Uv visible spectrophotometry and solution absorption

ASSIGN
BUSTER

All molecules absorb light at certain wavelengths. The absorption of light by a solution may be used to determine the concentration of a solute or a mixture of solutes in solution.

The Beer-Lambert law refers to the linear relationship between absorbance (A), and concentration (C) of an absorbing species.

According to the two fundamental principals that govern the absorption of light by a solution, the absorption of light passing through a solution is exponentially related to the number of molecules of the absorbing solute, and thus the solute concentration, and the length of the absorbing solution.

These principals are combined, and when working in concentration units of molarity, the Beer-Lambert law is as follows:

For part A of this experiment the Îµ value at the ï 🚺 ¬max for Vitamin B12 was determined by measuring the absorbance of a known concentration of Vitamin B12 and by using the above Beer-Lambert formula. Vitamin B12 is a compound of significant nutritional and clinical importance. Assaying and understanding absorption of vitamin B12 helps with diagnosis of defects in humans that can lead to hematological and neurological complications.

For part B of this experiment chlorophyll concentration of a leaf extract was calculated. In context to the experiment, eukaryotic green plants and algae, and prokaryotic cyanobacteria contain chloroplasts which have several pigment types, the most abundant of these being chlorophyll a.

Green and blue-green coloured chlorophyll a absorbs maximum light energy at the photosynthetic reaction centre (during the light reaction of

photosynthesis) at wavelengths in the blue (ï ¬max 420 nm) and red (ï ¬max 663 nm) regions of the visible spectrum.

The green-yellow coloured chlorophyll b is also present in all green plants and has an absorption spectrum (red ï ¬max 645 nm and blue ï ¬max 435 nm) slightly different from chlorophyll a.

Normally the ratio of chlorophyll a: b is 3: 1. As with most biological molecules chlorophyll is synthesised by biochemical pathways, and one intermediate molecule in the synthesis pathway is protochlorophyllide (ï ¬max 626 nm) which is eventually converted into chlorophylls a and b.

The amounts of chlorophyll and other pigments in plants can be determined using a spectrophotometer following extraction with various organic solvents.

Based on the Beer-Lambert Law and a knowledge of absorption coefficients of pigments dissolved in particular solvents, equations have been derived to directly determine the concentrations of common pigments following extraction by measurement of the absorbance (A) of the solution at a given wavelength (ï ¬max) in a cuvette.

For part 3 of the experiment, protein concentration was determined by use of UV and Visible spectrophotometry, and Construction of a Standard Graph.

The estimation of protein concentration is an important measurement in biological sciences. For pure samples of proteins absorbance measurements at 280 nm can be used to directly determine protein concentration; all

proteins absorb in this region of the spectrum due to their aromatic amino acid residues (tyrosine, tryptophan and phenylalanine).

For protein mixtures, very dilute solutions, or for proteins with interfering chromophores, colourimetric methods must be used. These involve subjecting a pure protein standard of known concentration to a colourimetric reaction, and measuring the absorbance of the coloured end product. The sample protein of unknown concentration is subject to the same colourimetric reaction. The concentration of the sample protein can be read directly from a standard curve.

The Lowry assay involves the production of a blue (phosphomolybdate-tungstate) chromophore, from a copper-protein complex.

In this part of the practical, Lowry and direct absorbance methods were compared for the determination of the concentration of lysozyme in solution. The first of the methods makes use of a Î» max in the UV part of the spectrum and the other in the visible part of the spectrum.

Aims

- To competently use a spectrophotometer and accociated cuvettes (cells)
- To relate absorbance of a solution to concentration using the Beer-Lambert law
- To determine the molar absorption (extinction) coefficient of vitamin B12 and compare its value with that from a standard reference table.

- To calculate the chlorophyll concentration in a leaf extract using absorbance values at defined wavelengths and a formula applicable to the solvent extraction medium.
- To measure protein concentration using direct absorbance and, following construction of a calibration curve, by a colourimetric method.

Methods

Part A

To begin the experiment, the spectrophotomer was calibrated in accordance to the information given in the instrumentation booklet (p. 35, viii). Using distilled water in a plastic cuvette at a wavelength of 550 nm the spectrometer was then placed on zero.

