7

Analytical spectroscopy

Analytical spectroscopy is the science of determining how much of a substance is present in a sample by accurately measuring how much light is absorbed or emitted by atoms or molecules within it. Different types of spectroscopy are available, depending on the type or wavelength of electromagnetic radiation absorbed or emitted by the atom or molecule. A detailed review of all types of modern instrumental analysis is beyond the scope of this book, but the use of spectroscopy in the analysis of drugs and medicines is very important and will be considered.

Light is a form of electromagnetic radiation, so called because it consists of an electric component and a magnetic component, which oscillate in mutually perpendicular directions and perpendicular to the direction of travel of the radiation through space (see Figure 7.1).

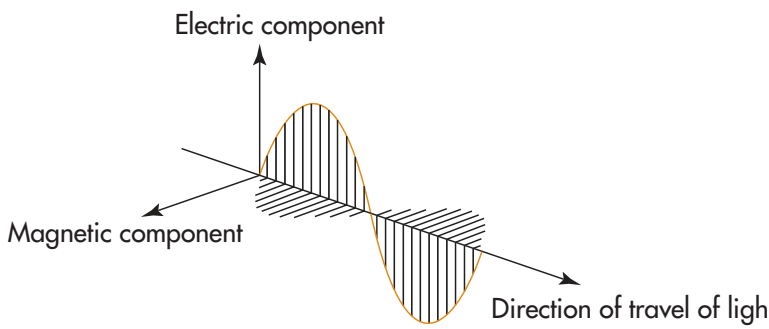
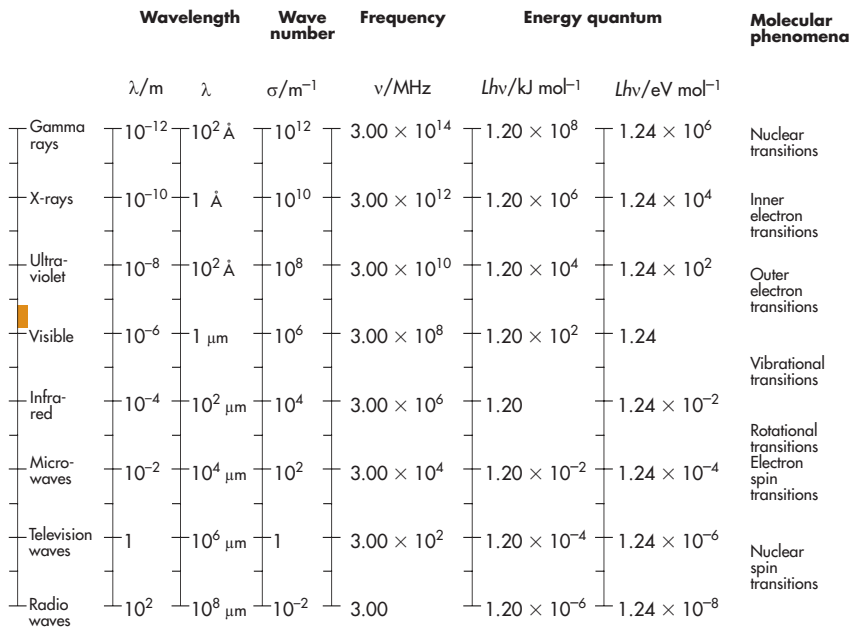


Figure 7.1 A diagrammatic representation of electromagnetic radiation.

The complete spectrum of electromagnetic radiation is shown in Figure 7.2 and ranges from low-energy radio and television waves through to very high-energy gamma rays. The tiny part of the electromagnetic spectrum that human eyes can detect (approximately 400–700 nm) is called the visible spectrum, and spectroscopy carried out at these wavelengths is termed visible spectroscopy or 'colorimetry'.



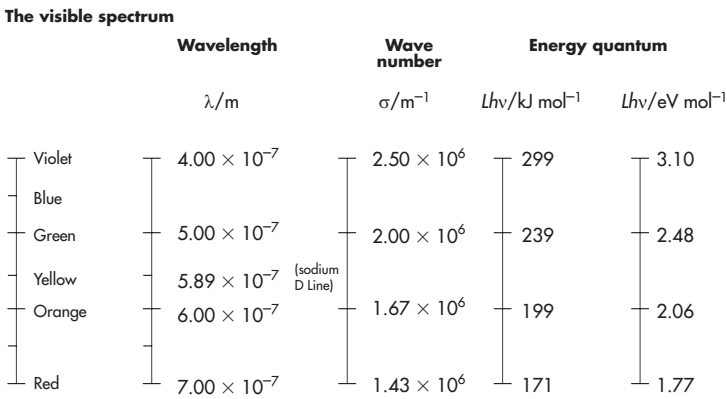


Figure 7.2 A diagram of the electromagnetic spectrum.

The part of the electromagnetic spectrum just beyond the red end of the visible spectrum is termed the *infrared* portion and has longer wavelength and lower energy than visible light. Similarly, the part of the spectrum beyond the violet end of the visible is called the *ultraviolet* portion and is of shorter wavelength and higher energy than visible light.

Electromagnetic radiation can be thought of as a wave-form travelling through space, and the type of radiation used in a particular experiment depends on the information required from the experiment.

One feature of the radiation, which is always quoted, is the *wavelength* of the light. The wavelength is defined as the distance from one wave crest to the next (or trough to trough) and is usually quoted in nanometres (nm, 10^{-9} m) to allow for reasonably sized numbers (Figure 7.3). The symbol for wavelength is λ the Greek letter 'lambda'. The energy contained in the individual quanta of energy (photons) of a beam of radiation of a given wavelength is inversely proportional to the wavelength. This means that radio waves with wavelengths of several hundred metres have low energies, while gamma rays and X-rays are high-energy, short-wavelength forms of radiation.

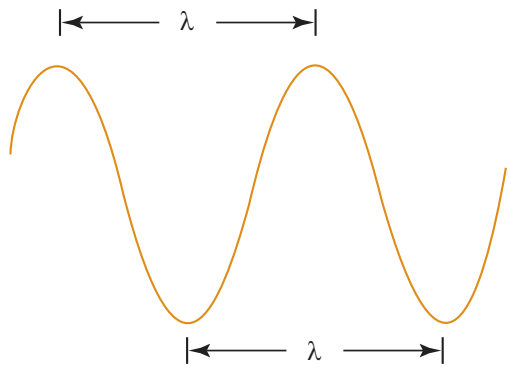


Figure 7.3 The wavelength of light.

Other terms used extensively in spectroscopy are the *wavenumber* and the *frequency*. The wavenumber is defined as the number of waves per unit of length (usually quoted in units of 'reciprocal centimetres' (cm^{-1}; where 1 cm = 10^{-2} m) and is the reciprocal of the wavelength in centimetres, i.e. $1/\lambda$. The use of wavenumber is usually confined to infrared spectroscopy.

The frequency is defined as the number of waves emitted from a source per second; the unit of frequency is the hertz (Hz; 1 Hz = 1 wave per second), and the symbol for it is ν (the Greek letter 'nu').

The frequency and the wavelength are related by a constant called the speed of light, symbol *c*. This value (approximately 3×10^8 m s^{-1}) is the product of the frequency and the wavelength, i.e.

$$\text{Velocity of light} = \text{frequency} \times \text{wavelength}$$

or

$$c = \nu \times \lambda$$

Since both frequency and wavenumber are inversely proportional to wavelength, the energy of a photon is directly proportional to both of these quantities.

When an atom or molecule is exposed to electromagnetic radiation, the energy can be absorbed in one of three ways:

1. The energy can promote an electron from a bonding orbital to a higher-energy antibonding orbital, a so-called *electronic* transition.
2. The energy can act to increase the vibration, or oscillation, of atoms about a chemical bond. This is termed a *vibrational* transition.
3. The energy can bring about an increase in the rotation of atoms about a chemical bond, which is a *rotational* transition.

In energy terms, the differences between these effects are enormous. It requires approximately 100 times more energy to bring about a vibrational transition than it does to produce a rotational one. Similarly, an electronic transition requires almost 100–fold more energy than is needed for a vibrational transition. This is important for two reasons: first, it means that each electronic transition must be associated with vibrational and rotational transitions; second, since electronic transitions require so much energy, only light of short wavelength is sufficiently energetic to bring them about. Thus, for example, infrared radiation can achieve increased vibration and rotation about chemical bonds, but has insufficient energy to promote an electron to an antibonding orbital and bring about an electronic transition. Ultraviolet or visible light is generally required to achieve electronic transitions.

Although spectroscopy can be carried out on different types of compounds, with different electronic configurations, most quantitative work (and all the examples in this book) will involve π ('pi') electron systems. The π electrons (the so-called 'mobile electrons') are the electrons found in multiple bonds. A carbon–carbon double bond contains one σ ('sigma') bond and one π bond, while a carbon–carbon triple bond consists of one σ bond and two π bonds. These π electrons are easily excited and promoted to a high-energy antibonding orbital. When the electron falls back down to the ground state, this energy is released and can be measured by a spectrophotometer.

The part of the molecule that is responsible for the absorption of light is called the *chromophore* (see Figure 7.4) and consists of a region of double or triple bonds, especially if the multiple bonds are *conjugated*, that is if the

structure contains alternating multiple and single bonds. The longer the run of conjugated double or triple bonds in the molecule, the more easily the molecule will absorb light. Aromatic compounds, which contain a benzene ring, will absorb ultraviolet light of wavelength 254 nm and this property is exploited in many spectroscopic analyses and in detectors for chromatographic systems. If the chromophore is more extensive, then the molecule will be excitable by light of lower energy, until, if the chromophore is very large, visible light will have sufficient energy to excite the electrons of the chromophore and the compound will absorb visible light.

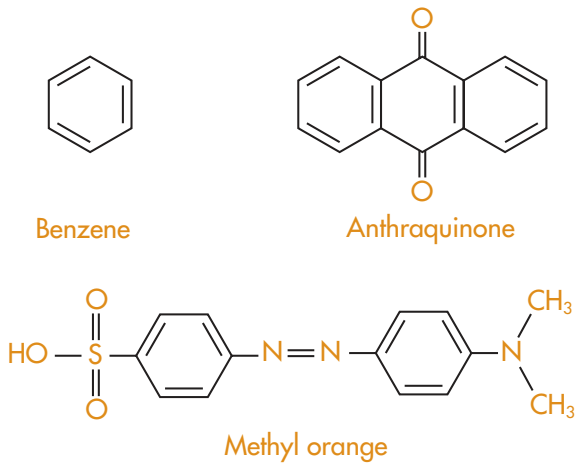


Figure 7.4 Examples of chromophores.

A molecule of this type, which absorbs light in the visible part of the electromagnetic spectrum, is said to be *coloured* because our eyes will detect the light reflected back from the compound, which will be the complementary colour to the light absorbed. White light, remember, is made up of all the colours of the rainbow, and can be split into its constituent colours by a prism or droplet of water. For example, if a dye molecule absorbs light of red, orange and yellow wavelengths, our eyes will detect the reflected blue, green and purple light and we will see the material as coloured blue. Similarly, a red dye will absorb the short-wavelength blue light and reflect the reds and oranges back to our eyes. This property is utilised in the use of indicators for titrations (see Chapter 6), where the absorption spectrum (and hence the colour) of the indicator changes with the pH of the solution.

Effect of pH on spectra

If a graph of the extent of light absorption (measured as the quantity termed 'absorbance', defined later on p. 174) is plotted against the wavelength, then the complete absorption spectrum of a molecule can be obtained (Figure 7.5). The wavelength at which the absorbance (*A*) is highest is called the λ_{max} (read as 'lambda max') and is a characteristic of a particular chromophore. The λ_{max} of a compound is sometimes used in the *British Pharmacopoeia* for identification of drugs and unknown compounds.

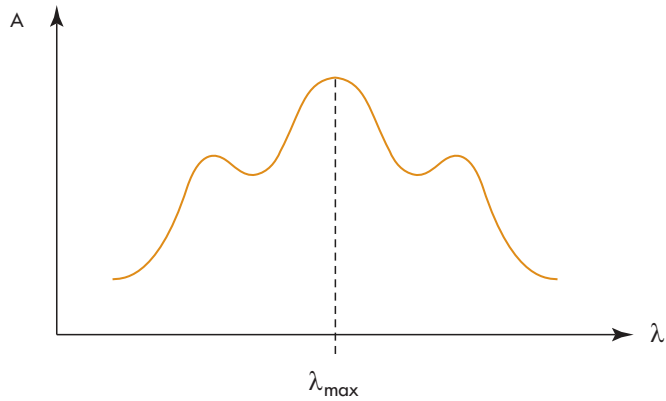


Figure 7.5 A plot of light absorbance vs λ.

