A theoretical and practical guide for spectrophotometric determination of protein concentrations at 280 nm

Introduction

Even though it was first reported in the 1950s [1], quantitation of protein concentration using direct measurements of absorbance at 280 nm is still one of the most widely used biochemical assays for protein measurement. This success is arguably related to several aspects: this assay is the fastest of all protein quantitation assays, no reagents are needed, and no incubation or other preparation steps are involved. It can typically be run without preparing any special protein standards, and the protein sample can be further recovered, if needed, for downstream applications. Because of these advantages, this assay is often the first choice for quick estimation of protein concentrations. Here we revisit the most important theoretical and practical aspects that are needed in order to successfully understand and perform this assay.

1. Basis for spectrophotometric quantitation of proteins at 280 nm

Direct spectrophotometric determination of proteins can be done at either 280 nm or 205 nm. In this note, only measurement at 280 nm will be discussed. At this wavelength, the aromatic amino acids tryptophan (Trp) and tyrosine (Tyr) exhibit strong light absorption, and to a lesser extent cysteine groups forming disulfide bonds (Cys–Cys) also absorb. Consequently, absorption of proteins and peptides at 280 nm is proportional to the content of these amino acids. However, the absorptivity of a given protein is not strictly dependent on amino acid content but also on buffer type, ionic strength, and the pH of the protein solution. Different preparations of the same protein can be highly heterogeneous with respect to protein conformations and posttranslational modifications (e.g., oxidation, glycosylation), which can further affect protein absorptivity.

The absorptivity coefficient, also known as the extinction coefficient (ε), can be established for any given protein. For each protein or peptide, the extinction coefficient can be experimentally determined or calculated from the amino acid sequence, based on the premise that the absorption of amino acids is additive. In section 2 of this note, we discuss different aspects related to extinction coefficients that can help clarify their use in practice.

Apart from their intrinsic absorptivity, proteins will absorb UV light in proportion to their concentrations. This relationship has been exploited for the spectrophotometric determination of protein concentrations, and it is defined by the Beer–Lambert law (or Beer's law). Beer's law describes the dependence of a protein's absorbance on its absorptivity coefficient, its concentration, and the pathlength of the incident light:

Equation 1

$$A = \varepsilon c L$$

where:

- A: absorbance of the sample (unitless)
- ε: 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 protein concentration based on the measured absorbance at 280 nm can be easily derived from the equation above:

Equation 2

$$c = \frac{A}{\varepsilon L}$$

Despite its mathematical simplicity, the application of this equation can be difficult in some cases, especially when different extinction coefficients are available or different types of samples and volumes are used. In this note, we provide practical guidance on how to overcome these challenges (sections 2–4).

2. Understanding and using extinction coefficients

The most reliable extinction coefficient for any given protein is obviously one that can be experimentally measured using a known concentration of protein dissolved in the same buffer intended for the subsequent downstream applications. However, several other estimation methods are quicker and still sufficiently accurate for determining protein concentrations for most laboratory applications.

For example, the extinction coefficient at 280 nm of any protein in water can be theoretically estimated by the weighted sum of the 280 nm absorptivity coefficients of the three amino acids mentioned earlier [2]:

Equation 3

$$\varepsilon = (nW \times 5500) + (nY \times 1490) + (nC \times 125)$$

where:

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

Currently these theoretical values are calculated with computing tools, and the only requisite or input needed is the primary sequence of the protein. Curated primary sequences of proteins, along with other functional information, are available in the Universal Protein Resource, known as UniProt (**uniprot.org**). For calculation of extinction coefficients, a popular and free solution is the ProtParam™ tool (**web.expasy.org/protparam**) provided by the Swiss Institute of Bioinformatics. The ProtParam tool calculates the extinction coefficients utilizing the equation described above. The program generates two values for extinction coefficients. The first value is calculated assuming all cysteine residues in the protein are forming disulfide bonds (C–C), whereas the second value is calculated assuming all cysteine residues are in a reduced state, i.e., no disulfide bonds are formed. This computation of extinction coefficients is quite reliable for tryptophan-containing proteins, but it is prone to errors for proteins that do not contain tryptophan residues.

Extinction coefficients of many different proteins have been compiled in the literature. For instance, they are reported in the *Practical Handbook of Biochemistry and Molecular Biology* [3] as well as in specialized scientific journals. Most of the measurements reported in the literature have been performed in aqueous buffers, e.g., phosphate, HEPES, or Tris-HCl buffers. Thus, a value obtained with the protein dissolved in a buffer similar to that of the sample of interest can be selected. Hence, it can be concluded that extinction coefficient data, either predicted or experimental, are available for most proteins.

