Method Development & Optimization

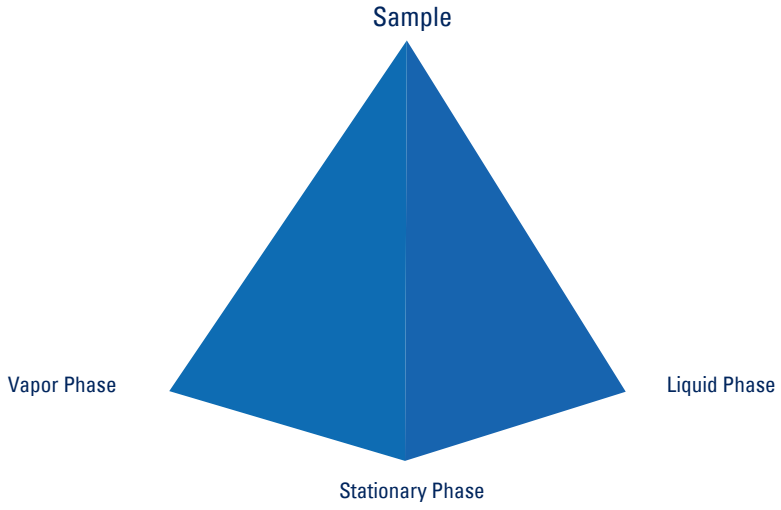
TLC Fundamentals

Basic principle:

TLC, compared to liquid chromatography on column, shows differences:

The mobile phase discovers the stationary phase while moving through the plate. The thin layer is not in equilibrium with the elution solvent, as it is the case in a column, but with the solvent vapors contained in the development chamber.

To set up a TLC analysis, 4 parameters have to be considered.

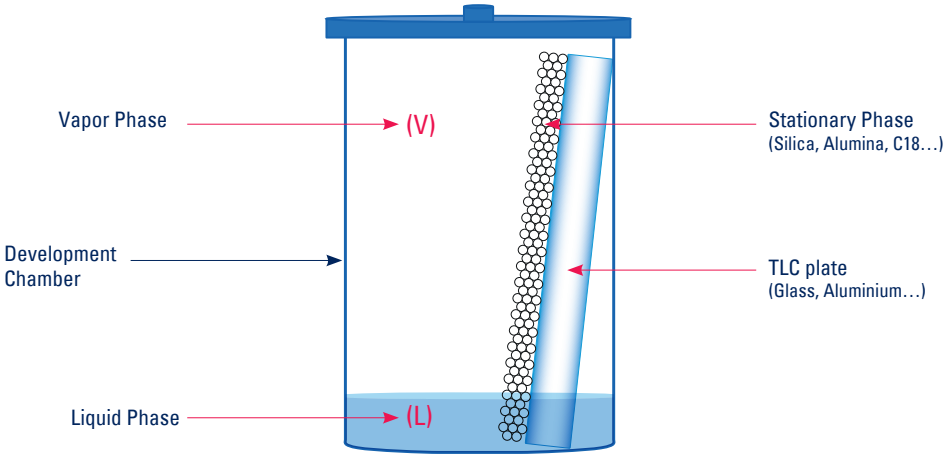


Basic principle:

TLC is a chromatography where each of the solutes remains the same time in contact with the mobile and the stationary phases. They travel different migration distances according to their interactions with the phases, whereas in an HPLC column, solutes go through the same total distance. They express a different residence time.

In TLC, the retention of each of the solutes is then characterized by the frontal ratio Rf, whereas on column it is characterized by the retention factor k.

(In the case of a preparative column, the volume of retention, of the mobile phase required to elute the solute, is a considered value.).



Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Basic principle:

Interactions description :

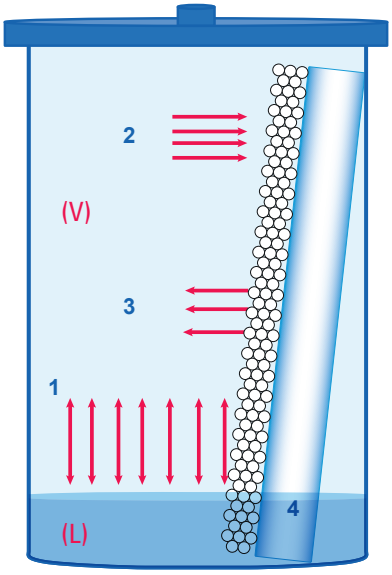
Specificity of the TLC related to evaporation phenomena

1: At the liquid (L) - vapor (V) balance, the mobile phase and the vapour phase compositions are not similar because the vapour pressure of the solvents used are generally not the same. At the liquid (L) - vapor (V) balance, according to the composition of the development phase and the respective vapor pressure of its components, the composition of the vapor phase is not the same as the development phase.

2: The dry stationary phase equilibrates with the vapor phase (V) (adsorption saturation). The vapors of polar solvents are much more adsorbed than those of apolar solvents. The composition of the adsorbed phase is different from those of the vapor phase (V) and the development phase (L).

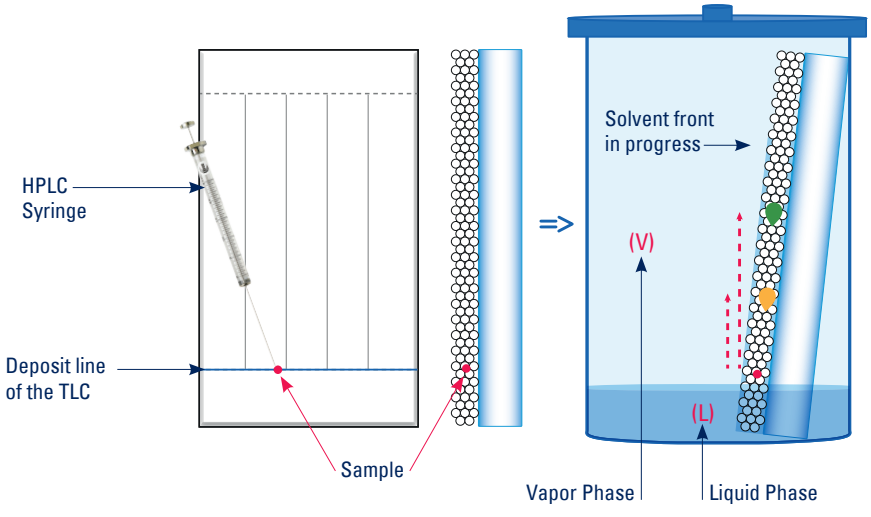
3: During migration the wet stationary phase is re-equilibrated with the vapor phase (V). This concerns the less polar solvents and the more volatile of the migrating liquid.

4 : During the migration the components of the mobile phase can be separated by the stationary phase which leads to secondary fronts.



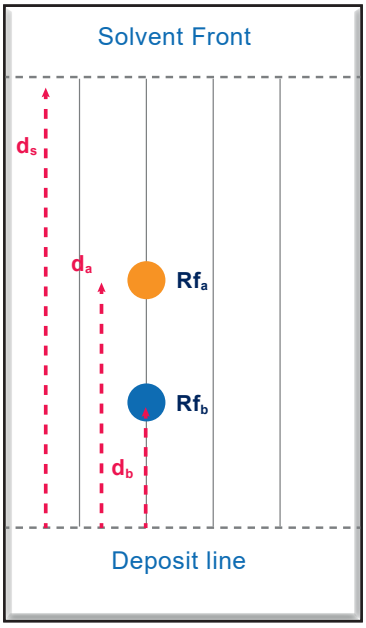
Basic principle:

The sample is dropped off, with a capillary, on the deposit line of the TLC plate which is then immersed in the tank containing the mobile phase. This one ascends through the stationary phase by capillarity carrying each compound which moves at its own velocity behind the solvent front according to its affinity for the stationary and the mobile phases.



Basic principle:

In TLC, the "retention factor" (Rf) is defined by the ratio of the distance traveled by the analyte (da) over the distance traveled by the solvent front (dS).



$Rf_a = d_a / d_S$
$Rf_b = d_b / d_S$

In practice, it is necessary to reason in the amount of mobile phase necessary to use to elute the solute out of the column. To take into account there different geometries, this retention volume is expressed relative to the void volume of the column used.

It is a dimensionless number identified by the acronym CV (also called Vs)

$Vs_a = CV_a = 1/Rf_a = 1 + k_a$

$Vs_b = CV_b = 1/Rf_b = 1 + k_b$

$\Rightarrow \Delta CV = CV_b - CV_a$

There is a mathematical relation between CV and the retention factor k in liquid HPLC =>

$k = K_{tr} x(1/Rf - 1)$ et avec $K_{tr} = cste = 1$

$\Rightarrow \Delta k = K_{tr} x[(1/Rf_b - 1) - (1/Rf_a - 1)]$

$\Rightarrow CV = \Delta k$

In practice, it is necessary to control the experimental conditions to obtain reproducible TLC analysis.

Operational modes:

Different operational modes are commonly used to make aTLC plate => Unsaturated, Saturated, Pre-Conditioning & Sandwich

For all of those, double tray chamber are recommended.

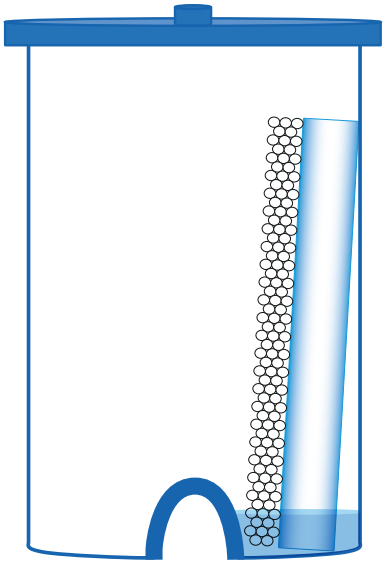
The equilibration of the stationary phase with the mobile phase has a strong influence on the repeatability of a thin-layer chromatography.

1. Unsaturated mode:

Compared to other modes, no equilibration leads to thinner spots (bands) and larger RFs.
However there are secondary fronts due to mobile phase de-mixing.

One tray is filled with the mobile phase.
Just after, TLC plate with "dropped off" compounds is placed as vertically as possible in the same tray.

This technique leads to low repeatability vs. others mode.



Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Operational modes:

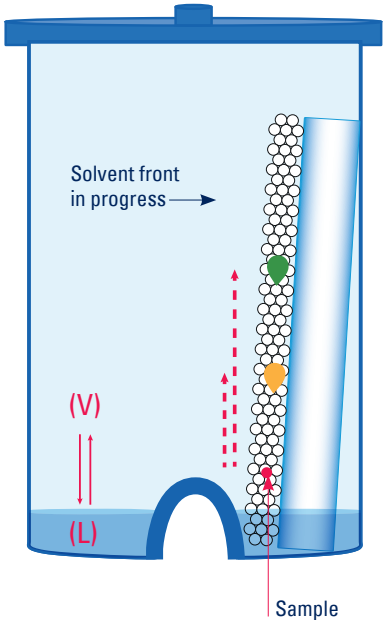
2. Saturation mode:
Equilibration of the development chamber with saturation by the solvent vapors.

The both trays are filled with the development phase.
A sheet of paper, vertically placed, dips into the mobile phase of one of the trays. Wait 20/30 minutes so that the mobile phase goes up by capillarity on the paper and is homogenized in vapor concentration.

By liquid-vapor equilibrium, the gas phase recondenses on the plate and wets it throughout its height.

After moving the lid laterally, the TLC plate is placed as vertically as possible in the second tray. Driven by the mobile phase, from the bottom of the chamber, solutes travel through the stationary phase in equilibrium with the adsorbed mobile phase.

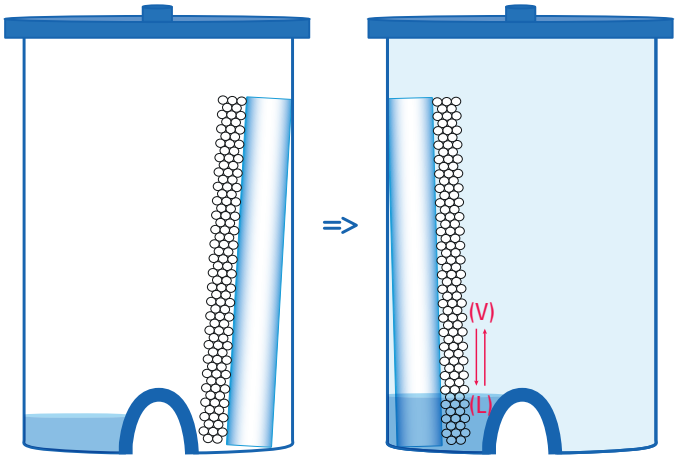
This technique gives better reproducibility and with closer HPLC conditions vs. others mode.



Operational modes:

3. Pre-conditioning mode:
The development phase is introduced into one of the tray of the chamber. Simultaneously, TLC plate is placed as vertically as possible in the second empty tray. 20 to 30 min are necessary to sature the chamber and homogenize vapor and liquid phase in concentration. At vapor equilibrium, the gas phase recondenses over the plate and wet it throughout its length. Then, the plate is switched from the tray to the other one.

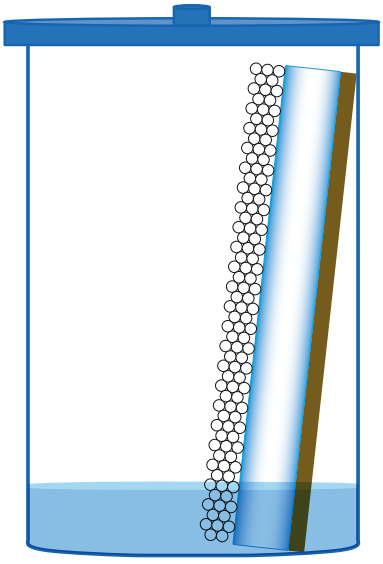
This technique leads to more diffuse spots and weaker Rf compared to the unsaturated mode.
However, it provides reproducible analysis with close HPLC conditions.



Operational modes:

4. Sandwich mode:
A counter-plate is placed a few mm from the TLC. To be more efficient a pad can be put vertically behind each of the two plates. The one behind the back plate must be shorter so that there is no transfer on the top of the separator plate. The more the equilibration between the liquid and vapor phase is reached, the more the adsorbed layer becomes homogeneous over the entire length of the plate.
It works under unsaturated, saturated or preconditioning mode either.

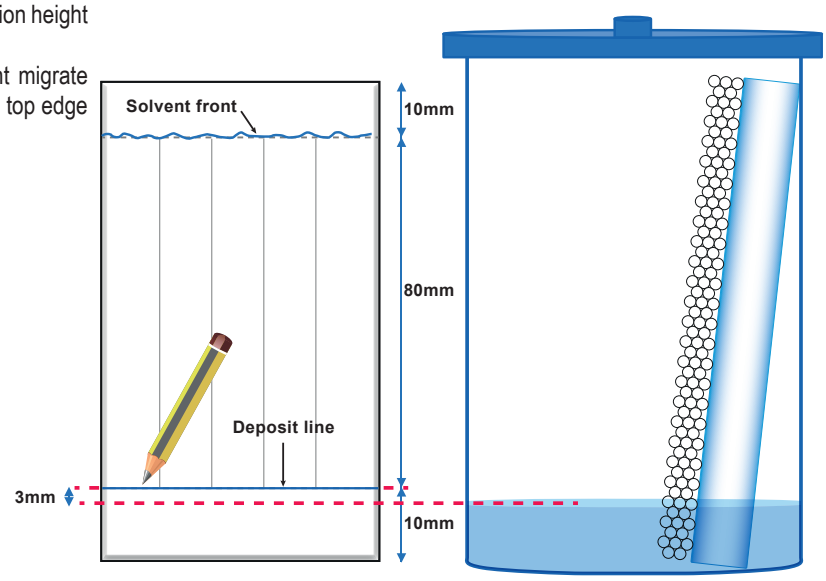
This technique leads to reproducible analysis with closer HPLC conditions.
Useful for complex analysis.



How to make a successful TLC plate:

1. Deposit line & Solvent front
Plot the deposit line with a pencil at 3 mm above the mobile phase level.

Recommended migration height is at least 8 cm.
Make the solvent front migrate at up to 1 cm from the top edge of the TLC plate.



Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

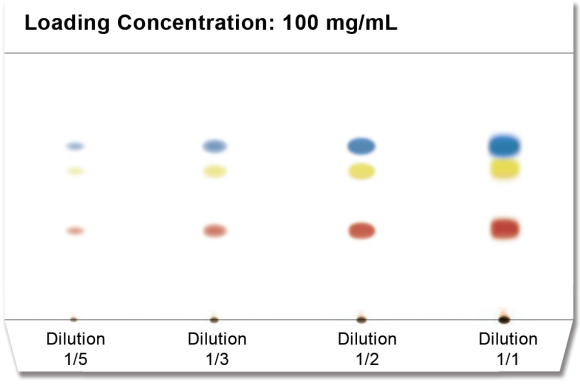
How to make a successful TLC plate:

2. How to make the deposit

a) Sample concentration
it is necessary to avoid over-loading

State of the art =>
for a standard: concentration of 0.1mg/L
for the sample: concentration must not exceed 2%

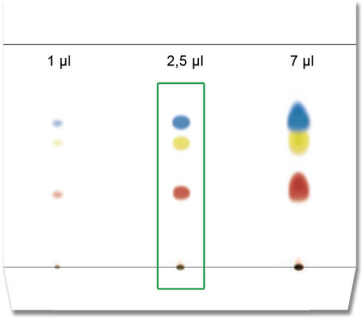
b) Deposit size
The deposit must be spreaded at the minimum possible (smallest possible volume) otherwise there is a loss of separation.



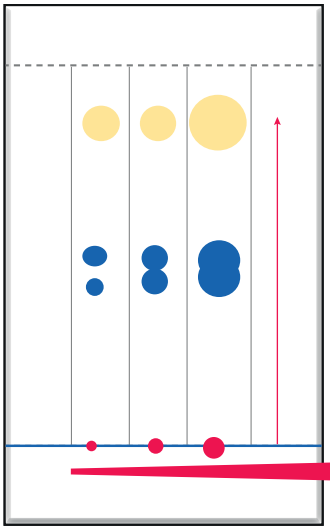
If the chemist wants to make several deposits, it is necessary to dry the plate between the successive deposits. The plate must be left to dry for 10min at room temperature before its development.

How to make a successful TLC plate:

Influence of the size of the deposit on the separation - Dimensions of the spots according to their Rf.



TLC calibration study in concentration is important to achieve the best optimization.



a) Spot ø increase with Rf

b) if the height of development is too important => significant dilution, detection difficulties

a large deposit => a loss in separation.

How to make a successful TLC plate:

3. Deposit of the raw sample

a) Spot mode:

By capillarity using an HPLC syringe or a capillary, there is a more or less deep penetration of the solute in the adsorbent layer. The finest deposit spots are obtained with a syringe rather than a capillary.

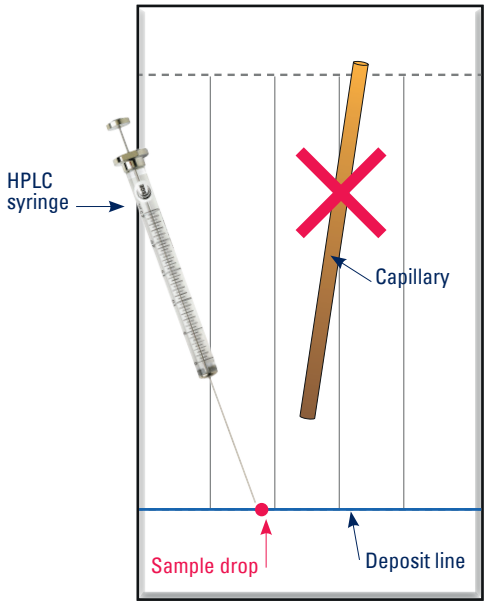
Using this mode of deposition mode, spots are more or less deformed

Load the smallest drop as possible to avoid dispersion caused by overloading which could affect the compounds resolution.

Avoid touching the TLC plate with the deposit tool to limit compounds penetration deep in the layer of the plate.

Spot density and dispersion may vary with solvent polarity.

Do not place the spot too close to the edges of the plate.



How to make a successful TLC plate:

3. Deposit of the raw sample

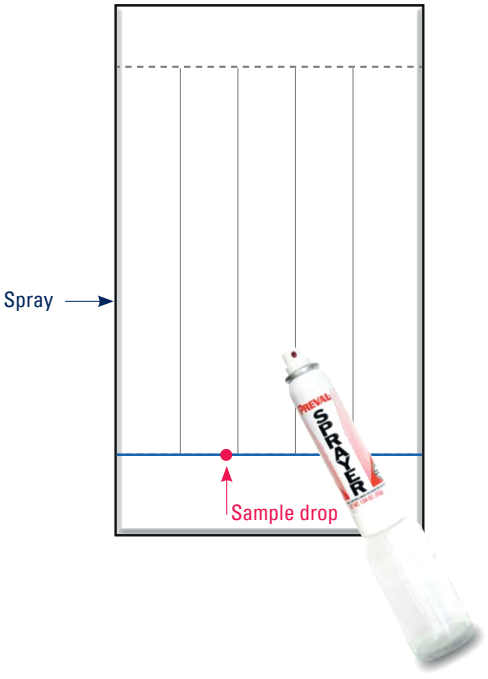
b) Spray mode:

By using a spray bottle, the spray speed must be slower than the evaporation velocity to avoid any projection.

Using this mode, there is little penetration of the solute into the adsorbent layer staying on the surface.

The eluted spots are less spreaded out.

Using this spray mode the separation of the migration spots is optimal.



Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

How to make a successful TLC plate:

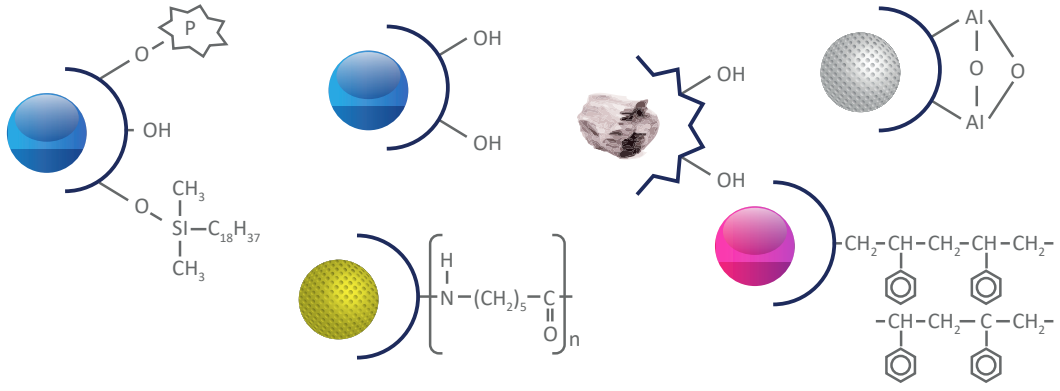
Solvent of dilution
It must have a weak elution force, be very pure.
It must be sufficiently polar to dissolve the sample (but not too much to be easily eliminated)
The use of bases and acids should be limited
Avoid viscous and high-boiling solvents (N,N-Dimethylformamide, DMSO, BuOH, water), as the migration time of the solvent will be longer.
It is necessary to dry the plate between two successive deposits

Solvent of elution
Adapt the elution strength according to the compounds polarity to keep Rf in the optimal zone (0.15 - 0.35).
The mobile phase velocity is not constant over the entire length of the plate.
Use the same mobile phase for TLC & Flash purification

Preparation of the mobile phase to ensure a perfect transfer to Flash purification =>
Solvents must be measured precisely in volume using separated flask (check the precision of the flask).
Low volume in % can be measured using syringe to ensure greater precision.

Stationary phase choice:

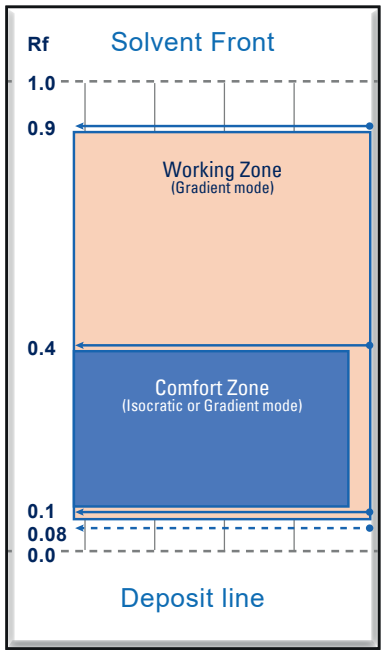
The choice of sorbent depends on the nature of the compounds to purify => polarity, functional groups.
The retention of compounds is very different depending on the sorbent used.
To avoid stain deformations, silica is generally chosen for the acidic compounds & alumina for the basic compounds.
Non-bonded polar stationary phases: silica, alumina, etc. are materials extremely eager of water.
If kept under open air, they lose their activities by quickly absorbing atmospheric water (50% in less than 3 min). This can lead to completely different separations in between two plates from the same batch that been left at ambient air and carried out at different times.
It is recommended to keep the plates in a desiccator, possibly under vacuum, in the presence of a desiccant.



Mobile Phase choice:

What is the ideal distribution of stains on a plate:
To get a good location accuracy of the center of the spots and to calculate the Rf it is necessary that they are distributed regularly in the Rf range from 0.08 to 0.9. The best is a spot distribution between 0.1 and 0.4 with a minimal ΔCV.

With silica and alumina the more the mobile phase is polar the more the solutes are eluted towards the front of the solvent, towards large values of Rf (Rf ≥ 0.6). Conversely, the more the mobile phase is none-polar, the less the solutes are entrained and the closer they stay to the deposit line with low values of Rf (Rf ≤ 0.1).



Mobile Phase choice:

The mobile phase has the following role:
- Dissolution of the sample
- Desorption of the sample from the stationary phase
- Transport of the sample at an acceptable migration distance

In general, the mobile phase must be:
- As simple as possible (maximum 3/4 components)
- None-toxic
- From a chromatographic quality
- Specific to not generate side reactions
- Selected to avoid demixing (vapor pressures, equivalent polarities)
- Having a low viscosity

Eluent strenght on different stationnary phases:
ε_0 silica = $0.77\varepsilon_0$ alumina
ε_0 diol = $0.3\varepsilon_0$ silica = $0.23\varepsilon_0$ alumina
ε_0 florisil = $0.52\varepsilon_0$ alumina
ε_0 magnesie = $0.58\varepsilon_0$ alumina

Polarity of mobile phases:
- The concept of polarity of the chemical species and the different scales of polarity are described in the purification chapter.

How to control retention:
Two solvents with total missibility parameter values δT , eluent force $\varepsilon°$or polarity P 'equal or very close will lead, for the same compound, to neighboring or equal retention parameters (even k, or even Rf).

How to change the separation by keeping the retention with same magnitude:
By cons for a pair of solutes with a slightly different polarity, the selectivity (separation of spots) will not be the same for two solvents with identical polarity (δT ou $\varepsilon°$ identical or similar) as they express different partial dominant polarity (Partial polarities of solvents must be taken into account).

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Mobile Phase choice:

The classification of solvents according to Trappe is expressed in eluotropic series classified by increasing eluent force:

- Based on the adsorption energy per unit area of the stationary phase
- Depends on the stationary phase
- The classification uses pentane as a reference.
- Eluotropic series on different adsorbents:

Solvents List	ε0 Silica Virgin	ε0 Alumina	ε0 Silica Diol	ε0 Silica CN	ε0 Silica NH_2	ε0 Silica C18.C4.C8.PH.RPAQ	ε0 Magnésie	ε0 Florisil
Acetone	0.470	0.560	0.141	0.470	0.470		0.325	0.291
Acetonitrile	0.501	0.650	0.150	0.501	0.501	0.577	0.377	0.338
Benzene	0.246	0.319	0.074	0.246	0.246		0.185	0.166
Butanol	0.550	0.714	0.165	0.550	0.550		0.414	0.371
Carbon tetrachloride	0.139	0.180	0.042	0.139	0.139		0.104	0.094
Chloroform	0.260	0.400	0.078	0.260	0.260		0.232	0.208
Cyclohexane	0.030	0.0400	0.000	0.000	0.000		0.023	0.021
Cyclopentane	0.000	0.05	0.000	0.000	0.000		0.029	0.026
1.2-Dichloroethane	0.339	0.490	0.102	0.339	0.339		0.284	0.255
Dichloromethane	0.323	0.420	0.097	0.323	0.323		0.244	0.218
Diethylamine	0.485	0.630	0.146	0.485	0.485		0.365	0.328
Diethyl ether	0.385	0.380	0.115	0.385	0.385		0.220	0.198
Diisopropyl ether	0.223	0.280	0.067	0.223	0.223		0.162	0.146
N.N-Dimethylformamide	0.640	0.831	0.192	0.640	0.640		0.482	0.432
Dimethyl sulfoxide	0.470	0.620	0.141	0.470	0.470		0.360	0.322
Dioxane	0.490	0.560	0.147	0.490	0.490		0.325	0.291
Ethanol	0.677	0.879	0.203	0.677	0.677		0.510	0.457
Ethyl acetate	0.380	0.580	0.114	0.380	0.380		0.336	0.302
Heptane	0.000	0.000	0.000	0.000	0.000		0.000	0.000
Hexane	0.000	0.010	0.000	0.000	0.000		0.006	0.005
Hexanol	0.385	0.500	0.115	0.385	0.385		0.290	0.260
Isooctane	0.000	0.010	0.000	0.000	0.000		0.006	0.005
Isopropanol	0.590	0.820	0.177	0.590	0.590		0.476	0.426
Isopropyl chloride	0.223	0.290	0.067	0.223	0.223		0.168	0.151
Methanol	0.732	0.950	0.219	0.732	0.732	0.450	0.551	0.494
Methyl acetate	0.393	0.510	0.118	0.393	0.393		0.296	0.265
Methyl ethyl ketone	0.393	0.510	0.118	0.393	0.393		0.296	0.265
Methyl tert-butyl ether	0.470	0.610	0.141	0.470	0.470		0.354	0.317
Pentane	0.000	0.000	0.000	0.000	0.000		0.000	0.000
Petroleum ether	0.000	0.010	0.000	0.000	0.000		0.006	0.005
Propanol	0.631	0.819	0.189	0.631	0.631		0.475	0.426
Pyridine	0.550	0.714	0.165	0.550	0.550		0.414	0.371
Tetrahydrofuran	0.346	0.449	0.104	0.346	0.346	0.726	0.261	0.234
Toluene	0.223	0.290	0.067	0.223	0.223		0.168	0.151
Water						0.000		

Mobile Phase choice:

Solvent selectivity according to Snyder chart:

The retention of compounds is different according to selectivity group.

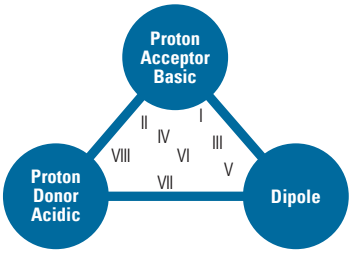
Based on the chemical structure of the compound choose, a solvent which interacts with compounds and is from different solvent group.

If the resolution is not achieved then try an alternative eluting solvent.

Based on the solvent selectivity, the choice of the solvent will be different for purification with normal phase, reversed phase or other technique.

Solvents	Polarity	Group
Hexane	Low	
Heptane		
Cyclohexane		
Chloroform		VIII
Toluene		VII
Dichloromethane		III
Tetrahydrofuran		V
Ethyl acetate		VI
Diethyl ether		I
Acetonitrile		VI
Acetone		VI
2-Propanol		II
Ethanol		II
Methanol		II
Water	High	VIII

Solvent Selectivity Group Triangle



Solvent Property	Example Solvent	Interacting Compounds
Dipole	Dichloromethane	Corbonyl, nitriles, sulphonates, amides
Proton acceptor	Amines, ammonia	Alcohols, acids, phenols
Proton donor	Alcohols, chloroform	Amines, sulphonamides

LR Snyder, J Chromatogr. 92 (1974) 223
LR Snyder, J Chromatogr. Sci 16 (1978) 223

Method for experimental determination of mobile phase composition:

1st stage => Make TLCs with 8 (10) pure solvents of increasing polarity

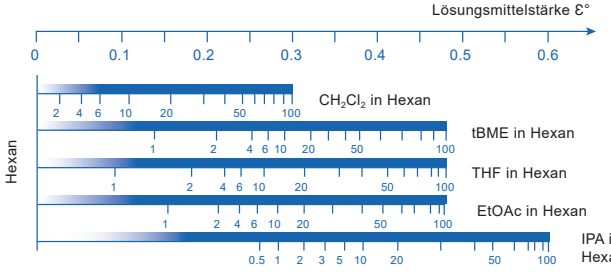
A solvent for which all the solutes are in the expected Rf range ($0.1 \leq Rf \leq 0.4$ (comfort Zone) or $0.08 \leq Rf \leq 0.9$ (working zone))

if separation considered correct => it is done

if separation considered to be incorrect => start the 2nd step

If with this first pure solvent the solutes are not all in the expected Rf range =>
find the solvent such that the 1st spot has the expected minimum Rf.
look for the solvent such that the last spot has an Rf equal to the maximum Rf desired.
Once these two solvents are fixed => start 3rd stage

2nd stage => the Ɛ° value of the solvent which placed all the solutes in the right zone but with a bad selectivity is known. Use abacuses to find different mobile phase of binary compositions with the same Ɛ° value



from M.D. Palamareva et V.R. Meyer, J. Chromatogr. 641, 391, (1993)

=>TLCs are made from which one finds the best mobile phase composition such that the separation of the least well separated spots is maximum.

