

Laboratory Chromatography Guide



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Abbreviations

TLC	Thin-layer chromatography
HPLC	High-performance liquid chromatography
$[C]_{\text{phase 1}}$	Concentration of the compound C in phase 1
GC	Gas chromatography
RPC	Reversed phase chromatography
S_i	Solvent strength
RI	Refractive index
S.I.	Symmetry index
F_m	Delivery rate
V_0	Dead volume
GLP	Good laboratory practice
MPLC	Medium pressure liquid chromatography
LC	Liquid chromatography
UV	Ultraviolet

Introduction

Chromatography has developed very rapidly over the past few years. It was a very long way from the first “capillary pictures” of Runge (1822–1850) through the early work of Tswett, the discoverer of Adsorption Chromatography (1903, separation of plant pigments) to modern HPLC from about 1967. Tswett had in fact adopted the name “Chromatography” for this separation technique (from the Greek chromos = colors, graphein = write).

However, the focal point of this enormous development was clearly in the area of analysis. In preparative chemistry, on the other hand, chromatographic separations are frequently carried out even today by a very simple method, i.e. with the aid of a simple glass column under hydrostatic pressure. The first publications on preparative chromatography under elevated pressure, so-called Flash Chromatography, only appeared towards the end of the seventies. This method too was subsequently further refined. This finally resulted in medium pressure liquid chromatography (called MPLC in the following), which is very efficient but nevertheless readily comprehensible and simple to carry out. At the same time, attempts were made to increase the size of the analytical HPLC systems and thus make them available also for preparative or at least semi-preparative work.

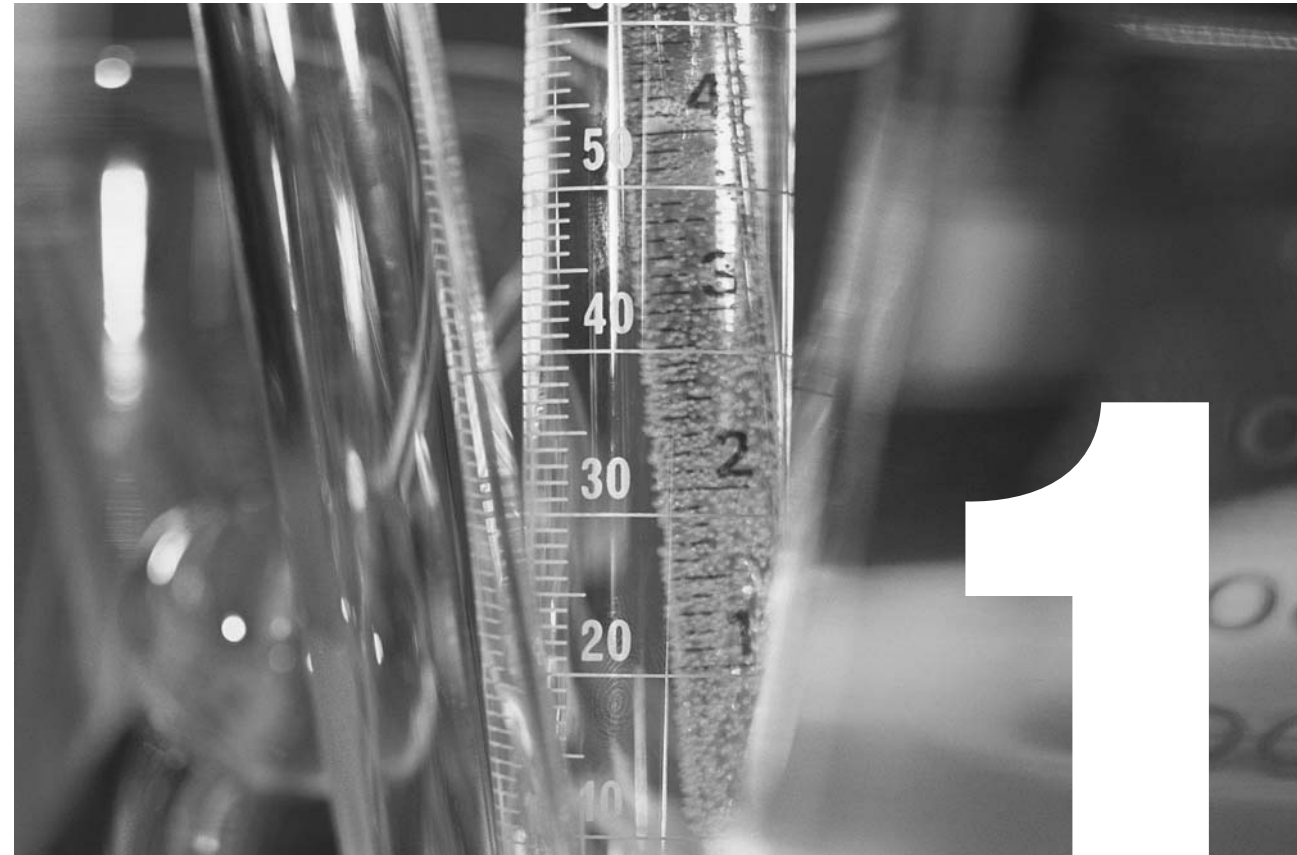
However, closer scrutiny reveals substantial differences between routine analysis and preparative separation. It is therefore essential for a preparative MPLC system to meet the specific requirements for such separations. The following factors must be noted in particular:

- Flexibility in the choice of column. The amount of substance and the required separating power differ for virtually every problem to be solved. Simple and economical adaptation to the particular separation problem must therefore be possible.
- High delivery of the pump. Large columns require large volume flows so that the desired linear flow rate can be achieved.
- Wide pressure range. The trend in preparative chromatography is clearly towards fine-grained adsorbents, which offer substantial resistance to flow.
- The apparatus must be simple to handle. In particular, filling and emptying of the columns as well as operation of the entire remaining system must be capable of being mastered immediately without a prolonged familiarization period. In the preparative laboratory, the liquid chromatography is in general not a specialized unit but rather a universal tool.

This booklet aims to provide both non-specialists and specialists with short and basic as well as with more detailed explanations of the different procedure steps encountered during a liquid chromatography separation.

The first part, “Quick Guide”, is a short, practice-oriented overview of liquid chromatography (LC) for quick reference searches and the second part provides a broader and deeper description of the process, under both practical and theoretical considerations.

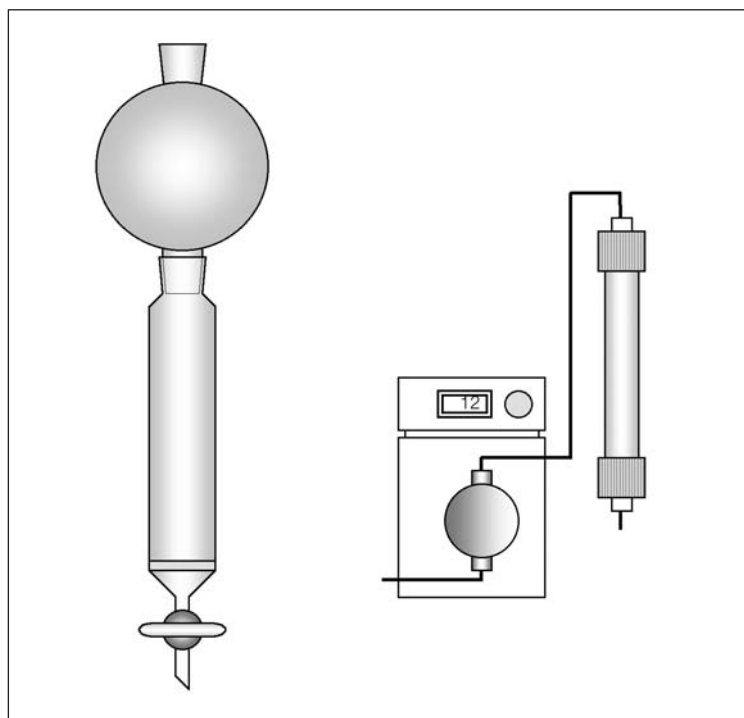
Flash Guide Basics



1 Introduction

Chromatography is a standard method used in preparative laboratories to isolate and purify substances. In the early days of chromatography simple glass columns were chiefly used, operated by means of the hydrostatic pressure of the solvent acting as an eluent. In a publication in 1978 Clark W. Still explored the possibility of accelerating the separation process in simple glass columns, which was until then the commonly used method, and thereby considerably increasing the efficiency of the technique. The results were convincing and the foundations of modern flash chromatography were laid. It triumphantly established itself in laboratories as an indispensable purification method in preparative chemistry. Flash chromatography has since undergone constant development, and has been adapted to meet present day expectations in terms of equipment and convenience.

