INSTITUTE OF MEDICAL BIOCHEMISTRY

Optical methods

in biochemistry

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Optical methods

Many techniques used in biochemistry are based on interaction between analyte and light. It can be change of colour or intensity of light, luminescence, fluorescence, change of rotation of polarised light or light scattering.

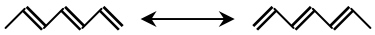
Colour of substances

Many compounds contain a valence electron that can be excited to a higher energetic shell by electromagnetic radiation. Such compounds absorb light of wavelength corresponding to the difference between energy of both electronic states. If the absorbed light is from visible range of spectrum the compound is coloured (it has colour complementary to the colour of absorbed light).

Absorbed wavelength	Colour of absorbed light	Colour of compound
400 – 435	violet	yellow green
435 – 480	blue	yellow
480 – 490	cyan	orange
490 – 500	blue green	red
500 – 560	green	magenta
560 – 580	yellow green	violet
580 – 595	yellow	blue
595 – 605	orange	cyan
605 – 670	red	blue green

It is very difficult to predict colour of a compound from its structure, and, vice versa, the structure cannot be determined only from the absorption spectrum of a compound. Coloured compounds nevertheless belong mostly to one of three groups:

1. Compounds containing **conjugated system of double bonds** with asymmetric molecule. A symmetric system of conjugated double bonds exist in energetically equivalent resonation states:



Asymmetric substituent brings energetic difference between bots states. This difference frequently corresponds to energy of visible light. Dyes containing polymethin chain (–CH=CH–CH=CH–) or azo-dyes (–N=N–) may serve as examples. Similarly, compounds, containing aromatic or heterocyclic structures bound to a central atom, are frequently coloured (e.g. triphenyl methane dyes).
2. Also *d* and *f* valence electrons are frequently responsible for colour of a compound. Such electrons can be found in **coordinative covalent bonds** of complexes. For example, anhydrous copper(II) sulphate $CuSO_4$ is colourless but its pentahydrate $CuSO_4 \cdot 5H_2O$ as well as aqueous solution are blue: copper forms a complex with water $[Cu(H_2O)_4]^{2+}$ in both cases. Similarly, complex compounds of other transitive metals (Fe, Cu, Cr, Mn, Ni, Co) are frequently coloured, complex-bound metal brings colour also to coloured proteins haemoglobin and cytochromes.
3. Ions which contain **transitive metal of high oxidation number** as central atom, are frequently coloured too – e.g. MnO_4^-, $Cr_2O_7^{2-}$.

Analytical methods used in medical biochemistry employ all three groups of coloured compounds. Systems of conjugated double bonds are readily formed in reactions where analyte is coupled with a suitable chromogen (e.g. creatinine with picric acid in Jaffé reaction, diazo-coupling reaction of bilirubin), or are formed by oxidising a chromogen that contain less double bonds (e.g. oxidation of benzidine derivatives in peroxidase reactions). Formation of coloured complexes is used e.g. in determination of protein by so called biuret reaction (complexes of Cu^{2+} with O and N of peptide bonds) or in detection of various compounds with $FeCl_3$. Colour change during reduction of Cr^{6+} to Cr^{3+} is employed in detection of ethanol in breath test.

Photometry and spectrophotometry

Estimation of properties of a solution, e.g. concentration of certain compound, based on absorption of light of a certain wavelength is called photometry. In case that not only one wavelength is used but measurement is rather done in some range of spectrum, we speak about spectrophotometry.

Transmittance

Amount of light that passed through a sample can be described with **transmittance**. Transmittance is defined as

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

where I_0 is intensity of light entering the sample and I is intensity of light emerging from sample.

It would be inpractical to measure both intensities accurately: besides light absorption of the sample, absorption and scattering of light on walls of the cuvette, in the photometer itself etc. influence them in a great deal. Thus, transmittance is usually measured with respect to a blank. First, intensity of light passing through a blank (reference solution) is measured. Blank should contain all components of the sample but the estimated compound. Then, light passing through the sample is measured under exactly the same conditions. In this case, transmittance is defined as

$$T_s = \frac{I_s}{I_b},$$

where I_s is intensity of light emerging from the sample and I_b is intensity of light emerging from blank.
Measuring transmittance in this way, non-specific loss of light intensity plays no role. Intensity of light passing through blank is considered 100% (in other words, transmittance of blank is 100%) and transmittance of samples at the same wavelength is always lower than 100%.

Transmittance of a solution containing a coloured compound depends on

1. properties of absorbing compound
2. wavelength of light
3. amount of absorbing compound, i.e. concentration of the solution and thickness of used cuvette.

August Beer (1825 – 1863) formulated dependence of transmittance on these values mathematically. Assuming that monochromatic light is used, it applies

$$T = 10^{-\varepsilon . l . c},$$

where T is transmittance, ε is molar decadic absorption coefficient (a constant characteristic for every compound at given wavelength), l is optical pathway (thikness of cuvette) and c is substance concentration of light-absorbing compound in solution.

