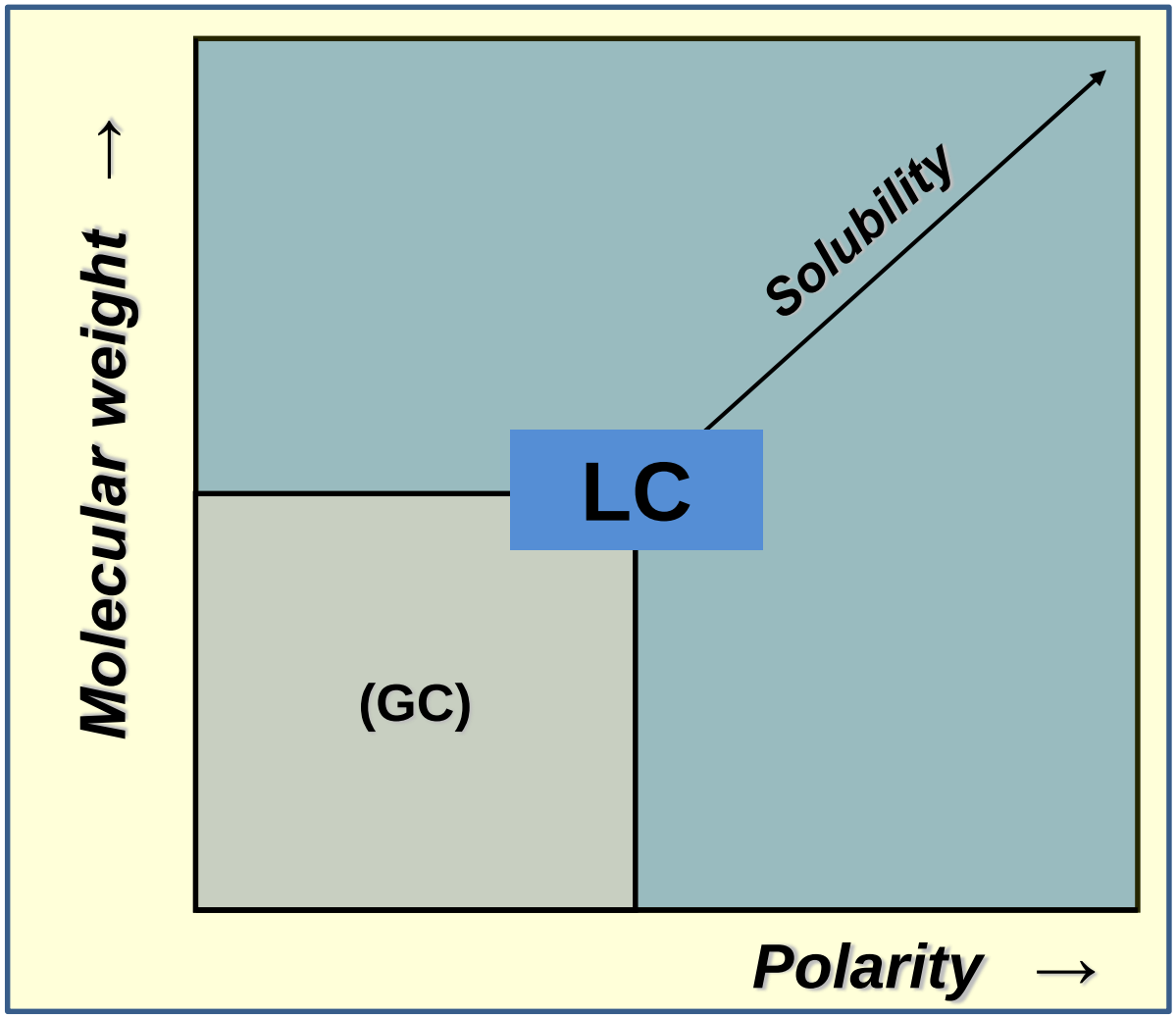
LIQUID CHROMATOGRAPHY (LC)

Partition between stationary and mobile phase
- based on differences in solubility and structure
(separated compounds show the different chromatographic affinity)

Suitable for:

- almost all compounds - nonreactive with others
- wide range of molecular weights and polarities
- samples with limited clean-up (higher ruggedness)
- non-targeted analysis

Estimation of compound suitability for LC



TECHNICAL REALIZATION OF LC

Source of solvents

Bottles with frits (2 μm)

Degasser

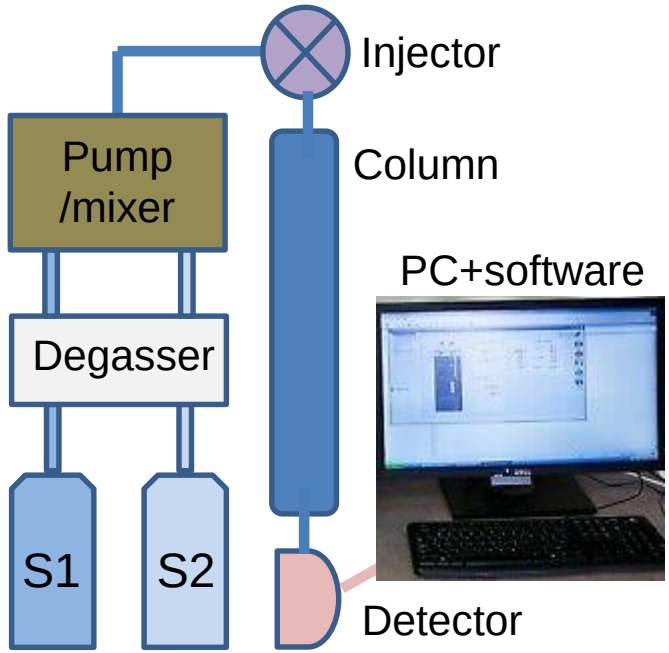
Inert gas (He), vacuum

Injector

6-valve, automatic

Column

Packed (capillary)



Detection – detector

Detector response/spectra - peak area or height/identification

Data processing - software

Various outputs

LC SEPARATION THEORY I

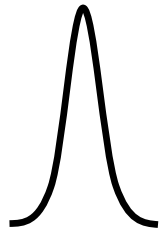
After introduction to column molecules are **partitioning between stationary (SP) and mobile phase (MP)**, system is almost in equilibrium – distribution between SP and MP is possible to describe using distribution constant $\mathbf{K_D}$

All molecules are moving only in mobile phase, all molecules go through the same path at different times according to their different affinity to the stationary phase.

