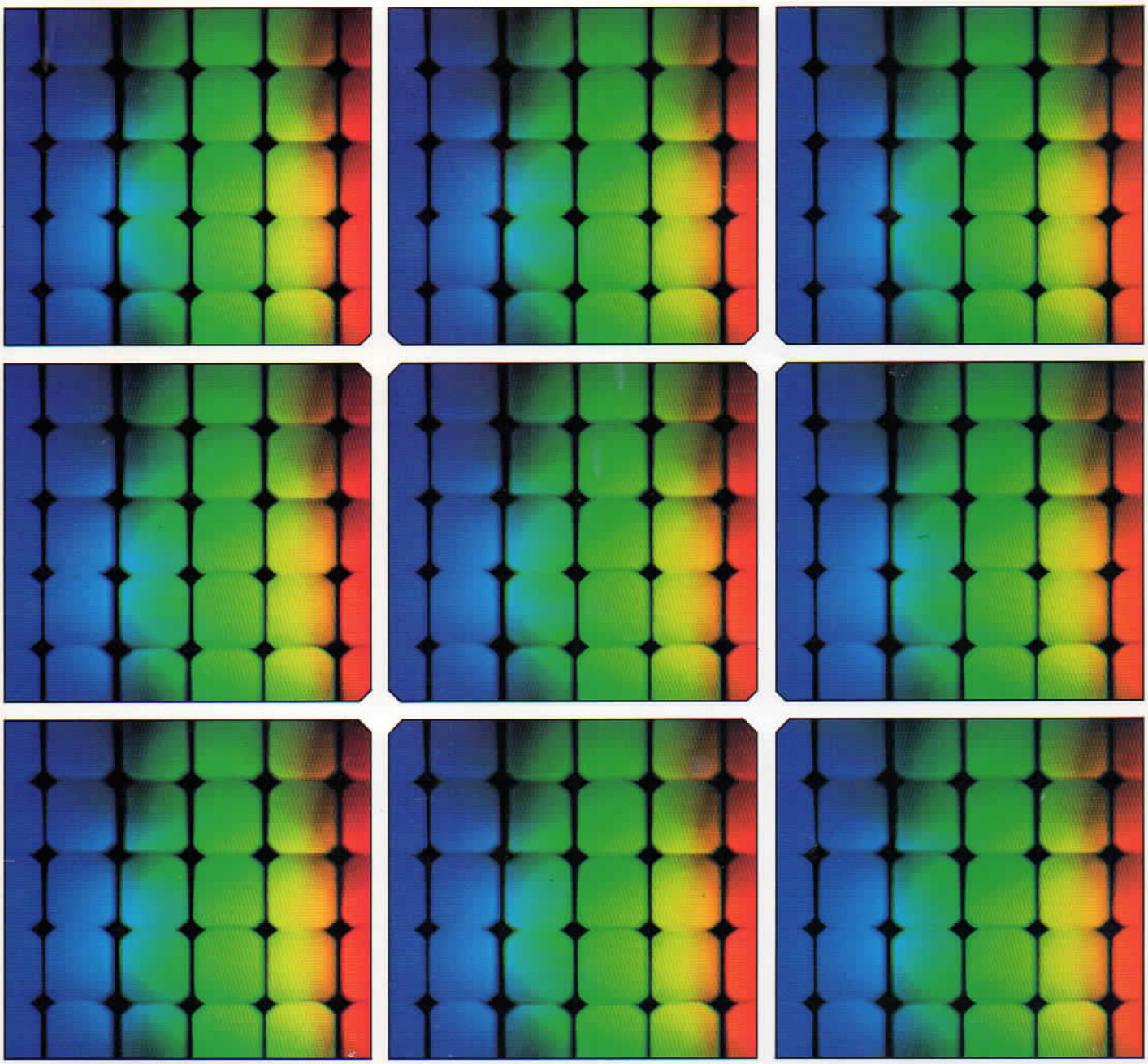
Introduction to Ultraviolet & Visible Spectrophotometry



PHILIPS Scientific & Analytical Equipment

PHILIPS

Introduction to Ultraviolet & Visible Spectrophotometry

Edited by J. E. Steward
with contributions from
C. T. Cottrell, D. Irish
and V. M. Masters

First edition published September 1975
—reprinted December 1975
July 1977
Second edition published July 1985

Published by Pye Unicam Ltd.
Printed by Heffers Printers Ltd, Cambridge.
Pye Unicam Ltd, York Street, Cambridge CB1 2PX Great Britain

Contents

Introduction

Ultraviolet and visible spectrophotometers are an almost indispensible part of the modern analytical laboratory's equipment. In many applications other techniques could be employed, but none surpass ultraviolet and visible spectrophotometry's unique combination of simplicity, versatility, speed, accuracy and cost-effectiveness.

Take the analysis of alloys as an example. The choice of techniques that we could use is very wide. If speed is an absolute priority, as in a foundry where it may be necessary to adjust the final composition of a melt before casting, then X-ray or arc emission techniques will be employed. Sample preparation is little more than a matter of solidification, cutting and polishing which, with a degree of mechanisation, enables results to be available in minutes. Both techniques require expensive instrumentation but can yield information on many elements simultaneously.

When time is not quite so pressing, perhaps in the quality control laboratory of a tool manufacturer which uses the products of the foundry, then atomic absorption might be used. Now the sample requires dissolution in acid before it can be sprayed into the flame of the spectrophotometer and only one element can be determined at a time. Consequently, a full check on any alloy is likely to take an hour or two but with the advantage of significantly lower investment in instrumentation.

Slightly further down the price scale we come to UV/visible spectrophotometry where the time to produce the results is even lengthier. This is due to the extra chemical steps required to produce a coloured solution whose intensity is proportional to the specific metal of interest and which is not affected by other, possibly quite similar, metals in the sample matrix. In these days no laboratory with any significant throughput of metallurgical samples would rely on UV/visible spectrophotometry for these assays but, despite this, few such laboratories can dispense altogether with the technique. UV/visible spectrophotometry still merits a place on the bench for its applicability to non-metallic elements and as a back-up to, or for cross-checking anomalous data from more dedicated equipment.

Metallurgy is unusual in the variety of analytical techniques that can be applied (we have neglected several others including polarography and gravimetry). In many other fields UV/visible spectrophotometry reigns supreme and has been so successful at some tasks that they have grown into quite distinct techniques of their own. Light of UV or visible wavelengths is used in clinical chemistry blood analysers, liquid chromatography detectors, densitometers and, more recently, plate readers employing enzyme-immunoassay chemistries for health-care and in the booming bio-technology field. Most of these techniques began by researchers building attachments for, or making modifications to, commercially available UV/visible spectrophotometers. Once the principle was proven, manufacturers made the hardware available as 'bolt-on' accessories until, as the technique gained acceptance, dedicated and optimised instruments became available. However, it is worthwhile remembering that whilst these dedicated devices usually pay scant acknowledgement to their origins in spectrophotometry, the quality of their performance is governed by the same general rules.

Most modern UV/VIS spectrophotometers can trace their ancestry back to the early 1940's when breakthroughs in amplifiers and detectors made precision spectrophotometry, as opposed to colorimetry and comparisons by eye, a practical proposition. In the years that have passed the available chemical methods library has grown so that a short search in one of the many text-books will reveal an assay for the determination of practically anything in anything else. UV/visible is overwhelmingly a quantitative technique and its potential for revealing structural information or qualitative information on the composition of totally unknown mixtures has largely been overtaken by techniques such as infrared spectrophotometry, mass spectrometry, NMR or one of the chromatographic processes. A few assays still remain of a qualitative nature, for example, an ultraviolet spectrum of a blood serum sample can reveal which of many barbiturates has been the cause of an overdose so that the appropriate remedy may be administered. However, the vast majority of all UV/visible assays are usually carried out at a fixed, well-documented wavelength.

Modern spectrophotometers have greatly benefited from the advances of our technological society and are now far more accurate, reliable and simple to operate than were their predecessors of only a few years ago. Most routine instrumentation now requires little in the way of operating skill other than the ability to press buttons in the correct order. However, spectrophotometers do not have finite boundaries to their operating envelopes and performance usually deteriorates gradually at the extremes of their wavelength ranges and as the transmission of the sample decreases. It is in these grey areas that even experienced spectroscopists may be misled by placing too great a trust in the infallibility of their instrumentation.

It is hoped that this booklet will assist the newcomer to ultraviolet and visible spectroscopy to avoid some of the pitfalls which may still be encountered when exploring the less well-trodden analytical paths. A booklet of this kind can never aspire to give the reader more than a brief introduction to the topic but it should help to establish a foundation of knowledge, which can be extended when circumstances dictate, by reference to more complete works on the subject.

1 Radiation and Light

(a) Definitions and Units

Radiation is a form of energy and we are constantly reminded of its presence via our sense of sight and ability to feel radiant heat. However, the human body is subject to a barrage of other forms of radiation, both natural and man-made. These kinds of radiation can only be detected with the help of devices which convert the energy into sensory signals which we can appreciate. For example, a Geiger counter enables us to detect radiation from radio-active rocks or from nuclear fallout while a television receiver converts radio-frequency energy into both visual and audio signals, hopefully for our entertainment.

Radiation may be considered in terms of a wave motion where the wavelength, λ, is the distance between two successive peaks. The frequency, ν, is the number of peaks passing a given point per second. These terms are related so that

$$c = \nu\lambda$$

where c is the velocity of light in vacuo.

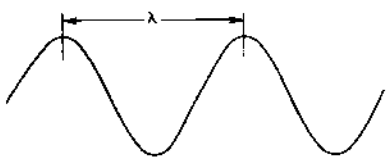


Fig. 1 The wavelength, λ, of Electromagnetic Radiation

The full electromagnetic radiation spectrum is continuous and each region merges slowly into the next. For spectrophotometric purposes we choose to characterise light in the ultraviolet and visible regions in terms of wavelength expressed in nanometres. Other units which may be encountered, but whose use is now discouraged, are the Ångstrom (Å) and the millimicron (mμ).

1 nm = 1 mμ = 10Å = 10^{-9} metres

For convenience of reference, definitions of the various spectral regions have been set by the Joint Committee on Nomenclature in Applied Spectroscopy:

Region	Wavelength, nm
Far ultraviolet	10–200
Near ultraviolet	200–380
Visible	380–780
Near infrared	780–3,000
Middle infrared	3,000–30,000
Far infrared	30,000–300,000
Microwave	300,000–1,000,000,000

The human eye is only sensitive to a tiny proportion of the total electromagnetic spectrum between approximately 380 and 780 nm and within this area we perceive the colours of the rainbow from violet through to red. If the full electromagnetic spectrum shown in *Fig. 2* was redrawn on a linear scale and the visible region was represented by a length of one centimetre, then the boundary between radio and microwaves would have to be drawn approximately 25 kilometres away!

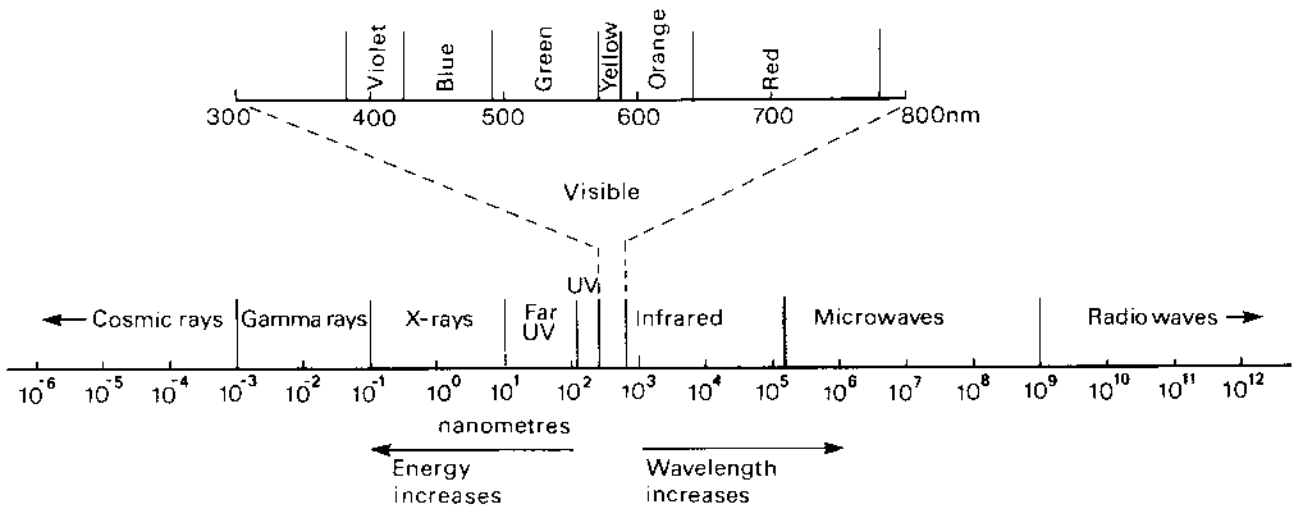


Fig. 2 The Electromagnetic Spectrum

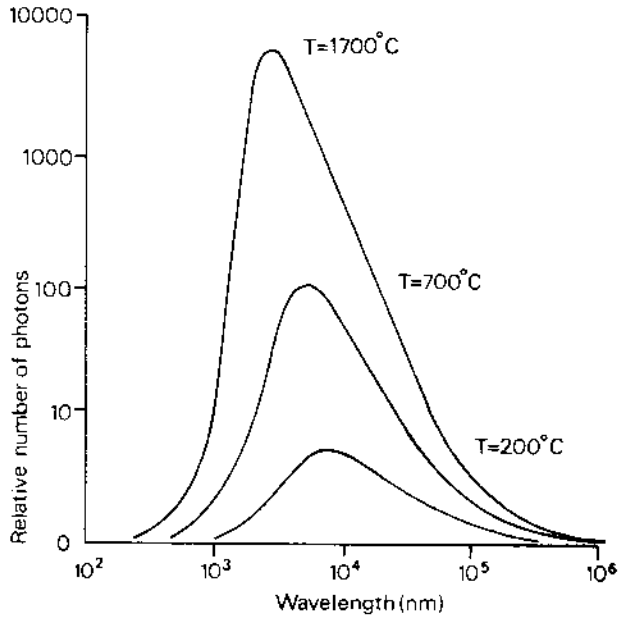


Fig. 3 Tungsten Filament Radiation

(b) Radiation Sources

Besides the sun, the most conveniently available source of visible radiation with which we are familiar is the tungsten filament lamp. If the current in the circuit supplying such a lamp is gradually increased from zero, the lamp filament at first can be felt to be emitting warmth, then glows dull red and then gradually brightens until it is emitting an intense white light and a considerable amount of heat.

The radiation from normal hot solids is made up of many wavelengths and the energy emitted at any particular wavelength depends largely on the temperature of the solid and is predictable from probability theory. The curves in *Fig. 3* show the energy distribution for a tungsten filament at three different temperatures. Such radiation is known as 'black body radiation'. Note how the emitted energy increases with temperature and how the wavelength of maximum energy shifts to shorter wavelengths.

2 Quantum Theory

To gain an understanding of the origins of practical absorption spectrophotometry a short diversion into quantum theory is necessary. For this purpose we must now think of radiation as a stream of particles known as photons instead of the waves considered earlier.

In our normal everyday world we are accustomed to objects being able to possess an infinite range of energy states, but the mechanics of atoms and molecules are different. They can only exist in a number of defined energy states or levels and a change of level requires the absorption or emission of an integral number of a unit of energy called a quantum, or in our context, a photon.

An analogy may serve to emphasise this difference between classical and quantum mechanics. In the normal world we could choose to scale the side of a building using a rope suspended from the roof. We can climb and stop at any number of points on the journey for a rest. There are no laws which govern where we can or cannot pause. However, if we were bound by the rules of quantum mechanics we would have to use a ladder where each time we climbed a rung we used a quantum of energy. We cannot stop between rungs but must expend a whole unit and continue to the next. The rungs of the ladder correspond to the fixed energy levels in the molecule or atom. While we can expend muscular energy, the molecule needs to absorb energy from a photon striking it in order to ascend one or more rungs. If we were to fall off the ladder and return to the ground state, we would probably emit some of our energy vocally, while the molecule would release a photon!

The energy of a photon absorbed or emitted during a transition from one molecular energy level to another is given by the equation

$$E = h\nu$$

where h is known as Planck's constant and ν is the frequency of the photon. We have already seen that

$$c = \nu\lambda$$

Therefore

$$E = hc/\lambda$$

Thus the shorter the wavelength the greater the energy of the photon and vice versa.

A molecule of any substance has an internal energy which may be considered as the sum of the energy of its electrons, the energy of vibrations between its constituent atoms, and the energy associated with rotation of the molecule.

The electronic energy levels of simple molecules are widely separated and usually only the absorption of a high energy photon, that is one of very short wavelength, can excite a molecule from one level to another. In complex molecules the energy levels are more closely spaced and photons of near ultraviolet and visible light can effect the transition. These substances, therefore, will absorb light in some areas of the near ultraviolet and visible regions.

The quantised vibration energy states of the various parts of a molecule are much closer together than the electronic energy levels and thus photons of lower energy (longer wavelength) are sufficient to bring about vibrational changes. Light absorption due only to vibrational changes occurs in the infrared region. The rotational energy states of molecules are so closely spaced that light in the far infrared and microwave regions of the electromagnetic spectrum has enough energy to cause these small changes.

Confining ourselves once again to ultraviolet and visible wavelengths, we should expect from this discussion that the absorption spectrum of a molecule, i.e. a plot of its degree of absorption against the wavelength of the incident radiation, should show a few very sharp lines. Each line should occur at a wavelength where the energy of an incident photon exactly matches the energy required to excite an electronic transition.

In practice we find that the ultraviolet and visible spectrum of most molecules consists of a few humps rather than sharp lines. These humps show us that the molecule is absorbing radiation over a band of wavelengths. One reason for this band, rather than line,

absorption is that an electronic level transition is usually accompanied by a simultaneous change between the more numerous vibrational levels. Thus, a photon with a little too much or too little energy to be accepted by the molecule for a 'pure' electronic transition can be utilised for a transition between one of the vibrational levels associated with the lower electronic state to one of the vibrational levels of a higher electronic state.

If we expand the ladder analogy, the electronic levels can be equated to the storeys of a building with each storey connected to the next by a ladder, the rungs of which are the vibrational levels. It is possible to change position from one storey to another, but it is also permitted to change from ground level to one rung up on the first storey. If the difference in electronic energy is 'E' and the difference in vibrational energy is 'e', then photons with energies of E, E + e, E + 2e, E − e, E − 2e, etc. will be absorbed.

Even with this explanation the spectrum of the molecule should be expected to consist of a major absorption line flanked by closely spaced, lesser lines known as 'vibrational fine structure' rather than the band absorption normally observed.

In fact, each of the many vibrational levels associated with the electronic states also has a large number of rotational levels associated with it. Thus, a transition can consist of a large electronic component, a smaller vibrational element and an even smaller rotational change. The rotational contribution to the transition has the effect of filling in the gaps in the vibrational fine structure.

In addition, when molecules are closely packed together as they normally are during spectrophotometric measurements, they exert influences on each other which slightly disturb the already numerous, and almost infinite energy levels and blur the sharp spectral lines into bands. These effects can be seen in the spectra of benzene as a vapour and in solution. In the vapour, the transitions between the vibration levels are visible as bands superimposed on the main electronic transition bands.