Algebraically deriving the previous equation, transmittance can also be expressed as

$$\log T = -\varepsilon \cdot l \cdot c \qquad \text{or} \qquad -\log T = \varepsilon \cdot l \cdot c.$$

Absorbance and Lambert-Beer law

Expression $-\log T$ is called **absorbance** A (literally, older name **extinction** E is used, too). From the equations above we get

$$A = -\log T = -\log \frac{I}{I_0} = \log \frac{I_0}{I}$$

$$A = \varepsilon \cdot l \cdot c$$

The last relation is called Lambert-Beer law (Johann Heinrich Lambert, 1728 – 1777). Importantly, absorbance is proportional to concentration of absorbing compound. From the previous equations it follows that sample that absorbs no light has absorbance equal to zero. Absorbance of 1 means that exactly one a tenth of light passed through sample, absorbance 2 one a hundredth and so on. Negative absorbance would mean that more light passed through the sample than through blank – usually due to some error in experimental design. Absorbance has no dimension.

Photometers and spectrophotometers

Photometers are used to measure absorbance. Devices capable of measuring at more wavelengths are called spectrophotometers.
In general, photometers are set up of four compartments:

1. source of light
2. monochromator
3. sample compartment
4. detector

A suitable incandescent or discharge lamp can be used as **source of light**.

Light bulbs and halogen lamps yield continuous spectrum in visible and infrared range but cannot be used in UV range. Hydrogen or deuterium lamps are most frequently used as sources of ultraviolet light. Both visible and ultraviolet light is produced by xenon discharge lamp, broad range of wavelengths is however on cost of several disadvantages: its light is composed of continuous and line spectrum with huge differences between intensities at different wavelengths. The lamp is quite expensive and its intensity is not very stable.

Polychromic light from the source gets to **monochromator**. Suitable **interference filter** inserted in the optical way is the simplest and cheapest choice.

Filters for almost any wavelength of visible and ultraviolet range are commercially available today. Several types of interference filters can be distinguished; a filter of required properties can be made by combining them. Low-pass filters transmit light of shorter wavelengths than a certain cut-off limit. On the other hand, high-pass filters transmit wavelengths longer than cut-off value. Band filters transmit light of some range of wavelengths. The wavelength limits are usually not sharp; therefore, wavelength at which the filter transmits 50% of light are usually quoted as cut-off values. Another possibility is to define the mean wavelength of the filter and the band-width (or width of halfband).

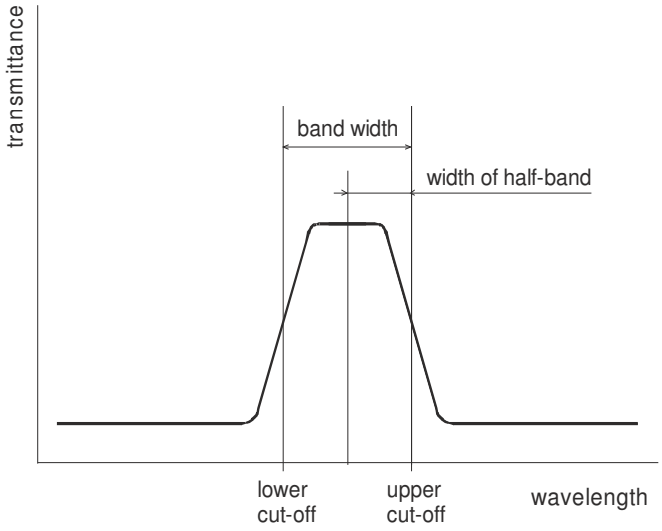


Fig. 1: Relations between band-width, width of half-band, lower and upper cut-off

Optical grid is most widely used as monochromator today. Declining it allows to continuously change the wavelength (e.g. so called Czerny-Turner monochromator). The band-width is determined by a **slit**, either permanent or adjustable.

The wider is the slit the higher is intensity of light, but the lower is specifity of measurement. On the other hand, narrower slit brings better definition of wavelength on the cost of lower intensity of light and worse signal-to-noise ratio.

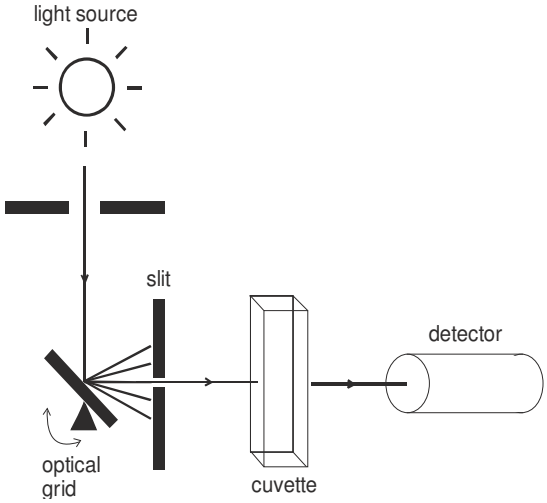


Fig. 2: Simplified chart of photometer.

