Chemistry 4631

Instrumental Analysis
Lecture 32
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
HPLC

**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).
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Instrumentation

Detectors

- Solute property
  - UV/vis
    - Fixed wavelength
    - Variable wavelength
    - Photodiode Array
  - Electrochemical
    - Amperometric
    - Coulometric
  - Fluorescence
- Bulk property
  - Refractive index
  - Conductivity
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
Vis - 350 – 700 nm
190 – 700 nm UV/Vis
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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.
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Instrumentation

Detectors - Absorbance Detectors

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

\[ A = \log \left( \frac{P_0}{P} \right) = \varepsilon bc \]

- \( A \) – absorbance of solution in the cell
- \( b \) – optical path – length through cell (cm)
- \( \varepsilon \) – molar absorptivity of the solute at a particular wavelength (l/mol·cm)
- \( C \) – molar concentration of the solute
- \( P_0 \) – light intensity from source
- \( P \) – light intensity transmitted
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Instrumentation

Detectors - Absorbance Detectors

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

<table>
<thead>
<tr>
<th>Source</th>
<th>Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercury</td>
<td>254, 280, 365, 405, 436, 546, 578 (most common 254)</td>
</tr>
<tr>
<td>Cadmium</td>
<td>229, 326</td>
</tr>
<tr>
<td>Zinc</td>
<td>214, 308</td>
</tr>
<tr>
<td>Magnesium</td>
<td>206</td>
</tr>
<tr>
<td>Deuterium</td>
<td>190-350 (continuous)</td>
</tr>
</tbody>
</table>
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).
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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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Detectors - Photodiode array (PDA)

Fig. 5.17. Diode-array detector optics. Reprinted with permission of Hewlett Packard.
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.
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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 $\pi$ 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$_2$H) decrease or quench fluorescence.
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Instrumentation

Detectors - Fluorescence Detectors
HPLC

Instrumentation

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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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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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. O₂ - common quenching agent.
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Detectors - Electrochemical Detectors

Application of electrical potential followed by measurement of current.

Common techniques are:
Voltammetry, amperometry, and coulometry.
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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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Detectors - Electrochemical Detectors

Working range is dependent on the nature of the working electrode, electrolyte used, pH of electrolyte.
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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.
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.
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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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Electrochemical Detectors
HPLC 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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Detectors - Refractive Index Detectors (RI)

Universal detector

Compare RI of the pure mobile phase with the presence of an analyte.
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Detectors - Refractive Index Detectors (RI)

Deflection type
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.
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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.
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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
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Instrumentation

Detectors

Liquid Chromatography / Mass spectrometry

Advantages:

- More definitive identifications
- Wide range of analytes can be studied
- Sensitivity (pg)
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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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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.
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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Figure 4. Electrospray ion source

Figure 5. Desorption of ions from solution
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.
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.
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, & 17
• HW Chapter 28 Due 4/25/18