Task 1

Acacia Honey

photo: Péter Vankó

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Szeged
Hungary

Acacia Honey

Robinia pseudoacacia is endemic to a few small areas in Northern America but it has been planted and naturalized all over the world and today in some areas it has become an invasive species. In Hungary, it was planted first at the beginning of the 18th century as a park tree but half a century later *Sámuel Tessedik* discovered that it can live on the sandy, saline soil and can be widely used for reforesting the Great Hungarian Plain. In the 19th century, it was already considered a "Hungarian tree", now its area reaches 4600 km^2.
In addition, acacia is also very popular with beekeepers. Acacia honey is popular because of its light colour, mild flavour, and acacia blossom scent. There are also many beekeepers in the Szeged area, and the acacia honey used in the measurement comes from here.

One of the main advantages of acacia honey is that it flows smoothly, it maintains its liquid state for years without any crystallisation. In the first problem of **Part A** this important property of acacia honey will be measured: the temperature dependency of its *viscosity*.
This high-value honey can be falsified with sugar beet molasses. In the second problem of this part and optical measurement will be analysed which can be used to measure the purity of the honey.

The three main phenolic compounds in *honey* (Figure 1A) are represented in Figure 1B-D, according to the studies by the research group of M. S. A Wahab (2014). The antioxidant activities of these molecules are different. Of the three phenolic molecules, ellagic acid has the highest, gallic acid has a medium, while quercetin has the lowest activity. Therefore, owing to the occurrence of numerous phenolic compounds, honey is a potent antioxidant food substance. These materials play natural roles as defenders against lipid oxidation, and they eliminate harmful free radicals. In **Part B** the antioxidant activity of three ingredients of honey will be investigated.

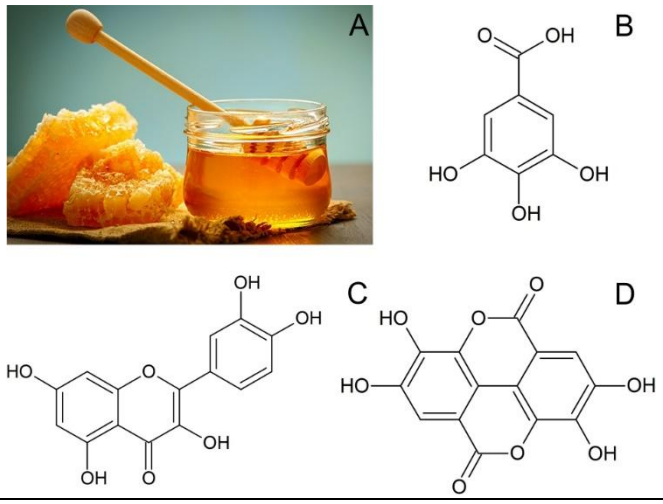


Figure 1. The three major phenolic compounds in *honey* (A) (B: gallic acid, C: quercetin, and D: ellagic acid).

Hungary is a Central-European country, which lies in the center of the Carpathian Basin. Due to its natural resources and traditions built during centuries of laborious work, this country is one of the most relevant honey exporters in the European Union. The country's honey production became so remarkable that even two types of honey – acacia honey and silkweed honey – were admitted to the "Collection of Hungarikums". The collection contains Hungarian values worthy of distinction, which carry features, uniqueness, peculiarities, and qualities that represent the high performance of Hungarian people. In **Part C** we will discuss the role of honey bees in honey production, their reproduction, the sugar content of honey, and the applicability of sugar.

Part A

In this part, there are two problems. In the first part, you will perform measurements to determine the temperature-dependent viscosity of your acacia honey sample.
In the second problem of this part you will study and evaluate a measurement made by fluorescence spectroscopy and you will determine the sugar beet molasses content of a falsified acacia honey sample.

Problem A1 *(75 points)*

Devices and materials for this problem:

- plastic kitchen tray, Bunsen stand with test tube clamps and cross clamps
- kettle, pot holder, kitchen measuring cup, ice cubes in a heat-insulating container
- beaker (1500-2000 mL volume)
- measuring cylinder (100 mL)
- acacia honey sample in a jar (a small amount of the honey will be used for Part C)
- lab thermometer
- jeweller's scale
- digital multimeter
- stopwatch
- calliper
- temperature sensor with banana plugs in a plastic bag
- 12 metal balls and a small plastic cap in a plastic bag
- mini funnel
- tesa film, paper towels, permanent marker, calculator, pens, pencil, rulers, ...

SAFETY WARNINGS!
Be careful with hot water. Use potholders to touch hot parts.
Work carefully with honey, clean the tray and devices with a wet paper towel if necessary.
Work with the small balls over the kitchen tray, otherwise, you can easily lose them.

Theoretical introduction:
Viscosity describes how thickly a liquid flows. It is caused by a kind of "internal" frictional force, which – unlike friction between solid bodies – depends on velocity. For ordinary (so-called Newtonian) fluids, in laminar flow, the frictional force is directly proportional to the velocity. If there is a relative velocity v between two parallel layers of fluid at a distance z apart, then there is a force F between the two layers: $F = \eta A \frac{v}{z}$, where A is the area of the layers and η is the viscosity. The SI unit of viscosity is Pa·s.

Viscosity depends on temperature. According to the theory $\eta = Ae^{\frac{B}{T}}$, where T is the absolute temperature, while A and B are constants specific to the material (e ≈ 2.7172 is the base of the natural logarithm).

If a solid body is moving in a medium (liquid or gas), the medium will impede the movement of the body. For high velocities, this is caused by vortices forming behind the body, but for low velocities, it is the viscosity of the medium that causes the restraining force. We are only concerned with the latter, as bodies can only move relatively slowly in high viscosity honey. In this case, the force acting on a *spherical* body is given by *Stokes' law*: $F = 6\pi r v \eta$, where r is the radius of the sphere, v is its velocity relative to the medium and η is the viscosity of the medium. This relationship is only valid in a very large medium, if a ball of radius r is moving in a tube of radius r_c, the force must be multiplied by a factor $(1 + 2.4\frac{r}{r_c})$.

Using Stokes' law, you can measure the viscosity of your acacia honey sample. If a spherical ball is dropped into a cylindrical vessel filled with honey, the ball – after a short acceleration – sinks in the liquid at a constant speed. In constant velocity motion, Newton's first law states that the resultant of the forces acting on the body – the *force of gravity*, the *buoyancy force*, and the *viscous drag force* – is zero.

> **Question A1.1** Write down the balance of forces acting on the ball sinking at constant speed in the honey in a cylindrical container. Give the forces using the following data: the radius r of the ball, the radius r_c of the cylindrical container, the density ρ_b of the ball, the density ρ_h of the honey, the gravitational acceleration g, the constant velocity v of the ball, and the viscosity η. Express the viscosity using the other data. Write your results in box **A1.1** on the *Answer sheet*.

Calibration of the temperature sensor.

The temperature of the honey will be measure by a *temperature sensor*. (The labour thermometer is too big, it would disturb the viscosity measurements.) This is an accurately manufactured platinum resistor that has a linear temperature dependence. It means that the $R(T)$ resistance of the sensor at T temperature is $R(T) = R_0(1 + \alpha T)$, where T is the temperature in °C, R_0 is the resistance of the resistor at $T = 0$°C, and α is a parameter of the resistor's material.

Your first task is to calibre your temperature sensor, i.e. to determine the value of R_0 and α.

> Fix the temperature sensor to the lab thermometer by tesa film. The sensor should be close to the ball of the thermometer. Do not cover the sensor by the film, fix it above the sensor. Attach the banana plugs to the multimeter. Use the 'COM' and the 'VmAΩ' inputs. Turn the selector switch to resistance mode (200Ω, 2kΩ, or 20kΩ), chose the appropriate range.
>
> Fix the lab thermometer by clamps on the Bunsen stand above the baker.
>
> Boil half a liter of water with the kettle and pour it into the baker. **WARNING! Be careful with hot water. Use potholders to touch the hot glass.**
>
> Adjust the height of the lab thermometer so, that the ball of the thermometer (and the temperature sensor) are underwater. Wait some time and read the temperature and the resistance of the sensor at (almost) the same time. Let cool the water (in the beginning it cools down rapidly) and repeat the reading. Later you can fasten the cooling by adding cold

water. If there is too much water in the baker, lift the thermometer, pour out a part of the water, and continue adding cold water.
On the end pour out again a part of the water, and add ice cubes. **WARNING! Use only half of the ice, because you need it later, too!**
Follow the cooling till ca. 5°C and regularly repeat the reading.

