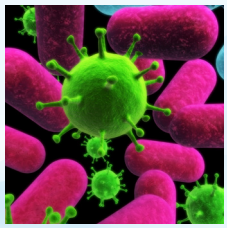
HORIBA Scientific

Fluorescence Spectroscopy

Experiment 3-11
Absorbance of Rhodamine B—Proper Use of Cuvettes



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For many samples (typically biological samples like reconstituted proteins), we do not have a sufficient quantity (volume) of the sample to fill a standard 1 cm (10 mm × 10 mm) cuvette (>2 mL). Also, dilution of the sample may not be an option and measurements have to be done in a much smaller volume. There are a few cuvette types that may allow measurements for sample volumes of 100 µL or even less. Examples of some such cuvettes are shown in the previous experiment (Figure 3-7.1). Typically, these kinds of cuvettes are made for fluorescence measurements and using a longer path for absorption is not possible without special modification. Using such cuvette for absorption measurement will lead to significant error. There are some specialized absorption cuvettes available for measuring small volume samples discussed in this and the next experiment.

The extinction coefficient of the dye (chromophore) is an intrinsic characteristic of the compound and should be independent of the type of cuvette used, spectrophotometer, and other experimental factors. So, data obtained from an absorbance measurement should be independent of the cuvette, as long as the cuvette is used properly. The initial Experiments 3-1 and 3-2 already discussed some aspects on how to properly measure transmittance/absorbance. But there are a few additional factors to be considered when using non-standard cuvettes. A cuvette may be misused when it is inserted into the cuvette holder at a wrong (not intended by manufacturer) angle (90° offset), not inserted completely, or just filled partially so that only part of the beam travels through the solution (meniscus in the beam path).

The goal of this exercise is to present some common errors made when using non-standard cuvettes. We will demonstrate the effects of misusing (using cuvette in not intended way).

The term “standard cuvettes” we use meaning cuvettes with exterior dimensions of 1.25 cm × 1.25 cm, and interior dimensions of 1 cm × 1 cm (10 mm × 10 mm). Most cuvettes used for absorption and fluorescence measurements will have standard external dimensions while having different internal sizes that refer to the size of the sample holding cavity. This is done on purpose to make sure that the cuvette will properly fit into the spectrophotometer’s (fluorimeter’s) standard sample holder and will be properly positioned in the light beam path. When using a non-standard size cuvette with external dimensions different from those of a standard cuvette, extra care should be taken to always position the cuvette properly in the beam path. These kinds of cuvettes typically use an “adapter” that is a metal or plastic element with external dimensions of the standard cuvette (12.5 mm × 12.5 mm) and internal dimensions to perfectly fit the cuvette and position it in the center.

Note: When talking about the use of a cuvette, the length through which the beam travels is written first.

Materials

- Rhodamine B
- Water
- 0.2 cm × 1 cm absorbance cuvette
- 0.2 cm × 1 cm frosted cuvette
- 0.2 cm × 1 cm clear cuvette
- 1 cm × 1 cm clear cuvette

Equipment

- Spectrophotometer

Methods

1. Prepare a solution of Rhodamine B (RhB) in water with a concentration of about 10 μM.
2. Use water as the baseline.
3. Measure baseline with the 1 cm × 1 cm cuvette filled with water.
4. Measure absorbance of rhodamine with above set up.
5. Measure baseline with the clear 0.2 cm × 1 cm cuvette filled with water using the 0.2 cm path.
6. Measure absorbance of rhodamine with above set up.
7. Measure baseline with the clear 0.2 cm × 1 cm cuvette filled with water using the 1 cm path.
8. Measure absorbance of rhodamine with above set up and cuvette positioning.
9. Measure baseline with the absorbance cuvette filled with water and 1 cm path.
   a. There is no 0.2 cm beam path since the black walls block all light.
10. Measure the absorbance of rhodamine with above set up, but filling the cuvette about halfway so that the entire beam does not pass through the solution.
11. Measure absorbance of rhodamine with above set up filling the cuvette all the way.
12. Measure baseline with the 0.2 cm × 1 cm frosted cuvette using the 1 cm path.
13. Measure absorbance of rhodamine with above set up.

Results

The above procedure should result in six spectra, three correct and three incorrect. The spectra are plotted in Figure 3-11.1. Short dashes are used for the clear 1 cm × 1 cm cuvette, dashes are used for the 0.2 cm × 1 cm cuvette, a solid line for the absorbance cuvette, and a dotted line for the frosted 0.2 cm × 1 cm cuvette.

In principle, all measurements along the 10 mm path should give similar (identical) results and in the 2 mm path measured absorption should be five times lower (see previous Experiment 3-7).

