Exploitation of Variable Pathlength Spectroscopy for the Spectroscopic Characterization and Quantitation of Proteins and Peptides

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Abstract: 393

The foundation of quantitative spectroscopy is

Beer's Law A = E c l

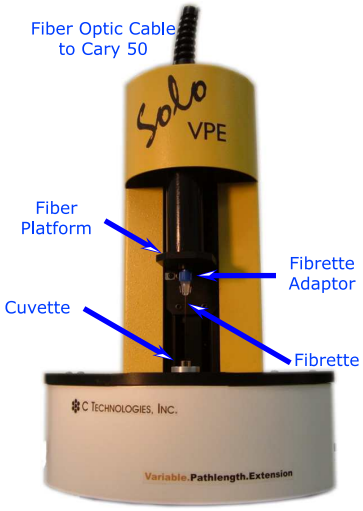
where A is the absorbance of a solution of thickness, l, containing mol cules at concentration, c. E is a property of the molecule, its extinction coefficient, and has units the reciprocal of c and l. Most users are familiar with its application for the measurement of concentration where A should be a linear function of c; however deviations from linearity do occur. These can arise from instrumental limitations of wh ch users are often unaware, or from changes in molecular properties in solution as c increases (e.g. light scattering losses, not accounted for in E). Most often dilutions of the sample are required to assure that one is in a linear region of A vs c. Beer's law is linear in both c and l, so that one could change the pathlength instead of dilution; how ver most laboratories have a very limited collection of cuvettes with different pathlengths (1 cm being the most commonly used cuvette). The SoloVPE permits the quick measurement of complete spectra at multiple pathlengths (pathlength scanning) using a SINGLE sample which in turn permits a unique approach to quantitation. For quantitation purposes one constructs a "section plot" of absorbance vs pathlength

To exploit section plots for quantitation we note that the slope (m) in a Beer's law plot should be a constant

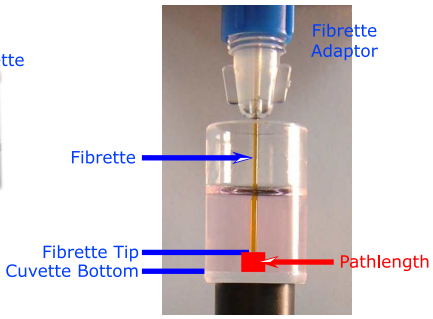
m = d(A)/d(l) = E c

In a section plot deviations from Beer's law are usually apparent in the data, permitting one to focus on a pathlength range in which the law is valid and eliminating the tedious task of doing multiple dilutions to ascertain the validity of t e measurement. The section plot slope is the product of E and c such that knowledge of either permits the other's calculation. The slope measurement provides redundant infor ation over what would be obtained at a single pathlength, improving accuracy.

We illustrate the application of variable pathlength spectroscopy for t e construction of a complete extinction spectrum of bovine serum albumin.

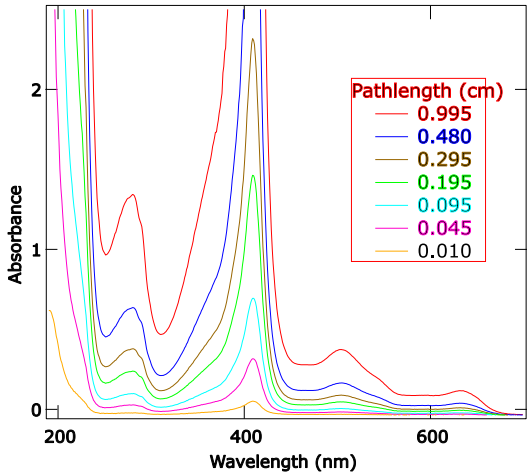


The SoloVPE is an accessory for a Cary 50 spectrophotometer. The Cary provides spectrally separated light to one end of a fiber optic cable which has its other end attached to the fiber platform in the SoloVPE. At the platform, the light is delivered to one end of a disposable "Fibrette" (a single piece of optical fiber) attached to the fiber platform by the Fibrette adaptor. The other end of this Fibrette is submerged within the sample in a cuvette located below the fiber platform. Light emerging from the Fibrette passes through the sample to a detector located beneath the cuvette. The detector returns an electrical signal to the Cary 50 proportional to the light intensity impinging on which is processed into an absorbance signal.



