Swiss Labs

Consulting & distribution for laboratories

Liquid Chromatography

Fundamentals and useful parameters

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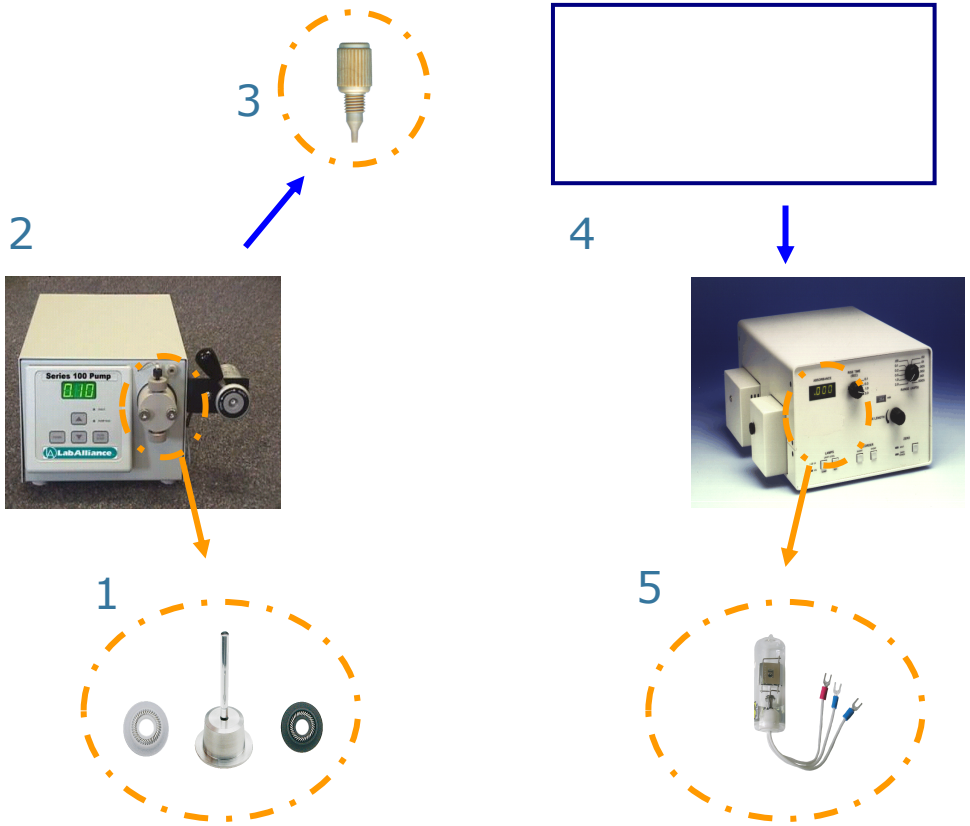
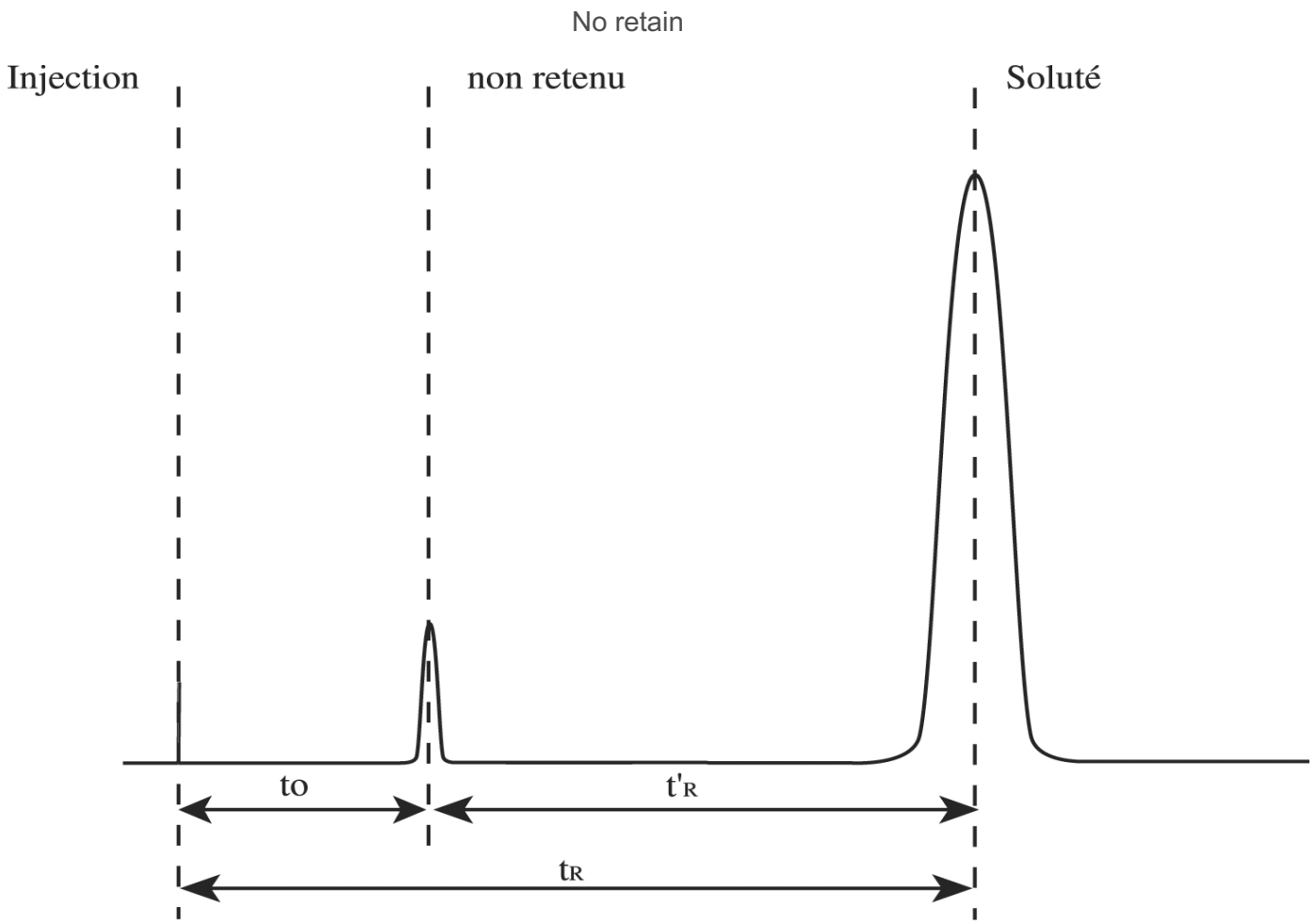
1. THE HPLC channel