Each molecule can enter stationary phase – one enters, one exits, the distribution between phases is kept in repeating equilibrations for each molecule.

If the position is occupied, the molecule travels further until it finds a free space. Another molecule may enter the released site. With a large number of molecules oversaturation of column can appear. Typical consequences of the column oversaturation is either fronting or tailing of peaks, in the extreme case, a significant reduction of retention times.

NORMAL



FRONTING

TAILING

LC SEPARATION THEORY II

Separation of 2 compounds is achieved, if they have different distribution between stationary and mobile phase.

IMPROVEMENT OF SEPARATION

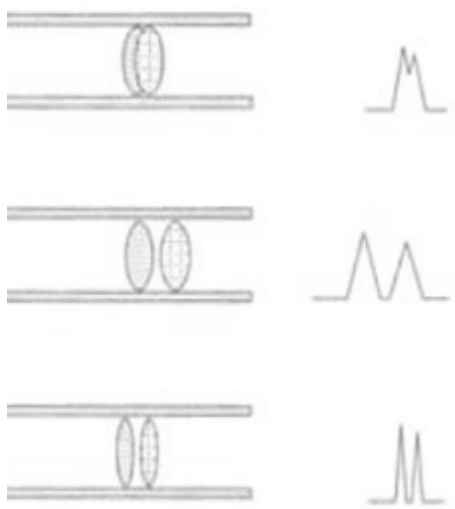
1. Increasing of differences in retention times

- affecting of interactions between analyte and SP
  = changes of **SP, MP (solubility)** and **temperature**

2. Narrowing of elution bands

- **column dimensions, temperature, flow rate**

narrow elution bands needs less separation than wide ones



MAJOR FACTORS AFFECTING SEPARATION

Stationary phase – higher solubility (affinity) of analyte in SP
⇒ higher retention

Analyte structure – differences in solubility of analytes in SP and MP

Solubility of analyte in MP – affects distribution between SP and MP

Temperature – affects speed of transition of molecules between SP and MP, speed of elution

↑ solubility of analyte in MP ⇒ ↑ number of molecules in MP
⇒ ↓ t_R and ↓ separation
⇒ narrower elution zones with lower retention times

Other factors: column dimensions, flow rate

Overview of separation parameters

N – number of theoretical plate (efficiency): $N = \frac{5{,}55 \cdot t_R^2}{w_{1/2}^2}$

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: $\alpha = k'_2 / k'_1$

Resolution (alt.): $R = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha} \right) \cdot \left(\frac{1 + k'_2}{k'_2} \right)$

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

Interpretation of separation parameters

N ~ stationary phase quality - particles size and sorting,
mobile phase composition, flow rate, column length, temperature
- characterizes quality of elution zone - wide, shape ...

- peak shape factor

k‘ ~ composition of stationary and mobile phase, temperature,
flow rate, column length
- characterizes retention under given conditions

- retention factor (time of analysis)

α ~ composition of stationary and mobile phase, temperature,
flow rate, column length
- characterizes separation (comparison of k‘)

- selectivity factor (analytes separation)

R ~ combined parameter - overall separation characteristics

Classification of liquid chromatography

- configuration: column, thin layer - sorbent, gel, paper
- separation parameters: efficiency, speed, capacity …
- stationary phases: elution, ionex, chiral …

COLUMNS

Materials – glass, metals (stainless steel, titanium)

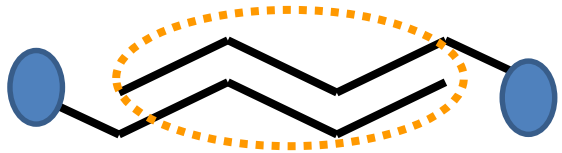
Dimensions – length (mm - tens of cm); diameter (mm)

Classification according to SP and MP

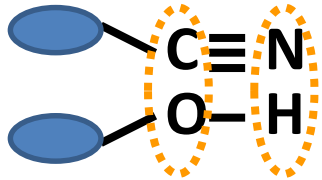
Combination of SP and MP	Particles size/sorting/porosity Applied pressure
Elution: - normal phase - reverse phase (classic x semipolar) - ion pair (on reverse phase) - hydrophilic interaction LC (HILIC) *Ion exchange (IE)* - catex, anex *Chiral* - cyclodextrin phases *Affinity*	*High Performance LC* HPLC: 3-10 µm - various - *p* up to 400 bar *Rapid Resolution LC* RRLC: 1.8 µm - porous mixed - *p* up to 600 bar Ultra Performance LC UPLC: 1.7 µm - porous unified - *p* up to 1000 bar Ultra High Pressure LC UHPLC: 1.0 µm - non-porous - *p* up to 5000 bar

Interaction occurring during separation

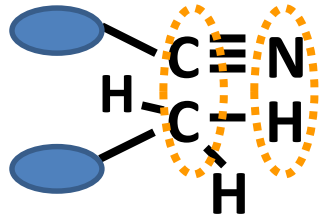
* van der Waalsovy dispersion force – *London's induced dipole – induced dipole*



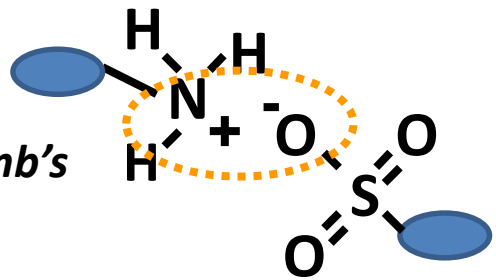
* van der Waalsovy orientation forces – *Keesom's dipole - dipole*