Because reporting of extinction coefficients has not been standardized in the literature, the calculation of protein concentrations may be somewhat challenging. For instance, absorbance values (A_{280}) for a 1% protein solution ($\varepsilon_{1\%}$ or $\varepsilon_{1\ percent}$) or a 0.1% solution ($\varepsilon_{0.1\%}$) are frequently reported, instead of the molar extinction coefficients shown in equation 2. The most commonly reported definitions of molar and mass extinction coefficients are listed below:

- ε_{molar} is the molar extinction coefficient or molar absorptivity of the protein, expressed as $M^{-1}\,cm^{-1}$
- $\varepsilon_{1\%}$ is the mass extinction coefficient or the percent solution extinction coefficient (absorbance values at 280 nm) for a 1% or 10 mg/mL solution of a reference protein measured in a 1 cm cuvette, expressed as $10\ (mg/mL)^{-1}\,(cm)^{-1}$
- $\varepsilon_{0.1\%}$ is the mass extinction coefficient or the percent solution extinction coefficient (absorbance values at 280 nm for a 0.1% or 1 mg/mL solution of a reference protein measured in a 1 cm cuvette; expressed as $(mg/mL)^{-1}\,(cm)^{-1}$

In the Beer's law equation (equation 1), the molar extinction coefficients are included (ε_{molar}) and the molar concentrations are obtained. Therefore, the molar concentration should be multiplied by the molecular weight of the protein to express the final protein concentration in mg/mL. In contrast, when using mass extinction coefficient values ($\varepsilon_{1\%}$ or $\varepsilon_{0.1\%}$) in the Beer's law equation, the results are directly expressed as mass percentages of 1% (10 mg/mL) or 0.1% (1 mg/mL). When using $\varepsilon_{1\%}$, an adjustment factor of 10 needs to be applied to express the final protein concentrations in mg/mL.

As an example, many extinction coefficients or absorptivity values are currently available for bovine serum albumin (BSA). With a quick online search, several values reported in the literature can be found (Table 1).

Table 1. Examples of extinction coefficients for BSA.

Absorptivity	Value	Source
ε_{molar}	43623 $M^{-1}\,cm^{-1}$	[4,5]
ε_{molar}	44300 $M^{-1}\,cm^{-1}$	[4,6]
ε_{molar}	47790 $M^{-1}\,cm^{-1}$	ProtParam tool (all reduced Cys residues)
$\varepsilon_{1\%}$	6.58	[4,5]
$\varepsilon_{1\%}$	6.68	[4,6]
$\varepsilon_{0.1\%}$	0.69	ProtParam tool (all reduced Cys residues)

When selecting values from the available data, extinction coefficients that have been determined experimentally are preferred; however, as explained above, the values can differ depending on the selected buffer as well as other factors. In general, ProtParam predictions made with reduced Cys residues can be used for most proteins.

For BSA, a $\varepsilon_{1\%}$ value of 6.7 (or $\varepsilon_{0.1\%}$ value of 0.67) at 280 nm is generally accepted. From the relationship between $\varepsilon_{1\%}$ and ε_{molar} seen in Equation 4 and assuming a molecular weight (MW) of 66400 for BSA, the extinction coefficient of 6.7 can be calculated to correspond to an ε_{molar} of 44488 $M^{-1}\,cm^{-1}$, which is close to the reports of Gill and von Hippel [4].

Equation 4

$$\varepsilon_{molar} = \frac{MW \times \varepsilon_{1\%}}{10}$$

where:

ε_{molar}: molar extinction coefficient or molar absorptivity of the protein ($M^{-1}\,cm^{-1}$)

$\varepsilon_{1\%}$: mass extinction coefficient or percent solution extinction coefficient (A_{280} for a 1% or 10 mg/mL solution of a reference protein measured in a 1 cm cuvette; expressed in $10\ (mg/mL)^{-1}\,(cm)^{-1}$

MW: molecular weight of the protein

Overall, mass extinction coefficients are easier numbers to handle, so they are frequently used. $\varepsilon_{0.1\%}$ is especially convenient because it provides absorbance values for a 0.1% (1 mg/mL) protein solution. In such cases, the Beer's law equation can be directly applied for the calculations of protein concentrations in mg/mL, without using an adjustment factor.

