BioInorganic Chemistry
Chemistry 2211a

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4) Physical methods used to study metallobiological molecules

1. **Introduction – Qualitative vs. Quantitative**
2. **Mass spectrometry – the answer to everything!**
3. **UV-visible spectroscopy**
4. **Separation of biological molecules - chromatography**
5. **Measurement of metal concentrations in biological molecules – atomic absorption spectrometry**

Recommended text Books

Principles of Bioinorganic chemistry by Lippard & Berg. TAYSTK QU 130.L765 1994 (On heavy demand (2-hour loan) at the Taylor Library and in the book store.)

Bioinorganic chemistry: a short course by Roat-Malone. QU130.R628b (On heavy demand (2-hour loan) at the Taylor Library and in the book store.)

Bioinorganic chemistry: inorganic elements in the chemistry of life: an introduction and guide by Kaim and Schwederski. (On heavy demand (2-hour loan) at the Taylor Library.)

The biological chemistry of the elements: the inorganic chemistry of life by da Silva and Williams. QU4.S586b 2001 (On heavy demand (1-day loan) at the Taylor Library.)

A good reference for this techniques unit is: Principles of Instrumental Analysis by Skoog, Holler and Nieman pub. Saunders. 5th Ed.

Rev: 16-1027-abC

(Brown text – for completeness NOT part of the course – no test questions from this text)

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Techniques used in BioInorganic Chemistry

Introduction – Qualitative vs. Quantitative

Aims

1. Proteins: Assessing purification
2. Proteins: Determining peptide mass
3. Determining the sequence of a peptide (1^0 structure)
4. Characterization of protein conformational status
5. Determining identity and concentration or number of metals present
6. Characterizing metal binding environments
7. Relating structure to function by measuring function for different structural forms

Techniques Bioinorganic Chemistry: Need to determine identity and amounts of proteins and the bound metals

1. Mass spectrometry - UV-visible spectroscopy – Circular dichroism spectroscopy; Emission spectroscopy; NMR; IR
2. Separation of biological molecules – Chromatography – Gas-liquid and Liquid-Liquid – for small molecules, amino acids and proteins in aqueous solutions
3. Measurement of metal concentrations in biological molecules – atomic absorption spectrometry – mass spectrometry

(We should omit: X-ray and synchrotron-based techniques (Sask – CLS) for metal binding properties – at the microscopic level – in vivo as well as in vitro)

The successes of bioinorganic chemistry rest on the availability of good analytical data to identify metals and ligands, and their respective stoichiometries, and on the chromophores in the metal binding site region (the metals and/or their ligands), because..?.

Overall, techniques to probe biological systems may be classified as: quantitative estimations of identity, mass, amount, concentration, stoichiometry, and size determinations, qualitative spectroscopic based information using available or added chromophores – that is using for the determination a property of the metal, or the protein bound to the metal, or of a ligand specially added to the metal-protein complex. Most techniques provide BOTH quantitative AND qualitative data.

Determination of the identity, mass, amount, concentration, stoichiometry, and size determinations

(A) Spectroscopic properties

1. Mass spectrometry – quantitative (numbers of metals; mass of peptide) and qualitative (concentrations)

2. UV-Visible absorption and emission- Backbone, aromatic amino acids (Phe, Tyr, Trp), co-factors (see spectra below)
   a. Typically measure in the regions: 200-300; 300-450; 400-700 nm
   b. Quantitative if standards available (absorbance and band maxima –identity); qualitative if no standards
3. Infrared/Raman- characteristic bond vibrations
4. Circular Dichroism (CD)- backbone conformation – α and β pleated sheet information: secondary structure
   a. Typically measure in the 180-300 nm region for structure
5. Fluorescence – fast, nanosecond lifetimes
   a. Intrinsic- Phe, Trp, Tyr (see below for examples)
   b. Attached dyes-
6. Phosphorescence - slow, millisecond lifetimes
   a. Mainly cofactors or prosthetic groups, but also metal-ligand states

(B) Chromatographic methods

1. Liquid-Liquid chromatography – for small molecules, amino acids, and proteins in aqueous solutions
   HPLC : high performance liquid chromatography – uses high pressures and packed columns
   Size exclusion chromatography (SEC) – liquid mobile phase – pores in the stationary phase

(C) Direct measurement of metals

1. Atomic absorption spectrometry – AAS, and other techniques
   Burns the sample in a flame and measures the colour … well, not quite that simple, we'll see

(A1) Mass spectrometry and, particularly, electrospray ionization mass spectrometry (ESI-MS)

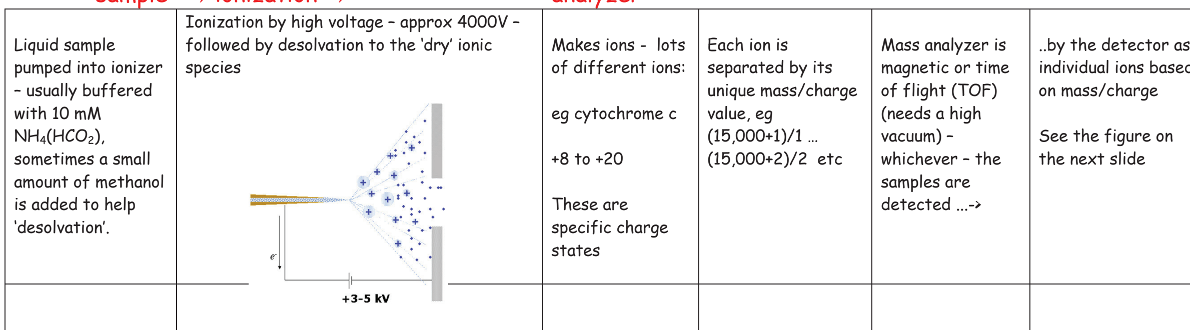
| Analyze solutions

No chemical modification needed, but is destructive! | Solutions can be quite dilute – to 1-10 μM

Qu. Calculate how many moles would be averaged if the signal for a 5 μM solution took 5 seconds to record at a flow rate of 3 μL/minute.
Ans:….. | Small volumes only needed, typically <100 μL (actually only 2-4 μL/minute flow rate but that's when you know where to look) | Must not contain appreciable amounts of alkali metals, especially, no Na^+ | Mass range 200 – 100,000 (!) .. only because…→ | Actually measure charge states – charge … the biological molecule is protonated first – see the cytochrome c example** |