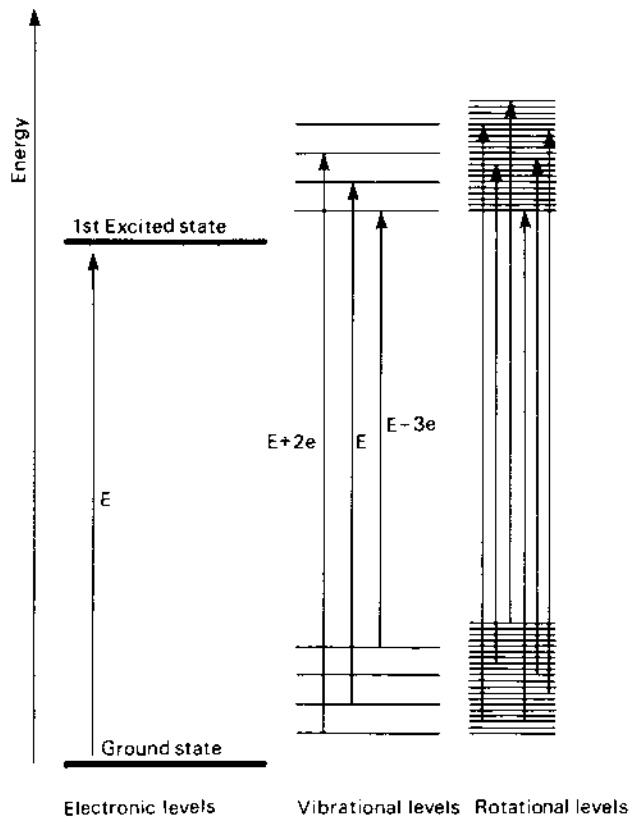


Fig. 4 Energy levels of a Molecule

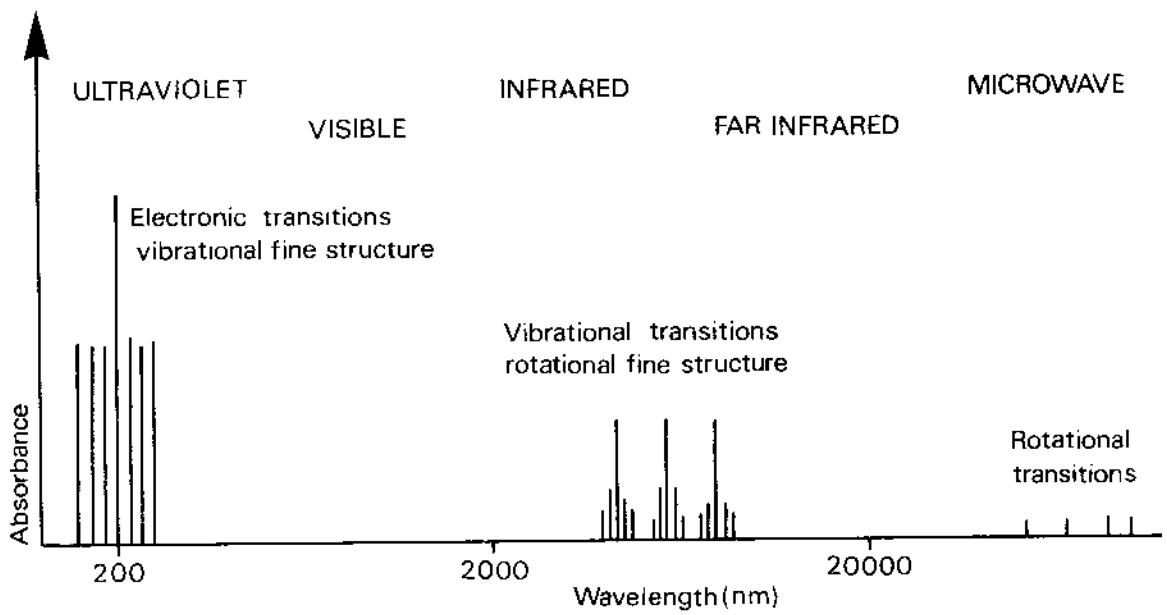


Fig. 5 Idealised Absorption Spectrum

In solution they merge together and at high temperature or pressure even the electronic bands can blur to produce single wide band such as that enclosed by the dotted line.

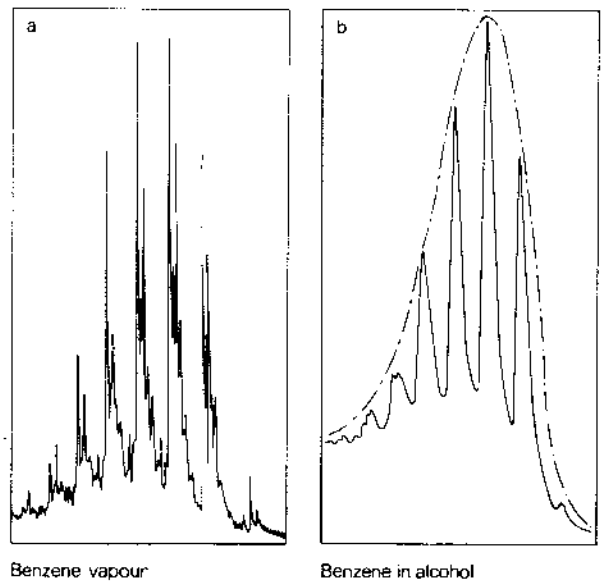


Fig. 6 Vapour and Solution Spectra of Benzene

3 Chemical Origins of Ultraviolet and Visible Absorption

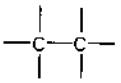
When white light falls upon a sample, the light may be totally reflected, in which case the substance appears white or the light may be totally absorbed, in which case the substance will appear black. If, however, only a portion of the light is absorbed and the balance is reflected, the colour of the sample is determined by the reflected light. Thus, if violet is absorbed, the sample appears yellow-green and if yellow is absorbed, the sample will appear blue. The colour is described as being complementary. However, many substances which appear colourless do have absorption spectra. In this instance the absorption will take place in the infra-red or ultraviolet and not in the visible region. Table 1 illustrates the relationship between light absorption and colour.

Colour Absorbed	Colour Observed	Absorbed Radiation (nm)
Violet	Yellow-Green	400–435
Blue	Yellow	435–480
Green-Blue	Orange	480–490
Blue-Green	Red	490–500
Green	Purple	500–560
Yellow-Green	Violet	560–580
Yellow	Blue	580–595
Orange	Green-Blue	595–605
Red	Blue-Green	605–750

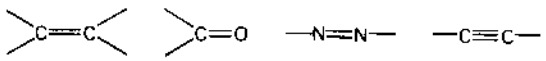
Table 1 Relationship between light absorption and colour.

A close relationship exists between the colour of a substance and its electronic structure. A molecule or ion will exhibit absorption in the visible or ultraviolet region when radiation causes an electronic transition within its structure. Thus, the absorption of light by a sample in the ultraviolet or visible region is accompanied by a change in the electronic state of the molecules in the sample. The energy supplied by the light will promote electrons from their ground state orbitals to higher energy, excited state orbitals or antibonding orbitals.
Potentially, three types of ground state orbitals may be involved:

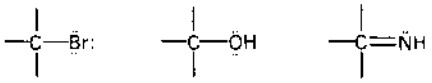
(i) σ (bonding) molecular orbital as in



(ii) π (bonding) molecular orbital as in



(iii) n (non-bonding) atomic orbital as in



In addition, two types of antibonding orbitals may be involved in the transitions:

(i) σ* (sigma star) orbital
(ii) π* (pi star) orbital

(There is no such thing as an n* antibonding orbital as the n electrons do not form bonds.)
A transition in which a bonding σ electron is excited to an anti-bonding σ orbital is referred to as an $\sigma \rightarrow \sigma^*$ transition. In the same way $\pi \rightarrow \pi^*$ represents the transition of a bonding π electron to an anti-bonding π orbital. An $n \rightarrow \pi^*$ transition represents the transition of one electron of a lone pair (non-bonding electron pair) to an anti-bonding π orbital. Thus the following electronic transitions can occur by the absorption of ultraviolet and visible light: $\sigma \rightarrow \sigma^*$, $n \rightarrow \sigma^*$, $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$. *Fig. 7* illustrates the general pattern of energy levels and the fact that transitions are brought about by

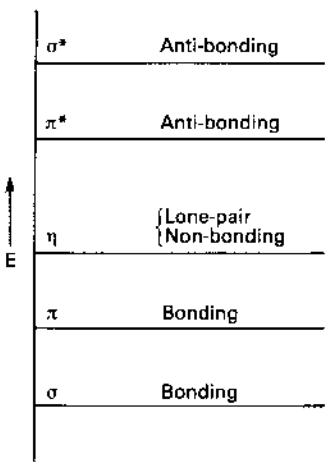


Fig. 7 Energy and Molecular Transitions

the absorption of different amounts of energy. Both $\sigma \rightarrow \sigma^*$ and $n \rightarrow \sigma^*$ transitions require a great deal of energy and therefore occur in the far ultraviolet region or weakly in the region 180–240 nm. Consequently, saturated groups do not exhibit strong absorption in the ordinary ultraviolet region. Transitions of the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ type occur in molecules with unsaturated centres; they require less energy and occur at longer wavelengths than transitions to σ^* antibonding orbitals. *Table 2* illustrates the type of transition and the resulting λ_{max}.

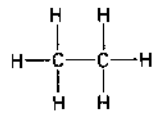
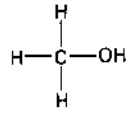
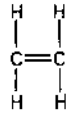
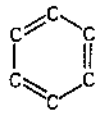
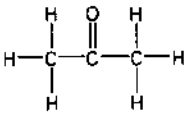
Molecule	Transition	λ_{max} (nm)
Ethane 	$\sigma \rightarrow \sigma^*$	135
Methanol 	$\sigma \rightarrow \sigma^*$	150
	$\eta \rightarrow \sigma^*$	183
Ethylene 	$\pi \rightarrow \pi^*$	175
Benzene 	$\pi \rightarrow \pi^*$	254
Acetone 	$\eta \rightarrow \pi^*$	290

Table 2 Examples of Transitions and Resulting λ max

It will be seen presently that the wavelength of maximum absorption and the intensity of absorption are determined by molecular structure. Transitions to π^* antibonding orbitals which occur in the ultraviolet region for a particular molecule may well take place in the visible region if the molecular structure is modified. Many inorganic compounds in solution also show absorptions in the visible region. These include salts of elements with incomplete inner electron shells (mainly transition metals) whose ions are complexed by hydration, e.g. $[Cu(H_2O)_4]^{2+}$. Such absorptions arise from a charge transfer process, where electrons are moved from one part of the system to another by the energy provided by the visible light.

(i) Correlation of Molecular Structure and Spectra

Conjugation

$\pi-\pi^*$ transitions, when occurring in isolated groups in a molecule, give rise to absorptions of fairly low intensity. However, conjugation of unsaturated groups in a molecule produces a remarkable effect upon the absorption spectrum. The wavelength of maximum absorption moves to a longer wavelength and the absorption intensity often increases greatly.

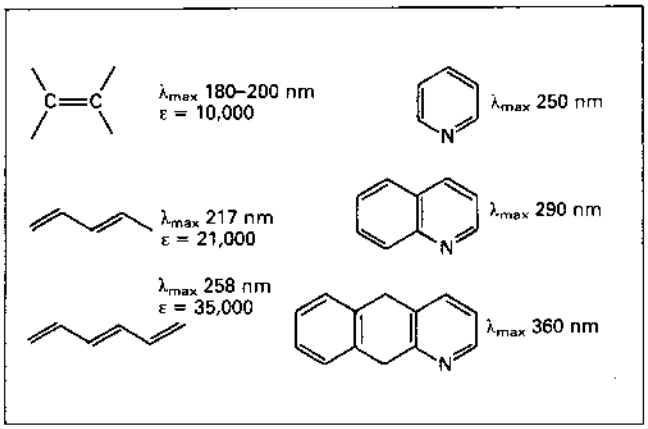
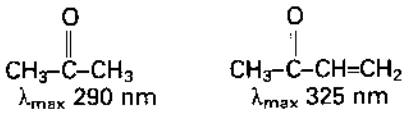
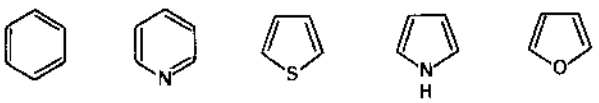


Table 3 The Effect of Increasing Conjugation on the Absorption Spectrum

The same effect occurs when groups containing n electrons are conjugated with a π electron group, e.g.



Aromatic systems, which contain π electrons, absorb strongly in the ultraviolet:

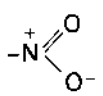
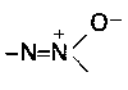


In general, the greater the length of a conjugated system in a molecule, the nearer λ_{max} comes to the visible region.

Thus, the characteristic energy of a transition and hence the wavelength of absorption is a property of a group of atoms rather than the electrons themselves. When such absorption occurs, two types of group can influence the resulting absorption spectrum of the molecule.

(ii) Chromophores

A chromophore (literally colour-bearing) group is a functional group, not conjugated with another group, which exhibits a characteristic absorption spectrum in the ultraviolet or visible region. Some of the more important chromophoric groups are:

Nitro		Azoxy	
Nitroso	–N=O	Carbonyl	C=O
Azo	–N=N–	Thiocarbonyl	C=S
Azo amino	–N=N–NH$^-$		

If any of the simple chromophores is conjugated with another (of the same or different type) a multiple chromophore is formed having a new absorption band which is more intense and at a longer wavelength than the strong bands of the simple chromophores.
This displacement of an absorption maximum towards a longer wavelength (i.e. from blue to red) is termed a bathochromic shift. The displacement of an absorption maximum from the red to ultraviolet is termed a hypsochromic shift.

(iii) Auxochromes

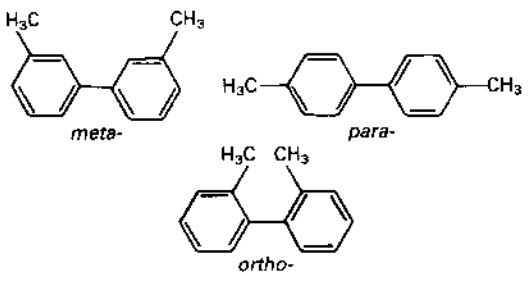
The colour of a molecule may be intensified by groups called auxochromes which generally do not absorb significantly in the 200–800 nm region, but will affect the spectrum of the chromophore to which it is attached. The most important auxochromic groups are OH, NH_2, CH_3 and NO_2 and their properties are acidic (phenolic) or basic.
The actual effect of an auxochrome on a chromophore depends on the polarity of the auxochrome, e.g. groups like CH_3–, CH_3CH_2–, and Cl– have very little effect, usually a small red shift of 5–10 nm. Other groups such as $-NH_2$ and $-NO_2$ are very polar and completely alter

the spectra of chromophores such as 

In general, it should be possible to predict the effect of non-polar or weakly polar auxochromes, but the effect of strongly polar auxochromes is difficult to predict.
In addition, the availability of non-bonding electrons which may enter into transitions also contributes greatly to the effect of an auxochrome.

(iv) Steric Effects

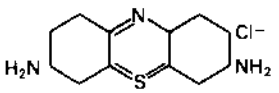
Steric hindrance will also affect the influence of an auxochrome on a chromophore. Electron systems conjugate best when the molecule is planar in configuration. If the presence of an auxochrome prevents the molecule from being planar then large effects will be noticed in the spectrum e.g. *m*- and *p*-methyl groups in the diphenyls have predictable but slight effects on the spectra compared with that of diphenyl itself. However, methyl groups in the *o*-position alter the spectrum completely.



Cis- and *trans*-isomers of linear polyenes also show differences in their spectra. The all-*trans* isomer has the longer conjugated system. λ_{max} is at a longer wavelength and ε_{max} (molar absorptivity or molar extinction coefficient – see Section 5) is higher than for the all-*cis* or mixed isomer.

(v) Visible Spectra

In general a compound will absorb in the visible region if it contains at least five conjugated chromophoric and auxochromic groups e.g.



Methylene blue
λ_{max} 660 nm

The ability to complex many metals, particularly the transition elements, with complex organic and inorganic molecules which absorb in the visible region provides the basis for their quantitative spectrophotometric analysis. The absorptions are due to movement of electrons between energy levels of the organo-metal complex. These complexing systems are termed spectrophotometric reagents. The most common are: dithizone, azo reagents (PAN, thoron, zincon), dithio carbamates, 8-hydroxyquinoline, formaldoxime and thiocyanate. In addition, many inorganic ions in solution also absorb in the visible region e.g. salts of Ni, Co, Cu, V etc. and particularly elements with incomplete inner electron shells whose ions are complexed by hydration e.g. $(Cu(H_2O)_4)^{2+}$.
Such absorptions arise from a charge transfer process where electrons are moved from one part of the system to another due to the energy provided by the visible light.

(vi) Solvents

The effect on the absorption spectrum of a compound when diluted in a solvent will vary depending on the chemical structures involved. Generally speaking, non-polar solvents and non-polar molecules show least effect. However, polar molecules exhibit quite dramatic differences when interacted with a polar solvent. Interaction between solute and solvent leads to absorption band broadening and a consequent reduction in structural resolution and ε_{max}. Ionic forms may also be created in acidic or basic conditions. Thus, care must be taken to avoid an interaction between the solute and the solvent.
Figure 8 illustrates the effect of *iso*-octane and ethanol on the spectrum of phenol, a change from hydrocarbon to hydroxylic solvent. The loss of fine structure in the latter is due to broad band H-bonded solvent-solute complexes replacing the fine structure present in *iso*-octane. This fine structure in the latter solvent illustrates the principle that non-solvating or non-chelating solvents produce a spectrum much closer to that obtained in the gaseous state.
Commercially available solvents of 'spectroscopic purity' are listed in *Table 4* accompanied by their cut-off wavelengths, based on a 10 mm pathlength.
Water and 0.1 N solutions of hydrochloric acid and sodium hydroxide are commonly used solvents for absorption spectrophotometry. Again care has to be taken to avoid interaction. Where methodology requires buffering, solutions have to be non-absorbing and generally both the composition and pH will be specified. However, if this information is not available

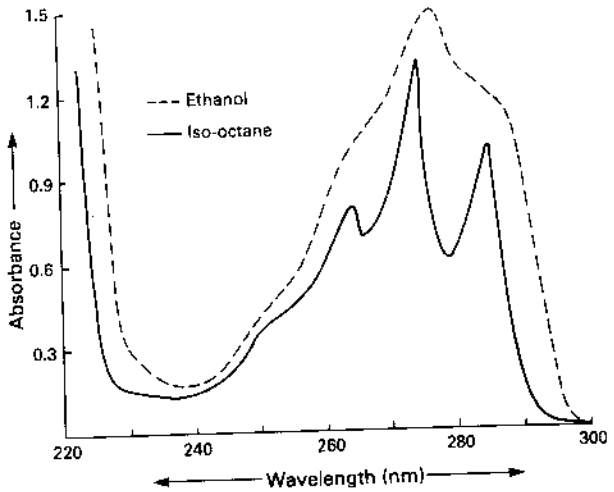


Fig. 8 Spectra of Phenol in Iso-octane and in Ethanol

lists can be found in the literature. For reactions in the 4.2 to 8.8 pH region, mixtures of 0.1 N dihydrogen sodium phosphate and 0.1 N hydrogen disodium phosphate are generally used.

Solvent	Cut-off, nm
Iso-octane	202
Ethyl alcohol	205
Cyclohexane	200
Acetone	325
Tetrachloroethylene	290
Benzene	280
Carbon tetrachloride	265
Chloroform	245
Ethyl ether	220
Isopropyl alcohol	210
Methyl alcohol	210

Table 4 Commonly used solvents and their 'cut-off' wavelengths

4 Interaction of Light and Matter

When a beam of radiation strikes any object it can be absorbed, transmitted, scattered, reflected or it can excite fluorescence. These processes are illustrated in *Fig. 9*. With scattering it can be considered that the radiation is first absorbed then almost instantaneously completely re-emitted uniformly in all directions, but otherwise unchanged. With fluorescence a photon is first absorbed and excites the molecule to a higher energy state, but the molecule then drops back to an intermediate energy level by re-emitting a photon. Since some of the energy of the incident photon is retained in the molecule (or is lost by a non-radiative process such as a collision with another molecule) the emitted photon has less energy and hence a longer wavelength than the absorbed photon. Like scatter, fluorescent radiation is also emitted uniformly in all directions.

The processes with which we are concerned in absorption spectrophotometry are absorption and

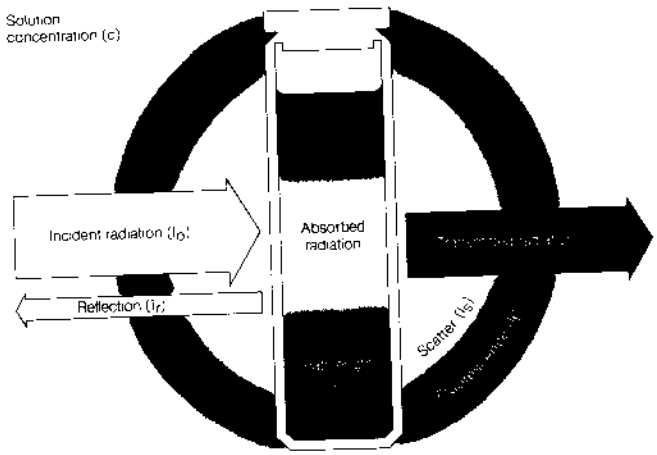


Fig. 9 Interaction of Light and Matter

transmission. Usually the conditions under which the sample is examined are chosen to keep reflection, scatter and fluorescence to a minimum.

In the ultraviolet and visible regions of the electromagnetic spectrum the bands we observe are usually not specific enough to allow a positive identification of an unknown sample, although this data may be used to confirm its nature deduced from its infrared spectrum or by other techniques. Ultraviolet and visible spectrophotometry is almost entirely used for quantitative analysis, that is, the estimation of the amount of a compound known to be present in the sample. The sample is usually examined in solution.

