Metallomics: Biomolecule Isolation and Identification

Roat-Malone's Chapter 3

Supplementary Reading:
Mounicou, S.; Szpunar, J.; and Lobinski, R. *Chem. Soc. Rev.*, 2009, *38*, 1119-1138

Prof. Arthur D. Tinoco
University of Puerto Rico, Rio Piedras Campus

General Approaches to Metallomics

1. ***In vivo***
   - Methods to analyze metal-containing biomolecules
   - Methods to characterize their bioactivity

2. ***In vitro***
   - Methods focused on DNA and protein mutations and studying the impact this may have on the metallome
   - Methods to analyze metal-containing biomolecules
   - Methods to characterize their bioactivity

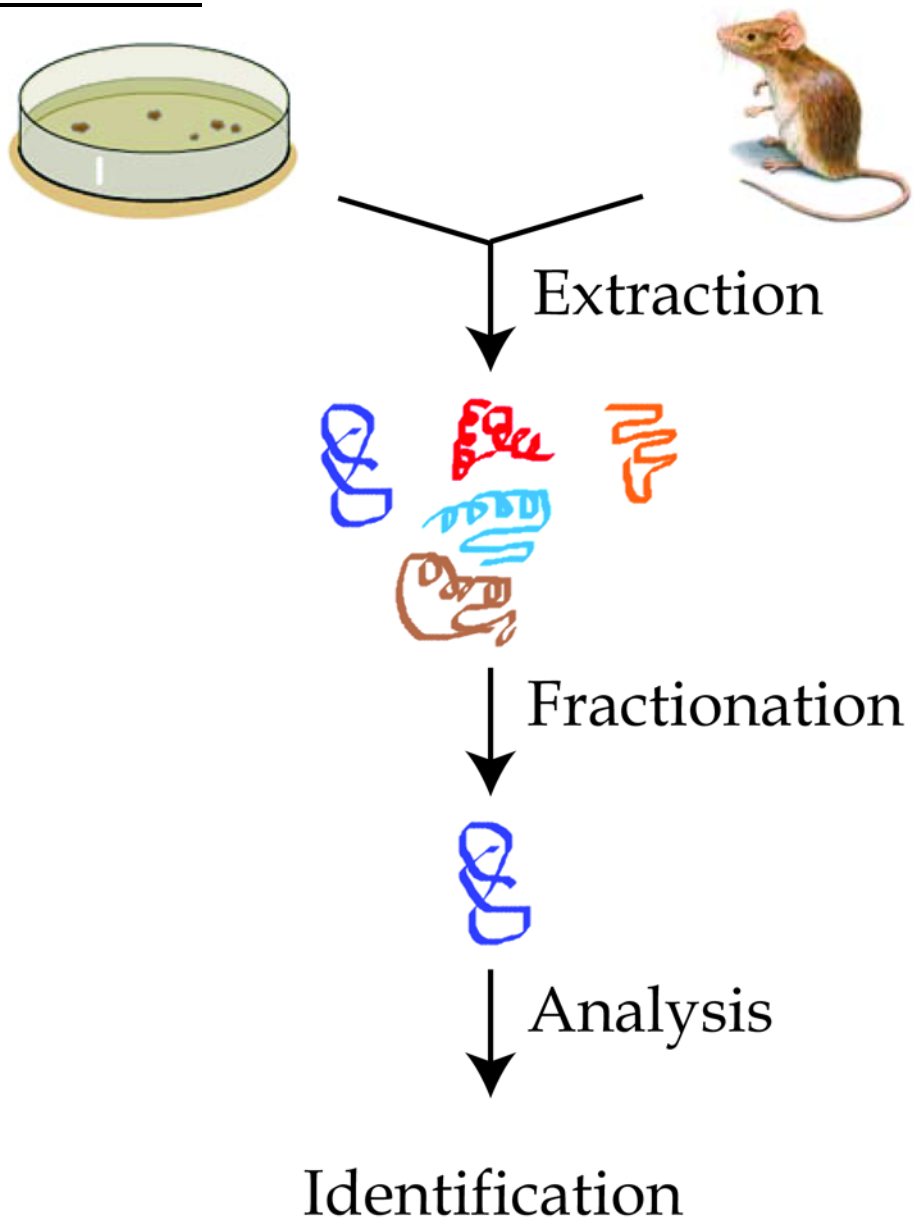
3. ***In silico*** (bioinformatics)

   - Methods focused on the connection between genome and protein sequences and metal binding and functionalization

To isolate or not to isolate?

That is the question!

Standard Workflow for Isolating Biomolecules



1. Extraction

A. Isolate molecules from blood, cells, or from tissue of an organism using a protocol specific to the type of molecules that you are interested in (i.e. lipids, nucleic acids, proteins, etc.)

There is no "one size fits all" when it comes to extractions.

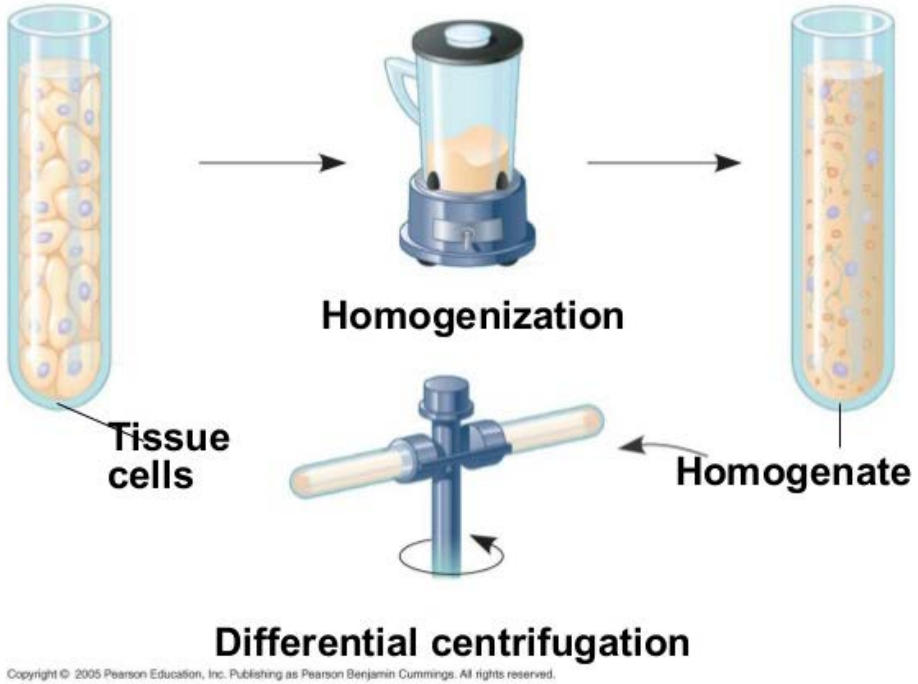
To get an adequate "*in vivo*" picture may require:

- **Detergents or organic solvents** to solubilize molecules of interest
  - Drawback: Can denature proteins
- **Chelators like EDTA** to inhibit metalloproteins (metalloproteases)
  - Without inhibition, the presence of active forms of these species may effect the levels of molecules of interests
- **Molecular weight cut-off filters** to isolate molecules in a certain size range

1. Extraction

B. Sample must be homogenized and if working with cells (in vitro), must be properly lysed

- Yields crude extract
- Must eliminate cellular/organ debris by centrifugation



2. Fractionation (purification)

- Use a biochemical property to purify the molecule of interest

- Usually requires more than one property for high purification (2-dimensional or multidimensional fractionation)

A. Column Chromatography

Technique that takes advantage of differences in molecular charge, size, binding affinity, and other properties. Consists of:

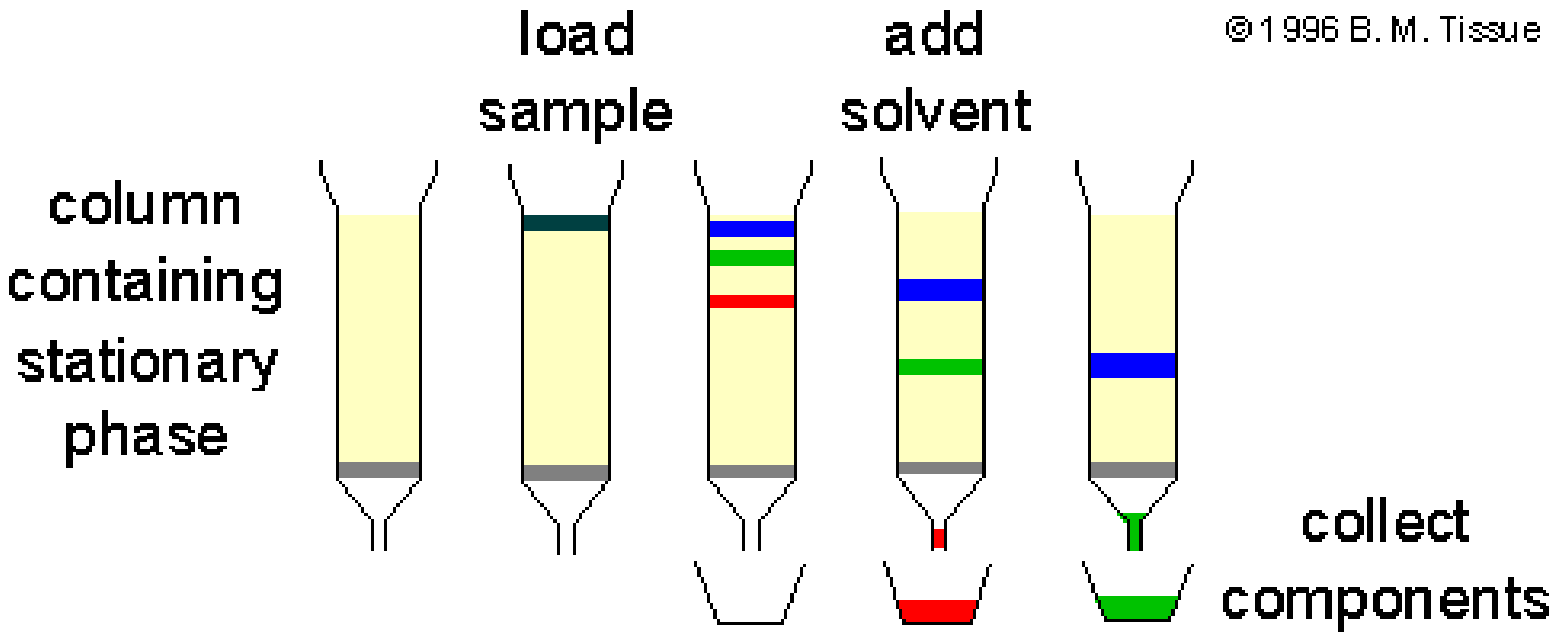
- **Stationary phase**: Porous solid matrix with appropriate chemical properties

- **Mobile phase**: A buffered solution percolates through the stationary phase and is not always constant

- The complex sample is layered on top of the column and the mobile phase causes the molecules to migrate faster or slower depending on their properties.

A. Column Chromatography

Technique that takes advantage of differences in molecular charge, size, binding affinity, and other properties. Consists of:



I. Ion Exchange Chromatography

a. Technique based on molecular charge (either cation or anion exchange)

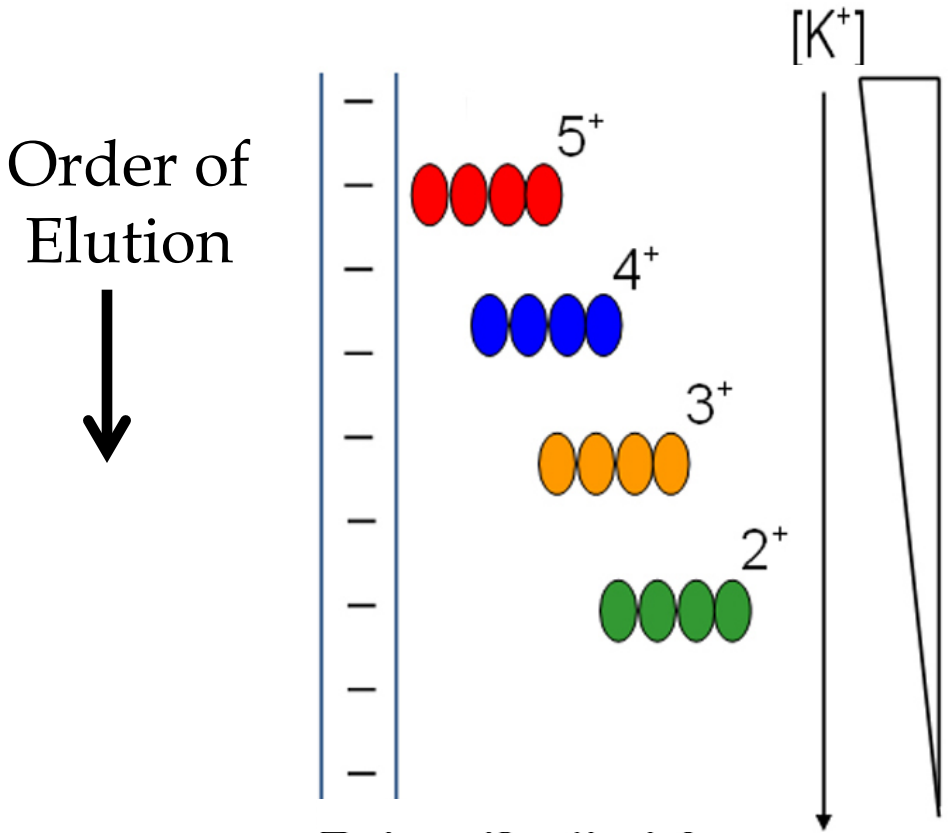
b. Stationary phase has charged groups

- Cation exchange: Charged groups are negative
- Anion exchange: Charged groups are positive

c. **Mobile phase** is initially the buffer and then transitions to a concentration gradient of charged ions opposite in charge to the stationary phase

- Cation exchange: K^+
- Anion exchange: Cl^-

i.e. Cation Exchange Chromatography



- Negatively charged molecules do not bind
- Elution in order of low to high positive charge

II. Affinity Chromatography

a. Technique based on binding affinity

b. **Stationary phase** contains a covalently attached chemical group

c. **Mobile phase** is initially buffer and then transitions to a concentration gradient of an agent with affinity for the chemical group or the free chemical group itself

d. Molecules will elute as the concentration of the agent or the free chemical group increases outcompeting the affinity constant of the molecule for the stationary phase

i.e. Purification of glucose-binding protein

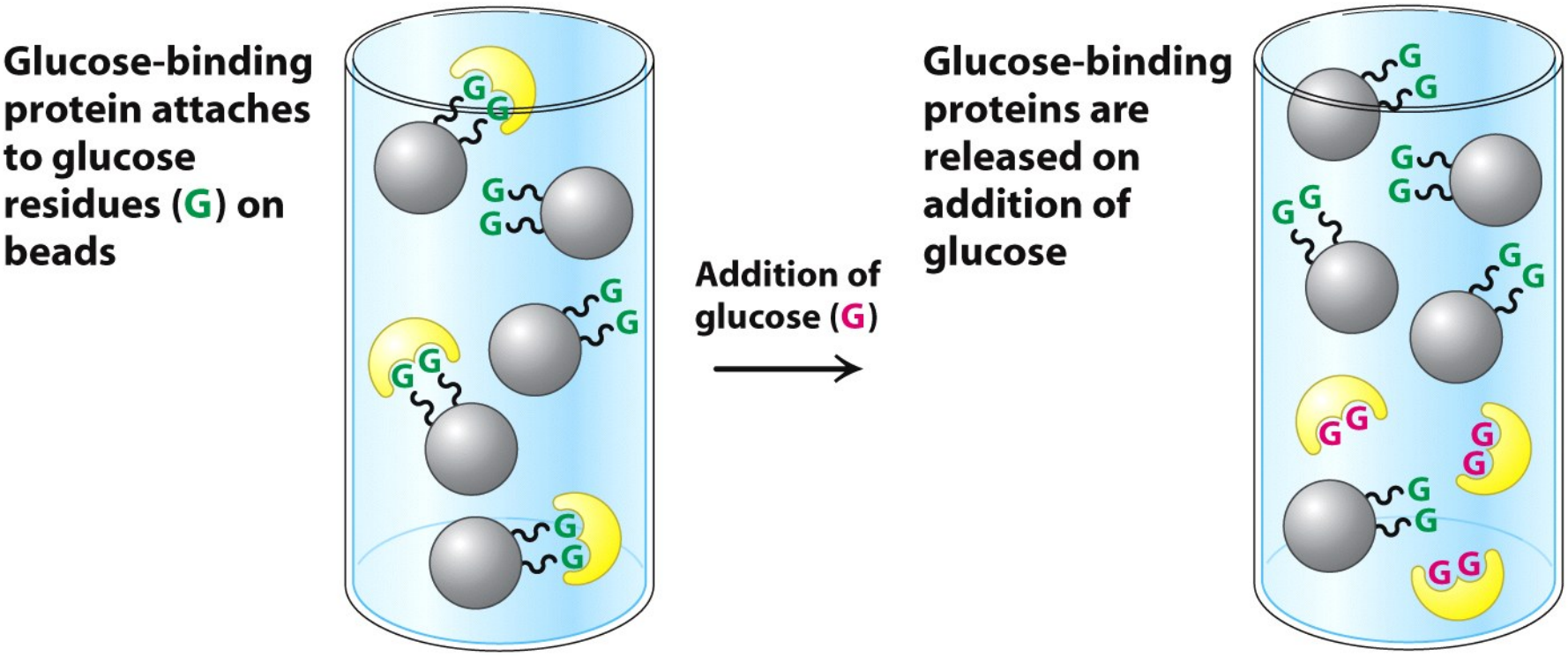


Figure 3.5
***Biochemistry*, Seventh Edition**

III. Chromatography based on hydrophobicity/ hydrophilicity

a. Technique based on the hydrophobicity/hydrophilicity of the molecules and performed with an HPLC

b. **Stationary phase**

- Normal phase: Polar groups
- Reverse phase: Nonpolar groups

c. **Mobile phase**

- Normal phase: Transitions from nonpolar to "polar"
- Reverse phase: Transitions from "polar" to nonpolar

d. **Elution**

- Normal phase: Least polar to most polar
- Reverse phase: Most polar to least polar

3. Analysis

A. NMR

- Solid/solution state studies requiring 2D approaches
- *Can be difficult to analyze*
- *Consumes lots of material*

B. Mass Spectrometry

- Solution state study (typically)
- Usually involves fragmentation of the sample before and/or during the experiment
  - Tandem mass spectrometry (MS MS) for fragmentation experiments
- Must use a pure standard to verify identification
  - If coupled with liquid chromatography (LC), retention times must be identical
- *Little material consumption*

3. Analysis

C. X-ray crystallography

- Solid-state study
- *Obtaining x-ray resolvable crystals can be difficult*

D. Activity assay

- Test an expected activity of a molecule
- *Reconstitution of sample is often necessary such as protein renaturation*

What if metals are bound to these biomolecules and are necessary for their function?