Question A1.2 Measure the resistance of the temperature sensor at different temperatures. Fill in table **A1.2** on the *Answer sheet*.

Question A1.3 Plot your $R - T$ data on a *graph paper*. Label the graph paper as '*graph A1.3*'. Fit a straight line on your data points and determine the parameters R_0 and α. For later work express the temperature T (in °C) in the function of the resistance R (in Ω) using the numerical values you have determined. Write your results in box **A1.3** on the *Answer sheet*.
Do not forget to attach '*graph A1.3*' to the answer sheet!

The next steps are to measure the parameters of the cylinder, the density of the honey, and the mass of the metal balls.

Question A1.4a Measure by calliper the inner diameter d_c of the 100 mL measuring cylinder. Calculate the inner radius r_c of the cylinder and write your result in field **A1.4** on the *Answer sheet*.

Question A1.4b Measure the distance s between the 10 mL and 90 mL markings on the wall of the measuring cylinder. Write your result in field **A1.4** on the *Answer sheet*.

Switch on the jeweller's scale by the leftmost button. The unit above the numbers must be 'g' (gram) – if not, you can change it by button 'M'. Put the small plastic cap on the scale and push the 'T' (Tare) button.

Question A1.5 Measure the total mass m_t of all (12) metal balls. Calculate the average mass m_b of a ball. Write your result in field **A1.5** on the *Answer sheet*.

Remove the temperature sensor from the lab thermometer and fix it at the *inside* wall of the 100 mL measuring cylinder at the 50 mL marking. Use tesa film to fix the wires above the sensor. Do not cover the sensor with the tape. Fix the wires at higher positions, too.
ADVICE: It is not easy to fix the sensor inside the thin cylinder. You can attach a small (1 -2 cm long) tesa film piece on the wires above the sensor, then carefully place the sensor in the proper place, and push the film against the cylinder with a pencil.

Question A1.6a Measure the mass m_e of the empty cylinder with the sensor. The banana plugs should lie on the table. Write the value in table **A1.6** on the *Answer sheet*.

Fill acacia honey in the measuring cylinder up to the 100 ml marking. **WARNING! Do not fill the honey above the scale. Fill the honey carefully and slow. Keep the cylinder slightly tilted, so the honey drips down on the inside wall of the cylinder and so fewer bubbles form in the honey. Clean the outside of the cylinder if necessary.**

Question A1.6b Measure the mass m_f of the cylinder with honey (and with the sensor). The banana plugs should lie on the table the same way as before. Write the value in table **A1.6** on the *Answer sheet*.

Question A1.6c Calculate the density ρ_h of the honey. Write your result in table **A1.6** on the *Answer sheet*.

You can now start to measure the viscosity of the honey. You should measure it at four or five different temperatures between 30°C and 15°C. Therefore first you have to warm up the honey to approx. 30°C. Follow the next procedure:

Fix the lab thermometer in the Bunsen stand above the baker and adjust its height so, that the ball of the thermometer is at the height of the 50 mL marking of the measuring cylinder. Boyle ca. 1 liter water in the kettle. Mix water at approx. 40°C in the baker, its level should be at the height of the 100 mL marking of the measuring cylinder.
Attach the banana plugs of the sensor to the multimeter and switch on the multimeter in the same way as earlier.
Place the measuring cylinder in the warm water. **BE CAREFUL! Do not immerse the mouth of the cylinder in the water.** Let warm up the honey for a few minutes. When the resistance of the sensor reaches the value corresponding to approx. 35°C (the sensor is at the wall, it becomes earlier warm, the middle of the honey is colder), remove the cylinder from the water, wipe it dry with a paper towel, and leave it to stand for about 10 minutes to ensure that the honey temperature becomes as even as possible.
ADVICE: During this time you can deal with Problem A2.

Question A1.7a Take a metal ball, measure its diameter d by the calliper. Write the value in the appropriate column of table **A1.7** on the *Answer sheet*.

Question A1.7b Read the resistance R of the sensor. Write the value in the appropriate column of table **A1.7** on the *Answer sheet*.

Question A1.7c Place the mini funnel in the top hole of the measuring cylinder to help you to drop the metal ball into the centre of the cylinder. Make the stopwatch in your hand and reset it. Drop the metal ball in the honey. Measure the time t the ball sink between the markings 90 ml and 10 mL, i.e. on a distance s, measured in **A1.4b**. Write the value in the appropriate column of table **A1.7** on the *Answer sheet*.

WARNING! Always let the balls in the bottom of the cylinder, do not try to take off them from the honey!

Question A1.7d At this temperature the ball sinks very rapidly. You should repeat the steps **A1.7a** - **A1.7c** *two more times* at the (almost) same temperature. Write the measured values in the appropriate columns of table **A1.7** on the *Answer sheet*.

Lift the thermometer, pour out the warm water, and fill the baker with cold tap water; the water level should be at the height of the 100 mL marking of the measuring cylinder.
Adjust the height of the thermometer again so, that the ball is at the height of the 50 mL marking of the measuring cylinder.
Place the measuring cylinder in the cold water. **Be careful! Do not immerse the mouth of the cylinder in the water.** Let cool down the honey for a few minutes. When the resistance of the sensor reaches the value corresponding to the temperature approx. 3°C below the wanted temperature (the sensor is at the wall, it becomes earlier cold, the middle of the honey is warmer), remove the cylinder from the water, wipe it dry with a paper towel, and leave it to stand for about 10 minutes to ensure that the honey temperature becomes as even as possible.
ADVICE: During this time you can deal with Problem A2.

Question A1.7e Repeat steps **A1.7a** - **A1.7c** *two times* at this second temperature. Write the measured values in the appropriate columns of table **A1.7** on the *Answer sheet*.

You can continue cooling down the honey in the same way. To make colder water use the rest of your ice cubes. The resistance of the sensor every time should correspond to the temperature approx. 3°C below the wanted temperature, as you remove the cylinder from the water. Always wipe it dry with a paper towel, and leave it to stand for about 10 minutes to ensure that the honey temperature becomes as even as possible.
ADVICE: During this time you can deal with Problem A2.

Question A1.7f Repeat steps **A1.7a** - **A1.7c** at two additional temperatures. In colder honey the sinking time is longer, one measurement is enough at every temperature.
WARNING! At lower temperatures, there can be bigger temperature differences inside the honey. In these cases, it is more accurate if you measure the (much longer) sinking time on a shorter distance, always around the temperature sensor, i.e. between the 70 mL and 30 mL, or at very low temperatures even between the 60 mL and 40 mL markings. Indicate your choice of distance (s, or $s/2$, or $s/4$) in the appropriate column of the table.
Write the measured values in the appropriate columns of table **A1.7** on the *Answer sheet*.

Question A1.7g Calculate the missing values (r, T, and v) in table **A1.7**. For calculating T use your expression derived in **A1.3**. Write the values in the appropriate columns on the *Answer sheet*.

Question A1.8 Calculate the average radius r_{avr} of the balls and determine the density ρ_{b} of the balls. Use the result of **A1.5**. Write the value in box **A1.8** on the *Answer sheet*.

Question A1.9 Calculate the viscosity η for every measurement by using the expression derived in **A1.1**. To do this safely and fast, first, change the units of your previous results to SI *base* units $(\mathrm{m}, \mathrm{kg}/\mathrm{m}^3, \mathrm{m}/\mathrm{s})$. Use $g = 9.81\, m/s^2$. Fill in the boxes in table **A1.9** on the *Answer sheet*.

As it was mentioned in the introduction, according to the theory the temperature dependence of the viscosity can be written in form $\eta = Ae^{\frac{B}{T}}$, where T is the absolute temperature (measured in K), while A and B are constants specific to the material. (e ≈ 2.7172 is the base of the natural logarithm.) Mathematical forms you may use: $\ln ab = \ln a + \ln b$, $\ln e^n = n$, $e^{\ln a} = a$.

Your last task in this problem is to show that data agrees with this equation and to determine the values of A and B.

Question A1.10 Linearize the relationship, i.e. find a suitable graph on which – based on the theory above – a line can be fitted to the data points. Write your choice in box **A1.10** on the *Answer sheet*.

Question A1.11 Calculate the values you want to plot. In the first line write the quantities you have chosen in **A1.10**. Fill out table **A1.11** on the *Answer sheet*.