The wavelength at which the λ_{max} occurs should be a constant for a given compound but, like many 'constants' in science, λ_{max} can move around and change. This is not entirely bad news, as a large amount of useful information can be obtained about a compound simply by observing any shifts that occur in λ_{max} when, for example, the compound is ionised.

A shift in λ_{max} towards longer wavelength is referred to as a *bathochromic* or red shift, because red is the colour at the long-wavelength end of the visible spectrum. A bathochromic shift usually occurs due to the action of an *auxochrome*. This is a functional group attached to the chromophore which does not absorb light energy itself but which influences the wavelengths of light absorbed by the chromophore.

Examples of auxochromes include the —NH_2, —OH and —SH groups. These functional groups possess lone pairs of non-bonded electrons that can interact with the π electrons of the chromophore and allow light of longer wavelength to be absorbed. A good example of this effect is to compare the λ_{max} values of benzene and aniline (also called phenylamine or aminobenzene), shown in Figure 7.6.

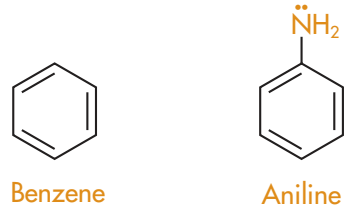


Figure 7.6 The structures of benzene and aniline.

The λ_{max} of benzene is 204 nm, whereas the λ_{max} of aniline is 230 nm. This is due to the lone pair of electrons on the NH_2 interacting with the ring electrons to increase the electron density throughout the ring, particularly at the *ortho* and *para* positions of the ring, as shown in Figure 7.7.

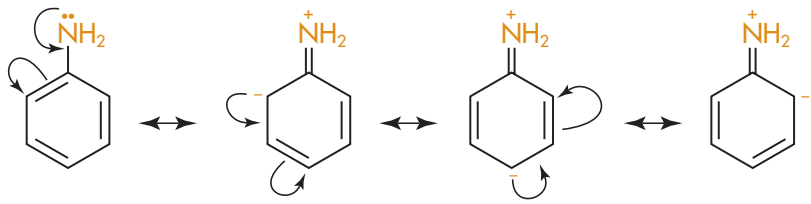


Figure 7.7 The +M effect of aniline.

This *mesomeric* (or M) effect is seen when aniline is placed in a solution of pH 8–14, i.e. when the basic aniline is unionised. When aniline is placed in a solution of pH < 7, the λ_{max} returns to virtually the value obtained for benzene (203 nm). What is happening is that aniline in acidic solution reacts to form the anilinium salt. The lone pair of electrons on the nitrogen is now involved in bond formation to an H^+ ion and can no longer function as an auxochrome. The structure of aniline hydrochloride is shown in Figure 7.8.

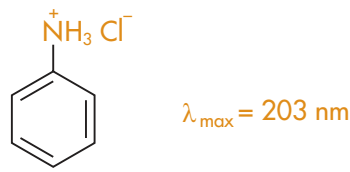


Figure 7.8 The structure of aniline hydrochloride and its λ_{max} value.

A shift in λ_{max} to shorter wavelength is called a *hypsochromic* effect, or blue shift, and usually occurs when compounds with a basic auxochrome ionise and the lone pair is no longer able to interact with the electrons of the chromophore. Hypsochromic effects can also be seen when spectra are run in different solvents or at elevated temperatures. Spectral shifts of this type can be used to identify drugs that contain an aromatic amine functional group, e.g. the local anaesthetic benzocaine (see Figure 7.9).

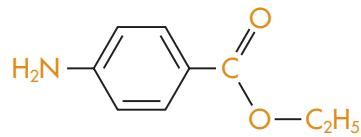


Figure 7.9 The structure of benzocaine.

Bathochromic and hypsochromic effects are seldom seen in isolation. Bathochromic effects are usually associated with increases in the intensity of light absorbed, while hypsochromic effects usually occur with decreases in absorbance. An effect that causes an increase in light absorbance is called a *hyperchromic* effect, while a decrease in the intensity of light absorbed is termed a *hypochromic* effect. The four words used to describe shifts in λ_{max} could almost have been chosen to cause maximum confusion among students trying to remember the terms. Perhaps the best way to remember the terms is to say that hyper- means an increase, hypo- a decrease, and that a shift to longer wavelength is a red shift while a shift to shorter wavelength is a blue shift, or, alternatively, commit to memory Figure 7.10. Hyperchromic effects are used in anticancer drug research to measure the extent of drug binding to DNA. If a solution of duplex, or double-stranded, DNA is gently heated, the double helix will start to unwind, exposing the heterocyclic bases in the centre of the duplex. This can be observed experimentally as the absorbance of the DNA solution at 260 nm will increase, causing a hyperchromic effect. Drugs that bind to DNA stabilise the molecule and reduce the extent of the observed hyperchromicity.

Drugs that contain phenolic groups, e.g. paracetamol (see Figure 7.11), also show spectral shifts on ionisation. In the case of phenols, which are weak acids with a pK_a of approximately 10, ionisation increases the intensity of light absorption and the position of λ_{max} moves to longer wavelength. This is because ionisation and loss of the H atom as an H^+ ion results in a full negative charge on the oxygen (a phenoxide ion), which can interact with the ring more effectively than the lone pair of electrons present in the unionised molecule. This is shown for phenol in Figure 7.11.

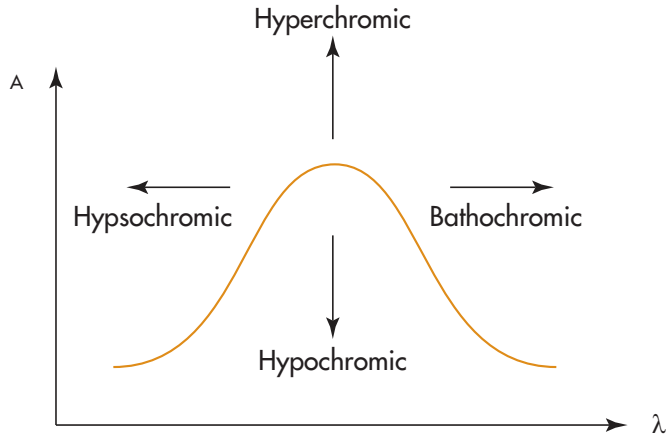


Figure 7.10 Changes that occur in λ_{max}.

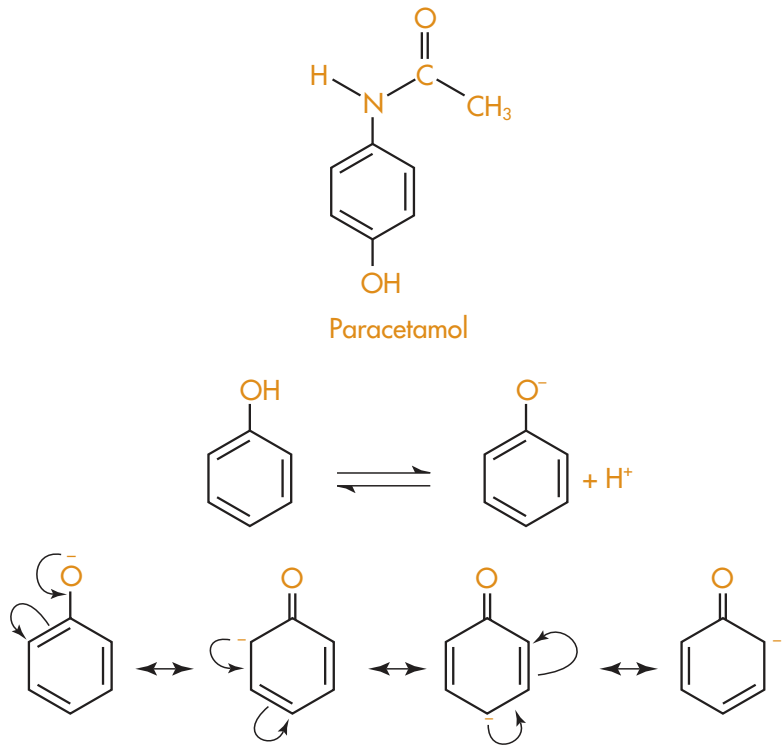


Figure 7.11 The structure of paracetamol and ionisation of phenols.

Instrumentation

An instrument that measures the intensity of light absorbed by atoms or molecules is called a *spectrophotometer*. Different types of spectrophotometers exist depending on whether they use a single beam of light or use separate reference and sample beams, and on whether they measure at a fixed wavelength or scan the absorption spectrum at many wavelengths. As with most analytical instruments, accuracy, precision and cost vary widely. In general, all spectrophotometers have a layout similar to the one shown in Figure 7.12.

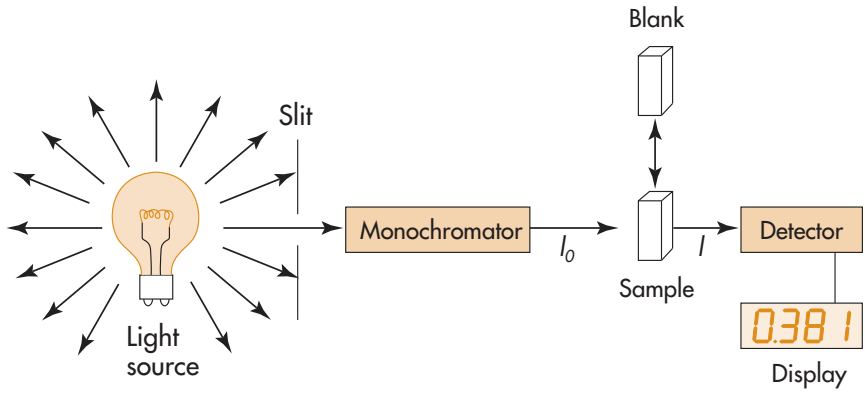


Figure 7.12 A schematic diagram of a spectrophotometer.

Light source

The source or lamp is really two separate lamps which, taken together, cover the whole of the visible and ultraviolet regions of the electromagnetic spectrum. For white visible light a *tungsten* lamp is used. This lamp is nothing more sophisticated than a light bulb with a filament made of the metal tungsten. You are probably reading this book by the light of one of these lamps. A tungsten lamp emits light of wavelengths 350–2000 nm and is adequate for colorimetry.

For compounds that absorb in the ultraviolet region of the spectrum, a *deuterium* lamp is required. Deuterium is one of the heavy isotopes of hydrogen, possessing one neutron more than ordinary hydrogen in its nucleus. A deuterium lamp is a high-energy source that emits light of approximately 200–370 nm and is used for all spectroscopy in the ultraviolet region of the spectrum.

Fixed-wavelength instruments allow the operator to select which lamp is required for an assay, whereas scanning instruments, which produce

a plot of the whole absorption spectrum of the sample, switch lamps automatically.

Monochromator

For most quantitative measurements, light must be *monochromatic*, i.e. of one particular wavelength. This is achieved by passing the polychromatic light (i.e. light of many wavelengths) through a monochromator. There are two types of monochromator in modern spectrophotometers: *prisms* or *diffraction gratings*.

A prism is a triangular piece of quartz that refracts (or bends) light passing through it. The extent of the refraction depends on the wavelength of the light, so a beam of white light can be split into its component colours by passage through a prism. The prism is then rotated to select a particular wavelength required for the assay (Figure 7.13). This effect is identical to the formation of a rainbow when light from the sun is split into its seven component colours (red, orange, yellow, green, blue, indigo and violet) by refraction through droplets of rain.

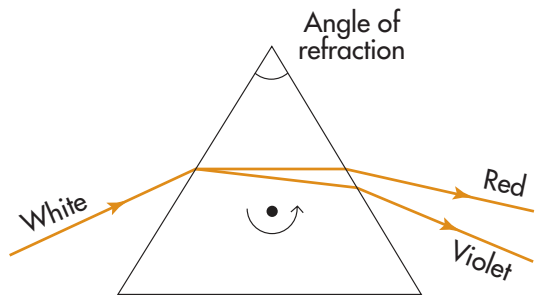


Figure 7.13 A diagram of a prism.

A diffraction grating is a small piece of mirrored glass onto which a large number of equally spaced lines have been cut, several thousand per millimetre of grating, to give a structure that looks like a small comb. The spaces between the cuts are approximately equal to the wavelengths of light and so a beam of polychromatic light will be resolved into its component wavelengths by the grating. The grating is then rotated to select the wavelength desired for assay (Figure 7.14).

