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Defining the HPLC Separations Categories

Amporn Wongcharoensatit

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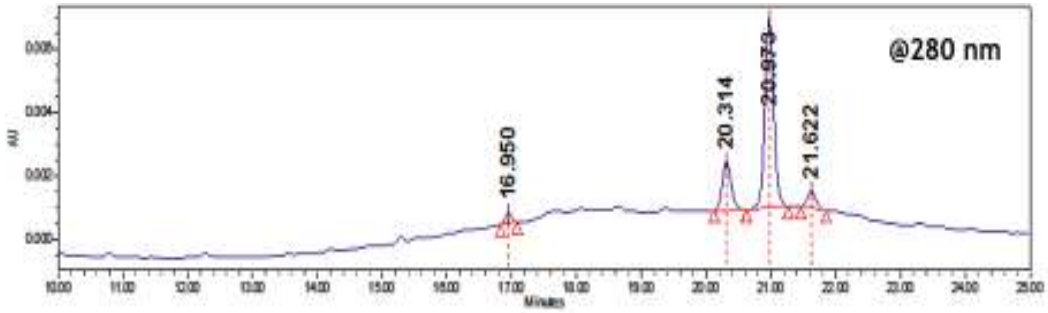

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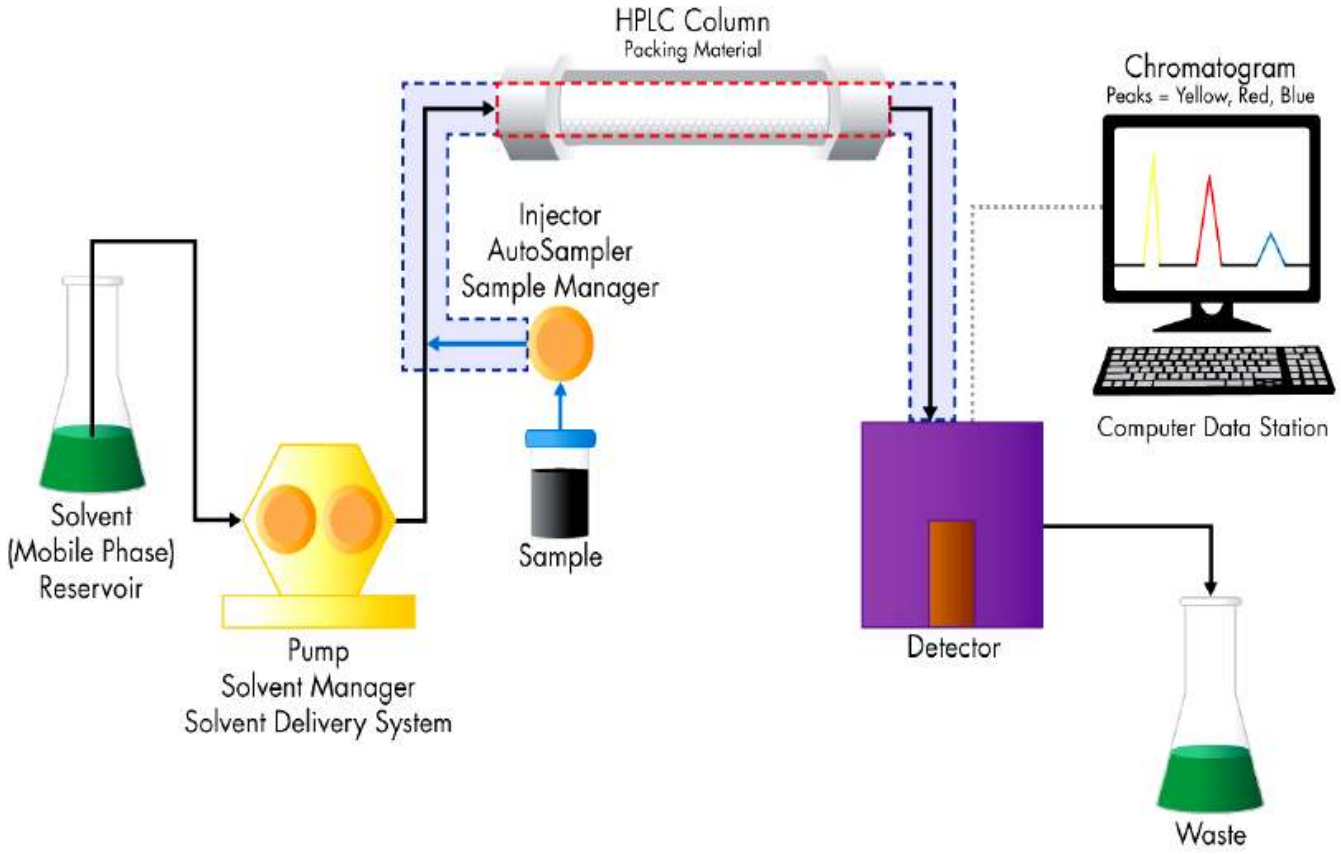
LC system



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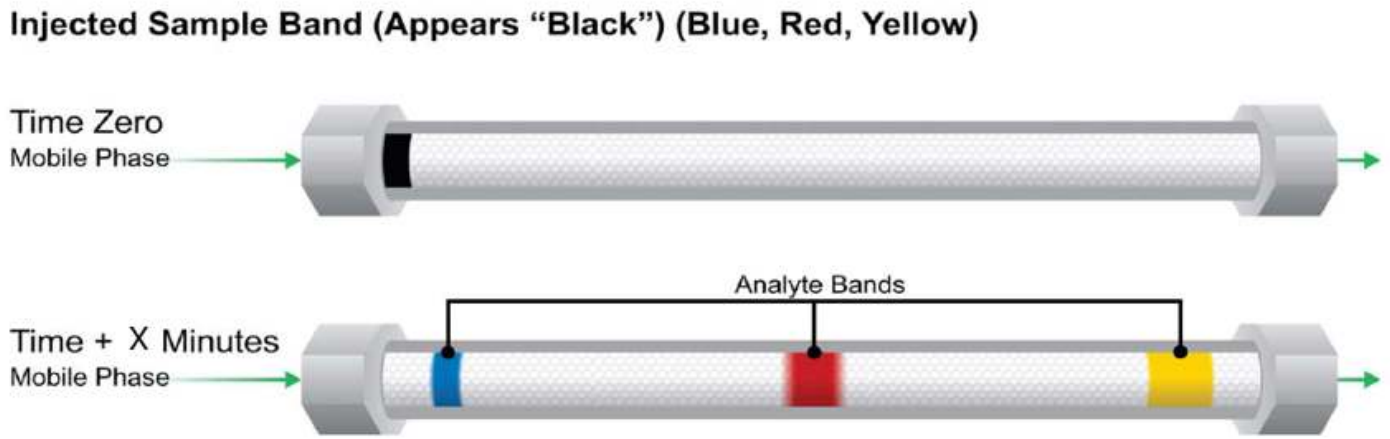
Understanding how a chromatographic columnmn work



- Yellow is the earliest eluting analyst "band" moving fastest it like the mobile phase
- Blue is well retained, move the slowest in the column it like particles

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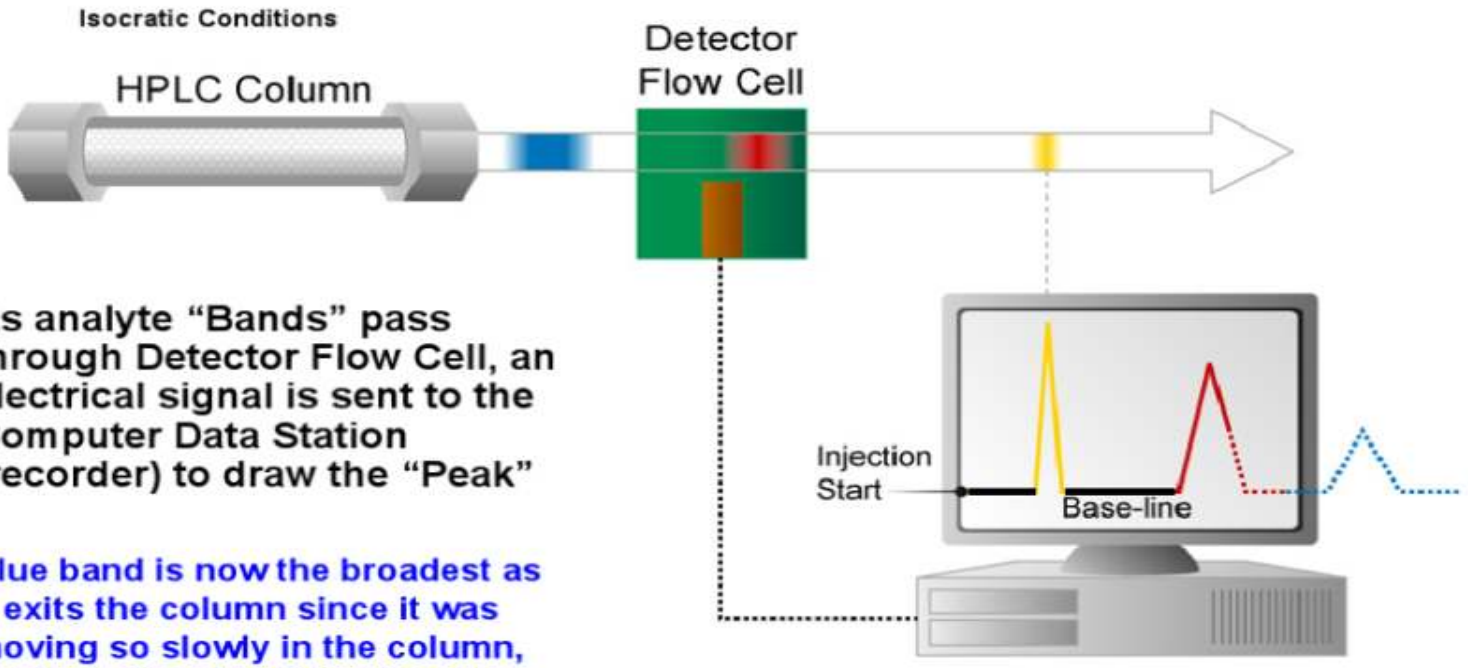
How are peaks created



As analyte "Bands" pass through Detector Flow Cell, an electrical signal is sent to the Computer Data Station (recorder) to draw the "Peak"

Blue band is now the broadest as it exits the column since it was moving so slowly in the column, It takes a lot of mobile phase to finally sweep it all out – this is why the latest eluting peaks are the broadest – it will be the most dilute

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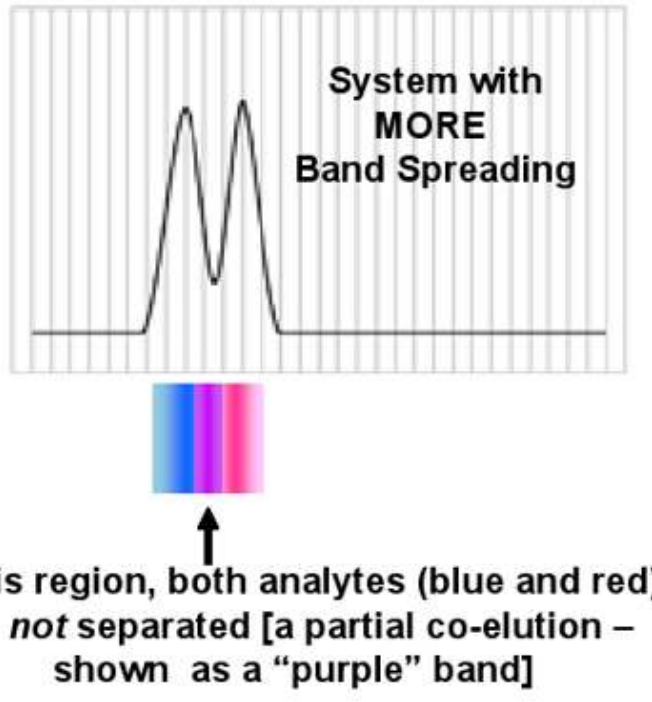
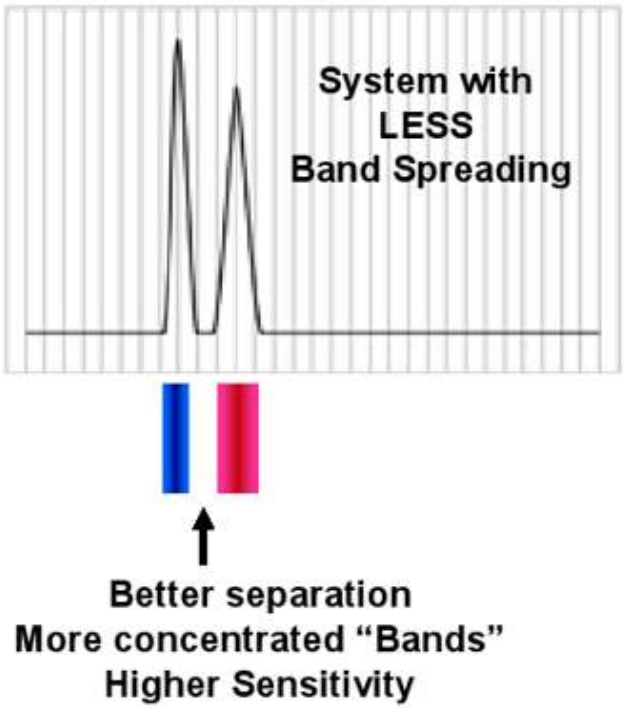
Band spreading and separating power



In this region, both analytes (blue and red) are *not* separated [a partial co-elution – shown as a "purple" band]



