1. Ultraviolet-Visible Spectroscopy (I)

Spectroscopy is based, principally, on the study of the interaction between radiation and matter. This interaction causes in the atom an electronic transition from a lower energetic level, \( m \), to a higher level, \( l \), occurring energy absorption from the atom equal to the energy difference between both levels, \( E_l - E_m \).

\[
\Delta E = h\theta = \frac{hc}{\lambda}
\]

where:
- \( h \) = Planck constant
- \( \theta \) = Frequency
- \( \lambda \) = Wavelength

A plot of these latter processes as a function of radiation wavelength is known as spectrum that offers information about the difference of energy involved in each electronic transition. Different types of spectroscopy can be found depending on the wavelength of the incident radiation as can be seen in the next table:

<table>
<thead>
<tr>
<th>( \lambda/\text{nm} )</th>
<th>1</th>
<th>10</th>
<th>10(^2)</th>
<th>10(^3)</th>
<th>10(^4)</th>
<th>10(^5)</th>
<th>10(^6)</th>
<th>10(^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )-RAY</td>
<td>X-RAY</td>
<td>UV</td>
<td>VIS</td>
<td>INFRARED</td>
<td>MICROWAVES</td>
<td>RADIOWAVES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear transitions</td>
<td>internal electrons</td>
<td>external electrons</td>
<td>vibrations</td>
<td>rotations</td>
<td>MNR</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1.** Electromagnetic spectrum as a function of the wavelength radiation.

UV-Visible spectroscopy (radiations with wavelengths between 10 and 1000 nm) offers information about the transition of the most external electrons of the atoms (figure 1). Since atoms or molecules absorb UV-visible radiation at different wavelength, spectroscopy/spectrometry is often used in physical and analytical chemistry for the identification of substances through the spectrum emitted from or absorbed by them. This technique is also used to assess the concentration or amount of a given species using the Lambert-Beer law.

**Lambert-Beer law**

This law relates the absorption of a radiation to the properties of the material through which is passing through. States that there is a logarithmic dependence between the transmission (or transmissivity), \( T \), of light through a substance and the product of the absorption coefficient of the substance, \( \alpha \), and the distance the beam travels through the material (i.e. the path length), \( l \). The absorption coefficient can, in turn, be written as a product of either a molar absorptivity of the absorber, \( \epsilon \), and the concentration \( c \) of absorbing species in the material. For liquids, these relations are usually written as:

\[
\log T = \log \frac{I}{I_0} = \epsilon lc
\]
where \( I \) and \( I_0 \) are the intensity of the incident and the transmitted beams, respectively. The transmission also can be expressed in terms of absorbance (A):

\[
A = -\log T
\]

And so, Lambert-Beer equation can be written finally as

\[
A = \varepsilon lc
\]

Either transmittance or absorbance can be measured experimentally with the spectrometer. Thus, if the path length and the molar absorptivity are known and the absorbance is measured, the concentration of the substance (or the number density of absorbers) can be deduced. Commonly, both parameters are constant for a given set of experiments, thus, a plot of the sample absorbance against the concentration of the absorbing substance should be a straight line. In practice, a calibration curve is obtained by plotting the measured absorbance of a series of standard samples as a function of their concentration. If the absorbance of an unknown sample is then measured, the concentration of the absorbing component can be determined from this graph.

However, the linearity of the Lambert-Beer law can be limited by chemical or instrumental factors as can be:

- deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between molecules in close proximity
- scattering of light due to particulates in the sample
- fluorescence or phosphorescence of the sample
- changes in refractive index at high analyte concentration
- shifts in chemical equilibria as a function of concentration
- non-monochromatic radiation, deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band
- stray light

Therefore, before each experiment it should be convenient to check the range of concentration in which the Law is satisfied.

**UV-Visible spectrometers**

Spectrophotometry involves the use of a spectrophotometer, which is a device to measure light intensity as a function of the wavelength of light.
Figure 2. Diagram of a double beam UV-Visible spectrometer.

The basic parts of a spectrophotometer are a light source, a sample holder, a diffraction grating or monochromator to separate the different wavelengths of light, and a detector. The radiation source is often a Tungsten filament (300-2500 nm), a deuterium arc lamp which is continuous over the ultraviolet region (190-400 nm), and more recently light emitting diodes (LED) and Xenon Arc Lamps for the visible wavelengths. The detector is typically a photodiode or a CCD. Photodiodes are used with monochromators, which permit than only light of a single wavelength reaches the detector. Diffraction gratings are used with CCDs, which collects light of different wavelengths on different pixels.

A spectrophotometer can be either single beam or double beam. In a single beam instrument, all of the light passes through the same sample cell. First, the reference, $I_0$, (generally the solvent) must be measured before the sample. This was the earliest design, but is still in common use in both teaching and industrial labs.

In a double-beam instrument, the light is split into two beams before it reaches the sample. One beam is used as the reference and the other as the sample. Some double-beam instruments have two detectors (photodiodes), and the sample and reference beam are measured at the same time.

Samples for UV/Vis spectrophotometry are most often liquids, although the absorbance of gases and even of solids can also be measured. Samples are typically placed in a transparent cell, known as a cuvette. Cuvettes are typically rectangular in shape, commonly with an internal width of 1 cm (this width becomes the path length, $l$, in the Lambert-Beer law.) They must allow radiation to pass over the spectral region of interest. The most widely applicable cuvettes are made of high quality fused silica or quartz glass because these are transparent throughout the UV, visible and near infrared regions. Glass and plastic cuvettes are also common, although glass and most plastics absorb in the UV, which limits their usefulness to visible wavelengths.

**Description of the Laboratory**

**Objective**

The principal objective is the application of this technique for the identification of substances dissolved in water and the estimation of its concentration using the Lambert-Beer law. To this aim, three experiments will be carried out:
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- Determination of the range of concentrations in which Lambert-Beer law in fulfilled.
- Determination of the concentration of a problem solution using a calibration curve.
- Experimental estimation of the pKₐ of bromocresol purple.

Experimental Procedure

a) Checking the Lamber-Beer law

First of all, students will collect the UV/Vis spectra of a Methyl Orange solution and determine the maximum absorbance wavelength. Afterwards, prepare a serie of solutions with different known concentrations of this latter indicator and measure its absorbance at the maximum absorbance wavelength (determined above for the maximum absorbance) in order to collect the calibration curve. Finally, students have to check if the Lambert-Beer law is fulfilled in the concentration range used.

Note: These indicators show strong absorption in the UV-Visible range, thus very dilute solutions should be used to satisfy the Lambert-Beer law. All the solutions will be prepared at pH=13.

b) Concentration of a problem solution using a calibration curve

Using the calibration curve previously determined in the concentration range where the law is satisfied, estimate the concentration (in mg/l) of a sample with an unknown concentration.

c) Estimation of the pKₐ of bromocresol purple:

The acid-base equilibrium for Bromocresol purple indicator is shown in figure 3. The transition betwee acid and base forms occurs at pHs between 5.2 and 6.8.

![Figure 3. Acid-base equilibrium for Bromocresol purple indicator. Transition range=5.2-6.8.](image)

The experimental procedure is the following:
1) Prepare a sample containing only the acid form and measure its absorbance between 200 and 800 nm.
2) Prepare a sample containing only the basic form and measure its absorbance between 200 and 800 nm.

Note: Adjust the pH with HCl and NaOH in each case, respectively.

On the basis of the collected spectra, ¿could you distinguish between both forms?
3) Prepare two new calibrations curves: one for the acidic form and other for the basic form.
4) Determine the concentration of the acidic ([HA]) and the basic ([A⁻]) forms for a sample with neutral pH by using the calibration curves. You need to measure the pH of this solution.

