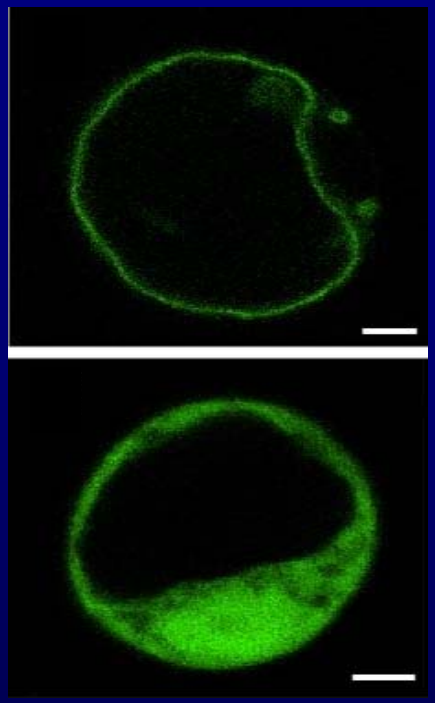
Isolation, Purification and Characterisation of Metalloproteins from Plants

In vivo study of proteins

Cellular localization of protein

Activity e.g. detection of metal transport by specific fluorescent dyes or patch clamping

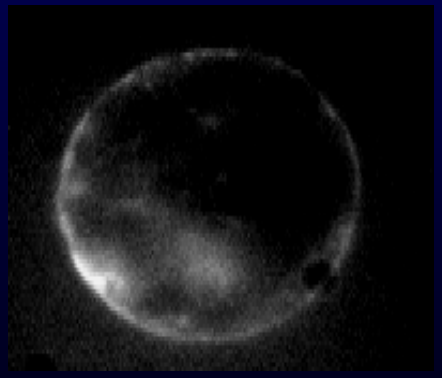


Kobae et al., 2004, PlantCell Physiol 45, 1749-58

Limitations :
No structural determination

No analysis of binding constant

No analysis of catalytic mechanism



Leitenmaier B, Küpper H (2011) Plant Cell & Environment 34, 208-219

I) Protein Isolation: Sources (expression) of plant proteins

Native expression (i.e. in intact organs or in tissue culture)

- Advantages:
  - Correct folding
  - Correct post-translational processing (e.g. insertion of active centre, phosphorylation, glycosylation, cleavage, ...)
  - No cloning needed

- Disadvantages
  - Usually lower yield
  - Often many similar proteins in the same organisms → difficulties with purification

root freezing in liquid nitrogen and hydroponic plant cultivation in Küpper lab

I) Protein Isolation: Sources (expression) of plant proteins

Heterologous expression (e.g. in bacteria, yeast or insect cells)

- Advantages:
  - Usually higher yield
  - Specific expression of one protein in large amounts and possibly with specific tag (e.g. His-tag) facilitates purification

- Disadvantages
  - Often problems with folding, in particular in the case of large proteins and integral membrane proteins
  - Insertion of complicated cofactors (e.g. iron-sulfur clusters) is difficult or impossible, other post-translational modifications may be missing or different compared to the native protein → possibly wrong conclusions about functions/mechanisms

I) Protein Isolation: Methods for isolation

Grinding in liquid nitrogen

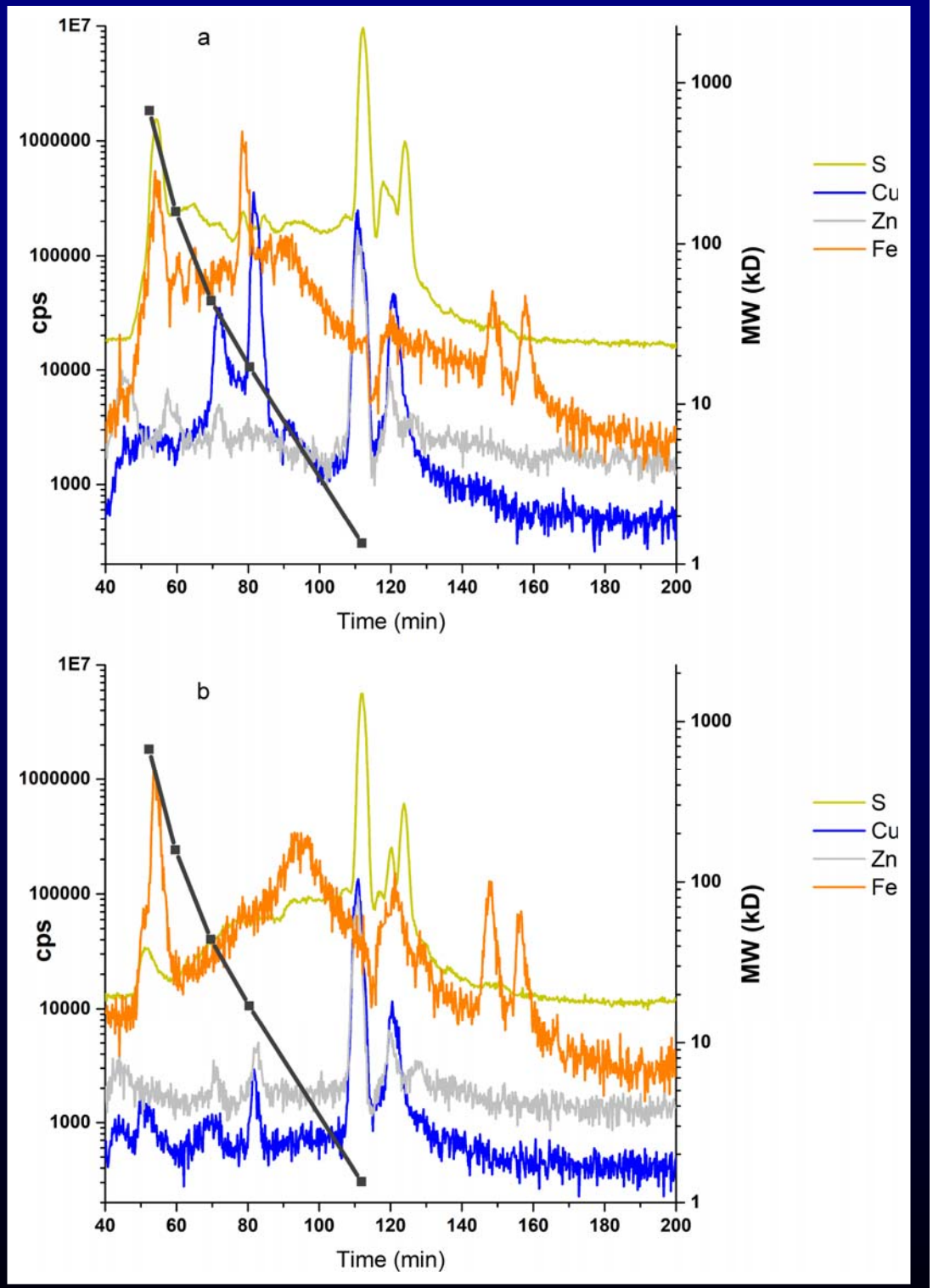
- Used for hard tissues and cells like roots, stems, but also for hard-walled cells like some algae and cyanobacteria
- Low temperature protects the proteins during grinding
- Time consuming (manual grinding) or requiring suitable machinery (expensive solution: lab mill, several thousand €, cheap solution: wheat mill, about 200 €)

Liquid nitrogen cooled wheat mill in Küpper lab

I) Protein Isolation: Methods for isolation

Ultrasound homogenization ... Can cause artefacts of denaturation

Many peaks of metalloproteins were strongly reduced and/or moved to low MW, showing severe protein denaturation, already by „gentle“ ultrasound treatment of protein extracts



Küpper H, Hussain Bokhari SN, Jaime Perez N, Lyubenova L, Ashraf N, Andresen E (2019) Analytical Chemistry 91, 1710961-10969

I) Protein Isolation: Methods for isolation

French Press

- Used for individual cells (plant cell culture, algae or bacteria) without or with soft walls
- Does not require freezing and thus may avoid artefacts of freezing
- Requires very expensive (usually many thousand €) machinery

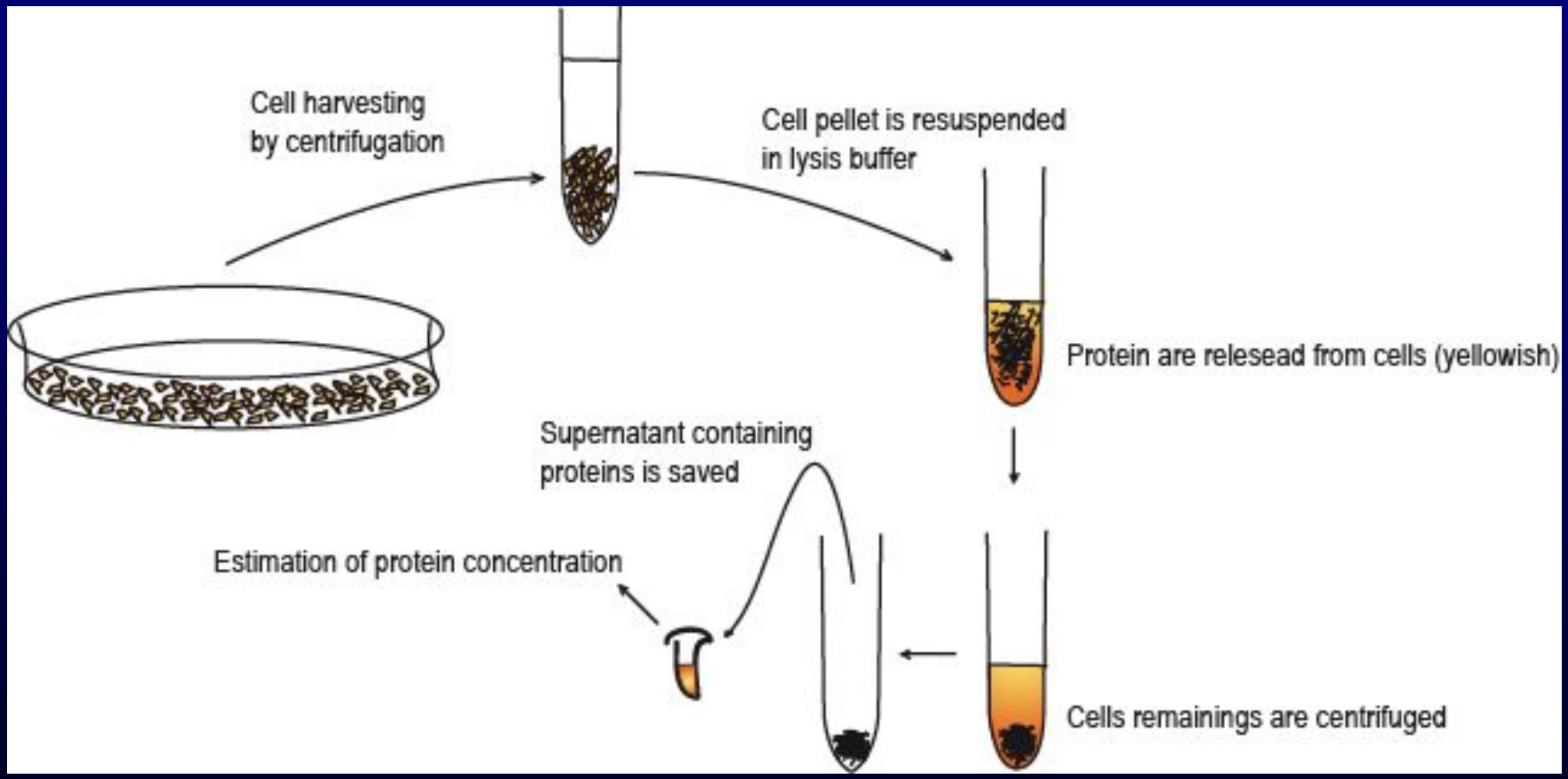
From: www.diversified-equipment.com

From: www.pegasusscientific.com

I) Protein Isolation: Methods for isolation

Lysis buffer

- Used only for bacteria or animal cells
- May cause degradation
- No machinery needed



From: bio-ggs.blogspot.com/2009/11/ggs-live-western...

II) Protein Purification: Overview of Principles

Separation by expression site

- Selective use of tissues or organelles
- Separation of soluble from membrane proteins by centrifugation

Separation by size

- Ultrafiltration
- Size exclusion chromatography
- Preparative native gel electrophoresis

Separation by charge

- Ion exchange chromatography
- Isoelectric focussing (as chromatography, in solution or in gel electrophoresis)

Separation by specific binding sites

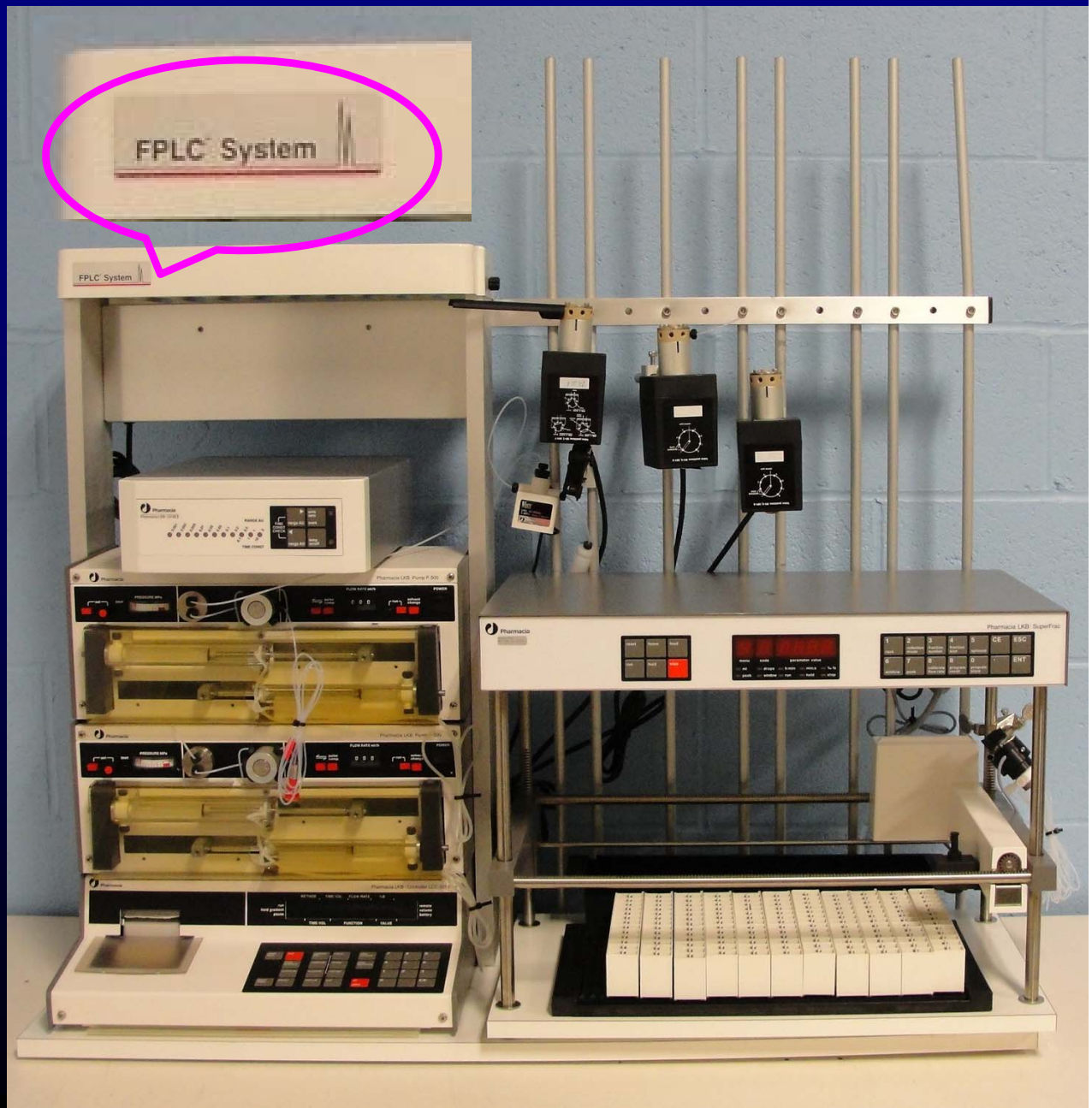
- Metal affinity chromatography using natural or artificial metal binding sites
- Immuno-Chromatography using immobilised antibodies
- Magnetic separation using magnetically tagged antibodies

Chromatography: Types

"Special" technique?

Fast protein liquid chromatography (FPLC)

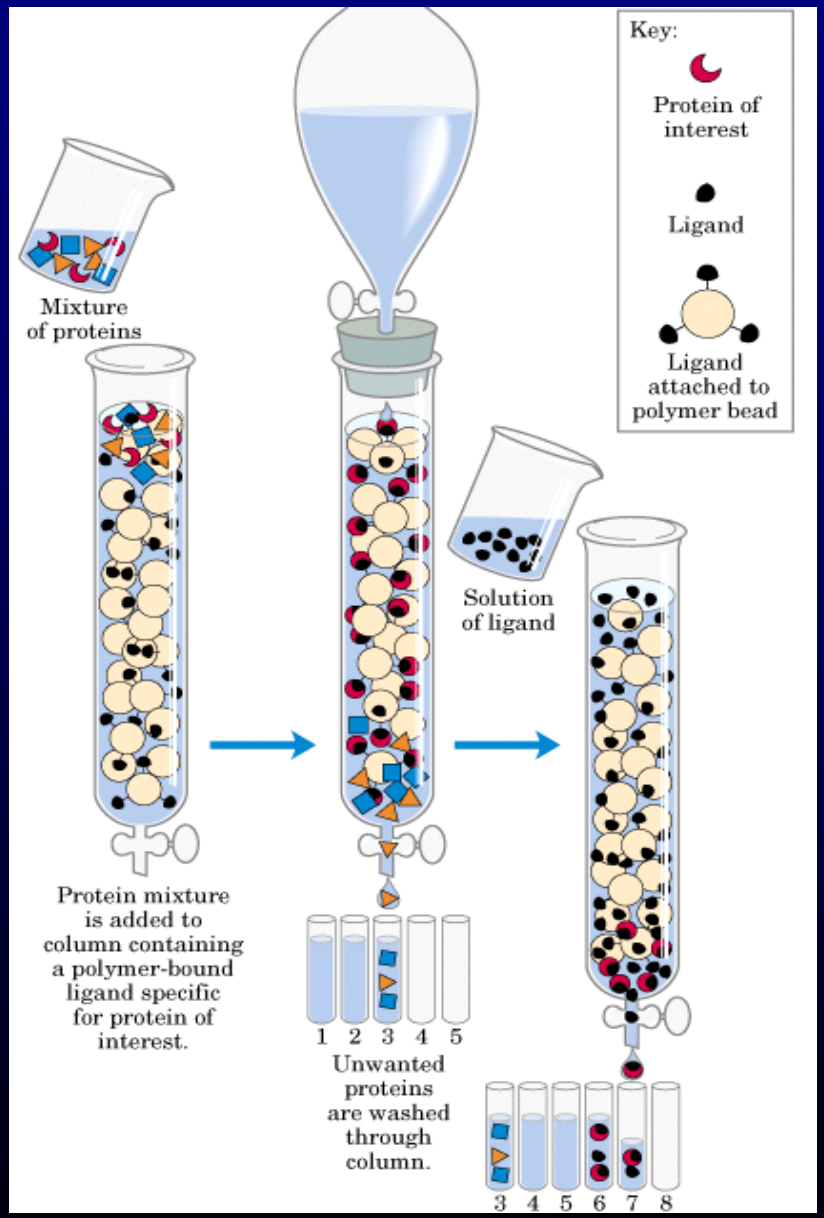
- Also called as fast performance liquid chromatography, a variant of HPLC
- Now often, wrongly, used as a synonym for HPLC of proteins, BUT: Originally a **trademark** of LKB→Pharmacia→Amersham→ GE healthcare→Cytiva
- Was designed by LKB/Pharmacia for protein purification in 1982
- Original FPLC operates at low pressure typically less than 5 bar, modern protein chromatography often up to 100 bar, i.e. typical HPLC range
- flow rate is relatively high, typically 1-5 ml/min.