Better separation
More concentrated "Bands"
Higher Sensitivity

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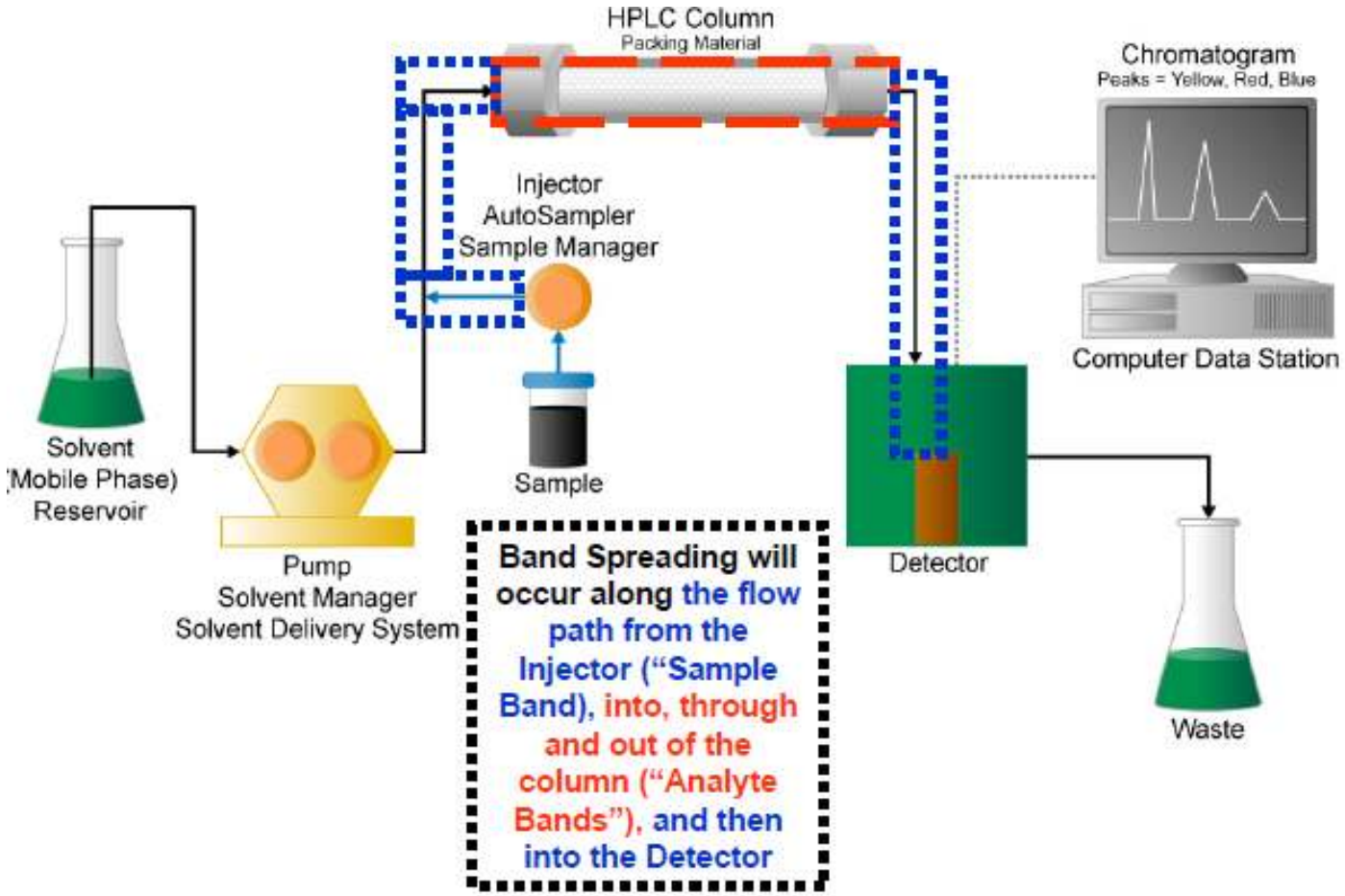
Potential for band spreading



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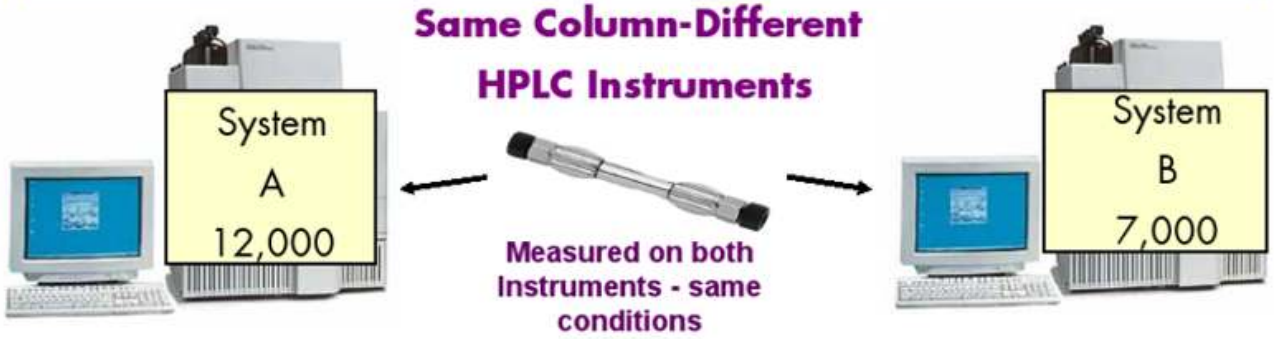
Meaning of a plate count

(Total system performance – Isocratic mode)

The Plate Count* is a Key Indicator of the ***COMBINED*** Band Spreading Performance of the Column AND Instrument ***as a System***



Instrument A has ***less*** ***Band Spreading Volume (70µL) HPLC*** MORE PLATES (N=12,000)

Instrument B has ***more*** ***Band Spreading Volume (130µL) HPLC*** LESS PLATES (N =7,000)

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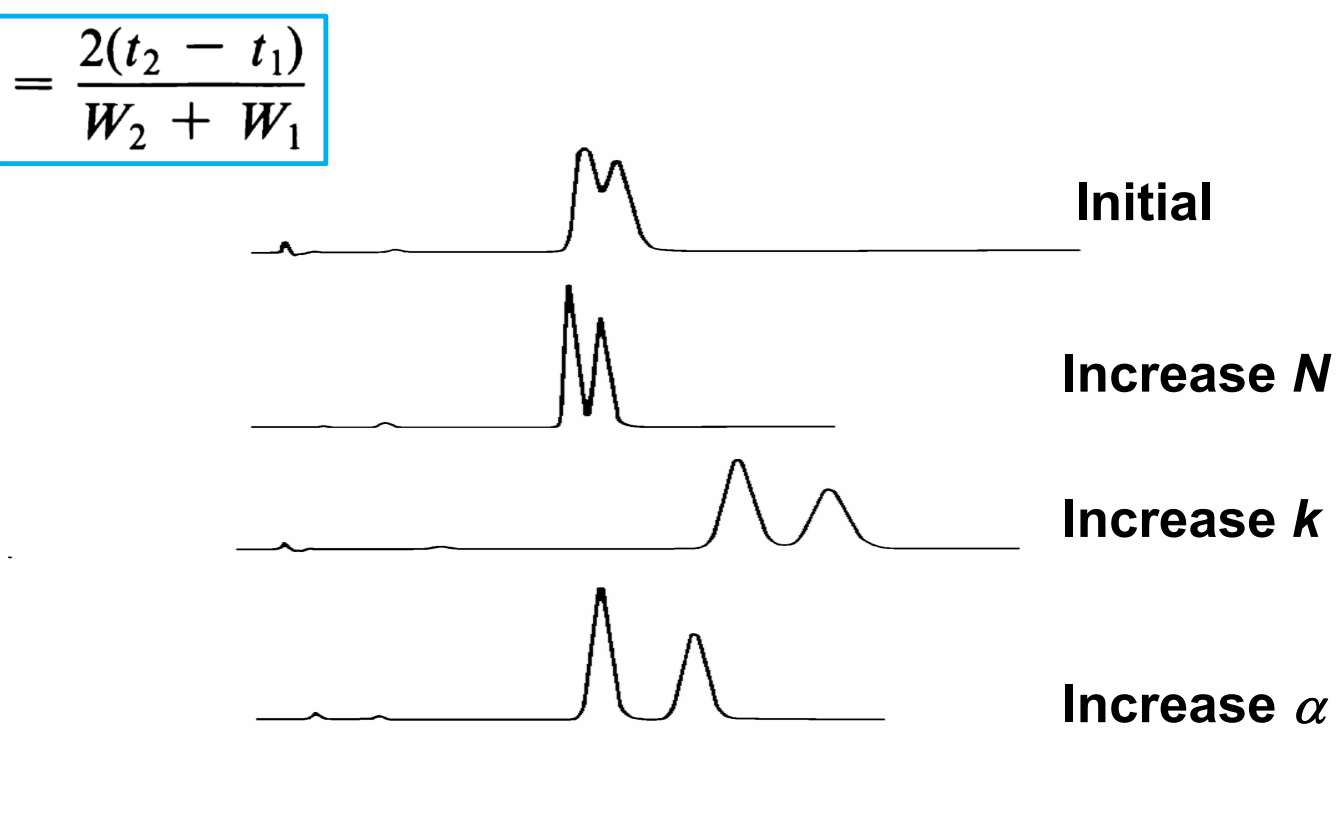
Principles of HPLC/UHPLC separations

Resolution effect of N, k, α

$$R = \frac{2(t_2 - t_1)}{W_2 + W_1}$$



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Resolution equation

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

Selectivity Efficiency Retention

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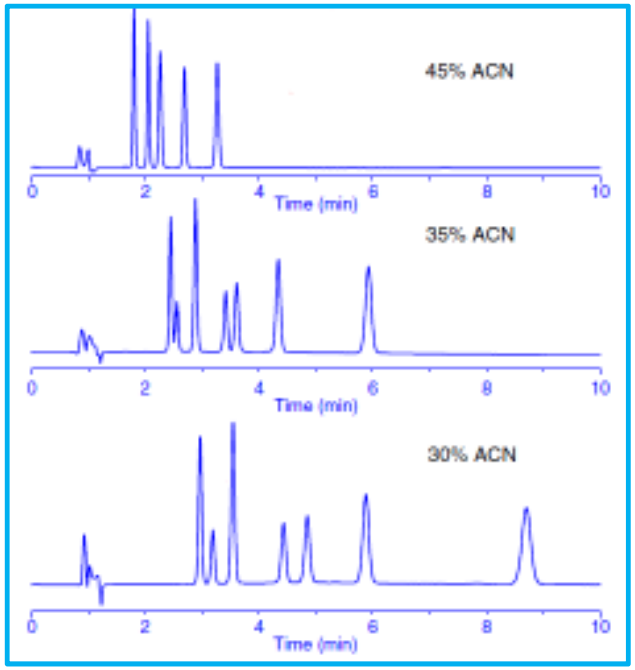
Resolution management

- **Retention**
  - No. of column volume to elute
  - iF k=0 Resolution (R_s) becomes zero
  - Affected by % strong solvent
  - Increase retention factor by decrease strong solvent in mobile phase
  - Typically 2 < k < 10

$$k = \frac{(t_R - t_0)}{t_0}$$



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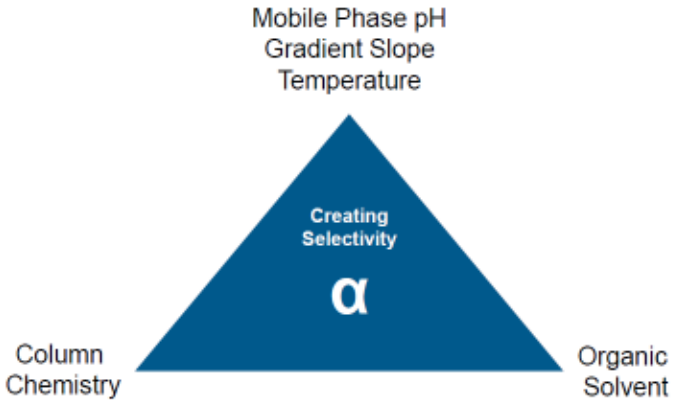
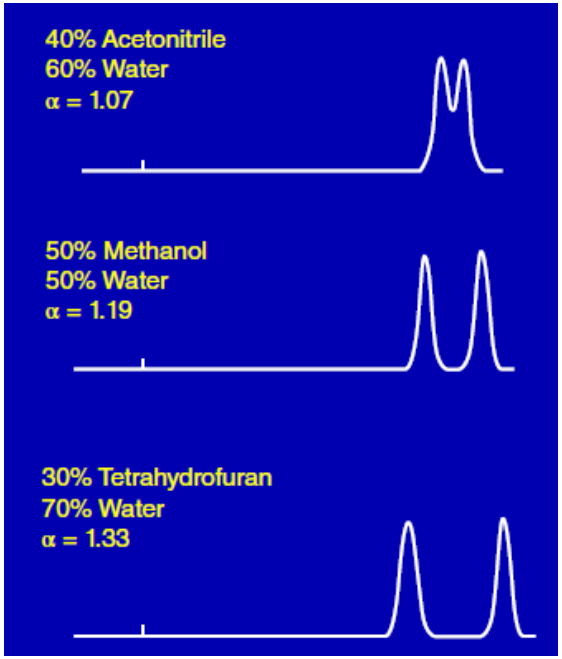
Resolution management

- **Selectivity**
  - How well two compounds can be resolved with respect to column volume
  - Affected by chemical selectivity : column type, solvent type, temperature, pH

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$





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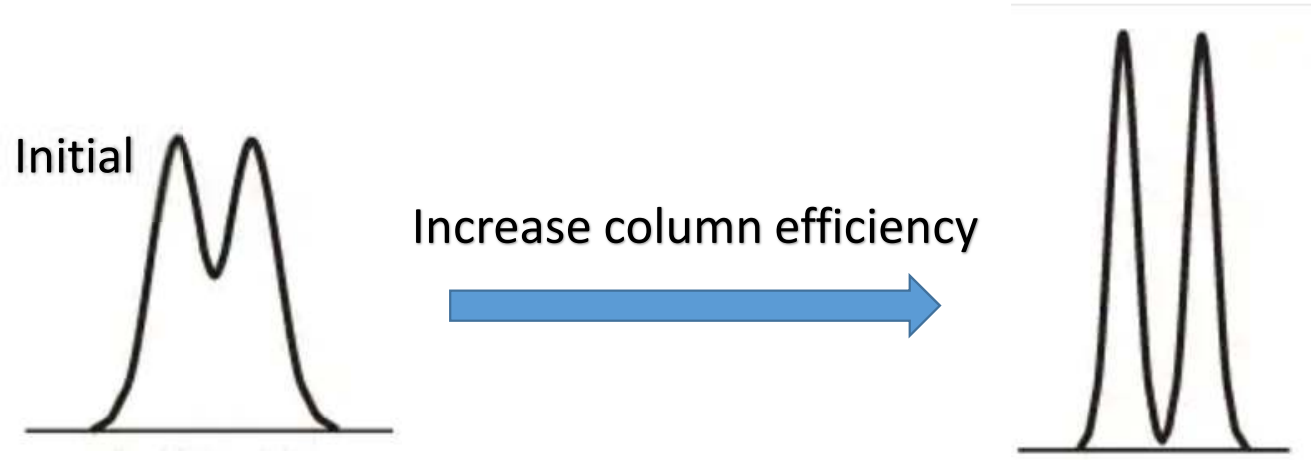
Resolution management

- **EFFICIENCY**
  - Improving resolution is to adjust the column's efficiency by increasing the number of theoretical plates

