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White Paper

Validating Slope Spectroscopy Methods: A Formula for Robust Measurements

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1.0 Abstract

Demands on measurement systems are ever increasing. UV-Vis-NIR measurements are not exempt from such pressures. With increasing investment in pharmaceutical research and development, combinatorial chemistry, biotech engineering and genetics research, the ubiquitous “vanilla” UV spec on the lab bench has never been more useful for various analytical methods. However, users are consistently seeking to make measurements more accurately, more precisely and more rapidly but with fewer mistakes. Most laboratories are performing more tests on more samples than ever before as the race to develop and patent new useful compounds and proteins plays out daily in laboratories around the globe. Frequently the amount of sample available for testing is small and value of the sample can easily exceed tens of thousands of dollars.

With so much at stake, scientists and technicians must carefully select what tests to run in order to gather all the desired information. And once selected, these tests must be conducted in such a way that there is absolute confidence in the measurement result. Numerous types of tests including: concentration, solubility, pH and others can be performed with UV-Vis-NIR spectroscopy and an emerging technique for making these measurements is Slope Spectroscopy®. Slope Spectroscopy techniques can be attractive for many reasons such as the reduced time and effort required to obtain a measurement result and applicability to small volumes. Perhaps one of the most compelling reasons to begin utilizing Slope Spectroscopy methods is the robustness of the measurement system. This paper will explore the reasons why Slope Spectroscopy methods are proving to be so mistake proof.

2.0 Introduction

Precise and accurate measurements in scientific analyses are critical to the success of all research endeavors. Much time and energy is spent developing detailed methods that are reproducible and repeatable from the preparation steps through to the analytical result. Ensuring the veracity of these methods both in definition and execution is very important. Frequently, analytical methods are developed based upon years of prior experience, techniques and de facto tradition. However, it is important from time to time to review other techniques, both new and pre-existing, for applicability and benefits in the laboratory, diagnostic and process environments. An emerging technique, recently made practical through advances and integration of spectroscopic hardware and software, is Slope Spectroscopy. Slope Spectroscopy methods are proving to be very robust in providing highly accurate results, rapidly across a wide range of samples and applications. Embracing slope algorithms may initially cause scientists to pause, because it appears different from the conventional spectroscopy techniques most have used since their first days working with a spectrophotometer. However, once it is understood that the technique of Slope Spectroscopy is fundamentally built upon the Beer-Lambert Law, analysts become very comfortable with the method. Additionally, when it is understood that the technique will allow them to avoid dilutions, use less material and get more accurate results faster, analysts go from being comfortable with the concept to finding applications and benefits in their work. To initially become comfortable with the concept and the terminology of Slope Spectroscopy, a review of its derivation and definitions is required.

3.0 Background

As suggested by its name, Slope Spectroscopy, is somehow dependent on a slope, but the slope of what? To explain the slope a quick review of the Beer-Lambert Law is necessary. In optics the Beer-Lambert Law is one of the most widely used empirical relationships that relates the absorption of light to the properties of material through which the light is travelling.[1] The most commonly seen representation of the Beer-Lambert law is as $A = \alpha \cdot l \cdot c$ where "A" is the measured absorbance, "α" is the wavelength dependent molar extinction coefficient, "*l*" is the pathlength, and "c" is the sample concentration.

Most spectroscopists are familiar with the standard curve which relates the change in absorbance to the change in concentration. This relationship is clearly defined by the Beer-Lambert Law which states that the absorbance changes proportionally with concentration. This fact is well known and repeatedly verified by chemistry students everywhere through dilutions and measurements in their lab experiments. Those experiments are of course based upon the assumption that pathlength is held constant, typically by using the same cuvette to make the measurements. There is another standard curve that is used much less frequently, because until recently, it was impractical to do so. The Beer-Lambert Law actually states that absorbance is proportional to concentration and pathlength, therefore if concentration is held constant, absorbance will vary linearly with changes in pathlength. This other standard curve can now be used to make very accurate measurements quickly. It takes only basic algebra to derive the Slope Spectroscopy Equation which is expressed as $m = \alpha \cdot c$.[2]

Slope Spectroscopy® Equation

$$m = \alpha \cdot c \quad \textit{where} \quad m = \frac{A}{l}$$

The standard curve that relates the change in absorbance to pathlength changes can be described by an equation of the format y = mx + b where "y" is the Absorbance, "m" is the slope of the line, "x" is the pathlength and "b" is the y-intercept of the line. The units of the slope term "m" are Abs per unit pathlength by definition. Additionally, by dividing both sides of this Beer-Lambert equation by the pathlength "*l*", the equation takes on the form $A/l = \alpha \cdot c$. A dimensional analysis of the above equation reveals that the left side of the equation (A/l) has units of Abs per Unit Pathlength, where absorbance (Abs) is a non-dimensional numerical result and the units of pathlength can be any unit of length measure though cm and mm are most commonly used. A simple substitution the slope term from the standard equation for the left hand side of the reconfigured Beer-Lambert Law equations gives us the Slope Spectroscopy Equation of $m = \alpha \cdot c$.

