

Measurement Techniques: What Should the Future Hold?

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Supra-Regional Assay Service

Centres for Analysis and Clinical Interpretation

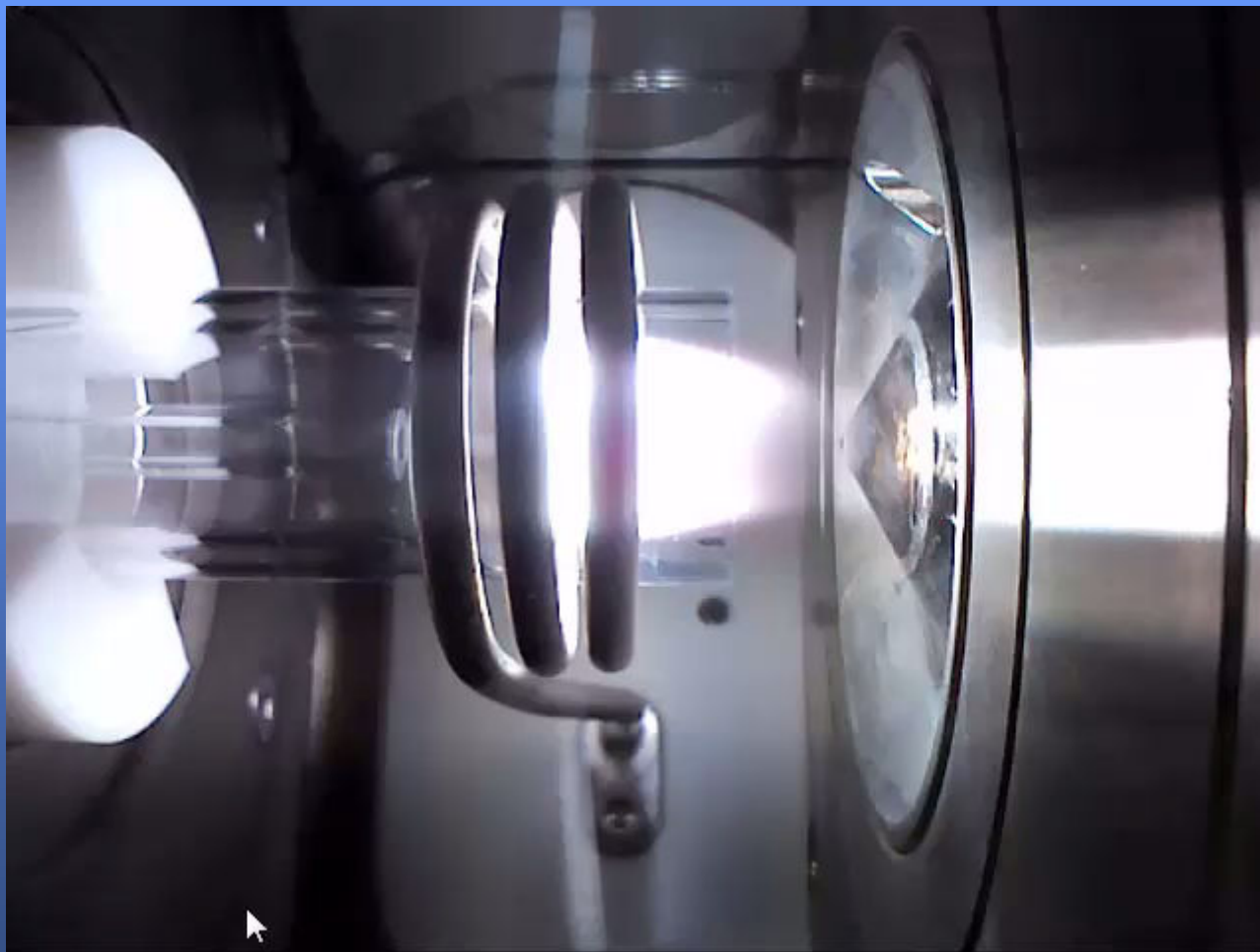
Overview of Presentation

- Current instrumentation and Cu indices.
- The power of mass spectrometry.
- Measurement of proteins.
- The need for better standardisation for Cp measurement and how this can be achieved.
- Development of high accuracy methods.