(a) Lambert's (or Bouguer's) Law

Lambert's Law states that each layer of equal thickness of an absorbing medium absorbs an equal fraction of the radiant energy that traverses it.

The fraction of radiant energy transmitted by a given thickness of the absorbing medium is independent of the intensity of the incident radiation, provided that the radiation does not alter the physical or chemical state of the medium.

If the intensity of the incident radiation is I_o and that of the transmitted light is I, then the fraction transmitted is:

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

The percentage transmittance is:

$$\%T = \frac{I}{I_0} \times 100$$

If we have a series of coloured glass plates of equal thickness, each sheet of which absorbs one quarter of the light incident upon it, then the amount of the original radiation passed by the first sheet is:

$$\frac{1 - \frac{1}{4}}{1} \times 100 = 75\%$$

and by the second sheet is 56.25%, i.e. 75% of 75%, and by the third sheet is 42.19%, i.e. 75% of 56.25%, and by the 'nth' sheet is $(0.75)^n \times 100\%$.

Now imagine a container with parallel glass walls 10 mm apart filled with an absorbing solution. If monochromatic light is incident on one face and 75% of

the light is transmitted, Lambert's Law tells us that if a similar cell is put next to the first the light transmitted will be reduced to 56.25%. If the contents of the two containers are evaporated to half their volume, thereby doubling the concentration, and then measured in a single container, it will be found that the transmission will again be reduced to 56.25%.
It can immediately be seen that if we wish to determine the concentration of an unknown sample we can plot the percentage transmittance of a series of solutions of known concentration or 'standards' and read the concentration of our unknown from the graph. It will be found that the graph is an exponential function which is obviously inconvenient for easy interpolation. See *Fig. 10(a).*

(b) The Beer-Lambert Law

The Beer-Lambert Law tells us that the concentration of a substance in solution is directly proportional to the 'absorbance', A, of the solution.
The law is only true for monchromatic light, that is light of a single wavelength or narrow band of wavelengths, and provided that the physical or chemical state of the substance does not change with concentration.
Mathematically, absorbance is related to percentage transmittance by the expression

$$A = \log \frac{I_0}{I} = \log \frac{100}{T} = kcL$$

where L is the length of the radiation path through the sample, c is the concentration of absorbing molecules in that path, and k is the extinction coefficient – a constant dependent only on the nature of the molecule and the wavelength of the radiation.
Now, in the example above, the transmittance of our sample fell from 75 to 56.25% when the concentration doubled. What happens to the absorbance in the same circumstance?

$$A = \log \frac{100}{T}$$
$$= \log 100 - \log T$$
$$= 2 - \log T$$

When $T = 75\%$, $A = 2 - 1.875 = 0.125$

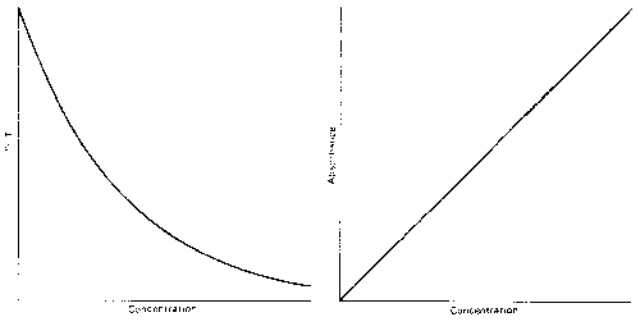
When $T = 56.25\%$, $A = 2 - 1.750 = 0.250$

Quite clearly, as the absorbance doubles for twice the concentration, it is far more convenient to work in absorbance than transmittance for the purposes of quantitative analysis.

It is useful to remember that

$$0\%T = \infty A$$
$$0.1\%T = 3.0A$$
$$1.0\%T = 2.0A$$
$$10\%T = 1.0A$$
$$100\%T = 0A$$

Absorbance in older literature is sometimes referred to as 'extinction' or 'optical density' (OD).



(a) (b)

Fig. 10 (a) %T v Concentration
(b) Absorbance v Concentration

5 Applications of UV/visible Spectrophotometry

Having looked, albeit very briefly, at the theory and origins of UV/visible spectra, let us now investigate how we can apply the technique to chemical analysis starting with consideration of sample state.
With solid samples it is usually found that the material is in a condition unsuitable for direct spectrophotometry. The refractive index of the material is high and a large proportion of the radiation may be lost by random reflection or refraction at the surface or in the mass. Unless the sample can be easily made as an homogeneous polished block or film, it is usual to eliminate these interfaces by dissolving it in a transparent solvent.
Liquids may be contained in a vessel made of transparent material such as silica, glass or plastic, known as a cell or cuvette. The faces of these cells through which the radiation passes are highly polished to keep reflection and scatter losses to a minimum. Gases may be contained in similar cells which are sealed or stoppered to make them gas tight.
With the sample now ready for measurement, the I_0 (incident intensity) can be set by moving the sample out of the beam and letting the light fall directly on the detector. On today's modern instrumentation, I_0 setting is generally accomplished by an 'autozero' command. In practice, such a method does not account for the proportion of radiation which is reflected or scattered at the cell faces. It also does not account for the radiation which is absorbed by any solvent and thus does not effectively pass through the sample. Therefore, it is usual to employ a reference or blank cell, identical with that containing the sample, but filled only with solvent and to measure the light transmitted by this reference as a true or practical I_0.
Having established the I_0 or reference position, the procedure adopted for the analysis will depend on the analytical information required. In general terms there are two major measurement techniques; how much analyte is in the sample (quantitative analysis) and which analyte is in the sample (qualitative analysis).

(a) Quantitative Analysis

For quantitative analysis we should normally choose to use radiation of a wavelength at which k, the extinction

coefficient, is a maximum, i.e. at the peak of the absorption band, for the following reasons:

(i) The change in absorbance for a given concentration change is greater, leading to greater sensitivity and accuracy in measurement.
(ii) The relative effect of other substances or impurities is smaller.
(iii) The rate of change of absorbance with wavelength is smaller, and the measurement is not so severely affected by small errors in the wavelength setting.

The extinction coefficient must have units relating to the units used for concentration and pathlength of sample.

It is usually written as:

$E^{1\%}_{1\ cm}$ the value of k for a 1% sample concentration of 1 cm thickness

or ε the molar absorptivity previously described as the molar extinction coefficient; the value of k for a sample concentration of 1 gram molecule per litre and a 1 cm thickness.

To carry out an analysis of a known material it should be possible in theory to measure the absorption of a sample at its absorption maximum, measure the thickness, obtain values of ε from tables and calculate the concentration. In practice, values of ε depend on instrumental characteristics, so published values from tables are not accurate enough, and a reliable analyst will usually plot the absorbance values of a series of standards of known concentration; the concentrations of actual samples can then be read directly from the calibration graph. Using today's modern microprocessor instruments, the analyst will usually only require one 'top' standard which can be used to calibrate the instrument so that it displays the concentration of subsequent samples directly in the required units. If the calibration curve is non-linear due to either instrumental or chemistry considerations, then some instruments will automatically construct a 'best-fit' calibration when presented with a number of standards.

(b) Kinetics

Reaction kinetics studies involve the measurement of the change in concentration of a participant in the reaction as a function of time. Whereas we have previously considered the measurement of a sample whose absorption characteristics are constant, spectrophotometric kinetics involves the measurement of a fall or rise in absorption at a fixed wavelength. This measurement provides information on the change in concentration of either the reactants or products.
The most widely encountered application of spectrophotometric kinetic techniques is for the study of enzymes (proteins that are present in all living tissue). Enzymes cannot be measured directly but their catalytic properties allow their estimation from the speed of the reactions which they catalyse. Enzymes have many uses as reagents or as 'labels' that can be attached to other molecules to permit their indirect detection and measurement. However, their widest use is in the field of clinical diagnostics as an indicator of tissue damage. When cells are damaged by disease, enzymes leak into the blood stream and the amount present indicates the severity of the tissue damage. The relative proportions of different enzymes can be used to diagnose diseases, say of the liver, pancreas or other organs which otherwise exhibit similar symptoms.
The reaction most used in these clinical assays is the reduction of nicotinamide adenine dinucleotide (NAD) to $NADH_2$. The spectra of NAD and $NADH_2$ are shown in *Fig. 11* and it can be seen that if the absorbance is monitored at the $NADH_2$ peak at 340 nm the reading will decrease as the reaction progresses towards NAD.

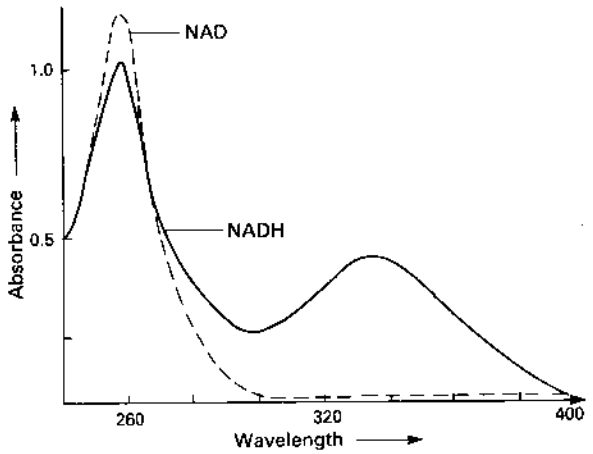


Fig. 11 Absorption Spectra of NAD and NADH

Other reactions can be monitored by coupling them together so that the product of one reaction feeds the next which can then be optically monitored.
The general equation for an enzyme reaction is:

$$A \xrightarrow{\text{enzyme X}} B$$

In this reaction enzyme X catalyses the conversion of compound A to B. A coupled reaction utilizes NAD in a secondary reaction, the completion of which depends on the concentration of a product of the primary reaction, e.g.:

$$\underset{\text{(Primary Reaction)}}{A \longrightarrow B} \qquad \underset{\text{(Indicator or Secondary Reaction)}}{B + NADH_2 \xrightarrow[\text{Enzyme}]{\text{Indicator}} C + NAD}$$

This equation, however, will only hold true when the reaction between B and $NADH_2$ is virtually instantaneous.
Consider the following coupled reaction:

$$\text{L-alanine} + \alpha\text{-ketoglutarate} \xrightarrow{\text{ALT}} \text{pyruvate}$$
$$+ NADH_2 \xrightarrow{\text{LDH}} \text{lactate} + NAD$$

The initial reaction is the conversion of L-alanine and α-ketoglutarate in the presence of alanine transaminase. The indicator reaction is the conversion of pyruvate (and $NADH_2$) to lactate which is catalysed by the enzyme lactate dehydrogenase.
The effect of this reaction on absorbance is illustrated in *Fig. 12*. The actual decrease in absorbance caused by the oxidation of $NADH_2$ can be seen in *Fig. 11*.
The rate of the reaction is determined by the average drop in absorbance over a short period of time following the addition of the α-ketoglutarate.
Other reactions involve the reduction of NAD to $NADH_2$

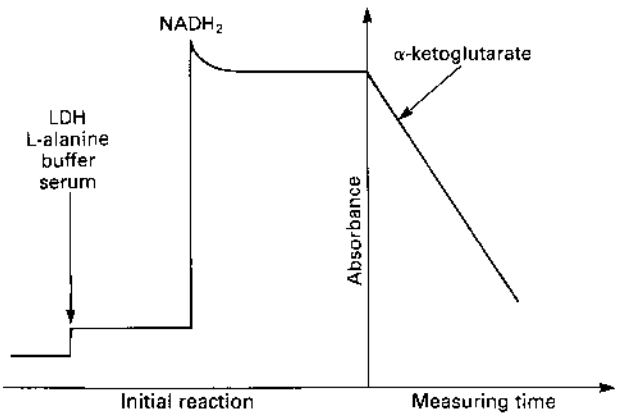


Fig. 12 Enzyme Reaction based on Oxidation NADH to NAD

with a resultant increase in absorbance. The conversion of glucose-6-phosphate to 6-phosphogluconolactone (a reaction catalysed by glucose-6-phosphate dehydrogenase in the presence of $NADH_2$) is an example of this type of reaction.
The equation for this reaction would be as follows:

$$\text{G-6-phosphate} + \text{NAD} \overset{\text{G-6-phosphate dehydrogenase}}{\rightleftharpoons} \text{6-phosphogluconate} + \text{NADH}_2$$

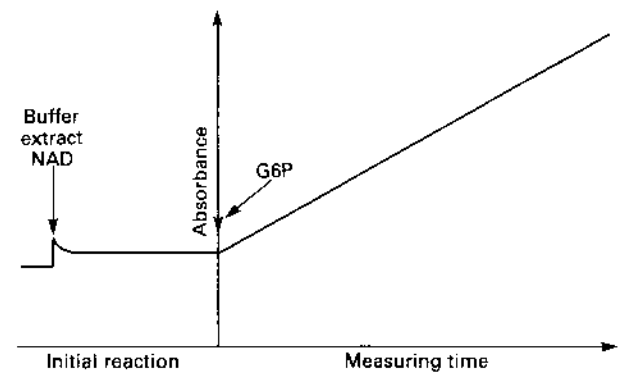


Fig. 13 Enzyme Reaction based on Reduction of NAD to NADH

The change in absorbance may be shown as in *Fig. 13.* In the case of reaction 1, i.e. alanine transaminase, the rate of reaction is calculated by measuring the average decrease in absorbance over a period of time and expressing it as an average drop in absorbance per minute. With reaction 2, the increase of absorbance is measured.
A variety of parameters will influence the rate of reaction. The majority of these i.e., substrate concentration, enzyme concentration and pH have to be accounted for in the chemistry. However, one of the most important is temperature and it is important to control the temperature of the sample and reagents precisely immediately prior to and during measurement. For this requirement, a whole range of thermostatted cell holders and temperature control systems is available.

(c) Analysis of Mixtures

It is relatively rare to find a practical problem in which one has a mixture to be analysed with only one component which absorbs radiation. When there are several such components which absorb at the same wavelength their absorbances add together, and it is no longer true that the absorbance of the sample is proportional to the concentration of one component (see *Fig. 14*).
In these cases, several approaches can be adopted with the most important being chemical reaction and multi-wavelength measurements.

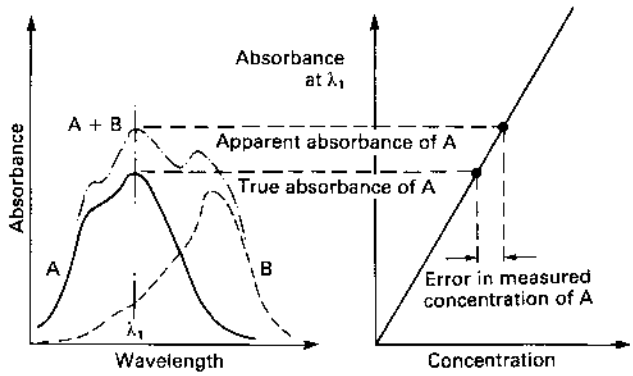


Fig. 14 Error Caused by Superimposed Absorption in a Mixture

(d) Chemical Reaction

A common method of analysis is to change the required component by adding a chemical reagent which reacts with it specifically to form a highly absorbing compound. An example of this is shown in *Fig. 15.* A quantity of the reagent added to the mixture reacts only with one component and both increases its absorption and changes the wavelength of the absorption maximum so that there is no longer interference between the components. The analysis is then reduced to a simple case and its sensitivity is improved. Many hundreds of such specific reagents are now available for all sorts of analyses and sample matrices and are thoroughly detailed in the literature.

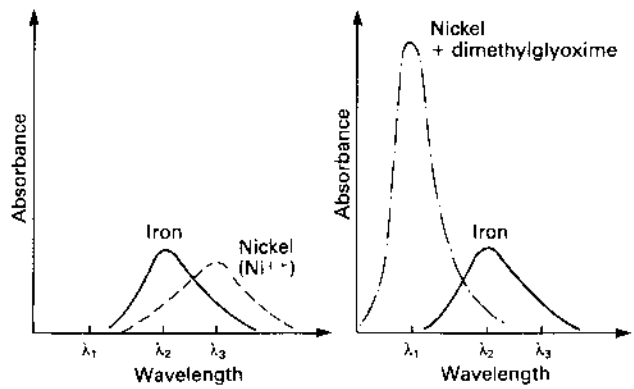


Fig. 15 Improving Sensitivity by Adding a Chemical Reagent

(e) Multi-Wavelength Measurements

In a mixture of components, the observed absorption at any wavelength is the sum of the individual absorption

spectra of the components thus:
Measured absorbance at λ_1

$$A_1 = E_A aL + E_B bL + E_C cL \ldots$$

Similarly at a second wavelength λ_2

$$A_2 = E'_A aL + E'_B bL + E'_C cL \ldots$$

Where E_A and E'_A are the absorptivity of component A at λ_1 and λ_2 and a is its concentration, etc.
The cell pathlength L is generally constant and therefore cancels. If we then take measurements at a number of different wavelengths equal to the number of components, and if the values of the absorptivities are known by measurement of the pure components at each of the wavelengths concerned, we may solve the simultaneous equations to find the required concentrations.
The multi-wavelength or multi-component analysis technique has seen a resurgence of interest over the last few years. This has been due to the development of microprocessor instruments with their ability to output data to low cost microcomputer systems. A variety of algorithms is available and the analyst is generally required to input the number of components, measurement wavelengths and concentration values of the standards. Having measured the standards, samples can be processed and results presented appropriately. Programs of this type, including measurement parameters, can be stored on disc and recalled and run with a minimum of operator intervention, providing results on complex mixtures containing up to 10 components.

6 Instrumentation

In earlier sections we have seen that the purpose of a spectrophotometer is to provide a beam of monochromatic radiation in a form suitable for illuminating a sample and to measure the ratio I/I_o. Any spectrophotometer will consist of the component parts illustrated in *Fig. 16*. There are many combinations of

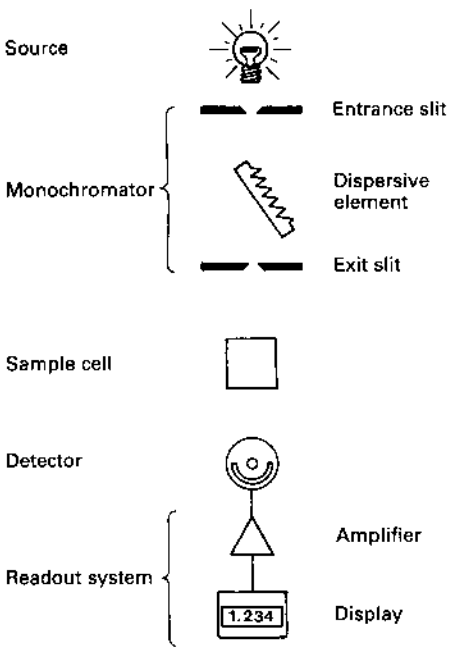


Fig. 16 Schematic Diagram of a Spectrophotometer

sources, monochromators, measuring systems etc. which can be assembled to form integrated spectrophotometers with varying degrees of accuracy and suitability for particular applications. It is hoped that the following discussion of alternative components will assist the reader to better understand what goes on under the covers of these instruments and help in the choice of new instruments or enable the best results to be obtained from existing equipment.

(a) Sources

(i) Tungsten Filament Lamp

The simplest method of providing visible radiation is by a tungsten lamp. It has the advantages that it is inexpensive, reliable, and a stable light intensity can be obtained with a relatively simple power supply. The life of the lamp is often limited by darkening of the glass envelope caused by evaporation of the tungsten filament. This energy loss may manifest itself as a noisy display some time before the lamp finally fails completely. The useful wavelength range of a tungsten lamp is from 330 nm to well into the near infrared. Outside this range, absorption by the glass envelope severely limits emission.

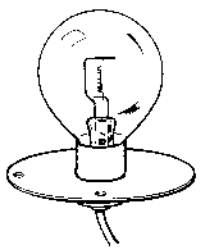


Fig. 17 Tungsten Filament Lamp

(ii) Tungsten Halogen Lamp

Virtually all modern spectrophotometers utilise a tungsten halogen (or quartz iodine) lamp. These lamps have a much more compact tungsten filament within a smaller quartz envelope and thus lend themselves more readily to being 'pre-aligned' on a suitable mount for the user to simply clip in place. It should be noted that when replacing any lamp, care must be taken not to leave fingerprints on the lamp envelope which will become 'burnt on' and absorb energy.
The tungsten halogen lamp has a number of advantages over the simple tungsten lamp. It operates at a higher filament temperature, so the UV output is

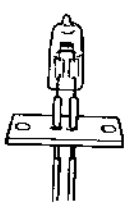


Fig. 18 Tungsten-halogen Lamp

higher, and obviously the quartz envelope absorbs less UV than a glass envelope. Moreover, they do not suffer from darkening of the envelope as the evaporated tungsten reacts with the halogen before reaching the envelope to form a volatile halide. On contact with the hot filament the halide decomposes again, depositing the tungsten back on the filament.