1. Test the metal binding affinity of the biomolecules (Post-isolation)

Immobilized metal affinity chromatography (IMAC):

- Chelating agents are immobilized on the **stationary phase**
  - A specific metal ion is loaded onto the chelator

- Purified molecule (or even complex mixture) is then loaded

- Binding is verified following elution using free chelator

Note: Typically applied for proteins because small molecules would likely overload these columns.

1. Test the metal binding affinity of the biomolecules (Post-isolation)

Disadvantages:

A. Only provides information on the presence of proteins with metal-binding sites
   - Does not indicate whether these complexes actually exist *in vivo*

B. Ineffective for proteins with deeply buried metal-binding sites or that require a metallochaperone

C. If isolated and unpurified proteins retain their physiological metals, then will not bind

D. May prove to be very inefficient unless interested in a specific metal-ligand interaction

2. Direct screening of covalently bound metals (During and post-isolation)

Note: Coordinate covalent bonding of metals as present in coordination compounds fit in this category.

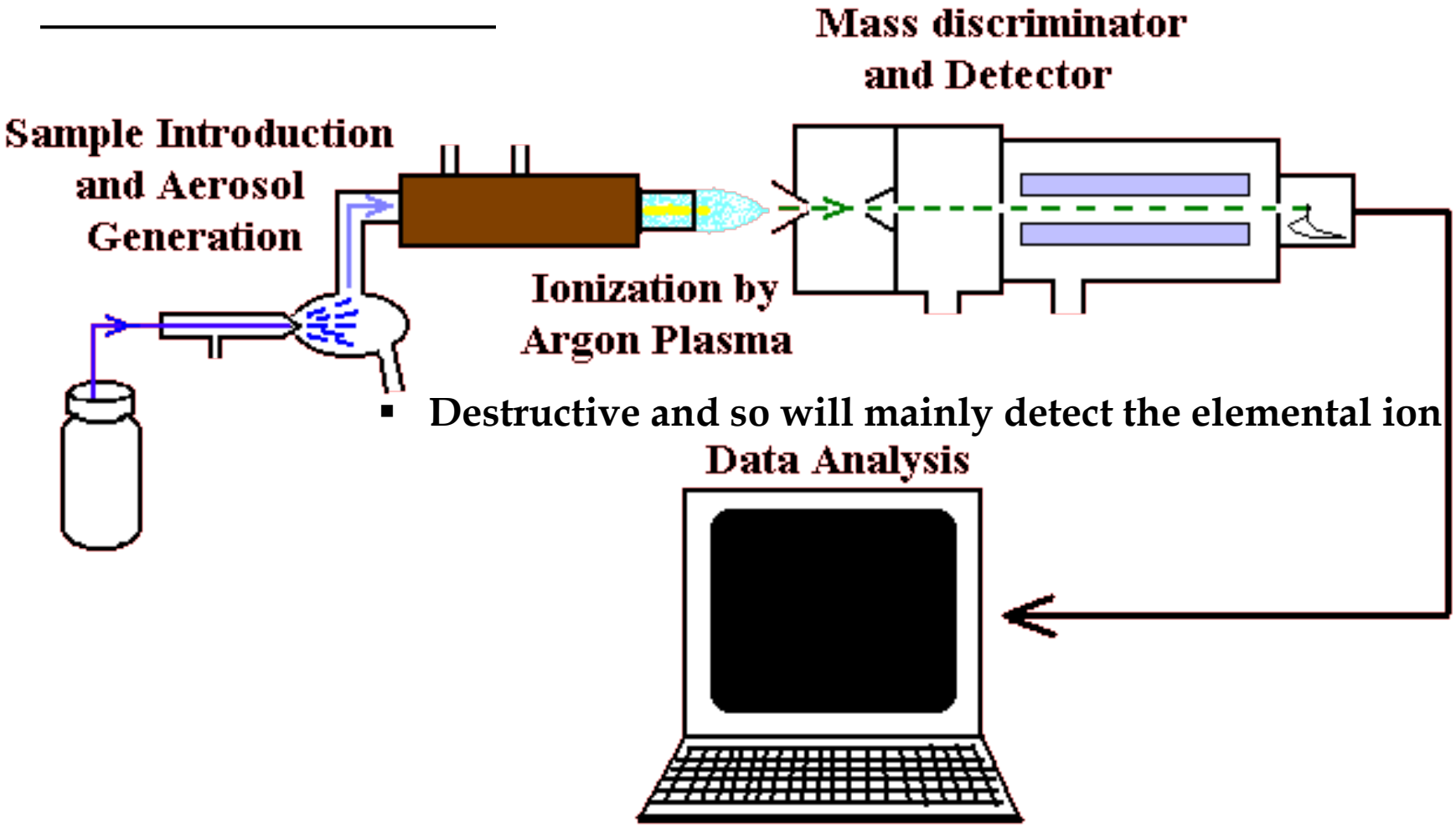
A. Couple an elemental analyzer as a co-detector during biomolecular fractionation

- HPLC-Inductively coupled plasma mass spectrometry (HPLC-ICP MS)

  ICP MS has the bonus of distinguishing between different oxidation states

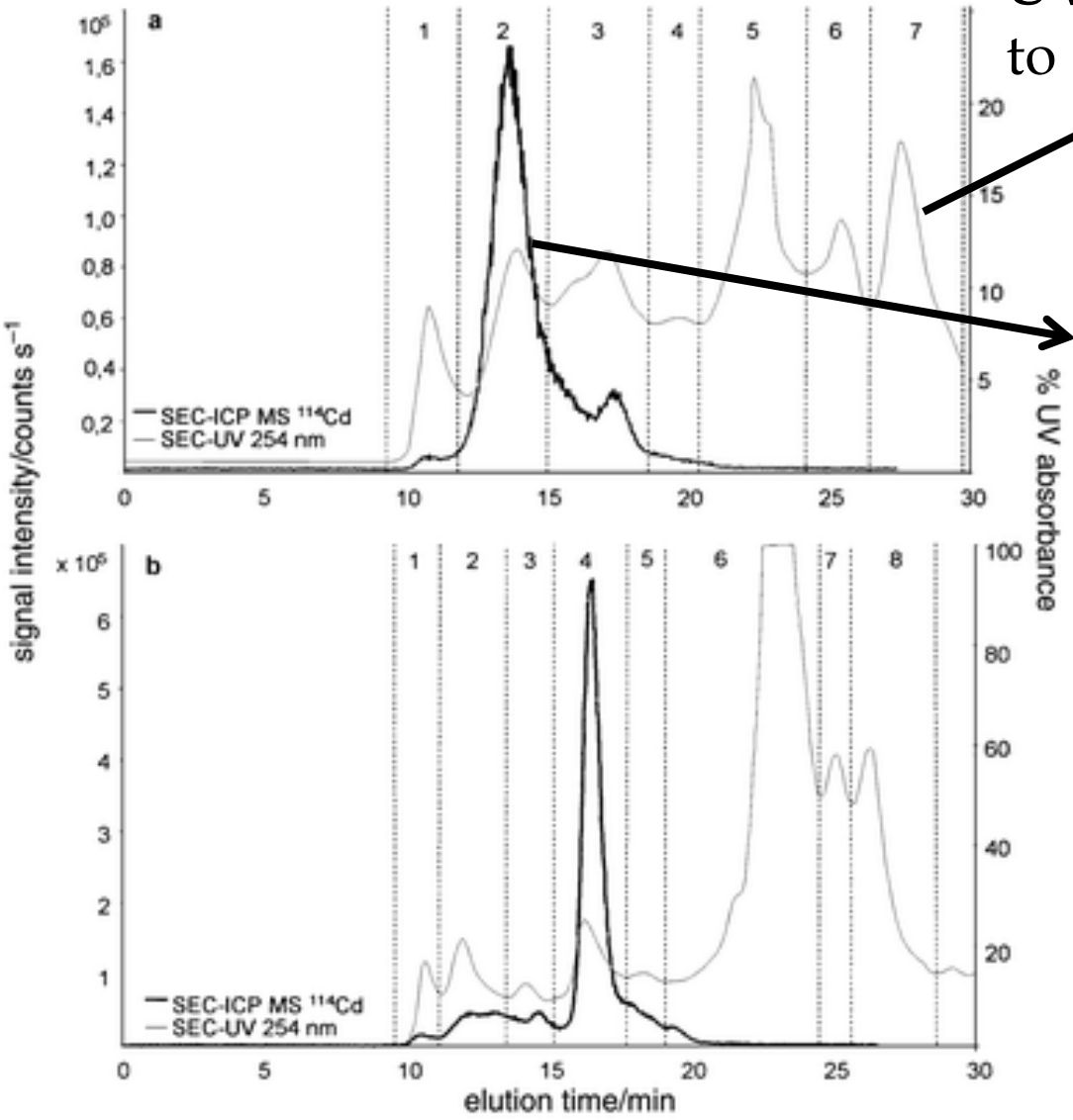
Anaerobic environment may be necessary to distinguish oxidation states.

