FE 315 Instrumental Analysis
HPLC

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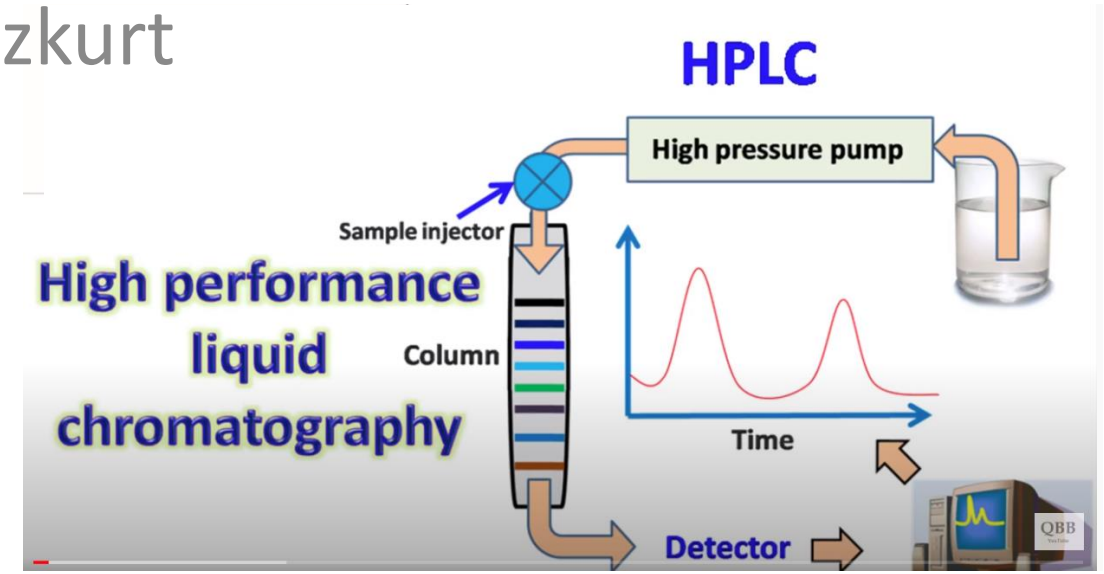


Introduction

- High Performance Liquid Chromatography (HPLC) is one mode of chromatography, the most widely used analytical technique.
- Chromatographic processes can be defined as separation techniques involving mass-transfer between stationary and mobile phases.
- Liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC) and ultra high performance liquid chromatography (UPLC)

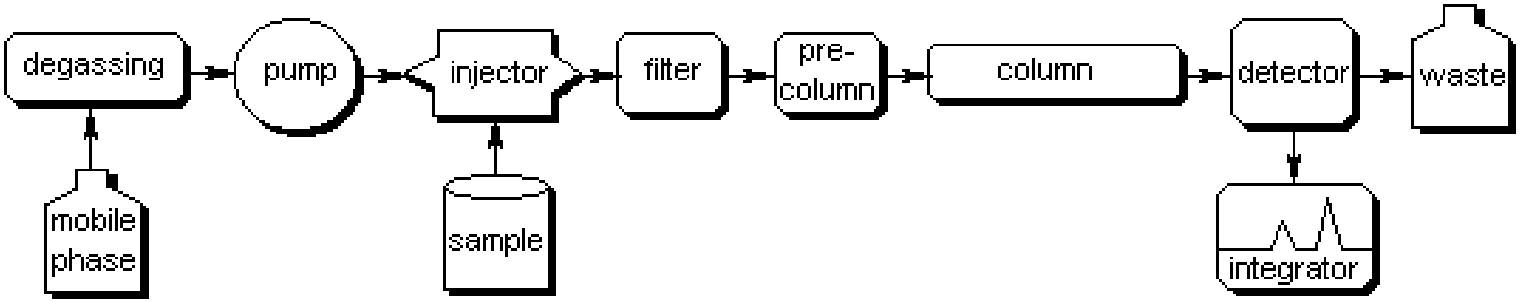
Introduction

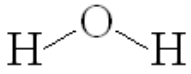
- **High-performance liquid chromatography** (or **high-pressure liquid chromatography**, **HPLC**) is a chromatographic technique that can separate a mixture of compounds, and is used in biochemistry and analytical chemistry to
  - identify,
  - quantify and
  - purify the individual components of the mixture.

Principles and parameters

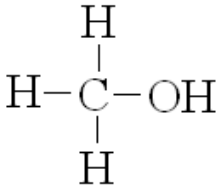
- Liquid chromatography is a well-established technique for the separation of substances. High performance liquid chromatography (HPLC) is a suitable method for the analysis of a wide range of application areas.
  - Principle of HPLC,
  - The most important components,
  - Factors that determine the success of a measurement.

Principles and parameters

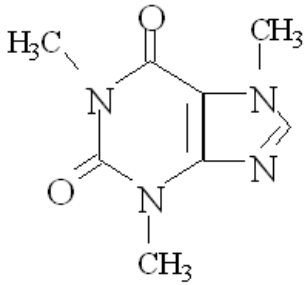




water



methanol



caffeine

Principle of HPLC System

- The separation principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time “on-column”. Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved.

Parts of HPLC

- HPLC utilizes
  - A liquid mobile phase to separate the components of a mixture. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under high pressure.
  - Pump moves the mobile phase(s) and analyte through the column.
  - Injection port or autosampler loading of sample into system.
  - In the column, the mixture is resolved into its components. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases.
  - A detection unit (e.g. UV detector) provides a characteristic retention time for the analyte. The detector may also provide other characteristic information (i.e. UV/Vis spectroscopic data for analyte if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase.

HPLC

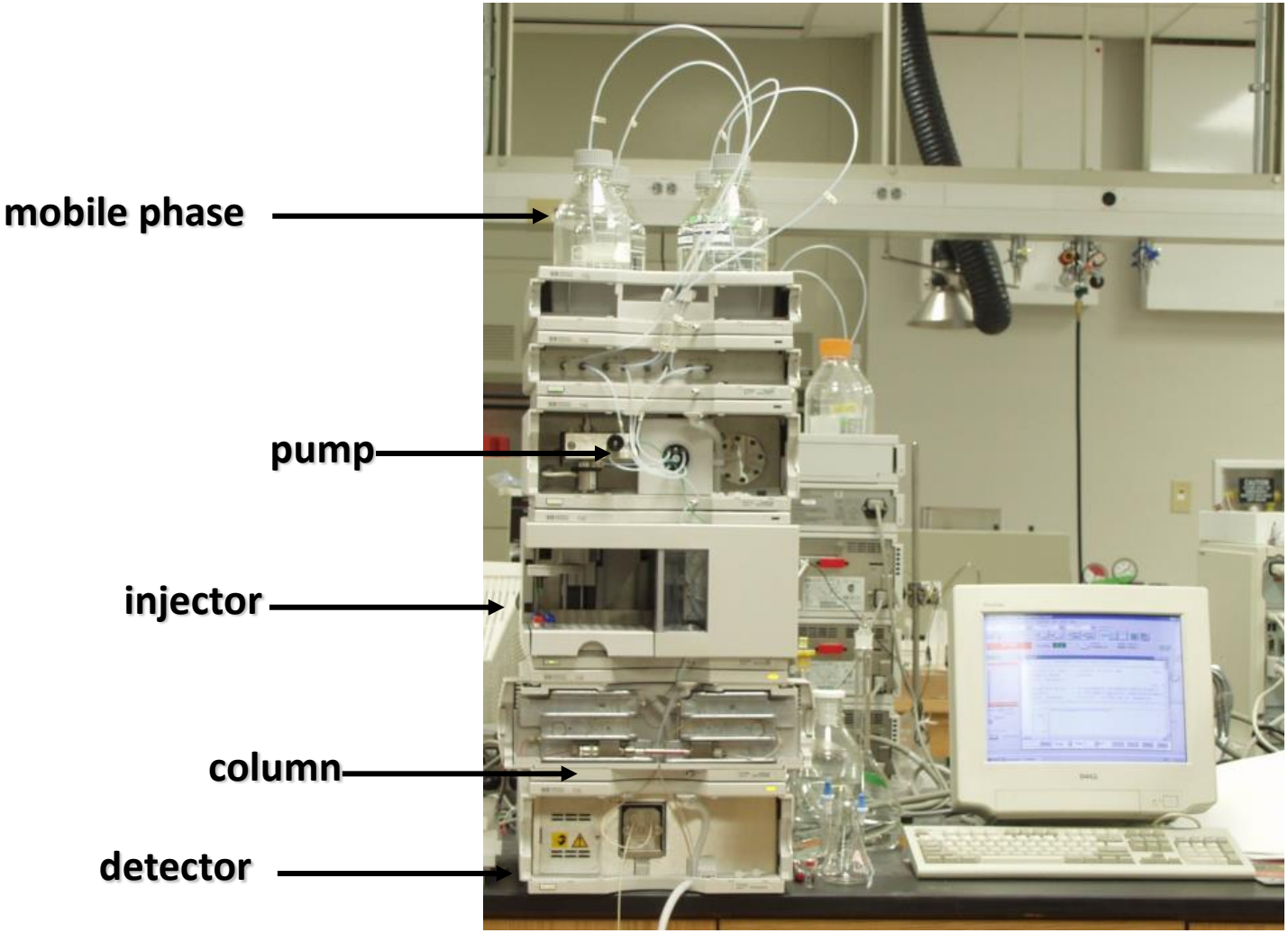
- In HPLC, components of a mixture are carried through the stationary phase by the flow of a mobile phase and separation is based on differences in migration rates among the sample components. Therefore, the nature of your analytes defines not only the method but also the HPLC system.