(iii) Deuterium Lamp
Below approximately 330 nm the most satisfactory source of ultraviolet radiation for spectrophotometric purposes is the deuterium arc lamp (*Fig. 19*). When thermally excited, deuterium gas emits a continuous spectrum below 400 nm plus a few intense emission lines in the visible region (*Fig. 20*). The lower boundary of normal ultraviolet spectrophotometry is 190 nm, below which atmospheric absorption of the radiation rather than the output of the lamp is the limiting factor. The envelope of the lamp is normally constructed of an optical grade of silica which exhibits excellent ultraviolet transmission but contributes heavily to the high replacement cost compared to visible light sources.

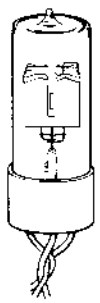


Fig. 19 Deuterium Arc Source

Before an arc can be established between the anode and cathode of the lamp the cathode is first raised to a few hundred degrees by a heating element when it then begins to emit electrons. At this point a high voltage is applied across the electrodes and the arc is established. The heating current is then switched off as the arc produces enough heat to maintain the emission. The arc is confined to a small volume within the lamp by a metal enclosure around the electrodes and the radiation escapes via a small aperture.
On modern instruments the striking procedure lasts only a few seconds and is carried out automatically either on power-up or at the press of a button.
As with tungsten halogen lamps, deuterium lamps are often prealigned by the instrument manufacturer. Reasonable care should be taken when replacing lamps not to look directly at the radiation as UV light can be harmful to the eyes.

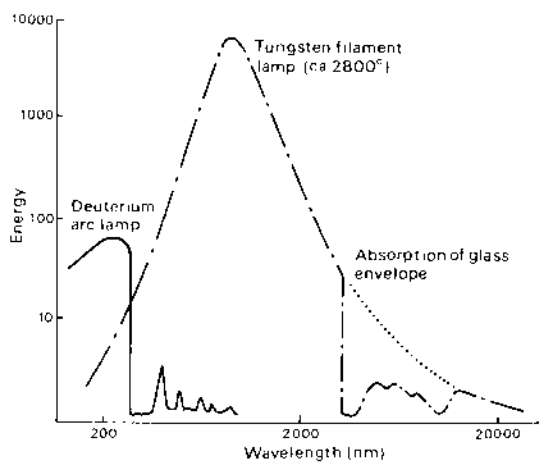


Fig. 20 Spectroscopic Sources

(iv) Mercury and Xenon Arcs
For some applications (commonly the related techniques such as fluorimetry), a deuterium arc is not sufficiently intense, and a mercury or xenon lamp may be used. Both lamps produce a continuum of radiation with many spectral lines (broadened by doppler and pressure broadening) superimposed on the continuum. Mercury lamps may also be used as accessories within the lamphouse for wavelength calibration. These lamps are usually quite low powered, compact lamps which give well defined spectral lines.

(v) Lasers
Lasers have yet to find wide application in conventional UV/visible spectrometry. There are as yet few commercially available which are useable over the required wavelength range; they are bulky, and more important – very expensive! It is unlikely they will ever supplant the sources described earlier.

(b) Focusing Elements
Having established a source of radiation, it is obviously necessary to collect as much of this radiation as possible and transfer it via the monochromator to the sample. This process of transferring the energy is accomplished by a series of lenses or mirrors.

(i) Lenses
Lenses were often used in older instruments, but are seldom found in present day instrumentation. Their chief drawback is chromatic abberation, caused by their rapidly varying refractive index in the UV. Thus the UV and visible portions of the spectrum may be focused up to 20 mm apart instead of being coincident and this poses obvious difficulties for the optimum cell holder position, especially for microcells.

(ii) Mirrors
The mirrors used in spectrophotometers are front surface mirrors coated with aluminium. Aluminium is one of the few materials which has high reflectivity throughout the UV-visible-near IR range. High reflectivity is very important, for if the spectrophotometer contains several mirrors, the energy loss will be considerable. Six mirrors, each reflecting 90% results in a total throughput of under 50%.

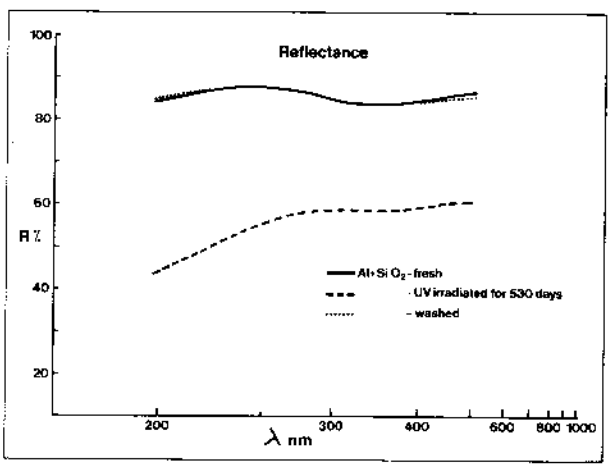


Fig. 21 The effect of UV irradiation and subsequent washing on the reflectance spectrum of Silica Coated Aluminium

Aluminium, especially when exposed to UV radiation in a lamphouse, ages and loses reflectivity. For this reason mirrors are often coated with a transparent layer. The best material to use is quartz (silica), which prevents an oxide layer from forming, and renders the surface sufficiently tough that it can withstand fairly vigorous washing. Indeed, washing will usually restore the reflectivity to its original value. All Pye Unicam mirrors (and even the diffraction gratings) are overcoated with quartz.

(c) Monochromators

The monochromator is the heart of a spectrophotometer and deficiencies in its performance cannot be made up by the use of higher quality components in other parts of the optical system. In practice, all but the most elementary monochromators usually consist of an entrance slit to confine the source radiation to a useable area, mirrors to pass the light through the system, a dispersing element to spread the source radiation into its component wavelengths and an exit slit to select the wavelength with which it is desired to illuminate the sample.

Before we can compare the relative performance of different monochromators we need a measure of their ability to select a narrow wavelength range. If the intensity of the radiation emerging from the exit slit is plotted against wavelength it will be found that the peak energy occurs at the nominal wavelength setting of the monochromator and the energy drops away over a few nanometres either side of the nominal setting. If a triangle is drawn which most closely fits the true energy distribution we may measure the peak intensity value at its apex. The range of wavelengths enclosed by the triangle at a point halfway between the base and apex is known as the spectral bandwidth or half bandwidth of the monochromator.

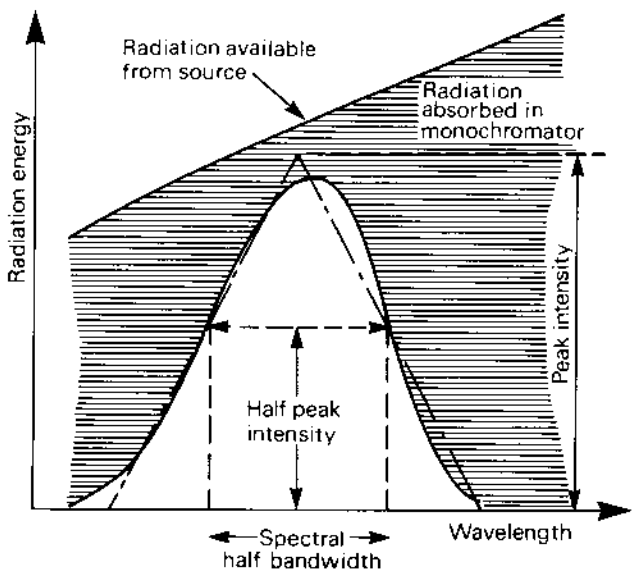


Fig. 22 Spectral Bandwidth

(i) Filters

The simplest monochromator is a piece of coloured glass or coloured gelatine sandwiched between glass plates to form a light 'filter'. The special term 'colorimeter' is reserved for instruments utilising only filters to isolate the desired band of wavelengths. These devices do not usually have a true entrance slit but the frame holding the filter serves that purpose. Similarly, the exit slit is usually a simple fixed mask to confine the beam to the dimensions of the sample holder.

To provide some degree of wavelength versatility, different filters are often located in a rotating disc or on a slider. These types of filter have a spectral bandwidth of the order of 25 nm or more and, consequently, colorimeters are less sensitive than a true spectrophotometer since it cannot always be arranged to measure at the absorbance peak. Similarly, they are less efficient at ignoring any interfering radiation at nearby wavelengths and hence, may not be truly linear with increasing concentration. Colorimeters are obviously impracticable for plotting absorbance spectra and are confined to the visible region due to the use of glass in constructing the filters.

Dielectric, or interference, filters are sometimes used in colorimeters. These are constructed by coating a piece of glass or silica with different thicknesses of materials of various refractive indices so that all wavelengths other than those required are either reflected or absorbed in the filter due to destructive interference of the light waves. Interference filters can be constructed with a spectral bandwidth of the order of 10 nm. However, whilst they are applicable to several regions of the spectrum they are generally expensive and it is often more economic to invest in a continuously tunable monochromator than purchase a range of interference filters.

A special type of interference filter, known as a wedge filter, can be made so that a continuous range of wavelengths may be selected. This is achieved by depositing a gradually increasing thickness of the material in which the interference occurs along the length, or around the circumference, of a glass base. Thus sliding or rotating such a filter brings a different thickness into the light beam and allows radiation of a new wavelength to reach the sample. This type of monochromator is not widely used as the wavelength range is still fairly restricted, and for comparatively little extra cost, superior performance may be provided by a low-cost grating.

(ii) Prisms

For many years, prisms were used as the standard dispersing element in spectrophotometers. A simple prism monochromator is shown in *Fig. 23*.

Light from the source is made into a parallel beam (collimated) by the mirror M1 and is reflected onto the prism. This has the property of deflecting light of different wavelengths by different amounts. The light is reflected back through the prism to double the dispersion or angular separation of the wavelengths. If the dispersed beam is now re-focused by mirror M2, the focal point for light of one wavelength will be physically displaced from that for light of any other wavelength. We may now select the required wavelength by moving the exit slit across the focal plane.

The optical systems most used in practice differ from that illustrated in *Fig. 23* for reasons of compactness, convenience and cheapness, but the principle is the

same. In most cases the prism is rotated to shift the focused spectrum across the exit slit rather than the slit being moved across the focal plane, and one mirror is used to provide the functions of M1 and M2 to save costs of high grade optical components.

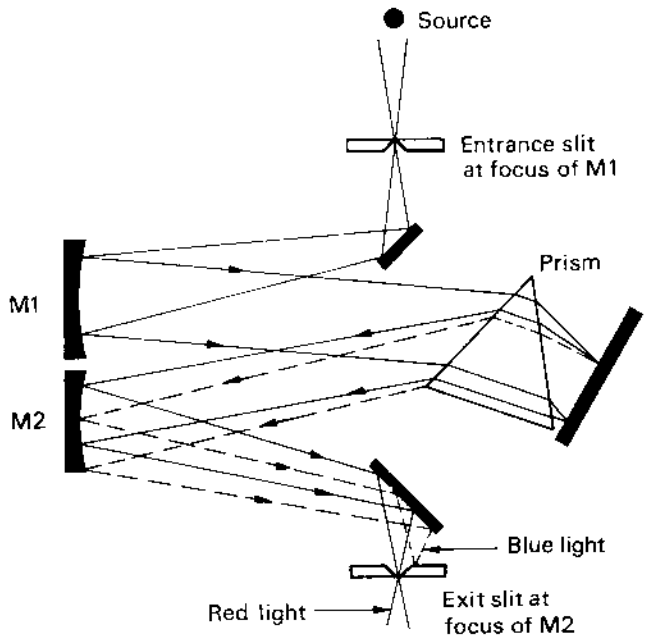


Fig. 23 Simple Prism Monochromator

Prisms presented the designer with a number of practical problems. There is a non-linear relationship between the angle through which the prism is turned and the wavelength emerging from the monochromator, so that an accurately machined cam and lever mechanism was required. Also, the angular dispersion is highly dependant on temperature, so some form of temperature control of the monochromator was often necessary.

The efficiency of prism monochromators is much better than that of filters and spectral half bandwidths of 1 nm or less are obtainable in the ultraviolet and visible regions of the spectrum. However, the dispersion of a prism changes with wavelength and if measurements are required to be made at a constant bandwidth then the width of the slit must be adjustable so that it can be closed where the spectrum from the prism is bunched up and opened where it is spread out.

(iii) Gratings

In most modern spectrophotometers the preferred dispersing element is the diffraction grating. These consist of a series of parallel grooves engraved on a reflecting surface. Good performance in the ultraviolet and visible regions requires as many as 1200 lines per mm.

Older instruments employed gratings replicated from master gratings. Masters could only be made on a very expensive ruling machine which had to be protected from the slightest temperature and vibrational fluctuation. The magnitude of the problem has been expressed by Hutley as equivalent to that of ploughing a furrow 10 km long with an overall straightness of 20 nm and a short term deviation of only 1 nm! Needless to say, there were few centres in the world capable of meeting such exacting conditions.

Modern instruments mostly employ holographic (or, more correctly, interference) gratings. These are manufactured by spinning a layer of photoresist onto a glass blank, and then exposing this resist to an interference pattern produced by a coherent light source which is usually a high powered laser. The blank is 'developed' to leave accurately parallel grooves corresponding to the interference pattern, which is then overcoated with aluminium to form a reflection diffraction grating. As with ruled gratings, copies can then be made if required, or if the process is well organised, sufficient masters can be produced to fit originals into instruments. All Pye Unicam instruments are fitted with master blazed holographic gratings.

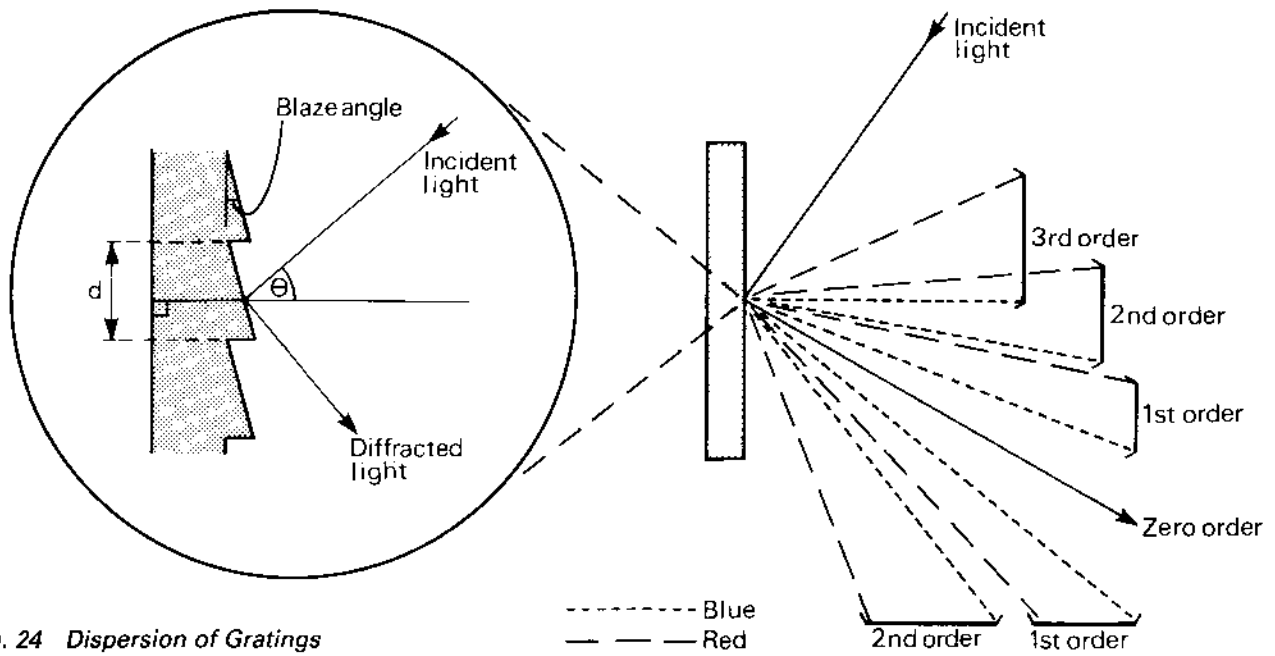


Fig. 24 Dispersion of Gratings

When a grating is put into a beam of parallel radiation so that one surface of the grooving is illuminated, this surface acts as a very narrow mirror. The reflected radiation from this groove mirror overlaps the radiation from neighbouring grooves and the waves interfere with each other.
When the wavelength of the radiation is such that the separation of the grooves in the direction of the radiation is a whole number of wavelengths, the waves are in phase and the radiation is reflected undisturbed. When the separation is not a whole number of wavelengths the waves cancel out and no radiation is propagated. By changing the angle θ at which the radiation hits the grating the wavelength which is reflected can be altered. The relationship between the wavelength and the angle at which it is reflected is most conveniently expressed in terms of d, the distance separating the grooves.

$$n\lambda = 2d \sin \theta$$

A diffraction grating in fact produces a whole series of overlapping spectra at angles determined by the value of n. Where n is 1 the spectrum is known as first order, where n is 2, as second order etc.
Diffraction gratings have the following advantages over prisms:

(A) By choosing a suitable value of d the separation of the wavelengths (i.e. the dispersion) can be made very large and superior to that obtainable with a prism above approximately 250 nm.
(B) The dispersion follows a geometric law and is not a property of the material. It also alters much less with wavelength so measurements at constant bandwidth are not a problem.
(C) They are much less temperature sensitive than prisms.

They also have the following disadvantages:

(A) At any given angle they reflect radiation of λ and also $\lambda/2$, $\lambda/3$ etc. – for example, the radiation of wavelength 1200 nm will be reflected at the same angle as that of 600 nm, 400 nm etc. This unwanted radiation has to be removed somehow, or it will appear as stray light. In practice, grating monochromators are usually fitted with 'order-sorting filters' which are inserted into the beam at appropriate wavelengths to absorb light from higher order reflections.
(B) The energy of the radiation is divided amongst the spectral orders. Since only one order is used in a monochromator a lot of the energy may be lost. This loss may be minimised by controlling the angle at which the grooves are engraved (blaze angle). For many years it was not possible to blaze holographic gratings, but now, using special techniques, this is possible. If a grating is said to be blazed at, say 300 nm, it means that the efficiency of the grating is highest at that wavelength.
(C) Besides providing the desired dispersion, the many grooves on the surface of gratings also scatter a proportion of the incident radiation and hence are a greater potential source of stray light than smooth-surfaced prisms. There is also quite a difference between conventional and holographic grating surface quality (*Fig. 25*) which accounts for the vastly improved stray light figures available with holographic gratings.

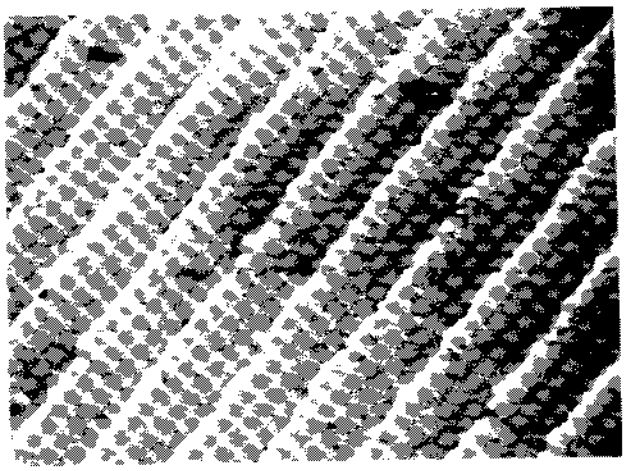


Fig. 25a A conventional ruled grating

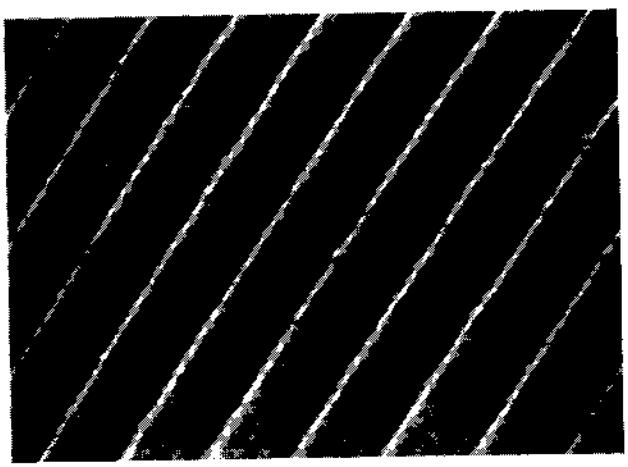


Fig. 25b A blazed holographic grating

(d) Sample Cells

Sample cells are available in a wide variety of shapes and sizes to suit almost any conceivable spectrophotometric measurement. Almost all instruments are fitted with holders for standard rectangular cells of 10 mm pathlength and generally provision is made for cells of other pathlengths and accessory holders for keeping cells at constant temperature. Holders must facilitate reproducible location of cells and ensure that the optical faces are held exactly perpendicular to the light beam.
Modern cells are normally constructed from pieces of glass or silica which are fused together to form a liquid-tight container. Glass is suitable for use in the region from 340–1000 nm but silica is necessary for work between 220 and 340 nm. Between 185 and 220 nm a special ultraviolet grade of silica must be used. Moulded polystyrene cells are now available as an alternative to glass. These have the advantage of being cheap enough to be disposable and consequently the chore of cell cleaning is avoided. However, fused cells are still to be preferred for measurements of the highest accuracy.
The optical faces of fused cells are polished very flat and have to be very parallel to avoid light losses at the surface due to reflection and scatter. The sides of the

cell usually have a ground surface which allows them to be marked with a soft lead pencil. This can be useful, for example, to indicate which cell contains the blank or solvent and which holds the standard solution during a series of repetitive measurements involving the removal of cells from the light beam.
Cells of 20 and 40 or 50 mm pathlength are useful when analytical sensitivity is limited and their use may avoid the need to perform a solvent extraction or concentration by evaporation step in the analysis. Conversely cells of 1, 2 and 5 mm pathlength may be needed when the sample has a high absorptivity and dilution is inconvenient.
Standard cells are usually supplied with a simple glass or plastic lid to prevent spillage when lifting them in or out of the sample compartment (*Fig. 26(a)*). When volatile solvents are to be used it is desirable to use cells with PTFE stoppers to avoid changes of absorbance due to evaporation and to prevent the escape of vapours into the spectrophotometer (*Fig 26(b)*). When using stoppered cells care should be taken to leave an air gap at the top or the cell may be ruptured by any build-up in pressure.