1.1 The scheme



1 - Piston and piston joints (sapphire, ceramics,...)
2 - Filter (acts on sample)
3 - HPLC Lamp
4 – Saturation precolonne (protects the separation column from the aggressiveness of the moving phase)

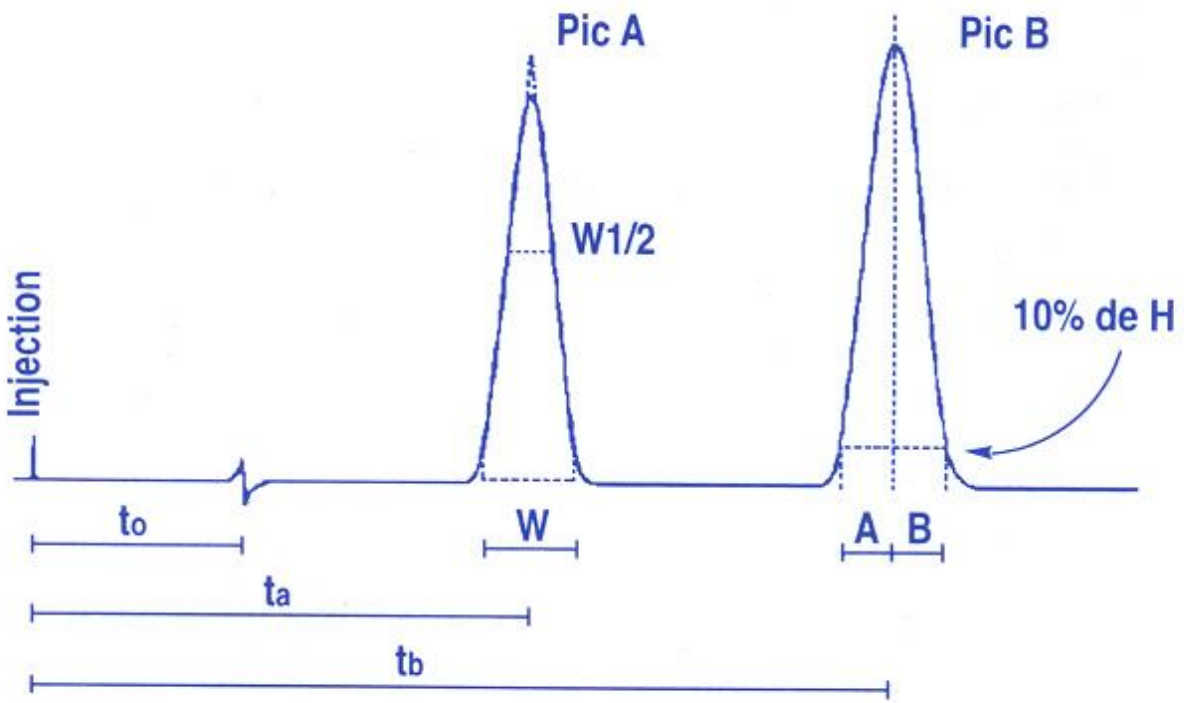
2. Retention settings



constant	definition	feedback
V_0	Dead (or empty mL) volume of the column $V_0 = D \times t_0$	This is the retention volume of the non-retained. It is related to the vacuum between and into the particles (for a silica of **5µm,** 50-75% of the geometric volume of the column)
t_0	Retention time of a non-retained compound (min)	An unre retained compound is a compound without interaction with the stationary phase (not to be confused with an excluded compound*)
D	Flow of vector fluid in the column (mL/min)	
t_R	Retention time measured at the top of the peak (min)	Time spent by the compound in the chromatographic apparatus but which is likely to vary depending on: flow (leakage), temperature (season), mobile phase (evaporation, stabilization additives)
t'_R	Reduced retention time $t'_R = t_R - t_0$	Time to stay of a compound spent in or on the stationary phase
k	Retention (or capacity) factor $k = (t_R - t_0) / t_0$	Fundamental parameter characterizing the retention of the compound by the chromatography system independent of the flow. For the same compound and 2 different analyses, if we observe: 2 $t_R \neq$ and 2 k = : leak problem before the column -2 $t_R \neq$ et 2 k $\neq$: mobile or stationary phase problem

Table 1:
Compound whose molecular volume prevents its diffusion into the pores of the gel