**why does this help?

sample → ionization → mass analyzer

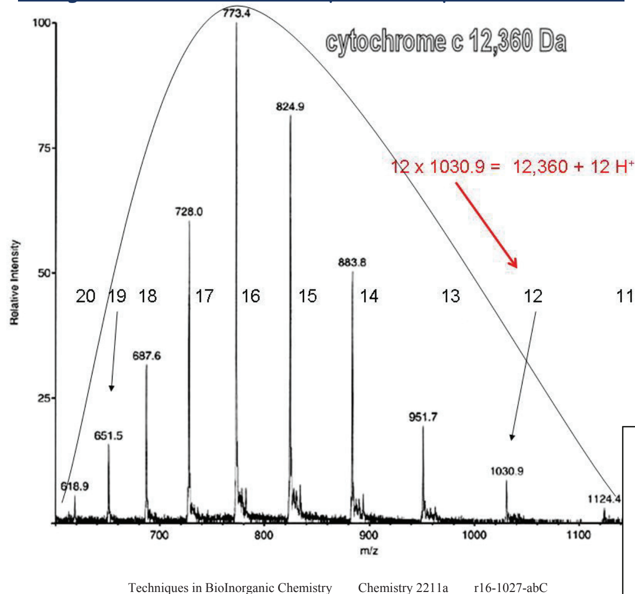


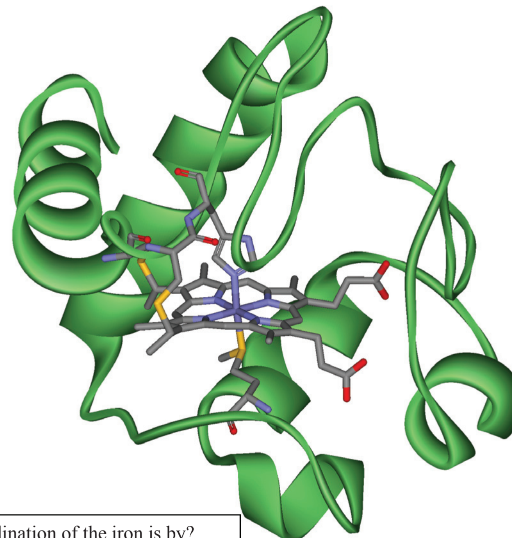
Liquid sample pumped into ionizer – usually buffered with 10 mM $NH_4(HCO_2)$, sometimes a small amount of methanol is added to help 'desolvation'.	Ionization by high voltage – approx 4000V – followed by desolvation to the 'dry' ionic species	Makes ions - lots of different ions: eg cytochrome c +8 to +20 These are specific charge states	Each ion is separated by its unique mass/charge value, eg (15,000+1)/1 … (15,000+2)/2 etc	Mass analyzer is magnetic or time of flight (TOF) (needs a high vacuum) – whichever – the samples are detected …->	..by the detector as individual ions based on mass/charge See the figure on the next slide

An example of a protein mass spectrum:

Cytochrome c is a heme protein with a mass of approx. 12,360 Da – it contains one heme molecule (heme c, not heme b of myoglobin and hemoglobin) with the iron coordinated in both 5th and 6th positions – the heme is locked in place. No ligand chemistry. This is a redox, electron exchange enzyme - the Fe switches between the ferrous and ferric states; the electron travels through the edge of the heme ring.

Charge states as measured by ESI-MS positive ion mode



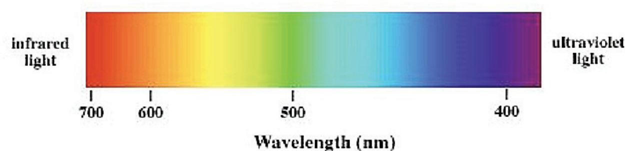


And, coordination of the iron is by?

Test the calculation for yourself – check the mass suggested by several charge state peaks…..

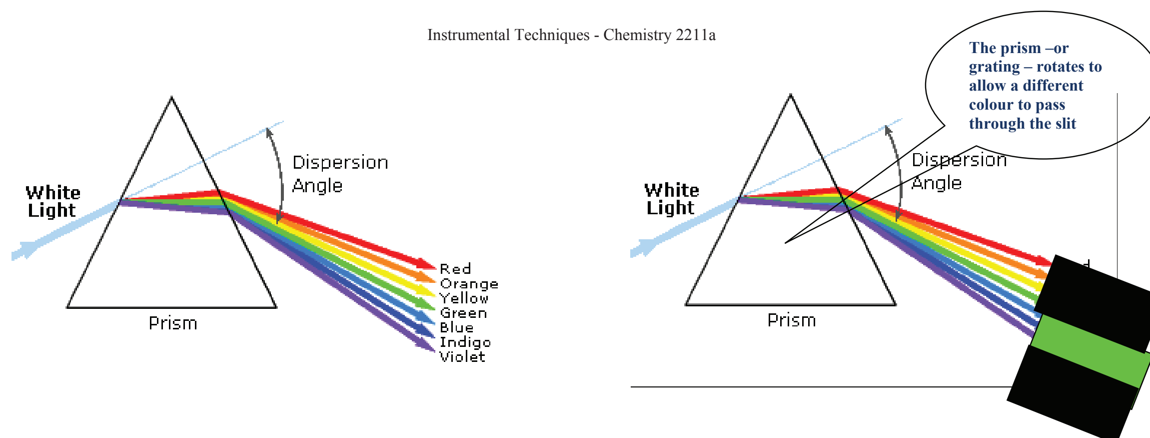
(A2) Optical spectroscopy …

Electromagnetic Spectrum



•	γ ray	< 1 nm ($10^4 – 10^6$ eV)
•	X ray	1 – 50 nm ($10^2 – 10^4$ eV)
•	far UV	10 – 200 nm (10 A = 1 nm)
•	near UV	200 – 350 nm (UVC – 180 – 280 nm; UVB – 280 -315 nm)
•	Visible	350 – 800 nm (UVA – 315-380 nm)
•	near IR	0.8 µm (800 nm) – 2,500 nm
•	IR	2.5 µm (2500 nm)
•	Microwave	400 µm – 30 cm (400,000 nm !)
•	radio wave	100 cm and above

Blue line: intensity at that λ
Red line: damaging effect vs λ



So how is the light used to make measurements?

