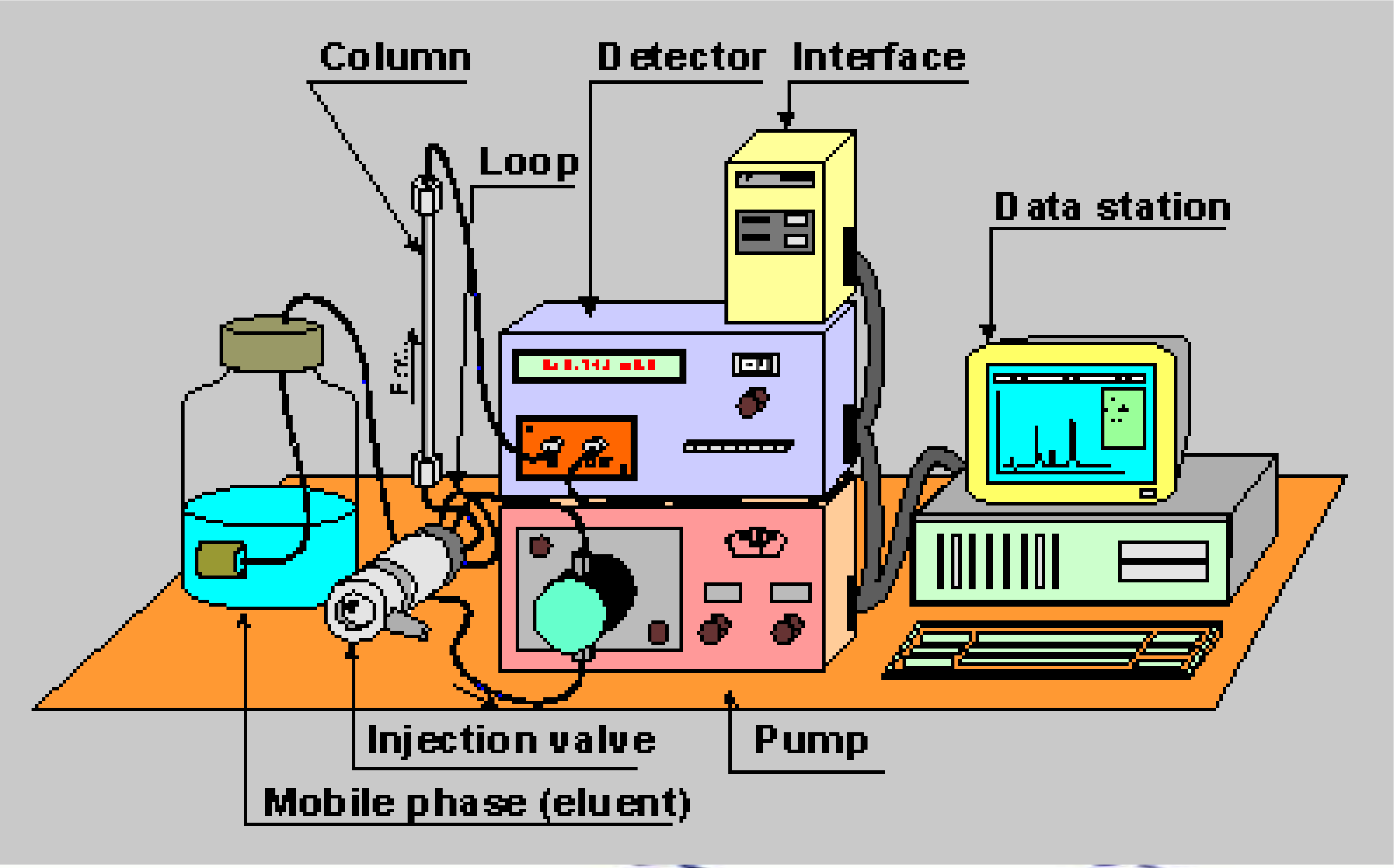
Chemistry 4631

Instrumental Analysis

Lecture 29

High Performance Liquid Chromatography (HPLC)

Instrumentation



HPLC

Instrumentation

Detectors

- Ideal HPLC Detector
  - high sensitivity
  - low baseline noise
  - large dynamic range
  - response independent of mobile phase composition
  - low dead volume
  - stable over long periods of operation

Instrumentation

Detectors

HPLC detectors are broadly classified as:

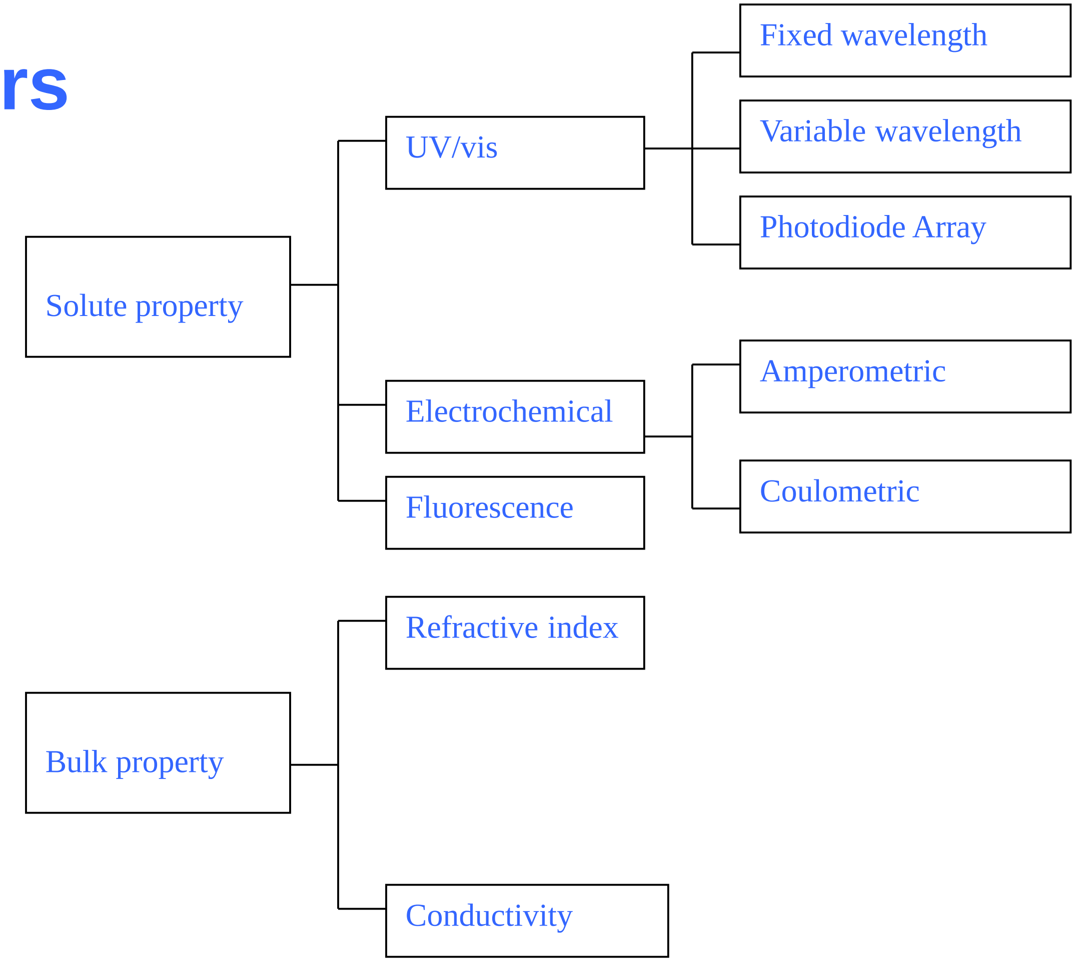
- **solute detectors (respond to a physical property of the solute not exhibited by the mobile phase),**