3. Settings related to the effectiveness of the equipmen



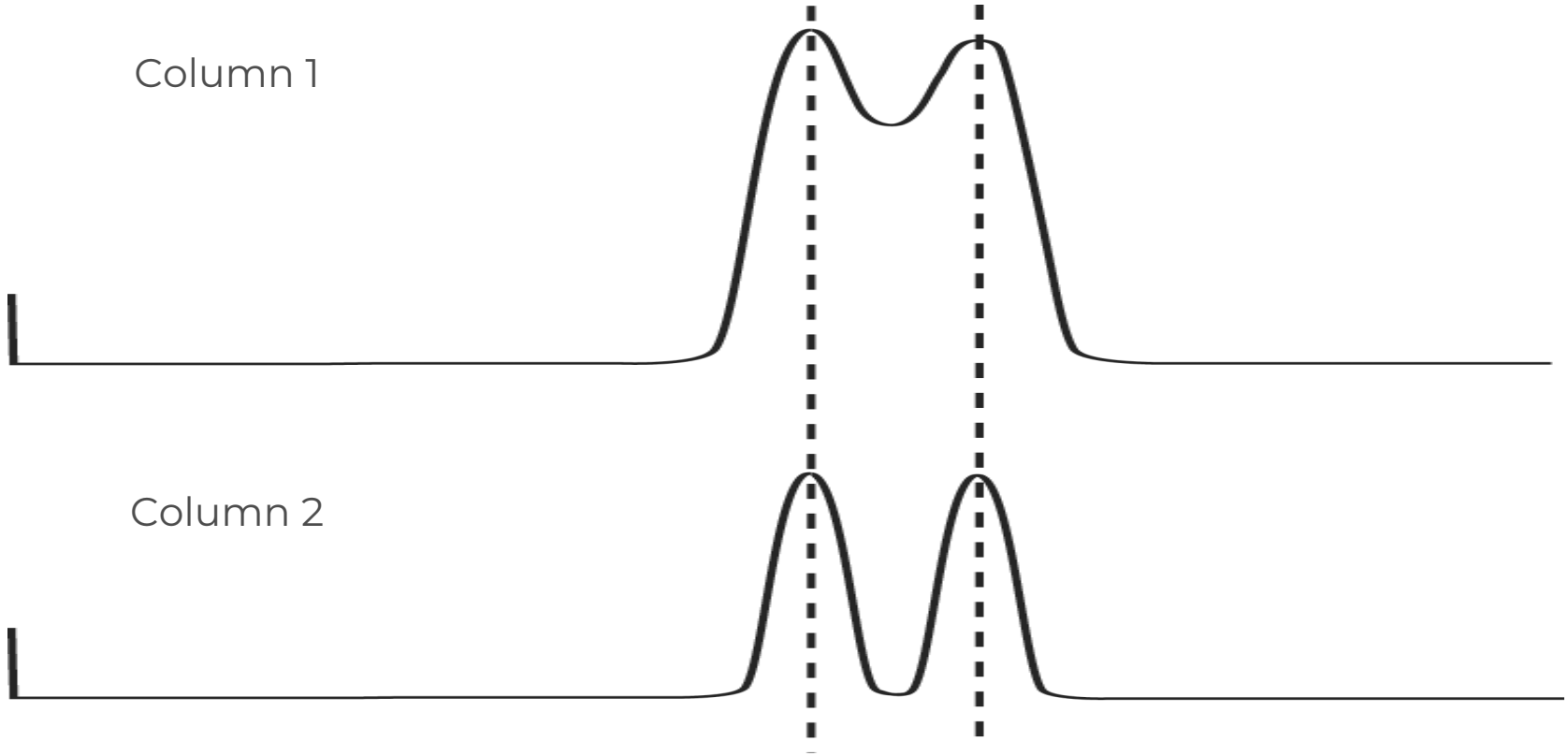
t_R : retention time

W_t : width of the peak at the base in a time unit

constant	definition	feedback
N (symmetrical peak) N (asymmetrical peak)	efficiency $N = 16 (t_R/W_t)^2$ $= 5.54 (t_R/W_{1/2t})^2$ $N=41.7*(tr/w_{0.1})^2)/(A/B+1.25)$	Theoretical plateau is the stationary phase portion in which a solute is in a balance of distribution between the moving and stationary phase (each tray can then be likened to a decanting bulb)
H	Height of a theoretical plateau $H = L / N$ (L: column length)	A low H means better efficiency
h	Height of a reduced theoretical plateau $h = H / d_p$	Fundamental size to compare two chromatographic systems, ignoring column geometry and filling.
d_p	Particle diameter	Generally referred to as "granulometry"
As	asymmetry $As = B/ A$	Peak asymmetry at 10% height
N (symmetrical peak) N (asymmetrical peak)	efficiency $N = 16 (t_R/W_t)^2$ $= 5.54 (t_R/W_{1/2t})^2$ $N=41.7*(tr/w_{0.1})^2)/(A/B+1.25)$	Theoretical plateau is the stationary phase portion in which a solute is in a balance of distribution between the moving and stationary phase (each tray can then be likened to a decanting bulb)

Table 2:
Efficiency (characterized by N) increases when L increases and when dp decreases for a fixed debit and temperature.

4. Separation-related settings



constant	definition	feedback
α	selectivity $\alpha = t'_{R2} / t'_{R1} = k_2 / k_1$	Details the relative positions of two adjacent peaks $\alpha \neq 1$: necessary but not sufficient condition for a good resolution. In addition, it takes a return to the baseline of the first peak before the second peak starts.
Rs	$R_s = \frac{\sqrt{N}}{4} \frac{\alpha - 1}{\alpha} \frac{k_2}{k_2 + 1}$	In analytical HPLC, it is estimated that the minimum resolution to detect two nearby height peaks must be 0.6 and 1.5 for a return to baseline.