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



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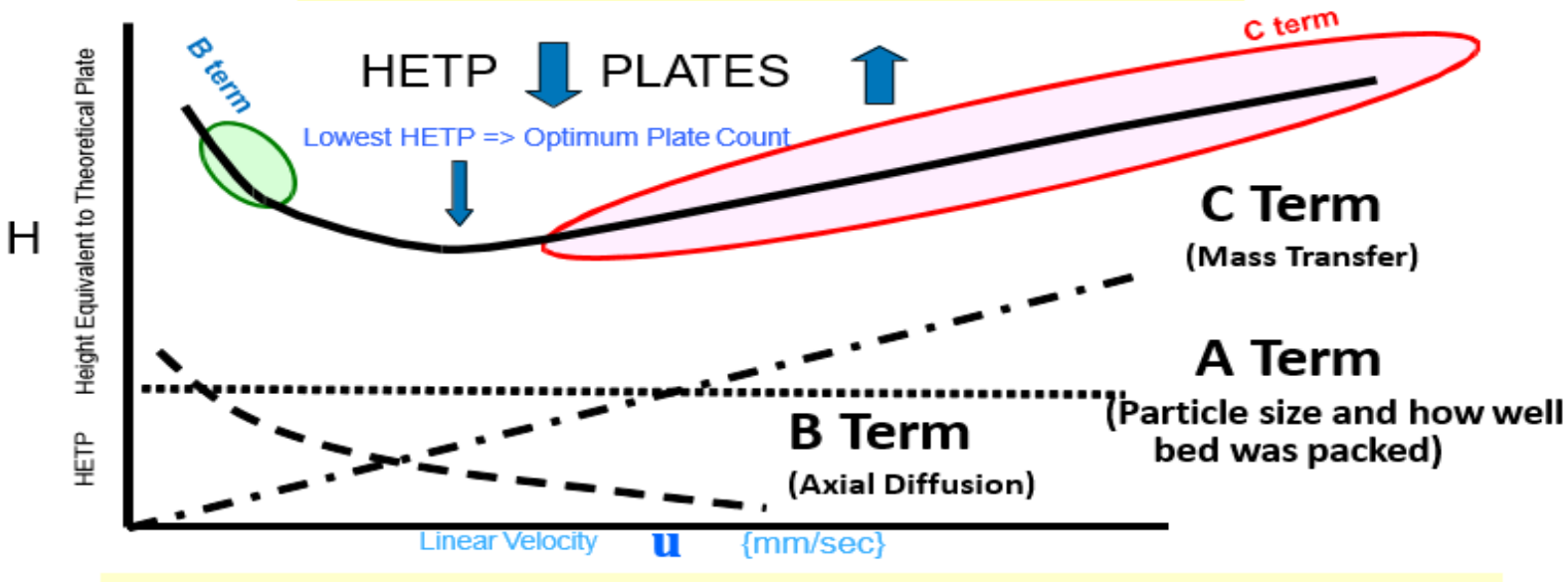
Van Deemter equation

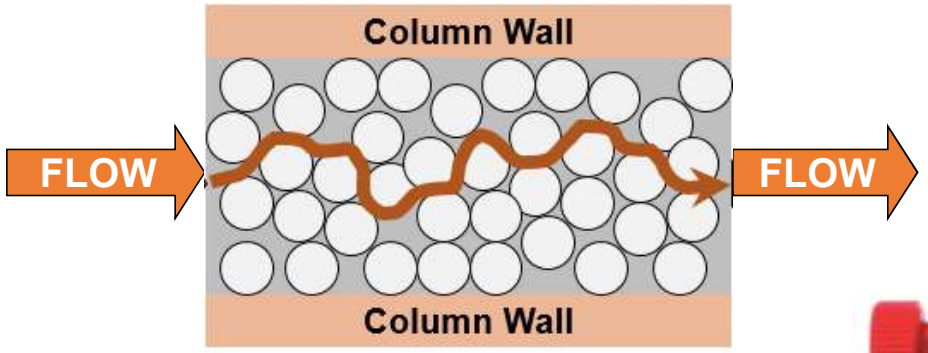
A term + B term + C term

$$H = a(dp) + \frac{b}{u} + c(dp)^2 u$$

HETP = Column Length / No. of Plates

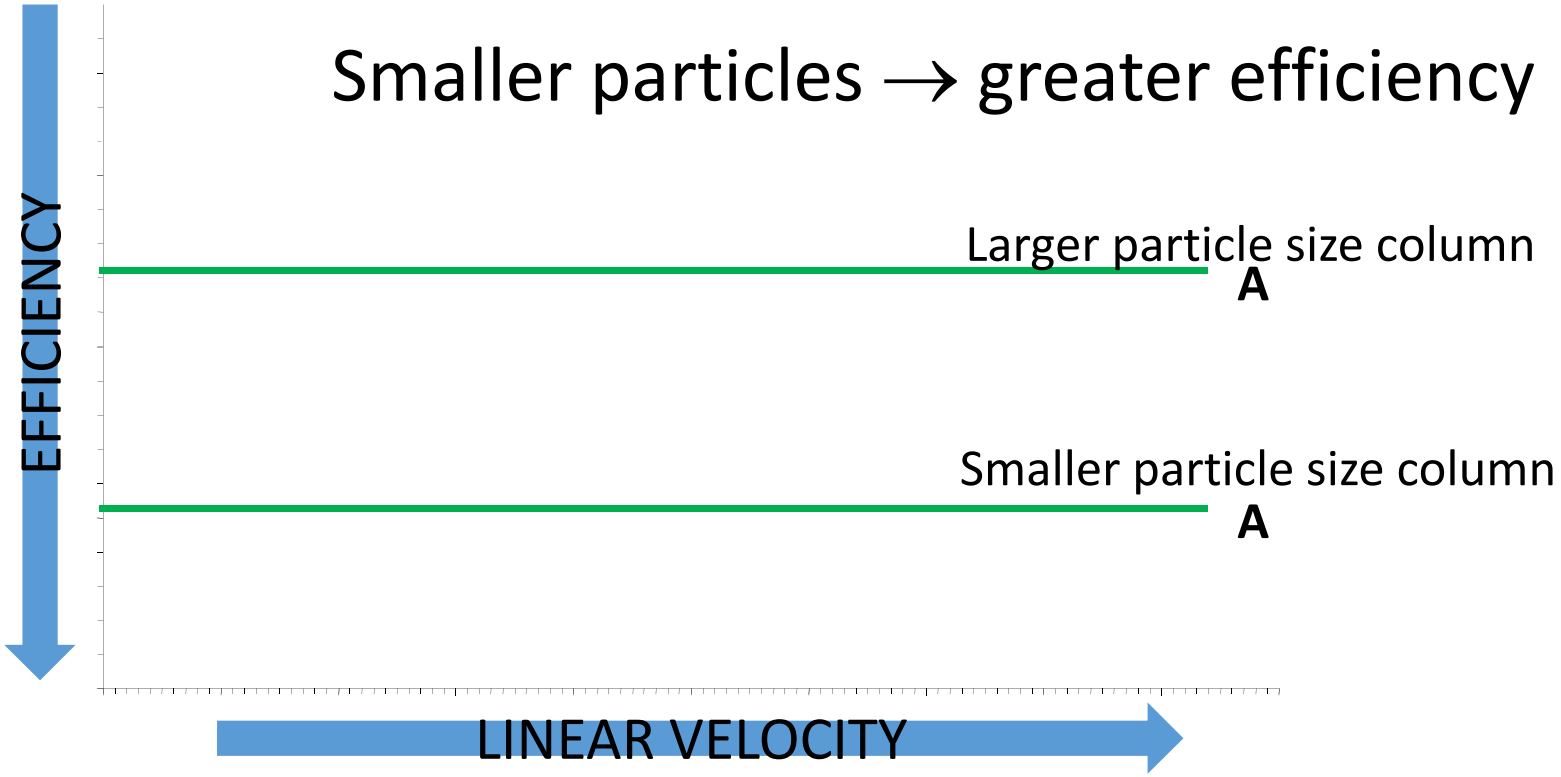


Eddy diffusion (A)



- Diffusion in the interstitial spaces of a packed column
- Flow independent
- Dependent on particle size, shape and packing efficiency

Effect of eddy diffusion on the van Deemter plot



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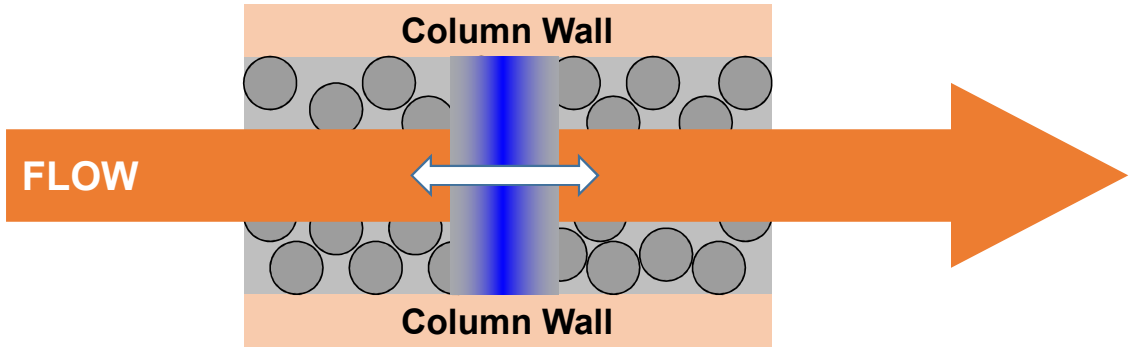
Longitudinal diffusion (B)



- Diffusion in or against flow direction
- Flow dependent
- Higher flows decrease longitudinal diffusion

Effect of longitudinal diffusion on the Van Deemter plot

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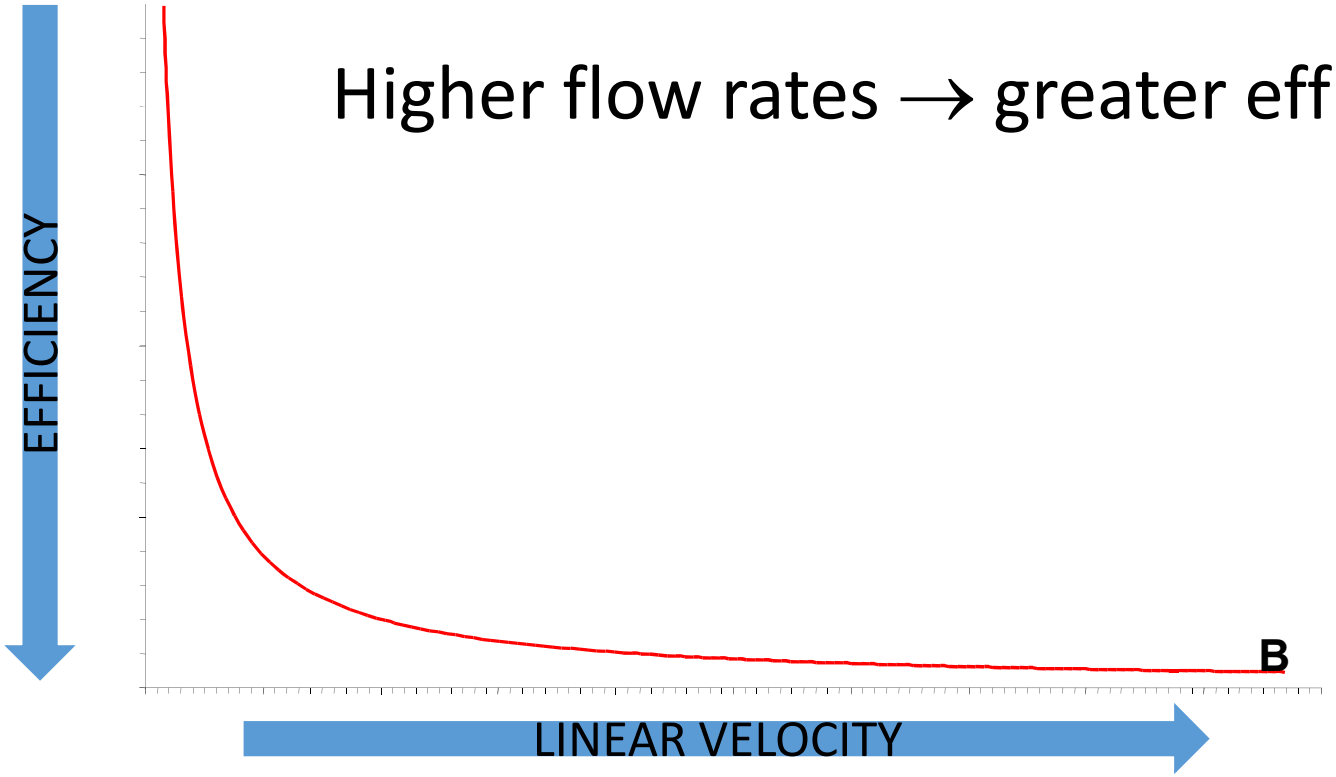
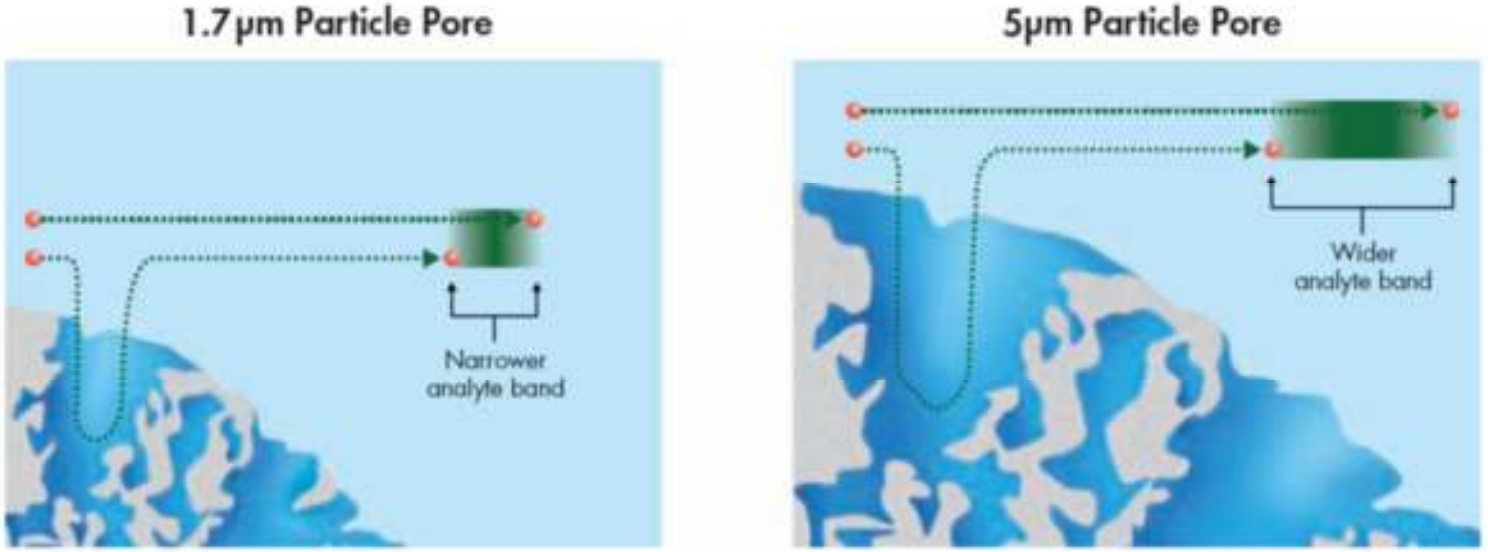


Higher flow rates → greater efficiency

Mass transfer (C)



- Mass transfer measures diffusion in and out of the pores
  - Flow dependent
  - Particle size dependent