Instrumentation and Indices

- **Copper in serum, urine and liver biopsy.**
 - Copper measured by **ICP-MS**, AAS, spectrophotometry (main clinical analysers).
 - EQA, IQC and standards available. **Traceability good.**
- **Ceruloplasmin in serum.**
 - Measured by immunochemistry and **spectrophotometry** using oxidase enzyme assay.
 - EQA, IQC and standards available, **traceability of Cp problematic** as no CRMs.
 - Immunochemistry overestimates Cp as not specific for holo-form.
 - Free copper calculation inaccurate due to poor Cp measurement.
- **Free/exchangeable copper measurement available.**
 - Terminology inconsistent.
 - Requires proper validation

Sunshine in Action: An Argon Gas Plasma



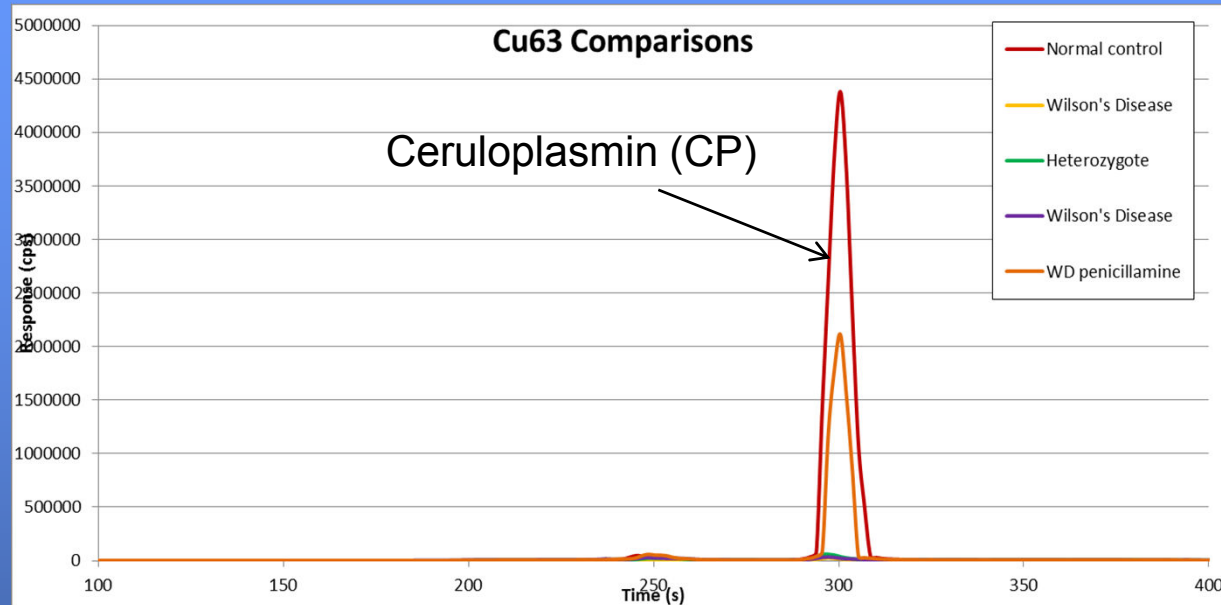
Metal Speciation and Metallomics

- Definition:
 - ..the **qualitative identification** and the **quantitative determination**, of the individual chemical forms that comprise the **total concentration** of a given trace element in a sample.
(NATO Workshop on Speciation, 1989).
- Significance:
 - The **toxicity** of metal(loid)s.
 - The **biogeochemistry** of metal(loid)s.
 - The **functionality** of biometallic species.