Every Spectrometer uses a Monochromator = Dispersion element plus slit

Some important details:

speed of light = wavelength x frequency

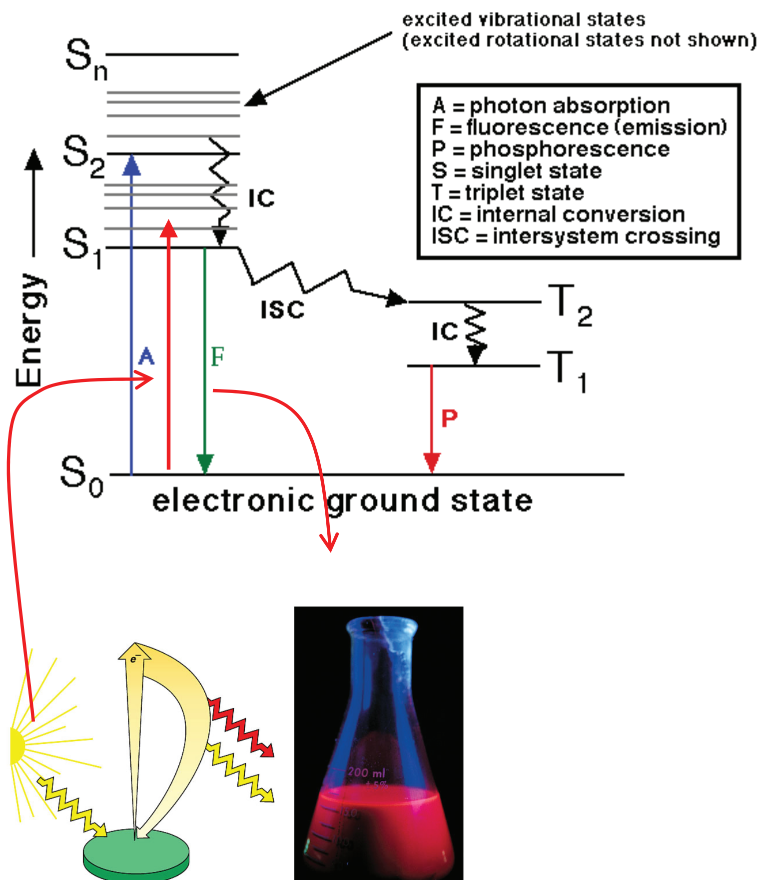
$$c = \lambda \upsilon$$

Wavenumber, ______, is the number of waves per cm. Units are cm^{-1}.
700 nm red light = 1.43×10^4 cm^{-1} 420 nm violet light = 2.38×10^4 cm^{-1}
Velocity of light changes in different substances.

Index of refraction of a substance, $\eta = c / v$
Speed of light in vacuum (c) divided by the speed of light in that substance.
(c= approx 3.0×10^8 m/sec)

Absorption vs. Emission of Light

Key features:
S_0 – the sum of all the electrons in the molecule – a state
$S_{1\rightarrow n}$ – the same sum but with one electron promoted to a higher energy ($1 \rightarrow n$) empty orbital – so a higher energy state
A – absorption is the endothermic energy required to promote an electron - - absorbs light to obtain this energy. Only light of exactly the correct energy can be absorbed – the energy gap must match the wavelength of the light
F & P – energy is given off – emitted when the electron drops back to the ground state. Energy seen as light = emission.
So for chlorophyll –looks **green** – so absorbs red (670 nm) leaving blue and yellow, the fluorescence (695 nm) tells us = **red**! But plants don't fluoresce (much) – why not????



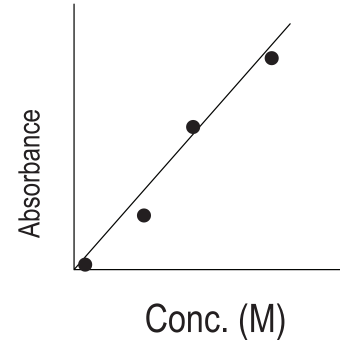
Quantitative measurements…

The Beer-Lambert Law

$$A = \varepsilon \times b \times c$$

where ε = molar extinction coefficient ($Lmol^{-1}cm^{-1}$)
or molar absorptivity ($Lmol^{-1}cm^{-1}$)
b = path length (cm)
c = concentration (mol/litre)

Units: A - unitless
b - cm
c - M



Therefore, absorbance (A) is directly proportional to the pathlength (b) and the sample concentration (c).

Can calculate concentrations given A, b, and ε --- this is a molar concentration.

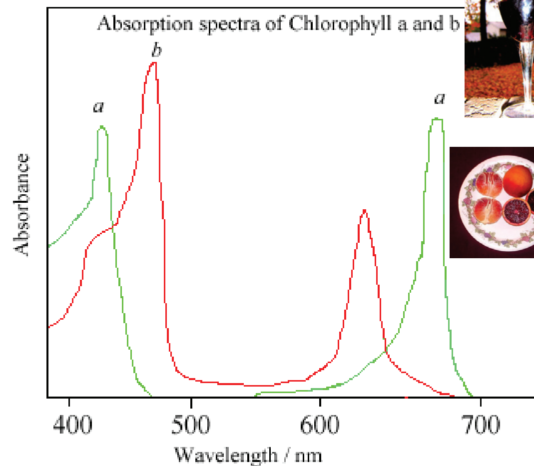
Theory used in atomic absorption spectrometry BUT the standards in AAS are in ppm so the ‘c’ is in ppm

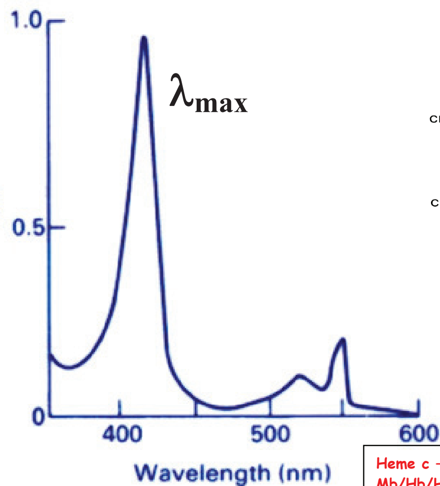
Remembering that 1 mL of water of a dilute salt has a mass of close to 1.0 g. So, 1 ppm is close to 1 μg/g (a 10^6 difference)

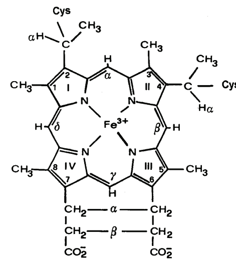
Must be able to recognize these absorption spectra – which comes from which molecule (well the heme c vs Chl a)?