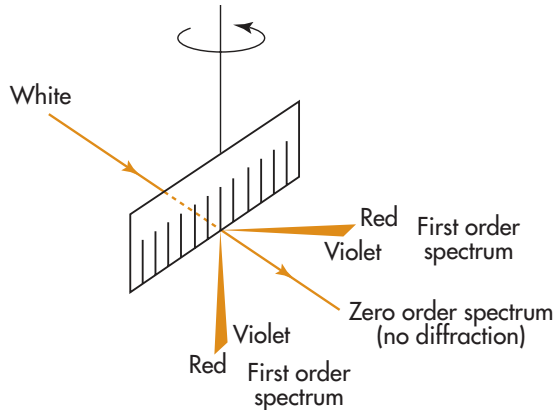


Figure 7.14 A diagram of a diffraction grating.

Detector

After light has passed through the sample, any decrease in intensity, due to absorption, is measured by a detector. This is usually a clever piece of electronics called a *photomultiplier tube* (see Figure 7.15), which acts to convert the intensity of the beam of light into an electrical signal that can be measured easily, and then also acts as an amplifier to increase the strength of the signal still further. Light enters the tube and strikes the cathode; this releases electrons, which are attracted to an anode above. When the electrons strike this anode they release more electrons, which are, in turn, attracted to the anode above that, where the process is repeated. In this way a cascade of electrons is generated and the signal is amplified.

Once the electrical signal leaves the photomultiplier tube, it is fed to a recorder if a printout is required, or, more usually, to a screen where the absorption spectrum can be displayed. Most modern spectrophotometers are now interfaced to a personal computer to allow storage of large amounts of data, or to allow access to a library of stored spectra on the hard drive of the machine. This allows comparison of stored spectra with the experimentally derived results from the laboratory and aids in the identification of unknown compounds.

Experimental measurement of absorbance

The sequence of events in making a measurement with a spectrophotometer is as follows.

1. The monochromator is set to the wavelength of measurement, the shutter is closed to prevent light reaching the detector, and the instrument is set to infinite

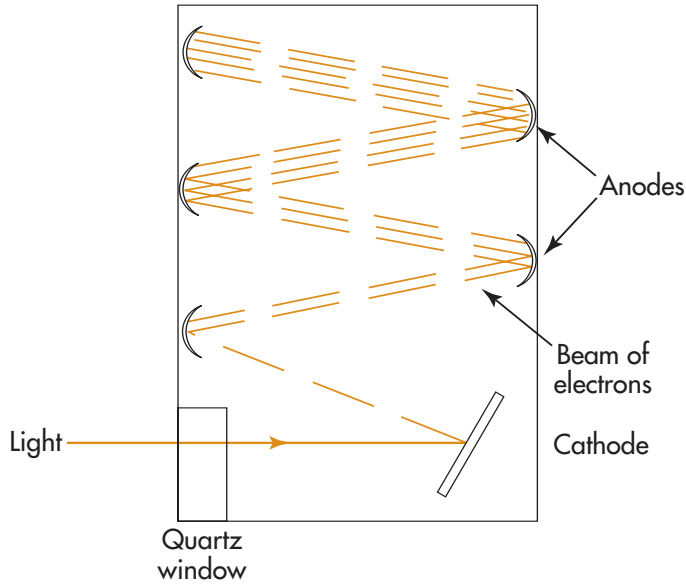


Figure 7.15 A diagram of a photomultiplier tube.

absorbance. This is often done automatically during the 'warm-up' by modern instruments.

2. The shutter is opened, the solvent (or 'blank') is placed in the light path and the instrument is set to zero absorbance. The blank is usually just the solvent for the assay but, strictly speaking, should be everything in the sample matrix except the sample being measured. This means that in complex assays the blank solution has to be made up to match exactly the composition of the solvent/medium in which the sample will be measured, and has to be extracted or otherwise treated in exactly the same way as the sample.
3. The sample solution (or 'test') is placed in the light path and the absorbance is read directly by the instrument.

Dilutions

The most important part of any spectroscopic assay is not the performance of the spectrophotometer (although the accuracy of the instrument is checked periodically). The crucial part of any experiment is the accurate preparation of the test and standard solutions. This often involves the accurate dilution of a stock solution using the volumetric glassware introduced in Chapter 6, namely the pipette and the volumetric flask.

A common procedure is to prepare a range of dilutions for use as a calibration graph as in the worked example below.

Worked example

You are presented with a stock solution containing a 50 μg mL^{-1} solution of a drug. Prepare 100 mL of solution to contain 5, 10, 20 and 30 μg mL^{-1} of drug.

The first step is to calculate how much of the 50 μg mL^{-1} stock solution will be required for each dilution. This can be done by using the relationship below

$$\frac{[\text{Required}]}{[\text{Stock}]} \times \text{volume required}$$

where [ ] represent the concentrations of drug. This relationship may be more easily remembered as

$$\frac{[\text{Want}]}{[\text{Got}]} \times \text{volume of flask}$$

Using this relationship, the 30 μg mL^{-1} solution is prepared from (30/50) × 100 = 60 mL of stock solution made up to 100 mL with solvent. The 20 μg mL^{-1} solution is prepared from (20/50) × 100 =40 mL of stock made up to 100 mL with solvent, and so on for all the dilutions.

The alternative way to prepare these dilutions is to prepare each dilution from the next most concentrated. This is called a *serial dilution* and is carried out as follows. The 30 μg mL^{-1} and 20 μg mL^{-1} solutions are prepared as above. The 10 μg mL^{-1} solution is prepared from the 20 μg mL^{-1} solution (50 mL of 20 μg mL^{-1} solution made up to 100 mL with solvent) and the 5 μg mL^{-1} solution is prepared from the 10 μg mL^{-1} solution in the same way. A serial dilution has the advantage of using less of the stock solution (100 mL compared to 130 mL in this example) and is used whenever the drug or reagent in question is expensive or in short supply.

Quantitative aspects of spectroscopy

Light passing through a substance decreases in intensity as a result of three processes:

1. reflection at phase boundaries (liquid/air, glass/liquid, etc.). This is caused by differences in the refractive index of the different materials through which the light is passing
2. scattering of light caused by non-homogeneity of the sample
3. absorbance by atoms or molecules in solution.

Loss of intensity due to point (1) can be compensated by use of an appropriate blank solution since phase boundary effects should be the same in the test and blank solutions.

The scattering effects in point (2) can be minimised by careful sample preparation, i.e. ensuring the sample dissolves completely in the chosen solvent, that there are no air bubbles adhering to the sample cell, and that there are no fingerprints, dust, mascara, dandruff or other unwanted material on the outside of the cell which will affect the accuracy of the absorbance measurements.

Losses in intensity due to point (3) are what we are interested in measuring.

Beer's and Lambert's laws

The quantitative aspects of spectrophotometry are based on two very similar laws. The first is Beer's law (Figure 7.16), which states that 'the intensity of a beam of parallel, monochromatic light decreases exponentially with the concentration of the absorbing molecules'. Beer's law (named after German chemist August Beer) can be expressed mathematically as

$$I = I_0 e^{-k'c} \tag{7.1}$$

where I_0 is intensity of light incident on the sample, I is intensity of light transmitted by the sample, k is a constant and c is the concentration of the sample.

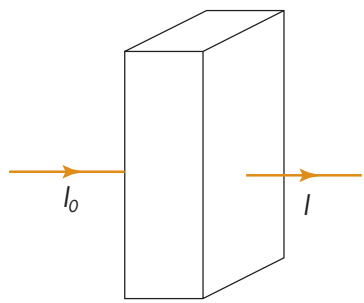


Figure 7.16 A diagram of Beer's law.

Taking logarithms,

$$\log \frac{I_0}{I} = k'c$$

Log (I_0/I) is a dimensionless quantity (strictly speaking, a logarithm of a ratio of light intensities) and is defined as *absorbance*. Absorbance is the quantity measured and plotted in spectrophotometry. Thus Beer's law states that absorbance is proportional to concentration.

The second relationship is Lambert's law, (named after the German physicist Johann Heinrich Lambert) which states that the 'intensity of a beam of parallel, monochromatic light decreases exponentially as the light travels through a thickness of homogeneous medium', expressed mathematically as

$$I = I_0 e^{-k''l} \tag{7.2}$$

where I and I_0 are as before, l is the thickness of the medium (or path length) through which the light passes and k'' is (another) constant.

Taking logarithms,

$$\log \frac{I_0}{I} = k''l$$

i.e. absorbance is proportional to path length.

These two fundamental equations are so similar that they can be combined into one relationship, the Beer–Lambert law or equation, which can be expressed as

$$\textbf{Absorbance} \quad \log \frac{I_0}{I} = kcl \tag{7.3}$$

Here k is yet another constant, the value of which depends on the units used for the concentration term, c, and on the path length, although this is usually 1 cm.

If the units of concentration are molarity (i.e. number of moles per litre), then the constant is ε (the Greek letter 'epsilon') and is known as the *molar absorptivity*, with units of L mol^{-1} cm^{-1}, although the units are seldom expressed. ε is equal to the absorbance of a 1 M solution in a cell of path length 1 cm and is usually a large number, approximately 10 000–20 000. In this case the Beer–Lambert equation is written as

$$A = \varepsilon cl \tag{7.4}$$

When the concentration of the sample is expressed in percentage weight in volume (% w/v) or g/100 mL, the constant used is $A\,1\%, 1$ cm, usually written as A_1^1, and is called the *specific absorbance*, with units of dL g^{-1} cm^{-1} although, again, the value is usually quoted without units. The A_1^1 value is very useful in pharmacy and pharmaceutical analyses where the molecular weight of the sample may be unknown (e.g. when analysing a macro-

molecule, such as a protein) or where a mixture of several components is being analysed in the same sample. This gives the most useful form of the Beer–Lambert equation:

$$A = A_1^1 cl \tag{7.5}$$

It follows from the derivations above that ε and A_1^1 are related, and either one can be calculated from the other using equation (7.6):

$$\varepsilon = \frac{A_1^1 \times \text{relative molecular mass}}{10} \tag{7.6}$$

As mentioned above, absorbance is defined as $\log I_0/I$; older textbooks refer to the term as *extinction*, while even older manuscripts call it *optical density*. All three terms mean the same, but 'absorbance' is the expression that should be used in all analytical spectroscopy.

Two other expressions of light intensity occur in spectroscopy:

- *transmittance*, defined as the ratio I/I_0
- *percentage transmittance*, which is the same ratio expressed as a percentage, i.e. $100\ I/I_0$.

The use of these two terms in analytical spectroscopy is limited to infrared spectroscopy since neither term, unlike absorbance, gives a linear relationship if plotted against concentration.

Methods of drug assay

There are two methods of using spectroscopic measurements in drug analysis, the *absolute* and the *comparative* methods of assay, and the one used depends on which side of the Atlantic Ocean you carry out the analysis.

In the UK and Europe the Beer–Lambert equation tends to be used in what is called the absolute method of assay. In this procedure the absorbance is measured experimentally and the Beer–Lambert equation is solved for c, the drug concentration. For this reason, the *British Pharmacopoeia* and *European Pharmacopoeia* quote A_1^1 values in drug monographs.

In the *US Pharmacopoeia*, the comparative method of assay is preferred. In this type of assay a standard solution of the drug to be analysed is prepared, the absorbance of the sample and the standard are measured under identical conditions, and the concentration of the sample is calculated from the relationship

$$\frac{A_{\text{test}}}{A_{\text{std}}} = \frac{[\text{test}]}{[\text{std}]}$$

where [test] is the concentration of the sample and [std] is the concentration of the prepared standard. The comparative method of assay has the advantage that it can be used even if the drug undergoes a chemical reaction during the assay (e.g. formation of a coloured derivative to allow measurement in the visible region of the spectrum), but suffers from the disadvantage that an authentic sample of the drug in question must be available for comparison.

When carrying out drug assays by spectroscopy it is often necessary to prepare a range of concentrations of a standard sample of the analyte and measure the absorbance of each solution. When these data are plotted, a straight line of positive slope should be obtained that passes through the origin. Constructing graphs of this type not only confirms that the Beer–Lambert law applies to the assay at the wavelength of measurement but also allows the graph to be used for calibration purposes. A solution of unknown concentration is prepared in exactly the same way as the standards and its absorbance is measured at the same wavelength as the standards. This absorbance is then read off the calibration graph and the concentration is calculated. Standard solutions prepared separately from the sample in this way are known as *external standards*.