Chromatography: Types

Affinity chromatography

Based on a highly specific interaction between analyte and stationary phase



dolly.biochem.arizona.edu/.../methods.html

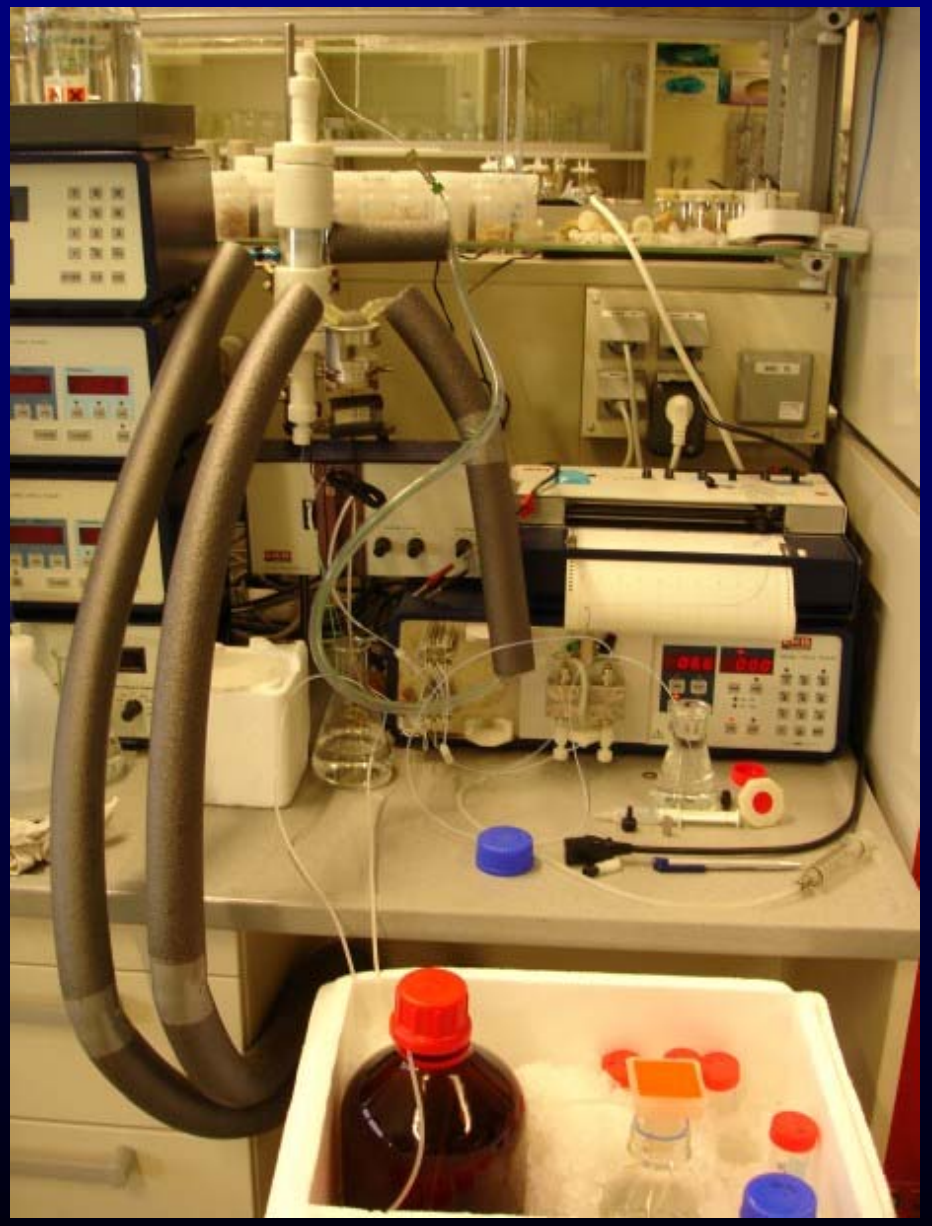
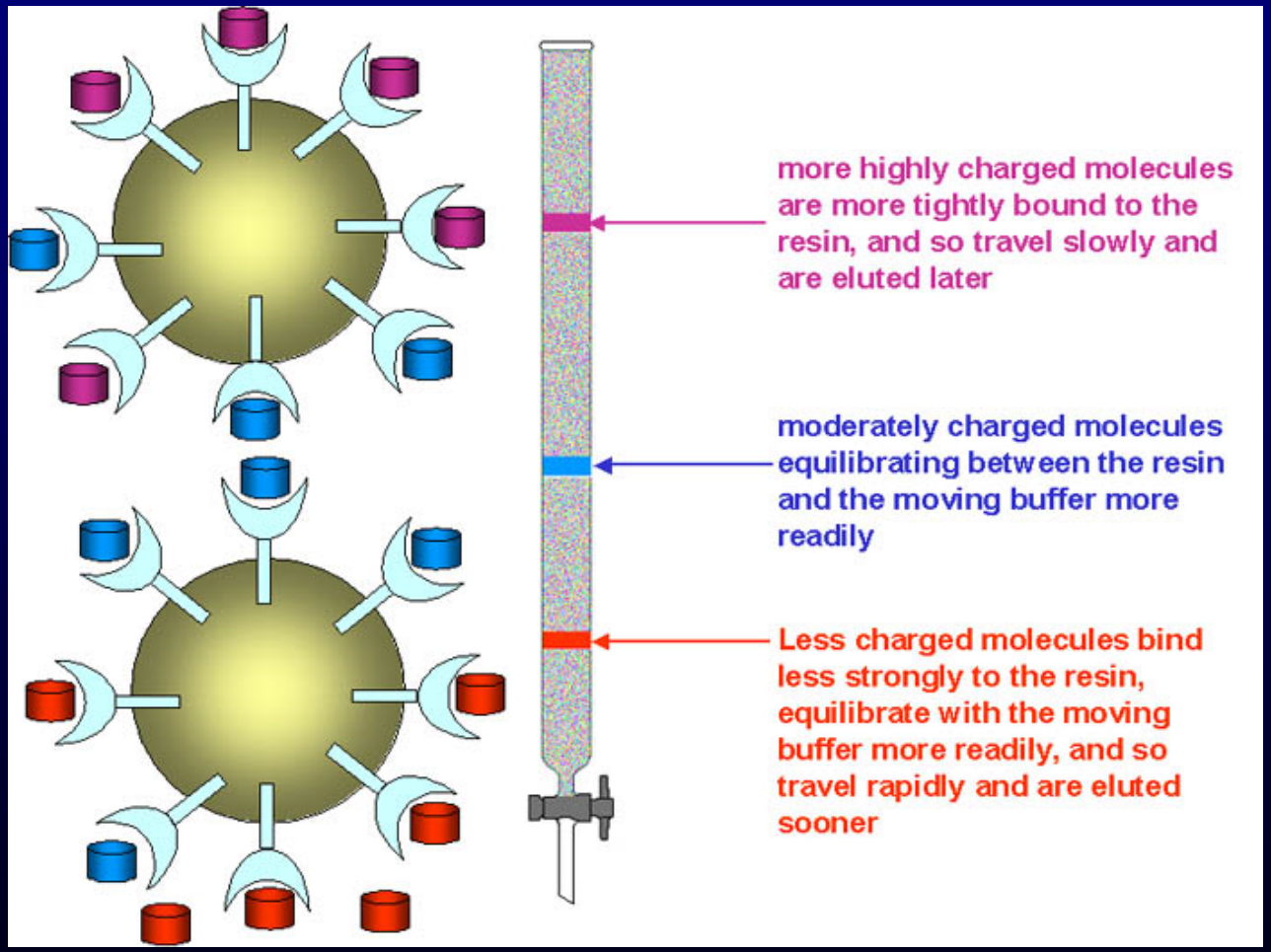


foto of TcHMA4 purification in the lab of H. Küpper on an IMAC column

Chromatography: Types

Ion exchange chromatography

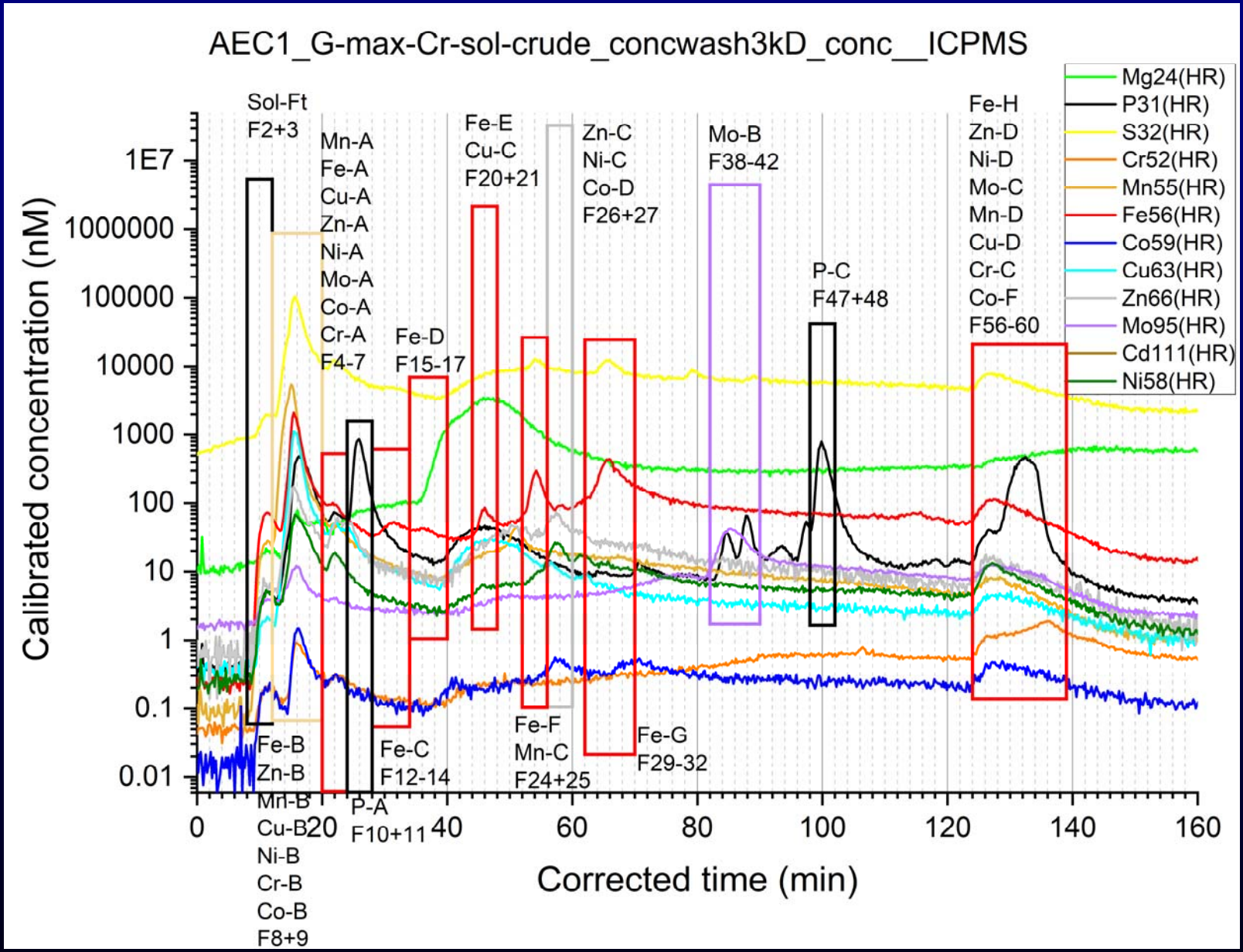
Principle: highly charged proteins bind stronger to the column material, so that they elute at higher salt concentrations in the buffer
than less charged proteins



From: www.ucl.ac.uk/~ucbcdab/enzpur/ionX.htm

foto of phycobiliprotein purification in the lab of H. Küpper on a MonoQ anion exchange column

Example of metalloprotein separation by anion exchange chromatography (*G. max* root soluble proteins)



Chromatography: Types

Hydrophobic Interaction Chromatography (HIC)

- Solute equilibrates between a solid hydrophobic stationary phase and the eluent
- separation is based on hydrophobicity of the protein surface
- choice of column important – different for membrane vs. soluble proteins!

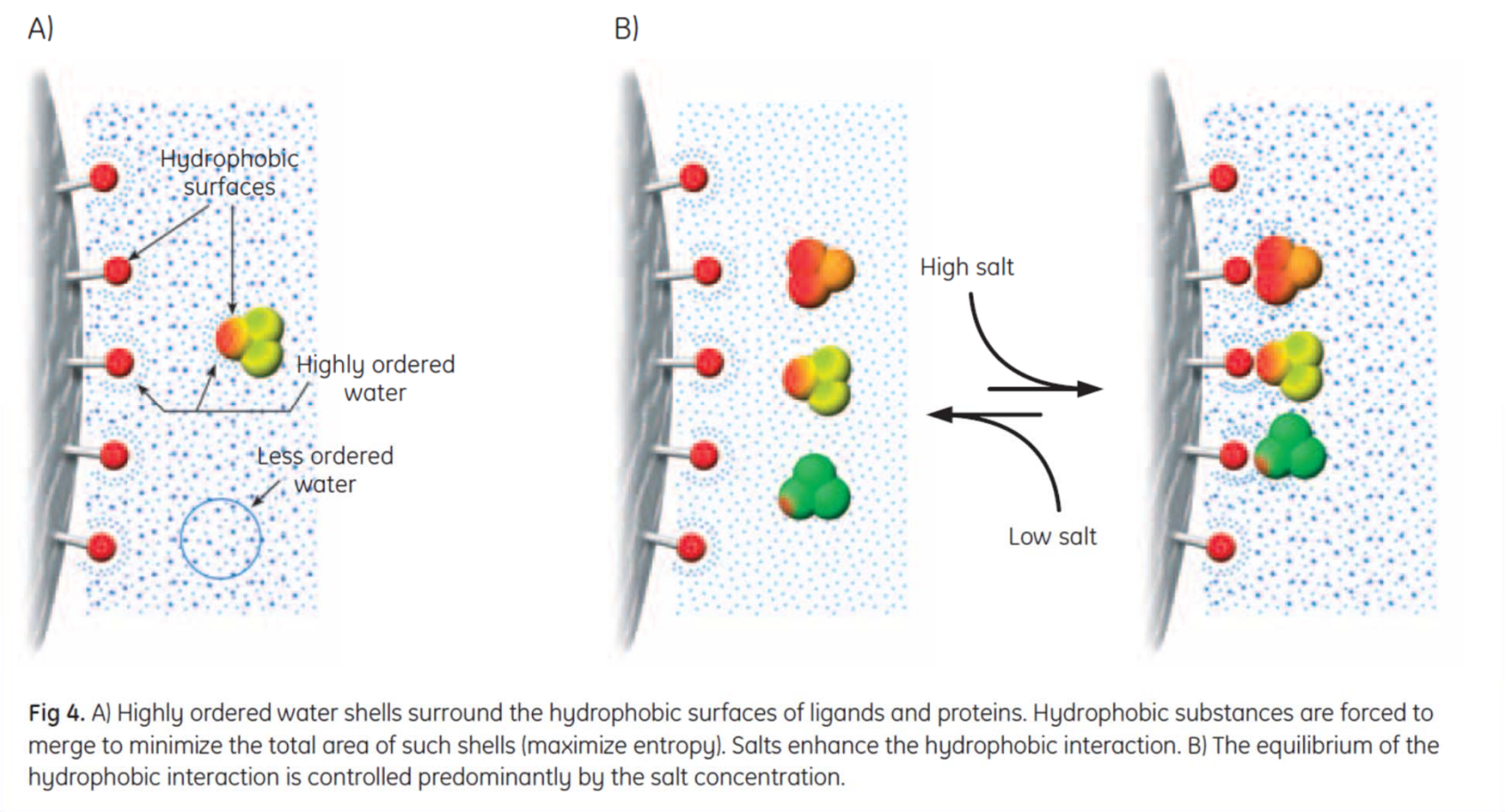


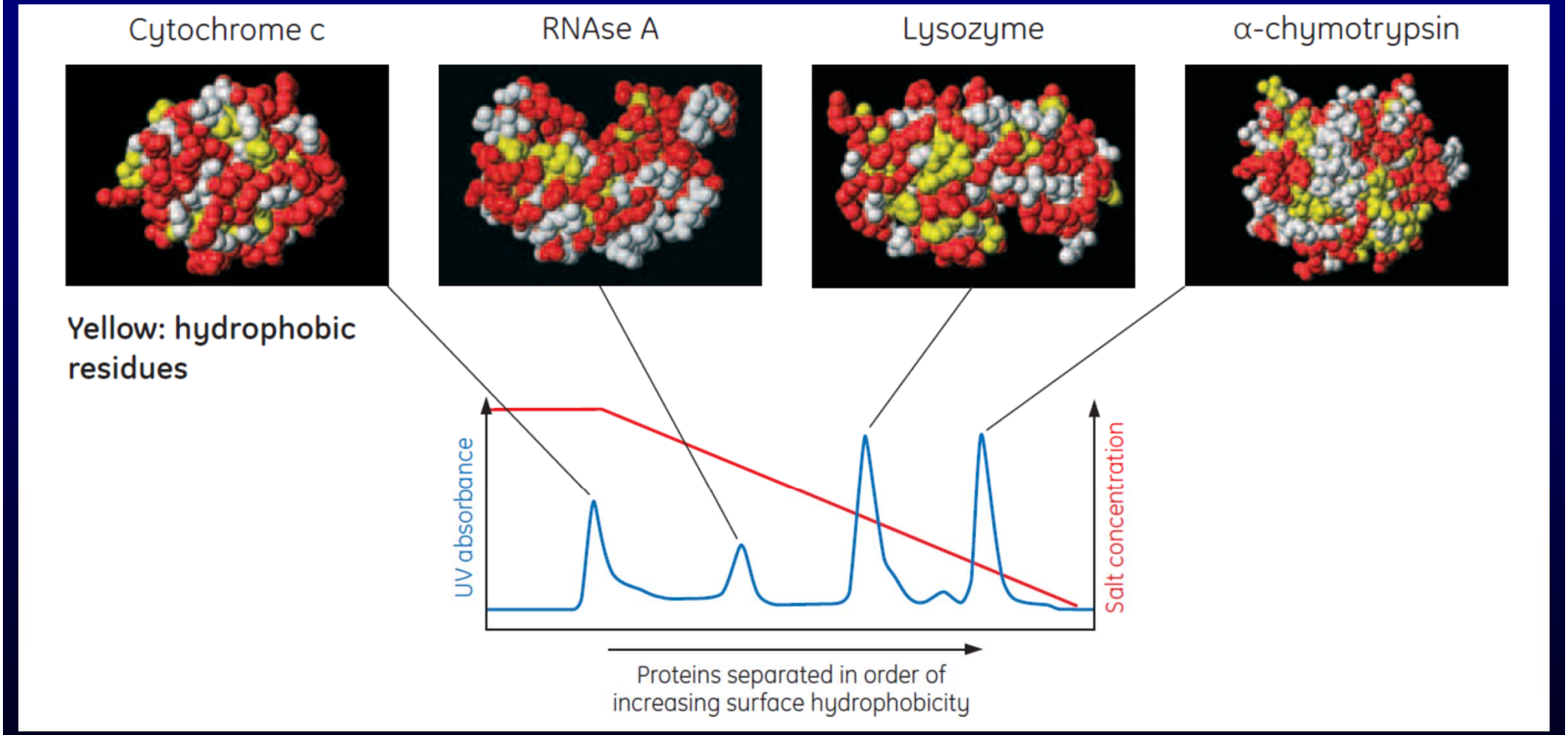
Fig 4. A) Highly ordered water shells surround the hydrophobic surfaces of ligands and proteins. Hydrophobic substances are forced to merge to minimize the total area of such shells (maximize entropy). Salts enhance the hydrophobic interaction. B) The equilibrium of the hydrophobic interaction is controlled predominantly by the salt concentration.