Now that is clear how the Slope Spectroscopy Equation is derived from the Beer-Lambert Law, the unique features of the relationship can be explored. The technique provides a very robust method for making determinations.

4.0 Solution

There are many benefits to employing Slope Spectroscopy techniques wherever UV-Vis-NIR methods are being used. The benefits include speed, simplicity, convenience and accuracy. One of the major practical benefits associated with leveraging slope based measurements is the elimination of required dilutions steps in defined methods. By avoiding dilutions you save time, money and sample material. Dilutions take time to make, require consumables for preparation and take up precious amount of sample available. Additionally, you eliminate the variability and errors associated with performing the dilutions. In many cases, multiple dilutions can be necessary when working with a new compound. By working with the raw solution you not only get more reliable results, but you obtain them with less effort and faster. The first question that enters the mind is "But how do I make measurements at multiple pathlengths on a sample which is so concentrated that it is outside the range of my instrument?" There are a variety of solutions available for taking measurements of highly concentrated solutions at small pathlengths, so the more appropriate question is "How do I take measurements at multiple pathlengths?"

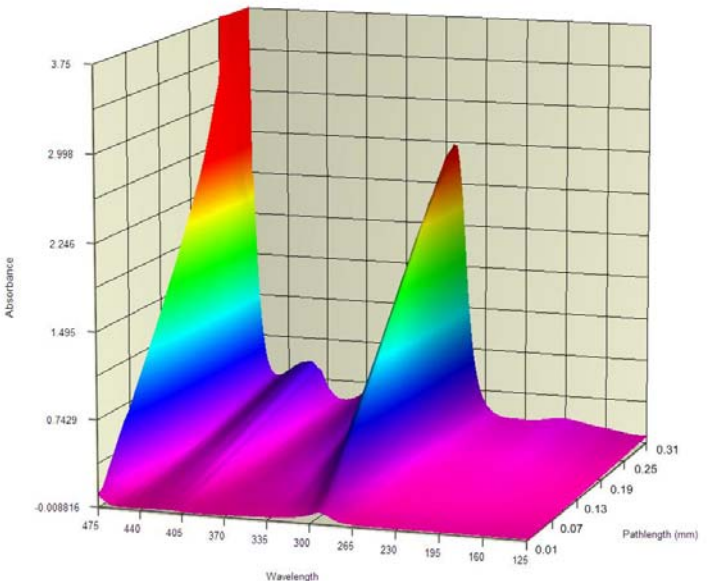

Figure 1: Three-dimensional Spectrographs

For purposes of this paper, imagine there is a way to measure virtually any pathlength desired. If it is useful to help in the understanding of the examples provided, these measurements could be made if the laboratory had a very large number of cuvettes made in 10 micron increments from as low as 10 microns up to 1 cm or more, or perhaps the laboratory has a Dial-A-Pathlength cuvette which can go to any pathlength at the press of a button. With the described measurement capability, one could envision being able to generate three dimensional spectrographic surfaces such as the those in Figure 1 which show plots of Absorbance versus Wavelength versus Pathlength. The power of such a device would provide tremendous measurement flexibility and enable the use of Slope Spectroscopy which will be shown to be a very robust measurement technique.

Consider a situation where you have a sample to be measured, the extinction coefficient is known and the concentration is not. The solution absorbs strongly in the UV making it difficult to get a measurement without resorting to very small pathlengths perhaps 1 mm or smaller. Most rely on existing solutions to make a single micro-pathlength measurement and then extrapolate what the absorbance value would be at 10 mm. But how confident should you be in that measurement? At least you avoided dilution errors, but there is a certain amount of uncertainty in the results based upon the magnitude of the signal, instrument noise, wavelength accuracy and of course pathlength. You have an absorbance value at one pathlength but how do you know if it is accurate or whether or not it is even within the linear range that can be measured? Yet that lone pathlength result is used to project what the absorbance will be much farther out the pathlength curve where the error can be greatly magnified.

