ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK

Spectrophotometry

Practical lesson in medical biochemistry

General Medicine

2022

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Translated to English by Jan Pláteník

Spectrophotometry

Spectrophotometry is one of the most widely used instrumental techniques in analytical practice. It is an optical method based on estimation of absorption of light either in the UV range having wavelength 200 – 400 nm, or in the visible light (VIS) range of wavelength 400 – 800 nm by molecules of the analyte in solutions.

The principle of spectrophotometry is interaction of electrons placed in the bonding or non-bonding orbitals with the photons of UV-VIS radiation. The energy of radiation excites the electrons in orbitals to a higher excited level; it is associated with absorption of certain quantum of radiation, which has definite energy contents, hence also a definite wavelength.

The method is able to estimate a particular analyte if at least part of the analyte molecule absorbs radiation. It means that it must contain a configuration of atoms or functional groups that are responsible for this absorption – these structural elements are called chromophores. The chromophores can consist of functional groups, or certain types of chemical bonds, such as the unsaturated bonds. Such chromophores are found especially in organic compounds. If a substance contains chromophores we can estimate absorption of radiation by the substance and in this way directly determine its concentration.

The absorption of light is quantitatively described by the Lambert-Beer law

$$\mathbf{A = \varepsilon . c . l} \qquad [\lambda]$$

A absorbance
ε molar absorption decadic coefficient, at the wavelength λ, unit is $l . mol^{-1}. cm^{-1}$
c concentration of the analyte in mol/l
l path length of the light (i.e. width of the cuvette containing the measured solution, through which the ray of UV-VIS radiation passes).

Absorbance tells how much of light is consumed by the measured solution. It is directly related to the absorption of radiation. If the absorption of light is zero, the absorbance is zero as well.

The Lambert-Beer law is valid for diluted solutions to concentration about 10^{-2} mol/l.

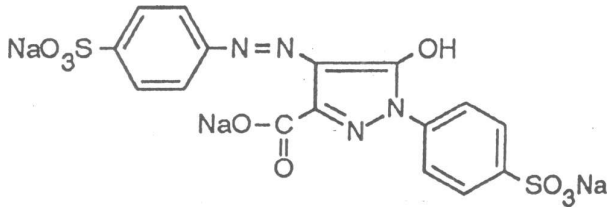
Food dyes

Various substances are added to foodstuffs to improve their taste, flavor, appearance or stability in storage. These food additives are denoted by E numbers. The codes E 100 to E 182 are reserved for food dyes. The Act No. 110/1997 Sb. defines dyes as substances that provide the foodstuffs with a color that would be absent without using the dye, or restore a color that was altered or attenuated during technological processing. The dyes can be classified to two groups – natural and synthetic dyes. In the practical lesson we will work with synthetic dyes.

The color of drinks is often caused by addition of synthetic dyes soluble in water. These substances can in sensitive persons induce allergic reactions and can be harmful if consumed in a higher quantity. That is why for various kinds of food and beverages limits have been set, to which the manufacturers are obliged to adhere. In flavored non-alcoholic beverages the maximal concentration of a dye should not exceed 100 mg/kg.

E-102, food yellow 4, $C_{16}H_9N_4Na_3O_9S_2$

Trisodium -hydroxy-l-(4-sulfonatophenyl)-4-[(4-sulfonatophenyl)diazenyl]-pyrazole-3-carboxylate,

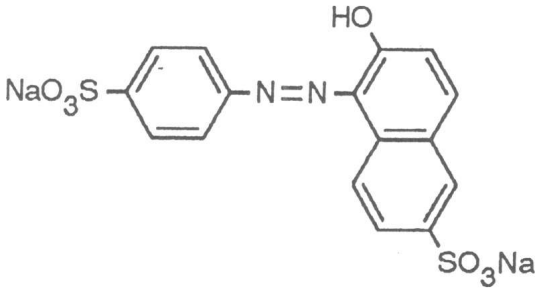


It is used in bakery and diary products, mustard, candy, non-alcoholic and even alcoholic beverages.

The substance can induce allergic reactions and asthmatic attacks in sensitive individuals. It has been associated with hyperactivity in infancy.