From product information of GE healthcare

Chromatography: Types

Hydrophobic interaction chromatography (HIC)

- separation is based on hydrophobicity of the protein surface: example

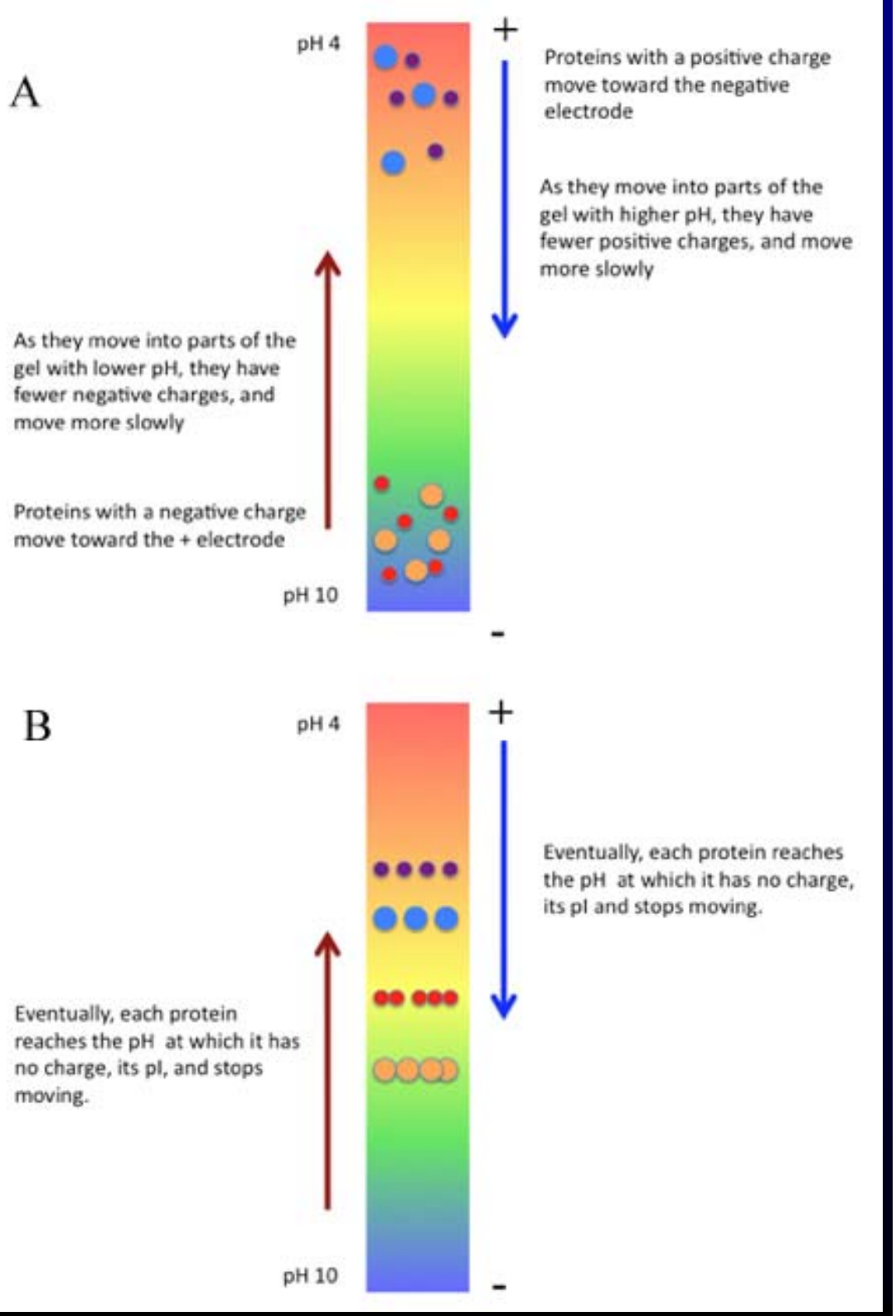


From product information of GE healthcare

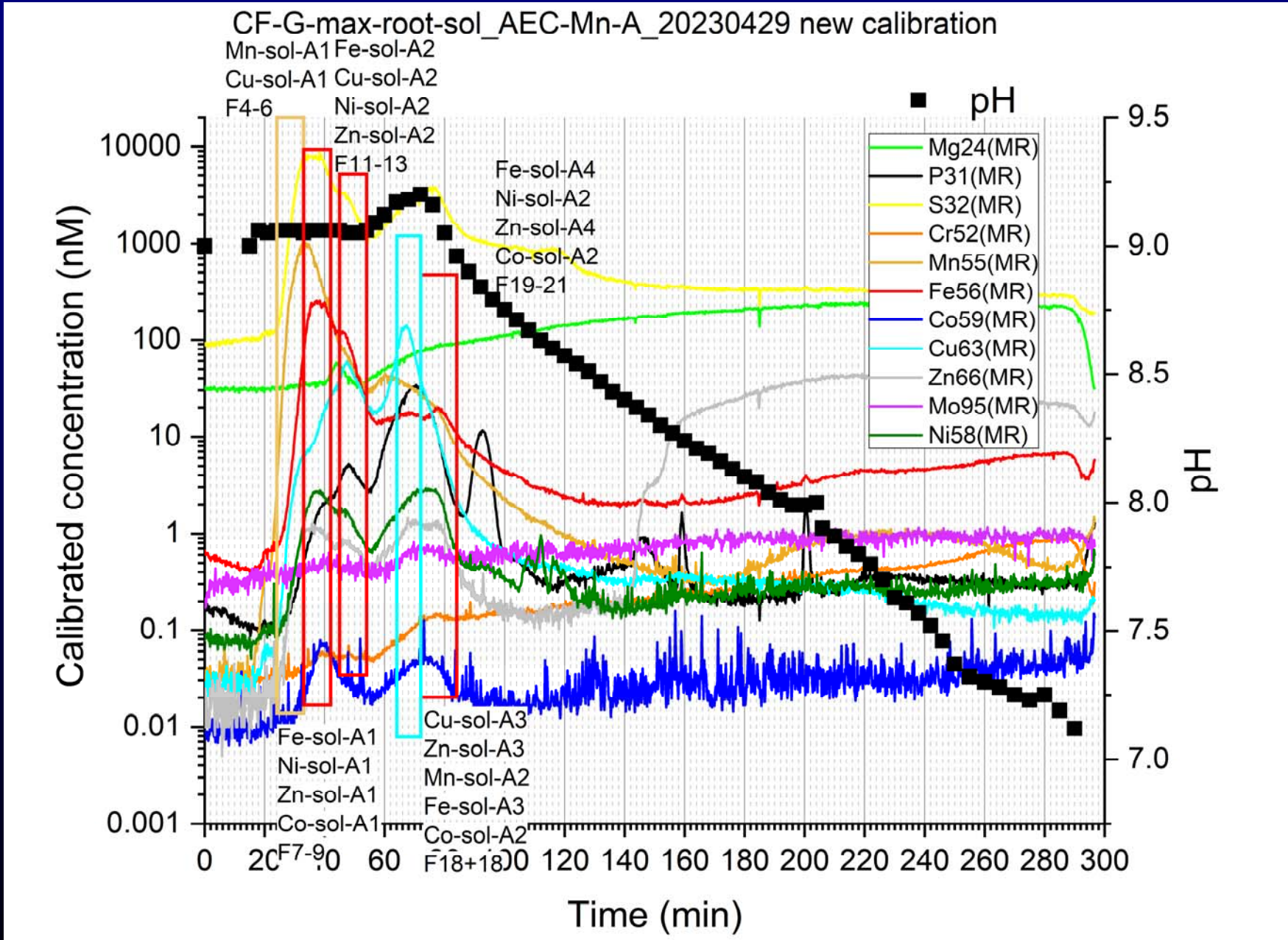
Isoelectric focussing chromatography = chromatofocussing

- Isoelectric focussing (IEF, using electric field) and chromatofocussing (CF, using HPLC) separate proteins by their isoelectric point. The proteins remain where the net charge of the protein is zero, i.e. balance between protonation of carboxyl groups and deprotonation of amino groups is achieved

- **Never done in combination with ICP-MS because traditional CF buffers are high-salt ICP-MS incompatible buffer formulations → test with volative buffers!**



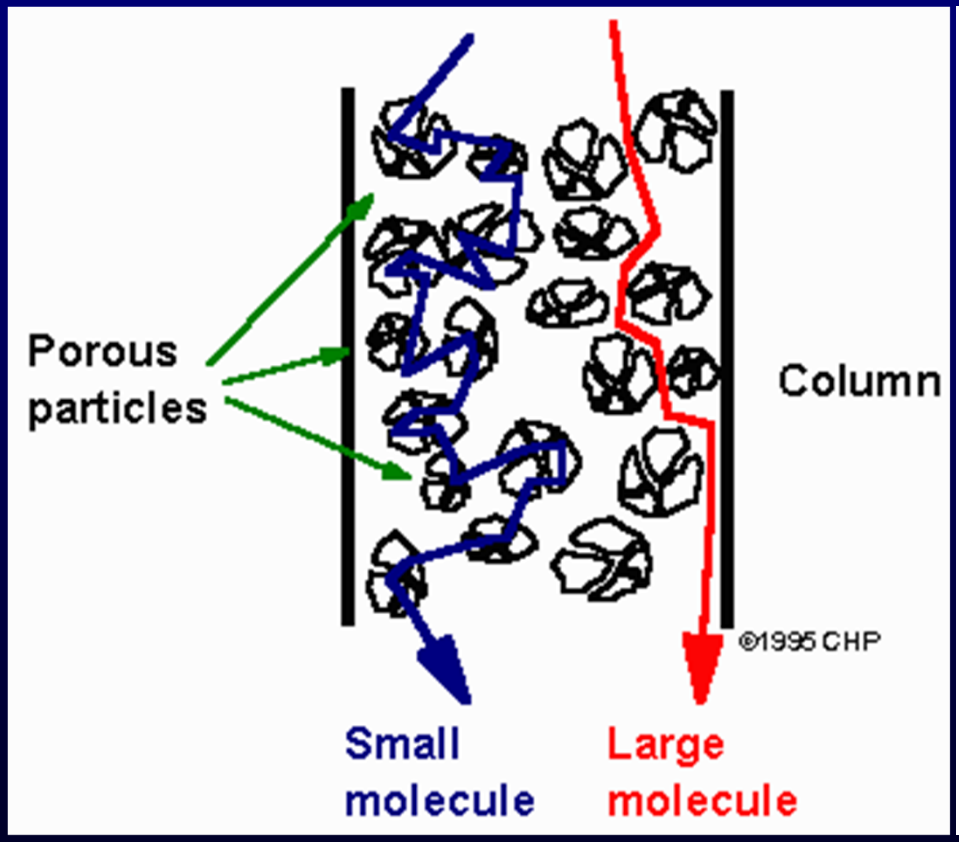
Example of metalloprotein separation by chromatofocussing chromatography (*G. max* root soluble proteins)



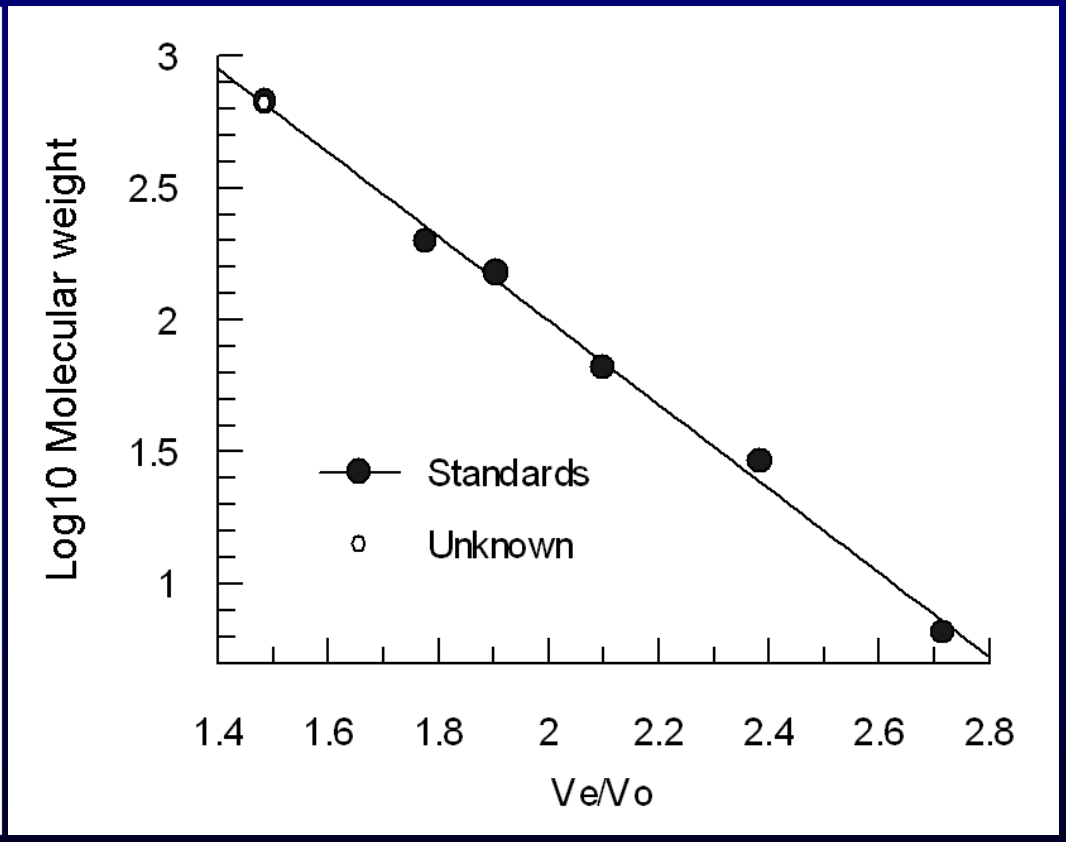
Chromatography: Types

size exclusion chromatography

Principle: Small proteins can enter more of the pores in the column material than large proteins, so that small proteins migrate **slower**



From: elchem.kaist.ac.kr



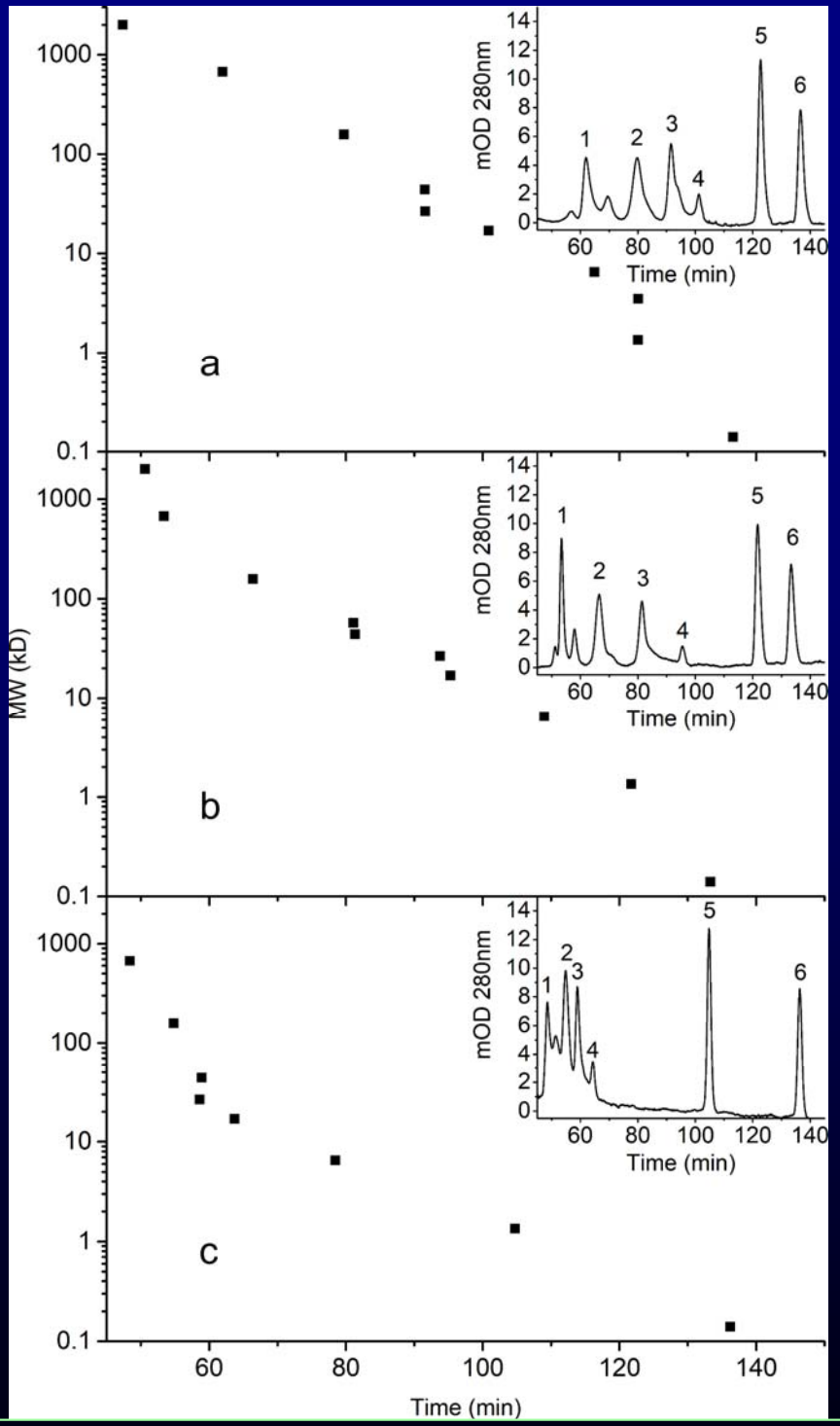
From: http://en.wikipedia.org/wiki

Chromatography: Types

size exclusion chromatography

Optimisation of column choice for different protein sizes.

(a) widest MW range with increased MW resolution in the centre: 1x Superose 6 increase, 1x Superose 12, 1x Superdex200 increase.
(b) focus on medium to high MW resolution: 1x Superdex75 increase, 2x Superdex200 increase.
(c) focus on ultra-low MW range but medium MW range covered: 2x Superdex 30 increase, 1x Superose 12.