A more rigorous technique involves the use of *internal standards*. An internal standard is a compound that is similar in chemical structure and physical properties to the sample being analysed. The internal standard should be added to the sample in question before extraction or assay commences and is then present in the sample matrix throughout the subsequent assay. In the assay of complex samples, some sample pre-treatment is usually required and the recovery of the sample from the extraction process may not be 100%. If an internal standard is used, losses in sample will be mirrored by similar losses in the standard and the ratio of sample to standard should remain constant. Internal standards are particularly used in chromatographic analysis (especially gas chromatography and high-performance liquid chromatography), where fluctuations in instrumental parameters (e.g. flow rate of mobile phase) affect accuracy.

In certain spectroscopic analyses a similar approach to the use of internal standards is employed. This is the technique of *standard additions* and involves addition of increasing volumes of a standard solution of the analyte to a fixed volume of the sample and construction of a calibration graph. The graph in a standard addition assay is of positive slope but intersects the y-axis at a positive value of absorbance. The amount of drug in the sample is found by extrapolation of the calibration graph back to the point where the line intersects the x-axis (i.e. when $y = 0$ in the equation of the line) as shown in Figure 7.17.

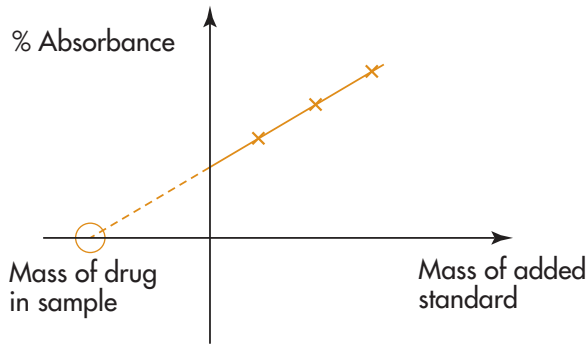


Figure 7.17 A calibration graph using method of standard additions.

The method of standard additions is widely used in atomic spectroscopy (e.g. determination of Ca^{2+} ions in serum by atomic emission spectrophotometry) and, since several aliquots of sample are analysed to produce the calibration graph, should increase the accuracy and precision of the assay.

Derivative spectroscopy

Occasionally during the assay of a medicinal product, excipients present in the formulation mask the absorbance of the active drug. This often occurs in liquid formulations such as syrups and linctuses where a small amount of a highly coloured dye is used to colour the mixture. If a simple dilution of a coloured mixture was carried out, the resulting absorbance measurements would not obey Beer's law and a non-linear graph of absorbance vs concentration would result.

In cases such as these, it is often possible to use the *mathematical derivative* of the absorbance spectrum to calculate the content of active drug. In calculus, the derivative of a function is the instantaneous rate of change of that function with respect to a variable. This can also be thought of as the slope of the graph or function at any point on the graph. Derivatising a simple plot of absorbance vs wavelength allows certain peaks within the total spectrum to be 'sharpened' and separated from the background absorbance of the excipients. The original spectrum of the drug is called the fundamental (or D^0) spectrum, the first derivative D^1, second derivative D^2, and so on until useful information becomes lost in the electronic noise of the spectrophotometer. If the original spectrum is a plot of absorbance vs wavelength (the D^0 spectrum) then the first derivative, D^1, is written as $dA/d\lambda$, the second derivative as $d^2A/d\lambda^2$ and so on. A diagram of D^0, D^1 and D^2 spectra is shown in Figure 7.18.

Derivative spectroscopy may be used in the assay of Ephedrine Elixir BP, which is used as a decongestant. This preparation is an oral solution containing 0.3% w/v of ephedrine hydrochloride in a suitably flavoured vehicle. The elixir is pink in colour and the presence of the pink dye interferes with the simple UV assay, but the preparation may be analysed as follows.

A number of standard dilutions of pure ephedrine hydrochloride are prepared and their D^2 spectra are measured. A calibration curve of D^2 peak height vs concentration is drawn. The elixir is diluted and the D^2 spectrum

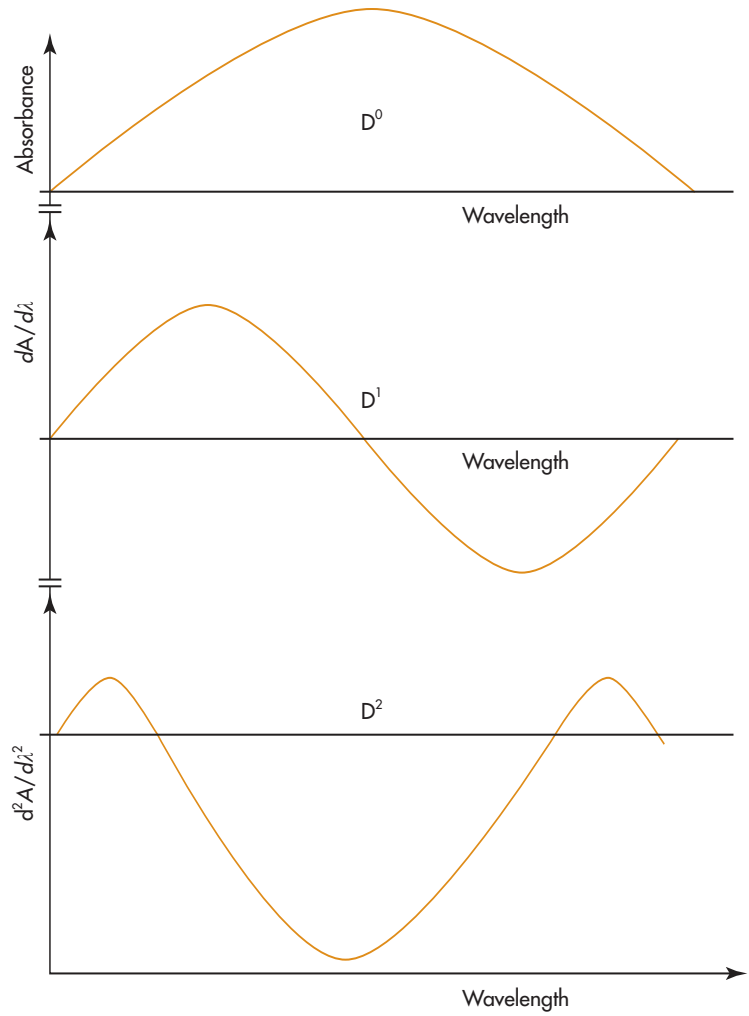


Figure 7.18 Higher-order spectra D^1 and D^2.

of the elixir is obtained. It is then a simple matter to read the ephedrine concentration off the standard curve. Ephedrine has a simple benzenoid absorbance (λ_{max} 263 nm; see structure in Chapter 1) which appears sharp and clear against the background absorbance when the D^2 spectrum is obtained. A sample D^2 trace obtained in this assay is shown in Figure 7.19.

Infrared spectroscopy

Infrared (IR) spectroscopy is a very useful technique for the identification of unknown compounds, e.g. products from a synthesis or urinary metabolites from an animal experiment, especially when used in conjunction with other structure elucidation techniques such as nuclear magnetic resonance and mass spectrometry. The infrared region of the electromagnetic spectrum refers to light of wavelength 2.5 to 15 μm (i.e. 2.5×10^{-6} to 15×10^{-6} m) and the absorption of this light by the molecule causes changes in the vibrational energy of the molecule in its ground state. As stated previously, vibrational transitions are always associated with changes in the rotation of atoms about chemical bonds. This is analogous to electronic transitions in the absorption of ultraviolet energy, which also result in vibrational and rotational transitions. The usefulness of IR stems from the fact that each peak on the spectrum can be assigned to a particular bond or functional group in the molecule. This often means that IR spectra are complex, with perhaps as many as 20 or 30 peaks on one spectrum.

Identification of chemical unknowns is made easier, however, because certain functional groups always appear in the same region of the IR spectrum. Single bonds (e.g. O—H, N—H, C—H) absorb in the high-frequency part of the spectrum (approximately 4000–2100 cm^{-1}). This is because the low mass of the hydrogen atom allows vibrations to occur at high frequency. Triple bonds (e.g. in organic nitriles, R—C≡N) absorb at approximately 2100–1900 cm^{-1}, while double bonds (e.g. C═O, C═C) absorb at approximately 1900–1500 cm^{-1}. The region of the IR spectrum corresponding to wavenumbers less than approximately 1500 cm^{-1} is due to stretching of the molecule as a whole and the peaks in this region are more difficult to assign accurately. This region of the spectrum is called the *fingerprint region*, since the pattern of peaks occurring in this region is characteristic of the compound in question *and no other*. Use is made of this property in the *British Pharmacopoeia* where two samples are said to be identical when the IR spectra, obtained under identical conditions, coincide completely – i.e. the same peaks are present in the same positions with the same intensities. Reference IR spectra of authentic samples of a drug are published in the BP to verify the identity of unknown samples.

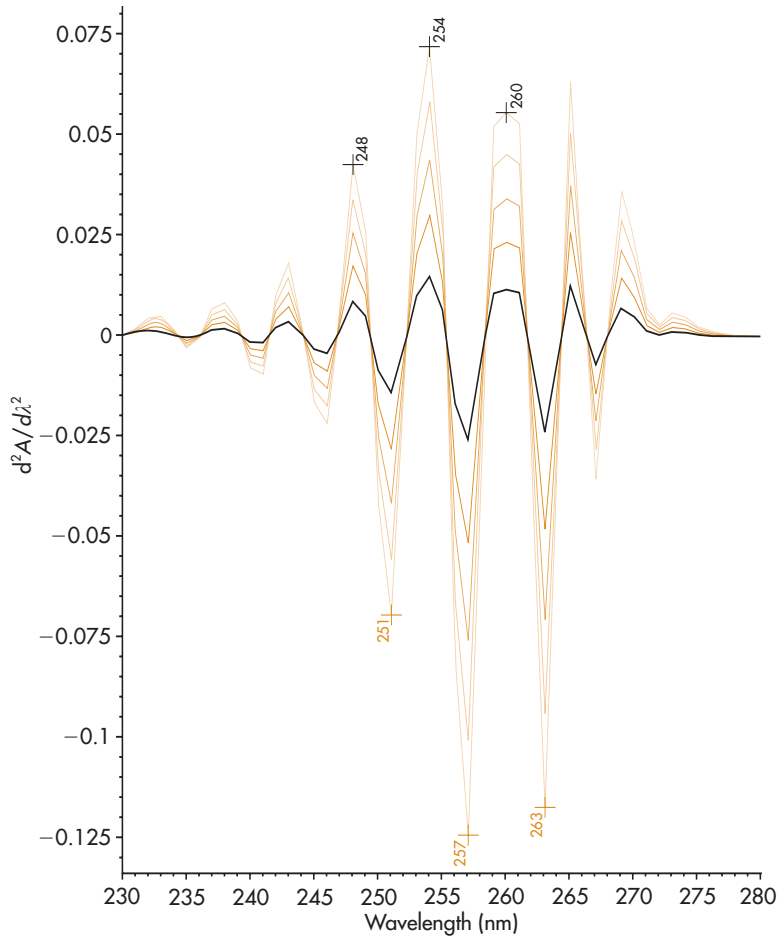


Figure 7.19 D^2 spectra from assay of Ephedrine Elixir BP.

Quantitative analysis using infrared spectroscopy

The Beer–Lambert rules derived above (equation 7.3) apply equally to absorption of infrared radiation by molecules. Moreover, infrared absorption spectra possess an advantage over the more common ultraviolet absorption in the greater number of bands present. It is often possible to select an absorption band for each component of a mixture such that little or no interference occurs between them. For these reasons, infrared spectroscopy is often used quantitatively in the analytical laboratory to determine drug concentrations in solution. A calibration curve for the assay may be obtained (and Beer's law confirmed) by converting the printed spectrum

into I_0 and I using the *baseline technique* as shown in Figure 7.20. The distance from the baseline to the bottom of the page is designated I_0, the radiation available for absorption, while the distance from the apex of the peak to the bottom of the page is designated I, the radiation transmitted through the sample. The logarithm (to base 10) of the ratio I_0/I is obtained as before to yield absorbance. Note that infrared spectra are usually plotted 'upside down' so that zero absorbance is at the top of the spectrum (as usually displayed) and 100% absorbance is at the bottom. Note also that infrared spectroscopy is idiosyncratic in using wavenumber ('reciprocal centimetres', cm^{-1}) instead of wavelength along the x-axis and percentage transmittance instead of absorbance on the y-axis.