E-110, food yellow 3, $C_{16}H_{10}N_2Na_2O_7S_2$

Disodium 6-hydroxy-5-[(4-sulfonatophenyl)diazenyl]-naphthalene-2-sulfonate

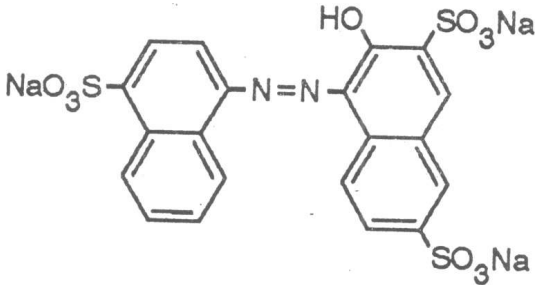


It is used in bakery and diary products, mustard, candy, non-alcoholic and even alcoholic beverages.

The substance can induce allergic reactions and asthmatic attacks in sensitive individuals. Also this substance has been linked to the hyperactivity in children.

E-123, food red 9, $C_{20}H_{11}N_2Na_3O_{10}S_3$

Trisodium -3-hydroxy-4-[(4-sulfonato-l-naphthyl)diazenyl]-naphthalene-2,7-disulfonate



In Czech Republic this dye is approved only for coloring of the following foodstuffs: aperitif wines, alcoholic liquors, fish eggs and milt.

In 1976 FDA (The Food and Drug Administration) banned this dye as a possible carcinogen. In addition, this substance causes allergic reactions in sensitive individuals.

Coordination compounds

Coordination compounds (complexes, coordination complexes) consist of a central atom M that is joined through at least one coordinate covalent bond to ligands L_{1-n}. The general formula of a complex ion is $[M(L_1)_{x1}\ldots(L_n)_z]^{n\pm}$

The central particles are usually atoms or ions of transition elements with vacant valence orbitals that can accept lone electron pairs; i.e., they are electron acceptors (Lewis acids). The *d*- and *f*-elements serve easily as coordination centers, whereas *p*-elements and especially *s*-elements are much less able of doing so.

Ligands are electron donors (Lewis bases); they can be anions such as Cl^- – chloro, Br^- – bromo, CN^- – cyano, OH^- – hydroxo, or even electroneutral molecules that possess an atom with a lone electron pair such as H_2O – aqua, NH_3 – ammin, NO – nitrosyl, CO – carbonyl.

Maximal number of univalent ligands around the central particle of a coordination compound is called **coordination number**; most often it is 6, 4, 8, or 2.

Stability constants of complexes

The term stability of a complex compound means its resilience to decomposition to free central atom and ligands. It can be also viewed as readiness with which the complex forms from the central atom and the ligands. In general, the more stable the complex compounds are, the more easily they form. Numerically, the complex stability constant can be expressed as a reciprocal value of the equilibrium constant for the reaction of complex dissociation.

Aqueous solutions of complex compounds reach a state of chemical equilibrium, for example

$$Cu^{2+} + 4\ NH_3 \rightleftharpoons [Cu(NH_3)_4]^{2+}$$

In this case the following relationship applies

$$K_k = \frac{\left[Cu(NH_3)_4\right]^{2+}}{\left[Cu^{2+}\right]\cdot\left[NH_3\right]^4}$$

where K_k is the **complex stability constant**. The higher the value of K_k is, the more stable is the complex, and vice versa.

Colors of complexes

Presence of ligands in various positions with respect to the central atom results in energetic differentiation of the (initially equivalent) *d*-orbitals of the central atom. The *d*-orbitals split to several energy levels with a small energy difference ΔE. Crystal field theory aims at explaining this phenomenon. It assumes that coordination bond is essentially an electrostatic attraction between positively charged central atom and a ligand that always bears a negative charge, in the form of a lone electron pair at least. This concept helps to explain many observed properties of complex compounds, such as their colors.

Color of coordination compounds reflects the degree of *d*-orbitals splitting, which, in turn, depends especially on:

- Complex geometry
- Central metal: *d*-orbitals of heavy metals (5^{th} and 6th period) split more than *d*-orbitals of metals of the first transitional row (4^{th} period).