5) Finally, determine the purple bromocresol indicator pKₐ using the next equation:

\[ pK_a = pH - \log \frac{[A^-]}{[HA]} \]

Final report

At the end of the course, a detailed description of the results will be presented as a report following the next scheme:

i) introduction
ii) experimental procedure
iii) results and discussion
iv) main conclusions
v) references and/or bibliography

Recommended books and WebPage:


3) [http://www.chemguide.co.uk/analysis/uvvisiblemenu.html#top](http://www.chemguide.co.uk/analysis/uvvisiblemenu.html#top)
2. Ultraviolet-Visible Spectroscopy (II)

Spectrophotometer consists of two instruments, namely a spectrometer for producing light of any selected color (wavelength), and a photometer for measuring the intensity of light. The instruments are arranged so that liquid in a cuvette can be placed between the spectrometer beam and the photometer. The amount of light passing through the tube is measured by the photometer. The photometer delivers a voltage signal to a display device, normally a galvanometer. The signal changes as the amount of light absorbed by the liquid changes.

If development of color is linked to the concentration of a substance in solution then that concentration can be measured by determining the extent of absorption of light at the appropriate wavelength. For example hemoglobin appears red because the hemoglobin absorbs blue and green light rays much more effectively than red. The degree of absorbance of blue or green light is proportional to the concentration of hemoglobin.

When monochromatic light (light of a specific wavelength) passes through a solution there is usually a quantitative relationship (Beer's law) between the solute concentration and the intensity of the transmitted light, that is, where $I_0$ is the intensity of transmitted light using the pure solvent, $I$ is the intensity of the transmitted light when the colored compound is added, $c$ is concentration of the colored compound, $l$ is the distance the light passes through the solution, and $k$ is a constant. If the light path $l$ is a constant, as is the case with a spectrophotometer, Beer's law may be written, where $k$ is a new constant and $T$ is the transmittance of the solution. There is a logarithmic relationship between transmittance and the concentration of the colored compound. Thus,

$$-\log T = \log \frac{1}{T} = kc = \text{optical density (OD)}$$

The O.D. is directly proportional to the concentration of the colored compound. Most spectrophotometers have a scale that reads both in O.D. (absorbance) units, which is a logarithmic scale, and in % transmittance, which is an arithmetic scale. As suggested by the above relationships, the absorbance scale is the most useful for colorimetric assays.

Using a spectrophotometer

The specific instructions will differ with the used model, but the principles remain.

1. The instrument must have been warm for at least 15 min. prior to use. The power switch doubles as the zeroing control.
2. Use the wavelength knob to set the desired wavelength. Extreme wavelengths, in the ultraviolet or infrared ranges, require special filters, light sources, and/or sample holders (cuvettes).
3. With the sample cover closed, use the zero control to adjust the meter needle to "0" on the % transmittance scale (with no sample in the instrument the light path is blocked, so the photometer reads no light at all).
4. Wipe the tube containing the reference solution with a lab wipe and place it into the sample holder. Close the cover and use the light control knob to set the meter needle to "0" on the absorbance scale.
5. Remove the reference tube, wipe off the first sample or standard tube, insert it and close the cover. Read and record the absorbance, not the transmittance.
6. Remove the sample tube, readjust the zero, and recalibrate if necessary before checking the next sample.

Why use a reference solution? Can't you just use a water blank? A proper reference solution contains color reagent plus sample buffer. The difference between the reference and a sample is that the concentration of the assayable substance in the reference solution is zero. The reference tube transmits as much light as is possible with the assay solution you are using. A sample tube with any concentration of the assayable substance absorbs more light than the reference, transmitting less light to the photometer. In order to obtain the best readability and accuracy, the scale is set to read zero absorbance (100% transmission) with the reference in place. Now you can use the full scale of the spectrophotometer. If you use a water blank as a reference, you might find that the assay solution alone absorbs so much light relative to distilled water that the usable scale is compressed, and the accuracy is very poor.

Colorimetric Assays

Here is a description of how one sets up and runs a colorimetric assay to determine the concentration of a substance that is in solution.

General approach

We cannot put material under a microscope and count the number of molecules per unit volume the way we can count number of cells per unit volume. We must find something that we can measure that is proportional to the concentration of the substance of interest. The measurement most commonly used in assays is absorbance of light. Beer's Law tells us that if a solute absorbs light of a particular wavelength, the
absorbance is directly proportional to the concentration of substance in solution. A device called a spectrophotometer is used to measure and display and/or record absorbance in quantifiable units. Often the substance by itself does not absorb light so as to allow for a practical assay. We may have to employ one or more reagents to produce colored compounds in proportion to the concentration of unknown.

Measuring absorbance of light by a sample tells us very little unless we have a standard for comparison. For example, if sample X shows absorbance of 0.5, what is the actual concentration of X? If we have a sample of known concentration, and that sample also gives absorbance of 0.5, then we are reasonably sure that the substance has that same concentration. Suppose that you have a number of samples, and their concentrations vary. It would be useful to have a number of standards that span the full range of likely concentrations of our unknown. That's where a standard curve comes into it. We prepare a series of standards of known concentration of X, ranging from low to high concentration. We run the assay and plot absorbance versus concentration for each standard. Using this standard curve we can read the concentration for an unknown given its absorbance reading.

**Controls**

When we run an assay we must ensure that only the substance we are assaying is responsible for absorbance of light in the wavelength range of interest. All conditions under which standards and unknowns are prepared should be kept identical. If solutes in the sample buffers affect absorbance, then we have a problem. We won't obtain accurate results if we vary the volumes in which we prepare and assay standards and unknowns. The timing of reading absorbance, temperature at which we keep the materials, and all other physical factors should be kept the same. Because it is not always practical to use identical buffers for all unknowns and standards, we need only ensure that none of the components of any of the buffers has a significant effect on absorbance.

When we use the same volume for all standards and unknowns we simplify the analysis considerably. The standard curve can plot absorbance versus amount of substance instead of concentration. It may be less confusing to work with amounts while doing an assay, especially if dilutions are required. As long as you know the original volume of sample that was used in an assay, determination of concentration is easy.

**Complication**

All assays have limits. Amounts of substance below some minimum will be undetectable. Beyond some maximum amount or concentration an assay becomes saturated, that is, increases in amount or concentration do not affect absorbance. We generally try to work within the linear range of an assay, that is, where absorbance is directly proportional to concentration. Ideally, we would set up standards that
encompass the entire useful range of an assay. That is, we optimize the range of the assay.

Often a sample is so concentrated that when you assay the prescribed volume of sample the result is off scale – the assay reagent is saturated. The solution then is to dilute the sample. For example, if the volume of each standard or sample is 1 ml, and 1 ml of your unknown gives a result that is off scale, you can add 0.1 ml sample to a test tube along with 0.9 ml buffer. If you read a concentration from the standard curve, then multiply the result by 10 to get the actual concentration in the sample. If you read an amount from the standard curve then simply divide that amount by 0.1 ml to get your concentration.

When samples are so concentrated that you cannot pipet a small enough amount accurately, you may have to conduct serial dilutions.

**Example: preparing a standard curve**

We will set up a hypothetical assay to measure substance X. When X is mixed with assay reagent a complex is formed that absorbs light at wavelength 400 nm. Our spectrophotometer requires that we put 2 ml volume in each cuvette. A cuvette is a transparent vessel to be placed in a light path for measurement of absorbance. To get the right proportion of assay reagent to sample, we make our sample volume 0.5 ml and add 1.5 ml color reagent to each tube. Set up in this manner the assay can detect amounts of X of as little as 10 micrograms (µg) to as much as 2 milligrams (mg).