Question A1.12 Plot your graph on a *graph paper*. Label the graph paper as '*graph A1.12*'. Fit a straight line on your data points and determine the parameters A and B. Write your results in box **A1.12** on the *Answer sheet*.
Do not forget to attach '*graph A1.12*' to the answer sheet!

Problem A2 *(25 points)*

Fluorescence

It can be observed that some special material can radiate light on a characteristic wavelength as a response to a shorter wavelength illumination. This phenomenon is called fluorescence, which is produced by some organic and inorganic structures. The wavelength spectrum of the emitted light depends on the material and the parameters of the excitation illumination. The wavelength of the emitted light is always longer than the wavelength of the incident light because of non-radiative transitions.

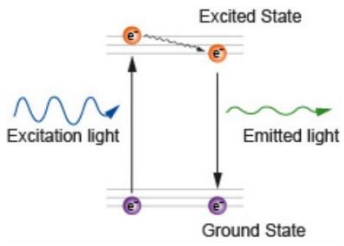


Fig.1 scheme of the fluorescence

We can explain the fluorescence by a simple model of light-material interactions. The energy quantum of the light called photon is proportional to the frequency of the light radiation. An incident photon can be absorbed by an atom or molecule. The energy of the photon converts the material system from the ground state to an excited state. As the material transforms back to the ground state spontaneously, the released energy is emitted by a photon (Fig.1).
Because the characteristic wavelengths of the emitted photons depend on the energy levels of the material, fluorescent light can be applied to analyse the structure and composition of different materials. Fluorescent spectroscopy is based on this principle, and it is widely used in chemical analysis, medicine, and food industry. In this task, you will analyse the cane sugar syrup doping of acacia honey by this method.

Integrated spectrometer

One of the most important parts of our fluorescent measurement is an integrated spectrometer. This spectrometer is applicable to analyse the wavelength composition of an arbitrary light. The schematic of the spectrometer is shown in Fig.2.
The light to be analysed get into the spectrometer by an optical fiber. The beam travels from the connector of the optical cable [1] to the mirror [4] which reflects the light to an optical grating [5]. The grating disperses the different wavelength components in different propagation directions. The dispersed light is reflected by the mirror [6] to a CCD detector matrix [7] which can measure the intensity of the different wavelength components. The electronically processed signals are transmitted through a USB cable to a computer. The measured spectrum can be plotted in real-time by software.

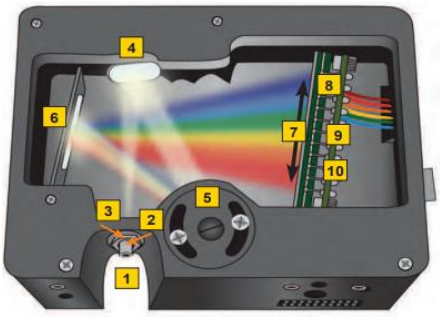


Fig.2 Inside schematics of the integrated spectrometer

Development of the measurement setup

To measure the fluorescence spectrum of the honey, an optical setup was built. The honey sample is put into a silica glass cuvette. A blue laser is used to illuminate the sample with a wavelength of 405 nm and power of 60 mW. The laser beam is orthogonal to the incident surface of the square-shaped cuvette. (Fig.3) We can observe the beam path in the honey as a turquoise line because the fluorescent light radiates in all directions from the excited volume of the sample.

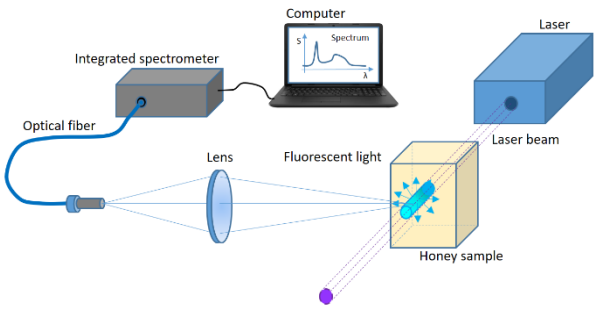


Fig.3 Schematics and of the measurement setup

We have to collect this fluorescent light to measure its spectrum. A converging lens is applied to project the light on the incident of an optical fiber. The focal length of the lens is 75 mm, both the object and image distances are set to 150 mm. The optical axis of the lens is perpendicular to the laser beam. The diameter of the incident aperture of the fiber is 0.6 mm. The fiber transmits the light into the integrated spectrometer.

Before further work, **watch the video** *'Honey_flour.mp4'* on the tablet/notebook provided.

Sample preparation

Before we qualify an unknown honey sample using its fluorescence spectrum, we have to calibrate our measurement system. To do this, we need to prepare honey samples with known cane sugar syrup concentration.

At first, the cane sugar syrup is prepared. A precision balance was used to measure the mass of the components. 10 pieces of conventional brown cane sugar cube were dissolved in 50°C water. The mass of the sugar was 37.81 g and the water was 37.75 g. It was mixing until the syrup reaches room temperature. This syrup is used to dope acacia honey.

Our goal is to prepare cane sugar syrup doped honey samples with different mass fractions between 0% and 100%. We put the empty cuvette on the balance and we set the scale to zero by pushing the 'tare' button to eliminate the mass of the cuvette. Then we pour some pure honey into the cuvette, and the mass of the honey (m_{honey}) is measured. Then the scale set to zero again using the tare button. Some cane sugar syrup is added to the honey, and the mass of the syrup is also registered (m_{syrup}) It was mixed to get a homogeneous mixture, during the outside of the cuvette was heated by 40°C water. This heating is necessary to decrease the viscosity of the honey. Before the measurement, the sample was cooled to room temperature (20°C). After these, the sample is ready to measure by the fluorescence spectrometer.

Sample No.	m_{honey} [g]	m_{syrup} [g]
0	0.00	4.91
1	0.51	4.38
2	1.24	4.97
3	2.05	4.77
4	2.85	4.26
5	3.49	3.47
6	2.72	2.01
7	2.13	0.92
8	3.53	0.87
9	4.87	0.61
10	3.88	0.00

Table 1

We repeat this sample preparation eleven times to get a set of mixtures with diverse mass fractions. m_{honey} and m_{syrup} data are logged in Table 1.

We define the mass fraction w of the doped honey samples by the following: $w = \frac{m_{syrup}}{m_{syrup}+m_{honey}}$

Question A2.1 Evaluate the mass fraction of the samples and fill the 'w' column of table **A2.1** on the *Answer sheet*.

Calibration measurements

After the sample preparation, we can start the fluorescence measurements. At first pure honey was put into the cuvette, and its fluorescence spectrum was measured in the setup shown in Fig.3. After that, the spectrum of the sugar cane syrup was also measured. The results are plotted in Fig.4a. Then we put pure water into the cuvette, and the spectrum of the water was also registered. We know that the water produces no significant fluorescence in the visible range, so this measurement gives information about the background. This background comes from the scattered light of the environment and the electronic noise of the spectrometer.