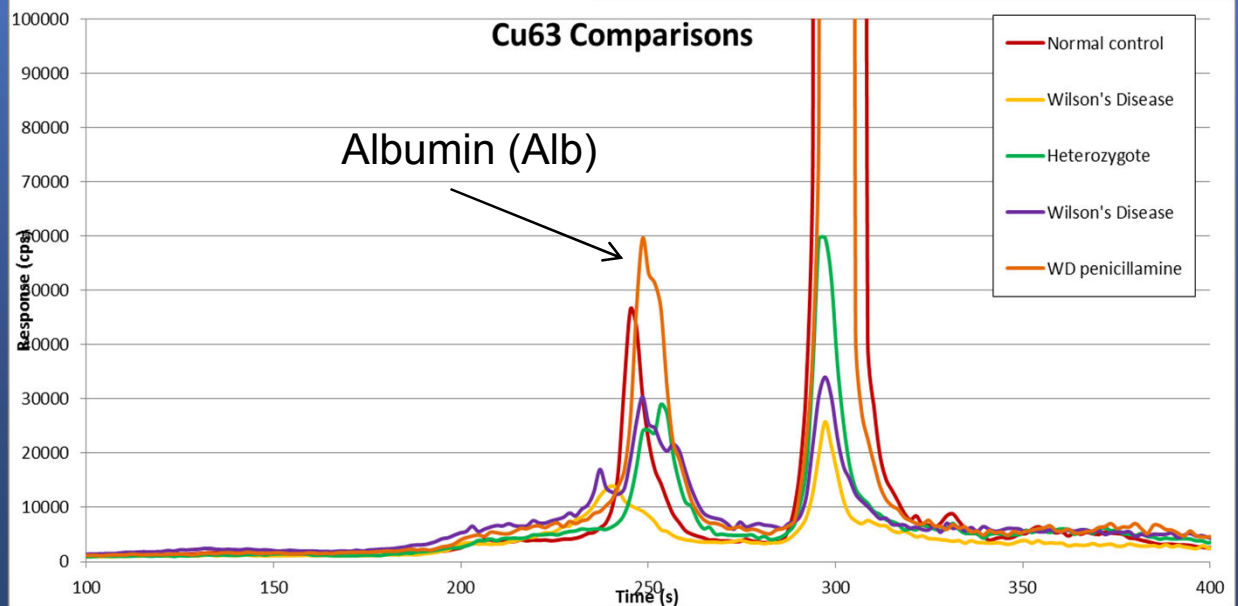
Research and Development Studies

- **Current Studies:**
- Development and validation of an in-house enzymatic method for ceruloplasmin.
 - Assay now available for send-away samples
- Investigation of free-copper assay by HPLC-ICP-MS to determine characteristics.
- **Future Work:**
- High accuracy analysis of ceruloplasmin to develop reference materials.
- Patient analysis for USA Registry in collaboration with Yale.

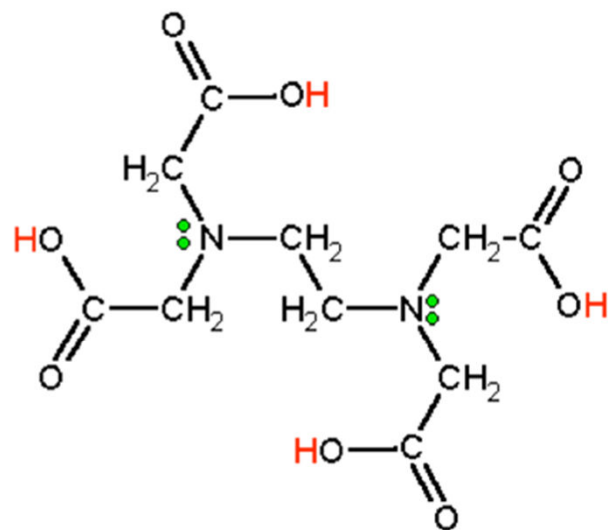
Cu-Proteins in Wilson's Disease



- Overcomes problems found with current CP assays.
- Apo-CP is not measured.