Monochromatic light interacts with the sample. In biochemistry, almost only solutions are addressed by photometry. They are filled into standard **cuvettes** (cells) with optical pathway of 1 cm (rarely shorter – in case of ultramicrocells for very small volumes of samples). Cuvettes can be made of various materials and differ in shape.

Cuvettes made of optical glass (usually denoted as OG, G and others) are suitable for measurements in visible range of spectrum. For measurements in the UV range, cuvettes made of quartz glass (denoted Q, UV etc.) are used. Other cuvettes made of special optical glasses (OS) are available; they are usable at wider range than cuvettes of "normal" glass and are cheaper than quartz cuvettes.

Measuring in cuvettes made of various types of glass is very accurate – absorbance of a sample can be determined even with precision of four to five decimal places. However, glass cuvettes are quite expensive, their lifetime is limited and their maintenance – especially cleaning – is sometimes laborious. Therefore, disposable cuvettes made of plastics are commonly used. They are inexpensive but less accurate – measurements with precision on the second to third decimal place are reliable (which is still fully sufficient for most applications). Mostly, they are made of polystyrene (PS) – for visible range, or polymethyl methacrylate (PMMA) – even for part of UV range.

Sometimes, factor of cuvette is quoted on its body. It indicates the real pathlength in cm. Ideally, it is equal to 1 but it may vary due to imprecision in manufacturing process. Then, factor is used to correct the measurement.

Standard spectrophotometric cuvettes ("macrocuvettes") have inner dimensions 1×1×3 to 4 cm. They are usually filled with approx. 3 mL of sample (exact volume depends on spectrophotometer – height of the light beam above the bottom of cuvette). As smaller and smaller samples are used in biochemistry, semimicrocuvettes widely replace the macrocuvettes. Semimicrocuvettes have a narrower space for sample and their filling volume is about 0.8 mL. Micro- and ultramicrocuvettes are available as well. Sometimes, on the cost of shorter optical pathlength, they can be used for much smaller volumes, even microlitres. Using semimicro-, micro- and ultramicrocuvettes, a big portion of light would pass through glass around the sample, increasing the background light intensity and influencing the measurement. Therefore, these cuvettes are frequently masked – glass around the sample area is blackened.

Other special types of cuvettes are used for special applications. Flow-through cuvettes that can be attached e.g. to chromatographs, thermostatic cuvettes, or spectrophotometric capillaries can serve as examples.

In the instrument, cuvette is placed to a **cell holder**. It ensures exact position, may be thermostated and sometimes contains a built-in magnetic stirrer. Frequently, several cuvettes can be placed to an automatic cuvette changer which subsequently puts them to the optical path.

Finally, the light emerging from sample reaches **detector**. It is usually a photodiode or another photoelectric element. Intensity of light is evaluated with a system of convertors, compared with blank and absorbance is calculated. The precision is influenced by **integration time** – the time of measuring absorbance. The longer it is the more accurate is the result, if the absorbing compound is not photosensitive (i.e. if it does not fade after longer illumination). Another disadvantages of longer integration times are longer time of measurement (especially important if a big number of samples are processed or when measuring at several wavelengths) and long integration times cannot be used when processing samples changing in time (kinetic measurements).

Besides single-beam spectrophotometers, in which blank is measured first and then the sample is placed to the same optical path, double-beam photometers equipped with two detectors can be used. Both blank and sample can be measured at once in two distinct optical paths.

Other setups of spectrophotometers

Absorption spectrum is measured on a spectrophotometer with optical grid in such a way that absorbance at one wavelength is measured, than the grid is moved, measurement at the second wavelength is taken and the procedure is repeated until the whole spectrum is measured. A long time is required to complete. It may be of disadvantage:

- the sample may change in time (especially in kinetic methods),
- photosensitive samples may bleach during measurement,
- when more samples are processed, it may be technically difficult to measure first and last of them under the same conditions,
- it consumes much time, throughput is low.

Another setup of spectrophotometer clears these difficulties. In **diode-array spectrophotometers** white light passes through sample. Then the light is scattered to individual wavelengths, usually with a fixed optical grid. Finally, a plate with a large number (an "array") of detectors – usually photodiodes – is illuminated with the scattered light. The diode array is constructed so that every photodiode is illuminated with a certain, quite narrow (e.g. 2 nm) range of wavelengths. The device contains no moving parts (it increases reproducibility and makes the instrument robust) and the whole spectrum is measured at once. Time required to measure absorption spectrum can therefore be shortened from minutes to fractions of a second. Moreover, diode-array spectrophotometers frequently reach higher accuracy compared to traditional spectrophotometers. They are also almost maintenance free, do not require calibrations etc. On the other hand, diode array spectrophotometers used

to be much more expensive than classical ones. However, as the price of miniature electronic components (including arrays of photodiodes) falls constantly, cost-effective instruments are commercially available today.

