University of Ss. Cyril and Methodius in Trnava
Faculty of Natural Sciences
Department of Biotechnology

LABORATORY EXERCISES IN BIOCHEMISTRY

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Foreword

The manual "Laboratory Exercises in Biochemistry" summarizes the basic principles and methods used in biochemistry. It provides a theoretical basis explaining the principles of selected biochemical techniques and laboratory exercises that have been validated in practice. This study material is intended primarily for students of biotechnology, as well as for anyone interested in learning the basic techniques commonly used in biochemistry. The intention of this text is to deepen the knowledge of biochemical methods and to enable the acquisition of experimental experience in this field.

Authors

Content

Safety at work in the laboratory

Laboratory regulations

The following safety rules must always be followed in the laboratory. The chemical laboratory is not necessarily a dangerous place. By taking reasonable precautions and having a proper understanding of the techniques, the chemistry laboratory can be just as safe as any other classroom.

1. The student is required to familiarize himself/herself with the laboratory regulations, safety and first aid rules before starting work in the laboratory.
2. The student is required to come to the laboratory on time and properly prepared. He/she must have prepared the necessary calculations, know the properties of the substances he/she will be working with, etc. The teacher will check the knowledge of the students before starting the exercise. If the student does not have sufficient knowledge to solve the assigned problem, the exercise will be carried out at an alternative time.
3. Any absence must be excused. If a student has serious personal reasons for not being able to attend a practical, he/she must notify the teacher in advance.
4. The student must wear a lab coat and appropriate footwear when working in the laboratory.
5. Students work in pairs. Any defects detected before or during the work should be reported immediately to the teacher.
6. Work must be done exactly as assigned and instructed by the teacher. Before using the apparatus, the student must first familiarize himself/herself with its operation.
7. The progress of the work and the results obtained should be recorded by each student in the laboratory notebook. At the end of the exercise, the results are handed over to the teacher.
8. At the end of the exercise, each student should hand in the prepared work protocol, which shall include the following information: a student's name, major field of study, date of the experiment, title of the task, brief principle of the task, brief procedure of the work, results (tables, figures), discussion and conclusion.
9. After finishing the work, the student is required to clean his/her workplace, turn off electrical appliances, properly wash the glassware, rinse it in distilled water, and place it in a drying rack.
10. The student may leave the laboratory only after the teacher has checked the results obtained and the condition of the workplace.
11. Any accidents or injuries must be reported immediately to the teacher and first aid must be administered immediately if necessary.

Work safety in the biochemical laboratory

1. The work is carried out only according to the teacher's instructions and work instructions.
2. Bags and clothing must be stored in cabinets outside the laboratory.
3. Under no circumstances is eating, drinking or smoking allowed in the laboratory. Hand washing after each laboratory activity is a necessity.
4. Chemical glassware is never to be used for eating or drinking (even outside the laboratory).
5. Chemicals are never to be touched, tasted, or their fumes inhaled. Do not mix the chemicals unless instructed to do so.

6. Work with toxic materials, volatile, odorous, and other harmful substances is done only in an operating fume hood.
7. Pipetting shall be done only with balloons, vacuum sources, or an automatic micropipette. Mouth pipetting is forbidden.
8. Repairs or modifications to electrical installations and apparatus should only be done by a qualified person.
9. When working with corrosives and other hazardous substances, the face and eyes must be protected by a protective shield, and the hands by protective gloves.
10. The workplace must always be kept tidy and clean. Care must be taken to ensure that the outside walls of containers or the work area are not spilt with chemicals. Before leaving the laboratory, all equipment and apparatus must be returned to its shelf.
11. Concentrated acids and bases are diluted by pouring the acid or base in a thin stream down a rod into the water while stirring and cooling, not the other way around.
12. When conducting experiments in test tubes, keep the mouth of the tube away from your face (and the faces of your co-workers).
13. Do not have an open flame nearby when working with combustibles. When distilling flammables, remove flammable storage containers from the vicinity and control the water flow in the cooler. Never heat combustibles using a direct flame but use baths or heating nests instead.
14. Practice utmost caution when handling Class I combustibles with a flash point up to 21 °C (acetone, ether, methanol, ethanol, gasoline, benzene, and toluene).
15. If a fire occurs, everyone must try to extinguish it with their own efforts (fire extinguishers, improvised fire extinguishers). Furthermore, it is necessary to turn off the electrical equipment and try to remove flammable substances (especially liquids) and compressed gases from the vicinity of the fire. If you are unable to control the fire on your own, you must call for help immediately (telephone number 150 or 112).
16. Splinters and other rubbish with sharp edges must be disposed of in containers specially designed for this purpose.
17. Residues of poisons and organic solvents must be disposed of according to the teacher's instructions. They should never be poured down the drain.
18. Safety precautions must be observed when working with ether (diethyl ether) (possibility of ignition even from hot components of other apparatus).
19. In the event of an accident (including minor incidents), the teacher must be informed immediately, and first aid must be given to the injured person. Any minor injury, headache, tinnitus, etc. should also be reported to the teacher. In any case, a record of the accident should be made in case of later complications.

First aid

If the skin is burned by strong alkali or acid, the affected area is immediately rinsed thoroughly with a stream of water. Neutralize sodium bicarbonate solution ($NaHCO_3$; 20 g/l) in the case of acid burns and dilute acetic acid (CH_3COOH; 5 g/l) in the case of alkali burns.

If you suspect that chemicals have entered your eye, begin flushing it out immediately with cold water or an eye wash and continue to do so for a minimum of approximately 15 minutes. In the case of eye contact with alkali, the eye is flushed out with water containing boron (boric acid solution, H_3BO_3; 30 g/l). If acid is involved, a borax solution ($Na_2B_4O_7$; sodium tetraborate) at a concentration of 20 g/l is used for lavage. In any case, a consultation with an ophthalmologist should be considered.

If the mucous membranes of the mouth are irritated, the mouth should be rinsed thoroughly with water and then neutralized by rinsing with dilute acetic acid (alkaline irritation) or sodium bicarbonate (acid irritation).
After ingestion of alkalis (NaOH; sodium hydroxide, KOH; potassium hydroxide, etc.), it is recommended to drink dilute acetic acid (0.5-2.0 g/l); after ingestion of acid, a suspension of magnesium oxide or aluminum hydroxide in water should be drank. After ingestion of poisons, the nature of first aid is specific to the type of poisoning, it is recommended to drink at least 0.5 l of water and induce vomiting. Professional medical treatment should always be sought.
Burning clothing is extinguished with a blanket or water. A fire extinguisher should be used to extinguish larger flames. For small burns, the affected area should be treated with burn ointment and covered with a sterile bandage. Larger burns should be treated by a doctor.
For glass incision wounds, the glass should be removed from the external wound, the site disinfected with a dilute hydrogen peroxide solution (H_2O_2; 3 %; *v/v*) and a sterile bandage applied. Larger wounds should be treated by a doctor.

Laboratory notebook and work protocol

Laboratory notebook

A laboratory notebook is a complete record of procedures (the actions you take), the reagents you use, the observations you make (these are the data), and the relevant thought processes that would enable another scientist to reproduce your observations. The pages in the notebook should be numbered for easy reference. A homework assignment should be written in the laboratory notebook from the materials provided prior to the laboratory exercise, summarizing all the information necessary for the smooth running of the laboratory exercise, including the objective of the work, the procedure written in text, flow chart or bullet points, the calculations necessary to prepare all the solutions and reagents needed for the exercise itself, indicating their expected consumption (including those prepared in the laboratory), and the design of tables to record the intended results to be measured during the exercise. Without this preparation, it will not be possible to complete the exercise.
Observations, partial results, calculations, and conclusions resulting from the experiment should be recorded in the laboratory notebook during the experiment. It does not rely on memory.

Work protocols

The pair will create their own work protocols and hand them in to the teacher at the next laboratory exercise. The students will prepare the work protocol according to the template given at the end of this manual (Appendix 1, 2). The work protocols should be brief, concise, and clear, without unnecessary duplication, and should include the following sections:

- **Aim of the work** - a precise definition of the aim of the work.
- **The procedure** of the work - describe in detail in your own words (past tense) what was actually done and how, including deviations from instructions, exact instructions, instruments, equipment used, measurement conditions, etc. - i.e., in such a way that the experiment can be repeated exactly according to the protocol. The number of valid decimal places must be observed - an error of 0.1 g is not the same as 0.10 g!

- **Calculations** - additions, dilutions of solutions, etc. required for the procedure (including the relationships and formulae needed to process the measured values).
- **Results** (+ their evaluation, graphs) - (only) what was observed or measured, or what the student calculated from the measured values. The results are presented as clearly as possible, preferably in a table form.
  The graphs must have all the necessary elements:
  - A title that makes it clear, without having to read any more text, what the dependency is. The title and a more detailed description should be given below the graph, e.g., Figure 1: Dependency...
  - Coordinate axes with description, i.e., label the quantity used including the appropriate unit (on the x-axis is the independent variable (a quantity that the student varies himself/herself - e.g., concentration of the solution), on the y-axis is the dependent variable (e.g., absorbance)).

  The value calculated/determined from the measured values must be rounded to account for the accuracy of the measurement methods used. This means that the number of valid decimal places of the result should respect the least accurate value of all from which you are adding to the formula - e.g., you need to consider how accurate the concentration of the calibration solutions was, how accurate the pipetted reagent additions were. This usually results in three or at most four valid numbers.
- **Discussion** - evaluation of the results; explanation of what the results imply, etc. Comparison of measured or calculated values with tabulated data, theoretically calculated data, etc., including the reason for any deviations. Comparison of results obtained using different methods - justify the strengths and weaknesses of each method. The result of the measurement may be biased by gross, systematic, or random errors. The discussion focuses mainly on possible sources of systematic errors reflecting the correctness of the whole measurement or method, i.e., errors that cannot be eliminated by repeated measurements under the same conditions - e.g., an inappropriately chosen procedure (when determining the concentration of one substance there may be interference with an impurity, etc.), poor calibration, etc.
- **Conclusion** - brief answer to the stated aim of the thesis.

1. Biochemical laboratory equipment

1.1. Glass

Laboratory glassware includes glass laboratory equipment you encounter in a chemical laboratory. Laboratory glassware has a high resistance to mineral acids and bases. The only exception is hydrofluoric acid (HF). HF is able to react with the compound silicon dioxide (SiO_2) found in glass, thus dissolving it. The most important of the physical properties of glass is its thermal expansion. In general, glasses that soften at higher temperatures have a greater resistance to sudden thermal changes. Three types of glass are mainly worked with in the laboratory:

1. **Soft glass (sodium-potassium-calcium)** – used to produce glassware that is not exposed to thermal stress. It has a significant coefficient of expansion, so it cannot withstand temperature fluctuations. It must be heated or cooled very carefully. It has a low melting point (550 °C), therefore, can be quickly melted in the burner flame. The softened glass can then be easily processed and can be bent. It has a characteristic blue-green color on the cut. Glassware made of this type of glass is used in the laboratory. Examples include tubes, stirring rods, watch glass (known as clock glass), plate glass, etc.
2. **Hard glass (borosilicate)** – used to produce glassware that can be heated directly over a flame. It is used to create most of the glass products in the laboratory (beakers, flasks, test tubes etc.). Borosilicate glass has a yellow-green color which is characterized by resistance to cracking, a high melting point (700 °C), and high chemical resistance. The most common brands are Sial, Simax, Duran, and Pyrex.
3. **Quartz glass –** characterized by high chemical and thermal resistance (melting temperature > 1400 °C). It is colorless, has a very low coefficient of thermal expansion compared to other types of glass, and therefore resists sudden temperature changes without breaking. But it is very fragile and is used exclusively for making unique vessels and equipment such as spectrophotometric cuvettes (UV-permeable).

1.2. Porcelain

Porcelain equipment (evaporating dishes, mortar, pestle, annealing crucibles, weighing boats, spoons, Büchner funnels) is often used in the chemical laboratory. Hard chemical porcelain has high mechanical and chemical resistance. It is sensitive to impacts and easily breaks, especially with sudden temperature changes. It is stable and resistant to atmospheric oxygen even at high temperatures. It does not bind water to its surface. It resists chemical agents at least as well as chemical glass.

1.3. Metalware and laboratory tools

Metals and metal alloys are used in the chemical laboratory as a general construction material. Compared to glass, metals have high mechanical strength, are not brittle, and have significantly higher thermal and electrical conductivity and thermal expansion. Metal vessels and equipment are used wherever glass, porcelain, or quartz glass cannot be used. The metalware and laboratory tools shown in Figure 1 are made from metals and alloys. For special applications, vessels and tools made of various metals (gold, platinum, silver, nickel, copper, chromium, etc.) are utilized.

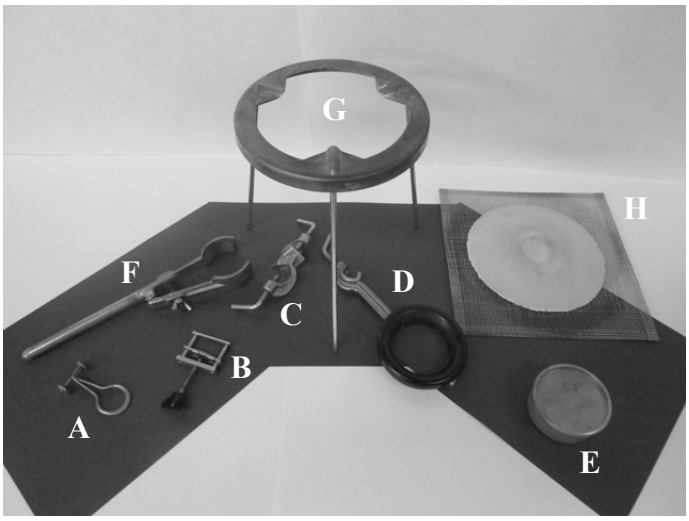


Figure 1: Laboratory metal tools: A – Mohr clip, B – Hoffman clip, C – clamp, D – filter ring (iron ring), E – aluminum moisture dish and cover, F – utility clamp, G – laboratory tripod stand, H – asbestos mesh (wire mesh gauze).

1.4. Laboratory equipment classification based on function

According to purpose and application, laboratory equipment can be divided into

- basic laboratory glassware,
- graduated glassware,
- containers used for storing chemicals,
- condensers (coolers),
- funnels and
- other types of laboratory equipment.

Basic laboratory glassware includes the basic glass vessels used in chemical laboratories, which include beakers, boiling flasks, distillation flasks, fraction flasks, Erlenmeyer flasks, test tubes, and crystallizing and evaporating dishes (see Figure 2).

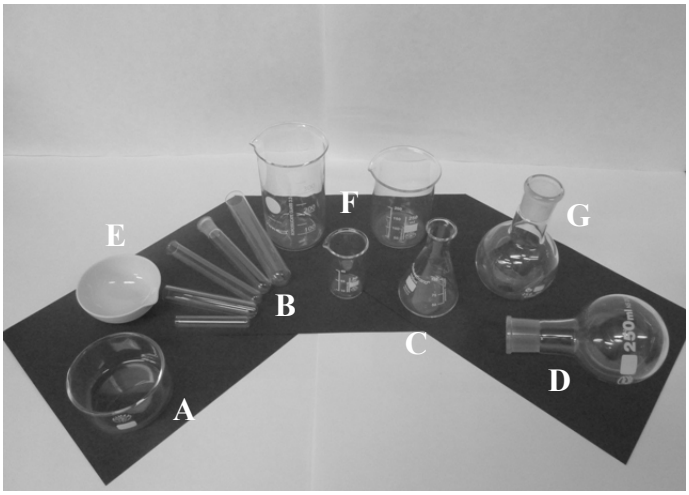


Figure 2: Basic laboratory glassware: A – crystallizing dish, B – various types of test tubes, C – Erlenmeyer flask, D – distillation flask with ground joint, E – evaporating dish, F – beakers with different volumes, G – boiling flask with a ground joint.

The group of **graduated glassware** includes graduated cylinders, volumetric flasks, pipettes, and burettes (see Figure 3).

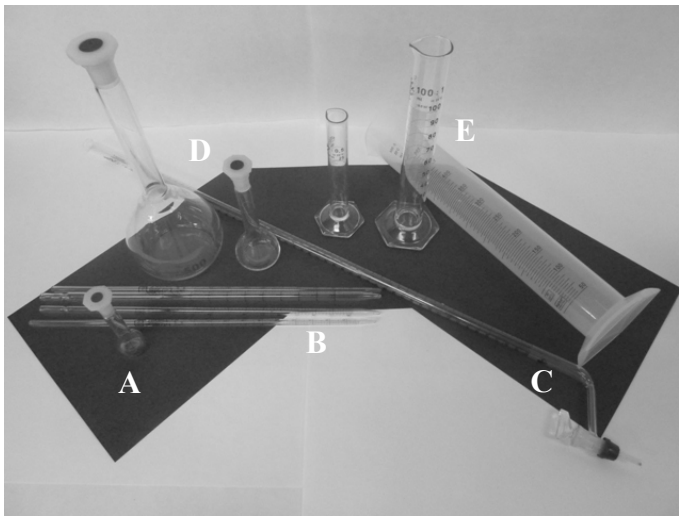


Figure 3: Graduated glassware: A, D – volumetric flasks (10, 50 and 500 ml), B – pipettes with different volumes, C – burette, E – glass and plastic graduated cylinders.

Graduated glassware is used to measure the volume of liquids or to dispense them accurately. The volume of the volumetric glassware is calibrated at a certain temperature, usually at laboratory temperature (20 °C).

- *Graduated cylinders* also known as measuring cylinders or mixing cylinders, are used to approximate the volume of liquids. Cylinders are calibrated for "IN" (inlet), which means the volume of liquid filled in corresponds exactly to the capacity printed on the measuring glass.
- *Volumetric flasks* (also called graduated flasks or measuring flasks) are used to prepare solutions of precise concentration, filled to the mark, and calibrated for "IN". The neck of the volumetric flasks is relatively narrow, with a fill mark around the circumference. The flask should be filled so that the lowest point of the meniscus touches the upper edge of the graduation mark. Like graduated cylinders, volumetric flasks are available in volumes from 5 ml to 2 l. The actual mixing of the solution's components is carried out when the flask is not entirely filled (approximately ¾ of its total volume). Allow the solution to reach laboratory temperature. The fill mark on the volumetric flask is for preparing solutions at the calibration temperature of the flask (the temperature at which the flask was calibrated, the value is printed on the flask and is usually close to the laboratory temperature, 20 °C). Once the solution reaches laboratory temperature, slowly add the solvent dropwise. When correctly filled, the meniscus should appear to rest on top of the etched fill mark. If too much solvent is added, the solution must be discarded, and a new solution should be prepared from the first step of the procedure.
- *Pipettes* are used for highly precise dosing, i.e., measuring a specific volume corresponding to the marked volume of the pipette (undivided pipettes). These pipettes are calibrated for "EX" (discharge), and the volume of liquid dispensed corresponds precisely to the capacity printed on the measuring glass. Part of the solution remains in the conical end of the pipette, which is already taken into account by the manufacturer when calibrating the pipette. Therefore, when the solution is discharged from the pipette, the remaining solution is not blown out. The pipettes are filled by aspirating the solution up to zero on the scale. All types of solutions that are not harmful to health can be aspirated by mouth, but because

of the possibility of contamination of glassware during other work in the laboratory, aspiration of the solution into the pipette by mouth is not recommended.

- *Burettes* are used for exact dosing of partial volumes and are calibrated for "EX". The smallest volume that can be dispensed with a burette is one drop, which, depending on the shape and size of the outlet valve, is approximately 0.03 ml. Burettes are filled by refilling or pressurizing (automatic burettes) to zero. They are emptied by gravity through a glass stopcock.

Another type of laboratory equipment is **containers for storing chemicals** (see Figure 4).

Figure 4: Containers for storing chemicals: A, B, D, E, F, G – reagent bottles, C – vials.

Condensers (coolers) are used to condense vapors. Condensers are routinely used in laboratory operations such as distillation, reflux, and extraction. Each condenser is a tube within a tube, rigidly connected, with one tube forming the condensing part (usually the inner one) and the other forming the cooling part – the cooling jacket (usually the outer one). Three basic types of condensers are known according to their ascending cooling effect:

- *Liebig condenser* (named after Justus von Liebig) with the simplest design, easy to build and inexpensive,
- *bulb condenser* (also known as Allihn condenser – named after Felix Richard Allihn) consists of a long glass tube and a series of bulbs on the tube, which increase the surface area on which the components of the vapor can condense, and
- *spiral condenser* with an internal spiral (see Figure 5).

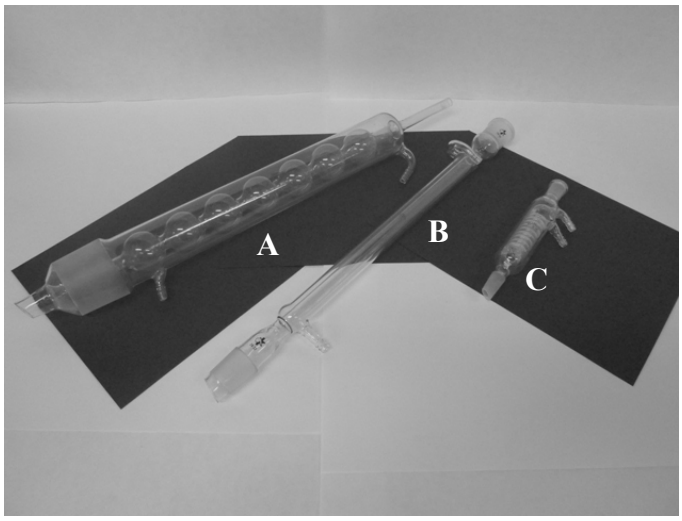


Figure 5: Condensers: A – bulb condenser, B – Liebig condenser, C – spiral condenser.

The word **funnel** refers to a larger number of laboratory equipment (see Figure 6).

- *Conventional funnels* are used for pouring liquids and simple filtration. They have smooth and equally thick walls.
- *Analytical funnels* are used for filtration and have thick walls at the top and thinner walls with projections at the bottom. The analytical funnel has a long and thin outlet tube - the stem.
- In addition to filter funnels mentioned above, *separatory funnels* are used to separate two liquids that are not readily miscible and may also be included in this group of laboratory equipment. Separatory funnels are used in many processes such as extraction, drying, dripping, etc.
- *Büchner funnels* are used to filter liquids under reduced pressure. They are made of porcelain and have a perforated bottom.
- *Fritted glass funnels* are used to filter liquids under reduced pressure, but instead of a perforated bottom, they have sintered glass to allow the passage of fluids.
- *Powder funnels* have a large bore, making them ideal for use with powders, larger particles, and viscous liquids. The funnel has contoured outer ribbing to prevent air lock.

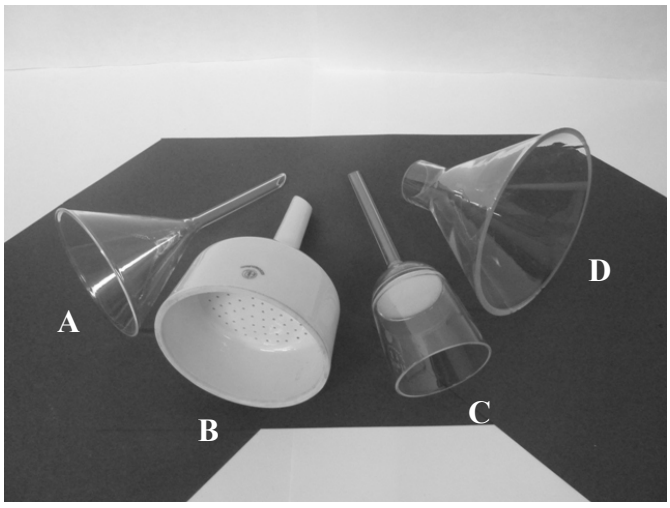


Figure 6: Funnels: A – filter funnel, B – Büchner funnel, C – fritted glass funnel, D – powder funnel.

Other types of laboratory equipment are summarized in Figure 7 and Figure 8. These include, for example *watch glasses* used in chemistry as a surface to evaporate a liquid, to hold solids while being weighed, for heating a small amount of substance and as a cover for a beaker.

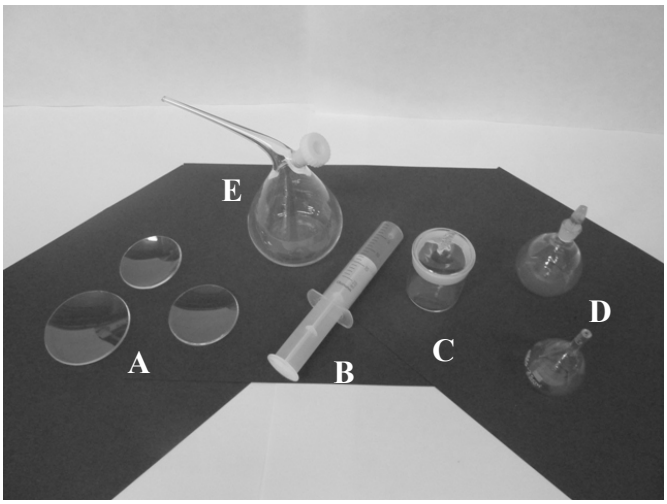


Figure 7: Other tools used in the laboratory: A – watch glasses, B – syringe, C – weighing bottle, D – pycnometers (specific gravity bottles), E – retort flask.

Pycnometer is a laboratory device used for measuring the volume and the density of solid objects in a non-destructive manner.
A retort flask is a convenient round bottom flask with a built-in air condenser used fot distilling substances placed inside and subjected to heat.

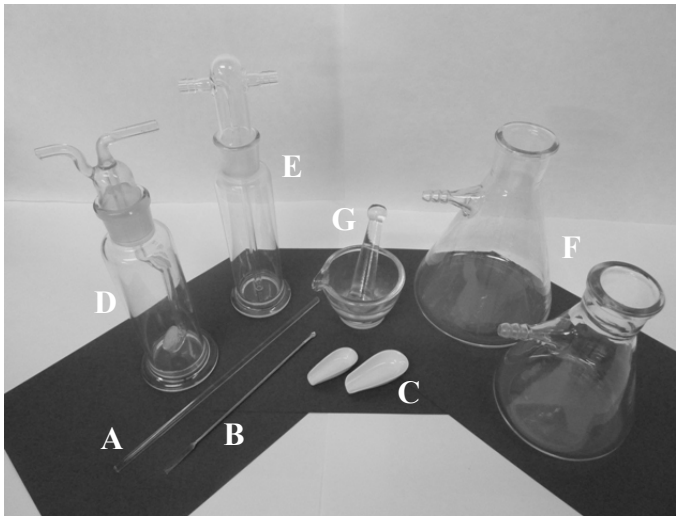


Figure 8: Other tools used in the laboratory: A – glass stirring rod, B – spatula, C – weighing boats, D, E – gas wash bottle, F – vacuum filtering flask (Büchner) with side arm socket, G – mortar and pestle.

Gas wash bottles are commonly used to dissolve gasses in a liquid or remove gaseous by-products of a reaction.
A *vacuum filtering flask*, also known as a Büchner flask, is a type of laboratory glassware used for vacuum filtration. It consists of a flat-bottomed flask with graduated volume

markings and a sidearm fitted with a vacuum adapter that allows for a vacuum pump to be attached.
Mortar and pestle are used in a chemical laboratory for grinding and crushing solid substances into powders.

1.5. Heat sources in the chemical laboratory

In laboratories where gas is introduced, we can use a gas burner as a heating source. A **burner** is a simple device used to mix air with gas. The resulting mixture burns and creates a flame at the end of the tube. If sufficient air is supplied to the gas, it burns with a *non-luminous flame*. This flame is more heating than a *luminous flame*, which we can obtain by preventing the air supply (see Figure 9). We always light the burner with the air supply closed.

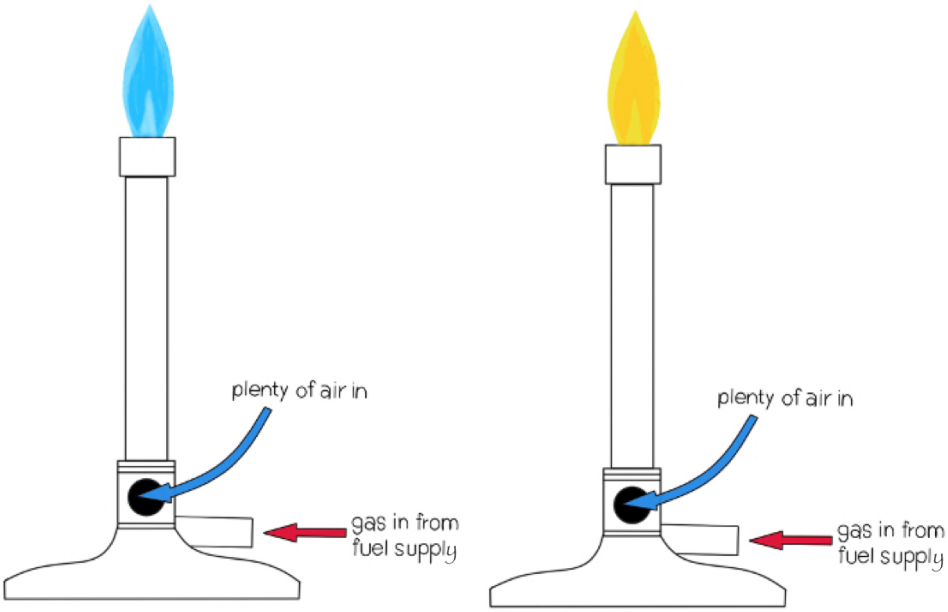


Figure 9: Types of burner flames.

In the laboratory, it is also possible to use an **alcohol burner** (spirit lamp), which has a lower calorific value (the amount of heat energy present in the fuel and which is determined by the complete combustion of a specified quantity at constant pressure and in normal conditions) than a gas burner. It is mainly used for test tube reactions and the sterilization of instruments intended for work with microorganisms.
Currently, **electric cookers** and **heating mantles** are used as the primary heat sources in chemical laboratories. An asbestos mesh (wire mesh gauze) is always placed on the electric cooker. Heating mantles are used to heat distillation flasks. It is an electric cooker in which a particular fabric is electrically heated and shaped into a hemisphere to cover the distillation flask. Care must be taken when working with a heating mantle so that the liquid does not enter the heating mantle.
Since any direct heating of glass vessels such as beakers or flasks places high demands on the properties of construction materials, transferring heat from the heating source through a bath is advantageous. According to the material forming the basis of the bath, it can be divided into air, water, oil, sand, metal, and salt.

- *Air baths* are used sparingly because air is the least conductive of all materials.

- The most frequently used is a *water bath*, which is suitable for heating substances up to the boiling point of water and for distilling liquids with a boiling point of approximately 80 °C.
- *Oil baths* are most commonly used to heat substances above the boiling point of water. The filling of an oil bath can be either mineral oil, which can be used up to a temperature of 250 °C, or silicone oil, which can be used up to temperatures of around 400 °C.
- *Salt* and *metal baths* are used at temperatures above 300 °C. A mixture of several salts is used, e.g., sodium and potassium nitrate with a melting point of 219 °C and low solidification alloys such as Wood's metal (an alloy of four metals, namely tin, lead, bismuth, and cadmium) with a melting point of 65 °C.
- *Sand baths* are no longer commonly used nowadays.

We use **electric resistance furnaces** for annealing and melting large quantities of substances. We use **tube furnaces** for syntheses in a gas stream or reactions in a vacuum, **crucible furnaces** for annealing in crucibles, and **crucible furnaces** for melting larger amounts of substances.

1.6. Vacuum and its sources

In laboratory practice working under reduced pressure is very often necessary. Reduced pressure (**vacuum)** is mainly used during distillation, filtration, or drying, i.e., in processes in which the volatility of substances is significantly increased due to reduced pressure, or the flow of liquids is directed and accelerated by removing the gas phase. A vacuum is a state of closed space in which the gas or vapor pressure is lower than the atmospheric pressure of the surrounding environment.

The device that creates the vacuum is called a **vacuum pump**. In the laboratory, we encounter vacuum pumps of several types, which differ in the pressure against which they pump, the level of vacuum that needs to be obtained, and the suction capacity. The simplest type of vacuum pump is a **water jet pump**. It is a narrow tube through which a stream of water flows. At the proximity of the nozzle mouth, the air is entrained in the direction of the water flow. While a discharge hose is connected to the tube, the other end is connected to the evacuated apparatus. The water jet pump, therefore, works directly against the atmospheric pressure. A safety container is always placed between the water jet pump and the vacuum device. If there is a drop in water pressure in the water pipe, water can be sucked from the vacuum pump into the vacuumed equipment. In addition to water jet pumps, we also know **rotary oil jet pumps** or **mechanical jet pumps**.

When working with a water jet pump, the main rule is to allow air back into the system after finishing work, and only then can the vacuum source be turned off. This will prevent water from entering the equipment or aggressive substances from being sucked into the vacuum cleaner and damaging it.

1.7. Temperature measurement

International temperature scales defined by water's freezing and boiling points were used to measure temperature. Other fixed points on the international scale are the boiling point of sulphur at 444.6 °C, the melting point of silver at 960.8 °C, and the melting point of gold at 1062.4 °C. All these temperatures are defined at the atmospheric pressure of one atmosphere (symbol: atm, one standard atmosphere is a unit of pressure equal to 101 325 Pa).

The temperature is measured by **thermometers**, which may be:

- *Liquid/gas dilation thermometers* use the expansion of liquid, solid, or gaseous substances and contain a capillary with a liquid or gas; the volume varies due to the temperature change. The oldest thermometers used are mercury in glass. Newer thermometers include non-mercury liquids in glass and digital and electronic devices that use a sensor to measure temperature.
- *Resistance thermometers* (resistance temperature detectors, RTDs) use the dependence of conductor resistance on temperature.
- *Thermocouples* (thermoelectrical thermometers) are based on the thermal effect (Seebeck effect) – two conductors made of two different metals connected at both ends. When the temperature changes, the thermocouple produces an electrical voltage directly proportional to the changed temperature.

1.8. Water in the biochemical laboratory

Tap water contains a high concentration of salts and is therefore unusable for any experiments in the biochemical laboratory. We only use it when it is not in contact with other reagents as a solvent (cooling or heating).

The primary method of water treatment is distillation. Each biochemistry laboratory is usually equipped with its distillation apparatus. **Distilled water** is sufficient for most biochemical operations, but its purity may not be sufficient for some particular purposes. Distilled water contains some cations from the components of the distillation apparatus, glassware, and/or electrodes. Its quality can be improved by repeated distillation, called re-distillation. Re-distillation is carried out in a special apparatus made of quartz glass which does not release cations.

For certain specific purposes, e.g., in the molecular biology laboratory, **deionized water** is used, which is then sterilized in an autoclave since even small contaminants in it can cause variations in the measured results. The preparation of deionized water is based on a combination of several separation methods that gradually remove individual groups of contaminants. The setup is usually based on sub-separators removing coarser impurities (filters), ions (ion exchangers), non-polar substances (adsorbent), and finally, a membrane filter. Some devices also work on the principle of reverse osmosis. However, the individual filters must be replaced after a few months of use, so the preparation of deionized water is costly.

The main criterion for the purity of water is its **specific conductivity**. For normal distilled water, this is approximately 10 $\mu S.cm^{-1}$. Prepared distilled or deionized water is usually stored in plastic containers. Glass containers are unsuitable for long-term storage because some cations are released back into the water. If conditions permit, the water is stored in a cool and dark place to avoid potential contamination by autotrophic organisms (organisms that synthesize organic matter from inorganic sources).

1.9. Chemicals and their storage

Industrially produced chemicals are mainly used as starting materials for work in the laboratory. The chemicals are delivered in a suitable package, so they need not be separated into additional packages. Suitable packaging is used, and the basic rules for storing chemicals are followed if necessary.

- Liquids are stored in a double-cap bottle (DualSeal).
- Hygroscopic substances are protected against atmospheric moisture by sealing the cap, e.g., with parafilm.

- Hydroxides and their solutions are not stored in vessels with ground glass stoppers.
- Light-sensitive substances are stored in dark containers in the dark.

The storage of chemicals in the laboratory has its fixed order, which is based on safety rules. Every chemical should have an identifiable storage place and should be returned to that location after use. Attention should be particularly oriented to flammable and explosive substances, which must never be stored together in large quantities. Poisons are kept in a special iron locker under lock and key. Attention is also paid to the storage of volatile substances in closed spaces (refrigerators), which should be stored so that their vapors are not released.

Chemicals are marked by the manufacturer with a label giving basic information about the chemical. The label provides the name of the chemical, its general formula, quantity, purity, and molecular weight. Chemicals present in the laboratory are considered hazardous if they are known to cause health problems, can instantaneously release pressure, if they can catch fire easily or are reactive. These chemicals have many needed uses but must be handled properly in order to avoid harmful side effects. Chemicals are classified based on their potential hazards. Since the adoption of Global Harmonization System (GHS), the hazard symbols or pictograms on chemical stock bottles have changed. Chemical manufacturers are required to identify potential hazards using GHS pictograms shown in Table 1**. Every student must know the meaning of these pictograms.**

Table 1: The hazard symbols on chemicals.

Pictogram	Name of pictogram	Pictogram	Name of pictogram
	**Explosive** - explosives - self-reactive - organic peroxides		**Corrosive** - skin corrosion/burns - eye damage - corrosive to metals
	**Fire Hazards** - flammables - pyrophoric - self-heating - emits flammable gas - self-reactive		**Acute Toxicity** - acute toxicity (fatal or toxic)
	**Oxidizer** - oxidizing gases, liquids, and solids		**Exclamation mark** - irritant (skin and eye) - skin sensitizer - acute toxicity (harmful) - narcotic effects - respiratory tract irritant

Table 1: The hazard symbols on chemicals (continued).

Pictogram	Name of pictogram	Pictogram	Name of pictogram
	**Compressed gas** - gases under pressure		**Health hazard** - carcinogen - mutagenity - respiratory sensitizer - reproductive toxicity - target organ toxicity - aspiration toxicity
	**Dangerous for the environment** (non-mandatory) aquatic toxicity		

Some important physical properties and safety information may also be listed. For solid substances, the given form is, e.g., crystalline (*crystalisatum*), anhydrous *(anhydricum*), or powdered (*pulveratum*). According to increasing **purity** (i.e., decreasing content of impurities), **chemicals are divided** into:

a. technical chemicals
   - raw (*crudum*)
   - technical (*technicum*)
   - pure (*purum*)

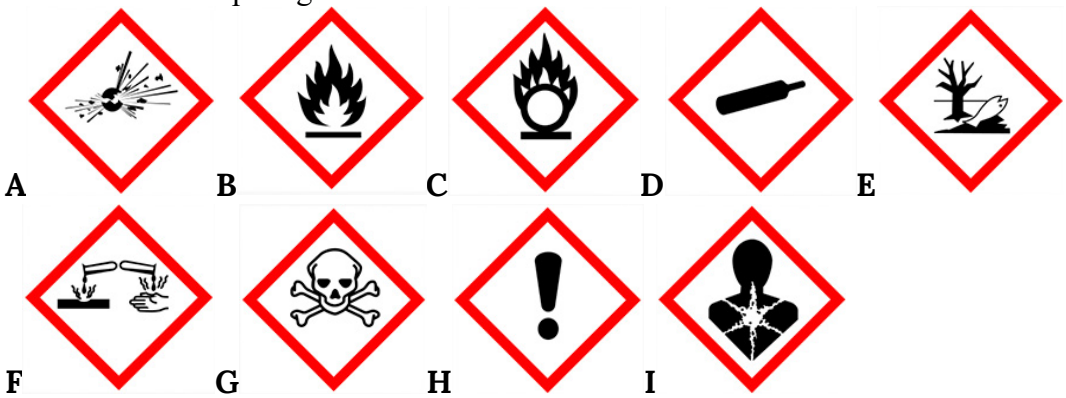
b. pure chemicals
   - purest (*purissimum*)
   - for analysis (p.a., *per analysis*)
   - chemically pure (*purissimum speciale*)

c. special chemicals - chemicals with higher purity are directly marked with the purpose for which they should be used, e.g., for UV spectrophotometry, molecular biology, HPLC analysis, etc. However, with the purity of chemicals, their price rises sharply.

Questions

1. What types of glass do we work with in the laboratory?
2. Give examples (3) of laboratory equipment made of porcelain.
3. Give examples (3) of laboratory equipment made of metals or their alloys.
4. How do we classify laboratory glassware according to its function?
5. Give examples (4) of graduated glassware.
6. How are graduated cylinders and pipettes calibrated?
7. What is a condenser? Name the three types of condensers.
8. Which condenser has the strongest cooling effect?
9. Give examples (4) of commonly used funnels.
10. What is a pycnometer?
11. What is the difference between a luminous and a non-luminous flame?
12. Name the types of baths used in the laboratory for heating.
13. What type of bath would you use to heat a solution with a boiling point of 150 °C? Explain.
14. What is a vacuum?
15. What is the pressure of one atmosphere? Express in kPa.

16. Name three types of thermometers used in the laboratory.
17. What is the difference between distilled water and deionized water?
18. What parameter determines the purity of the water?
19. Name the pictograms.



20. How do we classify chemicals according to their purity?

2. General laboratory procedures

2.1. Determination of weight and volume

2.1.1. Theory

Weight measurement

The biochemical laboratory uses the balances to determine the substances' weight (see Figure 10). The principle of weighing is based on a comparative method in which the unknown weight of a particular object (weighing boat, microtube with a sample) is compared with the known weight of a standard (weight).

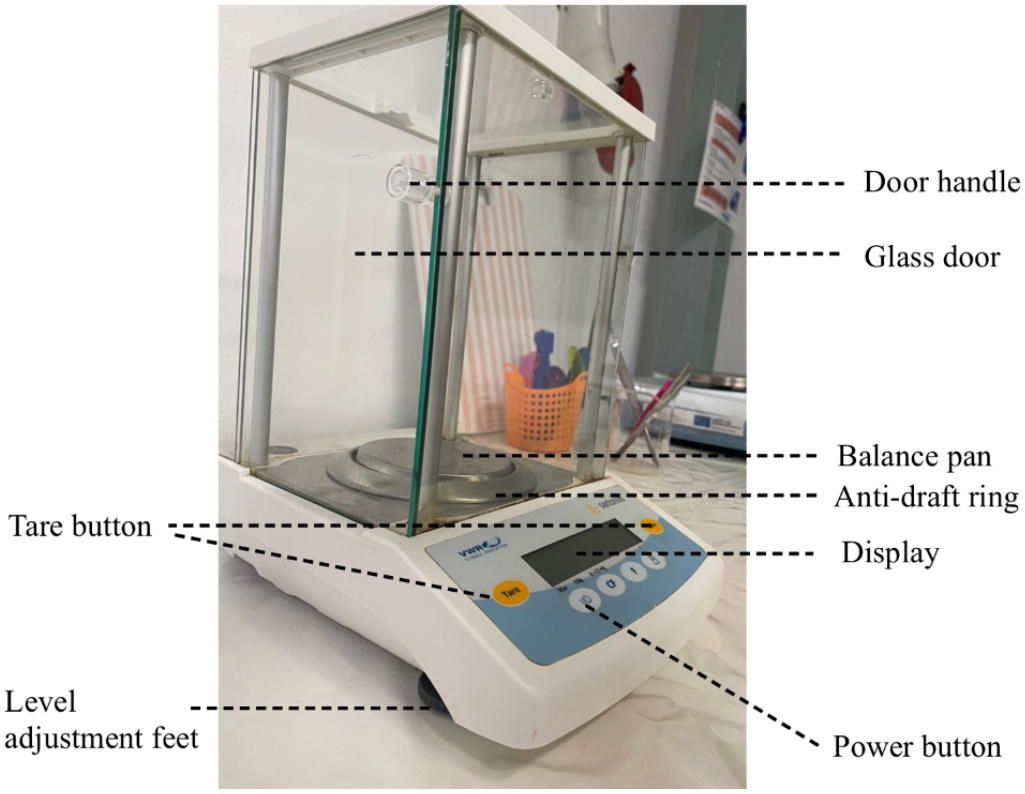


Figure 10: The analytical balance parts.

There are two different types of balance, which differ in maximum weighed mass (capacity) and accuracy of weighing:

1. **Technical balance** - depending on the model, they have a weighted mass (capacity) from 100 g to several kilograms. The accuracy is not more than 10^{-2} g.
2. **Analytical balance** - they usually have a weighed mass (capacity) of up to 100 g, and the accuracy of weighing is 10^{-4} g.

Pre-weighing scale is used to determine an approximate weight. They are used for weighing objects up to 200 g with a weighting accuracy of 0.1 g.

Most electronic and some optomechanical scales have a built-in tool for zeroing the balance (Tare button) after inserting the laboratory glassware to be used for weighing. It is not necessary to record the weight of this glass, but after inserting it, it is essential to let the balance stabilize and press the tare button. The balance is reset, and the required weight can then be weighed.

Basic rules apply to all balances:

- chemicals (liquid or solid) must not come into direct contact with the balance pan,

- handling of chemicals (addition and/or removal of the weighted sample) must be carried out outside the balance,
- any contaminant on the balance must be removed immediately with a brush. This will prevent damage to the balance and possible contamination of the following sample to be weighed.

Correct weighing procedure

1. Set the analytical balance to zero in the no-load condition by pressing the tare button.
2. Place a weighing boat, weighing paper, or another vessel in the center of the weighing pan and then close the glass door of the weighing chamber.
3. Check the value that was displayed after it was stabilized. To exclude the weight of the container from the measurement, press the tare button to reset the weight.
4. After removing the vessel from the balance, add the substance/chemical/sample to be weighed.
5. Wait 5 to 10 seconds (up to a minute) for the weight to stabilize, and then write down the value of the weighted object.

Volume measurement

Measuring cylinders are only used for the approximate measurement of liquids. For more precise volume measurement, **pipettes** (either for a specific volume/ undivided pipettes or divided pipettes) and **burettes** with the ability to regulate the liquid flow by tap or pressure are used. When filling pipettes, always ensure that the pipette's mouth is submerged below the liquid level. When it rises above the surface, the air is drawn into the pipette, which can also cause the pipetted liquid to enter the vacuum device (usually a rubber pipette bulb). Once the fluid has been drawn into the pipette above the mark indicating the desired volume, the upper end of the pipette is closed, and the liquid is then discharged dropwise. When reading its position on the scale, the eye should be on the same level as the mark. The curved surface of the liquid is called the meniscus. The bottom of the meniscus is always read (see Figure 11). Since the pipette is calibrated for "EX" (discharge), its content is never blown out.

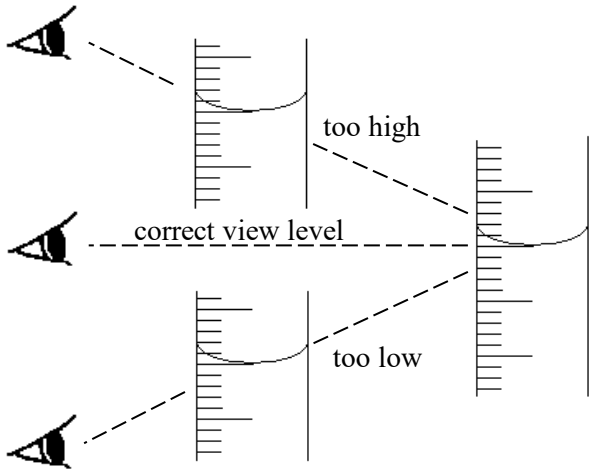


Figure 11: Reading the meniscus during pipetting.

Currently, **glass pipettes** are minimally used in biochemical or molecular biology laboratories. They have been entirely replaced by **automatic pipettes**, which allow more accurate and convenient measurement of a given volume (see Figure 12).

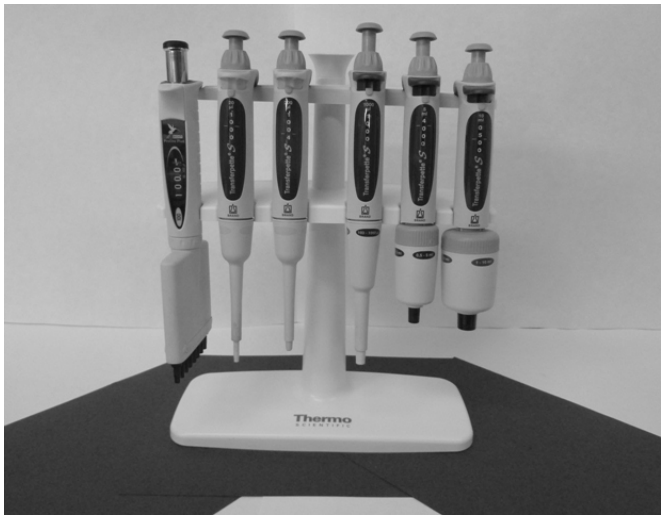


Figure 12: Multi-channel and single-channel mechanical pipettes.

Automatic pipettes are manufactured for different volume ranges (1-10 ml, 1-5 ml, 100-1000 µl, 20-200 µl, 2-20 µl and 0.2-2.0 µl). The volume is adjusted by turning the volume adjusting knob and displayed on the digital volume display (see Figure 13). The plunger is used to aspirate and dispense the desired volume of liquids.

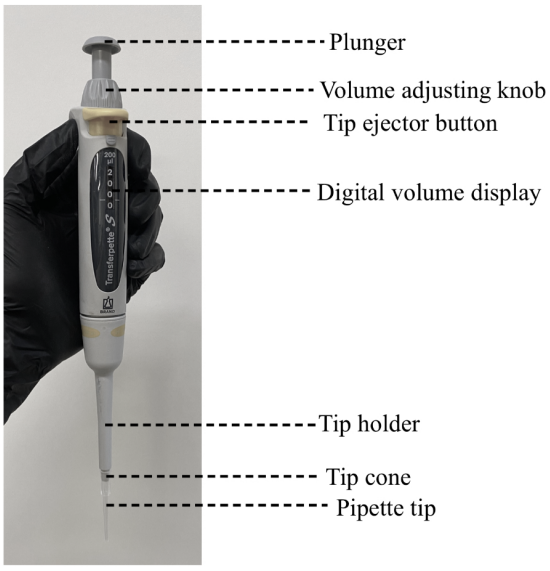


Figure 13: Parts of a single-channel pipette.

Pipettes are distinguished by their single-channel or multi-channel design. Single-channel pipettes are intended for pipetting one volume of liquid at a time, and multi-channel pipettes generally come with either 8 or 12 pipette heads, easily allowing a single device to fill multiple wells simultaneously. This enables the user to quickly and easily fill multi-well plates used in tissue culture, drug screening, or enzyme assays. Each channel in multi-channel pipettes has its piston, so using all channels at once is unnecessary. Hence it is possible to attach fewer than eight or twelve tips.

A plunger controls the aspiration and dispense of liquid through the attachable pipette tip. The plunger has three positions – rest position (ready position), first stop (first position), and second stop (second position).
The most commonly used **pipetting technique** is **forward pipetting**. In forward pipetting, a well-defined volume is aspirated into the tip, and in the next step, this volume is dispensed from the tip into the prepared vessel. The pipetting procedure is shown in Figure 14.
The forward pipetting procedure consists of several steps (see Figure 14):

1. Press the plunger to the first stop (1).
2. Dip the pipette tip into the solution to a depth of 1 cm and slowly release the plunger to the ready position (2). Wait 1-2 seconds and withdraw the tip from the liquid, touching it against the edge of the vessel to remove excess liquid.
3. Dispense the liquid into the receiving vessel by gently pressing the plunger to the first stop and then pressing the plunger to the second stop (3). This action will empty the pipette tip. Remove the tip from the vessel, sliding it up the wall of the vessel.
4. Release the plunger to the rest position (4).

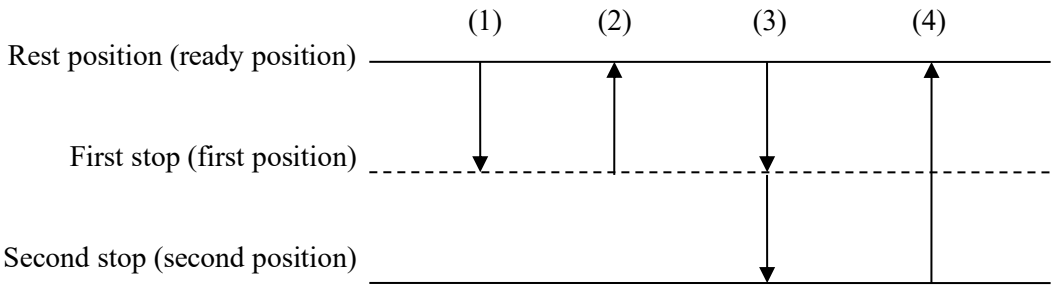


Figure 14: Positions of the pipette during **forward pipetting**. (1) pressing the plunger to the first stop (first position); (2) aspirating a given volume into the pipette tip while slowly (!) releasing the plunger; (3) dispensing the volume from the tip into the reaction mixture by pressing the plunger to the second stop (second position); and (4) slowly releasing the plunger and returning it to the rest position (ready position).

Pipetting small volumes (up to 50 µl) requires the use of the **reverse pipetting** technique. One drop has a volume of approximately 33 µl; therefore, even with the best pipette, it is impossible to dispense all liquid with a volume of less than one drop from the pipette tip. Especially with small volumes, this increases the relative error of your analysis using the forward pipetting technique.
The reverse pipetting procedure consists of several steps (see Figure 15):

1. Press the plunger of the pipette to the second stop (1).
2. Dip the pipette tip into the solution and slowly release the plunger to the ready position (2). Wait 1-2 seconds and withdraw the tip from the liquid, touching it against the edge of the vessel to remove excess liquid.
3. Dispense the liquid into the receiving vessel by gently pressing the plunger to the first stop (3). After pipetting, the remaining volume of liquid remains in the pipette tip.
4. Release the plunger to the rest position (4).

The relative error is significantly smaller with the reverse pipetting method than with the forward pipetting technique.

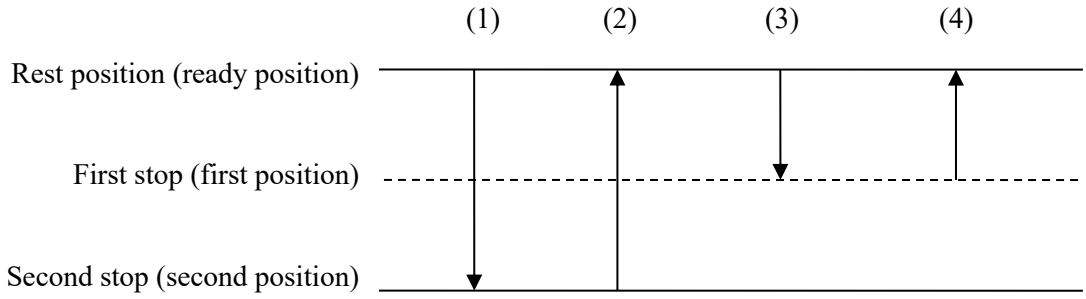


Figure 15: Positions of the pipette during **reverse pipetting**. (1) pressing the plunger to the second stop (second position); (2) aspirating a given volume into the pipette tip while slowly (!) releasing the plunger; (3) dispensing the volume from the tip into the reaction mixture by pressing the plunger to the first stop (first position); and (4) slowly releasing the plunger and returning it to the rest position (ready position).

2.1.2. Experimental part: Statistical analysis of experimental data

Principle

This laboratory exercise aims to learn how to weigh on technical and analytical balances and compare their accuracy in weighing different objects. In the second part of the work, we will focus on familiarizing ourselves with a set of single-channel pipettes and trying out the principles on which they work.

Errors occur randomly in every measurement. Their occurrence is irregular, and they are usually only minor deviations. The dependence of the probability of occurrence of errors on their absolute value is described by a **Gaussian curve**. The Gaussian curve (also known as the normal distribution) is a bell-shaped curve, and it is assumed that during any measurement, values will follow a normal distribution with an equal number of measurements above and below the mean value. The best estimate of the most probable value of the measurement is the **arithmetic mean $\bar{x}$** of the measured values:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

where x_i represents the ith value of variable x and n is the number of measurements.

The level of accuracy of a series of parallel results can be expressed as the **standard deviation (SD) σ** (sigma). SD is the dispersion of values around the arithmetic mean value (more precisely, half the width of the Gaussian curve at the inflection point). A low SD indicates that the values tend to be close to the arithmetic mean of the set, while a high SD indicates that the values are spread out over a wider range. A high SD usually means less precise measurements and errors during measurement.

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}}$$

The arithmetic mean and standard deviation have the same units of measure as the experimental values from which they were calculated.

Every measurement is burdened with errors due to imperfections in our observational skills, inaccuracies in instrumentation, defects in measurement technique, failure to adhere to experimental conditions, or the influence of unknown environmental

disturbances. This means physical quantities cannot be measured with absolute precision, so we use various statistical methods to get closer to the true value.
Errors can be divided into three main groups according to their origin:

1. **Gross errors** are caused by errors in measurement (analysis) or the evaluation of the result. Gross errors are errors that are so serious (large in magnitude) that they cannot be attributed to either systematic or random errors associated with the sample, instrument, or procedure. A considerable error value can cause inaccuracy and incorrectness of the result. Therefore, it is necessary to exclude the outlier from a data set (an outlier is a data point that is significantly different from another data point in a data set).
2. **Systematic errors** in repeated measurements made under the same conditions always distort the correct value of the measurand in the same way. Theoretically, these errors can be eliminated if they can be determined with more accurate instruments, and/or a correction can be introduced to refine the measurement method. In practice, however, this action is often difficult to implement. There are four types of systematic errors: observational (reaction time when measuring time data, limited discrimination ability of the eye), instrumental (an incorrectly calibrated or tared instrument), environmental (unpredictable changes in the experimental environment), and theoretical (simplifying conditions of the measurement method, approximation of the used relationships, inappropriateness of the used measurement method).
3. **Random errors** occur in every measurement and cannot be eliminated from the measurement. They manifest themselves in the fact that individual results differ from each other during repeated measurements, even under the same experimental conditions. This type of error is due to a large number of random environmental influences, the magnitude and origin of which cannot be accurately determined (an observer might make an error when measuring and recording a value that is too high, but because no one else was there when it was measured, the error went on unnoticed).

Experimental part A: Comparison of the accuracy of weighing objects on different types of balances

Laboratory requirements

Calculator, laboratory equipment (Eppendorf tube, beaker, weighing boat), technical balance, and analytical balance.

Procedure

Determine the weight of three identical objects (Eppendorf tube, beaker, weighing boat) successively on an analytical balance and a technical balance.
The procedure is as follows:

1. Set the zero value on the balance (use the tare button).
2. Weigh the selected object 5 times on the analytical balance. Record each weighed value of the object in your laboratory notebook. After each weighing, the balance must be set to zero value.
3. Weigh the selected object 5 times on the technical balance. Record each weighed value of the object in a laboratory notebook. After each weighing, the balance must be set to zero value.

Results
Calculate the arithmetic mean, standard deviation and compare the results with each other.

Experimental part B: Working with a single-channel pipette and statistical evaluation of results

Laboratory requirements
Single-channel pipette (variable volume 100-1000 µl), pipette tips, beakers, Eppendorf tubes (microtubes), and analytical balance.

Procedure
1. Pour distilled water into a beaker and prepare a series of labeled and **pre-weighed** Eppendorf tubes. Mark the tubes.
2. Pipette 1000 µl of distilled water into three Eppendorf tubes using a single-channel pipette, then pipette 2x 500 µl of distilled water into three more Eppendorf tubes and 10x 100 µl of distilled water into the last three Eppendorf tubes using a single-channel pipette.
3. Weigh the mass of all nine microtubes three times on an analytical balance, resetting the analytical balance each time to zero value. Record each weighted value of the Eppendorf tube with water in a laboratory notebook.

Results
Calculate the arithmetic means and standard deviations for aqueous solutions pipetted by different procedures (1x 1000 µl, 2x 500 µl and 10x 100 µl) and compare the results with each other.