**Reference**

To calibrate the spectrophotometer we need a reference tube that is identical in every respect to the standards and samples, except that it does not contain any substance X. With the light path blocked the spectrophotometer will be set to read infinite absorbance (no transmittance of light at all). With the reference tube in the light path we will set the spectrophotometer to read zero absorbance. That way, a sample containing X will give absorbance within that range. The reference tube is used to give us the maximum dynamic range.

For this hypothetical example the reference will contain 0.5 ml sample buffer and 1.5 ml color reagent.

**Standards**

This example describes a hypothetical assay for illustration purposes only.

We want the best accuracy that we can get, and our range spans two orders of magnitude, so one way to set up the standard curve is with a logarithmic progression of standards. We need standards from 0.01 mg to 2 mg. Let's try amounts of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2 mg. The last gap is rather wide, so let's toss in one standard
of, say, 1.5 mg. To prepare standards it is convenient to start with a concentrated stock solution of the substance. The largest amount that we need is 2 mg, in a volume of 0.5 ml. Just to give us a little "wiggle room" let's make a stock solution of 5 mg/ml substance X. The following table presents the calculations.

**Table 1.** Example of how to plan a standard curve. The concentration of protein in the stock solution was 5 mg/ml. This example is for illustration purposes only.

<table>
<thead>
<tr>
<th>amount of substance X (mg)</th>
<th>volume of stock solution (µl)</th>
<th>volume of buffer (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (reference)</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>0.01</td>
<td>2</td>
<td>498*</td>
</tr>
<tr>
<td>0.02</td>
<td>4</td>
<td>496*</td>
</tr>
<tr>
<td>0.05</td>
<td>10</td>
<td>490</td>
</tr>
<tr>
<td>0.1</td>
<td>20</td>
<td>480</td>
</tr>
<tr>
<td>0.2</td>
<td>40</td>
<td>460</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>300</td>
</tr>
<tr>
<td>1.5</td>
<td>300</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>100</td>
</tr>
</tbody>
</table>

*It is common to use pipettors that give us volumes that are accurate to no more than 2 significant figures.

The volume of buffer is not as critical as the volume of stock solution. Errors in pipetting buffer affect total volume and, thus, concentration of the color reagent. Errors of less than 1% will not have a significant effect on the results. In fact, if the volume of color reagent far exceeds the volume of sample (not in this case) we would not even need to equalize volumes by adding buffer.

Some labs are not equipped with pipettors that go below 5 µl with accuracy. It may be necessary to conduct a serial dilution to get, say, 2 or 4 µl stock solution into an assay tube.

**Sample preparation**

It helps to have a reasonable estimate of ranges of concentrations of sample that one can expect. Even with such an estimate it is good to prepare samples with a range of dilutions, in case a sample is so concentrated that its absorbance readings are out of range.

For the assay in the example, if we use 500 µl sample in an assay tube (the maximum volume), its concentration would have to be less than 4 mg/ml to give a readable absorbance. On the other hand, we would want that much if the sample was, say, ten times less concentrated. Knowing nothing about the concentration of a particular sample, we would load one tube with 500 µl to cover that range. Since the assay spans a
wide range of concentrations, we can use 50 µl in a second assay tube. Now the sample can be as concentrated as 40 mg/ml and we will still have 4 mg or less in the assay tube, giving a readable result. To cover all of the bases we can assay a third tube with just 5 µl sample.

**Run the assay**

When all of the standard and unknowns are ready we will have:

- 1 reference tube
- some number of standards that span the *full range* of the assay
- two or three assay tubes per sample representing a series of dilutions

It is time to conduct the procedure for color development, which may be as simple as adding a color reagent and letting the samples sit for a few minutes. When practical, treatment of each standard and sample should be timed so that absorbance is read following the same time interval for each tube. The instrument should be calibrated, then absorbances should be taken for each tube in order. A standard curve is obtained by plotting absorbance versus amount of substance X. If the relationship is clearly linear, a standard curve isn't even necessary. Amounts can be determined using interpolation. A curve should be constructed the first time an assay is used, to check for accuracy and linearity.

**Example of a standard curve**

Here is what the plot might look like in a lab notebook (the student obviously has excellent handwriting). The relationship is not perfectly linear, rather it shows a typical extinction pattern.
Since the range is so wide, for samples giving very low absorbance readings a student might want a second higher resolution plot.

**Determine the concentration of a sample**

A concentration is an amount of something per unit volume. We typically report protein concentrations in milligrams per milliliter (mg/ml), although it is sometimes convenient to use micrograms/microliter (µg/µl) or perhaps even µg/ml (for very small concentrations). For an unknown, we divide amount of substance (from the standard curve) by the volume of sample used in the assay. Note that this volume is not the assay volume, nor is it the diluted sample volume. Divide by the volume of undiluted sample that you placed in the assay tube.

Let us suppose that you prepared three assay tubes for sample #1, containing 500 µl, 50 µl, and 5 µl sample, respectively. Suppose they gave absorbance readings of 0.86, 0.12, and 0.01, respectively. The last absorbance is off scale, of course. The intercept should be zero, but we cannot count on very low absorbances giving us sufficiently accurate readings.

An absorbance of 0.86 corresponds with 1.7 mg substance X. The volume was 500 µl (0.5 ml), so we get a concentration of 3.4 mg/ml. Sounds good. Checking the other readable tube, absorbance of 0.12 indicates that the tube contained 0.20 mg substance X. The volume was 50 µl (0.050 ml). The concentration should be 0.20 mg/0.050 ml = 4.0 mg/ml. Which result do we use, or do we take an average?

It has been found that using the one absorbance reading that falls closest to the middle of the sensitive range gives the most accurate results. In the example above the
center is an absorbance of 0.5, corresponding to 0.1 mg of substance. The absorbance scale is logarithmic, so that even from a digital display the readings are more reliable at the low end of the scale. However, at very low absorbances one or more unknown factors, such as a defect in the sample tube or cuvette, will have a more profound effect on the absorbance value than at higher absorbances. At the upper end of the range the color reagent approaches saturation, so that not only do you have less resolution among absorbance readings, but the reagent is less sensitive to differences in protein concentration.

**Absorbance Assay (280 nm)**

**Considerations for use**

Absorbance assays are fast and convenient, since no additional reagents or incubations are required. No protein standard need be prepared. The assay does not consume the protein. The relationship of absorbance to protein concentration is linear. Because different proteins and nucleic acids have widely varying absorption characteristics there may be considerable error, especially for unknowns or protein mixtures. Any non-protein component of the solution that absorbs ultraviolet light will interfere with the assay. Cell and tissue fractionation samples often contain insoluble or colored components that interfere. The most common use for the absorbance assay is to monitor fractions from chromatography columns, or any time a quick estimation is needed and error in protein concentration is not a concern. An absorbance assay is recommended for calibrating bovine serum albumin or other pure protein solutions for use as standards in other methods.

**Principle**

Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, etc. can alter the absorbance spectrum.

**Equipment**

In addition to standard liquid handling supplies a spectrophotometer with UV lamp and quartz cuvette are required.

**Procedure**

Carry out steps 1-4 (280 nm only) for a very rough estimate. Carry out all steps if nucleic acid contamination is likely.

1. Warm up the UV lamp (about 15 min.)
2. Adjust wavelength to 280 nm
3. Calibrate to zero absorbance with buffer solution only
4. Measure absorbance of the protein solution
5. Adjust wavelength to 260 nm
6. Calibrate to zero absorbance with buffer solution only
7. Measure absorbance of the protein solution

**Analysis**

*Unknown proteins or protein mixtures.* Use the following formula to roughly estimate protein concentration. Path length for most spectrometers is 1 cm.

