Protein quantification and detection methods

1) Spectroscopic procedures

2) Measurement of the total protein content by colorimetry

3) Amino acid analysis

4) Other methods, eg. radiolabelling of proteins, Edman degradation, RP-HPLC

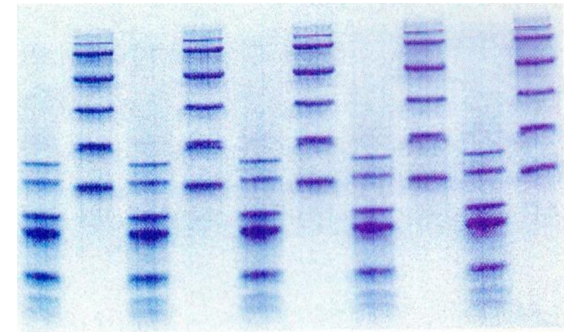
Practical aspects of protein measurement:

1. Monitoring the recovery of your protein during purification

This can be done by:

→ Measuring enzyme activity if it is an enzyme

→ Any other convenient method, eg electrophoresis or WB



2. Measuring the total amount of protein in your sample.

1. Spectroscopic procedures:
PROTEIN SPECTRA

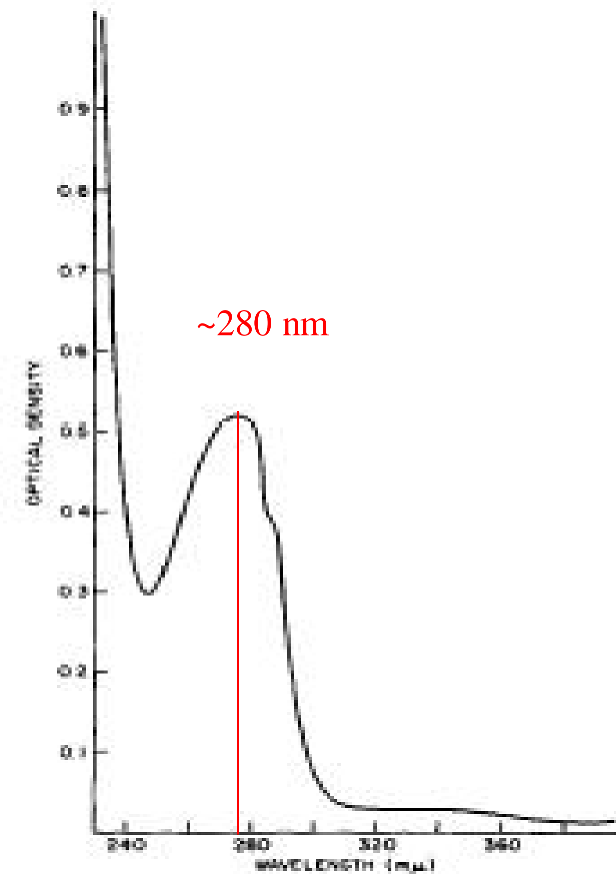
NAME: Thyrocalcitonin

SOURCE: Bovine

REFERENCE: H. Brewer, Jr., R. Schlueter, and J. Aldred, J. Biol. Chem., 245, 4232 (1970).

EXPERIMENTAL CONDITIONS AND COMMENTS

Solvent: 0.1 M acetic acid.



NAME: Myoglobin, Oxy- and Met-

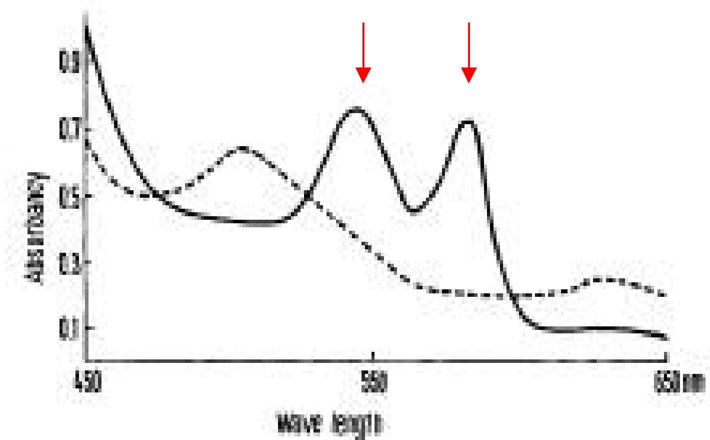
SOURCE: Chicken gizzard

REFERENCE: U. Groschel-Stewart, U. Jaroschik, and H. Schwalm, Experientia, 27, 512 (1971).

EXPERIMENTAL CONDITIONS AND COMMENTS

Solvent:

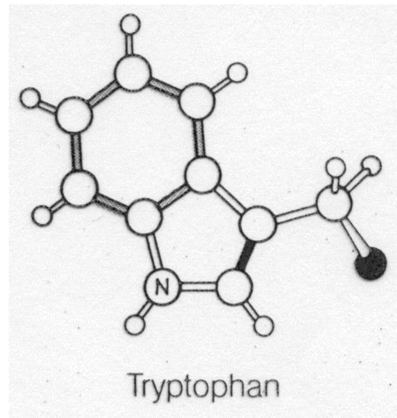
——— : Oxymyoglobin.
------ : Metmyoglobin.

