Technical Note

QUANTITATIVE ABSORBANCE MEASUREMENTS IN MICROPLATES

Beating the most common challenges

UV-VIS absorbance measurements have emerged as a standard technology to determine concentrations of substances in solution (or even in gas phase). As this technology provides absolute values, it is generally independent from the instrument or individual experiment setups. After having been used macroscopically in cuvette-based studies for decades, absorbance tests in the microplate format have gained increasing importance. Although absorbance studies appear to be straight forward at first glance, there are several challenges one needs to master in order to generate reliable experimental data. A deep understanding of the physical background of absorbance measurements and awareness of the strict boundary conditions that have to be fulfilled for a valid experiment are key for planning your experiment to result in accurate and reproducible data.

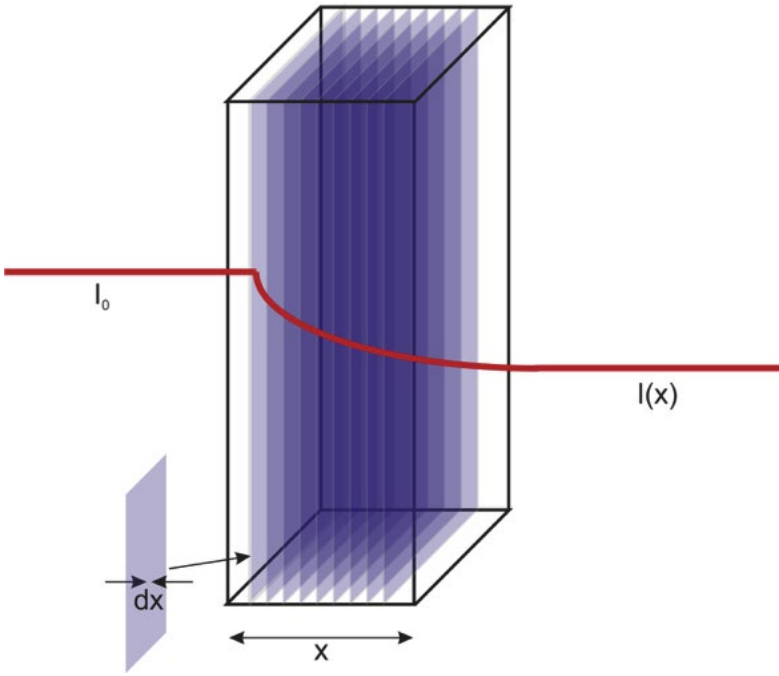

Figure 1: Light attenuation by passing through a solution of a given substance.

The physics of absorbance measurement

The basic principle of absorbance measurements is described by Lambert-Beer's law. Absorbance measurements are typically based on a transmission configuration[i] to determine the magnitude of intensity attenuation of incident light by an analyte [2]. The absorption of light with an initial intensity I_0 penetrating a diluted solution of a given substance over a length of x can be described by cutting the light path into infinitesimally small sheets of the width dx (Figure 1).

Dr. Frank Schleifenbaum & Dr. Thomas A. Schild,

Berthold Technologies GmbH Bad Wildbad, Germany

[i] Strictly speaking, the Lambert-Beer's law discribes the wave propagation of radiation through a medium [1]. For convenience, in this technical note we refer to a transmission configuration, which is commonly used in absorbance studies.

In each of these small sheets there may be molecules of the substance of interest present, which are equally distributed over the entire sample volume. To take into account that substances have different abilities to interact with irradiation of a certain wavelength, we may assign an area of a distinct diameter to each molecule and call this area the "absorbance cross-section" a (Figure 2). If light hits the cross-section area of a molecule, it will be completely absorbed[ii].