Table 3

Practical tips for developing an analysis method

Step 1: Uptiselect Kit

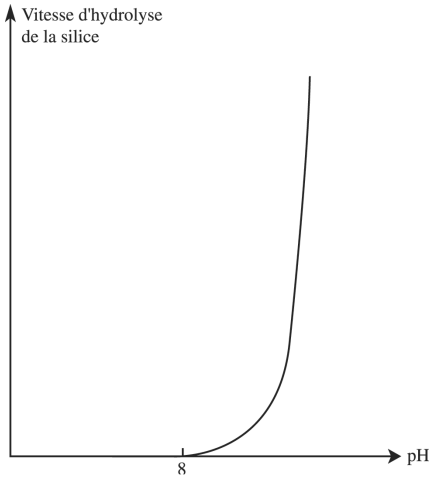
Step 2: Settings that influence the development and optimization of an HPLC method

2.1 Sample

Knowing the nature of the sample (miscibility, ionisable or non-ionisable compound, pKa, stability in relation to pH, polarity,...) is important to make the right choices in the next steps. If the composition of the mixture is known, it is strongly advised to obtain the standards corresponding to each of the different solutes of the mixture, in order to better understand their individual behavior.
If the mixture is unknown, it is important to think about the different steps that led to this mixture in order to get an idea of the type of solute that can be found there.

Influence of pH

Consider dabbling the eluent : keep in mind that a simple change in temperature can cause a change in pH if the medium is not buffered. However, a small change in pH can cause large variations in retention time (as shown in the graph below).

	acid	base
buffer	Stamp at a pH < of 2 or 3 units at pKa	2-unit pH > stamp at pKa
feedback	From pH 2, a short graft is more easily hydrolyzed! (e.g. C8 versus C18) → If the pKa of the product is too low, then you have to work on a pH such that the ionized form is always present. In this case, it is necessary to add a counter-ion that will form a neutral aggregate that will be retained on the column.	 From pH 8, silica is increasingly water soluble → If the product pKa is too high: use a counter-ion

Preparing the sample

It may be useful to extract the solute through a solid SPE extraction method (see our "SPE Guide"). Always filter the sample: in general, it is advisable to use a porosity filter 0.45 µm

2.2 Column Choice

Column choice based on mobile phase pH

pH	column
2 < pH < 8	Compatible with most chromatography support
pH < 2	Be careful with the use of low-chain alkyl grafted silica.
pH > 8	Use of a polystyrenedivinylbenzène gel (PSDVB, stable until pH12) or silica specially treated to support pH ranging from 8 to 10 (HSC type). To overcome the problem of silica dissolution, the mobile phase can be saturated in silica by introducing a saturation column between the pump and the injector.

Choosing a porous or non-porous silica

The use of porous silica increases the contact surface with the solute (it is used preferably, except in cases where the interaction of the solute with the stationary phase is already very strong). Pore diameter depending on the mass of the compound.

100A	Up to 2.5K Daltons
300A	1.5 K to 400 K Daltons
500A	Up to 1000 K Daltons
1000A	Up to about 1500 K Daltons
4000A	Up to 10,000 K Daltons (not mesured)

Choosing the type of silica

First-generation silica have a high metal residue, which makes residual sianols more acidic and therefore more reactive to basic compounds (hence spikes).
On the other hand, second-generation silicas are processed during their manufacturing processes: the residual metal level is extremely low, thus decreasing the spikes.
Example : Uptisphere: 99.995% pure with traces of metals less than 20ppm (Fe less than 1ppm).

It is therefore preferable for the analysis of basic compounds to use:

- Second-generation ultrapure silica (traces of metals in silica have been removed).
- End-crapped silica: End-crapped consists of a treatment of silica to cover residual silanols by a trimethyle or other grouping.
- Added AME (pure and distilled) in the eluent : AME has a strong affinity for residual silanols (more responsiveness than basic compounds) and has the advantage of not being detected in UV.
- Grafting having stical bulking ups so as to make access to residual silanols more difficult.
- Grafting with embedded polar clusters (shielded type)

Shielded column pattern

Porous graphite: The separation of non-polar compounds is similar to that observed on a C18, but with increased resolution and selectivity. An unexpected and increased retention of polar compounds was highlighted.

ODS, ODS1, ODS2, ODS3, ODS-AM, 

adécylyle with different chemistries.

