LIQUID CHROMATOGRAPHY

RECENT TECHNIQUES

HPLC – High Performance Liquid Chromatography

RRLC – Rapid Resolution Liquid Chromatography

UPLC – Ultra Performance Liquid Chromatography

UHPLC – Ultra High Pressure Liquid Chromatography

Principles of liquid chromatography

Principle generally

Separation based on different retention in chromatographic system - sorbent (stationary phase) and eluent (mobile phase)

Classification of methods

- configuration: column, thin layer ...

- separation parameters: efficiency, speed ...

- stationary phase: elution, ionex, chiral ...

Separation parameters

Resolution:

$$R = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{1 + k_2^{'}}{k_2^{'}}\right)$$

OR

$$R = \frac{2(t_{R1} - t_{R2})}{w_1 + w_2}$$

k′– capacity (retention) factor:

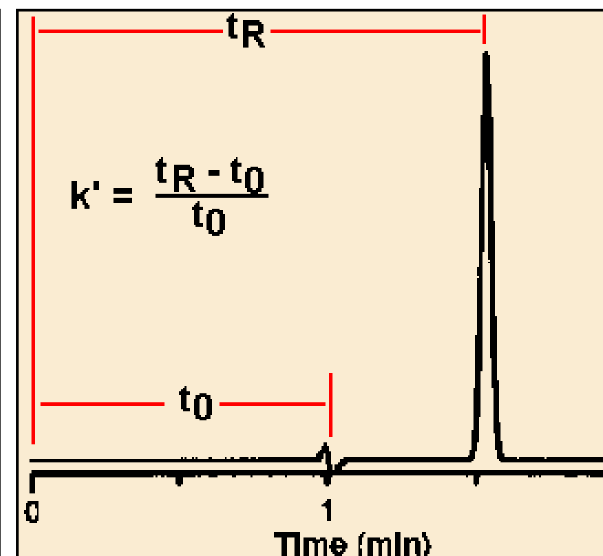
$$k^{'} = \frac{n_s}{n_m} = K_D \cdot \frac{V_s}{V_m} = \frac{t_R - t_0}{t_0}$$

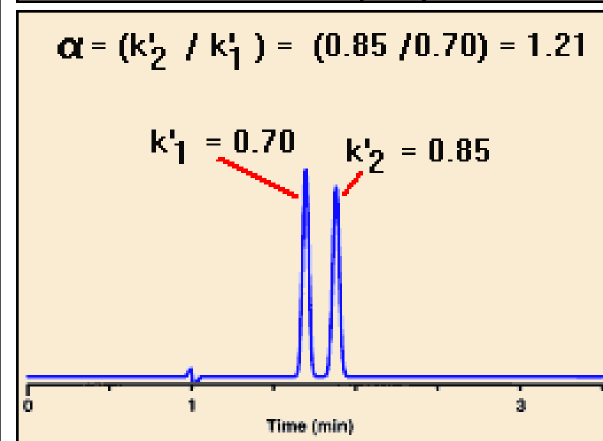
α – separation factor: k'_2/k'_1

N – number of theoretical plate (efficiency)

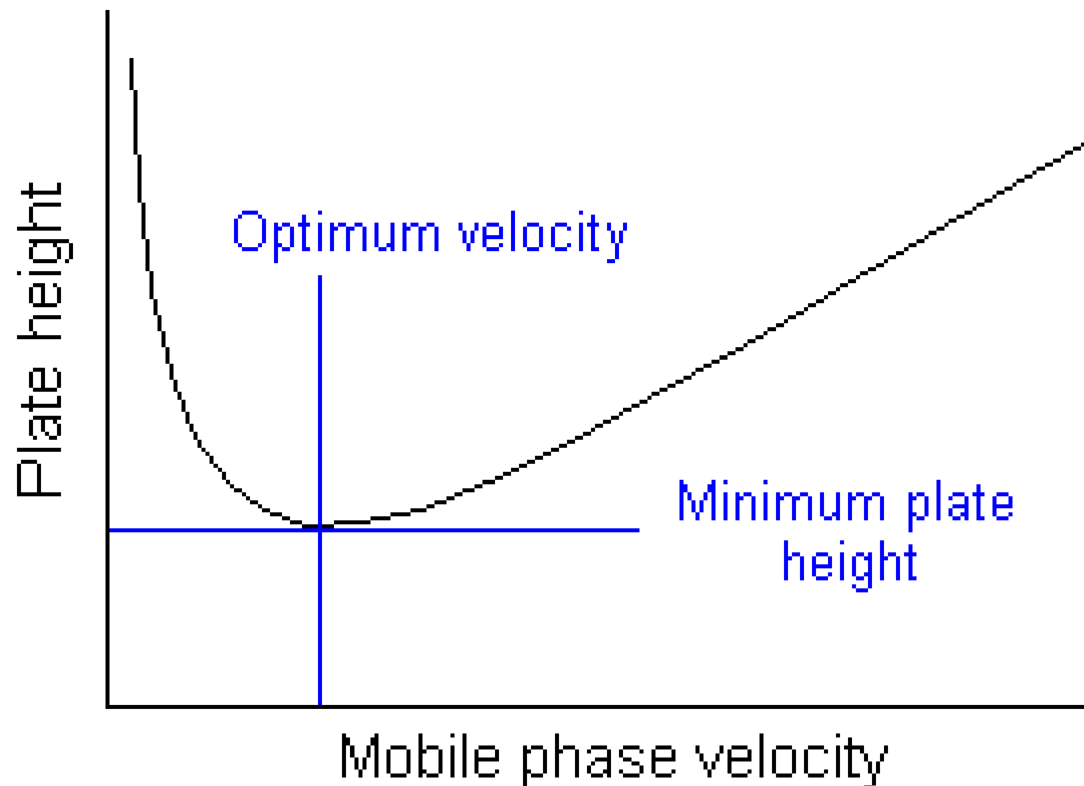
$$N = \frac{5{,}55 \cdot t_R^2}{w_{1/2}^2}$$

Dead Point
Theophyline
Chlomethiazol
Propanolol
Phenytoin
Carbamazapine
Retention Volume $(V)_r$
Corrected Retention Volume $(V')_r$
Peak Width at 0.607 (h) (2σ)
Peak Height
Dead Volume V_0
Peak Width at 0.5 (h)
Corrected Retention Time $(t')_r$
Dead Time t_0
Retention Time $(t)_r$
Peak Width at Base
Elution Point of Unretained Solute





R = 1,5 ⇒ baseline separation



HETP = L/N – height equivalent of a theoretical plate

Y - HETP

X - average linear velocity

Interpretation of separation parameters

k' ~ composition of stationary and mobile phase, temperature, flow rate, column length

- characterises retention under given conditions

- retention factor (time of analysis)

α ~ composition of stationary and mobile phase, temperature, flow rate, column length

- characterises separation (comparison of k‵)

- selectivity factor (analytes separation)

N ~ stationary phase quality - particles size and sorting, mobile phase composition, flow rate, column length, temperature

- characterises quality of elution zone - wide, shape ...