As a result of this process the light intensity is attenuated after it has passed through the sample, i.e. the number of photons that appear at the end of the sample volume is reduced compared to the number of photons entering the sample. To quantify this attenuation, we find that the light intensity depends on the number of molecules present in the light path n/A and on their absorbance cross-section. Hence, we can write for the local intensity in every of the small sheets

$$dI = -a \cdot n/A \cdot I\, dx$$

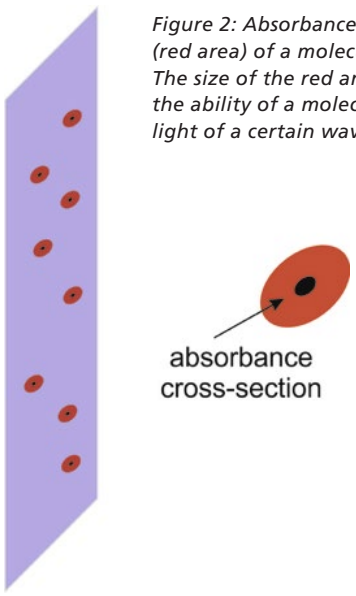

Figure 2: Absorbance cross-section (red area) of a molecule (black dot). The size of the red area represents the ability of a molecule to absorb light of a certain wavelength.

The density of the molecules in the complete sample, n/V, can be expressed as a molar concentration $c = n/N_A$ (N_A = Avogadro's number) and integration of this equation over x with the initial intensity I_0 yields:

$$\ln I = -a \cdot c \cdot x + \ln I_0$$

$$\ln \log \left(\frac{I_0}{I} \right) = - a \cdot cN_A \cdot x$$

or $$\ln \left(\frac{I}{I_0} \right) = - a \cdot cN_A \cdot x$$, respectively.

Commonly, this expression is found in decade notation and the conversion factor 2,303 is expressed together with the absorbance cross-section as the molar extinction coefficient $\varepsilon(\lambda)$. With the path length x written as d[iii] we obtain the common expression of Lambert-Beer's law for the absorbance A:

$$A = \log \left(\frac{I}{I_0} \right) = \varepsilon(\lambda) \cdot c \cdot d$$

Although appearing rather straight forward, Lambert-Beer's law is only valid under well-defined conditions and there are several challenges when applying the law in practice.

Wavelength dependency

Obviously, solutions of different molecules may appear in different colours. Accordingly, the selection of the measurement wavelength must be specified precisely [3]. Typically, one will chose a wavelength where the absorbance is maximal to obtain the highest sensitivity. Of similar importance is the spectral bandwidth $\Delta\lambda$ of the light. Let's consider a substance with a molar extinction coefficient $\varepsilon(\lambda)$ at a wavelength λ_1 which is

[ii] The model of a complete absorption of light by a single molecule accounts for the quantum nature of light. Here, single traveling photons are considered which can either be absorbed or my travel unaffected.

[iii] Note that for historic reasons d is given in centimeters (10^{-2} m).

irradiated with the intensity $I_{0,1}$ and a different molar extinction coefficient at a second wavelength λ_1 irradiated with the intensity $I_{0,2}$. For the two wavelengths Lambert-Beer's law yields:

$$I_1 = I_{0,1} \cdot 10^{-\varepsilon(\lambda_1)cd}$$

$$I_2 = I_{0,2} \cdot 10^{-\varepsilon(\lambda_2)cd}$$

The detected intensity represents the sum of the intensity of all wavelengths and one obtains:

$$A = \log \frac{I_1 + I_2}{I_{0,1} + I_{0,2}}$$

$$= \log \frac{I_1 + I_2}{I_{0,1} \cdot 10^{-\varepsilon(\lambda_1)cd} + I_{0,2} \cdot 10^{-\varepsilon(\lambda_2)cd}}$$

As a consequence, $A = \varepsilon(\lambda) \cdot c \cdot d$ is only true for $\varepsilon(\lambda_1) = \varepsilon(\lambda_2)$ and, strictly speaking, Lambert Beer's law is only valid for monochromatic light of one single wavelength as it is provided e.g. by laser sources. In practice, a spectral bandwidth of up to 10 – 15 nm nanometers is acceptable, especially if the absorbance spectrum is rather flat in the particular spectral window. For spectra exhibiting very sharp peaks it is recommended to choose a wavelength where the spectrum is smoother even if the absorbance is weaker at this wavelength (Figure 3).

