

Methods of Calculating Unknown Concentration – Spectrophotometry in Practice

Chapter Overview

In this chapter, we will focus on the methods by which the unknown concentration of a substance can be determined from experimental data. In the practical exercise, you will try out these procedures using spectrophotometry, where the concentration of a sample is derived from the measured absorbance. However, the principles of calculation are generally applicable to other analytical methods as well and represent a fundamental skill in laboratory work.

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1 Introduction to the Topic

In the laboratory, we often encounter situations where it is necessary to determine the **concentration of an unknown substance** in a solution. Typically, we measure a **physicochemical property** that changes depending on concentration. This could be, for example, the intensity of absorbed light, electrical potential, or migration time in chromatography.

One of the most common and accessible methods is **spectrophotometry**, which measures how a substance absorbs light at a specific wavelength. From the absorbance, the concentration can then be calculated using mathematical relationships. The key principle is the **Lambert–Beer law**, which describes the linear relationship between absorbance and concentration in diluted solutions.

More about spectrophotometry and optical methods can be found in the section

“Optical Methods – Spectrophotometry.”

There are several ways to determine concentration from absorbance. **The calibration curve method** is among the most accurate, as it relies on comparison with a series of standards of known concentrations. **The standard addition method** is faster and simpler, since it uses only one known standard, but it is generally less precise.

2 Methods of determining the concentration of a compound in solution

As part of the practical spectrophotometry exercises, you will determine the unknown concentration of a substance in a solution/sample. Some of the procedures are demonstrated in the following chapters.

2.1 Calibration curve

This method involves constructing a curve based on the absorbance of a series of standard samples with known concentrations. When measuring the standards, it is necessary to verify the validity of the Lambert–Beer law, which assumes a linear relationship between absorbance and concentration (hence the data points are fitted with a line). The obtained values are plotted in a graph, with concentration on the x-axis and absorbance on the y-axis.

Linear equation of the calibration curve:

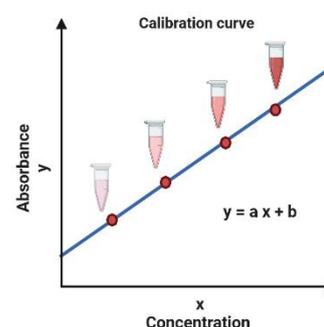
$$y = a \cdot x + b$$

y.... absorbance (plotted on the vertical axis)

x concentration (plotted on the horizontal axis)

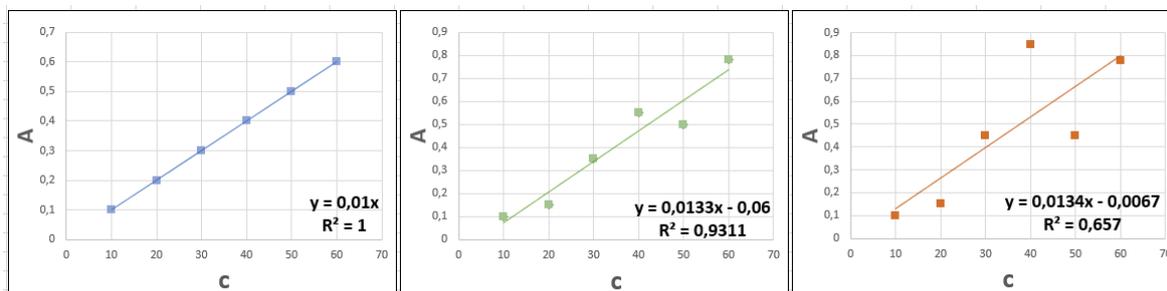
a slope of the line

by-intercept -absorbance value when the concentration is zero



Note: The calibration curve does not always pass through zero on the X or Y axis.

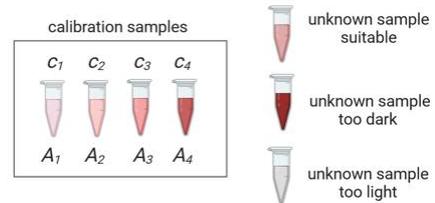
The linearity of a calibration curve is often gauged by its correlation coefficient, represented by the R² value. A R² value approaching 1 signifies a strong linear correlation (as seen in the blue plot). It's important to note that due to occasional experimental inaccuracies, the best-fit line might not pass directly through every data point. For an ideal curve, there should be an even spread of data points on both sides of this line, and a R² exceeding 0.9 is considered acceptable (as depicted in the green plot). Dispersed or scattered plot points, such as those in the orange plot, can hint at errors like inconsistent pipetting techniques. Thus, a minimum accepted R² value is typically set at 0.9.