Küpper H, Hussain Bokhari SN, Jaime Perez N, Lyubenova L, Ashraf N, Andresen E (2019) Analytical Chemistry 91, 1710961-10969

III) Protein Identification: Overview of Principles

Size determination

- Size exclusion chromatography or SDS PAGE
- Comparison with expected size of protein (known e.g. from reference or cDNA)

Western Blotting

- Binding to specific primary antibody, detected via labelled or enzymatically active secondary antibody

Biochemical assays in native gels

- Identification of enzymes by their characteristic activity
- Identification of metalloproteins by their metal content

Mass Spectrometry

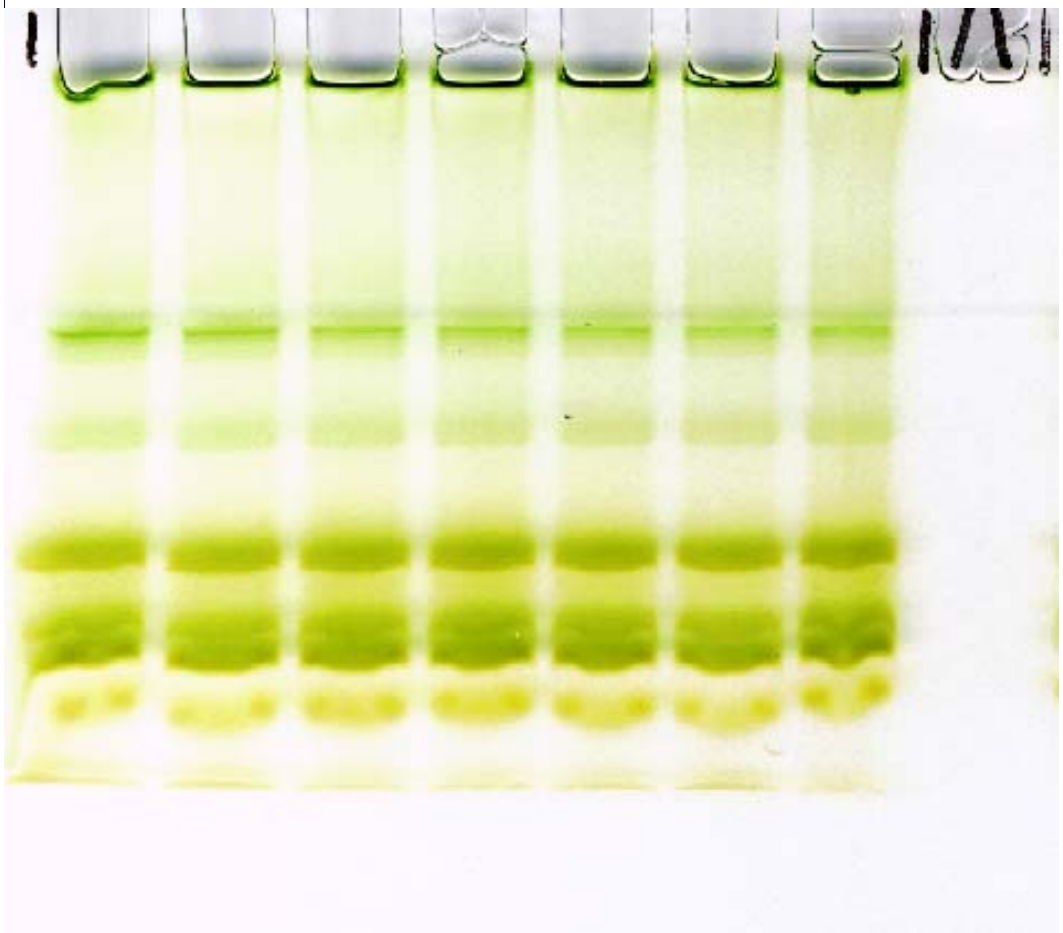
- Fragmentation of the protein, identification of fragment sizes, and subsequent comparison to a library of known fragmentation patterns

N-terminal Seqencing (Edman degradation)

- Sequential chemical removal of individual amino acids from the N-terminus

I) Separation by size in native gels

- Principle: Small proteins are less retained by the fibers of the gel than large proteins, so that small proteins migrate **faster**



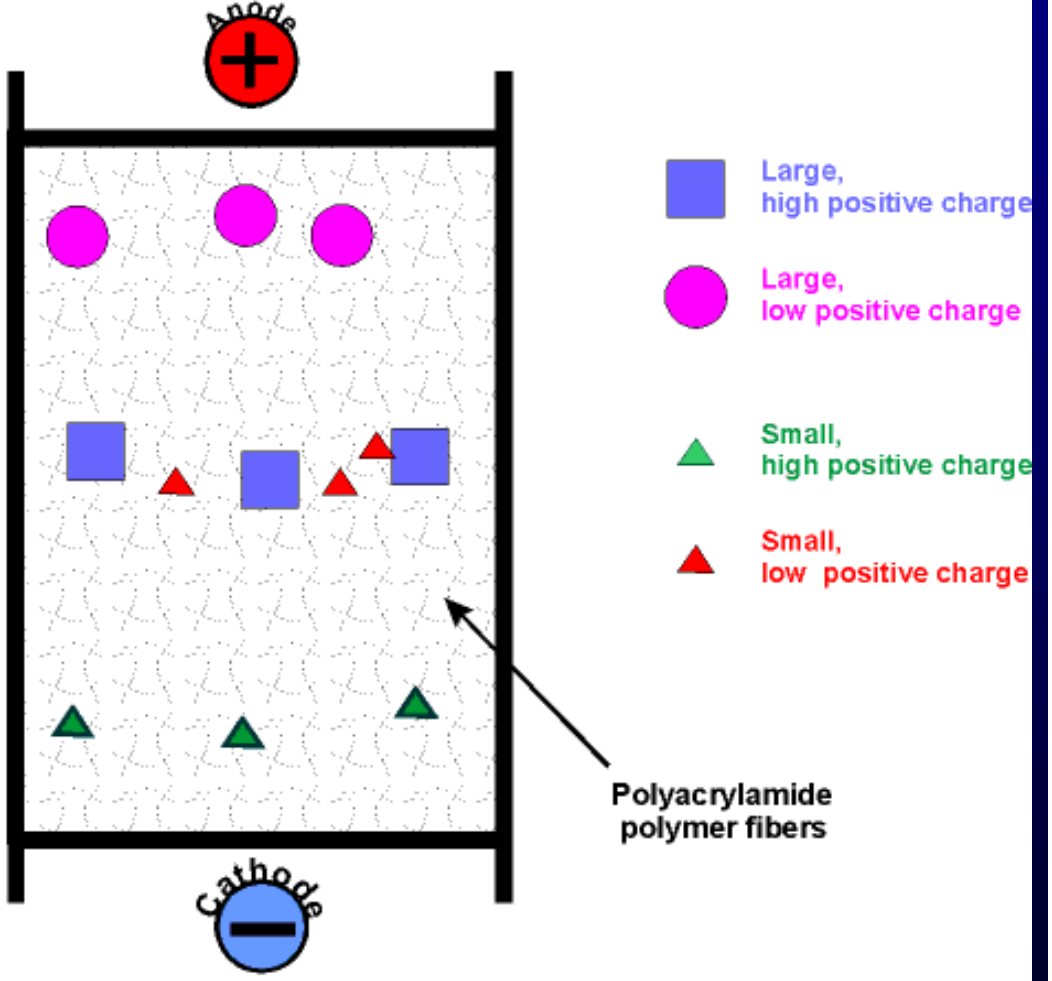
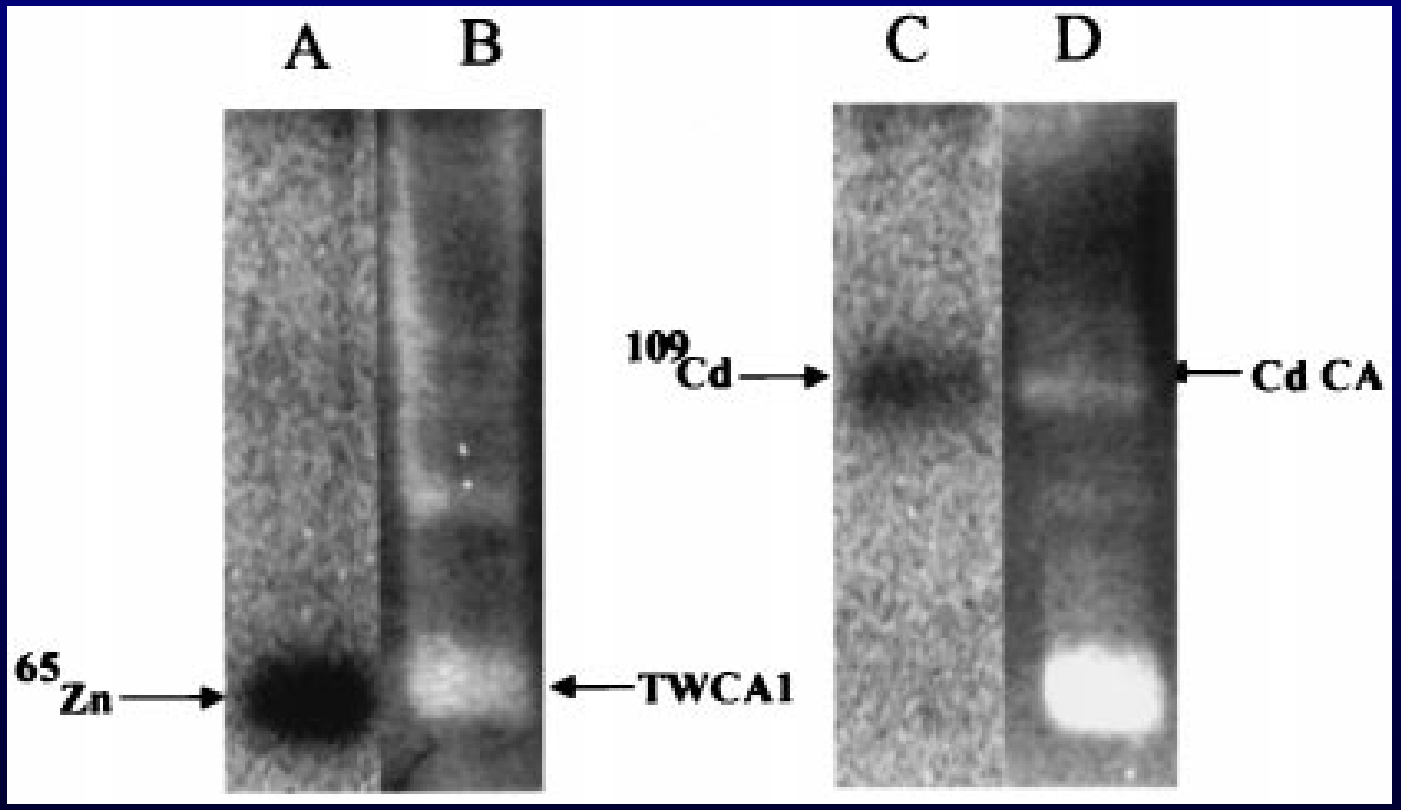


foto of a green gel (Küpper et al., 2003, Funct Plant Biol)

From: www.columbia.edu/.../c2005/lectures/lec6_09.html

III) Protein Identification by assays in native gels

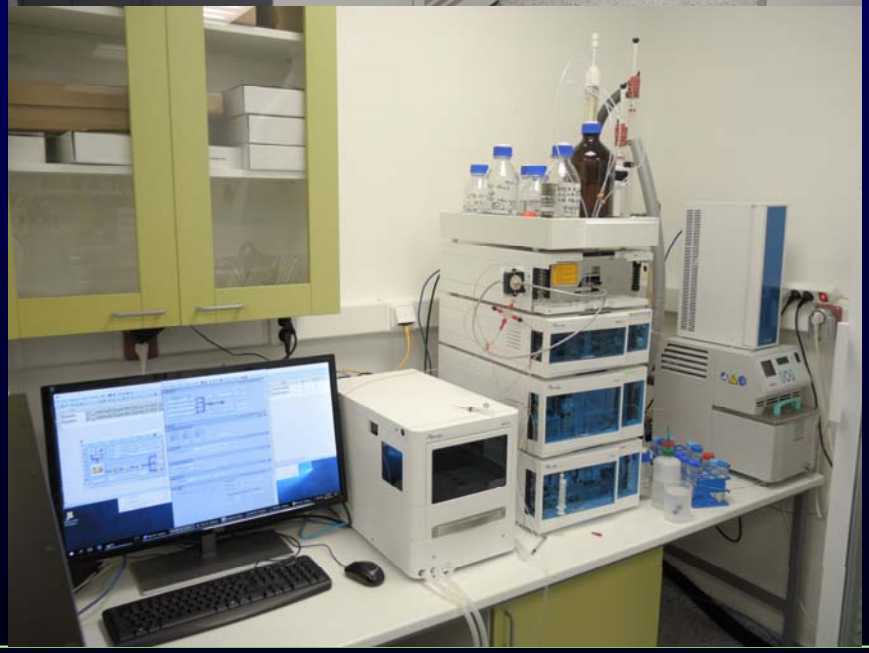
- Example of a biochemical assay in native gels: identification of Cd-Carboanhydrase by its metal content combined with an in-gel carboanhydrase assay

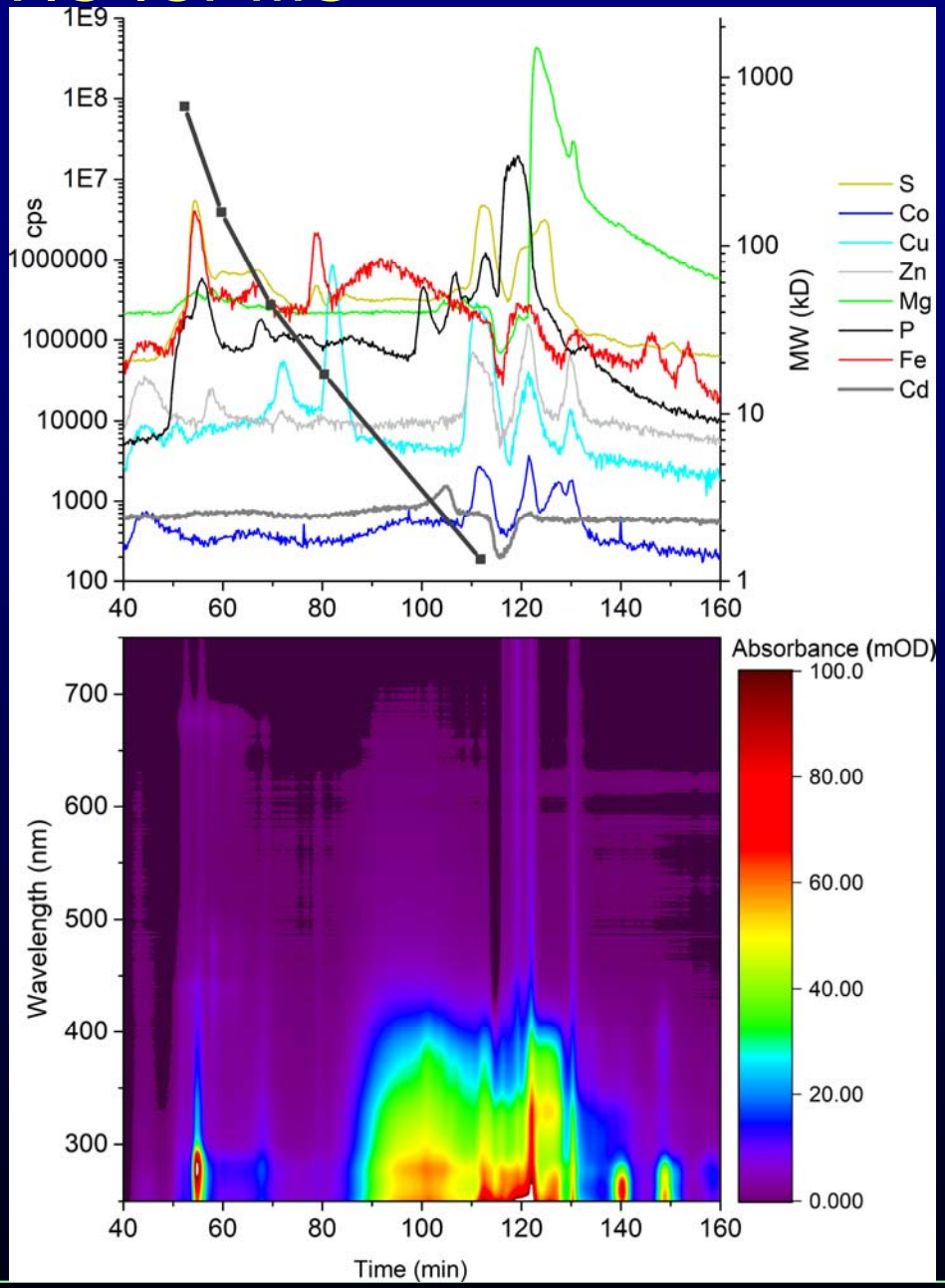


from: Lane TW, Morel FMM (2000) PNAS97, 4627-4631

III) Protein Identification by HPLC-ICPMS

HPLC-UVVIS-ICPMS



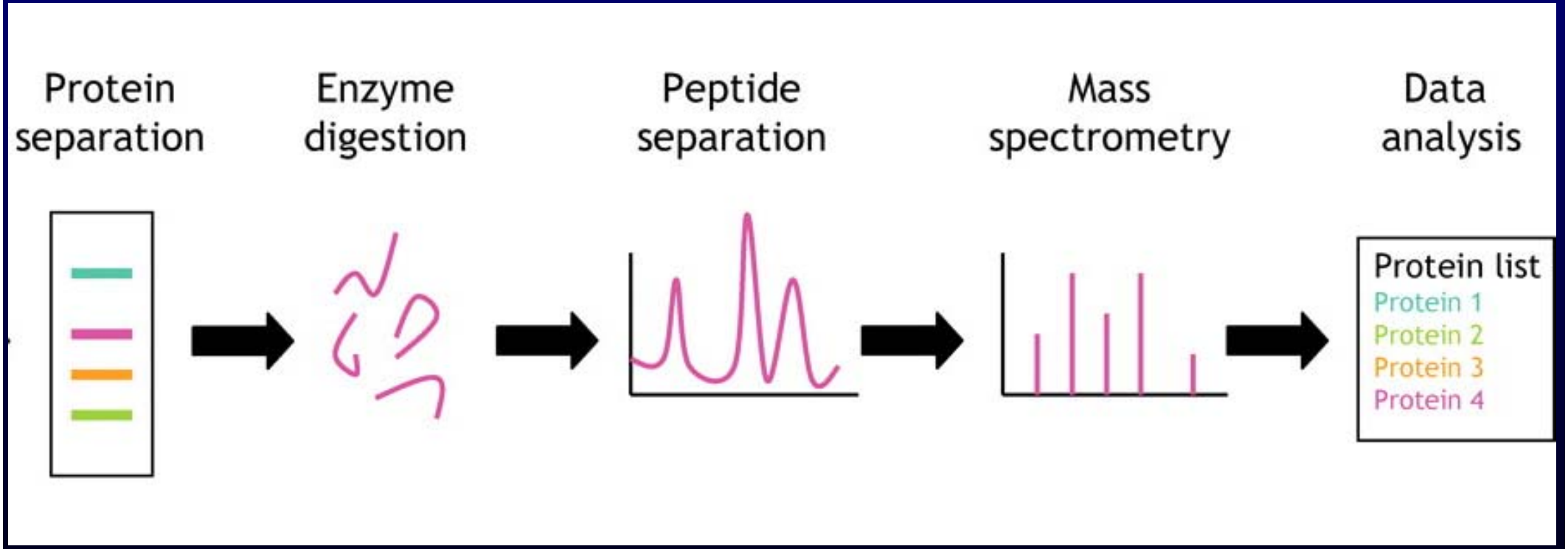


Left: Photo from our lab; *right:* Küpper H, Hussain Bokhari SN, Jaime Perez N, Lyubenova L, Ashraf N, Andresen E (2019) Analytical Chemistry 91, 1710961-10969

