PROTEIN CONCENTRATION BY UV ABSORBANCE AT 280NM

INTRODUCTION

Measuring the concentration of proteins in solution is routine practise for most biochemistry laboratories. This information sheet covers the use of UV Absorbance at 280nm for determining the concentration of proteins in solution. There is a section on the calculation used to convert an A_{280} reading to concentration followed by a section with notes on running the methods.

UV ABSORBANCE AT 280NM

The simplest and most common method to measure the concentration of a protein in solution is by using a spectrophotometer to measure the absorbance at 280nm. If you perform a wavelength scan between 200 and 350nm you can glean some additional information about your protein.

Other benefits of this technique is that it is simple and quick to perform, there is little sample preparation required. You may have to dilute your sample but otherwise there is no addition of other reagents and no need to incubate. Especially with precious samples, they can be recovered afterwards for other assays or uses.

However, the method does have some limitations. It is best for pure samples where the mass extinction coefficient is known or can be calculated from the amino acid sequence. Mixtures of proteins may well give an inaccurate reading. There can be interference from other chromophores, which includes nucleic acids that have significant absorption at 280nm.

The conversion equation is:-

$$\text{Concentration of protein sample in mg/mL} = \frac{\text{Measured } A_{280\text{-}320}}{\varepsilon_{0.1\%}} \quad \text{(assumes 1cm path length)}$$

Where

$A_{280\text{-}320}$ is the absorbance reading at 280nm minus the absorbance reading at 320nm (baseline)

$\varepsilon_{0.1\%}$ is the mass extinction coefficient or the percent solution extinction coefficient (absorbance values at 280nm for a 0.1% solution of a reference protein measured in a 1cm cuvette with units $(mg/mL)^{-1}\ cm^{-1}$). Note it is different to ε_{molar} the molar extinction coefficient or molar absorptivity of the protein (M–1 cm–1). With a 1cm pathlength $\varepsilon_{0.1\%} = \varepsilon_{molar}$ / molecular weight

The ProtParam tool [1] reports the $\varepsilon_{0.1\%}$ value as Abs 0.1% (= 1 g/l)

For a protein sample where the sequence is unknown, it is common to use an $\varepsilon_{0.1\%} = 1$ to give an estimate of concentration

Note: Care has to be taken as we have assumed a 1cm path length. If this is different in your spectrophotometer then that needs to be factored into your conversion. Some instruments, such as the nanodrop from ThermoFisher, that don't have a 1cm pathlength automatically normalise readings to reflect a 1cm pathlength.

The explanation for how this formula is derived is given below:-

Conversion of the absorbance measured at 280nm to a protein concentration is achieved by using the Beer-Lambert Law (or Beer's law) equation.

Equation 1

$$A = \varepsilon_{molar} cL$$

where:
A: absorbance of the sample at 280nm (unitless)
ε_{molar}: molar extinction coefficient or molar absorptivity of the protein ($M^{-1} cm^{-1}$)
c: concentration of the protein (molar units, M)
L: light pathlength (cm)

The absorption of radiation in the near UV is determined by a proteins' aromatic residue content. Mainly Tyr and Trp, but also Phe to a small extent. Disulphide bonds if present, also contribute a small portion to the absorption. ε_{molar} is calculated as a theoretical value from the sequence of the protein and the content of these residues (and there are a few different methods in the literature for calculating it). For example Equation 2.

Equation 2

$$\varepsilon_{molar} = (nW \times 5500) + (nY \times 1490) + (nC \times 125)$$

where:
W: tryptophan
Y: tyrosine
C: cysteine
n: number of each residue present in the protein
5500, 1490, and 125: are the molar absorptivity coefficients at 280 nm of W, Y, and C, respectively

A useful online tool to calculate ε_{molar} is the ProtParam tool [1] from Expasy. It requires the amino acid sequence of your protein and this can often be gained from Uniprot (Remember to include any modifications you have made to your particular construct such as tags, mutations, deletions etc)

However, in this form the equation 1 is not all that useful as generally we want to know the protein concentration in mg/mL. If we use equation 1 assuming a 1mg/mL (0.1%) protein concentration and convert the concentration term from M to mg/mL, the absorbance at A_{280} will be

$$A_{0.1\%} = \varepsilon_{molar} cL$$
$$A_{0.1\%} = \varepsilon_{molar} (1/MW)\, L$$

Equation 3

$$A_{0.1\%} = (\varepsilon_{molar}\, L)/MW$$

where:
$A_{0.1\%}$: measured absorbance of a 1mg/mL sample at 280nm (unitless)
MW: Is the molecular weight of the protein in Daltons (also reported by the ProtParam tool [1])

Assuming a standard 1cm pathlength cuvette (ε_{molar} L)/MW is sometimes simplified to $\varepsilon_{0.1\%}$ the mass extinction coefficient or the percent solution extinction coefficient (absorbance values at 280nm for a 0.1% solution of a reference protein measured in a 1cm cuvette with units $(mg/mL)^{-1}$ cm^{-1}).