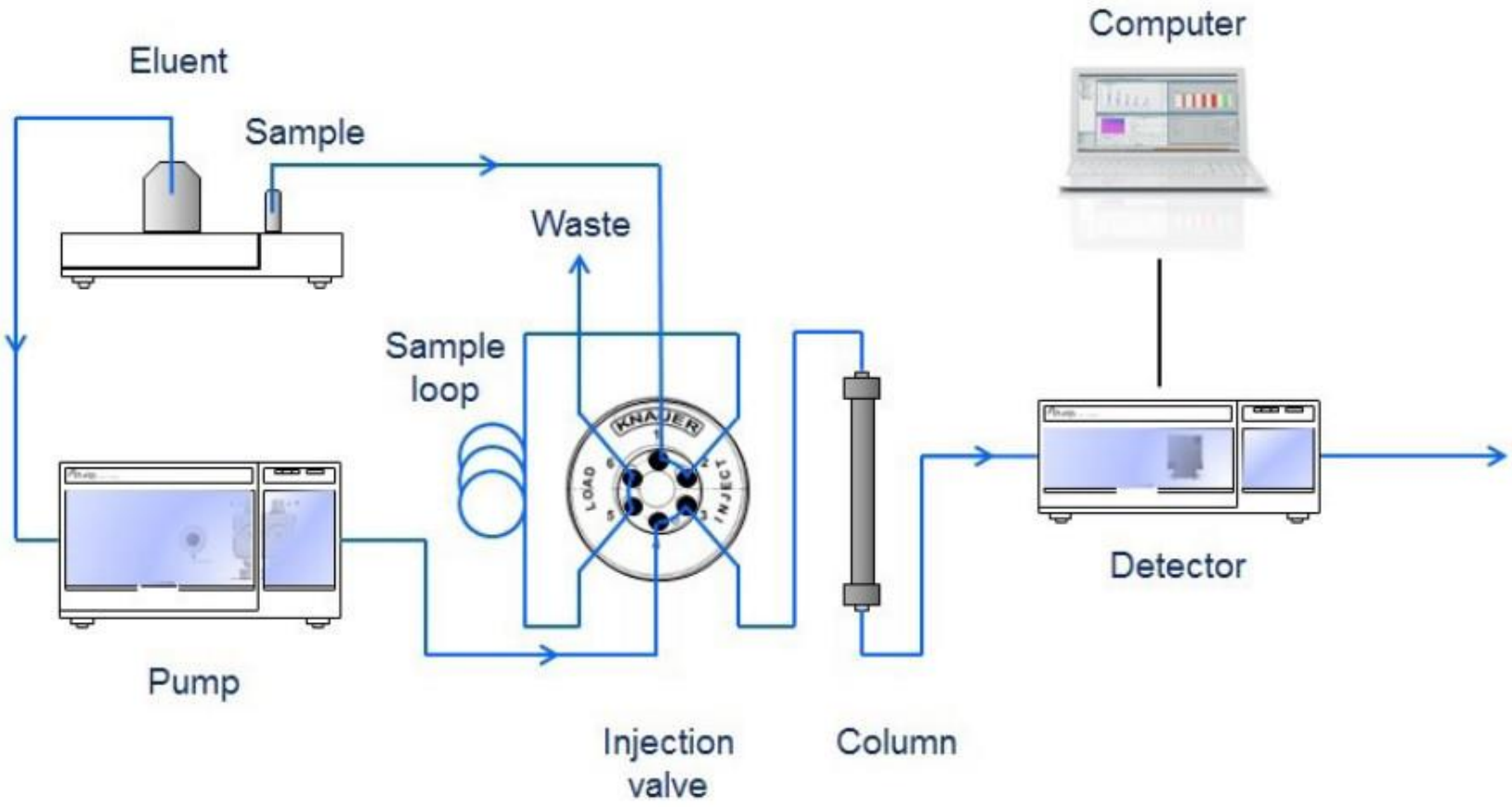
  Main characteristics of the analytes:

  MOLECULAR WEIGHT defines the pore size of the stationary phase

  SOLUBILITY defines the HPLC mode, chemistry of stationary phase and eluent.

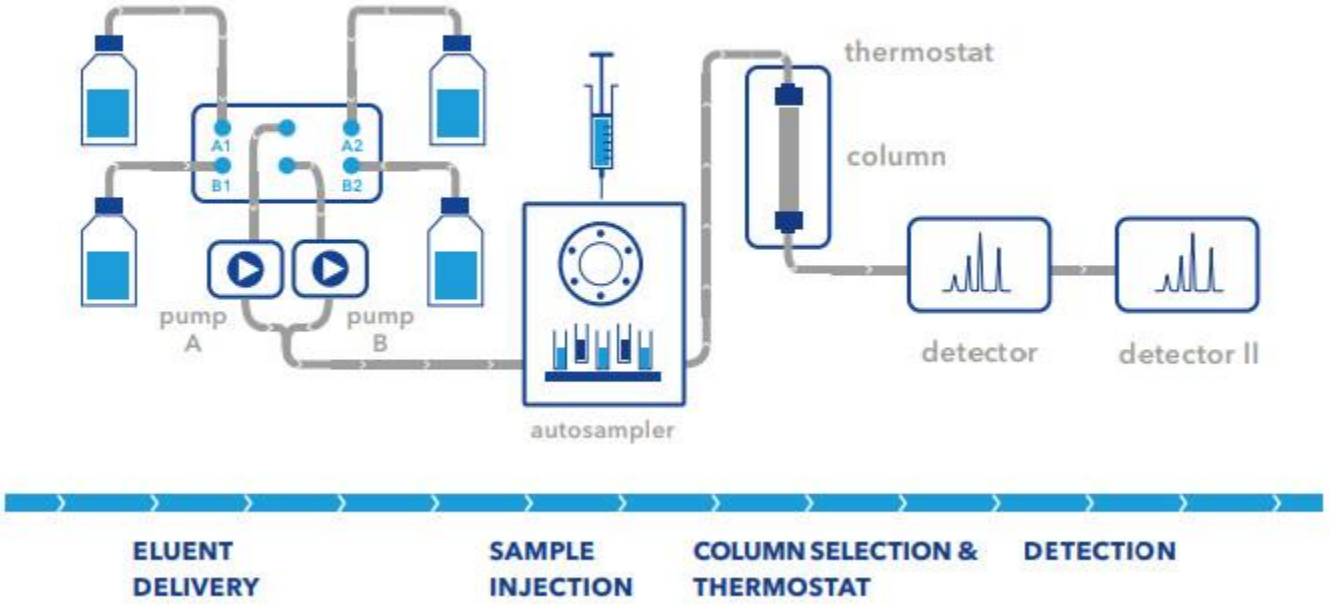
  CONCENTRATION AND MATRIX define the detection parameters and column dimensions.
- The solubility of your analytes defines the HPLC mode. The elutropic series defines the solvent strength for the most often used chromatography modes normal phase and reversed phase.
- The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. To keep the drift and noise of the detector signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve.


mobile phase
pump
injector
column
detector



Schematic layout of a HPLC system

UPLC


A1
B1
A2
B2
pump
A
pump
B
autosampler
thermostat
column
detector
detector II
ELUENT
DELIVERY
SAMPLE
INJECTION
COLUMN SELECTION &
THERMOSTAT
DETECTION

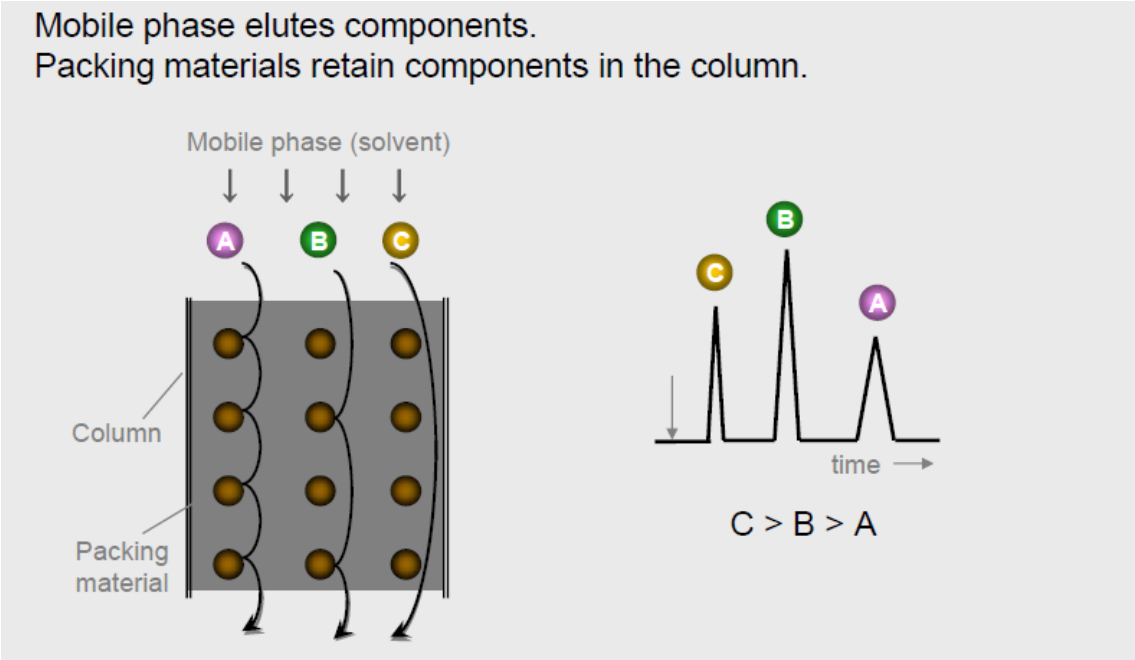
Mobile Phase

- Mixtures of water or aqueous buffers and organic solvents are used to elute analytes from a reversed phase column.
- The solvents have to be miscible with water and the most common organic solvents used are acetonitrile, methanol or tetrahydrofuran (THF). Other solvents can be used such as ethanol, 2-propanol (iso-propyl alcohol).
- Elution can be performed isocratic (the water-solvent composition does not change during the separation process) or by using a gradient (the water-solvent composition does change during the separation process).
- The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. Charged analytes can be separated on a reversed phase column by the use of ion-pairing (also called ion-interaction). This technique is known as reversed phase ion-pairing chromatography.