- **selective bulk-property detectors (compare an overall change in a physical property of the mobile phase with and without the analyte).**

HPLC

Instrumentation

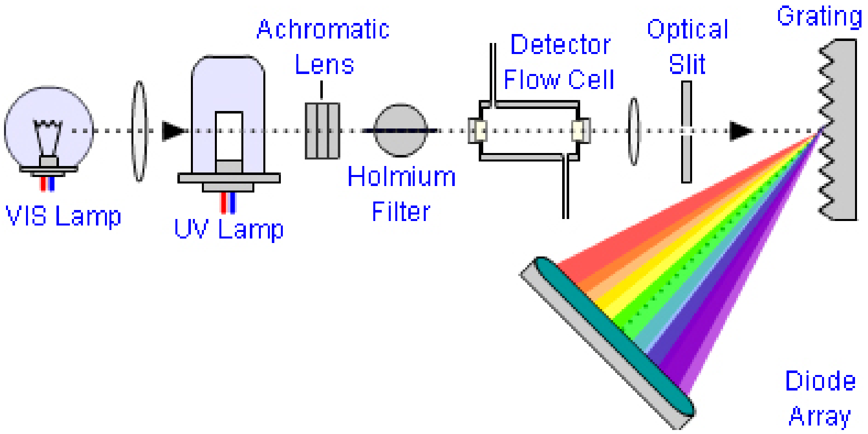
Detectors

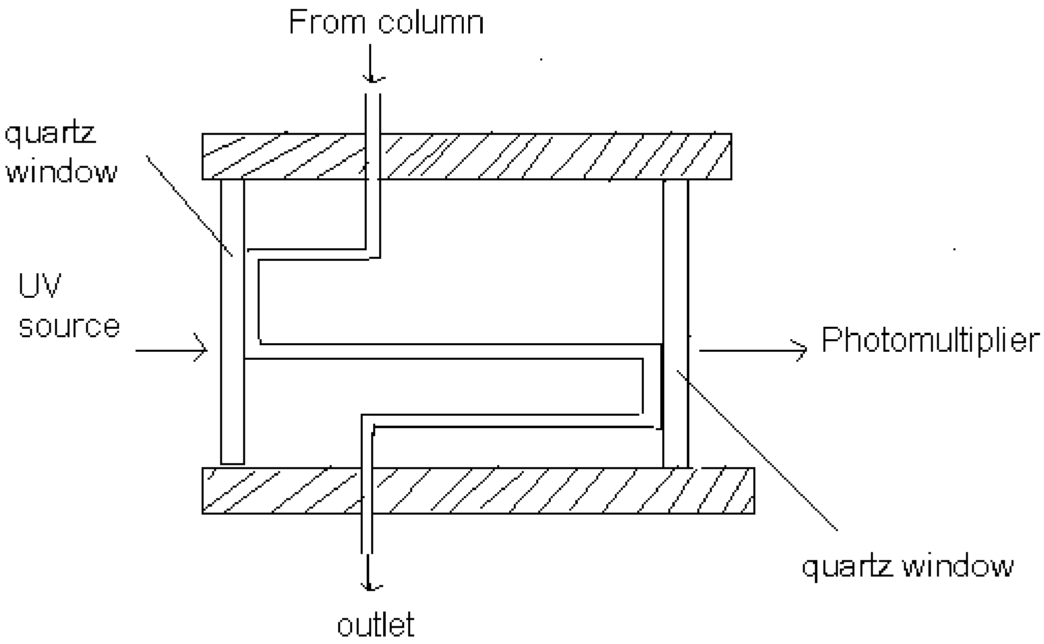


HPLC
Instrumentation

Detectors - Absorbance Detectors

Most common detector for HPLC





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Instrumentation

Detectors - Absorbance Detectors

Light from a radiation source is passed through a monochromating device (grating or filter) and through a cell which the mobile phase flows.

Amount of transmitted light is measured by a photodetector.

UV - 190 – 350 nm **190 – 700 nm UV/Vis**
Vis - 350 – 700 nm

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Instrumentation

Detectors - Absorbance Detectors

Light radiation is absorbed by particular electronic configurations of the analyte.

Compounds with one or more double bonds or unpaired electrons absorb in the UV.

Detector monitors light passing through the flowing stream, when a compound in the mobile phase passes through the cell it absorbs some of the light. Decrease in light is the peak.

HPLC

Instrumentation

Detectors - Absorbance Detectors

Solute concentration and intensity of light is related to Lambert-Beer Law.

$$A = \log(P_0/P) = \varepsilon bc$$

A – absorbance of solution in the cell
b – optical path – length through cell (cm)
ε – molar absorptivity of the solute at a particular wavelength (l/mol·cm)
C – molar concentration of the solute
P_o – light intensity from source
P – light intensity transmitted

Instrumentation

Detectors - Absorbance Detectors

Fixed-wavelength detector monitors one wavelength only. Noise is low, sensitive, inexpensive.
Common light sources used for fixed-wavelength detectors:

Source	Wavelength	
Mercury	254, 280, 365, 405, 436, 546, 578 (most common 254)	
Cadmium	229, 326	
Zinc	214, 308	
Magnesium	206	
Deuterium	190-350 (continuous)	(common)

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Instrumentation

Detectors - Absorbance Detectors

At 254 nm, majority of organic compounds can be detected.

Aromatic compounds, unsaturated compounds, absorb especially strong in this region.

Compounds with carboxylate functional group can be easily detected at 214 nm (Zn lamp).

Instrumentation

Detectors - Absorbance Detectors

Variable – wavelength detectors - more than one wavelength can be monitored during a run. Detectors use a light source with a continuous emission spectrum (Deuterium).