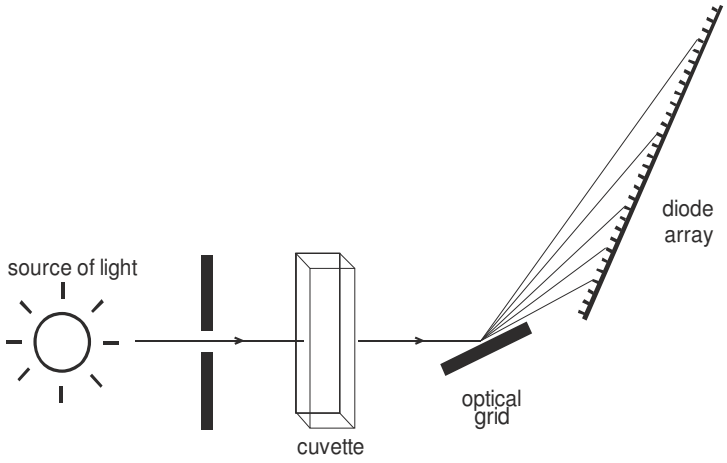


Fig. 3: Diode-array spectrophotometer

Traditional layout of photometry brings some other troubles in routine use as well:

- large volume of sample is necessary,
- low throughput, preparing of a large number of samples is laborious,
- cuvettes are expensive and demanding on maintenance,
- spectrophotometers are costly.

These disadvantages can be, in a great deal, overcome by use of a **plate reader** – a photometer for microtitration plates working with a **vertical beam**. Samples are filled into wells of polystyrene plates. Plates with 96 wells are most widely used but other formats (from 4 to 384 wells) are also available. Special instrumentation speeds up preparation of sample: multichannel pipettes allowing liquid delivery to several wells at once, repetitive pipettes etc. Compared to traditional photometers with horizontal beam and constant optical pathlength (given by thickness of the cuvette), plate readers work with vertical beam and the pathlength depends on the liquid level in the well. If a colourless solution is added to the well the concentration of light-absorbing compound decreases. However, liquid level goes up in the same time, i.e. the optical pathlength is increased proportionally and the resulting absorbance remains the same. On the other hand, if some solvent evaporates the sample gets more concentrated but, in the same time, the optical pathway is shortened and the result is again unchanged. It can be said that since absorbance depends on concentration of absorbing compound in traditional photometry, it corresponds with the number of moles of light-absorbing compound in vertical-beam photometry.

Much smaller volume of sample is required for vertical-beam photometry – typical filling volumes range from 100 to 300 μL. With respect to number of samples measured at once, microwell plates are also cheaper than plastic cuvettes. Usually just several seconds are necessary to measure all 96 wells. Plate readers are usually cheaper than traditional photometers because interference filters play usually the role of monochromator. Plate readers are commonly employed in immunochemical techniques, especially ELISA (hence the common name **ELISA-reader**).

In photometry with vertical beam, **optical density (OD)** is used besides absorbance. The relation between optical density and absorbance is $OD = A / l$. Thus, optical density depends only on sample properties, not on the optical pathlength.

At this point, we should at least mention another technique, **reflective photometry**, routinely employed in clinical biochemistry as a method of **dry chemistry**. Within dry chemistry, material is not examined in test-tubes or other vessels but it is applied onto a film impregnated with components of the reaction mixture. The reaction leads to change in colour of a field. The colour change can be quantified by reflective photometer – it measures lost of intensity of light reflected from the field. Light emitting diodes (LEDs) are frequently employed as sources of light of required wavelength, or light-bulbs and interfering filters are used. To increase sensitivity, the reflected light is concentrated to detector with a hollow mirror (so called Ulbricht's sphere). Analysis of urine using diagnostic strips may serve as a typical example of dry chemistry. The range of available methods however grows rapidly because they are easy to perform, cost effective and their accuracy is comparable with traditional tests.

Advanced spectrophotometric techniques

Quite often it happens that besides the measured compound, another one is absorbing in the same range of spectrum. Concentration of analyte cannot be determined directly in such a case because the absorbance is the sum of absorbances of both compounds. If the concentration of interfering substance is known or if it is constant, use of suitable blanks can solve the problem. In other cases so called **multicomponent analysis** is to be employed.

Absorbance is measured not only at one but at several wavelengths or in a whole continuous part of spectrum. If extinction coefficients (or absorption spectra) of all components are known the concentration of analyte can be calculated by solving a system of equations.

