Royal Society of Chemistry
Analytical Division
East Anglia Region

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School of Chemistry
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General Background to the Exercises

This year, the analytical exercises are based around assay of drugs and medicines. The background information provided in this document will hopefully help you to understand the basis of the analytical methods you will be using. Do not worry if you do not fully understand all the details. This will not stop you from completing the exercises on the day of the competition.

Medicines are familiar to all of us as products obtained from pharmacies or hospitals to treat a wide range of medical conditions. In general, we just take it for granted that the composition of the medicine is correct and fit for purpose, but have you ever thought about how we know that this is the case? How is the quality of medicines defined and controlled to ensure that you receive a safe and efficacious product? In practice, a great deal of chemical analysis occurs at all stages of the process of manufacturing medicines. All the raw materials (both the drug compounds and the excipients – the other ingredients that go to make up the finished medicine) have to be tested to ensure that they meet the rigorous quality standards defined in the British Pharmacopoeia (B.P.). The stability of the formulated products has to be tested to ensure that they have an acceptable shelf life (this is what defines the expiry date of every batch of medicine). The final products also have to be tested for both composition and uniformity of composition (e.g. does every tablet in a batch have the same amount of active ingredient, within defined limits).

A vast array of different techniques is used in pharmaceutical analysis. In research, many of the methods use highly sophisticated (and very expensive!) equipment, but in assaying raw materials and finished medicines many simpler and much more traditional techniques are used, such as titrations and UV/VIS spectrophotometry (see below). If carried out correctly, these methods are simple, precise, accurate and robust and are particularly suited to situations where you know the approximate composition of your sample. The reason for this is that such methods are not always very selective, so it is necessary to be sure that no other compounds are present that might interfere with your assay method and hence invalidate the result. This is true for the citric acid and paracetamol tablet assays described below. When more complex samples need to be analysed, it is often necessary to separate the mixture into its component parts before quantitating the analyte of interest. This avoids the problem of interference from other species present, but is more complex and more demanding in terms of techniques and instrumentation. The assay of caffeine in coffee (or blood serum or urine) is a good example of this. Caffeine is a very weak base that is difficult to assay by aqueous titration. It could be quantitated by UV/VIS spectrophotometry, but not in the presence of all the other components of coffee (many of which are highly coloured) since these would contribute to the absorbance. Other related compounds (e.g. theobromine, the biological precursor to caffeine) may also be present, so these would also need to be separated before quantitation. This rather complex task can be easily achieved using high performance liquid chromatography (HPLC) to separate the components of the mixture, prior to quantitation of the analyte of interest (in this case the caffeine).

Brief Theoretical Background to the Techniques you will use in the Analytical Exercises

Exercise 1
Citric Acid Estimation by Acid-Base Titration

Titrimetric methods are widely used in pharmaceutical analyses, despite the development of a vast array of complex instrumental methods, because of their robustness, cheapness and capability for high precision and accuracy. Numerous standard pharmacopoeial assays of unformulated drugs (raw materials), excipients (the other ingredients added to turn the drug into a pharmaceutical product, such as diluents, binders, flavours, preservatives etc.) and some formulated drugs exploit titrimetric methods. Very many different methods of titration have been developed to measure a wide range of different analytes using e.g. acid/base, redox and complexiметric methods. A weakness of titration is that it is not always easy to apply to complex mixtures, as one component present sometimes interferes with the titration of another component.

An analyte is chemically reacted with a standard solution of a reagent of known concentration (either because you made it up accurately or because you **standardised** it beforehand). The amount required to completely react with all of the sample is used to estimate the amount of analyte present. This information is often used in pharmaceutical assays to calculate purity, and hence define whether or not a batch of product meets a required specification. Some of the most common titrations are based on aqueous acid-base reactions. Methods for determining when the all the analyte has been consumed include the observation of an indicator colour change and the detection of a sudden change in pH using a pH meter.

