

Quantifying Protein Concentration using UV Absorbance Measured by the Ocean HDX Spectrometer

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Application Note

KEYWORDS

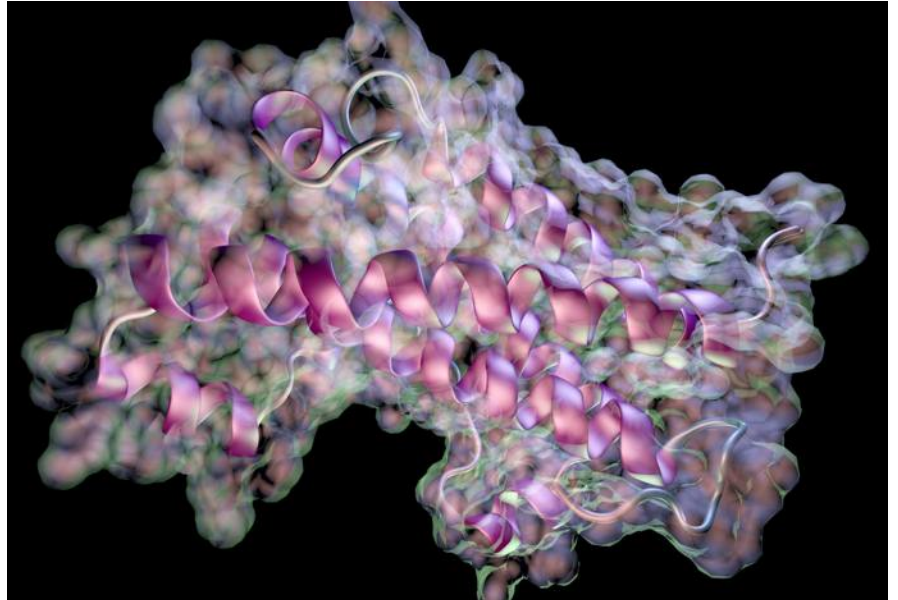
- Protein
- Bovine serum albumin
- Aromatic amino acids

TECHNIQUES

- UV absorbance
- Standard curve generation

APPLICATIONS

- Protein concentration determination
- Life sciences research



Proteins are a common sample in clinical, diagnostic and research labs. Studies involving protein extraction, purification or labeling, or working with proteins extracted from cells or labeled for study of the interactions between biomolecules, are common. Determination of protein concentration is a critical part of protein studies. In this application note, we use an Ocean HDX spectrometer to generate a standard curve for bovine serum albumin (BSA). High sensitivity in the UV, ultra-low stray light performance and upgraded optics make the Ocean HDX ideal for UV absorbance measurements.

Proteins are central to life. These intricate biomolecules play critical roles in cellular processes and metabolism, in catalyzing biochemical reactions and serving as structural elements, and so much more. As vital elements for all life, proteins are often the subject of biomedical diagnostic studies and life science research. Most research involving proteins, regardless of the answer being sought or the source of the protein, begins

with quantifying the amount of protein present in a sample.

Quantifying Protein Concentration

The methods for quantifying protein concentration fall into two groups – direct detection using UV absorbance or colorimetric visible absorbance assays requiring a reagent that reacts with the protein to make a colored product. Direct absorbance measurements at 280 nm – where aromatic amino acids Tryptophan, Tyrosine and Phenylalanine absorb – are relatively quick and the protein is not consumed by the measurement. Colorimetric assays using reagents can provide total protein concentration but protein impurities can affect the result.

Regardless of the method used for protein quantification, the first step is to measure absorbance for several dilutions of the protein of interest or a standard protein. In the case of the direct UV absorbance method, the absorbance at 280 nm is plotted versus the known concentration for each sample. According to Beer's Law (also called the Beer-Lambert law), the absorbance of a solution depends directly on the concentration of the absorbing molecules and the pathlength the light travels through the sample. This relationship enables us to use the standard curve generated for our known protein samples to determine the concentration of an unknown protein sample.