Concentration (mg/ml) = Absorbance at 280 nm divided by path length (cm.)

*Pure protein of known absorbance coefficient.* Use the following formula for a path length of 1 cm. Concentration is in mg/ml, %, or molarity depending on which type coefficient is used.

concentration = Absorbance at 280 nm divided by absorbance coefficient

To convert units, use these relationships:

Mg protein/ml = % protein divided by 10 = molarity divided by protein molecular weight

*Unknowns with possible nucleic acid contamination.* Use the following formula to estimate protein concentration:

Concentration (mg/ml) = (1.55 x A280) - 0.76 x A260

**Comments**

Cold solutions can fog up the cuvette, while warm solutions can release bubbles and interfere with the readings. For concentrated solutions (absorbance greater than 2) simply dilute the solution.

Absorbance coefficients of some common protein standards:

- Bovine serum albumin (BSA): 63
- Bovine, human, or rabbit IgG: 138
- Chicken ovalbumin: 70

**References**

Determination of the Extinction Coefficient for a Protein of Unknown Concentration

Considerations for use
The concentration can be determined for a solution of a pure protein with unknown extinction coefficient.

Principle
See the discussion for the 280 nm absorbance assay.

Equipment
In addition to standard liquid handling supplies a spectrophotometer with UV lamp and quartz cuvette are required.

Procedure
Dilute the solution about 30 fold for the reading at 205 nm and include 0.01% Brij 35 in the buffer to prevent adsorption of protein onto plastic or glass surfaces.

Analysis
Use the following formula to determine the extinction coefficient at 205 nm:

E(205 nm) = 27 + 120 x (A280 divided by A205)

The reading at 205 nm must be multiplied by the dilution factor before using the formula. Next, determine protein concentration:

Protein concentration (M) = A205 divided by E(205 nm)

You can now determine the extinction coefficient for 280 nm:

E(280 nm) = concentration (M) divided by A280

Comments
An abnormal phenylalanine content will throw off the result considerably. The accuracy of the technique depends on an average amino acid composition.

References

Bradford protein assay

Considerations for use
The Bradford assay is very fast and it is fairly accurate as samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis.

Assay materials including color reagent, protein standard, and instruction booklet are available from Bio-Rad Corporation. The method described below is for a 100 µl sample volume using 5 ml color reagent. It is sensitive to about 5 to 200 micrograms protein, depending on the dye quality. In assays using 5 ml color reagent prepared in lab, the sensitive range is closer to 5 to 100 µg protein. Scale down the volume for the "microassay procedure," which uses 1 ml cuvettes. Protocols, including use of microtiter plates are described in the flyer that comes with the Bio-Rad kit.

Principle
The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Equipment
In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum (no special lamp or filter usually needed). Glass or polystyrene (cheap) cuvettes may be used, however the color reagent stains both. Disposable cuvettes are recommended.

Procedure

Reagents

1. Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.
2. (Optional) 1 M NaOH (to be used if samples are not readily soluble in the color reagent).

The Bradford reagent should be a light brown in color. Filtration may have to be repeated to rid the reagent of blue components. The Bio-Rad concentrate is expensive, but the lots of dye used have apparently been screened for maximum effectiveness.
"Homemade" reagent works quite well but is usually not as sensitive as the Bio-Rad product.

Assay

1. Warm up the spectrophotometer before use.
2. Dilute unknowns if necessary to obtain between 5 and 100 µg protein in at least one assay tube containing 100 µl sample.
3. If desired, add an equal volume of 1 M NaOH to each sample and vortex (see Comments below). Add NaOH to standards as well if this option is used.
4. Prepare standards containing a range of 5 to 100 micrograms protein (albumin or gamma globulin are recommended) in 100 µl volume.
5. Add 5 ml dye reagent and incubate 5 min.
6. Measure the absorbance at 595 nm.

Analysis

Prepare a standard curve of absorbance versus micrograms protein and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

Comments

The dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. Obviously, the assay is less accurate for basic or acidic proteins. The Bradford assay is rather sensitive to bovine serum albumin, more so than "average" proteins, by about a factor of two. Immunoglogulin G (IgG - gamma globulin) is the preferred protein standard. The addition of 1 M NaOH was suggested by Stoscheck (1990) to allow the solubilization of membrane proteins and reduce the protein-to-protein variation in color yield.

References

3. Protein electrophoresis

Powerful electrophoretic techniques have been developed to separate macromolecules on the basis of molecular weight. The mobility of a molecule in an electric field is inversely proportional to molecular friction which is the result of its molecular size and shape, and directly proportional to the voltage and the charge of the molecule. Proteins could be resolved electrophoretically in a semi-solid matrix strictly on the basis of molecular weight if, at a set voltage, a way could be found to charge these molecules to the same degree and to the same sign. Under these conditions, the mobility of the molecules would be simply inversely proportional to their size.

It is precisely this idea which is exploited in PAGE to separate polypeptides according to their molecular weights. In PAGE, proteins charged negatively by the binding of the anionic detergent SDS (sodium dodecyl sulfate) separate within a matrix of polyacrylamide gel in an electric field according to their molecular weights. Polyacrylamide is formed by the polymerization of the monomer molecule-acrylamide crosslinked by N,N'-methylene-bis-acrylamide (abbreviated BIS). Free radicals generated by ammonium persulfate (APS) and a catalyst acting as an oxygen scavenger (-N,N,N',N'-tetramethylethylene diamine [TEMED]) are required to start the polymerization since acrylamide and BIS are nonreactive by themselves or when mixed together.

The distinct advantage of acrylamide gel systems is that the initial concentrations of acrylamide and BIS control the hardness and degree of crosslinking of the gel. The hardness of a gel in turn controls the friction that macromolecules experience as they move through the gel in an electric field, and therefore affects the resolution of the components to be separated. Hard gels (12-20% acrylamide) retard the migration of large molecules more than they do small ones. In certain cases, high concentration acrylamide gels are so tight that they exclude large molecules from entering the gel but allow the migration and resolution of low molecular weight components of a complex mixture. Alternatively, in a loose gel (4-8% acrylamide), high molecular weight molecules migrate much farther down the gel and, in some instances, can move right out of the matrix.

Separation by electrophoresis depends on differences in the migration velocity of ions or solutes through a given medium in an electric field. The electrophoretic migration velocity of an analyte is:

\[ v_p = \mu_p E \]

where \( E \) is the electric field strength and \( \mu_p \) is the electrophoretic mobility.
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The electrophoretic mobility is inversely proportional to frictional forces in the buffer, and directly proportional to the ionic charge of the analyte. The forces of friction against an analyte are dependent on the analyte's size and the viscosity (\(\eta\)) of the medium. Analytes with different frictional forces or different charges will separate from one another when they move through a buffer. At a given pH, the electrophoretic mobility of an analyte is:

\[
\mu_p = \frac{z}{6\pi\eta r}
\]

where \(r\) is the radius of the analyte and \(z\) is the net charge of the analyte.

Differences in the charge to size ratio of analytes causes differences in electrophoretic mobility. Small, highly charged analytes have greater mobility, whereas large, less charged analytes have lower mobility. Electrophoretic mobility is an indication of an analyte's migration velocity in a given medium. The net force acting on an analyte is the balance of two forces: the electrical force acting in favor of motion, and the frictional force acting against motion. These two forces remain steady during electrophoresis. Therefore, electrophoretic mobility is a constant for a given analyte under a given set of conditions.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate (SDS or sodium lauryl sulfate) is an anionic detergent which denatures proteins molecules without breaking peptide bonds. It binds strongly to all proteins and creates a very high and constant charge:mass ratio for all denatured proteins. After treatment with SDS, irrespective of their native charges, all proteins acquire a high negative charge.