As a rule of thumb, the FWHM[iiii] of the absorbance band $\Delta\lambda_{H,\varepsilon}$ should be greater than the FWHM of the incident light $\Delta\lambda_{H,meas}$ by a factor of 5. However, if a monochromator is used for wavelength selection, the spectral bandwidth might be set to the smallest value. It is important to note, that the maximum accessible OD value is reduced for small bandwidths for the benefit of a dramatic increase in the precision of concentration determination.

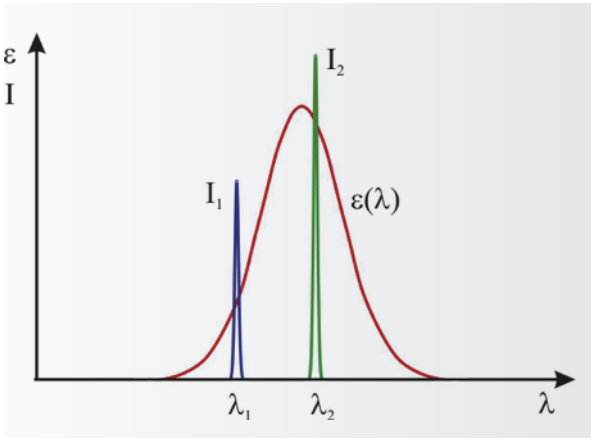

Figure 3: Influence of the spectral bandwidth of the irradiation light for different wavelengths.

Concentration dependency of ε

One highly important characteristic of the Lambert-Beer's law is that it is only valid for diluted solutions [3] with absorbance-values $A \lesssim 1.5$ corresponding to concentrations of about $c \lesssim 0.01$ mol/l for typical organic molecules. The reason for this limitation is given by the derivation Lambert-Beer's law as outlined above. Here, it was assumed that every molecule has the identical probability to absorb light. For higher concentrations, the mean free path (i.e. the average distance a molecule travels until it hits another molecule of its kind) between two analyte molecules is too small. This causes interactions between these molecules that may lead to e.g. aggregation, dissociation or the formation of complexes. Also, shadowing effects can have an impact on the measurement [4], [5]. Molecules can be shielded by neighbouring molecules so that they do not "see" the impinging light. Hence, reliable absorbance measurements should only be performed using diluted solutions and great care should be taken that the absorbance is not exceeding values of 1.5. Else, only qualitative conclusions may be given and exact information on concentration is no longer revealed.

[iiii] Full width at half maximum

Scattering and reflection

Another challenge in quantitative absorbance studies is a result of Lambert-Beer's law idealising the processes that occurs during a transmission measurement by considering exclusively light attenuation by molecular absorption. Especially losses due to scattering are not included in the theoretical description [6]. In general, scatter deflects part of the light beam so that it is not reaching the detector at all or at least not in a straight way. This scattering may occur at any objects in the beam path, e.g. at optical components such as lenses or shutters but also particulate contaminations on surfaces or in the sample solution itself can be the origin of optical scattering. Moreover, any dielectric surface partly reflects impinging light, causing an attenuation of e.g. up to 10% for glass surfaces. Therefore, in absorbance studies the unattenuated intensity I_0 is determined typically in a way that the beam path contains all elements of the actual measurement setup, including solvent and sample container, except for the actual analyte substance. This procedure guarantees that the measurement parameters are exactly the same as in the actual measurement. For classical photospectrometers using a cuvette as a sample container, this is accomplished either by providing a separate reference channel or by two successive measurements.

In instruments using microtiterplates as sample containers the situation is more complex. To facilitate an absorbance measurement in any of the plate wells the I_0 measurement is performed "through the air" by default. This means, the complete sample is bypassed and the unattenuated irradiation is measured. Although widely accepted, this procedure is not suited to determine absolute concentrations with highest accuracy. Thus, it is recommended to use a well in the microtiter plate that contains the solvent only, e.g. buffer, to measure I_0. In that case, the instrument takes the absorbance of that particular well as the I_0 value to calculate the concentration. This approach guarantees much more reliable data. However, the accuracy of a cuvette-based measurement still cannot be obtained. The reason for this restriction is due to the surface tension of the different solvents.