The ProtParm tool [1] reports this $\varepsilon_{0.1\%}$ value as Abs 0.1% (= 1 g/l)

This is much more useful as it gives us a conversion factor that we can use to convert a measured A_{280} reading to mg/mL. In that from this we can then say:-

Equation 4

$$\text{Concentration of protein sample in mg/mL} = \frac{\text{Measured } A_{280}}{\varepsilon_{0.1\%}} \quad \text{(assumes 1cm path length)}$$

Note: Care has to be taken as we have assumed a 1cm path length. If this is different in your spectrophotometer then that needs to be factored into your conversion. Some instruments, such as the nanodrop from ThermoFisher, that don't have a 1cm pathlength automatically normalise readings to reflect a 1cm pathlength.

For a protein sample where the sequence is unknown, it is common to use an $\varepsilon_{0.1\%}$ = 1 to give an estimate of concentration.

This formula in equation 4 can be improved by adding in a baseline subtraction term. The standard practise is to measure the UV absorbance of the sample at 320nm as well and subtract this from the 280nm reading. To give the final Equation 5

Equation 5

$$\text{Concentration of protein sample in mg/mL} = \frac{\text{Measured } A_{280\text{-}320}}{\varepsilon_{0.1\%}} \quad \text{(assumes 1cm path length)}$$

METHOD NOTES

IF USING A SPECTROPHOTOMETER

- Aim for a protein sample concentration of 0.1 – 1mg/mL.
- Remove any precipitation (filter or centrifuge at high speed (>10,000 xg).
- If using cuvette ensure that it is either quartz or made of a plastic that is transparent at 280nm and all other wavelengths being measured.
- No bubbles.
- Blank with buffer your protein sample is in (including any dilutions with water you have made to prepare it).
- Make sure the cuvette is sufficiently full so that light beam is passing through solution with the liquid height well above the beam so that there are no refraction effects from surface.

IF USING A NANODROP OR EQUIVALENT MACHINE

- Remove any precipitation (filter or centrifuge at high speed (14,000)
- Perform a few readings and take average. Our experience is that these instruments have a higher variability than a traditional cuvette spectrophotometer.

INTERFERING REAGENTS FOR UV ABSORBANCE 280NM

There are common buffer components that have chromophores that will absorb at 280nm. Always ensure that a buffer blank is used to zero the spectrophotometer and ideally dilute the sample to below the concentrations given in the table below.

Reagent	Conc'n above which will significantly absorb at 280nm
Ammonium sulphate	>50% (w/v)
Brij 35	1% (v/v)
DTT	3mM
EDTA	30mM
Glycerol	>40% (v/v)
KCl	100mM
ß mercaptoethanol	10mM
NaCl	>1M
NaOH	>1M
Phosphate buffer	>1M
SDS	0.1% (w/v)
Sucrose	2M
Tris Buffer	0.5M
Triton X-100	0.02% (v/v)
Urea	>1M

Taken from [2]

PROTEIN SOLUTIONS CONTAMINATED WITH NUCLEIC ACIDS

For a protein solution that is known to be contaminated with nucleic acids the following method can be used to give a reasonable approximation of the protein concentration.

This works for nucleic acid content up to 20% w/v or A_{280} / A_{260} < 0.6

- Zero spectrophotometer with a buffer blank.
- Read the absorbance of the sample at 280nm, 260nm and 205nm.
- Calculate that approximate protein concentration using one of the following equations.
    - Protein conc (mg/mL) = (1.55 x A_{280}) – (0.76 x A_{260})
    - Or Protein conc (mg/mL) =A_{205} / (27 + A_{280}/A_{205})

TYPICAL 280NM VALUES FOR PROTEINS COMMONLY USED AS STANDARDS

The true $\varepsilon_{0.1\%}$ of a protein (as opposed to the $\varepsilon_{0.1\%}$ calculated from its amino acid sequence) can be established in a number of ways. The most accurate way is by determining the concentration of a purified reference sample of the protein in question using amino acid analysis. Alternatively, comparison of the A_{280} with common protein standards can be used.

Protein	Typical A_{280} value for a 1mg/mL solution
Bovine Serum Albumin (BSA)	0.70
Immunoglobulin G (IgG)	1.35
Immunoglobulin M (IgM)	1.20

OTHER INFORMATION FROM A WAVELENGTH SCAN

There is other information that can be gained from a wavelength scan that is run between 220nm and 320nm:-

- An indication of protein aggregation will be seen by an increase in absorbance at 320nm.
- Because the peak absorbances of Phe, Tyr and Trp are at slightly different wavelengths around 280nm, a protein will have a “signature” shape to its main absorbance peak that is determined by the relative content of these 3 amino acids that it contains. Samples that give peaks that deviate from the expected “signature” shape should be further analysed for the presence of truncations, degradation products or other contaminants.
- DNA has a peak absorbance at 260nm, therefore the A_{280} / A_{260} ratio of a sample gives an indication of the amount of DNA that is present. A pure protein sample should have an A_{280} / A_{260} ratio of >1.8.

REFERENCES

[1] Expasy, ‘Protein Parameter Tool’. https://web.expasy.org/protparam/.

[2] C. M. Stoscheck, ‘[6] Quantitation of protein’, 1990, pp. 50–68.