Hemes high at 400, low at 550, Chl high in BOTH 400-450 AND near 700 nm – and what about that red colour in the Fall? 2 effects – loss of Chl – so carotenoids (yellow) are left and the leaf makes the bright red **anthocyanin** – which aborbs harmful blue light that might turn photosynthesis back on….

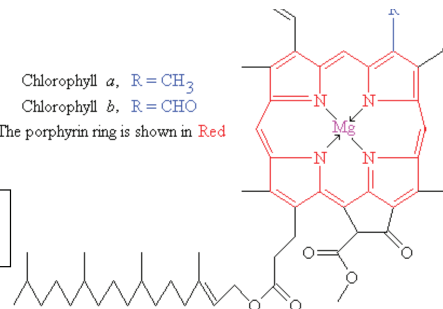
In humans: a powerful antioxidant – hence the ‘eat coloured vegetables and fruit’ statement (reported to reduce/cure: cancer; aging and neurological diseases;







Heme c – notice the difference wrt heme b in Mb/Hb/HRP) – esp CYS on 2 of the pyrroles.



9.7. Absolute absoption spectrum of reduced cytochrome c.

(B1) An Introduction to Chromatography: Major chromatographic techniques

Methods include:

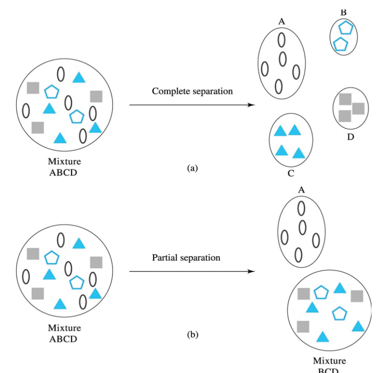
1. liquid/liquid (typically "HPLC" – with 'liquid' coated particles) separates by differential affinity of the solute with liquid coated particles (the Stationary Phase) and the solvent (the Mobile Phase). Molecule attracted more by solvent off FIRST - Molecule attracted by coating off LAST. Change solvent polarity to change separation. Polarity of the solvent is opposite polarity of the coating – in Reverse Phase (what most use) the polarity of the solvent is high – water.
2. liquid/solid – here the particles are not coated – the analyte adsorbs – or sticks to the surface of the particle
3. ion-exchange – the particles have one charge – so ions of the opposite charge will stick preferentially and slow down. Use cation or anion exchange columns.
4. size exclusion – small molecules will stick and come off last separates by size - Small off LAST - Large of FIRST. Choose 'pore' sizes to select the working range.

All separations can be described using the Partition Coefficient K_D that describes:

(Concn in the Stationary Phase liquid coating OR the small pores in SEC)/(Concn in the Mobile Phase)

In HPLC Reverse Phase the coating is a nonpolar "organic liquid" (C18 chains) and the mobile phase is "inorganic" polar (water, methanol, acetonitrile).

The basics of chromatography



Separations isolate the analyte from potentially interfering constituents. An interferent is a chemical species that causes a systematic error in an analysis by enhancing or attenuating the analytical signal or the background.

Here we see in (a) complete separation of 4 analytes. In (b) just separation of one.

Different separation methods are used – with different types of column chromatography – different mobile phases – different detectors.

The LC technique uses a mobile phase (the eluent) and a stationary phase (the packing).

Through differential interaction between each analyte in the mixture (A & B on the RHS) and the mobile & stationary phases, components in the mixture travel through the column at different rates = separated in time.

From the thesaurus "Any of various techniques for the separation of complex mixtures that rely on the differential affinities of substances for a gas or liquid mobile medium and for a stationary adsorbing medium through which they pass, such as paper, gelatin, or magnesia." – The stationary phases in LC are either silica, polymer or dextrose.

Mobile phase (M) and stationary phase (S)

S-fixed in place either in a column or in a planar surface

M-moves over or through the stationary phase, to carry the analyte with it.

The diagram shows the separation of a mixture of components A and B by column elution chromatography. Generally, the more the interaction with the stationary phase the slower the elution (eg B). The interaction can be related to time spent in the stationary phase, which is not moving. The time spent in the mobile phase moves all analytes at the same rate. The time in the mobile phase is the same for all analytes, all that changes is the time in the stationary phase.

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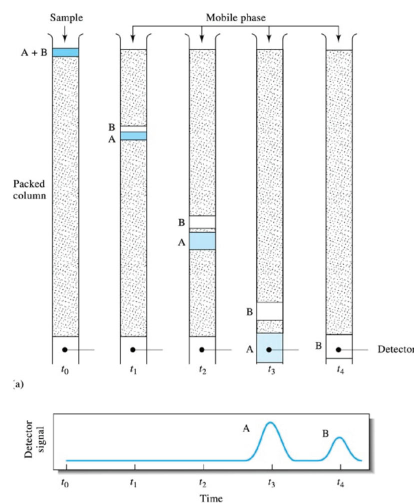


We will focus on chromatography that uses a liquid mobile phase (Liquid-Liquid chromatography (LLC) or Liquid-Solid chromatography LSC, etc.) rather than a gaseous mobile phase (GC, GLC, GSC)

In LLC (typically what is used in biological analyses) the elution process is the key to everything.

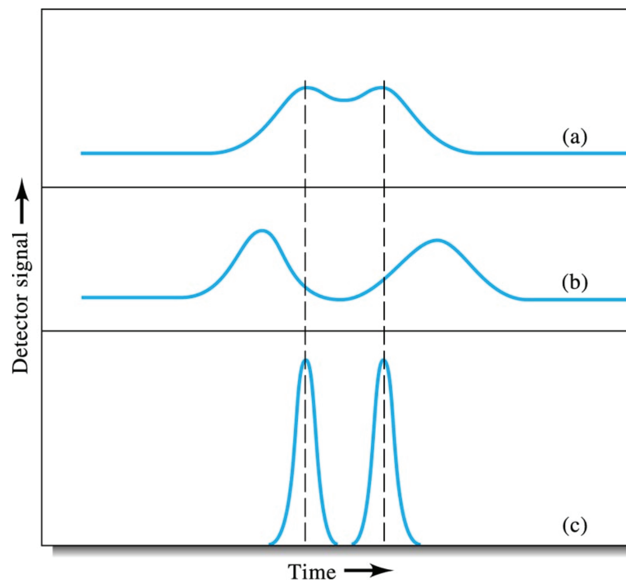
This is a process in which species are washed through a chromatographic column by adding fresh solvent, the eluent.

What is everything in chromatography?

Everything is in the …… resolution.

