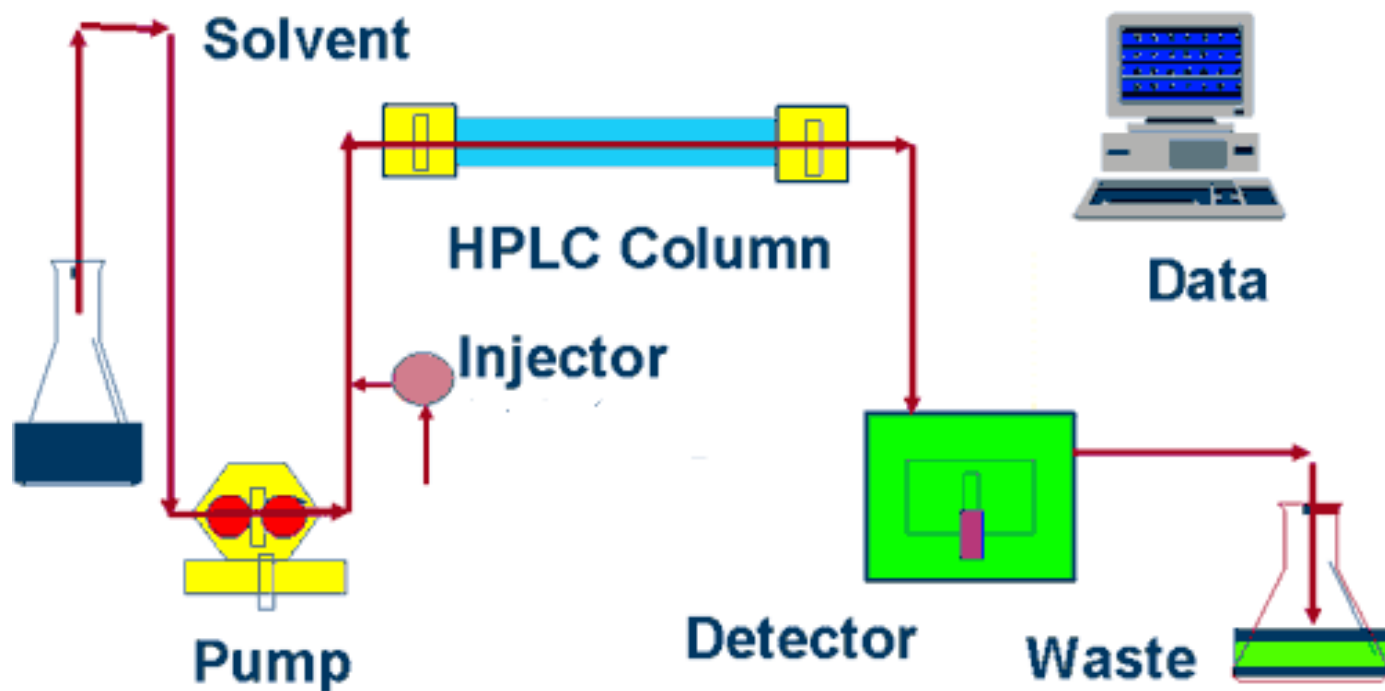
- Development of HPLC methods -

Csaba Hámori

HPLC



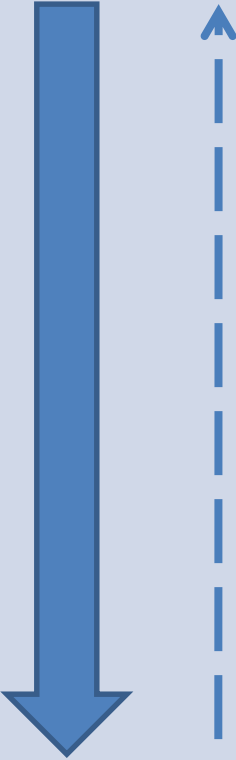
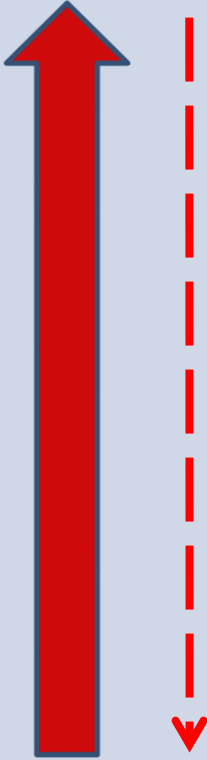
HPLC System



HPLC methods

- RP-HPLC
 - Non-polar stationary phase
 - Polar mobile phase
 - Water/buffer + organic solvents
- NP-HPLC
 - Polar stationary phase
 - Non-polar organic solvents
 - Silica gel stationary phase: Not use water!
 - HILIC

Eluent strenght, selectivity

NP-HPLC		RP-HPLC
	Hexane	
	CCl4	
	Chloroform	
	Dichloromethane	
	THF	
	Diethyl-ether	
	Ethil-acetate	
	Acetonitrile	
	2-PrOH	
	MeOH	
Water		

Requirement against solvents

- Purity
- Inertness
- Compatibility
 - Column
 - Detector
 - Sample
- Viscosity
- Miscibility
- Content of dissolved oxygen
- Toxicity
- Price



Common HPLC Solvents

Solvent	UV cut off (nm)	Viscosity (mPa s)	B.P. (°C)	Polarity (P) ¹
Water	200	1.00	100	9.0
Methanol	205	0.6	65	6.6
Acetonitrile	190	0.37	82	6.2
THF	215	0.55	65	4.2
IPA	210	2.35	83	4.3
Hexane	195	0.313	69	0

THF "Tetrahydrofuran" (not compatible with PEEK)

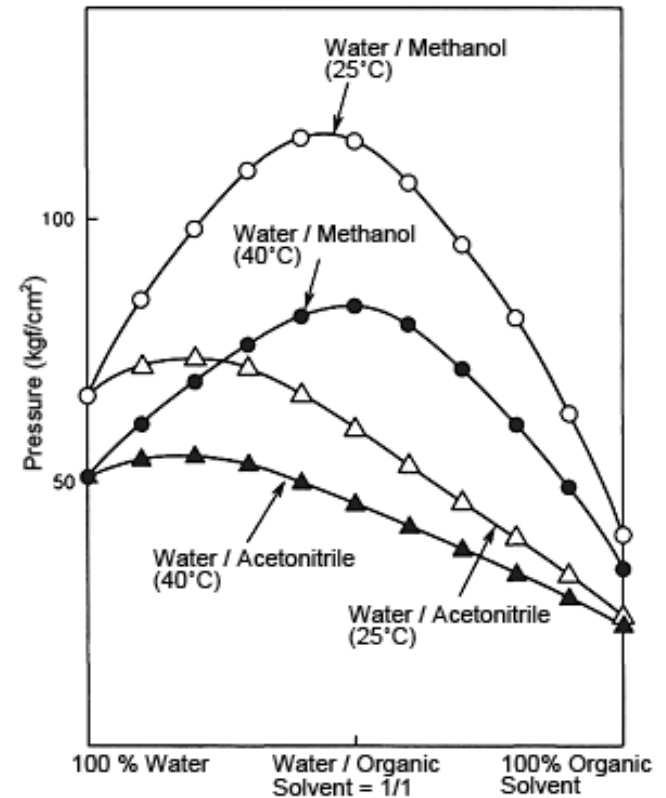
IPA "Isopropyl Alcohol"