Inductively Coupled Plasma MS



- Can detect elements (in some cases) as low as 1 part per trillion (could be picomolar)

i.e. Detection of protein bound cadmium by FPLC-ICP MS



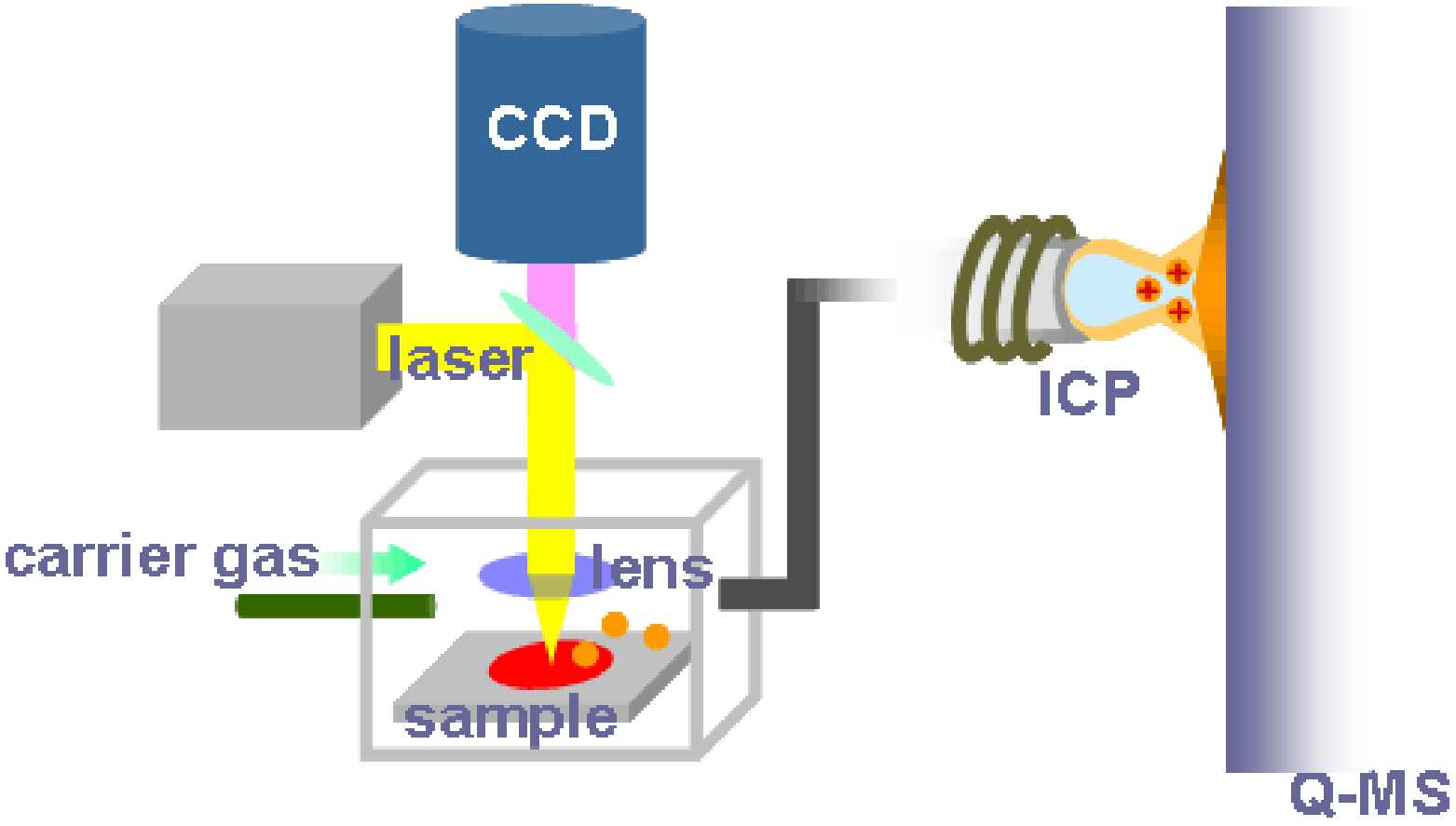
UV-Vis absorbance trace to detect eluting proteins.

Cd ICP MS trace to detect eluting proteins with bound Cd.

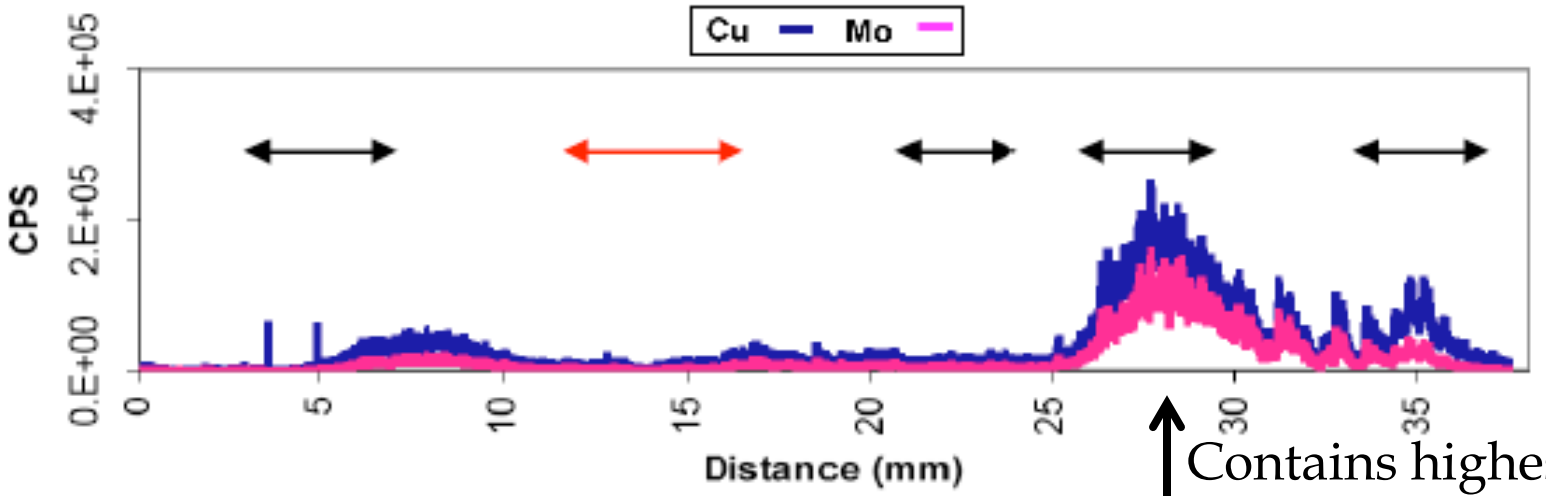
2. Direct screening of covalently bound metals

B. Can use elemental analyzer post-isolation

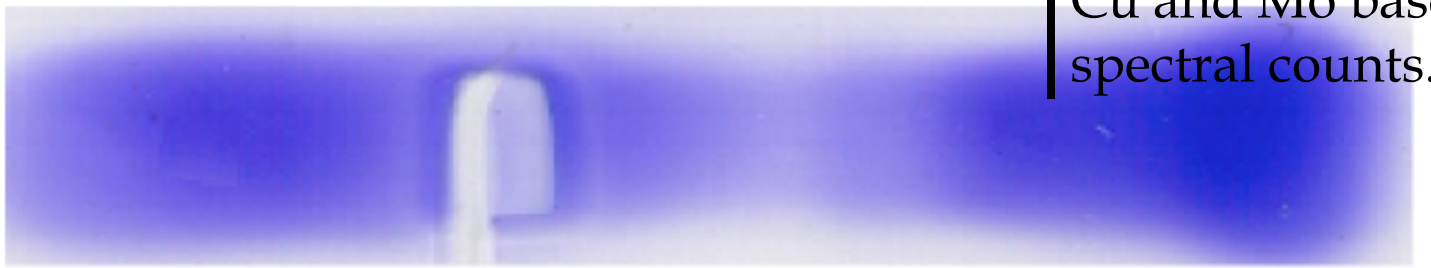
- Laser-ablation (LA)-ICP-MS



LA-ICP-MS of Cu and Mo containing proteins fractionated by gel electrophoresis



Contains highest levels of Cu and Mo based on spectral counts.



A lane on the protein gel. The dark bands are separated proteins.

