High-performance liquid chromatography

Detectors

Requirements for detectors (1)

- Equally sensitive towards all components.
  OR
  Sensitive towards components of interest.
- Gives as much information about the analyte as possible.
- Independent of eluent composition (gradient) and temperature.
- Detects very low concentrations (analysis of traces).
- Does not cause widening of the peaks (small flow cell).

Requirements for detectors (2)

- Fast, in order to "catch" very narrow and fast moving peaks.
- Stable and reproducible signal.
- Better if wide linear range (at least 3 orders of magnitude).
- Non-destructive.
- Easy to use, robust.
- Cheap.

Types of detectors (1)

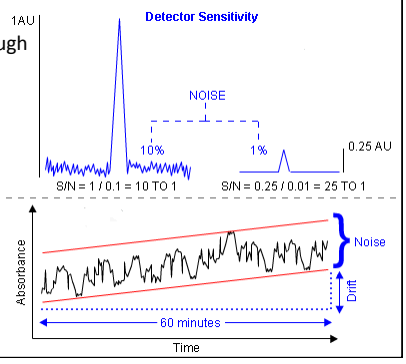
- Concentration and mass selective:
  - **Concentration selective** – signal is dependent of the analyte concentration in eluate.
    $S \propto c$ (g ml^{-1})
  - **Mass selective** – signal is dependent on the mass flow, amount of sample molecules in time.
    $S \propto n/\Delta t$ (g s^{-1})
- Eg when switching of the pump at the highest point in chromatographic peak – for concentration selective detector signal remains, for mass selective detector signal will fall to the base noise level.

Types of detectors (2)

- Selectivity of the measured properties:
  - Non-selective detectors react to the **bulk property of the passing solution**
    - Refractive index
    - Conductivity
  - Selective detectors measure a response due to specific property of the **analyte molecule**
    - UV absorbance
    - Fluorescence
    - Mass to charge ratio

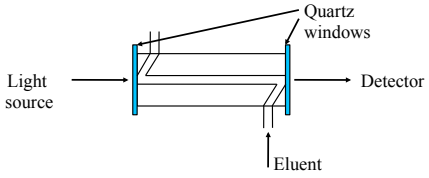
Sensitivity

For the estimation of sensitivity it is not enough to evaluate the signal of the analyte. Sensitivity is estimated through the signal to noise ration.

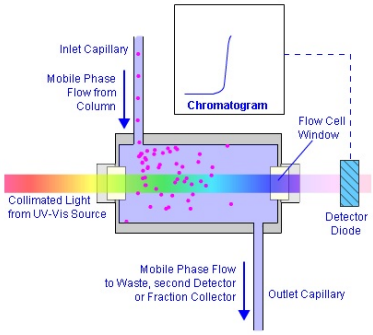


Flow cell (1)

- Cells with 1-15 µl volume are used:
  - Commonly used HPLC flow cells are 6-10 µl
  - UHPLC micro-flow cells are maximum 2.7 µl



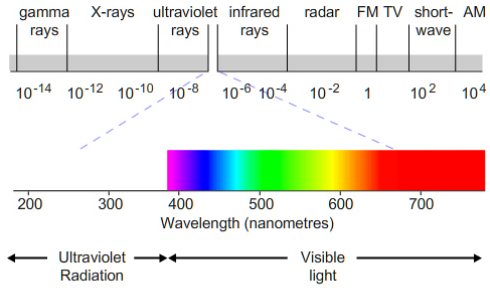
Flow cell (2)



UV-Vis detector

- Most widely used HPLC detector.
  - Selective detector (measures properties of the analyte).
  - Concentration selective.
  - Sensitive.
  - Wide linear range (4 orders of magnitude).
  - Not sensitive to temperature changes.
  - Suitable for gradient elution.

