Module 2. Determination of unknown NADPH concentration using absorbance spectrophotometry

1. **Purpose of the lab**

   The instructor will provide you with an Eppendorf tube containing a solution of NADPH (solute) in water (solvent).

   **Your goal is to design and conduct an experiment to determine the concentration (in mM) of NADPH in this tube.**

   Hint: To do this, you will use an absorbance spectrophotometer to measure the absorbance of a solution. Look at Beer’s law (see background) and note the following: If you have values for absorbance, path length, and the extinction coefficient, you can calculate the concentration.

2. **Agenda for the Day**

   - Show math moment calculations to instructor.
   - Instructor presentation of absorption spectroscopy, spectra (for NADPH for this experiment), Beer’s Law and dilution calculations
   - Groups review Math Moment Problems to prepare for experimental design.
   - Finalize Experimental design in groups. Each student hands in a step-by-step protocol today.
   - Conduct the experiment in groups. Each student individually records data in their personal laboratory notebook.
   - Clean up

3. **Background**

   - Useful information can be found Chapter 7A in the textbook (Boyer).
   - Extinction coefficient of NADPH at 340 nm is 6220 M⁻¹ cm⁻¹.
   - NADPH is a cofactor. NADPH is an unstable compound, keep it on ice throughout the lab.
   - Molecular weight of NADPH is 741 g/mole.
   - Approximately 50 mg NADPH powder (~50 mg +/- 15 mg) was dissolved in approximately 5 mL of water.
   - A math example:

     The “1/2” or “1 to 2” or “2 fold” dilution (these three terms are equivalent) can be achieved by mixing 1ml of sample and 1ml of buffer.

     Similarly, the “1/10” or “1 to 10” or “10 fold” dilution can be achieved by mixing 1ml of sample and 9 ml of buffer.

     How would you make
     - A 1/5 dilution?
     - A 1 to 3 dilution?
     - A 15 fold dilution?
• In this experiment, NADPH is your solute and water is your solvent.
• You must use a blank as a reference solution. The blank contains everything except the compound of interest which absorbs light. Since the NADPH was dissolved in water, use water as your blank.
• You will use absorbance spectroscopy to determine how much more light the analyte/solute/unknown (NADPH) will absorb compared to the pure solvent/blank (water).
• Use a plastic cuvette with a 1 cm path length.
• The linear range for the instrument is between absorbance values of A = 0.1 and A = 1.2 so your reading must be between these values.

Beer’s law is $A = \varepsilon l C$

\[ \varepsilon = \text{the extinction coefficient} \]
\[ l = \text{the path length} \]
\[ C = \text{concentration} \]
\[ A = \text{absorbance reading} \]

4. **Math Moment**

Reminders:
• We are using absorbance spectrophotometry to determine the concentration of NADPH in the tube provided by the instructor.
• Be careful with units. Decide to use only one unit (for example mL for all volumes) in dilution calculations in order to avoid mistakes. If your values are in different units, do a unit conversion so that all values are in the same units.
• Remember to provide units for all your calculations. The units are as important as the number.
• As you answer these “guiding questions”, think about how they relate to your experimental design for determining NADPH concentration. Discuss these questions with your group.

1. Copy Beer's Law here:

2. State the variables in Beer's Law with their units
   
   $A = \text{Absorbance}$
   $C = \varepsilon$
   $l =$

3. Solve the following problem using Beer’s law: When the absorbance of a Tyrosine sample with a concentration of $4.0 \times 10^{-4}$ M was measured, the absorbance was found to be 0.6. The cuvette had a 1 cm path-length and the wavelength used was 280 nm. What is the molar absorption coefficient (also known as the extinction coefficient) for Tyrosine (remember to provide units with your answer)?

4. For today's work, the instructor dissolved approximately 50 mg of NADPH powder into approximately 5 mL of water. What would the concentration (mg/mL) of NADPH be if exactly 50 mg of NADPH powder had been dissolved in exactly 5 mL of water in mg/mL?
5. Convert the answer to question 5 into units of mM? Hint: you will find the molecular weight in the background section.