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Method for the experimental determination of the composition of the mobile phase:

3rd stage =>

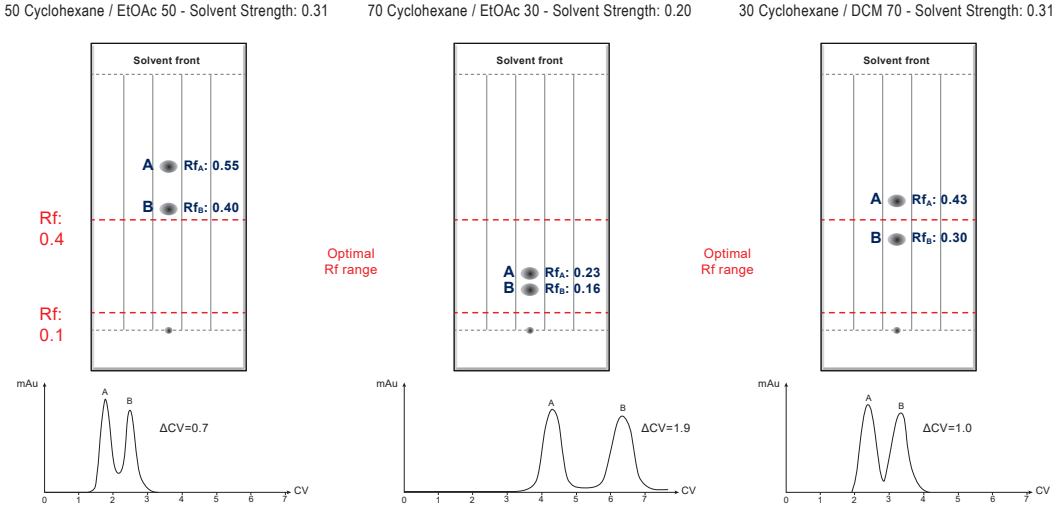
You know the domain ΔE° which places all the solutes in the expected Rf domain and you look for the value of eluent force E° which actually places all the solutes in the expected domain.

Make mixtures of the two mobile phases =>

less eluent	more eluent
95%	5%
90%	10%
85%	15%
80%	20%

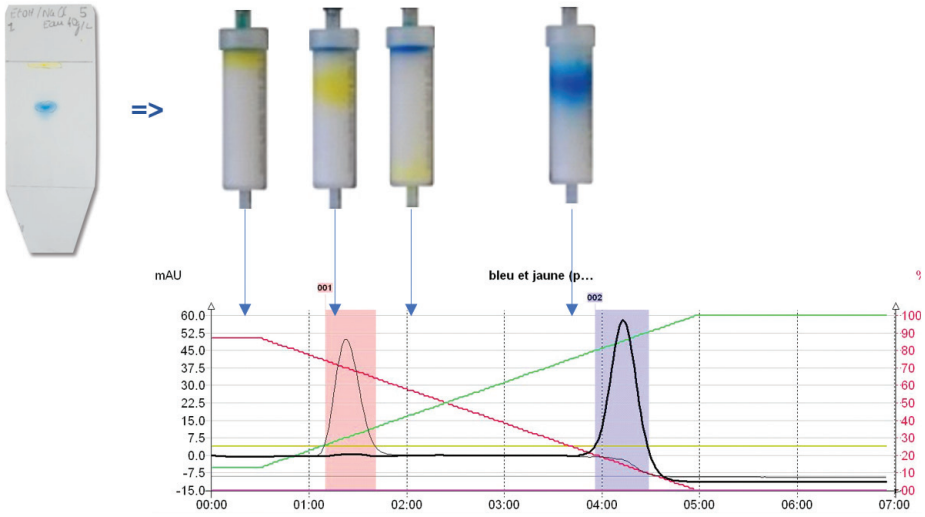
By iteration determine the good (better) composition and return to the situation of the 1st stage.

Impact of Mobile Phase selection:



First TLC shows 2 compounds which are not in the optimal Rf range, the separation is not achieved.
With the second TLC, 2 compounds are in the optimal Rf range and the resolution is better than the fisrt TLC. ΔCV is higher (1.9).
With the third TLC, Cyclohexane/Ethyl Acetate replaced by Cyclohexane/Dichloromethane (both 0.31 solvent strength). For a same eluent strength, the selectivity is different and the resolution is better but less important than the second TLC.

Method transfer from TLC's or HPLC's to Purification



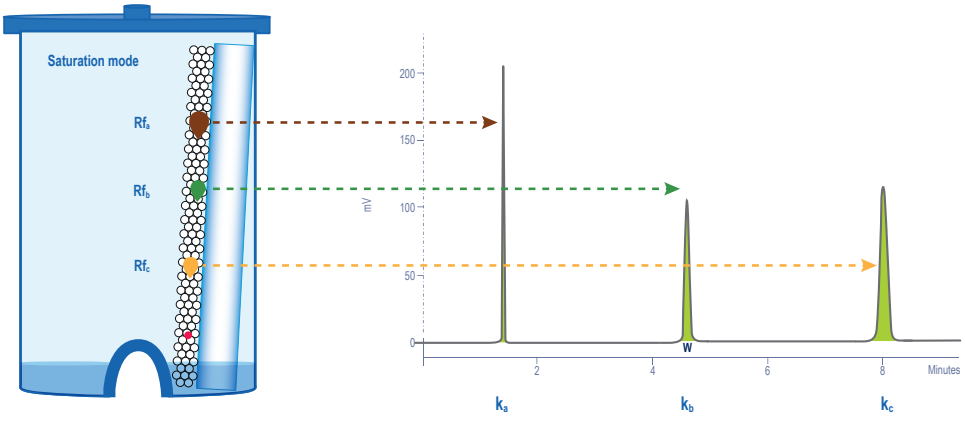
To be make possible any transpose from one chromatography mode ie, TLC, Open column, SPE, HPLC to another one ie, Flash, LC preparative, … without having the need to do any method adjustment, it is mandatory that:

a) Media of the two chromatographic modes must have the same surface chemistry.
b) Plates or columns must be stored under the same conditions to ensure the same degree of humidity.

As it is never the case, transfer laws are a guide but are never 100% reliable.

Method transfer from TLC's or HPLC's to Purification

In chromatography a mathematical relationship links the Rf to k and the mobile phase volume needed to elute the solute.
It is only valid for the same system => the same solute eluted at the same working temperature, by the same mobile phase on the same stationary phase with a saturation mode deposit (TLC) !



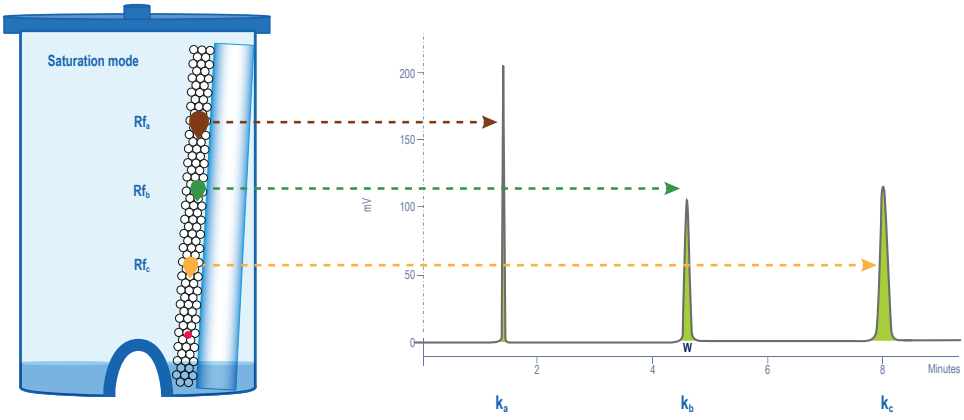
$k = K_{tr} \times [1/Rf - 1]$

Ktr ≈ 1 if medias are identical
(what is actually not exactly the case between silica from TLC and HPLC due to the binder and other modifiers.)

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Method transfer from TLC's or HPLC's to Purification

Pragmatically the chemist is led to reason in the amount of mobile phase necessary to use to elute the solute from the preparative column.
To take into account the different geometries of the preparative columns, this retention volume is expressed relative to the void volume of the column used.
It is a dimensionless number identified by the acronym Vs (also called CV).



$V_r = V_0 \times [1 + k]$
$V_s = V_r / V_0 = 1/Rf = [1 + k]$

Vs = mobile phase volume needed to elute a solute (expressed in V0 units of the flash column)

Method transfer from TLC's or HPLC's to Purification

a) The Rf ratio does not correspond to the ratio of k

If : $K_{tr} \approx 1$ $\alpha = k_b / k_a = [1 - Rf_b / 1 - Rf_a] \times [Rf_a / Rf_b]$

b) The resolution R is maximum for Rf = 0.3

$$R_{TLC} = .(2)\left[\left(\frac{Rf_B - Rf_A}{\omega_B + \omega_A}\right)\right]$$

$$R_{TLC} = .\left[\sqrt{\frac{N_A}{4}}\right]\left[\left(\frac{Rf_A - Rf_B}{Rf_B}\right)\right] = \left[\sqrt{\frac{N_B}{4}}\right]\left[\left(\frac{Rf_A - Rf_B}{Rf_A}\right)\right]$$

$$R_{TLC} = .(2)\left[\left(\frac{Rf_B - Rf_A}{\omega_B + \omega_A}\right)\right] \qquad R_{flash} = .(2V_0)\left[\left(\frac{Vs_B - Vs_A}{\omega V_B + \omega V_A}\right)\right]$$

for the same ΔRf:

1. the smaller the RFs are, the smaller the ω,
2. the greater ΔVs will be for the HPLC and flash, the higher the resolution will be in LC and flash.

Method transfer from TLC's to Purification

In practice, to transpose a TLC on a flash or a preparative column algorithms calculate from the Rf on the plate the retention factors of the solutes on the column in isocratic elution condition.

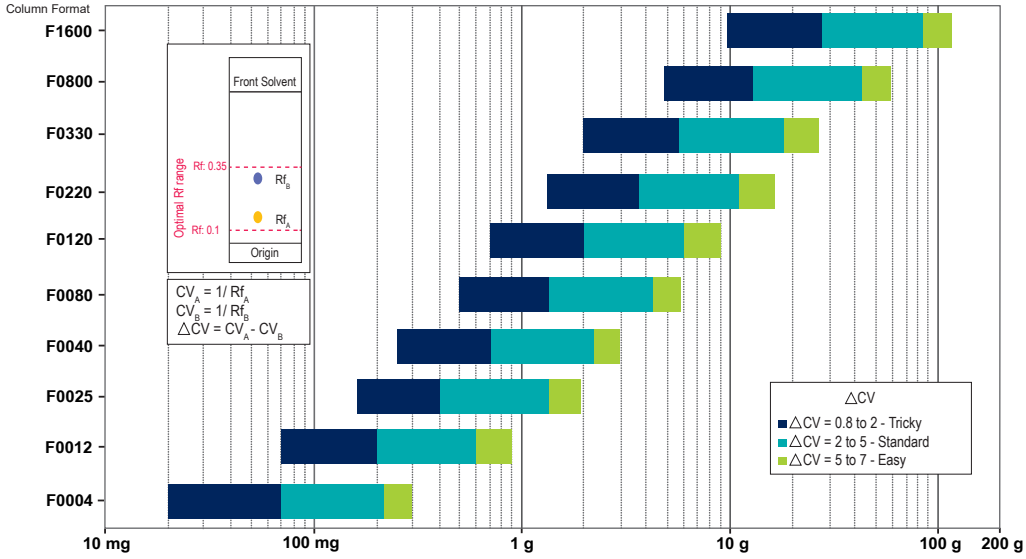
The minimal Δk which means the least well separated pair of peaks, identical to the ΔVs, evaluate whether the separation is easy or difficult.

ΔVs < 1.5	=> difficult purification
1.5 ≤ ΔVs < 4	=> standard purification
4 ≤ ΔVs < 10	=> easy purification

For the same Δk the separation is not the same as a function of the numerical value of k, Interchim® algorithms within the puriFlash® instrument give automatically the greater elution gradient program for each column from their V_0.

Method transfer from TLC's to Purification

Normal Phase Column Section Guide, loading chart based on 50µm irregular silica (worst case)



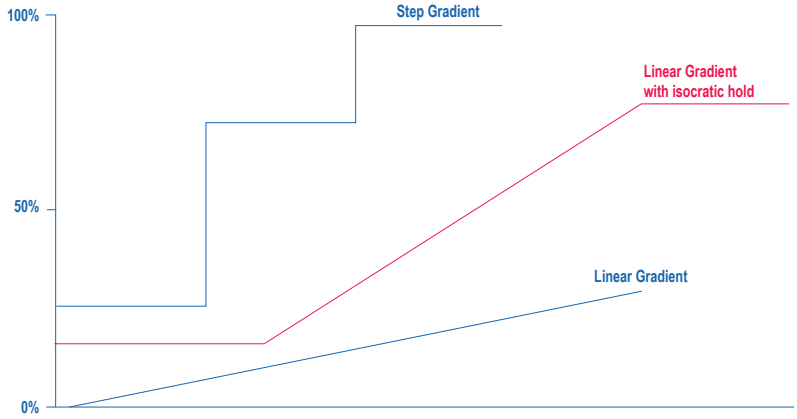
Average values for compounds < 800 MW
These data depend on the conditions of elution and the products to be purified.

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Method transfer from TLC's to Purification

How to choose the right Gradient conditions in function of ΔVs.

3 different modes can be considered => Isocratic, Linear Gradient, Step Gradient plus a combination of Linear Gradient/Isocratic



Isocratic mode:
The mobile phase has the same composition over the entire purification run.
Using an isocratic mode, TLC and flash operational conditions are directly correlated.

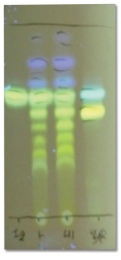
This mode is mainly used to purify compounds with Rf > 0.15 and ΔVs > 1. Compounds with Rf < 0.15 will be elute the latest with broad peaks.

Method transfer from TLC's to Purification

Example of Isocratic purification

1) TLC development:

Eluent :
50 Cyclohexan / 50 DCM

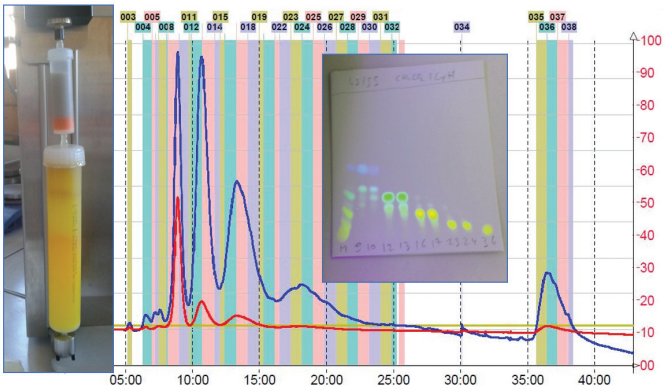


Optimization of TLC conditions
to get Rf between 0.05 & 0.35

Eluent : 55 Cyclohexan / 45 DCM

2) Choice of the column:
Crude sample : 800mg
(mixture of 8 compounds)
Column : PF-15SIHC/120g
Loading capacity: 0.6%

3) Flash condition:
Injection mode: Solid deposit with celite
Eluent : 55 Cyclohexan / 45 DCM
Flow rate : 60mL/min
254nm (red signal) + Scan : 230-450nm
(blue signal)
P= 12bar



Method transfer from TLC's to Purification

How to choose the right Gradient conditions in function of ΔVs.

Gradient mode (Step, Linear, Linear with isocratic hold):
This mode improves the peak smoothness compared to the isocratic mode and reduces the total analysis time, which makes possible to reduce the volumes of collection & the consumption of solvents.
The initial conditions of a gradient mode are deduced from the isocratic conditions found in TLC.
The % of initial strong solvent is a function of the chromatographic mode of the TLC (normal or reverse phase) and its value is to be determined according to the eluent force curves of the solvents used. The slope of the gradient plays a fundamental role, during the transposition, on the quality of separation (ΔVs or Δk).