Questions
1. How do we classify balances according to their accuracy of weighing?
2. State the difference between a measuring cylinder and a volumetric flask.
3. Define the following terms:
   a. forward pipetting
   b. reverse pipetting.
   Draw these techniques.
4. What pipetting technique would you use when pipetting 27 µl of the solution? Explain.
5. Calculate the arithmetic mean as well as the standard deviation for Eppendorf tubes marked Ep 2 - Ep 5. Write the results in the following table:

	Weight (g)					**Value (g)**	
Object	**I.**	**II.**	**III.**	**IV.**	**V.**	***Arithmetic mean (Average)***	***Standard deviation (SD)***
Ep 1	2.3267	2.3254	2.3247	2.3250	2.3253	2.325	0.001
Ep 2	2.3379	2.3382	2.3382	2.3380	2.3380		
Ep 3	2.3549	2.3550	2.3551	2.3351	2.3548		
Ep 4	2.3246	2.3247	2.3740	2.3247	2.3247		
Ep 5	2.3706	2.3704	2.3707	2.3706	2.3707		

6. Describe random, systematic, and gross errors.

7. Explain the terms:
   a. Gaussian curve,
   b. b. standard deviation,
   c. c. arithmetic mean.
8. What is the density of distilled water?

Example of calculation of arithmetic mean and standard deviation (SD)
You weighed the mass of the Eppendorf tube five times on an analytical balance and obtained the results shown in Table 2.

Table 2: Results of Eppendorf tube weight measurements on an analytical balance.

	Weight [g]				
Object	**I.**	**II.**	**III.**	**IV.**	**V.**
Eppendorf tube	2.3267	2.3254	2.3247	2.3250	2.3253

Calculation of the arithmetic mean:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} = \frac{(2.3267 + 2.3254 + 2.3247 + 2.3250 + 2.3253)}{5} = 2.3254 \text{ g}$$

Note: *n*- number of measurements.

Calculation of the standard deviation (SD):

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

$$\sigma = \sqrt{\frac{(2.3267 - 2.3254)^2 + (2.3254 - 2.3254)^2 + (2.3247 - 2.3254)^2 + (2.3250 - 2.3254)^2 + (2.3253 - 2.3254)^2}{5-1}}$$

$$\sigma = 0.0006$$

The arithmetic mean is 2.3254 g and SD is 0.0006 g. The standard way of writing the result is as follows: Eppendorf tube weighed 2.3254±0.0006 g.
The arithmetic mean and SD can also be calculated using the AVERAGE and STDEV functions in Microsoft Excel. For a detailed description of how to work with each function in Excel, see Attachment 4.

2.2. Spectrophotometry

2.2.1. Theory

Optical methods are used in the determination of many biochemically important substances. They are fast, sensitive, accurate, and, in addition, non-destructive, which means that after measurement, the solution can be further used and processed by other methods. Optical methods are based on the interaction of electromagnetic radiation with matter. Optical methods include:

- spectrophotometric methods based on the absorption or emission of radiation,
- methods based on light scattering, and
- polarimetric methods based on the measurement of the rotation of the plane of polarized light after interaction with optically active molecules (e.g., saccharides, most amino acids, etc.).

Absorption and emission

Absorption of radiation is a characteristic optical property of substances. When electromagnetic radiation of a suitable wavelength (see Figure 16) interacts with particles of matter (atoms and molecules), the absorbed energy (from a photon) is used to convert a molecule (atom) into an excited state (with higher energy than the ground state).

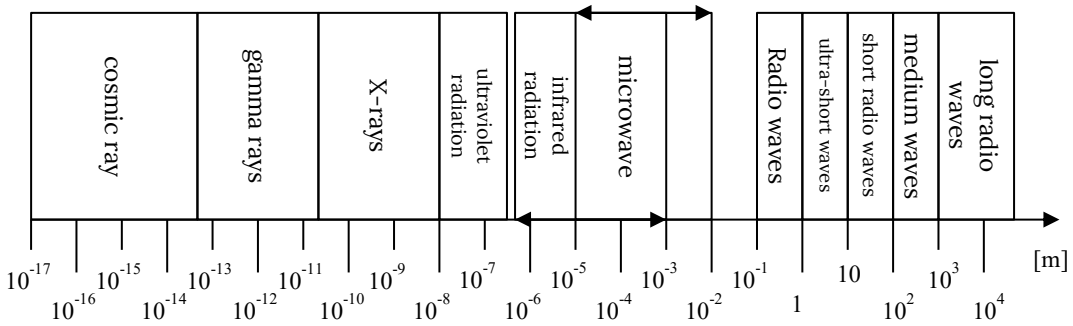


Figure 16: The electromagnetic spectrum.

Molecules or atoms absorb and emit radiation only at a certain frequency, which corresponds to the energy difference between the energy states (Planck's law state):

$$\Delta E = h.v$$

where v is the frequency (in reciprocal seconds – written s-1 or Hertz (Hz), 1Hz = 1 s^{-1}) and h is Planck's constant (6.6262 x 10^{-34} J.s).

Absorption of substances in the visible range of the spectrum (Electromagnetic radiation with a wavelength between 400 nm and 760 nm) leads to our eyes perceiving them as colored. The colored solution absorbs the light, which causes a part of the visible spectrum to disappear, and the rest of the reflected radiation represents the so-called complementary color (see Table 3), which we perceive as the coloring of the given substance (e.g., a solution that appears red absorbs light primarily in the blue-green portion of the visible spectrum and reflects).

Table 3: Visible light colors and complementary colors.

Absorbed wavelength λ [nm]	**Spectral color** (beam color)	**Complementary color** (color of solution)
400-435	violet	green-yellow
435-480	blue	yellow
480-490	blue-green	orange
490-500	cyan	red
500-560	green	purple (magenta)
560-580	yellow-green	violet
580-595	yellow-orange	blue
595-610	red-orange	green-blue
620-760	red	cyan

Estimation of the properties of a solution, e.g., the concentration of a compound, based on the absorption of light of a certain wavelength is called photometry. It is called spectrophotometry, where only one wavelength is not used, but rather the measurement is carried out over a specific range of the spectrum.

Beer-Lambert law

When a beam of radiation (light) passes through a solution capable of absorbing light, the intensity of the beam passing through this solution is less than the intensity of the entering beam. The amount of light that passes through a sample can be described with transmittance (*T).* Transmittance is defined as:

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

where I_0 is the intensity of light entering the sample (incident light), and I is the intensity of light that can pass through the material (transmitted light). Transmittance has no unit. The transmittance decreases exponentially with the path length (*l)* (the distance the light travels through the sample).

$$T = \frac{I}{I_0} = e^{-constant.l}$$

The Beer-Lambert law gives the relation between transmittance (T) and absorbance (A). According to Beer-Lambert law, the absorbance is formulated as:

$$A = -log\, T = -log \frac{I}{I_0} = \varepsilon_\lambda . c . l$$

where A is the absorbance, T is the transmittance, I is the intensity of the light passing through the solution, I_0 is the intensity of the incoming light, ε_λ is the molar absorption (extinction) coefficient, c is the concentration of the dissolved compound, and l is the path length (thickness of the absorbing layer, in practice, the thickness of the cuvette).

To determine the absorbance of a sample in a solution, I_0 is the absorbance of a **reference** (comparative) **solution** (also known as blank). The reference solution is usually the solvent used to dissolve the sample.

The molar absorption coefficient ε_λ is expressed as the absorbance of 1 mol/l solution of the measured substance at a given wavelength and a cuvette width of 1 cm. The value of the molar absorption (extinction) coefficient is usually tabulated, but it is a constant only under certain conditions. The most important of these is the requirement for a sufficiently low concentration of the dissolved compound in a solution.

The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a

fixed path length (cuvette length), UV/VIS spectroscopy can be used to determine the concentration of the substance in a solution. For this purpose, a **calibration curve** is used, which represents the dependence of absorbance at a certain wavelength on a known concentration of a substance. One should note that the Beer-Lambert law is obeyed by many substances mainly at low to moderate concentrations; therefore, dilute concentrations of the absorbing species should be measured. In practice, measuring absorbances in the range $0.1 < A < 1.0$ is advisable.

Photometry can also be used to distinguish between true and colloidal solutions. The **Tyndall effect** can be used for this purpose. The Tyndall effect is the effect of light scattering in colloidal dispersion while showing no light in a true solution. Tyndall effect can only occur on particles whose diameter is greater than a tenth of the wavelength of the light used ($\varphi > 0.1\ \lambda$). In laboratory practice, the Tyndall effect is used to determine the concentration of colloidal particles in solution by two methods:

- turbidimetry - turbidity measurement, measuring the intensity of a beam of light transmitted through the disperse system and
- nephelometry - measurement of the intensity of a beam of light transmitted through the sample.

In biochemistry, spectrophotometry is often used in the spectrum's ultraviolet (UV - ultraviolet; 200-380 nm) and visible (VIS - visible; 380-780 nm) ranges. The absorption of molecules in the visible and near ultraviolet regions is due to energy transitions of electrons of double and triple bonds and aromatic or heterocyclic rings. The functional groups responsible for the absorption of radiation are called chromophores. Almost all substances of biological interest (except saccharides) have characteristic absorption bands in the UV-VIS regions. They can be relatively easily quantified and qualitatively determined on the basis of their spectral properties.

Spectrophotometer

A spectrophotometer is an instrument for measuring light absorption as a function of wavelength. Therefore, the basis of the measurement is monochromatic radiation (electromagnetic radiation with a single constant frequency) or a photon stream with the same energy. In practice, monochromatic radiation is most often characterized by the wavelength λ, but its inverse – the wavenumber σ – or possibly the frequency ν can also be used. Frequency is the number of times a wave repeats in a sequence during a specific amount of time.

These quantities are related to each other by the following relationships:

$$\lambda \,.\, \nu = c \qquad\qquad E = h\nu = hc\sigma = \frac{hc}{\lambda}$$

where c is the speed of light ($2.9976.10^8$ m/s) and h is Planck's constant (6.6262×10^{-34} J.s).

The spectrophotometer consists of the following basic parts:

- *Light source* - three common broadband light sources used in UV and VIS regions are:
  - deuterium lamp – UV region (190-400 nm),
  - tungsten - halogen lamp - VIS region (320-1100 nm),
  - xenon Arc lamp – UV-VIS region (190-1100nm).
- *Monochromator* - comprises a dispersive element, an entrance slit and mirrors to create a parallel beam similar to sunlight, and an exit slit and mirrors to extract the monochromatic light (most often a combination of plane grating diffraction with prism refraction).

- *Cuvettes* are a type of vessel necessary for the precise spectral analysis of liquid samples. They must be transparent to the wavelengths used during the chemical analysis. In the instrument, a cuvette is placed in a cell holder.
    - Glass cuvettes have a decent transmission range from 340 to 2,500 nm. The glass performs well in spectrometers using VIS and NIR (near-infrared) light sources but will absorb light in the UV spectrum.
    - Quartz cuvettes have an extended transmission range from 190 to 2,500 nm, making them an appropriate choice for UV, VIS and NIR spectrums experiments.
- *The detector* is usually a photodiode or another photoelectric element.

Spectrophotometers measure light's wavelength distribution by providing the percentage of reflectance from an object, detecting the color of the sample. There are two types of beam spectrophotometers – single-beam and double-beam spectrophotometers.
Single-beam spectrophotometers first determine the reference sample (blank) and then the sample. The single light beam passes through the sample, and the single beam spectrophotometer measures the intensity of the light reflected from the reference (blank) to measure the sample (see Figure 17).

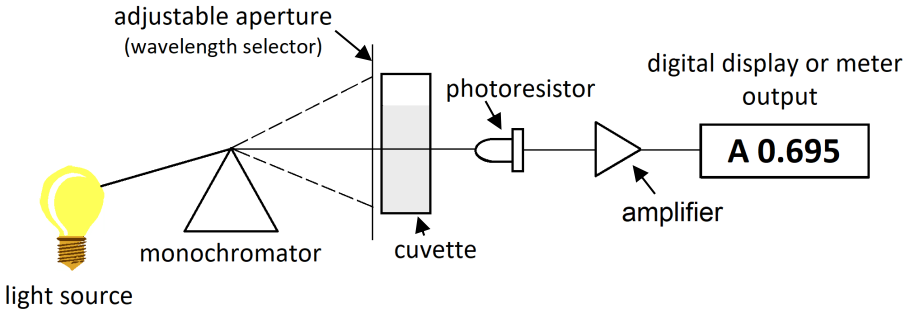


Figure 17: Schematic representation of the single-beam UV-VIS spectrophotometer.

Double-beam spectrophotometers measure color in a sample by using two or "double" beams. One beam passes through the sample, and the other passes through the reference (blank), so the reference and the sample can be read simultaneously without needing to recalibrate the instrument (see Figure 18).

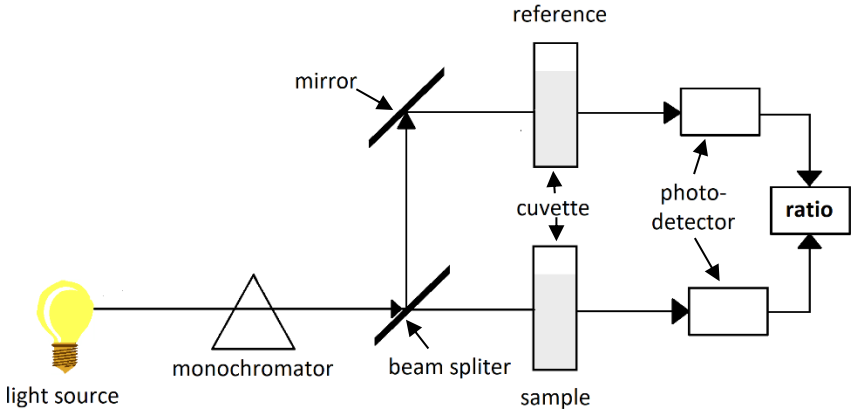


Figure 18: Schematic representation of the double-beam UV-VIS spectrometer.

The sample must be perfectly dissolved if we want to measure its absorbance in a spectrophotometer. If it is not perfectly dissolved, the undissolved parts present cause light scattering, which causes the light beam entering the sample solution to scatter and

not reach the detector. This may result in significant distortion effects on the measurements.
Spectrophotometric determination is usually done in a comparative manner because the value of the molar absorption coefficient of the substance to be determined at the selected wavelength is usually unknown. Therefore, a **standard solution** is also measured with the sample. A standard solution is a solution containing a precisely known concentration of an element or a substance. For the determination of proteins by the Lowry method, this is egg albumin, or for the determination of the concentration of reducing saccharides, this is glucose. However, it is not enough to prepare a standard solution (egg albumin, glucose, etc.) in only one concentration, but we have to use several concentrations of the standard. This procedure is referred to as the **calibration curve method**. In this procedure, the absorbance of these calibration solutions of the standard is measured in the same washed cuvette and at the same wavelength. The validity of the Beer-Lambert law can be verified from the obtained dependence of absorbance on concentration. The resulting dependence (the dependence of absorbance on the concentration of the standard) should be a straight line, which we call the **calibration curve** (see Figure 19).

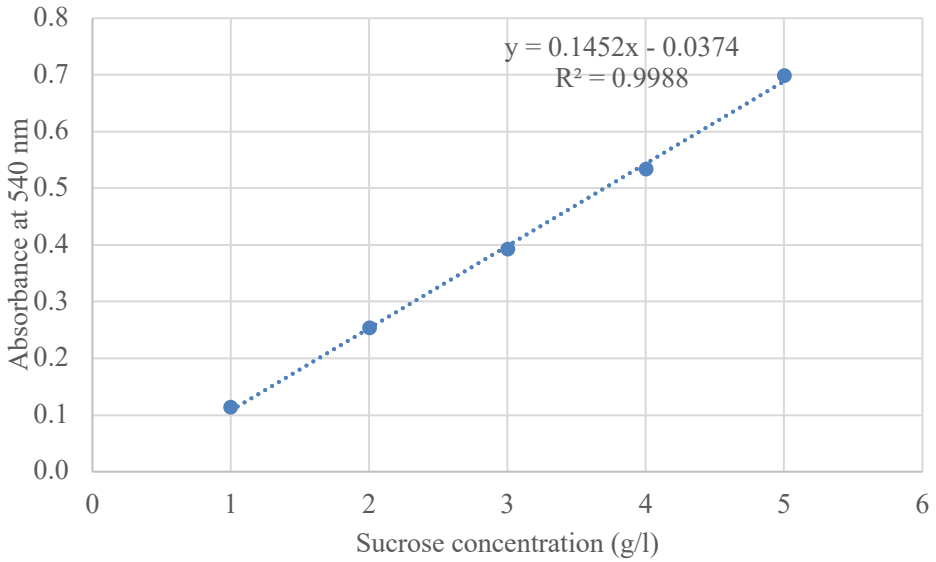


Figure 19: The dependence of absorbance at 540 nm on the concentration of the standard (sucrose).

The concentration of the solution is an independent variable (we can change it independently of the experiment) and is therefore shown on the x-axis. Absorbance is a dependent variable (it depends on the concentration of the substance in the solution) and is shown on the y-axis. If the concentration range of the standard solutions is set appropriately, it can be assumed that the line connecting the points will be linear. This correlation can be described by a linear regression, which is defined by the relationship:

$$y = ax + b$$

where y is a dependent variable (e.g., absorbance), x is an independent variable (e.g., concentration), b is the point at which the line intersects the y-axis (the absolute value, corresponds to the background signal of the matrix), and a is the direction of the line (represents the slope of the line - depending on its value, the angle between the calibration line and the x-axis changes).
Coefficients a and b are constants within the given regression, and y and x are variables. In Figure 19, the dependence of y on x is linear, therefore, it can be described by linear regression (y = 0.1452x - 0.0374). The R^2 value is referred to as the **coefficient of**

determination. It provides a measure of how the model replicates well-observed outcomes. In a simplified way, the coefficient of determination tells how well the fitted line describes the measured dependence of absorbance on concentration.
MS Excel or Origin, a less linear regression calculator, is used to construct the linear regression calibration curve. The calibration line should contain at least five points, and whether it passes through the origin [0,0] depends on the absorbance of the reference solution.

Example of calculating the unknown concentration of a substance using the calibration curve method
The DNS method was used to determine the glucose in the sample and the prepared solution's resulting absorbance was 0.513. Glucose solutions with a 2.5-15.0 g/l concentration were used as standard solutions. The absorbances of the glucose solutions prepared by the DNS method from the calibration solutions (standard solutions) measured at a wavelength of 540 nm are summarized in Table 4.

Table 4: Absorbance of glucose solutions prepared within the DNS method from standard solutions, measured at 540 nm in three parallel measurements.

Glucose concentration (g/l)	Absorbance at 540 nm			Average (Arithmetic mean)
	I.	II.	III.	
2.5	0.126	0.145	0.137	0.141
5.0	0.278	0.284	0.282	0.281
7.5	0.427	0.424	0.413	0.422
10.0	0.560	0.569	~~0.712~~	0.564
12.5	0.739	0.719	0.720	0.719
15.0	0.844	0.859	0.867	0.857

Plot the data with absorbance on the y-axis and concentration on the x-axis (see Figure 20-A). Examine the plot. The calibration curve looks linear, use statistical software to fit the data to a linear regression and obtain a coefficient of determination (see Figure 20-B).

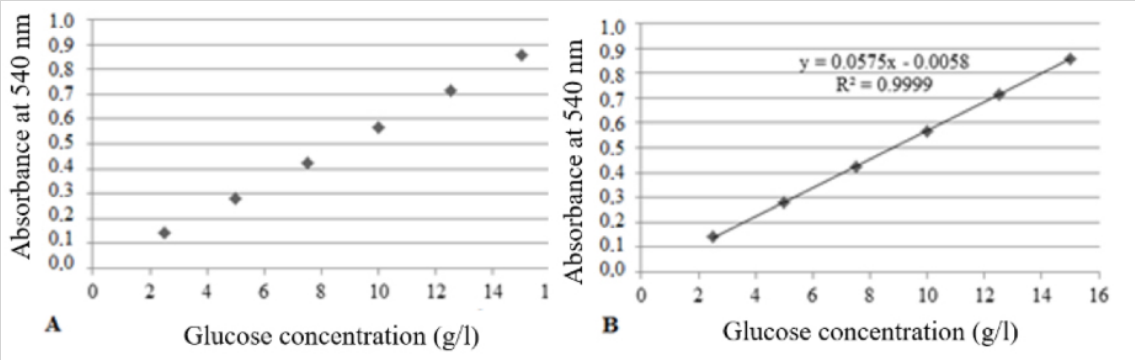


Figure 20: Dependence of absorbance at 540 nm on glucose concentration (g/l).
A – plotting the results in a graph and B – fitting a set of data using linear regression.

The equation describing the linear regression: $y = 0.0575x - 0.0058$, the value of x (concentration) is expressed:

$$y = ax + b \rightarrow x = \frac{y-b}{a} = \frac{y-(-0.0058)}{0.0575} = \frac{y+0.0058}{0.0575}$$

The sample with unknown glucose concentration had an absorbance of 0.513. Substitute this absorbance (y) into the linear regression equation for the absorbance of calibration solutions of glucose of known concentration:

$$x = \frac{0.513+0.0058}{0.0575} = 9.02\ g/l$$

The glucose concentration in the sample was found to be 9.02 g/l. The detailed procedure for preparing the calibration curve in Excel is given in Attachment 4 (Part C).

Work with a spectrophotometer

Spectrophotometry is an experimental technique used to measure the concentration of solutes in a solution by calculating the amount of light absorbed by these solutes. A spectrophotometer is a device used to analyze solutions in laboratory research (see Figure 21).

Figure 21: Parts of a spectrophotometer.

The steps in working with the spectrophotometer are as follows:

1. Press the power button (usually located on the back of the instrument} to the ON position.
2. Allow the device to warm up for 10 minutes before use.
3. Enter the desired wavelength.

4. Prepare a blank (usually distilled water or other solvent) in a cuvette.
5. Wipe off your cuvette containing the blank solution with a soft cloth (cotton wool) and place it in the cuvette holder. Make sure that the cuvette is aligned with the light source. Be sure to have the clear faces of the cuvette facing towards the front of the machine.
6. Close the sample door of the spectrophotometer.
7. Press the keyboard's AUTO ZERO button (or ENTER, depending on the instrument). This procedure sets the absorbance to 0.
8. Remove the blank. Wipe off the cuvette containing the sample with a soft cloth (cotton wool), insert it in the cuvette holder, and close the sample door.
9. Read the absorbance displayed and record it.

2.2.2. Experimental part: The relationship between the concentration of a substance in solution and its absorbance

Principle

The work aims to prepare a calibration curve of the dependence of absorbance on the concentration of Malachite Green dye (see Figure 22) and to calculate the molar absorption (extinction) coefficient of this dye.

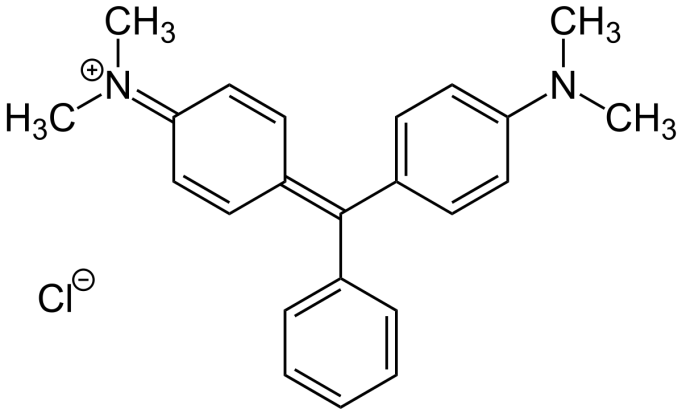


Figure 22: The structure of Malachite Green dye.

Laboratory requirements

Single-channel pipette (20-200 µl, 100-1000 µl), pipette tips, beakers, Eppendorf tubes (microtubes) (1.5 ml), volumetric flasks (100 ml), analytical balance, and spectrophotometer.

Materials and chemicals

- The stock solution of Malachite Green dye with a concentration of 150 mg/l (M = 364.911 g/mol, V = 100 ml).

Procedure

1. Prepare a stock solution of the dye with a concentration of 150 mg/l by weighing out 15 mg of the solid sample, transferring the quantitative to a 100 ml volumetric flask, and diluting to the mark with distilled water.
2. Through appropriate dilutions of the stock solution of the dye, prepare 9 calibration solutions as follows:

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
20.0	7.5	1.333	8.667
17.0	8.8	1.137	8.863
15.0	10	1.000	9.000
12.5	12	0.833	9.167
10.0	15	0.667	9.333
7.5	20	0.500	9.500
5.0	30	0.333	9.667
2.5	60	0.167	9.833
1.0	150	0.067	9.933

Prepare the dilute solutions (calibration solutions) in glass tubes. Dilute the stock solution of Malachite Green dye and distilled water using a one-channel pipette (see Figure 23). The total volume of dilute solution is 10 ml.

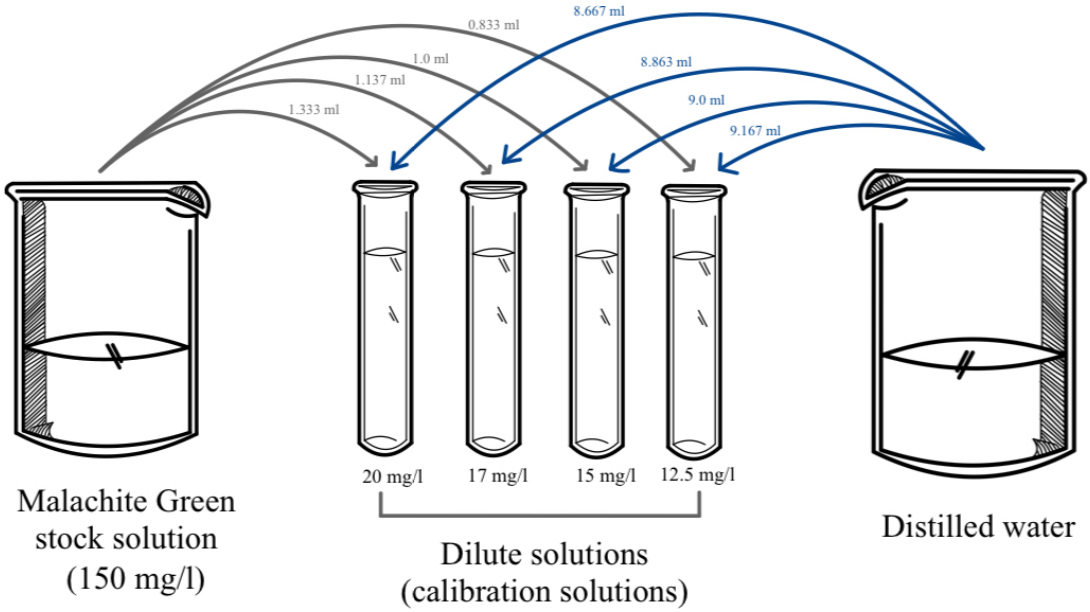


Figure 23: Simple dilution of a stock solution of Malachite Green dye to obtain a solution with a lower concentration (a dilute solution).

3. Then measure the absorbance of each calibration solution at 600 nm in three parallel measurements.
   a. Prepare a blank (distilled water) in a cuvette.
   b. Wipe off your cuvette containing the blank solution with a soft cloth (cotton wool).
   c. Place it in the cuvette holder.
   d. Close the sample door of the spectrophotometer.
   e. Press the AUTO ZERO button on the keyboard.
   f. Remove the blank. Wipe off the cuvette containing the sample (Malachite Green dye calibration solutions in the concentration range of 1-20 mg/l) with a soft cloth (cotton wool), insert it in the cuvette holder, and close the sample door.

4. Record the results in your laboratory notebook.

Results

Calculate for each calibration solution the average of absorbances and produce a plot of absorbance vs. actual concentration (the x-y relationship). Carry out a linear regression analysis to determine the equation of the relationship between absorbance and concentration ($y = ax + b$) as well as the coefficient of determination. Calculate the dye's molar absorption (extinction) coefficient and compare it with the literature.

Example of molar absorption (extinction) coefficient calculation

We use the Beer-Lambert law to calculate the extinction coefficient.

$$A = \varepsilon_\lambda . c . l \rightarrow \varepsilon_\lambda = \frac{A}{c . l}$$

It is evident from the equation that the calculation requires knowledge of the absorbance of the substance in a solution, its molar concentration, and the path length of the cuvette. The mass concentration of Malachite Green dye (M = 364.911 g/mol) was 20 mg/l (0.02 g/l) and the absorbance of this solution measured at 600 nm was 2.012. The path length of the cuvette (the thickness of the cuvette) was 1 cm. First, we need to convert the mass concentration (c_m) to molar concentration (c).

$$\textbf{\textit{I.}}\ c = \frac{n}{V}$$

$$\textbf{\textit{II.}}\ n = \frac{m}{M}$$

$$\textbf{\textit{III.}}\ c_m = \frac{m}{V} \rightarrow m = c_m . V$$

$$\textbf{\textit{IV.}}\ n = \frac{c_m . V}{M}$$

$$\textbf{\textit{V.}}\ c = \frac{\frac{c_m . V}{M}}{V} = \frac{c_m . \cancel{V}}{M . \cancel{V}} = \frac{c_m}{M}$$

$$\textbf{\textit{VI.}}\ c = \frac{0.02\ g/l}{364.911\ g/mol} = 5.48 . 10^{-5}\ mol/l$$

Mass concentration of Malachite Green dye is 20 mg/l is molar concentration of this solution is $5.48.10^{-5}$ mol/l.

Then we calculate the molar absorption (extinction) coefficient:

$$\varepsilon_\lambda = \frac{A}{c . l} = \frac{2.012}{(5.48.10^{-5}\ mol/l).\ 1\ cm} = 36{,}710\ \text{l.mol}^{-1}\ \text{cm}^{-1} = 36{,}710\ \text{M}^{-1}\text{cm}^{-1}$$

Malachite Green dye's molar absorption (extinction) coefficient is 36,710 $M^{-1}cm^{-1}$.

Questions

1. What optical methods are used in biochemistry? Describe their principle.
2. Define the excited state of a molecule (atom).
3. What does the Beer-Lambert law represent? Write the relationship between absorbance and concentration and define each variable.
4. Define the molar absorption (extinction) coefficient. In what units is it given?
5. What is the reference solution used for?
6. What reference solution would you use for the following solutions:
    a. violet 7 acid solution dissolved in distilled water,
    b. albumin dissolved in the saline solution,
    c. methanol solution of phenol red?

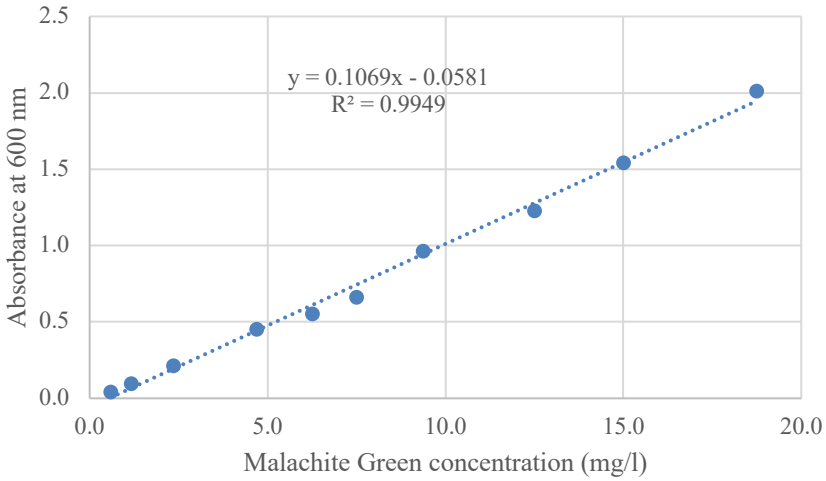
7. How do you use photometry to distinguish a true suspension solution from a colloidal solution?
8. Write the wavelength range for the UV and VIS regions.
9. What is the purpose of a spectrophotometer? What are the main parts of a spectrophotometer?
10. What is the meaning of monochromatic radiation?
11. Why are glass cuvettes not suitable for UV absorbance reading?
12. What are turbidimetry and nephelometry?
13. What is the calibration curve method?
14. State the linear regression equation and explain the symbols.
15. What is a chromophore?
16. How much Malachite Green should you use to make 100 ml with a concentration of 150 mg/l?
17. The stock solution of Malachite Green with a concentration of 150 mg/l should be diluted to a concentration in the range of 1-20 mg/l. Calculate the dilution factor, the volume of stock solution and the volume of distilled water needed to produce a solution of a given concentration. The total volume of dilute solution must be 10 ml.

Concentration (mg/l)	**Dilution factor (x)**	**Total volume of dilute solution**	
		Volume of stock solution (ml)	**Volume of distilled water (ml)**
20.0			
17.0			
15.0			
12.5			
10.0			
7.5			
5.0			
2.5			
1.0			

18. Convert the mass concentration of Malachite Green dye (M = 364.911 g/mol) to molar concentration.

Mass concentration (mg/l)	**Molar concentration (mol/l)**
20.0	
17.0	
15.0	
12.5	
10.0	
7.5	
5.0	
2.5	
1.0	

19. A sample containing an unknown concentration of Malachite Green has an absorbance of 0.542. Calculate its Malachite Green concentration using the linear regression equation in the figure.



20. Calculate the concentration of the synthetic dye Phenol Red if you know that its absorbance is 0.754, the path length of the cuvette is 1 cm, and the extinction coefficient at 480 nm is equal to 4460 $M^{-1}cm^{-1}$.

2.3. Buffer solutions and their capacity

2.3.1. Theory

A **solution** of a certain concentration is prepared either by weighing the exact amount of the given substance and adding the solvent to the precise volume. Alternatively, if a stock solution of the substance is available at a higher concentration than required, it should be prepared by diluting the stock solution.

There are different ways of expressing the concentration of solution (molar concentration, mass concentration, weight fraction (*w/w*), volume fraction (*v/v*), weight volume fraction (*w/v*), molarity (M), normality (N)).

Molar concentration (***c***) measures the concentration of a chemical species, particularly a solute in a solution, in terms of the amount of substance per unit volume of solution.

$$c = \frac{n_A}{V} = \frac{m_A}{V.M_A} \text{ [mol/l]}$$

where n_A is the amount of the solute in moles (amount of substance), V is the volume of the solution (in liters), m_A is the mass of solute (in grams), and M_A is the molar mass of solute (in grams to moles).

Mass concentration (***c_m***) is defined as the mass of dissolved substance per unit volume of solution.

$$c_m = \frac{m_A}{V} \text{ [g/l]}$$

where m_A is the substance's mass (in grams), and V is the solution's volume (in liters).

Weight fraction (***w***) is the number of grams of solute dissolved in 100 grams of solution**.**

$$w_A = \frac{m_A}{m}.100\ \%\ [\%\ (w/w)]$$

where m_A is the mass of solute (in grams) dissolved in 100 g of the solution (m).

Volume fraction (***φ***) is defined as number of milliliters of solute dissolved in 100 ml of solution.

$$\boldsymbol{\varphi}_A = \frac{V_A}{V}.100\ \%\ [\%(v/v)]$$

where V_A is the volume of the solute and V is the volume of the solution (both in liters).

Weight volume fraction is termed as how many grams of solute are dissolved in 100 ml of solution. A one percent solution (1 %, *w/v*) is defined as 1 gram of solute per 100 milliliters final volume. For example, 1 gram of sodium chloride, brought to a final volume of 100 ml with distilled water, is a 1 % (*w/v*) NaCl solution.

Molarity (M) is moles of solute contained in one liter of solution. Molarity is sometimes given by putting the formula of the substance in square brackets - the concentration of NaCl is given as [NaCl]. '1 molar aqueous solution' of NaCl is also often abbreviated to '1 M' NaCl. Solutions of the same molarity contain the same number of molecules of solute in one liter of solution; 1 mol of any substance contains a number of molecules equal to the Avogadro constant (6.023×10^{23}).

In the case of acids and bases, the term **normality** (***N***) can be found in the literature. This expression is based on the Arrhenius and Brönsted-Lowry theories of acids and bases. Normality enables an accurate expression of the concentration of hydrogen cations that can enter the acid-base reaction.

- In the case of monoprotic acids (which are able to donate one proton per molecule during the process of dissociation), the molarity value of the acid is equal to the normality of this acid because the concentration of molecules of a substance is exactly equal to the concentration of hydrogen cations.

  1 M (HCl) = 1 N (HCl)

- In the case of polyprotic acids, the normality is higher by a factor reflecting the amount of hydrogen cations in a given acid molecule.

$$1\ M\ (H_2SO_4) = 2\ N\ (H_2SO_4)$$
$$1\ M\ (H_3PO_4) = 3\ N\ (H_3PO_4)$$

If the concentration of a sulfuric acid solution is $c(H_2SO_4)$ = 1 mol/l (1 M), then its normality is 2 N. It can also be called a '2 normal' solution. Similarly, for a solution with $c(H_3PO_4)$ = 1 mol/l (1 M), the normality is 3 N because phosphoric acid contains 3 acidic hydrogen atoms.

To determine the concentration of a substance, knowledge of the relative molecular weight (M_r) is necessary. Therefore, mass concentration is often used for biologically active substances. The concentration of a substance, defined by weight or volume fraction, is essential always to indicate how a given percentage was calculated. This percentage can be determined in one of three ways: (1) the weight of the solute divided by the weight of the solution (*w/w*), (2) the volume of the solute divided by the volume of the solution (*v/v*), or (3) the weight of the solute divided by the volume of the solution (*w/v*).

When preparing solutions, the correct procedure is adding the solute to the solvent not vice versa (see Figure 24).

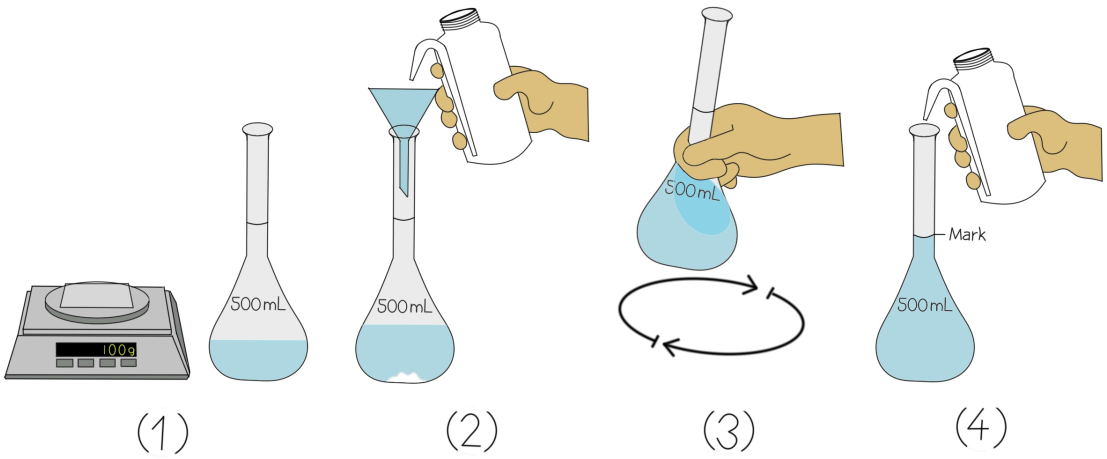


Figure 24: Preparation of a solution of known solute concentration.

To prepare a solution that contains a specified concentration of a substance, it is necessary to follow several steps.

(1) An amount of solute is weighed on an analytical balance, and then a portion of the solvent is added to the volumetric flask.
(2) Dissolve the desired number of moles of solute in enough solvent to give the desired final volume of solution. If the desired volume were 1.0 L, adding 1.0 L of water to 100 g of glucose would be incorrect because that would produce more than 1.0 L of solution. The solute occupies space in the solution; the solvent volume needed is almost always less than the desired volume of the solution.
(3) The mixture is swirled until all of the solute is dissolved.
(4) Additional solvent is added up to the mark on the volumetric flask. The flask is closed using a stopper. Mix the solution thoroughly by stoppering the flask securely and inverting it ten to twelve times.

In biochemistry, solutions with very low substance concentrations are often used. When working with a small amount of sample, the procedure is to weigh (accurately) the

approximate mass and calculate the volume of solvent that must be added to produce a solution of the exact concentration required.

The ion-product of water

In pure water and any solution, water molecules partially dissociate into H^+ (H_3O^+) and OH^-. In the equilibrium state, the dissociation of water is determined by the dissociation constant (K_w). The dissociation constant is defined as the ratio of dissociated ions (products) to the original acid or bases in the solution (reactants).

$$K_w = [H^+][OH^-]$$

The rate of water dissociation depends only on the temperature:

$$K_w^{25°C} \sim 10^{-14}\ mol^2 l^{-2}$$
$$K_w^{0°C} \sim 10^{-15}\ mol^2 l^{-2}$$

According to the ratio of $[H^+]$ and $[OH^-]$, the solutions are then divided into:

- an acidic solution in which the concentration of hydrogen ions is greater than the concentration of hydroxide ions ([H+] > [OH-]),
- a neutral solution in which the concentrations of hydrogen and hydroxide ions are equal ([H+] = [OH-]),
- a basic solution in which the concentration of hydroxide ions is greater than the concentration of hydrogen ions ([H+] < [OH-]).

The hydrogen exponent (pH)

The pH value is a measure of hydrogen ion activity. But in dilute solutions, the activity coefficient is a function of the ion concentration and approaches 1 as the solution becomes increasingly dilute. Therefore, hydrogen ion concentration and hydrogen ion activity are nearly equal in very dilute samples, then pH is defined as the negative logarithm of the hydrogen ion concentration:

$$pH = -\log[H^+] = -\log \frac{K_w}{[OH^-]}$$

The pH value of a solution is directly dependent on the temperature (e.g., for a neutral solution, $\mathrm{pH}^{25°C} = 7.0$ and $\mathrm{pH}^{0°C} = 7.5$). Every solution will change its pH value through temperature changes. If the pH falls as temperature increases, this does not mean that water becomes more acidic at higher temperatures. In the case of pure water, there are always the same concentrations of hydrogen ions and hydroxide ions, hence the water is still neutral (pH = pOH) - even if its pH changes.

Acids and bases

An acid is a substance capable of splitting off a hydrogen ion. A base is a substance capable of splitting off a hydroxyl anion (more specifically, taking up a hydrogen ion).

The strength of acids and bases can be characterized by the dissociation constant (K_D). The acid dissociation constant (K_A) and base dissociation constant (K_B) values represent how easily an acid or base dissociates into ions, in a solution.

There are two classes of acids and bases: strong and weak. Strong acids/bases ionize completely when dissolved in water. There are only a few strong acids as hydrochloric acid (HCl), hydrobromic acid (HBr), sulfuric acid (H_2SO_4), nitric acid (HNO_3), or perchloric acid ($HClO_4$). Examples of strong bases are sodium hydroxide (NaOH), lithium hydroxide (LiOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$), or barium hydroxide ($Ba(OH)_2$).

The degree of dissociation of a weak acid in water (e.g., acetic acid in water) is described by the acid dissociation constant (K_A).

$$CH_3COOH \leftrightarrow H^+ + CH_3COO^-$$
$$K_A = \frac{[H^+][CH_3COO^-]}{[CH_3COOH]} = 1.75.10^{-5}$$

$$pK_A = -\log K_A = -\log (1.75.10^{-5}) = 4.76$$

The larger the K_A, the more the acid dissociates (or reacts with water) to form H_3O^+ ions, and the stronger the acid. The smaller the K_A, the larger the pK_A. Stronger acids have the largest K_A but small pK_A values.

For hydrochloric acid:

$$HCl \rightarrow Cl^- + H^+$$

$$K_A = \frac{[H^+][Cl^-]}{[HCl]}$$

$$pK_A = -\log K_A$$

Below is a guide to identifying strong acids or bases based on their K_A/K_B and pK_A/pK_B values (see Table 5).

Table 5: Identifying the strength of an acid or base.

Type	K_A	pK_A
Strong acid	> 1	< 0 (negative)
Weak acid	< 1	> 0 (positive)
Type	K_B	pK_B
Strong base	> 1	< 0 (negative)
Weak base	< 1	> 0 (positive)

pH measurement

The measurement of the pH value is one of the basic tasks in chemical laboratories. Several methods are used to measure the pH value:

- spectral methods - the determination of the concentration of H^+ ions is based on the change in the structure of specific organic substances (acid-base indicators) and their spectral properties (absorbance or fluorescence) due to changes in the pH of the environment,
- electrochemical methods - are based on the change in electrochemical potential directly due to the concentration of H^+ ions.

Spectral methods

The simplest spectral methods for measuring pH are visual methods, where the indicator's color is monitored as a function of the change in pH. To determine the pH value, an indicator must be selected that changes color over a well-defined pH interval (see Table 6). These are suitable for indicating that the desired pH value has been reached, checking pH maintenance, etc.

Indicators may also be used to indicate the equivalence point in acid-base titrations. A titration is a procedure for carrying out a chemical reaction between two solutions by the controlled addition of one solution (the titrant) from the burette to the other, allowing measurements to be made through the reaction. In a reaction between an acid and a base, titration is useful for measuring the pH at various points in the reaction. A titration curve is a graph of the pH as a function of the amount of titrant (acid or base) added. The equivalence point of the titration is the point at which exactly enough titrant (a reagent prepared as a standard solution of known concentration and volume) has been added to react with all of the substance (a solution of the analyte, which may also be termed the titrand) being titrated. Reaching this point is the theoretical endpoint of the titration.

Table 6: Examples of acid-base indicators with their color and indication areas.

Indicator	The color of the indicator in the environment		Transition pH range
	acid	base	
Thymol Blue	red	yellow	1.2-2.8
Methyl Yellow	red	yellow	2.0-4.0
Methyl Orange	orange	yellow	3.2-4.4
Methyl Red	red	yellow	4.2-6.3
Bromothymol Blue	yellow	blue	6.0-7.6
Phenolphthalein	colorless	pink	8.2-10.0
Thymolphthalein	colorless	blue	9.4-10.6

The indicators are used in the form of an aqueous or ethanol solution (0.04-0.1 %, *w/v*), which is added to the sample to be monitored. pH indicators exist as liquid dyes and dye-infused paper strips. For versatile use, a universal indicator is used, made from a solution of several compounds that exhibit a variety of smooth color changes over a wide range of pH values.

Additionally, pH indicators are found in nature. Their presence in plants and flowers can indicate the pH of the soil from which they grow. These are anthocyanins, red and blue dyes, present in some flowers (e.g., rose, geranium, mallow) or vegetables (e.g., red cabbage). Anthocyanins are found as a glycoside that contains both sugar and non-sugar components. A mineral acid can hydrolyze them into one or more sugar portions (usually monosaccharides or disaccharides) and non-sugar moieties. The non-sugar moiety is called aglycone, whereas the sugar part is known as glycone. The anthocyanin turns red-pink in acids (pH 1-6), reddish-purple in neutral solutions (pH 7), and green in basic solutions (pH 8-14).

The color transition of the indicator can be more accurately monitored using spectrophotometric instruments. This also makes it possible to quantify the color transition when the pH changes (determination of the pK_A value). However, this method is not commonly used and is limited to exceptional cases (e.g., pH measurements inside cells and organelles or in non-aqueous solvents). When using fluorescent indicators, we are entirely dependent on measurements with fluorimeters. This method has no visual alternative.

Electrochemical methods

The most accurate measurement of pH is made with a pH meter. This is a potentiometric determination in which the pH is determined by measuring the potential of a suitable electrode. The most widely used method for measuring pH is the glass-electrode method. In this method, the known pH of a reference solution is determined by using two electrodes, a glass electrode and a reference electrode, and measuring the voltage (difference in potential) generated between the two electrodes. The difference in pH between solutions inside and outside the thin glass membrane (called the electrode membrane) creates an electromotive force in proportion to this difference in pH. The electrodes are inserted into the solution to be tested. The glass electrode and reference electrode can be constructed together in a single probe called as combined or combination electrode (see Figure 25).

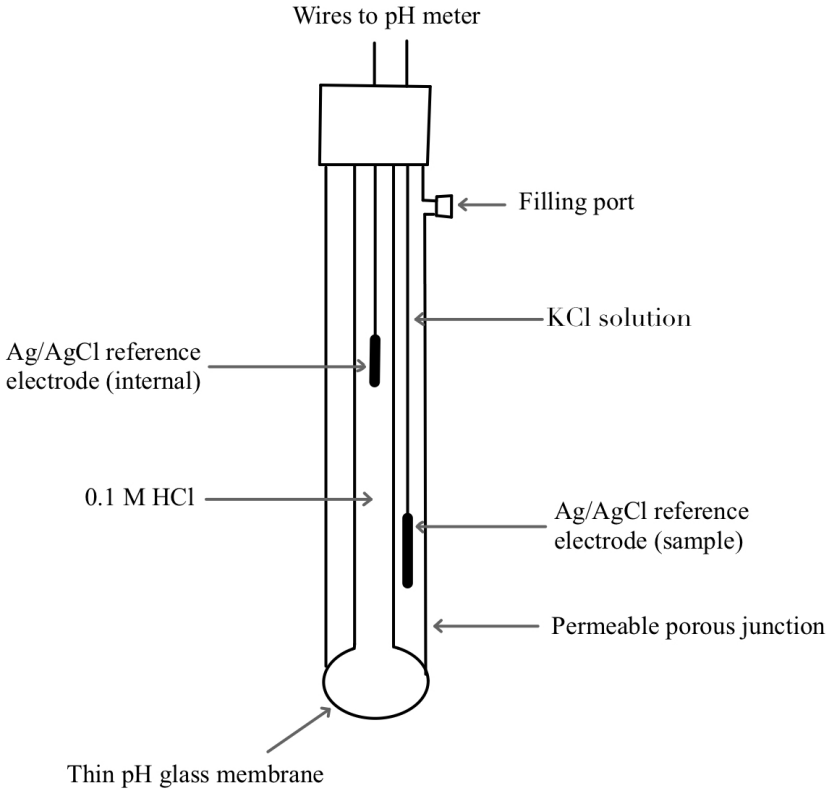


Figure 25: A schematic diagram of a combination glass pH electrode.

Most potentiometric methods employ one of two other common reference electrodes – the saturated calomel electrode (SCE) or the silver-silver chloride electrode (Ag/AgCl) (see Figure 26).

- The SCE is a half-cell composed of mercurous chloride (Hg_2Cl_2, calomel) in contact with a pool of mercury. These components are either layered under a saturated potassium chloride (KCl) solution or within a fritted compartment surrounded by the saturated KCl solution. A platinum wire is generally used to allow contact with the external circuit. A paste is prepared of Hg_2Cl_2, Hg, and saturated KCl solution. The solution over the paste is also saturated with KCl and the presence of some solid KCl crystals. Contact to the measurement cell is made through a porous glass frit or fiber, which allows the movement of ions, but not the bulk solution.
- Ag/AgCl is composed of a silver wire, sometimes coated with a layer of solid silver chloride, immersed in a solution saturated with potassium chloride and silver chloride.

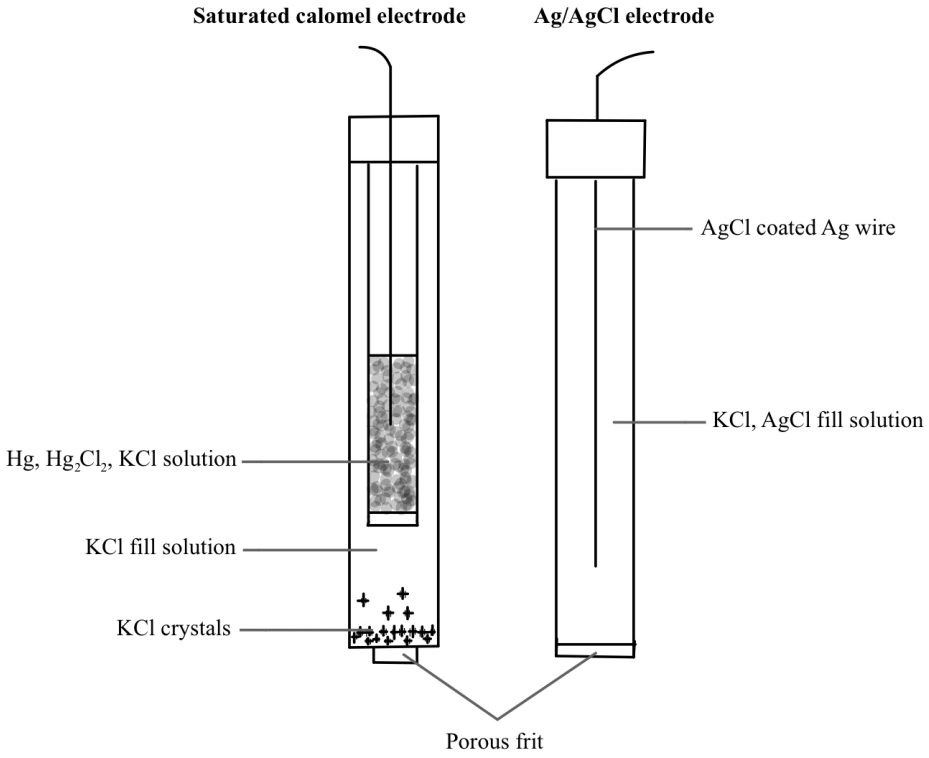


Figure 26: Common arrangement for saturated calomel and Ag/AgCl electrodes.

Using a pH meter

Follow the manufacturer's instructions when working with the pH meter. The general procedure and principles of work, especially the handling of the electrode, can be summarized in a few points:

I. Handle the electrode with care. The pH electrode is fragile, and one should not be tempted to use it as a stirring glass rod when adjusting pH. When using magnetic stirrers, take care to maintain an adequate distance between the pH electrode and the magnetic stirring pellet to prevent accidental breakage (check in advance). Be especially careful when measuring pH in a small volume of solution.

II. The pH electrode should never dry out. It is preserved by immersing it in a buffer solution with a pH at which it is most often used. Preserve it by immersing it in a buffer solution at the pH at which it is most commonly used. If not used daily, the evaporated water shall be checked and replenished. To prevent the solution from leaking and/or evaporating, the pH electrode should be stored in a 3 M potassium chloride (KCl) solution. If you will not be working with it for an extended period of time, it is better to rinse it with distilled water, dry it, and store it safely once dry.

III. The dry pH electrode (new or long stored) must be prepared according to the manufacturer's instructions before use. It is usually soaked for several hours in a hydrochloric acid (HCl) solution at a concentration of 0.1 mol/l.

IV. Before measuring the pH of the solution, the pH meter must be calibrated. The electrode is removed from the solution in which it is kept, rinsed with distilled water, the excess water drained off (with filter paper or cotton wool), and immersed in a standard buffer with a pH value closest to that of the solution to be measured (a set of standard buffers with pH 4.0, 7.0 and 10.0 is usually available). The reference electrode should be immersed at the same time. If a combination electrode is used, make sure that the side lead of the reference electrode is

completely submerged below the surface. The temperature correction is set to the buffer temperature, and the standard buffer's pH value is set on the scale or digital output using the calibration button. The electrode is then removed and, after rinsing with distilled water, immersed in the measuring solution. The pH value of the solution is displayed on the pH meter display. Calibration must be performed each time the instrument is switched on or if the samples' pH changes by more than 1.0. It is also checked occasionally during long-term measurements.

V. Some instruments allow or even require calibration to two or more buffers (a 2-point calibration, 3-point calibration, or more). In this case, it is possible to work over a wider pH range without always having to calibrate the instrument. Buffer solutions must be selected that limit the area in which to work. The default value is set to one of these. When the pH electrode is immersed in the second buffer, its value is set by another button that adjusts the potential guidance depending on the pH.

Once the pH meter has been successfully calibrated, it will be ready for testing. These are the necessary steps to ensure the most precise readings:

1. Switch on the pH meter.
2. The electrode should be washed with distilled/deionized water to clean it thoroughly and dried with a soft cloth (cotton wool) to prevent dilution of the test sample.
3. Then place the electrode in the solution, wait for the reading to stabilize, and take a reading.
4. Rinse the electrode, blot it dry, and switch it off.
5. After use, pH meters should ideally be kept in a suitable storage solution.

Buffers

In chemical practice, it is often necessary to set the pH of the solution and to ensure that this pH value does not change during the experiment. Solutions containing a weak acid or a weak base in combination with its salts are suitable for this purpose. These solutions are called buffer solutions or buffers. The value of the buffer is determined by the proportion in which the weak acid or base has been mixed with its salt. The Henderson-Hasselbalch equation is used to calculate the pH of the buffer solution formed by a weak acid and its salt, which can be derived as the negative decadic logarithm of the H^+ concentration expressed by the equation of the weak acid dissociation constant (K_A):

$$K_A = \frac{[H^+][A^-]}{[HA]}$$

$$[H^+] = \frac{K_A[HA]}{[A^-]}$$

$$pH = pK_A + \log\frac{[A^-]}{[HA]}$$

where pK_A is the negative decadic logarithm of the acid dissociation constant of a solution, *[A-]* is the concentration of salts (dissociated forms of the acid), and *[HA]* is the concentration of the (undissociated) acid. This equation is an approximation, with a specific region of validity. It is most reliable for buffers in which the ratio of acid and salt concentrations is close to 1.

Buffer capacity quantifies the ability of a solution to resist changes in pH by either absorbing or desorbing H^+ and OH^- ions. If a small amount of hydrochloric acid is added to a solution of a weak acid and its salt (e.g., acetic acid and sodium acetate), a new ionic equilibrium is created according to the law of dissociation of compounds. The part of the sodium acetate will form undissociated acetic acid (because this acid is weak), and the

pH of the solution will change only slightly; on the other hand, the addition of a base will cause the dissociation of part of the acetic acid, and the pH will not change either.
The examples of buffers are summarized in Table 7.

Table 7: The examples of buffers with their pK_A constant and buffer range.

Buffer	pK_A	Buffer range
Acetate buffer (Acetic acid/sodium acetate)	4.76	3.8-5.6
Phosphate buffer i. H_3PO_4/ NAH_2PO_4 ii. NAH_2PO_4/Na_2HPO_4 iii. Na_2HPO_4/Na_3PO_4	 2.1 (pK_{A1}) 7.2 (pK_{A2}) 12.3 (pK_{A3})	5-8
Citrate buffer (Citric acid/sodium citrate)	3.1 (pK_{A1}) 4.8 (pK_{A2}) 9.2 (pK_{A3})	1.2-6.6
Alkaline borate buffer (Boric acid/sodium borate)	9.2	7.8-10.6

The buffer capacity (β) expresses how the pH of a buffer changes when a small amount of strong acid or base is added. It is the measure of a buffer's ability to resist pH change. If activity coefficients are not considered, then the mathematical expression of buffering capacity can be written as follows:

$$\beta = \frac{\Delta B}{\Delta pH}$$

where ΔB is the gram equivalent of strong acid or base added to change the pH of 1 liter of buffer and ΔpH is the pH change caused by the addition of strong acid or base.
Most buffers are subject to microbial contamination (not only buffers composed of organic acids, but also e.g., phosphate buffer at 0-4 °C for 1 week). They can be preserved for some purposes (azide, toluene, thymol) but are usually stored in the refrigerator without preservation. Only small quantities of prepared buffers are stored in the refrigerator and are quickly used (within a week at most). If a larger quantity of buffer is prepared for stock, it is kept frozen in a plastic bottle. Before use, the entire quantity of buffer must be thawed and the solution mixed. Buffers can also be sterilized. The principles of working under aseptic conditions must be followed when handling such pre-treated solutions, and the buffers must not be re-contaminated (a non-sterile tip of the pipette).
The pH of the solution is affected by its dilution, especially at the extremes of the pH scale (below 3.0 and above 11.0), less so in the range of physiological values. Therefore, it is preferable to avoid preparing more concentrated stock solutions that would then be diluted to prepare a working solution. Temperature is another factor affecting the pH of the buffer by changing the pK_A value of the weak acid or base. References to pH values are presumed to be those at standard temperatures (18, 20, or 25 °C).
Common buffers used in biochemical procedures cover the neutral to slightly alkaline range with a pH of 6.0-9.0. Some operations are performed in more acidic or basic environments. The most common acids and bases used to prepare buffers in biochemistry are listed in Table 8.

Table 8: The most common acids and bases used to prepare buffers with their pK_A constant and pH range.