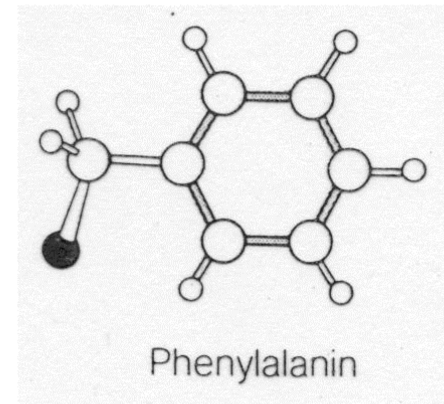


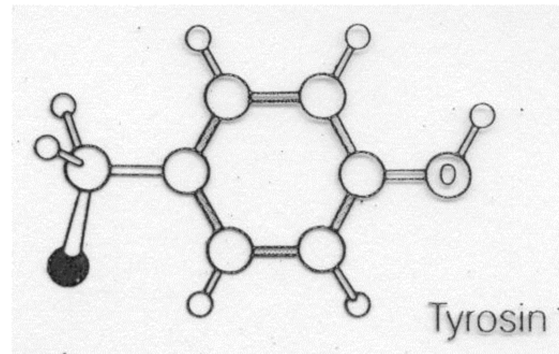
Spectroscopic procedures:

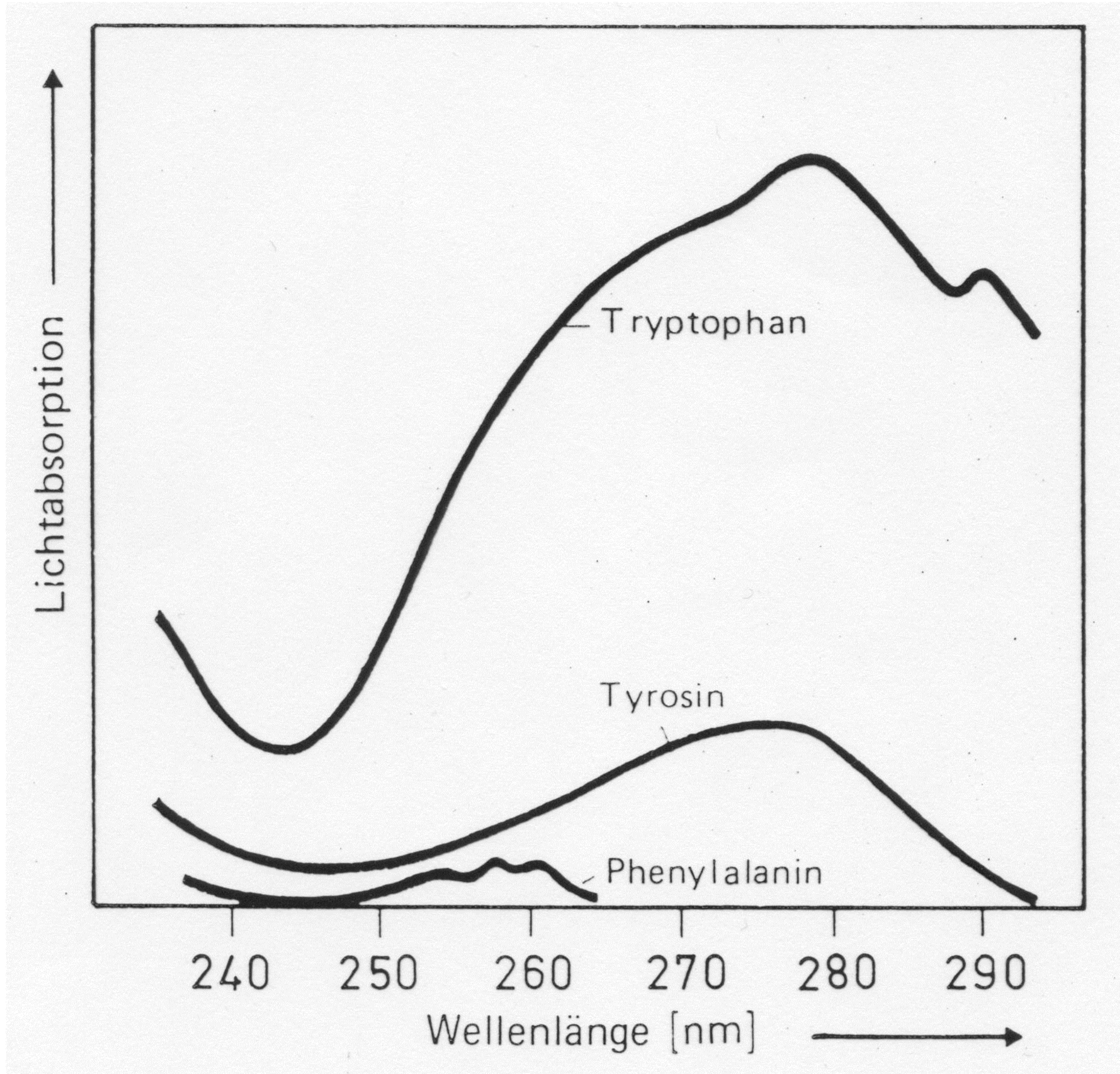
USING A_{280} TO DETERMINE PROTEIN CONCENTRATION

→ Measuring the absorbance of the aromatic amino acids








Lichtabsorption
Tryptophan
Tyrosin
Phenylalanin
240
250
260
270
280
290
Wellenlänge [nm]

Ref. Pace, C.N. et al, (1995) Protein Sci,. 4, p2411

Absorbance measured at 280 nm (A_{280}) is used to calculate protein concentration by comparison with a standard curve or published absorptivity values for that protein (ε_{280}).