Would the following not be an improvement? Go to the lab bench that holds the Dial-A-Pathlength device and as a first step, scan the pathlength range to find the pathlengths at which the sample absorbance levels are within the linear range of the instrument. Now that you know the pathlengths at which you can collect linear data, rather than just collect a

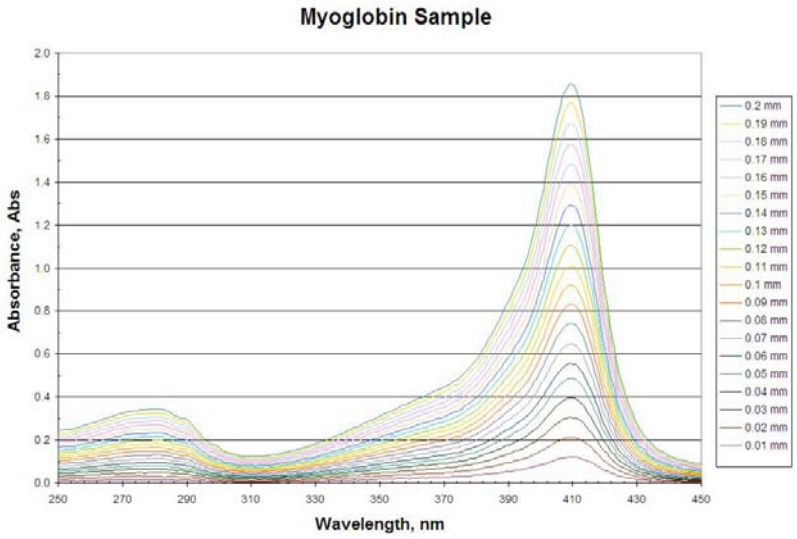

Figure 2: Myoglobin Spectra

single absorbance value and base your analysis on one point, use the power of the Dial-A-Pathlength and collect absorbance values at pathlength intervals of 100 microns, or maybe every 25 microns or perhaps you purchased the limited edition super high resolution Dial-A-Pathlength device that can collect data down to 10 micron pathlength steps. The sample is loaded. There is no additional work required. The incremental data is there for the taking. However, with that additional data you have immediate confirmation that the data you have acquired is consistent with the linear behavior predicted by the Beer-Lambert Law. You do not need to put your trust in a single data point collected at a single wavelength.

With this additional data in hand you could certainly go ahead and project what the absorbance would be at some distant pathlength, with more confidence that the result obtained is accurate. Well at least as accurate as your Dial-A-Pathlength device. When was the last time the Dial-A-Pathlength service tech was out to calibrate the instrument? *Hmm, how clean was the sample cuvette? And I noticed the other lab borrowed it last week to run some samples and two out of the last three times they borrowed equipment, they dropped it. The pathlengths we measure are usually very small, how accurate are those pathlengths? Is 100 micron truly 100 micron? Maybe I better starting making some dilutions.* STOP! Don't panic. Obviously measurement equipment and methods need to be validated and verifiable. Fortunately, the Dial-A-Pathlength is a very resilient measurement device and it is easy to confirm it is working properly.

The simple way to verify that your Dial-A-Pathlength is performing properly is to make a series of measurements at a variety of pathlengths on a solution which is linear within a specified pathlength range. When you plot Absorbance versus pathlength you should end up with a straight line and the changes in pathlength should be proportional to the changes in absorbance. It is the Beer-Lambert verification test. Such a test could be run daily or weekly if laboratory GLP requirements mandate it. However, there are other implications of the Dial-A-Pathlength device on measurement system accuracy.

Consider the following series of measurements on a sample of Myoglobin. The Dial-A-Pathlength was used to measure the Myoglobin sample at pathlengths from 10 micron up to 200 micron. This particular lab had sprung for the limited edition super high resolution version. A series of spectra were collected at wavelengths from 250 nm up to 450 nm. Those curves are shown in figure 2. Even though curve data was collected, the peak of interest is at 400 nm. Fortunately, the Dial-A-Pathlength software can create an Absorbance versus Pathlength curve at any wavelength desired. That curve is displayed in Figure 3. It is immediately clear that the Dial-A-Pathlength is moving uniformly and the pathlengths measured are within the linear range of the instrument because the straight line appears to behave exactly as the Beer-Lambert Law predicts. Using the simple regression utility, the equation of the best fit line is calculated and in what is another confirmation of the system and measurement integrity the Coefficient of Determination (R^2) is calculated to be 0.9997 very close to a perfect fit of R^2 = 1.0. Using the equation of that line, the absorbance at 10 mm can quickly be calculated and used to determine