Examples of calibration curves: If the data points align closely with the trendline, the R value would be close to 1, indicating a strong positive linear relationship between concentration and absorbance (blue plot). In the green plot the R square value was determined of 0.93. This suggests that 93% of the variability in absorbance is explained by the concentration. It indicates a strong positive linear relationship and validates the reliability of your calibration curve. In the orange plot R square value is 0.65, it would imply a weaker linear relationship, and the calibration curve might not be a reliable tool for determining concentrations due to improper liquid handling.

When applying the linear equation of a calibration curve, the absorbance (y) of an unknown sample can be used to calculate its concentration (x). However, certain pitfalls must be considered.

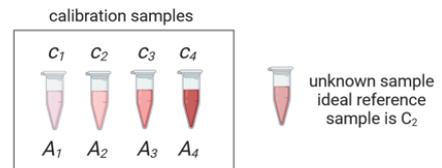
- If the sample’s absorbance exceeds the highest value on the curve, it may fall outside the linear detection range of the reagents or the spectrophotometer. In such cases, diluting the sample is recommended.
- Conversely, if the absorbance is lower than the starting point of the curve, there is a risk of interference from other components in the sample.



Under these circumstances, a more sensitive analytical method may be required (more details can be found in the study material *“Optical Analytical Methods with a Focus on Spectrophotometry”*).

2.2 Standard sample method

This method is quick and practical for routine use, but it is less accurate than working with a full calibration curve because it assumes perfect linearity of the signal within the given measurement range. In clinical biochemistry, however, it is one of the main approaches for determining an unknown concentration. Alongside the sample with an unknown concentration (c_x), a standard sample (c₁) with a known concentration is measured. The concentration of the unknown sample is then calculated from the absorbances of both samples and the known concentration according to the equation:



$$(c_1) : (A_1) = (c_2) : (A_2)$$

The concentration of the standard is chosen so that it lies in the middle of the calibration line (see figure).

? **Question:** Consider what advantages and disadvantages might arise from determining concentration using a calibration curve versus using a standard sample.

What is the concentration of a substance if the measured absorbance of our sample is A = 0.286 and our standard has a concentration of c = 5 mmol/L with an absorbance of A = 0.364?

3 Spectrophotometry in Practice

Although spectrophotometry is a very accurate and reliable method, errors can still occur. These may result from improper calibration, incorrect handling of the sample, or the presence of interferences in the sample. It is therefore important to understand the principles of correct evaluation and potential sources of measurement error.

Instrument calibration:

For the spectrophotometer to provide accurate values, it must be calibrated regularly. Calibration is performed using standard solutions of known concentration. Without proper calibration, results may show systematic errors, leading to underestimation or overestimation of the analyte concentration.

Blank sample:

Every proper measurement includes a so-called blank sample. This contains all components of the reaction mixture except for the analyte being determined. It serves to zero the absorbance of the solvent, presence of cuvette or reagents so that the result reflects only the signal from the analyte. Using a blank thus eliminates the influence of background and reagents on the final outcome.

Linear range:

The Lambert–Beer law applies only within a certain concentration interval where the relationship between analyte concentration and absorbance is linear. At very low concentrations, absorbance values approach zero and measurement accuracy is easily lost. Conversely, at high concentrations, almost no light passes through the cuvette, and the instrument can no longer correctly evaluate absorbance. Therefore, the optimal range is considered to be 0.1–1.0 absorbance. For concentrated samples, suitable dilution is necessary to ensure measurements fall within the linear range.

Interferences:

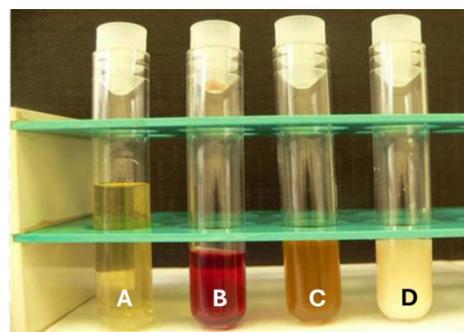
Various interferences, caused by substances that absorb light at similar wavelengths or scatter the light beam, can also be significant sources of error. For comparison, see the illustration showing different interferences that affect the measurement, alongside **normal serum (A)**.

Haemolysis (B) – the release of haemoglobin from red blood cells markedly increases absorbance and may distort results.

Icterus (C) – bilirubin present in serum absorbs light in the UV/VIS range and affects the outcomes of many biochemical tests.

Lipemia (D) – high lipid content causes serum turbidity and light scattering, artificially increasing absorbance.

Drugs and metabolites – some pharmaceuticals or their products have their own absorption maxima, or may react with reagents, leading to false results.