We can show the principle at a simple example. Let's suppose that a concentration of a compound is to be measured. Its absorption spectrum (Fig. 4, dotted line) is overlayed by spectrum of another compound in the mixture (dashed line). Measuring the whole mixture, we obtain a spectrum that is a sum of both absorption spectra (solid line). Even if we know that absorption maximum of measured compound is at λ_1, its concentration cannot be estimated directly because we cannot neglect absorption of the other compound at this wavelength. However, if the spectrum of contaminating compound is known another wavelength can be found at which the contaminating compound has the same absorption as at λ_1 (it is denoted λ_2), i.e. extinction coefficients of the contaminating compound at λ_1 and λ_2 are the same. Then, absorption of the sole estimated compound at λ_1 is $A = A(\lambda_1) - A(\lambda_2)$.

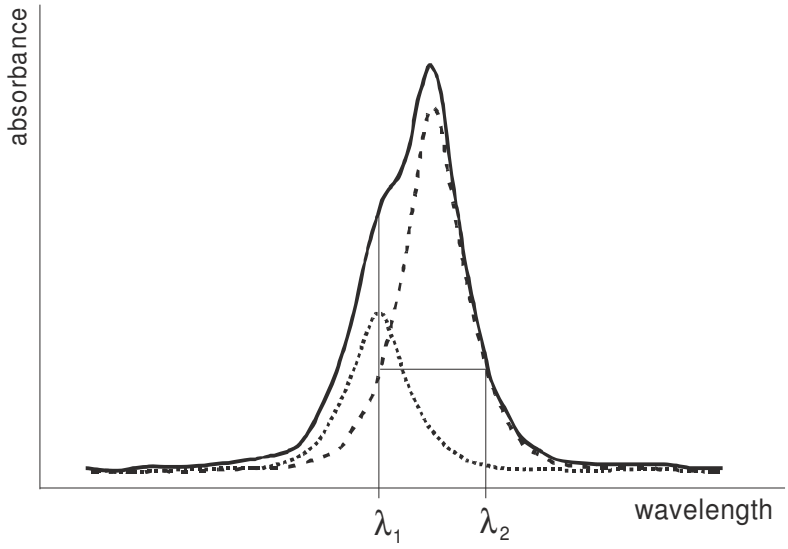


Fig. 4: Multicomponent analysis.

Another interesting situation is reaction of a compound that absorbs light, yielding a product with another absorbing spectrum, when both the spectrum of reactant and of product widely overlay. Spectra of NAD^+ and NADH may serve as example (estimation of these coenzymes by direct photometry is widely used to measure activity of many enzymes in so called Warburg optical test). Absorption maxima are apart each from the other so that it is easy to determine concentrations of both forms of the enzyme in this case. Interesting is the crossing of both spectra. In fact, all spectra for various NAD^+/NADPH ratios (at constant total concentration) cross at one point. The reason is that both NAD^+ and NADH have the same extinction coefficient at 281 nm. The intersection is called **isosbestic point** (from greek *isos* = the same and *sbennymi* = to quench). Total concentration of NAD^+ and NADH can be estimated by measuring absorbance at this point, without knowledge of actual ratio of both components.

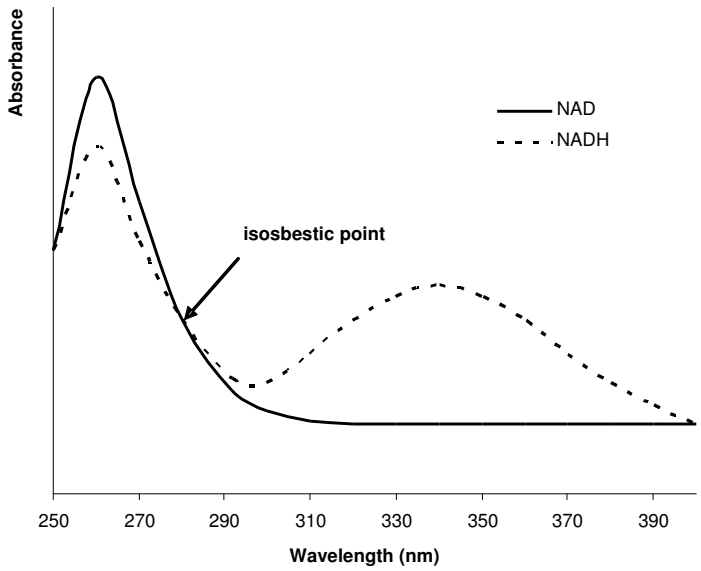


Fig. 5: Isosbestic point

Many other data can be obtained by mathematical processing of measured spectra. **Derivative spectrophotometry** is useful in more complicated situations. The first and second derivative of absorption spectrum on differential of wavelength can e.g. help in determination of precise position of absorption maximum. Mathematical processing is useful also for kinetic measurements.