The choice of graft is to be defined according to the solute

Compounds to be analyzed	Proposed grafting
Low hydrocarbon compounds	Normal phase (silica or diol)
Medium hydrocarbon volume compounds	Reverse phase (C18, C8, C4...)
High-volume hydrocarbon compounds	Reverse phase (C4, C8, C18...)
Amines	Ultra-pure "end-capped" silica recommended
Adehydes, ketones	Non-end-capped silica (residual silanols help with separation)
Phosphates	Non-ultra-pure silica
Compounds including an aromatic group	Phenyl graft
Compounds including a carboxylic grouping	Cyano graft
sugars	NH2 or NMe2 graft
If you want to work with an eluent who has water in the normal phase	Diol and non-virgin silica
Drug in biological fluid	"Mixed mode" (RP, SCX, ...)

As the C18s are the most frequently used columns, this guide devotes an entire chapter to them.

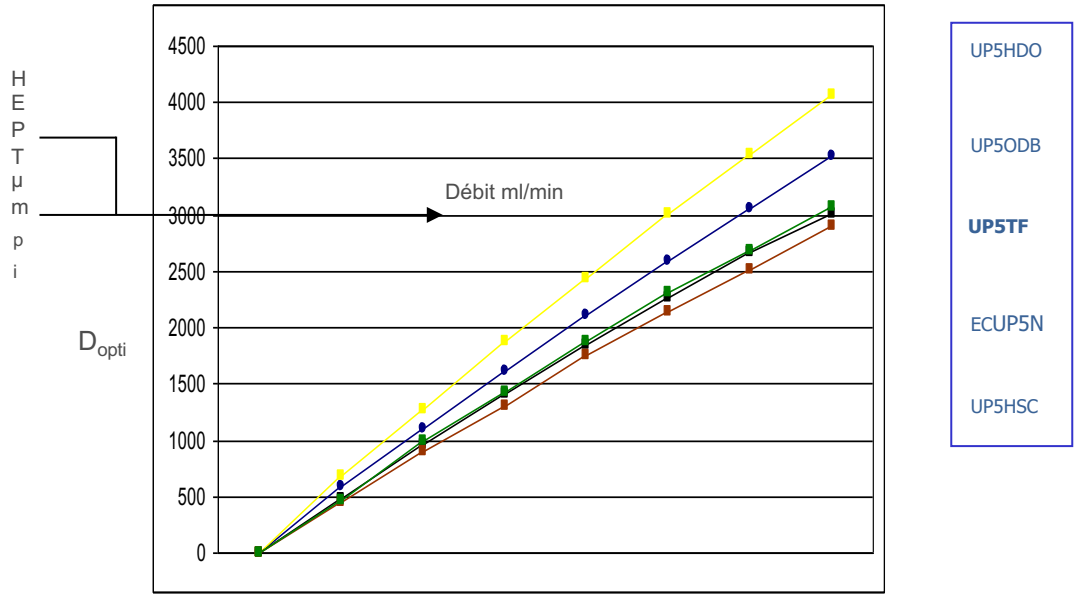
2.3 Pressure Limit

The HPLC columns (silica support) are filled under very high pressure (500-800 bars). Nevertheless, it is recommended not to use the columns at a continuous pressure higher than 3500psi (250 bars).
Working at high pressure limits the life of the spine and above all causes premature wear of the hpLC pump's high-pressure joints.

Typical Uptishere 250 x 4.6mm column value; 5 µm:
Mobile phase: 1mL/min, 25 degrees
ACN/H2O 60/30 100 bars
MeOH/H2O 70/20 160 bars

2.4 Debit Choice

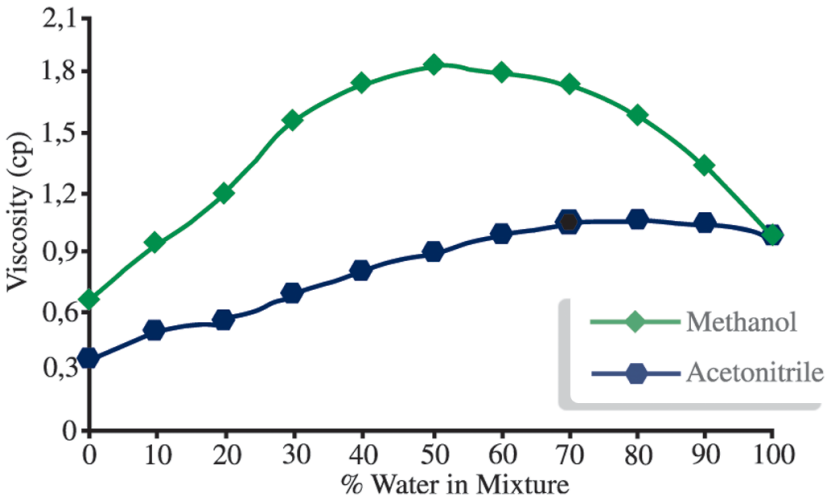
Silica-based HPLC columns can theoretically accept any flow as long as they respect correct pressure. Typical pressure relationship based on flow on Uptishere C18 columns:



2.5 Mobile phase choice and optimization

Difference between methanol and acetonitrile

In most cases, the problem boils down to a choice between methanol and acetonitrile.
Acetonitrile is less viscous, more efficient and allows you to work at higher mobile phase rates.



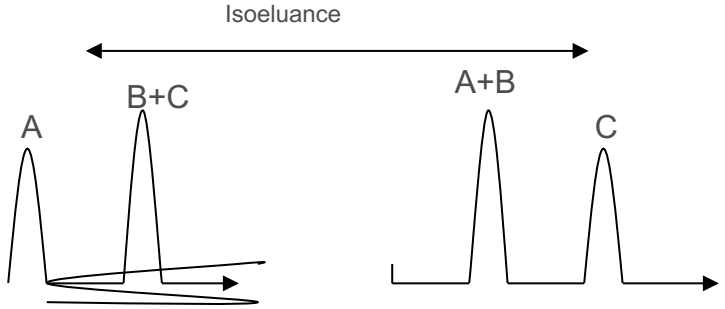
Variation in viscosity between methanol and acetonitrile

Isoelution rule : ϕ AcN = 0.78% ϕMeOH
ϕ THF = 0.62% ϕMeOH

For example: a mixture 50% MeOH - 50% water is equivalent)
39% can – 61% water
31% THF – 69% water

Mobile Phase 1 =
20/50MeOH/H2O

Mobile Phase 2 =
39/61 CAN/H20



2.6 Temperature Choice

It is important to thermostatize the column AND the tubing (especially to avoid a temperature gradient within the column itself).

In general, high-temperature work facilitates the separation, diffusion of compounds in both phases is favored, hence the increase in transfer speeds between the stationary and mobile phase and thus the number of exchanges.

2.7 Influence of injection loop volume

Uptisphere COLUMN ODB 150-4.6mm, 5m					
loop(µl)	10µL	20µL		50µL	
efficiency(N)	Nref		ΔN%		ΔN%
Toluene	9518	7116	-25%	2603	-72%
Naphthalene	9251	7276	-21%	2613	-71%

The volume to be injected should be less than 1/100 of the dead V0 volume of the column, knowing that V0 ≈ 65% of the geometric volume of the column. For good reproducibility, it is advisable to fill the injection loop with a volume five times that of the loop.

2.8 Influence of dead volume created by the turbulry between injector and column

Internal diameter (mm)	0.25	0.75		0.75	
Length (mm)	100	100		200	
Efficiency (N)	Nref		ΔN%		ΔN%
Toluene	9518	6819	-28%	4982	-27%
Naphthalene	9251	7030	-24%	5470	-22%

The table shows the loss of efficiency due to too much length and or internal diameter of the capillary.

2.9 Choice of turbulry diameter

Internal column diameter (mm)	Internal diameter of tubing (mm)	Flow (mL/min)
1.0-2.1	0.15	0.05-0.2
2.1-3.0	0.15-0.20	0.20-0.50
3.0-3.9-4.0-4.6	0.20-0.25	0.50-1
4.6-6.0-7.8-9.4-10	0.25	2-10
10-21.2	0.25-0.50	10-25
50	0.5-1	50-100