Acid	Base	pH range
KH_2PO_4	Na_2HPO_4	6.0 -8.0
HCl	TRIS[a]	6.8-8.6
HCl	Imidazole	6.0-8.0
MES[b]	NaOH	5.2-7.2
MOPS[c]	NaOH	5.2-7.1
TES[d]	NaOH	6.5-8.5
HEPES[e]	NaOH	6.5-8.5
HCl	5,5-diethylbarbiturate	6.8-9.6
Boric acid	sodium tetraborate	7.0-9.3
Tricine[f]	NaOH	7.2-9.1
HCl	Glycine	1.1-3.7
Glycine	NaOH	8.2-10.0
Citric acid	NaOH, KOH	2.2-6.2
$NaHCO_3$	Na_2CO_3	9.0-11.0
Na_2HPO_4	NaOH	11.0-12.0
$NaClO_4$	NaOH	1.8-12.0
HCl	NaOH	2.0-12.0
Citric acid	Na_2HPO_4	2.2-8.0

Legend: [a]Tris(hydroxymethyl)aminomethane, [b]2-(N-morpholino)ethanesulfonic acid, [c](3-(N-morpholino)propanesulfonic acid) , [d]2-{[1,3-Dihydroxy-2-(hydroxymethyl)propan-2-yl]amino}ethane-1-sulfonic acid, [e](4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and [f] N-(Tri(hydroxymethyl)methyl)glycine.

2.3.2. Experimental part: The preparation of buffers

Principle

The work aims to prepare two buffers (TRIS-acetic acid and K-phosphate buffers) with subsequent pH control of the prepared solutions.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, volumetric flasks, analytical balance, laboratory vortex, pH meter, magnetic stirrer, magnetic stirring pellet, and funnel.

Materials and chemicals

- Tris(hydroxymethyl)aminomethane (TRIS) at a concentration of 0.1 mol/l (M = 121.14 g/mol, V = 100 ml),
- potassium dihydrogen phosphate (KH_2PO_4) with a concentration of 0.2 mol/l (M = 136.09 g/mol, V = 250 ml),
- potassium hydrogen phosphate (K_2HPO_4) with a concentration of 0.2 mol/l (M = 174.18 g/mol, V = 250 ml),
- concentrated acetic acid (CH_3COOH).

Procedure

Preparation of TRIS-acetic acid buffer

1. Calculate the mass of the TRIS base required to prepare 100 ml of buffer with a concentration of 0.1 mol/l.

2. Weigh this quantity on an analytical balance, transferring quantitative to a 250 ml beaker.
3. Add approximately 60 ml of distilled water to the beaker, insert the magnetic stirring pellet, and stir on the magnetic stirrer until the solution is completely homogenized.
4. Using concentrated acetic acid, adjust the pH of the solution to 7.0 and check it with a pH meter. Add the acetic acid with a single-channel pipette (20-200 µl) while stirring the solution (add concentrated acetic acid drop by drop to the solution).
    a. The pH electrode should be washed with distilled water to clean it thoroughly and dried with a soft cloth (cotton wool) to prevent dilution of the test sample.
    b. Then place the pH electrode in the solution, wait for the reading to stabilize, and take a reading.
    c. Rinse the electrode, blot it dry and switch it off.
5. Quantitatively transfer the buffer solution from the beaker into a 100 ml volumetric flask using a funnel and carefully make up the volume to the mark with distilled water.
6. Transfer the buffer to a storage bottle.

Preparation of K-phosphate buffer

1. Calculate the mass of acid (KH_2PO_4) and basic (K_2HPO_4) phosphate required to prepare 100 ml of buffer at a concentration of 0.2 mol/l.
2. Weigh the amount of KH_2PO_4 on an analytical balance and transfer it quantitatively into a 100 ml beaker.
3. Add approximately 60 ml of distilled water to the beaker, insert the magnetic stirring pellet, and stir on the magnetic stirrer until the solution is completely homogenized.
4. Quantitatively transfer the buffer solution from the beaker into a 100 ml volumetric flask using a funnel and carefully make up the volume to the mark with distilled water.
5. Repeat the procedure for the second component of the buffer solution (K_2HPO_4).
6. Dilute the acid (KH_2PO_4) and basic (K_2HPO_4) phosphate to the pH value taught to you by your teacher according to the Table 9.

Table 9: Preparation of K-phosphate buffer.

pH	*x* 0.2 mol/l K_2HPO_4	*y* 0.2 mol/l KH_2PO_4
6.0	12.3	87.7
6.5	31.5	68.5
7.0	61.5	39.0
7.5	84.0	16.0

Legend: For a 0.2 mol/l buffer, mix *x* ml of 0.2 mol/l basic (K_2HPO_4) phosphate solution and *y* ml of 0.2 mol/l acid (KH_2PO_4) phosphate solution.

7. Check the pH of the solution. When the value on the pH meter is stable, write it down in your laboratory notebook.

Result

Check the pH value of the buffer solution on a pH meter and record the result in your laboratory notebook.

Questions

1. What are seven ways of expressing the concentration of a solution?
2. What is the unit and equation for:
    a. molar concentration,
    b. mass concentration,
    c. weight fraction,
    d. volume fraction?
3. What are the methods of measuring the concentration of H^+ ions?
4. How do you prepare a solution with known concentration? What are the steps for preparing a solution?
5. What is the dissociation constant?
6. What is an acid in terms of H^+ and OH^- ions?
7. What is the pK_A/pK_B and K_A/K_B value of:
    a. a strong acid/ a strong base
    b. a weak acid/ a weak base
8. What is a pH indicator?
9. What are four common pH indicators?
10. What are the uses of indicators?
11. What are the two types of reference electrodes?
12. What is the working procedure of the pH meter?
13. What is the density of distilled water at 20 °C?
14. What is buffer defined as?
15. What is buffer capacity and its equation?
16. What is the process of preparing a buffer solution?
17. What is a quantitative transfer?
18. How much TRIS should you use to make 100 ml with a concentration of 0.1 mol/l (M = 121.14 g/mol)?
19. How many g of KH_2PO_4 should you use to make 250 ml of a 0.2 mol/l solution (M = 136.09 g/mol)?
20. How many g of K_2HPO_4 should you use to make 250 ml of a 0.2 mol/l solution (M = 174.18 g/mol)?

3. Saccharides

3.1. Theory

Saccharides are permanent components of all cells in living organisms. Animal tissues and cells contain fewer saccharides than proteins or lipids (the human body contains approximately 2 % saccharides in dry matter), while plants contain 85-90 % saccharides in dry matter.

In cells, saccharides have different functions:

- They are a **source of energy** and **intermediate products of metabolism**. All animals require a suitable source of energy to meet their energy needs. The pathways by which organisms obtain this energy from food is important for understanding nutrition and normal metabolism. Death by starvation occurs when available energy reserves are exhausted. The storage of excess energy leads to obesity, which is one of the most common diseases of civilization.
- The saccharides ribose and deoxyribose form part of the **structural skeleton of RNA and DNA**. The conformational flexibility of these saccharides is important for the storage and expression of genetic information.
- Polysaccharides are **structural components of the cell walls of bacteria and plants,** and **form the skeleton of some animals**. Cellulose (the main component of plant cell walls) is the most abundant compound in the biosphere. Chitin is found in the cell wall of fungi and forms the outer skeleton of arthropods (crustaceans and insects).
- Saccharides **covalently bind** with **many lipids and proteins** to form glycolipids, proteoglycans, and glycoproteins.

Saccharide units on the cell surface play a key role in cell recognition. For example, fertilization of an egg begins when the sperm binds to a specific oligosaccharide on the egg surface. Another example of the important role of saccharides in recognition processes is the adhesion of lymphocytes to sites of damaged blood vessels and their return to the lymph nodes.

Monosaccharides are named according to their functional groups or number of carbon atoms. The basis of the monosaccharide molecule is a carbon chain with 3 to 9 carbon atoms, which determines the basic classification: 3 carbons have trioses, 4 - tetroses, 5 - pentoses, 6 - hexoses, 7 - heptoses, 8 – octoses and 9 - nonoses.

A monosaccharide with a ketone group is referred to as a *ketose*. A monosaccharide with an aldehyde group is called an *aldose*. Combining these designates such sugars as an aldotetrose or ketopentose. For example, aldotetrose is a monosaccharide containing both an aldehyde (an aldose) and four carbons (a tetrose) (see Figure 27).

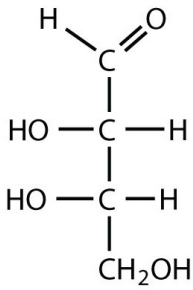


Figure 27: The structure of aldotetrose (L-erythrose) contains four carbon atoms, with the first carbon atom being part of the aldehyde functional group.

Disaccharides are carbohydrates formed by joining two monosaccharides via glycosidic bond. **Oligosaccharides** consist of short chains of monosaccharide units (usually 3-10 monosaccharides) via glycosidic bonds (trisaccharides, tetrasaccharides, and up to decasaccharides).
Polysaccharides are long polymers consisting of ten to thousands of monosaccharide units. The carbon atoms in a saccharide molecule are bonded to a hydroxyl group and an either aldehyde or ketone group. In other words, the saccharides are polyhydroxyaldehydes (carbon compounds with a high number of carbon atoms, containing several hydroxyl groups and an aldehyde group) or polyhydroxyketones (carbon compounds with a high number of carbon atoms, containing several hydroxyl groups and a ketone group).

3.1.1. Monosaccharides

Monosaccharides are those saccharides that cannot be hydrolyzed into simpler (smaller) saccharides. Monosaccharides are sometimes referred to as simple sugars or just sugars.
The most important hexose present in living organisms is **glucose** (grape sugar), which is an essential component of human blood and many extracellular fluids. Plants produce glucose in the process of photosynthesis from H_2O and CO_2; animals must obtain it through in their food. Other important monosaccharides are **fructose** (fruit sugar, primarily occurs naturally in many fruits) and **galactose** (brain sugar, it is a component of glycoproteins found in nerve tissue) derived from the breakdown of the disaccharide lactose (milk sugar) (see Figure 28).

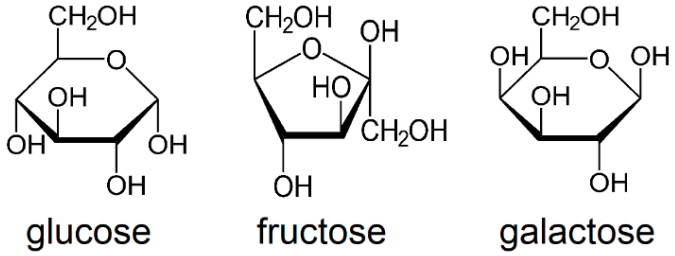


Figure 28: Structures of important monosaccharides: glucose, fructose, and galactose.

Overview of monosaccharides:

1. *Trioses:* Aldotriose is **glyceraldehyde** (a chiral compound with two enantiomers), ketotriose is **dihydroxyacetone** (an optically inactive compound with no chiral carbon).
2. *Tetroses:* **D-Erythrose** is an important intermediate in saccharide metabolism and at the same time a starting substrate in the biosynthesis of aromatic substances.
3. *Pentoses:* **D-arabinose, D-xylose**, **D-ribose, D-2-deoxyribose, D-ribulose,** and **D-xylulose**.
4. *Hexoses:* **D-mannose, D-galactose, D-glucose,** and **D-fructose**.

Projections of monosaccharides
Three types of structural projections are used to draw a monosaccharide molecule:

1. **Fisher projection** shows monosaccharides in their open-chain form. The carbon chain of a molecule is shown in linear form with free functional groups. The Fischer projection is not an accurate representation of the actual 3D configuration of these sugars.

2. **Tollens projection** shows the cyclic structure of the monosaccharide from a linear form through the reaction of a hydroxyl group with a carbonyl group to form a so-called hemiacetal (hemiketal) structure.
3. **Haworth projection** shows the structure of a sugar in the form of a simple circle. The monosaccharide molecule is viewed from the side and above the plane of the ring (see Figure 29).

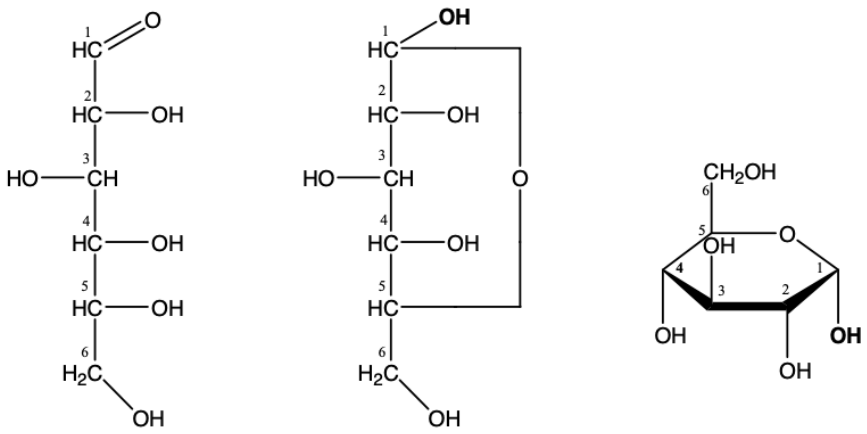


Figure 29: Structure of glucose according to Fisher, Tollens and Haworth projections.

Isomers of monosaccharides

Sugars exhibit various forms of isomerism. **Isomerism** means that the molecules with the same formula (the same number of the same kinds of atoms) have different chemical and physical properties. The presence of asymmetric carbon atoms (called chiral carbons) allows the formation of isomers. A chiral carbon has four different groups attached to it. A chiral compound does not have a plane of symmetry. If the number of asymmetric carbon atoms is known, the maximum possible number of stereoisomers for a given molecule can be calculated. If n is the number of asymmetric carbon atoms, then the maximum number of isomers is $2n$. For example, glucose with four asymmetric carbon atoms can form 16 isomers.

The important types of isomerism are as follows:

- D and L-isomerism,
- pyranose and furanose ring structures,
- α- and β-anomers,
- epimers, and
- aldose-ketose isomerism.

D- and L-isomerism (subject/mirror image). This is based upon a historical method for determining enantiomers using glyceraldehyde (see Figure 30). Glyceraldehyde is a three-carbon sugar with one chiral carbon. The orientation of the -OH group determines whether the saccharide belongs to the D- or L-form. If the -OH group on the single chiral carbon in the glyceraldehyde molecule is on the right, it is the D-isomer; if on the left, it is the L-isomer.

If the chiral carbon atom farthest from the carbonyl group of the monosaccharide has the same conformation as D-glyceraldehyde, it is called a D-monosaccharide, and if this chiral carbon atom has the same conformation as L-glyceraldehyde, it is an L-monosaccharide. The D- and L-isomers are two distinct conformations that are **the object (D-form) and its mirror image (L-form)** of each other. Monosaccharides commonly

found in organisms are in the D configuration, and the enzymes that catalyze their conversion are stereospecific for these isomers.

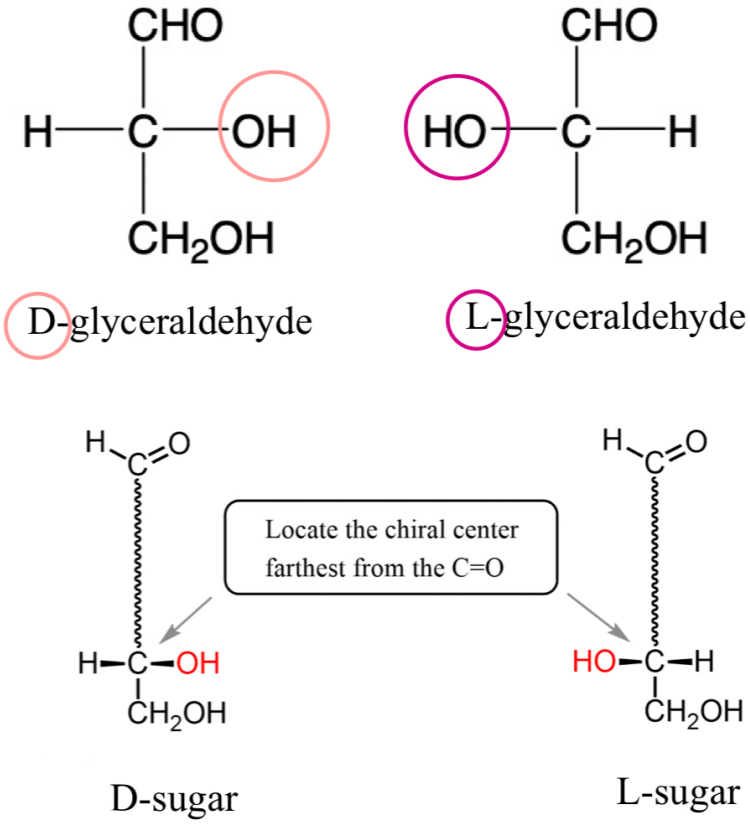
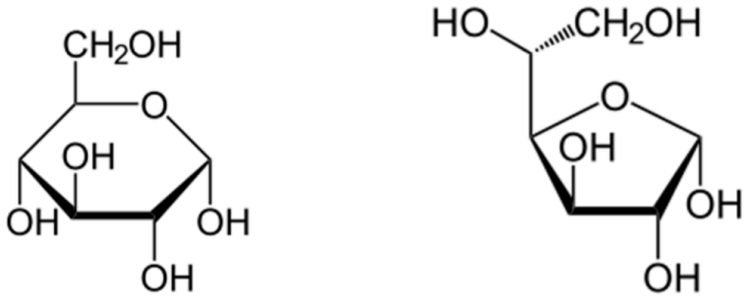


Figure 30: Configuration of D- and L-glyceraldehyde. The identification of the D- or L-form of the monosaccharide is based on the identification of the chiral carbon furthest from the C=O group and the position of the hydroxyl group on this carbon.

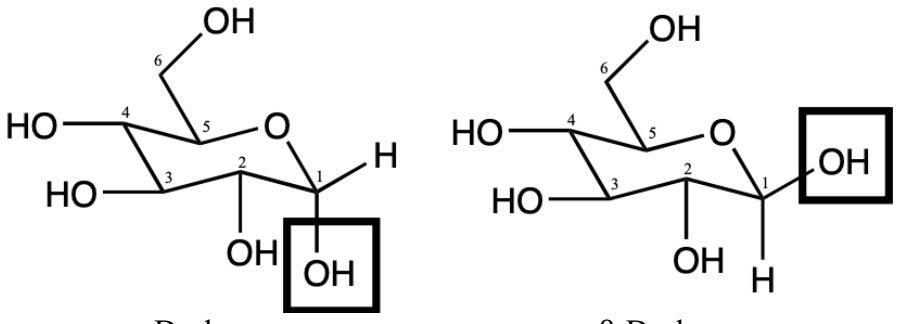
The presence of asymmetric carbon atoms is related to the **optical activity of the compound**. When a beam of plane-polarized light is passed through a solution of an optical isomer, it will be rotated either to the right ((+), or d-, dextrorotary) or to the left ((−), l-, levorotary). When both (+) and (-)-isomers are present in equal amounts, the mixture is called a racemic mixture. The direction of rotation is independent of the stereochemistry of the sugar, therefore, it may be designated as D(−), D(+), L(−), or L(+). **Pyranose and furanose ring structures**. Pentoses and hexoses can form cyclic molecules by the reaction of an aldehyde or ketone group with a hydroxyl group on the same molecule to form a hemiacetal (semiacetal) or a hemiketal (semiketal). The stable ring structure of the monosaccharide is similar to the ring structure of furan (five-membered ring) or pyran (six-membered ring). Glucose forms its cyclic form by the reaction of an aldehyde group with a hydroxyl group on the fourth carbon (to form glucofuranose) or the fifth carbon (to form glucopyranose). More than 99 % is the pyranose form of glucose and 1 % is its furanose form (see Figure 31).



α-D-glucopyranose α-D-glucofuranose

Figure 31: An example of the formation of hemiacetal in a glucose molecule.

α- and β-anomers. The cyclic form of the molecule is shown by Haworth projection. In the formation of the cyclic form of a monosaccharide, the carbon atom of the carbonyl group (C=O) becomes chiral, and a hydroxyl group (hemiacetal hydroxyl, anomeric hydroxyl group) is formed on it. The OH group on C-1 of sugars can have two possible configurations: an α-anomer and a β-anomer. They differ in the orientation of the -OH group in the ring (see Figure 32).



α-D-glucose β-D-glucose

Figure 32: The key difference between α-D- and β-D-glucose is the orientation of hydroxyl (-OH) group attached to the first carbon atom.

For glucose, the α-anomer has the anomeric -OH group on the opposite face of the ring to carbon-6, whereas the β-monomer has the anomeric -OH group on the same face of the ring as carbon-6. The existence of the α- and β-anomers made it possible to explain **mutarotation**. The optical rotation slowly changes over time in freshly prepared sugar solutions. The term mutarotation (known as change in rotation) refers to the observed change in the optical rotation of the α- and β-anomers of glucose upon dissolution in a solvent. Due to the ring-chain tautomerism, the α- and β-anomers are slowly exchanged until equilibrium is established.

Epimers. Isomers differ by changes in the configuration of -OH and -H on the carbon atoms, specifically in the case of glucose 2, 3 and 4. The most biologically important glucose epimers are mannose and galactose, which are formed by epimerization at carbons 2 and 4, respectively.

Aldose-ketose isomerism. Fructose has the same molecular formula as glucose ($C_6H_{12}O_6$), but they differ in its structural formula. Fructose is a ketose and glucose is an aldose.

3.1.2. Disaccharides

A disaccharide consists of two monosaccharide molecules that have been joined to one another through a glycosidic bond between the anomeric carbon (carbon 1, the hemiketal or hemiacetal carbon of the sugar) of one cyclic monosaccharide with the hydroxyl group (-OH) of a second monosaccharide. If two monosaccharides are linked through their anomeric centers, a *non-reducing disaccharide* is formed. If one monosaccharide is linked by one of its other hydroxyl groups, then the anomeric center is unsubstituted, and a *reducing disaccharide* occurs (see Figure 33). All monosaccharides are reducing saccharides.

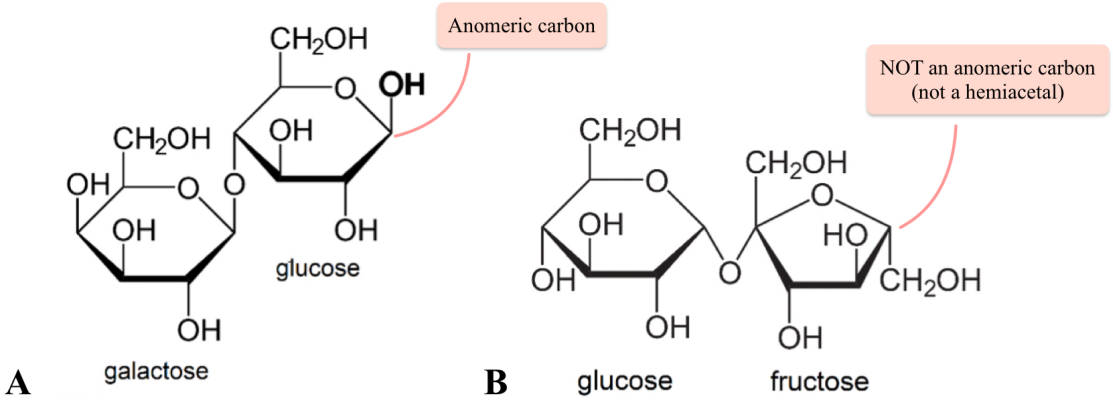


Figure 33: A - Reducing disaccharide – lactose (milk sugar) and B – non-reducing disaccharide – sucrose (table sugar).

Examples of reducing disaccharides include maltose, isomaltose, cellobiose and lactose, whereas non-reducing disaccharides include sucrose or trehalose.

Sucrose (saccharose, beet sugar, cane sugar, table sugar) is by far the most important saccharide. Sucrose is formed by D-glucose and D-fructose via the -OH group on the anomeric carbon of D-glucose and the OH group on the anomeric carbon of D-fructose. The bond between the two monosaccharides is α-1,β-2-glycosidic linkage. The hydrolysis of sucrose in dilute acid or through the action of enzymes (invertases) gives an equimolar mixture (invert sugar) of glucose and fructose (1:1). The hydrolysis has several practical applications. Sucrose readily recrystallizes from the solution, but invert sugar is much more likely to remain in the solution. In the manufacture of confectionery, recrystallisation of sugar is undesirable; moreover, fructose is sweeter than sucrose. **Lactose** (milk sugar) occurs in the milk of mammals but very rarely in the plant kingdom. Lactose consists of one molecule of D-galactose and one molecule of D-glucose joined by a β-1,4-glycosidic bond. The enzyme that hydrolyses lactose is referred to as lactase. **Trehalose** occurs in mushrooms and other fungi and consists of two D-glucose molecules linked by α,α-(1→1) glycosidic bonds. **Maltose** (malt sugar) is formed during the mashing of malt in brewing and serves as a substrate for yeast in alcoholic fermentation. It is also a component of high-maltose syrup. Two glucose molecules joined by an α-1,4-glycosidic bond form maltose. The enzyme catalyzing the hydrolysis reaction of maltose is maltase. **Cellobiose** is formed by the enzymatic hydrolysis of cellulose. It is derived from the condensation of a pair of β-glucose molecules forming a β(1→4) glycosidic bond.

3.1.3. Polysaccharides

Most saccharides occur in nature in the form of polysaccharides. These are substances of both plant and animal origin. Unlike mono- and disaccharides, they are very slightly

soluble in water and form colloidal solutions. They do not ionize in water and do not have a sweet taste. **Starch** in plants and **glycogen** in animals are polysaccharides from which glucose, can be rapidly released when needed.
Polysaccharides consist of a large number (more than 10) of monosaccharide units are linked by glycosidic bonds. The bond is formed between the hemiacetal hydroxyl of one molecule and the alcohol group of the other molecule. They can be divided into **homopolysaccharides** and **heteropolysaccharides**.

- Homopolysaccharides consist of only one type of monosaccharide unit (glucans, mannans, etc.).
- Heteropolysaccharides contain two or more types of monosaccharide units (e.g., bacterial wall glycan composed of N-acetylglucosamine and N-acetylmuramic acid). Hydrolysis results in the conversion of oligosaccharides to monosaccharides.

Homopolysaccharides
Starch is the most important plant's polysaccharide. It is a mixture of amylose (15-20 %) and amylopectin (80-85 %) (see Figure 34).

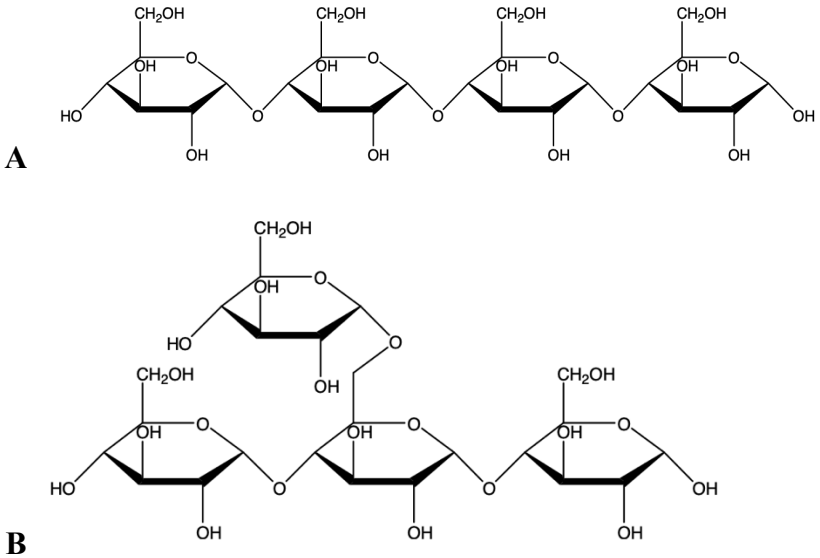


Figure 34: The structure of A – amylose and B - amylopectin.

It is the storage carbohydrate in plants. It is found in tubers (potatoes), roots (sweet potatoes), seeds, and fruits. Amylose consists of glucose molecules bound by an α-(1-4) glycosidic bond. The bond is formed between two α-glucose units, specifically between the first carbon of one glucose unit and the fourth carbon of the other glucose unit (Figure 34-A). The linear chain contains an average of 1000 glucose units and is twisted into a left-handed single helix. Amylopectin has a branched structure. The glucose molecules are bound via an α-(1-4) glycosidic bond and an α-(1-6) bond. The α-(1-6) glycosidic bond repeats for every 10-12 glucose units (see Figure 34-B). This structure makes it non-coiled, which leads to good water solubility, and the high amylopectin content of the starch makes it more easily soluble in hot water and cleavable by enzymes.
Starch is broken down by enzymes - amylases. Amylases are a group of hydrolytic enzymes consisting of α-amylase, β-amylase, pullulanase, and glucoamylases.

- The α-amylase (endoamylase) attacks the internal α-1,4-glycosidic linkages in starch to form glucose, maltose, and α-dextrins.

- β-amylase (exoamylase) hydrolyses α-1,4-glycosidic linkages of starch chain at the non-reducing end and produce maltose.
- Pullulanase is able to cleave α-1,6-glycosidic bonds in amylopectin, thereby disrupting its branched structure.
- Glucoamylases (exoamylase) cleave both α-1,4- and α-1,6-glycosidic bonds and release glucose molecules.

Glycogen is a storage animal polysaccharide. Its cellular content varies depending on its physiological state. It is composed of glucose units and has a more complex and irregular structure than starch. Glycogen forms a right-handed helix. It is soluble in water to a concentration of approximately 15-20 g/100 ml and insoluble in alcohols.

Cellulose is the most essential structural component of plant cell walls. It is a homopolymer of glucose. The glucose units in this polymer are linked by a β-(1-4) glycosidic bond. It is analogous to starch, except that the geometry of the glycosidic linkages is α-(1-4) for starch (more specifically amylose) and β-(1-4) for cellulose.

Chitin is found in the cell wall of fungi, in the exoskeletons of arthropods, such as crustaceans and insects, and in other protective structures of some organisms. It is an N-acetyl-glucosamine residue linked by a β-(1-4)-glycosidic bond. It has a low solubility in water.

Heteropolysaccharides

Glycosaminoglycans (mucopolysaccharides) are composed of chains of complex carbohydrates that are characterized by their amino acid and uronic acid content. If the chains are attached to a protein molecule, it is a proteoglycan (e.g., hyaluronic acid, chondroitin sulphate, and heparin sulphate).

Glycoproteins (mucoproteins) are found in body fluids, tissues and in cell membranes. They are proteins that contain carbohydrates in varying amounts, attached as short or long branched or unbranched chains.

Questions

1. What are the four main functions of saccharides in living cells?
2. What are monosaccharides, oligosaccharides, and polysaccharides? Give three examples from each group.
3. Divide the following monosaccharides among triose, tetrose, pentose, and hexose: xylose – glyceraldehyde – glucose – erythrose – fructose – arabinose – mannose.
4. What is the difference between ketose and aldose?
5. What are the major types of isomerism?
6. What is a chiral carbon?
7. What is the difference between D- and L-isomers?
8. What is the necessary condition for optical activity in a monosaccharide?
9. What is mutarotation?
10. What is the definition of reducing saccharides? What are 3 examples of reducing saccharides?
11. What is the definition of non-reducing saccharides? What are 3 examples of non-reducing saccharides?
12. How can one differentiate between reducing and non-reducing saccharides?
13. What are the two parts of
    a. sucrose
    b. lactose
    c. trehalose
    d. maltose
    e. cellobiose
14. What does starch consist of? Which enzymes are used to degrade starch?

15. What is the main component of glycogen and how does its structure compare to that of starch?
16. What is the primary role of cellulose in plants?
17. What type of organisms is chitin found in?
18. State the difference between homopolysaccharides and heteropolysaccharides. What are examples of homo- and heteropolysaccharides?

3.2. Experimental part A: Determination of reducing saccharides by the DNS method

Principle

The reducing properties of the free carbonyl group (free hemiacetal hydroxyl -C=O) can be used to determine the presence of reducing saccharides. This reaction involves oxidation of the aldehyde group of aldoses (e.g., glucose) to carboxyl and the ketone group of ketoses (e.g., fructose) to hydroxyl with chain scission. Oligosaccharides that do not have a free carbonyl group (free hemiacetal) do not react (e.g., sucrose and trehalose). Among the most commonly used reactions to detect the presence of a free carbonyl group is the reaction of reducing saccharides with the DNS (3,5-dinitrosalicylic acid) reagent. In this method, the reaction of the DNS reagent and a reducing saccharide produces an orange-colored product, 3-amino-5-nitrosalicylic acid (the reduced form of DNS) (see Figure 35). The intensity of the color depends on the concentration of the reducing saccharide in a solution and is measured at 540 nm. The reaction takes place in a basic medium. The test solution must be heated after the addition of the DNS reagent to increase the reaction rate.

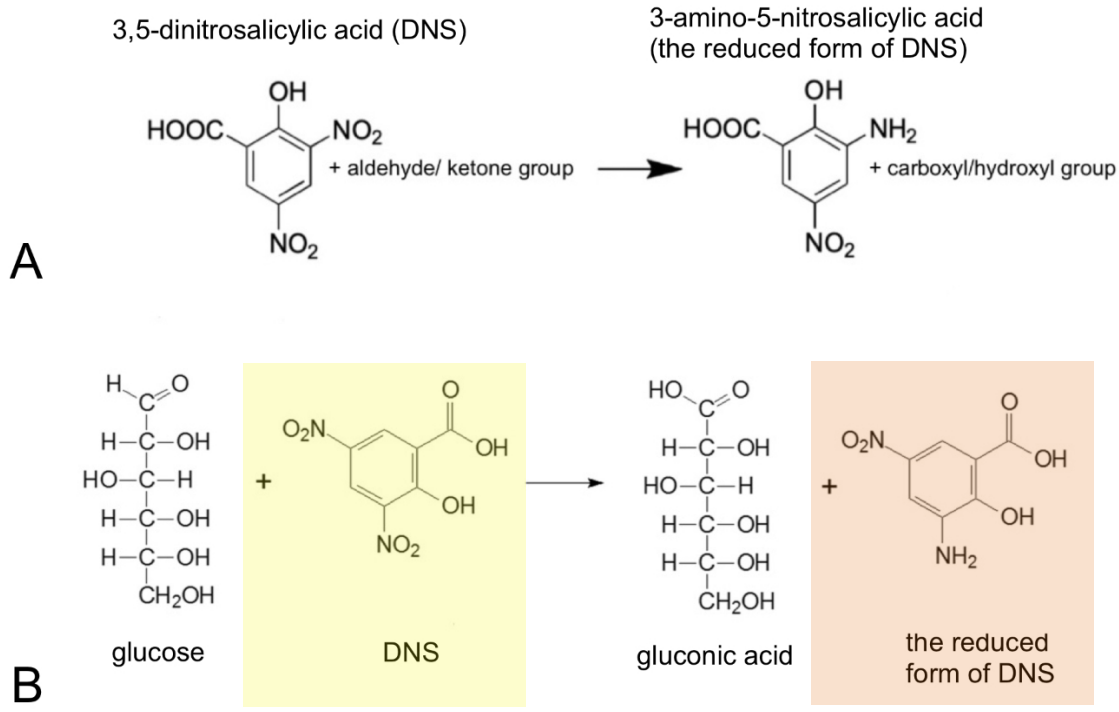


Figure 35: A – the reaction of an aldehyde/ketone group of a reducing saccharide with 3,5-dinitrosalicylic acid (DNS) to form 3-amino-5-nitro salicylic acid and a carboxyl/hydroxyl and B – the DNS reaction with glucose as the reducing sugar.

Different reducing saccharides generally yield different color intensities. It is necessary to calibrate each saccharide. Therefore, this method is not suitable for the determination of complex mixtures of reducing saccharides.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, Eppendorf tubes (1.5 ml), beakers, test tubes, volumetric flask (10 ml), water bath, hot plate, analytical balance, laboratory vortex, and spectrophotometer.

Materials and chemicals

- The sample with unknown glucose concentration,
- the stock solution of glucose with a concentration of 15 g/l (M = 180 g/mol, V = 10 ml),
- DNS reagent (dissolve 1 g DNS in 20 ml 2 mol/l aqueous sodium hydroxide solution, dilute to 50 ml with distilled water, add 30 g sodium hydroxide and make up to 100 ml with distilled water).

Procedure

The preparation of the stock solution of glucose and its dilute solutions

1. Prepare the stock solution of glucose with a concentration of 15 g/l by weighing 0.15 g of the solid sample, transferring the quantitative to a 10 ml volumetric flask and diluting to the mark with distilled water (see Figure 36-A).
2. Through appropriate dilutions of the stock solution of the glucose, prepare calibration solutions as follows:

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
12.5	1.2	0.833	0.167
10.0	1.5	0.667	0.333
7.5	2	0.500	0.500
5.0	3	0.333	0.667
2.5	6	0.167	0.833
1.0	15	0.067	0.933

3. Prepare the dilute solutions (calibration solutions) in Eppendorf tubes. Dilute the stock solution of glucose and distilled water using a one-channel pipette. The total volume of dilute solution is 1 ml (see Figure 36-B).

Determination of reducing saccharides by the DNS method

1. Mix 0.1 ml of the solution (a sample or glucose solutions in the concentration range of 1-15 g/l) with 0.8 ml of the DNS reagent in a test tube (Figure 36-C):

	Tube*	Glucose solution (ml)	Sample (ml)	Water (ml)	DNS reagent
	B (blank)	-	-	0.1 ml	0.8 ml
Glucose calibration solutions	1 (15 g/l)	0.1 ml	-	-	0.8 ml
	2 (12.5 g/l)	0.1 ml	-	-	0.8 ml
	3 (10 g/l)	0.1 ml	-	-	0.8 ml
	4 (7.5 g/l)	0.1 ml	-	-	0.8 ml
	5 (5 g/l)	0.1 ml	-	-	0.8 ml
	6 (2.5 g/l)	0.1 ml	-	-	0.8 ml
	7 (1 g/l)	0.1 ml	-	-	0.8 ml
	SAMPLE	-	0.1 ml	-	0.8 ml

**Work in three parallel measurements. Prepare a set of test tubes. Take 27 clean and dry test tubes to determine the absorbance of the sample or calibration solutions.*

3. Keep the test tube in a boiling water bath for 5 minutes (Figure 36-D).
4. Take the tubes and cool them to laboratory temperature.
5. Add 8 ml of distilled water to each test tube.

6. Prior to making all absorbance readings, a spectrophotometer must be calibrated using a blank solution, which comprises a cuvette filled with 2/3 full distilled water for this experiment.
7. Measure the absorbance of the cooled samples at 540 nm (see Figure 36-E).
8. Record the results in your laboratory notebook.

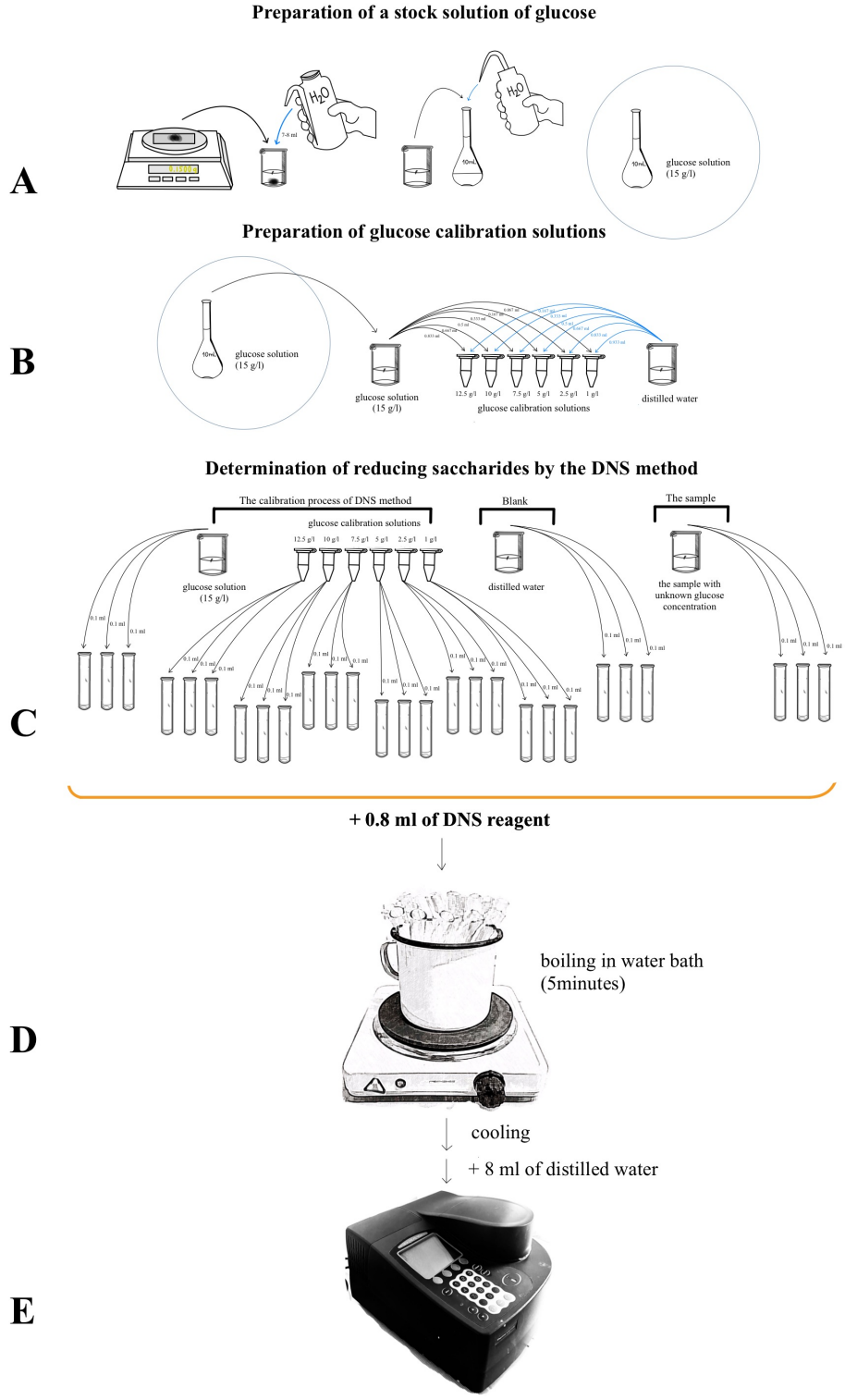


Figure 36: The spectrophotometric procedure for the determination of unknown glucose concentration in a sample by calibration of the DNS method with standard glucose solutions of known concentrations.

Results

Calculate for each calibration solution the average of absorbances, and produce a plot of absorbance vs. actual concentration (the x-y relationship). Carry out a linear regression analysis to determine the equation of the relationship between absorbance and concentration ($y = ax + b$) as well as the coefficient of determination. Calculate the concentration of glucose (g/l) in the sample.

Questions

1. What is a reducing saccharide?
2. Give examples of two reducing and two non-reducing saccharides.
3. What is the principle of reducing sugars by DNS method?
4. What is the product of the reaction between the DNS reagent and a reducing saccharide?
5. What wavelength is used to measure the color intensity of a solution in the DNS method? Explain your answer.
6. What reference solution will you use in this experimental part A for the determination of glucose by the DNS method?
7. How much glucose should you use to make 10 ml with a concentration of 15 g/l?
8. How much sodium hydroxide (M = 39.9971 g/mol) should you use to make 20 ml with a concentration of a 2 mol/l aqueous solution?
9. The stock solution of glucose with a concentration of 15 g/l should be diluted to the concentrations shown in the table. Calculate the dilution factor, the volume of stock solution and the volume of distilled water needed to produce a solution of a given concentration. The total volume of dilute solution must be 1 ml.

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
12.5			
10.0			
7.5			
5.0			
2.5			
1.0			

3.3. Experimental part B: Determination of sucrose by the DNS method

Principle

The DNS method can be used to determine reducing saccharides. All monosaccharides are reducing saccharides, while disaccharides can be classified as reducing or non-reducing. Lactose, maltose and cellobiose are reducing saccharides, with the exception of sucrose. **Sucrose** ($C_{12}H_{22}O_{11}$, saccharose, beet sugar, cane sugar, table sugar) is formed by D-glucose and D-fructose via the -OH group on the anomeric carbon of D-glucose and the OH group on the anomeric carbon of D-fructose. The reducing groups of glucose and fructose are involved in glycosidic bond formation, sucrose is a non-reducing saccharide. The DNS method can be used to determine sucrose concentration, but sucrose does not react with the DNS reagent, therefore it must first be broken down by hydrolysis into simple sugars such as glucose and fructose (see Figure 37). The hydrolysis of sucrose in dilute acid (acid hydrolysis) or through the enzymes (invertases, enzyme hydrolysis) gives an equimolar mixture (invert sugar) of glucose and fructose (1:1). These simple sugars can react with the DNS reagent to form the reduced form of DNS. The intensity of the color response of this product is measured at a wavelength of 540 nm.

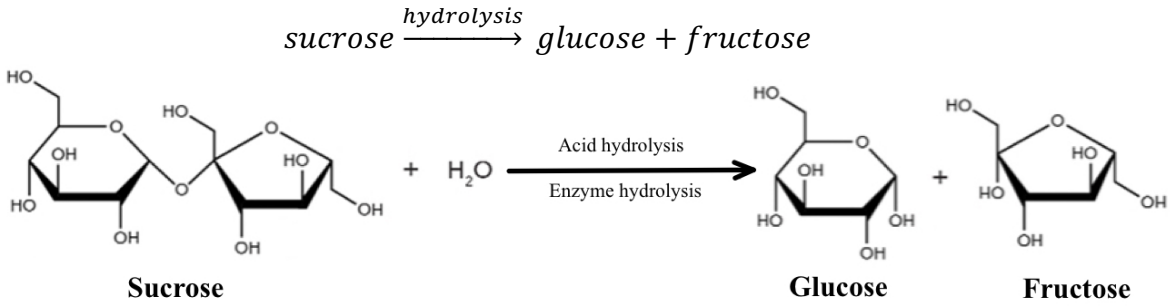


Figure 37: Hydrolysis of sucrose generates an equimolar mixture of glucose and fructose.

Laboratory requirements

Automatic pipettes (20 -200 µl, 100-1000 µl), glass pipette, pipetting tips, Eppendorf tubes (1.5 ml), beakers, test tubes, volumetric flask (10 ml), water bath, hot plate, analytical balance, laboratory vortex, and spectrophotometer.

Materials and chemicals

- The sample with unknown sucrose concentration,
- the stock solution of sucrose with a concentration of 5 g/l (M = 360 g/mol, V = 10 ml),
- concentrated hydrochloric acid (HCl, 36 % (*v/v*)),
- potassium hydroxide (KOH) with a concentration of 5 mol/l (M = 56.11 g/mol, V = 10 ml),
- DNS reagent (dissolve 1 g DNS in 20 ml 2 mol/l aqueous sodium hydroxide solution, dilute to 50 ml with distilled water, add 30 g sodium hydroxide and make up to 100 ml with distilled water).

Procedure

The preparation of the stock solution of sucrose and its dilute solutions

1. Prepare the stock solution of sucrose with a concentration of 5 g/l by weighing 0.05 g of the solid sample, transferring the quantitative to a 10 ml volumetric flask and diluting to the mark with distilled water.
2. Through appropriate dilutions of the stock solution of the sucrose, prepare calibration solutions as follows:

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
4.5	1.11	0.900	0.100
4.0	1.25	0.800	0.200
3.5	1.43	0.700	0.300
3.0	1.67	0.600	0.400
2.5	2.00	0.500	0.500
2.0	2.50	0.400	0.600
1.5	3.33	0.300	0.700
1.0	5.00	0.200	0.800

3. Prepare the dilute solutions (calibration solutions) in test tubes. Dilute the stock solution of sucrose and distilled water using a one-channel pipette. The total volume of dilute solution is 1 ml.

Acid hydrolysis of sucrose

1. Mix 1 ml of sucrose calibration solution (1-5 g/l of sucrose) or a sample with unknown sucrose concentration with 30 µl (1 drop) of concentrated HCl.
   Do not pipette a single drop of concentrated hydrochloric acid using a single-channel pipette. Use a glass pipette or glass dropper.
2. Keep the test tube with the solution in a boiling water bath for 5 minutes.
3. Take the tubes and cool to laboratory temperature.
4. Add 100 µl (3 drops) of KOH solution at a concentration of 5 mol/l to neutralize the acid.

Determination of reducing saccharides by the DNS method

1. Mix 0.1 ml of the solution (a sample or sucrose solution after hydrolysis) with 0.8 ml of the DNS reagent in a test tube. Do not forget to prepare a blank (0.1 ml of distilled water with 0.8 ml of the DNS reagent).
   Work in three parallel measurements. Prepare a set of test tubes. Take 33 clean and dry test tubes to determine the absorbance the sample or calibration solutions.
2. Keep the test tube in a boiling water bath for 5 minutes.
3. Take the tubes and cool to laboratory temperature.
4. Add 8 ml of distilled water to each test tube.
5. Prior to making all absorbance readings, a spectrophotometer must be calibrated using a blank solution, which comprises a cuvette filled with 2/3 full distilled water for this experiment.
6. Measure the absorbance of the cooled samples at 540 nm.
7. Record the results in your laboratory notebook.

Results

Calculate for each calibration solution the average of absorbances, and produce a plot of absorbance vs. actual concentration (the x-y relationship). Carry out a linear regression analysis to determine the equation of the relationship between absorbance and concentration ($y = ax + b$) as well as the coefficient of determination. Calculate the concentration of sucrose (g/l) in the sample.

Questions

1. What is the purpose of using hydrochloric acid in the hydrolysis of sucrose?
2. What is the standard for sucrose determination by the DNS method?
3. What is the blank for sucrose determination by the DNS method?
4. Why sucrose is a non-reducing saccharide?
5. How much sucrose should you use to make 10 ml with a concentration of 5 g/l? Express your result in grams.
6. How much potassium hydroxide (KOH, M = 56.11 g/mol) should you use to make 10 ml with a concentration of 5 mol/l?
7. The stock solution of sucrose with a concentration of 5 g/l should be diluted to the concentrations shown in the table. Calculate the dilution factor, the volume of stock solution and the volume of distilled water needed to produce a solution of a given concentration. The total volume of dilute solution must be 1 ml.

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
4.5			
4.0			
3.5			
3.0			
2.5			
2.0			
1.5			
1.0			

8. A sample containing an unknown concentration of sucrose has an absorbance of 0.492. Calculate its sucrose concentration using the linear regression equation $y = 0.0742x + 0.0045$ for sucrose solutions in the concentration range of 1-10 g/l.

4. Nucleic acids

4.1. Theory

One of the most fundamental properties of living matter is **heredity** (the ability to pass genetic information from parents to offspring). **Genetic information** is a set of instructions for all structural features and life processes of an organism. Individual instructions are written in a specific manner in the genetic material.

Nucleic acids are the essential components of living systems that serve to store genetic information. Their basic building blocks are **nucleotides**.

Each nucleotide consists of three basic components:

- a nitrogen-containing heterocycle (or nitrogenous base) (adenine, thymine, guanine, cytosine and uracil),
- a pentose sugar (2-deoxy-D-ribose or D-ribose),
- a phosphate group (inorganic phosphoric acid, H_3PO_4) (see Figure 38).

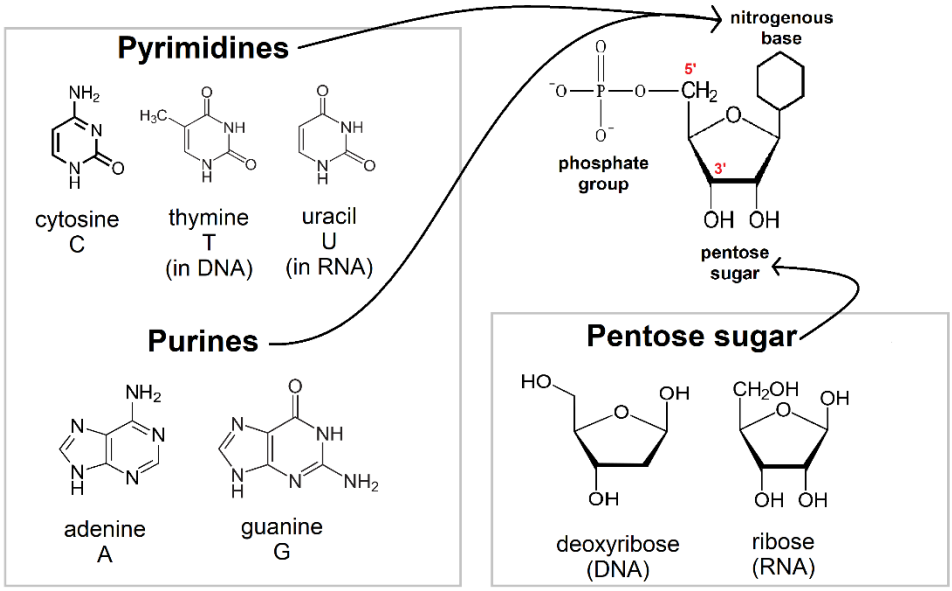


Figure 38: The chemical structure of a nucleotide composed from a nitrogenous base, a pentose sugar (2-deoxy-D-ribose or D-ribose), and a phosphate group.

All three parts are referred to as a nucleotide (Figure 38). A **nucleoside** is a derivative of a nitrogenous base that has a pentose sugar linked to a nitrogen ring without an attached phosphate group. The base binds with to the sugar via an N-glycosidic bond.

The combination of pentose sugar and a phosphate group forms the sugar-phosphate backbone of nucleic acid polymers. The nucleotides are linked to each other by a 3',5'-phosphodiester bond, between the phosphate group attached to the 5th carbon atom of the pentose on one nucleotide and the free hydroxyl group on the 3rd carbon atom of the pentose of the next nucleotide. The 3',5'-phosphodiester bond is formed as the result of this condensation reaction between phosphate group and hydroxyl group of two sugar groups. The 3',5'-phosphodiester bonds are the same in both DNA and RNA. In the polynucleotide chain formed, two ends could be distinguished: the 5'-end terminated with a phosphate group and the 3'-end terminated with a sugar -OH group.

Similar to proteins, in which the backbone is formed by an α-carbon with an amino group and a carboxyl group, the side chains of amino acids have a profound effect on the structure and biological activity of proteins, and the order of organic bases in the polynucleotide chain plays an important role in the encoding of genetic information in nucleic acids. The main nitrogenous bases of nucleic acids are adenine, guanine, cytosine,

thymine, and uracil. These bases form a link with other nucleic acid chains. The two strands are held together by hydrogen bonds between the **pairs of complementary nitrogenous bases**. The nucleotide chain is more stable and resistant to physical and chemical influences owing to these bonds. Nucleic acids are found in the cell nucleus, cytoplasm, and in various particles of cells (mitochondria, ribosomes, and chloroplasts). According to a pentose sugar molecule of nucleotides, nucleic acids are divided into **DNA** (deoxyribonucleic acid) and **RNA** (ribonucleic acid). The DNA molecule contains four organic bases: adenine, guanine, cytosine, and thymine, and in RNA, the base uracil takes the place of thymine. Guanine is the complementary base of cytosine, and adenine is the complementary base of thymine in DNA and of uracil in RNA.

4.1.1. DNA

The discovery of the structure of DNA was one of the most important discoveries in the last century. It was made in 1953 by Watson and Crick, based on data from the X-ray analysis of DNA. They found that the DNA molecule is shaped like a double helix (see Figure 39).

Figure 39: The structure of double-stranded DNA.

The double strand of the DNA twists like a spiral staircase. The outsides of the DNA molecule, the staircase rails, are composed of deoxyribophosphate residues and the steps of the staircase are made of nucleoside bases. The deoxyribose sugars alternating with phosphates is called as the backbone. The two polynucleotide chains run in opposite directions and are referred to as anti-parallel.

The DNA molecule contains four organic bases - adenine, guanine, cytosine, thymine, the pentose sugar molecule 2-deoxy-D-riboses and phosphate groups. The number of organic bases present in a DNA molecule varies depending on the encoding information. All DNA follows **Chargaff's rules** (given by Erwin Chargaff):

1. the amount of adenine is equal to thymine, **A/T = 1**,
2. the amount of guanine is equal to cytosine, **G/C = 1**.

This rule follows from the fact that DNA is a double-stranded polynucleotide chain composed of nucleotides containing these four bases. Both strands are connected by hydrogen bonds, which maintain the stability of DNA. In the DNA molecule,

- the thymine – adenine base pair is held together by two hydrogen bonds and
- the guanine – cytosine base pair is held together by three hydrogen bonds (see Figure 40).

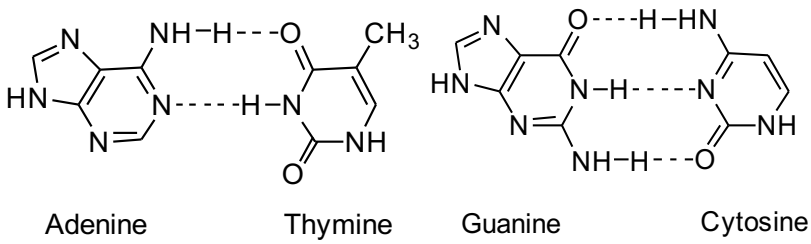


Figure 40: Formation of hydrogen bonds between the nitrogenous bases of complementary chains of single-stranded DNA.

This complementary base pairing rule (see Figure 41) is important in DNA because it allows base pairs to be arranged in the most energetically advantageous way. This allows DNA to replicate accurately. It also allows DNA to be transcribed accurately into RNA and then translated from RNA to amino acids.

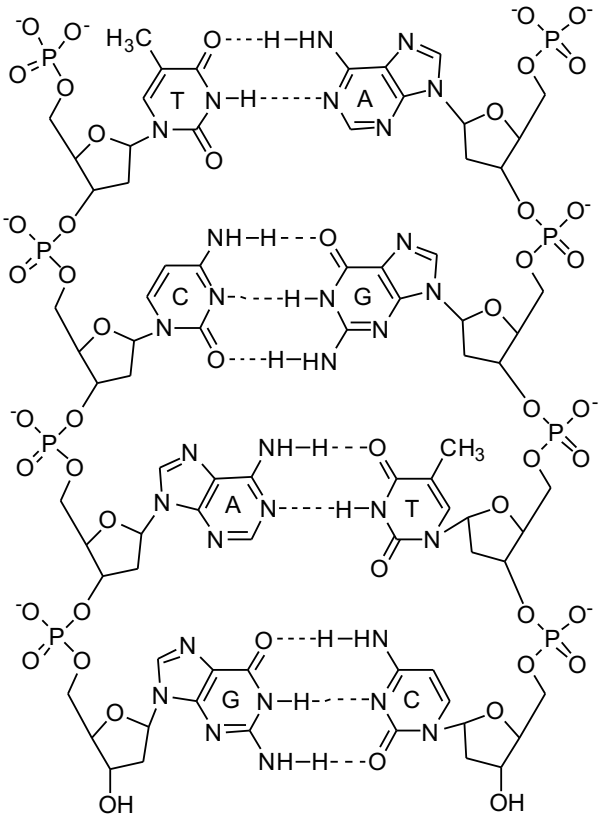


Figure 41: The complementary base pairing rule in the DNA molecule.

The main role of DNA in the cells is to maintain genetic information in an unchanged form. The entire set of DNA instructions found in a cell is called the **genome**. In the process of proteosynthesis, usually only one protein can be obtained from single **gene**. In practice, however, a single gene can encode multiple isoforms of a protein, depending on the specific exons (exons are coding sections of DNA in the eucaryotic genome) that are spliced. A gene is the basic functional unit of the genome. This is an essential function of DNA.

The DNA must be "decoded" to produce the characteristic primary structure of the protein. The analysis revealed that the number of nucleotides in the coding region of the gene is three times higher than the number of amino acids in the constructed protein. Three nucleotides (the triple codon) encode a single amino acid. Since there are 64 combinations of four nucleotides taken three at a time and only 20 amino acids, the code is degenerate (more than one codon per amino acid, in most cases). Codon tables, such as Table 10, list the amino acids encoded by mRNA codons, not DNA codons. If you have a DNA sequence, you must first transcribe it to make an mRNA, and then you can translate it into an amino acid sequence using a codon table. This is the central dogma of molecular biology (DNA ↔ RNA → protein).

Table 10: The genetic code for translating each nucleotide triplet in mRNA into an amino acid (AC) or a termination signal (stop codon) in a nascent protein.

