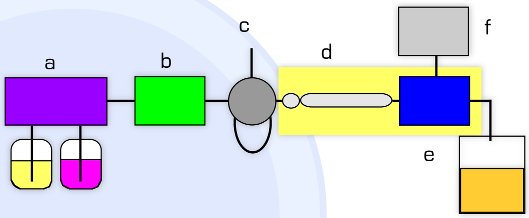
Basic HPLC equipment



a - gradient controller
b - pump/dampening system
c - sample introduction
d - column/pre-column
e - detector
f - data output

Basic HPLC equipment

Unlike GC equipment, many HPLC systems have a modular design - can simply add a new 'box' to change/extent capabilities.

There is also a wider range of how to do things like produce a flow or gradient.

We'll cover some of the basic approaches.

Modular HPLC

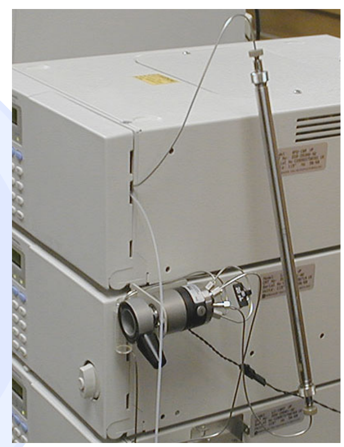
This unit is equipped with two pump units and a UV/Vis detector.

The gradient is controlled via the pump controllers.

Modular HPLC

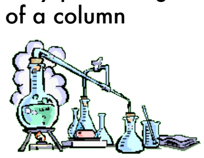
Unlike GC, LC methods are not as sensitive to temperature.

Columns are commonly mounted outside the instrument.



Solvents

- All solvents should be 'HPLC' grade.
  - This is a type of reagent grade material.
  - It has been filtered using a 0.2 μm filter.
- You can purchase it or produce it yourself.
- Filtered solvent helps extend pump life by preventing scoring. It also reduces the chances of a column plugging.



Solvent degassing

All solvents should be degassed prior to use. This reduces the chances of bubbles being formed in the column or detector. Oxygen present at high pressure can also cause a problem.

Methods that can be used

Displacement with a less soluble gas
Applying a vacuum
Heating the solvent.

Pumping systems

Basic types of systems
- Constant pressure
  - Pressurized vessel
  - Pressure intensifier
- Constant flow
  - Motor driven syringe
  - Piston
    - Reciprocating
    - Multiple reciprocating

Pumping systems

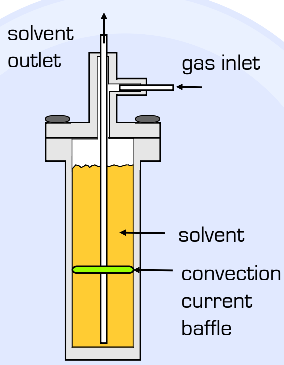
Each type of system has its own advantages and disadvantages.

Is the solvent reservoir limited?

Does it produce pressure pulses?

Can a gradient be produced?

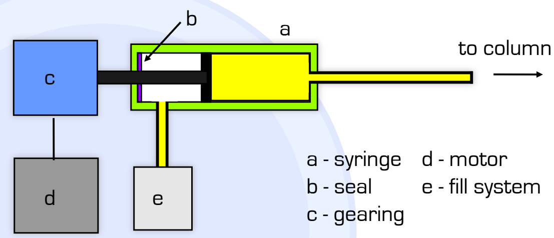
Direct pressure pump



Gas pressure is applied from an external gas tank using a high pressure regulator.

For this system
- No pressure pulses are produced.
- The solvent reservoir is limited.
- Low cost system
- Major problem is introduction of gas into the solvent