2. Direct screening of covalently bound metals

C. Can use elemental analyzer directly on tissue

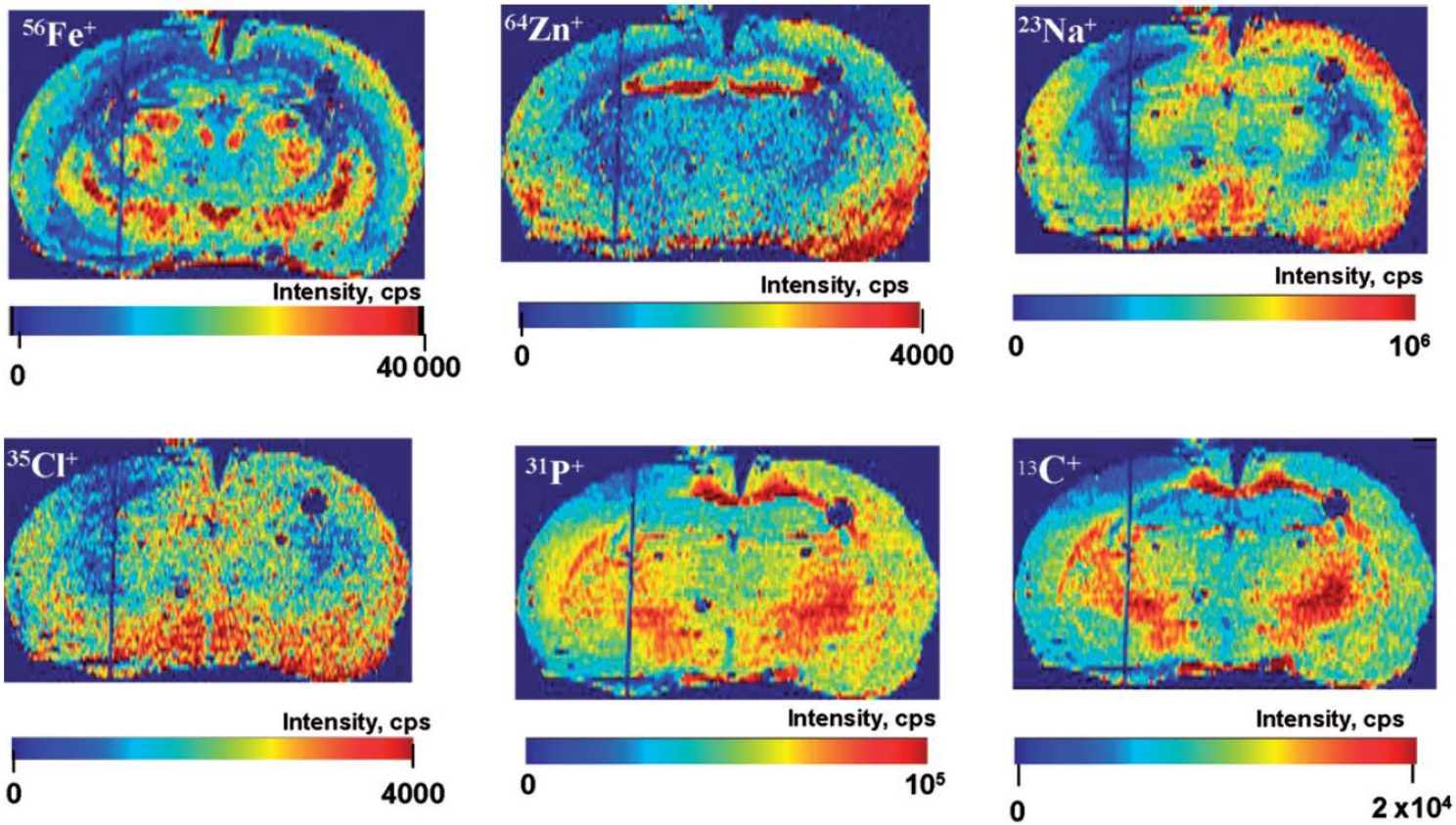


Image of selected metals and non-metals in a rat brain slice measured by LA-ICP-MS.

Becker, S.J. *et al. J. Anal. At. Spectrom.*, 2008, **23**, 1275-1280

3. Identification of metal-bound biomolecule

A. Mass spectrometry approaches

I. Can directly measure the mass spectrum of the intact metal-bound biomolecule (if the bond survives purification)

- "**Soft**" ionization is important to prevent loss of the metal

- Electrospray ionization techniques have been developed to detect intact metal-protein complexes but are limited to pure samples with low m/z resolution

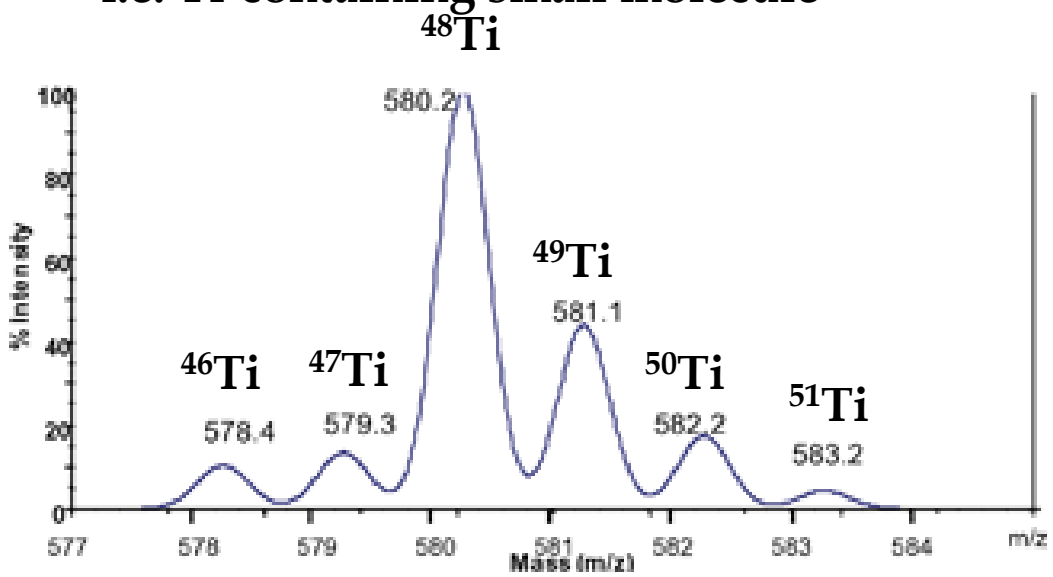
  i.e. Fe^{3+} bound to transferrin has been detected but often the synergistic anion does not survive the ionization

  Gumerov, D.R. and Kaltashov, I.A. *Anal. Chem.* 2001, *73*, 2565-2570.

3. Identification of metal-bound biomolecule

- MS of the parent ion must show an isotope distribution that is characteristic of the element of interest if good resolution is achievable

i.e. Ti-containing small molecule



Isotope	Natural Abundance (%)
^{46}Ti	8.25
^{47}Ti	7.44
^{48}Ti	73.72
^{49}Ti	5.41
^{50}Ti	5.18

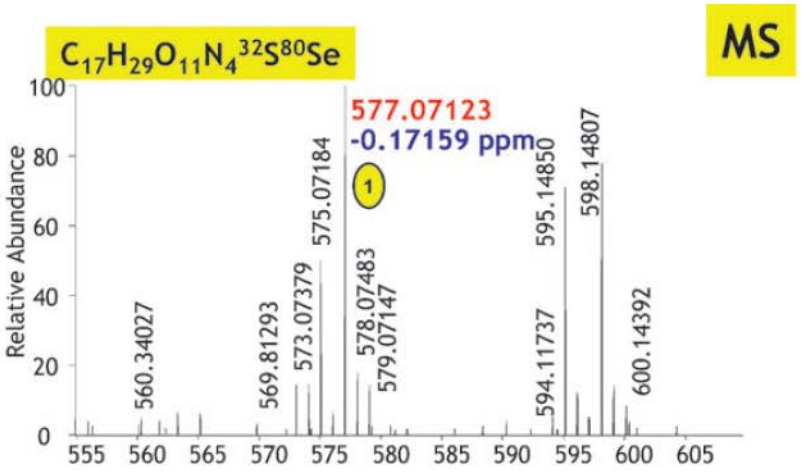
- MS of the parent ion is not sufficient if not compared to a pure standard

  Must couple with additional characterizations (i.e. NMR)

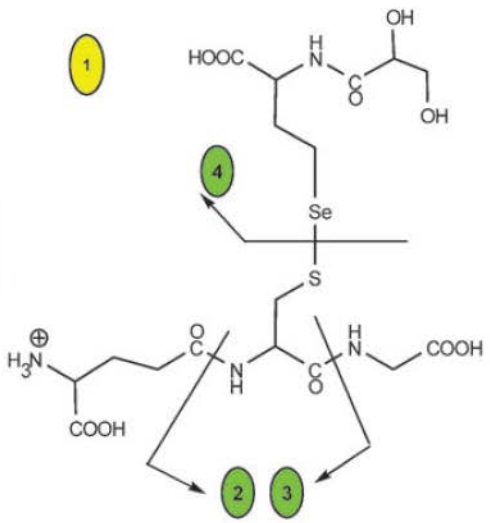
3. Identification of metal-bound biomolecule