Accuracy of photometric techniques

Most photometers are capable of measuring absorbance in the range of 0 to 3 or 4. However it is not reasonable to use the whole range. Assuming that the random error of detector is constant the relative error of absorbance against the real absorbance of sample is a curve plotted at Fig. 6 (on the right, it grows quickly). Apparently, best results can be obtained for **absorbances between 0.2 and 0.8**. Good results can be obtained when absorbance does not exceed 1.2; more concentrated samples should be diluted. Concrete values of errors depend on photometer, the shape of the curve remains however the same (only the scale changes).

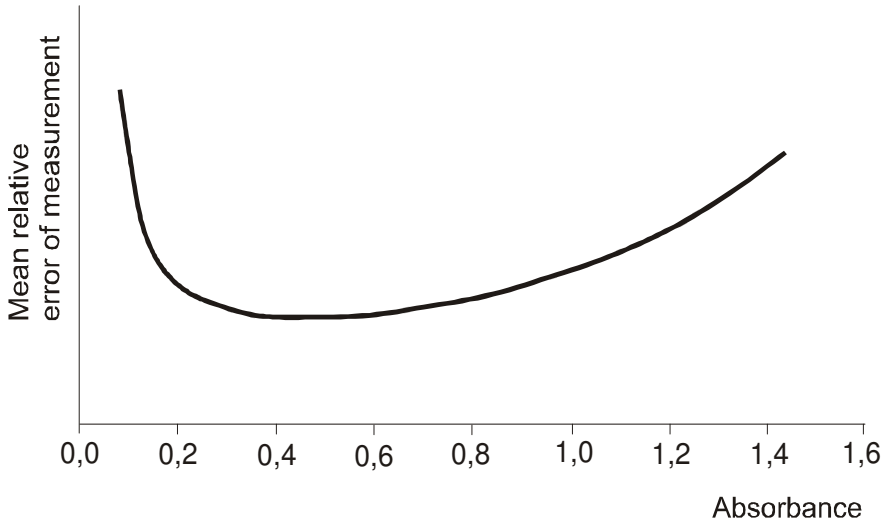


Fig. 6: Relation of relative error of measurement on absorbance of sample.

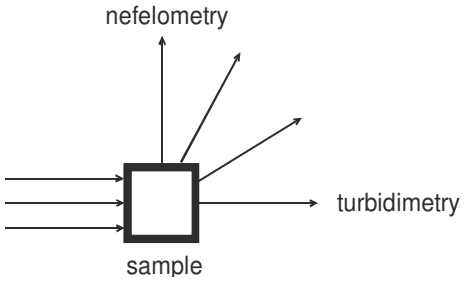
Accuracy is much influenced by preparation of sample and cuvette, as well. Because photometry is the most widely used analytical method in biochemistry today we will list at least the most important rules of accurate photometric measurement here:

- Suitable photometric cell must be used, the wavelengths must correspond to the working range of the cuvette.
- If more cuvettes are used they should have the same factor. Plastic cuvettes should be of the same lot.
- Cuvettes must be clean. When filled with distilled water they must have the same absorbance.

- Optical walls of the photometric cell must be kept clean (e.g. fingerprints must be avoided). The same applies for microwel plates.
- Measured solution must be homogenous; no air bubbles must be trapped in the cuvette. The outer surface of cuvette must be dry. A droplet on the surface, air bubble or a floating precipitate can be frequently recognised by unstable reading of absorbance.
- Cuvette must be filled with sufficient amount of the sample.
- If more samples are measured using one cuvette, remnants of preceding sample should not bring considerable error to the next one. The cuvette is usually rinsed with distilled water between samples and then thoroughly dried. Best results are reached when cuvette is pre-rinsed with a small amount of sample that is discarded and then it is filled with a fresh portion of the sample for measurement. If similar solutions are measured it is sometimes more accurate not to rinse the cuvette with distilled water but only pour off the cuvette and dry as thoroughly as possible.
- Concentration of the light absorbing compound determines the absorbance when cuvettes are used for measurement. Thus, it is important to observe the ratio of components of reaction mixture. It is advisable to arrange the experiment in such a way that all solutions are measured with one pipette, of a fixed volume if possible.
- On the other hand, number of moles of the absorbing compound determines absorbance when microtitration plates are used. Thus, the experiment should be arranged so that the volume of analyte is measured as exactly as possible. The geometry of liquid level may bring big differences to absorbance. Therefore, all samples should have the same affinity to the walls of microwell plate and the plate should be mixed before measurement, in order to douse even wall of the well above the liquid level.

