Calculation. Prepare a calibration curve from the mean of the readings obtained with the reference solutions by plotting the means as a function of concentration. Determine the concentration of the element in the test solution from the curve obtained.

METHOD II - STANDARD ADDITIONS

Add to at least 3 similar volumetric flasks equal volumes of the solution of the substance to be examined (test solution) prepared as prescribed. Add to all but 1 of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve, if possible. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument, using the same number of replicates for each of the solutions, to obtain a steady reading.

Calculation. Calculate the linear equation of the graph using a least-squares fit and derive from it the concentration of the element to be determined in the test solution.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than 4 reference solutions over the calibration range and a blank solution. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:

- the correlation coefficient is at least 0.99,
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and highest calibration level.

When the ratio of the estimated standard deviation of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

Recovery. For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. For other determinations, for example, for trace element determination the test is not valid if recovery is outside of the range 80 per cent to 120 per cent at the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) which is spiked with a known quantity of analyte (middle concentration of the calibration range).

REPEATABILITY

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

LIMIT OF QUANTIFICATION

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

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2.2.24. ABSORPTION SPECTROPHOTOMETRY, INFRARED

PRINCIPLE

Infrared absorption spectrophotometry (also known as infrared (IR) spectroscopy) is based on the interaction of infrared radiation with matter. As a result of interaction between a molecule and IR radiation, absorption of frequencies specific to that molecule can occur, and some intermolecular and intramolecular vibrations can be excited to higher vibrational levels. This results in an infrared absorption spectrum with characteristic bands that correspond to the functional groups of the molecule.

The infrared wavelength region can be further divided into 3 subregions, namely near-infrared, mid-infrared and far-infrared. These subregions have wavelength ranges that are generally accepted by convention to be 0.8-2.5 µm, 2.5-25 µm and 25-1000 µm respectively. However, in IR spectroscopy, wavenumber is more commonly used than wavelength, and can be calculated using the following equation:

$$\tilde{\nu} = \frac{1}{\lambda} \cdot 10^4$$

where $\tilde{\nu}$ is the wavenumber in reciprocal centimetres (cm^{-1}) and λ is the wavelength in micrometres. Thus 12 500-4000 cm^{-1} is near-infrared, 4000-400 cm^{-1} is mid-infrared and 400-10 cm^{-1} is far-infrared.

This chapter concerns only spectroscopy in the mid-infrared region, i.e. 4000-400 cm^{-1} (2.5-25 µm), which hereafter is referred to as infrared for simplicity. This region is where the fundamental molecular vibrations of functional groups appear in the spectrum as absorption bands. The region below 1500 cm^{-1} is known as the 'fingerprint region', a very complex and informative part of the spectrum which characterises the molecule being investigated.

The mid-infrared region is flanked by the near-infrared region, where overtones and combinations of fundamental vibrations, mainly C-H, N-H and O-H functional groups, are detected (*2.2.40*) and the far-infrared region, where absorption bands associated with crystal lattice modes, hydrogen bonds, angle deformation vibrations of heavy atoms and molecular rotations are observed.

APPLICATIONS

As the absorption bands in IR spectra are characteristic of the constituent functional groups of a compound, IR spectroscopy is widely used to identify substances and provide information on their structure. It can also be used for quantitative applications, which requires establishing a mathematical relationship between the intensity of the radiation absorbed by the sample and the concentration of the investigated component in the sample.

IR spectroscopy is widely used in the pharmaceutical field for chemical and physical analysis in the laboratory, and has a wide variety of applications during the manufacturing process as outlined below. IR spectroscopy thereby enables the application of Process Analytical Technology (PAT) as part of an advanced control strategy.

Chemical analysis:

- identification of active substances, excipients, dosage forms, manufacturing intermediates, chemicals and packaging materials;

– quality assessment of active substances, excipients, dosage forms, manufacturing intermediates and packaging materials, including batch-to-batch spectral comparison and supplier change assessment;
– quantification of active substances in a sample matrix, determination of water and solvent content;
– quantification of impurities, e.g. in gases, inorganic materials;
– reaction monitoring, e.g. chemical synthesis.