Effect of mass transfer kinetics on the Van Deemter plot

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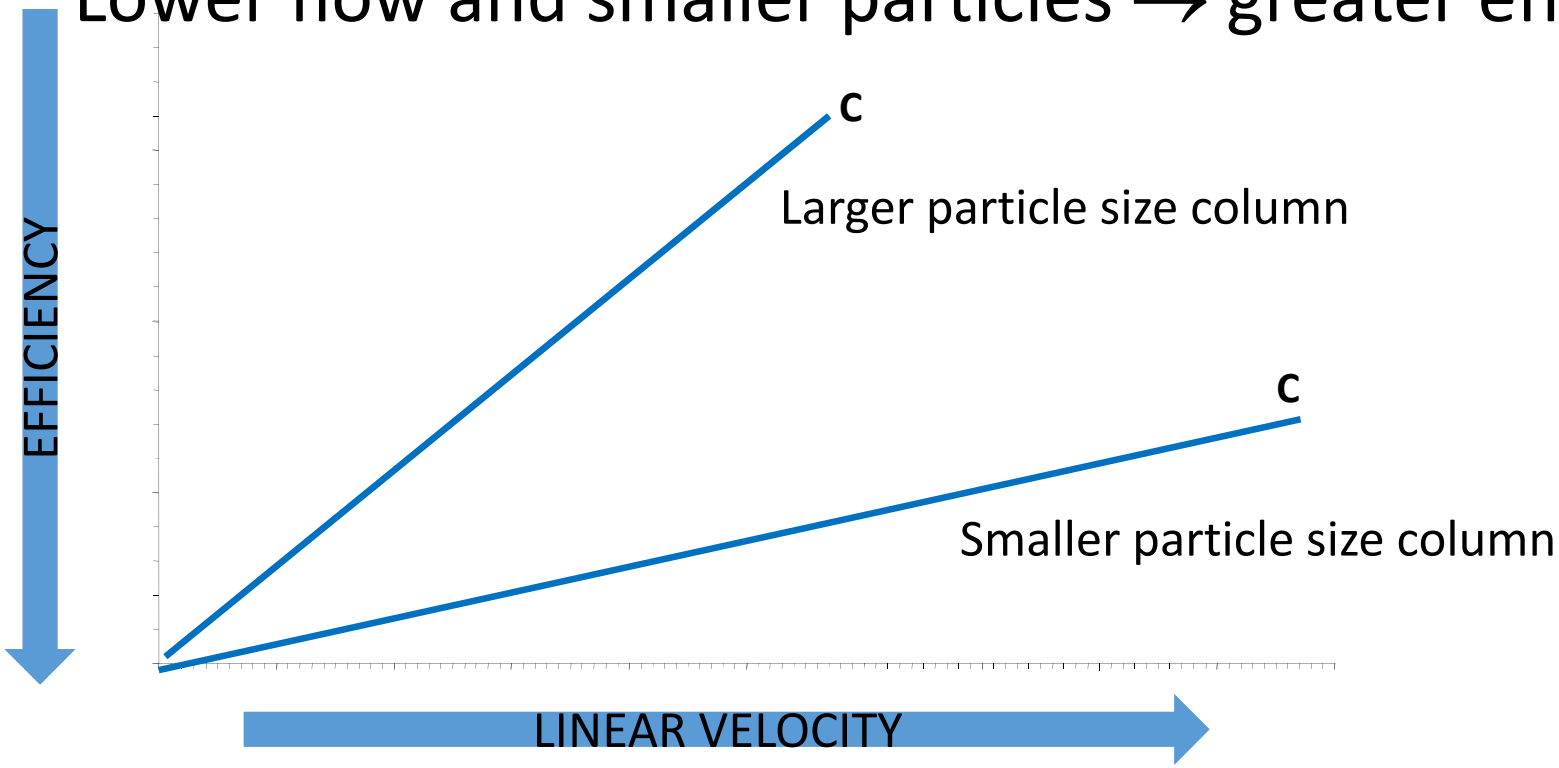
Lower flow and smaller particles → greater efficiency



Putting it all together

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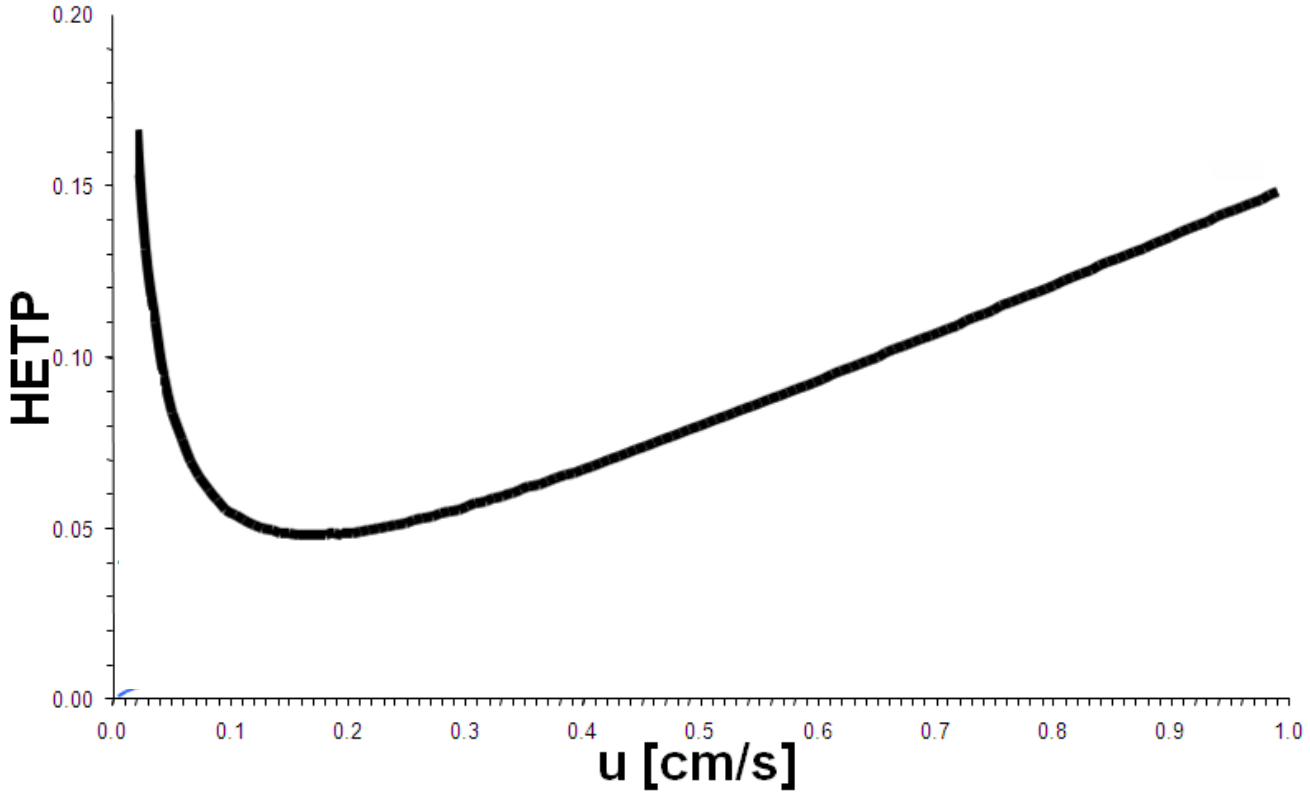

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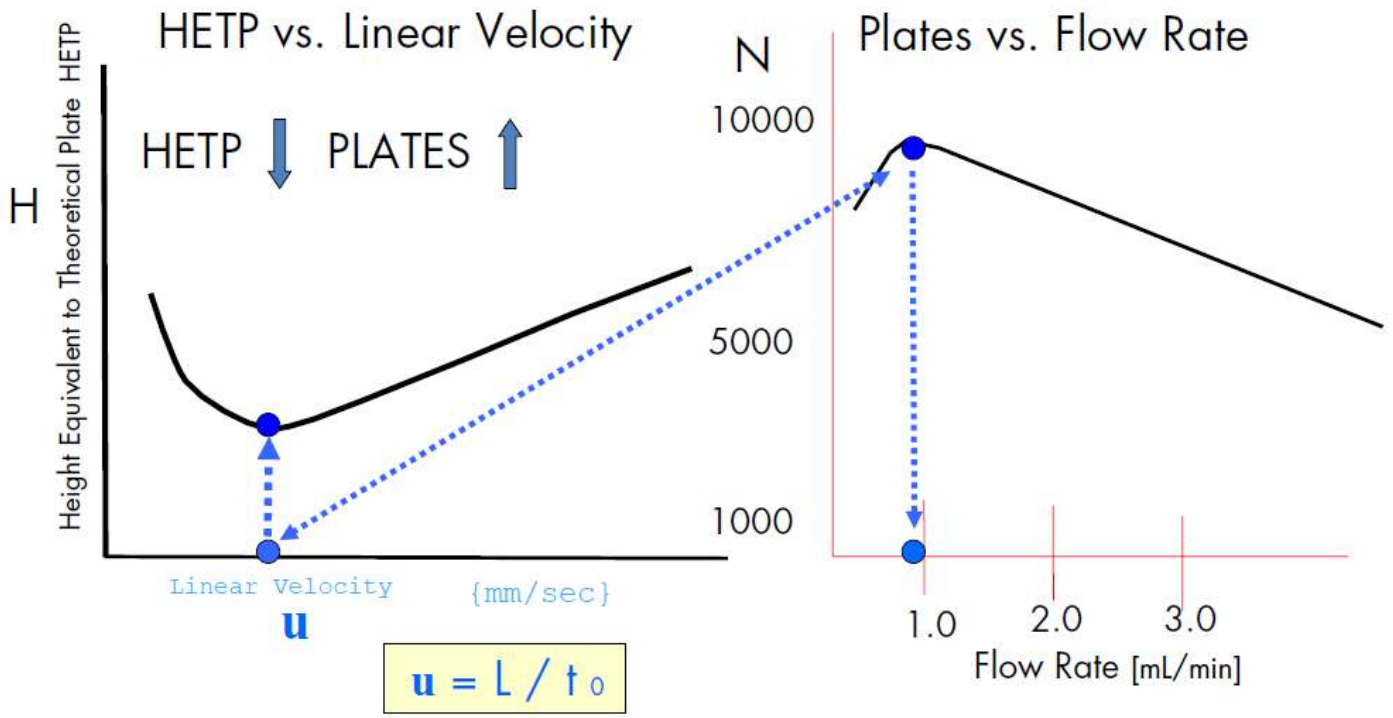
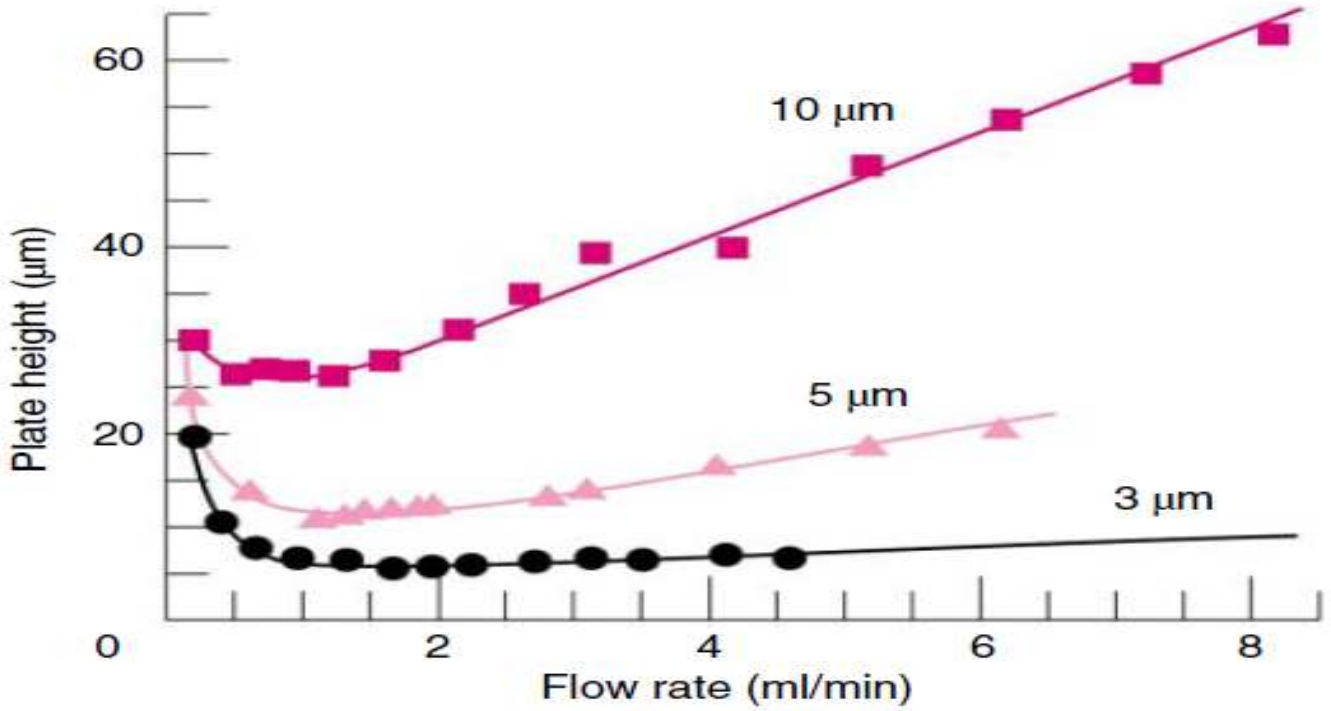
Van Deemter curve : plates & flow rate

Optimal Linear Velocity and Flow Rate



Van Deemter curve : column-particle size



Column-Particle size & Flow rate

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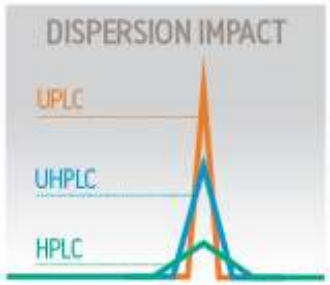
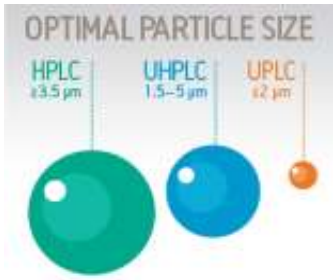
Defining the LC separations categories



Impact of the system dispersion on a chromatographic peak

Matching the right LC system to the right column will yield the best chromatographic results



Select the appropriate particle size to match the dispersion of your HPLC, UHPLC, or UPLC system

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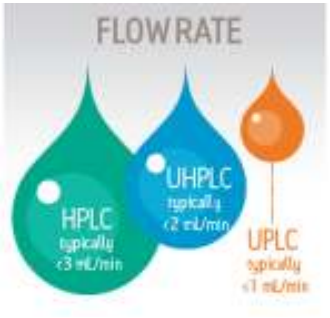
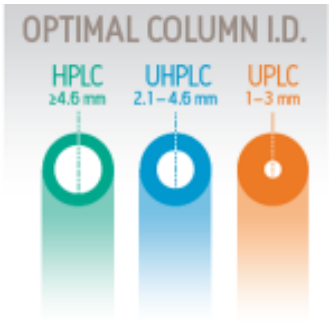
Defining the LC separations categories





Select a flow rate that gives the optimal linear velocity to maximize efficiency for your column characteristics (van Deemter)

Typical flow rates vs column id for optimal column efficiency vary according to the van Deemter equation.