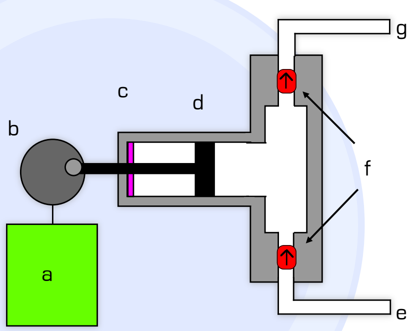
Motor driven syringe



a - syringe
b - seal
c - gearing
d - motor
e - fill system

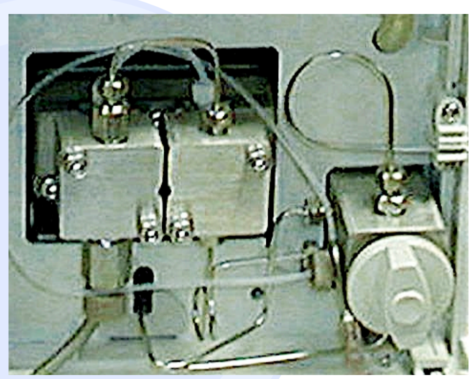
Another non-pulsating system with a limited reservoir.
Stepper motor/gear system allows for very fine flow control.

Reciprocating pump



a - motor
b - gear
c - seal
d - piston
e - solvent in
f - check valves
g - solvent out

Reciprocating pump

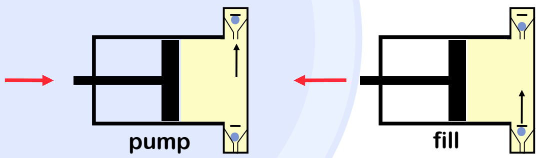


Reciprocating pump

One of the most common type of systems.

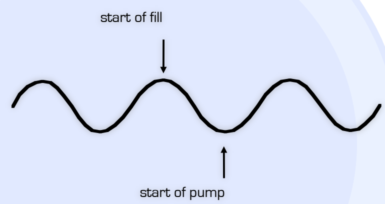
Unlimited reservoir system but expensive.

Another problem is that it produces variable pressure - must reverse stroke to refill.



Reciprocating pump

Since the pump must spend at least a portion of its time filling, the is a pressure drop during this phase.



This effect must be minimized or your peaks will all have pulses in them.

That would greatly affect your sensitivity and detection limit

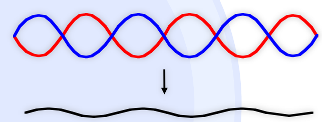
Reciprocating pump

One approach is to have a more rapid fill cycle compared to the pump cycle.

This does not eliminate the problem but does reduce it.

Reciprocating pump

One could also use two or more pumps working in tandem.



This is a more expensive option.

Pulse Dampeners

One approach to minimizing the pulses associated with reciprocating pump

Goal

Absorb the peak of the pressure pulse and minimize the trough.

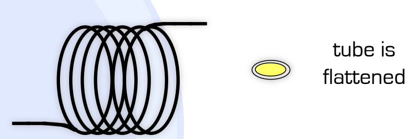
Several approaches can be used.

Pulse Dampeners

In-line metal coil system

Reduces pulse to +/- 3% at 240 psig.

Low cost system

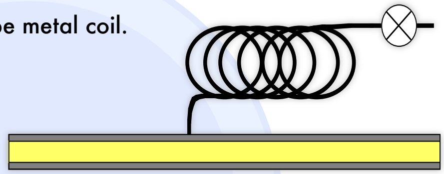


Flow passes through tube - possible contamination

Limited range - about +/- 50-100 psi.

Pulse Dampeners

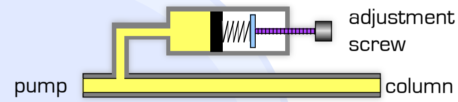
T type metal coil.



With this design, flow does not pass through the dampener.

It still has the previous limitations

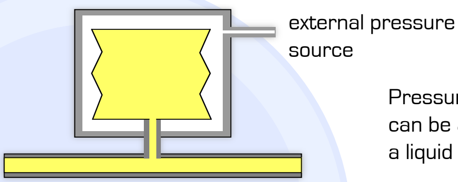
Adjustable spring loaded dampener



Allows the user to minimize pulsing under actual operational conditions.

Can reduce pulses to < 0.1 %

Bellows Dampener



Pressure source can be a gas or a liquid

Reduces pulses to < 0.1%

External pressure can be monitored and controlled by the system.

Most expensive approach but the best usually is.

Gradient controller

We've already covered the concepts of gradient elution.