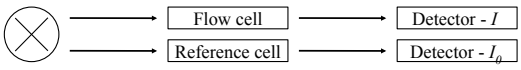
Electromagnetic spectrum



UV Cut-off - for common organic solvents used in reverse phase HPLC:
Acetonitrile 210nm / Methanol 210nm / THF 220nm

UV-Vis: principle

- Sometimes double-beam systems with reference light passing through the reference cell and filter are used.



$$A = \log \frac{I_0}{I}$$

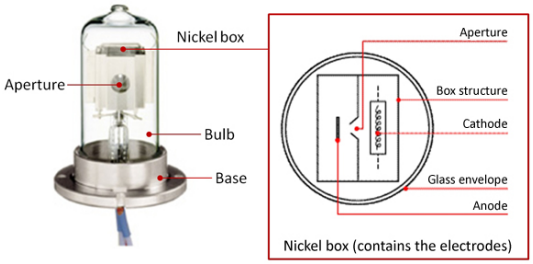
- Beer's law: $A=\varepsilon lc$ ehk $A=abc$,
where A – absorbance, ε (a) – molar absorptivity, l (b) – optical path length, c – analyte concentration.

UV-Vis: types and radiation sources
Fixed wavelength and filters (1)

- In case of low pressure Hg lamps wavelenght 254 nm is used.
- Cd-lamp – 229 nm.
- Zn-lamp – 214 nm.
  - Other wavelengths are filtrated out. (Why?)
  - Significantly more sensitive than detectors with changeable wavelength.

UV-Vis: types and radiation sources
Fixed wavelength and filters (2)

- Deuterium-lamp – constant UV-specter 190 nm to *ca* 340 nm.

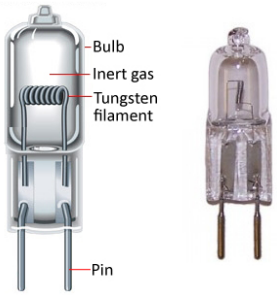


UV-Vis: types and radiation sources
Fixed wavelength and filters (3)

- Tungsten lamp – 340-850 nm.

Lamp bulb is made of quartz and can be used at temperatures as high as 900 C and pressure at 20 bars.

To lengthen the lifetime of lamp, the bulb is filled with a halogen such as Br or I.

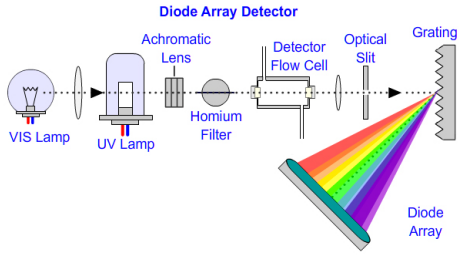


UV-Vis: applications

- Used for detection of very many compounds:
  - Double bond and free electron pair atom closeby X=Y-Z|
  - Br-, I- and S-containing compounds.
  - Carbonyl (C=O) or nitro (NO_2).
  - Conjugated double bond X=X-X=X.
  - Aromatic ring.
  - Anions: Br^-, I^-, NO_3^-, NO_2^-
- **Not suitable** for detection of saturated hydrocarbons and their amino and nitrile derivatives..

UV-Vis: types and radiation sources

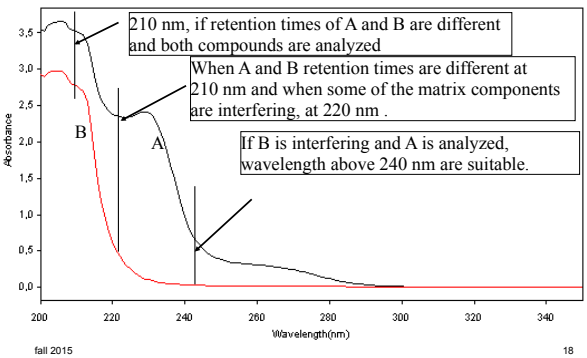
- With monochromator (D_2- and/or W-lamp)
- Diode array - PDA-photodiode array, DAD-diode array detector



UV-Vis: working modes

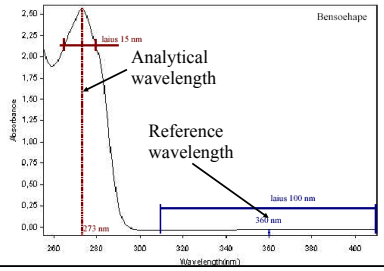
- Detection of chromatogram at fixed wavelength – all listed types.
- Changing the wavelength during detection of the chromatogram – most detectors with D_2- and W- lamps.
- Scanning (D_2 and W) detectors allow to stop the eluent flow and then register the spectrum.
- Diode array allows to detect **full spectrum** at each point in the chromatogram (with eluent flow).
  - In addition to sample wavelength, reference wavelength can be determined.