Some details -

An eluent is the solvent used to carry the components of a mixture through a stationary phase.



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Chromatogram

The analyte concentrations are represented by the measured signal vs. elution time or elution volume

In this two-component chromatogram we see two methods of improving separation of two overlapping peaks:

increase band separation or

decrease in the widths

In both cases resolution, R – see later-, is increased.

(Many detectors can be used – commonly a UV-visible absorption spectrometer)

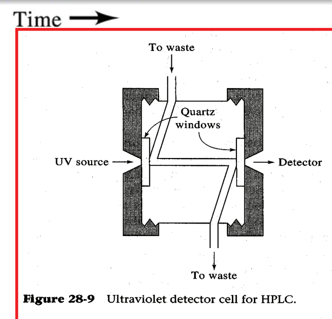
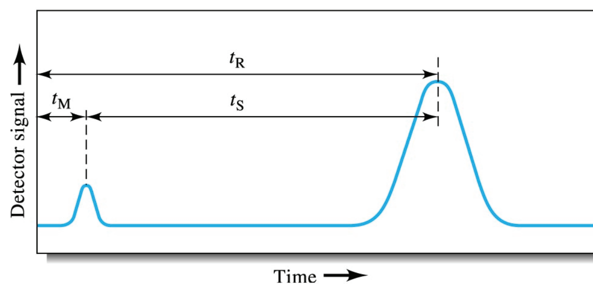


Figure 28-9 Ultraviolet detector cell for HPLC.

How do we quantify resolution then?

Well, how do we quantify band maxima as that's what we observe?

In Liquid-Liquid or Liquid-Solid chromatography - everything depends on the relative distribution of the analytes between the mobile phase (the eluent) and the solid (in LSC) or stationary phase packing (in LLC).



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Distribution constants

Solute A is distributed between the mobile and stationary phases:

Moles $A_{mobile} \leftrightarrow$ Moles $A_{stationary}$

(remember – prod/reactant)

$$K = \frac{C_S}{C_M}$$

the equilibrium constant, K, is called the distribution constant, the partition ratio, or the partition coefficient, which is equal to the ratio of its (the analyte's) molar concentration in the stationary phase to its molar concentration in the mobile phase:

note the order – Conc in stationary phases over Conc in mobile phase.

The average concentration of analyte in the stationary phase can be though of in terms of volumes and then in terms of velocity through the column then in terms of retention time.

Retention time – if the analyte does not elute with the solvent – then it is delayed – so, it is 'retained'. The retention time t_R is the time between injection of a sample and the appearance of a solute peak at the detector of a chromatographic column.

The solute spends a time t_s in the stationary phase and t_m in the mobile phase..

Therefore, the total time from start to finish is:

$$t_R = t_s + t_M$$

The average linear rate (the velocity the eluent moves through the column) of a analyte (v) and the mobile phase (u) are:

$$\bar{v} = \frac{L}{t_R} \qquad u = \frac{L}{t_M}$$

For the two species A and B, the Retention Factor is defined as: $k_A = (t_{R(A)} - t_M)/t_M$

the selectivity factor is defined as: $\alpha = \frac{k_B}{k_A}$ always >1

From the equation of the retention factor, $\alpha = \frac{k_B}{k_A} = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$

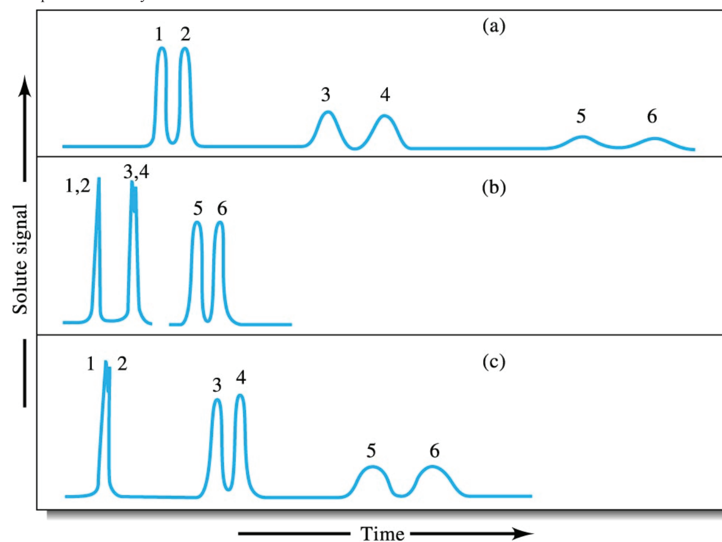
Intuitive – compare the normalised rates of passage through the column – remember t_M – is the system peak.

The general elution problem

This the compromise between elution time and resolution

The general elution problem arises whenever chromatograms are obtained on samples that contain species with widely different partition ratios.

When conditions are such that good separations of the more strongly held species are realized, lack of resolution among the weakly retained species is observed.



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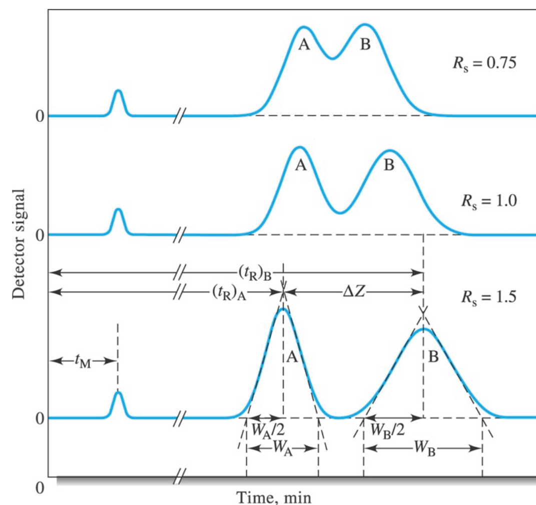
Conversely, when conditions are chosen that give satisfactory separations of the weakly retained compounds, severe band broadening and long retention times are encountered for the strongly bound species.

The general elution problem is often solved in liquid chromatography by gradient elution; temperature programming serves the same purpose in gas chromatography.

Calculating resolution, R - need to memorize this diagram

The resolution of a chromatographic column is a quantitative measure of its ability to separate analytes A and B



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$$R_s = \frac{\Delta Z}{\frac{W_A}{2}+\frac{W_B}{2}} = \frac{2\Delta Z}{W_A+W_B} = \frac{2\left[(t_R)_B-(t_R)_A\right]}{W_A+W_B}$$

Know this - $R = 2\,[(t_{RB}-t_{RA}) / (W_A+W_B)]$

From the following figure, it is evident that a resolution of 1.5 gives an essentially complete separation of A and B – that's at approximately 3/2 separation. Why should the W's be very similar do you think?.