Exchangeable Cu Methodology



1.5 g/L EDTA, 60 min incubation

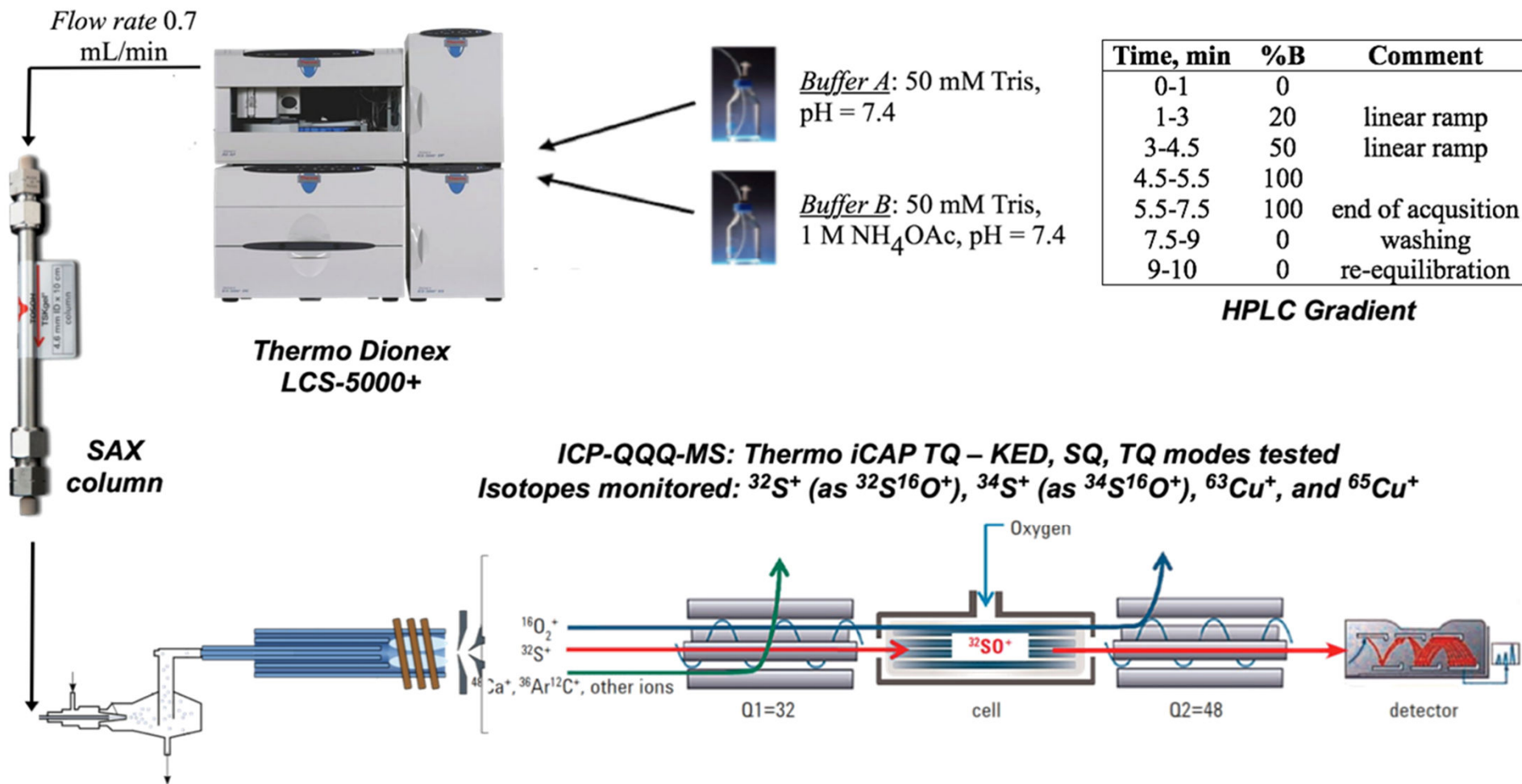


Amicon® 30 and 100 kDa cutoff filters

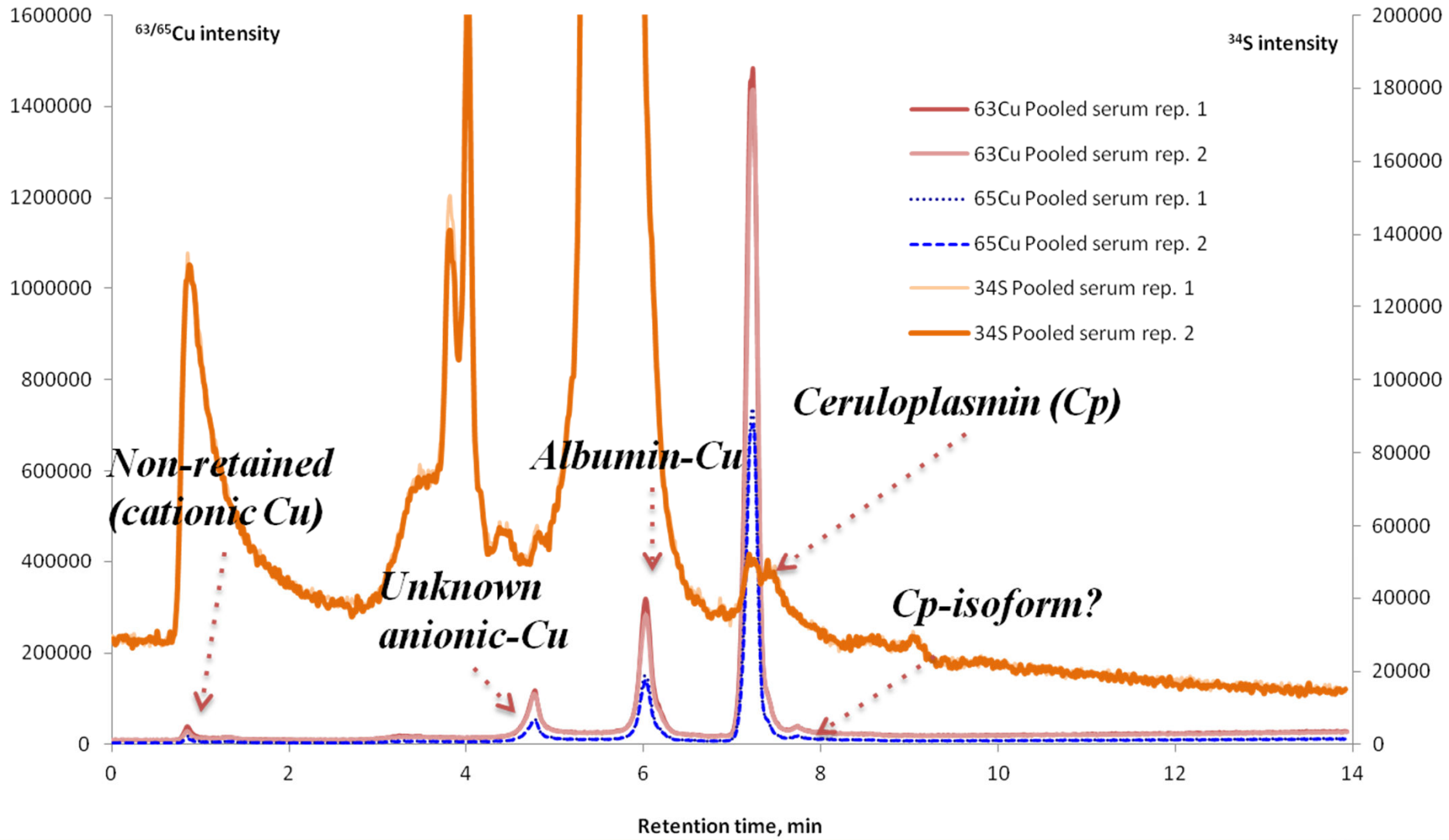
Questions to be answered:

- Measurement of a small conc _ requires low LOD
- Do the exchangeable Cu values look appropriate?
- Does membrane maintain Cp in upper layer?
- Does EDTA remove any Cu from Cp, inflating free-Cu?