III) Protein Identification: Mass Spectrometry

Principle

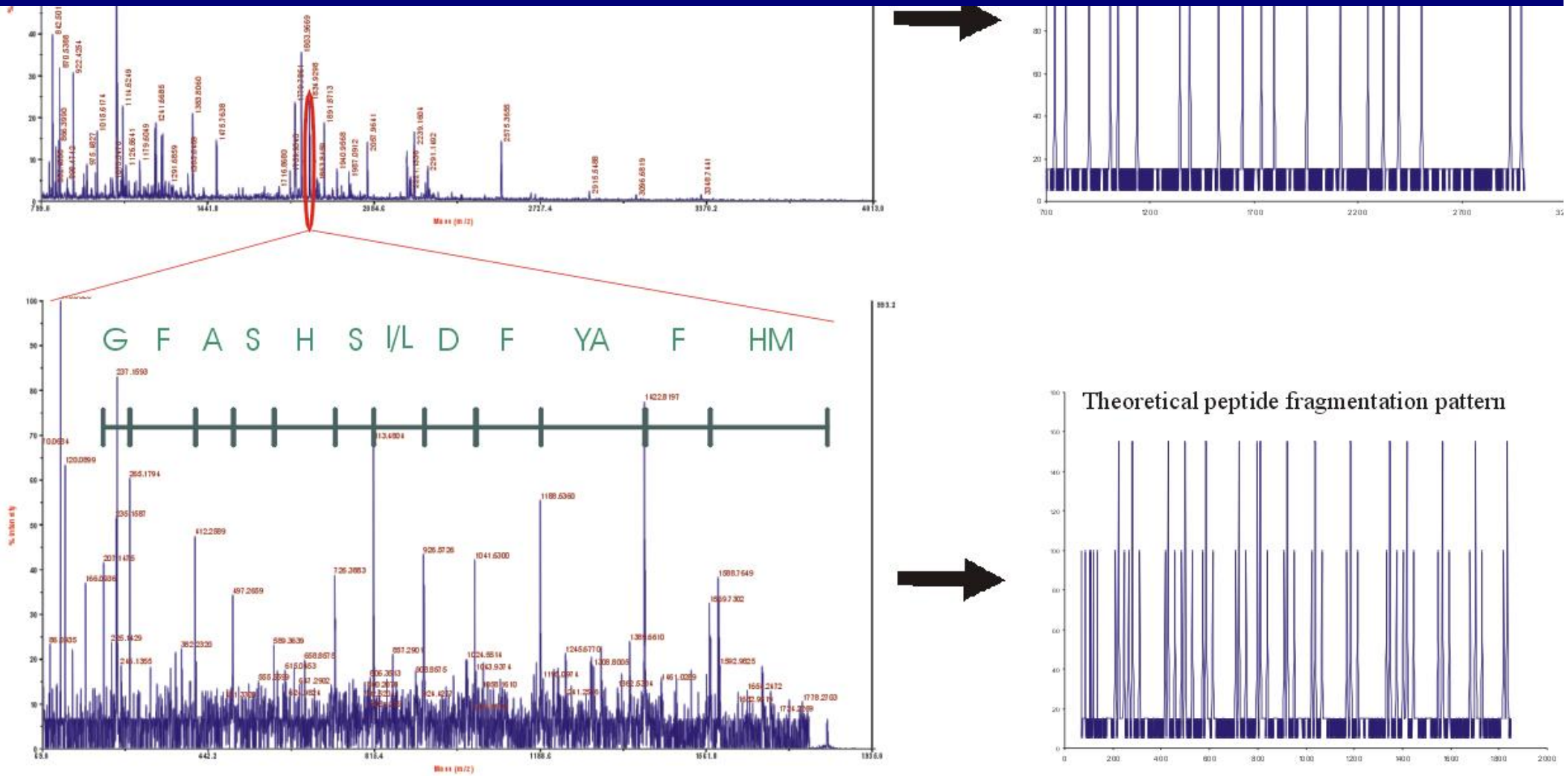
- 1) protein band is cut of out gel
- 2) protein is digested into peptides
- 3) mass spectrometry
- 4) comparison of the fragment sizes with a database
- 5) assignment of likely sequences to fragments
- 6) comparison of the fragment sequences with a database
- 7) result: list of proteins from the database that have a similar fragmentation pattern



from: Gingras AC et al, 2004, J Physiol 563, 11-21

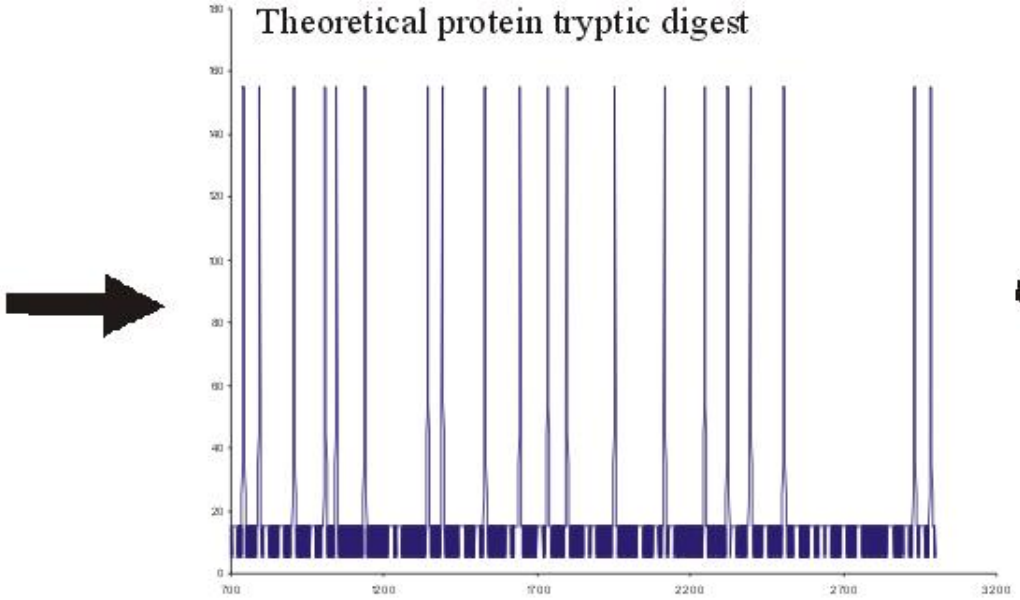
III) Protein Identification: Mass Spectrometry

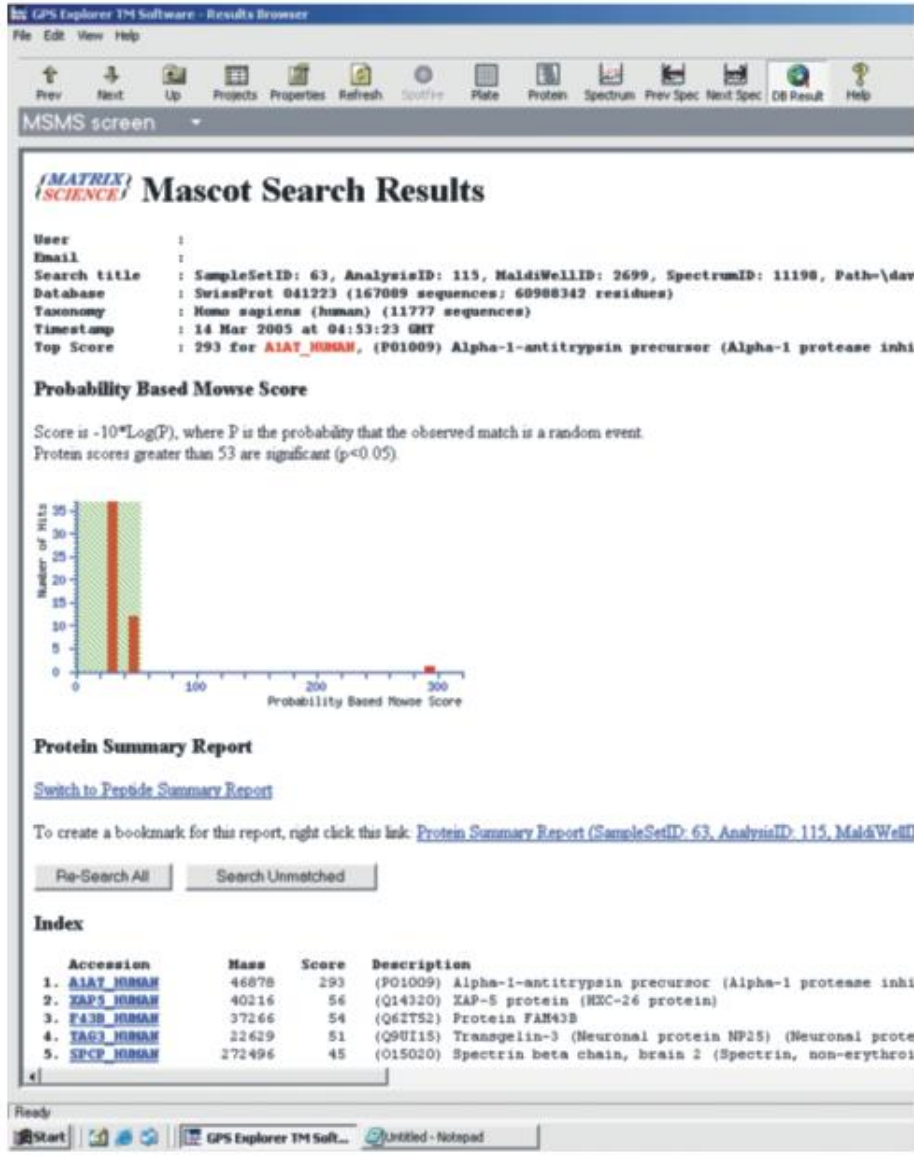
- 3) further fragmentation of one of the fragments from the digest in the mass spectrometer, then again mass spectrometry (MSMS)
- 4) comparison of the fragment sizes with a database



from: http://www.med.monash.edu.au/biochem/facilities/proteomics/maldi.html

III) Protein Identification: Mass Spectrometry





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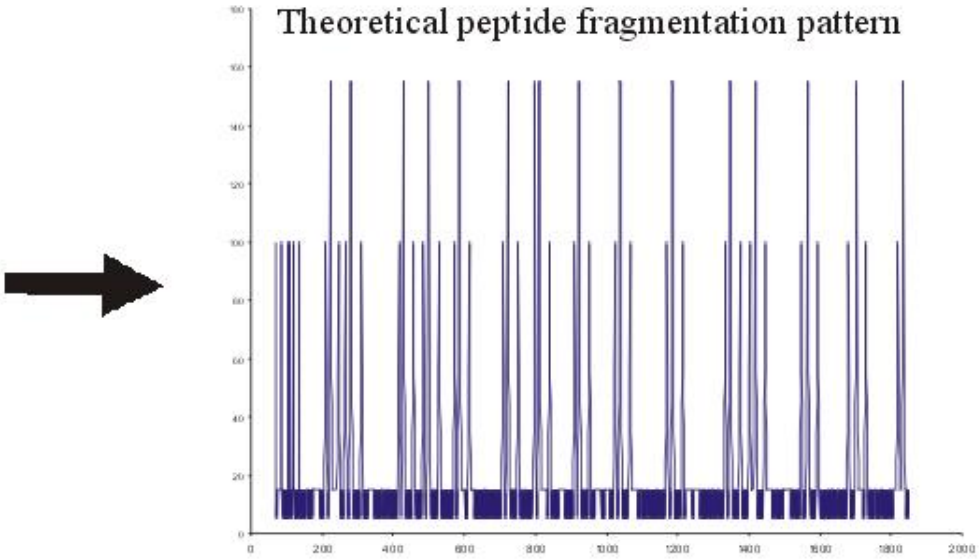
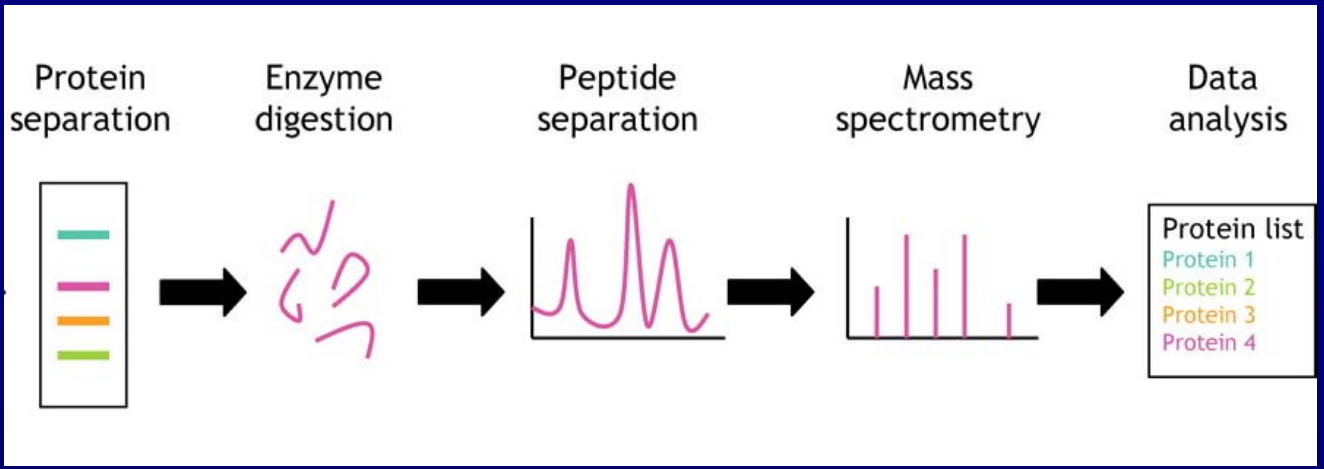


from: http://www.med.monash.edu.au/biochem/facilities/proteomics/maldi.html

III) Protein Identification: Mass Spectrometry



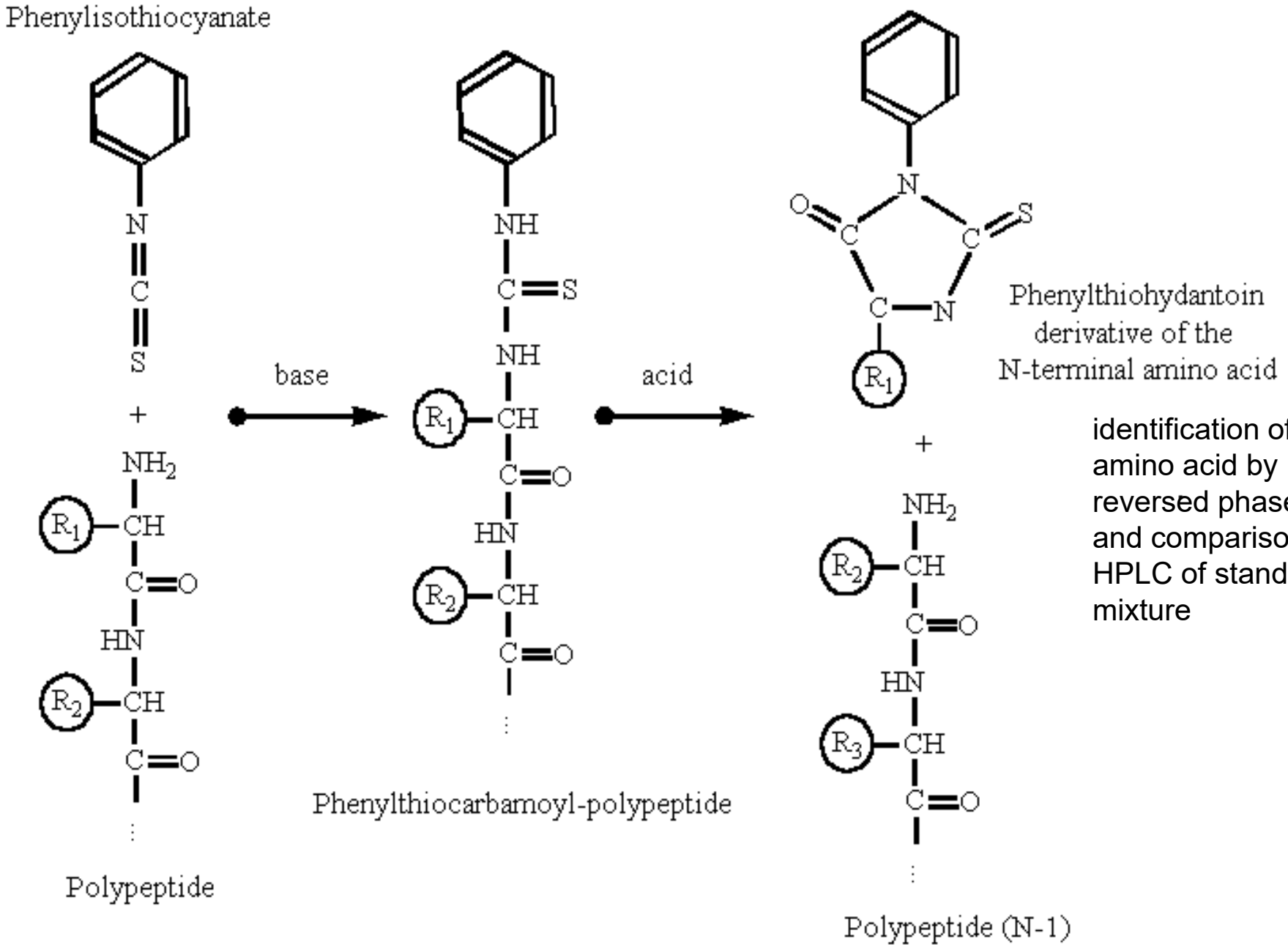
from: Gingras AC et al, 2004, J Physiol 563, 11-21

Limitations and artefacts

- contamination of gel bands with other proteins → identification of the contamination (very common: keratin from skin!) instead of the protein of interest
- not all proteins have the same detection efficiency (problems e.g. if many cysteines present) → even small contaminations sometimes lead to wrong identifications
- not all proteins are in the databases → database may show results that have a similar fragmentation pattern, but are otherwise unrelated

III) Protein Identification: N-terminal sequencing

Principle



identification of the amino acid by reversed phase HPLC and comparison with HPLC of standard mixture

from: www.ufrgs.br/depbiot/blaber/section4/section4.htm

III) Protein Identification: N-terminal sequencing

Sample preparation

- 1) run acrylamide gel (denaturating or native)
- 2) blot onto PVDF (NOT nitrocellulose) membrane using glycine-free buffer
- 3) stain the membrane with ponceau red (CBB and other stains also work)
- 4) submit for sequencing

Limitations and artefacts

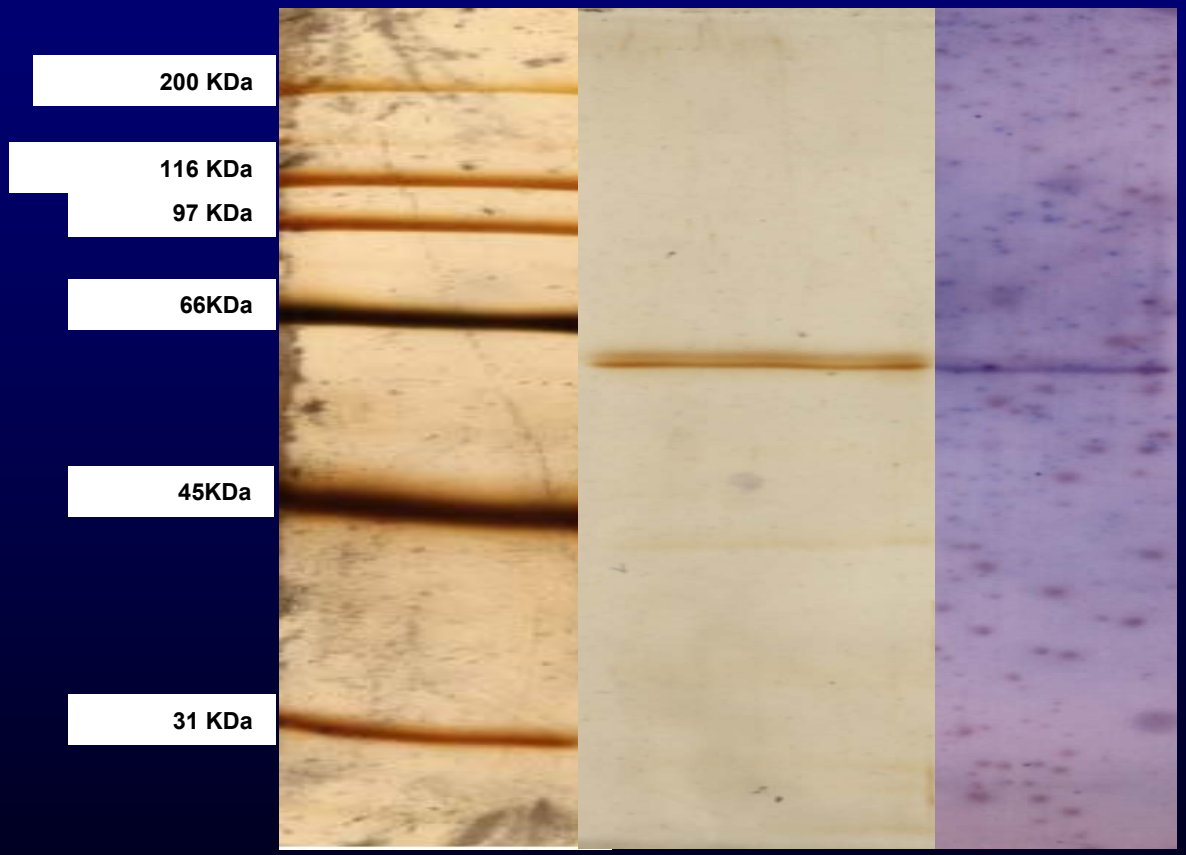
- problems with contaminations
- in eukaryotic proteins, the N-terminus is often blocked (e.g. by methylation), which required complicated de-blocking procedures. Also non-polymerised acrylamide remains in the gel can cause blocking, so let the gel polymerise over night
- Cys residues cannot be detected, and also glycosylated redidues may appear as blank spaces in the sequence

IV) Protein Characterisation: Overview of Principles

- Size determination
- Charge determination
- Analysis of cofactors
- Analysis of the 3-dimensional Structure
- Activity tests

IV) Protein Characterisation: Size determination

Comparison with predicted size by native and denaturating gel electrophoresis and by size exclusion chromatography can show native oligomerisation, post-translational modification but also artefactual degradation/aggregation