Denaturation of proteins is performed by heating them in a buffer containing a soluble thiol reducing agent (e.g. 2-mercaptoethanol; dithiothreitol) and SDS. Mercaptoethanol reduces all disulfide bonds of cysteine residues to free sulfhydryl groups, and heating in SDS disrupts all intra- and intermolecular protein interactions. This treatment yields individual polypeptide chains which carry an excess negative charge induced by the binding of the detergent, and an identical charge:mass ratio. Thereafter, the denatured proteins can be resolved electrophoretically strictly on the basis of size in a buffered polyacrylamide gel which contains SDS and thiol reducing agents.

SDS-PAGE gel systems are exceedingly useful in analyzing and resolving complex protein mixtures. Many applications and modifications of this technique are relevant to modern experimental biologists. Some are mentioned below. They are employed to monitor enzyme purification, to determine the subunit composition of oligomeric proteins, to characterize the protein components of subcellular organelles and membranes, and to assign specific proteins to specific genes by comparing protein extracts of wild-type organisms and suppressible mutants. In addition, the mobility of
polypeptides in SDS-PAGE gel systems is proportional to the inverse of the log of their molecular weights. This property makes it possible to measure the molecular weight of an unknown protein with an accuracy of +/- 5%, quickly, cheaply and reproducibly.

**Discontinuous SDS Polyacrylamide Gel Electrophoresis**

Disc gels are constructed with two different acrylamide gels, one on top of the other. The upper or stacking gel contains 4-5% acrylamide (a very loose gel) weakly buffered at pH 9.0. The lower resolving gel (often called the running gel), contains a higher acrylamide concentration, or a gradient of acrylamide, strongly buffered at pH 9.0. Both gels can be cast as tubes in glass or plastic cylinders (tube gels), or as thin slabs within glass plates, an arrangement which improves resolution considerably, and which makes it possible to analyze and compare many protein samples at once, and on the same gel (slab gels). Today, you will be constructing and running slab gels.

The ionic strength discontinuity between the loose stacking gel and the hard running gel leads to a voltage discontinuity as current is applied. The goal of these gels is to maximize resolution of protein molecules by reducing and concentrating the sample to an ultrathin zone (1-100 nm) at the stacking gel:running gel boundary. The protein sample is applied in a well within the stacking gel as a rather long liquid column (0.2-0.5 cm) depending on the amount and the thickness of the gel or tube. The protein sample contains glycerol or sucrose so that it can be overlaid with a running buffer. This buffer is called the running buffer, and the arrangement is such that the top and bottom of the gel are in running buffer to make a circuit.

As current is applied, the proteins start to migrate downward through the stacking gel toward the positive pole, since they are negatively charged by the bound SDS. Since the stacking gel is very loose, low and average molecular weight proteins are not impeded in their migration and move much more quickly than in the running gel. In addition, the lower ionic strength of the stacking gel (weak buffer) creates a high electrical resistance, (i.e., a high electric field V/cm) to make proteins move faster than in the running gel (high ionic strength, lower resistance, hence lower electric field, V/cm). Remember that applied voltage results in current flow in the gel through the migration of ions. Hence low ionic strength means high resistance because fewer ions are present to dissipate the voltage and the electric field (V/cm) is increased causing the highly polyanionic proteins to migrate rapidly.

The rapid migration of proteins through the stacking gel causes them to accumulate and stack as a very thin zone at the stacking gel/running gel boundary, and most importantly, since the 4-5% stacking gel affects the mobility of the large components only slightly, the stack is arranged in order of mobility of the proteins in the mixture. This stacking effect results in superior resolution within the running gel, where polypeptides enter and migrate much more slowly, according to their size and shape.
Separation by electrophoresis depends on differences in the migration velocity of ions or solutes through a given medium in an electric field. The electrophoretic migration velocity of an analyte is:

\[ v_p = \mu_p E \]

where \( E \) is the electric field strength and \( \mu_p \) is the electrophoretic mobility.

The electrophoretic mobility is inversely proportional to frictional forces in the buffer, and directly proportional to the ionic charge of the analyte. The forces of friction against an analyte are dependent on the analyte's size and the viscosity (\( \eta \)) of the medium. Analytes with different frictional forces or different charges will separate from one another when they move through a buffer. At a given pH, the electrophoretic mobility of an analyte is:

\[ \mu_p = \frac{z}{6\pi \eta r} \]

where \( r \) is the radius of the analyte and \( z \) is the net charge of the analyte.

Differences in the charge to size ratio of analytes causes differences in electrophoretic mobility. Small, highly charged analytes have greater mobility, whereas large, less charged analytes have lower mobility. Electrophoretic mobility is an indication of an analyte's migration velocity in a given medium. The net force acting on an analyte is the balance of two forces: the electrical force acting in favor of motion, and the frictional force acting against motion. These two forces remain steady during electrophoresis. Therefore, electrophoretic mobility is a constant for a given analyte under a given set of conditions.
Reagents and Recipes

Stacking Gel Buffer (0.5 M Tris)
12.1 g Tris up to 200 ml of H2O, PH 6.8 (store in 4C)

Separating Gel Buffer (1.5 M Tris)
36.3 g Tris, up to 200 ml of H2O, PH 8.8 (store in 4C)

20% Sodium Dodecyl Sulfate (SDS)
20 g SDS, up to 100 ml in H2O

1.5% Ammonium Persulfate (APS)
1.5 g APS, up to 100 ml in H2O (Aliquot and store in -20C)

5x Sample Buffer

10% w/v SDS
10 mM Dithiothreitol, or beta-mercapto-ethanol
20 % v/v Glycerol
0.2 M Tris-HCl, pH 6.8
0.05% w/v Bromophenolblue

Should add up to 8M urea for really hydrophobic proteins

1x Running Buffer:

25 mM Tris-HCl
200 mM Glycine
0.1% (w/v) SDS

1x Separating Gel Solution

For different applications increase your desired percentage acrylamide, make up thirty ml of running gel by selecting one of the following percentages and mixing the ingredients shown below. After adding TEMED and APS your gel will polymerize fairly quickly, so do not add these until you are sure you are ready to pour.
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<table>
<thead>
<tr>
<th></th>
<th>7%</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>15.3 ml</td>
<td>12.3 ml</td>
<td>10.2 ml</td>
<td>7.2 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>Acrylamide/Bis-acrylamide (30%/0.8% w/v)</td>
<td>6.9 ml</td>
<td>9.9 ml</td>
<td>12.0 ml</td>
<td>15.0 ml</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate (APS)</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
<td>0.02 ml</td>
</tr>
</tbody>
</table>

Stacking Gel Solution (4% Acrylamide):

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>3.075 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>Acrylamide/Bis-acrylamide (30%/0.8% w/v)</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate (APS)</td>
<td>0.025 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005 ml</td>
</tr>
</tbody>
</table>
MAKING A GEL AND ASSEMBLING THE GEL APPARATUS

Choose a percentage acrylamide based on the molecular weight range of proteins you wish to separate:

<table>
<thead>
<tr>
<th>% Gel</th>
<th>M.W. Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>50 kDa - 500 kDa</td>
</tr>
<tr>
<td>10</td>
<td>20 kDa - 300 kDa</td>
</tr>
<tr>
<td>12</td>
<td>10 kDa - 200 kDa</td>
</tr>
<tr>
<td>15</td>
<td>3 kDa - 100 kDa</td>
</tr>
</tbody>
</table>

Gel must be prepared in the gel caster. It must be poured in between to glass plates (the short plate and the spacer plate, with different spacer thickness).