(i) Matched Cells
The use of matched cells used to be very widespread, mainly because of their convenience in use. When measurements are made using unmatched cells allowance must be made for their slightly different transmission levels. Therefore, standard cells can usually be purchased as matched sets of two or four. Glass cells are selected to be within 0.5%T of each other at 365 nm and UV grade silica cells to within 1.5%T at 240 nm when filled with distilled water prepared in a silica still. However, modern microprocessor instruments with baseline memory facilities have reduced the need for matched cells.

(ii) Microcells
In some applications large volumes of sample may be difficult to obtain or expensive. For these reasons microcells are available having a typical working volume of 0.5 ml per 10 mm pathlength compared to 2.5 ml for standard cells. Microcells confine the liquid between walls only 2 mm apart, and it is important that the light beam travels only through the sample and not through the glass walls or a curved calibration relative to concentration will result.
Many spectrophotometers, therefore, require the use of a special holder for more precise alignment of the cell relative to the light beam and may even incorporate a mask and a lens to reduce the beam dimensions so that it will pass cleanly through the sample without touching the cell walls.
As an alternative to special holders, self-masking cells whose walls are constructed of black glass or silica may be used (*Fig. 26(c)*). These cells are more expensive than the clear-walled variety but have the vital advantage that it is impossible for any light to bypass the sample by travelling through the walls of a misaligned cell. Masks are often provided to reduce the beam width for use with clear-walled microcells but the diverging beam can still escape into the side walls part way through the cell. The problem gives rise to gross photometric non-linearity but often goes undetected especially in assays where a range of standards is not measured. The small premium for black walled cells is well worth the security that they offer.

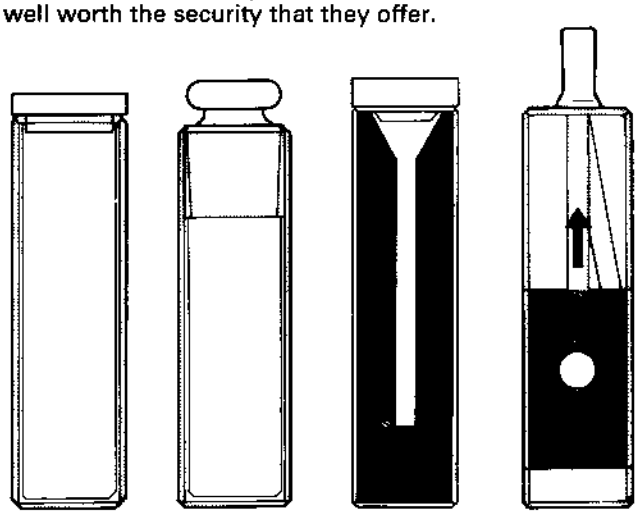


Fig. 26 Typical Sample Cells
(a) Rectangular Cell with Lid
(b) Rectangular Cell with Stopper
(c) Black-walled Microcell
(d) Flow Cell

(iii) Test Tubes
In many analyses where the highest degree of accuracy is not required it is sometimes convenient to use glass test tubes as the measuring vessels. Due to their circular cross-section, test tubes filled with liquid act as lenses and small variations in diameter and wall thickness will cause the beam to be focused at different points and illuminate a varying proportion of the detector surface. Special holders are normally required, but provided small differences in sample absorption are not significant, test tubes may offer a useful alternative to expensive cells and reduce analysis time.

(iv) Autocells
Devices for semi-automatically filling and emptying special cells, known as flow cells, serve to speed the analysis and reduce cell handling. As flow cells (see *Fig. 26(d)*) are made to the same high optical standards as ordinary types they do not suffer from the inaccuracies introduced by test tubes. However, unless an Autocell is designed to empty efficiently, droplets may remain in the cell and associated tubing and affect the measurement of the next sample. This phenomenon is known as cross-contamination or carry-over. Good designs ensure that the effect of one sample on the reading of the next is less than one percent.

(v) Cell Cleaning
In order to achieve the best results from any spectrophotometer it is essential to use chemically clean cells. Relatively high levels of contaminants, particularly those which absorb ultraviolet radiation, can remain undetected by the eye and severely affect the results. Cells which are left for relatively short periods on a laboratory bench can readily adsorb organic solvents onto their surfaces – as well as dust particles and grease. It is recommended that for results

of the highest accuracy cells be cleaned just before use. When working with aqueous solutions, it is normally sufficient to wash the cells out with plenty of tap water followed by several rinses in distilled or deionised water. A final rinse in a pure water-miscible solvent such as ethanol will speed the drying process. The cell should be dried either in a current of warm dry filtered air or under vacuum. The use of tissues or cotton wool is not advised for drying. If the cells have contained an organic solvent they should be washed out with fresh solvent and then treated with a water-miscible solvent which is compatible with the original solvent before proceeding as before.
Detergents, soap solutions and proprietary cleansing agents for glassware may be used to remove more obstinate contaminants. The manufacturer's dilution instructions for these agents should be followed as the concentrated solutions can chemically attack the surface of the cell. As a last resort a badly contaminated cell can be left soaking overnight in concentrated sulphuric acid in which a few crystals of potassium dichromate have been dissolved. After soaking, carefully drain off as much of the acid as possible into another beaker while retaining the cell with a glass rod, and then flood the drained beaker with water to remove the evolved heat and prevent local heating from fracturing the cell.
After cleaning and drying always store cells in the foam-lined box in which they are usually supplied or keep them immersed in distilled water in a covered container.

(e) Detectors

There are several types of detector differing in their wavelength range, speed of response, sensitivity etc. Their purpose is to convert the radiant energy falling upon them into an electrical signal which can then be processed and displayed in a way that the operator can interpret.

(i) Photocells

This detector is the simplest and least expensive of the available types of detector. It is constructed of a piece of metal coated with a light sensitive material. Radiation falling on this material, usually selenium, has sufficient energy to cause electrons to be released and a small current can be made to flow by connecting the front and rear faces of the photocell.
Photocells have the advantages of being inexpensive, robust, small and need no external power supply. They suffer the disadvantages of having a limited wavelength range of approximately 400 to 750 nm and comparatively low sensitivity. For this reason photocells are mainly confined to colorimeters where the large amount of energy transmitted by wide bandpass filters allows the use of a low sensitivity detector. Photocells have a slow response to changing light levels and are also prone to fatigue – the detector output gradually drops over a period of time if a high level of radiation is allowed to fall constantly on its surface. In addition, their output is very temperature dependent and often they are protected from the heat generated by the source by a heat filter.

(ii) Phototubes

Phototubes are slowly being supplanted by photodiodes as they basically rely on valve technology for their manufacture. They are constructed of a glass or silica envelope, according to the required wavelength range, which is evacuated to a near vacuum. Inside the envelope is a cathode coated with a photo-emissive alloy and a fine wire mesh as an anode. Light enters the phototube through the mesh of the anode and falls onto the cathode, liberating electrons in the process. These electrons are captured by the anode and the resultant current is then passed to an amplifier before being displayed.
The cathodes can normally be made sensitive to radiation from 190 to about 650 nm or from 600 to 1000 nm, so most spectrophotometers require two phototubes to cover their complete wavelength range.

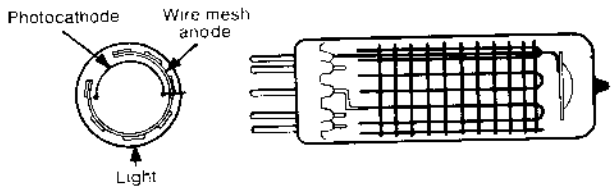


Fig. 27 Vacuum Phototube

Phototubes give an output, known as the 'dark current', even when no light is falling upon them and the magnitude of this current is dependent upon temperature. As the ambient temperature of most laboratories fluctuates during the day frequent resetting of the spectrophotometer '0%T' control may be necessary if measurement inaccuracies at high absorbance are not to be introduced. Like photocells, these detectors will fatigue if left subject to a high level of illumination. Therefore, instruments are fitted with a shutter of some kind to protect them from ambient lighting when the cell compartment is opened.
Although much more sensitive than photocells, phototubes require a power supply to maintain the voltage between the electrodes and an external amplifier for the signal. Due to their higher cost they are usually found in medium performance spectrophotometers.

(iii) Photodiodes

Silicon photodiodes have been available for many years, but it is only recently that the technology has advanced sufficiently for them to be incorporated as detectors in spectrophotometers. The photodiode consists of a reverse biased p–n junction. When light falls on the surface, the holes from the n layer migrate to the p layer and are annihilated, so increasing the conductance of the device. The usual photodiode has a peak sensitivity around 1000 nm, and virtually no sensitivity below 400 nm, making it unsuitable for a UV/visible spectrophotometer.
Recent advances, however, have allowed their response to be extended down to 200 nm, and the sensitivity profile to be tailored to reduce the red sensitivity, making them emminently suitable for spectrophotometers. Their sensitivity does not match that of photomultipliers and so they are not used in instruments where the energy levels are relatively low (e.g. very narrow bandpass instruments).
By making use of integrated circuit technology it is

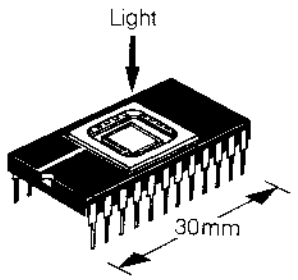


Fig. 28 Photodiode

possible to produce a device which has over one hundred diodes arranged in a linear array of only a few millimetres in length. If the spectrum is spread over this array it is possible to measure the intensity at any wavelength simply by monitoring the output of the appropriate diode element rather than by rotating the dispersive element. Very rapid wavelength scans (2–5s) can be accomplished purely by electronically interrogating the diodes in sequence. Such diode array spectrophotometers offer the speed necessary to characterise peaks eluting from a liquid chromatograph or the study of fast chemical reactions, but as yet, suffer considerable price disadvantages over conventional spectrophotometers of similar performance.

(iv) Photomultipliers

Photomultipliers are close relatives of phototubes but have the advantage of being much more sensitive and offer a wider wavelength range. They have a very fast response to changes of light level and are the preferred detector for double beam instruments, such as the PU8800 (see Section 7b), which shine light alternately from sample and reference beams onto the photosensitive surface.

Light passing through the silica envelope of a photomultiplier strikes an electrode known as a photocathode which emits electrons. These electrons are then attracted to a second electrode (the first dynode) which is maintained at a higher voltage than the cathode. Each electron reaching the first dynode causes two or more electrons to be emitted which fly off to hit the second dynode which is at a still higher potential. Thus, as electrons hit successive electrodes in the 'dynode chain' an avalanche effect is set up. With a chain of nine or more diodes a single photon striking the light sensitive surface can cause millions of electrons to be released by the last dynode. Therefore, a photomultiplier has a very useful internal amplification which eases the task of external circuitry. There are two main types of photomultiplier known as 'side-window' and 'end-on'. In the side-window variety the electrons follow a complicated path between curved dynodes before arriving at the anode. This arrangement has the advantage of being very compact but it is generally limited to about nine dynode stages. On the other hand, the end-on arrangement permits more dynode stages to be enclosed within the envelope providing extra amplification. Their photosensitive area can be made much larger than with side-window types and less sensitive to fluctuations caused by small shifts in position of the light patch. They are, however, more expensive to construct.

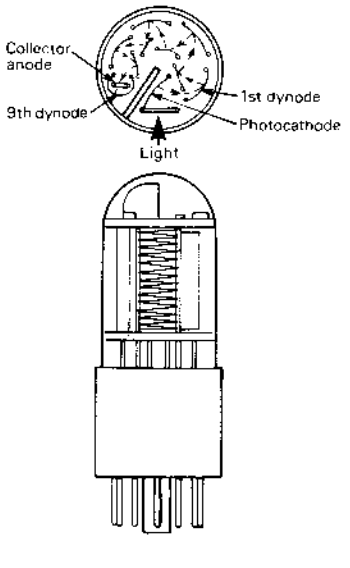


Fig. 29 Side-window Photomultiplier

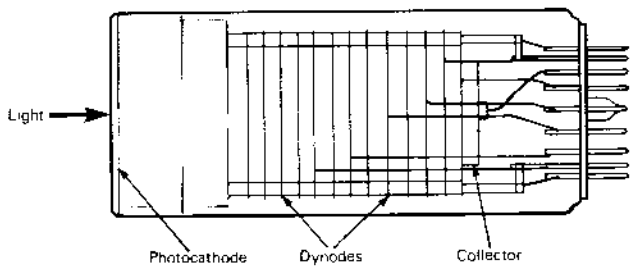


Fig. 30 End-on Photomultiplier

Photomultipliers are not as prone to fatigue as photocells and phototubes, but can be burnt out if daylight is allowed to fall upon them while working. Spectrophotometers utilising this type of detector are usually constructed so that the voltage across the dynodes is cut when the cell compartment is opened to avoid damage of this kind.

Photomultipliers can be made sensitive to light over the whole ultraviolet and visible range from approximately 190 to 900 nm, but they are expensive to produce and need a high voltage power supply. They are usually confined to high performance spectrophotometers with narrow bandwidths, consequently requiring the detection of low light levels, and to double beam instruments where rapid response to alternating light levels is important.

(f) Readout Systems

The final link in the chain is conversion of the signal from the detector into a form which the analyst can use. The term 'readout system' is used as this process involves the combination of electronic circuitry and a display unit to render the result visible.

The amplified signal from the detector is normally proportional to the percentage transmittance of the

sample and, to be more useful, this must be converted to absorbance. In older instruments, this conversion was effected by the exponential decay of a capacitor or the logarithmic characteristics of a diode. Modern microprocessor instruments may utilise a 'look-up' table to effect the transition. Microprocessors have, of course, revolutionised the electronics of spectrophotometers. Initially used purely to control the instrument, they are now often used to calibrate and check instrument functions, as well as providing the results for display on the instrument readout.
A detailed description of spectrophotometer electronics is beyond the scope of this booklet, but the interested reader can find further information in the operating or service manuals for most instruments. However, a discussion of the relative merits and shortcomings of the various types of display units should be beneficial to all potential operators.

(i) Meters
Until the early 1970s moving coil meters were the most widely used form of readout and many remain in service and continue to be offered on the lowest cost instrumentation. Most incorporate a mirrored surface behind the pointer so that its position relative to the printed scale could be read without errors due to parallax. The accuracy of reading is largely dependent upon the length of the scale – the longer the better. Meters are usually limited by their linearity which is seldom better than ±0.25% i.e. a meter which is exactly correct at zero deflection and full scale may indicate between 49.75 and 50.25% of full scale for a true 50% input, but this is perfectly adequate for most analytical purposes.
Meters are ideal for following the trend of a changing input signal as one can easily interpret the velocity of the pointer past the fixed scale. Similarly, when noisy signals are encountered, a sensible reading can often be obtained by estimating the mid-point of the pointer's vibrations.
The main disadvantage of meters is that they are subject to being misread. It is all too easy for a tired operator to ascribe the wrong value to a sample after a long series of repetitive measurements. Even greater mistakes can occur with meters having variable input sensitivities and different printed scales for each. Here the danger is that the pointer position can be, and often is, read off against entirely the wrong scale.

(ii) Digital Displays
Digital displays are inherently more linear than meters, and thus more accurate, and are not so subject to errors of misinterpretation. These advantages usually outweigh the fact that when the input signal is changing in magnitude or is noisy, the flickering digits make reading somewhat more difficult than with a meter. As these displays can be read accurately from some distance and the operator does not have to squint

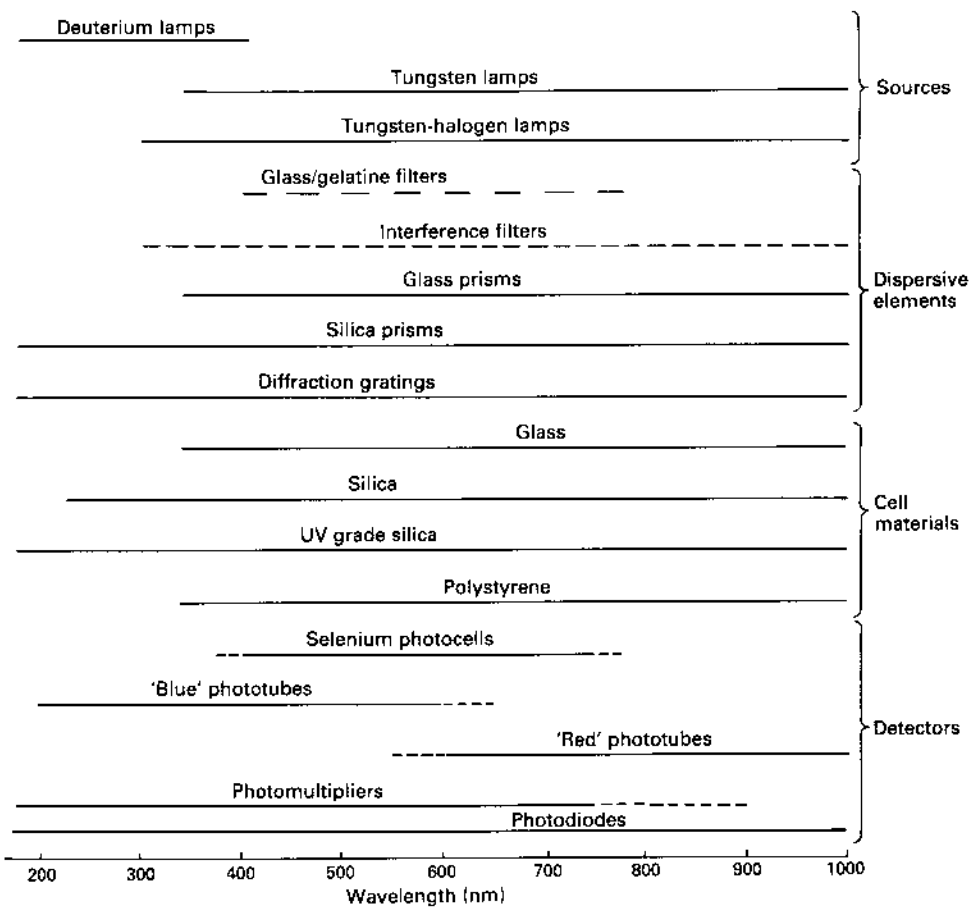


Fig. 31 Wavelength Range of Spectrophotometer Components

at an antiparallax mirror they are much less tiring to use for a series of measurements. Modern digital displays usually take the form of 7 bar displays. Each element (or bar) of a given number is a short light emitting diode (LED). Thus the display is robust, possesses solid state reliability and has low power consumption.

(iii) Visual Display Units
The most recent breakthrough in the man-machine interface has been the application of TV displays, otherwise known as CRTs (cathode ray tubes) or VDUs (visual display units), to the control of spectrophotometers and display of instrument status and results. Setting up such instruments is usually just a matter of choosing modes from the displayed menu of options and inputting information or carrying out actions in the sequence prompted by the instrument. These displays make operating manuals practically superfluous and dramatically reduce the complexity of the controls needed to drive even the most sophisticated spectrophotometer. Equally important, these screens are by far the best medium yet devised for continuous display of the instrument's status and make it possible to show results in both graphical and numerical formats.