마크 HPLC Solvent Grade Pyramid

Requirement against solvents

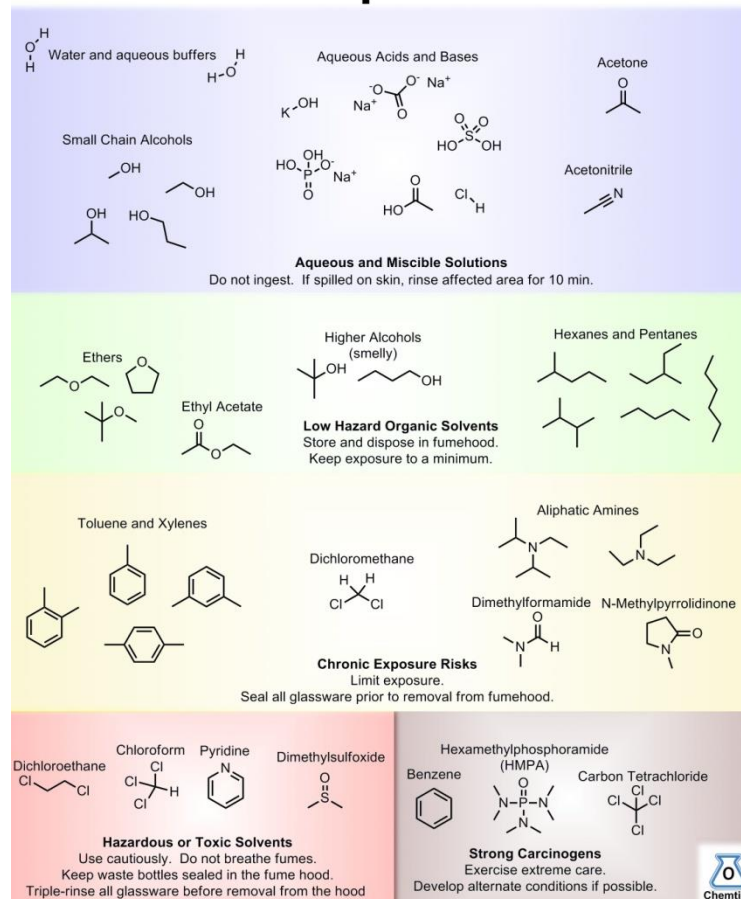
- Purity
- Inertness
- Compatibility
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 - Sample
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- Content of dissolved oxygen
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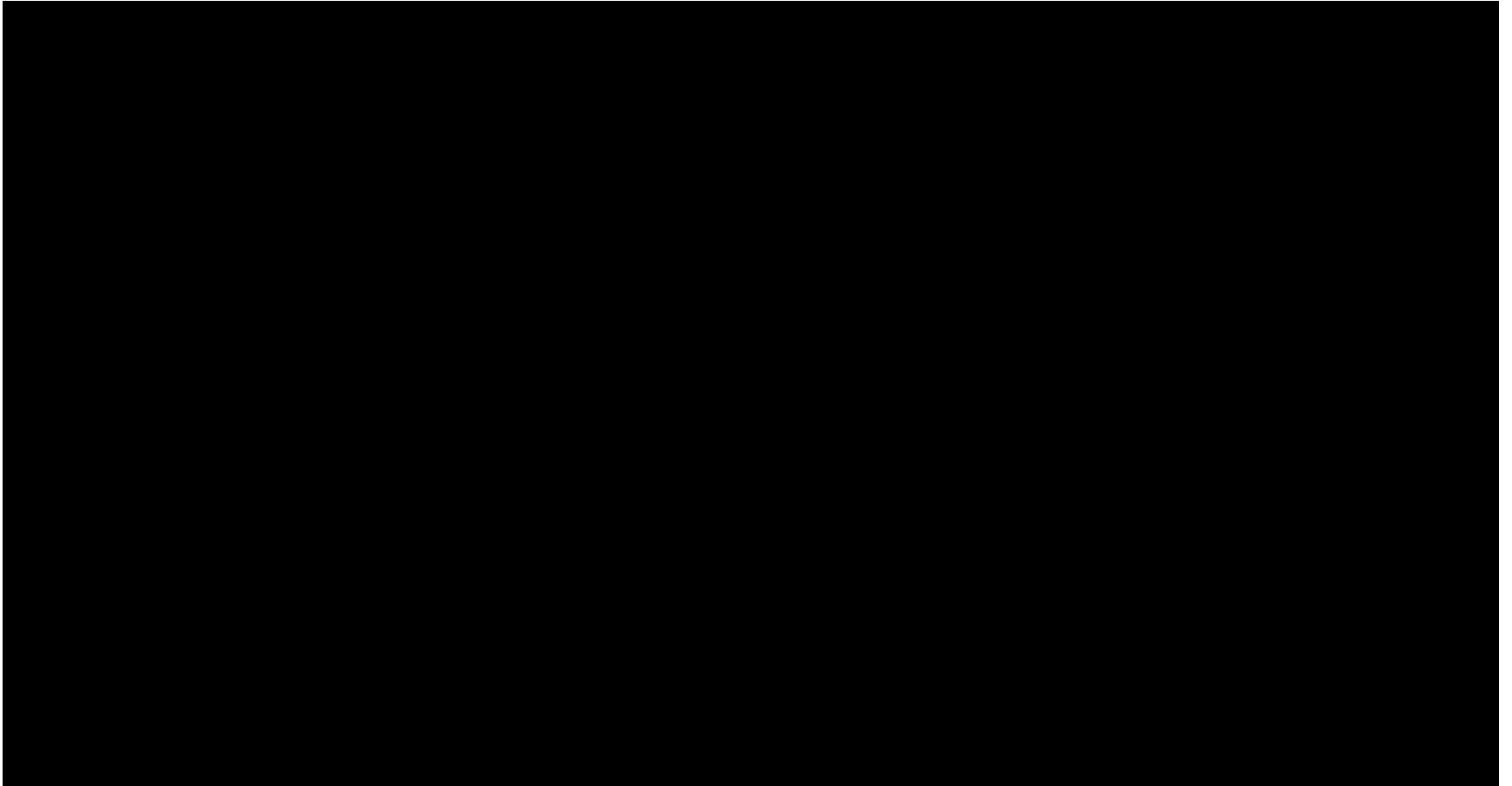
Requirements against solvents