Strong Anion Separation – ICP-MS



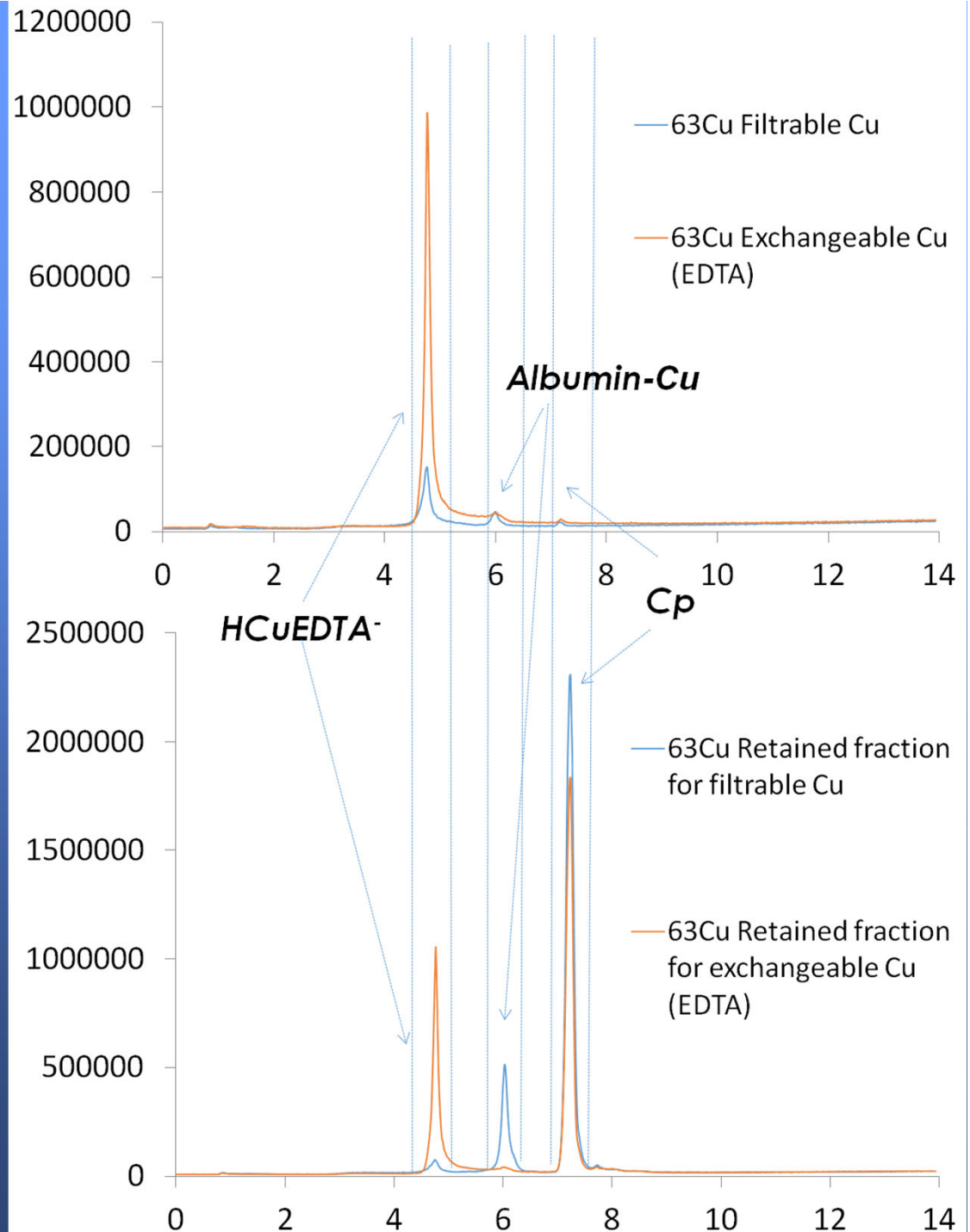
HPLC-ICP-MS of Pooled Normal Human Serum



Results show:

- Some Alb (6%) passes thro' filter (S results).