Example

Internal Dimension	3 µm	5 µm	10 µm
2.1 mm ID	0.3 mL/min	0.21 mL/min	-
3.0 mm ID	0.60 mL/min	0.43 mL/min	-
4.6 mm ID	1.50 mL/min	1.00 mL/min	0.7 mL/min

Pair the particle size with the column i.d. that best matches the dispersion of your chromatographic system

The system must be able to operate at the typical back pressures associated with the selected column

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Defining the LC separations categories

HPLC

Dispersion > 30 µL

Columns accepted:
- 3.0 – 4.6 mm ID
- 3 - 10 µm particles

Optimal:
- 4.6 mm ID, 5 µm

Typical operating pressure:
- < 6,000 PSI

UHPLC

Dispersion 12 - 30 µL

Columns accepted:
- 2.1 - 4.6 mm ID
- 1.7 - 5 µm particles

Optimal:
- 3.0 mm ID, 2.x µm

Typical operating pressure:
- 6,000 – 15,000 PSI

UPLC

Dispersion < 12 µL

Columns accepted:
- 1.0 - 4.6 mm ID
- 1.6 - 5 µm particles

Optimal column:
- 2.1 mm ID, 1.7 µm

Typical operating pressure:
- 9,000 – 20,000 PSI

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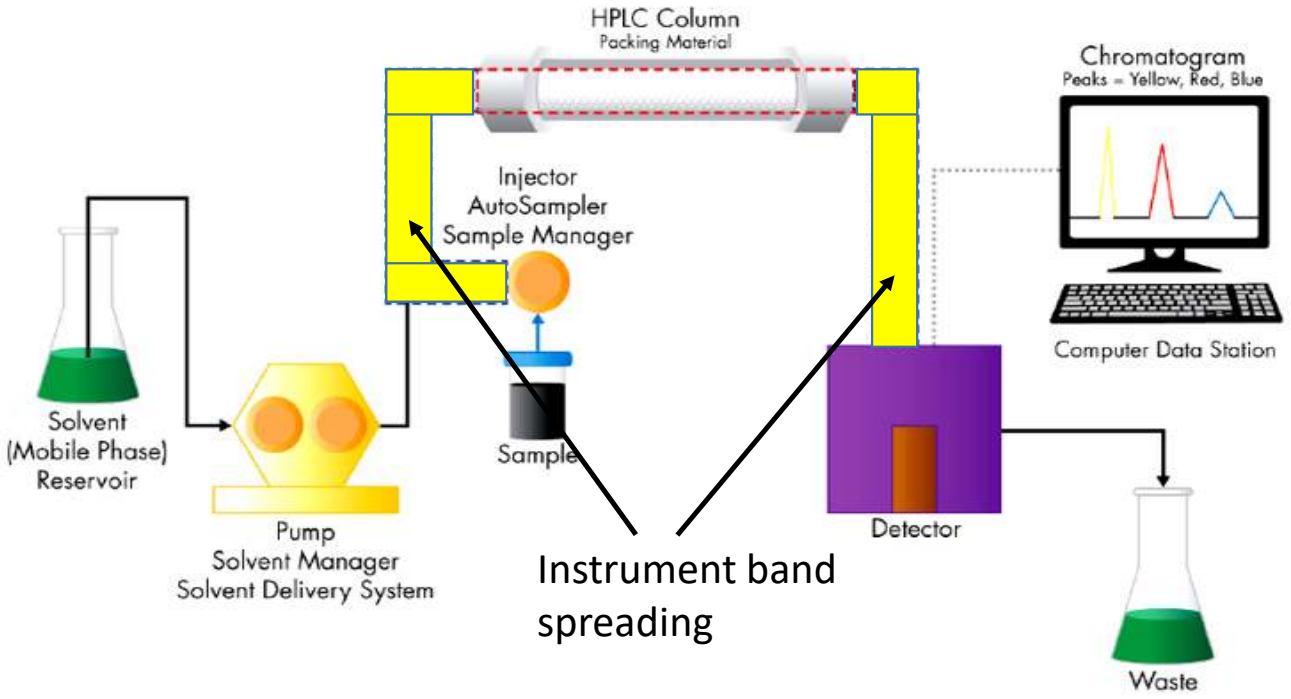
Instrument band spreading

Instrument band spreading or system dispersion



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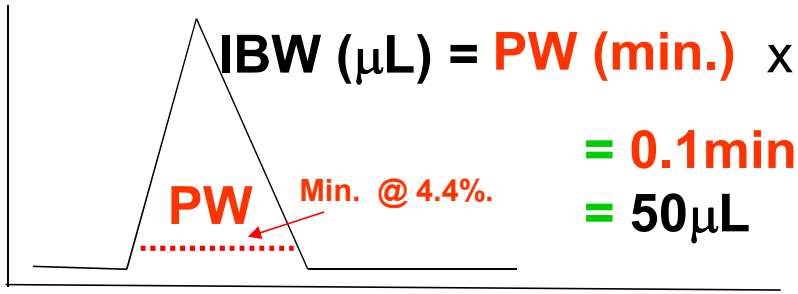
Measure instrumental band spreading

- Replace the column with a zero-dead-volume union
- Set the HPLC system to 0.5 ml/min, UV detection at 254 nm, data rate of 10 points per second
- Inject a 1-µl aliquot of a 0.5% caffeine or uracil solution
- Calculate the BS at 4.4% of peak height



IBW (µL) = PW (min.) x Flow Rate (µL/min.)

= 0.1min. x 500µL/min.

= 50µL

- Reduced BS replace connection tubing with shorter lengths of 0.005–0.007'' i.d. tubing

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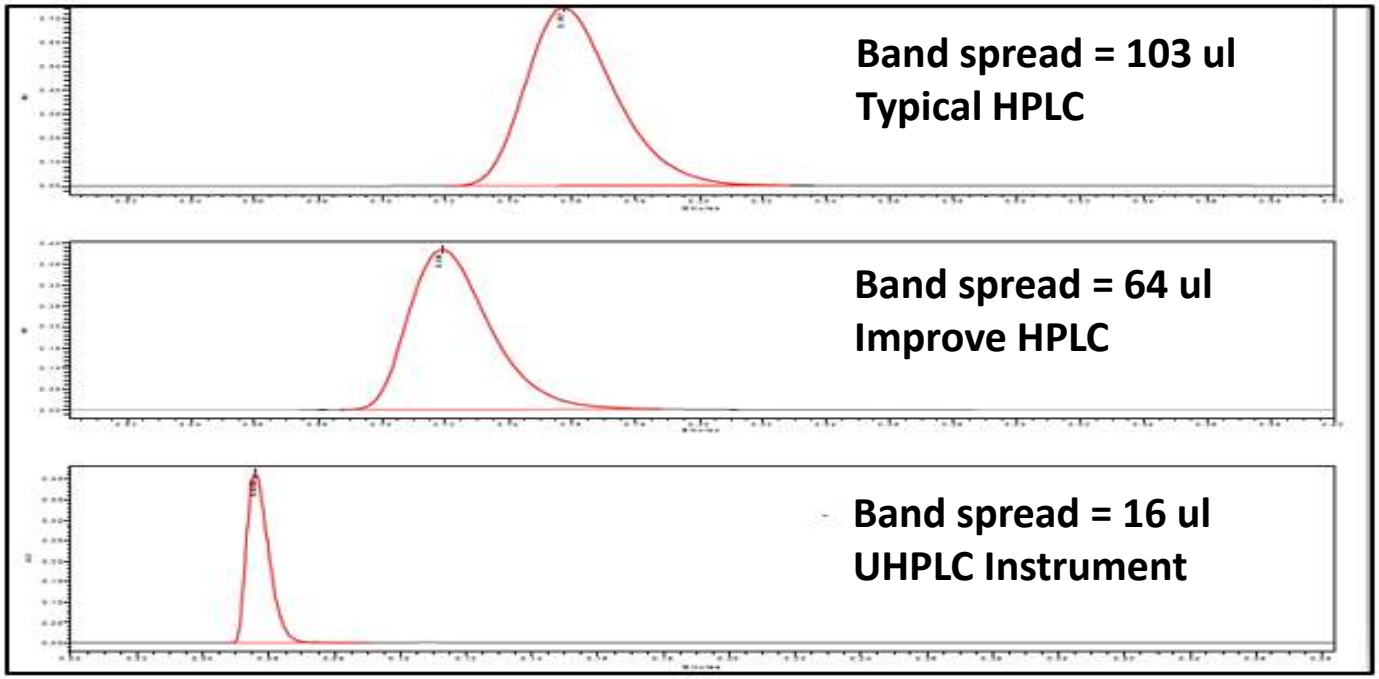
Band spreaded volume compairisons

Instrument band spreading or system dispersion



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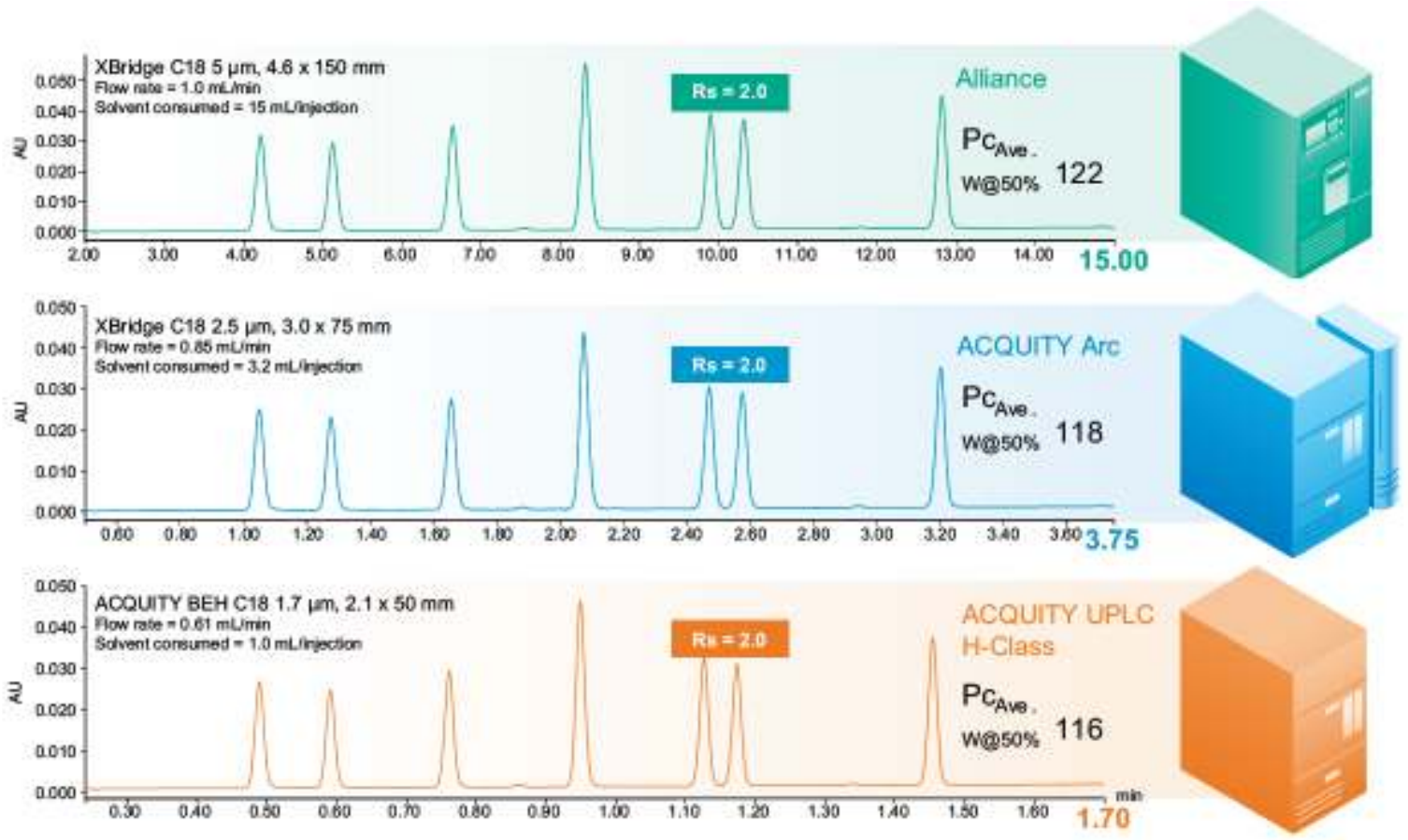
Matching the system to the column = best chromatographic performance