- peak shape factor

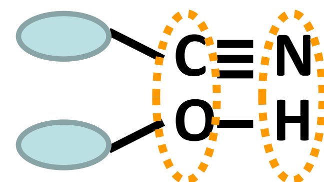
R ~ combined parameter

Stationary phase – type selection (selectivity) and particles size/sorting/porosity

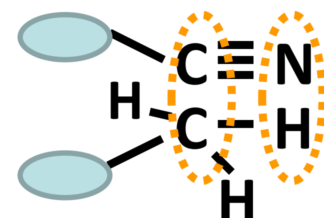
Elution: - normal phase (classic x HILIC) - reverse phase (classic x semipolar) - ion pair (reverse phase)	**HPLC: 3-10 μm - various** **- p up to 400 bar** **RRLC: 1.8 μm - porous mixed** **- p up to 600 bar**
Ion exchange (IE) - catex, anex	**UPLC: 1.7 μm - porous unified** **- p up to 1000 bar**
Chiral - cyclodextrin phases	**UHPLC: 1.0 μm - non-porous** **- p up to 5000 bar**
Affinity	

Interaction occurring during separation

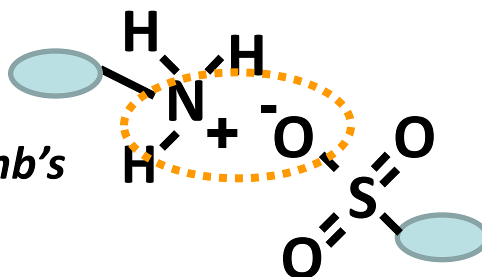
* van der Waalsovy dispersion force – *London's*
  induced dipole – induced dipole



* van der Waalsovy orientation forces – *Keesom's*
  dipole - dipole



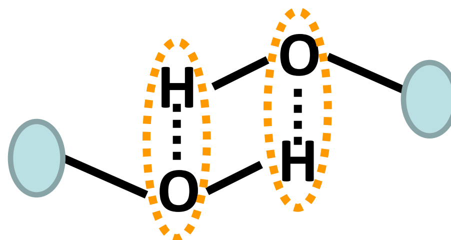
* van der Waalsovy induction forces – *Debye's*
  dipole – induced dipole

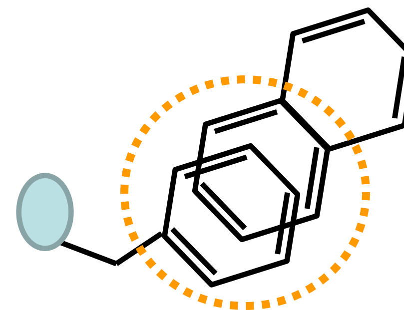


** electrostatic forces – *Coulomb's*

**** π - π interaction

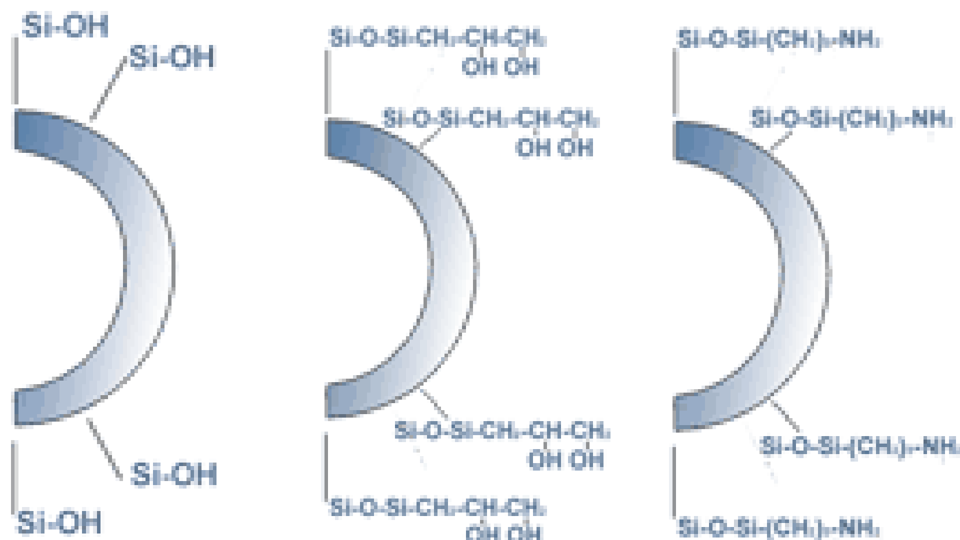
*** hydrogen bond

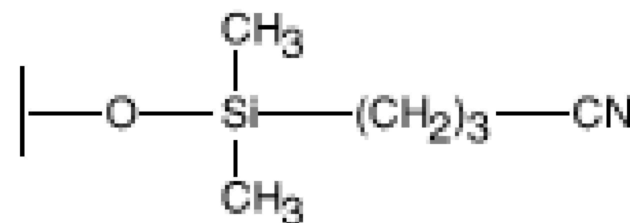


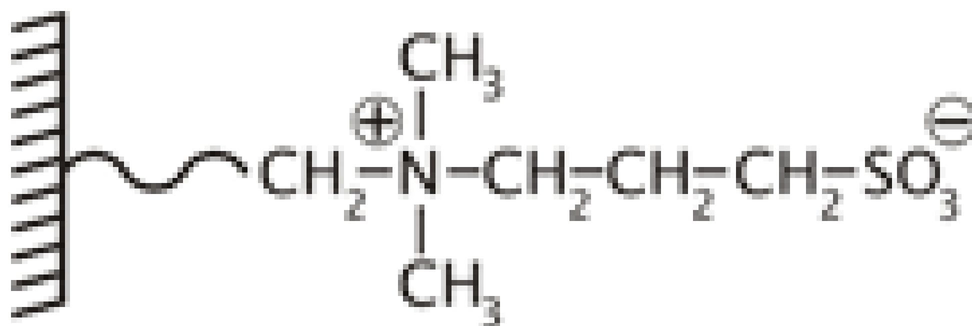


Normal phase chromatography:

Stationary phase - polar: silica gel, modified silica gel
Mobile phase - nonpolar (retention according to m.ph. comp.)