- Purity
- Inertness
- Compatibility
 - Column
 - Detector
 - Sample
- Viscosity
- Miscibility
- Content of dissolved oxygen
- Toxicity
- Price

Solvent Exposure Chart



Operation of autosampler



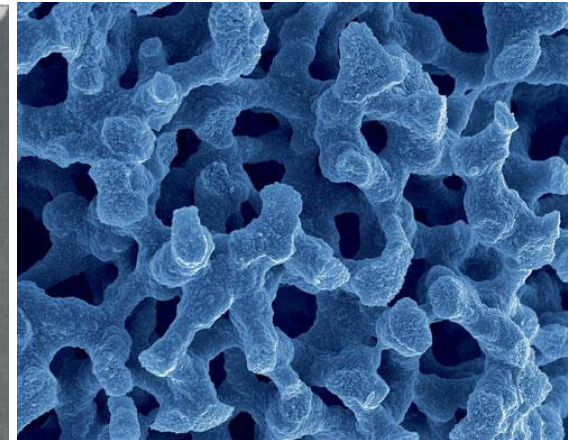
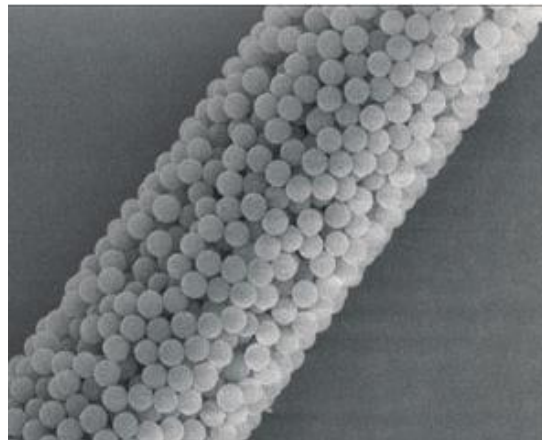
Stationary phases in HPLC

- Packed columns
 - Structure of particles
 - Size of particles
 - Distribution of particle size
 - Pore size, pore volume
 - Length, diameter

Type of usage:

- Analytical
- Preparative

- Polymer columns
 - Organic polymer
 - Monolithic



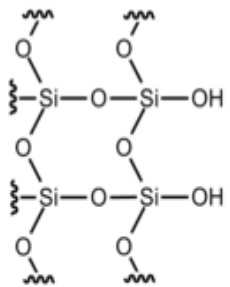
Stationary phases

- NP-HPLC

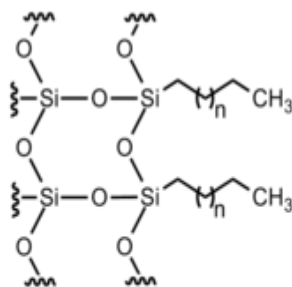
- Polar groups
- Silica gel (silanol groups)
- Modified silica gel

- RP-HPLC

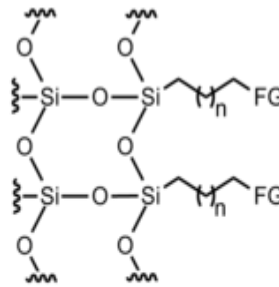
- Non-polar groups
- Modified silica gel



Normal phase silica

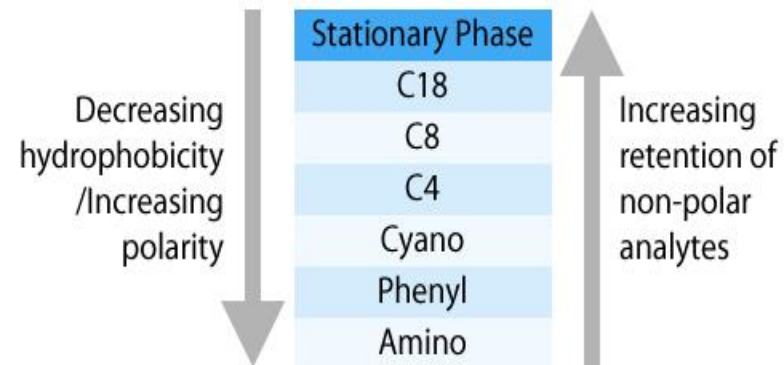


Reversed phase silica



Functionalized silica

FG = -NH₂
-CN



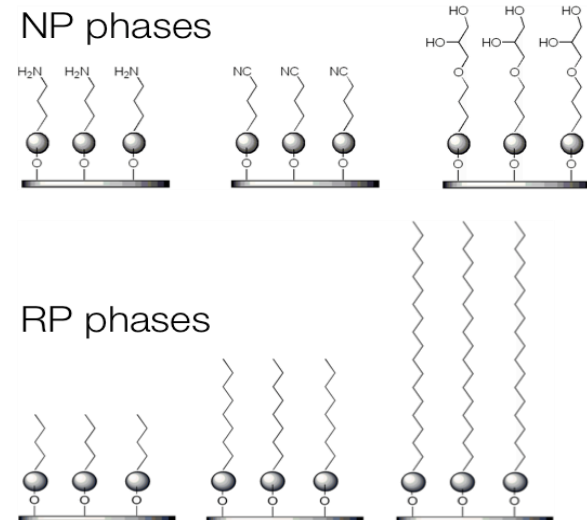
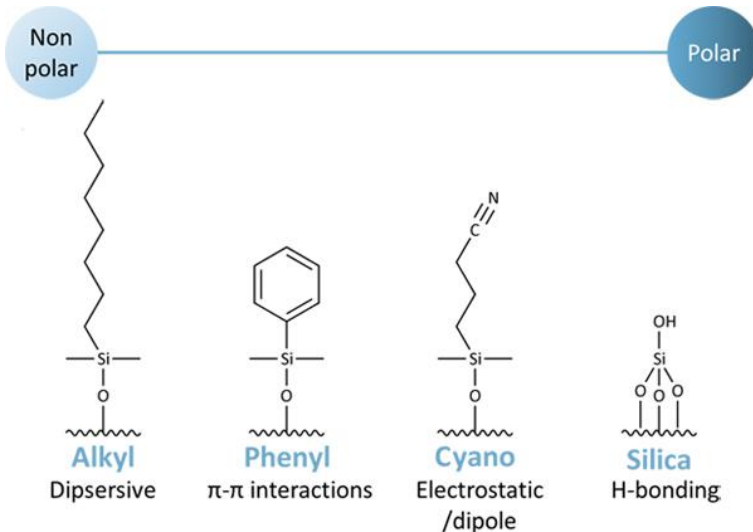
Stationary phases