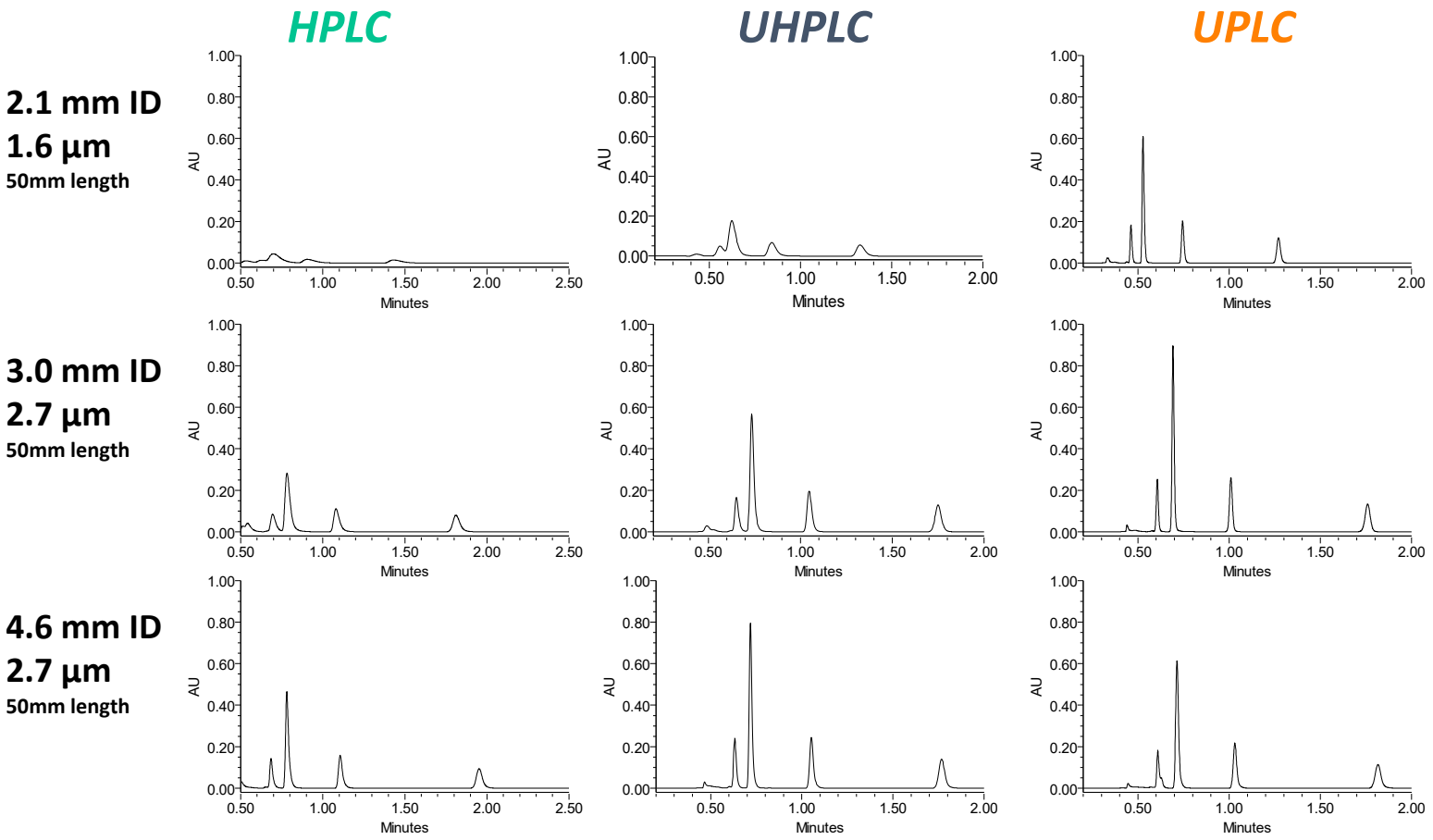


HPLC

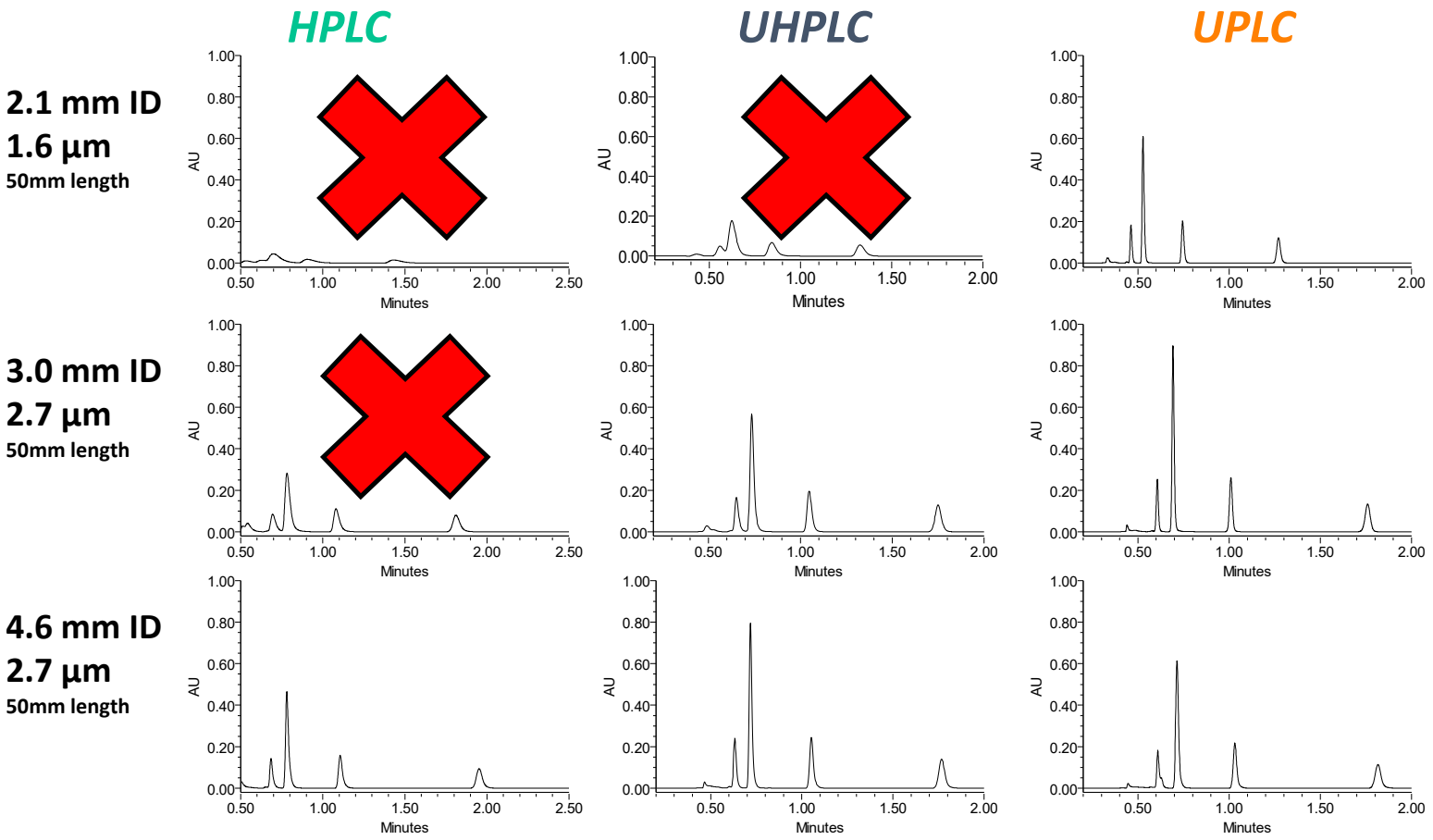
UHPLC

UPLC

Performance is impacted when system and column are not matched



Performance is impacted when system and column are not matched



Ratio of column length to particle diameter

L/dp ratio

Column Length/Particle Dimeter

$$\frac{300\ \text{mm}}{10\ \mu\text{m}} = 30{,}000$$

$$\frac{150\ \text{mm}}{5\ \mu\text{m}} = 30{,}000$$

$$\frac{100\ \text{mm}}{3\ \mu\text{m}} = 33{,}300$$

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Column length to particle size ratio

L/dp RATIO

$\frac{300mm}{10\mu m}$	=	30,000	**1970's**
$\frac{150mm}{5\ \mu m}$	=	30,000	**1980's**
$\frac{100mm}{3\ \mu m}$	=	33,300	**1990's**

Relationship

As	L/dp	↑	Resolution	↑
If	L/dp	SAME	Resolution	SAME
As	L/dp	↓	Resolution	↓

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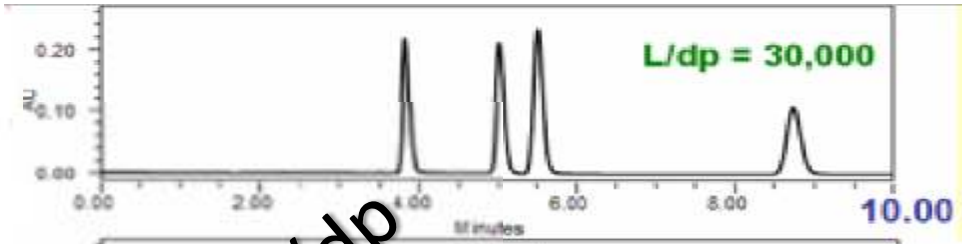
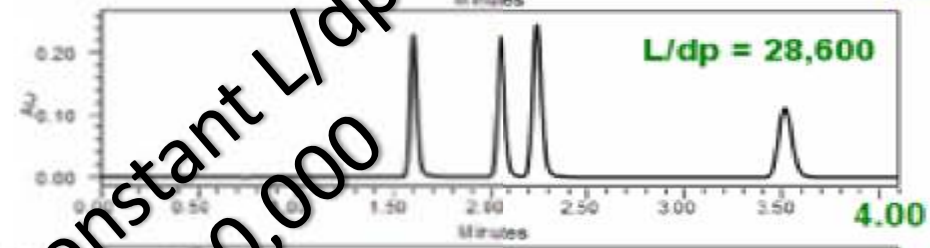
Scaling HPLC to UHPLC separations

HPLC

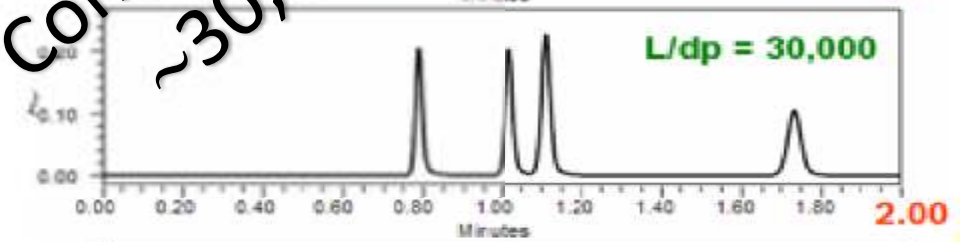


5 µm – 150 mm
Injection = 5.0 µL
Flow rate = 0.2 mL/min
Rs (2,3) = 2.28

Constant L/dp ~30,000

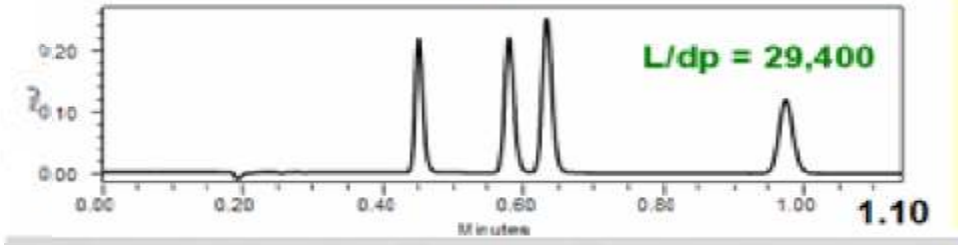


3.5 µm – 100 mm
Injection = 3.3 µL
Flow rate = 0.3 mL/min
Rs (2,3) = 2.32



2.5 µm – 75 mm
Injection = 2.5 µL
Flow rate = 0.5 mL/min
Rs (2,3) = 2.34

UHPLC



1.7 µm – 50 mm
Injection = 1.7 µL
Flow rate = 0.6 mL/min
Rs (2,3) = 2.29

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Selecting proper L/dp ration based on application difficulty

Application Difficulty	Example	Suggested L/dp Range
Extremely Difficult	Complex Matrix, Metabolite Identification	> 85,000
Difficult	Impurity Profile Degradation Study	> 50,000
Moderate Challenging	Related Compound Assay	> 30,000
Easy	Few Peaks, Well Separated (Fast) Content Uniformity, Dissolution	> 15,000

1.7 um 150 mm : L/dp 88,235

5 um 250 mm : L/dp 50,000
3.5 um 150 mm : L/dp 42,857
2.5 um 150 mm : L/dp 60,000
1.7 um 100 mm : L/dp 58,823

5 um 150 mm : L/dp 30,000
3.5 um 100 mm : L/dp 28,571

5 um 75 mm : L/dp 15,000
3.5 um 50 mm : L/dp 14,285

Analytical LC portfolio

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1220 Infinity II

Alliance

Nexera LC-40

Ultimate3000

HPLC : High Performance Liquid Chromatography

1260 Infinity II

Acquity A

Nexera LC-40 XR

Vanquish Core

UHPLC : Ultra-High Performance Liquid Chromatography

Nexera LC-40 X3

1290 Infinity II

Acquity H Class

Vanquish Flex

UPLC : Ultra Performance Liquid Chromatography

Performance ?

Feature benefit ?

Price ?

HPLC and UHPLC solvent delivery systems

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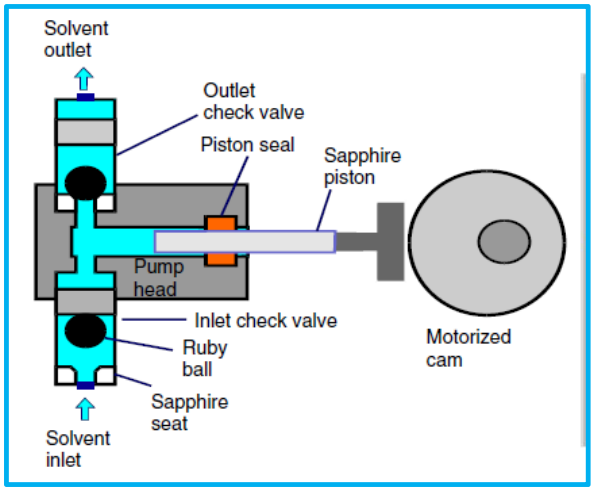
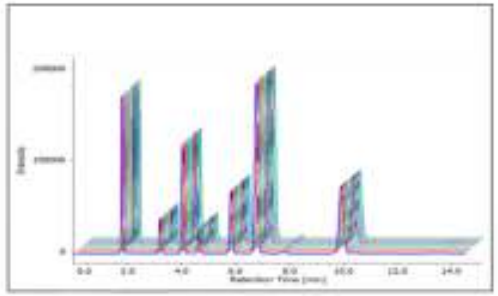
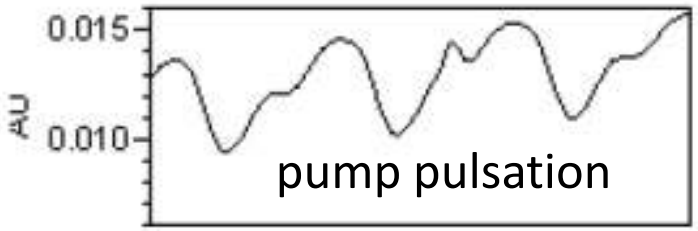
- Provides precise and pulse-free delivery of solvent
  - Typical flow rate range :
    - HPLC 0.01-10 ml/min
    - UHPLC 0.01-2-5 ml/min
  - Pressure limits :
    - HPLC 6000 psi
    - UHPLC 9000-12000 psi
    - UPLC 15000-22000 psi
- Compatible with common organic solvent, buffers, and salts
- Accurate blends solvents for isocratic or gradient operation







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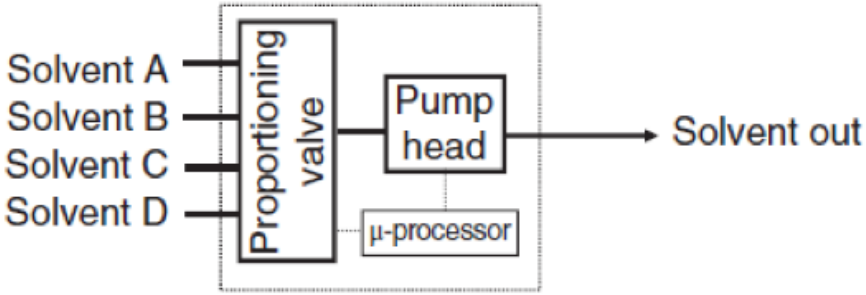
Low-pressure mixing designs pump

- All quaternary pumps use a low-pressure mixing design
- Solvent blending occurs inside the pump at low pressures
- Solvent degassing is mandatory to prevent outgassing of dissolved air during blending
- The advantages of low-pressure mixing pum
  sim