ε280nm (M-1cm-1) = (#Trp)(5500)+ (#Tyr)(1490)+(#Cys)(125)

If the ε_{280} of the protein is known: Calculate the unknown sample concentration from its absorbance.

In this equation A_{280} has units of ml/mg cm and *b* is the path length in cm.

$$\text{Prot. conc (mg/ml)} = \frac{A\ 280}{\varepsilon\ 280 \times b}$$

This assay (A 280) can be used to quantitate solutions with protein concentrations of 20 to 3000 ug/ml.

Spectroscopic procedures:

USING A_{205} TO DETERMINE PROTEIN CONCENTRATION

Determination of protein concentration by measurement of absorbance at 205 nm (A_{205}) is based on absorbance by the peptide bond. The concentration of a protein sample is determined from the measured absorbance and the absorptivity at 205 nm (a_{205}).

This assay can be used to quantitate protein solutions with concentrations of 1 to 100 ug/ml protein.

If the a_{205} *of the protein is known:* Use the 280 equation to calculate the concentration of the sample protein *except* substitute the appropriate values for A_{205} and ε_{205}.

If the a_{205} *is not known:* Estimate the concentration of the sample protein from its measured absorbance using:

$$\text{concentration (mg/ml)} = \frac{A\ 205}{31 \times b}$$

In this equation, the absorptivity value, 31, has units of ml/mg cm and *b* is the path length in cm.

Spectroscopic procedures:

USING FLUORESCENCE EMISSION TO DETERMINE PROTEIN CONCENTRATION

Protein concentration can also be determined by measuring the intrinsic fluorescence based on fluorescence emission by the aromatic amino acids tryptophan, tyrosine, and/or phenylalanine.

Usually tryptophan fluorescence is measured. The fluorescence intensity of the protein sample solution is measured and the concentration calculated from a calibration curve based on the fluorescence emission of standard solutions prepared from the purified protein.

This assay can be used to quantitate protein solutions with concentrations of 5 to 50 ug/ml.

Fluorescence characteristics of amino acids

	Lifetime	*Absorption*		*Fluorescence*	
		Wavelength	Absorptivity	Wavelength	Quantum
Tryptophan	2.6	280	5,600	348	0.20
Tyrosine	3.6	274	1,400	303	0.14
Phenylalanine	6.4	257	200	282 .	0.04

Lifetime, *nanoseconds*; wavelength, *nanometers*

- Generally, lifetimes are short and quantum yields are low for all three residues.
- A special case of fluorescence occurs in Green Fluorescent Protein. Fluorophore originates from s*erine-tyrosine-glycine* which is post-translationally modified
- Changes in intrinsic fluorescence can be used to monitor structural changes in a protein.

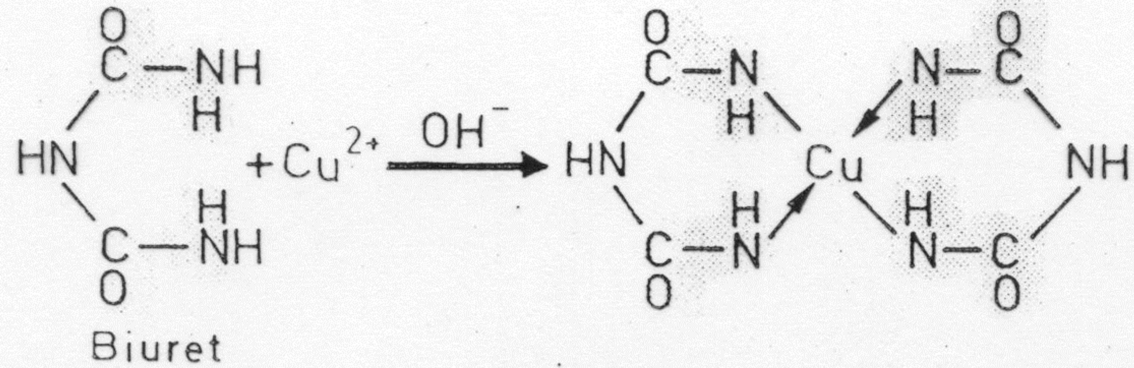
2. COLORIMETRIC ASSAYS

Biuret assay, Lowry assay, bis-cinchinonic acid (BCA) assay, Bradford assay.

Biuret method:

The assay is based on polypeptide chelation of cupric ion (colored chelate) in strong alkali. Compounds containing two or more peptide bonds react with cupric ions ($Cu2+$) in alkaline solution to produce a complex of reddish-purple colour.