		Second letter									
		U		C		A		G			
		code	AC	code	AC	code	AC	code	AC		
First letter	U	UUU UUC	Phe	UCU UCC UCA UCG	Ser	UAU UAC	Tyr	UGU UGC	Cys	U C A G	Third letter
		UUA UUG	Leu			UAA UAG	Stop	UGA	Stop		
								UGG	Trp		
	C	CUU CUC CUA CUG	Leu	CCU CCC CCA CCG	Pro	CAU CAC	His	CGU CGC CGA CGG	Arg	U C A G	
						CAA CAG	Gln				
	A	AUU AUC AUA	Ile	ACU ACC ACA ACG	Thr	AAU AAC	Asn	AGU AGC	Ser	U C A G	
		AUG	Met			AAA AAG	Lys	AGA AGG	Arg		
	G	GUU GUC GUA GUG	Val	GCU GCC GCA GCG	Ala	GAU GAC	Asp	GGU GGC GGA GGG	Gly	U C A G	
						GAA GAG	Glu				

The basic difference between prokaryotic and eukaryotic genomes is the organization of genetic information. **Prokaryotic** organisms have precisely defined individual parts of the gene:

- promoter – start codon – structural part of the gene – stop codon.

The **eukaryotic** genome is complex and consists of introns and exons. **Introns** form non-coding sequences of DNA (intruding sequences) within coding sequences (**exons**, expressing sequences).

An important feature of the eukaryotic DNA genome is that a large part of it consists of multiple repetitive sequences. These are shorter or longer homologous DNA fragments that are present in multiple copies in the genome. However, their functions remain unknown. Approximately 50 % of the human genome comprises repetitive sequences.

4.1.2. RNA

Ribonucleic acid (RNA) is present in the majority of living organisms and viruses. In terms of the primary structure, RNA is very similar to DNA. They differ in the pentose sugar part, with D-ribose instead of 2-deoxy-D-ribose, and a nitrogenous base part, with uracil instead of thymine. In the RNA molecule:

- the uracil – adenine base pair is held together by two hydrogen bonds and
- the guanine – cytosine base pair is held together by three hydrogen bonds (Figure 42).

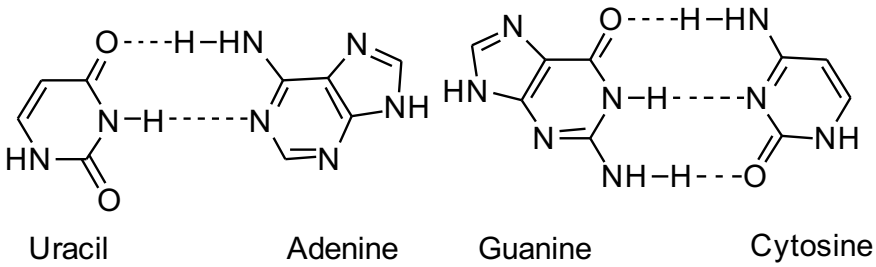


Figure 42: Single-stranded RNA can form secondary structures in which a single RNA molecule folds over and forms hairpin loops, stabilized by intramolecular hydrogen bonds between complementary bases.

RNA is a single-stranded nucleic acid polymer (Figure 43), so it does not form stable double-stranded structures like DNA and is therefore not subject to Chargaff's rules. The secondary and tertiary structures of RNA are less well known than those of DNA. This is due to the heterogeneous nature of RNA and the different spatial structures of certain type of RNA molecule.

Figure 43: Structure of single-stranded RNA.

The main function of RNA is the formation of proteins through translation. RNA carries genetic information that is translated by ribosomes into various proteins that are necessary for cellular processes. **mRNA, rRNA**, and **tRNA** are the three main types of RNA involved in protein synthesis. RNA also serves as the primary genetic material for viruses. Other functions include RNA editing, gene regulation, and RNA interference. These processes are carried out by a group of small regulatory RNAs, including small nuclear RNA, microRNA, and small interfering RNA. Individual ribonucleic acids differ significantly not only in terms of relative molecular weight and structure but also in terms of cell localization and biological function. The primary functions of some types of RNA are listed in Table 11.

Table 11: Different types of RNAs produced in cells and their functions.

Type of RNA	Primary function
messenger RNAs (mRNAs)	code for proteins
ribosomal RNAs (rRNAs)	form the basic structure of the ribosome and catalyze protein synthesis (essential in synthesis)
transfer RNAs (tRNAs)	serve as adapters for the translation of the information in the sequence of nucleotides of the mRNA into specific amino acids
small nuclear RNAs (snRNAs)	function in a variety of nuclear processes, including the splicing of pre-mRNA; splicing introns
small nucleolar RNAs (snoRNAs)	used to process and chemically modify rRNAs
microRNAs (miRNAs)	mainly involved in gene regulation

Table 11: Different types of RNAs produced in cells and their functions (continued).

Type of RNA	Primary function
small interfering RNAs (siRNAs)	inhibit gene expression through RNA interference
viral RNAs	occurs in plant and animal viruses; serves to store genetic information
other noncoding RNAs	function in diverse cellular processes, including telomere synthesis, X-chromosome inactivation, and the transport of proteins into the ER

4.1.3. Nucleoproteins

Nucleoproteins are complexes of nucleic acids with proteins. These include DNA complexes with histones, heterogeneous ribonucleoprotein particles, ribosomes, chromatin and viruses. These particles form complexes called megamolecules. Owing to their large molecular size, they are not soluble but form particles of different sizes.

Chromatin is a nucleoprotein complex composed of DNA, RNA, histones and non-histone proteins. Histones are the most abundant chromatin proteins, while non-histone proteins include enzymes involved in DNA replication (DNA topoisomerases). Chromatin is designed to regulate the transcription of genetic information encoded in the DNA strand. It represents the genetic material that makes up the chromosomes of eukaryotes.

4.1.4. Methods for the isolation of nucleic acids

DNA and RNA differ in their cellular location, function, structure and folding. The nucleic acid chains can be cleaved using either enzymatic or non-enzymatic methods. Non-enzymatic methods include alkaline hydrolysis and acid hydrolysis. Enzymatic hydrolysis is catalyzed by nucleases.

Alkaline hydrolysis

RNA is easily hydrolyzed under alkaline conditions. The hydrolysis involves breaking the phosphodiester bonds of RNA to form nucleotides (cyclic 2',3'-monophosphate nucleotides) that undergo further hydrolysis in water to form a mixture of 2'- and 3'-nucleoside monophosphates. The N-glycosidic bond is stable and does not undergo hydrolysis under these conditions. DNA is more resistant to alkaline hydrolysis (DNA is alkali resistant). DNA does not have the hydroxyl group at the 2' position of the pentose sugar, because deoxyribose in DNA has an -H instead of an -OH at the 2' position.

Acid hydrolysis

Mild acid hydrolysis (pH ~ 3) of nucleic acids produces a mixture of nucleotides, nucleosides and bases. Pyrimidine bases are more difficult to cleave than purine bases, so that purine bases predominate in the hydrolysate. In a strongly acidic environment and at a high temperature (6 M HCl, 175 °C), RNA and DNA are cleaved into their individual components. In addition, cytosine is deaminated to uracil, which must be considered when determining the ratio of bases in the polynucleotides.

The first step in isolating both DNA and RNA is to disrupt the cell membrane, and release the cell organelles into solution, followed by nucleic acid precipitation and isolation of this precipitate by centrifugation.

Centrifugation

Centrifugation is one of the most common operations performed in the biochemistry laboratory. It allows the separation of many or all components in a mixture based on density. This method is often faster and more convenient than the filtration method. In some cases, this leads to a better separation of the solid and liquid phases. Centrifugation can supplement or replace filtration (e.g., simple filtration techniques require time to separate the desired material) and also serves special preparative and analytical purposes (e.g., isolation of mitochondria from tissues and cultured cells by differential centrifugation at low- and high-speed).

A centrifuge operates by rotating at high speed and the substances are separated by centrifugal force. The centrifugal force (P) acting on the particles is expressed by the followed formula:

$$P = m \,.\, r \,.\, \omega^2$$

where m is the mass of the particles, r is the radius of gyration, and ω is the angular velocity. For practical calculations, it is more accurate to use the relative centrifugal acceleration (R). The relative centrifugal acceleration (also referred to as the centrifugal factor, *RCF*) represents the centrifugal force as a multiple of gravitational acceleration (g) and is given by the formula:

$$R = 1.118 \,.\, r \,.\, (RPM)^2 \,.\, 10^{-5}$$

where $1.118.10^{-5}$ is a constant, *RPM* is revolutions per minute and r is the radius of gyration (expressed in cm). *RPM* and *RCF* do not refer to the same thing. Both parameters are used to define the speed of the centrifuge. To avoid calculating the relative centrifugal acceleration in each case, manufacturers often supply centrifuge diagrams from which the *RPM* value can be easily derived (see Figure 44). To convert the maximum relative centrifugal force (*RCF*) to revolutions per minute (*RPM*), first measure the centrifuge's radius of rotation (in mm) by measuring the distance from the center of the centrifuge spindle to the bottom of the device when it is inserted into the rotor. Place a ruler or draw a line from the radius value in the right-hand column that corresponds to the maximum rated g force of the device. Then read the maximum value from column at left.

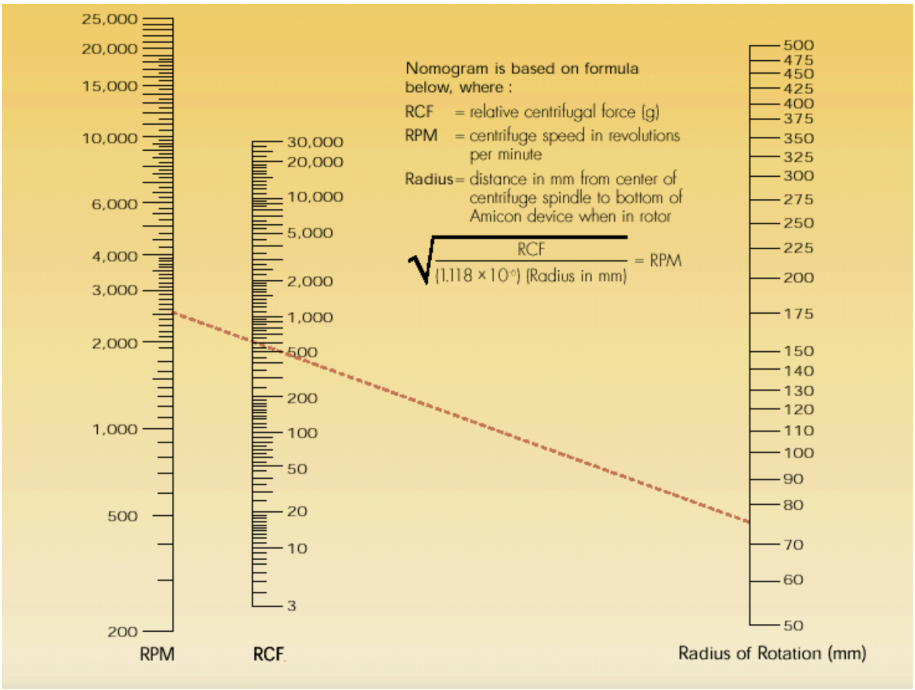


Figure 44: Nomogram for converting the maximum relative centrifugal force (*RCF*) to revolutions per minute (*RPM*).

Several types of centrifuges differ in size (microcentrifuges, small tabletop centrifuges, large-volume industrial centrifuges), the acceleration achieved (high-speed centrifuges, low-speed centrifuges, and ultracentrifuges) and rotor design (fixed angle centrifuges, swinging head centrifuges or continuous tubular centrifuges).
Fixed-angle centrifuges are designed to hold the sample containers at a constant angle (45-50°) relative to the central axis. Swinging head centrifuges have a hinge where the sample containers are attached to the central rotor. This allows all samples to swing outwards as the centrifuge rotates (see Figure 45). Continuous tubular centrifuges do not contain individual sample containers and are used for high volume applications.

Figure 45: A – fixed-angle centrifuge and B – swinging head centrifuge.

A special category is ultracentrifugation, in which a relative centrifugal acceleration of 100,000 x *g* or more can be achieved. The space around the rotor must be evacuated during centrifugation. Refrigerated centrifuges are required for most preparative and analytical procedures and for method validation. Centrifuges are cooled by cooling units that are integrated into the same operating unit as the centrifuge and controlled by a thermostat. The temperature ranges from -20 to -40 °C.

Using a centrifuge

Centrifuges are delicate, can break easily, and can be dangerous if they are not used properly. Following a few simple operating instructions should prevent any injuries during their use and the permanent damage of the centrifuge.

1. Before use, check the centrifuge bottles and tubes for cracks. Mark the tubes clearly for identification.
2. Close the tubes with the cap (if you are working with biohazardous material, wipe the outside of the tube with a disinfectant before inserting the tube into the centrifuge).
3. **The main rule when using a centrifuge is to make a counterbalance for the centrifuge tube** that you want to put in. Remember to balance the weights of the tubes, not their volumes. At high speeds, the centrifuge can easily become unbalanced if there are not equal weights placed opposite to each other in the rotor. Load the rotor symmetrically (see Figure 46) and ensure that the opposing

tube is not only the same type of tube but also filled with the same mass. Unbalanced tubes can cause permanent damage to a centrifuge.

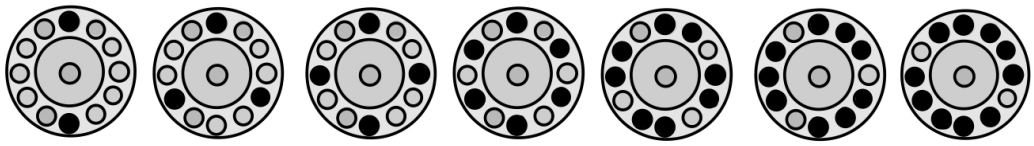
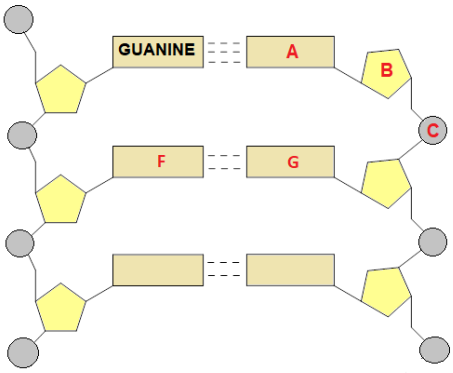


Figure 46: Correct symmetry when balancing a partial load.

4. Enter the centrifugation speed.
5. Keep a safe distance while the centrifuge is running. If the centrifuge wobbles, turn it off.
6. Open the lid only after the rotor has come to a complete stop and carefully remove the tubes.

Questions

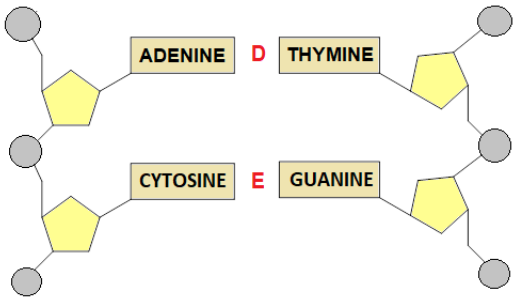
1. What is the genetic information?
2. What are nucleic acids? What are these two types of nucleic acids?
3. What is the building block of nucleic acids?
4. What are the three parts of a single nucleotide?
5. What is the difference between nucleotide and nucleoside?
6. What types of bonds are present in the nucleic acids?
7. What links 3',5'-phosphodiester bond in DNA/RNA?
8. What is the difference between the structure of DNA and RNA?
9. What are two Chargaff's rules?
10. How many hydrogen bonds are between
    a. A and T b. G and C?
11. What is the complementary base pairing rule for DNA?
12. What is the genome? What is the basic functional unit of the genome?
13. What does it mean that the genetic code is degenerate?
14. What is the central dogma of molecular biology?
15. Explain the difference between prokaryotic and eukaryotic genomes.
16. Does RNA follow Chargaff's rules? Explain your answer.
17. Name three main types of RNA involved in protein synthesis and their primary function in cells.
18. Complete the following picture:



    a. What parts of the nucleotide are labelled **A**, **B** and **C**?
    b. What is the part labelled **A**?
    c. Which parts, **A**, **B** or **C**, contain carbon?

d. What nitrogenous bases can be found at places **F** and **G**? Enter their full names.

19. Complete the following picture:



a. How many hydrogen bonds exist between nitrogenous bases at positions **D** and **E**? *

20. What is chromatin?
21. What are the two methods of cleaving nucleic acids?
22. Why does RNA degrade under alkaline condition, but DNA does not?
23. What is centrifugation?
24. How do you convert centrifuge RPM to RCF? Write the formula and name each variable.
25. Name the different types of centrifuges differ in their size, the acceleration achieved and rotor design.
26. What is the difference between fixed-angle and swinging head centrifuges?
27. What is one of the most important rules when using a centrifuge?

4.2. Experimental part A: Isolation of RNA from yeasts

Principle

A suitable material for RNA isolation is baker's yeast, which contains approximately 4 % (*w/w*) RNA. The yeast cell walls are disrupted with sodium hydroxide (NaOH) at elevated temperature to speed up extraction process. In this step, a mixture of oligo- and polyribonucleotides is obtained. The insoluble fraction, polysaccharides, and denatured proteins are separated by centrifugation. RNA is precipitated with isopropanol (isopropyl alcohol, IPA, C_3H_8O) at acidic pH, where the molecule loses its charge, and its concentration is determined spectrophotometrically. RNA, specifically the conjugated double bonds of heterocyclic nitrogenous (purine and pyrimidine) bases that make up RNA, absorbs radiation in the UV region with the maximum absorbance at 260-265 nm. The wavelength of 260 nm and the calibration curve method are used to determine the RNA concentration in each sample.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), glass rods, beakers (250 ml), graduated cylinder, mortar and pestle, volumetric flask (10 ml), centrifuge tubes, centrifuge, dye-infused paper strips (universal indicator paper, pH 1-14), filter paper, hot plate, asbestos mesh (wire mesh gauze), analytical balance, pre-weighing scale, and spectrophotometer.

Materials and chemicals

- Baker's yeast (fresh compressed yeast),
- sodium hydroxide, 10 % (*w/v*) aqueous solution (M = 39.9971 g/mol, V = 10 ml),
- concentrated acetic acid (CH_3COOH, 99 %, *v/v*),
- concentrated hydrochloric acid (HCl, 36 %, *v/v*),
- isopropanol ($(CH_3)_2CHOH$),
- acetone (CH_3COCH_3).

Procedure

RNA isolation procedure

1. Mix 20 g of baker's yeast (broken into small pieces) with 2 ml of 10 % (*w/v*) aqueous sodium hydroxide solution and 50 ml of distilled water. Grind in a mortar and transfer it quantitatively into a 250 ml beaker.
2. Place the beaker on a hot plate with an asbestos mesh (wire mesh gauze) and heat to 95 °C for 15 minutes, stirring occasionally.
3. Then carefully neutralize the mixture to pH 6.0 with acetic acid and cool with water.
   Check the pH value with a universal pH paper.
4. Centrifuge the yeast solution at 3,000 RPM for 15 minutes.
5. Carefully decant the opalescent supernatant from the sediment and add concentrated hydrochloric acid dropwise to pH 2.0.
   Check the pH value with a universal pH paper. Do not pipette a single drop of concentrated hydrochloric acid using a single-channel pipette. Use a glass pipette or glass dropper.
6. Mix the mixture with isopropanol in a 1:1 (*v/v*) ratio.
   Measure the volume of the mixture using a graduated cylinder and mix with an equal volume of isopropanol (e.g., mix 40 ml of the mixture with 40 ml of alcohol).
7. Put the tube in an ice bath, and after approximately 10 minutes, centrifuge the RNA precipitate (3,000 RPM, for 15 minutes).
   If you do not observe the formation of a precipitate, extend the time.

8. Carefully decant isopropanol from the RNA precipitate and wash the precipitate with 5 ml isopropanol. Centrifuge (3,000 RPM, for 15 minutes).
9. Carefully decant isopropanol and wash the RNA precipitate with 5 ml acetone. Centrifuge (3,000 RPM, for 15 minutes) in a pre-weighed tube.
10. Weigh the precipitate in the pre-weighted centrifuge tube (see Figure 47).

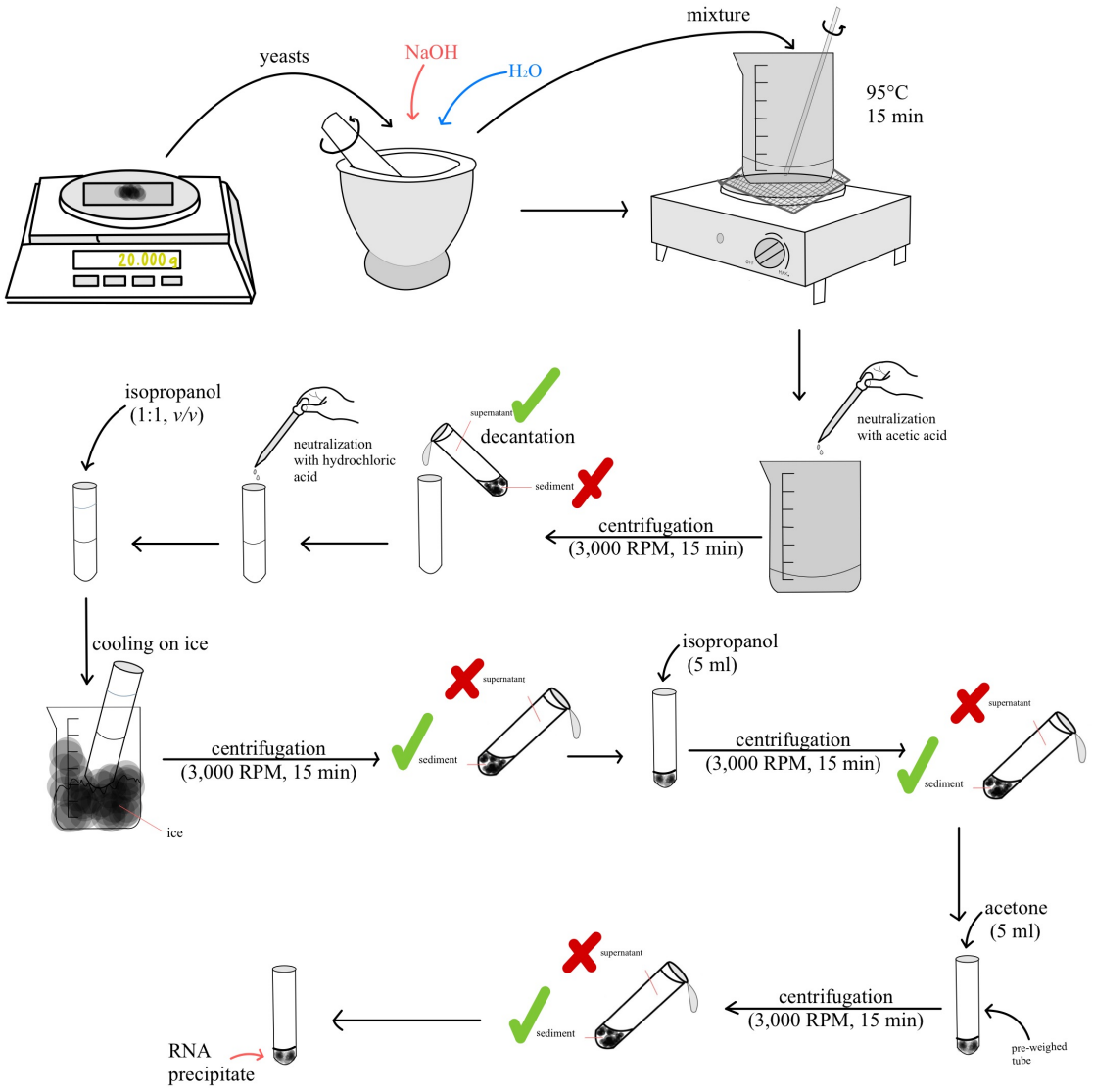


Figure 47: RNA isolation procedure.

11. Dissolve 5 mg of the air-dry precipitate in 5 ml of distilled water.
    Record the weight of the RNA precipitate.
12. Measure the spectrum of this solution in the UV region in the wavelength range of 200-300 nm.

Spectrophotometric determination of RNA

Use the A_{280}/A_{260} absorbance ratio to determine RNA (%) according to the method described by Warburg and Christian (see Table 12).

Table 12: *F* factor values according to Warburg and Christian.

A_{280}/A_{260}	% nucleic acids	F
1.75	0.00	1.116
1.63	0.25	1.081
1.52	0.50	1.054
1.40	0.75	1.023
1.36	1.00	0.994
1.30	1.25	0.970
1.25	1.50	0.944
1.16	2.00	0.899
1.09	2.50	0.852
1.03	3.00	0.814
0.939	4.00	0.743
0.874	5.00	0.682
0.846	5.50	0.656
0.822	6.00	0.632
0.804	6.50	0.607
0.784	7.00	0.585
0.767	7.50	0.565
0.753	8.00	0.545
0.730	9.00	0.508
0.705	10.00	0.478
0.671	12.00	0.422
0.644	14.00	0.377
0.615	17.00	0.322
0.595	20.00	0.278

The *F* factor is used to calculate the amount of ballast proteins present in the sample, based on the following formula:

$$proteins\ (mg/ml) = F.\ A_{280} / d$$

where *d* is the optical path of the cuvette expressed in cm and *F* is the factor from Table 12.

Results

Calculate the percent yield (%) of the RNA extraction procedure from yeast. For this purpose, you can use the amount of RNA quantification data from the literature (4 %, *w/w*). Calculate the amount of ballast proteins (mg/ml) obtained by spectrophotometric analysis of the precipitate.

Questions

1. Why is baker's yeast a suitable material for RNA isolation?
2. Is it possible to isolate DNA by alkaline hydrolysis?
3. Why is RNA isolated at acidic environment?
4. How are the insoluble fractions, polysaccharides and denatured proteins separated from the RNA?
5. What component of RNA is responsible for UV absorption?
6. How much potassium hydroxide (KOH) should you use to make 50 ml of its 1 % *(w/v)* solution?
7. Calculate the amount of ballast proteins (in mg/ml) and the percent yield of RNA (%) that were present in the precipitate if you know that the absorbance of the

solution at 280 nm was 0.726, at 260 nm was 0.624, and the path length of the cuvette was 1 cm. For the calculation of the yield, you can use the amount of RNA quantification data from the literature (4 %, *w/w*).

4.3. Experimental part B: Determination of RNA

Principe

The method is based on the reaction of ribose with orcin (orcinol, 5-methylresorcinol) in the presence of hot hydrochloric acid and ferric chloride to form a blue-green reaction product (see Figure 48). The concentration of this product can be determined at 670 nm. The intensity of the color change depends on the concentration of the hydrochloric acid, ferric chloride and orcin, heating time at 100 °C and the ribose concentration in the sample. Determination of RNA concentration using the orcin reagent is based on the Bial's test, which determines the presence of pentoses in the solution. Bial's test is useful for distinguishing pentoses sugar from hexoses sugars.

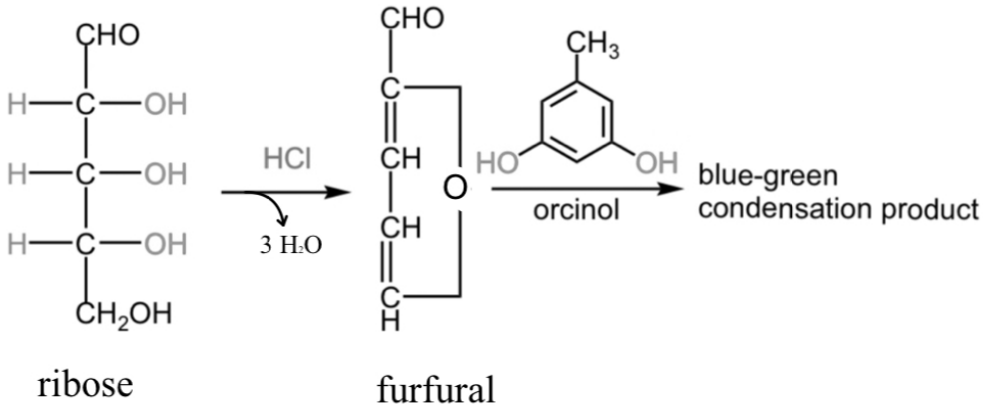


Figure 48: Ribose sugar form furfural in acidic medium which condense with orcinol in presence of ferric ions to give blue-green colored product.

Since the concentration of the sample is not known, it can be obtained by plotting a graph with ribose concentration on the x-axis and absorbance on the y-axis.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, test tubes, volumetric flasks (10 ml, 20 ml), water bath, hot plate, analytical balance, laboratory vortex, and spectrophotometer.

Materials and chemicals

- The sample with unknown RNA concentration from the previous laboratory exercise,
- the stock solution of ribose with a concentration of 1 g/l (M = 150.13 g/mol, 20 ml),
- the orcin reagent (add 10 mg of ferric chloride hexahydrate and 10 mg of orcin in 10 ml of concentrated hydrochloric acid, always prepare fresh).

Procedure

The preparation of the stock solution of ribose and its dilute solutions

1. Prepare the stock solution of ribose with a concentration of 1 g/l by weighing out 0.01 g of the solid sample, transferring the quantitative to a 10 ml volumetric flask and diluting to the mark with distilled water.
2. Through appropriate dilutions of the stock solution of the ribose, prepare calibration solutions as follows:

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
100	10.0	0.500	4.500
80	12.5	0.400	4.600
60	16.7	0.300	4.700
40	25.0	0.200	4.800
20	50.0	0.100	4.900
10	100.0	0.050	4.950

3. Prepare the dilute solutions (calibration solutions) in test tubes. Dilute the stock solution of ribose and distilled water using a one-channel pipette. The total volume of dilute solution is 5 ml.

Determination of RNA with the orcinol reagent

1. Mix 1 ml of the solution (a sample or ribose solution) with 1 ml of the orcinol reagent in a test tube.
   Work in three parallel measurements. Prepare a set of test tubes. Take 24 clean and dry test tubes to determine the absorbance the sample or calibration solutions.
2. Keep the test tube in a boiling water bath for 20 minutes in a fume hood.
3. Take the tubes and cool to laboratory temperature.
4. Add 4 ml of distilled water to each test tube.
5. Prior to making all absorbance readings, a spectrophotometer must be calibrated using a blank solution, which is comprised of a cuvette filled with 2/3 full distilled water for this experiment.
6. Measure the absorbance of the cooled samples at 670 nm.
7. Record the results in your laboratory notebook.

Results

Calculate for each calibration solution the average of absorbances, and produce a plot of absorbance vs. actual concentration (the x-y relationship). Carry out a linear regression analysis to determine the equation of the relationship between absorbance and concentration ($y = ax + b$) as well as the coefficient of determination. Calculate the concentration of RNA (mg/l) in the sample.

Questions

1. What pentose sugar is found in RNA?
2. What organic bases are found in RNA?
3. What is the principle of the orcinol test for RNA?
4. The stock solution of ribose with a concentration of 1 g/l should be diluted to the concentrations shown in the table. Calculate the dilution factor, the volume of stock solution and the volume of distilled water needed to produce a solution of a give concentration. The total volume of dilute solution must be 4 ml.

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
100			
75			
50			
25			
10			

5. A sample containing an unknown concentration of RNA has an absorbance of 0.392. The blank (distilled water with the orcinol reagent) has absorbance of 0.042. Calculate its ribose concentration using the linear regression equation $y = 0.0058x + 0.0419$ for ribose solutions in the concentration range of 10-100 mg/l.

4.4. Experimental part C: Isolation of DNA from yeasts

Principle

Deoxyribonucleic acid (DNA) binds to basic proteins in the form of deoxyribonucleoprotein (DNP) in eukaryotic cells. Its isolation is based on tissues that contain cells with large nuclei (e.g., the spleen) or cell biomass. Extraction is performed using sodium chloride (NaCl) solution. Sodium chloride helps to remove proteins that are bound to the DNA. It also helps to keep the proteins dissolved in the aqueous layer, so they do not precipitate in alcohol along with the DNA. After dilution of the solution with cold distilled water, the DNA is separated as a fibrous product. Cold water helps to keep the DNA intact during the extraction process. Moreover, cooling slows down the enzymatic reactions. This protects DNA from enzymes that can destroy it.

The concentration of DNA can be determined spectrophotometrically at the A_{280}/A_{260} absorbance ratio because the conjugated double bonds of heterocyclic nitrogenous bases (purine and pyrimidine) that make up DNA, absorbs radiation in the UV region, with the maximum absorbance at 260-265 nm.

Laboratory requirements

Beakers (250 ml), mortar and pestle, centrifuge tubes, wooden stick (skewer), filter paper, analytical balance, centrifuge, graduated cylinder, pre-weighing scale, and spectrophotometer.

Materials and chemicals

- Baker's yeast (fresh compressed yeast),
- sea sand (purified),
- sodium chloride (NaCl) with a concentration of 1 mol/l (M = 58.44 g/mol, V = 80 ml).

Procedure

DNA isolation procedure

1. Mix 10 g of baker's yeast (broken into small pieces) with a small amount of sea sand (purified) and 80 ml of 1 mol/l NaCl. Grind in a mortal for 10-15 minutes and transfer it quantitatively into a 250 ml beaker.
2. Centrifuge the viscous solution at 5,000 RPM for 10 minutes.
3. Measure the volume of the mixture using a graduated cylinder and very slowly add the cold water in a 1:6 (*v/v*) ratio.
4. The deoxyribonucleoprotein is precipitated as long fibers which are wound on a pre-weighed wooden stick (skewer, glass stick is not suitable). Dry the coiled precipitate between filter papers and weigh.
5. Dissolve 5 mg of the air-dry precipitate in 5 ml of distilled water.
   Record the weight of the DNA precipitate.
6. Measure the spectrum of this solution in the UV region in the wavelength range of 200-300 nm.

Spectrophotometric determination of DNA

Use the A_{280}/A_{260} absorbance ratio to determine DNA (%) according to the method described by Warburg and Christian (see Table 13).

Table 13: The *F* factor values according to Warburg and Christian.

A_{280}/A_{260}	% nucleic acids	F
1.75	0.00	1.116
1.63	0.25	1.081
1.52	0.50	1.054
1.40	0.75	1.023
1.36	1.00	0.994
1.30	1.25	0.970
1.25	1.50	0.944
1.16	2.00	0.899
1.09	2.50	0.852
1.03	3.00	0.814
0.939	4.00	0.743
0.874	5.00	0.682
0.846	5.50	0.656
0.822	6.00	0.632
0.804	6.50	0.607
0.784	7.00	0.585
0.767	7.50	0.565
0.753	8.00	0.545
0.730	9.00	0.508
0.705	10.00	0.478
0.671	12.00	0.422
0.644	14.00	0.377
0.615	17.00	0.322
0.595	20.00	0.278

The *F* factor is used to calculate the amount of ballast proteins present in the sample, based on the following formula:

$$proteins\ (mg/ml) = F.\ A_{280} / d$$

where *d* is the optical path of the cuvette expressed in cm and *F* is the factor from Table 13.

Results

Calculate the percent yield (%) of the DNA extraction procedure from yeast. For this purpose, you can use the amount of DNA quantification data from the literature (14 %, *w/w*). Calculate the amount of ballast proteins (mg/ml) obtained by spectrophotometric analysis of the precipitate.

Questions

1. Why is the purpose of adding water in DNA extraction?
2. What is the purpose of sodium chloride in DNA extraction?
3. How can we prevent DNA degradation by enzymes during extraction?
4. In which wavelength range does DNA absorb UV?
5. What component of DNA is responsible for UV absorption?
6. What is the rationale behind using the A_{260} A_{280} in the spectrophotometric measurements of DNA?
7. What pentose is found in DNA?
8. What bases are found in DNA?
9. How much NaCl should you use to make 80 ml of its solution with a concentration of 1 mol/l (M = 58.44 g/mol)?

4.5. Experimental part D: Determination of DNA

Principe

DNA is quantitatively depurinated in an extremely acidic environment, and subsequently the sugar is dehydrated to hydroxylevulinyl aldehyde. This aldehyde reacts with diphenylamine in an acidic environment to form a dark blue product with an absorption maximum at a wavelength of 595 nm (see Figure 49). The intensity of the blue color is proportional to the concentration of DNA. This method detects the deoxyribose of DNA and does not interact with the ribose in RNA.

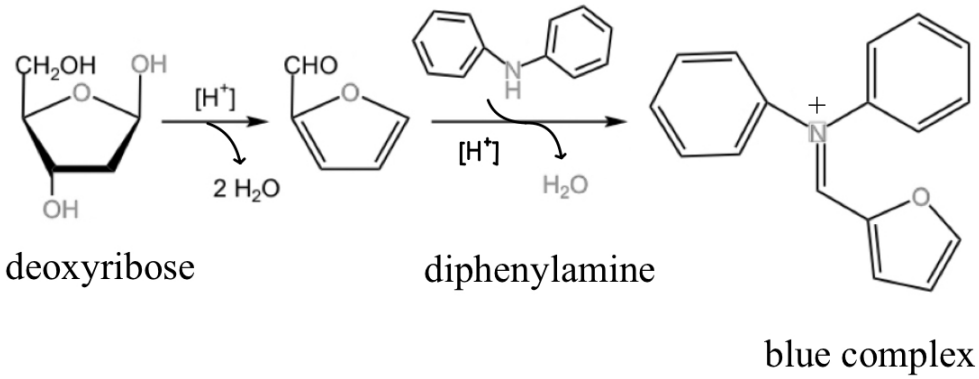


Figure 49: Deoxyribose sugar reacts with diphenylamine to produce a blue-green colored product.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), beakers (250 ml), volumetric flask (100 ml), analytical balance, hot plate, water bath, and spectrophotometer.

Materials and chemicals

- The sample with unknown DNA concentration from the previous laboratory exercise,
- the stock solution of deoxyribose with a concentration of 1 g/l (M = 134.13 g/mol, 20 ml),
- the diphenylamine reagent (mix 1 g of freshly recrystallized diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of concentrated sulfuric acid). Warm to room temperature before use.

Procedure

The preparation of the stock solution of deoxyribose and its dilute solutions

1. Prepare the stock solution of deoxyribose with a concentration of 1 g/l by weighing out 0.01 g of the solid sample, transferring it quantitative to a 10 ml volumetric flask and diluting to the mark with distilled water. Prepare a concentration of 1 mg/l by decimal dilution (see Figure 50).

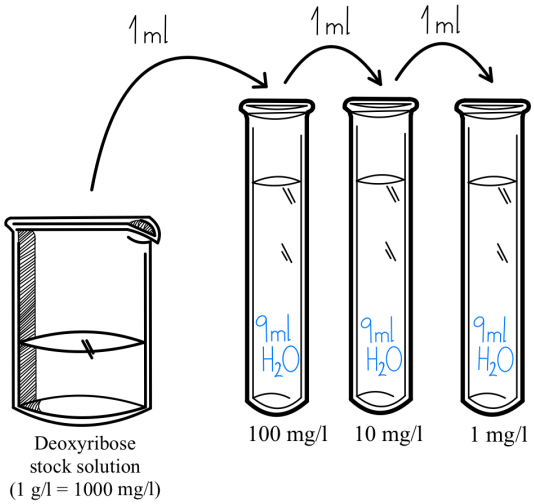


Figure 50: Schematic procedure of the decimal dilution of deoxyribose stock solution.

2. Through appropriate dilutions of the stock solution of the deoxyribose, prepare calibration solutions as follows:

Concentration (µg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
200	5	1.000	4.000
150	6.7	0.750	4.250
100	10	0.500	4.500
75	13.3	0.375	4.625
50	20	0.250	4.750
25	40	0.125	4.875
10	100	0.050	4.950

3. Prepare the dilute solutions (calibration solutions) in test tubes. Dilute the stock solution of deoxyribose and distilled water using a one-channel pipette. The total volume of dilute solution is 5 ml.

Determination of DNA with the diphenylamine reagent

1. Mix 1 ml of the solution (a sample or deoxyribose solution) with 2 ml of the diphenylamine reagent in a test tube.
   Work in three parallel measurements. Prepare a set of test tubes. Take 24 clean and dry test tubes to determine the absorbance of the sample or calibration solutions.
2. Keep the test tube in a boiling water bath for 10 minutes in a fume hood.
3. Take the tubes and cool to laboratory temperature.
4. Prior to making all absorbance readings, a spectrophotometer must be calibrated using a blank solution, which comprises a cuvette filled with 2/3 full distilled water for this experiment.
5. Measure the absorbance of the cooled samples at 595 nm.
6. Record the results in your laboratory notebook.

Results

Calculate for each calibration solution the average of absorbances, and produce a plot of absorbance vs. actual concentration (the x-y relationship). Carry out a linear regression

analysis to determine the equation of the relationship between absorbance and concentration ($y = ax + b$) as well as the coefficient of determination. Calculate the concentration of DNA (µg/l) in the sample.

Question

1. What is the principle of DNA determination using diphenylamine reagent?
2. How is the DNA concentration linked to the color intensity of the solution?
3. You had a solution containing 4 g of deoxyribose per liter. You diluted this original solution by adding 1 ml of its to 9 ml distilled water. Then, you made a 1:100 dilution of the resulting solution. What is the final concentration of a solution?

5. Lipids

5.1. Theory

Most components of biological systems are water-soluble. A cell is an organized biological system whose individual parts, in which different biochemical processes occur, are separated by a phase interface. The substances forming these interfaces are called **lipids** (from the Greek *lipos* = fat). Derivatives - esters or amides of higher carboxylic acids – are referred to as lipids but are generally compounds soluble in non-polar solvents (chloroform, ether, benzene) that can be extracted from biological material.

Lipids are esters and ethers of alcohols and carboxylic acids (mainly fatty acids) and are classified as simple and complex lipids. An ester bond binds the fatty acid to the hydroxyl group of the alcohol in the lipid. The alcohols most commonly found in lipids are glycol (C_2), glycerol (C_3), cetyl alcohol (C_{16}), ceryl alcohol (C_{26}), melissyl alcohol (C_{30}), and sphingosine (C_{18}). Carboxylic acids can be saturated with a linear or branched chain and unsaturated with one, two, or three double bonds, cyclic, and in specific lipids, hydroxy derivatives of fatty acids are also possible.

Lipids perform several **biological functions** in organisms, the most important of which are:

- **Source of energy and essential substances.** Fats and oils are the most concentrated forms of energy in food. They make food tastier and are important for slow digestion. Digestion does not begin in the stomach but in the small intestine. This group also includes fat-soluble vitamins and essential higher carboxylic acids found in foods with a natural fat and oil content.
- **Transmission of stimuli.** Polar lipids are found in tissues responsible for transmitting of nerve impulses (nerve tissue contains up to 40 % lipids).
- **Protective functions.** Some acylglycerols (fats) cover certain organs (e.g., kidneys) and protect them from mechanical damage. Subcutaneous fat acts as an insulating barrier, preventing excessive heat and water loss to the surroundings.
- **Structural functions.** Polar lipids spontaneously orient in mono- and bilayers and form the structural core of biomembranes. The structure of the biomembrane in the form of a phospholipid bilayer will be illustrated by the example of the cytoplasmic membrane (see Figure 51).

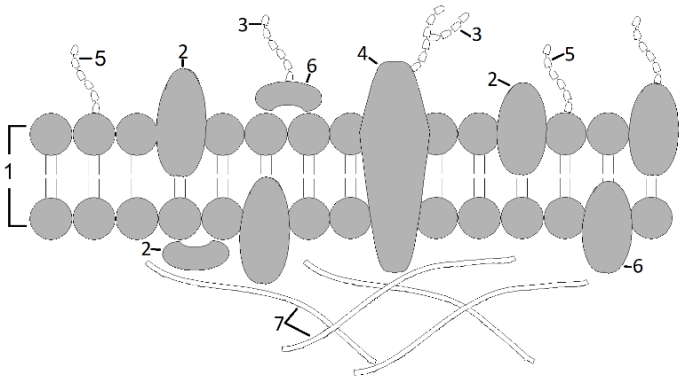


Figure 51: The structure of the cytoplasmic membrane (1- phospholipid bilayer, 2 - integral glycoprotein, 3 - oligosaccharide side chain, 4 - integral glycoprotein with a channel, 5 -oligosaccharide bound in glycolipid, 6 - peripheral proteins, and 7 - filaments (fibrous proteins) of the cytoskeleton in the surface cytoplasm.

5.1.1. Simple lipids

Simple lipids are esters of alcohols and carboxylic acids.

- **Fats** are esters of fatty acids with glycerol. Oils are fats in the liquid state.
- **Waxes** are esters of fatty acids with higher molecular weight monohydric alcohols. Waxes are non-polar, solid, plastic substances chemically stable in air. In plants, they form protective coatings on leaves and fruits that protect them from drying out. In animals, waxes are found in hair, wool, and fur, protecting these body parts from absorbing water.

Fatty acids occur mainly as part of the esters present in natural fats and oils. Fatty acids in natural fats usually have an even number of carbons because they are synthesized from two-carbon units. The chain may be saturated (containing no double bonds) or unsaturated (containing one or more double bonds) (see Figure 52).

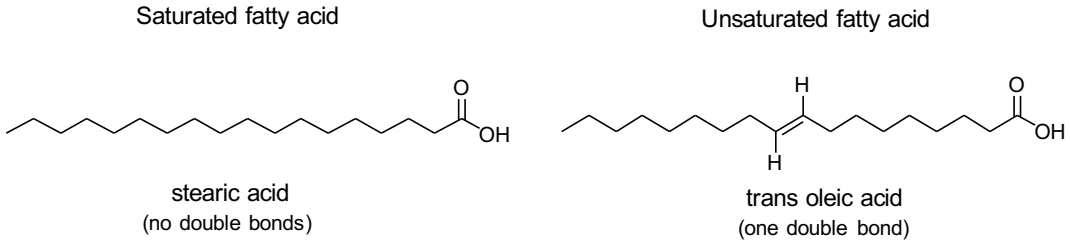


Figure 52: Differences between saturated and unsaturated fatty acids.

The physical properties of lipids vary depending on the length of the fatty acid chains. The melting point of fatty acids increases with increasing chain length and decreases with increasing number of double bonds (see Table 14).

Table 14: Common fatty acids with their chemical formulas and melting points.

common name	chemical formula	melting point
Saturated fatty acids		
lauric acid	$CH_3(CH_2)_{10}CO_2H$	45 ºC
myristic acid	$CH_3(CH_2)_{12}CO_2H$	55 ºC
palmitic acid	$CH_3(CH_2)_{14}CO_2H$	63 ºC
stearic acid	$CH_3(CH_2)_{16}CO_2H$	69 ºC
arachidic acid	$CH_3(CH_2)_{18}CO_2H$	76 ºC
Unsaturated fatty acids		
palmitoleic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7CO_2H$	0 ºC
oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7CO_2H$	13 ºC
linoleic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7CO_2H$	-5 ºC
linolenic acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7CO_2H$	-11 ºC
arachidonic acid	$CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2CO_2H$	-49 ºC

Natural lipids contain alcohols such as glycerol, cholesterol and higher alcohols (cetyl alcohol, most commonly found in waxes or dolichol) or fatty aldehydes, which are formed by reducing fatty acids. The most common are esters of the trisaturated alcohol glycerol and of some fatty carboxylic acids - triacylglycerols.

From a nutritional point of view, the term omega (ω) unsaturated fatty acids is used in the literature, especially omega-3 and omega-6 unsaturated fatty acids (the numbering is

derived from the position of the last double bond from the last, terminal, so-called omega carbon of the fatty acid chain).

Reactions of simple lipids

1. **Oxidation of lipids** takes place on double bonds of unsaturated carboxylic acids, leading to cleavage of the aliphatic chain to form aldehydes, ketones, and lower carboxylic acids. Oxidation of lipids in food is manifested by the yellowing of fats (rancidity) and subsequent off-odor/flavor, taste, and texture. Bacteria or oxidizing agents can cause the oxidation of fats in the presence of oxygen in a hot and humid environment. The parameter of the technological quality of lipids is peroxide number. High peroxide levels are a sign of lipid oxidation.
2. **The hydrogen addition to lipids** (catalytic hydrogenation of carboxylic acids in lipids) takes place on double bonds, converting unsaturated fatty acids to saturated ones. This process is used to prepare hydrogenated fats (also called trans-fatty acids) from vegetable oils. It prevents them from becoming rancid as well as keeps them solid at room temperature. This process is called fat hydrogenation.
3. **Hydrolysis of lipids** involves breaking the ester bond of acylglycerols, releasing carboxylic acids and glycerol molecules. Hydrolysis of lipids can be:
    a. *acid hydrolysis* (by strong mineral acids),
    b. *alkaline hydrolysis* (by hydroxides) and
    c. *enzymatic hydrolysis* (by enzymes called lipases).

The alkaline hydrolysis of fats produces sodium or potassium salts of fatty acids called soaps. Therefore, this reaction is also called saponification (see Figure 53).

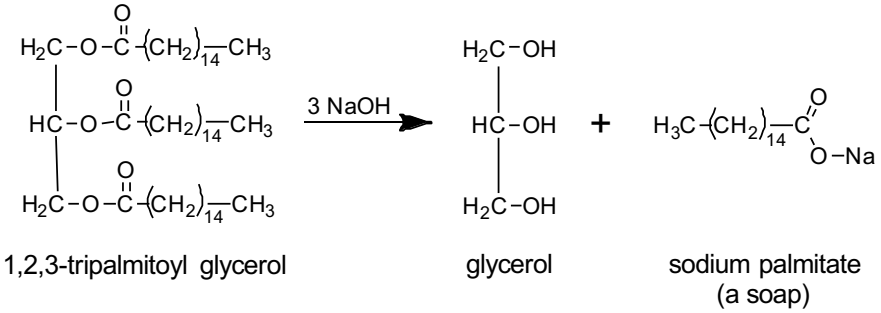


Figure 53: Alkaline hydrolysis of fats – saponification.

5.1.2. Complex lipids

Complex lipids are esters of fatty acids containing groups in addition to alcohol and fatty acids such as phospholipids, glycolipids, precursor, and derived lipids.

a. Phospholipids contain a phosphoric acid residue, fatty acid, and alcohol. They often have nitrogenous bases and other substituents and are divided into:
    - **glycerolphospholipids** with glycerol as the alcohol and
    - **sphingophospholipids** with the alcohol sphingosine.

b. Glycolipids (glycosphingolipids) contain fatty acids, sphingosine, and a saccharide component (e.g., glucose or galactose).

c. Other complex lipids are sulfolipids and aminolipids and may include lipoproteins.

Complex lipids are amphiphilic molecules and are an important component of cell membranes that ensure compartmental fragmentation of the environment in the cell. An **amphiphilic molecule** has one part of the molecule polar - hydrophilic - and another part non-polar - hydrophobic. These molecules are oriented at the interface of the oil and

aqueous phases, with polar groups facing the aqueous phase and non-polar groups facing the lipophilic phase.
Biological membranes form a natural barrier between the internal and external environment of the cell. Like the cell itself, cell organelles are bounded by specific intracellular membranes whose chemical diversity indicates the diverse function of the individual organelles. Biological membranes are composed of hundreds of different proteins and lipids. Lipids maintain optimal membrane permeability and serve as a support matrix for proteins anchored in membranes. Lipids are not only passive components forming an impermeable barrier but also dynamic regulators of many cellular processes. Cell membranes contain more types of lipids than those needed to form the lipid bilayer. Certain specific lipid compositions of biological membranes are required for the proper functioning of all membrane-related cellular processes. Changes in the composition and organization of membrane lipids significantly affect such vital processes as signal transduction and intercellular material exchange.

5.1.3. Degradation of complex lipids

Complex lipids are broken down in the digestive tract and in cells by appropriate enzymes. The enzymes involved in the hydrolysis of phospholipids are **phospholipases**. Due to their hydrolytic properties, phospholipases play an indispensable role in the process of membrane remodeling. The cell reuses most of the degradation products to synthesize new molecules. Many other phospholipid degradation products have essential signaling and regulatory roles in eukaryotic cells. As an example, phosphorylated forms of phosphatidylinositol and its derived molecules, inositol phosphates, play a vital function in intracellular signaling. Phospholipases are also essential in nutrient degradation, cell growth, secretion, respiration, cell cycle, differentiation, and cellular stress response.

Questions

1. What are lipids?
2. What are the main functions of lipids in the human body?
3. What are simple lipids? What are the 2 main types of simple lipids?
4. What are two examples of saturated and unsaturated fatty acids? What is the difference between saturated and unsaturated fatty acids?
5. Which are the three main reactions of simple lipids?
6. Write the reaction scheme for the alkaline hydrolysis of 1,2,3-tripalmitoylglycerol with sodium hydroxide.
7. What are complex lipids? What are three groups of complex lipids?
8. What is an amphiphilic molecule?
9. What is the enzyme for simple lipid degradation?
10. What is the enzyme for complex lipid degradation?

5.2. Experimental part A: Determination of free fatty acids (FFA)

Principle

The free fatty acids in the oil can be determined by titration with a stock solution of potassium hydroxide (KOH) in the presence of a phenolphthalein indicator. The content of free fatty acids is expressed by the **acid number (acid value)**, which represents the amount of potassium hydroxide (in mg) needed to neutralize the free fatty acids present in 1 g of the sample. The free fatty acid content is usually expressed as oleic acid equivalents. During storage, fats may go rancid due to the formation of peroxides on their double bonds by oxidation and their hydrolysis by microorganisms with the release of free acids. The number of free acids associated with the fat gives a sufficient image of its quality and age.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, Erlenmeyer flask (100 ml), volumetric flasks, burette, magnetic stirrer, magnetic stirring pellet, analytical balance.

Materials and chemicals

- Oil sample (linseed or rapeseed oil) (freshly opened and opened for several weeks),
- phenolphthalein, 1 % (*w/v*) indicator solution (M = 318.323 g/mol) in 96 % (*v/v*) ethanol (V = 10 ml),
- potassium hydroxide (KOH) with a concentration of 0.1 mol/l (0.1 N, M = 56.11 g/mol, V = 100 ml),
- isopropanol ($(CH_3)_2CHOH$), V = 25 ml).

Procedure

1. Add 10 g of oil to 25 ml of isopropanol in a 100 ml Erlenmeyer flask.
2. Insert the magnetic stirring pellet and stir on the magnetic stirrer for 10 minutes.
3. Add a few drops (2-3) of phenolphthalein solution (1 %, *w/v)*.
4. Titrate the contents against 0.1 N KOH. Shake constantly until a pink color, which persists for fifteen seconds, is obtained (see Figure 54).

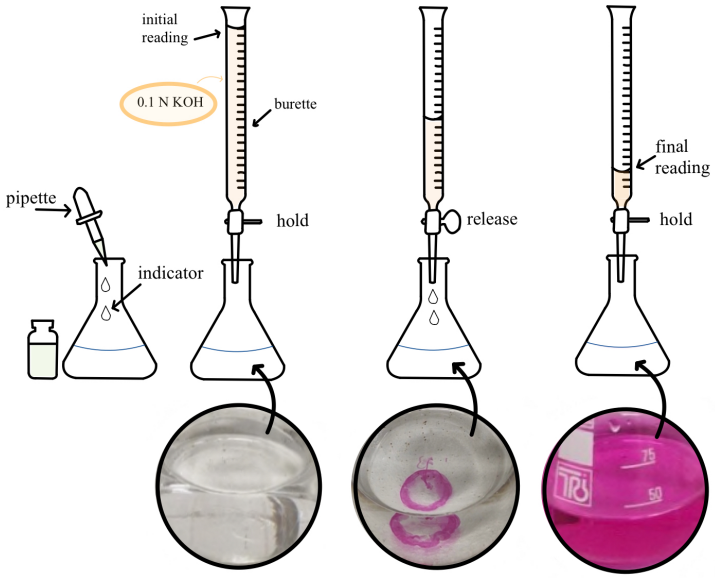


Figure 54: Determination of free fatty acids by titration method.

Acid number (acid value)
The acidity number of the selected oil is calculated as follows:

$$Acid\ number = \frac{56.1 \times N \times V}{sample\ weight\ (g)}\ [mg\ KOH/g\ of\ oil]$$

where N is the normality of the potassium hydroxide solution used in the titration, V is the consumption of potassium hydroxide solution required to produce a pink color, and *56.1* is the equivalent weight of potassium hydroxide.

Results
Compare the acid number of the freshly opened oil with the acid number of the oil opened for several weeks and then compare the results with the literature.

Questions
1. What is an acid number?
2. What are two examples of saturated and unsaturated fatty acids?
3. How much potassium hydroxide (KOH; M = 56.11 g/mol) should you use to make 100 ml with a concentration of 0.1 N?
4. How much phenolphthalein should you use to make 10 ml of its 1 % (*w/v*) solution?
5. Calculate the acid number of olive oil. The sample weighed 10.245 g, and the volume of KOH (0.15 N) consumed by the sample was 3.6 ml.
6. To determine the acid number of rapeseed oil, the experiment was carried out in triplicate as the following:
   Weight of sample 1-A = 10.023 g
   Weight of sample 1-B = 10.062 g
   Weight of sample 1-C = 9.982 g
   The volume of KOH (0.1 N) consumed by:
   Sample 1-A = 3.2 ml
   Sample 1-B = 3.3 ml
   Sample 1-C = 2.9 ml
   Calculate the arithmetic mean (average) of acid number and standard deviation.

5.3. Experimental part B: Saponification value

Principle
The oil is saponified under reflux in the presence of potassium hydroxide (KOH) in alcohol, and free fatty acids and glycerol are released. The refluxing with an alkali causes the hydrolysis of glyceryl esters, yielding glycerol and potassium salts of fatty acids (soaps) (see Figure 55).

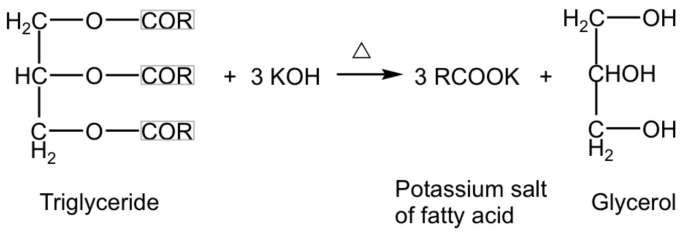


Figure 55: Alkaline hydrolysis of fats – saponification.

The test sample is titrated against HCl to determine the amount of KOH used in the saponification process. The excess of potassium hydroxide is determined by back titration with a stock solution of hydrochloric acid (HCl). The **saponification value** of an oil is defined as the number of mg of potassium hydroxide required to neutralize free and ester-bound fatty acids in 1 g of sample.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, Erlenmeyer flask (100 ml), volumetric flasks, burette, condenser, single-neck round bottom flask, water bath, hot plate, clamp, analytical balance.

Materials and chemicals

- Oil sample (linseed or rapeseed oil),
- hydrochloric acid (HCl) with a concentration of 0.5 mol/l (0.5 N, M = 36.46 g/mol, $\rho_{36\,\%}$ = 1.19 kg/l, V = 250 ml),
- phenolphthalein, 1 % (*w/v*) indicator solution (M = 318.323 g/mol) in 96 % (*v/v*) ethanol (V = 10 ml),
- potassium hydroxide (KOH) in isopropanol with a concentration of 40 g/l (KOH dissolves in isopropanol at 15 °C; the solution must be clear after dissolving KOH).

Procedure

1. Mix 5 g of the sample with 50 ml of potassium hydroxide in isopropanol in a round bottom flask.
2. Attach the flask to a reflux condenser (see Figure 56). Heat the flask in a boiling water bath for 1 hour.

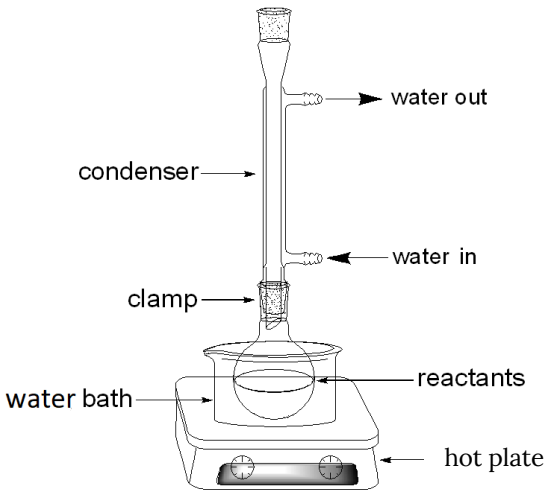


Figure 56: Schematic diagram of reflux setup.

3. Cool and add a few drops (2-3) of phenolphthalein solution (1 %, *w/v)*.
4. Titrate the contents against 0.5 N HCl. Shake constantly until the pink color disappears (see Figure 57).
5. Similar to the test, prepare a blank with the solvent (50 ml of potassium hydroxide in isopropanol) with a few drops (2-3) of phenolphthalein solution, without any oil sample.

