3.B.4 Using spectrophotometry to determine the concentration of a substance in a mixture.

Learning Objectives
* to calculate the concentration of a substance if A, ε and d are known.

* to calculate the concentration of a substance using a calibration line generated from a series of known concentrations.

There are two methods that can be used to determine the concentration of a substance in a mixture. Which one you use depends upon how accurate you wish to be.

The **first method** (see below) relies upon knowing ε and d and measuring A, then you can calculate C from A = εCd. This works well if you have a fairly pure solution and there is nothing else in there that may also absorb at the wavelength you have chosen.

A more accurate way is the **second method** (see below for an example). Here you prepare a series of known concentrations of the same molecule as you are trying to detect in your sample, in the same buffer solution (as close as possible anyway) as your sample. Then you measure the absorbance of those solutions alongside that of the sample with unknown concentration. You work out the equation for the line of known concentrations with their corresponding absorbances. Then you can plug in the value for the absorbance of your unknown sample and calculate its concentration.

First method.
We can get values of ε for a particular molecule from reference books with tables of the most commonly used molecules.

This is sometimes done with DNA solutions. Say you've extracted DNA from cheek cells and you want to know how much you've got.

For double-stranded DNA, ε = 20 $g^{-1}.cm^{-1}.L$.

If you measure the absorbance of your solution to be A = 0.089 when d = 1 cm, then

$$C = \frac{A}{\varepsilon d} = \frac{0.089}{20g^{-1}.L.cm^{-1} \times 1cm} = \frac{0.089}{20g^{-1}.L} = 0.00445 g.L^{-1} = 4.45 mg/L$$

Second method.
The second method is commonly used to work out the concentration of protein in a sample. Protein concentration can be measured using the blue-coloured dye called the Bradford reagent. In this experiment, a series of solutions are prepared with known concentrations of a protein such as bovine serum albumin. These solutions are then mixed with the Bradford reagent and a blue colour is formed. The intensity of the blue colour is proportional to the concentration of the albumin. Once we know how much "blue-ness" corresponds to which protein concentration, we can work out the protein concentration of our patient's urine sample.

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Let's look at some experimental results. We have used five tubes with different concentrations of albumin as shown in the table. Then we mixed them with the Bradford reagent and measured the absorbance at 595nm (A_{595} – 595nm is the most appropriate wavelength for this particular blue dye)

albumin standard (µg/ml)	A_{595}
10	0.124
20	0.149
50	0.225
100	0.346
200	0.609
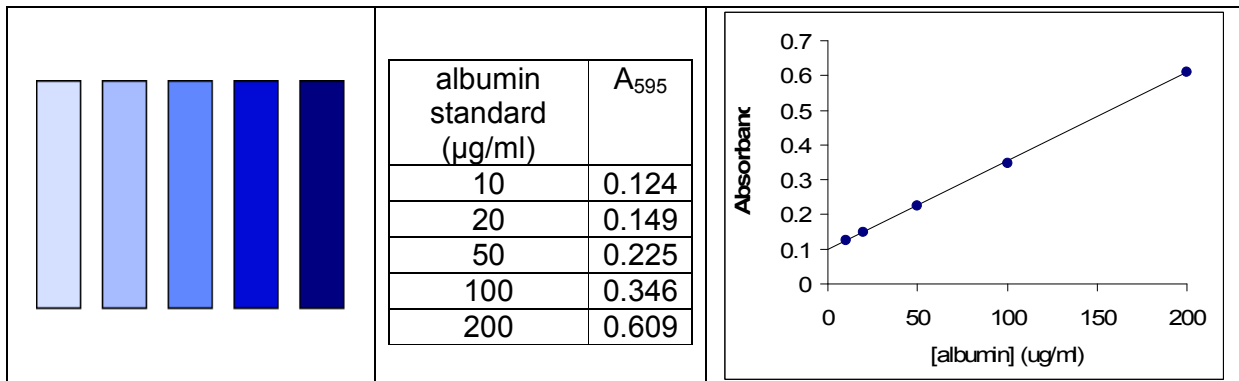
Plotting the data on a graph (sometimes called a calibration curve even though it is a straight line) … shows that the absorbance increases with albumin concentration in a straight line relationship.

However is doesn't go through the origin (0,0). This is because the Bradford Reagent has a little bit of blue colour even before the protein is added.

Some spectrophotometers have a "Zero" button which you press when you've put in the "Blank" sample (that is the one with no protein added), then the absorbance of that sample is subtracted from all the other samples making the line go through (0,0).

Alternatively, you can measure the absorbance of all of your samples including the zero albumin concentration sample, determine the equation for the line of A vs C. Then once you have that, you can put in your value for A of the unknown sample, and calculate the corresponding value for C.

the theory...	the example...
to get the equation for the line,	
choose two points on the line to get the slope	using $(x_1, y_1) = (100, 0.346)$ and $(x_2, y_2) = (200, 0.609)$ $$m = \frac{0.609 - 0.346}{200 - 100} = 0.00263 ml.\mu g^{-1}$$
then find the y-intercept c = y – mx	c = 0.346 – 0.00263 x 100 = 0.083
therefore the equation for the line is y = mx + c	the equation for the line is y = 0.00263x + 0.083 where x is given in µg/ml

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Starting with the equation for a straight line, rearrange the equation to **make x the subject of the equation**, and substitute in the values for m, c and y

y =0.421, c =0.083, m =0.00263

$$x = \frac{y-c}{m} = \frac{0.421 - 0.083}{0.00263} = 129\,\mu g.ml^{-1}$$

Therefore the protein concentration in the urine sample was 129 µg.ml^{-1}
(to three significant figures which is appropriate since the measurements were to three significant figures).