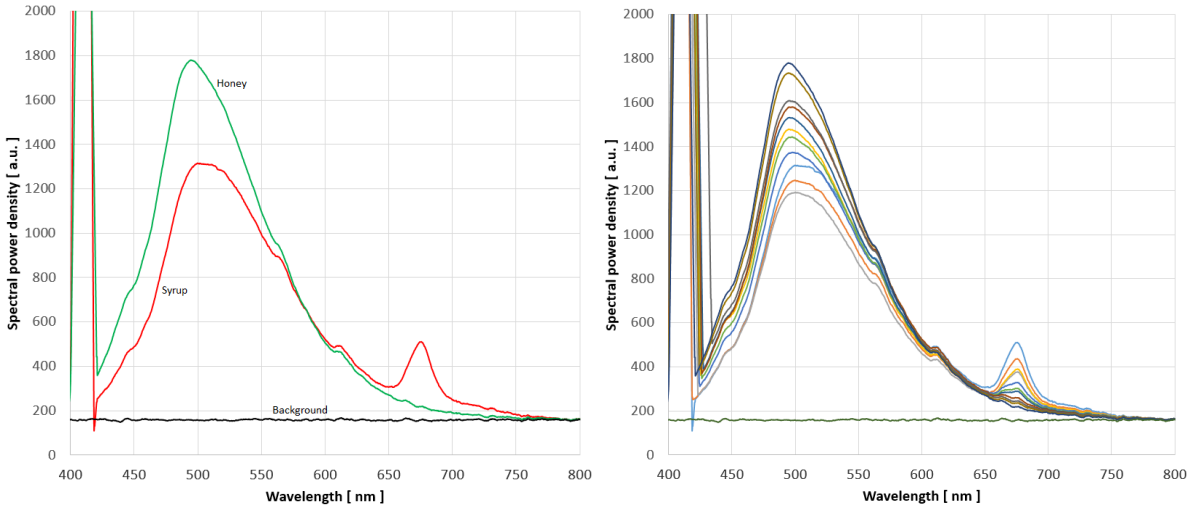
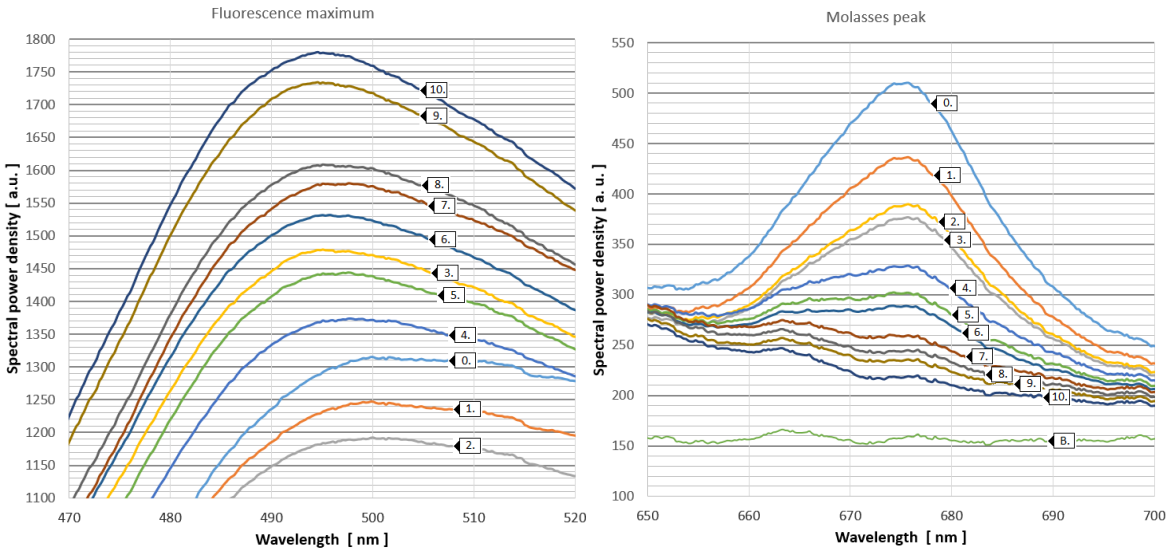


Fig.4 Fluorescence spectrum of the pure honey, the cane sugar syrup, and the background (a), spectra of the calibration samples (b)

We can see that the fluorescence of the pure honey and the syrup have similar spectra. A high and narrow peak can be detected around 405 nm, where the detector of the spectrometer is saturated. This high-intensity peak corresponds to the pump laser scattered on the sample material. In the next we analyse the longer wavelengths of the spectra correspond to the fluorescent emission. Both the syrup and the honey produce strong fluorescence in the middle of the visible regime. We can recognise a significant difference between the two spectra. At 675 nm there is a peak on the spectrum of the cane syrup, which peak is missing in the case of pure honey. This additional fluorescence light is produced by the molasses contained by the cane syrup. Detecting this “molasses peak” in the fluorescence spectrum of unknown honey gives information on whether the honey is doped by cane syrup. Evaluating the height of this spectral peak, we can estimate the syrup concentration of unknown doped honey.

To calibrate the concentration measuring method we measured the fluorescence spectra of the syrup doped honey samples. The profile of these spectra depends on the mass fraction w of the doping. The concentrations of these prepared samples are known from Question **A2.1**. The spectra are plotted in Fig.4b. We can see that the maxima of the fluorescent spectrum and the height of the molasses peak significantly depend on the mass fraction of the syrup. The set of curves on Fig.4b is too crowded to evaluate them, thus the relevant parts of the diagram are zoomed on Fig.5.



(a) (b)

Fig.5 Fluorescence spectra of syrup doped honey samples

Fig.5a shows the region of the maxima of the fluorescent spectra, Fig.5b shows the molasses peaks.

> **Question A2.2a** Read the maximum spectral power density values $F(w)$ correspond to the different syrup concentrations. Fill the column '$F(w)$' of table **A2.2** on the *Answer sheet*. Read the spectral power densities $M(w)$ correspond to the 675 nm molasses peak at each syrup concentration. Fill the column '$M(w)$' of table **A2.2** on the *Answer sheet*.

> **Question A2.2b** Subtract the background from each $F(w)$ and $M(w)$ values, and fill the column '$f(w)$' and '$m(w)$' of table **A2.2** on the *Answer sheet* with the reduced spectral power densities.

We can see that the $\alpha(w) = \frac{m(w)}{f(w)}$ ratio depends significantly on the mass fraction of syrup. This value can be used as the indicator of the mass fraction.

> **Question A2.2c** Evaluate $\alpha(w)$ for each sample. Fill table **A2.2** on the *Answer sheet*.

> **Question A2.2d** Plot $\alpha(w)$ in the function of the mass fraction w on a *graph paper*. Label the graph paper as '*graph A2.2'*. Fit a free handed regression curve on the point.
> **Do not forget to attach '*graph A2.2'* to the answer sheet!**

Analysis of an unknown honey sample

In this section, we have to determine the mass fraction of the sugar cane syrup in an unknown honey sample. The fluorescent spectrometer setup is used for this task which was calibrated above.
We pour the unknown honey sample into the cuvette and put it into the spectrometer setup. The measured spectrum is plotted in Fig. 6.

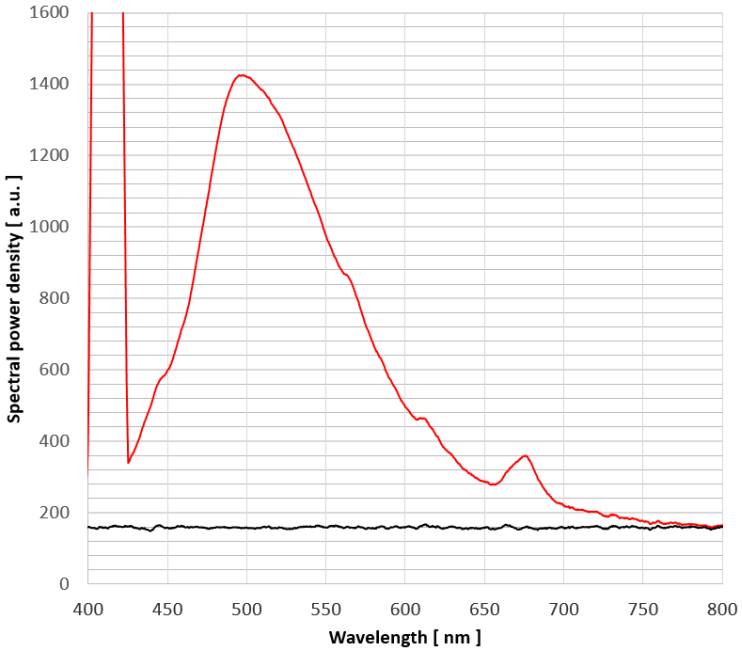


Fig.6 Fluorescence spectrum of the unknown honey sample

Question A2.3 Determine the syrup mass fraction of the unknown sample. Write the result in box **A2.3** on the *Answer sheet*.

Part B

In this part there are three problems concerning with antioxidant content of honey.
In the first part you will estimate the molar absorption coefficient of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and select the optimal wavelength. In the second problem you will determine the antioxidant activity of three main phenolic compounds of honey. In the third problem of this part, you will calculate the ascorbic acid equivalent of three antioxidants.

Problem B1 *(9 points)*

Every chemical compound can absorb, transmit or reflect light. Spectrophotometry is one of the most useful methods of quantitative analysis (e.g., chemistry, biology, clinical applications, etc), which based on the interaction between electromagnetic radiation and material. The basic structure of a spectrophotometer is shown in Figure 1. From the collimator white light arrives to the monochromator. After the monochromator splits the light to different wavelengths (λ), the wavelength selector will choose the required one. The detector can measure the intensity of the transmitted light, which will transform to transmittance or absorbance value.