In Figure 4 different surface tensions are indicated as they may occur for different solvents. Due to the differences in curvature the effective light pathway varies for the 4 examples shown in Figure 4.

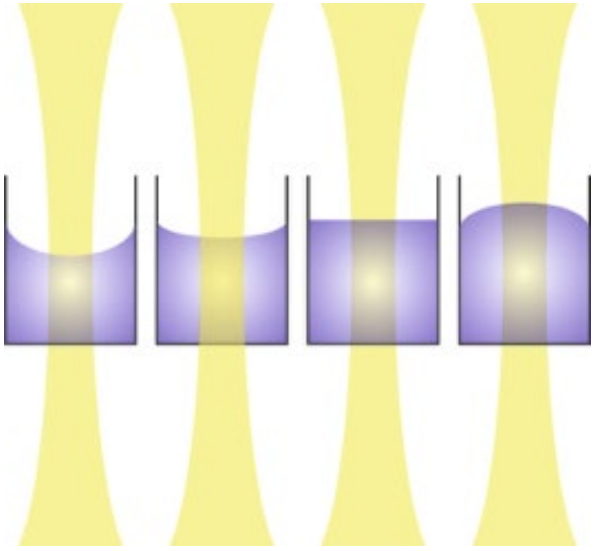

Figure 4:
Effect of surface tension on the effective light pathway

Using the absorbance values to determine the concentration of the analytes in the sample wells would lead to inaccurate results, because the value for the path length d in Lambert-Beer's law is not constant. Whereas the mean filling height can be determined by electronic means, curvatures in the liquid column cannot be corrected for. These curvatures, however, act like an optical lens that collimates or defocuses the light, respectively.

These effects can hardly be foreseen and corrected for. Thus, we recommend for concentrations measurements with highest accuracy to record a calibration curve prior to the actual measurement, using a dilution series of the analyte. Alternatively, BERTOLHD TECHNOLOGIES microplate readers also offer the possibility to use conventional cuvettes for absorbance measurements. Due to precisely parallel aligned glass surfaces the geometric limitations as discussed above are no longer valid and highly accurate absorbance measurements can be performed.

Important considerations summary

- Choose a measurement wavelength where the absorbance is maximal to obtain the highest sensitivity
- Use monochromatic light or light with a spectral bandwidth of <10 – 15 nm only
- The solution to be analysed should be diluted, resulting in absorbance values $A \lesssim 1.5$ or concentrations of about $c \lesssim 0.01$ mol/l
- Avoid scattering of light, e.g. due to pollutions in the sample
- When using microtiter plates run a buffer control
- For highest accuracy record a calibration curve prior to the actual measurement

References

[1] T. G. Mayerhöfer, H. Mutschke, J. Popp, ChemPhysChem 2016, 17, 1948 – 1955

[2] K. E. Peiponen, E. M. Vartiainen, T. Asakura, Dispersion, Complex Analysis and Optical Spectroscopy: Classical Theory, Springer, Berlin, Heidelberg, 1998.

[3] G. Gauglitz, Photophysical, photochemical and photokinetic properties of photochromic systems, Studies in organic chemistry 1990, 40, 15 – 63

[4] K. Buijs, M. J. Maurice, Anal. Chim. Acta 1969, 47, 469.

[5] P. Wittung, J. Kajanus, M. Kubista, B. G. Malmstrçm, FEBS Lett. 1994, 352, 37 – 40.

[6] G. Zaccanti, P. Bruscaglioni, J. Mod. Opt. 1988, 35, 229 – 242.

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Berthold Technologies GmbH & Co. KG
Calmbacher Straße 22
75323 Bad Wildbad
GERMANY
phone: +49 7081 177 0
email: bio@berthold.com

www.berthold.com