Acid base titration is probably a method that is familiar to you. It is a very good way of measuring the **concentration** of an acid or base, by titrating it against a base or acid of accurately known concentration The titration of citric acid is quite complex because it is a triprotic acid. This means that it can liberate 3 protons by stepwise dissociation.

Usually, with acids that are polyprotic, it is difficult to distinguish between the individual ionisations and only the overall process can be easily measured by titration (this is because the individual acid groups have similar pK_a values and hence their ionisation ranges overlap). In practice, this is not a problem, providing that the correct indicator is selected to ensure that all three acid groups have been completely titrated when the indicator end point is reached.

In this exercise you will titrate citric acid with sodium hydroxide solution using phenolphthalein as indicator (this is the official B.P. method of assay to determine the purity of citric acid). Sodium hydroxide is a very useful and very commonly used strong base for titration purposes, but it has one important drawback. It is not possible to make up standard solutions of accurately known concentration by gravimetric methods (i.e. by accurate weighing of the solid) due to two key problems:

- It is hygroscopic and hence picks up moisture from the air. This will depend on storage conditions and age of the reagent, and hence leads to uncertainty.
- It absorbs carbon dioxide from the air and reacts with it to form sodium hydrogen carbonate and sodium carbonate. This uses up some of the sodium hydroxide, leading to uncertainty of composition.

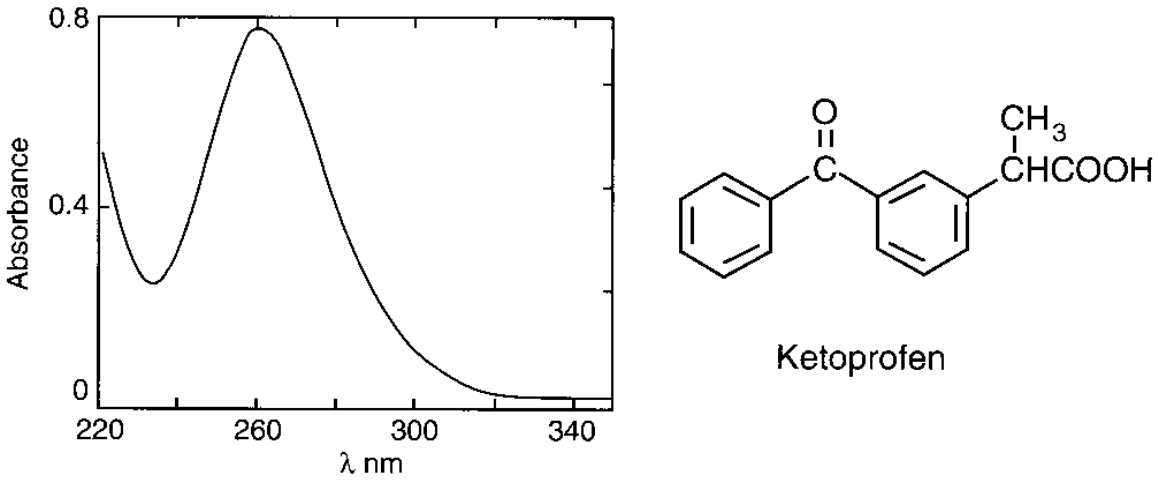
In an attempt to minimise these problems, sodium hydroxide is usually purchased in the form of pellets rather than powder or crystals, since this minimises the surface area available to react with carbon dioxide or pick up water, but even this is not sufficient to guarantee accuracy in titrations.

To overcome this limitation, a solution of sodium hydroxide is made up and **standardised** immediately before use by titration against an acidic compound which has a reliably known composition and can thus be used as a **primary standard** to make up solutions of accurately known concentration. The acid you will use in this exercise is potassium hydrogen phthalate. Once the concentration of the sodium hydroxide solution has been defined by titration against the primary standard, it can be used as a **secondary standard** to titrate other acids, such as the citric acid in this exercise.