II. Tandem MS (Fragmentation MS) can be helpful in determining biomolecular identification

Parent ion

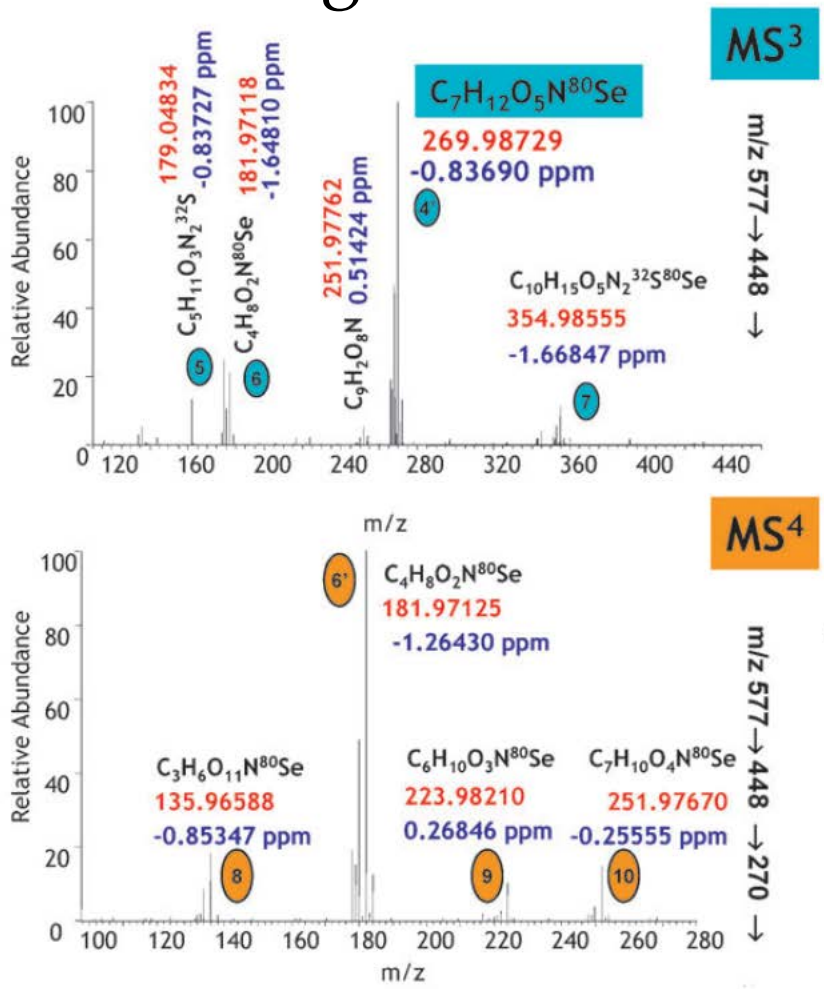


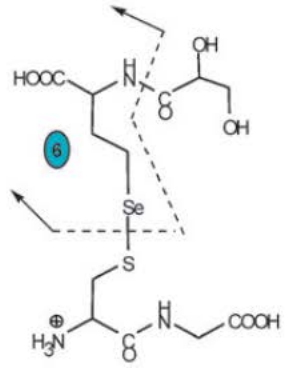
Daughter ions

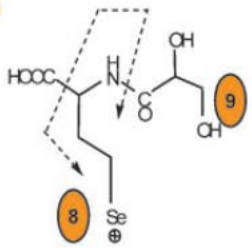


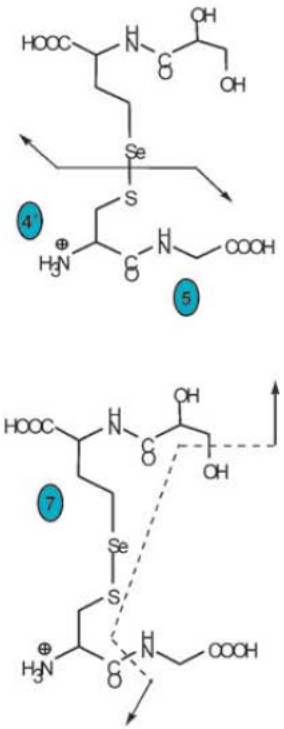
3. Identification of metal-bound biomolecule

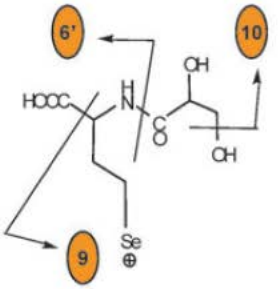
Granddaugther ions via additional fragmentation











3. Identification of metal-bound biomolecule

III. Limitations:

a. Some metals in protein samples (and small molecules) likely will not survive tandem MS fragmentation
   - Digestion of proteins would further complicate this problem

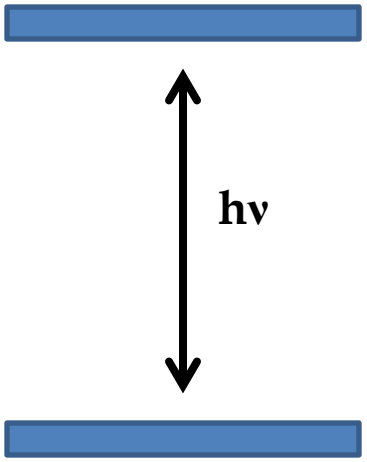
b. May need to couple *in vivo* experiments with *in vitro* experiments to verify that an isolated molecule actually does bind a specific metal

I am in charge of a MALDI TOF TOF at the Molecular Science Building if you are ever interested in performing MS or tandem MS!

3. Identification of metal-bound biomolecules

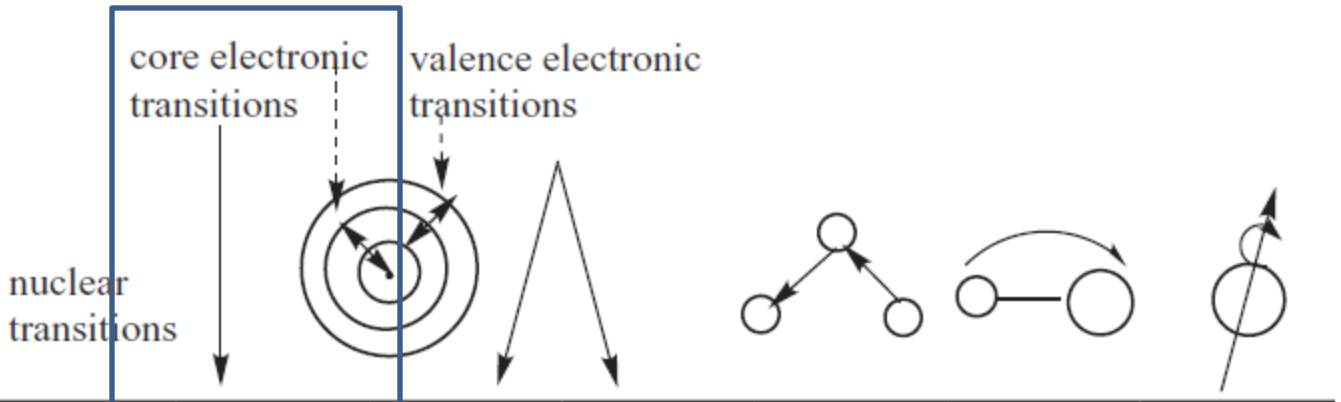
B. Spectroscopic Approaches

The absorption or emission of electromagnetic radiation is the basis for all spectroscopic methods used to characterized molecules. The total energy of the radiation consists of several components:



$$E_{total} = h\nu = \frac{hc}{\lambda} = E_{translation} + E_{rotation} + E_{vibration} + E_{electron\ spin} + E_{electron\ levels} + E_{nuclear\ spin} + E_{nuclear\ levels} + \ldots$$

h = Planck's constant
c = the speed of light
ν = the frequency of light in s^{-1} (Hz)
λ = the wavelength of light in meters (m)



λ (m)	10^{-12} - 10^{-10}	10^{-10} - 10^{-8}	10^{-8} - 10^{-6}	10^{-6} - 10^{-4}	10^{-4} - 10^{-2}	10^{-2} - 10^{+2}
ν (Hz)	10^{18} - 10^{20}	10^{16} - 10^{18}	10^{14} - 10^{16}	10^{12} - 10^{14}	10^{10} - 10^{12}	10^{6} - 10^{10}
$N_o h\nu^a$ ($Jmol^{-1}$)	10^{8} - 10^{10}	10^{6} - 10^{8}	10^{4} - 10^{6}	10^{2} - 10^{4}	10^{0} - 10^{2}	10^{-4} - 10^{0}

$^a N_o$ = Avogradro's number = 6.023×10^{23}

Figure 3.2 Illustrated energy transitions for several useful regions of the electromagnetic spectrum. (Adapted with permission of John Wiley & Sons, Inc. from Figure 2.2 of reference 3. Copyright 1997, Wiley-VCH.)