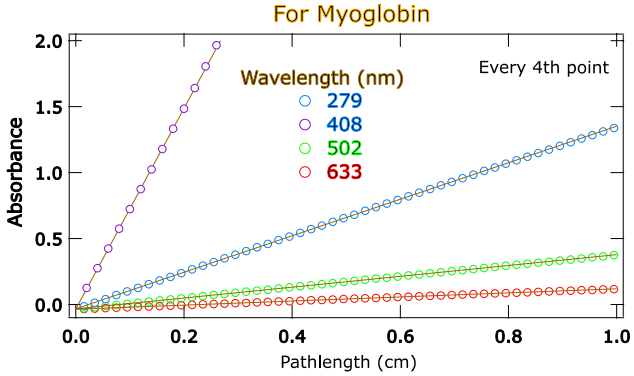
Pathlength is defined by the distance from the submerged tip of the Fibrette to the bottom, inside surface of the cuvette. This distance is controlled by the position of the fiber platform to which the Fibrette is attached via the Fibrette Adaptor. The vertical position of the fiber platform is itself determined by a software driven stepper motor; thereby permitting precise changes in the positioning of the Fibrette tip within the sample. This control places the pathlength used for any given measurement at the complete discretion of the user.

Spectra from SoloVPE Pathlength Scan of Myoglobin



One Sample Full Spectral Characterization

Section Plots at Various Wavelengths For Myoglobin



Quantitation using: m = d(A)/d(l) = E c

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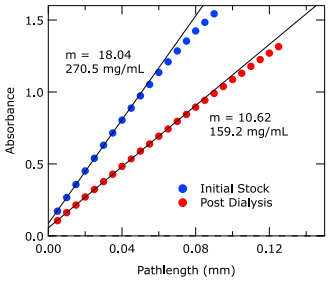
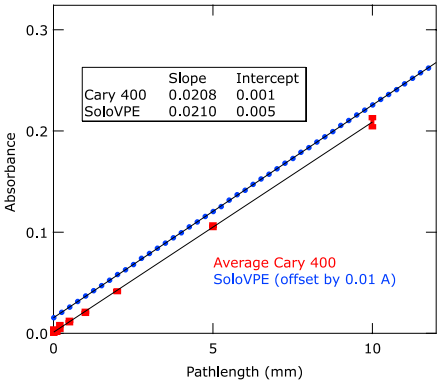
The Sample

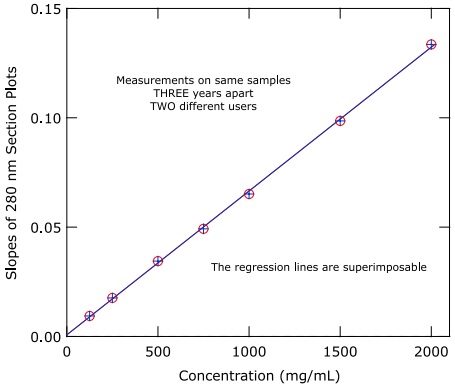
BSA was dissolved in PBS, and then dialyzed against several buffer changes at ~5C. The final solution was filtered and stored (the final buffer change was kept for use as reference). The goal was to exploit variable pathlength spectroscopy to measure the extinction coefficient at 280 nm, and use this to create a complete extinction spectrum of bsa. The figure below shows section plots for the initial stock solution and the final protein solution. Deviations from Beer's law are apparent above about 0.05 mm. An interesting feature to note is that because the extinction coefficient is a constant the ratio of the slopes provides a facile means to determining the actual dilution factors.



A solution of camphorsulfonic acid in water was used to create a "section" plot using various pathlength cuvettes in a Cary 400 spectrophotometer. A section plot was created for the same solution using the SoloVPE. There is excellent agreement between the two slopes. However, the Cary 400 data took several hours to complete; whereas, the SoloVPE data took under an hour.



Bovine serum albumin standards from Thermo-Fisher Scientific were examined using the SoloVPE. The reproducibility of the slopes is demonstrated to be excellent.