How to change the selectivity factor to increase R – important to know

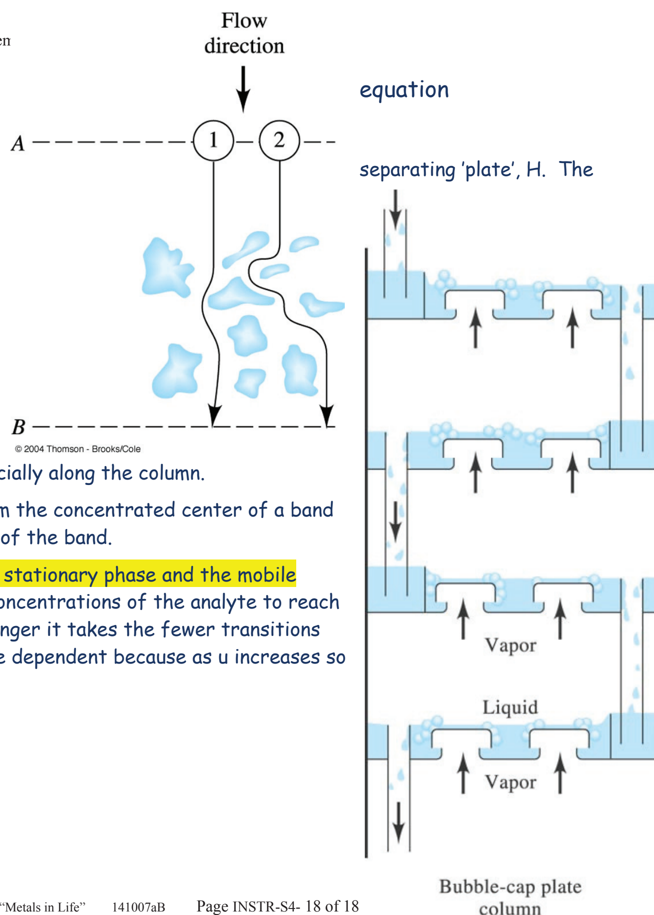
Increase α while maintaining k in the range of 1 to 10.

1. By changing the composition of the mobile phase
2. By changing the composition of the stationary phase
3. By using special chemical effects (complexation)

Theory of band broadening---the van Deemter equation

H=A+B/u+Cu

Provides a theoretical parameter, the height of a single separating 'plate', H. The smaller H the better, because smaller plates relate to a more frequent average separating activity (ie on and off the stationary phase), and where A, B and C are coefficients of multiple path effects, longitudinal diffusion and mass transfer



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A - paths Molecule 2 will arrive at B later than molecule 1 because it has taken a longer path

B/u - The longitudinal diffusion term

Diffusion is a process in which species migrate from a more concentrated part of a medium to a more dilute region – especially along the column.

Longitudinal diffusion results in the migration of a solute from the concentrated center of a band to the more dilute region on either side – behind and in front of the band.

Cu = average of ($C_s u + C_M u$) The mass transfer terms of the stationary phase and the mobile phase. The "C" term accounts for the time it takes for the concentrations of the analyte to reach equilibrium between the mobile and stationary phases. The longer it takes the fewer transitions possible during the flow through the column. This is flow rate dependent because as u increases so time in the column decreases.

Liquid-Liquid Chromatography

The column contains particles, like the SEC columns, but the outside of every particle is coated with a 'liquid'.

The solute molecules 'dissolve' into the liquid. The molecules that 'like' the liquid on the Stationary Phase more then the liquid of the Mobile Phase, are retained longer.

So, by changing the type of coating, it is possible to selectively delay specific types of molecules.

LLC is the correct name.

The objective is to decrease the band width as this increases the band height (same amount of compound) and therefore increases the resolution, R..

Choose:

- fast flow, less time on the column (less random diffusion)
- highly symmetric, very small particles or beads to reduce the number of different pathways - -> capillary flow

Review the van Deemter equation from above and see why this helps (the objective is the keep H small).

If the mobile phase is polar (eg water) then this is called Reverse Phase HPLC,

if the mobile phase has a low polarity (ether, hexane) then this is called Normal Phase HPLC.

The packing coating has the opposite polarity: nonpolar in Reverse Phase and polar in Normal Phase.

Like dissolves like so in Reverse Phase HPLC nonpolar analytes dissolve in nonpolar coatings and are slowed down. Polar analytes are not slowed down as much and elute first in Reverse Phase LC.

Size exclusion-1 – separates by size

Small off LAST Large of FIRST

In addition to First off is largest – there is an almost linear relationship between LOG_{10} (MolarMass) and Retention Time.

See the graph no need the memorize the molecules BUT you do need to memorize the trend and how to calculate the Molar Mass given the graph or the data (mass and retention time – so check you Log_{10} calculator key and the data here)

At low Retention Times – "Total Exclusion", means molecules this big simply flow down the column with the mobile phase, no separation – too large for pores.

Then there is the region of "Chromatographic Separation", the K_D region.

Then the region "Total Permeation", for these molecules, small ones of course, the pores are large enough that these molecules become trapped for ever! No separation.

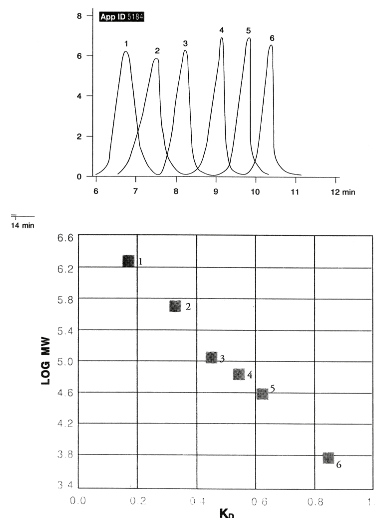
Used to separate molecules that are mixed together with masses roughly 1-10x each other – ie 1,000 to 10,000; 5,000 to 50,000 because the pore size in the packing can be changed to match the chromatographic separation centre. Choose the pore size to match the mid-point you want to separate.