Exercise 2

Quantitating Paracetamol in Paracetamol Tablets using UV-Visible Spectrophotometry

In this technique, radiation in the wavelength range 200-900 nm is passed through a solution of a compound. The electrons in the bonds within the molecule become excited so that they occupy a higher quantum state and in the process absorb some of the energy passing through the solution. The more loosely held the electrons are within the bonds of the molecule, the longer the wavelength (the lower the energy) of the radiation absorbed. The absorption spectrum of a compound typically contains one or several peaks at particular wavelengths. By scanning across a range of wavelengths this spectral profile can be recorded (see example below). The absorption spectrum is characteristic of the analyte being measured, but tends not to be very useful for identification purposes, due to the rather broad peaks and the great similarity of the spectra for groups of related compounds. Measurement is carried out in an instrument called a spectrophotometer. The sample is placed in a small (usually square section) cell called a cuvette, through which the beam of light is passed.



A typical UV absorption spectrum for a drug (Ketoprofen is an analgesic and an anti-inflamatory drug)

The measurement of light absorption by a solution of molecules at a specified wavelength is useful for analyte quantitation and is governed by the Beer-Lambert law as follows:

$$\log I_0/I_t = A = \varepsilon\, c.l$$

where I_0 is the intensity of incident radiation, I_t is the intensity of transmitted radiation, A is the absorbance and ε is a constant known as the molar extinction coefficient (at a defined wavelength), l is the pathlength of the cell in cm and c is the concentration of the analyte in moles.L^{-1}.

In pharmaceutical products, concentrations and amounts are usually expressed in g or mg rather than moles and thus the Beer-Lambert equation is written in the following form:

$$A = A\ (1\%,\ 1\ cm)\ c\ l$$

A is the measured absorbance, A (1%, 1 cm) is the absorbance of a 1% w/v (1 g/100 mL) solution in a 1 cm cell, l is the pathlength and c is the concentration of the sample in g/100 mL. Since measurements are usually made in a 1 cm cell, the equation can be written:

$$c = A / A\ (1\%,\ 1\ cm)$$

where c is the concentration of the analyte expressed as a % w/v (g/100 mL).

Analytical monographs in the British Pharmacopoeia often quote a standard A (1%, 1 cm) value for a drug at a specified wavelength, which is to be used in its quantitation. In this exercise you will be using absorption methods to check the amount of paracetamol present in commercial paracetamol tablets.

In this case we will not tell you what the A (1%, 1cm) value is, so you will have to determine it experimentally by making up a series of paracetamol standards of known