Figure 1:
From the simple glass
column to modern
flash chromatography.



Modern flash chromatography systems are popular nowadays because they are simple to handle, flexible and can be universally employed. The first part of this brochure aims to give simple, accessible advice, which should ideally instantly lead to effective laboratory elutions.

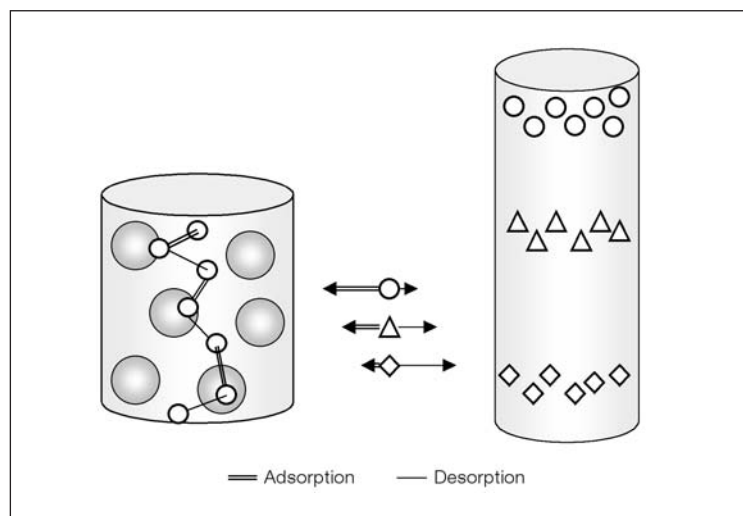
The following abbreviations are used in the first part:

TLC	Thin-layer chromatography
RP	Reversed phase, modified silica gels
NP	Normal phase polar silica gel phases
UV	Ultraviolet
S_i	Solvent strength (substitutes polarity)
% A	% solvent with low solvent strength
% B	% solvent with high solvent strength
R_f	Retention factor (from thin-layer chromatograms)
CV	Column volumes
ΔCV	Difference in column volumes
R_{f_1}	Retention factor of first substance (substance which spreads onto the TLC plate the quickest. The index increases according to the time the substance takes to spread).

2 Principle of chromatography

Chromatographic separation is based on a balanced state among the components to be separated, an adsorbent agent in the column (= stationary phase) and a solvent flowing through it (mobile phase). When a component settles on the stationary phase this is defined as adsorption, while detachment by the mobile phase is defined as desorption. A high adsorption capacity between the components of interest and the stationary phase means that there is a high retention of these components and that there is a considerable delay in elution from the column. The separation of a mixture into its individual components is only possible if the individual components in a combination of stationary and mobile phases have different adsorption/desorption properties.

Figure 2:
Adsorption und
Desorption, schematic
illustration of the
chromatographic
separation process.



3 Choice of the appropriate stationary phase

Chromatographic separation can be carried out on both polar and apolar stationary phases, and suitable sorbents are available from various manufacturers.

“Standard” chromatography requires the use of polar stationary phases such as silica gel and nonpolar solvents. The individual components are delayed as a result of a reaction between the polar function component groups and the polar groups of the sorbent. Low polarity substances are eluted first, followed by components of increasing size.

In “reversed phase” chromatography, however, the stationary phase is nonpolar and elution is by means of polar solvents. These stationary phases are produced by modifying silica gel with nonpolar groups such as C-18 or similar substances. Substances are eluted in order of decreasing polarity from reversed phase columns, i.e. the substance with the highest polarity appears first. Reversed phase materials are considerably more expensive than standard stationary phases, and this is one of the reasons why standard stationary phases are primarily used in flash chromatography. If the substance classes to be separated allow, modified stationary phases can nonetheless be used without restrictions or problems.