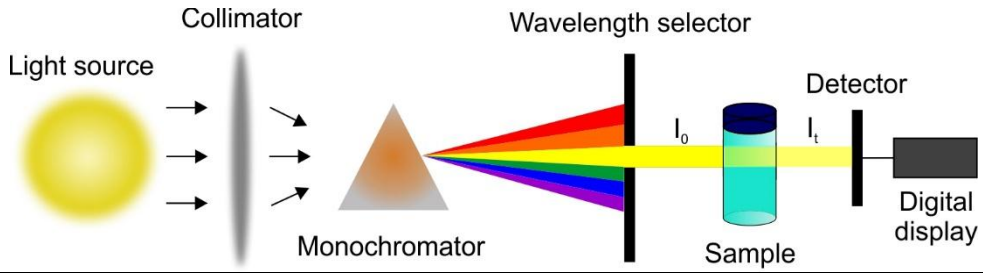


Figure 1. Schematic representation of a spectrophotometer

By detecting the change in incident light intensity, transmittance (T), the fraction of light which passes through the sample, could be calculated.

$$T = I_t/I_0$$

where I_0 is the intensity of the incident light and I_t is the intensity of the light after passing through the sample. Absorbance (A), the quantity of light absorbed by the sample, could be derived from transmittance.

$$A = - \log(T)$$

In visible spectrophotometry, the absorption or transmission of a certain substance originate from the color of the examined material. For instance, if the material absorbs blue light (~ 450 nm), it appears orange (~ 600 nm), the complementary color of blue.

Devices and materials for this problem:

- Volumetric flask, 50 mL
- Solid DPPH in plastic bottle (T1-B-P1/1)
- 500 mL Ethanol
- 1 plastic cuvette (T1-B)
- 1 cap for cuvette (T1-B)
- 50 mL beaker for DPPH solution
- 50 mL beaker for Ethanol
- Funnel
- Volumetric pipettes, 1 mL, 2 pieces
- Pipetting ball
- Calculator

SAFETY WARNINGS!

Be careful! During transport, the DPPH powder may have adhered to the wall of the sample holder.

Do not spill the DPPH solution, you will need it in Problem 2.

Pipette safety instructions:

1, Mouth pipetting is not allowed!

2, Insert the top of the pipette in the bottom of the pipetting ball. Be careful, don't break the glass pipette!

3, Look out! Do not draw liquid in the ball!

B1.1 Estimating the molar absorption of DPPH and wavelength selection

DPPH is a nitrogen-based radical, used to measure the antioxidant activity of molecular antioxidant, plant extracts, foods, etc. The molar absorption of DPPH and the optimal wavelength will be determined by spectrophotometric measurements. The concentration of a dissolved chromophore is dictated by the Beer-Lambert law.

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

where A is the absorbance of the solution (information given by the instrument), ε is the molar absorption coefficient of DPPH (a constant that describes the intensity of absorption) and c is the molar concentration of the chromophore and l is the width of the cuvette used for the measurement. Problem 1 is concerned with the estimation of the molar absorption (ε term in Beer-Lambert law) and absorption maximum of DPPH. Since absorbance is measured where it has a (local) maximum, first, you must determine the correct wavelength.

> Dissolve the solid DPPH powder with ethanol and wash it to the 50 mL volumetric flask. After completion to 50 mL with ethanol, mix 1 mL of this solution (contains 1.00×10^{-4} mol/L DPPH) with 1 mL ethanol in a cuvette.

The full spectrum of the diluted DPPH solution in the wavelength range of 400-700 nm is given in Figure 2. Select and indicate the wavelength, where absorption is maximal. Measurements were executed against a blank cuvette containing only ethanol.

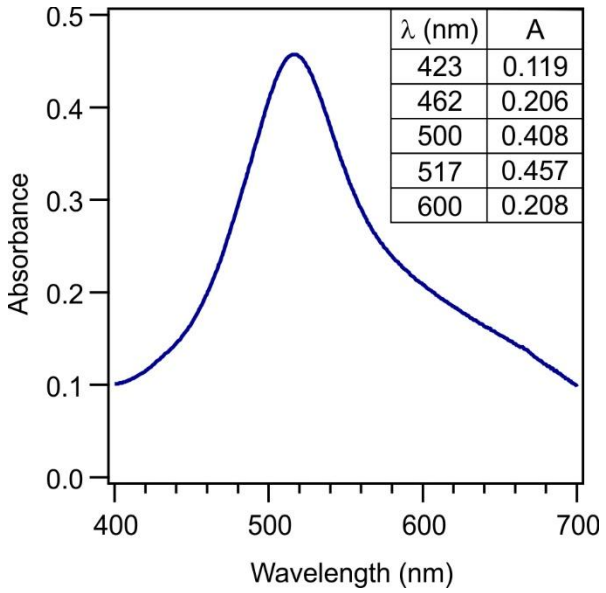


Figure 2. The full spectrum of the diluted DPPH solution (the inset shows the absorbance values at different wavelengths)

Question B1.1 What wavelength should be used for further measurements? Fill in the field **B1.1** on the *Answer sheet*.

Question B1.2 Calculate the concentration of the DPPH solution after dilution in the cuvette. Indicate the absorbance of this solution from the numerical data given with 2 decimal places at the absorption maximum. Using the Beer-Lambert law, calculate the molar absorption coefficient. Result should be given in dm^2/mol and $dm^3/(mol \cdot cm)$ dimensions. The width of the cuvettes is 10 mm. Fill in the field **B1.2** on the *Answer sheet*.

Problem B2 *(79 points)*

Estimation of antioxidant activity is possible through various reactions, in which the reducing agents are the antioxidants under consideration. The nature of oxidiser can be inorganic (such as nitrogen monoxide, metal ions) or organic (relatively stable free radicals). The present method relies on the reduction of DPPH radicals. The reaction scheme is displayed in Figure 3, involving DPPH and phenol as a model for phenolic antioxidants.

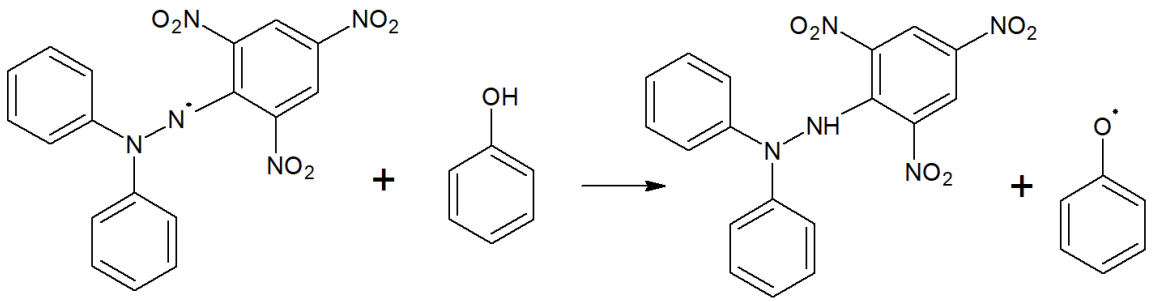


Figure 3. Scheme of the redox reaction between a DPPH radical and a phenol molecule.

DPPH is a relatively stable hydrazine-based radical with purple colour that diminishes into a pale-yellow colour after reduced to DPPH-H. It is apparent that after the antioxidants react with a radical (in the body, for instance), a new radical is formed, although these radicals are more stable than the counteracted radicals and thus, less harmful. The further oxidation of phenols is also possible provided they have unreacted phenolic hydroxyl groups (Figure 4).

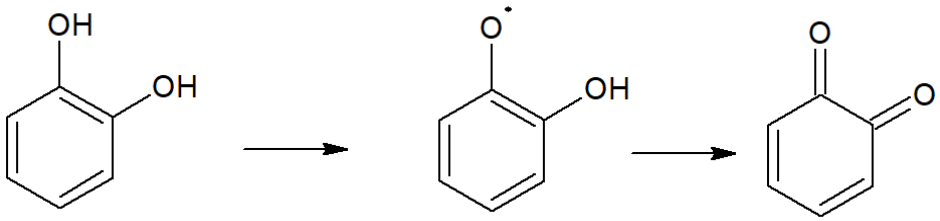


Figure 4. The complete oxidation of a diphenol (pyrocatechol) to a non-radical quinone (*o*-benzoquinone).

Devices and materials for this problem:

- Volumetric flask, 10 mL, 15 pieces
- Volumetric flask, 50 ml, 3 pieces
- Solid AO1 plastic bottle (T1-B-P2/1)
- Solid AO2 in plastic bottle (T1-B-P2/2)
- Solid AO3 in plastic bottle (T1-B-P2/3)
- DPPH solution (prepared in the volumetric flask in Problem B1)
- Graduated pipettes, 1 mL (2 pieces) and 5 mL (1 piece)
- Funnel
- Pipetting ball
- Plastic cuvettes, 3 mL, 15 pieces (T1-B)
- Cap for cuvettes, 15 pieces (T1-B)
- 250 mL beaker for waste
- 50 mL beaker for AO solutions and DPPH solution, 4 pieces
- 500 mL Ethanol
- Calculator
- Stopwatch, 1 piece
- Marker

SAFETY WARNINGS!