Photodiode array (PDA)

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Instrumentation

Detectors - Photodiode array (PDA)

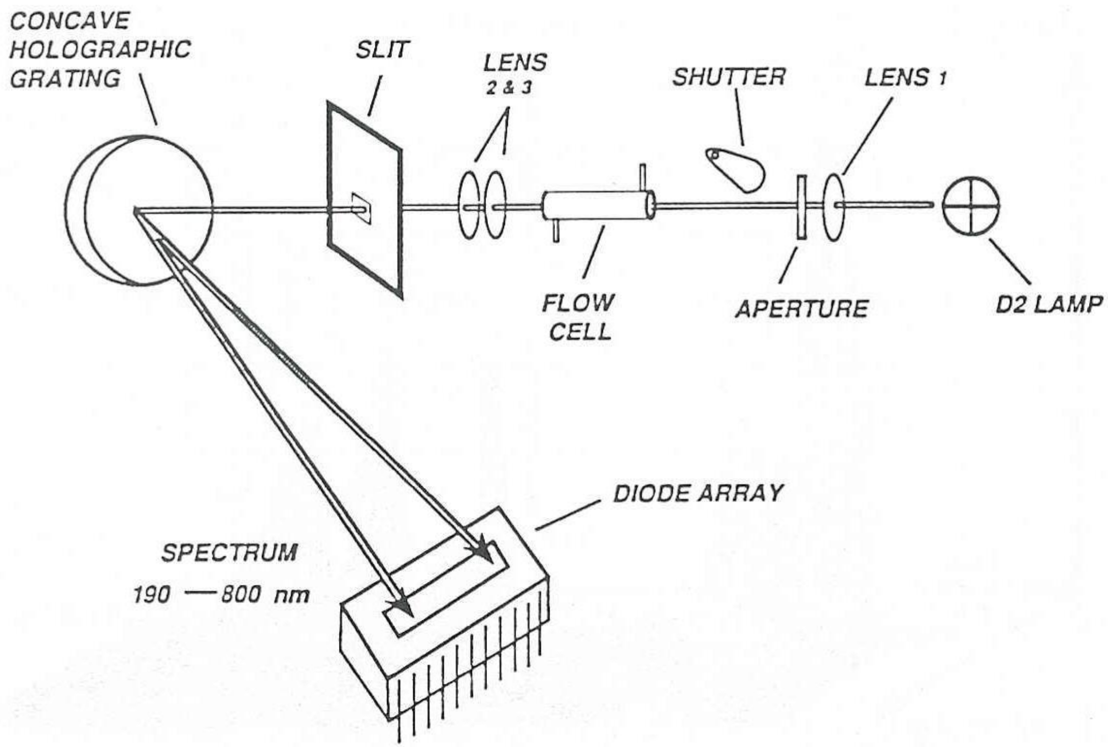

Fig. 5.17. Diode-array detector optics. Reprinted with permission of Hewlett Packard.

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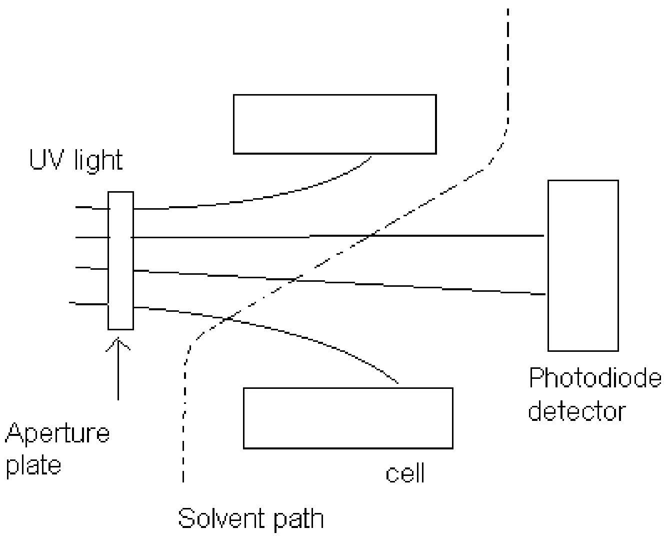
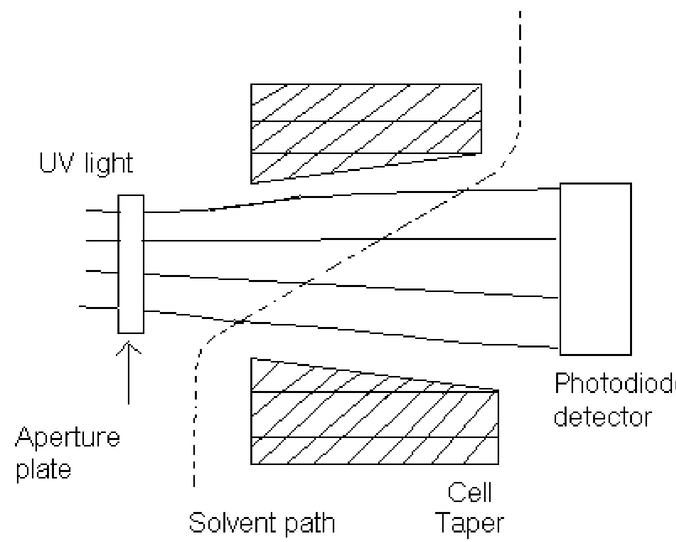
Instrumentation

Detectors - Absorbance Detectors

One problem with absorption detectors is that the refractive index of the solution passing through the cell may alter (different in RI of analyte and mobile phase), producing a “dynamic liquid lens” which distorts the light beam passing through the cell. This can be minimized by using a conical tapered cell.





Instrumentation

Detectors - Fluorescence Detectors

Fluorescence – instantaneous emission of radiation from a molecule which has attained an excited electronic state after the absorption of radiation.

Excitation and Emission

For excitation – absorption of a photon causes the solute molecule to move to an excited electronic state.

The excited molecule can lose energy through the emission of a photon (Fluorescence).

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Instrumentation

Detectors - Fluorescence Detectors

The excited molecule can lose energy through the emission of a photon (Fluorescence).

Compounds with delocalized π electrons that can easily be placed in low-lying excited single states are more fluorescent.

Electron – donating groups on a ring (-NH_2, -OH, -F, -OCH_3, -$N(CH_3)_2$) enhance fluorescence, while electron - withdrawing groups (-Cl, -Br, -I, -NO_2, -CO_2H) decrease or quench fluorescence.

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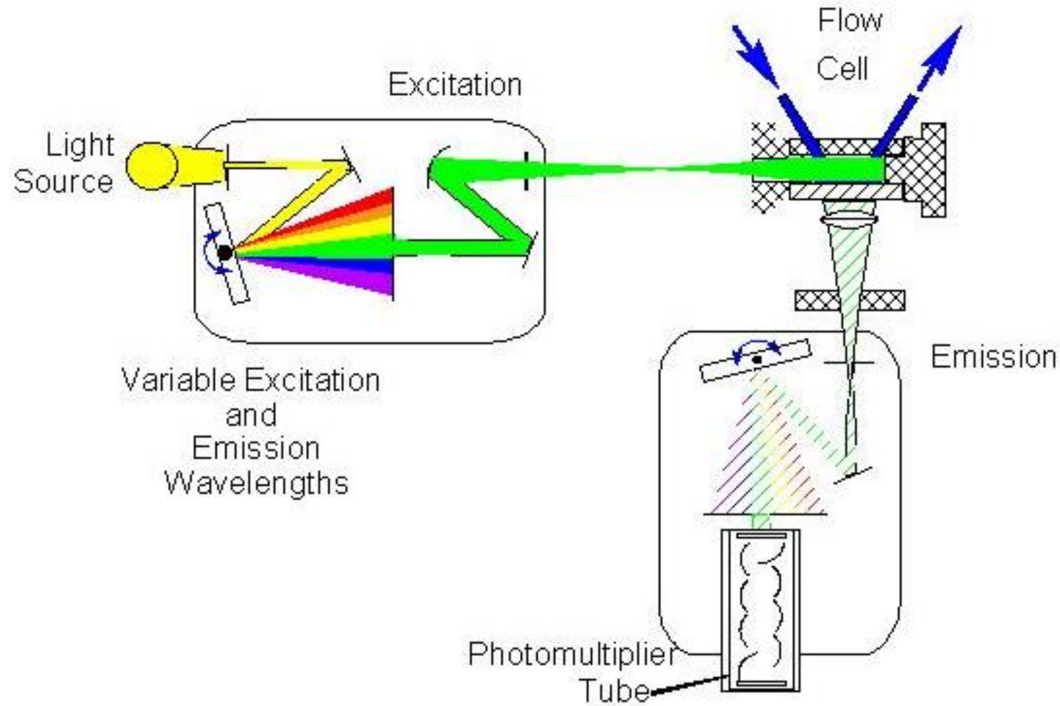
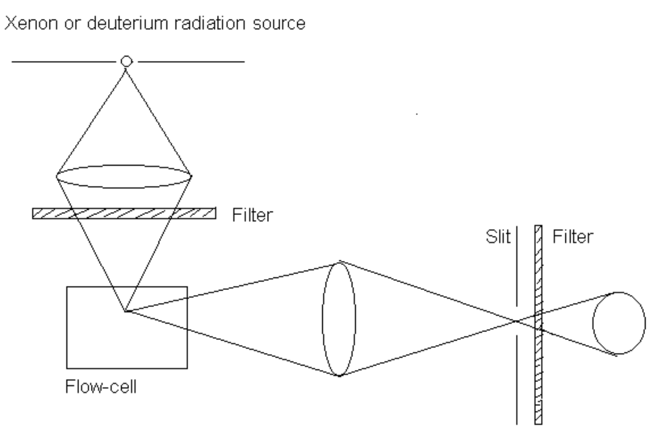
Instrumentation