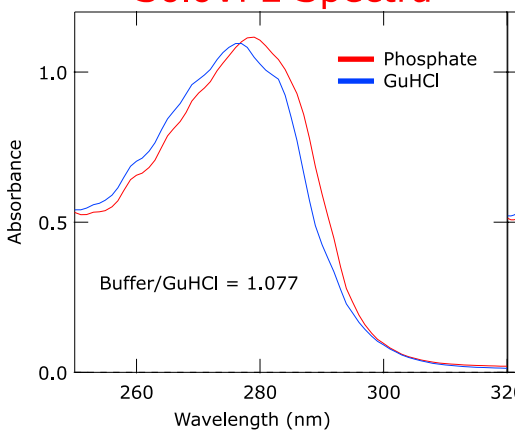
Extinction Coefficient Measurement

For most biologicals, dry weights are not readily determined. For prote ns and peptides, the dominant contributors to absorbance at 280 nm are the tryptophan (W) and tyrosine (Y) residues with a minor contribution rom the disulfide bonds (CC). Since the complete sequence of proteins, although the disulfide bonding pattern may not be fully characterized is usually known, we have an accurate accounting of the major contributors to the absorbance per polypeptide chain. Furthermore, most fully denature in 6 M Guanidine HCl (GuHCl) so that environmental effects arising from the folded protein are eliminated. Edelhoch, originally developed a method exploiting these facts to measure W and Y content of proteins based on extinction coefficients for W, Y and CC measured in 6 M GuHCl. Here th s approach coupled to the slope measurements made possible by the SoloVPE. Originally the procedure involved identical volumetric dilutions into b ffer and GuHCl and measuring the absorbance in the two solvents. The ratio of the absorbances is the ratio of extinction coefficients since the prote n concentration is identical in the two solutions. Using the SoloVPE slope data

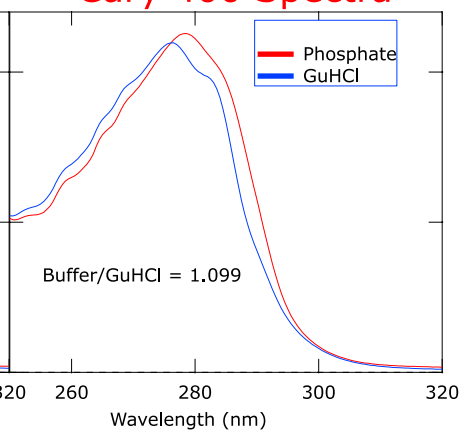
$$m_{buffer}/m_{GuHCl} = E_{buffer}[P]/E_{GuHCl}[P] = E_{buffer}/E_{GuHCl}$$

For BSA W=2, Y=20 and CC=17 leading to an extinction coefficient at 280 nm of 39000/M/cm in GuHCl. We show below the "classical" spectra approach with data at 1 cm for both the Cary 400 and the SoloVPE; and t e slope ratio approach. Using the latter ratio the extinction coefficient in PBS was calculated as 41,110/M/cm.

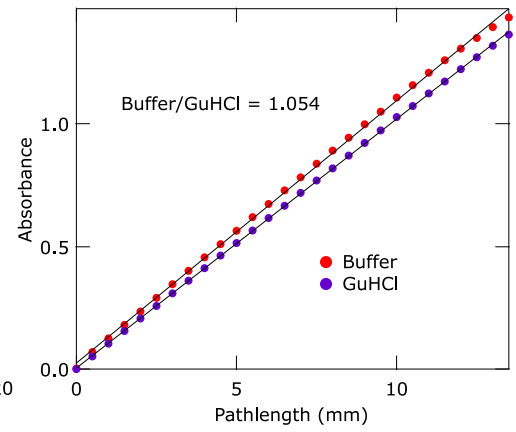
SoloVPE Spectra



Cary 400 Spectra

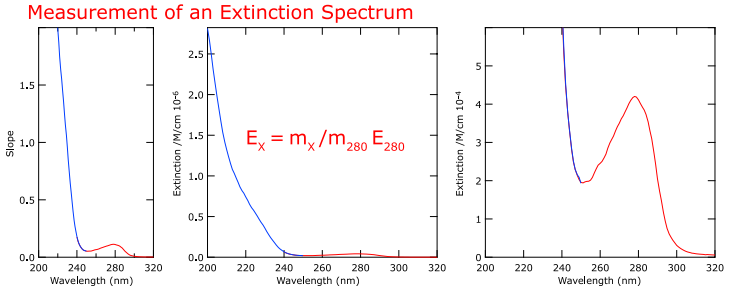


SoloVPE Section Plot



Measurement of an Extinction Spectrum

Once an extinction coefficient at a reference wavelength is determined, then a complete extinction spectrum can be readily determined, assuming that the absorbances at all other wavelengths are related to that at the reference wavelength. The ability of the SoloVPE to record spectra at various pathlengths eliminates the need to do numerous dilutions which would subsequently have to be corrected for increase the error in the final measurement. The process is simple, from a series of spectra recorded by the SoloVPE a section plot is made for each wavelength and its slope determined. As illustrated to the right the slope at each wavelength is divided by the slope at the reference wavelength and multiplied by the extinction coefficient for the reference wavelength. For these data the lower wavelengths (blue) were scanned over a different range and increment than the longer wavelength (red) due to the much higher extinction at the lower wavelength; yet the measurement still used only a single sample.



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The authors wish to thank C-Technologies, Inc for providing access to the instrument used in these studies. Further information on The SoloVPE can be found at http://www.solovpe.com/

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