Be careful! During transport, the antioxidant powder may have adhered to the wall of the sample holder.

B2.1 Determination of the antioxidant activity of phenolic compounds of honey

In the colorimetric method, the ethanolic solution of DPPH is mixed with the ethanolic solution of antioxidants in a wide concentration range. The reactive hydrogen atoms reduce the DPPH radicals, resulting in a less intensive coloured solution of the reduced DPPH and the oxidised phenolic compound.

Comparing the initial and final (after the reaction occurred) absorbances of the reaction mixture, it is possible to determine the remainder of DPPH radicals.

$$A(final)/A(initial) \times 100 = \text{Remaining DPPH\%}$$

Gallic acid, quercetin and ellagic acid are provided in plastic bottles labelled AO1, AO2 and AO3 in a random order. Dissolve the antioxidants in the sample holders, transfer each to 50 mL volumetric flasks and complete them with ethanol. The concentration of the as-prepared stock solutions is equally 1.00×10^{-4} mol/dm^3.

The reaction between DPPH and the antioxidants is followed at five different antioxidant doses. The antioxidant solutions required for the appropriate doses should be diluted with ethanol from the stocks into a 10 mL volumetric flask. This step is detailed in the following table.

Antioxidant dose number	Volume of stock solution to dilute in mL
1	0.4
2	1.0
3	1.6
4	2.2
5	2.8

Question B2.1 Calculate the antioxidant doses in mol/dm^3 units. Fill in the table **B2.1** on the *Answer sheet*.

In a typical test reaction, add 1 mL of antioxidant solution to 1 mL of DPPH solution in a cuvette. After homogenisation, set the cuvettes aside in the dark (in your drawer) for 20 minutes until reaction completion. The addition of the diluted AO solutions is recommended to do in parallel.

In the calculation of the remaining DPPH%, use the absorbance selected earlier for Q B1.1 for the initial absorbance of DPPH solution (0 antioxidant dose).

The absorbance of the reaction mixtures was measured against a blank cuvette containing only ethanol. Note that the antioxidants and their corresponding products do not absorb light in the 400-700 nm wavelength range. The measured absorbance data are shown in random order for the three antioxidants in the following table.

Antioxidant dose number	A_X	A_Y	A_Z
1	0.42	0.44	0.38
2	0.35	0.39	0.20
3	0.27	0.34	0.10
4	0.24	0.30	0.10
5	0.23	0.27	0.09

Question B2.2 Based on the color change after 20 minutes of the addition of antioxidant dose 3 to DPPH solution, give a rank in the antioxidant activity of AO1, AO2 and AO3. Fill in the field **B2.2** on the *Answer sheet*.

Question B2.3 Calculate the remaining amount of DPPH radicals. Fill in the table **B2.3** on the *Answer sheet*.

Question B2.4 Plot the remaining amounts of DPPH radicals as a function of initial antioxidant concentration on the same *graph paper*. Label the graph paper as *'graph B2.4'*. Fit a straight line on your linearly decreasing section of data points and determine the parameters of the fit line.
Calculate the antioxidant concentration required to decompose 50 % of the initial DPPH. This value is the so-called EC_{50} (effective concentration) of the antioxidant. Given the rank in the antioxidant activity of gallic acid (GA), quercetin (QC) and ellagic acid (EA). identify the materials (X, Y, Z) with the abbreviated name of the molecules. Determine the antioxidants in AO1, AO2 and AO3. Fill in the table **B2.4** on the *Answer sheet*.

After fitting the points on graph paper, the parameters of the fitted line can be determined. The standard form of a linear equation of two variables (x and y)

$$y = ax + b$$

where a is slope and b is the intercept. To calculate the slope, choose two points from both ends of line [$(x_1;y_1)$ and $(x_2;y_2)$] and replace it with the formula below.

$$a = (y_2-y_1)/(x_2-x_1)$$

Look out! Slope could be both negative and positive value!
From the slope and a coordinate of one point, the intercept can be easily calculated.

$$b = y_1 - ax_1$$

Problem B3 *(12 points)*

Ascorbic acid (or vitamin C), discovered in 1912 and isolated in 1928, is probably the most well-known and vital nutrient with various essential role in the human body. The ground-breaking investigations of vitamin C brought Nobel Prizes for Norman Haworth and Albert Szent-Györgyi. Szent-Györgyi spent several years in Szeged during his career and he is associated with the first isolation of ascorbic acid, the substance he briefly called godnose (for the analogy of sugars), as only God can know the identity of the new material. This name was rapidly swapped to hexuronic acid and then, to ascorbic acid.

B3.1 Determination of ascorbic acid equivalent

Ascorbic acid is a strong antioxidant, with an effective mass (mass calculated from the EC_{50} data obtained in an identical DPPH test that you performed today) value of 10.4 μg. It is widely used as reference molecule in antioxidant tests. With dividing the effective mass value of ascorbic acid by the effective mass value of the investigated antioxidant, ascorbic acid equivalent (AAEQ) could be easily determined.

AAEQ = effective mass(ascorbic acid)/effective mass(antioxidant)

Question B3.1 Determine the ascorbic acid equivalent of gallic acid, quercetin and ellagic acid using their effective mass values as follows. Calculate effective mass values of gallic acid, quercetin and ellagic acid in μg units (to 1 decimal points) using their molar mass below and the total volume of reaction mixture, then calculate the AAEQ value for each antioxidant. The required data are shown in the following table. Fill in the table **B3.1** on the *Answer sheet*.

Material	Molar mass (g/mol)	Total volume of DPPH tests
Gallic acid	170.1	
Quercetin	302.2	2 mL
Ellagic acid	302.2	

Part C

Hungary is a Central-European country, which lies in the center of the Carpathian Basin. Due to its natural resources and traditions built during centuries of laborious work, this country is one of the most relevant honey exporters in the European Union. The country's honey production became so remarkable that even two types of honey – acacia honey and silkweed honey – were admitted to the 'Collection of Hungarikums'. The collection contains Hungarian values worthy of distinction, which carry features, uniqueness, peculiarities and qualities that represent the high performance of Hungarian people.

Bees are flying insects with stout, furry bodies, that are closely related to ants and wasps. Honey is produced by Honey bees (*Apis mellifera*), by the extraction of nectar or other secretions of plants. After their collection, and the addition of self-secretions, the plant secretion is then stored and mellowed. Due to its composition, humankind has used honey since the prehistoric ages, not only as a nutrient source but also as a natural remedial delicacy. It has two major components: carbohydrates (fructose, glucose) and water (max. 20%). Proteins, minerals, vitamins and trace elements can also be found in honey, however only in minor proportions, less than 0.5% of its mass.

Problem C1 *(31 points)*

Honey bees

Based on taxonomic ranking, these animals belong to the phylum 'Arthropoda', with a specific physical appearance. On Figure 1., a Honey bee's typical physical appearance is presented, where each body part is labelled with a number.

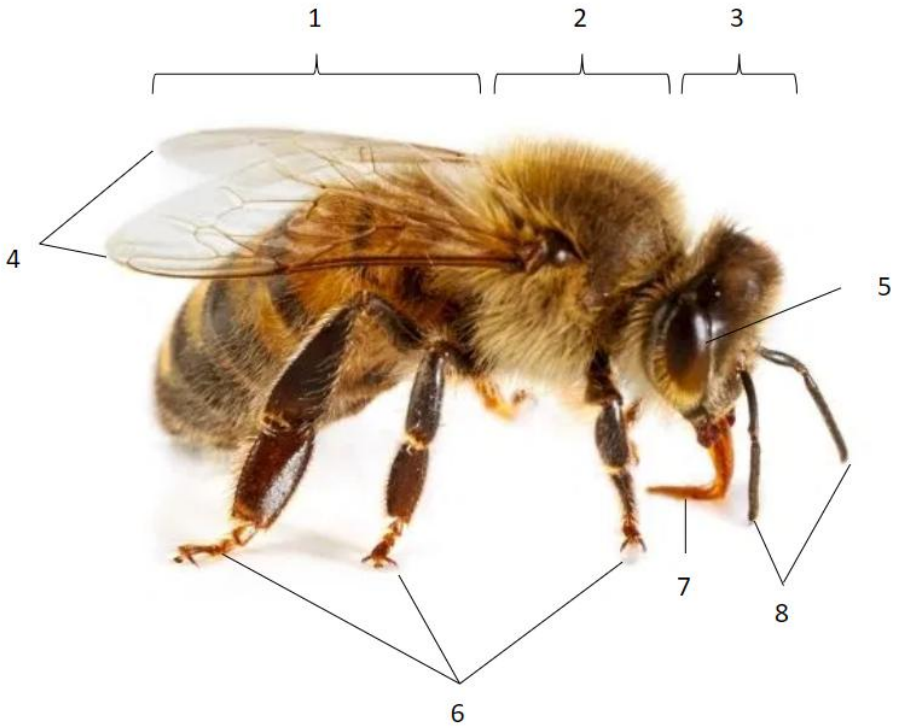


Figure 1.
Body parts of a Honey bee

Question C.1.1. Which body parts belong to the indicated numbers (Figure 1.)! Be careful, there are seven unnecessary answer options. Write your answers in the field **C.1.1** on the *Answer sheet*.