Size-Exclusion Chromatography of Dextrans on Biosep-SEC-S4000

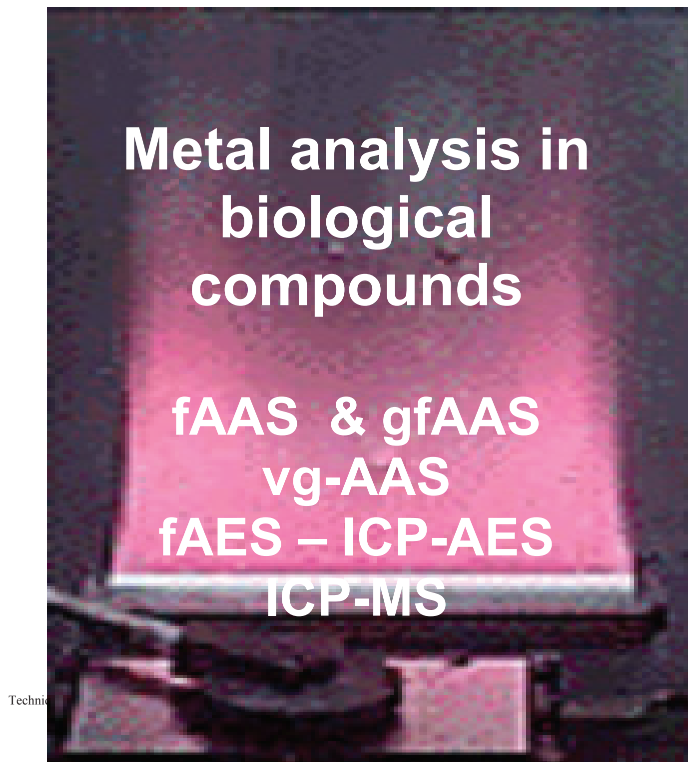
Dextrans of molecular weight ranging in size from 6000 to 2,000,000 daltons were separated on a BioSep-SEC-S4000 column to determine their separation profile. The plot of log MW vs. K_D showed an excellent linear relationship with a correlation coefficient r^2 of 0.993.

Column: BioSep-SEC-S4000
Dimensions: 300 x 7.8mm
Order No.: 00H-2147-K0
Mobile Phase: Water
Flow Rate: 1 mL/min
Detection: RI
Sample: Dextrans, 10µL
1. Blue dextran (2 x 10^6 daltons)
2. 500,000
3. 110,000
4. 70,000
5. 40,000
6. 6,000

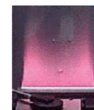
Dextran samples run individually, then overlaid



C. Metal content
– AAS, AES, ICP-AES, ICP-MS



Instrumental Techniques - Chemistry 2211a BioInorganic Chemistry



AAS-AES
Atom source chosen depends on the sample and the metal - flame, vapour generator, furnace

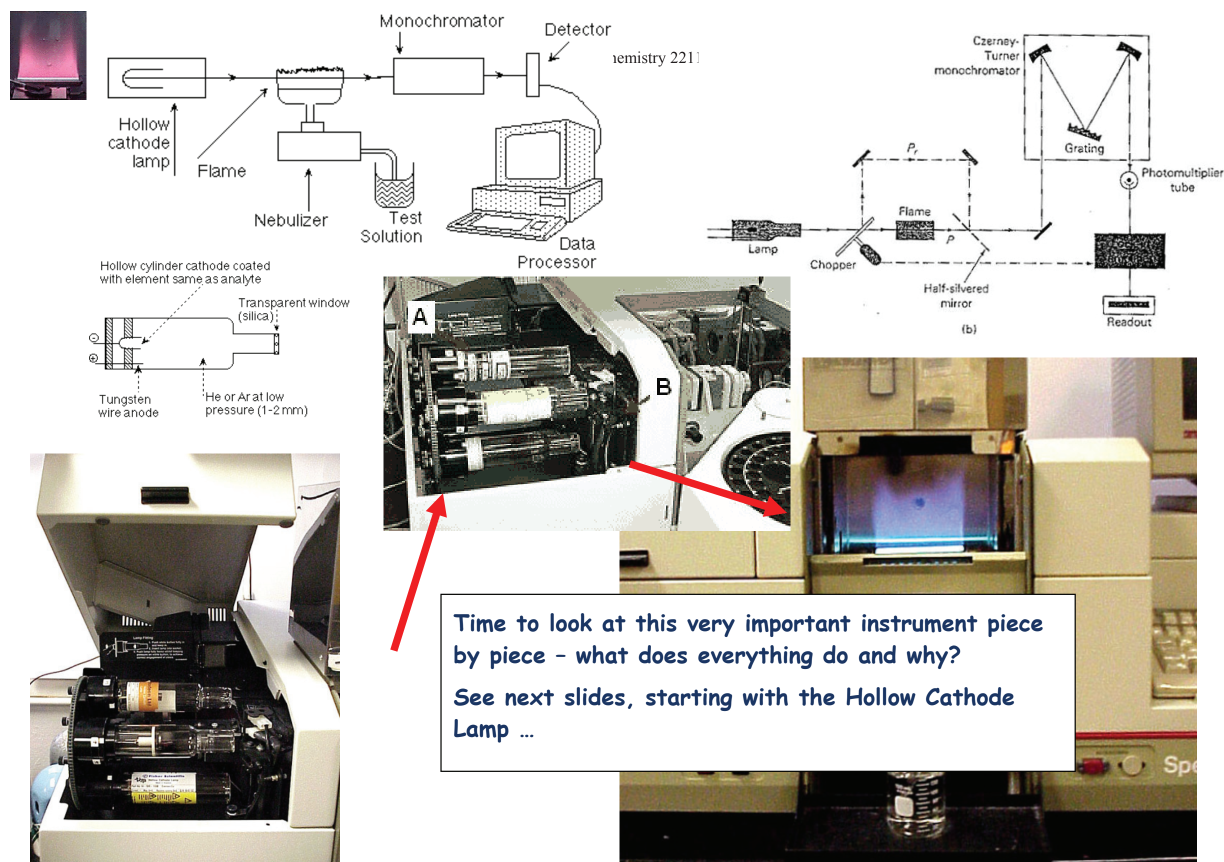
Detection method depends on metal:
absorption if atoms alone are produced in the flame;
emission if excitation results in excited states

- Sample **solution sprayed or aspirated** as fine mist into flame.
  Conversion of sample solution into an aerosol by nebulizer (scent spray) principle.
  No chemical change in the sample in this stage.
- **Heat of the flame vaporizes sample** constituents. Still no chemical change.
- By heat of the flame + action of the reducing gas (fuel), molecules & ions of the sample species are **decomposed and reduced to give ATOMS**. *e.g.* $Na^+ + e^- \rightarrow Na$
- Heat of the flame causes **excitation of some atoms** into higher electronic states, M* or **ionization** M^+. Ionization occurs most readily got group I, alkali metals, esp. heavy alkali metals, like Cs (ionization energy drops down the group).
- Excited atoms **revert to ground state** by **emission** of light energy, h , of characteristic wavelength; measured by detector.