Physical analysis:
– determination of solid-state properties such as polymorphism.

LIMITATIONS

Notable limitations to the use of IR spectroscopy include the following:
– it may be necessary to use additional techniques to unambiguously identify a substance;
– pure enantiomers of a substance cannot be discriminated;
– it may not be a suitable method for trace analysis;
– sample preparation conditions (e.g. pressure, solvent) may change the crystalline form of a substance that exhibits polymorphism;
– for heterogeneous samples, the limited sampling volume may be problematic.

MEASUREMENT MODES

IR measurements are based on passing radiation through or into a sample and measuring the attenuation of the emerging beam at various wavelengths. This corresponds to 2 main measurement modes, i.e. transmission and attenuated total reflection (ATR). However, other modes also exist for specific applications (e.g. diffuse and specular reflection).

TRANSMISSION MODE

This mode is based on determination of the transmittance (*T*), namely the ability of the sample to transmit IR radiation at a given wavelength (wavenumber). It is defined by the following ratio:

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

I_0 = intensity of incident radiation;
I = intensity of transmitted radiation.

The resulting spectrum is presented in terms of transmittance (*T*) on the y-axis versus wavelength or wavenumber on the x-axis. It can also be presented in terms of absorbance (*A*) on the y-axis, which is related to transmittance (*T*) by the following equation:

$$A = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right) = a \cdot b \cdot c$$

a = molar absorption coefficient of the sample, in square centimetres per mole ($cm^2 \cdot mol^{-1}$);
b = sample thickness, in centimetres;
c = sample concentration, in moles per cubic centimetre ($mol \cdot cm^{-3}$).

ATTENUATED TOTAL REFLECTION MODE

ATR mode is based on the phenomenon of total internal reflection. The sample, with a refractive index n_2, is brought into close contact with a crystal (diamond, germanium, zinc selenide or any other suitable material), having a refractive index n_1 which is greater than n_2. A beam of IR light is then passed through the crystal. When the angle α between the incident beam and the sample-crystal interface exceeds a critical value α_c, theoretically all of the radiation is reflected (total internal reflection). However, an evanescent wave is produced which slightly penetrates the sample and part of the energy is absorbed. The total reflection is attenuated, which makes it possible to generate an absorption spectrum. In practice, multiple internal reflections are often used to amplify the absorption intensity, although some accessories allow absorption measurements with a single reflection.
The penetration depth d_p is usually of the order of a few micrometres and is given for a wavelength λ by the following equation:

$$d_p = \frac{\lambda / n_1}{2\pi\sqrt{sin^2\ \alpha - (n_2 / n_1)^2}}$$

where d_p is the penetration depth, λ is the wavelength, α is the angle of incidence and n_1, n_2 are the refractive indices of the reflection element and the sample, respectively.

Due to the relationship between these parameters, the absorption intensity in ATR is greater at higher wavelengths (i.e. smaller wavenumbers) and slight band shifts occur compared to the corresponding transmission spectrum. It is therefore not advisable to compare ATR spectra with transmission spectra when identifying compounds.

EQUIPMENT

The most commonly used IR spectrometers are Fourier-transform (FT-IR) spectrometers which typically consist of:
– a suitable polychromatic light source, e.g. a conducting ceramic rod;
– an interferometer;
– a sample presentation accessory, e.g. a sample holder;
– a detector;
– appropriate software for controlling the spectrometer, and for spectral evaluation and data processing.

Other spectrometers based on alternative principles may also be used if the requirements described under Control of equipment performance are fulfilled.

IR spectrometers can also be used in association with a microscope for the study of a small part of the sample or for chemical imaging.

IR spectroscopy can be coupled to other analytical techniques such as thermal analysis or chromatography.

CONTROL OF EQUIPMENT PERFORMANCE

Accuracy of wavenumber scale and spectral resolution are critical parameters and must be verified. The tests described below can be used for the control of instrument performance and for qualification. They can also be used as system suitability tests.

These parameters are checked using suitable reference materials which are selected and presented depending on the measurement mode (e.g. transmission or ATR).

For quantitative analysis, appropriate assessment criteria for the control of absorption intensity must also be defined.