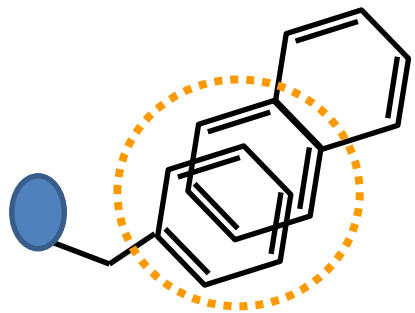
* van der Waalsovy induction forces – *Debye's dipole – induced dipole*



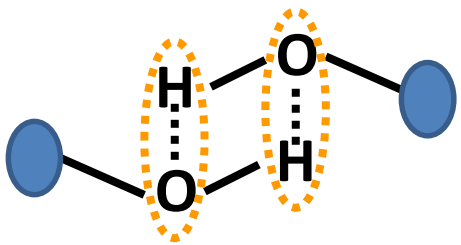
** electrostatic forces – *Coulomb's*



**** π - π interaction



*** hydrogen bond

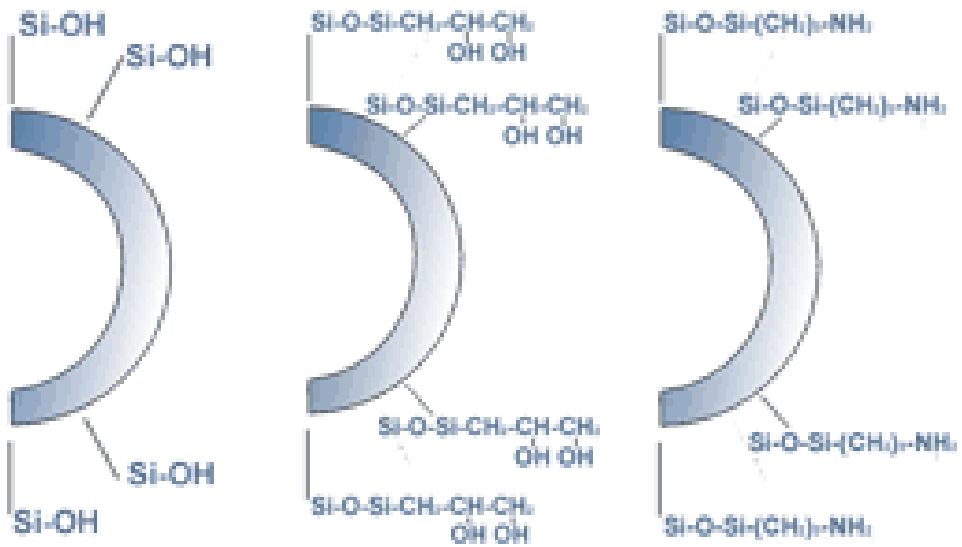


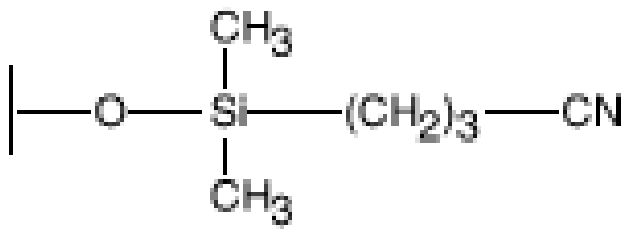
Normal phase chromatography (NP):

Stationary phase - polar: silica gel, modified silica gel

Mobile phase - nonpolar (retention according to MP comp.)

Classic silica (non-modified), diol-silica, amino-silica, cyano-silica (propyl-group)

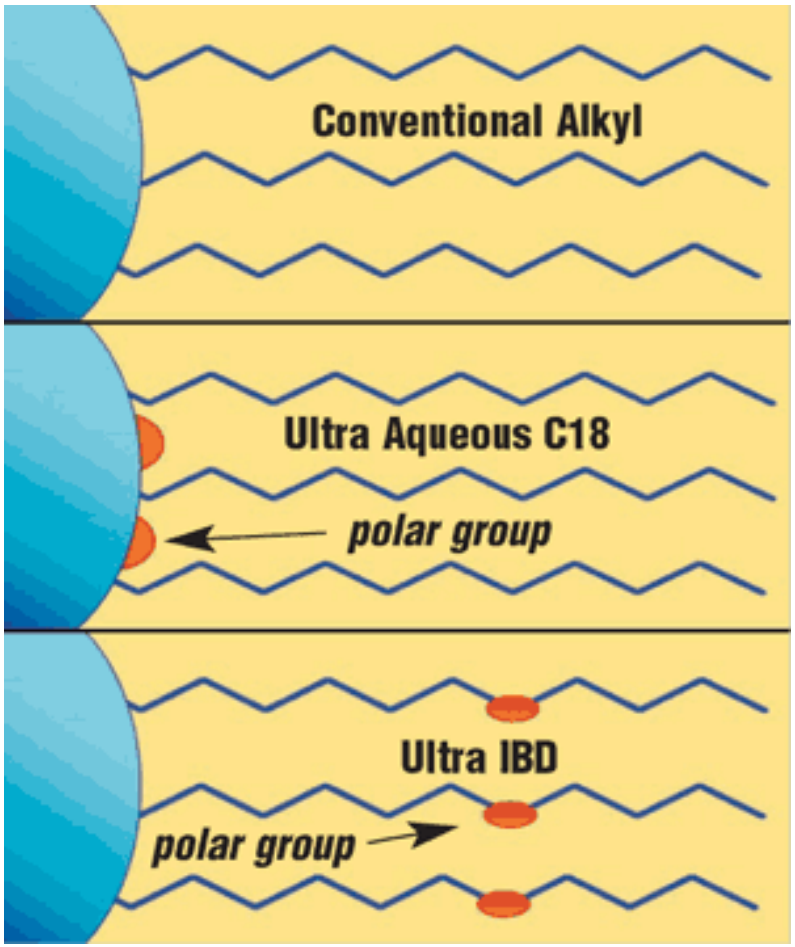




Reverse phase chromatography (RP):

Stationary phase - nonpolar: modified silica gel (C18 silica)

Mobile phase - polar (retention according to MP comp.)