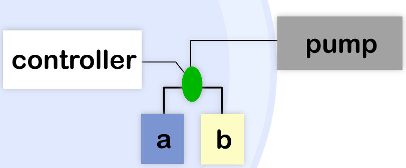
The controller is the device that allow you to create the gradient program.

Gradients are produced based on the type of pumping system you have.

Gradient controller

Single reciprocating pump systems

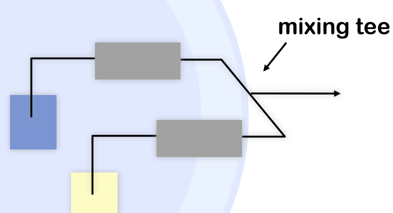
The gradient is produced by controlling a valve. The valve determines the relative amounts of each solvent pulled into the pump.



Gradient controller

Dual pumping systems.

A valve system can be used on each pump can provide a different solvent.



Injection systems

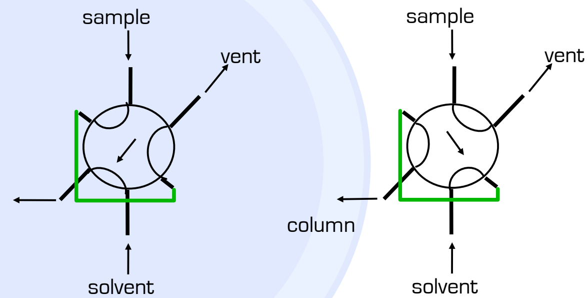
These can be a bit more complex than with GC systems.

If you attempted a manual syringe injection, expect to find the plunger shot into the ceiling - you might be working with pressures as high as 5000 psi.

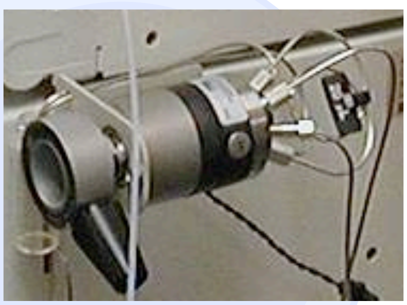
A simple approach would be to stop the flow and inject manually - not to good.

Injection systems

A very common approach is the use of sampling valves and loops.



Sampling valve.



Six port sampling valve and loop.

This valve is equipped with a switch to signal when an injection has occurred.

Injection systems

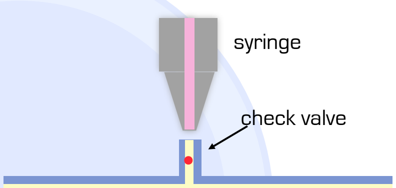
You must use 'zero dead volume' valves.

Manual and automated valve systems are available.

Major limitation is fixed sample size.
The loop must be changed in order to alter sample size - does not require that the flow be stopped.

Injection systems

Automated syringes



This method allow for adjustment of sample size. The motor driven syringe can provide sufficient pressure to inject sample past the check valve.

Guard column

A small column added between the injection system and the analytical column.

It helps prevent entry of materials that might want to stay on the column from your sample or solvent.

Used to extend column life

Should be the same packing as the analytical column.

The column

HPLC has seen significant improvement over the last 20 years primarily due to improved column technology.

Packings are more uniform and smaller.

Phases are commonly chemically bound to the packing.

Packing methods have improved.

Packings

Originally, these were irregular silica and alumina. A range of synthetic, regularly shaped packings are now available.

Porous - channels through packing

Superficially porous - rough surface

Smooth - bead like.

Packing size

As packing size is decreased, efficiency and pressure requirements are increased.

Common diameters for analytical work

diameter	plates
10 µm	5000
5 µm	9000
3 µm	15,000

All are for a 15 cm x 4.6 mm id column

Column body

Typically consist of stainless steel with a high precision internal bore.

Some manufacturers offer column inserts
- don't need to repurchase the column fittings.

Others offer columns where the external body can be compressed to improve packing efficiency.

HPLC column examples

Column stationary phases

Today, most packing fall into four classes.

Silica or alumina

Bound phases on either alumina or silica.

Gels

Controlled-pore glass or silica

Absorption phases

alumina

common mobile phases
hexane, chloroform, 2-propanol.
example application - amines.

silica

common mobile phases
hexane, chloroform, 2-propanol.
example applications - ethers, esters, porphyrins, fat-soluble vitamins.