3. Guide to understanding and working with the Beer–Lambert law

According to the Beer–Lambert law, the concentration of a protein is directly proportional to its absorbance, at a defined wavelength and at a constant pathlength, as seen in equation 2. In section 2, we discussed the meaning of extinction coefficients and factors to be considered when using molar or mass extinction coefficients for calculation of protein concentration. Another important factor to consider in this equation is the pathlength. The pathlength is the distance through which the light has to pass in order to reach the detector. When a sample is measured in a standard spectrophotometer, light passes through the sample horizontally through a cuvette (Figure 1), and the pathlength is typically 1 cm. However, when microvolumes are used in other formats, light passes through the sample vertically, and the pathlength is shorter and varies, as shown below:

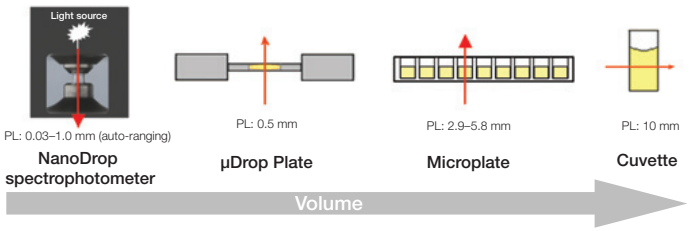

Figure 1. Examples of differences in pathlength (PL) among different sample formats and volumes. The pathlengths of the microplate are given as representative examples and they are based on volumes of 100 µL and 200 µL of TE buffer pipetted into a UV-quality 96-well plate.

The exact pathlength used for measurement of protein concentration is therefore an important factor that needs to be considered when using different sample formats and measurement platforms.

The autoranging pathlength technology of the Thermo Scientific™ NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer allows users to measure samples spanning a wide concentration range, thus eliminating the need for dilutions or consumables. This technology utilizes multiple pathlengths (ranging from 0.03 mm to 1.0 mm) that change in real time while measuring a 2 µL protein sample. By optimizing the pathlength based on the analyte absorbance, the instrument ensures that the absorbance stays within the linear range of the spectrophotometer.

The pathlength of a cuvette or the Thermo Scientific™ µDrop™ Plate is constant and provided by the vendor. For instance, the nominal pathlength of the µDrop Plate is 0.5 mm (or 0.05 cm), but each µDrop Plate has an individual pathlength that should be taken into account during calculations (see section 4). The situation becomes more complicated in the case of microplates. The pathlength of the liquid in the wells depends mainly on three variables: the liquid volume, microplate well dimensions, and the meniscus effect of the liquid surface. Therefore, the pathlength of the liquid cannot be calculated directly from the volume that has been dispensed or pipetted into the well. The microplate wells have a conical shape, which makes pathlength calculation complicated. Further, calculating the exact meniscus shape at the liquid surface is not trivial as it is strongly dependent on, among other factors, the liquid composition and the sample concentration. Overall, the well geometry and meniscus shape cause the measured absorbance to have a highly complex mathematical dependence on the liquid volume.

When measuring the pathlength in the individual wells in a microplate, first measure the absorbance of the sample buffer alone at 975 nm and 900 nm using a 1 cm cuvette. The subtraction $A_{975\ nm} - A_{900\ nm}$ is called the "K-factor", and it is simply used as a reference value that can be compared to the absorbance that is measured in microplates. Values of K-factor are available for many different aqueous buffers. Often, the K-factor of water (0.173) in a 1 cm cuvette measured at 25°C is used in calculations, as it is close enough to the values of low-concentration aqueous buffers and solvents. The liquid pathlength of each well is then calculated, and absorbance values are corrected accordingly, corresponding to the 1 cm pathlength, as shown in equation 5.

Equation 5

$$A_{corrected} = A_{raw} \times \left(\frac{\text{K-factor}}{A_{975}\,(well) - A_{900}\,(well)}\right)$$

where:

A_{raw}: original absorbance values measured for the sample in the microplate at 280 nm

A_{975}: absorbance values measured for the sample in the microplate at 975 nm

A_{900}: absorbance values measured for the sample in the microplate at 900 nm

K-factor: $A_{975} - A_{900}$ measured at 25°C in a 1 cm cuvette with the same aqueous buffer in which samples are dissolved (a K-factor value of 0.173 is often used)

To calculate the protein concentrations, these corrected absorbance values are then subsequently divided by the extinction coefficients, according to Beer's law (equation 2).