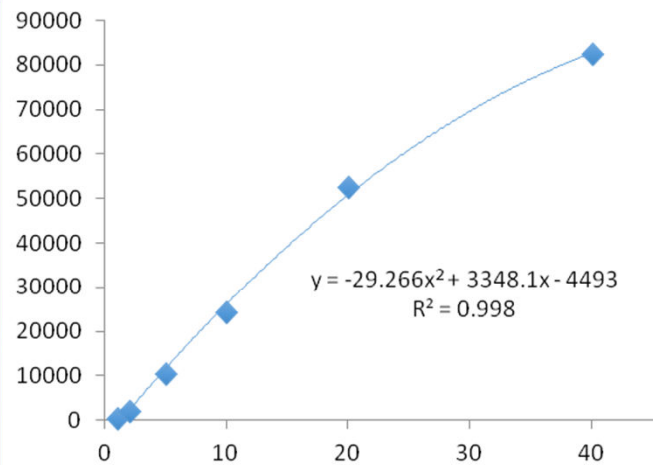
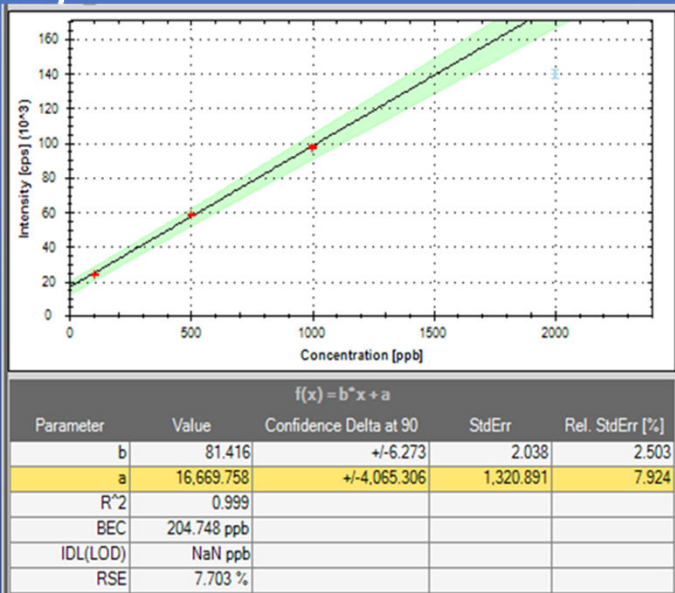
- EDTA pulls some Cu from Cp.



Ceruloplasmin by HPLC-ICP-MS

- Calibration using Cu-EDTA and S from cys and met amino acids mean an authentic std is not necessary for measurement.
- Cp can be measured directly using S.
- Results for direct measurement of Cp are lower than the ELISA method in most cases.

No.	SAC-ICP- QQQ-MS, mg/L	ELISA, mg/L
1	155	159
2	121	149
3	139	153
4	41	< 29
5	30	< 22
6	131	130
7	88	136
8	27	<i>n/m</i>
9	205	<i>n/m</i>
10	28	<i>n/m</i>
11	20	<i>n/m</i>
12	296	390
13	178	250
14	219	320
15	395	380



Copper concentration ug/L

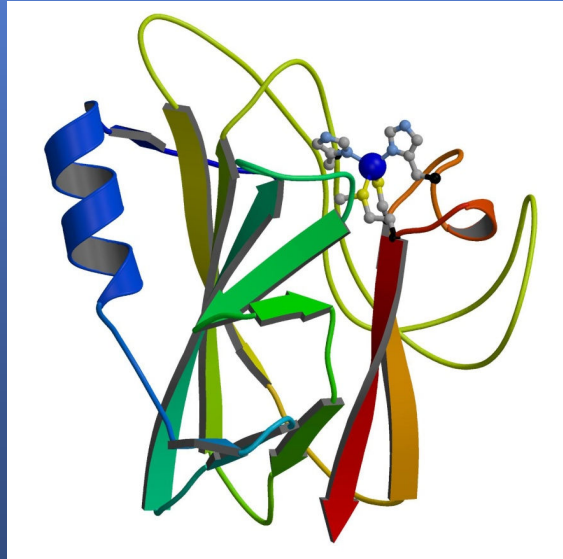
Sulfur concentration ng/L

The Future: Better Standardisation for Cp

- **Lack of well characterised standards.**
 - Number of Cu atoms.
 - Activity based conc rather than mass based.
- **No certified reference materials.**
 - Results in poor traceability.
 - Hampers inter laboratory comparisons.
- **Development of high accuracy methods.**
 - Production of isotopically enriched Cp.
 - Development of isotope dilution MS methods.
 - Overcomes loss of Cu or change in conformation of Cp during measurement.