Partition phases

Can be broken down into

Normal phase - polar materials bound to the support.

Reverse phase - non-polar materials bound to the support.

Mixed phase - may have some of each.

Partition phases

Normal

Amino	(-NH_2)
Cyano	(-CN)
Diol	(glycidoxy-ethylmethoxysilane)

Reverse

C-2 or RP-2	(-Si-CH_2CH_3)
C-8 or RP-8	(-Si-$(CH_2)_7CH_3$)
C-18 or RP-18	(-Si-$(CH_2)_{17}CH_3$)

Increasing the C number results in a thicker, more retentive phase

Ion exchange phases

Strong cation	- sulfonic acid group
Strong anionic	- quarternary amine
Weak anion	- primary amine
Weak cation	- COOH

Size exclusion phases

Gels - organic or aqueous based

Controlled-pore - silica or glass

Must be selected based on pressure requirements and size range required for your application.

Capillary and Microbore columns.

Several companies have begun offering columns with smaller ID.

Microbore column - 1 mm ID, packed column.

Capillary column - < 1 mm ID, internal bound phase.

These columns require smaller solvent flows, reduced sample size and improved detector design.

Capillary and Microbore columns.

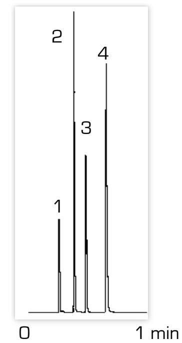
Example of some capillary HPLC columns.

Columns are basically the same as what is used in capillary GC, just shorter.

Capillary and Microbore columns.

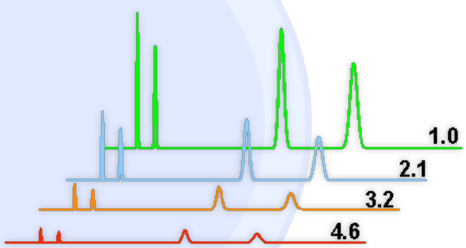
Aromatic Compounds
mobile phase 2% ethylacetate in hexane
flow rate 4 μl/min
column Fusica II, 300μm I.D. x 25 cm silica
sample
1. toluene
2. nitrobenzene
3. acetophenone
4. 2,6-dinitrobenzene

injection 60 nl
detection UV 254 nm



Capillary and Microbore columns.

By reducing column ID, you can obtain narrower peaks and better S/N ratios.



Capillary and Microbore columns.

Limits
- Reduced sample capacity
- Need improved detection
- Dead volume must be eliminated.

Advantages
- Reduced sample sizes
- Less solvent (5% or less compared to other HPLC methods)
- More suitable for interfacing to other methods like MS

Advantages/disadvantages similar to Capillary GC.
Relatively new method – not many applications – yet.

Detector Systems

Virtually every chemical and physical property that can be measured in solution has been look at.

Detectors fall roughly into two classes

Bulk property - measures an overall change in the mobile phase.

Solute property - measures a solute specific property.

Properties of a good detector

A detector must provide
high sensitivity, low detection limits, linearity, reproducibility.

This is true for any detector.

Each detector will have specific advantages and will vary as to peak shape and spread, noise and flow/temperature dependence they have.

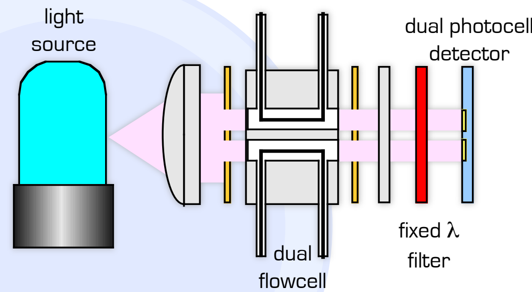
UV/Vis detector

A solute property detector.

Sample must exhibit absorption in UV/Vis range. Solvent must not absorb significantly at the measured wavelength.

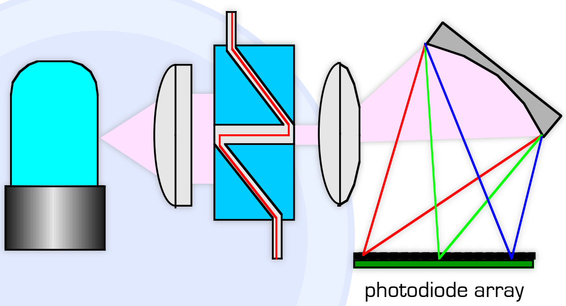
Types — Filter photometer - single λ
Variable wavelength
Multiwavelength.