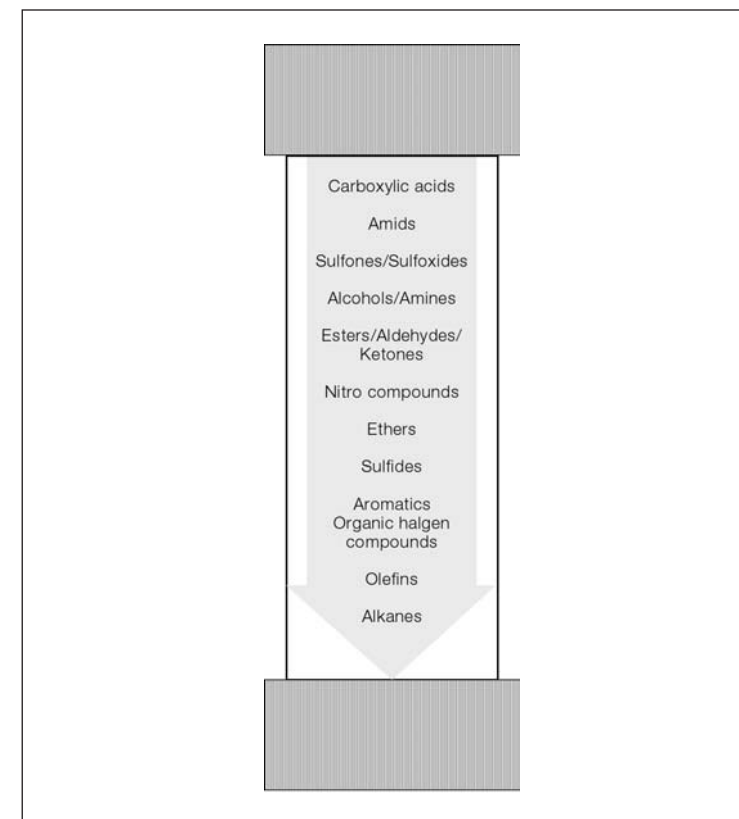


Figure 3:
Elution sequence for
normal silica gel.

**Preparative Column
Chromatography**
Theory and Practice



1 Starting point – Definition of the problem

The properties of the sample and the use of the purified compounds dictate the purification procedure to follow. Therefore, any separation should be carefully planned and targets clearly set before starting to avoid basic pitfalls and to make best use of available resources for an optimized purification.

1.1 Sample

Several characteristics of a sample must be considered before attempting a purification. The most important one is the sample's solubility. It must be ensured that the sample is completely soluble in the mobile phase; otherwise it will aggregate onto the column adsorbing material and make any purification attempts useless.

Other major features must also be considered, such as the

- origin (synthetic reaction mixture, biological crude extract)
- composition (known or unknown)
- matrix (chemical and physical properties)
- phase (gas, liquid or solid)
- concentration of the substance of interest (trace amounts, one or more major components)

The stability of the sample is also of great importance. The sample may degrade on the column or be oxygen- or light-sensitive.

A literature search can orient toward the appropriate system and conditions to be used with a known sample.

A judicious and simple sample pretreatment (i.e. filtration, extraction, ...) can often be useful to remove unwanted material from the original mixture, such as catalyst residue or reaction matrix, and thereby make the chromatographic separation an easier task. This is especially applicable to biological extracts and when working with expensive columns (RPC).

These considerations related to the sample's nature will decide if the sample requires a conditioning or a pre-treatment prior to its application onto the chromatography column.

1.2 Purity

The purity of a sample is limited by the ability to detect impurities therein or a lower activity thereof by available analytical means.

The required purity and the constraints of the further processing of the isolated compound(s) govern the conditions under which the chromatographic separation will be carried out. When purifying a substance to be used as a reference standard or as a drug to be tested on animals, the purity must be in excess of 99%. Usually, the higher the purity to be achieved, the closer the separation profile should be followed and the more carefully the procedure should be carried out. In the ideal case, some chromatographic separa-

tions may readily lead to sufficient purification without needing too much concern.

1.3 Others

Other logistical factors will also influence the procedure parameters when there is a choice between different conditions. Such factors are: time required (to maximize the throughput); difficulty of the procedure; cost; safety (solvents); procedure frequency and system capacity.

The safety concerns are not to be neglected. The hazard usually comes from the solvent used and from the sample. Since the use of large amounts of solvent can sometimes not be avoided with preparative-scale chromatography, the quantities also play a significant role when assessing the solvent's toxicity, not only its intrinsic toxicity. Careful consideration of the hazardous materials contained in the residue should be given before throwing any sample into the waste disposal.

The system capacity and the quantities to be processed must also be taken into account to limit the costs of the procedure.

2 Fundamentals – The basic principles

2.1 General

Chromatography is a powerful and extensively used method for chemical separations.