WAVENUMBER SCALE

The wavenumber scale is typically verified using a polystyrene film that exhibits IR absorption bands at the wavenumbers shown in Table 2.2.24.-1.

Table 2.2.24.-.1 - *Band positions and associated acceptable tolerances of the polystyrene film used to verify wavenumber accuracy*

Band position (cm^{-1})		Tolerance (cm^{-1})
Transmission	ATR	
906.6	906.1	± 1.0
1028.3	1027.7	± 1.0
1601.2	1601.0	± 1.0
3060.0	3059.7	± 1.0

For measurement modes other than transmission or ATR, reference materials must be defined by the user.

SPECTRAL RESOLUTION

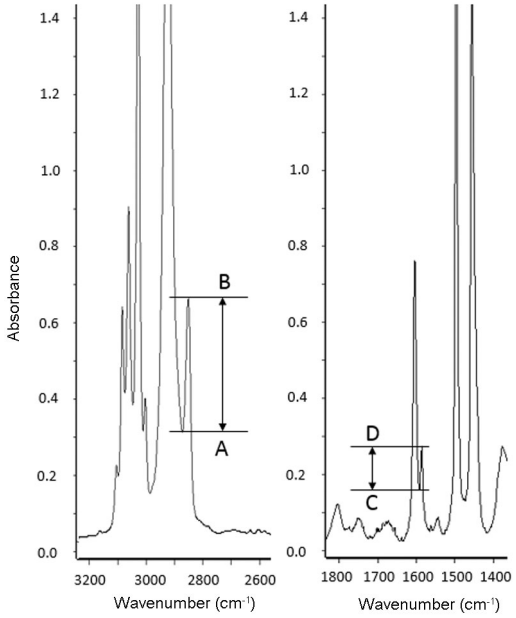

Figure 2.2.24.-1. – *Typical IR absorbance spectrum of polystyrene used to verify spectral resolution*

Spectra recorded in transmission mode. The spectral resolution is typically verified using a polystyrene film approximately 35 µm thick.

Acceptance criteria (see Figure 2.2.24.-1): the difference between the absorbance values at the absorption minimum at 2870 cm^{-1} (*A*) and the absorption maximum at 2849.5 cm^{-1} (*B*) is greater than 0.33; the difference between the absorbance values at the absorption minimum at 1589 cm^{-1} (*C*) and the absorption maximum at 1583 cm^{-1} (*D*) is greater than 0.08.

Spectra recorded in ATR mode. Appropriate assessment criteria for the control of spectral resolution according to the specifications of each instrument need to be defined.

For measurement modes other than transmission or ATR, reference materials have to be defined by the user.

PROCEDURE

SAMPLE PREPARATION AND PRESENTATION

Sample preparation and presentation vary according to the physical state of the sample and the measurement mode. Transmission mode is applied to transparent samples, such as neat liquids, solutions, gases or suitably prepared mulls and alkali halide discs. For liquids and gases, cells with fixed or variable pathlength and IR transparent windows can be used. For alkali-halide disks, specific sample holders are used. Reflection mode, e.g. ATR, is appropriate for the measurement of a wide range of samples in the solid and liquid state.

Some preparation modes (e.g. for discs and mulls in transmission mode or for solids in ATR mode) involve grinding and/or the application of pressure, which may induce unexpected crystal modifications.

Transmission mode

Prepare the substance by one of the following methods depending on the sample state (solid, liquid or gas). Sample bands in a spectrum have a minimum transmittance not lower than 5 per cent, unless otherwise justified.

Liquids. Examine liquids either in the form of a film between 2 plates transparent to infrared radiation or in a cell of suitable pathlength with windows that are transparent to infrared radiation.

Liquids or solids in solution. Prepare a solution of the substance to be examined in a suitable solvent. Choose a concentration and a pathlength that give a satisfactory spectrum. Generally, good results are obtained with concentrations of 10-100 g/L for a pathlength of 0.5-0.1 mm. The absorption due to the solvent is usually compensated by successively recording the spectra of the solvent and the sample solution and subtracting the solvent absorption bands from the spectrum of the sample solution.