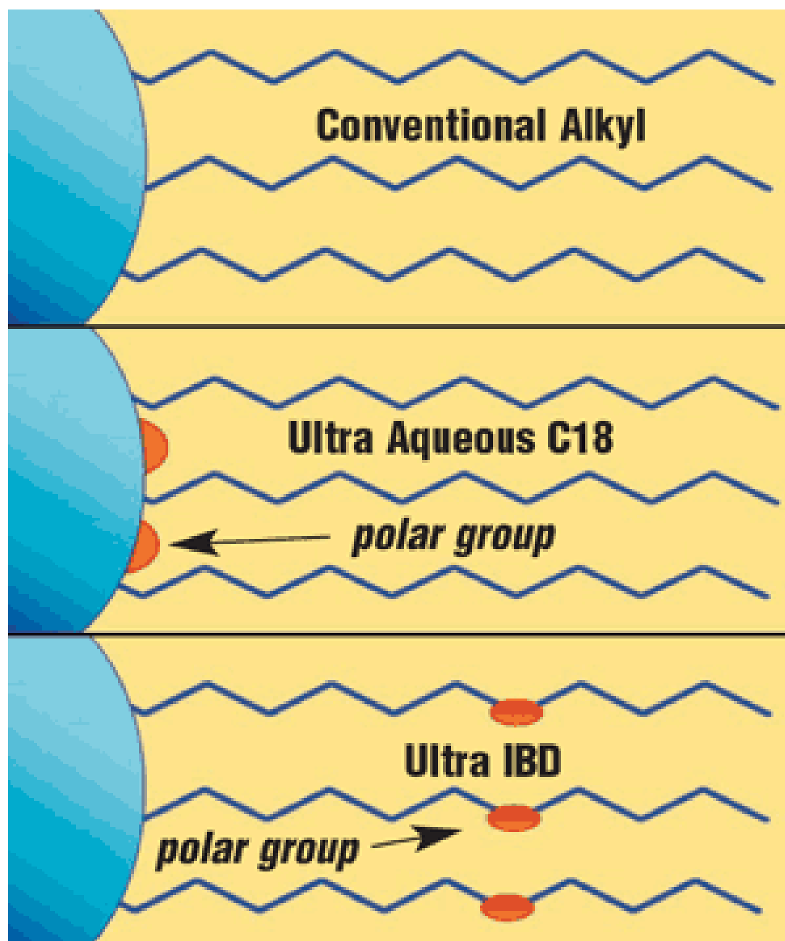


HILIC

Hydrophilic
Interaction
LIquid
Chromatography

Reverse phase chromatography:

Stationary phase - nonpolar: modified silica gel (C18)
Mobile phase - polar (retention according to m.ph. comp.)



ODS - octadecylated silica gel (C18)

Other modification: C4, C8, C30

Endcapping (Embedding):