- Oxidation state of the central metal: a more oxidized state has also more split levels of *d*-orbitals
- Type of ligand: according to order in the spectrochemical series of ligands:

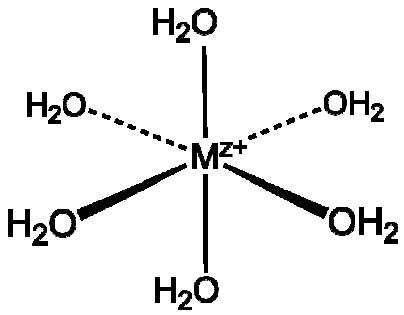
  $I^- < Br^- < S^{2-} < SCN^- < Cl^- < N^{3-} < F^- < NCO^- < OH^- < ONO^- < C_2O_4^{2-} \approx H_2O <$
  $NCS^- < NC^- <$ *pyridine* $< NH_3 <$ *ethylenediamine* $<$ *bipyridyl* $<$ *1,10-phenanthroline* $<$
  $NO^{2-} < CNO^- < CN^-$

The ability of ligands to split *d*-orbitals increases along this series, in relation to ligand binding properties.

Various degree of *d*-orbitals splitting manifests as a different color of complex compounds with the same central atom. Among two similar substances, the one with more split *d*-orbitals will absorb light of higher energy.

One of the commonest ligands is ubiquitous water. For example, anhydrous copper sulfate $CuSO_4$ is colorless, whereas its pentahydrate $CuSO_4 \cdot 5\ H_2O$ as well as its aqueous solution have a blue color. In both the latter cases, actually, copper engages in a complex with water. Likewise, aqua complexes of other transition metals (Fe, Cr, Mn, etc.) are colored.

Aqua complexes of metals are coordination compounds that contain metal ions and water molecules that serve as ligands. These complexes prevail in aqueous solutions of many metal salts, such as nitrates, sulfates, etc. The general stoichiometric formula is $[M(H_2O)_n]^{z+}$. The octahedral complexes of general formula $[M(H_2O)_6]^{2+}$ or $[M(H_2O)_6]^{3+}$ are the most common. Their spatial arrangement can be presented e.g. in this way:



Despite identical geometry and ligands, the aqua complexes of cations of various metals differ in the central atom and its charge and hence display different colors.

formula	$[Cr(H_2O)_6]^{2+}$	$[Cr(H_2O)_6]^{3+}$	$[Mn(H_2O)_6]^{2+}$	$[Fe(H_2O)_6]^{2+}$	$[Fe(H_2O)_6]^{3+}$	$[Cu(H_2O)_6]^{2+}$
color	blue	violet	light pink	pale green	pale yellow	blue

Aqueous solutions of metal aqua complexes are acidic because they can dissociate H^+ from the ligand:

$$[Cr(H_2O)_6]^{3+} \rightleftharpoons [Cr(H_2O)_5(OH)]^{2+} + H^+$$

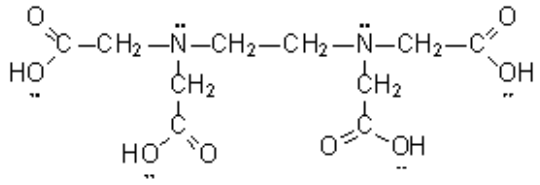
The pK_a of the chromium(III) aqua complex is approximately 4.3. It means that the aqua ion is a weak acid quite comparable to acetic acid ($pK_a = 4.76$). Aqua complexes of divalent metal cations are less acidic than the ones of trivalent cations.

Chelates

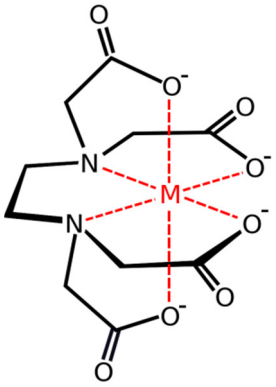
In the coordination compounds called chelate complexes (**chelates**) the ligand is an organic compound that simultaneously occupies several coordination sites on the central atom.

The name is derived form the action of the ligand that surrounds and "grips" the central atom like a claw (Greek chélé = claw). The chelate-forming (chelating) reagent is an organic substance with atoms of oxygen, nitrogen or sulfur in its molecule, and able to provide at least two lone electron pairs. The chelates formed by polydonor molecules of aminopolycarboxylic acids display the highest stability. For example EDTA (ethylenediaminetetraacetic acid) and its salts form very stable complexes with metal cations in ratio 1 : 1.