Detectors - Fluorescence Detectors





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Detectors - Fluorescence Detectors

Lamp of relatively high intensity passes light through a monochromator into a flow-cell.

Fluorescence radiation is collected at right angles to the excitation beam.

Monochromators can be simple filter or gratings. Flow cells are generally constructed from quartz.

Molecules that are strongly conjugated and have a rigid structure tend to exhibit fluorescence.

Ex. Aromatic and polyaromatic compounds (Aflatoxins, etc.)

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Instrumentation

Detectors - Fluorescence Detectors

Fluorescence detector drawbacks:

- **Fluorescence signal is dependent on mobile phase pH, components of the mobile phase, temperature, concentration of analyte and quenching effects.**
- **Fluorescence usually applicable over a narrow pH range – need buffer solutions.**
- **Decreasing temperatures minimizes vibrational relaxation processes that compete with fluorescence, thus causing increase in fluorescence output.**

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Instrumentation

Detectors - Fluorescence Detectors

Fluorescence detector drawbacks:

- **Concentration of analyte effect - emitted fluorescence can be reabsorbed by adjacent, unexcited analyte molecules (self-absorption). This process becomes greater as concentration of analytes increases. This gives a nonlinear calibration plot of fluorescent intensity vs analyte concentration.**

- **Quenching agents – interact with excited analyte molecule and enable it to relax without emission of radiation. O_2 - common quenching agent.**

Instrumentation

Detectors - Electrochemical Detectors

Application of electrical potential followed by measurement of current.

Common techniques are:
Voltammetry, amperometry, and coulometry.

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Instrumentation

Detectors - Electrochemical Detectors

Voltammetry - application of changing potential to a working electrode, followed by measurement of the current resulting from a reaction (redox).

Amperometry - fixed potential is applied to a working electrode and the current resulting from ox or red reactions is measured. Working electrodes for amperometry are small - so less than 10% of the analyte is oxidized or reduced.

Coulometry - is similar to amperometry except the analyte response is close to 100%.

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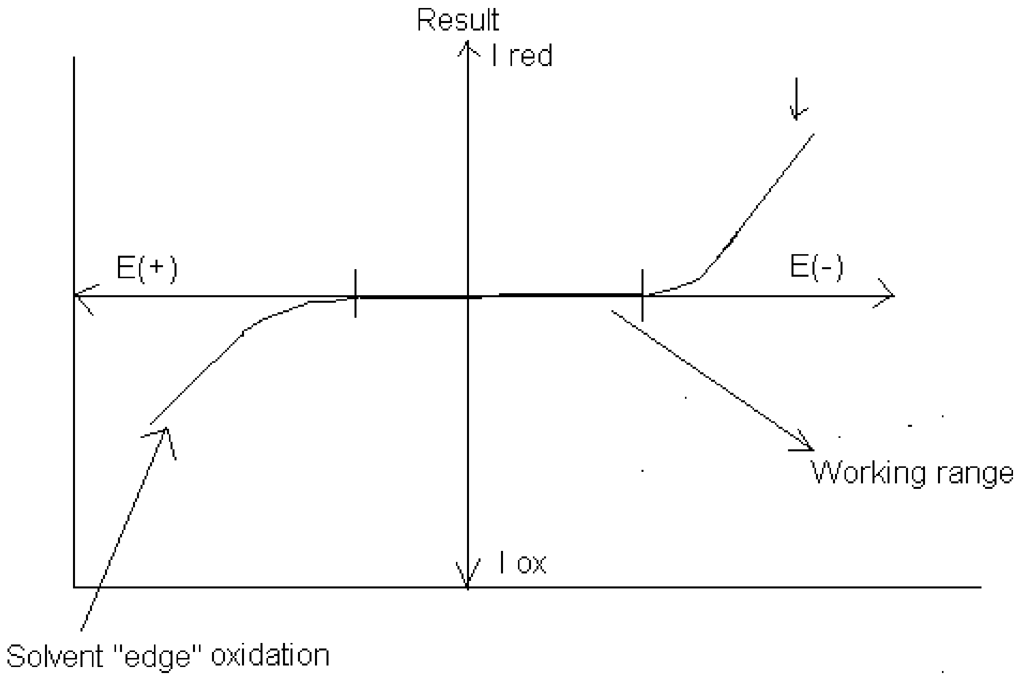
Instrumentation

Detectors - Electrochemical Detectors

Working range is dependent on the nature of the working electrode, electrolyte used, pH of electrolyte.



Instrumentation

Detectors - Electrochemical Detectors

To choose the correct potential for an analyte - can vary the potential and measure current, or for an amperometric instrument can measure individual currents over a range of potential and plot i's vs E's. ---- Hydrodynamic voltammogram.

Amperometry is more popular than coulometry - since for coulometry larger electrodes not only increase the faradaic current but the background current also - so there is little gain in sensitivity.