Reagent: alkaline Copper sulphate
sensitivity: -1 mg (very low)
Peptide bonds (A = 550nm)



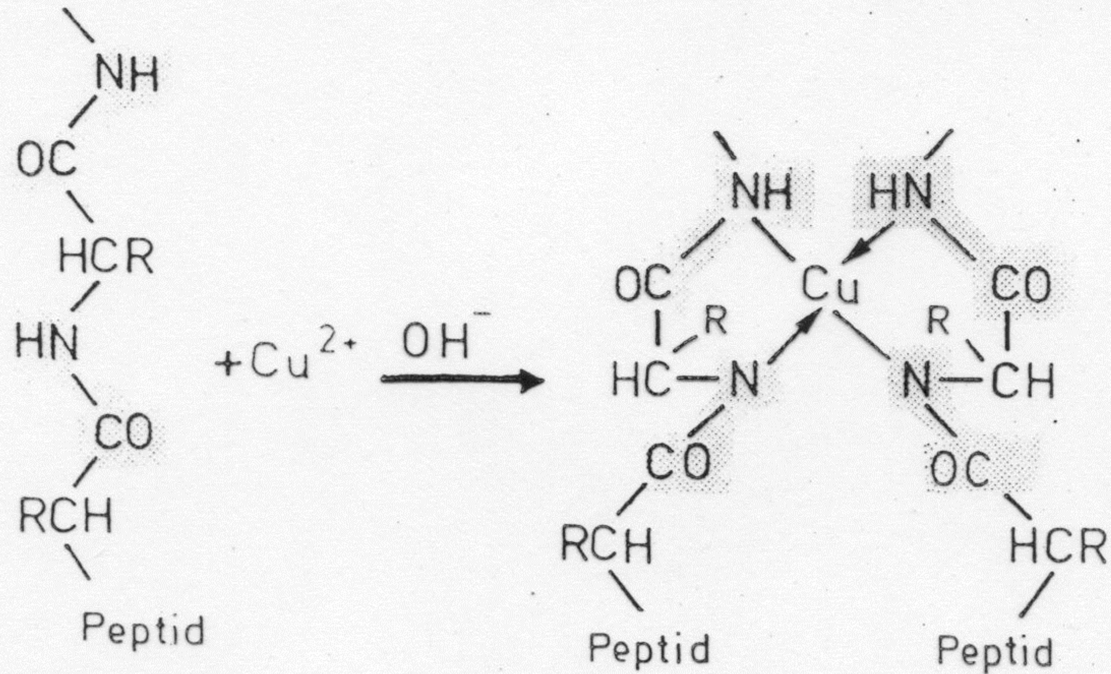


Abb. 5-47. Die Biuret-Reaktion

Lowry method:

The assay is a colorimetric assay based on reduction of the Cupric Cu^{2+} to cuprous ions Cu^{+} in alkaline pH when reacting with peptide. Cuprous ion and the phenolic group of Tyr; indole of Trp; -SH of Cys then react with Folin-Ciocalteau reagent to produce an unstable “molydenum blue”-type product (A=650nm).

Lowry-Folin-Ciocalteau reagent consits of phosphomolybdate and phosphotungstate which create the color when reduced. Most proteins contain little Trp or Cys. Therefore, the colour here is largely due to Tyr content.

Reagent: alkaline copper soln and F-C reagent
Sensitivity: A few micrograms but varies with different proteins.
Tyr (Trp/Cys)
A 650 nm

Bis-Cinchinonic Acid (BCA) method:

The bis-cinchinonic acid (BCA) assay for total protein is a spectrophotometric assay based on the alkaline reduction of the cupric ion to the cuprous ion by the protein, followed by chelation and color development by the BCA reagent. (A = 562 nm)