UV-Vis: choosing the wavelength



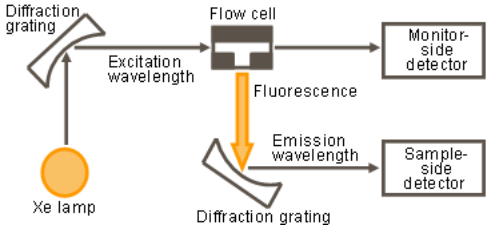
UV-Vis: Reference wavelength

- For diode array detector a reference wavelength (range) can be determined that corrects the drift for example from gradient.



FLD – fluorescence detector (1)

- FLD – excitation radiance is perpendicular to the detection:



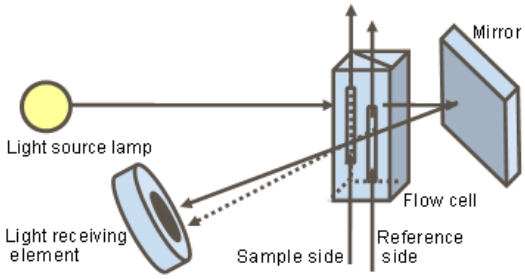
Hitachi High-Tech

FLD – fluorescence detector (2)

- Properties
  - Selective detector.
  - LOD is **1000 times** better than in UV-Vis.
  - For higher sensitivity, flow cell valume is *ca* 20 µl.
  - Sensitive to **fluorescence depleting** components in he mobile phase (eg dissolved oxygen).
  - Linear range depends of the system, but is narrow.

RI – refractive index detector (1)

- RI – *refractive index*



Hitachi High-Tech

RI – refractive index detector (2)

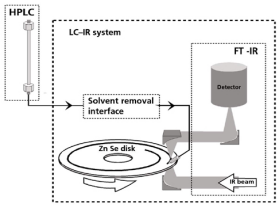
- Properties
  - Non-destructive detector (refractive index of the whole eluate).
  - LOD is 1000 times worse than for UV-Vis detector.
  - Very sensitive towards temperature changes – needs a thermostate.
  - It is necessary to have a reference cell with an eluent.
  - Not suitable for gradient elution.
  - Gases dissolved in eluent will influence the detection.

ELSD – *evaporative light scattering detector*

- ELSD – eluent with the dissolved analytes are nebulized and evaporated into tiny sample particles. These scatter a light beam (laser) and the extent of scattering is measured.
  - Non selective detector (sensitive towards **all non volatile** compounds).
  - More sensitive than RI.
  - Can be used for gradient elution if eluent contains volatile components.
  - Linear range is basically inexistent.

IR – infrared absorbance detector

- Based on the property of organic compounds to absorb infrared light.
  - Selective detector.
  - Mobile phase must be chosen so that it does not absorb at the same wavelength as the analyte.
  - Volatile buffers.
  - Good for polymer analysis.
  - Ability to differentiate secondary structure.

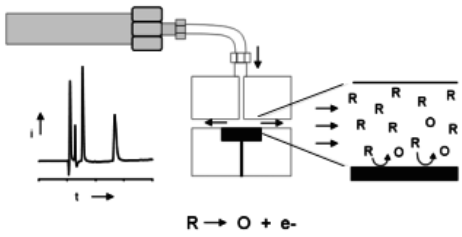


Electrochemical detectors

- Used for different types:
  - Amperometric
  - Polarographic
  - Culonometric
  - Conductometric

Amperometric (1)

- Amperometric detector measures electrical durrent resulting from oxidation or reduction reactions.

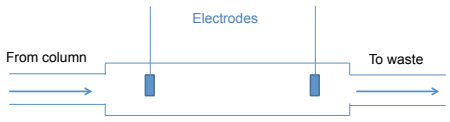


Amperometric (2)

- Properties
  - Selective detector (as low as fmole/L).
  - LOD is very low.
  - Simple and cheap.
  - Wide linear dynamic range (more than 6 orders of magnitude)
  - Mobile phase must conduct electricity, but does not have to be aqueous (non-polar eluents are not suitable).
  - Sometimes difficult to find the best conditions.

Conductivity detector (1)

- Classical detector for ion chromatography.
- Conductivity of the mobile phase is measured.