Beer's Law states ...

$$A(\lambda) = \epsilon(\lambda)cl$$

... where

- $A(\lambda)$ is the absorption of the solution as a function of wavelength
- $\epsilon(\lambda)$ is the molar absorptivity or extinction coefficient of the absorbing molecule as a function of wavelength (in L/mol·cm)
- c is the concentration of the solution (in mol/L)
- l is the pathlength traveled by light through the solution (in cm)

The unknown protein concentration is determined from a best-fit line drawn through the data points. Note that the linear portion of the standard curve is the only region used to determine the concentration of the unknown sample. Samples with measurements that fall outside the linear range of the standard curve must be diluted until the absorbance falls within the linear range. Concentration predictions based on data outside the concentration range measured or beyond the linear region of the plot are not valid.

We used an Ocean HDX spectrometer configured for UV absorbance measurements to generate a standard curve for bovine serum albumin (BSA). The high definition optics of the Ocean HDX make it ideal for UV absorbance measurements, with ultra-low stray light for higher maximum absorbance levels and upgraded optics to provide exceptional performance in a compact footprint.

Experimental Procedure

A 6 mg/mL BSA (Sigma P/N A2153) stock solution was prepared in water and diluted to 0.02 mg/mL to create a standard curve for predicting the concentration of unknown protein samples. Note that BSA is often used as a standard protein for the generation of calibration curves to predict protein concentration.



Absorbance was measured with the OCEAN-HDX-UV-Vis spectrometer, DH-2000-BAL balanced deuterium-tungsten halogen light source, CUV-UV cuvette holder, two QP230-2-XSR extreme solarization resistant fibers and the Absorbance Wizard in OceanView software. Since BSA does not absorb light in the visible region, only the deuterium lamp was used for the measurements. Limiting your light source to the region where absorbance occurs decreases stray light in the measurement, allowing for higher maximum absorbance levels for samples with higher concentrations. The spectrometer and light source were warmed up for 60 minutes to improve the stability of the measurements by eliminating the drift that occurs while the spectrometer and light warm up to a consistent temperature.

Measurements were made in a single quartz cuvette that was not removed from the cuvette holder between measurements. Dilutions were done directly in the cuvette by removing 0.5 mL sample and replacing it with 0.5 mL water. A disposable pipette was used to mix the sample after water was added to the cuvette. Dilutions were done down to 0.02 mg/mL.

Results

The protein absorbance spectra measured with the Ocean HDX are shown in **Figure 1**. The ultra-low stray light performance of the Ocean HDX enabled us to measure absorbance to almost 3 AU for the aromatic amino acid peak at 280 nm. Although the results are not shown here, we also achieved absorbance values above 3 AU at around 220 nm, where the peptide backbone absorbs.

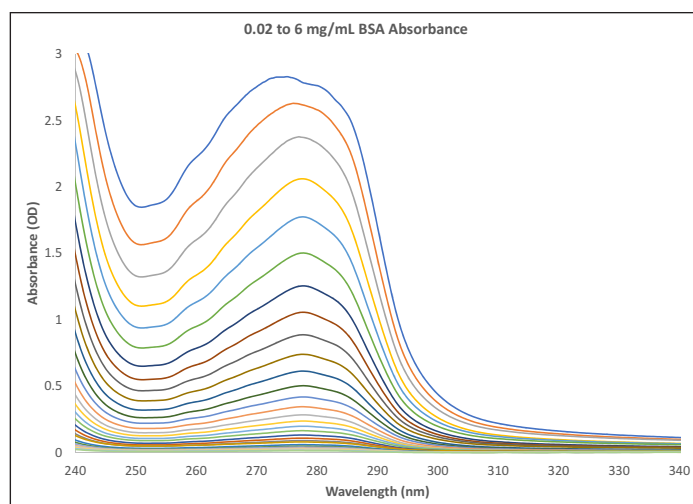


Figure 1: Absorbance for BSA diluted in water. As predicted by Beer's Law, absorbance increases linearly with concentration within the linear range for the instrument to ~2.5 AU.

The standard curve generated from the BSA absorbance data is shown in **Figure 2**. A leveling off or roll-off in the data is observed between 2.4 and 2.5 AU. This roll-off is indicative of stray light present in the measurement. Stray light (present in all absorbance measurements to varying degrees) limits the maximum absorbance value that can be measured with a system.