The migration of a mixture from a reaction or from more complex systems (i.e. biological crude extracts) together with a carrier mobile phase over a fixed bed of retardant and under the appropriate conditions promotes the separation of the mixture into its single components. Virtually any mixture that can be solubilized can be separated into its single components by chromatography.

Chromatography is used to separate mixtures at a preparative scale and is also extensively used for analytical goals such as qualitative substance identification and quantification. The goal of the separation rather than the quantity of sample being separated determines the analytical or the preparative nature of the process.

Preparative chromatography is usually performed on large scale batches with the sample saturating the stationary phase. This leads to different requirements in the detection devices. Analytical detectors will need a high sensibility that would be saturated at a preparative scale. Preparative detectors need to accommodate a high flow rate where a high sensibility does not play a major role.

Preparative purification enriches or purifies one or more components and also implies a further usage of the separated material, whereas analytical chromatography focuses mainly on the chromatogram or fingerprint and is usually not concerned about the sample's faith.

2.2 Adsorption chromatography

Chromatographic separations make use of the ability of compounds to adsorb, or to adhere to surfaces. Adsorption is a boundary reaction between a dissolved substance and a solid substance. Adsorption chromatography is mainly concerned with the weak, and therefore reversible, interactions between two phases. The formation of these weak bonds is called adsorption, and the breaking of these bonds is referred to as desorption.

2.2.1 Separation mechanisms in adsorption chromatography

Adsorption is based on the following interactions:

a) Dipole interactions

During bonding between two atoms of different electronegativities, there is an asymmetric arrangement of the bonding electron pair. The most electronegative atom pulls the bonding electron pair closer to itself; a bond dipole is formed, the strength of which can be measured. The charge distribution in the polar atom bond is marked with the symbols δ^+ and δ^- .

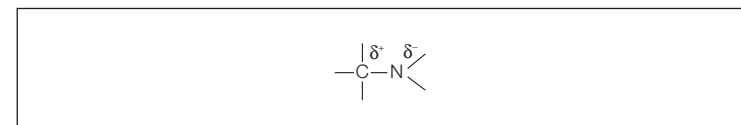


Figure 1:
Dipole interactions.

In the periodic table of elements, the positive charge on the nucleus, and hence the electronegativity, increases from left to right and decreases from top to bottom.

b) Hydrogen bridge bonds

Hydrogen bridges are bonds of a predominantly electrostatic nature between an H atom of one molecule and a strongly electronegative element of a second molecule (F, O, N, S). Such associates are stable in the solid state but unstable in the liquid phase, i.e. some of them break up while others re-form.

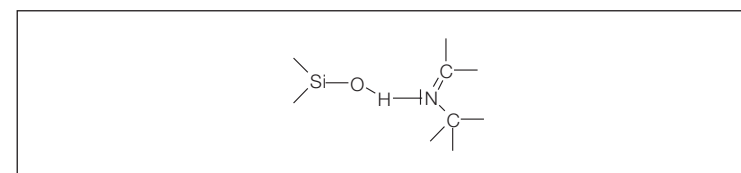


Figure 2:
Hydrogen-bond.

c) π -Complex

The π -complex is formed when an electrophilic partner with an electron hole (X^+) attacks a C = C double bond. The resulting loose adduct is called a π -complex.

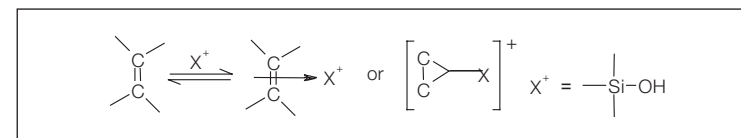


Figure 3:
 π -complex.

In the case of silica gel, the active partner in the adsorption chromatography is the silanol group, while in alumina this function is fulfilled by the Al centers and the linking O atoms.