Using the provided Aqueous Vitamin B12 (cyanocobalamin) solution at a stock concentration of 0. 15 g dm-3 (relative molecular mass = 1. 355 x 103 i. e. 1, 355 Daltons), The A value was measured and recorded at Î» max at 550 nm. The A value was Placed on the results sheet.

The vitamin B12 solution concentration was converted from g dm -3 to mol dm-3 and then using this data the Îµ value for Vitamin B12 was calculated (see calculations).

Part B

For the second part of the experiment a sample of pigments extracted from dandelion leaves homogenized in an aqueous acetone extraction medium (80%) was provided. A clear pigment solution was needed for the test and so

a check was carried out to ensure that there was no plant debris that may have interfered with light passage before the absorbance of the sample was measured.

Using a Pasteur pipette, the clear extract was transferred into a clean quartz cuvette. The spectrophotometer was placed on zero using a quartz cuvette filled with an aqueous acetone mixture (80%) set at a ï ¬max wavelength of 663 nm and the absorbance of the pigment solution was measured at 663 nm.

The spectrophotometer was again placed on zero using the acetone solution (80%), however it was set at a ï ¬max wavelength of 645 nm before the absorbance of the pigment solution was measured.

The spectrometer was placed on zero for a third time and set at ï ¬max wavelength of 626 nm. The absorbance of the pigment solution was again measured and all three sets of data were recorded.

Part C

(a) Direct absorbance

A quartz cuvette was filled to the level with H20 and used as a standard to set the spectrophotometer at zero. Using another quartz cuvette the A value of the lysozyme solution of “ unknown” concentration was measured at a Î» max of 280 nm. The value obtained was recorded.

Having measured the A280 value of the “ unknown” lysozyme sample, the concentration of lysozyme was calculated taking into consideration that Îµ280 of lysozyme = 3. 65 x 104 dm3 mol-1 cm-1 and using the Beer-

Lambert Law. The concentration of the lysozyme sample was then changed from mol dm-3 to ï gcm-3.

(b) Colourimetric Lowry Assay (Preparation and Use of a Standard Curve)

Using a stock reference standard BSA solution containing 250 ï g cm-3 protein, a series of dilutions of the stock were prepared accurately, as per the table below:

Tube No:

1

2

3

4

5

6

7

8

BSA stock

(cm3)

1. 0

1. 5

2. 0

2. 5

3. 0

3. 5

4. 0

5. 0

H_2O (cm^3)

4. 0

3. 5

3. 0

2. 5

2. 0

1. 5

1. 0

0. 0

Note that the dilution factors for each tube were used to enable calculations for final concentrations of BSA in tubes 1- 8 inclusive (see calculations). These values are then used to plot a standard curve.

Standard solution (1. 0 cm3) prepared in the above table was placed in 8 clean, dry test tubes. “ unknown” lysozome sample (1cm3) was placed into test tube 9, and H2O (1. 0 cm3) was placed in test tube 10 as a water/reagent blank control.

A solution of “ Lowry C” (alkaline copper reagent) was made up by mixing “ Lowry B1” (0. 5 cm3) with “ Lowry B2” (0. 5 cm3) and “ lowry A” (50 cm3). A solution of “ lowry D” (Folin & Ciocalteu’s phenol reagent) was then made up by diluting Folin reagent (5 cm3) with distilled H2O (10 cm3).

Lowry C reagent (5. 0 cm3) was added to all ten test tubes. The solution was mixed and left for 10 minutes. Lowry D reagent (1. 0 cm3) was then added to each test tube and mixed well. All tubes were left for 30 minutes at standard temperature (37oc) for reaction and colour development to occur, after which time the test tube contents were thoroughly mixed.

For test tubes 1-9, the A value at ï🚺¬max 750 nm was measured. Test tube 10 was not measured as it was used as a H2O/reagent blank to zero the spectrophotometer.

Calculations

Part A

Due to the fact that a known amount of solute has to be dissolved in a given volume of solvent to obtain a solution of the required concentration, the number of moles of the solid can be calculated from the following equation:

n = Mass of solute

Relative molecular mass

To convert the Aqueous Vitamin B12 (cyanocobalamin) solution from g dm -3 to mol dm-3 one must consider that the stock concentration is 0. 15 g dm-3, and the relative molecular mass of Vitamin B12 is 1. 355 x 103 .