X-Ray Absorption Spectroscopy (XAS)

X-rays

X-rays have wavelengths that are on the order of atomic dimensions and therefore these photons can be used to probe the molecular structure of materials.

i.e. Xray diffraction of crystalline material

1. XAS

XAS is a technique that is specific to an atom depending on the X-ray photon energy range used

In a typical X-ray absorption spectrum:

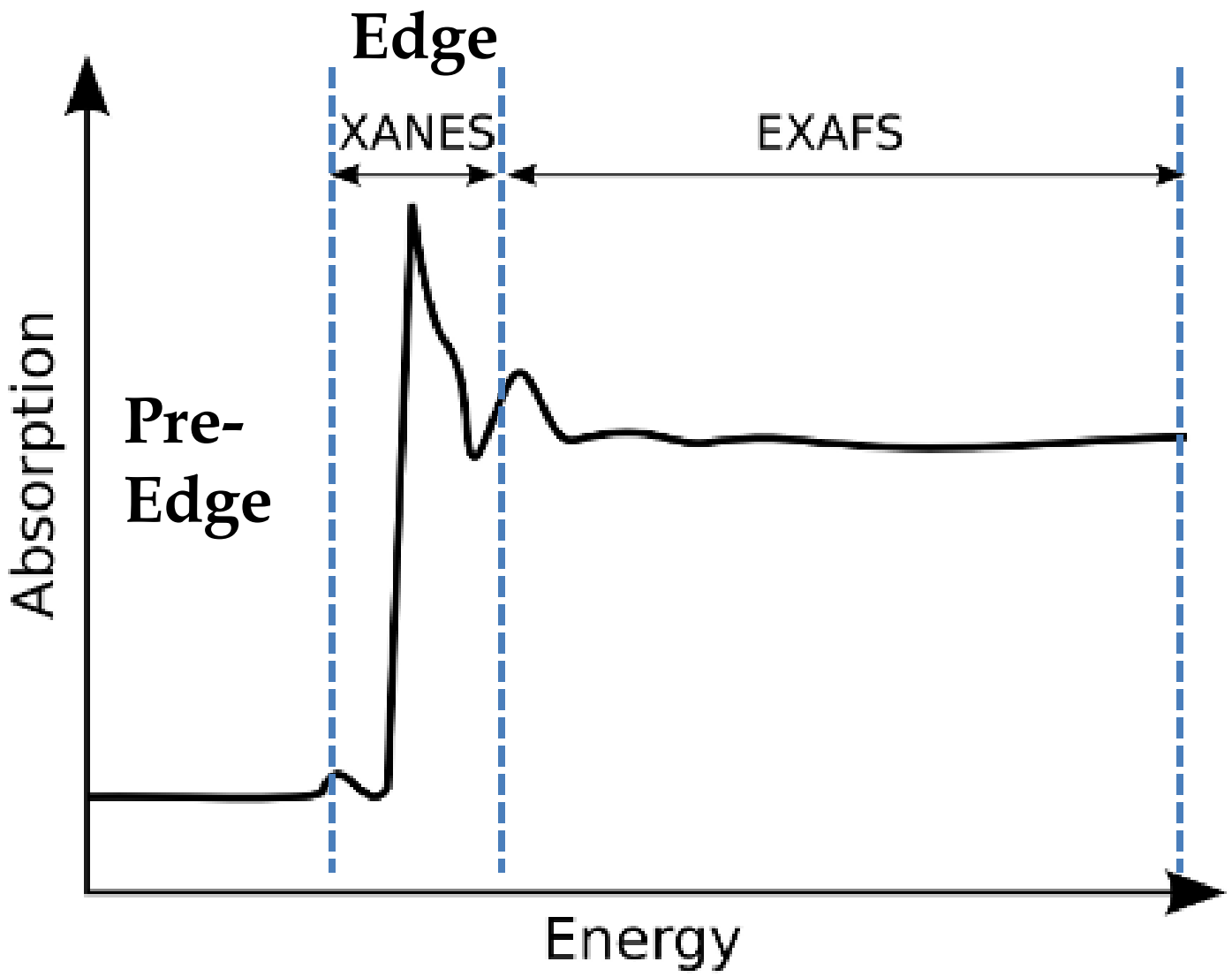
- A small rise in absorption coefficient is observed due to electronic transitions from the core level to valence levels just below the continuum. This is known as the **preedge**.

- A sharp rise in absorption coefficient is observed at a well-defined X-ray photon energy called an absorption **edge**

  - Due to core electron dissociation from the atom

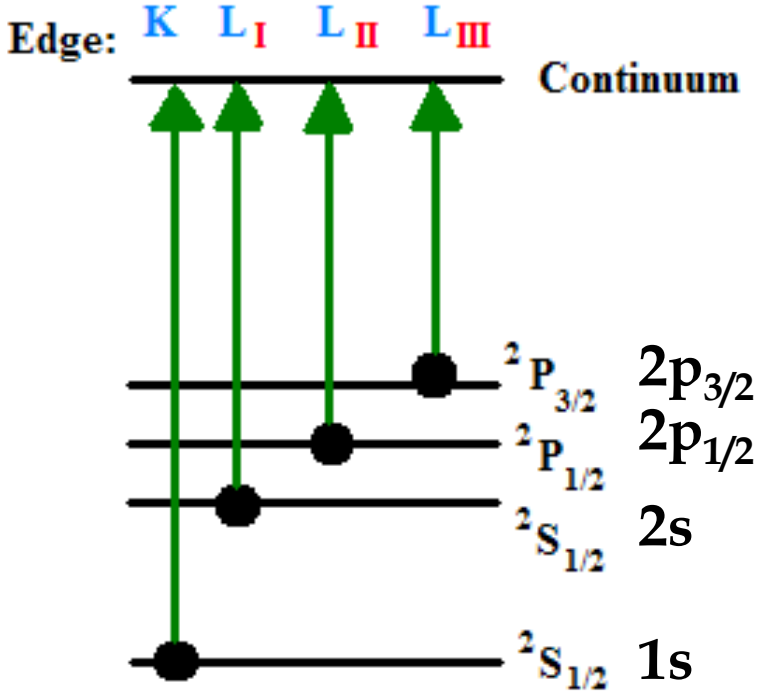
- Above the edge, there are quasiperiodic modulations in the X-ray absorption coefficient referred to as **EXAFS**

1. XAS



A. X-ray absorption near-edge structure (XANES)

I. The X-ray absorption spectrum of a given sample will exhibit an edge at a photon energy equal to the ionization potential of a bound electron in the constituent atom of the sample.



The edges are named for the shells of the Bohr atom:

K edge; n = 1
L edge; n = 2
M edge; n =3

A. X-ray absorption near-edge structure (XANES)

II. The accessible X-ray photon energy region is ~2-30 keV
-Lower limit defined by sample holders

K edges observable for P to Sn

L edges observable up to Lr

A. X-ray absorption near-edge structure (XANES)

III. Information obtainable from XANES

a. Oxidation number

- In general, the higher the oxidation state of the absorbing atom, the higher the energy of the X-ray absorption edge

Why?

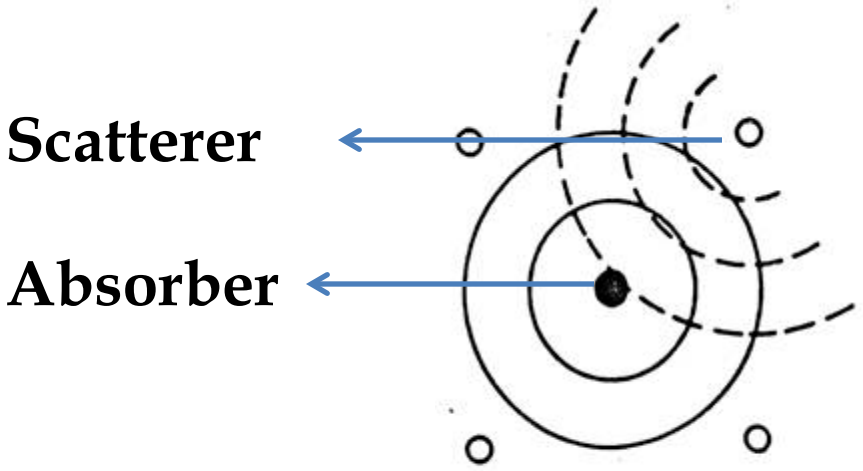
Harder to remove an electron from an atom with a higher positive charge

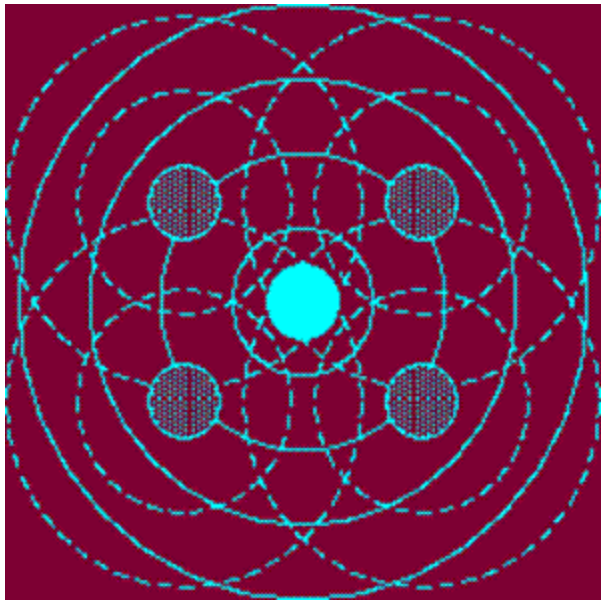
b. Covalence: The number of paired electrons

c. Molecular symmetry (coordination number)

B. Extended X-ray absorption fine structure (EXAFS)

I. The information content of the EXAFS technique arises from the physical interaction of the photodissociated core electron with electron density surrounding neighboring atoms.