- NP-HPLC

- Polar groups
- Silica gel (silanol groups)
- Modified silica gel

- RP-HPLC

- Non-polar groups
- Modified silica gel



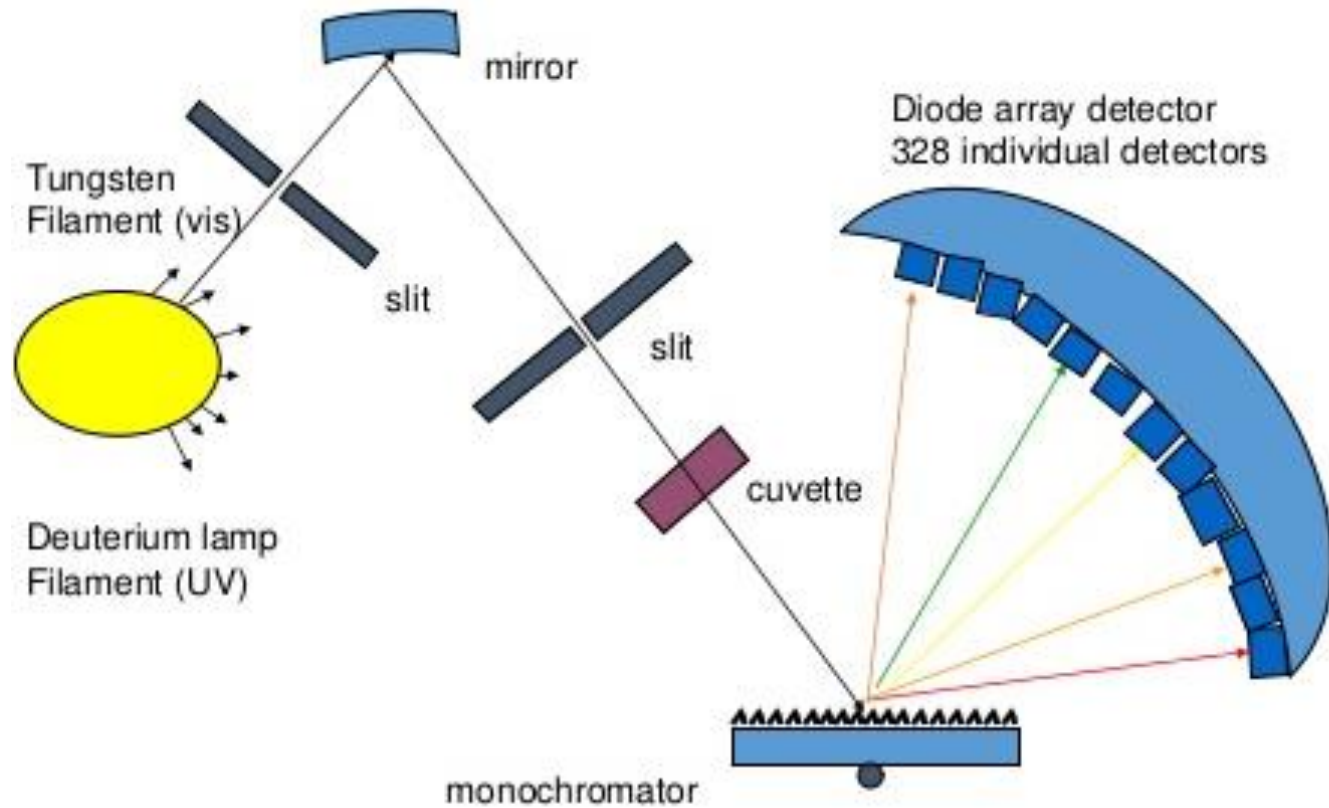
NP-HPLC vs. RP-HPLC

HPLC - Normal Phase HPLC vs Reverse Phase HPLC

Detectors

Name	Advantage	Disadvantage
UV-Vis	Works w/all molecules	Non-specific; complex samples; absorption wavelength
DAD	Works for all wavelengths	High LOD
Fluorescence	Very specific; low LOD	Not everything fluoresces
IR	Works w/all molecules	Many solvents IR active
Refractive Index	Works w/ <i>nearly</i> all molecules	Temperature sensitive; high LOD
Scattering	Uniform response; 5ng/25 μ L LOD	Non-specific; interference from solvent
Electrochemical	Commercially available	Non-specific; high LOD
Mass Spec	Low LOD; analyte identification	Ability to ionize analyte

PDA detectors



hplcsimulatorapp

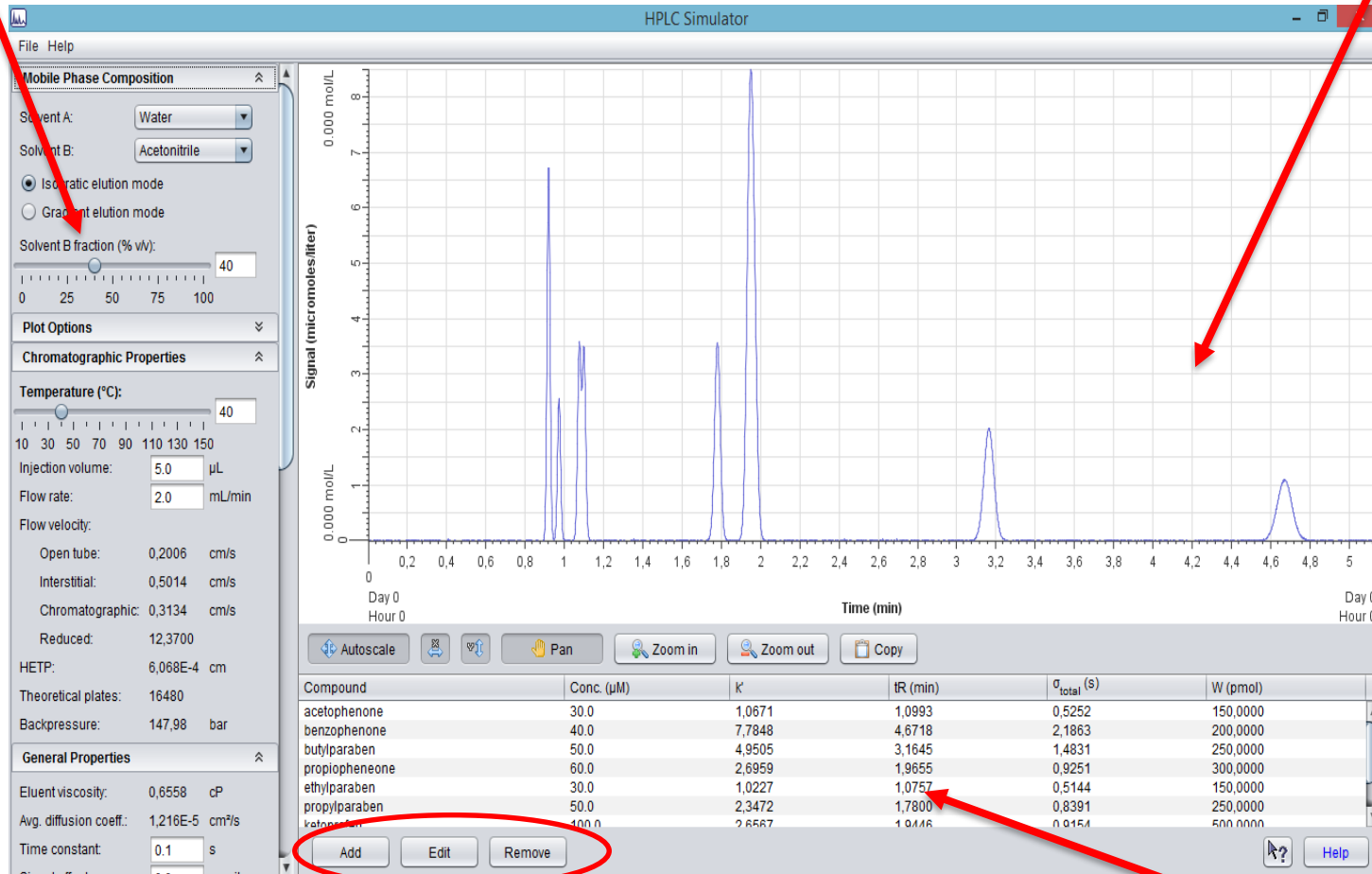
- Free software in English
- Requires Java software
- Components can be chosen free
- From real chromatographic datas
- All of the chromatographic parameters can be set up easily
- The results can be seen on the chromatogram immediately
- Installation set up and manual can be downloaded from e-learning website