An adapted slope will, theoretically, lead to improved separation with better selectivity, resolution, purity & loading capacity.

The CV calculated on the TLC isocratic is different with gradient elution mode on flash column. During the purification, the solvent strength increases so compounds are eluted rather with a Rf lower than predicted in isocratic TLC. By this mode, compounds with long retention times will come out earlier increasing the productivity.

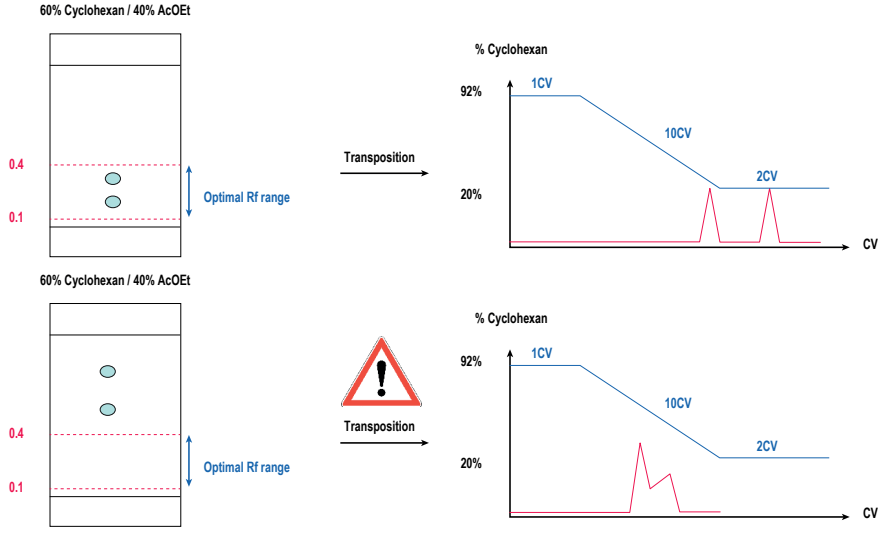
Linear Gradient + Isocratic hold:
This is the most gradient mode used in flash purification. The linear part of the gradient is the fastest way to separate complex mixture.

Method transfer from TLC's to Purification

Method #1 if ΔVs > 1

segment 1 :1/5 of the strong solvent of the TLC over 1V0
segment 2: from segment & over 10V0, reach 2x % of the strong solvent
segment 3: keep isocratic condition over 2V0

Example:



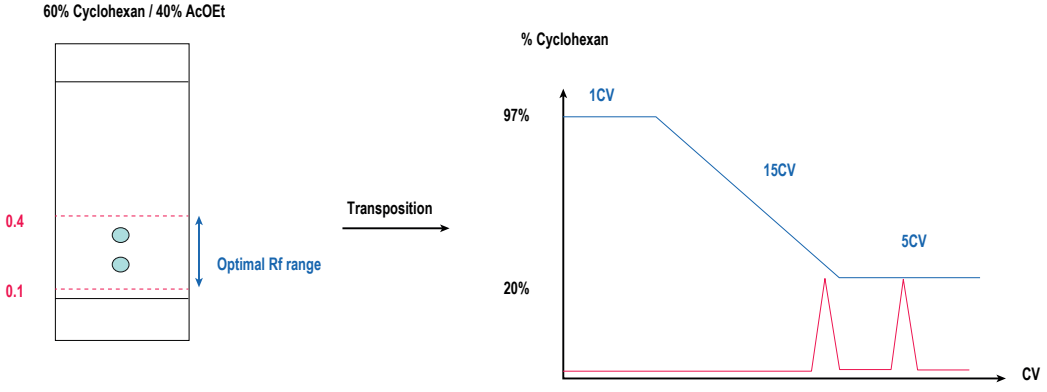
Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Method transfer from TLC's to Purification

Method #2 if 0.5 < ΔVs ≤ 1

segment 1: 3% of strong solvent over 1V0
segment 2 : from segment & over 20V0, reach 2x % of the strong solvent
segment 3: keep isocratic condition over 5V0

Example:

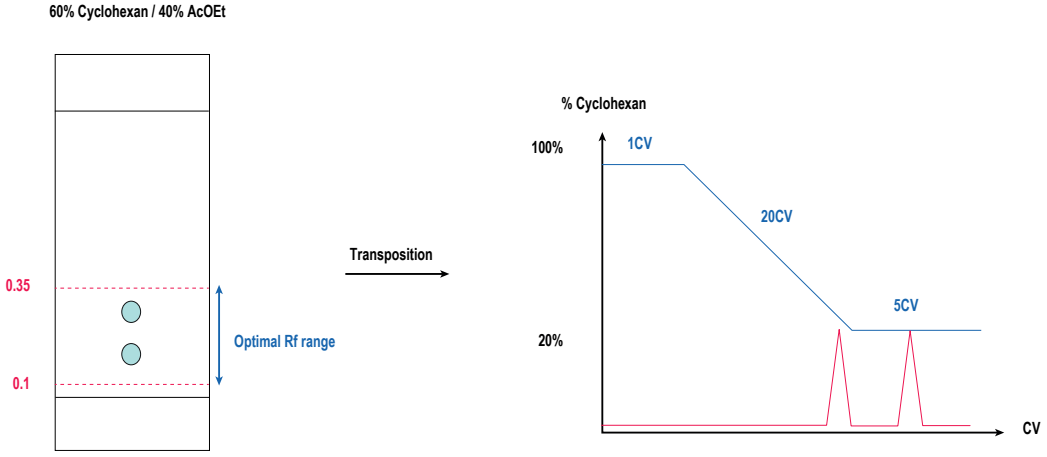


Method transfer from TLC's to Purification

Method #3 if ΔVs < 0.5

segment 1: 100% of weak solvent over 1V0
segment 2 : from segment & over 25V0, reach 2x % of the strong solvent
segment 3: keep isocratic condition over 5V0

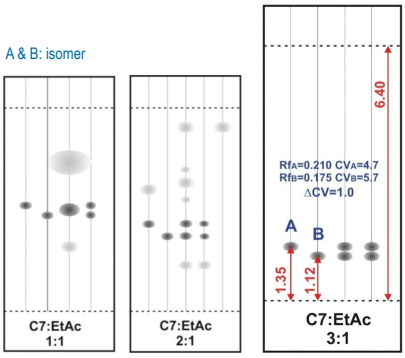
Example:



Method transfer from TLC's to Purification, Application example

1) TLC Development

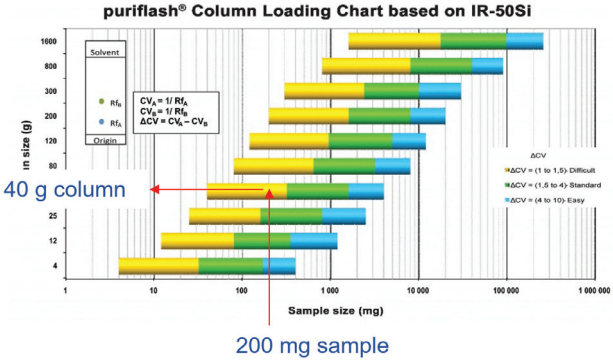
A & B: isomer



C7: Cyclohexane
EtOAc: Ethyl Acetate

2) Selection of column according to ΔCV mass of raw sample: 200mg

We choose to stack 2 columns PF-15SIHP-F0025 to increase the height of the silica bed in order to obtain a better efficiency / separation rather than use a single column PF-15SIHP-F0040.



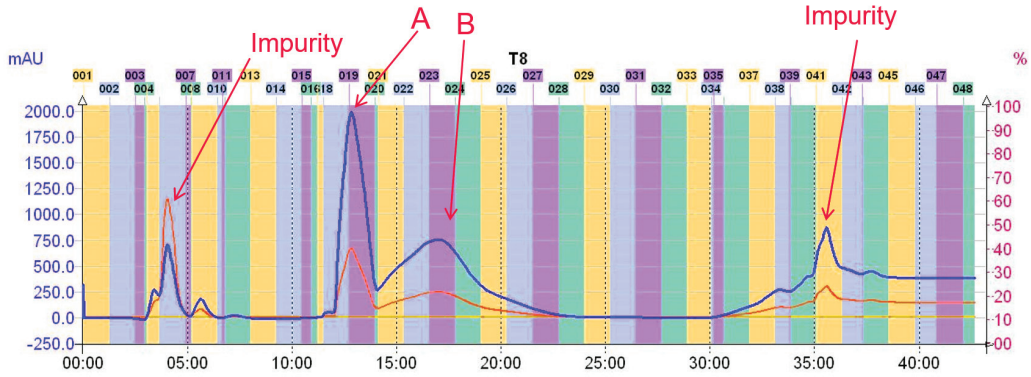
Method transfer from TLC's to Purification, Application example

3) Flash condition on puriFlash® 450

Solvents: A-Cyclohexane, B-Ethyl Acetate
Column: 2 x PF-15SIHP/25G
Flow rate: 20mL / min
Mode of injection: LiquidMass of crude sample: 200mg
UV detector: 232nm + Scan 220-600nm

Elution Gradient:

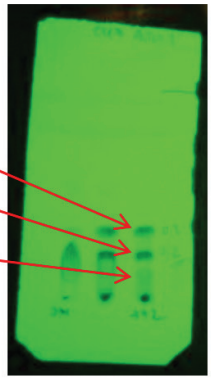
t (min)	%A	%B
0	80	20
26	80	20
32	50	50
42	50	50

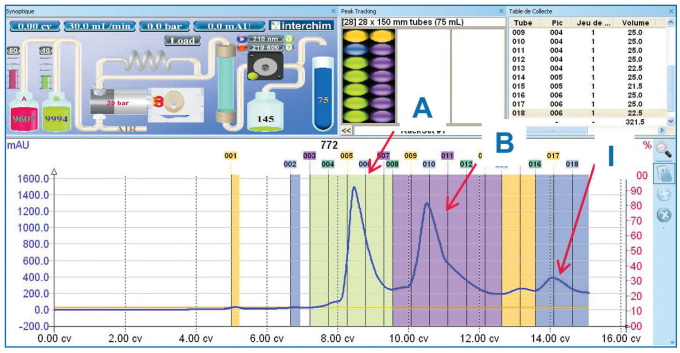


Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Method transfer from TLC's to Purification, Application example

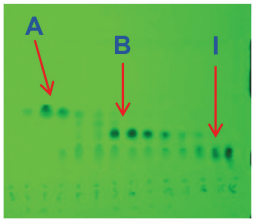
A) Cyclohexan 70%, B) AcOEt 30%
A and B: isomers





CV	%A	%B
0	100	0
5	80	20
10	70	30
14.25	40	60
15.08	5	95

Flash Condition on puriFlash® 450
Solid deposit (Dry-load 4g)
Column: PF-15SIHP/40G
M inj = 1g of crude sample
Flow rate: 30mL/min
UV: 254nm + Scan 210-600nm
Solvent: A-Cyclohexan, B-AcOEt
P = 6 bar



Method transfer from TLC's to Purification

Calculation of the essential analytical & preparative column parameters = >
a) Dead volume $V_0 = \pi(D^2/4) \times L \times \varepsilon$ - (ε: total column porosity, usually between 0.6 to 0.8)
b) Dead time $T_0 = V_0 / opt.F$ - (opt.F: Optimum flow rate depends on particle size and column I.D.)
Experimental method using Uracil a non retain compound or $NaNO_3$ can be use for the determination of T_0.

250 x 4.6mm
V_0 = 2.90mL
opt.F = 0.75mL/min
T_0 = 3.87min

250 x 10.0mm
V_0 = 13.70mL
opt.F = 3.50mL/min
T_0 = 3.87min

250 x 21.2mm
V_0 = 61.70mL
opt.F = 16.00mL/min
T_0 = 3.87min

250 x 30.0mm
V_0 = 123.60mL
opt.F = 32.00mL/min
T_0 = 3.87 min

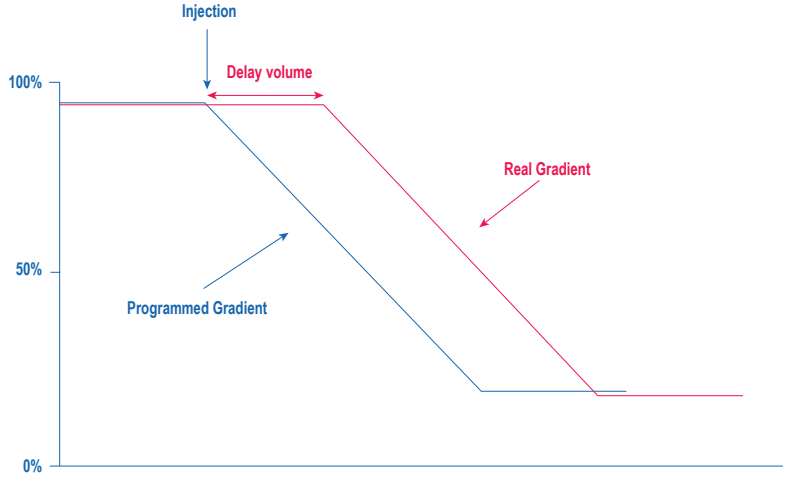
250 x 50.8mm
V_0 = 354.50mL
opt.F = 92.00mL/min
T_0 = 3.87min

Method transfer from HPLC's to Purification

Calculation of the essential system parameters = >

a) Delay volume

The delay volume is the time required for a change in the gradient to reach the column inlet.
Each instrument has its own delay volume. It can affect the results of the separation especially in terms of selectivity.
It is crucial to know its value to achieve an efficient method transfer. Usually for a preparative system delay volume is > 10mL.

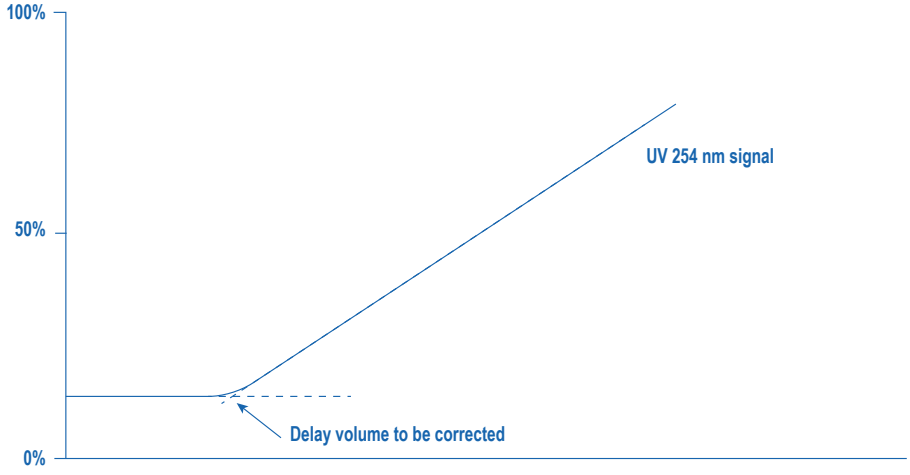


Method transfer from HPLC's to Purification

Calculation of the essential system parameters = >

a) Experimental measurement of the dead volume for an instrument

replace the column by an union
program a 0-100% B gradient in ~10 T_0 using (acetonitrile + 0.1% acetone) in solvent B
work at the flow rate at which subsequent experiments will be done
record the UV signal at 254nm



Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Method transfer from HPLC's to Purification

Calculation of the preparative conditions = >

First of all, we calculate conditions based on a direct scale-up.
Therefore, it is important between the analytical and preparative mode that :

a) The mobile phase (nature of the organic solvent,% organic solvent, pH, ionic strength, modifiers& temperature) and the stationary phase remain exactly the same.

b) To keep same efficiency N = L / (h x dp) - (L: column length, h: constant (depends on the quality of column filling, mobile phase flow and can also be negatively influenced by large volumes injected), dp: particle diameter). The ratio L /dp must be maintained constant.

c) The linear velocity (u) must be maintained constant so, adjusted according to the diameter of the column. Typically, for a 4.6mm id column at the optimum flow rate of 0.75mL/min the linear velocity is 1.07mm/s. The same linear velocity for a 30.0mm id column is obtained at a flow rate of 32.0mL/min.