Solids dispersed in a solid (disc). Grind the substance to be examined taking into consideration any possible changes (e.g. crystalline form) and mix with a suitable amount of finely powdered and dried *potassium bromide R* or *potassium chloride R*, unless otherwise specified. A mixture of a few milligrams (e.g. 1-2 mg) of the substance to be examined in a few hundred milligrams (e.g. 300-400 mg) of halide is normally sufficient to give a disc of 10-15 mm diameter and a spectrum of suitable intensity. If the substance is a hydrochloride salt, it is recommended to use *potassium chloride R*. Carefully grind the mixture, spread it uniformly in a suitable die and apply a suitable pressure. A compacting force of about 800 MPa is generally sufficient to prepare a disc. For substances that are unstable under normal atmospheric conditions or are hygroscopic, the disc may be pressed under vacuum. Several factors may cause the formation of faulty discs, such as insufficient or excessive grinding, humidity or impurities in the dispersion medium. For example, any water in either the sample or the potassium bromide will cause clouding of the disc and produce a low transmission spectrum. A disc is rejected if visual examination shows a lack of uniform transparency or when, in the absence of a specific absorption band, the transmittance is less than 60 per cent or the absorbance is more than 0.22 at about 2000 cm^{-1} (5 µm) and without compensation, unless otherwise prescribed.

Solids dispersed in a liquid (mull). Triturate a small quantity of the substance to be examined with the minimum quantity of *liquid paraffin R* or other suitable liquid. A mixture of a few milligrams (e.g. 5-10 mg) of the substance to be examined in 1 drop of *liquid paraffin R* is generally sufficient to make an adequate mull. Compress the mull between 2 plates transparent to infrared radiation. A mull is rejected if a visual examination shows lack of uniform transparency or where the spectrum shows features such as:

- low transmission at 4000 cm^{-1};
- a strongly sloping baseline between 4000 and about 2500 cm^{-1};
- a ratio of relative intensities of some absorption bands that is less than expected.

Molten solids. If prescribed in the monograph, make a film of a molten mass and fix it on a suitable mount.

Evaporated solution. If prescribed in the monograph, dissolve the substance to be examined in a suitable solvent. Prepare a film by evaporating the solvent on a suitable carrier and fix it on a suitable mount.

Gases. Use a suitable cell transparent to infrared radiation. Evacuate the air from the cell and fill to the desired pressure through a stopcock or needle valve using a suitable gas transfer line between the cell and the container of the gas to be examined. If necessary, adjust the pressure in the cell to atmospheric pressure using a gas transparent to infrared radiation (e.g. *nitrogen R* or *argon R*), or purge with carbon dioxide-free air. An appropriate measurement protocol must be followed to compensate for water, carbon dioxide or other atmospheric gases.

ATR mode

ATR is suitable for liquid and solid samples, and requires no preparation apart from simple treatments such as the grinding of large crystals and coarse material. Proceed as follows depending on the sample state (liquid or solid).

Liquids. Place the sample in contact with the crystal.

Solids. Ensure close and uniform contact between the substance to be examined and the whole crystal surface, either by applying pressure or by dissolving the substance in an appropriate solvent, then covering the crystal with the resulting solution and evaporating to dryness.

METHODS

Infrared spectroscopy is mostly used to identify substances, but it may also be carried out for quantitative applications. Quantitative analysis (based on the Beer-Lambert law, which relates the absorbance of a sample to its concentration) will not be described in this chapter.

The measurement is performed on an appropriately prepared sample. The data is then processed and evaluated, either to identify substances or quantify them (e.g. based on integration of IR-absorption bands).

Spectral quality may be enhanced by mathematical pretreatments. In practice, these are limited to spectral normalisation and subtraction of bands caused by carbon-dioxide and water vapour. The same pretreatments are performed on both the sample and the reference spectra.

Identification

Prepare the substance to be examined appropriately and record the spectra between 4000 and 650 cm^{-1}, unless otherwise prescribed.

Identification testing is performed by comparing the spectrum of the substance to be examined with the spectrum obtained from a Ph. Eur. chemical reference substance (CRS) or with a Ph. Eur. reference spectrum.

