**Absorbance spectroscopy**

The theory of quantitative spectroscopy is based upon Beer’s law which relates to the amount of radiation which is removed from a beam of radiation when it passes through a solution containing an absorbing species. The amount which is removed is termed the amount absorbed and is measured in terms of absorbance.

The process may be illustrated as:

Incident beam of radiation beam of transmitted radiation

Where ϵ is a constant related to the absorbing species

l is the path length of the cell containing the species

c is the concentration of the absorbing species

Given that ϵ and l remain constant throughout the analysis:

**Absorbance** is proportional to **concentration**.

It is because of this that we can perform quantitative analyses using absorbance spectroscopy. But… how can we do this?

**Calibration**

A key concept in analytical chemistry is calibration. All instruments give a response that changes depending on the concentration of the analyte (compound being analysed) in the sample. The problem is – how do we know which concentrations correspond to which response? As a result we must calibrate the instrument. This is performed by taking known concentrations of analyte and measuring the instrument response for each concentration. We can then plot these as a graph, with response on the y axis, and concentration on the x axis.

Absorbance

Compound concentration in mg/dm3

**Experiment 1 – Calculation of sunset yellow FCF concentration in Irn-Bru.**

Sunset Yellow FCF is a flavouring used in Irn-Bru that has a yellow-orange colour. The distinctive colouring allows us to measure sunset yellow in solution using absorbance spectroscopy. Firstly, we need to construct our calibration standards. Make up four solutions according to the table below.

|  |  |
| --- | --- |
| mL of SY | mL of water |
| 0.4 | 1.6 |
| 0.8 | 1.2 |
| 1.2 | 0.8 |
| 1.6 | 0.4 |

Now, take some Irn-Bru and pour into a beaker. Then place this into an ultrasonic bath for 5 minutes, to remove the bubbles. Pipette this degassed solution into a new sample vial and take this, along with the solutions you have already made to the lego-spectrophotometer.

First we’ll need to run a blank solution. Place water into spectrophotometer and record the absorbance. Next, analyse your remaining solutions and record absorbance for each one. Plot these values in the table below.

|  |  |
| --- | --- |
| Sample | Response |
| Water |  |
| STD 1 |  |
| STD 2 |  |
| STD 3 |  |
| STD 4 |  |
| Irn-Bru |  |

Questions

1.

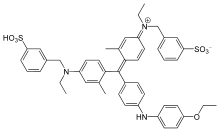
2.

3.

**Experiment 2 – Use of coomassie brilliant blue dye for protein quantification in milk**

Milk is one of the most widely consumed products in the world today. It consists mostly of water, and some additional constituents: carbohydrate, lipid, and protein. In this experiment we’re going to analyse protein content.

Proteins are biopolymers made up of long chains of amino acids that typically absorb in the UV range – around 280 nm; far below the range of our white light spectrophotometer. We do however; have a trick up our sleeves!



We’re going to make use of coomassie brilliant blue dye to stain the protein, shifting the absorbance of the compounds from 280 nm (undetectable) to 495 nm (detectable). This will then allow us to quantify protein in the same manner as experiment 1. Prepare solutions in accordance to the table below.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sample | Amount of CBB | Albumin | Water | Milk |
| Blank | 0.2 | 0 | 1.8 | 0 |
| Standard 1 | 0.2 | 0.2 | 1.6 | 0 |
| Standard 2 | 0.2 | 0.4 | 1.4 | 0 |
| Standard 3 | 0.2 | 0.6 | 1.2 | 0 |
| Standard 4 | 0.2 | 0.8 | 1 | 0 |
| Standard 5 | 0.2 | 1 | 0.8 | 0 |
| Milk | 0.2 | 0 | 0 | 1.8 |

Now these solutions are made up, leave them to stand for 10 minutes – this allows the reaction to take place. Then take them to the lego-spectrophotometer and analyse them…

Questions

1.

2.

3.

**Experiment 3 – Monitoring the kinetics of alkaline phosphatase and para-nitrophenylphosphate reactions**

Enzymes are the hidden heroes of our body’s systems. They facilitate reactions between chemical compounds that are in our body, and are implicated in all aspects of our biochemistry. The kinetics of enzyme reactions is hugely important for predicting the breakdown of products, and products could be anything! For instance, the study of enzyme mediated breakdown of drugs is important for ensuring a toxic level of drug is not reached in the body.

Here we’re going to be examining alkaline phosphatase – an enzyme responsible for dephosphorylation of various compounds in an alkaline environment. We’re going to study its kinetics by the use of the compound para-Nitrophenylphosphate (pNPP). This compound is undergoes a reaction, catalysed by alkaline phosphatase, to produce inorganic phosphate and another compound called para-nitrophenol (pNP). The good thing about pNP is that it’s yellow in colour, absorbing at 405 nm, and as such; we can analyse it! If we can monitor pNP over time we can see how alkaline phostphatase catalyses this reaction and the kinetics of it. This will give us a graph like the one below.

Absorbance of pNP

Time

For this experiment we’re going to be monitoring the formation of pNP. This means as soon as we make up our solution, we’re going to be analysing it. For that reason, we’ll make it up in the spectrophotometer. Place a 1 mL solution of alkaline phosphatase in the lego-spectrophotometer and start recording the absorbance. Now add 1 mL of pNPP and measure the change in absorbance over time.

We can now repeat this experiment at a different temperature – do you think this will change the kinetics?

Questions

1.

2.

3.