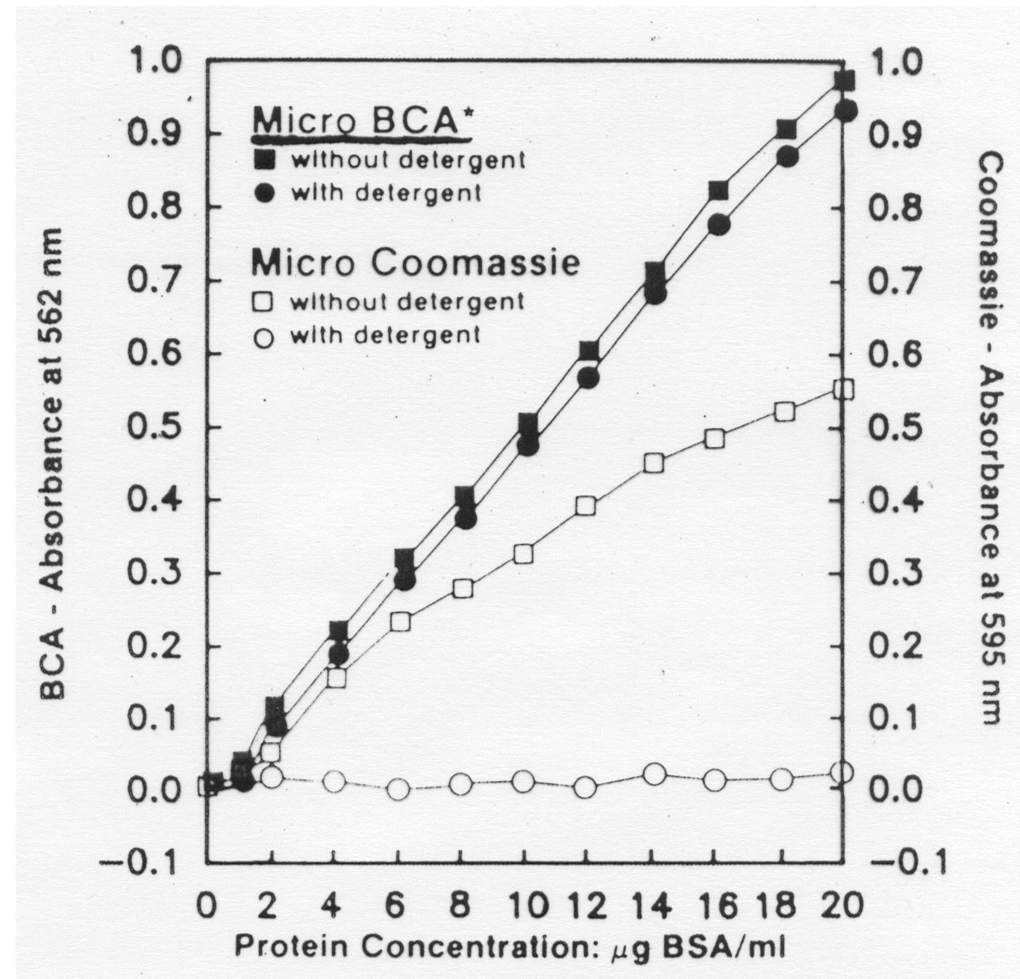
Cu^{2+} ions reduced to Cu^{+} ions by aromatic amino acids.
Cu^{+} ions + Bicinchoninic Acid ® Complex has absorption maximum at 562 nm.

BCA assay has a good combination of sensitivity and simplicity, slow without heating
Aromatic amino acids
A 562 nm

Bradford method:

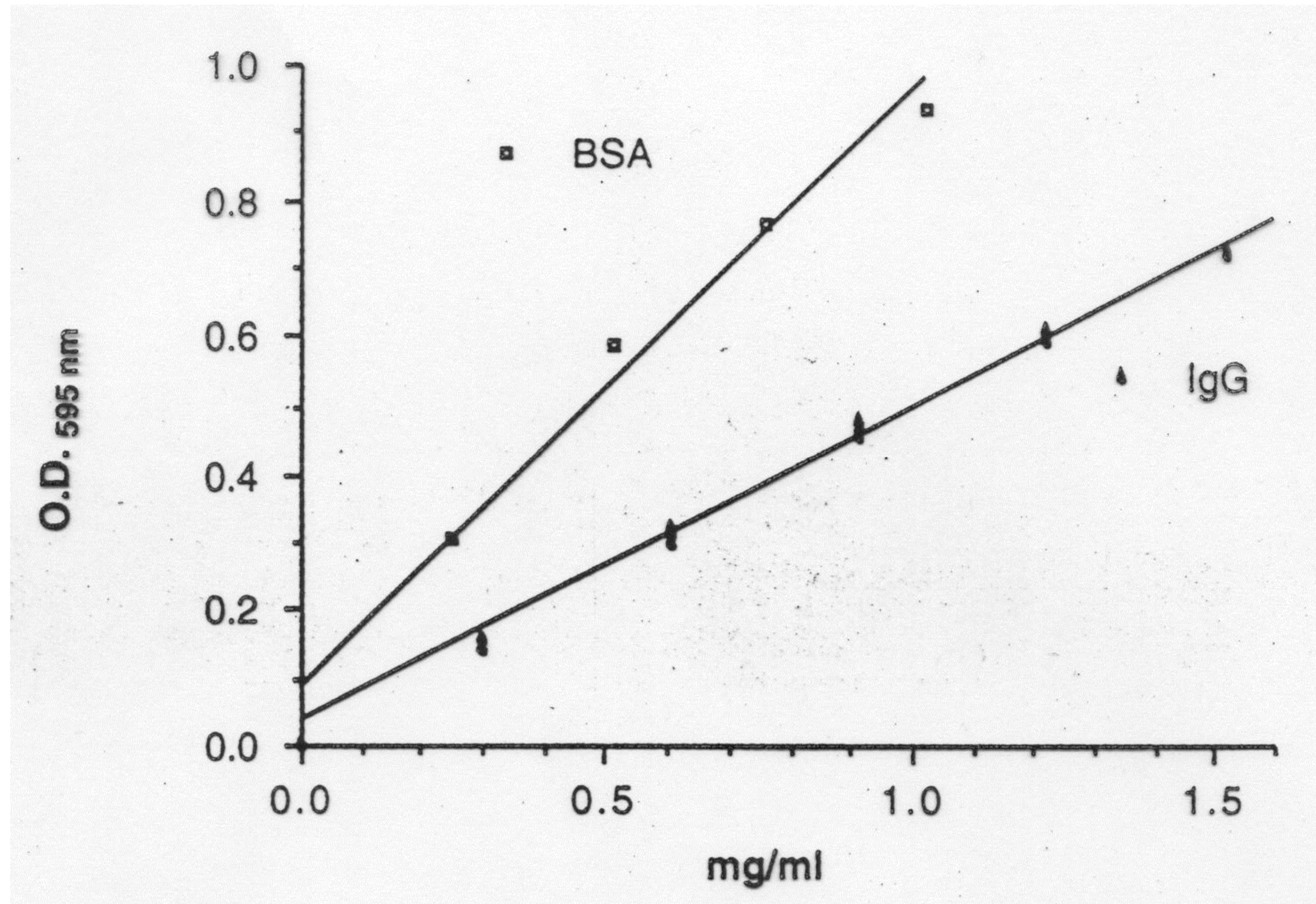
This assay is based on binding of Coomassie dye by protein (Brilliant blue G250).
Binds to aromatic, K,R,H
Shifts A465 max to A595 nm