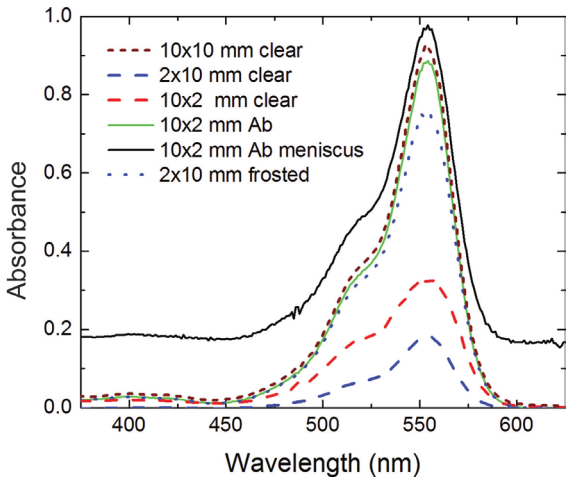

FIGURE 3-11.1 Measured absorption spectra for Rhodamine B solution in different cuvettes.

When looking at Figure 3-11.1, this is definitely not the case. A 10 mm × 10 mm cuvette is a standard cuvette that most probably gives proper result. A 2 mm × 10 mm cuvette should give 1/5 of the previous reading and the result is very close. A 10 mm × 2 mm absorption cuvette (cuvette with two black walls) should also give a very similar result. But looking at Figure 3-11.1, we notice both results are very close but differ by a few percents. We want to stress that with the 10 mm × 2 mm absorption cuvette, it is possible to get a result very close to the regular cuvette. We present average spectra from three randomized trials that we believe closely represents the average difference. The reason for the small deviation is the spectrophotometer we have been using for these measurements. Agilent 8463 spectrophotometers that have a relatively large beam size, so as explained later a significant part of the beam is cut off by the black cuvette walls. Since the sample holder is a little loose (typically intended for the sample to be easily mounted), putting the cuvette for a baseline measurement and taking it out and putting it back with the sample, the position slightly differs that leads to a different beam attenuation by the black cuvette walls. Similar measurement made in the Agilent Cary 60 that has a much smaller beam size will show a much smaller difference (less than 1%).

The rest of the measurements are definitely unacceptable. The 10 mm × 2 mm clear cuvette (this is fluorescence cuvette) shows artificially low absorption. The 10 mm × 2 mm frosted cuvette (this is fluorescence cuvette that has frosted edges) shows better results but is still far from what we would expect. Finally, the 10 mm × 2 mm absorption cuvette (black sidewalls cuvette) when we filled it not sufficiently and a small part of the beam hits the meniscus and travels over the sample solution which gives a strange result. A moderately good reading at the maximum absorption (~1) but completely off at low absorption reading (elevated baseline). We present this result since underfilling the cuvette is quite common when dealing with a small amount of the sample.

The lessons of Figure 3-11.1, we would like to take home are:

1. The same extinction coefficient should always be calculated if the absorbance is measured properly.
2. If the beam spot size is larger than the width of the facing sample cuvette side the measured absorbance will typically be too low.

The absorbance coefficient and optical density are related as

$$A(\lambda) = \varepsilon(\lambda)Cl$$

A is the quantity measured experimentally and presented in Figure 3-11.1, l is the distance the beam travels through the sample, 1 cm or 0.2 cm in this experiment, C is the concentration of the sample, and ε(λ) is the extinction coefficient. When the experiment is done correctly, the calculated extinction coefficient should always be the same regardless of the path length and used cuvette. In the table, we present calculated extinction coefficients at maximum absorption from the three closest measurements.

1 cm × 1 cm clear	$Ab(554\text{ nm}) = 0.93$	$\varepsilon(554\text{ nm}) = \frac{0.93}{10\mu\text{M}\times 1\text{cm}} = \frac{0.93}{10\mu\text{M cm}} = 93{,}000\ \mu\text{M}^{-1}\text{cm}^{-1}$
0.2 cm × 1 cm clear	$Ab(554\text{ nm}) = 0.184$	$\varepsilon(554\text{ nm}) = \frac{0.184}{10\mu\text{M}\times 0.2\text{cm}} = \frac{0.92}{10\mu\text{M cm}} = 92{,}000\ \mu\text{M}^{-1}\text{cm}^{-1}$
0.2 cm × 1 cm absorbance	$Ab(554\text{ nm}) = 0.89$	$\varepsilon(554\text{ nm}) = \frac{0.089}{10\mu\text{M}\times 1\text{cm}} = \frac{0.089}{10\mu\text{M cm}} = 89{,}000\mu\text{M}^{-1}\text{cm}^{-1}$

These values are relatively close, thus the calculated extinction coefficients are almost independent of cuvette used. As a comparison, the calculated extinction coefficient from the 1 cm × 0.2 cm cuvette measurement is 36,000 μM^{-1} cm^{-1}. Much different from the expected value of about 93,000 μM–1 cm–1.