0. 15 / 1. 355 x 103 = 0. 11 x 103

To find the Îµ value (wavelength absorption coefficient) of vitamin B12 the Beer-Lambert law must be applied:

A= 0. 827

L= 1cm

C= 0. 11 x 103 dm3 mol-1 cm-1 at Î» max of 550 nm

Îµ = “ unknown”

As A= ÎµLC, the equation can be rearranged as follows to make Îµ the subject:

Îµ = A/C

Therefore:

Îµ = 0. 827/0. 11 x 103 = 7. 51 x 103

Part B

Chlorophyll Concentration determination

The following formula was used to calculate the concentration of pigment in the extract.

Chlorophyll a = 12. 67A663 – 2. 65A645 – 0. 29A626

Chlorophyll b = 23. 6A645 – 4. 23A663 – 0. 33A626

Protochlorophyllide = 29. 6A626 – 3. 99A663 – 6. 76A645

The absorbance (A) is the respective wavelengths obtained directly from the spectrophotometer with the use of a 1cm light path length cuvette.

Chlorophyll a = (12. 67 x 0. 934) – (2. 65 x 0. 390) – (0. 29 x 0. 321) = 10. 71 ug cm-3.

Chlorophyll b = (23. 6 x 0. 934) – (4. 23 x 0. 390) – (0. 33 x 0. 321) = 20. 29 ug cm-3.

Protochlorophyllide = (29. 6 x 0. 934) – (3. 99 x 0. 390) – (6. 76 x 0. 321) = 23. 92 ug cm-3.

Part C

(a) Direct absorbance

Concentration of lysozyme was calculated using the Beer-Lambert law as follows:

A = 0. 177

Îµ = 3. 65 x 104 dm3 mol-1 cm -1

L = 1cm

C = “ Unknown”

The Beer-Lambert law can be rearranged, making C the subject of the equation. Therefore the value of C can be calculated as:

C = A / Îµ

C = 0. 177 / 3. 65 x 104 = 4. 84 x 10-6 mol dm-3

The concentration of the lysozyme sample was then changed from mol dm-3 to ï gcm-3

Using the following formula:

n= M / RMM

14. 31 x 103 x 4. 84 x 10-6 = 0. 069g

To change this from g to ï g it must be multiplied by 1000, 000 as follows:

0. 069 x 1000, 000 = 69000

To then change this calculation from dm-3 to cm-3 it must be divided by 1000 as follows:

69000 / 1000 = 69 ï g cm-3

(b). Preparation and Use of a Standard Curve for Lowry Assay:

Concentration (ï g cm-3) was calculated using the below figures:

Tube No:

1

2

3

4

5

6

7

8

BSA stock

(cm3)

1. 0

1. 5

2. 0

2. 5

3. 0

3. 5

4. 0

5. 0

H_2O (cm^3)

4. 0

3. 5

3. 0

2. 5

2. 0

1. 5

1. 0

0. 0

Test tube 1.

BSA stock (cm3) = 1. 0

H_2O (cm3) = 4. 0

1. 0 + 4. 0 = 5

1. 0 / 5 = 0. 2

0. 2 x 250 = 50

Test tube 2.

BSA stock (cm3) = 1. 5

H_2O (cm3) = 3. 5

1. 5 + 3. 5 = 5

1. 5/5 = 0. 3

0. 3 x 250 = 75

Test tube 3.

BSA stock (cm3) = 2. 0

H_2O (cm3) = 3. 0

2. 0 + 3. 0 = 5

2. 0 / 5 = 0. 4

0. 4 x 250 = 100

Test tube 4.

BSA stock (cm3) = 2. 5

H2O (cm3) = 2. 5

2. 5 + 2. 5 = 5

2. 5 / 5 = 0. 5

0. 5 x 250 = 125

Test tube 5.

BSA stock (cm3) = 3. 0

H2O (cm3) = 2. 0

3. 0 + 2. 0 = 5

4. 0 / 5 = 0. 6

0. 6 x 250 = 150

Test tube 6.

BSA stock (cm3) = 3. 5

H2O (cm3) = 1. 5

3. 5 + 1. 5 = 5

3. 5 / 5 = 0. 7

0. 7 x 250 = 175

Test tube 7.