www.hplcsimulator.org

hplcsimulatorapp

Parameters

Chromatogram



Separable components

Parameters

- **Parameters of column**
 - length
 - internal diameter
 - particle size
- **Solvent fractions %**
- **flow rate**
- injection volume
- **Isocratic/gradient elution**
- **temperature**
- **theoretical plates**
- HETP
- **Retention time (t_R)**
- **void time**
- **k prime (capacity factor) (k')**
- **backpressure**
- **Resolution (R)**
- **Selectivity (α)**
- **Width (σ) (not baseline)**

File Help

Mobile Phase Composition

Solvent A: Water

Solvent B: Acetonitrile

Isocratic elution mode
 Gradient elution mode

Solvent B fraction (% v/v): 40

Plot Options

Chromatographic Properties

Temperature (°C): 40

Injection volume: 5.0 μL

Flow rate: 2.0 mL/min

Flow velocity:

- Open tube: 0,2006 cm/s
- Interstitial: 0,5014 cm/s
- Chromatographic: 0,3134 cm/s
- Reduced: 12,3700

HETP: 6,068E-4 cm

Theoretical plates: 16480

Backpressure: 147,98 bar

Final time: 08.3405 s

Plot points: 3000

Column Properties

Stationary phase: Waters Acquity BEH C-18

Length: 100.0 mm

Inner diameter: 4.6 mm

Particle size: 3.0 μm

Interparticle porosity: 0.4

Intraparticle porosity: 0.4

Total porosity: 0,6400

Void volume: 1,0636 mL

Void time: 31,9085 s

Reduced Van Deemter terms:

- A: 1.0
- B: 5.0
- C: 0.05

Reduced plate height: 2,0227

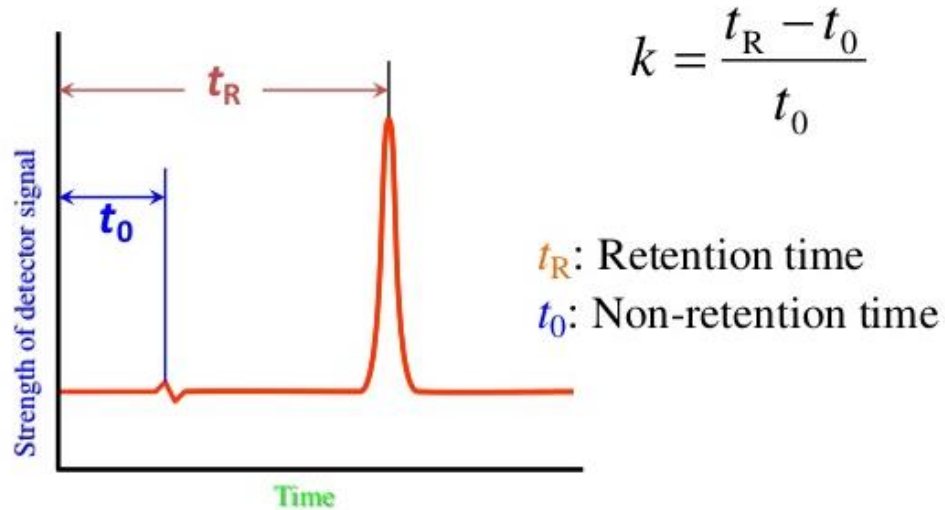
Autoscale Pan Zoom in Zoom out Copy

Compound	Conc. (μM)	k'	tR (min)	σ_{total} (s)	W (pmol)
acetophenone	30.0	1,0671	1,0993	0,5252	150,0000
benzophenone	40.0	7,7848	4,6718	2,1863	200,0000
butylparaben	50.0	4,9505	3,1645	1,4831	250,0000
propionophenone	60.0	2,6959	1,9655	0,9251	300,0000
ethylparaben	30.0	1,0227	1,0757	0,5144	150,0000
propylparaben	50.0	2,3472	1,7800	0,8391	250,0000
butylparaben	50.0	2,3472	1,7800	0,8391	250,0000