6. Give the extinction coefficient of NADPH (at 340 nm) in the units of mM$^{-1}$ cm$^{-1}$. Hint: the extinction coefficient is provided in the background section but you will need to do a unit conversion. Be careful here, students sometimes confuse whether a unit is in the numerator or in the denominator.

7. What would absorbance of the NADPH solution if exactly 50 mg of NADPH powder had been dissolved in exactly 5 mL of water in mg/mL in a 1 cm path-length cuvette? Hint: you can use Beer’s law. Be careful with units.

8. The value for absorbance for our device must be in the range of 0.1-1.2. This is the linear range for NADPH in this instrument. Does the absorbance in question 7 fall in this range?

*What would you do if the absorbance reading is >1.2?

*What would you do if the absorbance reading is < 0.1?

9. a) What should the concentration of NADPH be if we wanted the solution to have the low absorption value (A=0.1)? Hint: you can use Beer’s law.

b) What should the NADPH concentration be if we aimed to get a higher absorption value (A=1.2)?

10. Now we have a range for the concentration. Decide the concentration that you will aim for in your experimental design (avoid both extremes). Provide your answers for concentration and the absorbance on the lines below. Remember to provide units for concentration. Absorbance has no units.

The absorption for NADPH concentration of _______________ would be ________________.

11. Dilution Calculation. From your answer to question 10, you know what concentration of NADPH you want in the cuvette to obtain your absorbance measurement. For the experiment, you need a total volume of 1 mL in your cuvette. How much of your concentrated NADPH solution (the solution provided you instructor) will you need to add to the cuvette to get the desired NADPH concentration? How much water? Remember to provide units for your answers. Below is some information for dilution calculations if you need it. Otherwise just skip this and do the calculation in the
space provided below and provide final answers on the lines. Hint: for this question, you can use the C1V1 = C2V2
calculation. This is also sometimes described as M1V1 = M2V2 where instead of C for concentration M is used for
molarity. Molarity, of course, is one way to express concentration.

C1 = concentration of original, undiluted solution (solution provided
by instructor)
C2 = concentration of dilute solution (after NADPH is mixed with
water)
V1 = volume of original, undiluted solution used
V2 = final volume of the diluted solution

Note that C1 is always going to be a higher concentration that C2
and V2 is always going to be a larger volume than V1. See the
figure for an example.

An example: How may mL of 2.50 M NaOH solution are required to
make 525 mL of 0.150 M NaOH solution? See calculation below. Can you find V1, the mL required to make the solution? Note how
the units of concentration (M in this problem) cancel out. This is
why all the values must be in the same units before doing the
calculation.

\[ M_1V_1 = M_2V_2 \]
\[ (2.50 \text{ M})(V_1) = (0.150 \text{ M})(525 \text{ mL}) \]
\[ \frac{2.50 \text{ M}}{2.50 \text{ M}} \]

To make ____________ (remember units) concentration of NADPH, mix _________ uL of concentrated NADPH
(provided by instructor) with _______________ uL of water. The final volume will be 1 mL, which is equal to
_____________ uL.

12. What is the dilution factor for the dilution you planned in question 11? Hint: Dilution factor is equal to V2/V1 or C1/C2. You can check that you get the same answer both ways.

13. If a group adds 10 uL of NADPH (provided by instructor) and 990 uL of water in a cuvette and finds the concentration
in the cuvette to be 0.07 mM, what is the concentration in the tube provided by the instructor? Hint: Use C1V1 = C2V2.
Your have C2, V1, and V2. When you do your experiment, remember that you must calculate the concentration of the
solution provided by the instructor, not the concentration in the cuvette!