Elution

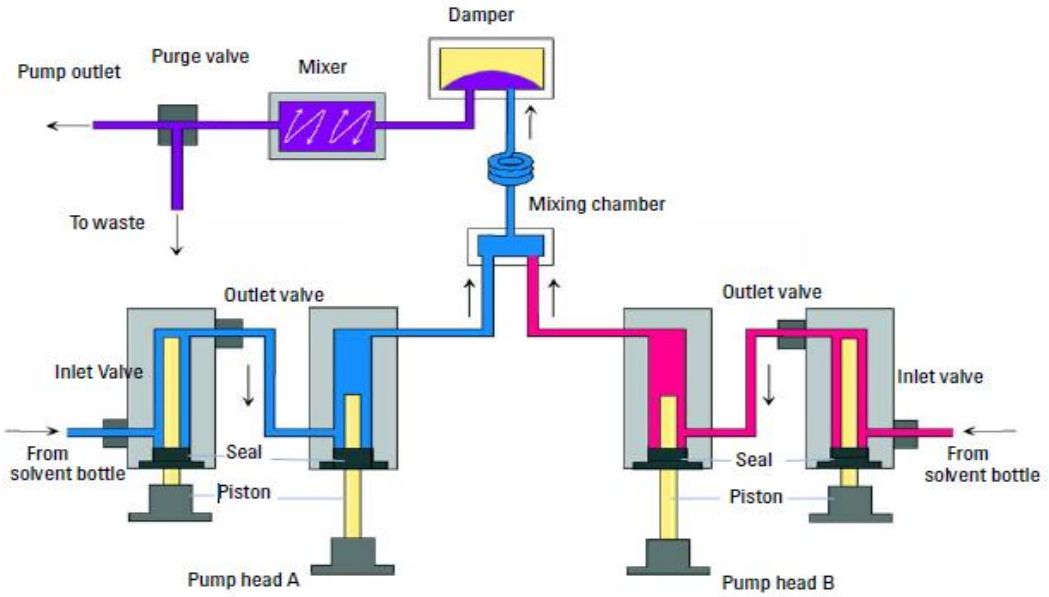
- Elution is a term used to describe the process of extracting one material from another by washing with a solvent (as in washing of loaded ion-exchange resins to remove captured ions).
- In a liquid chromatography experiment, for example, an analyte is generally adsorbed, or "bound to", an adsorbent in a liquid chromatography column. The adsorbent, a solid phase (stationary phase), is a powder which is coated onto a solid support. Based on an adsorbent's composition, it can have varying affinities to "hold" onto other molecules—forming a thin film on its outside surface (or on its internal surface if there are cavities within the compound).
- Elution then is the process of removing analytes from the adsorbent by running a solvent, called an "eluent", past the adsorbent/analyte complex. As the solvent molecules "elute", or travel down through the chromatography column, they can either pass by the adsorbent/analyte complex or they can displace the analyte by binding to the adsorbent in its place.

Elution



Retention also can be controlled by the polarity of the mobile phase, which competes with sample components for adsorption sites. Thus, a more polar mobile phase will more effectively displace adsorbed solute molecules and cause the retention time to decrease. A solvent of higher polarity will displace one lower in the polarity scale.

Pump



- With HPLC, a pump (rather than gravity) provides the higher pressure required to propel the mobile phase and analyte through the densely packed column.
- The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography

Pump

Gradient vs Isocratic

- Depending on the composition of the mobile phase, two different modes are generally applicable.

1. If the make up of the mobile phase remains constant during the separation process, the HPLC system is defined as an isocratic elution system.
2. When the composition of the mobile phase is changed during separation, the HPLC system is defined as a gradient elution system.

Using a gradient system, two different techniques are available:

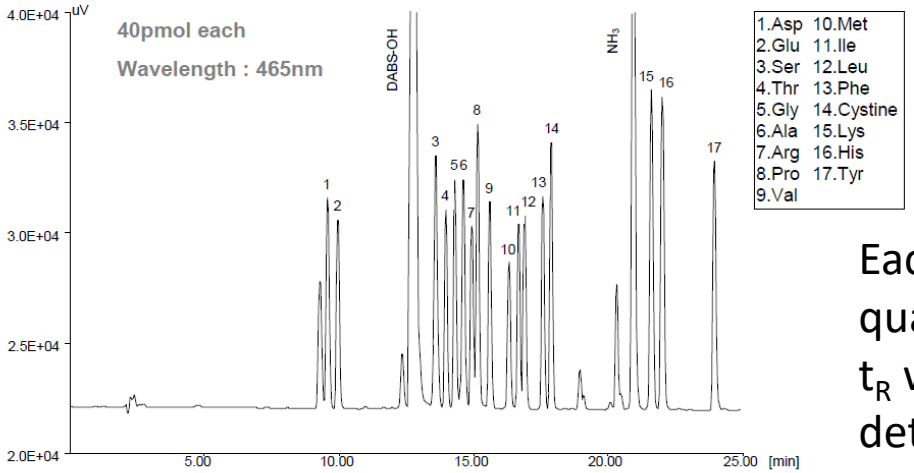
- a low-pressure gradient (LPG) and
- a high-pressure gradient (HPG).

A low-pressure gradient means that the mixing of the solvents is carried out upstream of the pump (suction side).

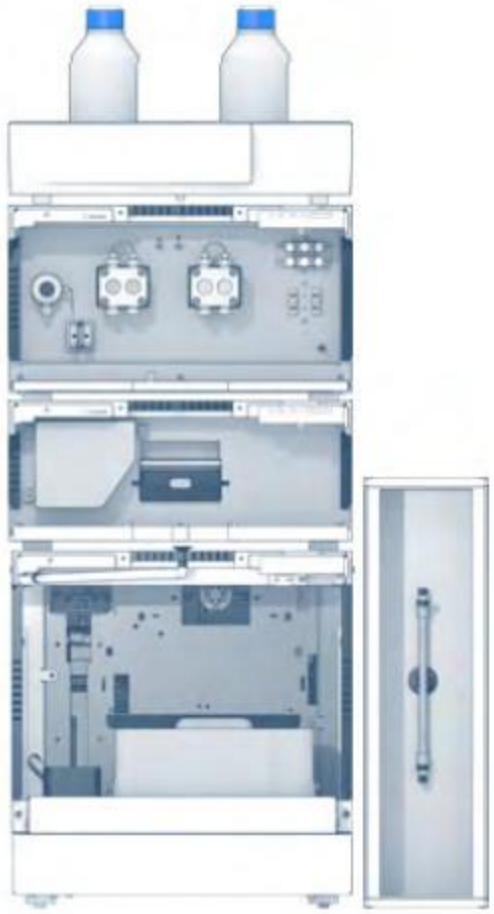
In a high-pressure gradient system, the different solvents are supplied by individual pumps and mixed after the pumps (discharge side).

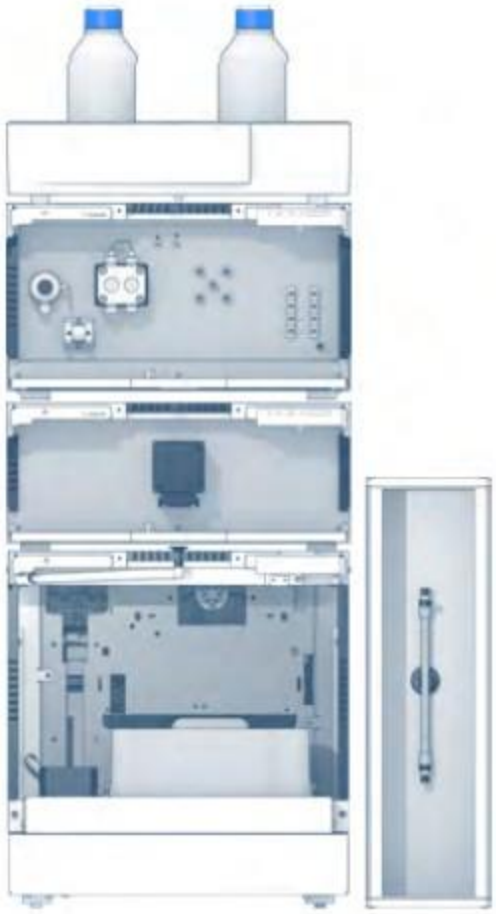
Gradient vs Isocratic

Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward. This also increases the peak height (the peak looks "sharper"), which is important in trace analysis. The gradient program may include sudden "step" increases in the percentage of the organic component, or different slopes at different times - all according to the desire for optimum separation in minimum time.