Add Edit Remove

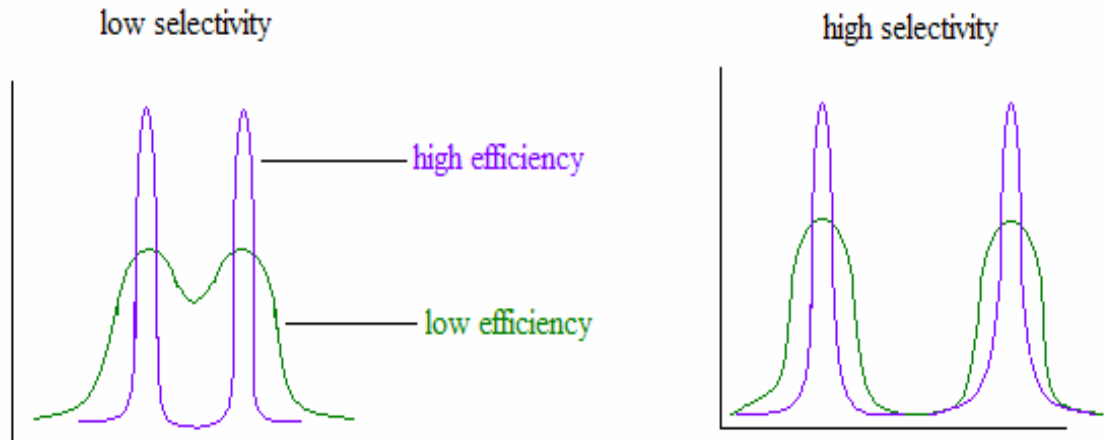
User Manuals Help

Retention Factor, k

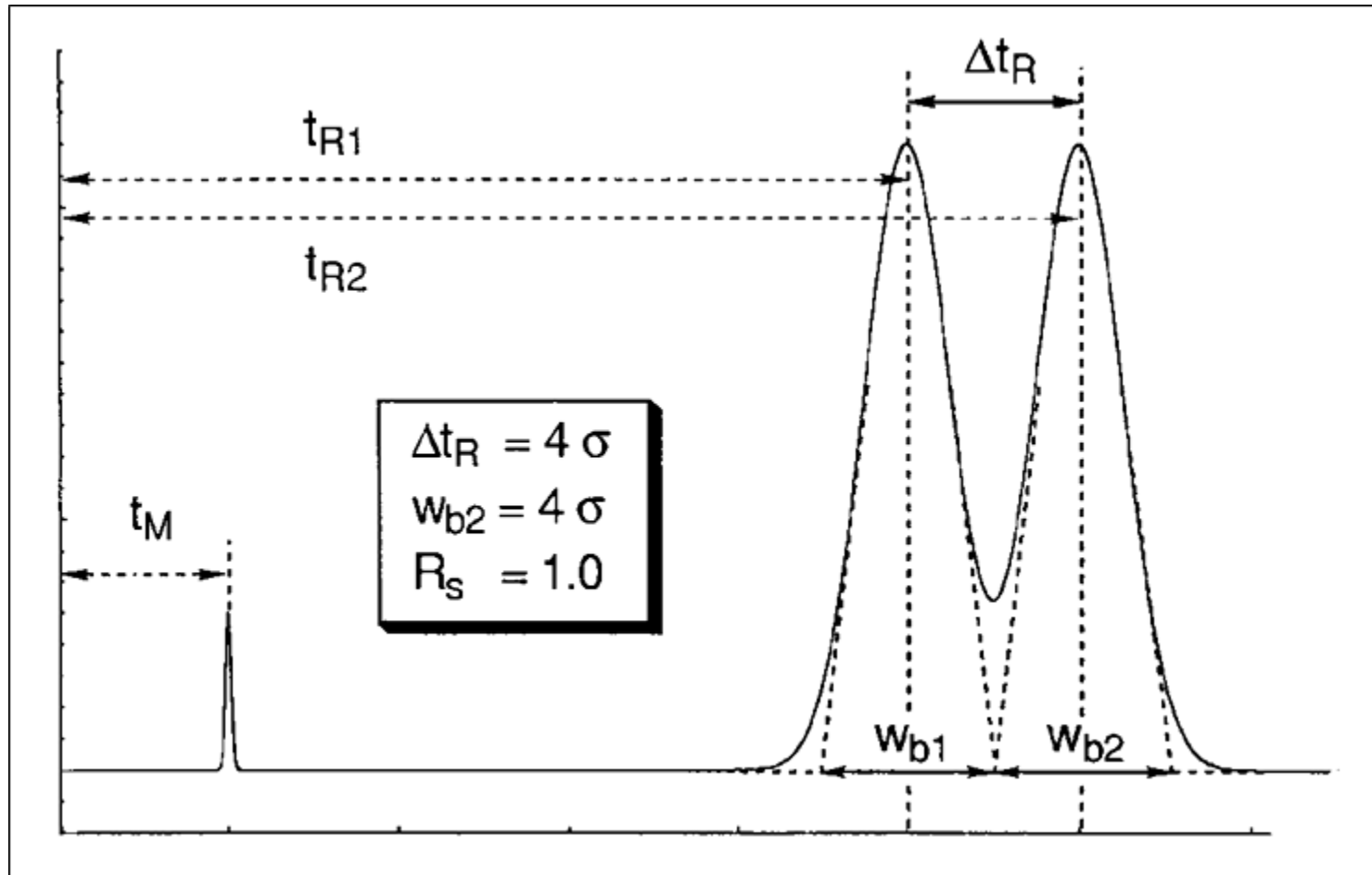


$$R_s = \frac{2(t_{R,2} - t_{R,1})}{(w_{b,1} + w_{b,2})}$$

$$\alpha = \frac{n_s}{n_m} = \frac{k_2}{k_1}$$

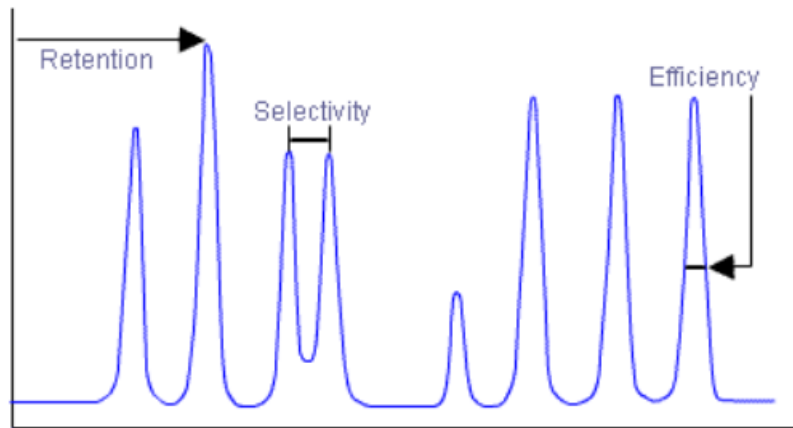


Resolution

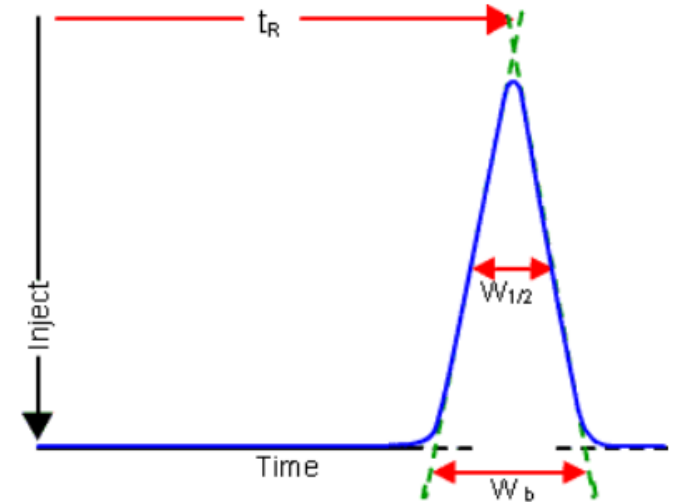


$$R_S = \underbrace{1/4\sqrt{N}}_{\text{Efficiency}} \times \underbrace{\frac{\alpha-1}{\alpha}}_{\text{Selectivity}} \times \underbrace{\frac{k}{1+k}}_{\text{Retention}}$$