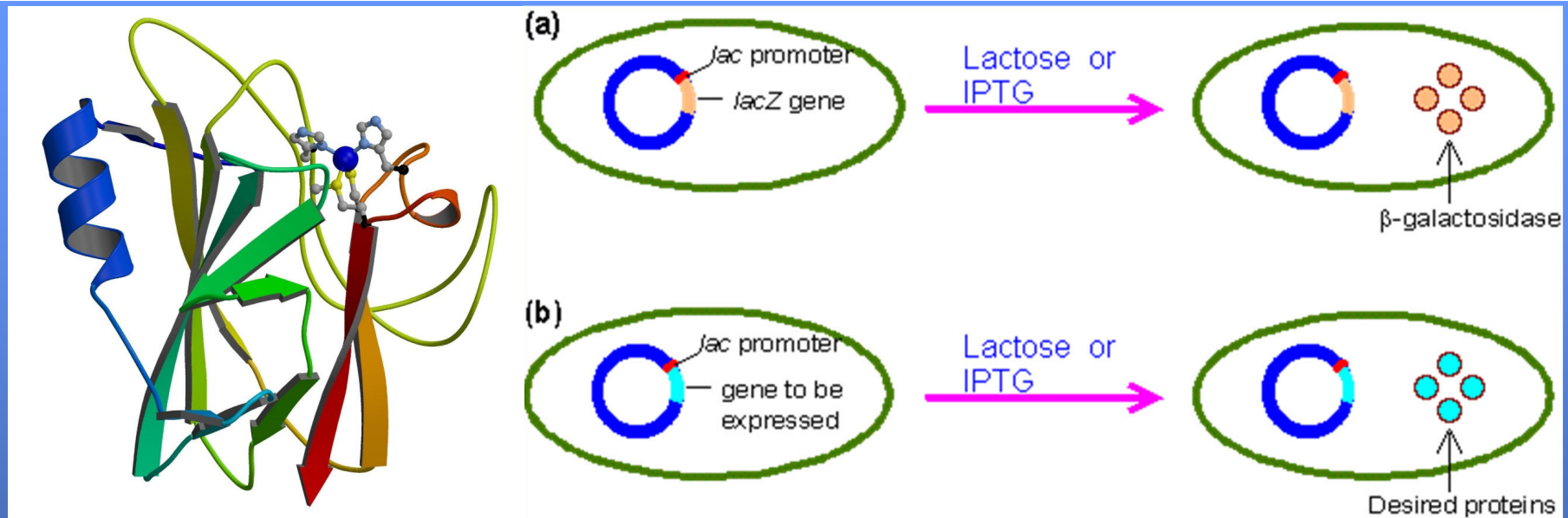
The Future: High Accuracy Analysis

- The measurement of ceruloplasmin is problematic due to the inaccuracy of the methods used.
- It also lacks good standards which means methods do not have the traceability required for good QC and QA controls.



- The best way to improve the situation is the use of high accuracy methods.
- This would involve the production of well characterised isotopically enriched ceruloplasmin standards.
- This has been achieved previously for the Cu containing protein Rusticyanin.
- Rusticyanin is a small (16 614 Da) copper containing protein isolated from *Acido-thiobacillus ferrooxidans*.

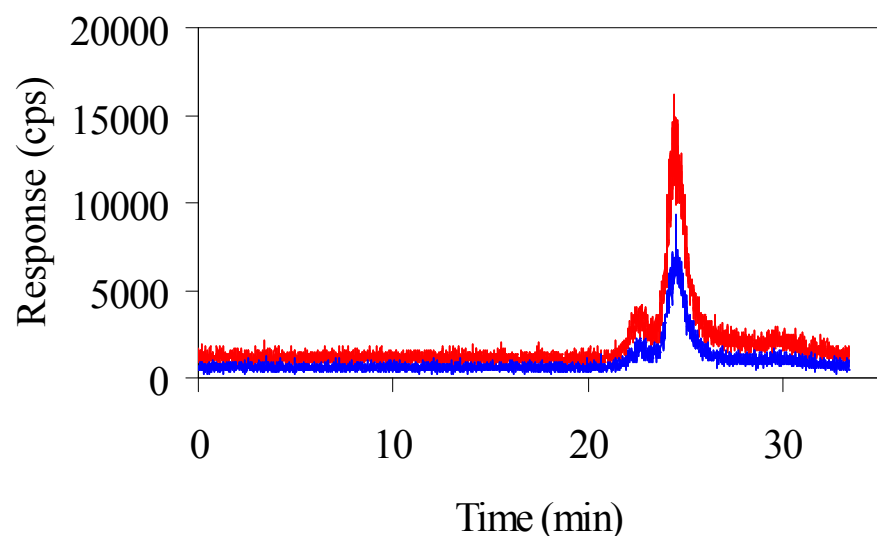
Production of Isotopically Enriched Standards



- **Rusticyanin** is a small (16 614 Da) copper containing protein isolated from *Acido-thiobacillus ferrooxidans*.
- If *lacZ* is replaced by the gene encoding for Rc, IPTG will stimulate the expression of Rc.
- Can produce **specific Cp isoforms** based on patient genes.
- Replace normal isotopic Cu with **enriched 65-Cu** and use as the “perfect internal standard”.