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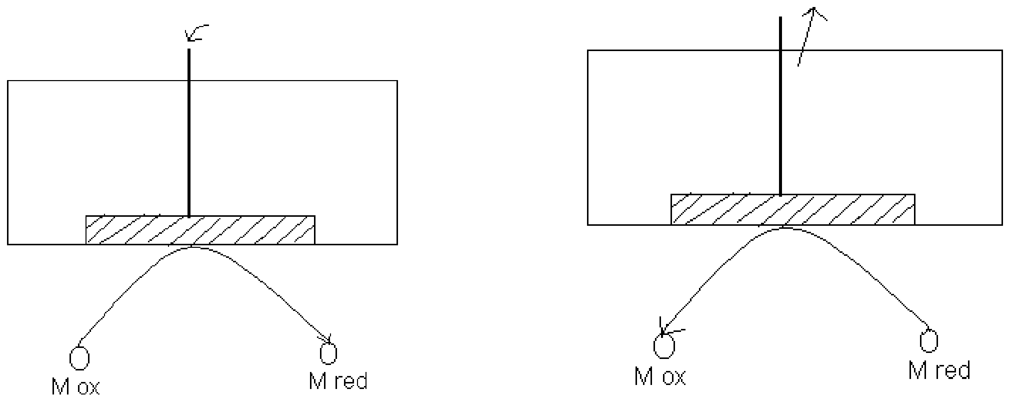
Instrumentation

Detectors - Electrochemical Detectors

In amperometry, a single potential is applied to the working electrode (w.e.).



The electrochemical reaction occurs at the interface between the w.e. and solution. One major problem - reaction products can accumulate and adhere to electrode surface (fouling, poisoning) eventually passivating the surface. Electrode must be removed and polished or cleaned by potential pulsing.

Instrumentation

Detectors - Electrochemical Detectors

Electrode Material:

Reference electrode (zero current flow)
- Ag/AgCl
- Saturated calomel electrode (SCE)
- Quasi reference - Pt

Auxiliary electrode - inert, situated close to w.e.
- Platinum
- Glassy Carbon
- Stainless Steel

Working electrode
- Mercury films (for reduction reactions)
- Glassy carbon
- Carbon paste
- Carbon fibers
- Pt, Au, Ni, Ag

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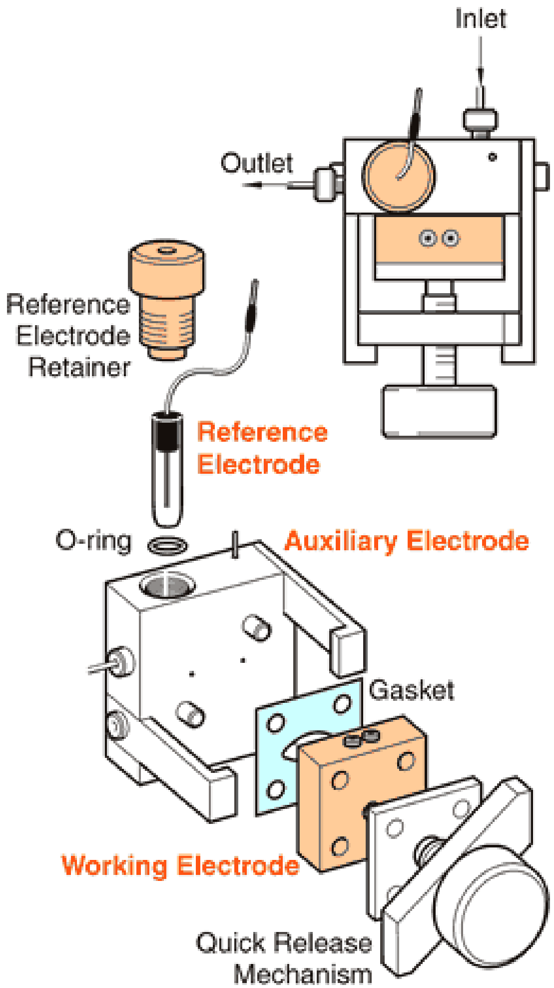
Instrumentation

Electrochemical Detectors



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Instrumentation

Detectors - Electrochemical Detectors

Cells:
Thin layer (parallel of series) and wall-jet

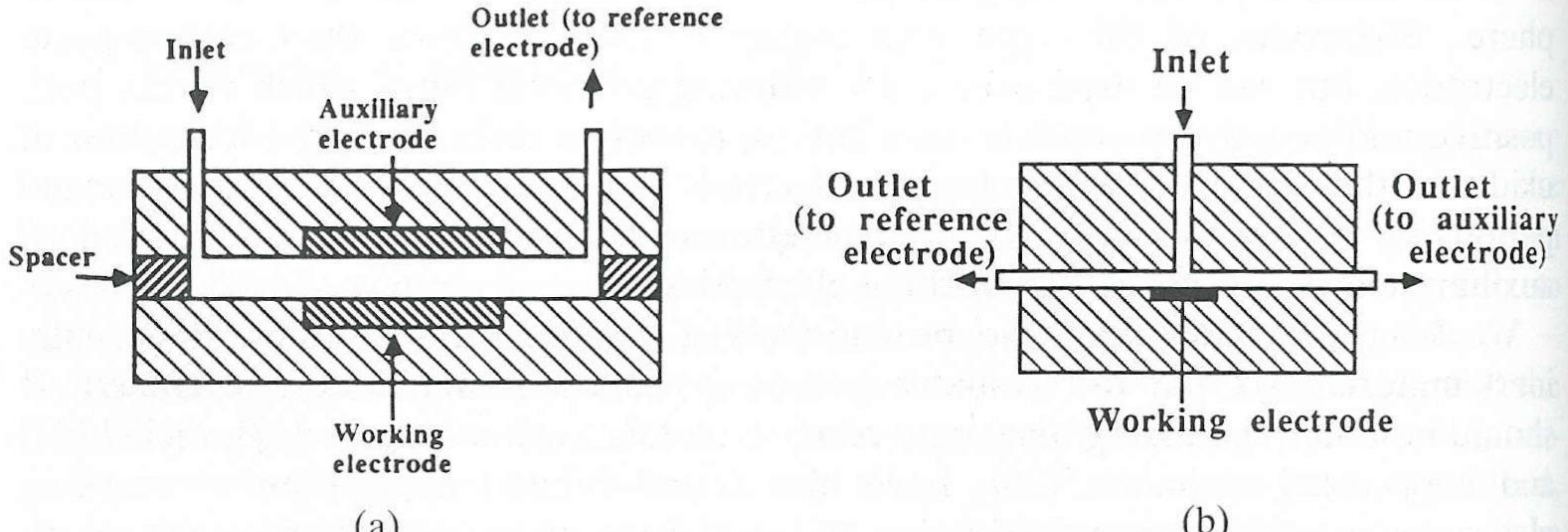

Fig. 5.27. Schematic illustration of (a) thin-layer and (b) wall-jet amperometric flow-cells.

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Detectors - Electrochemical Detectors

Electrochemical detectors used for detection of phenols and amines (neurotransmitters), drugs (penicillin), vitamins (ascorbic acid).

Extremely sensitive technique.

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Instrumentation

Detectors - Refractive Index Detectors (RI)

Universal detector

Compare RI of the pure mobile phase with the presence of an analyte.