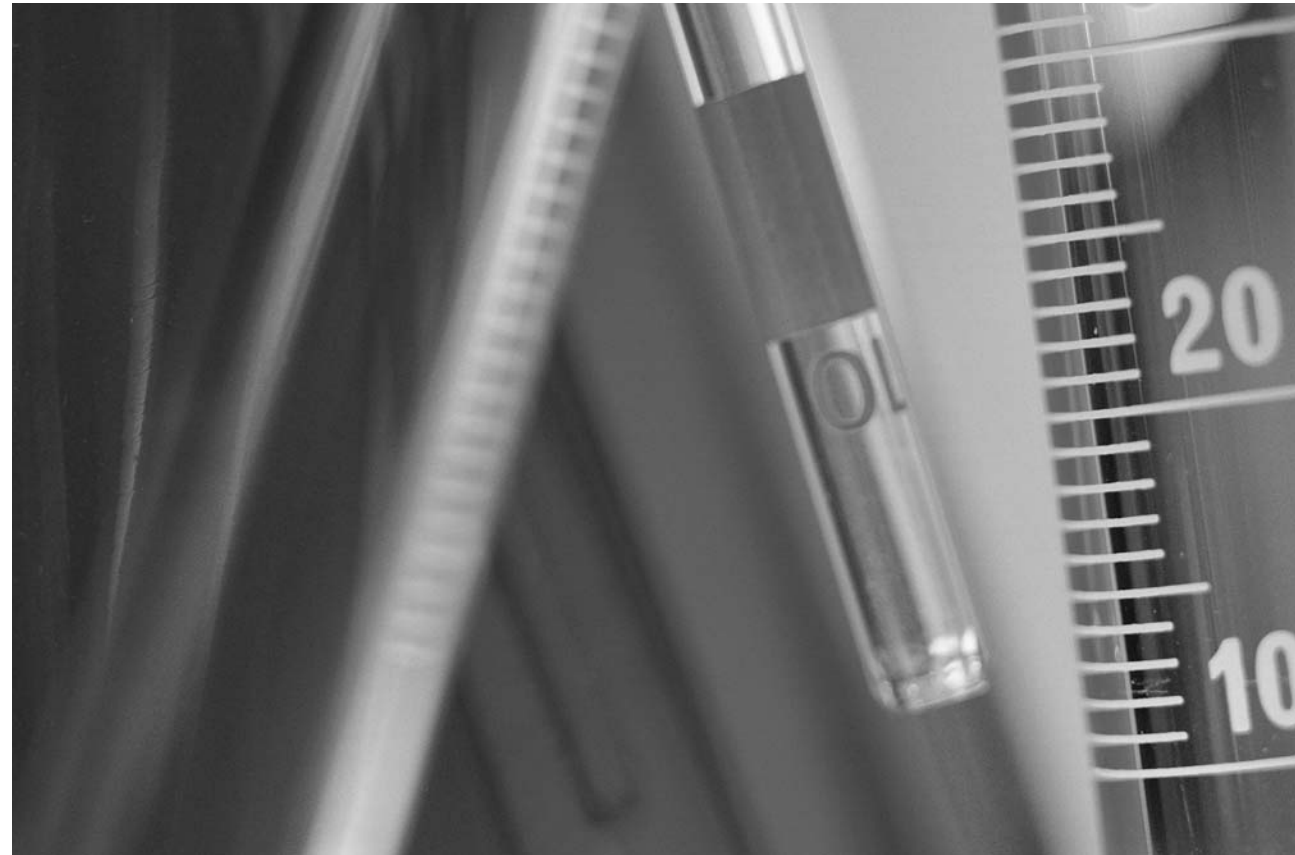
d) Charge-transfer complex

π -complexes in particular are referred to as charge transfer complexes. In this case, there is an interaction between systems in which the electron content has been greatly reduced (for example as a result of ionization effects) and another suitable π -electron system.

e) Steric effects

Apart from the mechanisms and interactions described above, spatial aspects of the molecules also play a role. Hence, molecules with sterically differing structures (isomers) can generally easily be separated by adsorption chromatography.

Appendix



1 Common formulae

Number of theoretical plates

Equation 42:
Number of theoretical plates (base-line width).

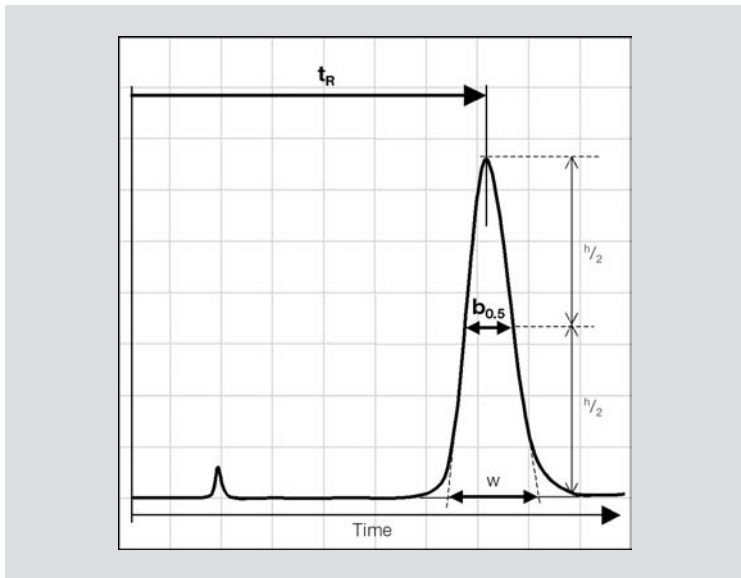
$$N = 16 \cdot \left(\frac{t_R}{w} \right)^2$$