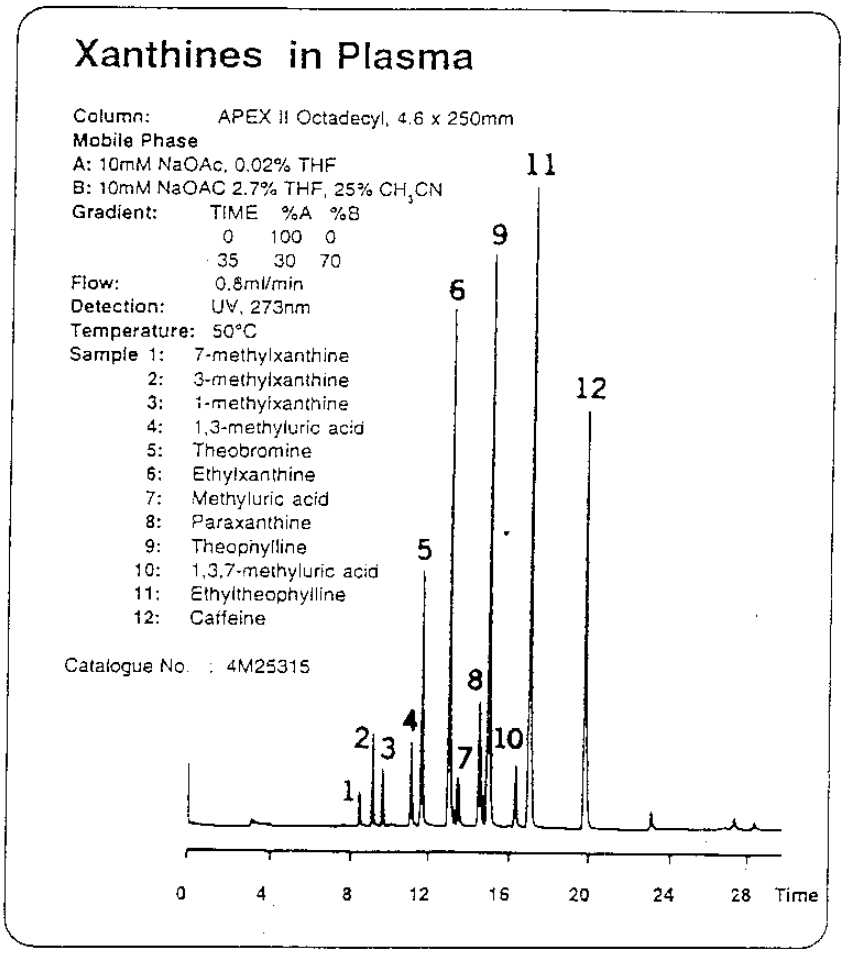
concentration and measuring the absorbance of each. By plotting a graph of absorbance against concentration (in g/100mL), the gradient will give the A (1%, 1cm) value, which can then be used to quantitate the paracetamol present in your tablet extracts, and hence what was present in the original tablets. Alternatively, you can use your calibration graph to read off the concentration of your unknown solution by interpolation.

Exercise 3
Caffeine Quantitation in Filter Coffee by HPLC Analysis

Chromatography is a generic term for a family of separation techniques including paper chromatography (which you might have done), thin-layer chromatography and the two main instrumental methods, gas chromatography (GC) and high performance liquid chromatography (HPLC). GC and HPLC are extremely powerful instrumental methods of analysis and are widely used in many analytical situations. This is because they can **separate** the various components present in complex mixtures (most analytical samples are complex mixtures of one sort or another), and also **quantitate** the different components present.

In all types of chromatography, there are two distinct phases, a mobile phase and a stationary phase. In HPLC, the mobile phase is a liquid, and the stationary phase is made up of very small porous particles packed into a column. A small sample (usually 10-20 μL - 1 μL = 1 mm^3) of the analytical mixture is injected into the system using a special sampling loop through which the mobile phase solvent (under high pressure - required to drive the solvent through the small particles in the column) is flowing at a constant rate. The components of the mixture partition between the mobile and stationary phase in a continuous on/off process as they are carried down the column by the mobile phase solvent. Compounds that have a high affinity for the stationary phase, spend more time on the stationary phase, and hence move more slowly along the column, compared with those that have a lower affinity. The affinity depends on the chemical structure of the analyte molecule. This results in separation of the components of the mixture as they move along the column. As each analyte emerges from the end of the column it passes through a detector, which signals its presence by recording a peak on the chromatogram. The whole chromatogram will contain several (maybe many) peaks, each of which signals the presence of at least one analyte.

A typical chromatogram is shown below.



The time taken for a peak to emerge from the column after injection, its **retention time**, is a measure of **analyte identity**, when compared with standard analytes run under the same chromatographic conditions. This is not foolproof, however, as it is possible that a different compound might coincidentally have the same retention time under these conditions. If you know something about the nature of your sample, however, you can be reasonably confident that the identification is correct if the retention time is the same as for the known standard.

The area underneath the peak recorded by the detector can be integrated to give a measure of the **amount** of analyte present. Again this needs to be **calibrated** using some kind of standardisation method. In this exercise you will be using the method of **standard addition**.

The method of standard addition

This is an alternative method to the more familiar technique of running a series of external standards of known concentration and plotting a calibration graph (as you will do for the estimation of paracetamol by UV/VIS spectrophotometry). Because it needs only two measurements, it is a very economical technique when only one or a few samples need to be analysed.