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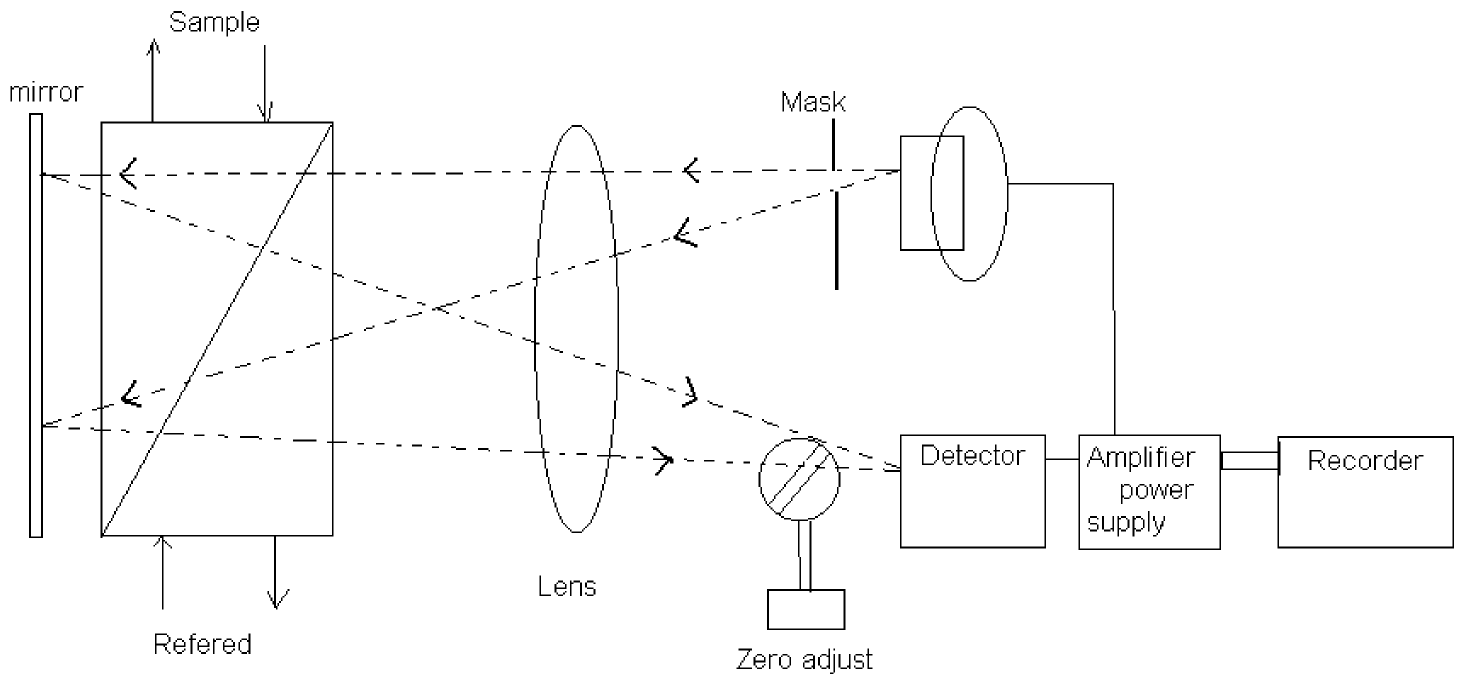
Instrumentation

Detectors - Refractive Index Detectors (RI)

Deflection type



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Detectors - Refractive Index Detectors (RI)

Deflection detectors measure deflection of a beam of monochromatic light passing through a double prism created by separating a rectangular cell into two compartments (with diagonal glass divider).

Column effluent passes through one compartment and pure mobile phase through (or fills) the other compartment.

Instrumentation

Detectors - Refractive Index Detectors (RI)

Light beam from collimated source passes through the prisms onto a beam splitter (mirror) which directs light to twin photomultipliers.

When no analyte is present signal is nulled to zero.

When analyte passes through cell the refractive index of sample compartment alters-causing refraction of the light beam and change in the angle at which beam strikes the beam splitter.

Relative amount of light falling on photomultipliers change.

Instrumentation

Detectors - Refractive Index Detectors (RI)

RI detectors will show response to most solutes - but magnitude and direction of response depends on difference in RI between mobile phase and the analyte.

Sensitivity is at a maximum when the difference is greatest.
Sensitivity at best is moderate – universal, not trace level detection.

Example: Tetrahydrofuran mobile phase (1.408)
Hexane (RI = 1.375) - give large negative peak
Nonane (1.408) - no peak
Decane (1.412) - small, positive peak

Instrumentation

Detectors

Liquid Chromatography / Mass spectrometry

Advantages:

- **More definitive identifications**
- **Wide range of analytes can be studied**
- **Sensitivity (pg)**

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Instrumentation

Detectors

LC-MS

Problems for LC-MS combination:

- **HPLC mobile phase – liquid w/ water or organics**
- **MS must be at 10^{-6} torr**
- **Most analytes separated by HPLC are thermally stable and non-volatile (unlike in GC) – so not ionized easily by EI or CI techniques**

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Instrumentation

Detectors - LC-MS

Ideal Interface:

- Has no reduction in chromatographic performance
- No chemical modifications
- High sample transfer
- Reliable and reproducible

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Detectors – LC-MS

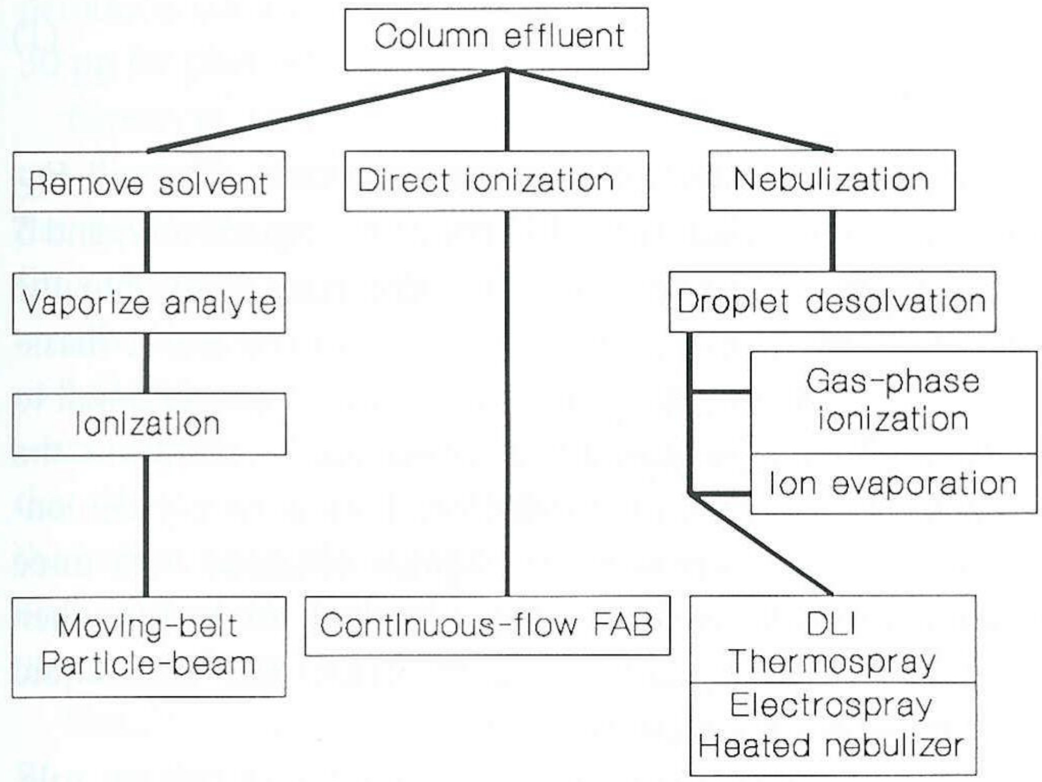

Fig. 1.5. Three general strategies to LC-MS interfacing.