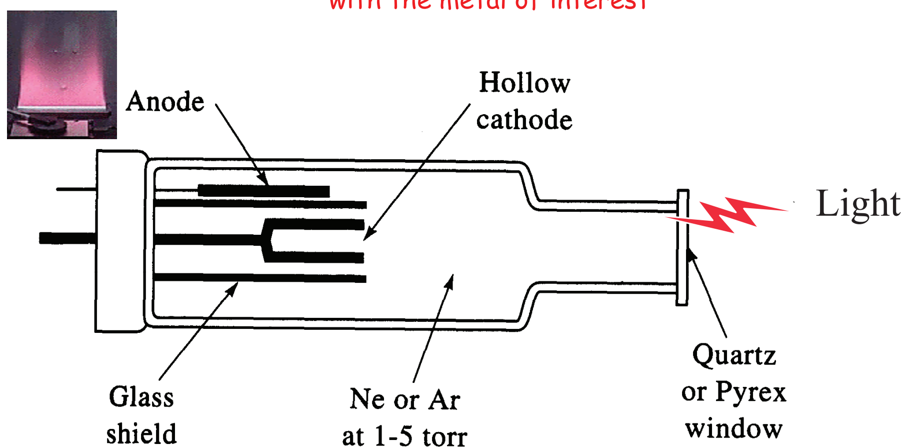
Atomic Line Spectra

Atoms in the vapour state give **LINE SPECTRA** (Not band spectra, because no covalent bonds hence no vibrational sub-levels to cause broadening).

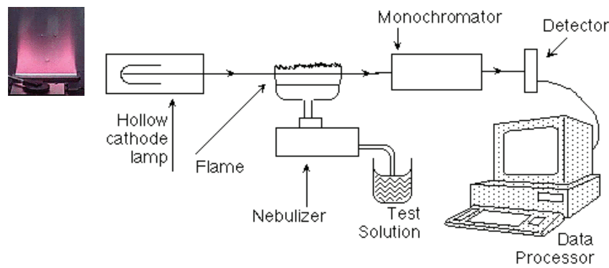
■**In summary ….** AA Spectroscopy requires atoms to be in their gas phase.
■As a result, the sample must be desolvated, vapourized and atomized at high temperatures**
■
** BUT the atom must not be in its excited state or worse, ionized for AAS.



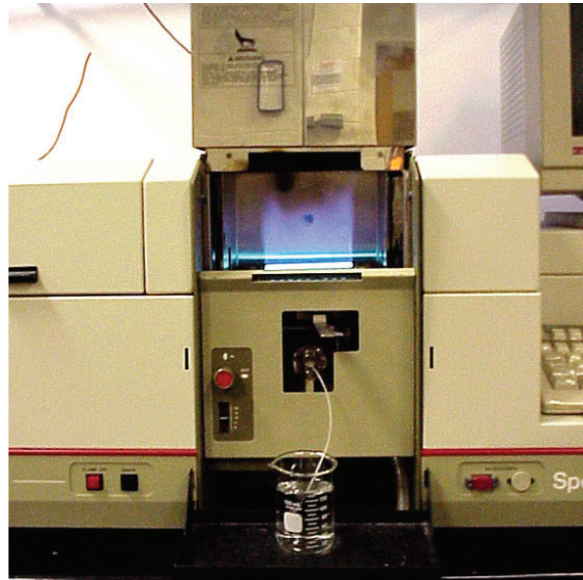
The hollow cathode lamp packed with the metal of interest

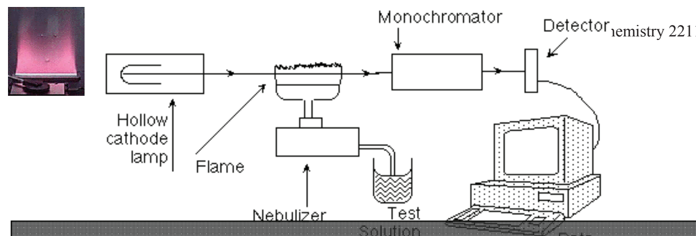


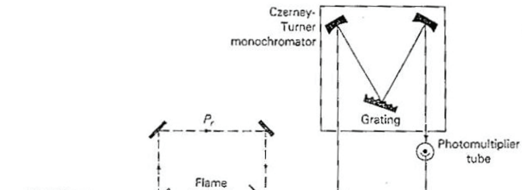
Ne – red colour; Ar – blue colour; even when no metal is left!



The flame







Hollow cathode lamps
Provide metal-specific light
Only as a line spectrum

Flame to make atoms
from the sample

Monochromator
to find the line

Detector to measure
light intensities

Compare the intensities with water (the blank) and the sample
What's absorbed must be absorbed by the metals in the sample
Use standards to find out the concentration.
Requires the Beer-Lambert Law to work – need calibration lines – Absorption vs. Concentration

Key points from this unit	
1	Qualitative vs Quantitative analysis – know examples of both – be able to explain which can be used for the answer to a specific question.
2	If we use SEC, what are the constraints? How do we obtain a molar mass for an unknown? Can you calculate that mass given the data from other molecules?
3	Electronic spectroscopy is used in UV-visible absorption and fluorescence as well as phosphorescence (seen any fireflies recently? What do they use?). What is the wavelength of blue light? Of red light? Where does chlorophyll absorb most strongly? Why? Where do heme proteins absorb greatest? What about the nucleic acids A, G, U and C? Why does sunbathing carry such a risk of cancer? Does fluorescence lie at higher or lower energy of the absorption band? For example, TRP –TYR and PHE – what type of amino acids are these? Which part of these molecules absorbs the lowest energy light? The wavelength range is where? (Very important to know this.) What does it mean to say we see 'fluorescence' or 'phosphorescence'? How we connect the speed of light with the wavelength and frequency of light? How is absorbance connected with concentration of the absorbing species?
4	AAS How does atomic absorption spectrometry determine concentrations? Draw a simplified diagram of the instrument. What are the key components? Why is the hollow cathode lamp so important? What happens in the flame? What is actually measured? What is 'colourless' to the AAS?