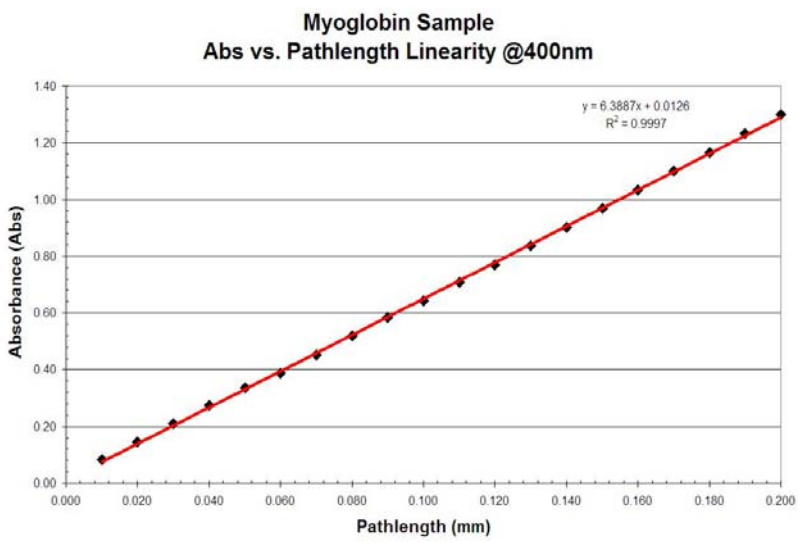

Figure 3: Absorbance vs. Pathlength @ 400 nm

the concentration. Because there are many data points and such strong correlation, confidence is very high in the concentration result. However, it is possible to save a step by using the Slope Spectroscopy Equation derived earlier. The concentration can be calculated directly from the slope term of the regression equation. The slope based calculation results in an accurate concentration result without relying on the theoretical absorbance at a pathlength that is not directly measurable. However, it requires that the desired measurement result is not a common absorbance value but a slope value. There are reasons why this type of measurement could be considered advantageous.

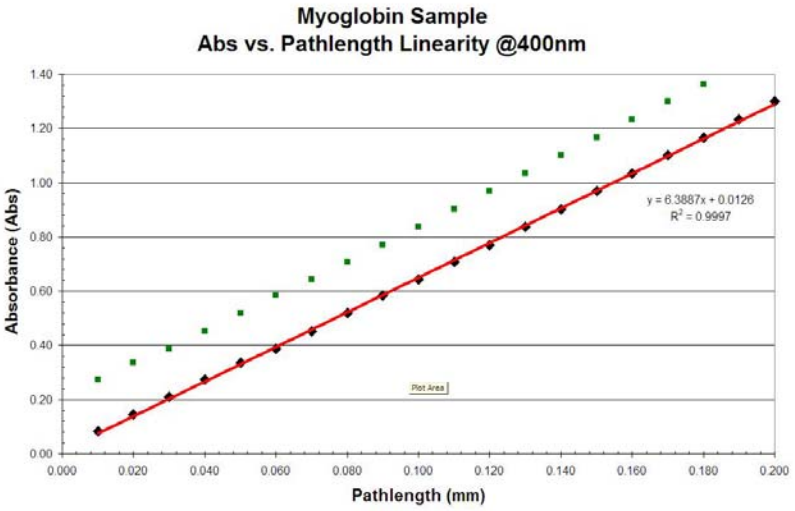

Figure 4: Absorbance vs. Pathlength @ 400 nm Pathlength Error Present

But what happens if the Dial-A-Pathlength was dropped by the borrowing lab technician and as a result the pathlength being reported was off by 30 microns. When the Dial-A-Pathlength is set for 10 microns the actual pathlength is 40 microns, when set for 50 microns, the actual pathlength is 80 microns, when set for 100 microns the actual pathlength is 130 microns and so on. That pathlength error is now in the measurement. As expected, if the pathlength changes, the absorbance changes as well. So when the Dial-A-Pathlength moves to 10 microns, which is actually 40 microns, the reported absorbance is measured at 40 microns, 20 microns yields the absorbance at 50 microns, 30 microns yields the absorbance at 60 microns and so on. However, if you look at Figure 4 which shows the original data with the data collected when the pathlength error was 30 microns, it is readily apparent that the data collected after the Dial-A-Pathlength was dropped is just shifted to the left. And as long as you have remained within the linear range of the instrument shifting the line does not substantially change the slope. The interesting and powerful consequence of this data set shift is that the absolute pathlength is not as critical when the calculations are based upon slope compared to when they are based on a single measurement value. That is not to say that pathlength does not matter at all, certainly the data must be within the linear range and complying with the Beer-Lambert Law, but it is not a critical requirement of the measurement system. The attractive feature of this measurement system is that linearity is immediately verified as part of the measurement and the slope will provide accurate concentrations reliably and consistently.