The Fundamental Resolution Equation



The Fundamental Resolution Equation and contributing parameters



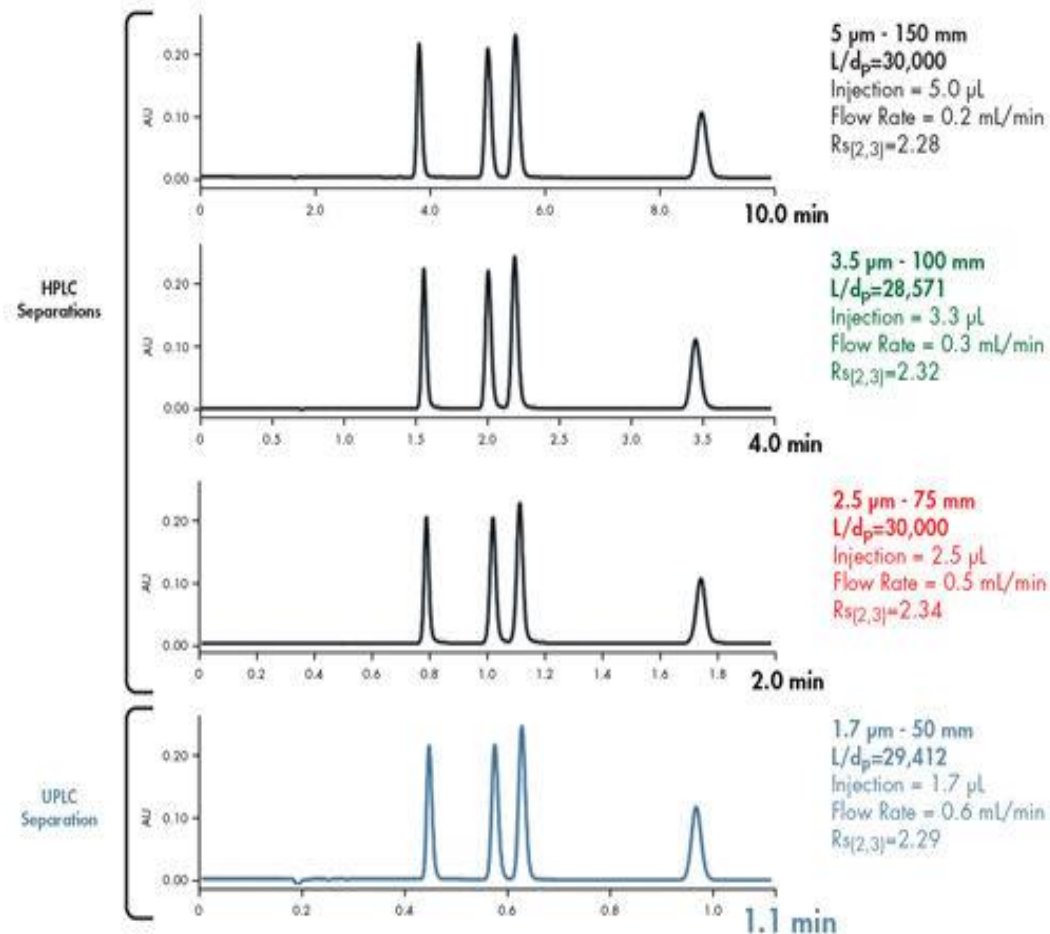
Determination of Efficiency (N)

$$N = 16 \left(\frac{t_R}{w_b} \right)^2 = 5.54 \left(\frac{t_R}{w_{1/2}} \right)^2$$

How can be changed theoretical plate (N)?

- Performance
- Descriptor parameter of width
- HEPT (H)
- How can be changed:
 - Length of column
 - Particle size
 - Flow rate
 - Injection volume
 - Temperature

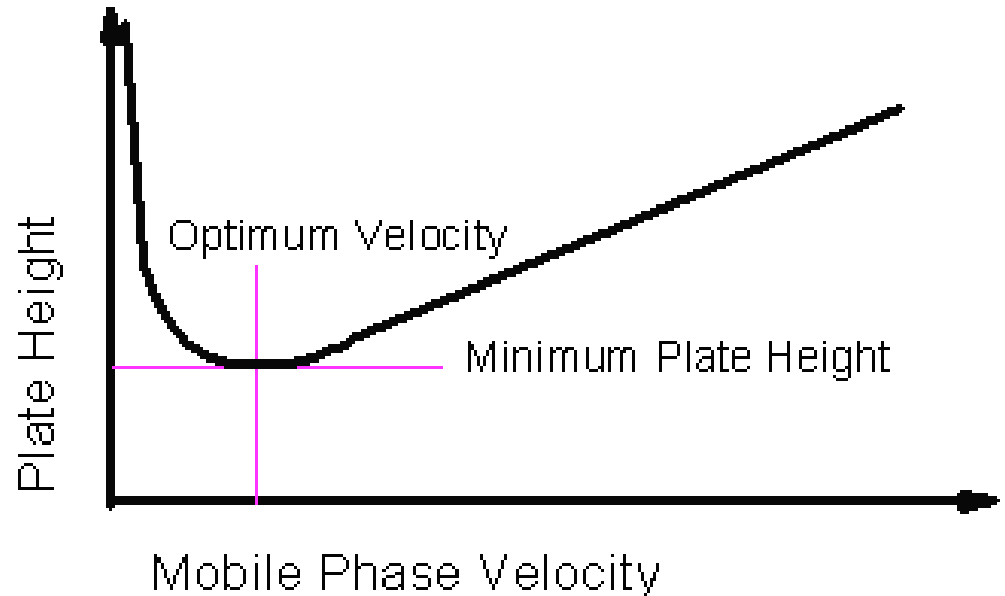
$$H = \frac{L}{N}$$



How can be changed theoretical plate (N)?