BSA stock (cm3) = 4. 0

H2O (cm3) = 1. 0

4. 0 + 1. 0 = 5

5. 0 / 5 = 0. 8

0. 8 x 250 = 200

Test tube 8.

BSA stock (cm3) = 5. 0

H2O (cm3) = 0. 0

5. 0 + 0. 0 = 5

5. 0 / 5 = 1

1 x 250 = 250

Results

1. Molar absorption coefficient of vitamin B12:

Absorbance reading at Î» max of 550nm (A550nm) 0. 827

2. Absorption

Pigment type

A Value

Absorption

Concentration

Chlorophyll a

A663nm

0. 934

10. 71

Chlorophyll b

A645nm

0. 390

20. 29

Protochlorophyllide

A626nm

0. 321

23. 92

Fig. 1. a table showing the A value of three different pigment types found in a leaf extraction, and the measured absorbance and calculated concentration of each.

3. Lysozyme Concentration Determination:

(a) Direct absorbance reading at Î» max of 280 nm (A280nm) 0. 177

(b). Preparation and Use of a Standard Curve for Lowry Assay:

Discussion

Part A

A). Using the data collected, the experimental the Îµ value (dm3 mol-1 cm-1) calculated during this experiment was compared to that of an Îµ value obtained from commercial standard references data.

Î»(nm)

Îµ(dm3 mol-1 cm-1)

Standard Îµ value

550nm

8. 55 x 103

Experimental Îµ value

550nm

7. 51 x 103

Fig. 4. a table to show a comparison between standard and experimental Îµ values.

As the above data indicates, the experimental Îµ value obtained during this experiment differs from that of the standard Îµ value.

B). Other than human and experimental errors, one possibility that could explain the differences in the Îµ values is that to a certain degree different spectrophotometers in the laboratory give different readings. If the standard Îµ value was recorded using a different spectrophotometer this could cause anomalies within the results.

A second possibility is that the solution used to find the experimental Îµ value was not at a stock concentration of exactly 0. 15 g. This would affect the calculations and hence a different result would be obtained to that of the standard Îµ value.

Part B

1(a). The % of chlorophyll a, chlorophyll b and protochlorophyllide in the leaf extract can be calculated in the following way:

chlorophyll a concentration = 10. 71 ug cm-3

chlorophyll b concentration = 20. 29 ug cm-3

protochlorophyllide concentration = 23. 92 ug cm-3

10. 71 + 20. 29 + 23. 92 = 54. 92

Percentage of chlorophyll a = 10. 71/54. 92 x 100 = 19. 5%

Percentage of chlorophyll b = 20. 29/54. 92 x 100 = 36. 9%

Percentage of protochlorophyllide = 23. 92/54. 92 x100 = 43. 6%

1(b). It can be seen from the above percentages that 43. 6% of the leaf extract is composed of protochlorophyllide. This is the largest percentage present within the leaf extract and therefore it can be considered as the predominant pigment type.

2(a). The percentages obtained can also be used to calculate the ratio of chlorophyll a: b in the leaf extract. In the case of this experiment the ratio of chlorophyll a: b in the leaf extract was 2: 1.

2(b). The determined value to be expected when calculating the ratio of chlorophyll a: b in the leaf extract was 3: 1. One would expect this as there are three pigments in the leaf extract, that ideally should contribute evenly. However, The results from this experiment vary from the standard data as they show a ratio of 2: 1 between chlorophyll a and b.

The most probable reason for this variation is not due to anomalies in results or calculations, but the fact that not all leaf extracts will contain the standard amount of pigments. In some cases pigments may be present in a higher percentage of one than the other, as is this experiment where protochlorophyllide was the predominant pigment type.

3). Although the above ratio is only dealing with percentages of chlorophyll a and b present in the leaf extract, one must consider that the presence of protochlorophyllide must be allowed for in the formulae and hence in the calculations. This is because there are three pigment types involved in the

leaf extract and so one must consider that the ratio of the whole leaf extract is actually 3: 2: 1 where the ratio of chlorophyll a: b is 2: 1.

4). The use of a formula related to a given extraction solvent is a convenient method for determining the concentration of chlorophyll. However, making use of the Beer-Lambert Law the concentration of chlorophyll could also be found by preparation and use of a standard curve.