A major difference between infrared and ultraviolet spectroscopy is in the concentrations required for assay. In infrared spectroscopy as much as a 10% w/v solution of sample must be prepared. This means that the path length of the cells used in infrared must be very short, usually 0.025–0.1 mm (otherwise absorbance values would be too high). Another problem with infrared spectra is that the solvent used in the assay (usually chloroform or dichloromethane) also possesses chemical bonds that will absorb infrared radiation in some part of the spectrum, obscuring the absorption by the sample at these wavelengths. Samples are prepared in solution, in a mull or paste made with liquid paraffin (Nujol), or in a solid disc prepared by trituration with dry potassium bromide followed by compression in a hydraulic press.

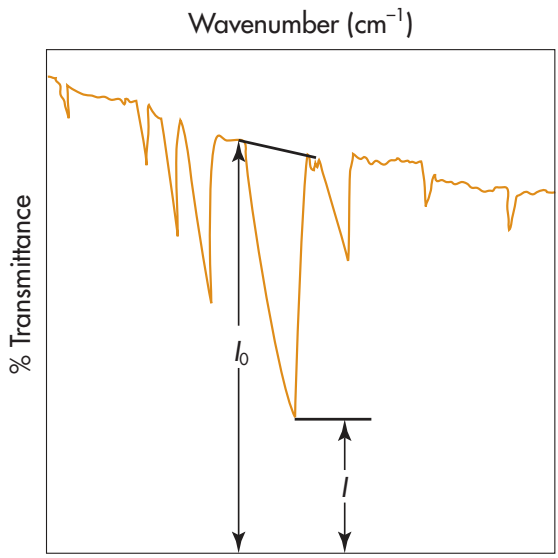


Figure 7.20 The application of the baseline technique.

Fluorimetry

Fluorimetry is an analytical technique that relies on the emission of electromagnetic energy by molecules. The chromophore of the molecule must be capable of absorbing light (usually in the ultraviolet region of the electromagnetic spectrum) and emitting it again (usually in the visible portion of the spectrum) to be measured by a detector. To do this, the chromophore (sometimes called the *fluorophore*) must be shielded from the normal processes that account for energy loss in the excited state (e.g. collision between molecules). *The light that is emitted by the sample is always of longer wavelength (i.e. lower energy) than the light absorbed by the molecule*. This is known as Stokes' law (after the Irish physicist Sir George Gabriel Stokes) and applies because the energy transfer process occurring within the excited state of the molecule is not 100% efficient. Some of the absorbing energy is lost (e.g. in vibrational transitions) and so the light emitted as fluorescence is of lower energy than the light absorbed.

The instruments used to measure fluorescence, spectrofluorimeters, require a high-energy light source (usually a xenon arc lamp) to deliver the energy required to excite the molecule, and the detector of the instrument is usually aligned at 90° to the source to minimise detection of light directly from the light source. A spectrofluorimeter also requires two monochromators, one to select the wavelength of excitation light and the other to select the wavelength of light emitted by the sample. Analytical spectrofluorimetry is widely used in pharmaceutical analysis, particularly for the assay of highly potent drugs present in medicines in tiny amounts.

There are two main advantages in the use of fluorimetry over ultraviolet spectroscopy:

1. The presence of two monochromators, and the fact that not all molecules with a chromophore fluoresce, means that fluorimetry is more specific than ordinary ultraviolet spectroscopy. This allows drugs that fluoresce to be assayed in the presence of other compounds that would interfere in an ultraviolet assay.
2. Fluorimetry is approximately 100 times more sensitive than ultraviolet spectroscopy and is ideal for the analysis of very small amounts of potent drugs. Examples are the steroids digoxin in Digoxin Tablets BP and the contraceptive agent ethinylestradiol, which is present at levels of only 30 µg per tablet.

Quenching

This phenomenon, as its name suggests, is a reduction in the intensity of light emitted during fluorescence. There are two types: self-quenching and quenching by other, non-fluorescent agents.

Self-quenching is seen at high sample concentrations (e.g. approximately 0.005% w/v) and is due to fluorescence concentrating at the irradiated face of the sample cell, instead of distributing throughout the cell. A plot of intensity of light emitted versus concentration should be linear (obeying the Beer–Lambert law). If the linearity of the graph falls off at high concentration, self-quenching should be suspected. If self-quenching of fluorescence is a problem during an assay, dilution of the sample (e.g. to 0.000 05% w/v) should eliminate the problem and restore linearity.

Quenching of fluorescence also occurs due to the action of other, non-fluorescent compounds. The most common quenching agents encountered in pharmaceutical analysis are halide ions (Cl^-, Br^-, I^-). The fluorescence of a drug such as quinine is much lower if the sample is dissolved in hydrochloric acid than if it is dissolved in sulfuric acid, even though the concentrations of the quinine and the pH of the solutions are kept constant. Quinine is an interesting compound (Figure 7.21). It is an alkaloid extracted from the bark of the *Cinchona* tree and was used extensively for the treatment of malaria. It is a very bitter substance and is responsible for the characteristic bitter taste of tonic water. An acidic solution of quinine displays a deep blue fluorescence, which can often be observed in the glass of someone drinking tonic water (with or without gin) in a bar or club with ultraviolet lights.

Structure elucidation

Frequently, during organic synthesis or drug metabolism studies, unknown compounds are isolated and their structure must be confirmed or, if they are totally unknown, solved using a combination of analytical techniques. Exercises of this type use *qualitative* spectroscopy rather than the *quantitative* aspects discussed so far. Whole university courses and many textbooks and online tutorials have been produced to cover elucidation of chemical structure, and a detailed treatment of the subject is beyond the scope of this book. However, a brief overview of the major techniques and their limitations may be useful.

Nuclear Magnetic Resonance (NMR)

This phenomenon was first discovered in 1946 by Felix Bloch and Edward Mills Purcell and won the 1952 Nobel Prize in Physics for the discoverers (who famously stated that they could not see an application for their discovery!).

Magnetic resonance arises because certain atomic nuclei can behave like small magnets. Only certain nuclei can exhibit this property (those with a nuclear magnetic spin that is an odd multiple of 1/2, not an integer), of

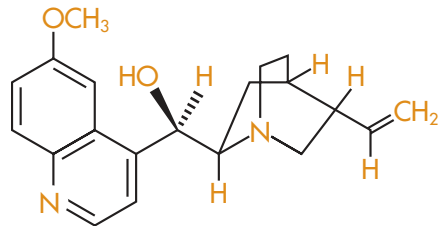


Figure 7.21 The structure of quinine.

which the most important are ^{1}H, ^{13}C, ^{15}N and ^{31}P, which have spin 1/2. The nuclei can exist with spins of +1/2 or −1/2 which are normally randomly oriented in space and have equal energy. However, if these nuclei are placed in an external magnetic field they may align themselves either *with* the direction of the external field (the low energy orientation) or *opposed* to the direction of the external field (a higher energy situation). If the nucleus is in the lower energy state (analogous to the 'ground state' in spectroscopy), and is irradiated with energy of the correct frequency, the nucleus can flip to the higher energy state. The energy required to bring about this effect is in the radiofrequency part of the electromagnetic spectrum. The nucleus can return to the lower energy state by losing energy to its surroundings – a process known as *relaxation*. Critically, the frequency of energy required to flip a nucleus from one state to another varies slightly depending on the electronic environment of the atom in question. This frequency is what is measured in a spectrophotometer and what gives rise to the NMR spectrum. It also means that NMR is exquisitely sensitive to tiny changes in the electronic environment of an atom – invaluable for the elucidation of unknown chemical structures.

Instrumentation

The apparatus required for obtaining an NMR spectrum comprises a powerful magnet capable of producing a homogeneous magnetic field, a radiofrequency oscillator and receiver and electronics capable of integrating and displaying spectra. Modern instruments are interfaced with a computer and the hard disc of the computer is used for data storage, a library of past spectra, and so on. A schematic diagram of an NMR spectrophotometer is shown in Figure 7.22.

Solvents for NMR

In NMR, the sample is usually analysed in solution (solid-state NMR does exist, but this is a specialised technique). Since hydrogen is active in NMR,

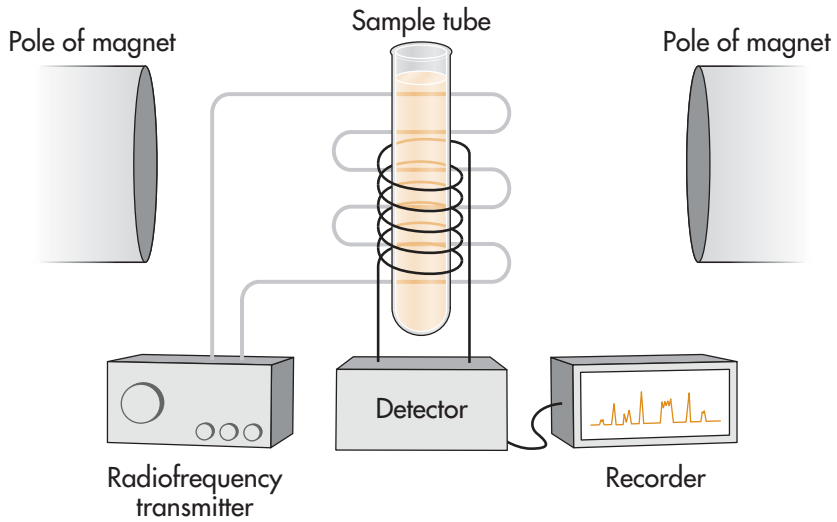


Figure 7.22 Diagram of an NMR spectrophotometer.

and most organic solvents contain hydrogen, the signal from the solvent would swamp the signal from the small amount of sample. To overcome this, use is made of *deuterium*, one of the heavy isotopes of hydrogen. Deuterium (symbol D) has one more neutron than 'ordinary' hydrogen (or 'protium') and a nuclear spin of 1. This means it is not active in NMR. A number of *deuterated solvents* are now available and these are used as solvents for NMR samples. Examples include deuterated water (D_2O), deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD). Needless to say, these solvents are considerably more expensive than their non-deuterated counterparts, but are otherwise chemically identical.

Internal standards

In no two machines will the magnets produce absolutely identical magnetic field strengths. This means that a peak with an absolute chemical shift of (say) 2.0 ppm measured in one instrument will not resonate at 2.0 ppm in another instrument even though it is nominally of the same field strength. To overcome this problem, use is made of an *internal standard* which is added to every sample and all measurements are made relative to the internal standard. There are a number of possible internal standards, but the most commonly used is *tetramethylsilane*, or TMS, $Si(CH_3)_4$. This substance has a number of advantages as an internal standard. It is chemically inert, so it will not react with the sample; it is volatile, so it can be removed easily; it has 12 identical hydrogen atoms, which give a strong

signal; and it naturally resonates at very high field position. It therefore appears at the right-hand side of a conventionally displayed spectrum. The instrument is zeroed on TMS and all measurements are made relative to the TMS peak.

Deuterium exchange

Deuterated water (deuterium oxide or D_2O) does not give rise to a signal in NMR and this can be used as a diagnostic test for certain types of hydrogen atom. Functional groups in which hydrogen is bonded to an electronegative atom such as oxygen (—OH), nitrogen (—NH_2) or sulfur (—SH) are termed 'exchangeable' because when D_2O is added to the spectrum, the weakly acidic hydrogen can exchange with a deuterium from the water according to the equation below:

$$\text{R-OH} + D_2O \rightleftharpoons \text{R-OD} + \text{DHO}$$

The effect of this is that the signal on the NMR due to the acidic hydrogen disappears when D_2O is added. The spectrum is run normally in a non-polar solvent such as deuterated chloroform; then, if the presence of exchangeable hydrogen atoms in the sample is suspected, a few drops of D_2O are added and the original spectrum is re-run. If the peak in question disappears in the presence of D_2O, this is proof of the presence of exchangeable hydrogen atoms in the molecule. An unfortunate side-effect of adding D_2O to the sample is that a large peak due to mono-deuterated water, DHO, appears in the spectrum. This peak can sometimes hide more interesting peaks in the spectrum.

Structure elucidation using NMR

When NMR is used to elucidate the structure of an unknown compound, there are three pieces of information which should be considered:

- the position of resonance of the peak (or *chemical shift*)
- the number of hydrogen atoms causing the signal (*integration*)
- the number of peaks constituting the signal (*multiplicity*).