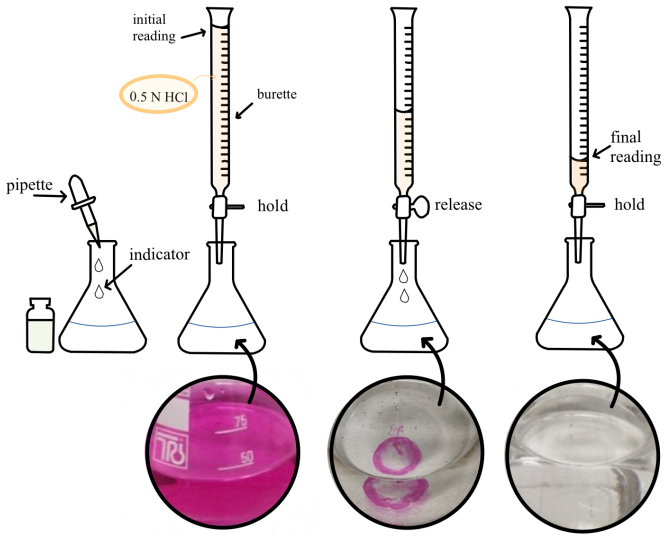


Figure 57: Determination of fatty acids released after alkaline oil hydrolysis by titration method.

Saponification number
The saponification value is calculated as follows:

$$Saponification\ value = \frac{28.05 \times (V_1 - V_2)}{sample\ weight\ [g]}\ [mg\ KOH/g\ of\ oil]$$

where V_1 is the volume (in ml) of hydrochloric acid consumed in the blank determination, V_2 is the volume (in ml) of hydrochloric acid consumed in the sample determination and 28.05 means 28.05 mg KOH corresponding to 1 ml 0.5 N HCl.

Results
Compare the saponification number of the oil with the literature.

Questions
1. Write the reaction scheme for the alkaline hydrolysis of triglyceride with potassium hydroxide.
2. Which indicator can be utilized in an acid-base titration?
3. What is a saponification number and how is it done?
4. What is the volume of concentrated HCl (36 %, v/v) required to prepare 250 ml of 0.5 N solution (M = 36.46 g/mol; $\rho_{36\%}$ = 1.19 kg/l)?
5. Calculate the saponification number of canola oil. The weight of a sample was 5.024 g, the volume of HCl (0.5 N) consumed by a sample was 52 ml, and consumed by a blank was 17.3 ml.

5.4. Experimental part C: Determination of the peroxide number

Principle
In edible oils, various titration determinations such as acid number, saponification number and peroxide number are used to assess the quality of the oil. The **peroxide value** indicates the content of primary lipid oxidation products, i.e., the rancidity of fats. The peroxide concentration is a sign of oxidation in the early phases of lipid degradation. In this method, the oxygen content of the sample is determined, with more oxygen increasing the peroxide value. The peroxide value is expressed as micro-equivalents of active

oxygen (i.e., iodine release from iodide) per 1 g of sample. The sample is treated in a solution with acetic acid and a suitable organic solvent and then with a potassium iodide solution. The iodine released is titrated using a standard solution of sodium thiosulphate.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, Erlenmeyer flask (100 ml), volumetric flasks, burette, analytical balance.

Materials and chemicals

- Oil sample (linseed or rapeseed oil),
- chloroform ($CHCl_3$),
- glacial acetic acid (CH_3COOH, a concentrated form of acetic acid solution that has less than 1 % of water content),
- saturated aqueous solution of potassium iodide (KI; dissolve 10 g KI in 10 ml water with heat and stir, continue to add 0.5 g KI at a time until solution no longer dissolves additional solid, the solution must be colorless or slightly yellowish) (V = 10 ml),
- starch indicator (mix 0.5 g of starch in 5 ml of water, then with 45 ml of boiling distilled water, boil for a few seconds, and then rapidly cool; the solution is always prepared fresh),
- sodium thiosulfate ($Na_2S_2O_3$) with a concentration of 0.01 mol/l (0.01 M, M = 158.11 g/mol, V = 100 ml).

Procedure

1. Mix 1 g of oil with 10 ml of chloroform and 10 ml of glacial acetic acid in an Erlenmeyer bank.
2. Add 1 ml of saturated aqueous solution of potassium iodide.
3. Stir the flask vigorously for one minute and then incubate it in the dark at laboratory temperature for 5 minutes, stirring occasionally.
4. Add 75 ml of distilled water and a few drops (4-5) of the starch indicator.
5. Titrate the contents against 0.01 mol/l sodium thiosulfate. Shake constantly until the blue color disappears (see Figure 58).

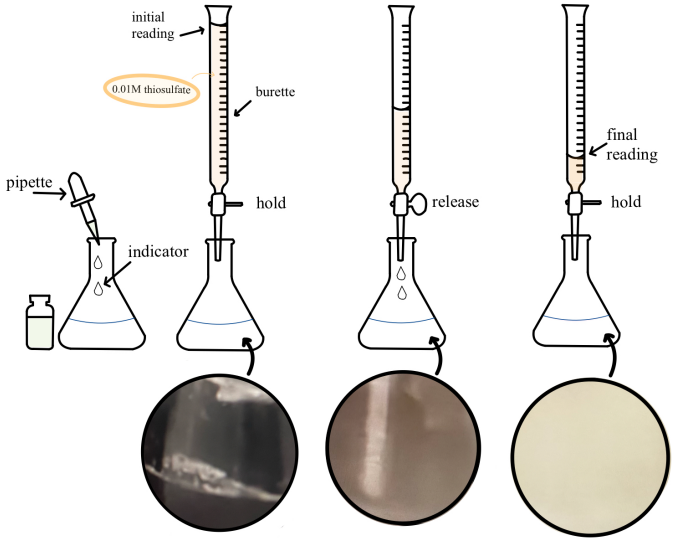


Figure 58: Determination of peroxide number by titration.

6. Similar to the test, prepare a blank with 10 ml of chloroform and 10 ml glacial acid, saturated aqueous potassium iodide solution, 75 ml distilled water, and a few drops of starch indicator without any oil sample.
   If iodine is not excreted at all, this may indicate that the solvents contain reducing agents.

Peroxide value
The peroxide value is calculated as follows:

$$Peroxide\ value = \frac{(V_1\ [\text{ml}] - V_2\ [\text{ml}])\ .\ M\ .\ 1000}{weight\ of\ the\ oil\ sample\ [g]}\ \ [mmol\ O_2/1\ kg\ or\ meq\ per\ 1\ kg]$$

where V_1 is the volume (in ml) of sodium thiosulfate consumed in the sample determination, V_2 is the volume (in ml) of sodium thiosulfate consumed in the blank determination, M is the molarity of sodium thiosulfate expressed in mol/l and 1000 is a conversion factor from g to kg.

Questions
1. What is a peroxide number?
2. Why is peroxide value used to indicate rancidity?
3. What does a high peroxide value mean?
4. How much sodium thiosulfate (M = 158.11 g/mol) should you use to make 100 ml with a concentration of 0.01 mol/l?
5. Calculate the peroxide number of linseed oil. The weight of a sample was 1.024 g, the volume of sodium thiosulfate (0.01 M) consumed by a sample was 0.6 ml, and consumed by a blank was 0.4 ml.

6. Proteins

6.1. Theory

Proteins are essential for nearly all biological processes and perform numerous functions in organisms. Proteins have six primary **biological functions** in living organisms:

1. **Structural function** - most structural proteins are fibrous proteins and insoluble in water. They provide structural components for bones, tendons, muscles, hair, skin, nails, horn, cartilage, etc., and form parts of cell membranes.
   Examples: keratin, and collagen.
2. **Catalytic function** - proteins act as biocatalysts of biochemical reactions. All enzymes are proteins that help speed up a chemical reaction by decreasing its activation energy.
   Examples: trypsin, saccharase, α-amylase, etc.
3. **Regulatory function** - proteins are the building blocks of many hormones and signaling molecules with specific functions, such as regulating body metabolism and the nervous system.
   Examples: insulin, glucagon, growth hormone, etc.
4. **Protective (defensive) function** - proteins are components of antibodies responsible for recognizing and destroying foreign substances from microbes or materials. Antibodies are protective proteins produced by the immune system.
   Examples: immunoglobulins and fibrinogen.
5. **Transport function** - some proteins are involved in the transport of essential substances throughout the body to their target destinations.
   Examples: haemoglobin and lipoproteins.
6. **Storage function** - these proteins store nutrients (metals or amino acids) in cells. Many proteins accumulate around the embryo to provide amino acids during its development. They are also found in seeds, eggs, milk, and legumes.
   Examples: casein, ferritin, gluten, ovalbumin, etc.

Proteins are biopolymers whose skeleton comprises polypeptide chain. The protein's properties are determined by its chemical composition, structure, and relative molecular weight. They are tasteless solids with varying water solubility, generally soluble in weakly alkaline environments. In the solutions, they are sensitive to salt concentration - at certain salt concentration, they precipitate out, and at another, they precipitate into solution (become soluble). These properties are used in their isolation.

6.1.1. Amino acids and peptide bonds

The primary structural unit of protein is an **amino acid**. An α-amino acid consists of an amino group ($-NH_2$), a carboxyl group (-COOH), a hydrogen atom, a central carbon atom called an α-carbon, and a side chain (-R) attached to the α-carbon atom (see Figure 59).

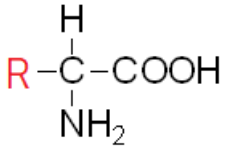


Figure 59: The structure of α -amino acid.

Amino acids are organic acids categorized based on the number of amino and carboxyl groups they contain:

- **neutral amino acids** have one carboxyl group and one amino group. Out of the 20 standard amino acids present in living organisms, there are 15 neutral amino acids (including glycine, alanine, leucine, isoleucine, valine, phenylalanine, proline, methionine, serine, threonine, tyrosine, cysteine, glutamine, asparagine, and tryptophan),
- **acidic amino acids** possess two carboxyl groups and one amino group (such as aspartic acid and glutamic acid), and
- **basic amino acids** have one carboxyl group and two amino groups (such as lysine, arginine, and histidine).

The total charge of an amino acid (the sum of all negatively and positively charged groups present) depends on the pH or proton concentration of the solution. The ability to change the charge of amino acids by changing the pH is used in protein separation. The pH value at which the amino acid has no charge (negative and positive charges are balanced) is known as the isoelectric pH, also called **the isoelectric point (pI)**.

Amino acids, except for glycine, are optically active substances because they have a chiral α-carbon, allowing them to rotate the plane-polarized light in the positive direction (D-), or in the negative direction (L-)). The L-forms of amino acids are physiologically usable. This geometric difference is depicted in Figure 60.

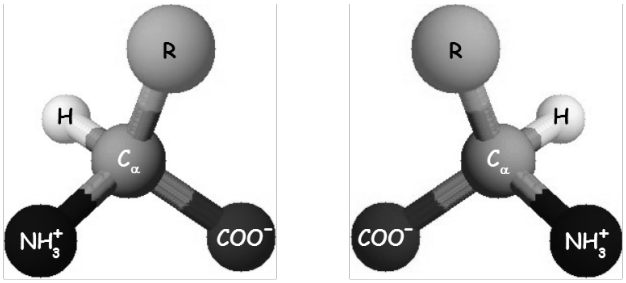


Figure 60: Geometry of L- (left) and D-forms (right) of amino acids.

Important α-amino acids include the standard **proteinogenic amino acids**. These are involved in proteosynthesis (protein synthesis) and are integrated into proteins during translation. While microorganisms and plants can synthesize all proteinogenic amino acids from inorganic compounds, animals need to obtain some, termed essential amino acids, from their diet. Humans require nine essential amino acids (such as valine, leucine, isoleucine, phenylalanine, tryptophan, lysine, methionine, histidine and threonine) in adequate amounts, as they can't be synthesized internally.

The structures and names of the **20 proteinogenic amino acids**, along with their one- and three-letter abbreviations and some distinctive features are given in Figure 61.

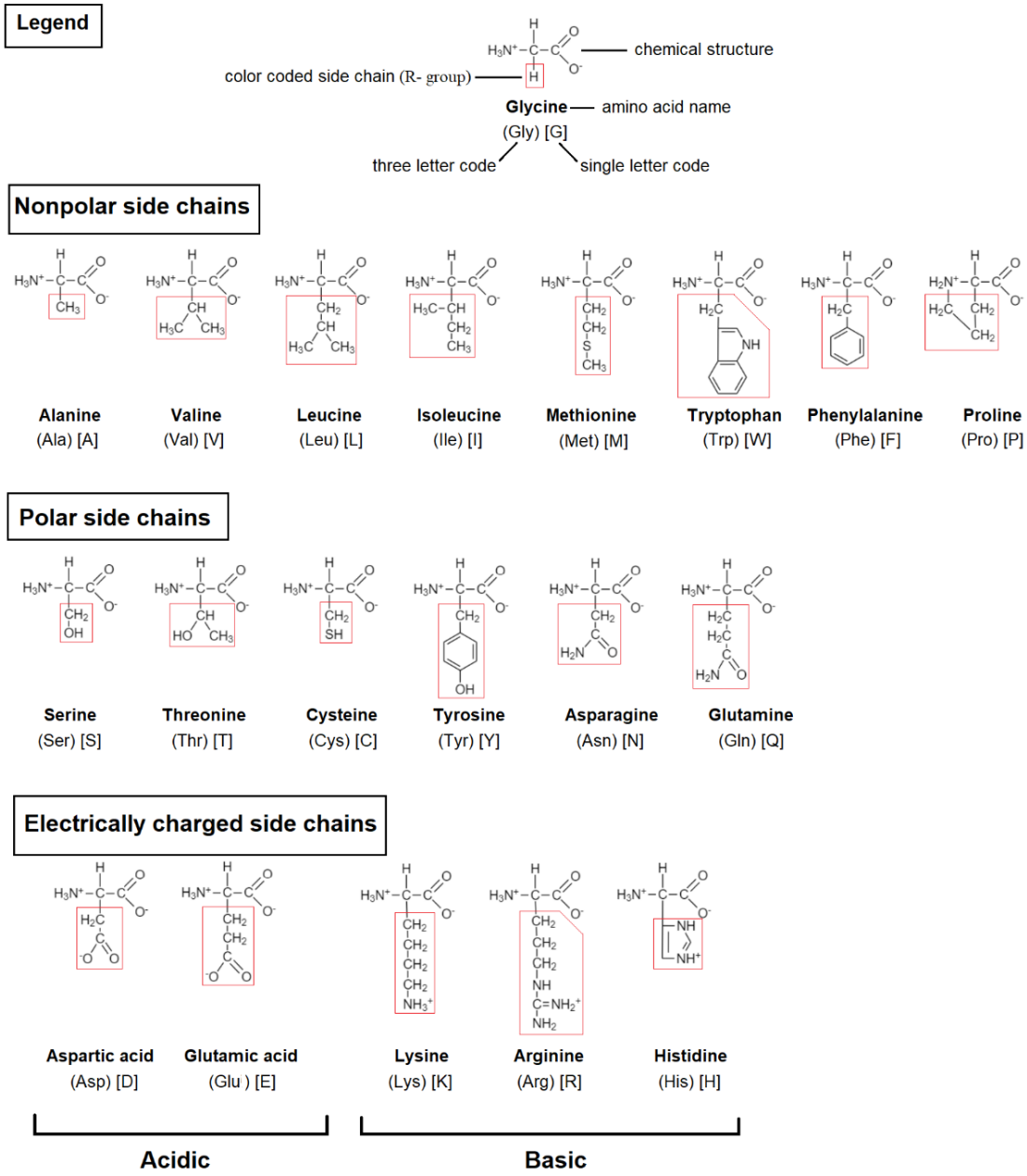


Figure 61: The proteinogenic amino acids present in proteins.

The most important reaction of amino acids is the formation of a **peptide bond**. This covalent bond is formed by a reaction between the α-amino group of one amino acid and the carboxyl group of a second amino acid, releasing a water molecule (see Figure 62).

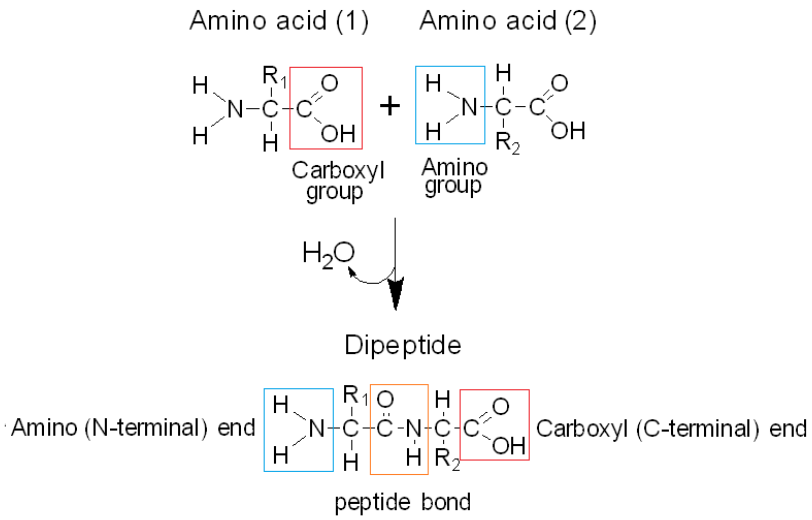


Figure 62: Formation of a peptide bond in a reaction between two amino acids.

6.1.2. Protein structure

Proteins are macromolecules whose spatial structure significantly determines their biological activity. Therefore, four hierarchical levels of structural organization (see Figure 63) of the polypeptide chain have been established for proteins:

1. **The primary structure** of a protein is the sequence of the amino acids in a linear polypeptide chain. The bond connecting the individual amino acids is the peptide bond. The overall composition of the protein molecule, as well as its resulting properties and biological function, are evaluated.
2. **The secondary structure** of a protein defines the spatial arrangement of the peptide chain. The formation of hydrogen bonds between the -CO- and -NH- groups, and the arrangement of atoms in its vicinity are crucial for the formation of the peptide bond. This structure reflects the geometric arrangement of the polypeptide chain, regardless of the nature of the side chains. Its secondary structure results from the folding of polypeptides into hydrogen-bonded motifs, such as the α-helix and the β-sheet (or β-strand).
3. **The tertiary structure** characterizes the spatial arrangement of the polypeptide chain, resulting from interactions between R-groups, such as hydrogen bonds and ionic bonds. The polypeptide chain further folds into a three-dimensional comprising one or more domains. The α-helix and β-sheets fold into a compact globular structure, primarily due to non-specific hydrophobic interactions. The tertiary structure refers to the entire 3D conformation of a polypeptide.
4. **The quaternary structure** depicts the spatial arrangement of a protein's subunits, which could either be identical or different. This structure outlines the relative positions of these subunits. A well-known example of this structure is haemoglobin - it consists of four peptide chains (twice α- and β-chains) that form a tetramer.

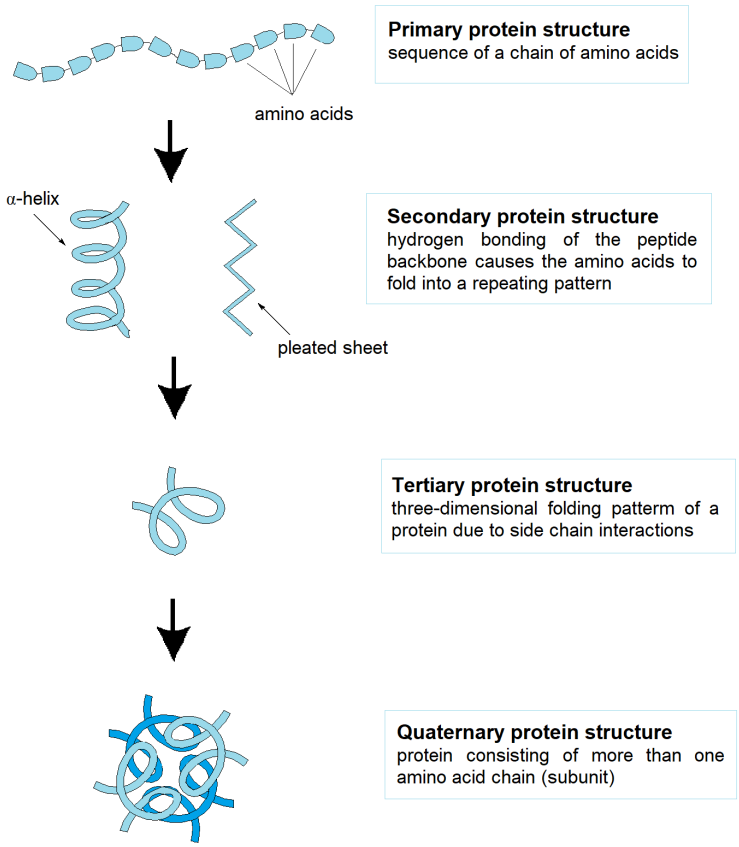


Figure 63: The hierarchy of protein structure.

A permanent change in the spatial arrangement of the secondary and tertiary structures of the peptide chain caused from the outside (high temperature, low or high pH), is termed **protein denaturation**. In this process, some biological properties of the protein are lost, e.g., the ability of enzymes to cleave the substrate or muscle contractility. Proteins denatured by cooking are easily digestible, their nutritional values remain unchanged.
There is **not universal classification system for proteins.** However, proteins can be classified on the basis of their solubility, shape or nutritional value.

1. Simple proteins are classified on their **solubility** as follows:
    a. *albumins* (soluble in water),
    b. *globulins* (insoluble in water but soluble in salt solutions),
    c. *glutenins* or *glutelins* (insoluble in water, soluble in dilute acids or bases), and
    d. *prolamins* or *gliadins* (insoluble in water and absolute alcohol but soluble in 70 - 80 % (*v/v*) alcohol).
2. Based on their shape, proteins can be divided into:
    a. *fibrous proteins* with elongated (stick-like) shapes, and
    b. *globular proteins* (include many enzymes) which have compactly folded polypeptide chains arranged in a sphere of a globe upon folding.
3. Considering nutritional value, proteins can be categorized as:
    a. *complete proteins, which* contain all nine essential amino acids, and
    b. *incomplete proteins*, where one or more essential amino acids are absent.

6.1.3. Protein synthesis and degradation

Amino acids are not stored in the body like other primary metabolites. They are continuously renewed (proteosynthesis - anabolism) and degraded from proteins (proteolysis - catabolism) through numerous and complex metabolic processes. The relationship between protein catabolism and anabolism is monitored by **the nitrogen balance**. The concept of nitrogen balance suggests that the difference between nitrogen intake and loss reflects the gain or loss of total body protein. In a healthy organism, the nitrogen balance is equilibrated, meaning the nitrogen intake balances with the nitrogen output. A **positive nitrogen balance** corresponds to growth periods when nitrogen uptake exceeds nitrogen loss, resulting in amino acids being incorporated into new tissues. Conversely, if more nitrogen is lost consumed, an individual is considered catabolic or exhibiting a **negative nitrogen balance**. This imbalance can arise during severe illness or in old age.

Synthesis of peptides

Peptides are formed by combining two or more amino acids through a condensation reaction, joining together through a covalent bond. This bond can be cleaved (hydrolyzed) either chemically (using dilute acids or bases) or enzymatically (using proteolytic enzymes). Each amino acid molecule can bind two other amino acid molecules. The polycondensation produces peptides with different numbers of amino acids and with different arrangements in the primary structure. Each peptide has two ends: the end with a free carboxyl group (the C-terminal amino acid) and the end with a free amino group (the N-terminal amino acid).

Peptides, depending on their composition, are synthesized by the same mechanism as proteins. However, some peptides are synthesized without using the cell's proteosynthetic machinery. In addition to the linear chain, the polycondensation of amino acids can also produce closed chains, which have neither a free carboxyl nor a free amino group. These are called **cyclic peptides**.

Cyclic peptides can be classified according to the types of bonds that comprise the ring.

- Homodetic peptides are composed exclusively of standard peptide bonds, and
- heterodetic peptides are cyclized between side chains or between a side chain and one of the terminal ends.

Depending to the number of amino acids in the chain, peptides are divided into:

- dipeptides - containing 2 amino acids,
- tripeptides - containing 3 amino acids,
- oligopeptides - with not more than 10 amino acids,
- polypeptides - with more than 10 amino acid units, up to 100 residues, and
- macropeptides - composed of more than 100 amino acids.

Peptide degradation

Peptide degradation is carried out by peptidases. Exopeptidases cleave the peptide bonds at the ends of the peptide molecule (from the C-terminal end - carboxypeptidases or from the N-terminal end - aminopeptidases) to release dipeptides or tripeptides. In contrast, endopeptidases break the peptide bond within the peptide molecule.

Protein synthesis

Proteins are formed by proteosynthesis on ribosomes. They are divided into simple and complex proteins. A simple protein consists solely of amino acids, while complex proteins, which are far more common, incorporate other non-amino acid groups in their

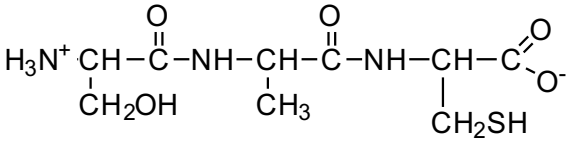
structure. These non-protein parts of protein molecules can include metal ions, saccharides, and various other substances.

Protein degradation

Protein degradation (catabolism) is a hydrolytic reaction that releases amino acids from a polypeptide chain. This reaction takes place mainly in the gastrointestinal tract via proteases. The breakdown of proteins into smaller polypeptides or their respective amino acids, are necessary for metabolic and cellular homeostasis. Proteolytic degradation can also occur utilizing various mechanisms, including intramolecular digestion and non-enzymatic methods. Proteolytic degradation mechanisms are required to obtain amino acids through degradation of digested proteins, to prevent the accumulation or abnormal concentrations of proteins, and to regulate cellular processes by removing proteins no longer needed.

Questions

1. What is a protein?
2. What are the six main functions of proteins?
3. What is the general formula for an α-amino acid?
4. What is an α-amino acid?
5. What is meant by isoelectric point of a protein?
6. Why is glycine not an optically active compound?
7. What are the 20 proteinogenic amino acids?
8. What are the nine essential amino acids for humans?
9. What is a peptide bond and how is it formed?
10. Draw the dipeptide of glycine and alanine.
11. What are the four types of protein structures? Briefly describe.
12. What is protein denaturation and how does it happen?
13. How are amino acids divided based on:
    a. the number of amino and carboxyl groups,
    b. solubility,
    c. shape, and
    d. nutritional value?
14. Draw the C- and N-terminus of this peptide structure:



15. What is proteosynthesis?
16. What is meant by nitrogen balance? What does a negative/positive nitrogen balance indicate?
17. What is a cyclic peptide?
18. What are the types of cyclic peptides?
19. How are peptides classified based on the number of amino acids?
20. What is the difference between of exopeptidase and endopeptidase?

6.2. Experimental part A: Determination of proteins by the Biuret method

Principle

Compounds containing two or more peptide bonds form a colored complex with Cu^{2+} ions in an alkaline medium. The Biuret reaction determines the presence of peptides and proteins within a concentration range of 1-6 g/l. Free amino acids do not interact directly with Cu^{2+} ions. The reaction is not dependent on the primary structure of the protein, as the copper (II) ions react with the peptide chain, not with the side groups (see Figure 64).

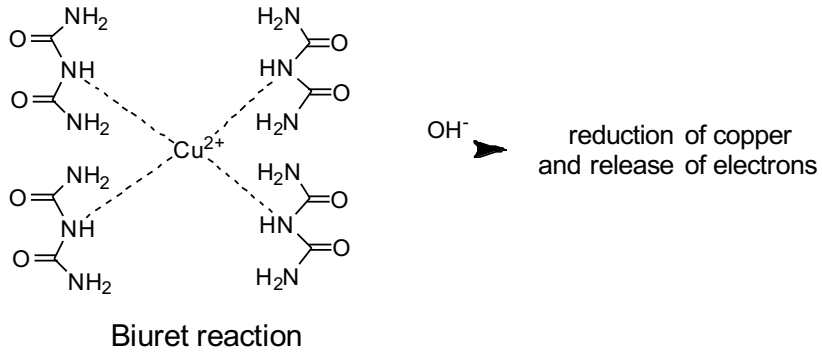


Figure 64: The Biuret reaction involves the reduction of copper ions, releasing electrons, and forming of a blue-violet color.

The reaction is based on the formation of a tetraammonium salt with a copper complex. The reaction is positive when the formed copper complex turns from blue to purple. The intensity of the color depends on the number of peptide bonds present in the sample. Polypeptides form a purple complex, whereas smaller peptides form a red complex. Glucose and other copper-reducing compounds, such as sulfhydryl groups of proteins (like keratin), high concentrations of ammonium salts, and some phosphates used in protein purification, can interfere with this determination, leading to false results.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, test tubes, volumetric flask (25 ml, 200 ml, 250 ml), glass rods, analytical balance, laboratory vortex, and spectrophotometer.

Materials and chemicals

- The sample with unknown protein concentration,
- the stock solution of egg albumin with a concentration of 5 g/l (V = 25 ml),
- saline solution; sodium chloride, 0.9 % (*w/v*) aqueous solution (M = 58.44 g/mol, V = 250 ml),
- biuret reagent (dissolve 0.6 g copper (II) sulfate pentahydrate and 2.4 g sodium potassium tartrate in 100 ml of 10 % (*w/v*) aqueous solution of sodium hydroxide and make up to 200 ml with distilled water).

Procedure

The preparation of the stock solution of egg albumin and its dilute solutions

1. Prepare the stock solution of egg albumin with a concentration of 5 g/l by weighing 0.125 g of the solid sample, transferring the quantitative to a 25 ml volumetric flask and diluting to the mark with distilled water.

2. Through appropriate dilutions of the stock solution of the egg albumin, prepare calibration solutions as follows:

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of saline solution (ml)
4.0	1.25	4	1
3.0	1.67	3	2
2.0	2.5	2	3
1.0	5.0	1	4
0.5	10.0	0.5	4.5

3. Prepare the dilute solutions (calibration solutions) in test tubes. Dilute the stock solution of egg albumin and saline solution using a one-channel pipette. The total volume of dilute solution is 5 ml.

Determination of proteins by the Biuret method

1. Mix 1 ml of the solution (a sample or egg albumin solutions in the concentration range of 0.5-5.0 g/l) with 3.0 ml of the Biuret reagent in a test tube.
   **Work in three parallel measurements. Prepare a set of test tubes. Take 24 clean and dry test tubes to determine the absorbance of the sample or calibration solutions.*
2. Mix the mixture on the vortex and let stand for 30 minutes at laboratory temperature.
3. Prior to making all absorbance readings, a spectrophotometer must be calibrated using a blank solution, which comprises a cuvette filled with 2/3 full with saline solution for this experiment.
4. Measure the absorbance of the samples at 540 nm.
5. Record the results in your laboratory notebook.

Results

Calculate for each calibration solution the average of absorbances, and produce a plot of absorbance vs. actual concentration (the x-y relationship). Carry out a linear regression analysis to determine the equation of the relationship between absorbance and concentration ($y = ax + b$) as well as the coefficient of determination. Calculate the concentration of proteins (g/l) in the sample.

Question

1. What is the principle of the biuret method?
2. What are the limitations of the Biuret method for proteins?
3. Do all proteins respond to the Biuret method?
4. How much sodium chloride (NaCl) should you use to make 250 ml of its 0.9 % (*w/v*) solution?
5. How much sodium hydroxide (NaOH) should you use to make 100 ml of its 10 % (*w/v*) solution?
6. The stock solution of egg albumin with a concentration of 10 g/l should be diluted to a concentration in the range of 1-7 g/l. Calculate the dilution factor, the volume of the stock solution and the volume of saline solution needed to produce a solution of a given concentration. The total volume of the dilute solution must be 5 ml.

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of saline solution (ml)
7.0			
6.0			
5.0			
4.0			
3.0			
2.0			
1.0			

6.3. Experimental part B: Determination of proteins by the Lowry method

Principle

The Lowry method is based on measuring the absorbance of two complexes that absorb light differently. The first is a biuret complex formed by Cu^{2+} ions in an alkaline solution. The second complex is a reduced phosphomolybdenum-phosphotungsten complex (the Folin-Ciocalteu reagent). The blue color arises from the reduction of phosphomolybdates and phosphotungstates present in the Folin reagent by aromatic amino acid residues (mainly tryptophan and tyrosine) present in the protein. The Lowry method (0.1-1.0 g/l) is about 10 times more sensitive than the Biuret method.

The reaction is very sensitive to the pH of the environment, hence, the pH should be maintained between 10.0 and 10.5, and the various time intervals for adding and mixing the reagents must be followed to obtain reproducible results.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, test tubes, volumetric flask, glass rods, analytical balance, laboratory vortex, and spectrophotometer.

Materials and chemicals

- The sample with unknown protein concentration,
- the stock solution of egg albumin with a concentration of 0.5 g/l (V = 50 ml),
- saline solution; sodium chloride, 0.9 % (*w/v*) aqueous solution (M = 58.44 g/mol, V = 250 ml),
- reagent A: sodium carbonate (2 %, *w/v*) in 0.1 mol/l aqueous solution of sodium hydroxide (M = 39.9971 g/mol, V = 50 ml),
- reagent B: copper (II) sulfate pentahydrate (0.5 %, *w/v*) in aqueous solution of sodium potassium tartrate (2 %, *w/v*) (V = 1 ml),
- reagent C: mix 3 ml of reagent B with 147 ml of reagent A prior to use (V = 150 ml),
- reagent D: mix 5 ml of the Folin-Ciocalteu reagent and 10 ml of distilled water (V = 15 ml).

Procedure

The preparation of the stock solution of egg albumin and its dilute solutions

1. Prepare the stock solution of egg albumin with a concentration of 0.5 g/l by weighing 0.025 g of the solid sample, transferring the quantitative to a 50 ml volumetric flask, and diluting to the mark with distilled water.
2. Through appropriate dilutions of the stock solution of the egg albumin, prepare calibration solutions as follows:

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of saline solution (ml)
400	1.25	4	1
300	1.67	3	2
200	2.5	2	3
100	5.0	1	4
50	10.0	0.5	4.5

3. Prepare the dilute solutions (calibration solutions) in test tubes. Dilute the stock solution of egg albumin and saline solution using a one-channel pipette. The total volume of dilute solution is 5 ml.

Determination of proteins by the Lowry method

1. Mix 1 ml of the solution (a sample or egg albumin solutions in the concentration range of 50-500 mg/l) with 5 ml of reagent C.
   **Work in three parallel measurements. Prepare a set of test tubes. Take 24 clean and dry test tubes to determine the absorbance of the sample or calibration solutions.*
2. Mix the mixture on the vortex and let stand for 10 minutes at laboratory temperature.
3. Add 0.5 ml of reagent D.
4. Mix the mixture on the vortex and let stand for 30 minutes at laboratory temperature.
5. Prior to making all absorbance readings, a spectrophotometer must be calibrated using a blank solution, which comprises a cuvette filled with 2/3 full saline solution for this experiment.
6. Measure the absorbance of the samples at 600 nm.
7. Record the results in your laboratory notebook.

Results

Calculate for each calibration solution the average of absorbances, and produce a plot of absorbance vs. actual concentration (the x-y relationship). Carry out a linear regression analysis to determine the equation of the relationship between absorbance and concentration ($y = ax + b$) as well as the coefficient of determination. Calculate the concentration of proteins (g/l) in the sample.

Question

1. What is the principle of the Lowry method?
2. What are the limitations of the Lowry method?
3. How much each component should you use to make:
   a. sodium carbonate (2 %, *w/v*) in 0.1 mol/l aqueous solution of sodium hydroxide (M = 39.9971 g/mol, V = 50 ml) (reagent A), and
   b. copper (II) sulfate pentahydrate (0.5 %, *w/v*) in aqueous solution of sodium potassium tartrate (2 %, *w/v*) (V = 1 ml) (reagent B)?

6.4. Experimental part C: Determination of proteins by the Bradford method

Principe

The Bradford method is based on the reaction of the triphenylmethane dye, Coomassie Brilliant Blue G-250 (see Figure 65), with proteins at an acidic pH. When the dye binds to the protein, a color change occurs, which is proportional to the amount of protein.

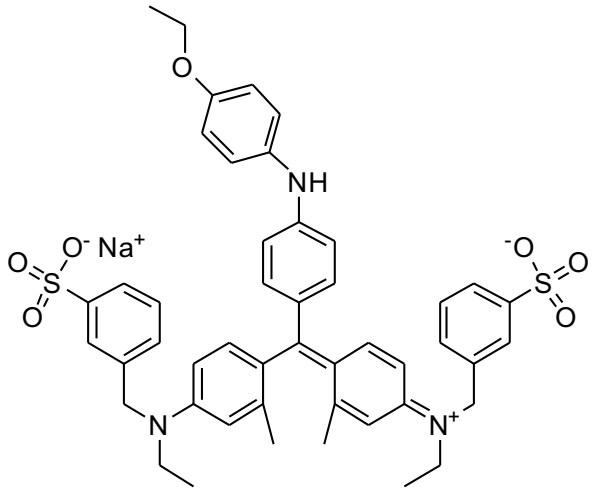


Figure 65: Structure of the triphenylmethane dye Coomassie Brilliant Blue G-250.

The color intensity is associated with the presence of certain basic amino acids (mainly arginine, lysine, and histidine) in the protein. Van der Waals forces and hydrophobic interactions are also involved in the binding of the dye to the protein. Free amino acids, peptides, and low-molecular weight proteins do not react with the dye. Consequently, this method is typically used to determine the concentration of high-molecular weight proteins.

The method is relatively sensitive (0.2-1.4 g protein/l) using the standard method, but protein concentrations in the range of 5-100 mg protein/l can be determined using microassays (by decreasing the volume of Bradford reagent and increasing the volume of the protein sample). The method is sensitive, simple, and rapid. The development of the blue color, which is stable for about an hour, does not take more than 2 minutes. Unlike other protein assays, the Bradford method is less susceptible to interference by various compounds, such assodium, potassium, or even saccharides that may be present in protein samples.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, test tubes, volumetric flask, glass rods, analytical balance, laboratory vortex, and spectrophotometer.

Materials and chemicals

- The sample with unknown protein concentration,
- the stock solution of egg albumin with a concentration of 0.1 g/l (V = 100 ml),
- saline solution; sodium chloride, 0.9 % (*w/v*) aqueous solution (M = 58.44 g/mol, V = 250 ml),
- Bradford reagent (dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 96 % (*v/v*) ethanol and add 100 ml of 85 % (*v/v*) phosphoric acid and make up to 1000 ml with distilled water).

Procedure

The preparation of the stock solution of egg albumin and its dilute solutions

1. Prepare the stock solution of egg albumin with a concentration of 0.1 g/l by weighing 0.01 g of the solid sample, transferring the quantitative to a 100 ml volumetric flask, and diluting to the mark with distilled water.
2. Through appropriate dilutions of the stock solution of the egg albumin, prepare calibration solutions as follows:

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of saline solution (ml)
85	1.18	8.5	1.5
65	1.54	6.5	3.5
45	2.22	4.5	5.5
25	4.0	2.5	7.5
5	20.0	0.5	9.5

3. Prepare the dilute solutions (calibration solutions) in test tubes. Dilute the stock solution of egg albumin and saline solution using a one-channel pipette. The total volume of dilute solution is 10 ml.

Determination of proteins by the Bradford method

1. Mix 2 ml of the solution (a sample or egg albumin solutions in the concentration range of 5-100 mg/l) with 2 ml of the Bradford reagent.
   **Work in three parallel measurements. Prepare a set of test tubes. Take 24 clean and dry test tubes to determine the absorbance of the sample or calibration solutions.*
2. Mix the mixture on the vortex and let stand for 5 minutes at laboratory temperature.
3. Prior to making all absorbance readings, a spectrophotometer must be calibrated using a blank solution, which comprises a cuvette filled with 2/3 full saline solution for this experiment.
4. Measure the absorbance of the samples at 595 nm.
5. Record the results in your laboratory notebook.

Results

Calculate for each calibration solution the average of absorbances, and produce a plot of absorbance vs. actual concentration (the x-y relationship). Carry out a linear regression analysis to determine the equation of the relationship between absorbance and concentration ($y = ax + b$) as well as the coefficient of determination. Calculate the concentration of proteins (g/l) in the sample.

Question

1. What is the principle of the Bradford method?
2. Which amino acids can be recognized by the Bradford method?

7. Organic acids

7.1. Theory

Organic acids encompass a broad spectrum of organic compounds that possess acidic properties. They are classified as important sensory and dietetic components of food. Medium-strength acids such as tartaric, citric, lactic, acetic, glutamic, fumaric, and others are utilized as food additives. Nonetheless, when using organic acids as food additives, it is essential to consider their effect on the organoleptic attributes of the products. For example, citric acid is more suitable for citrus-based beverages, tartaric acid is better for grape juices and beverages derived from them, malic acid is preferred for apple juices, and phosphoric acid is ideal for cola-type drinks.

The most important role of organic acids is to lower the pH and increase the titratable acidity of foods. Organic acids have the ability to dissociate and release the hydrogen cation (H^+). The amount of released hydrogen cations relies on the acid's concentration and its dissociation constant. Organic acids in foods are evaluated as the sum of all acidic food components by neutralizing the hydroxide to an appropriate indicator potentiometrically. Individual acids are determined either chemically or post-isolation through separation techniques or chromatographic methods.

An organic acid is an organic compound with acidic properties. The most common organic acids are the carboxylic acids. They are classified as water-soluble crystalline substances. A majority of these substances exhibit optical activity.

Glutamic acid

Glutamic acid ($C_5H_9NO_4$, 2-aminopentanedioic acid) is one of the dicarboxylic amino acids (see Figure 66). Glutamic acid is an α-amino acid that is used by nearly all living entities in the biosynthesis of proteins.

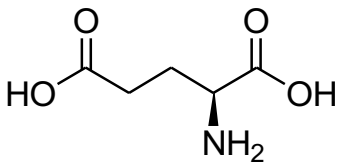


Figure 66: The structure of glutamic acid.

Both D- and L-forms of glutamic acid exist, which have the same physical properties, except for the rotation of the plane of polarized light. Only its L-form is observed in living organisms. Glutamic acid is used in the food industry as a dye stabilizer or as an antioxidant. Monosodium glutamate (MSG, a sodium salt of glutamic acid) is found naturally in some foods and is used as a flavor enhancer. This form creates the savory umami flavor of foods.

Citric acid

Citric acid ($C_6H_8O_7$, 2-hydroxypropane-1,2,3-tricarboxylic acid) (see Figure 67) is a weak tricarboxylic acid and prevalent in several fruits and vegetables. The highest concentration of citric acid was found in lemons and limes, which may contain up to 8 % by dry weight. It is also a key intermediate in the citric acid cycle (Krebs cycle). Its capacity to balance acidity and alkalinity, combined with its bacteriostatic properties, makes it valuable as a preservative in both food and cosmetics. It prevents fats and oils from becoming rancid and changing color undesirably. It is a component in an array of products like soft drinks, wine, fats, marmalades, frozen dairy products, mayonnaise, and

more. Additionally, citric acid is utilized in the pharmaceutical realm and for chemical production.

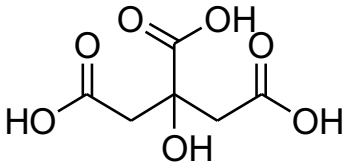


Figure 67: The structure of citric acid.

At laboratory temperature, citric acid is a white crystalline material. It occurs either anhydrous or as a monohydrate, which can be converted to the anhydrous acid by drying at 74 °C. Citric acid is very soluble in water (1330 g/l at 20 °C), and freely soluble in ethanol. From a chemical point of view, this acid shares properties with other carboxylic acids. Upon heating beyond 175 °C, it undergoes decomposition (decarboxylation) by shedding carbon dioxide and water. The fundamental physical properties of citric acid are summarized in Table 15.

Table 15: Physical properties of citric acid.

Physical property	Value with units
molar mass	192.124 g/mol
melting point	153 °C
decomposition temperature	175 °C
density (20 °C)	1.665 g/ml
solubility in water (20 °C)	1330 g/l

Malic acid

Malic acid ($C_4H_6O_5$, 2-hydroxysuccinic acid) is an optically active, colorless, crystalline substance detected in unripe fruits (see Figure 68). It is used as a preservative and acidifying agent. Flavoring substances adjust pH and strengthen the effects of antioxidants. It also prevents browning of the fruit. Malic acid exists in a natural L-form and a synthetic D-form. It occurs naturally mainly in apples, pears, and grape juice. It is also found in cheese, fruit nectars, and cosmetics.

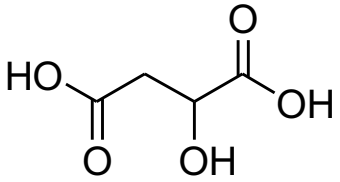


Figure 68: The structure of malic acid.

Acetic acid

Acetic acid ($C_2H_4O_2$, ethanoic acid) is a colorless liquid notable for its pungent aroma. In concentrations of 5-8 %, it is used in the food industry as vinegar (see Figure 69). It is formed by the oxidation of ethanol during acetic fermentation. It occurs as a natural metabolite in living organisms. Its active form is a complex with coenzyme A, known as acetyl-CoA. In plants, it occurs either in free form or in the form of salts.

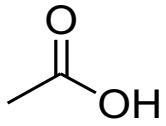


Figure 69: The structure of acetic acid.

Lactic acid

Lactic acid ($C_3H_6O_3$, 2-hydroxypropanoic acid) is one of the α-hydroxycarboxylic acids of which the most abundant in nature (see Figure 70). It is a white crystalline substance with good solubility. Lactic acid can be obtained from sour milk or sauerkraut, where it is produced by the lactic fermentation of saccharides. In the food industry, it serves as a bacterial growth inhibitor. Its applications extend to pharmaceuticals, cosmetics, and chemicals. Lactic acid, which contains carbon positioned chirally (asymmetrically), occurs in two isomeric forms, the L- and the D-form.

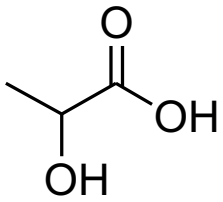


Figure 70: The structure of lactic acid.

Tartaric acid

Tartaric acid ($C_4H_6O_6$, 2,3-dihydroxysuccinic acid) is a white crystalline substance possessing two asymmetric carbon atoms. Thus, it exists as both a dextrorotatory D-form and a levorotatory L-form (see Figure 71). The D-form of tartaric acid is widespread in nature (e.g., in various types of fruits), while its L-form is rare. Its sour taste finds use in foods, and it also acts as an antioxidant. Like other polyhydroxyl compounds, tartaric acid forms complexes with heavy metals, like a bright blue complex with copper salts used in making Fehling's solution.

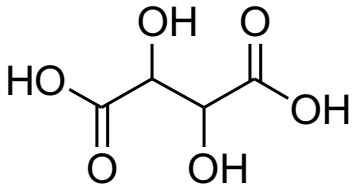


Figure 71: Structure of tartaric acid.

Questions

1. What is an organic acid in chemistry?
2. What is the significance of organic acids?
3. Which organic acids are used as food preservatives?
4. Draw the chemical structure of glutamic acid, citric acid, and tartaric acid.
5. Describe the properties and uses of glutamic acid and citric acid.
6. What is the function of citric acid in food?

7.2. Experimental part A: Isolation of glutamic acid from wheat flour gluten

Principle

Gluten can be easily isolated from the non-starch fraction of wheat flour. Gluten is known for its glutamic acid content, which is approximately 35 %. To isolate it, the starch must be removed from the wheat flour through repeated washing with water. The resulting preparation is then heated in an inorganic acid environment, vacuum evaporated, and crystallized.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, test tubes, glass rods, volumetric flasks, analytical balance, water bath, reflux condenser, Erlenmeyer flasks, round bottom flask, analytical funnel, rotary vacuum evaporator, and filter paper.

Materials and chemicals

- Wheat flour,
- concentrated hydrochloric acid (HCl, 36 %, *v/v*),
- acetone (CH_3COCH_3) (V = 15 ml),
- a mixture of ethanol:diethyl ether in a 1:1 (*v/v*) ratio (V = 10 ml),
- sodium hydroxide (NaOH) with a concentration of 1 mol/l (M = 39,997 g/mol; V = 10 ml).

Procedure

1. Mix 100 g of wheat flour with 300 ml of distilled water. Mix the mixture thoroughly and make a dough.
2. Carefully decant the white-colored water (starch) from the sediment and repeatedly add 300 ml distilled water.
3. Decant and wash again with distilled water (300 ml) until no more white-colored water (starch) is formed.
   We can consider gluten prepared in this way as starch-free gluten.
4. Mix 10 g of gluten with 30 ml of concentrated hydrochloric acid in a round bottom flask. Keep the flask with the solution in a boiling water bath until the gluten dissolves.
5. Attach the flask to a reflux condenser (see Figure 56). Heat the flask in a boiling water for 1 hour.
6. Cool and add 30 ml of distilled water, mix and filter (see Figure 72).

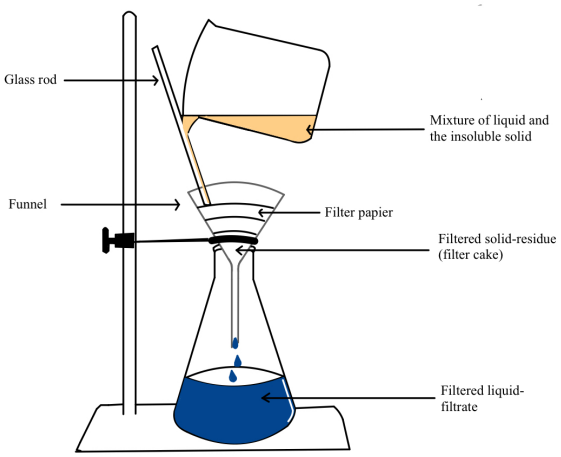


Figure 72: Filtration.

7. Vacuum evaporate the filtrate until 3/4 of its volume has evaporated.
8. Cool and crystallize the mixture at 4 °C for 2-3 days.
9. When glutamic acid hydrochloride crystals are formed, add 15 ml of ice-cold acetone and filter.
10. Wash the filter cake with an ice-cold mixture of ethanol and diethyl ether (1:1; *v/v*).
11. Dissolve the filter cake in a minimum volume of hot water, and then add 1 mol/l NaOH until a pH of 3.0 is attained.
    Check the pH value with a universal pH paper.
12. Vacuum evaporate the supernatant until a volume of about 6 ml remains, and subsequently leave it in the cold (4 °C) until glutamic acid crystallizes.
13. Filter and dry the obtained crystals.

Results

Calculate the percent yield (%) of glutamic acid per wheat flour and gluten. For the calculations, the maximum theoretical yield of glutamic acid isolated from wheat flour is 1.3 % (*w/w*) and isolated from wheat gluten is 35 % (*w/w*).

Question

1. Why is starch isolated from wheat flour before glutamic acid isolation?
2. Draw a block diagram of the isolation of glutamic acid from wheat flour.
3. Calculate the percent yield of glutamic acid (%) per wheat and gluten if you know that the weight of glutamic acid crystals was 0.487 g, wheat flour weight was 100.211 g and wheat gluten was 10.231 g. The maximum theoretical yield of glutamic acid isolated from wheat flour is 1.3 % (*w/w*) and isolated from wheat gluten is 35 % (*w/w*).

7.3. Experimental part B: Isolation of citric acid from lemon

Principle

Citric acid is a white crystalline substance soluble in water and slightly soluble in organic solvents. Its aqueous solution is slightly more acidic than acetic acid. Traces of citric acid are found in various plants and animals because it serves as an intermediate in the citric acid cycle (Krebs cycle).

Significant amounts of this compound are presented in the juice of citrus fruits, from which it can be precipitated by calcium ions. Calcium citrate is formed, and citric acid is subsequently converted back using dilute sulfuric acid (see Figure 73).

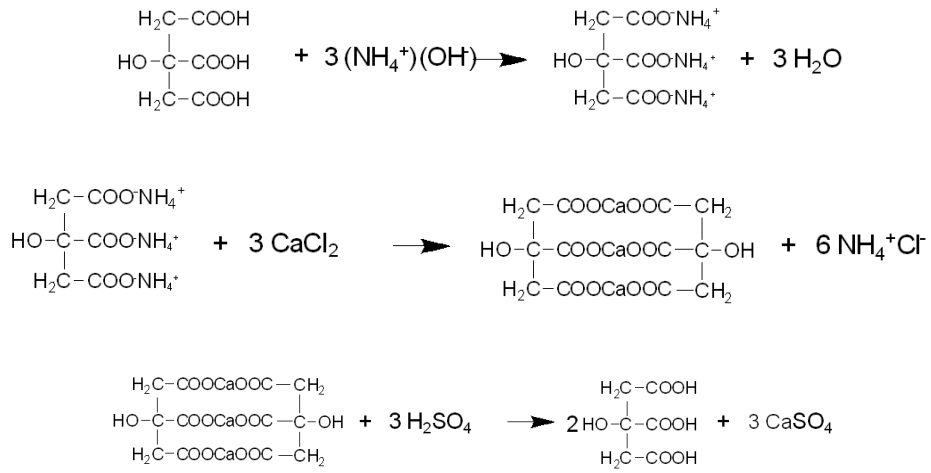


Figure 73: Principle of isolation of citric acid.

Citric acid is commercially obtained through the fermentation of saccharides by the filamentous fungus, *Aspergillus niger*. It is used as an additive in food and beverages to create a pleasantly sour taste. It is used in medicine, the textile industry (blueprinting) and as a polishing agent for metals.

Laboratory requirements

Automatic pipettes (20-200 µl, 100-1000 µl), pipetting tips, beakers, test tubes, glass pipettes, volumetric flasks, analytical balance, hot plate, asbestos mesh, vacuum filtering flask with side arm socket (Büchner flask), Büchner funnel, filter paper, desiccator, dye-infused paper strips (universal indicator paper, pH 1-14), pH meter, crystallizing dish, filter paper, and rotary vacuum evaporator.

Materials and chemicals

- Lemon,
- 26 % (*v/v*) ammonia solution,
- concentrated sulfuric acid (H_2SO_4, 98 %, *v/v*, M = 98,079 g/mol, $\rho_{98\ \%}$ = 1.836 g/ml),
- calcium chloride, 25 % (*w/v*) aqueous solution (M = 110.98 g/mol, V = 25 ml).

Procedure

1. Cut pre-weighted lemon into pieces and squeeze to get the juice.
2. Transfer the lemon juice to a 250 ml beaker, and tested its pH value.
   Check the pH value with a universal pH paper.

3. Neutralize the lemon juice with 26 % (*v/v*) aqueous ammonia solution until the pH becomes 7.1-7.5.
   Write down the exact ammonia consumption. A strongly alkaline environment is undesirable because precipitation of calcium chloride would produce calcium hydroxide, which would prevent subsequent isolation.
4. Filter the solution using the Büchner funnel (see Figure 74).
   When working with a water jet pump, the main rule is to allow air back into the system after finishing work, and only then can the vacuum source be turned off. This will prevent water from entering the equipment or aggressive substances from being sucked into the vacuum cleaner and damaging it.

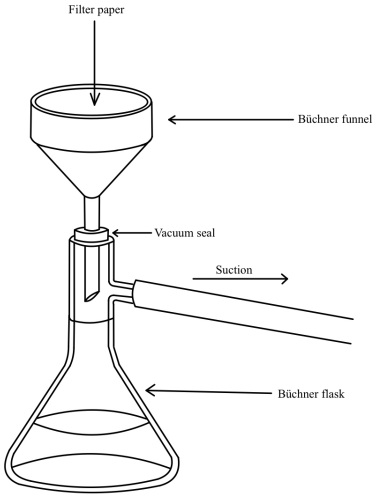


Figure 74: Vacuum filtration.

5. Transfer the filtrate to a 250 ml beaker. Place the beaker on a hot plate with an asbestos mesh (wire mesh gauze), and heat to a boil.
6. Add 25 ml of 25 % (*w/v*) aqueous solution of calcium chloride, and mix the solution thoroughly.
7. Filter the formed calcium citrate precipitate using the Büchner funnel.
8. Wash the filter cake with 40 ml of hot distilled water (prepare hot distilled water in advance).
9. Transfer the filter cake to a beaker and add 10 ml of distilled water.
10. **Calculate the required amount of sulfuric acid to neutralize the calcium citrate.**
11. Slowly, add the concentrated sulfuric acid to the solution.
12. Filter the solution using the Büchner funnel and concentrate the filtrate in a hot plate.
13. Kept the solution for 7 days to crystalize.
14. Isolate the crystals of citric acid by filtration.
15. Weight the isolated citric acid crystals.

Results

Calculate the percent yield (%) of citric acid. For the calculation, the maximum theoretical yield of citric acid isolated from lemon is 8 % (*w/w*).

Questions

1. How is citric acid isolated from lemon juice?
2. How does citric acid isolation affect pH?

3. What chemical reactions are involved in the extraction of citric acid from lemon juice?
4. How much calcium chloride ($CaCl_2$) should you use to make 25 ml of its 25 % (*w/v*) solution?
5. Calculate the required amount of sulfuric acid (98 %, *v/v*) to neutralize 250 µl of 26 % (*v/v)* ammonia used during the isolation of citric acid from lemon ($\rho_{(26\ \%\ ammonia)}$ = 0.904 g/ml; M_{NH3} = 17 g/mol; M_{H2SO4} = 98.079 g/mol; $\rho_{(98\ \%\ sulfuric\ acid)}$ = 1.84 g/ml).

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Attachment 1: Model protocol

Protocol name (Times New Roman, 14, bold, centre text, line spacing 1.5 lines)

Full name (Times New Roman, 12, right aligned)
Date of laboratory exercise

Determination of reducing saccharides by the DNS method (Times New Roman, 14, bold, centre text)

1. Aim (Times New Roman, 14, bold)

A precise definition of the aim of the work is needed (Times New Roman, 12, text is aligned with both margins).

2. Procedure (Times New Roman, 14, bold)

In your own words (past tense), state what was done and how it was done, including any deviations from instructions, etc. So that the experiment can be repeated exactly according to the protocol. The procedure does not include an evaluation of the result or an explanation of the principle of the method used (Times New Roman, 12, text is aligned with both margins).

3. Calculations (Times New Roman, 14, bold)

Outline the weights, dilutions of solutions, etc. required for the procedure (including the relationships and formulas needed to process the measured values) (Times New Roman, 12, text is aligned with both margins).

4. Results (Times New Roman, 14, bold)

(+ their evaluation, graphs) - (only) what you observed or measured, or what you calculated from the measured values (i.e., NOT how the experiment "should evolve", NOT what you conclude from the experiment - both are part of the discussion). Present the results as clearly as possible, preferably in a table or figure (Times New Roman, 12, text is aligned with both margins).

Processing the result in the form of a table or figure

The processing of measurement results usually begins with their graphical display. Displaying the result in the form of a table or figure allows to get an idea of the experiment, to point out some regularities and to point the way to further numerical processing.

The statistical data obtained are sorted and written in a clear and concise format in **tables.** A description of the table is always provided above the table. A legend for the symbols or abbreviations used is always given below the table.

Table x: Table title.

For example: **Table 2:** Absorbance of glucose solutions prepared by the DNS method, measured at wavelength of 540 nm in three parallel measurements.

Description of individual columns				
Glucose concentration [g/l]	**Absorbance at 540 nm**			**Average**
	I.	**II.**	**III.**	
2.5	0.126	0.145	0.137	0.141
5.0	0.278	0.284	0.282	0.281
13.0	0.739	0.719	0.720	0.719
14.0	0.844	0.859	0.867	0.857
	measured values such as			

A graph presents the mutual relations between two or more variables using clear symbols: points, segments, lines, squares, rectangles, circles, etc. The graph is the simplest representation of the dependence y = f(x). For the graph to be simple and easy

to read, it must have a concise title with the exact designation of the individual axes. It is most often constructed using the Excel program. Important attention must be paid to the scale when creating the graph to avoid distortion. The independent variable is indicated on the horizontal axis (x-axis), and the dependent variable on the vertical axis (y-axis). Figure 1 shows an example of an XY graph (dependency) with a description. The figure description always belongs below the figure, as well as the legend to the symbols or abbreviations used.