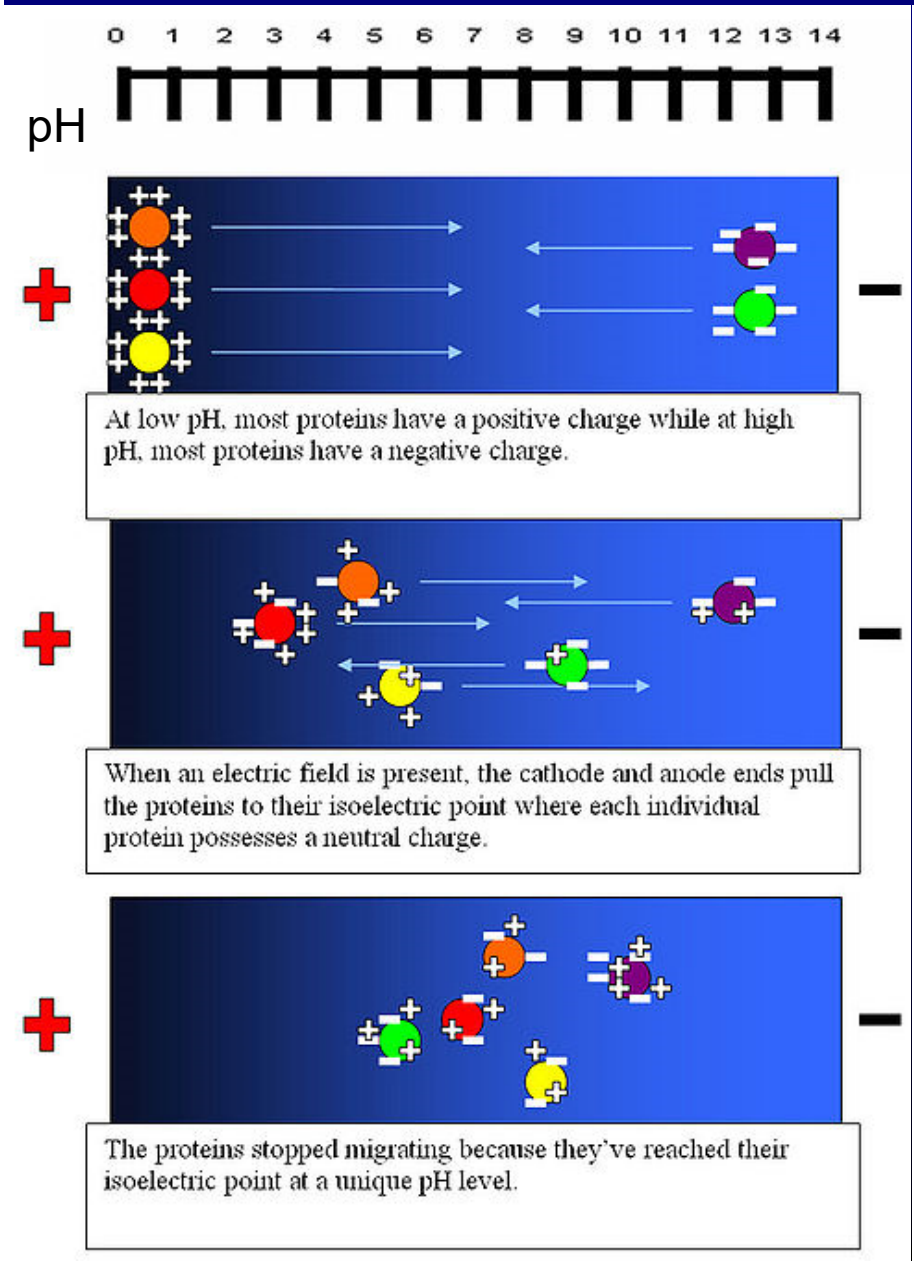
SDS gel and Western blot of TcHMA4

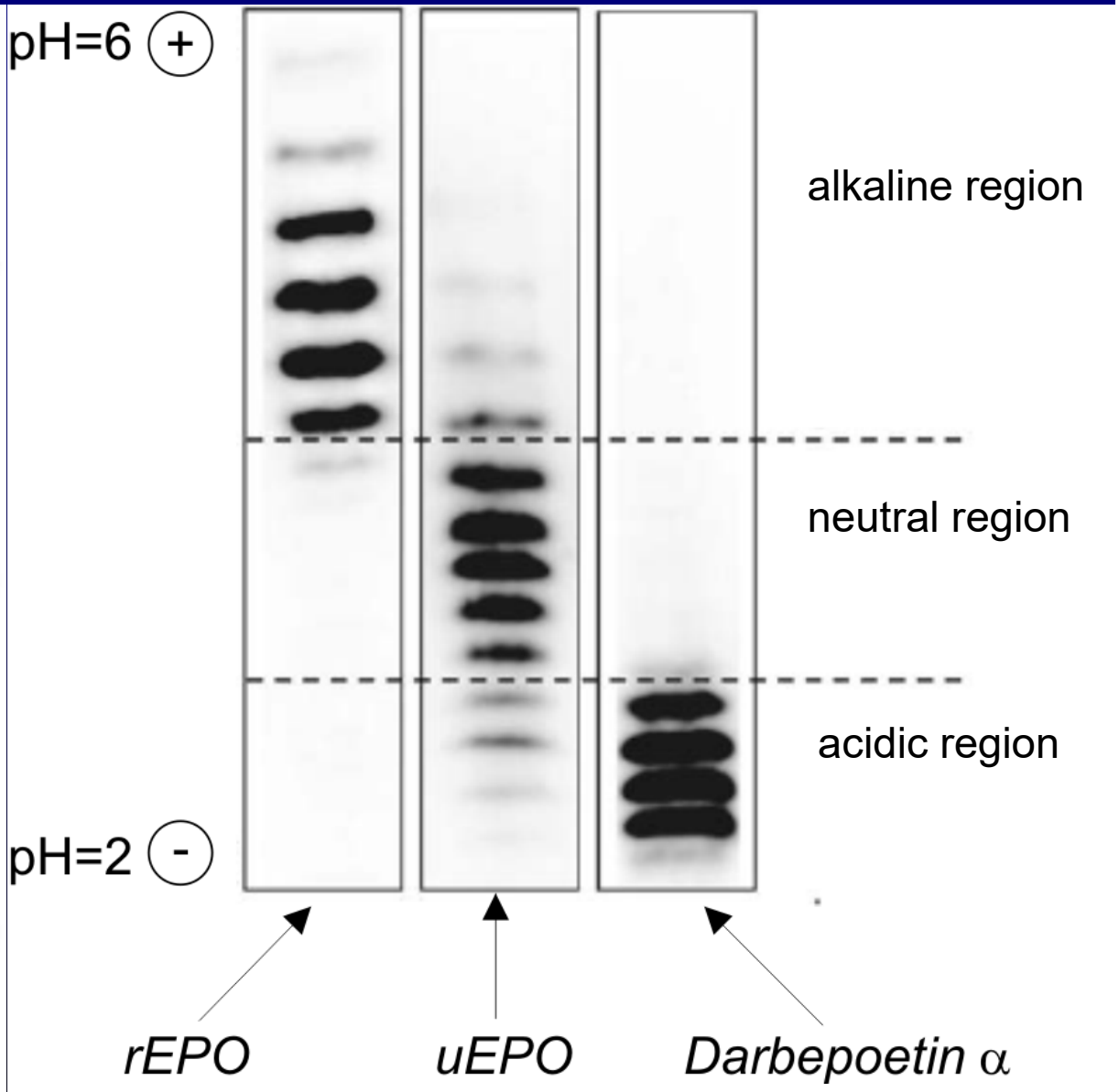
→ size shows post-translational processing as cDNA sequence predicts 128kDa

(Parameswaran, Leitenmaier et al., 2007, BBRC)

IV) Protein Characterisation: Charge determination

- Isoelectric focussing (IEF) can reveal the isoelectric point where the net charge of the protein is zero, i.e. balance between protonation of carboxyl groups and deprotonation of amino groups is achieved

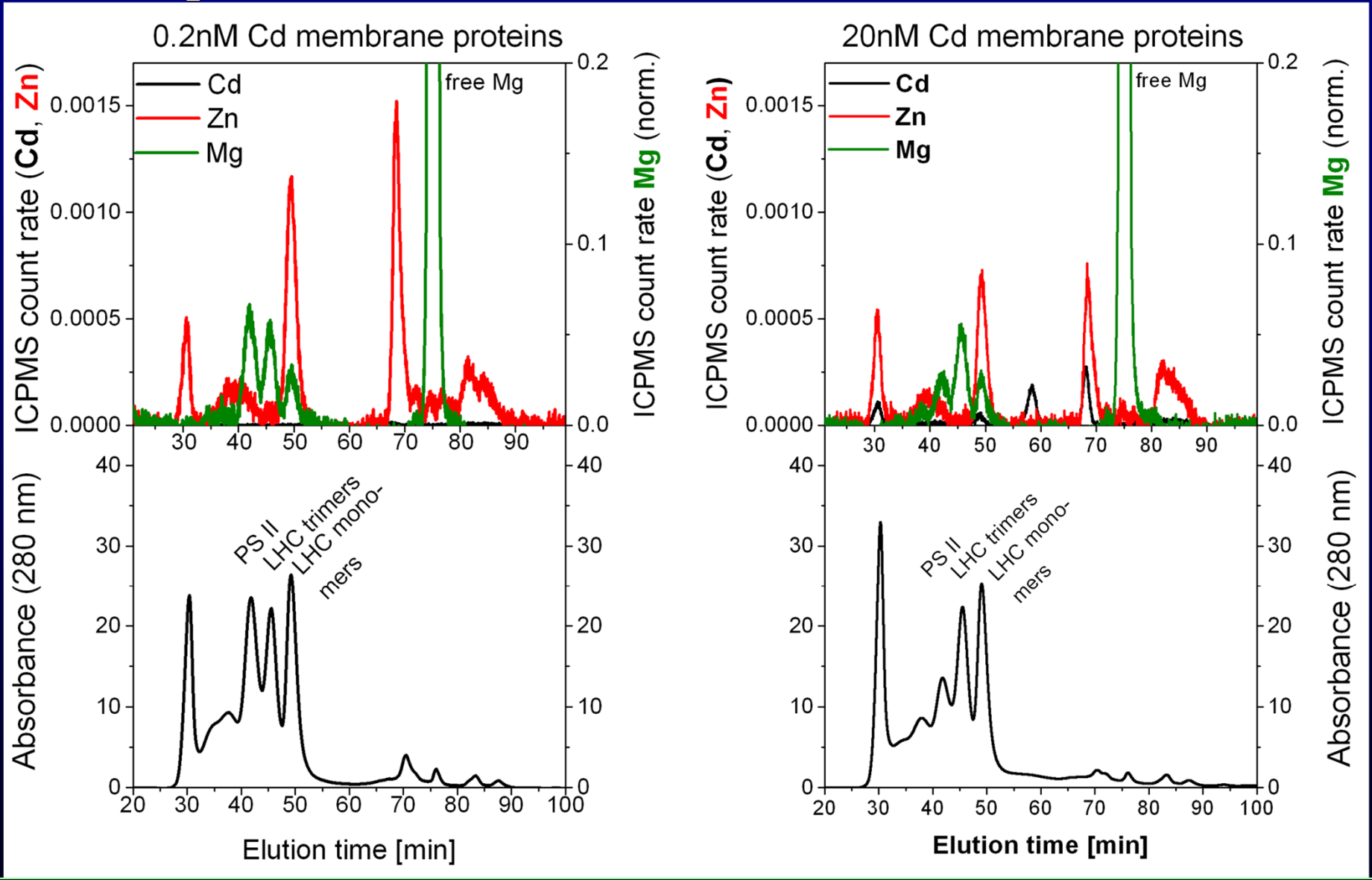




from: commons.wikimedia.org

IV) Analysis of cofactors by HPLC-ICPMS

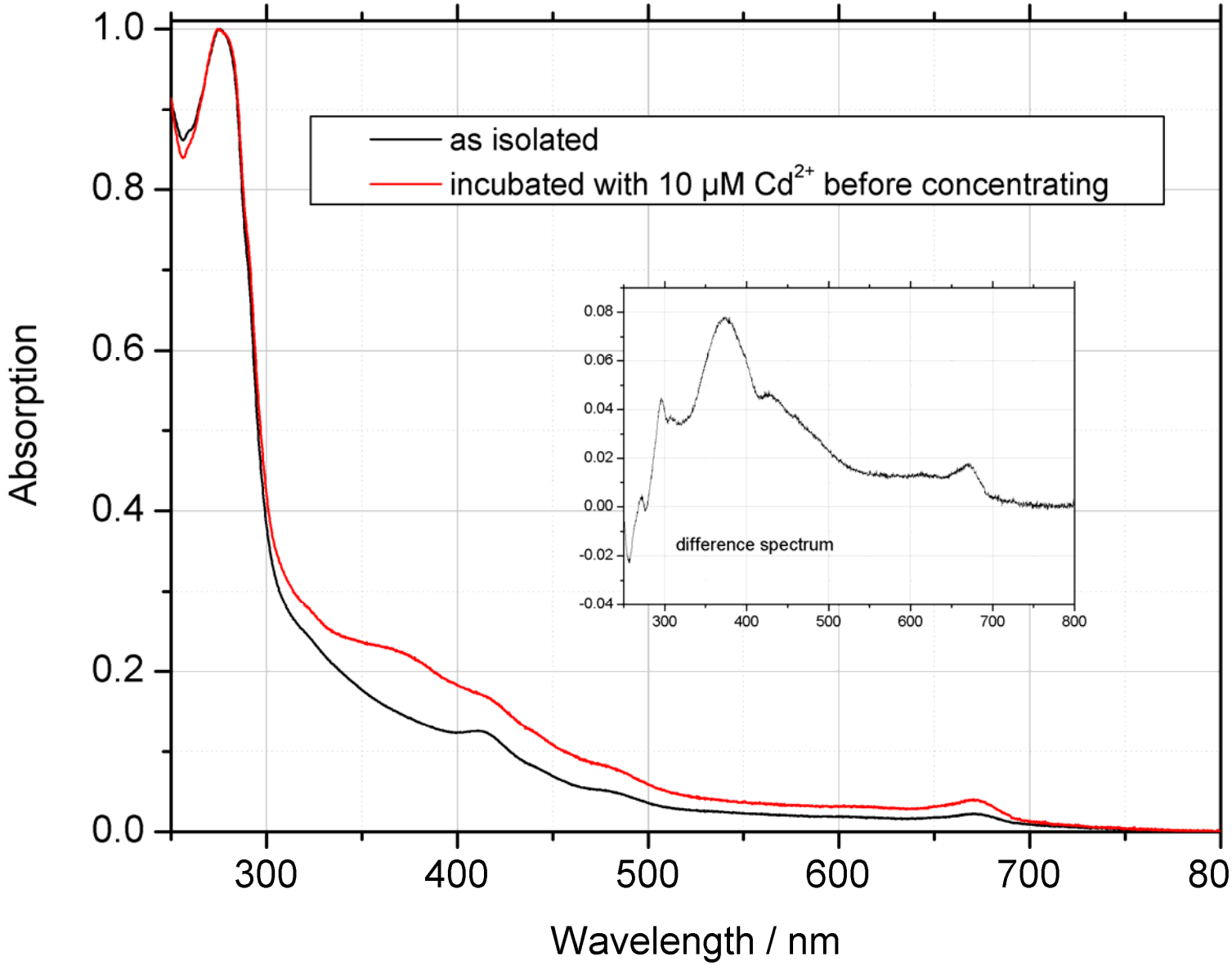
- Example of a metalloprotein characterisation by HPLC-ICPMS → Cd binding to main light harvesting antenna LHCII



Andresen E, Kappel S, Stärk HJ, Riegger U, Borovec J, Mattusch J, Heinz A, Schmelzer CEH, Matoušková Š, Dickinson B, Küpper H (2016) New Phytologist 210, 1244-1258.

IV) Analysis of cofactors by UV/VIS spectroscopy

- Identification and reactivity: UV/VIS Absorption and fluorescence Spectroscopy
- Metal content (about 30% of all proteins are metalloproteins!): AAS, ICP-MS/OES, EDX/PIXE/XRF



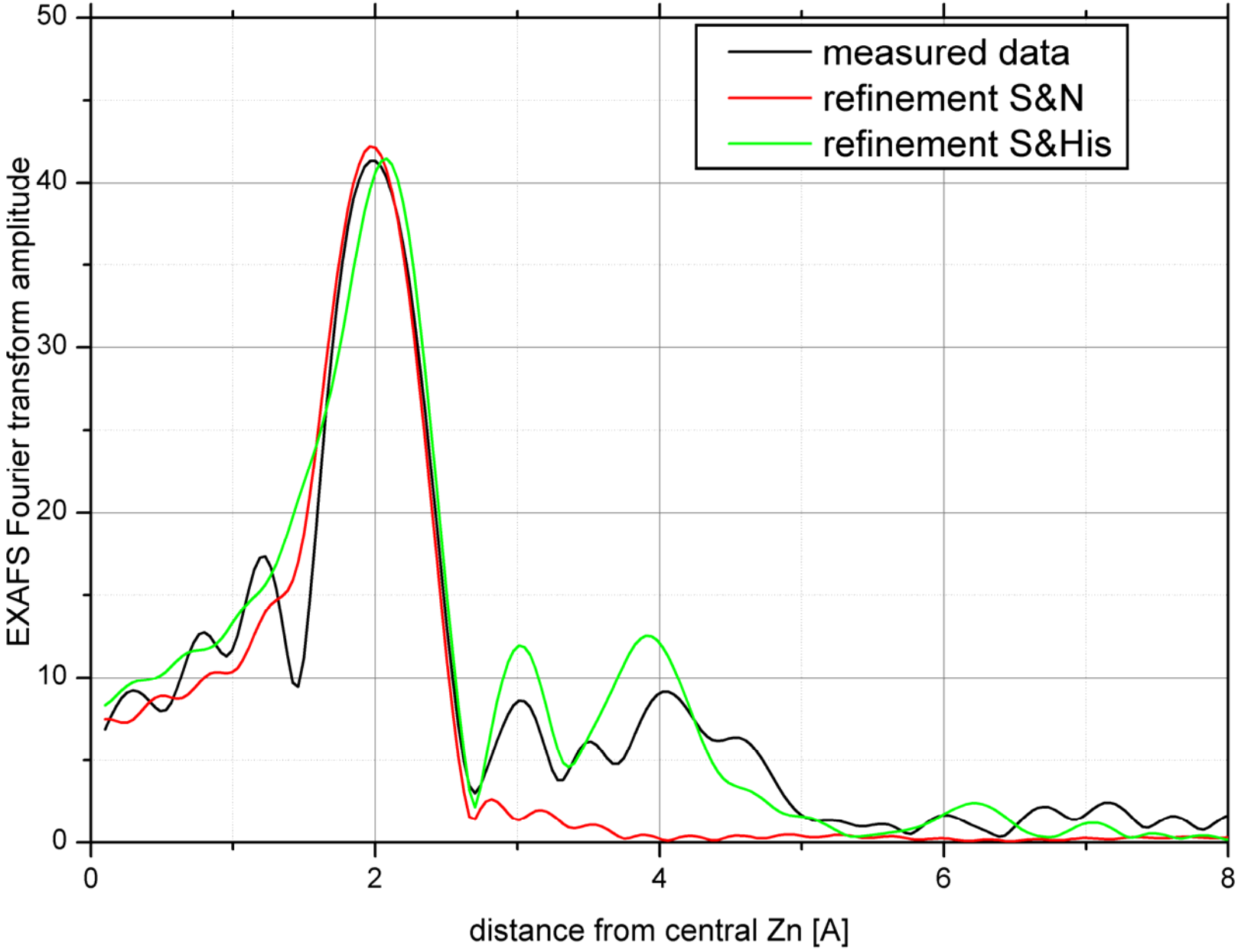
UV/VIS spectrum of TcHMA4

→ Cadmium binding causes ligand-metal-charge-transfer (LMCT) bands indicating cysteine ligation

Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta - Biomembranes 1808, 2591-2599.