Each component in a mixture can be qualitatively identified by its retention time t_R which is the time between injection and detection.



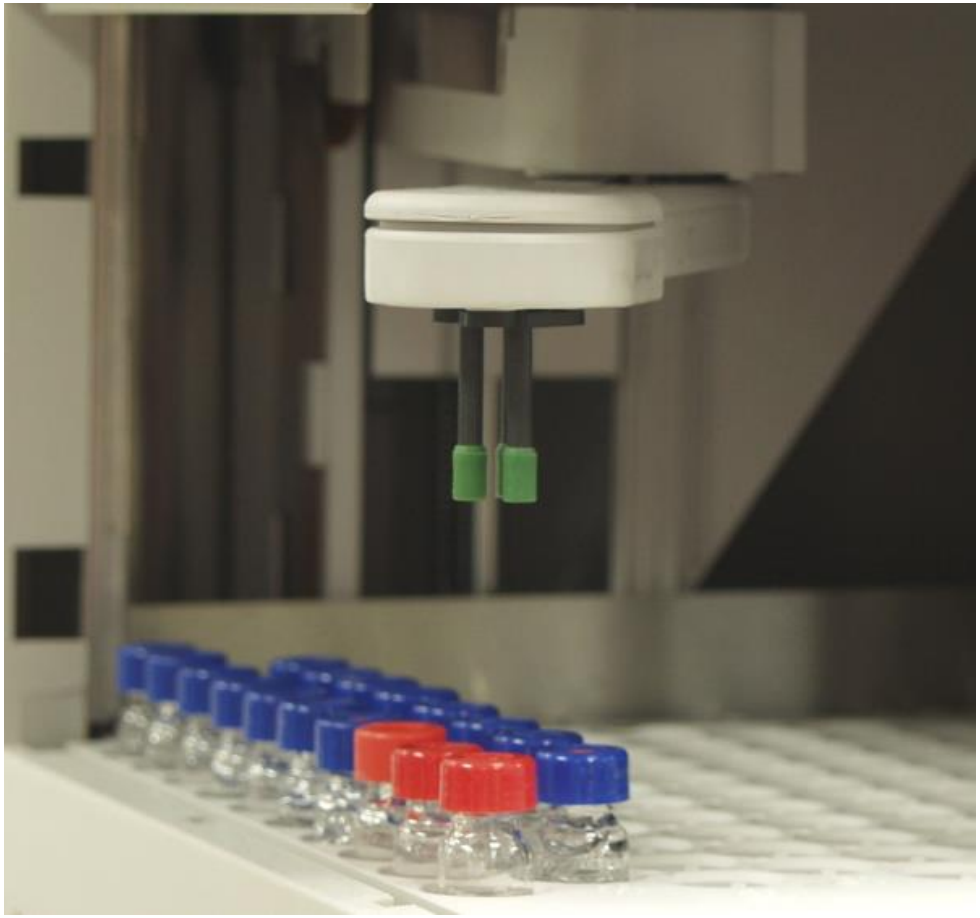


Autosampler or Injection port

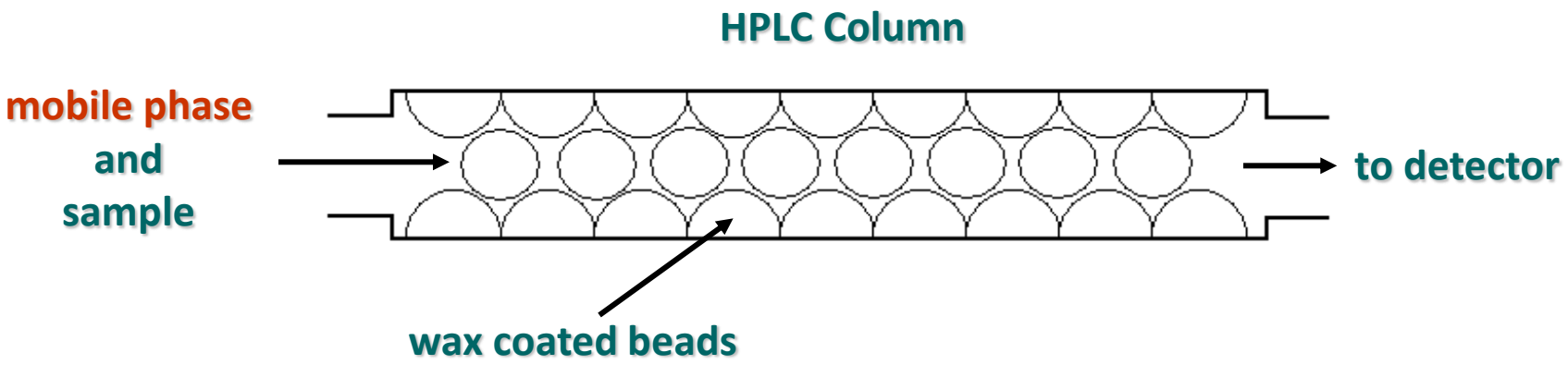
- HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (termed the mobile phase) that passes through a column packed with particles of a stationary phase.
- As in gas chromatography, separation of a mixture into its components depends on different degrees of retention of each component in the column.
- The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phases.
- A variety of HPLC separation techniques that utilize different stationary and mobile phases have been developed.