Equal quantities (weight or volume) of sample are added to two flasks of the same volume. To each flask is then added any necessary reagents or buffers. One flask is then made up to volume with the appropriate solvent (water in the coffee case). To

the second flask is added a known amount of the pure standard analyte. This flask is then also made up to volume with solvent.

Both flasks are then analysed. Assuming that the analytical signal (area under chromatographic peak in this case) is proportional to analyte concentration, we can express the analyte concentrations in the two flasks as follows

Sample flask $Y_0 = KC$

Standard addition flask $Y_1 = K(C + C_s)$

Where:
K is the sensitivity of the method (a constant - which you don't actually need to know…)
Y_0 and Y_1 are the analytical measurements
C is the concentration of analyte in the sample flask
C_s is the concentration of the added standard in the standard addition flask

By solving these two equations simultaneaously, it can be shown that:

$$C = \frac{Y_0 \times C_s}{(Y_1 - Y_0)}$$

From which C, the concentration of analyte in the sample flask, can be calculated from the known value of C_s and the measured values Y_0 and Y_1.

In this experiment you will determine the concentration of the drug caffeine in filter coffee. This is a convenient example of the assay of a drug in a complex mixture. You could do a very similar experiment to determine caffeine levels in human blood or urine, though you would have to take greater precautions in handling the samples due to the potential for infection (which is why we will not be doing it…) and make allowance for the fact that the concentrations present would be much lower.

Laboratory Experiments

Experiment 1: Assay of Citric Acid B.P.

A. Preparation of *0.1 M sodium hydroxide* solution

Weigh out approximately 2g of sodium hydroxide pellets using a top pan balance. Note the weight used (though this figure is not actually required for calculations). Add the pellets to a 500mL volumetric flask and dissolve in about 300mL distilled water. Make up to volume using a Pasteur pipette for the last bit, to ensure you do not overshoot. Mix thoroughly. Keep the solution stoppered when not in use to reduce absorption of carbon dioxide from the air

B. Preparation of standard ~0.1M potassium hydrogen phthalate solution

Accurately weigh (analytical balance) approximately 2g of potassium hydrogen phthalate and add it to a 100mL volumetric flask. Dissolve the solid in distilled water and make up to volume.

C. Standardisation of the 0.1 M sodium hydroxide solution

Measure accurately 25 mL of the potassium hydrogen phthalate solution into a 100 mL conical flask. Add a few drops of phenolphthalein as indicator. Fill a 50 mL burette with your ~0.1M NaOH solution. Ensure that no air bubbles are trapped below the tap by fully opening the tap and running a little of your solution out again. Take an initial burette reading. Titrate the potassium hydrogen phthalate solution by adding small aliquots of NaOH solution to the flask and swirling it until a pale pink colour is obtained that persists for at least 30 seconds. Remember to go slowly near the end, adding no more than 1 drop at a time. Take a new burette reading. Repeat the titration a further two times, refilling the burette as necessary. Calculate the average volume used and then use your average volume to calculate the exact concentration of the sodium hydroxide solution (see results/calculation sheet).

D. Preparation of an accurate ~0.05M citric acid solution

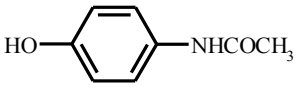
Accurately weigh approximately 2.4g of citric acid. Add to a 250 mL volumetric flask. Dissolve the solid in distilled water and make up to volume.

E. Titration of Citric Acid Solution

Pipette 25mL of citric acid solution into a conical flask. Add a few drops of *phenolphthalein* indicator to the conical flask. Place ~0.1 M NaOH solution into a 50 mL burette and take an initial volume reading. Titrate the citric acid by adding small aliquots of 0.1 M NaOH solution to the flask and swirling it until a permanent pink colour is obtained that persists for at least 30 seconds. Note the volume of 0.1M NaOH solution added. Repeat the titration two more times. Use the mean volume to calculate the exact amount of citric acid present, and from this the purity of the citric acid used (see results/calculation sheet) Does it conform to the B.P. specification?