Step 3: Sensors

REMINDER

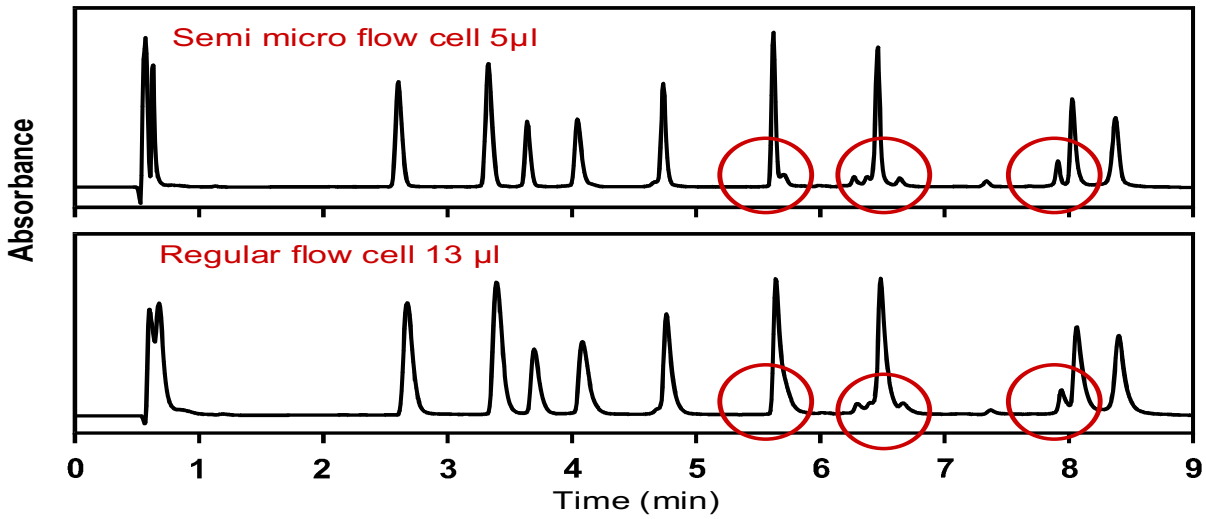
The limit height of detection of a compound is equivalent to three times the height of background noise.
The quantification limit of a compound is equivalent to ten times the height of background noise.

3.1 UV Detector (185-400nm)

For aromatic compounds
- UV Spectrophotometer
Detection is done at a given wavelength.

- Diode bar
Gets a chromatogram for each wavelength you choose

Influence of the volume of a UV spectrophotometer detection cell



The resolution is increased with a low volume detection cell but beware:

- Not to inject a solution too concentrated (risk of saturation)
- Overpressure if the flow is the same (risk of breaking the detection cell)

3.2 Fluorescence Detector

For compounds with many elections Π relocated such as poly-aromatic hydrocarbons, or rendered fluorescent by bypass

1000 times more sensitive than the U.V.

Trace analysis

3.3 Refraction Index Detector

For compounds that do not absorb in the visible U.V. or in series with other detectors.

Measures the difference in refraction index between the moving phase and the compound.

→Universal detector

→ Less sensitive than UV

→ Drift from baseline to the slightest change in temperature and pressure

3.4 Mass spectrometry detector

For all compounds with no limit of concentration and molecular weight.

→ Very good sensitivity

→ Information on the structure of the compound

→ Different Ions following the solvent of the mobile phase

→ Relatively complicated to set up

Step 4: Optimization

Column geometry, flow, the nature of the moving phase and the stationary phase are parameters that greatly influence separation and resolution in chromatography.

Their control requires a greaty knowledge and experience.
What is the majority and priority objective?

Performance: better mixing separation, minimal detection
Cost: analysis time, amount of solvent, lifespan of equipment

Ways to optimize resolution

It's about playing on these different parameters:

1/ Stationary settings

HPLC channel (injector, filter, pre-column, tubing, column,...)
Sensors (detection cell, detection mode,...)

2/ Dynamic settings

Increase k, change :
- Mobile phase composition
- The specific surface of the support
- The grafting rate

Increase α , change :
- The nature of the organic solvent
- The pH of the buffer

Increase N, change :
- The flow rate of the mobile phase
- The length of the column
- The particle diameter

All these parameters have been discussed in the previous paragraphs.

Step 5: Memo

Many parameters were mentioned in this guide, here are some useful reminders:

- Column length: Efficiency increases with the length of the column.

- Column diameter: the detection threshold increases as the column diameter decreases.

- Particle diameter: efficiency increases as particle diameter decreases.

- Mobile phase: acetonitrile is more effective than methanol.

- Bounding silica attention to pH.

- Buffer solution: pka-2< pH<pka-2

- If the pka is too low or too high consider using a counter-ion.

- For repeatable analyses it is necessary to stabilize the column by rinsing it with the mobile phase.

- The volume must be at least equal to the equivalent of :
10 or 20 x Vo in reverse phase mode
50 x Vo in normal live mode