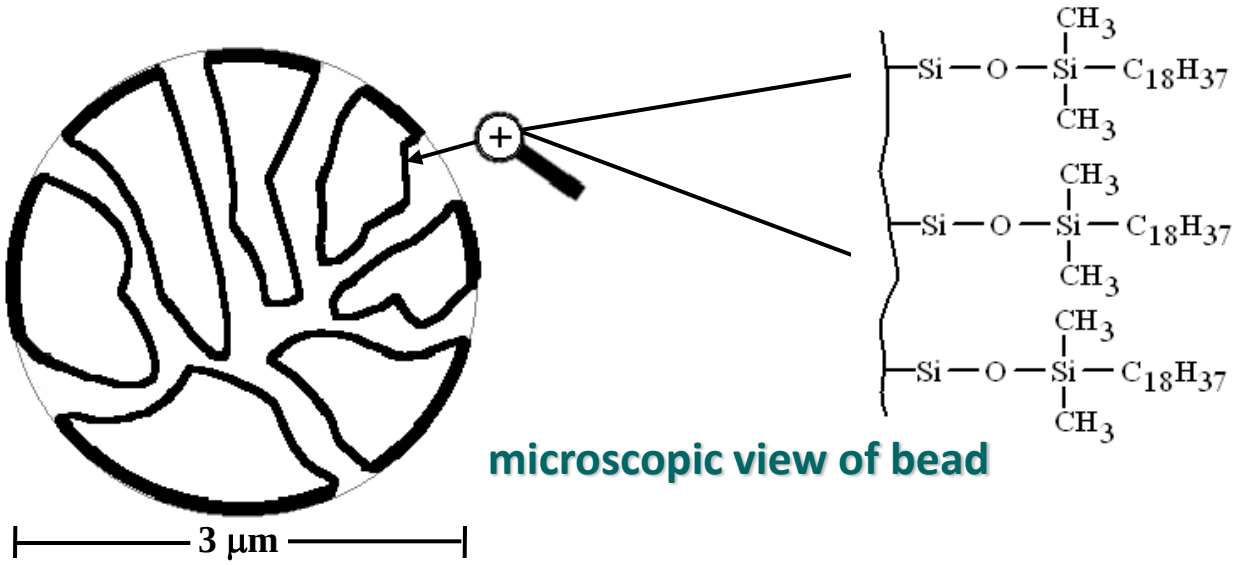
Autosampler/Injector

HPLC Autosampler and Injector

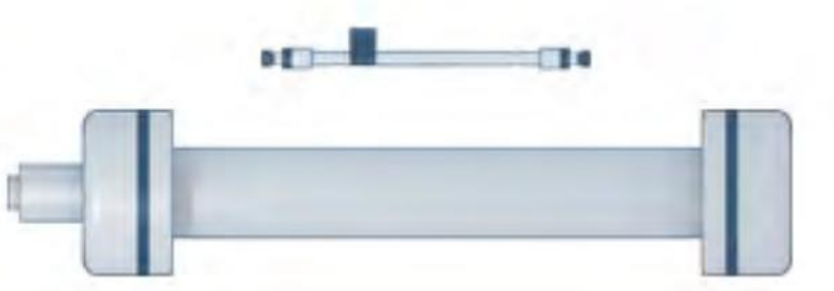


Column


HPLC Column
mobile phase
and
sample
to detector
wax coated beads


3 μm
microscopic view of bead

Column

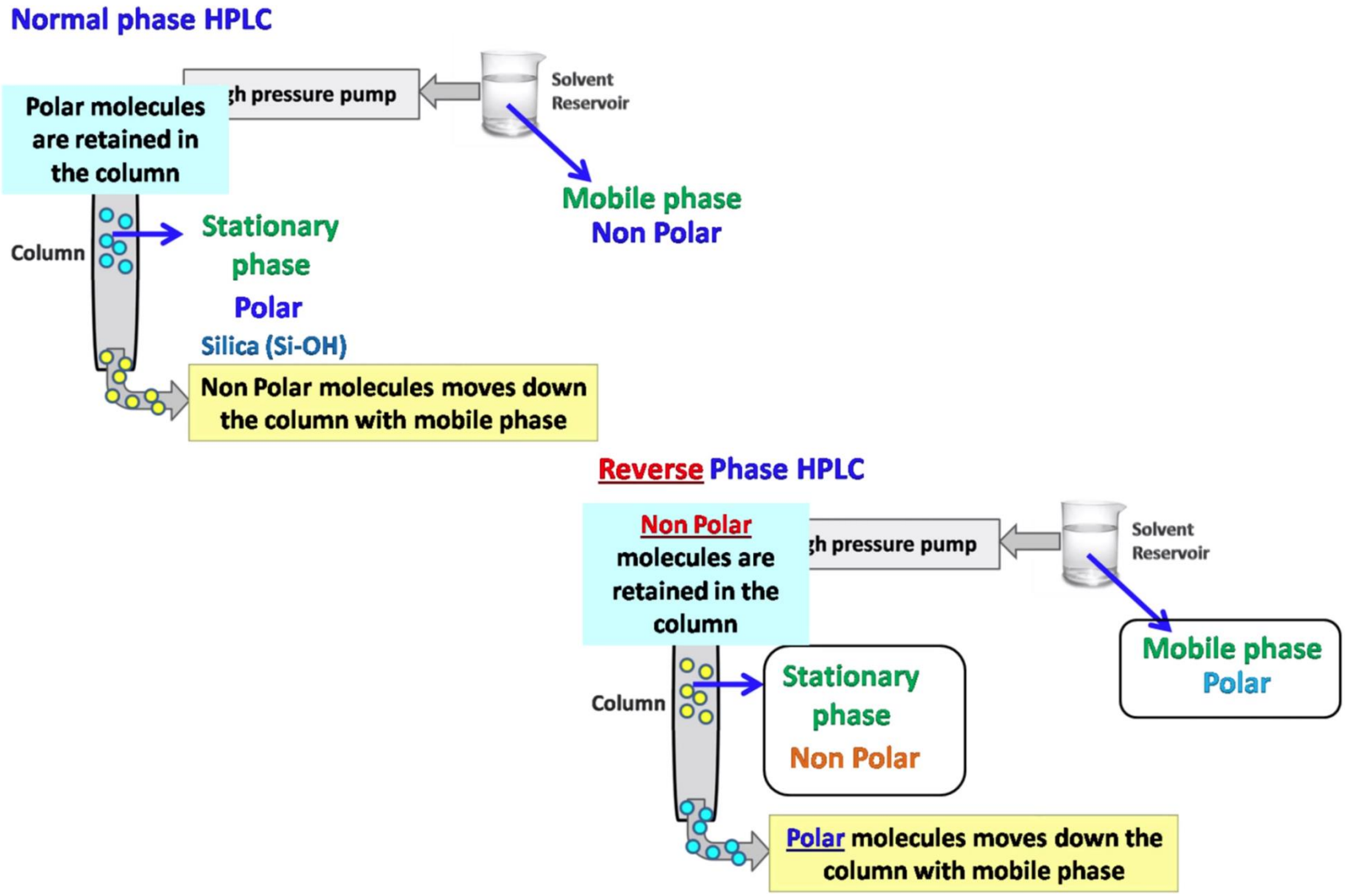


- The column represents the heart of any HPLC system.
- It is responsible for the adequate separation of the sample ingredients.
- The separation efficiency correlates with the column inner diameter, the length of the column and the type and particle size of the column packing material.
- Depending on the desired application, numerous HPLC columns are commercially available. Different packing materials support different separation mechanisms – common are materials for normal-phase, reversed-phase, size exclusion, ion exchange, affinity, chiral, or hydrophilic interaction HPLC.
- Technique in which the stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase) is called normal phase liquid chromatography (NPLC) and the opposite (e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is called reversed phase liquid chromatography (RPLC). Ironically the "normal phase" has fewer applications and RPLC is therefore used considerably more.

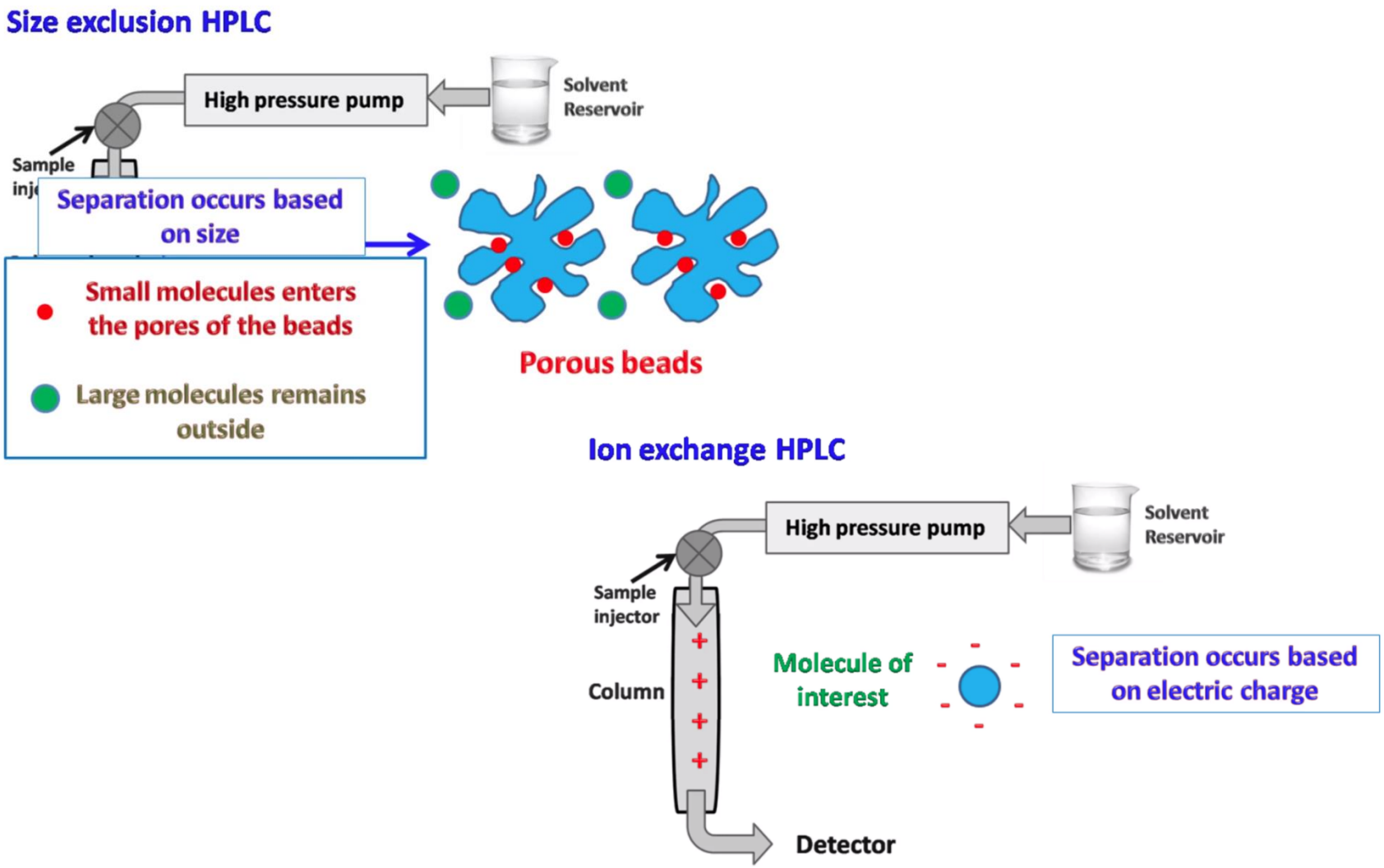
Separation mechanisms

- In **absorption chromatography**, the stationary phase is a solid of a polar nature such as particles of hydrated silica or alumina. The mobile phase and the solute (components in the sample) are in competition for active adsorption sites on the stationary phase particles. Thus, more strongly adsorbed components are retained longer than weakly adsorbed components. Because more polar compounds adsorb on a polar surface to greater degree than do less polar compounds, retention in the column is related to sample polarity.
- In **partition chromatography**, the solute is distributed between the liquid mobile phase and a second, immiscible liquid that is coated on or bonded to solid particles as the stationary phase. Compounds that partition more strongly into the stationary liquid phase are retained longer in the column. This type of chromatography is termed normal phase if the stationary phase is more polar than the mobile phase and reverse phase if the mobile phase is more polar than the stationary phase.
- **Ion exchange HPLC** is based on the partition of ions between a polar liquid phase and a stationary phase with ion exchange sites. The ion exchange sites are typically immobilized in small beads of resin that are formed by a cross-linked polymer.