(iv) Controls and Operation
In recent years, in the wake of the microprocessor revolution, knobs, levers and switches have fast given way to push-buttons and keypads. Now, instead of cranking a handle to change wavelength it is commonplace to simply key-in the desired value and a motor will drive the monochromator the correct distance. Once, mechanical end-stops would limit the travel of a handle and an abrupt halt to its manual rotation was the feedback to the operator that the wavelength limit had been reached. Now complex rules are held in software and every operator input is checked for validity before being actioned. Attempt to push the instrument into areas forbidden by the software and a flashing error message and a buzzer are likely to be revealed as the modern equivalent of the end-stop.
The coming of the microprocessor also suddenly made a lot more possible for the same outlay. Once upon a time operators were able to purchase a comparatively simple instrument and add 'black boxes' – each electronic module offering a separate function so that the instrument could be tailored to match exactly the lab's requirements. More boxes could be added as those needs changed or as more money became available. These facts of life suddenly changed with the advent of the microprocessor. All the essential functions and many frills could be included in the basic instrument without adding to the cost of manufacture. It became possible to add even more exotic capability simply by adding extra memory chips.
On the face of it, this situation should have been entirely to the benefit of the laboratory. However, the extra flexibility was often provided at the expense of ease of understanding and operation. Whereas one used to be able to inspect the status of an instrument simply by glancing at the relative positions of controls and indicators it now became necessary to interrogate each parameter in turn by pressing its key and reading the resulting displayed value. However, in the very latest instruments such problems have been overcome by careful attention to control panel layout and fool-proof control interlocks. Clear and constant display of the important parameter values is coupled with the masking or de-emphasising of rarely required functions so they do not intrude upon the setting-up of routine programs by inexperienced operators. Indeed, the ability to store routinely-used parameter sets for later recall at the touch of a button is, perhaps, the ultimate in operation simplification.
The first commercial microprocessor controlled UV/VIS spectrophotometers were introduced in the late 1970s. The costs of processor and associated memory at that time favoured their application in the more expensive wavelength scanning instruments but progress is such that within five years they could be found in equipment at every price level. They have provided the user with many benefits but also with a few new pitfalls for the unwary.
Fundamentally, a spectrophotometer is an optical device and its analytical performance will always be governed by the quality of its optical design. The purchaser must now be very careful not to lose sight of this when confronted with an instrument packed with the latest in fashionable software-generated features. No amount of data manipulation will conjure up spectral information which was never gathered by the optics in the first place. Wavelength and absorbance accuracy and reproducibility coupled with factors such as bandwidth, stray light and drift remain the hallmarks of quality to be inspected first in any specification.

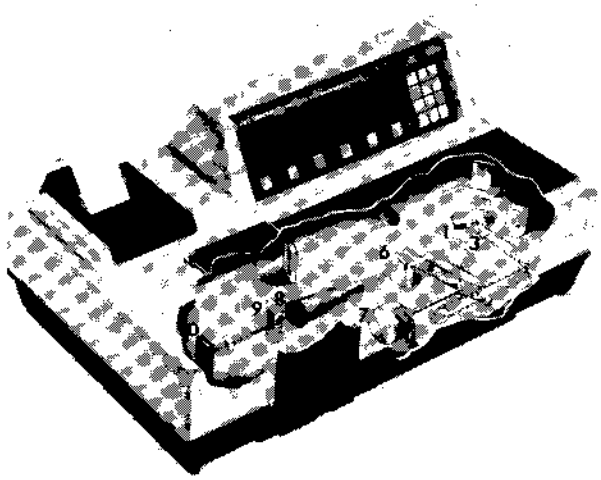
7 Typical Spectrophotometer Configurations

The spectrophotometer components discussed in the previous sections can be assembled in many alternative ways. We shall now consider two fairly typical configurations which will serve to illustrate the practical application of these components in instruments offering very different capabilities.

(a) Fixed Wavelength Instruments

The vast majority of UV/visible spectrophotometers are used only for the measurement of concentration or rate of change of absorbance with time at a fixed wavelength. Such requirements can be met by the use of a 'single beam' optical configuration. This terminology indicates that the light beam follows a single path between the source and the detector. We shall describe later a 'double beam' system where the light is split into two to provide a 'sample' and a 'reference' light beam. Modern, drift-free electronics mean that double beam systems are almost exclusively used on instruments with wavelength scanning capability.
The PU8600 Series of instruments is designed for routine quantitative analyses in fields of application which include biochemistry, pharmaceutical and food manufacture, water pollution, metallurgy, clinical chemistry and very many others. The design derives from the SP6 Series which sold in its thousands for many years to laboratories in every corner of the world. The addition of a microprocessor has permitted big

gains in operator convenience, confidence and precision.
The added convenience results from replacement of several manual controls (such as those for wavelength, zero and concentration) by touch buttons and the total elimination of source and detector change levers with their associated rules of use. Most laboratories have a group of core assays which are undertaken repetitively. The parameters for up to ten of these assays can be stored and then recalled at any time in the future at the touch of a button. A printer option not only provides hard copy of results but will also print the conditions under which they were obtained or provide a listing of the contents of the program stores.
If the operator is uncertain whether the PU8600 is set exactly on the absorbance peak then it can be commanded to search either side of the nominal wavelength and reset itself precisely on the maximum. Especially convenient for the life scientist is a special version with extra keyboard functions allowing kinetic results to be computed and for the temperature of the cell holder to be set.
Confidence is increased over its non-processor forerunner due to the ability of the PU8600 Series to carry out a self-test of its vital functions and its constant monitoring both of operator inputs and output data for validity. Precision has been enhanced by the use of the microprocessor to convert the digitised detector signal, which is linear in transmission, into absorbance or concentration by a mathematical log process. Earlier designs utilised the logarithmic characteristics of diodes or capacitors to produce absorbance readout and an amplifier to convert this into concentration. However, such circuitry was vulnerable to changes due to temperature, supply voltage and component ageing.



1 Tungsten-halogen lamp
2 Deuterium lamp
3 Lamp change mirror and stray light and order-sorting filters coupled to wavelength drive
4 Entrance slit
5 Mirror (silica coated for durability)
6 Master holographic grating with 600 lines/mm
7 Wavelength drive motor
8 Exit slit
9 Sample cell (pathlengths from 2 to 50mm may be used)
10 Photodiode detector

Fig. 32 PU8600 Single Beam Spectrophotometer

Signal averaging is another new facility which permits the noise encountered at high absorbances, especially towards the wavelength extremes of the instrument, to be smoothed over a few seconds to obtain a precise, flicker-free result.
The PU8600 Series spectrophotometers have tungsten-halogen and, in the UV versions, deuterium sources. The sources are mounted on pre-aligned bases so that replacement may be simply accomplished by the operator. A useful feature is the ability to display the number of hours that each lamp has been used and its energy relative to when it was new. The lamp change mirror and stray light and order-sorting filters are mounted on wheel mechanisms which are coupled directly to the wavelength drive so that necessary changes are made automatically whenever the operator keys in the new wavelength.
Light from the appropriate source is reflected through the entrance slit of the monochromator onto a spherical mirror and thence focused onto the exit slit via the grating. The monochromator used in the PU8600 Series is a focus-compensated version of the basic Monk-Gillieson arrangement i.e. a plane grating operating in a convergent light beam. In the simple configuration the dispersed light from a grating receiving a convergent beam is focused at a position which varies with wavelength. Thus the monochromator would be in focus and working efficiently at only one wavelength. However, in the PU8600 the grating is mounted on the end of a short arm linked to a motor-driven spindle so that as the spindle rotates, not only does the grating angle change, but the grating itself is moved along the optical axis of the system. By careful design, focusing errors in the PU8600 monochromator are thus virtually eliminated and very good performance is achieved in an economic way.
The master holographic grating has 600 grooves per millimetre and is blazed at 240 nm. The grating and the mirrors are coated with a tough evaporated layer of silica which protects their aluminised reflecting surfaces from the laboratory atmosphere. The etched entrance and exit slits are 0.35 mm wide and provide a bandpass of 8 nm which is ideally suited to the majority of routine quantitative analyses.
The light traverses the sample compartment, which will accommodate a very wide range of sample handling accessories, and is focused by a lens onto the detector. A shutter automatically swings in front of the lens whenever the cell compartment is open to protect the detector from ambient light. A diode detector covers the whole 195–900 nm range and replaces the vacuum technology phototube used by earlier versions of the instrument.
All detectors generate a current, known as the dark current, when no light is falling upon them. This current must be trimmed out if the output of the detector circuitry is to be linear with light intensity. On many single beam designs this is a manual operation, usually first requiring manipulation of a shutter to cut all light off from the detector. In the PU8600 Series this adjustment is automatic whenever the instrument is switched on, no shutter movement being necessary as the processor delays the illumination of the sources until measurement of the dark current is complete.
In the past, spectrophotometers have usually relied

upon factory setting of a mechanical stop or microswitch for their wavelength calibration, both of which could be subject to setting errors and changes with time due to wear on 'bedding-in'. Calibration on the PU8600 family is carried out automatically every time the instrument is powered-up or a Test program is executed. The instrument drives to 'zero' nanometres and automatically peaks-up on the white light, zero-order (mirror) reflection from the grating. From this known position the microprocessor generates the appropriate number of pulses for the stepper motor to obtain any required wavelength. A multi-vaned disc on the wavelength drive spindle rotates between a light-emitting diode and a photo-diode to provide feedback pulses to the processor. Should the number of pulses received by the microprocessor not check with those it outputs to the motor then a jam or slippage of the drive must have occurred and a wavelength error would be signalled to the operator.
Although the PU8600 has sufficient computing power to cope with the vast majority of applications it is fitted with an interface (to an internationally recognised standard known as RS232C) which permits it to output data to a computer and to receive commands to modify its measurement mode. Thus a computer program can be written to automatically take readings at several wavelengths to measure multicomponent mixtures or compensate for background turbidity. Microcomputer software will even allow modest resolution wavelength scans to be accomplished.

(b) Wavelength Scanning Spectrophotometers

The detector output of a single beam spectrophotometer varies rapidly with wavelength as it depends on the combined effects of the variation with wavelength of the source energy, grating efficiency, mirror reflectivity and detector photo-sensitivity. This means that a spectrum can only be obtained from simple single beam instruments by the tedious process of setting zero absorbance on a cell containing the solvent (blank or reference solution), moving the sample cell into the beam, measuring the absorbance, changing the wavelength and repeating the process for each data point required.
Linking a computer to the PU8600 can take the tedium out of this process and produce the data faster than any operator. The computer is simply told the start and stop wavelengths of the scan and the blank is placed in the beam. The computer then commands the PU8600 to drive to the start wavelength and output the absorbance which it stores in its memory. It then commands the PU8600 to go to the next wavelength and the process is repeated until the spectrum of the blank solution (and the cell containing it) is held in its memory. The blank solution is then emptied and the cell refilled with the sample solution and placed in the beam. The measurement sequences are repeated and at each wavelength the stored absorbance of the blank is subtracted by the computer from the new reading to yield the nett absorbance of the sample which is then displayed and plotted.
This principle of storing a spectrum of the blank and subtracting it from subsequent sample spectra has, with the reducing cost of memory and processing power, now become a practicable proposition for purpose-built single beam scanning instruments. However, the technique does have its limitations as it demands a high level of stability with time, both of the instrument and of the sample matrix. Little is gained if the instrumental readings drift between the time when the blank was measured and when the last sample is run. Similarly, if blanks and samples exhibit absorbance changes in time due to settling of particulate matter, variations in ambient temperature, sensitivity to light, etc., then the recorded differences will not be true.
All electronics drift, especially when warming to operating temperature. Consequently single beam scanners tend to be left powered-up night and day with only motors and sources shut-down when the instrument is not in use (reasonable in a routine scanning lab but unwise in research labs with patchy and unpredictable instrumental usage). Scanning rapidly is the only way of reducing the vulnerability to temporal changes in the sample. However, this involves other performance compromises and any apparent throughput gains are quickly swallowed by the necessity to produce hard-copy after the scan rather than simultaneously. Therefore, single beam scanning spectrophotometers are currently confined to applications where low to medium performance is acceptable. For the highest levels of performance the double beam concept must be utilised.
Double beam systems have the advantage of higher stability than single beam devices (despite some loud claims) and is achieved without the need to power the electronics continuously. As we shall see, the double

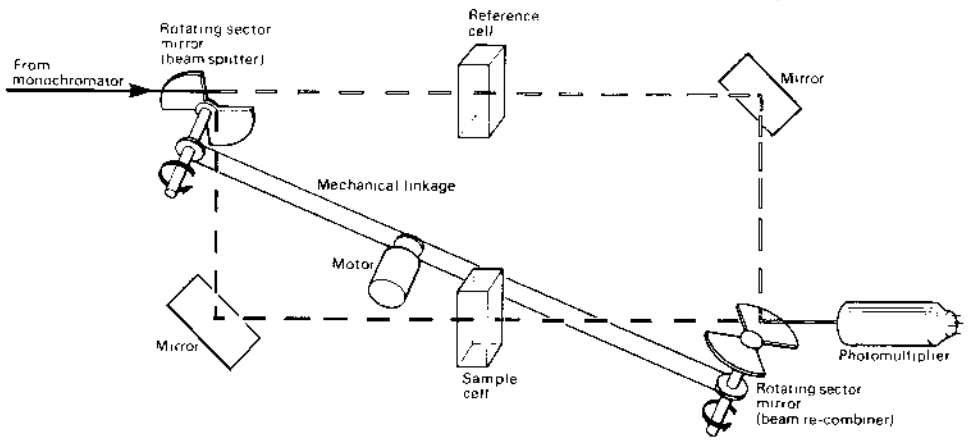


Fig. 33 Schematic Double Beam Optical System

beam concept permits essentially simultaneous measurement of blank and sample and is thus to be preferred whenever changes with time are suspected. The essentials of the double beam principle are shown in *Fig. 33.* Light emerging from the monochromator is split or 'chopped' into two alternating pulse trains of radiation. One of the pulse trains is diverted so that it passes through the sample cell and the other so it passes through the reference (blank). The beams are then recombined with another rotating mirror or, more usually, by arranging for sample and reference beams to overlap directly on the face of the detector.

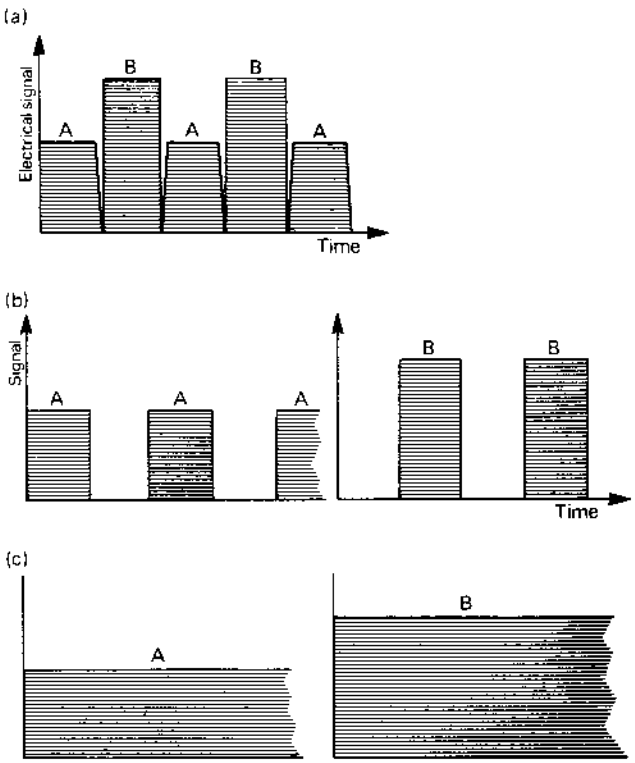


Fig. 34 Ratio Reading System

The signal from the detector is, therefore, as shown in *Fig. 34,* where A indicates the part of the signal passing through the sample, and B that due to the beam via the reference cell. The rotating beam splitter is usually fitted with a device which sends signals to the electronics so that, at any instant, it is known whether the detector signal arises from the sample or reference channel. In practice, the rotating chopper usually includes a blackened portion so that no light passes down either channel. This allows the dark current of the detector to be measured and compensated for on every rotation. The pulses are electronically resolved into separate a.c. signals before being rectified to two d.c. voltages proportional to sample (I) and reference (I_o) transmission. These voltages are ratioed, digitised and displayed as a transmission value or, after logarithmic conversion, as absorbance.

As the variations in source energy, detector sensitivity etc. affect both beams simultaneously (or almost simultaneously since the chopping frequency is typically between 50 and 100 Hz) the ratio of energies in the two beams remains unaltered unless an absorbing substance is placed in one of the beams. The monochromator may, therefore, be driven by a motor and the output from the electronics used to produce a spectrum of sample absorption against wavelength. In fact, a completely flat absorbance baseline against wavelength is difficult to obtain due to minor variations in mirror reflectivity and geometry between the two beams. In addition, the cells in which sample and reference solutions are contained are never perfectly matched so, for the highest possible standard of accuracy, modern double beam instruments allow these minor variations to be stored in memory and subtracted digitally from the subsequent sample scans. Such baseline memory functions are in essence the same as those used by the single beam scanning instruments but here they are a minor performance enhancement rather than an absolute necessity.

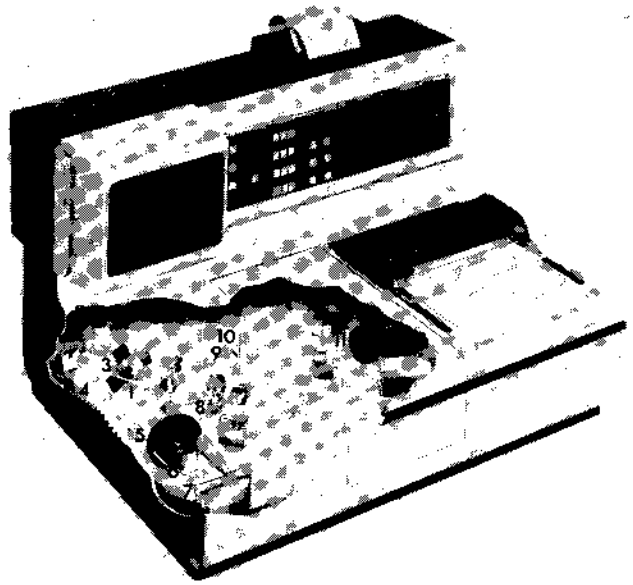
Let us now consider how the double beam principle is employed using the Pye Unicam PU8800 as a practical example. The PU8800 Series offers high and research levels of performance by a choice of single or double monochromator and side- or end-window photomultiplier. In the past such performance would have been accompanied by a daunting array of knobs, buttons and lights but the PU8800 uses its VDU to display instrument status and results whilst setting-up is simply a matter of following the menu of commands that is presented. Operation is thus exceptionally simple and is made even more convenient by the ability to store the laboratory's 10 most frequently used programs for near-instant recall at any time.

Like the PU8600, this instrument can also conduct a most comprehensive self-test program, even going to the extent of resetting its wavelength calibration using the sharp emission lines of the deuterium lamp and checking absorbance linearity by automatically inserting calibrated absorbance filters into the light beam. Results can be recorded on an integral, wavelength synchronised recorder or on an optional printer.

Tungsten halogen and deuterium sources are fitted and are changed at 317 nm by a mirror mounted on a motorised wheel which is under the command of the microprocessor. Also mounted on this wheel are the stray light and order-sorting filters which are inserted into the light beam at appropriate wavelengths. The light is normally focused by the next mirror onto the entrance slit of the monochromator but, on the double monochromator instrument, a supplementary grating and mirror are located ahead of the main monochromator. This compact secondary monochromator has the effect of reducing the level of stray light from typically 0.001 to 0.0001%T and thus permits analytical linearity to be maintained even on measurements of the most optically dense samples.

After passing through the entrance slit the light is collimated, i.e. made parallel, by one side of a concave mirror and directed onto the grating. The diffracted beam from the grating is focused by the other side of the concave mirror onto the monochromator exit slit. The entrance and exit slits are mounted as pairs in a motor-driven disc. Sets of slits can be selected simply by choosing the desired bandwidth on the VDU and they allow the operator to trade resolution for energy reaching the sample. The narrowest pair yield a

nominal bandwidth of 0.1 nm which gives the resolution necessary for producing vapour spectra. Most solution spectra are measured with the intermediate slits of 0.2, 0.5, 1 and 2 nm. When both sample and reference samples are highly attenuating (more than 2 or 3 A in both beams) or if special accessories are in use, high energy slits can be used. These have an effective bandwidth of 10 nm and provide an optical alternative to electronic signal averaging as a method of quenching the noisy signal. The master holographic grating has 1200 lines/mm giving a dispersion of 4.7 nm/mm at the exit slit, and is blazed at 240 nm. This type of monochromator is said to have an 'Ebert' configuration. Scanning is achieved by rotating the grating using a stepper motor and electronic hardware and software ensures perfect synchronism with the chart recorder and graphics display.