EDTA



Complex of EDTA with a metal cation



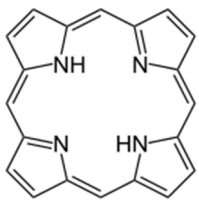
Coordination compounds in medicine

Complex compounds are often soluble in water, little dissociated and due to the presence of coordination bonds quite different from its original constituents both in color and solubility. Therefore, they are widely used in analytical chemistry. Coordination compounds can be present in reagents utilized for qualitative detection of various compounds: e.g. sodium nitroprusside for detection of oxo- group, Fehling reagent for detection of reducing substances. In other cases complexes originate in the reaction, such as in the biuret reaction (estimation of proteins), demonstration of Fe^{3+} with thiocyanate, and reaction of Fe^{2+} with ferrozine.

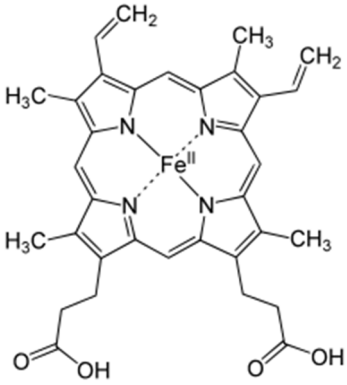
Some chelating agents are administered in medicine in cases of acute poisoning with cations of some di- or trivalent metals, in order to bind them and remove from the body. Ca-EDTA, DMSA (dimercaptosuccinic acid) or DMPS (dimercaptopropanesulfonate) are used. In iron-overload disease, e.g. due to repetitive blood transfusions, deferroxamine is the drug of choice.

Chelate structures of porphin derivatives are physiologically significant.

Porphin



Heme



Porphin is a chelating agent that can form coordination bonds between an iron ion (Fe^{2+} or Fe^{3+}) and nitrogens of the pyrrole rings. Porphyrins are derivatives of porphin. The most significant porphyrin chelate is heme, the component of hemoglobin, myoglobin and cytochromes.

Vitamin B-12 is another biologically important chelate. It is the only vitamin that contains an ion of cobalt(II), Co^{2+}.

1. Spectrophotometric estimation of molar concentration of food dyes

1.1 Absorption maximum of stock solution of food dye Demonstration

Reagents:

1. Stock solution of food dye E-102 of c = 55 μmol/l
2. Stock solution of food dye E-110 of c = 55 μmol/l
3. Stock solution of food dye E-123 of c = 62 μmol/l
4. Deionized water

Principle:

The absorption curve is obtained by continuous measurement of absorbance at various wavelengths and plotting these data to a graph, where the x axis contains the wavelength (independent variable) and the y axis is used for the absorbance (dependent variable). Regions with the highest absorbance values are called the absorption maxima.

Procedure:

a. According to the instructions for use of the spectrophotometer Lightwave II^+ set the wave scan range 350 – 600 nm.

b. Set the spectrophotometer reading to zero; using deionized water as the blank.

c. Into a cuvette put about 1 ml of stock solution of food dye and in the range of pre-set wavelengths measure the absorption curve. Record the wavelength of the absorption maximum.

1.2. Estimation of molar concentration of food dyes in given sample

Reagents:

1. Stock solution of food dye E-102 of c = 55 μmol/l
2. Stock solution of food dye E-110 of c = 55 μmol/l
3. Stock solution of food dye E-123 of c = 62 μmol/l
4. Deionized water

Explanation of evaluation methods:

Estimation of concentration of given substance requires a blind sample (reference sample, blank) and usually one or more standards. The blind sample contains all components

used in the assay except for the substance that is measured. The standard is a solution of the measured substance of known concentration.