Chemical shift

The position of a line in NMR spectroscopy is the most important piece of information when solving an unknown spectrum. The difference in frequency of resonance of different groups of atoms is a very small fraction of the total magnetic field and, as stated above, the absolute position of resonance cannot be determined due to variations in the strength of

magnets. To overcome this, all chemical shift values are measured relative to an internal standard (usually TMS) and expressed as 'chemical shift', represented by the dimensionless term δ, the Greek letter delta. Mathematically, this can be written as

$$\text{Chemical shift, } \delta = \frac{\text{shift observed}}{\text{oscillator frequency}} \times 10^6$$

The factor of 10^6 is introduced to give convenient, easily remembered values and this gives rise to the term 'ppm' as the unit for chemical shifts. This use of ppm should not be confused with its established use as a unit of concentration (μg ml^{-1} or μg g^{-1}) and arises because most resonances occur within ten millionths of the available field, or within 10 units from TMS.

When an atom is placed in a magnetic field, electrons start to circulate around the nucleus and this circulation induces a second, local magnetic field which opposes the applied external field. This process is called 'shielding'; the electron density around the nucleus increases and the nucleus will appear to resonate at high field position ('upfield' or at the right-hand side of a conventional spectrum). If a nucleus is attached to an electronegative atom such as oxygen or nitrogen, the inductive effect of the electronegative atom reduces the electron density around the nucleus and the atom is said to be 'deshielded' and will resonate to the left or 'downfield'. A table of common proton chemical shift values is shown in Table 7.1.

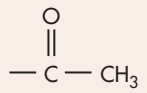
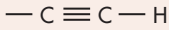
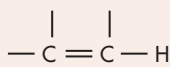
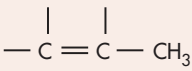
Integration

In proton NMR spectroscopy, after the spectrum has been run, it is usual to change to 'integration' mode and run the spectrum a second time. This gives rise to a second trace (often a different colour is used) where the trace jumps as it goes over a peak by an amount proportional to the number of hydrogen atoms causing the signal. Strictly speaking, the integration gives the *ratio* of hydrogen atoms responsible for the signal (i.e. the integration for two signals consisting of 3 and 2 protons would be the same as for 6 and 4), although this is not usually a problem as the total number of hydrogen atoms in the molecule may be known from another technique (such as mass spectrometry).

Multiplicity and spin–spin coupling

In NMR, the signal due to the resonance of a particular nucleus is often split into a number of peaks. This is termed the 'multiplicity' of the signal

Table 7.1 Typical values of proton NMR chemical shifts

Type of proton	Approximate δ value
Alkane—CH_3	0.9
Alkane—CH_2—CH_3	1.3
Alkane—CH—	1.4
	2.1
	2.5
R—CH_2—X (X = halogen, —O—	3.4
	5–6
	1.7
Ph—H	7.2
Ph—CH_3	2.3
R—CHO	9–10
R—COOH	10–12
R—OH	Variable, about 2–5
Ar—OH	Variable, about 4–7
R—NH_2	Variable, about 1.5–4

and arises due to the phenomenon of spin–spin coupling. When a sample is placed into a magnetic field, the NMR-active nuclei align themselves either with the external field (i.e. in the same direction) or opposed to the external field (in the opposite direction). This means that the field experienced by a nucleus depends on whether neighbouring nuclei align to augment the applied field or to decrease it. The result is that the original signal now arises from nuclei experiencing two different values of chemical shift and appears to split into two distinct signals. In proton NMR, when there is one neighbouring proton, the signal is split into two peaks of equal intensity (a 'doublet'); when there are two neighbouring protons, the signal is split into three signals (a 'triplet') with intensities 1 : 2 : 1; and if there are three neighbouring identical protons, the signal is split into four peaks (a 'quartet') with intensities 1 : 3 : 3 : 1, and so on. Figure 7.23 illustrates this effect for two neighbouring hydrogen atoms.

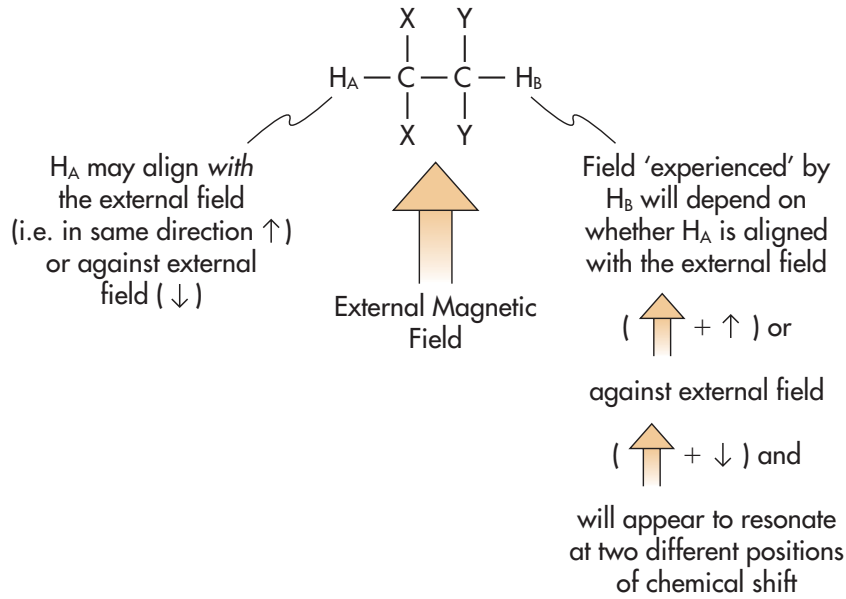


Figure 7.23 Spin–spin coupling.

For simple molecules, where there is no long-range coupling taking place, a proton NMR signal will be split into $(n + 1)$ peaks where n = the number of adjacent (magnetically equivalent) hydrogen atoms. It follows from this that a hydrogen atom without any neighbouring hydrogen atoms will resonate as a single peak; a hydrogen adjacent to one other will appear as a doublet, a hydrogen adjacent to two others as a triplet, and so on. The relative intensities of the peaks can be predicted, since the intensities follow the coefficients of Pascal's triangle (a geometric arrangement of numbers where each number is the sum of the two numbers above it) as shown below.

1
1 1
1 2 1
1 3 3 1
1 4 6 4 1

Part of Pascal's triangle

This type of simple treatment of coupling is sufficient for simple molecules. For more complex molecules, spin–spin coupling occurs between atoms and non-equivalent neighbouring atoms. If this is the case, it is often more useful to consider a 'tree diagram' to describe the splitting taking place. Figure 7.24 shows the splitting patterns obtained when an atom couples

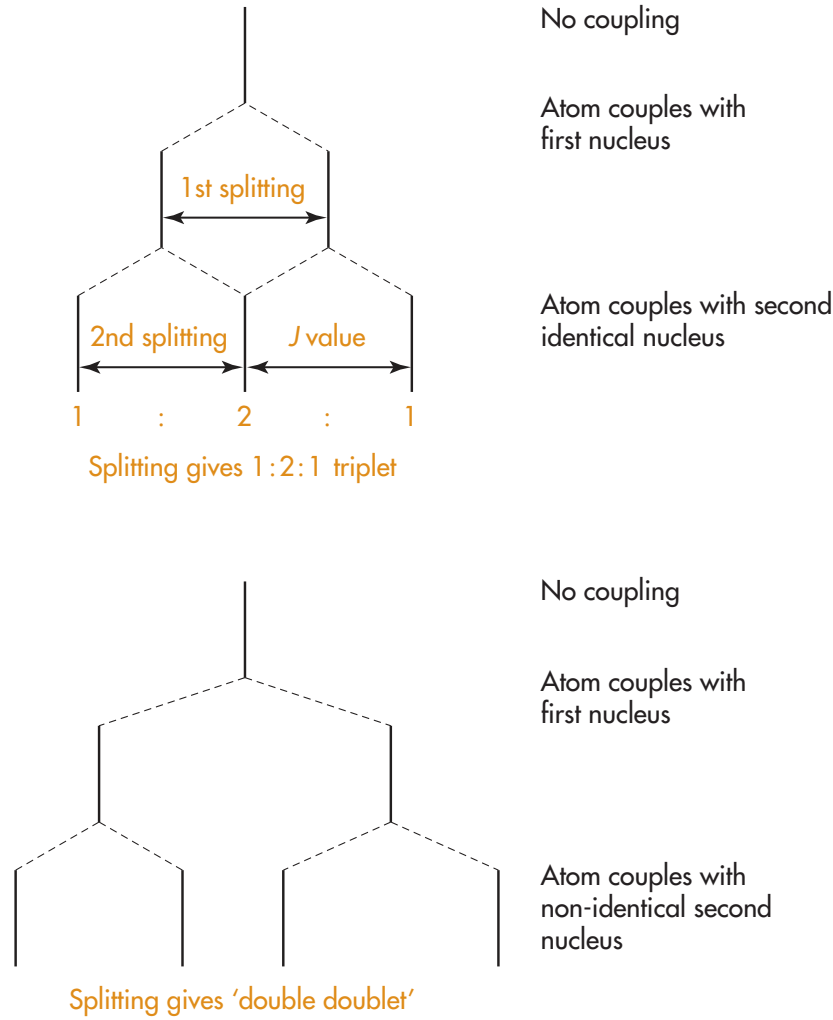


Figure 7.24 'Tree diagram' for spin–spin coupling.

with two identical neighbours (to give a triplet) and where coupling occurs with non-identical neighbours to give a 'double doublet'.

The extent to which a peak is split by coupling with a neighbouring atom is called the coupling constant (or *J* value) and is measured in hertz. The coupling constant may be calculated by multiplying the difference in chemical shift (in ppm) by the operating frequency of the instrument (in MHz), although modern instruments routinely display *J* values for all spectra.

A worked example of the use of NMR and other techniques of structure elucidation is presented as Tutorial example Q4 at the end of the chapter. This example should be studied closely to appreciate the strategies employed to solve unknown chemical structures.

Mass spectrometry

The final technique of instrumental analysis to consider in this chapter is *mass spectrometry*. This is not a spectroscopic technique (i.e. it does not involve the measurement of light radiation) but it is a very important technique for the elucidation of unknown chemical structures.

Mass spectrometry (or commonly 'mass spec') is an analytical technique in which the sample molecule is ionised either by chemical reaction (chemical ionisation, or CI), or through collision with a beam of high-energy electrons (electron ionisation, or EI). This fragments the sample into ions of different masses and these fragments are then accelerated into a vacuum chamber and separated according to their mass-to-charge ratio (m/z). The instrument which measures these molecular fragments is called a *mass spectrometer* and consists of three principal components, an ion source, a mass analyser and a detector system. A diagram of a mass spectrometer is shown in Figure 7.25.

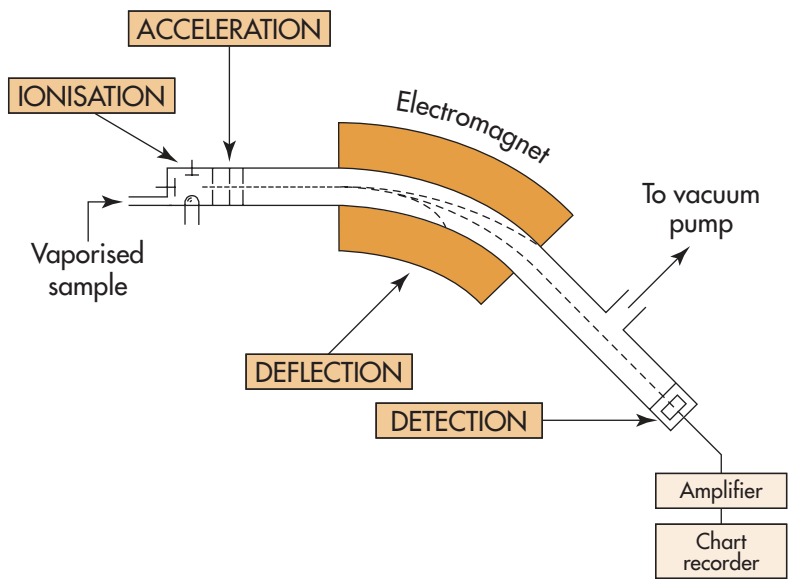


Figure 7.25 Diagram of a mass spectrometer.

The mass spectrometer plots the spectrum of the compound, in which the mass of the fragment divided by its charge (m/z) is plotted on the x-axis while the relative intensity of the fragment is plotted on the y-axis. The strongest peak in the spectrum is called the *base peak* and the intensities of all other peaks in the spectrum are measured relative to this peak. It is important for students to realise that the intensity of a peak measured in a mass spectrometer may be unrelated to the 'quantity' or 'amount' of an ion present in the sample and that mass spectrometry cannot predict, for example, the composition of a mixture. It merely identifies which fragments of the molecule are present in the chamber of the instrument.