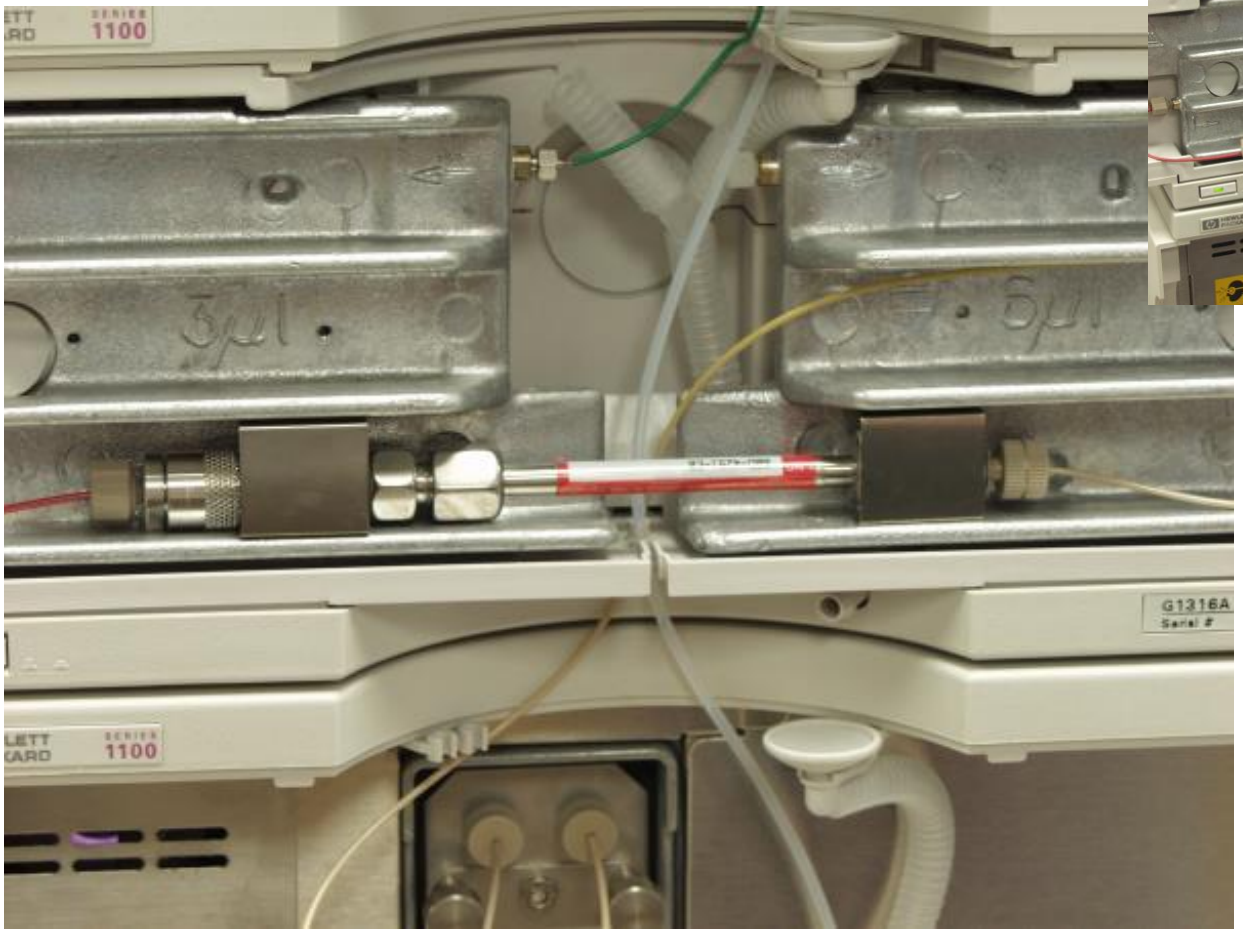
Normal vs Reversed Phase

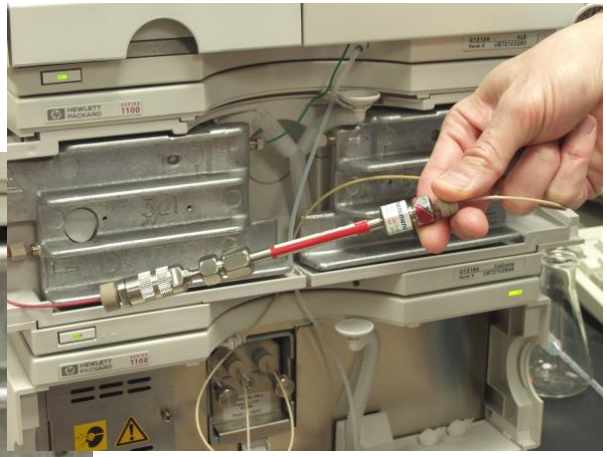


Size Exclusion vs Ion Exchange

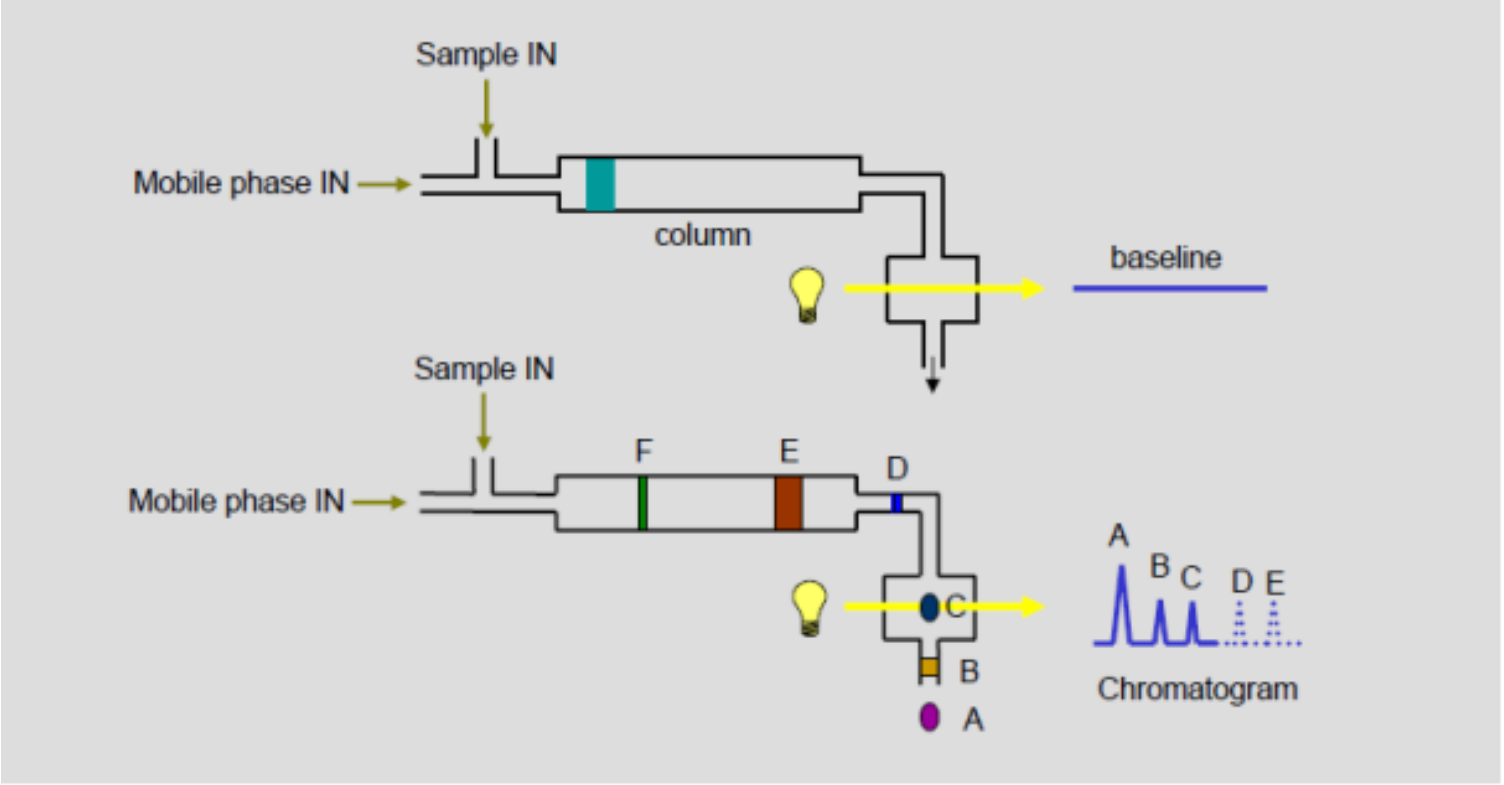


Column





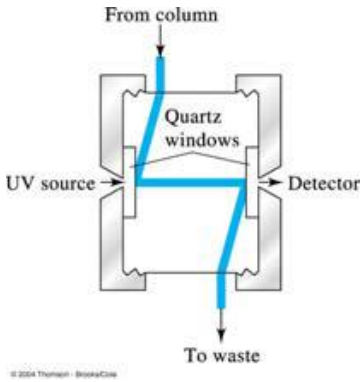
Column


Sample IN
Mobile phase IN
column
baseline
Sample IN
Mobile phase IN
F
E
D
C
B
A
A
B
C
D
E
Chromatogram

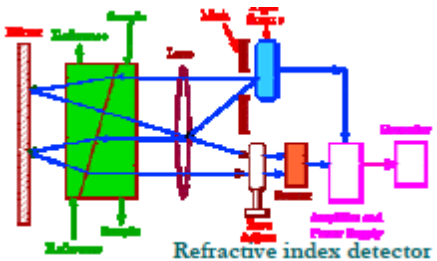
Detector

- The detector unit is to register the time and amount of a substance which is eluted from the column. The detector perceives the change in the composition of the eluent and converts this information into an electrical signal which is evaluated by the aid of a computer. A variety of detectors is available depending on the structural characteristics of the analyte. Common detector units are refractometric, UV/VIS, electrochemical and fluorescence detectors.