A) Elytron
B) Antenna
C) Cephalothorax
D) Thorax
E) Compound eyes
F) Chewing and lapping mouthpart
G) Membranous wings
H) Jumping legs
I) Jointed legs
J) Piercing-sucking mouthpart
K) Chambered eyes
L) Sponging mouthpart
M) Head
N) Eye spots
O) Abdomen

Question C.1.2. Which order of Insects do Honey bees belong to? Write your answer in the field **C.1.2** on the *Answer sheet*.

A) Hymenoptera
B) Lepidoptera
C) Beetles
D) Fly

Reproduction of Honey bees

The Honey bee is an eusocial species, meaning that it lives in large families (40.000-80.000 members), where the majority of members are not able to reproduce, but support the reproductive individuals. Eggs are laid by the queen, who is able to mate with males. Female bees (or workers) emerge from fertilized eggs (diploid) laid by the queen bee. These individuals are also capable of laying eggs from time to time. However, these eggs are infertile (haploid), from which male honey bees (or drones) emerge. The latter is a mode of asexual reproduction, reassuring the subsistence of the species without mating. In this case, ontogenetic development initiates from the unfertilized eggs without the need for a male gamete, or sperm. In either case, the laid eggs (both diploid and haploid) hatch after 3 days, and larvae are fed and nursed by the worker bees. After five to six days, larvae turn into pupae and 12 days later fully developed bees emerge.

Question C.1.3. Which interaction characterizes best the nursing act of worker bees? Write your answer in the field **C.1.3.** on the *Answer sheet*.

A) Altruism
B) Commensalism
C) Symbiosis
D) Competition
E) Predation

Question C.1.4. Which one is the proper term for the ontogenetic development of Honey bees? Write your answer in the field **C.1.4.** on the *Answer sheet*.

A) Epimorphosis
B) Metamorphosis
C) Anamorphosis
D) Hemimetamorphosis

Question C.1.5. What is the proper term for the asexual reproduction of Honey bees? Write your answer in the field **C.1.5.** on the *Answer sheet*. (one word)

A) Budding
B) Parthenogenesis
C) Hermaphroditism
D) Fragmentation

Question C.1.6. Based on the description above, how long does the embryonic development (egg state) last? Write your answer in the field **C.1.6.** on the *Answer sheet*.

A) 3 days
B) 5-6 days
C) 12 days
D) 21 days

Problem C2 *(22 points)*

Sweet honey: sugar content of the honey

Experiment 1.

Devices and materials for this problem:

- honey
- 10 x 50 ml Falcon tubes
- balance scale
- distilled water
- marker
- plastic chemical spoon
- test strip and color scale (T1-C-P2)

Determine the sugar content of honey with the help of test strips (T1-C-P2). For this, first prepare a 40% (m/V%, 40g/100mL) solution of honey in a final volume of 40 ml in distilled water (label it as "stock solution"). Measure 20 ml of stock solution into a tube labeled '1'. Prepare a two-fold serial dilution in Falcon-tubes in 20 ml final volumes and label each tube from '2.' to '8.' The 1. and stock solution concentration will be the same. For the appropriate mixing, close the Falcon tubes with their caps and shake them strongly. The honey content of the last tube should be 0.3125% (m/V%). Use

the scales on the Falcon tubes to measure the appropriate amounts. Determine the sugar content of **the last 3** two-fold serial dilutions as follows: immerse the test strip in a sugar solution for 1-2 seconds, then draw it by keeping a slow constant contact with the wall of the Falcon tube in order to drain the excess liquid. Due to a chemical reaction, the test strip will change colour. After 60 seconds, compare the colour of the test strip to the colour scale and determine the sugar concentration of the previously mentioned last 3 dilutions.

If you have difficulties preparing the serial dilutions, ask for help from a teammate first (no point deduction). If you still have difficulties with the task, ask a teacher for help. She/He will give you a detailed description to perform this task although this will cost you -15 points.

IMPORTANT! Do not trash the serial dilutions, you will need them later!

Question C.2.1. Determine the sugar concentration of the last 3 dilutions **in g/L** (6., 7., 8. Falcon tubes)! The test strip will determine sugar concentration in mmol/L. (Molecular mass of glucose and fructose is the same: 180 g/mol). The test strips do not differentiate between glucose and fructose (the main sugar components of honey) so the detected concentration refers to the whole sugar content. Write your answer letter in the field **C.2.1.** on the *Answer sheet*.

A) 0.025 g/l
B) 12.5 g/L
C) 0.1 g/L
D) 3.125 g/L
E) 0.5 g/L
F) 0.3125 g/L
G) 2.5 g/L
H) 1.25 g/L
I) 10 g/L
J) 1 g/L
K) 0.625 g/L
L) 5 g/L
M) 0.25 g/L
N) 6.25 g/L
O) 0.05 g/L

Question C.2.2. Considering the serial dilutions/measured sugar concentrations, calculate the original (undiluted) sugar content of the honey in g/L. **(**Molecular mass of glucose and fructose is the same: 180 g/mol). The test strips do not differentiate between glucose and fructose (the main sugar components of honey) so the detected concentration refers to the whole sugar content. Which is the correct answer? Write your answer in the field **C.2.2.** on the *Answer sheet*.

A) 320 g/L
B) 1760 g/L
C) 800 g/L
D) 640 g/L
E) 4400 g/L

Problem C3 *(47 points)*

Attention: Experiment 3. requires 30 min of waiting time and 20-30 min of observation.

Sweet honey: its sugar content and the applicability of sugar

In the previous section, we concluded that honey has a considerably high sugar content. Certain microorganisms, such as the yeast *Saccharomyces cerevisiae,* are able to produce alcohol and carbon dioxide (CO_2) from glucose. While the alcohol production ability of yeasts is used for example beer production, their CO_2 production ability is beneficial for the production of bread and bakery products as it „blows'' the dough. The available amounts of sugar, however, greatly determines the activity of *Saccharomyces cerevisiae.* On the one hand, high sugar content of the culturing broth medium results in substrate inhibition, meaning that it does not allow yeast cells to effectively reproduce and blocks their metabolic processes. Although, it does not lead to total growth inhibition (in this case honey at concentrations higher than 45% (m/V%) causes this effect). On the other hand, low sugar content of the culturing broth medium does not supply enough nutrition for yeasts, hence they stop growing, and reach a stationary phase earlier with lower OD_{600} value.

In a laboratory, the growth curve of a microorganism is usually determined with a photometric method. During this method, after microbe inoculation (when microbes are inoculated in fresh culturing broth medium) we take samples from microbial cultures at given time points and measure their optical density at 600 nm (OD_{600}). With this method, a growth curve can be drawn, based on only the time points of the samplings (x axis) and the correspondingly measured OD_{600} values (y axis). Environmental parameters, such as the above mentioned sugar concentration, or temperature, pH, etc. also greatly influence the growth of *Saccharomyces cerevisiae*.For this reason, now we understand why it is important that sugar is present in proper amounts in the culturing broth. Given the proper amount, yeast cells find their ideal conditions to reproduce effectively with active and fast metabolism, meanwhile producing also CO_2 in high amounts.

The growth curve on Figure 2. represents the reproduction rate of microorganisms.