Ensure that everything is clean (Best way to ensure no leakage!)

Mix the ingredients needed for the chosen percentage and pour the solution quickly into your gel casting form - be sure to leave a some room for the stacking gel - Usually leave about 2 centimeters below the bottom of the comb for the stacking gel. You can do this by inserting the comb into the dry form, and marking a region below the comb for the height of the stacker you want. Look for bubbles and remove them, then layer the top of the gel with water saturated butanol or, very carefully, with water. This will remove bubbles at the top of the gel and will ensure this part does not dry out. Wait for about 30 minutes for the gel to polymerize completely. (If you always use fresh ammonium persulfate, you're gel may polymerize more quickly and reliably.)

While waiting mix the reagents for the stacking gel, but LEAVE OUT the APS and TEMED until you are ready to pour this gel; stacking gels will polymerize more quickly than desired sometimes while one is trying to add combs to make wells.

When the running gel is polymerized wash out the water or butanol completely or your stacker may separate from the gel and you will get ugly running artifacts. Mix in the polymerizing reagents and pour the stacking gel on top of the running gel. Insert your combs trying not to get bubbles stuck underneath and allow another 30 min - 1 hour for complete polymerization. Your gels are ready!
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Pour separating gel:

1. Combine Acrylamide, water, SDS, Separating gel buffer
2. Mix by swirling (don't create bubbles)
3. Add APS and TEMED (swirl to mix)
4. Pipet mixture between plates to about 2/3 up with enough room for a few cm's from the bottom of the comb (move quickly)
5. Add water or 70% EtOH to make top smooth.
6. Wait about 20 min.

Pour stacking gel:

1. Pipet Upper Gel displacing water/EtOH to about 1-2cm from top.
2. Insert comb displacing the rest of the water/EtOH.
3. Let sit for about 15 min.

PREPARING YOUR SAMPLE

Mix your protein 5:1 with the sample buffer. Heat your sample by either:

a) Boiling for 5-10 minutes (Works for most proteins)
b) 65 degrees C for 10 minutes (If you have smearing using the above procedure)
c) 37 degrees for 30 minutes (Membrane proteins or others that do not enter the gel otherwise may benefit from this type of sample preparation)

RUNNING YOUR GEL

Clamp in your gel and fill both buffer chambers with gel running buffer according to the instructions for your specific apparatus. Pipet your sample into the gel adjusting the volume according to the amount of protein in your sample. If you are going to stain using Coomassie, don't use much more than 5μg of your protein of interest to get a nicely defined band. Be sure to include a lane with molecular weight standards.

Now attach your power leads and run the gel until the blue dye front reaches the bottom. I prefer to run at 250 V constant which in a four to twenty percent mini gel needs about 30 minutes total run time, but adjust to the thickness of your gel, the power supply used and the resolution desired. Remove the gel for the power supply and process further - Visualize your proteins using Coomassie Brilliant Blue, Silver stain, or any of the other protein stains.
Once protein bands have been separated by electrophoresis, they can be visualized using different methods of in-gel detection. Over the past several decades, demands for improved sensitivity for small sample sizes and compatibility with downstream applications and detection instrumentation have driven the development of several basic staining methods over the years. Each method has particular advantages and disadvantages, and an number of specific formulations of each type of method provide optimal performance for various situations. This article discusses the general principles of protein gel staining and describes several types of staining methods.

**GEL STAINING**

The first step after performing denaturing polyacrylamide gel electrophoresis (SDS-PAGE) is to disassemble the gel cassette and place the thin (1 mm thick) polyacrylamide gel in a tray filled with water or buffer. The electrophoresed proteins exist as concentrated "bands" embedded within each lane of the porous polyacrylamide gel matrix. Typically, the proteins are still bound to anionic SDS detergent, and the entire gel matrix is saturated in a particular buffer.

To make the proteins visible, a protein-specific, dye-binding or color-producing chemical reaction must be performed on the proteins within the gel. Depending on the particular chemistry of the stain, various steps are necessary to hold the proteins in the matrix and to facilitate the necessary chemical reaction. All steps are done in solution, i.e., with the gel suspended in a tray filled with one liquid reagent or another.

Given the common constraints of this format, most staining methods involve some version of the same general incubation steps:

- A water-wash to remove electrophoresis buffers from the gel matrix
- An acid- or alcohol-wash to condition or fix the gel to limit diffusion of protein bands from the matrix
- Treatment with the stain reagent to allow the dye or chemical to diffuse into the gel and bind (or react with) the proteins
- Destaining to remove excess dye from the background gel matrix

Depending on the particular staining method, two or more of these functions can be accomplished with one step. For example, a dye reagent that is formulated in an acidic buffer can effectively fix and stain in one step. Conversely, certain functions require several steps. For example, silver staining requires both a stain-reagent step and a developer step to produce the colored reaction product.

**Coomassie Dye Stains**

The most common method for in-gel protein detection is staining with coomassie dye. Several coomassie stain reagent recipes exist in the literature and use either the G-250 (“colloidal”) or R-250 form of the dye. Colloidal coomassie can be formulated to effectively stain proteins within one hour and require only water (no methanol or acetic acid) for destaining.
In acidic buffer conditions, coomassie dye binds to basic and hydrophobic residues of proteins, changing from dull reddish-brown to intense blue (see previous images on this page). As with all staining methods, coomassie dye reagents detect some proteins better than others based on their chemistry of action and differences in protein composition. Thus, coomassie dye reagents can detect as few as 8-10 nanograms per band for some proteins and 25 nanograms per band for most proteins.

Coomassie dye staining is especially convenient because it involves a single, ready-to-use reagent and does not permanently chemically modify the target proteins. An initial water wash step is necessary to remove residual SDS, which interferes with dye-binding. Then stain reagent is added, usually for about 1 hour; finally, a water or simple methanol:acetic acid destaining step is used to wash away excess non-bound dye from the gel matrix. Because no chemical modification occurs, excised protein bands can be completely destained and the proteins recovered for analysis by mass spectrometry or sequencing.

**Silver Stains**

Silver staining is the most sensitive colorimetric method for detecting total protein. The technique involves the deposition of metallic silver onto the surface of a gel at the location of protein bands. Silver ions (from silver nitrate in the stain reagent) interact and bind with certain protein functional groups. Strongest interactions occur with carboxylic acid groups (Asp and Glu), imidazole (His), sulfhydryls (Cys), and amines (Lys). Various sensitizer and enhancer reagents are essential for controlling the specificity and efficiency of silver-ion binding to proteins and effective conversion (development) of the bound silver to metallic silver. The development process is essentially the same as for photographic film; silver ions are reduced to metallic silver, resulting in brown-black color.

Silver staining protocols require several steps that are affected by reagent quality as well as incubation times and thickness of the gel. An advantage of commercially available silver staining kits is that the formulations and protocols are optimized and consistently-manufactured, helping to minimize the effects of minor differences in day-to-day use. Kits with optimized protocols are robust and easy to use, detecting less than 0.5 nanograms of protein in typical gels.

**References:**


4. Centrifugation

Centrifugation is one of the most important and widely applied research techniques in biochemistry, cellular and molecular biology, and in medicine. Current research and clinical applications rely on isolation of cells, subcellular organelles, and macromolecules, often in high yields.

A centrifuge uses centrifugal force (g-force) to isolate suspended particles from their surrounding medium on either a batch or a continuous-flow basis. Applications for centrifugation are many and may include sedimentation of cells and viruses, separation of subcellular organelles, and isolation of macromolecules such as DNA, RNA, proteins, or lipids.