UV/Vis detector - filter type



If the filter is replaced by a monochrometer, you end up with a variable wavelength UV/Vis system

Photodiode array detector

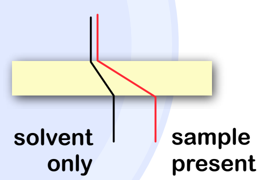


The photodiode array allow you to simultaneously monitor a range of λ or obtain complete spectra.

Refractive index detector

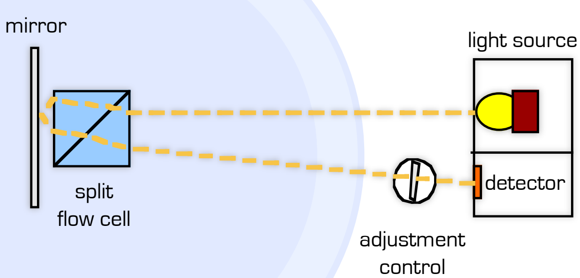
Bulk property detector - general purpose.

Based on refraction of light as it passes from one media to another. Presence of a solute changes the refractive index of the solvent.



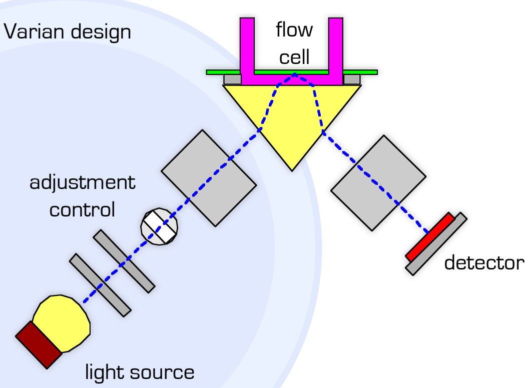
Refractive index detector

Waters design



Refractive index detector

Varian design



Refractive index detector

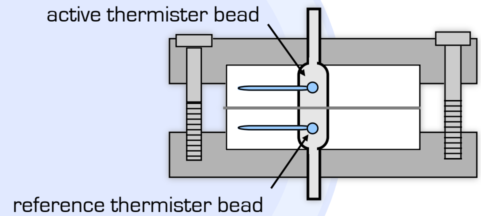
Temperature effect
Dependent on magnitude of refractive index and thermal expansion coefficient of solvent.

Temperature must be maintained to +/- 0.0001 °C for optimum performance.

This requirement can be relaxed somewhat if a reference cell is used.

Heat of absorption detector

A small amount of heat is released when a sample absorbs on a suitable surface. This detector can measure this.



Electrochemical detectors

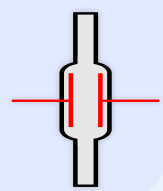
A number of properties have been evaluated

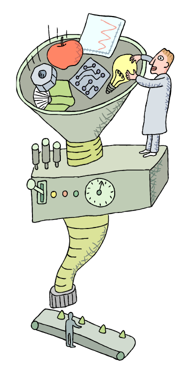
Detector types
- Dielectic constant
- Amperometric
- Conductometric
- Polarographic
- Potentiometric

We'll only look briefly at the first three.

Dielectric constant detector

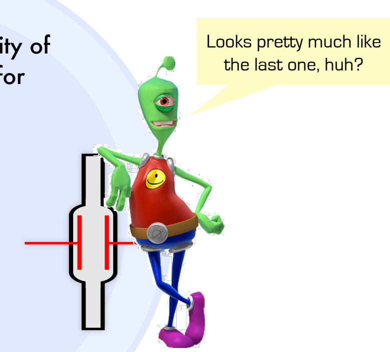
Bulk property detector.
Measures changes in polarity of the liquid phase passing through the cell.