Nevertheless, a huge amount of chemical information may be obtained from a mass spectrum, and for the purposes of structure elucidation, the most important piece of information is the presence or absence of a *molecular ion* peak. This peak is obtained when some of the sample ionises but does not fragment. The molecular ion peak corresponds to the loss of one electron from the intact molecule (to give a species called a *molecular ion radical*) and is usually the fragment of highest mass in the spectrum. The mass-to-charge ratio of this ion is important as it usually provides the relative molecular mass of the sample molecule. Occasionally, the molecule may ionise twice, and since the instrument measures the mass-to-charge ratio for fragments, a molecule which ionises twice will appear at a m/z value of half of the expected value for the molecular ion.

Recent advances in mass spectrometry have produced a number of 'soft' ionisation techniques such as fast atom bombardment (FAB) or electrospray ionisation. The major advantage of these techniques is that they are less likely to break the sample into small fragments and are more likely to produce a molecular ion. This is particularly important in the analysis of macromolecules such as proteins and nucleic acids.

Another recent development in mass spectrometry is the advent of the so-called 'hyphenated' techniques. In these a mass spectrometer is coupled to another analytical technique such as gas or liquid chromatography. In gas chromatography–mass spectrometry (GC-MS) a gas chromatograph is used to separate a mixture into individual components which are then injected directly into the mass spectrometer for detection and analysis. Liquid chromatography–mass spectrometry (LC-MS) is a similar technique in which liquid chromatography is used to separate the components of a mixture prior to introduction into the mass spectrometer. These techniques are very powerful but have to overcome the huge technical difficulties associated with removing relatively large amounts of gas or liquid mobile phases from the sample prior to injection into the vacuum chamber of the mass spectrometer.

There is a technique called tandem mass spectrometry (MS-MS) which allows for multiple cycles of mass selection or analysis. In MS-MS, two or more mass spectrometers are connected together to allow separation of one particular molecular fragment (e.g. a drug metabolite or a pharmacologically active peptide) from a complex mixture. This molecule is then injected into a second mass spectrometer where fragmentation occurs and either all of the fragments are identified or the presence of one particular fragment is monitored. The sensitivity of this technique is extremely high and allows the measurement of tiny amounts of substance from a complex background matrix.

Mass spectrometers have even made it into space, where modified GC-MS equipment has been installed in space probes to measure the atmospheric composition of distant planets and their moons.

Tutorial examples

Q *1 Five standard solutions of a drug (relative molecular mass 288.4) were prepared in spectroscopy-grade ethanol and the absorbance of each solution was measured at 285 nm in 1 cm cells.*

Concentration (mg/100 mL)	*Absorbance*
1.25	0.697
1.00	0.562
0.75	0.421
0.50	0.281
0.25	0.140

(a) Is Beer's law obeyed for this drug at this wavelength?
(b) Calculate the A_1^1 and molar absorptivity for this drug at 285 nm.
(c) *Calculate the % transmittance given by a 0.5 mg/100 mL solution in a 2 cm cell.*

A 1(a) Whenever a column of numbers appears in an examination question it is crying out for a graph to be plotted. In this case, a plot of absorbance versus concentration yields a straight line through the origin and confirms Beer's law for this drug at this (and only this) wavelength.
(b) The value is simply determined from the gradient of the graph obtained in (a) if the units of concentration are converted

to percentage weight in volume. The path length of the cell is 1 cm, so the Beer–Lambert equation simplifies to a $y = mx$ type of equation with the slope A_1^1.

Alternatively, the value can be obtained by calculation:

$$1 \text{ mg}/100 \text{ mL} = 0.001 \text{ g}/100 \text{ mL}$$

$$= 0.001\% \text{ w/v}$$

$$A = A_1^1 cl$$

Hence,

$$0.562 = A_1^1 \times 0.001 \times 1$$

Therefore,

$$A_1^1 = 562$$

In practice, A_1^1 would be calculated for each solution and the answers averaged, although the graphical method above is probably more accurate and quicker.

The molar absorptivity, ε can be calculated as follows.

$$\frac{1 \text{ mg}}{100 \text{ mL}} = 0.01 \text{ g L}^{-1}$$

$$= \frac{0.01}{288.4 \text{ mol L}^{-1}}$$

$$A = \varepsilon cl$$

Hence,

$$0.562 = \varepsilon \times \frac{0.01}{288.4 \text{ mol L}^{-1}} \times 1$$

Therefore,

$$\varepsilon = 16\,210$$

Note that ε, the absorbance of a 1 M solution, is much greater than A_1^1, the absorbance of a 1% w/v solution.

(c) The first step in calculating the percentage transmittance is to calculate the absorbance of the 0.5 mg/100 mL solution.

Using A_1^1 and converting the units of concentration to percentage weight in volume,

$$A = 562 \times 0.0005 \times 2$$

$$A = 0.562$$

Unsurprisingly, this is the same absorbance as given by twice the concentration measured in a cell of half the thickness.
Hence,

$$\log \frac{I_0}{I} = 0.562$$

Therefore,

$$\frac{I_0}{I} = 3.648$$

and

$$\% \text{ Transmittance} = 100 \frac{I_0}{I} = 100 \times \frac{1}{3.648} = 27.4\%$$

i.e. for this solution, 72.6% of available light is absorbed and 27.4% is transmitted.

Q

2 The A_1^1 of cocaine at its λ_{max} is 430. In an experiment, 11.20 mg of cocaine was weighed and made up to 1 litre with 0.1 M HCl. If the measured absorbance in a 1 cm quartz cell was 0.470, calculate the purity of the sample of cocaine.

A

2 The first step is to solve the Beer–Lambert equation:

$$A = A_1^1 cl$$

$$= 430 \times c \times 1$$

Therefore,

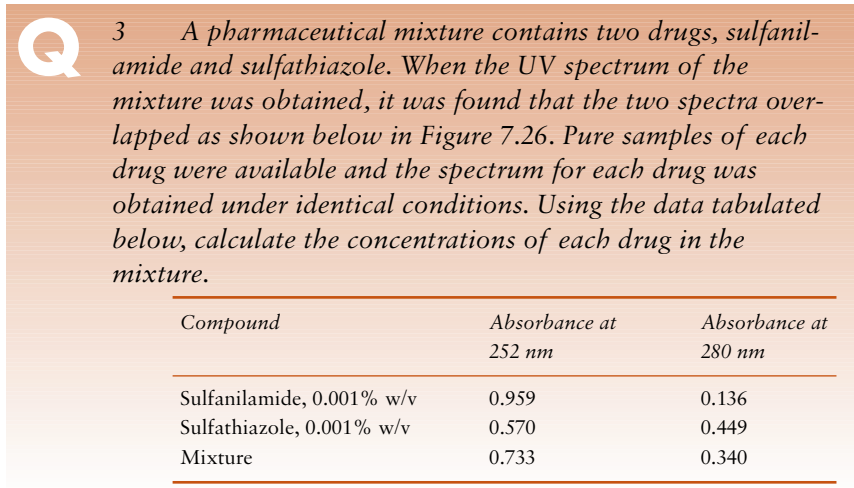
$$c = \frac{0.470}{430} = 0.001093\% \text{ w/v}$$

Note that if is used, the units of concentration must be percentage weight in volume, i.e. g/100 mL:

$$0.001093 \text{ g}/100 \text{ mL} = 10.93 \text{ mg L}^{-1}$$

However, 11.20 mg was weighed; therefore,

$$\% \text{ Purity of sample} = \frac{10.93}{11.20} \times 100 = 97.6\%$$



3 A pharmaceutical mixture contains two drugs, sulfanilamide and sulfathiazole. When the UV spectrum of the mixture was obtained, it was found that the two spectra overlapped as shown below in Figure 7.26. Pure samples of each drug were available and the spectrum for each drug was obtained under identical conditions. Using the data tabulated below, calculate the concentrations of each drug in the mixture.

Compound	*Absorbance at 252 nm*	*Absorbance at 280 nm*
Sulfanilamide, 0.001% w/v	0.959	0.136
Sulfathiazole, 0.001% w/v	0.570	0.449
Mixture	0.733	0.340

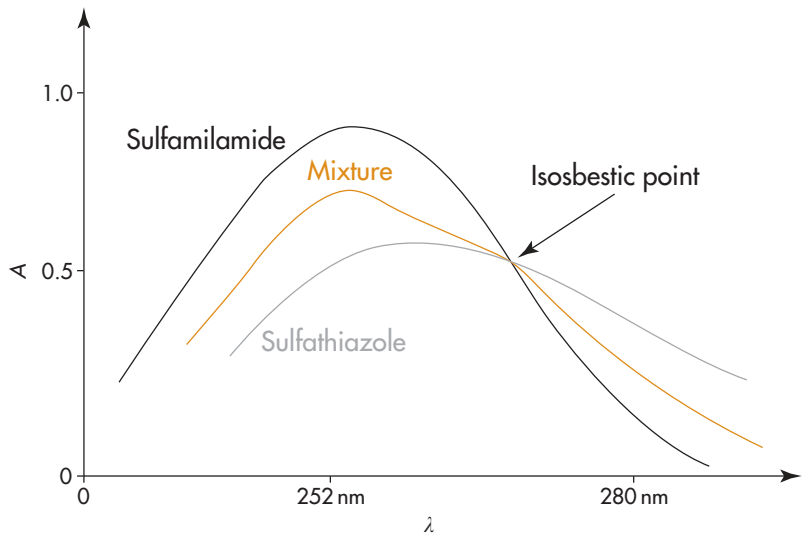


Figure 7.26 Spectra from two-component mixture.

A 3 Sulphonamides (see Chapter 3) are a group of antibacterial drugs widely used for the treatment of infection, although their use has declined in recent years with the advent of newer antibiotics such as penicillins. In this assay, a simple dilution cannot be used as the absorbance spectra of the two drugs overlap. We can, however, make use of the additive nature of absorbance measurements and use simultaneous equations to solve the problem. This method of analysis is used extensively for mixtures of components when the absorption characteristics of each substance separately are known. The absorption curves of the two separate compounds are plotted and from the traces obtained, two suitable analytical wavelengths are chosen. If possible, these wavelengths should coincide with reasonably flat regions of the absorption curve to avoid errors due to inaccurate selection of wavelength by the monochromator. Usually, the top of an absorbance peak is chosen, but any flat area (such as a peak shoulder or even a trough) can be used. In this example, wavelengths of 252 and 280 nm were selected.

The first task in solving the composition of this mixture is to calculate the A_1^1 value of each drug at each wavelength, using the Beer–Lambert equation. The results obtained from this are shown below:

Compound	*Calculated A_1^1 values*	
	At 252 nm	At 280 nm
Sulfanilamide	959	136
Sulfathiazole	570	449

If each component of the mixture obeys the Beer–Lambert law, then the total absorbance at each wavelength is given by the sum of each drug's contribution, or

$$A_1 = [(A_1^1)_x\, c_x\, l] + [(A_1^1)_y\, c_y\, l] \qquad \text{(equation 1)}$$

$$A_2 = [(A_1^1)_x\, c_x\, l] + [(A_1^1)_y\, c_y\, l] \qquad \text{(equation 2)}$$

where

A_1 is the total absorbance of the mixture at wavelength 1

A_{1x}^1 is the A_1^1 of substance x

A_{1y}^1 is the A_1^1 of substance y

A_2 is the total absorbance of the mixture at wavelength 2

c_x is the concentration of substance x (%w/v)

c_y is the concentration of substance y (%w/v)

l is the path length in cm

Using the data obtained above and since, in this example, path length = 1 cm,

Total absorbance at 252 nm, $0.733 = 959c_x + 570c_y$ (equation 1)

Total absorbance at 280 nm, $0.340 = 136c_x + 449c_y$ (equation 2)

Multiplying equation (1) by 136 and equation (2) by 959 yields

$99.688 = 130\,424c_x + 77\,520c_y$ (equation 3)

$326.06 = 130\,424c_x + 430\,591c_y$ (equation 4)

Subtraction of equation (3) from equation (4) gives

$226.372 = 353\,071c_y$

Therefore

$c_y = 0.00064\%$ **w/v**

Substituting this value of c_y into equation (1) gives

$c_x = 0.00039\%$ **w/v**

Q *4 Determine the structure of the unknown compound from the spectral data shown in Figure 7.27.*

A 4 There are many ways to approach a structure elucidation problem of this type and as long as the student solves the structure correctly (and explains the assignments made) marks will be awarded. This example makes greatest use of NMR data to solve the structure but it is equally acceptable to use MS or IR data to solve the unknown.