The following test demonstrates how the actual absorbance value is de-emphasized through the use of Slope Spectroscopy techniques. Using the Dial-A-Pathlength a sample of Potassium Dichromate solution will be measured. The certified concentration of the solution is 2 mg/ml. The measurement will be repeated two times. The first measurement will be made using a quartz sample vessel with a vessel thickness of 1.5 mm. The second measurement will be made using a plastic sample vessel with a vessel thickness of 0.2 mm. In both cases no baseline subtraction will be made. Under these test conditions, the absorbance values are expected to be different for each measurement. Using the Absorbance vs. Pathlength plot generator on the Dial-A-Pathlength, absorbance vs. pathlength plots were created at 235 nm, 257 nm, 313 nm and 350 nm. The plots from the quartz vessel measurement are shown in Figure 5 and the plots from the plastic vessel measurement are shown in Figure 6. Linear regression was performed on each data set and the slopes of the best fit lines were compared. The concentration could be calculated but for this experiment there is no point. The same sample is being measured in different vessels so the concentration and the extinction coefficient are known to be equal. Additionally, the absorbance will not be equal because there is no baseline correction and different vessel materials with different thicknesses are being used. The only difference we are concerned about is how data is changing with pathlength or the slope value of the

Table 1

	Slope Value (Abs / mm)			
λ (nm)	**Quartz**	**Plastic**	**Delta**	**% Error**
235	3.0825	3.0454	0.0371	**1.20%**
257	3.6591	3.6707	-0.0116	**-0.32%**
313	1.1006	1.0856	0.0150	**1.36%**
350	2.4060	2.3934	0.0126	**0.52%**

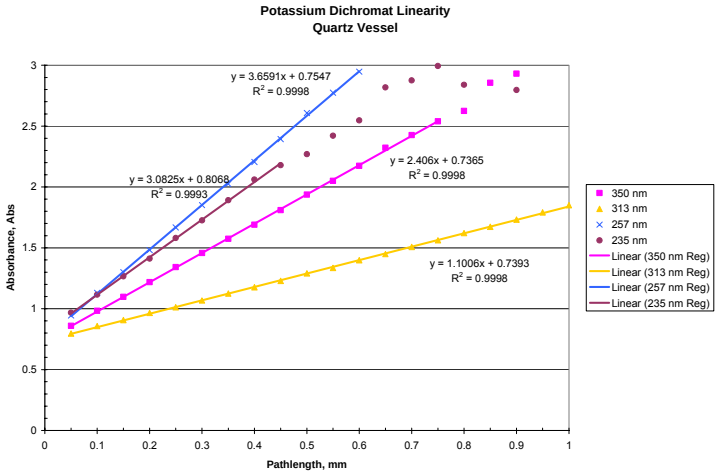

Figure 5: Quartz Vessel Measurements

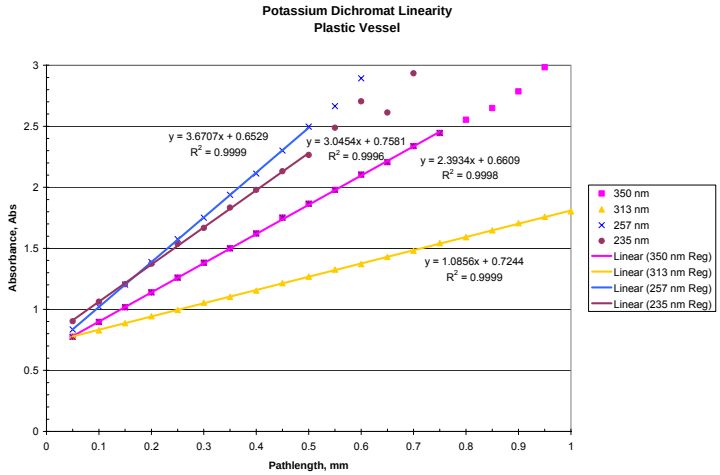

Figure 6: Plastic Vessel Measurements

regressed absorbance vs. pathlength data set. Table 1 clearly shows that even in light of all of the difference between to the two measurements, the slope remains reliably consistent for making determinations. The maximum difference in the slope was only 1.36% even with the difference material and vessel configuration and no baseline correction.