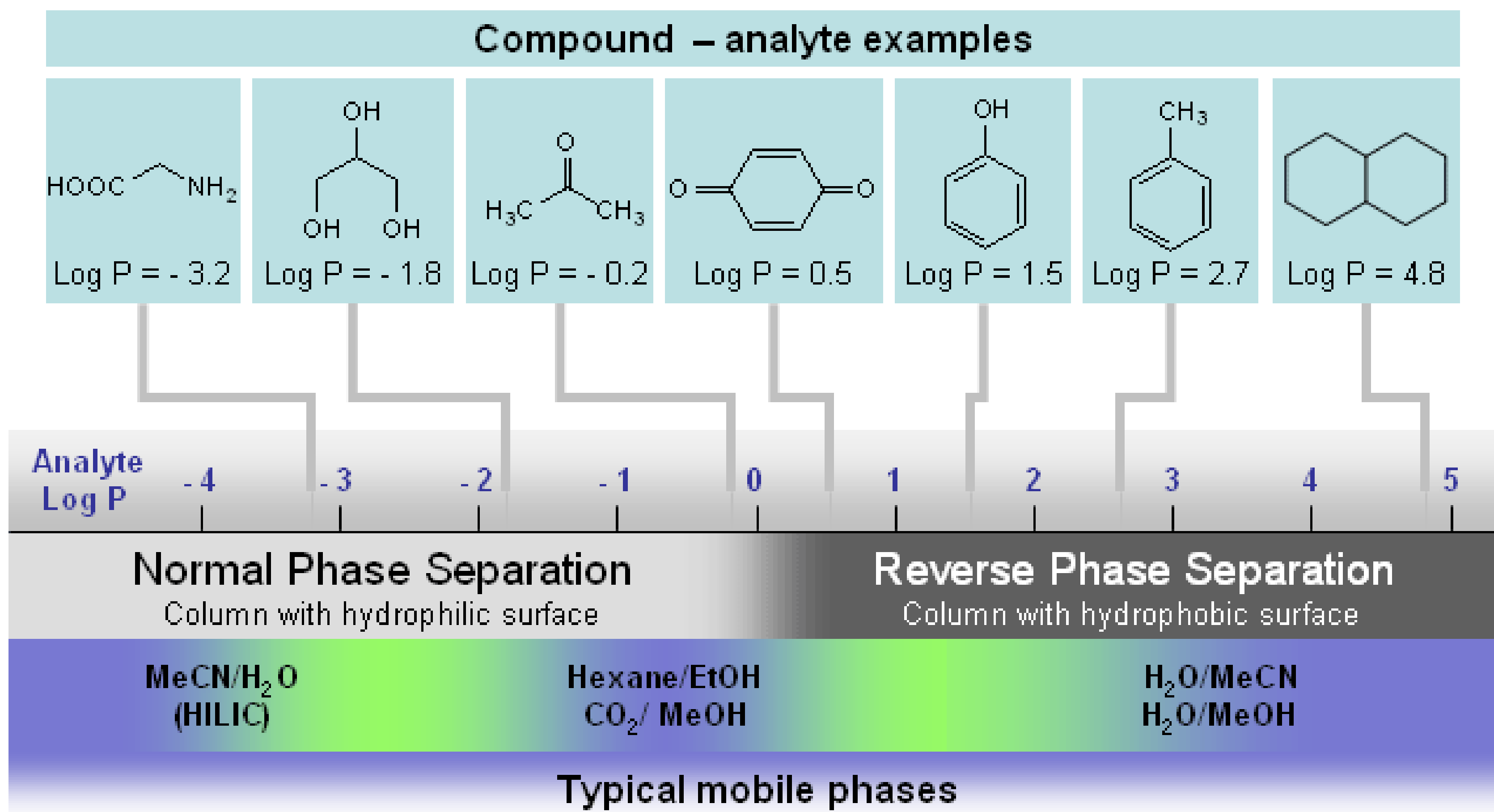
Classic - 100% nonpolar

Polar – partially polar groups

Porous x nonporous

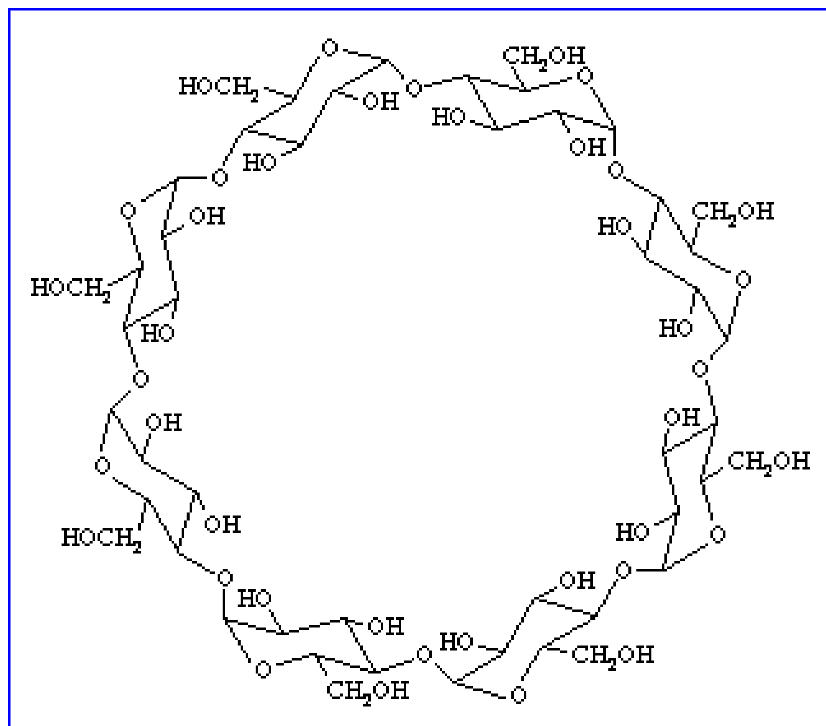
Monomeric X polymeric (cross-linked)

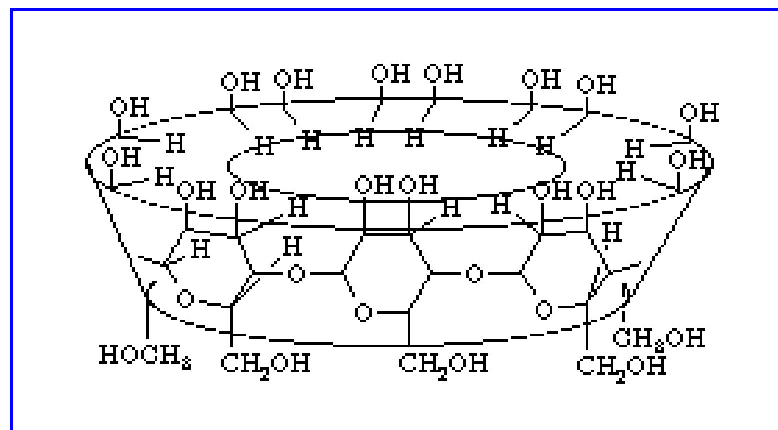
Comparison of HILIC, NP a RP chromatography

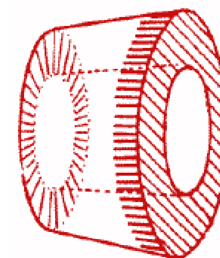


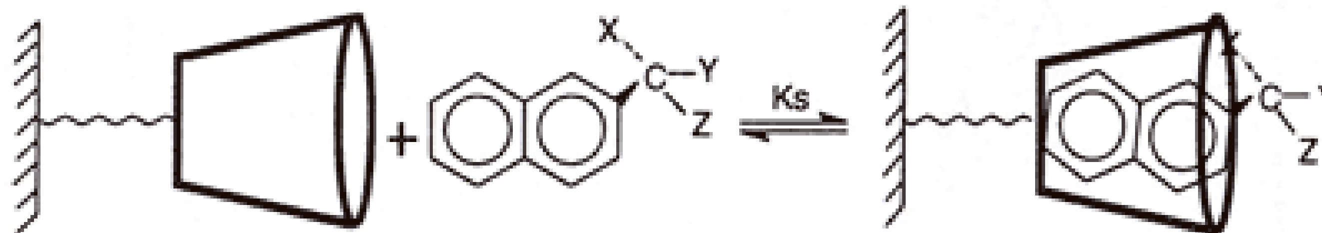
Chiral chromatography:

Stereoisomers (enantiomers, diastereoisomers ...)
- separation interaction with chiral phase