The IR spectrum is fairly straightforward (suggesting a simple molecule) with a strong peak at 1725 cm^{-1}. This peak is due to a carbonyl group.

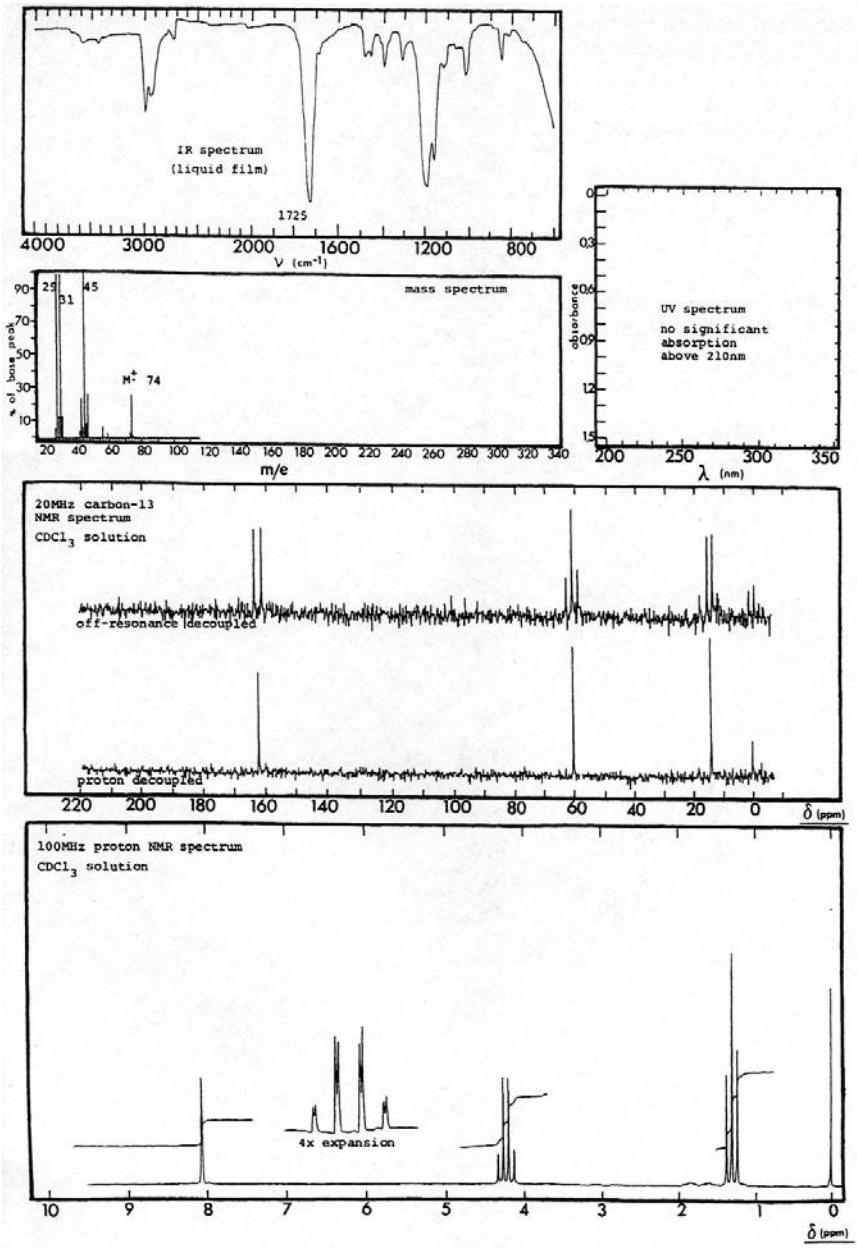


Figure 7.27 Data for Tutorial Example Q4.

No significant UV absorption >210 nm implies the absence of a chromophore and therefore precludes the presence of conjugated double bonds or aromatic rings in the molecule.

The mass spectrum shows a molecular ion radical at 74 mass units, which is the relative molecular mass of the compound. The smaller fragments may be used to confirm the structure once it is solved.

The proton NMR shows three distinct populations of hydrogen atoms, a triplet centred at 1.3 ppm, a quartet centred at 4.2 ppm and a single peak at 8.1 ppm. The sharp peak at zero is due to the internal standard, TMS.

A chemical shift of 1.3 suggests a methyl group. If this is true, the height of the integration trace must equal three hydrogen atoms. It follows from this that the signal at 4.2 is due to two hydrogen atoms and the signal at 8.1 is due to one hydrogen.

The methyl signal is split into three peaks and must therefore be adjacent to two other hydrogens ($3 = n + 1$), while the signal at 4.2 is split into four, so must be next to three hydrogens. These signals are splitting each other and are due to an ethyl group (CH_3CH_2—). The molecular mass of an ethyl group and a carbonyl equal 57 daltons, so only 17 mass units remain to be found, one of which is due to the downfield hydrogen resonating at 8.1. The missing atom must therefore be oxygen and since the chemical shift of the CH_2 is well downfield at 4.2, the oxygen must be attached to the CH_2 of the ethyl group. The solved structure is therefore the ethyl ester of formic acid (ethyl formate or ethyl methanoate):

$$H—COO—CH_2CH_3$$

The most common error in this example is to propose propanoic acid (CH_3CH_2COOH) as the answer. Although propanoic acid is isomeric with ethyl formate and does possess a carbonyl, the IR of the acid would show extensive hydrogen bonding, the NMR signal for the CH_2 would not be so far downfield, and the NMR peak due to the acidic hydrogen would resonate further downfield at approximately 11–12 ppm.

Problems

Q7.1 The assay for Diazepam Tablets BP is as follows.

> Weigh and powder 20 tablets. To a quantity of the powder containing 10 mg of diazepam, add 5 mL of water, mix and allow to stand for 15 minutes. Add 70 mL of a 0.5% w/v solution of sulfuric acid in methanol, shake for 15 minutes, add sufficient of the methanolic sulfuric acid to produce 100 mL and filter. Dilute 10 mL of the filtrate to 50 mL with the same solvent and measure the absorbance of the resulting solution at the maximum at 248 nm. Calculate the content of $C_{16}H_{13}ClN_2O$ taking 450 as the value of A_1^1 at this wavelength.

(a) Draw the part of the molecule responsible for the absorption of light in this assay (see Figure 7.28). What is this part of the molecule called?

(b) What assumptions are made in this assay?

(c) When this assay was carried out on 5 mg diazepam tablets, the following results were obtained:

> Weight of 20 tablets = 7.4878 g
>
> Weight of sample taken = 0.7450 g
>
> Absorbance of a 1 cm layer at 284 nm = 0.848

Calculate the content of diazepam in a tablet of average weight and hence calculate the percentage of the stated amount of diazepam in the tablets.

(d) Suggest another assay method for the determination of diazepam in Diazepam Tablets.

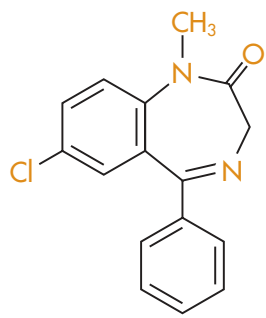


Figure 7.28 The structure of diazepam.

Q7.2 Mepyramine (Figure 7.29) is an antihistamine used in the treatment of insect bites and stings. The BP assay for 50 mg Mepyramine Tablets is as follows.

> Weigh and powder 20 tablets. To a quantity of the powder containing 0.1 g of mepyramine, add 75 mL of water and 5 mL of 2 M hydrochloric acid, shake vigorously for 15 minutes, and dilute to 100 mL with water. Centrifuge and dilute 10 mL of the clear supernatant liquid to 100 mL with water. To 10.0 mL of this solution add 10 mL of 0.1 M hydrochloric acid and dilute to 100 mL with water. Measure the absorbance of the resulting solution at the maximum at 316 nm taking 206 as the value of A_1^1.

This assay was carried out and the following data were obtained:

> Weight of 20 tablets = 2.1361 g
>
> Weight of sample used in assay = 0.2214 g
>
> Absorbance at 316 nm (measured in 1 cm cell) = 0.225

(a) Calculate the content of mepyramine in a tablet of average weight and the % stated amount.
(b) Why was the sample centrifuged prior to assay?
(c) Using the structure shown in Figure 7.29 as a guide, classify mepyramine as acidic, basic or neutral and hence explain the role of the hydrochloric acid in this assay.
(d) Explain fully how the blank solution for this assay would be prepared.

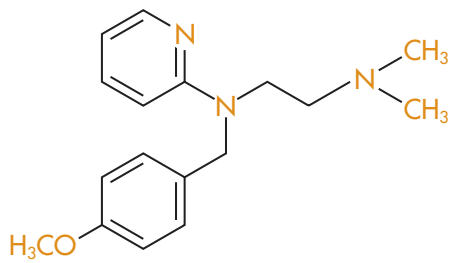


Figure 7.29 The structure of mepyramine.

Q7.3 Determine the structure of the unknown compound from the spectral data shown in Figure 7.30.

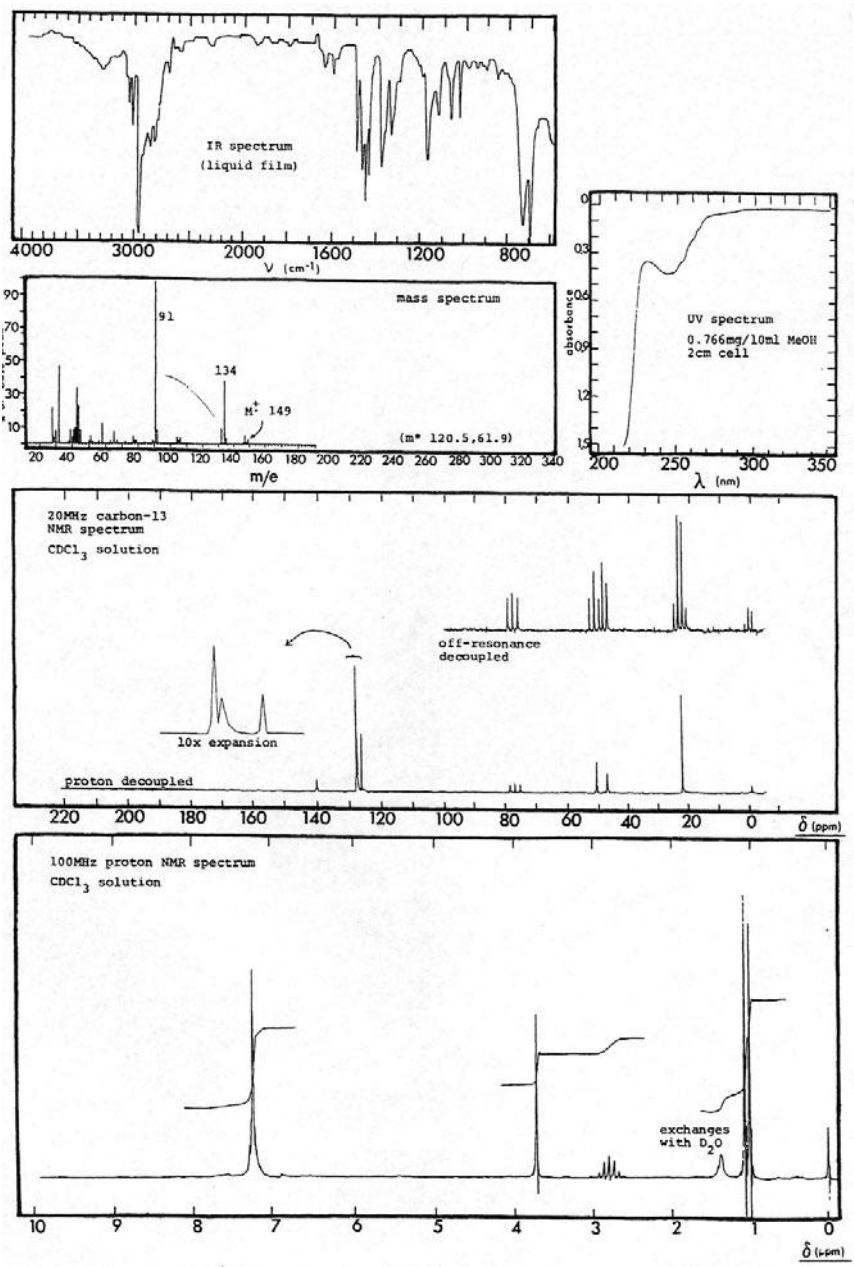


Figure 7.30 Data for Problem Q7.3.

(Answers to problems can be found on p. 263–264.)