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Detectors - LC-MS Interfaces

- Moving-belt interface 1977
- Direct-liquid-introduction interface 1980
- Thermospray interface 1983
- Frit FAB/continuous-flow FAB interface 1985/1986
- Atmospheric-pressure chemical ionization interface 1986
- Particle-beam interface 1988
- Electrospray interface 1988

Instrumentation

LC-MS Interfaces

Electrospray interface 1988

High performance liquid chromatography is an effective technique for the separation of compounds of high molecular weight. However, two major problems for the study of this type of molecule have severely limited the application of LC–MS. Specifically,:

- (a) The inability to ionize, in an intact state, many of the labile and/or involatile molecules involved.
- (b) Should ionization be possible, the lack of appropriate hardware to allow the mass analysis and efficient detection of the ions of high m/z ratio involved.

- Electrospray is an ionization method that overcomes these problems.

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Electrospray – 1st generation

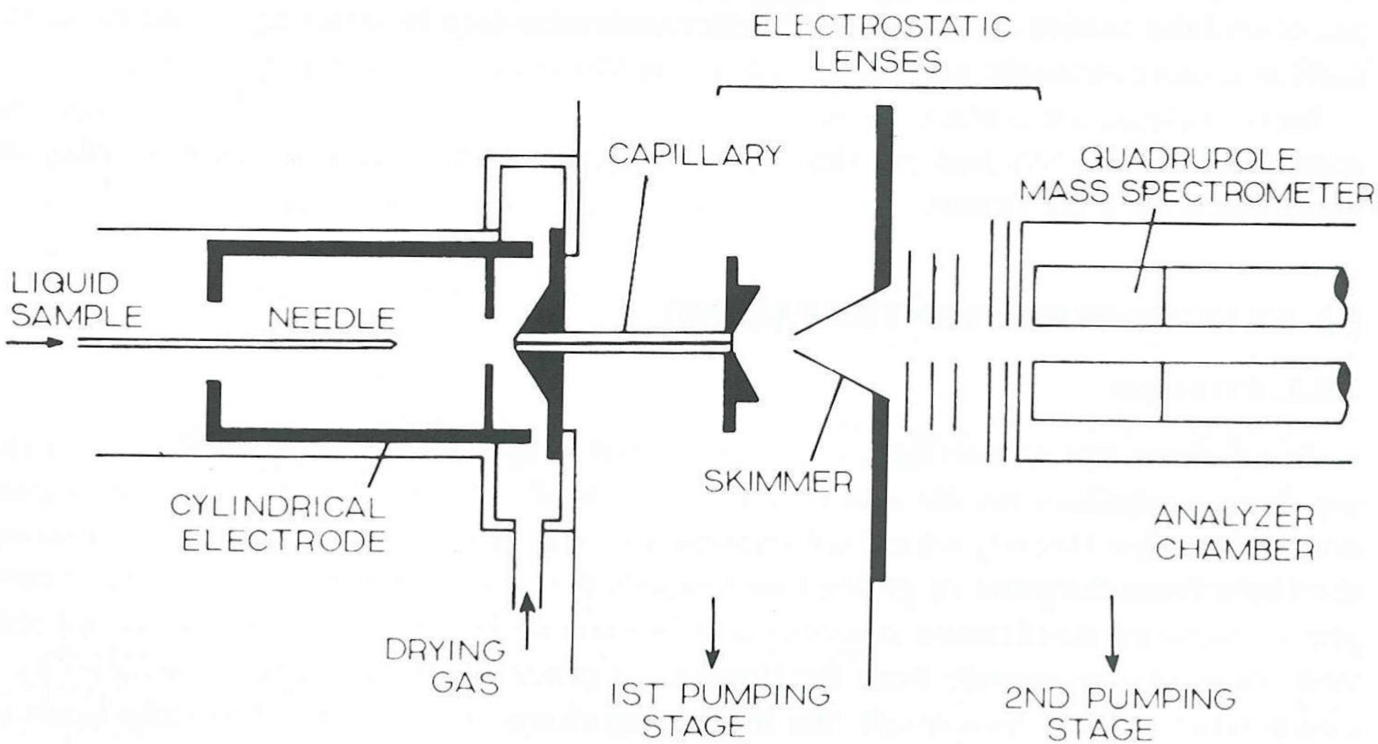

Fig. 1.13. Schematic diagram of the first-generation electrospray LC-MS interface as described by Whitehouse et al. [216]. Reproduced from Ref. [216] with permission. © 1985, American Chemical Society.

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Electrospray

- A liquid, in which the analyte(s) of interest have been dissolved, is passed through a capillary (typically stainless steel), at atmospheric pressure, maintained at high voltage (3 to 4 kV).

- The liquid stream breaks up with the formation of highly charged droplets which are desolvated as they pass through the atmospheric-pressure region of the source towards a counter electrode.