Stray light is light from outside the designed light path that unintentionally lands on any part of the detector, giving a false reading. The detector does not distinguish between wavelengths landing on a given pixel of the detector, it simply measures the intensity of light striking the detector. For this reason, if light lands on the detector at a pixel (wavelength) where it shouldn't, the detector will erroneously output a reading at that wavelength. This stray light is typically from the intended source but scatters within the spectrometer and lands on the wrong part of the detector, but it may also be from a different source entirely. This light sets a working limit on the maximum absorbance that can be achieved with a system and reduces the signal to noise ratio by limiting how dark the system can be. Common sources of stray light include higher



order reflection off the grating, defects in the grating, internal reflection within the spectrometer, and light leaks in the spectrometer housing. The Ocean HDX has the lowest stray light and highest maximum absorbance level of spectrometers its size.

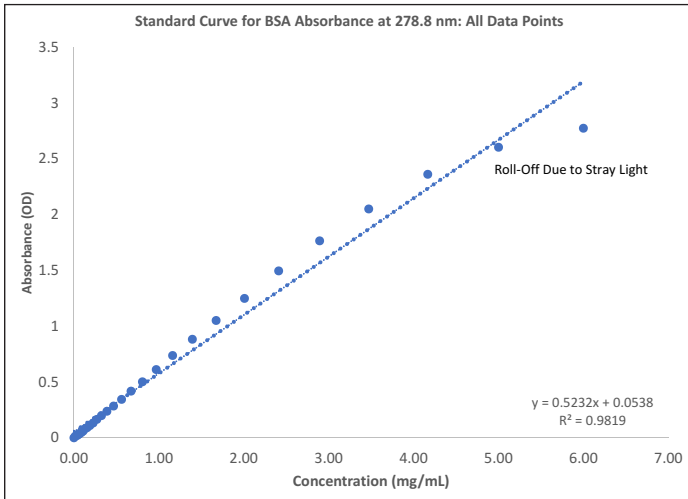


Figure 2: BSA standard curve including more than 30 data points from 0 to 6 mg/mL concentration. Note the roll-off or leveling of the spectrum between 2.4 and 2.5 AU where stray light limits the maximum absorbance.

In **Figure 3**, the data points from the highest protein concentrations have been removed from the plot to evaluate the linear range for the measurements. The improvement in the fit of the line as evidenced by the improvement in the R2 value indicates that the equation for this line is a better fit and will provide more accurate values of protein concentrations. Some roll-off is still observed at 2.5 AU. Additional measurements at concentrations between 4 and 4.5 AU are needed to assess linearity in this region.

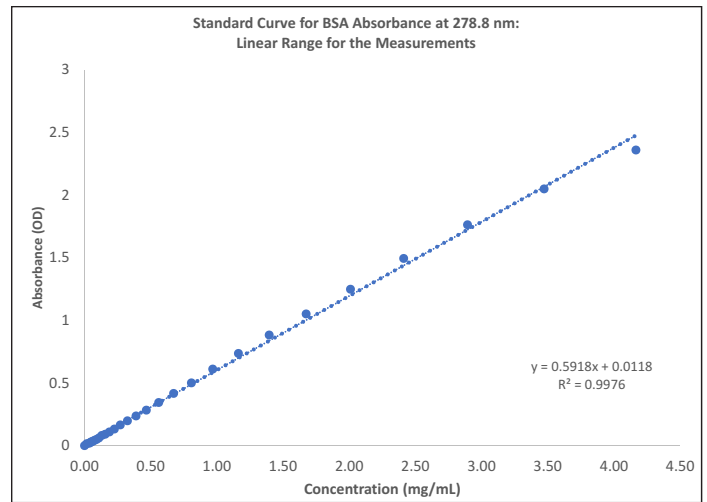


Figure 3: BSA standard curve showing the approximate linear range for the absorbance measurements.

Using this graph, the concentration of unknown BSA or other protein samples is calculated using the equation of the best-fit line where y is the absorbance value measured for your unknown sample.

Conclusions

The generation of a standard curve (also known as a calibration curve) is a typical procedure in many biomedical, diagnostic and life science research laboratories. An accurate determination of protein concentration is the first step in assays involving proteins. With ultra-low stray light, upgraded optics and high UV sensitivity, the Ocean HDX is ideal for UV absorbance measurements including those required for determining the concentration of an unknown protein.



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