1 Tungsten-halogen lamp

2 Deuterium lamp

3 Lamp change mirror and stray light and order-sorting filters on motor-driven wheel

4 Location of optional secondary monochromator (not shown)

5 Pairs of monochromator entrance and exit slits on motor-driven disc provide bandwidths from 0.1 to 10mm

6 Master holographic grating with 1200 lines/mm (rotation mechanism not shown)

7 Collimating mirror (all mirrors and the grating are silica coated for durability)

8 Rotating chopper mirror

9 Sealing windows

10 Sample and reference cells (the large sample compartment will accommodate a wide range of sample handling accessories)

11 End-window photomultiplier (side-window is optional)

Fig. 35 PU8800 Double Beam Spectrophotometer

The light emerging from the exit slit is directed onto the rotating beam splitter by a plane mirror. Light which is reflected from the mirrored sectors of the splitter is directed into the reference channel. Light passing through the splitter is directed into the sample channel. Both beams are focused at the sample holder positions and recombined on the face of the photomultiplier by mirrors.

As befits a research performance instrument, the very large sample compartment will accommodate a vast range of attachments for unusual samples or for using associated analytical techniques such as densitometry, fluorescence and reflectance. One attachment allows 10 mm cells to be mounted at the secondary foci of the beams, immediately ahead of the end-window photomultiplier. Scattered light from turbid samples located at these foci will be gathered over a very wide angle by the detector thus providing much higher analytical sensitivity and linearity than if they were mounted in the normal position. This holder is particularly valuable to microbiologists and other life scientists who study suspensions or extracts from plant and animal tissue.

A novel aspect of the PU8800 design is the care taken to avoid secondary reflections in the optical path which can be shown to affect the absorbance accuracy of other instruments. It is possible that light traversing the cell could be reflected at its rear face and pass back along the beam. If this light then met another reflecting surface, a small proportion could return through the cell to give a false reading at the photomultiplier. Such multiple passes are eliminated in the PU8800 by angling the cell compartment sealing windows so that reflected rays are deflected.

Similarly, light from one beam could be reflected back into the other beam off the face of the photomultiplier. This rogue light can then pass through the cell, be reflected at the front face and return. The unwanted light is eliminated because the mirrors immediately ahead of the photomultiplier are tilted so that they direct light downwards onto it. The photomultiplier is mounted slightly below the plane of the optical system, so that any light reflected from the detector face thus continues on its downward path and is lost.

The superb optics of the PU8800 coupled with advanced software features, not described here, yield a level of performance which is unmatched by other commercial spectrophotometers. Consequently, it has rapidly established itself as a reference instrument in the most discerning establishments including pharmaceutical, medical and university research centres where no compromise can be afforded in the search for accuracy and precision.

8 Instrumental Performance and Errors

It is important in dealing with spectrophotometry not to lose sight of the purpose behind the instrumentation. We have discussed in Section 5 what spectrophotometry is required to do, in other sections we have considered how it may be done in theory and in Section 7 how it is done in practice. In this section we shall mention some of the ways in which practical instruments fall short of theoretical perfection and how instrument specifications should be assessed.

There are two main features a user should always assess in a spectrophotometer (though often features such as speed, simplicity, versatility and design may be important).

(i) Photometric Accuracy and Reproducibility

In quantitative work the user wishes to measure the concentration of a substance to a certain degree of accuracy. The accuracy he can achieve depends partly upon the accuracy with which the spectrophotometer measures the values of $\log (I_o/I)$. It also depends upon the accuracy with which he can collect and prepare the

sample and, sometimes, the errors involved in these processes are so large that it is pointless to demand a high accuracy from the instrument. Often it may be sufficient for him to know whether the concentration of the substance is within certain wide limits. (For example, in medicine it is quite normal for a patient to have a blood sugar level anywhere between 0.6 and 1 g litre^{-1} as his diet varies from day to day, and it is only concentrations outside these limits that are of concern). Usually, however, the user requires maximum possible accuracy. In qualitative work, photometric accuracy is not of great importance though it is still desirable.

(ii) Wavelength Accuracy and Reproducibility
In qualitative work on identification the user is comparing the measured wavelengths of bands with standard published values and will be led astray if the wavelengths are not correct. In qualitative comparisons and in quantitative analyses the reproducibility of wavelengths is important though the actual accuracy may not be.
Of the several factors which influence these two requisites we shall define and describe only the following important ones:

(a) Spectral Bandwidth and Resolution

The definition of spectral bandwidth has already been given in Section 6(c), as a criterion of the monochromator performance. It can affect both the photometric accuracy and wavelength accuracy of the spectrophotometer. The absorbance value measured by a spectrophotometer at a wavelength λ is an average value for the absorbance of the sample over the wavelength range of the light which has been passed through the monochromator. The narrower this wavelength range is, the nearer is the measured value to the true figure. A spectrophotometer with a broad bandwidth will therefore give spectra in which the bands are smoothed out and absorbance differences appear smaller than they should (i.e. the sensitivity of the instruments is diminished).
Fig. 36 illustrates this effect and also illustrates how the instrument loses the power to discriminate between (or to resolve) two closely spaced bands. This means that in some cases absorption bands may be missed altogether and also that interference problems in mixtures are worsened. Moreoever, when the width of the absorption band is small by comparison with the bandwidth of the spectrophotometer, Beer's Law is no longer followed exactly and a curved calibration will be obtained.

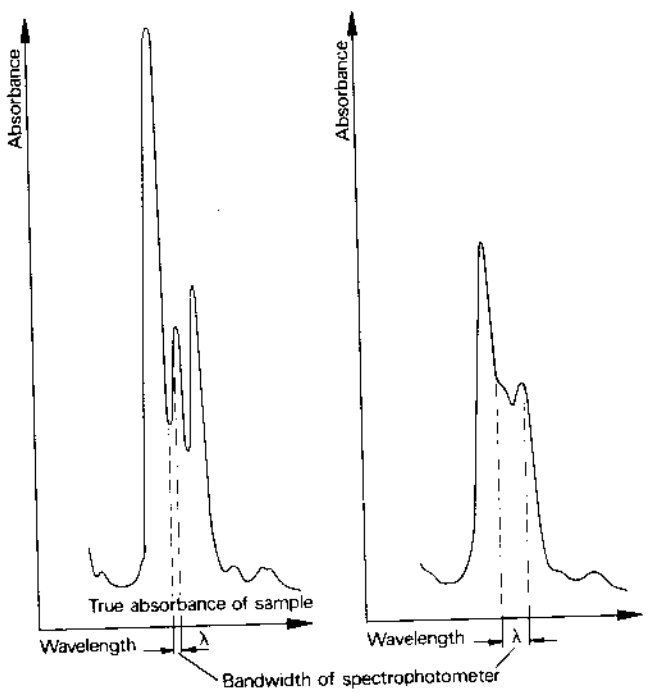


Fig. 36 Effect of Bandwidth

(b) Stray Light

Light of a wide range of wavelengths enters the monochromator from the source and, to be useful, only a very narrow band of wavelengths should emerge from the exit slit. Hopefully, the unwanted radiation is totally absorbed within the monochromator enclosure and the internal surfaces of the enclosure are normally blackened to assist this absorption. Unfortunately, no monochromator is ever perfect and some radiation of unwanted wavelengths usually manages to escape via the exit slit. This radiation is known as stray light.
Stray light is most frequently encountered at the extremes of a spectrophotometer's wavelength range where either the source output or the detector sensitivity is low, or near 200 nm, where the oxygen in the atmosphere absorbs strongly. Consider a spectrophotometer with a photomultiplier detector and tungsten filament source. If we were to plot the photomultiplier output against wavelength we should obtain a function similar to that in *Fig. 37*. At short wavelength the output is low because the source energy has fallen (see *Fig. 3*) even though the photomultiplier has adequate sensitivity, whilst at longer wavelengths the detector sensitivity drops, rapidly outweighing the rising source energy.
If we were to measure a sample with a reasonable transmittance at λ_2 we should have a large detector signal and a small amount of stray light of shorter or

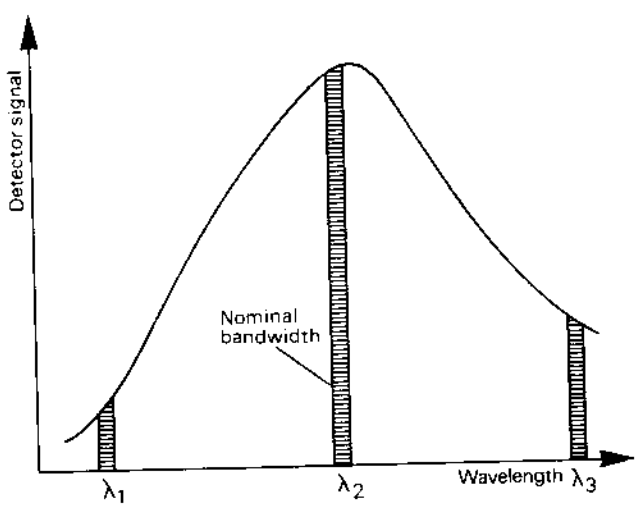


Fig. 37 Stray Light

longer wavelength would produce only a slight increase in signal. Now, if we measure a similarly transmitting sample at λ_1 or λ_3, we shall have a comparatively small detector signal but even a small proportion of stray light from wavelengths near λ_2 will produce a significant increase in the detector output.
The effect of stray light becomes worse as the sample absorbance increases since a progressively larger proportion of the detector signal is due to light of other wavelengths escaping from the monochromator and remaining unabsorbed by the sample. Thus, if the sample absorbance is high enough, stray light can be troublesome at any wavelength. The analyst's attention is normally drawn to the presence of stray light by the curved calibration it causes (see *Fig. 38*). In most cases the analysis may still be carried out but at some small loss in convenience and precision as direct readout in concentration is no longer possible. A calibration graph must be constructed, and highly absorbing samples diluted to avoid working on the most curved portions of the graph.

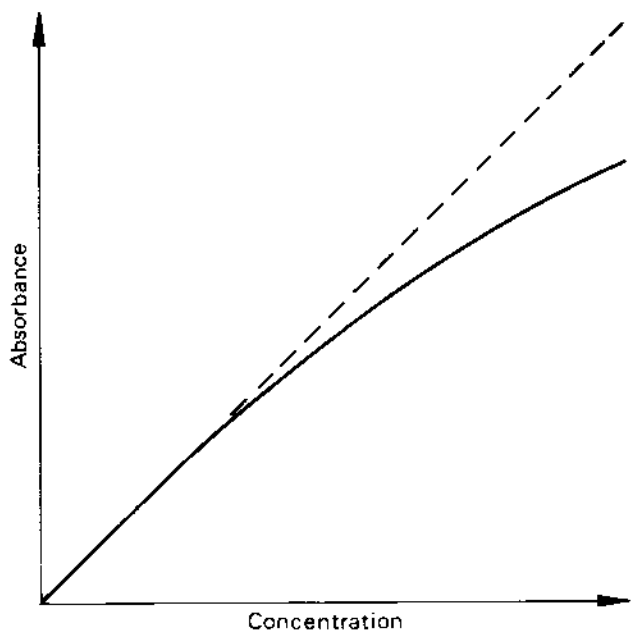


Fig. 38 Effect of Stray Light

Deviations from linearity are not only caused by stray light escaping the clutches of the monochromator. Ambient light can enter the spectrophotometer through damaged cell compartment lids or loose covers. This can easily be checked by setting the instrument to a visible wavelength and shining a handlamp on any gaps in the lids or covers. Any light leaks will be revealed by a jump in the instrument reading.
Deviations may also be blamed on stray light but which could be due to changes in the solution with concentration caused by complex formation with the solvent, polymerisation, change of pH etc. This may be checked by making up a solution of the sample which gives a reading of about 0.5A in a 10 mm pathlength cell and then measuring the same solution in a 40 mm cell. If one reading is not four times the other, after correcting for the different absorbances of the cells when filled with pure solvent, then stray light is likely to be the culprit. However, spectrophotometers are less accurate at high absorbance and small deviations from a four to one ratio could be explained by inaccuracies in the logarithmic conversion process.
Other causes of curved calibrations include fluorescent or turbid solutions. If the solution is fluorescent, part of the incident energy is absorbed and re-emitted in all directions as radiation of a different wavelength. If the sample in the cell compartment is moved so that it is closer to the detector a lower, and more inaccurate, absorbance reading will be obtained as more of the fluorescent radiation is collected by the photosensitive surface. If, on the other hand, the solution is turbid, a proportion of the incident radiation is lost by scattering rather than by any absorption so that the reading is too high. Moving the sample closer to the detector will again give a lower but, in this instance, more accurate result.

(c) Noise

The beam of radiation reaching the detector consists of photons, arriving not in a steady stream but at random. Further random fluctuations rise in the measuring electronics, again due to the quantum nature of matter. When the signal from the detector is large the small fluctuations, or noise, superimposed upon it are seldom a problem. However, when the detector is receiving light of wavelengths at the edge of its photosensitive range or when, even at the detector's most sensitive wavelength, the sample is absorbing much of the light, then the noise becomes a significant proportion of the total signal.
Consequently, a low noise specification is a vital characteristic of a precision spectrophotometer. Unfortunately most manufacturers have chosen to quote their performance only under absolutely ideal circumstances, that is in the 500–550 nm region (where maximum detector output can be expected) with wide (usually 2 nm) slits, several seconds signal smoothing, and no sample in position. In such circumstances, all spectrophotometers can exhibit an impressive string of zeros in their noise figures but it is a quite useless figure for comparison purposes. True quality shows itself in low noise quotations under more demanding conditions. Pye Unicam spectrophotometers have additional specifications at 340 nm and 2 A which, whilst demanding, are also practical conditions frequently encountered during enzyme assays. Unfortunately, until other manufacturers follow this example, the purchaser's only protection is a pre-sale evaluation using a reasonably absorbing sample at a range of wavelengths. Another trap for the unwary is the method of quoting noise, most manufacturers using peak to peak values but with a few stating RMS (root-mean-square) figures which are approximately five times lower.
What can be done to reduce noise? If high absorbance is the sole cause then dilution or use of a shorter pathlength may be the cure but, failing that, opening the slits will help. Doubling their width will usually halve the noise for photomultiplier i.e. 'shot noise' limited instruments. However, we must be aware that we are worsening the resolution and may depress the results. In practice, this is usually only a problem for rare, vapour studies or sharp bands of some organic

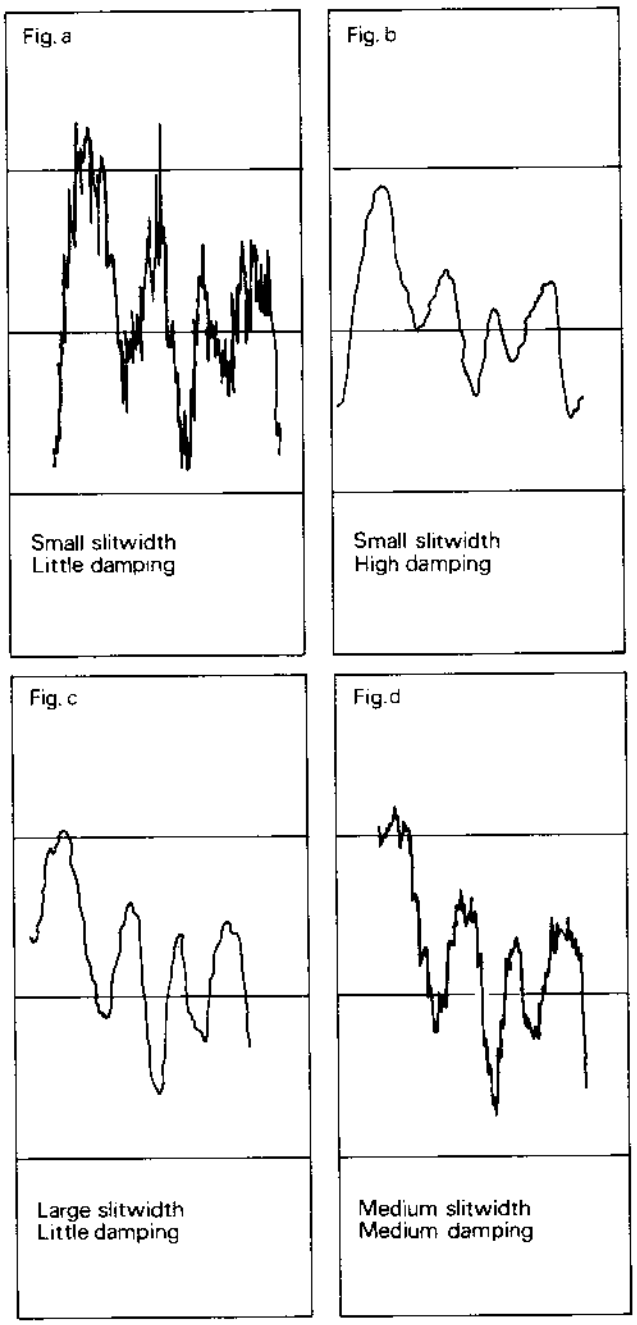


Fig. 39 Noise and Response

compounds in the UV. Widening the slits is the obvious choice for normal colorimetric assays of solutions with broad absorbance bands where standards are employed.

If the instrument has fixed slits, or for other reasons we are determined to hang onto all the available resolution, then we must look to electronic hardware and/or software methods to smooth our signal. Resistor/capacitor networks will store voltages and allow them to be smoothed whilst software routines will permit rapid repetitive measurements to be accumulated and then statistically averaged. In older analogue instruments a control (usually labelled response, period, time constant, or damping) would change the circuit's component values. In modern microprocessor instruments either the time over which the data is to be averaged or the required number of data points is input. The parameter may be labelled with one of the familiar 'analogue' names or with terms such as integration or averaging time. In any event the result is that speed is traded for precision, the improvement being proportional to the difference between the square roots of the old and new times. When the object of the assay is to perform a concentration or rate assay at a fixed wavelength, the benefits of precision versus speed of sample throughput are simple to grasp and balance to individual requirements. However, when the object is to produce a wavelength scan, increasing the time over which measurements are smoothed can produce unwanted cropping and slewing of peaks. We must realise that bandwidth, noise and now, scan speed, are all interrelated and a compromise must usually be sought between resolution, precision and time.

(d) Scan Speed

All measuring and display circuits take a finite time to respond to any change. If their input changes faster than their ability to respond then the full magnitude of the displacement will not be recorded and the output signal will lag behind the input. In the case of a spectrophotometer this means that, by scanning too fast, peaks can be much reduced in height and distorted in shape with the peak shifted in the direction of the scan. If noise on the spectrum is obscuring the position and magnitude of fine detail, the temptation is to increase the applied smoothing but, if this is not accompanied by an appropriate reduction in scan speed, the detail can be lost anyway. Obviously, the broader the absorption band the lower the rate of change of signal and the faster the scan can be. However, a higher concentration of the same sample can be enough to push the rate of change beyond the capability of the system and a reduced peak height recorded without the operator being aware. Achievement of the optimum compromise is an art. However, the novice operator need not be deterred but simply heed the adage 'slow but sure' and keep to a low speed. The PU8800, discussed earlier, offers a novel solution to the dilemma – it can automatically change to lower speeds as a peak is approached and speed up again as it returns to the flat baseline. In this way the highest resolution is obtained in the shortest possible time with no cropping or shifting of peaks. Probably the limiting component in the spectrophotometer system, as far as response is concerned, has been the recorder or plotter. This is because of all the problems associated with the inertia of a pen drive or moving print head. Now it is feasible to avoid these mechanisms and substitute the velocity of the electron beam on a VDU to produce a spectrum – thus permitting faster scanning under any given conditions. Unfortunately the total time advantage gained is slight since, to obtain a hard-copy, the recording is now done consecutively to the scan and not concurrently. Most scans are, in fact, over a hundred nanometers or so using standard conditions and, in these circumstances, the throughput of such 'rapid' scanners can actually be lower than conventional devices.

9 Data Processing

UV/Visible spectrophotometers are becoming ever more powerful in their on-board data processing power. In general, the facilities that are judged beneficial to the majority of the target users are built in as standard and, those that appeal to small but significant groups, are usually available as plug-in memory options. Beyond this are users whose requirements are so specific that manufacturers are unable to warrant production of standard software but who can tailor their own system using the power of a microcomputer connected to the spectrophotometer. In the largest and most advanced establishments the data from many and varied analytical instruments is passed directly or via a micro-computer to a mini-computer at the heart of a complete laboratory management system (e.g. Philips PALM system). With the rapid developments in the field of data processing the equilibrium between these three levels will alter constantly so any discussion here will not only be a generalisation but will also be valid only temporarily. Accepting those qualifications, let us examine each level of sophistication in turn.