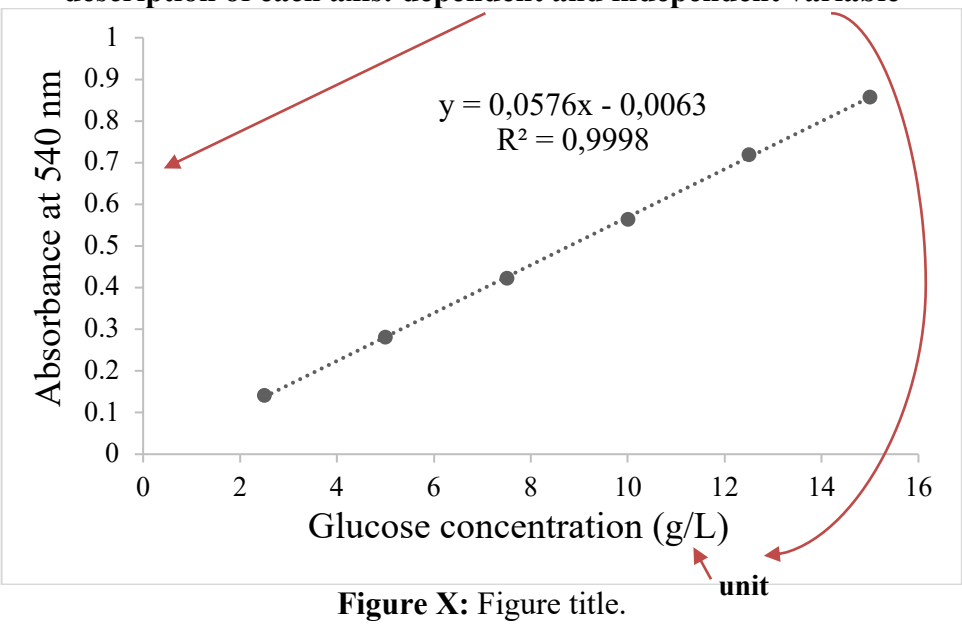


Figure X: Figure title.

For example: **Figure 1:** The dependence of absorbance at 540 nm on the glucose concentration.

5. Discussion (Times New Roman, 14, bold)

Evaluation of the results achieved, explanation of the implications of the results, etc. Comparison of measured or calculated values with those that are tabulated, theoretically calculated, etc. Comparison of results obtained by different methods - justification of the strengths and weaknesses of each method (Times New Roman, 12, text is aligned with both margins).

6. Conclusion (Times New Roman, 14, bold)

Brief response to the aim of this laboratory work is required (Times New Roman, 12, text is aligned with both margins).

Attachment 2: The laboratory protocols

Statistical analysis of experimental data

1. Aim

The aim of this laboratory exercise was to learn how to weigh objects using technical and analytical balances, and to compare their accuracy in weighing different objects. In the second part of the work, we focused on familiarizing ourselves with a set of single-channel pipettes and exploring the principles on which they operate.

2. Procedure

We determined the weight of three objects. namely an Eppendorf tube, a beaker (25 ml), and a test tube, using both n analytical balance and a technical balance. We calculated the arithmetic mean (average) as well as the standard deviation.

Working with a single-channel pipette and statistical evaluation of results

a. We poured distilled water into a beaker and prepared a series of labelled and pre-weighed Eppendorf tubes.
b. We pipetted 1000 µl of distilled water into three Eppendorf tubes using a single-channel pipette. Subsequently. we pipetted 2 x 500 µl of water into another three tubes, and 10 x100 µl of distilled water into the final three tubes.
c. Each Eppendorf tube was weighed three times on an analytical balance. We calculated the arithmetic mean and the standard deviation.

3. Calculations

Calculation of the arithmetic mean (average):

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} = \frac{(1.0992 + 1.0987 + 1.0987 + 1.0984 + 1.0986)}{5} = 1.0987 \text{ g}$$

Calculation of the standard deviation (SD):

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \text{ x}$$

σ

$$= \sqrt{\frac{(1.0992 - 1.0987)^2 + (1.0987 - 1.0987)^2 + (1.0987 - 1.0987)^2 + (1.0984 - 1.0987)^2 + (1.0986 - 1.0987)^2}{5 - 1}}$$

$$\sigma = 0.0003$$

Note: *Round the result to the same number of decimal places as the number with the fewest number of decimal places. In this case, as the analytical balance provides the weight of the object to four decimal places, round your result to four decimal places. Therefore, round to four decimal places.*

4. Results

The following tables summarize the results of weighing three different objects on both analytical (Table 1) and technical balances (Table 2).

Table 1: The results of Eppendorf tube weight measurements on an analytical balance.

Object	Weight [g]					Arithmetic mean	SD
	I.	II.	III.	IV.	V.		
Eppendorf tube	1.0992	1.0987	1.0987	1.0984	1.0986	1.0987 g	0.0003 g
Beaker (25 ml)	28.7472	28.7466	28.7470	28.7468	28.7471	28.7469 g	0.0003 g
Test tube	5.7310	5.7309	5.7311	5.7312	5.7312	5.7311 g	0.0001 g

Table 2: The results of Eppendorf tube weight measurements on a technical balance.

Object	Weight [g]					Arithmetic mean	SD
	I.	II.	III.	IV.	V.		
Eppendorf tube	1.099	1.100	1.099	1.099	1.100	1.099 g	0.001 g
Beaker (25 ml)	28.758	28.758	28.757	28.758	28.758	28.758 g	0.000 g
Test tube	5.734	5.731	5.734	5.733	5.735	5.733 g	0.002 g

Table 3 shows the calculated means and standard deviations of 1 ml of water weighed on the analytical balance and pipetted into Eppendorf tubes by different procedures.

Table 3: The results of working with a single-channel pipette and pipetting 1 ml of distilled water in different ways.

Procedure	Weight of an empty tube (g)	Weight of a tube with water (g)			Weight of water (g)			Mean	SD
		I	II	III	I	II	III		
1x1000 µl	1.1245	2.1261	2.1259	2.1263	1.0016	1.0014	1.0018	1.0022 g	0.0021 g
	1.1212	2.1214	2.1212	2.1216	1.0002	1.0000	1.0004		
	1.1155	2.1204	2.1202	2.1206	1.0049	1.0047	1.0051		
2x500 µl	1.1121	2.1207	2.1205	2.1209	1.0086	1.0084	1.0088	1.0080 g	0.0012 g
	1.1071	2.1135	2.1133	2.1137	1.0064	1.0062	1.0066		
	1.1315	2.1404	2.1402	2.1406	1.0089	1.0087	1.0091		
10x100 µl	1.112	2.2297	2.2295	2.2299	1.1177	1.1175	1.1179	1.1043 g	0.0101 g
	1.1067	2.2030	2.2028	2.2032	1.0963	1.0961	1.0965		
	1.1108	2.2098	2.2096	2.21	1.099	1.0988	1.0992		

5. Discussion

From the results of weighing different objects, it is clear that analytical balance (Table 1) is more accurate than the technical balance (Table 2), providing lower standard deviations and more accurate weighing results.

When pipetting the same volume (1 ml) of distilled water using a single-channel pipette, the results are more accurate, with the lowest observed standard deviation (1.0022±0.0021 g). The least accurate results were obtained by pipetting 10 x100 µl of distilled water (1.1043±0.0101 g) (Table 3).

6. Conclusion

For very accurate weighing of objects, it is preferable to use an analytical balance. In the case of pipetting a certain volume of solution, it is recommended to use a single-channel pipette with a suitable range of volumes.

The relationship between the concentration of a substance in solution and its absorbance

1. Aim

The aim of this work was to prepare a calibration curve of the dependence of absorbance on the concentration of Malachite Green dye* and to calculate the molar absorption (extinction) coefficient of this dye.

***Note:** * Specify your dye.*

2. Procedure

a. We prepared a stock solution of the dye with a concentration of 150 mg/l and 9 calibration solutions through appropriate dilutions.

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
20.0	7.5	1.333	8.667
17.0	8.8	1.137	8.863
15.0	10	1.000	9.000
12.5	12	0.833	9.167
10.0	15	0.667	9.333
7.5	20	0.500	9.500
5.0	30	0.333	9.667
2.5	60	0.167	9.833
1.0	150	0.067	9.933

b. We measured the absorbance of each calibration solution at 600 nm in three parallel measurements.

c. We plotted the dependence of absorbance on actual concentration, performed a linear regression analysis and calculated the molar absorption (extinction) coefficient under defined conditions.

3. Calculations

Preparation of the Malachite Green stock solution (V = 100 ml, c = 150 mg/l)

c = 150 mg/l

m = ? g

150 mg …. 1000 ml

x mg …… 100 ml

x = (100x150)/1000 = **15 mg**

Calculation of dilution factor

Dilution factor (x) = 150 mg/l / 1 mg/l = 150x

Calculation of the volume of stock solution and distilled water for the preparation of a dilute solution (1 mg/l)

$$Volume\ of\ stock\ solution\ transferred\ = \frac{10\ ml}{150\ x} = \mathbf{0.067\ ml}$$

$$Volume\ of\ distilled\ water\ = 10\ ml - 0.067\ ml = \mathbf{9.933\ ml}$$

Calculation of the arithmetic mean (average) of absorbances at 600 nm:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} = \frac{(2.012 + 2.010 + 2.014)}{3} = 2.012$$

Calculation of the molar concentration of Malachite Green dilute solution

$$c = \frac{c_m}{M}$$

c = 0.001 g/l / 364.911 g/mol = **$0.27.10^5$ mol/l**

Calculation of the molar absorption (extinction) coefficient for Malachite Green

$$A = \varepsilon_\lambda . c . l \rightarrow \varepsilon_\lambda = \frac{A}{c . l}$$

ε_λ = *0.097/ (0.27.10^5 mol/l x 1 cm) = 35,393 l.mol^{-1} cm^{-1}* = **35,393 $M^{-1}cm^{-1}$**

4. Results

Based on the measured absorbances recorded in Table 1, we constructed a calibration curve and calculated a linear regression equation (see Figure 1).

Table 1[A]: Measured absorbance values of dilute solutions of Malachite Green at a concentration of 1-20 mg/l at 600 nm.

Malachite Green concentration (mg/l)	Molar concentration (mol/l)	Absorbance			Average value
20.0	$5.48.10^5$	2.012	2.010	2.014	2.012
17.0	$4.55.10^5$	1.545	1.543	1.547	1.545
15.0	$4.11.10^5$	1.231	1.229	1.233	1.231
12.5	$3.43.10^5$	0.963	0.961	0.965	0.963
10.0	$2.74.10^5$	0.663	0.661	0.665	0.663
7.5	$2.06.10^5$	0.553	0.551	0.555	0.553
5.0	$1.37.10^5$	0.455	0.453	0.457	0.455
2.5	$0.69.10^5$	0.214	0.212	0.216	0.214
1.0	$0.27.10^5$	0.097	0.095	0.099	0.097

Notes:

A- *The table description always above the table, while the figure description is placed below the figure. The description of the table, as well as the table itself, must be on the same page of the protocol. Do not separate them.*

B- *You can remove any absorbance measurements that obviously do not make sense, as they may have been caused by inaccurate pipetting, poor homogenisation, etc. You will not include this value when calculating the average.*

C- *Working with Excel for calculations. Do not subtract each number in turn using a calculator.*

We also converted the mass concentration of each dilute solution to a molar concentration (see Table 1). This conversion is necessary for calculating the extinction coefficient (see Table 2).

Table 2: The calculation of molar absorption (extinction) coefficient under defined conditions.

Malachite Green concentration (mg/l)	Malachite Green concentration (g/l)	Molar concentration (mol/l)	Molar absorption (extinction) coefficient ($M^{-1}cm^{-1}$)	Average
20.0	0.020	$5.48.10^5$	36,710	
17.0	0.017	$4.55.10^5$	33,164	
15.0	0.015	$4.11.10^5$	29,947	
12.5	0.0125	$3.43.10^5$	28,113	
10.0	0.010	$2.74.10^5$	24,194	30,986
7.5	0.0075	$2.06.10^5$	26,906	
5.0	0.005	$1.37.10^5$	33,207	
2.5	0.0025	$0.69.10^5$	31,236	
1.0	0.001	$0.27.10^5$	35,396	

Figure 1 shows the dependence of absorbance at 600 nm on the Malachite Green concentration.

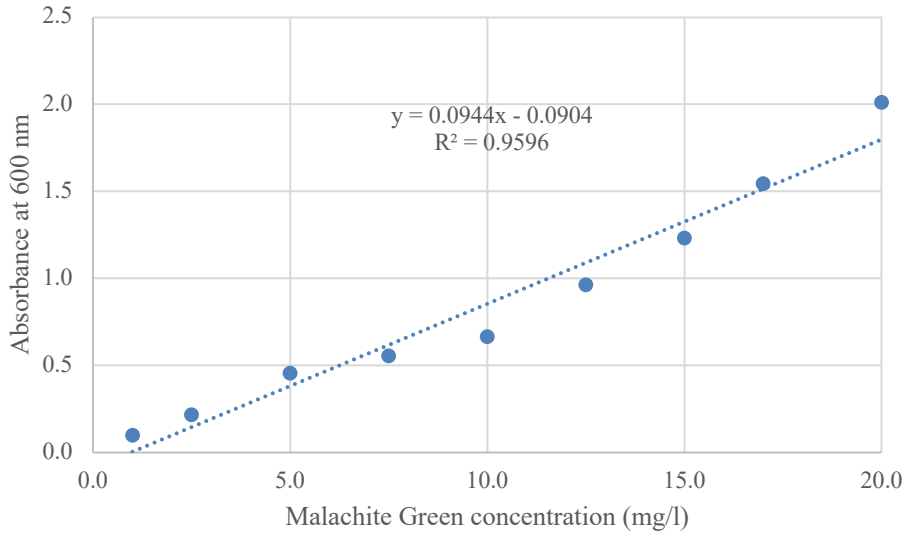


Figure 1: The dependence of absorbance at 600 nm on the Malachite Green concentration.

Note for the graph: *Each graph must have a description of the x and y axes with units (unless it is a dimensionless number).*

5. Discussion

Based on the measured absorbance results of the dilute solutions, we prepared a graph of absorbance versus Malachite Green dye concentration. The linear regression equation was as follows: 0.0944x - 0.0904. The value of the coefficient of determination (R^2) was 0.9596, which indicates that we could improve our accuracy. The lower value of this coefficient could be due to inaccurate pipetting when diluting the solutions.

The calculated value of the molar absorption (extinction) coefficient of Malachite Green dye was 30,986 $M^{-1}cm^{-1}$. According to the literature[1], the molar extinction coefficient

for Malachite Green dye is 14,899 $M^{-1}cm^{-1}$ or 140,000 $M^{-1}cm^{-1}$ at 618 nm. The extinction coefficient is affected (i) the amount of light absorbed by the substance for a specific wavelength, (ii) the distance that the light travels through the solution, and (iii) the concentration of the absorbing solution per unit volume. The extinction coefficient for a molecule is not the same for all wavelengths. We determined the extinction coefficient of Malachite Green dye at a wavelength of 600 nm. In the case of one literature source[2], this coefficient was determined at a wavelength of 618 nm.

6. Conclusion

We prepared a calibration curve of the dependence of absorbance on the concentration of Malachite Green dye (y =. 0.0944 x – 0.0904, $R^2 = 0.9596$). Malachite Green dye 's he molar absorption (extinction) coefficient of is 30,986 $M^{-1}cm^{-1}$.

References

[1] https://www.aatbio.com/resources/extinction-coefficient/malachite_green
[2] https://pdf.sciencedirectassets.com/

The relationship between the concentration of a substance in solution and its absorbance

1. Aim

The aim of the laboratory exercise was to prepare two buffers (TRIS-acetic acid and K-phosphate buffers) with subsequent pH control of the prepared solutions. K-phosphate buffer should have a pH value of 6.0*.

Note: ** Specify pH value of K-phosphate buffer you should have prepared.*

2. Procedure

Preparation of TRIS-acetic acid buffer

a. We calculated the mass of TRIS base required to prepare 100 ml of buffer with a concentration of 0.1 mol/l.
b. Using concentrated acetic acid (1.2 ml), we adjusted the pH of the solution to 7.0.

Preparation of K-phosphate buffer

a. We calculated the mass of acid (KH_2PO_4) and basic (K_2HPO_4) phosphate required to prepare 100 ml of buffer at a concentration of 0.2 mol/l.
b. We diluted the acid (KH_2PO_4) and basic (K_2HPO_4) phosphate to the pH 6.0*.

Note: ** Specify pH value of K-phosphate buffer you should have prepared.*

Table 1: Preparation of K-phosphate buffer.

pH	*x* 0.2 mol/l K_2HPO_4	*y* 0.2 mol/l KH_2PO_4
6.0	12.3	87.7

Legend: For a 0.2 mol/l buffer, mix *x* ml of 0.2 mol/l basic (K_2HPO_4) phosphate solution and *y* ml of 0.2 mol/l acid (KH_2PO_4) phosphate solution.

c. We checked the pH of the solution on the pH meter.

3. Calculations

Calculation of TRIS mass

$$m = c \cdot V. M = 0.1 \text{ mol/l} \times 0.1 \text{ l} \times 121.14 \text{ g/mol} = \mathbf{1.2114 \text{ g}}$$

Calculation of KH_2PO_4 mass

$$m = c \cdot V. M = 0.2 \text{ mol/l} \times 0.25 \text{ l} \times 136.09 \text{ g/mol} = \mathbf{6.8045 \text{ g}}$$

Calculation of K_2HPO_4 mass

$$m = c \cdot V. M = 0.2 \text{ mol/l} \times 0.25 \text{ l} \times 174.18 \text{ g/mol} = \mathbf{8.709 \text{ g}}$$

4. Results and discussion

To prepare the TRIS-acetic acid buffer, we needed 1.2 ml of dilute acetic acid.

To prepare the K-phosphate buffer, we obtained a solution with pH 6.2 instead of a solution of pH 6.0. This means that we did not weigh the individual components accurately or we prepared the measuring solutions of the individual components incorrectly. It is also possible that the equipment used (balance, pipettes and pH meter) was not property calibrated and lacked sufficient accuracy.

5. Conclusion

The TRIS-acetic acid buffer had pH 7.0 and the K-phosphate buffer had pH 6.2.

Determination of glucose concentration by the DNS method

1. Aim

The aim of this work is to determine the glucose concentration in the sample 1* by the DNS method.

Note: ** Specify your sample number.*

2. Procedure

a. We mixed 0.1 ml of the solution (either the sample or standard solution with known glucose concentration) with 0.8 ml of the DNS reagent.
b. We boiled the mixture in a boiling water bath for 5 minutes.
c. After this time, we added 8 ml of distilled water and stirred mixture on a vortex.
d. We measured the absorbance of the cooled samples at a wavelength of 540 nm.
e. We calibrated the DNS method using glucose solutions of varying concentrations (1-15 g/l*).

Note: ** Indicate the concentrations you worked with during your experimental work in the laboratory.*

3. Calculations

Calculation of glucose concentration in sample 1

c = (0.623* + 0.0274)/0.1307 = 4.84 g/l

Note: ** Here, provide the linear regression equation you obtained by plotting the dependence of absorbance on the known glucose concentration. Use the standard curve to calculate the concentration of a solution. Don't forget to subtract the average of the blank (i.e., DNS reagent and water)! Calculate the concentration of glucose in the sample for each absorbance since we are working with three parallel measurements. Then calculate the average glucose concentration in the sample as well as the standard deviation.*

4. Results

Based on the measured absorbances recorded in Table 1, we constructed a calibration curve (see Figure 1).

Table 1[A]: Measured absorbance values of standard glucose solutions with a concentration of 1-15 g/l at a wavelength of 540 nm.

Glucose concentration (g/l)	Absorbance			Absorbance after subtraction of the average value of the blank			Average value
0	0.019	0.021	0.018	**Average:** 0.019			
1	0.121	0.119	0.342	0.102	0.100	~~0.323~~[B]	0.101
2.5	0.313	0.315	0.311	0.294	0.296	0.292	0.294
5	0.631	0.655	0.672	0.612	0.636	0.653	0.633[C]
7.5	0.974	0.970	0.978	0.955	0.951	0.959	0.955
10	1.304	1.291	1.300	1.285	1.272	1.281	1.279
12.5	1.634	1.641	1.628	1.615	1.622	1.609	1.615
15	1.902	1.960	1.968	1.883	1.941	1.949	1.924
SAMPLE 1	0.623	0.501	0.625	0.604	~~0.482~~	0.606	-[D]

Notes: ***A-*** *The table description always above the table, while the figure description is placed below the figure. The description of the table, as well as the table itself, must be on the same page of the protocol. Do not separate them.*
B- *You can remove any absorbance measurements that obviously do not make sense, as they may have been caused by inaccurate pipetting, poor homogenization, etc. You will not include this value when calculating the average.*
C- *Working with Excel for calculations. Do not subtract each number in turn using a calculator.*
D- *The concentration of glucose in the sample must be calculated separately from each absorbance. Only after this operation can the arithmetic mean(average) and standard deviation be calculated.*

Figure 1 shows the dependence of absorbance at 540 nm on the glucose concentration.

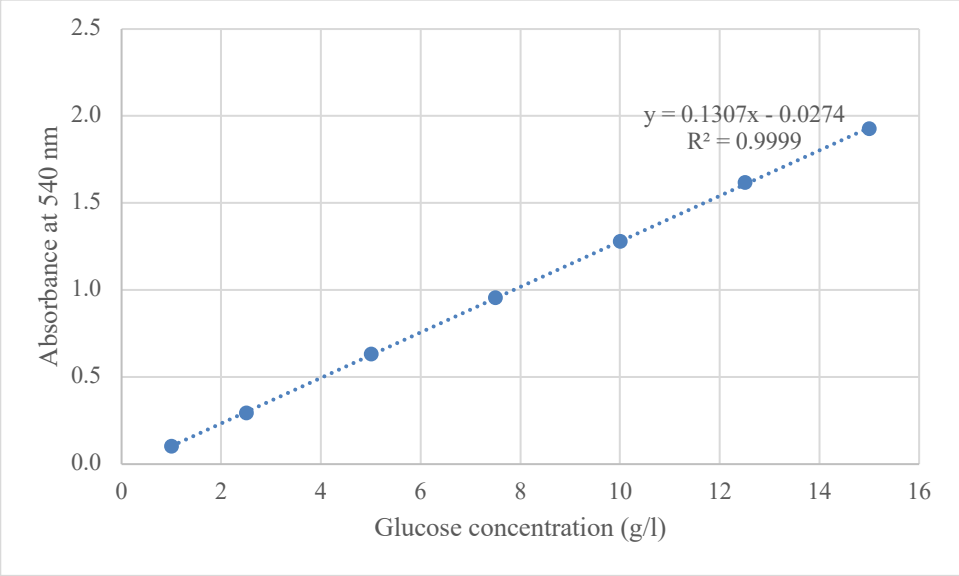


Figure 1: The dependence of absorbance at 540 nm on the glucose concentration.
Note for the graph: *Each graph must have a description of the x and y axes with units (unless it is a dimensionless number).*

5. Discussion

Based on the results presented in Figure 1, we determined the linear regression equation is y = 0.1307x - 0.0274, with R^2 of 0.9999. We found that the glucose concentration in sample 1 is 4.84±0.01 g/l*.
Note: ** You determined the absorbance of the sample in three repetitions. The standard deviation should therefore be given here.*

6. Conclusion

The experimentally determined glucose concentration is 4.84±0.01 g/l*.
Note: ** The accuracy of your result will be assessed by the teacher. The teacher will know the exact concentration of glucose in the sample.*

Determination of sucrose concentration by the DNS method

1. Aim

The aim of this work is to determine the sucrose concentration in the sample 3* by the DNS method.

Note: ** Specify your sample number.*

2. Procedure

a. We mixed 1 ml of sucrose solution or a sample with 30 µl (1 drop) of concentrated HCl (36 %; *v/v*).
b. We boiled the mixture in a boiling water bath for 5 minutes.
c. We neutralized the acid by the addition of 100 µl (3 drops) of 5 mol/l aqueous KOH solution.
d. We mixed 0.1 ml of the solution (either the sample or standard solution with known sucrose concentration) with 0.8 ml of the DNS reagent.
e. We boiled the mixture in a boiling water bath for 5 minutes.
f. After this time, we added 8 ml of distilled water and stirred on a vortex.
g. We measured the absorbance of the cooled samples at a wavelength of 540 nm.
h. We calibrated the DNS method using different concentrations of sucrose solutions (1-5 g/l*) after acid hydrolysis and neutralization.

Note: ** Indicate the concentrations you worked with during your experimental work in the laboratory.*

3. Calculations

Calculation of KOH solution

c = 5 mol/l
V = 100 ml = 0,1 l
M = 56.1056 g/mol

$c = n/V \rightarrow n = c \cdot V$
$n = m/M \rightarrow m = n \cdot M = c \cdot V \cdot M = 5\ mol/l \cdot 0{,}1\ l \cdot 56.1056\ g/mol = 28.053\ g$

Calculation of sucrose concentration in sample 3

$c = (0.382^{*} + 0.0373)/0.1449 = 2.89\ g/l$

Note: ** Here, provide the linear regression equation you obtained by plotting the dependence of absorbance on the known sucrose concentration. Use the standard curve to calculate the concentration of a solution. Don't forget to subtract the average of the blank (i.e., DNS reagent and water)! Calculate the concentration of sucrose in the sample for each absorbance as we are working with three parallel measurements. Then calculate the average sucrose concentration in the sample as well as the standard deviation.*

4. Results

Based on the measured absorbances recorded in Table 1, we constructed a calibration curve and calculated a linear regression equation (see Figure 1).

Table 1[A]: Measured absorbance values of standard sucrose solutions after acid hydrolysis and neutralization at a concentration of 1-5 g/l at 540 nm.

Sucrose concentration (g/l)	Absorbance			Absorbance after subtraction of the average value of the blank			Average value
0	0.019	0.021	0.018	**Average:** 0.019			
1	0.133	0.131	0.376	0.114	0.112	~~0.357~~[B]	0.113
1.5	0.1995	0.2035	0.325	0.181	0.185	~~0.306~~	0.183
2	0.266	0.276	0.274	0.247	0.257	0.255	0.253
2.5	0.3345	0.348	0.3435	0.316	0.329	0.325	0.323
3	0.403	0.420	0.413	0.384	0.401	0.394	0.393
3.5	0.477	0.4855	0.4845	0.458	0.467	0.466	0.463
4	0.551	0.551	0.556	0.532	0.532	0.537	0.534
4.5	0.6225	0.636	0.6475	0.604	0.617	0.629	0.616
5	0.694	0.721	0.739	0.675	0.702	0.720	0.699[C]
SAMPLE	0.401	0.392	0.421	0.382	0.373	0.402	-[D]

Notes: ***A-*** *The table description always above the table, while the figure description is placed below the figure. The description of the table, as well as the table itself, must be on the same page of the protocol. Do not separate them.*

B- *You can remove any absorbance measurements that obviously do not make sense, as they may have been caused by inaccurate pipetting, poor homogenization, etc. You will not include this value when calculating the average.*

C- *Working with Excel for calculations. Do not subtract each number in turn using a calculator.*

D - *The concentration of sucrose in the sample must be calculated separately from each absorbance. Only after this operation can the arithmetic mean(average) and standard deviation be calculated.*

Figure 1 shows the dependence of absorbance at 540 nm on the sucrose concentration.

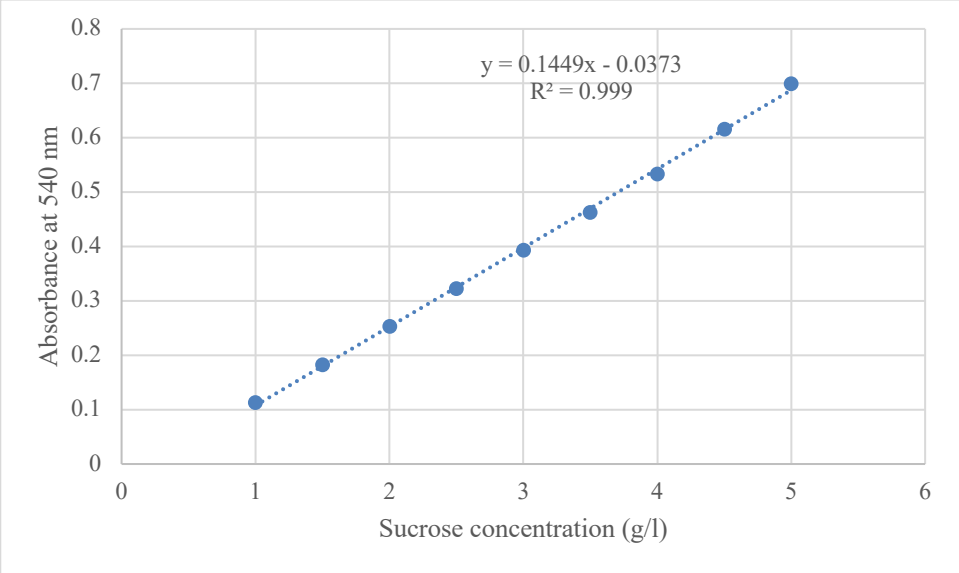


Figure 1: The dependence of absorbance at 540 nm on the sucrose concentration.

Note for the graph: *Each graph must have a description of the x and y axes with units (unless it is a dimensionless number).*

5. Discussion

Based on the results presented in Figure 1, we determined the linear regression equation $y = 0.1449x - 0.0373$, where R^2 is 0.9988. We found that the sucrose concentration in sample 3 is 2.92±0.10 g/l*.

***Note:** * You determined the absorbance of the sample in three repetitions. The standard deviation should therefore be given here.*

6. Conclusion

The experimentally determined sucrose concentration in the sample is 2.92±0.10 g/l*.

***Note:** * The accuracy of your result will be assessed by the teacher. The teacher will know the exact concentration of sucrose in the sample.*

Isolation of RNA from yeasts and determination of its concentration by the orcinol reagent

1. Aim

The aim of this work is to determine the actual yield (%) of the RNA extraction procedure from yeast, the quantity of ballast proteins (mg/ml) and the concentration of RNA (mg/l) using the orcinol reagent. We also compared the determined RNA concentrations in the sample obtained by the two methods.

2. Procedure

RNA isolation procedure

1. We mixed 20.021 g [A] of yeast (broken into small pieces) with 2 ml of 10 % (*w/v*) aqueous sodium hydroxide solution and 50 ml of distilled water.
2. We heated the prepared mixture for 15 minutes at 95 °C on a hot plate with an asbestos mesh, stirring occasionally.
3. Subsequently, we neutralized the mixture to pH 6.0 with acetic acid and cooled with water. We checked the pH using a universal pH paper.
4. We centrifuged the yeast mixture at 3,000 RPM for 15 minutes.
5. We poured the opalescent solution into a beaker and added hydrochloric acid drop by drop to pH 2.0. We checked the pH using a universal pH paper.
6. We added 35 ml of isopropanol to the mixture, cooled it with ice, and after about 10 minutes, we centrifuged the separated RNA precipitate again (3,000 RPM, for 15 minutes).
7. We decanted isopropanol and we washed the RNA precipitate with 5 ml of isopropanol and centrifuged again (3,000 RPM, for 15 minutes).
8. Finally, we washed the precipitate with 5 ml of acetone and centrifuged again (3,000 RPM, for 15 minutes) in a pre-weighed tube.
9. We dissolved 8 mg [B] of the precipitate in 8 ml [B] of distilled water and measured the spectrum in the UV region in the wavelength range 200-300 nm.

Notes: ***A-*** *Record the exact value of the weighted sample.*
B- *Record the exact weight of the precipitate and the volume of distilled water used to dissolve them. The precipitate should be dissolved in the distilled water at a 1:1 (v/v) rations.*

Determination of RNA using orcinol

1. We mixed 1 ml of the solution (either the sample or ribose solution) with 1 ml of the orcinol reagent.
2. We boiled the mixture in a boiling water bath for 20 minutes in a fume hood.
3. We took the tubes and cooled them to laboratory temperature.
4. After this time, we added 4 ml of distilled water to each test tube and stirred on a vortex.
5. We measured the absorbance of the cooled samples at a wavelength of 670 nm.
6. We calibrated the RNA determination using ribose solutions with different concentrations (10-100 mg/l).

3. Calculations*

Note: ***** *All values in the calculations, as well as the amount of precipitate, are made up. The calculation serves as a template to guide you in performing the process correctly.*

<u>The percent yield of RNA</u>

m (precipitate) = 18.2 mg[A]

A_{280} = 0.326

A_{260} = 0.250

$$\text{absorbance ratio} = \frac{A_{280}}{A_{260}} = \frac{0.326}{0.250} = 1.304$$

F = 0.970

% nucleic acids = 1 %

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \text{ x } 100\ \%$$

$$\text{Percent yield} = \frac{1\ \%}{4\ \%} . 100\ \% = 25\ \%$$

The amount of ballast proteins

proteins (mg/ml) = F. A_{280} / d

F = 0.970

A_{280} = 0.326

d = 1 cm

proteins (mg/ml) = 0.970. 0.326 / 1

proteins (mg/ml) = 0.32 mg/ml

Note: A- *Here, you should enter the weight of the precipitate, which refers to the amount of nucleic acid and protein you obtained after the last acetone washing step. To do this, you should have weighed the empty tube and then weighed it again with the precipitate.*

Calculation of RNA concentration

c = (0.241 - 0.0389)/0.0058 = 34.84 mg/l = 34.8 mg/l = 0.0348 g/l

0.0348 g of RNA ……. in 1000 ml of a solution

x g of RNA ……. In 100 ml of a solution

x : 0.0348 = 100 : 1000

$$\frac{x}{0.0348} = \frac{100}{1000}$$

$$x = \frac{100\ x\ 0.0348}{1000} = 0.00348 \text{ g/100 ml} = 0.004\ \%\ (w/v)$$

Note: *Here, you should provide the linear regression equation you obtained by plotting the dependence of absorbance on the known ribose concentration. Use the standard curve to calculate the concentration of a solution. Do not forget to subtract the average of the blank (i.e., the orcinol reagent and water)! Calculate the concentration of RNA in the sample for each absorbance since we are working with three parallel measurements. Subsequently, calculate the average RNA concentration in the sample.*

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \text{ x } 100\ \%$$

$$\text{Percent yield} = \frac{0.004\ \%}{4\ \%} . 100\ \% = 0.1\ \%$$

4. Results

Based on the measured absorbances recorded in Table 1, we constructed a calibration curve and calculated a linear regression equation (see Figure 1).

Table 1[A]: Measured absorbance values of standard ribose solutions at a concentration of 10-100 g/l at 670 nm.

Ribose concentration (mg/l)	Absorbance			Absorbance after subtraction of the average value of the blank			Average value
0	0.031	0.033	0.032	**Average:** 0.032			
10	0.590	0.581	0.610	0.622	0.613	0.642	0.626
20	0.445	0.455	0.439	0.477	0.487	0.471	0.478
40	0.300	0.299	0.260	0.332	0.331	0.292	0.318
60	0.155	0.160	0.169	0.187	0.192	0.201	0.193
80	0.068	0.060	0.066	0.100	0.092	0.098	0.097
100	0.590	0.581	0.610	0.622	0.613	0.642	0.626
SAMPLE	0.273	0.277	0.253	0.241	0.245	0.221	-[D]

Notes: ***A-*** *The table description always above the table, while the figure description is placed below the figure. The description of the table, as well as the table itself, must be on the same page of the protocol. Do not separate them.*

B- *You can remove any absorbance measurements that obviously do not make sense, as they may have been caused by inaccurate pipetting, poor homogenization, etc. You will not include this value when calculating the average.*

C- *Working with Excel for calculations. Do not subtract each number in turn using a calculator.*

D- *The concentration of ribose in the sample must be calculated separately from each absorbance. Only after this operation can the arithmetic mean(average) and standard deviation be calculated.*

Figure 1 shows the dependence of absorbance at 670 nm on the ribose concentration.

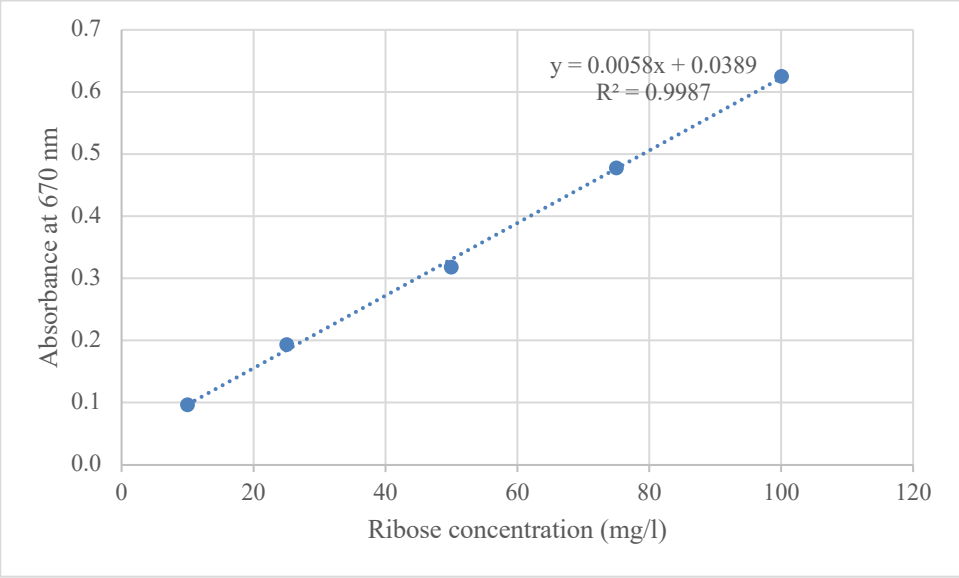


Figure 1: The dependence of absorbance at 670 nm on the ribose concentration.

Note for the graph: *Each graph must include a description of the x and y axes with units (unless it is a dimensionless number).*

Based on the measured absorbance values at 260 and 280 nm, we calculated the *F* factor and the quantity of ballast proteins present in the precipitate. The estimated amount of RNA is 1 %, and the amount of ballast proteins is 0.32 mg/ml.
Subsequently, we determined the amount of RNA using an orcin reagent. We calculated the amount of RNA, which was 0.004 %.

5. Discussion

Based on the results, we found that the calculated quantity of RNA from the precipitate likely contained other ballasts that interfered with the wavelengths used (260 and 280 nm). The determination of RNA using the orcin reagent was less accurate and the percent yield of RNA was 0.1 % compared to 25 % percent yield using the A_{280}/A_{260} absorbance ratio.

6. Conclusion

The actual yield (%) of the RNA extraction procedure from yeast was 25 %, the amount of ballast proteins was 0.32 mg/ml and the concentration of RNA (mg/l) by the orcinol reagent was 34.8 mg/l. We also compared the determined RNA yield in the sample obtained by the two methods and the determination of RNA using orcinol reagent was less accurate (0.004 %) than the determination of RNA using the A_{280}/A_{260} absorbance ratio (1 %).

Isolation of DNA from yeasts and determination of its concentration by the diphenylamine reagent

1. Aim

The aim of this work is to determine the percent yield (%) of the DNA extraction procedure from yeast, the quantity of ballast proteins (mg/ml) and the concentration of DNA (mg/l) using the diphenylamine reagent. We also compared the DNA yields determined in the sample obtained by the two methods.

2. Procedure

DNA isolation procedure

1. We mixed 10.013 g [A] of baker's yeast (broken into small pieces) with small amount of sea sand (purified) and 80 ml of 1 mol/l NaCl.
2. We centrifuged the viscous solution at 5,000 RPM for 10 minutes.
3. We measured the volume of the mixture using a graduated cylinder and very slowly added the cold water in a 1:6 (*v/v*) ratio.
4. We wounded DNA fibers on a pre-weighed wooden stick. We dried the coiled precipitate between filter papers and weighed it.
5. We dissolved 10 mg [B] of the air-dry precipitate in 10 ml [B] of distilled water.
6. We measured the spectrum of this solution in the UV region in the wavelength range of 200-300 nm.

Notes: ***A-*** *Record the exact value of the weighted sample.*
B- *Record the exact weight of the precipitate and the volume of distilled water used to dissolve it. The precipitate should be dissolved in the distilled water at a 1:1 (v/v) rations.*

Determination of DNA using diphenylamine

1. We mixed 1 ml of the solution (a sample or ribose solution) with 2 ml of the diphenylamine reagent.
2. We boiled the mixture in a boiling water bath for 10 minutes in a fume hood.
3. We took the tubes and cooled them to laboratory temperature.
4. We measured the absorbance of the cooled samples at a wavelength of 595 nm.
5. We calibrated the DNA determination using deoxyribose solutions with different concentrations (0-200 μg/l).

3. Calculations*

Note: ** All values in the calculations, as well as the amount of precipitate, are made up. The calculation serves as a template to guide you through the correct process.*

The percent yield of DNA

m (precipitate) = 18.2 mg[A]

A_{280} = 0.426

A_{260} = 0.351

$$\text{absorbance ratio} = \frac{A_{280}}{A_{260}} = \frac{0.426}{0.341} = 1.249$$

F = 0.944

% nucleic acids = 1.5 %

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \text{ x } 100\ \%$$

$$\text{Percent yield} = \frac{1.5\ \%}{14\ \%} . 100\ \% = 10.7\ \%$$

The amount of ballast proteins
proteins (mg/ml) = F. A_{280} / d
F = 0.944
A_{280} = 0.351
d = 1 cm
proteins (mg/ml) = 0.944. 0.351 / 1
proteins (mg/ml) = 0.33 mg/ml

Note: A- *Here, you should enter the weight of the precipitate, which refers to the amount of nucleic acid and protein you obtained after the last acetone washing step. To do this, you should have weighed the empty tube and then weighed it again with the precipitate.*

Calculation of DNA concentration
c = (0.741 - 0.044)/0.0058 = 120.17 µg/l x 50**B** = 6.01 mg/l = 0.006 g/l

0.006 g of DNA ……. in 1000 ml of a solution
x g of DNA ……. in 100 ml of a solution
x : 0.006 = 100 : 1000

$$\frac{x}{0.006} = \frac{100}{1000}$$

$$x = \frac{100\ x\ 0.006}{1000} = 0.0006\ \text{g/100 ml} = 0.0006\ \%\ (w/v)$$

Note: *Here, provide the linear regression equation you obtained by plotting the dependence of absorbance on the known deoxyribose concentration. Use the standard curve to calculate the concentration of a solution. Don't forget to subtract the average of the blank (i.e., the diphenylamine reagent and water)! Calculate the concentration of DNA in the sample for each absorbance, as we are working with three parallel measurements. Then calculate the average DNA concentration in the sample.*
B- *We had to dilute the sample 50 times and this dilution factor is taken into account in the calculation.*

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}}\ \text{x}\ 100\ \%$$

$$\text{Percent yield} = \frac{0.0006\ \%}{14\ \%} . 100\ \% = 0.4\text{x}10^{-4}\ \%$$

4. Results

Based on the measured absorbances recorded in Table 1, we constructed a calibration curve and calculated a linear regression equation (see Figure 1).

Table 1[A]: Measured absorbance values of standard deoxyribose solutions at a concentration of 0-200 µg/l at 595 nm.

Deoxyribose concentration (mg/l)	Absorbance			Absorbance after subtraction of the average value of the blank			Average value
0	0.049	0.048	0.05	**Average:** 0.049			
10	0.149	0.148	0.160	0.100	0.099	0.111	0.103
25	0.236	0.240	0.233	0.187	0.191	0.184	0.187
50	0.381	0.380	0.381	0.332	0.331	0.332	0.332
75	0.526	0.510	0.534	0.477	0.461	0.485	0.474
100	0.671	0.688	0.680	0.622	0.639	0.631	0.631
150	0.961	0.959	0.966	0.912	0.910	0.917	0.913
200	1.251	1.241	1.255	1.202	1.192	1.206	1.200
SAMPLE	0.790	0.794	0.770	0.741	0.745	0.721	-[D]

Notes: ***A-*** *The table description always above the table, while the figure description is placed below the figure. The description of the table, as well as the table itself, must be on the same page of the protocol. Do not separate them.*

B- *You can remove any absorbance measurements that obviously do not make sense, as they may have been caused by inaccurate pipetting, poor homogenization, etc. You will not include this value when calculating the average.*

C- *Working with Excel for calculations. Do not subtract each number in turn using a calculator.*

D- *The concentration of deoxyribose in the sample must be calculated separately from each absorbance. Only after this operation can the arithmetic mean(average) and standard deviation be calculated.*

Figure 1 shows the dependence of absorbance at 595 nm on the deoxyribose concentration.

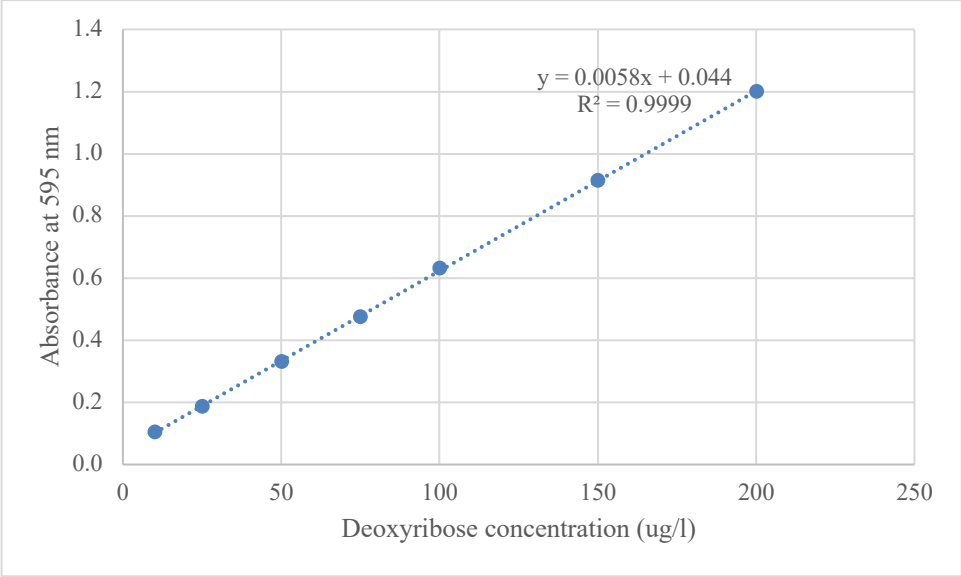


Figure 1: The dependence of absorbance at 595 nm on the deoxyribose concentration.
Note for the graph: *Each graph must include a description of the x and y axes with units (unless it is a dimensionless number).*

Based on the measured absorbance values at 260 and 280 nm, we calculated the *F* factor and the quantity of ballast proteins present in the precipitate. The estimated amount of DNA is 1.5 % and the amount of ballast proteins is 0.33 mg/ml.
Subsequently, we determined the amount of DNA using an orcin reagent. We calculated the amount of DNA, which was $0.4x10^{-4}$ %.

5. Discussion

Based on the results, we found that the calculated amount of DNA from the precipitate likely contained other ballasts that interfered with the wavelengths used (260 and 280 nm). The determination of DNA using the diphenylamine reagent was less accurate and the percent yield of DNA was $0.4.10^{-4}$ % compared to 10.7 % percent yield using the A_{280}/A_{260} absorbance ratio.

6. Conclusion

The percent yield (%) of the DNA extraction procedure from yeast was 10.7 %, the amount of ballast proteins was 0.33 mg/ml and the concentration of DNA (mg/l) determined by the diphenylamine reagent was 6.05 mg/l. We also compared DNA concentrations determined in the sample obtained by the two methods, and the determination of DNA using diphenylamine reagent was less accurate ($0.4x10^{-4}$ %) than the determination of DNA using the A_{280}/A_{260} absorbance ratio (10.7 %).

Determination of oil quality using acidity number, saponification number and peroxide number

1. Aim

The aim of this work is to determine the quality of the freshly opened olive oil [A] using the acid number, saponification number, and peroxide number to compare the quality of the oil. Another objective was to compare these results with those from other members of the group, specifically: freshly opened sunflower oil, rapeseed oil opened for two to three weeks, and freshly opened linseed oil [B]).

Note: **A-** *Specify your sample of oil.*

B- *Specify oil samples to your colleagues.*

2. Procedure

Determination of acid number

1. We added 10.023 g* of oil to 25 ml of isopropanol in a 100 ml Erlenmeyer flask.
2. We inserted the magnetic stirring pellet and stirred on a magnetic stirrer for 10 minutes.
3. We added a few drops (three) of phenolphthalein solution (1 %, *w/v)*.
4. We titrated the contents against 0.1 N KOH until a pink color appeared.

Note: ** Give the exact value of weighted sample.*

Determination of saponification number

1. We mixed 5.120 g* of the sample with 50 ml of potassium hydroxide in isopropanol in a round-bottom flask.
2. We attached the flask to a reflux condenser and heated it (in the flask) in a boiling water bath for 1 hour.
3. We cooled the solution and added a few drops (two) of phenolphthalein solution (1 %, *w/v)*.
4. We titrated the contents against 0.5 N HCl until a pink color disappears.
5. We also prepared a blank using the solvent (50 ml of potassium hydroxide in isopropanol), with a few drops (two) of phenolphthalein solution (1 %, *w/v*), without any oil sample and titrated the contents against 0.5 N HCl.

Note: ** State the exact value of the sample you weighed.*

Determination of peroxide number

1. We mixed 1.024 g* of oil with 10 ml of chloroform and 10 ml of glacial acetic acid in an Erlenmeyer bank.
2. We added 1 ml of a saturated aqueous solution of potassium iodide.
3. We stirred the flask vigorously for one minute, then incubated it in the dark at laboratory temperature for 5 minutes, stirring occasionally.
4. We added 75 ml of distilled water and a few drops (five) of starch indicator.
5. We titrated the contents against 0.01 mol/l sodium thiosulfate until a blue color disappears.
6. We prepared a blank with 10 ml of chloroform, 10 ml of glacial acid, a saturated aqueous solution of potassium iodide, 75 ml distilled water, and a few drops of starch indicator (five), without any oil sample, and titrated it against 0.01 mol/l sodium thiosulfate.

Note: ** State the exact value of the sample you weighed.*

3. Calculations

Determination of acid number

$$\text{Acid number} = \frac{56.1 \times \text{N} \times \text{V}}{\text{sample weight (g)}} = \frac{56.1 \times 0.1 \times 1.3^{\mathbf{A}}}{10.023^{\mathbf{B}}} = 0.728 \text{ mg KOH/g of oil}$$

Determination of saponification number

$$\text{Saponification value} = \frac{28.05 \times (\text{V}_1 - \text{V}_2)}{\text{sample weight [g]}} = \frac{28.05 \times (61-42.5)^{\mathbf{A}}}{5.120^{\mathbf{B}}} = 101.35 \text{ mg KOH/g of oil}$$

Determination of peroxide number

$$\text{Peroxide value} = \frac{(\text{V}_1 \text{ [ml]} - \text{V}_2 \text{ [ml]}) \,.\, \text{M} \,.\, 1000}{\text{weight of the oil sample [g]}} = \frac{(0.6-0.4)^{\mathbf{A}} \,.\, 0.01 \,.\, 1000}{1.024^{\mathbf{B}}} = 1.9 \text{ mmol } \text{O}_2/\text{kg}$$

Notes: ***A-*** *Give the values of your measurements.*
B- *Give the exact value of weighted sample.*

4. Results

The results of the oil quality are summarized in Table 1.

Table 1: The acid number, saponification number and peroxide number of tested oils.

Oil	Acid number (mg KOH/g oil)	Saponification number (mg KOH/g oil)	Peroxide number (mmol O_2/kg)
Olive oil$^{\mathbf{C}}$	0.728$^{\mathbf{A}}$	101.35$^{\mathbf{A}}$	1.9$^{\mathbf{A}}$
Sunflower oil$^{\mathbf{D}}$	0.532$^{\mathbf{B}}$	…$^{\mathbf{B}}$	…$^{\mathbf{B}}$
Rapeseed oil$^{\mathbf{D}}$	0.412$^{\mathbf{B}}$	…$^{\mathbf{B}}$	…$^{\mathbf{B}}$
Linseed oil$^{\mathbf{D}}$	…$^{\mathbf{B}}$	…$^{\mathbf{B}}$	…$^{\mathbf{B}}$

Notes: ***A -*** *Give the values of your measurements.*
B *- Enter the values of your colleagues' calculations.*
C - *Specify your sample of oil.*
D - *Specify oil samples to your colleagues.*

5. Discussion*

Under the current legislation, the limit value for refined oils is set at 0.6 mg KOH/g. Olive oils are categorized differently, so the limit for virgin olive oil is 2.0 mg KOH/g, while for extra virgin olive oil is 0.8 mg KOH/g. For the sunflower oil, the packaging indicates that it is refined. However, our experiment showed that this limit was exceeded (see Table 1). continue….

The saponification numbers for the selected oils, according to the literature, are given in Table 2.

Table 2: The saponification number of selected oils.

Oil	Saponification number (mg KOH/g oil)
Olive oil	187 – 196
Sunflower oil	186 – 194
Rapeseed oil	166 – 198
Linseed oil	180–196

According to the values in Table 1, it is evident that the olive$^{\mathbf{A}}$ oil does not align with the range presented in the literature data (see Table 2). Our studied olive$^{\mathbf{A}}$ oil sits at the lower

end of the saponification number range (see Table 2)[B]. Alternatively, our olive oil (see Table 1) is in the middle of the saponification number range[B]. continue….

Note: ***A-*** *Specify your type of oil.*
B- *Select the option that corresponds to the comparison of the observed measurements and the measurements obtained from the scientific literature.*

Under the current legislation, there are specified limits for the peroxide values of refined fats and oils, virgin fats and oils, and virgin palm oil (see Table 3). However, in Slovakia, these limits do not extend to olive oils. Generally speaking, the maximum peroxide value for various types of olive oil (including extra virgin, virgin, and Lampante virgin olive oil) is set at 20 mmol O_2/kg. The literature-based peroxide values for selected oils are provided in Table 3.

Table 3: The upper limit of the peroxide number of fats and oils.

Oil	Peroxide number (mmol O_2/kg)
Refined fats and oils	≤ 10
Virgin fats and oils	≤ 15
Virgin palm oil	≤ 15
All types of olive oil	≤ 20

[B]Our sample of olive[A] oil exhibits a value that is lower/comparable/higher value than the data presented in the literature (see Table 3). This suggests that… (an elevated peroxide level indicates that the oil has been damaged by free radicals and is beginning to turn rancid/ ….).

Don't forget to compare the results of the quality of the oils determined by your colleges.

Note: ***A-*** *Specify your type of oil.*
B- *Select the option that corresponds to the comparison of the observed measurements and the measurements obtained from the scientific literature.*

6. Conclusion

The acid number of our sample of olive oil was 0.728 mg KOH/g of oil, the saponification number was 101.35 mg KOH/g of oil, and the peroxide number was 1.9 mmol/kg. This indicates that the quality of our oil was good/bad[*].

Note: ** Select the correct option that describes the quality of the oil.*

Determination of proteins using the Biuret method

1. Aim

The aim of this work is to determine the protein concentration in the sample 1* using the Biuret method.

Note: ** Specify your sample number.*

2. Procedure

a. We mixed 1 ml of the solution (either the sample or egg albumin solutions in the concentration range of 0.5-5.0 g/l*) with 3.0 ml of the Biuret reagent.
b. We vortexed the mixture and allowed it to stand for 30 minutes at laboratory temperature.
c. We measured the absorbance of the samples at a wavelength of 540 nm.
d. We calibrated the Biuret method using egg albumin solutions of varying concentrations (0.5-5.0 g/l*).

Note: ** Indicate the concentrations you worked with during your experimental work in the laboratory.*

3. Calculations

Calculation of protein concentration in the sample 1:

c = (0.234* - 0.0972)/0.1296 = 1.06 g/l

Note: ** Here, provide the linear regression equation obtained by plotting the dependence of absorbance on the known egg albumin concentration. Use the standard curve to calculate the solution's concentration. Don't forget to subtract the average of the blank (i.e., the Biuret reagent and saline solution)! Calculate the protein concentration in the sample for each absorbance, since we are working with three parallel measurements. Then, calculate the average protein concentration in the sample and the standard deviation.*

4. Results

Based on the measured absorbances recorded in Table 1, we constructed a calibration curve (see Figure 1).

Table 1[A]: Measured absorbance values of standard egg albumin solutions with a concentration of 0.5-5.0 g/l at a wavelength of 540 nm.

Egg albumin concentration (g/l)	Absorbance			Absorbance after subtraction of the average value of the blank			Average value
0	0.019	0.021	0.018	Average: 0.019			
0.5	0.121	0.119	0.342	0.102	0.100	~~0.323~~[B]	0.101
1	0.242	0.251	0.249	0.223	0.232	0.230	0.228
2	0.366	0.382	0.375	0.347	0.363	0.356	0.355
3	0.501	0.721	0.505	0.482	~~0.702~~	0.486	0.484
4	0.631	0.655	0.672	0.612	0.636	0.653	0.633[C]
5	0.731	0.755	0.772	0.712	0.736	0.753	0.733
SAMPLE 1	0.253	~~0.501~~	0.253	0.234	~~0.482~~	0.234	-[D]

Notes: ***A-*** *The table description always above the table, while the figure description is placed below the figure. The description of the table, as well as the table itself, must be on the same page of the protocol. Do not separate them.*

B- *You can remove any absorbance measurements that obviously do not make sense, as they may have been caused by inaccurate pipetting, poor homogenization, etc. You will not include this value when calculating the average.*
C- *Working with Excel for calculations. Do not subtract each number in turn using a calculator.*
D- *The concentration of proteins in the sample must be calculated separately from each absorbance. Only after this operation can the arithmetic mean(average) and standard deviation be calculated.*

Figure 1 shows the dependence of absorbance at 540 nm on the egg albumin concentration.

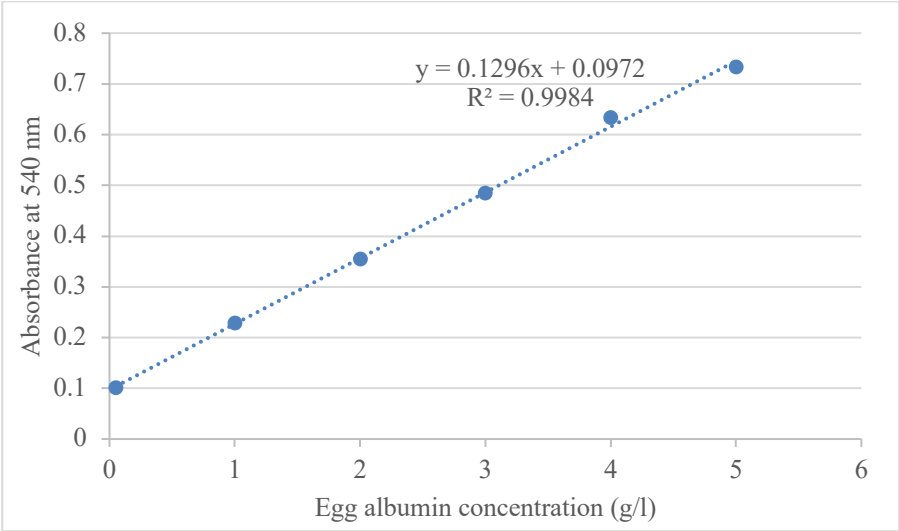


Figure 1: The dependence of absorbance at 540 nm on the egg albumin concentration.
Note for the graph: *Each graph must have a description of the x and y axes with units (unless it is a dimensionless number).*

5. Discussion

Based on the results shown in Figure 1, we determined that the linear regression equation is y = 0.1296x + 0.0972, with R^2 value of 0.9984. We found that the protein concentration in the sample 1 is 1.06±0.05 g/l*.
Note: ** You determined the absorbance of the sample in three repetitions. The standard deviation should therefore be given here.*

6. Conclusion

The experimentally determined protein concentration is 1.06±0.05 g/l*.
Note: ** The accuracy of your result will be assessed by the teacher. The teacher will know the exact concentration of proteins in the sample.*

Determination of proteins using the Lowry method

1. Aim

The aim of this work is to determine the protein concentration in the sample 1* using the Lowry method.

Note: ** Specify your sample number.*

2. Procedure

a. We mixed 1 ml of the solution (either the sample or egg albumin solutions in the concentration range of 50-500 mg/l*) with 5 ml of reagent C.
b. We vortexed the mixture and allowed it to stand for 10 minutes at laboratory temperature.
c. We added 0.5 ml of reagent D.
d. We vortexed the mixture and allowed it to stand for 30 minutes at laboratory temperature.
e. We measured the absorbance of the samples at 600 nm.
f. We calibrated the Lowry method using egg albumin solutions of varying concentration (50-500 mg/l*).