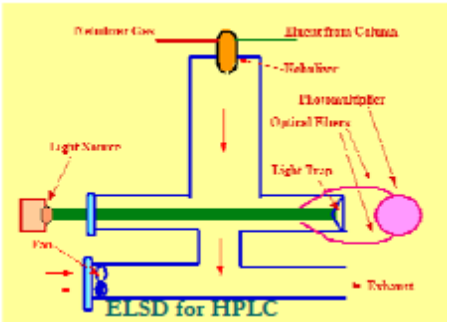
HPLC Detector	Available	(typical)	(decades)
Absorbance	Yes	10 pg	3–4
Fluorescence	Yes	10 fg	5
Electrochemical	Yes	100 pg	4–5
Refractive index	Yes	1 ng	3
Conductivity	Yes	100 pg–1 ng	5
Mass spectrometry	Yes	<1 pg	5
FTIR	Yes	1 μg	3
Light scattering	Yes	1 μg	5
Optical activity	No	1 ng	4
Element selective	No	1 ng	4–5
Photoionization	No	<1 pg	4



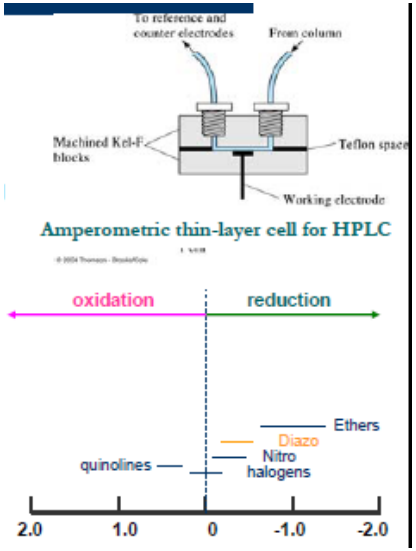
UV



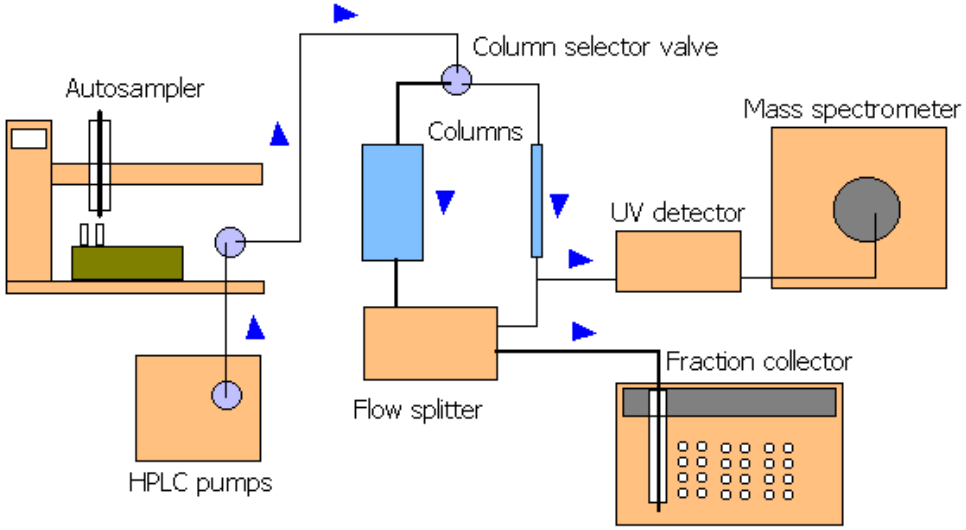
RI



Evaporative light scatteringdetector



Electrochemical



Mass

Chromatographic parameters

- The separated analytes which are transported by the mobile phase are recorded as signal peaks by the detector unit. The total amount of all peaks is called chromatogram. Each individual peak provides qualitative and quantitative information of the analyte. Qualitative information is given by the peak itself (e.g.: shape, intensity of the signal, time of appearance in the chromatogram). In addition, the area of a peak is proportional to the concentration of the substance. Hence, the chromatography data management software can calculate the concentration of the sample by integration. This provides quantitative information. Ideally the peaks are recorded as a Gaussian bell-shaped curve.

```
Injection Date  :
Sample Name     :
Inj Volume      : 10 µl
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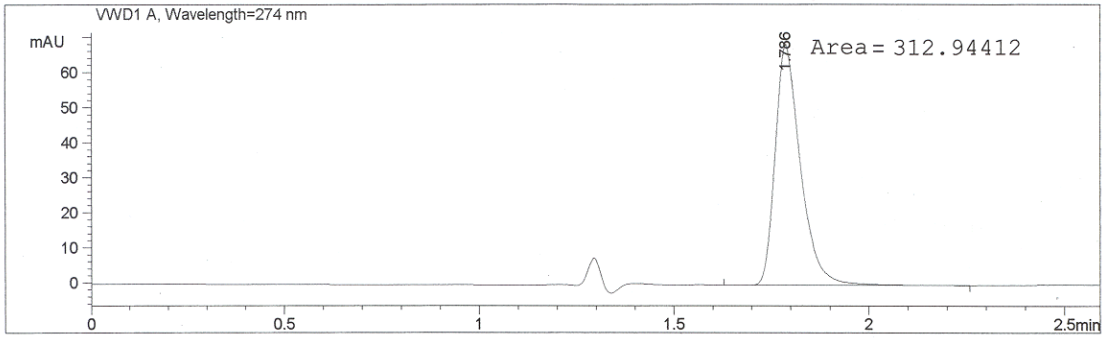
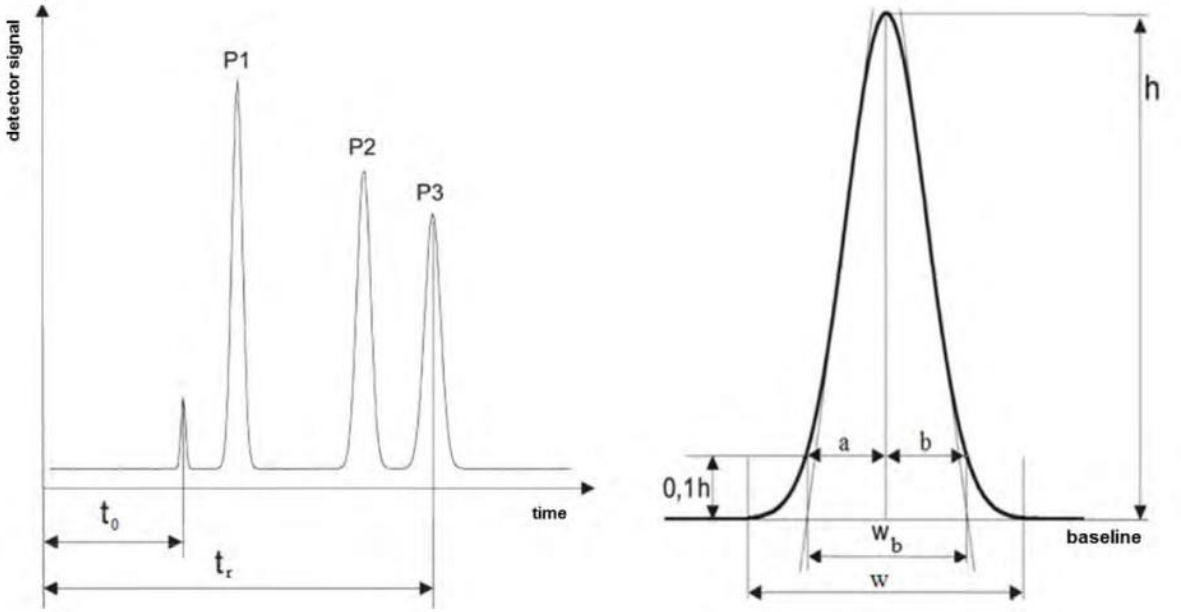


Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	1.786	MM	0.0759	312.94412	68.69455	100.0000

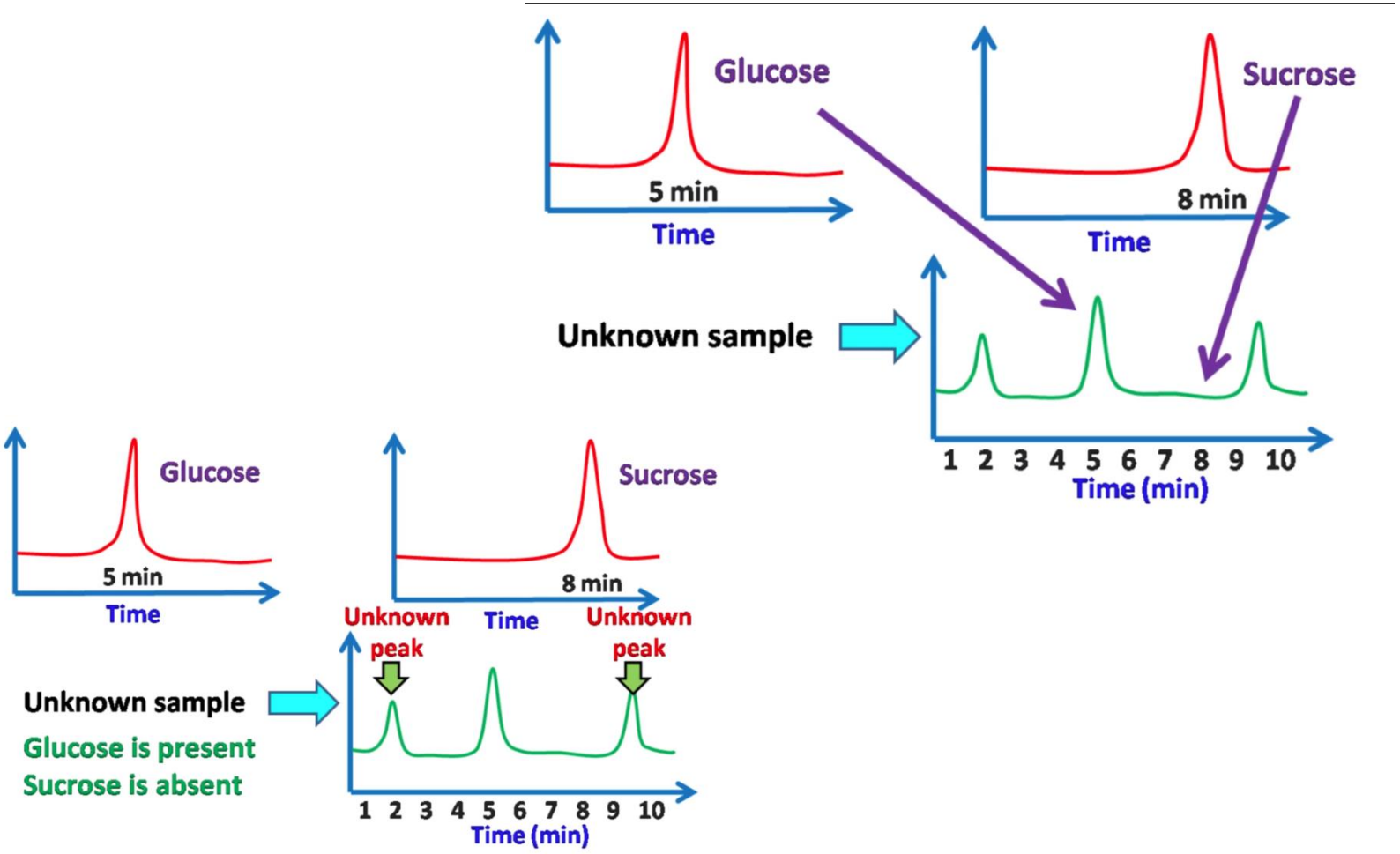


Delay time (t0)
The delay time refers to the time which is required for a non-retarded compound to be transported from the injection site to the detector unit (where the compound is recorded). During this time, all sample molecules are exclusively located in the mobile phase. In general, all sample molecules share the same delay time. The separation is caused by differing adherence of the substances with the stationary phase.

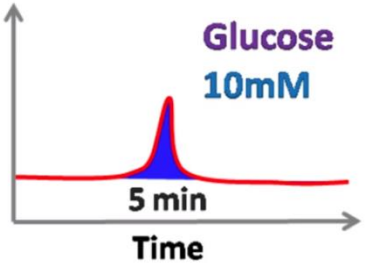
Retention time (tR)
The retention time refers to the time which is required for a compound from the moment of injection until the moment of detection. Accordingly, it represents the time the analyte is in the mobile and stationary phase. The retention time is substance-specific and should always provide the same values under the same conditions.

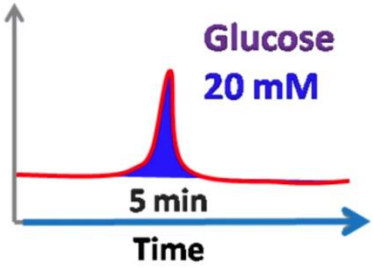
Peak width (w)
The peak width covers the period from the beginning of the signal slope until reaching the baseline after repeated drop in the detector signal.