Method transfer from HPLC's to Purification

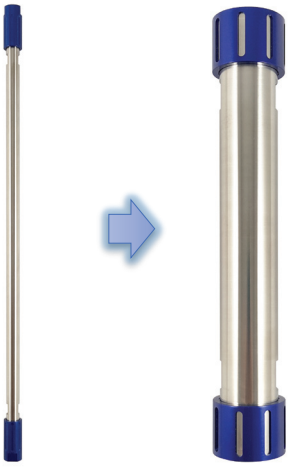
Calculation of the preparative conditions = >

a) Flow rate (F)
It must be adjusted by keeping the linear velocity constant between the analytical and the transferred preparative method, taking into account the particle size and the geometry of the column.

$$F_{prep} = F_{ana} \times \left(\frac{id^2_{prep}}{id^2_{ana}}\right) \times \left(\frac{d_{p\ ana}}{d_{p\ prep}}\right)$$

Example:

$$F_{prep} = 0.75 \times \left(\frac{30.0^2}{4.6^2}\right) \times \left(\frac{5}{15}\right) = 11.0\text{mL/min}$$



5µm, 250 x 4.6mm
opt.F = 0.75mL/min

15µm, 250 x 30.0mm
F = 11.0mL/min

Method transfer from HPLC's to Purification

Calculation of the preparative conditions = >

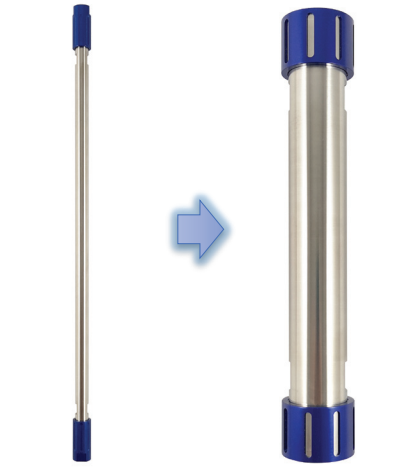
a) Injected volume (V)

It must be adapted according to the volume of the phases to obtain the same chromatographic efficiencies.
The injected volumes are usually higher than those at the analytical scale to increase the loading capacity. Under overloading conditions, asymmetric peaks and a change in retention time are observed.

$$V_{prep} = V_{ana} \times \left(\frac{id^2_{prep}}{id^2_{ana}}\right) \times \left(\frac{L_{prep}}{L_{ana}}\right)$$

Example:

$$V_{prep} = 5 \times \left(\frac{30.0^2}{4.6^2}\right) \times \left(\frac{250}{250}\right) = 213\mu L$$



5µm, 250 x 4.6mm
V_{inj} = 5µL

15µm, 250 x 30.0mm
V_{inj} = 213µL

Method transfer from HPLC's to Purification

Calculation of the preparative conditions = >

a) Gradient conditions, isocratic step

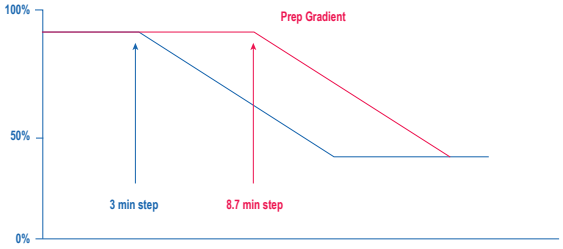
It is necessary to keep the ratio isocratic time / dead time of the column constant in analytic / preparative to keep a number of percolated column volumes equivalent.

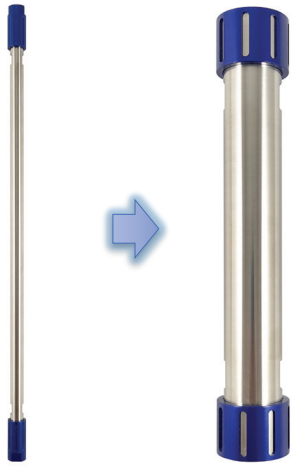
$$T_{prep} = T_{ana} \times \left(\frac{id^2_{prep}}{id^2_{ana}}\right) \times \left(\frac{L_{prep}}{L_{ana}}\right) \times \left(\frac{F_{ana}}{F_{prep}}\right)$$

T = time of the isocratic step

Example:

$$T_{prep} = 3 \times \left(\frac{30.0^2}{4.6^2}\right) \times \left(\frac{250}{250}\right) \times \left(\frac{0.75}{11}\right) = 11.0\text{mL/min}$$





5µm, 250 x 4.6mm
opt.F = 0.75mL/min

15µm, 250 x 30.0mm
F = 11.0mL/min

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Method transfer from HPLC's to Purification

Calculation of the preparative conditions = >

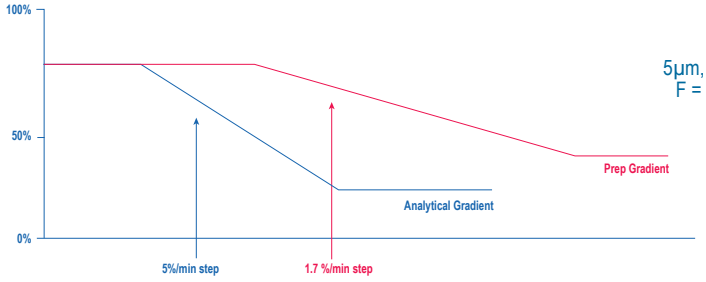
d) Gradient conditions, gradient slope
The initial and final compositions must remain the same during the transfer.
The new gradient slope is calculated by keeping the product
(slope x dead time) constant to keep the number of column volumes constant.

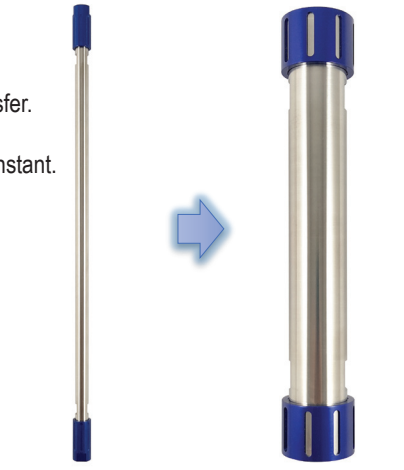
$$S_{prep} = S_{ana} \times \left(\frac{id^2_{ana}}{id^2_{prep}}\right) \times \left(\frac{L_{ana}}{L_{prep}}\right) \times \left(\frac{F_{prep}}{F_{ana}}\right)$$

S = slope of the gradient

Example:

$$S_{prep} = 5\%/min \times \left(\frac{4.6^2}{30.0^2}\right) \times \left(\frac{250}{250}\right) \times \left(\frac{11}{0.75}\right) = 1.7\%/min$$





5µm, 250 x 4.6mm
F = 0.75mL/min

15µm, 250 x 30.0mm
F = 11.0mL/min

Method transfer from HPLC's to Purification

Calculation of the preparative conditions = >

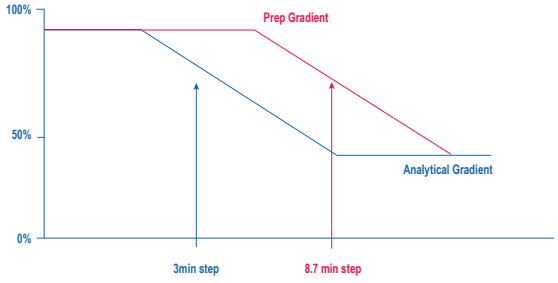
e) Gradient conditions, gradient slope
The initial and final compositions must remain the same during the transfer.
The new gradient slope is calculated by keeping the product (slope x dead time) constant to keep the number of column volumes constant.

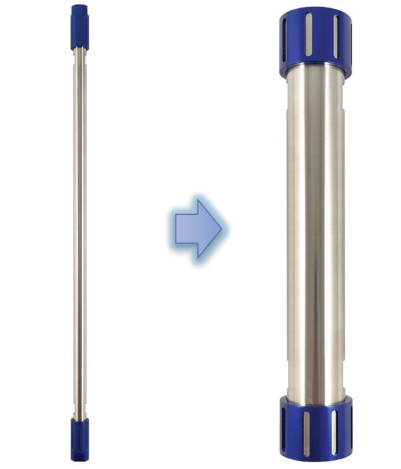
$$S_{prep} = S_{ana} \times \left(\frac{id^2_{ana}}{id^2_{prep}}\right) \times \left(\frac{L_{ana}}{L_{prep}}\right) \times \left(\frac{F_{prep}}{F_{ana}}\right)$$

S = slope of the gradient

Example:

$$S_{prep} = 5\%/min \times \left(\frac{4.6^2}{30.0^2}\right) \times \left(\frac{250}{250}\right) \times \left(\frac{11}{0.75}\right) = 8.7\%/min$$





5µm, 250 x 4.6 mm
F = 0.75mL/min

15µm, 250 x 30.0mm
F = 11.0mL/min

Method transfer from HPLC's to Purification

Calculation of the preparative conditions = >

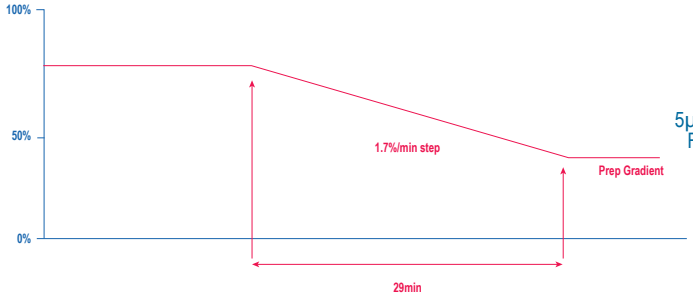
f) Gradient conditions, gradient slope time
The initial and final compositions must remain the same during the transfer.
The new gradient slope is calculated by keeping the product
(slope x dead time) constant to keep the number of column volumes constant.

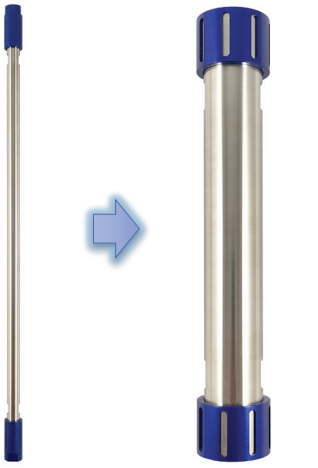
$$T_{prep} = \left(\frac{\%B\ final_{ana} - \%B\ final_{ana}}{S_{prep}} \right)$$

T = time of the gradient slope

Example:

$$T_{prep} = \left(\frac{75 - 25}{1.7} \right) = 29min$$





Method transfer from HPLC's to Purification

Calculation of the preparative conditions = >

g) Gradient conditions, additional comments

#The column reconditioning step generally consists of a rapid return to initial conditions and stabilization for about 5 to 10 column volumes.

#The delay volume creates an isocratic step at the beginning of the analytical and preparative gradient. The ratio between the delay time (Td) and the dead time of the column (T0) must remain constant, same number of percolated column volumes during the delay time. To compensate for the differences of Td and T0 it is recommended to reduce an existing isocratic plateau or to add an additional isocratic step.

#The analysis time is proportional to the dead time of the column.
The pressure is inversely proportional to dp3 and to the length of the column.
The solvent consumption is proportional to the internal diameter and the length of the column.

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

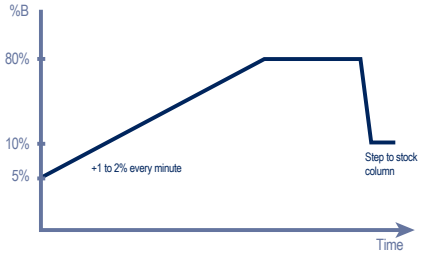
Reverse Phase mode

Interactions between stationary phase and hydrophobic parts of the peptide. Use C18 (-N or -T) or C8-N column according to polarity and length of the peptides.

Solvents are often Water+ACN+0.1%TFA (to modify selectivity, it's also possible to use methanol, and isopropanol for hydrophobic peptides).

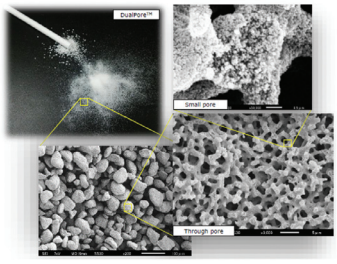
Typical process: Increase % of organic solvent of 1 - 2% every minute. To increase the resolution, a second isocratic step can be done when the interest peptide is eluted.

Optimization parameters: Temperature, isocratic gradient, change of media (C8, PhC4, C4), porosity (100, 200, 300Å), modify pH.



Benefit of Interchim® peptides monolith

Monolith is C18 bounded, and can be used as a conventional Reverse Phase.
- Selectivity is comparable to conventional silica
- Works for small and large molecules
- Lower generation of back pressure, allow to use high viscosity solvent like isopropanol
- Provide high resolution, 30µm DualPoreTM columns provide comparable resolution than 15µm conventional silica
- Can work at higher flow rate and save until 80% time => Ultra High throughput



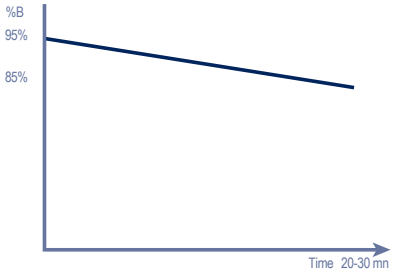
HILIC mode

Used for the separation of highly polar peptides. Use C18-N or C8-N columns according to length of the peptides.

Solvents are the same than RP mode, and in this case, water is the strong solvent.

Typical process: Start method with 95% of organic solvent to 85% in few minutes.

Optimization parameters: temperature, isocratic step, change of media (C8, PhC4, C4), porosity (100,200,300Å) modify pH.



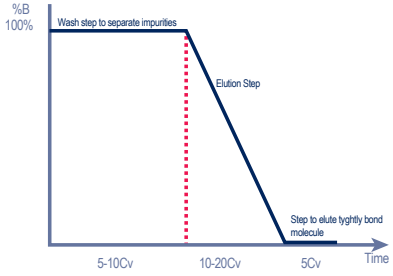
HIC mode

Hydrophobic interactions between solutes and a stationary phase with low or medium hydrophobicity. The separation is based on the reversible interaction between a peptide and the hydrophobic surface of a chromatographic media. Use 45-RP column.

Solvents are often a solution of Na_2HPO_4+ 1-4M of antichaotropic salt (Ammonium sulfate, NaCl, Na_2SO_4…).

Typical process: Start with buffer solution of 1.5-4M (Na_2HPO_4) + ammonium sulfate, and decrease the concentration of ammonium sulfate.

Optimization parameters: Salt concentration, try other salts, modify pH, test other columns (C8, C4…).



See all stationary phases for peptides method development & purification from page C.28

Dry-Load Injection

Dry-Load Injection

The dry-load injection is a convenient technic when the polarity of the reactional mix (or the extract) to purify is close to the polarity of stationary phase or, when it contains solutes with extreme opposite polarity.
It should also be consider when:

- the compound of interest is a lot more retained than the other compounds we would like to separate.
- the sample contains one or several compounds having low solubility with the eluent.

Compare to liquid injection, the dry-load injection improves the efficiency, the resolution, and the final purity.

Sample in solution

Injection Loop

Limited injection volume
due to sample solubility

Solid Sample

Dry injection

Allow to large sample amounts

High pressure dry-load

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

How to prepare the Dry-load cell for injection

1. Adsorb the dissolved sample in a "better solvent" on a small amount of stationary phase (Silica, C18 or Celite).
2. Evaporate the deposit solvent with a rotary evaporator until a "dry" powder is obtained. (If the volume of the dissolved sample is small, it can be poured onto the silica, and the partially impregnated silica mixed up to obtain a homogeneous dry powder, thus avoiding the passage to the rotary evaporator)
4. Place the mixture over the inlet frit of the column, once it has possibly been equilibrated with the elution solvent.
5. Add a sintered frit over the mixture, then a closure system or the piston of the column (for equipped systems) and tamp the mixture slightly to obtain a perfectly homogeneous deposit thickness.
6. Proceed with elution.

Technical tips

The volume of the dry-load must not exeed 5% to 10% of the purification column volume to keep sufficient resolution between fractions.

If possible, wet the dry-load with 100% of the less eluent solvent before to start the purification run.

Caution : Adapt your step in function of the back pressure and the acceptable flow rate.

The dry-load can generate air bubbles creating disruptions that hide the first peaks. (UV detector)

Celite exhibit advantages:

It does not generate back pressure due to its large particle size
It does not interact with the compounds that arrive at the same time at the top of the column
It improves separation and are compatible with both NP and RP mode

Liquid Injection

Liquid injection

This technique permit to dissolve the crude sample in minimum solvent to prevent peak broadening (dispersion phenomenon). The solvent which have the least affinity with the crude sample must be used (Ex: Cyclohexane for normal phase purification and water for reversed phase purification).
If you do a liquid injection, check that the sample is soluble at the starting condition of the run otherwise a cristalization can occure. A strong dissolving solvent impact the resolution. To avoid the lost of resolution, Interchim® advise to dissolve the crude sample at the starting condition of the run.

The dissolving solvents have an impact on the quality of the purification. The dispersion of the crude sample by the dissolving solvent decrease the peak resolution. The volume of the dissolving solvent must be less than 5% of the column volume to conserve the resolution.