Nephelometry and turbidimetry

Light can be not only absorbed by the sample but also scattered, especially if the sample is a dispersion or a colloid solution. Light scattering can be measured using **nephelometry** (from greek *nephele* = cloud) and **turbidimetry**. In nephelometry, intensity of light scattered under a certain angle is measured while turbidimetry measures intensiy of non-scattered light passing through sample in the original direction. Turbidimetry and nephelometry are, in some way, "mirroring" techniques. Here, turbidimetry will be described: usual photometric equipment can be used to measure turbidance and loss in light intensity by scattering in the sample can be expressed using quantities usual in photometry.



Obr. 7: Nefelometrie a turbidimetrie

The amount of scattered light depends on:

1. Concentration of particles. This relation is linear in a wide range of concentrations and therefore analogy to photometry with Lambert-Beer law is applicable. The quantity analogic to absorbance is called turbidance.
2. Size of particles. The amount of scattered light is approximately reciprocal to molecular weight of particle.
3. Wavelength of light used for measurement. The shorter the wavelength is the more light is scattered (Tyndal's phenomenon). Intensity of scattered light is approximately proportional to the forth power of reciprocal value of wavelength. Practically, light from range of 340 to 450 nm is most widely used. Shorter wavelengths are absorbed by proteins that are commonly found in biologic samples.

Formation of a stabile suspension that is not changing during measuring is often the most difficult task. **Protective colloids**, most frequently polyethyle glycol, are therefore added to the reaction mixture.

Turbidimetry and nephelometry are widely used in immunochemical methods for evaluation of immunoprecipitation where antigen-antibody complexes are formed.

Flame emission photometry

Estimation of some elements, e.g. natrium or lithium, can be carried out by measuring intensity of light of a certain wavelength that is emmited after exciting the sample in flame. Measured solution is atomised first – fine spray of it is formed. The spray gets to a colourless flame (from propane or acetylene burner). An inner standard (usually, salts of lithium or potassium) must be used to ensure reliable results. This technique is demanding and it is difficult to automate it. Therefore, flame photometry is not very common in clinical biochemistry today.

Atomic absorption photometry

Atomic absorption photometry is another optical technique suitable for determination of metal elements. Light from visible or UV range is absorbed by free atoms of the element. Also here, sample is atomised first, usually at high temperatures, and then light of a certain wavelength passes through it. A photomultiplier usually serves as detector. This method is not very common due to demands on instrumentation. It is however indispensable for determination of trace elements like copper or zinc.

Techniques assessing luminescence

Methods measuring intensity of light emitted by addressed compound are more demanding than photometry but very sensitive. Flame emission photometry mentioned above could be classed with this group because it is based on the common principle of these methods – an electron is excited to a higher energetic state. When it returns to its original energetic level some energy is emitted in form of light. In most of these techniques, an electron is excited by radiation (photoluminescence, employed in fluorimetry, and phosphorescence) or a compound with an excited electron is formed in course of a chemical reaction (chemiluminescence, used in luminimetry).

Fluorimetry and spectrofluorimetry

Fluorimetry is based on photoluminescence. Fluorescent compound is excited with a monochromic light. An electron is excited in this way and gets to a higher energetic shell. Returning back, a portion of energy is dissipated as heat, another portion is radiated as a photon. Energy of emitted light is therefore always lower than energy of excition light. In other words, emitted light has longer wavelength than exciting one. Emitted light is usually measured in a direction perpendicular to the excition beam. It passes through emission monochromator and its intensity is measured using photomultiplier.

Interference filters play the role of both excitation and emission monochromator in most fluorimeters. More precious instruments are equipped with optical grids allowing adjusting wavelengths continuously. In this case we speak about spectrofluorimetry.

Compared to photometry, spectrofluorimetry is more specific: intensity of light illuminating detector depends not only on absorption (i.e. excitation) spectrum but also on emission spectrum. It is also more sensitive: photomultiplier allows detection of very low intensities of light and perpendicular direction of excitation and emission together with different wavelengths of both lights ensures that emitted light is not "polluted" with the excitation one. Unfortunately, this technique conceals many technical difficulties: fluorescent compounds are sensitive to little changes in pH, ionic strength or polarity of solvent, oxidative reagents or trace amounts of so called quenchers (compounds that allow descend of the excited electron to the basic state without emitting light – e.g. many of transitive metals). Moreover, costly and complicated instrumentation is necessary.

Analytical capabilities of spectrofluorimetry can be further broadened by introduction of polarisation filters to both excitation and emission optical path – **polarising fluorimetry**. This technique is based on the fact that excited electron returns to the basic state with some delay. If this delay was negligible and the fluorescent compound was excited with polarised light emitted light would remain in the same polarisation plane. However, as the delay is significant, emitted light, loses its polarised character because excited molecules may rotate in solution. Small molecules rotate faster than large ones; therefore, polarising fluorimetry allows determining the size of fluorescent molecule. This is employed e.g. in immunochemistry where it allows to distinguish between a small, fre fluorescent-labeled antibody and a big complex of the same antibody and an antigen.