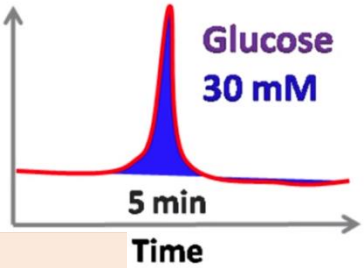
Integration of chromatogram

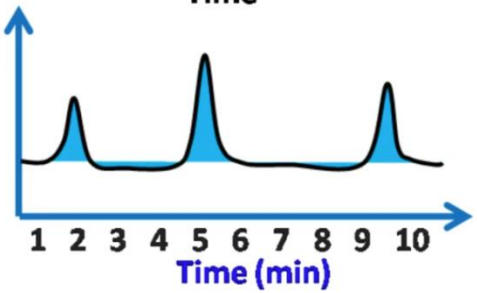


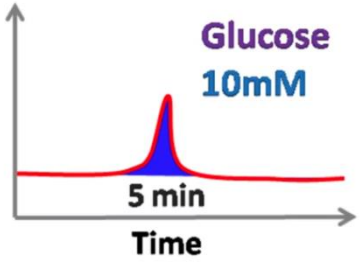
Finding of concentration

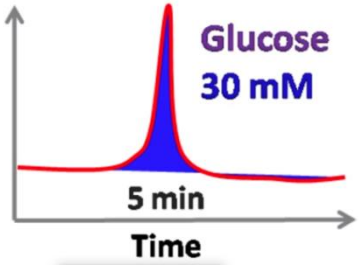




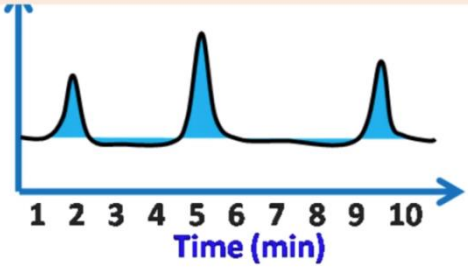


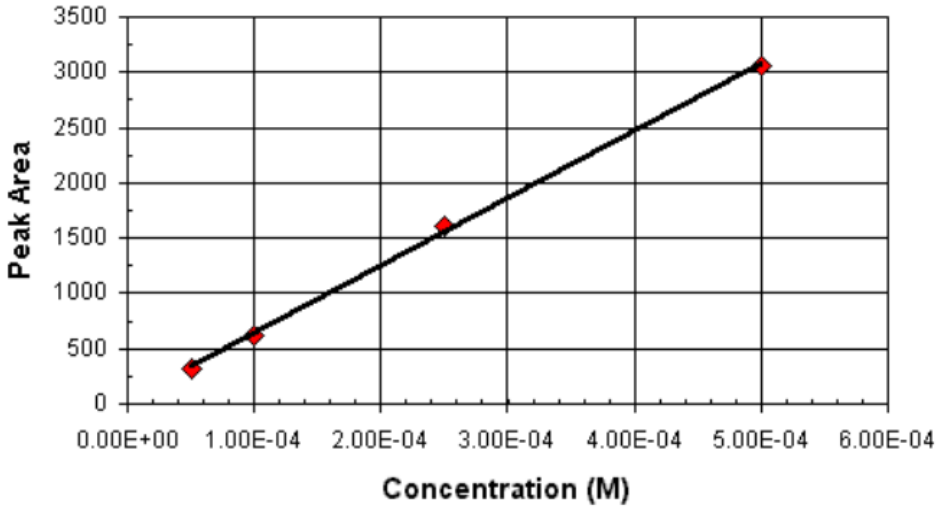






By measuring <u>area of glucose</u> peak in unknown sample, and <u>comparing it with area of standard</u> (glucose), the concentration of glucose in unknown sample can be estimated





By using the calibration curve, concentration of a compound can be found.

Biogenic Amine determination

- In this experiment we want to determine amount and types of biogenic amine and their precursors of amino acids in kashar cheese.
- Biogenic amines could be found in meat, sausages, milk, chocolate, cheese, fishes and some beverages.

- Biogenic amines are toxic substances; can cause nausea, respiratory distress, hot flushes, sweating, heart palpitation, bright red rash, oral burning, gastric, intestinal problems, and hyper– or hypotension.

- Histamine intake of 8-40 mg, 40-100 mg and higher than 100 mg, may cause slight, intermediate and intensive poisoning, respectively

Biogenic Amine

- The allowable maximum level of tyramine in foods is 100-800 mg/kg and 1080 mg/kg of tyramine is toxic.

- Spermine, spermidine and cadaverine have not adverse health effect, but

  – they may react with nitrite to form carcinogenic nitrosamines and

  – also can be proposed as indicators of spoilage.

Procedure

- Grinding about 70 g of kashar cheese
- Homogenize by mixing
- Take 50 g of homogenized and ground cheese into 250 ml erlenmayer
- Add 150 ml of pure water
- Extract soluble compounds by using ultrasound for 30 min.
- Centrifuge at 7000 rpm for 10 min
- Take supernatant
- Filtrate supernatant with syringe filter
- Load 10 µL into HPLC system

Calibration curve preparation for biogenic amines and amino acids

- For calibration curve prepare different concentrations of histamine, tyramine, histidine, and tyrosine were prepared.
- Data obtained from same procedure for sample as given above by HPLC system for 10 µL injection are given below.

Concentration ppm	Area			
	Histamine	Tyramine	Histidine	Tyrosine
0	0	0	0	0
20	221.2	838.1	363.8	710
50	661.9	2122	987.4	1765
100	1299.10	4459.9	1861.8	2481.5
150	1983.5	6347.2	2686.2	3755.6
200	2645	8025.9	3984.7	4675.4
300	4402.7	12973.8	5637.3	6991.8
500	7685.6	21338.9	9446	12625.9

Compound	Rt
Histamine	11.6
Tyramine	27.7
Histidine	5.2
Tyrosine	6.2

- DATA SHEETS ARE ON THE WEB PAGE PAGE.
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