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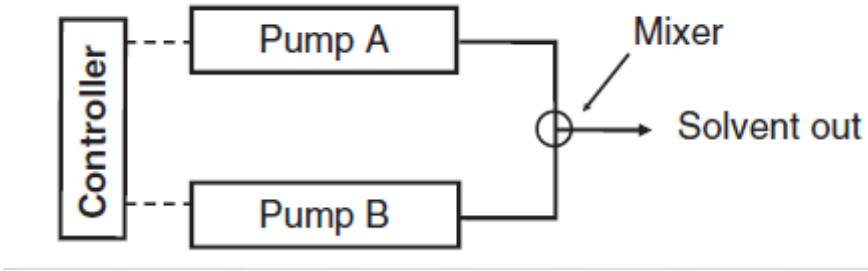
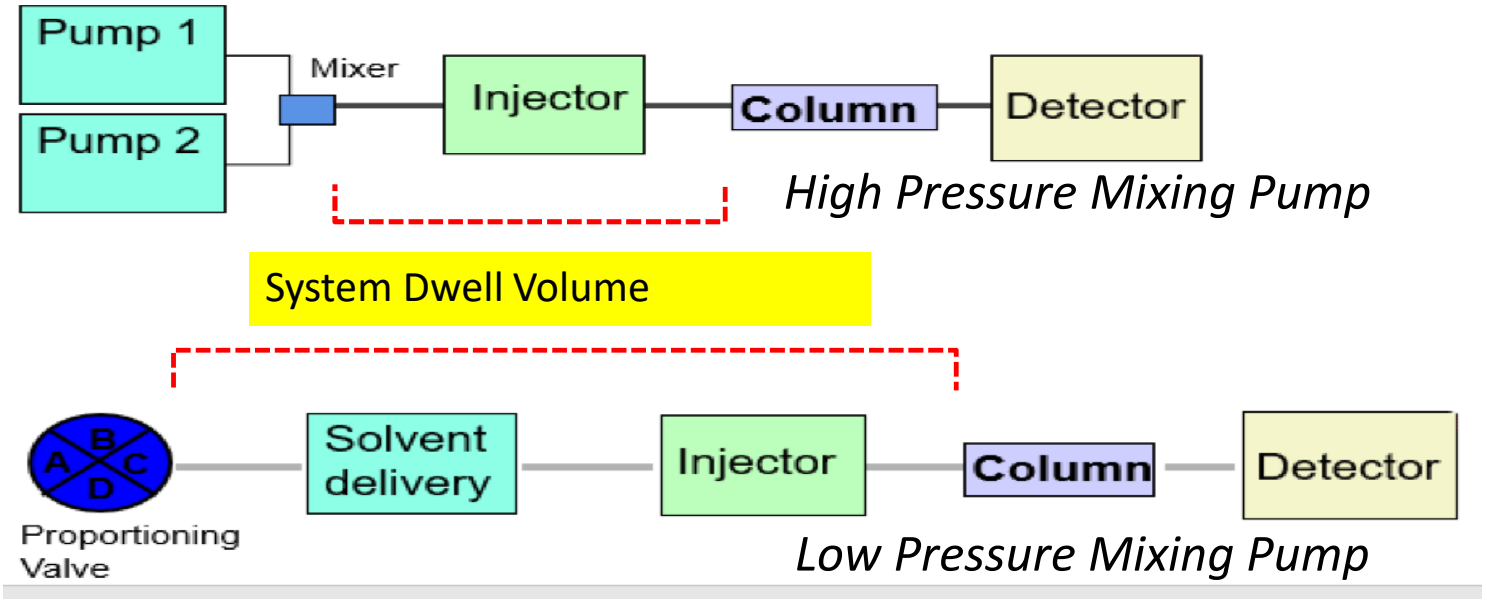
High-pressure mixing designs pump

- Two separate pumps are used to mix solvents at high pressures
- Flow rate change of each pump is used to generate different isocratic blends or gradient profiles
- An external mixer is required to ensure adequate mixing of the two solvents
- Binary pumps cost more but have the advantage of lower dwell volumes for applications using small-diameter columns



System Dwell volume

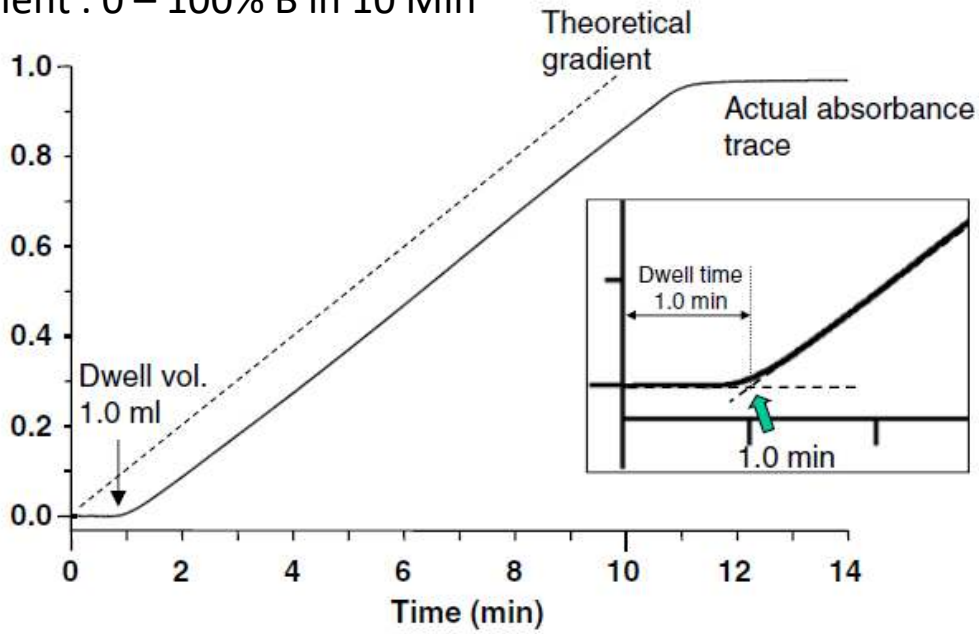
The volume of HPLC system from the point of solvent mixing to the inlet of the column



Dwell volume is inconsequential in isocratic analysis but becomes important in gradient analyses

System Dwell volume

Gradient : 0 – 100% B in 10 Min



- Typical Dwell volume of a low-pressure mixing HPLC system is ~1ml
- UHPLC systems typically have much lower dwell volumes than conventional HPLC
  - Binary system ~0.1-0.4 ml
  - Quaternary system ~0.4-0.8 ml

Dwell volume is inconsequential in isocratic analysis but becomes important in gradient analyses

System Dwell volume & gradient method

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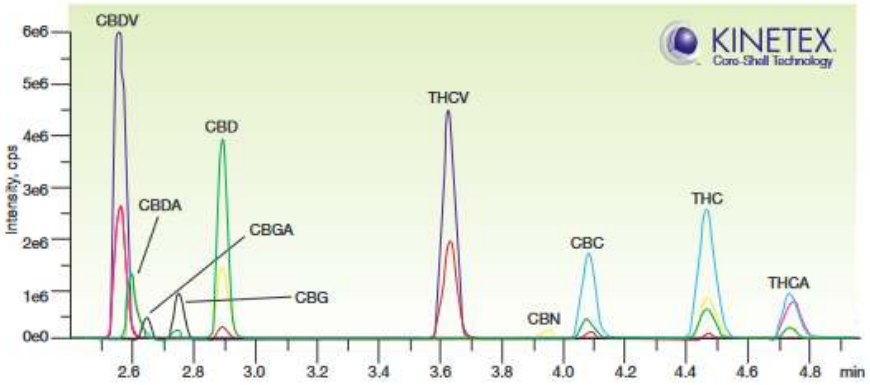
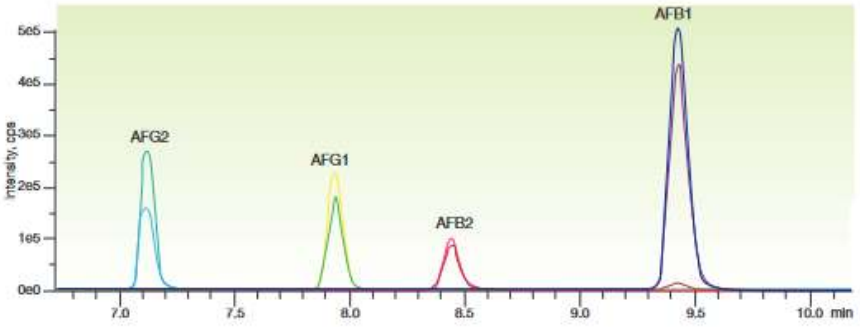
10 Cannabinoids using Kinetex Biphenyl by LC-MS/MS



4 Aflatoxins using Kinetex Biphenyl by LC-MS/MS



Conditions for both applications

Column: Kinetex 2.6 µm Biphenyl
Dimension: 150 x 4.6 mm
Part No.: 00F-4622-E0
Mobile Phases: A: Water + 5 mM Ammonium acetate + 0.1% Formic acid
B: Methanol/Water (98:2) + 5 mM Ammonium acetate

Gradient:	Time (min)	% B	
	0.75	5	
	4	50	Segment1
	5	60	
	5.01	78	
	8	88	Segment2
	10	92	
	12	100	
	13.80	100	
	13.90	5	
	16	0	

Flow Rate: 1 mL/min
Injection: 2 µL
Temperature: Ambient
Detector: SCIEX QTRAP® 6500+

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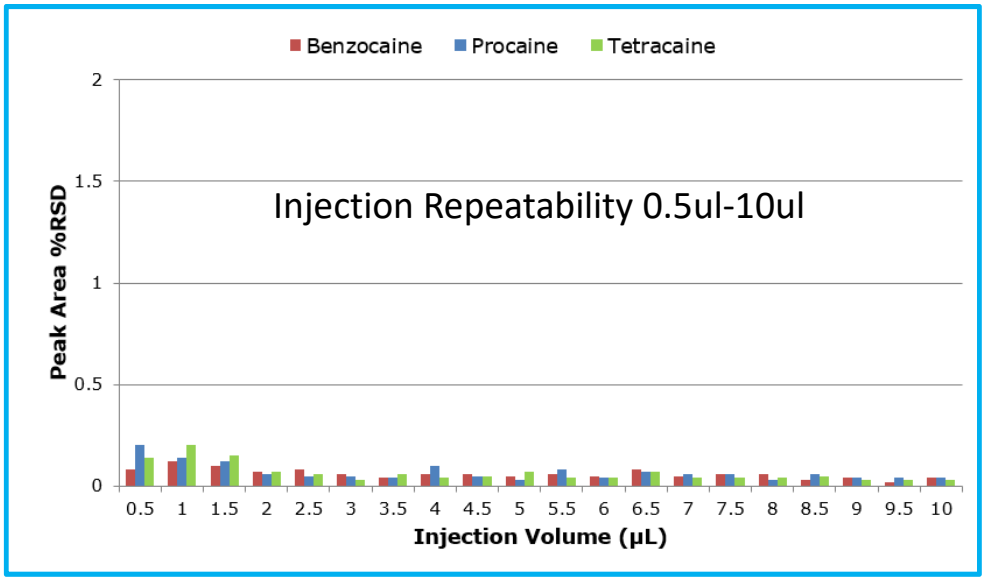
Autosamplers

The autosampler must have :

- Excellent precision at small injection volumes (%RSD <0.2)
- Injection volume range
  - Minimum for HPLC ~ 5 µl, UHPLC ~ 1 µl
- Low carryover
- High-pressure rating
- Lower dispersion
- Fast operation



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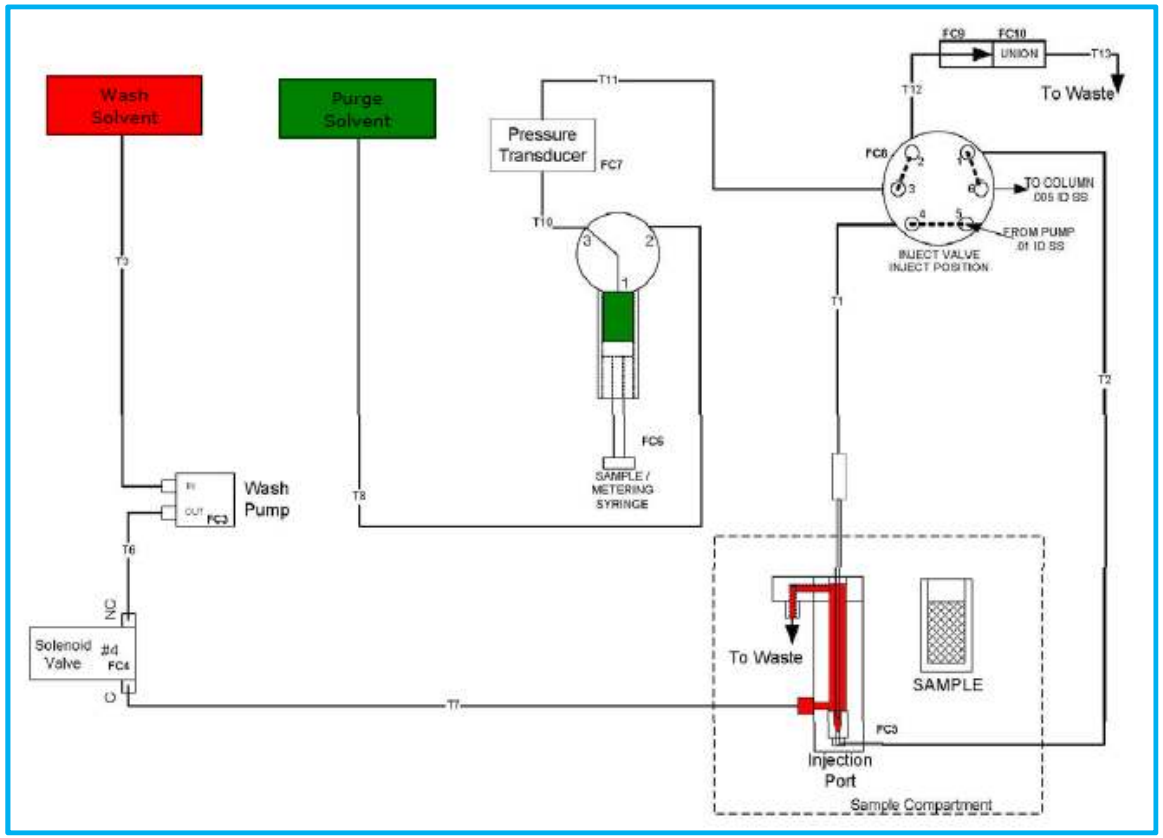
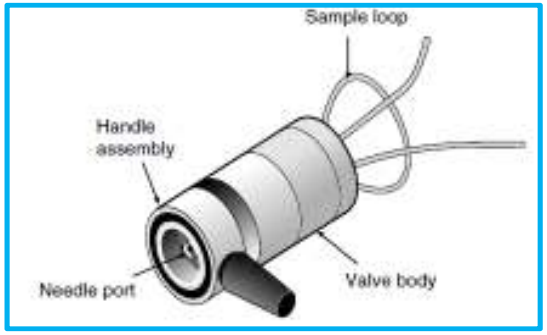
Injector flow diagram



Acquity H-Class Autosampler



Rheodyne Injector

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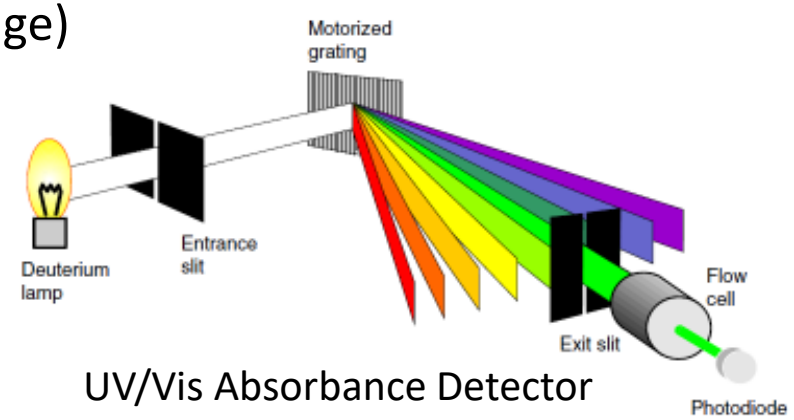
Detectors

Detector measures the concentration (or mass) of eluting analytes :

(sensitivity (noise), drift, and linear dynamic range)