A Method for Validating Slope Spectroscopy Equipment:

Typically, when validating equipment whether it is for an IQOQPQ, preventative maintenance or routine validation for GLP/GMP, there is a process for verifying the proper functionality, a simple controlled test where the answer is known and a quick comparison confirms everything in functioning normally. Equipment that enables Slope Spectroscopy techniques requires these tests as well. However, they are different from the standard type of tests run on spectroscopy equipment. Tests of accuracy, repeatability and reproducibility are necessary, but the validation of Slope Spectroscopy equipment requires different types of tests. Consider the types of validation tests that need to be performed on the Dial-A-Pathlength. Tests which confirm absorbance accuracy and pathlength accuracy are necessary. These can be accomplished by measuring a spectroscopic standard and the measurement result at the published wavelength and pathlength. To confirm the repeatability and reproducibility multiple measurements can be made in order to confirm a consistent results across the multiple runs. However, because the system is dynamic, it is not as simple as pressing the Start button again after a measurement has been completed. The system must be prepared for the repeated measurement to obtain controlled results. Perhaps the most unique validation test is to confirm that the pathlength changes are performing as required. The most appropriate validation technique to confirm this is the Beer-Lambert verification test described earlier. Pathlength validation can be obtained by making slope measurements across the pathlength range of the Dial-A-Pathlength. This may require multiple standards in order to cover the pathlength range since it is critical that the validation measurement occur within the linear range of the standard. Verification is accomplished by confirming behavior consistent with the Beer-Lambert Law, specifically that the data is linear across the pathlength range and the coefficient of determination (R^2) approaches1.0. For this validation the confirming value is an R^2 that exceeds an acceptable threshold (e.g. ($R^2 \geq 0.998$) which confirms the strength of the linear relationship.

It is through the combination of these validation techniques that the Dial-A-Pathlength of any other piece of equipment capable of Slope Spectroscopy type measurements can be validated. These techniques should be incorporated in any installation, operation and performance qualifications as well as routine preventative maintenance and standard GLP/GMP compliance checks.

5.0 Conclusion

What is most clear from the above examples is that when Slope Spectroscopy techniques are being used, the absolute absorbance value is not the critical measurement result. The slope is the critical measurement result and this value is used to determine the concentration. The implication of relying on the slope value means controlling variables related to obtaining an accurate absorbance value are not as important as controlling variables related to how the pathlength and associated absorbance changes. Essentially, the absorbance values themselves do not matter, only the absorbance differences between pathlengths truly matter. This robust measurement technique would prove truly useful in numerous applications in the laboratory environment, in process monitoring and in quality control settings. The savings in time and money combined with improved accuracy resulting from eliminated dilution steps would be features most laboratory managers and technicians would be interested in exploring.

Though the theory of using pathlength varied slopes for spectroscopic determination is easily derived from the Beer-Lambert Law with simple algebra, any practical implementation would require the three dimensional spectroscopy techniques made possible by the Dial-A-Pathlength. Fortunately, there is a new measurement system available that brings the Dial-A-Pathlength and Slope Spectroscopy out of the realm of theory and into the lab. That device is the SoloVPE (Variable Pathlength Extension) from C Technologies, Inc. In fact, this device was used to collect and analyze the data presented in this paper. The beauty of the Slope Spectroscopy techniques is that it relies on continuity across a range of pathlengths and so long as a series of measurements can be obtained in the linear range of the instrument, the derived slope can be used for determinations. With its pathlength measurement range of 10 micron up to 20 millimeters and a resolution of 5 microns, the SoloVPE is capable of making multiple measurements even on very highly concentrated solutions thus enabling the use of Slope Spectroscopy. With versions coming out for longer pathlengths, up to 60 mm and online versions to be used in online flow and process environments, the Variable Pathlength product line from C Technologies, Inc. is making the use of slope spectroscopy possible. *For more information about the SoloVPE and related products, please visit the website at www.solovpe.com.*

6.0 References

(1) Wikipedia. “Beer Lambert Law” Available at: http://en.wikipedia.org/wiki/Beers_law. Accessed 27 February 2008

(2) Shih, Eric, Salerno, Mark. “The Power of Slope Spectroscopy” Available At: http://www.solovpe.com. Accessed 27 February 2008