Easy to use compared with Biuret, Lowry, and BCA but more sensitive for other substances, eg detergents

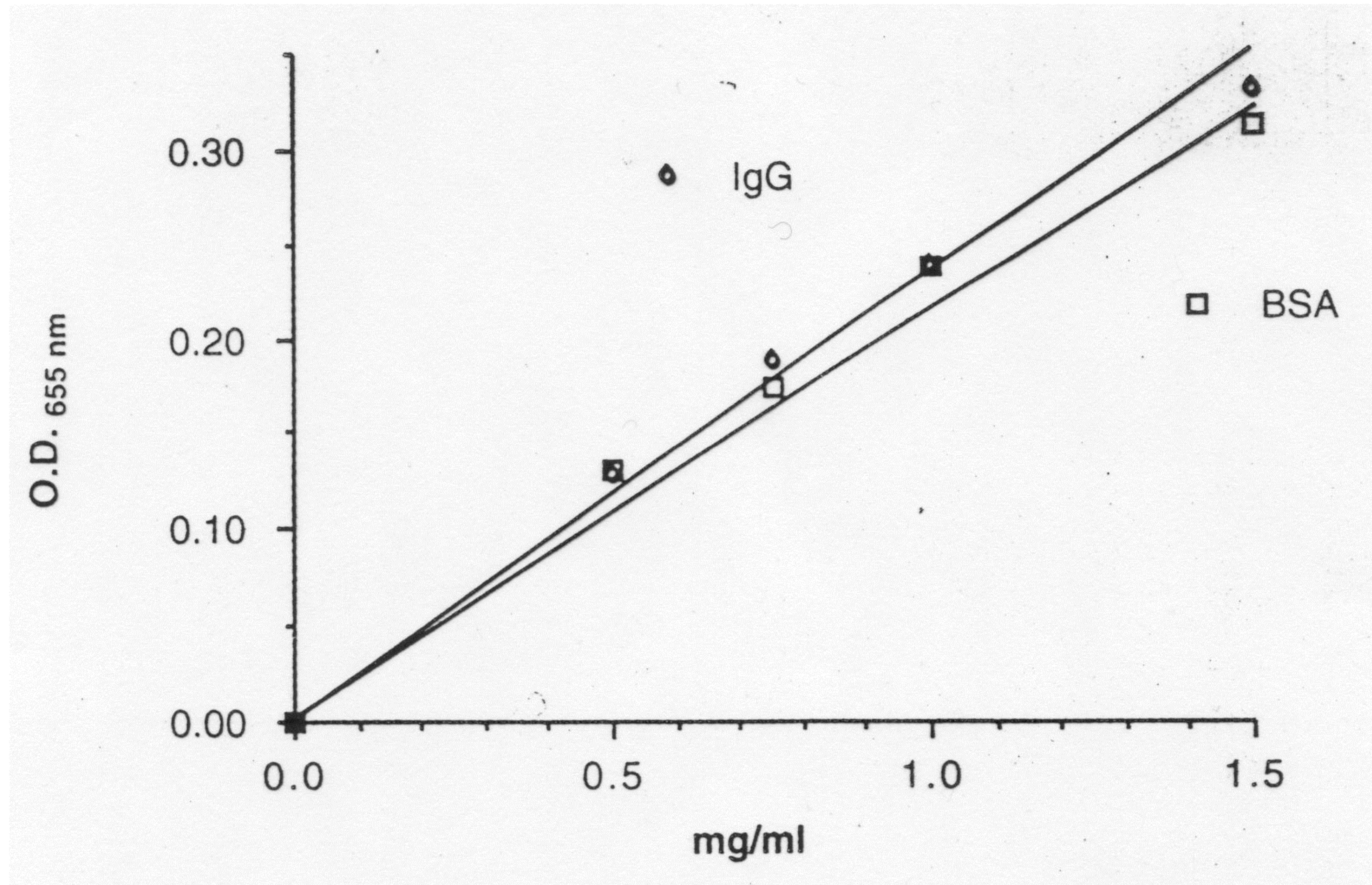


Comparison of the reliability of colorimetric assays:

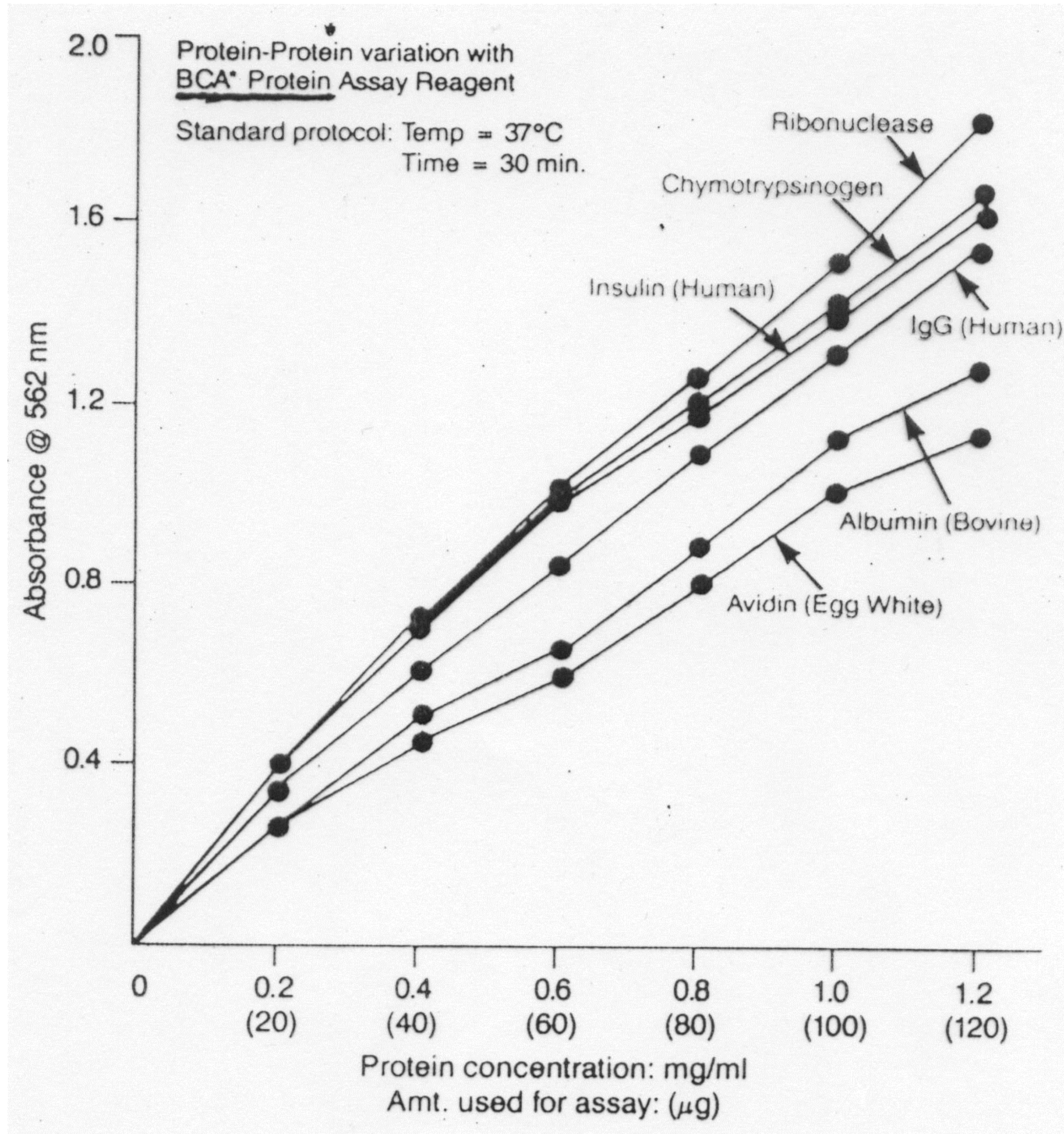
Coomassie (Bradford)(A = 595 nm)



Lowry method (A = 650 nm)



BCA method (A = 562 nm)



Substances causing trouble in average assays:

Things that bind Coomassie blue:

Detergents, TX 100, SDS, CHAPS, ampholytes

Things that affect Cu-ions:

Reducing agents DTT, BME; chelating agents like tiourea, urea, EDTA

Remember to check with a control!!

2D Quant Kit – commercial kit of GE

Developed in a need to measure protein concentration in detergent / urea solutions.

Uses a precipitation step prior to quantification

Reverse quantification: precipitated proteins solubilized in alkaline solution of cupric ions. Colorimetric reagent binding the unreacted cupric ions results in orange color (A= 480 nm). The intensity is inversely proportional to protein concentration in the sample.

<50 ug, little protein-to protein variation. Not dependent of aa composition of sample.