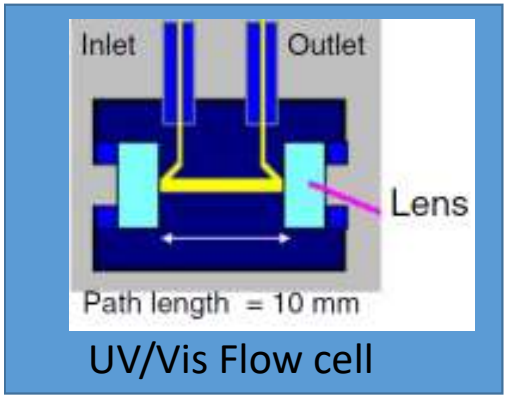
- UV/Vis absorbance
- Photo diode array (PDA)
- Fluorescence (FLD)
- Refractive index (RID)
- Evaporative light scattering (ELSD)
- Electrochemical (ECD)
- Conductivity



UV/Vis Absorbance Detector

Flow cell, analytical, 500nL, 10-mm path length
Flow cell, high sensitivity, 2400nL, 25-mm path length

	Volume (μl)	Path length (mm)
Analytical cell	10	10
Semi-prep cell	2.6	3
Microbore cell	2.6	3
Inert (titanium) cell	10	10



UV/Vis Flow cell

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Chromatography data systems (CDS)

Data acquisition

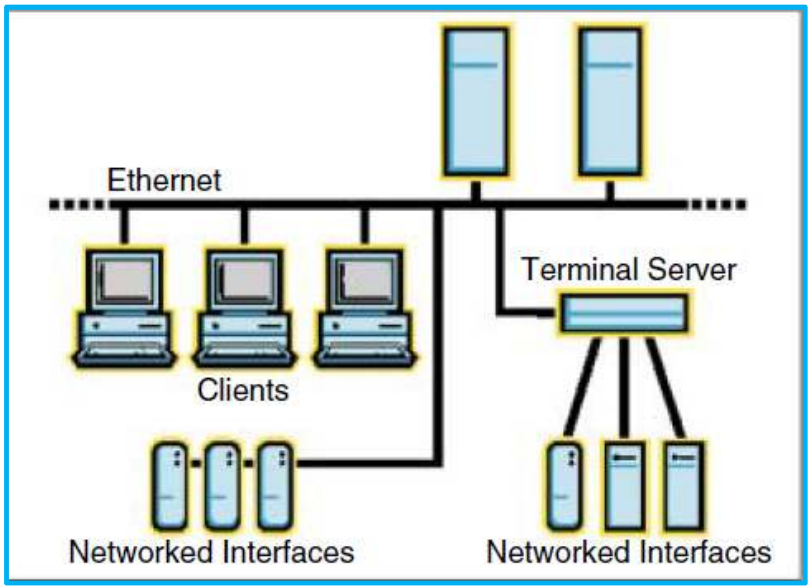
Integration
Calibration
Quantitation

Report formatting
Custom calculation

Raw data

Results

Reports



Client server CDS network

High detector sampling rate (acquisition > 20 Hz), fast detection constants (< 0.1 s)

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What System is right for my Laboratory ?

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In your laboratory…

1. Do you perform routine HPLC analysis ?	☐
2. Do you transfer chromatographic methods between laboratories and other global facilities ?	☐
3. Are you looking for tools to improve laboratory efficiency ?	☐
4. Do you analyze complex samples that require increased chromatographic resolution ?	☐
5. Do you perform method development, or are you considering method modernization ?	☐

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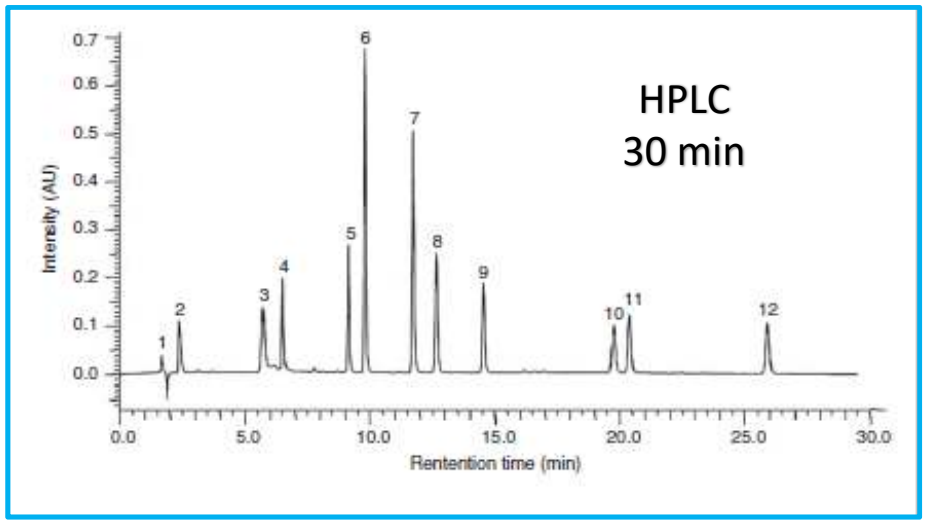
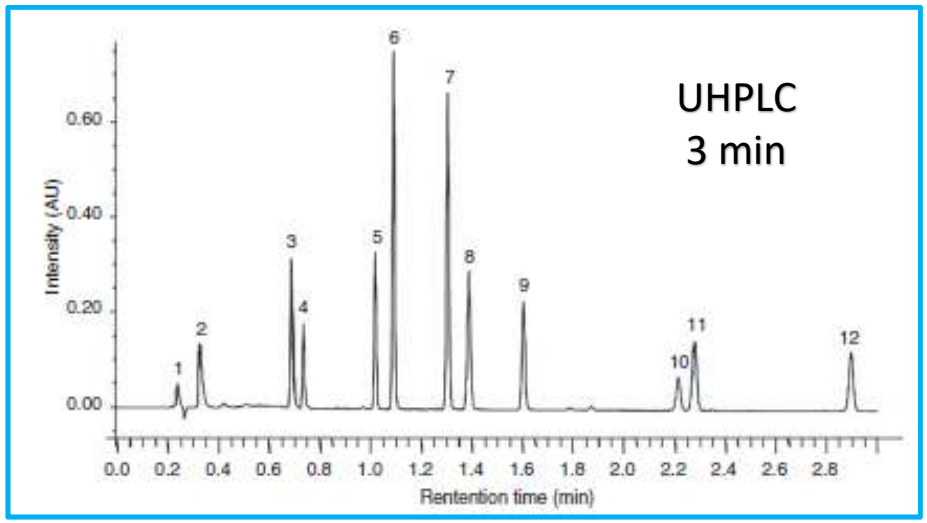
Benefits of UHPLC

- ***Fast separations with good resolution***
  - ✓Smaller columns packed with sub-2 or sub-3 µm particles
  - ✓Low-dispersion UHPLC Instruments





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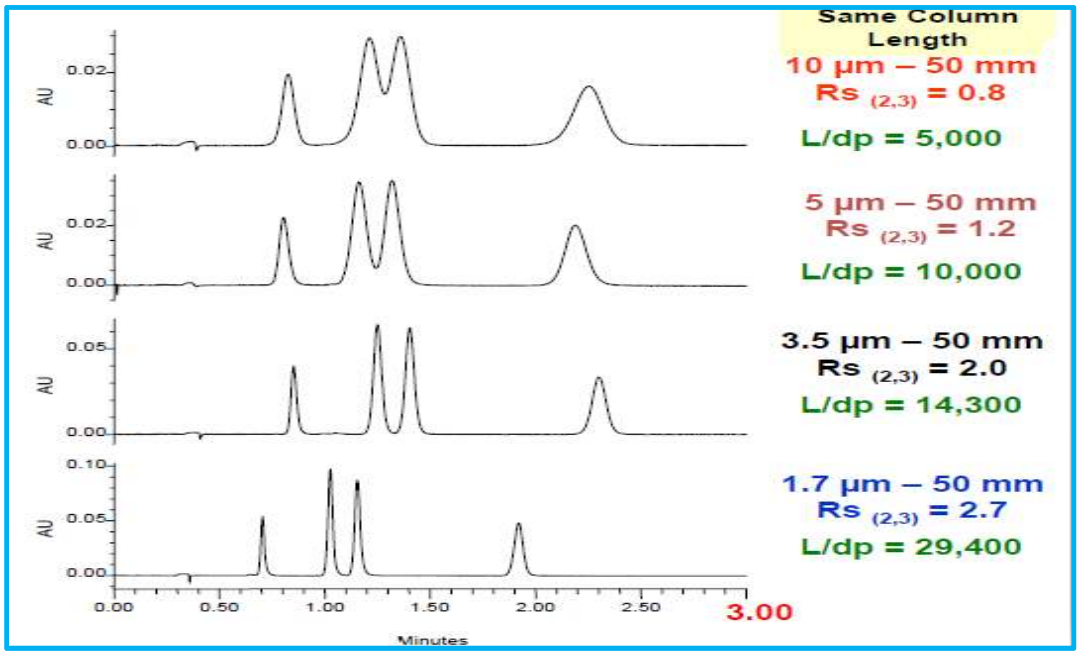
Benefits of UHPLC

- ***High-resolution analysis of complex samples***

  ✓Can used more high efficiency column

  (more L/dp)



Column Length mm	Particle Size µm	L/dp
250	5	50,000
250	3.5	71,400
150	2.5	60,000
150	1.7	88,200

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Benefits of UHPLC

- ***Rapid method development***
  - ✓Shorter analysis time and quicker column equilibration
- ***Solvent saving, higher precision, higher mass sensitivity***

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Column equilibration time

Column type	Column volume : $Vc\ hplc = 0.80\pi r^2L$ $Vc\ uhplc = 0.65\pi r^2L$ (ml)	Equilibration time : 10Vc (min)	Solvent used: (ml)
HPLC Column : 4.6x250 mm 5u	**3.32**	**22.1** (@Flow rate 1.5 ml/min)	**33.2**
UHPLC Column : 2.1x100 mm 1.7u	**0.23**	**4.6** (@Flow rate 0.5 ml/min)	**2.3**

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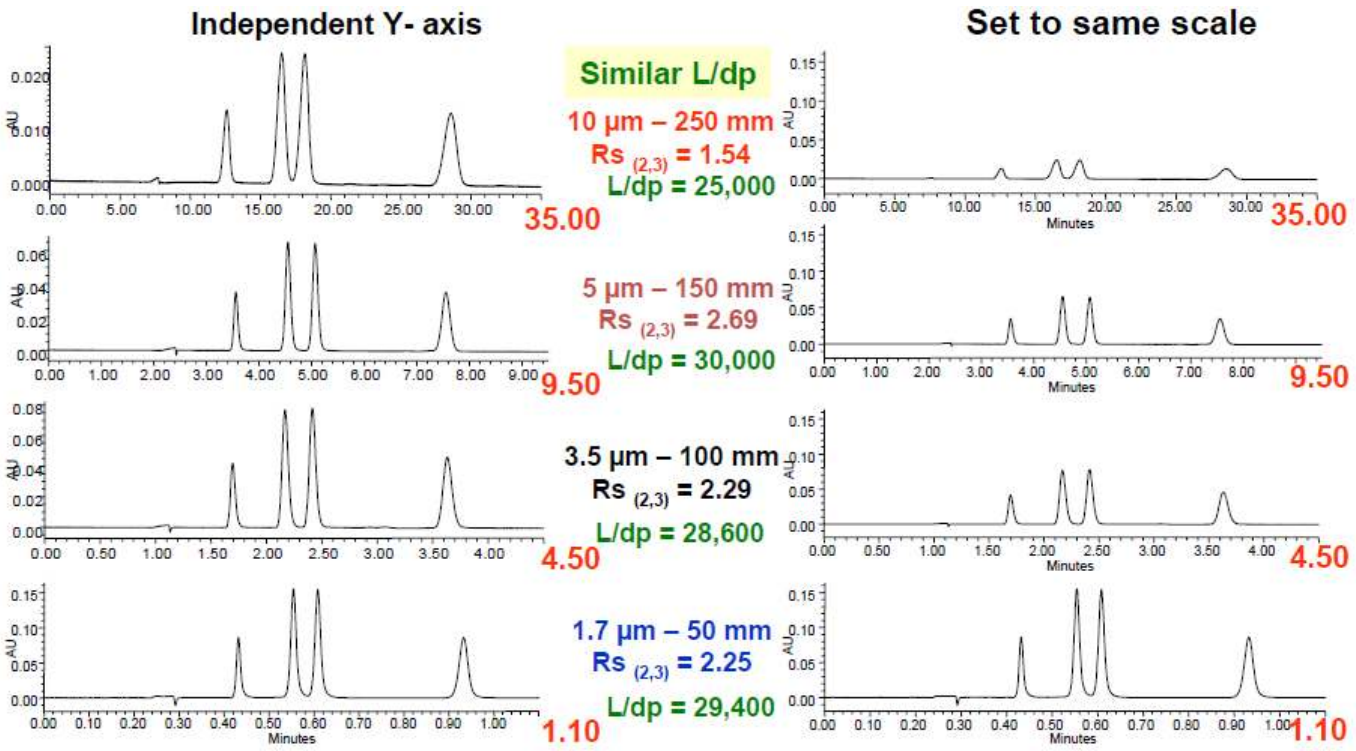
Increased resolution, same speed



Increase in sensitivity as particle size is decreased

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Instrumentation for UHPLC

- Robust pumping and injection modules working at high pressures
- Small gradient delay volume
- Fast injection cycles (< 1 min)
- Low sample carryover
- Minimum system dispersion
- More system accuracy/precision (flow rate & injection volume)
- High detector sampling rate (acquisition> 20 Hz)

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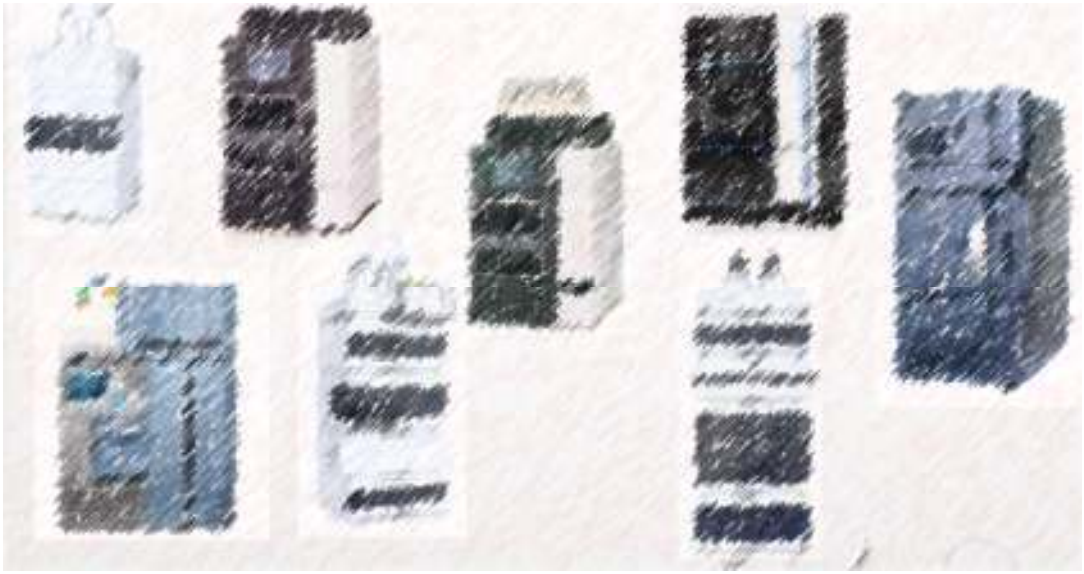
What System is right for my Laboratory ?



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Questions ?