Equation 43:
Number of theoretical plates (peak width at half height).

$$N = 5.55 \cdot \left(\frac{t_R}{b_{0.5}} \right)^2$$

t_R = Retention time
 w = Base-line width
 $b_{0.5}$ = Peak width at half height
 All values in mm, min or sec
 (always use the same units)

Figure 56:
Number of theoretical plates.



Resolution

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

Equation 44:
Resolution (base-line width).

$$R = \frac{1.177 \cdot (t_{R2} - t_{R1})}{b_{0.5(1)} + b_{0.5(2)}}$$

t_R = Net retention time
 w = Base-line width
 $b_{0.5}$ = Peak width at half height
 All values in mm, min or sec
 (always use the same units)

Equation 45:
Resolution (peak width at half height).

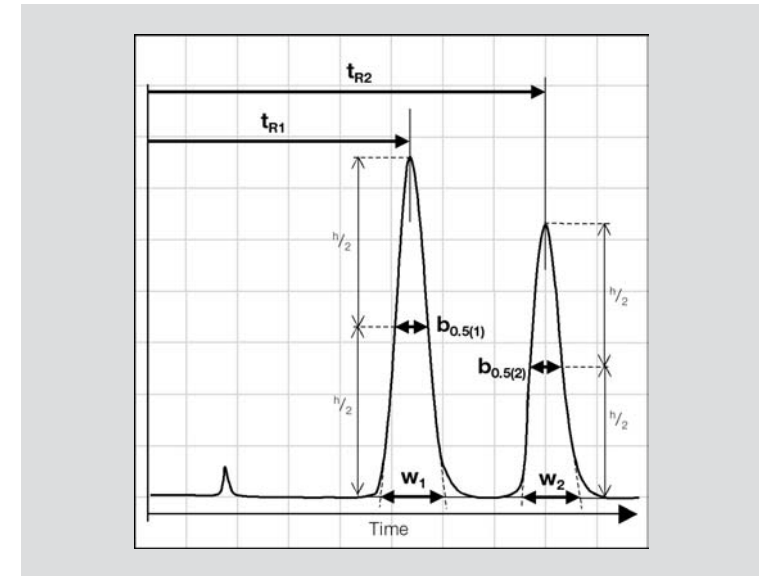


Figure 57:
Resolution.

Linear flow rate

Equation 46:
Linear flow rate.

$$u = \frac{F_m}{6A} = \frac{2 \cdot F_m}{3 \cdot d^2 \cdot \pi}$$

F_m = Delivery of the pump, in ml/min
 A = Base area of the column, in cm^2
 d = Internal diameter of the column, in cm

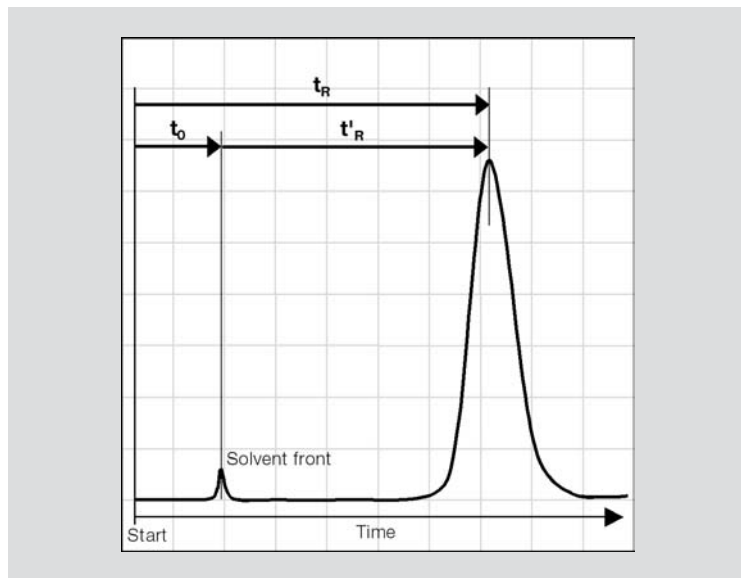
Net retention time

Equation 47:
Net retention time.

$$t'_R = t_R - t_0$$

t'_R = Net retention time
 t_R = Total retention time
 t_0 = Dead time

Figure 58:
Net retention time.