ODS - octadecylated silica gel (C18)

Other modification: C4, C8, C30

Endcapping (Embedding):

Classic - 100% nonpolar

Polar – partially polar groups

Porous x nonporous

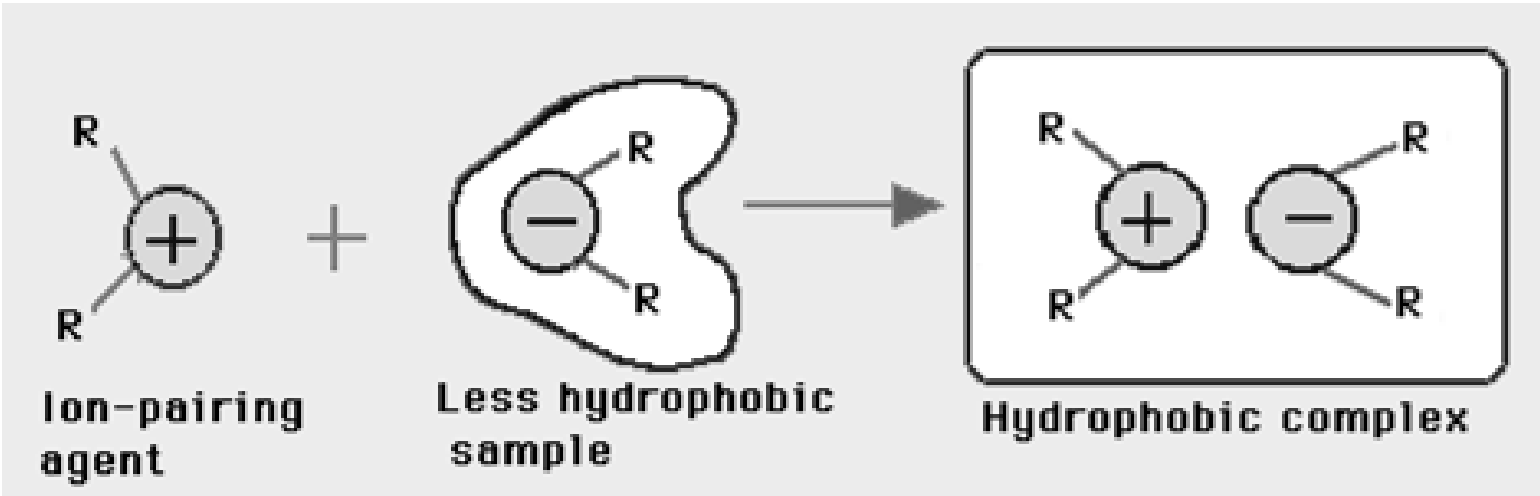
Monomeric X polymeric (cross-linked)

Ion pair chromatography:

Polar analyte + polar agent (ion pair agent)

⇒ nonpolar complex with retention on nonpolar SP

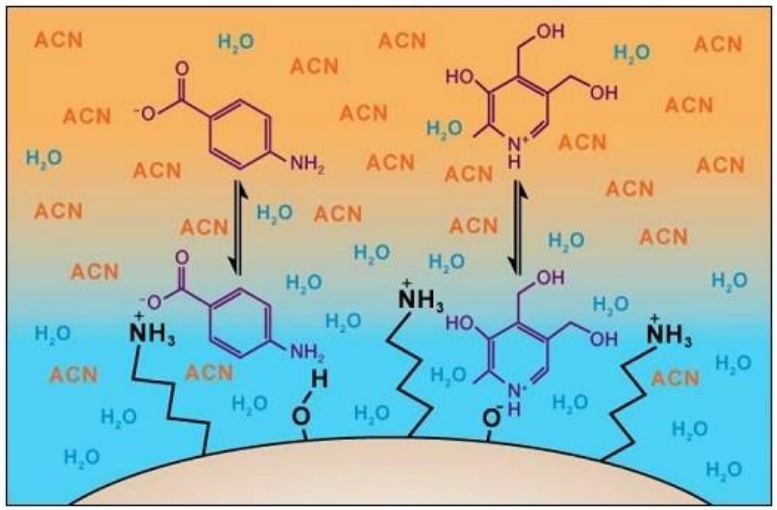
- realization on reverse phase

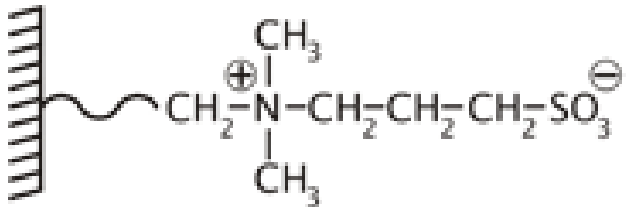


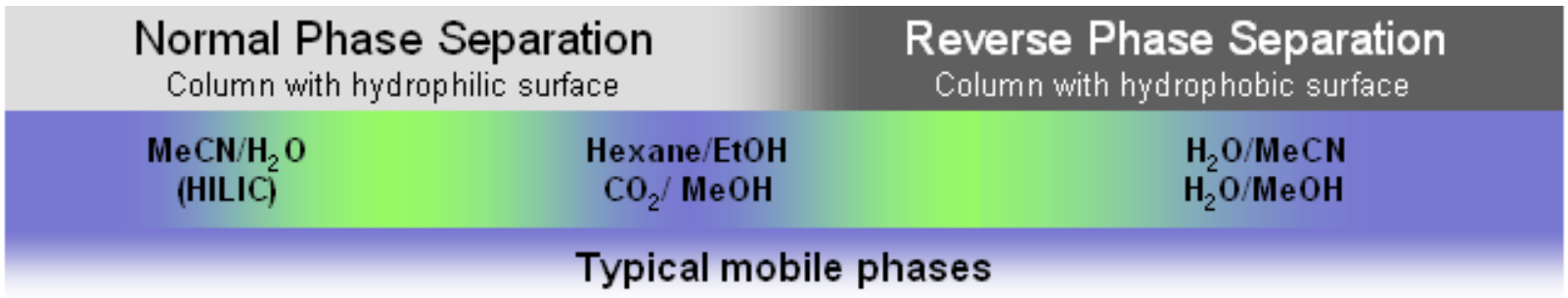
Hydrophilic Interaction LIquid Chromatography (HILIC):