Most spectrophotometers now read directly in concentration (plus rate units if aimed at the life science market) and, if they scan, have a baseline memory and 1st/2nd derivative capability. Beyond this one will often find extra functions offered in the form of plug-in PROMs (Programmable Read-Only Memories). In this category we encounter multi-wavelength measurements (e.g. Allen Correction and λ ratios), corrections for curved calibrations (i.e. mathematical correction for stray light or immuno-assay computations) and other minor enhancements. To progress beyond this it is usually necessary to add an interface so that data can be output to a microcomputer. Several types of interface are available of which only two are common – IEEE 488 and RS232C, with the latter having become the defacto industry standard.

Most current spectrophotometers utilise an 8 bit microprocessor which usually limits the amount of memory to 64K. Once a microcomputer is interfaced not only can its RAM (Random Access Memory) be tapped but disc storage conjures up near infinite possibilities. Data can be stored and then manipulated by the most complicated algorithms prior to plotting in a format that exactly matches the laboratory's documentation system.

Applications that can now be accomplished include colour measurement, tablet dissolution and multi-component analysis. The laboratory can store standards and retrieve them from disc to compare with current results. Data can be treated statistically for quality control purposes and discs can be posted to other laboratories for comparison or archiving.

Besides receiving data, the microcomputer can also send commands to the spectrophotometer. Most spectrophotometers can now store a few programs on board but, using the microcomputer's ability to control, the suite of stored analytical programs can be expanded infinitely. Interactive programs can be written and, together with automatic sample changers, open up enormous possibilities. For example, the appropriate programs can be recalled at given sample numbers thus permitting economic automation of very small batches. Alternatively many samples can be screened rapidly at a fixed wavelength and, should a pre-set limit be exceeded, a diagnostic scan carried out automatically on just that sample before reverting to the screening mode – true reporting by exception.

Linking many different analytical instruments to a single computer poses many difficult problems but with many potential laboratory management benefits. When a sample is received in the laboratory and a priority assigned, the computer will be able to integrate it into the work lists or schedules for each instrument in the most efficient manner. As the data is transmitted from each instrument it is stored and collated by the computer and, ultimately, the report is generated. At any time the laboratory staff can call up the sample on the VDU and inspect its progress whilst the computer constantly monitors and alerts the operator to any anomalies or bottle-necks so that immediate action may be taken.

Inevitably, over the coming years the instrumentation for UV/visible spectrophotometry, and for that matter all other analytical techniques, will be changed dramatically by developments in data processing and information technology. Whilst the prospect is exciting, we must guard against the temptation that will arise to treat the instrument as just another 'black-box' computer peripheral for, without a proper understanding of the principles outlined in this booklet, we may lose our ability to distinguish valid data from that yielded by faulty equipment or procedures.

10 Associated Techniques

We have been concerned up until now solely with absorbed and transmitted radiation. The following sections briefly describe the properties of reflection, scatter and fluorescence.

(a) Reflection

When a light beam impinges upon a surface, the total reflected radiation is made up of two components. The first is due to the specular, or regular reflection. In this case the reflection of the light beam from the surface obeys Snell's Law such that the angle of incidence is equal to the angle of reflection, as shown in *Fig. 40.*

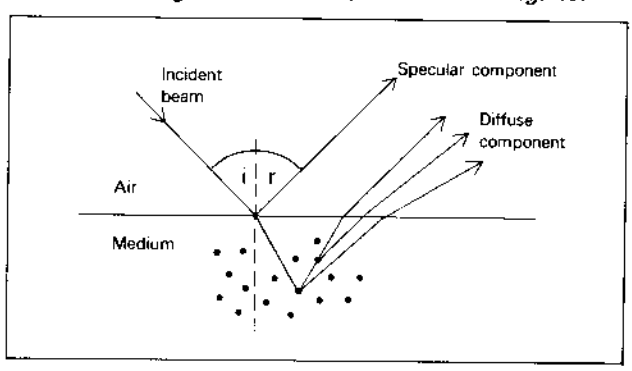


Fig. 40 Specular and Diffuse Reflection of a Light Beam

The second component of reflection comes about because the radiation, which penetrates the surface of the medium, undergoes multiple scattering at the surfaces of the individual particles. A portion of the radiation is returned to the surface by the scattering

process to emerge as the diffuse reflection. The total reflection from a surface is thus comprised of both the specular and diffuse components. The reflectance characteristics of a sample are dependent upon its physical properties, such as the surface gloss and how much light energy the material absorbs.

(i) Specular Reflection

Knowledge of the specular reflectance properties of materials is important in many areas of production and finishing e.g. paints, plastics, ceramics, metal finishing and printing industries. For example, variation in reflectance with angle of incidence is a commonly measured parameter for glazed surfaces. Absolute reflectance data is important in the production of mirrors and in their calibration and quality control. This parameter can be easily measured with modern instrumentation.

Measurements taken with varying angles of incidence are very useful in the non-destructive determination of thin film thicknesses, for example epitaxial silicon films used in the production of microcircuitry. The principle of the thickness determination, using specular reflectance, is the measurement of the separation of the interference fringe pattern produced when the sample is scanned over a range of wavelengths.

The interference pattern is caused by interference between the light beam reflected from e.g. the air-oxide boundary and the emergent, refracted light beam which has undergone reflection at the oxide-silicon boundary (see *Fig. 41*). The emergent beam is in or out of phase with the reflected light beam, thereby giving rise to either constructive or destructive interference. If the wavelength of the incident beam is continuously varied, the difference in phase of the reflected and refracted beams will be altered. This will give rise to a series of maxima at wavelengths where constructive interference occurs and minima at wavelengths where destructive interference occurs. The thickness of the oxide film is calculated using the wavelength difference between the maxima or minima of a reference fringe and a selected fringe.

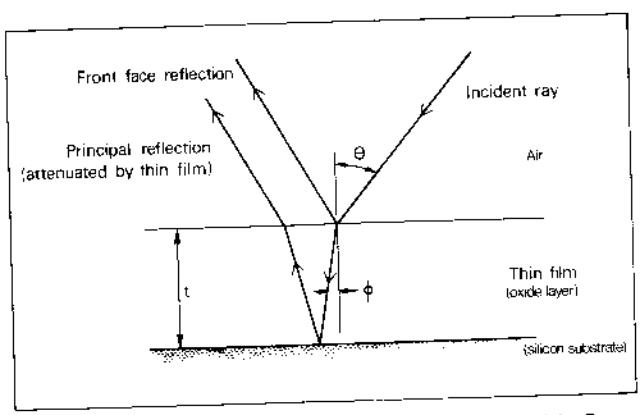


Fig. 41 Reflection and Refraction at the Air-oxide Boundary of a Silicon Dioxide Film

(ii) Diffuse Reflectance

Measurement of diffuse reflectance is most commonly used to determine the colour of a sample.

Today, with heightened awareness of colour differences through the increased range of dyes and pigments, and the need for standardisation in mass production, a system is required for precise measurement and specification of colour.

The Commission Internationale de l'Eclairage (CIE) first tackled the specification of colour on a mathematical basis in 1931.

Colour can be defined in terms of three parameters:

1 The observer
2 The illuminant
3 The sample reflectance.

Firstly we must define our 'observer'. The CIE based their system on the work of Guild and Wright, who averaged the visual characteristics of a number of people and defined the 'standard observer'. Three primary colours: red, green and blue were mixed by each of the panel of observers to match various spectral colours. The arbitrary primaries chosen by the CIE are called X, Y and Z and can be used to match any colour including monochromatic radiation (unlike the original red, green and blue).

The 1931 CIE data were all derived for observers looking at a field subtending an angle of 2° at the eye, and were thus concerned only with the response of the central (foveal), region of the retina, representing a much smaller area than is normally used for general vision. In 1964, the CIE supplementary standard observer (or 10° observer) was defined and gives improved agreement with most observers for general viewing, particularly in the blue region of the spectrum.

Coloured objects depend for their appearance on the lighting conditions under which they are viewed as well as their intrinsic colour as shown by their spectral reflectance curves. A white light whose energy is constant at all wavelengths does not exist. In order to define the appearance of a coloured surface, it is necessary to specify the nature of the light under which it will be viewed. The CIE has defined four standard light sources, illuminants A, B, C and D. A is based on the light given by a gas filled tungsten filament lamp and B and C were obtained from the same source by means of filters. They represent noon sunlight and average daylight respectively.

Recently, a new series of standard illuminants, the D

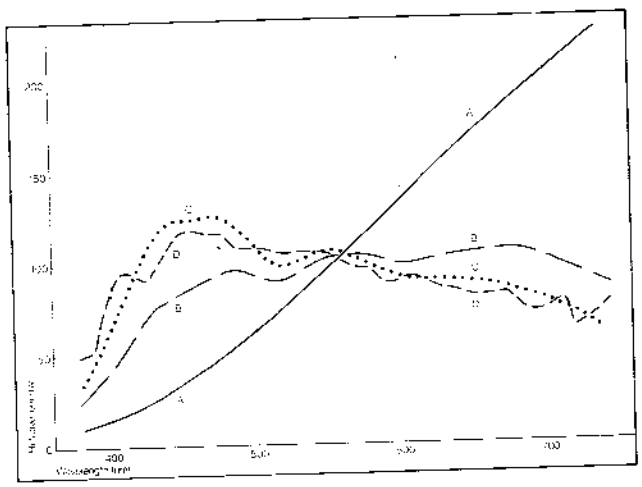


Fig. 42 C.I.E. Standard Illuminants

Series, has been defined to represent daylight of various colour temperatures. D65 represents daylight with a correlated colour temperature of 6500°K and is most frequently used. *Fig. 42* illustrates the spectral compositions of illuminants A, B, C and D65.
A coloured surface can now be defined by the three tristimulus values X, Y and Z in terms of:

(a) the three arbitrary primaries and their effect on the eye of the observer,
(b) the type of illuminant and
(c) the sample reflectance.

Thus:
$$X = \Sigma\, \bar{x}\, E\lambda\, R\lambda$$
$$Y = \Sigma\, \bar{y}\, E\lambda\, R\lambda$$
$$Z = \Sigma\, \bar{z}\, E\lambda\, R\lambda$$

Where $\bar{x}$, $\bar{y}$ and $\bar{z}$ are the values corresponding to the response of the eye to the three primaries, $E\lambda$ is the energy distribution of the illuminant and $R\lambda$ is the reflectance of the sample. Most books on colour measurement give tables of $E\lambda$, $\bar{x}$, $\bar{y}$ and $\bar{z}$ versus wavelength for various illuminants and both 2° and 10° observers.
When a colour-matcher is asked to produce material to a given standard he will be interested in the closeness of match between standard and sample. At first sight the CIE system would appear to afford a very simple means of specifying these tolerances in terms of X, Y and Z. Unfortunately the colour space as defined there is visually non-uniform.

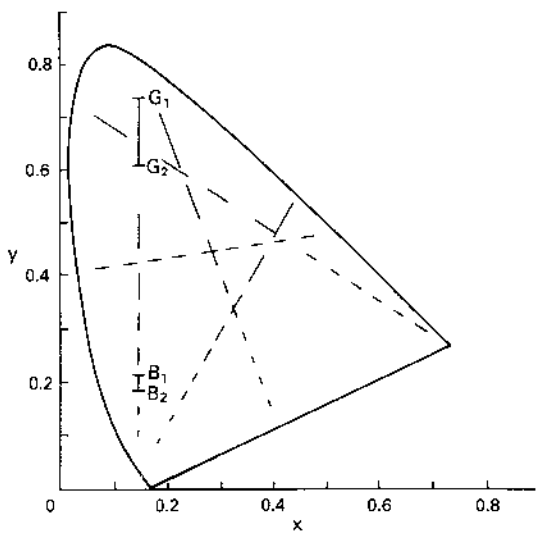


Fig. 43 Chromaticity Diagram showing Visually Equal Colour Differences

Suppose that two blue samples were just noticeably different in colour, so that on the chromaticity diagram in *Fig. 43* they were represented by two points, one arbitrary unit apart. If two green samples with the same, just, noticeable difference apart, are placed on the chromaticity diagram these would appear four or five units apart. Numerous attempts have therefore been made at deriving a more uniform colour space, by mathematical transformation. This transformation mathematically 'squeezes' the colour space shown in *Fig. 43* so that the two greens and two blues mentioned above will both appear the same number of units apart. Two such formulae are the ANLAB 40 system (widely used in the textiles industry) and the more recent CIE 1976 formulae. In both these formulae, transformation is made in L, a, b space in which the three axes are represented by: L (lightness), a (redness-greenness) and b (yellowness-blueness). The colour difference ΔE between two samples is then the distance between them in L, a, b space. To give an idea of scaling, 1 ΔE unit represents about five times the smallest difference the human eye can detect.
As well as determining the colour of solids by reflection, solutions can, of course, be measured using the transmission mode of a spectrophotometer. Such measurements are most important in the food colourants, dyestuffs and ink industries and for wines, spirits and other beverages.

(b) Turbidimetry and Nephelometry

To remain at least partially in the world of liquids, there are two techniques which use the property of light scattering to quantitatively determine the number of particles in a liquid. These are the techniques of turbidimetry and nephelometry.

(i) Turbidimetric Measurements

These assays determine the amount of light blocked by particulate matter in the sample as light passes through. Several problems are inherent in making turbidimetric measurements, and these problems are associated mostly with sample and reagent preparation rather than with the operation of the instrument. The amount of light that is blocked or scattered away from the detector by a suspension of particles depends not only upon the number of particles present, but also upon the cross-sectional area of each particle. If the particle size of the standards is not the same as the particle size of the samples being measured, errors in turbidimetric measurements will result. Also, the length of time between sample preparation and measurement must be kept as short as possible. Particles may aggregate or settle out of solution while the measurements are being made. Control of the rate of settling can be accomplished by using gum arabic or gelatin, which provide a viscous medium that retards settling. Preparation of very fine suspensions also helps minimise this effect. An alternative is to pump a stirred suspension through a flow cell and to integrate the reading over a period of 5 or 10 seconds in order to obtain an average measurement. Turbidimetric measurements can be accurate provided the number of particles and their size are within a reasonably narrow range.

(ii) Nephelometric Measurements

These are similar to turbidimetric measurements except that the light scattered by the small particles or colloids in the sample is measured at right angles to the incident beam. The arrangement is similar to that used in fluorimetry. The amount of scatter that occurs is related to the number and size of the particles in the light beam. This technique provides an order of magnitude increase in sensitivity over measurements made by turbidimetry. Thus, the ability to obtain measurements at much lower concentrations is the main advantage of using nephelometric measuring

techniques, especially if a high powered source, such as a laser, is used.
The applications of turbidimetry and nephelometry are diverse, but they are of greatest importance in any assay that results in a quantitative and specific precipitant e.g. sulphate by barium precipitation or vice versa, proteins by immuno-precipitation and the estimation of haze in highly coloured solutions. Other applications include the analysis of enzyme controlled polymerisation reactions and bacterial counting.
The optical configuration required for nephelometry is also used for the measurement of fluorescence.

(c) Fluorescence

The process of fluoresence involves the near instantaneous emission of light from a molecule which has previously absorbed light. *Fig. 44* illustrates the routes available for excited state electrons and how energy transfers within the system can occur. Every molecule possesses a series of energy levels. The electrons of that molecule can travel from a position of lower energy (e.g. S_0, ground state), to a position of higher energy (e.g. S_1 or S_2, excited states), by the absorption of a discrete quantum of light energy.

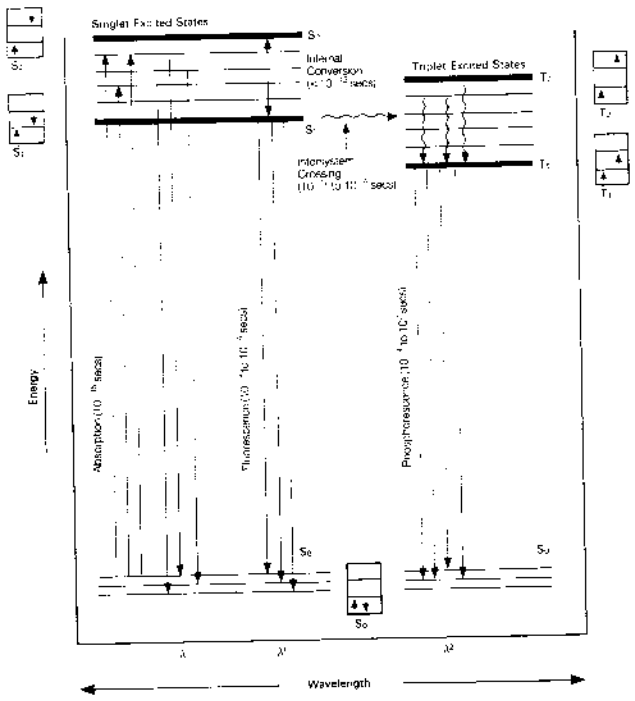


Fig. 44 Excited State Deactivation Routes

An excited molecule will lose its energy rapidly by one or more of a number of pathways. Within about 10^{-12} seconds, the processes of internal conversion and vibrational relaxation will bring the molecule to the lowest vibrational level in the first excited singlet electronic level S_1. Here the molecule may remain for about 10 nanoseconds before returning to the ground state via fluorescence. The emitted radiation will have a longer wavelength i.e. a lower energy than the absorption transition. The wavelength difference is sometimes called the 'Stokes shift'.

A molecule in the S_1 excited state may also undergo 'intersystem crossing' to the lowest triplet state, T_1, which has a lower energy than S_1. The radiative transition from T_1 to S_0 is called phosphorescence. Because S-T transitions are so called 'forbidden', the lifetime of phosphorescence is very long (10^{-3}–10^{2} s). This phenomenon is generally observed at very low temperatures (77 °K) and, at room temperature, it will only be observable in practice when the sample is adsorbed on a solid surface, or when the molecule is protected from collisional quenching.
Most users of fluorimetry are attracted by the great sensitivity of the methods. In solution studies, pg ml^{-1} levels can often be determined, in contrast to the µg ml^{-1} levels that can be detected in absorption spectroscopy. Fluorescence techniques are generally more selective than absorption methods, since two distinct wavelengths (those of absorption and emission) can be used to characterise a sample.
There are however, many factors which affect the fluorescence intensity e.g. pH, temperature, viscosity, solvent and quenching. A large number of fluorescent species contain ionisable groups. In such cases it is common to find that only one ionic form of the molecule is fluorescent, and therefore pH control is important. Increasing the temperature will normally reduce the intensity of fluorescence because of increased collisional quenching. In some cases the temperature dependence of fluorescence is substantial – about 5% per degree. In practice it is easy to thermostat the sample cell.
Increasing the viscosity of the solvent will generally lead to increased fluorescence, since collisional interactions will be reduced. This procedure is only deliberately used in studies of fluorescence polarisation where the orientation of the molecules is important.
Solvents may have large and unpredictable effects on both the intensity and the wavelength of fluorescence. Intensity effects may occur as a result of alterations in the relative energies of the electronic states, or as a result of quenching phenomena.
Quenching may be defined as the molecular interactions that reduce fluorescence e.g. quinine fluorescence is quenched by the presence of halide ion despite the fact that the absorption spectrum and extinction coefficient of quinine is identical in 0.5M H_2SO_4 and 0.5M HCl.
If care is taken to minimise these 'disadvantages' then the technique of fluorescence can be a very powerful tool in chemical analysis.

There are many instruments dedicated to these 'associated techniques'. However, many standard UV/visible spectrophotometers such as the PU8800 described in Section 7b, have a range of accessories which enable measurements to be taken using these techniques and which can be very rapidly fitted to and removed from the basic spectrophotometer.

Acknowledgements
The authors would like to express their thanks to Sandra Tucker for deciphering their handwriting, Rosemary Holgate for bringing order to the drafts and Christine Lewandowska for final editing.

Other articles available include:

Derivative and Log Spectrophotometry
Colour Measurement
Fluorimetry and Nephelometry
Densitometry
Specular Reflectance
Design and Applications of an End-Window PM Optical System
Measurement of T_m Values of DNA
The PU8600 Series and Microcomputers
The PU8600 in the 'PC' Environment
The PU8800 Series and Microcomputers
The PU8800 in the 'PC' Environment
Stray Light in UV/VIS Spectrophotometers

PHILIPS

Pye Unicam Ltd

A SCIENTIFIC AND INDUSTRIAL COMPANY OF PHILIPS
York Street Cambridge CB1 2PX Gt. Britain
Telephone (0223) 358866 Telex 817331

Printed in Great Britain 9498 905 92531