Part C

Determination of Protein Concentration by UV and Visible Spectrophotometry, & Construction of a Standard Graph

Proteins have aromatic side chains such as tryptophan tyrosine and phenylalanine which absorb light at 280nm.

The Lowry method is based upon a combination of the biuret method and the oxidation of tyrosine and tryptophan residues.

The biuret reaction involves the binding of Cu^{2+} under alkaline conditions to nitrogen found in the peptide bonds of proteins. This reaction gives off a deep blue colour. The folin reagent contains phosphomolybdotungstate acids which are reduced to tyrosine, tryptophan and polar amino acids. This creates an intense blue-green colour.

(a). The data collected was used to create a graph, plotting a standard curve of A (Y axis) against BSA concentration (X axis) in ï 🚺 g cm-3 (See fig. 3). This graph, shown in fig. 3., was effectively constructed assuming that the relationship between absorbance (A) and concentration (C) must be linear to satisfy the Beer-Lambert law. However, the Beer-Lambert relationship

between absorption and concentration deviates from lineariy in the case of more concentrated solutions.

Linear BSA standard curves are only obtained at low protein concentration and so therefore to decrease possible anomilies in the results, timing of both residue addition and mixing were crucial.

Using the A value from test tube 9, it was possible to use the graph to determine the concentration of the “ unknown” lysozyme sample in ï g cm-3. Results from the graph show that….

(b) By examining the lysozyme concentration results obtained (in ï g cm-3), it is possible to make a comparison between the results for the colourimetric assay and the direct absorption technique.

Results show that Lysozyme concentration for colormetric assay were ——– (m/rmm thing)

Lysozyme concentration results for direct absorbance technique were.. (graph)

These results are same/different.

Due to the fact that different proteins have widely varying characteristics, there may be considerable errors within the data. With the colormetric assay any non-protein component of the solution that absorbs UV light could interfere with the assay, resulting in the production of colour by substances other than the analyte of interest. This would cause the results to vary from that of the direct absorbance technique.

(c). For this experiment three different methods were used for concentration determination, each of which had different strengths and weaknesses with respect to their sensitivity, accuracy and convenience.

The first of these methods was the use of a formula, to determine chlorophyll concentration.

Using a formula gives a very accurate theoretical result but it is not particularly convenient as for calculations to be correct it can take a great deal of time and effort. Obviously with such calculations, they are not sensitive as there is no outside interference to affect results.

Direct absorbance is not as sensitive as the colormetric method, but as it requires the use of a spectrophotometer, it is an accurate assay method. This also makes the method relatively convenient for determining the concentration of lysozyme present in a given solution as changes in absorbance of the lysozyme could be clearly seen and recorded using the spectrophotometer at a particular wavelength.

The colourimetric method was also used to determine the concentration of lysozyme during this experiment. One benefit of using the Colormetric method is that it is extremely sensitive (down to a protein content of 20ug ml-1) and it is also moderately constant from one protein to another. However, with respect to accuracy, this method is subject to interference from a wide range of non-protein substances including many organic buffers. The choice of an appropriate standard is important as the intesnsity of colour produced for a particular protein is dependant on the number of aromatic proteins.

As different proteins have a different number of aromatic residues, the Lowry assay is considered more of a qualitative measure of protein content more than quantitative method of determining protein concentraion.

This method is not as convenient as the direct absorbance method in that it takes a lot longer to perform and there is a higher frequency of anomalies that must be accounted for.

(d). The measurement of protein levels is of significant diagnostic importance in both clinical and veterinary medicine. In clinical medicine there are a wide variety of biomedical tests involving the measurement of protein levels, such as the detection of abnormal protein levels in cerebrospinal fluid (CSF), suggesting that there is an abnormal process occurring in the central nervous system.

Protein levels in urine samples are tested to monitor and evaluate kidney function, and essentially to detect and diagnose kidney damage and disease at and early stage. Serum protein tests are also important as they concern measurement of protein levels of albumin and globulin in the blood.

Such tests are also important in veterinary medicine. According to reports from Cornell universities college of veterinary medicine, protein tests have been developed to accurately indicate canine liver failure caused by the toxin aflatoxin.

(e). Another way in which protein concentration can be measured, other than by the use of a formula or a spectrophotometer is gel electrophoreses. This

technique uses charged protein molecules to separate physical properties, as they are forced through a gel by an electrical current.