4. Solutions for direct protein quantitation

Due to the long-term tradition and consistent high quality of Thermo Scientific™ spectrophotometers, our instruments have become indispensable tools that help scientists in their daily evaluation of protein samples. Thermo Scientific™ instruments cover the broadest array of sample formats and volumes, and sample throughput needs. Many of our instruments have been designed with preprogrammed methods to speed up protein determination workflows. Several measurement platforms are also available—from microvolume to microtiter plates, and cuvettes. In this section, we will discuss two instruments, the Thermo Scientific™ NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer and the Multiskan™ Sky Microplate Spectrophotometer. Both instruments provide various options to select predefined extinction coefficients, which are then used to automatically calculate protein concentrations. These instruments also provide several sample types for protein quantitation, as well as guidance for selection criteria for a suitable sample type (Table 2).

Table 2. Sample types available in the NanoDrop One/One^C and Multiskan Sky spectrophotometers.

Sample type	Extinction coefficient	Recommended application
1 Abs = 1 mg/mL	$\varepsilon_{1\%} = 10$	For uncharacterized or novel proteins
BSA	$\varepsilon_{1\%} = 6.7$	Bovine serum albumin reference; unknown samples are calculated assuming $\varepsilon_{1\%} = 6.7$
IgG	$\varepsilon_{1\%} = 13.7$	IgG reference; unknown samples are calculated assuming $\varepsilon_{1\%} = 13.7$; can be used with most antibodies, as they have $\varepsilon_{1\%}$ value in the range of 12–15
Lysozyme	$\varepsilon_{1\%} = 26.4$	Unknown samples are calculated assuming $\varepsilon_{1\%} = 26.4$

In addition, users can also select their own extinction coefficients, which can be given either as molar extinction coefficients (ε_{molar} and MW) or as mass extinction coefficients ($\varepsilon_{1\%}$ or $\varepsilon_{0.1\%}$). In general, if the true value of the extinction coefficient is not known, a value of $\varepsilon_{1\%} = 10$ is commonly applied.

4.1 Measuring microvolume samples with the NanoDrop One/One^C spectrophotometer

To measure protein samples on the NanoDrop One/One^C spectrophotometer, users must first select the extinction coefficient they will use for calculation of the protein concentration (Table 2), and whether to have the instrument perform a baseline correction at a particular wavelength. It is recommended that baseline correction be performed at 340 nm to ensure the most accurate results. However, if the protein buffer or the label attached to the protein has significant absorbance at 340 nm, we recommend that the baseline correction be turned off or performed at a different wavelength. To perform protein measurements, users can simply follow the on-screen instructions and absorbance can be measured using 2 µL of the protein sample. To clean the measurement pedestals, simply wipe away the sample with a dry laboratory tissue. The NanoDrop One/One^C instrument collects full spectral data in approximately 8 seconds. The absorbance data are automatically normalized to reflect a 10 mm (1 cm) pathlength, and the concentration is calculated using the following equation:

Equation 6

$$c = \left(\frac{A_{280}}{\varepsilon_{1\%}} \times \frac{1}{cm} \right) \times 10$$

where:

c = protein concentration in mg/mL

A_{280} = 10 mm equivalent absorbance at 280 nm (after baseline correction at 340 nm)

$\varepsilon_{1\%}$ = published percent solution extinction coefficient 10 $(mg/mL)^{-1}$ $(cm)^{-1}$

4.2 Measuring protein samples with the Multiskan Sky Microplate Spectrophotometer using the µDrop Plate

The µDrop Plate provides a tool for analyzing low-volume samples in the range of 2–10 µL. The sample is pipetted to the low-volume area of the plate, which accommodates up to 16 samples. A quartz slide is then placed over the sample area and used to spread the sample, creating a pathlength of approximately 0.5 mm; however, the specific pathlength may vary slightly between µDrop Plates. Thus, the pathlength of the µDrop Plate needs to be specified before measurements can be taken with the Multiskan Sky Microplate Spectrophotometer. If A_{280} is above 3.0, then the sample needs to be diluted and measured again. Researchers can select between 0, 1, or 2 blanks, according to the on-screen instructions. In all cases, a background subtraction is performed at 320 nm.