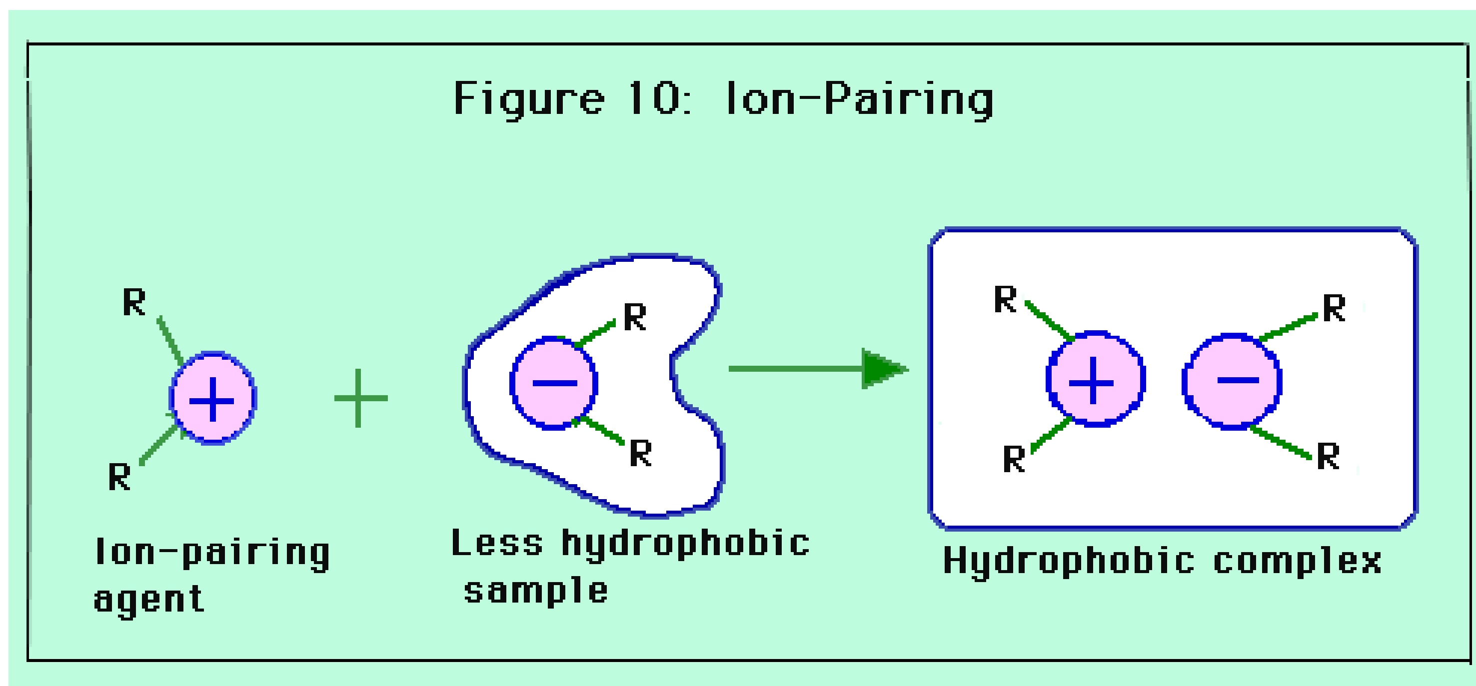






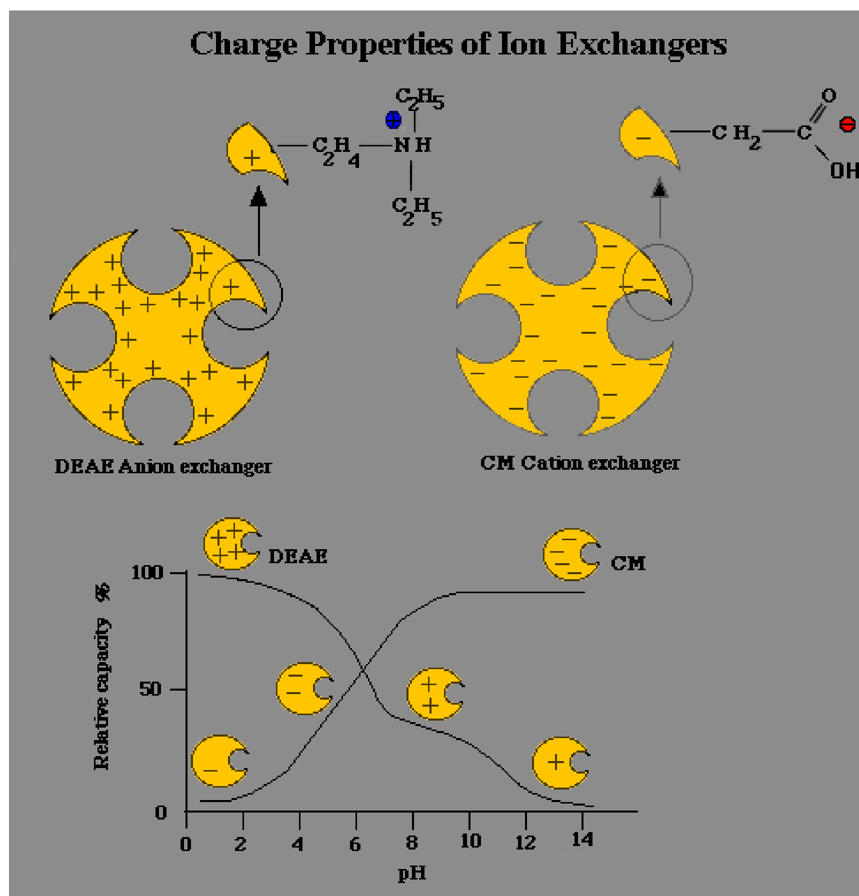
Ion pair chromatography:

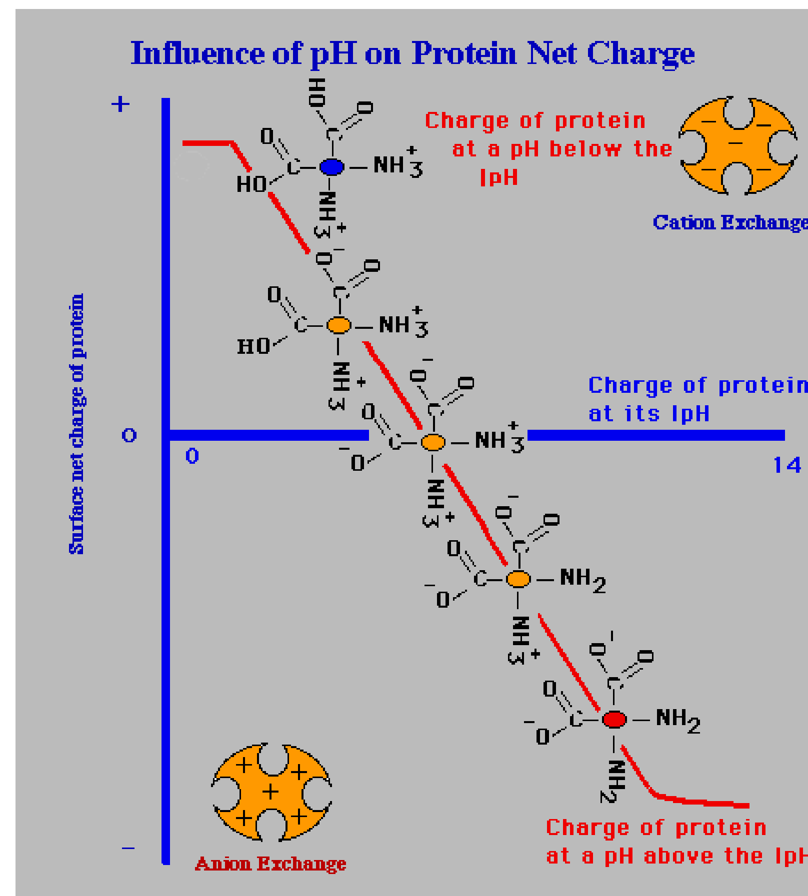
Polar analyte + polar agent (ion pair agent)
⇒ nonpolar complex with retention on nonpolar st.ph.
- realisation on reverse phase



Ion exchange chromatography:

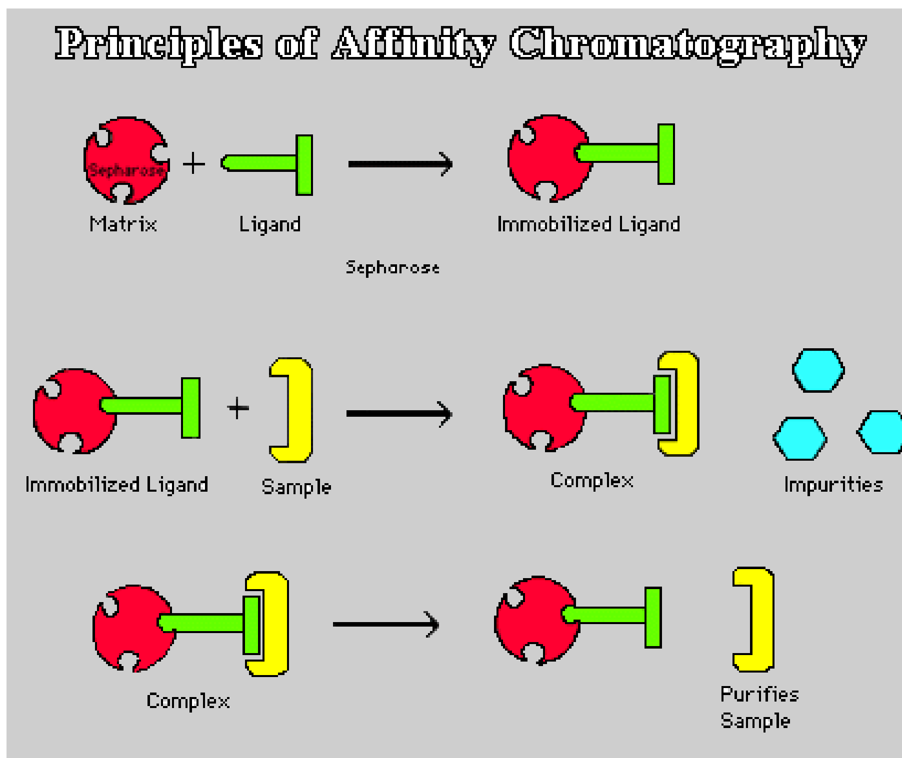
Stationary phase - embedding ⊕ or ⊖ ; counter ions are mobile (retention according to m.ph. composition)