Many other modifications of spectrofluorimetry were developed and are in use especially in research. Sample can be illuminated with very short gleams of light and course of fluorescence in time is recorded. Spectrofluorimeter can be attached to a fluorescence microscop, chromatograph, special microwell plates can be used etc.

Luminimetry

Electrons are excited in course of a chemical reaction in chemiluminescence. The process must be strongly exergonic, it can be almost only some oxidation. Released energy must not be freed as heat. Some compounds can radiate energy as a photon directly and a short flash of light appears. Other compounds can transfer energy to another compound that emits light, usually in course of several seconds or minutes. Synthetic luminophors are used, or a naturally occuring enzyme, luciferase of fire-fly – in this case the method is called **bioluminiscence**. Oxidation of some compounds can also be performed electrically on an anode in case of **electroluminescence**.

Luminimetric techniques are quite sensitive. Luminimeters are technically similar to emission part of fluorimeter. Interference filters are usually used as monochromator, a photomultiplier is usually employed as the detector.

Polarimetry

Some compounds are optically active, i.e. they rotate the plane of optically polarised light. Chemically, these compounds contain a center of chirality – in biochemically important molecules a carbon atom that is bound to four different substituents (it means that the compound is not symmetric).

It is difficult to explain why asymmetric molecules rotate the plane of polarised light without deeper physical commentary. Here, we will only state that plane-polarised light results from interaction of two circular-polarised radiations that differ in direction of circulation. Field of force of an asymmetric molecule is also asymmetric and thus interacts with every of the two circular-polarised lights in a different way. Every of these two radiations spreads with different speed. In other words, optically active environment has various reflective indexes for light polarised clockwise and counter-clockwise. When both radiations are put together again after passing through the sample, plane polarised light is obtained again, the plane of polarisation is however rotated. It can be showed that the angle of rotation is

$$\alpha = \frac{180 \cdot \mathsf{l} \cdot \Delta \mathsf{n}}{\lambda},$$

where α is the angle in degrees, l is thickness of optically active environment, Δn is difference in refraction indexes for clockwise and anti-clockwise circular polarised light and λ is wavelength of light.

Optical activity can be measured with a polarimeter. Monochromic light (e.g. from a sodium lamp) passes through a polarising filter (*polariser*) and then through a cuvette filled with the measured solution. Optical activity of the sample is evaluated using another polarising filter (*analyser*) fixed in a revolving holder. Intensity of light in the eyepiece of the instrument depends on the position of analyser and the plane of polarised light emerging from sample. The analyser is rotated until this intensity reaches its maximum and position of analyser with respect to polariser is read on a scale. To make it easier, the light that passed the whole system illuminates only a part of the field of view in some devices. The rest is illuminated with light that bypassed the polariser (but passed through analyser). When the analyser is set correctly, both parts of the field are illuminated with the same intensity.

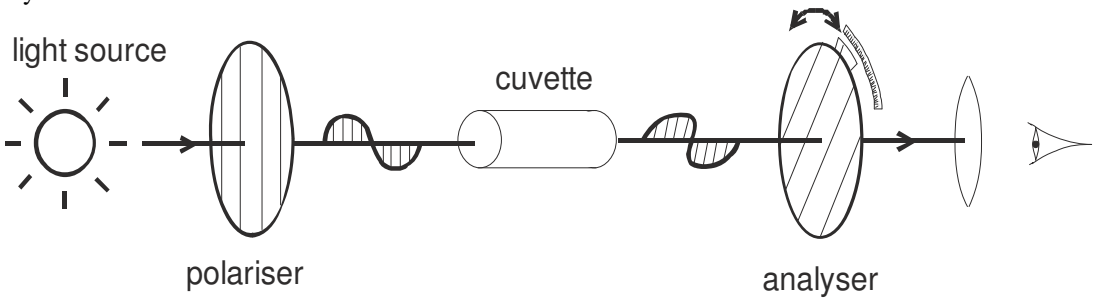


Fig. 8: Polarimetry

To compare optical activity of various compounds, the angle of rotation of polarised light is referred to unity of concentration. Depending on the way how concentration is expressed, specific and molar rotation can be defined. *Specific rotation* [α] is the ratio of the angle of rotation α over optical pathlength l and mass concentration w

$$[\alpha] = \frac{\alpha}{\mathsf{l} \cdot \mathsf{w}},$$

Molar rotation [Φ] is analogy with substance concentration c

$$[\Phi] = \frac{\alpha}{l \cdot c} \ .$$

Both constants for a given compound depend on temperature and wavelength of light. Their dimensions are °$kg^{-1}m^2$, or °$mol^{-1}m^2$ respectively. In tables, they are usually listed with 100× smaller units.