- Performance
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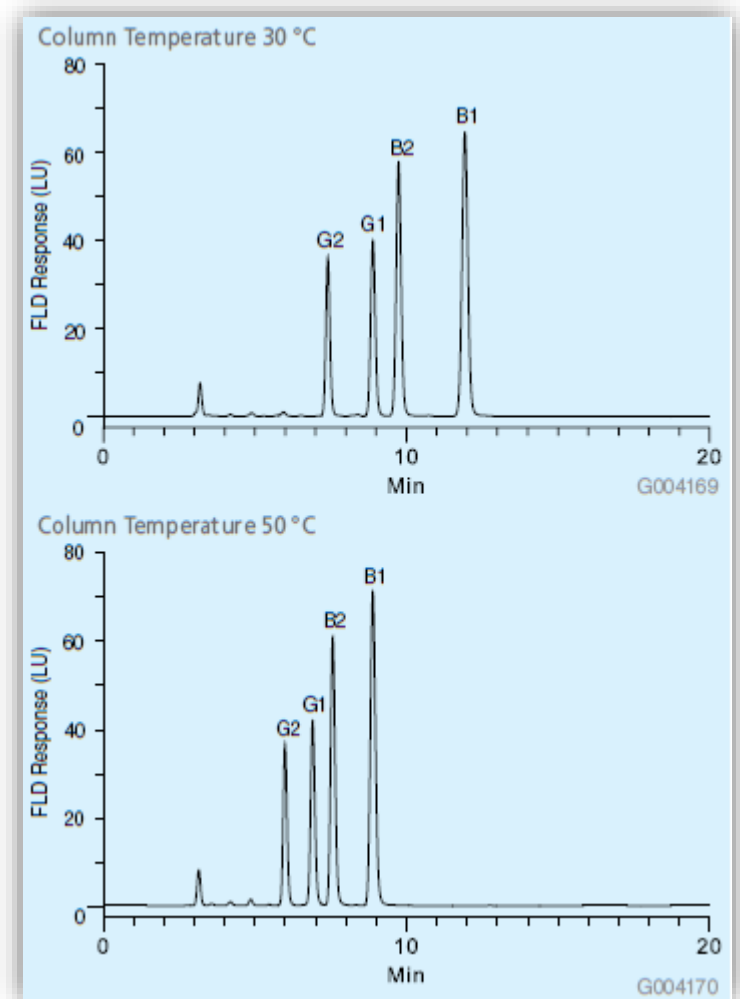
$$H = \frac{L}{N}$$



How can be changed theoretical plate (N)?

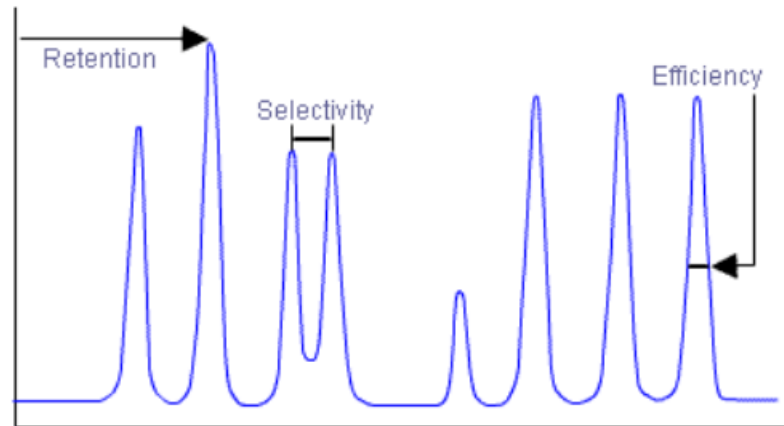
- Performance
- Descriptor parameter of width
- HEPT (H)
- How can be changed:
 - Length of column
 - Particle size
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$$H = \frac{L}{N}$$



$$R_S = \underbrace{1/4\sqrt{N}}_{\text{Efficiency}} \times \underbrace{\frac{\alpha-1}{\alpha}}_{\text{Selectivity}} \times \underbrace{\frac{k}{1+k}}_{\text{Retention}}$$

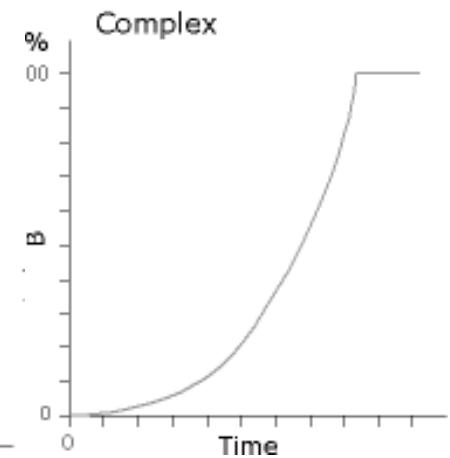
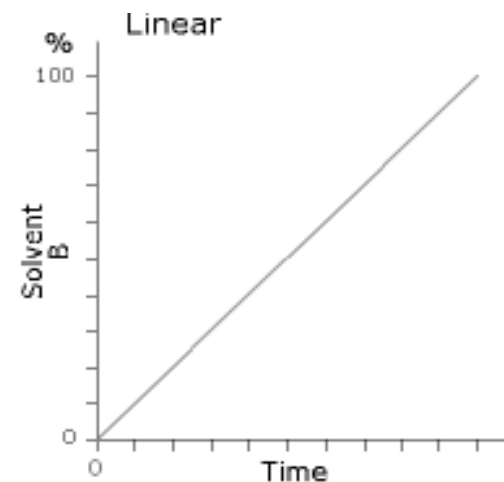
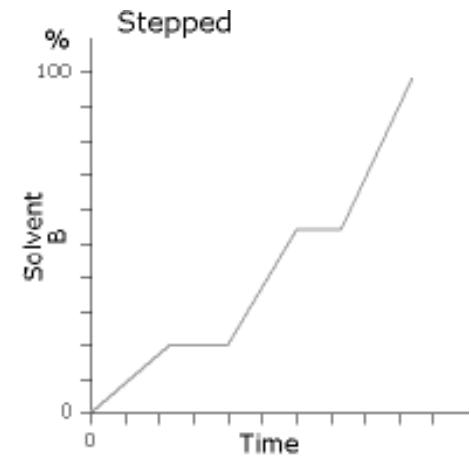
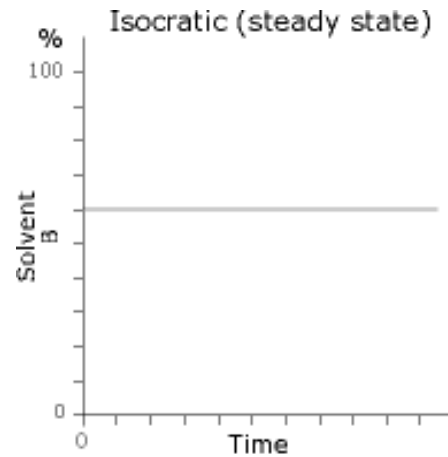
The Fundamental Resolution Equation



The Fundamental Resolution Equation and contributing parameters

Isocratic/gradient elution

- Similar/different components
- Increase of eluent strength under the measurement
- Different gradient profiles
- Equilibration time



Practical exercise

- Put the separable components in order by polarity
- Calculations of parameters
- Examination of effect of main parameters
 - Examination of eluent composition
 - Examination of flow rate
 - Effect of change column parameters
 - Effect of temperature
 - Optimization of gradient elution
- Summary

Lab manual: datas, pictures, explanations and summary!

Limit of parameters

$$1 < k < 10 \quad (2 < k < 5)$$

$$R \geq 1,5$$

($R < 1,5$ bad separation)

$$\alpha > 1,05$$

($\alpha = 1$ no separation)

$$N > 1000$$