Conductivity detector (2)

- Properties
  - Non-selective mass sensitive detector.
  - Signal is dependent on of the ionic component in the mobile phase.
  - **Sensitivity is decreased** if eluent is conducting electricity (suppression columns are used).
  - Sensitive to temperature.
  - Linear range is not very wide.

MSD – mass spectrometric detector

- MS as a detector is so powerful and special that the whole method is abbreviated as LC-MS (LC/MS).
- MS has developed so much in the last decade that options presented in older books must be taken with care.
- For MS detection, analyte must be in ionic form, therefore MS is splitted into to parts:
  - Ion source
  - Mass spectrometer

MSD properties

- **Selectivity** – Coeluting peaks can be separated/isolated accoring to mass, even when they are not chromatographically separated.
- **Detection of peaks** – specific to chemical compounds.
- **Information on molecular mass** – Identification of known and unknown compounds.
- **Information on the structure of the compound** – Fragmentation provides additional information.
- **Faster method development** – Fast and easy analyte detection without retention time validation.
- **Differentiating sample matrix from analyte** – may reduce the time spent on sample preparation.
- **Quantification** – Both quantitative and qualitative analysis.

MSD ion sources

- Roles of ionization source:
  - Ionization of the analyte.
  - Separation of analyte from the eluent.
  - Make sure that only analyte enters the mass spectrometer.
- Atmospheric ionization sources (API):
  - ESI (*electrospray*)
  - APCI (*AP chemical ionizaton*)
  - APPI (*AP photoionization*)
  - AP-MALDI (AP *matrix-assisted laser desorption/ionization)*

MSD: ESI

- Principle of ESI is electrospray – eluent sprayed into high voltage creates an aerosol. Small drops in the aerosol will aquire a charge.
- Eluent flow introduced to ESI is sprayed into fine droplets with gaseous N_2 and electric field. Analyte molecules aquire a charge (eg addition of H^+) and due to electric field move to MS.
- ESI
  - **Very soft** ionization, even biomolecules don't decompose.
  - Most commonly used ionization source.
  - Concentration sensitive.

MSD: APCI

- In case of APCI eluent flow is sprayed to droplets with the help from gaseous N_2. Eluent is evaporated through heating.
- Corona discarge is used to ionize N_2, which will ionize eluent molecules and which in turn with ionize analyte.
- APCI
  - **Very soft** ionization, which allows to analyze **less polar compounds** than ESI.
  - Most common after ESI.
  - Can be said that mass sensitive.

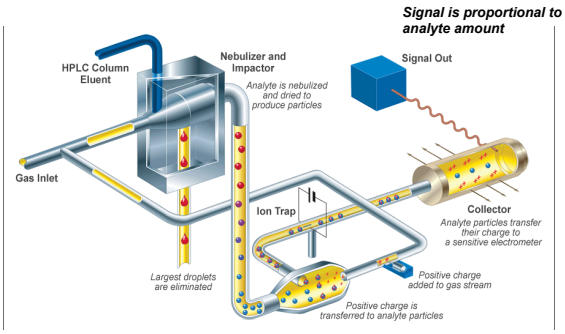
MSD: mass spectrometers

- Quadrupole MS.
  - Single quadrupole is quite sensitive with good LOD but it does not allow fragmentation and is therefore less selective than other MS types.
  - Triple quadrupole (3Q) is more sensitive than single quadrupole. MS^2 allows to get structural information and therefore raises the selectivity.
- Ion trap MS
  - Very sensitive MS detector; allows at least MS^4, which adds significant amout of structural information.
- Time of flight (TOF) MS
  - Very exact mass.
- Orbitrap MS
  - Most sensitive MS detector, very exact for small and large molectules. Fast detection, wide linear range and allows MS^n which gives a lot of structural information.

More detectors

- Photoconductivity detector
- Radioactivity detector
- Light scattering detector
- Dielectric constant detector
- Corona Charged Aerosol Detector: http://www.esainc.com/products/type/hplc_systems/detectors/coronacad

CCAD (1)



CCAD (2)

- Excellent sensitivity (in nanograms)
- Wide dynamic range
- Very reproducible signal
- Can detect analytes with very different properties (also without chromophores)
- Robust and easy to use