Polar analytes with weak non-polar interactivity interacts with polar groups in SP

Polar components of MP (water) shows similar behavior



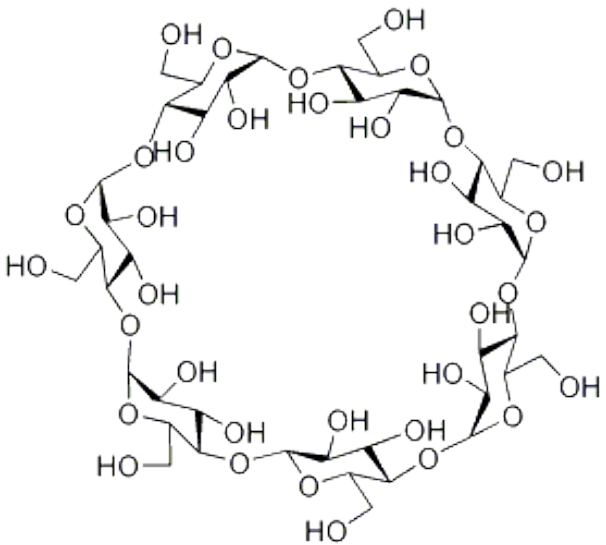


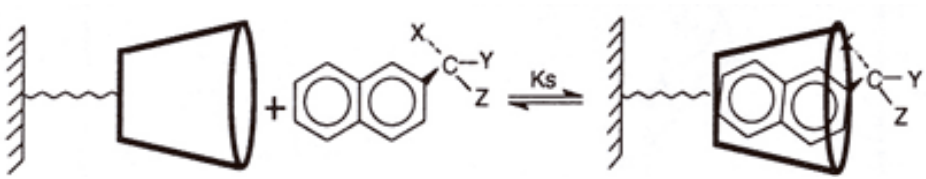


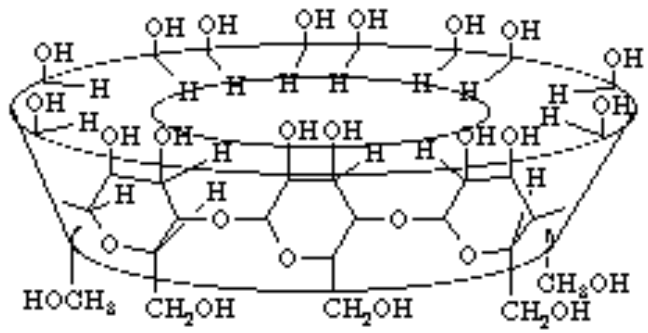
Chiral chromatography:

Separation of stereoisomers (enantiomers, diastereoisomers ...)

- interaction with chiral phase – cavity - toroid (inside hydrophobic)
- typically composed of cyclodextrins (6, **7**, 8 units = α, **β**, γ)
- **β-cyclodextrins are mostly used for separation**







Ion exchange chromatography:

Stationary phase - embedded ⊕ or Θ charged groups;
counter ions are retained and released
according to MP composition

Modes of separation:

1. Retention of analytes on SP due to interaction of oppositely charged ions
2. Release of analytes to MP by change of pH or concentration increase of competitive ions (displacement of analytes)

Strong IEC: CATEX - propyl/benzene-sulfonic acid
ANEX - quaternary amine, Ca^{2+} for polyols (sugars)

Weak IEC: CATEX - carboxylic acid
ANEX - amines (secondary, tertiary)

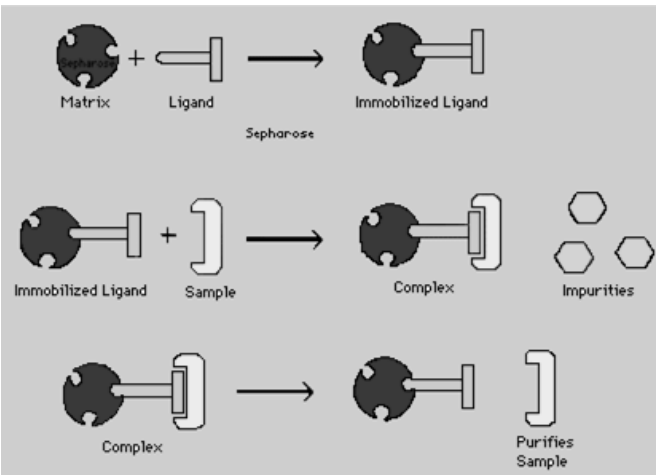
Affinity chromatography:

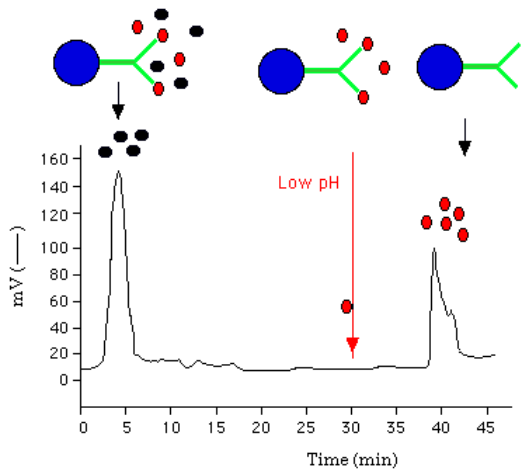
Stationary phase – immobilization of ligands (biologically active compounds);

Sample specifically interacts with SP – creating complex and subsequently is released by change of MP

Modes of separation:

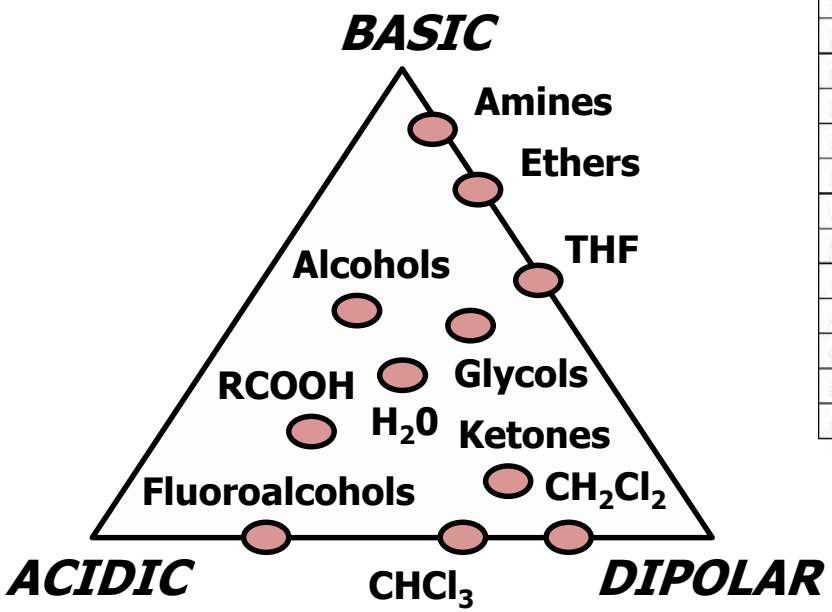
1. Retention of analytes on SP due to interaction with SP
2. Release of analytes to MP by change of pH or concentration increase of competitive compound (displacement of analytes)





Mobile phase - polarity, elution techniques:

SELECTION:
Sample solubility
Compatibility: SP, materials
Elution: isocratic or gradient,
flow rate



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

Mobile phase – elution strength

Polarity (Snyder's polarity index) - P'
Eluotropic value – elution strength (SP type, primary for NP) - ε^0
Solubility parameters - δ

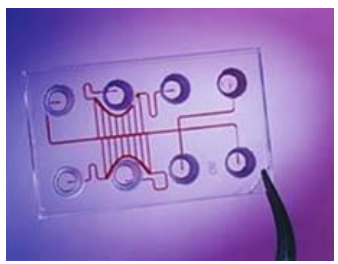
Solvent	P'	ε^0 (silica gel)	ε^0 (C18)	δ
Hexane	0.1	0	-	7.3
Acetone	5.1	0.53	8.8	9.6
Acetonitrile	5.8	0.52	3.1	12.7
Methanol	5.1	0.7	1.0	14.5
H_2O	10.2	-	-	23.5

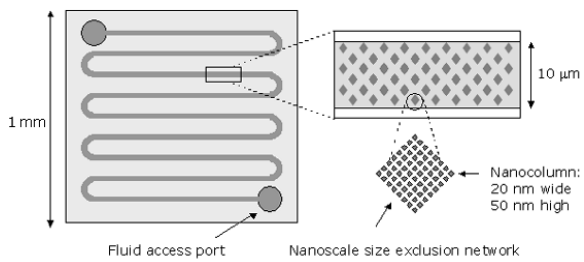
Overview of typical combinations of columns, sorbents and flow rates according to LC type

Column dimensions	*Particles*	*Flow rate*	*Chromatography*
10-25 cm x 3-4.6 mm	3-1 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

(FIGURES: Agilent Technologies)