The spectrum of the current batch of the Ph. Eur. CRS may be recorded for immediate use or stored, for example, in a spectral library for future consultation. A stored spectrum may be used, provided traceability to the current batch of CRS is ensured.

In the case of substances that are not covered by individual monographs, a suitable reference standard may be used.

In all cases, spectra must be recorded using the same operating conditions and procedure, and especially the same measurement mode.

When comparison of the spectra recorded in the solid state show differences (see below), treat the substance to be examined and the reference substance in the same manner so that they recrystallise or are produced in the same crystalline form, or proceed as prescribed in the monograph, then record the spectra again. However, this procedure must only be done for substances where the monograph does not cover a particular form of a substance that exhibits polymorphism.

Several comparison procedures may be used, and the analyst must document and justify the method used and the specific acceptance criteria that allow a conclusion for identification. The spectra can be compared either by overlaying the spectra (in the whole spectral range or in the region of interest specified in the monograph) or by using mathematical calculations from the software. It is possible for example to perform:

- visual comparison based on band positions and relative intensities unless otherwise specified - the transmission minima (or absorption maxima) in the spectrum obtained with the substance to be examined correspond in position and relative size to those of the reference;
- calculation of the correlation coefficient between the 2 spectra - this value is calculated by the software and the identification threshold is defined by the user;
- evaluation by chemometric methods (e.g. Euclidean distance, Mahalanobis distance, classification methods); these methods involve the set-up, assessment and validation of the chemometric model by the analyst (see *5.21. Chemometric methods applied to analytical data*).

Impurities in gases

For the analysis of impurities, use a cell transparent to infrared radiation and of suitable optical pathlength (e.g. 1-20 m). Fill the cell as prescribed under Gases. For detection and quantification of the impurities, proceed as prescribed in the monograph.

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2.2.25. ABSORPTION SPECTROPHOTOMETRY, ULTRAVIOLET AND VISIBLE

PRINCIPLE

Ultraviolet and visible (UV-Vis) spectroscopy (or spectrophotometry) is based on the ability of atoms, molecules and ions to absorb light at specific wavelengths in the ultraviolet (approximately 180-400 nm) and visible (approximately 400-800 nm) range. This absorption is associated with changes in electronic energy in the form of temporary transitions of electrons to an excited state at a higher energy orbital. As each energy level of a molecule or molecular ion also has associated vibrational and rotational sub-levels, this results in many permitted transitions, which are generally impossible to separate, thereby producing absorption bands rather than sharp lines. These bands are characteristic of the functional groups and bonds in a molecule.

UV-Vis spectroscopy measurements involve exposing a sample to light and measuring the attenuation and/or scattering of the emerging (transmitted or reflected) light either at a single wavelength or over a specified wavelength range.

APPLICATIONS

UV-Vis spectroscopy is traditionally used for the quantitative and qualitative analysis of liquid samples, but is also suitable for solid and gaseous analytes and has other applications such as the determination of physical or chemical properties.

UV-Vis spectroscopy as described in this chapter can be applied in various ways:

- when a monograph or general chapter refers to this chapter, the requirements described in the relevant paragraphs of this chapter are mandatory;
- when used as the detection method in chromatographic systems as described in general chapter *2.2.46*, the requirements listed in the relevant paragraphs of this chapter are mandatory;
- when used as a process analytical technology (PAT) tool for PAT applications similar to the applications described in this chapter, the provisions herein apply; for other PAT applications, the principles are the same, however the criteria are established bearing in mind the intended purpose of the analysis, using a risk-based approach.

EQUIPMENT

Spectrophotometers used for carrying out measurements in the UV-Vis region typically consist of:

- a suitable light source (such as a deuterium lamp for the UV region, a tungsten-halogen lamp for the visible region or a xenon lamp to cover the entire UV-Vis range); UV-Vis spectrophotometers often have 2 sources;
- a monochromator such as a grating system;
- other optical components, such as lenses or mirrors, that relay light through the instrument and that may also be used to generate more than one beam of light, i.e. in double-beam spectrophotometers, as opposed to single-beam spectrophotometers;