Note: ** Indicate the concentrations you worked with during your experimental work in the laboratory.*

3. Calculations

Calculation of protein concentration in sample 1

c = (0.234* - 0.0671)/0.0014 = 119.21 mg/l

Note: ** Here, provide the linear regression equation obtained by plotting the dependence of absorbance on the known egg albumin concentration. Use the standard curve to calculate the solution's concentration. Don't forget to subtract the average of the blank (i.e., reagents C and D and saline solution)! Calculate the protein concentration in the sample for each absorbance since we are working with three parallel measurements. Then, calculate the average protein concentration in the sample and the standard deviation.*

4. Results

Based on the measured absorbances recorded in Table 1, we constructed a calibration curve (see Figure 1).

Table 1[A]: Measured absorbance values of standard egg albumin solutions with a concentration of 50-500 mg/l at a wavelength of 600 nm.

Egg albumin concentration (mg/l)	Absorbance			Absorbance after subtraction of the average of the blank			Average
0	0.019	0.021	0.018	Average: 0.019			
50	0.121	0.119	0.342	0.102	0.100	~~0.323~~[B]	0.101
100	0.242	0.251	0.249	0.223	0.232	0.230	0.228
200	0.366	0.382	0.375	0.347	0.363	0.356	0.355
300	0.501	0.721	0.505	0.482	~~0.702~~	0.486	0.484
400	0.631	0.655	0.672	0.612	0.636	0.653	0.633[C]
500	0.731	0.755	0.772	0.712	0.736	0.753	0.733
SAMPLE 1	0.253	~~0.501~~	0.253	0.234	~~0.482~~	0.234	-[D]

Notes: ***A-*** *The table description always above the table, while the figure description is placed below the figure. The description of the table, as well as the table itself, must be on the same page of the protocol. Do not separate them.*

B- *You can remove any absorbance measurements that obviously do not make sense, as they may have been caused by inaccurate pipetting, poor homogenization, etc. You will not include this value when calculating the average.*

C- *Working with Excel for calculations. Do not subtract each number in turn using a calculator.*

D- *The concentration of proteins in the sample must be calculated separately from each absorbance. Only after this operation can the arithmetic mean(average) and standard deviation be calculated.*

Figure 1 shows the dependence of absorbance at 600 nm on the egg albumin concentration.

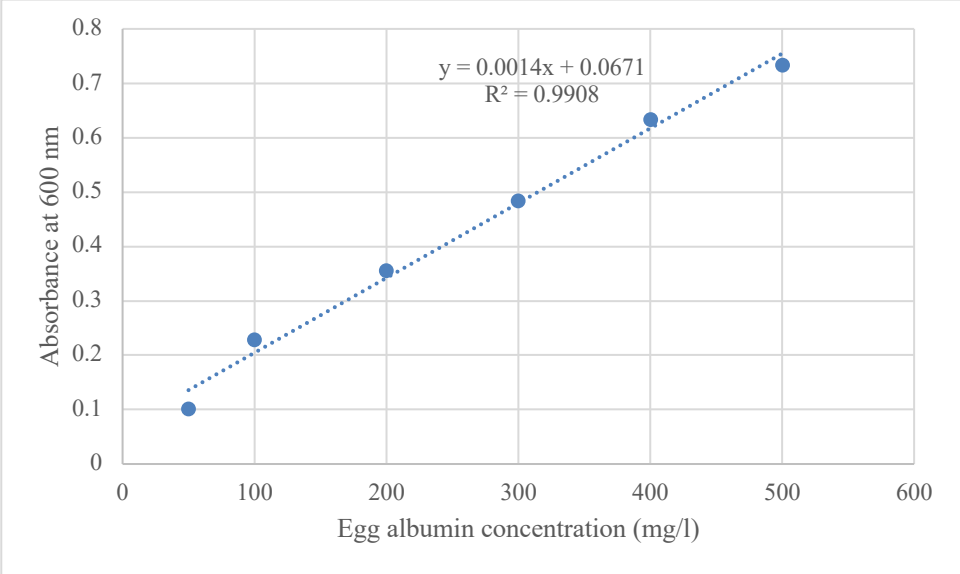


Figure 1: The dependence of absorbance at 600 nm on the egg albumin concentration.
Note for the graph: *Each graph must have a description of the x and y axes with units (unless it is a dimensionless number).*

5. Discussion

Based on the results shown in Figure 1, we determined the linear regression equation is y = 0.0014x + 0.0671, with R^2 of 0.9908. We found that the protein concentration in the sample 1 is 119.21±0.05 mg/l*.

Note: ** You determined the absorbance of the sample in three repetitions. The standard deviation should therefore be given here.*

6. Conclusion

The experimentally determined protein concentration is 119.21±0.05 mg/l*.

Note: ** The accuracy of your result will be assessed by the teacher. The teacher will know the exact concentration of proteins in the sample.*

Determination of proteins using the Bradford method

1. Aim

The aim of this work is to determine the protein concentration in the sample 1* by the Bradford method.

***Note:** * Specify your sample number.*

2. Procedure

a. We mixed 2 ml of the solution (either the sample or egg albumin solutions in the concentration range of 5-100 mg/l*) with 2 ml of the Bradford reagent.
b. We vortexed the mixture and allowed it to stand for 5 minutes at laboratory temperature.
c. We measured the absorbance of the samples at 595 nm.
d. We calibrated the Bradford method using egg albumin solutions of varying concentration (5-100 mg/l*).

***Note:** * Indicate the concentrations you worked with during your experimental work in the laboratory.*

3. Calculations

Calculation of protein concentration in sample 1

c = (0.234* - 0.0611)/0.0067 = 25.81 mg/l

***Note:** * Here, provide the linear regression equation obtained by plotting the dependence of absorbance on the known egg albumin concentration. Use the standard curve to calculate the solution's concentration. Don't forget to subtract the average of the blank (i.e., the Bradford reagent and saline solution)! Calculate the protein concentration in the sample for each absorbance, since we are working with three parallel measurements. Then, calculate the average protein concentration in the sample as well as the standard deviation.*

4. Results

Based on the measured absorbances recorded in Table 1, we constructed a calibration curve (see Figure 1).

Table 1[A]: Measured absorbance values of standard egg albumin solutions with a concentration of 5-100 mg/l at a wavelength of 595 nm.

Egg albumin concentration [mg/l]	Absorbance			Absorbance after subtraction of the average of the blank			Average
0	0.019	0.021	0.018	Average: 0.019			
5	0.121	0.119	0.342	0.102	0.100	~~0.323~~[B]	0.101
25	0.242	0.251	0.249	0.223	0.232	0.230	0.228
45	0.366	0.382	0.375	0.347	0.363	0.356	0.355
65	0.501	0.721	0.505	0.482	~~0.702~~	0.486	0.484
85	0.631	0.655	0.672	0.612	0.636	0.653	0.633[C]
100	0.731	0.755	0.772	0.712	0.736	0.753	0.733
SAMPLE 1	0.253	~~0.501~~	0.253	0.234	~~0.482~~	0.234	-[D]

***Notes:** **A-** The table description always above the table, while the figure description is placed below the figure. The description of the table, as well as the table itself, must be on the same page of the protocol. Do not separate them.*

B- *You can remove any absorbance measurements that obviously do not make sense, as they may have been caused by inaccurate pipetting, poor homogenization, etc. You will not include this value when calculating the average.*
C- *Working with Excel for calculations. Do not subtract each number in turn using a calculator.*
D- *The concentration of glucose in the sample must be calculated separately from each absorbance. Only after this operation can the arithmetic mean(average) and standard deviation be calculated.*

Figure 1 shows the dependence of absorbance at 595 nm on the egg albumin concentration.

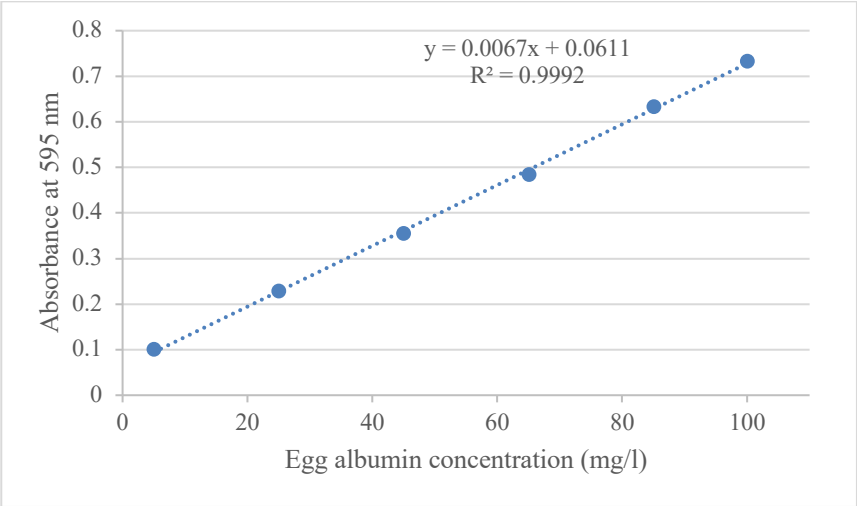


Figure 1: The dependence of absorbance at 595 nm on the egg albumin concentration.
Note for the graph: *Each graph must have a description of the x and y axes with units (unless it is a dimensionless number).*

5. Discussion

Based on the results shown in Figure 1, we determined the linear regression equation is y = 0.0067x + 0.0611, with R^2 of 0.9992. We found that the protein concentration in the sample 1 is 25.81±0.05 mg/l*.
Note: ** You determined the absorbance of the sample in three repetitions. The standard deviation should therefore be given here.*

6. Conclusion

The experimentally determined protein concentration is 25.81±0.05 mg/l*.
Note: ** The accuracy of your result will be assessed by the teacher. The teacher will know the exact concentration of proteins in the sample.*

Isolation of glutamic acid from wheat flour gluten

1. Aim

The aim of this work is to determine the percent yield (%) of the glutamic acid extracted from wheat flour* and gluten. Another objective is to compare these results with those from other group members.

Note: **Specify your type of wheat flour.*

2. Procedure

a. We mixed 100.021 g [A] of wheat flour with 300 ml of distilled water.
b. We decanted the white-colored water (starch) from the sediment four-times, each time adding 300 ml of distilled water.
c. We continued decanting and washing with distilled water (300 ml) until no more white-colored water (starch) appeared (four times).
d. We mixed 10.065 g [A] of gluten with 30 ml of concentrated hydrochloric acid in a round-bottom flask and dissolved the gluten.
e. We heated the flask in boiling water for 1 hour under reflux.
f. After cooling, we added 30 ml of distilled water, mixed, and then filtered.
g. We vacuum-evaporated the filtrate until 3/4 of its volume had evaporated.
h. We cooled and crystallized the mixture at 4 °C for 7 days.
i. We continued decanting and washing with distilled water (300 ml) until no more white-colored water (starch) appeared (four times).
j. We washed the filter cake with an ice-cold mixture of ethanol and diethyl ether (1:1; *v/v*).
k. We dissolved the filter cake in a minimum volume of hot water, then added 1 mol/l NaOH until a pH of 3.0 was reached.
l. We vacuum-evaporated the supernatant until about 6 ml remained, cooled, and crystallized.
m. We filtered and dried the obtained crystals.

Notes: ***A-*** *Record the exact value of the weighted sample.*

3. Calculations*

Note: ** All values in the calculations, as well as the weight of glutamic acid crystals, are made up. The calculation serves as a template to guide you in performing the process correctly.*

The percent yield of glutamic acid

m (wheat flour) = 100.021 g
m (glutamic acid crystals) = 0.321 g
m (gluten) = 10.065 g

The percent yield of glutamic acid per wheat flour

0.321 g of glutamic acid ……. in 100.021 g of wheat flour
x g of glutamic acid ……. in 100 g of wheat fluor

$$x : 0.321 = 100 : 100.021$$

$$\frac{x}{0.321} = \frac{100}{100.021}$$

$$x = \frac{100 \text{ x } 0.321}{100.021} = 0.321 \text{ g}/100 \text{ g} = 0.321 \text{ \% } (w/w)$$

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \text{ x } 100 \text{ \%}$$

$$\text{Percent yield} = \frac{0.321\ \%}{1.3\ \%}\ .100\ \% = 23.92\ \%$$

The percent yield of glutamic acid per gluten

0.321 g of glutamic acid ……. in 10.065 g of gluten
x g of glutamic acid …..…. in 100 g of gluten

x : 0.321 = 100 : 10.065

$$\frac{x}{0.321} = \frac{100}{10.065}$$

$$x = \frac{100\ x\ 0.321}{10.065} = 3.19\ \text{g/100 g} = 3.19\ \%\ \text{(w/w)}$$

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}}\ x\ 100\ \%$$

$$\text{Percent yield} = \frac{3.19\ \%}{35\ \%}\ .100\ \% = 9.1\ \%$$

4. Results

The results of the glutamic acid yields are summarized in Table 1.

Table 1: Percent yield (%) of glutamic acid per wheat flour and gluten.

Flour	Percent yield per wheat flour (%)	Percent yield per gluten (%)
Group 1[C]	23.92[A]	9.1[A]
Group 2[D]	…[B]	…[B]
Group 3 [D]	…[B]	…[B]

Notes: ***A -*** *Give the values of your measurements.*
B - *Enter the values of your colleagues' calculations.*
C - *Specify your wheat flour sample.*
D - *Specify the wheat flour samples used by your colleagues.*

5. Discussion

According to the values in Table 1, it is evident that our sample of wheat flour … The percent yield of glutamic acid is 23.92 % per 100 g of wheat flour and 9.1 % per 100 g of gluten. This suggests … The low yield might be due to the shorter heating duration of the mixture under reflux. The literature recommends 8 hours instead of the 1 hour we used. …[**continue discussion**]……

6. Conclusion

The percent yield of glutamic acid was 23.92 % per wheat flour and 9.1 % per gluten.

Isolation of citric acid from lemon

1. Aim

The aim of this work is to determine the percent yield (%) of the citric acid extracted from lemon.

2. Procedure

a. We cut a pre-weighted lemon into pieces and squeezed it to extract the juice.
b. We neutralized the lemon juice with a 26 % (*v/v*) aqueous ammonia solution (150 µl)**A** until the pH reached 7.2.
c. Using a Büchner funnel, we filtered the solution and then heated it to boiling.
d. We added 25 ml of a 25 % (*w/v*) aqueous calcium chloride solution and mixed thoroughly.
e. We filtered the formed calcium citrate precipitate using the Büchner funnel.
f. We washed the filter cake with 40 ml of hot distilled water.
g. We transferred the filter cake to a beaker, adding 10 ml of distilled water and 56 µl**B** concentrated H_2SO_4.
h. We filtered the solution using the Büchner funnel, concentrated the filtrate on a hot plate, and allowed it to crystalized.

Notes: ***A-*** *Give the exact volume of the 26 % (v/v) aqueous ammonia solution used.*
B- *Enter the exact volume of the 98 % (v/v) sulfuric acid used.*

3. Calculations*

Note: ** All values in the calculations, as well as the weight of citric acid crystals, are made up. The calculation serves as a template to guide you in performing the process correctly.*

The percent yield of citric acid

m (citric acid crystals) = 1.152 g
m (lemon) = 54.065 g

The percent yield of citric acid

1.152 g of citric acid ……. in 54.065 g of lemon
x g of citric acid ……... in 100 g of lemon

x : 1.152 = 100 : 54.065

$$\frac{x}{1.152} = \frac{100}{54.065}$$

$$x = \frac{100\ x\ 1.152}{54.065} = 2.13 \text{ g/100 g} = \underline{2.13\ \%\ (w/w)}$$

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \text{ x } 100\ \%$$

$$\text{Percent yield} = \frac{2.13\ \%}{8.0\ \%} . 100\ \% = 26.63\ \%$$

4. Results

The results of the citric acid yields are summarized in Table 1.

Table 1: Percent yield (%) of citric acid.

Flour	Percent yield (%)
Group 1[C]	26.63[A]
Group 2[D]	…[B]
Group 3 [D]	…[B]

Notes: ***A -*** *Give the values of your measurements.*
B *- Enter the values of your colleagues' calculations.*
C - *Specify your wheat flour sample.*
D - *Specify wheat flour samples used by your colleagues.*

5. Discussion

According to the values in Table 1, it is evident that our sample of … The percent yield of citric acid is 26.63 %. This suggests … [**continue discussion**] …

6. Conclusion

The percent yield of citric acid form lemon was 26.63 %.

Attachment 3: Practical problems and answers to the practice problems

GENERAL LABORATORY PROCEDURES

1. **Calculate the arithmetic mean as well as the standard deviation for Eppendorf tubes marked Ep 2 - Ep 5. Write the results in the following table:**

	Weight (g)					Value (g)	
Object	**I.**	**II.**	**III.**	**IV.**	**V.**	***Arithmetic mean (Average)***	***Standard deviation (SD)***
Ep 1	2.3267	2.3254	2.3247	2.3250	2.3253	2.325	0.001
Ep 2	2.3379	2.3382	2.3382	2.3380	2.3380		
Ep 3	2.3549	2.3550	2.3551	2.3351	2.3548		
Ep 4	2.3246	2.3247	2.3740	2.3247	2.3247		
Ep 5	2.3706	2.3704	2.3707	2.3706	2.3707		

Calculation of the arithmetic mean (average):

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} = \frac{(2.3379 + 2.3382 + 2.3382 + 2.3380 + 2.3380)}{5} = \mathbf{2.3381\ g}$$

Note: *n*- number of measurements.

Calculation of the standard deviation (SD):

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

$$\sigma = \sqrt{\frac{(2.3379 - 2.3381)^2 + (2.3382 - 2.3381)^2 + (2.3382 - 2.3381)^2 + (2.3380 - 2.3381)^2 + (2.3380 - 2.3381)^2}{5-1}}$$

$$\sigma = \mathbf{0.0001\ g}$$

Eppendorf tube 2 weighed 2.3381±0.0001 g.

Results

	Weight (g)					Value (g)	
Object	**I.**	**II.**	**III.**	**IV.**	**V.**	***Arithmetic mean (Average)***	***Standard deviation (SD)***
Ep 1	2.3267	2.3254	2.3247	2.3250	2.3253	2.3254	0.0006
Ep 2	2.3379	2.3382	2.3382	2.3380	2.3380	2.3381	0.0001
Ep 3	2.3549	2.3550	2.3551	2.3351	2.3548	2.3510	0.0089
Ep 4	2.3246	2.3247	2.3740	2.3247	2.3247	2.3345	0.0221
Ep 5	2.3706	2.3704	2.3707	2.3706	2.3707	2.3706	0.0001

2. **How much Malachite Green dye should you use to make 100 ml with a concentration of 150 mg/l?**

150 mg of a dye ……. in 1000 ml of a solution
x mg ……. 100 ml

$$x : 150 = 100 : 1000$$

$$\frac{x}{150} = \frac{100}{1000}$$

$$x = \frac{100 \text{ x } 150}{1000} = \mathbf{15\ mg}$$

Result
For the preparation of 100 ml of a solution with a concentration of 150 mg/l, it is necessary to weigh 15 mg of Malachite Green dye.

3. **The stock solution of Malachite Green with a concentration of 150 mg/l should be diluted to a concentration in the range of 1-20 mg/l. Calculate the dilution factor, the volume of the stock solution and the volume of distilled water needed to produce a solution of a given concentration. The total volume of the dilute solution must be 10 ml.**

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
20.0			
17.0			
15.0			
12.5			
10.0			
7.5			
5.0			
2.5			
1.0			

The example of how to calculate the dilution factor, the volume of the stock solution and the volume of distilled water to produce dilute solution at a concentration of 20 mg/l

Method 1

First, calculate the dilution factor by dividing the concentration of the stock solution by the concentration of the solution you want to make. This will tell you how much more dilute your new solution will be.

$$\text{dilution factor (x)} = \frac{\text{concentration of the stock solution}}{\text{concentration of the dilute solution}}$$

$$\text{dilution factor (x)} = \frac{150 \text{ mg/l}}{20 \text{ mg/l}} = \mathbf{7.5\ x}$$

Because the solution will be 7.5 times more dilute, you will need to use 7.5 times less of it. To find out how much stock solution to add, divide the total volume of the solution you will make by the dilution factor.

The volume of dilute solution is set to 10 ml in the practical example.

$$\text{Volume of stock solution transferred } = \frac{10 \text{ ml}}{7.5 \text{ x}} = \mathbf{1.333 \text{ ml}}$$

You will add 1.333 ml of the stock solution, then dilute it with distilled water to get the total volume that you need (10 ml). The total volume minus the transferred volume will give you the volume of distilled water you need to add.

$$\text{Volume of distilled water } = 10 \text{ ml} - 1.333 \text{ ml} = \mathbf{8.667 \text{ ml}}$$

Method 2

Use the following equation to work out the volume of stock solution to transfer.

$$c_1 \,.\, V_1 = c_2 \,.\, V_2$$

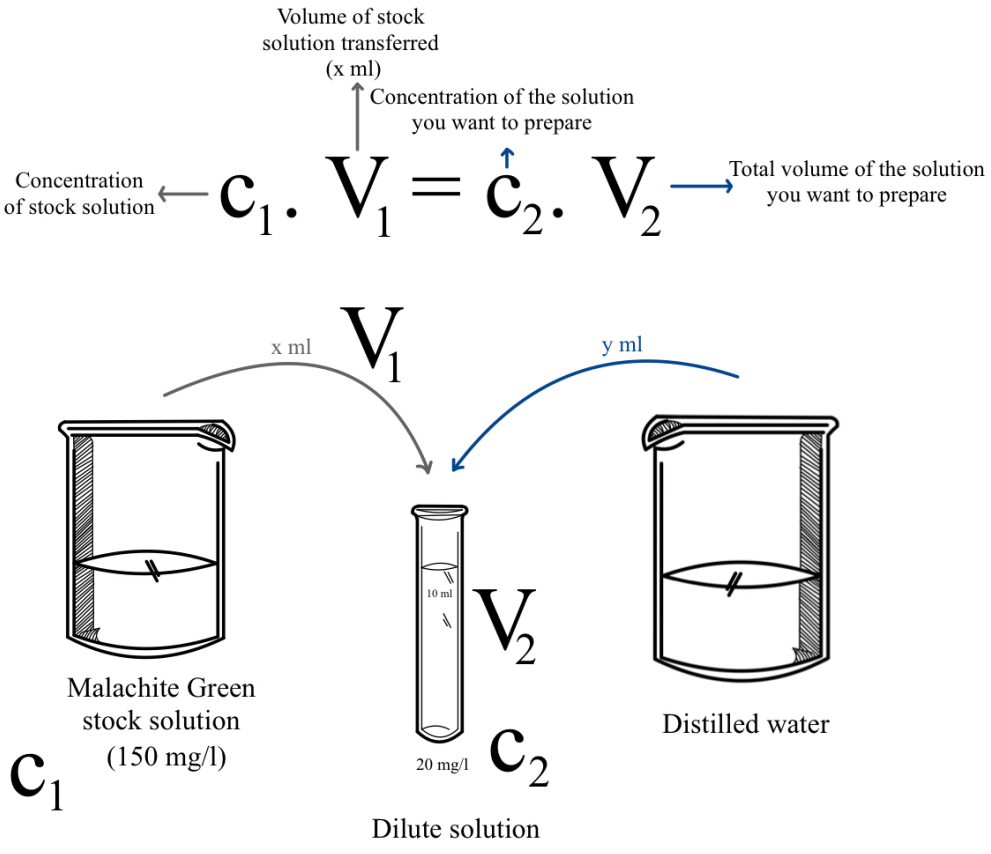
where c_1 is the concentration of the stock solution, V_1 is the volume of the stock solution transferred, c_2 is the concentration of the solution you want to prepare and V_2 is the total volume of the solution you want to prepare.

<u>The example of calculation for a dilute solution concentration of 20 mg/l:</u>

V_1 = ? ml
c_1 = 150 mg/l (the stock solution of Malachite Green dye)
V_2 = 10 ml (the total volume of dilute solution)
c_2 = 20 mg/l (the dilute solution of Malachite Green dye)

$$c_1 \,.\, V_1 = c_2 \,.\, V_2 \rightarrow V_1 = \frac{c_2 \,.\, V_2}{c_1}$$

$$V_1 = \frac{c_2 \,.\, V_2}{c_1} = \frac{20 \text{ mg/l} \,.\, 10 \text{ ml}}{150 \text{ mg/l}} = \mathbf{1.333 \text{ ml}}$$



Next, calculate the volume of distilled water to add to make up the final volume (10 ml) of the dilute solution.

$$V_2 = V_1 + V_3$$

where V_1 is the volume of the stock solution transferred, V_2 is the total volume of the solution you want to take and V_3 the volume of distilled water.

$$V_3 = V_2 - V_1$$

$$V_3 = 10\ \text{ml} - 1.333\ \text{ml} = \mathbf{8.667\ ml}$$

Results

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
20.0	7.5	1.333	8.667
17.0	8.8	1.137	8.863
15.0	10	1.000	9.000
12.5	12	0.833	9.167
10.0	15	0.667	9.333
7.5	20	0.500	9.500
5.0	30	0.333	9.667
2.5	60	0.167	9.833
1.0	150	0.067	9.933

4. **Convert the mass concentration of Malachite Green dye (M = 364.911 g/mol) to molar concentration.**

Mass concentration (mg/l)	Molar concentration (mol/l)
20.0	
17.0	
15.0	
12.5	
10.0	
7.5	
5.0	
2.5	
1.0	

We need to convert the mass concentration (c_m) to molar concentration (c) using the following equations:

$$\textbf{I.}\ \ c = \frac{n}{V}$$

$$\textbf{II.}\ \ n = \frac{m}{M}$$

$$\textbf{III.}\ \ c_m = \frac{m}{V} \rightarrow m = c_m \,.\, V$$

$$\textbf{IV.}\ \ n = \frac{c_m \,.\, V}{M}$$

$$\textbf{V.}\ \ c = \frac{\frac{c_m \,.\, V}{M}}{V} = \frac{c_m \,.\, V}{M \,.\, V} = \frac{c_m}{M}$$

The relationship between mass and molar concentration is as follows:

$$c = \frac{c_m}{M}$$

Example of calculating the molar concentration for the Malachite Green mass concentration of 20 mg/l:

$$c = 20 \text{ mg/l} = 0.02 \text{ g/l}$$

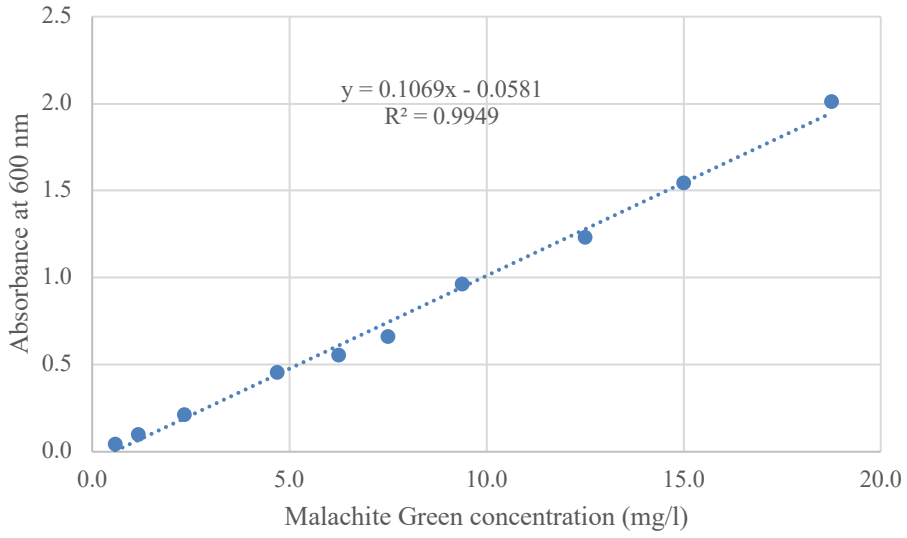
$$M_{\text{Malachite Green}} = 364.911 \text{ g/mol}$$

$$c = \frac{0.02 \text{ g/l}}{364.911 \text{ g/mol}} = 5.48 \,.\, 10^{-5} \text{ mol/l}$$

Results

Mass concentration (mg/l)	Molar concentration (mol/l)
20.0	$5.48.10^5$
17.0	$4.55.10^5$
15.0	$4.11.10^5$
12.5	$3.43.10^5$
10.0	$2.74.10^5$
7.5	$2.06.10^5$
5.0	$1.37.10^5$
2.5	$0.69.10^5$
1.0	$0.27.10^5$

5. A sample containing an unknown concentration of Malachite Green has an absorbance of 0.542. Calculate its Malachite Green concentration using the linear regression equation in the figure.



The equation describing the linear regression: $y = 0.1069x - 0.0581$, the value of x is expressed:

$$y = ax + b \rightarrow x = \frac{y-b}{a}$$

$$x = \frac{y-(-0.0581)}{0.1069} = \frac{y+0.0581}{0.1069}$$

where y is absorbance (0.542) of a sample containing an unknown concentration of Malachite Green (x).

The sample with unknown Malachite Green dye concentration had an absorbance of 0.542. Substitute this absorbance (y) into the linear regression equation for the absorbance of calibration solutions of known Malachite Green concentration, we get:

$$x = \frac{0.542+0.0581}{0.1069} = \mathbf{5.61\ mg/l}$$

The result is given in mg/l, because also in the plot of absorbance versus Malachite Green concentration, the concentration of the dye is in the range of 0.6-18.8 mg/l.

Result
The Malachite Green dye concentration in the sample is 9.02 mg/l.

6. **Calculate the concentration of the synthetic dye Phenol Red if you know that its absorbance is 0.754, the path length of the cuvette is 1 cm and, the extinction coefficient at 480 nm is equal to 4460 $M^{-1}cm^{-1}$.**

To calculate the concentration of the synthetic dye Phenol Red, we can use the Beer-Lambert law:

$$A = \varepsilon_\lambda . c . l \rightarrow c = \frac{A}{\varepsilon_\lambda . l}$$

This equation indicates that the calculation requires knowledge of the absorbance of the substance in a solution, its molar absorption (extinction) coefficient and the path length of the cuvette.
For Phenol Red dye, the molar absorption (extinction) coefficient is 4460 $M^{-1}cm^{-1}$ and the absorbance of this solution measured at 480 nm is 0.754. The path length of the cuvette (the thickness of the cuvette) is 1 cm. Using these values, we can calculate the concentration as follows:

$$c = \frac{A}{\varepsilon_\lambda . l} = \frac{0.754}{4460\ M^{-1}cm^{-1}.\ 1\ cm} = 0.000169\ M$$

This can be further converted as follows:

$$0.000169\ M = 0.000169 \frac{mol}{l} = 0.169 \frac{mmol}{l} = \mathbf{0.2 mmol/l}$$

Result
The concentration of the synthetic dye Phenol Red is 0.2 mmol/l.

7. **How much TRIS should you use to make 100 ml with a concentration of 0.1 mol/l (M = 121.14 g/mol)?**

V = 100 ml = 0.1 l
c = 0.1 mol/l
M_{TRIS} = 121.14 g/mol
m = ? g

I. $c = \frac{n}{V}$

II. $n = \frac{m}{M}$

III. $c = \frac{\frac{m}{M}}{V} \Rightarrow m = c . V . M$

m = 0.1 mol/l x 0.1 l x 121.14 g/mol = **1.2114 g**

Result
For the preparation of 100 ml of a solution with a concentration of 0.1 mol/l, it is necessary to weigh 1.2114 g of TRIS.

8. **How many g of KH_2PO_4 should you use to make 250 ml of a 0.2 mol/l solution (M = 136.09 g/mol)?**

V = 250 ml = 0.25 l
c = 0.2 mol/l
M_{KH2PO4} = 136.09 g/mol
m = ? g

$$c = \frac{\frac{m}{M}}{V} \Rightarrow m = c \,.\, V \,.\, M$$

m = 0.2 mol/l x 0.25 l x 136.09 g/mol = **6.8045 g**

Result
For the preparation of 250 ml of a solution with a concentration of 0.2 mol/l, it is necessary to weigh 6.8045 g KH_2PO_4.

9. **How many g of K_2HPO_4 should you use to make 250 ml of a 0.2 mol/l solution (M = 174.18 g/mol)?**

V = 250 ml = 0.25 l
c = 0.2 mol/l
M_{K2HPO4} = 174.18 g/mol
m = ? g

$$c = \frac{\frac{m}{M}}{V} \Rightarrow m = c \,.\, V \,.\, M$$

m = 0.2 mol/l x 0.25 l x 174.18 g/mol = **8.709 g**

Result
For the preparation of 250 ml of a solution with a concentration of 0.2 mol/l, it is necessary to weigh 8.7009 g KH_2PO_4.

SACCHARIDES

1. **How much glucose should you use to make 10 ml with a concentration of 15 g/l?**

15 g of glucose ……. in 1000 ml of a solution
x g ……. 10 ml

x : 15 = 10 : 1000

$$\frac{x}{15} = \frac{10}{1000}$$

$$x = \frac{10 \, x \, 15}{1000} = 0.\underline{15 \text{ g}}$$

Result
For the preparation of 10 ml of a solution with a concentration of 15 g/l, it is necessary to weigh 0.15 g of glucose.

2. **How much sodium hydroxide (M = 39.9971 g/mol) should you use to make 20 ml with a concentration of a 2 mol/l aqueous solution?**

V = 20 ml = 0.02 l
c = 2 mol/l
M_{NaOH} = 39.9971 g/mol
m = ? g

$$c = \frac{\frac{m}{M}}{V} \Rightarrow m = c \,.\, V \,.\, M$$

m = 2 mol/l x 0.02 l x 39.9971 g/mol = **1.5999 g**

Result
For the preparation of 20 ml of a solution with a concentration of 2 mol/l, it is necessary to weigh 1.5999 g NaOH.

3. **The stock solution of glucose with a concentration of 15 g/l should be diluted to the concentrations shown in the table. Calculate the dilution factor, the volume of the stock solution and the volume of distilled water needed to produce a solution of a given concentration. The total volume of the dilute solution must be 1 ml.**

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
12.5			
10.0			
7.5			
5.0			
2.5			
1.0			

The example of how to calculate the dilution factor, the volume of the stock solution and the volume of distilled water to produce dilute solution at a concentration of 12.5 g/l
Method 1

$$\text{dilution factor (x)} = \frac{\text{concentration of the stock solution}}{\text{concentration of the dilute solution}}$$

$$\text{dilution factor (x)} = \frac{15 \text{ g/l}}{12.5 \text{ g/l}} = \mathbf{1.2\ x}$$

The volume of dilute solution is set to 1 ml in the practical example.

$$\text{Volume of stock solution transferred} = \frac{1 \text{ ml}}{1.2 \text{ x}} = \mathbf{0.833\ ml}$$

$$\text{Volume of distilled water } = 1 \text{ ml} - 0.833 \text{ ml} = \mathbf{0.157\ ml}$$

Method 2

<u>The example of calculation for a dilute solution concentration of 12.5 g/l:</u>

V_1 = ? ml
c_1 = 15 g/l (the stock solution)
V_2 = 1 ml (the total volume of dilute solution)
c_2 = 12.5 g/l (the dilute solution)

$$c_1 . V_1 = c_2 . V_2 \rightarrow V_1 = \frac{c_2 . V_2}{c_1}$$

$$V_1 = \frac{c_2 . V_2}{c_1} = \frac{12.5 \frac{g}{l} . \ 1 \text{ ml}}{15 \frac{g}{l}} = \mathbf{0.833\ ml}$$

Calculate the volume of distilled water:

$$V_3 = 1 \text{ ml} - 0.833 \text{ ml} = \mathbf{0.167\ ml}$$

Results

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
12.5	1.2	0.833	12.5
10.0	1.5	0.667	10.0
7.5	2	0.500	7.5
5.0	3	0.333	5.0
2.5	6	0.167	2.5
1.0	15	0.067	1.0

4. How much sucrose should you use to make 10 ml with a concentration of 5 g/l? Express your result in grams.

15 g of sucrose ……. in 1000 ml of a solution
x g ……. 10 ml

x : 5 = 10 : 1000

$$\frac{x}{5} = \frac{10}{1000}$$

$$x = \frac{10\ x\ 5}{1000} = 0.\underline{05}\ g$$

Result
For the preparation of 10 ml of a solution with a concentration of 5 g/l, it is necessary to weigh 0.05 g of sucrose.

5. How much potassium hydroxide (KOH, M = 56.11 g/mol) should you use to make 10 ml with a concentration of 5 mol/l?

V = 10 ml = 0.01 l
c = 5 mol/l
M_{KOH} = 56.11 g/mol

m = ? g

$$c = \frac{\frac{m}{M}}{V} \Rightarrow m = c \,.\, V \,.\, M$$

m = 5 mol/l x 0.01 l x 56.11 g/mol = **2.8055 g**

Result
For the preparation of 10 ml of a solution with a concentration of 5 mol/l, it is necessary to weigh 2.8055 g NaOH.

6. The stock solution of sucrose with a concentration of 5 g/l should be diluted to the concentrations shown in the table. Calculate the dilution factor, the volume of the stock solution and the volume of distilled water needed to produce a solution of a given concentration. The total volume of the dilute solution must be 1 ml.

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
4.5			
4.0			
3.5			
3.0			
2.5			
2.0			
1.5			
1.0			

The example of how to calculate the dilution factor, the volume of the stock solution and the volume of distilled water to produce a dilute solution at a concentration of 3.0 g/l
Method 1

$$\text{dilution factor (x)} = \frac{\text{concentration of the stock solution}}{\text{concentration of the dilute solution}}$$

$$\text{dilution factor (x)} = \frac{5\,\frac{g}{l}}{3.0\,\frac{g}{l}} = \mathbf{1.66\overline{6}\ x}$$

In this practical example, the volume of a dilute solution is set to 1 ml.

$$\text{Volume of stock solution transferred} = \frac{1\text{ ml}}{1.66\overline{6}\text{ x}} = \mathbf{0.6\ ml}$$

$$\text{Volume of distilled water} = 1\text{ ml} - 0.6\text{ ml} = \mathbf{0.4\ ml}$$

Method 2
<u>The example of calculation for a dilute solution concentration of 12.5 g/l:</u>
V_1 = ? ml
c_1 = 5 g/l (the stock solution)
V_2 = 1 ml (the total volume of dilute solution)

c_2 = 3.0 g/l (the dilute solution)

$$c_1 . V_1 = c_2 . V_2 \rightarrow V_1 = \frac{c_2 . V_2}{c_1}$$

$$V_1 = \frac{c_2 . V_2}{c_1} = \frac{3.0 \frac{g}{l} . \ 1 \text{ ml}}{5 \frac{g}{l}} = \mathbf{0.6 \text{ ml}}$$

The volume of distilled water:

$$V_3 = 1 \text{ ml} - 0.6 \text{ ml} = \mathbf{0.4 \text{ ml}}$$

Results

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
4.5	1.11	0.900	0.100
4.0	1.25	0.800	0.200
3.5	1.43	0.700	0.300
3.0	1.67	0.600	0.400
2.5	2.00	0.500	0.500
2.0	2.50	0.400	0.600
1.5	3.33	0.300	0.700
1.0	5.00	0.200	0.800

7. A sample containing an unknown concentration of sucrose has an absorbance of 0.492. Calculate its sucrose concentration using the linear regression equation *y = 0.0742x + 0.0045* for sucrose solutions in the concentration range of 1-10 g/l.

The equation describing the linear regression: *y = 0.0742x + 0.0045,* the value of *x* is expressed:

$$y = ax + b \rightarrow x = \frac{y-b}{a}$$

$$x = \frac{y-0.0045}{0.0742} = \frac{y-0.0045}{0.0742}$$

where *y* is the absorbance (0.492) of a sample containing an unknown concentration of sucrose (*x*).

The sample with unknown sucrose concentration had an absorbance of 0.482. Substitute this absorbance (*y*) into the linear regression equation for the absorbance of calibration solutions of sucrose of known concentrations:

$$x = \frac{0.492-0.0045}{0.0742} = \mathbf{6.57 \text{ g/l}}$$

The result is given in g/l, using the linear regression equation *y = 0.0742x + 0.0045* for sucrose solutions in the concentration range 1-10 g/l.

Result

The sucrose concentration in the sample is 6.57 g/l.

NUCLEIC ACIDS

1. **How much potassium hydroxide (KOH) should you use to make 50 ml of its 1 % *(w/v)* solution?**

   Weight volume fraction *(w/v)* means how many grams of solute are dissolved in 100 ml of a solution. A one percent solution (1 %, *w/v*) is defined as 1 gram of solute per 100 milliliters of the final volume.

   1 g of KOH ….. in 100 ml of a solution
   x g of KOH …. in 50 ml of a solution

   x : 1 = 50 : 100

   $$\frac{x}{1} = \frac{50}{100}$$

   $$x = \frac{1\ x\ 50}{100} = \mathbf{0.5\ g}$$

   Result
   For the preparation of 50 ml of its 1 % (*w/v*) KOH solution, it is necessary to weigh 0.5 g of KOH.

2. **Calculate the amount of ballast proteins (in mg/ml) and the percent yield of RNA (%) that were present in the precipitate if you know that the absorbance of the solution at 280 nm was 0.726, at 260 nm was 0.624, and the path length of the light beam passing thorough the cuvette was 1 cm. For the calculation of the yield, you can use the amount of RNA quantification data from the literature (4 %, *w/w*).**

   The percent yield of RNA
   First, you need to determine the A_{280}/A_{260} absorbance ratio.

   $$\text{absorbance ratio} = \frac{A_{280}}{A_{260}} = \frac{0.726}{0.624} = 1.163$$

   Then find the *F* factor and the percentage of nucleic acids from Table 12.

A_{280}/A_{260}	% nucleic acids	F
1.75	0.00	1.116
1.63	0.25	1.081
1.52	0.50	1.054
1.40	0.75	1.023
1.36	1.00	0.994
1.30	1.25	0.970
1.25	1.50	0.944
1.16	2.00	0.899
1.09	2.50	0.852
…	…	…
…	…	…
0.874	5.00	0.682

   The precipitate contains 2 % (*w/w*) nucleic acids (specifically RNA, because we used alkaline hydrolysis for the isolation procedure). This value is referred as the actual yield. The maximum amount of product (RNA) from the literature represents the theoretical yield (4 %, *w/w*).

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}} \text{ x } 100\ \%$$

$$\text{Percent yield} = \frac{2\ \%}{4\ \%} . 100\ \% = 50\ \%$$

The amount of ballast proteins

proteins (mg/ml) = F. A_{280} / d

F = 0.899
A_{280} = 0.726
d = 1 cm

proteins (mg/ml) = 0.899. 0.276 / 1
proteins (mg/ml) = 0.65 mg/ml

Result
The amount of ballast proteins was 0.65 mg/ml and the percent yield of RNA was 50 %.

3. **The stock solution of ribose with a concentration of 1 g/l should be diluted to the concentrations shown in the table. Calculate the dilution factor, the volume of stock solution and the volume of distilled water needed to produce a solution of a given concentration. The total volume of dilute solution must be 4 ml.**

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
100			
75			
50			
25			
10			

Note: *c* = 1 g/l = 1000 mg/l

The example of how to calculate the dilution factor, the volume of the stock solution and the volume of distilled water to produce dilute solution at a concentration of 75 mg/l
Method 1

$$\text{dilution factor (x)} = \frac{\text{concentration of the stock solution}}{\text{concentration of the dilute solution}}$$

$$\text{dilution factor (x)} = \frac{1000\ \text{mg/l}}{75\ \text{mg/l}} = 13.33\bar{3}$$

The volume of dilute solution is set to 4 ml in the practical example.

$$\text{Volume of stock solution transferred} = \frac{4\ \text{ml}}{13.33\bar{3}\ \text{x}} = \mathbf{0.3\ ml}$$

$$\text{Volume of distilled water} = 4\ \text{ml} - 0.3\ \text{ml} = \mathbf{3.7\ ml}$$

Method 2

<u>The example of calculation for a dilute solution concentration of 75 mg/l:</u>

V_1 = ? ml

c_1 = 1 g/l = 1000 mg/l (the stock solution)

V_2 = 4 ml (the total volume of dilute solution)

c_2 = 75 mg/l (the dilute solution)

$$c_1 . V_1 = c_2 . V_2 \rightarrow V_1 = \frac{c_2 . V_2}{c_1}$$

$$V_1 = \frac{c_2 . V_2}{c_1} = \frac{75 \frac{\cancel{mg}}{l} . \ 4 \text{ ml}}{1000 \frac{\cancel{mg}}{l}} = \mathbf{0.3 \text{ ml}}$$

The volume of distilled water is calculated as follows:

$$V_3 = 4 \text{ ml} - 0.3 \text{ ml} = \mathbf{3.7 \text{ ml}}$$

Results

Concentration (mg/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of distilled water (ml)
100	10.0	0.400	3.600
75	13.3	0.300	3.700
50	20.0	0.200	3.800
25	40.0	0.100	3.900
10	100.0	0.040	3.960

4. A sample containing an unknown concentration of RNA has an absorbance of 0.392. The blank (distilled water with the orcinol reagent) has absorbance of 0.042. Calculate its ribose concentration using the linear regression equation *y = 0.0058x + 0.0419* for ribose solutions in the concentration range of 10-100 mg/l.

The equation describing the linear regression: *y = 0.0058x + 0.0419,* allows us to express the value of *x*:

$$y = ax + b \rightarrow x = \frac{y-b}{a}$$

$$x = \frac{y-0.0419}{0.0058}$$

where *y* is the absorbance (0.392) of a sample containing an unknown concentration (*x*) of RNA.

The sample with an unknown RNA concentration had an absorbance of 0.392. However, in the example specification, the absorbance of the blank is also given (0.042). This needs to be subtracted from the sample absorbance to account for the measurement background.

$$y = 0.392 - 0.042 = 0.350$$

Substitute this absorbance (*y*) into the linear regression equation for the absorbance of calibration solutions of ribose of known concentration:

$$x = \frac{0.350 - 0.0419}{0.0058} = \mathbf{53.12\ mg/l}$$

The result is given in mg/l using the linear regression equation *y = 0.058x + 0.0419* for ribose solutions in the concentration range 10 - 100 mg/l).

Result
The RNA concentration in the sample is 53.12 mg/l.

5. **How much NaCl should you use to make 80 ml of its solution with a concentration of 1 mol/l (M = 58.44 g/mol)?**

V = 80 ml = 0.08 l
c = 1 mol/l
M_{KOH} = 58.44 g/mol
m = ? g

$$c = \frac{\frac{m}{M}}{V} \Rightarrow m = c \,.\, V \,.\, M$$

m = 1 mol/l x 0.08 l x 58.44 g/mol = **4.6752 g**

Result
For the preparation of 80 ml of a solution with a concentration of 1 mol/l, it is necessary to weigh 4.6752 g NaCl.

6. **You had a solution containing 4 g of deoxyribose per liter. You diluted this original solution by adding 1 ml of its to 9 ml distilled water. Then, you made a 1:100 dilution of the resulting solution. What is the final concentration of a solution?**

$$\text{dilution factor (x)} = \frac{\text{total volume}}{\text{sample volume}}$$

where the sample volume is the volume of the solution being diluted, and the total volume is the sample volume in addition to the volume of the diluent.

You dilute this original solution by adding 1 ml of its to 9 ml distilled water.

$$\text{dilution factor (x)} = \frac{10\ \text{ml}}{1\ \text{ml}}$$

Our sample volume is 1 ml. The total volume is the sum of our sample volume (1 ml) and the volume of water (9 ml). The total volume is found to be 10 ml.

$$\text{dilution factor (x)} = 10$$

The dilution factor je 10×. Now let's find the dilution of the sample.

$$\text{dilution} = \text{sample volume} : \text{total volume}$$
$$\text{dilution} = 1 : 10$$

The dilution of the sample is 1:10.

In the first step, we decreased the concentration of the deoxyribose solution by 10×. In this step, the concentration of the deoxyribose solution is reduced tenfold, meaning the diluted solution's concentration is 10 times lower than the original.

$$\text{dilution factor (x)} = \frac{\text{concentration of the stock solution}}{\text{concentration of the dilute solution}}$$

$$10\ \text{x} = \frac{4\ \text{g/l}}{\text{concentration of the dilute solution}} = \frac{4\ \text{g/l}}{10} = 0.4\ \text{g/l}$$

Subsequently, we prepared a 1:100 dilution of this solution. The dilution factor for this step is following:

$$\text{dilution factor (x)} = \frac{100}{1}$$
$$\text{dilution factor (x)} = 100$$

In the second step, we decreased the concentration of the deoxyribose solution by 100×. This means the new diluted solution's concentration is 100 times lower than the previous concentration.

Using the dilution factor formula again:

$$\text{dilution factor (x)} = \frac{\text{concentration of the stock solution}}{\text{concentration of the dilute solution}}$$

from which:

$$100\ \text{x} = \frac{0.4\ \text{g/l}}{\text{concentration of the dilute solution}} = \frac{0.4\ \text{g/l}}{100} = \mathbf{0.004\ g/l}$$

Result
The final concentration of the deoxyribose solution is 0.004 g/l.

LIPIDS

1. **How much potassium hydroxide (KOH, M = 56.11 g/mol) should you use to make 100 ml with a concentration of 0.1 N?**

V = 100 ml = 0.1 l
c = 0.1 N = 0.1 mol/l
M_{KOH} = 56.11 g/mol
m = ? g

$$c = \frac{\frac{\text{m}}{\text{M}}}{\text{V}} \Rightarrow m = c\,.V\,.M$$

m = 0.1 mol/l x 0.1 l x 56.11 g/mol = **0.5611 g**

Result
For the preparation of 100 ml of a solution with a concentration of 0.1 mol/l, it is necessary to weigh 0.5611 g KOH.

2. **How much phenolphthalein should you use to make 10 ml of its 1 % (*w/v*) solution?**

$$\begin{array}{c} \uparrow \text{1 g of phenolphthalein ….. in 100 ml of a solution} \uparrow \\ \text{x g of phenolphthalein …. in 10 ml of a solution} \end{array}$$

$$x : 1 = 10 : 100$$

$$\frac{x}{1} = \frac{10}{100}$$

$$x = \frac{1\,x\,10}{100} = \underline{0.1\ g}$$

Result
For the preparation of 10 ml of its 1 % (*w/v*) phenolphthalein solution, it is necessary to weigh 0.1 g of this chemical.

3. **Calculate the acid number of olive oil. The sample weighed 10.245 g, and the volume of KOH (0.15 N) consumed by the sample was 3.6 ml.**

$$\text{Acid number} = \frac{56.1 \times N \times V}{\text{sample weight (g)}}\ [\text{mg KOH/g of oil}]$$

where *N* is the normality of the potassium hydroxide solution used in the titration, *V* is the consumption of potassium hydroxide solution required to produce a pink color, and *56.1* is the equivalent weight of potassium hydroxide.

m = 10.245 g
N = 0.15 N = 0.15 mol/l
V = 3.6 ml

$$Acid\ number = \frac{56.1 \times 0.15 \times 3.6}{10.245} = 2.96\ \text{mg KOH/g of oil}$$

Result
The acid number of olive oil is 2.96 mg KOH/g of oil.

4. **To determine the acid number of rapeseed oil, the experiment was carried out in triplicate as the following:**
Weight of sample 1-A = 10.023 g
Weight of sample 1-B = 10.062 g
Weight of sample 1-C = 9.982 g
The volume of KOH (0.1 N) consumed by:
Sample 1-A = 3.2 ml
Sample 1-B = 3.3 ml
Sample 1-C = 2.9 ml
Calculate the arithmetic mean (average) of acid number and standard deviation.

$$SAMPLE\ 1\text{-}A:\ Acid\ number = \frac{56.1 \times 0.1 \times 3.2}{10.023} = 1.79\ mg\ KOH/g\ of\ oil$$

$$SAMPLE\ 1\text{-}B:\ Acid\ number = \frac{56.1 \times 0.1 \times 3.3}{10.062} = 1.84\ mg\ KOH/g\ of\ oil$$

$$SAMPLE\ 1\text{-}C:\ Acid\ number = \frac{56.1 \times 0.1 \times 2.9}{9.982} = 1.63\ mg\ KOH/g\ of\ oil$$

Calculation of the arithmetic mean (average):

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} = \frac{(1.79 + 1.84 + 1.63)}{3} = 1.75 \text{ mg} KOH/g\ of\ oil$$

Note: *n*- represent the number of measurements.

Calculation of the standard deviation (SD):

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n - 1}}$$

$$\sigma = \sqrt{\frac{(1.79 - 1.75)^2 + (1.84 - 1.75)^2 + (1.63 - 1.75)^2}{3 - 1}}$$

$$\sigma = 0.11 \text{ mg KOH/g of oil}$$

Result
The acid number of olive oil is 1.75±0.11 mg KOH/g of oil.

7. What is the volume of (36 %, v/v) of concentrated HCl required to prepare 250 ml of 0.5 N solution (M = 36.46 g/mol; $\rho_{36\%}$ = 1.19 kg/l)?

Note: HCl is a monoprotic acid. Hence, n-factor (also known as the equivalence factor) of HCl = 1.
Normality (N) is related to molarity (M) by the equation N = n × M. Since HCl is monoprotic acid (n = 1), the normality and molarity are equivalent for this acid. Thus, 0.5 N HCl is indeed the same as 0.5 M HCl, which means the solution has a concentration of 0.5 moles per liter.
0.5 N HCl = 0.5 M HCl = 0.5 mol/l

First, you need to determine the number of moles of HCl contained in 250 ml of a 0.5 mol/l HCl solution.

I. $c = \frac{n}{V} \rightarrow n = c.\ V = 0.5\ mol/l\ .\ 0.25\ l = 0.125\ mol$

Next, determine the weight of HCl that corresponds to 0.125 mol HCl.

II. $n = \frac{m}{M} \rightarrow m = n\ .\ M = 0.125\ mol\ .\ 36.46\ g/mol = 4.5575\ g\ HCl\ (100\ \%,\ v/v)$

You need 4.5575 g of 100 % (*v/v*) HCl, but you have a solution of its 36 % (v/v) HCl. To determine the mass of the 36 % (*v/v*) HCl solution required, you can set up a proportion:

III.

4.5575 g of HCl ….. 100 %
x g of HCl……36 %

x : 4.5575 = 100 : 36

$$\frac{x}{4.5575} = \frac{100}{36}$$

$$x = \frac{4.5575\ x\ 100}{36} = \underline{12.66 \text{ g HCl (36 \%, } v/v)}$$

If you have a less concentrated acid (like 36 % (*v/v*) HCl), you must weigh more than with concentrated acid (100 % (*v/v*) HCl. This is an inverse proportionality.

Finally, since the density of the 36 % (*v/v*) HCl solution is 1.9 kg/l, you can determine the volume of the 36 % (*v/v*) HCl needed:

$\rho_{36\%}$ = 1.19 kg/l = 1.19 g/ml

IV. $\rho = \frac{m}{V} \rightarrow V = \frac{m}{\rho} = \frac{12.66\ g}{1.19\ g/ml} = 10.64$ ml

Result
For the preparation of 0.5 mol/l HCl you need 10.64 ml of 36 % (v/v) HCl.

8. **Calculate the saponification number of canola oil. The weight of a sample was 5.024 g, the volume of HCl (0.5 N) consumed by a sample was 52 ml, and consumed by a blank was 17.3 ml.**

$$\text{Saponification value} = \frac{28.05 \times (V_1 - V_2)}{\text{sample weight [g]}} \text{ [mg KOH/g of the oil]}$$

where V_1 is the volume (in ml) of hydrochloric acid consumed in the blank determination, V_2 is the volume (in ml) of hydrochloric acid consumed in the sample determination, and 28.05 refers to the 28.05 mg KOH corresponding to 1 ml of 0.5 N HCl.

m = 5.024 g
V_1 = 52 ml
V_2 = 17.3 ml

$$\textit{Saponification value} = \frac{28.05 \times (52 - 17.3)}{5.024} = 193.7\ \textit{mg KOH/g of oil}$$

Result
The saponification value of canola oil is 193.7 mg KOH/g of oil.

9. **How much sodium thiosulfate (M = 158.11 g/mol) should you use to make 100 ml with a concentration of 0.01 mol/l?**

V = 100 ml = 0.1 l
c = 0.01 mol/l
$M_{Na2S2O3}$ = 158.11 g/mol
m = ? g

$$c = \frac{\frac{m}{M}}{V} \Rightarrow m = c \,.\, V \,.\, M$$

m = 0.01 mol/l x 0.1 l x 158.11 g/mol = **0.1581 g**

Result
For the preparation of 100 ml of a solution with a concentration of 0.01 mol/l, it is necessary to weigh 0.1581 g thiosulfate.

10. **Calculate the peroxide number of linseed oil. The weight of a sample was 1.024 g, the volume of sodium thiosulfate (0.01 M) consumed by a sample was 0.6 ml and consumed by a blank was 0.4 ml.**

$$\text{Peroxide value} = \frac{(V_1\ [\text{ml}] - V_2\ [\text{ml}])\ .\ M\ .\ 1000}{\text{weight of the oil sample [g]}}\ \ [\text{mmol } O_2/1\ \text{kg or meq per 1 kg}]$$

where V_1 is the volume (in ml) of sodium thiosulfate consumed in the sample determination, V_2 is the volume (in ml) of sodium thiosulfate consumed in the blank determination, and M is the molarity of sodium thiosulfate, expressed in mol/l.

m = 1.024 g
V_1 = 0.6 ml
V_2 = 0.4 ml
M = 0.01 M

$$\text{Peroxide value} = \frac{(0.6 - 0.4)\ .\ 0.01\ .\ 1000}{1.024} = \mathbf{1.95\ mmol\ O_2/1\ kg}$$

Result
The peroxide value of linseed oil is 1.95 mmol O_2/kg.

PROTEINS

1. **How much sodium chloride (NaCl) should you use to make 250 ml of its 0.9 % (*w/v*) solution?**

0.9 g of NaCl ….. in 100 ml of a solution
x g of NaCl …. in 250 ml of a solution

x : 0.9 = 250 : 100

$$\frac{x}{0.9} = \frac{250}{100}$$

$$x = \frac{0.9\ x\ 250}{100} = \mathbf{2.25\ g}$$

Result
For the preparation of 250 ml of its 0.9 % (*w/v*) NaCl solution, it is necessary to weigh 2.25 g of NaCl.

2. **How much sodium hydroxide (NaOH) should you use to make 100 ml of its 10 % (*w/v*) solution?**

10 g of NaOH ….. in 100 ml of a solution
x g of NaOH …. in 100 ml of a solution

x : 10 = 100 : 100

$$\frac{x}{10} = \frac{100}{100}$$

$$x = \frac{10\ x\ 100}{100} = \mathbf{10\ g}$$

Result
For the preparation of 100 ml of its 10 % (*w/v*) NaOH solution, it is necessary to weigh 10 g of NaOH.

3. **The stock solution of egg albumin with a concentration of 10 g/l should be diluted to a concentration in the range of 1-7 g/l. Calculate the dilution factor,**

the volume of the stock solution and the volume of saline solution needed to produce a solution of a given concentration. The total volume of the dilute solution must be 5 ml.

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of saline solution (ml)
7.0			
6.0			
5.0			
4.0			
3.0			
2.0			
1.0			

The example of how to calculate the dilution factor, the volume of the stock solution and the volume of saline solution to produce dilute solution at a concentration of 5.0 g/l.