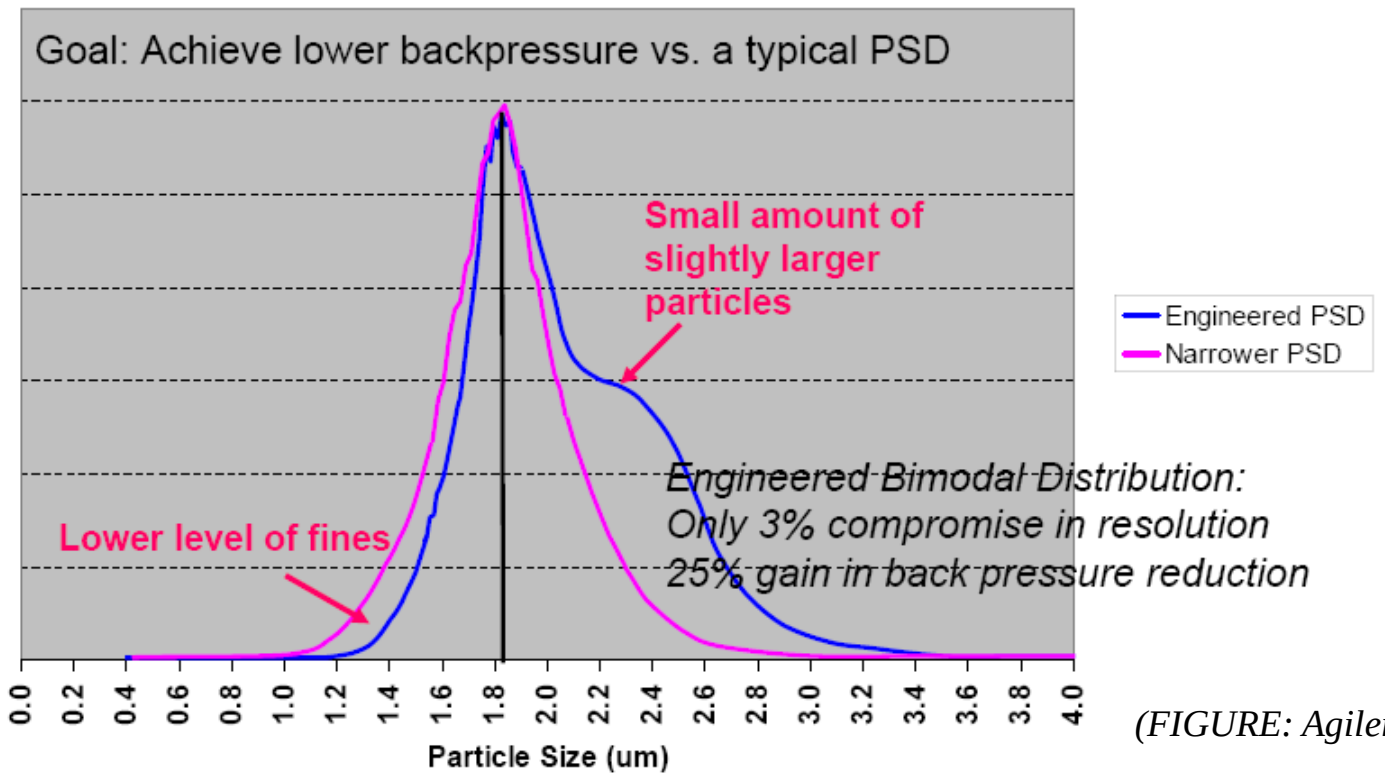




Sorbent sorting - back pressure effect

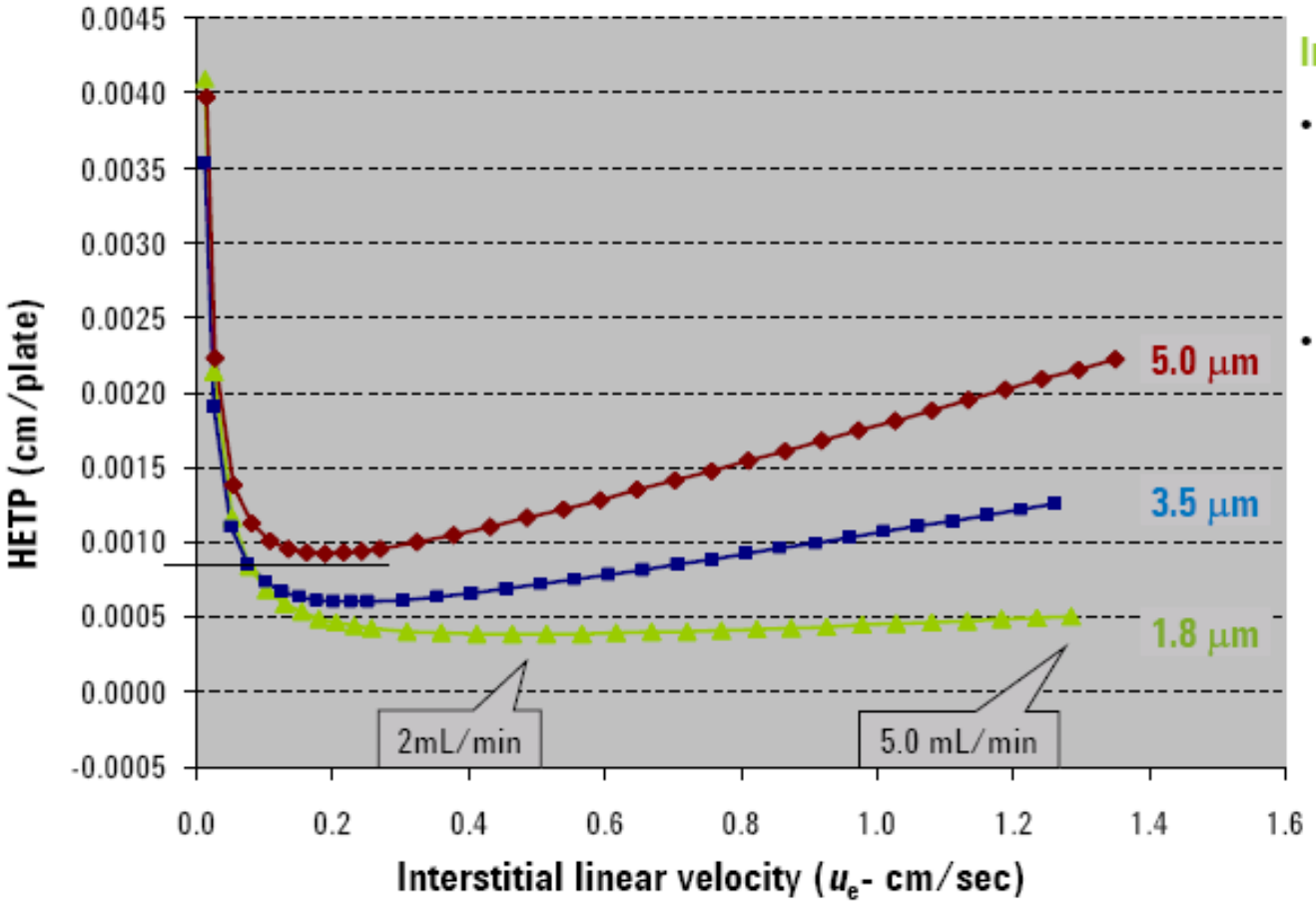
- higher permeability ⇔ porosity
- can be higher than sorbent particles

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



(FIGURE: Agilent Technologies)