After selecting the extinction coefficients (Table 2) and the appropriate number of blanks, protein concentrations are calculated by the instrument as follows (equation 7):

Equation 7

$$c = \left[\frac{(A_{280} - A_{280\ blank}) - (A_{320} - A_{320\ blank})}{L} \right] X \frac{1}{\varepsilon_{1\%}} X\ 10$$

where:

c = protein concentration in mg/mL

A_{280}, $A_{280\ blank}$: absorbance at 280 nm for the samples and blank solutions, respectively

A_{320}, $A_{320\ blank}$: absorbance at 320 nm for the samples and blank solutions, respectively

L: light pathlength of the µDrop Plate (cm)

$\varepsilon_{1\%}$: published percent solution extinction coefficient 10 $(mg/mL)^{-1}$ $(cm)^{-1}$

If the user only has molar extinction coefficients (ε_{molar}), ε_{molar} can be easily converted to $\varepsilon_{1\%}$ as shown in equation 8, which is derived from equation 4:

Equation 8

$$\varepsilon_{1\%} = \frac{\varepsilon_{molar} \times 10}{MW}$$

These values are used by the instrument to calculate protein concentration as described for equation 7.

4.3 Measuring microtiter plates with the Multiskan Sky Microplate Spectrophotometer

To simultaneously assay a large number of samples with volumes in the microliter (µL) range, microtiter plates can be utilized. Light absorption by polystyrene or polypropylene plates in the visible region is very low, and thus their background contribution is negligible when performing colorimetric assays. However, these plates tend to absorb light in the UV range, making it imperative to use UV-compatible or quartz plates for direct spectrophotometric determination of protein concentrations. A general recommendation is to use volumes over 70 µL in a UV-compatible microplate. Assuming a flat meniscus, this volume would roughly correspond to a pathlength of 2 mm. Larger volumes will result in larger pathlengths and thus better sensitivity. For instance, with the same assumption of a flat meniscus, a volume of 150 µL will result in a pathlength of 4.3 mm.

Because most protein solutions are prepared in aqueous buffers, using a K-factor of 0.173 is acceptable for the calculations. The Multiskan Sky Microplate Spectrophotometer automatically corrects the pathlength for each individual well, using the strategy that was described in section 3. Select between 0, 1, or 2 blanks, and in all cases, a background subtraction is made at 320 nm.

When blanks and extinction coefficients (Table 2) are selected, calculation of protein concentrations is performed by the Multiskan Sky Microplate Spectrophotometer as follows (equation 9):

Equation 9

$$c = \left[\frac{(A_{280} - A_{280\ blank}) - (A_{320} - A_{320\ blank})}{\varepsilon_{1\%}} \right] X \frac{1}{cm} X \frac{K\text{-factor}}{A_{975} - A_{900}} X\ 10$$

where:

c = protein concentration in mg/mL

A_{280}, $A_{280\ blank}$: absorbance at 280 nm for the sample and blank solutions, respectively

A_{320}, $A_{320\ blank}$: absorbance at 320 nm for the sample and blank solutions, respectively

$\varepsilon_{1\%}$: published percent solution extinction coefficient 10 $(mg/mL)^{-1}$ $(cm)^{-1}$

K-factor: a value of 0.173 is often used (exact calculation for specific solvents is described in section 3)

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User-entered molar extinction coefficients (ε_{molar}) can easily be converted to $\varepsilon_{1\%}$ (equation 7), and these values can be used to calculate the protein concentrations as already described above (equation 6).

4.4 Measuring protein samples with the NanoDrop One^C or Multiskan Sky spectrophotometers using cuvettes

Both the NanoDrop One^C and Multiskan Sky spectrophotometers have cuvette ports that allow A_{280} measurements using cuvettes. Key factors to consider when measuring the absorbance of a protein sample in a cuvette are:

- The cuvettes must be UV-transparent (quartz or UV-transparent plastic)
- The use of masked cuvettes is preferable; unmasked cuvettes can allow light that has not passed through the sample to hit the detector, which can lead to significant measurement error
- The z-height of the cuvette should be 8.5 mm for both instruments

For the measurements, follow the on-screen instructions, and inclusion of blank samples is recommended. The absorbance data are converted to the protein concentration values using equation 10:

Equation 10

$$c = \left(\frac{A_{280}}{\varepsilon_{1\%}} \times \frac{1}{cm} \right) \times 10$$

where:

c = protein concentration in mg/mL

A_{280} = 10 mm equivalent absorbance at 280 nm (after baseline correction at 340 nm or 320 nm, depending on the instrument)

$\varepsilon_{1\%}$ = published percent solution extinction coefficient 10 $(mg/mL)^{-1}$ $(cm)^{-1}$

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