IV) Analysis of cofactors by advanced spectroscopies

Metal ligands: EXAFS, EPR, heteronuclear NMR provide information about ligand types and their spatial arrangement around the active site (each of these techniques has different strenghts)



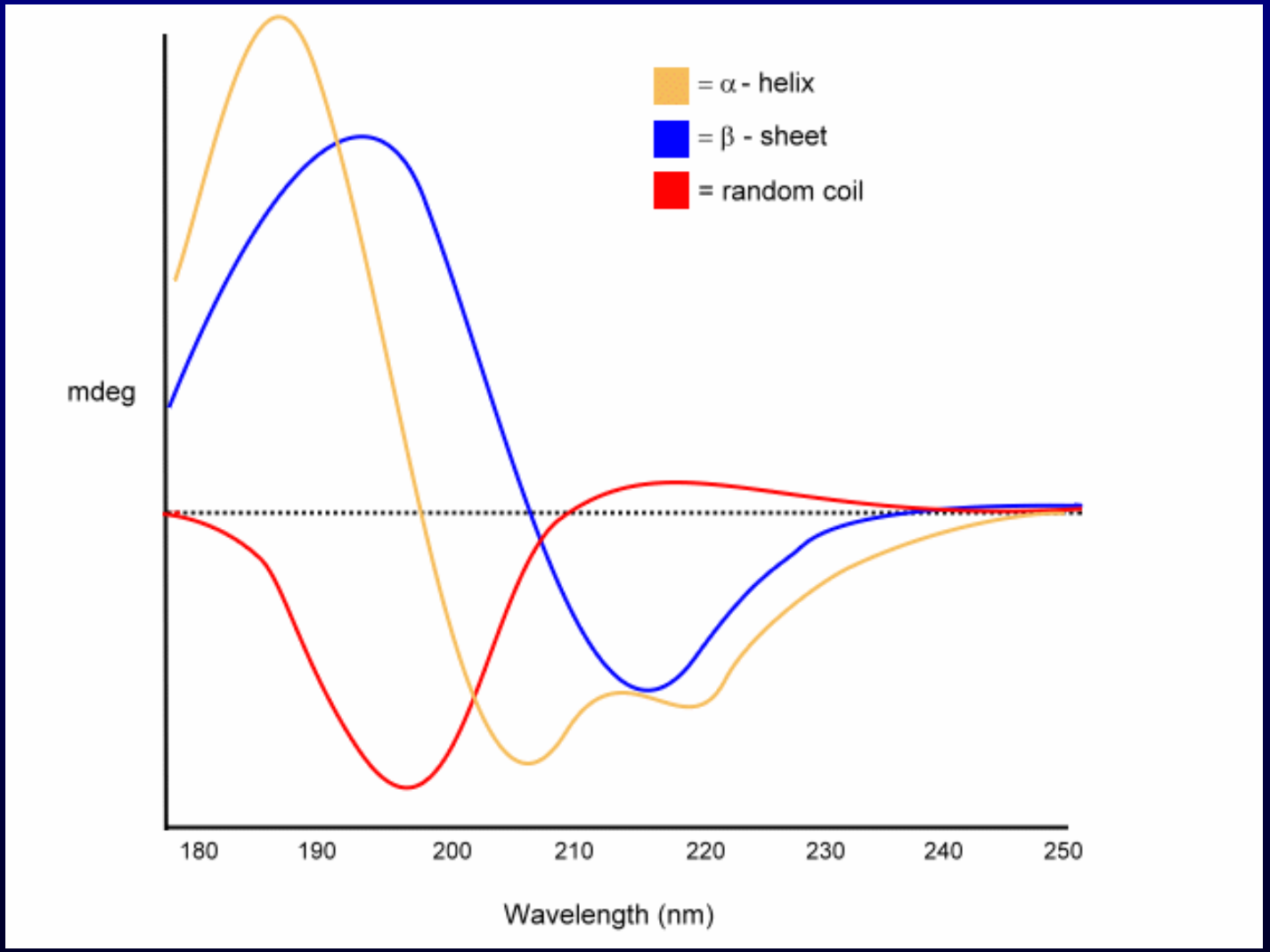
EXAFS spectrum of Zn-TcHMA4

→ mixed S/N ligands, multiple scattering shows histidine contribution

(Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta - Biomembranes 1808, 2591-2599.)

IV) Protein Characterisation: Analysis of the 3D Structure

Circular Dichroism (CD) Spectroscopy



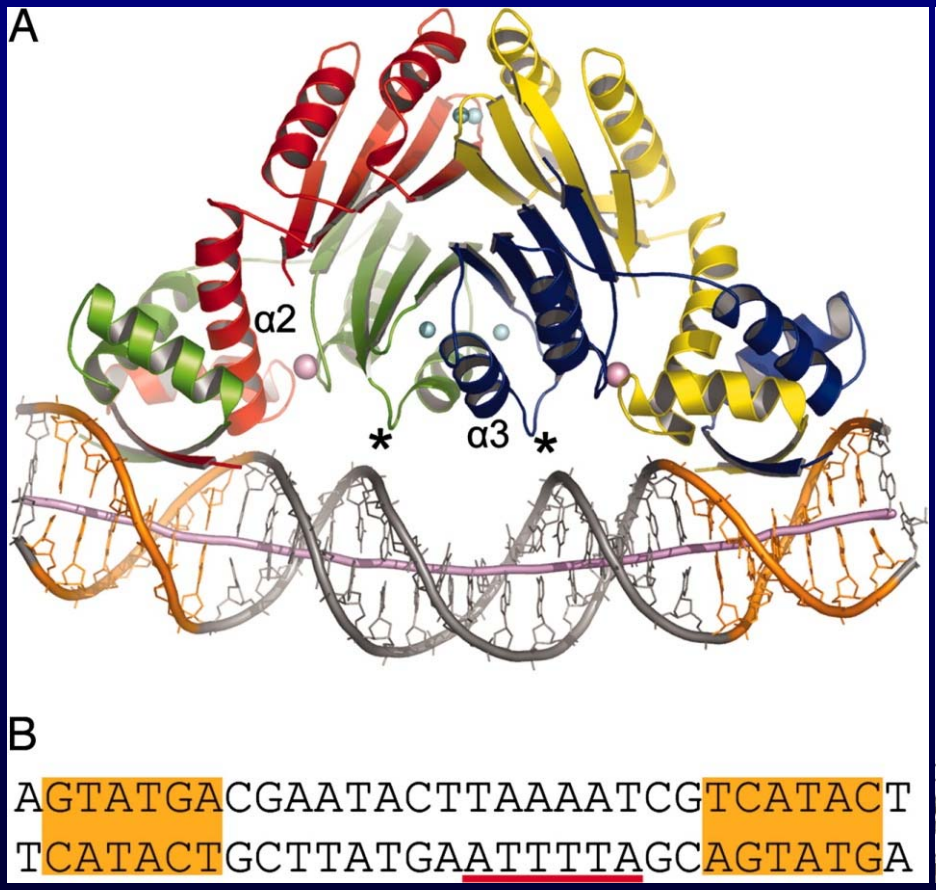
From: www.proteinchemist.com/cd/cdspec.html

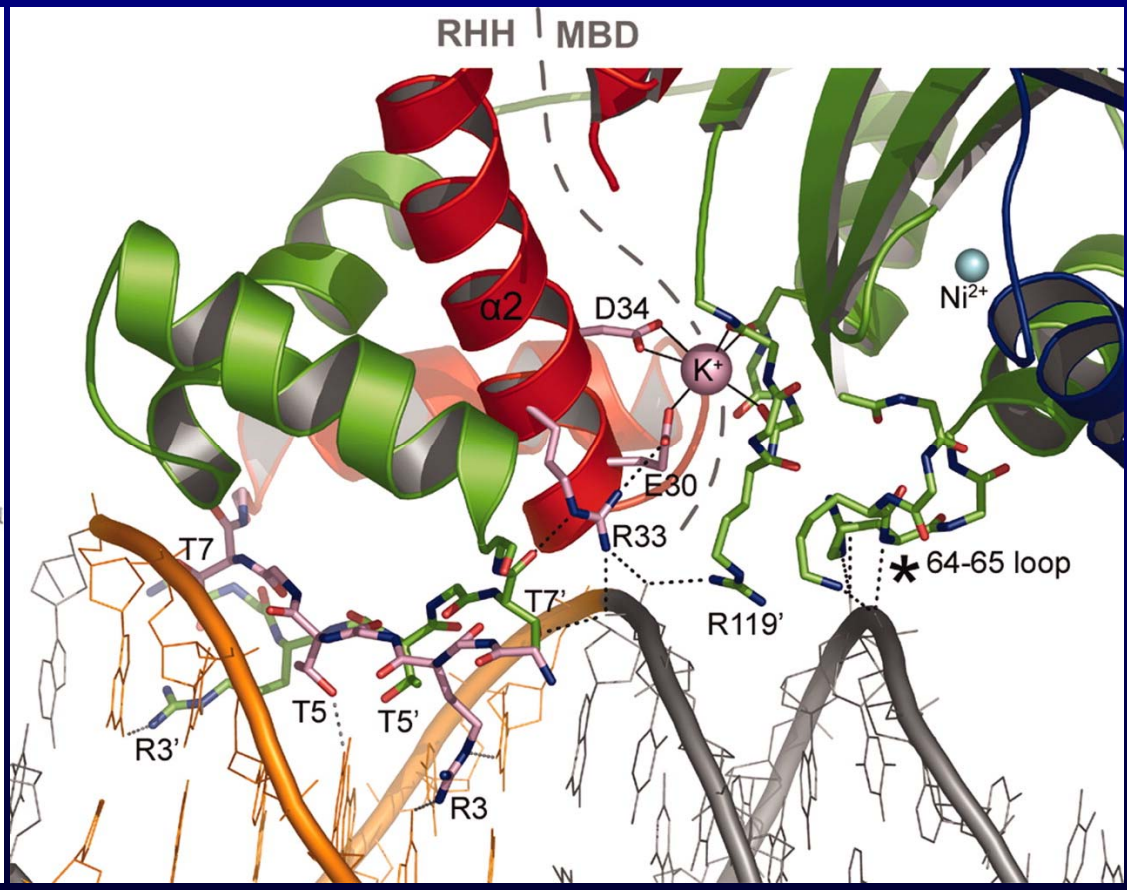
- information about proportions of secondary structure types in a protein
- particularly useful when X-ray crystallography and NMR are not applicable

IV) Protein Characterisation: Analysis of the 3D Structure

X-ray crystallography

Example: in the nickel-binding transcription factor NikR, the mechanism was concluded from the X-ray structure



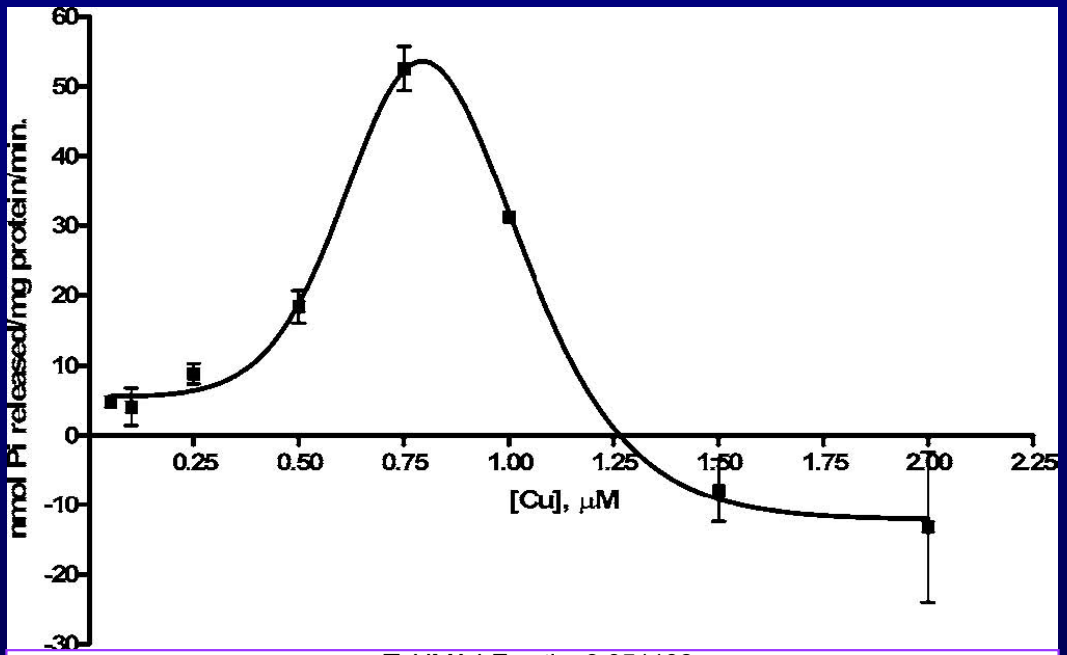


From: Schreiter ER et al., 2006, PNAS103, 13676-81

- Ni binding causes RHH domains to rotate about the flexible interdomain linkers to orient their antiparallel -strands toward the same face of the repressor, allowing each to occupy the DNA major groove of an operator palindrome half-site
- Binding of Ni creates a surface of the MBD suitable for interacting with DNA by stabilization of a helix and a loop

IV) Protein Characterisation: Activity tests

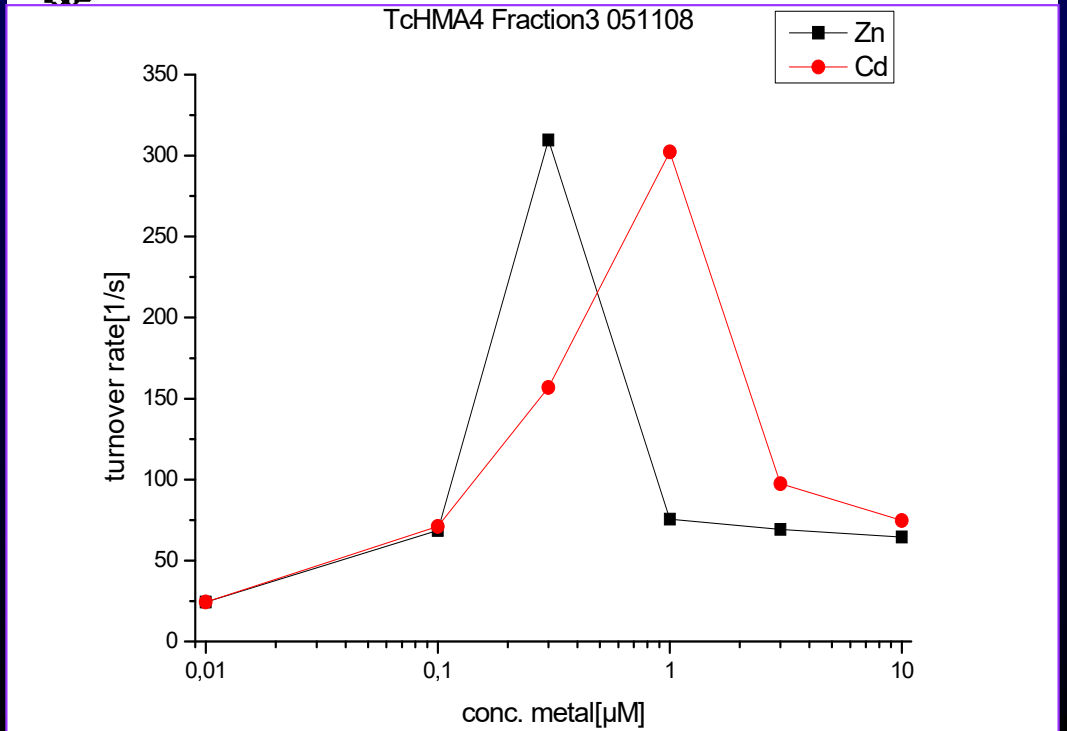
Titration of an enzyme with its substrate(s) reveals binding constants and possible substrate inhibition



Activation of a human Cu-ATPase

Hung et al. Biochem.J. 2007, 401

Both are P_{1B}-Type ATPases showing the same activation pattern by „their“ metal after reconstitution into artificial lipid vesicles

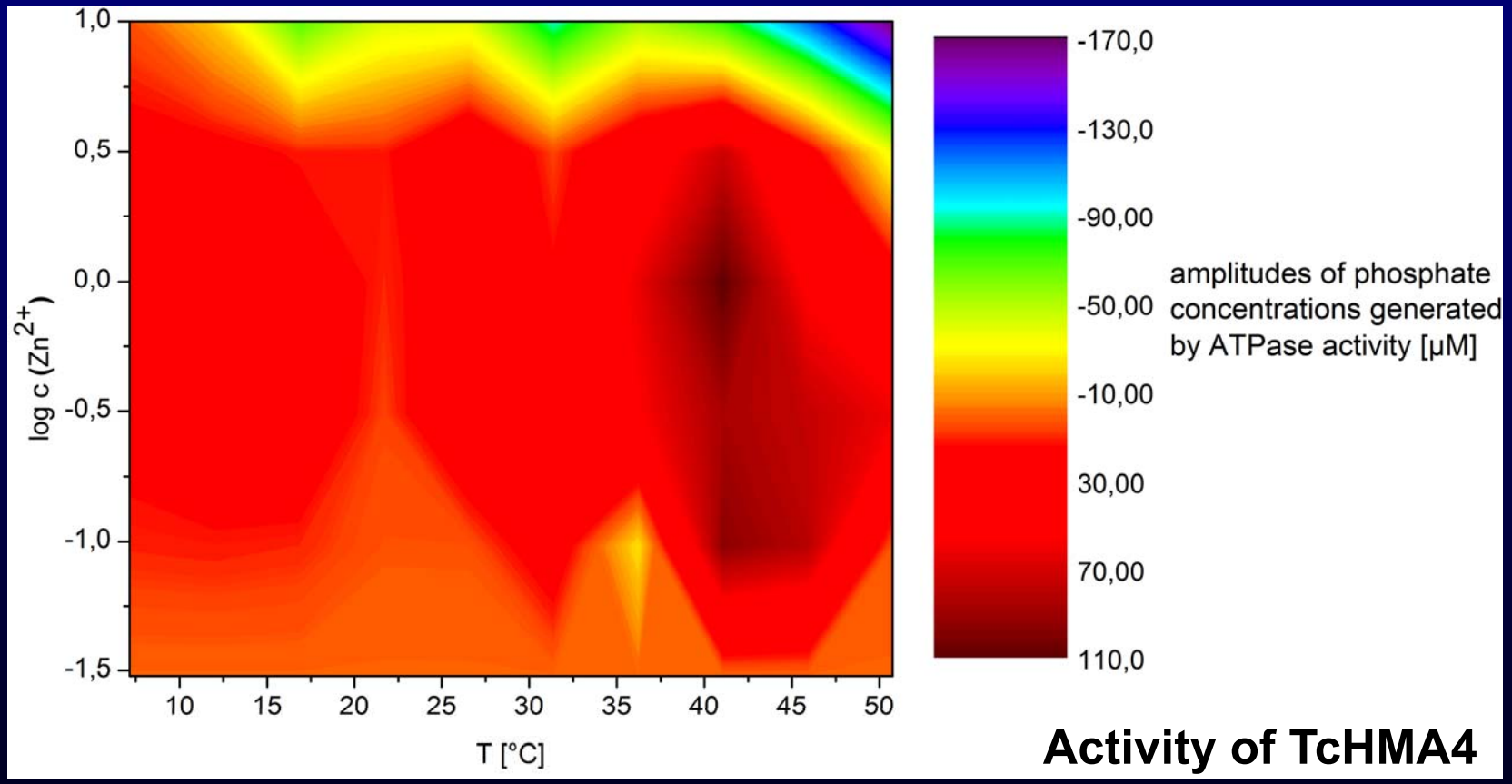


Activity of TcHMA4

(Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta - Biomembranes 1808, 2591-2599)

IV) Protein Characterisation: Activity tests

Two-dimensional data, e.g. substrate concentration and temperature reveal insights into interactions between factors that are decisive for enzyme activity, e.g. temperature dependence of substrate binding and influence of the substrate on the thermostability of the enzyme