Outgoing photoelectron waves (solid lines) propagate to neighboring atoms represented by open circles. The backscattered waves (dashed lines) modify the wave function at the central atom and give rise to the EXAFS modulations.

B. Extended X-ray absorption fine structure (EXAFS)

II. EXAFS gives radial distance and helps you answer:

How many of what type of atom are at what distance from the absorbing atom?

a. Identity of the ligand atoms

b. Bond lengths

- Radial structural information within a 4-5 Å radius around the absorbing atom

c. Coordination number

B. Extended X-ray absorption fine structure (EXAFS)

III. The EXAFS data has to be processed before fitting it to different models of proposed "shells" of atoms

- See data reduction handout of EXAFS analysis
- In general, the number of "shells" that you use to fit EXAFS will depend on the number of unique atoms around the absorbing atom

- If 4 unique atoms, then use 4 "shells" of atoms to fit the data

- But bond order is important:

  A single bond to an oxygen would be different from a double bond to an oxygen

- It is sometimes difficult to distinguish between oxygen and nitrogen and they may be counted as the same shell of atoms

C. XAS Limitations

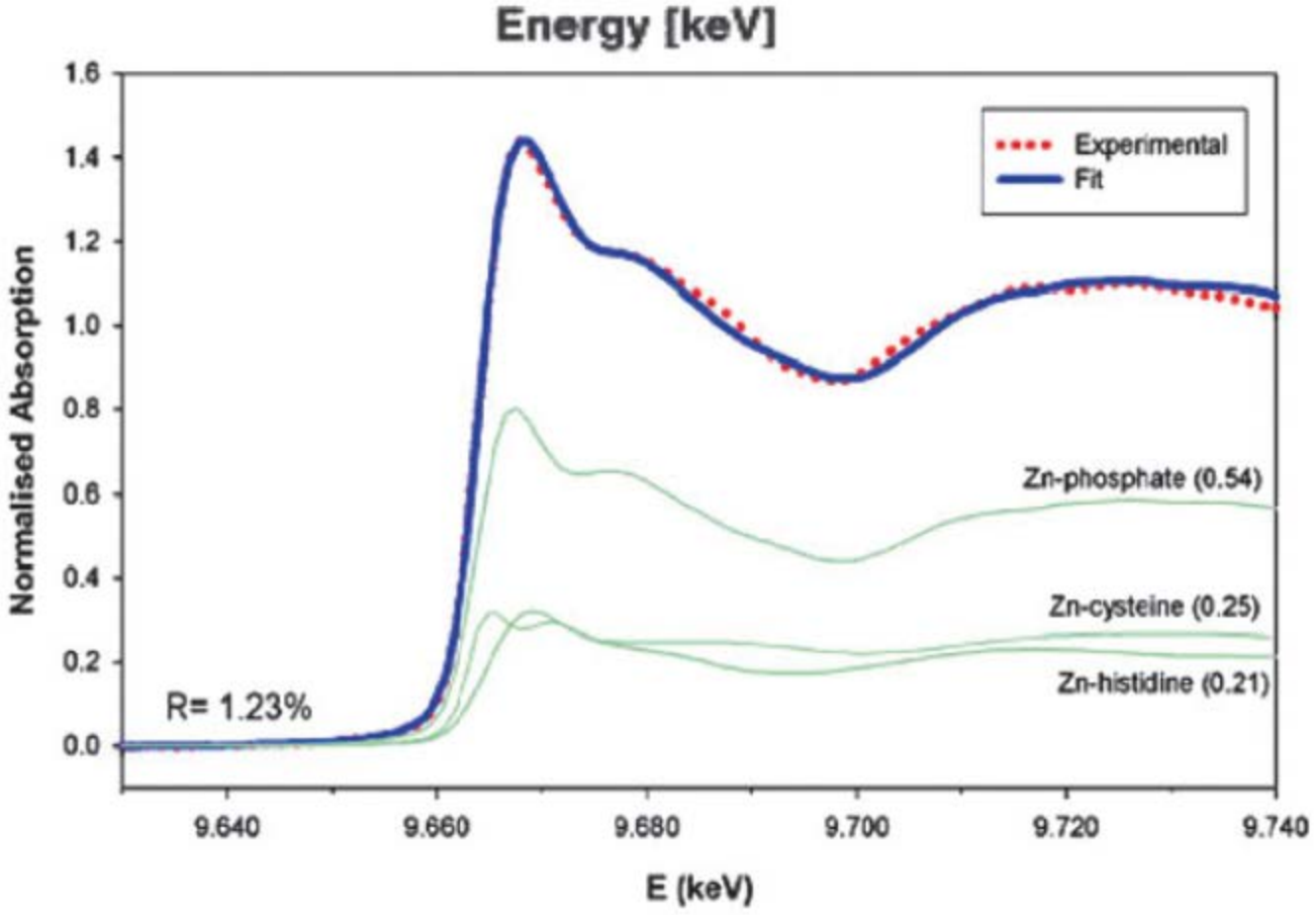
Getting access to an XAS is not difficult. You just have to write a convincing proposal to gain access to one. The Brookhaven National Synchroton Light Source II is an example.

However,

1. You need to use pure samples
2. You can use *in vivo* samples, but if you have a mixture of metal complexes then it would be hard to deconvolute them without the use of standards of **expected species**

How to make XAS work for you?

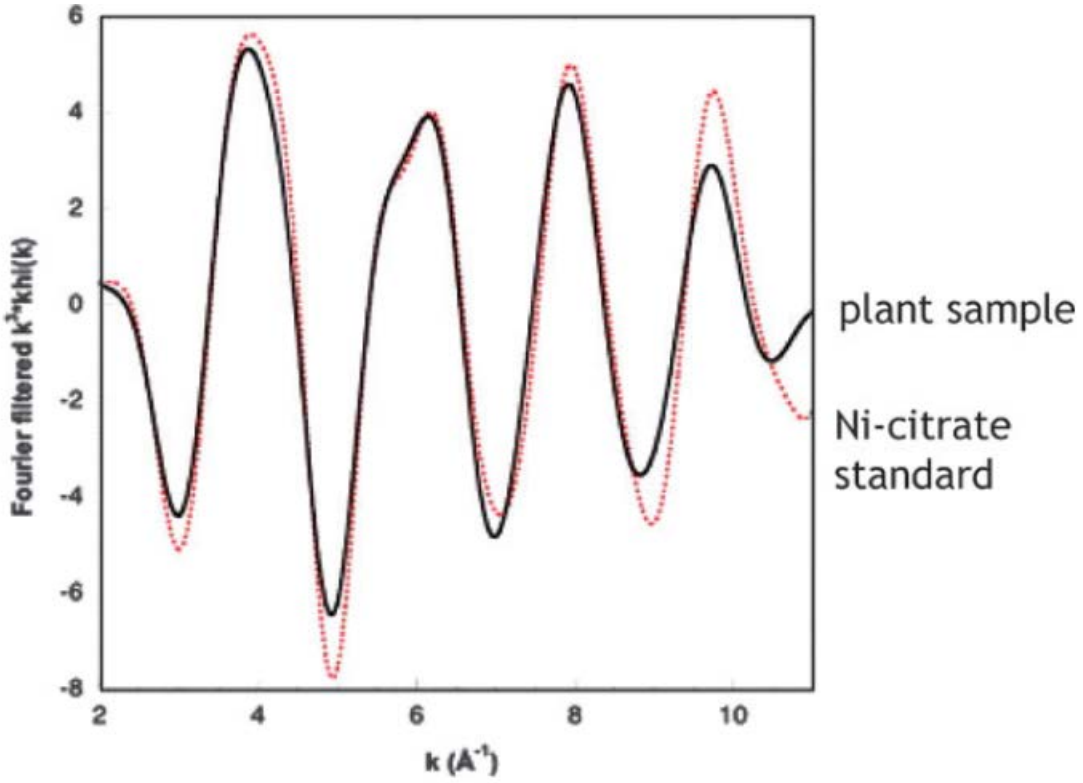
XANES was used here to deconvolute the distribution of Zn-phosphate, Zn-cysteine, and Zn-histidine in leaves using standards of these species.



Terzano, R. *et al. J. Agric. Food Chem.* 2008, *56*, 3222.

How to make XAS work for you?

EXAFS was used here to confirm the dominance of a Ni-Citrate species in a plant using a standard.



Montarges-Pelletier, E. *et al. Phytochemistry*. 2008, *390*, 1739.

How to make XAS work for you?

For metal-bound proteins, you can also use small molecule standards to predict the metal coordination in the metal binding site.

- If you have a pure sample, then by fitting the EXAFS data one can determine the primary coordination of metals even with the difficulty of distinguishing between O and N.
- Use educative guesses as to the amino acids involved coupled with *in vitro* experiments