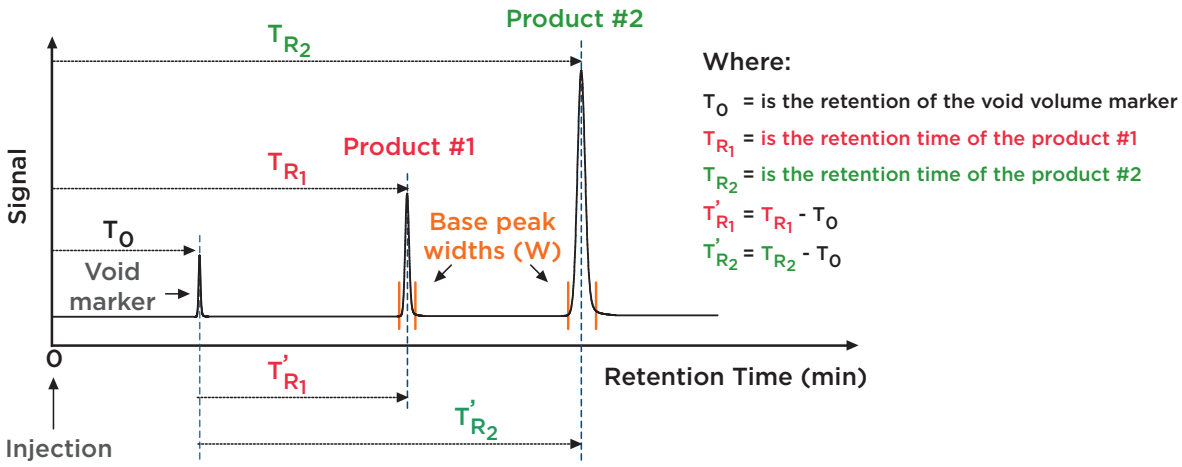
Important HPLC Definitions and Equations

Typical Chromatogram in liquid chromatography



Capacity Factor or Retention Factor (*k'*) is measured by the retention factor of the analyte compared to an unretained peak (*void volume marker*) using the following equation:

$$k' = \frac{(T_R - T_0)}{T_0}$$

Where:

T_R: is the retention time of the analyte

T_0: is the retention time of the unretained product

Efficiency (*N*) is usually measured by the plate count (*N or also called theoretical plate number*) using various equations. The most popular ones are:

By USP (*United States Pharmacopeia*)

$$N = 16 \times \left[\frac{t}{W}\right]^2$$

Where:

N: is the number of theoretical plates

t: is the retention time of the analyte

W: is the width at the base of the analyte

By DAB (*German Pharmacopeia*)

$$N = 5.54 \times \left[\frac{t}{W_{0.5}}\right]^2$$

Where:

N: is the number of theoretical plates

t: is the retention time of the analyte

$W_{0.5}$: is the width-at-half-height of the analyte

Selectivity (α) is measured by the retention factor ratio between two similar compounds.

$$\alpha = \frac{k'_2}{k'_1}$$

Where:

K_1': is the retention factor of product #1

K_2': is the retention factor of product #2

Separation's difficulty based on the selectivity value. If the selectivity is:

≥ 2:	Easy separation
1.5 - 2:	Possible separation*
1.2 - 1.5:	Difficult separation
≤ 1.2:	Very difficult separation**

* Method adjustment could be required

** Selectivity's optimization may be required

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Resolution (R) can be expressed using the two following equations

$$R = \frac{\sqrt{N}}{4} \times \left(\frac{\alpha - 1}{\alpha}\right) \times \left(\frac{1 + k'_2}{k'_2}\right)$$

Where:

N: is the number of theoretical plates

α: is the selectivity

K_2': is the retention factor of product #2

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

Where:

T_1: is the retention time of the product #1

T_2: is the retention time of the product #2

W_1: is the width at the base of the product #1

W_2: is the width at the base of the product #2

Summary of Influencing Factors in HPLC

To chose the most suitable HPLC column, various parameters need to be taken into account: the desired selectivity and the sample load as well as the efficiency and the resolution. All these parameters are influenced by different factors in HPLC summarized in the table below.