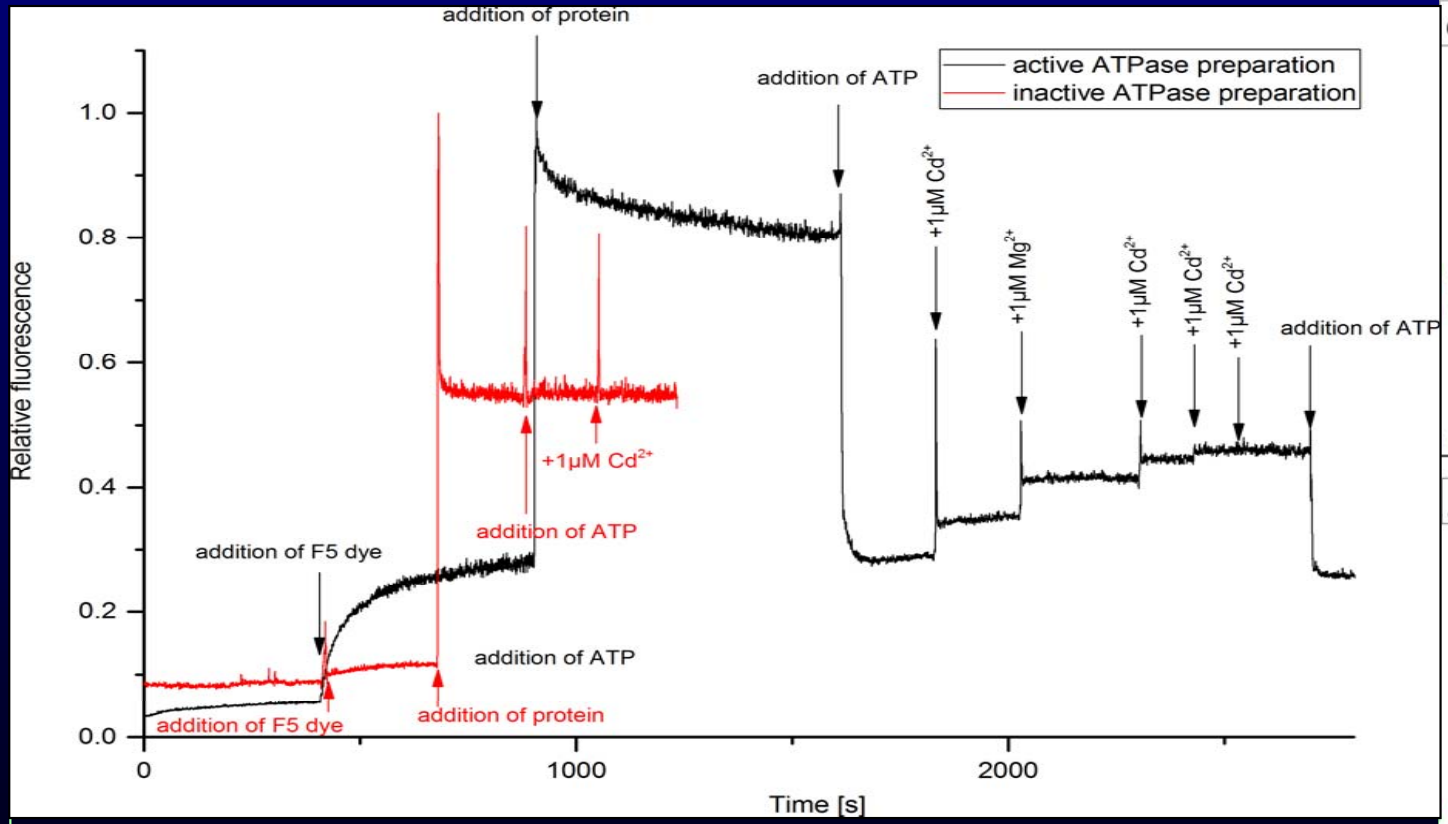


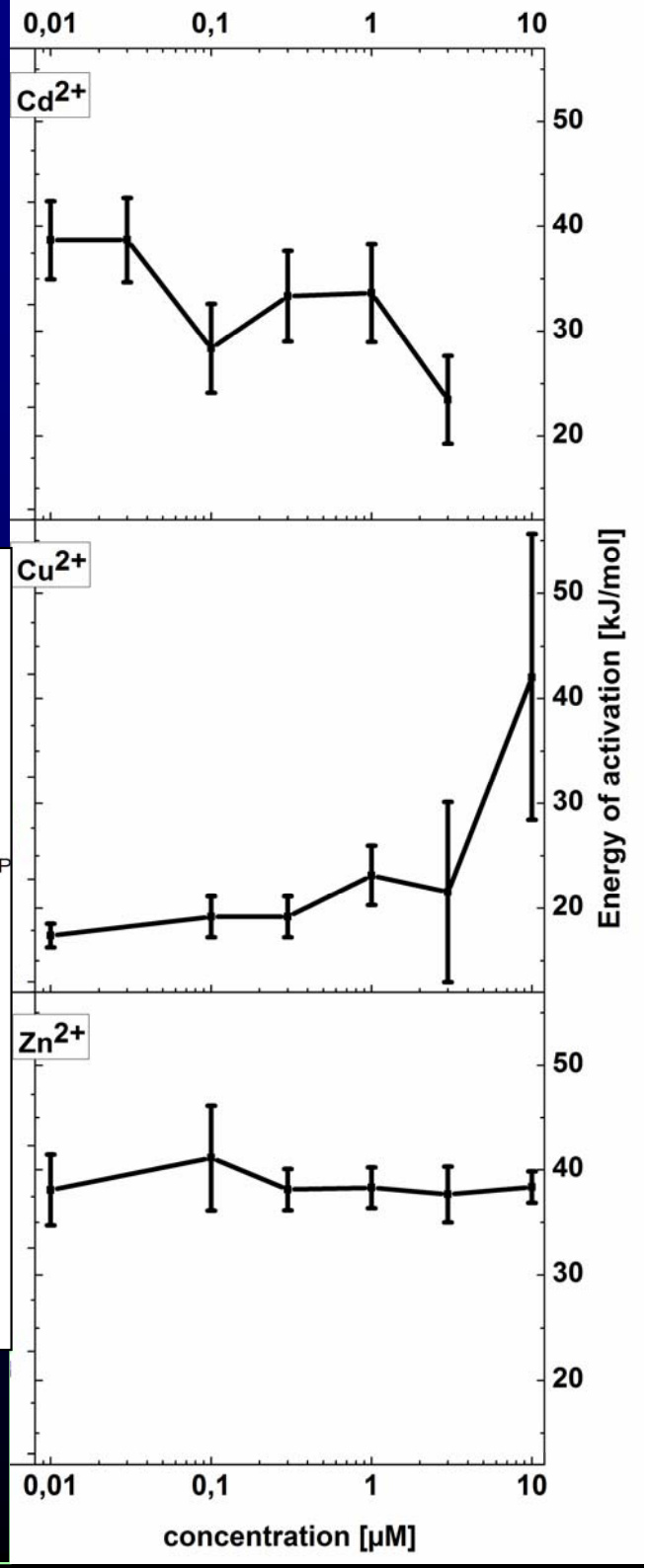
(Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta - Biomembranes 1808, 2591-2599)

IV) Metal-dependent differences in energetics of NcHMA4

- Activation energy changes with the concentration and type of the metal to be pumped.

- Maximal activity after saturation of all high-affinity Cd binding sites



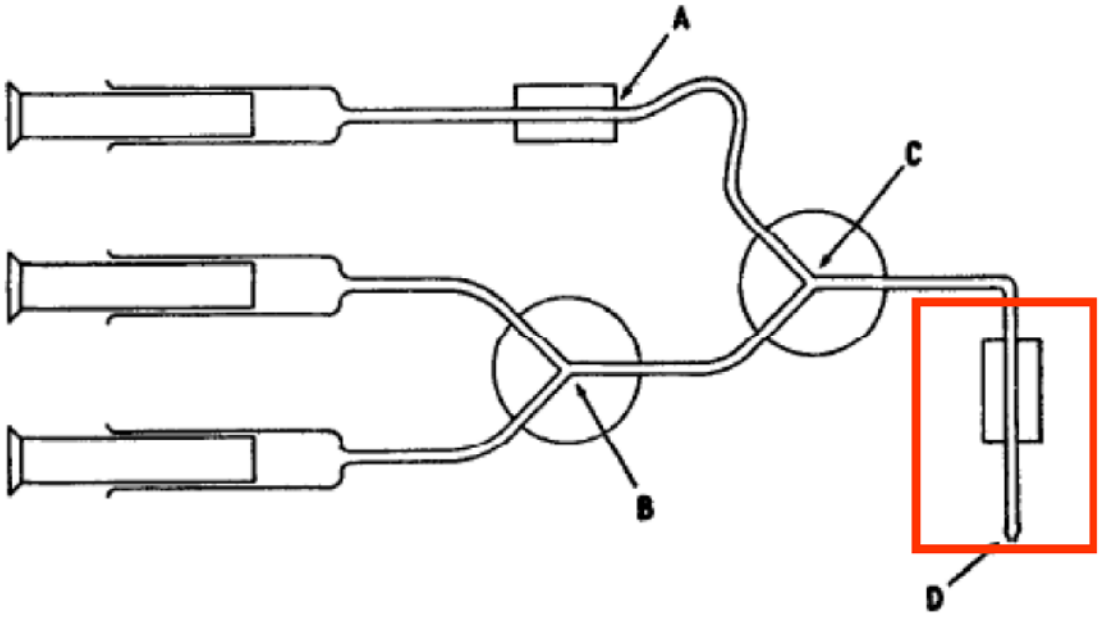


top: Mishra S, Mishra A, Küpper H (2017) Frontiers in Plant Scince, https://doi.org/10.3389/fpls.2017.00835

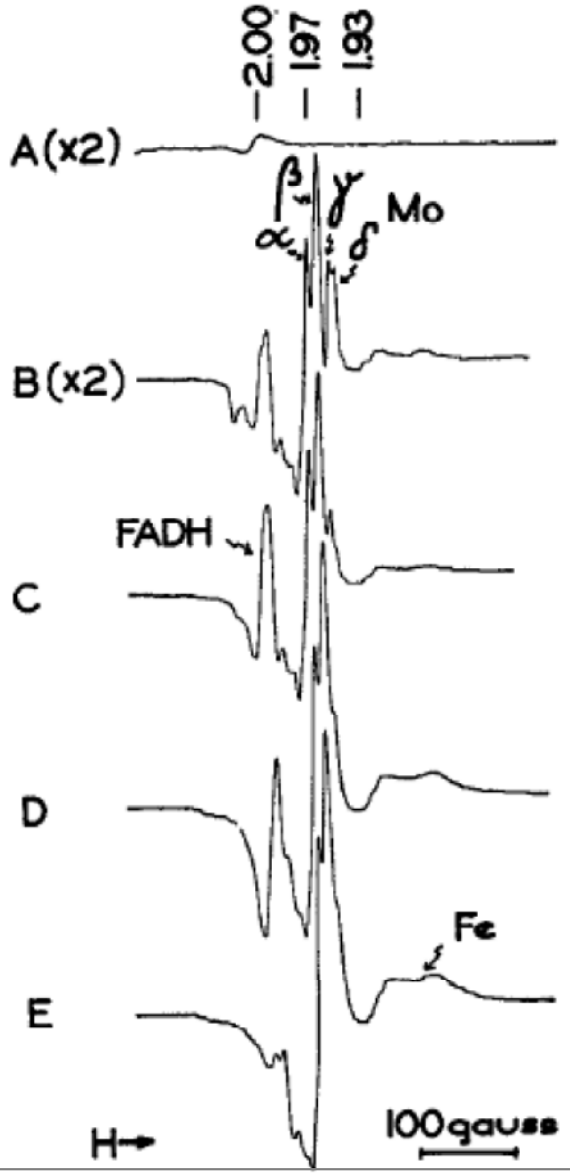
right: Leitenmaier B, Witt A, Witzke A, Stemke A, Meyer-Klaucke W, Kroneck PMH, Küpper H (2011) Biochimica et Biophysica Acta (Biomembranes) 1808, 2591-2599

Rapid-Freeze EPR (Triple Mix) – 26-1400 ms

Palmer et al., J. Biol. Chem., 239, 2657, 1964

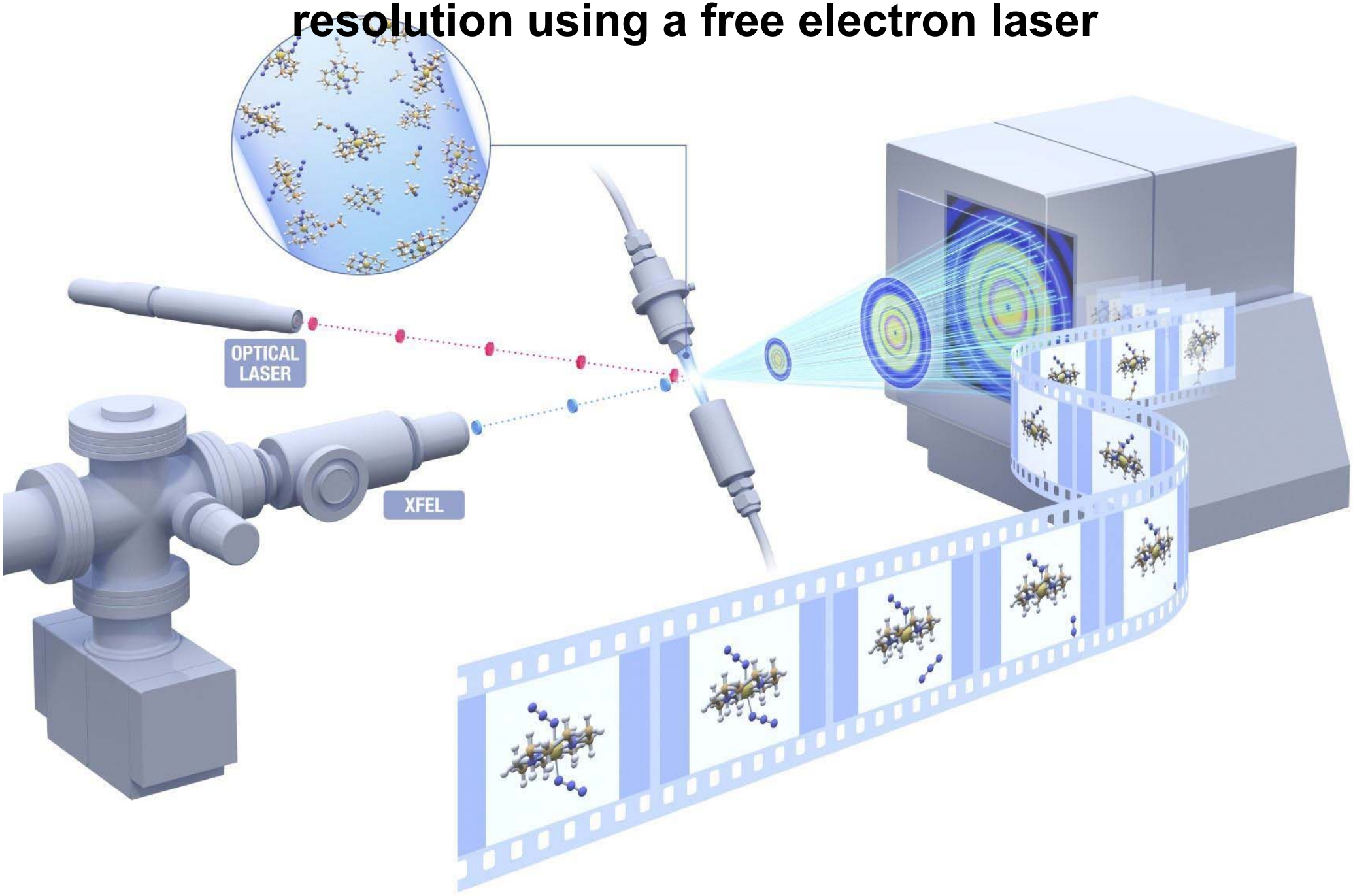


Isopentane Bath at minus 140-150 C°



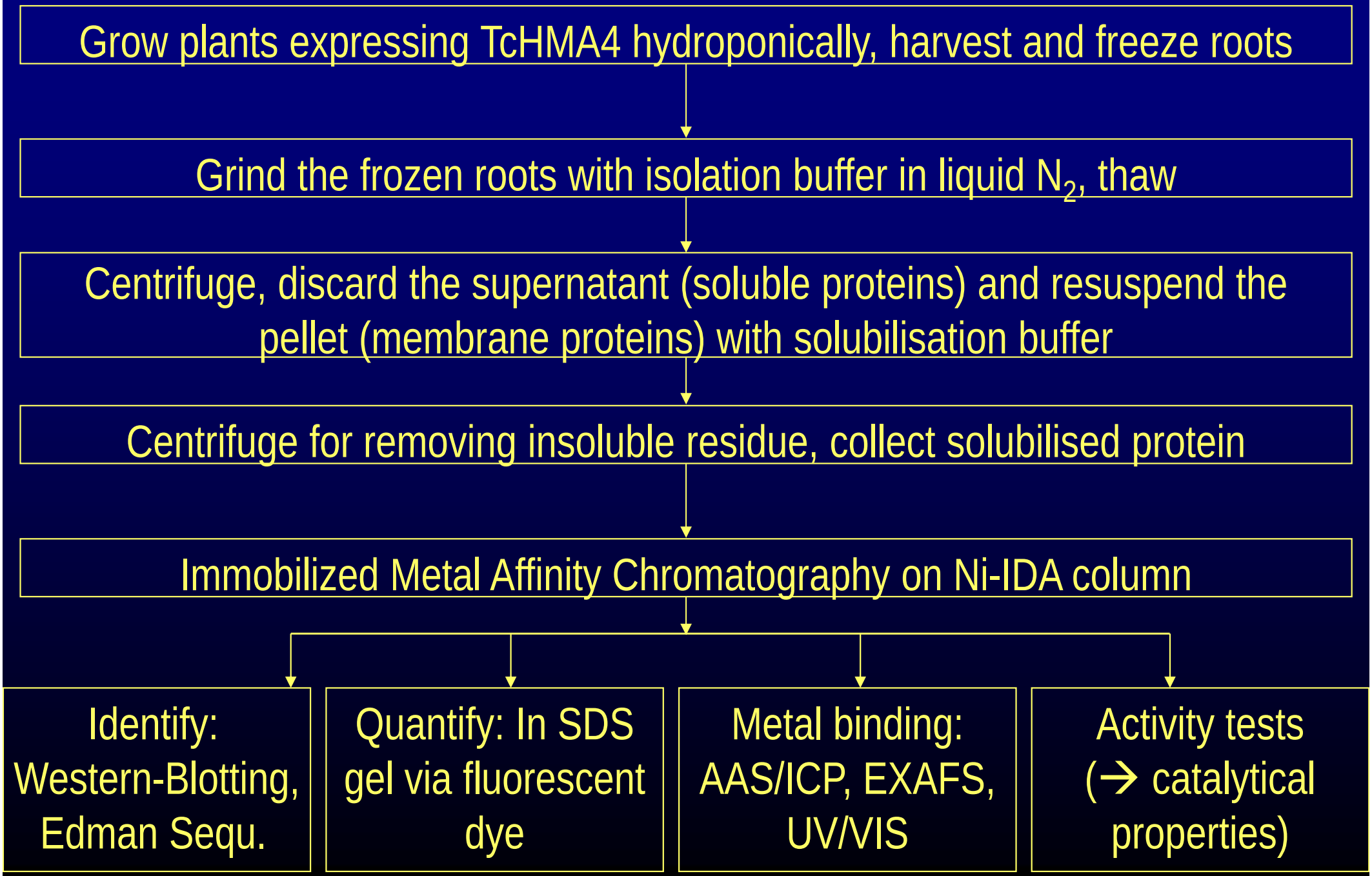
Lecture slide from Peter Kroneck

The future: Filming chemical reactions with femtosecond resolution using a free electron laser



Information from DESY (Germany)

Example: Flowchart of expression, isolation, purification and characterisation of the Cd/Zn-ATPase TcHMA4



All slides of my lectures can be downloaded from my workgroup homepage

Biology Centre CAS → Institute of Plant Molecular Biology → Departments → Department of Plant Biophysics and Biochemistry,

or directly

http://webserver.umbr.cas.cz/~kupper/AG_Kuepper_Homepage.html