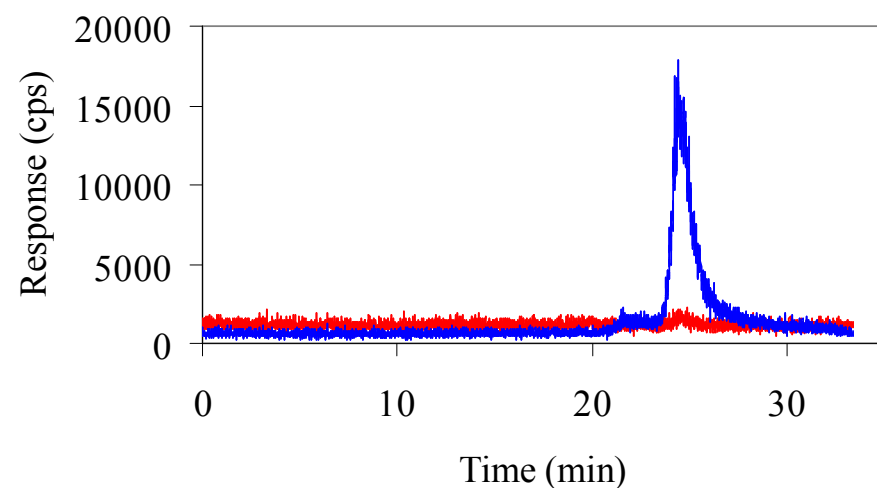
Harrington et al. Analytical Chemistry, 2005, 77, 4034 - 4041

HPLC-ICP-MS Analysis: Natural-Cu Rc



Natural-Cu Rc

- Shown in red is the response for ^{63}Cu and in blue for ^{65}Cu .
- These are in the ratio 2.2:1 reflecting the natural abundance of Cu.

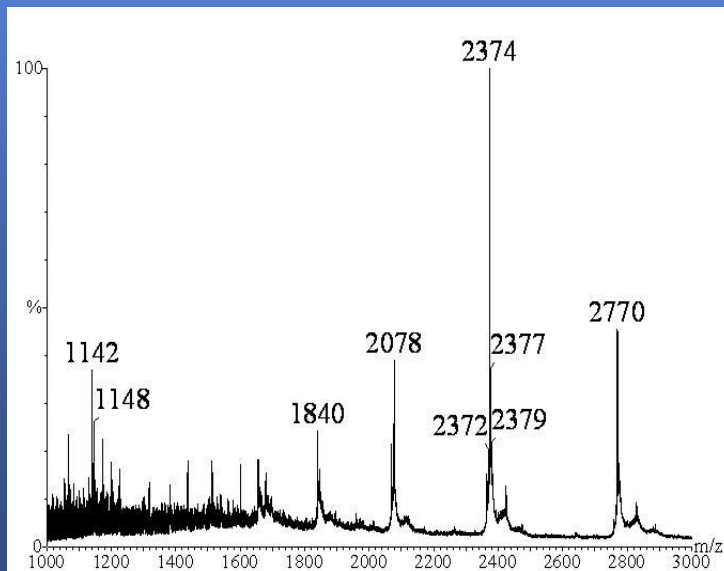


Enriched-Cu Rc

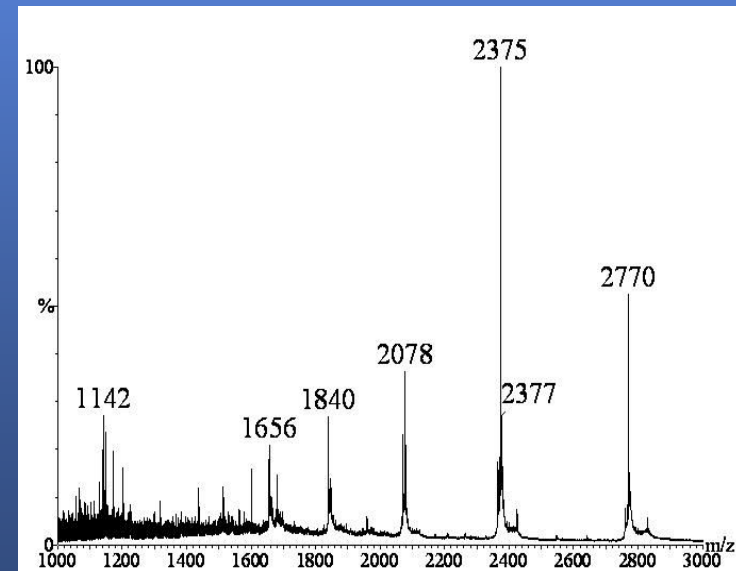
- An enriched Cu isotope is inserted into the protein.
- ^{63}Cu 0.4 atom % and ^{65}Cu 99.6 atom%.
- This can be used for high accuracy IDMS measurements.

Why Not Organic IDMS?

- No difference was observed for the two proteins using electrospray MS. Tris pH 7.0 buffer.
- Would require an instrument with a resolution of 17000.
- By using elemental MS a quadrupole instrument with **unit mass resolution** can be used.



Natural-Cu Rc



Enriched-Cu Rc

Concluding Remarks

- Exchangeable Cu method requires further investigation.
- Using HPLC-ICP-MS can give a direct measurement of Cp.
- High accuracy methods could be used to produce Cp standards for better traceability.

Acknowledgements

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Thanks for Listening!!



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