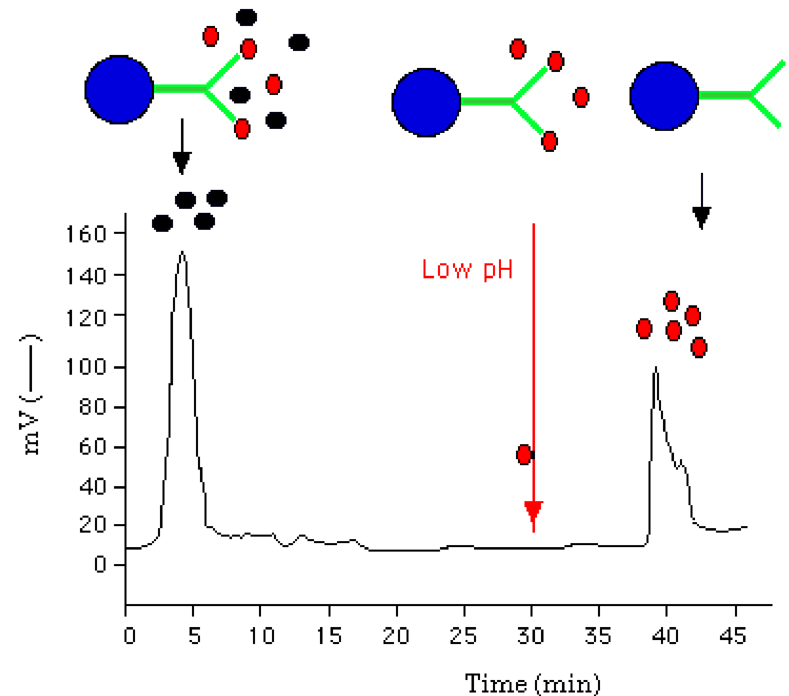




Affinity chromatography:

Stationary phase – immobilisation of ligand (biologically active compounds); sample is bonded to complex and released by the change of mobile phase)





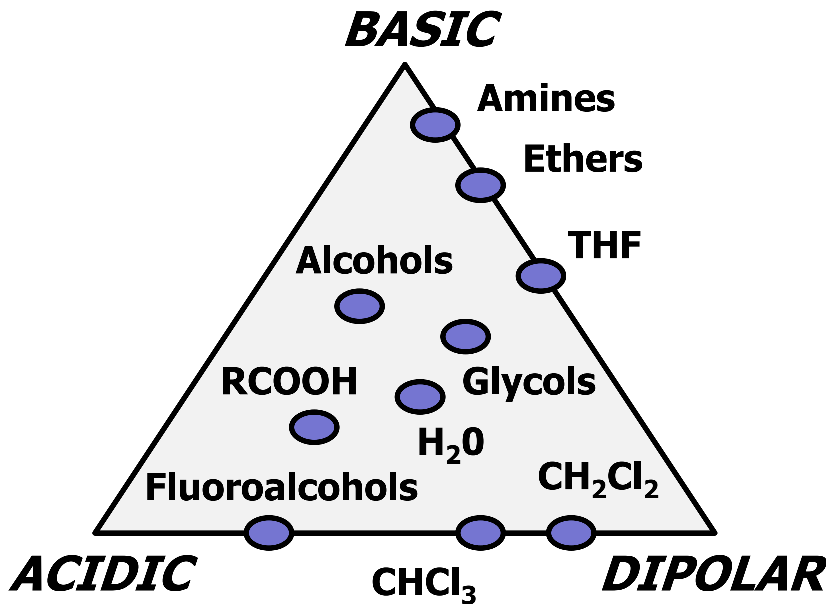
Mobile phase - polarity, ion strength, elution technique

SELECTION:

Sample solubility

Compatibility with stationary phase and system

Elution: isocratic …X… gradient



Solvent Miscibility Table

Dielectric Constant	UV (nm) Cutoff	Refractive Index	Viscosity (℃)	Boiling Point(℃)	Solvent	iso-Octane	n-Hexane	n-Heptane	di-Ethyl ether	Cyclohexane	Ethyl acetate	Toluene	Chloroform	Tetrahydrofuran	Benzene	Acetone	Dichloromethane	Dioxane	n-Propanol	Ethanol	Dimethylformamide	Acetonitrile	Acetic Acid	Dimethyl Sulfoxide	Methanol	Water
1.94	210	1.3914	0.50	98	iso-Octane																					
1.88	195	1.3749	0.31	69	n-Hexane																					
1.92	200	1.3876	0.41	98	n-Heptane																					
4.33	218	1.3524	0.24	34	di-Ethyl ether																					
2.02	200	1.4262	1.00	80	Cyclohexane																					
6.02	256	1.3724	0.45	76	Ethyl acetate																					
2.38	284	1.4969	0.59	110	Toluene																					
4.81	245	1.4458	0.57	60	Chloroform																					
7.58	212	1.4072	0.55	65	Tetrahydrofuran																					
2.27	278	1.5011	0.65	80	Benzene																					
20.70	330	1.3587	0.36	56	Acetone																					
8.93	233	1.4241	0.44	40	Dichloromethane																					
2.25	215	1.4224	1.37	101	Dioxane																					
20.33	210	1.3856	2.30	98	n-Propanol																					
25.80	210	1.3610	1.20	78	Ethanol																					
36.70	268	1.4305	0.92	153	Dimethylformamide	X	X	X																		
37.50	190	1.3411	0.38	82	Acetonitrile	X	X	X		X																
6.30	230	1.3720	1.26	118	Acetic Acid																					
4.70	260	1.4830	2.24	189	Dimethyl Sulfoxide	X	X	X	X																	
32.70	205	1.3284	0.55	65	Methanol	X	X	X		X																
81.10	210	1.3330	1.00	100	Water	X	X	X	X	X	X	X	X		X		X									

Mobile phase – selection according to elution strength

Polarity (Snyder's polarity index) …….. P′

Eluotropic value – elution strength (stationary phase type, primary for NP) - ε^0