Height equivalent to a theoretical plate (HETP, H)

$$HETP = \frac{L}{N} = \frac{L}{5.5} \cdot \left(\frac{b_{0.5}}{t_R}\right)^2 = \frac{L}{16} \cdot \left(\frac{w}{t_R}\right)^2$$

L = Length of column, in mm
 N = Number of theoretical plates
 t_R = Total retention time*
 w = Base-line width*
 $b_{0.5}$ = Peak width at half height*
 * in mm, min or sec

Equation 48:
HETP.

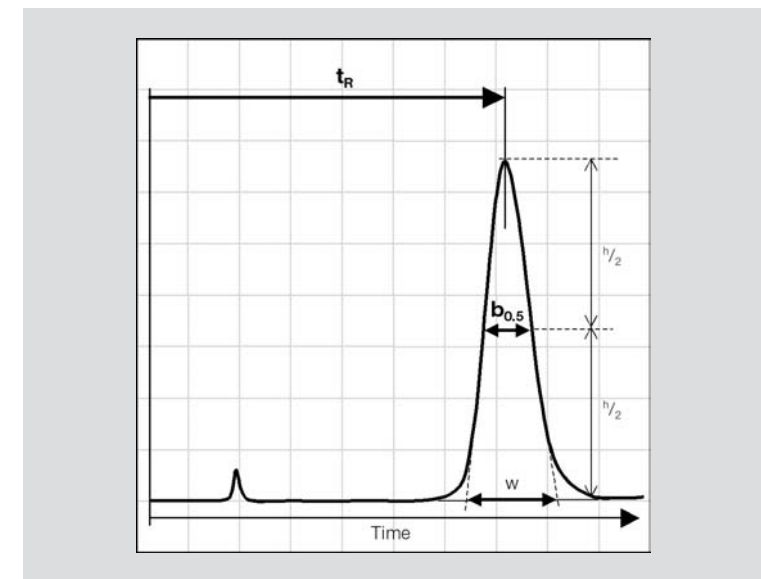


Figure 59:
HETP.

Peak symmetry T (or symmetry index S.I.)

$$SI = T = \frac{a}{b}; \quad a \geq b$$

Equation 49:
Peak symmetry.

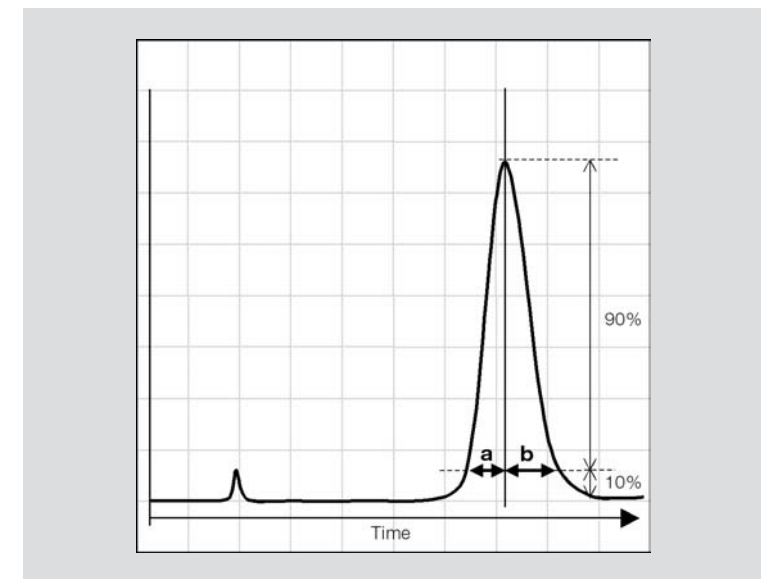


Figure 60:
Peak symmetry.

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