Liquid Chromatography Influencing Factors

Properties	Typical Parameters	Affected Influencing Factors	Limitations
Chromatographic Conditions	Solvent	Retention, Efficiency	Back-pressure & phase stability
	pH	Selectivity, Resolution & Retention	Phase stability
	Flow Rate	Analysis Time, Efficiency & Resolution	Back-pressure & phase stability
Packing Characteristics	Chemistry (*SiO_2, C18, etc.*)	Selectivity, Resolution & Retention	Solvent used
	Pore Size (Å)	Sample Load & Selectivity	Size of the molecule
	Particle Size (µm)	Back-pressure, Efficiency & Resolution	Back-pressure & flow rate
HPLC Column Dimensions	Internal Diameter	Sample Load & Sensitivity	Back-pressure & flow rate
	Length	Analysis Time & Resolution	Back-pressure & analysis time too long

HPLC Method Scaling Up or Scaling Down Theory

When your experimental conditions are well optimized to get the most suitable purification, it is possible to scale up/down your method by keeping the same particle size and sorbent using these two equations:

Adjustment of the Sample Load

$$x_2 = \frac{x_1 \times r_2^2 \times C_L}{r_1^2} \quad \text{where} \quad \left[C_L = \frac{L_2}{L_1}\right]$$

Where:

x_1: is the maximum sample load in initial column

x_2: is the maximum sample load in final column

r_1: is the radius of the initial column

r_2: is the radius of the final column

L_1: is the length of the initial column

L_2: is the length of the final column

Adjustment of the Flow Rate

$$V_2 = \frac{V_1 \times r_2^2}{r_1^2}$$

Where:

V_1: is the flow rate use with the initial column

V_2: is the flow rate use with the final column

r_1: is the radius of the initial column

r_2: is the radius of the final column

Acceptable Modifications to an HPLC Validated Method

Even if you are using an FDA validated or a USP recommanded method, some operating conditions can be adjusted if the modifications respect the acceptable specifications proposed by Pharmacopeias[1-3] and the FDA[4]. A side-by-side comparison of both the original and the adjusted method needs to be performed to demonstrate that the method's accuracy and precision is not affected by these modifications.

Acceptable Modifications to an HPLC Validated Method

Parameters	Allowable modification	Examples of possible modifications
Mobile phase pH	± 0.2 units	Validated pH: 7.0 Allowed pH range: 6.8 – 7.2
Concentration of salts in buffer	± 10%	Validated concentration: 20 mM Allowed concentration range: 18 - 22 mM
Ratio of components in mobile phase	Only the minor components can be adjusted by ± 30% or ± 2% absolute (*i.e.: in regards to the total mobile phase*), whichever is the larger but should never exceed ± 10% absolute or removed totally.	**Binary mixtures:** Validated ratio: 50/50 Allowed ratio: 40/60 to 60/40 Validated ratio: 95/5 Allowed ratio: 93.5/6.5 to 96.5/3.5 **Ternary mixtures:** Validated ratio: 60/35/5 Allowed % of the 1st component: 60% Allowed % of the 2nd component: 25 – 45% Allowed % of the 3rd component: 3.5 – 6.5% The total of the three components together need to be 100%.
Wavelength of UV detector	No modification allowed.	n/a
Column length	± 70%	Validated length: 150 mm Allowed length range: 45 - 255 mm
Column inner diameter	± 50%	Validated inner diameter: 4.6 mm Allowed inner diameter range: 2.3 – 10.6 mm
Flow rate	± 50%	Validated flow rate: 1.00 mL/min Allowed flow rate range: 0.5 – 1.5 mL/min
Injection volume	May be increased to as much as 2 times if no adverse effects on LOD and repeatability.	n/a
Particle size	No increase permitted. May be decreased by as much as 50%.	Validated particle size: 5 µm Allowed particle size range: 2.5 - 5 µm
Column temperature	± 20%	Validated temperature: 23°C Allowed length range: 18.4 – 27.6°C

1. USP. USP 32–NF 27, Chromatography <621>. Rockville, MD: USP; 2009:227.
2. USP. Second Supplement to USP 32–NF 27. Rockville, MD: USP; 2009:4147.
3. USP. USP 32–NF 27, Verification of Compendial Procedures <1226>. Rockville, MD: USP; 2009:736.
4. ORA Laboratory Procedure, Food and Drug Administration, modification criteria.