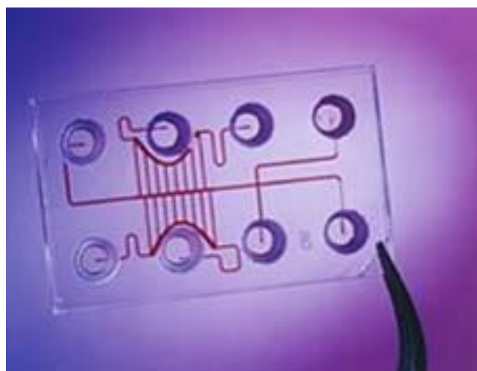
Solubility parameters … δ

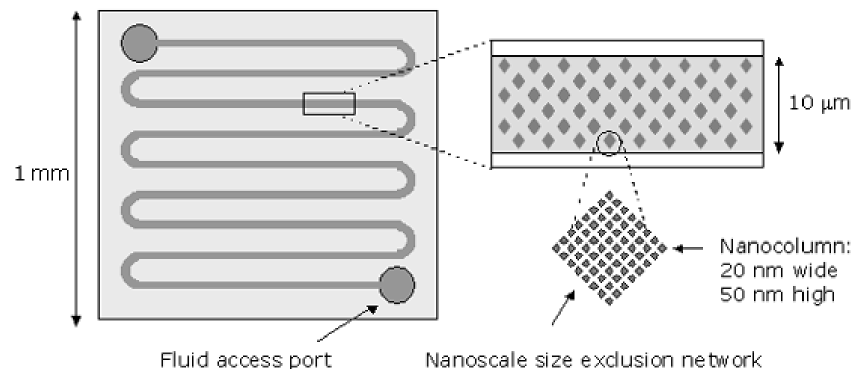
Solvent	P′	ε^0 (silica gel)	ε^0 (C18)	δ
Hexane	0.1	0	-	7.3
Acetone	5.1	0.53	8.8	9.6
Acetonitrile	5.8	0.52	3.1	12.7
Methanol	5.1	0.7	1.0	14.5
H_2O	10.2	-	-	23.5

Typical column and sorbents - technical parameters

Column dimensions	*Particles*	*Flow rate*	*Chromatography*
10-25 cm x 3-4.6 mm	3-10 µm	0.1-2 mL/min	Conventional (classic)
0.5-7.5 cm x 3-4.6 mm	3-10 µm	0.1-2 mL/min	fast
5-25 cm x ± 2 mm	3-10 µm	0.05-1 mL/min	narrow x micro-bore 2
15-100 cm x ± 1 mm	3-10 µm	30-60 µL/min	narrow x micro-bore 1
10-200 cm x 0.2-0.5 mm	1-5 µm	1-10 µL/min	capillary (packed)
100-200 cm x 0.04-0.08	--------	< 1 µL/min	capillary (open tubular)
0.5-2 cm x 2 mm	1.8 µm	0.5-2.5 mL/min	RRLC
5-15 cm x 2 mm	1.7 µm	0.1-1.0 mL/min	UPLC
5-15 cm x < 1-2 mm	1.0 µm	< 0.1-0.5 mL/min	UHPLC
Grooves on a chip	2.5-10 µm	< 1 µL/min	nano-LC (Lab on Chip)

Lab on Chip





Sorbent sorting - back pressure effect

- higher permeability ⇔ porosity
- can be higher than sorbent particles

- lower permeability ⇔ porosity
- must be less than sorbent particles
  ⇒ corresponding back pressure

Goal: Achieve lower backpressure vs. a typical PSD

Small amount of slightly larger particles

Engineered PSD
Narrower PSD

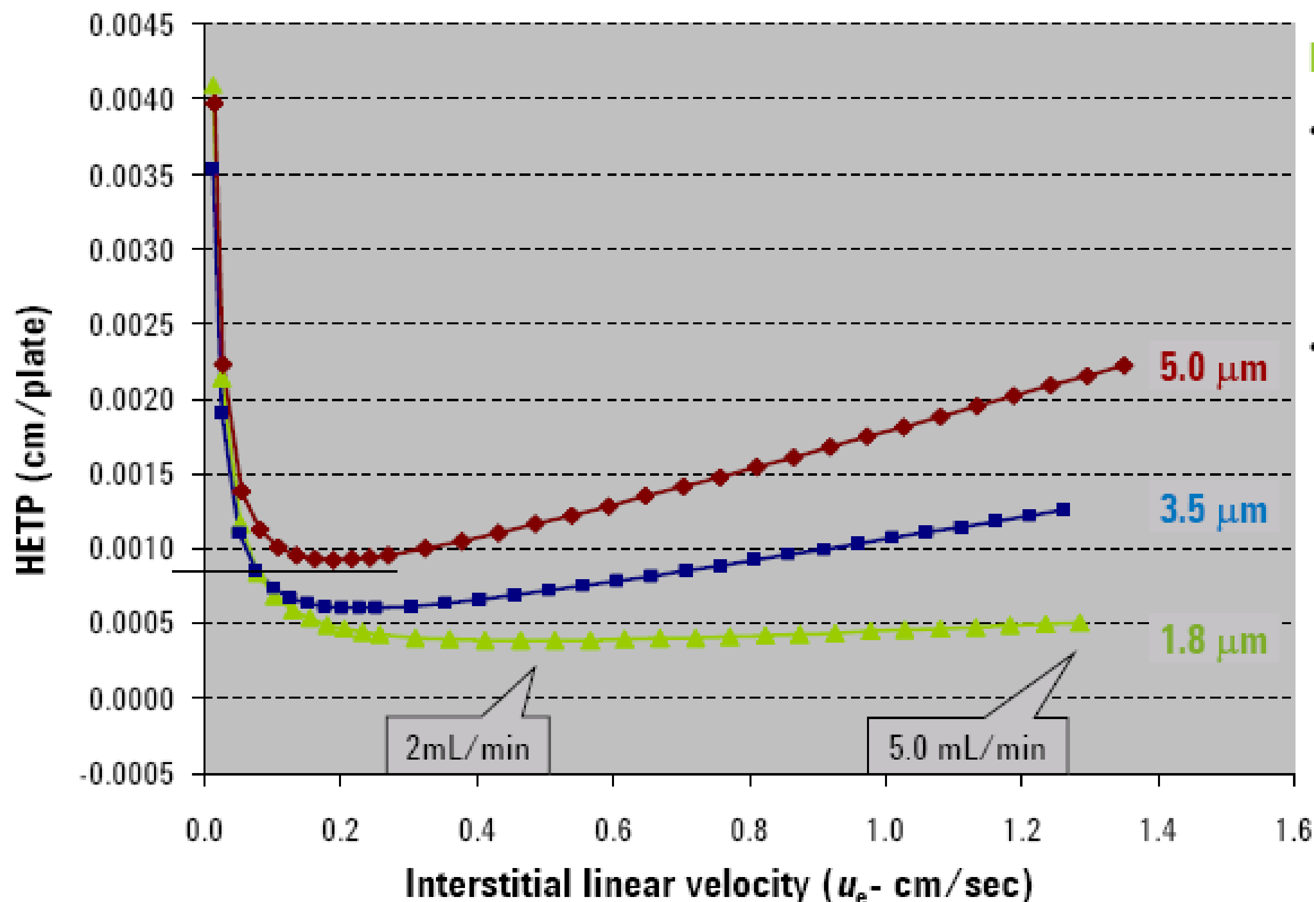
Engineered Bimodal Distribution:
Only 3% compromise in resolution
25% gain in back pressure reduction