5. **Supplies Provided**

- UV-VIS spectrophotometer (one is located in SH317, one in SH318 next door)
- 1 mL cuvettes that work for the wavelength of 340 nm
- Pipettors (P20, P200, P1000)
- Pipettor tips
- Eppendorf tubes
- Distilled water
- Sample of NADPH (100 µL) with unknown concentration
6. **Experimental Design**

With your group, plan out how you will conduct your experiment and come to an agreement about the plan. The goal is to determine the concentration of an unknown sample of NADPH provided by the instructor using the supplies listed above. Each student writes a 1 page experimental protocol (step-by-step directions). Hand in the protocol at the end of this lab (include the names of all group members, the date, class, section, and instructor name). Consider the number of times the experiment should be repeated (minimum of three but depends on results you get) for accurate results. At the end of the protocol, write down a few notes about what was confusing or difficult about designing this experiment.

7. **Common Mistakes and Some Advice**

The absorbance of the solution provided would be above what the spectrophotometer can measure and so you will need to dilute the solution before measuring the absorbance (see math moment calculations). A common mistake by students is to determine the diluted concentration in the cuvette instead of the concentration in the Eppendorf vial provided by instructor. See a diagram of a 10-fold dilution in the figure; the dots represent molecules. If the concentration in the diluted tube (on the right) is 0.7 mM, what is the concentration in the more concentrated tube (on the left)? The answer is 7 mM because the dilution was 10 X. So if you were asked for the concentration of the original solution provided, 0.7 mM would be incorrect and 7 mM would be correct.

Be careful with units. Decide to use only one unit (for example mL for all volumes or mM for all concentrations) in dilution calculations in order to avoid mistakes. If your values are in different units, do a unit conversion so that all values are in the same units.

Practical mistakes some students make include:
- Insufficient mixing of the diluted solution before measuring the absorbance. After preparing a diluted solution, mix the solution thoroughly by pipetting up and down or inverting the vessel.
- Insufficient number of replicate measurements (repeat measurements). You must always do a minimum of 3 measurements. If the values are very different from each other, do more measurements.
- Pipetting errors. Remember to check the volume of the pipettor before you aspirate. You need to check the volume every time you use it! Keep an eye on the liquid level in the tip to make sure that you draw in and expel the correct volume each time.

8. **Vocabulary**

NADPH, cuvette, absorbance, extinction coefficient, molar absorptivity, blank, wave-length, dilution, dilution factor, solvent, solute

9. **Safety**

You must wear safety glasses when conducting the experiment. You must never eat or drink in the laboratory. Any observed violations of these rules will result in lower final grade and/or removal from the lab. These safety items are solely the responsibility of the student.

10. **Clean up**

For clean-up, return the remaining original solution to the instructor. Discard dilutions in sink. Wash cuvettes and leave them to the box labeled “used cuvettes”. Put pipettors in the correct boxes, the last person puts the boxes in the cabinet in the back of the lab. Dry your ice buckets and place them back in the cabinet. Place all other items where you got them from. Make sure they are clean. Leave your bench ready for the next class to start working.
11. **Data Sheet (Homework)**

For this experiment, a 1 page data sheet (one per group) with data and calculations is handed in one week after the completion of the Module. This is a team project, make sure to plan meetings. Include names of group members, date, class, section and instructor name. Provide the data sheet in professional format, include correct units, data labels etc. Do not use excessive significant figures and **present your data in tables when possible**. Remember to include the following:

- Absorbance values from each measurement (for each, include wavelength, path-length and dilution made)
- Sample calculation of how you converted absorbance values to concentration values
- Concentration values of the solutions in the cuvette from each measurement
- Sample calculation of how you used the concentration in the cuvette to determine the concentration of the original solution (for each measurement)
- Concentration values of the original solution provided by instructor based on each measurement
- For the concentration values of the original solution, determine the average and standard deviation.
- For each value, remember to include units. Please, show data in tables when possible.
- Make sure your final value +/- standard deviation in mM is predominantly displayed.
- Your table should have a figure number and figure caption (a short paragraph that describes what is shown)