Increasing the effect of gravity: the centrifuge

Many particles or cells in a liquid suspension, given time, will eventually settle at the bottom of a container due to gravity (1 x g). However, the length of time required for such separations is impractical. Other particles, extremely small in size, will not separate at all in solution, unless subjected to high centrifugal force. When a suspension is rotated at a certain speed or revolutions per minute (RPM), centrifugal force causes the particles to move radially away from the axis of rotation. The force on the particles (compared to gravity) is called Relative Centrifugal Force (RCF). For example, an RCF of 500 x g indicates that the centrifugal force applied is 500 times greater than Earth’s gravitational force. Table 1 illustrates common centrifuge classes and their applications.

Table 1. Classes of centrifuges and their applications.

<table>
<thead>
<tr>
<th>Pelleting applications</th>
<th>Centrifuge Classes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lowspeed</td>
</tr>
<tr>
<td>Maximum Speed (rpm x10^3)</td>
<td>10</td>
</tr>
<tr>
<td>Maximum RCF (x10^5)</td>
<td>7</td>
</tr>
</tbody>
</table>

Table: Pelleting applications and centrifuge classes.

- **Bacteria**: Yes, Yes, (Yes)
- **Animal and plant cells**: Yes, Yes, (Yes)
- **Nuclei**: Yes, Yes, (Yes)
- **Precipitates**: Some, Most, (Yes)
- **Membrane fractions**: Some, Some, Yes
- **Ribosomes/Polyosomes**: - , - , Yes
- **Macromolecules**: - , - , Yes
- **Viruses**: - , Most, Yes

( ) = can be done but not usually used for this purpose.
Types of Centrifugal Separations

1. Differential centrifugation.

Separation is achieved primarily based on the size of the particles in differential centrifugation. This type of separation is commonly used in simple pelleting and in obtaining partially-pure preparation of subcellular organelles and macromolecules. For the study of subcellular organelles, tissue or cells are first disrupted to release their internal contents. This crude disrupted cell mixture is referred to as a homogenate. During centrifugation of a cell homogenate, larger particles sediment faster than smaller ones and this provides the basis for obtaining crude organelle fractions by differential centrifugation. A cell homogenate can be centrifuged at a series of progressively higher g-forces and times to generate pellets of partially-purified organelles.

When a cell homogenate is centrifuged at 1000 x g for 10 minutes, unbroken cells and heavy nuclei pellet to the bottom of the tube. The supernatant can be further centrifuged at 10,000 x g for 20 minutes to pellet subcellular organelles of intermediate velocities such as mitochondria, lysosomes, and microbodies. Some of these sedimenting organelles can obtained in partial purity and are typically contaminated with other particles. Repeated washing of the pellets by resuspending in isotonic solvents and re-pelleting may result in removal of contaminants that are smaller in size (Figure 1). Obtaining partially-purified organelles by differential centrifugation serves as the preliminary step for further purification using other types of centrifugal separation (density gradient separation).

![Differential Centrifugation](image)

2. Density gradient centrifugation

Density gradient centrifugation is the preferred method to purify subcellular organelles and macromolecules. Density gradients can be generated by placing layer after layer of
gradient media (Table 2) such as sucrose in a tube with the heaviest layer at the bottom and the lightest at the top in either a discontinuous or continuous mode. The cell fraction to be separated is placed on top of the layer and centrifuged. Density gradient separation can be classified into two categories. 2a. Rate-zonal (size) separation. 2b. Isopycnic (density) separation.

2a. Rate zonal (size) separation

Rate-zonal separation takes advantage of particle size and mass instead of particle density for sedimentation. Figure 2 illustrates a rate-zonal separation process and the criteria for successful rate-zonal separation. Examples of common applications include separation of cellular organelles such as endosomes or separation of proteins, such as antibodies. For instance, Antibody classes all have very similar densities, but different masses. Thus, separation based on mass will separate the different classes, whereas separation based on density will not be able to resolve these antibody classes.

Certain types of rotors are more applicable for this type of separation than others. Please See rotor categories (below) and Table 2.

Criteria for successful rate-zonal centrifugation:

Density of the sample solution must be less than that of the lowest density portion of the gradient.

- Density of the sample particle must be greater than that of the highest density portion of the gradient.
- The pathlength of the gradient must be sufficient for the separation to occur.
- Time is important. If you perform too long runs, particles may all pellet at the bottom of the tube.

2b. Isopycnic separation

In this type of separation, a particle of a particular density will sink during centrifugation until a position is reached where the density of the surrounding solution is exactly the same as the density of the particle. Once this quasi-equilibrium is reached, the length of centrifugation does not have any influence on the migration of the particle. A common example for this method is separation of nucleic acids in a CsCl gradient. Figure 3 illustrates the isopycnic separation and criteria for successful separation. A
variety of gradient media can be used for isopycnic separations and their biological applications are listed in Table 2.

Criteria for successful isopycnic separation:

- Density of the sample particle must fall within the limits of the gradient densities.
- Any gradient length is acceptable.
- The run time must be sufficient for the particles to band at their isopycnic point. Excessive run times have no adverse effect.

Table 2. Applications of density gradient media for isopycnic separations.

<table>
<thead>
<tr>
<th>Gradient media</th>
<th>Cells</th>
<th>Viruses</th>
<th>Organelles</th>
<th>Nucleoproteins</th>
<th>Macromolecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars (e.g. sucrose)</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Colloidal silica</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Iodinated media</td>
<td>++++</td>
<td>++</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Alkali metal salts</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>++++</td>
</tr>
</tbody>
</table>

+ + + = excellent, + + = good, + = good for some applications, + = limited use, - = unsatisfactory

Rotor categories

Rotors can be broadly classified into three common categories namely swinging-bucket rotors, fixed-angle rotors, and vertical rotors (Figure 4, Table 3). Note that each type of rotor has strengths and limitations depending on the type of separation.

1. swinging-bucket
2. fixed-angle
3. vertical

Other rotors include continuous flow and elutriation rotors.

Table 3. Types of rotors and their applications.

<table>
<thead>
<tr>
<th>Type of rotor</th>
<th>Pelleting</th>
<th>Rate-zonal Sedimentation</th>
<th>Isopycnic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed-angle</td>
<td>Excellent</td>
<td>Limited</td>
<td>Variable*</td>
</tr>
<tr>
<td>Swinging-Bucket</td>
<td>Inefficient</td>
<td>Good</td>
<td>Good**</td>
</tr>
<tr>
<td>Vertical</td>
<td>NS</td>
<td>Good</td>
<td>Excellent</td>
</tr>
<tr>
<td>Zonal</td>
<td>NS</td>
<td>Excellent</td>
<td>Good</td>
</tr>
</tbody>
</table>

NS = not suitable
*Good for macromolecules, poor for cells, and organelles
**Good for cells and organelles, caution needed if used with CsCl
In swinging bucket rotors, the sample tubes are loaded into individual buckets that hang vertically while the rotor is at rest. When the rotor begins to rotate the buckets swing out to a horizontal position (Figure 4). This rotor is particularly useful when samples are to be resolved in density gradients. The longer pathlength permits better separation of individual particle types from a mixture. However, this rotor is relatively inefficient for pelleting. Also, care must be taken to avoid “point loads” caused by spinning CsCl or other dense gradient materials that can precipitate.

In fixed-angle rotors, the sample tubes are held fixed at the angle of the rotor cavity. When the rotor begins to rotate, the solution in the tubes reorients (Figure 4). This rotor type is most commonly used for pelleting applications. Examples include pelleting bacteria, yeast, and other mammalian cells. It is also useful for isopycnic separations of macromolecules such as nucleic acids.