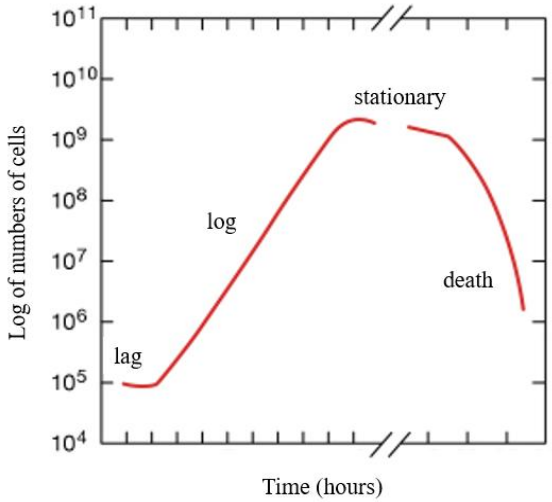


Figure 2.
The growth curve of a microorganism.

Within a fresh culturing broth, mircoorganisms first adapt to their environment. In optimal case it takes just a few hours (max. 10 hours). This phase we call as 'lag' phase. After this, cells begin to rapidly proliferate, hence the cell count excessively doubles (logarithmic (log) or exponential phase). If the OD_{600} value of a data point is 40% higher than the OD_{600} value of a previous data point, the

culture is in the logarithmic phase." (E.g. if the difference between two consecutive data points is higher than 40%, then the earliest data point means the end of the lag phase, and the latest data point the start of the log phase). If the OD_{600} value is higher with 40% than the previously measured OD_{600} value, the culture is in the logarithmic phase. Then microbes reach a maximum cell amount, where the cell proliferation and cell death events balance each other (stationary phase). If the OD_{600} value difference is less than 10% between two sampling points, the culture is in the stationary phase. After this, the culture reaches a decay phase (shown as 'death' on Figure 2.), when cell death events overcome the proliferation events, that will ultimately result in a decrease in cell numbers.

Experiment 2

Devices and materials for this problem:

- plotting paper and Datasets (A and B), (T1-C-P3/1)
- ruler
- pen
- pencil
- rubber

Based on the data provided in the sent paper (T1-C-P3/1; Dataset "A" and "B"), illustrate the growth curves of the *Saccharomyces cerevisiae* cultures in conditions "A" and "B" (different sugar containing culturing media), in terms of sampling time (x axis) and OD_{600} measurements (y axis). Illustrate the 'A' and "B" dataset on the sended plotting papers.

Dataset "A"	
Time (hour)	OD_{600}
0	0.1
5	0.12
10	0.24
15	0.48
20	0.96
25	1.4
30	1.48
35	1.52
40	1.52
45	1.54

Dataset "B"	
Time (hour)	OD_{600}
0	0.1
5	0.12
10	0.15
15	0.18
20	0.25
25	0.42
30	0.84
35	1.34
40	1.52
45	1.53

Question C.3.1. On both graphs (drawn from the datasets "A" and "B") and according to the information provided in the introductory text, determine approximately at which sampling time point does the lag phase end! Write the appropriate answer in the field **C.3.1.A. in the case of Dataset "A" and C.3.1.B. in the case of Dataset "B"** on the *Answer sheet*. (Choose one letter for each dataset!)

A) 5 hour
B) 10 hour
C) 15 hour
D) 20 hour

Question C.3.2. On both graphs (drawn from the datasets "A" and "B") and according to the information provided in the introductory text, determine approximately at which sampling time point does the *S. cerevisiae* culture first reach the stationary phase! Write the appropriate answer in the field **C.3.2.A. in the case of Dataset "A" and C3.2.B. in the case of Dataset "B"** on the *Answer sheet*. (Choose one letter for each dataset!)

A) 15 hour
B) 20 hour
C) 25 hour
D) 30 hour
E) 35 hour
F) 40 hour
G) 45 hour

Question C.3.3. According to the added information (Problem C3) and comparing both growth curves (A and B), which one would you determine as optimal? Write your answer in the field **C.3.3.** on the *Answer sheet*.

A) "A" growth curve
B) "B" growth curve

Question C.3.4. Which condition (A or B) better resembles substrate inhibition? Write your answer in the field **C.3.4.** on the *Answer sheet*.

A) "A" growth curve
B) "B" growth curve

Question C.3.5. Based on the description above (Problem C3), what could have caused the growth inhibition at the beginning of the growth? Write your answer in the field **C.3.5.** on the *Answer sheet*!

A) The available sugar (major carbohydrate nutrient) was consumed
B) High sugar content of the culturing medium inhibited growth
C) Cell death of *S. cerevisiae*
D) Product inhibition (e.g. alcohol)

Experiment 3

Devices and materials for this problem:

- honey dilutions in Falcon tubes (marked with numbers 1 to 8) from the previous experiment (‘Experiment 1.’)
- granulated yeast (“Élesztő”; T1-C-P3/2)
- chemical spoon
- weighing boat
- balance scale
- balloons
- timer

Add 1- g of yeast to each of the two-fold serial dilutions prepared in ‘Experiment 1’ (use the weighing boat, chemical spoon and a balance scale), then close the Falcon tubes and turn the samples up and down until the pellet disappears and the solutions are completely homogenized. Then, remove the lid, and place a balloon on each Falcon tube as shown on the images below (Figure 3.). The balloon’s neck should be placed right on the neck of the Falcon-tube (topside), where the lid closes if tightened. Squeeze the air out from the balloon before putting it on! (If necessary, you may ask for help from a team member or a teacher. This will not cost you point deduction.) After 30 minutes, revisite the experiment and follow the changes for the next 20-30 minutes! Take notes for yourself during the experiment! Observe the outcome and answer the questions below.

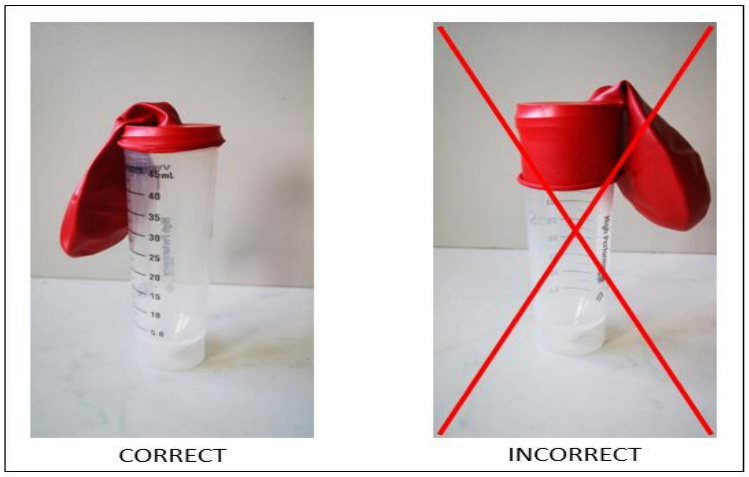


Figure 3.
Correct position of the balloon on the Falcon-tube.

Devices and materials for this problem:

- honey dilutions in Falcon tubes (marked with numbers 1 to 8) from the previous experiment (‘Experiment 1.’)
- granulated yeast (“Élesztő”; T1-C-P3/2)
- chemical spoon
- weighing boat
- balance scale
- balloons
- timer

Question C.3.6. Based on the growth of *Saccharomyces cerevisiae*, which tubes were the most likely optimal according to the CO_2 production observed? Write the appropriate tube numbers in the field **C.3.6.** on the *Answer sheet*.

A) 4. Tube
B) 5. Tube
C) 6. Tube
D) 8. Tube

Question C.3.7. In which case did you observe the least optimal growth based on the CO_2 production? Write the number(s) of the corresponding Falcon-tubes in the field **C.3.7.** on the *Answer sheet*.

A) 1. Tube
B) 4. Tube
C) 5. Tube
D) 8. Tube

Question C.3.8. Based on the experiment (Exp. 2 and 3) and the description above determine which dilution (Tube number) could the growth curves (Dataset "A" and "B") belong to most likely! Indicate your answer in the field **C.3.8.A. in the case of Dataset "A" and C.3.8.B. in the case of Dataset "B"** on the *Answer sheet*. (Choose one letter for each dataset!)

A) 1. Tube
B) 4. Tube
C) 6. Tube
D) 8. Tube

Question C.3.9. What could be the reason that in the case of Tube 8. you could observe only a little gas production?

A) The reproduction of *S. cerevisiae* was inhibited by pH
B) The carbohydrate active enzymes of *S. cerevisiae* were inhibited
C) The available sugar (major carbohydrate nutrient) was too little
D) Product inhibition (e.g. alcohol)