Conductometric detector

Measures conductivity of the solvent. Useful for solutions of ions



Amperometric detectors

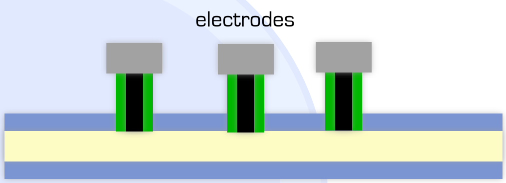
Most frequently applied type of electrochemical detector.

A known potential is applied across a set of electrodes - typically a glassy carbon type.

Ability to oxidize or reduce a species can be measured.

Typically limited to working with a specific class of materials per analysis.

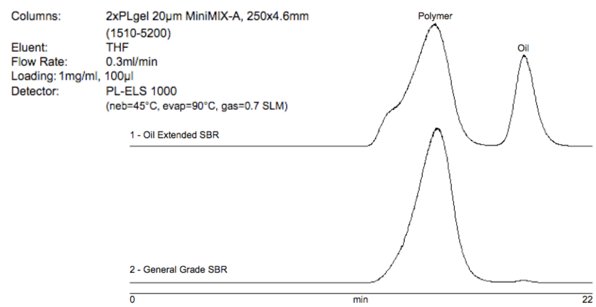
Amperometric detectors



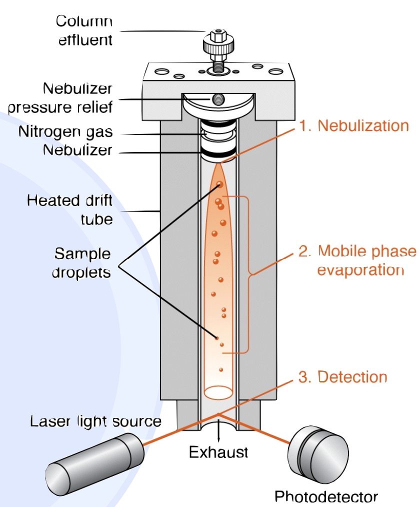
Several electrodes and combinations can be used. Allows for some interesting data.

ELSD (Evaporative Light Scattering)

Universal, destructive, large linear range.
Useful for large molecules.
Molecules are desolvated in the detector.
Pass light through sample stream
Measure reduction in light intensity as a result of scattering.

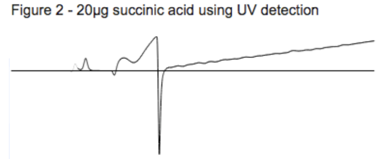


ELSD (Evaporative Light Scattering)



Succinic acid example

Figure 2 - 20μg succinic acid using UV detection



Sample:	1mg/ml
Column:	PLRP-S 300Å 8μm, 150x4.6mm (1512-3801)
Eluent A:	1% ACN, 0.1% TFA, 99% Water
Eluent B:	100% ACN, 0.1% TFA
Gradient:	5-80% B in 20 mins
Flow Rate:	1.0ml/min
Detectors:	UV, 215nm PL-ELS 1000 (evap=85°C, neb=85°C, gas=1.0 SLM)

Figure 3 - 20μg succinic acid using ELS detection

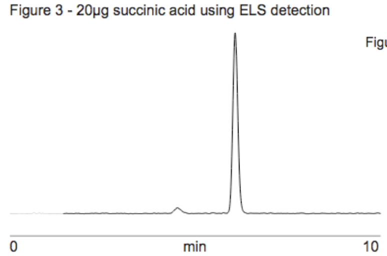
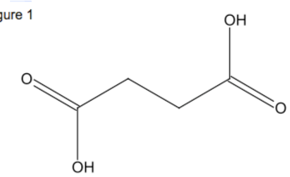


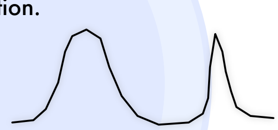
Figure 1



Detectors and peak shapes

Based on type of detector used.

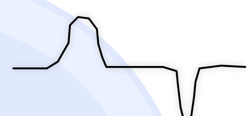
A UV or UV/Vis detector gives typical Gaussian shaped peaks. Absorption is proportional to concentration.



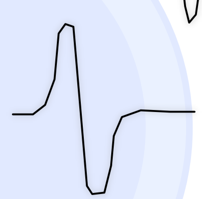
This is not true for all detectors.

Detectors and peak shapes

RI detector



Absorption detector



Your data system must be able to correct for this