In vertical rotors, sample tubes are held in vertical position during rotation. This type of rotor is not suitable for pelleting applications but is most efficient for isopycnic (density) separations due to the short pathlength. Applications include plasmid DNA, RNA, and lipoprotein isolations.

**Selection of Centrifuge Tubes**

Table 4 and Table 5 illustrate properties of centrifuge tubes and the proper rotors in which they should be used.

**Selection of the appropriate centrifuge tube:**

· Prevents sample leakage or loss

· Ensures chemical compatibility

· Allows easy sample recovery

**Major factor in selection of a tube (plastic) material:**

· Clarity

· Chemical resistance

· Sealing mechanism (if needed)
check product guide pages or tube packaging for notes on recommended sample volume and maximum speed.

always run thin-walled, sealed tubes full in a fixed angle or vertical rotor. Examples:
- open top tube with multiple sealing assembly
- Re-seal tubes
- Ultracrimp® and Clearcrimp® tubes

autoclave tubes only if absolutely necessary and only at 121°C for 15 min.

avoid cleaning plastic tubes in automated dishwashers or glassware washers, which may produce excessively hot temperatures.

we recommend that you clean tubes with a mild laboratory detergent in warm water, rinse, and thin air dry.

tube must be carefully matched with rotor type to prevent sample loss and/or failure as illustrated in Table 5 below.

To prolong tube life and avoid breakage or collapse:

Table 5 – Tube Type and Rotor Compatibility

<table>
<thead>
<tr>
<th>Tube type</th>
<th>Rotors Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fixed-angle</td>
</tr>
<tr>
<td>Thin wall open top</td>
<td>No</td>
</tr>
<tr>
<td>Thick wall open top</td>
<td>Yes</td>
</tr>
<tr>
<td>Thin wall sealed</td>
<td>Yes</td>
</tr>
<tr>
<td>Oak ridge</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Common Centrifugation Vocabulary and Formulas

- **Pellet**: hard-packed concentration of particles in a tube or rotor after centrifugation.

- **Supernatant**: The clarified liquid above the pellet.

- **Adapter**: A device used to fit smaller tubes or centrifugal devices in the rotor cavities.
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- **RPM**: Revolutions Per Minute (Speed).
- **R_max**: Maximum radius from the axis of rotation in centimeters.
- **R_min**: Minimum radius from the axis of rotation in centimeters.
- **RCF**: Relative centrifugal Force. $RCF = 11.17 \times \frac{R_{\text{max}} \times \text{(RPM)}^2}{1000}$
- **K-factor**: Pelleting efficiency of a rotor. Smaller the K-factor, better the pelleting efficiency.
  
  \[ K = \frac{2.53 \times 10^{11} \ln(R_{\text{max}}/R_{\text{min}})}{(\text{RPM})^2} \]

- **S-value**: the sedimentation coefficient is a number that gives information about the molecular weight and shape of the particle. S-value is expressed in Svedberg units. The larger the S-value, the faster the particle separates.

For more information about sedimentation coefficients, please refer to the section on references and suggested readings in this article.

- **Pelleting time**: time taken to pellet a given particle. $T = K/S$ where $T = \text{pellet time in hours}$. $K = \text{K-factor of the rotor}$, and $S = \text{sedimentation coefficient}$.
- **Rotor conversion formula**: If the time to pellet a sample in your “old” rotor is known, one could determine the time it would take for the same sample to pellet in a “new” rotor. The formula for this determination is as follows:

  \[ \frac{T_1}{K_1} = \frac{T_2}{K_2} \quad \Rightarrow \quad T_1 = T_2 \left( \frac{K_1}{K_2} \right) \]

Where:
- $T_1 = \text{Time to pellet in the “new” rotor}$
- $T_2 = \text{Time to pellet in the “old” rotor}$
- $K_1 = \text{K-factor of the “new” rotor}$
- $K_2 = \text{K-factor of the “old” rotor}$

Example of a rotor conversion:

**Old Rotor (Beckman® JA-10)  New Rotor (Sorvall® SLC-1500)**

- $T_2 = 20 \text{ min;} \quad K_2 = 3610$
- $T_1 = (\ ?) \text{ min;} \quad K_1 = 1676$

**Old Pelleting Time = 20 min  New Pelleting Time = 9.2 min**
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Ultracentrifugation

Ultracentrifuges can be operated at much greater speeds (up to 65,000 or 75,000 rpm). Due to these high speeds the rotor chamber must be evacuated of air to reduce friction and permit accurate rotor temperature control. In most ultracentrifuges the rotor is driven either by a motor and a set of gears or by an oil or air turbine system.

Airfuge® Ultracentrifuge (Beckman)

References

2. Subcellular Fractionation: A Practical Approach, by John M. Graham (Editor) and D.
5. Dynamical Light Scattering

Dynamical light scattering has gained over the last years a deserved importance as an non-invasive analytical tool very useful for protein crystallization and crystal growth laboratories and also in industrial and laboratory studies of small molecular compounds like drugs and minerals. Its wide application is based on simple physical phenomena and more complicated analysis that allow this technique to yield information about the size distribution of a suspension of particles/proteins, about the surface solution chemistry through zeta potential measurements, and more. However, the technique needs adequate training, as all the final results is the consequence of many analysis and fittings of the same raw data, and the possibility of artefacts and misinterpretations are high. In this lab you will learn the fundaments of the technique so that you can have a rational and knowledge-based approach to this very useful technique. (See Study Material DLS I and II for more details).

Description of the lab

Objectives

After finishing this lab the student should be capable of designing, performing and analysing a size distribution measurement with DLS of mono and polydisperse samples. Additionally, they should have understood the physical fundaments of the technique as well as the basics of the theoretical approximations and numerical analysis that lies behind the size measurement. The students will also learn to perform z-potential measurements and its main applications.

Experimental Procedure

The lab comprises four blocks of 5-6 hours of duration. The first block will be entirely devoted to a theoretical introduction of the technique. To prepare for this session you will receive study material that should be read at least once (preferably twice) before the first block. During this block the teacher will review and expand the concepts included in the material and will try to spur a discussion/debate.

a) Size distributions

Firstly, the students will calibrate the system (ZNanosizer) by using a size standard of latex spheres.

Afterwards, they will also check for effects of the presence of salts on the latex spheres size distributions.

This block will be completed with the size distribution measurement of a colloidal system proposed by the students.
b) Z-potential measurements

The protocol for this block will be as described for a. Firstly, the DLS device will be calibrated for z-potential measurements with a standard sample and then, the students will measure the z-potential of different samples. In this block, it will be also evaluated the effect of the salt (NaCl) concentration on the z-potential values of a colloidal systems.

Final report

At the end of the course, a detailed description of the results will be presented as a report following the next scheme:

i) introduction
ii) experimental details
iii) results
iv) conclusions
v) references and/or bibliography
6. Refractive index measurements

The estimation of the refractive index of a liquid through the measurement of the total internal reflection angle is a common procedure. For crystal growth studies it is useful because ad hoc calibration curves of refraction index against concentration of a solute species in a cocktail can be used to determine the concentration of that solute in an unknown solution, for example after a solubility experiment.

Goals of the lab

After finishing this lab you should know what is a refractometer, what does it measure, and how can it be useful to determine concentration of solutes in solutions.

Schedule of the lab

The lab comprises 2 blocks of 5-6 hours of duration. The first half of the first block will be devoted to a small theoretical introduction about some initial concepts. In the second half you will be presented to the measurement equipment and will learn the calibration and measuring routines.

In the second block you will design and plan experiments in order to obtain calibration curves for concentration of sodium chloride, ammonium sulfate and a polyethylene glycol. And then determine and “unknown” with it. The results will be discussed with the teacher.