Different injection modes

Direct injection on column head

Injection with an automatic valve

Injection pump

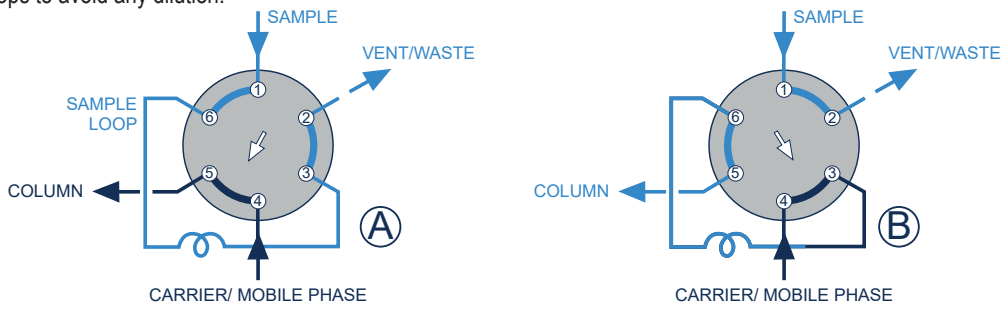
Autosampler

- Injection on a dry column without previous equilibration.
- Injection on a pre-conditioned column. This method gives the best results, because the column absobent offers a regular flow, and the sample follows exactly the flow of the solvent in the column. This injection mode allows the purification of the compounds with high Rf values: Rf<0.7. You can work directly with the optimum flow rate.
- Direct injection on column head: the use of a Luer-Rock connector at the entrance of the column allows to use a syringe and inject rapidly the sample, without any cross contamination risk or product loss.
- Injection through an external pump for a greater volume.
- Injection through an autosampler to automat purifications.
- Injection with an automatic valve: this method improves the reproducibility of the injections, increases productivity and is less time consuming.

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Injection with a 6-way 2 positions automatic valve

With the valve in position A, the sample is loading into the loop from the injection port while the mobile phase flows directly through to the column. When the valve is switched to position B, the sample contained in the sample loop is displaced by the mobile phase and is carried onto the column. The flow direction of the mobile phase through the loop is opposite to the flow direction during the loading. This is espacially critical for partially-filled loops to avoid any dilution.

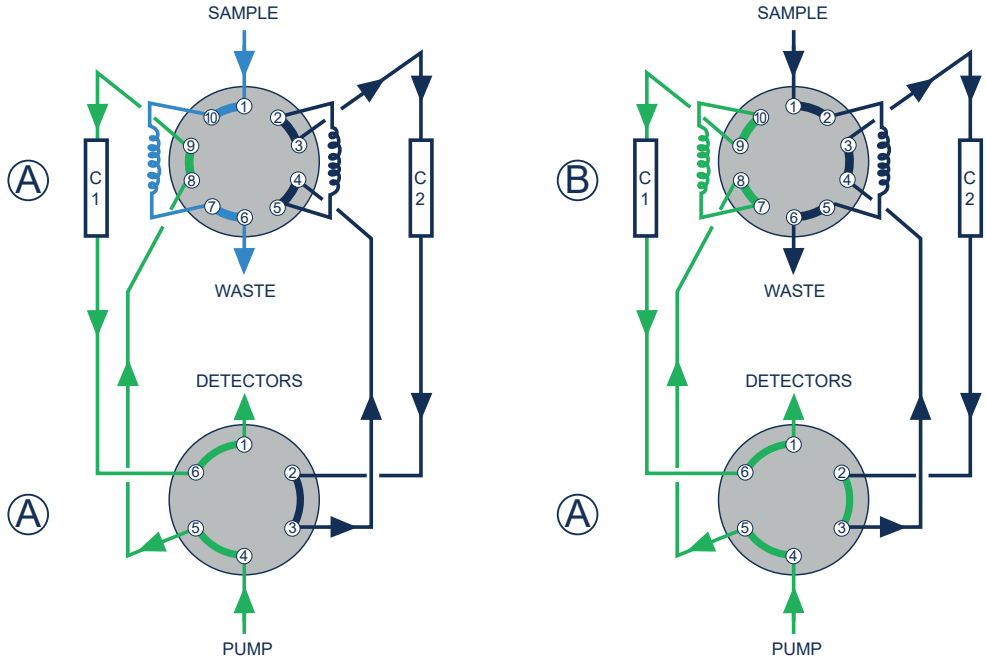


Injection with a 10-way 2 positions automatic valve on 2 different columns

With this coupling of two valves, each of the two column is linked to is own loop.

When the Valve 1 is in position A and valve 2 in position A the the sample is loading into the loop 1 from the injection port while the mobile phase flows directly through to the column 1.

After the column equilibration the valve 2 switch in position B. The sample contained in the sample loop 1 is displaced by the mobile phase and is carried onto the column 1.



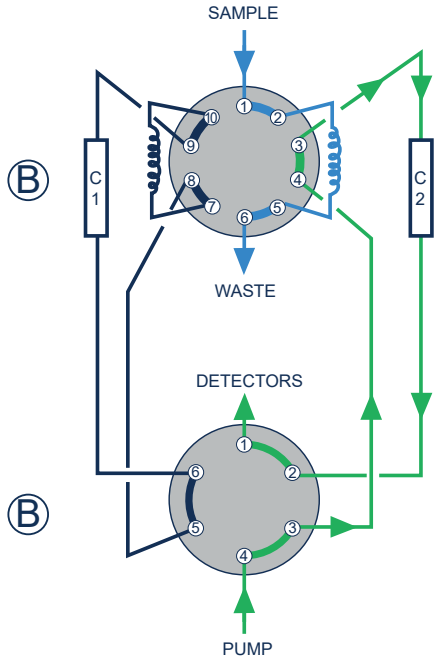
Equilibration column 1
Loading sample loop 1

Inject sample column 1

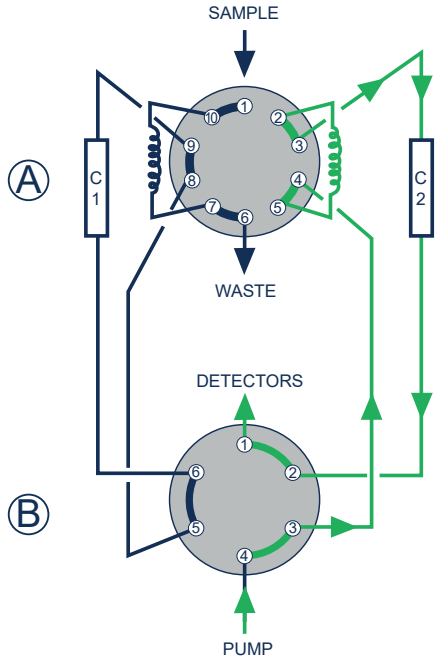
Injection with a 10-way 2 positions automatic valve on 2 different columns

At the opposite, when the Valve 1 is in position B and valve 2 in position B the the sample is loaded into the loop 2 from the injection port while the mobile phase flows directly through the column 2.

After the column equilibration the valve 2 toggles in position A. The sample contained in the sample loop 2 is displaced by the mobile phase and is carried onto the column 2.



Equilibration column 2
Loading sample loop 2

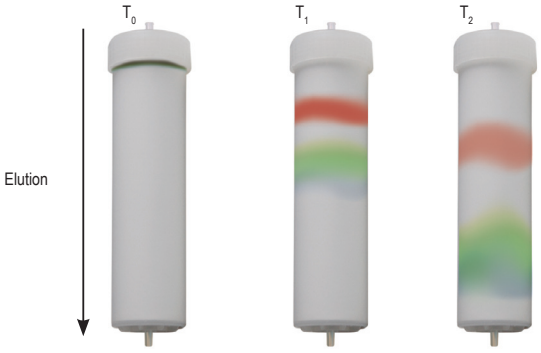


Inject sample column 2

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

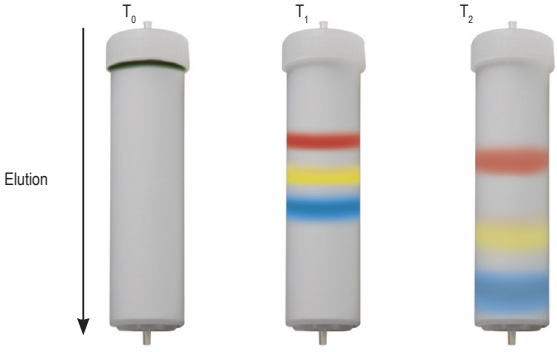
Injection example 1: Liquid deposit

Liquid deposit on dry columns
Starting condition too eluent (20% strong solvent)



Poor separation

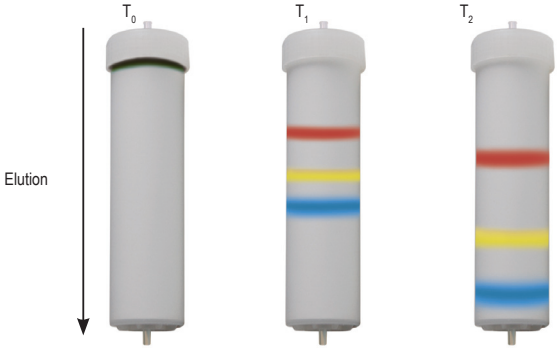
Liquid deposit on pre conditioned columns with the same starting eluent condition



Correct separation

Injection example 2: Liquid injection

Liquid injection with syringe on pre-conditionned column (same starting eluent condition than example 1)



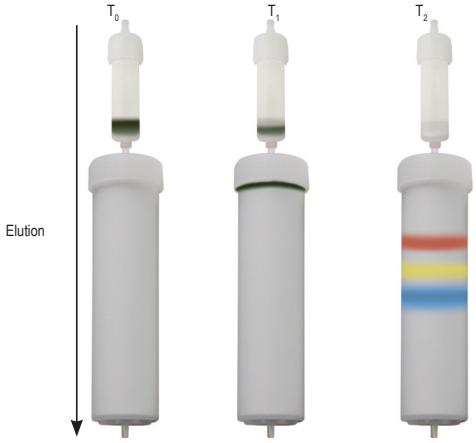
Best separation

Injection example 3: Liquid injection on dry column

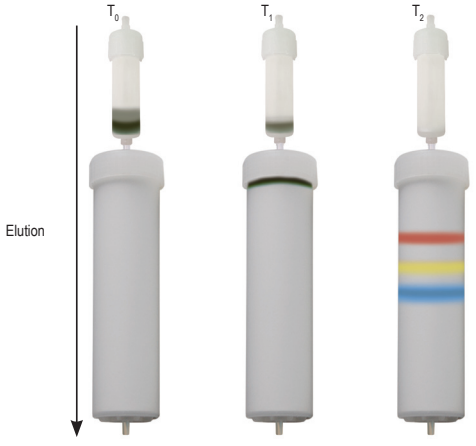
System not cleaned with starting eluent condition

Injection example 4: Dry-load injection on pre-conditionned column

Dry-load equilibration with 20% of strong solvent



Dry-load equilibration with 100% of weak solvent

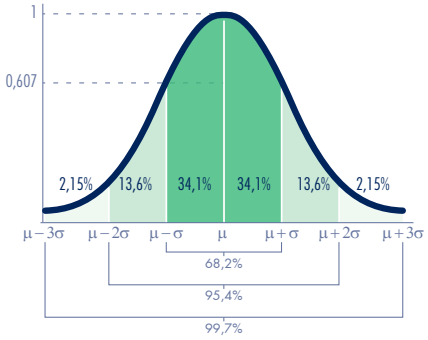


Best separation

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Peak shape

A peak can be assimilated to a Laplace Gauss curve with different amounts of species (percentage of the surface) according to the standard deviation of this function.



As the goal is a clear separation of different molecules we can easily guess that the more the peaks are separated the more the compounds are pure.

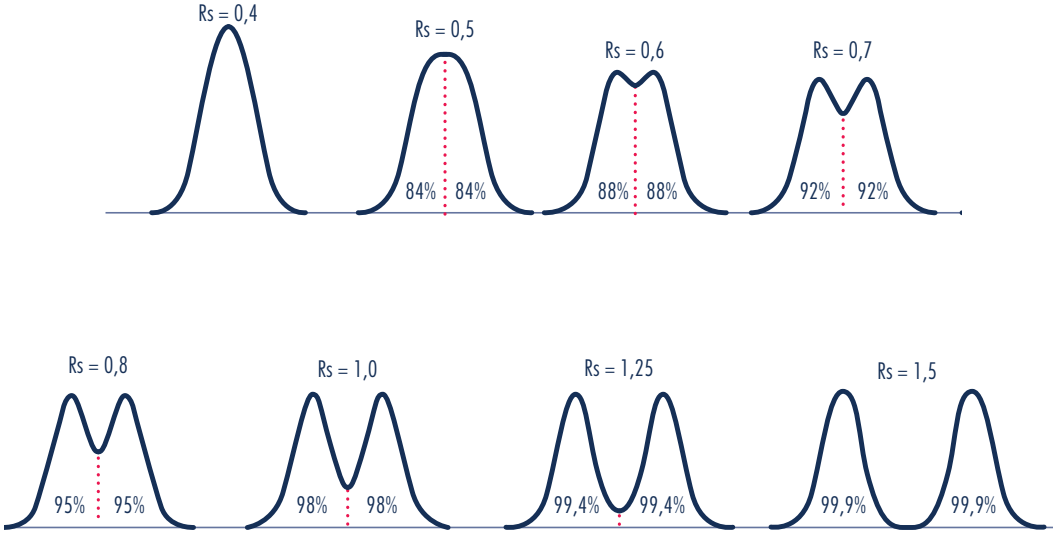
μ : average value
σ : standard deviation

Peak separation

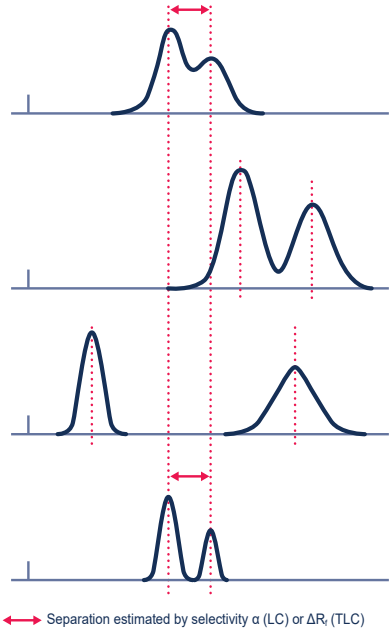
In that way, some parameters must be enhanced to reach the best compromize between elution time (quantity of solvent) and separation (resolution).

These parameters interact to lead to a measurable separation in terms of resolution Rs.

Different resolution values correlated to the peaks separation.



Peak separation



High diameter of particles
Overlapped peaks
How to improve ?

- Decrease solvent strenght to increase retention time
- Use more packing material (size of the column)
- Try a new packing material

- Improve selectivity by suitable choice of conditions (gradient, proportion or nature of solvents).

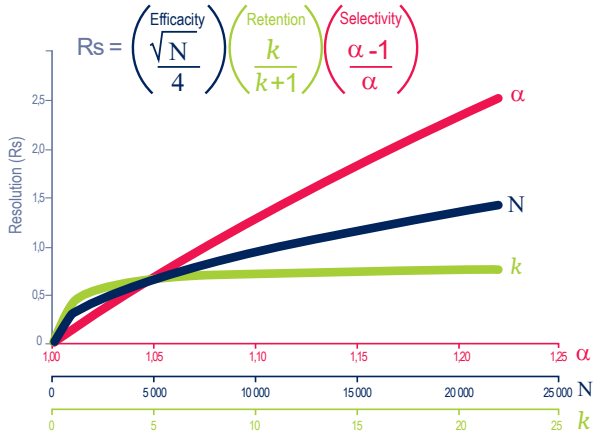
- Increase efficiency (plate number N) (smaller column-packing particles)

Separation parameters

The 3 main parameters ruling the chromatographic technic are:

Efficiency (plates number) : **N**
Retention factor : **k**
Selectivity : **α**

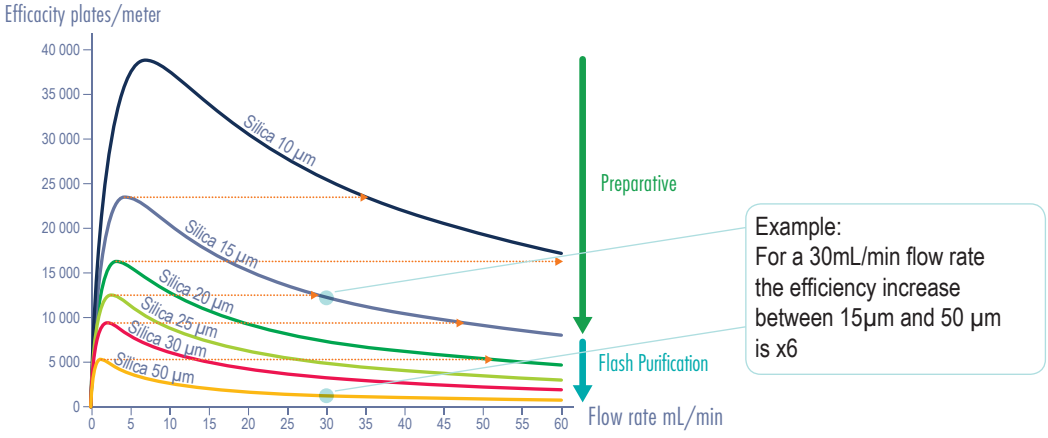
Their influence on resolution is shown in the side diagram.



Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Efficiency (N) influence

The efficiency parameter (N) is usually the first one that many operators want to change. But we must take into account that only the square root of this value influences the resolution.
However, as shown in the diagram, smaller particles allow to strongly impact the resolution.



Efficiency (N) influence: Example

The application below is highlighting the benefit of smaller particle sizes in terms of resolution and cost of purification. High efficiency (N) is giving better separation and allows a huger charge of crude sample.

PF-15SIHP vs IR-50SI columns

The Ultra Performance Flash Purification (UPFP) concept achieve accelerating the throughput by reducing the time and cost per sample of the purification process with increased confidence. What differentiates UPFP from Flash purification is the combination of advanced machine technology, built to last and mastery of small particles spherical silica puriFlash® columns which offers significant benefits over the traditional flash grade silica.

Conditions:
Device: puriFlash® 450
Solvents: A-Cyclohexane, B-Ethyl acetate
Injection Mode: Liquid injection
Crude sample mixture: 400mg of each Phthalate
Injection volume: 3.2mL
UV Detection: 254nm
Eluent conditions:

IR-50SI-F0080

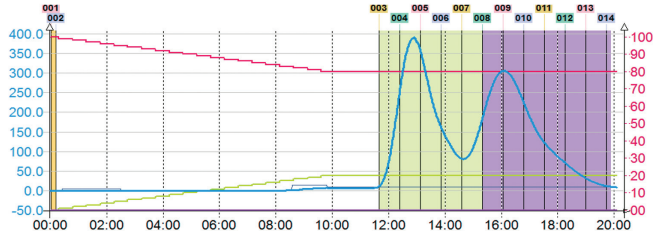
Step	CV	Time	%A	%B
1	0	0	100	0
2	3.28	09:50	80	20
3	5.63	16:51	80	20

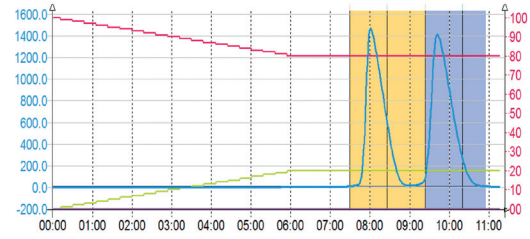
Loading capacity: 1%

PF-15SIHP-F0040

Step	CV	Time	%A	%B
1	0	0	100	0
2	3.28	06:03	80	20
3	5.63	10:21	80	20

Loading capacity: 2%





Purification cost PF-15SIHP vs IR-50SI columns

Flash column	IR-50SI	PF-15SIHP
Qty of silica per column	80g	40g
Silica Ratio		50% less
Flow rate	34mL/min	26mL/min
Cyclohexane consumption (equilibration & run time)	1032mL	418mL
Ethyl Acetate consumption (run time)	94mL	40mL
Total volume	**1126mL**	**458mL**
Consumption Ratio		59% less
Total Purification time	**20min**	**11min**
Time Saving		45%
Labor time: Edit method	5min	5min
Labor time: Analysis & Collection of collected fraction	25min	9min
Total Labor time	**30min**	**14min**
Time Improvement		114%
Column Cost (Cat. price)	15.10€	35.17€
Solvent Cost	27.66€	11.24€
Labor Cost	37.50€	17.50€
Waste recycling (Solvent & Column)	0.466€	0.232€
Total Cost of Purification per run	**80.73€**	**64.14€**
Cost Saving		26%

- Cylclohexane 1L price (Cat. price): 25.10€
- Ethyl Acetate 1L price (Cat. price): 18.70€
- Labor cost per hour: 75€
- Solvent recycling without halogen compound (Cat. price): 0,00035€/mL
- Silica columns recycling (Cat. price): 0,0009€/mL

Conclusion :

A 15µSIHP-F0040 column gives a better result with greater resolution, efficiency, loading capacity and improved retention versus a IR-50SI column. Using a 15µSIHP, reduce run time by 45%, improve in time for the purification by 114%, reduce the solvent consumption by 59% and improve in cost reduction for the purification by 26%. Lower collection volume means reduced evaporation time.

If the sufficient selectivity is reached, the 15µSIHP allows to achieve greater fraction purity. The best ratio cost/productivity is obtained with 15µm silica.

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Selectivity (α) influence

The selectivity (α) is an important parameter, occuring from the interaction of compounds with the stationary and mobile phases. The goal consists to find the best elution conditions leading to retention times farthest from each other.
It is a ratio between the K value of two compounds so directly related to their own retention time. **α = K2/K1**

For a column type F0025,
15µm silica gel,
113mm bed length

By changing the elution conditions and keeping the same column:

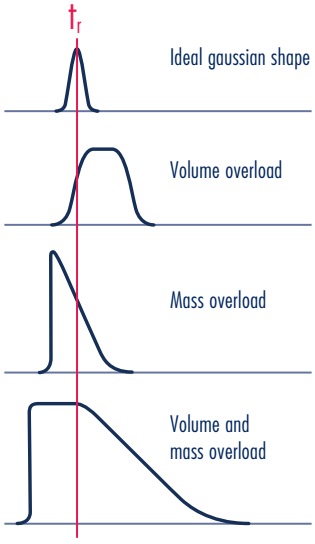
Selectivity (α) increase => x1.16 & Resolution (Rs) increase => x1.82

Retention (k) influence

On the other hand, the retention factor (k) has a real measurable effect up to 5.
After this value, his contribution on resolution is quite weak.
There isn't any advantage to aim very long retention times as they lead to a big collection volume of the compounds.

Loading capacity

The loading capacity of a column has also a great importance on the purification success.
A silica gel or any other adsorbent show a specific surface area, linked to its hability to develop interactions with the compounds.
In order to insure a smooth process of separation, once usually takes care to not exceed the ratio charge/surface.
This theorical value can be overtaken but that gives rise to peaks distorsion which reduces the resolution.



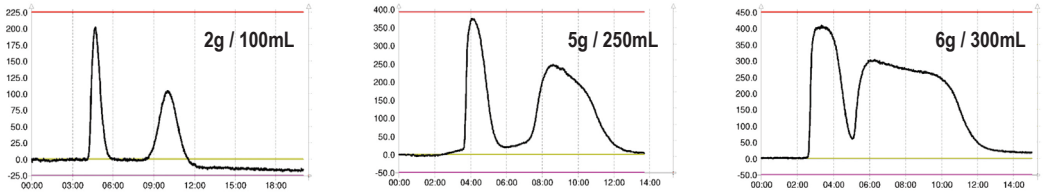
Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Mass overloading

Commonly, most of users say that a virgin silica gel bears a loading capacity around 10% of its weight. This is obviously less for a bonded one. That must be correlated with :
- the distance between two peaks
- their relative position
- their own surface

Mass over loading example

This example reveals how a column, according to a good resolution, can be loaded with a high amount of sample.
Purification of Guaifenesin enantiomers



Purify 6g under 12min

Flash Conditions :
Device: puriFlash®430
Solvents: 80% Hexane/20% EtOH
Column: CHIRALPAK OD 20µm 250x30mm glass column
Flow rate: 200mL/min
Injection mode: liquid injection by external injection pump
Sample concentration: 20g/L
Injection volume: 100mL (2g); 250mL (5g); 300mL (6g)
UV Detection

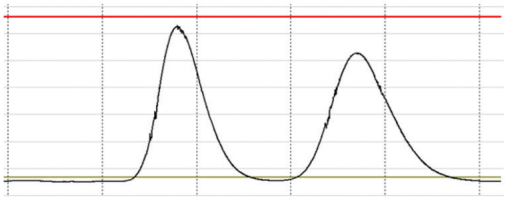
Volume overloading

In any case it is necessary to take into account the injected volume which creates a significant distortion of the peaks beyond **10% of the pore volume** of the column.
Due to these parameters the loading amount will vary widely.
This rule is valide for liquid and dry-load injection modes.

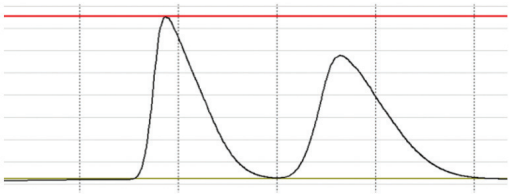
The following applications reveal the influence of the volume overloading.

Volume overloading example

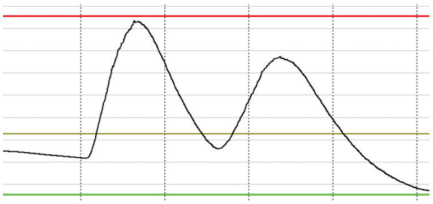
Liquid injection concentration 91mg/mL



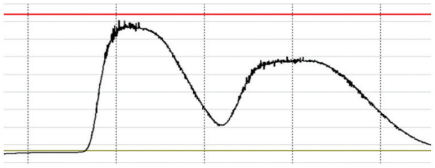
Injection volume: 0.7mL



Injection volume: 1.6mL

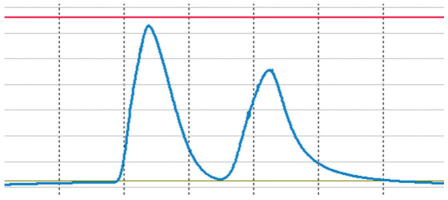


Injection volume: 2.3mL

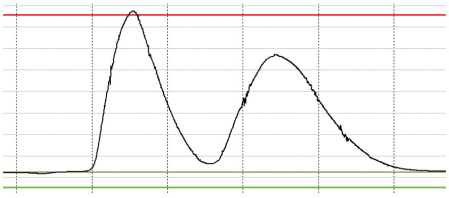


Injection volume: 3.3mL

Dry-Load injection 4G packed with Celite®



Injection on conditioned Dry-Load
Crude sample: 300mg



Injection on unconditioned Dry-Load
Crude sample: 300mg

Silica gel 50µm column
TLC elution: Heptane / Ethyl Acetate (95/(5)
Compounds: Methyl Phtalate, Ethyl Phtalate
Gradient elution

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Loading capacity values for virgin silica gel

The below quantities are given as an indication for virgin silica gels and may vary depending on the injection method, conditions of elution and the compounds to be purified.

ΔCV or Δk around 7												
column type		F0001	F0004	F0012	F0025	F0040	F0080	F0120	F0220	F0330	F0800	F1600
15µm	HP	0.10g	0.45g	1.4g	2.8g	4.5g	9.1g	14g	25g	37g	---	---
	HC	0.11g	0.52g	1.6g	3.2g	5.2g	10.4g	16g	29g	43g	---	---
20µm	IR	0.08g	0.38g	1.1g	2.4g	3.8g	7.6g	11g	21g	31g	---	---
25µm	HC	0.09g	0.40g	1.2g	2.5g	4.0g	8.0g	12g	22g	33g	80.0g	160.0g
30µm	HP	0.07g	0.34g	1.0g	2.1g	3.4g	6.7g	10g	18g	28g	67g	134g
50µm	IR	0.06g	0.29g	0.9g	1.8g	2.9g	5.8g	9g	16g	24g	58g	116g
	HP	0.06g	0.30g	0.9g	1.9g	3.0g	6.0g	9g	17g	25g	60g	120g
	HC	0.07g	0.35g	1.0g	2.2g	3.5g	6.9g	10g	19g	29g	69g	138g

ΔCV or Δk around 5												
column type		F0001	F0004	F0012	F0025	F0040	F0080	F0120	F0220	F0330	F0800	F1600
15µm	HP	0.09g	0.42g	1.3g	2.6g	4.2g	8.5g	13g	23g	35g	---	---
	HC	0.10g	0.48g	1.5g	3.0g	4.8g	9.7g	15g	27g	40g	---	---
20µm	IR	0.07g	0.35g	1.0g	2.2g	3.5g	7.0g	10g	19g	29g	---	---
25µm	HC	0.08g	0.36g	1.1g	2.3g	3.6g	7.3g	11g	20g	30g	73g	146g
30µm	HP	0.06g	0.29g	0.9g	1.8g	2.9g	5.7g	9g	16g	24g	57g	114g
50µm	IR	0.04g	0.21g	0.6g	1.3g	2.1g	4.2g	6g	11g	17g	42g	84g
	HP	0.05g	0.22g	0.7g	1.4g	2.2g	4.4g	7g	12g	18g	44g	88g
	HC	0.05g	0.25g	0.7g	1.6g	2.5g	5.0g	7g	14g	21g	50g	100g

ΔCV or Δk around 2												
column type		F0001	F0004	F0012	F0025	F0040	F0080	F0120	F0220	F0330	F0800	F1600
15µm	HP	0.04g	0.18g	0.5g	1.1g	1.8g	3.5g	5g	10g	14g	---	---
	HC	0.04g	0.20g	0.6g	1.3g	2.0g	4.0g	6g	11g	17g	---	---
20µm	IR	0.03g	0.14g	0.4g	0.9g	1.4g	2.7g	4.1g	7g	11g	---	---
25µm	HC	0.03g	0.14g	0.4g	0.9g	1.4g	2.9g	4g	8g	12g	29g	58g
30µm	HP	0.02g	0.10g	0.3g	0.6g	1.0g	2.0g	3g	5.5g	8.2g	20g	40g
50µm	IR	0.015g	0.07g	0.20g	0.4g	0.7g	1.3g	2g	3.7g	5.5g	13.5g	27g
	HP	0.015g	0.07g	0.21g	0.4g	0.7g	1.4g	2.1g	3.9g	5.8g	14.0g	28g
	HC	0.017g	0.08g	0.24g	0.5g	0.8g	1.6g	2.4g	4.4g	6.7g	16.0g	32g

IR: Irregular silica gel - Average values for compounds < 800MW
HP: High Performance silica gel - Average values for compounds < 800MW
HC: High Capacity silica gel - Average values for compounds < 500MW

Loading capacity values for virgin silica gel

The below quantities are given as an indication for virgin silica gels and may vary depending on the injection method, conditions of elution and the compounds to be purified.

ΔCV or Δk around 0.8												
column type		F0001	F0004	F0012	F0025	F0040	F0080	F0120	F0220	F0330	F0800	F1600
15µm	HP	0.014g	0.07g	0.20g	0.41g	0.7g	1.3g	2.0g	3.6g	5.5g	---	---
	HC	0.016g	0.08g	0.23g	0.47g	0.8g	1.5g	2.3g	4.2g	6.2g	---	---
20µm	IR	---	0.05g	0.15g	0.30g	0.5g	1.0g	1.5g	2.7g	4.0g	---	---
25µm	HC	---	0.05g	0.16g	0.32g	0.5g	1.0g	1.6g	2.8g	4.3g	10.5g	21.0g
30µm	HP	---	0.04g	0.11g	0.22g	0.4g	0.71g	1.1g	2.0g	2.9g	7.0g	14.0g
50µm	IR	---	---	0.07g	0.15g	0.24g	0.5g	0.7g	1.3g	2.0g	4.8g	9.6g
	HP	---	---	0.08g	0.16g	0.25g	0.5g	0.8g	1.4g	2.1g	5.0g	10.0g
	HC	---	---	0.09g	0.18g	0.29g	0.6g	0.9g	1.6g	2.4g	5.8g	11.6g

ΔCV or Δk around 0.4												
column type		F0001	F0004	F0012	F0025	F0040	F0080	F0120	F0220	F0330	F0800	F1600
15µm	HP	---	0.04g	0.12g	0.25g	0.40g	0.8g	1.2g	2.2g	3.3g	---	---
	HC	---	0.05g	0.14g	0.29g	0.46g	0.9g	1.4g	2.5g	3.8g	---	---
20µm	IR	---	---	0.09g	0.18g	0.29g	0.6g	0.9g	1.6g	2.4g	---	---
25µm	HC	---	---	0.09g	0.19g	0.31g	0.6g	0.9g	1.7g	2.6g	6g	12g
30µm	HP	---	---	0.06g	0.13g	0.21g	0.4g	0.6g	1.2g	1.7g	4.2g	8.5g

IR: Irregular silica gel - Average values for compounds < 800MW
HP: High Performance silica gel - Average values for compounds < 800MW
HC: High Capacity silica gel - Average values for compounds < 500MW

Loading capacity for bonded phases (RP & NP)

Loading capacity for bonded phases as a percentage of the adsorbent mass in the column						
		Δk = 0.4	Δk = 0.8	Δk = 2	Δk = 5	Δk = 7
15µm	60Å < pore size < 120Å	0,12%	0,20%	0,55%	1,30%	1,40%
	200Å < pore size < 300Å	0,06%	0,10%	0,25%	0,65%	0,70%
30µm	60Å < pore size < 120Å	0,07%	0,10%	0,30%	0,90%	1,10%
	200Å < pore size < 300Å	0,03%	0,06%	0,15%	0,45%	0,60%
50µm	60Å < pore size < 120Å	...	0,08%	0,20%	0,70%	0,90%
	200Å < pore size < 300Å	...	0,04%	0,10%	0,35%	0,50%

These values are given as an indication and may vary depending on the molecules and adsorbents used.

Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

Gradient mode

Isocratic vs Gradient

An other way to enhance the separation consists in increasing the amount of the strong solvent all along the elution, starting with a low percentage of this one.

A gradient can be modeled in two ways: linear or incremental.

Depending on the difficulty of the separation one may be more suitable than the other. Interchim® has developed an algorithm that automates the composition of the gradient according to the difficulty of the separation and the particle size of the columns used.

Compared with an isocratic method, a well-developed gradient makes it possible to significantly reduce the width of the peaks and thus considerably increase the mass of crude to be purified.

This influence is very clear in areas of Rf between 0.1 and 0.7.Isocratic elution will not allows good selectivity in a restricted Rf zone between 0.1 and 0.3.

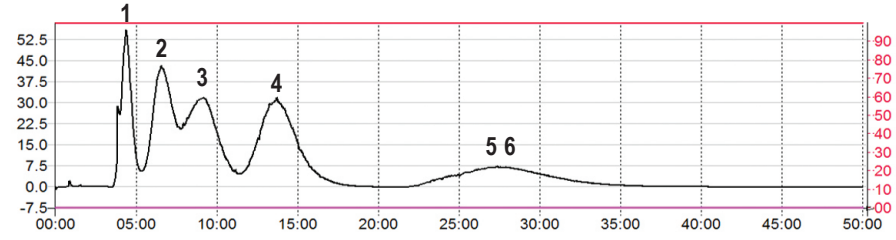
According to the following purification:

Compound 2: Rf = 0.52

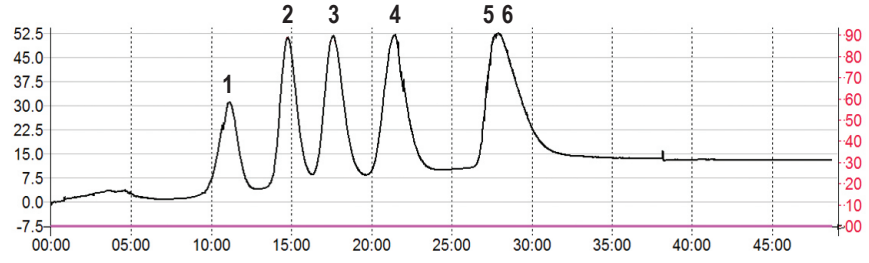
Compound 3: Rf = 0.33

Eluent: cyclohexane/ethyl acetate column type F0025 IR 50µm flow rate 15mL/min

Isocratic **91%/9% (original TLC eluent composition)**



Gradient **slope 2% => 17%**



Conclusion

According to those explanations, it is easy to understand that a longer column or a smaller particle size gives more efficiency and enhance greatly the separation.

By choosing the right silica gel and the right eluent, the compounds will interact advantageously and will be retained differently.

The gradient elution mode will also greatly improve the separation all along the purification.

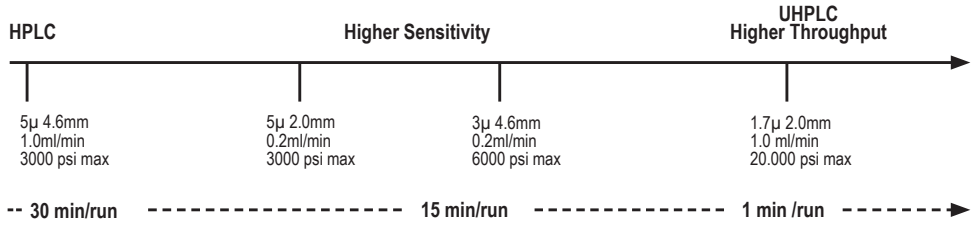
Finally the column size must be related to the quantity of crude sample to purify and the difficulty of the separation.

Ultra Performance Flash Purification
or how to do high throughput purification?

Liquid chromatography is a technique that first requires finding the right selectivity to properly separate the compounds. The purpose of preparative liquid chromatography is to recover the compound of interest with an aim of purity, quantity and productivity.

Improvements in the analytical techniques of liquid chromatography over the last thirty years have mainly focused on the transition from the use of irregular silicas to spherical silicas, the increase in the supply of stationary phases, in particular for the reverse phase and finally the benefit of reducing the size of the silica particles.

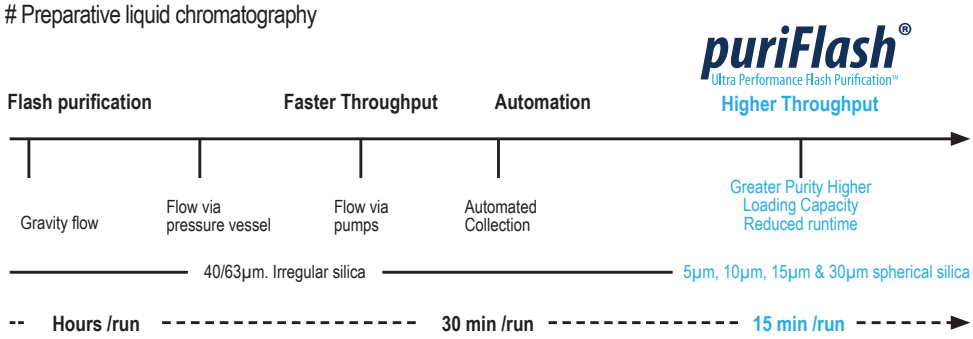
Analytical liquid chromatography



This evolution has led to a high increase of productivity by a significant reduction of the runtime, tens of minutes to min.

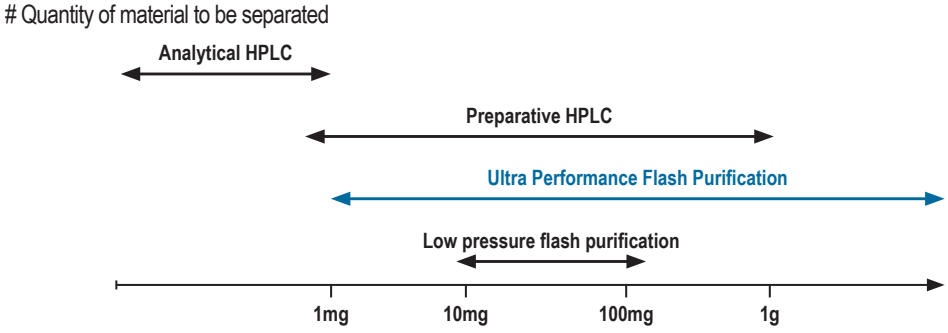
It is always a challenge and often a compromise to obtain the desired purity, loading and throughput. Since years, Interchim® has pushed to scientists a similar approach, as for the analytical field, called " Ultra Performance Flash Purification" to help them to achieve their day to day challenge.

Preparative liquid chromatography



Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

This concept has established the Ultra Performance Flash Purification as an innovative solution that offers a larger spectrum of purification versus other techniques.



a) Irregular vs. Spherical silicas

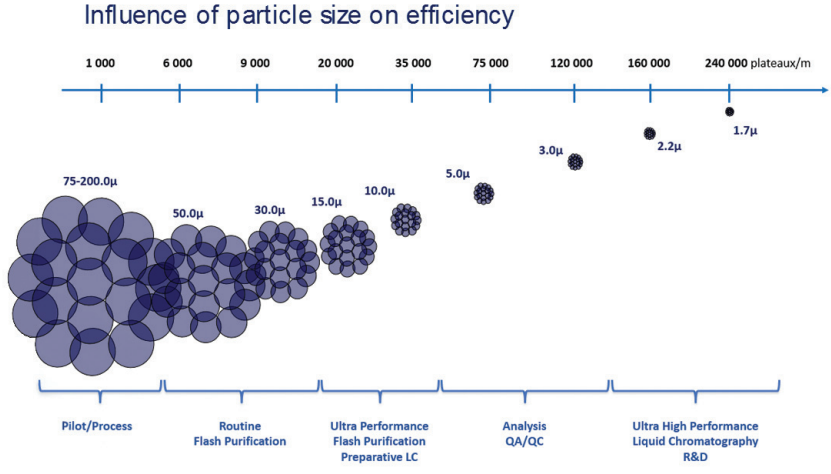
Usually spherical silicas are purer and have narrow particle and pore size distribution than irregular one. These advantages make them easier to pack in column with an optimum bed density. The benefit for the user is an optimized and reproducible recovery, a lower collection volume and a reduced evaporation time.

b) Stationary Phases

A wide range of selectivities must be available to cover all the different polarities of samples to be purified. Interchim offers more than 50 selectivities for normal and reverse phase, ion exchange, hilic, … and for the purification of peptides and polypeptides.

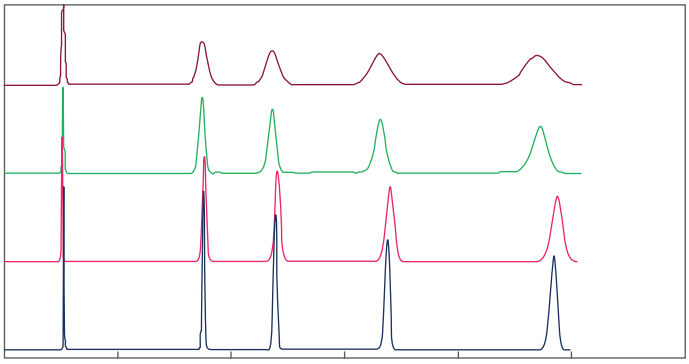
The benefit of reduced particles size

As the particle size of the silica beads reduced, the efficiency increase while the related back pressure of a packed column with such particle increase.

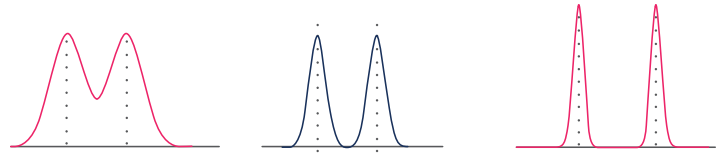


The benefit of reduced particles size

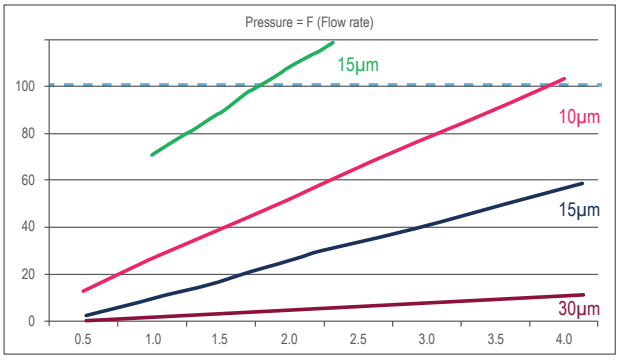
One of the benefit of greater efficiency is the direct impact on the peak shape. The higher the efficiency is the shaper the peak is. Which means for a single peak, smaller collection volume and less evaporation time.



The impact is far greater when the question is to separate two peaks. For the same selectivity, the higher the efficiency is, the higher the resolution is.



This lead to give to the scientist more flexibility by either to collect a product with higher purity or to increase the loadability or to reduce the runtime. Finally, it increases the global productivity of the purification.
The counter-part is the back pressure generated by the reduction of the particle size.



"Optimum" flow rate @ 0.75mL/min for a 4.6mm i.d. column

The above experiments have been done on a 5, 10, 15 and 30 micron Uptisphere Strategy C18HQ packed into a 4.6x250mm columns.
At the optimum flow rate for a 21.2mm id (21mL/min), a 28mm id (37mL/min) and a 50mm id (118mL/min) the back pressure generated, under MeOH/H_2O (50/50) is for a:
30 micron = 3 bar – 15 micron = 6 bar – 10 micron = 20 bar – 5 micron = 60 bar

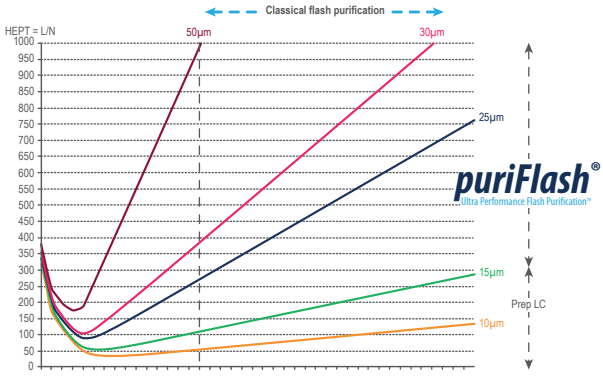
Tel.: +33 4 70 03 88 55 | Hotline: +33 4 70 03 73 01 | E-mail: interfine@interchim.fr | Fax: +33 4 70 03 82 60 | WWW.INTERCHIM.COM

The benefit of reduced particles size

Interchim® has developed over the years high quality pump able to handle, with accuracy and repeatability, such a pressure range to satisfy the scientist purification objective of purity, loading capacity or runtime.

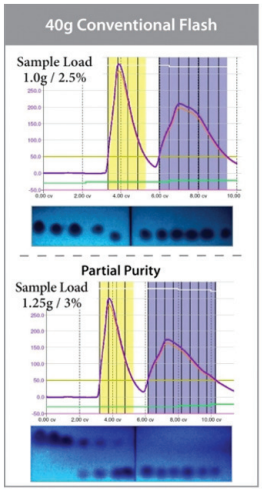
Increasing flow rate without compromising with resolution is also a benefit of using smaller particle size:

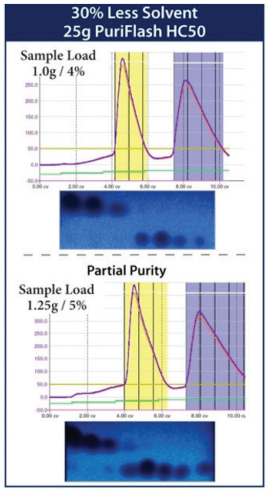
H = Fion (flow rate) for a 25g column

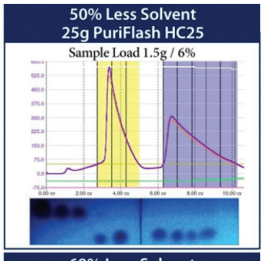


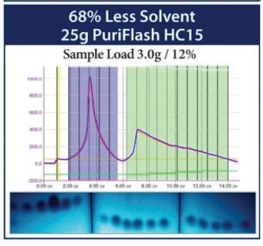
4.6x250mm col	
Paticle size	Opt flow rate
5.0µm	0.710mL/min
10.0µm	0.370mL/min
15.0µm	0.240mL/min
20.0µm	0.190mL/min
25.0µm	0.160mL/min
30.0µm	0.130mL/min
50.0µm	0.087mL/min

Example of the advantages & benefit of the Ultra Performance Flash Purification concept









100% Purity				
Column	Sample Load	Tubes	Collected Volume	Solvent Consumed
40g Conventional	1.0g	11	263mL	480mL
25g puriFlash® HC50	1.0g	7	144mL	340mL
25g puriFlash® HC25	1.5g	9	199mL	360mL
25g puriFlash® HC15	3.0g	17	367mL	460mL

Partial Purity / Overload Condition				
Column	Sample Load	Tubes	Collected Volume	Solvent Consumed
40g Conventional	1.25g	13	298mL	540mL
25g puriFlash® HC50	1.25g	8	186mL	360mL

Test Conditions

Sample: 50mg/mL dibutyl and diethyl phtalate UV: 254nm
Flow rate: 20mL/min Tube volume: 25mL
Solvents: A:Heptane B: EtOAc
Gradient: 0 - 7 CV 5% B, 7 - 13 CV 5 - 8% B