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Instrumentation

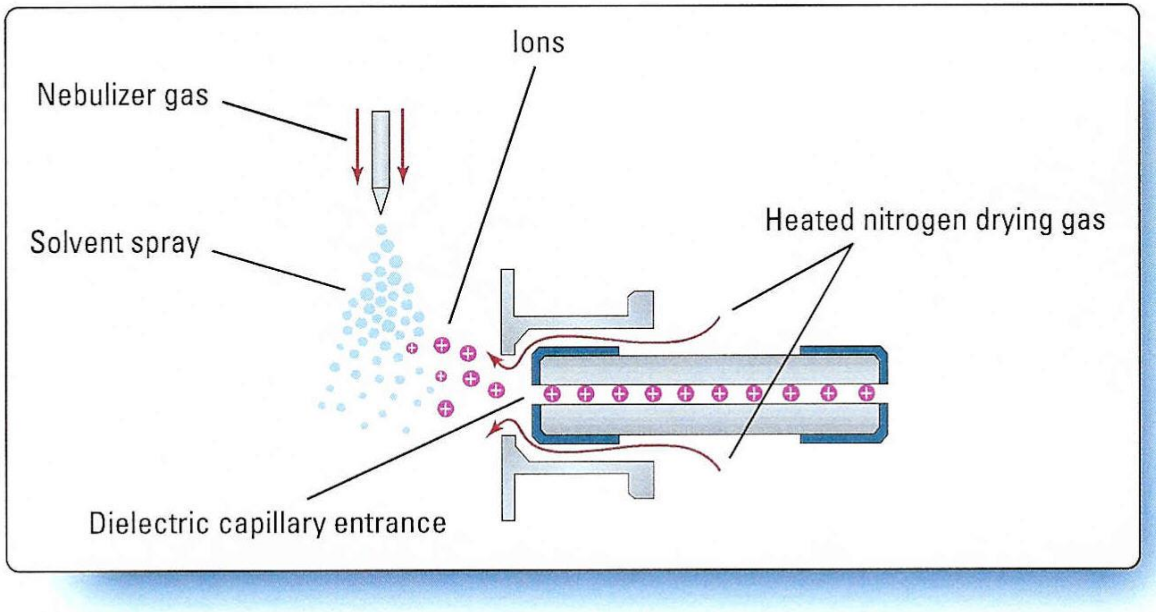

Figure 4. Electrospray ion source

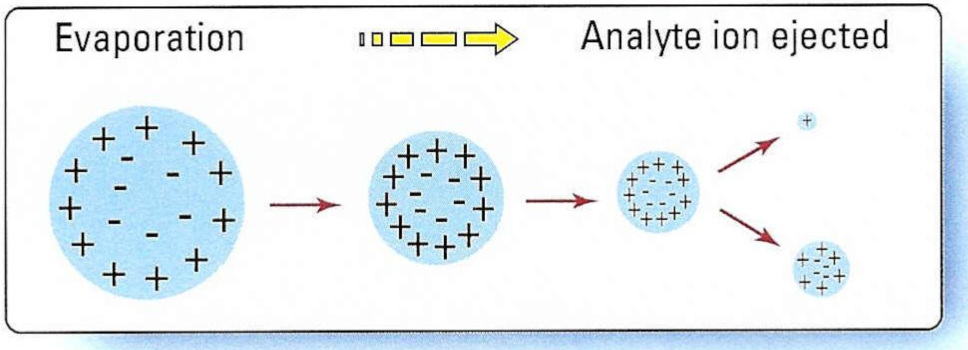

Figure 5. Desorption of ions from solution

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Electrospray

- Desolvation is assisted by a stream of a drying gas, usually nitrogen, being continually passed into the spraying region.
- As the droplets shrink, the charge concentration in the droplets increases. The repulsive force between ions with like charges exceeds the cohesive forces and ions are ejected (desorbed) into the gas phase.
- Analyte ions are obtained from these droplets which then pass through two differentially pumped regions into the source of the mass spectrometer.

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Electrospray – Disadvantages

- **Electrospray is not applicable to non-polar or low-polarity compounds.**
- **The mass spectrum produced from an analyte depends upon a number of factors and spectra obtained using different experimental conditions may therefore differ considerably in appearance.**
- **Suppression effects may be observed and the direct analysis of mixtures is not always possible. This has potential implications for co-eluting analytes in LC–MS.**

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Electrospray – Disadvantages

- **Electrospray is a soft-ionization method producing intact molecular species and structural information is not usually available.**

- **Electrospray sources are capable of producing structural information from cone-voltage fragmentation but these spectra are not always easily interpretable. Experimentally, the best solution is to use a mass spectrometer capable of MS–MS operation but this has financial implications.**

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Electrospray – Advantages

- **Ionization occurs directly from solution and consequently allows ionic and thermally labile compounds to be studied.**

- **Mobile phase flow rates from nl min^{-1} to in excess of 1 ml min^{-1} can be used with appropriate hardware, thus allowing conventional and microbore columns to be employed.**

Electrospray – Advantages

- Electrospray ionization, in contrast to the majority of other ionization methods, produces predominantly multiply charged ions of the intact solute molecule. This effectively extends the mass range of the mass spectrometer and allows the study of molecules with molecular weights well outside its normal range.

- For high-molecular-weight materials, an electrospray spectrum provides a number of independent molecular weight determinations from a single spectrum and thus increased precision.

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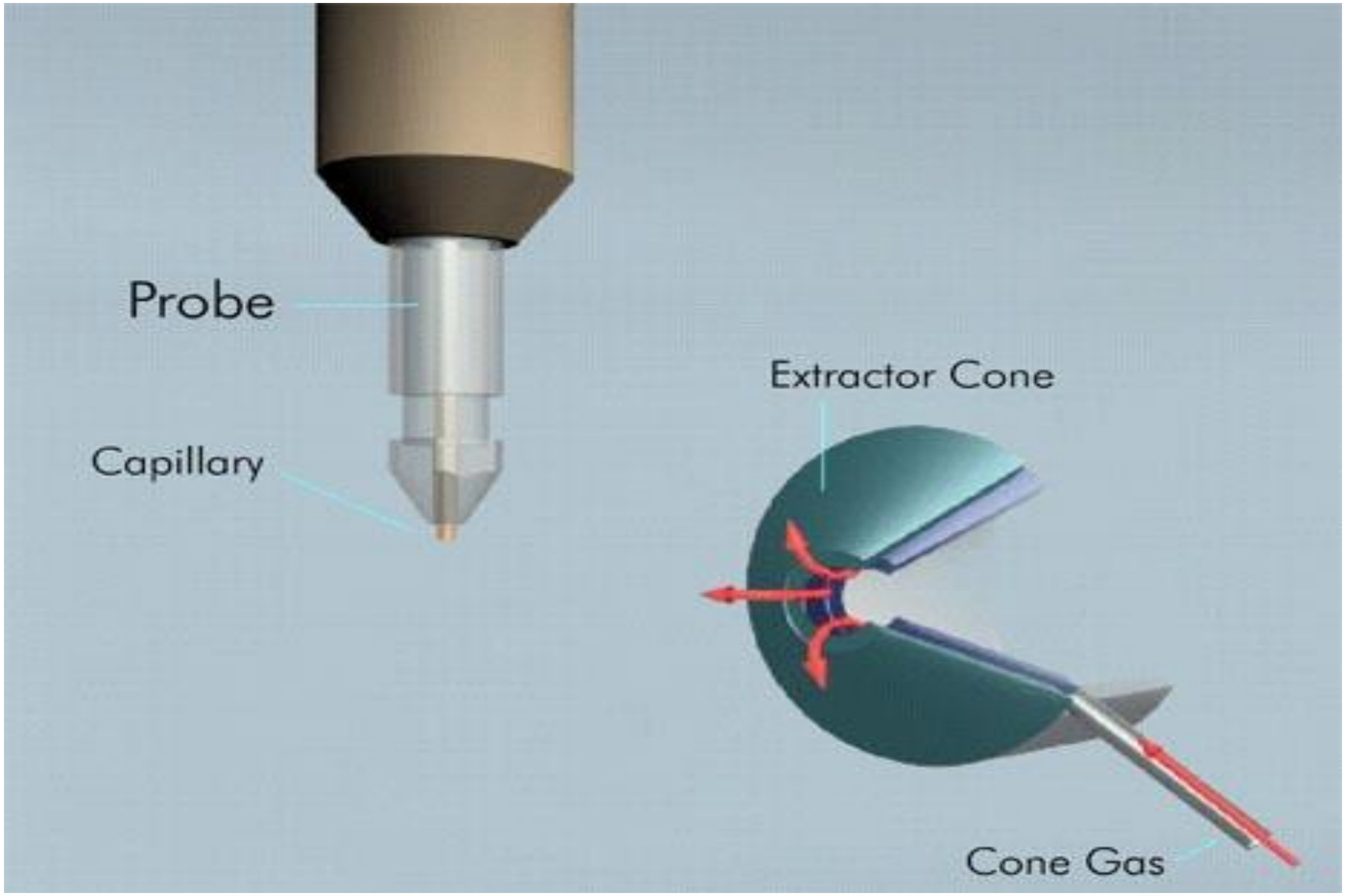
Electrospray interface - orthogonal



Assignment

- Read Chapter 28
- Homework Chapter 28: 2-12, & 15
- HW16 Chapter 28 Due 4/19/24

- Test 4