Lower level of fines

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6 3.8 4.0

Particle Size (um)

Small particles – sorbents application

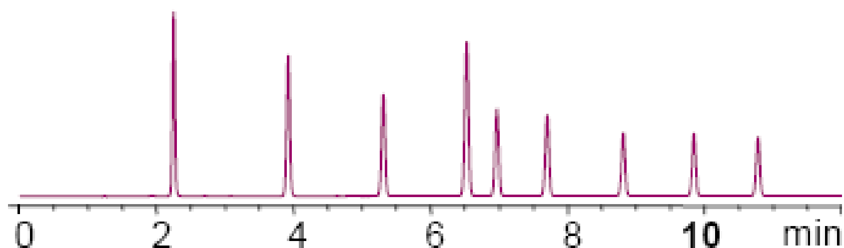


Increased Speed

- Run short columns with small particle size at high linear velocities
- Speed gains: 5-10x

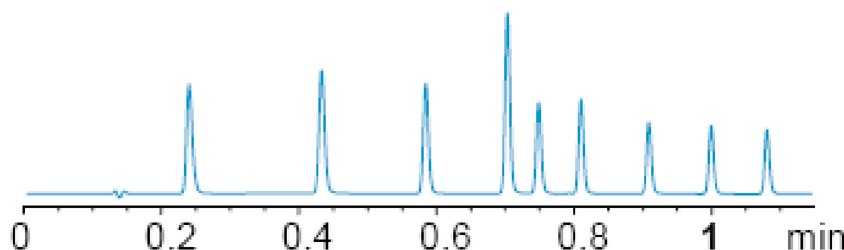
Application of rapid resolution (RRLC)

4.6 x 150mm, 5µm
1.20ml/min, 40°C
Analysis Time = 11min



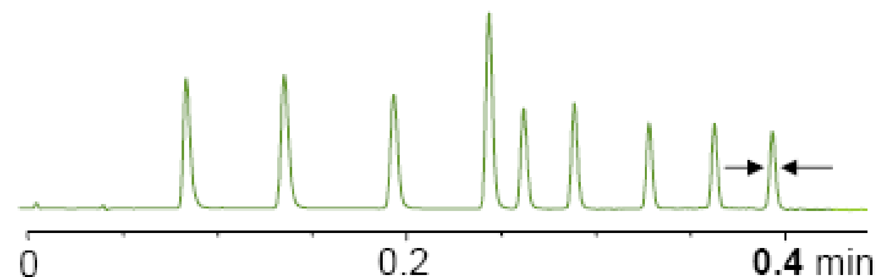
HPLC, 40°C
PW = 3.4sec

2.1mm x 50mm 1.8µm
1.00ml/min, 40°C
Analysis Time= 1.1min



RRLC, 40°C
10x faster
PW = 0.5 sec

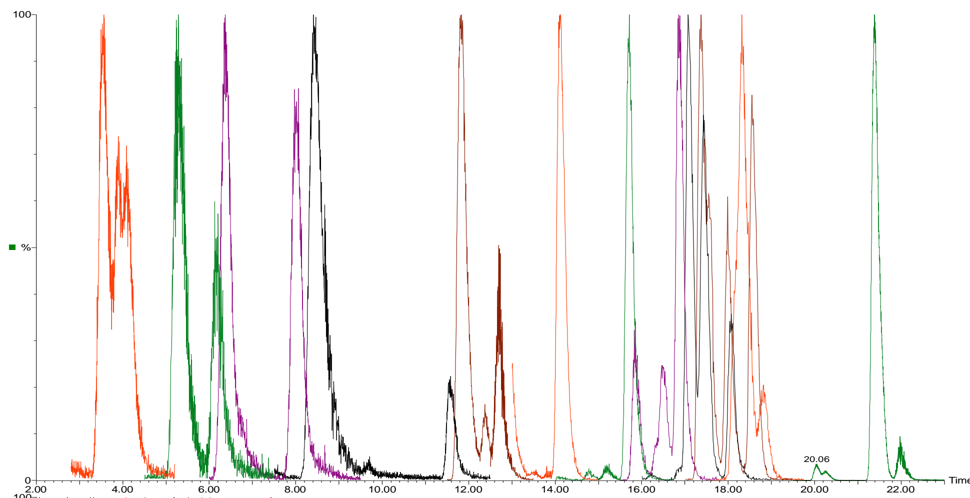
2.1mm x 50mm 1.8µm
2.40ml/min, 95°C
Analysis Time: 0.4min



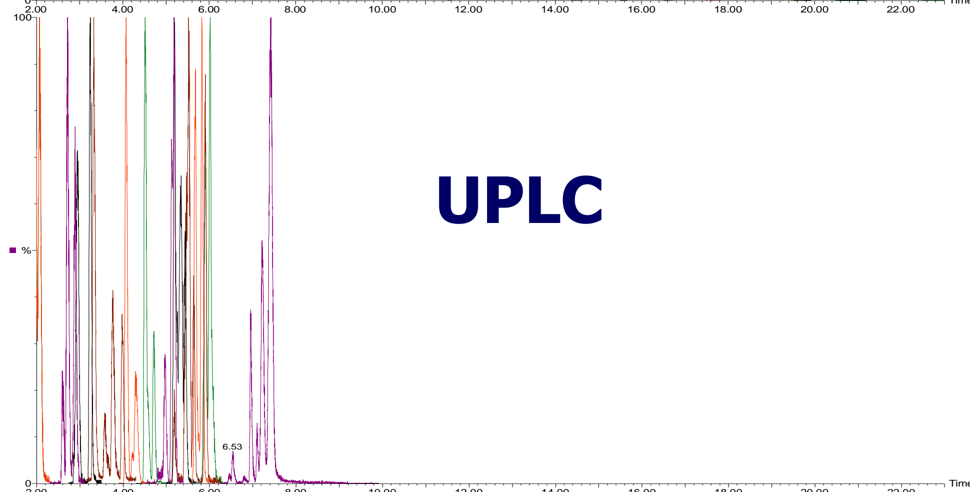
RRLC, 95°C
27x faster
PW = 197msec

150mm > 50mm: 3x
1.2ml/min on 4.6 > 2.4ml/min on 2.1: 10x
3 x 10 = 30x

Application of UPLC - multiresidue pesticide analysis



HPLC



UPLC

Flow rate: 0.3 mL/min (in both systems)

UPLC 10 min

HPLC 50 min