When the absorbances of the sample (A_{sa}) and standards (A_{st}) are measured, the value of concentration of the measured substance can be obtained in several ways:

1. **Calculation from the Lambert-Beer law**,

   if the molar absorption coefficient is known. The most commonly used unit for the molar absorption coefficient is $l \cdot mol^{-1} \cdot cm^{-1}$. This unit is convenient because usually cuvettes with the light path 1 cm are used. Then a simple calculation according to the formula

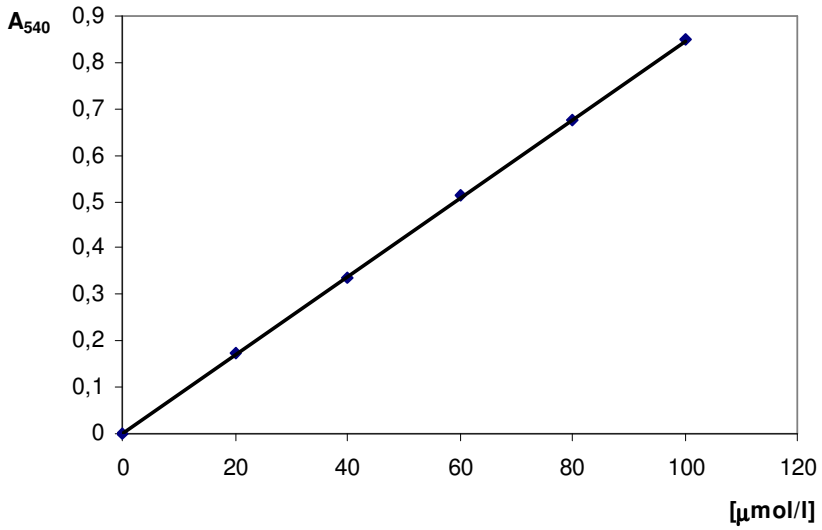
   $$\mathbf{A = \varepsilon \cdot c}$$

   gives molar concentration of the solution (mol/l).
   Another used unit is $cm^2 \cdot mol^{-1}$. This unit is 1000-times smaller than the $l \cdot mol^{-1} \cdot cm^{-1}$.

2. **Calibration graph method**.

   For construction of calibration graph we use the measured absorbances of the standards, i.e. solutions of known concentrations of the estimated substance.
   On the x axis we plot the standard concentration (independent variable) and on the y axis the standard absorbance (dependent variable). The graphical expression of the relationship is a straight line with slope 'k' that passes through the origin.

   Example: Calibration graph for the photometric estimation of NO_3^-



3. **Calibration factor method**

   Calibration factor (f) is the reciprocal value of the straight line slope (k).

   $$\mathbf{f = \frac{1}{k}}$$

   The factor can be calculated from the values obtained for the calibration line: by dividing the value of concentration c_α with the corresponding measured value of absorbance A_α.

   $$\mathbf{f_\alpha = \frac{c_\alpha}{A_\alpha}} \quad \text{where} \quad \mathbf{\alpha = 1...n}$$

From the values $f_1 - f_n$ the arithmetic mean is calculated that gives the value of the calibration factor f. The absorbance of the sample whose concentration is unknown is then multiplied with the factor. In practice it is possible to store the factor in the spectrophotometer memory and read directly concentrations of the analyzed samples. This method is therefore suitable for routine processing of high number of unknown samples.

4. **Standard sample method**

In addition to the sample of unknown concentration (c_{sa}), a standard sample (c_{st}) whose concentration is known is processed as well. The standard concentration is chosen so that it would be in the middle of the calibration line, or at the upper limit of the physiological range for the given analyte. The unknown sample concentration is calculated from the absorbances of both samples and the known concentration.
The absorbance is directly proportional to the concentration of the absorbing substance:

$$\frac{c_{sa}}{A_{sa}} = \frac{c_{st}}{A_{st}} \quad \text{then} \quad c_{sa} = \frac{c_{st}}{A_{st}} \cdot A_{sa}$$

A_{st} standard absorbance c_{st} standard concentration
A_{sa} sample absorbance c_{sa} sample concentration

Procedure:

a. Prepare and mark 6 test tubes (1 - 6).

b. Choose the stock solution of one of the food dyes (E-102, E-110, E-123 of given concentrations) and successively dilute it with distilled water, always to the concentration that is one half of the previous solution. Repeat 5-times in total (it is geometrical dilution).
Proceed according to the following table:

Test tube No.	**Deionized water** (ml)	**Solution of food dye** (ml)	Note:
1		2	*Stock solution*
2	1	1	*From tube 1*
3	1	1	*From tube 2*
4	1	1	*From tube 3*
5	1	1	*From tube 4*
6	1	1	*From tube 5*

c. Calculate the resulting molar concentrations of the diluted standards solutions (round to two significant figures) and fill in the table in your report.

d. From the provided graph of the food dyes absorption spectra choose a wavelength suitable for absorbance measurement of the given food dye.

e. On your spectrophotometer set the measurement of absorbance and the chosen wavelength. Insert a cuvette filled with deionized water (the blank sample) and set the absorbance to zero.

f. Measure the absorbance of an unknown sample (obtained from your instructor) and record the absorbance value to your report.

g. Using the same settings measure the absorbances of the diluted standard solutions 1-6 and fill the values to the table in your report.

h. Evaluation: as explained above, estimate concentration of the food dye in the unknown sample using

A) Reading from the calibration graph
B) Calculation with the calibration factor
C) Calculation with the standard

i. In your conclusion summarize results obtained with all three methods of evaluation. Try to compare precision of all the used methods.

2. Preparation of coordination compounds and measurement of their spectral curves in the visible spectral range

Reagents:

1. Copper sulfate crystalline 
2. Iron(III) chloride anhydrous 
3. Aqueous ammonia 2 mol/l 
4. Sodium thiocyanate 5g/l 
5. Chelaton III (ethylenediaminetetraacetic acid disodium salt dihydrate) 

   saturated solution
6. Deionized water

2.1. Complexes of Cu^{2+}

Principle:

$$Cu^{2+} + 2\,NH_4OH \rightarrow Cu(OH)_2 \downarrow + 2NH_4^+$$

$$Cu(OH)_2 + 2\,NH_4^+ + 2\,NH_4OH \rightarrow \left[Cu(NH_3)_4\right]^{2+} + 4\,H_2O$$

$$\left[Cu(NH_3)_4\right]^{2+} + EDTA + 4\,H_2O \rightarrow \text{complex } Cu^{2+}EDTA + 4\,NH_4OH$$

Note:
Cu^{2+} as well as other metal ions occurs in the aqueous solution as an aqua ion. However, in the equations above the water ligands are omitted.

Procedure:

N.B.: It is qualitative analysis – use the plastic droppers

a. Dissolve crystalline $CuSO_4$ in deionized water to light blue color.

b. Measure spectrum of the $CuSO_4$ solution in range 350 – 900 nm.

c. Add aqueous ammonia drop wise. First few drops are likely to produce a pale blue precipitate, which in further ammonia dissolves to tetraammincopper sulfate.

d. Measure spectrum of the tetraammincopper sulfate solution.

e. To the solution of tetraammincopper sulfate add drop wise a saturated solution of Chelaton III until the mixture changes/loses color.

f. Measure spectrum of the resulting complex of EDTA with cation Cu^{2+}.

g. Write all results into the table in your report.

2.2. Complexes of Fe^{3+}

Principle:

$$\left[Fe(H_2O)_6\right]^{3+} + SCN^- \rightarrow \left[Fe(H_2O)_5(SCN)\right]^{2+} + H_2O$$

$$\left[Fe(H_2O)_5(SCN)\right]^{2+} + EDTA \rightarrow \text{complex } Fe^{3+}EDTA + SCN^- + 5\,H_2O$$

Procedure:

a. Dissolve anhydrous $FeCl_3$ in deionized water to a light yellow color. Add a few drops of this solution to another test tube with deionized water, the resulting diluted $FeCl_3$ solution should have a very pale yellow color. Use the diluted solution for the subsequent steps.

b. Measure spectrum of the $FeCl_3$ solution in the range 200 – 800 nm.

c. Take about 2 ml of sodium thiocyanate solution in a test tube, and add 1-2 drops of the $FeCl_3$ solution.

d. Measure spectrum of the resulting sodium pentaaquathiocyanoferrate(III) solution.

e. To the solution of sodium pentaaquathiocyanoferrate(III) add drop wise a saturated solution of Chelaton III until the mixture changes/loses color.

f. Measure spectrum of the resulting complex of EDTA with cation Fe^{3+}.

g. Write all results into the table in your lab report.