(Source: <https://www.vovcr.cz/odz/zdrav/189/page09.html>)

3.1 Example Calculation 1

Principle of Glucose Determination

In biochemistry, the determination of glucose is often based on an enzymatic reaction that produces a colored product. The most common approach uses a combination of enzymes:

1. **Glucose oxidase** catalyzes the conversion of glucose into gluconolactone with the simultaneous formation of hydrogen peroxide (H₂O₂).
2. **Peroxidase** uses H₂O₂ to oxidize a chromogenic substrate (e.g., o-dianisidine, ABTS, or POD-dye), resulting in a colored product.

The intensity of the color (measured spectrophotometrically) is directly proportional to the glucose concentration in the sample.

Procedure

1. **Preparation of the standard**
 - Prepare a glucose solution of known concentration, e.g., 5 mmol/L.
 - Measure its absorbance after performing the enzymatic reaction (e.g., at $\lambda = 505$ nm).
2. **Measurement of the unknown sample**
 - Perform the same reaction with the patient's serum.
 - Measure the absorbance of the product.
3. **Calculation of concentration**
 - If only one standard is used, apply the relationship:

$$\frac{c_1}{A_1} = \frac{c_2}{A_2}$$

where c_1 , A_1 are the concentration and absorbance of the standard, and c_2 , A_2 are the corresponding values of the sample.

- If a calibration curve is available, the sample concentration can be read directly from the graph (absorbance vs. concentration) or calculated precisely using the regression equation.

Example Calculation

- Glucose standard: $c_1 = 5$ mmol/L, $A_1 = 0.364$
- Patient sample: $A_2 = 0.286$

Calculation as follow:

$$\frac{5}{0,364} = \frac{c_2}{0,286}$$
$$c_2 = \frac{5 \times 0,286}{0,364} = 3,93 \text{ mmol/l}$$

Interpretation

- Measured glucose concentration = **3.9 mmol/L**.
- This value is at the lower end of the physiological fasting range (\approx 3.9–5.6 mmol/L).
- The result may indicate **hypoglycemia**, but clinical context must always be considered for confirmation.

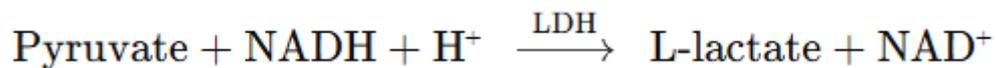
3.2 Example Calculation 2

Principle of Enzyme Activity Determination Using NADH/NAD⁺ (Warburg Optical Test)

In the previous section, we introduced two basic approaches for determining an unknown concentration. The same principle applies to enzymes – with one key difference: instead of a single concentration value, we are interested in **how fast the concentration changes over time**. In other words, Lambert–Beer’s law is applied **dynamically**.

Principle of Lactate Dehydrogenase (LDH) Determination

LDH catalyzes the conversion of **pyruvate** → **lactate**, with **NADH** as the cofactor:



- **NADH** absorbs light at **340 nm**, while **NAD⁺** does not.
- As the reaction progresses, **absorbance at 340 nm decreases**.
- If a calibration curve for NADH is prepared, the decrease in absorbance over time can be converted into the corresponding decrease in NADH concentration. This decrease is directly proportional to the **enzyme activity**.

Example Procedure Using a Calibration Curve

1. Calibration (linearity check)

- Prepare a series of NADH standard solutions of known concentration (e.g., 0.05–0.5 mmol/L).
- Measure their absorbance at 340 nm.
- Plot the calibration curve (**Absorbance vs. concentration**).
- Verify linearity ($R^2 > 0.99$).

2. Sample measurement

- Add patient serum (containing LDH) to the reaction mixture (pyruvate, buffer, NADH).
- Record the decrease in absorbance over time (e.g., every 30 seconds for 2–3 minutes).

3. Calculation of NADH concentration

- For each absorbance value, determine the corresponding NADH concentration using the calibration curve.
- Create a data table: **time (min) vs. NADH concentration (mmol/L)**.

Summary

The goal of this chapter is to show how to calculate an unknown concentration from a measurable signal (for example, absorbance). In practice, we use two approaches: a **calibration curve** (the most accurate, based on a series of standards) or comparison with a **single standard** (quick, but less precise and reliable only within a verified linear range). The reliability of measurement depends on correct instrument **calibration**, use of a **blank**, working within the **linear range** (typically $A \approx 0.1\text{--}1.0$), and checking for **interferences** (hemolysis, icterus, lipemia, drugs). These principles are not limited to spectrophotometry but apply to any method where the signal is proportional to concentration.

Control Questions

1. What is the Lambert–Beer law, and what does each term represent?
2. What is the difference between a calibration curve and the standard sample method?
3. How is the linearity of a calibration curve evaluated?
4. Why is it sometimes necessary to dilute the unknown sample before measurement?
5. What are the advantages and disadvantages of the standard sample method?
6. Which factors can lead to a violation of the Lambert–Beer law?
7. What are the clinical applications of the Lambert–Beer law?