Experiment 2: Estimation of Paracetamol in Paracetamol Tablets

A. Calibration curve for *paracetamol*



Weigh accurately (analytical balance) about 60 mg (note exact weight) of paracetamol. Place into a 100 mL volumetric flask and add about 70 mL of 0.05 M acetic acid. Mix until dissolved and then adjust the volume to 100 mL with more 0.05 M acetic acid, ensuring you mix the flask thoroughly. Using graduated pipettes, prepare dilutions as follows: take 0.5 mL, 1 mL, 2 mL, 3 mL and 4 mL of the stock paracetamol solution in 5 separate 100 mL volumetric flasks and adjust the volumes to 100 mL with 0.05 M acetic acid.

Measure the absorbance of each solution at 243 nm using a spectrophotometer (the demonstrators will show you how to do this). Take two readings of each dilution. Draw a suitable calibration graph (see results/calculation sheet for instructions on how to do this).

B. Assay of Paracetamol in Paracetamol Tablets B.P.

Press two tablets out of the packaging and weigh them accurately (analytical balance - note exact weight). Powder the two tablets with a pestle and mortar. Weigh accurately (analytical balance) about 140mg of the powder and add this sample to a 500 ml volumetric flask. About half fill the flask with 0.05 M acetic acid. Shake for 10 minutes then adjust the volume to 500 mL with more 0.05 M acetic acid and mix thoroughly. Filter ~50 mL of the solution through a filter paper into a 100 mL conical flask. Transfer 3 separate 5 ml aliquots (accurately measured – use a bulb pipette) of the filtrate to 100 ml volumetric flasks and adjust the volumes to 100 ml with 0.05 M acetic acid. Take your solutions to the spectrophotometer and make two absorbance readings of each dilution at 243 nm. Use these values to calculate the % w/w

paracetamol in the tablets (see results/calculation sheet for instructions on how to do this).. Compare the value with the stated tablet content. Is it correct?

Experiment 3: Estimation of Caffeine in Filter Coffee by High Performance Liquid Chromatography (HPLC)

A. Making up the Caffeine Standard Solution

In a weighing boat, accurately weigh about 100mg of caffeine (you will need to use the four figure analytical balances for this). Record the exact weight of the caffeine.

Transfer the caffeine into a 250 mL volumetric flask, using distilled water to wash any residues in the weighing boat into the flask. Make up to just below the line and shake the flask until all the caffeine has dissolved. Make up to exact volume using distilled water and a Pasteur pipette. Mix thoroughly again.

Using a 25 mL pipette, transfer 25 mL of the caffeine solution into a 250 mL volumetric flask, make up to volume with distilled water and mix thoroughly. This solution is your CAFFEINE STANDARD SOLUTION.

B. Making up the solutions for HPLC analysis

Using a 5 mL bulb pipette, transfer 5 mL of coffee extract into a 50 mL volumetric flask. Make up to exact volume with distilled water (add it carefully to avoid foaming) and mix thoroughly. This is your coffee sample for analysis. Label the flask

Using a 5 mL pipette, transfer 5 mL of coffee into a second 50 mL volumetric flask Using a 25 mL pipette, transfer 25 mL of caffeine standard solution (see above) into the flask. Make up to exact volume with distilled water (add it carefully) using a Pasteur pipette and mix thoroughly. This is your coffee sample with added standard. Label the flask.

C. HPLC Analysis of the Samples

Take your samples to the instrument lab, where Dr Kate Bowman will show you how to inject your samples into the HPLC instrument. Please be patient if you have to

wait a few minutes while other samples are run. Once you have the chromatograms and data for your two samples, you can calculate the amount of caffeine in your coffee extract (see results sheet for instructions on how to do this).

(If you have to wait for the instruments to become free, I suggest that one team member should do this, while the others start the next exercise).