Application of sorbents with small particles - very high speed in RRLC

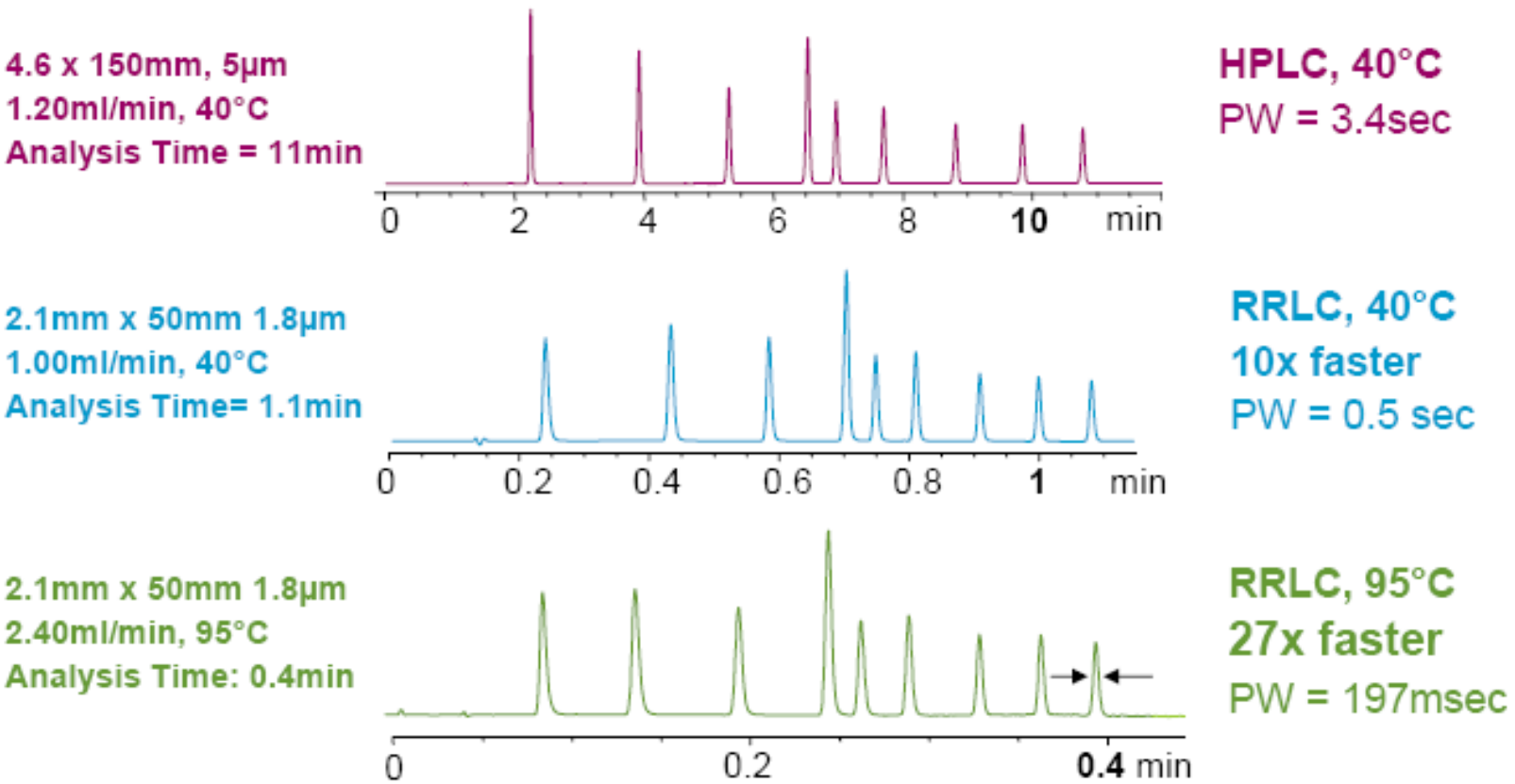


Increased Speed

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

(FIGURE: Agilent Technologies)

Application of rapid resolution LC (RRLC)



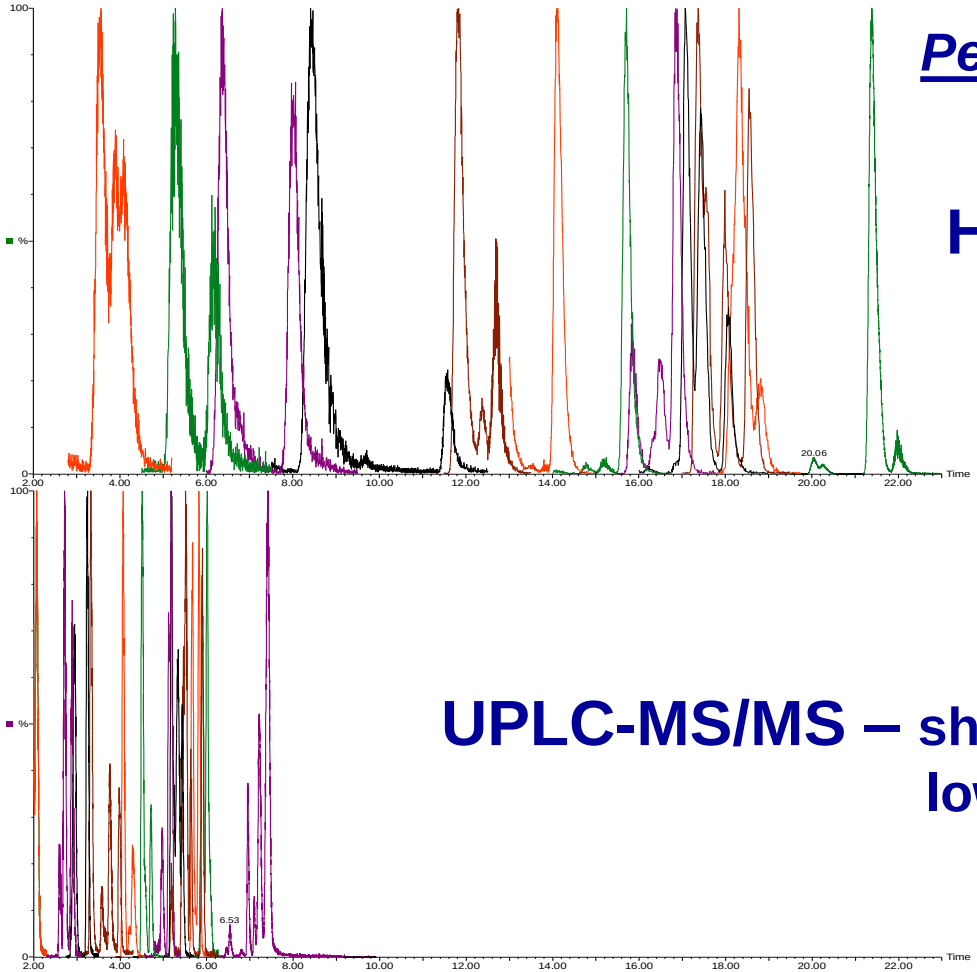
150mm > 50mm: 3x

1.2ml/min on 4.6 > 2.4ml/min on 2.1: 10x

3 x 10 = 30x

(FIGURE: Agilent Technologies)

Application of ultra performance LC (UPLC)



Pesticides multi residue analysis

HPLC-MS/MS

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

UPLC-MS/MS – shorter time
lower solvents consumption

UPLC 10 min

HPLC 50 min