The goal of the following considerations is to explain what may lead to significant errors in absorption measurements when using a non-standard cuvette. Understanding this will allow prevention of such mistakes in different experiments.

The most common error in using a cuvette comes from allowing parts of the beam to reach the detector without traveling through the sample solution. The baseline measurement even if it is taken with the exact same set up will not prevent the problem. Two common causes of this are using a thin clear cuvette with a thin side facing the beam or not filling the cuvette high enough for the whole beam to travel through the sample. If the level is so low that the beam does not hit the sample at all, the resulting measurement would be near zero absorbance with no clear absorption profile. This would be easy to spot as an incorrect result since that would mean there are no chromophores in the sample (at least in the wavelength range selected). In that case, it is best to test over the entire accessible wavelength range of the spectrophotometer.

In Figure 3-11.2, we are showing diagrams of three types of cuvettes that most of the readers will be familiar with. The first is a regular square cuvette 10 mm × 10 mm. Second is a fluorescence cuvette 2 mm × 10 mm but for absorption, we are using it as a 10 mm × 2 mm cuvette (the light is traveling along 10 mm path), and the third one is a 10 mm × 2 mm absorption cuvette.

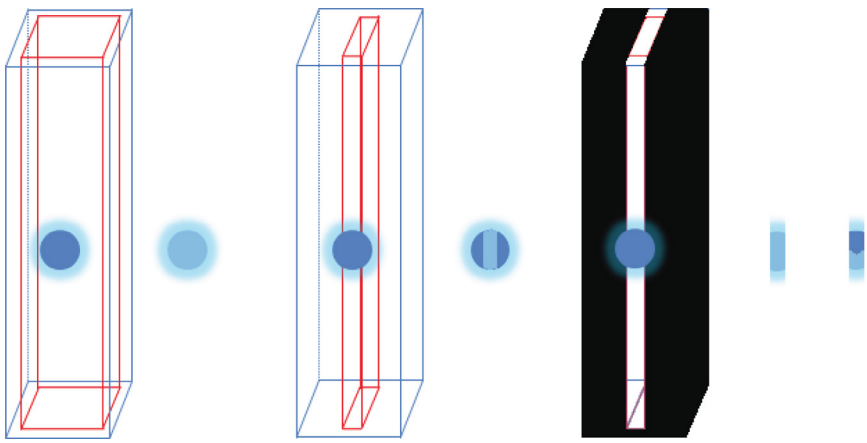

FIGURE 3-11.2 Cuvettes and schematic of beam spot before and after traveling through the cuvette.

This cuvette has two black (nontransparent) walls and the transparent path is only along the 10 mm length. This cuvette cannot be used for fluorescence. On a side of each cuvette, we schematically have drawn the profile of the transmitted beam. For the 10 mm × 10 mm standard cuvette, the beam profile is unchanged only the intensity of the beam decreased proportionally to the sample absorption. Measurement of absorption will be correct. For the second 10 mm × 2 mm fluorescence cuvette, the beam size is larger than 2 mm width and part of the beam travels through cuvette glass. On the right is the beam profile after pathing through the cuvette. The part of the beam that travels through the sample will be adequately attenuated but parts of the beam traveling through the glass will be practically unchanged. In this case, the spectrophotometer will see artificially higher intensity (not the whole beam was attenuated) leading to artificially lower absorption reading (see red dashed line in Figure 3-11.1). This will happen even if the baseline (blank) measurement was done using the same cuvette filled with buffer. For the third cuvette, the only part of the beam that has been transmitted is the open 2 mm space. The beam profile shows that the intensity is adequately attenuated and absorption measurement will give a proper result. Important: The baseline must be taken with the same cuvette filled with buffer and the cuvette should be positioned in exactly the same way for the blank and sample measurement. Typically the beam profile in the spectrophotometer is not uniform and attenuation will depend on the cuvette position and shifting the cuvette slightly left or right will significantly affect the reading. In such a case, it is typically very difficult to position the cuvette exactly the same way for baseline and sample measurement. A small fraction of the millimeter displacement will affect the reading. For this reason, in Figure 3-11.1, we can see a small (2–3%) difference between the expected value and measured one in an absorbance cuvette (two black walls).

Conclusions

Any absorption/transmission measurement will be corrupted if cuvettes are not used properly. It is also important to make sure that if different cuvettes are used for the sample and the reference it should be matched cuvettes (identical cuvettes). This can be checked by measuring the transmission of both cuvettes as described in Experiment 3-4. Transmission of Cuvettes—An Introduction to Absorbance.

Acknowlegements

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