Protein Concentration Determination

In nearly any biochemistry research situation, it will be necessary for you to accurately determine the concentration of proteins in solution. Several methods are available, each having particular advantages and disadvantages. The method ultimately chosen will depend on such factors as the speed of analysis, accuracy required, sensitivity required, the composition of the solution containing the protein, and the need to recover the protein sample.

1) **Amino acid analysis**: HPLC analysis of amino acid mass after acid hydrolysis would enable you to determine a protein concentration. This however requires specialized instrumentation, is time consuming and is not really used as a protein determination method.

2) **Kjeldahl nitrogen determination**: This method also requires specialized equipment and large amounts of ammonium sulfate. The ammonium solution is acidified and the amount of ammonia released is determined by a titration. This is not used routinely in biochemistry.

3) **Biuret reaction**: In alkaline solutions, cupric ion complexes with the peptide bonds of proteins and peptides to form a purple charge transfer complex ($\lambda_{\text{max}} = 540$ nm). The intensity of the color is proportional to the protein concentration. This reaction occurs only with the peptide bond and not with the amino acid side chains. Because the number of peptide bonds per given unit weight is approximately the same for all proteins, this method is generally applicable and reasonably accurate, irrespective of the composition of the protein mixture. Other advantages of this method are that the color development time is relatively short and the color intensity remains constant for a reasonable amount of time (at least 30 minutes). A major disadvantage of this assay is its lack of sensitivity, the lower limit being 2 mg of protein. Greater sensitivity can be achieved by measuring the absorbance of the protein–cupric ion complex at 310 nm rather than at 540 nm; however, because so many substances found in crude protein solutions absorb in the near-ultraviolet region, this approach is usually impractical, even when appropriate blanks are included. Another disadvantage with this assay is that some compounds used in the laboratory such as Tris buffer and ammonium sulfate, as well as endogenous compounds in crude extracts, can interfere with color development or generate colored complexes themselves. These interferences can be minimized by analyzing protein precipitates.

4) **Lowry (Folin-Lowry) method**: This is the most cited method of protein analysis. The method relies on the color development from the Biuret reaction and from the reduction of an arsenomolybdate reagent (the Folin-Ciocalteau reagent) by the tyrosine and tryptophan residue in the treated protein. Both reactions generate intense blue colors which are additive. The major advantage of this method is its sensitivity, being 100-fold more sensitive than the Biuret assay. There are however, several major disadvantages to this method.

1) The absorbance values per milligram of protein are dependent on the composition of the protein. Proteins which contain larger mole fractions of tyrosine and tryptophan will give higher absorbance values than those devoid of or containing lower levels of those amino acids. Thus the concentration of an unknown mixture will be incorrectly estimated if an inappropriate protein standard is used or as mixture composition is altered.

2) The absorbance of the reaction mixture is not strictly proportional to protein concentration.

3) Numerous substances found in biological and laboratory samples can interfere with the Lowry analysis. Examples include tryptophan and tyrosine (if present as free amino acids), most phenols, uric acid, xanthine, Tris buffer, ammonium sulfate (concentration > 0.15%), glycine (>0.5%), hydrazine (>0.5%), EDTA, and reducing agents such as 2-mercaptoethanol. Nucleic acids, urea, guanidine hydrochloride, perchloric acid (neutralized) trichloroacetic acid (neutralized), ethanol, and acetone are some commonly used materials that do not interfere with color development or produce color themselves.

4) Color development is slow and fades relatively rapidly, so reasonable precise reaction times and temperatures are required. This can be a problem when analyzing a large number of samples.
5) **Dye-binding method, (Bradford method, Bio-Rad protein assay):** This method has gained popularity steadily since its discovery in 1976 by Bradford. It is as sensitive as the Lowry, fast, easy to perform and is less susceptible to interference by contaminants. In this assay, the dye Coomassie Blue G-250 is dissolved in an acidic solution causing it to absorb at 465 nm (reddish brown). When the dye (negatively charged) binds to the positively charged protein molecule the absorbance undergoes a shift to 595 nm (blue). This shift in absorption maximum is proportional to protein concentration over a broad range. The shift is fast and relatively stable, however, it can be quite dependent on the composition of the protein, varying as much as 6-fold between individual pure proteins. Care must be taken in analyzing complex mixtures. Suitable standards must be chosen for each particular application. Alterations in dye or phosphate concentrations may help to minimize this variation. Most buffers, including Tris, have little effect on the color change. A minor disadvantage of this assay is that the dye adsorbs to glassware, cuvettes, skin, and clothing. Dye can be removed from glassware by dilute solutions of the detergent sodium dodecyl sulfate.

6) **BCA Protein Assay:** The BCA Protein Assay has been advertised as an alternative to the Lowry assay. The key component in this assay is bicinchoninic acid (BCA) which reacts with cuprous ions to generate an intense purple color at 562 nm. Cuprous ions are produced by the reduction of cupric ions by proteins in alkaline solutions. The chromophore produced is more stable than that produced in the Lowry assay. Additional advantages include high sensitivity (100 times greater than the Biuret), a simple two-solution reagent which is commercially available, ease of procedure, and compatibility with a large number of extraneous materials commonly found in protein preparations.

7) **UV absorbance:** Protein concentrations can be determined directly by ultraviolet spectroscopy because of the presence of tyrosine and tryptophan which absorb at 280 nm. Because the levels of these two amino acids vary greatly from protein to protein, the UV absorbance per milligram protein is highly variable. The extinction coefficient (usually expressed as E1%, i.e., the absorbance at 280 nm of a 1% solution (10 mg/ml)) will generally fall between 4.0 and 15; however, examples of proteins at either extremes have been observed, eg. parvalbumin (0.0), serum albumin (5.8), trypsin (14.3) and lysozyme (26.5). Thus, the absorbance at 280 nm will only give an estimation of the protein concentration unless the extinction coefficient for a pure protein has been accurately determined (by dry weight or by amino acid analysis). Alternatively, the absorbance in the far-ultraviolet region (190-220 nm) can be used. This method is much more sensitive (E1% = 110 or greater) and is less dependent on the amino acids composition because the absorbance is dominated by the peptide bond transition. The major advantages of this method include its high sensitivity, ease of performance, and the fact that the method is nondestructive so valuable protein samples can be recovered. Major disadvantages include the requirement of uv spectrophotometers and quartz cuvettes and the fact that virtually everything including commonly used buffers absorb in the UV regions. Nucleic acids also absorb strongly in the UV region (260 nm). A ratio of absorbance (280/260) can be used to correct for the presence of nucleic acids. This method is often used to monitor the growth of yeast and cell suspensions.

References:

**Biuret method**

**Lowry method**

**Bradford assay**

**UV Absorbance**

**BCA Assay**