Method 1

$$\text{dilution factor (x)} = \frac{\text{concentration of the stock solution}}{\text{concentration of the dilute solution}}$$

$$\text{dilution factor (x)} = \frac{10\ \text{g/l}}{5.0\ \text{g/l}} = \mathbf{2\ x}$$

In this practical example, the volume of a dilute solution is set to 5 ml.

$$\text{Volume of stock solution transferred} = \frac{5\ \text{ml}}{2\ \text{x}} = \mathbf{2.5\ ml}$$

$$\text{Volume of saline solution} = 5\ \text{ml} - 2.5\ \text{ml} = \mathbf{2.5\ ml}$$

Method 2

<u>The example of calculation for a dilute solution concentration of 5.0 g/l:</u>

V_1 = ? ml

c_1 = 10 g/l (the stock solution)

V_2 = 5 ml (the total volume of dilute solution)

c_2 = 5.0 g/l (the dilute solution)

$$c_1 . V_1 = c_2 . V_2 \rightarrow V_1 = \frac{c_2 . V_2}{c_1}$$

$$V_1 = \frac{c_2 . V_2}{c_1} = \frac{5.0\ \text{g/l} .\ 5\ \text{ml}}{10\ \text{g/l}} = \mathbf{2.5\ ml}$$

The volume of saline solution:

$$V_3 = 5\ \text{ml} - 2.5\ \text{ml} = \mathbf{2.5\ ml}$$

Results

Concentration (g/l)	Dilution factor (x)	Total volume of dilute solution	
		Volume of stock solution (ml)	Volume of saline solution (ml)
7.0	1.43	3.5	1.5
6.0	1.67	3.0	2.0
5.0	2.0	2.5	2.5
4.0	2.5	2.0	3.0
3.0	3.33	1.5	3.5
2.0	5.0	1.0	4.0
1.0	10.0	0.5	4.5

4. **How much individual components should you use to make:**
   a. **sodium carbonate (2 %, *w/v*) in 0.1 mol/l aqueous solution of sodium hydroxide (M = 39.9971 g/mol, V = 50 ml) (reagent A), and**
   b. **copper (II) sulfate pentahydrate (0.5 %, *w/v*) in aqueous solution of sodium potassium tartrate (2 %, *w/v*) (V = 1 ml) (reagent B)?**

Reagent A

2.0 g of Na_2CO_3 ….. in 100 ml of a solution
x g of Na_2CO_3 …… in 50 ml of a solution

x : 2.0 = 50 : 100

$$\frac{x}{2.0} = \frac{50}{100}$$

$$x = \frac{2.0 \; x \; 50}{100} = \mathbf{1.0\ g}$$

V = 50 ml = 0.05 l
c = 0.1 mol/l
M_{NaOH} = 39.9971 g/mol
m = ? g

$$c = \frac{\frac{m}{M}}{V} \Rightarrow m = c \,.\, V \,.\, M$$

m = 0.1 mol/l x 0.05 l x 39.9971 g/mol = **0.2 g**

Results
For the preparation of 50 ml of reagent A, it is necessary to weigh 1.0 g Na_2CO_3 and 0.2 g NaOH.

Reagent B

0.5 g of $CuSO_4 \,.\, 5\ H_2O$ ….. in 100 ml of a solution
x g of $CuSO_4 \,.\, 5\ H_2O$ …. in 1 ml of a solution

x : 0.5 = 1 : 100

$$\frac{x}{0.5} = \frac{1}{100}$$

$$x = \frac{0.5\ x\ 1}{100} = \mathbf{0.005\ g}$$

2.0 g of sodium potassium tartrate ….. in 100 ml of a solution
x g of sodium potassium tartrate …. in 1 ml of a solution

x : 2.0 = 1 : 100

$$\frac{x}{2.0} = \frac{1}{100}$$

$$x = \frac{2.0\ x\ 1}{100} = \mathbf{0.02\ g}$$

Results
For the preparation of 5 ml of reagent B, it is necessary to weigh 0.005 g $CuSO_4$. 5 H_2O and 0.02 g sodium potassium tartrate.

ORGANIC ACIDS

1. **Calculate the percent yield of glutamic acid (%) per wheat and gluten if you know that the weight of glutamic acid crystals was 0.487 g, wheat flour weight was 100.211 g and wheat gluten was 10.231 g. The maximum theoretical yield of glutamic acid isolated from wheat flour is 1.3 % (*w/w*) and isolated from wheat gluten is 35 % (*w/w*).**

The percent yield of glutamic acid
m (wheat flour) = 100.211 g
m (glutamic acid crystals) = 0.487 g
m (gluten) = 10.231 g
The maximum theoretical yields for glutamic acid isolated from:
- wheat flour: 1.3 % (*w/w*)
- wheat gluten: 35 % (*w/w*)

The percent yield of glutamic acid per wheat flour
Given that there is 0.487 g of glutamic acid in 100.211 g of wheat flour, for 100 g:

0.487 g of glutamic acid ……. in 100.211 g of wheat flour
x g of glutamic acid ……. in 100 g of wheat flour

x : 0.487 = 100 : 100.211

$$\frac{x}{0.487} = \frac{100}{100.211}$$

$$x = \frac{100\ x\ 0.487}{100.211} = 0.486\ \text{g}/100\ \text{g} = 0.486\ \%\ (w/w)$$

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}}\ \text{x}\ 100\ \%$$

$$\text{Percent yield} = \frac{0.486\ \%}{1.3\ \%}\ .100\ \% = \mathbf{37.38}\ \%$$

The percent yield of glutamic acid per gluten
Given that there is 0.487 g of glutamic acid in 10.231 g of gluten, for 100 g:

0.487 g of glutamic acid ……. in 10.231 g of gluten
x g of glutamic acid ..…… in 100 g of gluten

x : 0.487 = 100 : 10.231

$$\frac{x}{0.487} = \frac{100}{10.231}$$

$$x = \frac{100\ x\ 0.487}{10.231} = 4.76\ \text{g}/100\ \text{g} = 4.76\ \%\ (\text{w/w})$$

$$\text{Percent yield} = \frac{\text{Actual yield}}{\text{Theoretical yield}}\ \text{x}\ 100\ \%$$

$$\text{Percent yield} = \frac{4.76\ \%}{35\ \%}\ .100\ \% = \mathbf{13.6}\ \%$$

Results
The percent yield of glutamic acid per wheat flour is 37.38 % and per gluten is 13.6 %.

2. **How much calcium chloride ($CaCl_2$) should you use to make 25 ml of its 25 % (*w/v*) solution?**

25 g of $CaCl_2$ ….. in 100 ml of a solution
x g of $CaCl_2$ …. in 25 ml of a solution

$$x : 25 = 25 : 100$$

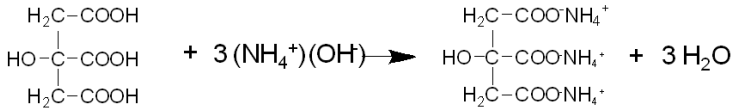
$$\frac{x}{25} = \frac{25}{100}$$

$$x = \frac{25\ x\ 25}{100} = \mathbf{6.25\ g}$$

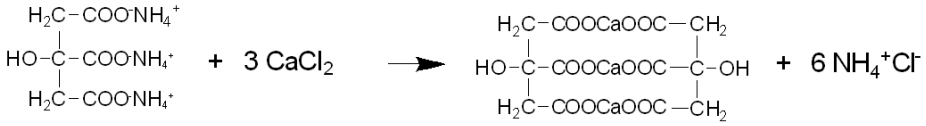
Result
For the preparation of 25 ml of its 25 % (*w/v*) $CaCl_2$ solution, it is necessary to weigh 6.25 g of $CaCl_2$.

3. **Calculate the required amount of sulfuric acid (98 %, *v/v*) to neutralize 250 µl of 26 % (*v/v*) ammonia used during the isolation of citric acid from lemon ($\rho_{(26\ \%\ \text{ammonia})} = 0.904$ g/ml; $M_{NH3} = 17$ g/mol; $M_{H2SO4} = 98.079$ g/mol; $\rho_{(98\ \%\ \text{sulfuric acid})} = 1.84$ g/ml).**

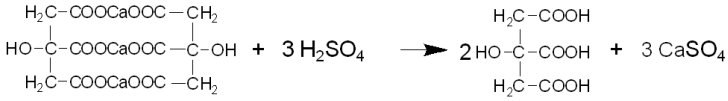
First, establish the relationship between the amount of sulfuric acid ($n_{(H2SO4)}$) and the amount of ammonia ($n_{(NH3)}$)



$$\text{I.}\quad \frac{n_{\text{ammonia}}}{n_{\text{ammonium citrate}}} = \frac{3}{1} \rightarrow n_{\text{ammonia}} = 3\ .\ n_{\text{ammonium citrate}}$$



$$\text{II.}\quad \frac{n_{\text{ammonium citrate}}}{n_{\text{calcium citrate}}} = \frac{2}{1} \rightarrow n_{\text{ammonium citrate}} = 2\ .\ n_{\text{calcium citrate}}$$



III. $\frac{n_{\text{calcium citrate}}}{n_{\text{sulfuric acid}}} = \frac{1}{3} \rightarrow n_{\text{calcium citrate}} = \frac{1}{3} \cdot n_{\text{sulfuric acid}}$

$n_{\text{ammonia}} = 3 \cdot n_{\text{ammonium citrate}} = 3 \cdot 2 \cdot n_{\text{calcium citrate}} = \cancel{3} \cdot 2 \cdot \frac{1}{\cancel{3}} \cdot n_{\text{sulfuric acid}}$

Combining the relationships: $n_{\text{ammonia}} = 2 \cdot n_{\text{sulfuric acid}}$

Calculations

Based on the known values for the volume and density of 26 % (v/v) NH_3, calculate its mass from the density formula, which is then converted to the mass of 100 % NH_3.

$$\rho = \frac{m}{V} \rightarrow m_{26\,\%\,NH_3} = V_{26\,\%\,NH_3} \cdot \rho_{26\,\%\,NH_3}$$

$$m = V \cdot \rho = 0.25\ \text{ml} \cdot 0.904\ \text{g/ml} = 0.226\ \text{g}\ (26\,\%\ NH_3)$$

Considering the concentration:

If you have a more concentrated base (like 100 % (v/v) NH_3), you must weigh less than with dilute base (26 % (v/v) NH_3). This is an inverse proportionality.

0.226 g of NH_3 …… 26 % (*v/v*) of solution
x g of NH_3 ……..…… 100 % (*v/v*) of solution

x : 0.226 = 26 : 100

$$\frac{x}{0.226} = \frac{26}{100}$$

$$x = \frac{26\ x\ 0.226}{100} = 0.0588\ \text{g}\ 100\,\%\ (v/v)\ NH_3$$

Now, calculate the amount of NH_3 (100 %, v/v).

$$n_{\mathbf{100\,\%\,NH_3}} = \frac{m}{M} = \frac{0.0588\ \text{g}}{17\ \text{g/mol}} = 3.456 \cdot 10^{-3}\text{mol}$$

From the relationship between the amount of sulfuric acid ($n_{(H2SO4)}$) and the amount of ammonia ($n_{(NH3)}$), calculate the amount of sulfuric acid.

$$n_{NH_3} = 2 \cdot n_{H_2SO_4} \rightarrow n_{H_2SO_4} = \frac{n_{NH_3}}{2}$$

$$n_{H_2SO_4} = \frac{3.456 \cdot 10^{-3}\text{mol}}{2} = 1.73 \cdot 10^{-3}\text{mol}\ (100\%\ (v/v)\ H_2SO_4)$$

Calculate the weight of sulfuric acid (100 %, v/v).

$$n = \frac{m}{M} \rightarrow m_{\mathbf{100\,\%\,H_2SO_4}} = n \cdot M$$

$$m = n \cdot M = 1.73 \cdot 10^{-3}\text{mol} \cdot 98.079\ \text{g/mol} = 0.16959\ \text{g}\ (100\,\%\ H_2SO_4)$$

Have 98% sulfuric acid to dispose of, so calculate the weight of sulfuric acid (98%, v/v).

If you have a less concentrated acid (like 98 % (v/v) H_2SO_4), you must weigh more than with concentrated acid (100 % (v/v) H_2SO_4). This is an inverse proportionality.

0.16959 g of H_2SO_4 … 100 % (*v/v*) of solution
x g of H_2SO_4 ……..…. 98 % (*v/v*) of solution

x : 0.16959 = 100 : 98

$$\frac{x}{0.16959} = \frac{100}{98}$$

$$x = \frac{100\ x\ 0.16959}{98} = 0.173\ \text{g}\ 98\ \%\ (v/v)\ H_2SO_4$$

Sulfuric acid is a liquid, calculate the volume of the required amount of 98 % (v/v) sulfuric acid.

$$\rho = \frac{m}{V} \rightarrow V_{98\ \%\ H_2SO_4} = \frac{m_{98\ \%\ H_2SO_4}}{\rho_{98\ \%\ H_2SO_4}}$$

$$V = \frac{m}{\rho} = \frac{0.173\ \text{g}}{1.84\ \text{g/ml}} = 0.094\ \text{ml} = \mathbf{94\ \mu l\ 98\ \%\ (v/v)\ H_2SO_4}$$

Result
The required amount of sulfuric acid to neutralize the calcium citrate is 94 µl.

Attachment 4: Functions and graphs in Excel

Functions in Excel are used to perform complex mathematical operations. Each function has the form NAME.FUNCTION() .... The name of the function is always followed by parentheses (), which contain any arguments to the function. In the following sections, we will describe each step in more detail. These are functions that are needed to evaluate the results of your work in the laboratory as calculating the arithmetic mean (average), and standard deviation.

Part A: the AVERAGE function

The AVERAGE function expresses the middle value, which is the position in the middle of a group of numbers in a statistical distribution. In other words, it is an arithmetic mean and is calculated by adding a group of numbers and then dividing by the number of those numbers. For example, the average of the numbers 5, 8, 3, 7, 9 and 15 is 47 (sum) divided by 6 (number), i.e., the result is 7.83.

The AVERAGE function expresses the mean, which is the position in the middle of a group of numbers in a statistical distribution. In other words, it is the arithmetic mean and is calculated by adding a group of numbers and then dividing by the number of those numbers. For example, the average of the numbers 5, 8, 3, 7, 9 and 15 is 47 (the sum) divided by 6 (the number), i.e., the result is 7.83.

Detailed procedure in MS Excel

1. Calculate the arithmetic mean for Eppendorf tubes marked Ep 1 - Ep 5.

Table A1: The results of Eppendorf tube weight measurements on an analytical balance.

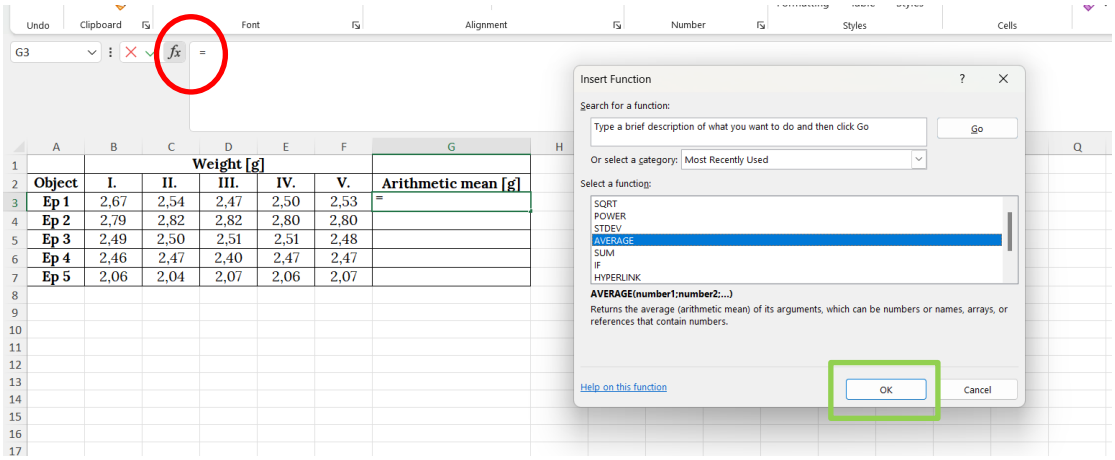
	Weight [g]					
Object	**I.**	**II.**	**III.**	**IV.**	**V.**	**Arithmetic mean [g]**
Ep 1	2.3267	2.3254	2.3247	2.3250	2.3253	
Ep 2	2.3379	2.3382	2.3382	2.3380	2.3380	
Ep 3	2.3549	2.3550	2.3551	2.3351	2.3548	
Ep 4	2.3246	2.3247	2.3740	2.3247	2.3247	
Ep 5	2.3706	2.3704	2.3707	2.3706	2.3707	

2. Create a table in MS Excel according to the template below:

	A	B	C	D	E	F	G	H
1		Weight [g]						
2	**Object**	**I.**	**II.**	**III.**	**IV.**	**V.**	**Arithmetic mean [g]**	
3	**Ep 1**	2,67	2,54	2,47	2,50	2,53		
4	**Ep 2**	2,79	2,82	2,82	2,80	2,80		
5	**Ep 3**	2,49	2,50	2,51	2,51	2,48		
6	**Ep 4**	2,46	2,47	2,40	2,47	2,47		
7	**Ep 5**	2,06	2,04	2,07	2,06	2,07		
8								
9								

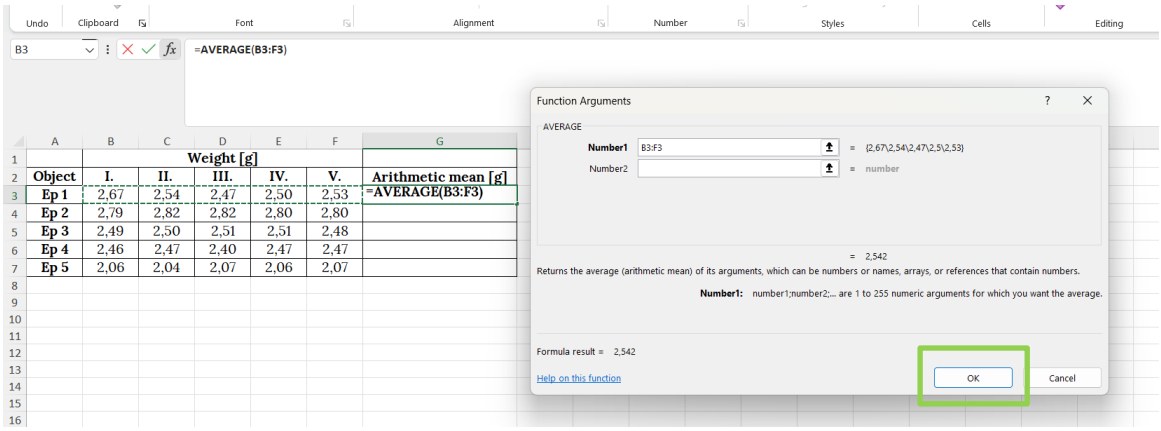
3. Click in the cell G3 in which we want to calculate the average of the values for Ep 1.

4. In the marked area (the red circle) insert the AVERAGE function, which you select from the Statistical functions category by clicking on *fx*.



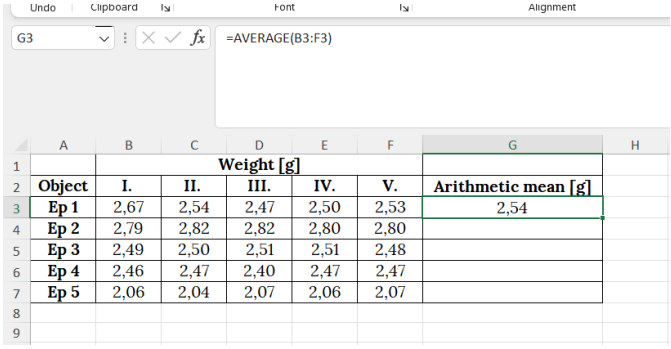
	A	B	C	D	E	F	G
1		Weight [g]					
2	Object	I.	II.	III.	IV.	V.	Arithmetic mean [g]
3	Ep 1	2,67	2,54	2,47	2,50	2,53	=
4	Ep 2	2,79	2,82	2,82	2,80	2,80	
5	Ep 3	2,49	2,50	2,51	2,51	2,48	
6	Ep 4	2,46	2,47	2,40	2,47	2,47	
7	Ep 5	2,06	2,04	2,07	2,06	2,07	

5. Confirm the function (the green rectangle) and open the function dialog via *fx*. Click OK.
6. Select the cells with the values you want to average. In our case, these are cells A3-F3.



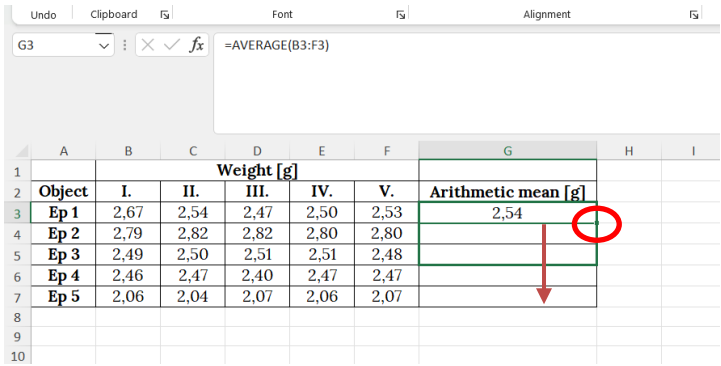
	A	B	C	D	E	F	G
1		Weight [g]					
2	Object	I.	II.	III.	IV.	V.	Arithmetic mean [g]
3	Ep 1	2,67	2,54	2,47	2,50	2,53	=AVERAGE(B3:F3)
4	Ep 2	2,79	2,82	2,82	2,80	2,80	
5	Ep 3	2,49	2,50	2,51	2,51	2,48	
6	Ep 4	2,46	2,47	2,40	2,47	2,47	
7	Ep 5	2,06	2,04	2,07	2,06	2,07	

7. Click OK (the green rectangle), and the average value of the individual measurements for the observed object Ep 1 will then be displayed in cell G3.



	A	B	C	D	E	F	G	H
1		Weight [g]						
2	Object	I.	II.	III.	IV.	V.	Arithmetic mean [g]	
3	Ep 1	2,67	2,54	2,47	2,50	2,53	2,54	
4	Ep 2	2,79	2,82	2,82	2,80	2,80		
5	Ep 3	2,49	2,50	2,51	2,51	2,48		
6	Ep 4	2,46	2,47	2,40	2,47	2,47		
7	Ep 5	2,06	2,04	2,07	2,06	2,07		

8. Click in the bottom right corner (a red circle) of cell G3 and drag the function into cells G4-G7 to calculate the average values for the remaining objects (Ep 2 – Ep 5).



9. The resulting table with calculated average values for weighed objects will look like this:

	Weight [g]					
Object	**I.**	**II.**	**III.**	**IV.**	**V.**	**Arithmetic mean [g]**
Ep 1	2,67	2,54	2,47	2,50	2,53	2,54
Ep 2	2,79	2,82	2,82	2,80	2,80	2,81
Ep 3	2,49	2,50	2,51	2,51	2,48	2,50
Ep 4	2,46	2,47	2,40	2,47	2,47	2,45
Ep 5	2,06	2,04	2,07	2,06	2,07	2,06

Part B: the STDEV function

The standard deviation expresses how the values differ from the average value. It is used to measure statistical dispersion. It indicates how dispersed the values are in each set. In Excel, the STDEV function is used to calculate the standard deviation.

Detailed procedure in MS Excel

1. Calculate the standard deviation for Eppendorf tubes marked Ep 1 - Ep 5.

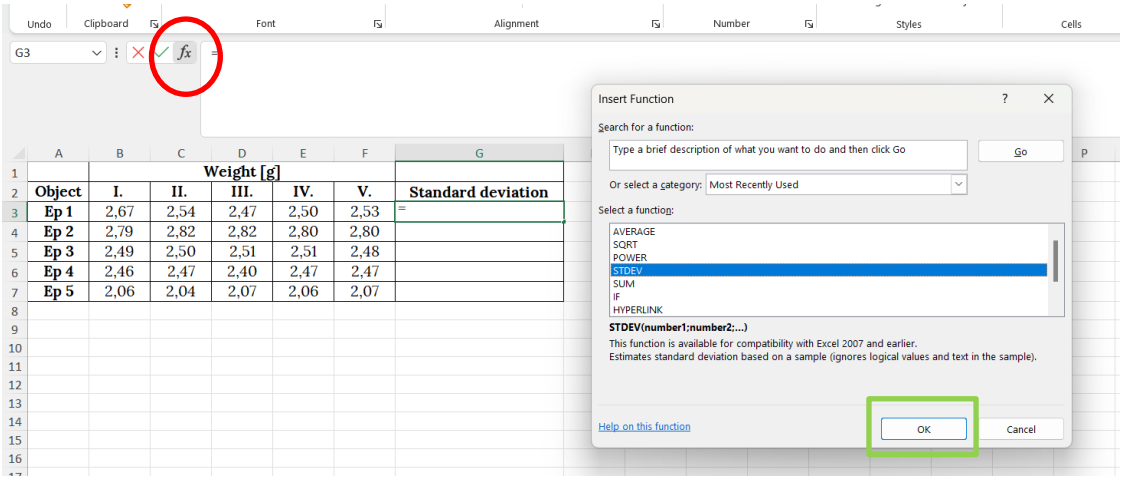
Table A1: The results of Eppendorf tube weight measurements on an analytical balance.

	Weight [g]					
Object	**I.**	**II.**	**III.**	**IV.**	**V.**	**Standard deviation [g]**
Ep 1	2.3267	2.3254	2.3247	2.3250	2.3253	
Ep 2	2.3379	2.3382	2.3382	2.3380	2.3380	
Ep 3	2.3549	2.3550	2.3551	2.3351	2.3548	
Ep 4	2.3246	2.3247	2.3740	2.3247	2.3247	
Ep 5	2.3706	2.3704	2.3707	2.3706	2.3707	

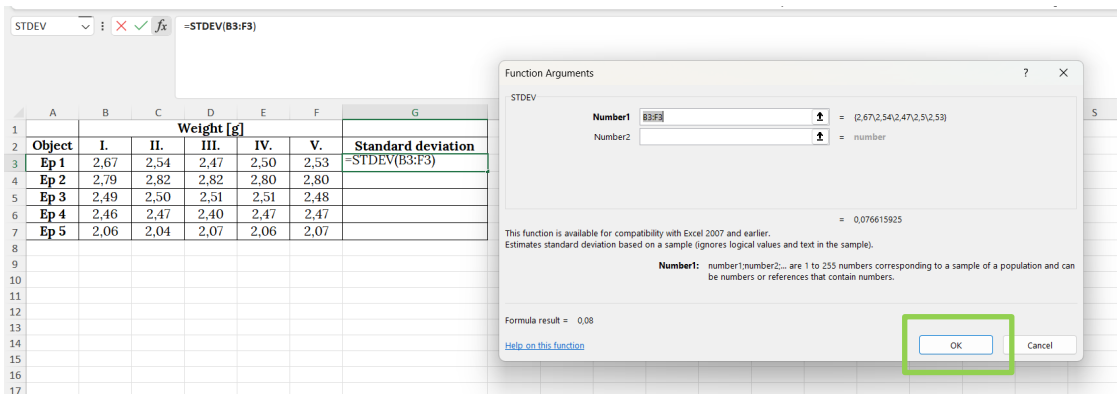
2. Create a table in MS Excel according to the template below:

	A	B	C	D	E	F	G	H
1		Weight [g]						
2	**Object**	**I.**	**II.**	**III.**	**IV.**	**V.**	**Standard deviation**	
3	**Ep 1**	2,67	2,54	2,47	2,50	2,53		
4	**Ep 2**	2,79	2,82	2,82	2,80	2,80		
5	**Ep 3**	2,49	2,50	2,51	2,51	2,48		
6	**Ep 4**	2,46	2,47	2,40	2,47	2,47		
7	**Ep 5**	2,06	2,04	2,07	2,06	2,07		
8								
9								

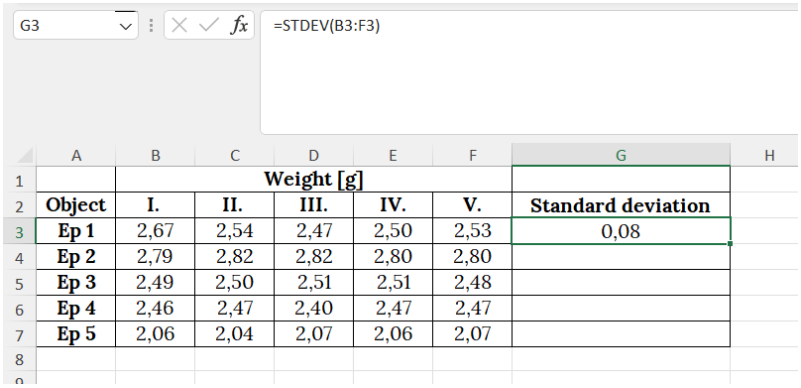
3. Click in the cell G3 in which we want to calculate the standard deviation of the values for Ep 1.
4. In the marked area (the red circle) insert the STDEV function, which you select from the Statistical functions category by clicking on *fx*.



5. Confirm the function (the green rectangle) and open the function dialog via *fx*. Click OK.
6. Select the cells with the values from which you want to calculate the standard deviation. In our case, these are cells A3-F3.

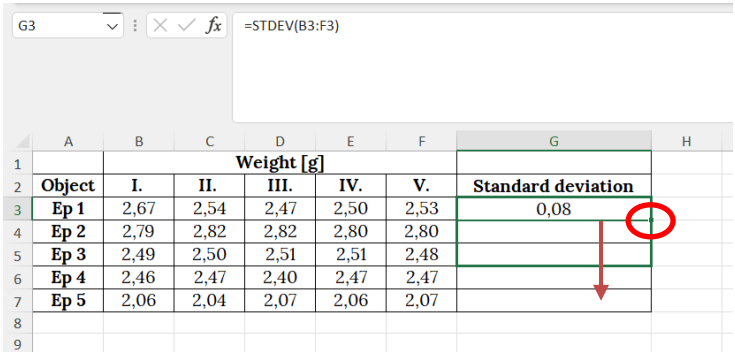


7. Click OK (the green rectangle), and the standard deviation value of the individual measurements for observed object Ep 1 will then be displayed in cell G3.



	A	B	C	D	E	F	G	H
1		Weight [g]						
2	**Object**	**I.**	**II.**	**III.**	**IV.**	**V.**	**Standard deviation**	
3	**Ep 1**	2,67	2,54	2,47	2,50	2,53	0,08	
4	**Ep 2**	2,79	2,82	2,82	2,80	2,80		
5	**Ep 3**	2,49	2,50	2,51	2,51	2,48		
6	**Ep 4**	2,46	2,47	2,40	2,47	2,47		
7	**Ep 5**	2,06	2,04	2,07	2,06	2,07		
8								

8. Click in the bottom right corner (a red circle) of cell G3 and drag the function into cells G4-7 to calculate the standard deviations for the remaining objects.



	A	B	C	D	E	F	G	H
1		Weight [g]						
2	**Object**	**I.**	**II.**	**III.**	**IV.**	**V.**	**Standard deviation**	
3	**Ep 1**	2,67	2,54	2,47	2,50	2,53	0,08	
4	**Ep 2**	2,79	2,82	2,82	2,80	2,80		
5	**Ep 3**	2,49	2,50	2,51	2,51	2,48		
6	**Ep 4**	2,46	2,47	2,40	2,47	2,47		
7	**Ep 5**	2,06	2,04	2,07	2,06	2,07		
8								
9								

9. The resulting table with calculated standard deviation values for weighed objects Ep 1 – Ep 5 will look like this:

	A	B	C	D	E	F	G	H
1		Weight [g]						
2	**Object**	**I.**	**II.**	**III.**	**IV.**	**V.**	**Standard deviation**	
3	**Ep 1**	2,67	2,54	2,47	2,50	2,53	0,08	
4	**Ep 2**	2,79	2,82	2,82	2,80	2,80	0,01	
5	**Ep 3**	2,49	2,50	2,51	2,51	2,48	0,01	
6	**Ep 4**	2,46	2,47	2,40	2,47	2,47	0,03	
7	**Ep 5**	2,06	2,04	2,07	2,06	2,07	0,01	
8								
9								

Part C: Construction of a calibration line using linear regression in MS Excel

When measuring the interdependence of two quantities x and y, we obtain a set of pairs (x_i, y_i). The graph is a set of points in the coordinate plane that all are solutions to the equation. If all variables represent real numbers one can graph the equation by plotting enough points to recognize a pattern and then connect the points to include all points. Many mathematical relationships are linear relationships.

Linear regression is defined by the relationship:

$$y = ax + b$$

where y is a dependent variable (e.g., absorbance), x is an independent variable (e.g., concentration), b is the point at which the line intersects the y-axis (the absolute value, corresponds to the background signal of the matrix), and a is the direction of the line (represents the slope of the line - depending on its value, the angle between the calibration line and the x-axis changes).

The methodology we use as an example to explain the construction of the calibration curve is based on the fact that the solution becomes colored upon the addition of a suitable reagent. While the intensity of the coloring is directly proportional to the concentration of the substance to be determined (e.g., glucose concentration, sucrose concentration, protein concentration) in the solution and can be determined using a spectrophotometer.

Detailed procedure in MS Excel

PART 1: ***Preparation of the calibration curve***

1. Generate a calibration curve in MS Excel for measured values:

Table A3: Measured absorbance values of standard glucose solutions with a concentration of 2.5-15 g/l at a wavelength of 540 nm.

Glucose concentration [g/l]	Absorbance at 540 nm			Average
	I.	II.	III.	
2.5	0.126	0.145	0.137	
5.0	0.278	0.284	0.282	
7.5	0.427	0.424	0.413	
10.0	0.560	0.569	0.568	
12.5	0.739	0.719	0.720	
15.0	0.844	0.859	0.867	

2. Create a table in MS Excel according to the template below:

	A	B	C	D	E	F
1	Glucose concentration [g/l]	Absorbance at 540 nm			Average	
2		I.	II.	III.		
3	2,5	0,126	0,145	0,137		
4	5,0	0,278	0,284	0,282		
5	7,5	0,427	0,424	0,413		
6	10,0	0,560	0,569	0,568		
7	12,5	0,739	0,719	0,72		
8	15,0	0,844	0,859	0,867		
9						

3. Click in the cell E3 to calculate the average of the values for the glucose concentration of 2.5 g/l (see part A the AVERAGE function) and for the other glucose concentrations.

	A	B	C	D	E
1	Glucose concentration [g/l]	Absorbance at 540 nm			Average
2		I.	II.	III.	
3	2,5	0,126	0,145	0,137	0,136
4	5,0	0,278	0,284	0,282	0,281333
5	7,5	0,427	0,424	0,413	0,421333
6	10,0	0,560	0,569	0,568	0,565667
7	12,5	0,739	0,719	0,72	0,726
8	15,0	0,844	0,859	0,867	0,856667
9					

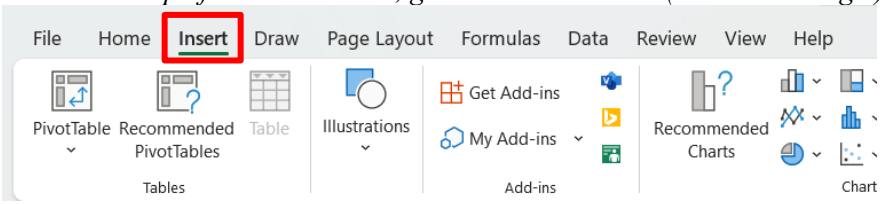
4. Graphical display will allow to check visually that all your data points are on the curve. Initial data consist of different concentrations of glucose (*x,* independent

variable) and their absorbances (*y*, dependent variable). Use chart wizard to generate calibration curve, select "(XY) scatter".

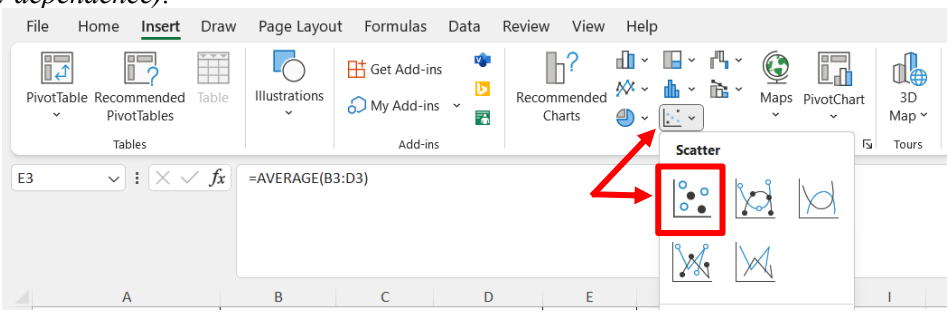
First, select the cells in the column A (x-values). Now press the Ctrl key and then click on the cells of the column E (y-values).

	A	B	C	D	E
1	Glucose concentration [g/l]	Absorbance at 540 nm			Average
2		I.	II.	III.	
3	2,5	0,126	0,145	0,137	0,136
4	5,0	0,278	0,284	0,282	0,281
5	7,5	0,427	0,424	0,413	0,421
6	10,0	0,560	0,569	0,568	0,566
7	12,5	0,739	0,719	0,72	0,726
8	15,0	0,844	0,859	0,867	0,857

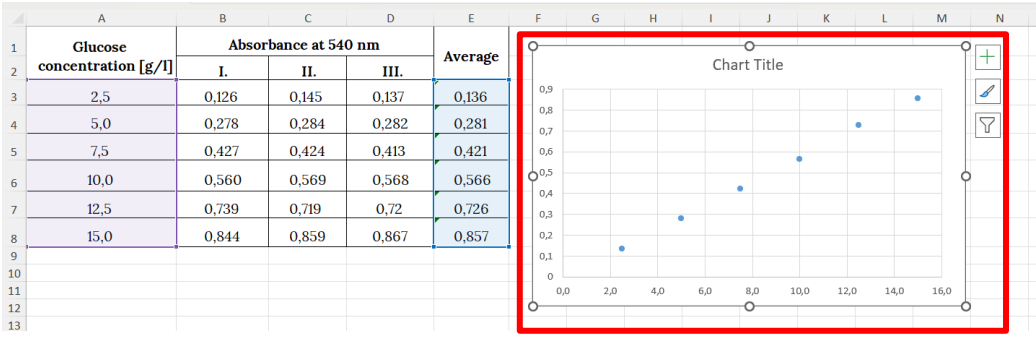
Then at the top of the Excel menu, go to the "Insert" tab (the red rectangle).



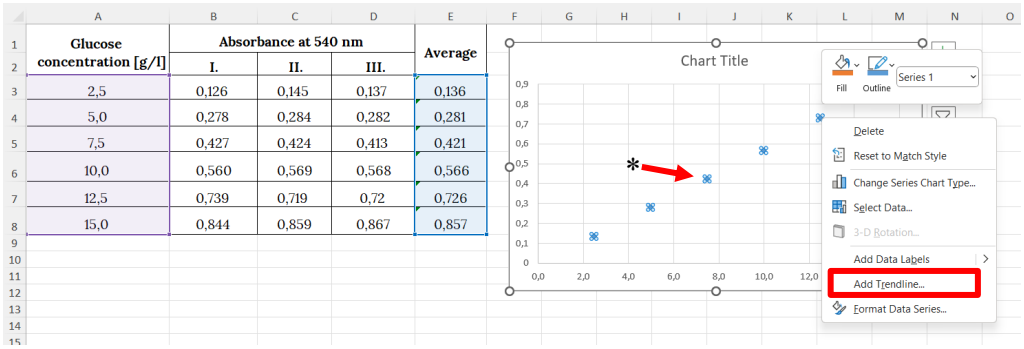
Go to the "Charts" menu and select the first option in the drop-down list "Scatter" (x,y dependence).



5. A chart containing the data points from the selected two columns is displayed.



6. Select the data (clink on the blue dots) and right click to get menu and select "add trendline".



Glucose concentration [g/l]	Absorbance at 540 nm			Average
	I.	II.	III.	
2,5	0,126	0,145	0,137	0,136
5,0	0,278	0,284	0,282	0,281
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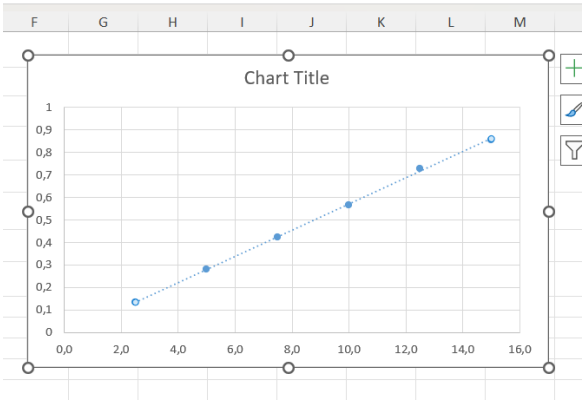
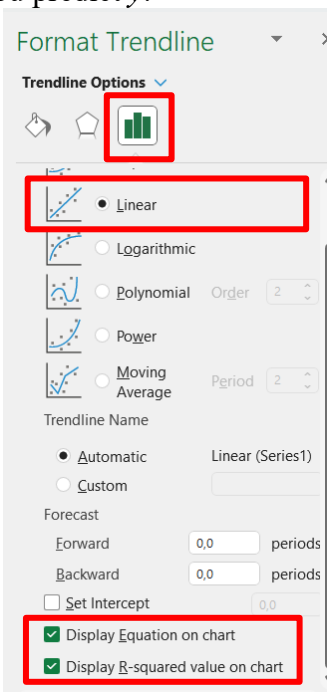
12,5	0,739	0,719	0,72	0,726
15,0	0,844	0,859	0,867	0,857

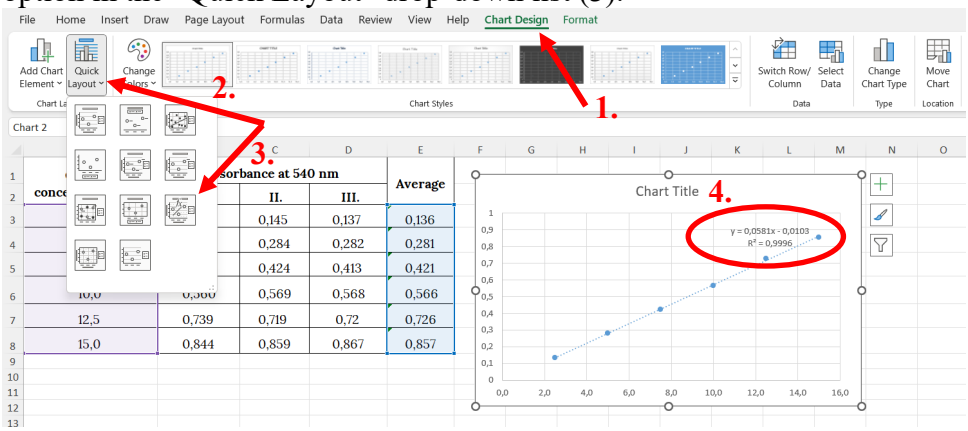
7. The result is the calibration curve.



8. Now we need to show the equation and R^2. We have two options for displaying them on the graph.
Option 1: The "Format Trendline" menu will appear on the right side of the screen. Check the boxes next to "Show equation in graph" and "Show square root in graph". The R-squared value is a statistic measure of how close the data are to the fitted regression line. When the r-squared value equals 1.0, all points lie exactly on a straight line with no scatter. An R^2 value of 0.0 means that knowing *x* does not help you predict *y*.

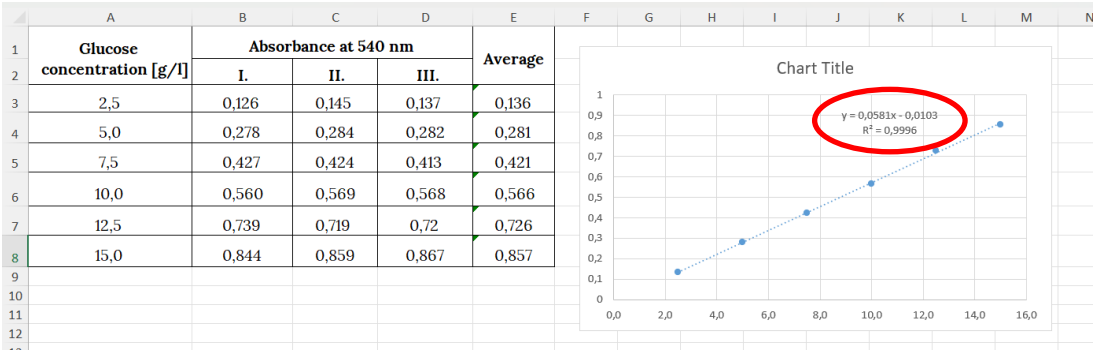


Option 2: Another option for adding the equation and R^2 (**4**) to the graph is to go to the tab "Chart Design"(**1** at the top of the Excel menu (left-click on the chart to see this option). Go to the "Quick Layout" menu (**2**) and select the 9th option in the "Quick Layout" drop-down list (**3**).



Glucose concentration [g/l]	Absorbance at 540 nm I.	II.	III.	Average
2,5	0,126	0,145	0,137	0,136
5,0	0,278	0,284	0,282	0,281
7,5	0,427	0,424	0,413	0,421
10,0	0,560	0,569	0,568	0,566
12,5	0,739	0,719	0,72	0,726
15,0	0,844	0,859	0,867	0,857

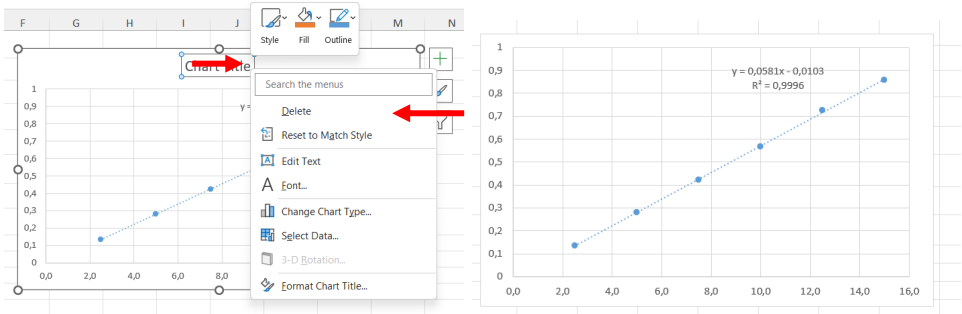
9. The result is the calibration curve, and the equation and the R-squared value. *Note that the data correlation is very good in our example, with an R-squared value of 0.9996. The concentration in the unknown sample can be calculated from the equation describing the linear regression ($y = 0.0581x - 0.0103$). When y is the dependent variable (absorbance), and x is the independent variable (concentration). The resulting value will have the same unit as the standard samples used to construct the calibration curve.*



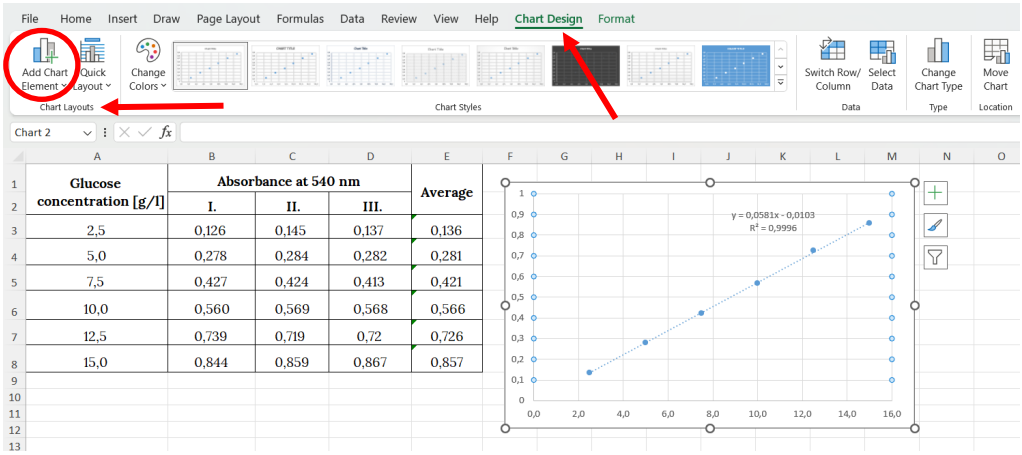
PART 2: ***Insert description for axes in chart***

Now that the calibration is complete, let's work on customizing the chart by editing the title and adding axis names.

1. In the first step, remove the graph name by left-clicking on it, right-clicking when the menu appears, and selecting “Delete”.



2. To add labels to the x-axis and y-axis, first left-click on the graph, a menu will appear at the top, and go to Chart design → Chart layouts → Add chart element.



Glucose concentration [g/l]	Absorbance at 540 nm			Average
	I.	II.	III.	
2,5	0,126	0,145	0,137	0,136
5,0	0,278	0,284	0,282	0,281
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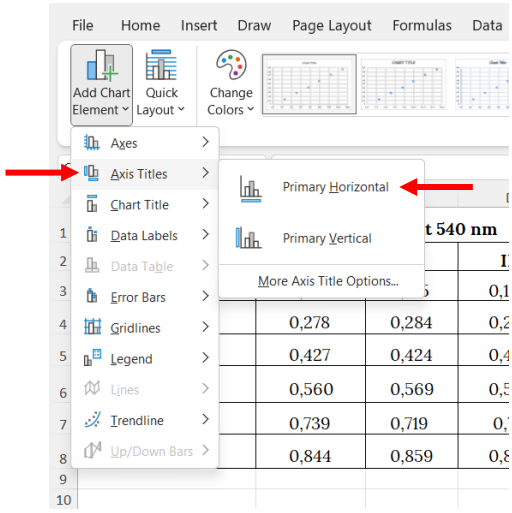
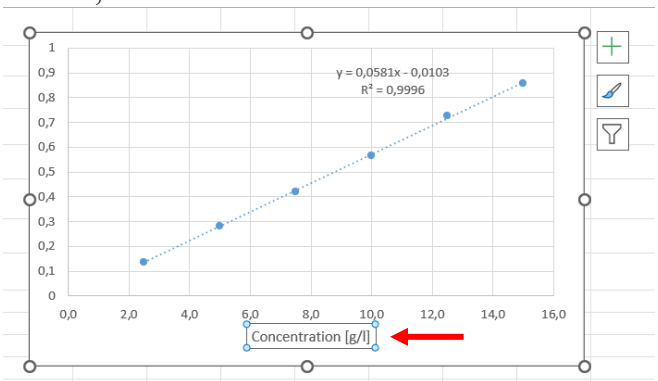
12,5	0,739	0,719	0,72	0,726
15,0	0,844	0,859	0,867	0,857

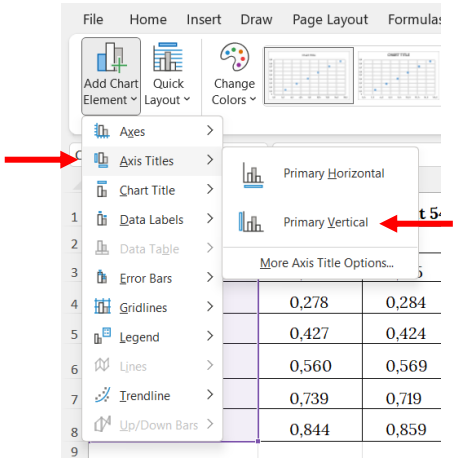
3. Now go to Axis titles → Primary horizontal.



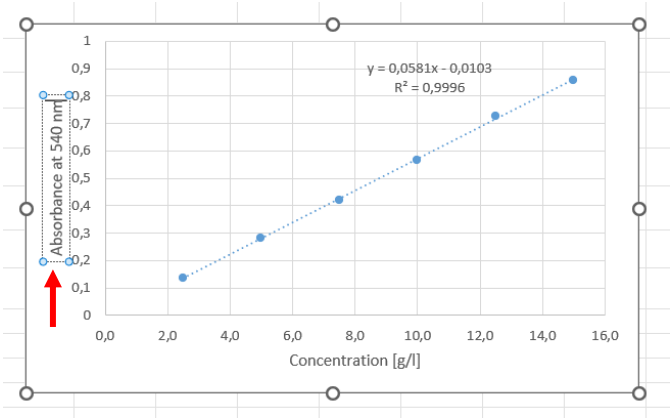
4. To rename an axis name, first select the text and then enter the new text.



5. To add label to the y-axis, first left-click on the graph, a menu will appear at the top, and go to Chart design → Chart layouts → Add chart element. Then go to Axis titles → Primary vertical.



6. To rename an axis name, first select the text and then enter the new text.



7. The final graph that will be added to the protocol.

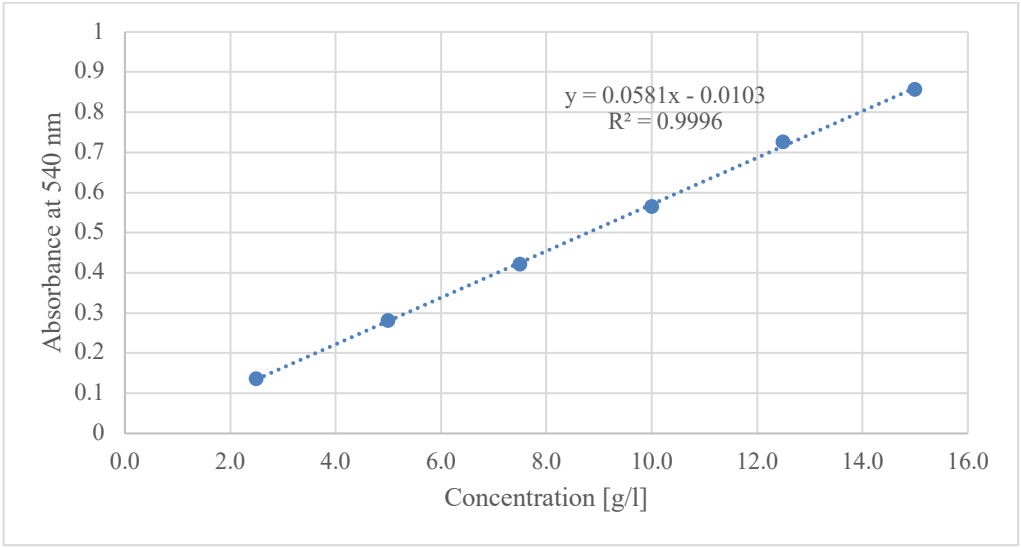


Figure 1A: The dependence of absorbance at 540 nm on the glucose concentration.

Attachment 5: Test your knowledge

SACCHARIDES

1. **Which of the following is a monosaccharide?** (1 point)
   a) sucrose b) glucose c) lactose d) maltose

2. **The bond that links two monosaccharides of a disaccharide is called the _____ bond.** (1 point)
   a) ionic b) glycosidic c) hydrogen d) acidic

3. Which of the following are **NOT** reducing disaccharides? (1 point)
   a) trehalose b) lactose c) sucrose d) isomaltose

4. **Divide the following monosaccharides** (*D-xylose, glyceraldehyde, D-glucose, erythrose, D-fructose, D-arabinose, and D-mannose*) **between:** (1 point)
   a) triose:
   b) tetrose:
   c) pentose:
   d) hexose:

5. **A monosaccharide consisting of 6 carbons, one of which is in a ketone group, is classified as a(an) _____**. (1 point)
   a) aldohexose b) aldopentose c) ketohexose d) ketopentose

6. **Polysaccharides are:** (1 point)
   a) polymers formed from monosaccharide subunits.
   b) monomers formed from monosaccharide subunits.
   c) two monosaccharides linked by a glycosidic bond.
   d) two disaccharides linked by hydrogen bonding.

7. **The units of saccharides which cannot be further hydrolyzed to simpler compounds are_____**. (1 point)
   a) Disaccharides b) polysaccharides c) monosaccharides d) oligosaccharides

8. **Explain the terms:** (1 point)
   a) *reducing saccharides*:

   b) *non-reducing saccharides*:

9. **State the principle for the determination of reducing saccharides by the DNS method.** (1 point)

10. **Calculate the weight of glucose (M = 180 g/mol) to prepare 35 ml of a solution with a concentration of 5 g/l. Express your result in grams.** (1 point)

NUCLEIC ACIDS

1. **Which technique was used to determine the double-helical structure of DNA?** (1 point)
   b) electrophoresis b) chromatography
   c) centrifugation d) X-ray crystallography

2. **Nucleic acids can be experimentally analyzed using their:** (1 point)
   b) molecular weights b) absorption of visible light
   c) absorption of UV light d) none of the options

3. **The saccharide that forms the backbone of DNA is called?** (1 point)
   b) β-D-ribose b) β-D-fructose
   c) β-D-galactose d) β-D-2-deoxyribose

4. **Which of the following are pyrimidine bases found in DNA:** (1 point)
   a) uracil and thymine b) thymine and cytosine
   c) adenine and thymine d) cytosine and uracil

5. **Pairs of bases are chemically boded in DNA by:** (1 point)
   e) hydrogen bonds between complementary bases.
   f) none of the options.
   c) polar covalent bonds between complementary bases.
   d) saccharide bonds between complementary bases.

6. **Nucleotides are linked together by bonds between 5' and 2' carbons of two different pentose saccharides. Explain your answer.** (1 point)
   b) true b) false
   Explanation:

7. The research of Erwin Chargaff was essential in the discovery of the structure of DNA by Crick and Watson. Chargaff analyzed the base composition of DNA from a wide range of organisms. He reported his findings using the initial letter of each base to stand for the number of that base found in an organism's genome.
   A = number of adenine bases G = number of guanine bases
   C = number of cytosine bases T = number of thymine bases
   Which of the following relationships did he find within the genome of each organism he studied? (1 point)
   a) A = G C = T b) A+T = C+G c) A+G = T+C d) A = C G = T

8. **What is difference between nucleotide and nucleoside?** (1 point)
   c) *nucleotide*:

   d) *nucleoside*:

9. **Do Chargaff's rules also apply to the RNA molecule? Explain your answer.** (1 point)

10. **Calculate how much potassium hydroxide (KOH) you will need to prepare 35 ml of its 1.5 % (w/v) solution.** (1 point)

LIPIDS

1. **What are lipids?** (1 point)

2. **Write the reaction scheme for the alkaline hydrolysis of 1,2,3-tripalmitoylglycerol with sodium hydroxide.** (2 points)

3. **What are the main functions of lipids in the body?** (1 point)

4. **Which indicator can be used in an acid-base titration to determine the acid number of oil?** (1 point)

5. **The saponification number indicates the number of mg of hydrochloric acid required to neutralize free and ester-bound fatty acids in 1 g of sample. Explain your answer.** (1 point)
   a) true b) false
   Explanation:

6. **What are two examples of:** (1 point)
   - *saturated fatty acids:*
   - *unsaturated fatty acids:*

7. **What are complex lipids? What are the main types of complex lipids?** (2 points)

8. **Calculate the weight of potassium hydroxide that you will need to prepare 125 ml of a solution with a concentration of 0.5 mol/l (M = 56.11 g/mol).** (1 point)

PROTEINS

1. **Which of the following statements is not correct? Explain.** (1 point)
   a) Amino acids have N-terminus, C-terminus, and R-groups.
   b) The charge on a protein is a function of the pH of the solution.
   c) Proteins are most soluble in a solution with the pH at their isoelectric point.

2. **What structures are recognized in proteins? Briefly describe.** (2 points)

3. **What are the six main functions of proteins?** (1 point)

4. **Glycine is:** (1 point)
   a) optical active
   b) hydrophilic, basic, charged at neutral pH
   c) hydrophobic
   d) hydrophilic, acidic, charged at neutral pH
   e) optical inactive
   f) none of the options

5. **The unfolding of a protein by heat or chemical treatment is referred to as:** (1 point)
   a) destruction b) renaturation c) denaturation d) restructuring

6. **Fill in the missing words in the sentence.** (1 point)
   The most important reaction of amino acids is the formation of a _____bond, which is formed between the _____ group of one amino acid and the _____ group of another amino acid, while one ____ molecule is releasing.

8. **Fill in the missing spaces in the sentences with the following options**. **Note:** some options may be extra. (2 points)
   a) Lowry method b) 1 – 6 mg/ml c) 0.1 – 1 mg/l d) biuret
   e) does not occur f) less g) does occur h) Biuret method
   i) more j) reduced phosphomolybdenum-phosphotungsten

   __is used to determine the presence of peptides and proteins in concentrations of __. This reaction __with free amino acids and there is little dependence on the primary structure of the protein, as the copper reacts with the peptide chain and not the side groups.
   __is based on measuring the absorbance of two complexes (__and __complex) that absorb light differently. Lowry method (in concentration of __) is __ sensitive than Biuret method.

9. **Calculate the weight of each reagent you will need to determine proteins using the Lowry method:** (1 point)
   Solution A: 1.5 % (*w/v*) sodium carbonate in 0.2 mol/l aqueous sodium hydroxide solution (**V = 75 ml**).
   - **$m_{(sodium\ carbonate)}$= ? g** ($M_{(Na2CO3)}$= 105.9888 g/mol)
   - **$m_{(sodium\ hydroxide)}$= ? g** ($M_{(NaOH)}$= 39.997 g/mol)

LABORATORY EXERCISES IN BIOCHEMISTRY

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