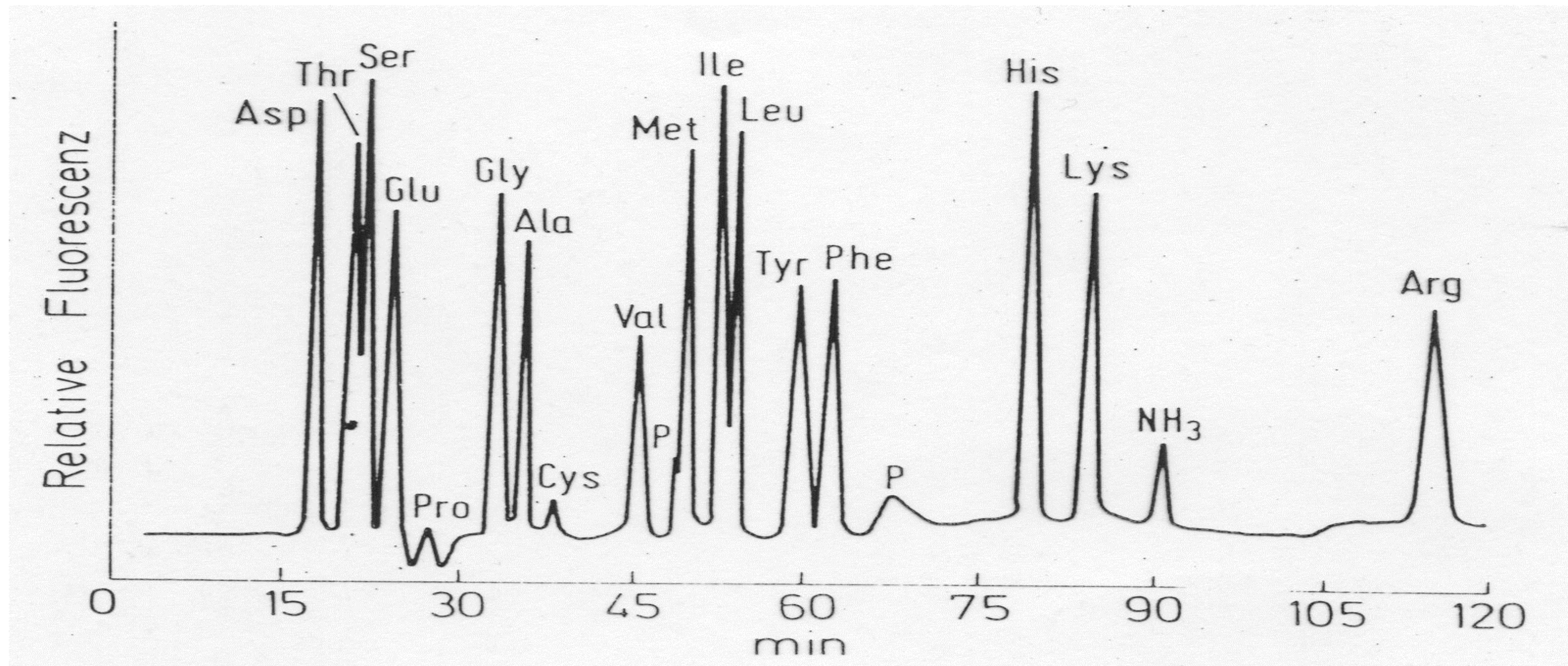
3. AMINO ACID ANALYSIS

The use of quantitative total amino acid analysis in the characterization of protein samples is often overlooked

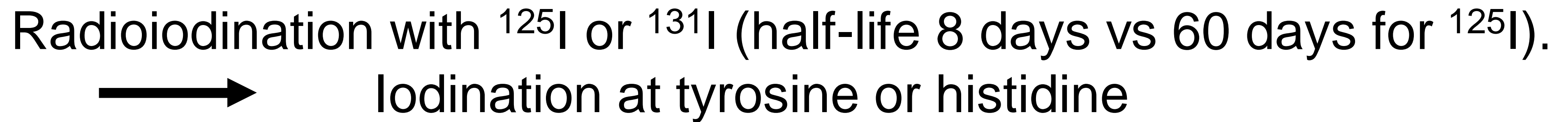
If you have the possibility to do this, it is the most accurate method for to determine the amount of protein in a sample.

The amount of protein required for detection and quantitative analysis could be as low as 0.05 nmol, or 2.5 ug.

Boil your sample in 10N HCl at 120 C o/n. Label the released amino acids either pre- or post column with a proper dye (e.g. fluorescent or Dabsyl-Cl). Perform RP-HPLC analysis and compare your sample to standards. You need sophisticated instrumentation for this method.



4. RADIOLABELLING AND LABELLING WITH OTHER TAGS



Radioiodination with ^{125}I or ^{131}I (half-life 8 days vs 60 days for ^{125}I).

→ Iodination at tyrosine or histidine

Peptides or proteins can be labeled also with ^{14}C or ^{3}H

Two methods are available to label a peptide without altering its structure:

^{3}H labeling by catalytic reduction of (nonradioactive) ^{127}I-labeled peptide and de novo peptide synthesis using a radiolabeled form of any amino acid

Fluorescent labels or other tags, like iTRAQ, can also be used to label proteins for quantification. Usually reacts with Lys / Cys

OTHER METHODS

- Edman degradation
- RP- chromatography